The Journal of American Science

ISSN 1545-1003

Volume 6 - Number 12 (Cumulated No. 33), December 1 2010, ISSN 1545-1003

Marsland Press, Michigan, The United States

The Journal of American Science

The ***Journal of American Science*** is an international journal with a purpose to enhance our natural and scientific knowledge dissemination in the world. Any valuable paper that describes natural phenomena and existence or any reports that convey scientific research and pursuit is welcome. Papers submitted could be reviews, objective descriptions, research reports, opinions/debates, news, letters, and other types of writings that are nature and science related. All the manuscripts will be processed in a professional peer review. After the peer review, the journal will make the best efforts to publish all the valuable works as soon as possible.

Introductions to Authors

1. General Information

(1) Goals: As an international journal published both in print and on internet, *The Journal of American Science* is dedicated to the dissemination of fundamental knowledge in all areas of nature and science. The main purpose of *The Journal of American Science* is to enhance our knowledge spreading in the world. It publishes full-length papers (original contributions), reviews, rapid communications, and any debates and opinions in all the fields of nature and science.

(2) What to Do: *The Journal of American Science* provides a place for discussion of scientific news, research, theory, philosophy, profession and technology - that will drive scientific progress. Research reports and regular manuscripts that contain new and significant information of general interest are welcome.

(3) Who: All people are welcome to submit manuscripts in any fields of nature and science.

(4) Distributions: Web version of the journal is opened to the world. The printed journal will be distributed to the selected libraries and institutions. For the subscription of other readers please contact with: editor@americanscience.org or americansciencej@gmail.com or editor@sciencepub.net

(5) Advertisements: The price will be calculated as US$400/page, i.e. US$200/a half page, US$100/a quarter page, etc. Any size of the advertisement is welcome.

2. Manuscripts Submission

(1) Submission Methods: Electronic submission through email is encouraged and hard copies plus an IBM formatted computer diskette would also be accepted.

(2) Software: The Microsoft Word file will be preferred.

(3) Font: Normal, Times New Roman, 10 pt, single space.

(4) Indent: Type 4 spaces in the beginning of each new paragraph.

(5) Manuscript: Don't use "Footnote" or "Header and Footer".

(6) Cover Page: Put detail information of authors and a short title in the cover page.

(7) Title: Use Title Case in the title and subtitles, e.g. "**Debt and Agency Costs".**

(8) Figures and Tables: Use full word of figure and table, e.g. **"Figure 1.** Annul Income of Different Groups**", Table 1. Annual Increase of Investment".**

(9) References: Cite references by "last name, year", e.g. "(Smith, 2003)". References should include all the authors' last names and initials, title, journal, year, volume, issue, and pages etc.

Reference Examples:

Journal Article: Hacker J, Hentschel U, Dobrindt U. Prokaryotic chromosomes and disease. Science 2003;301(34):790-3.

Book: Berkowitz BA, Katzung BG. Basic and clinical evaluation of new drugs. In: Katzung BG, ed. Basic and clinical pharmacology. Appleton & Lance Publisher. Norwalk, Connecticut, USA. 1995:60-9.

(10) Submission Address: editor@sciencepub.net, Marsland Company, P.O. Box 21126, Lansing, Michigan 48909, The United States, 517-980-4106.

(11) Reviewers: Authors are encouraged to suggest 2-8 competent reviewers with their name and email.

2. Manuscript Preparation

Each manuscript is suggested to include the following components but authors can do their own ways:

(1) Title page: including the complete article title; each author's full name; institution(s) with which each author is affiliated, with city, state/province, zip code, and country; and the name, complete mailing address, telephone number, facsimile number (if available), and e-mail address for all correspondence.

(2) Abstract: including Background, Materials and Methods, Results, and Discussions.

(3) Key Words.

(4) Introduction.

(5) Materials and Methods.

(6) Results.

(7) Discussions.

(8) References.

(9) Acknowledgments.

Journal Address:
Marsland Press
PO Box 180432, Richmond Hill, New York 11418, USA
Telephones: 347-321-7172; 718-404-5362; 517-349-2362
Emails: editor@americanscience.org; americansciencej@gmail.com; sciencepub@gmail.com
Websites: http://www.americanscience.org; http://www.sciencepub.net; http://www.sciencepub.org

The Journal of American Science

ISSN 1545-1003

Volume 6, Issue 12, Cumulated No. 33, December 1, 2010

Cover Page, Introduction, Contents, Call for Papers, am0612

Welcome to send your manuscript(s) to: americansciencej@gmail.com.

CONTENTS

	3. Faculty of Veterinary Medicine and Pharmacology, University of Maiduguri, P.M.B 1069, Maiduguri, Borno State, Nigeria. E-mail: joechemakan@yahoo.com **ABSTRACT:** Acute and sub-acute toxicities of aqueous extract of Vitex doniana was carried out in rats. The LD50 following intraperitoneal administration estimated at 95% confidence interval was 980 mg/kg. The oral administration of the extract for 21 days at 50,100 and 200mg/kg had beneficial effects on the haematological parameters. There were significant (P<0.05) increases in red blood cell count (RBC) haemoglobine (HB) concentration and packed cell volume (PCV) values in treated rats. The treated animals had leucocytosis, which may be due to increase lymphocyte count observed. The i.p LD50 (980 mg/kg) indicated that the extract is moderately toxic, though the prolong oral administration of the extract under the condition of this study shows that the extract may be toxic at higher doses. Nevertheless, the extract appear to be more beneficial at lower doses and significantly (p< 0.05) improves RBC, HB and PVC values and this effect has potential application as anti-anaemic agent. This seems to provide justification for its use as anti-anaemic agent in African traditional medicine. [Journal of American Science. 2010;6(12):8-12]. (ISSN: 1545-1003). **Keywords:** Vitex doniana, Red blood Cell Count, Haemoglobin Concentration, Packed Cell Volume, Anaemia, Aqueous Extract		
3	**Evaluation of Antioxidant Effect of Nigella sativa oil on Monosodium Glutamate-Induced Oxidative Stress in Rat Brain** Neveen, A. Noor* and Iman, M. Mourad Zoology department, Faculty of science, Cairo University, Egypt; *neveen.nour5@gmail.com Abstract: Oxidative stress is a characteristic feature in a number of neurodegenerative disorders. The present study evaluates the antioxidant effect of Nigella sativa oil (NSO) in comparison to that of vitamin C (vit.C) in the cortex and hippocampus of rats pretreated with monosodium glutamate (MSG) as an animal model of oxidative stress. The intraperitoneally injected MSG (4 mg/g body wt.) for 6 consecutive days induced significant decreases in cortical and hippocampal catalase activity and cortical glutathione-S-transferase (GST) activitity and glutathione reduced (GSH) level after 4 weeks. Oral administration of vit.C (200 mg/kg) to stressed rats restored catalase activity, increased GST activity and decreased malondialdehyde (MDA) level after 4 weeks in the cortex. Oral administration of NSO (1 ml/kg) for 4 weeks to MSG-treated rats increased cortical and hippocampal catalase activity and cortical GSH content but significantly inhibited GST activity and increased MDA level in the cortex. Combined administration of vit.C and NSO revealed nonsignificant changes in cortical and hippocampal parameters, as compared to control levels, except for a significant decrease in hippocampal GSH content. In conclusion, although there are some antioxidant effects of NSO, the pro-oxidant effect of NSO cannot be ruled out in the present MSG model of oxidative stress. [Journal of American Science. 2010;6(12):13-19]. (ISSN: 1545-1003). **Key words:** Oxidative stress, monosodium glutamate, vitamin C, Nigella sativa oil, cortex, hippocampus	Full Text	3
4	**Modulating Effect of Carvedilol on Doxorubicin-Induced Cardiomyopathy and Hepatic Damage** Safinaz S. Ibrahim*, Maged A. Barakat and HebaTullah S. Helmy Biochemistry Department, Faculty of Pharmacy, Cairo University, Cairo, Egypt. * dr_safinaz_747@hotmail.com **Abstract:** Background: Doxorubicin is an anthracyclin antibiotic that is considered as one of the most effective antitumor agents. The clinical use of doxorubicin soon proved to be hampered by such serious problems as hepatotoxicity and most notably cardiomyopathy. Objectives: The current study aims at evaluating the efficiency of carvedilol as an adjuvant therapy with doxorubicin to protect against doxorubicin - induced cardiomyopathy and hepatic damage. Materials and Methods: Animals were divided into normal group and doxorubicin -treated group injecting doxorubicin as a dose of 2.5 mg/kg/twice weekly/ 3 weeks. Doxorubicin - treated animals were divided into two groups, one kept without further	Full Text	4

	treatment (doxorubicin group) and second group, (doxorubicin + carvedilol), received carvedilol 1mg/kg/ 7 times over a period of 4 weeks including a dose before doxorubicin 1st dose. Creatine phosphokinase, lactate dehydrogenase, as cardiac damage markers, and alanine aminotransferase, as indicator of hepatic damage, were measured. Malondialdehyde and nitric oxide levels, as cardiac oxidative status indices, glutathione content, glutathione peroxidase, glutathione-S–transferase and superoxide dismutase activities, as measures for cardiac antioxidant capacity, were also investigated. Histopathological changes in cardiac and hepatic tissues of all groups were examined. Results and Conclusions: Our results revealed that doxorubicin caused oxidative stress which plays a major role in doxorubicin -induced cardiomyopathy and hepatic damage. Co-administration of carvedilol in concomitant with doxorubicin caused protection against doxorubicin–induced cardiomyopathy; however, it augmented doxorubicin -induced hepatic damage. Histopathological examination of cardiac and hepatic tissues supported the previous biochemical results. [Journal of American Science. 2010;6(12):20-32]. (ISSN: 1545-1003). **Keywords:** Doxorubicin, carvedilol, cardiomyopathy, hepatic damage		
5	**Effect of Water Stress and Ascorbic Acid on Some Morphological and Biochemical Composition of Ocimum basilicum plant.** 1Soha E. Khalil, 2 Nahed G. Abd El- Aziz and 1Bedour H. Abou Leil 1Departments of Water Relation and Field Irrigation, National Research Centre, Dokki, Cairo, Egypt 2Department of Ornamental and Woody Trees, National Research Centre, Dokki, Cairo, Egypt Soha_khalil2001@yahoo.com **Abstract:** Basil (Ocimum basilicum c.v. Thai Magic) is an annual herb plant belonging to the lamiaceae family that used as drug, mainly cultivated for leaves and flowering topes, the plant yield have an essential oil on steam distillation. The experiment was conducted to study the effect of different levels of water stress (30, 50 and 70% depletion of available soil moisture), different concentrations of ascorbic acid (0, 100, 150 and 200 ppm) and spraying time (at vegetative or vegetative plus flowering stages) on some morphological and biochemical characteristics of basil plant. A pot experiment was conducted in a split-split plot design with 24 treatments and three replicates in greenhouse. The results of staticall analysis showed that water stress, ascorbic acid concentrations and spraying time have significant effect on morphological and biochemical characteristics. Plant height, number of branches, number of leaves, leaf area, fresh and dry weights of the first cut showed significant increase under 50% soil moisture level while further increase in water stress level showed significant decrease in previously mentioned parameters. The same tendency was observed for relative water content % as well as photosynthetic pigments concentrations (chla, chlb, total chla+b and carotenoids). While in the second cut, the previously mentioned characters showed progressive decrease with increasing water stress level (except for photosynthetic pigments which revealed the same trend as in the first cut). Reveres trend observed for oil% and proline content. The data also indicated that the application of ascorbic acid in different concentrations showed significant increase in all growth parameters, fresh and dry weights, relative water content, oil % and photosynthetic pigments compared with control treatment and revealed decrease in proline accumulation. [Journal of American Science. 2010;6(12):33-44]. (ISSN: 1545-1003). **Keywords:** Water Stress; Ascorbic Acid; Biochemical Composition; Ocimum basilicum	Full Text	5
6	**Isolation and Screening of a Feather-Degrading Keratinolytic Actinomycetes from Actinomyces sp** T.Jayalakshmi 1, P. Krishnamoorthy 1,, G.Ramesh kumar 2, P.Sivamani 3 1 Dept of Bioinformatics, Bharath University, Chennai,Tamilnadu - 600073, India. 2 Dept of Bioinformatics, MIT Campus, Anna University, Chennai, Tamilnadu-600044, India. 3 Microlabs, Vellore, Tamilnadu-632521, India. jayamaniraaja07@gmail.com	Full Text	6

	ABSTRACT: The Actinomycetes comprise a ubiquitous order of bacteria which exhibits wide physiological and morphological diversity. These microorganisms are particularly abundant in alkaline soils rich in organic matter. Keratin is an insoluble structural protein of skin, and its derivatives (e.g. feather, hair, wool and horn) are known for their high stability. Actinomycetes producing keratinases are having high applications in feed, fertilizer, leather and also for pharmaceutical and biomedical applications. Actinomyces species newly isolated, thermo tolerant feather degrading bacterial strain was investigated for its ability to produce keratinase enzyme. Maximum keratinolytic activity was observed at 28°C and pH 7.5. Keratin-containing materials (feather, hair, wool, etc.) are abundant in nature but have limited uses in practice since they are insoluble and resistant to degradation by the common proteolytic enzymes. Keratinous wastes represent a source of valuable proteins and amino acids and could find application as a fodder additive for animals or source of nitrogen for plants. Actinomycetes have the ability to break down many different varieties of organic compounds. The keratinase production by the thermophilic actinomycete strain Actinomyces was induced by chick feather as the sole source of carbon and nitrogen in the cultivation medium and characterization studies were carried out for the identification of the specific strain. [Journal of American Science. 2010;6(12):45-48]. (ISSN: 1545-1003). **Keywords:** Keratin, Actinomycetes, Antibacterial activity, Actinomyces		
7	**Denaturation and Viscosity of Whey Proteins Solutions as Affected by Frozen Storage** Soliman, T.N.*1, A.F. Farrag1; A. Shendy2 and El-Sayed, M.M.1 1Dairy Dept. National Research Centre, 2Dairy Dept. Faculty of Agriculture, Al-Azhar University, Cairo, Egypt. Tariknour.nrc@gmail.com **Abstract:** Concentrated solutions of whey proteins (WPC) were prepared from sweet whey by ultrafiltration technique, and stored at – 18°C up to three months. Denaturation degree and viscosity of WPC solutions were assessed. Denaturation degree of whey protein solutions increased significantly ($P<0.05$) as affected by duration of frozen storage and protein content. The highest degree of denaturation was found at pH 5.0 and 7.0 after one month of storage. Denaturation percentages of heated and thawed WPC solutions increased significantly ($P<0.05$) as function of storage, protein content and pH. The flow properties of unheated WPC solutions exhibited a time-independent non-Newtonian behaviour as shear-thickening (dilatants) properties with an increase in the apparent viscosity with increasing the shear rate. Heated thawed WPC solutions behaved as thixotropic fluids with a decrease in the apparent viscosity with increasing shear rate. Apparent viscosities of unheated and heated WPC solutions greatly affected by frozen storage, protein content and pH. [Journal of American Science. 2010;6(12):49-62]. (ISSN: 1545-1003). **Key words:** WPC, Frozen storage, Denaturation, Viscosity	Full Text	7
8	**Evolution and Development Towards 4th Generation (4G) Mobile Communication Systems** M. Junaid Arshad, Amjad Farooq, Abad Shah Department of Computer Science and Engineering, U.E.T., Lahore-Pakistan junaidarshad@uet.edu.pk amjadfarooq@uet.edu.pk abadshah@uet.edu.pk Abstract: It is the need of hour to get ourselves acquainted with the communication technology, its tools and its trends. Mobile communication is an important technology in this regard and mobile phone has become the most common tool of communication over the recent years. As a number of innovative improvements in the field of mobile communication technologies have been made by developing various multiple-access schemes used for wireless communication (such as TDMA, FDMA, CDMA, WCDMA, EDGE etc) but a big challenge is to select the right technology for the applications and systematically identify the factors that influence the overall performance. In this research paper, we present the detail comparison of the different generations of the mobile communication technologies in a tabular form to have a better knowledge and understanding in the advancement of mobile communication systems. The survey presented here will be helpful for designing the new strategies for the development of 4th	Full Text	8

	generation mobile communication systems. This research work can steer all those learners who are trying to enhance their acquaintance in the field of mobile communication system, and also for such mentors and researchers who desire to have a foundation for further research and study in this field. [Journal of American Science. 2010;6(12):63-68]. (ISSN: 1545-1003). Keywords: Mobile Communication, Evolution, Generations, Comparison, Wireless Technologies		
9	**A Layered approach for Similarity Measurement between Ontologies** Amjad Farooq, M. Junaid Arshad and Abad Shah Computer Science and Engineering Department, University of Engineering and Technology, Lahore-Pakistan amjadfarooquet@gmail.com **Abstract:** With the vision of Semantic Web, the ontology operations such as aligning, merging and mapping have gained much importance. The measuring of similarity between concepts of source ontologies is preprocessing of all these operations. Several techniques have been proposed for measuring similarity between concepts based on their lexical, taxonomic and elementary characteristics but a very little attention has been given on their non-taxonomic relations. We have observed that lexically similarity between concepts is mandatory in order to their taxonomic similarity. Furthermore, the taxonomic similarity between two concepts is pre-requisite of their non-taxonomic similarity. This motivates that if the similarity measurement process is made in layered fashion then it will become more efficient. In this paper, a new technique is proposed that includes non-taxonomic relations of concepts along with their lexical and taxonomic characteristics while measuring their similarities. The proposed technique works in a layered fashion that enables the measuring process more efficient. [Journal of American Science. 2010;6(12):69-77]. (ISSN: 1545-1003). **Keywords:** Ontology Matching, Lexical Similarity, Taxonomic Similarity, non-taxonomic similarity	Full Text	9
10	**Physicochemical Parameters in Soil and Vegetable Samples from Gongulon Agricultural Site, Maiduguri, Borno State, Nigeria** 1J. C. Akan, 1F.I. Abdulrahman, 2O.A. Sodipo, 1A. G. Lange 1. Department of Chemistry, University of Maiduguri, P.M.B 1069, Maiduguri, Nigeria. 2. Department of Clinical Pharmacology and Therapeutics, College of Medical Sciences, University of Maiduguri, Maiduguri, Nigeria. E-mail: joechemakan@yahoo.com **ABSTRACT:** Anthropogenic activities are a leading cause of metal emission, often associated with high elevated soil and plant metal concentrations. The accumulation of heavy metals and anions in soil and vegetables in the vicinity of Gungulung agricultural site were investigated. Soil samples were collected at depths of 0-5 cm, 5-10 and 10-20 cm. Soil properties including pH, electrical conductivity (EC), organic matter, organic carbon, cation exchange capacity (CEC) and heavy metals content were determined using standard procedures. Vegetable samples (spinach, Amaranthus caudatus; carrot, Daucus carota; lettuce, Lactuca sativa; cabbage, Brassica oleracea; tomato, Lycopersicon sculenetum; waterleaf, Talinum Triangulare and onion Allium cepa were used for this research. The plant samples were prepared for heavy metals and anions determination using standard procedures. Results show that the soil metal content, conductivity and organic carbon decreased with depth, suggesting anthropogenic sources of contamination while pH, organic matter and CEC decreased with depth. The results obtained from this analysis revealed that Zn and Mn show the highest concentrations, Ni shows the lowest levels. Similarly, the results also revealed that Fe, Zn and Cu show the highest concentrations, while Pb shows the lowest levels in the whole vegetables parts studied. The leaves contained much higher concentrations of heavy metals and anions than roots and stems. The concentrations of the above parameters in the vegetable samples were	Full Text	10

	higher than the FAO, WHO/EU and FAO/WHO allowed limit. The high values might be attributed to the use of wastewater from river Ngada and application of sewage sludge by farmers for the irrigation of these vegetables. The results of this study suggest that the vegetables grown in the vicinity of Gugulung agricultural site are subjected to anthropogenic activities. Thus, the high values of these metals in the vegetable samples could put the consumers of these vegetables at health risk with time due to bioaccumulation. [Journal of American Science. 2010;6(12):78-87]. (ISSN: 1545-1003). **Key words:** Physicochemical, Parameters, Soil, Vegetables, Bioavailability, Uptake		
11	**Data Networks' Design and Optimization through MPLS VPNs using BGP** Mohammad Junaid Arshad 1, Tauqir Ahmad 2, Amjad Farooq 3 1, 2, 3 Department of Computer Science & Engineering, University of Engineering and Technology Lahore, Pakistan. junaidarshad@uet.edu.pk **Abstract:** The key strong points of the Internet have been its vast scalability and flexibility to provide accommodation to the variety of applications. In this context, MPLS (Multi Protocol Label Switching) is the newest technology being employed today's in the Internet core, which is continuously growing to meet the increasing demands of bandwidth and connectivity. In this research work, we provide a survey of MPLS, BGP (Border Gateway Protocol) and both layer-2 and layer-3 VPNs (Virtual Private Networks). We address the issues (such as speed, scalability and security) of traditional IP-based VPNs. Since layer-2 VPNs are efficient but not so intelligent and scalable, while layer-3 VPNs are intelligent and scalable but not so efficient. Thus, we propose a new design scheme for MPLS/BGP-VPNs in such a way that the features of layer-3 as scalability and intelligence are merged with the efficiency of layer-2 to deal with today's evolving demands of speed, scalability and security. The proposed design of optimized data networks through MPLS/BGP-VPNs is implemented in Dynagen simulator for the better understanding the system. This research work will be helpful for adding new security features in core networks in future and provides a guideline for network engineers towards the world of network security. [Journal of American Science. 2010;6(12):88-95]. (ISSN: 1545-1003). **Keywords:** BGP-Border Gateway Protocol, MPLS-Multi Protocol Label Switching, QoS-Quality of Service and VPN-Virtual Private Network	Full Text	11
12	**GRIAS: GUI-Based Real-Time Industrial Automation Software** Mohammad Junaid Arshad 1, Amjad Farooq 2 1, 2 Department of Computer Science & Engineering, University of Engineering and Technology Lahore, Pakistan. amjadfarooq@uet.edu.pk junaidarshad@uet.edu.pk **Abstract:** Industry has a great importance in the development of a country. These days a country cannot progress and prosper without industrial development. Industrial revolution has changed the fortune of many western countries. In the fast moving world of today, the industrial plants have become very complicated and many new technologies have been introduced in the market to overcome these complications by automating the industrial plants. This work proposes an industrial automation software called GRIAS (GUI-Based Real-Time Industrial Automation Software) that can be used for any industrial plant in which OPC (OLE (Object Linked Embedding) for Process Control) compliant hardware devices are used. This generic software has the ability to interact with an OPC server which is responsible to retrieve runtime data from the hardware device. The data provided by the server can be used by the software to monitor the running industrial plant. It can also be used in critical industrial units where it is very difficult to manually control the machinery. The industry has been looking for such software which can meet up their requirements, thus, this new industrial automation software will surely be able to realize their dreams into reality. The purpose of this automation software is not only to eliminate the perils and hazards involved in industries but also to speedup the process of manufacturing and production in such a	Full Text	12

	way that it is no more error prone. [Journal of American Science. 2010;6(12):96-101]. (ISSN: 1545-1003). **Keywords:** Graphical User Interface (GUI), Human Machine Interface (HMI), Design, Industrial Automation, OPC, Solution		
13	Arch Dam Failure Diagnosis Applying Micro-Planes Damage Based Framework Mojtaba Labibzadeh 1 1. Department of Civil Engineering, Faculty of Engineering, Shahid Chamran University, Ahvaz, Iran Labibzadeh_m@scu.ac.ir Abstract: A recently new developed set of constitutive equations which simulating the mechanical behavior of plane concrete have been implemented for monitoring the probability of cracking phenomenon within an arch concrete dam . The applied constitutive model was build on the basis of combination of micro-plane theory and damage framework. This model had been verified through comparing numerical results with experimental ones. The case study is a high elevated concrete arch concrete dam entitled Liroo dam. Obtained analysis results demonstrated that under proposed earthquake excitations, dam experiences some cracks near its middle crest. [Journal of American Science. 2010;6(12):102-107]. (ISSN: 1545-1003). **Keywords:** Arch dam, Micro-planes, Cracks, Constitutive relations, Concrete	Full Text	13
14	**Exploring the Potential and Constraints to Implementing the International Best Practice Principles of EIA Follow-up: The Case of Pakistan** Obaidullah Nadeem1 , Rizwan Hameed2 Department of City and Regional Planning, University of Engineering and Technology, Lahore, Pakistan. 1obaidnadeem@yahoo.com; 2d_rizwan@hotmail.com **Abstract:** Every Environmental Impact Assessment (EIA) carried out for development projects in Pakistan includes a long list of mitigation measures and an environmental management plan (EMP). The environmental approvals also contain numerous conditions including implementation of EMP during construction and operation phases of development projects. Without appropriate follow-up and compliance monitoring the entire exercise may go waste. That is why follow-up is considered essential to ensure positive outcome of EIA by protecting the environment and learning lessons for its improvement. In this regard, the International Association for Impact Assessment has suggested best practice guiding and operating principles. This paper attempts to explore the potential and constraints to implementing these principles in Pakistan. Various data sources including interviews with the officials of environmental protection agencies, project proponents, EIA consultants and representatives of some of the affected communities as well as review of EMPs have been used to provide empirical evidence for this purpose. This paper identifies some potential but overall it argues that a lot more is needed to be done to bridge the gap between the international best practice principles and the current state of EIA follow-up in Pakistan. Some imperative steps have also been suggested in this context to improve follow-up and hence strengthen the overall process for EIA. It is expected that other developing EIA regimes may also benefit from the suggestions. [Journal of American Science. 2010;6(12):108-121]. (ISSN: 1545-1003). **Keywords:** EIA follow-up; Best practice principles; Pakistan.	Full Text	14
15	**Effect of Trichoderma Species on Damping off Diseases Incidence, Some Plant Enzymes Activity and Nutritional Status of Bean Plants** Abd-El-Khair *1, H., R. Kh. M. Khalifa 2and Karima, H. E. Haggag3 1*Plant Pathology Department, 2Fertilization Technology Department, National Research Centre, and	Full Text	15

	3Pest Rearing Department, Central agricultural Pesticides Laboratory, Dokki, Giza, Egypt. **Abstract:** Fusarium solani and Rhizoctonia solani are the common causal pathogens causes the damping off disease of beans (Phaseolus vulgaris L.) in Egypt. The antagonistic effect of four Trichoderma species, i.e. Trichoderma album, Triechoderma hamatum, Trichoderma harzianum and Trichoderma viride, was tested against F. solani and R. solani in vitro, in greenhouse and in field. In vitro tests, all Trichoderma spp. significantly reduced the mycelial growth of two pathogenic fungi. In greenhouse experiment, T. album, T. hamatum, T. harizianum and T. viride, as soil treatments, significantly reduced the pre- and post-emergence damping off disease incidence under artificial infection with F. solani and R. solani. Soil treatments with four Trichoderma species significantly reduced the incidence of damping off disease where the percentages disease incidence were in the range of 7.0 -20.0% and 2.4 – 6.5%, compared to 25.7 and 13.5% in control plants, at pre- and post-emergence stages ,respectively. The best protection to damping off disease was obtained by T. hamatum, followed by T. viride, T. album and T. harzianum, respectively. The treatments gave the highest plant survival (%) and improved the growth and yield parameters. Results showed that the levels of chitinase, peroxidase and polyphenol oxidase activities highly increased in treated bean plant compared in untreated plants. The macro- and micro-elements content in treated bean plants was affected by Trichoderma species treatments compared to elements content in untreated plants. The relationship between plant nutrient content and some plant enzymes activity was studied. [Journal of American Science. 2010;6(12):122-134]. (ISSN: 1545-1003). **Key words:** Fusarium solani, Rhizoctonia solani, Phaseolus vulgaris, Trichoderma spp., biological control, nutritional atatus		
16	**Phase I Trial: Mesenchymal Stem Cells Transplantation in End Stage Liver Disease** El-Ansary M,1 Mogawer Sh,2 Abdel-Aziz I,3* and Abdel-Hamid S,1 Department of Clinical Pathology1, Department of Hepatology and Gastroenterology2 Cairo University, *3Department of Haematology (Theodor Bilharz Research Institute), Cairo, Egypt. *iman_khaled@yahoo.com **Abstract:** Background, End-stage liver disease and in particular human liver cirrhosis represents a worldwide health problem. Currently, liver transplant is the only effective treatment, but it is affected by many problems including relative lack of donors, operative damage, risk of rejection and high costs. Stem cell therapy is very attractive in this setting because it has the potential to help tissue regeneration while providing minimally invasive procedures and few complications. The aim of this study was to evaluate the effect of autologous transplantation of bone marrow derived mesenchymal stem cells in cirrhotic patients following chronic hepatitis C virus infection. Methods, Twelve patients with Child C liver cirrhosis, Model of End Stage Liver Disease (MELD) score > 12 were included. They divided into 2 groups according to method of MSCs injection, 1st group was injected intrasplenic and 2nd group was injected through the peripheral blood. First group patient's ages ranged from 32 to 69 years, mean value was 48.50 ±11.09, they were 4 males (67%) and 2 females (33%). Second group patient's ages ranged from 43 to 59 years, mean value was 50.83 ±6.88, they were 5 males (83%) and 1 female (17%). Fifty ml bone marrow was aspirated from the iliac bone for separation of MSCs. Surface expression of CD271 and CD34 were analyzed using flowcytometry. Finally approximately 10 million MSCs/ 5ml saline were infused intrasplenic or peripherally in one session. There was highly statistical significant difference between CD271 before and after culture, p value was <0.01. Results, Monthly Follow up of patients for 6 months revealed partial improvement of liver function tests with decline of elevated bilirubin and liver enzymes and elevation of prothrombin concentration and serum albumin levels. There was statistically significant difference between total bilirubin, direct bilirubin, MELD score and creatinine level before and after MSCs injection in both groups, p value was <0.05. Conclusion, MSCs are the most potent component of bone marrow cells in its ability to differentiate into hepatocytes thus, MSC transplantation can be used as a potential treatment for liver cirrhosis. The dose, frequency and route of administration of this treatment are still to be defined. [Journal of American Science. 2010;6(12):135-144]. (ISSN: 1545-1003). **Keywords:** End-stage liver disease; liver cirrhosis; liver transplant; autologous transplantation; bone marrow; mesenchymal; stem cell	Full Text	16

17	Role of Hepcidin in Anemia of Chronic Hepatitis C Patients Salwa Toima1, Abeya Saleh*1, Mona Madkour1, Olfat Hammam2 and Emad EL-Din Baioumi3 1Hematology Department, 2Pathology Department, 3Hepatology and Gastroenterology Department, Theodor Bilharz Research Institute, Cairo, Egypt. dr.abeyasaleh@hotmail.com **Abstract:** This study was done to clarify the role of hepcidin in the regulation of iron homeostasis and development of anemia in chronic hepatitis C (CHC) patients targeting the differentiation of the type of anemia. Patients and methods: This study was conducted on 70 CHC patients. Iron profile and soluble transferrin receptor (sTfR) were measured. Transferrin saturation and transferrin receptor ferritin (TfR-F) Index were calculated. Serum prohepcidine hormone and IL6 levels were measured (ELISA). Histopathological examination and immunohistochemical detection of hepcidin were done. According to the iron profile patients were reclassified into iron deficiency anemia (IDA) group, anemia of chronic disease group (ACD) and combined anemia group (COMBI). Results: 64.3% of patients were of the COMBI group, 10% had ACD and 25.7% had IDA. Hepcidin was increased in Child C group ($P<0.05$). Hepatic expression of hepcidin showed reduced expression in Child A, B and C groups. Hepcidin level was found to be increased in ACD and COMBI group in comparison to control and IDA group. Stepwise logistic regression demonstrated that sTfR was the most predictive parameter for IDA while hepcidin was the most predictive parameter for ACD and COMBI in CHC patients. Conclusion: hepcidin plays an important role in the pathogenesis of anemia in CHC patients. The role of hepcidin in discriminating different types of anemia in CLD is comparable to that of sTfR/logFn index. An appropriate combination of both tests provides evidence for iron depletion or reflects excessive production of hepcidin which will help to establish a correct diagnosis of IDA, ACD or combined anemia in patients with CHC. [Journal of American Science. 2010;6(12):145-154]. (ISSN: 1545-1003). **Key words:** Hepcidin, CHC, IDA, ACD, Anemia	Full Text	17
18	**Controversial Role of Two Different Local Haemostatic Agents on Bone Healing** Ali Sawan 1 Yousry Elhawary2 Mohamed Zaghlool Amer*3 and Mohamed Abdel Rahman4 1 Professor Of Oral & Maxillofacial Surgery-,2Associate professor Of Oral Biology, 3 Lecturer of Oral & Maxillofacial Surgery- Faculty of Dentistry-Mansoura University, 4 B.D.S 2002- Ministry of Health, Mansoura –Egypt. *norhanmohammed910@yahoo.com **Abstract:** Controversial role of different local haemostatic agents on bone healing represented a major challenge for oral & maxillofacial surgeons. So, this study was directed to evaluate the effect of water soluble alkylene copolymer hemostat (ostene) versus bone wax on bone healing. Material & Methods: Forty five adult male rabbits weight 1kg were divided into three equal groups. A surgical bone defect was created into the anterior mandibular area. In 1st group the surgical defects were not subjected to any of local haemostatic agents. In 2nd group water soluble alkylene copolymer was applied within surgical defect and bone wax was applied within the 3rd group. Postoperatively, 3 animals were sacrificed from each group at 1, 2, 3, 6 and 12 weeks for histological assessment through H&E and Trichrome stain Results: Water soluble alkylene copolymer hemostat treated defects showed faster healing rate in 1st, 2nd weeks than defects left untreated. Ostene was disappeared from surgical defect at 1st week without presence of inflammatory cells in the defect. In 3rd group, the defects showed large empty vacuoles, representing bone wax remnants with inflammatory cells infiltration that interfere bone healing. Conclusion: Water soluble alkylene copolymer is biodegradable material that does not interfere with bone healing in contrast with bone wax which causes foreign body reaction, leading to interference of bone healing. [Journal of American Science. 2010;6(12):155-163]. (ISSN: 1545-1003). **Key words:** Local Haemostatic Agents- Bone wax- Ostene	Full Text	18
19	**A Framework for Testing Software Product**	Full Text	19

	Amjad Farooq[1], M. Junaid Arshad[1] and Muhammad Abuzar Fahiem[2] [1, 2]Computer Science and Engineering Department, UET, Lahore [2]Department of Computer Science, Lahore College for Women University, Lahore, Pakistan amjadfarooq@uet.edu.pk **Abstract:** There is a growing need of frameworks for automatic testing of software product because manual testing of huge software product is very time-consuming and costly. Furthermore, the manually testing of complex software becomes more difficult and a challenging activity. However this can be easily achieved through automatic testing strategies. In this paper we propose a framework for testing software automatically. Now errors and bug finding become simpler and easier. It takes less time to test the whole application rather than testing application modules separately. The proposed framework provides programmatic access to most user interface elements. The main propose of our framework is to make testing phase easier and cost efficient. We validate our framework through a case study. By analyzing the results of testing the correctness and completeness of framework is proved. [Journal of American Science. 2010;6(12):164-173]. (ISSN: 1545-1003). **Keywords:** Software testing; test automation; test framework		
20	**Effect of Mineral, Organic Nitrogen Fertilization and Some Other Treatments on vegetative growth of Picual Olive Young Trees.** Laila, F. Hagag1 ; H. S. A. Hassan1; M. Abou Rawash2; H. El-Wakeel2 and A .Abdel-Galel 1 1-Pomology Department, National Research Center, Cairo, Egypt 2- Dept. of Hort. Fac. of Agric. Ain Shams Univ., Shobra El-Khiema, Cairo, Egypt **Abstract:** This study was carried out through two successive seasons (2007& 2008) on cultivated Picual olive young trees grown at the Research Station Farm of National Research Center, El Nobarya, El Behera governorate. The investigation aimed to study the effect of applying mineral, organic fertilizers and some other treatments on growth parameters at the first two years of planting. Planting holes were prepared for control plants in the first season only. Each treatment received 100 g actual nitrogen/plant/year as recommended by M.A.R.L. (2007). The following treatments were applied: T1 : control (mineral nitrogen + planting hole preparation), T2(100%mineral nitrogen), T3(100% organic N as cattle manure), T4(50% mineral N + 50% organic N as chicken manure), T5 (100%mineral nitrogen + humic acid as soil application), T6(100% mineral nitrogen + activated dry yeast as soil application), T7 (100%mineral nitrogen + GA3 spray) and T8 (100% mineral nitrogen + sea algae as soil application).At the end of each season, plant height, stem diameter, lateral shoot number, lateral shoot length, leave numbers per plant, Percentage of plant height increment, whole plant dry weight were determined and recorded. The obtained results revealed that as follow: plant height, shoot number, shoot length, leaves number and stem diameter were not affected by different treatments in both seasons. Meanwhile, whole plant dry weights were improved by humic acid treatment compared with control and all other treatments in Picual cv. [Journal of American Science. 2010;6(12):174-179]. (ISSN: 1545-1003). **Keywords:** Nitrogen Fertilization; vegetative growth; Picual Olive	Full Text	20
21	**Response of Picual Olive Young Trees to Mineral, Organic Nitrogen Fertilization and Some Other Treatments** M. Abou Rawash2; H. El-Wakeel2; Laila, F. Hagag1 ; H. S. A. Hassan1 and A. Abdel-Galel1 1-Pomology Department, National Research Center, Cairo, Egypt	Full Text	21

	2- Dept. of Hort. Fac. of Agric. Ain Shams Univ., Shobra El-Khiema, Cairo, Egypt **Abstract:** This study was carried out through two successive seasons (2007& 2008) on a cultivated Picual olive young trees grown at the Research Station Farm of National Research Center, El Nobarya, El Behera governorate. The investigation aimed to study the effect of applying mineral, organic fertilizers and some other treatments on leaf mineral contents at the first two years of planting. Planting holes were prepared for control plants in the first season only. Each treatment received 100 g actual nitrogen/plant/year as recommended by M.A.R.L. (2007). The following treatments were applied: T1 : control (mineral nitrogen + planting hole preparation), T2(100%mineral nitrogen), T3(100% organic N as cattle manure), T4(50% mineral N + 50% organic N as chicken manure), T5 (100%mineral nitrogen + humic acid as soil application), T6(100% mineral nitrogen + activated dry yeast as soil application), T7 (100%mineral nitrogen + GA3 spray) and T8 (100% mineral nitrogen + sea algae as soil application).At the end of each season, leaves dry weight per plant, and leaf mineral content were determined and recorded. The obtained results revealed that as follow: Effect of treatment on Leaves dry weight (g) per plant, fifth treatment with humic acid and sixth treatment with activated dry yeast gave the highest significant values in the first season, meanwhile in the second season fourth treatment with 50% cattle manure and fifth treatment with humic acid recorded higher significant values. Leaf nitrogen content revealed that first, fifth, sixth and seventh treatments showed higher significant values respectively than those of other treatments in the first season. In the second season, the first treatment had higher significant leaf nitrogen content compared with most of other treatments. [Journal of American Science. 2010;6(12):180-186]. (ISSN: 1545-1003). **Keywords:** Picual Olive; Organic Nitrogen; Treatments		
22	**Effect of Bud Load on Bud Behavior, Yield, Cluster Characteristics and some Biochemical Contents of the Cane of Crimson Seedless Grapevines** 1Fawzi M. I. F. 1Shahin M. F. M. and 2Kandil E. A. 1Pomology Department, National Research Center. 2Horticulture Research Institute agriculture Research Center, Giza, Egypt **Abstract:** This study was conducted through the seasons of 2007 and 2008 to determine the optimum bud loads/ vine for Crimson seedless "grapevines. Eight years old uniform vines were chosen and pruned to six different levels of bud load, namely 75, 91, 104, 117, 130 and 143 buds/ vine. Number of buds was fixed at 13 bud/cane. The results showed that the number of bursted buds was increased significantly by increasing bud load /vine in the two seasons of the study, while the percentage of bursted buds decreased. The bud fertility and fruitfulness were decreased by increasing bud load. Data also indicated that 104 or 117 buds/ vine were more suitable for Cirimson seedless grapevines to produce good yield and fruit quality. On the other hand, 78 or 143 buds/vine was unfavorable science it produced rather compact clusters. Increasing bud load increased number of cluster/vine and yield but reduced cluster weight. Vines pruned to 117 bud/vine gave the greatest cluster weight, length, rachis weight, berry weight, berry firmness, adherence, T.S.S and total sugars. Increasing bud load on the vine significantly increased total carbohydrates and protein contents of the canes during the dormant season. In this respect, vines pruned to 143 bud/vine showed higher percent of both total carbohydrate and protein contents than the other levels of bud load. [Journal of American Science. 2010;6(12):187-194]. (ISSN: 1545-1003). **Key words:** Grapevine, winter pruning Crimson seedless, bud load, fruit quality.	Full Text	22
23	**The Effect of some Slow Release Nitrogen Fertilizers on Growth, Nutrient Status and Fruiting of "Mit Ghamr" Peach Trees** 1 Kandil, E. A., 2M. I. F. Fawzi, and 2M. F. M. Shahin 1Hortcultural Research Institute, 2 National Research Center, Dokki, Giza, Egypt. **Abstract:** This study was conducted for comparing three slow release N fertilizers namely, urea – formaldehyde, phosphorus – coated urea and sulphur coated- urea and that fast release nitrogen namely (urea) at 500, 750 and 1000g/tree/year for vegetative growth, leaf mineral content, yield and fruit quality of	Full Text	23

	"Mit Ghamr" peach tree grown in a private orchard Aga city Dakahlia Governorate, Egypt, during 2008 and 2009 seasons, were studied. Urea was added at two times at the start of spring growth and after fruit set, while slow – release N fertilizers applied once at the start of spring growth. Results showed that supplying the tree of "Mit Ghamr" peach with the three slow release N fertilizers were superior to the application of the fast one in improving shoot length, leaf area, percentage of leaf N, as well as physical and chemical characteristics of the fruits. Application of sulphur – coated urea (SCU), phosphorus- coated urea (PCU) and urea- formaldehyde in a descending order was very favorable. Generally, "Mit Ghamr" peach trees once with sulphur coated urea at 500-750g/trees/year was the best results on vegetative growth, yield nutritional status of trees and fruit quality. In addition saving nitrogen fertilization cost and reducing nitrate pollution. [Journal of American Science. 2010;6(12):195-201]. (ISSN: 1545-1003). **Keywords**: N fertilizers; urea; formaldehyde; phosphorus; sulphur		
24	**Influence of Foliar Application of some Nutrient (Fertifol Misr) and Gibberellic Acid on Fruit Set, Yield, Fruit Quality and Leaf Composition of "Anna" Apple Trees Grown in Sandy Soil** 1Shahin M. F. M. 1Fawzi M. I. F. 2Eman. A. K and 2kandil E. A. 1Pomology Department, National Research Center, 2Horticulture Research Institute agriculture Research Center, Giza, Egypt. **Abstract:** The effect of Fertifol Misr (N, P, K, Mg zn, Fe, Mu, Cu, Mo & B) and gibberellic acid on fruit set, drop percentage, yield, fruit quality and leaf chemical composition on "Anna" Apple trees were studied during 2007 and 2008 seasons. Results showed that, fruit set%, drop%,, yield, leaf minerals & chlorophyll contents as well as physical and chemical characters of the fruit were positively effected by single or combined application of Fertifol Misr and gibberellic acid compared to unspraying .There was a slight promotion on such characters with increasing Fertifol Misr concentration from 1.5 – 2.5 g/l. The best results with regard to yield and fruit quality were obtained due to spraying "Anna" apple trees three times with a mixture containing Fertifol Misr at 2.5 g/l and gibberellic acid at 20ppm. [Journal of American Science. 2010;6(12):202-208]. (ISSN: 1545-1003). **Key words:** Anna apple, foliar application, nutrients, gibberellic acid.	Full Text	24
25	**Anti-Ulcer Effect of Cinnamon and Chamomile Aqueous Extracts in Male Rats** Amr, A. Rezq* and Maysa, M. Elmallh Nutrition and Food Science Department, Faculty of Home Economics, Helwan University, Cairo, Egypt. *dr.amr_rezq@yahoo.com **Abstract:** Peptic ulcer disease is a problem of the gastrointestinal tract. Nowadays, drugs are expensive and have many side effects during treatment of any disorders. Therefore, our study aimed to investigate and compare antiulcer effect of cinnamon and chamomile aqueous extracts at doses of (100, 200, 300, 400mg/kg of body weight) with antiulcer drug (Zantac ™ Ranitidine). Fifty male rats weighing 160±5g were distributed into ten groups. Group I serves as a positive group. Group II serves as control group (treated with drug). Groups III, IV, V and VI were administered orally the different doses of cinnamon aqueous extract (CIAE). Groups VII, VIII, IX and X were administered orally the different doses of chamomile aqueous extract (CHAE). Values of pH and volume of gastric juice, ulcer area and curative ratio were estimated as well as histopathological examination of gastric. Results revealed that treatment with Zantac and CIAE or CHAE was associated with significant increase in the pH values compared to the respective value of the positive group. CHAE was superior to that of CIAE. Oral administration of CIAE or CHAE was associated with significant reduction in the volume of gastric juice compared to positive and control groups. Curative ratios of gastric ulcer were better in rats given CIAE or CHAE over those given Zantac. Furthermore, CHAE was superior over CIAE in its curative ratios of gastric ulcer. Histological examination showed necrosis of gastric mucosa associated with congestion of submucosal blood vessels, submucosal edema and hemorrhage in the stomachs of positive rats. The stomachs of group receiving Zantac showed necrosis of gastric mucosa associated with hemorrhage. Whereas, higher dosages of CIAE	Full Text	25

	(300 and 400 mg/kg of body weight) and CHAE dosages i.e., 200, 300 or 400 mg/kg of body weight were efficient to arrest histopathological changes in the stomachs. Conclusion: our finding concluded that water extracts of cinnamon and chamomile had potential antiulcer effect, which was superior to the respective effect observed with Zantac. Chamomile extracts were more superior to cinnamon in its protection of the stomach. The antiulcer curative ratios were dose dependent with no adverse effects. [Journal of American Science. 2010;6(12):209-216]. (ISSN: 1545-1003). **Keywords:** Chamomile- Cinnamon-Peptic ulcer		
26	**Botanical Studies On Phaseolus Vulgaris L. Ii- Anatomy Of Vegetative And Reproductive Organs** 1 Rania M. A.Nassar, 2 Mohamed S. Boghdady and 3 Yasser M. Ahmed 1- Department of Agricultural Botany, Faculty of Agriculture, Cairo University, Giza, Egypt. 2- Department of Agricultural Botany and Plant Pathology, Faculty of Agriculture, Zagazig University, Egypt. 3- Department of Vegetable Crops, Faculty of Agriculture, Cairo University, Giza, Egypt. **Abstract:** The present study is concerned with the histological features of Kidney bean plant. The anatomical structure of different vegetative and reproductive organs was investigated fortnightly throughout the whole growing season. Studied organs included main root, main stem (represented by apical and median internodes), different types of foliage leaves developed on the main stem and on lateral shoot; including lamina and petiole, flower bud, fruit and seed. Histological features of various organs of Kidney bean plant were analyzed microscopically and photomicrographed. [Journal of American Science. 2010;6(12):217-229]. (ISSN: 1545-1003). **Keywords:** Phaseolus vulgaris L., Kidney bean, Fabaceae, Anatomy, Vegetative organs, Reproductive organs	Full Text	26
27	**Efficacy of Intercropping Mango, Mandarin or Egyptian Clover Plants with Date Palm on Soil Properties, Rhizospere Microflora and Quality and Quantity of Date Fruits** *1H. F. H. Abouziena, 2Elham Z. Abd El-Motty, 3Youssef, R. A. and 4Sahab, A. F. 1Botany Department, 2Pomology Research Department, 3Soils and Water Use D Department, 4Plant Pathology Department, National Research Center, Dokki, Cairo, Egypt. *abouzainah@yahoo.com **Abstract:** Intercropping is claimed to be one of the most significant cropping techniques in sustainable agriculture; to its utilization a number of environmental benefits, from promoting land biodiversity to diversifying agricultural outcome. This model integrates low, medium, and tall plants, as well as plants of short, medium, and long life cycles, including trees. Therefore, a study was carried out to evaluate the impact of intercropping mango (Mangifera indica L.), Balady mandarin (Citrus reticulate Blanco) and Egyptian clover (Trifolium alexandrinum L) crops with date palm on soil chemical properties and quality and quantity of date fruits, in comparison with date palm sole. Rhisosphere of palm (pure stand) had a high concentration of N compared to palms intercropped with mango or mandarin. Intercropped mandarin with palms caused a depletion of N from soil by 14.3%, relative to date palm pure stand. High levels of Zn and Mn in soil were recorded in rhisosphere of clover and palms intercropped with mandarin. The effect of intercropping on occurrence and enumeration of microorganisms in the rhizosphere of trees was also studied. The results indicated that the colony count of fungi and bacteria in date palm rhizosphere were fluctuated according to plantation method. Intercropping date palm with mandarin decreased the total fungal count from 21.17 cfu x 103g-1 in the non- intercropped roots to 16.00 cfu x 103g-1 (24.4% decrease) in date palm root intercropped with mandarin. While, intercropping date palm with mango and clover increased the total fungal count to 118.32 cfu x 103g-1 and 52.00 x103g-1 in date palm root intercropped with mango and clover, respectively. Growing mango or mandarin under date palm resulted in the highest fruit yield/palm. However, intercropping Egyptian clover with date palm caused a significant reduction in fruit diameter. Intercropping mango gave the highest net profit ($8213/ha/yr), followed by the	Full Text	27

	same area intercropped with mandarin ($3992/ha/yr). Evaluation of growing mango, mandarin or Egyptian clover with date palm indicated that growing mango with date palm could be used for combating desertification in sandy soil in arid lands regions and gave the highest net return per unit area. [Journal of American Science. 2010;6(12):230-238]. (ISSN: 1545-1003). **Key words**: intercropping, date palm, Egyptian clover, mango, mandarin, fruit		
28	**Study the Effect of some Metallic Additives on the Physical Properties of the Commercial Pure Aluminum Metal** E.M.Sakr*1, A.Nassar2, N.Tawfik2 and M.Soliman2 1 Physics Department- Faculty of Girls for Arts, Science & Education- Ain Shams University, 2 Physics Division- National Research Center (NRC), Cairo, Egypt. *Elham.sakr@yahoo.com **Abstract:** The aim of the present work is to develop the 6201 alloy, which is the most used for conductor cables by adding different amount of Ce into Al-Mg-Si alloy namely (0.0, 0.024, 0.043, 0.054, 0.133, 0.166 and 0.194 wt% Ce) concentration. Sample alloys were homogenized by annealing at 540º C for various duration in range (½ to 5 hours), followed by water quenching. Tensile tests, hardness, electrical conductivity tests, microstructure characterization in Scanning Electron Microscope (SEM) have all been investigated as-cast and annealing. The results indicate that the alloys with Ce content make a more refined structure of grains and have higher tensile properties especially in range (0.043 to 0.054 wt% Ce) content and also hardly increase resistivity rather than the alloy which is free of cerium. [Journal of American Science. 2010;6(12):239-252]. (ISSN: 1545-1003). **Keywords:** Tensile test, hardness, electrical conductivity, microstructure characterization	Full Text	28
29	**The Role of some Natural Soil Conditioner and AM Fungi on Growth, Root Density and Distribution, Yield and Quality of Black Monukka Grapevines Grown on Calcareous Soil.** *1Mervat, S. Rizk-Alla and 2Hager, I. Tolba 1*Hortcultural Research Institute, 2Microbiology Dep., Soils, Water & Environment Research Institute, Agricultural Research Center, Giza, Egypt. **Abstract**: The current research was carried out during two successive seasons (2007 and 2008) on ten years old Black Monukka grapevines to disclose the role of some natural soil conditioners namely, humic acid (HA), Nile fertile (NF) and AM fungi (AM) in a single application or in combined mixture growth, root density and distribution, yield and quality of Black Monukka grapevines grown under calcareous soil in a private vineyard in Nobaria at Cairo-Alexandria Desert Road; the results showed that all different soil conditioners were effective but the treatment of humic acid at 15 ml/ vine (HA1) + Nile fertile at 200 g/ vine (NF1) + AM fungi gave the best results in comparison with other treatments and control. This treatment enhanced the growth characters namely total leaf area/ vine, shoot diameter and coefficient of wood ripening, total chlorophyll, NPK% of the leaves and total carbohydrates of the canes. Also, the vines of this treatment produced the heighest fibrous root fresh weight, larger number and longest fibrous root. With respect to microbiological activity in the rhizosphere, it was noticed that the best AM infection %, no. of AM spores /g dry soil, total microbial count, phosphatase and dehydrogenase enzymes activity were obtained by the same treatment. From the economic point of view, this treatment was accompanied by the highest yield and best its components namely physical and chemical characteristics of bunches and berries. Under such promising treatment the adverse effects of calcareous soil on growth and production of vines could be overcome. [Journal of American Science. 2010;6(12):253-263]. (ISSN: 1545-1003). **Keywords:** grapevine; humic acid (HA); Nile fertile (NF); AM fungi (AM); microbiological activity	Full Text	29
30	**Microbial Bio-Fertilization Approaches to Improve Yield and Quality of Washington Navel Orange and Reducing the Survival of Nematode in the Soil**	Full Text	30

	Abdelaal Shamseldin1; Mohamed H. El-Sheikh2; H. S. A. Hassan*3 and Kabeil S.S.4 1Environmental Biotechnology Department, 2Horticulture Department at Arid lands Cultivation and Development Institute (ARADI); Mubarak City for Scientific Research and Technology applications, New Borg-Elarab, Alexandria, Egypt, *3 Pomolgy Department, National Research center, Cairo, Dokki-Egypt. 4Protien Research Department at Genetic Engineering and Biotechnology Research Institute (GEBRI) Hsasm2000@yahoo.com **Abstract:** To test the ability of microbial strains Pseudomonas flourescence strain 843 and Azospirillum brasilense strain W24 to improve Washington navel orange fruit quality and to control the persistence of nematode in the soil, strains were applied one time monthly during the period of experiment to trees at two levels 300 ml and 500 ml per tree with 10-8 cells ml-1. Bio-fertilizer inoculation with strain Pseudomonas fluorescence strain 843 growth promoting rhizobacteria was significantly improve fruit quality as well as increased fruit yield, fruit weight, fruit length, TSS and juice volumes, while inoculation with strain Azospirillum brasilence strain W24 increase but not significantly improve fruit quantity and quality of Washington navel orange. Commonly, three types of nematode were detected in the roots including Tylenchulus Spp, saprophytic nematode and Pratylenchulus while the dominant species was Tylenchulus semipenetrans. Generally there is a reduction in the number of nematode with the two examined strains while the addition of Pseudomonas f. strain 843 was successfully greater to inhibit the growth of nematode than Azospirillum b. strain W24 suggesting that this strain can be use as a bio-fertilizer for promoting citrus growth and bio-control for reducing the distribution and propagation of nematode associated with citrus. Enhancement and maintenance of soil fertility and conservation of the soil's health through bio-fertilizer applications will be a vital role and occupy significant concern for many of researcher in the future as a unique key for sustainable agriculture in developing countries. [Journal of American Science. 2010;6(12):264-271]. (ISSN: 1545-1003). **Key words:** Citrus, Bio-fertilizers, Azospirillum brasilence, Pseudomonas fluorescence, Tylenchulus semipenetrans and biological control		
31	**Experimental Natural Prints And The Re-Calculated General Equations Of The Electrical Parameters For Buried Bare Pipe -Soil- Earth System With And Without Applying Cathodic Protection System]** Dr. Ashraf Abdel Raouf Mohamed Fouad Ahmed Canadian International College CIC – Egypt Ashrafahmed9000@yahoo.com **Abstract:** The rate of discharge through the stray electrolytic capacitor between the pipe and the remote earth is to be considered as the corrosion current. The electrochemical properties of the soil, which are the soil resistivity of the soil volume, the relative permittivity of the soil layer around the pipe and the chemical properties which could be considered as the pH of the soil film layer around the pipe, are affected directly by the humidity change. When considering the room temperature and by neglecting the effect of CO2 content in the soil, these values of the electrochemical properties of any soil returns back to its initial conditions after soil dryness to its initial condition. This means that corrosion rate will also be changed during the humidity change around the pipe segment. So, when considering the fact that the pipeline will not be changed or replaced and the surrounding medium around it will not be changed or replaced by another kind of soil, then the behavior of the electrical parameters (stray electrolytic capacitance, stray potential, surface created charge) of the pipe-soil-earth system will act as a print of this combination of this pipe and this soil. This paper recalculates the general form of the equations of the electric parameters and obtains the print curves & constants at natural condition with and without applying cathodic protection system in terms of the electrochemical properties around the pipe. The average error reduced to be less than ± 5%. This will help to study both the corrosion problem and cathodic protection by an electric concept with an electric analogue circuit which is the aim of this study. [Journal of American Science. 2010;6(12):272-283]. (ISSN: 1545-1003).	Full Text	31

	Keywords: Electrical study of pipe – soil – earth system		
32	**A Systematic Approach for Mobile Agent Design Based on UML (Class and Sequence Diagrams)** M. S. Al_Kholy, A. R. Khalifa and M. G. Alsaied Systems and Computer Engineering Department, Faculty of Engineering, Al-Azhar University, Cairo, Egypt **Abstract:** Agent researchers are still trying to determine useful ways to represent agents and agent-based systems. So, this paper presents a proposal for a Systematic Approach for Agent Design by using a Unified Modelling Language (UML) diagram. Here we illustrate notions for the behavior of an agent using and extending UML class diagrams. Focus on representing the agent migration from take requests and between other hosts. In this case study, we explain one variant of notation that is the most suitable for given scenario, show that it is easier to design agent applications based on agent UML, by developing software for our case study generated by UML software package. [Journal of American Science. 2010;6(12):284-290]. (ISSN: 1545-1003). **Keywords:** Mobile Agent Design, Class Diagram ,Sequence Diagram, UML, A Systematic Approach	Full Text	32
33	**EFFECT OF VANADIUM TOXICITY IN CLARIAS LAZERA** Mona S. Zaki[1]; Nevin E.Sharaf[2] and Mostafa H. Osfor[3] [1]Head of Department of Hydrobiology, National Research Center, Cairo, Egypt. [2]Department of Environmental and Occupational Medicine, National, Research Center, Cairo, Egypt . [3]Department of Nutrition, National, Research Center, Cairo, Egypt dr_mona_zaki@yahoo.co.uk **Abstract:** The effect of dietary carbohydrates and vanadium toxicity on haematalogical profile, blood chemistry and hormonal level was studied in cat fish Clarias Lazera. Fish were divided into 3 groups (n=10) and exposed to different doses of vanadium sulfated and carbohydrate. Group1 was served as control, group 2 was fed with carbohydrate and vanadium sulfate (10 mg/ Kg diet ration), group 3 was fed with carbohydrate and vanadium sulfate (15 mg/Kg diet ration). There is a significant decrease in hemoglobin and P.C.V in group (3). There is a significant increase in serum cortisol, cholestrol, AST, ALT, urea, creatinine and alkaline phosphatase in group (3), also there is a significant decrease in serum phosphorous, sodium and potasium in treated fish. There is a significant high level of vanadium content in kidney muscles, heart and spleen in group (3) suggesting toxic effects of vanadium on cat fish Clarious Lazara. The total viable count of bacteria identified higher in fish fed on carbohydrate vandium. Predominate bacteria were identified as Aeromonas, E. coli, Staph aureus. Pseudomonas, Fluorscences and Lacto bacilus species. We emphasize the finding that increase in carbohydrate concentration causes harmful pathological effects which reduces humoral immune responses and enhances dietary vanadium toxicity. [Journal of American Science. 2010;6(12):291-296]. (ISSN: 1545-1003). **Keywords:** Clarias Lazera, Vanadium Pollution, Haematological, Biochemical, Clinicopathological, Bacterial count.	Full Text	33
34	**Cubic Nonpolynomial Spline Approach to the Solution of a Second Order Two-Point Boundary Value Problem** W.K. Zahra, F.A. Abd El-Salam, A.A. El-Sabbagh and Z.A. ZAki* 1Department of Engineering Mathematics and Physics, Faculty of Engineering, Tanta University, Tanta, Egypt	Full Text	34

	2Department of Engineering Mathematics and Physics, Faculty of Engineering, Benha University, Shoubra, Cairo, Egypt. Zahmed_2@yahoo.com* **Abstract:** Third and fourth order convergent methods based on cubic nonpolynomial spline function at midknotes are presented for the numerical solution of a second order two-point boundary value problem with Neumann conditions. Using this spline function a few consistency relations are derived for computing approximations to the solution of the problem. Convergence analysis of these methods is discussed two numerical examples are given to illustrate practical usefulness of the new methods. [Journal of American Science. 2010;6(12):297-302]. (ISSN: 1545-1003). **Keywords:** Cubic nonpolynomial spline; two-point boundary value problem; Neumann boundary conditions		
35	**The Numerical Solution of Linear Third Order Boundary Value Problems using Nonpolynomial Spline Technique** F.A. Abd El-Salam, A.A. El-Sabbagh and Z.A. ZAki* Department of Engineering Mathematics and Physics, Faculty of Engineering, Benha University, Shoubra, Cairo, Egypt. Zahmed_2@yahoo.com* Abstract: Second and fourth order convergent methods based on Quartic nonpolynomial spline function are presented for the numerical solution of a third order two-point boundary value problem. The proposed approach gives better approximations than existing polynomial spline and finite difference methods and has a lower computational cost. Convergence analysis of the proposed method is discussed; two numerical examples are included to illustrate the efficiency of the method. [Journal of American Science. 2010;6(12):303-309]. (ISSN: 1545-1003). Keywords: Quartic nonpolynomial spline; third order two-point boundary value problem; convergence analysis, finite difference	Full Text	35
36	**The Numerical Solution of Linear Fourth Order Boundary Value Problems using Nonpolynomial Spline Technique** F.A. Abd El-Salam and Z.A. ZAki* Department of Engineering Mathematics and Physics, Faculty of Engineering, Benha University, Shoubra, Cairo, Egypt. Zahmed_2@yahoo.com* **Abstract:** In this paper we develop a class of accurate methods based on quartic nonpolynomial spline function at midknots for the numerical solution of a fourth order two point boundary value problems associated with plate deflection theory. Using this spline function a few consistency relations are derived for computing approximations to the solution of the problem. Existing second and fourth order finite difference and spline functions based methods developed at midknots become special cases of the new approach. Convergence analysis of the proposed method is discussed. Two numerical examples are included to illustrate the practical usefulness of our method. [Journal of American Science. 2010;6(12):310-316]. (ISSN: 1545-1003). **Keywords:** Quartic nonpolynomial spline; two point boundary value problem; plate deflection theory; convergence analysis.	Full Text	36
37	**Lambda, the pyrethroid insecticide as a mutagenic agent in both somatic and germ cells.** Abdel Aziz K.B. and Abdel Rahem H.M. Cell Biology Department, National Research Center, Cairo, Egypt.	Full Text	37

	k.badrakhan.yahoo.com **Abstract:** Cytogenetic evaluations of pyrethroid insecticide cyhalothrin (lambda) were investigated in mice *in vivo* by recording chromosomal aberrations in bone marrow cells and in primary spermatocytes. Cyhalothrin (lambda) insecticide was orally administrated with 2, 2.5, 5 mg/kg b.wt. (1⁄10, 1⁄8, 1⁄4 LD50 doses respectively) for repeated treatment. Cyhalothrin (lambda) was found to produce a significant structural and numerical chromosomal damage after subacute treatment in both bone marrow cells and primary spermatocytes. This effect was dose and time-dependent. For studying sperm abnormalities, mice were orally treated with the highest dose, 1⁄4 LD50. Cyhalothrin (lambda) insecticide was found to induce a significant increase in the percentage of sperm abnormalities which was mainly in the head. The present study clearly indicates that Cyhalothrin (lambda) insecticide is genotoxic to the different kinds of cells analyzed. Accordingly, much more care should be taken during the use of these pesticides. [Journal of American Science. 2010;6(12):317-326]. (ISSN: 1545-1003). **Keywords:** Pyrethroid insecticides; Lambda-cyhalothrin; chromosomal aberrations; Sperm abnormalities; genotoxicity		
38	**Chlorophyll-a dynamics in relation to environmental parameters in a tropical lagoon** [1]P. C. Onuoha, [2]D.I. Nwankwo and [3]Vyverman, W. [1]Department of Fisheries and Marine Biology, Federal College of Fisheries and Marine Technology, Bar-beach Victoria Island, Lagos Nigeria. E-mail- hydro_vision@yahoo.com [2]Department of Marine Sciences University of Lagos, Akoka, Lagos, Nigeria [3]Protistology and Aquatic Ecology Research Laboratory, University of Ghent, Belgium **Abstract:** The chlorophyll-*a* dynamics and environmental factors of the Ologe lagoon, Lagos were investigated for 2 years (Feb., 2002 – Jan., 2004). The environmental indices reflected seasonal changes related to rainfall distributive pattern and tidal seawater incursion. Air temperature (27-34 ^{0}C), surface water temperature (25-32^{o}C), transparency (24-76cm), total dissolved solids (48-294mg/l), salinity (0-0.5‰), conductivity (83-631µS/cm), pH (5.8-8.1), total alkalinity (42-162mg/l), biochemical oxygen demand (0-28mg/l), chemical oxygen demand (6-39mg/l), total hardness (62-342mg/l), cations, and heavy metals recorded increasing values in the dry season than the wet months, while dissolved oxygen (7-12.7mg/l), total suspended solids (7-378mg/l), nitrate-nitrogen (0.02-1.02mg/l), phosphate-phosphorus (0.03-1.79mg/l) and silicate (2.05-9.54mg/l) had higher values in the wet season than the dry season. Estimation of phytoplankton biomass by chlorophyll-*a* concentration ranged from 0.1 to 64.5ug/l with mean value of 16.99ug/l. Values for chlorophyll-*a* were higher in the dry than wet season for the lagoon. Analysis, using Pearson correlation co-efficient recorded positive relationship between chlorophyll-a values and air temperature, surface water temperature, salinity, conductivity, total dissolved solids, pH, transparency, biochemical oxygen demand, chemical oxygen demand, alkalinity, total hardness and cations. Analysis using ANOVA showed significant differences in the sample means of physico-chemical parameters of effluent discharge station (OL4) and the other stations within the lagoon at 5% level of probability. Recorded chlorophyll-*a* values placed the Ologe lagoon between the mesotrophic and eutrophic status. It is suggested that increasing tidal influence associated with reduction in rain events may have encouraged elevated salinities and created conditions for the development of more algal cells, hence higher chlorophyll *a* records. [Journal of American Science 2010;6(12):327-337]. (ISSN: 1545-1003). **Keywords**: Chlorophyll-a, environmental factors, mesotrophic, eutrophic, Ologe	Full Text	38
39	**Effect of Mineral, Organic Nitrogen Fertilization and some other Treatments on Vegetative Growth of Kalamata Olive Young Trees.** Hassan, H. S. A[*1]; Laila, F. Hagag[1]; H. El-Wakeel[2]; M. Abou Rawash[2] and A. Abdel-Galel[1] [1]Pomology Department, National Research Center, [2] Dept. of Hort. Fac. of Agric. Ain Shams Univ.,	Full Text	39

	Shobra El-Khiema, Cairo, Egypt *Hsasm2000@yahoo.com **Abstract:** This study was carried out through two successive seasons (2007& 2008) on Klamata olive young trees grown at the Research Station Farm of National Research Center, El Nobarya, El Behera governorate. The investigation aimed to study the effect of applying mineral, organic fertilizers and some other treatments on growth parameters at the first two years of planting. Planting holes were prepared for control plants in the first season only. Each treatment received 100 g actual nitrogen/plant/year as recommended by M.A.R.L. (2007). The following treatments were applied: T1 : control (mineral nitrogen + planting hole preparation), T2(100%mineral nitrogen), T3(100% organic N as cattle manure), T4(50% mineral N + 50% organic N as chicken manure), T5 (100%mineral nitrogen + humic acid as soil application), T6(100% mineral nitrogen + activated dry yeast as soil application), T7 (100%mineral nitrogen + GA_3 spray) and T8 (100% mineral nitrogen + sea algae as soil application).At the end of each season, plant height, stem diameter, lateral shoot number, lateral shoot length, leaves numbers per plant, percentage of plant height increment, whole plant dry weight were determined and recorded. The obtained results revealed that plant height, shoots number, shoot length, leaves number and stem diameter were not affected by different treatments. However the fifth treatment with humic acid and seventh treatment with GA_3 spray gave highest significant values of leaf numbers per plant compared with all other treatments in the first season, but in the second one, the differences among treatments lake significance. As for Whole plant dry weight, no significant differences among treatments could be noticed in both seasons. [Journal of American Science 2010;6(12):338-343]. (ISSN: 1545-1003). **Keywords:** Klamata olive; mineral fertilizer; organic fertilizer; growth parameter; plant		
40	**The Proposed Electric Circuit Diagram Of The Buried Bare Pipe Segment- Soil - Earth System With And Without Applying Cathodic Protection System** Dr. Ashraf Abdel Raouf Mohamed Fouad Ahmed Canadian International College CIC – Egypt. Ashrafahmed9000@yahoo.com **Abstract:** Based on proposed electrical concept of corrosion process, it is possible to simulate buried bare pipe segment with the surrounding soil medium by an electric circuit where the circuit electric quantities are function of the electrochemical properties of the soil as 4^{th} degree polynomial equations. The equivalent cylindrical electrolytic capacitor between the pipe and the remote earth and the potential across it, verifies the equation that charge $Q = C \times V$ at natural condition with & without applying cathodic protection system. The created positive charges consists with an equivalent negative charge (electrons losses) a charged stray electrolytic capacitor between the pipe and the earth through thin film soil layer around the pipe as cylindrical capacitor. The amounts of these charges are depending on the electrochemical properties of the soil which are surrounding the pipe segment, the length of the pipe segment and its diameter. The rate of discharge (equivalent to capacitor self discharge) is to be considered as the corrosion current. That's beside the facts deduced before that all electrical parameters prints & equations are function of the electrochemical properties of soil medium around the pipe at different cathodic protection levels. The error of these new equations of the electrical parameters reduced to be less than ± 5%. This will help to study both the corrosion problem and cathodic protection for a complete pipeline by an electric concept with an electric analogue circuit which is the aim of this study. This will help, in the future, in the choice of pipeline route, pipeline cathodic protection design and cathodic protection maintenance process for the pipe line along its route, however long it is. [Journal of American Science 2010;6(12):344-354]. (ISSN: 1545-1003). **Keywords:** Electrical study of pipe – soil – earth system	Full Text	40
41	**Molecular Markers for New Promising Drought Tolerant Lines of Rice under Drought Stress *via* RAPD-PCR and ISSR Markers** Youssef; M. A., Mansour A. and Solliman S. S Genetics Department, Faculty of Agriculture, Zagazig University, Zagazig, Egypt,	Full Text	41

	bakr2000us@yahoo.com **Abstract:** Random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) and inter simple sequence repeats (ISSRs) markers were performed to detect the genetic diversity among 6 new rice lines and 4 cultivars with different responses to drought tolerance and establish specific DNA markers associated with drought tolerance. Among 16 RAPD primers tested, only 5 produced bands polymorphic between lines with an average of 5.2 bands per primer (ranging from approximately 252 to 1232 bp) and 73.02 % were polymorphic. Among the tested ISSR primers, only five amplified polymorphic ISSR loci with an average number of 4.4 bands per primer (ranging from approximately 80 to 813 bp) and the mean percentage of ISSR polymorphism was 90.91. Based on band polymorphisms generated by RAPD-PCR and ISSR after using the primers, the highest similarity value (0.93) was found between P-5-3-b line and P-5-3-a line and the lowest value (0.44) was found between P-5-3-b line and Giza 172. The dendrogram separated all cultivars and new lines into two clusters and indicated that the cross of tolerant line (P-5-3-b) and susceptible cultivar (Giza 172) is suggested as the most suitable cross for drought tolerance analysis studies as they have the lowest similarity value (0.44) and also grouped in distinct cluster. Since two fragments of about (315 and 505 bp) were visualized using HP15 primer in the genomic DNA of the drought tolerant lines while were absent in the sensitive cultivars, they can be considered as positive drought tolerant markers. [Journal of American Science 2010;6(12):355-363]. (ISSN: 1545-1003). **Key words:** RAPD-PCR, ISSRs, rice, drought stress, dendogram		
42	**Genetic Evaluation and Molecular Markers for Heat Tolerance in Tomato (*Lycopersicon esculentum* Mill.)** M.A. Kamel*[2], S.S.Soliman[1], A.E. Mandour[1] and Mahassen S. S. Ahmed[1] [1]Genetics Department, Fac. Agric., Zagazig University, Egypt, [2] Samtrade trials station, Samtrade, Samir Fahmy Group, Egypt. *kamel_moh77@yahoo.com **Abstract:** Genetic evaluation was performed on twenty three genotypes of tomato (*Lycopersicon esculentum* Mill.) under high temperature at summer season to determine the variation between them for heat tolerance. Heat tolerance related criteria, i.e., pollen viability, fruit setting, osmotic pressure and fruit yield per plant. LSSS1, Homestead 24, Black Russian Plum, Super Marmand and Money Maker possess more tolerance of heat. In contrast, Super Stain B, Castle Rock, Cherokee Purple, Moskvich and Nicholevna Pink were more susceptible of heat. The pollen grain viability and fruit setting criteria consider as suitable morphological markers for heat tolerance than other heat tolerant related criteria as osmotic pressure. Heritability was high and moderately whereas, the genetic improvement of new strains could be done. From previous evaluation, Lsss1 as tolerant line and Super Strain B as sensitive cultivar of heat tolerance was crossed for study of molecular markers related to heat tolerance by using bulk segregant analysis (BSA). Crossing was carried out between these two genotypes to obtain the F_1 seeds which were left for selfing to obtain the F_2 seeds. The two selected genotypes, their F_1 and F_2 plants were evaluated for their response to heat stress by recording some heat stress related traits. Bulk of the two extremely F_2 plants (most tolerant and most sensitive F_2 groups), the two contrasting parents and their F_1, were used to develop some molecular genetic markers associated with heat tolerance in tomato by using ten RAPD and seven ISSR primers. two RAPD markers (with molecular sizes of 100 bp for primers A16 and 500 bp for primer Z13)and one ISSR marker(with molecular size of 650 bp) were considered as reliable markers for heat tolerance as well as susceptible genotypes possessed eight RAPD markers (with molecular sizes 500 and 1500 bp for primer C02, 1750 and750 bp for primer C03, 2400 bp for primer C05, 550 bp for primer C08, 400 bp for primer C14 and 850 bp for primer C15). [Journal of American Science 2010;6(12):364-374]. (ISSN: 1545-1003). **Keywords:** Tomato, Heat stress, Heat related traits, Molecular markers, RAPD-PCR, ISSR-PCR. Bulked segregant analysis (BSA), Marker assisted selection (MAS)	Full Text	42
43	**The Risk of Primary Open Angle Glaucoma and Glutathione S-Transferase M1 and T1 Polymorphism among Egyptians** Hoiyda A Abdel Rasool[1], Shahira Riad Nowier[2], Moataz Gheith[3], Ahmed T.S. Saif [4] and Somaia Ismail[5]	Full Text	43

	Dept. of Clinical and Chemical Pathology, Fayoum University[1], Dept. of Genetics, Research Institute of Ophthalmology[2], Dept. Ophthalmology, Research Institute of Ophthalmology[3], Dept. Ophthalmology, Fayoum University[4] Dept. Medical genetics, National Research Center[5], Cairo, Egypt **Abstract:** Purpose: Glaucoma, the second leading cause of blindness, is characterized by changes in the optic disc and visual field defects. The elevated intraocular pressure was considered the prime factor responsible for the glaucomatous optic neuropathy involving death of retinal ganglion cells and their axons. Extensive investigations into the pathophysiology of glaucoma now reveal the role of multiple factors in the development of retinal ganglion cell death. Genetic factors and oxidative damage have been shown to have a role in the development of primary open angle glaucoma (POAG). Glutathione S-transferases (GSTs) are a family of enzymes that inactivate xenobiotics and endogenous end products formed as secondary metabolites during oxidative stress. In humans, GSTT1 and GSTM1 deletion genotypes are associated with a variety of pathologic processes including certain ophthalmologic diseases. The aim of this study was to determine the effects of genetic polymorphisms of glutathione S transferase GSTM1 and GSTT1 on the risk of POAG in an Egyptian population. Methods: We compared the prevalence of GSTT1 and GSTM1 deletion genotypes, which were determined by multiplex polymerase chain reaction, in 32 patients with primary open angle glaucoma to 16 age, sex, and ethnically matched controls. Results: The GSTM1 positive genotype had an increased risk of developing POAG ($p< 0.05$, OR 4.681, 95% CI 1.190 – 18.412). The risk of glaucoma also increased significantly in subjects with a combination of GSTM1 positive and GSTT1 null genotypes ($p< 0.05$, OR 4.700, 95% CI 0.959 – 23.033). Conclusion: The GSTM1 positive genotype or the combination of both GSTM1 positive and GSTT1 null genotypes may be associated with the increased risk of development of POAG in the Egyptian population. The overall results indicate a possible variable association between various GSTT1 and GSTM1 genotypes and primary open angle glaucoma. Decreased GST function might interfere with the metabolism of oxidative intermediates and exacerbate the direct or indirect damaging effects of oxidative stress on the optic nerve. It is possible that these GST polymorphisms may be risk factors for primary open angle glaucoma [Journal of American Science 2010;6(12):375-381]. (ISSN: 1545-1003). **Keywords:** Glaucoma; optic disc and visual field defects; primary open angle glaucoma (POAG); Glutathione S-transferases (GSTs)		
44	**Determination of milk urea nitrogen for the Egyptian cattle fed the summer and winter diets.** Ahlam El Shewy; Sobhy Kholif; Tarek Morsy Dairy Sci. Dept. National Research Center, Dokki, Giza, Egypt. ahlam58aa@yahoo.com **Abstract:** Milk urea nitrogen (MUN) equilibrates with and is proportion to blood urea nitrogen. So, it is an excellent indicator of urea nitrogen status in dairy cows. The objective of this study was to determine the MUN during the summer (with a temperature range of 35-40°C) and winter (with a temperature range of 18-22°C) seasons. Forty hetero- parity lactating cattle twenty of each cows and buffaloes, at different stages of lactation were used to collect milk samples. All animals received the diet consisting of concentrate, fodder, and rice straw as 2:1:1 on DM basis. The fodder was berseem(*Trifolium alexandrium*) and rayana corn(*Zea mays mexicana*) in the winter and summer, respectively. The dietary crude protein was 11.38 and 8.97 % and the dietary gross energy was 3.86 and 3.83 Mcal/kg DM for the winter and summer diets, respectively. The results indicated, milk protein content was 3.06 and 3.18 % and MUN was 24.57 and 28.00 mg/dl for cows, while milk protein was 3.96 and 2.67 % and MUN was 19.60 and 28.03 mg/dl for buffaloes during the winter and summer seasons, respectively .This study revealed that the heat- summer significantly ($P<.05$) increased MUN of lactating buffaloes and this phenomenon needs further studies. [Journal of American Science 2010;6(12):382-384]. (ISSN: 1545-1003). **Keywords**: dietary protein, cow, buffaloes, milk urea nitrogen	Full Text	44
45	**In Vitro Propagation of *Tylophora indica*-Influence of Explanting Season, Growth Regulator Synergy, Culture Passage and Planting Substrate** Sulekha Rani, J S Rana*	Full Text	45

	Department of Bio & Nano Technology, Guru Jambheshwar University Of Science & Technology Hisar, Haryana (India) -125001, jogenderrana@yahoo.co.uk **Abstract:** An efficient protocol for rapid clonal propagation of an endangered medicinal plant, *Tylophora indica* (Burm. *f.*) Merrill through *in vitro* culture is described. High frequency bud break (85%) and multiple shoot formation were induced from nodal segments explanted between September through November and cultured on MS medium supplemented with 2.0mg/l BAP. Although callus- free multiple shoot formation was a function of cytokinin activity alone, faster bud break coupled with enhanced frequency of shoot development (95%) and internode elongation were dependent on the synergistic effect of GA_3(0.2mg/l). By repeated sub culturing of nodal segments harvested from the newly formed axenic shoots, prolific shoot cultures, free of proximal callusing, showing a high frequency multiplication rate were established within three months. The percentage shoot multiplication as well as the number of shoots per node attained the highest values (100%, 7 shoots/node) during the first two culture passages; beyond this there was a gradual decline in shoot bud differentiation. Rooting of the excised shoots from secondary or subsequent cultures was best induced on ½ strength MS medium containing 0.5 mg/l IBA. Vermi-compost was the most suitable planting substrate for hardening and its use ensured high frequency survival (96%) of regenerated plantlets prior to outdoor transfer. Regenerated plants get established in pots containing garden soil followed by their transfer to natural soil under full sun. The *in vitro* regenerated plants were uniform and identical in growth characteristics and morphology to the donor plants. [Journal of American Science 2010;6(12):385-392]. (ISSN: 1545-1003). **Keywords:***Tylophora indica,* medicinal plant, micropropagation, culture media, explants, growth regulators		
46	**Kinetic and Electrolytic Conductivity of C.I. Acid Orange 15 and C.I. Acid Red 97 dyes in Different Media** A.M. Gamal Chemistry Department, Faculty of Science, Al-Azhar University (Girls), Nasr City, Cairo, Egypt **Abstract:** The electrolytic conductivities for Acid Orange 15 and C.I. Red 97 have been studied. The electrolytic conductivities have been analyzed by Deby HUckel-Onsager theory, The degree of ionization, α, Van't Hoffs factor, i, and thickness of ionic atmosphere, X^{-1}. were calculated. Each value diminishes with increasing dye concentration. The dye anion transport number, t, mobility's, μ were also computed at infinite dilution. The results provide evidence for the presence of interionic attraction and association. Furthermore the kinetics of two acid dyes has been studied using spectrophotometric and conductimetric methods. The former study was carried out at 28°C at different percentage of solvents. The results revealed that the reaction rate was govemed by a pseudo-first order. [Journal of American Science 2010;6(12):393-399]. (ISSN: 1545-1003). **Keywords :** C.I. Acid Orange 15, C.I. Acid Red 97, solvents, electrolytic conductivity, spectrophotometry, kinetics	Full Text	46
47	**Adsorption of Cadmium (II) and Mercury (II) onto Natural Adsorbent Rice Husk Ash (RHA) from Aqueous Solutions: Study in Single and Binary System** A.G. El-Said, N.A. Badawy, and S.E. Garamon Chemistry Department. Faculty of Science, Al-Azhar University (Girls), Nasr City, Cairo, Egypt **Abstract:** The present study deals with the competitive adsorption of cadmium (Cd(II)) and mercury (Hg(II)) ions onto rice husk ash (RHA) from single component and binary systems. Equilibrium adsorption is affected by the initial pH (pH_0) of the solution. The $pH_0 \approx 6.0$ is found to be the optimum for the individual removal of Cd(II) and Hg(II) ions by RHA. The pH of the system, however, increases during the initial sorption process for about 60 min and, thereafter, it remains constant. The equilibrium adsorption data were obtained at different initial concentrations (C_0 = 10–100 mg/l), 6 h contact time, 25 °C	Full Text	47

	temperature, RHA dosage of 10 g/l at pH_0 6. The single ion equilibrium adsorption data were fitted to the non-competitive Langmuir and Freundlich isotherm models. The Freundlich models represent the equilibrium data better than the Langmuir model in the studied initial metal concentration range (10–100 mg/l). The adsorption capacity of Cd(II) is higher than that for Hg(II) for the binary metal solutions and is in agreement with the single-component adsorption data. The equilibrium metal removal decreases with increasing concentrations of the other metal ion and the combined action of Hg(II) and Cd(II) ions on RHA is generally found to be antagonistic. Equilibrium isotherms for the binary adsorption of Cd(II) and Hg(II) ions onto RHA have been analyzed by using Langmuir and Freundlich models.. Desorption with various solvents showed that the nitric acid is the best solvent; the maximum elution being about 28.41 % for Cd(II) and about 31.53 for Hg(II). [Journal of American Science 2010;6(12):400-409]. (ISSN: 1545-1003). **Keywords:** Mercury (II); Cadmium(II); Binary adsorption; Rice husk ash (RHA); Simultaneous metal removal; Adsorption isotherms		
48	**Impact of Gibberellic Acid Enhancing Treatments on Shortening Time to Budding of Citrus Nursery Stocks** HODA, M.MOHAMED; ABD EL-RAHMAN, G.F. and ABD EL-RAHEEM, M.E. Horticultural institute, Agricultural Research Center, GIza, Egypt. **Abstract:** Screen house experiment was conducted to study the application of gibberellic acid (GA_3) at different concentrations on budding shortening time of Volkamer lemon (*C.Volkameriana* Ten & Pasq) and Sour orange (*C.aurantium L.)* rootstocks in two seasons (2008-2009). Shortening the period to reach suitable diameter for budding seedling would benefit nurserymen by reducing various production inputs and their costs. The results indicated that, the highest success rate of suitable seedlings for budding was in mid-July. This time led to shortening the period for budding about 8 months, whereas, resulting seedlings could be budded because their stem diameter reached of a pencil size (5.4 mm) or larger. Also, this study revealed that, Volkamer lemon rootstock was superior as compared to sour orange rootstock in terms of vegetative growth, root distribution, leaf mineral content and percent of suitable seedlings for budding, while leaves of sour orange contained higher chlorophyll and total carbohydrate. It could be recommended to use T_5 (Soaked seeds and seedling treated with GA_3 at 200 ppm) for giving the best vegetative growth and suitable seedlings for budding in mid July. [Journal of American Science 2010;6(12):410-422]. (ISSN: 1545-1003). **Keywords:** Screen house; gibberellic acid (GA_3); lemon; vegetative growth	Full Text	48
49	**Prognostic Impact of Elevated Serum Hyulronic Acid, Ferritin and Interleukin-6 in Patients with Acute Myeloid Leukemia** **Nabila Abd El Maksoud,[1] Halla M. Ragab[1], Maha M. Abd El Latif[2] and Sh. Abdalla[3]** [1]Department of Biochemistry, Genetic Engineering and Biotechnology Division, [2]Department of research and applications of supplementary medicine, National Research Centre, and [3]Department of Clinical Pathology, El-Sahel Teaching Hospital, Cairo, Egypt . hmragab@yahoo.com **Abstract: Background:** Acute myeloid leukemia (AML) is a clonal disease of hematopoiesis with poor clinical outcome despite recent improvements in chemotherapy and stem cell transplantation regimens. It is the most common acute leukemia in adults. Hyaluronic acid, ferritin and Interleukin-6 are involved in the pathogenesis of acute myeloid leukemia , but their prognostic significance in these diseases is unknown. In the current study, the authors assessed the serum levels of these parameters in different stages of the disease to predict their prognostic value, which might therefore represent interesting target for immunotherapy in patients with different hematological malignancies. **Methods:** Serum levels of. hyaluronic acid, ferritin and Interleukin-6 were measured using a commercially available sandwich Enzyme Linked Immune Sorbent Assay (ELISA) kit in patients with AML who were attending for	Full Text	49

	treatment at National Cancer Institute, Cairo University from September 2006 through January 2009. **Results:** Newly diagnosed and relapsed patients with AML had significantly higher serum levels of hyaluronic acid, ferritin and Interleukin-6 compared with both control group and leukemic patients in remission stage. Serum levels of hyaluronic acid, ferritin and interleukin–6 in patients with AML (at diagnosis and at relapse) correlated inversely with the hemoglobin concentration. While their serum levels correlated positively with both total leukocyte count and with the % of blast cells in bone marrow in patients with AML. **Conclusions**: It could be concluded that serum levels of hyaluronic acid, ferritin and Interleukin-6 can be used as prognostic markers at diagnosis of adult AML and it could be used as follow up parameters for early detection of relapse. Furthermore, they might represent interesting target for immunotherapy in patients with different hematological malignancies. [Journal of American Science 2010;6(12):423-432]. (ISSN: 1545-1003). **Keywords:** Acute myeloid leukemia (AML), Hyaluronic acid (HA), Ferritin (Fe), Interleukin–6 (IL-6).		
50	**The Effect of Green, Roasted and Decaffeinated Coffee on Serum Glucose, Insulin and Serum Lipid Profile in Diabetic Experimental Animals** **Eman A.Sadeek[*1] , Hala, A. Abd El;-Rahman[2] and Waffa, Sh. Ali[3]** [1]Department of Biochemistry & Nutrition -Women's College –Ain –Shams University. [2] Food Tech. Res. Ins. Agric. Res. Center. [3]College of Home Economics, Helwan University. Cairo, Egypt [*]dr_emansadeek@yahoo.com **Abstract:** Aim of the work: Assessing the Effect of green, roasted and decaffeinated coffee on serum glucose, insulin and serum lipid profile in diabetic rat models. Methods: Design of the Study: Thirty female wistar rats weighing 124.5 ±5.41g (mean ±S.D) were divided into 5 groups. The first group served as a control and consumed a standard diet according to (AIN – 93). The other 4 groups were injected intraperitoneally with 105 mg / kg body weight of alloxan . One group was kept without further treatment and served as a positive diabetic control. Groups 3, 4, 5 consumed 5% green, roasted and decaffeinated coffee in drinking water, respectively. The feeding trial continued for four weeks. At the end of the experiments, the animals were sacrificed, blood samples were collected, and the liver, kidney, spleen and heart were separated, washed, dried and weighed. Laboratory investigations Consisted of serum glucose, insulin, calcium, phosphorus and complete lipid profile was determined to test the magnitude of antioxidant potential green, roasted and decaffeinated coffee. Results: The present study show a significant difference ($p < 0.05$) in body weight gain and food intake between all treatment groups , with non significant difference in water intake , relative weight of organs including liver , kidney , spleen and heart . the study also shows significant elevation ($p < 0.05$) in serum glucose and insulin in diabetic control group as compared to normal control group. This indicates uncontrolled hyperglycemia in alloxan diabetic rats. While consumption of green, roasted and decaffeinated coffee resulted in a decrease in serum glucose and insulin ($p < 0.05$) .There is a significant decrease ($p < 0.05$) in serum calcium and serum phosphorus in groups 3,4 and 5 fed green, roasted and decaffeinated coffee respectively indicating an association between coffee consumption and bone health. our results also shows that alloxan injection produced a significant increase($p < 0.05$) in serum total- cholesterol(TC); triacylglycerol (TAG); LDL-C ; VLDL-C and in LDL\ HDL ratio and TC \ HDL ratio however a significant decrease ($p < 0.05$) in serum HDL-C is observed ; In diabetic rats compared to normal control .green, roasted and decaffeinated coffee resulted in a significant decrease ($p < 0.05$) in triacylglycerol (TAG); LDL-C ; VLDL-C and in LDL\ HDL ratio and TC \ HDL ratio .on the other hand a significant increase ($p < 0.05$) in serum HDL-C is observed in green, roasted and decaffeinated coffee groups compared to diabetic rats compared to normal control with the highest value for green coffee .Non significant effect on serum total- cholesterol(TC) reported in this study. Conclusion: The observed improvement in glucose, insulin profile, triacylglycerol and HDL-C confirm the potent biological action of green, roasted and decaffeinated coffee and suggest that chlorogenic acid (a component in coffee) might have an antagonistic effect on glucose transport. Suggesting a novel function of coffee on lowering the risk factors of diabetes and delaying the progress of diabetes complications as well. [Eman A.Sadeek, Hala, A. Abd El;-Rahman and Waffa, Sh. Ali. The Effect of Green, Roasted and Decaffeinated Coffee on Serum Glucose, Insulin and Serum Lipid Profile in Diabetic Experimental	Full Text	50

	Animals. Journal of American Science 2010;6(12):433-441]. (ISSN: 1545-1003). **Keywords:** Green, roasted, decaffeinated coffee, glucose, insulin and lipid profile		
51	**Combined at-admission estimation of plasma gelsolin and injury severity score could predict the outcome of multiple trauma patients** Adel F. Al-Kholy [1], Mosad M Odah [1], Jehan Sabry [2], Ehab El-Shahat [3], Ehab Said [3] [1] Department of Medical Biochemistry, Faculty of Medicine, Benha University [2] Department of Clinical Pathology, Faculty of Medicine, Benha University [3] Department of Anesthesia & ICU, Faculty of Medicine, Benha University *adeladel59@yahoo.com* **Abstract**: To estimate plasma gelsolin levels in multiple trauma patients and its predictability for their outcome in relation to clinical data. The study included 70 multiple trauma patients and 20 healthy adult controls for blood donation as control group for the plasma level of gelsolin. All patients underwent history taking, time elapsed since trauma inflection and amount of external bleeding if present. Clinical evaluation included both Acute Physiology and Chronic Health Evaluation II (APACHE II) and Injury Severity Scores (ISS). Patients were evaluated daily throughout their ICU or hospital stay for the development of secondary morbidities and/or mortality. Venous blood samples were obtained at 12 hours after ICU admission for ELISA estimation of plasma gelsolin level. During hospital stay, 20 patients (28.6%) developed secondary morbidities and 8 patients (11.4%) died. Mean plasma gelsolin levels were significantly lower in patients compared to control levels with significantly lower levels in non-survivors compared to controls and survivors. Development of secondary morbidities showed a positive significant correlation with at admission ISS score and a negative significant correlation with plasma gelsolin. Survival rate showed positive significant correlation with plasma gelsolin level and negative significant correlation with both time since trauma inflection and ISS score. ROC curve analysis, defined prolonged time since trauma inflection as the significant sensitive predictor for both morbidity and mortality, while plasma gelsolin level was significant specific predictor for development of secondary morbidity and combined with ISS score were significant specific predictors for mortality. Conclusion: At admission plasma gelsolin level is a specific independent marker for prediction of the development of secondary morbidities that may progress to endanger patients' life and time since trauma inflection was found to be significant sensitive parameter for the patients' survival irrespective of development of these morbidities. [Adel F. Al-Kholy, Mosad M Odah, Jehan Sabry, Ehab El-Shahat, Ehab Said**. Combined at-admission estimation of plasma gelsolin and injury severity score could predict the outcome of multiple trauma patients**. Journal of American Science 2010;6(12):442-447]. (ISSN: 1545-1003). **Keywords**: Gelsolin, Trauma, Sepsis, Morbidity, Mortality	Full Text	51
52	**Serum levels of placental growth factor and retinol-binding protein-4 in pregnancy-induced hypertensive women** Adel F. Al-Kholy [1], Mamdouh Z. Abadier [1], Ebrahem M. Rageh [2], Hany El-Kallaf [3] [1] Department of Medical Biochemistry, Faculty of Medicine, Benha University [2] Department of Clinical Pathology, Faculty of Medicine, Benha University [3] Department of Obstetrics & Gynecology, Faculty of Medicine, Benha University *adeladel59@yahoo.com*	Full Text	52

	Abstract: To investigate the relationship between clinical parameters of pre-eclampsia (PE) and serum levels of Retinol binding protein4 (RBP4) and Placental growth factor (PlGF). Patients and Methods: The study included 90 pregnant women categorized as Group I: Control group (n= 20), included pregnant women who continued their pregnancy without development of PE manifestations, Group II: included patients had Mild PE (n=56) and group III included patients had Severe PE (n=14). After clinical evaluation and ultrasonographic examination, samples of maternal peripheral blood were obtained either at time of diagnosis of PE in groups II and III or at time of delivery in control group for ELISA estimation of serum RBP4 and PlGF. Results: PE patients had significantly lower serum PlGF, but significantly higher serum RBP4 levels when compared to the corresponding levels of the control group. Serum levels of PlGF showed negative correlation with systolic and diastolic blood pressures (SBP and DBP) and extent of proteinuria, but showed positive significant correlation with birth weight, while serum levels of RBP4 showed positive significant correlation with DBP, extent of proteinuria and patients' body weight measures. Conclusions: RBP4 and PlGF were associated with the development and severity of PE. [Adel F. Al-Kholy, Mamdouh Z. Abadier, Ebrahem M. Rageh, Hany El-Kallaf. **Serum levels of placental growth factor and retinol-binding protein-4 in pregnancy-induced hypertensive women**. Journal of American Science 2010;6(12):448-455]. (ISSN: 1545-1003). **Keywords**: Pre-eclampsia, Placental growth factor, Retinol-binding protein		
53	**Soil Plant Nutrients and Maize Performance as Influenced by Oilpalm Bunch Ash plus NPK Fertilizer** Ojeniyi, S. O.[1], Awanlemhen, B. E.[2] and Adejoro, S. A.[1*] [1.]Department of Crop, Soil and Pest Management, Federal University of Technology, PMB 704 Akure, Nigeria. [2.]Nigeria Institute for Oilpalm Research, Benin City, Nigeria. solomonadejoro@yahoo.com **ABSTRACT:** The work investigated the effects of combined application of oilpalm bunch ash (OPBA) with NPK fertilizer (NPK) on soil and plant nutrient content and maize performance at two sites in southern Nigeria. Six treatments: control, OPBA at 4 t/ha, NPK (15-15-15) at 300 kg/ha, 75% NPK + 25% OPBA, 50% NPK + 50% OPBA, 25% NPK + 75% OPBA were applied to maize at Nigeria Institute for Oilpalm Research (NIFOR) Benin and Ekiadolor in rainforest zone of Nigeria. Relative to control, other treatments increased soil organic matter (OM), N, P, K, Ca, Mg and pH, and plant nutrients content, growth and cob yield. The effects were generally significant except in case of OPBA alone. The NPK, 75% NPK + 25% OPBA and 50% NPK + 50% OPBA gave significantly high and similar values of the parameters. The treatments increased cob yield by 20 – 22%, OPBA most increased soil pH and K. [Ojeniyi, S. O, Awanlemhen, B. E, Adejoro, S. A. Soil Plant Nutrients and Maize Performance as Influenced by Oilpalm Bunch Ash plus NPK Fertilizer. Journal of American Science 2010;6(12):456-460]. (ISSN: 1545-1003). **Key words:** oilpalm bunch ash; nutrient; maize	Full Text	53
54	**The Protective Effect of White Ginseng against Biochemical and Pathological Changes Induced by Aflatoxins in Rats** Abdel- Fattah, Sh. M.[*1]; Sanad, M.I[2].; Safaa,M.A[2] and Ragaa F. F.Ghanem [1]Department of Food Toxins and Contaminants, Dokki, Cairo, Egypt.	Full Text	54

[2]Department of Biochemistry, Faculty of Agriculture, Mansoura University, Mansoura, Egypt.

shaabanmostafa@yahoo.com*

Abstract: The objective of the present study was to explore modification in toxico-pathological responses of rats toward aflatoxins (AF) in the presence of white ginseng. The dietary supplementation with white ginseng (WG) at levels of of 0.0, 1 and 2 % (W/W) of the composition of daily rations, on the performance and toxicity of female Albino rats received aflatoxins-contaminated diets (1.011 mg/kg ration, of dry matter basis), were successively examined for six weeks, as attempt to prevent or minimize the negative probabilities due to ingesting aflatoxin(s) contaminated food. Thirty native apparently healthy female Albino rats with average weight of 100± 3.4 gm., were put under observation for two weeks, then they were divided into five equal groups of six rats each according to their live body weight for performing feeding trials. An exposure study extended for two different stages was conducted using female Albino rats. The 1st stage (pre-treatment) was suggested to compare the performance of animal groupings under the normal conditions before receiving any treatment, either level of contamination(s) or dosage(s) of additive, such stage extended for 2 weeks. The 2nd stage (treatment), the animals received different levels of aflatoxin(s) and the food additive (white ginseng), such stage extended for 4 weeks. Rats treated with AF-contaminated diet alone showed depression, decrease in feed intake, body weight and loose feces. The activities of serum ALT, AST enzymes, which are reflecting liver function, were obviously affected during exposure to aflatoxins, but such levels came back to normal as the level of the WG in the ration increased. Serum urea and creatinine concentrations had also severed and such severe effects came back to moderate when receiving the proposed additive. Livers exhibited fatty change, necrosis and newly formed bile ducts. Lesions in kidney included tubular necrosis and pink homogeneous tubular casts. Rats fed white ginseng only had no significant differences compared to the negative control group (fed on a sole diet without any additives). A concurrent treatment of AF with white ginseng indicated a potentiation of the animal performance reflected by decreased severity of clinical signs and inccreased body weight gains. The studied food additive minimized and reduced significantly the deterioratsion of such performance which obviously observed in animal grouping received AF-contaminated diet. Female rats were responding to contaminated diets and to the food additive as well. Thus, our data strongly suggested that deleterious effects of AF could be overcome or, at least, significantly were diminished by WG. Moreover, this plant by itself did not show any toxic effects.

[Abdel- Fattah, Sh. M.; Sanad, M.I; Safaa,M.A and Ragaa F. F.Ghanem. **The Protective Effect of White Ginseng against Biochemical and Pathological Changes Induced by Aflatoxins in Rats**. Journal of American Science 2010;6(12):461-472]. (ISSN: 1545-1003).

Keywords: Ginseng; Aflatoxins; Histopathological changes; Food additives

55

Akhond Khorasani's Viewpoints towards the Modern Concepts of Freedom and Justice

Alireza Soroush[1] , Sarvinder Kaur Sandhu[2] , Hamed Alaei[3]

[1] Department of Government and Civilization Studies, Faculty of Human Ecology, Universiti Putra Malaysia,43300 Serdang, Selangor Darul Ehsan, Malaysia, Email: soroush_alireza@yahoo.ca

[2] Department of Government and Civilization Studies, Faculty of Human Ecology, Universiti Putra Malaysia,43300 Serdang, Selangor Darul Ehsan, Malaysia, Email: sarvinder@putra.upm.edu.my

[3]Graduate School of Management, Universiti Putra Malaysia,43300 Serdang, Selangor Darul Ehsan, Malaysia, Email: hamedala@yahoo.com

Abstract: This article seeks to understand the mentality of Akhond Khorasani, the leader of the Iranian constitutional revolution (1905-1911) regarding the political and social concepts such as freedom and justice. In the Iranian society, concepts such as freedom and justice were always affected by various kinds of understanding and comprehension. These concepts were never interpreted based on their original and true meanings which are essentially the principles of democracy. In other words, the Iranian society was faced some problems and difficulties in absorbing these concepts and it seems even nowadays these concepts do not possess their true meaning in the political social culture of Iran and everybody explain

Full Text

55

	them based on their own personal assumptions and subjectivity. It is for this reason that Iran has not had much of a practical experience from the existence of these concepts and achieving such and experience needs more time. Understanding the opinions of Akhond Khorasani (the revolution's leader) vis-à-vis these concepts can be an indication of the formation of democracy's pillar in Iran and also an indication of how the clergy faced these concepts, understand them and what practical ways they used to realize them. The theoretical framework of this article is based on the modernity theory. In essence, modernity comprises the theoretical aspects of the entire social, political, economical and cultural issues and guide human societies through the passageway of tradition to the modern world. The methodology used in this study is the unobtrusive research methodology, since this is a qualitative and historical research. The content analysis method which is one of the methods used in qualitative and historical researchers has also been implemented in this research. [Alireza Soroush, Sarvinder Kaur Sandhu, Hamed Alaei. Akhond Khorasani's Viewpoints Towards Modern Concepts such as Freedom and Justice. Journal of American Science 2010;6(12):473-479]. (ISSN: 1545-1003). **Keywords:** Iran; politics; modernity; freedom; justice; democracy		
56	**Chemical Composition and Potential Application of *Spirulina platensis* Biomass** Aly, M. S[*1] and Amber. S., Gad[2] Agriculture microbiology Dept, [2]Chemistry of Natural and Microbial products Dept., NRC, Cairo, Egypt. [*]mohamed_saad_1@hotmail.com **Abstract**: Submerged batch cultures, Semi -pilot scale cultivations and Outdoor biomass production were performed to increase Spirulina platensis biomass which is naturally grown in El Khadra lake water body. Comparing Chlorella vulgaris and Spirulina platensis showed higher protein contents of Spirulina as it reached 64 % (w/w) so, it may be used in agriculture as a nitrogen biofertilizer and as an animal and fish growth promoter. Bio-chemical analysis of Spirulina biomass showed presence of 17 amino acids, 10% (w/w) carbohydrates, 8 %(w/w) fibers and 8 %(w/w) lipids. The biomass of Spirulina contained 0.04 ppm Mg, 0.3 ppm Ca, 0.16 ppm Mn, .0.8 ppm Fe, 0.16 ppm Zn, 11.3 ppm Na, 0.003 ppm Se and 5.6 ppm K. It also contained 1 ppm Cu, 0.04 ppm Hg, 0.03 ppm Ni, 0.9 ppm Cr, 0.1 ppm Cd, and 0.6 ppm Co. [Aly, M. S and Amber. S., Gad. **Chemical Composition and Potential Application of *Spirulina platensis* Biomass**. Journal of American Science 2010;6(12):480-488]. (ISSN: 1545-1003). Key words**: *Spirulina platensi*,, El Khadra lake, biofertilizer**	Full Text	56
57	**Microbial load as Pollution Indicator in Water of El-Khadra Lake at Wadi El-Natroun, Egypt** Ali, M. S. [1]and Osman, G. A. [2] [1]Agriculture Microbiology Department, National Research Centre, Cairo, Egypt. [2]Bacteriology Lab., Water Pollution Research Department, National Research Center, Cairo, Egypt. [*]mohamed_saad_1@hotmail.com gamalosmanali2005@yahoo.com **Abstract:** Occurrence and survival of some classical bacterial indicators, (salmonellae group, total staphylococci and *Pseudomonas spp.)* in water samples at surface and one meter depth of El-Khadra lake have been studied as well as, cyanobacteria and fish lagoons were included for comparison. The results showed that, fecal streptococci and *Pseudomonas spp.* are not present in surface and deep lake water samples respectively, while other bacteria tested are presented. Similarly, salmonellae group and fecal coliform were absent in all water samples from the fish lagoon and the deep lake samples. In addition, the high and low log average counts of total viable bacteria incubated at 37 ºC for 24 hours were 7.5 and 3.4 /100m in cyanobacteria lagoon and surface lake water samples respectively. On the other hand, the high log average counts of total viable bacterial incubated at 22 ºC for 48 hours was 7.3 /100m in cyanobacteria	Full Text	57

	lagoon, while the low recorded 3.67 /100m in surface water samples. The statistical analysis (log average) showed that, some factors such as human activity, sun ray and sedimentation as well as biological activity play role on the bacterial distribution in all water samples tested. [Ali, M. S. and Osman, G. A. **Microbial load as Pollution Indicator in Water of El-Khadra Lake at Wadi El-Natroun, Egypt**. Journal of American Science 2010;6(12):489-496]. (ISSN: 1545-1003). **Key words:** Lake water, Classical bacterial indicators, Salmonellae group, Total staphylococci and *Pseudomonas spp*		
58	**Effect of Different Rates of Cobalt on some Macro-Micronutrients and Heavy Metals Contents in Lettuce under Different Types of Recently Reclamation Soils** **Abdel Fattah. M. S and Khaled. S.M.** Plant Nutrition Dept. National Research centre-Cairo, Egypt. **Abstract:** The main objective of this research work is to assess the influence of cobalt element addition on the uptake of some macronutrients (N, P and K) and some heavy metals (Cd, Ni and pb) in two different reclaimed soils. The first soil was sandy from (Abu- Rwash) region, the second soil was calcareous from (El Noboria) region. Cobalt was added with different rates (10, 15 and 20) ppm after plantation stage. Nitrogen was added by rate 100 ppm N at form amonium nitrate NH_4NO_3. Moreover, Dihydrogen potassium phosphate H_2KPO_4 at rate 200 ppm as source of phosphours and potassium was added at the same time. Lettuce plant of class (lactuca sativa var capitata). The obtained results can be summarized as follows: In sandy soil a positive connection between rates of cobalt and (N,P,K) contents, negative contact was found between cobalt concentrations and heavy metals contents (Cd, Ni, pb). Dry weight gave a positive contact with cobalt treatments, all differences were significantly to each of chlorophyll concentration and all trace elements contents except Mn were a positive relationship with cobalt treatments. All differences between treatments were significantly. In calcareous soil negative contact was found between rates of cobalt and nitrogen, while potassium a positive contact was found with phosphorus, concerning the heavy metals (Cd, Ni, pb) contents, positive contact was found with rates of cobalt. All this connections were significantly. Dry weight gave a negative connection with cobalt treatments but not significantly. Chlorophyll concentration and trace elements contents were in a positive relationship with cobalt treatments. All differences between treatments were significantly. Dry weight gave a negative connection with cobalt treatments but notsignificantly. Chlorophyll concentration and trace elements contents were in a positive relationship with cobalt treatments. All differences between treatments were significantly. [Abdel Fattah. M. S and Khaled.S.M. **Effect of Different Rates of Cobalt on some Macro-Micronutrients and Heavy Metals Contents in Lettuce under Different Types of Recently Reclamation Soils**. Journal of American Science 2010;6(12):497-502]. (ISSN: 1545-1003). **Key words:** Cobalt – lettuce plant – Sandy- Calcareous soil- Macronutrients – Heavy metals – Trace elements - Chlorophyll- Dry weight	Full Text	58
59	**Phenotypic Stability Analysis, Heritability and Protein Patterns of snake Cucumber Genotypes.** **AbdEl-Salam,M.M.M[1].; I.S. El-Demardash[*2], and A.H.Hussein[1]** [1]Dep. of vegetable – Hort. Res. Inst., Agric.Res. Center, Giza, Egypt. [2]National Research Center, Genetic Section, Giza, Egypt. [*]lola_El-Demardash@yahoo.com **Abstract:** Stability analysis was carried out for six traits in snakecucumber by growing 5 genotypes (1,2,3,4,5) collected from different regions of Egypt (Assiut,Ismialia, El-kalyoubia, Domiat and Fayoom) respectively, in 3 years at El-kassaseen region, Ismailia. Genotypes × environment interaction was significant for all studied traits; the linear component of genotype × environment interaction was significant for number of fruits plant, yield / Fadden and fruit shape index. Environments (linear) were significant for yield / plant, yield / Fadden, fruit diameter and fruit shape index . The linear regression on	Full Text	59

	environmental means (bi) close to unite with significant for genotypes (2,3,5,) for number of fruits / plant and (3,4,5,) for fruit diameter . Broad sense heritability was high for number of fruits / plant, yield / plant, fruit length and fruit shape index, but it was moderate for yield / Fadden and fruit diameter. The figure genotypes showed different patterns in presence of bands, the maximum number of band (6) in genotype (2) and the minimum number (3) was present in genotype (6), there are non resemblance between any genotypes, each genotype was characterized by a unique Finger print, except genotype (2) was monomorphic . [**AbdEl-Salam,M.M.M; I.S. El-Demardash, and A.H.Hussein. Phenotypic Stability Analysis, Heritability and Protein Patterns of snake Cucumber Genotypes**. Journal of American Science 2010;6(12):503-507]. (ISSN: 1545-1003). **Keywords:** Phenotypic Stability; Analysis; Heritability; Protein; snake; Cucumber; Genotype		
60	**Bio-removal of nitrogen from wastewaters-A review** Gaber Z. Breisha[1], Josef Winter[2] [1.] Department of Agricultural Microbiology, Faculty of Agriculture, Minia University, Minia, Egypt [2.] Institut für Ingenieurbiologie und Biotechnologie des Abwasser, Universität Karlsruhe, Germany gaberbresha@yahoo.com, Josef.Winter@iba.uka.de **Abstract:** If the present large volumes of nitrogen-containing wastewater of domestic and industrial origin are discharged into the environment without proper treatment, they lead to extensive soil and water pollution. Proper elimination of pollutants from these effluents is essential in industrialized countries and is becoming increasingly important from an environmental and human health point of view in developing and emerging countries. Beside the conventional nitrogen removal process (lithoautotrophic nitrification and denitrification), novel and cost-effective biological nitrogen elimination processes have been developed, including simultaneous nitrification and denitrification, anaerobic ammonium oxidation (Anammox), and its combined system (completely autotrophic nitrogen removal over nitrite, Canon). This review summarizes the recent studies dealing with agricultural, domestic and industrial wastewaters regarding their nitrogen content. Traditional and novel biological nitrogen elimination technologies are reviewed. Furthermore, recent studies dealing with temperature, dissolved oxygen, nitrate concentration, salinity, pH or the free ammonia concentration as factors affecting the nitrogen removal efficiency have also been incorporated. [Gaber Z. Breisha, Josef Winter. Bio-removal of nitrogen from wastewaters-A review. Journal of American Science 2010;6(12):508-528]. (ISSN: 1545-1003). **Keywords:** wastewaters; nitrogen removal; salinity; free ammonia; temperature; dissolved oxygen	Full Text	60
61	**Comparison of Electrostatic and Spinning-discs Spray Nozzles on Wheat Weeds Control** Ali Esehaghbeygi[1], Ali Tadayyon[2], Shahin Besharati[2] [1]Department of Agricultural Engineering, [2]Department of Agronomy and Plant Breeding College of Agriculture, Shahrekord University, Shahrekord, Iran, 115 esehaghbeygi@cc.iut.ac.ir **Abstract:** Electrostatic spraying is the method that is noted for improving the spraying efficiency and droplet deposition. The efficacy of electrostatic charge and spinning-discs spraying were assessed for the	Full Text	61

	application of 2, 4-D to control weeds in irrigated wheat. Sprayer nozzle performance was evaluated in terms of wheat grain yield (*Ghods* variety), weed shoot biomass, and wheat residual (straw) at the research farm of Shahrekord University in 2007 and 2008. The results indicated that electrostatic spraying gave better weed control. Spray penetration through dense weeds enhanced with electrostatic charging. The spinning disc nozzle decreased water use and so was cheaper to operate, but it did not significantly improve herbicide efficacy, especially in dense canopies compared with the electrostatic charge. [Ali Esehaghbeygi, Ali Tadayyon, Shahin Besharati. **Comparison of Electrostatic and Spinning-discs Spray Nozzles on Wheat Weeds Control.** Journal of American Science 2010;6(12):529-533]. (ISSN: 1545-1003). http://www.americanscience.org **Keywords:** electrostatic, spinning disc, wheat, weed		
62	**The Effect of work support and family support on Work- Family Conflict (W-FC) Among Married Female Nurses in Shiraz-Iran** Hajar Namayandeh, Siti Nor Yaacob, Rumaya Juhari Faculty of Human Ecology, University Putra Malaysia. h_nemayandeh@yahoo.com **Abstract:** The present study highlights the significance of work support (supervisor and coworker support) on work- family conflict. Furthermore, this paper also examines the effects of family support (husband and family members/relatives support) on work-family conflict. This study consists of 198 married female nurses in Shiraz-Iran. The findings revealed that reducing support from husband, family members/relatives and supervisor might increase perceived conflict between work and family. Unlike previous studies, the finding also indicates that there is no significant relationship between the respondents' support from co-worker with work- family conflict, which may be explained by the specific cultural context in Iran. Implications are discussed and recommendations are made regarding future researches in this area. [Hajar Namayandeh, Siti Nor Yaacob, Rumaya Juhari. **The Effect of work support and family support on Work- Family Conflict (W-FC) Among Married Female Nurses in Shiraz-Iran**. Journal of American Science 2010;6(12):534-540]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** Work- family conflict, Work support, Family support	Full Text	62
63	**Effect of using pectin on lead toxicity** Dalia, M. El-Nahal Special Food and Nutrition Dep., Food Technology Research Institute, Agric. Res. Center, Giza, Egypt daliaelnahal@hotmail.com **ABSTRACT:** Lead has many undesired effects on humans and animals, including neurological, behavioral, respiratory, visual, growth retardation, hematological immunological, renal, hepatic. **The aim of** the present study was to investigate the alterations in biochemical parameters in serum and blood due to lead retention in blood, organs and estimating the role of low and high esterified pectin in alleviating the negative effects of lead. **Material and Methods**: Sixty male *albino* rats which were divided into ten groups (6 rats for each). The first group (was fed on basal diet ;normal control). Groups 1,2 and 3 [which were fed on basal diet and administrated lead acetate (LA) daily once a time for 30 days by gavages at three different concentrations 61.94, 30.97 and 15.49 mg /Kg bw (1/4, 1/8, and 1/16 of lead acetate LD_{50};positive control]. Groups 4,5 and 6 [were fed on basal diet containing 10% low esterified pectin (LEP, DE 31%) and administered the same LA doses]. Groups 7,8 and 9 [were fed on basal diet containing 10% high esterified pectin (HEP, DE 73.5%) with the administration of the same LA doses]. **Results** obtained showed that LA significantly induced a decrease in body weight, serum total protein, albumin, globulin, total billirubin, direct billirubin, indirect billirubin, RBCs and WBCs counts, blood haemoglobin (Hb), heamatocrite values (PVC), serum triidothyronine (T3)and thyroxin (T4) levels. In the contrary, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AlP), gamma	Full Text	63

	glutamyl transferase (γ-GT) activities, serum urea, uric acid and creatinine were significantly increased in positive control rat groups. Additionally, treatment of rats with LA led to a considerable increase in accumulation of the metal in the blood, liver, kidney, brain, heart and bones compared with the normal group. LEP and HLP significantly decreased the effect of LA on the tested parameters and level of lead in different organs. Histopathological examination clearly indicated that LEP or HEP eliminated from the harmful effect of LA on liver, kidney and brain tissues. **In conclusion**, LEP and HLP have beneficial effects which could be able to antagonize lead toxicity. Moreover, LEP was contributed to fast elimination of the lead acetate to blood, organs and bones, whereas HEP removed lesser amount of lead. It could be recommended that LEP has a good effect to bind material of lead and should be incorporated into human food to reduce the hazards toxicity of lead pollution of food and water. [Dalia, M. El-Nahal. **Effect of using pectin on lead toxicity.** Journal of American Science 2010;6(12):541-554]. (ISSN: 1545-1003). http://www.americanscience.org. **Key words**: Lead toxicity, esterified pectin, Histopathological examination		
64	**Chemopreventive effect of celecoxib and expression of cyclooxygenase-2, Casapase-3 and AGNOR on chemically- induced rat submandibular salivary gland neoplasm.** **Mohamed Zayed** Lecturer, Oral Biology, Oral Histopathology Department, Misr International University (MIU). dr_zayed2@yahoo.com **Abstract: BACKGROUND:** Cyclooxygenase-2 (COX)-2 inhibitor (Celecoxib) is a non-steroidal anti-inflammatory drug (NSAIDs) and over-expression of COX-2 protein and mRNA has been reported in various cancer tissues. Therefore, it has been suggested that COX-2 is related to carcinogenesis. **METHODS:** Twenty five albino rats were used .They were divided into 3 groups; group I (normal control) and group II and III which was delivered 4-NQ in the drinking water .Meanwhile group III was given 1500 ppm celecoxib. Submandibular salivary glands were obtained after 32 weeks. Immuno-histochemical staining for COX-2 was performed to determine the COX-2 level and Caspase-3 immuno-expression was done for detection of apoptosis and silver nitrate staining of nucleolar organizer regions (AgNORs) was done for estimating the proliferating cells. The data were analyzed using Student's independent t-test and one-way analysis of variance (ANOVA). **RESULTS:** The group II and III showed pathological evidence of cancer. COX-2 immuno-staining was stronger in group II than in Group III. Caspase-3 immuno-reaction was statistically highly significant in group III ($p<0.05$) .Meanwhile proliferation estimated by AgNOR nuclear count was statistically highly significant group II ($p<0.05$). **CONCLUSION:** The COX-2 expression was increased in group II (untreated group) than group III. Administration of celecoxib demonstrated the chemo-preventive potential against the carcinogenesis through induction of apoptosis and suppression of tumor growth and proliferation. [Mohamed Zayed. Chemopreventive effect of celecoxib and expression of cyclooxygenase-2, Casapase-3 and AGNOR on chemically- induced rat submandibular salivary gland neoplasm. Journal of American Science 2010;6(12):555-363]. (ISSN: 1545-1003). http://www.americanscience.org. **Key words:** Oral cancer, Cyclooxygenase-2, submandibular salivary gland	Full Text	64
65	**The Outcomes of Concomitant Radiation Therapy plus Capecitabine for Refractory Locally Advanced Breast Cancer Patients Pre-Treated with Anthracycline Based Regimens** **Fatma Zakaria Hussen; Hanan Shawky Gamal El-Deen*; Amr Abd- El Aziz Ghanam; Samar Galal U** **and Omnia Abd –El-Fatah G.** Clinical Oncology Department, Faculty of Medicine, Tanta University Hospital, Tanta, Egypt	Full Text	65

hannshawky@yahoo.com*

Abstract: Purpose: Anthracycline based chemotherapy is the first line treatment for most of patients with locally advanced breast cancer (LABC). However, some patients fail to respond to these regimens and no established second line treatment. Effective treatments options for patients with LABC resistant to anthracyclins based regimens are limited. We have conducted a phase II trial of capecitabine concomitant with radiation therapy to assess the safety, tolerability and efficacy of this regimen as a second line for down staging those inoperable patients with LABC. Patients and methods: Between February 2008 and September 2009, 27 patients with infiltrating ductal carcinoma, locally advanced breast cancer , who were refractory to first line anthracycline based regimens were planed to receive radiation therapy (50Gy/25f) and concomitant capecitabine (850 mg/m^2) twice daily for 14 days every 3 weeks, at Clinical Oncology Department , Faculty Of Medicine, Tanta University Hospital. All patients were assessed for objective response rate (ORR), progression –free survival (PFS), overall survival (OS), safety and tolerability. Results: Eighty five percent of patients (23 out of 27) became operable. The remaining four patients didn't undergo surgery because of progressive disease. Objective response rates (ORR) including those with complete clinical response 0.0% and partial clinical response in 10 (37%) patients. A complete pathological response for primary tumor and axillary lymph nodes was seen in 1 patient (3.7%). Pathologically negative axillary lymph nodes were seen in 5 patients (18.5%). The median follow up period was 16 months (range 6-26 months), the median PFS for all patients was 10 months (range 2-22 months), the one –year PFS was 29%. The median OS was not reached, the mean OS was 20.8 months (95% CI 17.78 - 23.84) and the two-year OS rate was 69.5%. Positive significant correlations were observed for PFS in patients with age ≥ 45 years , postmenopausal , +ve estrogen receptors (ER), +ve progesterone receptors (PR), -ve human epidermal growth factor receptors (HER-2), non triple negative patients, patients with ER/PR positive tumors, non inflammatory breast cancer (IBC) patients and those with axillary lymph node ratio (ALNR) <50%. There were no grade 3 or 4 adverse events with study protocol. Conclusion: The results of this phase II trial prove that concomitant capecitabine and radiation therapy is safe and effective in down staging of inoperable locally advanced breast cancer patients resistant to primary anthracycline based regimens. We are ongoing trial to use capecitabine as a maintenance monotherapy in patients with advanced breast cancer.

[Fatma Zakaria Hussen; Hanan Shawky Gamal El-Deen; Amr Abd- El Aziz Ghanam; Samar Galal U and Omnia Abd –El-Fatah G. The Outcomes of Concomitant Radiation Therapy plus Capecitabine for Refractory Locally Advanced Breast Cancer Patients Pre-Treated with Anthracycline Based Regimens. Journal of American Science 2010;6(12):564-574]. (ISSN: 1545-1003). http://www.americanscience.org.

Key Words: Locally advanced breast cancer, radiosensitizing agents, neoadjuvant treatment, capecitabine

66	**Modulation of ochratoxin-induced oxidative stress, genotoxicity and spermatotoxic alterations by *Lactobacillus rhamnosus* GG in male Albino mice**	Full Text	66

Farag, I.M. [a]; Abdel-Aziz, K.B.[a]; Nada, S.A.[c]; Tawfek, N.S.[b]; Farouk, T.[b] and Darwish, H.R.[a]

[a] Cell Biology Dept., National Research Centre, , Cairo , Egypt.

, [b] Zoology Dept., faculty of Science, Al-Mania Uni., Egypt.

[c] Pharmacology Dept. National Research Centre, , Cairo , Egypt

Abstract: The mycotoxin ochratoxin A (OTA) is a widespread contaminant in human food and animal feed. It is a carcinogenic, genotoxic, teratogenic, immunotoxic, and hepatonephrotoxic agent. Therefore, the present study was designed to assess the possible protective effect of *Lactobacillus rhamnosus* GG (LGG) against OTA-induced toxicity in mice. Four groups of 30 mice each were used: control group, LGG-treated group (1 × 10^{10} CFU), OTA-treated group (1.8 mg/kg b.w.) and a group of mice given LGG two hours before OTA gavage. The levels of malondialdehyde (MDA), glutathione (GSH) and superoxide dismutase (SOD) activity were measured in of liver and kidney. Bone marrow micronucleus test and chromosomal aberrations in spermatocytes, as well as mitotic and meiotic activities were performed to assess the genotoxicity; besides sperm parameters were evaluated. Results showed that OTA significantly decreased the body weight. OTA significantly elevated the tissue levels of MDA, whereas the levels of

	GSH as well as SOD activity were significantly decreased in both liver and kidney. OTA increased statistically the frequencies of MNPCEs in bone marrow and structural and numerical aberrations in spermatocytes. In addition, mitotic and meiotic activities of somatic and germ cells were declined significantly. Also, OTA caused a high significant reduction in cauda epididymal sperm count, sperm motility and increased sperm abnormalities, as compared to control. In mice received LGG before OTA gavage, a significant amelioration in LPO in liver and kidney, by increasing the contents of GSH and SOD activity, have been occurred. Cytogenetic analyses revealed that LGG administration before OTA gavage significantly reduced frequencies of MNPCEs in bone marrow and chromosomal aberrations in spermatocytes, and recovered mitotic and meiotic activities as well. Moreover, gavage LGG before OTA intoxication caused significant recovery in all sperm parameters studied. In conclusion, LGG was found to be safe and successful agent counteracting the oxidative stress and protected against the genotoxicity induced by OTA, in addition to reduction in spermatotoxic alterations. [Farag, I.M.; Abdel-Aziz, K.B.; Nada, S.A; Tawfek, N.S.; Farouk, T. and Darwish, H.R. **Modulation of ochratoxin-induced oxidative stress, genotoxicity and spermatotoxic alterations by *Lactobacillus rhamnosus* GG in male Albino mice**. Journal of American Science 2010;6(12):575-587]. (ISSN: 1545-1003). **Key words:** ochratoxin A, *Lactobacillus rhamnosus*, oxidative stress, micronucleus, spermatocytes, sperm		
67	**Study of Risk Factors Involved in the Progression of Non Alcoholic Fatty Liver Disease in Egyptian Patients** **Elsayed A. Wasfy[1], Nadia M. Elwan[*1], Shreif L. Bayomi[2], Thanaa F. El- Sheikh[3], Sahar A. El-yamani[1] and Boshra E. Talha[1]** Tropical Medicine[1], Bathology[2]and Biochemistry[3] Departments, Tanta University, Tanta, Egypt *nadiaelwan@yahoo.com **Abstract:** Nonalcoholic fatty liver disease (NAFLD) includes hepatic steatosis, nonalcoholic steatohepatitis (NASH), fibrosis, and cirrhosis .NAFLD has also the potential to progress to hepatocellular carcinoma (HCC) or liver failure. NAFLD is linked to obesity, insulin resistance, hyperlipidaemia and genetic factors. *Our objective was* to study the risk factors that involved in the progression of non alcoholic fatty liver disease. Subjects and methods: Thirty-three patients and ten healthy controls were included in our study. Patients were classified into 3 groups. Group I included12 patients with simple liver steatosis. Group II included 11 patients with NASH. Group III included 10 patients with cirrhosis most probably a late sequel of NASH. Results: BMI, fasting blood glucose, insulin and HOMA-IR were significantly higher in patients with fatty liver, NASH and cirrhosis, also, NASH patients showed a significant high serum triglycerides and ALT. All previous parameters were significantly increased with the increased severity of histopathological score in patients with fatty liver and NASH. Serum AST levels and AST / ALT ratio were significantly increased in NASH and cirrhotic patients as compared to patients with steatosis alone and controls. Mitochondrial ATP levels in patients with fatty liver and NASH showed a statistically significant decrease. Also patients with NASH showed a statistically significant decrease when compared to patients with fatty liver. Finally, patients with fatty liver and NASH showed a significant decrease in mitochondrial ATP with increased BMI and histopathological score. Conclusion: Increased BMI, hyperglycemia, hypertriglyceridaemia, insulin resistance and depletion of mitochondrial ATP in hepatocytes can be considered risk factors involved in the development and progression of fatty liver to NASH and cirrhosis. [Elsayed A. Wasfy, Nadia M. Elwan, Shreif L. Bayomi, Thanaa F. El- Sheikh, Sahar A. El-yamani and Boshra E. Talha. Study of Risk Factors Involved in the Progression of Non Alcoholic Fatty Liver Disease in Egyptian Patients. Journal of American Science 2010;6(12):588-]. (ISSN: 1545-1003). http://www.americanscience.org. Key words: BMI, insulin resistance, mitochondrial ATP, NAFLD	Full Text	67
68	**Optimization of Cadmium, Zinc and Copper biosorption in an aqueous solution by *Saccharomyces***	Full Text	68

cerevisiae

[1]Salem M. Hamza [2*]Hanan F. Ahmed [3] Ehab A. M. [2]Mohammad F. M.

[1] Faculty of Science-Chemistry Department. El-Menoufia University, El-Menoufia, Egypt

[2] Food Technology Res. Institute, Agric. Res. Center, Giza, Egypt.

[3] Food Science Department, Faculty of Agriculture. Cairo University, Cairo, Egypt

*hanan_fawzy29@yahoo.com

Abstract: Optimization of Cd (II), Zn (II) and Cu (II) biosorption from contaminated water were performed as function of parameters (pH, contact time, initial metal ions concentration and yeast dose). The experimental results showed that the highest equilibrium adsorption capacity at the optimum pH were 8.5 for Cd (II) and 6 for Zn (II) and 6 for Cu (II). Optimum pH values were carried out to evaluate other parameters. Results demonstrate that removal efficiency increased with increased contact time for the three metal ions. Results indicated that removal efficiency increased with increased yeast dose up to 2 g/ 100ml, and removal efficiency decreased with increased yeast dose from 2.2 g/100ml to 4 g/100ml. The results also showed that increasing removal efficiency from 1 to 20 mg/L concentration for the three metal ions and the removal efficiency decreasing with increasing initial concentration from 25 to 50 mg/L. It is evident that the highest removal efficiency for Cd (II) ion compared to Zn (II) and Cu (II). This study revealed that use of baker's yeast is suitable for removal of these ions from contaminated water in order Cd > Zn > Cu at these conditions. The negative values of the standard free energy change (ΔG°) indicate spontaneous nature of the process. Competitive biosorption of (Zn and Cu) ions was investigated in terms of sorption quantity. The amount of Cu metal ion adsorbed onto unit weight of biosorbent (q_e) decreased with increasing the competing metal ion (Zn), in contrast, the amount of Zn ion adsorbed onto unit weight of yeast has been increased with increasing the competing metal ion (Cu). The binding capacity for Zn (II) is more than for Cu (II). Ion exchange is probably one of the main mechanism during adsorptive process.

[Salem M. Hamza, Hanan F. Ahmed Ehab A. M., Mohammad F. M. Optimization of Cadmium, Zinc and Copper biosorption in an aqueous solution by *Saccharomyces cerevisiae*. Journal of American Science 2010;6(12):597-604]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Optimization; Cadmium; Zinc; Copper; biosorption; *Saccharomyces cerevisiae*

69

Neuro Fuzzy Modeling Scheme for the Prediction of Air Pollution

Tharwat E. Alhanafy[*1], Fareed Zaghlool[1], and Abdou Saad El Din Moustafa [3]

[1]Computer and System Engineering Department, Al-Azhar University, Cairo, Egypt

[2]Director General, Arab Co. for Engineering & Systems Consultations (AEC) Certified Lead Auditor.

dr. s_ewiss@yahoo.com inf@aecegypt.org

Full Text

Abstract: The techniques of artificial intelligence based in fuzzy logic and neural networks are frequently applied together. The reasons to combine these two paradigms come out of the difficulties and inherent limitations of each isolated paradigm. Hybrid of Artificial Neural Networks (ANN) and Fuzzy Inference Systems (FIS) have attracted the growing interest of researchers in various scientific and engineering areas due to the growing need of adaptive intelligent systems to solve the real world problems. ANN learns from scratch by adjusting the interconnections between layers. FIS is a popular computing framework based on the concept of fuzzy set theory, fuzzy if-then rules, and fuzzy reasoning. The structure of the model is based on three-layered neural fuzzy architecture with back propagation learning algorithm. The main objective of this paper is two folds. The first objective is to develop Fuzzy controller, scheme for the prediction of the changing for the NO2 or SO2, over urban zones based on the measurement of NO2 or SO2 over defined industrial sources. The second objective is to develop a neural net, NN; scheme for the

69

	prediction of O3 based on NO2 and SO2 measurements. [Tharwat E. Alhanafy, Fareed Zaghlool, and Abdou Saad El Din Moustafa. Neuro Fuzzy Modeling Scheme for the Prediction of Air Pollution. Journal of American Science 2010;6(12):605-616]. (ISSN: 1545-1003). **Keywords:** Neuro Fuzzy Modeling Scheme for the Prediction of Air Pollution		
70	**Ester Phosphate of Discarded Palm Oil from Potato Chip Factories as Fat-Liquoring Agent** **M. G. Megahed[1] and El-Shahat H. A. Nashy[2*]** [1]Department of Fats and Oils, National Research Centre, Dokki, Cairo, Egypt. [2]Department of Chemistry of Tanning Materials and Leather Technology, National Research Centre, Dokki, Cairo, Egypt. [*]nashy_eha@yahoo.com dr_mgmegahed@hotmail.com **Abstract:** In Egypt most potato chip factories used palm oil for frying. The quantity of palm oil resulting from frying processes as discarded represents more than half of the total other oils used in the Egyptian food factories. Discarded palm oil resulting from frying processes was preliminary treated by purification and bleaching as well as characterized via its physico-chemical properties and fatty acids composition. Therefore, this work was devoted to explore the application of the discarded palm oil in leather industry as fat-liquoring agent. Fat-liquors help to prevent the loosening of leather grain and ugly appearance of chrome tanned leather after drying. In addition, fat-liquoring process improves leather characters such as soft handle, full, flexibility, and pliability as well as enhancement its mechanical properties. The study involved preparation of discarded palm fat-liquor via phosphoration process. The importance of the prepared fat-liquor is due to their environmentally friendly nature, relatively safe utilization by human being, in addition to their economical feasibility. The fat-liquored leather led to an improvement in the mechanical properties of the leather e.g. tensile strength, elongation at break and tear strength. In addition a great enhancement in the texture of the treated leather by discarded palm fat-liquor as indicated from the scanning electron microscope (SEM). [M. G. Megahed and El-Shahat H. A. Nashy. Ester Phosphate of Discarded Palm Oil from Potato Chip Factories as Fat-Liquoring Agent. Journal of American Science 2010;6(12):617-626]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords**: Discarded Palm Oil, Fatty Acids, Fat-liquor, Phosphoration, Chrome Tanned Leather, FT-IR, HLB, Strength Properties, Scanning Electron Microscope, Frying wastes	Full Text	70
71	**Reuse of Industrial Materials in Buildings to Activate their Application in Egypt** **Nermin Mokhtar Mohamed** Civil and Architectural Department ,Engineering division, National research centre, Egypt Nermin_farag@yahoo.com **Abstract:** Increasingly stringent rules and regulations on construction and demolition waste, diminishing landfill space and depletion of natural resources are all reasons for the push for industrial byproduct materials recovery. In Egypt, Industrial byproduct materials are generated in large volumes every day that are potentially usable materials, and must be disposed of. The main goal of this paper is to change the way Egyptians' think about waste—to see the value of a used material as a product or commodity, not as a waste, and encourage the use and recycling of these rich, largely untapped resources. Positive economic rewards and environmental results are moving our partners toward more waste reduction and materials	Full Text	71

	management. This paper summarizes the proposed Egyptian industrial materials waste management guidelines to reuse in building, which cover: (1) Identify the parties involved and the distribution of responsibilities; (2) Complementarily of roles of parties(owner, engineer, designer, and contractor) involved in the process of re-use to remove the causes that hinder the management of such material in Egypt; and (3) Participation of the Parties to the proposed project to achieve sustainable development fields at the actual application of the project. [Nermin Mokhtar Mohamed. **Reuse of Industrial Materials in Buildings to Activate their Application in Egypt.** Journal of American Science 2010;6(12):627-639]. (ISSN: 1545-1003). http://www.americanscience.org. **KEYWORD:** reuse –industrial byproduct materials, waste management, sustainability, Egypt		
72	**Mitochonderial Cytochrome C Oxidase Subunit 1 (*cox 1*) Gene Sequence of the *Hymenolepis* Species.** **Omnia M. Kandil*, Mona S. Mahmoud, Nesreen A.T. Allam, Amira H. El Namaky** Parasitology and Animal Diseases Department, National Research Center, Dokki, Giza, Egypt, kandil_om@yahoo.com* **Abstract:** In the current study, Mitochondrial Cytochrome *c* oxidase gene especially codons within subunit 1 (*cox1*) of *H. diminuta and H. nana* Egyptian isolates from two stages (adult worms and eggs) and hosts origin (human and rat) were amplified, sequenced and aligned. PCR products were approximately 700 bp, 702 bp and 715 bp of *H. nana* rat isolates, *H. diminuta* rat isolates, and *H. nana* human isolates, respectively. Moreover, despite their host susceptibility differences they all gathered in one cluster with three genbank published isolates of *H. nana*; AB033412.1, AB494472.1 and AY121842.1), forming one clade with 100% similarity, which was non significantly decreased on internal nodes. In addition, clearly far away from *H. diminuta* published sequence AB033412.1 who's assumed to be genetically closely related to Egyptian *H. diminuta* than all other *H. nana* isolates. Both Egyptian murine isolates of Hymenolipidid; *H. diminuta* and *H. nana*, were closer to each other than being to *H. nana* of human origin. The annotated sequences of Egyptian isolates were deposited in GenBank under the following accession numbers; *H. diminuta* (GU433102), *H. nana* rat isolate (GU433103), and *H. nana* human isolate (GU433104). Finally, the development of effective control strategies will only be possible if complete understanding of the epidemiology of infection is elucidated. [Omnia M. Kandil, Mona S. Mahmoud, Nesreen A.T. Allam, Amira H. El Namaky. Mitochonderial Cytochrome C Oxidase Subunit 1 (*cox 1*) Gene Sequence of the *Hymenolepis* Species. Journal of American Science 2010;6(12):640-647]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** Hymenolepidid, Phylogeny, Cytochrome *c* oxidase subunit 1 gene (*cox1*), Sequencing	Full Text	72
73	**Synthesis and Characterization of Poly (Acrylamide - co - Acrylic acid) Hydrogel Containing Silver Nanoparticles for Antimicrobial Applications** **Fatma S. Aggor [1] ; Enas M. Ahmed[1]*, A.T. El-Aref [2] and M. A. Asem[3]** [1] Department of Chemical Engineering & Pilot Plant [2] Department of Pre-treatments and Finishing. [3] Department of Chemistry of Natural and Microbial Products, National Research Center, Dokki, Cairo, Egypt *elarefenas123@yahoo.com **Abstract:** Acrylamide was copolymerized with acrylic acid at different ratios using potassium persulphate initiation system in presence of a crosslinking agent and different doses of silver nitrate to yield hydrogels containing silver nanoparticles upon post treatment with sodium hydroxide. Swelling capacity and kinetics of swelling of these hydrogels were studied. Size and distribution of the nanoparticles and their dependence	Full Text	73

	on acrylamide / acrylic acid ratios as well as on the dose of silver nitrate were also studied using Transmission Electron Microscopy (TEM). Furthermore, the antimicrobial and antifungal activities of the hydrogels in correlation with TEM results were reported. Hydrogels samples having relatively large number of Ag nanoparticles and widely distributed smaller particle size inhibit bacterial and fungal growth. [Fatma S. Aggor; Enas M. Ahmed, A.T. El-Aref and M. A. Asem. **Synthesis and Characterization of Poly (Acrylamide - co - Acrylic acid) Hydrogel Containing Silver Nanoparticles for Antimicrobial Applications.** Journal of American Science 2010;6(12):648-656]. (ISSN: 1545-1003). http://www.americanscience.org. **Key words: hydrogel;** silver nanoparticles; kinetic study; antimicrobial activity		
74	**Novel Switching H_2/H_∞ Control: Combination of Dwell Time Switching Signal and Multiple Lyapunov Function** Fatemeh Jamshidi [1], Mohammad Taghi Hamidi Beheshti [1] [1] Communication and Control Lab, School of Computer and Electrical Engineering, Tarbiat Modares University, Tehran, Iran Fjamshidi59@yahoo.com, mbehesht@modares.ac.ir **Abstract:** In this paper, a switching strategy is employed to solve the H_2/H_∞ multi objective controller design. Two controllers are designed to meet the H_2 and H_∞ performance specifications. Linear matrix inequalities are used in the controller design process. New switching signal is defined which is the combination of dwell time switching signal and multiple Lyapunov function such that stability of closed loop system is guaranteed as well as desired performance. Simulation results show that proposed switching strategy improves the performance of the controller and reduces the conservation in comparison with the common H_2/H_∞ controller. [Fatemeh Jamshidi, Mohammad Taghi Hamidi Beheshti. Novel Switching H_2/H_∞ Control: Combination of Dwell Time Switching Signal and Multiple Lyapunov Function. Journal of American Science 2010;6(12):657-663]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** Asymptotical Stability, Dwell time, H_2/H_∞ control, Multiple Lyapunov function, Switching signal	Full Text	74
75	**Investigation of Groundwater quality for Domestic and Irrigation purposes around Gubrunde and Environs, northeastern Nigeria** [1]Arabi, Suleiman Abdullahi; [1]Funtua, Idris Isa; [1]Dewu, Bala .Muhammad; [2]Zabosrki, Peter; and [2]Alagbe, Solomon .A [1]Centre for Energy Research and Training, Ahmadu Bello University, Zaria [2]Department of Geology, Ahmadu Bello University, Zaria-Nigeria Email: arabisuleiman@gmail.com **Abstract:** Fourteen groundwater samples were collected from boreholes, springs and hand dug wells in and around Gubrunde in Borno State north-eastern Nigeria to investigate its quality for domestic and irrigation uses. The area investigated falls within longitude 11° 35' - 12° 05' and latitude 10° 10' - 10° 31'. The samples were analyzed using Atomic Absorption Spectrometer (AAS), multi-analyte photometer and Flame photometer while interpretation of the results was carried out with RockWare Aq•QA software, a spreadsheet for water analysis. Six of the samples investigated are of NaCl water type while fourteen were CaCl water types. Sodium Adsorption Ratio (SAR) values recorded ranges from 0.80 – 2.84, Exchangeable Sodium Ratio (ESR) 0.33 – 1.78, Magnesium hazard (MH) 5.19 – 47.9, Residual Sodium Carbonate (RSC)	Full Text	75

	0.00, Hardness 0.65 – 221.48 and Total Dissolved Solid (TDS) ranges from 130 – 407308mg/l. Twelve of the samples analyzed had medium Salinity Hazard (SH), and one each for high and low Salinity Hazard (SH). For water with high salinity hazard, adverse effect is expected on crops, medium salinity hazard has detrimental effects only on crop that are sensitive to salinity while waters with low salinity hazard is suitable for all crops. The variation in chemical composition of groundwater in the study area may be due to leaching of terrestrial salts, extensive use of chemical fertilizers and ion exchange between water and the host rock. The result of samples analyzed indicates that all the samples are undersaturated in calcite and aragonite, while most of the major anion and cations falls within World Health Organization and Nigeria Industrial Standard for Drinking water Values. Nine samples had NO_3 values ranging from 53 – 106mg/l exceeding the 50mg/l standards. NO_3 values exceeding 50mg/l has the tendency of causing asphyxia to infants less than three months old. A plot of SO_4, HCO_3 and Cl indicates that the groundwater samples are from intermediate water category (neither fresh nor old). Generally, the groundwater quality is fairly suitable for agricultural uses and suitable for domestic utilization. [Arabi, Suleiman Abdullahi; Funtua, Idris Isa; Dewu, Bala .Muhammad; Zabosrki, Peter; and Alagbe, Solomon .A. Investigation of Groundwater quality for Domestic and Irrigation purposes around Gubrunde and Environs, northeastern Nigeria. Journal of American Science 2010;6(12):664-672]. (ISSN: 1545-1003). **Keywords**: Adverse effects; Sodium Adsorption Ratio; Exchangeable Sodium Ratio, asphyxia		
76	**Effect of the Type of Aggregate on the Properties of Alumina Refractory Concrete** **S.A. Ghonaim[1], H.B.G. Ghazal[2], and M.F. Abadir[*3]** [1] Egyptian Organization for Standardization and Quality, [2]The High Institute of Engineering, Shorouk [3]Chemical Engineering Department, Faculty of Engineering, Cairo University, Cairo, Egypt. [*]magdi.abadir@yahoo.com **Abstract:** Low cement refractory concrete samples were prepared by mixing cement (containing 50% alumina) in percentages ranging from 10 to 20% with some aggregates and the necessary amount of water. Two types of refractory aggregate were used: Bauxite containing 81% alumina and grog containing 52% alumina. Four particle sizes of each aggregate were used. The cast samples were left in their moulds for 24 hours in a 100% relative humidity cabinet. The de–molded specimens were left in an open air until their moisture content reaches 3–6%, then kept in a drying oven at (110 ± 5) ºC until reaching constant weight. They were then tested for phase constitution, water absorption, bulk density, apparent porosity and cold crushing strength (after 28 days curing). It was found that bauxite based samples gave better results than those prepared with grog. It was also found using statistical analysis that the percent cement used affects all properties much more than does the particle size of aggregate. [S.A. Ghonaim, H.B.G. Ghazal, and M.F. Abadir. Effect of the Type of Aggregate on the Properties of Alumina Refractory Concrete. Journal of American Science 2010;6(12):673-684]. (ISSN: 1545-1003). **Key Words:** Refractory concrete – Alumina – Grog – Sodium citrate – Bauxite	Full Text	76
77	**Osteoporosis in Diabetic Children** **Enas R. Abdel Hameed[*1], Hisham W. Badr[1], Azza A.Abdallah [1], Wagdi M. Hanna[1]and Nehal Salah[2]** [1]Child Health Department and [2]Clinical Pathology department, National Research Center, Cairo, Egypt [*]enas_raafat@hotmail.com **Abstract:** Background: Osteoporosis is a disease characterized by low bone mass and deterioration of bone structure that causes bone fragility and increases the risk of fracture. . Children and adolescents with	Full Text	77

	type 1 (insulin-dependent) diabetes mellitus (T1DM) show several impairment of bone metabolism and structure, resulting in a higher risk of decreased bone mass and its related complications later in life. Objective: to analyze whether bone mineral density (BMD) with bone status are influenced in children with T1DM and evaluate their relationships with clinical status, age and duration. Patients and Methods: Forty cases (age 7.5 $\pm$ 3.4 and duration of disease 3.7 $\pm$ 2.5 years) were studied. BMD expressed as Z-score was measured at neck of femur and Lumbar spines (L_2 – L_4) using dual energy x-ray absorptiometry (DEXA) for 15 cases. Urinary excretion of deoxypyridinoline (DPD) was measured by radio immunoassay and was corrected by creatinine (Cr). Serum levels of osteocalcin, osteoprotegrin, procollagen and rankle – markers of bone formation and resorption were measured. They were matched by age and sex for another 40 normal children as control. Results: there was a significant decrease in serum level of osteocalcin in 12 of our patients,all cases showed significant increase in serum rankle with significant difference $P < 0.05$ compared to control. Mean values of procollagen showed no significant difference compared to controls. As regard DPD mean values of cases showed a significant increase compared to control. BMD – expressed as Z-score-by DEXA revealed 10 cases with mild degree osteopenia, while the other 5 cases showed moderate degree. Conclusion: pediatric patients with T1DM appear to constitute a population at risk of developing osteopenia. Age-optimizing of metabolic control in growing diabetic children may prevent osteoporosis in later life. [Enas R. Abdel Hameed, Hisham W. Badr, Azza A.Abdallah, Wagdi M. Hanna and Nehal Salah. **Osteoporosis in Diabetic Children.** Journal of American Science 2010;6(12):685-690]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** Osteoporosis- Type 1 Diabetes Mellitus-Dual energy x-ray absorptiometry (DEXA), Bone Mass density – Osteopenia		
78	**Studies on Antimicrobial and Antioxidant Efficiency of Some Essential Oils in Minced Beef** **Amany, M. Salem[*], Reham, A. Amin and Gehan, S. A. Afifi** Food Control Department, Fac. Vet. Med., Benha University, Benha, Egypt dr_amany40@yahoo.com[*] **Abstract:** In this study, the antioxidant and antibacterial effect of garlic (G), thyme (T) and lemon grass (L) oils were investigated in refrigerated minced beef. It is noticed that, all essential oils used had considerable effectiveness in decreasing aerobic plate count (APC), *Enterobacteriaceae* count, Coliform count and *Staphylococci count*, as well as chemical indices as pH, total volatile nitrogen (TVN) and thiobarbituric acid (TBA). Sensory analysis indicated significant advantages in using lemon grass and thyme oils in refrigerated minced beef. In addition, a highly significant differences ($P<0.05$) between the different oils were noticed. Also, results indicated that the bacterial counts, pH, TVN and TBA values decrease as the concentration of the oil increases since the concentration (1.5%) gives the best effectiveness. The antioxidant and antibacterial activities of the added essential oils followed the order lemon grass oil > thyme oil > garlic oil. The treated minced beef samples extend the shelf life of the treated samples more than the control samples by 6 days. In conclusion, lemon grass, thyme and garlic oils can play an important role as antioxidant and antibacterial agents in refrigerated minced beef, but lemon grass oil is the best one. [Amany, M. Salem, Reham, A. Amin and Gehan, S. A. Afifi. Studies on Antimicrobial and Antioxidant Efficiency of Some Essential Oils in Minced Beef. Journal of American Science 2010;6(12):691-700]. (ISSN: 1545-1003). **Keywords:** Antimicrobial; Antioxidant; Efficiency; Oil; Beef	Full Text	78
79	**Recent Techniques used for Isolation and Characterization of *Staphylococcus Aureus* from Mastitic Cows.** **[1]El-Seedy,F.R [2] El-Shabrawy, M; [2]Hakim, A. S; [2*]Dorgham,S.M. [2]Ata, S. Nagwa; [2]Bakry, M.A and**	Full Text	79

	[2]**Osman, N.M.N.** [1]Bact. Mycol. and Immuno. Dept. Vet. Med. Beni-Suef University, Beni-Suef, Egypt [2]Micro. and Immuno. Dept. National Research Centre, Cairo, Egypt Sohad_dorgham@yahoo.com **Abstract:** A total of 152 cows was examined in this study for Staphylococcus species, it was found that 44.3% of cows and were clinically mastitic whereas 14.5% were subclinically mastitic respectively. The identification of Staphylococcus species revealed that *S. aureus, S. epidermidis, S. intermedius* and *S. hyicus* for cows were (17.2%, 7.5%, 3.9% and 1.6%) respectively. *Staphylococcus aureus* isolates were confirmed after biochemical identification by API test. The study of virulence factors of total *S. aureus* isolates from mastitic cows revealed that lipase, fibrinolysin, DNase and protein A production were presented as percentage 67.3, 74.0, 85.6 and 84.6 respectively. The antibiotic sensitivity for *S. aureus* revealed that 96.2% of cow isolates were methicillin sensitive which considered the drug of choice for these isolates. The study also included the identification of *S. aureus* enterotoxins using set-RPLA and multiplex PCR. The incidence of enterotoxins C,A,B and D by set-RPLA were 36.5%, 14.4%, 10.6% and 2.9% respectively. Meanwhile the results of multiplex PCR were 7 isolates as enterotoxin C,4 isolates as enterotoxin E and one isolate for each A,B, and D respectively. The identification of MRSA of cow's isolates using PCR revealed that 3 isolates out of 5 isolates were positive. [El-Seedy,F.R, El-Shabrawy, M; Hakim, A. S; Dorgham,S.M.m Ata, S. Nagwa; Bakry, M.A and Osman,N.M.N. Recent Techniques used for Isolation and Characterization of *Staphylococcus Aureus* from Mastitic Cows. Journal of American Science 2010;6(12):701-708]. (ISSN: 1545-1003). http://www.americanscience.org. **Key words:** *Staphylococcus aureus*; mastitis; methicillin sensitive; set- RPLA, multiplex PCR		
80	**Biosynthesis and Characterization of *Aspergillus Niger* AUMC 4301 Tannase.** **M. Z. El-Fouly[1]; Z. El-Awamry[2]; Azza A.M. Shahin[1]; Heba A. El-Bialy[1]; E. Naeem[2] and Ghadeer E. El-Saeed[1]*** [1] National Center for Radiation Research and Technology (NCRRT), Radiation Microbiology Department, Atomic Energy Authority, Cairo, Egypt. [2] Faculty of Science, Azhar University, Cairo, Egypt. **Abstract:** A study on biosynthesis and characterization of an extracellular tannase from *Aspergillus niger* AUMC 4301 was carried out. *A. niger* AUMC 4301 was selected out of one hundred and thirty fungal isolates have the ability to grow in the presence of tannic acid. Maximum enzyme synthesis under solid state fermentation was attained in the presence of 3% tannic acid and 0.2% ammonium nitrate after five days incubation at 30°C. Effect of different carbon and nitrogen sources on tannase formation was also investigated. Crude tannase had maximum activity at pH 4.8, 60°C and 20 min as a function of reaction time. The catalytic action of biosynthesized tannase was directly proportional to the amount of enzyme in the reaction mixture. Using tannic acid as substrate, the K_m value for tannase was 2.50 mM. Gallic acid was shown to be a competitive inhibitor to tannase and the inhibition constant (K_i) was 1.35 mM. Effect of EDTA and some metal salts on enzyme activity was also studied. [M. Z. El-Fouly; Z. El-Awamry; Azza A.M. Shahin; Heba A. El-Bialy; E. Naeem and Ghadeer E. El-Saeed. **Biosynthesis and Characterization of *Aspergillus Niger* AUMC 4301 Tannase.** Journal of American Science 2010;6(12):709-721]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** *Aspergillus niger*, tannase, tannins, gallic acid, solid state fermentation	Full Text	80
81	**Growth, Yield and Fruit Quality of Sweet Pepper Plants (*Capsicum annuum* L.) as Affected by**	Full Text	81

	Potassium Fertilization **El-Bassiony, A.M*.; Z.F. Fawzy; E.H. Abd El-Samad and A.A.Ghoname** Vegetables Crop Research Dept., National Research Centre, Dokki, Cairo, Egypt *mohsin2eg@yahoo.com Abstract: Two field experiments were conducted during the two successive summer seasons of 2009 and 2010 at the Experimental Farm of the National Research Centre in Nobaria region, Behira Governorate, to investigate the response of sweet pepper plants cv. California wonder to different rates of potassium fertilization (50, 100 and 200 kg/fed.) as potassium sulfate in addition to foliar application by potassium oxide (2 and 4 cm/L) and potassium humate (4 gm/L) as a stimulative dose. Potassium foliar applications were made 3 times in a 15 days interval with the same doses during the growing period (30, 45 and 60 days after transplanting). The highest potassium fertilization rate (200 kg/fed.) gave the tallest sweet pepper plants, the highest number of leaves and branches per plants and the highest fresh and dry weights of leaves as well as the highest total yield. Also, the obtained results reported that the fruit measurements expressed as fruit length, average fruit weight and vitamin C content, as well as leaves chemical composition (N, P, K and total chlorophyll) were increased with increasing potassium fertilization rate. On the other hand, spraying sweet pepper plants with potassium humate at rate of 4 gm/L markedly increased vegetative growth, yield, fruit quality and chemical composition. The favorable effects of the potassium on the growth, total yield and fruit parameters were obtained when sweet pepper plants fertilized with 200 kg/fed. potassium sulfate plus foliar application of potassium humate 4 gm/L followed statistically by 200 Kg/fed. potassium sulfate with foliar application of either 2 or 4 gm/L potassium oxide with no significant differenced between them but both of them were significantly higher than control. [El-Bassiony, A.M.; Z.F. Fawzy; E.H. Abd El-Samad and A.A.Ghoname. Growth, Yield and Fruit Quality of Sweet Pepper Plants (*Capsicum annuum* L.) as Affected by Potassium Fertilization. Journal of American Science 2010;6(12):722-729]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** Potassium stimulative dose, Potassium humate, Potassium oxide, Foliar spraying, Vegetative growth, Total yield, Fruit quality, Chemical composition.		
82	**How Do University Students Spend Their Time On Facebook? An Exploratory Study** Alimohammad Aghazamani Faculty of Technology and Science, Karlstad University, Karlstad, Sweden' Aghazamani99@yahoo.com **Abstract:** Despite major productive uses of Internet technology in today's digital world, users prefer to spend much more time on social networking sites (SNSs) like Facebook. The objective of this study is to determine student motives for using Facebook. A close-ended questionnaire was administered to 595 University students who were recognized as users of the site at Karlstad University in Sweden. Male users spend more time on the site than female users during both weekdays (p-value=0.9238) and weekends (p-value=0.9953). The survey showed that undergraduate students login more times per day than graduate students (p-value=0.2138). In addition, friendship was named the most favorite activity among male users (p-value=0.8883) and also among undergraduate students comparing with graduate students (p-value=0.2045). If users were asked to pay a membership fee to use the site, the results showed that male users (p-value=0.9991) and undergraduate students (p-value=0.9884) were more likely to pay the charge than other groups (females and graduate students). It is apparent that using Facebook can be seen as an important part of daily life among University students and its phenomenon spread out inevitably. [Alimohammad Aghazamani**. How Do University Students Spend Their Time On Facebook? An Exploratory Study.** Journal of American Science 2010;6(12):730-735]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** Internet; Facebook; Global village; Social networking	Full Text	82

83	**Succession Planning In Iranian Governmental Agencies** Masoud Porkiani[1], Malikeh Beheshtifar[2], Mahmood Nekoie-Moghadam[3] [1.] Assistant Professor, Management Department, Islamic Azad University, Kerman Branch, Kerman, Iran [2.] Member of scientific board, Management Department, Islamic AZAD University, Rafsanjan Branch, Rafsanjan, Iran [3.] Associate Professor, Management Department, Kerman University of Medical Sciences, Kerman, Iran m.beheshtifar@yahoo.com **Abstract:** It is becoming increasingly challenging for organizations to obtain qualified and talented staff. Succession planning is often introduced as a way to attract and employ such staff. Succession planning is a process of recruitment and development of employees for vital roles within the organization. Implementation of succession planning is central to certain organizational requirements. This research surveyed organizational requirements in Iranian governmental agencies and their relation to the implementation of succession planning. This study used descriptive methods with correlation. The statistical population consisted of two groups, experts and managers of Iranian governmental agencies, and data was collected using three questionnaires. The findings of this study demonstrated a meaningful relationship between organizational requirements such as managers' commitment, organizational culture, organizational readiness, and managers' competencies with the implementation of succession planning. By considering these organizational requirements in their management practices, managers are more likely to be successful in recruiting, evaluating, training and developing talent as dimensions of the implementation of succession planning. [Masoud Porkiani, Malikeh Beheshtifar, Mahmood Nekoie-Moghadam. **Succession Planning In Iranian Governmental Agencies.** Journal of American Science 2010;6(12):736-741]. (ISSN: 1545-1003). http://www.americanscience.org.	Full Text	83
84	**Identification Of Fungi Prevalent On Environmental Labour Ward Of General Hospital Umuguma And Umezuruike Hospital Labourward** **Ijioma B. C. Ph.D[2], Nwachukwu C. U. Ph.D[2], Akobundu, C.[1]** Department of Biology, Alvan Ikoku Federal College of Education, Owerri, Imo State, Nigeria[2]. Department of Microbiology, Federal University of Technology, Owerri, Imo State, Nigeria[1] nwachukwucu2005@yahoo.co.uk **Abstract:** There has been an increase in the frequency of fungal infections over the past decade. Nosocomial transmission of fungal pathogens and the recognition of resistance to antifungal agents pose a significant problem. This study identified the fungi species prevalent in the labour ward of the general hospital Umuguma and Umezuruike Hospital, Owerri Imo State Nigeria. Fungi are eukaryotic cells and therefore more complex than bacteria. The data available shows that Mucor Species and Rhizopus Species are the predominate species found in both hospitals in decreasing order. Fungal infection are often severe, rapidly progressive and difficult to diagnose or treat, therefore a thorough appreciation and understanding of fungi infections, including diagnostic and therapeutic modalities are needed among clinicians and microbiologists to provide a better patient care. [Ijioma B. C. Ph.D, Nwachukwu C. U. Ph.D, Akobundu, C. **Identification Of Fungi Prevalent On Environmental Labour Ward Of General Hospital Umuguma And Umezuruike Hospital Labourward.** Journal of American Science 2010;6(12):742-746]. (ISSN: 1545-1003). http://www.americanscience.org.	Full Text	84

	Key Words: Nosocomial infections, fungi, Mucor, Rhizopus, Penicillin, Hospital		
85	**A modified Algorithm to Model Highly Nonlinear System** **Tharwat O. S. Hanafy** Al_Azhar University,Faculty of Engineering, Systems and Computers Department s_ewiss@yahoo.com **Abstract:** In this paper, the Fusion of neural and fuzzy Systems will be investigated. Membership Function Generation and its mapping to Neural Network are introduced. An adaptive network fuzzy inference system (ANFIS) is introduced, and Multiple Inputs /Outputs Systems (Extended ANFIS Algorithm) is implemented. A Modification algorithm of ANFIS, Coupling of ANFIS called coactive neuro fuzzy system (CANFIS), is introduced and implemented using Matlab. The software of the modified algorithm of MIMO model identification is built. To test the validity of the modified algorithm ANFIS (CANFIS algorithm), an example is simulated from the numerical equation. The result of modified algorithm (CANFIS) showed a conformance with the simulated example and the root mean square (RMSE) is very small. [Tharwat O. S. Hanafy. A modified Algorithm to Model Highly Nonlinear System. Journal of American Science 2010;6(12):747-759]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** A modified Algorithm to Model Highly Nonlinear System	Full Text	85
86	***In vitro* and *in vivo* Activity of some Antibiotics against Staphylococcal Biofilm and Planktonic Cells Isolated from Diabetic Foot Infections.** **A. Abd El-Aziz[1], T. El-Banna[1], A. Abo-Kamar[1], A. Ghazal[2], and R. AboZahra[*3]** [1]Microbiology Department, Faculty of Pharmacy, Tanta University, Tanta, Egypt [2]Microbiology Department, Medical Research Institute, Alexandria University, Alexandria, Egypt. [3]Microbiology Department, Faculty of Pharmacy, Pharos University, Alexandria, Egypt [*]rania_abozahra@yahoo.com **Abstract:** The diabetic foot syndrome is clearly one of the most important complications of diabetes and is the most common cause of hospitalization among diabetic patients. *Staphylococcus aureus* is found to be the commonest pathogen present in diabetic foot infections. The aim of the present study is to determine activities of three kinds of antibiotics against Staphylococcal biofilm and planktonic cultures in vitro, and to indicate the difference in wound healing between staphylococcal planktonic and biofilm stage of colonization in vivo by using diabetic rat models. Biofilm forming staphylococci were identified by using the modified microtiter plate method. And the effect of different concentrations of several antibiotics (including ciprofloxacin, gentamycin and amoxicillin-clavulanic acid) on eight isolates was determined. The result showed that out of 86 Staphylococcal isolates, eight strains were found to be strong biofilm forming. It was found that the preformed biofilm was very difficult to remove with most isolates even with multiples of the MIC and that the biofilm MBC reached 46 times the planktonic MBC in some isolates. This was also noticed in case of the diabetic foot infection of the rat model, as the treatment was more efficient when it started immediately after infection, before the formation of the biofilm, as the bacterial infection was eliminated within 3-4 days, while it could not be completely eliminated when treatment started after the biofilm formation. This was also observed from the rate of healing and confirmed by histological examination. [**A. Abd El-Aziz, T. El-Banna, A. Abo-Kamar, A. Ghazal, and R. AboZahra**. *In vitro* and *in vivo* Activity of some Antibiotics against Staphylococcal Biofilm and Planktonic Cells Isolated from Diabetic	Full Text	86

	Foot Infections. Journal of American Science 2010;6(12):760-770]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** In vitro- in vivo- diabetic foot- staphylococcus- biofilm		
87	**Women Participation in Agro-allied Small and Medium Scale Enterprise and Its Impact on Poverty Alleviation in Oyo State Nigeria** Fajimi F.O and Omonona B.T Department of Agricultural Economics, University of Ibadan, Ibadan, Nigeria **ffovivid@yahoo.com** Abstract: This study examined the impact of women participation in agro-allied small and medium scale enterprises (SME) on poverty alleviation. Data were collected using the multistage sampling technique from 119 respondents in the study area made up of 59 participants and 60 non-participants. Data generated were analysed using descriptive statistics, FGT – weighted poverty measures and Probit regression analysis. Results from the study showed that the non-participants have the highest poverty level (51%), while the participants have poverty level of (17%) and the non-participants contribute greatly to whole group poverty. The estimated probit regression analysis showed that marital status, household size and women status in the family are poverty enhancing while educational status participation in Small and Medium Enterprises, income and monogamous family type are poverty reducing. Hence participation in agro-allied Small and Medium Enterprises is antidote to reducing poverty among women. [Fajimi F.O, Omonona B.T. Women Participation in Agro-allied Small and Medium Scale Enterprise and Its Impact on Poverty Alleviation in Oyo State Nigeria. Journal of American Science 2010;6(12):771-780]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** Poverty, SMEs, Agro-allied, Women, Participation	Full Text	87
88	**Studies on Probiotic Effects of Lactic Acid Bacteria Against *Vibrio vulnificus* in freshwater Prawn *Macrobrachium rosenbergii*** **Mona M. Ismail[1] and Waleed S. E.Soliman[2]** 1-Fish Disease and managment Department Fac. Vet. Med. Suez Canal Univ.Ismailia,Egypt 2- Hydrobiology Department National Research Center Dokki, Egypt soliman.waleed@ymail.com **Abstract:** Cell free extracts of three strains of Lactic acid bacteria (LAB) viz. Lactobacillus. acidophilus, Streptococcus.cremoris and Lactobacillus bulgaricus inhibited growth of Vibrio vulnificus in nutrient broth. The antagonism of LAB to Vibrio vulnificus was further confirmed by streak plating wherein suppression of growth of Vibrio was obtained. Juveniles of Macrobrachium rosenbergii (average weight 0.985 ± 0.1 g) on administering orally a moist feed base containing 5 × 106 cells·g of the three LAB probionts for a period of four weeks showed better survival (77 to 93%) when challenged with V. vulnificus by intra-muscular injection of 0.1 ml containing 3 × 109 cells·ml. Animals maintained on a diet devoid of bacterial biomass exhibited 95% mortality. No external or internal pathological changes were observed in shrimp fed with the LAB incorporated diets. Results showed inhibition of V. vulnificus by LAB and stimulation of the non-specific immune response resulting in resistance to disease in the prawn fed on LAB incorporated diets. [Mona M. Ismail and Waleed S. E.Soliman. **Studies on Probiotic Effects of Lactic Acid Bacteria Against *Vibrio vulnificus* in freshwater Prawn *Macrobrachium rosenbergii*.**. Journal of American Science 2010;6(12):781-	Full Text	88

	787]. (ISSN: 1545-1003). http://www.americanscience.org. **Key words:** Macrobrachium rosenbergii- Lactic acid bacteria - Vibrio vulnificus- immune response		
89	**Coag-flocculation studies of *Moringa olifera* coagulant (MOC) in brewery effluent; Nephelometric approach.** ***Menkiti Matthew .C. and Onukwuli Okechukwu .D.** Department of Chemical Engineering, Nnamdi Azikiwe University, Awka, Nigeria. *Corresponding author Department of Chemical Engineering, Nnamdi Azikiwe University, Awka, Nigeria. E-mail: cmenkiti@yahoo.com; Telephone: +234 8037441882 **ABSTRACT:** The coag-flocculation behavior of MOC in respect of pH variation in brewery effluent has been investigated at room temperature using various dosages of unblended MOC. Coag-flocculation parameters such as order of reaction α, rate constants (K and K_s), coagulation period $\tau_{1/2}$ e.t.c were determined. Turbidity measurement was carried out using the single angle (90°) nephelometric standard jar test while MOC processing was based on work reported by Ghebremichael. Microsoft excel package was employed in the evaluation of simulated parameter K_s. The maximum MOC performance are recorded at K of 6.6667 x $10^{-4}m^3$/kg.s, dosage of 0.4kg/m^3, pH of 4 and $\tau_{1/2}$ of 289.2614s while the minimum are recorded at K of 1.3333 x $10^{-4}m^3$/kg.s, dosage of 0.5kg/m^3, pH of 2 and $\tau_{1/2}$of 1446.6419s. The least value of E (%) recorded after 30 minutes is > 78%, thus confirming MOC as effective coag-flocculant. In general, the parameter obtained lie within the range of previous works, confirming that the theory of perikinetics holds for coag-flocculation of brewery effluent using MOC at the conditions of the experiment. [Menkiti Matthew. C. and Onukwuli Okechukwu. D. **Coag-flocculation studies of *Moringa olifera* coagulant (MOC) in brewery effluent; Nephelometric approach**. Journal of American Science 2010;6(12):788-806]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** *Moringa olifera;* coag-flocculation; coal effluent; kinetics; coagulation	Full Text	89
90	**Association between inflammation and the risk of cardiovascular disorders in atherogenic male rats: Role of virgin and refined olive oil** **Azza M. EL Wakf[*1]; Hamdy A. Ebraheem[1]; Hanaa A. Serag[1]; Hanaa A. Hassan[1]; Hussein S. Gumaih[2]** [1]Faculty of Science, Mansoura University, Mansoura, Egypt, [2]Faculty of Education, Sana'a University, Sana'a, Yemen [*]dr_azzaelwakf@yahoo.com **Abstract:** The aim of the present study was to determine changes in inflammatory markers, lipid profile and vascular wall integrity, (monitored as nitric oxide levels) in the male rats with experimental atherosclerosis. Also, to evaluate the role of two olive oils (virgin and refined) in these changes. Experimental atherosclerosis was induced by feeding rats normolipidemic diet (NLD) supplemented with (4% cholesterol, 1% cholic acid and 0.5% thiouracil, w/w) for three months. Feeding atherogenic diet (AD) exhibited marked elevation in serum total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), very low density lipoprotein cholesterol (VLDL) and triglycerides (TG), along with decreased high density lipoprotein cholesterol (HDL-C). Besides, an elevation in serum level of the two inflammatory markers, tumor necrosis factor-α (TNF-α) and fibrinogen was demonstrated with a lowered	Full Text	90

	nitric oxide (NO) levels in both aorta and cardiac tissues, indicating impaired vessel wall integrity and development of cardiovascular disorders in response to hyperlipidemia and enhanced inflammation. Subsequently, marked elevations in total leucocytes and other inflammatory mediators, including monocytes and lymphocytes have been recorded in the atherogenic diet fed rats. In addition, a significant reduction in erythrocytes count, hemoglobin (Hb) content and other hematologic indices was demonstrated, indicating further signs of inflammation. However, administration of olive oil (OO) [(in particular virgin olive oil (VOO)] to atherogenic rats exhibited improved inflammatory status, lipid profile and NO levels. Therefore, VOO might be a good candidate to replace other fats in the functional food for retarding atherosclerosis and risk of cardiovascular disorders. [Azza M. EL Wakf; Hamdy A. Ebraheem; Hanaa A. Serag; Hanaa A. Hassan; Hussein S. Gumaih. **Association between inflammation and the risk of cardiovascular disorders in atherogenic male rats: Role of virgin and refined olive oil**. Journal of American Science 2010;6(12):807-817]. (ISSN: 1545-1003). http://www.americanscience.org. **Key words:** Atherogenic diet, inflammatory markers, nitric oxide, vascular wall injury		
91	**Response of Wheat to Magnesium and Copper Foliar Feeding under Sandy Soil Condition** **EL-Metwally, A.E.[1]; F.E. Abdalla[2]; A.M. El-Saady[2]; S.A. Safina[1] and Sara S. EI-Sawy[2]** [1.] Agronomy Dept., Fac. of Agric., Cairo University, Cairo Egypt [2]. Fertilization Technology Dept., National Research Centre (NRC), Dokki – Cairo Egypt **Abstract:** Two field experiments were conducted during the winter seasons of 2007/2008 and 2008/2009 at Ismailia Experimental Station, Agriculture Research Center, Ismailia Governorate, to study the influence of foliar feeding with magnesium (Mg), copper (Cu) either as single nutrient or in combination on growth of wheat (*Triticum aestivum* L.) cv. Sakha 94. Nine treatments were applied: two levels of Mg, two levels of Cu and four combined treatment (Mg + Cu), in addition to control treatment. Results showed that the highest positive significant effect on flag leaf area, chlorophyll contents and dry matter/m^2 were achieved by spraying the highest Mg level + the highest Cu level (6.72 kg Mg + 1.68 kg Cu/fed.) Results also, showed that most of both macro and micronutrients content increased markedly due to the same previous treatment. [EL-Metwally, A.E.; F.E. Abdalla; A.M. El-Saady; S.A. Safina and Sara S. EI-Sawy. **Response of Wheat to Magnesium and Copper Foliar Feeding under Sandy Soil Condition**. Journal of American Science 2010;6(12):818-823]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords**: Wheat, *Triticum aestivum* L., Magnesium, Copper and Sandy soil	Full Text	91
92	**Effect of yeast (*Saccharomyces cerevisiae*) on reduction of aflatoxicosis, enhancement of growth performance and expression of neural and gonadal genes in Japanese quail** **Mariam G. Eshak[1], Wagdy K.B. Khalil[1], Eman M. Hegazy[2], Ibrahim M. Farag[1], M. Fadel[3] and Farid K.R. Stino[4]** [1]Cell Biology Department, National Research Centre, Dokki, Giza, Egypt. [2]Food Toxicology and Contaminants Department, National Research Centre, Dokki, Giza, Egypt. [3]Microbial Chemistry Department, National Research Centre, Dokki, Giza, Egypt. [4]Faculty of Agriculture, Cairo University, Egypt. mgergis@yahoo.com	Full Text	92

	Abstract: The present investigation was designed to evaluate the role of yeast, *Saccharomyces cerevisiae* (SC) in the reduction of aflatoxicosis induced by aflatoxin B_1 (AFB_1) in Japanese quail. Sixty male quail were used and distributed into six groups. The first group received basal diet. The other five groups received the basal diet plus 0.5 mg AFB_1/kg diet. Four of them received increasing levels of SC (0.5, 1.0, 2.0 and 2.5 gm/kg diet, respectively). All groups received their prospective diets for 35 days. The birds were weighed weekly to determine body weight (BW) and body weight gain (BWG). The results showed that addition of the SC to AFB_1-containing diet significantly reduced the adverse affect of AFB_1 on quail BW and BWG. The concentrations of AFB_1 had been lowered in the breast muscle and liver samples of quail fed diet containing AFB_1 plus SC than those found in such quail organs of AFB_1 group. The expression levels of neural and gonad genes were significantly up-regulated in quail fed diet containing AFB_1 plus high levels of SC compared to those of AFB_1 group. It could be concluded that SC supplementation to quail diets suppressed the aflatoxicosis in quail tissues leading to improvement of growth performances and enhancement of expression levels of neural and gonadal genes. Thus, the use of HPLC and gene expression analysis might contribute in detecting aflatoxin contamination in the poultry industry in Egypt. [Mariam G. Eshak, Wagdy K.B. Khalil, Eman M. Hegazy, Ibrahim M. Farag, M. Fadel and Farid K.R. Stino. **Effect of yeast (*Saccharomyces cerevisiae*) on reduction of aflatoxicosis, enhancement of growth performance and expression of neural and gonadal genes in Japanese quail**. Journal of American Science 2010;6(12):824-838]. (ISSN: 1545-1003). http://www.americanscience.org. **Key words:** Quail; body weight; growth rate; yeast; aflatoxin B_1; sqRT-PCR; gene expression		
93	**Perceived Family-Supportive Work Culture, Affective Commitment and Turnover Intention of Employees** Aminah Ahmad, Zoharah Omar Department of Professional Development and Continuing Education, Faculty of f Educational Studies, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia. aminah@ace.upm.edu.my **Abstract:** The objective of this research is to examine the role of perceived family-supportive work culture in reducing turnover intention of employees and the mediating role of affective commitment in the relationship between perceived family-supportive work culture and turnover intention. The subjects in this study constituted 693 employees from 20 private service organizations in the Klang Valley, Malaysia. Results of multiple regression analyses indicate that perceived family-supportive work culture is positively related to turnover intention of employees and employees' affective commitment mediate the relationship between perceived family-supportive work culture and turnover intention. The results imply the need for employers to understand how employees view the family-friendly programs in terms of the support provided and the values they place on the programs as captured in perceived family-supportive work culture. Positive perceptions would help reduce turnover intention as well the affective commitment of employees. [Aminah Ahmad, Zoharah Omar. Perceived Family-Supportive Work Culture, Affective Commitment and Turnover Intention of Employees. Journal of American Science 2010;6(12):839-846]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** Perceived family-supportive work culture; turnover intention; affective commitment	Full Text	93
94	**Synbiotic Tarhana as a functional food** ***Shreef G N Gabrial, ** Ahmed H Zaghloul, ***Abd El-Rahman M Khalaf-Allah, ***Nagwa M El-Shimi, *Rasha S Mohamed and *Gamal N Gabrial** * Food Science and Nutrition Department, National Research Centre, Dokki, Cairo, Egypt. **Dairy Science Department, National Research Centre, Dokki, Cairo, Egypt.	Full Text	94

***Food Science Department, Faculty of Agriculture, Cairo University, Giza, Egypt.

snoub_2000@yahoo.com

Abstract: In the present study formulated synbiotic tarhana (Turkish fermented cereal food) was produced as a functional food from the fermentation of wheat flour, some spices [salt, pepper, dill and sweet marjoram (Organum majorana)], some vegetables [tomato (Lycoprsicum esculentum), pepper (Capsicum annum) and onion (Allium cepa)], and synbiotic yoghurt which prepared with prebiotic (inulin and lactose each 3%) and different concentrations of the probiotic culture (0.5, 1.5, 3, 4.5% DVS-ABT2 containing Streptococcus thermophilus, Lactobacillus acidophilus and Bifidobacterium bifidum). After fermentation (3 days), tarhana dough was dried in the sun. The effect of the fermentation (0, 1, 2 and 3 days) and the probiotic culture concentration on the chemical composition and the probiotic population of the wet tarhana were evaluated. The effect of the probiotic culture concentration on the chemical composition, the probiotic population and the sensory attribute of dried tarhana were evaluated. Also the effect of dried tarhana (prepared from yoghurt which was fermented by 4.5% probiotic culture) on the plasma lipid profile of human subjects was studied. The results showed that the pH value decreased while the acidity increased, acetaldehyde and diacetyl values increased during the fermentation period and by increasing the probiotic culture concentration of the wet and the dried tarhana. Neither the fermentation nor the concentration of the probiotic culture of wet and dried tarhana affected the crude protein, ether extract, crude fibre, and ash values. The numbers of probiotic bacteria increased until the second day of fermentation. However, in the following day, with an increase of the acid content their number decreased. Generally the increasing of the probiotic culture concentration increased the numbers of probiotic bacteria of the wet and dried tarhana. Also the concentration of the probiotic culture didn't affect the sensory attributes of dried tarhana. Subjects supplemented with dried tarhana showed significant reduction in total plasma cholesterol, low density lipoproteins (LDL-C) and triglycerides, while high density lipoprotein (HDL-C) increased.

[Shreef G N Gabrial, Ahmed H Zaghloul, Abd El-Rahman M Khalaf-Allah, Nagwa M El-Shimi, Rasha S Mohamed and Gamal N Gabrial. **Synbiotic Tarhana as a functional food**. Journal of American Science 2010;6(12):847-857]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: Tarhana, functional food, fermented food, probiotic, synbiotic yoghurt, serum lipids

95

Response of Wheat to Different Rates and Ratios of Organic Residues on Yield and Chemical Composition under Two Types of Soil

Yassen, A.A*; Khaled, S.M and Sahar, M. Zaghloul and Habib, A.M.

Plant nutrition Dept., National Research Centre, Dokki, Giza, Egypt. *azimyassen@yahoo.com

Abstract: Two field experiments were conducted in two successive seasons (2007-2008 and 2008-2009) at Atta, Giza –Governorate and Nubaria region to study the effect of different rates and ratios of organic residues (Farmyard manure and filter mud) on yield and chemical composition of wheat under two types of soils (sandy and Calcareous soil). Results showed that, application of farmyard manure and filter mud residue gave a significant increase in grain and straw weight, total yield, crop index, harvest index, curd protein, N, P and K compared to the control treatment. Data also, indicated that significant increase grain, straw and total yield in sandy soil compared with calcareous soil under study in all treatments. On the other hand, the addition of organic materials (Farmyard manure and filter mud) were effective either individual or mixed with other. The pronounced increase in grain and straw weight, N, P and K content and uptake was noticed when farmyard manure was combined with filter mud at the rate of 2% compared with 1% of organic residues.

[Yassen, A.A; Khaled, S.M and Sahar, M. Zaghloul and Habib, A.M. **Response of Wheat to Different Rates and Ratios of Organic Residues on Yield and Chemical Composition under Two Types of Soil**. Journal of American Science 2010;6(12):858-864]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: wheat plant - organic residues –yield –N, P, K

Full Text

95

96	**Radioprotective activity of L- Carnitine and α -Lipoic acid against whole body γ- irradiation in rats** **Sally S. Alam[1*], Aziza M. Hassan[1], Nermine K. El Halawaney[1], Dalia E. El-Nashar[2], Mona G. Abd El-Azeem[2]** [1]Cell Biology Department, National Research Center, 12622 Dokki, Giza, Egypt. [2]Health Radiation Research, National Center for Radiation Research and Technology (NCRRT). Atomic Energy Authority, Cairo, Egypt. dr_Sallyalam@yahoo.com **Abstract:** The present study was designed to investigate the radioprotective efficacy of naturally occurring antioxidants, L - carnitine (LC) and α -Lipoic acid (LA) on radiation-induced bone marrow and liver damages in a rat model. The cellular changes were estimated by evaluation the expression of antioxidant enzymes, superoxide dismutase (SOD) and glutathione peroxidase (GPx), genes using RT-PCR and DNA damage in bone marrow and liver cells. The histopathological and ultrastructural changes were also determined. To evaluate the effects of the above antioxidants, adult rats were treated with LC (300 mg/kg b wt) and LA (150 mg/kg b wt) after exposure to whole-body γ-rays (6 Gy) for 10 days, or treated with LC & LA for 7 consecutive days and one hour after the last administration, animals irradiated a single dose of whole-body γ-rays (6 Gy) and received again LC & LA in same dose for 10 days. The obtained data revealed that γ –irradiation significantly decreases the expression of SOD and GPx genes and increases DNA fragmentation in liver cells as well as the incident of micronuclei in bone marrow cells. In addition, different histological and ultrastructural alterations in the liver of irradiated animals were recorded. These alterations were varied from hemorrhage, congestion in blood vessels, pyknosis and necrosis as well as complete degenerated area in the liver electron micrographs recorded swollen mitochondria, fragmented endoplasmic reticulum, distorted nuclei and cell membrane. Treatment with LC & LA post-exposure to radiation attenuated most of these changes. Whereas pre- and post- treatment with LC & LA to γ-irradiation normalized the expression of the antioxidant genes enzymes, decreased the DNA fragmentation and micronuclei formation with a normal restoration of histopathologial and ultrastrucure liver architecture. Thus, our results suggested that pre-treatment with LC & LA offers protection against γ-irradiation induced cellular damage. [Sally S. Alam, Aziza M. Hassan, Nermine K. El Halawaney, Dalia E. El-Nashar, Mona G. Abd El-Azeem. **Radioprotective activity of L- Carnitine and α -Lipoic acid against whole body γ- irradiation in rats**. Journal of American Science 2010;6(12):865-879]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** L-Carnitine, α-Lipoic acid, γ-irradiation, DNA fragmentation, Antioxidant gene expression, Ultrastructure**,** Histopathology	Full Text	96
97	**Fresh Water (The Nile And Its Branches) As One Of The Ways For The Development Of Fish Protein Sources In Egypt** **Saber Mostafa Mohamed, Mahmoud Khalifa Ahmed and l. A. E l Karyony** National Institute of Oceangraphic & fishries (NIoF) Alexandria. **saber_mostafa0@yahoo.com** **Abstract:** This study aimed to identify the freshwater fisheries in Egypt in terms of its evolution of fish production, the economic significance of the geographical distribution of the fish with identifying the seasonal productivity and measuring the impact of effort done on the fish production with emphasis on ways of development of those fisheries. The study had been adopted to achieve its goals on both economic analysis and descriptive statistical. The most important results were as follows: The fish production increased from river Nile fisheries from 57.8 thousand tons in 1995 to 79.7 thousand tons in 2008, after interest in the development of this source to provide fry tilapia and carp used in the development of water bodies.The study has been identified on the most important species and their relative importance, which represents about 72.3% of the average fish production during the study period, estimated at approximately	Full Text	97

	87500 tons. The tilapia, catfish, carp, and bayad are the most important varieties of high production are estimated the relative importance of 32.5%, 17.8%, 12.7%, and 9.8% respectively. While, the order comes after that in the arrangement, Nile perch, Shelan, (unicornfish) Albesaria, Nile lebeo, Eel, and barbal with an estimated relative importance of 3.9%, 3.6%, 3.3%, 2.1%, and 0.8%, respectively. The 96% of the annual variability production is due to changes in the productivity of varieties perch, catfish and tilapia. The middel Delta region (Desouk, Kafr El-Zayat, Menouf, Qanatier, and Benha) of the most important productive areas for fish in the River Nile, where a production of about 39.1%, followed by the region of the Nile Valley, which includes (Cairo, Giza, Fayoum, Beni Suef, Minya, and Assiut) represents 26.1%, while the production of Aswan region, represent 16.3%, which include (Sohag, Qena, Aswan).With regard to the employment and fishing boats, has decreased from 16400 boats in 1990 to about 11800 boats in 2008. While, the employment of fishing has decreased at high rates, which dropped from 51.5 thousand fishermen in 1990 to about 7.9 thousand fishermen in 2008, mostly working through the primitive ways, which have lacked in the safety manner. Furthermore, the number of boats licensed reflects the non reality where, the manual boat needed two or more person to complete the various operations on the boat, which indicates an increase in employment of fishing, non-licensed in those fisheries. The average production of the boat has increased with an average annual increase of productivity of 0.28 kg, while the average annual increase of productivity of a fisherman about 0.72 kg per year. However, the number and the productivity of boats are affected by 98% due to the annual changes of the production. Regarding, the examining of seasonal productivity and using seasonality index after excluding the effect of the general trend shows that, production is more than the overall average in the months of May, June, August and December. Whilst, the production is lower than of the overall average in the rest of the year months. Now there are a lot of efforts for the development of freshwater fisheries, through a variety of development programs (i.e. protect fisheries from pollution, fishery Seed supply, determine the appropriate fishing effort, and re-evaluate the characteristics and working methods of fishing). The targeted development plans to increase fish production through the overall development and coordination between the various parties to prevent the pollution of water resources and expansion in the construction and clearing waterways of plants, and re-stocking, especially carp fish, the Nile fish varieties, which became extinct with the quiet water stream, and made use of fish production. [Saber Mostafa Mohamed, Mahmoud Khalifa Ahmed and l. A. E l Karyony. Fresh Water (The Nile And Its Branches) As One Of The Ways For The Development Of Fish Protein Sources In Egypt. Journal of American Science 2010;6(12):880-888]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** Fresh water; Fish protein; tilapia, catfish, carp		
98	**Role of lactic acid bacteria as a biopreservative agent of Talbina** **Amal S. Hathout*, Soher E. Aly** Toxicology & Food Contaminant Dept., Food Industries & Nutrition Division, National Research Centre, Dokki 12622, Cairo, Egypt. amal_hathout@hotmail.com **Abstract:** Talbina is a mixture of barley flour and milk. The aim of this study is to evaluate the role of probiotic bacteria (*L. gasseri*, *L. reuteri*) compared to yoghurt starter bacteria (*S. thermophilus* and *L. delbreukii* sub sp. *bulgaricus*) as a biopreservative agent of Talbina samples. Shelf life of refrigerated Talbina processed by lower count (ratio 1:3 LAB : Talbina) of *L. gasseri* or *L. reuteri* increased and reached over 21 days at 6±2°C, compared to yoghurt starter bacteria which ranged between 6 and 14 days depending on the type of milk used. Storage temperatures are considered the main factors for biopreservation action of lactic acid bacteria (LAB). Increasing storage temperature to 12±2°C increased total fungal count and greatly changed fungal profile. It could be concluded that the potential of LAB to inhibit the growth of common food spoiling fungi opens up new perspectives for the bio-preservation of food products. [Amal S. Hathout, Soher E. Aly. **Role of lactic acid bacteria as a biopreservative agent of Talbina**. Journal of American Science 2010;6(12):889-898]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** lactic acid bacteria; Talbina; barley flour; fungi; bio-preservation	Full Text	98

99	**Immunohistochemical Study of Heat Shock Protein 70 in Psoriasis Vulgaris** Amina Gamal el Din[1]*, Hanan M Saleh[2], Nermeen Abdel Fattah[2] and Abdel Maksoud A[3] [1].Pathology Department, Medical Research Division, National Research Centre,Dokki, Cairo-12622 (Egypt)* [2.]Dermatology & Venereology Department, Faculty of Medicine, Ain Shams University, Abbasia, Cairo-12622 (Egypt). [3]Dermatology and Venereology Department, Medical Research Division, National Research Centre, Dokki, Cairo-12622 (Egypt). hassaneinamina@yahoo.com **Abstract:** Psoriasis, a common skin disease in Egypt, has drawn much attention to study the potential role of immunity in its pathogenesis.. Exposure of skin to microbial antigens and other stressful stimuli can induce heat shock proteins (HSPs) expression. HSPs comprise a large number of antigens against which immune responses are directed, owing to their cytokine-like effects and immunomodulatory properties. The potential role of HSP70 in pathogenesis of psoriasis is under investigation. We aimed at evaluating the differential immunohistochemical expression of HSP 70 in psoriatic skin and correlating the results with disease severity ; to elucidate its potential role in pathogenesis of psoriasis. Skin biopsies were taken from 20 patients with different severity of untreated chronic plaque-type psoriasis and from 20 healthy volunteers. Antibodies to HSP70 were analyzed immunohistochemically. Immunoreactivity intensity distribution index (IRIDI) scores including the proportion of immunoreactive cells and their staining intensity were calculated in the basal, suprabasal, superficial as well as the whole epidermal layers of patients and controls. Differential and total IRIDI scores for HSP70 expression showed highly significant higher values in psoriatic patients compared to controls. Statistical differences were found between the different groups of patients; according to their disease severity and controls. Positive correlations also existed between IRIDI scores of patients and disease severity. Based on the findings of the present study, HSP70 is suggested to play a role in the pathogenesis of psoriasis and to correlate with disease severity. Further studies on immunotherapeutic intervention are recommended, aiming at inhibiting events in an ongoing immune response which may provide new therapeutic and perhaps preventive approaches for psoriasis. [Amina Gamal el Din, Hanan M Saleh, Nermeen Abdel Fattah and Abdel Maksoud A. **Immunohistochemical Study of Heat Shock Protein 70 in Psoriasis Vulgaris**. Journal of American Science 2010;6(12):899-908]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** psoriasis, heat shock protein 70, immunohistochemistry	Full Text	99
100	[Journal of American Science 2010;6(12):904-909]. (ISSN: 1545-1003). http://www.americanscience.org. 6	Full Text	100
101	**Assessment Removal of Heavy Metals Ions from Wastewater by Cement Kiln Dust (CKD)** **Taha, A. Waly[1]; A. M. Dakroury[2]; G. O. El-Sayed[3] and S. A. El-Salam[1]** [1]Egyptian Fuel Manufacturing Pilot Plant (FMPP), [2]Hot Lab. Center and Waste Management, Atomic Energy. Authority, Egypt. [3]Department of Chemistry, Faculty of Science, Benha University. Benha, Egypt [*] aishaw95@yahoo.com **Abstract:** The effective removal of HM ions from aqueous wastes is among the most important issues for many industrialized countries. The present work has been carried out to study the adsorption of Cd(II),	Full Text	101

	Al(III), Co(II) and Zn(II), by adsorption technique using CKD which, are both wastes and are pollutants. The sorption process was examined in terms of its equilibria and kinetics. Batch adsorption experiments were conducted to evaluate the removal of Cd(II), Al(III), Co(II) and Zn(II), onto CKD waste over a wide range of operating conditions of sorbat concentration, pH, contact time, sorbent dose. The batch experiments showed that the most effective pH range was found from 5.5 to 8. Time-dependent experiments for the removal efficiency of HM ions showed that Al(III) required a shortest contact time, for Zn(II) and Cd(II), binding to the CKD was rapid and occurred within 20 to 40 min and completed for Co(II) within 4 hrs. High sorption capacities were observed for the four HM ions. The binding capacity experiments revealed the following amounts of HM ions bound per gram of CKD: 165.994 mg/g, 75.389 mg/g, 64.296 mg/g and 108.875 mg/g for Zn(II), Al(III), Co(II) and Cd(II), respectively. The equilibrium data for HM ions fitted both Langmuir and Freundlich models and based on Langmuir constant. The adsorption isotherm studies clearly indicated that the adsorptive behavior of HM ions on CKD satisfies not only the Langmuir assumptions but also the Freundlich assumptions, i.e. multilayer formation on the surface of the adsorbent with an exponential distribution of site energy. [Taha, A. Waly; A. M. Dakroury; G. O. El-Sayed and S. A. El-Salam. **Assessment Removal of Heavy Metals Ions from Wastewater by Cement Kiln Dust (CKD)**. Journal of American Science 2010;6(12):910-917]. (ISSN: 1545-1003). http://www.americanscience.org. **Key words:** Sorbat, Sorbent, Adsorption, Freundlich, Langmuir and Neutralization. Cement kiln dust (CKD)		
102	**Evaluation of Avian Influenza Vaccines used in Broiler Flocks in Egypt.** **Lebdah, M.A and *Shahin, A.M.** Avian and Rabbit Medicine Department; Faculty of Vet. Med.; Zagazig University *abeer.shahin@gmx.de **Abstract:** This study was carried out to investigate the efficacy of different types of commercial Avian Influenza Vaccines (H5N1 &H5N2) used in Egypt. Three – hundred and fifty day-old broiler chicks were divided into 7 groups. Six groups of chickens were vaccinated with H5N1 and H5N2 AI vaccines at 1, 7, and 14 days-old. The chickens of group 7 were kept as negative control. All groups were fed adlibtum and kept under observation. Serum Samples were collected at day-old to evaluate the maternal immunity and after 7 weeks post vaccination with both types of vaccines from all chickens. This study revealed that, the challenge virus was highly pathogenic for control group as causing 100 % mortalities 24 hours after challenge with 10^6 EID_{50}/ 0.2 ml intranasal. Challenge of other groups showed difference in pathogenicity of the virus and immune response of the chickens according to type of vaccine and age of birds at vaccination. It could be concluded that H5N2 AI vaccine was more protective than H5N1 AI vaccine as the protection percentage and GMHI titer of experimentally broiler chicks vaccinated at day-old and fourteen days-old with H5N2 higher than chicks vaccinated with H5N1. Moreover, the vaccination of the chicks at seven days-old showed higher GMHI titer and protection percentage than vaccination at one day-old. [Lebdah, M.A and Shahin, A.M. **Evaluation of Avian Influenza Vaccines used in Broiler Flocks in Egypt**. Journal of American Science 2010;6(12):918-926]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** Evaluation of Avian Influenza Vaccines used in Broiler Flocks in Egypt	Full Text	102
103	**Her 2/neu Gene and VEGF in Bladder Cancer in Egypt: Relationship to Schistosomiasis** **Olfat A. Hammam [1], Iman Abdel Aziz [2],Ola Mahmoud [2], Manal Zahran [2], Amr Alkholy [3], Ahmed Abdel Hadi[1], Maha Akl [1], Mohamed Wishahi [3]. Bruno Voss[4] and Sabine Boehm[4]** Departments of Patholog [1], Hematology [2], Urolog[3], Theodor Bilharz Research Institute, El-Nil Street, Giza,	Full Text	103

	Egypt Research Institute for Occupational Diseases[4], Ruhr University Bochum, Germany. *totoali1@hotmail.com **Abstract:** The aim of the current study was to assess Her2/neu protein on paraffin tissue sections and serum VEGF in carcinoma of the urinary bladder in a cohort of Egyptian patients. Furthermore, they were correlated to the schistosomal-associated and non-schistosomal associated bladder cancer as well as tumor types and disease stages. Immunohistochemical (IHC) procedure for Her 2/neu, FISH for detection of Her2/neu gene and serum level of VEGF by EIISA were performed in 35 patients with chronic cystitis (10 patients with nonschistosomal chronic cystitis and 25 patients with chronic schistosomal cystitis), 135 were schistosomal-associated malignant patients (75 cases of squamous cell carcinoma and 60 cases of urothelial carcinoma) and 50 cases of non schistosomal-associated urothelial carcinoma. In addition to 20 normal blood donor volunteers act as control. IHC results for Her 2/neu was overexpressed in malignant group compared to control and chronic schistosomal cystitis groups ($p<0.01$). In malignant group it was 1^+ in 33 (30%), 2^+ in 45 (40.9%) and 3^+ in 32 (29.09%). Her 2/neu incidence was significantly higher in urothelial carcinoma group 80/110 (72.2%) compared to SCC 30/75 (40%) with ($p<0.01$) and in high grade tumors than low grade tumors with ($p< 0.01$). FISH results in SCC showed that the signal ratio were 0-1.0 in 2 (6.6%), 1.1-2.0 in 18 (60%), and ≥2.0 in 10 (33.35%), which were considered positive for Her 2/neu gene amplification. In urothelial carcinoma the signal ratio was 0-1.0 in 10 patients (12.5%), 1.1-2.0 in 25 (22.3%), and ≥2.0 in 45 (58.2%). Overexpression of Her2/neu gene was significantly higher in high grades; 31 (63.6%) than in low grades; 14(56%) tumor with ($p< 0.01$), also Her2 /neu gene was significantly ($p< 0.01$) higher in invasive tumors; 45 cases (78.9%) than in non invasive tumors 10 (43.3%) with high significance ($p< 0.01$). The serum VEGF levels showed higher levels for SCC, urothelial carcinoma patients, chronic cystitis patients compared to normal controls, they were 94.7% (71/75), 89% (98/110), and 22.9% (8/35), 5% (1/20) respectively. Our results suggest that Her 2/neu overexpression might provide additional prognostic information in patients with bladder carcinomas. Because 50% of our patients harbor Her 2/neu overexpressing those patients may potentially benefit from molecular targeted therapy targeting Her 2/neu for bladder carcinoma and they should be identified by gene amplification analysis using FISH in IHC 2+ patients. In addition association between increased serum VEGF levels with high grades and invasive bladder cancer patients indicates that serum VEGF may play a role in the invasion and metastasis of cancer and may serve as an indicator of tumor progression and future recurrence and may be a candidate as a new noninvasive diagnostic tool. [Olfat A. Hammam, Iman Abdel Aziz,Ola Mahmoud, Manal Zahran Amr Alkholy, Ahmed Abdel Hadi, Maha Akl [1], Mohamed Wishahi [3]. Bruno Voss[4] and Sabine Boehm. **Her 2/neu Gene and VEGF in Bladder Cancer in Egypt: Relationship to Schistosomiasis**. Journal of American Science 2010;6(12):927-936]. (ISSN: 1545-1003). http://www.americanscience.org. **Key Words**: Her 2/neu protein; Her 2/neu Gene; IHC- FISH; Serum VEGF		
104	**Ultrasonic Comparative Assessment for Biodiesel Production from Rapeseed** **N.N. Ibiari*, S.A. Abo El-Enin, N.K. Attia and G. El-Diwani** Chem. Eng. and Pilot Plant Dept., National Research Centre, Egypt **Abstract:** The application of ultrasound during extraction and trans-esterification of oil from rapeseed was evaluated. Two methods of extraction were used, batch wise extraction and soxhlet extraction. In batch wise extraction procedure, ground rapeseeds were added to solvent and ultra-sonicated either by cleaning bath or ultrasonic generator. Conventional soxhlet extraction assisted in the soxhlet chamber by ultrasound has been developed. Ultrasonic technique reduced time required to extract oil. Using batch wise extraction procedure, percent recovery of oil increased almost 17.83% and 20.99% by using cleaning bath and ultrasonic generator respectively rather than control after 2hrs.While in using soxhlet extraction percent recovery reached 85% after 1.5 hr in case of ultrasonic and after 4 hrs without using ultrasonic. Physical and chemical properties of rapeseed oil were tested. Then the alkaline trans-esterification of rapeseed oil with methanol and potassium hydroxide for production of biodiesel was studied, using ultra-sonication and magnetic stirring. In trans-esterification the use of ultra-sonication and magnetic stirring led to similar high	Full Text	104

	yields of 90% of methyl esters after approximately 10 min. of reaction time. Comparison between biodiesel obtained and standard biodiesel and diesel fuel was done. [N.N. Ibiari, S.A. Abo El-Enin, N.K. Attia and G. El-Diwani. **Ultrasonic Comparative Assessment for Biodiesel Production from Rapeseed**. Journal of American Science 2010;6(12):937-943]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** Ultrasound, extraction, biodiesel, rapeseed oil, transesterification		
105	**Application of Different Methods of Natural Aeration of Wastewater and their Influence on the Treatment Efficiency of the Biological Filtration** **Tarek Ismail Sabry [1], Walid Hamdy[*2] and Saleem S. AlSaleem[3]** [1] Sanitary Engineering, Ain Shams University, Egypt. [2] Sanitary Engineering, Helwan University, Egypt. [3]Al Qassim Research Station, King Abdulaziz City for Science and Technology, KSA. awawalid@yahoo.com **Abstract:** The main objective of the proposed study is to examine the performance and the feasibility of using three different natural aeration methods (AM). The first two methods are the spray aerator (AM1) and the cascade aerator (AM2). The third method, the curtain aerator (AM3), is a new aeration technique that has different dynamic movement of wastewater falls (physical scrubbing action of aeration) for the aerobic treatment of raw sewage. The study investigates the most effective and suitable natural aeration system among these three methods for use in rural areas of developing countries where high costs of construction, operation, and maintenance of high-rate energy-intensive conventional aeration system technologies are the main bottleneck. The influences of the investigated natural aeration systems on the biological filtrations system are also investigated. The experimental results indicate an increasing in the aeration during the whole experimental by 21 %, 29 %, and 27 % for the AM1, AM2, and AM3 aeration systems, respectively; in respect with dissolved oxygen saturation. The results also showed that the amount of DO added to wastewater was influenced by both the surface loading rate ($m^3/m^2.hr$) of the aeration method and the splash movement of wastewater through the surface of the different methods As well, it was observed that cascade aerator (AM2), and the curtain aerator (AM3) had better removal efficiency in BOD, COD, and TSS compared with the spray aerator (AM1). [Tarek Ismail Sabry, Walid Hamdy and Saleem S. AlSaleem. **Application of Different Methods of Natural Aeration of Wastewater and their Influence on the Treatment Efficiency of the Biological Filtration**. Journal of American Science 2010;6(12):944-952]. (ISSN: 1545-1003). http://www.americanscience.org. **Key Words:** Natural aeration; wastewater treatment; biological filter; low-cost technology; rural developing countries	Full Text	105
106	**Biochemical Changes in Glutathione Redox System and Glucose Regulation in Late Pregnant Ossimi Ewes** **Ali Hafez El-Far[*1], Mohamed K. Mahfouz[2] and Hussein A. Abdel maksoud[2]** [1] Department of Biochemistry, Faculty of Veterinary Medicine, Alexandria University, Damanhour Branch (Al-Bostan), Egypt. [2]Department of Biochemistry, Faculty of Veterinary medicine, Moshtohor, Banha University, Egypt.	Full Text	106

	*aboufares90@yahoo **Abstract:** Pregnancy is the more prevalent stress in under feeding small ruminant with multiple bearing. Fifty Ossimi ewes of two years old and their body weight ranging between 35 and 50 kg were allotted into three groups; Group I: contains ten non pregnant non lactating ewes were used as control group. Group II: contains twenty single pregnant ewes* and Group III: contains twenty twin pregnant ewes used as experimental animals. Our study focused on the comparison between single and twin bearing ossimi ewes in the last four weeks of pregnancy and the day of parturition by measurement of reduced glutathione (GSH) level and the activities glutathione peroxidase (GSH-Px); glutathione reductase (GR-ase); glutathione-S-transferase (GST) and total superoxide dismutase (t-SOD) in erythrocytic haemolysate. In addition, glucose, non esterfied fatty acid (NEFA), Beta hydroxyl butyric acid (BHBA), cortisol, insulin and protein electrophoric patterns were measured in serum. Our results concluded that, In erythrocytic haemolysate the mean values of GSH-Px and GST in group II and III during the period of 2nd and last week before parturition and at the day of parturition were high significantly increased. While, GSH and t-SOD were high significantly decreased (P<0.01) and GR-ase activities were significantly decreased. While serum insulin level decreased while serum NEFA, BHBA and cortisol were increased in single and twin but in twin the values is more significant. The data showed that twin bearing ewes are more susceptible to pregnancy toxemia than single bearing that may be influence the productivity and performance of those animals. [Ali Hafez El-Far, Mohamed K. Mahfouz and Hussein A. Abdel maksoud. **Biochemical Changes in Glutathione Redox System and Glucose Regulation in Late Pregnant Ossimi Ewes**. Journal of American Science 2010;6(12):953-959]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords**: pregnancy, glutathione, single bearing, twin bearing, ewes		
107	**Economic Return of Recycling the Agricultural Wastes in Egypt and Spain** **Mohamed G.M. Abou El- Azayem* and Salah S. Abd El-Ghani** Department of Agricultural Economics, National Research Centre, Dokki, Cairo, Egypt. **Abstract:** Animal wastes and plant wastes are an important resource in Sustainable Agricultural Development and organic crops production for healthy food for life, when it is recycled to produce organic fertilizer (compost). It is clear that through the study of The economic returns to rotate some animal wastes and plant wastes in Egypt and Spain, And to identify. The quantity and value of losses in the content of animal wastes and plant wastes fertilizer elements (N, P, K) And also to identify Economic returns to recycling plant wastes for the production of industrial organic fertilizer (compost). Sustainable waste management means using resources efficiently to cut down on the amount of waste produced and where waste is generated, dealing with it in a way that contributes to the economic, social and environmental goals of sustainable development. [Mohamed G.M. Abou El- Azayem and Salah S. Abd El-Ghani. **Economic Return of Recycling the Agricultural Wastes in Egypt and Spain**. Journal of American Science 2010;6(12):960-970]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** Economic Return; Recycling; Agricultural Waste; Egypt; Spain	Full Text	107
108	**Role of S-100B as a Serum Biochemical Marker for Brain Injury in Egyptian Patients with Phenylketonuria** Mohga S Abdallah[1], Hayat M Sharada[1], Esmat Ashour[2]*,Lamiaa Mageed [2], Nagwa Meguid [3] [1]Biochemistry Division, Chemistry Dept., Faculty of Science , Helwan University [2]Biochemistry Dept., Genetic Engineering & Biotechnology Division, National Research Centre, Dokki, Giza, Egypt	Full Text	108

	[3] Children with Special Needs Dept., Medical Reseach Division, National Research Centre, Dokki, Giza, Egypt drmohgashafik@yahoo.com., hayatsharadamk@gmail.com.,esmatashour3@yahoo.com., malakalhoda@yahoo.com., Meguidna@yahoo.com **Abstract :Background:** Phenylketonuria (PKU) is a metabolic disorder characterized by high phenylalanine (Phe) levels in blood. Tissue accumulation of L-phenylalanine (Phe) is the biochemical hallmark of human phenylketonuria (PKU), an inherited metabolic disorder clinically characterized by mental retardation and other neurological features. The mechanisms of brain damage observed in this disorder are poorly understood. S-100B protein is highly specific for nervous tissue where its role is not yet fully understood. **Objective:** The aim of our study was to determine the diagnostic value of measuring S-100b in the serum of PKU patients as a marker for brain lesion. Additional validity should be acquired by a comparison with plasma levels of phenylalanine. **Methods:** Nineteen PKU patients from 15 families were selected from the clinic for special needs at the National Research Centre. Their age ranged between 2 and 20 years in addition to 15 healthy controls with same age. Blood samples were drawn to investigate circulating serum levels of S-100b using ELISA technique for all the studied cases. **Results:** Statistical significant increase of serum S-100B concentrations was present in PKU patients compared to controls. Regarding sensitivity and specificity, PKU patients, serum neural protein S-100b showed high sensitivity and specificity values. In addition, there was non significant negative correlation between S100B and Phe **Conclusion:** we concluded that serum S-100B blood could be a useful peripheral marker of nervous system damage in patients with phenylketonuria. [Mohga S Abdallah, Hayat M Sharada, Esmat Ashour, Lamiaa Mageed, Nagwa Meguid. **Role of S-100B as a Serum Biochemical Marker for Brain Injury in Egyptian Patients with Phenylketonuria**. Journal of American Science 2010;6(12):971-978]. (ISSN: 1545-1003). http://www.americanscience.org. **Key Words:** Phenylketonuria, Serum neural protein S-100B		
109	**Changes in Biochemical and Isozymes Components of Watermelon seeds during accelerated Ageing Technique** Magdy M. Rashed[1]; Magdy shallan[1]; Mahmoud Fathy[2]; and Anisa I. Ismail[2] [1]Bio Chemistry Department, Faculty of Agriculture, Cairo University, Giza,Egypt [2]Department of Seed Technology, Horticulture Research Institute, A R C, Ministry of Agriculture, Giza, Egypt **Abstract: The aims** of this work was to study some changes in the total content of storage components of watermelon (*citrullus lanatus*) seeds during accelerated ageing technique and its relation to seeds viability. **Materials and Methods**: Before the experiment, seeds were stored for two years in store house at 25C° in the start experiment, ageing at 50°C with 17% moisture up to 24, 48, 72, and 96 hours respectively. Germination percentage was decreased, a reduction in the total content of storage components such as proteins, carbohydrates, in addition, increasing oils and decreases in the activities of various esterase enzymes under the same condition were observed. **Results:** It was clearly that 50°C with 17% moisture content could be used as a good ageing seed testing condition for watermelon seeds In the present study The treatments watermelon seeds could be identified by Biochemical analysis (Esterase isozyme and Protein) banding pattern. [Magdy M. Rashed; Magdy shallan; Mahmoud Fathy; and Anisa I. Ismail. **Changes in Biochemical and Isozymes Components of Watermelon seeds during accelerated Ageing Technique.** Journal of American Science 2010;6(12):979-985]. (ISSN: 1545-1003). http://www.americanscience.org. **Key words**: Watermelon, accelerated ageing seed, seed germination	Full Text	109
110	**Effectiveness of Low Power Laser Therapy and Betamethasone in Minimizing Postoperative Edema**	Full Text	110

and Trismus after Third Molar Surgery: a Clinical Trial

Dalia A. Radwan[1], Nermeen H. Mohammed[1], Ahmed A. Zaky[2]

[1] Oral and Maxillofacial Surgery, Faculty of Oral and Dental Medicine, Cairo University.

[2]Dental Laser Application, National Institute for Laser Enhancement Science, Cairo University

Corresponding author: dradwan@link.net

Abstract: Purpose: In this study the therapeutic low-power laser (LPL) and Betamethasone (as an anti-inflammatory) were compared in terms of their effects on edema and trismus associated with surgical removal of impacted mandibular third molars. **Material and methods:** 20 healthy patients divided into two equal groups were included in the study. Group (I) received LPL irradiation (energy output 6 J/cm^2 with constant power density of 100 mW, wavelength 980 nm) on the 1st and 3rd postoperative days. Group (II) received a single dose of 4 mg systemic intramuscular Betamethasone Sodium Phosphate (Diprofos) into the gluteal region immediately after suturing of the surgical wound. Both groups received the usual medical and physical postoperative recommendations. **Results:** LPL irradiation (group 1) showed reduction of postoperative edema on the 3rd postoperative day. In addition, no significance difference resulted on comparing this effect between both groups. Postoperative trismus was nearly the same in both groups. No adverse effects of the procedure or medication were observed. **Conclusion**: LPL therapy is effective than systemic Betamethasone in reducing postoperative edema after third molar surgery without statistical significant differences. However both treatment modalities have the same effect on postoperative trismus.

[Dalia A. Radwan[1], Nermeen H. Mohammed[1], Ahmed A. Zaky. Effectiveness of Low Power Laser Therapy and Betamethasone in Minimizing Postoperative Edema and Trismus after Third Molar Surgery: a Clinical Trial. Journal of American Science 2010;6(12):986-989]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: edema, trismus, low power laser therapy, Betamethasone

111

Comparism Of The Quality Parameters Of The Seed And Condiment Oil Of Adansonia Digitata

Full Text

I.Y Chindo1*, J.S Gushit2, P.N Olotu3, J. Mugana1and D.N. Takbal1

1Chemistry Programme, Abubakar Tafawa Balewa University, Bauchi, Nigeria.

2Department of Science Laboratory Technology, University of Jos, Nigeria.

3Department of Pharmacognosy, University of Jos, Plateau State, Nigeria.

istifanuschindo@yahoo.com

Abstracts: The oil quality parameters of the seed and condiment oil of Adansonia digitata were evaluated. The Iodine value, Peroxide value, Saponification value and percentage Free Fatty Acid (FFA) were 98.07g/100g, 1.4mEq/Kg, 122.60mg/g and 0.21% respectively for seed oil and 71.06g/100g, 10.20mEq/Kg, 142.80mg/g and 6.37% respectively for the condiment oil. The variation in the parameters from seed oil to condiment oil observed include increased in peroxide value, FFA and Saponification value and decreased in Iodine value. The changes have been interpreted to be due to some structural changes in the Triglyceride leading to the formation of new chemical properties and products. The Infra Red (IR) spectra have also given an identification of Rancidity of the condiment oil due to bands observed at 3400-2700 and 1705 cm-1 indicating the possible formation or absence of acid and aldehyde respectively; which are products of oxidative Rancidity.

[I.Y Chindo, J.S Gushit, P.N Olotu, J. Mugana and D.N. Takbal. Comparism Of The Quality Parameters

111

	Of The Seed And Condiment Oil Of Adansonia Digitata. Journal of American Science 2010;6(12):990-994]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** Rancidity, Adansonia digitata, seed oil, condiment oil, Saponification & Infra Red		
112	**Distribution of Gamma-Emitting Radionuclides in Soils around the Centre for Energy Research and Training (CERT) Ahmadu Bello University, Zaria, Zaria-Nigeria** Muhammad, Musa Auwal; Idris, Isa Funtua; Simon, Peter Malam and Arabi, Suleiman Abdullahi Muhammad, Musa Auwal Reactor Engineering Section, Centre for Energy Research and Training (CERT), Ahmadu Bello University, P.M.B. 1014 Zaria, Nigeria, Email: mamyola@yahoo.com Idris, Isa Funtua Material Science and Development Section, Centre for Energy Research and Training (CERT), Ahmadu Bello University, P.M.B. 1014 Zaria, Nigeria, Email: iifuntua@yahoo.com Simon, Peter Malam Health Physics and Radiation Biophysics Section, Centre for Energy Research and Training (CERT), Ahmadu Bello University, P.M.B. 1014 Zaria, Nigeria Arabi, Suleiman Abdullahi Material Science and Development Section, Centre for Energy Research and Training (CERT), Ahmadu Bello University, P.M.B. 1014 Zaria, Nigeria, Email: arabisuleiman@gmail.com **Abstract:** A portable HPGe spectrometer has been employed to characterised, *in-situ* gamma activity concentration from the primordial Radionuclides ^{238}U, ^{232}Th ^{40}K in the soil at 12 monitoring points (MPs) in the environment in and around the Centre for Energy Research and Training (CERT), Ahmadu Bello University, Zaria, Nigeria. The MPs were marked-out using Global Positioning System (GPS) navigation. The measured activity concentrations due to ^{238}U range from 4.8 ± 3.0 to 11.9 ± 2.0 Bq kg^{-1} with an average of 8.3 ± 2.6 Bq kg^{-1}, ^{232}Th range from 15.5 ± 4.3 to 46.4 ± 3.5 Bq kg^{-1} with an average of 34.3 ± 3.4 Bq kg^{-1} and ^{40}K range from 317.2 ± 8.4 to 985.3 ± 7.0 Bq kg^{-1} with an average of 641.8 ± 7.3 Bq kg^{-1}. **[**Muhammad, Musa Auwal; Idris, Isa Funtua; Simon, Peter Malam and Arabi, Suleiman Abdullahi. **Distribution of Gamma-Emitting Radionuclides in Soils around the Centre for Energy Research and Training (CERT) Ahmadu Bello University, Zaria, Zaria-Nigeria.** Journal of American Science 2010;6(12):995-1001]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** In-situ gamma, activity concentration, primordial Radionuclides	Full Text	112
113	**An Introduction of OSCE versus Traditional Method in Nursing Education: Faculty Capacity Building &** **Students' Perspectives** [1*]Shadia A. Eldarir, [2]Hanan A. El Sebaae, [2]Hanaa A. El Feky, [3]Hewida A. Hussien, [1]Nagwa Abd El Fadil and [4]Inas H. El Shaeer [1]Maternal-Newborn Health Nursing Dept., [2] Medical Surgical Nursing Dept., [3] Pediatric Nursing Dept., [4] Community Health Nursing Dept., Faculty of Nursing Cairo University, Cairo, Egypt	Full Text	113

	*shadiaeldarir@yahoo.com **Abstract:** Background Assessment of clinical competence is of great importance when evaluating the expected learning outcomes of nursing education. Increasing number of students enrolled at Egyptian nursing faculties might increase the chances of malpractice that compromise patients' conditions. Therefore it is challenging to have such an objective assessment tool to comprehensively assess students' clinical competencies especially with increased students' number. Aims of the current project are building capacity of nursing faculties and staff members for OSCE; establishing simulated learning experiences (OSCE) in nursing practice; comparing the feasibility, utility, and effectiveness of using OSCE versus traditional clinical assessment; examining faculty and students perspectives for OSCE; and evaluating the effectiveness of OSCE versus traditional clinical assessment. Method: To achieve aims of this study needs' assessment of faculty members were carried out during conduction of raising awareness seminar about OSCE which attended by 72 faculty and staff members from both Cairo and Ain Shams Universities. A total of 7 workshops were held to build up their capacities on the scheme of OSCE and clinical scenario writings .One-hundred and forty faculty and staff members were attended and pre-post tests a were administered. Out of the 140, 31 were trained as data collectors. . Implementation of the OSCE was carried out on 400 second and third year students at the areas of critical care units. Comparison of students' achievements at traditional and OSCE methods were carried out. Faculty's and students' perspectives were investigated. Results: Needs' assessment revealed that 57% of faculty members knew nothing about OSCE and 98.6% of them had no experience in using OSCE; also a high statistical significant differences between OSCE and traditional assessment groups in the first and second trial ($t = 2.423$, $p= 0.016$), and ($t= 6.23$, $p= 0.000$) respectively. The students' achievements were better with OSCE. Faculty staff members indicated that, OSCE saves time (76.3%), prepares highly qualified competent students (62.5%) and improve students' performance (62.5%). Conclusion OSCE examination offers an attractive option for assessment of students' competency. It provided particular strengths in terms of faculty staff objectivity and reliability of the assessment process for all students, especially when compared with other methods of assessing practice. [Shadia A. Eldarir, Hanan A. El Sebaae, Hanaa A. El Feky, Hewida A. Hussien, Nagwa Abd El Fadil and Inas H. El Shaeer. An Introduction of OSCE versus Traditional Method in Nursing Education: Faculty Capacity Building & Students' Perspectives. Journal of American Science 2010;6(12):1002-1014]. (ISSN: 1545-1003). http://www.americanscience.org. **Key Words:** Assessment, OSCE, traditional method, Faculty capacity building, students' perspectives		
114	**Effect of Type of Aggregate on the Properties of Refractory Concrete** **S.A. Ghonaim*, H.B.G. Ghazal**, and M.F. Abadir*** [+]** * Egyptian Organization for Standardization and Quality ** The High Institute of Engineering, Shorouk *** Chemical Engineering Department, Faculty of Engineering, Cairo University [+] Corresponding author: Magdi F. Abadir The Chemical Engineering Department, Faculty of Engineering, Cairo University, Giza, Cairo, Egypt Area Code: 12613; Tel: 202 35678617; Fax: 202 35678625; E–mail: magdi.abadir@yahoo.com **ABSTRACT:** Low cement refractory concrete samples were prepared by mixing cement (containing 50% alumina) in percentages ranging from 10 to 20% with aggregate and the necessary amount of water. Two types of refractory aggregate were used: Bauxite containing 81% alumina and grog containing 52% alumina. Four particle sizes of each aggregate were used each time. The cast samples were left in their moulds for 24 hours in a 100% relative humidity cabin. The de–molded specimens were left in open air until their moisture content reaches 3–6%, then put in a drying oven at (110 ± 5) ºC until reaching constant	Full Text	114

	weight. They were then tested for phase constitution, water absorption, bulk density, apparent porosity and cold crushing strength (after 28 days curing). It was found that bauxite based samples gave better results than those prepared with grog. It was also found using statistical analysis that the percent cement used affects all properties much more than does the particle size of aggregate. [S.A. Ghonaim, H.B.G. Ghazal, and M.F. Abadir. **Effect of Type of Aggregate on the Properties of Refractory Concrete.** Journal of American Science 2010;6(12):1015-1027]. (ISSN: 1545-1003). http://www.americanscience.org. **Key Words:** Refractory concrete – Alumina – Grog – Sodium citrate		
115	**Effect of Beta Radiation on Extraocular Muscles** Mohamed A. Marzouk ,MD*, Hossam E. Sayed*, Ayman A . Shoman , MD* , Hisham A .Hashim ,MD*. * Ass. Professor – Research Institute of Ophthalmology – Giza – Egypt. **Abstract: Purpose: T**o evaluate the effect of different Beta radiation doses on frogs extraocular muscles. **Methods:** 50 frogs of species Rana Ridibunda were used in this study, they were divided into 5 groups, every group was treated with a different dose of radiation, and the first group was taken as control. **Results:** The estimation of soluble protein content in extraocular muscles of Beta radiated eyes showed a gradual decrease with the increase of dose. **Conclusion:** Significant changes in extraocular muscles were detected with the increase of Beta radiation dose. **[**Mohamed A. Marzouk, Hossam E. Sayed, Ayman A. Shoman, Hisham A. Hashim. **Effect of Beta Radiation on Extraocular Muscles.** Journal of American Science 2010;6(12):1028-1033]. (ISSN: 1545-1003). http://www.americanscience.org. ***Keywords:*** *Effect; Beta Radiation; Extraocular; Muscle*	Full Text	115
116	**Chronic Asthmatic Chest Troubles And Their Effects On Cognitive Functions, Psychosocial Behaviour And Academic Achievment Among Children In Egypt** **Samiha Samuel*, Mai Safwat*, William Morkos**, Samar Salem**, Tarek El-Adly*and Abeer Mohammed.** *Department of Paediatrics, Faculty of Medicine, Cairo University **Department of Childhealth, National Research Center **ABSTRACT:** Chronic illness is clearly an important factor affecting psychosocial state of children and adolescents. This case-control study is an effort to clarify the effect of chronic asthmatic chest troubles, as chronic illnesses, on the cognition and psychological aspects of such chronically ill children. This study was executed in the Chest Clinic of the Abou El-Reesh Children's Hospital, Cairo University. The Study was carried out on 23 children suffering from chronic asthmatic chest troubles (13 boys and 10 girls) with an age range of 6-15 years (mean age ± SD = 9.6±2.67). Twenty three age and sex matched children not suffering from any disease and living under the same socioeconomic conditions were taken as controls. WISC-R and PSCL were used to assess the cognitive and psychosocial adjustment among children while the mid-year scores for Mathematics and Arabic language were used to evaluate the academic performance. Our results indicated that chronic asthmatic disease has a negative effect on cognitive abilities, psychosocial behavior and academic achievement of such children. [Samiha Samuel, Mai Safwat, William Morkos, Samar Salem, Tarek El-Adly and Abeer Mohammed. Chronic Asthmatic Chest Troubles And Their Effects On Cognitive Functions, Psychosocial Behaviour And Academic Achievment Among Children In Egypt. Journal of American Science 2010;6(12):1034-	Full Text	116

	1043]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** Chronic; Asthmatic; Chest; Cognitive Functions; Psychosocial Behaviour		
117	**Effect of different sources of potassium fertilizers on growth yield, and chemical composition of *Calendula Officinalis*** *Yassen, A.A; **Habib, A. M.; *Sahar, M. Zaghloul, and *Khaled, S.M. *Plant Nutrition Dept., National Research Centre, Dokki, Giza, Egypt. **Ornamental plant Dept., Faculty of agriculture, Cairo University **azimyassen@yahoo.com** **ABSTRACT:** A field experiments were carried out during the two successive seasons of 2007/2008 and 2008/2009, in Qualubia Governorate, Egypt, to study the effect of different source of potassium fertilizer (banana residue and potassium sulphate) on yield, and chemical composition of herbs and flowers of *Calendula Officinalis***.** It had been deduced that) that application of potassium fertilizer from different sources; potassium sulphate and banana residue were effective in increasing all tested growth yields compared with unfertilized treatment. Data also, showed that mixing potassium sulphate or/ and banana residue led to a marked increase in fresh and dry weight of herbs and flowers as compared with application of potassium sulphate or/ and banana residue solely in both seasons**.** Data also, showed that mixing potassium sulphate or/ and banana residue increased N and P and K content and uptake as compared with the control, potassium sulphate and banana residue alone. [Yassen, A.A; Habib, A. M.; Sahar, M. Zaghloul, and Khaled, S.M. Effect of different sources of potassium fertilizers on growth yield, and chemical composition of Calendula Officinalis. Journal of American Science 2010;6(12):1044-1048]. (ISSN: 1545-1003). http://www.americanscience.org. **Key words**: *Calendula Officinalis* - potassium fertilizer - banana residue - growth – yield - NPK	Full Text	117
118	**Effect of packing on extension of self life of retail meat** **[*]Khalafalla, F.A; Nagwa, S.S. Ata; Mona. A.E. Elshabrauy, Azza, S.M. Abu Elnaga Dorgham,S.M and Khairy, A. E** **[*]Faculty of vet. Medicine. Beni-Suef University, Egypt. National Research Center. Dokki, Egypt.** **Abstract:** The packing of meat in retail markets plays important role in controlling of microbial load. Trails for extension of shelf-life of meat was studied during chilling. The comparative between the different types of packing as well as compared with fresh and chilled meat have low available data. Therefore, this study was carried out to assessment the effect of packing (Aerobically and anaerobically) on chilled meat as compared with fresh ones in retail market. [Khalafalla, F. A; Nagwa, S.S. Ata; Mona. A.E. Elshabrauy, Azza, S.M. Abu Elnaga Dorgham, S.M and Khairy, A. E. **Effect of packing on extension of self life of retail meat.** Journal of American Science 2010;6(12):1049-1058]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** packing; meat; retail market; microbial load	Full Text	118
119	**Serum Level of Cartilage Oligomeric Matrix Protein as a Screening Modality for Osteoarthritis among Knee Joint Pain Patients** **Ahmed M. Awadallah [*1], Gehan H.Sabry[1] and Tarek M.Khater[2]**	Full Text	119

[1]Departments of Clinical Pathology Faculty of Medicine, Benha University, Benha, Egypt

[2] Orthopedic Surgery Faculty of Medicine, Zagazig University, Zagazig, Egypt

[*]a_mamdouh8@hotmail.com

Abstract: Objectives: This study aimed to evaluate the diagnostic yield of estimation of serum cartilage oligomeric matrix protein (COMP) as a screening tool for osteoarthritis (OA) among patients with knee joint pain. Patients & Methods: The study included 140 female patients with knee pain and 20 volunteers to donate blood as a control group for laboratory findings. All patients underwent full history taking, clinical examination for evaluation of pain severity using a visual analogue scale (VAS) and extent of patient mobility using mobility score (MS) and had knee anteroposterior radiographs that were scored using the Kellgren-Lawrence scoring (K-L score) system. Patients were classified according to K-L scores into: group A: pain plus no radiographic findings (K-L score=1), group B: pain plus doubtful or minimal radiographic findings (K-L score=1) and group C: pain plus radiographically determined OA (K-L score≥2). Venous blood samples were obtained from all patients and controls for erythrocyte sedimentation rate (ESR) determination and ELISA estimation of serum COMP and high-sensitivity C-reactive protein (hsCRP) levels. Results: Group C patients had significantly higher pain scores and lower MS compared to groups A and B. Mean patients' serum COMP levels was significantly higher compared to control levels and in group C compared both to controls and to groups B and A levels with significantly higher levels in group B compared to controls and group A. However, serum COMP levels were non-significantly higher levels in group A compared to control levels. There was a positive significant correlation between serum COMP levels and body mass index (BMI), pain VAS score and radiological grade and a negative significant correlation with MS. ROC curve analysis revealed that elevated serum COMP is a sensitive predictor and high BMI is a specific predictor for the presence of OA. Serum COMP at 1097.5 ng/ml was the best cutoff point with high sensitivity (87.7%), positive predictive value (PPV, 92.6%) and accuracy (84.3%) for differentiation between patient with and without OA radiological manifestations and serum COMP at 1290 ng/ml showed 100% specificity and PPV and accuracy rate of 65.7% for diagnosis of the presence of radiological findings of OA. Conclusion: Estimation of serum COMP level could be considered as screening modality for patients with knee pain and using cutoff point of 1097.5 ng/ml helps to define patients free of OA and cutoff of 1290 ng/ml could define patients with OA.

[Ahmed M. Awadallah, Gehan H. Sabry and Tarek M. Khater. **Serum Level of Cartilage Oligomeric Matrix Protein as a Screening Modality for Osteoarthritis among Knee Joint Pain Patients.** Journal of American Science 2010;6(12):1059-1066]. (ISSN: 1545-1003). http://www.americanscience.org.

120

Biochemical Changes in Glutathione Redox System and Glucose Regulation in Late Pregnant Ossimi Ewes

Ali Hafez El-Far[*1], Mohamed K. Mahfouz[2] and Hussein A. Abdel maksoud[2]

[1] Department of Biochemistry, Faculty of Veterinary Medicine, Alexandria University, Damanhour Branch (Al-Bostan), Egypt.

[2]Department of Biochemistry, Faculty of Veterinary medicine, Moshtohor, Banha University, Egypt.

[*]aboufares90@yahoo

Abstract: Pregnancy is the more prevalent stress in under feeding small ruminant with multiple bearing. Fifty Ossimi ewes of two years old and their body weight ranging between 35 and 50 kg were allotted into three groups; Group I: contains ten non pregnant non lactating ewes were used as control group. Group II: contains twenty single pregnant ewes* and Group III: contains twenty twin pregnant ewes used as experimental animals. Our study focused on the comparison between single and twin bearing ossimi ewes in the last four weeks of pregnancy and the day of parturition by measurement of reduced glutathione (GSH) level and the activities glutathione peroxidase (GSH-Px); glutathione reductase (GR-ase); glutathione-S-transferase (GST) and total superoxide dismutase (t-SOD) in erythrocytic haemolysate. In addition, glucose, non esterfied fatty acid (NEFA), Beta hydroxyl butyric acid (BHBA), cortisol, insulin and protein electrophoric patterns were measured in serum. Our results concluded that, In erythrocytic

Full Text

120

	haemolysate the mean values of GSH-Px and GST in group II and III during the period of 2nd and last week before parturition and at the day of parturition were high significantly increased. While, GSH and t-SOD were high significantly decreased (P<0.01) and GR-ase activities were significantly decreased. While serum insulin level decreased while serum NEFA, BHBA and cortisol were increased in single and twin but in twin the values is more significant. The data showed that twin bearing ewes are more susceptible to pregnancy toxemia than single bearing that may be influence the productivity and performance of those animals. [Ali Hafez El-Far, Mohamed K. Mahfouz and Hussein A. Abdel maksoud. **Biochemical Changes in Glutathione Redox System and Glucose Regulation in Late Pregnant Ossimi Ewes.** Journal of American Science 2010;6(12):1067-1073]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords**: pregnancy, glutathione, single bearing, twin bearing, ewes		
121	**A Typical Bacteria in Ventilator Associated Pneumonia; an Egyptian University Hospital Experien** **Nadia Abdel-Salam Mokhless[1,3] , Malaka Farouk El-Mofty[1,3] , Nesrine Fathy Hanafi[* 1,3], Akram Muhammad Fayed[2,3] and Sara Lotfy Asser[1,3].** [1]Medical Microbiology and Immunology, [2]Critical Care Medicine, [3]Faculty of Medicine University of Alexandria, Egypt. drnesra1@hotmail.com* **Abstract:** Background Ventilator-associated pneumonia (VAP) is the most common hospital acquired infection seen in ICU in patients on mechanical ventilation. A diversity of microbes can cause VAP, causative agent differ according to patient populations and types of ICUs. Atypical bacteria not cultured by routinely used methods , have been implicated as causes of VAP, still no sufficient studies to assess size of their role as causative agent in VAP. In this study we aim at estimation of the potential role of atypical bacteria as Mycoplasma pneumoniae, Chlamydia pneumoniae and Legionella pneumophila in ventilator-associated pneumonia in the intensive care units of Alexandria Main University Hospital. Materials and methods: 60 endotracheal aspirates were collected from VAP ICU patients. Samples were subjected to routine culture as well as PCR amplification using specific primers for detection of the following atypical bacteria : Mycoplasma pneumoniae, Chlamydia pneumoniae and Legionella pneumophila. Results: Out of the 60 endotracheal aspirate, routine culture revealed growth of: enterobacteriaecae in 14 (23.3%) aspirate, pseudomonas in 13(21.7%), candida in14(23.3%) , and MRSA in 10 (16.7%) . In 19 (31.7%) endotracheal aspirates, no growth was encountered on routine culture. PCR reaction was positive for Atypical bacteria in 9 (15%) out of 60 samples, five were positive for mycoplasma, three for Legionella, and only one was positive for Chlamydia. Atypical bacteria positive results were encountered in 4 (21%) out of 19 aspirates with no growth culture results. Conclusion: Our results point that atypical bacteria are not an uncommon cause for VAP. This finding has to be taken into consideration while tailoring the empiric antimicrobial coverage of patients diagnosed with VAP. [Nadia Abdel-Salam Mokhless, Malaka Farouk El-Mofty, Nesrine Fathy Hanafi, Akram Muhammad Fayed and Sara Lotfy Asser. **A Typical Bacteria in Ventilator Associated Pneumonia; an Egyptian University Hospital Experience.** Journal of American Science 2010;6(12):1074-1079]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** Bacteria; Ventilator; Pneumonia; Egyptian; Hospital	Full Text	121
122	**Synthesis of Some New Benzoxazole Acetonitrile Derivatives and Evaluation of Their Herbicidal Efficiency.** M.A.youssef[a], S.M.A.Sherif[b], A.M.A.Elkady[c] and S.E.S.Hamouda[c]. a Chemistry Department , Faculty of Science , Helwan University. b Chemistry Department , Faculty of Science , Cairo University . c Central Agricultural Pesticides Lab. (CAPL), Agriculture Research Center (ARC), Cairo, Egypt.	Full Text	122

	Abstract: Twenty three new 2-cyanomethyl benzoxazole derivatives were synthesized by different methods. Their structures were elucidated by many ways as elemental analysis, spectroscopic analysis and chemical methods. The herbicidal activity of the newly synthesized compounds was evaluated against wheat as pattern for monocotyledonous plants, three plant parameters were studied, seed gerimination, root and shoot growth under laboratory conditions. Compounds that showed an observable inhibition on one or more of the growth parameters under study were considered as promising compounds and needs more studies from the toxicological, soil, environmental and formulation points of view to stand on the most potent derivative that can be formulated in a suitable formulation form to be used in the field of pest control. Compounds (**16a**),(**16b**),(**16f**),(**13b**),(**10a**),(**7a**) and (**3b**) inhibited all growth parameters under study by different degrees. While compounds (**13b**) and (**13a**) were more effective on root and gerimination respectively. Most synthesized compounds inhibited markedly shoot growth. [M.A.youssef, S.M.A.Sherif, A.M.A.Elkady and S.E.S.Hamouda. **Synthesis of Some New Benzoxazole Acetonitrile Derivatives and Evaluation of Their Herbicidal Efficiency.** Journal of American Science 2010;6(12):1080-1090]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** 2-cyanomethyl benzoxazole , 2-arylidene cyanomethyl benzoxazole, herbicidal activity, growth parameters, wheat and monocotyledonous plants		
123	**Designing a Reliable Supply Chain Network Model under Disruption Risks** Nader Azad, Hamid Davoudpour Department of Industrial Engineering, Amirkabir University of Technology, P.O. Box 15875-4413, Tehran, Iran. n.azad@aut.ac.ir **Abstract:** In this paper, we consider random disruption risks in designing a reliable distribution network model. We consider the disputations in the location and the capacity of the distribution centers. In our model, the probability of disruption in distribution centers is dependent to the amount of investment for opening and operating them. We show that this problem can be formulated as a non-linear integer programming model, and then for obtaining optimal solution, we linearize the mentioned model. In the following to solve the model in large-sized instances, a tabu search algorithm is developed. The results indicate that the tabu search method is efficient for a wide variety of problem sizes. [Nader Azad, Hamid Davoudpour. **Designing a Reliable Supply Chain Network Model under Disruption Risks**. Journal of American Science 2010;6(12):1091-1097]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** Supply chain network, Distribution network, Facility location, Random disruption risks, Tabu search	Full Text	123
124	**Serum Fetuin-A in Chronic Renal Disease Patients: Contribution to Endothelial Dysfunction and Hemostatic alteration** Nadia A. Hussein[1], Ola M. Mahmoud[1], Manal Y. Zahran[1], Manar A. Rafaat[2] [1]Hematology and [2]Nephrology Departments, Theodor Bilharz Research Institute, Giza, Egypt nadhussein@hotmail.com **Abstract: Background/Aim:** Fetuin-A is a circulating calcium-regulatory glycoprotein that inhibits vascular calcification. In the present study, serum fetuin-A was studied as a novel risk factor for the development of endothelial dysfunction (ED) and hemostatic alteration in patients with chronic renal disease (CRD). **Patients and Methods:** 15 CRD patients on conservative treatment, 15 end stage renal	Full Text	124

	disease (ESRD) patients on regular hemodialysis (HD) treatment and 15 healthy volunteers were enrolled in the study. Fetuin-A, thrombomodulin (TM), von Willebrand factor (vWF), tissue plasminogen activator (t-PA), plasminogen activator inhibitor (PAI-1), D-dimer, high sensitivity CRP (hs CRP) and IL-6 were measured by ELISA. **Results:** There was a significant reduction in Fetuin-A levels in CRD and HD patients compared to controls. A significant decrease was also detected in HD group when compared to CRD group. The inflammatory markers, hs CRP and IL-6, were significantly increased in CRD and HD patients in comparison to controls. The increase was also significant on comparing HD group to CRD group. A strong inverse correlation was found between serum fetuin-A and each of hs CRP and IL-6. In addition, regression analysis revealed that hs CRP is an independent determinant of serum fetuin-A level. The traditional markers of ED, TM and vWF, were significantly increased in CRD and HD patients compared to controls. The increase was also significant when HD patients were compared to CRD patients. The significant inverse correlation between fetuin-A and each of TM and vWF supports the hypothesis that low serum fetuin-A with subsequent vascular calcification could be one of the contributing factors for the development of ED in CKD and HD patients. The fibrinolytic parameters tPA, PAI-1 and D-dimer levels were significantly higher in CRD and HD compared to controls. HD patients had significantly higher values of the previously mentioned parameters in comparison to CRD patients. t-PA, PAI-1 and D-dimer were significantly correlated to fetuin-A in CRD and HD patients. **Conclusion:** The results of this study demonstrate that in CKD and HD patients inflammatory processes are increased and linked to low fetuin-A and vascular calcification which represents a novel risk factor for the development of ED. The interplay of these phenomena could be responsible for the development and progression of accelerated thrombogenesis that is peculiar to renal patients. [Nadia A. Hussein, Ola M. Mahmoud, Manal Y. Zahran, Manar A. Rafaat. **Serum Fetuin-A in Chronic Renal Disease Patients: Contribution to Endothelial Dysfunction and Hemostatic alteration**. Journal of American Science 2010;6(12):1098-1105]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** Serum Fetuin; Chronic Renal Disease; Patients; Endothelial Dysfunction; Hemostatic alteration		
125	**Electrogastrographic Findings in Cerebral Palsy Patients** **Nassar M.F.[1] Aly R.H.[1], Mahmoud N.H.[2], El-Batrawy, S.R.[3] and Abdel-Kereem N.[1]** [1]Pediatrics Department and [2]Clinical Pathology Department, Faculty of Medicine, Ain Shams University, [3]Department of Anthropology, National Institute of Research, Cairo, Egypt. [*]maie_nassar@yahoo.co.uk **Abstract:** Objectives: This work was designed to detect any changes in the gastric electrical activity and gastrin levels in infants with cerebral palsy (CP) and correlate them to the clinical findings. Patients and methods: The study was conducted on 30 CP patients in comparison to 12 age and sex matched clinically healthy infants. All enrolled infants and children were initially subjected to complete history taking with special emphasis on gastrointestinal symptoms, clinical examination and routine laboratory procedures as well as total serum gastrin hormone by ELISA. Electrogastrographic (EEG) recording for gastric electrical activity was performed for all subjects upon enrollment. Results: The initial power ratio was non-significantly higher in CP patients compared to the controls while the dominant frequency (DF) was non-significantly lower. Regarding the initial visual analysis of the EGG, 13 patients (43.3%) were normogastric compared to bradygastria in 16 (53.3%) of them. Initial serum gastrin was higher in CP patients compared to the controls. The regression analysis revealed that gastrin was the most determinant factor for dominant frequency values followed by the power ratio in the CP patients. Conclusion: In conclusion, CP patients have disturbed gastric motility which explains the different proximal gastrointestinal clinical manifestations experienced by our patients and this should be considered during their nutritional rehabilitation programs. [Nassar M.F. Aly R.H., Mahmoud N.H., El-Batrawy, S.R.and Abdel-Kereem N. **Electrogastrographic findings in cerebral palsy patients.** Journal of American Science, 2010; 6(12): 1106-1113]. (ISSN: 1545-1003). http://www.americanscience.org.	Full Text	125

	Key words: electrogastrography; gastrin; cerebral palsy		
126	**Significance of Angiopoietin-2 as a Serum Marker for Hepatocellular Carcinoma** Shereen Shoukry Hunter*[1], Maha Sayed Hassab-Allaah[1], Mahmoud Ahmad El-Ansary[2], Faten M El Shanawani [3] and Mona M Hassan[3] [1]Tropical Medicine Department, Faculty of Medicine, Cairo University, Cairo, Egypt [2]Hepatology and Gastroenterology Department, Theodor Bilharz Research Institute, Cairo, Egypt [3]Clinical Chemistry Department, Theodor Bilharz Research Institute, Cairo, Egypt *shereenhunter@hotmail.com **Abstract:** Background and study aims: Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide and one of the major causes of death. The aim of this study was to investigate the potential role of Angiopoietin-2 as a non-invasive marker for HCC. Patients and Methods: This study was conducted on 30 patients with documented HCC and 30 cirrhotic patients with no evidence of HCC; as well as 30 healthy subjects who served as control group. The levels of alfa fetoprotein (AFP) and angiopoietin-2 (Ang-2) were measured for all cases together with full clinical assessment, liver biochemical profile, viral markers, ultrasound, abdominal triphasic computerized tomography (CT) scan and guided liver biopsy for HCC cases with atypical triphasic CT pattern. Results: There was a statistically highly significant elevation ($p< 0.001$) in the mean serum AFP in HCC group (155.5 ± 271.5 ng/ml) when compared with the control group (6.3 ± 2.4 ng/ml) and also a highly significant elevation ($p<0.01$) when compared to the cirrhosis group (29.3 ± 31.2 ng/ml). There was a statistically highly significant elevation ($p< 0.001$) in the mean serum Ang-2 in HCC group (10855 ± 5321.92 pg/ml) when compared with both the control (480.67 ± 202.3 pg/ml) and cirrhosis (5578.33 ± 2928.21 pg/ml) groups. The diagnostic sensitivity of AFP at a cutoff of 200 ng/ml was 24% and the specificity was 100%. The cutoff level of Ang-2 for diagnosis of HCC in this study was 8100 pg/ml, with a sensitivity and specificity of 70% and 80% respectively. Serum Ang-2 was significantly elevated in HCC patients with portal vein thrombosis than those without. There was a significant positive correlation between the number of hepatic focal lesions and the serum level of Ang-2. The combined use of the two markers (AFP and Ang-2) led to an increase in the sensitivity of AFP from 53.3% to 83.3%. Conclusion: Serum Ang-2 is elevated in patients with cirrhosis and further elevated in patients with HCC, so its use as an independent tumor marker in the diagnosis of HCC is to be considered. Simultaneous measurement of serum AFP and Ang-2 may enhance the sensitivity of HCC detection. [Shereen Shoukry Hunter, Maha Sayed Hassab-Allaah, Mahmoud Ahmad El-Ansary, Faten M El Shanawani and Mona M Hassan. **Significance of Angiopoietin-2 as a Serum Marker for Hepatocellular Carcinoma**. Journal of American Science 2010;6(12):1114-1123]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** Hepatocellular carcinoma (HCC)**,** Alpha-fetoprotein (AFP)**,** Angiopoietin-2 (Ang-2)	Full Text	126
127	**Double -Control, Randomized Study of Antibiotic Prophylaxis during Standard Dose Chemotherapy in Cancer Patients** Fatma Zakaria*[1] and Mohamad Zakaria[2] Departments of Clinical Oncology[1] and Microbiology[2], Faculty of Medicine, Tanta University, Tanta, Egypt **Abstract:** Background: Dilemma of antibacterial prophylaxis after chemotherapy still opened. Patients and methods:Double, control trial in patients who were receiving cyclic chemotherapy for solid tumors or lymphoma and who were at risk of temporary, sever neutropenia (fewer than 500 neutrophils/ml). Patients were randomly divided into two group, the first groups assigned to receive oral 500 mg of quinolone once daily for seven days during the expected neutropenic period, while the second group received no	Full Text	127

	prophlaxis (control group). The primary end point was the incidence of clinically documented febrile episodes (FE) (temperature of more than 38°C) due to infection. Assessment of the risk of FE in controll group on first versus non first cycles with or without first cycle FE in the light of different pretreatment factors. Secondary end point included the incidence of all infections, severe infections, hospitalization and cost. Results: A total of 403 patients randomly divided into 201 patients received antibacterial prophylaxis quinolone (levofloxacin®) and 202 patients as control group. The tumors included breast cancer 238 (59.1 percent), lung cancer 82 (20.3%), testicular cancer 34 (8.4%) and lymphoma 49 (12.2%). During the first cycle of chemotherapy, 3.5% of patients in the quinolone group had at least one febrile episode, as compared with 8.4% in the control group (P=0.009).The per- cycle FE rate for the first cycle was 8.4% compared with 4.4% in non first cycles in control group. During the entire chemotherapy course, 9.5% of patients in the quinolone prophylactic group had at least one febrile episode; as compared with 16.3% in the control group (P ≤0.005).There was significant reduction in the rate of G3&G4 neutropenia in quinolone group (52%). The respective rates of infections were 33.8% and 42.1% (p=0.098) for quinolone versus control group. Hospitalization was required for treatment of infection in 3% of patients in the quinolone group and 7% of patients in the control group (P≤0.05). Respective rates of reduction of cost and length of stay (LOS) were 51.8% and 51.6% for infections in quinolone prophylactic group. Respective rates of sever infections were 1.0% and 2.0% (p≤0.06), for quinolone and control group, with one infection related death in each group. An organism was isolated in 194/250 cycles (77.6% of infections). Conclusions: Quinolone prophylaxis (levofloxacin is preferred) should be offered to those receiving standard dose chemotherapy for solid tumors and lymphomas to reduce incidence of fever, infection, hospitalization and cost with rational selection of patients for antibacterial prophylaxis with first cycle chemotherapy. [Fatma Zakaria[*1] and Mohamad Zakaria. **Double -Control, Randomized Study of Antibiotic Prophylaxis during Standard Dose Chemotherapy in Cancer Patients**. Journal of American Science 2010;6(12):1124-1135]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** Antibiotic Prophylaxis; standard Dose; Chemotherapy; Cancer; Patient		
128	**Protective Effect of Broccoli and Red Cabbage Against Hepaocellular Carcinoma Induced by N-Nitrosodiethyamine in Rats** Aml F. M. Morsy*[a], Hodaa S. Ibrahim[a] and M. A. Shalaby[b] [a] Nutrition and Food Science Department, Faculty of Home Economics, Helwan University, Egypt. [b] Pharmacology Department Faculty of Veterinary Medicine, Cairo University, Egypt. * dr_aml_fawzy@yahoo.com **Abstract:** The hepatoprotective effect of broccoli and red cabbage extracts against hepatocellur carcinoma induced by N- Nitrosodiethyamine (NDEA) in male rats were studied. Four groups of rats were used; group (1) was used as a negative control (normal), while rats of the other groups were given NDEA as a single interperoitenial dose with subcutaneous injection of carbon tetrachloride (CCl4) once weekly for six weeks to induce hepatocellular carcinoma. Group (2) was left as a positive control, while groups (3) and (4) were pretreated with broccoli and red cabbage 10%extract, for 12 weeks, respectively. At the end of the experiment, blood samples were taken for biochemical analysis and liver tissues were histopathologically examined. The obtained results revealed that rats with hepatocellur carcinoma (HCC) had significant increase in serum levels of AST, ALT, ALP, total protein, albumin, total and direct bilirubin and malondialdehyide (MDA), as well as significant decrease in reduced glutathione (GSH), glutathione peroxidase (GPX), superoxide dismutase (SOD) and catalase (CAT) enzymes, compared to the normal control group. Liver sections of rats with HCC showed fatty infiltration of hepatocytes, cytomegaly with karyomegaly as well as vesicular active nuclei and presence of more than one nucleolus in some hepatocytes. Oral administration of broccoli and red cabbage extracts caused significant reduction in serum levels of AST, ALT, ALP, total protein, albumin, total and direct bilirubin as well as MDA and produced significant increase in GSH, GPX, SOD and CAT, compared to the positive group. Liver of these rats revealed only slight hydropic degeneration of hepatocytes, while other sections showed apparent normal hepatocytes. This study concluded that broccoli and red cabbage have a protective effect against	Full Text	128

	hepatocellular carcinoma in rats, therefore this study recommends increased dietary intake of broccoli and red cabbage may be beneficial for patients with liver cancer as a preventative measures. [Aml F. M. Morsy, Hodaa S. Ibrahim and M. A. Shalaby. **Protective Effect of Broccoli and Red Cabbage Against Hepaocellular Carcinoma Induced by N- Nitrosodiethyamine in Rats**. Journal of American Science 2010;6(12):1136-1144]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** Broccoli; Red cabbage; Liver cancer; Biochemistry; Histopathology; Rat		
129	**Eating Behavior and Problems in Egyptian Adolescents; Relation to Dietary Intake** Zeinab M. Monir*; Abla G Khalifa; Fawzya Hassaballa; Sawsan Tawfeek; Mohamed Abdelmonem, Moönes Abu Shady and Manal Mansour Child Health Department, National Research Centre, Dokki, Giza, Egypt. *zeinab_monir@yahoo.com **Abstract:** Objective of this study is to examine the presence of disordered eating (ED) behavior among Egyptian adolescent boys and girls and detect the influence of obesity, body image, depression, somatic symptoms, binging and weight teasing by peers and family members as well as assessing dietary intake of macronutrients and micronutrients and its correlation to obesity and eating disorder. *Subjects and Methods*: The sample consisted of 1124 adolescents (642 girls & 482 boys) aged from 14-17 years, divided according to their BMI into four groups. The questionnaires used were EAT, ACDI, body image, and teasing, 24hr- dietary recall. and sociodemographic data were collected. *Results*: we found that 25.5% & 38.6% of boys and girls reported ED that was significantly correlated to body image, bad eating habits, depression and somatic symptoms. ED is more prevalent among overweight-obese adolescents of high social class. Adolescents have deficient intake of vitamin A, calcium, thiamine and niacin; girls are more deficient in iron and boys are deficient in vitamin C. On assessing weight teasing by peers and family member by weight status and ED after adjustment for socioeconomic standard; there was statistically significant association with obesity in girls & boys. *Conclusion*: Social back ground, obesity, negative body image and depression and teasing are the main risk factors for developing ED. Early detection and intervention for ED by biological and psychological approaches, treatment of overweight and obesity using family based treatment; early detection of depression and encouraging sports practice are recommended. [Zeinab M. Monir; Abla G Khalifa; Fawzya Hassaballa; Sawsan Tawfeek; Mohamed Abdelmonem, Moönes Abu Shady and Manal Mansour. **Eating Behavior and Problems in Egyptian Adolescents; Relation to Dietary Intake**. Journal of American Science 2010;6(12):1145-1159]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords**: eating disorder (ED), obesity, depressions, body image, teasing	Full Text	129
130	**Raising Awareness of Deaf Students and their School Care-Givers about First Aid Intervention in Medical Emergencies** Rabab El-Sayed Hassan*[1] and Samar Elhoseiny Abd-Elraouf[2] [1]Pediatric Nursing, [2]Community Health Nursing, Faculty of Nursing, Mansoura University. Mansoura, Egypt, *biboelsayed@yahoo.com **Abstract:** Objectives: To assess and raise the awareness of deaf students and their school care-givers about first aid intervention in medical emergencies. Participants: All deaf students in both the preparatory and secondary levels of education in a school for the deaf, who were under 18 years old (n = 44), in addition to school dormitories care-givers (n = 2) were participated in this study. Research Hypothesis: An implementation of first aid intervention program would have a positive outcome on raising awareness of deaf students and their school care-givers in medical emergencies. Methods: Participants' knowledge and skills were assessed using pre and post test questionnaire sheet contained thirty seven quiz multiple choices statement questions in Arabic language. Moreover, post-test intervention didactic and practical learning sessions consisted of six video films on DVD-ROM are presented to the participants accompanied by sign language translation in order to achieve the research objectives. Results: An intervention program showed	Full Text	130

	a clear positive outcome on raising awareness of deaf students and their school care-givers about first aid intervention in medical emergencies. The highest percentage of deaf students (61.4%) obtained the lowest sum score lies between zero to less than 25% in the pre-intervention phase, while about half of them (45.5%) obtained sum score lies between 50 to less than 75%, and more than tenth (11.3%) obtained the highest sum score that lies between 75 to 100% in the post-intervention phase, which revealed statistical significant differences in the participants' knowledge of skills at p=0.001 and 0.000. Similarly, pre knowledge sum scores of the two school care-givers about first aid skills rose from 43.2% and 63.2% respectively reached to the mastery level of 100% in response to the study intervention programmed. Conclusion: Although not enough for all items to be statistically significant, first aid intervention program raised the awareness of deaf students and their school care-givers. [Rabab El-Sayed Hassan and Samar Elhoseiny Abd-Elraouf. Raising Awareness of Deaf Students and their School Care-Givers about First Aid Intervention in Medical Emergencies. Journal of American Science 2010;6(12):1160-1168]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** Deaf children; First aid; school-age; Care-givers		
131	**Sudeck's Atrophy, Hyperhydrosis and other Hypersympathetic Syndromes, what is the Recent Proper Surgical Management?** Abulazaym A.A.[1*] and Horria H.[2] [1]Neurosurgery Department, Faculty of medicine, Cairo University, Cairo, Egypt. [2]General surgery Department, Faculty of medicine, Mansoura University, Mansoura, Egypt. abaaaza51@yahoo.com* **Abstract:** Twenty eight patients with upper limb Sudeck's atrophy (minor causalgia), hyperhydrosis (palmar and axillary) and causalgia were submitted to endoscopic transthoracic sympathectomy as a definitive treatment. There were 9 patients with Sudeck's atrophy, 16 patients with upper limb hyperhydrosis and 3 patients with major causalgia. The procedure was successful in curing 26 patients (92.86%) and gave mild improvement in two patients (7.14%) whom belonged to the Sudeck's atrophy (minor causalgic) group because of the advanced dystrophic changes in their limbs. The commonest side effects were compensatory sweating. The procedure is effective, very simple, and required only two nights stay, and is recommended as a method of choice for the surgical treatment of hypersympathetic syndromes of the upper limbs as Sudeck's atrophy, hyperhydrosis and major causalgia. [Abulazaym A.A. and Horria H. **Sudeck's Atrophy, Hyperhydrosis and other Hypersympathetic Syndromes, what is the Recent Proper Surgical Management**. Journal of American Science 2010;6(12):1169-1174]. (ISSN: 1545-1003). http://www.americanscience.org. Keywords: **Sudeck's Atrophy, Hyperhydrosis and other Hypersympathetic Syndromes, what is the Recent Proper Surgical Management**	Full Text	131
132	**Reconstructive Cervical Laminoplasty with the Preserved Fixed Spinous Processes Row as an Intervening Bone Graft; a Successful Novel Surgical Approach.** Abulazaym A.A.[*1] and Meziad M.[2] [1]Neurosurgery Department, Faculty of Medicine, Cairo University, Cairo, Egypt [2]Orthopedic Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt abaaaza51@yahoo.com* **Abstract: Study design:** A prospective study to evaluate the outcome of a novel operation for cervical	Full Text	132

	myelopathy secondary to cervical canal stenosis; the Reconstructive Cervical Laminoplasty with the preserved fixed spinous processes row as an intervening bone graft. **Objective:** To explore a more effective, less invasive and more physiological operative technique for cervical myelopathy of cervical spinal canal stenosis. **Background:** The popular two cervical laminoplasties for the nowadays becoming common in elderly people cervical myelopathy of cervical spinal canal stenosis, i.e. open door laminoplasty and double doors laminoplasty are plagued with many drawbacks such as around 50% diminution in the range of cervical movements, 25% occurrence of kyphotic deformity, laminar fusions, from 10% to 50% chronic axial neck pain and nuchal musculature atrophy. A more physiological modification of this very beneficial operation is badly needed. We presented our novel reconstructive cervical laminoplasty with the preserved fixed spinous processes row as an intervening bone graft to avoid such drawbacks. **Methods:** This prospective preliminary study included 14 patients who underwent the novel reconstructive cervical laminoplasty with the preserved fixed spinous processes row as an intervening bone graft operation for their cervical myelopathy. **Results:** The novel operation is proved to be easier, more physiological and succeeded to avoid to a great extent the aforementioned drawbacks of the two popular cervical laminoplasties; only about 30% diminution of cervical movements occurred, no kyphotic deformities, post-operative axial neck pain was moderate and occurred in only 21% of the patients and the post operative nuchal musculature atrophy was avoided. **Conclusion:** Cervical myelopathy secondary to cervical spinal canal stenosis can be managed adequately with our novel cervical reconstructive myelopathy with the preserved fixed spinous processes row as an intervening bone graft. This technique obtained satisfactory outcomes and avoided the drawbacks of the popular laminoplasty operations. It can be a standard procedure for the surgical treatment of this nowadays becoming common disease. [Abulazaym A.A. and Meziad M. **Reconstructive Cervical Laminoplasty with the Preserved Fixed Spinous Processes Row as an Intervening Bone Graft; a Successful Novel Surgical Approach**. Journal of American Science 2010;6(12):1175-1180]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** Reconstructive Cervical Laminoplasty; Spinous; Processes Row; Intervening Bone Graft; Novel		
133	**The Effectiveness of the Intervention Program on the Attitude and Self-Concept of Students with Dyslexia** Zeinab, Mihandoost[1], Prof. Habibah Elias[2], Prof. Sharifah Nor[3], Dr. Rosnaini Mahmud[4] [1]Faculty of Educational Studies, University Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia. [2] Faculty of Educational Studies, University Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia. [3] Faculty of Educational Studies, University Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia. [4] Faculty of Educational Studies, University Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia. xozns2006@yahoo.com **Abstract:** The purpose of this study is to investigate the effect of the Barton Intervention Program on the attitude and self-concept of dyslexic students. The Dyslexia Screening Instrument (DSI), and Reading Text were employed in order to identify the dyslexic students in schools in Ilam, Iran. The population of the study included 138 dyslexic students studying in elementary schools in Ilam, Iran and from this population, 64 students were selected randomly and assigned equally to an experimental group and a control group (32 students in each group). The experimental group was taught for 36 sessions using the Barton method, in two levels, and ten lessons were provided to improve their reading skills. Reading attitude and self-concept to read instruments were employed to measure their attitude and self-concept, before and after the	Full Text	133

	intervention program. The reliability of the reading attitude and self-concept were confirmed. The content validity of the scales was investigated using the judgment of 10 psychology experts. The analysis of the finding through independent t-test showed a significant difference between the control group and the experimental group after the intervention, at $\rho<0.000$. [Zeinab, Mihandoost, Prof. Habibah Elias, Prof. Sharifah Nor, Dr. Rosnaini Mahmud. **The Effectiveness of the Intervention Program on the Attitude and Self-Concept of Students with Dyslexia**. Journal of American Science 2010;6(12):1181-1191]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** Intervention program; attitude; self-concept; dyslexia		
134	**Evaluation of two different implant designs for immediate placement and loading in fresh extraction sockets** Amr Zahran[1], Hisham Samy[2], Basma Mostafa [3], Ramy Rafik[4] 1. Professor, Department of Periodontology, Cairo University, Cairo, Egypt 2. Assistant Professor, Department of Periodontology, Cairo University, Cairo, Egypt 3. Researcher, Department of Surgery and Oral Medicine, National Research Centre, Cairo, Egypt 4. Assistant Researcher, Department of Surgery and Oral Medicine, National Research Centre, Cairo, Egypt **Corresponding author:** Dr. Basma Mostafa Zaki, BDS, MDS, PhD Researcher, Department of Surgery and Oral Medicine, National Research Centre, 33 El Bohouth Street Dokki- Cairo- Egypt-12311, Phone: +202 0124901019; E-mail: boshta@hotmail.com **Abstract:** This study was conducted to compare between two self-tapping, self-drilling tapered one-piece implant designs used for immediate post-extraction placement with the immediate loading protocol. **Materials and Methods**: Ten patients (6 males and 4 females), with a mean age of 28.5 years (range 18-39 years) were included in this study. All selected patients had two or more maxillary unrestorable hopeless anterior or premolar teeth indicated for extraction. Each patient received two implants of different designs (The OsteoCare™ Midi and Maxi-Z implants) which were placed in fresh extraction sockets and immediately loaded. Clinical criteria were survival rate, papillary bleeding index, probing depth, gingival index, Periotest M values, crestal bone level and bone density. An overall survival rate of 100% was attained. **The results** showed no significant difference in both the bleeding index and gingival index scores and also in the probing depth values, bone density measurements and crestal bone level for both implant designs after 3 and 6 months. The mean and the standard deviation of the Periotest M values (PTMV) for the Midi and the Maxi-Z implants immediately post operative were (-1.83±0.8) and (-2.57±0.9) and after 6 months were (-3.06±0.7) and (-3.11±0.7) showing a significant difference immediately postoperative and no significant difference after 6 months. Surface area analysis revealed that there is a direct relation between the initial stability and the surface area. **Conclusion**;It can be concluded that the immediate implant placement and loading using both designs is a successful treatment modality and the prognosis depends on proper case selection and treatment planning. [Amr Zahran, Hisham Samy, Basma Mostafa, Ramy Rafik. **Evaluation of two different implant designs for immediate placement and loading in fresh extraction sockets**. Journal of American Science 2010;6(12):1192-1199]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** Dental implants, immediate implant, immediate loading, two implant designs	Full Text	134

135	**Grape seed extract alleviate reproductive toxicity caused by aluminium chloride in male rats** Hala, A.H. Khattab [a], Inas, Z.A. Abdallah[a] and Gehan, M. Kamel [b] [a] Nutrition and Food Science Department, Faculty of Home Economics, Helwan University [b] Pharmacology Department, Faculty of Veterinary Medicine, Cairo University haya_khattab@hotmail.com **Abstract:** Natural dietary antioxidants are studied for their ability to protect cells from miscellaneous damage. Grape seed extract (*Vitis vinifera* L., Vitacease) is a potent antioxidant. The present study aimed to investigate the protective effect of grape seed extract (GSE) against the possible testicular dysfunction caused by aluminium chloride (AlCl3) in male rats. Twenty sexually mature male albino rats were divided into four equal groups, the first served as negative control, the second received AlCl3 (20 mg/kg bw, 1/ 20 LD 50), the third administered GSE (75 mg/kg bw), and the fourth received AlCl3 and treated with GSE. Doses were given once daily via gavage for 70 consecutive days. The results revealed that, AlCl3 induced significant decrease in final body weight, sex organs relative weight, sperm concentration, motility and viability, serum testosterone concentration and superoxide dismutase (SOD) activity, with significant increase in sperm abnormalities and thiobarbituric acid reactive substance (TBARS) concentrations. Moreover, AlCl3 induced apparent alteration in the histological structure of the testis. Treatment with GSE ameliorated the harmful effects of AlCl3, this was also proved histopathologically by the noticeable improvement in the testis tissues . It may be concluded that GSE may be promising as a natural therapeutic agent in AlCl3-induced reproductive toxicity and oxidative stress in the male rat testes. [Hala, A.H. Khattab, Inas, Z.A. Abdallah and Gehan, M. Kamel. **Grape seed extract alleviate reproductive toxicity caused by aluminium chloride in male rats**. Journal of American Science 2010;6(12):1200-1209]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** Grape seed extract- aluminium chloride- reproductive- experimental animals	Full Text	135
136	**Assessment of Body Composition, Fat Distribution and Serum lipid Profile in Obese School Children** Nayera E. Hassan[1], Sahar A. El-Masry[*1], Rokia A. El Banna[1], Mona Salam[2], Azza M Sarry El-Din[1], Tarek S Ibrahim[2] and Mona Anwar[3] [1]Biological Anthropology Dept., [2] Child Health Dept., [3]Medical Biochemistry Dept., National Research Centre, Dokki, Giza, Egypt [*]masrysa@yahoo.com **Abstract:** Objective*:* To determine the relationship between body compositions, fat distribution and blood lipid profile in obese school children aged 7 to 18 years. Methodology*:* In this cross-sectional study, 150 pupils between the ages of 7 to 18 years were included. Anthropometric measures of adiposity (BMI, waist circumference, waist-to-hip ratio, peripheral adiposity: as the sum of triceps and biceps skinfold thickness, central adiposity: as the sum of sub scapular, suprailiac and abdominal skinfold thickness), body composition and serum total lipids profile were assessed. Results*:* There are significant sex differences in ages 7 -18 years regarding BMI, abdominal skinfold thickness and TC/ HDL-C, and in peripheral adiposity at young age (7-11 years) and central one at adolescents (12-18 years). Body composition and fat distribution showed significant sex differences in adolescent period only; and in fat distribution in young age period. For young age, triglycerides and HDL-C are correlated to most of the body composition and anthropometric parameters in boys and not in girls. For adolescents, there is no correlation between any one of the lipid profile and the body composition and anthropometric parameters in either gender. Conclusion*:* This study has shown that in comparison to girls, the correlation of body composition, fat distribution and lipid profiles were higher in boys aged 7 – 11 years only, with a tendency to develop the higher risk level of cardio vascular disease. Particular attention should be focused on the time prevention of	Full Text	136

	childhood obesity. [Nayera E. Hassan, Sahar A. El-Masry, Rokia A. El Banna, Mona Salam, Azza M Sarry El-Din, Tarek S Ibrahim and Mona Anwar. **Assessment of Body Composition, Fat Distribution and Serum lipid Profile in Obese School Children**. Journal of American Science 2010;6(12):1210-1217]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** Body composition, Anthropometry, fat distribution, lipid profile, School children, Risk of cardiovascular disease		
137	**Evaluate of Head Loss, Sediment Value and Iron Removal in Rapid Sand Filter** Hossein Banejad [1], Reza Pirtaj Hamedany [1], Navab Daneshi [1] [1.] Department of Water Engineering, Faculty of Agriculture, Bu-Ali Sina University, Hamedan, Iran Hossein_banejad@yahoo.com **Abstract:** Quality and appropriate quantity of water is necessary for human kind to survive. Along with the technology development and increasing consumption of water resources, we are experiencing low qualities in the mentioned resources. Iron is the fixed element found in the crust of the earth. This metal found variously in water resources and industrial activities. Therefore, it needs to treat the water resources from these excessive amounts. Different methods have used for this reason but the most used method during recent years has been the absorption by economic absorbers such as sand. Rapid sand filters usually used in water treatment plants for water clarification. In this research, a single layer gravity rapid sand filter has used to reduce different concentrations of iron. sediment value and head loss arising from it specially oxidized iron sediments in filter media is simulated by using combination of Carman-Kozeny, Rose and Gregory models in different discharges of rapid sand filter. Results have shown that with increasing in discharge and decreasing in input iron concentration, arriving time to given head loss, is increasing. In addition, results demonstrated that with increasing in iron concentration in influent, removal efficiency is decreasing somewhat. Results of this research can applied in (1) appropriate design of rapid sand filter to iron removal, (2) prediction of rapid sand filter ability to iron removal and (3) estimation of arising head loss during filter work thus evaluating of time interval backwash. [Hossein Banejad, Reza Pirtaj Hamedany, Navab Daneshi. Evaluate of Head Loss, Sediment Value and Iron Removal in Rapid Sand Filter [Hossein Banejad, Reza Pirtaj Hamedany, Navab Daneshi. **Evaluate of Head Loss, Sediment Value and Iron Removal in Rapid Sand Filter**. Journal of American Science 2010;6(12):1218-1226]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** Sand filter, Iron concentration, Removal efficiency, Head loss	Full Text	137
138	**Comparative Study Of Isocratic And Gradient Elution Chromatography In Stability Indicating Assay Of An Antihypertensive Drug Combination.** M. Abdel Kawy*, A.E. El-Gendy**, E.S. Shokry** * Faculty of pharmacy, Cairo university ** Faculty of Pharmacy, Misr International University **ABSTRACT:** Inspite that chromatographers are cautioned to avoid gradient elution when isocratic elution will do. In this work, the analytical properties of gradient and isocratic elution applied to separation of a complex sample of (fosinopril, hydrochlorothiazide and their degradation products) which can be done under isocratic condition are compared. Procedures were developed for determining fosinopril and hydrochlorothiazide in presence of each other and their degradation products by HPLC in the gradient	Full Text	138

	elution mode using methanol- 20 mM KH_2PO_4 (PH 2.4) containing 0.1% triethyl amine. In the isocratic mode, the same mobile phase composition was applied in a constant ratio of 60: 40 (Buffer: methanol). Separation was achieved on a cyanopropyl column (4 x 250 mm, 5 µm) known for its high selectivity for polar and hydrophilic compounds and the least retentive of hydrophobic compounds which do not normally elute on standard C18 or C8 columns. The present work shows that gradient elution gave a shorter overall analysis time with similar resolution of the critical pair without sacrificing repeatability in parameters, so many of the reasons given to avoid gradient elution deserve serious reconsideration especially for those samples that can be separated isocratically. [M. Abdel Kawy, A.E. El-Gendy, E.S. Shokry. **Comparative Study Of Isocratic And Gradient Elution Chromatography In Stability Indicating Assay Of An Antihypertensive Drug Combination.** Journal of American Science 2010;6(12):1227-1236]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** Comparative Study; Isocratic And Gradient Elution Chromatography; Stability; Antihypertensive Drug Combination		
139	**Effect of *Rhazya stricta* extract on rat adiponectin gene and insulin resistance.** Nabih A. Baeshen[1], Sahira A. Lari[2], Huda A. R. Al Doghaither[1] and Hassan A. I. Ramadan[1, 3] [1]Department of Biological Sciences, Faculty of Sciences, King Abdul-Aziz University, Jeddah [2]Department of Biochemistry, Faculty of Sciences, King Abdul-Aziz University, Jeddah [3]Department of Cell Biology, National Research Centre, Dokki, Egypt **Abstract:** *Rhazya stricta* plants have always played a major role in the treatment of human and animal diseases. The aim of this study was to study the effect of different doses of *Rhazya stricta* extract administered orally to rats, treatment period, effect on adiponectin protein, insulin resistance and finally its effect on exon 3 of adiponectin gene. Oral administration of aqueous leaves extracts of *Rhazya stricta* evoked fluctuations in adiponectin levels during eighteen weeks period of treatment. Serum adiponectin levels showed a significant increase after 2 and 4 weeks of treatments. Also a highly significant increase in adiponectin level, compared with the control group, was detected in rats treated with 0.125 gm/ml and 0.150 gm/ml after eighteen weeks of treatment. Insulin resistance is an important risk factor for type II diabetes mellitus and cardiovascular disease. Therefore, we performed HOMA-IR to check the degree of insulin resistance in rats. The results showed an inverse highly significant correlation between adiponectin levels and insulin resistance degrees after two weeks of treatment with *Rhazya stricta*. Studies published to date indicate that polymorphisms at the adiponectin gene (exon 3) are indeed predictors of circulating adiponectin levels. However, our results showed a significance increase in adiponectin levels, we did not detect any rare mutation in this locus using CSGE technique. The effects of *Rhazya stricta* extract on the increase of adiponectin levels concentrations could be promising issue (after avoiding its possible mutagenic effects) in treating diabetes, carbohydrate metabolism, hypertension, as well as inflammatory conditions. [Nabih A. Baeshen, Sahira A. Lari, Huda A. R. Al Doghaither and Hassan A. I. Ramadan. **Effect of *Rhazy stricta* extract on rat adiponectin gene and insulin resistance**. Journal of American Scienc 2010;6(12):1237-1245]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** *Rhazia stricta*; rat adiponectin gene, exon 3; insulin resistance; mutagenicity	Full Text	139
140	**Biomonitoring Of Aquatic Ecosystem With Concept And Procedures Particular Reference To Aquatic Macro invertebrates** Shailendra Sharma, Praveen Sharma *Department of Zoology, Shari Umiya Girls College, Mandleshwar,(M.P.) India	Full Text	140

	Department of Chemistry, Narmada Samagra, Bhopal,(M.P.) India shailendra.b.sharma@gmail.com **ABSTRACT: The "biological monitoring" has been widely used to assess the environmental impact of pollutant discharges. The methodology must be evaluated in terms of false positives and false negatives. A false positive is an indication that an excursion beyond previously established quality control conditions (i.e., unacceptable conditions) has occurred when, in fact, one has not. A false negative is an indication that conditions are acceptable when, in fact, they are not. Statistics must play a more important role in biological monitoring because they are capable of explicit statements of confidence in the biological monitoring results. With appropriate statistical evaluation of the data, professional judgment on whether to initiate immediate action or wait for more confirming data will be more objective and reliable. In order to optimize the usefulness of biological monitoring, the selection of biological monitoring methodology shall not be based on the investigator's favorite organism or group of organisms. Neither can be a convenient methodology adopted by regulatory agencies. The selections must be based on the compatibility of data generated with the decision making process, including the statistical establishment of confidence in the result obtained. [Shailendra Sharma, Praveen Sharma. **Biomonitoring Of Aquatic Ecosystem With Concept And Procedures Particular Reference To Aquatic Macro invertebrates**. Journal of American Science 2010;6(12):1246-1255]. (ISSN: 1545-1003). http://www.americanscience.org. **Key words:** Biomonitaring, bioindicator, diversity indices, saprobic indix, macroinvertebrates		
141	**Effect of different phosphatic fertilizers on growth attributes of wheat (Triticum aestivum L.)** Muhammad Bilal Khan[1], Muhammad Iqbal Lone[1], Rehmat Ullah[2*], Shuaib Kaleem[3] and Muhammad Ahmed[3] [1]Department of Soil Science & SWC, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi [2]Soil and Water Testing Laboratory, Rajanpur-Punjab-Pakistan [3]Agriculture Adoptive Research Complex, Dera Ghazi Khan, Punjab-Pakistan rehmat1169@yahoo.com **Abstract:** Among all the elements required by a plant, phosphorus (P) is one of the most important nutrients for crop production and emphasis is being given on the sufficient use of P fertilizer for sustainable crop production. A pot experiment was conducted in green house at the Department of Soil Science and SWC, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi during Rabi season, 2007. Phosphorus was applied at the rate of 40 and 80 kg P ha^{-1} in the form of SSP, TSP, NP and DAP. A basal doze of 100 kg N and 60 kg K ha^{-1} was applied as urea and murate of potash (MOP) respectively. All the growth parameters of wheat were significantly improved by addition of P application. It was concluded from the study that phosphorus application at the rate of 80kg P ha^{-1} as single super phosphate (SSP) showed better results as compared to triple super phosphate (TSP), nitrophos (NP) and diammonium phosphate (DAP) on phosphorus deficient soil of Balkasr area of Tehsil Chakwal. [Muhammad Bilal Khan, Muhammad Iqbal Lone, Rehmat Ullah, Shuaib Kaleem and Muhammad Ahmed. **Effect of different phosphatic fertilizers on growth attributes of wheat (Triticum aestivum L.)**. Journal of American Science 2010;6(12):1256-1262]. (ISSN: 1545-1003). http://www.americanscience.org. **Key Words:** Phosphorus, Wheat, Growth Attributes, P Fertilizer, calcareous soil	Full Text	141
142	**A new categorization of construction materials based on sources of waste across supply chain**	Full Text	142

Mohamad Reza Parsanejad[1], Mansor Momeni[2], Ahmad Jafarnejad[3], Ali Mohaghar[4]
[1,2,3,4] Department of Industrial Management, Faculty of Management, University of Tehran parsanejad@ut.ac.ir

Abstract: Construction industry is an important part of any economy. But it does not have an appropriate performance especially in the productivity of materials. Statistics show production of billions of tons of construction waste per year in the world, and these issues threaten all beneficiaries of this industry. Thus, convenient strategies should be founded for improving waste production. This will not be achieved unless we recognize waste sources across construction supply chain. Also each material has its own source of waste, therefore exact identification of any material and after that its source will help to develop waste minimization strategies. In this research 30 questionnaires were distributed between experts. At first we prioritized waste sources, and by following the question about impact of sources on selected material, using binominal test, it observed that a category of sources had impact on some of material and another sources on another materials. Analysis of these two types of materials showed us that this result was not accidental and those materials when use in building, their dimensions is important (like brick, block, tile and etc.), those sources have impact on their waste that emphasize design parameters of building. Those material when use in building, their weight are important (like cement, gypsum, sand and etc.), those sources have impact on their waste that emphasize purchasing level of ordering and purchasing. Therefore materials categorized by their sources of waste across supply chain.
[Mohamad Reza Parsanejad, Mansor Momeni, Ahmad Jafarnejad, Ali Mohaghar. **A new categorization of construction materials Based on sources of waste across supply chain**. Journal of American Science 2010; 6(12):1263-1273]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Waste, Source of waste, Construction supply chain, Non-coordination, Dimensional, Weight based

143

Effect of miso (A soybean fermented food) on some human cell lines; HEPG2, MCF7 and HCT116

Full Text

Abeer Abu Zaid [1, 2] and Nahla S. El-Shenawy[2,3*]

[1]Food Technology Research Institute, Agriculture Research Center, Giza, Soy product Processing Center, Egypt. [2]Biology department, Faculty of Science, Taif University (Qurwa), Taif, Saudi Arabia.

[3]Zoology Department, Faculty of Science, Suez Canal University, Ismailia 41522, Egypt

elshenawy_nahla@hotmail.com

Abstract: The study was conducted to investigate the antitumor activity of miso, storage at different period or prepared with different starters, on human cell lines {HEPG2 (liver carcinoma), MCF7 (breast carcinoma), and HCT116 (colon carcinoma)}. The highest inhibitory effect on liver and breast carcinoma was seen when miso used after fermentation/aging zero time without storage period. Miso with different storage period (zero, 6 months and 5 years) has the same effect on colon carcinoma. Preparation of miso with different mixture of starters was also investigated on the same human tumor cell lines in culture. Miso prepared with *A. oryzae* and *Bacillus subtilis* starters inhibited the proliferation of human tumor cell lines culture with a wide variation in LC_{50} values (2.97, 3.37 and 3.37 µg/ml for MCF7, MCT116 and HEPG2, respectively). Miso prepared with *Aspergillus oryzae* and *Pleurotus ostreaus* starters inhibited human tumor cell line cultures with different LC_{50} values (10.9, 17.5 and 24.3 µg/ml for MCF7, MCT116 and HEPG2, respectively). The miso prepared with *A. oryzae* and *Rhizopus oryzae* effect only on MCF7 and HEPG2 with high LC_{50} values (25.5 and 35.8 µg/ml, respectively). We can conclude that the mixture of *A. oryzae* and *Bacillus subtilis* has the best effect among the other mixture of starters. The results indicated that all of fermented soybeans products with different mixture of starters contained higher isoflavones compounds than unfermented cooked soybeans. Moreover, soybean fermented with *B. subtilis* showed highest amount of isoflavones. Therefore, miso can be used as anticancer.

[Abeer Abu Zaid and Nahla S. El-Shenawy. **Effect of miso (A soybean fermented food) on some human cell lines; HEPG2, MCF7 and HCT116**. Journal of American Science 2010;6(12):1274-1282]. (ISSN:

143

	1545-1003). http://www.americanscience.org. **Keywords:** Miso; Human cell lines; Amino acid; Fatty acid; Isoflavones; *Aspergillus oryzae*; *Bacillus subtili; Rhizopus oryzae*; *Pleurotus ostreaus*		
144	Oxidative Stress in Brains of Rats Intoxicated With Aluminum and the Neuromodulating Effect of Different Forms of Sage EL-Kholy, W.M.; EL-Habibi, E.M. and Mousa, A.T. Zoology Dept., Faculty of Science, Mansoura University, Mansoura, Egypt dr_mona_zaki@yahoo.co.uk **Abstract:** The present study was designed to investigate the role of oxidative stress and the status of antioxidant system in the management of aluminum chloride (AlCl3) induced brain toxicity in rats and further to elucidate the potential role of three forms of *Salvia officinalis* (sage) in alleviating such negative effects. The results revealed that the levels of thiobarbituric acid reactive substances (TBARS) and protein carbonyl (PC) were significantly increased, however, the activities of superoxide dismutase (SOD) and catalase (CAT) as well as the reduced glutathione (GSH) content were significantly decreased in the cerebral cortex (Co) and hippocampus (Hip) of rats intoxicated with $AlCl_3$. Inhibition, the lipid profile, total lipids (TL), total cholesterol (TC) and triglycerides (TG) were significantly increased in serum and the mentioned brain regions, while phospholipids (PL), total protein (TP) and serum HDL-C were significantly decreased in $AlCl_3$ group. Additionally, serum and brain regions acetylcholinesterase (AChE) as well as alkaline phosphatase (ALP) and acid phosphatase (ACP) activities were significantly increased. On the other hand, the results exhibited that, sage when given in any form along with $AlCl_3$ was able to regulate the mentioned parameters and the values returned close to the normal ones. It can be concluded that Al-induced neuronal oxidative stress and inhibition of the antioxidant system, accompanied with disturbed lipid profile, total protein and enzyme activities could be the cause of $AlCl_3$ neurotoxicity. In addition there different sage forms, by their antioxidant constituents, could be able to antagonize Al neurotoxicity perhaps by reducing the oxidative stress and improving the antioxidant status and particularly by inhibiting the acetylcholinesterase activity, thus may improve memory and other brain cognitive activities. [EL-Kholy, W.M.; EL-Habibi, E.M. and Mousa, A.T. Oxidative Stress in Brains of Rats Intoxicated With Aluminum and the Neuromodulating Effect of Different Forms of Sage. Journal of American Science 2010;6(12):1283-1297]. (ISSN: 1545-1003). http://www.americanscience.org. **Key words:** Aluminum neurotoxicity- Alzheimer's disease- *Salvia officinalis* - Lipid peroxidation - Antioxidants-acetylcholinesterase	Full Text	144
145	**First Record of Microsporidium *Neonosemoides* Sp. and some Ciliates Infecting *Chrysichthus Auratus* (Bagridae) from the Damietta Branch of River Nile, Egypt** Enayat Salem Ahmed Reda Department of Zoology, Faculty of Science, Mansoura University, Mansoura, Egypt enayatSalem40@yahoo.com **Abstract:** The present study was carried out as a general survey for the possible ectoparasites that can infect the Nile fish *Chrysichthus auratus*. A total of 52 fish specimens were collected from Damietta branch of River Nile. Examination of the investigated fish revealed that, fish were infected with four ectoparasitic species belonging to three genera. These species were: *Neonosemoides* sp., *Scyphidia* sp. 1, *Scyphidia* sp. 2 and *Ichthyophthirius multifillis*. The first three species were recorded for the first time in Egypt. The recovered parasites have pathological effects on the host fish with subsequent economic losses were discussed. [Enayat Salem Ahmed Reda. First Record of Microsporidium *Neonosemoides* Sp. and some Ciliates	Full Text	145

	Infecting *Chrysichthus Auratus* (Bagridae) from the Damietta Branch of River Nile, Egypt. Journal of American Science 2010;6(12):1298-1305]. (ISSN: 1545-1003). http://www.americanscience.org. **Key words:** *Neonosemoides* sp., Ciliates, *Chrysichthus auratus*, River Nile. Egypt.		
146	**Biochemical studies on some cotton by products Part I- Chemical constituents and cellulose extraction of Egyptian cotton stalks** *****Fouad A. Ahmed; *Nadia M. Abdel-Moein ; **Amal S. Mohamed and **Shaimaa E. Ahmed *Agric. Biochemistry Dep., Fac. Agric., Cairo University ** Cotton Research Institute, Agricultural Research Center, Giza, Egypt shrose22@yahoo.com **ABSTRACT:** The main objectives of the current investigation are to compare some chemical constituents, mainly cell wall components (cellulose and lignin), of stalks of five Egyptian cotton cultivars, as a step to convert a low valued bio-wastes of cotton plant stalks into highly value product as pure cellulose, which will, also, contributed in solving major environmental and health problem in Egypt. Lignocellulosic raw material cultivars; Giza 80, Giza 85, Giza 89, Giza 86, and Giza 90 were used in this study. They were obtained from Cotton Research Institute experimental fields. As first stage, chemical analysis comparison among aforementioned cultivars was conducted. The results showed that, there were significant differences among the five studied cultivars in moisture, Lipids, wax, crude fibers and β cellulose contents. As coincides, ash, protein, holocellulose, hemicellulose, α cellulose and lignin percentages exhibits no significant differences among cultivars. The highest percentages of moisture estimated in Giza 89 (7.74%), also in ash and lignin (3.39% and 25.75%, respectively), but it was the lowest cultivar in wax percentage (2.43%). Giza 86 showed the highest percentage in lipids and crude fibers (1.96% and 46.92, respectively), also in protein and holocellulose percentages (5.12 and 77.26 %, respectively), but it was the lowest cultivar in β cellulose (1.11%) as well as ash (2.95 %). The highest percentage in wax and β cellulose estimate (3.67% and 2.72%, respectively) was in Giza 90, but it was the lowest cultivar in Lipids (0.96%) and hemicellulose (40.04%). The highest percentages in α cellulose (49.21%) was in Giza 80 which reflected the lowest percentage in the crude fibers (38.75%). The second stage was the preparation of cellulose by removing the waxes, lignin, and hemicellulose, since cotton stalk consists of 75±2% holocellulose percentage and 44±5% α cellulose %. The third stage was conducting physical test by analyzing the sample that was prepared by X-ray, then comparison with standard cellulose sample chart to confirm its structure as cellulose. [Fouad A. Ahmed; *Nadia M. Abdel-Moein; **Amal S. Mohamed and **Shaimaa E. Ahmed. Biochemical studies on some cotton by products Part I- Chemical constituents and cellulose extraction of Egyptian cotton stalks. Journal of American Science 2010;6(12):1306-1313]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** Cotton; stalk; Delignification; Hemicellulose; Cellulose; X-ray	Full Text	146
147	**Bacterial infections affecting marine fishes in Egypt** M. Moustafa [1], Laila. A. Mohamed[2], M.A. Mahmoud[3], W.S , Soliman[2], , A.E. Eissa [1] and M.Y. El-gendy[2] [1] Department of Fish Diseases and Management, Faculty of Veterinary Medicine, Cairo University. Giza, Egypt [2] Department of Hydrobiology, National Research Center, Dokki, Egypt [3] Department of Pathology, Faculty of Veterinary Medicine, Cairo University. Giza, Egypt [4] Corresponding Author	Full Text	147

	Abstract: Marine fishes are suffering from continuous depletion due to bacterial pathogens infections triggered by devastating environmental changes at their native aquatic environment. Qarun Lake and Suez Gulf are among the most vulnerable areas. 600 fish samples of Six different fish species; *Epinephelus tuvina*, *Sisganus rivulatus*, and *Dedlechilus labiosus* native to Suez-gulf at Suez governorate; *Mugil capito* , *Solea vulgaris* and *Tilapia zilli* native to Qarun Lake at El-Fayoum governorate were examined throughout the different year seasons. Gram positive and negative fish pathogenic bacteria were isolated from a total of 245 fish sample. Among those samples, the following bacteria were retrieved in the following percentages respectively, 17.55% (Vibrio*. anguillarum),* 16.73% (*Vibrio. alginolyticus*), 15.51% (*Pasteurella. piscicida*), 15.91% (*Pseudomonas. fluorescens),* 13.46% *(Streptococus. fecalis*), 11.02% (*Aeromans . hydrophila*), 6.12% (*Aeromans. sobria)* and 3.67% were infected with *Staph. aureus*. The *Siganus rivulatus* was the highest infected fish species with a prevalence of 8.33%, while *Mugil capito* was the lowest infected species (5.67 %). The highest total prevalence of bacterial infection was recorded in summer season (40.81%) while the lowest was recorded in winter (15.91%). The aforementioned bacterial isolates were successfully re-isolated from experimentally infected fish. The retrieved isolates were confirmed by semi-automated (API 20 E) and conventional biochemical tests. [M. Moustafa, Laila. A. Mohamed, M.A. Mahmoud, W.S, Soliman, A.E. Eissa and M.Y. El-gendy. **Bacterial infections affecting marine fishes in Egypt**. Journal of American Science 2010;6(12):1314-1324]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords**: Marine fishes, Bacterial diseases ,Diagnosis, seasonal variation		
148	**Clinical Prespective Of Repeat Breeding Syndrome In Buffaloes** Ahmed W.M., El-khadrawy H.H., Emtenan M. Hanafi , Amal H. Ali , Shalaby S.A. Department of Animal Reproduction and Artificial Insemination, National Research Centre Dokki,Cairo, Egypt. (wahidmma@hotmail.com) **ABSTRACT:** Local meat production in Egypt is in continuous decrease and can not meet the local market requirement. So this study was designed to throw light on true repeat breeding syndrome (RBS) as one of the reproductive disorders that hinders the buffalo meat and milk production. A field survey was carried out on 1358 female buffaloes which were subjected to clinical and gynecological examination , and blood samples were collected for carrying out some relevant analyses. Treatment trials were practiced using different ways to control the condition and the economic impact of this syndrome has been studied. Results revealed that the incidence of clinical repeat breeding (RB) in the examined buffalo cows was 4.34 %. Typical repeat breeders represented 7.25 % of total reproductive disorders in female buffaloes. Serum progesterone level was 1.44 ± 0.39 and 3.66±0.84 in RB and normal buffaloes (NB), respectively. Oxidant/antioxidant markers in RB buffalo-cows showed increased malondialdehyde (MDA) and nitric oxide (NO) and decreased catalase (CAT), superoxide dismutase (SOD), ascorbic acid (ASCA), reduced glutathione (R-GSH) and total antioxidant capacity (TAC). Serum zinc, copper,iron and selenium values were lower in repeat breeder cows compared to normal animals. Repeat breeder buffalo-cows responded to the treatments with mineral mixture, GnRH and Lugol‘s solution with recovery rates; 63.64, 61.54 and 60.00%, respectively. The study concluded that special care should be paid for food additives to control this syndrome. [Ahmed W.M., El-khadrawy H.H., Emtenan M. Hanafi , Amal H. Ali, Shalaby S.A. **Clinical Prespective Of Repeat Breeding Syndrome In Buffaloes**. Journal of American Science 2010;6(12):1325-1331]. (ISSN: 1545-1003). http://www.americanscience.org. **Key words:** Repeat breeding buffaloes - progesterone - oxidant/antioxidants and trace elements	Full Text	148
149	**Efficiency of Natural Minerals in Presence of Different Nitrogen Forms and Potassium Dissolving Bacteria on Peanut and Sesame Yields** Gehan H. Youssef, Wafaa M. A. Seddik and Mona A. Osman	Full Text	149

Soil, Water and Environ. Research Institute, agricultural Research Center (ARC), Giza, Egypt

ABSTRACT: A field experiment was carried out for two summer seasons at Ismailia Agric. Res. Station to study the effect of some natural minerals combined with potassium dissolving bacteria inoculation in the presence of different nitrogen forms on chemical properties of soil, nutritional status and yield of peanut-sesame. Each experiment was designed in a split-split design with three replications. Three forms of nitrogen fertilizer were included along with two natural minerals, in a presence of potassium dissolving bacteria inoculation, as well as one mineral fertilizer as source potassium fertilizer. Furthermore, data show high significant increases in available N due to the application of ammonium nitrate in combination with feldspar, and calcium nitrate in combination with potassium sulfate in a presence of inoculation for peanut and sesame, respectively. However, application of calcium nitrate combined with potassium sulfate, and ammonium nitrate in combination with feldspar, in a presence of inoculation, led to significant increases in K available in soil for peanut and sesame, respectively. Oppositely, the pH values, different to those of EC, decreased either for inoculation or non-inoculation as compared to control. In spite of that, the values of EC and pH of soil were higher with application of either bentonite or bentonite + feldspar in a presence of all nitrogen fertilizer forms. Generally, the highest EC values in soil, after the two studied seasons were encountered with calcium nitrate fertilizer as well as bentonite mineral. Moreover, applying feldspar mineral and ammonium nitrate treatments had recorded the highest values of yield components as well as nutrient (N and K) uptake by either peanut or sesame crops, particularly in the presence of inoculation as compared to those given by other treatments.

[Gehan H. Youssef, Wafaa M. A. Seddik and Mona A. Osman. **Efficiency of Natural Minerals in Presence of Different Nitrogen Forms and Potassium Dissolving Bacteria on Peanut and Sesame Yields**. Journal of American Science 2010;6(12):1332-1345]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Natural Mineral; Nitrogen; Potassium; Bacteria; Peanut; Sesame

150

Mitochoderial cytochrome c oxidase subunit 1 (*cox 1*) gene sequence of the *Hymenolepis* species.

Full Text

Omnia M. Kandil, Mona S. Mahmoud, Nesreen A.T. Allam, Amira H. El Namaky

Corresponding author: Kandil, O.M., Prof. of Parasitology, Parasitology and Animal Diseases Department, Veterinary Division, National Research Center, Dokki, Giza, Egypt, P.O. Box 12622, Fax +20233370931, Mobile +20105414113 kandil_om@yahoo.com

Mahmoud, M.S., Prof. of Parasitology, Parasitology and Animal Diseases Department, Veterinary Division, National Research Center, Dokki, Giza, Egypt, P.O. Box 12622, monasaid3000@yahoo.com

Allam, N. A. T., Researcher of Molecular Biology and Animal Diseases, Parasitology and Animal Diseases Department, Veterinary Division, National Research Center, Dokki, Giza, Egypt, P.O. Box 12622, nesreenallam_nrc@yahoo.com

El Namaky, A.H., Researcher of Parasitology, Parasitology and Animal Diseases Department, Veterinary Division, National Research Center, Dokki, Giza, Egypt, P.O. Box 12622, amiraelnamaky@g.mail.com

Abstract: *Hymenolepis nana* and *H. diminuta* are the most common cestodes in humans, domestic and wild rodents. Since isolates of *H. nana* species are morphologically identical, the way they can be reliably distinguished is comparing the parasite in each host using molecular techniques. In the current study, Mitochondrial Cytochrome *c* oxidase gene especially codons within subunit 1 (*cox1*) of *H. diminuta and H. nana* Egyptian isolates from different developmental stages (adult worms and eggs) and hosts origin (human and rat) were amplified, sequenced and aligned. PCR products were approximately 700 bp, 702 bp and 715 bp of *H. nana* rat isolates, *H. diminuta* rat isolates, and *H. nana* human isolates, respectively. Moreover, despite their host susceptibility differences they all gathered in one cluster with three genbank published isolates of *H. nana*; AB033412.1, AB494472.1 and AY121842.1), forming one clade with 100% similarity, which was non significantly decreased on internal nodes. In addition, clearly far away from *H. diminuta* published sequence AB033412.1 who's assumed to be genetically closely related to Egyptian *H. diminuta* than all other *H. nana* isolates. Both Egyptian murine isolates of Hymenolipidid; *H.*

150

	diminuta and *H. nana*, were closer to each other than being to *H. nana* of human origin. The annotated sequences of Egyptian isolates were deposited in GenBank under the following accession numbers; *H. diminuta* (GU433102), *H. nana* rat isolate (GU433103), and *H. nana* human isolate (GU433104). Finally, the development of effective control strategies will only be possible if complete understanding of the epidemiology of infestation is elucidated. [Omnia M. Kandil, Mona S. Mahmoud, Nesreen A.T. Allam, Amira H. El Namaky. **Mitochoderial cytochrome c oxidase subunit 1 (*cox 1*) gene sequence of the *Hymenolepis* species**. Journal of American Science 2010;6(12):1346-1353]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** Hymenolepidid, Phylogeny, Cytochrome *c* oxidase subunit 1 gene (*cox1*), Sequencing		
151	**Eusyllinae, Anoplosyllinae, and Exogoninae (Polychaeta: Syllidae) for the Mediterranean Coasts of Egypt, Together the Description of One New Species** F. A. Abd-Elnaby[*1] and G. San Martín[2] [1]National Institute of Oceanography and Fisheries, Alexandria, Egypt [2] Departamento de Biología (Zoología), Facultad de Ciencias, Universidad Autónoma de Madrid, calle Darwin, 2, 28049 Madrid, Spain. [*]faiza_abdelnaby@yahoo.com **Abstract:** In this paper, 18 species of the subfamilies Exogoninae, Anoplosyllinae, and Eusyllinae (Syllidae, Polychaeta) are reported from the Mediterranean Egyptian coasts, 8 of them are new records for the area: *Odontosyllis fulgurans* (Audouin and Milne Edwards, 1833); *Syllides japonicus* Imajima, 1966; *Salvatoria clavata* (Clapare de, 1863); *Salvatoria euritmica* (Sardá, 1984); *Sphaerosyllis glandulata* Perkins, 1981; *Parapionosyllis labornica* Cognetti, 1965; *Sphaerosyllis* sp.; and *Prosphaerosyllis* sp. Five species were reported previously in the area. Four species are new records for Mediterranean Sea: *Palposyllis prosostoma* Hartmann-Schröder, 1977; *Paraehlersia weissmaniodes* (Augener,1913); *Streptosyllis compoyi* Brito, Núñez and San Martín, 2000; and *Exogone africana* Hartmann-Schröder, 1974); *P. weissmaniodes* and *Exogone africana* are two widely distributed Indo-Pacific species, so they could be considered as Lessepsian migrants. Finally, one new species is described, *Parapionosyllis aegyptia*. [F. A. Abd-Elnaby[*1] and G. San Martín. **Eusyllinae, Anoplosyllinae, and Exogoninae (Polychaeta: Syllidae) for the Mediterranean Coasts of Egypt, Together the Description of One New Species**. Journal of American Science 2010;6(12):1354-1363]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** Eusyllinae**,** Anoplosyllinae, Exogoninae, Taxonomy, Mediterranean, Egypt, New species	Full Text	151
152	**Association of Depression and Anxiety Disorders with Weight Status among Egyptian School Children: Giza Governorate** Zeinab M Monir[a]; Abla G Khalifa[a] & Manal Mansour[b] *The child health department; National Research Center, Dokki, Giza; Egypt,* abla_ncr@yahoo.com. a Professor Child Health; b Assistant Professor Child Health *abla_ncr@yahoo.com. Tel.: 0123723398 zeinab_monir@yahoo.com. Tel.: 0122149533.	Full Text	152

mnlsalem@yahoo.com Tel.: 0101492207

Address: National Research Centre EL-Bohooth Street, Dkki,Giza, Egypt. Postal cod: 12311

Abstract: **Background:** The prevalence of childhood obesity is rapidly increasing, and many obese children suffer from emotional and behavior problems .**The aim** of this study was to explore the relationship between nutritional obesity and psychosocial behavior among school –children in their natural setting. ; and to examine whether social backgrounds play a role in this relationship. Target population was third; fourth and fifth grade primary school children (n=861; mean age10±0.72) attending 3 public elementary schools at Dokki District; in Giza Governorate. **Measurements:** Weight status was assessed through measurements of Body Mass Index percentiles (BAP) for age& sex using World Health Organization Growth Standards. Familial backgrounds**&** academic school achievements of the children were recorded from school files. Data on anxiety and depressive symptoms of children was assessed using standardized methods. **Results:** 23.5% of boys and18.7% of girls showed signs of depression; whereas anxiety was prevalent among 54%of boys and 52% of girls. Calculation of odds ratio (OR) showed that depression and anxiety is higher in low school achievers in girls ($p<0.05$) and boys ($p<0.01$). In a multiple regression model; depression was predicted by anxiety, age and academic achievements ($R^2=0.53$; $P\leq0.001$). Anxiety was predicted by BAP and birth order ($R^2=0.38$; $P\leq0.003$)**. Conclusion:** Obesity affects psychosocial adjustment of children raising the importance of early detection and prevention of obesity in the form of nutritional and health awareness programs and training of school health personnel.

[Zeinab M Monir[a]; Abla G Khalifa[a] & Manal Mansour. Association of Depression and Anxiety Disorders with Weight Status among Egyptian School Children: Giza Governorate. Journal of American Science 2010;6(12):1364-1373]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: Obesity - Children - Depression -Anxiety - Academic Performance

153

Effect Of Exercise On Plasminogen Activator Inhibitor-1(PAI-1) Level In Patients With Metabolic Syndrome

Seraag Esmat[*],Randa F Abd Al Salam[*],Lila Rashed[**]

[*]Department of Internal Medicine,Faculty of medicine,Cairo University.

[**]Department of Medical and Clinical Biochemistry,Faculty of medicine,Cairo University.

randa.salam@live.com

Abstract: *Back ground and aim:* Metabolic syndrome is a group of interrelated risk factors, accurate clinical diagnosis and treatment is mandatory for detection of population at risk for coronary heart disease and diabetes. Physical exercise protects against the development of cardiovascular disease, partly by lowering plasmatic total cholesterol, LDL and increased HDL .In addition it is now established that increased C - reactive protein (CRP) and plasminogen activator inhibitor-1(PAI-1) play a role in the maintenance of an inflammatory state and in the development of cardiovascular disease.This study aimed to compare plasma levels of LDL, HDL, CRP, and PAI-1 in patients with metabolic syndrome before and 6 months after moderate intensity exercise. **Methods:** Forty five obese non smoker, males with metabolic syndrome living sedentary life were included in the study .Blood samples were collected at the beginning of the study and 6 months later. However only 42 patients completed in our study The plasma lipid profile (Triglycerides, HDL, LDL, totalcholesterol), fasting blood glucose, C - reactive protein and PAI-1 levels were determined. Body weight and BMI were also measured before and after the exercise. **Results:** Total cholesterol ,LDL,HDL,triglycerides,CRP,PAI-1 levels were lower after moderate intensity exercise in relation to levels before moderate intensity exercise($p<0.05$).In addition we observed a positive correlation between PAI-1 and LDL after exercise($r=0.301$,$p=0.053$),PAI and triglycerides after exercise($r=0.286$,$p=0.066$), negative correlation between HDL and PAI-1($r=-0.315$,$p=0.042$). These results indicates that moderate intensity exercise induces favorable changes in metabolic syndrome in lowering lipid profile and PAI-1 levels and may reduce risk of cardiovascular diseases.

[Seraag Esmat, Randa F Abd Al Salam, Lila Rashed. **Effect Of Exercise On Plasminogen Activator Inhibitor-1(PAI-1) Level In Patients With Metabolic Syndrome**. Journal of American Science

Full Text

153

	2010;6(12):1374-1380]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** Metabolic syndrome, PAI-1, exercise		
154	**Reuse of Industrial Materials in Buildings To activate their application in Egypt** Nermin Mokhtar Mohamed Researcher in Civil and Architectural Department, Engineering division, National research centre, Egypt **ABSTRACT:** Increasingly stringent rules and regulations on construction and demolition waste, diminishing landfill space and depletion of natural resources are all reasons for the push for industrial byproduct materials recovery. In Egypt, Industrial byproduct materials are generated in large volumes every day that are potentially usable materials, and must be disposed of. The main goal of this paper is to change the way Egyptians' think about waste—to see the value of a used material as a product or commodity, not as a waste, and encourage the use and recycling of these rich, largely untapped resources. Positive economic rewards and environmental results are moving our partners toward more waste reduction and materials management. This paper summarizes the proposed Egyptian industrial materials waste management guidelines to reuse in building ,which cover: (1) Identify the parties involved and the distribution of responsibilities; (2) Complementarily of roles of parties(owner, engineer, designer, and contractor) involved in the process of re-use to remove the causes that hinder the management of such material in Egypt ; and (3) Participation of the Parties to the proposed project to achieve sustainable development fields at the actual application of the project. [Nermin Mokhtar Mohamed. **Reuse of Industrial Materials in Buildings To activate their application in Egypt**. Journal of American Science 2010;6(12):1381-1393]. (ISSN: 1545-1003). http://www.americanscience.org. **KEYWORD:** reuse –industrial byproduct materials, waste management, sustainability, Egypt	Full Text	154
155	**Molecular Biological And Biochemical Studies On Avian Influenza Virus Receptors In Different Avian Species** Hussein I. El-Belbasi; Mohamed F. Dowidar and Safaa I. Khater Department of Biochemistry, Faculty of Veterinary Medicine, Zagazig University,Egypt H_elbelbasi2003@yahoo.com **Abstract:** Avian influenza viruses are considered to be the key contributors to the emergence of human influenza pandemics. A major determinant of infection is the presence of virus receptors on susceptible cells to which the viral haemagglutinin is able to bind. Avian viruses preferentially bind to sialic acid α 2,3-galactose (SA α 2,3-Gal) linked receptors, whereas human strains bind to sialic acid α 2,6-galactose (SA α 2,6-Gal) linked receptors. Although ducks are the major reservoir for influenza viruses, they are typically resistant to the effects of viral infection, in contrast to the frequently severe disease observed in chickens In order to understand whether differences in receptors might contribute to this observation, we studied the expression of influenza receptors in upper and lower respiratory organs of ducks and chickens (expression of ST3Gal-III sialyltransferase and ST6Gal-I sialyltransferase genes) using semi quantitative RT-PCR. There was a marked difference in the expression of primary receptor type in the trachea of chickens and ducks. In chicken trachea, SA α 2,6-Gal was the dominant receptor type whereas in ducks SA α 2,3-Gal receptors were most abundant. This suggests that chickens could be more important as an intermediate host for the generation of influenza viruses with increased ability to bind to SA α 2,6-Gal receptors and thus greater potential for infection of humans. Chicken tracheal and intestinal epithelial cells also expressed a broader range of SA α 2,3-Gal receptors in contrast to ducks, which suggests that they may be able to support infection with a broader range of avian influenza viruses. [Hussein I. El-Belbasi; Mohamed F. Dowidar and Safaa I. Khater. **Molecular Biological And Biochemical Studies On Avian Influenza Virus Receptors In Different Avian Species**. Journal of	Full Text	155

	American Science 2010;6(12):1394-1401]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** Host receptors, influenza, , chicken, duck, ST3Gal-III sialyltransferase, ST6Gal-I sialyltransferase, gene expression		
156	**Synbiotic Tarhana as a functional food** *Shreef G N Gabrial, ** Ahmed H Zaghloul, ***Abd El-Rahman M Khalaf-Allah, ***Nagwa M El-Shimi, *Rasha S Mohamed and *Gamal N Gabrial * Food Science and Nutrition Department, National Research Centre, Dokki, Cairo, Egypt. **Dairy Science Department, National Research Centre, Dokki, Cairo, Egypt. ***Food Science Department, Faculty of Agriculture, Cairo University, Giza, Egypt. E-mail: snoub_2000@yahoo.com **Abstract**: In the present study formulated synbiotic tarhana (Turkish fermented cereal food) was produced as a functional food from the fermentation of wheat flour, some spices [salt, pepper, dill and sweet marjoram (Organum majorana)], some vegetables [tomato (Lycoprsicum esculentum), pepper (Capsicum annum) and onion (Allium cepa)], and synbiotic yoghurt which prepared with prebiotic (Inulin and lactose each 3%) and different concentrations of the probiotic culture (0.5, 1.5, 3, 4.5% DVS-ABT2 containing Streptococcus thermophilus, Lactobacillus acidophilus and Bifidobacterium bifidum). After fermentation (3 days), tarhana dough was dried in the sun. The effect of the fermentation (0, 1, 2 and 3 days) and the probiotic culture concentration on the chemical composition and the probiotic population of the wet tarhana were evaluated. The effect of the probiotic culture concentration on the chemical composition, the probiotic population and the sensory attribute of dried tarhana were evaluated. Also the effect of dried tarhana (prepared from yoghurt which was fermented by 4.5% probiotic culture) on the plasma lipid profile of human subjects was studied. The results showed that the pH value decreased while the acidity increased, acetaldehyde and diacetyl values increased during the fermentation period and by increasing the probiotic culture concentration of the wet and the dried tarhana. Neither the fermentation nor the concentration of the probiotic culture of wet and dried tarhana affected the crude protein, ether extract, crude fibre, and ash values. The numbers of probiotic bacteria increased until the second day of fermentation. However, in the following day, with an increase of the acid content their number decreased. Generally the increasing of the probiotic culture concentration increased the numbers of probiotic bacteria of the wet and dried tarhana. Also the concentration of the probiotic culture didn't affect the sensory attributes of dried tarhana. Subjects supplemented with dried tarhana showed significant reduction in total serum cholesterol, low density lipoproteins (LDL-C) and triglycerides, while high density lipoprotein (HDL-C) increased. [Shreef G N Gabrial, Ahmed H Zaghloul, Abd El-Rahman M Khalaf-Allah, Nagwa M El-Shimi, Rasha S Mohamed and Gamal N Gabrial. **Synbiotic Tarhana as a functional food**. Journal of American Science 2010;6(12):1402-1412]. (ISSN: 1545-1003). http://www.americanscience.org. **Key words:** Tarhana, functional food, fermented food, probiotic, synbiotic yoghurt, serum lipids	Full Text	156
157	**Relationships between Personal, Indoor, and Outdoor PM_{10} in the Residential Environment in Damietta, Egypt** Omnya A. El-Batrawy Environmental Sciences Dept., Fac. of Sci., Damietta Branch, Mansoura Univ., Egypt. Om_elbattrawy@mans.edu.eg **Abstract:** The relationship between indoor and outdoor air pollution levels is important as people spend about 90% of their time indoors. This may underline the importance of particulate as an environmental	Full Text	157

	health risk and the consequence need for monitoring them particularly in indoor microenvironment. The current study measured the concentrations of PM_{10} in the personal (P), indoor (I), and outdoor (O) air of buildings located in three residential areas at Damietta Governorate, Egypt, during the summer and the winter of 2009. Twenty- four homes were included in this study. The outdoor PM_{10} concentrations ranged from 975.9 – 512 μgm^3 in summer and from 1184 to 555.6 μgm^3 in winter. The indoor PM_{10} concentrations ranged from 997.1n to 65302 μgm^3 in summer and from 1198.8 to 705.6 μgm^3 in winter. The personal PM_{10} concentrations ranged from 1008.4to 334.52 μgm^3 in summer and from 1164.48 to 642.6 μgm^3 in winter. It was apparent that there is a general pattern of increasing levels from winter to summer, and similarly from indoor to outdoor air PM_{10} measured in this study. The indoor/outdoor (I/O) ratios varied between in summer (1–1.5) and winter (1–1.3). The I/O ratios obtained were linked to the indoor activities using occupant's diary entries. Results from the regression analysis showed a relatively strong correlation between the indoor and personal concentrations, between personal and outdoor PM_{10} concentrations and between indoor and outdoor concentrations at both summer and winter. The only exception was the correlation between the concentrations in summer season. Whereas statistically significant correlations were observed between outdoor and personal concentrations in winter, the correlations observed in summer were relatively low. The strongest correlations were found between indoor and personal concentrations, indicating that personal PM_{10} exposures were significantly affected by indoor PM_{10} than by ambient PM10. This shows that the contribution of outdoor pollutants to indoor pollution is higher in winter than summer. The estimated F_{inf} of the studied homes in summer and winter were 0.65 and 0.89, respectively. [Omnya A. El-Batrawy. **Relationships between Personal, Indoor, and Outdoor PM_{10} in the Residential Environment in Damietta, Egypt.** Journal of American Science 2010;6(12):1413-1422]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** Personal; Indoor; Outdoor PM_{10}; Residential Environment; Egypt		
158	**Atypical Bacteria in Ventilator Associated Pneumonia; an Egyptian University Hospital Experience** Egypt **Background** Ventilator-associated pneumonia (VAP) is the most common hospital acquired infection seen in ICU in patients on mechanical ventilation. A diversity of microbes can cause VAP, causative agent differ according to patient populations and types of ICUs. Atypical bacteria not cultured by routinely used methods , have been implicated as causes of VAP, still no sufficient studies to assess size of their role as causative agent in VAP. In this study we aim at estimation of the potential role of atypical bacteria as Mycoplasma pneumoniae, Chlamydia pneumoniae and Legionella pneumophila in ventilator-associated pneumonia in the intensive care units of Alexandria Main University Hospital. **Materials and methods:** 60 endotracheal aspirates were collected from VAP ICU patients. Samples were subjected to routine culture as well as PCR amplification using specific primers for detection of the following atypical bacteria : Mycoplasma pneumoniae, Chlamydia pneumoniae and Legionella pneumophila. **Results:** Out of the 60 endotracheal aspirate, routine culture revealed growth of: enterobacteriaecae in 14 (23.3%) aspirate, pseudomonas in 13(21.7%), candida in14(23.3%) , and MRSA in 10 (16.7%) . In 19 (31.7%) endotracheal aspirates, no growth was encountered on routine culture. PCR reaction was positive for Atypical bacteria in 9 (15%) out of 60 samples, five were positive for mycoplasma, three for Legionella, and only one was positive for Chlamydia. Atypical bacteria positive results were encountered in 4 (21%) out of 19 aspirates with no growth culture results. **Conclusion:** Our results point that atypical bacteria are not an uncommon cause for VAP. This finding has to be taken into consideration while tailoring the empiric antimicrobial coverage of patients diagnosed with VAP. [Egypt. **Atypical Bacteria in Ventilator Associated Pneumonia; an Egyptian University Hospital Experience**. Journal of American Science 2010;6(12):1423-1428]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** Atypical Bacteria; Ventilator; Pneumonia	Full Text	158
159	**The neuroprotection role of heat shock protein 70 (HSP70) against microwave radiation induced DNA damage in male Wistar rat brain**	Full Text	159

Magda Mohamed El-Ezabi

Department of Zoology – Faculty of Science – Benha University- Egypt

E-mail address:dr.rosa@hotmail.com

Abstract : This investigation aims to study the effect of radiofrequency (RF) radiation on DNA damage in brain cells of male Wistar rats using the comet assay and to investigate the role of HSP70 as a protective molecular chaperone that increases stress tolerance of brain cells. Male Wistar rats (118±20g) were divided into three groups. The 1st (exposed) group was subdivided into three subgroups and exposed for 15 min to activated cell phone emitting a frequency radiation of 900 MHz, at non-thermal specific absorption rate (SAR) of 2.9×10^{-3} W/Kg. The 2nd (exposed) group was also subdivided into three subgroups but was exposed for 30 min to the cell phone. The third group was the sham-exposed (control). Animals in each group were sacrificed after 1, 3 and 7 days recovery period. The comet assay parameters showed significantly increased DNA damage in brain cells after 1 and 7 days in the first group and after 7 days in the second group. The HSP70 showed significantly increased levels after 7 days in both exposure groups. Meanwhile, HSP70 showed significantly decreased levels after 1 day in the second group. The results of the present study demonstrate a damaging effect of RF radiation on DNA of the brain cells. This damaging effect initially inhibits the synthesis of HSP70; But after a 7 day recovery period, the levels of HSP70 increase significantly possibly due to powerful capacity of the cells for recovery.

[Magda Mohamed El-Ezabi. **The neuroprotection role of heat shock protein 70 (HSP70) against microwave radiation induced DNA damage in male Wistar rat brain**. Journal of American Science 2010;6(12):1429-1435]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: comet assay, DNA damage, brain cells, heat shock protein 70, radiofrequency radiation

160

Perception Of Student Nurses Towards The Use Of Portfolio In A Faculty Of Nursing

Full Text

*****Salwa S. Kamal and **Nagwa R. Attia

***Assistant Professor Medical Surgical Nursing, Faculty of Nursing, Ain Shams University**

****Lecturer Medical Surgical Nursing, Faculty of Nursing Tanta University**

Sal232@hotmail.com

Abstract: The use of portfolio for learning and assessment within nursing education has recently increased. The main purposes of developing a portfolio is to link understanding about clinical experiences and theoretical knowledge, promotion of student-centered learning and reflective learning. It is important that students developing a portfolio understand the process. Unless portfolio is perceived by students to be relevant and useful, they will not be committed to using portfolios to their full potential. This paper aimed to identify perception of student nurses towards the use of portfolio and to compare the perception of first and second year student nurses towards the use of portfolio. The sample of the study composed of 376 first and second year students studying medical surgical nursing, in a faculty of nursing. Students were asked to respond voluntary to portfolio perception questionnaire, which was developed by researchers. The results of the present showed that students stated that portfolio encourage their independent learning, understanding and utilization of basic concepts. The results showed also some discrepancies between first and second year students in their perceptions toward portfolio. Students expressed how the portfolio process could be improved and they recommended the continued use of portfolio in subsequent study years.

[Salwa S. Kamal and Nagwa R. Attia. **Perception Of Student Nurses Towards The Use Of Portfolio In A Faculty Of Nursing**. Journal of American Science 2010;6(12):1436-1446]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Portfolio, Perception, Nursing education

160

161	**Utilization Of Alfalfa And Atriplex For Feeding Sheep Under Saline Conditions Of South Sinai, Egypt** Afaf M. Fayed, Abeer, M. El- Essawy, E.Y. Eid, H. G. Helal, Ahlam, R. Abdou & H. M. El Shaer* 1) Department of Animal & Poultry Nutrition, Desert Research Center, Mataria, Cairo, Egypt **ABSTRACT:** Atriplex nummularia (At) can be an effective fodder component in mixed diets for livestock. Alfalfa (alf) protein is poorly utilized by ruminants due to its rapid degradation in rumen. The objective of this study was to assess the influence of replacing alfalfa by Atriplex as roughage fed to animals. Twenty four adult Barki lambs weighed an average 49± 77 kg and age 3 years were used in six digestibility trials (4 animals each). Experimental diets were made of alfalfa and Atriplex nummularia in different ratios to each other as follow:R1: 75 % alf + 25% At, R2: 50 % alf + 50 % At, R3: 25 % alf + 75 % At, R4: 100 % At, T5: 100 % alf furthermore R6: berseem hay (BH) + CFM. All animals were fed on barley at 25 % of energy requirements. Results obtained indicated that: all experimental diets had comparable values of DM. Mixing of plants affecting chemical composition of feed ingredients where highest CP was recorded in R5 and least one was in R4. CF values were decreased as follow in R6, R5, R1, R2, R3 and R4, respectively. R6 and R4 had comparable values of condensed tannins while highest saponin levels were recorded in R4. Animals fed on R5 showed highest DM intake and TDN g/kg BW followed by those fed on R1 and R2. Nitrogen intake showed higher values in R5 followed by R1, R2, R3, R6 then R4. Nitrogen retention also was maximum in R5 and minimum in R6. Animals fed on At alone showed highest water intake with significant ($P< 0.05$) differences when compared with other treatments. There is a sampling time effect (zero and 6 hrs post feeding) on serum metabolites, liver enzymes and some minerals. Indeed the prefeeding rumen parameters (NH3 – N) and TVFA,s were increased significantly ($P\leq 0.05$) to reach the peak value at 8 hr post feeding. Some minerals Na, K, Ca and P were analyzed. Finally R2 is nutritious despite the generally low the nutritive value and energy content. [Afaf M. Fayed, Abeer, M. El- Essawy, E.Y. Eid, H. G. Helal, Ahlam, R. Abdou & H. M. El Shaer. **Utilization Of Alfalfa And Atriplex For Feeding Sheep Under Saline Conditions Of South Sinai, Egypt**. Journal of American Science 2010;6(12):1447-1461]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords**: salt tolerant fodders, digestibility, antinutirtional factors, intake, rumen and blood metabolites, sheep	Full Text	161
162	Oxidative stress in brains of rats intoxicated with aluminum and the neuromodulating effect of different forms of sage EL-Kholy, W.M.; EL-Habibi, E.M. and Mousa, A.T. Zoology Dept., Faculty of Science, Mansoura University, Mansoura, Egypt **ABSTRACT:** The present study was designed to investigate the role of oxidative stress and the status of antioxidant system in the management of aluminum chloride (AlCl3) induced brain toxicity in rats and further to elucidate the potential role of three forms of *Salvia officinalis* (sage) in alleviating such negative effects. The results revealed that the levels of thiobarbituric acid reactive substances (TBARS) and protein carbonyl (PC) were significantly increased, while the activities of superoxide dismutase (SOD) and catalase (CAT) as well as the reduced glutathione (GSH) content were significantly decreased in the cerebral cortex (Co) and hippocampus (Hip) of rats intoxicated with $AlCl_3$. Regarding the lipid profile, total lipids (TL), total cholesterol (TC) and triglycerides (TG) were significantly increased in serum and the mentioned brain regions, while phospholipids (PL), total protein (TP) and serum HDL-C were significantly decreased in $AlCl_3$ group. Additionally, serum and brain regions acetylcholinesterase (AChE) as well as alkaline phosphatase (ALP) and acid phosphatase (ACP) activities were significantly increased. On the other hand, the results exhibited that, sage when given in any form along with $AlCl_3$ was able to regulate the mentioned parameters and the values returned close to the normal ones. It can be concluded that Al-	Full Text	162

	induced neuronal oxidative stress and inhibition of the antioxidant system, and the consequent disturbed lipid profile, total protein and enzyme activities could be the mechanisms of $AlCl_3$ neurotoxicity. Moreover, the results suggested that the different sage forms, by their antioxidant constituents, could be able to antagonize Al neurotoxicity perhaps by reducing the oxidative stress and improving the antioxidant status and particularly by inhibiting the acetylcholinesterase activity, thus may improve memory and other brain cognitive activities. [EL-Kholy, W.M.; EL-Habibi, E.M. and Mousa, A.T. Oxidative stress in brains of rats intoxicated with aluminum and the neuromodulating effect of different forms of sage. Journal of American Science 2010;6(12):1462-1474]. (ISSN: 1545-1003). http://www.americanscience.org. **Key words:** Aluminum neurotoxicity- Alzheimer's disease- *Salvia officinalis* - Lipid peroxidation - ntioxidants-acetylcholinesterase		
163	**Study on immune response of quail for avian influenza vaccines** M.A.saad, A.I.Abd-Elhady, A.EL-nagar Central laboratory for evaluation of veterinary biologics Quail influenza **Abstract:** This study was a trial to evaluate: The immune responses of quails vaccinated with common AI commercial vaccines in Egypt The results revealed that: There were high to moderate levels of maternal immunity against AIV (H5N1and H5N2) on the 1st, 5th day of age and low levels on the 7th day of age. There was no significant difference concerning the immune response of H5N1 and H5N2 AI vaccines ($P < 0.05$) in vaccinated quails. Vaccination at 8-days of age with 0.5ml of vaccine, gave satisfactory titers, on the 3rd week post vaccination. By the 4th week post vaccination quails exhibited highest titers and continued to the 5th week post vaccination(age of slaughter or marketing of quail) against AIV. [M.A.saad, A.I.Abd-Elhady, A.EL-nagar. **Study on immune response of quail for avian influenza vaccines**. Journal of American Science 2010;6(12):1475-1478]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** immune response; quail; avian influenza; vaccines	Full Text	163
164	**First Record Of Microsporidium *Neonosemoides* Sp. And Some Ciliates Infecting *Chrysichthus Auratus* (Bagridae) From The Damietta Branch Of River Nile, Egypt** Enayat Salem Ahmed Reda Department of Zoology, Faculty of Science, Mansoura University, Mansoura, Egypt **Abstract:** The present study was carried out as a general survey for the possible ectoparasites that can infect the Nile fish *Chrysichthus auratus*. A total of 52 fish specimens were collected from Damietta branch of River Nile. Examination of the investigated fish revealed that, fish were infected with four ectoparasitic species belonging to three genera. These species were: *Neonosemoides* sp., *Scyphidia* sp. 1, *Scyphidia* sp. 2 and *Ichthyophthirius multifillis*. The first three species were recorded for the first time in Egypt. The recovered parasites have pathological effects on the host fish with subsequent economic losses were discussed. [Enayat Salem Ahmed Reda. **First Record Of Microsporidium *Neonosemoides* Sp. And Some Ciliates Infecting *Chrysichthus Auratus* (Bagridae) From The Damietta Branch Of River Nile, Egypt.** Journal of American Science 2010;6(12):1479-1482]. (ISSN: 1545-1003). http://www.americanscience.org. **Key words:** *Neonosemoides* sp., Ciliates, *Chrysichthus auratus*, River Nile. Egypt	Full Text	164
165	**A Novel Self-adaptive Behavior Quantum Evolutionary Algorithm**	Full Text	165

	Hassan K. Khalafi School of Mathematics and Computer Science, Damghan University, Damghan, Semnan, P.O. Box 36715-364 Iran h_kadkhodaei@du.ac.ir **Abstract:** In accordance with tradition quantum evolutionary algorithms can obtain excellent results in the optimization of Multi-peak functions. In any case, they are easy to be trapped to hurriedness. In this article, A Novel Self-adaptive Behavior Quantum Evolutionary Algorithm is recommending on the basis of the concepts and tenet of quantum evolutionary algorithms in order to enhance the efficiency. Firstly, Self-adaptive Behavior triploid chromosome is constructed to keep the population variety; Secondly, double mutation is used to make sure the variety of the swarm, then individual chromosome cross will be imported into this new algorithm in order to achieve the information communication between the chromosomes and enlarge the search scope in the available space. Experiments on test functions of varied intricacies are implemented and compared with other EAs. The result indicates that the new algorithm in this article can search and get the global most efficient solution in a shorter time. [Hassan K. Khalafi. **A Novel Self-adaptive Behavior Quantum Evolutionary Algorithm**. Journal of American Science 2010;6(12):1483-1486]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** Self-adaptive Behavior; Evolutionary Algorithm; QEA; Double Mutation; Discrete Cross		
166	**The Factors For Free Flow Speed On Urban Arterials – Empirical Evidences From Nigeria** Ibrahim Tunde Yusuf Department of Civil Engineering, University of Ilorin, P.M.B. 1515, Ilorin, Kwara State of Nigeria (zip code: 234). Email**:** ityusuf@yahoo.com; tunde_aroes@hotmail.com **ABSTRACT:** Many generic factors of the weather, environment, vehicles (machines), fixed facilities (roadway) characteristics, humane (driver) and traffic streams either singly or in combination influence the free flow speed. The quantitative measures of these factors are desirable for reliable system design, analysis and evaluation for effectiveness, especially as reflect the typical humane-machine-environment system prevailing in Nigeria. This paper therefore presents the outcome of the quantitative evaluation of the influence of some factors on the free flow speed on an arterial in a medium sized urban settlement in Nigeria with a view to determining the probable analytical values for towns of similar hierarchy in Nigeria. Instantaneous speeds of forty test vehicles were observed in-vehicle at lull periods on the 7.1km Offa Garage-Emir's Market urban Road, Ilorin with simultaneous collection of data on age of driver, age of vehicle, passenger occupancy, roadside packed vehicles and businesses. The geometric properties of the arterial ware earlier established and segmented to four uniform sections. The data were computed using the category and statistical analysis approach. The results of the study indicated that the three factors of the environment (weather), humane and roadway geometry have negative influences on the free flow speed on an urban arterial. Estimates of the reduction of the various factors were detailed in the paper which was recommended for adoption for design and analysis of traffic stream in Nigeria and other medium sized towns in Nigeria. [Ibrahim Tunde Yusuf. **Factors for Free Flow Speed on Urban Arterials – Empirical Evidences from Nigeria**. Journal of American Science 2010;6(12):1487-1497]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** Free flow, instantaneous speed, lull period, roadway geometry, environment, in-vehicle data**,** time speed and traffic stream	Full Text	166

167	**Biochemical and Molecular genetic Evaluation of some conifers genetic resources** Soliman.M.H.,⋆ Gad,Mervat,M.A.⋆⋆ Hussein, H.Mona, ⋆ Mohamed,A.S.⋆⋆ ⋆ Department of Genetics, Faculty of Agriculture, Cairo University, Giza, Egypt. ⋆⋆ Horticulture Research Institute, Agricultural Research Center , Giza. **Abstract:** Genetic polymorphism was investigated in six conifers representing four Pinus species, i.e (*P.halepensis, P.canariensis, P.pinea, and P.roxburghii)which belong to family Pinaceae and two members of family Taxodiaceae, i.e. (Sequoia sempervirens and Taxodium distichum).* In this respect, genetic biochemical (proteins and isozymes), as well as molecular (RAPDs and ISSRs) analysis were investigated. Proteins and peroxidase banding patterns resulted in extensive polymorphism among conifers under investigation, however, Adh isozyme banding patterns were not satisfactory in this concern. RAPD analysis exhibited a total of 66 bands, out of them 25 bands were polymorphic (37.88%). Five ISSR primers generated reproducible and informative amplified products. those were used to distinguish between the six conifers, since 38 bands were polymorphic out of total 81 bands with 47.95% of polymorphism which can be considered as useful markers for identifying conifers. Based on combined data obtained by proteins, peroxidase, RAPD and ISSR analysis, it was possible to discriminate between the six conifer trees under investigation. The present study indicates that the application of biochemical and molecular fingerprinting of the six conifers provided a solid ground that will allow an easier and faster genetic identification of other woody trees species. [Soliman.M.H., Gad,Mervat,M.A. Hussein, H.Mona, Mohamed, A.S. **Biochemical and Molecular genetic Evaluation of some conifers genetic resources.** Journal of American Science 2010;6(12):1498-1509]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** Conifers, Pinus, Sequoia, Taxodium, fingerprint, RAPD, ISSR, SDS-PAGE, Peroxidase, alcoholdehydrogenase	Full Text	167
168	**Study on immune response of quail for avian influenza vaccines** **M.A.saad, A.I.Abd-Elhady, A.EL-nagar** Central laboratory for evaluation of veterinary biologics Quail influenza Saad940@yahoo.com **Abstract:** This study was a trial to evaluate: The immune responses of quails vaccinated with common avian influenza (AI) commercial vaccines in Egypt The results revealed that: There were high to moderate levels of maternal immunity against AIV (H5N1and H5N2) on the 1st, 5th day of age and low levels on the 7th day of age. There was no significant difference concerning the immune response of H5N1 and H5N2 AI vaccines ($P < 0.05$) in vaccinated quails. Vaccination at 8-days of age with 0.5ml of vaccine, gave satisfactory titers, on the 3rd week post vaccination. By the 4th week post vaccination quails exhibited highest titers and continued to the 5th week post vaccination(age of slaughter or marketing of quail) against AIV. [M.A.saad ,A.I.Abd-Elhady, A.EL-nagar. **Study on immune response of quail for avian influenza vaccines**. Journal of American Science 2010;6(12):1510-1514]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords**: immune response avian influenza - vaccines	Full Text	168
169	**Modification of Silk for Improvement of Weighting and Properties**	Full Text	169

	S.M.Gawish*, A.M.Ramadan, S.M.Abo El-Ola Textile Research Division, National Research Centre, Tahrir Street, Dokki, Cairo, Egypt *Samihagawish@yahoo.com **Abstract:** Silk was modified by 2-hydroxy ethyl methacrylate (HEMA) and glycidyl methacrylate (GMA) and GMA/ derivatives to increase the weight, improve silk properties, antibacterial and fungicidal activities. Thus silk was grafted using a chemical method to different percentage add-on HEMA and GMA. Modified silk / GMA were further reacted with Diethylene triamine (DETA) at 85ºC for one hour to yield bactericidal and fungicidal silk fabrics. The weight of silk was increased and the properties were improved including moisture regain, crease recovery angles, abrasion resistance, whiteness and decrease of yellowness index. Characterization of modified fabric was done by FTIR, thermal gravimetric analysis (TGA) and SEM. [S. M. Gawish, A. M. Ramadan, S. M. Abo El-Ola. Modification of Silk for Improvement of Weighting and Properties. Journal of American Science 2010;6(12):1515-1520]. (ISSN: 1545-1003). http://www.americanscience.org . **Key words:** modified silk, chemical redox method, HEMA, GMA/derivatives, weighting moisture regain, crease recovery angle, abrasion resistance, whiteness, yellowness index, bactericidal and fungicidal activities		
170	Different Bone Resorption Levels Effect on Stresses Distribution for Different Implant Design Mohamed M. EL Zawahry[1], Mohamed I. El-Anwar[2], and Ahmed F. El-ragi[3] [1] Researcher, Prosthodontic Dept., National Research Centre, Egypt. [2] Researcher, Mechanical Engineering Dept., National Research Centre, Egypt. [1,2] Member Scientific Excellence Center (Applied Programming Network), [3] Ass. Professor, Civil Engineering Dept., Faculty of Engineering, Fayoum University, Egypt. **Abstract: Aim** :Different bone resorption levels effect, and better understanding of the effect of implant design parameters such as implant length on the stability of implants, stress values and distribution in surrounding bone are targeted in this study. **Materials and Methods**: Nine cases (implant-bone conditions) were numerically analyzed in 3D by Finite Element Method (FEM). Three bone levels were tested versus three implant lengths, while one type of loading was applied. **Results** showed that implant stability decreases as bone level decreases. The level of instability depends on implant design parameters. Bone stresses increase as bone level decreases with varying values depending on implant parameters. Approximate design curves were obtained. [Mohamed M. EL Zawahry[1], Mohamed I. El-Anwar[2], and Ahmed F. El-ragi. Different Bone Resorption Levels Effect on Stresses Distribution for Different Implant Design. Journal of American Science 2010;6(12):1521-1525]. (ISSN: 1545-1003). http://www.americanscience.org. **Key words:** Bone resorption, implants	Full Text	170
171	**Diagnosis of Egyptian Bovine Meat Borne Zoonosis** **Nawal, A. Hassanain; Mohey, A. Hassanain; Raafat, M. Shaapan; Hassan, A. Fadaly and Ashraf, M. Barakat** Department of Zoonotic Diseases, National Research Center,	Full Text	171

	nnawalah@yahoo.com **Abstract:** Food borne zoonoses have a major health impact in both industrialized and developing countries. Meat might be infected with some bacterial and parasitic agents; that could be threatening on human health. One hundred and eight meat samples (20 buffaloes and 88 cows) were collected from different Cairo abattoirs and examined parasitologically and bacteriologically. Results showed that 16.67% and 34.26% of the examined meat samples were infected with bacteria and parasites, respectively. The bacterial isolates were non typhoid *Salmonella* (50%), *E. coli* (38.89%) and *Mycobacterium bovis* (1.11%). Three out of the *E. coli* isolates (16.67%) were identified as *E. coli* O157:H7. The liberated parasites were *Cysticercus bovis* (51.35%) and *Toxoplasma gondii* (48.65%). ELISA results showed that seroprevalence of toxoplasmosis was 47, 22.7 and 38.42% in human, cows and buffaloes, respectively. The immunoreactive profiles of *C. bovis* (167.82, 137.32, 88.839, 66.859, 59.851, 54.660 and 48.480 KDa) and *T. gondii* local tachyzoite (158, 111, 102, 86, 55 and 33 KDa) antigens probed with rabbit hyper immune serum showed one immunoreactive band at 55 KDa. While those of *E. coli* (182.01, 144.90, 72.558, 60.324, 28.312 and 18.392 KDa) and non typhoid *Salmonella* (91.967, 60.955 and 20.031 KDa) antigens displayed one common immunoreactive band at 60 KDa. It can be concluded that although immunoblotting help in identification of strains and detection of common cross reactive epitopes between different pathogens, there still exist many challenges and opportunities to improve the current technology of food pathogen detection. [Nawal, A. Hassanain; Mohey, A. Hassanain; Raafat, M. Shaapan; Hassan, A. Fadaly and Ashraf, M. Barakat. **Diagnosis of Egyptian Bovine Meat Borne Zoonosis**. Journal of American Science 2010;6(12):1526-1533]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords**: Meat borne, human health, parasites, bacteria, ELISA, SDS-PAGE, immunoblotting		
172	**Effect of Titanium oxide toxicity on Biochemical, Haematological and clinicopathological Changes in *Clarias lazera* Present in the River Nile** **Mona, S. Zaki[*1], Refat A. Yossef [2] and Nadia M. Taha[3]** Department of uHydribuilogy[1], National Research Centre, Cairo, Egypt Soil and Water Uses Department[2] National Research Centre, Cairo, Egypt Department of Physiology[3], Faculty of Verti. Medicine Cairo University [*]dr_mona_zaki@yahoo.co.uk. **Abstract:** The effect of dietary carbohydrates and titanium oxide on haematalogical profile, blood chemistry and hormonal level was studied in cat fish *Clarias lazera*. Fish were divided into 3 groups (n=10) ant exposedto different doses of titanium oxide and carbohydrate. Groupi was served as control. Group 2 was fed with carbohydrate and titanium oxide (10 mg Kg^{-1} diet ration), group 3 was fed with carbohydrate and titanium oxide (1 5 rng Kg^{-1} diet ration). There is a significant decrease in hemoglobin and P .C.V in group (3). There is a significant increase in serum corlisol, cholesterol, AST, ALT, urea, creatinine and alkaline phosphorous in r group (3), also there is a significant decrease in serum phosphorous, sodium and potassium in treated fish. There is a significant high level of titanium content in kidney muscles, heart and spleen in group (3) suggesting toxic effects of titanium on cat fish *Clarias lazera*. The total viable count of bacteria identified higher in fish fed on carbohydrate titanium. Predominate bacteria were identified as Aeromona, E. coli, Streptococcous, Pseudomonas, Fluorscences and Lacto bacilus species. We emphasize the finding that increase carbohydrate concentration causes harmful pathological effects which reduces humoral immure responses and enhances dietary titanium toxicity. [**Mona, S. Zaki, Refat A. Yossef and Nadia M. Taha. Effect of Titanium oxide toxicity on Biochemical, Haematological and clinicopathological Changes in *Clarias lazera* Present in the River Nile**. Journal of American Science 2010;6(12):1534-1539]. (ISSN: 1545-1003).	Full Text	172

	http://www.americanscience.org. **Keywords:** *Clarias lazera*, titanium pollution, haematalogical, biochemical, clinicopathological, Bacteria account		
173	The Influence of Technological Changes on Labour Availability: A Case of Cocoa Farming Households in Ogun State, Nigeria. [1]Oluyole, Kayode A and [2] Egbetokun, Olugbenga A [1]Economics and Statistics Division, Cocoa Research Institute of Nigeria, PMB 5244, Ibadan, Nigeria [2]Institute of Agricultural Research and Training, PMB 5029, Apata, Ibadan **greatjoge@yahoo.com** **Abstract:** This study examined the effects of technological changes on labour availability. Primary data was collected using structured questionnaires administered to a purposive sample of eighty cocoa farmers in Ogun state of Nigeria. The data collected was analyzed using descriptive statistics, Analysis of Variance (ANOVA) and Multi-variate regression analysis. Descriptive analysis revealed that some technologies such as improved spacing and fertilizer application require the employment of more labour while some technologies like mechanization and herbicide application displace labour. The result of the ANOVA shows that there is significant difference in the magnitude of labour used in different technological groups. Multi-variate regression analysis revealed that availability of labour is influenced by the extent of cultivation as well as the expenditure on improved technologies ($P<0.01$). The study recommended that small scale processing industries should be established in the rural areas to take the advantage of the available excess rural labour resulting from the displacement by some technologies thereby eliminating the problem of unemployment that is likely to be generated as a result of the adoption of the technologies. [Oluyole, Kayode A and Egbetokun, Olugbenga A. **Toxic Effects of *Grewia mollis* Stem Bark in Experimental Rats**. Journal of American Science 2010;6(12):1540-1543]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords**: Effects, technological changes, labour availability, cocoa farming households, Nigeria, Analysis of Variance, multivariate regression analysis	Full Text	173
174	**Toxic Effects of *Grewia mollis* Stem Bark in Experimental Rats** Wilson Obidah[†], Julius L. Godwin[†], Joseph Z. Fate[#] , Mike A. Madusolumuo[†] [†]Department of Biochemistry, Federal University of Technology Yola, P.M.B.2076 Yola, Nigeria. [#]Department of Science Laboratory Technology, Federal Polytechnic Mubi, P.M.B. 35 Mubi, Nigeria. domwam@yahoo.com **Abstract:** *Grewia mollis* stem bark used locally in Nigeria as food additive was mixed with the normal diet at 0, 1, 5 and 10% and fed male albino Wister rats over a four week period. No deaths or remarkable changes in general appearance or behaviour were observed in treated animals. Significant increases ($p< 0.05$) in serum transaminases activities, accompanied by decreased food intake were observed in rats fed the stem bark at 10% dietary level. Treatments had no effect on serum alkaline phosphatase activity, urea, creatinine, triglycerides, cholesterol, glucose concentrations and body and organ weights determined. These findings suggest that dietary exposure of rats to *Grewia mollis* stem bark at high concentrations may cause some adverse effects, especially liver injury. [**Toxic Effects of *Grewia mollis* Stem Bark in Experimental Rats. Toxic Effects of *Grewia mollis* Stem Bark in Experimental Rats**. Journal of American Science 2010;6(12):1544-1548]. (ISSN: 1545-1003). http://www.americanscience.org.	Full Text	174

	Key words: *Grewia mollis;* stem bark ; additive; toxicity; rats		
175	**Rapid leaf Area Estimation of *Crytorchid monteiroae*** **Olosunde, M.A[1]. Dauda, T.O[2] and. Aiyelaagbe, I.O.O[1].,** 1. Department of Horticulture Unversity of Agriculturre, Abeokuta, Nigeria. Institute of Agricultural Research and Training, Obafemi Awolowo University, PMB 5029, Moor Plantation, Ibadan, Nigeria. taofikdaud@yahoo.com, taofik.iart@gmail.com **Abstract:** Leaf area measurement of *Crytorchid monteiroae* was carried out using non –destructive methods at the University of Agriculture, Abeokuta, Nigeria in 2008. The objective of this study was to assess rapid leaf area estimation from both destructive and nondestructive sampling method for *Crytorchid monteiroae*. Leaf samples were randomly selected from lower, middle and upper parts of the plant. Leaf length, leaf width leaf dry weight and leaf area from the graphical method were determined. The results showed that leaf width has the minimum variance (2.083) while leaf length ***x*** leaf with had the maximum variance (428.497). Also, all the considered growth indices were directly and significantly correlated. Of the entire investigated model, cubic model of the relationships between leaf area and the leaf length ***x*** leaf width gave the best result in term of minimum residual variance and highest coefficient of determination. [Olosunde, M.A. Dauda, T.O and. Aiyelaagbe, I.O.O. **Rapid leaf Area Estimation of *Crytorchid monteiroae***. Journal of American Science 2010;6(12):1549-1553]. (ISSN: 1545-1003). http://www.americanscience.org. **Key words**- Exponential model, cubic model, residual variance, monopodial	Full Text	175
176	[Journal of American Science 2010;6(12):1554-1564]. (ISSN: 1545-1003). http://www.americanscience.org. 11	Full Text	176
177	**Including Effectiveness of the blue crab (*portunus pelagicus*) antioxidants inhibit oxidative stress** Ashraf Jazayeri[1*], Ahmad savari[1], Mehran Hossein-Zadeh[2], Forough papan[3], Manijeh kadkhodaei[2] 1. Marine Science and Technology of Khorramshahr University 2.Professor of medical Sciences, Jondi Shapur University of Ahwaz 3. Professor of marine biology, Shahid Chamran university of Ahwaz [*]Corresponding Arthur: *jazayeriashraf@yahoo.com* **Abstract:** today, the role and importance of the human body antioxidant defense system in prevention of many diseases has been proved completely on the other hand the need to strengthen the immune system is inevitable that the consumption of synthetic antioxidants in numerous adverse effects Experts are always following the use of natural antioxidants in this regard must recommend extensive research to find natural resources and the needs of modern human antioxidant is ongoing results of many studies have shown that marine resources are rich in antioxidants are the blue swimmer crab research in this respect were fractions antioxidant extracted from muscle tissue extracts such, antioxidant capacity showed significant antioxidant addition fractions such significant effects in inhibiting oxidative stress, including inhibition hemolysis of red blood cells and protection of Thiol groups of blood showed in a laboratory. [Ashraf Jazayeri, Ahmad savari, Mehran Hossein-Zadeh, Forough papan, Manijeh kadkhodaei. **Including Effectiveness of the blue crab (*portunus pelagicus*) antioxidants inhibit oxidative stress.** Journal of .http://www.americanscience.orgAmerican Science 2010;6(12):1565-11569]. (ISSN: 1545-1003).	Full Text	177

	Keywords: antioxidant capacity, blue crab, oxidative stress		
178	**Production of *Potato Spindle Tuber Viroid*-Free Potato Plant Materials *in Vitro*** Sherin A. Mahfouze [*1]; Kh. A. El-Dougdoug[2] and E. K. Allam[2] [1]Genetic Engineering and Biotechnology Division, Genetic and Cytology Department, National Research Center, El-Beehoth street, 12622, Dokki, Giza, Egypt . [2]Microbiology Department (Virology lab.), Faculty of Agricultue, Ain Shams University, Shoubra, El-Kheima, Cairo, Egypt. Sherinmahfouze@yahoo.com **Abstract:** PSTVd-$_{EG}$ strain was isolated from infected potato plants cv. Diamond during autumn season. The PSTVd-$_{EG}$ was eliminated from these plants by different methods. The meristem-tip culture with size (0.25 mm) gave the high percentage of plantlets PSTVd-$_{EG}$-free was 83.33%. The chemotherapy with ASA, 2-TU and Virazole was applied in culture media with concentrations 10, 20, 30, 40 and 50 ppm. It was found that, the percentage of PSTVd-$_{EG}$-free plantlets was increased by increasing chemical concentrations. The thermotherapy of plantlets in jars (21, 3-4, 5, 8 and 21 $^{\circ}$C /4 mon. due to PSTVd-$_{EG}$ elimination). In addition to, the combination cold treatment of tubers plus meristem-tip culture is more effective for PSTVd-elimination *in Vitro*. As well as, the exposure of the tubers for electricity 5/5, 5/10, 10/5, 10/10, 15/5 and 15/10 mA/min. due to PSTVd-$_{EG}$ elimination particularly the exposure at 10/10; 15/5 and 15/10 mA/min. The results were confirmed by dot-blot hybridization assay. **[**Sherin A. Mahfouze; Kh. A. El-Dougdoug and E. K. Allam. **Production of *Potato Spindle Tuber Viroid*-Free Potato Plant Materials *in Vitro*.** Journal of American Science 2010;6(12):1570-1577]. (ISSN: 1545-1003). http://www.americanscience.org. **Key words:** Potato, PSTVd, Meristem-tip, Chemotherapy, Thermotherapy, Electrotherapy	Full Text	178
179	**The Environmental Impact of Industrial Agriculture: The Case of Mulindi Tea Plantations in Rwanda** JIWEN GE[1,2] , MUPENZI JEAN DE LA PAIX[1]*, GABRIEL HABIYAREMYE，JEAN DE DIEU BAZIMENYERA [1]China university of Geosciences, Institute of Ecology and Environmental Sciences School of Environmental Studies, 388 Lumo Road, Hongshan Administrative District, Wuhan, Hubei 430074, China [2]Hubei Wetland Evolution & Ecological Restoration Key Laboratory, 388 Lumo Road, Hongshan Administrative District,Wuhan, Hubei 430074, China E-mail: jeandelapaixmup@yahoo.fr, gejiwen2002@yahoo.com.cn **Abstract:** The aim of this study is to assess the impact of industrial agriculture on the environment in Rwanda taking at Mulindi tea plantations as a case study. Soil samples collected in three zones of Mulindi Valley were analyzed in the laboratory through PH Meter and the results showed that pH of all soil samples is less than 5 (pH<5), which implies that the soil in that valley is acidic. During this study, soil erosion caused by deforestation has been noticed and the sediments carried down and deposited in valley were became a peat after process of acidification. On the another hand, the analysis of water samples from the tank in polyethylene of three streams of Mulindi using spectroscopic techniques revealed a high concentration of elements like: Na, Ca NO^{3-}, H^{+}, H_2NO_3, Cu and S. and elements with low concentration : Fe, NO_3, K, and $al^{3.}$ This pollution may be due to agrochemicals used. Finally we proposed the methods	Full Text	179

	which can be applied in the country in order to ensure a sustainable tea agriculture and better environmental conservation. [JIWEN GE, MUPENZI JEAN DE LA PAIX, GABRIEL HABIYAREMYE, JEAN DE DIEU BAZIMENYERA. **The Environmental Impact of Industrial Agriculture: The Case of Mulindi Tea Plantations in Rwanda**. Journal of American Science 2010;6(12):1578-1590]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords**: Environment,;Soil degradation,; Deforestation; Tea and Water pollution; Rwanda		
180	**Antimicrobial Activity Of *Waltheria Indica*** *Zailani, A. Hauwa , #Jada, S, Mahmud and +Wurochekke, U Abdullahi. Department of Biochemistry, Federal University of Technology, P.M.B Yola, Adamawa State, Nigeria. +wchekke@yahoo.co.uk; #amjnennelushi@yahoo.com; *howwrkoulou@yahoo.com **Abstract:** *Waltheria indica* is used in Nigeria traditional medicine for the treatment of diarrhoea, infertility, skin diseases, gonorrhea and for relieving pains. Phytochemical analysis revealed the presence of steroids, tannins, saponins, and cardiac glycosides in all parts of the plant; flavonoids were detected only in the leaves and stem, while terpenoids and alkaloids were detected in the leaves only. No part of the plant showed the presence of anthraquinones. Antimicrobial activity of different parts of the plant on *E.coli, Pseudomonas aeroginosa and Salmonella typhi* showed the leaves having highest activity against *E.coli and pseudomonas aeroginosa* with the stem having the lowest activity against the three organisms. Column chromatography of crude extracts of the leaves gave fractions I, II and III that were eluted with ethylacetate/methanol benzene/methanol and acetic acid/methanol respectively. Of these extracts, fraction III showed highest activity against *E.coli and Salmonella typhi.* These findings support the traditional use of the plant as an anti diarrhoeal agent. [Zailani, A. Hauwa , Jada, S, Mahmud and Wurochekke, U Abdullahi. **Antimicrobial Activity Of *Waltheria Indica***. Journal of American Science 2010;6(12):1591-1594]. (ISSN: 1545-1003). http://www.americanscience.org. **Key words:** *Waltheria indica,* phytochemical analysis, antimicrobial activity, *E.coli, Pseudomonas aeroginosa,* and *Salmonella typhi*	Full Text	180
181	**Allelopathic effect of leaf extract of *Azardirachta indica* and *Chromolaena odorata* against post harvest and transit rot of tomato (*Lycopersicum esculentum* L)** [1*] Ijato James Yeni, [1]Oyeyemi Sunday Dele, [2]Ijadunola John Ademola, [3]Ademuyiwa Justus Adeniran [1]Department of Plant Science, Faculty of Science, University of Ado–Ekiti, P.M.B 5363. Nigeria. E-mail: jamesyeni@yahoo.com; GSM: 08067335124 [2]Federal College of Agriculture, Institute of Agricultural Research and Training, Moor Plantation, Ibadan, Nigeria. [3]Department of Statistics, Federal Polytechnic, Ado-Ekiti, Nigeria. **Abstract:** The aim of the present research was focused on the allelopathic effects of *Azardirachta indica* and *Chromolaena odorata* via *in vitro* approach. The aqueous and organic solvents (water and ethanol) extracts from leaves of *Azardirachta indica* Adr.Juss (*Meliaceae*) and *Chromoolaena odorata* (*Asteraceae*) where tested against fungal pathogens of rotten tomato (*Aspergillus niger, Fusarium oxysporum, Rhizopus stolonifer* and *Geotrichum candidium*) by poisoned food method. The results showed promising antifungal activity against the fungi tested. Among the various solvents with varying concentrations, aqueous extracts	Full Text	181

	of 80% *Azardirachta indica* was found to have more inhibitory effect (65.20%) against *Rhizopus stolonifer* compared with other concentrations of 80% *Chromolaena odorata* (52.60%). Ethanol extracts of 30% *Azardirachta indica* had the best inhibitory effect (83.30%) against *Aspergillus niger* followed by 30% ethanol extract of *Chromolaena odorata* (80.00%) against *Geotrichum candidium* comparatively, 20% ethanol extract of *Azardirachta indica* (75.20%) against G. candidium inhibited than 20% ethanol extract of *Chromolaena odorata* (69.80%) against *Geotrichum candidium.* This finding proved the potentiality of the plant extracts for the control of post harvest and transit fungal rot of tomato fruit. Ijato James Yeni, Oyeyemi Sunday Dele, Ijadunola John Ademola, Ademuyiwa Justus Adeniran. **Allelopathic effect of leaf extract of *Azardirachta indica* and *Chromolaena odorata* against post harvest and transit rot of tomato (*Lycopersicum esculentum* L).** Journal of American Science 2010;6(12):1595-1599]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** *Azadirachta indica, Chromolaena odorata,* allelopathy, tomato fungal rot, Pathogens		
182	**Purification and properties of alanine aminopeptidase from water buffalo kidney** Mahmoud A. Ibrahim[1], Abdel-Hady M.Ghazy[1], Mohamed N. Mosaad[2] and Doaa A. Darwish[1] [1.] Molecular Biology Department, National Research Centre, Dokki, Cairo, Egypt [2.] Zoology Department, Faculty of Science, Banha University ibrahimm70@hotmail.com **Abstract:** Aminopeptidases participate in the development of flavour in food products. The present study aims at production of aminopeptidase(s) from the safe mammalian locally available rich sources. Three forms of alanine aminopeptidase AAP1, AAP2 and AAP3 isoenzymes were purified to homogeneity from the kidney cortex of water buffalo. The purification procedures involved anion exchange chromatography on DEAE-cellulose column and gel filtration through Sephacryl S-300 column. All of the purified isoenzymes turned out to be homogeneous as judged by native polyacrylamide gel electrophoresis. The molecular weights of the native isoenzymes AAP1, AAP2 and AAP3 were determined by gel filtration to be 120, 400 and 310 kDa. AAP1 was a homodimer of 60 kDa subunits. AAP2 was a homohexamer of 67 kDa subunits. AAP3 was a homohexamer of 53 kDa. AAP1, AAP2 and AAP3 displayed their maximum activity at pH 8, 7.8 and 7.8 and their isoelectric point (pI) values at pH 6.4, 6.2 and 6.6 respectively. The type of inhibition of AAP1 by dithiothreitol and AAP2 and AAP3 by 1,10 phenanthroline was found to be competitive. One binding site was deduced on each isoenzyme for its corresponding inhibitor. [Mahmoud A. Ibrahim, Abdel-Hady M.Ghazy, Mohamed N. Mosaad and Doaa A. Darwish. Purification and properties of alanine aminopeptidase from water buffalo kidney. Journal of American Science 2010;6(12):1560-1613]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** Alanine aminopeptidase; water buffalo; kidney	Full Text	182
183	**Engineering aspects and associated problems of flood plain deposits in Sohag Governorate, Upper Egypt** EL-SAYED SEDEK ABU SEIF* AND EL-SHATER, A.A. *Geology Department, Faculty of Science, Sohag University, 82524 Sohag, Egypt.* *e-mail: elsayed_71@Yahoo.com **Abstract:** The flood plain sediments occurred on both sides of the River Nile course which are dominated by alluvial sediments. Signs of deterioration have been seen which characterized by cracking of the superstructures. This due to low bearing capacity, ground settlement and shrinkage and swelling of these soils. The sediments of the floodplain were accumulated during the annual inundation of the Nile causing	Full Text	183

	deposition of fine materials before the construction of the High Dam. Five types of clay minerals were identified throughout the studied sequence, namely smectite and kaolinite were the predominant clay minerals present in all samples, mixed layer (smectite-illite), chlorite and illite. In general, for each unit the SPT "N" values increase downwards with depth. The high C_C values of the studied clayey soil (A-Unit) is ranged between 0.24 and 0.32, that indicated to the loose and very high compressible nature of this type of soil. The geotechnical associated problems of the River Nile flood plain sediments area: the low bearing capacity of the sediments, ground settlement and Shrinkage and swelling. [EL-SAYED SEDEK ABU SEIF AND EL-SHATER, A.A. **Engineering aspects and associated problems of flood plain deposits in Sohag Governorate, Upper Egypt**. Journal of American Science 2010;6(12):1614-1623]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords**: Geotechnical problems; Standard penetration test (SPT); Undrained shear strength (S_U); Coefficient of compressibility (Co); Nile flood plain sediments; Clay minerals		
184	**A Multi-Objective Approach for Multi Capacity warehouse Location within Distribution Supply Chain Problem** Mehrdad Rezazadeh [1], Sajjad Farahani [2] [1.]School of Industrial Engineering, Islamic Azad University - South Tehran Branch, Tehran, Iran. [1.] Industrial Engineering department at Amirkabir University of Technology, Tehran, Iran. Sajad.farahani@gmail.com **Abstract:** In this paper, we propose a mixed integer programming formulation for a location distribution problem. We have a two layer supply chain, central warehouses/stocks, regional warehouses and customers. Stocks should satisfy the multi-commodity customers demand. Our objectives are to minimize transportation cost of goods, from stocks to regional warehouses and from regional warehouses to customers, and installing cost of warehouses and to maximize average service level of customers. Our model determines a set of Pareto optimal solution for considering these two conflicting objectives. We have a three type alternatives for both stocks and regional warehouses with varying installing costs and capacities. Regarding the long term decision making for a location problem, we consider time value of money to have more assumptions of real worlds. As a result, a case study is indicated to show efficiency of model to solve the industrial problems; a sensitivity analysis is also implemented upon the rate of return and the life of cycle of the supply chain system. [Mehrdad Rezazadeh, Sajjad Farahani. **A Multi-Objective Approach for Multi Capacity warehouse Location within Distribution Supply Chain Problem**. Journal of American Science 2010;6(12):1624-1628]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** Supply chain, facility location, mixed integer programming, time value of money	Full Text	184
185	**Patient Satisfaction And Its Related Factors Within Emergency Care Departments: A Study Of Iranian Military Hospitals** Ahmad Ameriyon[1],Mohammadkarim Bahadori[1*], Mohammad Meskarpour Amiri[2], Hosein Amini Anabad[2] [1.] Health Management Research Centre, Baqyattallah University of Medical Sciences, Tehran, Iran [2.] Department of Health Economics, School of Medicine, Shahed University, Tehran, Iran bahadorihealth@gmail.com	Full Text	185

	Abstract: Background: Today, researchers pay special attention to patient satisfaction with emergency care services, the first line of hospital healthcare services. However, the nature of emergency medicine (EM) has changed significantly in recent years, and related factors in patient satisfaction have changed over time. The aim of this study was assessment of patient satisfaction and its related factors with emergency care services in six Iranian military hospitals. **Materials and Methods:** In this cross-sectional study, the satisfaction levels of 360 patients of emergency care services in six military hospitals of Iran in 2007 were assessed. After discharge from the emergency ward, a checklist of basic information and a 12-item questionnaire about satisfaction levels was completed for each patient. A 5-level Likert scale was used for the responses. Scores from 20-100 were allocated to each response (completely dissatisfied to completely satisfied), respectively. **Results:** 3,559/4,220 responses (82.4 percent) were completely satisfied or satisfied. In respect to priority, "Observation of ethical issues," "giving information “and” behavior of reception personnel" had the highest scores. "Variety of medical specialists," "emergency ward facilities," and "speed in calling doctor" scored the lowest. The total satisfaction score reported by patients older than 35 year (p=0.022), insurance coverage (p=0.002) and with history of previous referring to that emergency ward (p=0.017) was significantly higher than others. Gender, marital status, and educational level had no statistical correlation with the total satisfaction score (p>0.05). **Conclusion:** The findings of this study revealed favorable satisfaction levels for patients receiving emergency care services at military hospitals. However, using a variety of expert physicians and more facilities and also improving the process of calling doctors into the emergency ward are aspects that need more attention from healthcare managers in emergency centers. [Ahmad Ameriyon, Mohammadkarim Bahadori, Mohammad Meskarpour Amiri, Hosein. **Patient Satisfaction And Its Related Factors Within Emergency Care Departments: A Study Of Iranian Military Hospitals**. Journal of American Science 2010;6(12):1629-1635]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** satisfaction; emergency departments; military hospital		
186	**Growth Pattern in Anemic Children and Adolescents, aged 12-14 years** **Sanaa Kamal *; Moushira Erfan *; Shams Mohamad Kholoussi**; and karima Abd Elfatta Bahgat***** *Biological anthropology Department, Medical Research Division, National Research Centre (NRC) **Immuno-genetics Department, Human Genetics and Genome Research Division, NRC ***Pediatric Department faculty of Medicine (Girls), Al-Azhar University. moushiraz@yahoo.com **Abstract:** Iron deficiency anemia among children and adolescents is a large health problem worldwide. Adolescence is characterized by a large growth spurt and the acquisition of adult phenotypes and biologic rhythms. During this period, iron requirements increase dramatically in both boys and girls. Anemia due to iron deficiency often coexists with zinc deficiency. Deficits in macronutrients or micronutrients can impair growth. Menstruation increases the risk for iron deficiency anemia among girls throughout their adolescence. The aim of the present work is to assess growth pattern in anemic boys and girls and to study relations between anthropometric parameters and hemoglobin, iron and zinc levels. The sample consisted of 60 anemic children and adolescents aged from 12- 14 years (30 boys and 30 girls) and 30 normal healthy children (15 boys and 15girls). Weight, height, mid upper arm circumference (MUAC), waist and hip circumferences were measured and body mass index (BMI) was calculated. Sex- and age-independent SD scores (SDS) were calculated for all anthropometric measurements with the use of the Egyptian reference data. Hemoglobin concentration, serum ferritin, iron and zinc were measured for patients and control. Anemic girls showed significant association between height SDS, weight SDS, BMI SDS and hemoglobin concentration level and also between MUAC SDS and zinc level. Anemic boys showed less marked growth delay. The study showed that growth delay was pronounced among anemic girls during adolescent growth spurt. Thus, age and sex are the factors most predicative of growth delay among Egyptian adolescents. The study emphasized that iron and zinc are essential micronutrients for normal growth and anemia has a negative impact on growth. The study suggests regular nutrition assessment of adolescents	Full Text	186

	and recommends behavior modification to get dietary change among adolescents. The inhibited growth rate, induced by the iron-deficient diet could be reversed by giving a diet supplemented with iron. [Sanaa Kamal; Moushira Erfan; Shams Mohamad Kholoussi; and karima Abd Elfatta Bahgat. **Growth Pattern in Anemic Children and Adolescents, aged 12-14 years**. Journal of American Science 2010;6(12):1636-1646]. (ISSN: 1545-1003). http://www.americanscience.org. **Key words**: Iron deficiency, phenotypes, Growth Pattern		
187	**Effect of Spraying with some Nutrient Elements on Tolerance Beachilyfolia Pear Rootstock to Salinity** **Faten, H. M. Ismaeil[*1] and Wahdan, M. T. [2]** [1]Agric. Botany. Dept. Fac. of Agric. Benha Univ. Benha, Egypt [2] Hort. Dept. Fac. of Agric. Suez Chanel Univ. Egypt fatenesmaeil@yahoo.com[*] **Abstract:** The present investigation was carried out during 2008 and 2009 seasons in the experimental farm belonging to El-Kanater Horticultural Research Station, Kalyubeia Governorate Egypt to study effect of some nutrient elements on tolerance beachilyfolia pear rootstock to salinity. The following measurements were recorded: vegetative growth, nutritional status and some physiological properties of Beachilyfolia pear rootstock, irrigated with saline solution at 6000 ppm with 6 SAR and high chloride level (Cl : So4). Zinc at 50 ppm, Potassium at 250 ppm and Phosphorus at 250 ppm were used in this study to give more explanation about the protect against salt injury. The results revealed that, foliar spray treatments caused a significant increase of some growth measurements (stem height, root length, number of branches & leaves, leaf area, stem diameter and fresh & dry weights of plant organs), leaf photosynthetic pigments content (chlorophyll A, B and carotenoids), leaf mineral content (N, P, K, Na, Fe Mn and Zn), physiological properties (leaf succulence grade, leaf water potential and leaf relative turgidity) of beachilyfolia pear rootstock transplants during 2008 and 2009 consecutive seasons. On the contrary, leaf sodium and proline contents and leaf osmotic pressure took the other way around during the study. [Faten, H. M. Ismaeil and Wahdan, M. T. **Effect of Spraying with some Nutrient Elements on Tolerance Beachilyfolia Pear Rootstock to Salinity**. Journal of American Science 2010;6(12):1647-1654]. (ISSN: 1545-1003). http://www.americanscience.org. Key wards: **Beachilyfolia, pear, rootstock, nutrient elements, salinity**	Full Text	187
188	**Black Tea Forestalls Sodium Fluoride-Induced Neurobehavioral Toxicity in Laboratory Rats** **Mervat M. Kamel[1*], Heba S. El-lethey[1], Iman B. Shaheed[2], and Gehan M. Kamel[3]** [1]Department of Animal Hygiene and Management, [2]Department of Pathology, [3]Department of Pharmacology, Faculty of veterinary Medicine, Cairo University, Cairo, Egypt, mevy58@yahoo.com[*] **Abstract:** The present study aimed to investigate the main effects as well as the interaction effect of supplemental Na-F and black tea on emotional reactivity and learning and memory capacities in rats using a variety of behavioural tasks. Eighty weanling 32-days old Wistar male rats randomly distributed into four groups of 20 animals each, were supplemented with Na-F at 100 ppm and 2% black tea in a factorial pattern to constitute 4 experimental treatments. Brain tissue specimens, representing all treatments, were taken for biochemical and histopathological investigations. In the open field test, Na-F-treated rats displayed higher levels of anxiety that were significantly reduced when black tea was concomitantly administered. Marked impairment in habituation was a significant remark for Na-F group. A superior learning and memory ability was recorded for black tea-supplemented rats during classic maze test, where black tea significantly recovered the intervention observed in Na-F-exposed rats. Moreover, black tea significantly enhanced spatial cognition learning ability and successfully alleviated Na-F-induced spatial	Full Text	188

	memory impairment. Rats administered Na-F displayed distinct neurodegenerative changes of nerve cells especially in hippocampus, accompanied by inhibition of brain acetylcholinesterase (AChE) activity with increased oxidative stress. Administration of black tea along with Na-F was able to afford protection against these Na-F-induced alterations. Our findings suggest a profound ameliorative effect of black tea on Na-F-induced adverse alterations in the brain of rats as indicated by hindrance of learning and memory performance, and argue for concurrent administration of black tea to Na-F-exposed individuals in order to help alleviate fluoride intoxication. [Mervat M. Kamel, Heba S. El-lethey, Iman B. Shaheed, and Gehan M. Kamel. **Black Tea Forestalls Sodium Fluoride-Induced Neurobehavioral Toxicity in Laboratory Rats**. Journal of American Science 2010;6(12):1655-1673]. (ISSN: 1545-1003). http://www.americanscience.org. **Key words:** Sodium fluoride, black tea, SOD, TBARs, learning, memory, anxiety-like behaviour		
189	**Epidemiological Studies of Urinary Tract Infection (UTI) among Post-menopausal Women in Uyo Metropolis, South-South, Nigeria.** **[1]Akinjogunla, O. J., [2]Odeyemi, A. T. and [3]Olasehinde, G. I.** [1]Department of Microbiology, Faculty of Science, University of Uyo, P.M.B. 1017, Uyo, Akwa Ibom State, Nigeria. [2]Department of Microbiology, Faculty of Science, University of Ado-Ekiti, P.M.B.5363, Ado-Ekiti, Ekiti State, Nigeria. [3]Department of Biological Sciences, College of Science and Technology, Covenant University, Ota, Ogun State, Nigeria. papajyde2000@yahoo.com **ABSTRACT:** Cross-sectional studies of UTI among post menopausal women were carried out between January and June, 2009 using standard microbiological techniques. The result obtained showed that 42 (39.6%) out of 106 postmenopausal women had urinary tract infections with highest prevalence among women aged 56-60 and lowest among those aged ≥ 61 . Microscopic examinations of forty-two (42) mid-stream urine samples revealed the presence of 13(30.9%) epithelial cells, 5 (11.9%) phosphate crystals, 16 (38.1%) pus cell , 9 (21.4%) yeast cells, 7(16.7%) red blood cells and eggs of *Schistoma haematobium* 2(4.8%). Bacteria isolated were: *Escherichia coli* 20 (25.3%), followed by *Staphylococcus aureus* 16 (20.3%), *Pseudomonas aureginosa* 10 (12.7%), Coagulase negative *Staphylococcus* spp 9 (11.4%), *Streptococcus pyogenes* 6 (7.6%), *Serratia marcescens* 6 (7.6%), *Enterobacter* spp 5 (6.3%). *Klebsiella* spp. 4 (5.1%) and *Enterococcus faecalis* 3(3.8%). *E. coli* showed low percentage resistance to ciprofloxacin, ceftazidime and ceftriaxone. *Enterobacter* spp. were susceptible to ciprofloxacin and cotrimoxazole in 80%, respectively. Between 60-80% of *Pseudomonas aeruginosa* and *Enterobacter* spp were susceptible to all the tested antibiotics, while 4(66.7%) *Streptococcus pyogenes,* 6 (66.7%) CON-*Staphylococcus* spp and 4(66.7%) *Serratia marcescens* were sensitive to ceftazidime. All the *Enterococcus faecalis* and *Klebsiella* spp isolated were sensitive to ciprofloxacin. The phenotypic determination identified a low ESβL rate of 28.8 % (13 of 45 isolates). ESBLs were detected among the following species: 5 *Escherichia coli* (25.0%), 3 *Pseudomonas* spp (30.0%), 1 *Klebsiella* spp (25.0%), *Serratia marcescens*2 (33.3%) and *Enterobacter* spp. 2 (40.0%). The result also showed that 18.9 % of the bacteria were resistant to at least 3 antibiotics with (MAR) index ranging from 0.2 to 0.8. The results obtained in this study are statistically significant (*p≤0.05*). However, continuous surveillance to monitor the prevalence of UTI and antimicrobial resistance among post menopausal women is overwhelmingly necessary. [Akinjogunla, O. J., Odeyemi, A. T. and Olasehinde, G. I. **Epidemiological Studies of Urinary Tract Infection (UTI) among Post-menopausal Women in Uyo Metropolis, South-South, Nigeria.** Journal of American Science 2010;6(12):1674-1681]. (ISSN: 1545-1003). http://www.americanscience.org. **Key Words:** Post-menopausal, Prevalence, Infection, Susceptibility, ESβL, Uyo	Full Text	189
190	**Morphology And Wall Structure Of Some Turonian Rudists (Bivalvia, Hippuritoida) Of Gabal**	Full Text	190

	Yelleg, Northern Sinai, Egypt **Hosni Hamama** Geology Department, Faculty of Science, Mansura University, Mansura 35516, Egypt. **hamamahem@hotmail.com** **Abstract:** The Turonian succession exposed at the northern extremity of Gabal Yelleg at Northern Sinai yields many rudists. Most of these rudists exhibit polymorphism. Identification, systematic, wall structure and the biostratigraphy of the rudists are made. Rudists encountered are found to belong to: Family RADIOLITIDAE Gray which includes species related to subfamily RADIOLITINAE Gray: 1948: *Radiolites* cf *polyconilites* Orbigny, *Radiolites peroni* (Choffat), *Radiolites sauvagesi* (d Holmis-Firmas), *Gorjanovicia costata* Polsak and *Praeradiolites biskraensis* (Coquand); subfamily BIRADIOLITINAE Douville : *Milovanovicia heraki* Polsak 1968; Subfamily SAUVAGESIINAE Douville: *Suvagesia sharpie*(Bale), *Durania* gaensis (Dacque), *Suvagesia nicaisei* (Coquand), *Durania barakatnsis* nov. sp, *Durania cornupastoris* (Des Moulins) and *Durania arnaudi* (Choffat) and subfamily LAPEIROUSIINAE K u¨hn: *Lapeirousella aumalensis* (Douville). From the family HIPPURITIDAE Gray, 1948 only species *Hippurites (Hppuritella)* cf. *castroi* Vidal was identified. One species among the rudists of Gabal Yelleg is suggested as new species: *Durania barakatnsis* nov. sp. Fourteen thin sections representing the described Turonian rudists were prepared to study the wall structure of rudists, and the evaluation such structure in classification of the studied rudists is discussed. [Hosni Hamama. **Morphology And Wall Structure Of Some Turonian Rudists (Bivalvia, Hippuritoida) Of Gabal Yelleg, Northern Sinai, Egypt**. Journal of American Science 2010;6(12):1682-1701]. (ISSN: 1545-1003). http://www.americanscience.org. **Key words:** Turonian rudists, Bivalvia, Hippuritoida, Gabal yelleg		
191	**Barremian And Aptian Mollusca Of Gabal Mistan And Gabal Um Mitmani Al-Maghara Area, Northern Sinai, Egypt** Hosni Hamama Geology Department, Faculty of Science, Mansura University, Mansura 35516, Egypt; E-mail: hamamahem@hotmail.com, Hamama@mans.edu.eg **ABSTRACT:** A very rich assemblage of 40 Molluscan species were identified from the Lower Cretaceous succession of Gabal Mistan and Gabal Lagama lying at the extremity of the northern flank of Gabal Al-Maghara, northern Sinai. These are used to date the investigated material as Barremian and Aptian. Comparison of the Sinai material with coeval deposits in the northern Caucasus and Western Europe signifies a possible direct marine connection between these areas. [Hosni Hamama. **Barremian And Aptian Mollusca Of Gabal Mistan And Gabal Um Mitmani Al-Maghara Area, Northern Sinai, Egypt**. Journal of American Science 2010;6(12):1702-1714]. (ISSN: 1545-1003). http://www.americanscience.org. **Key words:** Barremian, Aptian, Albian**,** Mollusca, north Sinai	Full Text	191
192	**Phenotypic and Gene-technological Methods for the Identification of Clinically Isolated *Streptococcus pneumoniae* from Egyptian Children** **Fouz Mohamed El-Antably[*1], Salah Abdalla[2], Alaa El-Dien MS Hosney[3] and Gehan Saddik El-Hadidy[4]** [1]Ministry of Heath, Egypt, [2]Department of Microbiology & Immunology, Faculty of Pharmacy, Suez Canal University, Egypt, [3]Department of Microbiology & Immunology, Faculty of Pharmacy, Cairo University, Egypt, [4] Department of Microbiology & Immunology, Faculty of Medicine, Suez Canal	Full Text	192

	University, Egypt fouzelantably@yahoo.com* **Abstract:** *Streptococcus pneumoniae* is an important human pathogen that causes both serious invasive infections, such as septicemia, meningitis and pneumonia, as well as mild upper respiratory infections. The purpose of the study was to identify the *Streptococcus pneumoniae* using the conventional phenotypic methods and the PCR assay; especially, to evaluate their usefulness in the identification of suspected pneumococcal isolates lacking one or more of their typical phenotypic characteristics. A total of 123 nasopharyngeal specimens obtained from children under five years of age, with acute upper respiratory tract infection were subcultured and identified by conventional and gene-technological methods. Forty-one isolates were identified as *Streptococcus pneumoniae*. Approximately (7.31%) were found to be atypical optochin-resistant, while, (4.87%) were bile insoluble. A 209-bp fragment indicative the pneumolysin (ply) gene was obtained from all typical and atypical isolates. The bile solubility test is more specific than the optochin test for identification of *Streptococcus pneumoniae*. Genetic test (PCR) for ply could be used to evaluate any isolates giving questionable results by any of the other phenotypic methods. [Fouz Mohamed El-Antably, Salah Abdalla, Alaa El-Dien MS Hosney and Gehan Saddik El-Hadidy. **Phenotypic and Gene-technological Methods for the Identification of Clinically Isolated *Streptococcus pneumoniae* from Egyptian Children**. Journal of American Science 2010;6(12):1715-1719]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords**: *Streptococcus pneumonia*; optochin susceptibility test; bile solubility; *pneumolysin* PCR		
193	**Relationship between Nursing Procedures and Oxygen Saturation Level of Preterm Infants with Respiratory Distress Syndrome** **Randa El-Sayed Mohammed El-Sayed*; Gamalat El-Sayed Mansy; Bahaa Salah El-Din Hamad and Nehad Sabry Basiouny** Pediatric Nursing, Faculty of Nursing, Alexandria University, Alexandria, Egypt *randa_7373@yahoo.com **Abstract:** The present study was conducted to determine the relationship between nursing procedures (Suctioning, change of position, Heel stick) and blood oxygen saturation level, using pulse oximter monitoring. Fifty preterm infants with respiratory distress syndrome were monitored during performing the nursing procedures at the Neonatal Intensive Care Unit, in Maternity University Hospital at EI-Shatby in Alexandria. An assessment sheet was developed for monitoring the oxygen level before, during, and after each of the three nursing procedures. The main results were the preterm neonates with respiratory distress syndrome reacted to nursing care procedures with decrease in oxygen saturation (SPO_2) during different positioning and repositioning, suctioning and heelstick. After the procedures, all preterm neonates returned to pre-procedure average of oxygen saturation except after repositioning from side- lying to supine, from supine to prone position, and after suctioning. The supine position contributed to a slight decrease in oxygenation. Both prone position and suctioning contributed to an increase in oxygenation after the procedures. The main recommendation is that continuous monitoring of oxygen saturation before, during and after performing the nursing procedures is mandatory. [Randa El-Sayed Mohammed El-Sayed*; Gamalat El-Sayed Mansy; Bahaa Salah El-Din Hamad and Nehad Sabry Basiouny. **Relationship between Nursing Procedures and Oxygen Saturation Level of Preterm Infants with Respiratory Distress Syndrome**. Journal of American Science 2010;6(12):1720-1732]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** Relationship; Nursing; Oxygen Saturation; Preterm Infants; Respiratory Distress Syndrome	Full Text	193
194	**Overcoming Early Shoot Senescence of *Colutea istria* Miller Propagated *In Vitro***	Full Text	194

Ghada Abd El-moneim Hegazi and Mahdia Farid Gabr

Tissue Culture Unit, Plant Genetic Resources Department, Ecology and Dry Land Agriculture Division, Desert Research Center, 11753 El-matarya, 1 Mathaf El-matarya St., El-matarya, Cairo, Egypt.

com.hegazi_ghada@yahoo or com.hegazighada@yahoo :Corresponding author

Abstract: Seedlings of the leguminous shrub; *Colutea istria* Mill. were used as explants for the micropropagation of this vulnerable species. Cotyledonary nodes, stem node sections and shoot tips from the *in vitro* germinated seedlings were examined for micropropagation. The *in vitro* establishment of explants was attempted by using various concentrations of 6-benzylaminopurine (BA), Thidiazuron (TDZ) and N6-(2-isopentenyl) adenine (2iP) (0.5, 1, 2 mg/L each) in combination with NAA at 0.1 and 0.2 mg/L incorporated into Murashige and Skoog (MS) and Gamborg's (B5) media, in addition to the MS and B5 media without plant growth regulators (PGRs). The best results were obtained on MS medium supplemented with NAA and BA, in addition to PGRs free MS medium. And the best average number and length of shoots were obtained by using cotyledonary nodes as explants. For multiplication, the explants were cultured on MS medium containing BA at concentrations of 0.25, 0.5 and 1 mg/L either individually or in combination with 2iP at a concentration of 0.5 mg/L. The combination of BA and 2iP is recommended for multiplying the established shoots produced from colyledonary nodes and stem node sections due to the significantly higher average number of shoots/explant comparing to the media containing BA singly. However, BA is better for the multiplication of shoot tip explants. When axillary shoots were subcultured on the same medium, the shoots failed to multiply and began to senesce. The senescence progressed to the entire shoot, and growth ceased. Reducing of the duration of the subculture to 3 weeks is necessary to prevent this problem. Explants rooted on MS medium containing 0.5 mg/L of both Indole-3-butyric acid (IBA) and NAA and plantlets with well developed shoots and roots transferred to soil and grew normally without loss of green colour and wilting.

[Ghada Abd El-moneim Hegazi and Mahdia Farid Gabr. **Overcoming Early Shoot Senescence of *Colutea istria* Miller Propagated *In Vitro***. Journal of American Science 2010;6(12):1733-1738]. (ISSN: 1545-1003). http://www.americanscience.org.

Key Words: *Colutea Istria*, Leguminosae, micropropagation, *in vitro* culture, seedlings, yellowing, senescence

195

The influence of amaryl on genetic alterations and sperm abnormalities of rats with alloxan-induced hyperglycemia

Full Text

Abeer H Abd El-Rahim [1], Hasnaa A Radwan [1], Omaima M Abd El-Moneim [1], Ibrahim M Farag [1], Somaia A Nada [2]

1- Cell Biology Department, National Research Centre, Dokki, Cairo, Egypt.
2- Pharmacology Department, National Research Centre, Dokki, Cairo, Egypt.

Corresponding author: faragimdiab@yahoo. Com

Abstract: Amaryl (Glimepiride) is the third generation antidiabetic sulphonylurea known to possess the antioxidant effect in streptozotocin (STZ) induced diabetes. In this study, the antimutagenic activity of amaryl (0.03mg/kg.po daily for 30 days) was evaluated against the cytotoxic effect of alloxan (150mg/kg) on somatic cells using bone marrow (for chromosome abnormality and micronucleus tests) and liver samples (for DNA fragmentation test). As well as, germinal cells (using sperm abnormality test) cells in albino rats. The present results showed that the glucose levels significantly increased in alloxan diabetic rats compared to those found in the control. The alloxans diabetes of rats (males or females) had higher frequencies of structural and numerical chromosome aberrations compared to normal control. The diabetic condition in both male and female rats also increased the populations of each of micro nucleated erythrocytes and DNA fragmentation. Moreover, the diabetic condition of male rats significantly increased the sperm shape abnormalities besides significant reducing of caudal sperm count. On the other hand, the administrations of amaryl (glimepiride) to the alloxan diabetic rats had reduced the blood glucose level, abnormalities of genetic materials (chromosomal aberrations, the population of micro-nucleated erythrocytes, DNA fragmentation) and sperm shape abnormalities besides enhancing the sperm count. In

195

	conclusion, the present findings add that the antioxidant property of amaryl could have contributed for its ability to decrease the alloxan mediated defects in somatic and germinal cells. [Abeer H. Abd El-Rahim, Hasnaa A. Radwan, Omaima M. Abd El-Moneim, Ibrahim. M. Farag and Somaia A. Nada. **The influence of amaryl on genetic alterations and sperm abnormalities of rats with alloxan-induced hyperglycemia**. Journal of American Science 2010;6(12):1739-1748]. (ISSN: 1545-1003). http://www.americanscience.org. **Key words;** Hyperglycemia, amaryl, alloxan, genetic alterations, sperm abnormalities, rats		
196	**Comparative study of three calcium hydroxide based root canal sealers using different cultivating techniques.** Omar N.*, Negem M. **, Kataia M. **, and Zaazou M. * * Restorative and dental material department, National Research center, Cairo, Egypt. ** Department of endodontics, Faculty of Oral and Dental Medicine Cairo University. mohamedzaazou@yahoo.com **Abstract: The aim** of this study was to evaluate the antimicrobial properties of three calcium hydroxide based endodontic sealers: Apexit sealer (Ivocalr/Vivdent, Schaan and Liechtenstein), CRCS sealer (Coltene-Whaledent, U.S.A) and Sealapex sealer (Kerr Co, Italy) against *Streptococcus α haemolyticus* (facultative anaerobic bacteria). **Materials and Methods**: Three methods were used to evaluate the antibacterial activities of the three root canal sealers against *Streptococcus α haemolyticus* using Agar diffusion test . The strains were prepared and inoculated into 5ml broth and incubated at 37^{o}C for 24 h. The freshly mixed sealers were placed into the prepared wells of agar plates (inoculated with the test microorganisms). The antimicrobial effect of each sealer was determined by measuring the diameter of zones of inhibition in millimeters at one and three days period. Five plates were prepared for every sealer in each method. **Results** showed that Sealapex gave the highest mean of inhibition zone diameter. This was followed by CRCS and Apexit showed the lowest mean of inhibition zone after one day. While at the three days period, the Sealapex gave the largest inhibition zone diameter and there was no statistically significant difference between CRCS and Apexit groups. The three methods used confirm these results. [Omar N., Negem M., Kataia M., and Zaazou M. Comparative study of three calcium hydroxide based root canal sealers using different cultivating techniques. Journal of American Science 2010;6(12):1749-1753]. (ISSN: 1545-1003). http://www.americanscience.org. **Key words:** Root canal sealers, *Streptococcus α haemolyticus*, Agar diffusion test	Full Text	196
197	**Influence of the Etiological Factors for Gingival Enlargement on some Angiogenic and Inflammatory Mediators: An immunohistochemical study** Abeer Gawish [a], Amira M. Gamal-Eldeen [b*], Salah H.Sherif [c] Amany Neamat [d] [a] Assoc. prof.of Oral Medicine, Periodontology, Oral Diagnosis and Radiology, Faculty of Dental Medicine (Girls'), Al-Azhar University, Cairo, Egypt [b] Assoc. prof. in Cancer Biology Laboratory, Centre of Excellence for Advanced Sciences, National Research Centre, Cairo, Egypt [c] prof. of Oral pathology. Dean of Faculty of dentistry Misr international University d Prof of Oral Pathology in Department of Surgery and Oral Medicine Researches, National Research Centre, Cairo, Egypt **Corresponding Author:** dr_amany@hotmail.com	Full Text	197

	Abstract: Inflammatory gingival enlargement is the most common inflammatory gingival disease and is associated with multiple factors including inflammation due to bacterial plaque colonization, as aside-effect of systemic medications, prolonged orthodontic treatment, and other etiological factors. This study investigated the effect of different etiological causes of gingival enlargement; including the treatment with cyclosporine A, the plaque, and the orthodontic treatment; on the angiogenic inflammatory mediators such as vascular endothelial growth factor (VEGF), cycloxygenase-2 (COX-2), 5-lipoxygenase (5-LO), and tumor necrosis factor-α (TNF- α), using histopathological and immune-histochemical analysis. The results of the immune-histochemical analysis of different angiogenic inflammatory mediators in the gingival enlargement samples indicated that in cyclosporine A-induced enlargement neither of VEGF, COX-2, 5-LO, nor TNF-α were affected, while there is a remarkable general over-expression of VEGF, COX-2, and 5-LO in the parakeratinized epithelial surface, the epithelial layer, connective tissue and in the fiber bundles regions of plaque-induced enlargement gingival. Additionally, orthodontic treatment samples indicated that there is a very high expression of VEGF in the epithelial layer of gingival but not in the connective tissue nor in the fiber bundles regions with no change in COX-2, 5-LO, nor TNF-α expression. In conclusion, this report indicated that the expression of different angiogenic and inflammatory mediators in gingival enlargement is influenced by the etiological factor that initially induced this enlargement.
[Abeer Gawish, Amira M. Gamal-Eldeen, Salah H.Sherif, Amany Neamat. Influence of the Etiological Factors for Gingival Enlargement on some Angiogenic and Inflammatory Mediators: An immunohistochemical study. Journal of American Science 2010;6(12):1754-1760]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: gingival enlargement, plaque, cyclosporine A, orthodontic treatment; nickel, angiogenic, inflammatory, VEGF, COX-2, 5-LO, TNF-α, immunohistochemical | | |
| 198 | **Technical knowledge of biological plants America and localizes it for energy production from agricultural residues in IRAN (Khuzestan province)**

Ashraf jazayeri[1*], Tayeb Saki Nejad[2], Sorosh zarrin abadi[3]
1- Department of biology, Shahid Chamran University

2-Assistant Professor Department of Agronomy Physiology, Islamic Azad University, Ahvaz branch

3- Assistant Professor Department of science, Islamic Azad University, Ahvaz branch

jazayeriashraf@yahoo.com[*]Corresponding Arthur:

Abstract: Power generation business in the United States with approximately 9,733 MW of installed capacity from agricultural waste and non-agricultural sector, the largest source of non-renewable water power in the world has created. The capacity of 9733 MW including 5,886 MW of forest plants and agricultural residues, 3,308 MW power generation capacities of 539 MW waste urban and other materials, such as the gas is obtained from buried waste. Maximum electricity production from biomass to electricity load based on the existing electricity distribution system is used. More than 200 companies from non-wood products and food industries in the United States produce electricity biomass. Four power generation systems using biomass there. Direct fuel, the fuel mixture (with coal), and plants gasify module. Most biomass power plants are direct systems such as traditional fossil fuel power plants often act. Biomass production in North America is 180 million tons of which 43 percent of the amount of agricultural residues in plants using advanced biological anaerobic bacteria and gas production and energy production are a combination of fuel between the available biomass Potential country of Iran 22 million is a system of energy production from residue agriculture often is that this residue in a tank Amplifier as burnt is fuel ash and gas artificial is that gas result can be thermal energy used or by the generator to electrical energy to become today the ability to produce 15 billion cubic meters of gas household artificial residue agriculture there is fuel derived from technologies convert biomass or state gas (Environmental gas) or liquid (methanol, ethanol and biodiesel), which for produce electricity and heat are used. It is estimated that if only 10 percent of farms and forests to provide and providing allocated biomass, annual production of energy from biomass, equivalent to four-fifths of world energy consumption will be present. Developing communities that almost three-quarters of the world's population are included, 35 percent of energy consumption comes from biomass. If the process can be used to power advanced production techniques | Full Text | 198 |

	such as biological America, collecting, etc. in areas such as agriculture in Khuzestan, which remains almost "Between 25-18 percent of products is very high figure is in addition to performing and indigenous on energy production, burning farms and destruction of ecosystems, soil, water, air and will prevent .... Use of biomass resources, one of the best and most economical solutions to provide basic energy needs of people in remote areas, and environmental benefits this type of environment, renewable energy and its development, its application, is reasonable and affordable. [Ashraf jazayeri, Tayeb Saki Nejad, Sorosh zarrin abadi. **Technical knowledge of biological plants America and localizes it for energy production from agricultural residues in IRAN (Khuzestan province). Journal of American Science 2010;6(12):1761-1760]. (ISSN: 1545-1003). http://www.americanscience.org.** **Keywords**: biological plants, infrastructure, agriculture remains		
199	**Optical and mechanical effects of different bleaching regimens on enamel surface** Nagy S *, Zoghbi A M El **, Zaazou MH.*, Abd alsabour K K ***, Taher HA ** *Department of restorative dentistry and dental materials ,national research center; ** Department of operative dentistry, faculty of oral and dental medicine, Cairo University; ***Laser Institute, Cairo university. Corresponding author :mohamedzaazou@yahoo.com **Abstract: Purpose:** This study was designed to assess the effect of 3 different bleaching regimens on color and microhardness of enamel. **Materials and Methods**: Ninety bovine upper central incisors were divided into three main groups according to the bleaching regimen used; chemically activated (Viva Style Paint on Plus), photo activated (Zoom 2), and laser activate (Opalescence X-Boost) bleaching agents. Each group was stained with (tea, carbonated beverage or a combination of both tea and carbonated beverage for 1 day or 6 days. Color was assessed using computerized image analysis in terms of grey scale, while Vickers microhardness tester was used to assess change in enamel microhardness. **Results:** Computerized image analysis revealed statistically significant decrease in the mean grey scale value of all teeth immersed in the three staining solutions used. The results also revealed that color change become intense as the immersion time increased. After bleaching with the three bleaching regimens the results revealed increase in the mean grey scale value of all the three bleaching regimens used with statistically significant increase in the mean grey scale value of both photo and laser activated bleaching agents than did chemical activated bleaching agent. Microhardness results revealed that there was statistically significant decrease in enamel microhardness after immersion in the three solutions, where the carbonated beverage group showed the lowest mean microhardness value than did the tea and the combination solutions. After bleaching with the three bleaching regimens enamel revealed a significant decrease in its microhardness. For all groups, no correlation was found between color change of enamel surface and its microhardness. **Conclusion**: Tea and Carbonated beverages have the ability to discolor teeth and alter their microhardness. Different bleaching regimens are lightening the color of discolored teeth but adversely affect enamel microhardnes. [Nagy S, Zoghbi A M El, Zaazou MH, Abd alsabour KK, Taher HA. **Optical and mechanical effects of different bleaching regimens on enamel surface.** Journal of American Science 2010;6(12):1766-1773]. (ISSN: 1545-1003). http://www.americanscience.org. **Keywords:** Optical and mechanical effects of different bleaching regimens on enamel surface	Full Text	199
200	**Evaluation of Conical Self-tapping One-piece Implants for Immediate Loading of Maxillary Over-dentures** Amr Zahran[1], Mona Darhous[2], Mohamed Sherien[3], Tarek El-Nimr[4], Basma Mostafa[5], Tamer Amir[6] 1. Professor of Periodontology, Cairo University, Cairo, Egypt. 2. Professor and Chairman of the Department of Periodontology, Cairo University, Cairo, Egypt. 3. Professor of Periodontology, Cairo University, Cairo, Egypt.	Full Text	200

4. Lecturer of Maxillofacial Surgery, MSA University, Cairo, Egypt.

5. Researcher, Department of Surgery and Oral Medicine, National Research Center, Cairo, Egypt.

6. Researcher, Department of Periodontology, Cairo University, Cairo, Egypt.

Corresponding author: Dr. Basma Mostafa Zaki, BDS, MDS, PhD, Researcher, Department of Surgery and Oral Medicine, National Research Centre, E-mail: boshta@hotmail.com

Abstract: Studies of maxillary overdentures supported by conventional implants often show a high implant failure rate. It was believed that maxillary implants should be splinted to retain a removable maxillary overdenture in order to maintain osseointegration. **Materials and Methods:** The present study evaluated the clinical performance of new generation of OsteoCare's Midi self-tapping self-drilling one-piece (ball type) implants for the support of maxillary overdentures. Seventy five implants were placed in the anterior maxillary region of 14 patients. A transmucosal flapless procedure was used to place four to six implants for each patient and followed by immediate delivery of an overdenture. The patients were evaluated at 6-month intervals for a follow-up period of 18 months. The clinical criteria to be checked were survival rate, Periotest values, radiographic crestal bone level and patient satisfaction. The **results** showed that 73 implants had successfully osseointegrated as indicated by the clinical and radiographic examinations. Implant survival rate of 97.3% was attested. The accumulated mean marginal bone loss was 0.88mm at the end of the follow-up period. Patients showed a very high degree of satisfaction of the treatment outcome due to the highly improved retention with partial palatal coverage using horse shoe designed maxillary over-dentures. This procedure has many advantages which include implant placement with minimally invasive transmucosal flapless surgery, decreased postoperative pain and a decreased cost of treatment. Single-stage one-piece implant placement, immediate loading, and transmucosal flapless surgery can result in high success rates when proper techniques are utilized with appropriate patient selection. In **conclusion**, the use of the Osteocare's Midi one-piece (ball type) implants is a valid unique simple treatment modality to support maxillary over-dentures.

[Amr Zahran, Mona Darhous, Mohamed Sherien, Tarek El-Nimr, Basma Mostafa, Tamer Amir[6]**Evaluation of Conical Self-tapping One-piece Implants for Immediate Loading of Maxillary Over-dentures.** Journal of American Science 2010;6(12):1774-1781]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Midi one-piece implants, immediate loading, maxillary over-dentures

Simultaneous Removal of Iron and Manganese from Ground Water by Combined Photo-Electrochemical Method

Hala A. Talaat [1], Montaser Y. Ghaly [1], Eman M. Kamel [2], Enas M. Ahmed [1] , Ahmed M. Awad [1 *]

[1] Chemical Engineering and Pilot Plant Department, [2] Photo-Chemistry Department, National Research Centre - Dokki, Cairo – Egypt, * awadx1@yahoo.com

Abstract: Ground water is highly important source of drinking water in Egypt. Some provinces suffer from high content of iron and manganese in ground water which threat human health. Different processes, such as electrochemical (EC), photo (UV), and combined photo-electrochemical (CPE) methods were used to attain both metals content to the level in accordance to reports of World Health Organization (WHO). A cell containing aluminium electrode as anode, graphite electrode as cathode and UV lamp were used and filled with waste water enriched with iron and manganese as an electrolytic solution. A limited quantity of sodium chloride salt was added to enhance the electric conductivity through the solution. A comparison between different methods was undertaken to evaluate the applied conditions and the efficiency of Fe and Mn removal at different times and initial concentrations. The results revealed that CPE method was the best choice for the simultaneous removal of both iron and manganese in a short time < 10 min. [Journal of American Science. 2010;6(12):1-7]. (ISSN: 1545-1003).

Keywords: Ground water; Egypt; electrochemical (EC); photo (UV); combined photo-electrochemical (CPE); World Health Organization (WHO).

1. Introduction:

Some regions in Egypt suffer from the contamination of ground water with high concentration of iron and manganese, which threat human health and leading to chronic diseases. Groundwater pollution can occur in various ways, in addition to natural or geochemical contamination, by leaks in pipelines, from landfill leachates, etc. It can be divided into three main contamination categories, by organic compounds, by microorganisms, and inorganic pollutants. The contamination of groundwater with metals of inorganic pollution comprises a danger environmental problem due to the fact that metals are not biodegradable and can cause severe adverse effects on human health [1].

The presence of iron and manganese compounds in groundwater, and eventually in drinking water, is a serious environmental problem. When iron and manganese compounds are present in both surface and groundwater, even at low concentrations, they can be linked to various water quality problems and their removal is essential.

The Safe Drinking Water Act (SDWA) secondary standards for iron in drinking water is 0.3 parts per million (ppm) and for manganese it is 0.05 ppm. Iron and manganese are both known to stain the water supply. They can make water appearance red or yellow, create brown or black stains, and give off an easily detectable metallic taste. Several years ago, it was believed that incumbent soil layers, acting as natural filters and protected ground waters, but actually it was found that soil ores of iron and manganese can be easily dissolved into ground water particularly at highly acidic medium [2].

Iron in water supplies causes aesthetic and operational problems, such as bad taste and colour, staining and deposition in the water distribution system leading to high turbidity. Manganese is a very common compound that can be found everywhere on earth and it is one of the most abundant metals in soils, where it occurs as oxides and hydroxides, and cycles through its various oxidation states. Manganese is one out of three toxic essential trace elements, which means that it is not only necessary for humans to survive, but it is also toxic when too high concentrations are present in a human body [3].

Manganese is one of the most abundant metals in soils, where it occurs as oxides and hydroxides, and it cycles through its various oxidation states. Manganese occurs principally as pyrolusite (MnO_2), and to a lesser extent as rhodochrosite ($MnCO_3$).

Manganese, in the form of potassium permanganate, may be used in drinking water treatment to oxidize and remove iron, manganese, and other contaminants [4-6].

Manganese in ground water is difficult to remove by using normal methods, where it required a high potential to overcome its high activation energy required for manganese oxide formation, where MnO_2 is formed by highly oxidizing and high pH conditions [7, 8]

In recent years, various treatment technologies have been employed to enhance water quality by

removing inorganic and organic contaminants. Both photo and electro-chemical oxidation technologies recently have become more popular for water treatment. Doan and Saidi [9] used combined electrochemical and photochemical oxidation for the removal of inorganic contaminants like Zn and Ni, and organic contaminants like alkylbenzene sulfonate. They found that the results of combined system are at comparable levels to those obtained in the sole electrochemical system. Peralta-Hernández et al [10] designed an annular tube reactor of combined photo- electrochemical system for the generation of H_2O_2 and Fenton reagent in situ, the rate of oxidation was increases substantially when the semiconductor anode was illuminated as compared to the same processes carried out in the dark. These processes are considered as attractive options in solving the issues concerning iron and manganese removal from water particularly, if other compounds such as ammonia, total dissolved solids or natural organic matter (NOM) are found [11]. To solve this problem, Fe^{+2} or Fe^{+3} can be introduced into the system, constructing an electro-Fenton's reagent as one of a special class of oxidation techniques defined as advanced oxidation processes (AOPs) [12]. These processes are characterized by the capability of exploiting the high reactivity of free hydroxyl radicals. Free hydroxyl radical ($OH^{\bullet}$) is a non selective and very powerful oxidant agent able to oxidize organic and inorganic pollutants in water and is generated from chemical, electro and photochemical (by using light irradiation) processes. Electro-Fenton process can be enhanced in presence of UV radiation [13]. Stephen and Charlotte were used electrolytic cell containing aluminum and iron electrodes of high surface area relative to the volume of electrolyte for the generation of fine flocs of Al(OH)3 acting as colloids and adsorption centers for contaminants dispersed in waste water [14].

In the present study an approach was studied and evaluated for the removal of heavy metals like, iron and manganese to avoid their harm to human health. Combined photo electrochemical oxidation technique was investigated for the removal of iron and manganese from water, since a little information is available on this approach. The removal of iron and manganese from synthetic solution using bench-scale CPE oxidation system was evaluated using different concentrations levels of both iron and manganese at different conditions.

2 - Materials and Experimental:

2.1. Materials

Ferrous sulfate heptahydrate ($FeSO_4.7H_2O$) were used as a source of iron in form of Fe (II) was supplied by S.D. Fine Chem. Ltd. Manganese sulfate mono-hydrate ($MnSO_4.H_2O$) was used as sources of manganese in form of Mn (II) was supplied by S.D. Fine Chem. Ltd. Pure sodium chloride (NaCl) was used as electrolyte, purchased from Merck. Distilled water was used throughout. Analar Sulfuric acid 98 % purchased from ADWIC.

2.2. Experimental Set-up

A laborator combined photo electrochemical unit was used for the batch experiments. The schematic diagram of the experimental set-up used is shown in Fig.1. It consists of a cylindrical quartz photo reactor (1.2 L), with a coaxial and immersed medium pressure UV mercury lamp used as the UV emitter and light source (Heraeus TQ150, input energy of 150 W) emitting a polychromatic radiation in the range from 100 to 280 nm wavelength. The UV lamp was equipped with a cooling water jacket to maintain the temperature of the reaction of wastewater treatment at room temperature.

The reaction vessel was filled with authentic solution containing both iron and manganese. The electrochemical characterization of the solution was carried out by using DC power supply GW 3030 an two electrodes, graphite cathode and aluminium anode. The measurements were performed at room temperature and the mixing was accomplished by using continuous magnetic stirrer.

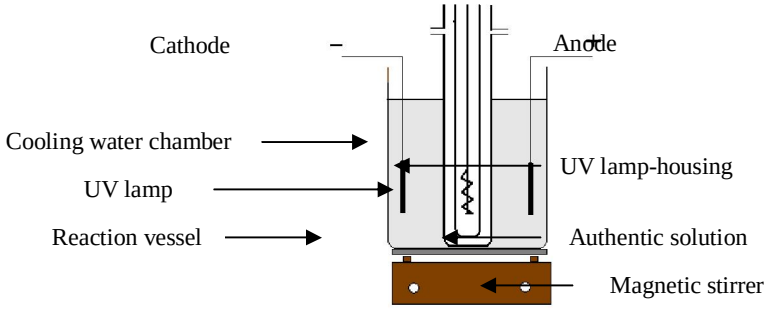


Figure (1): The schematic diagram of the experimental set-up

2.3. Procedures and Analysis

Workers have developed an approach for the removal of heavy metals like, iron and manganese. Three techniques were examined for the removal of iron and manganese namely, Electrochemical (EC), photochemical (UV), and combined photo-electrochemical (CPE) methods, where the study comprised a comparison between all techniques to track the most efficient one by determination of removal efficiency.

Authentic solutions of different iron and manganese concentrations are prepared as model of ground water by dissolving definite concentrations of both a mixture $FeSO_4.7H_2O/MnSO_4.H_2O$ in distilled water. Definite amounts of NaCl were added to improve the conductivity and ionic mobility through the electrolyte. The solution was acidified by drops added of prepared dilute sulfuric acid 15 % to pH 3. The efficiency of the process was evaluated by measuring the metal removal from samples at the end of each experiment. Samples are filtered before the measurement of metals by using atomic adsorption (Percken Elmar 1100B, Germany) [15]

3. Results and Discussion:

3.1. Comparative removal behaviour of different methods

Before iron and manganese can be filtered, they need to be oxidized to a state in which they can form insoluble complexes. Oxidation involves the transfer of electrons from the iron and manganese to the oxidizing agent. Oxidation methods using additives like chlorine, ozone, air; or those using an oxidizing filter media fall to oxidize iron and manganese.

UV oxidation, electrochemical oxidation (EC) and combined photo-electrochemical (CPE) methods were used for removal of both dissolved iron and manganese from authentic solution. Fig.2 represents a comparison between the removal efficiency of dissolved iron and dissolved manganese by using combined CPE method from a mixture solution of concentration 5 ppm $FeSO_4.7H_2O$ and 5 ppm $MnSO_4.H_2O$. A highly removal efficiency was achieved after a short time, where the concentration of Fe was decreased from 5 ppm to less than 0.1 ppm after 5 min (R=98%), while the removal of Mn was decreased from 5 ppm to 1.7 ppm after 5 min. (R= 66 %), and it was highly decreased to 0.2 ppm after 20 min (R= 96%). It is obviously shown that the removal efficiency of Mn was less than Fe after the same time, where the removal of Mn required an oxidation potential higher than iron, so the removal efficiency of Mn can be improved by using high potential and adequate time.

This indicates that the electrochemical potential supported by UV irradiation (CPE) enhanced the oxidation of both soluble Fe^{2+} and Mn^{2+} to insoluble Fe^{3+} and Mn^{4+} ions in a short time, which can be adsorbed on the surface of formed $Al(OH)_3$ floc.

The lowest concentration obtained of Mn after CPE treatment was 0.2 ppm (R = 96 %), but it still higher than the recommended standard values according to the reports of WHO. So, the second treatment may be required for the removal of residue of Mn to attain the standard concentration 0.05 ppm (R = 99.6 %),

Another method was carried out by using electrochemical oxidation, where the solution was totally exposed to electric field between anode and cathode. Some reactions were taken place at the surface of electrodes and in the bulk of electrolytic solution, where Al^{3+} ions were generated by anodic oxidation, reduction of hydrogen ions at the surface of cathode and water molecules were electrolyzed to OH^- and H^+.

$$Al \rightarrow Al^{3+} + 3e^- \quad (1)$$

$$H_2O \rightarrow OH^- + H^+ \quad (2)$$

$$Al^{3+} + OH^- \rightarrow Al(OH)_3 \quad (3)$$

The concentration of dissolved iron was decreased sharply from 5 to 0.1 ppm within 10 min of oxidation time as shown in Fig.3 (R= 98 %). The concentration of Mn was decreased slowly from 5 to 2.2 ppm after 5 min (R = 56%) and by elongation of time, the removal efficiency was improved to (R= 76%), where the concentration of manganese was decreased from 5 to 1.2 ppm after 20 min.

In the electrochemical method, the formed Al $(OH)_3$ floc gave a high important role as a suspended colloids having an electrostatic adsorption capability of agglomeration of dissolved Fe^{2+} and Mn^{2+} ions.

On the other hand, one can observe the comparative removal of iron and manganese of different methods. As shown in Fig.4, a quite slow removal of 5 ppm of Fe by using UV light after 20 min was occurred to attain the lowest concentration 0.5 ppm after 20 min (R=90%), while the lowest concentration residue of Mn was 3.5 ppm (R= 30 %) was obtained after 20 min. This indicates the low energy produced and consequently low effect of UV irradiation, where the generation of a very small concentration of the main oxidant $OH^{\bullet}$ radical from the decomposition of water.

Also, it was obviously observed that the removal efficiency values of (EC) method was approximately similar to (CPE) method; i.e. the oxidation activation energy of (EC) method >> the

oxidation activation energy of (UV) irradiation method. This indicates the low effect of UV irradiation for metal oxidation process by CPE method.

On the other hand, the removal efficiency of iron by all methods was generally higher than the removal efficiency of manganese; this behavior can be attributed to the higher oxidation potential required for oxidation of Mn, where the insoluble form MnO_2 is formed by high potential (1.05 V) and high pH value 9. While a high removal efficiency of iron was achieved due its lower oxidation potential (0.77 V) required for the formation of Fe $(OH)_3$.

UV irradiation participates marginally in the oxidation of Fe and Mn, while the participation of electrochemical oxidation was proved in Fig.2. The role of photo oxidation can be outlined in the following points: (i) It accelerates the oxidation of Fe and Mn to the insoluble high oxidation state, (ii) In the presence of photo oxidation $^{\bullet}OH$ free radical can be formed by the photolysis of H_2O and (iii) it can be excellently used for the disinfection of municipal water.

In the electrochemical oxidation, hydroxyl radicals may be produced over the surface at high-oxygen over voltage anode from water oxidation and electro generation of hydrogen peroxide (H_2O_2) formed from the two-electron reduction of O_2 at a graphite cathode [16]. The oxidizing H_2O_2 can be enhanced in the presence of catalytic Fe^{2+}, due to the formation of hydroxyl radicals from the classical Fenton's reaction between both species.

$$H_2O \rightarrow OH^{\bullet} + H^+ + e^- \quad (4)$$

$$O_{2\,(g)} + 2H^+ + 2e^- \rightarrow H_2O_2 \quad (5)$$

It can be seen from Fig.2, the quicker iron removal found by combined photo electrochemical oxidation where 96.8 % iron removal was obained within 10 minutes reaction time due to the generation of more hydroxyl radicals with the enhancement of electrochemical oxidation in the presence of UV light (CPE).

Moreover, in both EC oxidation and CPE oxidation processes, by using aluminum as anode in the electrolytic cell, aluminum ions (Al^{3+}) are formed which reacts with hydroxyl ions (OH^-) and forms aluminum hydroxide as flocs which adsorb Fe^{2+} and Mn^{2+} ions and co-precipitate together.

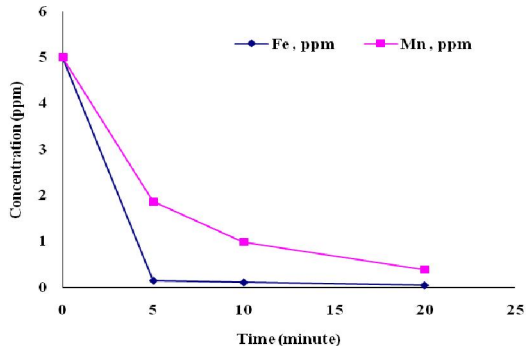


Fig. 2: Removal efficiency of Fe and Mn by (CPE) oxidation method from a mixture authentic solution of (Fe&Mn) [Initial concentration 5 ppm, 150W, 0.25 A]

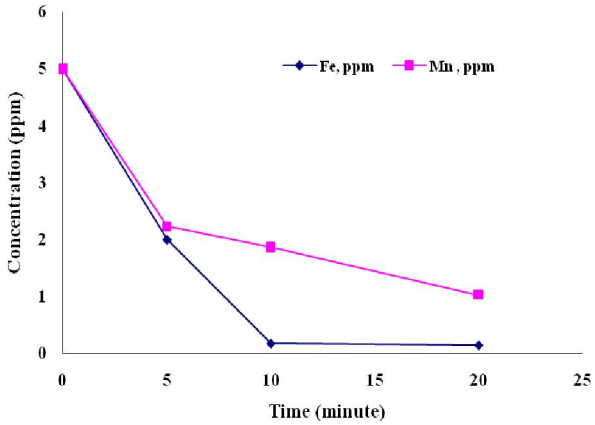


Fig.-3 : Removal efficiency of Fe and Mn by (E) oxidation method from a mixture authentic solution of (Fe&Mn) [Initial concentration 5 ppm, 0.25 A]

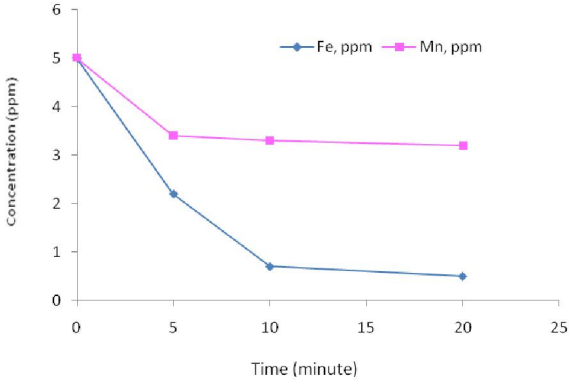


Fig.-4 : Removal efficiency of Fe and Mn by (UV) oxidation process from a mixture authentic solution of (Fe&Mn) [Initial concentration 5 ppm, 150W].

3.2. Effect of initial concentration on the removal of Fe and Mn by CPE

The presence of high concentration of dissolved metals into ground water gives an advantage for the treatment by electrochemical or combined CPE method, where the dissolved salts increase the electric conductivity and the ionic mobility of ions through the solution toward or backward both electrodes.

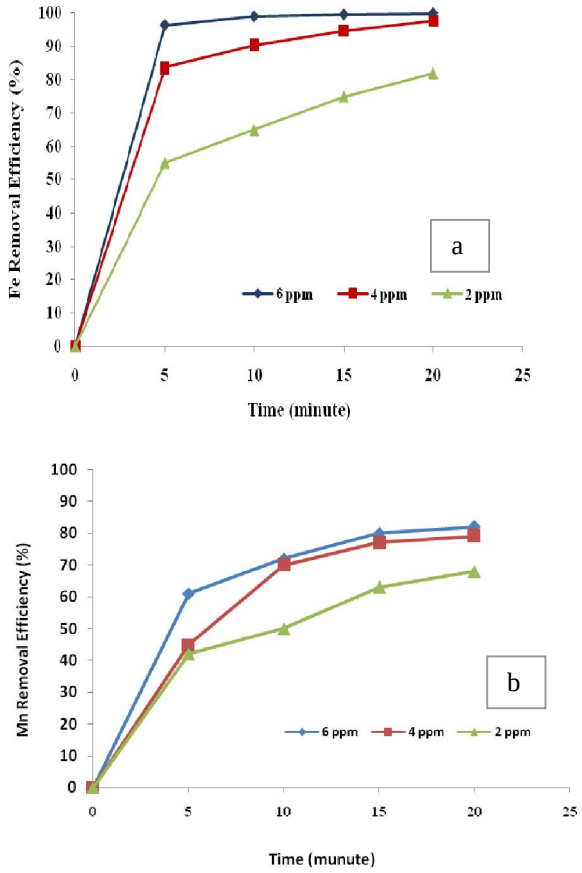


Fig. 5: Removal Efficiency of (a) Fe and (b) Mn from mixture solutions of different concentrations 2, 4, and 6 ppm by (CPE) method.

As shown in Fig. 5, the removal efficiency was highly affected by the initial concentrations of mixture solution Fe/Mn. Fig.3a showed the rapid and high removal efficiency of iron (R= 96 %) from high concentration 6 ppm of mixture Fe/Mn solution after 5 min, while after the same time, the treatment of less concentrations 2 and 4 ppm by CPE method showed a lower removal efficiencies 54 and 82 % respectively. Generally, the removal efficiency was enhanced with time in low concentration. Removal efficiency of manganese in a mixture solution Fe/Mn as shown in Fig. 5b revealed lower removal efficiencies than Fe, this is attributed to the high activation energy required for oxidation of Mn more than Fe, so removal of Mn needs a long time and high potential more than Fe, where R of Mn of concentration 6 ppm was 63 % and 89 % after 5 min and 20 min, respectively. While at low concentration 2 ppm 54% was achieved after 20 min.

It can be observed that the removal efficiency of high concentrations 6 ppm of dissolved Fe and Mn revealed better values more than less concentrations 2 ppm, where the high concentrations strengthen the electrochemical reactions and overcome the resistance of ionic mobility through the solution.

3.2. Effect of current on the removal of Fe and Mn by CPE

The applied electric current played a big role in the removal efficiency enhancement, where at high electric current there is a better condition for the electrolysis of water molecules to produce OH^- and anodic oxidation of aluminium to produce Al^{3+} . Finally, a high content of $Al(OH)_3$ flocs were formed which are a strong adsorbed electrostatic particles.

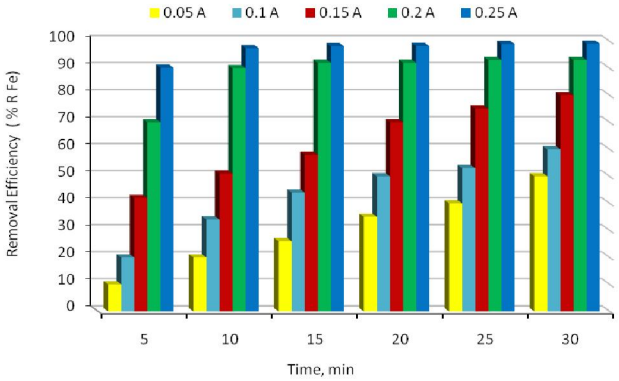


Fig.6 : Effect of current density on the removal efficiency of iron by CPE method at different time

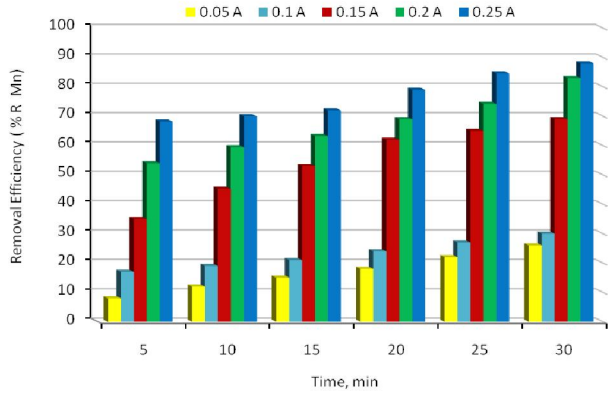


Fig.7 : Effect of current density on the removal efficiency of manganese by CPE method at different time

As shown in Fig.6, according to the applied electric current, the removal efficiency was varied , where at low applied electric current, a low values of % R was obtained, but by elongation of time, the removal efficiency was improved. At low time 5 min, the removal efficiency was less than 10% at low current 0.05 A, and it did not exceed 68 % by increasing current to 0.25 A. Removal efficiency was increased with time from 5 to 30 min, where at low electric current 0.05A the removal efficiency was 10 % after 5 min and 49 % after 30 min, while at high electric current 0.25 A, the removal efficiency was 89 % and 97% after 5 min and and 30 min, respectively. The study of removal of dissolved Mn by CPE method showed that the removal efficiency was less than Fe at the same concentration, pH, temperature in spite of incresing of electric current or time.

As shown in Fig.7, the removal efficiency of Mn at low current 0.05 A was less than 6 % and 24 % after 5 min and 30 min , respectively. While at higher current 0.25 A, the removal efficiency was 68 % and 87 % after 5 min and 30 min , respectively.

Thus, it was ascertained that the removal efficiency of iron was higher than manganese at the same conditions, this phenomenon is attributed to the high oxidation potential of Mn (1.05 V) more than iron (0.44 V). Moreover, because the oxidation of iron at low potential takes place before Mn, the major $Al(OH)_3$ flocs were consumed in the adsorption of Fe^{2+} ions before the adsorption of Mn^{2+}.

3.2. Effect of NaCl dose added on the removal of Fe and Mn by CPE

In both CPE and EC methods, the addition of salt to the solution such as sodium chloride has an important role to reduce the resistivity and improve the ionic mobility through the solution and hence electrochemical reactions are going forward.

Sodium chloride was added according to the limits of standard WHO values, where the concentration added was not exceed 45 ppm. Without addition of salts, the final pH was not varied where pH was 3 in the beginning of treatment.

An additional advantage of using NaCl is the bacteriacidal effect by the chlorination, where Iron and manganese bacteria are destroyed. Moreover, Hypochlorite and chlorate anions can be formed by oxidation of chloride ions, forming strong oxidizing agents in the solution as the following:

$$2Cl^- \longrightarrow Cl_2 + 2e^-$$
$$Cl_2 + H_2O \longrightarrow HOCl + Cl^- + H^+$$
$$HOCl \longrightarrow OCl^- + H$$
$$2HClO + OCl^- \longrightarrow ClO_3^- + 2\,HCl$$

Fig. 8 shows the effect of NaCl added where, as the dose of NaCl increased from 15 to 45 ppm, pH was varied from 5.5 to 7.2 and also current intensity was increased from 120 to 260 mA.

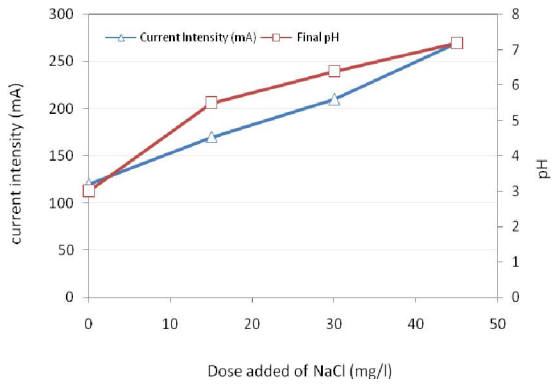


Fig.8: Effect of addition of NaCl on both current intensity and final pH

Furthermore, without addition of NaCl, the removal efficiency was very low (R=60%), while it was increased from 77 to 92 as the dose of NaCl added from 15 to 45 ppm. The removal efficiency was increased as the dose added of NaCl increased, this indicate the importance of salt addition to the solution when the conductivity is not adequate for the ionic transfer between the bulk solution and the surface of electrodes.

The presence of manganese into the solution of iron may behave as a catalyst for the oxidation of Fe^{2+} to Fe^{3+} and Mn^{2+} is oxidized to Mn^{4+} and co-precipitated together.

So, it can be considered that the presence of two or several types of dissolved metals is more applicable in the electrochemical or CPE methods, where the dissolved salts enhance the conductivity and hence, reduce the potential required. They behave as a catalyst and mediators for oxidation according to their redox potential.

4. Conclusion:

A combined photo electrochemical method was used for oxidation of soluble forms Fe^{2+} and Mn^{2+} to the insoluble forms Fe^{3+} Mn^{4+}. The combined method (CPE) revealed more efficiency than a sole EC and UV methods. The presence of both dissolved iron and manganese has the advantage of less resistivity of the solution of waste water. Low concentration of NaCl (15-45 ppm) was added to increase the conductivity and electric current beside its bactericide effect after electrolysis to chlorine. The effect of Fe^{2+} and Mn^{2+}

concentrations revealed that the higher concentration of dissolved iron and manganese ions, the higher removal efficiency obtained.

The study showed the rapid oxidation of Fe^{2+} more than Mn^{2+} due to the lower oxidation potential of iron than manganese and the catalytic oxidation behaviour of manganese may accelerate the oxidation of iron.

Corresponding Author
Ahmed M. Awad
Engineering and Pilot Plant Department
Dokki, Cairo – Egypt
awadx1@yahoo.com

5. References:

1- F.R. Spellman, Handbook for Waterworks Operator Certification, Vol. 2, Technomic Publishing Company Inc., Lancaster, USA, 2001, 6–11, 81–83.
2- U.S. Environmental Protection Agency, Office of Water (4304T), Health and Ecological Criteria Division, Washington, DC 20460, *http://www.epa.gov/safewater/*, EPA-822-R-04-003, January, 2004.
3- EC-Official Journal of the European Communities Council Directive, Drinking water quality intended for human consumption, Brussels, Belgium, 98/83/EC L 330/32, 1998.
4- ATSDR. 2000. *Toxicological Profile for Manganese*. U.S. Department of Health and Human Services, Public Health Service, Agency for Toxic Substances and Disease Registry, Atlanta, GA.
5- Shacklette, H.T., and J.G. Boerngen. 1984. Element oncentrations in soils and other surficial materials of the coterminous United States. USGS Paper 1270. Washington, DC: US Govern. Printing Office, p. 6.
6- WHO. 1981. Environmental Health Criteria 17: Manganese. World Health Organization. Geneva, Switzerland.
7- Crerar, D.A., and H.L. Barnes. 1974. Deposition of deep-sea manganese nodules. Geochim. Cosmochim. Acta 38:270–300.
8- Sikora, F.J., L.L. Behrends, G.A. Brodie, and H.N. Taylor. 2000. Design criteria and required chemistry for removing manganese in acid mine drainage using subsurface flow wetlands. Water Environ. Res. 72(5):536–544.
9- Doan HD, Saidi M, "Simultaneous removal of metal ions and linear alkylbenzene sulfonate by combined electrochemical and photocatalytic process." Journal of Hazardous Materials , Volume 151, Issues 2-3, 1, 2008, 306-315.
10- J. M. Peralta-Hernández, Yunny Meas-Vong, Francisco J. Rodríguez, Thomas W. Chapman,Manuel I. Maldonado and Luis A. Godínez, "In situ electrochemical and photo electrochemical generation of the Fenton reagent: A potentially important new water treatment technology", Journal of Hazardous Materials, 143, 8, 2007, 198-205.
11- M. Zaw, B. Chiswell, (1999), Iron and manganese dynamic in lake water, Water Res. 33, 1900.
12- E. Brillas, B. Boye, I. Sirés, J.A. Garrido, R.M. Rodr´ýguez, C. Arias, P. Cabot, C. Comninellis, (2004), Electrochemical destruction of chlorophenoxy herbicides by anodic oxidation and electro-Fenton using a borob-doped diamond electrode. Electrochimica Acta 49, 4487–4496.
13- E. Brillas, R. Sauleda and J. Casado, (1998), Degradation of 4-chloropheno by anodic oxidation, electo-Fenton, photoelectron-Fenton, and peroxi-cagulation processes. J. Electrochem. Soc. 145, 759–765.
14- A. Rojo. Stephen and N.C. Charlotte, US 4149953, 1979.
15- H.D. Chapman, P.E. Pratt, Method of Analysis for Soil, Plant and Water. Univ. of California, Dept. of Agric. Sci., 1978, Priced Publication 4034.
16- G. Busca, S.Berardinelli, C. Resini, L. arrighi, "Technologies for the removal of phenol from fluid streams: A short review of recent developments", J. of Hazardous Materials, 160, 265-288 (2008).
17- G. S. R. Krishnamurti , P. M. Huang, "Influence Of Mn $^{2+}$ And Ph On The Formation Of Iron Oxides From Ferrous Chloride And Ferrous Sulfate Solutions", Clays and Clay Minerals, Vol. 37, No. 5, 451-458, 1989.

6/20/2010

Effect of Aqueous Root-Bark Extract of Vitex Doniana Sweet on Haematological Parameters in Rats

F. I. Abdulrahman[1], J.C. Akan, O.A.Sodipo[2] and P.A. Onyeyili[3]

1. Department of Chemistry, University of Maiduguri, P.M.B 1069, Maiduguri, Borno State, Nigeria
2. Department of Clinical Pharmacology and Therapeutics, College of Medical Sciences, University of Maiduguri, P.M.B 1069, Maiduguri, Borno State, Nigeria.
3. Faculty of Veterinary Medicine and Pharmacology, University of Maiduguri, P.M.B 1069, Maiduguri, Borno State, Nigeria

E-mail: joechemakan@yahoo.com

ABSTRACT: Acute and sub-acute toxicities of aqueous extract of Vitex doniana was carried out in rats. The LD50 following intraperitoneal administration estimated at 95% confidence interval was 980 mg/kg. The oral administration of the extract for 21 days at 50,100 and 200mg/kg had beneficial effects on the haematological parameters. There were significant ($P<0.05$) increases in red blood cell count (RBC) haemoglobine (HB) concentration and packed cell volume (PCV) values in treated rats. The treated animals had leucocytosis, which may be due to increase lymphocyte count observed. The i.p LD50 (980 mg/kg) indicated that the extract is moderately toxic, though the prolong oral administration of the extract under the condition of this study shows that the extract may be toxic at higher doses. Nevertheless, the extract appear to be more beneficial at lower doses and significantly ($p< 0.05$) improves RBC, HB and PVC values and this effect has potential application as anti-anaemic agent. This seems to provide justification for its use as anti-anaemic agent in African traditional medicine. [Journal of American Science. 2010;6(12):8-12]. (ISSN: 1545-1003).

Keywords: Vitex doniana, Red blood Cell Count, Haemoglobin Concentration, Packed Cell Volume, Anaemia, Aqueous Extract.

1. INTRODUCTION

There is little doubt the positive roles that herbal medicines have and continue to have on the lives of Nigerians and of all people throughout the world. Herbs are known to have sustained mankind not only as sources of food but also as medicines and poisons utilized in various ways for varied purposes (Abdu-aguye, 1997). The use of medicinal plants in West Africa is probably as old as the duration of human settlement in the region.

Vitex doniana is one of the agents used for folklore medicinal purposes. Although parts of the plant are used by traditional healers for the treatment of various ailments (Akiniyi and Sultanbawa 1987) information on the toxicity of the extract in man animal are lacking. The present study investigated the acute and chronic toxicity of the aqueous extract of vitex doniana in rats. This is important since science requires the validation of drugs by medicinal practitioners and drug regulatory authorities demand that all potential drugs should pass through a rigorous series of study and scrutiny (Abdulrahman, 2004).

2. MATERIALS AND METHODS

2.1 Plant collection and preparation

The root-bark of *Vitex doniana* was collected from the outskirts of Maiduguri, Borno State and identified by Prof. S.S. Sanusi a plant taxonomist in the Biological Sciences Department of University of Maiduguri, Nigeria. The air-dried root-bark was subsequently ground into powder. Five hundred grammes (500g) of the powdered root- bark was exhaustively soxhlet extracted with distilled water (Mittal *et al.,* 1981 and WHO, 1992). The extract was concentrated *in vacuo* and stored at $4^{O}C$ until required. A fresh solution was prepared from the residue on each day of extract administration.

2.1.1 Animals

Rats of both sexes weighing between 94. 8-l 20 g was used for the experiments. They were obtained from a colony of rats maintained at the animal house of the Institute for Trypanosorniasis Research, Vom, Nigeria. The animals were housed in clean plastic cages and had access to feed (ECWA Feeds Nigeria LTD, Jos, Nigeria) and water ad libitum. They were allowed to acclimatize for two weeks in the Veterinary Physiology and Pharmacology Laboratory before the commencement of the studies. The animals were handled according to the international guiding principles for Biochemical Research (CIOMS, 1985) as certified by the Animal ethics committee of the Faculty of Veterinary Medicine, University of Maiduguri, Maiduguri, Nigeria.

2.2 EXPERIMENTAL PROCEDURE

2.2.1 Acute Toxicity of Aqueous Extract of *Vitex doniana* in Rats

Six groups of five rats each were used. The animals in groups A to E were dosed intraperitoneally (i.p.) with varying doses (400, 600, 1000, 1200 and 1600 mg/kg) of aqueous extract of *vitex doniana* while the rats in group F (control) received distilled water only by same route. The rats were allowed access to food and water *ad libitum* and observed over a period of 24 hours for clinical signs and death. The LD_{50} with 95% confidence interval was determined using the arithmetic methods of Aliu, and Nwude (1987).

2.2.2 Effect of Extract on Haematology

The rats (100) were separated into four equal groups. Animals in groups I-III were treated with 50, 100 and 200 mg/kg of the root-bark extract orally respectively for three (3) weeks, while group IV served as control and was given distilled water only by the same route. Clinical signs and haematological parameters were used to assess the effects of the various doses of the extract on the rats following prolonged administration. The animal were bled weekly and the blood used for the determination of the total red blood cell counts (RBC), packed cell volume (PCV), haemoglobin concentration (Hb), white blood cell counts (WBC) and differential leucocyte counts (DLC) using standard procedure (Kelly, 1977 and Pflanzer, 1990).

3 RESULTS AND DISCUSSIONS

3.1 Acute Toxicity Study

The dose of water root-bark extract of *Vitex doniana* that produced mortality was 3000 mg/kg, while the dose that causes 100% death was 1600 mg/kg (Table 1). The symptoms of toxicity observed with extract administration were dose dependent. Ten to fifteen minutes after administration of extract all the rats in the various groups were very weak. Those that received 600 mg/kg dose and above were deeply sedated and slept. Signs observed before death included loss of appetite, lethargy, paralysis of hind limb, which progressed to fore limbs, difficulties in respiration and coma. Mortality was recorded five hours after 1600 mg/kg extract of *Vitex doniana* treatment. The $LD_{5}o$ with 95% confidence limit of the extract was estimated to be 980 mg/kg.

Table 1. The dose of water root-bark extract of *Vitex doniana*

Group (n=5)	Extract dose (mg/kg)	Number of death	% Mortality
A	400.00	0	0.00
B	600.00	0	0.00
C	1000.00	3	60.00
D	1200.00	4	80.00
E	1600.00	5	100.00
F	Control (distilled water)	0	0.00

3.2 Effect on haematology

The rats treated with 100 and 200mg/kg of the water extract of Vitex dtmiana were depressed and less active compared to the rats in the other groups. The percentage weight gain decreased significantly with increase in extract dose (Table 2). Twenty one days following administration of the extract at 50, 100 and 200 mg/kg respectively to rats, there was a percentage weight gain of 56.0, 40.0 and 5.6 percent, Fourteen days following termination of treatment resulted in percentage weight gain of 52.3, 53.4 and 28.9 percent respectively in the group treated with 50, 100 and 200 mg/kg of Vitex doniana extract. The control group showed a percentage weight gain of 112.8 percent by the end of the experimentation period. The mean RBC counts of rats treated with the aqueous extract of the root back of vitex doniana orally for 21 days (3 weeks) are shown in Table 3. The RBC counts of rat treated with the extract increased significantly ($p<0.05$) and at 21 days of treatment the percentage increase were 93.3, 38.1 and 36.7 percent respectively in groups treated with 50, 100 and 200 mg/kg of the extract. RBC counts decreased with the termination of treatment but were still above the pre - treatment values.

The mean Hb concentration (Table 4) increased from the pretreatmcnt values of 1 1.4 ± 0.11, 12.2 ±0.17, 13.2±013g/dl respectively in groups treated with 50, 100, 200mg/kg of the root bark extract to 14.6 ± 0.13, 15.0 + 0.37 and 14.4 ±0.38g/dl at 21days of treatment. Following the termination of extract treatment there was gradual decrease in Hb

concentration.

The mean PVC value of rats treated with various doses of the root back extract of vitex doniana showed that at 21 days of treatment the PCV value were significantly (P<0.05) increased (36.4±0.13, 37.4±0.27 and 37.4±0.34, however at 14 days post-treatment the values decreased to 30.0 ±0.21, 28.0 ± 0. 44 and 29.0 ± 0.55 per cent in the groups treated with 50, 100 and 200 mg/kg of extract doses (Table 5).

Table 2: Effect of aqueous extract of the root-back of Vitex doniana on mean body weight of albino rats percent respectively) for the treatments

	Days of Treatment					
Extract Dose (mg/kg)	0	7	14	21	28	35
50.0	68.2±3.2 0	11.0±10.0	102.5±2.41	106.4±1.54	114.8±2.60	104.0±4.54
100.0	110.0±0.82	134.0±7.33	148.0±4.80	154.0±4.20	164.0±8.00	160.7±1.48
200.0	115.6±2.86	120.0±5.40	120.50±4.60	122.5±2.10	137.5±2.80	134.0±3.70
Control	48.4±2.32	62.2±1.70	57.20±8.70	74.0±2.00	90.0±2.00	103.0±4.70

Table 3: Effect of aqueous extract of the root-back of Vitex doniana on red blood cells (RBC) count

	Days of Administration of extract					
Extract Dose (mg/kg)	0	7	14	21	28	35
50.0	3.26±0.34	3.18±0.65	4.11±0.12*	6.30±0.25*	5.72±0.52 *	5.26±0.29*
100.0	5.30±0.28	4.90±0.34	7.04±0.24*	7.32±0.51*	5.60±0.50*	5.40±0.17*
200.0	4.96±0.19	4.94±0.56	5.52±0.28 *	6.78±0.38*	5.82±0.46*	5.84±0.44*
Control	4.96±0.19	4.28±0.60	4.36±0.25	4.24±0.54	4.34±0.59	4.44±0.15

*Significant increased (p<0.05) compared with mean of the control
Day 0= immediate ly before extract administration Day 34 – 14 days post treatment
Control = Given distilled water only

Table 4: Effect of aqueous extract of the root-back of *Vitex doniana* on haemoglobin concentrations in rats

	Days of Extract Treatment					
Extract Dose (mg/kg)	0	7	14	21	28	35
50.0	11.4±0.11	11.6±0.23	14.2±0.09*	14.6±0.13*	13.4±0.30	12.74±0.15
100.0	12.2±0.17	12.4±0.42	14.6±0.35 *	15.0±0.37*	13.2±0.19*	12.6±0.19
200.0	13.2±0.13	13.2±0.35	14.2±0.14*	14.4±0.38 *	13.0±0.07	12.8±0.27
Control	12.6±0.10	13.0±0.15	12.8±0.13	12.7±0.29	12.6±0.37	13.0±0.23

*Significant increased (p<0.05) compared with mean of the control
Day 0= immediate ly before extract administration Day 34 – 14 days post treatment
Control = Given distilled water only

Table 5: Effect of aqueous extract of the root-back of *Vitex doniana* on packed cells volume (PCV)

	Days of Extract Treatment					
Extract Dose (mg/kg)	0	7	14	21	28	35
50.0	29.9±0.21	29.0±0.33	33.6±0.17 *	36.4±0.13*	30.0±0.33	30.0±0.21
100.0	30.7±0.14	31.8±0.40 *	34.0±0.41*	37.4±0.27*	29.6±0.51	28.0±0.44
200.0	30.8±0.29	13.8±0.40	36.2±0.42 *	37.4±0.34*	29.2±0.38	29.0±0.55
Control	29.8±0.30	31.0±0.37	31.0±0.14	31.0±0.02	31.0±0.11	29.8±0.06

*Significant increased (p<0.05) compared with mean of the control
Day 0= immediate ly before extract administration Day 34 – 14 days post treatment
Control = Given distilled water only

Table 6: Effect of aqueous extract of the root-back of *Vitex doniana* on white blood cells count in rats

Extract Dose (mg/kg)	Days of Extract Treatment 0	7	14	21	28	35
50.0	4.34±0.55	4.36±0.67	4.88±0.94	4.85±0.78	6.34±0.56*	4.02±0.99**
100.0	4.28±0.44	4.16±0.49	6.08±0.08*	6.48±0.71*	6.78±0.38*	3.50±0.60**
200.0	4.86±0.29	4.88±0.57	6.12±0.77*	6.34±0.57*	6.53±0.47*	4.36±0.58**
Control	4.74±0.38	4.64±0.37	4.72±0.76	4.69±0.51	4.68±0.51	4.74±0.76

*Significant increased ($p<0.05$) compared with mean of the control
** Significant decreased ($p<0.05$) compared with mean of the control
Day 0= immediate ly before extract administration Day 34 – 14 days post treatment
Control = Given distilled water only

The total leucocyte (WBC) counts (Table 6) in rats treated with 100 and 200mg/kg doses of the water extract of Vitex doniana root-back were significantly increased ($P<0.05$) from day 14 of treatment. However, 14 days following withdrawal of the extract the high leucocyte values were reduced and were below the values for the control. The mean WBC counts of the control were statistically the same throughout the experimentation period. The differential leucocyte counts (DLC) of rats treated with various doses of the aqueous extract of root-bark of Vitex doniana showed some fluctuations. Following treatment with the extract, neutrophile significantly decreased ($P<0.05$), while the percentage lymphocytes increased significantly ($P<0.05$) when compared to the control. When treatment was withdrawn the percentage lymphocyte/neutrophil ratio improved. The percentage basophil, monocyte and eosinophil were also decreased following extract treatment but improved with the termination of treatment.

The LD50 of the aqueous extract of vitex doniana following intraperitoneal administration was 980 mg/kg using the arithmetic method of Aliyu and Nwude (1987).The calculated LD50 showed that the exlract is not very toxic. Clark and Clark (1977) were of the opinion that any substance whose LD50 in rats that falls between 50-500 mg/kg should be regarded as very toxic, while substances with LD50 above 500 mg/kg but below 1000 mg/kg are classified as being moderately toxic. The signs observed before death following the administration of Vitex doniana extract in this study included loss of appetite, paralysis of hind limbs. This progressed to fore limbs, dyspnea and corna. The toxicity observed may have resulted from the various organic chemicals like saponins, tannins, glycosides and phenolic compounds present in the extract (Abdulrahman, 2004).

The administration of the extract to rats for 21 days at 100 and 200 mg/kg doses produced depression, with the animals being less active. The percentage weight gain also decreased with increasing extract dose. The overall weight gain in the control rats was much higher than those of the extract treated animal. The decreased weight gain in extract treated rats may be due to decreased feed consumption since the animals were depressed and inactive. It also may be due to the indirect effect of saponin present in the extract. Saponins are known to cause bloating, thereby reducing the appetite of the treated animals (Trease and Evans, 1992).

The aqueous root-back extract of vitex doniana administered at various doses for 21 days to albino rats appears to have some beneficial effects on haematological parameters. The results of the haematological study revealed significant increase in RBC, Hb and PCV values of the treated rats (Table 3, 4 and 5) when compared with the control. The increase in haematological parameters also appears to decrease with increased concentration of the extract. This may be an indication that the extract could be more beneficial at low dosages than at higher concentration. The improvement of RBC, Hb and PCV values of treated animals is an indication of the anti-anaemic effect of the extract. Substance with anti-anaemic effect is known to stimulate increase production of RBC and improve the values of Hb and PCV (Brown, 1976). The higher percentage increase in RBC of rats treated with 50 mg/kg of the aqueous extract of vitex doniana compared to those of rats treated with 100 and 200 mg/kg of the extract could be an indication of the concentration of saponin absorbed following treatment.

It is suggested that the saponin component of the extract at 50 mg/kg would be very low to exact any appreciable effect after administration on RBC. However, at 100 and 200 mg/kg doses higher saponin contents occur and could be responsible for the lower percentage RBC values when compared to the group with 50 mg/kg dose. Saponins are known to cause lysis of the RBC and/or inhibition of blood cell synthesis (Effraim, et al., 1999 and Irvine, 1961). Furthermore orally administered saponins are known to indirectly affect the haematological parameters by

reducing the appetite of the animals (Trease and Evans, 1989). Interaction of saponins with micronutrients makes the nutrients unavailable and could also affect haemopoesis (Xing et al., 1995).

The improvement of RBC, Hb and PCV values of treated rats in this study may be an indication that the extract could be useful for treatment of anaemia, hence the justification for its use by natives.

The administration of the extract at various doses stimulated increased production of WBC significant ($P<0.05$) from day 14 of treatment. This could be a result of possible stimulation of immune system (Kashinath, 1990). Furthermore, reports have shown that persistent antigen load in the body results in lymphocytosis (Schalm, et al., 1975). Lymphocytosis may be primarily responsible for the increases in WBC count in the present study.

4. Conclusion

In conclusion, the aqueous extract although used by traditionalists for treatment of ailments in man was observed in rats under the conditions of this study to be toxic. Therefore, caution must be exercised in its usage especially in high doses. In low dosage the extract improved the haematological parameters and could be used to treat anaemia.

Corresponding Author:
Dr. Joseph Clement Akan
Department of Chemistry, University of Maiduguri,
Maiduguri, P.M.B 1069,
Maiduguri, Borno State, Nigeria.
Tel: +2348036000506

References

1. Abdu-aguye I. Medicinal herbal research in West Africa. Annual Regional Conference of West Africa society of Pharmacognosy, Usmanu Danfodio University, Sokoto, Nigeria. 1997; 22-25.
2. Abdulrahman FI. Studies on the chemical and pharmacological activities of the root back extract of vitex doniana Guill and pers. (Black Plum). Ph.D Thesis, Department of chemistry, University of Maiduguri, Maiduguri, Nigeria. 2004; pp 1-4.
3. Akiniyi JA, Sultanbawa MUS. A grossary of kanuri names of plants with botanical names, distribution and uses. Annals of Borno. 1987;1:85-98.
4. Aliu YO, Nwude N. Veterinary pharmacology and toxicology experiments. 1st Ed. Baraka press and Publishers, Zaria. 1987; pp 43-45.
5. Brown BA. Haematological, principles and procedures. 2nd ed. Lea and Ferbinger, Philadelphia. 1976; pp 56-81
6. Clark E. GC, Clark ML. Veterinary toxicology. 2nd ed. Nailliere Tindall, New York. 1977; pp10
7. CIOMS: Council for International Organizations of Medicinal Sessions. International Guiding Principles for Biomedical Research Involving Animals C/O WHO 1211, Geneva 27, Switzerland. 1985.
8. Effraim K.D, Salami HA, Nwafor P. The effect aqueous seed of Trigonelli foenum graecom (Fenugreek) on haematological parameter in albino rats. Afri. J. Biomed. Res. 1999; 47-51.
9. Irvine FI. Woody plants of Ghana with special reference to their uses. Oxford University Prsss, London. 1961; pp868
10. Kelly WR. Veterinary clinical diagnosis. Baelliere Tindall, London. 1977; pp 271-282.
11. Kashinath RT. Hypolipimedic effect of disulphide in rate fed with high lipid and/or ethanol. Ph.D Thesis, University of Bangalore. 1990; pp 221-225.
12. Mittal GC, Aguwa CN, Akube AA. Preliminary Pharmacological studies on the anti-venom action of Diochia scandens leaves. Nig. J. Pharm. 1981;12: 432-436.
13. Pflanzer RG. Experimental and applied physiology 4th ed. W.M.C Brown publishers, London. 1990; pp 187-218.
14. Schalm OW, Jain NC, Carol, EJ. Veterinary haematology. 3rd ed. Lea and Ferbinger Philadelphia. 1975; pp 200-280.
15. Trease GE, Evans WC. Pharmacognosy. Brailliere Tindal, London. 1989; pp 687-689.
16. Trease GE, Evans WC. Pharmacognosy. 11th ed. Brailliere Tindal. London, 1992; pp 176-180.
17. WHO. Quality control methods for medicinal plants materials. Geneva. 1992; pp 26.
18. Xing D, Du K, Ung L, (1995) Effects of ginseng flower saponin on blood and levels of five metals elements in tissue of dogs with hemorrhagic shock. Zhongyno Bingli Shengli Zazhilas. 1995; 11: 83-85.

15/06/2010

Evaluation of Antioxidant Effect of *Nigella sativa* oil on Monosodium Glutamate-Induced Oxidative Stress in Rat Brain

Neveen, A. Noor* and Iman, M. Mourad
Zoology department, Faculty of science, Cairo University, Egypt
*neveen.nour5@gmail.com

Abstract: Oxidative stress is a characteristic feature in a number of neurodegenerative disorders. The present study evaluates the antioxidant effect of *Nigella sativa* oil (NSO) in comparison to that of vitamin C (vit.C) in the cortex and hippocampus of rats pretreated with monosodium glutamate (MSG) as an animal model of oxidative stress. The intraperitoneally injected MSG (4 mg/g body wt.) for 6 consecutive days induced significant decreases in cortical and hippocampal catalase activity and cortical glutathione-S-transferase (GST) activitity and glutathione reduced (GSH) level after 4 weeks. Oral administration of vit.C (200 mg/kg) to stressed rats restored catalase activity, increased GST activity and decreased malondialdehyde (MDA) level after 4 weeks in the cortex. Oral administration of NSO (1 ml/kg) for 4 weeks to MSG-treated rats increased cortical and hippocampal catalase activity and cortical GSH content but significantly inhibited GST activity and increased MDA level in the cortex. Combined administration of vit.C and NSO revealed nonsignificant changes in cortical and hippocampal parameters, as compared to control levels, except for a significant decrease in hippocampal GSH content. In conclusion, although there are some antioxidant effects of NSO, the pro-oxidant effect of NSO cannot be ruled out in the present MSG model of oxidative stress. [Journal of American Science. 2010;6(12):13-19]. (ISSN: 1545-1003).

Key words: Oxidative stress, monosodium glutamate, vitamin C, *Nigella sativa* oil, cortex, hippocampus.

1. Introduction

Oxidative stress is a characteristic feature in a number of neurodegenerative disorders such as stroke, Parkinson's disease and Alzheimer's disease.[1,2] The brain is particularly vulnerable to oxidative stress injury because of its high rate of oxidative metabolic activity, high content of polyunsaturated fatty acids, relatively low antioxidant capacity, the abundance of redox-active transition metal ions,[3] and non-replicating nature of its neuronal cells.[4]

Certain brain regions are highly enriched in non-heme iron, which is catalytically involved in the production of oxygen free radicals,[5] thus increasing the risk of neurodegenerative disease. It has been shown that the regions like cortex and hippocampus are more susceptible to oxidative damage when compared to cerebellum. [6,7]

Reactive oxygen species (ROS) are generated continuously in nervous system during normal metabolism and neural activity. When the balance between ROS (for example: superoxide, hydrogen peroxide, hydroxyl radicals and singlet oxygen) and antioxidant system is lost, oxidative stress results.[8,9]

In order to scavenge ROS, different defense systems exist in the brain, such as enzymatic (superoxide dismutase, glutathione peroxidase and catalase), non enzymatic (glutathione and uric acid) and dietary (vitamins A, E and C, β – carotene, quinones and flavons) antioxidants. If ROS are not effectively eliminated, they can cause peroxidation of cell membrane phospholipids, proteins (receptors and enzymes) and DNA.[9]

Monosodium glutamate (MSG), the sodium salt of glutamate, is commonly used as a flavor enhancer,[10-12] to increase palatability and food selection in a meal.[13] Many people throughout the world ingest large doses of MSG and in many countries there are no limitation on the amount of MSG that can be added to food.[14]

Glutamate is the main excitatory neurotransmitter in rat brain.[15] It has been shown that MSG administration during neonatal period increase lipid peroxidation in the midbrain of adult rats.[16] Bawari *et al*[16] suggested that oxidative stress caused by excitotoxin- generated free radicals sustained and progressed during development until adulthood. In addition, Park *et al*.[17] reported that MSG could impair memory retention and induce damage in the hypothalamic neurons in mice. Moreover, alteration due to MSG in mitochondria lipid peroxidation and antioxidant status in different brain regions is well documented.[18]

Vitamin C (ascorbic acid) is an essential micronutrient required for normal metabolic functioning of the body. It is a well known antioxidant in animal tissues.[19] It decreases the adverse effects of ROS implicated in chronic diseases including neurodegenerative diseases.[20] Neurons maintain relatively high intracellular concentrations of ascorbic acid.[21] The reduced form of the vitamin C (ascorbate) is an especially effective antioxidant owing to its high

electron – donating power and ready conversion back to the active reduced form,[7] in addition to the stability and low reactivity of the ascorbyl radical formed when ascorbate scavenges a reactive oxygen or nitrogen species.[22]

Nigella sativa, commonly known as black seeds, belongs to the botanical family of *Ranunculaceae*. In recent years, the plant has been investigated to justify its broad traditional therapeutic value.[9] Many favorable biological properties of *Nigella sativa* have been reported such as anti-inflammatory,[23] antioxidative,[24,25] antitumour,[26] antiulcerogenic,[27] and hepatoprotective,[28] properties. The biological activity of *Nigella sativa* seeds is attributed to its essential oil components.[23]

The aim of the present study is to evaluate the antioxidant properties of *Nigella sativa* oil (NSO) in comparison to that of the established antioxidant, vit. C and whether the combination of both treatments could increase the effect of each other or not. The activities of catalase and glutathione-S-transferase (GST), as well as, the levels of glutathione reduced (GSH) and malondialdehyde (MDA) were measured in the cortex and hippocampus of rats pre-treated with monosodium glutamate (MSG) as an animal model of oxidative stress and after treatment with vit. C and/or NSO.

2. Materials and Methods:

Animals

Male albino rats (*Rattus norvegicus*) weighing 120-180 g were used as experimental animals. The animals were obtained from animal house of the National Research Center. They were maintained on stock diet and kept under fixed appropriate conditions of housing and handling. All experiments were carried out in accordance with research protocols established by the animal care committee of the National Research Center, Egypt.

Chemicals

L- glutamic acid sodium salt monohydrate (MSG), was purchased from WINLAB, U.K. and vitamin C **(**L-ascorbic acid) was from SAS chemicals co. India. Phosphate buffer and reagent kits were purchased from Bio-diagnostic Company, Egypt. *Nigella sativa* oil was obtained by pressing of seeds on cold to keep the active constituents unaltered.

Experimental design

The animals were divided into 5 groups. Animals of group (1) served as control and were daily administered distilled water throughout the experimental protocol. Animals of groups 2, 3, 4 and 5 were injected intraperitoneally (i.p.) with 4 mg/g body wt. MSG for six consecutive days.[29] At the 7th day, animals of the 2nd, 3rd, 4th and 5th groups were daily administered orally with distilled water, vit.C (200 mg/kg)[30], NSO (1 ml/kg)[31] and both vit C (200 mg/kg) and NSO (1 ml/kg) respectively, for 4 weeks.

Handling of tissue samples

Both treated and control animals were sacrificed after being fasted overnight. The brain of each animal was quickly removed and rapidly transferred to an ice-cold Petri dish and dissected to obtain the cortex and hippocampus.[32,33] Each brain area was weighed and frozen until analyzed.

The hippocampus and cortex were homogenized in 4 and 6 ml of ice cold phosphate buffer (50 mM pH 7.4, 0.1% tritonX and 0.5 mM EDTA), respectively. The homogenate was centrifuged at 1753 g for 15 minutes at 4ºC using a high speed cooling centrifuge (Type 3K-30, Sigma, Germany). The clear supernatant was separated and used for analysis. Catalase activity,[34,35] GST activity,[36] GSH level,[37] and Thiobarbituric acid reactive substances (expressed as malondialdehyde, MDA content),[38] were determined by using reagent kits.

Statistics

The data were expressed as mean ± SEM. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test for multiple comparisons. Analysis was made using the SPSS statistical software. The *p*-values less than 0.05 were considered statistically significant. Percentage difference representing the percent of variation with respect to the control was calculated.

% difference = (treated mean–control mean/control mean)×100.

3. Results

The data represented in Table (1) showed a significant decrease in cortical and hippocampal catalase activity in MSG-injected group as compared to control group. These decreases in the enzyme activity persisted after treatment of MSG-injected rats with vit.C, being nonsignificant and significant in the cortex and hippocampus, respectively as compared to control animals. However, the oral administration of NSO to MSG-treated rats induced a significant increase in the enzyme activity in both cortex and hippocampus in comparison to the control, MSG and vit.C-treated groups. The combined administration of vitamin C and NSO to MSG-treated rats returned the enzyme activity to nearly control values in the cortex and hippocampus.

Regarding GST activity (Table 2), the present data showed that the cortical GST activity decreased significantly after MSG injection. The oral administration of vit. C alone and vit. C+ NSO to MSG-treated animals induced significant and

nonsignificant increases in GST activity, respectively in comparison to control group. Meanwhile, the oral administration of NSO to MSG-treated rats did not alter the significant decrease in the enzyme activity due to MSG injection alone. On the other hand, GST activity in the hippocampus did not show any significant changes during the experimental period.

As shown in Table (3), the cortical GSH level decreased significantly after MSG treatment. However, this decrease in GSH level persisted significantly after vitamin C administration in comparison to the control. The oral administration of NSO alone and vit. C+NSO induced significant and non significant increases in GSH levels, respectively relative to the control group. In contrast to the cortex, the data did not record any significant changes in the hippocampal GSH content after MSG injection. The combined administration of both vit. C and NSO showed a significant decrease in the GSH content as compared to the control value.

Data in Table (4) revealed a nonsignificant change in the cortical lipid peroxidation after MSG injection as determined by MDA formation. However, vit. C administration to MSG-injected rats induced a significant decrease in MDA level. Meanwhile, NSO administration provoked a significant increase in MDA level as compared to control, MSG and vit.C-treated groups. The combined administration of vit.C and NSO to MSG-treated rats did not induce any significant change in MDA level in relation to control and MSG-treated groups. MDA content in the hippocampus presented a significant decrease due to MSG injection. Administration of vit.C did not change the decrease in MDA level obtained as a result of MSG injection alone. Nonsignificant changes were observed in MDA level as a result of NSO and vit.C+NSO treatments to MSG-treated rats as compared to control group.

Table (1): Effect of daily oral administration of vit. C and/or NSO on catalase activity (U/g tissue) in the cortex and hippocampus of rats treated with MSG.

Area	Control	MSG		Vit.C		NSO		(Vit. C+ NSO)		F-test
		Value	% D	value	% D	value	% D	value	% D	
Cortex	A 2.74±0.07 (7)	B 1.93±0.06 (7)	-29.56	AB 2.42±0.10 (8)	-11.68	C 3.53±0.28 (6)	+28.83	A 2.87±0.13 (8)	+4.74	*
Hippocampus	A 9.70±0.58 (7)	B 7.16±0.63 (5)	-26.19	B 7.67±0.29 (7)	-20.93	C 11.58±0.15 (6)	+19.38	AC 10.65±0.18 (6)	+9.79	*

Values represent mean±S.E.M. with the number of animals between parentheses.
*: $p < 0.05$ significant.
A, B and C: different letters mean significant changes.
%D: percentage difference between treated and control.

Table (2): Effect of daily oral administration of vit. C and/or NSO on GST activity (U/g tissue) in the cortex and hippocampus of rats treated with MSG.

Area	Control	MSG		Vit.C		NSO		(Vit. C+ NSO)		F-test
		value	%D	value	% D	value	% D	value	% D	
Cortex	A 0.30±0.03 (6)	B 0.09±0.01 (7)	-70.00	C 0.50±0.04 (6)	+66.67	B 0.10±0.02 (6)	-66.67	AC 0.46±0.06 (6)	+53.33	*
Hippocampus	0.10±0.01 (6)	0.06±0.01 (6)	-40.00	0.08±0.02 (7)	-20.00	0.10±0.02 (6)	0.00	0.06±0.01 (6)	-40.00	n.s.

Values represent mean±S.E.M. with the number of animals between parentheses.
n.s.: $p > 0.05$ nonsignificant. *: $p < 0.05$ significant.
A, B and C: different letters mean significant changes.
%D: percentage difference between treated and control.

Table (3): Effect of daily oral administration of vit. C and/or NSO on GSH content (mg/g tissue) in the cortex and hippocampus of rats treated with MSG.

Area	Control	MSG		Vit.C		NSO		(Vit. C+ NSO)		F-test
		Value	% D	Value	% D	Value	% D	Value	% D	
Cortex	A 26.36±0.95 (5)	B 13.64±0.97 (6)	-48.25	B 16.59±1.45 (7)	-37.06	C 36.78±1.85 (6)	+39.53	AC 32.39±2.18 (6)	+22.88	*
Hippocampus	A 53.64±1.16 (6)	A 52.75±1.31 (6)	-1.66	AB 47.68±1.34 (6)	-11.11	A 51.98±1.59 (6)	-3.09	B 44.03±2.31 (6)	-17.92	*

Values represent mean±S.E.M. with the number of animals between parentheses.

*: $p < 0.05$ significant.
A, B and C: different letters mean significant changes.
%D: percentage difference between treated and control.

Table (4): Effect of daily oral administration of vit. C and/or NSO on MDA content (nmol/g tissue) in the cortex and hippocampus of rats treated with MSG.

Area	Control	MSG		Vit.C		NSO		(Vit. C+ NSO)		F-test
		value	%D	Value	%D	Value	%D	Value	%D	
Cortex	A 197.75±2.84 (6)	A 194.91±4.93 (5)	-1.44	B 163.43±1.94 (6)	-17.36	C 230.23±7.86 (6)	+16.42	AB 180.89±4.14 (6)	-8.53	*
Hippocampus	AC 175.52±2.51 (6)	B 155.03±4.07 (6)	-11.67	B 156.73±4.02 (6)	-10.71	AB 170.58±4.20 (5)	-2.81	C 191.93±4.17 (6)	+9.35	*

Values represent mean±S.E.M. with the number of animals between parentheses.
*: $p < 0.05$ significant.
A, B and C: different letters mean significant changes.
%D: percentage difference between treated and control.

4. Discussion:

The present data showed that the i.p. injection of MSG for 6 consecutive days to adult rats induced significant decreases in cortical and hippocampal catalase activity as well as cortical GST activity and GSH content after 4 weeks, as compared to control rats. Singh *et al*.[29] reported that repeated monosodium glutamate doses showed prolonged and delayed effects on the mitochondrial free radical scavenger system and the consequential membrane damage as inferred from altered levels of Mn-superoxide dismutase (Mn-SOD), catalase, glutathione peroxidase (GPx), glutathione reduced (GSH), lipid peroxidation and uric acid content in mitochondria in different brain regions of male rats.

Thus, it could be suggested that the i.p. injection of MSG administration may provide a successful model of oxidative stress and hence may be used to investigate the antioxidant properties of several natural products.

Decreased catalase activity has been shown to be associated with oxidative stress in brain regions.[39,40] It functions to catalyze the decomposition of H_2O_2 to water and oxygen.[41]

Singh *et al*.[29] investigated the effect of i.p. MSG administration for 6 consecutive days on mitochondrial lipid peroxidation and antioxidant status in adult rat cerebral hemisphere after 30 and 45 days. In the present study, the effect of MSG is investigated in the cerebral cortex and hippocampus of adult male rats and the decrease in catalase activity and glutathione reduced content in the cerebral cortex is in accordance with the results of Singh *et al*.[29] in the cerebral hemisphere. Thus, the cerebral cortex could be the target area for MSG effects in the cerebral hemisphere. Furthermore, the present results agree with the more recent study of Farombi and Onyema,[12] who indicated a decrease in brain GSH content and catalase activity after i.p. injection of adult rats with MSG for 10 days.

Kono and Fridovich,[42] suggested that increased production of free radicals may lead to depletion or inactivation of catalase enzyme. Further reports have shown that mitochondria generate superoxide and related ROS during glutamate receptor over activation.[43,44] During oxidative stress, the superoxides destroy the iron-sulfur centers and thereby irreversibly deactivate the iron containing enzymes.[45,46]

Thus, the decrease in cortical and hippocampal catalase activity in MSG-injected group may be due to depletion or irreversible deactivation of the catalase enzyme by superoxide generation.

GSH (a tripeptide comprised of glutamate, cysteine and glycine) plays a critical role in protecting cells from oxidative stress and xenobiotics. GSH can function as an antixodant in many ways. It can react non-enzymatically with superoxide,[47] nitric oxide,[48] hydroxyl radical,[49] and peroxynitrite.[50] Thus, it functions directly as a free radical scavenger. Therefore, the decrease in cortical GSH content observed in the present study may be due to its consumption in scavenging the generated ROS.

Glutathione-S-transferases (GSTs) are a family of enzymes that catalyze the addition of the tripeptide glutathione to endogenous and xenobiotic substrates which have electrophilic functional groups.[51] Subsequently, the observed decline in the cortical GST activity, in the present study, may be ascribed to the reduction in the level of its substrate, GSH.

Lipid peroxidation is an autocatalytic mechanism leading to oxidative destruction of cellular membranes.[52] MDA is the major

aldehyde metabolite of lipid peroxidation.[53] The present nonsignificant change in cortical MDA level in MSG-treated group may confirm the delayed effect of MSG exposure and this result is in line with the results of Singh *et al*.[29] as they found significant increase in the level of mitochondrial lipid peroxidation in the cerebral hemisphere at the 45th day of MSG administration and not at the 30th day.

In the present study, it was found that the oral administration of vit. C to MSG-injected rats restored cortical catalase activity to nearly control level, whereas it reversed the significant decrease in cortical GST activity due to MSG injection to a significant increase (+66.67%). Meanwhile, GSH content in the cortex was not affected by vit. C treatment to stressed rats. However, cortical MDA level was decreased significantly after vit. C administration as compared to control group.

Vitamin C is a well known antioxidant and has been shown to protect various tissues against the damage caused by ROS.[19] In addition, many studies have indicated that vitamin C may be of benefit in chronic diseases such as cardiovascular disease, cancer and cataract, probably through antioxidant mechanisms.[54] Moreover, Huang *et al*.[55] reported that vitamin C (500 mg ascorbate/day) supplementation in non-smokers reduced lipid peroxidation.

Therefore, the present increase in GST activity and restoration of catalase activity to nearly control level in addition to the decrease in MDA content in the cortex could indicate that the antioxidant properties of vit. C may be mediated on one hand by an increase in antioxidant enzymatic activity and by a decrease in lipid peroxidation on the other hand.

Within the brain, ascorbate levels are not homogenous; in humans, the highest levels are found in the hippocampus, amygdala, and hypothalamus.[56] Therefore, the mild oxidative state observed, in the present study, in the hippocampus due to MSG administration may be due to the inherent high content of vit. C in this brain area.

Treatment of oxidatively stressed rats, in the present study, with NSO increased catalase activity in the cortex and hippocampus as well as the GSH content in the cortex. Meanwhile, a significant inhibition of cortical GST activity accompanied by an increase in MDA was obtained.

Thymoquinone (TQ) is the main constituent of NSO. It was intensively studied and ascribed to possess antioxidant properties.[57] On the other hand, Khader *et al*.[58] reported that TQ like other quinone compounds, can be considered to be a redox-cycler which is metabolized *in vitro* to hydroquinones or semiquinone radicals by cellular oxidoreductases leading to the production of ROS. This may explain the present increase in cortical MDA content after treatment of MSG-injected rats with NSO.

It has been reported that the exposure to ROS and nitrogen species may raise the GSH content by increasing the GSH synthesis rate.[59,60] Thus, the increase in cortical GSH content may be an attempt to counteract the increase in MDA level as a defense mechanism by brain cells against free radicals generation.

Cortical and hippocampal catalase activity showed significant increases due to NSO treatment of stressed rats. In agreement with the present result, Kanter *et al*.[61] found that NSO prevented inhibition of catalase activity following experimental spinal cord injury in rats. Moreover, Al-Majed *et al*.[62] found that thymoquinone is beneficial in restoring declined hippocampal SOD and catalase due to ischemia insult.

Although, there are some antioxidant effects of NSO which are represented in the increase of GSH content and catalase activity, the pro-oxidant effect of NSO cannot be ruled out in the present MSG model of oxidative stress.

Vitamin C is considered the most important water-soluble antioxidant in extracellular fluids,[63] and must have neutralized the ROS generated in the aqueous phase before lipid peroxidation was initiated.[12]

Therefore, it could be concluded that the combined administration of vitamin C and NSO to stressed rats, in the present study, may represent a synergestic antioxidant effect between the water soluble vit. C and lipid soluble components of NSO. Moreover, vit. C may counteract the pro-oxidant effects of NSO. However, further studies are recommended for the evaluation of the antioxidant properties of NSO in different models of oxidative stress.

Corresponding author
Neveen, A. Noor
Zoology department, Faculty of science, Cairo University, Egypt
neveen.nour5@gmail.com

5. References:

1. Andersen JK (2004) Nat. Med. 10 Suppl, S18-S25
2. Ji B-S, Ji H & Liu G-Q (2004) *Acta Pharmacol Sin* 25, 297-300
3. Butterfield DA & Stadtman ER (1997) *Adv Cell Aging Gerontol* 2, 161-191
4. Evans PH (1993) *Br Med Bull* 49, 577-587
5. Hill JM & Switzer RC (1984) *Neuroscience* 11, 595-603

6. Mandavilli BS & Rao KS (1996) *Biochem Mol Biol Int* 40, 507-514.
7. Venkataraman P, Muthuvel R, Krishnamoorthy G, Arunkumar A, Sridhar M, Srinivasan N, Balasubramanian K, Aruldhas M M & Arunakaran, J (2007) *Neuro Toxicol* 28, 490-498
8. Allen RG (1991) *Proc Soc Exp Biol Med* 196, 117-129
9. Ilhan A, Gurel A, Armutcu F, Kamisli S & Iraz M (2005) *Neuropharmacology* 49, 456-464
10. Kurihara K & Kashiwayanagi M (1998) *Ann NY Acad Sci* 855, 393-397
11. Chaudari N & Roper SD (1998) *Ann NY Acad Sci* 855, 398-405
12. Farombi EO & Onyema OO (2006) *Hum Exp Toxicol* 25, 251-259
13. Bellisle F, Dalix AM, Chappuis AS, Rossi F & Fiquet P (1996) *Appetite* 26, 267-276
14. Diniz YS, Faine LA, Galhardi C.M, Rodrigues HG, Ebaid GX, Burneiko RC, Cicogna, AC & Novelli, ELB (2005) *Nutrition* 21, 749-755
15. Rotta LN, Schmidt AP, Mello e Souza T, Nogueira, CW, Souza KB, Izquierdo IA, Perry ML & Souza DO (2003) *Neurochem Res* 28, 1181-1186
16. Bawari M, Babu GN, Ali MM & Misra UK (1995) *Neuroreport* 6, 650-652
17. Park CH, Choi SH, Piao Y, Kim S, Lee YJ, Kim HS, Jeong, SJ, Rah JC, Seo JH, Lee JH, Chang K, Jung YJ & Suh YH (2000) *Toxicol Lett* 115, 117-125
18. Singh K & Ahluwalia P (2003) *J Nutr Sci Vitaminol[Tokyo]* 49, 145-148
19. Kajo S (2004) *Curr Med Chem* 11, 1041-1064
20. Muthuvel R, Venkataraman P, Krishnamoorthy G, Gunadharini DN, Kanagaraj P, Jone Stanley A, Srinivasan N, Balasubramanian K, Aruldhas MM & Arunakaran J (2006) *Clin Chim Acta* 365, 297-303
21. Qiu S, Li L, Weeber EJ & May JM (2007) *J Neurosci Res* 85, 1046-1056
22. Tsao CS (1997) In *Vitamin C in health and disease* (Packer L & Fuchs J, eds), pp. 25-58, Marcel Dekker Inc. New York
23. Hajhashemi V, Ghannadi A & Jafarabadi H (2004) *Phytother. Res* 18, 195-199
24. Burits M & Bucar F (2000) *Phytother Res* 14, 323-328
25. Meral I, Yener Z, Kahraman T & Mert N (2001) *J Vet Med A Physiol Pathol Clin Med* 48, 593-599
26. Salomi MJ, Nair SC & Panikkar KR (1991) *Nutr Cancer* 16, 67-72
27. Kanter M, Demir H, Karakaya C & Ozbek H (2005) *World J Gastroenterol* 11, 6662-6666
28. Kanter M, Coskun O & Budancamanak M (2005) *World J Gastroenterol* 11, 6684-6688
29. Singh P, Mann KA, Mangat HK & Kaur G (2003) *Mol Cell Biochem* 243, 139-145
30. Ramanathan K, Balakumar BS & Panneerselvam C (2002) *Hum Exp Toxicol* 21, 675-680
31. Ali BH (2004) *Am J Chin Med* 23, 49-55
32. Zeman W & Innes JRM (1963) Graigie's Neuroanatomy of The Rat. Academic Press, New York, London
33. Glowinski J & Iversen LL (1966) *J Neurochem* 13, 655-669
34. Fossati P, Prencipe L & Berti G (1980) *Clin Chem* 26, 227-231
35. Aebi, H (1984) *Methods Enzymol* 105, 121-126
36. Habig WH, Pabst MJ & Jakoby WB (1974) *J Biol Chem* 249, 7130-7139
37. Beutler E, Duron O & Kelly BM (1963) *J Lab Clin Med* 61, 882-888
38. Ohkawa H, Ohishi N & Yagi K (1979) *Anal Biochem* 95, 351-358
39. Arivazhagan P, Shila S, Kumaran S & Panneerselvam C (2002) *Exp Gerontol* 37, 803-811
40. Shila S, Kokilavani V, Subathra M & Panneerselvam C (2005) *Toxicology* 210, 25-36
41. Chelikani P, Fita I & Loewen PC (2004) *Cell Mol Life Sci* 61, 192-208
42. Kono Y & Fridovich I (1982) *J Biol Chem* 257, 5751-5754
43. Dugan LL, Sensi SL, Canzoneiro LMT, Handran SD, Rothman SM, Lin TS, Goldberg MP & Choi, DW (1995) *J Neurosci* 15, 6377-6388
44. Patel M, Day BJ, Crapo JD, Fridovich I & McNamara JO (1996) *Neuron* 16, 345-355
45. Gardner PR & Fridovich I (1991) *J Biol Chem* 266, 1478-1483
46. Qing-You L, Pederson C, Day BJ & Patel M (2001) *J Neurochem* 78, 746-755
47. Winterbourn CC & Metodiewa D (1994) *Arch Biochem Biophys* 314, 284-290
48. Clancy RM, Levartovsky D, Leszczynska-Piziak J, Yegudin J & Abramson SB (1994) *Proc Nat Acad Sci USA* 91, 3680-3684
49. Bains JS & Shaw CA (1997) *Brain Res Rev* 25, 335-358.
50. Koppal T, Drake J, Yatin S, Jordan B, Varadarajan S, Bettenhausen L & Butterfield DA (1999) *J Neurochem* 72, 310-317
51. Yousef MI (2004) *Toxicology* 199, 47-57
52. Cheeseman, KH (1993) *Mol Aspects Med* 14, 191-197.

53. Paradis V, Mathurin P, Kollinger M, Imbert-Bismut F, Charlotte F, Piton A, Opolon P, Holstege A, Poynard T & Bedossa P (1997) Journal of Clinical Pathology 50, 401-406
54. Carr AC & Frei B (1999) *Am J Clin Nutr* 69, 1086-1107.
55. Huang HY, Appel LJ, Croft KD, Miller ER, Mori TA & Puddey IB (2002) *Am J Clin Nutr* 76, 549-555/
56. Hornig D (1975) *Ann NY Acad Sci* 258, 1293-1398.
57. Houghton PJ, Zarka R, de las Heras B & Hoult JR (1995) *Planta Med* 61, 33-36.
58. Khader M, Bresgen N & Eckl PM (2009) *Food Chem Toxicol* 47, 129-133.
59. Rahman I, Bel A, Mulier B, Lawson MF, Harrison DJ, Macnee W & Smith CA (1996) *Biochem Biophys Res Commun* 229, 832-837.
60. Moellering D, Mc Andrew J, Patel RP, Forman HJ, Mulcahy RT, Jo H & Darley-Usmar VM (1999) *FEBS Lett* 448, 292-296.
61. Kanter M, Coskun O, Kalayci M, Buyukbas S & Cagavi F (2006) *Hum Exp Toxicol* 25, 127-133
62. Al-Majed AA, Al-Omar FA & Nagi MN (2006) *Eur J Pharmacol* 543, 40-47.
63. Singh RP, Sharad S & Kapur S (2004) *JIACM* 5, 218-225.

6/9/2010

Modulating Effect of Carvedilol on Doxorubicin-Induced Cardiomyopathy and Hepatic Damage

Safinaz S. Ibrahim*, Maged A. Barakat and HebaTullah S. Helmy

Biochemistry Department, Faculty of Pharmacy, Cairo University, Cairo, Egypt
* dr_safinaz_747@hotmail.com

Abstract: Background: Doxorubicin is an anthracyclin antibiotic that is considered as one of the most effective antitumor agents. The clinical use of doxorubicin soon proved to be hampered by such serious problems as hepatotoxicity and most notably cardiomyopathy. Objectives: The current study aims at evaluating the efficiency of carvedilol as an adjuvant therapy with doxorubicin to protect against doxorubicin - induced cardiomyopathy and hepatic damage. Materials and Methods: Animals were divided into normal group and doxorubicin -treated group injecting doxorubicin as a dose of 2.5 mg/kg/twice weekly/ 3 weeks. Doxorubicin - treated animals were divided into two groups, one kept without further treatment (doxorubicin group) and second group, (doxorubicin + carvedilol), received carvedilol 1mg/kg/ 7 times over a period of 4 weeks including a dose before doxorubicin 1st dose. Creatine phosphokinase, lactate dehydrogenase, as cardiac damage markers, and alanine aminotransferase, as indicator of hepatic damage, were measured. Malondialdehyde and nitric oxide levels, as cardiac oxidative status indices, glutathione content, glutathione peroxidase, glutathione-S–transferase and superoxide dismutase activities, as measures for cardiac antioxidant capacity, were also investigated. Histopathological changes in cardiac and hepatic tissues of all groups were examined. Results and Conclusions: Our results revealed that doxorubicin caused oxidative stress which plays a major role in doxorubicin -induced cardiomyopathy and hepatic damage. Co-administration of carvedilol in concomitant with doxorubicin caused protection against doxorubicin–induced cardiomyopathy; however, it augmented doxorubicin -induced hepatic damage. Histopathological examination of cardiac and hepatic tissues supported the previous biochemical results. [Journal of American Science. 2010;6(12):20-32]. (ISSN: 1545-1003).

Keywords: Doxorubicin, carvedilol, cardiomyopathy, hepatic damage.

1. Introduction

Almost all clinically used antitumor drugs exhibit toxic side effects affecting heart function. Because of cardiotoxicity during anticancer chemotherapy, effective doses of cytostatics have to be limited, which may worsen antitumor efficacy. Doxorubicin (Dox) is an anthracyclin antibiotic that is considered as one of the most effective antitumor agents. Dox is an essential component of treatment of breast cancer (1), soft tissue sarcomas (2) and many other cancers (3). The immense value of Dox in treating a variety of malignant conditions is unquestioned. However, the clinical use of Dox soon proved to be hampered by such serious problems as the development of resistance in tumor cells (4) and toxicity in healthy tissues, in the form of central nervous system toxicity (5), nephrotoxicity (6) and most notably in the form of cardiomyopathy and congestive heart failure (7). These adverse effects of the drug can preclude its use in some patients and limit the duration of its use in many others (8).

Carvedilol is non cardioselective β-blocker which lacks intrinsic sympathomimetic activity. In addition, it has blocking effects at vascular α1-receptors, antioxidant, and calcium antagonist properties (9). The antioxidant activity of carvedilol was examined in a variety of *in vitro* and *in vivo* assay systems, including physicochemical, biochemical and cellular models. The data indicate that carvedilol prevents electron adduct formation in both aqueous or lipid environments containing either superoxide- or hydroxyl-radical generating systems (10). Furthermore, carvedilol and several of its metabolites are as effective in inhibiting lipid peroxidation in brain and heart membranes (11). The cardioprotective effect of carvedilol has been shown in a variety of *in vitro* and *in vivo* models. The efficacy of carvedilol has been observed with anthracyclin cardiomyopathy and ischemia/ reperfusion (12). One most likely mechanism of cardioprotection by carvedilol is the antioxidant effect (13-15).

The current study aims at evaluating the efficiency of carvedilol as a protective agent against cardiomyopathy and hepatic damage induced by Dox.

2. Materials and Methods:

A- Animals:

We used a total of 32 male albino rats of the Wister strain, weighing 170-200 g, that were obtained from the central animal facility at the Faculty of Pharmacy, Cairo University, Cairo, Egypt. All rats were housed in a room with a controlled environment,

at a constant temperature of 23 ± 1^0 C, humidity of 60% ± 10%, and a 12 hr light/dark cycle. The animals were housed in groups and kept at constant nutritional conditions throughout the experimental period. The experimental protocols were approved by the Ethical Committee of Faculty of Pharmacy, Cairo University.

B- Drugs and chemicals:

Doxorubicin HCL was obtained from Pharmacia & Upjohn, Milan, Italy. Carvedilol was obtained from Cadila Pharmaceuticals Limited, India. Other chemicals in the experiments were of analytical pure grade and supplied by British Drug House (BDH, UK) and Sigma Chemical Company (USA).

C- Experimental design:

Animals were divided into a normal control group (10 rats), receiving the appropriate volume of saline i.p, and Dox-treated group (22 rats). Dox was dissolved in saline and injected i.p. as total cumulative dose of 15 mg/kg, divided into 6 equal doses, each of 2.5 mg/kg. They were injected twice weekly/ 3 weeks (16). The Dox-treated animals were divided into two groups, one was kept without further treatment termed Dox-group(12 rats), and a second group (10 rats), termed Dox + carvedilol group, received carvedilol as an i.p dose of 1mg/kg / 7 times over a period of 4 weeks including a dose before the 1st Dox dose (17).

D- Serum and Tissue sampling:

Twenty four hours following the last Dox injection, rates were sacrificed by decapitation. A blood sample of each animal was collected into a dry centrifuge tube. Serum was separated by centrifugation at 3000 r.p.m/15 minutes and used to determine creatine phosphokinase (CPK), lactate dehydrogenase (LDH) and alanine aminotransferase (ALT). Serum CPK activity was determined using a kit provided by STANBIO, USA (18). Serum LDH activity was determined using a kit provided also by STANBIO, USA (19). Serum ALT activity was determined, using kit provided by Quimica Clinica Aplicada, Spain (20).

For determination of the biochemical parameters and histopathological changes, hearts and livers were removed by dissection, washed by ice-cold saline and blotted between filter papers.

Histopathological study:

Samples were taken from hearts and livers of rats in different groups and fixed in 10% formol saline for 24 hours. Washing was done in tap water, then serial dilutions of alcohol (methyl, ethyl and absolute ethyl) were used for dehydration. Specimens were cleared in xylene, embedded in paraffin at 56^O C in hot air oven for 24 hours. Paraffin bees wax tissue blocks were prepared for sectioning at 4 microns thickness by sledge microtome. The obtained tissue sections were collected on glass slides, deparaffinized and stained by hematoxylin and eosin stains (21) for histopathological examinations using light microscope.

Biochemical parameters:

The remainder of heart tissue of each rat was weighed and homogenized in ice-cold saline for 1 minute, using an ice-cold Potter Elvejhem glass homogenizer, forming 10% w/v homogenate.

Estimation of cardiac glutathione (GSH) content

A portion of homogenate was mixed with ice-cold 7.5% sulfosalicylic acid in a ratio 1:1 and centrifuged at 3000 r.p.m/15 minutes. The resulted supernatant was used for the determination of GSH (22), depending on the reaction between GSH and 5, 5'- dithio-bis, 2-nitrobenzoic acid to yield a stable yellow colour which can be measured colourimetrically.

Estimation of cardiac malondialdehyde (MDA) level:

Another portion of homogenate was mixed with ice-cold 2.3% KCL (1:1), centrifuged at 3000 r.p.m/15 minutes. The level of MDA was determined in the supernatant depending on measuring the coloured complex formed between thiobarbituric acid reagent and MDA in acidic medium (23).

Preparation of cytosolic fraction for the estimation of glutathione peroxidase (GPx), glutathione-S–transferase (GST) and superoxide dismutase (SOD) activities:

Part of the homogenate was mixed with equal volume of ice-cold Tris- EDTA buffer (pH=7.6), centrifuged at 39.000 r.p.m/4ºC/ 20 minutes. The resulted supernatant was used for the determination of GST; GPx and SOD activities. Determination of GST activity (24, 25) depends on the ability of GST to catalyze the formation of GSH- adduct with 1-chloro-2,4- dinitrobenzene(CDNB). This adduct was measured by noting the net increase in absorbance at 340 nm. Determination of GPx activity (26) is based on following the rate of oxidation of GSH by H_2O_2 in the presence of GPx. Oxidized GSH was determined by following up the decrease in absorbance of the reaction medium at 340 nm, as NADPH was converted to NADP. SOD activity was determined (27) depending on the fact that the spontaneous autoxidation of pyrogallol, at alkaline pH less than 9.5, produces superoxide anion, which in turn enhances further oxidation of pyrogallol with a resultant increase in the absorbance at 420 nm. The

presence of SOD in the reaction medium inhibits pyrogallol autoxidation by scavenging the formed superoxide anion. Protein content of the supernatant was determined (28) using bovine serum albumin as standard.

Estimation of nitric oxide (NO) (NO2- / NO3-):

An aliquot of the homogenate was centrifuged at 17.000 r.p.m/ 4ºC/ 20 minutes. The resulted supernatant was used for the determination of NO. Determination of NO radical itself is difficult because of its radical nature and very short half-life. Therefore, determination of the stable oxidation products of NO radical, nitrite ($N0_2^-$) and nitrate ($N0_3^-$) concentrations is used as a measure for the production of NO radical. NO content was determined (29, 30) depending on the colourimetric detection of nitrite with Griess reagent after the enzymatic reduction of nitrate to nitrite using nitrate reductase enzyme. The Griess reaction involves the reaction of nitrite with sulfanilamide in an acidic solution to yield a diazonium salt, followed by coupling with N-(1-naphthyl) ethylenediamine to yield a colored azo dye that can be measured colourimetrically at 540 nm.

Statistical analysis

Results were analyzed statistically by one-way analysis of variance (ANOVA test) with subsequent multiple comparisons using Tukey test. Differences were considered statistically significant at p less than 0.05. Results are presented as the mean ± standard error of the mean (SEM), with the number of observations (n) given in parentheses. All data obtained were submitted to a computerized statistical treatment using SPSS statistical package, version 17. Tables were represented by Microsoft Excel computer program.

3. Results:

Effect of doxorubicin (Dox), separately or in combination with carvedilol, on serum lactate dehydrogenase (LDH) and creatine phosphokinase (CPK) activities in rats:

Results of table (1) revealed that Dox caused significant increase in serum levels of LDH and CPK amounting to 182.4% and 183.6%, respectively, as compared to the normal values. Carvedilol co-administration caused normalization of LDH as well as significant decrease in CPK serum levels reaching 119.4% of the control value.

Effect of doxorubicin (Dox), separately or in combination with carvedilol, on cardiac malondialdehyde (MDA) and nitric oxide (NO) levels in rats:

Table (2) illustrated that, Dox caused significant increase in MDA and NO levels amounting to 183.36% and 177.7%, respectively, of the control values. Carvedilol co-administration caused normalization of both MDA and NO levels.

Effect of doxorubicin (Dox), separately or in combination with carvedilol, on cardiac glutathione (GSH) content, glutathione peroxidase (GPx), glutathione-S-tranferase (GST) and superoxide dismutase (SOD) activities in rats:

As shown in table (3), Dox administration caused a significant decrease in cardiac GSH level reaching to 64% of the normal value. Co-administration of carvedilol significantly raised GSH content to about 92% compared to the control value. Results of the same table showed significant increase in cardiac activities of GPx, GST and SOD in the Dox-treated rats amounting to 410%, 184% and 225%, respectively, compared to the normal values. Co-administration of carvedilol produced normalization of GST and significant decrease in GPx and SOD activities, accounting to157%, 151% respectively, compared to the control values.

Effect of doxorubicin (Dox), separately or in combination with carvedilol, on serum alanine aminotransferase (ALT) activity in rats:

Results of table (4) revealed that Dox administration caused significant elevation in the serum ALT level to reach 118% of the normal value. Co-administration of carvedilol caused significant elevation in the same enzyme level compared to both Dox-treated and normal group values, reaching 128.8% of the normal control level.

Histopathological examination of the cardiac tissues:

Histopathological examination of the control cardiac section showed normal structure of the myocardium (Figure 1). Sections obtained from rats administrated Dox showed hyalinization the myocardial bundles associated with either inflammatory cells infiltration only or inflammatory cells and oedema in focal manner in between the bundles (Figures 2, 3). With respect to cardiac sections obtained from rats administrated combined therapy of Dox+ carvedilol, no histopathological alterations, compared to normal section, were observed (Figure 4).

Histopathological examination of hepatic tissues:

Examination of liver sections of the different groups illustrated that liver tissue of the normal group showed hepatic lobules with normal architecture (Figure 5). In case of liver sections of rats

administrated Dox, congestion was observed in the central vein in addition to kupffer cells proliferation in diffuse manner between the fatty degenerated hepatocytes (Figures 6, 7). In rats administrated Dox + carvedilol, livers were most affected, congestion was observed in both the central and portal veins associated with diffuse kupffer cells proliferation in between the hepatocytes. Moreover, the portal area showed inflammatory cells infiltration (Figures 8, 9).

Table (1): Effect of doxorubicin, separately or in combination with carvedilol, on serum lactate dehydrogenase (LDH) and creatine phosphokinase (CPK) activities in rats:

Group	LDH(U/L)	CPK(U/L)
Control	581.17±13 (9)	616.5±20.8 (9)
Doxorubicin	1060.2±29.4 [a, c] (10)	1131.9±32 [a, c] (10)
Doxorubicin + Carvedilol	598.5±23.6 [b] (9)	736.3±41.2 [a, b] (9)

Values are given as means ± SEM (No. of observations are given in parentheses)
a: Significant difference from control group at P<0.05
b: Significant difference from doxorubicin group at P<0.05
c: Significant difference from doxorubicin+ carvedilol group at P<0.05

Table (2): Effect of doxorubicin, separately or in combination with carvedilol, on cardiac malondialdehyde (MDA) and nitric oxide (NO) levels in rats:

Group	MDA nmole /gm tissue	NO nmole / gm tissue
Control	57.6±1.9 (9)	175±11 (9)
Doxorubicin	105.8±4.6 [a, c] (10)	311±18 [a, c] (10)
Doxorubicin + Carvedilol	59.1±3.5 [b] (9)	184±8 [b] (9)

Values are given as means ± SEM (No. of observations are given in parentheses)
a: Significant difference from control group at P<0.05
b: Significant difference from doxorubicin group at P<0.05
c: Significant difference from doxorubicin + carvedilol group at P<0.05

Table (3): Effect of doxorubicin, separately or in combination with carvedilol, on cardiac glutathione (GSH) content, glutathione peroxidase (GPx), glutathione-S-tranferase (GST) and superoxide dismutase (SOD) activities in rats:

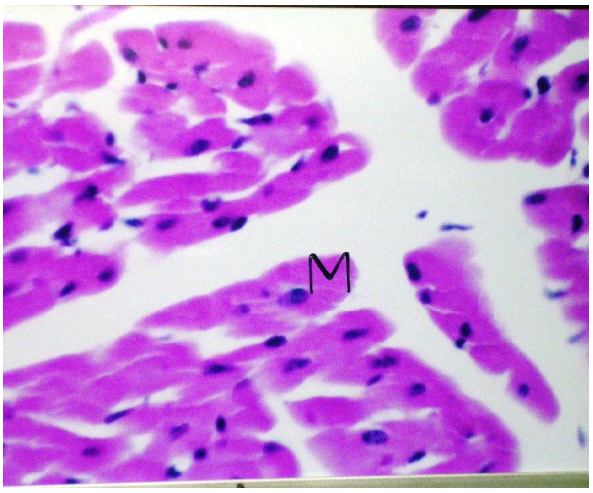
Group	GSH µg/gm tissue	GPx nmoles /min /mg protein	GST nmoles /min/mg protein	SOD U/mg protein
Control	64.3±1.5 (9)	33.6±1.9 (9)	29.6±1.0 (9)	21.3±1.4 (8)
Doxorubicin	41.2±1.1a,c (9)	137.8±7.1a,c (10)	54.7±2.9a,c (9)	48.1±2.2a,c (10)
Doxorubicin +Carvedilol	59.7±2.0a,b (9)	53.0±2.7a,b (8)	36.6±1.59 b (9)	32.3±1.8a,b (9)

Values are given as means ± SEM (No. of observations are given in parentheses)
a: Significant difference from control group at P<0.05
b: Significant difference from doxorubicin group at P<0.05
c: Significant difference from doxorubicin + carvedilol group at P<0.05

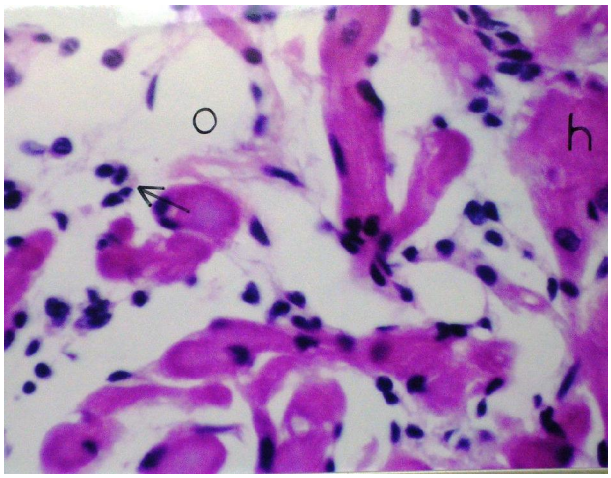
Table (4): Effect of doxorubicin, separately or in combination with carvedilol, on serum alanine aminotransferase (ALT) activity in rats:

Group	ALT (U/L)
Control	52.4±1.8 (9)
Doxorubicin	60.2±1.5[a,c] (10)
Doxorubicin + Carvedilol	69.6±2.6[a,b] (9)

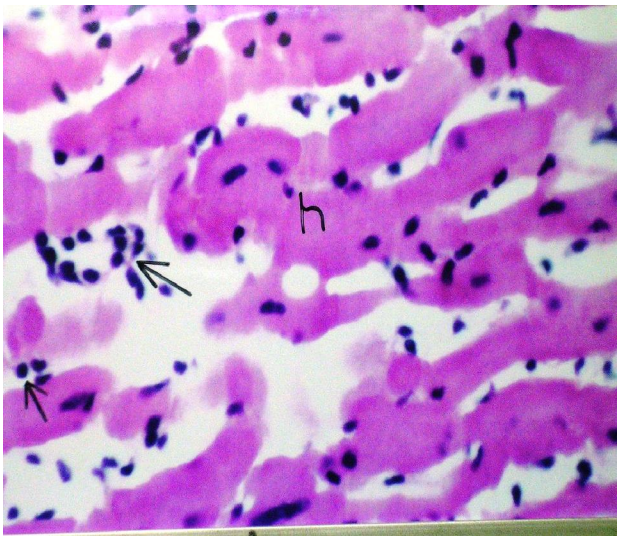
Values are given as means ± SEM (No. of observations are given in parentheses)
a: Significant difference from control group at P<0.05
b: Significant difference from doxorubicin group at P<0.05
c: Significant difference from doxorubicin + carvedilol group at P<0.05



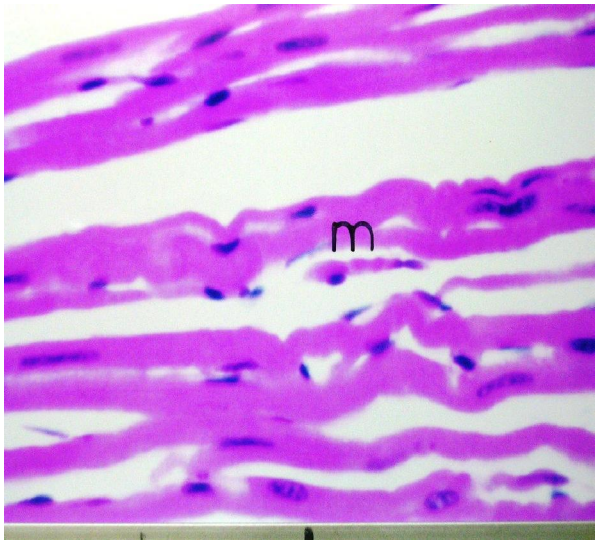
Figure(1): A photomicrograph of cardiac muscle fibers of control group showing normal histological structure of myocardium(M) (H&E x160)



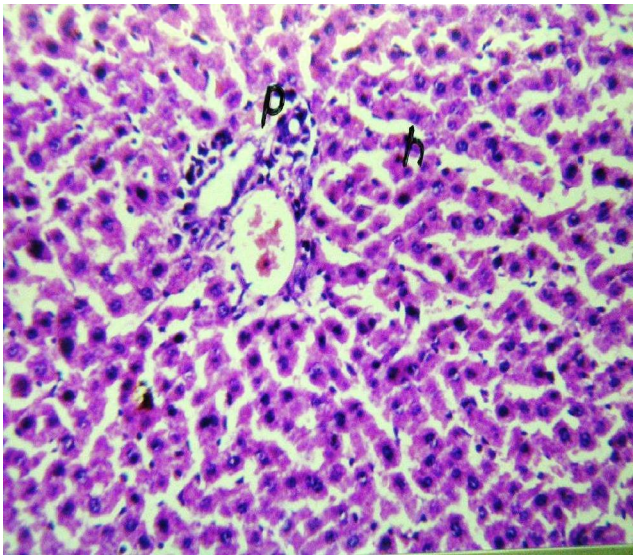
Figure(3): A photomicrograph of cardiac muscle fibers of Dxo group showing oedema(o) with inflammatory cells infiltration (arrow) in focal manner between the myocardial bundles (h). (H&E x160)



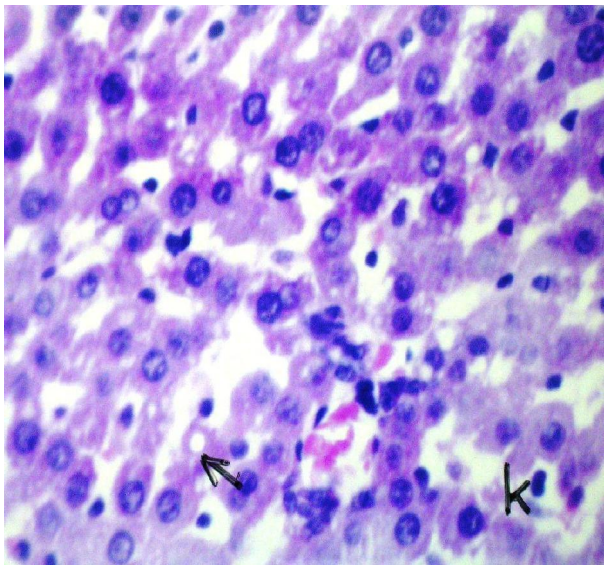
Figure(2): A photomicrograph of cardiac muscle fibers of Dox group showing the inflammatory cells infiltration (arrow) inbetween the hyalinized myocardial bundles(h) (H&E x160)



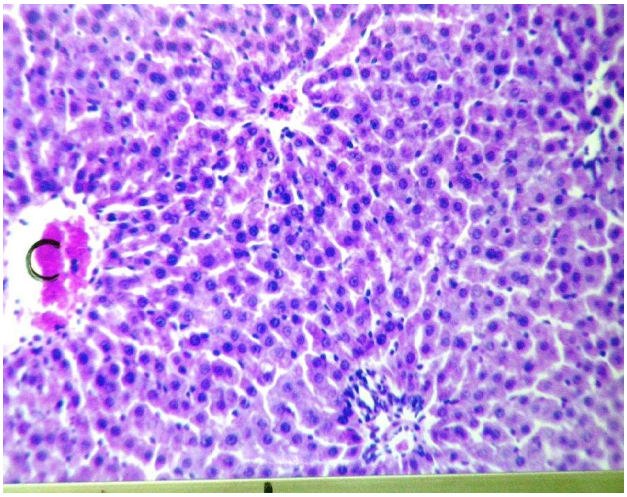
Figure(4): A photomicrograph of cardiac muscle fibers of Dox+ carvedilol group showing intact histological structure of myocardium (m) (H&E x160)



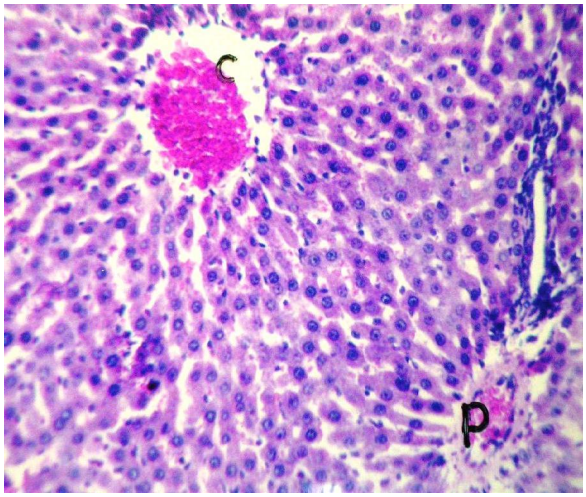
Figure(5): Photomicrograph of liver of normal group showed hepatic lobules (h) and portal vein (p) with normal architecture (H&E x64)



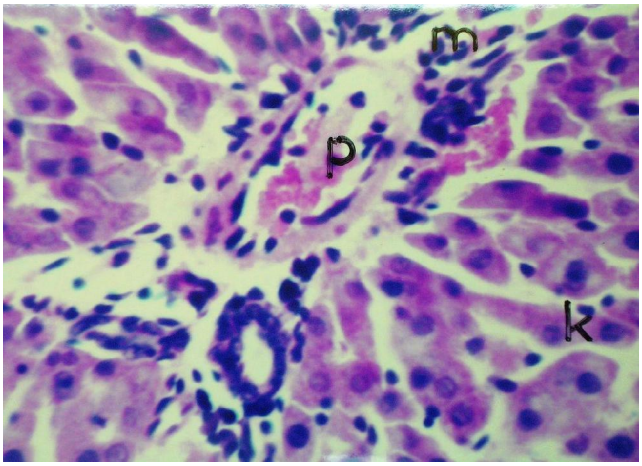
Figure(7): Photomicrograph of liver of Dox group showing diffuse kuffer cells proliferation (k) inbetween the fatty degenrated hepatocytes (arrow) (H&E x160)



Figure(6): Photomicrograph of liver of Dox group showing congestion in central vien(c) (H&E x64)



Figure(8): Photomicrograph of liver of Dox + carvedilol group showing congestion in the central (c) and portal(p) viens. (H&E x64)



Figure(9): Photomicrograph of liver of Dox + carvedilol group showing inflammatory cells infiltration in the portal area (m) around the portal vein (p) with diffuse kuffer cells proliferation (k) inbetween the hepatocyte. (H&E x160)

4. Discussion:

Doxorubicin (Dox) is a potent anticancer drug that is used in treating both hematological and solid tumors. However, severe cardiomyopathy and heart failure have been observed in Dox-treated cancer patients, which limit the clinical dosage of Dox in cancer treatments (31). Oxidative stress is generally held as the mediating mechanism in the multiple biological processes leading to Dox cardiomyopathy, e.g. redox mediated superoxide radical production (32), tissue-specific mitochondrial DNA damage (33) and disturbances of calcium (34) or iron (35). Results of the present study revealed that 15 mg/kg total cumulative dose of Dox induced cardiomyopathy and hepatotoxicity manifested biochemically as significant increase in serum levels of LDH; CPK and ALT. In addition, Dox caused elevation in cardiac NO, MDA levels, SOD, GPx, GST activities, and reduction in GSH content. Histopathological examination of heart and liver sections of Dox-treated animals supported these biochemical results.

Results of table (1) revealed that Dox caused significant increase in the serum levels of LDH and CPK, considered as important markers of cardiac injury. Many previous studies have demonstrated similar elevations in cardiac enzymes activities in rats following Dox administration (36, 37). Leakage of cardiac enzymes directly correlates to ultrastructure damage of heart tissues examined by electron microscope. Dox-induced cardiomyopathy is mainly attributed to increase oxidant production in heart. Dox may undergo a one-electron reduction through a metabolic activation by NADPH-cytochrome P-450 reductase. This reduction leads to the formation of the free radical semiquinone, which in turn can produce a variety of active ROS/RNS, including H2O2, •OH and ONOO (38). These species can attack the cardiomyocyte membrane, damage several macromolecular cellular components, cause protein and lipid peroxidation and consequently lead to cardiomyocyte apoptosis or death (39). This effect would compromise the cellular integrity and potentially account for the leakage of heart enzymes, LDH and CPK, through the membranes and increase their serum activity.

Regarding the effect of Dox administration on cardiac oxidative status, Dox caused significant increase in MDA level (table 2), which is in agreement with previous study (40) who used similar drug regimen. This elevation might be attributed to Dox mediated oxidative stress. The first targets of Dox-mediated free radical damage are various cellular membranes, which are rich in lipids prone to peroxidation. This radical damage results in production of many relatively stable and highly toxic aldehydes, such as MDA. These aldehydes can easily diffuse within the cell, or even cross the plasma membrane, and attack macromolecular targets far from where they were generated and thus act as "second cytotoxic messengers" (41). Table (2) revealed also a marked increase in cardiac NO level in those rats received Dox. This finding is in harmony with previous study (42), which used a model of Dox-induced cardiomyopathy similar to that used in our study. The increase in NO level can be explained on the basis of the ability of Dox to mediate the induction of nitric oxide synthase (NOS) expression and, hence, NO release in heart (43). Previous studies suggested that stimulation of endothelial cells with calcium-mobilizing agents activates and dissociates the membrane-bound eNOS (44). Because Dox-induced toxicity is mediated by intracellular H_2O_2 as well as calcium influx, Dox treatment causes an increase in eNOS transcription and protein activity in aortic endothelial cells and thus NO synthesis.

Dox administration, as shown in table (3), caused a significant decrease in cardiac GSH content, which is quiet compatible with previous studies (40, 45). The overproduction of ROS, caused by Dox administration, can account for this decrease in GSH

content, as these species are detoxified by endogenous antioxidants mainly GSH causing their cellular stores to be depleted (46). The observed decrease of cardiac GSH content may also be attributed to the enhanced activities of GSH metabolizing enzymes, as shown in the present study. One is GPx which reduces H_2O_2 and various peroxides using GSH as reducing agent, the other is GST which consumes GSH in the conjugation of Dox toxic metabolites (47).

Table (3) showed, also, significant increase in cardiac activity of SOD in the Dox-treated rats, which is consistent with some studies (40, 48). The increase in SOD activity can be explained on the basis that the redox cycling of Dox between quinone and semiquinone forms generates large amounts of O_2 (38), which in turn stimulate SOD as an adaptive response to counteract oxidative stress (49). Also, it was previously showed that ROS can upregulate and induce the synthesis of SOD protein (50). The observed increase in SOD activity might lead to overproduction of hydroperoxides. In consequence, cardiac GPx activity might be stimulated in response to the accumulated peroxides which can subsequently lead to the formation of highly toxic •OH radical through Fenton reaction catalyzed by iron (51). This assumption was supported by our results which showed a significant enhancement in cardiac GPx activity in the Dox-treated group. Another assumed explanation for such increased GPx activity is that multiple doses of Dox alter the activities of antioxidant enzymes in the heart so as to protect against Dox cardiotoxicity (42, 49). Additionally, GPx have been reported to be over expressed in Dox-treated cells, especially those tumor resistant ones (52). This can be considered as intracellular detoxification process for free radical end products (53). In our study, we assumed that this detoxification mechanism can occur also in cardiac myocytes, exposed to Dox administration, to prevent the accumulation of peroxides and the propagation of peroxidation. Table (3), additionally, revealed significant increase in cardiac GST activity in rats treated with Dox, which is in agreement with previously reported results (54). The increase in cardiac GST activity might be related to the fact that GSTs are family of dimeric proteins that posses a multitude of functions including the enzymatic conjugation of GSH to electrophilic xenobiotics (55). It has been reported that cellular exposure to xenobiotics and antioxidants leads to coordinated induction of a battery of genes encoding detoxifying enzymes including GST (56). Indeed, it has been known that Dox is metabolized, via alkedoreductases, yielding C13 hydroxyl derivative, doxorubicinol. This metabolite is actually more polar and toxic than Dox itself. Doxorubicinol accumulates in the heart and contributes significantly to chronic cumulative cardiotoxicity induced by Dox (57). In brief, GST has showed elevation after Dox injection to detoxify Dox and its metabolites and to attenuate the elevated oxidative stress (58). Moreover, Dox-treated cancer cell lines often show elevated GST expression and activity, which is in consistence with our result. Such increased activity of GST enzyme almost certainly leads to increased resistance to Dox treatment, and hence, creation of one of the most problems of Dox therapy (59).

Our results showed also an elevation in serum ALT upon Dox administration (table 4). This result agrees with previous study (60). Leakage of hepatic ALT into the serum is due to Dox-induced oxidative damage to hepatocytes (61). Dox-induced hepatotoxicity may be less severe than its cardiotoxicity. This can be related to the fact that liver mitochondria, unlike cardiac mitochondria, lack the NADH-related pathway of reducing equivalents from the cytosol to the respiratory chain, as a result, liver mitochondria do not generate significant amounts of Dox semiquinones (62).

The previously mentioned biochemical results produced by Dox administration are supported by the obtained cardiac histopathological results which showed that in the cardiac sections of the Dox-treated rats, serious morphological changes were observed in the myocardium. Also, the examination of liver sections of the same group illustrated that congestion was observed in the central vein in addition to kupffer cells proliferation in diffuse manner between the fatty degenerated hepatocytes.

From the previously mentioned discussion, it has been well established that oxidative stress plays a major role in Dox-induced cardiomyopathy and hepatotoxicity. Thus, the importance of antioxidant co-administration as an adjuvant therapy with Dox in preventing these damages has been emphasized.

Carvedilol is an 1-blocking agent and a potent antioxidant, with a 10-fold greater activity than vitamin E. Some carvedilol metabolites found in human plasma also exhibit antioxidative activity approximately 50- to 100-fold greater than carvedilol. These unique properties of carvedilol may be important in preventing progressive deterioration of left ventricular dysfunction and chronic heart failure (63). Results of the present study revealed that co-administration of carvedilol with Dox could attenuate the cardiotoxicity manifested biochemically by normalization of LDH as well as significant decrease in CPK serum levels. Also, such combined therapy caused normalization of cardiac MDA and NO levels, as well as, significantly raised GSH content. Moreover, co-administration of carvedilol produced normalization of GST, as well as, significant

decreased in cardiac activities of GPx and SOD. Histopathological examination of heart sections supported the alleviating effect of carvedilol on Dox-induced cardiotoxicity. On the contrary, carvedilol caused significant elevation in the serum ALT level compared to both normal and Dox-treated groups. Histology of liver sections showed that liver in this group was most damaged compared to other groups.

Our results showed that, carvedilol effectively prevented cardiomyocyte damage caused by Dox, evidenced biochemically by its ability to reduce the leakage of cardiac enzymes LDH and CPK into the serum. This finding is in harmony with previous results (64). This observed result is attributed to the antioxidant capacity of carvedilol and its ability to protect cellular membranes against oxidative damage preserving their integrity. The protective effect of carvedilol is strongly supported by the present ultra structural results, as carvedilol completely relieved cardiac histopathological damage induced by Dox. This observation is in harmony with results reported previously who related the cardioprotective effect of carvedilol to its positive impact on cardiac mitochondrial function, since, carvedilol prevented the inhibitory effect of Dox on mitochondrial respiration in heart, and also prevented the decrease in mitochondrial Ca^{2+} loading capacity and the inhibition of the respiratory complexes of heart mitochondria caused by Dox. Thus, co-administration of carvedilol decreased the extent of cellular vacuolization and cell death in cardiac myocytes (65, 66).

Our results revealed also that MDA was greatly reduced by the administration of carvedilol concomitantly with Dox, reaching to the normal level. This result is quiet consistent with many previous studies (67, 68). Earlier report (69) showed that carvedilol reduced plasma lipid peroxidation in patients with heart failure. Another possible mechanism is the direct antioxidative property of carvedilol which is attributed to the carbazole moiety of the drug (70). Carvedilol inhibited Fe^{2+}-initiated lipid peroxidation via scavenging free radicals (71) or by sequestering ferric ion (72). Carvedilol can also scavenge lipid radicals directly, thus breaking the chain reaction in membranes (73). On the other hand, as shown in our study, carvedilol caused significant reduction in cardiac NO level, reaching to normal value. This finding is in harmony with previous report (74) demonstrated the effect of carvedilol as a NO quenching agent. Carvedilol protected nitrosylation of intracellular molecule by exogenous NO and reduced intracellular concentration of NO produced by NO donors. Moreover, carvedilol is able to inhibit ROS generation by leukocytes (75) and decrease phagocyte degranulation and the amount of free myeloperoxidase (76), which at the sites of inflammation may function as a catalytic sink for NO (77).

As shown in our results, carvedilol produced remarkable inhibition of the observed GSH depletion in rats treated with Dox, which is in harmony with previous results (78, 67) that used carvedilol dose similar to that used in the current study. This result can be explained depending on the fact that carvedilol is potent antioxidant, since it reduced the production of free radicals in heart and thus the consumption of endogenous antioxidants especially GSH (46). Moreover, a reduced energy breakdown offered by carvedilol is expected to maintain cellular viability, thus avoiding membrane damage, alteration of ionic homeostasis and the occurrence of oxidative stress. Therefore, the positive effects on glutathione and –SH group metabolism which were found after treatment with carvedilol are most likely the consequence of a generalized cardiac protection due to the β-blocking-mediated energy saving (64).

Our study revealed also that carvedilol caused significant decrease in SOD and GPx activities in heart when administered concomitantly with Dox. This effect is, to some extent, similar to previous observation (67). Carvedilol protects a variety of cultured cells from oxygen radical- induced damage when subjected to either artificial oxygen-radical generating systems, such as Fe2+/vitamin C, or endogenous oxygen radical generating systems, such as xanthine-xanthine oxidase which involves the release of superoxide ions. This effect of carvedilol may result from its ability both to scavenge superoxide ions and to inhibit the production of superoxide radicals, the latter being inferred from the observation that carvedilol inhibits superoxide release from phorbol ester-activated neutrophils (79). The reduction in superoxide radical production may in turn relief the adaptive increase in SOD activity preceding Dox administration, as shown in our results. This decrease in cardiac SOD activity might be considered as a direct mechanism for the concomitant decrease in cardiac GPx activity observed in the present study, since no more H2O2 to be declared by GPx.

Regarding the effect of carvedilol on Dox-induced elevation in cardiac GST, the present study showed that carvedilol caused significant decrease in such enzymatic activity, reaching to the normal level. This finding might be related to previous observations that carvedilol could reverse cellular drug resistance by inhibiting MRP1 drug efflux system (80, 81). GST catalyzes the conjugation of Dox and its metabolites to GSH (47). Glutathione conjugates of Dox show high affinities toward the MRP1 pump, a drug efflux protein mediates efflux of GSH conjugates (82). The inhibitory effect of carvedilol on MRP1 can, in turn, reduce the efflux of GSH conjugates of Dox and thus

down regulates the production of the conjugating enzyme, GST.

Our results revealed that in the group administered a combined therapy of Dox+carvedilol, significant elevation in serum ALT level, with respect to both control and Dox- administrated group levels, was shown. Histopathological examination of liver sections of this group supported the biochemical result in that liver tissues of rats belonging to this group were shown to be most damaged compared to other groups, given that, congestion was observed in both the central and portal veins associated with diffuse kupffer cells proliferation in between the hepatocytes and the portal area showed inflammatory cells infiltration.

5. Conclusion:

Oxidative stress plays a major role in Dox-induced cardiomyopathy and hepatic damage. Carvedilol could effectively attenuate the cardiomyocyte damage caused by Dox as evidenced by the biochemical measurements and histopathological examination of cardiac tissue. Unfourtiontly, carvedilol augmented Dox-induced hepatic damage as evidenced by the present biochemical result and the histopathological examination of the hepatic tissue.

Competing Interests:

The authors declare that they have no competing interests.

Authors' Contributions :

SSI: participated in designing the point of research and the plan of work; followed up all the practical experiments; reviewed the statistical analysis; participated in analysis and interpretation of data; drafted the manuscript; the corresponding author for article publication; read and approved the version to be published.

MMB: participated in designing the point of research and the plan of work, read and approved the version to be published.

HSH: carried out the experimental works and performed the statistical analysis.

Acknowledgements:

We are thankful to Dr. Mohamed A Ali, Assistant professor of Histology, Faculty of Medicine, Cairo University, for his professional help in carrying out the histopathological examination.

Corresponding author

Safinaz S. Ibrahim

Biochemistry Department, Faculty of Pharmacy, Cairo University, Cairo, Egypt

dr_safinaz_747@hotmail.com

5. References:

1. Bonadonna G, Valagussa P: Sequential or alternating doxorubicin and CMF regimens in breast cancer with more than three positive nodes. *JAMA*. 1996; 237:542-547.
2. Bacci G, Ferrari S, Delepine N: Predictive factors of histologic response to primary chemotherapy in osteosarcoma of extremity. *J.Clin.Oncol*. 1998;16:658-663.
3. Kufe DW, Holland JF, Frei E: American Cancer Society: Cancer medicine 6 edition. Edited by BC Decker, Hamilton, 2003:344-367.
4. Lage M, Helbach H, Dietel M, Schadendorf D: Modulation of DNA topoisomerase II activity and expression in melanoma cells with acquired drug resistance. *Br J Cancer* 2000; 82:488 – 491.
5. Tangpong J, Cole M, Sultana R: Adriamycin-mediated nitration of manganese superoxide dismutase in the central nervous system: Insight into the mechanism of chemobrain. *J. Neurochem.* 2007; 100: 191–201.
6. Shri N, Mohammed A, Xiaogu D: Amelioration of doxorubicin-induced cardiac and renal toxicity by pirfenidone in rats. *Cancer Chemother. Pharmacol*. 2004; 53:141–150.
7. Weiss R: The anthracyclines: will we ever find a better doxorubicin? *Semin Oncol.* 1992; 19: 670–686.
8. Bryant J, Picot J, Levitt G: Cardioprotection against the toxic effects of anthracyclines given to children with cancer: a systematic review. *Health Technol Assess*. 2007; 11: 1-84.
9. Opie L, Yusuf S: Beta-blocking agents. In: Drugs for the Heart. 6th ed. Edited by Opie LH, Gersh B. Philadelphia, Pensylvania: W.B. Saunders Company, 2005: 1–32.
10. Qin F, Shite J, Mao W: Selegiline attenuates cardiac oxidative stress and apoptosis in heart failure: association with improvement of cardiac function. *Eur J Pharmacol*. 2003; 461: 149–158
11. Bakris GL, Fonseca V, Katholi RE: Metabolic effects of carvedilol vs metoprolol in patients with type 2 diabetes mellitus and hypertension: a randomized controlled trial. *JAMA*. 2004; 292: 2227–36
12. Spallarossa P, Garibaldi S, Altieri P: Carvedilol prevents doxorubicin-induced free radical release and apoptosis in cardiomyocytes in vitro. *J. Mol. Cell. Cardiol*. 2004; 37(4):837-846.
13. Nakamura K, Kusano K, Nakamura Y: Carvedilol decreases elevated oxidative stress in human

failing myocardium. *Circulation*. 2002; 105: 2867-2871.
14. de Groot AA, Mathy MJ, van Zwieten PA: Antioxidant activity of nebivolol in the rat aorta *J Cardiovasc Pharmacol*. 2004; 43: 148-153.
15. Giordano FJ: Oxygen, oxidative stress, hypoxia and heart failure *J Clin Investig*. 2005; 115: 500-508.
16. Matsui H, Morishima I, Numaguchi Y: Protective effect of carvedilol against doxorubicin-induced cardiomyopathy in rats. *Life Sci*. 1999; 65(12): 1265-74.
17. Santos DL, Moreno AJ, Leino RL: Carvedilol protects against doxorubicin-induced mitochondrial cordimyopathy. *Toxicol Appl Pharmacol*. 2002; 185 (3): 218-27.
18. Rosalki SB: Kinetic Method for the Determination of Serum CPK. *J Lab Clin Chem*. 1977; 23: 646-649.
19. Buhl SN, Jackson KY: Kinetic Method for the Determination of Serum LDH. *Clin Chem*. 1978; 24: 828-832.
20. Reitman, S and Frankel, S: A colorm etric method for the determination of serum glutamic oxaloacetic acid and glutamic pyruvic transaminases *Am. J. Clin. Pathol*. 1957; 28: 56-59.
21. Banchroft J, Stevens A, Turner D: Theory and practice of histopathological techniques. 4 ed. Chuchil Livingstone, New York, London, Sanfrancisco, Tokyo. 1996; 332-336.
22. Beutler E, Duron O, Kelly B: Improved method for the determination of blood glutathione *J Lab Clin Med* 1963; 61: 882-888.
23. Uchiyama M, Mihara M: Determination of malondialdehude precursor in tissues by thiobarbituric acid test. *Anal Biochem*. 1978; 86: 271-288.
24. Habig W, Pabst M, Jakoby W: Glutathione S-transferase: The first enzymatic step in mercapturic acid formation. *J. Biol. Chem*. 1974; 249: 7130-7139.
25. Gawai K, Pawar S: Purification and characterization of glutathione S-transferase from liver cytosol of phenobarbital treated rabbits. *Xenobiotica*. 1984; 14: 605-607.
26. Paglia DE, Vatentine WN: Studies on the quantative and qualitative characterization of erythrocyte glutathione peroxidase. *L Lab Clin Med*. 1967; 70: 158-161.
27. Marklund S, Marklund G: Involement of superoxide anion radical in autoxidation of pyrogallol and convenient assay for superoxide dismutase. *Eur J Biochem*. 1974; 47: 469-474.
28. Lowry OH, Rosebrough NJ, Farr AL, Ranadall RJ: Protien measurement with the folin phenol reagent *J Biol Chem*. 1951; 193: 265-275.
29. Tracey WR, Tse J, Carter G: Lipopolysaddharide-induced changes in plasma nitrite and nitrate concentrations in rats and mice: pharmacological evaluation of nitric oxide synthase inhibitors. *J Pharmacol Exp Ther*. 1995; 272: 1011-1015.
30. Grandari M, Verrecchia C, Revaud ML, Allix M, Boulu RG, Plotkine M: Calcium-independent NO-synthase activity and nitrites/nitrates production in transient focal cerebral ischemia in mice. *Br J Pharmacol*. 1997; 122: 625-630.
31. Horan PG, McMullin MF, McKeown PP: Anthracycline cardiotoxicity. *Eur Heart J*. 2006; 27: 1137–1138.
32. Vasquezvivar J, Martasek P, Hogg N, Masters B: Endothelial nitric oxide synthase-dependent superoxide generation from adriamycin. *Biochemistry*. 1997; 36: 293– 297.
33. Lebrecht D, Kokkori A, Ketelsen UP, Setzer B: Tissue-specific mt DNA lesions and radical-associated mitochondrial dysfunction in human hearts exposed to doxorubicin. *J of Pathology*. 2005; 207: 436–444.
34. Solem LE, Henry TR, Wallace KB: Disruption of mitochondrial calcium homeostasis following chronic doxorubicin administration. *Toxicology and Applied Pharmacology*. 1994; 129: 214–222.
35. Kotamraju S, Chitambar CR, Kalivendi SV, Joseph J: Transferrin receptor-dependent iron uptake is responsible for doxorubicin-mediated apoptosis in endothelial cells - role of oxidant-induced iron signaling in apoptosis. *J. of Biological Chemistry*. 2002; 277: 179–187.
36. Fadillioglu E, Erdogan H: Effects of erdosteine treatment against doxorubicin-induced toxicity through erythrocyte and plasma oxidant/antioxidant status in rats. *Pharmacol Res*. 2003; 47: 317-22.
37. Yagmurca M, Fadillioglu E, Erdogan H, Ucar M, Sogut S: Erdosteine prevents doxorubicin-induced cardiotoxicity in rats. *Pharmacol Res*. 2003; 48: 377-82.
38. Takemura G, Fujiwara H: Doxorubicin-induced cardiomyopathy from the cardiotoxic mechanisms to management. *Prog Cardiovasc Dis*. 2007; 49: 330-352.
39. Menna P, Minotti G, Salvatorelli E: In vitro modeling of the structure-activity determinants of anthracycline cardiotoxicity. *Cell Biol Toxicol*. 2007; 23: 49-62.
40. Balachandar AV, Malarkodi KP, Varalakshmi P: Protective role of DL alpha-lipoic acid against adriamycin-induced cardiac lipid peroxidation. *Hum Exp Toxicol*. 2003; 22: 249-54.
41. Luo X, Evrovsky Y, Cole D, Trines J: Doxorubicin-induced acute changes in cytotoxic

aldehydes, antioxidant status and cardiac function in the rat. *Biochim Biophys Acta* , 1997; 136: 45–52.
42. Saad S, Najja T, Al-Rikabi A: The preventive role of deferoxamine against acute doxorubicin - induced cardiac, renal and hepatic toxicity in rats. *Pharmacol Res.* 2001; 43: 211–218.
43. Fogli S, Nieri P, Breschi MC: The role of nitric oxide in anthracycline toxicity and prospects for pharmacologic prevention of cardiac damage. *FASEB J* . 2004; 18: 664-75.
44. Drummond G, Cai H, Davis M, Ramasamy S, Harrison D: Transcriptional and Posttranscriptional Regulation of Endothelial Nitric Oxide Synthase Expression by Hydrogen Peroxide. *Circ. Res.* 2000; 86:347–354
45. Arafa HM, Abd-Ellah MF, Hafez HF: Abatement by naringenin of doxorubicin-induced cardiac toxicity in rats. *J Egypt Natl Canc Inst* . 2005; 17(4):291-300.
46. Thomas JA. Oxidative stress: Oxidant defense and dietary constituents. In: Modern Nutrition in Health and Disease. Edited by Maurice E, Shils ME, Olson JA, Shike M. Challotesville, Universuty of Virginia: Awaverly Company. 1994:501-512.
47. O'Brien ML, Tew KD: Glutathione and related enzymes in multidrug resistance. *Eur J Cancer.* 1996; 32A:967–78.
48. Signal PK; Siveski-Iiiskovic, Hill M, Thomas T, Li T: Combination therapy with probucol prevents adriamycin-induced cardiomyopathy. *J Mol.Cell Cardiol* . 1995; 27: 1055 -1063.
49. Childs AC, Phaneuf SL, Dirks AJ, Phillips T: Doxorubicin treatment in vivo causes cytochrome C release and cardiomyocyte apoptosis, aswell as increased mitochondrial efficiency, superoxide dismutase activity, and Bcl-2:Bax ratio. *Cancer Res* . 2002; 62(16):592-8.
50. Li T, Singal P: Adriamycin-induced early changes in myocardial antioxidant enzymes and their modulation by probucol. *Circulation.* 2000; 102: 2105-2110.
51. Alegria AE, Samuni A, Mitchell JB, Riesz P: Free radicals induced by adriamycin-sensitive and adriamycin resistant cells;A spin-trapping study. *Biochemistry.* 1989; 28:653-658.
52. Akman S, Forrest G, Chu F, Esworthy R, Doroshow J: Antioxidant and xenobiotic-metabolizing enzyme gene expression in doxorubicin-resistant MCF-7 breast cancer cells, *Cancer Res.* 1990; 50: 1397 - 1402.
53. Benchekroun M, Pourquier P, Schott B, Robert J: Doxorubicin-induced lipid peroxidation and glutathione peroxidase activity in tumor cell lines selected for resistance to doxorubicin. *Eur J Biochem.* 1993; 211(1-2):141-6
54. Paranka NS, Dorr RT: Effect of doxorubicin on glutathione and glutathione-dependent enzymes in cultured rat heart cells. *Anticancer Res.* 1994; 14(5A):2047-52.
55. Hayes, JD, and Pulford DJ: The glutathione 5-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. *Crii. Rev. Biochem. Mol. Biol* . 1995; 30: 445-600.
56. Venugopal R, Joseph P, Jaiswal A: Gene expression of DT-diadhorase in cancer cells. In :Oxidative stress and signal induction. Edited by Forman, H.j. and Cadenas, E. Chapman & Hall, New York.1997: 441-448.
57. Wang C, Wang S, Shiah H, Lin J: Effect of ethanol on hepaypyoxicity and hepatic DNA-binding of aflatoxin B1 in rats. *Biochem. Pharmacol* . 1990; 40(4): 715-712.
58. L'Ecuyer T, Allebban Z, Thomas R, Heide R: Glutathione S -transferase overexpression protects against anthracycline-induced H9C2 cell death. *Am J Physiol Heart Circ Physiol* . 2004; 286: 2057–2064.
59. Harbottle A, Daly AK, Atherton K, Campbell FC: Role of glutathione S-transferase P1, P-glycoprotein and multidrug resistance-associated protein 1 in acquired doxorubicin resistance. *Int J Cancer.* 2001; 92(6): 777-83.
60. Deepa PR, Varalakshmi P: Protective effect of low molecular weight heparin on oxidative injury and cellular abnormalities in adriamycin-induced cardiac and hepatic toxicity. *Chemico-Biological Interactions.* 2003; 146:201–210.
61. Llesuy G, Arnaiz H: Hepatotoxicity of mitoxantrone and doxorubicin. *Toxicology,* 1990; 63(2): 187-98
62. Nohl H, Gille L, Staniek K: The exogenous NADH dehydrogenase of heart mitochondria is the key enzyme responsible for selective cardiotoxicity of anthracyclines. *Z Naturforsch.* 1998; 53: 279–285.
63. Feuerstein G, Ruffolo R R, Jr: Carvedilol, a novel vasodilating beta-blocker with the potential for cardiovascular organ protection. *European Heart Journal* . 1996; 17: 24-29
64. Cargnoni A, Ceconi C, Bernocchi P, Boraso A: Reduction of oxidative stress by carvedilol: role in maintenance of ischaemic myocardium viability. *Cardiovasc Res.* 2000; 47(3):556-66.
65. Santos DL, Moreno AJ, Leino RL, Froberg MK: Carvedilol protects against doxorubicin-induced mitochondrial cardiomyopathy. *Toxicol Appl Pharmacol.* 2002; 185(3):218-27.

66. Oliveira PJ, Bjork JA, Santos MS, Leino RL: Carvedilol-mediated antioxidant protection against doxorubicin-induced cardiac mitochondrial toxicity. *Toxicol Appl Pharmacol.* 2004;200(2):159-68.
67. Huang H, Shan J, Pan XH, Wang HP, Qian LB: Carvedilol protected diabetic rat hearts via reducing oxidative stress. *J Zhejiang Univ Sci B*. 2006;7(9):725-31.
68. Wang R, Miura T, Harada N, Kametani R, Shibuya M, Fukagawa Y, et al: Pleiotropic effects of the beta-adrenoceptor blocker carvedilol on calcium regulation during oxidative stress-induced apoptosis in cardiomyocytes. *J Pharmacol Exp Ther.* 2006;318(1):45-52.
69. Kukin ML, Kalman J, Charney RH: Prospective, randomized comparison of effect of long-term treatment with metoprolol or carvedilol on symptoms, exercise, ejection fraction, and oxidative stress in heart failure. *Circulation*. 1999;99:2645–2651
70. Feuerstein G, Shusterman NH, Ruffolo RR: Carvedilol update. IV. Prevention of oxidative stress, cardiac remodeling and progression of congestive heart failure. *Drugs Today*.1999;33:453–473.
71. Yue T, Cheng H, Lysko P, McKenna PJ, Feuerstein R, Gu J, et al: Carvedilol, a new vasodilator and beta-adrenoceptor blocker antagomsts is an antioxidant and free radical scavenger. *J Pharmacol Exp Ther.* 1992;263:92-8
72. Noguchi N, Nishino K, Niki E: Antioxidant action of the antihypertensive drug, carvedilol, against lipid peroxidation. *Biochem Pharmacol* 2000;59(9):1069–1076.
73. Yue TL, Mckenna PJ, Gu JL, Cheng HY: Carvedilol, a new antihypertensive agent, prevents lipid peroxidation and oxidative injury to endothelial cells. *Hypertension*. 1993;22(6):922-8.
74. Yoshioka T, Iwamoto N, Tsukahara F, Irie K, Urakawa I, Muraki T: Anti-NO action of carvedilol in cell-free system and in vascular endothelial cells. *Br J Pharmacol.* 2000;129(7):1530-5.
75. Nosal R, Jancinova V, Ciz M, Drabikova K: Inhibition of chemiluminiscence by carvedilol in the cell-free system, whole human blood and blood cells. *Scand J Clin Lab Incest*. 2005;65:55-64.
76. Pecivova J, Macickova T, Lojek A: In vitro effect of carvedilol on professional phagocytes. *Pharmacology.* 2007;79:86-92.
77. Eiserich JP, Baldus S, Brennan ML: Myeloperoxidase, a leukocyte-derived vascular NO oxidase. *Science.* 2002;296:2391-2394.
78. Ma XL, Lopez BL, Liu GL, Christopher TA, Gao F: Hypercholesterolemia impairs a detoxification mechanism against peroxynitrite and renders the vascular tissue more susceptible to oxidative injury. *Circ Res*. 1997;80(6):894-901.
79. Yue TL, McKenna PJ, Ruffolo RR Jr, Feurerstein G: Carvedilol, a new adrenergic antagonist and vasodilator antihypertensive drug, inhibits superoxide release from human neutrophils. *Eur J Pharmacol*. 1992;214:277-80.
80. Kakumoto M, Sakaeda T, Takara K, Nakamura T., Okumura K: Effects of carvedilol on MDR1-mediated multidrug resistance: comparison with verapamil. Cancer Sci. 2003;94(1):81-6.
81. Takara K, Sakaeda T, Okumura K: Carvedilol: a new candidate for reversal of MDR1/P-glycoprotein-mediated multidrug resistance. *Anticancer Drugs.* 2004;15(4):303-9.
82. Loe DW, Ahmquist KC, Deeley RG: Multidrug-resistance protein (MRP)-mediated transport of leukotriene C-4 and chemotherapeutic-agents in membrane-vesicles-demonstration of glutathione-dependent vincristine transport. *J Biol Chem*. 1996;271:9675-82.

7/1/2010

Effect of Water Stress and Ascorbic Acid on Some Morphological and Biochemical Composition of Ocimum basilicum plant.

1Soha E. Khalil, 2 Nahed G. Abd El- Aziz and 1Bedour H. Abou Leil

1Departments of Water Relation and Field Irrigation, National Research Centre, Dokki, Cairo, Egypt
2Department of Ornamental and Woody Trees, National Research Centre, Dokki, Cairo, Egypt
Soha_khalil2001@yahoo.com

Abstract: Basil (*Ocimum basilicum c.v. Thai Magic*) is an annual herb plant belonging to the lamiaceae family that used as drug, mainly cultivated for leaves and flowering topes, the plant yield have an essential oil on steam distillation. The experiment was conducted to study the effect of different levels of water stress (30, 50 and 70% depletion of available soil moisture), different concentrations of ascorbic acid (0, 100, 150 and 200 ppm) and spraying time (at vegetative or vegetative plus flowering stages) on some morphological and biochemical characteristics of basil plant. A pot experiment was conducted in a split-split plot design with 24 treatments and three replicates in greenhouse. The results of staticall analysis showed that water stress, ascorbic acid concentrations and spraying time have significant effect on morphological and biochemical characteristics. Plant height, number of branches, number of leaves, leaf area, fresh and dry weights of the first cut showed significant increase under 50% soil moisture level while further increase in water stress level showed significant decrease in previously mentioned parameters. The same tendency was observed for relative water content % as well as photosynthetic pigments concentrations (chla, chlb, total chla+b and carotenoids). While in the second cut, the previously mentioned characters showed progressive decrease with increasing water stress level (except for photosynthetic pigments which revealed the same trend as in the first cut). Reveres trend observed for oil% and proline content. The data also indicated that the application of ascorbic acid in different concentrations showed significant increase in all growth parameters, fresh and dry weights, relative water content, oil % and photosynthetic pigments compared with control treatment and revealed decrease in proline accumulation. [Journal of American Science. 2010;6(12):33-44]. (ISSN: 1545-1003).

Keywords: Water Stress; Ascorbic Acid; Biochemical Composition; *Ocimum basilicum*

1. Introduction

Water stress is the most influential factors affecting crop yield particularly in irrigated agriculture in arid and semi arid regions, it is necessary to get maximum yield in agriculture by using available water in order to get maximum profit form per unit area because existing agricultural land and irrigation water are rapidly diminishing due to rapid industrialization and urban development. Optimizing irrigation management due to water scarcity together with appropriate crops for cultivation is highly in demand; the cost of irrigation pumping and inadequate irrigation scheme capacity as well as limited water sources is among the reasons that force many countries to reduce irrigation applications. Potential of water stress tolerance and the economical value of medicinal and aromatic plants, make them suitable alternative crops in dry lands (Ghanbari et al., 2007).

Ocimum basilicum plant is one of the most important aromatic plants which used to flavor foods and in traditional medicines (Yusuf et al., 1994). In aromatic plants, growth and essential oil production are influenced by various environmental factors, such as water stress (Burbott and Loomis, 1969). Efforts are being made to overcome this problem primarily by studding the tolerance of different plants to water stress or by using hormones, chemical and physical treatments as well as biological methods (El Saidi, 1997). Water stress is one of the important limiting factors of plant growth that has limited the production of 25% of world lands (Levitt, 1980). Solinas and Deiana (1996) reported that secondary products of plants can be altered by environmental factors and water stress is the major factor affecting the synthesis of natural products. Water stress resulted in significant reduction of fresh and dry matter, nutrient content and essential oil yield (Mirsa and Strivastov, 2000). Fresh and dry weights of Ocimum bacilicum L. were decreased as plant water deficit increased (Simon et al., 1992). The linalool and methyl chavicol contents of sweet basil as percentage of total essential oil increased as water stress increased (Simon et al., 1992) the essential oil yield of basil was increased by subjecting plants to water stress just before harvesting (Baeck et al., 2001). Essential oil, total carbohydrates and proline contents were pronouncedly increased with increasing stress levels of Salivia officinalis L. (sage) plants (Hendawy and khalid, 2005) Moreover, Ashraf and Foolad (2007) reported that osomoprotectants such as proline and glycine betaine

were increased under drought stress. Also, Tawfik (2008) indicated that osmoprotectants such as total soluble sugars, proline and glycine betaine increased in plants subjected to water stress.

Ascorbic acid is one of the water soluble reductants which is very important antioxidant which protect plants by suppressing oxidative injury, by affecting many enzymes activities and also is required for regeneration of x-tocopheral (Smirnoff, 1995). Ascorbate occurs in the cell wall where it is a first line of defense against ozone; Ascorbate also has been implicated in regulation of cell division and photosynthesis. Ascorbate has benefits for human nutrition and possibly for tolerance of plants to photo oxidative stresses (Foyer et al., 1993; Smirnoff, 1995 and Abou-Leila 1994).

Therefore, we aim in this investigation to study the effect of water stress, ascorbic acid concentrations and spraying time on vegetative growth, essential oil % and chemical content of Ocimum basilicum which is economically important plant in Egypt.

2. Material and Methods

The experiment was conducted during the two successive seasons of 2008 and 2009 in the greenhouse of National Research Centre (NRC), Giza, Egypt. Seeds of Ocimum basilicum c.v. Thai Magic were sown in the second week of April in plastic pots (30 cm diameter), each pot was filled with 10 kg of air dried soil, physical and chemical properties of the soil used are presented in Table (1) using the standard method described by Klute (1986).

Seeds of Ocimum basilicum c.v. Thai Magic were provided by the Department of Medicinal and Aromatic plants, Ministry of Agriculture, Giza, Egypt. Ocimum seeds were irrigated regularly with tap water for three weeks until seedling emergency, then seedlings were thinned to two plants per pot, all pots received recommended does of NPK fertilizers.

The experiment including 24 treatments which were the combination between three levels of soil moisture (30, 50, and 70 % depletion of available soil water) and four concentrations of ascorbic acid spray (0, 100, 150 and 200 ppm.) which sprayed two times (at the vegetative stage or at vegetative plus flowering stages). The treatments arranged in a split- split plot design with three replicates, water stress was assigned at random in the main plots, while sub-plots were devoted to ascorbic acid concentrations and spraying time were allotted in the sub-sub plots. All pots were weighed daily and the needed amount of water was added. All plants received the soil moisture levels after three weeks from planting. The different concentrations of ascorbic acid were added for the first group at the vegetative stage only; the addition was twice, the first after 35 days from planting and the other two weeks later. While for the second group the addition of ascorbic acid was at vegetative plus flowering stages (four times) where the third addition was applied at the begging of flowering stage and the fourth two weeks later. The spraying process was foliar and always performed early in the morning; the plants were sprayed until run off.

The plants were harvested two times (first and second cuttings) by cutting plants 5 cm above the soil with three replications in each season, the first cut on the first week of July and the second cut on the first week of October. The growth parameters which recorded for each cut were plant height (cm), number of leaves/plant, number of branches/plant, leaf area (cm2) and fresh and dry weights (gm) of herb yield. The amount of chlorophyll (a, b, a+b and carotenoids) was determined according to Metzener et al., (1965).

The fresh plants were collected from each treatment during the first and second cuttings and weighed to extract the essential oil, the fresh plant material from each replicate of all treatments was subjected to steam distillation for 3h using petroleum ether, which was removed carefully and the essential oil was obtained according to (Guenther, 1961). Proline was determined in dry leaves in the first and second cuts using the method of Troll (1995). The relative water content was also measured according to Weatherly (1962). The averages of data from two seasons were tested by analysis of variance according to (Snedecor and Cochran 1980) and the means separations were compared by using Least Significant Difference (LSD) at 5% level.

Table (1): Mechanical and chemical analyses of the tested soil.

Mechanical characteristics	first season	second season
Clay %	17.00	16.00
Sand %	23.75	25.25
Chemical Properties		
PH (1:2.5)	7.25	7.9
E.C. (1:5)	1.1 dsm-1	1.0
Available macro nutrients (ppm)		
Na	3.22	5.01
N	169.10	172
P	3.04	4.00
K	242.25	244.15
Ca	62.15	65.21
Mg	63.18	65.22
Available micro nutrient (ppm)		
Fe	12.14	15.21
Mn	18.81	19.32
Zn	1.18	1.34
Cu	1.00	1.31
Cl	0.58	0.66
Texture Sandy Soil		

3. Results

Effect on growth criteria:

Data presented in Tables 2&3 revealed the effect of water stress, ascorbic acid concentrations, spraying time and their interactions on growth criteria of ocimum plants in both cuts. For the effect of water stress, the data of the first cut showed that all growth characters increased significantly so as to reach their maximum values under 50% soil moisture level followed by decrease under 30% depletion of the available soil moisture level. While for the second cut, the data showed progressive increase in growth criteria with increasing soil moisture level so as to reach their maximum values under 30% depletion of the available soil moisture level. Moreover, all growth criteria were significantly reduced under the highest stressed level (70% depletion of the available soil moisture level) in both cuts.

Data in the same tables revealed also that plants treated with 150 ppm ascorbic acid showed the highest significant increase in growth criteria of the first cut compared with control treatment, followed by 100 ppm where the difference between the two concentrations was insignificant in most cases. While in the second cut, the highest significant increase in growth criteria appeared in plants sprayed with 200 ppm ascorbic acid compared with control treatment, followed by 150 ppm where the difference between the two treatments was insignificant for plant height, number of branches and dry weight.

Spraying plants with ascorbic acid at the vegetative stage only showed the highest significant means of growth criteria in the first cut. While in the second cut, spraying plants with ascorbic acid at vegetative plus flowering stages revealed the highest significant means of growth criteria compared with the other treatment.

In addition, the data of interaction between water stress and ascorbic acid concentrations indicated that the highest significant increase in growth criteria of the first cut observed under 50% soil moisture level interacted with 150 ppm ascorbic acid, followed by 100 ppm under same moisture level where the difference between the two treatments was insignificant, except for number of branches/plant which revealed insignificant increase. While in the second cut, the highest significant means observed under 30% depletion of the available soil moisture level interacted with 200 ppm ascorbic acid.

The data of interaction between water stress and spraying time showed that plants grown under 50% soil moisture level and sprayed with ascorbic acid at the vegetative stage only revealed the highest significant means in the first cut. While, plants grown under 30% depletion of the available soil moisture level and sprayed with ascorbic acid at vegetative plus flowering stages showed the highest significant means in the second cut.

Also, the data of interaction between ascorbic acid concentrations and spraying time revealed in the first cut that the highest significant means in number of branches/plant, fresh and dry weights reached when plants sprayed with 100 ppm ascorbic acid twice at the vegetative stage only, while for plant height, number of leaves/plant and leaf area the highest significant increases observed when plants sprayed with 150 ppm ascorbic acid twice at the vegetative stage only. For the second cut, spraying plants twice with 200 ppm ascorbic acid at vegetative plus twice at flowering stage showed the highest significant means of growth criteria compared with the other treatments.

The interaction between the three studied factors showed that the highest significant means in growth criteria of the first cut observed when plants grown under 50% soil moisture level and sprayed twice with 150 ppm ascorbic acid at the vegetative stage only. While for the second cut, the highest significant means observed in plants grown under 30% depletion of the available soil moisture level and sprayed twice with 200 ppm ascorbic acid at vegetative plus twice at flowering stage.

Table (2): Effect of water stress, ascorbic acid concentrations, spraying time and their interactions on growth criteria of Ocimum basilicum plant in the first cut (combined analysis of two seasons)

Charact. / Treatments	Plant height (cm)	No of leaves /plant	No of branches /plant	Fresh weight /plant (g)	Dry weight /plant (g)	Leaf area (cm2)
Water stress						
70%	37.58	71.75	2.46	28.63	7.26	1.06
50%	44.38	83.50	3.38	40.08	10.78	1.44
30%	42.42	79.33	3.08	39.58	10.72	1.40
LSD0.05	1.46	1.37	0.73	2.47	0.66	0.18
Ascorbic acid concentrations						
0	35.83	60.28	2.17	26.39	6.66	1.05
100ppm	42.44	88.56	3.44	40.93	11.07	1.32
150ppm	45.61	92.28	3.56	41.12	11.13	1.49
200ppm	41.44	71.67	2.72	35.96	9.49	1.34
LSD0.05	1.36	4.20	0.71	2.73	0.74	0.18
Spraying time						

Vegetative		43.94	84.08	3.19	39.22	10.37	1.62
Vegetative+Flowering		38.72	72.31	2.75	32.98	8.80	0.99
LSD0.05		0.61	2.10	0.23	1.48	0.41	0.08
W.S	ASC	Water stress X Ascorbic acid con.					
70%	0	29.50	56.17	2.00	20.94	5.09	0.92
	100	33.50	79.50	2.33	33.71	8.75	0.91
	150	44.17	95.17	3.00	31.47	8.06	1.24
	200	41.67	56.17	2.50	28.41	7.14	1.18
50%	0	40.33	59.83	2.00	23.38	5.78	1.09
	100	47.33	98.50	4.17	48.54	13.63	1.61
	150	47.67	99.67	4.83	49.28	13.88	1.72
	200	42.17	76.00	2.50	37.14	9.88	1.36
30%	0	37.67	64.83	2.50	34.84	9.10	1.15
	100	46.50	86.50	3.50	41.12	11.00	1.33
	150	45.00	83.17	3.17	42.03	11.32	1.61
	200	40.50	82.83	3.17	42.34	11.45	1.49
LSD0.05		2.36	7.27	N.S	4.73	1.28	0.32
W.S	Spraying time	Water stress X Spraying time					
70%	Vegetative	39.42	72.17	2.58	30.56	7.62	1.41
	Veg.+Flow.	35.00	71.33	2.33	26.71	6.90	0.72
50%	Vegetative	46.42	95.17	3.83	46.00	12.62	1.79
	Veg.+Flow.	42.33	71.83	2.92	33.16	8.95	1.10
30%	Vegetative	46.00	85.75	3.17	41.10	10.87	1.65
	Veg.+Flow.	38.83	72.92	3.00	39.07	10.57	1.14
LSD0.05		1.06	3.63	0.40	2.57	0.70	0.14

Cont. Table 2.

Charact. / Treatments			Plant height (cm)	No of leaves /plant	No of branches /plant	Fresh weight /plant (g)	Dry weight /plant (g)	Leaf area (cm2)
ASC	Spraying time		Ascorbic acid conc. X Spraying time					
0	0		35.84	60.28	2.17	26.39	6.66	1.06
100	Vegetative		44.00	97.22	4.22	45.25	12.27	1.64
	Veg.+Flow.		40.89	79.22	2.89	36.99	9.99	1.00
150	Vegetative		47.33	101.22	3.89	44.14	11.63	1.82
	Veg.+Flow.		43.89	83.33	3.00	37.71	10.52	1.16
200	Vegetative		45.67	81.33	2.56	40.28	10.65	1.66
	Veg.+Flow.		37.22	62.00	2.89	31.64	8.33	1.02
LSD0.05			1.23	4.19	0.46	2.97	0.81	0.17
W.S	ASC	Spraying time	Water stress X Ascorbic acid con.X Spraying time					
70%	0	0	29.5	56.17	2.00	20.95	5.09	0.92
	100	Vegetative	34.33	83.67	2.33	37.56	9.65	1.24
		Veg.+Flow	32.67	75.33	2.33	29.86	7.85	0.59
	150	Vegetative	45.67	102.33	2.67	34.40	8.52	1.60
		Veg.+Flow	42.67	88.00	2.33	28.55	7.59	0.88
	200	Vegetative	46.00	57.33	2.67	29.27	7.26	1.44
		Veg.+Flow	37.33	55.00	2.33	27.55	7.03	0.91
50%	0	Vegetative	44.00	63.67	2.00	25.15	6.16	1.35
		Veg.+Flow	36.67	56.00	2.00	21.61	5.39	0.84
	100	Vegetative	48.33	112.33	4.67	53.53	14.86	2.01
		Veg.+Flow	47.67	84.33	3.33	40.42	11.11	1.35
	150	Vegetative	50.00	115.00	6.33	56.66	16.15	2.08
		Veg.+Flow	47.00	84.67	3.67	45.02	12.83	1.20
	200	Vegetative	45.67	97.33	2.67	48.68	13.30	1.71
		Veg.+Flow	38.67	54.67	2.33	25.59	6.45	1.00
30%	0	Vegetative	40.67	66.33	2.67	35.45	9.57	1.33
		Veg.+Flow	34.67	63.33	2.33	34.23	8.64	0.97
	100	Vegetative	47.00	101.33	4.00	41.53	11.00	1.62
		Veg.+Flow	43.00	71.67	3.00	40.71	11.00	1.04
	150	Vegetative	48.00	89.00	3.67	44.49	11.50	1.84
		Veg.+Flow	42.00	77.33	2.67	39.56	11.13	1.38
	200	Vegetative	45.33	89.33	3.67	42.91	11.51	1.83
		Veg.+Flow	35.67	76.33	2.67	41.78	11.39	1.15
LSD0.05			2.13	7.26	0.79	5.14	1.41	0.29

Table 3: Effect of water stress, ascorbic acid concentrations, spraying time and their interactions on growth criteria of *Ocimum basilicum* plant in the second cut (combined analysis of two seasons)

Charact. / Treatments		Plant height (cm)	No of leaves /plant	No of branches /plant	Fresh weight /plant (g)	Dry weight /plant (g)	Leaf area (cm2)
Water stress							
70%		37.19	218.12	7.04	68.82	13.97	3.57
50%		53.37	382.08	9.50	92.75	21.81	4.77
30%		53.65	465.25	10.79	101.57	25.32	5.16
LSD0.05		0.59	22.56	1.50	12.04	2.27	0.19
Ascorbic acid concentrations							
0		41.87	274.83	6.78	73.72	16.04	3.96
100ppm		48.44	358.33	9.00	88.09	20.21	4.42
150ppm		50.98	384.00	9.94	92.46	22.29	4.60
200ppm		50.99	403.83	10.72	96.57	22.91	5.02
LSD0.05		2.18	19.63	0.81	3.61	1.63	0.13
Spraying time							
vegetative		42.21	306.56	7.61	73.35	15.51	4.30
veg.+lowering		43.93	403.94	10.61	102.07	25.22	4.70
LSD0.05		1.23	14.59	0.75	2.80	1.21	0.09
W.S	ASC	Water stress X Ascorbic acid conc.					
70%	0	31.25	189.83	5.17	61.41	11.98	3.34
	100	39.15	206.50	7.00	66.70	13.62	3.57
	150	40.48	222.17	7.67	68.10	12.47	3.69
	200	37.87	255.17	8.33	79.07	17.80	3.69
50%	0	50.33	303.00	7.33	76.33	17.15	4.14
	100	51.25	324.17	9.67	90.43	20.99	4.64
	150	54.27	351.33	9.00	88.91	20.75	4.44
	200	58.23	549.83	12.00	115.31	28.34	5.66
30%	0	44.02	331.67	7.83	83.42	18.99	4.40
	100	54.92	544.33	10.33	107.14	26.03	5.06
	150	56.30	406.50	11.83	95.33	22.61	5.54
	200	58.77	578.50	13.17	120.38	33.65	5.84
LSD0.05		3.79	34.00	1.40	6.25	2.83	0.22
W.S	Spraying time	Water stress X Spraying time					
70%	vegetative	33.80	202.00	5.75	49.11	9.55	3.49
	veg.+Flowering	40.58	234.83	8.33	88.53	18.38	3.66
50%	vegetative	49.22	351.00	8.25	83.94	18.07	4.51
	veg.+Flowering	57.98	413.17	10.75	101.55	25.55	5.02
30%	vegetative	48.76	366.67	8.83	87.01	18.90	4.91
	veg.+Flowering	58.09	563.83	12.75	116.13	31.73	5.42
LSD0.05		2.13	25.27	1.29	4.86	2.09	0.16

Cont. Table 3.

Charact. / Treatments			Plant height (cm)	No of leaves /plant	No of branches /plant	Fresh weight /plant (g)	Dry weight /plant (g)	Leaf area (cm2)
ASC	Spraying time		Ascorbic acid conc.X Spraying time					
0	0		41.87	274.84	6.78	73.77	16.04	3.96
100	vegetative		44.88	299.33	7.56	75.43	15.96	4.26
	veg.+Flowering		52.00	417.33	10.44	100.74	24.46	4.59
150	vegetative		43.67	313.89	7.89	74.00	15.76	4.48
	veg.+Flowering		54.09	434.22	12.00	109.40	27.65	4.78
200	vegetative		47.87	373.44	9.22	83.74	18.18	4.72
	veg.+Flowering		58.32	454.11	12.22	110.93	28.81	5.27
LSD0.05			2.46	29.17	1.50	5.61	2.42	0.19
W.S	ASC	Spraying time	Water stress X Ascorbic acid conc. X Spraying time					
70%	0	0	31.25	189.835	5.17	61.41	11.99	3.34
	100	vegetative	36.30	195.00	6.33	47.46	9.06	3.53
		veg.+Flowering	42.00	218.00	7.67	85.93	18.17	3.61
	150	vegetative	28.67	201.00	5.67	50.23	9.84	3.51
		veg.+Flowering	52.30	243.33	9.67	85.97	15.09	3.88
	200	vegetative	40.73	232.33	7.00	58.21	11.61	3.68
		veg.+Flowering	35.00	278.00	9.67	99.93	23.98	3.71
	0	vegetative	47.67	255.33	6.67	65.75	13.35	3.99
		veg.+Flowering	53.00	350.67	8.00	86.90	20.94	4.30

50%	100	vegetative veg.+Flowering	48.00 54.50	292.67 355.67	8.00 11.33	87.59 93.57	18.74 23.23	4.35 4.93
	150	vegetative veg.+Flowering	49.67 58.87	298.00 404.67	7.00 11.00	78.36 99.47	16.53 24.94	4.15 4.73
	200	vegetative veg.+Flowering	51.53 63.80	558.00 541.67	11.33 12.67	104.06 126.57	23.64 33.04	5.54 5.54
30%	0	vegetative veg.+Flowering	40.70 47.33	283.67 379.67	6.67 9.00	74.44 92.40	15.34 22.63	3.86 4.93
	100	vegetative veg.+Flowering	50.33 59.50	410.33 678.33	8.33 12.33	91.24 123.03	20.07 31.98	4.90 5.22
	150	vegetative veg.+Flowering	52.67 61.27	442.67 483.00	11.00 14.33	93.40 101.70	20.92 25.93	5.78 5.97
	200	vegetative veg.+Flowering	51.33 66.00	330.00 714.33	9.33 15.33	88.95 147.37	19.28 46.37	5.11 6.14
LSD0.05			4.26	50.53	2.59	9.71	4.19	0.33

2- Effect on some physiological process: Photosynthetic pigments content:-

Data presented in Tables 4 & 5 showed the effect of water stress, ascorbic acid concentrations, spraying time and their interactions on photosynthetic pigments content of basil leaves. The obtained data revealed that the concentration of photosynthetic pigments i.e. chla, chlb and total chl (a+b) as well as carotenoids was higher in plants grown under 50% soil moisture level and with significant difference in both cuts.

The data in same tables also indicated that plants sprayed with ascorbic acid showed significant increases in photosynthetic pigments content compared with control plants. Where the highest records in photosynthetic pigments content of the first cut observed under 150 ppm ascorbic acid and 200 ppm in the second cut compared with control plants.

It could be also noticed that spraying plants with ascorbic acid at the vegetative stage only during plant life revealed the highest significant increases in chla, chlb, chl (a+b) as well as carotenoids of the first cut. Furthermore, spraying plants with ascorbic acid at vegetative plus flowering stages during plant life showed the highest significant increases in photosynthetic pigments of the second cut.

Concerning the effect of interaction between water stress and ascorbic acid concentrations, the data demonstrated that plants grown under 50% soil moisture level and sprayed with 150 ppm ascorbic acid showed the highest significant increases in photosynthetic pigments of the first cut and 50% soil moisture level interacted with 200 ppm ascorbic acid for the second cut.

Also, the data of interaction between water stress and spraying time showed that the highest significant increases in photosynthetic pigments recorded in plants grown under 50% soil moisture level in both cuts, spayed with ascorbic acid at the vegetative stage only for the first cut and sprayed with ascorbic acid at vegetative plus flowering stages for the second cut.

Irrespective to water stress, the data revealed that the highest significant increases in photosynthetic pigments obtained in plants sprayed with 150 ppm ascorbic acid twice at the vegetative stage only for the first cut. While for the second cut, the highest significant increases in photosynthetic pigments obtained in plants prayed with 200 ppm twice at vegetative plus twice at flowering stage.

The data of tri-interaction indicated that the best treatment for the first cut observed when plants grown under 50% soil moisture level and sprayed twice with 150 ppm ascorbic acid at the vegetative stage only and with significant difference compared with the other treatments. While, the highest significant records in photosynthetic pigments of the second cut obtained when plants grown under 50% soil moisture level and sprayed with 200 ppm ascorbic acid twice at vegetative plus twice at flowering stage.

Relative Water Content (RWC %):

Data in Tables 4 & 5 also revealed the effect of water stress, ascorbic acid concentrations, spraying time and their interactions on RWC % of basil leaves. The obtained data revealed that the highest records for the RWC % observed under 50% depletion of the available soil moisture in the first cut and 30% depletion of the available soil moisture for the second cut.

The obtained data also indicated that all ascorbic acid concentrations showed significant increase in RWC % compared with untreated plants of both cuts. Where the highest significant increase in RWC % obtained in plants sprayed with 150 ppm ascorbic acid in the first cut and 200 ppm for the second cut.

For the effect of spraying time, the data revealed that spraying plants with ascorbic acid at the vegetative stage showed the highest significant means in RWC % of the first cut compared with the other treatment, while for the second cut, spraying plants with

ascorbic acid at vegetative plus flowering stages revealed the highest means in RWC%.

Regarding the effect of interaction between water stress and ascorbic acid concentrations, the data illustrated that the highest significant means of RWC% obtained under 50% soil moisture level combined with 150 ppm ascorbic acid in the first cut, and 30% depletion of the available soil moisture combined with 200 ppm ascorbic acid for the second cut. It was also clear from data that spraying plants with different concentrations of ascorbic acid caused increase in RWC % under different soil moisture levels and with significant difference.

The data of interaction between water stress and spraying time revealed that plants grown under 50% soil moisture level and sprayed with ascorbic acid at the vegetative stage only showed the highest significant means of RWC % in the first cut and under 30% depletion of the available soil moisture combined with two sprays of ascorbic acid at the vegetative plus two at flowering stage in the second cut.

Irrespective to water stress, the data illustrated that spraying plant twice with 150 ppm ascorbic acid at the vegetative stage proved to be effective in increasing RWC % significantly in the first cut. While for the second cut, spraying plants with 200 ppm twice at vegetative and twice at flowering stage revealed the highest significant means of RWC % in the second cut.

The combined effect between the three studied factors showed that the highest significant values of RWC% attained when plants grown under 50% soil moisture level and sprayed twice with 150 ppm ascorbic acid at the vegetative stage of the first cut. While for the second cut, the highest significant means observed in plants grown under 30% depletion of the available soil moisture level and sprayed twice with 200 ppm ascorbic acid at the vegetative and flowering stages.

Proline content:-

Examination of data in Tables 4 & 5 showed that increasing water stress level caused progressive and significant increase in proline content of basil leaves. Moreover, the highest significant increase in proline accumulation in both cuts obtained under 70% depletion of the available soil moisture level, while the lowest accumulation in both cuts observed under 30% depletion of the available soil moisture level combined with 150 ppm.

Treated basil plants with different concentrations of ascorbic acid revealed significant decrease in proline accumulation compared with untreated plants. For the first cut, the lowest means obtained under 150 ppm ascorbic acid and the highest means observed under the control treatment. While for the second cut, the difference between the different concentrations of ascorbic acid was insignificant while the highest means observed under control plants also.

Spraying plants with ascorbic acid at the vegetative stage only revealed the lower means in proline accumulation in both cuts, compared with the other treatment.

Concerning the bi-interaction between water stress and ascorbic acid concentrations, the obtained data revealed that the different concentrations of ascorbic acid caused significant decrease in proline accumulation under different water stress levels compared with control plants. Furthermore, the highest significant increase in proline accumulation obtained in control treatment of 70% depletion of the available soil moisture level, while the lowest accumulation obtained under 30% depletion of the available soil moisture level, these results were true for both cuts.

The data of interaction between water stress and spraying time also proved that plants sprayed with ascorbic acid at the vegetative stage only revealed the lowest means in proline accumulation under different soil moisture levels compared with the other treatments, this result was true for both cuts.

It could be also observed from the data of interaction between ascorbic acid concentration and spraying time that the highest accumulation of proline obtained in untreated plants (control plants) in both cuts, while the lowest accumulation obtained when plants treated with different concentrations of ascorbic acid at the vegetative stage only.

The effect of tri-interaction illustrated that the lowest significant means in proline accumulation obtained when plants grown under 30% depletion of the available soil moisture level and sprayed twice with 150 ppm ascorbic acid at the vegetative stage only in both cuts, while the highest means obtained in control plants of 70% depletion of the available soil moisture level.

Oil percent:

The results in Tables 4 & 5 showed also the effect of water stress, ascorbic acid concentrations, spraying time and their interactions on oil % of basil leaves. The data of both cuts indicated that water stress induced significant and progressive increase in oil %, where the highest significant increase in oil % of both cuts obtained in plants grown under 70 % depletion of the available soil moisture level.

Ascorbic acid treatments caused significant increase in oil % compared with untreated plants in both cuts, where 150 ppm ascorbic acid proved to be the most effective concentrations that affected oil % significantly in both cuts.

A significant increase was also recorded when plants treated with ascorbic acid twice at vegetative and flowering stages in both cuts.

In addition, the interaction between water stress and ascorbic acid concentrations indicated that oil % increased significantly with increasing stress levels, also spraying plants with ascorbic acid induced significant increase in oil % under different soil moisture levels, where the highest significant increase in oil % appeared in plants grown under 70% depletion of the available soil moisture and sprayed with 150 ppm ascorbic acid in both cuts.

For the interaction between water stress and spraying time the data in both cuts illustrated that the highest significant means of oil % obtained in plants grown under 70% depletion of the available soil moisture level and sprayed with ascorbic acid twice at vegetative and twice at flowering stage.

Moreover, the data of interaction between ascorbic acid concentrations and spraying time showed that the highest significant means of oil % in both cuts appeared when plants sprayed twice (at vegetative and flowering stages) with 150 ppm.

The effect of tri-interaction indicated that 70% depletion of the available soil moisture level interacted with 150 ppm ascorbic acid when sprayed twice at both vegetative and flowering stages proved to be the most effective treatment in oil % compared with the other treatments and with significant difference, this result was true for both cuts.

Table (4): Effect of water stress, ascorbic acid concentrations, spraying time and their interactions on some physiological process of *Ocimum basilicum* plant in the first cut (combined analysis of two seasons).

Charact. / Treatments		Photosynthetic pigments				RWC %	Proline content	Oil %
		Chl.a	Chl.b	Chl.a+b	Carot.			
Water stress								
70%		1.61	1.57	3.18	0.49	55.29	0.18	0.22
50%		2.58	2.31	4.89	1.53	67.95	0.16	0.13
30%		2.15	1.95	4.10	0.99	63.07	0.13	0.07
$LSD_{0.05}$		0.05	0.11	0.16	0.04	1.76	0.04	0.003
Ascorbic acid concentrations								
0		1.41	1.59	3.00	0.73	58.40	0.18	0.08
100ppm		2.52	2.04	4.55	1.13	65.46	0.15	0.16
150ppm		2.63	2.14	4.77	1.25	66.79	0.14	0.20
200ppm		1.90	2.02	3.92	0.91	61.17	0.15	0.12
$LSD_{0.05}$		0.03	0.07	0.11	0.03	1.01	0.03	0.002
Spraying time								
Vegetative stage		2.22	2.17	4.39	1.03	64.29	0.15	0.13
Veg.+flowering stages		2.01	1.72	3.73	0.98	61.81	0.16	0.15
$LSD_{0.05}$		0.02	0.05	0.08	0.01	0.49	0.01	0.001
W.S	ASC	Water stress X ascorbic acid con.						
70%	0	1.33	1.25	2.58	0.36	52.91	0.21	0.11
	100	1.77	1.62	3.39	0.50	54.67	0.18	0.25
	150	2.01	1.71	3.71	0.69	56.46	0.16	0.32
	200	1.34	1.71	3.05	0.42	57.12	0.16	0.21
50%	0	1.58	2.02	3.60	1.01	61.48	0.17	0.07
	100	3.05	2.39	5.44	1.88	71.94	0.15	0.15
	150	3.32	2.43	5.75	1.91	73.57	0.15	0.21
	200	2.37	2.40	4.77	1.35	64.80	0.15	0.08
30%	0	1.33	1.50	2.83	0.82	59.06	0.15	0.06
	100	2.46	2.00	4.47	1.00	67.31	0.12	0.08
	150	2.83	2.01	4.84	1.18	70.34	0.11	0.09
	200	2.00	2.30	4.29	0.95	65.81	0.13	0.06
$LSD_{0.05}$		0.05	0.13	0.20	0.05	1.75	0.05	0.003
W.S	Spraying time	Water stress X spraying time						
70%	vegetative	1.72	1.63	3.35	0.63	57.37	0.17	0.21
	veg.+flow.	1.50	1.52	3.02	0.36	53.22	0.18	0.24
50%	vegetative	2.86	2.70	5.56	1.77	71.65	0.14	0.10
	veg.+flow.	2.29	1.91	4.20	1.29	64.25	0.17	0.15
30%	vegetative	2.31	2.18	4.49	1.29	68.02	0.13	0.07
	veg.+flow.	2.00	1.72	3.72	0.69	63.25	0.13	0.08
$LSD_{0.05}$		0.04	0.09	0.14	0.07	0.85	0.07	0.004

Cont. Table (4).

Charact. / Treatments		Photosynthetic pigments				RWC %	Proline content	Oil %
		Chl.a	Chl.b	Chl.a+b	Carot.			
ASC	Spraying time	Ascorbic acid con.X Spraying time						
0	0	1.41	1.59	3.00	0.73	58.41	0.18	0.08
100	vegetative	2.61	2.05	4.66	1.34	65.60	0.13	0.16
	veg.+flow.	1.95	1.98	3.93	1.16	64.75	0.17	0.17

150	vegetative		3.31	2.46	5.77	1.36	67.98	0.13	0.16
	veg.+flow.		2.42	1.65	4.07	0.91	66.17	0.15	0.24
200	vegetative		1.95	1.81	3.76	1.02	62.31	0.15	0.09
	veg.+flow.		1.85	2.42	4.27	0.79	60.04	0.15	0.14
$LSD_{0.05}$			0.04	0.11	0.16	0.06	0.99	0.06	0.006
W.S	ASC	Spraying time	Water stress X Ascorbic acid con. X Spraying time						
70%	0	0	1.33	1.26	2.59	0.37	54.67	0.22	0.12
	100	vegetative	2.31	1.71	4.02	0.77	58.25	0.16	0.24
		veg.+flow.	1.72	1.53	3.25	0.62	54.68	0.20	0.26
	150	vegetative	2.22	1.95	4.17	0.77	58.98	0.15	0.24
		veg.+flow.	1.31	1.48	2.79	0.22	55.27	0.16	0.40
	200	vegetative	1.48	1.72	3.20	0.60	53.79	0.17	0.18
		veg.+flow.	1.21	1.70	2.90	0.24	52.03	0.15	0.24
50%	0	vegetative	1.88	2.48	4.36	1.12	70.63	0.15	0.06
		veg.+flow.	1.28	1.56	2.83	0.86	52.33	0.20	0.07
	100	vegetative	3.85	2.62	6.47	2.03	72.23	0.13	0.16
		veg.+flow.	2.25	2.24	4.49	1.72	71.04	0.18	0.18
	150	vegetative	3.92	2.89	6.81	2.83	75.73	0.15	0.11
		veg.+flow.	2.71	1.95	4.66	0.98	71.40	0.14	0.25
	200	vegetative	2.40	2.83	5.22	1.58	68.57	0.13	0.05
		veg.+flow.	2.33	1.92	4.25	1.11	61.04	0.17	0.11
30%	0	vegetative	1.48	1.68	3.16	0.86	64.88	0.16	0.05
		veg.+flow.	1.18	1.31	2.49	0.78	53.24	0.13	0.06
	100	vegetative	3.78	2.20	5.98	1.53	67.32	0.11	0.06
		veg.+flow.	1.88	1.80	3.68	0.83	67.30	0.12	0.10
	150	vegetative	2.89	2.49	5.38	1.53	73.54	0.10	0.07
		veg.+flow.	2.03	1.53	3.56	0.46	67.15	0.13	0.10
	200	vegetative	2.75	2.76	5.51	1.24	66.33	0.14	0.05
		veg.+flow.	2.24	1.83	4.07	0.66	65.29	0.13	0.07
$LSD_{0.05}$			0.08	0.19	0.27	0.09	1.71	0.09	0.008

Table (5): Effect of water stress, ascorbic acid concentrations, spraying time and their interactions on some physiological process of *Ocimum basilicum* plant in the second cut (combined analysis of two seasons).

Charact. / Treatments		Photosynthetic pigments				RWC %	Proline content	Oil %
		Chl.a	Chl.b	Chl.a+b	Carot.			
Water stress								
70%		0.50	0.29	0.79	0.29	58.73	0.17	0.26
50%		0.96	0.53	1.49	0.49	67.64	0.12	0.16
30%		0.67	0.38	1.05	0.38	70.23	0.12	0.11
$LSD_{0.05}$		0.03	0.10	0.13	0.04	1.51	0.03	0.04
Ascorbic acid concentrations								
0		0.63	0.34	0.97	0.35	53.04	0.16	0.13
100ppm		0.70	0.42	1.12	0.39	67.82	0.13	0.19
150ppm		0.73	0.40	1.13	0.40	70.18	0.13	0.21
200ppm		0.78	0.44	1.22	0.41	71.08	0.13	0.18
$LSD_{0.05}$		0.02	0.07	0.11	0.03	0.97	0.02	0.03
Spraying time								
Vegetative stage		0.43	0.25	0.68	0.27	64.67	0.11	0.15
Veg.+flowering stages		0.99	0.55	1.52	0.50	66.40	0.17	0.20
$LSD_{0.05}$		0.02	0.07	0.07	0.01	0.65	0.01	0.01
W.S	ASC	Water stress X Ascorbic acid con.						
70%	0	0.45	0.32	0.78	0.27	47.11	0.19	0.22
	100	0.47	0.31	0.78	0.29	67.03	0.16	0.27
	150	0.49	0.20	0.69	0.28	60.35	0.15	0.30
	200	0.60	0.33	0.93	0.32	60.43	0.16	0.24
50%	0	0.85	0.48	1.33	0.50	56.37	0.15	0.10
	100	0.94	0.55	1.49	0.44	66.49	0.12	0.19
	150	1.00	0.54	1.54	0.50	73.70	0.12	0.21
	200	1.06	0.55	1.61	0.51	73.99	0.12	0.16
30%	0	0.60	0.21	0.81	0.29	55.64	0.14	0.07
	100	0.69	0.42	1.11	0.40	69.95	0.11	0.14
	150	0.68	0.44	1.12	0.42	76.40	0.11	0.14
	200	0.71	0.45	1.15	0.43	78.91	0.13	0.10
$LSD_{0.05}$		0.04	0.13	0.20	0.05	1.68	0.03	0.05
W.S	Spraying time	Water stress X Spraying time						
70%	Vegetative	0.27	0.20	0.47	0.16	55.44	0.11	0.22
	Veg.+flow.	0.73	0.38	1.11	0.42	62.02	0.22	0.29

50%	Vegetative	0.65	0.35	1.00	0.35	65.84	0.10	0.14
	Veg.+flow.	1.25	0.71	1.96	0.62	69.44	0.14	0.19
30%	Vegetative	0.37	0.19	0.56	0.30	69.13	0.10	0.09
	Veg.+flow.	0.97	0.57	1.45	0.47	71.33	0.14	0.12
$LSD_{0.05}$		0.03	0.13	0.13	0.07	1.12	0.04	0.07

Cont. Table (5).

Treatments \ Charact.			Photosynthetic pigments				RWC %	Proline content	Oil %
			Chl.a	Chl.b	Chl.a+b	Carot.			
ASC	Spraying time		Ascorbic acid X Spraying time						
0	0		0.63	0.34	0.97	0.35	54.04	0.16	0.13
100	Vegetative		0.43	0.20	0.63	0.30	67.78	0.10	0.17
	Veg+Flow.		0.97	0.60	1.57	0.52	67.87	0.15	0.21
150	Vegetative		0.51	0.26	0.77	0.26	68.65	0.10	0.16
	Veg+Flow.		0.94	0.54	1.48	0.31	71.59	0.15	0.24
200	Vegetative		0.41	0.27	0.69	0.46	70.58	0.10	0.15
	Veg+Flow.		1.15	0.65	1.75	0.54	71.71	0.16	0.21
$LSD_{0.05}$			0.03	0.15	0.15	0.06	1.30	0.03	0.06
W.S	ASC	Spraying time	Water stress X Ascorbic acid X Spraying time						
70%	0	0	0.46	0.32	0.78	0.27	47.11	0.22	0.22
	100	Vegetative	0.24	0.10	0.34	0.16	66.34	0.10	0.23
		Veg.+flow.	0.70	0.51	1.21	0.42	67.72	0.19	0.30
	150	Vegetative	0.31	0.16	0.47	0.10	59.71	0.12	0.26
		Veg.+flow.	0.66	0.24	0.90	0.45	61.15	0.19	0.33
	200	Vegetative	0.28	0.15	0.44	0.25	54.69	0.11	0.20
		Veg.+flow.	0.91	0.51	1.43	0.39	66.00	0.21	0.29
50%	0	Vegetative	0.55	0.27	0.82	0.35	53.16	0.11	0.07
		Veg.+flow.	1.14	0.69	1.84	0.64	59.57	0.18	0.13
	100	Vegetative	0.62	0.31	0.93	0.37	65.26	0.11	0.16
		Veg.+flow.	1.26	0.74	2.00	0.64	67.72	0.13	0.21
	150	Vegetative	0.78	0.34	1.12	0.33	68.88	0.10	0.17
		Veg.+flow.	1.21	0.63	1.95	0.35	78.34	0.14	0.24
	200	Vegetative	0.63	0.46	1.09	0.52	69.65	0.10	0.14
		Veg.+flow.	1.49	0.78	2.27	0.67	78.53	0.13	0.19
30%	0	Vegetative	0.28	0.10	0.38	0.14	53.03	0.11	0.06
		Veg.+flow.	0.91	0.32	1.24	0.43	58.25	0.18	0.08
	100	Vegetative	0.42	0.19	0.61	0.38	67.89	0.10	0.10
		Veg.+flow.	0.96	0.64	1.60	0.49	72.01	0.12	0.17
	150	Vegetative	0.45	0.27	0.72	0.34	75.93	0.09	0.09
		Veg.+flow.	0.96	0.62	1.59	0.49	76.88	0.12	0.10
	200	Vegetative	0.32	0.21	0.53	0.32	78.71	0.10	0.12
		Veg.+flow.	1.04	0.67	1.71	0.47	79.12	0.15	0.15
$LSD_{0.05}$			0.05	0.26	0.31	0.09	1.30	0.08	0.09

4. Discussions

From the results, it was clear that the highest water stress level (70% depletion of the available soil moisture level) caused an observed adverse action on growth characters, fresh and dry weights, relative water content % as well as photosynthetic pigments of basil plants in both cuts. Previous results were supported by Fatima *et al.*, (1999); Mirsa and Strivastava (2000); Khalid (2006) and Tawfik (2008). This result could be due to that one of the first signs of water shortage was the decrease of turgor which resulted in decrease in growth and development of cell especially in stem and leaves (Alishah *et al.*, 2006). When the leave level decreased the plant lose less water through transpiration so the restriction of leaves level could be the first mechanism against drought (Levitt, 1980). Farooqi *et al.*, (1998) and Fatima *et al.*, (1999) supported previous results. Moreover, when the leaf level decrease the light attraction decrease and the total capacity of photosynthesis decrease so plant growth became less and plant performance decrease (Hsiao, 1973), which leads also to the decrease in dry matter production (Cox and Joliff, 1987), this result agrees with Fatima *et al.* (1999); khalid (2006) and Alishah *et al.*,(2006). Drought stress made chloroplast break down and the amount of chlorophyll decrease, therefore formation of chlorophyll a, b and carotenoids decrease. Our finding was in harmony with Cox and Jolliff (1987); Begum and Apaul (1993); Sepehri and Modarres (2003) and Alishah *et al.*, (2006). Furthermore, proline content showed significant increase with increasing water stress level and these results agree with Blum and Ebercon (1976) who indicated that proline is regarded as a source of energy, carbon, and nitrogen for recovering tissue, so it increased under water shortage, Aspinal and Paleg (1981) stated that under water deficit condition the concentration of amino acid proline increase. Since

chlorophyll and proline are both synthesis from the same substance therefore the increase in synthesis of proline leads to the decrease in synthesis of chlorophyll under drought conditions. Bajji *et al*, (2001); Begum and Paul (1993); Irigoyen *et al.,* (1992); khalid (2006) and Tawfik (2008) reached the same conclusions. Moreover, the essential oil percentage was increased significantly with increasing water stress level; these results were in line with those of Sabih *et al*., (1999); Baher *et al*., (2002) and Khalid (2006). Our results also indicated difference between results of two cuts, since the results of the first cut revealed that 50% soil moisture level showed the highest records in growth parameters, fresh and dry weights, RWC % and photosynthetic pigments which may results from increasing total capacity of photosynthesis as result of increasing photosynthetic pigments under this soil moisture level. While in the second cut, the data revealed progressive decrease in previously mentioned characters with increasing stress levels (except for photosynthetic pigments) which may due to that plants in the second cut exposed to higher temperature degrees than plants in the first cut because of summer season which revealed increase in the amounts of water that loss through transpiration and evapotranspiration and increasing needs for more water.

Water stress causes various physiological and biological changes in plants, one of which is the accumulation of reactive oxygen species in the cell, the reactive oxygen radicals are toxic and may result in a series of injuries to plant metabolism, it damages photosynthetic components, inactivates protein and enzymes, destroys cell membrane structure and permeability by causing lipid peroxidation, also excess accumulation of reactive oxygen species results in a series of oxidative injuries to plant prolines, polysacchorides and nucleic acids (Price and Henry, 1987, 1989, 1991, Winston, 1990), as a result normal cell metabolism can be seriously disturbed. The results of the present study indicated that ascorbic acid reduced the harmful effects of reactive oxygen species and improved plant resistance to water stress. In brief, ascorbic acid treatment reduced the damaging action of drought and decreased enzyme activity due to scavenging of reactive oxygen species; thereupon it may be effective for improvement of stressed plants in arid and semi-arid regions (Dolatabadian *et al*, 2009).

References

1. Abou–Leia, B.H., M.S. Aly, and N.F. Abdel-Hadey, 1994. Effect of foliar application of GA and Zn on Ocimum basilicum L. grown in different soil types. Egypt. J. Physiol. Sci. 18, No. 2, pp. 365-380.
2. Alishah, H.M., R.Heidari, A. Hassani, and A.Dizaji, 2006. Effect of water stress on some morphological and biochemical characteristics of purple Basil (Ocimum basilicum).Jornal of Biological Sciences 6(4):763-767.
3. Ashraf, M. and M.R. Foolad, 2007. Roles of glycine betaine and proline in improving plant a biotic stress resistance. Environ. and Exp. Botany, 59: 206-216.
4. Aspinal, D. and L.G. Paleg, 1981. Proline accumulation In: Physiology and biochemistry of drought resistance in plants: (Eds.), Academie press, New York. Physiol – Aspects. pp: 205-240.
5. Baeck, H., P.Kuenwoo, H.W.Baeck, and K.W.park, 2001. Effect of watering on growth and oil content of basil (ocimum americanu L.l). Korean J. Hort. Sci. Tech., 19, 1, 81-86.
6. Baher Z.F., M. Mirza, M.Ghorbanil, and M.Z. Rezail, 2002. The influence of water stress on plant height, herbal and essential oil yield and composition in Satucja hartensis L., Flavor and Fragrance J., 17,275-277.
7. Bajji, M. S. Lutts and J. M. Kinet, 2001. Water deficit effects on solute contribution to osmotic adjustment as a function of leaf ageing in three durum wheat (Triticum durum Desf.) cultivars performing differently in arid conditions. Plant Sci., 160: 669-681.
8. Begum, F.A., and N.Y. paul, 1993. Influence of soil moisture on growth, water use and yield of mustard. J. Agon crop Sci., 170:136-141.
9. Blum, A. and A.Ebercon, 1976. Genotype responses in sorghum to drought stress III. Free proline accumulation and drought resistance. Crop. Sci, 16:379-386.
10. Burbott, A.J., and D. Loomis, 1969. Evidence for metabolic lumver monoter pene in pepper mint. Plant Phsiol., 44, 173-179.
11. Cox, W.J. and G.D. Joliff, 1987. Water relations of Sunflower and Soybean under irrigated and dry land conditions. Crop SCi., 27: 553-557.
12. Dolatabadian A., S. A. M. Modarres Sanavy and M. Sharifi, 2009. Alleviation of Water Deficit Stress Effects by Foliar Application of Ascorbic Acid on Zea mays L. Journal of Agronomy and Crop Science. 195 (5):347-355.
13. El-Saidi, M.T., 1997. Salinity and its effect on growth, yield and some physiological processes of crop plants. In "Strategies for Improving Salt Tolerance in Higher Plants" P. K. Jaiwal, R. P. Singh and Anju Gulati (Eds). Oxford and IBH Publishing Co. Pvt. Ltd. New Delhi, Calcutta and Enfield (USA), P. III.
14. Farooqi, A.H.A., S. R. Ansari, R. Kumar, S. Sharma and S. Fatima, 1998. Response of different genotypes of citronellajava (Cymbopogon winterianus Jowitt) to water stress. Plant Physiol. Biochem., 25:172-175.

15. Fatima, S., A.H.A. Farooqi, S.R. Ansari, and S. Sharma, 1999. Effect of water stress on growth and essential oil metabolism in Cymbopogon marlini (palmarosa) cultivars. J. Essent. Oil Res., 11:491-496.
16. Foyer, C.H. R.G. Alscher and J.L. Hess, 1993. Antioxidants in higher plants. pp. 31-58. CRC press, Inc. Florida.
17. Ghanbari, F. Nadjafi, S. Shabahang and A. Ghanbari, 2007. Effects of irrigation regimes and row arrangement on yield, yield components and seed quality of pumpkin (Cucurbita pepo L.) .Asian J. Plant Sci.,6:1072-1079.
18. Guenther, E., 1961. "The Essential oils" D- van Nostrond comp. Inc. New York, Vol. 1, 3 and 4.
19. Hendawy, S.F., and k.H.A. khalid, 2005. Response of sage (Salvia officinalis L.) plants to zinc an application under different salinity levels. J. Appl. Sci. Res., 1 (2): 147-155.
20. Hsiao, T. C., 1973. Plant responses to water stress. Annu Rev. Plant Physiol., 24:519-570.
21. Irigoyer, J.J., D.W. Emerich, and M. Sanchez – Diaz, 1992. Water stress induced changes in concentrations of proline and total soluble sugars in nodulated alfalfa (Medicago sativa) plants. Physiol. Plant., 84: 55-60.
22. Khalid, Kh.A., 2006. Influnce of water stress on growth, essential oil, and chemical composition of herbs (ocimum sp.). Int. Agrophysics. 20:289-296.
23. Klute, A., 1986. "Method of solid Analysis", 2nded. Part 1. Physical and Mineralogical Methods. Part 2. Chemical and Microbiological methods. Properties. Modifon, Wiscon fin. U.S.A.
24. Levitt, J., 1980. Responses of plants Environmental stress. Academic press. New York, vol. 2.
25. Metzner, H., Rau, H. and senger, H., 1965. Unler, Suchunger zur. Synchronizer Barkiteinsieher– pigmentmangel. Mutanten von chlorella planta 65, 180.
26. Mirsa, A., and Strivastava, N.K, 2000. Influence of water stress on Japanese mint. J. Herb, Spices and Med. Plants, 7, 1, 51-58.
27. Price, A. H., and G.A.F. Henry, 1991. Iron-catalyzed oxygen radical formation and its possible contribution to drought damage in nine native grasses and three cereals. Plant, Cell and Environmental 14: 477-474.
28. Price, A.H., and G.A.F. Henry, 1989. Stress and the role of activated oxygen scavengers and protective enzymes on plants subjected to drought. Biochem. Soc. Trans. 17: 493.
29. Price, A.H., and G.A.F. Henry, 1987. The significance of the tocopherois in stress survival in plants In: C. Rice- Evens (eds.) free radicals,, oxygen stress and drug action. Pp443-450. Richlieu Press, London.
30. Sabih F., Abab Farooki A.H., Ansari S.R., and S. Sharama, 1999. Effect of water stress on growth and essential oil metabolism in Cymbopogon martini (palmrosa) cultivars, J. Essential Oil Res., 1, 151-157.
31. Sepheri, A. and S.A.M. Modarres, 2003. Water and nitrogen stress on maize photosynthesis. Biol. Sci., 3: 578-584.
32. Simon, J.E., B.D. Reiss, R.J. Joly, and D.J. Charles, 1992. Water stress induced alternations in essential oil content of sweet basil J. Essential Oil Res., 1, 71-75.
33. Smirnoff N., 1995. Antioxidant systems and plant response to the environment. In: N. Smirnoff (eds.) environment and plant metabolism: flexibility and acclimation–BIOS scientific publishers. Oxford, UK.
34. Snedecor, G.W., and W.G. Cochran, 1980. "Statistical Methods".7th ed. Iwa State Uni., press, Ames, Iwa, U.S.A.
35. Solinas, V., and S. Deiana, 1996. Effect of water and nutritional conditions on the Rosmairnus afficinalis L., phenolic fraction and essential oil yield. Ital. Eposs., 19, 189-198.
36. Tawfik, K.M, 2008. Effect of water stress in addition to potassiomag application on Mungbean. Australian Journal of Basic and Applied Sciences, 2(1): 42-52.
37. Troll. W.J. Lindsley, 1995. A photometric method for determination of proline. J. Biol. Chem. 215: 655-660.
38. Weatherly P. E., 1962. Examination of the moisture stress on carbohydrate development and growth in plants. Am. J. Bot. 41, 316-320.
39. Wiston. G.W., 1990. Physiochemical basis for free radical formation in cells: production and defenses, pp 57-86. In: R.G. Alscher and J. F.R. Cummings (eds). Stress responses in plants. An adaptation and acclimation mechanisms. Wileytiss, New York.
40. Yusuf, Chowdhury, J.U., M.A.Wohab, and J. Bequm, 1994. Medicinal plants of Bangladesh. BCSIR, Dhaka, Bang.

6/6/2010

Isolation and Screening of a Feather-Degrading Keratinolytic Actinomycetes from *Actinomyces sp*

T.Jayalakshmi 1, P. Krishnamoorthy 1,, G.Ramesh kumar 2, P.Sivamani 3

1 Dept of Bioinformatics, Bharath University, Chennai,Tamilnadu - 600073, India.
2 Dept of Bioinformatics, MIT Campus, Anna University, Chennai, Tamilnadu-600044, India.
3 Microlabs, Vellore, Tamilnadu-632521, India.
jayamaniraaja07@gmail.com

ABSTRACT: The Actinomycetes comprise a ubiquitous order of bacteria which exhibits wide physiological and morphological diversity. These microorganisms are particularly abundant in alkaline soils rich in organic matter. Keratin is an insoluble structural protein of skin, and its derivatives (e.g. feather, hair, wool and horn) are known for their high stability. Actinomycetes producing keratinases are having high applications in feed, fertilizer, leather and also for pharmaceutical and biomedical applications. *Actinomyces* species newly isolated, thermo tolerant feather degrading bacterial strain was investigated for its ability to produce keratinase enzyme. Maximum keratinolytic activity was observed at 28°C and pH 7.5. Keratin-containing materials (feather, hair, wool, etc.) are abundant in nature but have limited uses in practice since they are insoluble and resistant to degradation by the common proteolytic enzymes. Keratinous wastes represent a source of valuable proteins and amino acids and could find application as a fodder additive for animals or source of nitrogen for plants. Actinomycetes have the ability to break down many different varieties of organic compounds. The keratinase production by the thermophilic actinomycete strain *Actinomyces* was induced by chick feather as the sole source of carbon and nitrogen in the cultivation medium and characterization studies were carried out for the identification of the specific strain. [Journal of American Science. 2010;6(12):45-48]. (ISSN: 1545-1003).

Keywords: Keratin, Actinomycetes, Antibacterial activity, *Actinomyces*.

1. INTRODUCTION

Actinomycetes sometimes cause bio-deterioration of materials and are often responsible for spoilage of hay, straw, cereal grains, seed, wood paper, wool, hydrocarbon, rubber and plastics. In nature, biodegradation by acinomycetes plays an extremely useful role in waste removal and is an integral part of the recycling of materials. Most actinomycetes live in aerobic soils, where they biodegrade organic substrate. Number of actinomycetes living in soils often exceed in one million per gram. The keratin chain is tightly packed in the helix and then substrate turns into a supercoiled polypeptide chain resulting in mechanical stability and resistance to common proteolytic enzymes such as pepsin, trypsin and papain (Parry and North, 1998). Keratin is having more protein and aminoacid source Chicken feather mainly contains keratin, which is an insoluble protein with high stability and is indigestible by common proteases (Parry and North., 1998; Kreplak *et al.*, 2004). Keratinase enzyme are reported to produce by some species of bacteria especially Microbacterium sp (Thys *et al*., 2004) and by actinomycetes such as Bacillus and Streptomyces (Williams *et al.*, 1990)and by some keratinolytic Fungi (Yamamura *et al*., 2002). In this study, keratinolytic actinomycetes present in the soil environment were analyzed and isolation techniques were carried out.

2. MATERIALS AND METHODS

2.1 Collection of soil

The soil samples were collected from various poultry and chick farm.

2.2Cleaning of glassware

The glassware of borosil grade was used in all the experiments. The glassware was cleaned by soaking in chromic acid solution (100g potassium dichromate dissolved in one liter water with 500ml concentrate sulphuric acid) for two hours and washed in water.

2.3Chemicals used

All the chemicals were used of high purity and whenever necessary sigma grade chemicals were used.

2.4 Sterilization techniques

All the glass wares were sterilized in a hot air oven at 180^{o}C for two hours. All the prepared media and water blanks were sterilized in an autoclave at 1atm for 95 minutes. All the antibiotics were filter sterilized using sintered glass filter the isolation, purification, inoculation and other microbiological

works were carried out in a laminar Air flow chamber (Air Flow, India).

2.5. Isolation and screening of keratinolytic actinomycetes

A basal medium, containing 0.5g $M_gSO_4.7H_2O$, 1.0g K_2HPO_4, 3.0g $CaCO_3$, 20g agar per liter and 1ml trace element solution was used to test the ability of salt – loving actinomycetes to grow on sterile feathers. The feathers were supplied in small pieces on the surface of the solidified medium and were the sole source of carbon and nitrogen.

The basal medium was fortified with 100gm/LNacl to adjust salinity to 10%, and the initial pH of the medium was adjusted to 7.5-7.6. The plates were seeded with spore suspensions of the actinomycetes strains, and were incubated at 28^{o}C. Plates were examined for growth and colonization on feather pieces. Strains that showed visible growth on the feathers were considered to be potentially keratinophillic.

Keratinolytic potential of selected salt-loving strains

The keratinolytic potential of the two newly described salt-loving actinomycetes species, *Nocardiopsis halotolerans* and *Saccharomonospora halophila* (Al-Zarban *et al*.2002), were tested by a modification of the diffusion method of minimal agar medium containing 0.15% chicken feather flour (ball-milled) was poured in Petri plates. After solidification 4mm wells were cut in the agar, filled with culture fluid (initially grown in starch nitrate broth plus 10% Nacl) and incubated at 28^{o}C. The positive utilization of the feather flour as the sole source of carbon and nitrogen was assessed by the formation of a clear zone around the wells.

3.Physiological characteristics of Actinomycetes

3.1 Effect of pH

Modified Bennett broth was prepared and sterilized. The pH of broth was adjusted to 5, 7, and 8.5 using 0.1 N HCL and NaoH. The actinomycete cultures were inoculated in the broth. The tubes were incubated at room temperature for 7 days & 14 days after incubation the growth was recorded (Ivanko and Varbanets *et al*., 2004).

3.2 Effect of temperature

Modified Bennett broth was prepared and sterilized. The Actinomycetes cultures were inoculated in the broth. The tubes were incubated at 37° C & 45° C for 7 and 14 days, then at 4^0C & 10^0C for 2 and 4 weeks respectively. After incubation the growth was recorded. The pH and temperature optima were determined to be 8.6 and 70^{o}C (Ignatova *et al*., 1999).

3.3 Effect of Inhibitory compounds

Modified Bennett broth was prepared and sterilized. Inhibitory compound such as crystal violet (0.0001 gm/100ml), phenol (0.1 gm/100ml), sodium azide (0.01, 0.02 gm/100ml) sodium chloride (4, 7, 10, 13 gms/100ml) were added. Actinomycetes cultures were inoculated into the broth then the tubes were incubated at room temperature for 7 and 14 days. After incubation the growth was recorded.

3.4 Utilization of carbon source

Modified Bennett broth was prepared with carbon source like Dextrose, sucrose, Lactose, Maltose, Mannitol, L-arabinose, D-arabinose, D-Galactose, L-Rhamnose & starch. The tubes were sterilized at 121^0C for 15 lbs, 10 minutes Actinomycetes cultures were inoculated into the broth. Then tubes were incubated at room temperature for 7, 14 and 21 days after which the tubes were examined for the growth (Al-Musallam *et al*., 2003).

3.5 Utilization of Nitrogen source

Modified Bennett broth was prepared with Nitrogen source such as L-cysteine, L-histidine, L-phenylalanine, L-serine, L-Threonine, L-Glycine and L-valine Actinomycetes cultures were inoculated into the broth. Then the tubes were incubated at room temperature for 15 days. After incubation the growth was recorded (Chao *et al*., 2007).

3.6 RESISTANCE TOWARD SODIUM CHLORIDE:

The basal medium was prepared in 5 batches which are supplemented with NaCl (g/lit) 2, 7, 10, 30, 50, 70, and120 separately, the medium was autoclaved and poured into the plates.

An agar plate surface was divided into 4 sections. Each section was streaked with an actinomycete culture and incubated for 5 days at 30°C. Inhibition of growth from strong to moderate level was tested based on the observation of nature of growth compared to control.

Antibacterial property of the purified product was determined by overlay method. In this method spores were streaked on Petri plates containing 15ml of arginine glycerol salt agar medium and incubated for 5days at 30°C. 10ml (0.75% agar) of test bacteria were mixed with 0.1ml of cell/spores suspension (10^2 ml^{-1}) and overlaid on 5 days old growth of isolated actinomycetes. The plates were further incubated at 30°C for 24 hrs.

4. RESULTS

Streptomyces and *Actinomyces* partially degraded native chicken feather at 50°C (Bockle *et al*., 1995).

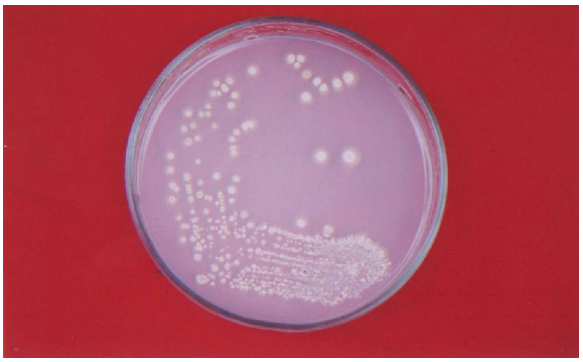


Figure: 4.1. Plate shows the Cultural characteristics of *Streptomyces spp* in Starch Caesin agar

5. DISCUSSION:

The soil samples were collected from various poultry and chick farm wastes was collected from Namakkal district and in and around Arcot, Tamil Nadu, India and the 64 isolates of actinomycetes were obtained from them is shown in Table 5.1.

Table 5.1. Total Actinomycetes population in different soil

S. No	Nameof The Soil	Number Of Actinomycetes Obtained
1	Poultry soil	28
2	Sheep soil	18
3	Cow soil	12
4	Buffalo soil	06
TOTAL		**64**

Total actinomycetes colonies were differentiated by cultural characteristics. The Actinomycetes isolates population was shown in Table 5.2, which were mainly Actinomyces and Streptomyces species.

Table 5.2. Selection of Keratinolytic Actinomycetes isolates

S. NO	NAMEOF THE SOIL	NUMBER OF DIFFERENT ISOLATES OBTAINED
1	Poultry soil	16
2	Sheep soil	12
3	Cow soil	8
4	Buffalo soil	3
TOTAL		39

SUMMARY AND CONCLUDING REMARKS

The present study mainly involved in the isolation of Actinomyces based on its morphology and identification based on the cultural characteristics. The Actinomyces species isolation was mainly based on the physiological characters and its potentiality were identified. Further work should be focused in species identification and on the keratinolytic potential of some dermatophytic fungi such as Trichophyton and Microsporum (Asahi et al., 1985, Qin *et al*., 1992, Filipello Marchisio 2000, Moallaei *et al*., 2006).

Acknowledgements:
The authors are grateful to thank Bharath University, Chennai, Tamilnadu for their support to carry out this work.

Corresponding Author:
T. Jayalakshmi
Dept of Bioinformatics, Bharath University,
Chennai,Tamilnadu - 600073, India.
E-mail: jayamaniraaja07@gmail.com

References:

1. **Al-Musallam AA, Al-Zarban SS, Al-Sarawi HK, Kroppenstedt RM, Stackebrandt E, Fasasi YA.** 2003. Influence of nutritional supplements on keratinolysis by *Amycolatopsis keratinophila*. Indian J Exp Biol. Aug; 41(8):870-4.
2. **Asahi M, Lindquist R,Fukuyama K, APODACA g, Epstein WL, Mc Kerrow JH** (1985) Purification and Characterisation of major extracellular proteinases from *Trichophyton rubrum*. Biochem J 232:139-144.
3. **Bockle B. Galunsky B, Muller R,** 1995. Characterization of Keratinolytic Serine Proteinase from *Streptomyces Pactum* DSM 40530 [J]. Appl environ microbial, 61(10):3705-3710.
4. **Chao YP, Xi (e FH, Yang J, Lu JH, Qian SJ.** 2007. Screening for a new *Streptomyces* strain capable of efficient keratin degradation. J Environ Sci (China); 19(9):1125-8.5.
5. **Filipelo Marchisio V** (2000) Keratinophilic fungi:their role in nature and degradation of keratinic substrates .In: Kushwaha RKS,Guarro J (eds) Biology of dermatophytes and other keratinophilic fungi. Revista Iberoamericana de Micologia,Bilbao,pp 86-92.
6. **Ignatova Z, Gousterova A, Spassov G, Nedkov P**. 1999. Isolation and partial characterization of extracellular keratinase from a wool degrading thermophilic actinomycete strain

Thermoactinomyces candidus. Can J Microbiol. Mar; 45(3):217-22.

7. **Ivanko OV, Verbanets' LD**.2004. keratinolytic activity of *Streptomyces* sp. 1382.Mikrobiol Z. Jan-Feb; 66(1):3 -9.
8. **Kreplak L, Doucet J, Dumas P, Broki F**,(2004) New aspects of the alpha helix of beta sheet transition in stretched hard alpha keratin fibres . Biophys J 87:640-647.
9. **Moallaaei H, Zaini F, Larcher G,Beucher B**, **Bouchara JP** (2006) Partial purification of a 37 Kda extrecellular proteinase from *Trichophyton vanbreuseghemii.* Mycopathologia 161:369-375.
10. **Parry D A D,North A C T**, 1998.Hard keratin intermediate filament chains: substructure of the N- and c-terminal domains and the predicted structure and function of the c-terminal domains of type1 and type 2 chains[J].J struct Biol, 122(1):67-75.
11. **Qin LM, Dehio S, Jidoi J** (1992) some biochemical characteristics of a partially purified extracellular keratinase from *Trichophyton schoenleinii*. Zentralbl Bacteriol 277:236-244.
12. **Thys RC, Brandelli A**. 2006. Purification and properties of a keratinolytic metalloprotease from *Microbacterium sp.* J Appl Microbiol. Dec; 101(6):1259-68.
13. **Williams C M,Richter C S, Mackenzie J M** et al., 1990. Isolation, identification and characterization of a feather degrading bacterium [J]. Appl Environ Microbiol, 56(6):1509-1515.
14. **Yamamura S, Morita Y, Hasan Q** et al., 2002. Keratin degradation: a co-operative action of two enzymes from *Stenotrophomonas sp.*[J]. Biochemical and Biophysical Research communications, 294(5):1138-1143.

6/20/2010

Denaturation and Viscosity of Whey Proteins Solutions as Affected by Frozen Storage

Soliman, T.N.[*1], A.F. Farrag[1]; A. Shendy[2] and El-Sayed, M.M.[1]

[1]Dairy Dept. National Research Centre, [2]Dairy Dept. Faculty of Agriculture, Al-Azhar University, Cairo, Egypt.
Tariknour.nrc@gmail.com

Abstract: Concentrated solutions of whey proteins (WPC) were prepared from sweet whey by ultrafiltration technique, and stored at – 18°C up to three months. Denaturation degree and viscosity of WPC solutions were assessed. Denaturation degree of whey protein solutions increased significantly (P<0.05) as affected by duration of frozen storage and protein content. The highest degree of denaturation was found at pH 5.0 and 7.0 after one month of storage. Denaturation percentages of heated and thawed WPC solutions increased significantly (P<0.05) as function of storage, protein content and pH. The flow properties of unheated WPC solutions exhibited a time-independent non-Newtonian behaviour as shear-thickening (dilatants) properties with an increase in the apparent viscosity with increasing the shear rate. Heated thawed WPC solutions behaved as thixotropic fluids with a decrease in the apparent viscosity with increasing shear rate. Apparent viscosities of unheated and heated WPC solutions greatly affected by frozen storage, protein content and pH. [Journal of American Science. 2010;6(12):49-62]. (ISSN: 1545-1003).

Key words: WPC, Frozen storage, Denaturation, Viscosity

1. Introduction

Whey proteins are used as functional ingredients in many food products not only for their nutritional properties, but also for their functional and technological properties. The functional properties of whey proteins may be refered to as: (a) hydration properties that have an important effect on wettability, swelling, adhesion, dispersibility, solubility, viscosity, water absorption and water holding; (b) interfacial properties including emulsification and foaming characteristics; (c) aggregation and gelation properties which are related to protein–protein interactions (Kresic, et al. 2006). These functionalities can be affected by either heat treatment (Mulvihill & Donovan, 1987) or pressure treatment (Patel, et al. 2005).

Preservation of the valuable cheese whey proteins in the form of a whey protein concentrate (WPC) powder has been increasing in recent years. Studies on the functional properties of dried WPC (Harper, 1984; Mangino, 1992; de Wit, 1989; Morr & Foegeding, 1990) indicate variability depending upon the source of whey, extent of protein denaturation during processing, presence of non-protein components and failure to standardize pre-treatment and processing conditions for manufacture.

Storage at freezing temperature is a well established technique for long-term preservation of many foods and other commodities. Surprisingly, very little attention has been paid to the possibility of using frozen storage for long preservation of protein solutions. Freezing of foods may result in undesirable changes, including textural damage, protein denaturation, destruction of cellular membranes mainly due to the freeze-concentration phenomenon. Bhargava & Jelen (1995) investigated the effects of freezing on viscosity of concentrated WPC solutions. They found that a small but statistically highly significant difference in viscosity, showing an effect of the slow freezing rate.

Denaturation of WPI results from a complex mechanism dominated by the denaturation of β-lactoglobulin which has been explained by Simmons *et al.,* (2007) and Schokker *et al.* (2000), by a two-step process. The first step is endothermic; which consists of protein unfolding and changes in the equilibrium between protein dimers and native and non-native monomers, associated with reversible or irreversible intramolecular rearrangements (e.g. disruption of hydrogen bonds). The second step corresponds to aggregation, resulting mainly from an intermolecular –SH to S–S exchange and, to a lesser extent, from non-covalent interactions. Aggregation starts with the formation of non-native dimers and oligomers which rapidly grow as a function of chemical environment and temperature, mainly by incorporation of monomers and smaller aggregates (Le Bon et al., 1999).

The apparent viscosity of WPC determines the potential application of these ingredients with excellent technological functionality and high nutritional value in liquid food preparation as textural ingredients (Patocka et al., 2006).

The main objective of the present work was therefore to investigate the influence of frozen storage followed by heat treatment on the WPC denaturation degree and viscosity of WPC solutions.

2. Materials and Methods:

Materials

Sweet whey from Mozzarella cheese manufacture (pH 5.9 – 6.0) was obtained from Arab Dairy products Co. Kaha, Kalubia. Residual fat and curd were removed from the whey by a cream separator. Clarified whey was directly concentrated by ultrafiltation using a 50,000 molecular weight cut off zirconium oxid membrane installed in a Carbosep pilot plant (modules S 151 UF system, Nova-Sep France). Ultrafiltration was carried out in a batch mode at 45-50°C, inlet and outlet pressure of 5.5 and 3.5 bar, respectively. Ultrafiltration was continued to a concentration factor 20. The retentate (whey protein concentrates) was diluted with an equal volume of water and diafiltered three times to remove most of lactose and minerals from whey retentate.

The WPC solutions were packaged in polyethylene sacs and stored frozen at ─18°C. A sample sac was removed from freezer after 1, 2, and 3 months of storage and thawed in a refrigerator at 4°C for analysis.

After thawing WPC was diluted with distilled water to obtain three WPC solutions which containing of different protein ratios. These solutions were adjusted to pH 3.0, 5.0, 7.0 and 8.6 using 1N HCl or NaOH, heated to 80°C for 10 min, and cooled rapidly. Undenatured whey proteins and viscosity for WPC preparing solutions were determined before and after heating.

Methods:

1. Chemical analysis:

Whey protein concentrate solutions were analysed for total solids by dry oven at 105°C for 6 hrs as described in AOAC (1990). Fat by Gerber bytrometer and protein nitrogen fractions by micro Kjeldahl method and pH according to Ling (1963). Lactose content by the phenol-sulphuric method of Barrnet and Tawab (1957).

2. Determination of denaturation of WPC

The degree of denaturation of WPC was determined according to Andersen *et al.* (1983). Aliquot of the WPC solution was adjusted to pH 4.6 using 1N acetic acid and 1N sodium acetate solutions respectively. The precipitate was removed by filtration and the total nitrogen was determined in the filtrate. Trichloroacetic acid (TCA) solution was added to the supernatant to give 12% TCA in the mixture. The precipitate was removed by filtration, and NPN was determined in the filtrate. The percentage of undenatured whey proteins (UDW%) was calculated as:

$$\% \text{ UDW} = \frac{\text{Percentage of 4.6 soluble N – NPN}}{\text{Total WPC N before heat treatment - NPN}} \times 100$$

3. Viscosity of WPC

The viscosity of WPC was measured according to Farrag *et al.* (2006). The apparent viscosity of the thawed and heated WPC solutions were measured using a Bohlin coaxial cylinder viscometer (Bohlin Instrument Inc., Sweden) attached to a work station loaded with software V88 viscometery programme. The system C30 was filled with the WPC solution at the measurement temperature of 20°C. The viscosity was carried out in the up mode at shear rate ranging from 37 to 910 1/s.

The heated WPC solutions samples at 80°C for 10 min were kept at refrigerator at 5-6°C for 24 hrs to examine renaturation of whey proteins again.

Statistical analysis

The data were analysed according to Statistical Analysis System (SAS, 1998). Duncan multiple range test was carried out for separation among means. All experiments were replicated 3 times.

3. Results and Discussion

The chemical composition of whey and WPC solutions are shown in table (1). WPC solutions contained total solids (TS) of 9.50, 4.75 and 3.20% for WPC 1, WPC 2 and WPC 3 respectively. Its contained total proteins (TP) of 5.09, 2.5 and 1.7% in the same order. Although the WPC solutions had been subjected to diafiltration, the solutions still contained residual lactose and fat.

Table 1: Chemical composition of whey protein concentrates (WPC) solutions of different protein content.

Concentration Test	Whey	WPC 1	WPC 2	WPC 3
TS %	7.25	9.50	4.75	3.20
TN %	0.220	0.798	0.385	0.259
TP %	1.40	5.09	2.50	1.70
Fat %	0.1	1.8	0.9	0.6
Lactose %	5.5	2.40	1.23	0.83
pH	5.99	5.85	5.75	5.75

Whey protein denaturation as affected by frozen storage

Degree of denaturation of the whey protein concentrates solutions as affected by freezing storage are given in Table 2.

After one month of frozen storage no visible changes was observed in the WPC solutions at all pHs on freezing and thawing. The percentage of denaturation increased significantly ($p<0.05$) compared

with fresh or zero time samples. The highest degree of denaturation was found at pH 5.0 and 7.0 being 48.71 and 48.94% compared with that found at pH 3.0 and 8.6 namely 45.10 and 46.46% at WPC solution containing 5.09% protein. The percent of denaturation decreased with decrease in the protein concentration. It was decreased from 45.10 for WPC1 to 41.94% for WPC3 at pH 3.0 and from 48.71 for WPC1 to 39.96 % for WPC3 at pH 5.0. The same trend was found at different pHs. These results were agreement with that reported by Farrag *et al.* 1997.

After two and three months of frozen storage, thawed WPC exhibited protein destabilization with flocculated protein aggregates. At pH 5.0 solutions, a uniform small protein aggregates could be seen in the WPC solution. These observations agreement with that finding by (Bhargava & Jelen, 1995). They found that the protein aggregates seen after thawing appeared to consist of larger clumps along with small floating fragments, indicating possible minor effects of the freezing storage at pH 5.0.

After two months of frozen storage the denaturation degree of WPC solutions increased significantly ($p<0.05$) compared with fresh or one month samples. After three months freezing storage the highest denaturation was found at pH 8.6 of 56.12, 52.16 and 51.61% for WPC1, WPC2 and WPC3 respectively. Previous studies reported good protein stability in WPC solution stored below -20°C (Antifantakis *et al.*, 1980; Bastian, 1994, Young, 1985). Koschak *et al*. (1981) reported that frozen bovine milk and milk concentrates stored at -20°C or lower remain stable for long periods of time, but stability decreases greatly as the temperature is raised above -20°C.

Heat denaturation of thawed WPC solutions:

Table 3 shows the denaturation percentages of heated WPC solutions as function of freezing storage, protein content and pH. At pH 3 heating denaturation degree increased significantly ($P<0.05$) from 71.43% at zero time to 85.19% after 3 months of freezing storage of WPC 1 solution. At pH5.0 degree of denaturation increased significantly ($P<0.05$) of 70.00, 74.44, 82.50 and 85.37% after zero time, 1 month, 2 months and 3 months for WPC2 solution respectively. At pH 7.0 the same trend were found. On the other hand at pH 8.6 no significant differences were found in the denaturation percent of WPCs solutions (different protein content) after 3 months of freezing storage. Higher pH values caused formation of soluble whey proteins aggregates. Vasbinder and de Kruif (2003). Heat treatment at higher pH caused a clear formation of whey protein aggregates, indicating a pH dependent aggregation mechanism. Anema and Klostermeyer (1997) demonstrated using ultracentrifugation that at higher pH more whey proteins remained soluble than at lower pH.

Table (2): Effect of frozen storage on the denaturation % of WPC solutions at different pHs.

	A	B	C
(a) pH 3.0			
Fresh	30.85^{f}	29.17^{f}	28.12^{f}
1 month	45.10^{d}	40.44^{e}	41.94^{e}
2 months	48.91^{bc}	47.81^{bcd}	46.63^{cd}
3 months	52.75^{a}	51.09^{ba}	50.00^{ba}
(b) pH 5.0			
Fresh	30.00^{g}	26.53^{h}	27.29^{h}
1 month	48.71^{cd}	38.30^{f}	39.96^{f}
2 months	52.81^{b}	46.82^{de}	44.79^{e}
3 months	55.68^{a}	50.02^{c}	48.24^{cd}
(c) pH 7.0			
Fresh	26.45^{f}	25.51^{f}	25.81^{f}
1 month	48.94^{b}	39.57^{d}	35.74^{e}
2 months	53.93^{a}	44.68^{c}	39.32^{d}
3 months	55.32^{a}	47.83^{bc}	44.83^{c}
(d) pH 8.6			
Fresh	27.47^{f}	29.65^{f}	30.30^{f}
1 month	46.46^{cd}	41.67^{e}	43.75^{de}
2 months	50.47^{b}	50.00^{b}	48.39^{bc}
3 months	56.12^{a}	52.16^{b}	51.61^{b}

A: WPC 1 Containing 9.50% TS
B: WPC 2 Containing 4.75% TS
C: WPC 3 Containing 3.20% TS

Means with different superscript in the same row are significant a, b, c, and d ($P<0.05$); Means with different superscript in the same column are significant e, f, g and h ($P<0.05$).

Means with different superscript in the same row are significant a, b, c and d ($P<0.05$); Means with different superscript in the same column are significant e, f, g and h ($P<0.05$).

Table (3) : Denaturation % of heated thawed WPCs solutions at 80°C/10 min as affected by frozen storage and pHs.

	A	B	C
(a) pH 3.0			
Fresh	71.43^{e}	74.27^{cd}	70.00^{e}
1 month	74.44^{cd}	72.50^{de}	75.84^{c}
2 months	79.07^{b}	81.08^{b}	79.29^{b}
3 months	85.19^{a}	84.20^{a}	85.17^{a}
(b) pH 5.0			
	A	B	C

Fresh	73.09^{fg}	70.00^{h}	71.19^{gh}
1 month	76.78^{de}	74.44^{ef}	77.78^{d}
2 months	79.31^{cd}	82.50^{ab}	80.75^{bc}
3 months	83.33^{ab}	85.37^{a}	84.62^{a}
(c) pH 7.0			
	A	B	C
Fresh	73.61^{d}	71.41^{e}	70.37^{e}
1 month	75.29^{c}	73.67^{d}	73.05^{d}
2 months	80.69^{b}	80.55^{b}	79.98^{b}
3 months	84.71^{a}	83.77^{a}	83.98^{a}
(d) pH 8.6			
	A	B	C
Fresh	72.73^{cd}	70.57^{d}	73.33^{cd}
1 month	75.19^{c}	73.01^{cd}	79.29^{b}
2 months	79.78^{b}	79.99^{b}	82.10^{b}
3 months	85.71^{a}	85.76^{a}	85.71^{a}
A: WPC 1 Containing 9.50% TS B: WPC 2 Containing 4.75% TS C: WPC 3 Containing 3.20% TS			

By varying the pH prior to heat treatment the mechanism of denaturation is influenced. The degree of denaturation is rather constant (Law & Leaver, 2000), but heat treatment at higher pH results in the formation of more whey protein aggregates while heat treatment at lower pH results in more association of the whey proteins with the casein micelle (Anema & Klostermeyer, 1997; Corredig & Dalgleish, 1996).

However, heating does have a definite effect on the whey protein fractions, which consists mainly of β-lactoglobulin (β-lg) and α-lactalbumin (α-la). Upon heating, a reactive thiol group is exposed in β-lg due to conformational changes of the molecule. This reactive thiol group can form disulfide links with other proteins having a reactive thiol group or through thiol group-disulfide bridge exchange reactions. The reaction makes the denaturation process irreversible, in contrast to the reversible denaturation of porcine β -lg which lacks free thiol groups (Burova, et al., 2002; Ugolini *et al.*, 2001).

Renaturation

Table 4 showed that the ability of denatured heated whey proteins to relation its nature after keeping in refrigeration temperature for 24 hrs. The results showed that slight decrease in the ratio of denatured proteins.

The process of denaturation and subsequent aggregation of bovine β -lg resembles a polymerisation process, in which the unfolding represents the initiation step (de Kruif et al., 1995; Roefs & de Kruif, 1994). α-La cannot initiate the polymerization process due to the absence of a free thiol group; however as it has four disulfide bridges it is irreversibly denatured in the presence of β-lg due to thiol group-disulfide bridge exchange reactions (Mulvihill & Donovan, 1987).

According to de Wit (1989), the solubility of whey protein is impaired by heating above 70°C when the pH is 4.0-6.5 due to irreversible unfolding, while at pH 6.8 or 7.0, the increase in aggregation and visible turbidity does not result in the formation of sedimentable particles (Britten *et al.*, 1993). For pH 5.0 samples, the precipitate was in the form of loose aggregates of denatured protein particles; indicating the lack of any major impairment of the structural network formed after heating by the preceding freezing step.

Table (4): Renaturation of Denatured heated WPCs solutions at 80°C/10 min after 24 hrs keeping in refrigerator 4-6°C.

(a) pH 3.0			
	A	B	C
Fresh	69.45^{de}	68.61^{e}	67.83^{e}
1 month	73.04^{c}	70.25^{de}	72.00^{cd}
2 months	78.31^{b}	79.40^{b}	76.90^{b}
3 months	83.12^{a}	82.84^{a}	82.59^{a}
(b) pH 5.0			
	A	B	C
Fresh	71.62^{de}	67.57^{ef}	66.67^{f}
1 month	75.28^{cd}	71.82^{de}	73.91^{cd}
2 months	77.65^{bc}	78.42^{bc}	78.26^{bc}
3 months	81.48^{ab}	83.78^{a}	81.79^{ab}
(b) pH 7.0			
	A	B	C
Fresh	71.83^{ef}	67.63^{g}	67.97^{g}
1 month	73.52^{de}	71.41^{ef}	69.53^{fg}
2 months	78.16^{bc}	76.47^{bcd}	76.00^{cd}
3 months	81.71^{a}	79.46^{ab}	79.17^{abc}
(c) pH 8.6			
	A	B	C
Fresh	70.31^{e}	69.70^{e}	70.37^{e}
1 month	72.22^{de}	70.59^{e}	74.97^{cd}
2 months	78.42^{b}	78.18^{b}	77.78^{bc}
3 months	82.93^{a}	83.86^{a}	81.46^{a}
A: Containing 9.50% TS B: Containing 4.75% TS C: Containing 3.20% TS			

Means with different superscript in the same row are significant a, b, c and d ($P<0.05$); Means with different superscript in the same column are significant e, f, g and h ($P<0.05$).

Viscosity

Apparent viscosity of WPC samples at different pHs showed different patterns. For all the fresh WPC solutions at different pHs, the apparent viscosities were increased with increasing shear rate.

Also, the apparent viscosities of WPC solutions were increased with increase in the whey protein content. As shown in Fig. 1, at pH values above 5.0 all apparent viscosities were low with little variation, but below this pH at pH 3.0 values increases in apparent viscosity occurred, accompanied by the development of 'greasy' textures (Rattray & Jelen 1995). At shear rate of 185 1/s the apparent viscosities of WPC 1 sample of 4.8, 2.9, 3.6 and 3.2 mPas at pH 3.0, 5.0, 7.0 and 8.6 respectively (Fig 1). On the other hand at decreasing total solids content as sample WPC 3 no differences were records at different pHs uses. At pH 5.0 and 7.0 the highest apparent viscosity was recorded and its increased to 56 and 34.4 mPas for heated WPC 1at shear rate of 185 1/s (Fig. 2). However, at pH 5.0 all WPC solutions were found to be thixotropic fluids as there was a decrease in the apparent viscosity with increasing shear rate. These results were agreement with finding of Howard, (1991).

After one month of frozen storage the apparent viscosity of WPC solution increased at all pHs compared to fresh WPC solutions (Fig. 3). For WPC1, at pH 5.0 the viscosity of 13.3 mPas decreased to 11, 8.4 and 6.6 mPas at pH 3.0, 7.0 and 8.6 respectively at shear rate of 37 1/s. Also apparent viscosities were decreased with decrease of protein content of WPC solutions. Viscosities of heated thawed WPC solutions were illustrated in Fig. 4. The results showed significant differences in the viscosities of different pHs heated WPC solutions stored frozen as compared to the unfrozen control samples. Apparent viscosities were increased significantly of 308, 264 and 269 mPas for WPC 1 at pH 5.0, 7.0 and 8.6 respectively at shear rate of 37 1/s. On the contrary, at pH 3.0 the WPC solutions were observed significantly decreased in apparent viscosity as compared to other pHs. The increase in the viscosity of WPC coincide with the degree of denaturation, Kresic *et al.* (2008) and Meza, *et al.*, (2009) which was confirmed in the present study. The impact of protein denaturation on the development of high apparent viscosities is noticeable. Conceivably, heat treatments of WPC solutions led to some protein denaturation, rendering the proteins more pronounced to pH. (Rattray & Jelen 1995). Fig. 5 and 7 showed the apparent viscosity of WPC solutions after two and three months of frozen storage. Apparent viscosity was increased with increase shear rate. No significant differences in viscosity values were recorded between WPC solutions after two or three months from freezing storage.

Apparent viscosities of heated WPC solutions after two and three months of freezing storage were illustrated in Fig. 6 and 8.

After two months of freezing storage significantly differences were obsereved in apparent viscosity of heated WPC solutions as affected by protein content and pHs (Fig. 6). Viscosities values of WPC1 (highest protein content) record of 8.2, 39.6, 62.4 and 107 mPas at pHs of 3.0, 5.0, 7.0 and 8.6 at shear rate of 185 1/s. These values decreased to 6.5, 8.0, 9.3 and 6.5 for WPC3 sample (lowest protein content) in the same order. The presence of large number of high molecular weight aggregates increase the resistance to flow which, in turn, increases the apparent viscosity (Rattray and Jelen, 1995).

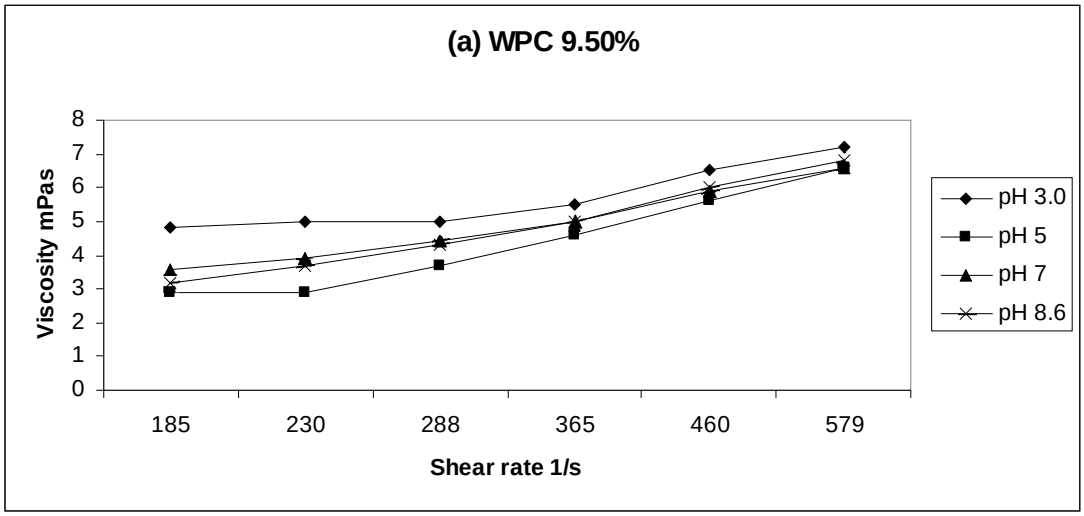
On the other hand, further freezing storage to three months led to increase in the viscosity values of 13.8, 18.7 and 18.5 mPas for heated WPC2 and of 7.0, 10.1 and 8.3 mPas for heated WPC3 at pHs of 5.0, 7.0 and 8.6 respectively (Fig. 8). On contrary, apparent viscosity of heated WPC1 was decreased to 49.0, 64.0 and 56.0 mPas compared to that finding in two months of freezing storage.

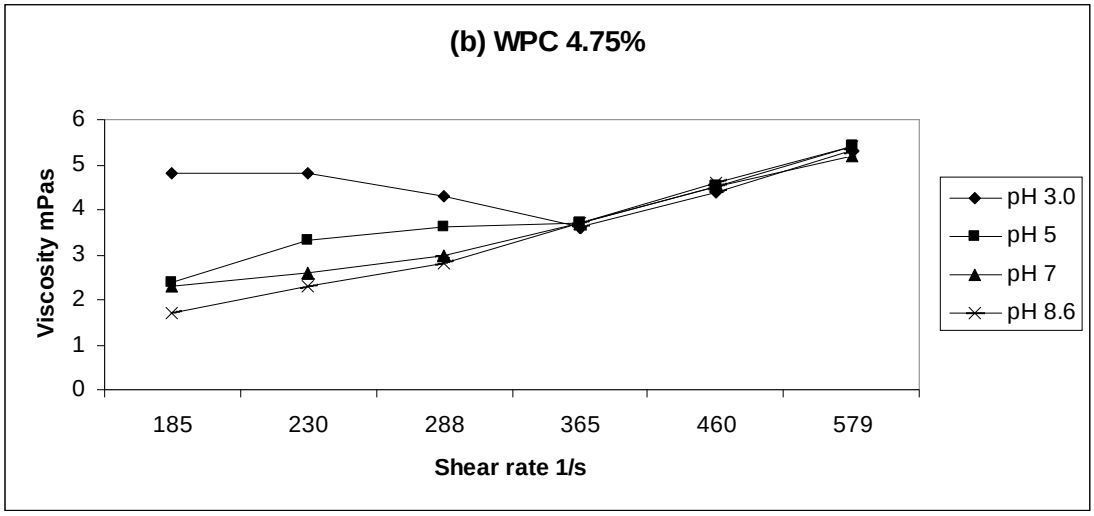
From the summarized over all viscosities results showed that the apparent viscosities of unheated WPC solutions exhibited a time-independent non-Newtonian character what should be considered as shear-thickening (dilatants) properties. In this type the increase in shear rate results in an increase in apparent viscosity (Kresic et aL. 2008). On the other hand, heated thawed WPC solutions were found to be thixotropic fluids as there was a decrease in the apparent viscosity with increasing shear rate. Generally viscosities results showed that the apparent viscosities of unheated and heated WPC solutions greatly affected by protein content and pH degree. The investigation carried out on the WPC of different protein concentration (Lelas and Herceg, 2002), revealed that the intensity of mentioned increase of water binding properties is proportional to protein concentration.

4. Conclusion:

From the obtained results it can be concluded that frozen storage increased the denaturation degree and viscosity of WPC. However, the protein content and pH play a role in the observed changes in the denaturation and viscosity of stored WPC.

Corresponding author
Soliman, T.N
Dairy Dept. National Research Centre
Cairo, Egypt.
Tariknour.nrc@gmail.com





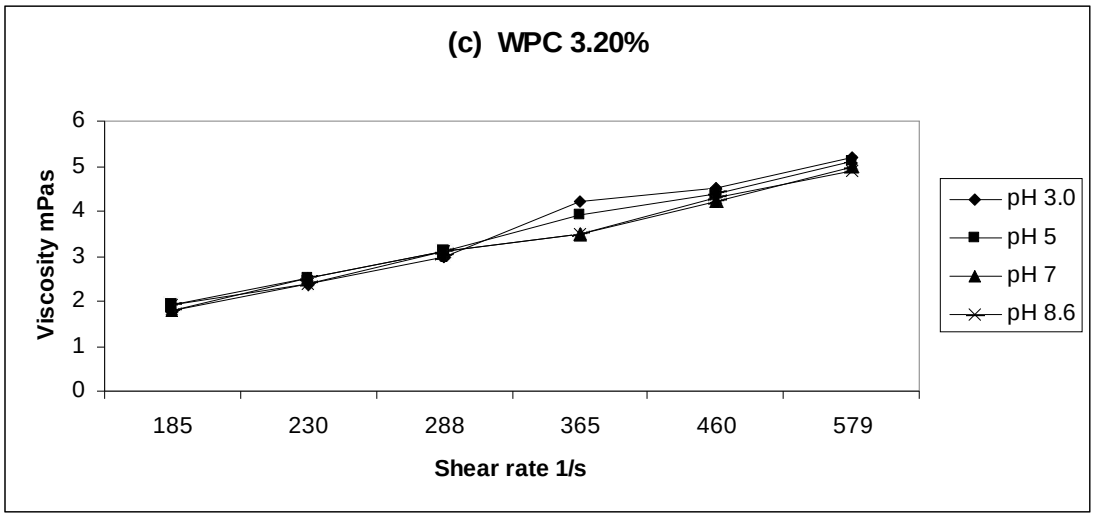


Fig. (1): Viscosity of fresh WPC solutions at different concentration before freezing storage at different pHs

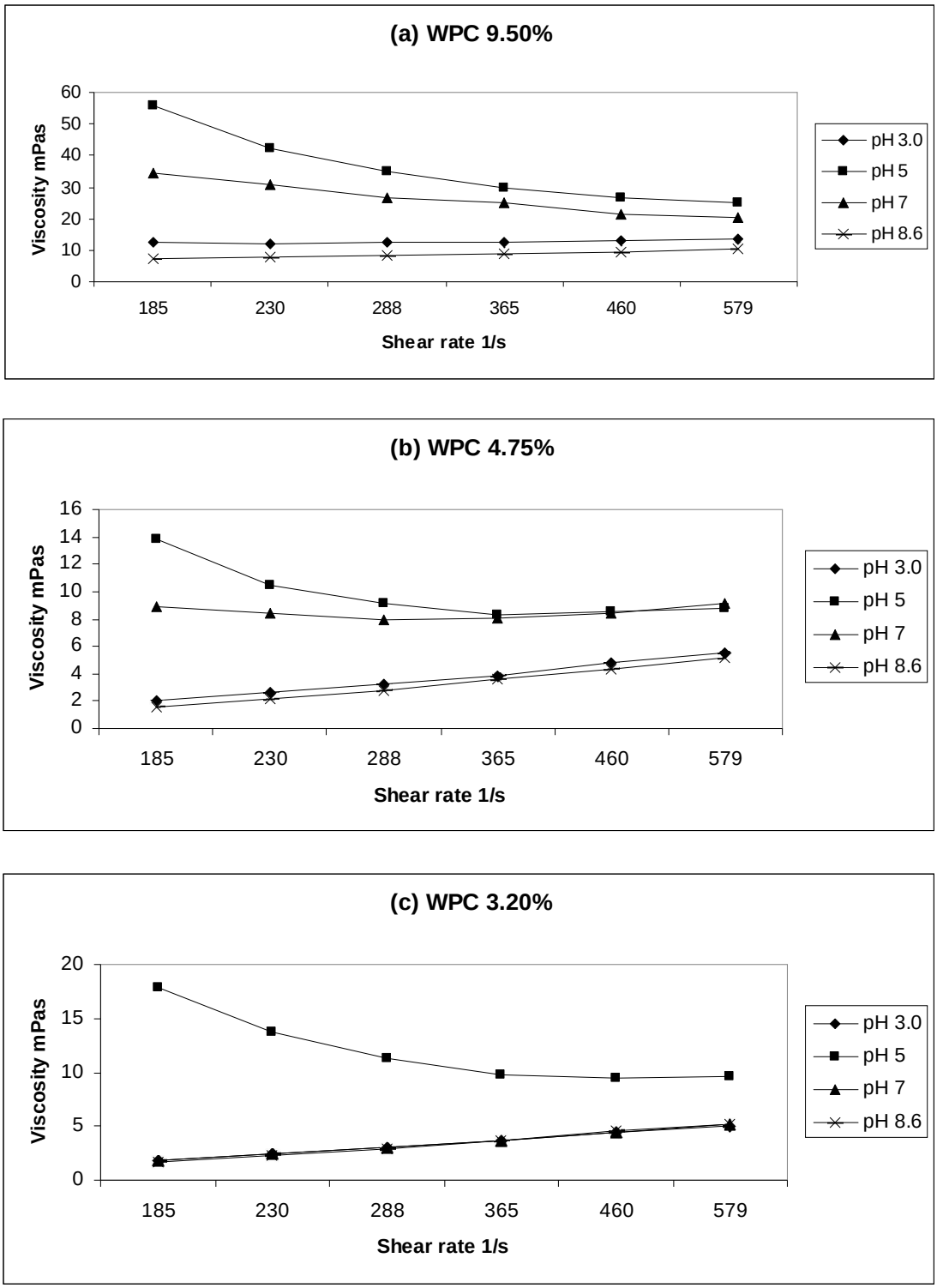


Fig. (2): Viscosity of heated fresh WPC solutions at different concentration before freezing storage at different pHs

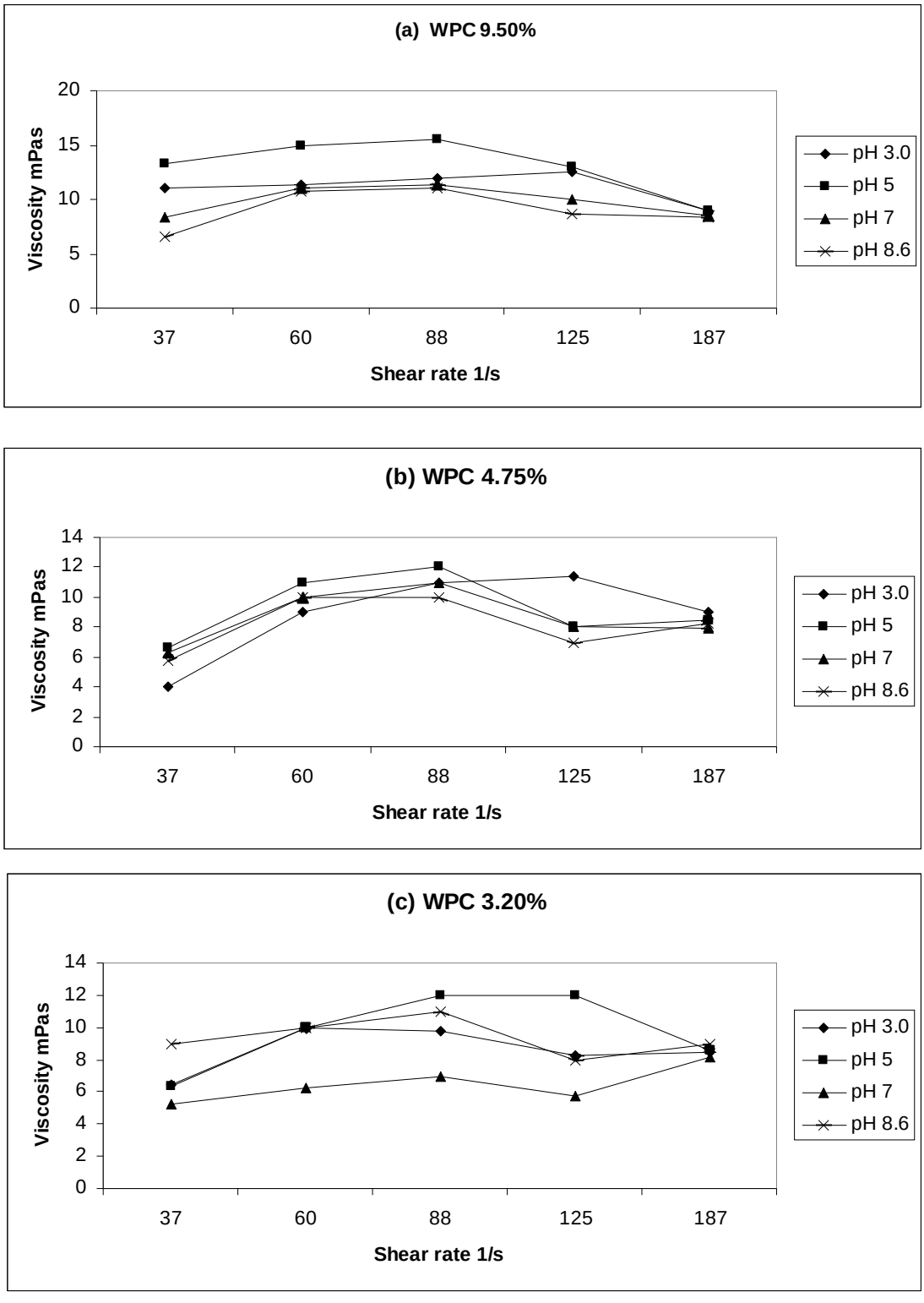


Fig. (3): Viscosity of WPC solutions at different concentration after one month freezing storage

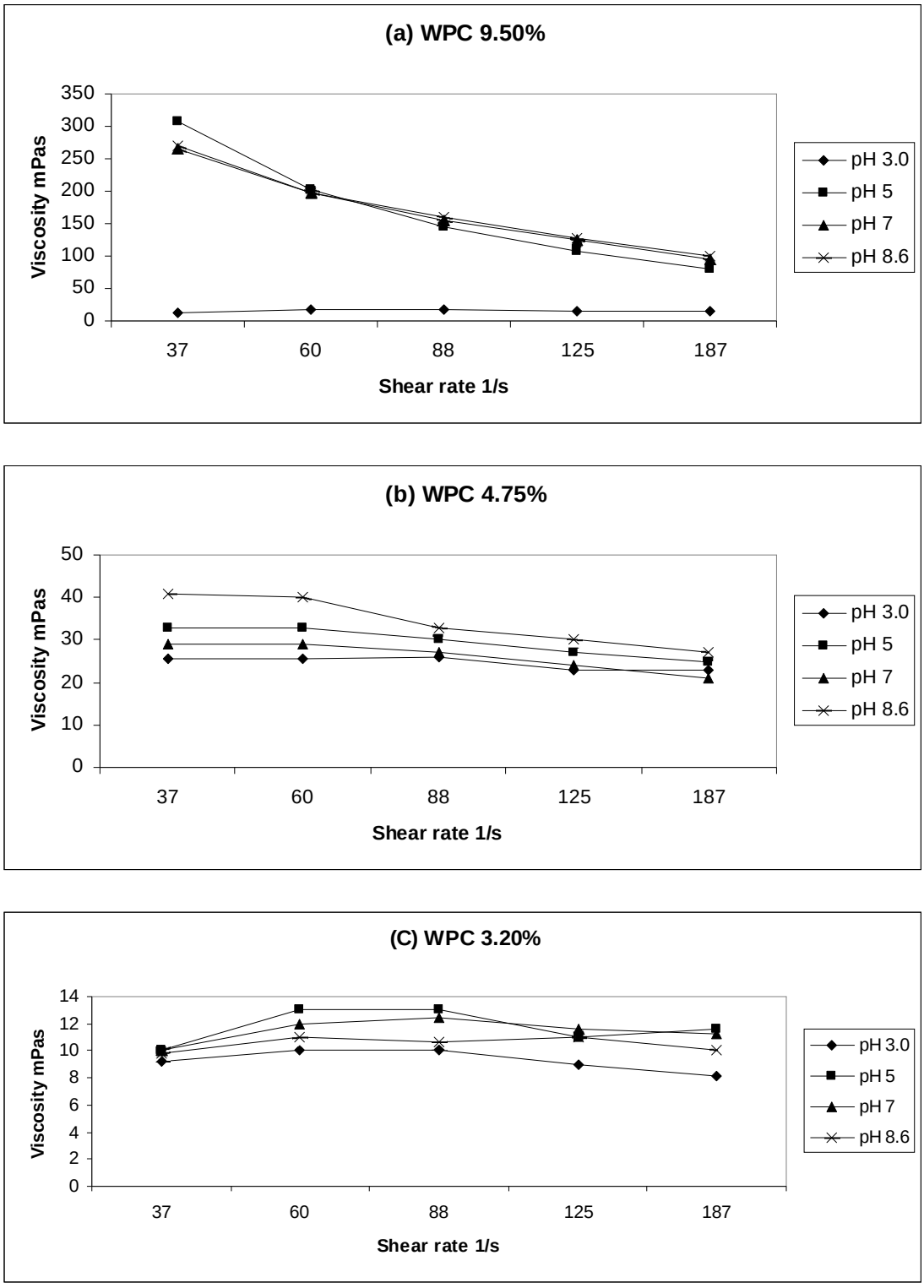


Fig. (4): Viscosity of heated WPC solutions at different concentration after one months freezing storage

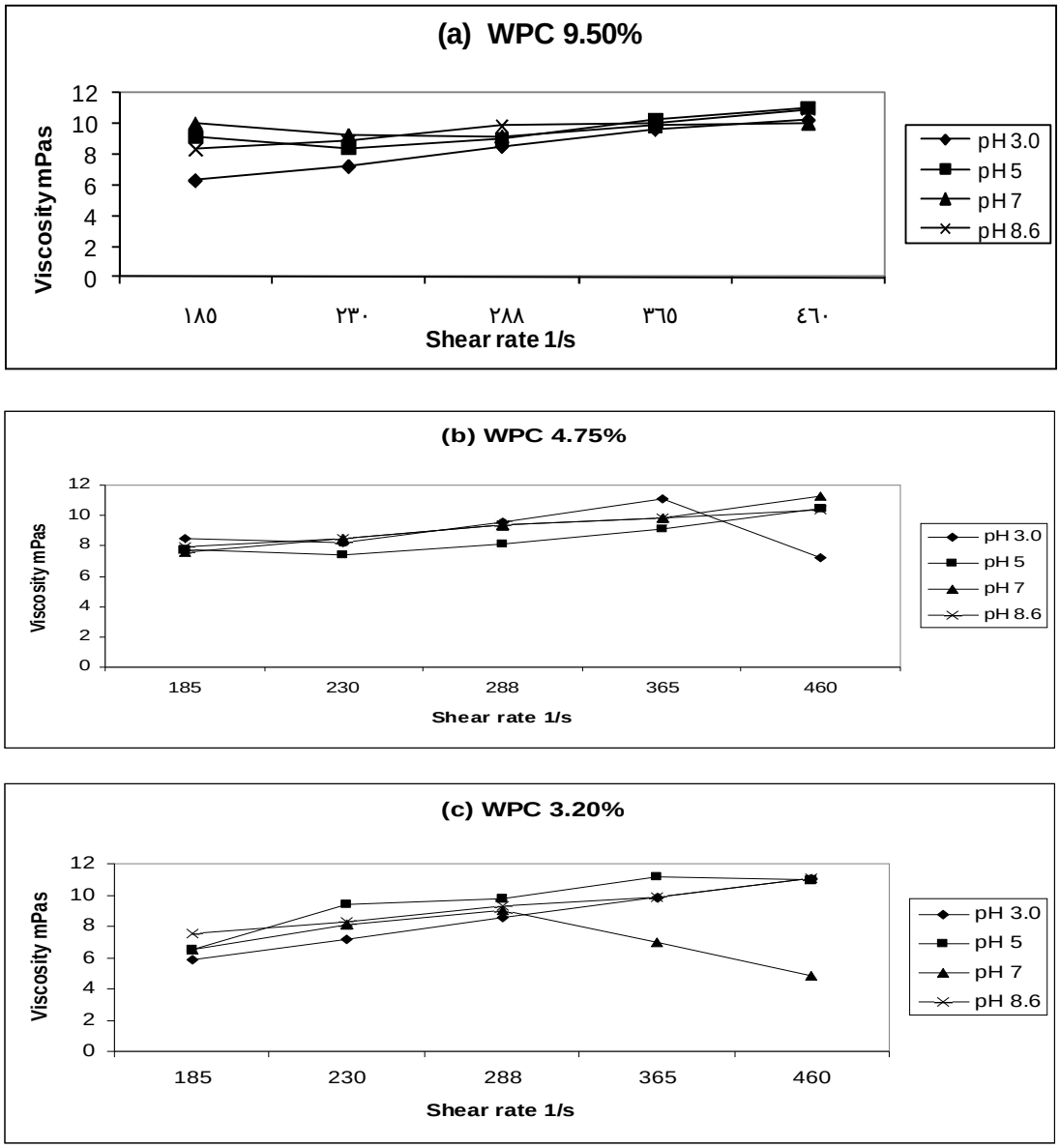


Fig. (5): Viscosity of WPC solutions at different concentration after two months freezing at different pHs

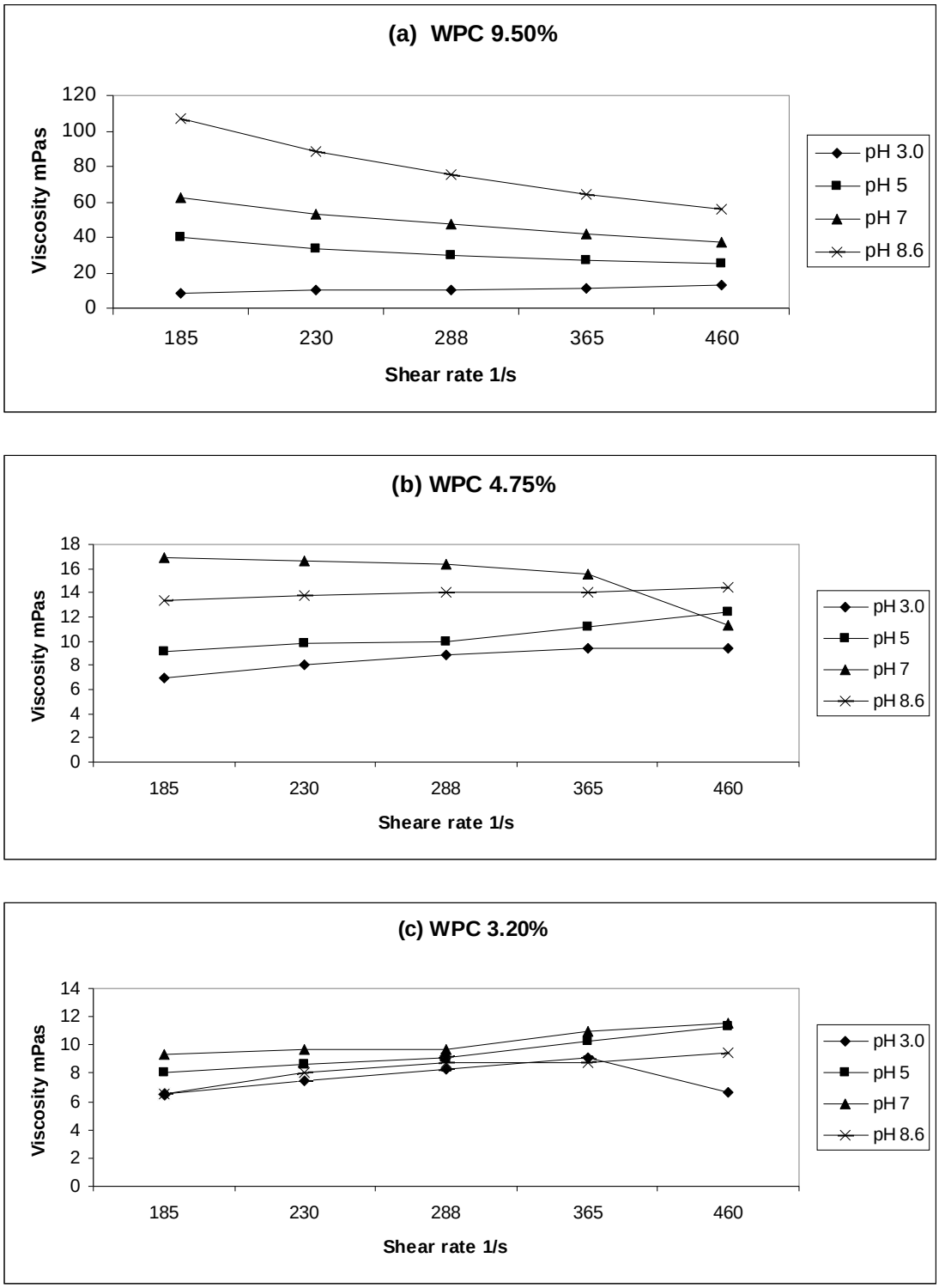
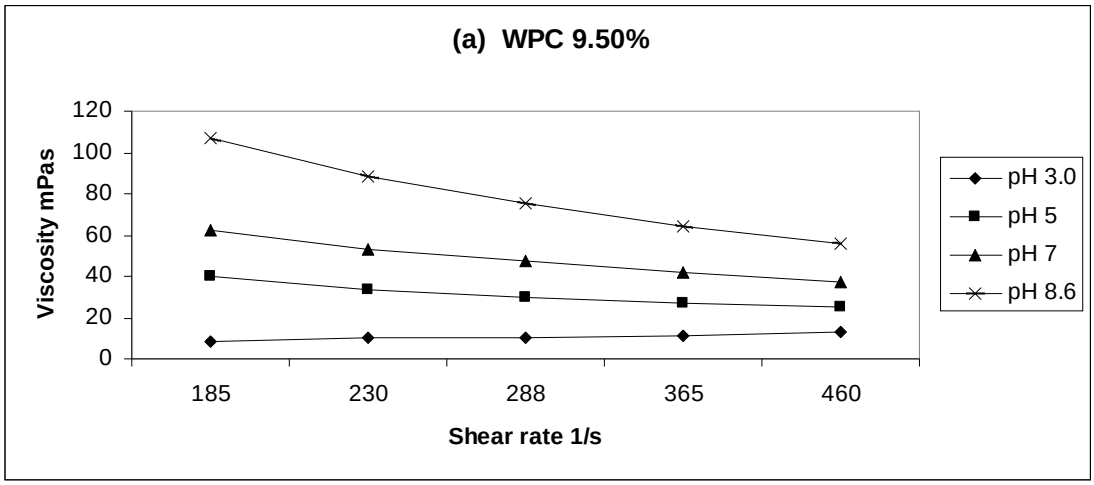
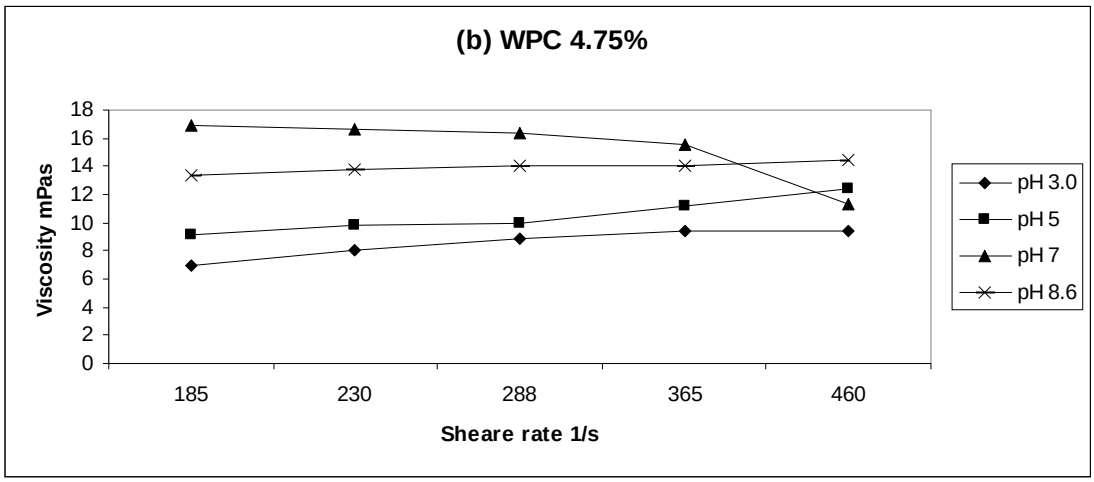


Fig. (6): Viscosity of heated WPC solutions at different concentration after two months freezing at different pHs





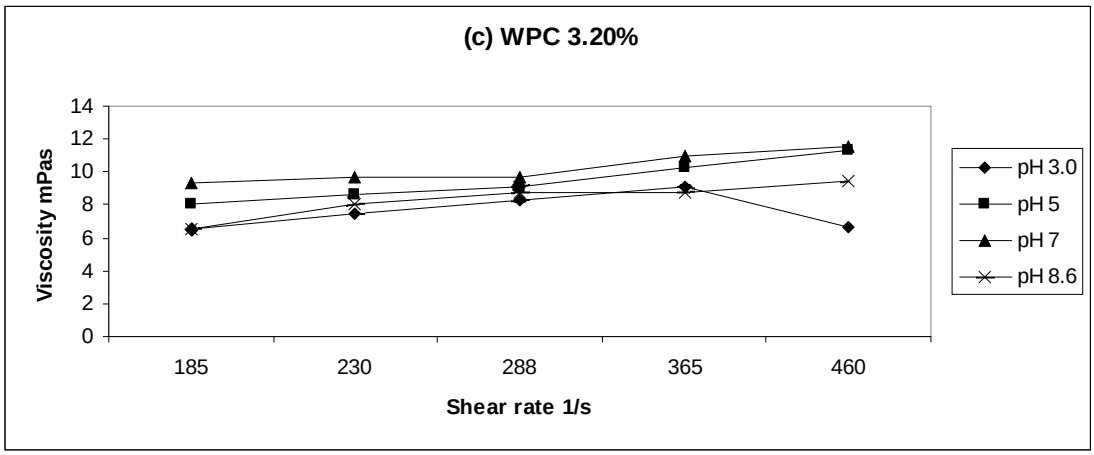


Fig. (7): Viscosity of WPC solutions at different concentration after three months freezing at different pHs

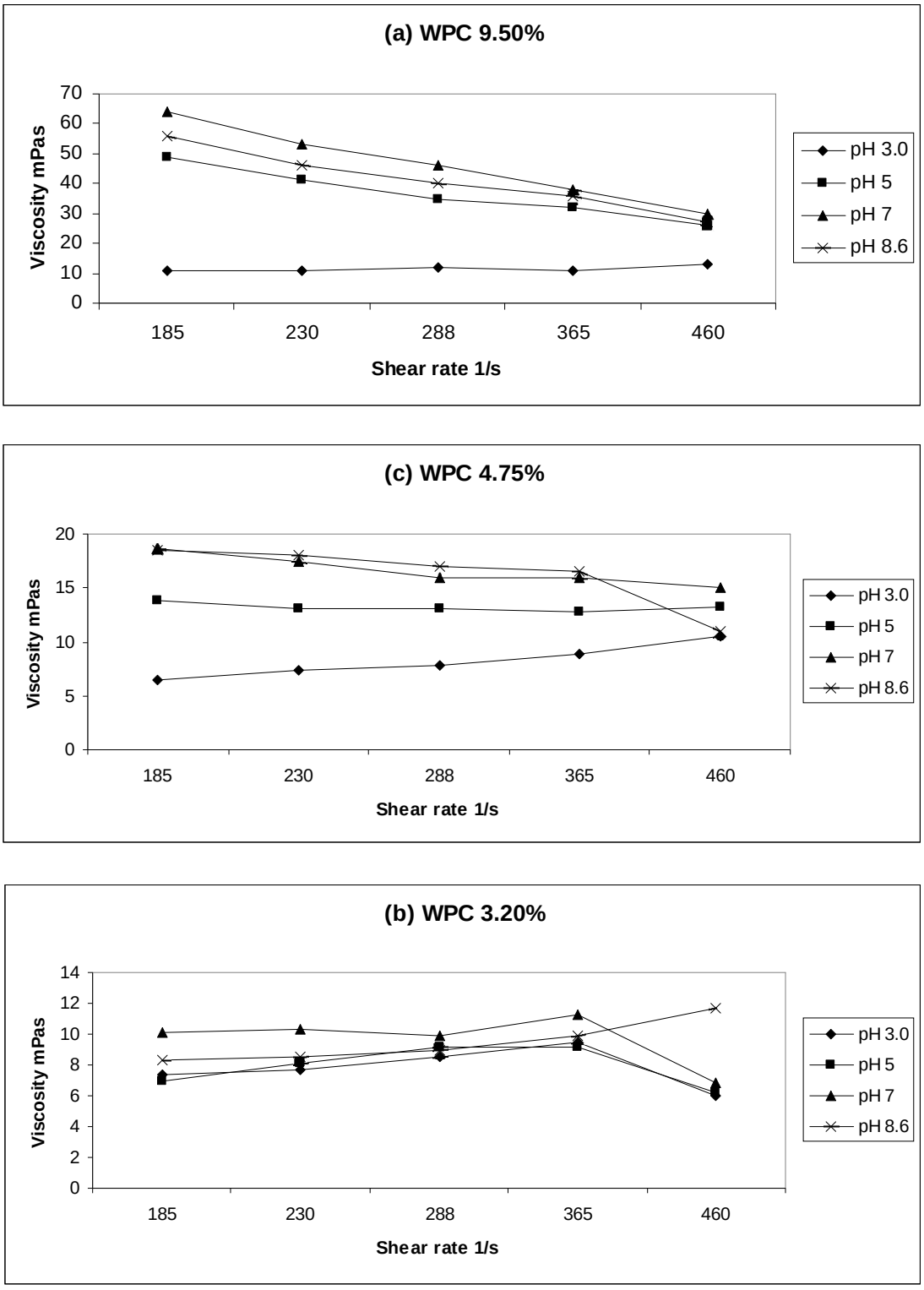


Fig. (8): Viscosity of heated WPC solutions at different concentration after three months freezing at different pHs

5. References:

1. Andersen, G.G.; Bech, A.M. and Munksgaard, L. (1983). Methods used for determination of functional properties of protein products at the Danish Government research Institute for Dairy Industry. Proceedings of IDF Symposium "Phisco-Chemical aspects of dehydrated protein rich milk products". Holsinger Denmark 17th -19th May 1983 PP .
2. Anema, S. G., & Klostermeyer, H. (1997). Heat-induced, pH dependent dissociation of casein micelles on heating reconstituted skim milk at temperatures below 100_C. Journal of Agricultural and Food Chemistry, 45, 1108–1115.
3. Antifantakis, E., C. Kehagias, I. Kotouza, and G. Kalatzopoulos. (1980). Frozen stability of sheeps milk under various conditions. Milchwissenschaft 35: 80-82.
4. AOAC, (1990). Offecial Methods of Analysis. 15th Ed. Association of Official analysis Chemists INC. Suite 400, 2200 Wilson Boulevard, Arlington, Virginia 22201, USA.
5. Barrent, A.J.G. and Tawab, A. (1957). J. Sci. Food Agric. (8) 437-441
6. Bastian, E. D. 1994. Sheep milk coagulation: Influence of freezing and thawing. Cultured Dairy Prod. J. 29(4):18-21.
7. Bhargava, A. And Jelen, P. (1995). Freezing of Whey Protein Concentrate Solutions and its Effect on Protein Functionality Indicators. Int. Dairy Journal (5) 533-541
8. Britten, M., Giroux, H. J., Jean, Y. & Rodrigues, N. (1993). Composite blends from heat denatured and undenatured whey proteins: emulsifying properties. Int. Dairy J., 4, 25-36.
9. Burova, T. V., Grinberg, N. V., Visschers, R. W., Grinberg, V. Ya., & de Kruif, C. G. (2002). Calorimetric study of porcine blactoglobulin at different pH. European Journal of Biochemistry, 269, 3958–3968.
10. Corredig, M., & Dalgleish, D. G. (1996). Effect of temperature and pH on the interactions of whey proteins with casein micelles in skim milk. Food Research International, 29(1), 49–55.
11. de Kruif, C. G., Hoffmann, M. A. M., van Marle, M. E., van Mil, P. J. J. M., Roefs, S. P. F. M., Verheul, M., & Zoon, P. (1995). Gelation of proteins from milk. Faraday Discussion, 101, 185–200.
12. de Wit, J. N. (1989). Functional properties of whey proteins. In Developments in Dairy Chemistry, ed. P. F. Fox. Elsevier, London, Vol. 4, pp. 305-6.
13. Farrag, A.F.; El-Garawany, G. and Abd El-Salam, M.H. (2006). Flow properties of concentrated soultions of casein glycomacropeptide. International Journal of Dairy Science 1 (2) 161-166.
14. Farrag, A.F.; Asker, A.A.; El-Din, H.M.F. and Abd El-Salam, M.H. (1997). Heat denaturation of whey protein comcentrate as affected by some factors. Milchwissenschaft 52 (4): 294 - 208.
15. Harper, W. J. (1984).Whey proteins. Food Technol. New Zealand, 19(3), 21
16. Howard, D. W. (1991). A look at viscosity. Food Technol., 45(7), 81-96.
17. Koschak, M. S., O. Fennema, C. H. Amundson, and J. Y. Lee. (1981). Protein stability of frozen milk as influenced by storage temperature and ultrafiltration. J. Food Sci. 46:1211-1217.
18. Kresic, G., Lelas, V., Herceg, Z & Rezek, A. (2006). Effects of high pressure on functionality of whey protein concentrate and whey protein isolate. Lait, 86, 303-315.
19. Kresic, G., Lelas, V., Jambrak, A. R., Herceg, Z. and Brncic, S. R. (2008). Influence of novel food processing technologies on the rheological and thermophysical properties of whey proteins. Journal of Food Engineering 87 (2008) 64–73
20. Law, A. J. R., & Leaver, J. (2000). Effect of pH on the thermal denaturation of whey proteins in milk. Journal of Agricultural and Food Chemistry, 48, 672–679.
21. Le Bon, C., Nicolai, T., & Durand, D. (1999). Growth and structure of aggregates of heat-denatured b-lactoglobulin. International Journal of Food Science and Technology, 34, 451–465.
22. Lelas, V., Herceg, Z., (2002). Influence of tribomechanical treatment on the phase transition temperatures of whey proteins model systems. In: Proceedings of the international conference on innovation in food processing technology and engineering, Bangkok, Thailand, pp. 24–34.
23. Ling, E. R. (1963). A Text Book of Dairy Chemistry Vol. II3 rd Ed. Chapman and Hall Ltd., London.
24. Mangino, M. E. (1992). Gelation of whey protein concentrates. Food Technol., 46(l), 114
25. Meza, B. F., R.A. Verdini, R. A. And Rubiolom, A.C. (2009). Viscoelastic behaviour of heat-treated whey protein concentrate suspensions Food Hydrocolloids (23) 661–666
26. Morr, C. V. & Foegeding, E. A. (1990). Composition and functionality of commercial whey and milk protein concentrates and isolates: a status report. Food Technol., 44(4), 100
27. Mulvihill, D.M., & Donovan, M. (1987). Whey proteins and their thermal denaturation – a review. Irish Journal of Food Science and Technology, 11, 43–75.
28. 3Patel, H. A., Singh, H., Havea, P., Considine, T., & Creamer, L. K. (2005). Pressure-induced unfolding and aggregation of the proteins in whey protein concentrate solutions. Journal of Agricultural and Food Chemistry, 53, 9590–9601.
29. Patocka, G., Cervenokova, R., Narine, S., Jelen, P., (2006). Rheological behaviour of dairy products as affected by soluble whey protein isolate. International Dairy Journal 16, 399–405.
30. Rattray, W. and Jelen, P. (1995). Viscous Behaviour of Whey Protein Concentrate Dispersions. Int. Dairy Journal 5 (1995) 673-684
31. Roefs, S. P. F. M., & de Kruif, C. G. (1994). A model for the denaturation and aggregation of b-lactoglobulin. European Journal of Biochemistry, 226, 883–889.
32. SAS, (1998) Statistical Analysis System. SAS User`s Guide Statistics. SAS Institute Inc., Editors, Cary, NC.
33. Schokker, E. P., Singh, H., & Creamer, L. K. (2000). Heat-induced aggregation of blactoglobulin A and B with a-lactalbumin. International Dairy Journal, 10, 843–853.
34. Simmons, M.J.H., Jayaraman, P., Fryer, P.J., (2007). The effect of temperature and shear rate on the aggregation of whey proteins and its implications for milk fouling. Journal of Food Engineering 79, 517– 528.
35. Ugolini, R., Ragona, L., Siletti, E., Fogolari, F., Visschers, R. W., Alting, A. C., & Molinari, H. (2001). Aggregation, stability and electrostatic potential properties of porcine b-lactoglobulin. European Journal of Biochemistry, 268, 4477–4488.
36. Vasbinder, A. J. and de Kruif, C. G. (2003). Casein–whey protein interactions in heated milk: the influence of pH. International Dairy Journal 13 (2003) 669–677
37. Young, P. (1985). The freezing of sheep milk for storage and transport. Sheep Dairy News 2(2):7-8.

6/10/2010

Evolution and Development Towards 4th Generation (4G) Mobile Communication Systems

M. Junaid Arshad, Amjad Farooq, Abad Shah
Department of Computer Science and Engineering, U.E.T., Lahore-Pakistan
junaidarshad@uet.edu.pk amjadfarooq@uet.edu.pk abadshah@uet.edu.pk

Abstract: It is the need of hour to get ourselves acquainted with the communication technology, its tools and its trends. Mobile communication is an important technology in this regard and mobile phone has become the most common tool of communication over the recent years. As several innovative improvements regarding mobile communication technologies have been made by developing various multiple-access schemes used for wireless communication (such as TDMA, FDMA, CDMA, WCDMA, EDGE etc) but a big challenge is to select the right technology for the applications and systematically identify the factors that influence the overall performance. In this research paper, we present the detail comparison of the different generations of the mobile communication technologies in a tabular form to have a better knowledge and understanding in the advancement of mobile communication systems. The survey presented here will be helpful for designing the new strategies for the development of 4th generation mobile communication systems. This research work can steer all those learners who are trying to enhance their acquaintance in the field of mobile communication system, and also for such mentors and researchers who desire to have a foundation for further research and study in this field. [Journal of American Science. 2010;6(12):63-68]. (ISSN: 1545-1003).

Keywords: Mobile Communication, Evolution, Generations, Comparison, Wireless Technologies

1. Introduction

The Internet is the network of large-scale group of connected computers around the world that sends out data using packet switching technique based on the TCP/IP stack. (Wright and Steven, 1995) With a continuous improvement in the field of communication technologies (ITU-R, 2000) and infrastructures by means of enhancing the functionalities of the wired/wireless protocols, the Internet has achieved the massive success and popularity. Over the time with the growing and accelerating progresses in communication patterns (wired and wireless) and wildly demands for spare capacity and connectivity, the Internet in almost every aspect frequently experiences modifications and changes in order to bring up-to-date. Along with the requirements supporting the Internet has been an implausible progress in the field of wire-less and mobile technologies. Since during the last few years, a countless fast improvements have been observed in the area of mobile and wireless technologies, and these two drastically rising user-driven service demands have significantly and intensely revolutionized the spirit and nature of worldwide telecommunications (Freeman and Roger, 2004) in these millenniums and have developed the modern telecom territory.

In this research work, we present the detail survey of the different generations of the mobile communication systems and our purpose is not to choose a victor because many of the technologies are still in progress. Somewhat, we anticipate to get in detail knowledge of the situations wherein different technologies would work better, and the sources of performance degradation.

The remainder of this paper is structured as follows. In Section 2, we give an overview of the different wireless technologies. In Section 3, we present the comparative analysis of 4th generation mobile communications system to the earlier generations (i.e., 1G, 2G and 3G) and progressively analyze their characteristics, and elaborate the various visions for the future utilization of 3G and 4G technologies. Finally in Section 4, we present the conclusions of this work.

2. Review of Literature

2.1 Introduction to 1G, 2G, 3G and 4G

The list of wire-less air interface protocols which follows signifies the most familiar standards in exercise around the world nowadays. They're structured almost by means of generations (i.e., 1st G, 2nd G, 3rd G and 4th G) of development and include various derivations or alternate terms used to describe them.

1st Generation (1G): 1G of technology was analog (voice-only) i.e., 1G wireless phones exercised analog technology. These devices were weighty and exposure was unreliable, however they effectively

presented the inherent easiness of mobile communications.

2nd Generation (2G): The 2G of technology is digital – in the United States, mostly devices & services are digital. Digital cellular services are vastly deployed world-wide. They presented a significant development in the quality of voice (since analog information is much subject to distortions as that of digital information) and enhanced capacity as well, as voice calls in a more efficient way can be multiplexed. It provides the different services like some degree of web-access facility, digital voice calling and short message service (SMS). The GSM, TDMA and CDMA are the few cell phone standards of 2G.

3rd Generation (3G): The 3G (Smith et al., 2000) of wireless technology is committed to provide reasonably speedy wireless communication to support more useful services such as data, video and multimedia as well as voice. The wire-less mobile communication technology in it's present form is often known as 2.5 G (Gozalvez et al., 2001) (called EDGE technology) but simply existing right networks of 3G in the United States are EVDO & UMTS (with HSPDA) offered by (Sprint/Verizon) and Cingular carriers, respectively. The 3G offers next to future advances into the business/private wire-less technology, particularly in a field of mobile communications and some expected capabilities and features of 3G systems are:

- Enhanced features for multimedia communications (i.e., digital data & voice, video & remote controls system)
- Supporting to utilize onto all advanced modes (i.e., electronic-mail, fax, paging, cellular-telephones, web-surfing, video-conferencing etc)
- Extensive bandwidth & high-speed capability (upwards of 2 Mbps)
- Providing flexibility for Routing(repeaters, satellites, LANs etc)

4th Generation (4G): The 4G (Woerner, 2001) of wireless technology is still underway and stands to be the upcoming wireless devices standard. The key difference between 4G and 3G technologies is the improved data transfer rates and security, like it is for 3G over 2.5G or 2G. The 4G is looked forward to offer more enhanced versions of the same advancements promised by 3G (e.g., improved-multimedia, video-streaming, global-access and worldwide-portability through all kinds of devices).

Normally, a generation is defined by the result of technology grows over a time-frame of ten-to-fifteen year, hence, 4G would turn to whatever is deployed in the 2010 period onward, supposing 3G exploitation covers the 2000-2010 period.

Somewhat, 4G would provide customers with on demand first-class video and audio by utilizing OFDM (Robertson, 1999) (Orthogonal Frequency Division Multiplexing) technique and would able to better allocate network resources to multiple clients by making use of multiple channels simultaneously. Unlike the 3G networks which are a mixture of packet-switching and circuit-switching networks, 4G (Hui et al., 2003) will be based on packet switching only and might actually connects the whole world and be operable from any place above or on the surface of the globe.

2.2 Code Division Multiple Access (CDMA)

CDMA (Gharavi et al., 2001) is a type of multiplexing which doesn't share the channels by frequency or time (like FDMA or TDMA), however it en-codes information by some unique codes related to each channel and exploits dynamic interferences effects of a particular code to do multiplexing. In addition, it refers to a digital cellular telephony system which utilizes these multiple access schemes. CDMA has since been applied in a number of communication systems (i.e., Omni-TRACS satellite system (Freeman et al., 2004) and Global Positioning System (GPS) (Hatch et al., 2002)). Figure 1 presents a general architecture of a CDMA system.

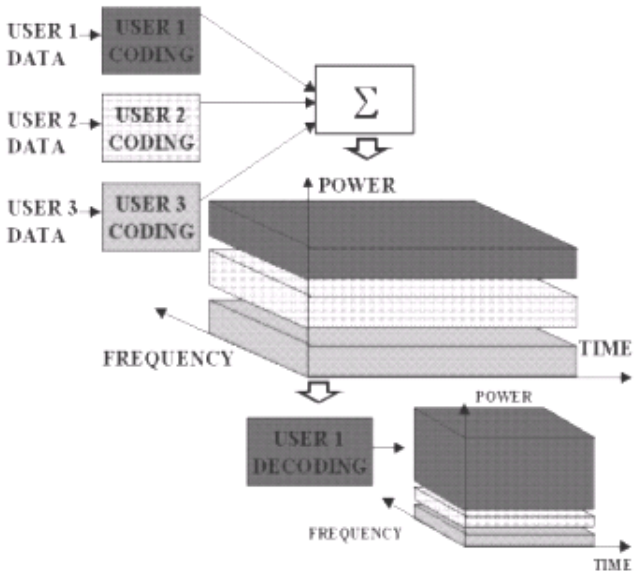


Figure 1. Working of CDMA system

2.3 Wideband Code Division Multiple Access (WCDMA)

WCDMA (Bidaud, 2001) is a type of wideband digital radio-access technology. In 1998, the European Telecommunications Standard Institute (ETSI) used it to support 3G multimedia applications for wideband digital radio-access. WCDMA offers an innovative service capability, improved network speed and low-cost for services (data and voice) compared to 2G technologies. It is the foremost worldwide 3G-standard preferred by eight of the world's ten largest service-providers. Operators can softly progress from GSM on the way to WCDMA, hence saving investments via reutilizing the GSM setups and services (Gozalvez et al., 2001).

2.4 Global System for Mobile Communications (GSM)

For mobile communications the most well-liked standard in the world is GSM (Bach, 2000). More than 2-billion people across the world (over 250 countries/territories) use the services offered by the GSM. It distinguishes much from its ancestors given that collectively speech and signaling channels are digital, indicating that it is assumed as the 2G mobile communication system. GSM now becomes an open standard that is presently proposed by the 3GPP. According to the consumer judgment, the GSM system's main advantage has been high-quality digital-voice channels and reduced price alternative to make calls (like SMS) and from a network operator prospective, it has been the potential to employ equipments as of various providers as the open standards accepts simple inter-compatibility. In addition, these standards permitted networks providers to allow roaming facilities that means the customers can utilize their phones whole across the world. Figure 2 illustrates the general architecture of a GSM system.

2.5 General Packet Radio Service (GPRS)

The GSM mobile phones users get benefit from a new mobile data service called GPRS (Bratton et al., 2001). The GPRS is an imperative step in the evolution of 2G mobile systems towards 3G systems. It offers a reasonable data transfer speed, via utilizing unoccupied TDMA channels in the GSM networks. It provides friendly billing system, high capacity channel and reduced call setup time. The GPRS system is optimized for the packet switched data networks and is transition totally from the cellular network of circuit switched to packet switched. Figure 3 describes the general architecture of a GPRS system. GPRS improves GSM data services providing:

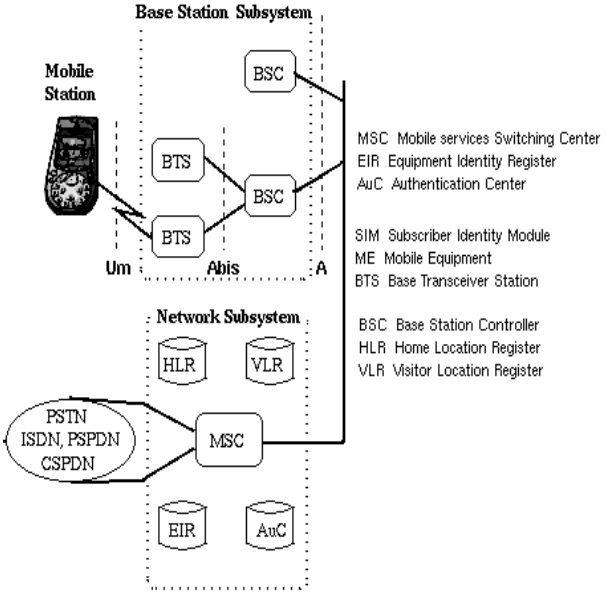


Figure 2. GSM system's architecture

- offer variety of services at reasonably low-cost to support flexible co-existence using GSM voice
- provide bursty traffic support
- possible support for connectivity to the Internet
- providing fast-access time
- utilize network and radio resources efficiently

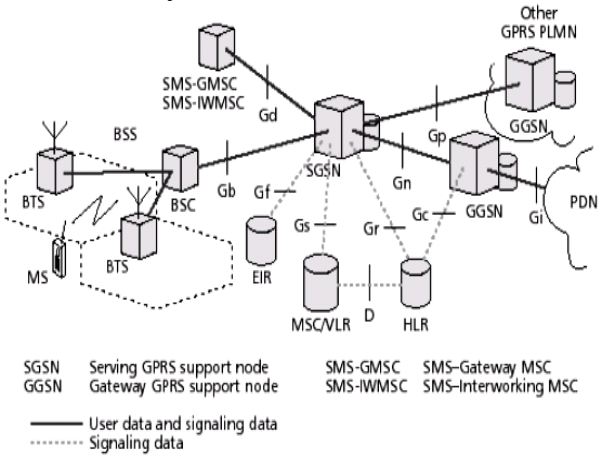


Figure 3. GPRS system's architecture

3. Comparison of Technologies

3.1 Tabular Comparison of 1G, 2G and 3G Technologies

Table 1 shows the comparison of the three technologies (i.e., 1G, 2G and 3G) in terms of data services currently offered by the different carriers.

3.2 Tabular Comparison of 3G and 4G Technologies

The 3G is the next step leaving beyond the 2G standards (for instance CDMA and GSM). The important aspects of 3G consist of the skills to merge the cellular mobile networks with the internet, adding multimedia applications to a wireless computer/mobile phone by improving the range and QoS of voice and data services. In near future, the 4G is the upcoming technology of wire-less networks which will switch from 3G to 4G networks. To understand both the technologies more clearly their detail comparison are as follows (Table 2):

Table 1: Comparison of 1G, 2G and 3G technologies

Generation	Technology	Features
1st Generation (1G)	AMPS (Advanced Mobile Phone Service)	Support voice service: Analog, data services: No
	CDMA (Code Division Multiple Access)	Analog Cellular (Discontinued) Speed: 9.2-kbits/sec
2nd Generation (2G)	Time Division Multiple Access (TDMA) Personal Digital Cellular (PDC)	PDC & TDMA (only support one-way data transmission) Offers advance calling options as caller ID Not constantly on data connections
	Code Division Multiple Access (CDMA)	CDMA carriers: Sprint, Verizon Technology: 1xRTT Digital voice service Speed: 128-Kbits/sec
	Global System for Mobile Communications (GSM)	GSM carriers: Cingular, Nextel, T-Mobile Technology: GPRS, EDGE, UMTS Speed: 40-160-kbits/sec
3rd Generation (3G)	Wideband Code Division Multiple Access (WCDMA)	Carriers: Cingular Technology: UMTS Excellent voice qualities About 2-Mbits/s Constantly on data connection
	CDMA-2000	CDMA carriers: Sprint, Verizon Technology: EV-DO Speed: 500-700-kbits/sec Based on the Interim Standards (95) CDMA standards
	Time-Division Synchronous Code Division Multiple Access (TD-SCDMA)	Supports broad-band data services (such as multimedia & video), Improved roaming features

Table 2: Comparison of 3G and 4G technologies

Key Parameters	3rd Generation (3G)	4th Generation (4G)
(1) Speed	**3G has the ability to utilize circuit/packet data at higher bit rates** 144 kb/s or higher in high capacity vehicular-traffic. 384 kb/s for pedestrian traffic. 2 Mb/s or greater for indoor-traffic.	**4G can support data rates up to 20 to 100 Mbps in mobile mode** A developed wireless corporation NTT-DoCoMo is evaluating 4 G tech over 100 Mb/s (when moving) & 1 Gbit/s (when it is still)
(2) Bandwidth	**3G uses 5to20 MHz Bandwidth** A radio signal bandwidth is defined as being the difference b/w the upper/lower frequencies of the signal. The bandwidth amount required for 3G service would be as more as 15to20 MHz.	**4G has the absolute bandwidth-range of 100MHz or above** The bandwidth could be as much as (100MHz) and data could be sent at much highly rates. The data sending cost could be relatively much low and worldwide mobility could be probably 100MHz or above.
(3) Switching Design Basis	**3G is relay on packet switching or circuit switching** A few 3G aspects also utilize packet switching. Circuit switching leads PSTN-public switched telephone network. Networks	**4G utilizes packet-switching** Packet-switching dominating the data-networks such as the internet. Through circuit switching, whole packets send directly towards the receiver in an arranged manner, over a single track in sequence. Remaining

	resources establish calls through the most capable route.	packets from other calls race upon such circuits too, making the mostly use of each path or flow, pretty different the circuit switching calls which engage a single path to the omission of all others.
(4) Access Technologies	**3G utilizes CDMA-2000 & WCDMA as access technologies** WCDMA offers speeds b/w 384kb/s & 2Mb/s. If this protocol is deployed over a WAN, the maximum-speed is 384kb/s. If it is employed in a LAN, the upper-speed is 2Mb/s. It is approved also by the ITU. The others important 3G standards are CDMA2000 that is product of the initial 2G CDMA IS-95 standard. The different transmission technology utilized in CDMA2000 that are 1xRTT, CDMA-2000-1xEV_DV & 1xEV_DO.	**4G is based on OFDM/OFDMA** 4G uses OFDM/OFDMA to better distribute networks resources among the available users. 4G enable equipments permit to utilize available bandwidth and to make utilizing multiple channels parallely. In OFDM, pulse making task & modulation can be done via an easy IDFT that can be deployed much better as that of IFFT. Thus, in the receiver we requie only a FFT for reversing this process.
(5) Frequency Band	**Country dependent/continent (1800to2400 MHz)** In Europe, the satellite services utilize the 1980to2010 MHz for uplink-bands & 2170to2200 MHz for downlink. In United States, 45MHz of capacity in the 1710to1755 MHz band & 45MHz of space in the 2110to2170 band for 3G applications are available.	**High frequency bands capability (2to8 GHz)** Mobile communication 4G systems will have to exploit the frequency band as competently as feasible, with the minimum possible transmission power.
(6) Network Architecture	Cell-Based Wide-Area (WAN)	Wire-less LANs Hybrid Integrations (WiFi/Bluetooth) & Wide Area
(7) Forward Error Correction	Convolutional rate: (1/2), (1/3)	Concatenated Coding Schemes
(8) Component Design	Optimized antenna design, multi-bands adapter	Smart Enabled Antenna, softwares multi-bands & wide-band radio
(9) Major Requirement Driving Architecture	Pre-dominantly voice driven: data was constantly add-on	Converged data & VoIP (Voice Over IP)
(10) Internet Protocol	Numerous air link protocols, plus IP 5.0	Up to (IP 6.0)
(11) Backup Compatibility	3G CDMA-2000 is backward compatible to the 2G IS-95 standards. It supports less compatibility.	4G enhances the 3G capacity/capability by an order of magnitude.
(12) Market Overview	**(a) Lacks of Demand** The 3G services market diffusion has been slow as estimated initially because of fewer demands for developed services lately exploitation by the service providers & challenges relevant to QoS and convergence. **(b) Other Challenges** Other challenges are operational costs that are considerably greater than in a 2G/2.5G operation and doubt surrounding nearby demand for non-voice qualities	**(a) High-Speed Multimedia Service Demand** **(b) Fixed-Mobile Convergence** **(c) Issues of Spectrum** **(d) Issues of Standards and Certification** **(e) Technological Challenges** **(f) Alternative Services/Applications for 4G**
(13) Mobile Top Speeds	200 kilometer per hour	200 kilometer per hour
(14) Cost Comparison	It's working costs that are considerably larger than in a 2G-2.5G system and doubt nearby estimate requirement for non-voice qualities.	At this phase, no certified 4G networks devices have been commercialised that's why costs are not determined. Though, it is sensible to guess that unverified latest technologies will initially be a focus for less demand hence, costs will be comparatively large & will only reduce slowly as demand raises. Because of the beginning price of CPEs, 4G vendors are assuming to provide leasing contracts.

4. Conclusions

In this research work, we have surveyed four wireless technologies namely 1G, 2G, 3G and 4G. We conclude that the 4G mobile technologies will stimulate subscriber interest in broadband wireless applications because of its ability and flexibility towards the world of wireless mobile communications. A concentrated effort seems to categorize how wire-less mobile technologies can accompaniment a more user focused world of wire-less. Finally the report elaborates the different Mobile Communication Technologies that have been developed in the past and their evolution and development towards 4th generation communication systems. Their detail comparison with each other has been discussed to have a better knowledge and understanding about the technological advancement made towards the evolution and development of 4th generation communication systems. In this research work, we have tried to gather as much information as possible and assemble it in such a manner that the reader can gain maximum knowledge of the topic.

Acknowledgements:

This work was supported by the Directorate of Research Extension and Advisory Services, University of Engineering and Technology (UET), Lahore-Pakistan.

Corresponding Author:
Dr. M. Junaid Arshad
Department of Computer Science & Engineering,
University of Engineering and Technology, Lahore-Pakistan. Email: junaidarshad@uet.edu.pk

References

1. Wright, G., and Stevens, W. TCP/IP Illustrated, Volume 2: The Implementation, Addison-Wesley, Boston, MA., January, 1995.
2. (ITU–R) M.1645: Framework and overall objectives of the future development of IMT–2000 and systems beyond IMT–2000.
3. Smith, C., and Collins, D. 3G Wireless Networks, page. 136, 2000. http://searchnetworking.techtarget.com/sDefinition/0,,sid7_gci214486,00.html
4. Woerner, B. Research Directions for Fourth Generation Wireless. Proceedings of the 10th International Workshops on Enabling Technologies: Infrastructure for Collaborative Enterprises (WET ICE). Massachusetts Institute of Technology, Cambridge, MA, USA. June 2001.
5. Freeman, and Roger, L. Telecommunication System Engineering, John Wiley & Sons, Inc., 3rd Edition, 2004.
6. Robertson, P., and Kaiser, S. The effects of Doppler spreads in OFDM(A) mobile radio systems, Vehicular Technology Conference, 1999. (VTC 1999 - Fall. IEEE VTS). OFDM, http://www.xilinx.com/publications/products/v2/xc_pdf/wireless.pdf
7. Hui, SY., Yeung, KH. Challenges in the migration to 4G mobile systems. Communications Magazine, IEEE (City Univ. of Hong Kong, China), December 2003.
8. Gharavi, H., Millington, R., and Chin, F. CDMA-2000 Reverse-link Evaluation. Proceedings of the IEEE International Conference on Third Generation Wireless and Beyond, San Francisco May 2001. [CDMA, http://www.webopedia.com/TERM/C/CDMA.html]
9. Hatch, Ron et al., Starfire: A Global high Accuracy Differential GPS System, Navcom Technology, Inc. November 2002.
10. Bidaud, Bertrand. First Asia/Pacific 3G Auction Completed: Gartner Dataquest Analysis, Telecommunications Televiews, Issue 4, January 25, 2001. http://www.ericsson.com/technology/tech_articles/WCDMA.shtml
11. Bach, D. International Cooperation and the Logic of Networks: Europe and the Global System for Mobile Communications (GSM), Proceeding of 12th International Conference of Europeanists, Chicago IL. April 1, 2000. (GSM), http://en.wikipedia.org/wiki/GSM
12. Gozalvez et al,. An Overview of the GSM System. Communications Division, Department of Electronic and Electrical Engineering, University of Strathclyde, Glasgow, Scotland. Link:http:www.comms.eee.strath.ac.uk/~gozalvez/gsm/gsm.html. May, 22, 2001.
13. Bratton, W., Jameson, J., and Pentland, S. Analysis: 3G madness – time for some moderation! Totaltele.com, July 16, 2001. (GPRS), http://en.wikipedia.org/wiki/GPRS

6/26/2010

A Layered approach for Similarity Measurement between Ontologies

Amjad Farooq, M. Junaid Arshad and Abad Shah

Computer Science and Engineering Department
University of Engineering and Technology, Lahore-Pakistan
amjadfarooquet@gmail.com

Abstract: With the vision of Semantic Web, the ontology operations such as aligning, merging and mapping have gained much importance. The measuring of similarity between concepts of source ontologies is preprocessing of all these operations. Several techniques have been proposed for measuring similarity between concepts based on their lexical, taxonomic and elementary characteristics but a very little attention has been given on their non-taxonomic relations. We have observed that lexically similarity between concepts is mandatory in order to their taxonomic similarity. Furthermore, the taxonomic similarity between two concepts is pre-requisite of their non-taxonomic similarity. This motivates that if the similarity measurement process is made in layered fashion then it will become more efficient. In this paper, a new technique is proposed that includes non-taxonomic relations of concepts along with their lexical and taxonomic characteristics while measuring their similarities. The proposed technique works in a layered fashion that enables the measuring process more efficient. [Journal of American Science. 2010;6(12):69-77]. (ISSN: 1545-1003).

Keywords: Ontology Matching, Lexical Similarity, Taxonomic Similarity, non-taxonomic similarity

1. Introduction

Nowadays the web has become the main source of information but the semantic heterogeneity is its main bottleneck in the retrieval of relevant information. The semantic web proposed its solutions but still the problem is not fully solved. Semantic web is mainly based on ontologies whereas ontologies themselves suffer from heterogeneity when simultaneously used in some integrating processes such as merging, aligning and mapping. Those ontologies may contain some lexically similar concepts belonging to different context and likewise some contextually similar concepts may have different roles or granularities in their respective ontologies (Farooq and Shah, 2010). When such ontologies are required to reuse simultaneously in some operations for sharing and acquiring of information, the heterogeneity usually arises and then it is required to find the similarity between their concepts to handle the situation.

With respect to ontology, a concept is defined as a class of objects or individuals with some common elementary, taxonomic and non-taxonomic characteristics. A concept has a certain name with some synonyms. Usually, it is known by its taxonomic characteristics (parents, children and siblings), and the non-taxonomic characteristics it keeps in a certain domain in addition to its name or synonyms.

Motivations

- Usually a concept is known by the role it keeps in its respective domain rather than by its parent, sub and/or sibling concepts, therefore the similarity of concepts based on their roles should be properly measured.
- Some pairs of similar concepts are discarded during the measuring of their taxonomic similarity because they have un-similar immediate parent, sub or sibling concepts. This motivates that there is a need of change in the measuring process of taxonomic similarity.
- Some pairs of similar concepts are discarded during the measuring of their lexical similarity because the terms used to name them are not similar. This motivates that there is a need of change in the measuring process of lexical similarity.
- There may some concept those are lexically similar but taxonomically they are not similar but vive versa is not true. Similarly taxonomically similar concepts may be un-similar with respect to their roles but again the vice versa is not true. This motivates that the measuring process should be in some layered fashioned to make it efficient.

- There should be an integrated language-independent technique for measuring lexical, taxonomic and role-based similarities between concepts of ontologies whereas the measuring process should be at conceptual levels of ontologies to make it language independent.

In this paper we propose an integrated language-independent technique, for measuring similarity between concepts of two ontologies by taking into consideration the above motivations to achieve the following objectives: (a) None of similar pair of concepts should remain undetected or eliminated. (b) The role-based similar concepts between ontologies should be determined. (c) The measure process should be more efficient, complete and realistic.

The paper is structured as follows. The related work is briefly overviewed in Section 2. The proposed technique is given in Section 3 and it is validated through a case study in Section 4. Finally the paper is concluded with future directions in Section 5.

2. Background and Related Work

In lexical similarity, the terms used to represent concepts, are compared. Different techniques such as (i) edit-distance (ii) prefix (iii) suffix and (iv) n-gram as surveyed in (Lee et al., 2001) are used to measure the degree of similarities between terms. A method (Muller et al., 2006) known as edit distance is mostly used for measuring the similarity between two terms. In this method, the similarity is measured based on the number of insertions, deletions and substitutions to transform one term into other. The degree of similarity between two concepts based on their terms can be measured using a metric as proposed in (Madhavan et al., 2001), based on (Muller et al., 2006) and that metric is:

$DoS_{Lex}=$

$$Max(0, \frac{Min(Length(a), Length(b)) - NoOfIDS(a,b)}{Min(Length(a), Length(b))}) \in [0,1] \quad (1)$$

In above Equation, the *NoOfIDS* is a function that returns integer-value equal to the number of insertion, deletion or substitutions to transform term *a* into *b* or vice versa. In some scenarios, the Equation 1 *does not give accurate results* e.g. the degree of similarity between terms *Deptt* and *Department* of respective ontologies *A and B* is 0.25 i.e. these are partially similar according to this equation, although both terms represent the same concept.

The two concepts are rendered similar taxonomically (Miller, 1995; Noy and Musen, 2001; Giunchiglia et al., 2007; Aleksovski et al., 2006) if i) their direct super-concepts are similar; ii) their sibling-concepts are similar; iii) their direct sub-concepts are similar; iv) their descendant-concepts are similar; v) their leaf-concepts are similar and vi) concepts in the paths from the root to those concepts are similar. Irrespective of the structural aligning technique used, we have observed that *certain pairs of similar concepts are categorized dissimilar* because of bias of above mentioned criteria towards those concepts whose siblings-concepts, sub-concepts or direct super-concepts are not similar. Secondly, the roles of concepts represented via non-taxonomic relations are not properly incorporated in the similarity measuring process.

The non-taxonomic relations of a concept represent its roles and in most of domains, a concept is known by the role it keeps. However, in some domains the concepts have no intellectual properties e.g. in ontology of a furniture domain, the concepts like chair, table and desk have only taxonomic (i.e. parent, child, sibling) and elementary (i.e. color, type, etc.) characteristics. For such situation the granularities of concepts should be used for measuring semantic relations.

In (Erhard and Philip, 2001; Lambrix and Tan, 2006; Shvaiko and Euzenat, 2005; Hariri et al.,, 2006), the similarities between concepts are measured based on their taxonomic properties (parents, siblings and children concepts) and the degree of similarity between two ontologies may decrease because of over-looking of some pairs of similar concepts in these approaches. The measuring of similarities of concepts based on different criteria is discussed in (Lambrix and Tan, 2006) where a software package WordNet (Miller, 1995) has been used to measure the semantic similarity between a pair of concepts through their synonyms (Giunchiglia et al., 2007). If the similarity score is above a given threshold then the concepts are considered to be similar. In order to identify semantic equivalence between concepts of different ontologies, only SubClassOf, Generalize, partOf and InstanceOf relationships with predefined semantics have been considered. Several ontology alignment tools are reviewed and a new tool for ontology alignment is described in (Isabel et al., 2007). Mostly these tools have XML-schema orientation. That is, the ontologies are represented into XML trees. XML nodes are taken as concepts. Their similarities are computed on the bases of similarities of their respective parents and sub-nodes.

In (Maedche and Staab, 2002), a set of similarity measures for ontologies at lexical and conceptual levels of their concepts have been proposed. Similarity measures at lexical level compare the terms used for

concepts in ontology but at conceptual level the similarity is computed from hierarchical relations existing between those terms. Schema-based matching techniques and systems have been surveyed in (Erhard and Philip, 2001), in which techniques are grouped into terminological, structural and semantic categories. The terminological techniques are further divided into string-based and language-based categories. Structural category includes all taxonomy-based and graph-based techniques whereas the semantic category includes all model-based techniques such as propositional satisfiability and description logics reasoning techniques. In (Aleksovski et al., 2006), the background knowledge of domain has been used via ontology to determine similarity between concepts of two ontologies, especially for those concepts which are not lexically and structurally similar. A similar work was presented in (Aleksovski et al., 2006), and it has been evaluated by matching a medical ontology to another, while using comprehensive medical domain ontology as background knowledge. The key consideration of this technique is if source ontologies are missing some non-taxonomic or logical relations between concepts, then for those logical relations, the third ontology i.e. the comprehensive domain ontology can be consulted while measuring similarity for those concepts. This technique is well suited for those ontologies having very poor taxonomic and non-taxonomic relations between concepts.

3. Proposed Technique

As stated earlier:

(i) Usually a concept is also known by the non-taxonomic relations it keeps in its respective domain in addition to its other characteristics; therefore the non-taxonomic relation based similarity of concepts should be measured.

(ii) To make result more complete and accurate, the taxonomic similarity of two concepts should be based on the similarity of their respective parents only whereas the similarity of sub and sibling concepts should be relaxed to determine all pairs of similar concepts.

(iii) The lexical similarity should be measured via domain-vocabulary of respective system instead of using existing techniques such as edit-distance, prefix, suffix and n-gram to make result more complete and accurate.

(iv) To make measuring process more efficient, the similarity should be measured in a layered fashion because there is no need to measure the contextual similarity for primarily un-similar concepts. Furthermore, there is no need to measure role-based similarity for those concepts which are contextually un-similar.

(v) There should be an integrated technique for measuring primarily, contextual and role-based similarities between concepts of ontologies whereas the measuring process should be at conceptual levels of ontologies to make it language independent.

The proposed technique fulfills these requirements as said above. It works in three phases as shown in Figure 1. First of all we describe the main terms used in the proposed technique:

Primary similarity may be called as conceptual similarity or 1st level of similarity and it is updated form of lexical similarity. Since in ontologies, the concepts are represented via terms, therefore while measuring primarily similarity we identify the corresponding terms between source ontologies, representing the exactly-same or similar concepts in addition to representing entirely different concepts.

Taxonomic similarity: Two concepts are contextually similar if and only if they possess primarily similarity and there are one or more common concepts in their respective parent-concepts. It may also be called 2nd level of similarity and it is updated form of taxonomic similarity.

Non-taxonomic similarity: This is a 3rd level of similarity between concepts. Two concepts possess 3rd level of similarity if and only they have second level similarity and they have similar roles i.e. their interaction with concepts other than parent, children and sibling concepts, in their respective domains.

The input ontologies are taken in triple-forms where each triple consists of three parts i.e. subject, predicate and object. There are some preprocessing activities of acquiring concepts, their super-concepts and their roles. The details of preprocessing are omitted here just for sake of simplicity. The concepts of source ontologies *A* and *B* are taken into sets CS_A and CS_B as mathematically represented in Equations 1 and 2 respectively.

$$CS_A = \{a_i \mid \forall\ a_i \in A\} \qquad (1)$$

$$CS_B = \{b_j \mid \forall\ b_j \in B\} \qquad (2)$$

Since contextual similarity of two concepts is based on the similarity of their respective parent concepts, therefore in order to it we need the parent-concepts of each concept. The parents of each concept of *A* and *B* ontologies are separately acquired in two sets i.e. C^PS_A and C^PS_B, formally defined as:

$C^PS_A = \{(a_i, p_i) \mid \forall\ a_i, p_i \in A \wedge p_i\ isParentOf(a_i)\}$ (3)

$C^PS_B = \{(b_j, p_j) \mid \forall\ b_j, p_i \in B \wedge p_j\ isParentOf(b_j)\}$ (4)

Similarly, to measure the role-based similarity we need to acquire the roles of concepts. The roles of each concept of *A* and *B* ontologies are separately acquired in two vectors i.e. C^RS_A and C^RS_B, formally defined as:

$C^RS_A = \{(a_i, r_i) \mid \forall\ a_i, r_i \in A \wedge r_i\ isRoleOf(a_i)\}$ (5)

$C^RS_B = \{(b_j, r_j) \mid \forall\ b_j, r_j \in B \wedge r_j\ isRoleOf(b_j)\}$ (6)

Phase-1: Measurement of Primary Similarity

The primarily similarity as defined earlier, is not the same as terminological similarity as reported in literature because we focus is on concepts rather than terms used to represent them.

We measure the first-level similarity between concepts via a domain-specific vocabulary that contains the terms-names, abbreviated-names, synonyms and hyponyms of those concepts. While populating synonyms and hyponyms of a concept the WordNet can be used as helping aid.

The measuring process of first-level of similarity is given in algorithmic form in Figure 1. Let DV be the domain-specific vocabulary of source ontologies A and B whose similarity is to be determined. Each element of *DV* has four components: (i) *name* (term that is exactly the same spelled as concept); (ii) *aName* - the abbreviated-names, (iii) *sName* - the synonyms and (iv) *hName* - the hyponyms of a concept. The output of this phase is a vector containing pairs of similar concepts with semantic relations exist concepts of each pair separately.

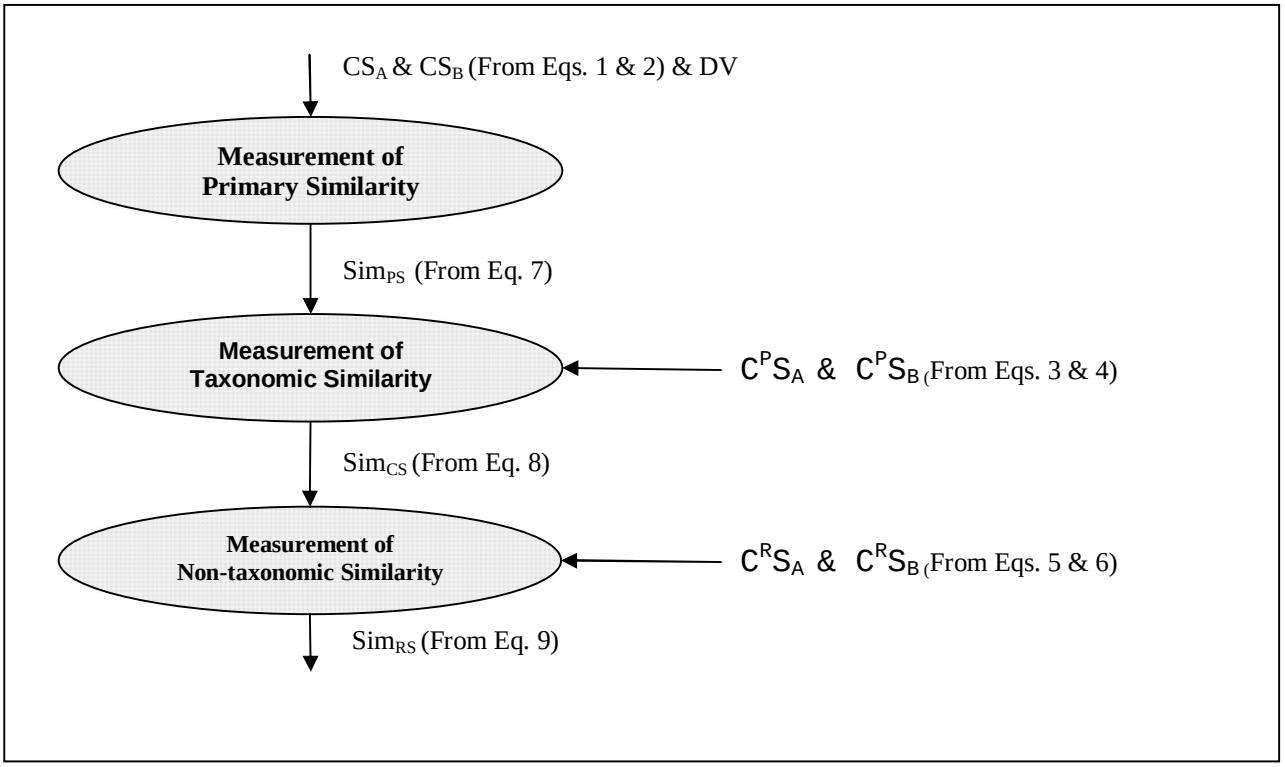


Figure 1: Outline of proposed technique

The terms used for concepts in both source ontologies A and B, as obtained in sets CS_A, CS_B (from Eqs. 1 & 2) is input and a set as formally defined in Equation. 7, containing pairs of primarily similar concepts is obtained as output of this phase. A slice of pseudo code of this phase is given in Figure 3.

$Sim_{PS} = \{(a_i, b_j, SR) \mid \forall a_i \in CS_A \wedge b_j \in CS_B\}$ (7)

The terms a_i and b_j holds a semantic relation *SR* and this may be equal (=), more generic ($\supseteq$) or more specific ($\subseteq$), i.e. ai = bi or ai $\supseteq$ bj or ai $\subseteq$ bj.

$$DoS_{PS} = LexSim(A:ai, B) \ return \ P$$

The *P*, in above expression, is a vector containing pairs of terms a_i and b_j with semantic relation *SR* i.e., $P = (a_i, b_j, SR)$

There may be no b_j exactly similar to a_i, and there may be multiple b_j*s* that are more specific that a_i and/or multiple b_js that are more generic than b_js. In that cases, we have opted two strategies i.e. up-ward and down-ward strategies. In up-word strategy, we choose a pair (a_i, b_j) with *SR* such that b_j is least granular in all b_js. Similarly in down-ward strategy we choose a pair with b_j having the maximum granularity. If there is no b_j similar to a_i then a_i is declared entirely different term. In that case p = (a_i, null, null) will be returned and this pair is not included in the resultant vector and it is simply discarded. For mapping, aligning and merging of ontologies, the correspondence between their similar concepts are required. It is required to find exactly equivalent, and the semantic relations between similar concepts

Phase-2: Measurement of Taxonomic Similarity

This phase takes C^pS_A, C^pS_B (from Eqs. 3 & 4) and Sim_{Lex} (from Eq. 7) as input and returns a set Sim_{CS}, formally defined in Eq. 8, containing pairs of taxonomically similar concepts as output.

$$Sim_{CS} = \{(a_i, b_j, SR) \mid \forall a_i, b_j \in Sim_{PS} \wedge \exists \ isSameParent(C^pS_A(a_i), C^pS_B(b_j)))\} \quad (8)$$

Taxonomic similarity is based on taxonomic positions of a_i and b_j. To measure this similarity, we need to measure the similarity between their respective parents. A slice of pseudo code of measuring process of taxonomic similarity is given in Figure 4.

```
Algorithm: Measurement of primary similarity

Input:  two vectors CSA, CSB (from Eqs. 1 & 2), DV (Domain Vocabulary)
Output: SimPS (From Eq. 7); a vector containing pairs of primarily similar concepts
Begin
For each a in CSA
  For each d in DV
   IF d.name.equal(a)OR d.aName.found(a)OR d.sName.found(a)
   THEN {tempA.add(d.rowId, a, 1); break;}
   Else IF a.hName.found(a)
   THEN {tempA.add(d.rowId, a, 2); break;}
  Next
Next
For each b in CSB
For each a in tempA
IF (a.level=1) AND ( DV[a.rowId].name.equal(a) OR
    DV[a.rowId].aName.found(b) OR DV[a.rowId].sName.found(b)
THEN { SimPS.add(a, b, '='); break;}
ELSEIF (a.level=1) AND  DV[a.rowId].hName.found(b)
THEN      { SimPS.add(a, b, '⊇'); break;}
ELSEIF (a.level=2) AND (DV[a.rowId].name.equal(a) OR
       DV[a.rowId].aName.found(b)  OR  DV[a.rowId].sName.found(b)
THEN { SimPS.add(a, b, '⊆'); break;}
ELSE      // a and b are dissimilar
Next
Next
End
```

Figure 2: A slice of pseudo code for measuring primary similarity

```
Algorithm: Measurement of taxonomic similarity
Input:(i) Two vectors   CpSA, CpSB (From Eqs. 3 & 4);
      (ii) SimPS vector (From Eq. 7)
Output: SimCS (From Eq. 8); a vector containing pairs of taxonomically
similar concepts
Begin
      For each p in LexSim
            parentCa = CpSA.getParents(p.Ca)
            parentCb = CpSB.getParents(p.Cb)
            same = isSameParent(parentCa, parentCb)
            If same Then SimCS.add(p)
      Next
         -  - -
         -  - -
         -  - -
      End.
```

Figure 4: A slice of pseudo code for measuring taxonomic similarity

```
Algorithm: Measurement of non-taxonomic similarity
Input:(i) CRSA, CRSB (From Eqs. 5 & 6);
      (ii) SimTax (From Eq. 8)
Output: SimNonTax (From Eq. 9); a vector containing pairs of non-
taxonomically similar concepts.
Begin
For each p in SimTax
      NTRCa= CRSA.getNTRs(p.Ca)
      NTRCb= CRSB.getNTRs(p.Cb)
      same = isSame(NTRCa, NTRCb)
      If same Then  SimNonTax.add(p)
Next
         -  - -
         -  - -
         -  - -
End.
```

Figure 5: A slice of pseudo code for measurement of non-taxonomic similarity

Phase-3: Measurement of Non-taxonomic Similarity

It is based on roles of concepts. In a domain, usually a concept is known by the roles it keeps, in addition to its parents, children, siblings and attributes. The non-taxonomic relations represent roles of concepts and their parts as well. If some pairs of concepts have no intellectual characteristics then they may have no roles. In that case those pairs of concepts possess third level of similarity implicitly. Figure 5, depicts a slice of pseudo code of measuring process of non-taxonomic similarity.

The C^RS_A, C^RS_B (from Eqs. 5 & 6) and Sim_{CS} (from Eq. 8) is the input and a set Sim_{RS}, formally defined

in Eq. 9, containing pairs of similar concepts based on their roles, is output of this phase.

$$Sim_{RS} = \{(a_i, b_j, SR) \mid \forall a_i, b_j \in Sim_{CS} \wedge \exists isSameRole(C^{R}S_{A}(a_i), {}^{R}S_{B}(b_j))\} \quad (9)$$

4. Case study

Using various ontologies we validated our proposed technique for its both cases i.e. some ontologies may have only taxonomic relations whereas some other ontologies may have both taxonomic and non-taxonomic relations at the same time. Two ontologies about university domain developed by different groups were used. Concepts along with non-taxonomic relations of O_1 and O_2 are listed in Tables 2 and 3 respectively. A set of sample concepts selected from both ontologies is shown in Figure 6. For sake of simplicity, we have just show the similarity status in terms or true of false rather than semantic relation in Table 3.

Table 1: A slice of non-taxonomic relations from ontology O_1 of a university domain

	Subject	Predicate (InverseOf)	Object
a1.	Faculty	teacherOf (hasTeacher)	Student
a2.	Faculty	demonstratorOf (hasDemostrator)	LabExperiment
a3.	Faculty	developerOf (hasDeveloper)	DevProject
a4.	Faculty	ResearcherOf (hasResearcher)	ResProject
a5.	SoftwareEngineer	developerOf (hasDeveloper)	DevProject
a6.	SoftwareEngineer	demonstratorOf (hasDemostrator)	LabExperiment
a7.	Consultant	consutantOf (hasConsultant)	DevProject
a8.	Consultant	consultantOf (hasConsultant	ResProject
a9.	Consultant	consultantOf (hasConsultant)	Education
a10.	Consultant	consultantOf (hasConsultant)	Network
a11.	Consultant	consultantOf (hasConsultant)	HumanResource
a12.	Director	directorOf (hasDirector)	DevProject
a13.	Director	directorOf (hasDirector	ResProject
a14.	Director	directorOf (hasDirector)	Sport
a15.	Director	directorOf (hasDirector)	Transport
a16.	Manager	managerOf (hasManager)	Network
a17.	Manager	managerOf (hasManager)	HumanResource
a18.	Manager	managerOf (hasManager)	Transport
a19.	Manager	managerOf (hasManager)	DevProject
a20.	Manger	managerOf (hasManager)	ResProject
a21.	Convener	convenerOf (hasConvener)	AdmissionCommittee
a22.	Convener	convernerOf (hasConvener)	LibraryCommittee
a23.	Convener	convernerOf (hasConvener)	DisciplinaryCommittee
a24.	Course	hasInstructor	Faculty
a25.	Course	hasBook	Book
a26.	Course	hasContent	Content
a27.	University	hasDepartment	Department
a28.	University	hasResearchCentre	ResearchCentre
a29.	ResearchPaper	publishIn	Book
a30.	Conference	isA	Event

Table 2: A slice of non-taxonomic relations from ontology O_2 of a university domain

	Subject	Predicate (InverseOf)	Object
b1.	Faculty	teacherOf (hasTeacher)	Student
b2.	Faculty	demonstratorOf (hasDemostrator)	LabExperiment
b3.	Faculty	developerOf (hasDeveloper)	DevProject
b4.	Faculty	ResearcherOf (hasResearcher)	ResProject
b5.	SoftwareEngineer	developerOf (hasDeveloper)	DevProject
b6.	SoftwareEngineer	demonstratorOf (hasDemostrator)	LabExperiment
b7.	Consultant	consutantOf (hasConsultant)	DevProject
b8.	Consultant	consultantOf (hasConsultant	ResProject
b9.	Consultant	consultantOf (hasConsultant)	Education
b10.	Consultant	consultantOf (hasConsultant)	Network
b11.	Consultant	consultantOf (hasConsultant)	HumanResource
b12.	Director	directorOf (hasDirector)	DevProject
b13.	Director	directorOf (hasDirector	ResProject
b14.	Director	directorOf (hasDirector)	Sport
b15.	Director	directorOf (hasDirector)	StundentAffair
b16.	Manager	managerOf (hasManager)	Network
b17.	Manager	managerOf (hasManager)	HumanResource
b18.	Manager	managerOf (hasManager)	Transport
b19.	Manager	managerOf (hasManager)	DevProject
b20.	Manger	managerOf (hasManager)	ResProject
b21.	Convener	convenerOf (hasConvener)	AdmissionCommittee
b22.	Convener	convernerOf (hasConvener)	LibraryCommittee
b23.	Convener	convernerOf (hasConvener)	DisciplinaryCommittee
b24.	Course	hasInstructor	Faculty
b25.	Course	HasBook	Book
b26.	Course	hasContent	Content
b27.	Department	hasResearchCentre	ResearchCentre
b28.	University	hasDepartment	Department
b29.	ResearchPaper	publishIn	Book
b30.	ResearchPaper	publishIn	Journal
b31.	ResearchPaper	publishIn	Conference

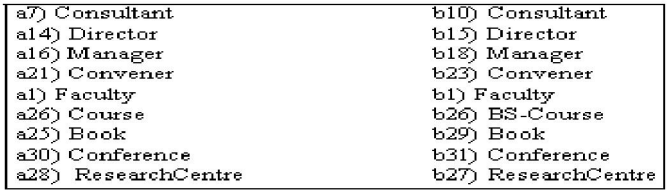


Figure 6: A sample set of concepts from O1 and O2

Table 3: Similarity of pairs of concepts with different criteria

Pair\Criteria	Primarily Similar	Taxonomically Similar	Non-taxonomically Similar
(a7,b10)	Y	Y	N
(a14,b15)	Y	Y	N
(a16,b18)	Y	Y	N
(a21,b23)	Y	Y	N
(a1,b1)	Y	Y	Y
(a26,b26)	Y	Y	Y
(a25,b29)	Y	N	N
(a30,b31)	Y	N	N
(a28,b27)	Y	N	N
(a14, b29)	N	N	N

5. Conclusion and Future Work

The proposed technique measures similarity in a layered fashion. The conceptual schemas of two ontologies are taken as input (technique is language-independent). Concepts with their super-concepts and non-taxonomically relating concepts along with synonyms of concepts are acquired in phase-1. Concepts are short-listed in phase-2, based on their primarily similarity so-called lexical similarity. Only those concepts, short-listed in phase-2, are tried to find their taxonomic similarity i.e. Concepts are short-listed based on their taxonomic similarity in phase-3. Only those concepts, short-listed in phase-3, are tried to find their non-taxonomic similarity in phase-4. We validated the technique by a case study. The current test case study includes small ontologies. Although the similarities between concepts of large realistic ontologies are difficult to obtain however, they are necessary for better evaluation of proposed technique. A framework is needed to realize its full potential and completeness.

Acknowledgement

This research work has been supported by the “Higher Education Commission of Pakistan”, and the University of Engineering and Technology, Lahore.

References

1. Aleksovski, Z., Kate, W., and Harmelen, F., ”Exploiting the structure of background knowledge used in ontology matching”, Proceedings of International Workshop on Ontology Matching collocated with the 5th International Semantic Web Conference, pp. 13-24 , USA, 2006.
2. Aleksovski, Z., Klein, M., Kate, W., and Harmelen, F., “Matching Unstructured Vocabularies Using Background Ontology”, Proceedings of 15th International Conference on Knowledge Engineering and Knowledge Management Managing Knowledge in a World of Networks”, pp. 182-197, Czech Republic, 2006.
3. Duchateau, F., Bellahsene, Z., and Roche, M., “Context-based Measure for Discovering Approximate Semantic Matching between Schema Elements", Proceedings of The International Conferences on Research Challenges in Information Science (RCIS), Morocco, 2007.
4. Ehrig, M., Koschmider, A., and Oberweis, A., “Measuring Similarity between Semantic Business Process Models”, Proceedings of the Fourth Asia-Pacific Conference on Conceptual Modelling, pp. 71-80, Australia, 2007.

5. Erhard, R. and Philip, B.A., "A survey of approaches to automatic schema matching", The VLDB Journal, Volume 10, No. 4, pp. 334-350, Springer Berlin, 2001.
6. Euzenat, J., Le Bach, T., and Barasa, J., "State of the art on ontology alignment", Technical Report IST-2004-507482, University of Karlsruhe, 2004.
7. Farooq, A., and Shah, A.," Similarity Identification and Measurement between Ontologies", The Journal of American Science 2010; 6(4):67-85.
8. Giunchiglia, F., Shvaiko, P., and Yatskevich, M., "Semantic schema matching", Proceedings of International Conference on Cooperative Information Systems, pp. 347-365, Cyprus, 2005.
9. Giunchiglia, F., Yatskevich, M., Shvaiko, P., "Semantic Matching: Algorithms and Implementation**",** LNCS Journal on Data Semantics Volume 9 (2007), pp. 1-38, Germany, 2007.
10. Hariri, B., Abolhassani, H., and Khodaei, A., "A new Structural Similarity Measure for Ontology Alignment", In proceedings of the 2006 International Conference on Semantic Web & Web Services, pp.36-42 , USA, 2006.
11. Isabel, F. C., Sunna, W., Makar, N., and Bathala S., "A visual tool for ontology alignment to enable geospatial interoperability", Journal of Visual Languages and Computing, Volume 18, No.3, pp. 230–254, UK, 2007.
12. Jeong, B., Lee, D., Cho, H., and Lee, J., "A novel method for measuring semantic similarity for XML schema matching", Expert Systems with Applications, Volume 34, No. 3, pp. 1651–1658, Elsevier, 2008.
13. Lambrix P., and Tan H., "SAMBO - A System for Aligning and Merging Biomedical Ontologies", Journal of Web Semantics, Volume 4, No. 3, pp. 196-206, www.semanticwebjournal.org, 2006.
14. Lee, T. B., Hendler, J., and Lassila, O., "The Semantic Web", Scientific America, Volume 284, No.5 pp.34-43, USA, 2001.
15. Madhavan, J., Bernstein, P., and Rahm, E., "Generic schema matching using Cupid", Proceedings of. 27th International Conference on Very Large Data Bases, pp. 48-58, Roma, 2001.
16. Maedche, A., and Staab, S., "Measuring similarity between ontologies", Proceedings of the International Conference on Knowledge Engineering and Knowledge Management (EKAW), pp. 251–263, Spain, 2002.
17. Miller, G. A., "WordNet: A lexical Database for English", Communications of the ACM, Volume 38, No. 11, pp. 39-41, http://cacm.acm.org, 1995.
18. Muller, P., Hathout, N., and Gaume, B., "Synonym Extraction Using a Semantic Distance on a Dictionary", Proceedings of the workshop on TextGraphs: Graph-based algorithms for Natural Language Processing, pp.65-72, New York, 2006.
19. Noy, N., and Musen, M., "Anchor-prompt: using non-local context for semantic matching", Proceedings of the workshop on Ontologies and Information Sharing at the International Joint Conference on Artificial Intelligence (IJCAI), pp. 63–70, USA, 2001.
20. OntoWeb Consortium, "Deliverables 1.3 (A survey on ontology tools) and 1.4 (A survey on methodologies for developing, maintaining, evaluating and reengineering ontologies)", www.ontoweb.org, 2002.
21. Shvaiko, P., and Euzenat, J., "A Survey of Schema-based Matching Approaches", LNCS, Journal on Data Semantics, Volume 4 (2005), pp. 146-171, Germany, 2005.

6/29/2010

Physicochemical Parameters in Soil and Vegetable Samples from Gongulon Agricultural Site, Maiduguri, Borno State, Nigeria

[1]J. C. Akan, [1]F.I. Abdulrahman, [2]O.A. Sodipo, [1]A. G. Lange

1. Department of Chemistry, University of Maiduguri, P.M.B 1069, Maiduguri, Nigeria.
2. Department of Clinical Pharmacology and Therapeutics, College of Medical Sciences, University of Maiduguri, Maiduguri, Nigeria.
E-mail: joechemakan@yahoo.com

ABSTRACT: Anthropogenic activities are a leading cause of metal emission, often associated with high elevated soil and plant metal concentrations. The accumulation of heavy metals and anions in soil and vegetables in the vicinity of Gungulung agricultural site were investigated. Soil samples were collected at depths of 0-5 cm, 5-10 and 10-20 cm. Soil properties including pH, electrical conductivity (EC), organic matter, organic carbon, cation exchange capacity (CEC) and heavy metals content were determined using standard procedures. Vegetable samples (spinach, Amaranthus caudatus; carrot, Daucus carota; lettuce, Lactuca sativa; cabbage, Brassica oleracea; tomato, Lycopersicon sculenetum; waterleaf, Talinum Triangulare and onion Allium cepa were used for this research. The plant samples were prepared for heavy metals and anions determination using standard procedures. Results show that the soil metal content, conductivity and organic carbon decreased with depth, suggesting anthropogenic sources of contamination while pH, organic matter and CEC decreased with depth. The results obtained from this analysis revealed that Zn and Mn show the highest concentrations, Ni shows the lowest levels. Similarly, the results also revealed that Fe, Zn and Cu show the highest concentrations, while Pb shows the lowest levels in the whole vegetables parts studied. The leaves contained much higher concentrations of heavy metals and anions than roots and stems. The concentrations of the above parameters in the vegetable samples were higher than the FAO, WHO/EU and FAO/WHO allowed limit. The high values might be attributed to the use of wastewater from river Ngada and application of sewage sludge by farmers for the irrigation of these vegetables. The results of this study suggest that the vegetables grown in the vicinity of Gugulung agricultural site are subjected to anthropogenic activities. Thus, the high values of these metals in the vegetable samples could put the consumers of these vegetables at health risk with time due to bioaccumulation. [Journal of American Science. 2010;6(12):78-87]. (ISSN: 1545-1003).

Key words: Physicochemical, Parameters, Soil, Vegetables, Bioavailability, Uptake.

1. INTRODUCTION

Several studies have indicated that vegetables, particularly leafy crops, grown in heavy metal contaminated soils have higher concentrations of heavy metals than those grown in uncontaminated soil (Guttormsen el al. 1995; Dowdy and Larson 1995). A major pathway of soil containing through atmospheric deposition of heavy metals from point sources such as: metaliferous metal smelting and industrial activities. Other non point sources of contamination affecting agricultural soils include inputs such as, fertilisers, pesticides, sewage sludge and organic (Singh 2001). Additionally, foliar uptake of atmospheric heavy metals emissions has been identified as an important pathway of heavy metal contamination in vegetable crops (Salim et al. 1992). Vegetable growing areas are often situated in, or near sources of deposits, and thus have an elevated risk of potential contamination. There have been a number of studies which have investigated atmospheric deposition in soil and/or vegetables growing in the vicinity of industrial areas (Gzyl 1995). These studies indicate high concentrations of heavy metals in vegetables grown in the vicinity of industries and polluted areas and identify leafy vegetables at greatest risk of accumulating elevated concentration. Each plant species has its nutritive requirements differing from others. Thus different plants supported by identical solutions will contain varying concentrations of minor and macro elements. Application of industrial effluent decreases the budding and growth rate of vegetables (Ihekeronye and Ngoddy 1985). Leafy vegetables occupy a very important place in the human diet, but unfortunately constitute a group of foods which contributes maximally to nitrate and other anions as well as heavy metals consumption. The excessive application of nitrogen and other inorganic fertilizers and organic manures to these vegetables can accumulate high levels of nitrate and other anions as well as heavy metals. Consequently their consumption by humans and animals can pose serious health hazards. Although some heavy metals such as Cu, Zn, Mn and Fe are essential in plant nutrition, many of them do not play any significant

role in the plant physiology. The uptake of these heavy metals by plants especially leafy vegetables is an avenue of their entry into the human food chain with harmful effects on health (Ihekeronye and Ngoddy 1985).

Although the nutrient content of wastes makes them attractive as fertilizers, when untreated wastes are used in crop production, consumers risk to contact diseases like cholera and hepatitis, or to undergo heavy metal contamination (Drechsel et al., 1999). In fact, large amounts of the waste comprise organic material, but there are considerable proportions of plastic, paper, metal rubbish and batteries which are known to be real sources of heavy metals (Lisk, 1988; Zhang et al., 2002; Pasquini and Alexander, 2004). Heavy metals and non-biodegradable materials can accumulate in soils to toxic concentrations that affect plant and animal life. Contamination of soils by heavy metals can be caused by many factors such as metal-enriched parent materials, mining or industrial activities, non point sources of metals, especially automotive emission, and use of metal-enriched materials, including chemical fertilizers, farm manures, sewage sludge, and wastewater irrigation (Freedman and Hutchinson, 1981). However, soil contamination by heavy metals and toxic elements due to parent materials or point sources often occurs on a limited area and is easy to identify (He et al., 2005). In agricultural production systems, soil contamination of heavy metals is mainly related to input and accumulation of these elements through repeated use of metal enriched chemicals such as fungicides, farm manures, chemical fertilizers and biosolids (Webber, 1981). Biosolids and/or municipal composts made of biosolids and yard wastes often contain higher concentrations of Cu, Zn, Cd, Cr and Ni than those found in soils (He et al., 2001). Several works have been done in developed countries and showed excessive concentrations of heavy metals in agricultural soils and plants (Alloway, 1995).

The effect of pH on heavy metal availability to plants has been reported by many researchers and it is accepted that as pH decreases, the solubility of cationic forms of metals in the soil solution increases and, therefore, they become more readily available to plants (Gray et al., 1998; Salam and Helmke, 1998; Oliver et al., 1998, Singh et al., 1995; Evans et al., 1995; Filius et al., 1998; Mann and Ritchie, 1995; Chlopecka et al., 1996; Vigerust and Selmer-Olsen, 1985). Evans (1989) explained that pH has a major effect on metal dynamics because it controls adsorption and precipitation, which are the main mechanisms of metal retention to soils. Metal solubility in the solution depends on the solubility product of the solid phase (precipitate) containing the metal.

He and Singh (1993) found that application of sludge increased the cation exchange capacity (CEC) value of the soil (that is the ability of the soil to retain metals). The movement of heavy metals down the soil profile is often evident in high applications of heavy metals, usually in sewage sludge, in soils with low organic matter and clay contents, acidic conditions, and when high rainfall or irrigation water rates have been applied. The movement occurs through soil macropores or cracks which is also referred to as preferential flow (Dowdy and Volk, 1983).

Since organic matter plays an important role in metal binding, some researchers have tested whether organic carbon (OC) compounds influence metal leaching. Fotovat et al. (1996) reported that metals such as Cd, Ni and Zn may be influenced in their solubility characteristics from the presence of OC. LaBauve et al. (1988) applied synthetic waste water to soils and measured the soluble metals. It was found that the synthetic material increased the solubility of metals, especially Cd and Ni, and this was particularly attributed to the soluble organic matter of the waste.

Gongulon is an agricultural site located in Maiduguri Metropolis, Borno State, Nigeria along the coast of River Ngada. Vegetables are grown in this area of Gongulon for commercial purposes. The river receives copious amounts of wastes from residence houses and abattoirs sited along its course. Urban waste management and garbage disposal practices in the city are very poor. Process water from the Municipal waste and Abattoir located near the river contains large amounts of heavy metals. The contaminated water from river Ngada is used extensively for the irrigation of these vegetables particularly at the agriculture site in Gongulon. Hence, this poses significant effect on the soil and vegetable crops thereby exposing consumers of these vegetable crops to bioaccumulation of trace metals and anions with time. This study is aimed at determining the levels of some physicochemical parameters in vegetable and soil samples.

2. MATERIALS AND METHODS

2.1 Sample Areas

Soil and vegetable samples were collected from the agricultural sites of Gongulon located within Maiduguri Metropolis, Borno State, Nigeria. In these areas of study, sewage sludge and waste water from river Ngada are used by farmers to improve soil fertility for the growth of vegetables.

2.2 Sample Collections and Preparations

In the field, soil samples were collected from twelve plots. In each plot, soil samples were collected

at three depths (0-5 cm, 5-10 cm and 10-15 cm), by using spiral auger of 2.5cm diameter. Soil samples from the Agricultural site were randomly sampled and bulked together to form a composite sample. In all cases, soil samples were put in clean plastic bags and transported to the laboratory. Soil samples were then air-dried, crushed and passed through 2mm mesh sieve. The samples were then put in clean plastic bags and sealed. Soil samples were analysed for the following parameters: pH, electrical conductivity, organic matter, organic carbon, cation exchange capacity and heavy metals.

Vegetables (spinach, *Amaranthus caudatus;* carrot, *Daucus carota;* lettuce *Lactuca sativa*; cabbage, *Brassica oleracea*; tomato, *Lycopersicon sculenetum; waterleaf, Talinum Triangulare* and onion *(Allium cepa)* from the Gongulon agricultural site were freshly harvested from twelve farms and packaged into labelled paper bags, and transported to the laboratory awaiting analysis. The vegetable samples were collected and divided into root, stem and leaf. Soil and vegetable samples were collected four times a month from the period of January to July, 2008.

2.3 Soil sample analysis

The pH was measured using a 1:2 soil: water ratio (Mclean, 1982); electrical conductivity was determined using the aqueous extraction (1/5) method (Mathieu and Pieltain, 2003). Organic matter and organic carbon (OC) were determined using Anne method (modified Walkey-Black method) (Mathieu and Pieltain, 2003). Cation exchange capacity (CEC) was determined using standard method taken from Rowell (1994). The cation used in this method to saturate the soil solution is Na. Five gramme (5g) of soil were weighed into a 50 ml plastic centrifuge tube and 30 ml of 1 M NaOAc pH 8.2 were added. The sample was shaken at an end-to-end shaker at $21^{O}C$ for 5 minutes and was then centrifuged for 10 minutes at 4000 rpm. The supernatant was discarded and 30 mL of 1 M NaOAc pH 8.2 was added the sample was resuspended and the procedure was repeated for 2 more times. After the supernatant was discarded for the third time 30 ml of 95 % ethanol solution were added, the sample was resuspended and another 3 cycles were conducted. At the end of the third cycle, 30 ml of NH_4OAc pH 7 were added, the sample was resuspended and a new phase of 3 cycles was commenced. This time the supernatants were filtered through a filter paper, Whatman No 42, and collected into a 100 mL volumetric flask. At the end of this, the flask was made to the volume with NH_4OAc pH 7 solution. The samples were kept at $4\ ^{O}C$ until Na was measured on the FAAS according to standard procedure. CEC value was then determined by the formular

$$\text{CEC, cmol}_c\ \text{kg}^{-1}\ \text{soil} = \frac{10 * \text{Na concentration in mg L}^{-1}}{\text{Mass of sample (g)}}$$

2.4 Determination of Heavy Metals in Soil Sample

Two grammes of the soil samples were weighed into acid-washed glass beaker. Soil samples were digested by the addition of $20cm^3$ of aqua regia (mixture of HCl and HNO_3, ratio 3:1) and $10cm^3$ of 30% H_2O_2. The H_2O_2 was added in small portions to avoid any possible overflow leading to loss of material from the beaker. The beakers was covered with a watch glass, and heated over a hot plate at $90^{O}C$ for two hours. The beaker wall and watch glass were washed with distilled water and the samples were filtered out to separate the insoluble solid from the supernatant liquid. The volumes were adjusted to $100cm^3$ with distilled water. Blank solutions were handled as detailed for the samples. All samples and blanks were stored in plastic containers. The results were expressed in mg/kg dry weight. All statistical analyses were carried out with the program SPSS 12.3 for windows.

2.5 Sample Preparation and Digestion of Vegetables for Heavy Metals Determination

The vegetables samples were weighed to determine the fresh weight and dried in an oven at $80^{O}C$ for 72 hours to determine their dry weight. The dry samples were crushed in a mortar and the resulting powder digested by weighing 0.5g of oven-dried ground and sieved (<1mm) into an acid-washed porclain crucible and placed in a muffle furnance for four hours at $500^{O}C$. The crucibles were removed from the furnance and cooled. 10ml of 6M HCl were added covered and heated on a steam bath for 15minute. Another 1ml of HNO_3 was added and evaporated to dryness by continuous heating for one hour to dehydrate silica and completely digest organic compounds. Finally, 5ml of 6 M HCl and 10ml of water were added and the mixture was heated on a steam bath to complete dissolution. The mixture was cooled and filtered through a Whatman no. 541 filter paper into a 50ml volumetric flask and made up to the mark with distilled water.

2.6 Elemental Analysis of Samples

Determination of Cu, Zn, Co, Mn, Mg, Fe, Cr, Cd As, Ni and Pb in soil and vegetable samples were made directly on each of the final solution using Perkin-Elmer AAnalyst 300 Atomic Absorption Spectroscopy (AAS).

2.7 Determination Of Nitrate, Nitrite, Sulphate And Phosphate In The Vegetable Samples

2.7.1 ***Determination of nitrate and nitrite***

The concentration of nitrate and nitrite analyzed in each of the vegetable samples were carried out using smart spectro Spectrophotometer (LaMotte 2000). Vegetable samples solutions were prepared by chopping each sample into smaller sizes. A known amount (1g) of the chopped sample was transferred into 100ml flask and soaked with 50ml of distilled water. The flask was capped and shaken for 30 minutes, then filtered into another 100ml volumetric flask and the volume made to the mark with distilled water (Radojevic and Bashkin 1999). Nitrate was determined spetrophotometrically using standard cadmium reduction method 3649 – SC (Lamotte, 2000), while Nitrite was determined using standard diazotization method 3650 – SC (Lamotte, 2000).

2.7.2 *Determination of Phosphate*

Each of the vegetables samples was chopped into small pieces. The chopped samples were then air-dried. The air-dried samples were ground and sieved with a siever of mesh 1mm. A known amount (1g) of each of the ground and sieved samples was weighed into acid-washed porcelain crucibles. The crucibles were labelled and 5ml of 20% (w/v) magnesium acetate were added and evaporated to dryness. The crucibles were then transferred into the furnace and the temperature was raised to 500^0C. The samples were ashed at this temperature for four (4) hours, removed and cooled in desiccators.

Ten (10) ml of 6 M HCl were then added to each of the crucible and covered, then heated on a steam bath for fifteen minutes. The contents of each crucible were completely transferred into different evaporating basins and 1ml of concentrated HNO_3 was added. The heating was made to continue for 1 hour to dehydrate silica. 1ml of 6M HCl was then added, swirled and then followed by the addition of 10ml distilled water and again heated on the steam bath to complete dissolution. The contents of the evaporating basins were cooled and then filtered through a Whatman no.1 filter paper into 50ml volumetric flasks and the volumes made up to the marks with distilled water (Radojevic and Bashkin 1999). Phosphate was determined using Hach Direct Reading 2000 Spectrophotometer.

2.7.3 *Determination of Sulphate*

For sulphate determination, 5ml of magnesium nitrate solutions were added to each of the ground and sieved samples in the crucibles. These were then heated to 180^0C on a hot plate. The heating process was allowed to continue until the colour of the samples changed from brown to yellow (Kenneth, 1990). The samples were then transferred to the furnace at a temperature of 500^0C for four hours. Magnesium nitrate was added to prevent loss of sulphur. The contents of each crucible were carefully transferred to different evaporating basins. 10ml of concentrated HCl were added to each of them and covered with watch glasses. They were boiled on a steam bath for 3 minutes. On cooling, 10ml of distilled water were added to each of the basins and the contents of each were filtered into 50ml volumetric flasks and the volumes made up to the marks with distilled water (Radojevic and Bashkin 1999). Sulphate was determined using Smart spectro Spectrophotometer (2000).

3. RESULTS

3.1 Soil Properties

The soil properties had a wide range of values for measured soil properties (Figures 1). The soil pH values range from acidic (5.98) to moderately alkaline (7.26) and varied with depth. Conductivity values ranged from 2.03 µS cm^{-1} to 2.54 µS cm^{-1}. Organic carbon ranged from 1.03% to 2.11% and decreased with depth. Cation exchange capacity values were 20.45 to 23.54 C.mol/kg, while organic matter ranged from 8.56 to 10.55 %.

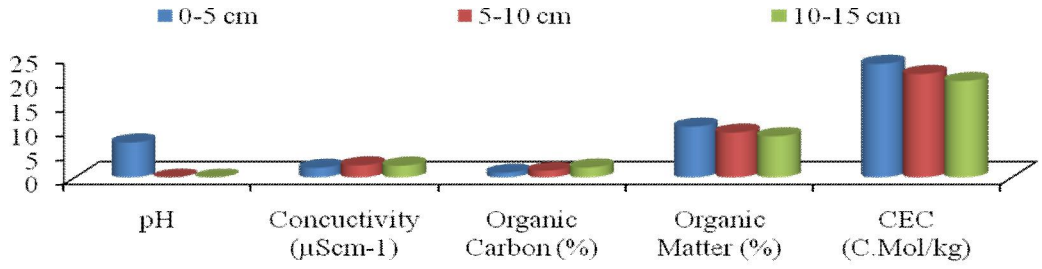


Figure 1: Mean concentration of chemical and physical properties of soil sample from Gongulon agricultural site

3.2 Distribution of Heavy Metals in Soil Profiles

At the Gongulon farming area, heavy metals concentrations increased significantly ($p < 0.05$) with depth

(Figure 2) suggesting anthropogenic sources of contamination. The concentrations of Cr in the soil sample from different depths were 2.21 to 5.32 mg/kg; Co ranged from 0.12 mg/kg (0-5cm) to 3.43 mg/kg (10-15cm). Fe concentrations in the soil ranged from 2.54 mg/kg to 4.21 mg/kg with depth; 0.98 to 1.22 mg/kg Ni; 6.75 to 14.54 mg/kg Pb; 23.75 to 33.92 mg/kg Zn; 8.94 to 15.97 mg/kg Cd; 6.88 to 7.65 mg/kg Cu; 1.03 to 1.76 mg/kg As and 13.76 to 19.96 mg/g Mn.

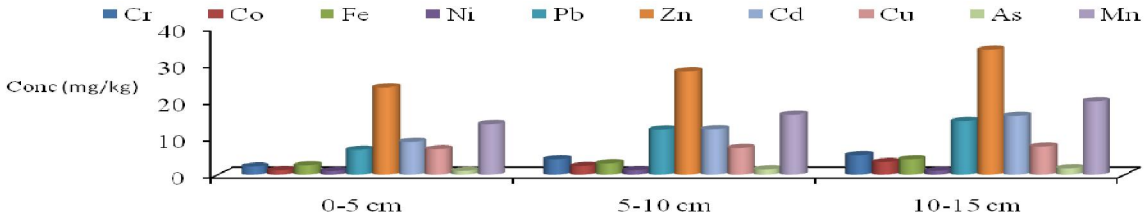


Figure 2: Mean concentration of heavy metals in soil samples from Gongulon agricultural site with depth

3.3 Heavy Metals in Vegetables

The concentrations of heavy metals in all the vegetable samples are presented in Figure 3a and b. The concentration of Cr ranged from 0.12 to 1.02 mg/kg; 0.11 to 0.72 mg/kg Mn; 0.33 to 3.21 mg/kg Fe; 0.11 to 1.21 mg/kg Cu; 0.11 to 0.53 mg/kg As; 0.11 to 2.04 mg/kg Ni; 0.11 to 0.39 mg/kg Pb; 0.11 to 1.44 mg/kg Zn and 0.11 to 0.66 mg/kg Cd.

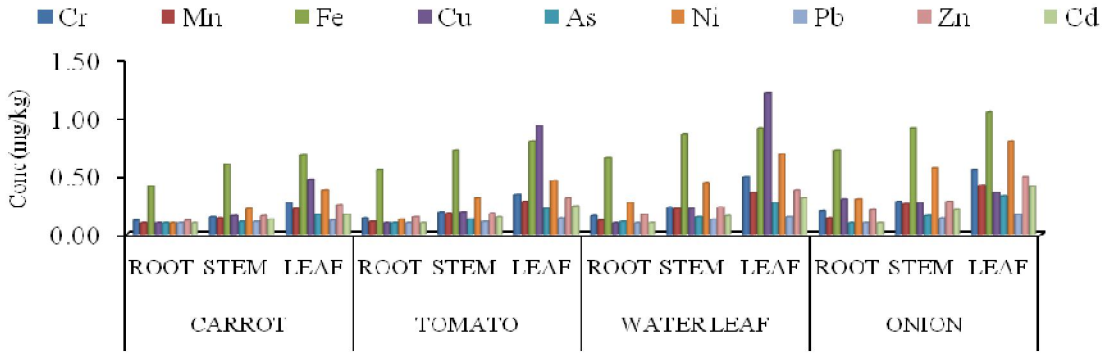


Figure 3b: Mean concentration of heavy metals in differnet of vegetable samples from Gongulon agricultural site

3.4 Anions in Vegetable Samples

The mean concentrations of anions for all the organs of different vegetable samples are as presented in Figure, 4, 5, 6 and 7. The concentrations of sulphate (Figure 4) ranged from 267.67 to 388.76 mg/kg carrot; 678.33 to 989 mg/kg spinach; 456.44 to 807.09 mg/kg lettuce; 378.66 to 487.66 mg/kg water leaf; 312.23 to 411.12 mg/kg cabbage; 217.81 to 294.55 mg/kg tomato and 422.45 to 566.70 mg/kg onion. For phosphate concentrations Figure 5, carrot ranged between 43.45 and 65.34 mg/kg; 134.77 and 187.99 mg/kg spinach; 118.45 and 154.33 mg/kg lettuce; 78.94 and 92.45 mg/kg water leaf; 56.23 and 74.00 mg/kg cabbage; 33.27 and 58.44 mg/kg tomato and 98.05 and 123.68 mg/kg onion. The levels of nitrate ranged from 210.03 to 359.67 mg/kg carrot; 421.22 to 674.22 mg/kg spinach; 322.56 to 587.33 mg/kg lettuce; 234.56 to 388.90 mg/kg cabbage; 289.00 to 412.33 mg/kg water leaf; 177.89 to 288.43 mg/kg tomato and 310.33 to 466.78 mg/kg onion Figure 6. Nitrite concentration ranged between 196.33 and 311.02 mg/kg carrot; 311.21 and 543.54 mg/kg spinach; 277.33 and 453.44 mg/kg lettuce; 211.02 and 398.77 mg/kg cabbage; 263.19 and 387.34 mg/kg water leaf; 167.88 and 281.07 mg/kg tomato and 233.23 and 428.11 mg/kg onion Figure 7. From figure 4, the maximum concentration of sulphate was found in spinach (678.33 to 989.01 mg/kg) and the minimum in tomato (217.81 to 294.55 mg/kg). Phosphate had the maximum concentration in spinach (134.77 to 187.99 mg/kg) and minimum in tomato (33.27 to 58.44 mg/kg) Figure 5. Nitrate content was higher in spinach (421.22 to 674.22 mg/kg) while tomato shows the least values (177.89 to 288.43 mg/kg) Figure 6. Nitrite showed the maximum concentrations in spinach (311.21 to 543.54 mg/kg) and the minimum concentrations in tomato (167.88 to 281.07 µg/g) Figure 7.

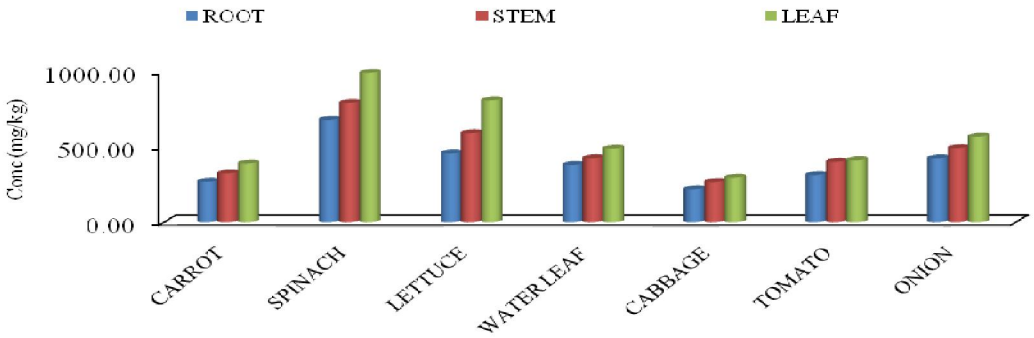


Figure 4: Mean concentration of Sulphate in different organs of vegetable samples from Gongulon agricultural site

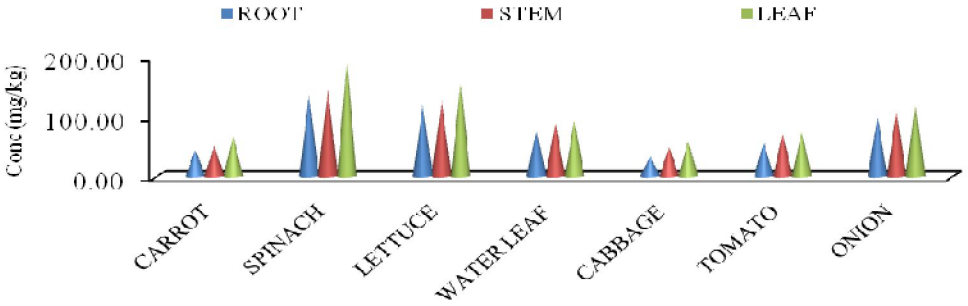


Figure 5: Mean concentration of phosphate in different organs of vegetable samples from Gongulon agricultural site

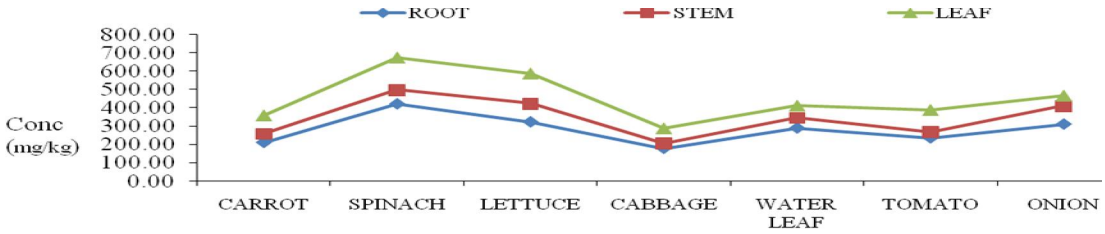


Figure 6: Mean concentration of Nitrate in different organs of vegetable samples from *Gongulon agricultural site*

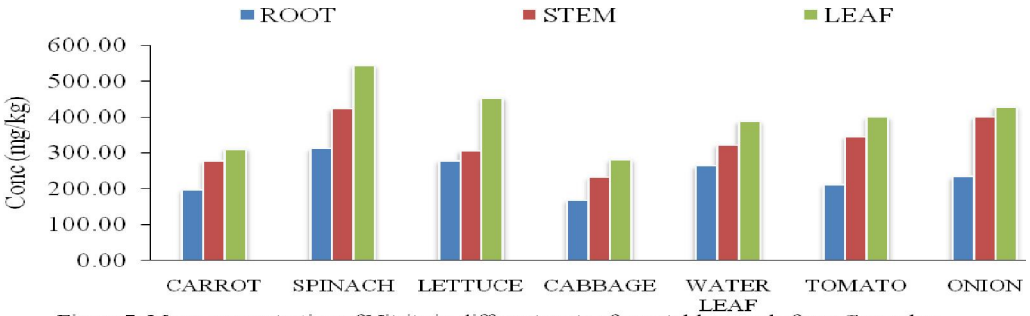


Figure 7: Mean concentration of Nitrite in different parts of vegetable sample from Gongulon Agricultural site

4. DISCUSSION

The sequence of heavy metals in the cultivated soil samples from the Gongulon agricultural site was in the order of Zn > Mn > Cd> Pb > Cu > Cr > Fe > Co > As >Ni Figure 1. The concentrations of heavy metals showed spatial and temporal variations, which may be ascribed to the variation in heavy metal sources and the quantity of heavy metals in irrigation water and sewage sludge. This trend suggests that continuous application of sewage sludge and municipal wastewater influenced the soil physicochemical properties (Willett *et al.,* 1984). The levels of organic carbon in the soil sample increased significantly with depth, while organic matter decreased. OC also increased with the increase in the water rate Davis *et al.,* 1988). This may be of significant environmental consequences, because it was shown that higher rates of applied water (irrigation) during the study periods increased the amounts of OC Figure 2, and this also influence the solubility and availability of heavy metals.

Evidence that heavy metals may move in the soil profile was provided by Lund *et al.,*(1976), in their field experiment the researchers used sludge with a high content of heavy metals and found that Zn had moved down to 50 cm, Cd to 17 cm while Ni to 75 cm. Davis *et al.,* (1988) measured the metal distribution in the soil profile in a field experiment where sludge had been applied at a rate of 40 t ha-1 and rainfall rate was around 560 mm per annum over a period of 4 years. They found a significant movement of Cd, Ni, Pb and Zn to a depth of 10 cm. Also Schirado *et al.,* (1986) reported that heavy metals had a uniform distribution in the soil profile to a depth of 1 m, due to their movement. Results such as these tend to have been obtained from the present study, where movement of heavy metals down the soil profile (leaching) to a depth of 15 cm due to application of sewage sludge and waste water from river Ngada were observed Figure 1. The concentrations of heavy metals in the soil samples obtained during the present study were higher than the FAO standard.

Soil pH was significantly greater and degreased with depth. pH is one of the factors which influence the bioavailability and the transport of heavy metals in the soil and according to Smith and Giller (1992) heavy metal mobility decreases with increasing soil pH due to precipitation of hydroxides, carbonates or formation of insoluble organic complexes. In the present study, it was observed that heavy metals increase significantly with decrease in pH ($p< 0.05$) Figures 1 and 2. The soil electrical conductivity (EC) also varied significantly with depth ($p< 0.05$). By comparism, Boulding (1994) classified EC of soils as: non saline <2; moderately saline 2-8; very saline 8-16; extremely saline >16. From the result of the study, the EC is classified as moderately saline. The amount of heavy metals mobilized in soil environment is a function of pH, properties of metals, redox conditions, soil chemistry, organic matter content, clay content, cation exchange capacity and other soil properties (Arun and Mukherjee, 1998; Kimberly and William, 1999; Sauve *et al.,* 2000). Heavy metals are generally more mobile at pH < 7 than at pH > 7. The pH of the soils from the Gongulon agricultural sites ranged from 5.98 to 7.26. This is therefore hazardous for agricultural purposes since crops are known to take up and accumulate heavy metals from contaminated soils in their edible portions (Wei *et al.,* 2005).

Leaves contained higher concentrations of heavy metals than roots and stems. Similar study carried out by (Santamaria *et al.,* 1999) shows that the heavy metal content of various parts of plant differs. They reported that in vegetable organs the concentrations of heavy metals are in the order of leaf> stem> root> tuber> bulb> fruit> seed. Amusan *et al.,* 1999, studied plant uptake of heavy metals on a similar site at University of Ife dump site and reported that Pb uptake by water leaf (*Talinum triangulare*), okra (*Albennucus esculentus*) increased in leaves and roots of water leaf and in the fruit of okra relative to those grown in the non-dump sites. Similar work by Ademoroti (1996) reported that vegetables accumulate considerable amount of heavy metals especially Pb, Cr, Cu and Zn in roots and leaves. The concentrations of heavy metals in all the vegetable samples analysed were higher than the FAO/WHO guideline values of 0.1-0.2 mg/kg Cr, 0.3 mg/kg Fe; 0.1 mg/kg Pb; 0.1 mg/kg Cu; 0.1 mg/kg Zn; 0.1 mg/kg Ni; 0.02 mg/kg Cd and 0.3 mg/kg Mn. Results from present and earlier reports (Liu *et al.,* 2005; Muchuweti *et al.,* 2006 and Sharma *et al.,* 2007) demonstrated that plants grown on wastewater-irrigated soils are contaminated with heavy metals and pose health concern. Absorption and accumulation of heavy metals in plant tissues depend upon many factors. These include temperature, moisture, organic matter, pH and nutrient availability, while the presence of organic matter has been reported to increase the uptake of zinc, chromium, lead, iron and copper in the wheat plant. (Rupa *et al.,* 2003). In the present study many soil factors such as pH, organic matter and organic carbon have interacted to impact on uptake. The acidic range of soil is known to increase the mobilization of heavy metals, thus increasing their uptake. The field data support this argument in that the soil pH was acidic.

The values of sulphate, phosphate, nitrate and nitrite in the vegetable samples show that the leaves are rich in this anion content than other organs studied. Similar study was carried out by Santamaria *et al.,* (1999) stated that nitrate and nitrite contents of

various parts of a plant differ in the order of leaf> stem> root> tuber> bulb> fruit> seed. Zhou *et al.,* (2000) reported that vegetables that are consumed with their roots, stems and leaves have a high nitrate and nitrite accumulation, whereas melons and those vegetables with only fruits as consumable parts have a low nitrate accumulation This observation was also noted by Hunt and Turner (1994) where leaf and stem accumulate the most nitrate, sulphate and nitrite followed by stem and roots. The concentrations of these anions were higher in the leafy vegetables (spinach and lettuce) than in tomato. Results of analysis of variance (ANOVA) showed that variation between vegetables and organs were statistically significant ($p<0.05$).

5. Conclusion

The levels of soil and plants contamination in the agricultural site of Gongulon appear to be as a result of anthropogenic activities within the area. The levels of heavy metals, pH and organic carbon increased significantly ($p < 0.05$) to a depth of 15 cm, while conductivity, organic matter and CEC, decreased to a depth of 15 cm. The results indicate that all the vegetable samples analyzed in this study had high levels of heavy metals. Heavy metal levels were higher than those recommended by Food and Agricultural Organization (FAO) and the WHO/EU joint limits. The high levels of these heavy metals might place the consumers of these and other vegetable crops grown within the vicinity of the area at health risk with time.

Corresponding Author:
Dr. Joseph Clement Akan
Department of Chemistry, University of Maiduguri,
Maiduguri, P.M.B 1069, Maiduguri,
Borno State, Nigeria.
Tel: +2348036000506

REFERENCES

1. Ademoroti CMA. Standard method for water and Effluents Analysis. Foludex pressLtd, Ibadan. 1996: pp.22-23, 44-54, 111-112.
2. Alloway BJ. The origins of heavy metals in soils. In: Heavy Metals in soils. Second edition (ed. Alloy B.J.) Balckie Academic & Professional. 1995: pp. 39-57.
3. Amusan, A.A., Ige, P.V. and Olawale, R. Preliminary investigation on the use of municipal waste dump for farming Paper presented at the 25th annual conference of soil science society of Nigeria held November 21-25, 1999, Benin city, Nigeria
4. Arun B, Mukherjee. Nickel: a review of occurrence, uses, emissions, and concentration in the environment in Finland. *Environ. Rev.* 1998; *6: 1-15.*
5. Boulding JR. Description and sampling of Contaminated Soils. AIfeild Guide. 2nd Edn. Lewis Publishers, Boca Raton, FL. 1994. Chapter 5.
6. Chlopecka A, Bacon JR, Wilson MJ, Kay J. (1996), Forms of cadmium, lead and zinc in contaminated soils in Southwest Poland. *Journal of Environmental Quality. 1996;* 25; 69- 79.
7. Davis RD, Charlton-Smith CH, Stark JH, Campbell JA. Distribution of metals in grassland soils following surface applications of sewage sludge. *Environmental Pollution. 1988;* 49: 99-115.
8. Dowdy RH, Larson, WE. The availability of sludge-borne metals to various vegetables. *Journal of Environmental Quality. 1995; 4;* 278-282.
9. Dowdy RH, Volk, VV. Movement of heavy metals in soils. In: *Chemical Mobility and Reactivity in Soil Systems.* Soil Science Society of America and American Society of Agronomy, Madison. 1983; pp.229-240.
10. Drechsel P, Quansah C, Penning, DVF. Urban and peri-urban agriculture in West Africa. Characteristic, challenges, and need for action. In: Olanrewaju BS (ed) Urban Agriculture in West Africa, Canada. 1999; p. 210.
11. Evans LJ. (1989), Chemistry of metal retention by soils. *Environmental Science and Technology. 1989;* 23: 1046-1056.
12. Evans LJ, Spiers GA, Zhao G. Chemical aspects of heavy metal solubility with reference to sewage sludge-amended soils. *International Journal of Environmental and Analytical Chemistry. 1995;* 59: 291-302.
13. Filius A, Streck T, Richter J. Cadmium sorption and desorption in limed topsoils as influenced by pH: Isotherms and simulated leaching. *Journal of Environmental Quality1998;* 27: 12-18.
14. Fotovat A, Naidu R, Oades JM. The effect of major cations and ionic strength on desorption of native heavy metals in acidic and sodic soils. In: 1st International Conference on *'Contaminants in the soil Environment'*, Adelaide, Australia. 1996; pp. 193-194.
15. Freedman B. Hutchinson TC. Sources of metal and elemental contaminants of terrestrial environments. In: Lepp NW (ed) Effect of heavy metal pollution on plants: Metals in the environment, vol. II, London and New- Jersey, Applied Sci Publ. 1981; pp. 35-94
16. Gray CW, McLaren RG, Roberts AH, Condron LM. Sorption and desorption of cadmium from some New Zealand soils: Effect of pH and

contact time. *Australian Journal of Soil Research. 1998);* 36: 199-216.
17. Guttormensen G, Singh BR, Jeng, AS. Cadmium concentration in vegetable crops grown in sandy soil as affected by Cd levels in fertilizer and soil pH. *Fertilizer Research. 1995;* 41:27-32.
18. Gzyl J. Ecological impact and remediation of contaminated sites around lead smeltering. *Journal of Geochemical Exploration. 1995;* 52:251-258.
19. He QB, Singh R. Effect of organic matter on the distribution, extractability and uptake of cadmium in soils. *Journal of Soil Science1993;* 44: 641-650.
20. He ZL, Xiaoe EY, Stoffella PJ, Calvert DV. Plant nutrition benefits of phosphorus, potassium, calcium, magnesium, and micronutrients from compost utilization. In: Stoffella PJ, Kahn BA (eds) Compost utilization in horticultural cropping systems. Boca Raton, FL: CRC Press. 2001; pp. 307-317.
21. He ZL, Xiaoe EY, Stoffella PJ. Trace elements in agroecosystems and impacts on the environment. *J. Trace. Ele. Med. Bio. 2005; 19: 125-140.*
22. Hunt J. Turner, MK. A survey of nitrite concentrations in retail fresh vegetables. *Food Additive and Contaminations.* 1994; 11: (3) 327-332.
23. Ihekeronye AI, Ngoddy PO. Integral Food Science and technology for the Tropics. Macmillian Education Ltd. Oxford and London. 1985; p 293.
24. Kenneth, H (1990), Official Method of Analysis of AOAC 5th Edition. AOAC Inc. Arlington USA. 1990; p234
25. Kimberly MFH, William H. Trace metals in Montreal urban soils and the leaves of Teraxacum officinale. Can. J. Soil Sci. 1999; 79: 385-387.
26. LaBauve JM, Kotuby-Amacher J. and Gambrell RP. The effect of soil properties and a synthetic municipal landfill leachate on the retention of Cd, Ni, Pb and Zn in soil and sediment materials. *Journal of World Pollution Control Federation,* 1988; 379-385.
27. Lisk DJ. (1988), Environmental implications of incineration of municipal solid waste and ash disposal. Sci. Total. Environ. 1988; 74: 39-66.
28. Liu WH, Zhao JZ, Ouyang ZY, Soderlund L, Liu GH. Impacts of sewage irrigation on heavy metals distribution and contamination in Beijing, China. Environ. Int. J. 2005; 31: 805-812.
29. Lund LJ, Page AL, Nelson CO. Movement of heavy metals below sewage disposal ponds. *Journal of Environmental Quality. 1976;* 5: 330-334.
30. Mann SS, Ritchie GSP. Form of cadmium in sandy soils after amendment with soils of higher fixing capacity. *Soil Science and Plant Nutrition. 1995,* 87: 23-29.
31. Mathieu C, Pieltain F. Chemical analysis of soils. Selected methods. France. 2003; pp.387.
32. Maynard DN, Barker, Minotti A.V, Peck, NH. Nitrate accumulation in vegetables. *J.Adva. Agro.* 1978; 28 71-118.
33. Mclean EO. Soil pH and lime requirement. In: A.L. Page, R.H. Miller, and D.R. Keeney (eds.), Methods of Soil Analysis. Part 2: Chemical and Microbiological Properties. 2nd ed. American Society of Agronomy, Madison, WI.1982; pp. 199-224.
34. Muchuweti MJ, Birkett JW, Chinyanga E, Zvauya R, Scrimshaw MD, Lester, JN. Heavy metal content of vegetables irrigated with mixture of wastewater and sewage sludge in Zimbabwe: Implications for human health. *Agric. Ecosyst. Environ. 2006; 112: 41-48.*
35. Oliver DP, Tiller KG, Alston AM, Cozens GD, Merry RH. Effect of soil pH and applied cadmium on cadmium concentration in wheat grain. *Australian Journal of Soil Research. 1998;* 36: 571-583.
36. Pasquini MW, Alexander MJ.(2004),Chemical properties of urban waste ash produced by open burning on the Jos Plateau: implications for agriculture. *The Science of the Total Environment.2004; 319: 225–240.*
37. Radojevic M, Bashkin VN. Practical Environmental Analysis. The Royal Society of Chemistry, Cambridge. 1999; pp 466.
38. Rowell DL. *Soil Science: Methods and Applications*. Longman, Harlow.1994; p 114
39. Rupa TR, Sinivas RC, Subha RA, Singh M. Effects of farmyard manure and phosphorus on Zinc transformation and phytoavailability in two altosol of India. *Bioresour. Technol.2003; 87(3): 279-288*
40. Salam AK. and Helmke PA. The pH dependence of free ionic activities and total dissolved concentrations of copper and cadmium in soil solution. *Geoderma. 1998;* 83: 281-291.
41. Salim R, Al-Subu MM, Douleh A, Chenavier L, Hagetneyer J. Effects of root and food treatment or carrot plants with lead and cadmium on the growth, uptake and the distribution in treated plants *Journal of Environmental Science and Health Part A* 27. 1992; 1739- 1758.
42. Santamaria P, Elia A, Serio F, Todaro E. A suevey of nitrate, metals and oxalate content in retail fresh vegetables. *J.Sci. Food. Agric. 1999;*

79: 1882-1888.
43. Sauve S, Henderson W, Allen HE. Solid-solution portioning of metals in contaminated soils: dependence on pH, total metal burden, and organic matter. Env. Sci. Techn. 2000; 34: 1125-1131.
44. Schirado T, Vergara I, Schalscha, EB, Pratt PF. Evidence for movement of heavy metals in a soil irrigated with untreated wastewater. *Journal of Environmental Quality. 1986;* 15: 9-12.
45. Sharma RK, Agrawal M, Marshall F. Heavy metal contamination of soil and vegetables in suburban areas of Varanasi, India. Ecotoxicol. Environ. Safety. J.2007; Doi: 10.1016/jecenv
46. Singh BR, Narwai RP, Jeng AS, Almas A. Crop uptake and extractability of cadmium in soils naturally high in metals at different pH levels. *Communications of Soil Science and Plant Analysis. 1995;* 26: 2123-2142.
47. Singh B. Heavy metals in soils sources, chemical reactions and forms. In 'GeoEnviron Proceedings of the 2nd Australia and New Zealand Conference on Environmental Geotechnology, Newcastle, New South Wales' Eds D Smith, S Fityus and M Allman. 2001; pp. 77-93.
48. Smith SR, Giller KE. Effective *Rhizombium leguminosarum* biovar *Trifolii* present in five soils contaminated with heavy metals from long-term applications of sewage sludge or metal mine spoil. *Soil Biology and Biochemistry. 1992;* 24: 781-788.
49. Vigerust E, Selmer-Olsen, AR. Basis for metal limits relevant to sludge utilisation. In: *Directorate - General Science, Research and Development, Environmental Research Programme*. Elsier Applied Science Publishers, London and New York: 1985; pp.26-41
50. Webber J. Trace metals in agriculture. In: Lepp NW (editor) Effect of heavy metal pollution on plants: Metals in the environment, vol. II. London and New Jersey: Applied Sci. Publ. 1981; 159-184.
51. Wei S, Zhou Q, Xin W. Identification of weed plants excluding the uptake of heavy metals. Environment International. 2005; 31: 829-834.
52. Willett I. R, Jakobsen P, Malafant KWJ, Bond WJ. Effect of land disposal of lime treated sewage sludge on soil properties and plant growth. Division of Water and Land Resource. CSIRO. Canberra. Div. Rep. 1984; 84/3:56.
53. Zhang FS, Yamasaki S Nanzyo M. Waste ashes for use in agricultural production: I. Liming effect, contents of plant nutrients and chemical characteristics of some metals. *Sci. Total. Environ.2002; 284: 215-225*
54. Zhou ZY, Wang MJ, Wang JS. Nitrate and nitrite contamination in vegetables in china. *Food. Rev. Int. 2000; 16, 61-76.*

15/06/2010

Data Networks' Design and Optimization through MPLS VPNs using BGP

Mohammad Junaid Arshad [1], Tauqir Ahmad [2], Amjad Farooq [3]

[1, 2, 3] Department of Computer Science & Engineering, University of Engineering and Technology Lahore, Pakistan
junaidarshad@uet.edu.pk

Abstract: The key strong points of the Internet have been its vast scalability and flexibility to provide accommodation to the variety of applications. In this context, MPLS (Multi Protocol Label Switching) is the newest technology being employed today's in the Internet core, which is continuously growing to meet the increasing demands of bandwidth and connectivity. In this research work, we provide a survey of MPLS, BGP (Border Gateway Protocol) and both layer-2 and layer-3 VPNs (Virtual Private Networks). We address the issues (such as speed, scalability and security) of traditional IP-based VPNs. Since layer-2 VPNs are efficient but not so intelligent and scalable, while layer-3 VPNs are intelligent and scalable but not so efficient. Thus, we propose a new design scheme for MPLS/BGP-VPNs in such a way that the features of layer-3 as scalability and intelligence are merged with the efficiency of layer-2 to deal with today's evolving demands of speed, scalability and security. The proposed design of optimized data networks through MPLS/BGP-VPNs is implemented in Dynagen simulator for the better understanding the system. This research work will be helpful for adding new security features in core networks in future and provides a guideline for network engineers towards the world of network security. [Journal of American Science. 2010;6(12):88-95]. (ISSN: 1545-1003).

Keywords: BGP-Border Gateway Protocol, MPLS-Multi Protocol Label Switching, QoS-Quality of Service and VPN-Virtual Private Network

1. Introduction

The purpose of this work is to analyze and identify the features and advanced requirements of core networks (i.e., MPLS/BGP-VPNs (Previdi, 2000)). MPLS/BGP-VPNs aim to provide secure, reliable and consistent communication. Some of the aspect of the world most popular core networks design is miserably handled and due to which they are being compromised time after time.

MPLS aim to provide enhanced Traffic Engineering (TE) (Awduche, 1999) mechanisms for IP-based networks to facilitate the ISPs for capably monitoring, assessing and fulfilling a variety of service provisions all through their networks backbone. Like L3-VPN PE-based (Rosen et al., 1999) technology, MPLS/BGP-VPN employs BGP for VPN routes advertisement and utilizes MPLS to send VPN packets over the provider backbone networks. MPLS/BGP-VPN has flexible networking modes, good extensibility and convenient support for MPLS QoS (Lee et al., 2003) and MPLS TE (Swallow, 1999), that's why it is extensively employed.

When building a VPN (Ferguson et al., 1998) based on p-to-p overlays, connection-oriented (like ATM or frame relay, tunneling-on-IP techniques) scalability is a main problem, while VPNs based on MPLS are used to address scalability issues (as they are purely designed on the basis of connection-less, peer-based architecture). Since, a customer-site in a peer-based architecture requires the peer simply within a single provider-edge router in place of the entire customer-edge/provider-edge routers which are associated with the VPN that results in the reduction of large number of VCs. In addition, MPLS-based VPNs naturally use connectionless approach. The Internet is to be obliged its worth to its fundamental approach which is based on connection-less, packet-switching network topology (i.e., TCP/IP). Thus, it does not require any prior act to make association possible in a flexible and useful way among the hosts. In an IP-based connection-less setup the traditional VPNs require initial connection establishment process over p-to-p, connection-oriented overlay networks. When it utilizes under a connection-less environment it still can not get benefit from the connection simplification and service expandability offered by connection-less network. In contrast, if a connection-less VPN is built, to guarantee the network privacy the use of tunnels and encryptions are not required, hence it eliminates the considerable complications.

In this work, the basic goal is also to highlight drawbacks in traditional IP-based VPNs (Callon, 2002) and show how MPLS/BGP VPNs (Alawieh et al., 2008) are used to handle these issues. The conventional IP VPNs in core networks have the following issues:

- **Quality of Service (QoS) Problem**

IP-based applications do not have any straight mechanism to state QoS, as many users and clients are uneasy with independently desirable QoS, because it requires extra charging on behalf of additional QoS category adopted. The regulations for policy managing to create QoS are achievable which are related to customers, servers and associations; however, the dilemma is the volume of the organization tasks. A better policy in simple is to give the matter of QoS headed for the whole VPN (e.g., the working of an ATM/frame-relay network etc). But it is hard to do this through IP-based services, for the reason that the OSPF protocols utilized for constructing routing table cannot share QoS statistics, in other words information concerning resource utilization of the specified trunks or nodes.

- **Scalability Problem**

A very large number of associations can be easily supported by a huge VPN network, and lots of millions can be easily supported by the Internet. So it requires a huge number of VCs which generally makes the process so weak. When every service-link-toward-partner relation is evaluated onto a VC, then the networks having C links of service will generate C(C-1)/2 VCs.

- **Security Problem**

Conventional IP-based Virtual Private Networks (VPNs) have been broadly employed all over the world for remote connectivity; however they are usually vulnerable by multifarious client software and complexities in handling the health position of remote clients. A lot of worms and viruses broadcast through these abandoned VPN endpoints causing destruction of the internal security of the networks. Thus with the Internet, one of the most important issue is the VPN's security, particularly for those which depend upon the publicly designed Internet used for transportation. The IP-based network (unlike ATM/frame relay or private-line services) doesn't allocate the constant logical/physical pipes to the special sites, applications or protocols. To address the Internet security, IPSec (Davis, 2001) is the latest IETF (Internet Engineering Taskforce) solution that was initially proposed for the IPv6 (Deering et al., 1998) protocol; however, it has been used in the current's IPv4 networks. Since, it describes a framework for giving a powerful security in support of network transport over the IP-based environments.

1.1 Contribution and Scope of the Proposed System

To meet high-level demands of security and efficiency in the backbone networks, MPLS aim to offer advanced IP network TE mechanisms these will facilitate the ISPs for easily evaluating, examining and meeting a variety of their service necessities a-cross the backbone. By the use of intelligent routers and speedy switches MPLS provides a technique for mapping IP segments with connection-based transport (such as frame relay or ATM) more efficiently. This supports the QoS definition inside the header of MPLS (Rosen et al., 2001; Rosen et al., 1999) as well. Using routing statistics of layer 3, MPLS distributes resources and builds forwarding tables for routing, while it utilizes layer 2 for switching or forwarding the information through the right link or route. Each IP packet includes a label of MPLS which is subsequently linked with a specific entry inside the forward routing table that identifies the upcoming hop. The network-flows with similar requirements for level of service and routing decisions, commonly keep the same pathway/route across the network resulting in a consistency of service-level for network-flows which having higher priority. MPLS is required to deploy the Label Switching Routers (Das et al., 2003) in the networks that will affect the momentum whereupon MPLS based solution is deployed. At the moment, MPLS is at target in favor of deployment in the backbones first.

We have implemented the MPLS/BGP VPNs in such a way that the features of layer 3 as scalability and intelligence are merged with the efficiency of layer 2 to cope up with almost all modern demands of speed, scalability and security. The proposed MPLS/BGP VPN design is implemented in Dynagen simulator (Dynagen, 2007) for easily understanding the system. Dynagen simulator is easily available and supports variety of network designs. In the proposed system five routers are used in which two routers belong to customer network and the rest belong to MPLS core network. BGP is used for route

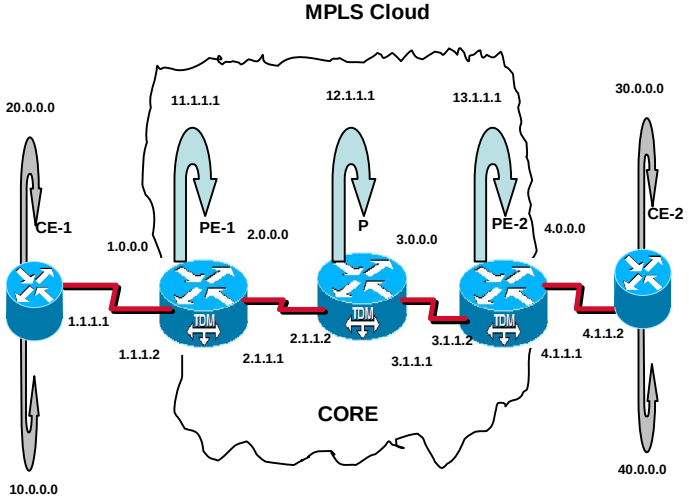


Figure 1. Network Topology

advertisement. After creating MPLS core testing is performed on customer routers which meet stated requirements.

2. MPLS/BGP VPNS Implementation

2.1 Network Topology

The proposed system MPLS/BGP VPN in a network core is implemented using Dynagen simulator in which the network topology consists of five routers that are as follows (also shown in Figure 1):

- CE-1 Customer Edge router at the one end of the network.
- CE-2 Customer Edge router at the other edge of the network.

Table 1. Proposed Network Design Configuration

Customer Edge (CE-1)	Customer Edge (CE-2)	Provider Edge (Core Router P)	Provider Edge (PE-1)	Provider Edge (PE-2)
config t	config t	config t	config t	config t
host CE-1	host CE-2	host P-1	host PE-1	host PE-2
no ip routing	no ip routing	no ip routing	no ip routing	no ip routing
ip routing	ip routing	ip routing	ip routing	ip routing
no ip domain-loo	no ip domain-loo	no ip domain-loo	no ip domain-loo	no ip domain-loo
no ena sec	no ena sec	no ena sec	no ena sec	no ena sec
enable pass cisco	enable pass cisco	ena pass cisco	ena pass cisco	ena pass cisco
int s1/0	int s1/1	ip cef	mpls label protocol ldp	mpls label protocol ldp
ip address 1.1.1.1 255.0.0.0	ip address 4.1.1.2 255.0.0.0	mpls label protocol ldp	mpls ldp router-id loop 0 force	mpls ldp router-id loop 0 force
no shutdown	no shutdown	mpls ldp router-id loop 0 force	ip vrf vpn1	ip vrf vpn1
k	k	int s1/0	rd 1:1	rd 1:1
no en	no en	mpls ip	route-target 1:1	route-target 1:1
clock rate 128000	clock rate 128000	ip address 3.1.1.1 255.0.0.0	int s1/0	int s1/0
int loop 0	int loop 0	no sh	ip address 1.1.1.2 255.0.0.0	mpls ip
ip address 10.1.1.1 255.0.0.0	ip address 30.1.1.1 255.0.0.0	k	no shutdown	ip address 3.1.1.2 255.0.0.0
int loop 1	int loop 1	no en	k	no sh
ip address 20.1.1.1 255.0.0.0	ip address 40.1.1.1 255.0.0.0	clock rate 128000	no en	k
router rip	router rip	int s1/1	clock rate 128000	no en
ver 2	ver 2	mpls ip	ip vrf forwarding vpn1	clock rate 128000
no auto	no auto	ip address 2.1.1.2 255.0.0.0	ip address 1.1.1.2 255.0.0.0	int s1/1
network 1.0.0.0	network 4.0.0.0	no shutdown	int s1/1	ip address 4.1.1.1 255.0.0.0
network 10.0.0.0	network 30.0.0.0	k	mpls ip	no shutdown
network 20.0.0.0	network 40.0.0.0	no en	ip address 2.1.1.1 255.0.0.0	k
line v 0 4	line v 0 4	clock rate 128000	no sh	no en
no login	no login	int loop 0	k	clock rate 128000
privi l 15	privi l 15	ip address 12.1.1.1 255.0.0.0	no en	ip vrf forwarding vpn1
line c 0	line c 0	router rip	clock rate 128000	ip address 4.1.1.1 255.0.0.0
no login	no login	ver 2	int loop 0	int loop 0
privi l 15	privi l 15	no auto	ip address 11.1.1.1 255.0.0.0	ip address 13.1.1.1 255.0.0.0
		network 2.0.0.0	router rip	router rip
		network 3.0.0.0	ver 2	ver 2
		network 12.0.0.0	no auto	no auto
		line v 0 4	network 2.0.0.0	network 3.0.0.0
		no login	network 11.0.0.0	network 13.0.0.0
		privi l 15	address-family ipv4 vrf vpn1	address-family ipv4 vrf vpn1
		line c 0	ver 2	ver 2
		no login	no auto	no auto
		privi l 15	network 1.0.0.0	network 4.0.0.0
			redistribute bgp 100 metric transparent	redistribute bgp 100 metric transparent
			router bgp 100	router bgp 100
			no auto -summary	no auto -summary
			no synchronization	no synchronization
			neighbor 13.1.1.1 remote-as 100	neighbor 11.1.1.1 remote-as 100
			address-family vpnv4	address-family vpnv4
			neighbor 13.1.1.1 activate	neighbor 11.1.1.1 activate
			neighbor 13.1.1.1 send-community both	neighbor 11.1.1.1 send-community both
			neighbor 13.1.1.1 next-hop-self	neighbor 11.1.1.1 next-hop-self
			address-family ipv4 vrf vpn1	address-family ipv4 vrf vpn1
			no auto -summary	no auto -summary
			no synchronization	no synchronization
			redist rip	redist rip
			line v 0 4	line v 0 4
			no login	no login
			privi l 15	privi l 15
			line c 0	line c 0
			no login	no login
			privi l 15	privi l 15

- PE-1 Provider Edge router by the side of one end of the MPLS cloud. Interface of this router with IP address 2.1.1.1 is a part of MPLS/BGP VPN while the interface with IP address 1.1.1.2 does not belong to VPN.
- PE-2 Provider Edge router by the side of other end of the MPLS cloud. Interface of this router with IP address 3.1.1.2 is a part of MPLS/BGP VPN while the interface with IP address 4.1.1.1 does not belong to VPN.
- P is a Core router.

2.2 Network Configuration

Following configurations are made on each router in the proposed network design as given in Table 1:

2.3 Simulation Results

2.3.1 Operation 1

The following operation (Figure 2) is to perform that the designed MPLS/BGP VPN has been established and showing the VPNs basic feature of security against the unauthorized access. An attempt

to access interface 1.1.1.2 of router PE-1 from CE-1 gets 100 percent success while for the 2.1.1.1 is totally denied. Similarly, all attempts to access router P and 3.1.1.2 interface of PE-2 from CE-1 are refused. But all attempts to access 4.1.1.2 interface of router PE-2 and router CE-2 are again perfectly successful. So, it shows that MPLS BGP VPN exists consisting of three routers.

2.3.1.1 Results

The interfaces of the routers lying within the VPN are inaccessible by any external host but traffic destined through these is communicated properly which is an ultimately key security feature of VPN. As above configuration is of MPLS/BGP VPN so it becomes obvious that MPLS/BGP VPN is a dedicatedly secure channel for traffic transmission.

2.3.2. Operation 2

The following operation shows how labels are assigned to the routes in MPLS based networks (i.e., edge and core routers) and what is the role of these routers in traffic forwarding treat the routes.

2.3.2.1 Router PE-1

Tagging and Label Distribution

The following output (Figure 3) shows the labels being received and forwarded by the provider's edge router PE-1.

MPLS Forwarding & BGP Routing Table

The output displayed in Figure 4 shows the basic operation of provider edge router PE-1. It pops up the tags from all of routes received form P while it tags up the routes properly coming from CE-1 and directly connected to it. It shows the detail of valid and best routs along with their next hops. The presence of VPN named as VPN1 expresses the establishment of Tunnel.

2.3.2.2 Router P

MPLS Forwarding Table

The output displayed in Figure 5 shows the basic operation of provider core, it pops up the tags from all of routes received form PE-1 and PE-2.

Tagging and Label Distribution

Figure 6 and Figure 7 show the labels being received and forwarded by the provider core router P.

2.3.2.3 Router PE-2

Tagging and Label Distribution

Figure 8 shows the labels being received and forwarded by the provider edge router PE-2.

MPLS Forwarding & BGP Routing

The output displayed in Figure 9 shows the basic operation of provider edge router PE-2, it pops up the tags from all of routes received form P while it tags up the routes properly coming from CE-2 and directly connected to it. It shows the detail of valid and best routes along with their next hops. The presence of VPN named as VPN1 expresses the establishment of tunnel.

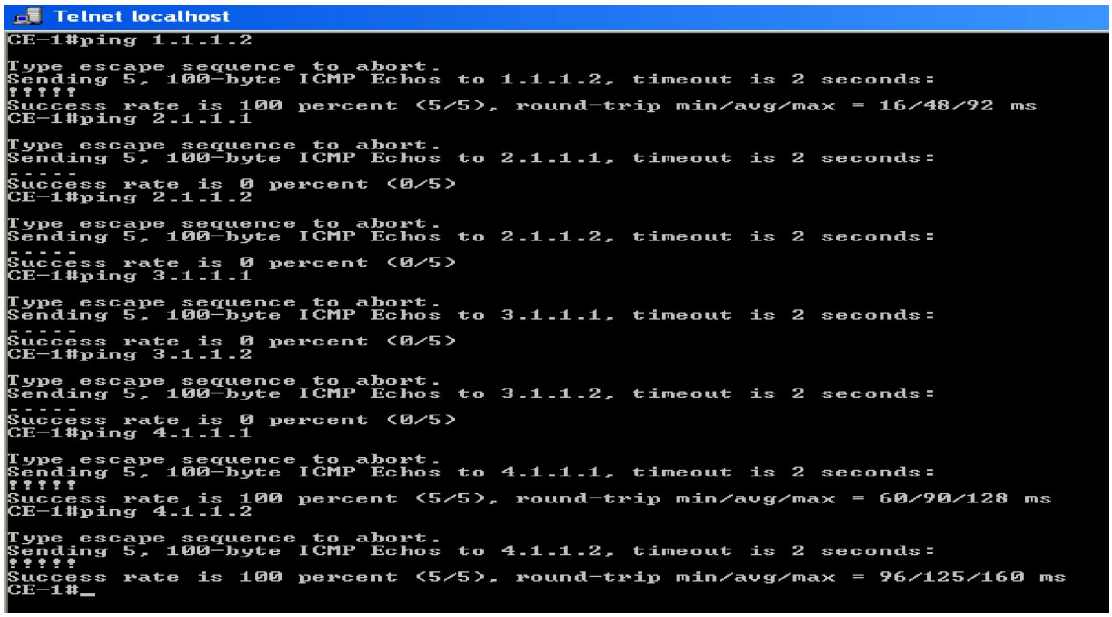


Figure 2(a). MPLS BGP VPN Test

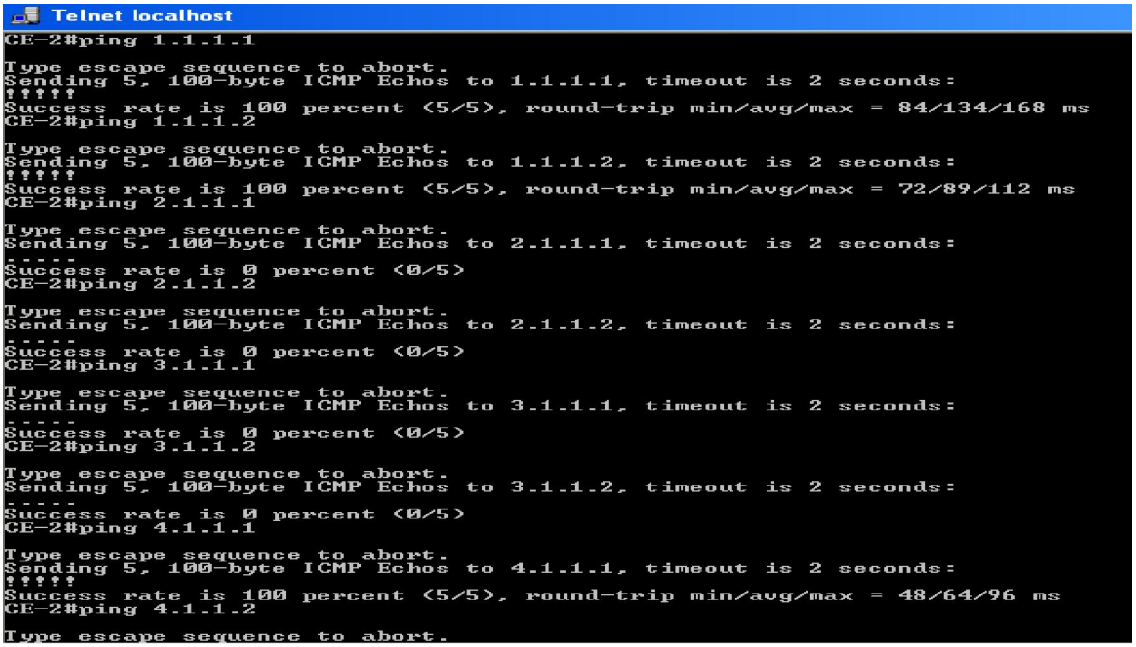


Figure 2(b). MPLS BGP VPN Test

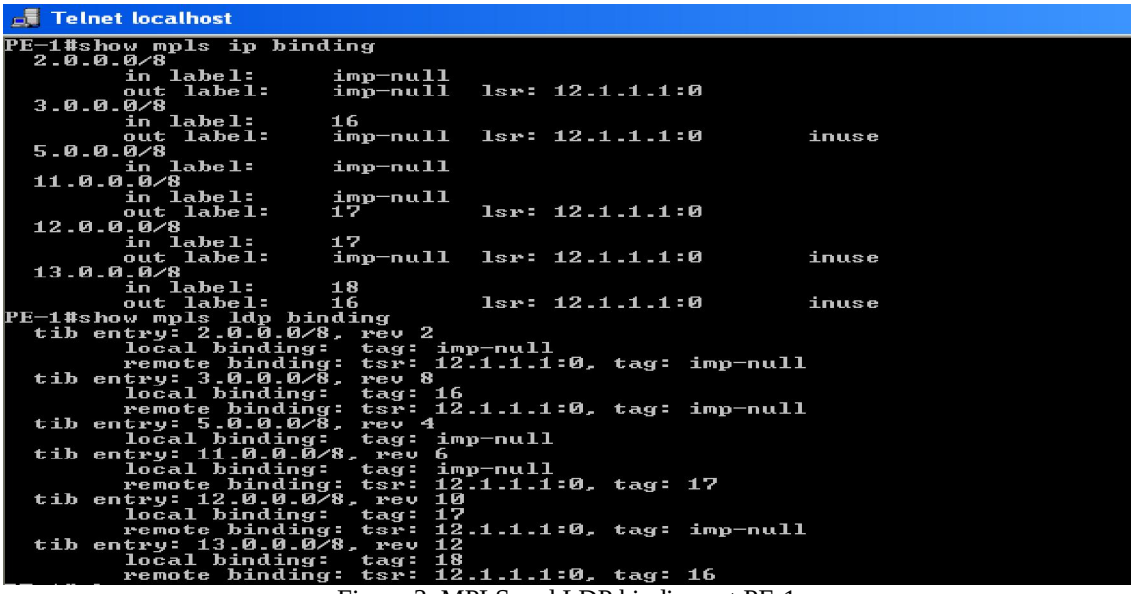


Figure 3. MPLS and LDP bindings at PE-1

2.3.3 Results

- The provider edge router PE-1 assigns labels to its directly connected networks and the traffic coming from CE-1 as starting edge router of MPLS cloud and sends it to core route P. The router which receives the outside traffic at the edge of MPLS VPN and forwards it to the core after labeling is called Ingress Router (IR). Similarly, it pops up the tags of traffic coming from core as ending edge router of MPLS cloud and sends it to C-1.
- The edge router which receives tagged traffic from the core and forwards this traffic after un-tagging is called Egress Router (ER). So PE-1 is ingress for the traffic coming form CE-1 and being forwarded to the core and is egress for the traffic coming from core and being forwarded outside the MPLS cloud.
- The PE-2 (provider edge router) is ingress for the traffic coming from CE-2 while it is egress for the traffic coming from core.
- The core router just switches the tags which causes a huge enhancement in traffic forwarding.

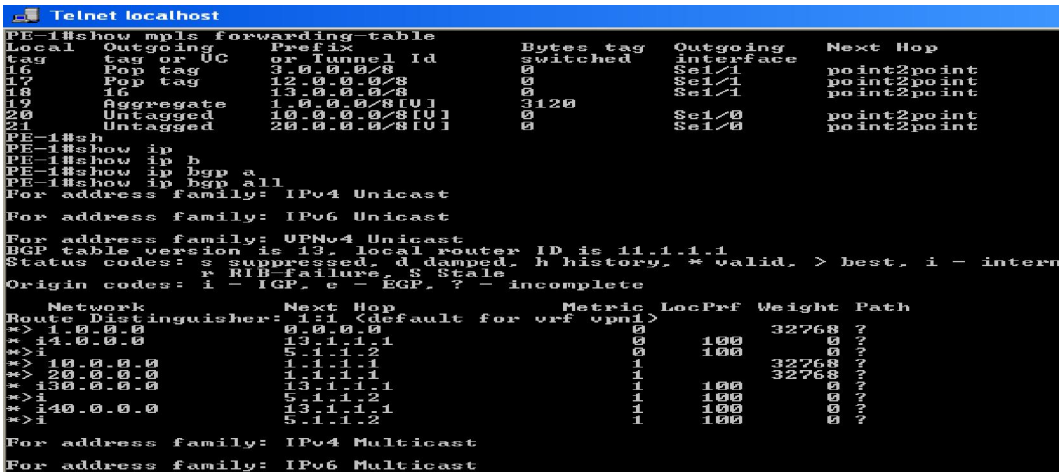


Figure 4. MPLS Forwarding & BGP Routing Table of PE-1

Figure 5. MPLS Forwarding Table of P

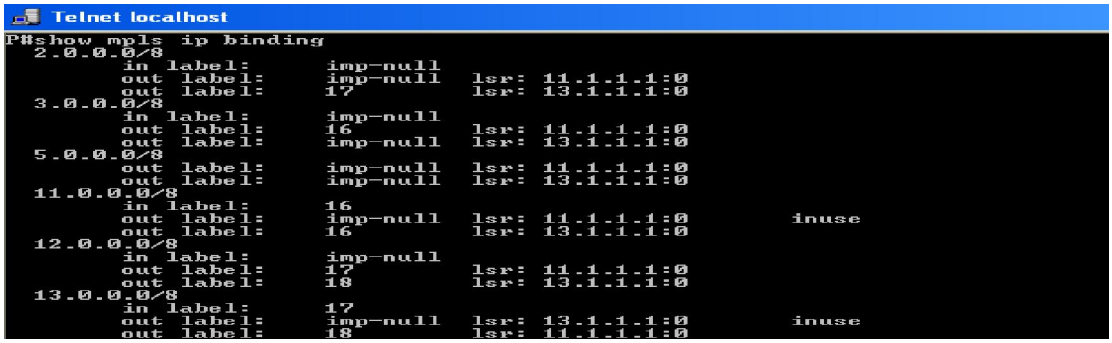


Figure 6. MPLS labeling on core router P

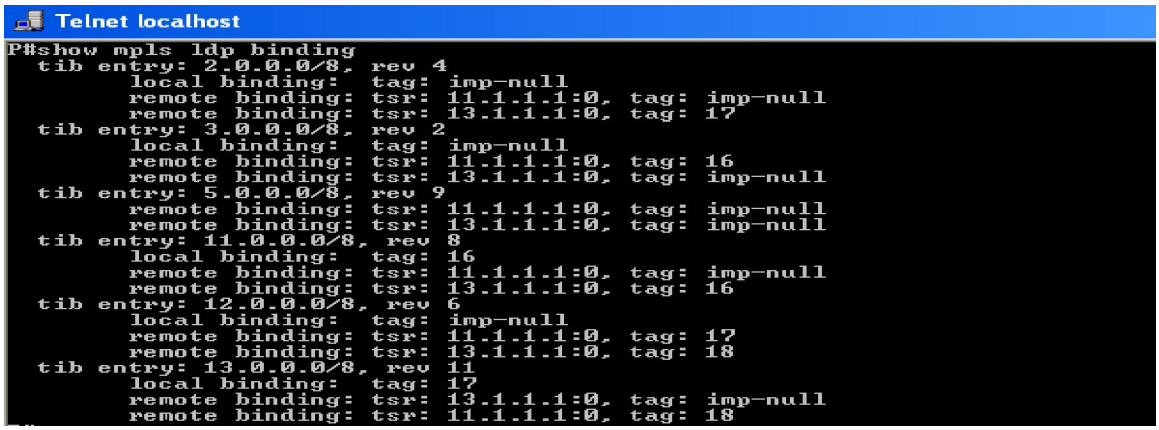


Figure 7. LDP binding on Core router P

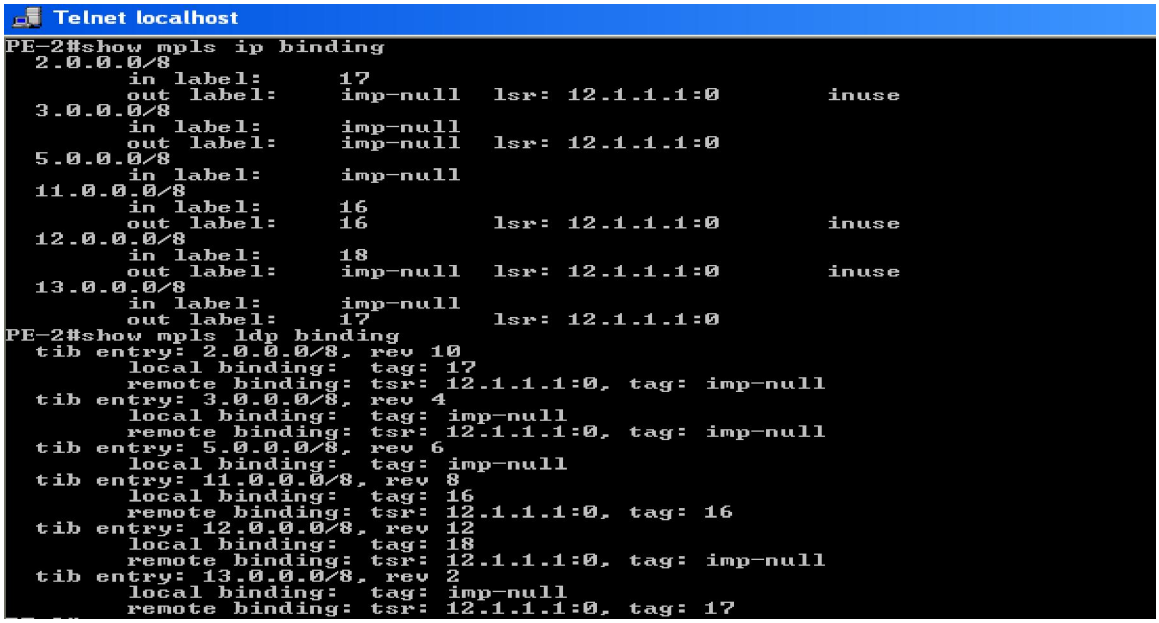


Figure 8. MPLS & LDP bindings at PE-2

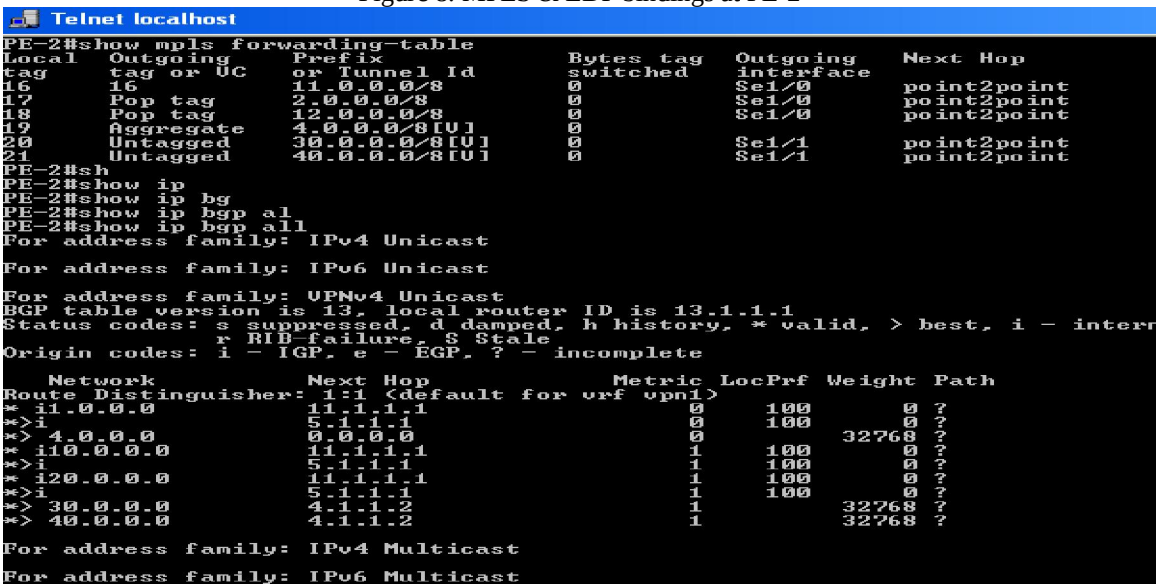


Figure 9. MPLS Forwarding & BGP Routing Table of PE-2

3. Conclusions and Future Work

In this research work firstly, we have comprehensively presented an overview of MPLS, BGP and, both layer 2 and layer 3 VPNs. In particular, IP VPNs issues such as speed, scalability and security are discussed in detail. Secondly, we have proposed a new design scheme for MPLS/BGP-VPNs by merging the features of layer-3 (such as scalability and intelligence) with the features of layer-2 (such as efficiency and simplicity), to deal with the today's evolving demands of network speed, quality of service, scalability and security.

We have discussed in detail the challenges when these two architectures will be merged to provide another infrastructure and we have also provided the solution to some of these challenges to make this new concept worthy and an asset to the current research world. We have presented a network simulation architecture that helps us to assess the security constraints for MPLS/BGP-VPNs.

Further this research can be enhanced to traffic engineering (TE), end-to-end performance, security and path management. Traffic engineering (TE) includes schemes and methods that are applied to force routed traffic to pass through the network on a path, except one which is selected on the basis of standard routing. This system will be helpful for adding new security features in core networks in future and provides a guideline for network engineers towards the world of network security.

In our future work, we will discuss the various issues regarding the implementation of MPLS/BGP-VPNs in multihomed (Junaid and Saleem, 2010; Junaid & Saleem, 2008) environments.

Acknowledgments:

This work was supported by the Directorate of Research Extension and Advisory Services, University of Engineering and Technology (UET), Lahore-Pakistan.

Corresponding Author:
Mohammad Junaid Arshad, PhD
Department of Computer Science & Engineering,
University of Engineering and Technology,
Lahore-Pakistan-54890.
Email: junaidarshad@uet.edu.pk

References

1. Previdi, S. Introduction to MPLS-BGP-VPN, Proceeding of MPLS Forum 2000, Cisco, April, 2000.
2. Awduche, D.O. MPLS and Traffic Engineering in IP Networks, IEEE Communication Magazine, Volume 37, No. 12, pp. 42-47, New Jersey, USA., December, 1999.
3. Rosen, E., and Rekhter, Y. BGP/MPLS VPNs, RFC 2547, IETF (draft-rosen-rfc2547bis-03.txt), March, 1999.
4. Lee, H., Hwang, J., Kang, B., and Jun, K. End-To-End QoS Architecture for VPNs: MPLS VPN Deployment in a backbone Network, Proceedings of IEEE International Workshop on Parallel Processing, Volume 5, No. 1, pp. 479-483, Toronto, Canada, August, 2003.
5. Swallow, G. MPLS Advantages for Traffic Engineering, IEEE Communication Magazine, Volume 37, No. 12, pp. 54-57, New Jersey, USA. December, 1999.
6. Ferguson, P., and Huston, G. What is VPN, The Internet Protocol Journal, Volume 1, No. 2, San Jose, USA., September, 1998,
7. Callon, R. A framework for layer 3 provider provisioned virtual private networks, IETF (draft-ietf-ppvpn-frame-work-06.txt), October, 2002.
8. Alawieh, B., Ahmed, R.E., and Mouftah, HT. Security impacts on establishing MPLS/BGP VPNs, Journal of Security and Communication Networks, Volume 1, No. 4, pp. 269-275, John Wiley & Sons, New Jersey, USA., July, 2008.
9. Davis, C. IPsec: Securing VPNs, McGraw-Hill, Berkley, pp. 1-432, California, USA. April, 2001.
10. Deering, S., and Hinden, R. Internet Protocol Version 6 (IPv6) Specification, RFC 2460, December, 1998.
11. Rosen, E., Viswanathan, A., and Callon, R. Multiprotocol Label Switching Architecture", IETF RFC 3031, January, 2001.
12. Rosen, E., and Rekhter, Y. BGP/MPLS VPNs, RFC 2547, March, 1999.
13. Das, S.K., Venkataram, P., and Biswas, J. MPLS-BGP based LSP Setup Techniques, Proceedings of the 28th Annual IEEE Conference on Local Computer Network (LNC), Volume 17, No. 1, pp. 279-280, Singapore, July, 2003.
14. Dynagen Simulator, http://dynagen.org/ [online, April, 2007].
15. Junaid, M., and Saleem, M. A Simulation-Based Study of FAST TCP Compared to SCTP: Towards Multihoming Implementation Using FAST TCP. Journal of Communications and Networks (JCN), VOL. 12, NO. 3, pp. 275-284, June 2010.
16. Arshad, MJ., and Saleem, M. Issues of Multihoming Implementation Using FAST TCP: A Simulation Based Analysis, IJCSNS International Journal of Computer Science and Network Security, VOL. 8, No. 9, pp.104-114, August 2008.

6/28/2010

GRIAS: GUI-Based Real-Time Industrial Automation Software

Mohammad Junaid Arshad [1], Amjad Farooq [2]

[1, 2] Department of Computer Science & Engineering, University of Engineering and Technology Lahore, Pakistan
amjadfarooq@uet.edu.pk junaidarshad@uet.edu.pk

Abstract: Industry has a great importance in the development of a country. These days a country cannot progress and prosper without industrial development. Industrial revolution has changed the fortune of many western countries. In the fast moving world of today, the industrial plants have become very complicated and many new technologies have been introduced in the market to overcome these complications by automating the industrial plants. This work proposes an industrial automation software called GRIAS (GUI-Based Real-Time Industrial Automation Software) that can be used for any industrial plant in which OPC (OLE (Object Linked Embedding) for Process Control) compliant hardware devices are used. This generic software has the ability to interact with an OPC server which is responsible to retrieve runtime data from the hardware device. The data provided by the server can be used by the software to monitor the running industrial plant. It can also be used in critical industrial units where it is very difficult to manually control the machinery. The industry has been looking for such software which can meet up their requirements, thus, this new industrial automation software will surely be able to realize their dreams into reality. The purpose of this automation software is not only to eliminate the perils and hazards involved in industries but also to speedup the process of manufacturing and production in such a way that it is no more error prone. [Journal of American Science. 2010;6(12):96-101]. (ISSN: 1545-1003).

Keywords: Graphical User Interface (GUI), Design, Industrial Automation, Human Machine Interface (HMI), OPC, Solution

1. Introduction and Problem Statement

The sole super power of the world is basically an industrial power. Industry has the importance of a backbone in the economy of a country. In the fast moving world of today, the industrial plants have become very complicated and also very dangerous to the environment and the human life. A single industrial unit which is not functioning properly can prove to be a source of great peril to all human population in the nearby area.

The industrial trends have changed at a very fast pace and these days due to the presence of internet trends are changing on a day to day basis. The industrial trends are in a transition from traditional based business to business transactions, to web based and e-commerce. The most important parameters in the industrial manufacturing are the consumer interaction, product characteristics, rapidity, costs, strategic assets and productions.

The automation software has totally changed the industry trends. These days no industry can think of increase in productivity and competing in the market without the automation software. The automation software provides the competitive edge to the company using it, because it increases both efficiency and the productivity. Traditionally, without the presence of automation softwares, the speed of placing an order was too much. It took weeks to place an order and even months to receive the confirmation.

There are a large number of industrial automation technologies which are widely used in the market to automate an industrial plant/unit. These technologies cannot work in isolation and usually a blend of technologies is used to prepare an automation solution. Some of these are physical interface, data acquisition, databases, GUI/visualization etc. The various vendors have developed various tools which based on any one of these technologies. These tools provide an integrated development environment (IDE) which helps to develop a software solution in a very efficient way. The various software technologies are the SCADA (Supervisory Control and Data Acquisition) (Daneels et al., 1999), Human Machine Interface (HMI) (Hagenmeyer, 2005), OPC (OLE for Process Control) (DeltaV, 2007) and so on.

1.1. Overview of OPC

OPC is an industry standard for hardware devices used in critical industrial units. All OPC devices follow certain specifications which are laid down in the OPC standards papers (Draft-3, 1998; OPC; Zheng et al., 2002). These specifications force all the devices to use the same technology so that any software written for one OPC compliant device should be able to work with another. OPC is based on

OLE technologies. It characterizes standard objects, schemes and functions for real-time information servers (such as historian, PLCs (Baresi et al., 2000), DCS and other software applications). The end users by utilizing the OPC specifications may select the client application which best fulfills their demands. Prior to OPC an end user used particular client software that offered an interface for a specific control device only, but by means of OPC, any client application which is OPC compliant can interface to a control device with an OPC compliant server, thus, the end user finds the best way out for a specific assignment.

In this context, this work also proposes new automation software (i.e., GRIAS) to automate any OPC (OLE for Process Control) compliant device. The GRIAS automation software actually interacts with an OPC server which is responsible to retrieve runtime data from the hardware device. This data provided by the server is used by the software to monitor the running industrial plant. This data may be the temperature in a certain boiler or the pressure at a certain valve etc. The software is also able to generate commands to the hardware device through the OPC server in order to keep the industrial unit under control. These commands may result in lowering the pressure or temperature to a certain degree or the opening of a valve etc.

The GRIAS has two types of users; a higher level user (or the administrator) is able to configure the software for a particular industrial unit. The administrator can also perform certain administrative tasks like creating or deleting the operators and setting their privileges and logins. The second type of user is the operator who is assigned the task of monitoring the runtime data coming from the industrial unit. An operator may also issue the commands if required.

It also includes a manual to provide a systematic and right approach for making and maintaining automation software. The various design steps are explained with the help of market standard UML diagrams. The various use-case, sequence and collaboration diagrams are also included to give a clear view of the functionalities and the sequence in which various actions are taken by the software.

The most important feature of this work is that it throws light on the different development phases of software engineering which are necessary for developing quality software.

Thus, this research work proposes such GUI-Based Real-Time Industrial Automation Software that will be able to control an industrial plant which uses an OPC complaint hardware device. It will be used to monitor the industrial units and issue the required commands on time. This will make the overall process of industrial manufacturing and production very efficient and will certainly reduce the cost of business. Its implications will also include the safety of human lives as everything will be controlled by the software and no human interaction with the industrial plants will be needed.

1.2. Assumptions and Dependencies

In the initial software it will be assumed that the PLC (Programmable Logic Controller) (Baresi et al., 2000) device is available and the OPC server is also available. Since a PLC device is very expensive and a group of students without any funding cannot purchase it, thus it will be assumed that a PLC device is present and working. A simulator will be used instead of a PLC device; the simulator will help to generate the data for the software just like a PLC device produces. The data produced by the simulator will be used by the application and it will appear as if data is really coming from the hardware device that is the PLC.

2. Software Analysis and Design

In this section, data flow diagrams (DFD) are used to represent the flow of data in the system. The zero level DFD in Figure 1 shows the top level view of the proposed system. The first level DFD in Figure 2 then shows the various processes hidden in a zero level diagram and demonstrates the interaction of various external entities with these processes. The next level DFD in Figure 3 represents the processing of data at different levels and interaction of various processes with the data stores.

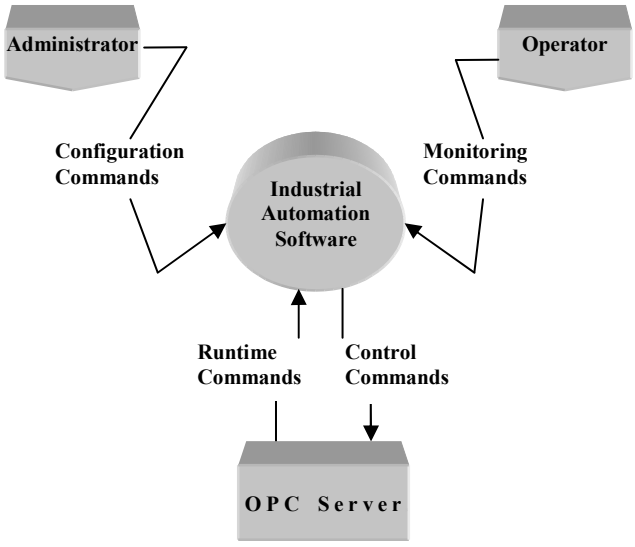


Figure 1. A top level view of the proposed system

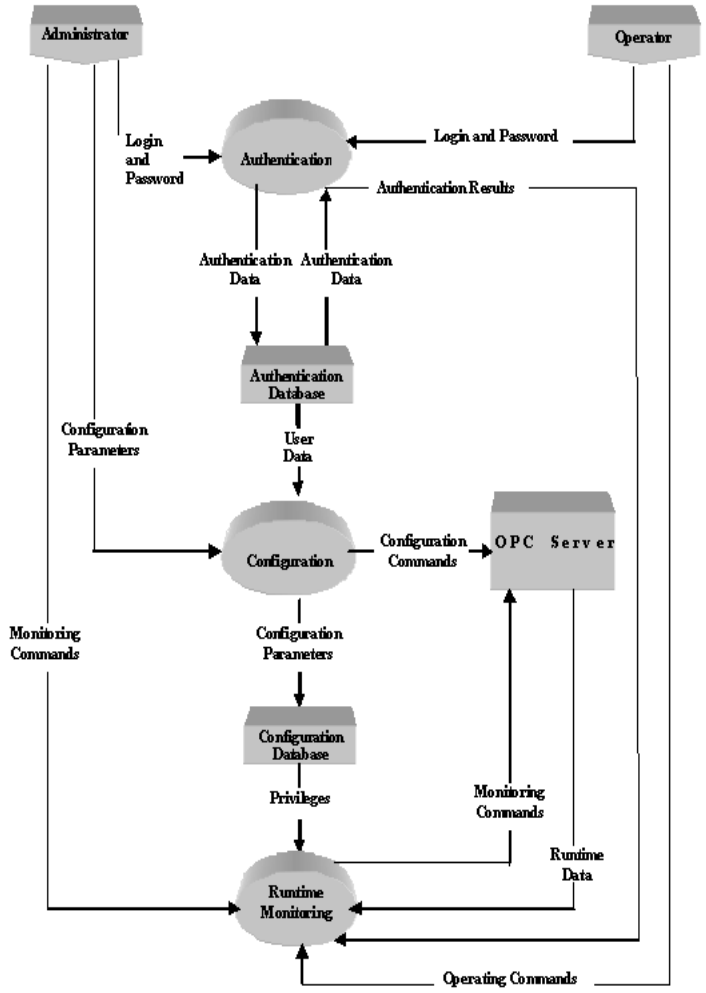


Figure 2. First level DFD of the system

3. Proposed Software's Features

3.1. Object-Oriented Architecture

This software involves some basic steps to build a process. Initially choose the object's type that the developer wants to make from software's big library having display, driver, control & data handling objects. Next, you organize the objects through software's advanced features and lastly you attach these objects. A developer is able to make remote/complex connection, or direct connection which perform calculation or check logical conditions. After creating the objects selected from software's extensive library, all the driver objects are now arranged to write and read from the field hardware attached to your system. You are even able to create a basic process also within an aggregate object to reuse with other processes that save your time.

3.2. Event-Driven Performance

Events drive the industrial automation software, meaning that whenever a change happens, it is instantly reported. As compared to loop-based softwares it responds to events more rapidly.

3.3. Online Configuration Features

A developer can build, modify and stop a process while its application is in progress. With the help of online configuration feature you can perform the maintenance without having any gap in your application data, event, alarm and records.

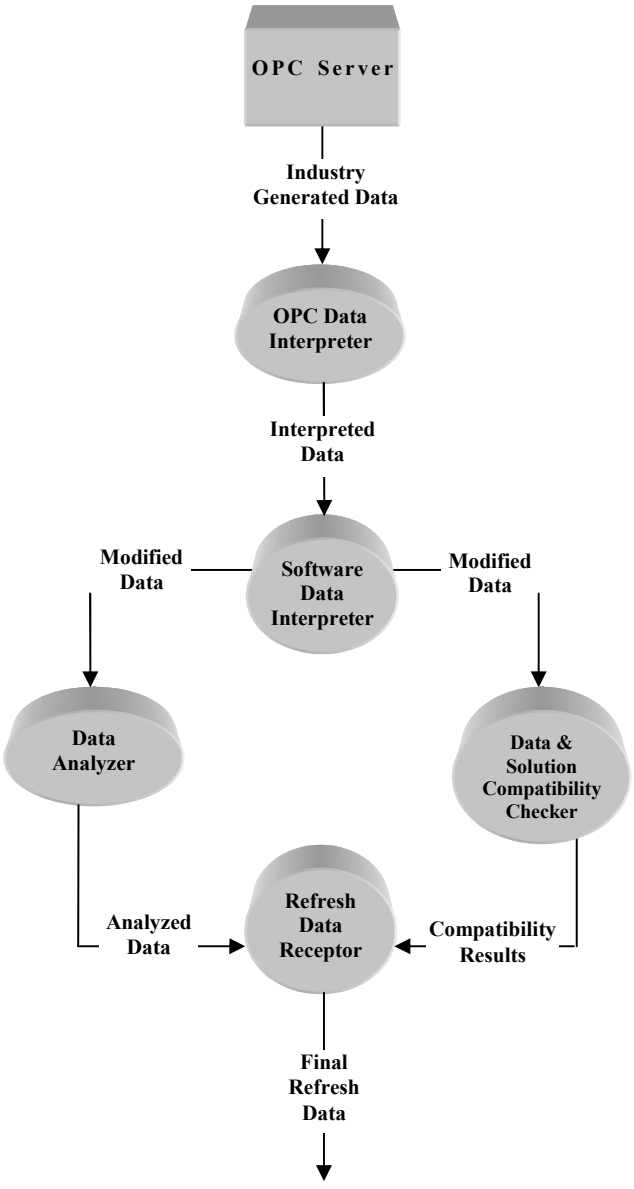


Figure 3. Second level DFD of the system

3.4. Redundancy Capabilities

GRIAS provides enhanced objects which can simply be used to produce urgent backing system to work at the time when the system breakdowns. A developer can apply objects as well to build and sprint several processes as of a single process model–all made the simple Industrial Automation Software way.

3.5. Web Capabilities

It also supports the web-connectivity and by the means of this feature; a developer is able to examine and supervise automation software's process by any workstation connecting to the Internet. You can rapidly construct a basic webpage by means of an easy export module and then via Internet Explorer the client process can be accessed. Also you can generate the HTML reports pages simply for observing.

3.6. Graphics and Visualization

GRIAS offers a simple visual interface for controlling and monitoring industrial applications. It allows the developers to apply graphics to visually illustrate their processes at control panels. This software contains a wide graphics library which includes not only animated figures but static pictures as well that can improve your process via graphically presenting your system and the variations that take place, as shown in Figure 4. Also your own graphics (for example auto-cad documents or photographs) for added customizing can be imported. This automation software also supports .wmf and .bmp files. The graphics and visualization add to the look and feel of the entire application that makes it more attractive.

3.7. Multi-Client/Multi-Server Networking

A developer can attach several computers, which are connected through Industrial Automation Software's server and client processes, as shown in Figure 6. The TCP/IP (Stevens, 1996) networking protocol integrated into Industrial Automation Software to interconnect networks in a simple-way like to drag a data-point on a control-panel view or typing into a URL.

3.8. Security

Automation software allows you to set different levels of security, with varying levels of write/read access derived from these levels of security.

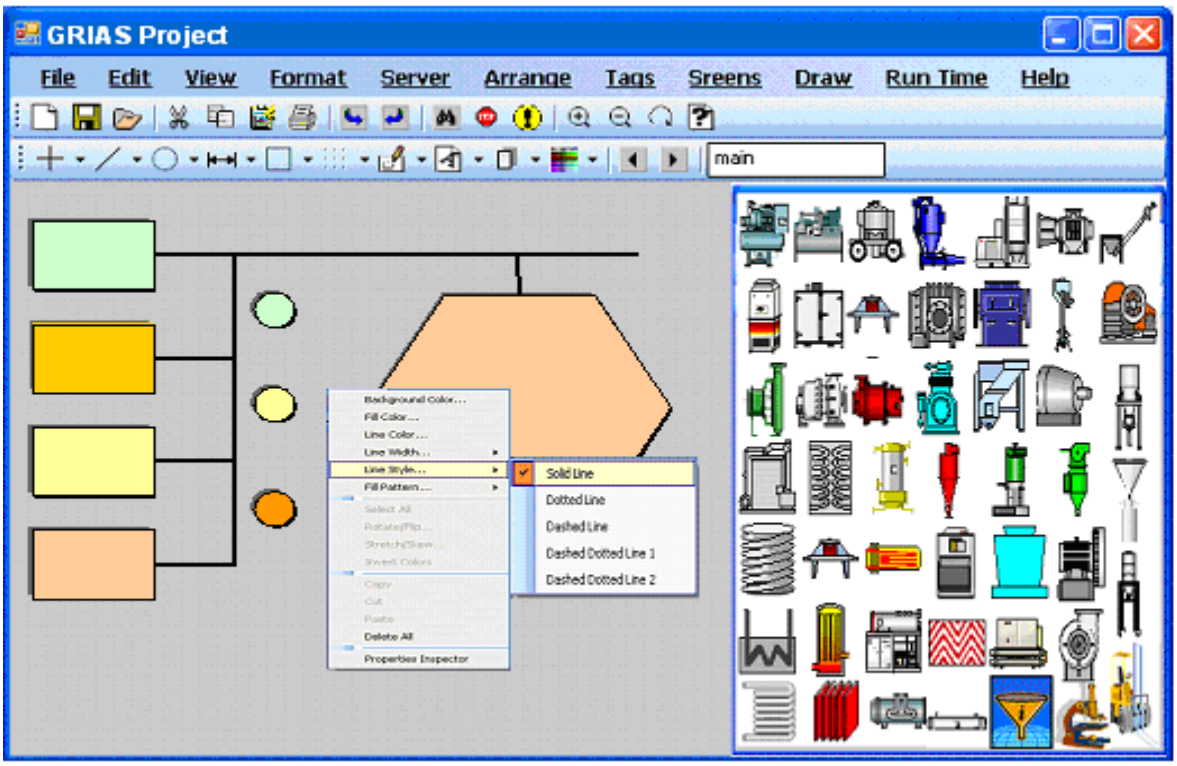


Figure 4. Screen shots of the GRIAS software

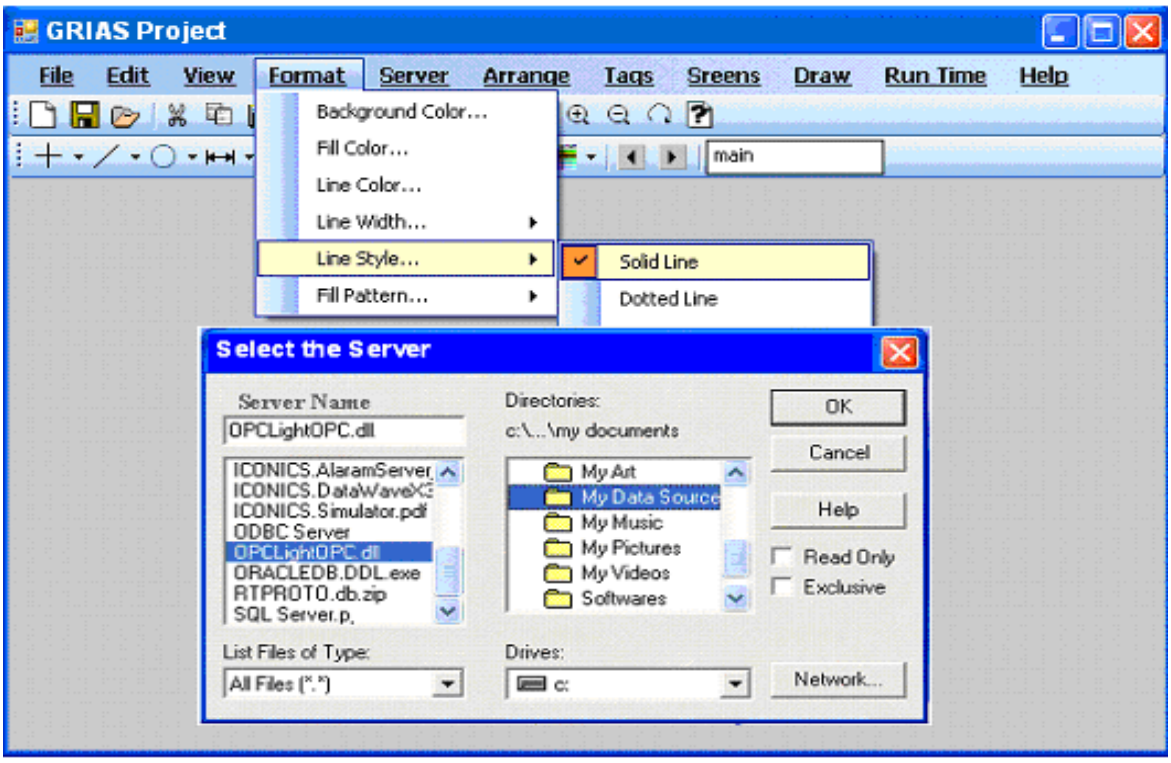


Figure 5. Screen shots for selecting a server

You are able to apply the security options restricting panel to retrieve and view, particular object handling or whether closing or minimizing the Automation Software. You can apply the limit over users and groups accessibility, including permission to a particular folder and individual object. Even you have the authority to impose accessing limit by means of IP-addresses.

In addition, Automation Software includes the following other capabilities:

Distributed Data-Logs- the GRIAS logs event, alarm and data to the software's personal data-base.

Trends & Charts- it facilitates the user to represent the graphics-data from several different sources at once in parallel.

Report Generation- it provides the facility to generate custom reports using your favorite spreadsheet package.

Telemetry- it supports the various protocols to utilize the single serial-port.

Hardware Connectivity- it provides notable integration for National Instruments (NI) hardware, for example, Field-Point & Data Acquisition (DAQ) (Hayles, 2006) equipments.

Technical Support- the software not only performs the automation but also helps in management purposes (e.g., it helps in maintaining session information).

Operating Systems Used- it supports different operating systems according to the requirements of an industry.

4. Conclusions

In this research work, we have proposed the GUI-Based Real-Time Industrial Automation Software to control and maintain an industrial plant which uses an OPC complaint hardware device. We have described the proposed system in terms of its interface to the process hardware and structural design, and presented its functionalities/facilities it provides for application development.

In some developing countries like Pakistan, there are still hundreds of small or medium size industries that are neither automated nor computerized, because they cannot afford to buy such costly automation software currently available in the market. Thus, through the proposed GRIAS software, the small or medium size industry of Pakistan that is running its system manually will be encouraged to use and maintain its system at the least possible cost. That tolerates significant progress in the competitive

development of a country via the use of real-time (and even web-enabled) automation software.

This research work will also be helpful for the researchers or whether you are an IT supervisor in an industrial unit or a novice who is interested in automating his industrial plants to give a boost to his productions.

Acknowledgements:

This work was supported by the Directorate of Research Extension and Advisory Services, University of Engineering and Technology (UET), Lahore-Pakistan.

Corresponding Author:
Mohammad Junaid Arshad, PhD
Department of Computer Science & Engineering,
University of Engineering and Technology, Lahore-Pakistan
Email: junaidarshad@uet.edu.pk

References

1. Hagenmeyer, Marberger, C., Bekiaris, E., and Nikolaou, S. Preliminary Guidelines for the Personalization of the HMI of Hypovigilance-Management-Systems, In Proceedings of Human Computer Interaction International, Mahwah, 2005.
2. White Paper (DeltaV). OLE for Process Control (OPC) Overview, pp. 1-13, March, 2007.
3. Daneels, A., and Salter, W. What is SCADA, In Proceedings of International Conference on Accelerator and Large Experimental Physics Control Systems, pp. 339-343, Italy, 1999.
4. OPC Foundation, OPC Overview, Version 1.0 (Draft 3), October 1, 1998.
5. Foundation, OPC Data Access Automation Interface Standard, Version 2.02, February 4, 1999.
6. Zheng, L., and Nakagawa, H. OPC (OLE for process control) specification and its developments, In Proceedings of the 41st SICE Conference, Volume 2, pp. 917-920, August 5, 2002.
7. Baresi, L., Mauri, M., Monti, A., and Pezze, M. PLCTOOLS: Design, formal validation, and code generation for programmable controllers, In Proceedings of the IEEE SMC, 2000.
8. Stevens, WR. *TCP/IP Illustrated: Volume 1; The Protocols*, Addison-Wesley Professional Computing Series, Addison-Wesley, Reading, Massachusetts, 1996.
9. Hayles, T. Developing Networked Data Acquisition Systems with NI-DAQ, National Instruments, 2006 http://www.natinst.com/pdf/instrupd/appnotes/an116.pdf.

6/26/2010

Arch Dam Failure Diagnosis Applying Micro-Planes Damage Based Framework

Mojtaba Labibzadeh 1

1. Department of Civil Engineering, Faculty of Engineering, Shahid Chamran University, Ahvaz, Iran
Labibzadeh_m@scu.ac.ir

Abstract: A recently new developed set of constitutive equations which simulating the mechanical behavior of plane concrete have been implemented for monitoring the probability of cracking phenomenon within an arch concrete dam . The applied constitutive model was build on the basis of combination of micro-plane theory and damage framework. This model had been verified through comparing numerical results with experimental ones. The case study is a high elevated concrete arch concrete dam entitled Liroo dam. Obtained analysis results demonstrated that under proposed earthquake excitations, dam experiences some cracks near its middle crest. [Journal of American Science. 2010;6(12):102-107]. (ISSN: 1545-1003).

Keywords: Arch dam, Micro-planes, Cracks, Constitutive relations, Concrete

1. Introduction

In the field of structural analysis of concrete arch dams subjected to hydrostatic, thermal and earthquake loads many efforts have been performed until today (Noorzaie, et. al., 2002, Sadrnejad and Labibzadeh, 2006, Labibzadeh et. al., 2010, Labibzadeh and Khajehdezfuli, 2010, Labibzadeh, 2010). However, these analyses mostly done based on the hypothesis of macroscopic stress and strain fields. Mentioned assumption leads to some extent of limitation in obtaining desired level of accuracy of analysis results mainly due to the application of stress and strain invariants in derivation of material constitutive relations. Holding this issue in mind, in this study an attempt has been made to overcome such shortcoming through using a meso scale behavioral model named micro-planes in simulation. Micro planes model has been proposed for the first time by (Taylor, 1938) according to the plasticity model and after that (Bažant, et. al., 1984, 1985, 1988, 1997, 2000) has expanded the micro planes theory with strain softening and showed its ability in explanation of cracking and strain softening damages in brittle materials such as concrete. In this model the main concern is to present behavior of materials of different type in a scale between macro and micro scales called meso scale. In this paper, a recently developed micro-planes damage based model (Labibzadeh and Sadrnejad, 2006, Sadrnejad and Labibzadeh, 2006) has been implemented to failure analysis of an arch concrete dam named Liroo dam. Such a work had been done just one time in the past (Labibzadeh and Sadrnejad, 2007) and satisfactory results had been obtained which demonstrated the applicability of the proposed model. Liroo dam is a high elevated (height of the dam=200m) concrete arch dam which has been designed by Dez-Ab consulting engineers company and it is going to be constructed in north of the Khouzestan Province of Iran in near future.

2. Materials and Methods

Figure 1 outlines the procedure which should be followed to obtain the constitutive modulus matrix used in the fundamental stress-strain relation in material modeling. This flowchart has been pursued through any iteration in a nonlinear iterative solution method used in a developed finite elements code to evaluate the concrete behavior at any integration sampling point. The sequences which must be proceeded to the proposed three dimensional finite elements code have been shown schematically in figure 2. In each load step, the procedure described in figure 1 will be followed through any iteration until craving approximation may be achieved. According to devoted algorithms outlined in figures 1 and 2, a computer program has been developed based on the well-known F.E.M approximation method. This code then would have been implemented for analyzing the Liroo arch dam. The plan of the arches, the crown cantilever and profile of the line centers of the dam were shown in figures 3 and 4. As it can be seen from those two pictures the dam is a two-centered double curvature arch dam. The height of the dam equals to 220m from its base. The thickness of the crest and base of the dam are 10m and 35m respectively. The modulus of elasticity for the mass concrete was inserted as 25000 MPa into program and Poison's ratio was considered as 0.17.

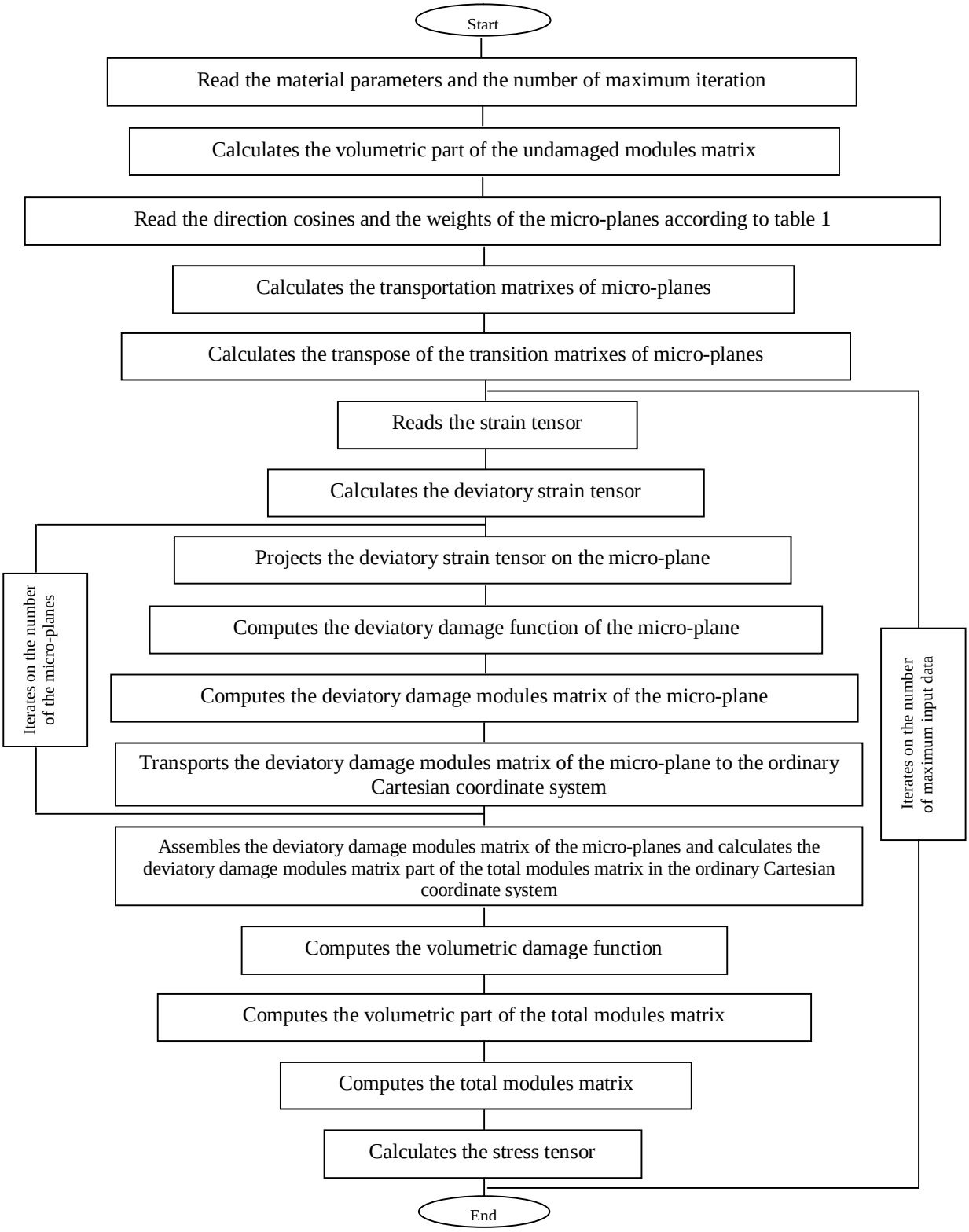


Figure 1. Operation sequence of the proposed micro-plane damage model developed in the VISUAL FORTRAN computer language

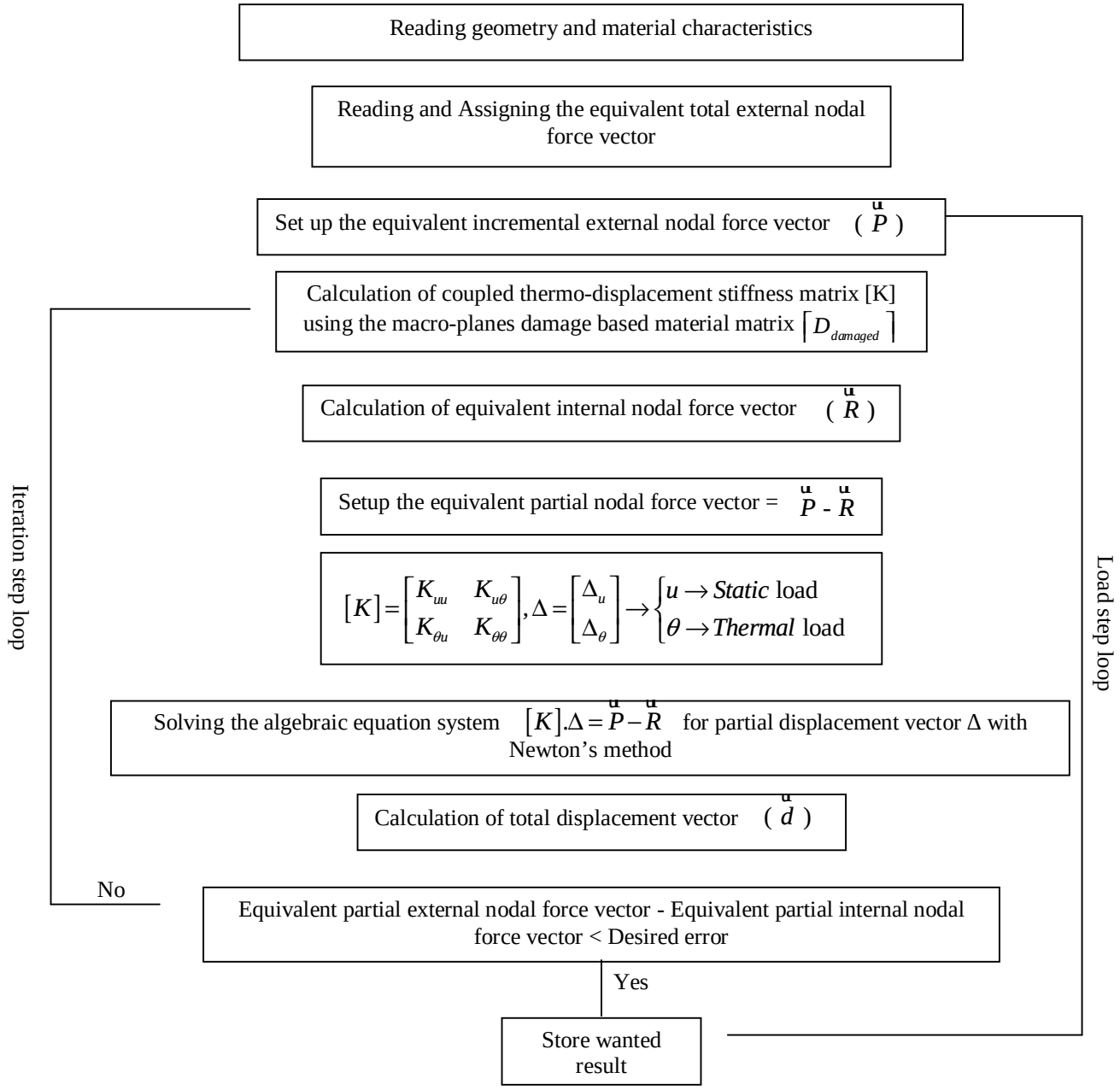


Figure 2. Operation sequence of the proposed F.E. solution method

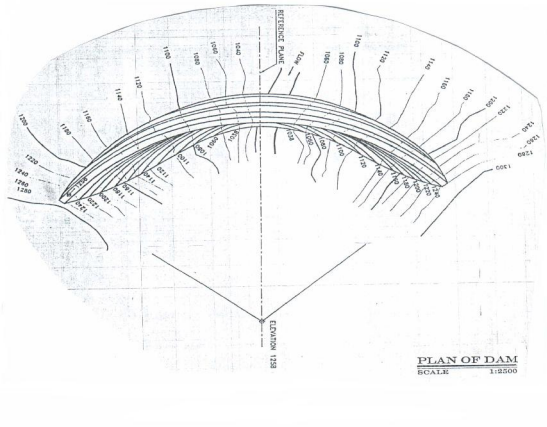


Figure 3: Liroo dam: Arches plan

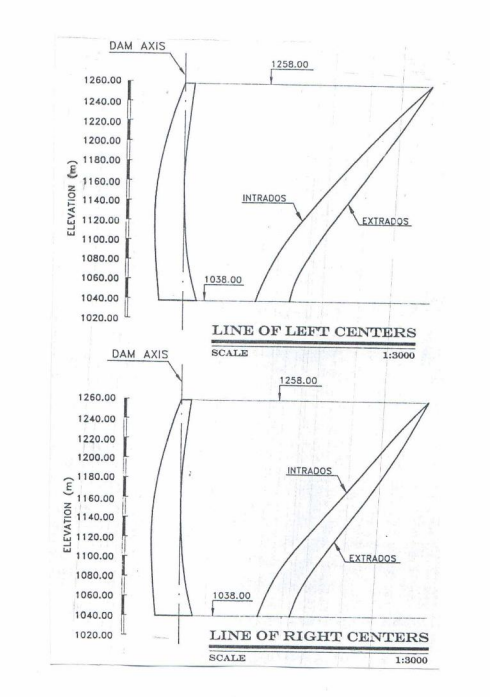


Figure 4: Liroo dam: Lines of the centers

3. Results and Discussions

Before applying the developed computer program for dam analysis, its ability for concrete failure simulation has been verified through analyzing some benchmark problems. One of them was an anti-symmetric four point shear test. The thickness of the beam was 38 mm. figure 5 shows the beam and its mesh. A total number of 428 brick 8-noded elements were used in the model.

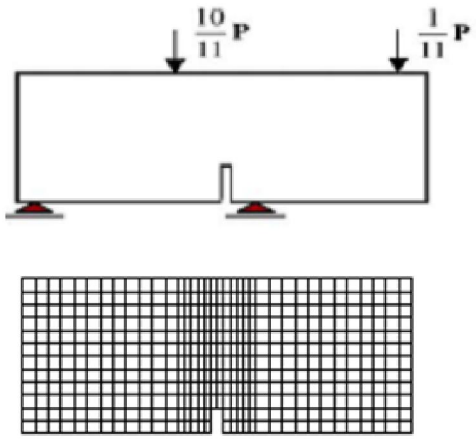


Figure 5. Single-edge-notched beam subjected to anti-symmetric four-point shear loading.

Material parameters are $E = 27000\,MPa$ and $\upsilon = 0.2$. As the load (P) increases, a crack starts growing from the left corner of the notch and continues its growth upwards to the left side of the loading platen. Figure 8 shows the crack path in the actual test. As it can be seen there is a good conformity between model result and experimental ones.

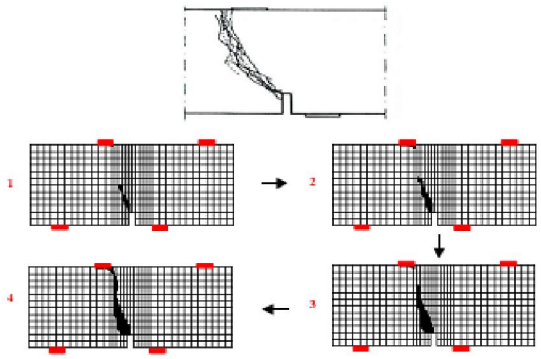


Figure 6. Comparison between simulated crack path and actual crack path

After getting the confidence on accuracy and correctness of model calculations, the Liroo dam was modeled by it. The finite elements model of the dam and its abutments has been viewed in figure 7. Total number of 652 brick elements and 1146 nodes are consumed in dam modeling. The modulus of elasticity of dam concrete was considered 30000 MPa and that of foundation was assigned 20000 Mpa. The Poison's ratio for both of the dam and its foundation was considered as 0.2.

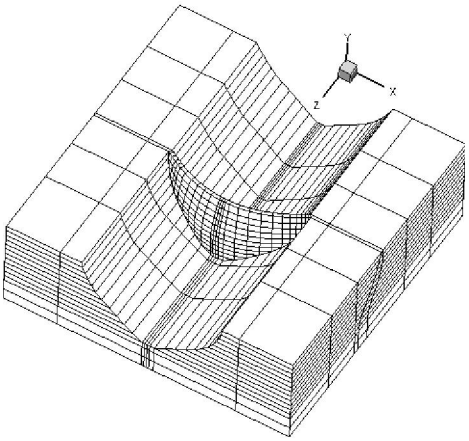


Figure 7. Liroo arch dam F.E. model

After completing the F.E. model of the dam, the gravity and hydrostatic loads were being applied to the model. Then, failure analysis was performed according to the manner discussed earlier and the following results were obtained.

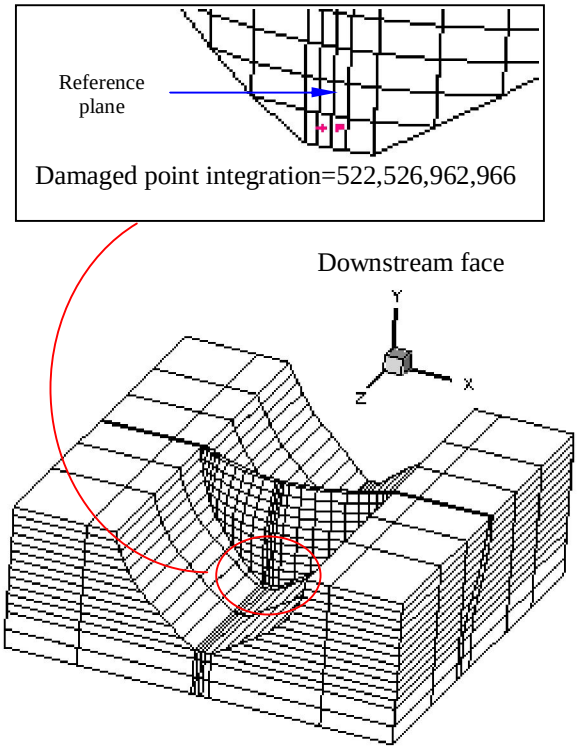


Figure 8. Damaged or failure points of the dam

As it is cleared from figure 8, at four integration points on the upstream face of the dam adjacent to the foundation (approximately 4m above the contact line), fracture phenomenon can be happened. Figure 9 shows the position and direction of the fractures. These cracks align in a plane that it is normal to the faces of dam and propagate through the thickness direction. The angle between crack lines and faces of the dam is about 45 degrees.

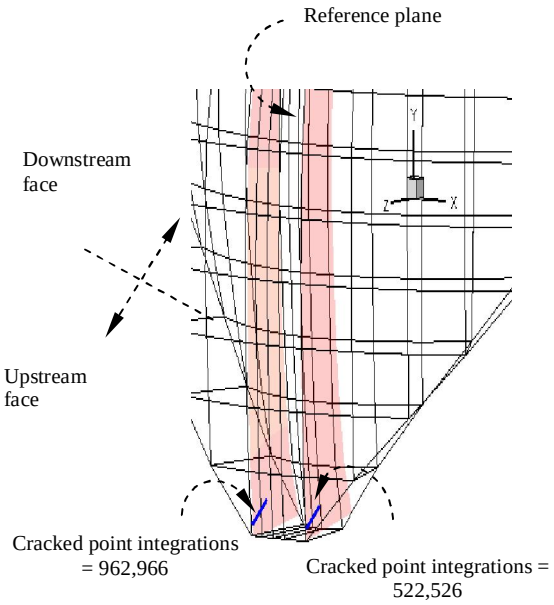


Figure 9. Position and direction of the cracks

It is also necessary to say that in all four cracked points the fractured micro-planes are as micro-planes number 9 and 10. These micro-planes have been illustrated in Figure 10.

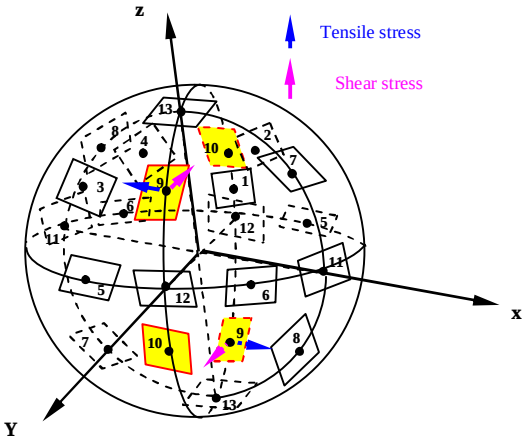


Figure 10. Damaged micro-planes

At these micro-planes there exists a combination of the tensile and shear stresses. So, it can be derived that the main reason of cracking of the dam in specified region would be the cotemporary

actions of tension and shear forces. This can be interpreted as follows: under the action of the hydrostatic loads resulted from the reservoir of dam, there would be a strong value of tension at the upstream face of the dam in vicinity of the foundation and concomitant to that strong shears through the thickness of the dam . However, from the action of the gravity loads, there would be a large quantity of compression right there at the same time. So, under the combined effects of these two main mentioned loads, there would be planes i.e. micro-planes on which the worse situation can be occurred from the stress point of view. This situation is the action of tensile and shear stresses and the planes would be plane designated as 9 and 10. Resultant stress becomes greater than the maximum allowable tensile strength of plane concrete and consequently the failure would be happened.

4. Conclusion

After reviewing the results it was cleared that under the action of hydrostatic and gravity loads, Liroo dam can be exposed to fracture on upstream face of the dam adjacent to the foundation in combined tensile-shear mode and the design should be reviewed exactly again.

Acknowledgements:

Author is so grateful to Dez-Ab consulting engineering company for their aid in gathering data.

Corresponding Author:
Dr. Mojtaba Labibzadeh
Head of Civil Department,
Faculty of Engineering, Shahid Chamran University,
Ahvaz, Iran.

E-mail: labibzadeh_m@scu.ac.ir

References

1. Noorzaie, J., Ghafoori, H., and Labibzadeh, M. Dynamic response of the arch dams using finite, infinite and joint elements. Proceedings of International Conference of Hydraulic Structures, Kerman, Iran, 2002; 2: 15-22.
2. Sadrnejad, S. A., and Labibzadeh, M. Dynamic solution code for structural analysis upon joint element. Journal of Computer Science, 2006; 2 (5) :401-409.
3. Labibzadeh, M., Sadrnejad, S. A., and Khaajeh dezfuli, A. Thermal assessment of Karun-1 dam. Trends in Applied Sciences Research, 2010; 5(4): 251-266.
4. Labibzadeh, M. and Khaajeh dezfuli, A. Effect of vertical contraction joints on thermo-static stability of Karun-1 arch dam. Trends in Applied Sciences Research, 2010; 6(1): 31-42.
5. Labibzadeh, M. Hydro-thermal safety control of Karun-1 dam under unusual reservoir level reduction. The Journal of American Science; 2010; 6(11): 179-184.
6. Taylor, G. I. Plastic strains in metals. J. Inst. Metals. 1938; 62: 307-324.
7. Bažant, Z. P., and Gambarova, P. G. Crack shear in concrete: Crack band micro-plane model. J. Struct. Eng., ASCE, 1984; 110: 2015-2036.
8. Bažant, Z. P., and Oh. B. H. Microplane model for progressive fracture of concrete and rock. Journal of engineering mechanics, ASCE, 1985; 111:559-582.
9. Bažant, Z. P., and Prat, P. Microplane model for brittle plastic materials. Part I&II. Journal of engineering mechanics, ASCE, 1988; 114:1672-1702.
10. Carol, I. and Bažant, Z. P. Damage and plasticity in microplane theory.International Journal of Solids & Structures. 1997; 34:1173-1191.
11. Bažant, Z. P., and Caner, F. C. Microplane M4 for concrete: II. Algorith and calibration. Journal of engineering mechanics, ASCE, 2000; 126:954-961.
12. Labibzadeh, M. and Sadrnejad, S. A. Mesoscopic Damage Based Model for Plane Concrete under Static and Dynamic Loadings. American Journal of Applied Sciences, 2006, 3 (9): 2011-2019.
13. Sadrnejad, S. A. and Labibzadeh, M. A continuum/discontinuum micro plane damage model for concrete. International Journal of Civil Engineering. 2006; 4: 296-313.
14. Labibzadeh, M. and Sadrnejad, S. A. Crack analysis of concrete arch dams using micro-planes damage based constitutive relations. American Journal of Applied Science. 2007; 4(4): 197-202.

6/21/2010

Exploring the Potential and Constraints to Implementing the International Best Practice Principles of EIA Follow-up: The Case of Pakistan

Obaidullah Nadeem[1] , Rizwan Hameed[2]

Department of City and Regional Planning, University of Engineering and Technology, Lahore, Pakistan.
[1]obaidnadeem@yahoo.com; [2]d_rizwan@hotmail.com

Abstract: Every Environmental Impact Assessment (EIA) carried out for development projects in Pakistan includes a long list of mitigation measures and an environmental management plan (EMP). The environmental approvals also contain numerous conditions including implementation of EMP during construction and operation phases of development projects. Without appropriate follow-up and compliance monitoring the entire exercise may go waste. That is why follow-up is considered essential to ensure positive outcome of EIA by protecting the environment and learning lessons for its improvement. In this regard, the International Association for Impact Assessment has suggested best practice guiding and operating principles. This paper attempts to explore the potential and constraints to implementing these principles in Pakistan. Various data sources including interviews with the officials of environmental protection agencies, project proponents, EIA consultants and representatives of some of the affected communities as well as review of EMPs have been used to provide empirical evidence for this purpose. This paper identifies some potential but overall it argues that a lot more is needed to be done to bridge the gap between the international best practice principles and the current state of EIA follow-up in Pakistan. Some imperative steps have also been suggested in this context to improve follow-up and hence strengthen the overall process for EIA. It is expected that other developing EIA regimes may also benefit from the suggestions. [Journal of American Science. 2010;6(12):108-121]. (ISSN: 1545-1003).

Keywords: EIA follow-up; Best practice principles; Pakistan.

1. Introduction

Follow-up is internationally considered essential to determine the outcome of EIA through evaluating environmental performance of projects (Marshall et al., 2005; Morrison-Saunders et al., 2007). It is also termed as monitoring and auditing. Several authors have highlighted the significance of monitoring and auditing as important tools to evaluate the effectiveness of EIA during post-decision stages (see for example, Arts and Noteboom, 1999; Glasson et al., 1999; Arts et al., 2001; Morrison-Saunders et al., 2001; Wood, 2003). Theoretically, EIA follow-up is said to constitute a set of four activities: monitoring, evaluation, management and communication. These involve monitoring baseline conditions and environmental impacts during operation of project, evaluating impact significance and conformance with standards, preparing and implementing environmental management plan (EMP) including mitigation measures and communicating follow-up outcome to the key stakeholders (see Arts et al., 2001, p.176 for further detail). Matching the impacts predicted in the EIA report of a project with those actually arising during operation of that project is also stated as one of the benefits of follow-up to improve EIA practice. Thus, follow-up can be called a panacea for the EIA system as a whole.

Recent literature also suggests international best practice principles of EIA follow-up, as listed in Box-1(Morrison-Saunders et al., 2007; Marshall et al., 2005). These principles are organized on the basis of their core values as guiding principles indicating **'why'** follow-up is needed and identifying role of various stakeholders as **'who'** are responsible to undertake follow-up. The operating principles suggest the kind of follow-up activities as **'what'** to undertake; and the way **'how'** the follow-up should be conducted (Marshall et al., 2005, p.178). So far, the research on EIA follow-up is 'largely piecemeal' and focussed on its 'need and benefits', role and stakes, approaches and techniques, follow-up of socio-economic impacts as well as follow-up design etc (Glasson et al, 1994; Morrison-Saunders et al., 2001; Macharia, 2005; Burdge, 2003; Morrison-Saunders and Arts, 2005; Jha-Thakur at al., 2009). This paper draws attention towards the need to look into the potential and constraints to implementing EIA follow-up best practice principles in the context of overall EIA system and practice in a country. To this end it presents the case of Pakistan where EIA has become one time activity and follow-up practice is scrawny, similar to what the literature suggests in case of both the developed and developing EIA regimes (Wood, 2003; Bond et al, 2003; Glasson et al., 2005; Noble and Storey; 2005; Ahammed and Nixon, 2006; Jha-Thakur et al., 2009).

Box 1. EIA Follow-up International best practice principles

Guiding Principles
Why?
1. Follow-up is essential to determine EIA (or SEA) outcomes.
2. Transparency and openness in EIA follow-up is important.
3. EIA should include a commitment to follow-up.
What?
4. Follow-up should be appropriate for the EIA culture and societal context.
5. EIA follow-up should consider cumulative effects and sustainability.
6. EIA follow-up should be timely, adaptive and action oriented.

Operating Principles
Who?
7. The proponent of change must accept accountability for implementing EIA follow-up.
8. Regulators should ensure that EIA is followed up.
9. The community should be involved in EIA follow-up.
10. All parties should seek to co-operate openly and without prejudice in EIA follow-up.
11. EIA follow-up should promote continuous learning from experience to improve future practice.
How?
12. EIA follow-up should have a clear division of roles, tasks and responsibilities.
13. EIA follow-up should be objective-led and goal oriented.
14. EIA follow-up should be fit-for-purpose.
15. EIA follow-up should include the setting of clear performance criteria.
16. EIA follow-up should be sustained over the entire life of the activity.
17. Adequate resources should be provided for EIA follow- up.

Source: Morrison-Saunders et al. (2007, pp.1-4)

The next section indicates data sources used for gathering empirical evidence and qualitative analysis. The nature of legislative provision and guidelines for EIA follow-up in Pakistan is then described. The penultimate section presents analysis of the current state of EIA follow-up practice in the country with respect to the international best practice principles. Lastly, conclusions have been drawn identifying potential and constraints as well as necessary steps to improve the follow-up practice.

2. Data Sources

The Pakistan Environmental Protection Act (PEPA) 1997 and Initial Environmental Examination (IEE)/EIA Regulations 2000 of the Pakistan Environmental Protection Agency have been reviewed to explore the provisions for EIA follow-up in the country (GoP, 1997; GoP, 2000). The input data for qualitative analysis have been drawn from interviews with EIA officials of Federal and Provincial Environmental Protection Agencies (EPAs) in the country, EIA consultants, academics and proponents.

To gather empirical evidences of EIA follow-up, baseline environmental and socio-economic conditions, predicted impacts, proposed mitigation measures as well as environmental management plans (EMPs) of 18 projects were analysed. These projects relate to various development sectors viz. industrial, transport infrastructure, oil exploration and hydroelectric power generation dams. Only those projects were selected which had been granted EIA/EMP approval by the regulators/federal and provincial environmental protection agencies of Pakistan (the EMP of an industrial estate is presented in Appendix III as an example).

Concerned management employees of 4 (out of the 18) development projects located in the biggest province Punjab including two from industrial sector and two from transport infrastructure sector were also interviewed. The industrial projects include an industrial estate spread over an area of 1600 acres designed for nearly 700 medium and large industries, directly affecting about 10000 people. The other is spread over an area of 400 acres meant for a cement plant with a production capacity of 6000 tons per day, directly affecting about 21000 people. The transport infrastructure projects consist of a 120 meter wide and 100 km long motorway covering 3000 acres of predominantly agricultural land, directly affecting nearly 250 families including demolition of about 650 structures. Another project comprises 7.5 meters widening of 14 km long road after cutting about 2000 trees (see Nadeem and Fischer 2010 for further detail).

The purpose of undertaking detailed investigation of these four projects was to determine how far the mitigation measures suggested in EMPs and conditions of EIA approval for these projects were being implemented. In this regard, roles of key stakeholders i.e. regulators, proponent and affected communities have also been critically examined. From the aforementioned analysis, potential and

constraints to effective EIA follow-up in Pakistan are identified and improvement measures suggested.

3. Nature of legislative provisions and guidelines for EIA follow-up in Pakistan

The Pakistan Environmental Protection Agency (Review of IEE and EIA) Regulations (GoP, 2000) provide the basis for EIA follow-up in the country. The follow-up programme is not chalked out separately but in the form of EMP as well as EIA approval conditions. These two have been used in this paper as alternative to EIA follow-up.

Four out of twenty four sections of the said Regulations deal with various aspects of follow-up. Section 13 (2) clause (b) requires that prior to starting the operation; proponent of every project should obtain from the competent authority (Federal or Provincial EPA) a written confirmation of compliance with the conditions of EIA approval. The request should substantiate that the conditions related to project design; its construction and necessary mitigation measures have been implemented. Section 14(1) states that an EMP should also be included in every EIA report also indicating impact monitoring and auditing arrangements.

The competent authority is empowered under Section 18 to send its authorized staff for verifying the site characteristics and the extent to which conditions of EIA approval have been followed. For this purpose, the staff may also examine built up structures at project site and its plant machinery. Over and above, it is mandatory to submit every year a monitoring report regarding the project's environmental performance and impact management and adoption of mitigation measures. Under Section 20, the competent authority is empowered to cancel EIA/environmental approval, if it is found that the approval conditions have not been followed.

The major steps involved for EIA follow-up as suggested by the Pakistan Environmental Protection Agency are presented in Appendix I. Other than the legal requirements of obtaining written confirmation of compliance with the EIA approval conditions and submitting monitoring report, the proponent is required to develop a proper environmental management system and make follow-up as a continuous process. The implementation phase does not only include monitoring of predicted and un-expected impacts but also design review of EMP and environmental assessment audit involving post project analysis. These steps for EIA follow-up can prove to be helpful for project proponents, if properly followed up.

4. Analysis of EIA Follow-up Practice in Pakistan

This section presents the analysis and discussion on the of EIA follow-up practice in Pakistan with respect to the international best practice principles.

4.1 Use of follow-up to determine EIA outcome

Theoretically, follow-up is considered essential for positive outcome of EIA or to minimize adverse impacts of development projects and to improve quality of assessment (Morrison-Saunders et al., 2001; Wood, 2003; Glasson et al., 2005; Marshall et al, 2005; Noble and Storey, 2005; Morrison-Saunders et al., 2007). Interviews with officials of competent authorities, project proponents and EIA consultants in Pakistan also suggested fairly similar stance. Within the local context, it was strongly linked with the adequacy of technical and financial resources. Such limitations are hampering the consistency of follow-up on part of both the regulators and proponents**.** Thus after granting EIA approval, the regulators keep busy with processing other EIAs until someone files a complaint against the negative impacts of the project compelling them (the regulators) to take some action. On the other hand, the 'convenience' and 'suitability' are the factors considered by the project proponents to implement whatever deemed necessary out of the EMP which they got prepared very well through consultants by paying a 'handsome' remuneration. Given the above situation, the consequences of EIA decision making largely remain unknown.

4.2 Degree of transparency and openness

One of the pre-requisites for transparency and openness of any EIA system is easy access to information (Boyle, 1998; Beierle and Cayford, 2002; Rajvanshi, 2003). During the EIA follow-up, these are important at both pre and post-decision stages. In Pakistan, EIA report including data on baseline environmental conditions remain confidential, initially with the EIA consultants and then with concerned EPA. Stakeholders are not provided with access to such information except during public review of EIA report for 30 days. Even this regulatory requirement is not fulfilled properly. There are many examples of projects the EIA reports of which are either placed at locations far away from the affected public or made available for a period less than what was legally required (Nadeem, 2010). After the approval of EIA report and beginning of operation of projects, particularly for industrial development, even stakeholders' representatives are not allowed to access the EMP or know about the monitoring outcome.

4.3 Degree of commitment by the key stakeholders

Out of the triangle of stakeholders in EIA follow-up, comprising of project proponent, regulator and the community or affected/interested public (see Figure 1), the foremost key player i.e. the proponent has serious lack of commitment. According to EPA officials, the very reason is that proponents often regard EIA as a legal and technical barrier to development. But being a requirement, the proponents get the follow-up programme/EMP prepared and commit to implement the same along with conditions of EIA approval. However, they are not yet convinced that implementing EMP/mitigation measures can save cost, as the experience elsewhere suggests (Glasson et al., 1999; Morrison-Saunders et al., 2001; Aschemann, 2004). Rather, majority of proponents felt that implementing EMP would incur heavy cost and in turn it will increase the cost of production.

Contrary to this, the proponents of selected projects of industrial development claimed in their interviews that they were implementing conditions of approval/mitigation measures as and when the need aroused. But the project site visits and interviews with the nearby living communities unveiled totally different scenario (Nadeem, 2010). For instance, due to emission of raw cement, it was difficult to breathe even 2 kilometres away from a cement factory project which was installed after getting EIA clearance from the Punjab EPA. Similarly, in case of a paper and board mill, the community living close to the factory, though unaware of its EIA approval, protested directly to the proponent against the sawdust emitting out of its chimneys. In response, the proponent installed water sprinklers. Other than this type of apparent environmental pollution, the members of the community were unaware of other problems like decrease in ground water table as a result of continuous extraction and its contamination due to untreated effluent discharge from the paper mill.

Still there are some encouraging examples of public sector proponents who implemented many commitments made in the EMP of a project. For example, installation of effluent treatment plant worth Rs. 40 million (US$ 0.48 million) (1 US$ = 84 Rupees) and construction of landfill site have been made by the proponent organization of an industrial estate project. Not only that, provision of sewerage system and maintenance of roads in and around the villages near the estate at an estimated cost of Rs.20 million is also in progress. However, such examples are very rare. In most of the other development projects constructed after getting EIA approval, the communities are neither involved in EIA follow-up nor these actively pursue the concerned EPAs against environmental and socio-economic impacts, as found by Hussain and Ahmad (2009) in the case of paper mill and lather tannery projects.

4.4 Nature of accountability for implementing follow-up

It is suggested that "the proponent of change must accept accountability for implementing EIA follow-up" (Marshal et al., 2005, p.179). Under the Pak-EPA's IEE/EIA Regulations (GoP, 2000), the project proponent is held responsible for implementing EMP to mitigate adverse impacts as mentioned earlier (see Section 3). However, except in one or two cases, the project proponents with approved EIA are not held responsible as per spirit of the said regulations. So far very few cases against such proponents have been sent to Environmental Tribunals. Generally speaking, it is assumed that the proponents shall implement mitigation measures and submit yearly monitoring report, the authenticity of which is never checked as confirmed by the officials of concerned EIA during the interview. On the other hand, interviews with the affected communities revealed that most of the mitigation measures were not being implemented.

4.5 Role of regulators to ensuring follow-up

It is the responsibility of the regulator to ensure implementation of EIA approval conditions/ mitigation measures as committed by the project proponent. In most of the cases, EPAs field staff (regulators) do not routinely check the compliance with EIA approval conditions presumably due to lack of capacity and staff. It is left on the discretion of the proponent to implement whatever he considers appropriate. However, the EPAs do take quick action if some members of aggrieved community lodge a complaint against the proponent/project causing environmental pollution. In other words, whatever follow-up is pursued sometimes by EPA is generally as a response to complaints against severe impacts of development projects commissioned after EIA clearance (Nadeem and Hameed, 2008). For instance, while responding to several complaints by the representatives of the directly affected communities of a cement factory concerned EPA's field staff carried out environmental monitoring. The report revealed that

> "the unit along with other two units of cement factories located in close proximity were violating environmental laws by mismanaging natural resources, contaminating water ponds, disposing of untreated wastewater towards farm land, blocking conventional routes and disturbing socio-economic conditions of local

people. Large volumes of suspended particulate matter and toxic gases being emitted by the factory without any treatment or air filtration were also observed. The report further stated that about 4,060 acres of privately owned land and green hills used by locals as grazing fields for their livestock have been allotted to this factory for limestone quarry" (Nadeem, 2010, p.194).

The above report was prepared by the concerned District Officer Environment as a response to the complaints by the affected communities. But the communities' representatives revealed that after this report, EPA just issued a warning to the said factory administration simply asking for implementing mitigation measures. Although some mitigation measures have been adopted, but this situation arises off and on as the factory administration does not consistently ensure implementation of mitigation measure/EMP to avoid expenditures.

4.6 Extent of community involvement

Community involvement is considered vital for the continuity and acceptability of the follow-up in Pakistan (GoP, 1997a). But, the affected and interested public is informed about the EMP during public hearing and their comments are just responded to by the proponents. This means that the EMPs once prepared are not revised. Interviews with affected communities of two of the four development projects revealed that they were kept in dark about the conditions of EIA approval (Nadeem and Fischer, 2010). In fact, the EIA follow-up process provides the affected communities with an opportunity to know about the EMPs and conditions of EIA approval since these more often suggest to forming environmental monitoring committees which must include representatives of affected communities (Hussain and Ahmad, 2009; Nadeem, 2010). But no example was found in which the communities or even Environmental Non-Government Organizations (ENGOs) had played any role in EIA follow-up. Thus EIA follow-up in Pakistan can be called a uni-faceted mechanism revolving around the whims and mood of project proponents.

4.7 Nature of co-operation among the parties involved

The international principles envisage that "[a]ll parties should seek to co-operate openly and without prejudice in EIA follow-up" (Morrison-Saunders et al., 2007, p.3). This suggestion seems more idealistic than pragmatic. Practically, the proponent, the regulator and the community are three different corners of a triangle more often having competing interests; especially those of the proponent and of the affected community (see Figure 1). The proponent always tries to minimize the time and resources involved in mitigating adverse environmental and socio-economic impacts. On the other hand, the affected community may not even accept the project at proponent's desired location as such situation aroused in the cases of a cement factory and an industrial estate (See for example Nadeem and Fischer, 2010).

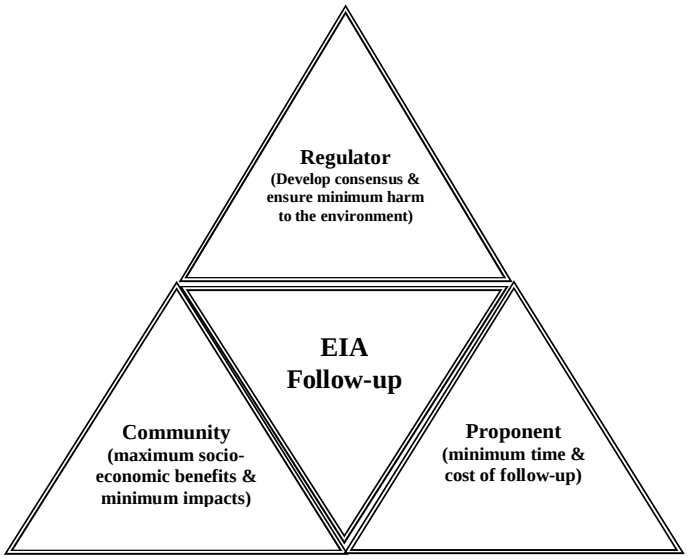


Figure 1. Competing interests of key stakeholders in EIA follow-up
Adapted from Morrison-Saunders et al. (2001,p.293)

The role of regulator in an EIA regime without consistent practice of accountability may not be impartial. However, it may act as a facilitator and builder of consensus over the procedures and methods of implementing follow-up programme which may be acceptable to the other two parties of a particular project and cause minimum harm to the environment. But the nature of cooperation among the parties involved in EIA follow-up in Pakistan is far from this notion.

4.8 Compatibility with EIA and social context

EIA follow-up in Pakistan is independent of the project planning process. It is essentially a proponent driven exercise without involvement of the other two stakeholders viz the competent authority/ regulator and the affected community. Once the project is planned, commissioned and EIA approved, follow-up is done independently to the extent whatever is considered essential by the proponent. The EMP is considered an important part of EIA

report. It is reviewed by the EPAs in Pakistan before granting EIA approval. In most of the cases EMPs have also been criticised by the affected and interested stakeholders during public hearings. There are instances where an EPA got the entire EIA report including the EMP revised (Nadeem, 2010). Despite these efforts, implementation of EMP or follow-up outcome is rarely reported to the competent authority, though it is required to be submitted annually. Out of the 18 cases of approved EIA by various EPAs in the country scrutinized by the first author, only 3 were found having annual monitoring reports. Those reports were submitted by the proponents of such projects whose cases were in the courts and affected communities had lodged complaints against adverse impacts of such projects. No one from the concerned EPA ever bothered to verify the authenticity of such monitoring reports.

Community organization is strong in rural areas of the country as compared to that in urban areas. There is a culture of frequent interaction among the community members through congregations in mosques and punchayats. It is not difficult to involve people or representatives of the affected communities in the EIA follow-up and monitoring activities. But the community organizations as well as the socio-economic and cultural circumstances do not have explicit links with follow-up. Except follow-up in a few cases like that of an industrial estate, it is a technical exercise limited to controlling air and noise pollution through installing electrostatic precipitators and mufflers at chimneys of industrial plants. On the other hand, majority of the people with low literacy rate are not aware about the indirect consequences of environmental impacts on human health and overall quality of life. ENGOs can help raising awareness among the masses about environmental and socio-economic impacts of development projects and form pressure groups to ensure positive outcome of EIA (World Bank, 2001; Lehrack, 2006; Morrison-Saunders, 1998). Unfortunately the country lacks such NGOs to do this job.

4.9 Consideration of cumulative impacts and sustainability

Consideration of cumulative impacts and overall sustainability of the environment is more relevant to strategic level of planning (including policy, plan and programme) and follow-up. Perhaps due to this reason, cumulative impacts and sustainability considerations are weak in EIA in Pakistan. Major emphasis of follow-up where pursued in reality remains on monitoring and implementation of mitigation measures to control air and noise pollution, or at the most, contamination of ground water due to liquid emissions during project operation. However, there are cases of industrial development projects in the EIA of which cumulative impacts were indicated in the form of qualitative statements without suggesting mitigation measures. Analysis of public hearing proceedings of 2 industrial and 2 road sector projects suggests that stakeholders have been emphasising the need to consider cumulative impacts but the proponents declared it beyond the scope of EIA and the competent authority accepted this stance of proponents (Nadeem and Fischer, 2010).

4.10 Timing and adaptability

Pak-EPA's guidelines suggest that follow-up should begin early in the project development process (GoP, 1997a). The practice indicates that EMP is ignored during construction of projects and the public has to suffer from severe environmental impacts during this phase. One of the very reasons is that EIA process is generally initiated after procurement of project site and beginning construction works (Nadeem and Hameed, 2008). Whilst the monitoring data for baseline environmental conditions is, of course, recorded during preparation of EIA, it is done occasionally during operation of projects. Proponents are not generally concerned with the variations in the air and noise emissions or other impacts rather they adopt mitigation measures should there be serious reaction or complaint from the communities adversely affected by the project. Adaptability of follow-up also pertains to the flexibility of its design. New situations or unforeseen impacts may arise during the construction as well as operation of projects. It is very rare that a mechanism or alternative measures are suggested in the EMPs of development projects in Pakistan to handle such situations except to deal with fire hazard.

4.11 Extent of learning from experience to improve follow-up

Learning from experience has increasingly been suggested to improve EIA follow-up practice. This can be done by matching the impacts arising during construction and operation of the project with those predicted in its EIA report (Glasson et al., 1999; Morrison-Saunders et al., 2003). In developed EIA regimes this is practiced to some extent but in developing countries this very important aspect of follow-up is neglected. Resultantly, stereotype EMPs are produced in almost all EIA reports of projects, particularly belonging to same development sector. The situation is no different in this regard were EIA approval conditions for most of the projects are mostly similar in nature in Pakistan (Nadeem and Fischer, 2010). The monitoring reports submitted to

EPAs are made part of EIA case file without analysing the nature of impacts arising during operation of project or matching them with those impacts predicted in its EIA. However, proponents do modify their EMPs during operation of projects as and when the need arises without informing the concerned regulating agency.

4.12 Division of follow-up tasks and responsibilities by proponents

The follow-up tasks are identified and responsibilities to execute the tasks are worked out by EIA consultants and made part of every EMP of development project which gets environmental clearance in Pakistan. Even the cost of implementing various mitigation measures is estimated by the consultants. Moreover, the project proponents are rarely involved in this very important matter which can directly influence the success of follow-up implementation. It appears that the division of tasks, responsibilities and calculation of cost of implementing follow-up provisions are suggestive in nature to fulfil the requirement of getting EIA approval. Once is approved, the proponents implement whatever is financially viable from their point of view and hence most of the suggestive exercise remains futile. This happens because the expert staff and required financial resource are often lacking or the proponents are reluctant to invest million of rupees on EIA follow-up. This is evident from thorough investigation of two projects including one of leather tannery and one of paper and board mill located in the Punjab (Hussain and Ahmad, 2009). There was a significant gap between the tasks and responsibilities assigned in their respective EMPs as compared to the presence of trained staff and mitigation measures which were actually being implemented.

4.13 Clarity of goal and objectives of follow-up

The overall goal of EIA follow-up in Pakistan is to ensure the implementation of mitigation measures for protecting the natural resources and the people. The Pak-EPA's guidelines for preparation and review of environmental reports (GoP, 1997a) clearly suggest five objectives of follow-up as presented in Box 2. It can be argued that the competent authorities have done a good paper work. But some of the objectives as suggested in the international principles for EIA follow-up are not explicitly indicated in the said guidelines. For instance, maintaining flexibility and promoting adaptive management; improving community awareness and acceptance of projects (Morrison-Saunders et al., 2007). The current state of EIA follow-up in the country, as portrayed in rest of the sections of this paper, also suggests a significant gap between these objectives and the actual practice.

Box 2. Objectives of EIA Follow-up in Pakistan

- monitoring the impacts actually arising during construction and operation of projects
- maintaining anticipated impacts within the levels predicted
- mitigating unanticipated impacts before becoming unmanageable
- ensuring that environmental management contributes to protecting the environment and achieving sustainability
- improving knowledge of project impacts prediction, management and EIA review process

Source: GoP (1997a, p.35)

4.14 Fit for purpose nature of follow-up

EIA follow-up in Pakistan is, to some extent, 'fit for purpose' in terms of project type, specific design, location, affected communities as well as availability of financial resources. For instance, the EMP of an industrial estate located close to villages suggested installation of combined effluent treatment plant while that of a leather tannery located away from residential areas relied only upon primary treatment of effluents being a single industry with limited financial resource. Similarly, electrostatic precipitator was suggested and installed to control the emissions of a cement factory but water sprinklers and fans for a paper and board mill (Hussain and Ahmad, 2009). The on-going scoping, as suggested in the follow-up principles, is done and reported to project management to limit the follow-up to the 'art of the possible'. On the other hand, about 60% of the EIA approval conditions suggested by the regulator for one type of projects are general and similar in nature except a few conditions specific to the project.

4.15 Clarity of performance criteria

Review of EMPs included in EIA reports of 18 development projects and interviews with the technical staff of proponents of 2 industrial and 2 road projects suggests that a general performance criterion of follow-up is to maintain the pollution levels within acceptable limits prescribed in the National Environmental Quality Standards (NEQS) of Pakistan. The NEQS further define parameters for noise level, liquid effluents and gaseous emissions (GoP, 1993, GoP, 2000a; GoP, 2009). Parameters for ambient air quality are not included in the said NEQS but have recently been drafted and public

consultation is currently going on. In this regard, the USEPA standards are referred to.

Methodologies to meet the said standards are usually defined as generic mitigation measures. For instance, a cement factory EMP suggests that noise would be brought within NEQS level by maintenance and repair of noise producing equipment and installation of low NOx preclaciner vessel to minimize emission of NOx from kiln. The EMP of an industrial estate suggests to creating air pollution barrier by planting trees and using high rise chimneys for minimizing the adverse impacts of air emissions from industries. However, the same EMP also suggests installation of combined effluent treatment plant to avoid contamination of ground water.

4.16 Sustainability of follow-up over lifespan of projects

The follow-up programme including EMP is normally formulated during the preparation of EIA report in Pakistan. It includes impact mitigation and management measures for construction and operation phases of development projects. The EMPs neither discuss de-commissioning phase nor suggest environmental management measures to be taken in case the project is closed or shifted somewhere else in future. The EIA approving authorities or the regulators do not emphasize the need to include such measures.

However, strategies/specific measures are identified to manage short-term as well as long-term environmental changes. For instance, EMP of an industrial estate suggested that the combined effluent treatment plant will be surrounded by 15 meter wide buffer zone to protect the environment from short and long term impacts and that the wastewater will be disposed off after treatment to recharge the aquifer. The EMP of a landfill site project suggested that groundwater monitoring well have been installed up and down stream of the site and will be monitored regularly. Similarly, the EMPs of road remodeling and motorway construction projects suggested planting 4 new trees in place of every tree to be cut and plantation of trees in 15 meter wide strips on both sides of motorway to control environmental and noise pollution. The outcome of even these types of measures depends on how rigorously these are implemented by the proponents and pursued by the EPAs.

4.17 Adequacy of technical and financial resources

Inadequacy of technical staff and financial resources for follow-up is one of the major impediments being faced both by the project proponents and the environmental protection agencies (EPAs) in Pakistan. The EMPs included in the EIA reports appear to allocate adequate resources. But in reality most of the mitigation measures are not implemented. Interviews with the proponents suggest that EIA follow-up was not their priority as it required extra staff and finances. During operation of the projects, the stakeholder's representatives are given all okay report and that efforts are going on to protect the environment. Some encouraging examples also exist. For instance, proponent of an industrial estate project employed qualified environmentalist and spent millions of rupees on EIA follow-up. The very reason appears to be the realization about social and economic benefits of protecting the environment.

However, interviews with concerned officials of the EPAs/regulators in Pakistan revealed that technical field staff to monitor the EIA follow-up activities and equipment to verify the pollution levels was far less as compared to that actually required for this purpose. Tantamount to that, EPA did not have sufficient financial resources even to get the EIA reports reviewed by experts. But recently the federal and provincial governments have allocated funds in their annual development plans for various capacity building projects. These include, training of officials of EPAs and line departments both at federal and provincial levels; establishment of environmental laboratories in six cities of the Punjab and more than one hundred new posts of field and office staff. But these are 2 to 3 years programmes. An overall summary of the potential and constraints with respect to the international best practice principles of EIA follow-up is presented in Appendix II.

5. Conclusions

EIA without follow-up can be termed as a futile exercise. The international principles provide a thorough understanding of necessary ingredients and qualities of the best EIA follow-up practice. These can also be used for evaluating the practice of EIA follow-up in other developing and developed countries as well as to determine the international applicability of these principles. Examining the EIA system of Pakistan with respect to these principles formed a basis to determine the potential and constraints to successful follow-up.

Legal provisions for EIA follow-up in Pakistan categorically put the responsibility of implementing mitigation measures including EMP and conditions of approval on the project proponents. The regulators are bound to undertake compliance monitoring. In these respects, the guidelines clearly spell out follow-up mechanism and roles of key stakeholders including the affected and interested communities. It is encouraging that every EIA report submitted to EPAs in the country includes EMP at the outset. The EMP is presented along with potential

impacts and mitigation measures of development projects during public hearing to the interested and affected communities or to their representatives whosoever attend the hearing. The conditions of EIA approval also include implementation of EMP and the proponent is required to submit an undertaking in this regard. The EMP provides a framework for EIA follow-up with clear division of tasks and responsibilities and even the cost of implementing each management measure.

But overall, EIA follow-up is lagging far behind the best practice principles. The dilemma is that EMPs solely prepared by EIA consultants generally lack consideration of public input and the proponent's willingness to implement. The EPAs/regulators in Pakistan are lacking in technical and financial resources. Their role in ensuring EIA follow-up is reactive and spontaneous rather than proactive and consistent. There is no effective accountability of regulators and proponents. Even the commitments for mitigating specific impacts are not fulfilled what to talk of cumulative impacts and sustainability which are considered beyond the scope of EIA by the proponents. Several other developing countries are also facing almost similar constraints.

To make EIA a useful exercise for protecting the environment as well as socio-economic rights of people, it is utmost important to launch a campaign for convincing project proponents that how EIA follow-up through implementing EMP could ultimately save their cost of operation and maintenance in the long run. Besides, financial incentives like tax exemption on importing environmental management equipment and award on better environmental performance can also help motivate proponents. In fact, the Ministry of Environment has already started this practice of recognizing the efforts of environmentally friendly projects through an award scheme.

In addition, capacity building of EPAs/regulators is urgently needed by provision of adequate technical staff and financial resources for this purpose. The need for this has been highlighted in nearly every study amid at strengthening EPAs in the country and gradual efforts have been started. If the government intends to save its natural resources and the people, it has to give top priority to EIA follow-up and make handsome investment particularly in capacity building to reap long term benefits. Lastly, it is expected that similar measures may also prove to be effective in other developing EIA regimes for successfully implementing the intentional best practice principles of EIA follow-up.

Acknowledgements

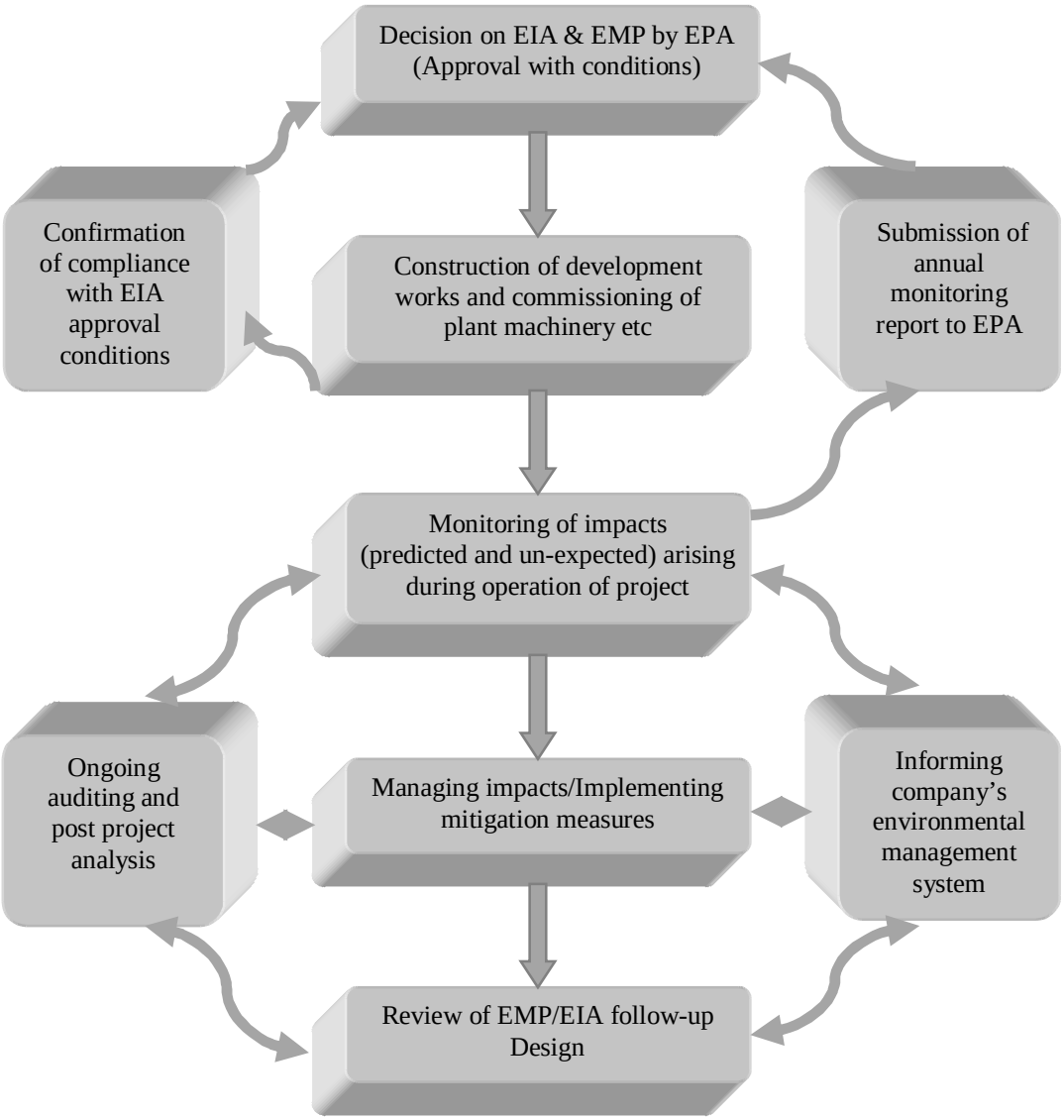
The authors are grateful to the interviewees of this research for sparing their precious time. Thanks are also due to the authorities of University of Engineering & Technology, Lahore, Pakistan for providing access to online sources of literature.

Corresponding Author:
Dr. Obaidullah Nadeem
Department of City & Regional Planning
University of Engineering & Technology, Lahore, Pakistan. Email: obaidnadeem@yahoo.com

References

1. Ahammed AKMR, Nixon BM. Environmental impact monitoring in the EIA process of South Australia. Environ Impact Assess Rev 2006;26:426-47.
2. Arts EJMM, Nooteboom S. Environmental impact assessment monitoring and auditing. In: Petts J, editor. Handbook of environmental impact assessment, vol. 1. Oxford: Blackwell; 1999. p.229–51.
3. Arts J, Caldwell P, Morrison-Saunders A. Environmental impact assessment follow-up: good practice and future directions-findings from a workshop at the IAIA 2000 conference. Impact Assess Proj Appraisal 2001;19:175-85.
4. Aschemann R. Public participation and consultation: the experience of Europe. World Bank; 2004. Available at: http://info.worldbnak.org/etools/library/latestvesrion.asp?35155.
5. Bierele TC, Cayford J. Democracy in practice: public participation in environmental decisions. Washington DC: Resources for the Future; 2002.
6. Bond A, Langstaff L, Ruelle C, Zwetkoff C. Post-evaluation procedures: monitoring and follow-up. Task 3.5 report on SUIT project in the EU programme environment and sustainable UNIWA and SUIT, 2003 Available at: http://www.lema.ulg.ac.be/research/suit/Reports/public/SUIT3.5_Report.pdf.
7. Boyle J. Cultural influences on implementing environmental impact assessment: insights from Thailand, Indonesia and Malaysia. Environ Impact Assess Rev 1998;18:95–116.
8. Burdge R. Editorial on special issue on the practice of social impact assessment. Impact Assess Proj Appraisal 2003;21:166-67.
9. Glasson J, Therivel R, Chadwick A. Introduction to environmental impact assessment: principles and procedures, process, practice and prospects. London: UCL Press; 1994.

10. Glasson J, Therivel R, Chadwick A. Introduction to environmental impact assessment. Second ed. London: UCL Press; 1999.
11. Glasson J, Therivel R, Chadwick A. Introduction to environmental impact assessment. 3rd ed. London: Routledge; 2005.
12. Government of Pakistan (GoP). National environmental quality standards**.** Islamabad: Gazette of Pakistan; 1993.
13. Government of Pakistan (GoP). Pakistan environmental protection act, 1997. Islamabad: Gazette of Pakistan; 1997.
14. Government of Pakistan (GoP). Guidelines for preparation and review of environmental reports. Islamabad: Pakistan Environmental Protection Agency; 1997a.
15. Government of Pakistan (GoP). Pakistan environmental protection agency (review of IEE and EIA) regulations, 2000. Islamabad: Pakistan Environmental Protection Agency; 2000.
16. Government of Pakistan (GoP). National environmental quality standards for municipal and liquid industrial effluents and gaseous emissions**.** Islamabad: Gazette of Pakistan; 2000a.
17. Government of Pakistan (GoP). National environmental quality standards for motor vehicle exhaust and noise**.** Karachi: Gazette of Pakistan; 2009.
18. Hussain Z. Ahmad W. EIA follow-up of industrial projects in Lahore: the case studies of a leather tannery and paper mill. BSc Thesis, University of Engineering and Technology, Lahore, 2009.
19. Lehrack D. Environmental NGOs in China-partners in environmental governance. Wissenschaftszentrum Berlin für Sozialforschung (WZB);2006. Available at: http://skylla.wzb.eu/pdf/2006/p06-009.pdf.
20. Macharia SN. A framework for best practice environmental impact assessment follow-up: a case study of the Ekati Diamond Mine, Canada. MA Thesis, University of Saskatchewan, Saskatoon, 2005. Available at: http://library2.usask.ca/theses/available/etd-05192005-093452/unrestricted/sarah_macharia.pdf.
21. Marshall R, Arts J, Morrison-Saunders A. International principles for best practice EIA follow-up. Impact Assess Proj Appraisal 2005;23:175–81.
22. Morrison-Saunders A, Arts J. Learning from experience: emerging trends in environmental impact assessment follow-up. Editorial. Impact Assess Proj Appraisal 2005;23:170–174.
23. Morrison-Saunders A, Arts J, Baker J, Caldwell P. Roles and stakes in environmental impact assessment follow-up. Impact Assess Proj Appraisal 2001;19:289–96.
24. Morrison-Saunders A, Baker J, Arts J. Lessons from practice: towards successful follow-up. Impact Assess Proj Appraisal 2003;21:43–56.
25. Morrison-Saunders A, Marshall R, Arts J. EIA follow-up international best practice principles. Special publication series No. 6. Fargo: IAIA;2007.
26. Morrison-Saunders A. The effect of public pressure during environmental impact assessment on environmental management outcomes. Paper presented at IAIA‘98 Sustainability and the Role of Impact Assessment in the Global Economy Conference, Christchurch, New Zealand; 1998.
27. Nadeem O, Fischer TB, An evaluation framework for effective public participation in EIA in Pakistan. Environ Impact Assess Rev 2010;doi:10.1016/j.eiar.2010.01.003.
28. Nadeem O, Hameed R. Evaluation of environmental impact assessment system in Pakistan. Environ Impact Assess Rev 2008;28:562-71.
29. Nadeem O. Public participation in environmental impact assessment of development projects in Punjab, Pakistan. PhD Thesis, University of Engineering and Technology, Lahore, 2010.
30. Noble B, Storey K. Towards increasing the utility of follow-up in Canadian EIA. Environ Impact Assess Rev 2005;25:163–80.
31. Rajvanshi A. Promoting public participation for integrating sustainability issues in environmental decision-making: the Indian experience. J Environ Assess Policy Manag 2003;5:295–319.
32. Jha-Thakur U, Fischer TB, Rajvanshi A. Reviewing design stage of environmental impact assessment follow-up: looking at the open cast coal mines in India. Impact Asses Proj Appraisal 2009;27:33–44.
33. Wood CM. Environmental impact assessment: a comparative review. Second ed. Harlow: Prentice Hall; 2003.
34. World Bank. China: air, land and water. Washington DC: The World Bank; 2001.



Appendix I. EIA follow-up guidelines in Pakistan
Based on GoP (1997a, p.36)

Appendix II. Summary of potential and constraints in Pakistan with respect to principles of EIA follow-up

EIA Follow-up Principles	Potential and Constraints in Pakistan
1. Use of follow-up to determine EIA outcome	• Notionally considered essential by the proponents and regulators for positive outcome of EIA • Practically constrained due to lack of technical and financial resources
2. Degree of transparency and openness	• Low degree of transparency and openness • After one month availability of EIA report during public review and hearing, EMP cannot be seen or known by stakeholders • Monitoring report is a confidential document • Stakeholders are not provided with any information on follow-up issues or monitoring outcome
3. Degree of commitment by the key stakeholders	• Low degree of commitment among the key players • After getting EIA approval, proponents do not implement most of the mitigation measures in order to save short term cost • Due to protest by the affected communities some proponents were forced to implement mitigation measures
4. Nature of accountability for implementing follow-up	• No formal accountability of project proponents except in response of complaints, if any • No formal accountability of EPA officials
5. Role of regulators to ensuring follow-up	• Field staff of the regulators does not routinely check compliance of EIA approval conditions • Monitoring is done, if serious environmental impacts are reported. Only warning is issued in such cases
6. Extent of community involvement	• Community consultations take place after preparation of EMP • No involvement of community in follow-up • No information to community about follow-up outcome
7. Nature of co-operation among the parties involved	• No cooperation among proponents and affected community due to opposing interests • Regulators attempt to build consensus but cooperation with proponent is not transparent
8. Compatibility with EIA and social context	• EIA is independent of project planning process • Political interference acts as a constraint to EIA follow-up • Community organization is strong in rural areas but weak in urban areas • Environmental awareness among general public is increasing but still poor in people with low literacy rate • Environmental NGOs are very few
9. Consideration of cumulative impacts and sustainability	• No consideration of cumulative impacts of developments at strategic/plan level • Where cumulative impacts are indicated those are just based on qualitative statements • Overall consideration of sustainability of affected environment is weak

EIA Follow-up Principles	Potential and Constraints in Pakistan
10. Timing and adaptability	• Initiated very late in the EIA process • No proper follow-up during construction and closure of projects • Monitoring data is recorded occasionally • Follow-up design is not flexible and lacks alternative measures for unforeseen impacts
11. Extent of learning from experience to improve follow-up	• Monitoring reports are not analysed by regulators to learn from follow-up outcome • No formal practice to match the impacts predicted in EIA report with those actually arising during operation of that project
12. Division of follow-up tasks and responsibilities by proponents	• Clearly indicated in most of the EMPs included in EIA reports • Proponents not involved in division of tasks and estimating cost of mitigation measures but EIA consultants do • Practically, expert staff is lacking
13. Clarity of goal and objectives of follow-up	• Goal and objectives are clearly defined but lacking in adaptive management • Follow-up of goals and objectives is weak
14. Fit for purpose nature of follow-up	• Generally stereotype EMPs are presented in EIA reports • Review of follow-up design on the basis of feedback is weak
15. Clarity of performance criteria	• Detailed performance criteria are not set • General criteria are set to meet the NEQS and USEPA standards
16. Sustainability of follow-up over lifespan of projects	• Follow-up programme/EMPs are formulated during EIA preparation • Cover construction and operation phases • Decommissioning phase not covered • Include generalized strategies for managing short and long-term environmental changes
17. Adequacy of technical and financial resources	• Employment of inadequate technical resources by most of the project proponents • A few encouraging examples also exist • Regulators are lacking technical staff and equipment • Regulators are provided with inadequate financial resources

Appendix II. Continued from previous page

Appendix III. Environmental management plan for monitoring of impacts during construction and operation of an industrial estate project in Pakistan

Concern/Impact Component	Considerations/ parameters	Applied Standards	Location	Monitoring Frequency	Duration	Responsibility
Groundwater	pH, turbidity, colour, TDS, hardness, sulphate, fluoride, iron, faecal coliforms etc.	NEQS	Construction site, effluent treatment plant and landfill site.	Quarterly	-	Environment Manager/ Resident Engineer
Wastewater	Effluent flow, pH, BOD, COD, TSS, Chromium, Copper and Zinc etc.	NEQS	Offices, Effluent treatment plant and landfill site.	Monthly	-	Manger Treatment Plant
Air Emissions	CO, NOx, SOx, PM10	USEPA air quality standards	3 points near the main entrance, treatment plant site and landfill site in downwind direction.	Quarterly	8 hours	Environment Manager/ Resident Engineer
Noise Levels	Noise levels on dB(A) scale	NEQS	7.5 meter from the vehicles at 6 points near construction site, generator room, treatment plant site.	Quarterly	15 minutes at each point	Environment Manager/ Resident Engineer
Solid Waste	Source, type, generation, used oil, discarded mechanical parts etc.	-	Construction site, administrative buildings, industrial sites.	Daily	-	Chief Sanitary Supervisor/ Incharge Landfill Site

Source: EIA Report/EMP of an industrial estate project in Pakistan.

Note: This also represents most of the EMPs of development projects in the country. However, some EMPs also include environmental management measures for flora, fauna, soil conditions, health and safety of workers and resettlement action plan (if needed) indicating targets and mitigation measures.

6/25/2010

Effect of *Trichoderma* Species on Damping off Diseases Incidence, Some Plant Enzymes Activity and Nutritional Status of Bean Plants

Abd-El-Khair [*1], H., R. Kh. M. Khalifa [2] and Karima, H. E. Haggag[3]

[1*]Plant Pathology Department, [2]Fertilization Technology Department, National Research Centre, and [3]Pest Rearing Department, Central agricultural Pesticides Laboratory, Dokki, Giza, Egypt.

Abstract: *Fusarium solani* and *Rhizoctonia solani* are the common causal pathogens causes the damping off disease of beans (*Phaseolus vulgaris* L.) in Egypt. The antagonistic effect of four *Trichoderma* species, i.e. *Trichoderma album, Triechoderma hamatum, Trichoderma harzianum* and *Trichoderma viride*, was tested against *F. solani* and *R. solani in vitro*, in greenhouse and in field. *In vitro* tests, all *Trichoderma* spp. significantly reduced the mycelial growth of two pathogenic fungi. In greenhouse experiment, *T. album, T. hamatum, T. harizianum* and *T. viride*, as soil treatments, significantly reduced the pre- and post-emergence damping off disease incidence under artificial infection with *F. solani* and *R. solan*i. Soil treatments with four *Trichoderma* species significantly reduced the incidence of damping off disease where the percentages disease incidence were in the range of 7.0 -20.0% and 2.4 – 6.5%, compared to 25.7 and 13.5% in control plants, at pre- and post-emergence stages ,respectively. The best protection to damping off disease was obtained by *T. hamatum*, followed by *T. viride, T. album* and *T. harzianum*, respectively. The treatments gave the highest plant survival (%) and improved the growth and yield parameters. Results showed that the levels of chitinase, peroxidase and polyphenol oxidase activities highly increased in treated bean plant compared in untreated plants. The macro- and micro-elements content in treated bean plants was affected by *Trichoderma* species treatments compared to elements content in untreated plants. The relationship between plant nutrient content and some plant enzymes activity was studied. [Journal of American Science. 2010;6(12):122-134]. (ISSN: 1545-1003).

Key words: *Fusarium solani*, *Rhizoctonia solani*, *Phaseolus vulgaris*, *Trichoderma* spp., biological control, nutritional atatus.

1. Introduction

Beans (*Phaseolus vulgaris* L.) are considered one of the most important economic legumes in Egypt. Bean is very important as a human food, animal feed and its beneficial effects in improving the soil fertility (Anonymous, 2005 and Broughton *et al*., 2003). *Fusarium* root rot on beans is caused by the fungus *Fusarium solani* f. sp. *phaseoli*. The fungus can attack older seedlings, and is most severe on plants growing under stressful conditions. The pathogen usually survives as thick-walled chlamydospores in soil. *Rhizoctonia* root rot, caused by *Rhizoctonia solani,* is common throughout the world. It is one of the most economically important root diseases of beans. It has a broad host range that includes most annual and many perennial plants. Generally, *Rhizoctonia* survives between crops as sclerotia or as fungal mycelia in the soil. Young plants are more susceptible to infection than older plants. Application of the fungicides is not economical in the long time because they pollute the environment, leave harmful residues and can lead to the development of resistant strains of the pathogen with repeated use (Vinale *et al.,* 2008). Replacement of fungicides with bio-control agents is an alternative mean to; manage the plant pathogens, produce safety food and reduce the environment pollution (Barakat and Al-Masri, 2005). One of the most important bio-control agents is *Trichoderma* spp. that the most frequently isolated soil fungi and present in plant root ecosystems (Harman *et al*., 2004).*Trichoderma* spp. also are commercially marketed as biopesticides, bio-fertilizers and soil amendments. The use of *Trichoderma* fungi in agriculture can provide numerous advantages ; 1) colonization of the root and rhizosphere of plant, 2) control of plant pathogens by different mechanisms such as parasitism, antibiosis production and induce systemic résistance , 3) improvement of the plant health by promote plant growth , and 4) stimulation of root growth (Harman *et al.*,2004).

The antagonistic activity of the genus *Trichoderma* to *F. solani* and *R. solani* has been widely demonstrated (Lewis *et al*., 1998). *Trichoderma harzianum* protected the bean seedlings against pre-emergence damping off infection, reduced the disease severity and increased the plant growth in the presence of *R. solani* pathogen (Paula *et al*., 2001). El-Kafrawy (2002) reported that the *T. harzianum, Trichoderma hamatum, Trichoderma pseudoknonningii* and *Trichoderma polysporum* inhibited the radial mycelial growth of *R. solani in*

vitro test from 59.6 to 78.4 %. Soil treatment with *T. hamatum*, *T. harzianum* and *T. viride* gave the maximum protection against pre- and post-emergence damping off and reduced the disease incidence from 50 to 6.6, 10 and 10%, respectively, compared to fungicide Rizolex (tolclofos-methyl) at 10%. The bio-control agent treatments improved the plant heights, fresh and dry weight and increased dry seeds yield comparing with the control (El-Kafrawy, 2002). The seed treatment with the bio-control agents was less than soil treatment. Gonzalez *et al*. (2005) showed that the field application with *T. viride* and/or *T. harzianum* as soil application gave the same effectiveness (99%) against *R. solani* pathogen, comparing with the seed immersion. Soil amendments with *T. harzianum* significantly increased the heights and weight of plants and significantly reduced the *R. solani* infection (Malik *et al.*, 2005). Application of *T. harzianum* as seed treatment significantly reduced the incidence of damping-off diseases some leguminous crops, i.e. faba bean, lentil, and chickpea, when planted in a soil naturally infested with *Fusarium* spp. and *R. solani* (Abou-Zeid *et al*., 2003).*T. harzianum*, *Trichoderma koningii* and *T. viride*, as seed dressing, improved the seedling emergence and health of runner bean (*Phseous coccineus* cv. Eureka) [Pieta *et al*., 2003]. Seeds of common bean were dressed, prior to sowing; with conidia of *T. harzianum* protected the germinating seedlings and plants against infection by soil borne pathogenic fungi, i.e. *Fusarium* spp. and *R. solani* (Pieta and Pastucha, 2004).

Soil provides the medium for root development and with the exception of carbon, hydrogen, oxygen and some nitrogen, plants depend on soil for all other nutrients and water. The soil microbes that include bacteria, fungi, actinomycetes, protzoa and algae play a significant role in the nutrient cycling (Nannipieri *et al.*, 2003). Interactions between plant root systems and bio-control agents such as rhizobacteria are able to generate a wide array of secondary metabolites which can have a positive influence on plant growth, enhancing the availability of minerals and nutrients, improving nitrogen fixation ability and improving plant health through the bio-control of phytopathogens (Sturz and Christie, 2003). Applying biological control agents to infected plants increase mineral levels [(nitrogen (N), phosphorous (P), potassium (K) and magnesium (Mg)], and both chlorophyll biosynthesis and photosynthetic activity, which in turn led to the accumulation of metabolites i. e., carbohydrates and proteins (Mahmoud *et al*.,2004). *Bacillus subtilis* and *T. viride* only or combined were significantly increased the values of NPK concentration on tomato plants compared to control treatments (Henry *et al*., 2009 and Morsy *et al*., 2009).

The objective of this search was to evaluate the antagonistic potential effect of *Trichoderma* species (spp.), i.e. *T. album, T. hamatum, T. harzianum* and *T. viride* as bio-control agents against *F. solani* and/or *R. solani* the causal organisms of damping off disease and nutritional atatus in bean plants. The antagonistic activity of *Trichoderma* spp. was tested *in vitro*, in pot and in field. Role of bio-agents in enhance of some enzymes (chitinase, peroxidase and polyphrnol oxidase) related to disease control in plant was detected. The relationship between nutritional status of bean plants and *Trichoderma* spp. application was tested.

2. Materials and Methods

1- Plant material:

Bean seeds (*Phaseolus vulgaris* L.) cv. Pulista was obtained from Vegetable Crops Research Depart., Agricultural Research Centre, Giza, Egypt, to produce the host plants in this search. Health test of bean seeds was made. Seeds were placed on sterile cotton and filter paper moistened with sterile distilled water in Petri dishes and incubated at 25 ºC. Ten replicates of 20 seeds were used for each dish .No infected bean seeds were recorded through the test (Coskuntuna and Özer, 2008).

2- Pathogens:

Fusarium solani and *Rhizoctonia solani* were isolated from naturally infected bean plants, showing damping off and root rot symptoms, cultivated in Qalyubiya Governorate, Egypt. The isolated fungi were identified on the basis of cultural and microscopic morphological characters according to the key given by Barnett & Hunter (1972) and Booth (1985). Pathogencity of isolated fungi toward bean plants (cv. Pulista) was estimated (Sallam *et al*., 2008). Artificial inoculum of pathogenic fungi was prepared by growing each fungus on sorghum - sand medium as described by Abd El- Khair and El-Mougy (2003). The most aggressive isolate of each pathogenic fungus was used *in vitro* and in pot experiments.

3- Isolation of *Trichoderma* spp.:

Four fungi of *Trichoderma* species, i.e. *T. album, T. hamatum, T. harzianum* and *T. viride* were isolated from healthy bean plants rhizosphere collected from Qalyubiya Governorate , using dilution plate technique . All *Trichoderma* spp. fungi were purified by hyphal tip technique and identified on the basis of cultural and microscopic morphological characters (Barnett & Hunter, 1972 and Bissctt, 1991) in Plant Pathology Department,

National Research Centre. The fungi of *Trichoderma* spp. were used *in vitro*, in pots and in field experiments.

4- Preparation of *Trichoderma* spp. inoculums:

The propgules (colony forming unit, cfu) suspension of each *T. album*, *T. hamatum*, *T. harzianum* and *T. viride* fungus was prepared in sterile distilled water from 7-days-old-culture on potato dextrose agar [PDA] (Rojo *et al*., 2007). The fungal inoculum was harvested by flooding the culture with SDW and then rubbing the culture surface with a sterile glass rod. The fungal propgules concentration in each suspension was determined by counting using a haemocytometer slide (Adjusted at 10^8 cfu / ml). A mixture of milted soybean and talc powder (1:1, w: w) was used as a carrier mixture for antagonistic fungal propgules. A carrier mixture was added at rate of 50% to fungal suspension and mixed to even distribution of fungal propgules (Abd El-Khair and El -Mougy, 2003).

5- Evaluation of antagonistic activity of *Trichoderma* species:

5.1- In dual culture technique (*in vitro*):

The antagonistic effect of *T. album*, *T. hamatum*, *T. harzianum* and *T. viride* against *F. solani* and *R. solani* pathogens *in vitro* was evaluate using the dual culture technique (Coskuntuna and Özer, 2008). Each *Trichoderma* spp., *F. solani* and *R. solani* were cultured, separately, on PDA medium for 7 days at 25ºC. Disc (5mm- diameter) from each bio-control fungus was inoculated on surface of PDA medium in side of Petri dish. A disc (5 mm - diameter) of *F. solani* and *R. solani*, separately, was inoculated at equal distance of the opposite side of Petri dish. Petri dishes were inoculated with each pathogenic fungus only as control. Four Petri dishes for each bio-control - pathogenic fungus treatment, as well as the control, were used as replicates. The inoculated Petri dishes were incubated at 25 ºC at 7 days. Antagonistic effect of *Trichoderma* spp., as decrease of the mycelial growth of pathogenic fungi, was determined using the following formula.

$$\text{Antagonistic effect} = A-B/A \times 100$$

Where, A: The diameter of mycelial growth of pathogenic fungus in control and

B: The diameter of mycelial growth of pathogenic fungus with *Trichoderma* fungus.

5.2- In pot experiment:

Antifungal activity of *T. album*, *T. hamatum*, *T. harzianum* and *T. viride* against *F. solani* and *R. solani* pathogens was evaluated in pots under artificially infestation conditions. The experiment were designed under greenhouse conditions in Pest Rearing Department, Central Agricultural Pesticides Laboratory, using pots (40 cm – diameter) containing 4 kg of sterilized loamy clay soil. First, soil was infested with each pathogenic fungus grown on sorghum-sand medium at rate of 5 g/ kg soil in different pots and then the pots were irrigated for 7 days before bio-control agent inoculation. Next, soil was inoculated with each *Trichoderma* fungus at 5 g/ kg soil, and then pots were watered for 7 days before sowing. Ten of bean seeds (cv. Pulista) were sown in each pot. Five pots as replicates were used for each treatment as well as the control. The experiment included the following treatments; 1) non-infested soil (control), 2) soil treated with *F. solani* only, 3) *F. solani* + each *Trichoderma* fungus, separately, 4) soil treated with *R. solani* only and 5) *R. solani* + each *Trichoderma* fungus , separately. Pots were kept under greenhouse conditions till the end of the experiment. Disease incidence of pre- and post-emergence of damping off disease incidence and survival (%) of bean plants were recorded after 15, 30 and 45 days, respectively.

5.3- In field experiment:

The efficacy of soil treatment with *T. album*, *T. hamatum*, *T. harzianum* and *T. viride*, in separated block, against the incidence of damping off disease, were evaluated in a commercial bean field with a previous history of the disease in Qalyubiya Governorate. The field trial (20 plots) was designed in complete randomized block with four replicates. Each plot was 3x3 m in area and had four rows of 3m in length and 75 cm in width. First, each bio-control agent inoculum was applied at rate of 50g/1 m^2 of soil was incorporated with the 20 cm of the soil surface. Next, the soil was irrigated before 7 days before sowing. Bean seeds (cv. Pulista) were planted at rate of 3 seeds / hole at 20cm space. The field treatments were as follows; 1) soil naturally infected (control), 2) soil treated with *T. album*, 3) soil treated with *T. hamatum*, 4) soil treated with *T. harzianum* and 5) soil treated with *T. viride*.

Effect of the tested *Trichoderma* spp on:

5.3.1- Disease assessment:

Effect of the tested *Trichoderma* spp. in reducing the damping off disease incidence at pre- and post-emergence stages as well as the percentages of the survival of healthy plants were recorded after 15 , 45 and 60 days.

5.3.2- Plant growth and yield parameters.

Random samples of ten bean plants were collected at 60 days of sowing for each bio-control agent treatment as well as the control plants. The plant growth parameters as number of branches per plant, plant height, and number of leaves per plant and fresh weight of plant were determined. The yield parameters also recorded as number of pods per plant and the average pod weight were recorded.

5.3.3- Some plant enzymes activity:

Effect of *Tricoderma* spp. application on the activity of chitinase, peroxidase and polyphenol oxidase enzymes related to plant defense against pathogens infection were determined in leaves of bean plants.

Extraction of enzymes:

Plant tissue (g) was homogenized with 0.2 Tris Hcl buffer (pH 7.8) containing 14mM B-Mercaptoethanol at the rate of 1/3 w/v. The homogenate was centrifuged at 3000 rpm for 15 minutes. The supernatant was used to determine enzyme activities (Tuzun *et al*., 1989).

5.3.3.1-Chitinase assay:

Colloidal chitin was prepared from chitin powder (Sigma Co.) according to the method described by Ried and Ogryd –Ziak (1981).Twenty five grams of chitin powder suspended in 250 ml of 85 % phosphoric acid (H_3PO_4) and stored at 4 ^{O}C for 24 h., then blended in 2 liter of distilled water using blender. The suspension was centrifuged. This washing procedure was repeated twice. The colloidal chitin suspension was adjusted at PH 7 with (1N) NaOH and re-centrifuged. The pelleted colloidal chitin was stored at 4 c until used. Determination of enzyme activity was carried out according to the method of Monreal and Reese (1969).One ml of colloidal chitin in 0.05 M citrate phosphate buffer (pH 6.6) in test tubes. One ml of enzyme extract was added and mixed by shaking. Tubes were incubated in a water bath at 37 ^{O}C for 60 min., then cooled and centrifuged before assaying. Reducing sugars were determined in 1 ml of the supernatant by dinitro-salysilic acid (DNS). Optical density was determined at 540 nm.

5.3.3.2 - Peroxidase assay:

Proxidase activity was measured by incubation 0.1 of enzyme extract with 4 ml of guaiacol for 15 minutes at 25 ^{O}C and absorbance at 470 nm was determined. The guaiacol solution consisted of 3 ml of 0.05 M potassium phosphate. PH 7, 0.5 ml of 2 % guaiacol and 0.5 ml of 0.3 % H_2O_2 (Abeles *et al*., 1971). Peroxidase activity was expressed as the increase in absorbance at 470 nm/gram fresh weight/15 minutes.

5.3.3.3 - Polyphenol oxidase assay:

Activity of polyphenol oxidase was determined using the colorimetric method described by Matta and Dimond (1963). The reaction mixture contained 1.0 ml of crude enzyme extract, 1.0 ml 0.2 M sodium phosphate at pH 7.0 and 1.0 ml of 10 M catchall brought to final volume of 6.0 ml with distilled water (Morsy, 2005). The activity of polyphenol oxidase was expressed as the optical density at 475 nm.

5.3.4 - Macro- and micro-nutrient elements content:

Representative soil sample (0-30 cm) was taken before cultivation to determine some physico-chemical characteristic (Table, 1) according to standard methods described by Jackson (1973) and Lindsay & Norvel (1978). At early F1 (pods <10 cm long) growth stage, young mature blade from terminal and green pods were taken for determine their macro- and micro-elements content. The dry aching technique as described by Chapman and Pratt (1978) was used to extract macro- and micronutrients from dried leaves and pods.Total nitrogen (N) in leaves was determined by Buechi-320 apparatus, while potassium (K) and calcium (Ca) were measured using flame photometer, Genway instrument and phosphorus (P) was measured by using Spectrophotometer, Perkin Elemer instrument. The leaves content of iron (Fe), manganese (Mn), zinc (Zn) and magnesium (Mg) were measured by using Atomic Absorption Spectrophotometer, Perkin, Elemer, and Model 1100B.

5.4- Statistical analysis:

Data obtained were subjected to Computer Statistical Package (CO-STATE) originated by Anonymous (1989).

3. Results:

1 - *In vitro* tests:

The inhibitory effect of *T. album, T. hamatum, T. harizianum* and *T. viride* against the mycelial growth of *F. solani* and *R. solani in vitro* test are shown in Table (2). The antagonistic effects of *Trichoderma* spp. against *F. solani* were in the range of 38.9 – 57.5 %. *T. hamatum* gave the highest effect about 57.5 %, followed by *T. album* (49.1%), *T. harzianum* (44.4%) and *T. viride* (38.9 %), respectively. Results showed that the growth inhibition of *R. solani* by *Trichoderma* spp were in the range of 41.7 – 70.3 %. *T. hamatum* also highly inhibited the mycelial growth of *R. solani*, where the

growth inhibition was 70.3 %, followed by *T. harzianum* (61.1%), *T. album* (53.7%) and *T. viride* (41.7%), respectively. Results showed that the best growth inhibition against two pathogenic fungi was obtained by *T. hamatum*, while the lowest one was obtained by *T. viride* (Table, 2).

Table (1) : Some physico-chemical properties of soil sample from the experimental site.

Properties	**Items**
Sand %	29.2
Silt %	25.0
Clay %	25.2
Texture	Clay loam
pH (1:2.5 soil : water)	8.36
E. C. (1:2.5 soil: water) dS/m	0.30
$CaCO_3$	1.20
Organic matter %	1.50
Available macronutrients (mg/100 g soil)	
P	3.24
K	55.86
Mg	289
Co	693
Na	36.40
Available micronutrients (mg/Kg soil)	
Fe	7.40
Mn	3.30
Zn	1.80
Cu	0.90

Table (2): Effect of *Trichoderma* species treatments aganist the leaner mycelial growth of *Fusarium solani* and *Rhizoctonia solani* in *vitro* tests.

Trichoderma species	Antagonistic effect against			
	Fusarium solani		*Rhizoctonia. solani*	
	Mycelial diameter (cm)	Reduction %	Mycelial diameter (cm)	Reduction %
Trichoderma album	4.9 c(1)	49	4.2 c	54
Trichoderma hamatum	3.8 d	58	2.7 e	70
Trichoderma harzianum	5.0 c	44	3.5 d	61
Trichoderma viride	5.5 b	39	5.3 b	42
Control	9.0 a	-	9.0 a	-

(1) Means in each column followed by the same letter are not significantly different according to LSD test (P = 0.05).

2 - In greenhouse experiment:

Soil treatments with *T. album, T. hamatum, T. harizianum* and *T. viride* significantly reduced the pre- and post-emergence damping off disease incidence under artificial infection with *F. solani* and *R. solan*i in greenhouse conditions (Table, 3). The damping off disease incidence caused by *F. solani* under application of *Trichoderma* spp. were in the range of 9.5 – 19.0 % and 2.5 - 7.5% ,compared to 48.5 and 55.8 % at pre- and post-emergence stages ,respectively**.** At pre-emergence**,** *T. hamatum* gave the highest reduction to disease incidence about 81%, followed by *T. viride* (71 %), *T. album* (69%) and *T. harzianum* (61%). At post-emergence stage, *T. album* gave the best growth reduction to disease incidence about 96%, followed by *T. harzianum* (90%), *T. viride* (90%) and *T. hamatum* (87 %), respectively. The percentages of survival bean plants were in the range of 92.5 – 97.5 % compared to 44.2 % healthy bean plants in the control treatment. *T. album* gave 97.5 % healthy plants, followed by *T. harzianum* (94.5%), *T. viride* (94.3%) and *T. hamatum* (92.5%) where the percentages of the increase of healthy plants were 121 , 114, 113 and 109% ,respectively (Table,3).

The damping off disease incidence caused by *R. solani* under application of *Trichoderma* spp. were in the range of 4.0 – 16.0 % and 2.1 – 5.9% , compared to 35.0 and 52.5 % in the control plants , at pre- and post-emergence stages (Table,3). *T. hamatum* gave the highest reduction (80%) of disease incidence, followed by *T. viride* (63%), *T. album* (62%), and *T. harzianum* (54%) at pre-emergence stage. While at post-emergence, *T. album* gave the highest reduction (96%) against damping off disease, followed by *T. harzianum* (90%), *T. hamatum* (90%) and *T. viride* (89%), respectively (Table,3). The percentages of healthy bean plants were in the range of 94.1 – 97.9 %. *T. album* produced the highest percentage of healthy bean plants (97.9%), followed by *T. harzianum* (94.8%), *T. viride* (98%) and *T. hamatum* (95%), compared to 47.5 % in the control plants, where the percentages of the increase were 106, 100, 98 and 95, respectively.

3 - In field experiment:

Effect of *Trichoderma* spp. on:

3.1- Damping off disease incidence:

Soil treatments with four *Trichoderma* species, i..e., *T. album*, *T. hamatum*, *T. haezianum* and *T. viride*, significantly reduced the incidence of damping off disease in the field comparing with the control plants (Table, 4). Results showed that the percentages of damping off disease incidence were in the range of 7.0 -20.0% and 2.4 – 6.5%, compared to 25.7 and 13.5%, at pre- and post-emergence stages, respectivily. The best protection against the disease obtained by *T. hamatum*, where the disease incidence reduction was 73 %, followed by *T. viride* (53%), *T.*

album (38%) and *T. harzianum* (22%) at pre-emergence, respectively (Table, 3). At post-emergence, *T. album* gave the highly reduction (82%) to damping off incidence, followed by *T. viride* (65%), *T. harzianum* (63%) and *T. hamtum* (52%), respectively. Application of *Trichoderma* spp. also reduced the root rot disease of bean plants where the disease incidence were in the range of 0.0 – 2.2 % compared to 10.7 % in control bean plants.

Table (3): Effect of *Trichoderma* species treatments on the percentage of damping-off disease of bean plants under greenhouse condition (artificially infection) .

Trichoderma species	Disease assessment					
	Damping-off				Survival %	
	pre-emergence		Post-emergence			
	Incidence %	Reduction %	Incidence %	Reduction %	Healthy plants %	Increase %
Fusarium solani						
Trichoderma album	15.0 c[(1)]	69	2.5 e	96	97.5 a	121
Trichoderma hamatum	9.0 e	81	7.5 b	87	92.5 d	109
Trichoderma harzianum	19.0 b	61	5.5 d	90	94.5 b	114
Trichoderma viride	14.0 d	71	5.7 c	90	94.3 c	113
Control	48.5 a	-	55.8 e	-	44.2 e	-
Rhizoctonia solani						
Trichoderma album	14.0 bc	62	2.1 c	96	97.9 a	106
Trichoderma hamatum	7.0 d	80	5.5 b	90	92.5 d	95
Trichoderma harzianum	16.0 b	54	5.2 b	90	94.8 b	100
Trichoderma viride	13.0 c	63	5.9 b	89	94.1 c	98
Control	35.0 a	-	52.5 a	-	47.5 a	-

(1) Means in each column (for each pathogenic fungus) followed by the same letter are not significantly different according to LSD test (P = 0.05).

Trichoderma spp. also significantly increased the percentages of healthy plants compared to control plants (Table, 4). Results showed that the survival bean plants were in the range of 91.3 – 97.6%, while in control bean plants was 75.5%. Application of *T. album* produced the highest percentage of healthy plants (97.6%), followed by *T. viride* (95.3%), *T. harzianum* (92.5%) and *T. hamatum* (91.3%), respectively.

Table (4): Effect of *Trichoderma* species treatments on the percentage of damping-off disease of bean plants under field applications (natural infection) .

Trichoderma species	Disease assessment						
	Damping-off %				Root-rot %	Survival %	
	Pre-emergence		Post-emergence				
	Inc. %	Red. %	Inc. %	Red. %		Healthy plants %	increase %
Trichoderma album	16.0 c [(1)]	38	2.4 e	82	0.0	97.6 a	29
Trichoderma hamatum	7.0 e	73	6.5 b	52	2.2 b	91.3 d	21
Trichoderma harzianum	20.0 b	22	5.0 c	53	2.2 b	82.5 c	22
Trichoderma viride	12.0 d	53	4.7 d	65	0.0	95.3 b	26
Control	25.7 a	-	13.5 a	-	10.7 a	75.8 e	-

(1) Means in each column followed by the same letter are not significantly different according to LSD test (P = 0.05).

3.2- Growth and yield parameters:

Results revealed that the average of bean plant height with *Trichoderma* application were in the range of 46.0-49.8 cm compared to 37.3 cm in the control plants (Table,5).*T. hamatum* gave the highest increase of plant height (34%) , followed by *T. harzianum* (26%) , *T. viride* (26%) and *T. album* (23%). No significant differences were recorded among *Trichoderma* treatments, while significant once were recorded between *Trichoderma* treatments and the control plants. The branches number average per plant as result for application of *Trichoderma* spp. were in the range of 5.0 – 6.3 branch/plant, compared to 3.7 branch /plant in control treatment. *T. harzianum* significantly increased the branches number average (70%), followed by *T. viride* (49%), *T. hamatum* (41%) and *T. album* (35). The leaves number average in treated bean plants were in the range of 11.5 – 15.2 leaves/plant; while in untreated plants were 9.5. *T. harzianum* significantly increased number of leaves (60%) , followed by *T. hamatum* (56%) , *T. viride* (50%) and *T. album* (21%).Results also showed that the fresh weight average without pods were in the range of 43.1 – 77.4% g , compared to 42.5g in the control plants (Table,5). *T. hamatum* significantly increased the fresh weight of bean plant (82%), followed by *T. harzianum* (36%), *T. viride* (21%) and *T. album* (2%).

Table (5): Effect of *Trichoderma* species treatments on some growth and yield parameters of bean plants under field applications (natural infection).

Growth and yield prameters		*Trichoderma* spp.				
		Trichoderma album	*Trichoderma hamatum*	*Trichoderma harzianum*	*Trichoderma virde*	control
Growth parameters						
Plant height (cm)	Average	46.0 a (1)	49.8 a	47.0 a	46.8 a	37.3 b
	Increase %	23	34	26	26	-
Branches no./ plant	Average	5.0 b	5.2 b	6.3 a	5.5 ab	3.7 c
	Increase %	35	41	70	49	-
Fresh weight / plant	Average	43.1 c	77.4 a	57.9 b	51.2 bc	42.5 c
	Increase %	2	82	36	36	21
Leaves no./ plant	Average	11.5 b	14.8 ab	15.2 a	14.2 b	9.5 c
	Increase %	21	56	60	50	-
Yield parameters						
Pods no./ plant	Average	15.7 a	20.0 a	17.5 a	15.2 a	10.8 b
	Increase %	45	85	62	41	-
Pods weight (g)	Average	3.1 b	4.0 a	2.7 c	2.7 c	2.7 c
	Increase %	15	48	0	0	-
Pods fresh yield	Average	3.3 b	5.1 a	3.2 b	3.0 b	1.6 c
	Increase %	106	219	100	99	-

(1) Means in each row followed by the same letter are not significantly different according to LSD test (P = 0.05).

Application of *Trichoderma* spp. significantly increased the pods number average per plant where the number were in the range of 15.2 – 20.0 pods/plant ,compared to 10.8 pods/plant in the control plants (Table,5). No significant differences were recorded among *Trichoderma* treatments. *T. hamatum* significantly increased the pods number average (85%), followed by *T. harzianum* (62%), *T. album* (45%) and *T. viride* (41%).Results indicated that *T. hamatum* and *T. album* significantly increased the pod weight average about 48 and 15 % , respectively, comparing with other *Trichoderma* treatments as well as the control plants(Table,5).

3.3 - Some plant enzymes activity:

All *Trichoderma* spp. treatments stimulated the activity of chitinase, Perxidase and polyphenol oxidase enzymes, comparing with the control treatment (Table, 6). The optical density of chitinase enzyme activity in treated bean plants were in the range of 0.271 – 0.620, compared to 0.117 in control plants. The increases of chitinase enzyme activity were in the range of 132-430 %. *T. viride* gave the highest enzymatic activity of chitinase (430%), followed by *T. album* (174%), *T. harzianum* (150%) and *T. hamatum* (132%). The optical density of peroxidase enzyme activity was in the range of 0.437- 0.775 in bean plants under *Trichoderma* application, compared to 0.346 in untreated bean plants. The peroxidase enzymatic activity was in the

range of 26-124 % with *Trichoderma* treatments application. *T. harizanum* significantly increased the activity of peroxidase activity about 124 %, followed by *T. hamatum* (100%), *T. viride* (34%) and *T. album* (26%).Results showed that the optical density of polyphenol oxidase were in the range of 0.231 – 0.518 in treated bean plants , compared to 0.146 in control plants (Table,6). *Trichoderma* application enhanced the activity of polyphenol oxidase enzyme in bean plants from 58 to 255 %. *T. harizanum* significantly increased the enzyme activity (255%), followed by *T. viride* (108%), *T. hamatum* (103%) and *T. album* (58%), respectively (Table, 6).

Table (6): Enzymatic activity of chitinase, peroxidase and polyphenol oxidae, in bean plants treated with *Trichoderma* species in field applications.

Trichoderma species	Enzymatic activities					
	Chitinase		Peroxidase		Polyphenol oxidase	
	Activity	Increase %	Activity	Increase %	Activity	Increase %
Trichoderma album	0.321 b[(1)]	174	0.437 c	26	0.231 d	58
Trichoderma hamatum	0.271 e	132	0.693 b	100	0.296 c	103
Trichoderma harzianum	0.293 c	150	0.775 a	124	0.518 a	255
Trichoderma viride	0.620 a	430	0.465 c	34	0.303 b	108
Control	0.117 d	-	0.346 d	-	0.146 e	-

(1) Means in each column followed by the same letter are not significantly different according to LSD test (P = 0.05).

3.4 - Macro- and micro-nutrient elements content:

3.4.1 – In bean leaves:

Results showed that the *Trichoderma* spp. treatments affected on the level of nutrients leave content from macro- and micro-elements (Table, 7). The *Trichoderma* spp. treatments significantly decreased the level of nitrogen and phosphorus content in bean leaves in treated plants .The level of nitrogen content was in the range of 2.19 – 2.60 %, compared to level of 2.73 % in untreated leaves. *T. harzianum* highly decreased the level of nitrogen content, where the level was 2.19 %, followed by *T. album* (2.21%), *T. hamatum* ((2.36 %) and *T. viride* (2.60 %). No significant differences were found between *T. album* and *T. harizanium* treatments. The level of phosphorus in leaves of treated plants was in the range of 15 – 17 %, compared to 0.21% in untreated control. The level of phosphorus was 15, 16, 16 and 17% in leave plants treated with *T. hamatum*, *T. album*, *T. viride* and *T. hariznum*, respectively. No significant differences were noticed among *T. hamatum*, *T. album* and *T. viride* (Table, 6).Results also revealed that the treatments increased the levels of potassium, magnesium, calcium and sodium in treated bean leaves than untreated one (Table,7). The level of potassium was in the range of 1.73 – 2.88% in treated leaves, compared to 1.39 % in untreated plants. *T. harzianum* significantly increased the level of potassium (2.88%) in treated leaves, followed by *T. viride* (20.16%), *T. album* (1.87%) and *T. hamatum* (1.73%). Treatment with *T. album*, *T. hamatum* and *T. viride* increased the level of magnesium in bean leave plants to 0.91, 0.88 and 0.84 %, except *T. harzianum* treatment decreased the level to 0.68 %, compared to level of 0.80% in untreated control. Results also showed that the level of calcium was in the range of 2.80 – 3.25 % in treated plants, compared to level of 2.75 % in controls. *T. viride* significantly increased the level of calcium in leaves (3.25%), followed by *T. album* (3.15%), *T. hamatum* (3.10%) and *T. harzianum* (2.80%). Treatment with both *T. hamatum* and *T. harzianum* significantly increased the level of sodium to 0.088 and 0.039 % in treated leaves, respectively, compared to the control (0.023%).

Effect of *Trichoderma* spp. treatments on the content of bean leaves in treated plants from micro-elements i.e. iron, manganese, zinc and cupper as shown in Table (7). The treatments significantly increased the levels of both iron and manganese in leave of treated plants. On the other hand, the same decreased the levels of zinc and cupper (Table, 7). The level of iron was in the range of 1250 – 2150 ppm .*T. hamatum* significantly increased the level of iron element (2150 ppm), followed by *T. album* (1550 ppm), *T. viride* (1425 ppm) , while *T. harzianum* significantly decreased the level of iron to 1250 ppm , compared to level of 1350 ppm in control . The level of manganese was in the range of 75 – 84 ppm, compared to level of 71 ppm in control plants. *T. viride* treatment only significantly increased the level of cupper in treated leaves. It is clear that *Trichoderma* spp. significantly increased the levels of potassium, calcium, iron and manganese in leaves in

treated bean plants. *T. hamatum* gave the highest values of magnesium, iron and manganese in bean leaves.

Table (7) : Effect of *Trichoderma* species on the nutrient elements content in bean leaves in field application (Average of two season).

Nutrient elements	Nutrient content of leaves with *Trichoderma* species				
	Trichoderma album	*Trichoderma hamatum*	*Trichoderma harzianum*	*Trichoderma viride*	Control
Macro-elements (%)					
Nitrogen	2.21 d[(1)]	2.36 c	2.19 d	2.60 b	2.73 a
Phosphorus	0.16 bc	0.15 c	0.17 b	0.16 bc	0.21 a
Potassium	1.87 c	1.73 d	2.88 a	2.16 b	1.39 e
Magnesium	0..91 a	0.88 ab	0.68 d	0.84 bc	0.80 c
Calcium	3.15 b	3.10 c	2.80 d	3.25 a	2.75 e
Sodium	0.023 c	0.088 a	0.039 b	0.022 c	0.023 c
Micro-elements (ppm)					
Iron	1550 b	2150 a	1250 e	1425 c	1350 d
Manganese	75 b	84 a	74 b	82 a	71 c
Zinc	29 bc	28 c	31 b	31 b	34 a
Cupper	9 cd	9 cd	8 d	13 a	10 b

(1) Means in each row followed by the same letter are not significantly different according to LSD test (P = 0.05).

3.4.2 – In fresh bean pods:

Results showed that *Trichoderma* spp. treatments significantly decreased the levels of both nitrogen and phosphorous in bean pods of treated plants, compared to control treatments (Table, 8).The level of nitrogen was in the range of 1.79 – 3.09 % , while the level of phosphorous was in the range of 0.42 – 0.50% , compared to 3.37 and 0.56 % in untreated pods. *T. harzianum* significantly increased the level of potassium to level of 4.42 %, while other *Trichoderma* spp. significantly decreased it as compared to level of 3.65 % in treated pods. The level of magnesium was in the range of 0.32 – 0.38 % in treated pods, compared to level of 0.43 % in untreated pods. *T. harzianum* only significantly increased the level of calcium and sodium to 0.35 and 0.022 % in treated pods, compared to levels of 0.25 and 0.014 %, respectively.

Effect of *Trichoderma* spp. on the levels of micro-elements in bean leaves as shown in Table (8). The treatments of *Trichoderma* significantly increased the content of pods from zinc*;* the level of element was in the range of 49 – 58 ppm, compared to level of 44 ppm in control. *T. harzianum* only significantly increased the levels of iron (550) and cupper (10 ppm) in pods of treated bean plants, compared to level of 475 and 6 ppm in untreated pods (Table, 8).

Table (8): Effect of *Trichoderma* species on nutrient elements content in bean green pods in field application (Average of two season).

Nutrient elements	Nutrient content of bean pods with *Trichoderma* species				
	Trichoderma album	*Trichoderma hamatum*	*Trichoderma harzianum*	*Trichoderma viride*	Control
Macro-elements (%)					
Nitrogen	2.03 d [(1)]	2.13 c	3.09 b	1.79 e	3.37 a
Phosphorus	0.45 d	0.42 e	0.48 c	050 b	0.56 a
Potassium	3.60 c	2.26 e	4.42 a	3.55 d	3.65 b
Magnesium	0.38 b	0.32 c	0.38 b	0.37 b	0.43 a
Calcium	0.25 c	0.28 b	0.35 a	0.26 c	0.25 c
Sodium	0.017 b	0.015 c	0.022 a	0.016 b	0.014 c
Micro-elements (ppm)					
Iron	245 e	390 d	550 a	407 c	475 b

Manganese	19 d	20 c	22 b	26 a	26 a
Zinc	50 b	49 b	51 b	58 a	44 c
Cupper	6 c	6 c	10 a	7 b	6 c

(1) Means in each row followed by the same letter are not significantly different according to LSD test (P = 0.05).

3.4.3 – Correlation coefficient:

The correlation coefficient between macro- and micro-element nutrients concentration and some plant enzymes i.e. chitinase, peroxidase and polyphenol oxidase which related to plant disease resistance in bean leaves as average of two seasons are shown in Table (9). Results indicated that the significantly correlation was found between chitinase activity and the leaves content from phosphorous, potassium, calcium, manganese and cupper). The positive correlations were found between potassium, calcium, manganese and cupper, while the highly significant negatively one was found with phosphorous (Table, 9). Results showed that negatively correlation was noticed between the activity of peroxidase enzyme and macro-elements of nitrogen, phosphorus, magnesium, zinc and copper while positive correlation was detected with potassium and sodium. The activity of polyphenol enzyme in bean plants was negatively correlated with nitrogen, phosphorus and magnesium, while the significantly positive correlation was found with potassium. It is clear that the activity of peroxidase was highly correlated with nutrients elements, followed by chitinase and polyphenol oxidase. The highly significant positive correlation was recorded between the potassium and the activity of studied plant enzymes which may reflect emphatic role of potassium in plant resistance. The activity of polyphenol oxidase was correlated with micro-elements.

Table (9) : Correlation coefficient between nutrient elements content in bean leaves and enzymatic activity in leaves (Average of two seasons).

Nutrients content In bean leaves	Enzymatic activity in leaves		
	Chitinase	Peroxidiase	Polyphenol oxidase
Macro-elements (%)			
Nitrogen	0.0010(NS)	– 0.6489**	– 0.6148 **
Phosphorus	– 0.5174 **	– 0.4641 **	– 0.3671 *
Potassium	0.3904 *	0.6958 **	0.9547 **
Magnesium	0.1568 (NS)	– 0.4415 *	– 0.6449 **
Calcium	0.7629 **	– 0.1073 (NS)	– 0.1210 (NS)
Sodium	– 0.2055 (NS)	0.6556 **	0.2125 (NS)
Micro- elements (ppm)			
Iron	– 0.0696 (NS)	0.2685 (NS)	– 0.1905 (NS)
Manganese	0.5566 **	0.3103 (NS)	0.1205 (NS)
Zinc	– 0.2094 (NS)	– 0.3840 *	– 0.1934 (NS)
Cupper	0.6092 **	–.4507 *	– 0.2837 (NS)

(NS) = Non significant * , ** = Significant at the probability levels of 0.05 and 0.01,respectively r 0.05 = 0.361 r 0.01 = 0.463

4. Discussion:

Our results revealed that the *T. album*, *T. hamatum*, *T. harzianum* and *T. viride*, which obtained from the rhizosphere of healthy bean plants, have bean reported as the best antagonists for damping off disease caused by *F. solani* and *R. solani* under laboratory, pot and field conditions. All *Trichoderma* spp. treatments reduced the mycelial growth of two pathogenic fungi. It is very important, especially the chemical methods are not economical in the long run, because the pollution the atmosphere, damage the environment, leave harmful residues, and can lead to the development of resistant strains among the target organisms with repeated use. Results indicated that all *Trichoderma* spp. significantly reduced the disease incidence at pre- and post-emergence stages in pot and field experiments. These results agree with those recorded by Abou-Zeid *et al*. (2003) and Pieta and Pastucha (2004). They reported that *T. harzianum*, *T. koningii* and *T. viride* protected the germinating bean seedlings against *Fusarium* spp. and *R. solani* infection. Our results, based on soil treatments with the tested *Trichoderma* spp. demonstrated a significant reduction to incidence of damping off disease in bean under pot and field infection (El-Kafrawy, 2002, Gonzalez *et al*., 2005 and Malik *et al*., 2005).

Our results showed that the use of *Trichoderma* spp. as bio-control agents induced the accumulation of some enzymes such as chitinase,

peroxidase and polyphenol oxidase which play an important role in plant defense mechanisms against pathogens infection. Results cleared that the enzymatic activity in treated bean plants increased than in untreated one. Nawar and Kuti (2003) reported that there are positive relationships between peroxidase and resistance development in plants. Caruso *et al*. (2001) also experimentally supported the idea that peroxidase play a defense role against invading pathogens. Hassan *et al*. (2007) recorded the lowest percentages of chocolate spot disease severity and the highest levels of peroxidase activities. Treatments with *Trichoderma* spp. gave the highly protection o bean seedlings against damping off disease at post-emergences stage comparison with per-emergence one. It is may be related to the ability of *Trichoderma* spp. to stimulate the enzymes in bean plants associated with increased the protection against disease. Our results revealed that *Trchoderma* spp. treatments increased some macro- and micro-elements content in leave and pods of bean and decreased the content of nitrogen, phosphorus and magnesium. These results agree with those reported by Snoeijers *et al* (2000). They reported that the successful colonization of plants by pathogens requires efficient utilization of nutrient resources available in host tissue. Our results revealed that the *Trichoderma* treatments highly increased the activity in bean leaves than pods. The treatments significantly increased the macro-elements of potassium, magnesium, and calcium and micro-element of iron which play an important role in defense plant tissues against plant pathogen infection. Results indicated that the activity of plant enzymes (chitinase, peroxidase and polyphenol oxidase) was correlated with level of macro- and micro-elements in bean plants.

Trichoderma is listed both in Europe and USA as an active principal ingredient permitted for use in organic farming for plant disease control. *Trichoderma* spp utilize various mechanisms including nutrient competition, antibiosis, antagonism, inhibition of pathogen or plant enzymes; processes of biodegradation, carbon and nitrogen cycling; complex interactions with plants in the root zone of the rhizosphere, which involve various processes such as colonization, plant growth stimulation, bio-control of diverse plant pathogens, decomposition of organic matter, symbiosis, and nutrient exchange (Howell,, 2003 and Harman, 2006). Recent studies indicate that these fungi can induce systemic resistance in plants, thus increasing the plant defense response to diverse pathogen attack (Harman *et al.*, 2004).

5. Conclusion

The previous results concluded that the soil treatments with *Trichoderma* spp significantly reduced the incidence of damping off disease .Results showed iit was positive correlation between the activity of chitinase , peroxidase and polyphenol oxidase and the level of nutrient elements especially potassium. Results recommended that the determine the nutritional status of plant under field treated with bio-control agents helps to select the suitable fertilization programs as well as diagnose nutritional deficiencies

Corresponding author
Abd-El-Khair'
Plant Pathology Department National Research Centre, Dokki, Giza, Egypt.

6. References

1. Abd El- Khair, H. and Nehal, S. El- Mougy (2003). Field biological approach under organic cultivation conditions for controlling garlic black mould disease infection during storage. Egypt. J. Appl. Sci., 18 (6): 50-69.
2. Abeles, F. B., R. B. Bpsshart, L. E. Forrence and W. H. Habig (1971). Preparation and purification of glucanase and chitinase from bean leaves. Plant Physiology, 47; 129 – 134.
3. Abou-Zeid, N. M., M.K. Arafa and S. Attia (2003). Biological control of pre- and post-emergence diseases on faba bean, lentil and chickpea in Egypt. Egyptian J. Agriculture Research , 81 (4) : 1491 – 1503.
4. Anonymous (1989). Cohort Soft Ware Crop. Co-State User Manual Version 3.03, Barkley Co., USA.
5. Anonymous (2005).Food and Agriculture Organization of the United Nations. FAO Production, Yearbook, Rome, Italy.
6. Barakat, R. M. and M. I. Al-Masri (2005). Biological control of gray mold diseases (*Botrytis cinerea*) on tomato and bean plants by using local isolates of *Trichoderma harzianum*. Dirasat Agricultural Science, 32 (2):145 – 156.
7. Barnett, H. L. and B. B. Hunter (1972).Illustrated Genera of Imperfect Fungi .Burgess Publishing Company, Minneapolis, pp241.
8. Bissett, J. (1991). A revision of the genus *Trichoderma*. II: Infragenic classification. Can. J. Bot., 69:2357 – 2317.

9. Booth, C. (1985). The Genus *Fusarium* .Kew, Surrey. Commonwealth Mycol. Inst., 2nd Ed., 237 pp.
10. Broughton ,W. J. ,G. Hernάndeaz , M. Blair , B. Beebe , P. Gepts and J. Vanderlryden (2003). Beans (*Phaseolus* spp.) – model food legumes. Plant and Soil , 252 :55-128.
11. Caruso ,C. ,G. Chilosi , L. Leonard , L. Bertin , P. Magro , V. Buonocore and Caporale (2001). A basic peroxidase from wheat kernel with antifungal activity. Phytochemstry , 72 : 248 – 254.
12. Chapman, H. D. and P. F. Pratt (1978). Methods of Analysis for Soils, Plants and Water . pp. 309. Univ. of California , Dept. of Agric. Sci. , USA.
13. Coskuntuna, A. and N. Özer (2008). Biological control of onion basal rot disease using *Trichoderma harzianum* and induction of antifungal compounds in onion set following seed treatment. Crop Protection, 27: 330 – 336.
14. El-Kafrawy, A. A. (2002). Biological control of bean damping-off caused by *Rhizoctonia solani* . Egyptian Journal Agricultural Research, 80 (1): 57-70/
15. Gonzalez, R. M., L. G. Castellanos, M. F. Rames and G. G. Perez (2005). Effectiveness of *Trichoderma* spp. for the control of seed and soil pathogenic fungi in bean crop. Fitosanidad, 9 (1):37-41.
16. Harman. G. E. (2006). Overview of mechanisms and uses of *Trichoderma* spp., Phytopathology 96, 190–194.
17. Harman, G. E., C. R. Howell, A. Viterbo , I. Chet and M. Lorito (2004). *Trichoderma* species-opportunistic, avirulent plant symbionts. Nature Review Microbiology, 2:43-56.
18. Hassan, E. M. Maggie , Saieda ,S. Abd El-Rahman , I. H. El-Abbasi and M. S. Mikhail (2007).Changes in peroxidase activity due to resistance induced against faba bean chocolate spot disease. Egypt. J. Phytopathology ,35 (1): 35 – 48.
19. Henry , A. , P.J. A. Kleinman and J. P. Lynch (2009). Phosphorus runoff from a phosphorus deficient soil under common bean (*Phaseolus vulgaris* L.) and soybean (*Glycine max* L.) genotypes with contrasting root architecture. Plant Soil, 317:1–16
20. Howell, C. R. (2003). Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: The history and evolution of current concepts, Plant Dis. 87, 4–10.
21. Jackson, M. L.(1973). Soil chemical analysis .Prentice- Hall of Indian Private Limited , New-Delhi.
22. Lewis, J. A., R. P. Larkin and D. L. Rogers (1998). A formulation of *Trichoderma* and *Gliocladium* to reduce damping-off caused by *Rhizoctonia solani* and saprophytic growth of the pathogen in soil less mix. Plant Diseases, 82:501-506.
23. Lindsay ,W. L. and W. A. Norval (1978). Development of DTPA- micronutrients soil tests for zinc , iron , manganese and cupper Soil Sci. ,Am. J. ,42:421-428..
24. Mahmoud,Y. A. G. , M. K. H. Ebrahim and M. M. Aly (2004). Influence of some plant extracts and micro-bioagents on some physiological traits of Faba bean infected with *Botrytis fabae*. Tuekish Journal Botany, 28 (6):519 – 528.
25. Malik ,G. , S. Dawar , A. Sattar and A. Dawar (2005). Efficacy of *Trichoderma harzianum* after multiplication on different substrates in the control of root rot fungi. International Journal Biology and Biotechnology, 2 91: 237-242.
26. Matta, A. and A. E. Dimond (1963) .Symptoms of *Fusarium* within relation to quantity of fungus and enzyme activity in tomato stems .Phytopathology ,53 : 544 – 578 .
27. Monreal, J. and E. T. Reese (1969).The chitinase of *Sarratia marcescens*. Canadian J. Microbiology, 15: 689 – 696.
28. Morsy , M. Ebtsam ,K. A. Abdel-Kawi and M. N. A. Khalil (2009). Efficiency of *Trichoderma viride* and *Bacillus subtilis* as bio-control agents against *Fusarium solani* on tomato plants. Egypt. J. Phytopathol. , 37 (1):47 – 57.
29. Morsy, K.M.M. (2005). Induced resistance against damping-off, and wilt diseases of lentil. Egypt. J. Phytopathol., 33(2):53-63.
30. Nannipieri , P., J. Ascher , M. T. Ceccherini, L. Landi, G. Pietramellara and G. Renella (2003) .Microbial diversity and soil functions. Eur. J. Soil Sci., 54:655–670
31. Nawar, H. F. and J. D. Kuti (2003). Wyerone acid phytoalexin synthesis and peroxidase activity as markers for resistance of broad bean to chocolate spot disease. J. Phytopathol.., 151 : 564 – 570.
32. Paula, T. J. de, C. Rotter and B. Han (2001). Effect of soil moisture and panting date on *Rhizoctonia* root rot of beans and its control

by *Trichoderma harizanum*. Bulletin OILB/SROP, 24(3):99-102.
33. Pieta, D. , A. Pastucha and E. Patkowska (2003).The use of antagonistic microorganisms in biological control of bean diseases. Sodininlyste ir Darzininkyste, 22 (3):401-406.
34. Pieta, D. and A. Pastucha (2004). Biological methods of protecting common bean (*Phaseolus vulgaris*, L.) Folia Universitaris Agriculturae Stetinensis Agricultura (95):301 – 305.
35. Ried, J. D. and D. M. Ogryd-Ziak (1981).Chitinase over producing mutant of *Serratia marcescens* .Appl. and Environ.Microbiol.41:664-669.
36. Rojo, F. G., M. M. Reynoso, M. Ferez, S. N. Chulze and A. M. Torres (2007). Biological control by *Trichoderma* species of *Fusarium solani* causing peanut brown root rot under field conditions. Crop Protection, 26: 549-555.
37. Sallam, M. N. Nashwa, K. A. M. Abo-Elyousr and M. A. E. Hassan (2008). Evaluation of *Trichoderma* species as biocontrol agents for damping off and efficacy of suggested formula. Egypt. J. Phytopathol., 36 (1-2):81-93.
38. Snoeijers , S. S. , A. P. Garcia , M. H. A. J. Joosten and P. J. G. M. DE Wit (2000). The effect of nitrogen on disease development and gene expression in bacterial and fungal plant pathogen. European J. Plant Pathology, 106:493 – 506.
39. Sturz, A. V. and B.R. Christie (2003).Beneficial microbial allelopathies in the root zone: the management of soil quality and plant disease with rhizobacteria. Soil & Tillage Research, 72: 107–123.
40. Tuzun , S., M. N. Rao, U. Vogeli, C. L. Schardl and J. Kuc (1989). Induced systemic resistance to blue mould; Early induction an accumulation of B-1,3-glucanase , chitinase and other pathogenesis proteins (b- proteins) in immunized tobacco . Phytopathology , 79 : 979 – 983
41. Vinale, F., K. Sivasithamparam, E. L. Ghisalberti, R. Marra, S, L. and M. Lorito (2008). *Trichoderma* - plant pathogens interactions. Soil Biology & Biochemistry, 40: 1-10.

6/23/2010

Phase I Trial: Mesenchymal Stem Cells Transplantation in End Stage Liver Disease

El-Ansary M,[1] Mogawer Sh,[2] Abdel-Aziz I,[3*] and Abdel-Hamid S,[1]

Department of Clinical Pathology[1], Department of Hepatology and Gastroenterology[2] Cairo University,
[*3]Department of Haematology (Theodor Bilharz Research Institute), Cairo, Egypt.
[*]iman_khaled@yahoo.com

Abstract: Background**,** End-stage liver disease and in particular human liver cirrhosis represents a worldwide health problem. Currently, liver transplant is the only effective treatment, but it is affected by many problems including relative lack of donors, operative damage, risk of rejection and high costs. Stem cell therapy is very attractive in this setting because it has the potential to help tissue regeneration while providing minimally invasive procedures and few complications. The aim of this study was to evaluate the effect of autologous transplantation of bone marrow derived mesenchymal stem cells in cirrhotic patients following chronic hepatitis C virus infection. Methods, Twelve patients with Child C liver cirrhosis, Model of End Stage Liver Disease (MELD) score > 12 were included. They divided into 2 groups according to method of MSCs injection, 1st group was injected intrasplenic and 2nd group was injected through the peripheral blood. First group patient's ages ranged from 32 to 69 years, mean value was 48.50 ±11.09, they were 4 males (67%) and 2 females (33%). Second group patient's ages ranged from 43 to 59 years, mean value was 50.83 ±6.88, they were 5 males (83%) and 1 female (17%). Fifty ml bone marrow was aspirated from the iliac bone for separation of MSCs. Surface expression of CD271 and CD34 were analyzed using flowcytometry. Finally approximately 10 million MSCs/ 5ml saline were infused intrasplenic or peripherally in one session. There was highly statistical significant difference between CD271 before and after culture, p value was <0.01. Results, Monthly Follow up of patients for 6 months revealed partial improvement of liver function tests with decline of elevated bilirubin and liver enzymes and elevation of prothrombin concentration and serum albumin levels. There was statistically significant difference between total bilirubin, direct bilirubin, MELD score and creatinine level before and after MSCs injection in both groups, p value was <0.05. Conclusion, MSCs are the most potent component of bone marrow cells in its ability to differentiate into hepatocytes thus, MSC transplantation can be used as a potential treatment for liver cirrhosis. The dose, frequency and route of administration of this treatment are still to be defined. [Journal of American Science. 2010;6(12):135-144]. (ISSN: 1545-1003).

Keywords: End-stage liver disease; liver cirrhosis; liver transplant; autologous transplantation; bone marrow; mesenchymal; stem cell

1. Introduction

Cirrhosis represents a late stage of progressive hepatic fibrosis characterized by distortion of hepatic architecture and formation of regenerative nodules. This results in many clinical manifestations including ascitis, variceal hemorrhage and encephalopathy (1).

Cirrhosis is the terminal outcome of viral hepatitis (particularly hepatitis C) *in our country* and alcoholic liver diseases. Other less frequent causes include some parasitic infections such as shistosomiasis, some metabolic disorders, toxic chemicals and unknown conditions. Egypt has the highest world wide prevalence of Hepatitis C reaching 20% in some areas. This is apparently due to past parenteral antischistosomal therapy. HCV infection was found to be a major cause of liver cirrhosis in Egypt (2).

Although liver cirrhosis carries a poor prognosis, the only very dated treatment for advanced liver cirrhosis is liver transplantation (3). Liver transplantation has become a procedure with a relatively good 5-year survival. Yet, organ donation has not kept up with the demand because of many problems, including relative lack of donors, operative complication, risk of rejection and high cost (4). Furthermore, it is expected that over the next few years there will be a 5- fold increase in the need for liver transplantation. For all these reasons, there is an urgent need to develop alternative strategies for the treatment of advanced liver disease (4).

Owing to the ability of stem cells to repopulate and differentiate at the engrafted site, stem cell-based therapy has received attention as a possible alternative to organ transplantation (5).

Bone marrow is a reservoir of various stem cells including hematopoietic (HSCs) and non-hematopoietic stem cells variously referred to as mesencymal stem cells or marrow stromal cells (MSCs) (6).While MSCs have been shown to be capable of mesodermal and neuro-ectodermal differentiation, they have the potential of endodermal differentiation; their differentiation into functional hepatocyte-like cells has also been demonstrated in vivo (7) and in vitro by continuous exposure to cytokine cocktail (8).

Both HSCs and MSCs have the ability to trans-differentiate to hepatocytes, but MSCs are the most potent component of bone marrow cells in hepatic differentiation. Thus, bone marrow stem cell transplantation, particularly MSC transplantation can be a potential treatment for liver cirrhosis (9).

Aim of work:

The aim of this study was to evaluate the effect of transplantation of autologous bone marrow derived mesenchymal stem cells into cirrhotic patients in improving liver function tests and patient's quality of life as a possible alternative to organ transplantation.

2. Subjects and Methods

Subjects & Methods:

Subjects:

The present study included 12 patients with chronic hepatic failure due to hepatitis C virus infection. According to modified Child Pugh scoring all our patients were Child's C liver cirrhosis, (MELD) score was > 12. They divided into 2 groups according to method of MSCs injection, the 1st group was injected intrasplenic and the 2nd group was injected through the peripheral blood. These patients were selected among cases referred from the medical department in Kasr EL- Aini hospitals; a written informed consent was taken from all patients.

Selection of the patients will be based on
Inclusion Criteria:

- Age 20-70 years
- Chronic hepatic failure due to hepatitis C or hepatitis B virus infection
- Child C liver cirrhosis
- Model of End –Stage Liver Disease (MELD) score > 12

Exclusion criteria:

History of moderate to severe hepatic encephalopathy or variceal bleeding during the last 2 months before enrolment ,presence of hepatic, portal or splenic vein thrombosis on Doppler ultrasonography, history of autoimmune diseases ,presence of active untreated infectious diseases, severe respiratory or cardiac diseases, presence of any types of malignancy, use of hepatotoxic drugs within the last 6 month before enrolment, severe bleeding and life threaten bleeding disorder.

The diagnosis was based on detailed history taking**,** complete clinical examination with special emphasis on abdominal examination**,** laboratory investigations including complete blood picture, Liver function tests, prothrombin time and concentration, alpha fetoprotein, kidney function tests, HCV Ab and HBs Ag using 4th generation ELISA technique, HCV (RNA) by RT-PCR and HBV (DNA) by PCR, radiological investigations including abdominal ultrasonography, doppler and duplex study of portal system.

Methods:

1. *Sampling*:

Fifty ml bone marrow was aspirated from the iliac bone after local anesthesia and placed in sterile tubes contains preservative free heparin.

2. *Mononuclear cells (MNCs) isolation:*

The bone marrow aspirate was diluted with phosphate buffer saline containing 2mM EDTA (PBS/EDTA buffer). MNCs were separated by density gradient centrifugation.

3. *Mesenchymal stem cells (MSCs) separation:*

The MNCs were plated in Dulbecco`s modified Eagle's medium (DMEM) and were cultured at 37˚C in 5% CO_2. After one day, non adherent cells were removed and adherent cells were cultured in presence of mesenchymal media for 3 weeks. After reaching 80% confluence the mesenchymal stem cells (MSCs) were harvested by incubation with trypsin / EDTA and counted on hemocytometer then flowcytometric analysis of surface expression of MSCs using anti CD271 and antiCD34 monoclonal antibodies was done. Finally, in a single session ten million MSCs in 5ml saline were injected percutaneously through the splenic vein under computerized tommograpy guidence.

4. Follow up:

Follow up of patients for 1-6 months by clinical assessment and laboratory work was done.

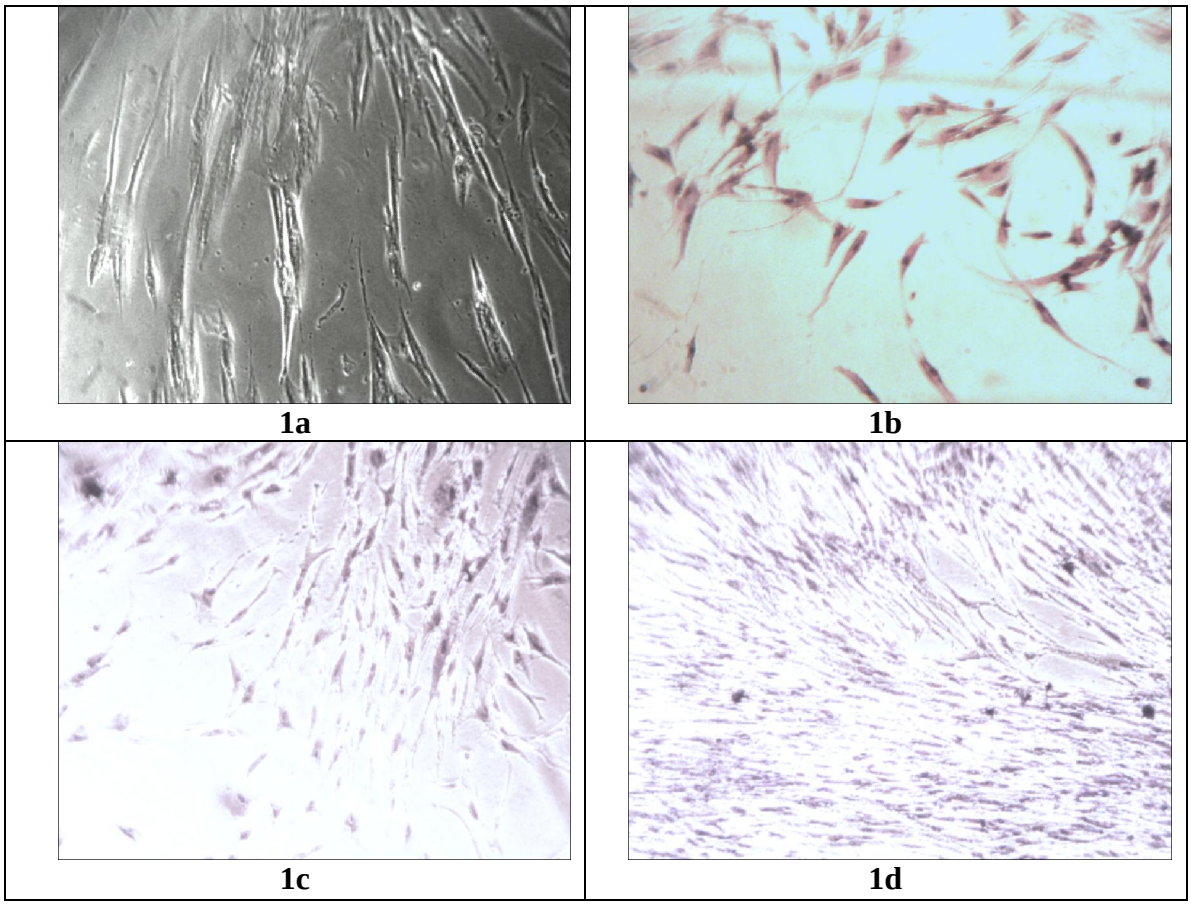


Figure (1): Photography of MSCs culture; 1a unstained MSCs 20% confluence, 1b stained MSCs 20% confluence, 1c stained MSCs 50% confluence and 1d stained MSCs 90% confluence.

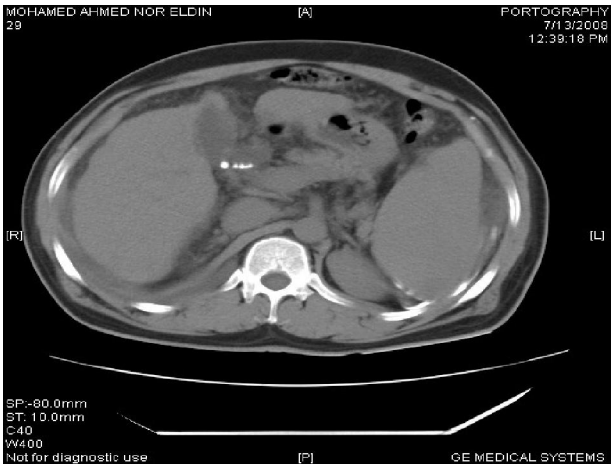


Figure (2): CT photography taken after MSCs injection into the spleen

Statistical analysis:

Quantitative values are expressed as mean ± S.D, and were compared using Student's *t*-test. Qualitative data were compared using Qui Square test. A *p* value <0.05 was considered a significant. A *p* value <0.01 was considered as highly significant. Pearson's correlation coefficients for the different variables were calculated. The SPSS statistical package was used

3- Results:

First group (injected intrasplenic) patient's ages ranged from 32 to 69 years, mean value was 48.50 ±11.09, they were 4 males (67%) and 2 females (33%). Second group (injected through PB) patient's ages ranged from 43 to 59 years, mean value was 50.83 ±6.88, they were 5 males (83%) and 1 female (17%).

- On clinical examination before MSCs injection:

All patients of 1st and 2nd group had clinical evidence of decompensated liver cirrhosis. Three

patients (50%) of 1st group and 2 patients (33%) of 2nd group had a past history of at least one attack of encephalopathy, 5 patients (83%) of 1st group and 4 patients (67%) of 2nd group presented with jaundice, 2 patients (33%) of both groups had a history of hematemesis and/or melena, 5 patients (83%) of 1st group and 4 patients (67%) of 2nd group had lower limb edema during their initial assessment and 3 patients (50%) of both groups had mild to moderate ascitis. Splenomegaly was found in all patients.

- On clinical examination after MSCs injection:

In both groups of patients, only 1 patient (17%) show improvement of encephalopathic manifestation and ascitis, 3 patients (50%) show marvelous decline of jaundice, 2 patients (33%) show improvement of lower limb edema, however non of patients show improvement of bleeding manifestation (Table 1).

Statistical comparison between the 2 groups as regards clinical data before and after MSCs injection: On comparing the 2 groups regarding clinical data before and after MSCs injection there was no statistical significant difference, p value >0.05.

In both groups there was statistically significant difference between total bilirubin, direct bilirubin, MELD score, creatinine level before and after MSCs injection, p value was <0.05, also there was a highly statistically significant difference between CD271 before and after culture, p value was <0.01. However, comparison revealed statistically non significant difference as regards other laboratory data before and after injection, p value was >0.05, table-2 & table-3.

Correlation of CD271 expression after MSCs culture with clinical & fold change of laboratory data of patients before and after injection:

There was no statistical significant correlation between CD271 expression after culture with age, sex, clinical data and fold change of laboratory data before and after culture in both groups.

Statistical comparison between the 2 groups as regards laboratory data before and after MSCs injection:

On comparing the 2 groups regarding laboratory data before and after MSCs injection there was no statistical significant difference, p value >0.05.

Table (1): Clinical data of all patients before and after MSCs injection

Items	**Patients** (No. 12)			
	Group 1 (No. 6)		**Group 2** (No. 6)	
Age (years)				
• **Range**	32-60		43-59	
• **Mean ± SD**	48.50 ± 11.09		50.83 ± 6.88	
Sex				
• **Male (No ;%)**	4 ; 67 %		5; 83%	
• **Female (No ;%)**	2 ; 33 %		1; 17%	
Clinical data	**Before**	**After**	**Before**	**After**
Encephalitis (No ;%)				
• **Present**	3 ; 50 %	2 ; 33 %	2 ; 33 %	1 ; 17 %
• **Absent**	3 ; 50 %	4 ; 67 %	4 ; 67 %	5 ; 83 %
Jaundice (No ;%)				
• **Present**	5; 83 %	2; 33 %	4; 67 %	1; 17%
• **Absent**	1; 17 %	4; 67 %	2; 33 %	5; 83 %
Hematemesis and/or melena (No ;%)				
• **Present**	2; 33 %	2; 33 %	2; 33 %	2; 33 %
• **Absent**	4; 67 %	4; 67 %	4; 67 %	4; 67 %
LL edema (No ;%)				
• **Present**	5 ; 83 %	3 ; 50 %	4 ; 67 %	2 ; 33 %
• **Absent**	1; 17 %	3; 50 %	2; 33 %	4; 67 %
Ascitis (No ;%)				
• **Present**	3; 50 %	2; 33 %	3; 50 %	2; 33 %
• **Absent**	3; 50 %	4; 67 %	3; 50 %	4; 67%

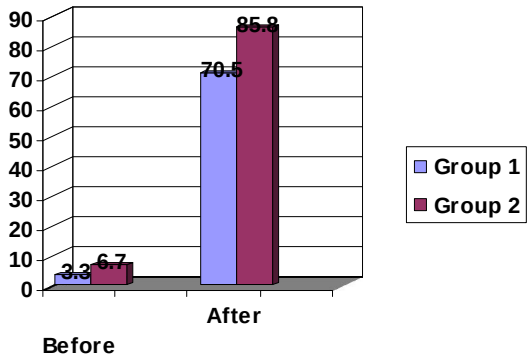


Figure (3): Comparison of mean value of CD271 % before and after MSCs culture in the 2 groups

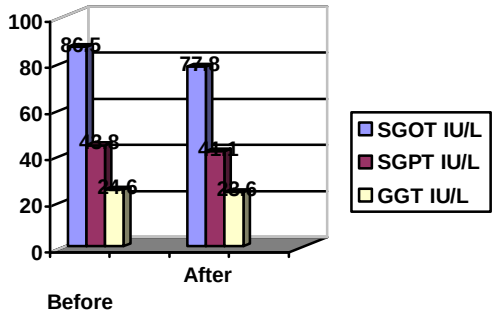


Figure (4): Comparison of mean value of SGOT, SGPT and GGT before and after MSCs injection in group 1

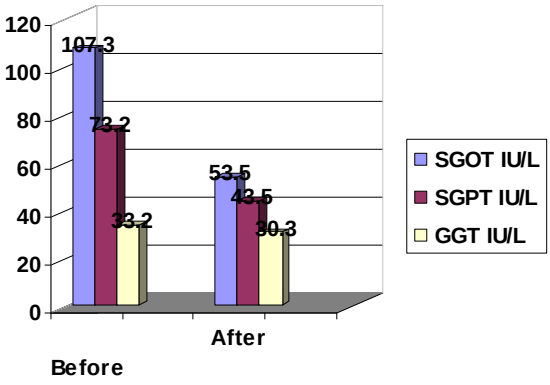


Figure (5): Comparison of mean value of SGOT, SGPT and GGT before and after MSCs injection in group 2

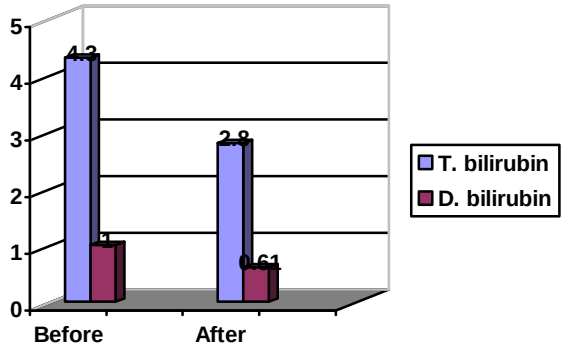


Figure (6): Comparison of mean value of total and direct bilirubin before and after MSCs injection in group 1

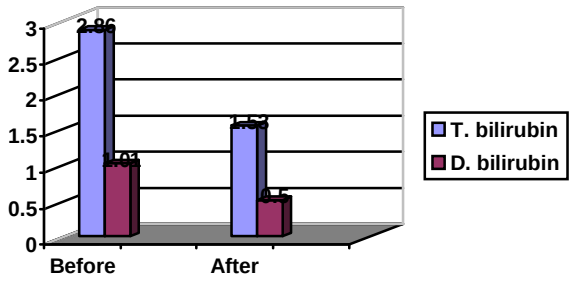


Figure (7): Comparison of mean value of total and direct bilirubin before and after MSCs injection in group 2

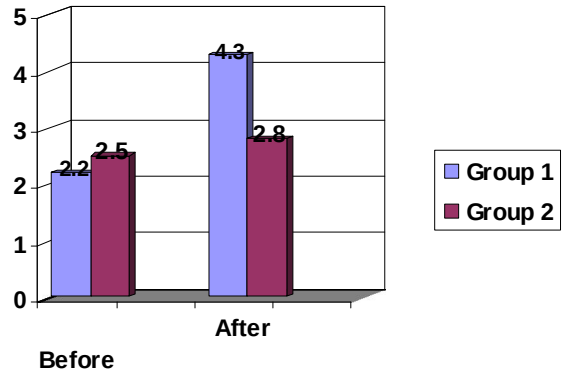


Figure (8): Comparison of mean value of albumin before and after MSCs injection in the 2 groups

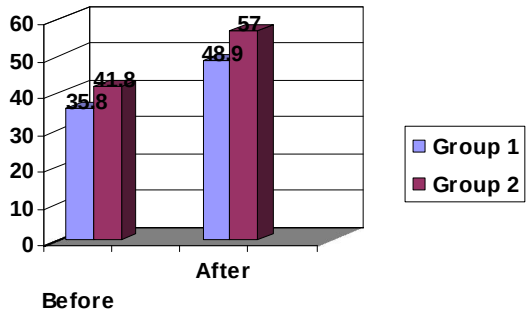


Figure (9): Comparison of mean value of prothrombin concentration before and after MSCs injection in the 2 groups

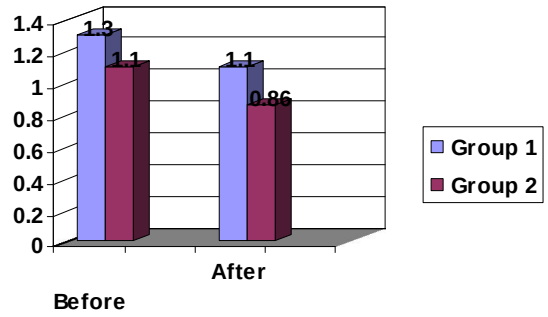


Figure (11): Comparison of mean value of creatinine before and after MSCs injection in the 2 groups

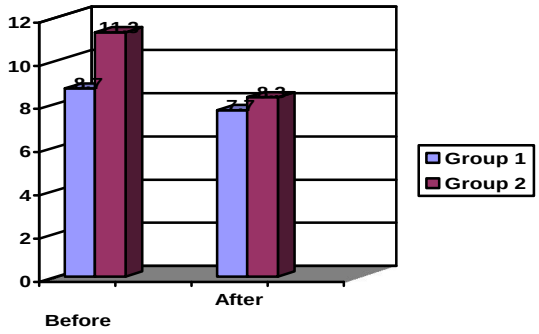


Figure (10): Comparison of mean value of alpha fetoprotein (AFP) before and after MSCs injection in the 2 groups

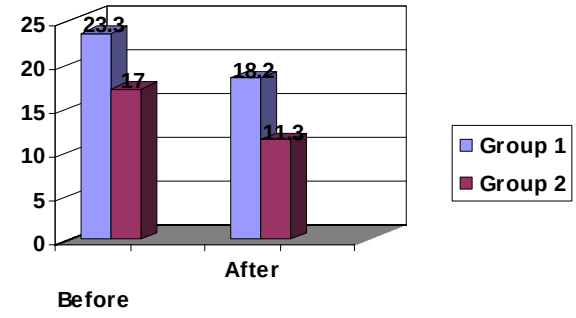


Figure (12): Comparison of mean value of MELD score before and after MSCs injection in the 2 groups

Table (2): Laboratory and Statistical comparison of laboratory data before and after MSCs injection

Items	Patients (No. 12)					
	Group 1 (No. 6)			Group 2 (No. 6)		
	Before	After	P value	Before	After	P value
Hb (g/dl) • **Range** • **Mean ± SD**	8.00- 13.40 10.76 ± 1.83	7.10- 13.90 10.63 ± 2.46	0.873 NS	10.90- 12.30 11.73 ± 0.45	11.00-12.90 11.71 ± 0.76	0.56 NS
TLC (x10³ / cmm) • **Range** • **Mean ± SD**	2.00– 6.20 3.56 ± 1.59	2.50– 7.70 3.76 ± 1.98	0.684 NS	3.00-8.30 5.34 ± 2.10	2.90-8.50 5.00 ± 2.21	0.93 NS
Platelets (x10³ / cmm) • **Range** • **Mean ± SD**	44.00 – 296.00 92.83 ± 99.70	36.00 – 176.00 85.00 ± 50.19	0.769 NS	34.00-179.00 94.16 ± 48.79	48.00-160.00 93.00 ± 44.93	0.96 NS
SGOT (u/L) • **Range** • **Mean ± SD**	12.00- 179.00 77.83 ± 58.12	33.00- 234.00 86.50 ± 78.98	0.811 NS	57.00-157.00 107.33 ± 46.02	37.00-79.00 53.50 ± 16.37	0.02 S
SGPT (u/L) • **Range** • **Mean ± SD**	19.00– 83.00 43.83 ± 23.13	23.00– 77.00 41.16 ± 23.03	0.898 NS	39.00-138.00 73.17 ± 35.80	23.00– 77.00 43.50 ± 11.76	0.08 NS

Alkaline phosphatase (u/L) • **Range** • **Mean ± SD**	52.00– 162.00 89.83 ± 40.48	53.00– 160.00 103.00 ± 38.06	0.243 NS	53.00– 160.00 103.00 ± 38.06	32.00-60.00 103.00 ± 38.06	0.69 NS
Total bilirubin (mg/dl) • **Range** • **Mean ± SD**	2.00- 6.00 4.30 ± 1.73	1.30- 4.30 2.79 ± 1.21	0.02 **S**	1.50-4.00 2.86 ± 0.86	0.90-1.95 1.53 ± 0.39	0.01 **HS**
Direct bilirubin (mg/dl) • **Range** • **Mean ± SD**	0.30- 1.60 1.00 ± 0.46	0.20- 1.00 0.61 ± 0.38	0.04 **S**	0.30-1.50 1.01 ± 0.42	0.20- 0.80 0.50 ± 0.22	0.02 **S**
GGT (u/L) • **Range** • **Mean ± SD**	14.00– 32.00 24.66 ± 6.86	12.00– 39.00 23.66 ± 9.95	0.658 NS	19.00-41.00 33.17 ± 8.73	20.00-40.00 30.33 ± 8.26	0.57 NS
Alphafeto protein (Iu/ml) • **Range** • **Mean ± SD**	2.70– 33.00 8.71 ± 11.98	2.16– 30.00 7.71 ± 10.95	0.167 NS	9.00-15.00 11.33 ± 2.25	5.00-15.00 8.33 ± 3.98	0.13 NS
Albumin (g/dl) • **Range** • **Mean ± SD**	2.10- 2.40 2.20 ± 0.12	1.90- 2.90 2.43 ± 0.39	0.128 NS	2.20-2.80 2.48 ± 0.28	2.4-3.00 2.76 ± 0.28	0.11 NS
Prothrombin conc. (%) • **Range** • **Mean ± SD**	31.90- 43.00 35.81 ± 4.89	36.60- 73.00 48.93 ± 13.06	0.087 NS	22.00-67.00 41.83 ± 16.52	40.00-75.00 57.00 ± 11.30	0.09 NS
MELD score • **Range** • **Mean ± SD**	19.00- 35.00 23.33 ± 5.95	15.00- 27.00 18.16 ± 4.49	0.01 **HS**	13.00-22.00 17.00 ± 3.41	8.00-14.00 11.33 ± 2.16	0.01 **HS**
Creatinine (mg/dl) • **Range** • **Mean ± SD**	0.50- 2.50 1.26 ± 0.73	0.30- 2.30 1.10 ± 0.74	0.01 **HS**	0.90-1.20 1.10 ± 0.11	0.70-1.00 0.86 ± 0.09	0.01 **HS**

Table (3): Statistical comparison of CD271% of all patients before and after MSCs culture

Items	**Patients** (No. 12)					
	Group 1 (No. 6)			**Group 2** (No. 6)		
	Before	**After**	**P value**	**Before**	**After**	**P value**
CD271 (%) • **Range** • **Mean ± SD**	2.00- 5.00 3.33 ± 1.21	46.00- 96.00 70.50 ± 20.83	0.01 **HS**	4.00-9.00 6.67 ± 2.07	83.00-94.00 85.83 ± 5.30	0.01 **HS**

4. Discussion:

End-stage liver disease, and in particular human liver cirrhosis, represents a worldwide health problem. Cirrhosis is the terminal outcome of viral hepatitis (particularly hepatitis C) and alcoholic liver diseases. Egypt has the highest prevalence of Hepatitis C Virus (HCV) in the world, up to 20% in some areas. HCV infection is a major cause of liver cirrhosis in Egypt (2). Currently, liver transplant is the only effective treatment, but it is affected by many problems, including relative lack of donors, operative damage, risk of rejection, and high costs. Stem cell therapy is very attractive in this setting because it has the potential to help tissue regeneration while providing minimally invasive procedures and few complications (4).

The bone marrow (BM) contains at least two populations of stem cells, haematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs), which provide stromal support for HSCs. It also contains many other haematopoietic cell types involved in immune surveillance, inflammatory responses and pathogen removal. It has long been proposed that bone marrow, a known source of stem cells, might be able to contribute to the repair of other organs (10).

Both HSCs and MSCs have the ability to trans-differentiate to hepatocytes, but MSCs are the most potent component of bone marrow cells in hepatic differentiation (9). MSCs are present in low numbers in BM and have a capacity to differentiate into a wide range of mesenchymal tissue types, including cartilage, bone, muscle, stroma, fat, tendon, and other connective tissues. Their differentiation

into functional hepatocyte-like cells has also been demonstrated in vivo (7) and in vitro by continuous exposure to cytokine cocktail (8).This term more recently has been applied to plastic adherent fibroblastic cells that are isolated from the bone marrow and other tissues that show mesenchymal multipotency. Unlike HSC, once isolated, these mesenchymal stromal cells can be grown in culture for many population doublings and now have been shown also to have a much broader potential, including neural differentiation. In some studies, the surface phenotype of an MSC has been investigated. They are negative for markers that include CD34, CD45, and CD14 and positive for CD166, CD105, CD29, and CD44 (11).

Upon liver injury, the typical repair process involves two distinct phases: a regenerative phase, in which injured liver cells are replaced with regenerated hepatocytes; and a phase known as fibroplasias or fibrosis, in which connective tissue replaces normal parenchymal tissue. Although initially beneficial, the repair process becomes pathogenic when it is not controlled appropriately. Extensive accumulation of ECM components can ultimately lead to cirrhosis and liver failure (12). Moreover, fibronectin, a component of ECM, has been proved to promote the MSC-induced cytoprotection following transplant for liver disease (13). The ideal strategy to treat liver injury is to generate new hepatocytes replacing damaged cells without causing excessive ECM deposition.

The first demonstration of the existence of putative liver stem cells in the bone marrow was reported by Petersen et al. (14). They showed that bone marrow cells transplanted into lethally irradiated mice engrafted in the recipient's liver and differentiated into liver stem cells (oval cells) or mature hepatocytes. These in vivo results were confirmed in animal models and in patients who received bone marrow transplantation for hematological disorders (15).

Hepatic stellate cells (HSCs) are the main ECM-producing cells in the injured liver. When a liver injury occurs (e.g., viral hepatitis), HSCs proliferate and undergo a dramatic phenotypical alteration, which is characterized by the acquisition of a proliferative, contractile, migratory, fibrogenic and inflammatory phenotype. Activated HSCs secrete large amount of ECM proteins, including collagen (I, III, and IV), fibronectin, undulin, elastin, laminin, hyaluronan, and proteoglycans (16). The accumulating interstitial ECM constituents that collectively form the hepatic scar replace the low-density type IV collagen with the normal subendothelial space of Disse. These interstitial fibril-forming collagens (especially types I and III collagens) become distributed primarily in the connective septa surrounding the regenerative hepatic nodules. A cirrhotic liver may contain up to six times more collagen and proteoglycan than a healthy organ (17). In addition to the resident HSCs, periportal fibroblasts, bone marrow-derived fibrogenic cells, epithelial-mesenchymal transition, and possibly circulating fibrocytes can contribute to the fibrogenesis in the liver. MSC have a significant impact on hepatic fibrogenesis through their ability of inhibiting activated HSC and re-regulating the fibrogenic process. The interventions of mesenchymal stem cells (MSC) include: (1) inhibit HSC proliferation; (2) stimulate HSC apoptosis; (3) inhibit ECM accumulation; (4) stimulate endogenous hepatocyte regeneration; and (5) hepatocyte-like differentiation (18).

MSC paracrine-mediated hepatic regeneration from endogenous liver stem cells may also contribute to the hepatocyte replication and recovery of hepatic function (19).

The aim of this study was to evaluate the effect of transplantation of autologous bone marrow derived mesenchymal stem cells into cirrhotic patients in improving liver function tests and patient's quality of life as a possible alternative to organ transplantation. The present study included 12 patients with chronic hepatic failure due to hepatitis C virus infection. The degree of hepatic affection was determined according to modified Child Pugh scoring. All our patients were Child's C liver cirrhosis, MELD score was > 12. They divided into 2 groups according to method of MSCs injection, the 1st group was injected intrasplenic and the 2nd group was injected through the peripheral blood. These patients were selected among cases referred from department of Internal medicine in Kasr EL Eini hospitals in Cairo University; a written informed consent was taken from patients. Bone marrow was aspirated from the iliac bone for separation of MSCs then 10 million MSCs in 5 ml saline were infused intrasplenic or peripherally in one session.

After 1-6 months follow up of patient's we observed that 1 patient (17%) show improvement of encephalopathic manifestation and ascitis, 3 patients (50%) show marvelous decline of jaundice, 2 patients (33%) show improvement of lower limb edema, however non of patients show improvement of bleeding manifestation. Partial improvement of liver function tests with decline of elevated bilirubin and liver enzymes and elevation of prothrombin concentration and serum albumin levels was noticed in both groups. There was statistical significant difference between total bilirubin, direct bilirubin, MELD score, creatinine level before and after injection in both groups, p value <0.05. Also there

was highly statistical significant difference between CD271 before and after culture, p value <0.01. However, comparison revealed no statistical significant difference as regards other laboratory data before and after injection of our patients following laboratory culturing of MSC, p value >0.05. On comparing the 2 groups regarding laboratory data before and after MSCs injection there was no statistical significant difference, p value >0.05 that might be explained by small number of patients, so large scale population study is required. There are only a handful of clinical trials in the field of regenerative cell therapy specifically in the field of hepatology, all of which are small-scale, uncontrolled safety and feasibility studies.

Our results were more or less in consistent with Terai et al. (20) who implemented a clinical trial on nine patients with decompensated liver cirrhosis. These patients were infused with $5.2 \pm 0.63 \times 109$ autologous bone marrow cells into the peripheral vein. At 24 weeks after transplantation, significant improvements were observed. These improvements included total protein, serum albumin, Child-Pugh scores, and α-Fetoprotein and proliferating cell nuclear antigen expression in liver biopsy tissues.

Also Gordon et al. (21) evaluated the effects of CD34+ hematopoietic stem cell intrahepatic injection and whole bone marrow peripheral infusion in five patients with liver cirrhosis. Their results showed a decrease in serum bilirubin and an improvement in serum albumin in three and four of the five patients, respectively, with the disappearance of ascites observed in one patient. They concluded that bone marrow stem cells are able to improve the residual liver function in cirrhotic patients.

More recently, Mohamadnejad et al. (22) performed two small scaled clinical studies, in their first trial, four patients with decompensated liver cirrhosis were infused 31.73×106 (mean) MSCs through a peripheral vein. At the end of follow-up (after 12 months), MELD scores of two patients improved by four points and by three points. The mean physical and mental component scales were more than doubled by the end of follow-up. Computed tomography (CT) showed the increase of liver volumes of three patients by the sixth month. However, the results of their second trial were not satisfactory. Four patients received 5.25×106 (mean) autologous bone marrow-hematopoietic stem cells infused through hepatic artery. Only marginal improvements were observed in some patients. The results of their MSC transplantation were more promising than the study of hematopoietic stem cell transplantation. They also indicated that hepatic artery delivery of stem cells was not a safe procedure. Because of the lack of reliable means of identifying transplanted stem cells in the human body, they recommended caution during the evaluation of the clinical outcomes.

Kuo et al. (23), Xu and Liu (24) and Dai et al. (25) described some parameters governing the success of using MSCs and characteristics of various delivery approaches in their recent papers. We can conclude from our results and others that MSCs are the most potent component of bone marrow cells in hepatic differentiation thus, bone marrow stem cell transplantation, particularly MSC transplantation can be a potential treatment for liver cirrhosis. Also from our results there was no difference in clinical and laboratory improvement regarding the route of administration. However, the dose, frequency and route of administration of this treatment are still to be defined.

Corresponding author
Abdel-Aziz I
Department of Haematology (Theodor Bilharz Research Institute), Cairo, Egypt.
iman_khaled@yahoo.com

5. References

1. Iredale JP (2003): Cirrhosis: new research provides a basis for rational and targeted treatments, BMJ; 327, pp. 143-7.
2. Darwish MA, Faris R, Darwish N, Shouman A, Gadallah M, El-Sharkawy MS, Edelman R, Grumbach K, Rao MR, Clemens JD (2001): Hepatitis C and cirrhotic liver disease in the Nile delta of Egypt: a community-based study.Am J Trop Med Hyg; 64, pp. 147-53.
3. Lorenzini S and Andreone P (2007): Stem cell therapy for human liver cirrhosis:a caution analysis of the results, Stem cell; 25(9), pp. 2383-4.
4. Forbes JS. (2008): Stem cell therapy for liver disease-choosing the right tools for the job. Gut; 57, pp. 153-155
5. Yagi K, Kojima M, Oyagi S, Ikeda E, Hirose M, Isoda K, Kawase M, Kondoh M, Ohgushi H (2008): Application of mesenchymal stem cells to liver regenerative medicine, Yakugaku Zasshi;128(1), pp. 3-9.
6. Abdel Aziz MT, Atta HM, Mahfouz S, Fouad HH, Roshdy NK, Ahmed HH, Rashad LA, Sabry D, Hassouna AA, Hasan NM (2007): Therapeutic potential of bone marrow derive mesenchymal stem cells on experimental liver fibrosis, Clinical Biochemistry; 40, pp. 893-899.
7. Fang B, Shi M, Liao L,Yang S, Liu Y, Zhao RC (2004): Systemic infusion of FLK1 mesenchymal stem cells ameliorates carbon

tetrachloride-induced liver fibrosis in mice,Transplantation; 78, pp. 83-8.
8. Sarah Synkers, Tamora Vanhaecke, Peggy Papeleu, Aernout Luttun, Yuehuga Jiang (2006): Sequential Exposure to Cytokines Reflecting Embryogenesis: The Key for in vitro Differentiation of Adult Bone Marrow Stem Cells into Functional Hepatocyte-like Cells, Toxicological Sciences; 94 (2), pp. 330-341.
9. Sato Y, Araki H, Kato J, Nakamura K, Kawano Y, Kobune M, et al (2005): Human mesenchymal stem cells xenografted directly to rat liver are differentiated into human hepatocytes without fusion, Blood; 106, pp. 756 – 763.
10. Kale S. et al. (2003): Bone marrow stem cells contribute to repair of the ischemically injured renal tubule, J. Clin. Invest. 112, pp. 42–49.
11. Yokoo T. et al. (2005): Human mesenchymal stem cells in rodent whole-embryo culture are reprogrammed to contribute to kidney tissues, Proc Natl Acad Sci U S A 102; 3296–3300.
12. Wynn TA. (2008): Cellular and molecular mechanisms of fibrosis, J. Pathol. 214, pp. 199–210.
13. Kao CL, Lin HT, Chen YW, Chen YC, Tsai FT, Chang YL, Chiou SH, Sheu DC, & Ku HH. (2007): Fibronectin suppresses lipopolysaccharide-induced liver damage and promotes the cytoprotection abilities of hepatocyte-like cells derived from human bone marrow mesenchymal stem cells, Transplant Proce. 39, pp. 3444–3445.
14. Petersen BE, Bowen WC, Patrene KD, Mars WM, Sullivan AK, Murase N, Boggs SS, Greenberger JS, and Goff JP. (1999): Bone marrow as potential source of hepatic oval cells, Science 284, pp. 1168–1170.
15. Alison MR, Poulsom R, Jeffery R, Dhillon AP, Quaglia, Jacob J, Novelli M, Prentice G, Williamson J. and Wright NA. (2000): Hepatocytes from non-hepatc adult stem cells, Nature 406, pp. 257.
16. Bataller R, and Brenner D. (2005): Liver fibrosis, J. Clin. Invest. 115, pp. 209–218.
17. Friedman SL. (2008): Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver, Physiol. Rev. 88, pp. 125–172.
18. Zhang B., Inagaki M., Jiang B., Miyakoski M., Arikura J., Ogawa K. & Kasai Sh. (2009): Effects of BM and hepatocyte transplantation on liver injury, Journal of surgical research, Article on press.
19. Parekkadan B, Poll D V, Suganuma K, Carter EA, Berthiaume F, Tilles AW, and Yarmush ML. (2007): Mesenchymal stem cell-derived molecules reverse fulmimant hepatic failure, PLoS One 2 (9), p. 941.
20. Terai S, Ishikawa T, Omori K, Aoyama K, Marumoto Y, Urata Y, Yokoyama Y, Uchida K, Yamasaki T, Fujii Y, Okita K, and Sakaida I. (2006): Improved liver function in patients with liver cirrhosis after autologous bone marrow cell infusion therapy, Stem Cells 24, pp. 2292–2298.
21. Gordon MY, Levicar N, Pai M et al. (2006): Characterization and clinical application of human CD34+ stem/progenitor cell populations mobilized into the blood by granulocyte colony-stimulating factor. STEM CELLS; 24:1822–1830.
22. Mohamadnejad M, Namiri M, Bagheri M, Hashemi SM, Ghanaati H, Zare Mehrjardi N, Kazemi S, Malekzadeh R, and Baharvand H. (2007): Phase 1 human trial of autologous bone marrow-hematopoietic stem cell transplantation in patients with decompensated cirrhosis, World J. Gastroenterol. 13, pp. 3359–3363.
23. Kuo TK, Hung SP, Chuang CH, Chen CT, Shih YR, Fang SC, Yang VW, and Lee OK. (2008): Stem cell therapy for liver disease: parameters governing the success of using bone marrow mesenchymal stem cells, Gastroenterology 134, pp. 2111–2121.
24. Xu YQ, and Liu ZC. (2008): Therapeutic potential of adult bone marrow stem cells in liver disease and delivery approaches, Stem. Cell Rev. 4, pp. 101–112.
25. Dai LG, Li HY, Guan LX, Ritchie and Jeff X. Zhou JX. (2009): The therapeutic potential of bone marrow-derived mesenchymal stem cells on hepatic cirrhosis. Stem Cell Res. 2 (1), pp. 16-25.

6/8/2010

Role of Hepcidin in Anemia of Chronic Hepatitis C Patients

Salwa Toima[1], Abeya Saleh[*1], Mona Madkour[1], Olfat Hammam[2] and Emad EL-Din Baioumi[3]

[1]Hematology Department, [2]Pathology Department, [3]Hepatology and Gastroenterology Department, Theodor Bilharz Research Institute, Cairo, Egypt.
dr.abeyasaleh@hotmail.com

Abstract: This study was done to clarify the role of hepcidin in the regulation of iron homeostasis and development of anemia in chronic hepatitis C (CHC) patients targeting the differentiation of the type of anemia. Patients and methods: This study was conducted on 70 CHC patients. Iron profile and soluble transferrin receptor (sTfR) were measured. Transferrin saturation and transferrin receptor ferritin (TfR-F) Index were calculated. Serum prohepcidine hormone and IL6 levels were measured (ELISA). Histopathological examination and immunohistochemical detection of hepcidin were done. According to the iron profile patients were reclassified into iron deficiency anemia (IDA) group, anemia of chronic disease group (ACD) and combined anemia group (COMBI). Results: 64.3% of patients were of the COMBI group, 10% had ACD and 25.7% had IDA. Hepcidin was increased in Child C group ($P<0.05$). Hepatic expression of hepcidin showed reduced expression in Child A, B and C groups. Hepcidin level was found to be increased in ACD and COMBI group in comparison to control and IDA group. Stepwise logistic regression demonstrated that sTfR was the most predictive parameter for IDA while hepcidin was the most predictive parameter for ACD and COMBI in CHC patients. Conclusion: hepcidin plays an important role in the pathogenesis of anemia in CHC patients. The role of hepcidin in discriminating different types of anemia in CLD is comparable to that of sTfR/logFn index. An appropriate combination of both tests provides evidence for iron depletion or reflects excessive production of hepcidin which will help to establish a correct diagnosis of IDA, ACD or combined anemia in patients with CHC. [Journal of American Science. 2010;6(12):145-154]. (ISSN: 1545-1003).

Key words: Hepcidin, CHC, IDA, ACD, Anemia

1. Introduction

The prevalence of hepatitis C virus (HCV) infection varies throughout the world, with the highest number of infections reported in Egypt [1]. Anemia of diverse etiology occurs in about 75% of chronic liver disease (CLD) patients and this frequent association provides a rationale for examining the contribution of the liver in the development of anemia in those patients [2].

Anemia of chronic illness is frequent among patients with inflammatory disease without apparent blood loss e.g. rheumatoid arthritis, renal failure or chronic hepatitis [3].

Increased iron requirements, limited external supply, and increased blood loss may lead to iron deficiency (ID) and iron-deficiency anemia (IDA). In chronic inflammation, there is hypoferremia and iron-restricted erythropoiesis, despite normal iron stores (functional ID). Anemia of chronic disease (ACD) can evolve into combined ACD and true ID (COMBI anemia) [4].

The liver performs three main functions in iron homeostasis. It is the major site of iron storage, it regulates iron traffic into and around the body through its production of the peptide hepcidin, and it is the site of synthesis of major proteins of iron metabolism such as transferrin and ceruloplasmin [5].

At the core of iron homeostasis is hepcidin, a small acute phase anti-microbial peptide that now appears to synchronously orchestrate the response of iron transporter, ferroportin and regulatory genes [6]. Liver hepcidin is regulated by a number of factors such as iron overload, inflammation, hypoxia and anemia [7].

The effects of inflammation on hepcidin levels are best understood and are mediated at least in part by interleukin 6 (IL6) [8]. Thus, in chronic inflammation, the excess of hepcidin decreases iron absorption and prevents iron recycling [9].

Laboratory tests provide evidence of iron depletion in the body, or reflect iron-deficient red cell production. The appropriate combination of these laboratory tests help to establish a correct diagnosis of ID status and anemia [3].

Measurement of serum ferritin is currently the accepted laboratory test for diagnosing iron deficiency, and a ferritin value of 12 µg/l is a highly specific indicator of iron deficiency. Other commonly used laboratory tests such as serum iron, total iron-binding capacity, mean corpuscular volume, and transferrin saturation provide little additional diagnostic value over ferritin [10]. However, serum ferritin levels are increased in several pathological conditions associated with chronic inflammatory conditions.

The transferrin receptor (TfR) mediates cellular uptake of iron by binding the iron carrier-protein

transferrin (Tf). The soluble form of TfR in serum is used as a surrogate marker of bone marrow erythropoiesis and red cell production [11]. Because serum ferritin reflects the storage iron compartment and sTfR reflects the functional iron compartment, these two values can be combined into a ratio, which is reciprocally regulated. The special value of the sTfR/log ferritin index (TfR-F Index) in the differential diagnosis of IDA has already been documented [12].

The aim of this study was to clarify the role of hepcidin (serum and tissue) in the regulation of iron homeostasis and development of anemia in patients with CHC targeting the differentiation of the type of anemia (ID, ACD or combined) in those patients. We also aimed at investigating the iron profile and tissue iron score and its relevance to the diagnosis of anemia in CLD patients with different clinical stages in an attempt to choose the best diagnostic markers for type of anemia.

2. Patients and Methods

This study was conducted on 70 CLD patients admitted to Hepatology Department Theodor Bilharz Research Institute in addition to 20 healthy controls who had normal biochemical tests, CBC, liver function tests and were negative for hepatic viral infection both by ELISA and by polymerase chain reaction (PCR) in addition to normal ultrasonograph configuration of the liver Patients were enrolled in this study if they had CLD as evidenced by 2-3 folds increased liver function tests for more than 6 months and the etiology of CLD was chronic hepatitis C (CHC) virus as diagnosed by HCV antibody detection in serum (ELISA) and PCR viral detection both quantitative and qualitative tests. Patients were excluded if they had hepatitis B or schistosomiasis co-infection or history of hematemesis or melena in the last 21 days. None of the patients received blood or blood components transfusion therapy in the past 21 days. Patients were classified according to modified Child Pugh classification (1973) [13] into child A (25 patients), Child B (17 patients) and Child C (28 patients).

After taking their written consent according to Helsinki guidelines of ethical research, Samples from all patients and controls were withdrawn in suitable vacutainers and subjected to liver function tests (AST, ALT, Albumin, Bilirubin and Prothrombin time), complete blood count (CBC) including Hb concentration, MCV, MCH, MCHC and RDW using hematology analyzer Celtac-MEK 8118 (Nihon Kohden, Japan), HCV antibody by ELISA and PCR detection of HCV both qualitative and quantitative. Liver biopsies of which pathological examination, tissue iron quantification and immunohistochemical detection of prohepcidine expression using monoclonal antibody was done. The same procedures were done on liver biopsies taken from patients during cholecystectomy operation after taking their written consents. Those acted as control for immunohistochemistry. Biopsies from controls were negative for hepatitis markers both B and C and didn't show any evidence of inflammation.

Iron profile (serum iron, TIBC, serum ferritin and transferrin) was measured by ELISA technique using commercially available kits (Bayer Diagnostics, U.K.) Transferrin saturation was calculated as serum iron (µg/dl) x 70.9/ serum transferrin (mg/dl). sTfR receptor (sTfR) level was measured by ELISA using Quantikin human sTfR kit (R&D systems Inc, USA). Calculation of the ratio TfR/log ferritin (TfR-F Index) was used as a determinant of body iron stores. If the Index was equal or more than 2, the patients were considered iron deficient. Patients were considered iron replete if Index was less than or equal to1 [14].

Serum prohepcidine hormone levels were measured by using DRG hepcidin prohormone ELISA kit (DRG, Germany). IL6 was evaluated by ELISA using Quantikine IL-6 (Quantikine IVD, minipolos, USA) test kits. Blood samples were collected from all patients at the same time during routine sampling to avoid diurnal variation of hepcidin.

According to the results of the iron profile and markers of inflammation, patients were regrouped into 3 groups; group 1: patients with iron deficiency anemia (IDA, n=18). Criteria of inclusion in this group were: low Hb (male<13 g/dl and female <12 g/dl), TSAT (<20%) and ferritin concentrations (<30 ng/ml). Group 2: patients with anemia of chronic disease (ACD, n=7). Criteria of inclusion in this group were: evidence of chronic inflammation (high CRP level>1mg/dl), Hb concentration <13 g/dl for male and<12 g/dl for female and low TSAT <20% but normal or increased serum ferritin concentration (>100 ng/ml) or low serum ferritin concentration (30-100 ng/dl) and sTfR/ log ferritin ratio< 1. Group 3 was compromised of patients who had combined ID and ACD (COMBI, n=45). Criteria of inclusion were: chronic inflammation (high CRP level >1mg/dl), hemoglobin concentration < 13 g/dL for male and < 12 g/dL for female, low TSAT < 20%, serum ferritin concentration > 30 and < 100 ng/mL, and a sTfR/log ferritin ratio > 2 [4].

Histopathological examination and tissue Iron evaluation:

Histological sections were processed and stained with hematoxylin and eosin (H&E) and Masson trichrome was used to examine the histopathological changes. Perls Prussian blue staining for iron was performed. Liver specimens were scored for grade of inflammatory activity according to the classification of Desmet et al. [15] The histological quantification of

iron was done according to Deugnier et al. [16] by scoring iron separately within hepatocytes (hepatic iron score (HIS) 0 to 36), sinusoidal cells (sinusoidal iron score (SIS) 0 to 12), and portal tracts or fibrotic tissue (portal iron score (PIS) 0 to 12). The total iron score (TIS) 0 to 60, was the sum of these scores.

Immunohistochemical procedure:

The standard avidin-biotin immunoperoxidase technique was used [17]. Hepcidin was detected using a primary antibody against hepcidin (Santa Cruz Biotechnology, Santa Cruz, CA., USA). The number of positively stained cells with positive intracytoplasmic brown stain was recorded within ten successive fields (X400), the final value represented 10 readings per case. Zero percentage was given to unstained sections. Hepcidin expression sites were examined in hepatocytes, intra-lobularly and in peri-portal areas.

Statistical methods:

All data are reported as mean +/- SD. The data were analyzed by computer using the statistical package SPSS for windows version 11 (software). Comparing means was performed by one-way ANOVA Post HOC LSD test. Correlations between different parameters were determined by bivariate Pearson correlation test. The linear relationship between variables was assessed by linear regression analysis. To determine the best predictors of ID, ACD and COMBI anemia multivariate analysis was done using stepwise logistic regression and odd ratios were estimate for each of the independent variables in the model. For all tests P values <0.05 were considered statistically significant.

3. Results:

The results of the biochemical tests of the control and patient groups are shown in table 1

The hematological results of the control and patients groups are shown in table 2.

Results of the iron profile (serum iron, TIBC, transferrin saturation and serum ferritin), sTfR, sTfR/Fn index, prohepcidin hormone, IL6, tissue expression of hepcidin, tissue iron scores and liver histology in the control, Child A, Child B and Child C groups are shown in Table 3.

Histopathological grading of inflammation and hepcidin detection:

In Child A, all cases were with mild inflammatory activity. All Child B cases showed moderate inflammatory activity and all 28 cases in Child C expressed severe activity.

Tissue expression of hepcidin was mainly in hepatocytes, expressed as cytoplasmic brownish stain (figures 1, 2 and 3). Figure 4 shows iron stain in a Child C patient.

Correlation studies:

In Child A group, there was a negative correlation between hepcidin and sTfR (r=-0.52) and a positive correlation between hepcidin and Fn (r= 0.059). Negative correlations were found between Fn index and hepcidin (r=-0.58) and between sTfR and Fn (r=-0.57) in Child A. In Child B group, there were two positive correlations between hepcidin and each of IL6 (r=0.54) and ferritin (r=0.49) while IL6 revealed a negative correlation with sTfR (r=-0.66) and a positive correlation with ferritin (r=0.93) in the same group. Correlation studies also showed an inverse correlation between sTfR and ferritin (r=-0.71) and between ferritin index and IL6 (r=-0.76) in Child B group. In Child C group there was a positive correlation between ferritin index and IL6 (r=0.39). The results of IDA group, ACD group and COMBI group are shown in table 4.

In Child A group, 10/25 (40%) of patients were categorized under IDA group, 5/25 (20%) under ACD group while 10/25 (40 %) were of the COMBI group. In Child B group 8/17 (47%) were found to have IDA, 2/17 (11.7%) were found to be in the ACD group while 7/17 (41.1%) were found to be of the COMBI group. As regard Child C group, all 28 patients (100%) were found to have combined IDA and ACD. In all diseased groups 18/70 (25.7%) had IDA, 7/70 (10%) had ACD and 45/70 (64.3%) had combined anemia.

Correlation studies revealed a negative correlation between hepcidin and sFe (r=-0.83) in ACD group. In COMBI group, positive correlations were expressed between IL6 and ferritin (r=0.37), Fn index and hepcidin (r=0.36) while negative correlations were noted between hepcidin and TfR (r=-0.46), IL6 and serum Fe (r=-0.32), Fn index and serum Fe (r=-0.42).

Univariate regression analysis comparing variables other than those used for classification of IDA, ACD and COMBI anemia revealed that sTfR (P<0.001) and ferritin (P<0.001) were the most predictive parameters for IDA, while hepcidin was the most predictive parameter for both ACD (P<0.05) and COMBI group (P<0.001). Stepwise multivariate logistic regression analysis demonstrated that sTfR was the most predictive parameter for IDA anemia while hepcidin was the most predictive parameters for ACD and COMBI in CLD patients. Odd ratios (OR) estimated for sTfR and hepcidin were considered significant. The 95% confidence interval (CI) for OR was 1.6 [lower bound (L) 1.2 and upper bound (U) 1.84] and 0.003 (Lower 0.0001and Upper 0.68) respectively with P<0.0014 and 0.043 respectively. This was confirmed by stepwise discriminate analysis (p<0.0001 for both parameters).

Table 1: Results of the biochemical tests of the control and patient groups.

	Control (n=20)	Child A (n=25)	Child B (n=17)	Child C (n=28)
Albumin gm/dl	4.08±0.32	4.48±. 35	3.43±0.48	2.53±. 76
Prothrombin concentration%	90.04±11.37	84.4±15.7	73.18±9.6	51.14±9.63*
Total bilirubin mg/dl	1.17±. 67	1.46±1.02	2.86±1.54	4.78±2.49
Direct bilirubin mg/dl	1.06±. 3	1.05±1.02	1.981+0.7	2.75±1.78
Alkaline phosphatase u/L	227.6±120.22	227.08±82.93	223.12±109.23	287.57±42.45*°
ALT u/L	97.8±61.34	101.72±60.79	112.94±65.75	134.96±31.02*°◊
AST u/L	105.6±99.9	118.96±42.35	129.88±80.45	142.78±39.3*°◊
Alpha feto protein	0.78±0.26	0.93±0.58	2.66±0.46	11.6±1.5
PCR IU	_	150 000±5000	300 000±100 000	600 000±150 000
Gamma GT	120±74.75	162±72.4	117.29±57.23	139.7±83.34
CRP mg/dl	0.2±0.7	2.3±0.6	4.7±1.3	12.4±1.5

* Statistical significant difference compared to control group (P<0.05).
° Statistical significant difference compared to Child A group (P<0.05).
◊ Statistical significant difference compared to Child B group (P<0.05).

Table 2: Hematological results of the control and patients groups.

	Control (n=20)	Child A (n=25)	Child B (n=17)	Child C (n=28)
WBC (×103/L)	8.6±0.75	6.25±0.59*	6.48±1.02*	5.41±0.54*°◊
RBC (× 1012/L)	5.2±0.24	4.9±0.4*	4.5±0.54*	3.93±0.38*°◊
Hb g/dl	13.8±0.27	11.69±0.37*	10.61±0.42*°	8.74±0.41*°◊
RDW %	15± 0.34	18±0.98*	19±0.67*	19±1.12*
MCV (fl)	86.45±3.49	76.4±3.7*	72.42±2.78*	67.76±3.95*°◊
MCH pg/L	28.53±1.23	25.67±1.24*	23.26±1.25*°	21.54±2.12*°◊
MCHC g/dl	34.14±1.1	33.02±1.2	30.23±1.53*	29.98±0.85*°
Platelet (×103/L)	200.2±8.28	186.16±21.11*	150±2.9±25.39*°	100.5±33.18*°◊

* Statistical significant difference compared to control group (P<0.05).
° Statistical significant difference compared to Child A group (P<0.05).
◊ Statistical significant difference compared to Child B group (P<0.05).

Table 3: Results of the iron profile, sTfR, sTfR/Fn index, prohepcidin hormone, IL6, tissue expression of hepcidin, tissue iron scores and liver histology in the control and diseased groups.

	Control(n=20)	Child A (n=25)	Child B (n=17)	Child C (n= 28)
Serum iron ug/dl	94.78±15.59	82.65±13.74*	78.84±13.3*	70.62±13.98*°◊
TIBC	345±69.96	355.44±56.56*	409.18±63.26*°	430.18±64.53*°
TSAT%	37.28±1.45	19.12±2.32*	15.24±3.13*	11.58±1.71*
s ferritin ng/ml(Fn)	133.07±21.9	128.82±105.17	104.78±94.98	82.64±19.52*
sTfR nmol/L	3.2±1.03	4.04±1.25*	3.95±1.43*	4.6±1.18*
sTfR/log Fn index	1.53	2.14*	0.95*	2.36*
Serum prohepcidin	76.88±11.45	74.91±19.95	81.15±23.17	91.88±9.05*°
IL6 (pg/mL)	9.28±1.27	9.91±1.38	11.75±4.97	13.49±2.2*°◊
Tissue expression of hepcidin	0.0	70.9±9.9*	57.06±8.9*°	26.32±8.2*°◊
Tissue iron score	0.0	2.4±03*	5.2±2.2*°	8.4±3.2*°◊
Inflammatory activity	10/0/0/0	0/25/0/0	0/0/17/0	0/0/0/28

*Statistically significant difference compared to Control group (P<0.05).
° Statistically significant difference compared to Child A group (P<0.05)
◊ Statistically significant difference compared to Child B group (P<0.05).
Intensity of necro-inflammatory lesions: 0, no histological activity; 1, mild activity; 2, moderate activity; 3, severe activity.

Table 4: The results of control, IDA, ACD and COMBI groups

	Control (n=20)	IDA (n=18)	ACD (n=7)	COMBI (n=45)
CRP mg/dl	0.20±1.4	0.3±0.12	0.5±0.4	2.5±1.2*°◊
Hb g/dl	13.8±0.27	11.0±2.3*	8.5±2.1*°	10.8±1.5*◊
sFe µg/dl	101.58±42.06	79.1±11.47*	72.71±9.23*	81.16±17.67*
Fn ng/ml	118.8±83.19	63.89±11.38*	296.31±78.01*°	79.77±12.24*◊
Ferritin index	0.9±5.5	2.51±0.6*	0.95±0.3°	2.43±0.59*◊
sTfR mg/L	3.5±1.03	4.4±0.95*	2.3±0.74*°	4.63±1.08*
prohepcidin(ng/mL)	77.08±14.37	55.23±11.65*	97.44±7.88*°	91.1±8.5*°
IL-6 (pg/mL)	9.16±3.28	9.27±1.6	13.74±*	10.25±1.6°◊

*Statistically significant difference compared to Control group (P<0.05).
° Statistically significant difference compared to Child A group (P<0.05)
◊ Statistically significant difference compared to Child B group (P<0.05).

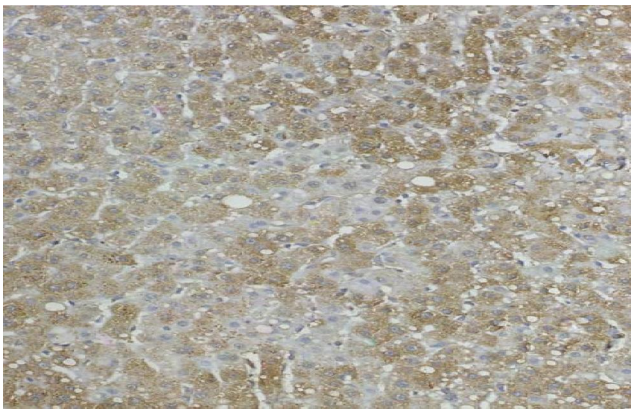


Figure 1: A case of HCV, Child A, showing expression of Hepcidin in about 60% of hepatocytes as brownish cytoplasmic colour (IHC, DABx200) (20mcm).

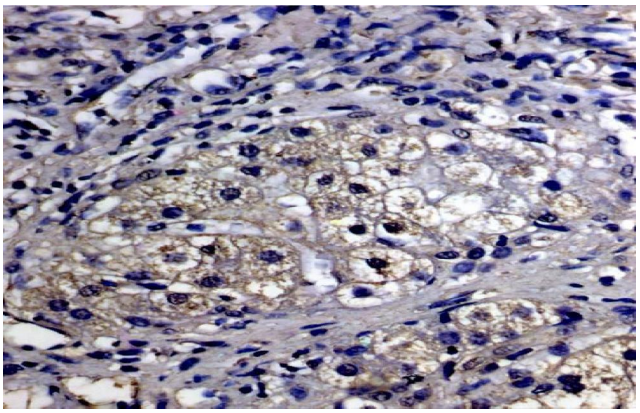


Figure 3: A case of HCV, Child C, showing cirrhotic nodule, expressing Hepcidin in about 40% of hepatocytes as brownish cytoplasmic color (IHC, DABx400) (50mcm).

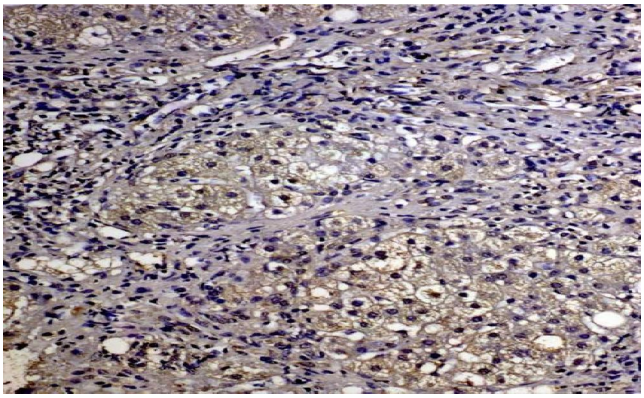


Figure 2: A case of HCV, Child C showing cirrhotic nodule expressing Hepcidin in about 40% of hepatocytes as brownish cytoplasmic color (IHC, DABx200) (20mcm).

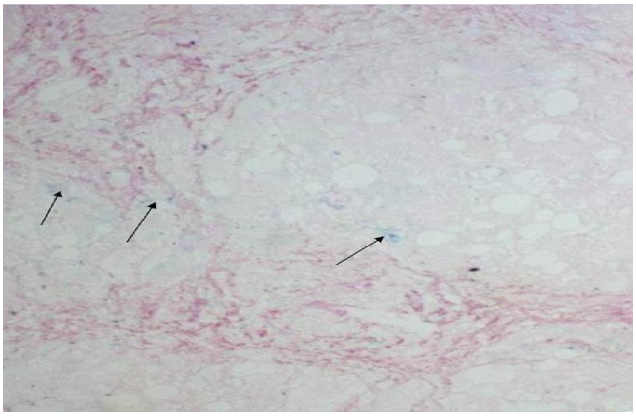


Figure 4: A case of HCV, Child C, showing some hepatocytes revealed positive expression of iron as bluish intracytoplasmic granules (Perl's iron stain, x100) (10mcm).

4. Discussion:

Hepatitis C virus (HCV) infection is a major cause of liver diseases [18] in which anemia is a prevalent feature. Hepcidin is the central regulator of systemic iron homeostasis. Hepcidin deficiency is the cause of iron overload in hepatitis C while hepcidin excess is associated with anemia of inflammation and iron-refractory iron deficiency anemia [19].

According to the Hb and RBCs indices, patients had microcytic hypochromic anemia with anisocytosis in all diseased groups. Since active bleeding was excluded in these patients, anemia could be attributed to iron deficiency either functional (FID) or true (TID). True iron deficiency in CLD could be due to increased requirements or diminished supplies. This may result from hidden blood loss or low-grade gastrointestinal bleeding which also may be compounded by decreased oral iron absorption because of dietary restrictions. Blood loss could be caused by defects of blood coagulation as a consequence of endothelial dysfunction, thrombocytopenia or deficiencies of coagulation factors. Anemia could also be due to secondary hemolysis caused by splenomegaly, which is usually caused by portal hypertension in patients with CLD. In some patients, bone marrow failure and aplastic anemia develop after an episode of hepatitis. Finally, anemia could be due to ribavirin-induced hemolysis as a complication of treatment of CHC with a combination of interferon and ribavirin [2].

The iron profile denotes that patients suffered from decreased iron in all groups. A significant reduction in ferritin was noted only in Child C. Low ferritin levels due to iron deficiency could be counteracted by increased ferritin synthesis which is induced by inflammatory cytokines [20]. The inflammatory state detected in those patients induces ferritin synthesis, probably through hepcidin secretion, masking the reduced ferritin levels due to iron deficiency [21]. Elevated serum ferritin and hepatic iron concentrations are frequently observed in CHC which may be related to hepcidin [22].

The level of tissue iron showed a statistical significant increase in all diseased groups. Increased serum hepcidin levels explain the inappropriate low hepatic hepcidin expression in an iron-over loaded cirrhotic liver. While the cirrhotic liver tissue is unable to secrete hepcidin, extra hepatic sources of hepcidin increases. Irrespective of the source of hepcidin, the downstream consequences are likely to be internalization and degradation of the cellular iron export protein ferroportin in the background of an elevation in the cellular iron import proteins such as transferrin receptor 1. This culminates in cellular iron accumulation; a phenotype which have previously been reported in HCV patients. This raises the possibility that hepcidin may have a dual effect contributing to the systemic anemia whilst acting locally at liver cells leading to iron accumulation. [23].

The soluble form of TfR (sTfR) is proportional to the total amount of surface TfR [24] and the erythroblasts rather than reticulocytes are the main source of serum sTfR [25]. The interpretation of the iron state according to sTfR has been controversial by many authors. Some believe that increased sTfR concentrations indicate ID even during ACD, and sTfR levels, was found to be considerably elevated in iron deficiency anemia but remain normal in the anemia of inflammation [24]. Others claim that elevated sTfR levels are also the characteristic feature of functional iron deficiency (FID) [25]. Increased sTfR may also indicate increased erythropoietic activity without ID, whereas lower sTfR concentrations may reflect decreased numbers of erythroid progenitors [24].

sTfR was found to be significantly increased in all patients compared to the controls ($P<0.05$). According to these results, all Child groups suffered from iron deficiency either solely or on top of ACD. It might also indicate the increased erythroid synthesis compensating for hemolysis or hidden gastrointestinal bleeding.

Statistical studies showed that in Child A and C most of the patients (80% and 100% respectively) suffered from iron deficiency either solely or combined with ACD which explains the high ferritin index values. In Child B group although still the percentage of patients suffering from iron deficiency was higher than those with ACD (88.3 versus 11.7), yet the marked high ferritin level of patients with ACD explains the decreased index value in this group. On regrouping patients into IDA, ACD and COMBI we found that after excluding patients with active bleeding the main type of anemia present in CHC patients is a combined form of ACD and IDA (COMBI anemia). All patients in Child C had COMBI anemia denoting that the more the progress of the disease the more patients are liable to acquire both types of anemia probably as a result of increased amount of chronic blood loss together with increased degree of inflammation caused by increased viral influence.

Statistical significant increase was detected in serum hepcidin in Child C group compared to control and Child A groups ($P<0.05$). On the contrary, hepatic expression of hepcidin showed reduced expression in diseased groups compared to the control group.

High level of hepcidin was detected in the presence of chronic hepatitis C (CHC) in many studies [26, 27]. The discrepancy between serum levels and hepatic expression levels could be due to the fact that hepcidin

expression was also localized in other tissues other than liver as kidney tubular system [28] and macrophages [29]. There is evidence that hepcidin is also expressed in the heart, kidney, adipose tissue, pancreas and haematopoietic cells [30].

Although there was an inflammatory state detected in all patients as proved by inflammatory index and CRP, significant increase in IL6 was detected only in Child C and in COMBI group. The lack of correlation between IL6 and hepcidin except in Child B group denotes that there are other factors contributing to increased hepcidin levels. The inflammatory stimulus to hepcidin secretion could be mediated through other stimulatory cytokines as IL1.

Four putative upstream regulatory pathways are generally thought to control liver hepcidin production: iron store, erythropoietic activity, inflammation, and a mandatory signaling pathway. All are found to interact with liver cells to initiate the production of sufficient hepcidin for correct maintenance of iron homeostasis [31].

Hepcidin transcription is stimulated by iron overload as well as by inflammation. Iron can accumulate in the liver in a variety of conditions, including anemia of chronic disease, hepatitis C and liver-specific iron accumulation of uncertain pathogenesis in cirrhosis [32].

A reduced hepcidin synthesis indeed might be one of the mechanisms leading to iron overload in advanced liver disease of any origin [33]. In a further step, progressive iron retention in the liver induces hepcidin formation to counterbalance hepatic iron accumulation by reducing duodenal iron absorption [34].

The liver plays a central role in maintaining body iron homeostasis not only as a storage tissue and a site of hepcidin production, but also by a relatively specific expression of several other iron-related genes including the hereditary hemochromatosis protein called HFE, transferrin receptor 2, hemojuvelin, bone morphogenetic protein 6, matriptase-2 and Transferrin [29]. Apart from these genes with a relatively specific expression, the liver expresses other genes and molecules involved in cellular iron transport, which are also present in other tissues. They include transferrin receptor 1, divalent metal transporter 1 and ferroportin [35]. The deregulated expression of these proteins in the diseased liver could result in aberrant hepcidin production [36].

Hepatic hepcidin repression might be the result of high circulating erythropoietin level due to ongoing hemolysis and oxidative stress due to accumulation of iron [23]. Hypoxia, anemia, increased erythropoiesis and reduced iron stores all negatively regulate hepcidin expression. The fact that elevated erythropoiesis increases iron absorption regardless of body iron loading could be explained by the presence of a direct connection between EPO and the suppression of hepcidin expression in hepatocytes, which express EPO receptors [29]. Anemia could mediate hepcidin suppression through multiple mechanisms including increased EPO, increased iron demand or liver hypoxia. The nature of the erythropoietic regulator of hepcidin is still uncharacterized, but may include one or more proteins released during active erythropoiesis. Possible candidate proteins include GDF15, secreted by erythroblasts, or soluble hemojuvelin, secreted by skeletal muscle [37]. Hepcidin is suppressed by hypoxia but the mechanisms are still uncertain and conflicting. Hypoxia-inducible factor (HIF)-1 and reactive oxygen species (ROS) have been both implicated [35].

The ongoing hemolysis, hidden blood loss, depressed bone marrow and decreased dietary intake or poor absorption from the intestine might lead to deteriorated iron supply in CHC patients. Iron deficiency state might cause anemia and suppress hepcidin synthesis in those patients [35]. In uncomplicated iron deficiency anaemia, both the anaemia per se and the absent iron stores provide a message to stop production of hepcidin [10].

Despite the fact that hemolysis down regulates hepcidin, Vokurka et al observed an increase in hepcidin expression despite severe hemolytic anemia after suppression of erythropoiesis [35]. This could be the case in patients with CHC in whom erythropoisis could be suppressed due to the presence of immune antibodies. Interferon based therapy can also suppress bone marrow production of erythrocytes [1] thus contributing to increased hepcidin production.

In addition, Merle et al showed that, in contrast to the liver, in which hypoxia results in down-regulation of hepcidin expression, cardiac hepcidin expression is significantly up regulated in response to hypoxia [38]. Extra hepatic sources of hepcidin could be up regulated due to hypoxia resulting in the observed increased levels.

This illustrates potentially differential effects of stimulation of hepcidin perhaps via inflammation and hepatic iron accumulation opposed by repression mediated by increased erythropoisis, anemia, hypoxia, reduced iron stores and oxidative stress. The effect of heamolysis and hypoxia on hepcidin repression could be reversed in our patients due to inhibition of erythropoisis due to interferon therapy and shifting the source of hepcidin to the extra hepatic sites where synthesis of hepcidin is induced by hypoxia. Increased hepatic iron is a double edged weapon which increases hepcidin synthesis as a compensatory mechanism to suppress iron absorption but in the same time it causes

decreased hepatic hepcidin synthesis due to accumulation of ROS.

The level of hepcidin in the three types of anemia is complementary to the pathophysiology of each type. In IDA the decreased iron levels lead to depressed hepcidin secretion while in ACD and COMBI anemia groups increased hepcidin secretion could be a cause or a result of increased hepatic iron accumulation.

It should not be forgotten that chronic disease anemia is a complex phenomenon and hepcidin is not the single molecule playing part in this condition. Numerous cytokines, particularly tumor necrosis factor alpha, which does not induce hepcidin m-RNA, can play an important role as well and the coexistance of iron deficiency may further complicate the process [39].

Univariate analysis study revealed that TfR is the most predicting parameter for the presence of IDA while hepcidin is the most predictive parameter for ACD. COMBI anemia was best predicted by hepcidin. The fact that hepcidin level was increased in ACD and COMBI groups without any statistical difference between these two groups while ferritin index was statistically increased in COMBI compared to ACD group could be used to discriminate between the two types of anemia. This denotes that a combination of the two tests is critical for the accurate determination of the type of anemia.

A positive correlation was found between hepcidin and ferritin levels in Child A and B groups. Hepcidin shifts the iron from the functional compartment to the stored one, part of which is ferritin. In addition ferritin could be the stimulus for hepcidin synthesis. Hepcidin could be the missing link between IL6 and ferritin explaining the positive correlation between the later two detected in Child B and COMBI groups. The negative correlation between hepcidin and ferritin index in Child A group is explained by the increased ferritin levels resulting from increased hepcidin level. The negative correlation between ferritin and sTfR in Child A and B and that between hepcidin and TfR in Child A and COMBI is due to the decreased erythroid synthesis manifested by decreased receptor levels in the presence of high ferritin level due to hepcidin production in ACD patients. The negative correlation between IL6 and TfR in Child B group is linked by increased hepcidin levels as a result of increased IL6 levels. The negative correlation between IL6 and ferritin index in Child B group and between IL6 and serum Fe in COMBI group is due to the stimulatory effect of IL6 on hepcidin production. Hepcidin in return lowers the serum iron level by shifting it to the storage compartment. The negative correlation between hepcidin and serum iron in ACD (r=-0.83) emphasizes the role of hepcidin in controlling the serum iron level in anemia of chronic disease.

5. Conclusion:

It could be concluded that hepcidin plays an important role in the pathogenesis of anemia in CHC patients. Accumulation of iron in hepatocytes together with inflammation induced by IL6 or other factors may cause increased hepcidin secretion whether from normal hepatic tissue or extra hepatic origins. The effect of anemia, hypoxia, ROS and erythropoisis counterbalances increased hepcidin levels. In CHC, combined ACD and ID is the main type of anemia after excluding active bleeding. Hepcidin is a good tool in discrimination of the type of anemia and is comparable to that of sTfR/logFn index. An appropriate combination of both tests provides evidence of iron depletion or reflects excessive production of hepcidin which will help to establish a correct diagnosis and appropriate therapy.

Corresponding author
Abeya Saleh
Hematology Department
Theodor Bilharz Research Institute, Cairo, Egypt.
dr.abeyasaleh@hotmail.com

6. References

1. El-Zayadi A. Hepatitis C co morbidities affecting the course and response to therapy. World J Gastroenterol, 2009 October 28; 15(40): 4993–4999.
2. Gonzalez-Casas R, Jones E, Moreno-Otero R. Spectrum of anemia associated with chronic liver disease. World J Gastroenterol, 2009 October 7; 15(37): 4653–4658.
3. Muñoz M, Villar I, García-Erce J. An update on iron physiology. World J Gastroenterol, 2009 October 7; 15(37): 4617–4626.
4. Theurl I, Aigner E, Theurl M, et al. Regulation of iron homeostasis in anemia of chronic disease and iron deficiency anemia: diagnostic and therapeutic implications. Blood, 2009; 113: 5277–5286.
5. Anderson G, Frazer D. Hepatic iron metabolism. Semin Liver Dis. 2005 Nov; 25(4):420-32.
6. Bayele H, Kaila S. Genetic variation in hepcidin expression and its implications for phenotypic differences in iron metabolism. Haematologica, 2009 September; 94(9): 1185–1188.
7. Nemeth E, Ganz T. Hepcidin and iron-loading anemias. Haematologica, 2006; 91:727–732.
8. Ganz T. Molecular control of iron transport. J Am Soc Nephrol, 2007; 18(2):394-400.
9. Delaby C, Pilard N, Goncalves A, et al. Presence of the iron exporter ferroportin at the plasma

membrane of macrophages is enhanced by iron loading and down-regulated by hepcidin. Blood, 2005; 106:3979–3984.
10. Leszek Rams, Lena Drozdowska-Rams, Jacek Małyszko, Krystyna Pawlak, Jolanta Małyszko. Prohepcidin and its possible role in anaemia of pregnancy. Arch Med Sci 2008; 4, 3: 305–309.
11. Koulaouzidis A, Said E, Cottier R, Saeed A. Soluble Transferrin Receptors and Iron Deficiency, a Step beyond Ferritin. A Systematic Review. J Gastrointestin Liver Dis, September 2009; 18(3), 345-352.
12. Suominen P, Punnonen K, Rajamäki A, Irjala K. Serum Transferrin Receptor and Transferrin Receptor-Ferritin Index Identify Healthy Subjects With Subclinical Iron Deficits. Blood, 1998 Oct; 92 (8): 2934-2939.
13. Pugh R, Murray-Lyon I, Dawson J, Pietroni M, Williams R. Transection of the oesophagus for bleeding oesophageal varices. Br J Surg. 1973 Aug; 60(8):646-9.
14. Koulaouzidis A, Cottier R, Bhat S, Said E, Linaker BD, Saeed AA. Ferritin level >50 microg/L is frequently consistent with iron deficiency. Eur J Intern Med. 2009 Mar; 20(2):168-70.
15. Desmet V, Gerber M, Hoofnagle H, et al. Classification of chronic hepatitis: Diagnosis, grading and Staging. Hepatology, 1994; 19: 1513-1520
16. Deugnier Y et al. Liver pathology in genetic hemochromatosis: a review of 135 homozygous cases and their bioclinical correlations. Gastroenterology, 1992;102:2050-9.
17. Hsu SM, Raine L, Fauger H. Use of avidin-biotin peroxidase complex (ABC) in immunoperoxidase technique: A comparison between ABC and unlabelled antibody (PAP) procedure. J Histochem, 1981; 29: 577-580.
18. Suwantarat N, Tice AD, Khawcharoenporn T, Chow DC. Weight loss, leukopenia and thrombocytopenia associated with sustained virologic response to Hepatitis C treatment. Int J Med Sci. 2010 Jan 22; 7(1):36-42.
19. Nemeth E, Ganz T. The role of hepcidin in iron metabolism. Acta Haematol. 2009; 122(2-3):78-86.
20. Testa U. Recent developments in the understanding of iron metabolism. The hematology journal, 2002; 3:63-89.
21. Matsumoto M, Tsujino T, Lee-Kawabata M, Naito Y, Akahori H, Sakoda T, Ohyanagi M, Tomosugi N, Masuyama T. Iron regulatory hormone hepcidin decreases in chronic heart failure patients with anemia. Circ J. 2010 Feb; 74(2):301-6.
22. Nagashima M, Kudo M, Chung H, Ishikawa E, Hagiwara S, Nakatani T, Dote K. Regulatory failure of serum prohepcidin levels in patients with hepatitis C. Hepatol Res., 2006 Dec; 36(4):288-93.
23. Valenti L, Pulixi E, Arosio P, Cremonesi L, Biasiotto G, Dongiovanni P, Maggioni M, Fargion S, Fracanzani A. Disorders of Iron Metabolism Relative contribution of iron genes, dysmetabolism and hepatitis C virus (HCV) in the pathogenesis of altered iron regulation in HCV chronic hepatitis. Haematologica, 2007; 92 (8)
24. Gómez M, Campos Garríguez A, García Erce JA, Ramírez Ramírez G. Fisiopathology of iron metabolism: diagnostic and therapeutic implications. Nefrologia, 2005; 25:9–19.
25. Beguin Y. Soluble transferrin receptor for the evaluation of erythropoiesis and iron status. Clin Chim Acta. 2003 Mar; 329(1-2):9-22.
26. Lin T, Liao L, Chou J, Liu S, Wang C. Serum prohepcidin levels correlates with hepatic iron stores in chronic hepatitis C patients. Hepatogastroenterology, 2009; 56(93): 1146-51.
27. Nagashima M, Kudo M, Chung H, Ishikawa, Higiwara S, Nakatani T, Dote K. Regulatory failure of serum prohepcidin levels in patients with hepatitis C. Hepatol Res, 2006; 36(4): 288-93.
28. Kulasiz H, Theilig F, Bachmann S, Gehrke S, Rost D, Janetzko A, Cetin Y, Stremmel W. The iron regulatory peptide hormone hepcidin: expression and cellular localization in the mammalian kidney. Journal of endocrinology, 2005; 184: 361-370.
29. Zhang A, Enns C. Molecular mechanisms of normal iron homeostasis. *Hematology, 2009, American society of hematology, Education program book.*
30. Piperno A, Mariani R, Trombini P, Girelli D. Hepcidin modulation in human diseases: From research to clinic. World J Gastroenterol, 2009 February 7; 15(5): 538–551.
31. Kemna E, Tjalsma H, Willems H, Swinkels D. Hepcidin: from discovery to differential diagnosis. Haematologica; 93 (1): 90-97.
32. Batts K. Iron overload syndrome and the liver. Modern Pathology, 2007; 20: S31–S39.
33. Tai A, Benita Y, Peng L, Kim S, Sakamoto N, Xavier R, Chung R. A Functional Genomic Screen Identifies Cellular Cofactors of Hepatitis C Virus Replication. Cell Host Microbe, 2009 March 19; 5(3): 298–307.
34. Aigner E, Theurl I, Theurl M, Lederer D, Haufe H, Dietze O, Strasser M, Datz C, Weiss G.

Pathways underlying iron accumulation in human nonalcoholic fatty liver disease. American Journal of Clinical Nutrition, 2008 May; 87 (5), 1374-1383.
35. Vokurka M, Krijt J, sulc K, Necas E. Hepcidin mRNA Levels in Mouse Liver Respond to Inhibition of Erythropoiesis. Physiol Res 2006; 55: 667-674.
36. Bergmann O, Mathahs M, Broadhurst K, Weydert J, Wilkinson N, Howe J, Han O, Schmidt W, K Brown. Altered expression of iron regulatory genes in cirrhotic human livers: clues to the cause of hemosiderosis? Laboratory Investigation, 2008; 88: 1349–1357.
37. Krjt J, Fujikura Y, Luděk E, Vokurka M, Hlobeňová T, Nečas E. Hepcidin downregulation by repeated bleeding is not mediated by soluble hemojuvelin. Physiol Res, 2009 Feb 27 [Epub ahead of print] PMID: 19249912
38. Merle U, Fein E, Gehrke S, Stremmel W, Hasan Kulaksiz. The Iron Regulatory Peptide Hepcidin Is Expressed in the Heart and Regulated by Hypoxia and Inflammation. Endocrinology, 2007; 148 (6): 2663-2668.
39. Demirag M, Haznedaroglu S, Sancak B , Konca C , Gulbahar O, Ozturk M, Goker B. Circulating Hepcidin in the Crossroads of Anemia and Inflammation Associated with Rheumatoid Arthritis. Inter Med, 2009; 48: 421-426.

6/27/2010

Controversial Role of Two Different Local Haemostatic Agents on Bone Healing

Ali Sawan [1] Yousry Elhawary[2] Mohamed Zaghlool Amer*[3] and Mohamed Abdel Rahman[4]

[1] Professor Of Oral & Maxillofacial Surgery-,[2]Associate professor Of Oral Biology, [3] Lecturer of Oral & Maxillofacial Surgery- Faculty of Dentistry-Mansoura University, [4] B.D.S 2002- Ministry of Health, Mansoura - Egypt

*norhanmohammed910@yahoo.com

Abstract: Controversial role of different local haemostatic agents on bone healing represented a major challenge for oral & maxillofacial surgeons. So, this study was directed to evaluate the effect of water soluble alkylene copolymer hemostat (ostene) versus bone wax on bone healing. Material & Methods: Forty five adult male rabbits weight 1kg were divided into three equal groups. A surgical bone defect was created into the anterior mandibular area. In 1st group the surgical defects were not subjected to any of local haemostatic agents. In 2nd group water soluble alkylene copolymer was applied within surgical defect and bone wax was applied within the 3rd group. Postoperatively, 3 animals were sacrificed from each group at 1, 2, 3, 6 and 12 weeks for histological assessment through H&E and Trichrome stain Results: Water soluble alkylene copolymer hemostat treated defects showed faster healing rate in 1st, 2nd weeks than defects left untreated. Ostene was disappeared from surgical defect at 1st week without presence of inflammatory cells in the defect. In 3rd group, the defects showed large empty vacuoles, representing bone wax remnants with inflammatory cells infiltration that interfere bone healing. Conclusion: Water soluble alkylene copolymer is biodegradable material that does not interfere with bone healing in contrast with bone wax which causes foreign body reaction, leading to interference of bone healing. [Journal of American Science. 2010;6(12):155-163]. (ISSN: 1545-1003).

Key words: Local Haemostatic Agents- Bone wax- Ostene

1. Introduction

Development of topical haemostatic agents has greatly improved surgeon's ability to achieve and maintain homeostasis, reduce patient blood loss, and decrease injury to the surrounding tissue that may occur with conventional haemostatic techniques. An ideal haemostatic agent has yet to be developed; however, with increased understanding and continued research on the coagulation mechanism, a number of new, commercially available products have been introduced to meet the criteria for the ideal agent .[1]

Over the years, bone wax products with various ingredients were developed in several countries. Bone wax has no haemostatic quality, but its effect is to tamponade marrow spaces[2]. Several reports have shown that bone wax residues are not resorbed and produce a foreign body giant cell reaction and inhibit bone reformation[3,4]. Furthermore, *Von Arx et al., in 2006* [5] detected that due to its adverse effects on tissue healing, it should no longer be used for homeostasis control in surgery.

Other haemostatic agents such as gelatin foam, oxidized cellulose and microfibrillar collagen are absorbable, less prone to tissue reaction, do not interfere with bone healing and have platelet stimulating effects. Unlike bone wax, however, because of their physical properties, they are not ideal to seal the bleeding cancellous surfaces[6].

Many efforts were made to obtain material with physical properties of bone wax to seal bleeding cancellous surface and also not interfere with bone healing[7]. A soft bone haemostatic wax (ostene) comprised of water-soluble alkylene oxide copolymers approved as local haemostatic material with this properties. This material comprises a sterile mixture of water-soluble alkylene oxide copolymers, derived from ethylene oxide and propylene oxide. These compounds are not metabolized, but eliminated from the body unchanged [8].

Form this point of view, this study was carried out to evaluate the effect of water-soluble alkylene oxide copolymers (ostene) versus conventional bone wax on bone healing process.

2. Materials and Methods:

Normal adult forty five rabbits Weight 1 kg each were used in this study. They were divided into three equal groups each group contained fifteen animals. *In 1st group,* a cortical bony defect was done in the anterior mandibular area of rabbit. The edges of the defects in this group of animals were not coated with any of local haemostatic agents.

In 2nd group, a cortical bony defect was done in the anterior mandibular area of rabbit.

The edges of the defects in this group of animals were coated with a commercially available water soluble alkylene oxide copolymers (fig. 1) (Ostene, absorbable haemostatic material, cermed, inc company. U. S. A). Conventional bone wax (bone wax, non absorbable sealant, synergy sutures, a member of matrix health care) was applied within the created surgical defect in anterior mandibular area of rabbits in the *3rd group.*

For each group, it was equally subdivided into five subgroups for histological assessment after 1, 2, 3, 6, and 12 weeks respectively.

Fig. 1: Showing the sterile package contain ostene.

Anesthesia and surgical procedures:-

All the rabbits included in this study were anaesthetized by using diazepam (0.5mg/kg) (valpam, amoun Co, Cairo, Egypt) and ketamine hydrochloride (20mg/kg) injection (ketamine hydrochloride; ketalar, amoun Co, Cairo, Egypt). Mepecaine hydrochloride with 1: 20000 levonordefrin (Mepecaine hydrochloride with 1: 20000 levonordefrin; Alexandria Co, Egypt) as local anesthetic agent was injected in the proposed area of surgery to improve the homeostasis and to provide post operative analgesia. The sub mental area of the mandible was disinfected using sterile pellet soaked with povidone iodine (Betadin) (Betadin; Nile Co, Cairo, Egypt).

Extra oral submental incision was made, and then mucoperiosteal elevator was used for flap elevation. No.3 rose head surgical bur was used to induce bony cavity of the same size (involving the cortex and spongiosa) under efficient coolant using normal saline. To ensure standardization of the bony defect, the same size of the bur was used making its head contained in the bone defect, at the same speed of the micro motor device for all animals.

In the 1st group, the edges of the cortical bony defect were not coated by any material. In the 2nd group, Ostene was manipulated between the finger till reach the body temperature to be soft and easy for application then was applied to the edges of the cortical bony defect. In the 3rd group, bone wax was applied to the edges of the cortical bony defect and the excess was removed.

The two edges of the incision were approximated and sutured by using 3/0 black silk.The incision line was painted with garamycin **(**Memphic Co, Cairo, Egypt) cream as local antibiotic.

Histological Evaluation:

Three rabbits within each subgroup were scarified at the different time intervals 1, 2, 3, 6, and 12 weeks respectively and the mandible was dissected out using a heavy scissor then fixed in 10% buffered formalin, decalcified and processed for paraffin sectioning. Sections were stained with hematoxylin for routine examination and trichrome stain for collagen.

3. Results

Histological observation using Hematoxylin and Eosin stain & Masson's Trichrome stains:-

In control group; the bony defect specimens contained granulation tissue, at the end of the first week (fig. 2). During second and third weeks, there were abundant less organized bony trabecullae with maturation of granulation tissue into connective tissue (fig. 3, 4), in addition; collagen bundles was seen running through the defect (fig. 5, 6). From three weeks to twelve weeks there were gradual increase in numbers, thickness coalescence (fig. 7, 8) and maturation of bony trabeculae (fig. 9).

In group II (ostene group) at the end of first week, the specimens of the bony defects revealed that more organized granulation tissue were formed with collagen bundles diffused through them, in comparison with the control group. Also there were new bony trabeculae formed (fig. 10, 11).

From two to six weeks, bony trabeculae were increased in number, thickness and organization when compared with control group. There was maturation of granulation tissue to connective tissue when compared with control group (fig. 12, 13, 14, 15, 16).

At the end of twelve weeks, the bone defects were completely filled with bony tissue which could not be distinguished from the surrounding normal bone (fig. 17, 18).

In group III (Bone wax), the histological specimens after one week revealed that the defect remained unchanged and there were some fibers at the base of defects, and the other part remained empty (fig.19). After two weeks, there were no bony trabeculae formation, but there were some inflammatory cells. By the time and after three

weeks, there were vacuoles between the fibers formed and more inflammatory cells (fig. 20). After six weeks, more bone destruction and inflammtory cells appeared (fig. 21, 22). After 12 weeks, the histological specimens showed micro abscess formation, increased inflammatory cells and more bone destruction (fig. 23).

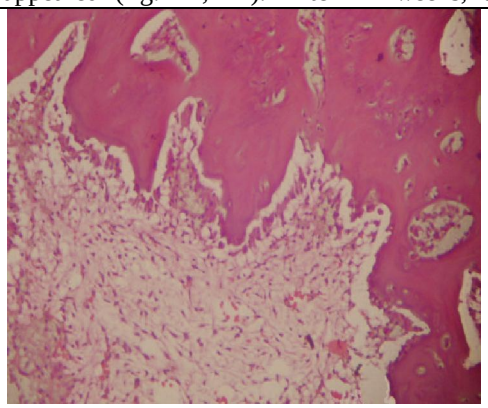


Fig. 2: Photomicrograph of bony defect in control group after one week from surgery showing, granulation tissue formation with newly formed blood vessels (H&E X100).

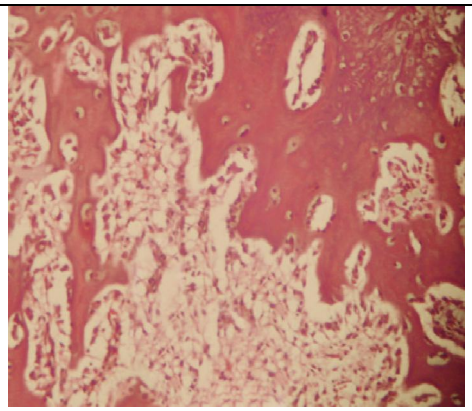


Fig. 3: Photomicrograph of bony defect in control group after two weeks showing, some bony trabeculae formation with granulation tissue appeared (H&E X100).

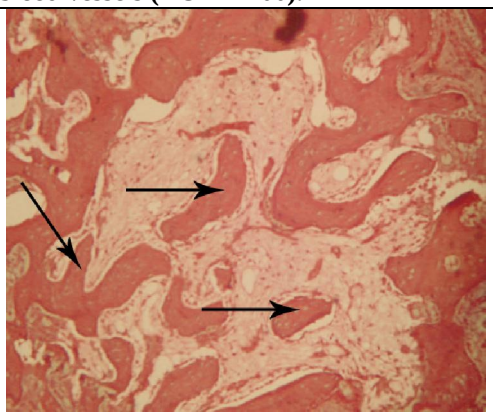


Fig. 4: Photomicrograph of bony defect in control group after three weeks showing, increased numbers of bony trabeculae (H&E X100).

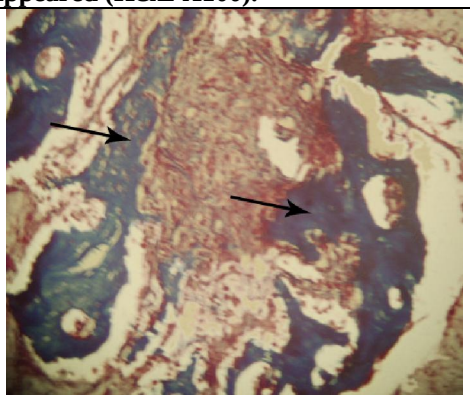


Fig. 5: Photomicrograph of bony defect in control group after two weeks showing, the collagen fibers of granulation tissue and newly formed bony trabeculae were seen (trichrom x 100).

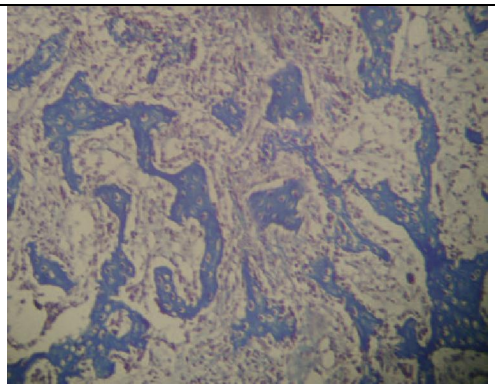


Fig. 6: Photomicrograph of bony defect in control group after three weeks showing, more bony trabeculae were formed (trichrom x 100).

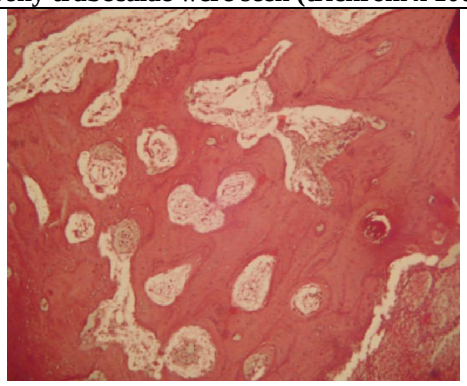


Fig. 7: Photomicrograph of bony defect in control group after six weeks showing, increased numbers and organization of bony trabeculae

(H&E X100).

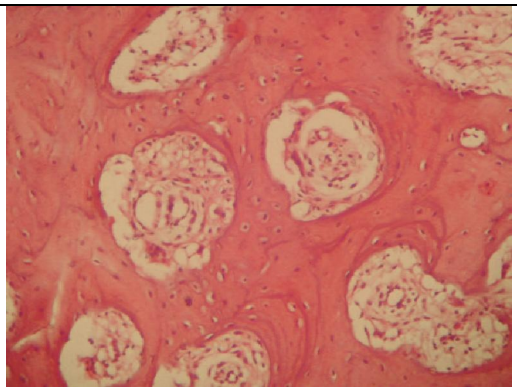


Fig. 8: Photomicrograph of bony defect in control group after twelve weeks showing well organized bony trabeculae with reversal lines (H&E X100).

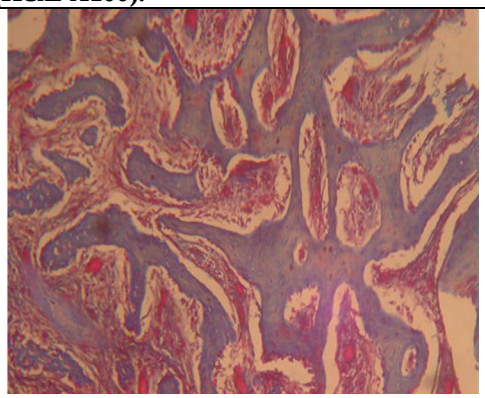


Fig. 9: Photomicrograph of bony defect in group I (control) after six weeks showing increased numbers of bony trabeculae with some degree of maturity (trichrom x 100).

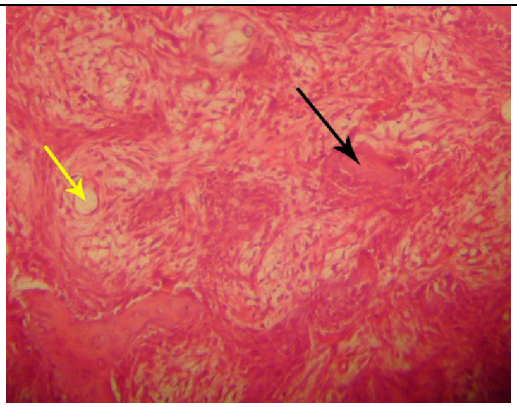


Fig. 10: Photomicrograph of bony defect in group II (Ostene) after one week showing, granulation tissue formation with newly formed blood vessels and newly formed bony trabeculae (H&E X100).

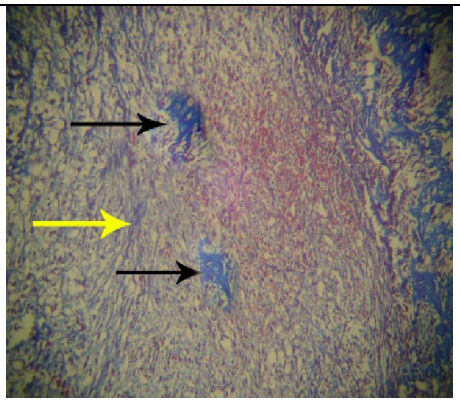


Fig. 11: Photomicrograph of bony defect in group II (Ostene) after one week showing, formation of collagen fibers with beginning of new bony trabeculae formation (trichrome x 100).

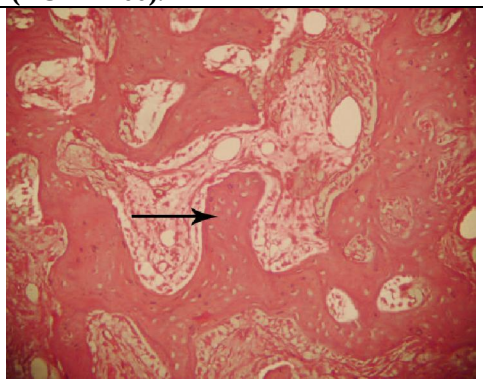


Fig. 12: Photomicrograph of bony defect in group II (Ostene) after three weeks showing increased numbers and maturity of bony trabeculae (H&E X100).

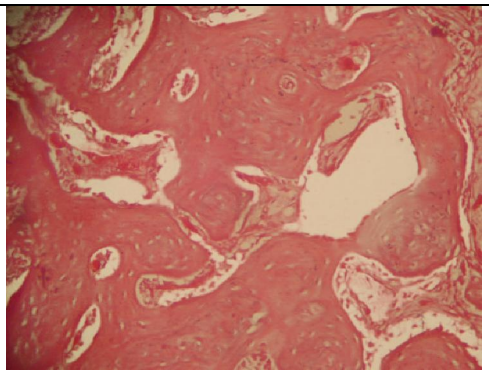


Fig. 13: Photomicrograph of bony defect in group II (ostene) after six weeks showing, more organization and maturity of bony trabeculae (H&E X100).

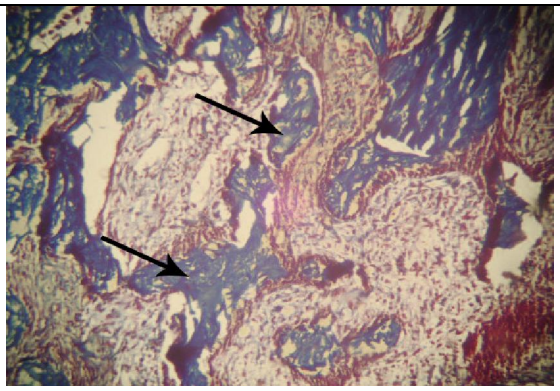


Fig. 14: Photomicrograph of bony defect in group II (Ostene) after two weeks showing, more collagen and bony trabeculae formation (trichrom x 100).

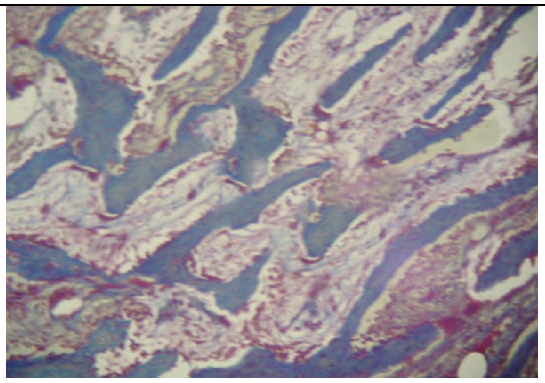


Fig. 15: Photomicrograph of bony defect in group II (Ostene) at three weeks showing increased numbers and maturity of bony trabeculae (trichrome x 100).

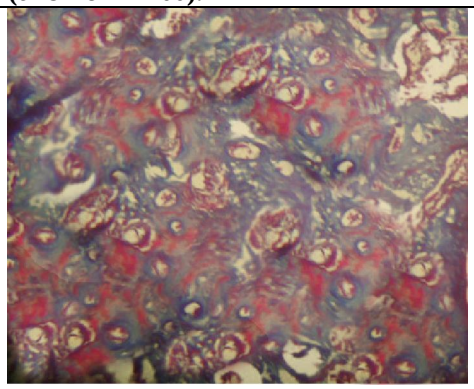


Fig. 16: Photomicrograph of bony defect in group II (ostene) after six weeks showing increased numbers of bony trabeculae with high degree of maturity (trichrome x 100).

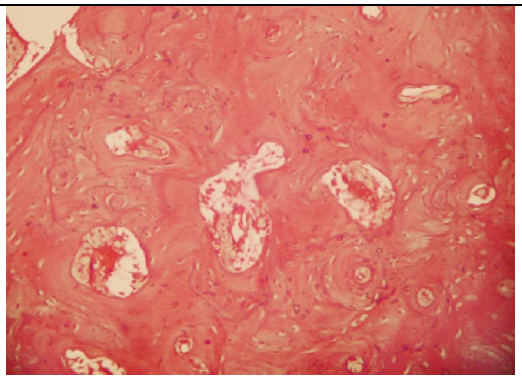


Fig. 17: Photomicrograph of bony defect in group II (ostene) after twelve weeks showing well maturated bony trabeculae (H&E X100)

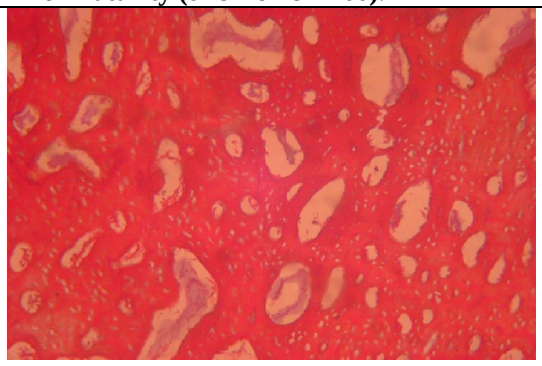


Fig. 18: Photomicrograph of bony defect in group II (Ostene) after 12 weeks showing, well organized bony trabeculae (trichrome x 100).

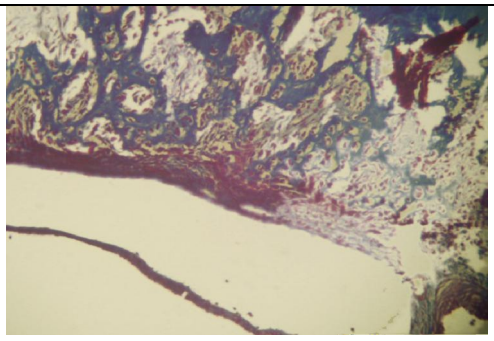


Fig.19: Photomicrograph of bony defect in group III (Bone wax) after one week showing, minimal fibers formed at the border of the cavity (trichrome x 100).

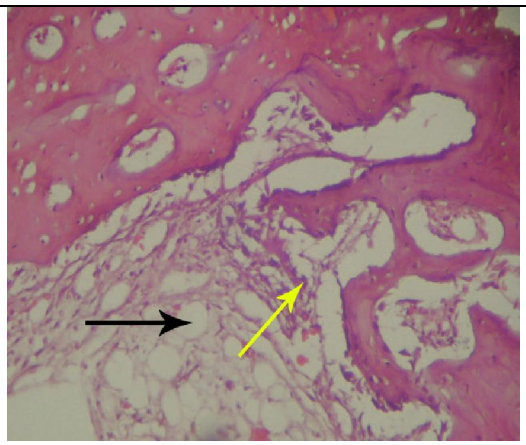


Fig. 20: Photomicrograph of bony defect in group III (Bone wax) after three weeks showing, no bone trabeculae formation, increased numbers of inflammatory cells and there were vacuoles between the fibers formed (H&E X100).

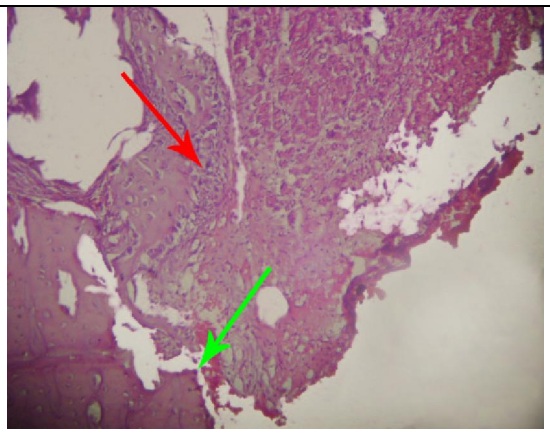


Fig. 21: Photomicrograph of bony defect in group III (Bone wax) after six weeks showing, more inflammatory cells and more destruction of bone (H&E X100).

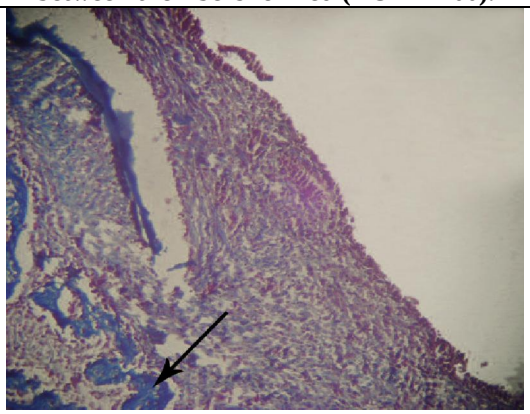


Fig. 22: Photomicrograph of bony defect in group III (Bone wax) after six weeks showing, no bony trabeculae formation with destruction of bone surrounding the defects (trichrom x 100).

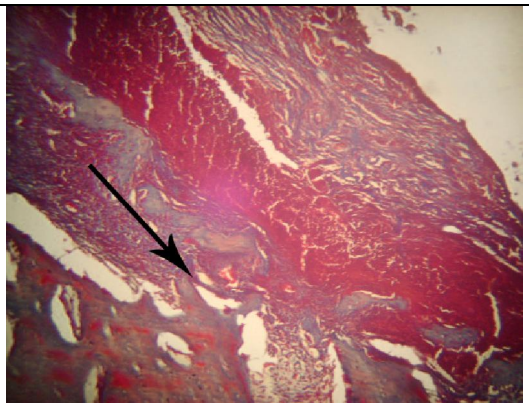


Fig. 23: Photomicrograph of bony defect in group III (Bone wax) after 12 weeks showing, micro abscesses were formed with destruction of bone surrounding the defects (trichrome x 100).

4. Discussion

In the last decade, the biotechnology has resulted in an explosive growth of topical haemostatic agents which are available to the modern surgeon[9]. One of the earliest topical haemostatic agents was cotton, in the form of gauze sponges. They are not absorbed by the body, and upon removal, the clot may be dislodged, leading to further bleeding[10].

Passive topical haemostatic agents can absorb up to several times their own weight in fluid. The expansion of a passive topical haemostatic agent can result in complications such as pressing nerves in surrounding tissue against bone or hard tissue; an extreme case of this has resulted in spinal cord compression leading to paraplegia[11-13]. Therefore, it is recommended that minimum amount of haemostatic agent necessary to achieve homeostasis be used and as much of the agent as possible be removed after homeostasis is achieved when a passive topical haemostatic agent is used on or near bony or neural spaces. Also, passive topical haemostatic agents do not adhere strongly to wet tissue and have little impact on actively bleeding wounds[14].

The physical presence of passive topical haemostatic agents can lead to confusion on subsequent diagnostic imaging because it may be difficult to distinguish between residual local topical homeostatic product and a tumor or abscess. Any residual product that may remain at the site also can

possibly potentiate a foreign-body reaction, chronic inflammation, or infection, which could promote granuloma formation and prevent optimal healing. Granulomas have been reported at a number of different sites after the use of passive topical haemostatic agents[14].

Absorbable topical haemostatic agents have been developed and provide useful adjunctive therapy when conventional methods of homeostasis are ineffective or impractical. *Wang et al., in 2001*[15] first described the use of a 'Pluronic copolymer blend' as a biocompatible and absorbable haemostatic alternative agent to bone wax.

Clinically, no signs of infection or inflammation were detected at the surgical site during the time of operation in all groups. This indicated that surgical technique was carried out under aseptic condition and there was no affection of bone vitality. In 2nd group, the water soluble alkylene copolymer material has no inflammatory effect on bone or surrounding tissue throughout all time intervals of follow up. These results were in accordance with the histological result of *Wellisz et al., in 2008*[16] that made a study on rabbit's tibias and the created defects were treated with the polymer, and observed that all of the cortical defects in the animals healed without evidence of infection. This might be due to tolerability and tissue compatibility of the water soluble alkylene copolymer material (ostene).

In 3rd group, there were no signs of infection or inflammation at the surgical site at the time of operation, after first week, but after the second week there were signs of infection and inflammation which include swelling at the surgical site appeared as cortical expansion, and this swelling increase with time to reach maximum level after 12 weeks. This result indicated that there was chronic infection which increases with time due to foreign body reaction. These results were in agreement with *Von Arx et al., in 2006* [5] who conducted a study in the calvarium of six rabbits. Standardized bony defects were trephined, and different haemostatic agents were applied and compared with control defects. Bone wax was one of these materials. He observed that bone wax residues were not resorbed and produce a foreign body giant cell reaction.

Furthermore, *Eser et al., in 2007* [2] reported that bone wax may cause a foreign body reaction and fibrosis and may increase the incidence of infection**.** Historically, several studies have described that, bone wax was noted to potentially cause chronic inflammation[17,18]**.** *Geary and Frantz, in 1950*[19] stated that, "in the series of control animals in which ordinary bone wax was used, no significant change in the implant was noted in the first 24 hours. At the 7th day, all showed an intense inflammatory reaction, three having cysts containing sterile fluid and numerous particles of wax and a fourth presenting a single, large encapsulated mass of wax".

In 3rd group the histological sections after one week showed large empty space at the bottom of the defects, representing bone wax remnants (dissolved during the embedding procedure). These results are in agreement with *Von Arx et al., in 2006* [5]who revealed large empty vacuoles; represent bone wax, which was applied in bony cavity created in rabbit calvarium model. Furthermore, *Sudmann et al.,in 2006* [20] demonstrated microscopically that bone wax remnants seen in 17of 18 sterna cadavers who prior to death had undergone surgery with median sternotomy and bone wax was used as haemostatic.

Histological sections of the control group and group II showed absence of inflammatory cells, no evidence of foreign body reaction was observed. These results were in accordance with *Lee et al., in 2009* [21] who reported that water soluble alkylene local haemostatic material did not cause local or systemic inflammation. This indicates the inert property of soluble alkylene copolymer material and its biocompatibility.

Histological examination of 2nd group after one week showed complete disappearance of the material at the application site, this indicate the biodegradable nature of material. These results are in agreement with *Wang et al., in 2001* [15] that used the same material, and had proved its effectiveness in allowing homeostasis at the bleeding sites of bone and can be absorbed within 24 to 48 hours.

During a comparison of bone healing between 1st versus 2nd group, bony trabeculae were formed in 2nd group and no bony trabeculae were evident in 1st group after first week. After second and third weeks, bony trabeculae in 2nd group were more in numbers, size, and maturation than 1st group. This revealed that healing started faster in 2nd group, at first, second, and third weeks. However, there was no remarkable difference in number, distribution, maturation, and coalescence of the bony trabeculae in both groups after six and twelve weeks. This indicated that bone healing levels occurred during later periods were nearly the same.

These results may be attributed to the nature of water soluble alkylene copolymer material which dissolved rapidly and eliminated from the body within days after surgery.

The previous results were in agreement with the observation of *Wellisz et al., in 2008* [16] that made his study on the tibia of rabbits and revealed that the use of the water-soluble polymer did not affect bone healing compared with controls. All of the cortical defects in the animals without radiographic evidence of infection had histological evidence of bone healing. *Lee et al., in*

2009 [21] showed that Ostene was effective in achieving bone homeostasis and absorbable in the body of rabbits. It did not inhibit new bone formation at the cut surface of the bone and did not cause local or systemic inflammation.

Furthermore, *Magyar et al., in 2008* [22] made a circular non critical-sized defect in the calvariae of rats. Alkylene oxide copolymer material was applied. He revealed that healing after 3 weeks was faster in defects with alkylene oxide copolymer material than defects without any material, and healing rate in the two groups were the same after six and twelve weeks from surgery**.**

During a comparison of bone healing between 3rd group versus 1st group, there were retardation and inhibition of bone healing. This demonstrated by absence of collagen fibers formation or bony trabeculae. This indicated that, bone wax inhibit osteogenesis. This result in agreement with number of studies, which reported that bone wax act as a mechanical barrier to bone regeneration [23,24]**.**

In 3rd group the histological findings showed inflammatory reaction, abscess formation, and destruction of bone at the border of cavity. Such findings might be attributed to the bone wax which remained in the defect, not absorbed, and acting as nidus for infection. The body reacted with these remnants as foreign body, it might lead to stimulation of chronic inflammatory cells and foreign-body reaction occurred. It might reduce bacterial clearance in cancellous bone and increase the liability of infection by decreasing the amount of bacteria needed to produce Staphylococcus aureus osteomyelitis.

Eser et al., in 2007 [2] demonstrated the presence of inflammatory granulation tissue as a reaction due to the presence of bone wax remnants, which was used as a local hemostat. Bone wax was seen within granulation tissue. Histopathological examination revealed infiltration of inflammatory cells and a foreign body granuloma in the connective tissue. The inflammatory cells were composed of lymphocytes, macrophages, and foreign body–type multinucleated giant cells.

To the best of our knowledge and as a result of the findings of this study, we believe that prevention of bone wax application as a local haemostatic agent is mandatory with replacement of it by such recent local haemostatic agent.

5. Conclusion:

Water soluble alkylene copolymer is biodegradable material that does not interfere with bone healing in contrast with bone wax which causes foreign body reaction, leading to interference of bone healing.

Corresponding author
Mohamed Zaghlool Amer
Oral & Maxillofacial surgery
Faculty of Dentistry- Mansoura University, Mansour, Egypt.
norhanmohammed910@yahoo.com

6. References:

1. Jay B. Erne and Fred Anthony Mann: Surgical Hemostasis. Small Animal/Exotics. 2003; 25(10).
2. Eser O, Cosar M, Aslan A, Sahin O: Bone Wax as a Cause of Foreign Body Reaction after Lumbar Disc Surgery: A Case Report. *Adv Ther*. 2007 May-Jun;24(3):594-7.
3. Solheim E, Pinholt EM, Bang G, Sudmann E: Effect of local hemostatics on bone induction in rats: a comparative study of bone wax, fibrin-collagen paste, and bioerodible polyorthoester with and without gentamicin. *J Biomed Mater Res.* 1992 Jun;26(6):791-800..
4. Allison RT. Foreign body reactions and an associated histological artefact due to bone wax. *Br J Biomed Sci*. 1994 Mar;51(1):14-7.
5. Von Arx T, Jensen SS, Hänni S, Schenk RK.: Haemostatic agents used in periradicular surgery: an experimental study of their efficacy and tissue reactions. *Int Endod J.* 2006 Oct;39(10):800-8.
6. Lavigne M, Boddu Siva Rama KR, Doyon J, Vendittoli PA.: Bone-wax granuloma after femoral neck osteoplasty. *Can J Surg.* 2008 Jun;51(3):E58-60.
7. Peng T: Biomaterials for Hemorrhage Control. *Trends Biomater. Artif. Organs.*2010; 24(1):27-68
8. Grindel JM, Jaworski T, Emanuele RM, Culbreth P.: Pharmacokinetics of a novel surface-active agent, purified poloxamer 188, in rat, rabbit, dog and man. *Biopharm Drug Dispos.* 2002 Apr;23(3):87-103.
9. Achneck HE, Sileshi B, Jamiolkowski RM, Albala DM, Shapiro ML, Lawson JH. A Comprehensive Review of Topical Hemostatic Agents Efficacy and Recommendations for Use. *Ann Surg.* 2010 Feb;251(2):217-28.
10. -Bochicchio G, Dunne J, Bochicchio K, Scalea T. The combination of platelet-enriched autologous plasma with bovine collagen and thrombin decreases the need for multiple blood transfusions in trauma patients with retroperitoneal bleeding. *J Trauma*.2004; 56(1):76-79.
11. Sabel M, Stummer W.: The use of local agents: Surgicel and Surgifoam. *Eur Spine J.* 2004 Oct;13 Suppl 1:S97-101. Epub 2004 May 15
12. Banerjee T, Goldschmidt K. "Surgiceloma" manifested as cauda equina syndrome. *South Med J. 1998; 91*(5):481-483.

13. Brodbelt AR, Miles JB, Foy PM, Broome JC. Intraspinal oxidized cellulose (Surgicel) causing delayed paraplegia after thoracotomy—a report of three cases. *Ann R Coll Surg Engl.*2002; 84(2):97-99.
14. Tomizawa Y.: Clinical benefits and risk analysis of topical hemostats: a review. *J Artif Organs.* 2005;8(3):137-42.
15. Wang MY, Armstrong JK, Fisher TC, Meiselman HJ, McComb GJ, Levy ML: A new, pluronic-based, bone hemostatic agent that does not impair osteogenesis. *Neurosurgery.* 2001 Oct;49(4):962-7; discussion 968.
16. Wellisz T, An YH, Wen X, Kang Q, Hill CM, Armstrong JK: Infection rates and healing using bone wax and a soluble polymer material. *Clin Orthop Relat Res.* 2008 Feb;466(2):481-6. Epub 2008 Jan 10.
17. Howard TC, Kelley RR: The effect of bone wax on the healing of experimental rat tibial lesions. *Clin Orthop Relat Res.* 1969; 63: 226–232.
18. Brightmore TG, Hayes P, Humble J, Morgan AD. Haemostasis and healing following median sternotomy. *Langenbecks Arch Chir.* 1975;Suppl:39-41.
19. Geary JR, Frantz VK.: A new absorbable hemostatic bone wax; preliminary report. *J Neurosurg.* 1950 Sep;7(5):383-4.
20. Sudmann B, Bang G, Sudmann E.: Histologically verified bone wax (beeswax) granuloma after median sternotomy in 17 of 18 autopsy cases. *Pathology.* 2006 Apr;38(2):138-41.
21. Lee TC, Chang NK, Su FW, Yang YL, Su TM, Lin YJ, Lin WC, Huang HY.: Systemic and local reactions of a water-soluble copolymer bone on a bony defect of rabbit model. *Surg Neurol.* 2009 Dec;72 Suppl 2:S75-9; discussion S79. Epub 2009 Aug 7.
22. Magyar CE, Aghaloo TL, Atti E, Tetradis S.: Ostene, a new alkylene oxide copolymer bone haemostatic material, does not inhibit bone healing. *Neurosurgery.* 2008 Oct;63(4 Suppl 2):373-8; discussion 378.
23. Rockwood C, Perkins JC, Roberts L, Dixon D: Reaction of bone wax on bone and muscle. *J Bone Joint Surg.* 1968; 50:837–838.
24. -Sorrenti SJ, Cumming WJ, Miller D.: Reaction of the human tibia to bone wax. *Clin Orthop Relat Res.* 1984 Jan-Feb;(182):293-6.

6/22/2010

A Framework for Testing Software Product

Amjad Farooq[1], M. Junaid Arshad[1] and Muhammad Abuzar Fahiem[2]

[1, 2]Computer Science and Engineering Department, UET, Lahore
[2]Department of Computer Science, Lahore College for Women University, Lahore, Pakistan
amjadfarooq@uet.edu.pk

Abstract: There is a growing need of frameworks for automatic testing of software product because manual testing of huge software product is very time-consuming and costly. Furthermore, the manually testing of complex software becomes more difficult and a challenging activity. However this can be easily achieved through automatic testing strategies. In this paper we propose a framework for testing software automatically. Now errors and bug finding become simpler and easier. It takes less time to test the whole application rather than testing application modules separately. The proposed framework provides programmatic access to most user interface elements. The main propose of our framework is to make testing phase easier and cost efficient. We validate our framework through a case study. By analyzing the results of testing the correctness and completeness of framework is proved. [Journal of American Science. 2010;6(12):164-173]. (ISSN: 1545-1003).

Keywords: Software testing; test automation; test framework

1. Introduction

The manually testing of complex software is a very difficult and a challenging activity. However this can be easily achieved through automatic testing strategies (Yingxiang, 2008). Nowadays, the user interface (UI) testing automation has become an integral part of software development in all big corporations (Jovic and Hauswirth, 2010). UI Automation means testing any User Interface application in an automated fashion. This method of testing is far more effective than just doing manual testing as it is best to catch the last minute bugs and basically improves overall product quality (Mathew and Spraetz, 2009). The automation runs faster and the benefit is that it doesn't require human input like manual testing. By this large application testing become simpler, easier and less time consuming [9]. As for the evolution of the testing field, there have been some innovations over the years. What's striking about the innovations, however, is how few people know about them, and even fewer people are using them. In terms of evolution, here in Pakistan, we're in the Dark Ages of testing. Many test teams work in isolation, knowing little of the existing literature on the subject, and providing little input to improve UI testing. Due to the importance of testing, most probably in few years it will evolve into a proper engineering discipline.

The rest of paper is structured as follows: The background and the related work are given in Section 2. The functionalities, and design description of proposed framework is given in Section 3 whereas; the different test scenarios used to validate the proposed framework are also listed in the same Section. Finally, the paper ends with conclusion in Section 4.

2. Background and Related Work

There is a growing need of frameworks for automatic testing of software (Riungu et al,2010; Berner et al, 200). Both, the functional and non-functional requirements of the software need to be tested but their manual testing consumes a lot of time and budget resources (Jovic and Hauswirth, 2010; Sarkar et al., 2009; Mesbah and van Deursen, 2009). Furthermore, it rises multiple times for a complex software testing. Furthermore, to measure the quality of software the execution-based testing has its own importance (Viswanathan and Peters, 2010). In (Wang and Damata, 2009), GUI testing toolset is described. This toolset supplements the basic testing tasks required in the common GUI testing process. It generates test cases and has a reporting mechanism. Although it is a good toolset but the target software is not a web-based. The Force.com (Mathew and Spraetz, 2009) framework includes some testing utilities to create test cases and those test cases are applied for the automation of individual modules of software. AUTOWEB (Chai et al., 2009) is an interesting tool, it test the online- assignments submitted by students. It generates demo of failed test cases automatically. It aims to provide help to students in order to solve their assignments.

3. Proposed Work

The basic architecture of our framework is given in Figure 1. There is a UI Automation Tester class which contains all the test cases. User firstly has to start the target UI application. The user task then is to select the test case to which he/she wants to run.

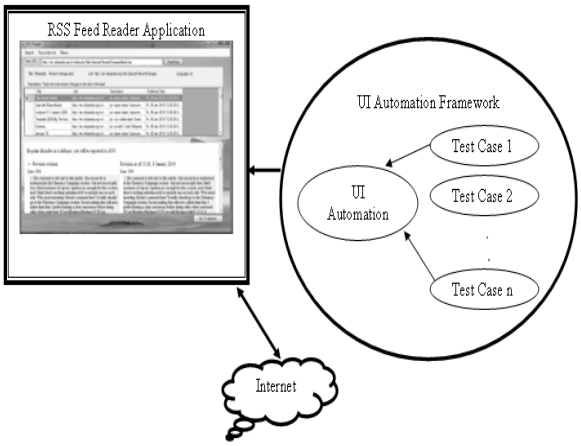


Figure 1: System Architecture of our Proposed Framework

Internet connection should be established to test RSS Feed Reader. RSS Feed Reader needs internet to read latest feed which are continuously upgraded on the sites, regarding the URL specified. The layered architecture of our framework is as given below:

Test Cases
Operational Logics
UI Wrappers – Physical Layer

Figure 2: Layered Architecture of our Proposed Framework

Test cases layer contain the test cases for the application organized by the area names and test names. Each test case represents a user scenario. Some scenarios are higher in priority than others. A test case is collection of a bunch of logical calls that live in logical layer. The test cases use / consume the logical layer in it or interacts with the Logical Layer. Some examples of test case: Reading an RSS Feed and making sure that correct feed is displayed; create a new word document and making sure that a plain document is created without errors.

Logical layer is collection of methods that represents user actions. These are logical methods that do actions needed to complete a test case. For example in order to complete the above mentioned test cases logical methods will be needed to make sure that application is in running state; to read RSS Feed that takes in a custom RSS Feed location. A method in logical layer is merely a collection of calls to physical layer that does physical action e.g. to create a document the physical action that test code will do by clicking the File Menu and then New Document Menu.

Physical layer is also called direct UI Wrappers. Physical layer consists of the wrappers over all the UI Controls that we have in the application. It gives us an interface that we can use to call actions on the UI Controls in the application e.g. Click Read Feed Button, set value in a Location Text box. The mechanism is limited to Testing UI. Applications only developed in C# or VB.Net. The software is application specific; test cases are specific to the application that you want to test. The overall workings of proposed framework have shown in Figure 2 and 3.

For sake of simplicity, the following we have considered the following assumptions and constraints.

- Software is developed for testing a UI application which is RSS feed reader.
- Test cases are specific to the application.
- Test cases are not generic or couldn't run on other UI applications for testing.
- We have developed our software in C# and it is capable of testing UI application.
- We have not purchased any type of software and hardware equipment for our project.

The class diagram of proposed framework is given in Figure 4. We have TestExecution Client App that runs our tests; you can pick a test and run it that class is represented by ExecuteScriptClient. it contains the UI for our execution engine and methods like start app, execute script etc. When a test is selected and executes script is called then we move to Script class or TestCases class. Script class has all our test cases. Script class interacts with Logical Class and Logical Class contains RSS ReaderFunction. RssReaderFuction Class is the one that contains our logical actions. Rss Reader has an association relationship with physicalObjects class.Physical Objects contains wrappers for all the UI controls. Then Physical Objects calls into ScriptFunctons class that has all the methods that help us do actions on these UI controls e.g. click, set text Toggle, Set Value etc.

A selective list of graphical user interfaces used for different requirements are given in the Figures 5-9 followed by their respective input, processing and output. We have used different test scenarios to check the correctness of proposed framework. Some of the scenarios are listed in the Tables 1-8 respectively.

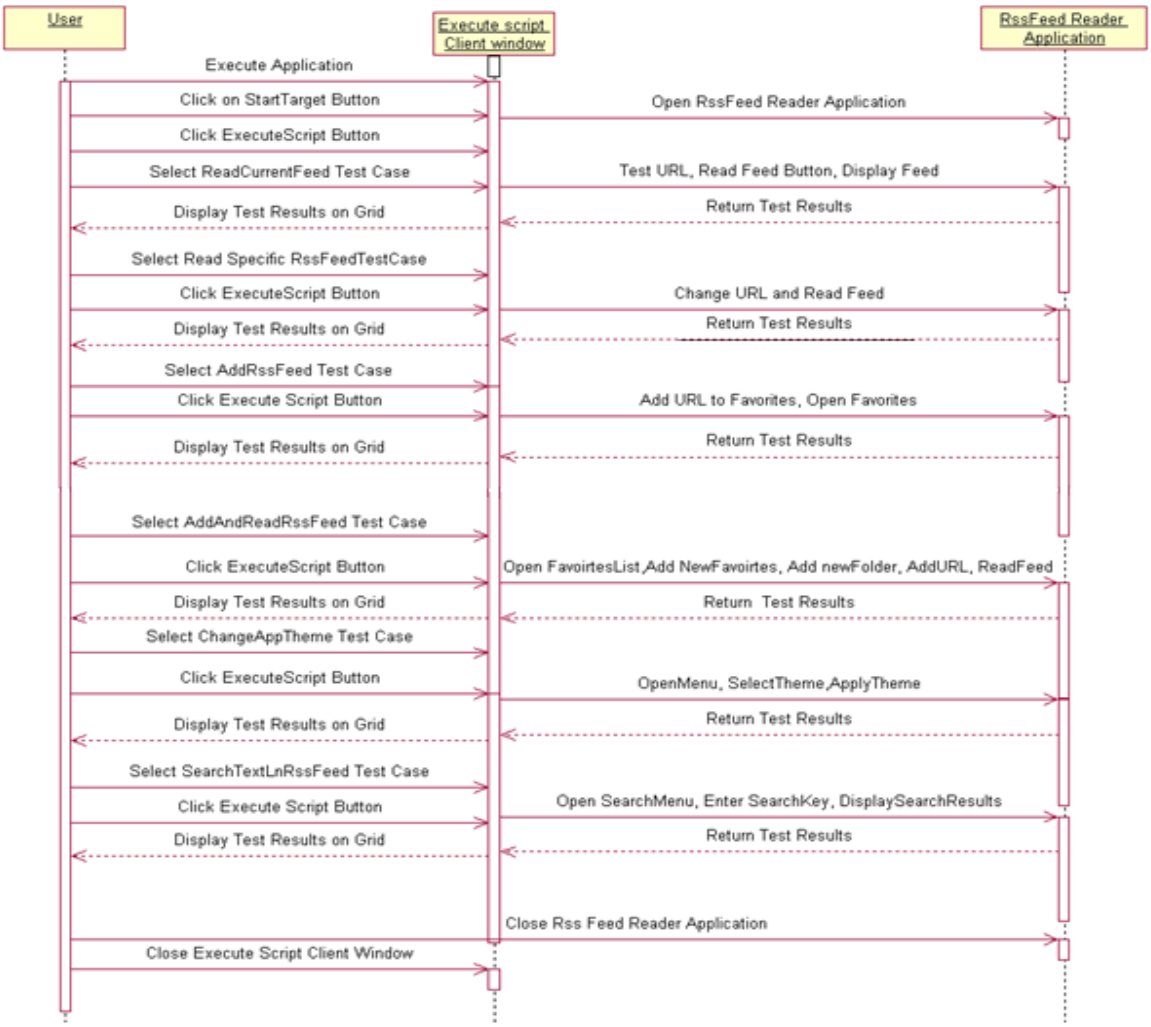


Figure 2: System sequence diagram of proposed framework

Figure 5:Graphical User Interface for adding a Favourite

Input	• Automatically click on Favorites List. • Click on Favorites List. • Write URL in name text box. • Specify folder name in folder text box or select already created folder from drop down list. • Click on Add button. • To cancel this window click on cancel button.
Output	URL added to the favorites list in the specified folder.
Processing	Create folder if specified. Add URL to the favorites list. And close the window.

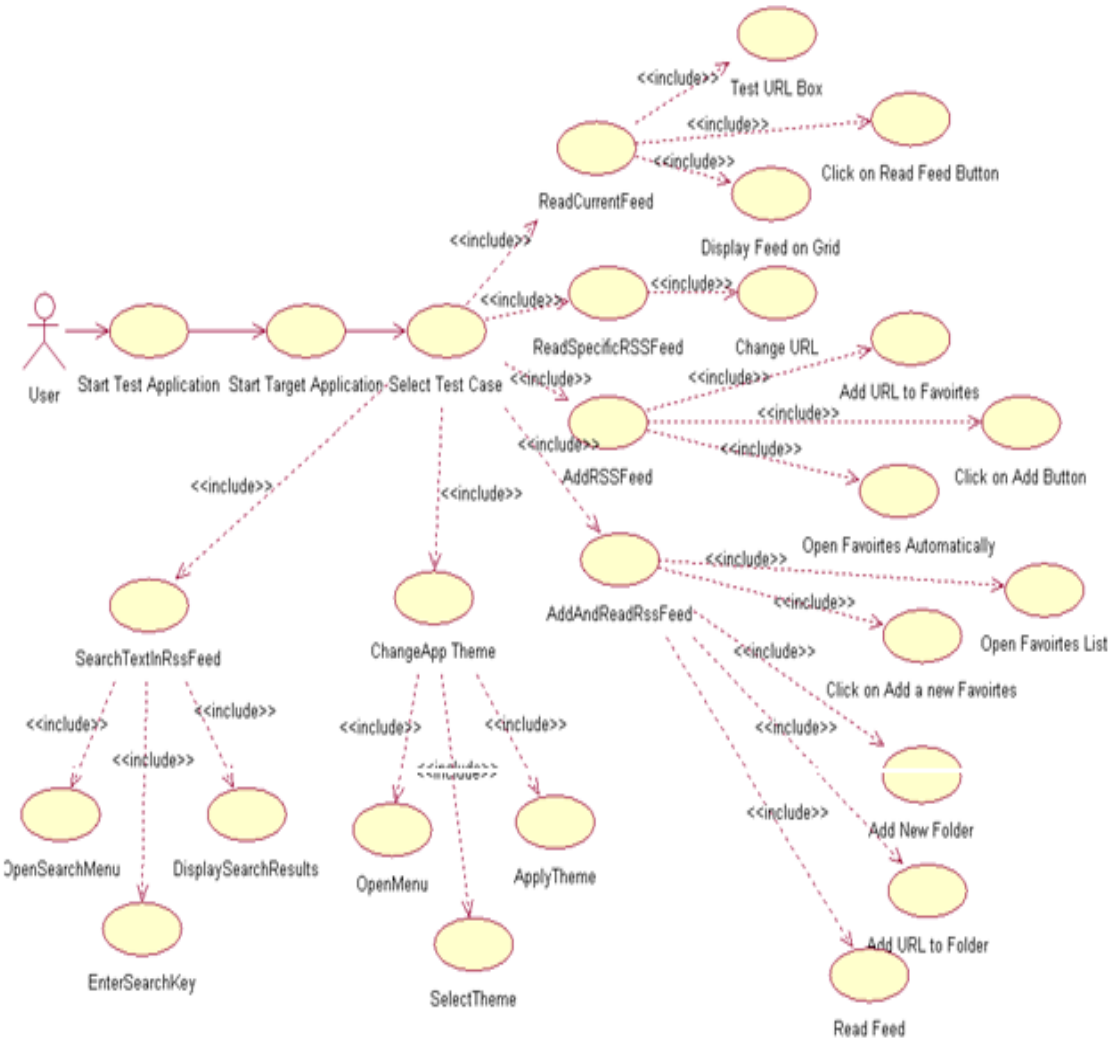


Figure 3: Operations of proposed framework

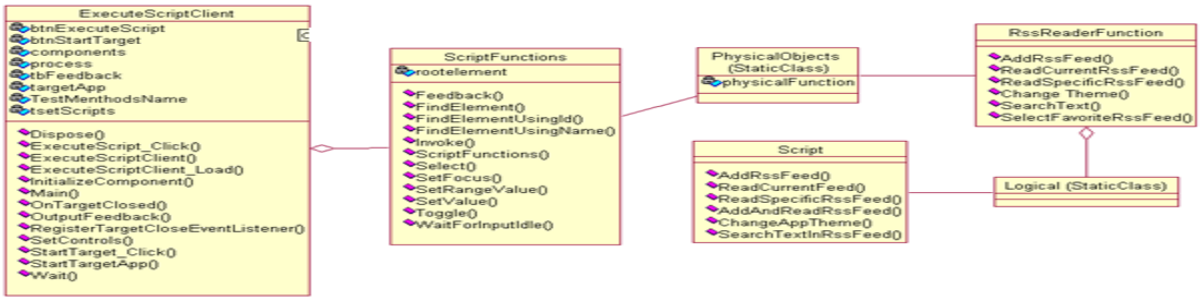


Figure 4: Class Diagram of proposed framework

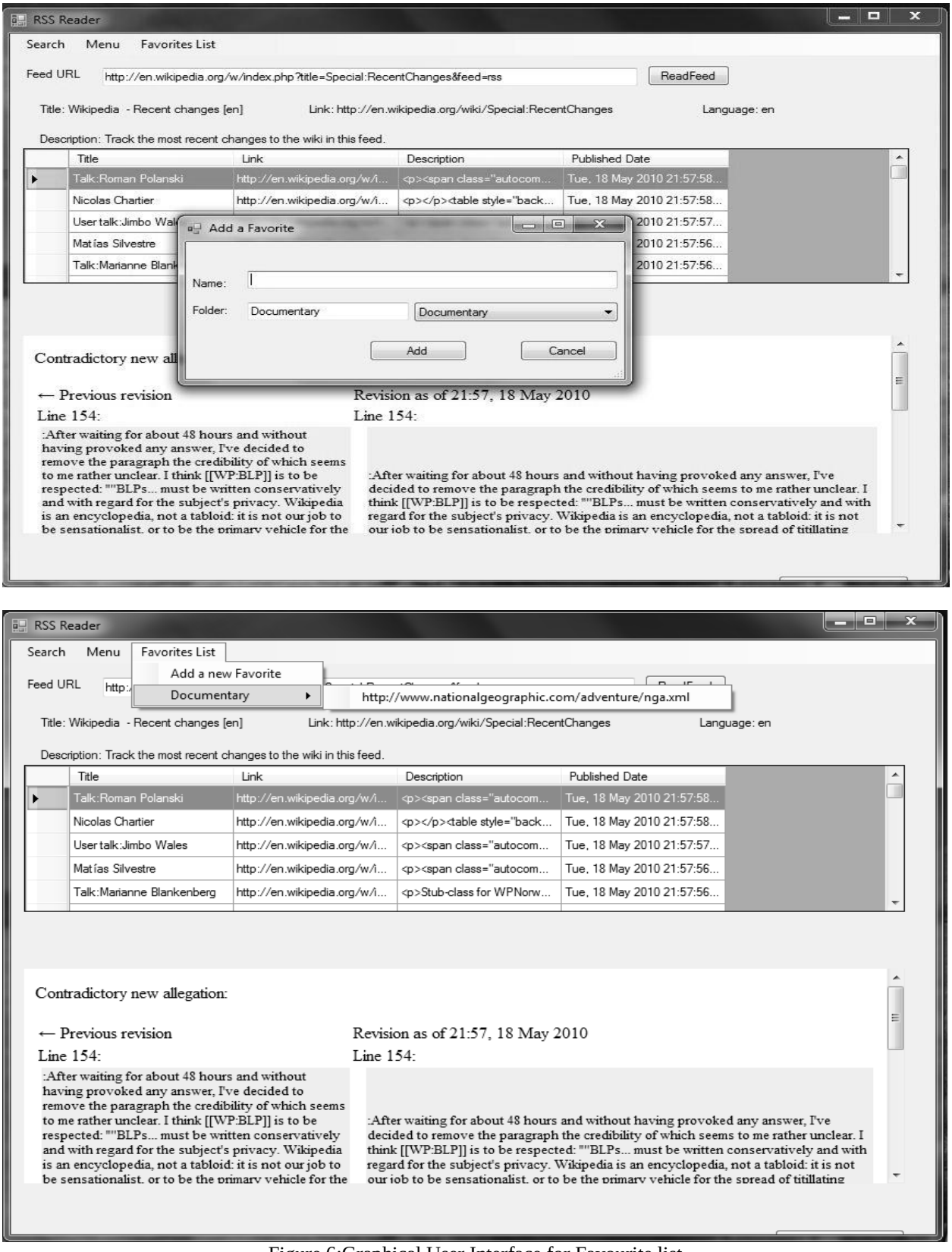


Figure 6:Graphical User Interface for Favourite list

Input	Automatically click on Favorites List from menu bar and then go to the Documentary folder from the list.
Output	Display selected URL in the text box.
Processing	Select the URL from the Documentation list.

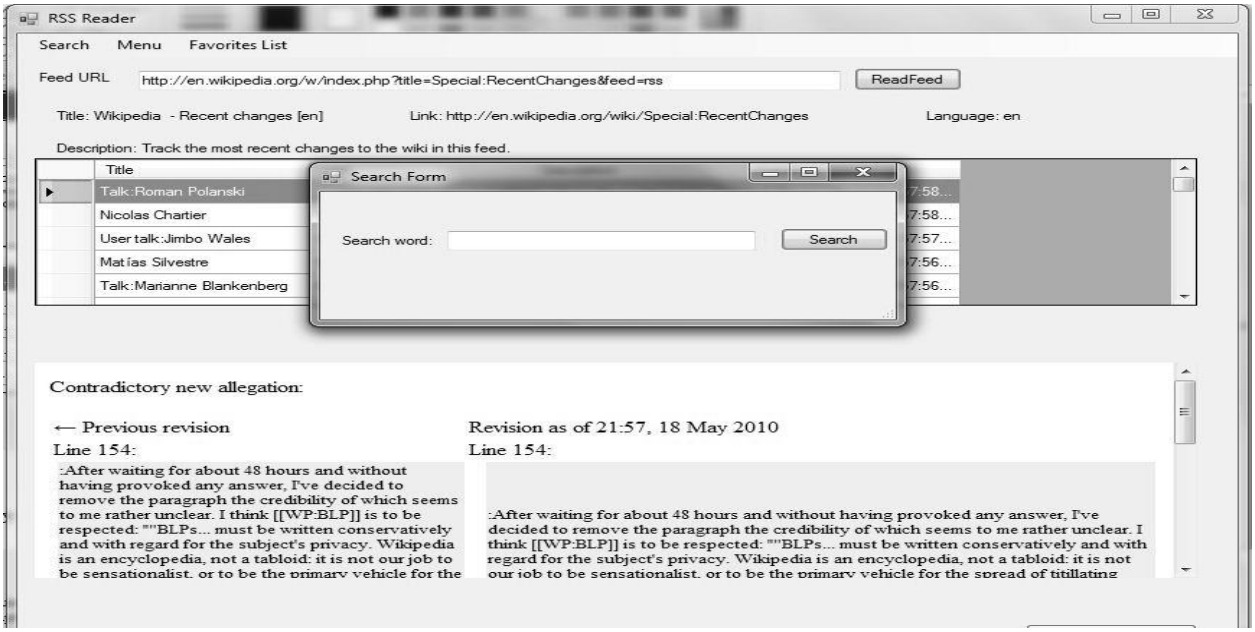


Figure 7:Graphical User Interface for searching

Input	• Automatically click on the search from menu list. • Input what you want to search. • Click on Search button.
Output	Show search results.
Processing	Read input from search word text box and display find the results.

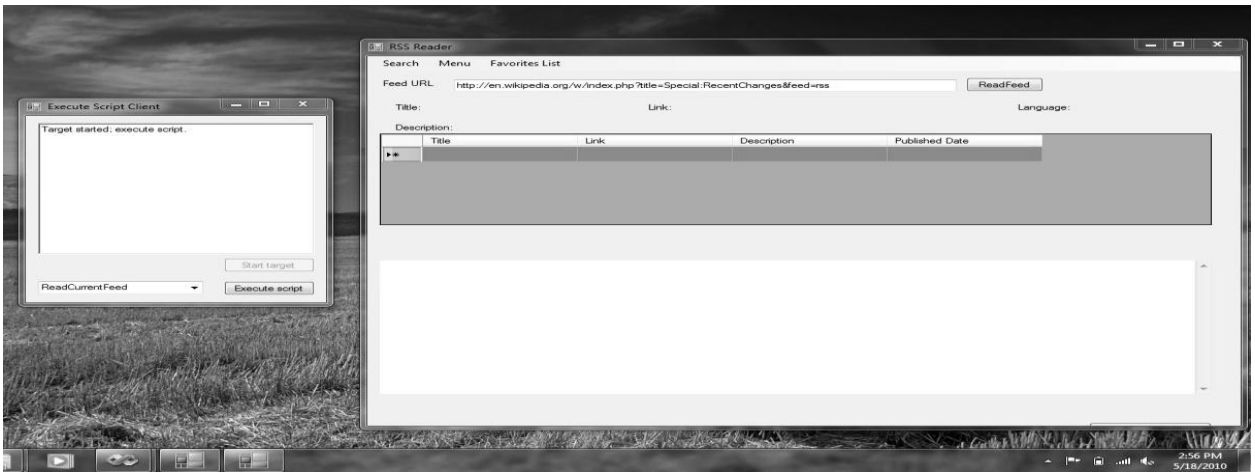


Figure 8:Graphical User Interface for starting the target application

Input	Click on Start Target button.
Output	Display RSS Feed Reader window.
Processing	Open the target application.

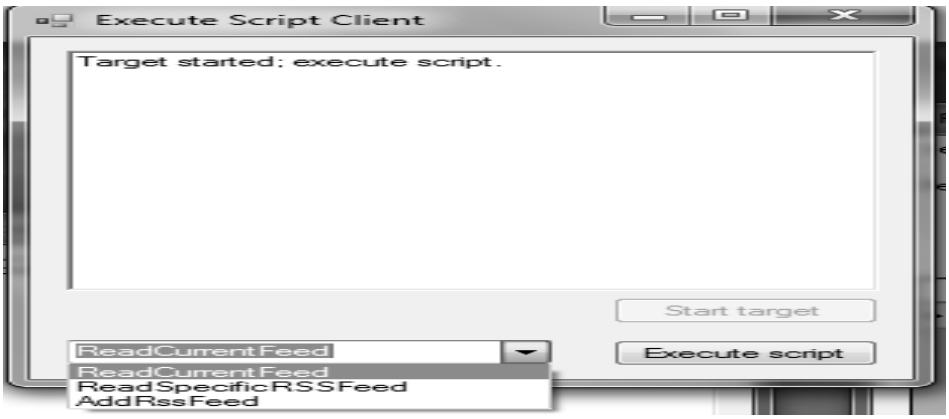


Figure 9:Graphical User Interface for executing script

Input	Select any test case which u wants to run and click on Execute script button.
Output	Display test results on the Data Grid Box.
Processing	Run selected test case to test the target application.

Table 1: Start Test Application

UC-01: Start Test Application		
Actors: User		
Feature: Start application		
Use Case Id:	UC-01	
Pre Condition:	Application should be debugged for starting the application.	
Scenarios		
Step #	**Action**	**Software Reaction**
1.	Start target application.	Software will open target application.
2.	Select test cases from drop down menu.	Run the selected test case and display results in grid box.
Alternative Scenarios:		
Target application should be started before selecting test cases.		

Table 2: Start Target Application

UC-02: Start Target Application		
Actors: User		
Feature: User will start the target application.		
Use Case Id:	UC-02	
Pre Condition:	User should be click on the start target application button.	
Scenarios		
Step #	**Action**	**Software Reaction**
1.	User presses the start target application button.	Software will open the RSS Feed Reader application. Execute Script Client Window will displays a message in grid box “Target started; execute script”.
Alternative Scenarios:		
User should have to click the start target application button in each and every condition.		

Table 3: Select Test Case

UC-03: Select Test Case		
Actors: User		
Feature: User will select test cases from the drop down menu.		
Use Case Id:	UC-03	
Pre Condition:	User should select the test case in order to test the application.	
Scenarios		
Step #	**Action**	**Software Reaction**
1.	User selects the desired test case from the drop down list. Available test cases are: • Read Current Feed • Read specific RSS Feed • Add RSS Feed	
Alternative Scenarios:		
User may directly close the application without running test cases.		

Table 4: Read Current Feed

UC-04: Read Current Feed		
Actors: User, Execute Script Client		
Feature: User will select the "Read Current Feed" test cases from the drop down menu.		
Use Case Id:	UC-04	
Pre Condition:	User should press the Execute Script button in order to run the test case.	
Scenarios		
Step #	**Action**	**Software Reaction**
1.	User will press the Execute script button to run the test case.	Software will automatically test the application and performs the following functionalities. • Test URL Box • Click on Read Feed Button • Display Feed on Grid
Alternative Scenarios:		
User may select any other test case rather than selecting this test case.		

Table 5: Read Specific RSS Feed

UC-05: Read Specific RSS Feed		
Actors: User, Execute Script Client		
Feature: User will select the "Read Specific RSS Feed" test cases from the drop down menu.		
Use Case Id:	UC-05	
Pre Condition:	User should press the Execute Script button in order to run the test case.	
Scenarios		
Step #	**Action**	**Software Reaction**
1.	User will press the Execute script button to run the test case.	Software will automatically test the application and performs the following functionalities. • Change URL
Alternative Scenarios:		
User may select any other test case rather than selecting this test case.		

Table 6: Add RSS Feed

UC-06: Add RSS Feed		
Actors: User, Execute Script Client		
Feature: User will select the "Add RSS Feed" test cases from the drop down menu.		
Use Case Id:	UC-06	
Pre Condition:	User should press the Execute Script button in order to run the test case.	
Scenarios		
Step #	**Action**	**Software Reaction**

1.	User will press the Execute script button to run the test case.	Software will automatically test the application and performs the following functionalities. • Open Favorites Automatically • Click on Add Button • Add URL to Favorites
Alternative Scenarios:		
User may select any other test case rather than selecting this test case.		

Table 7: Execute Client Script

Test Case ID: T-01	**Engineer:** Faiza Aziz,Iram Waheed ,Farah Amjad
Application Name: Testing via UI Automation	**Use Case Id:** UC-Start Test Applcation-01
Purpose: To start the application.	
Scenario: To test target application.	
Environment: Visual Studio .NET 2008, IE 8.0	
Pre-Request: User should debug the program in order to test the application.	
Strategy: 1. User will run the Program. 2. Execute Client Script window will open. 3. Press starts target application. 4. Select required test case from drop down list. 5. Click on execute script button.	
Expected Results: 1. Execute Client Script window will open. 2. RSS Feed Reader window will open. 3. Automated testing regarding the specific test case will start.	
Observations: The testing will start properly and less time is consumed on testing. All results will be displayed in the Data Grid Box.	
Results: No error found. Client Script Window opened properly. All Test cases execute properly and will check all controls of target application RSS Feed Reader. For Example, it will show errors if URL text box is empty.	

Table 8: RSS Feed Reader

Test Case ID: T-02	**Engineer:** Faiza Aziz,Iram Waheed ,Farah Amjad
Application Name: Testing via UI Automation	**Use Case Id:** UC-Start Target Applcation-02
Purpose: To start the target application for testing.	
Scenario: To run all test cases on RSS Feed Reader.x	
Environment: Visual Studio .NET 2008, IE 8.0	
Pre-Request: User should press the Start Target Application button in order to test the application.	
Strategy: 1. Select ReadCurrentFeed from drop down list and click on Execute Script button. 2. Select ReadSpecificRS Feed from drop down list and click on Execute Script button. 3. Select AddRSSFeed from drop down list and click on Execute Script button. 4. Select AddAndReadRSSFeed from drop down list and click on Execute Script button. 5. Select SearchTextlnRSSFeed from drop down list and click on Execute Script button. 6. Select ChangeApp Theme from drop down list and click on Execute Script button.	
Expected Results: 1. RSS Feed Reader window will open. 2. When Read Current Feed test case selected following actions performed automatically ○ Test URL Box ○ Click on Read Feed Button ○ Display Feed on Grid 3. When Read Specific RSSS Feed test case selected following actions performed automatically ○ Change URL 4.When Add RSS Feed test case selected following actions performed automatically ○ Open Favorites Automatically	

○	Click on Add Button
○	Add URL to Favorites
Observations: Different URL is changed to check that whether RSS Feed Reader is properly getting latest feeds from the URL. Different URL is also added to the favorites list to check that it is properly maintaining favorites List.	
Results: No error found. All Test cases execute properly and will check all controls of target application RSS Feed Reader. For Example, it will show errors if URL text box is empty etc.	

5. Conclusion and Future Work

The proposed framework is purely independent and self contained. And there is no other related application or any third party component involved for the development of the framework. We have developed the framework in C# and enabled to test various C# UI Applications. The proposed framework is capable of testing UI application automatically. In our framework user have to start the application, after the UI Automation starts user task now is to start the target application(RSS Feed Reader), which is the application to which user wants to test. After the target application is selected, now user has to select any test case which he/she wants. After the desire test case is selected automated testing will start by testing all controls of the application. It will tell about all bugs/errors in the application automatically. As manual testing was more time consuming, so our application not only reduces time complexity but also make it efficient and convenient for users.

Acknowledgements

Authors are thankful to the University of Engineering and Technology, Lahore, and HEC – Pakistan for financial support for doing this work.

References

1. Appasami S. User Interface Accessibility and Test Automation for Silverlight Applications. International Journal of Computational Intelligence Research 2009, 5(2), Print ISSN: 0973-1873. Online ISSN: 0974-1259.
2. Berner S, Weber, R., and Keller, R. K. 2005. Observations and lessons learned from automated testing. In Proceedings of the 27th international Conference on Software Engineering (St. Louis, MO, USA, May 15 - 21, 2005). ICSE '05. ACM, New York, NY, 571-579. DOI= http://doi.acm.org/10.1145/1062455.1062556
3. Chai T, Wang Z and Wang J. Automated Universal Testing and tutoring system for WEB application. Computer Science and Information Technology, International Conference on, pp. 188-192, 2009 2nd IEEE International Conference on Computer Science and Information Technology, 2009.
4. Jovic M and Hauswirth M. Performance Testing of GUI Applications. icstw, pp.247-251, 2010, Third International Conference on Software Testing, Verification, and Validation Workshops.
5. Mathew, R. and Spraetz, R. 2009. Test Automation on a SaaS Platform. In Proceedings of the 2009 international Conference on Software Testing Verification and Validation (April 01 - 04, 2009). ICST. IEEE Computer Society, Washington, DC, 317-325. DOI= http://dx.doi.org/10.1109/ICST.2009.46
6. Mesbah A and van Deursen, A. 2009. Invariant-based automatic testing of AJAX user interfaces. In Proceedings of the 31st international Conference on Software Engineering (May 16 - 24, 2009). International Conference on Software Engineering. IEEE Computer Society, Washington, DC, 210-220. DOI= http://dx.doi.org/10.1109/ICSE.2009.5070522
7. Riungu L, Taipale O, and Smolander K. Software Testing as an Online Service: Observations from Practice. icstw, pp.418-423, 2010 Third International Conference on Software Testing, Verification, and Validation Workshops, 2010.
8. Sarkar C, Soderston C, Klementiev D, and Bell E. Remote Automated User Testing: First Steps toward a General-Purpose Tool. A chapter in book: Software Engineering Research, Management and Applications 2010.
9. Viswanathan S, and Peters J. C. Automating UI guidelines verification by leveraging pattern based UI and model based development. In Proceedings of the 28th of the international Conference Extended Abstracts on Human Factors in Computing Systems (Atlanta, Georgia, USA, April 10 - 15, 2010). CHI EA '10. ACM, New York, NY, 4733-4742. DOI= http://doi.acm.org/10.1145/1753846.1754222.
10. Wang M and Damata, L. 2009. TAO Project: An Intuitive Application UI Test Toolset. In Proceedings of the 2009 Sixth international Conference on information Technology: New Generations (April 27 - 29, 2009). ITNG. IEEE Computer Society, Washington, DC, 796-800. DOI= http://dx.doi.org/10.1109/ITNG.2009.86
11. Yingxiang Z. Ingrid - An automated testing system for telephony software - A case study. UVic Subject Index::Sciences and Engineering::Applied Sciences::Computer science, 2008.

7/1/2010

Effect of Mineral, Organic Nitrogen Fertilization and Some Other Treatments on vegetative growth of Picual Olive Young Trees.

Laila, F. Hagag[1] ; H. S. A. Hassan[1]; M. Abou Rawash[2]; H. El-Wakeel[2] and A .Abdel-Galel [1]

1-Pomology Department, National Research Center, Cairo, Egypt
2- Dept. of Hort. Fac. of Agric. Ain Shams Univ., Shobra El-Khiema, Cairo, Egypt

Abstract: This study was carried out through two successive seasons (2007& 2008) on cultivated Picual olive young trees grown at the Research Station Farm of National Research Center, El Nobarya, El Behera governorate. The investigation aimed to study the effect of applying mineral, organic fertilizers and some other treatments on growth parameters at the first two years of planting. Planting holes were prepared for control plants in the first season only. Each treatment received 100 g actual nitrogen/plant/year as recommended by M.A.R.L. (2007). The following treatments were applied: T1 : control (mineral nitrogen + planting hole preparation), T2(100%mineral nitrogen), T3(100% organic N as cattle manure), T4(50% mineral N + 50% organic N as chicken manure), T5 (100%mineral nitrogen + humic acid as soil application), T6(100% mineral nitrogen + activated dry yeast as soil application), T7 (100%mineral nitrogen + GA_3 spray) and T8 (100% mineral nitrogen + sea algae as soil application).At the end of each season, plant height, stem diameter, lateral shoot number, lateral shoot length, leave numbers per plant, Percentage of plant height increment, whole plant dry weight were determined and recorded. The obtained results revealed that as follow: plant height, shoot number, shoot length, leaves number and stem diameter were not affected by different treatments in both seasons. Meanwhile, whole plant dry weights were improved by humic acid treatment compared with control and all other treatments in Picual cv. [Journal of American Science. 2010;6(12):174-179]. (ISSN: 1545-1003).

Keywords: Nitrogen Fertilization; vegetative growth; Picual Olive.

1. Introduction

The olive tree (*Olea europaea* L.) Family Oleaceae is a widely distributed tree grown in many arid areas of the world. The Mediterranean region is its native habitat. Olive is adapted to extremely arid conditions because of its special leaf structure and ramified root system.The olive tree is an evergreen, one of the oldest cultivated tree, about 8000 years ago.

World olive production perform an important role in the economy of many countries such as Spain, Italy, Greece, Turkey and Tunisia.The olive tree yield has two main products:oil and table olives, produced from several cultivars such as Coratin,Klamata,Picual. Total cultivated area in Egypt with Picual cvs. Represent about60% of total olive cultivation. The Egyptian olive production reached about 507053 tons produced from 110764 Feddan according to the staticics of M.A.L.R (2007a). This investigation aimed to study the effect of some mineral and organic nitrogen fertilization sources as well as some other treatments (humic acid, activated dry yeast, GA3 and sea algae) on some growth parameters and leaf mineral contents of Kalamata young trees at the first two years of planting. However, it is hope also to find out a fertilization program can replace the mineral nitrogen one which will be beneficial for organic production of olives and save human health and environment.

Xiloyannis *et. al.* (2000) working on mineral nutrient uptake from the soil in irrigated olive trees, cultivar Coratina, over six years after planting they recorded that, the nutrient demand was relatively steady during the different stages of the year. The results showed that demand for P and K is minimal during the first four years after planting and can be fulfilled by naturally supplied soils. Low doses of N should be applied through localized fertilization during the year. Nawaf and Yara (2006) found that, young olive trees benefit from low levels of NPK and N alone and additional fertilizers would not be significant. However, NPK are considering being essential element for plant growth and development. The 16 g NPK and 32 g N significantly gave the highest shoot and root dry weight, this probably due to nitrogen concentration which increased dry matter accumulation in roots and decreased shoot: root ratio. Monge *et. al.* (2000) reported that, organic wastes fertilization did not lead to significant increases in olive mineral leaf concentrations in the first year trial. Hegazy *et. al.*(2007) studied the effect of organic and bio-fertilization on vegetative growth and flowering of Picual olive trees, they recorded that, N and K contents in leaf increased significantly with applying 100% organic fertilization (poultry manure), but no significant difference was observed on leaf P content in both seasons. The same treatment gave the highest Fe leaf content in both seasons and Mn in the second

season, while leaf Zn content increased in second season with using 100% mineral fertilization.

Fernández-Escobar *et. al.* (1999) mentioned that, under field conditions, foliar application of leonardite extracts (humic substances extracted) stimulated shoot growth and promoted the accumulation of K, B, Mg, Ca and Fe in leaves. However, when leaf N and leaf K values were below the threshold limit for the sufficiency range, foliar application of humic substances was ineffective to promote accumulation of these nutrients in leaves. Abdel Fatah *et. al*.(2008) mentioned that, soil drench application of humic acid to Tifway Bermodagrass hybrid improved growth parameters and NPK leaves cotents.

Mostafa and Abou Raya (2003) recorded that, all dry yeast soil application improved growth parameters of Grand Nain banana cv. Compared with control without dry yeast treatment.

Smith and Schwabe (1984) recorded that, top growth of *Quercus robur* could be further accelerated by application of gibberellic acid (GA_3) as foliar spray. Eman and Abd-Allah (2008) reported that, progressive increase on percentages of N, P, and K in the Superior grapevine leaves was observed as a result of increasing concentration of algae till 50%.

This investigation aimed to study the effect of mineral and organic nitrogen fertilization sources and some other treatments (humic acid, activated dry yeast, GA_3 and sea algae) on growth parameters of Picual young trees at first two years of planting. That to improve and push tree growth through these years.

2. Material and Methods

This study was carried out through two successive seasons (2007& 2008) on newly cultivated Picual olive cv. young trees in the Expermental research station of National Research Center at El Nobarya, El Behera governorate Egypt. The investigation aimed to study the effect of applying mineral, organic nitrogen fertilizers and some other treatments on young Picual cv. trees at the first two years of planting. The soil was characterized by: pH = 8.82, EC =1.11 dS/m, organic matter = 0.31%, $CaCO_3$ =12.8 %, Sand = 63 %, Silt = 13 % and clay = 3%. The soil texture grade was sandy. Drip irrigation system was applied using river Nile water. Planting distance was 5 × 5 meters apart.

In control plots, planting holes were prepared by adding 50 kg cattle manure, 1kg super phosphate, 1/4 kg potassium sulfate and 1/2 kg agricultural sulfur and each treatment received 100 g actual nitrogen/plant/year in each season as recommended by M.A.R.L. (2007a).

3. Results and discussion

The following treatments were applied:

1- Control: recommendation of M.A.R.L. (2007a) (100g actual nitrogen 500 g ammonium sulfate as mineral nitrogen source) + planting holes preparation.
2- Mineral nitrogen only 100 %.
3- Organic nitrogen source 100 % (cattle manure 100g actual nitrogen).
4- Mineral nitrogen source 50 % + organic nitrogen source 50 % (chicken manure).
5- Mineral nitrogen source 100 % + humic acid (monthly doses from March to November each 20 ml/plant).
6- Mineral nitrogen source 100 % + actived dry yeast as drench treatment three times in March, July and October each at 30 g/plant.
7- Mineral nitrogen source 100 % + one spray of GA_3 acid at 50 ppm in March.
8- Mineral nitrogen source 50 % + sea algae in March and June each at 50 g/plant.

- Cattle manure analysis was: N = 1.6%, P = 0 46% and K = 0.51%.
- Chicken manure analysis was: N =3.47%, P =0.67% and K = 0.64%.
- Sea algae analysis: N =8%, P = 2%, K = 4%, chelate microelements = 4% and traces of vitamins + amino acids.

Ammonium sulfate was divided into five equal doses through growing season. All these treatments were repeated in the second season except holes preparation with control plants only in the first season. The treatments were arranged in randomized complete block design in a simple experiment with four replicates for each treatment and each replicate was represented by one plant. At the end of each season at mid November four plants as replicates for each treatment were removed genteelly with their root system to estimate and record the following data for each cv individually:

1- Plant height (cm).
2-Stem diameter (cm) was measured at 10 cm above soil surface.
3- Lateral shoots length average (cm).
4- Leaf number per plant.
5- Lateral shoot number per plant.
6-Percentage of plant height increment
7- Whole plant dry weight (g).

Data obtained throughout this study were statistically analyzed using the analysis of variance method as reported by (Snedecor and Cochran, 1980), and the differences between means were differentiated by using Duncan's range test.

Effect of treatments on growth characters:

Plant height fig. (1) show that, insignificant differences were recorded among treatments in both seasons. But the tallest plants were recorded by control treatment in the first season (85.8 cm) and in the second season (141 cm). while the lowest plant height in both seasons was obtained by cattle manure supply alone (67.8 cm & 137 cm) respectively.

Stem diameter fig. (2) show that, the eighth treatment with sea algae had the lowest significant value (1.23 cm) compared with most other treatments in the first season whereas, in the second season the differences lake significance among treatments .However, the lowest value in the second season was recorded by 100%cattle manure (3.55 cm).

Shoot number fig.(3) show that, number of shoot per plant showed insignificant differences among treatment in both seasons. But we can notice that , control treatment recorded the highest value in first season(18.3) .In the second season the eighth treatment with sea algae had the highest value (38.3) and the lowest value in both seasons was recorded by 100% cattle manure (15.5&37 respectively).

Shoot length fig.(4) show that, average of shoot length is obvious that the sixth treatment with dry yeast recorded the highest significant value .(29.0 cm)as compared with the third one in the first season but in the second season no significant differences could be noticed among treatments. However in the second season the highest value was achieved by fifth treatment with mineral nitrogen+humic acid (57.5 cm) and the lowest value was recorded by the first treatment (37 cm).

Leaf number fig.(5) show that, leaf number per plant, result indicated in table 1 during the first season that the highest number of leaves per plant were significant for treatment of mineral nitrogen only (treatment2) compared to all organic treatments, while humic acid treatment gave the best results compared to improve the vegetative growth of other, however, in second season sea algae was less impact on number of leaves per plant.

Percentage of plant height increment Fig. (6) show that, the seventh treatment recorded the highest significant values in both seasons compared with most of other treatments (120.6% & 89.22% respectively).

Whole plant dry weight fig.(7) show that, the fifth treatment with humic acid showed the highest significant value (183.5 g.) compared with all other treatment in the first season. In addition, in the second season the same treatment had higher significant value (873.3 g.) than those of the third and eighth treatments.

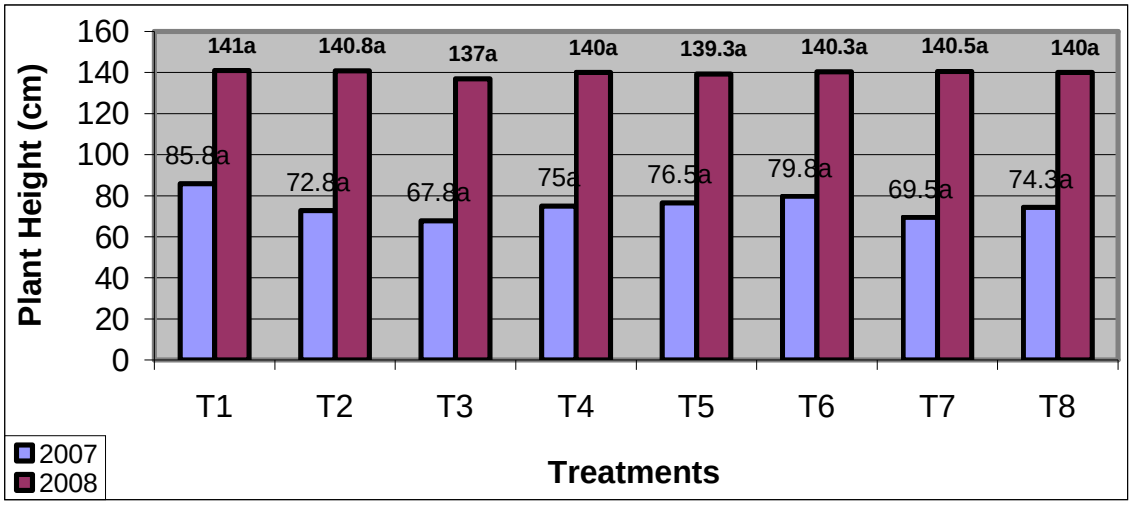


Fig (1): Effect of mineral, organic nitrogen and some other treatments on plant height (cm) of Picual olive cv. young trees in 2007 and 2008 seasons.

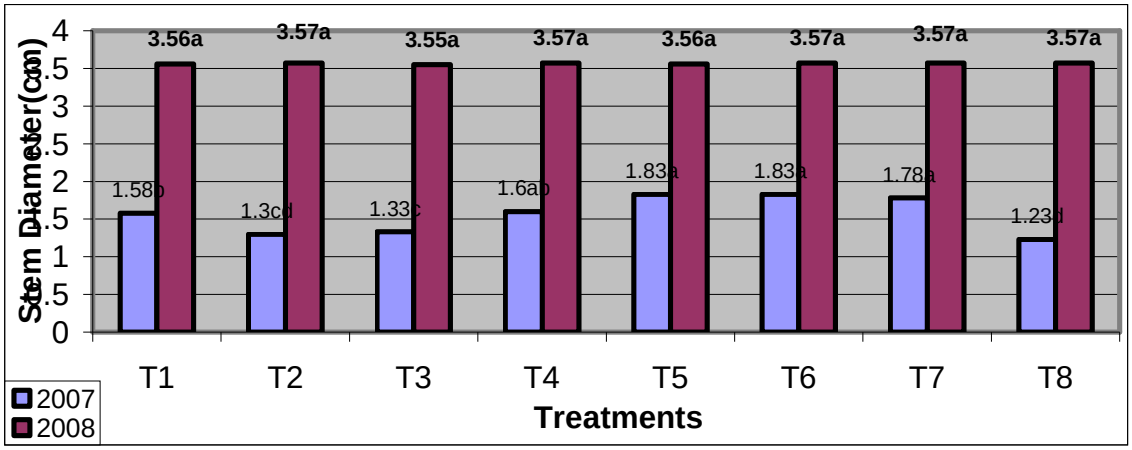


Fig (2): Effect of mineral, organic nitrogen and some other treatments on stem diameter(cm) of Picual olive cv. young trees in 2007 and 2008 seasons.

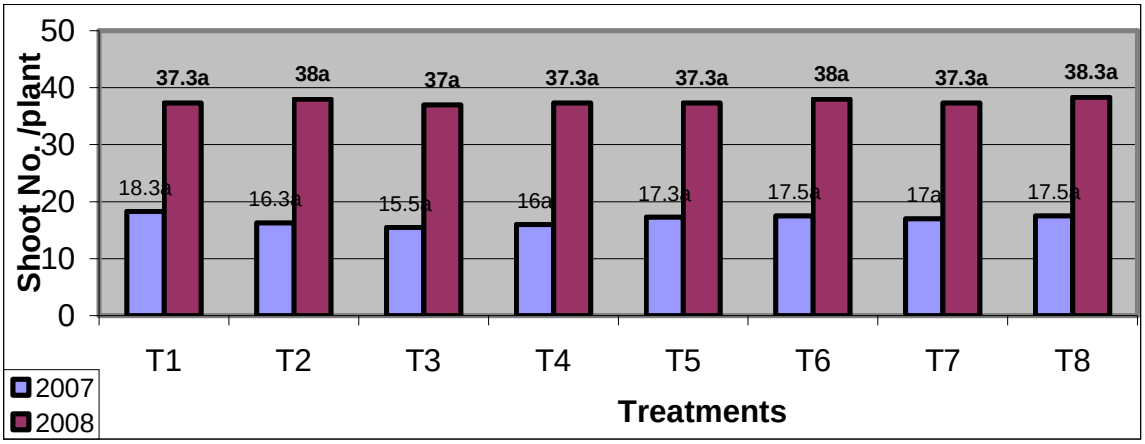


Fig (3): Effect of mineral, organic nitrogen and some other treatments on shoot No./plant of Picual olive cv. young trees in 2007 and 2008 seasons.

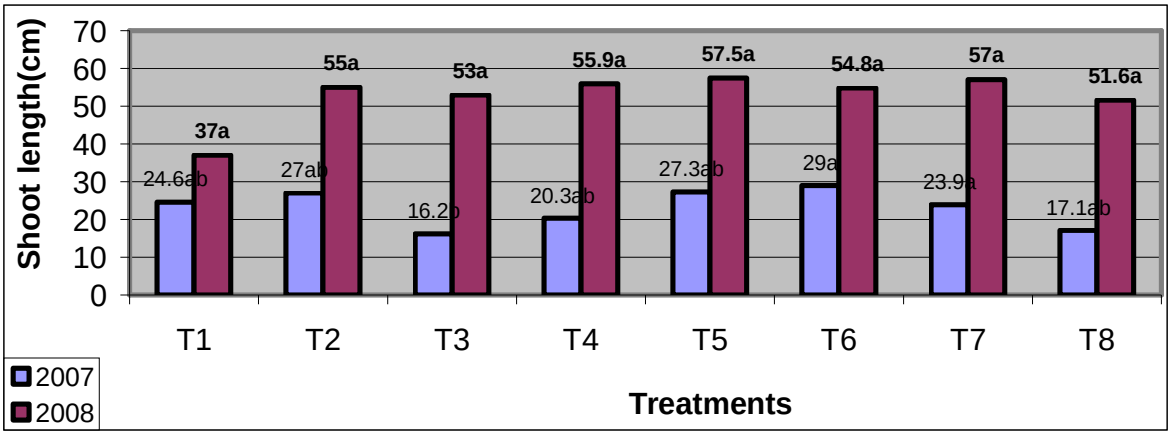


Fig (4): Effect of mineral, organic nitrogen and some other treatments on average shoot length(cm) of Picual olive cv. young trees in 2007 and 2008 seasons.

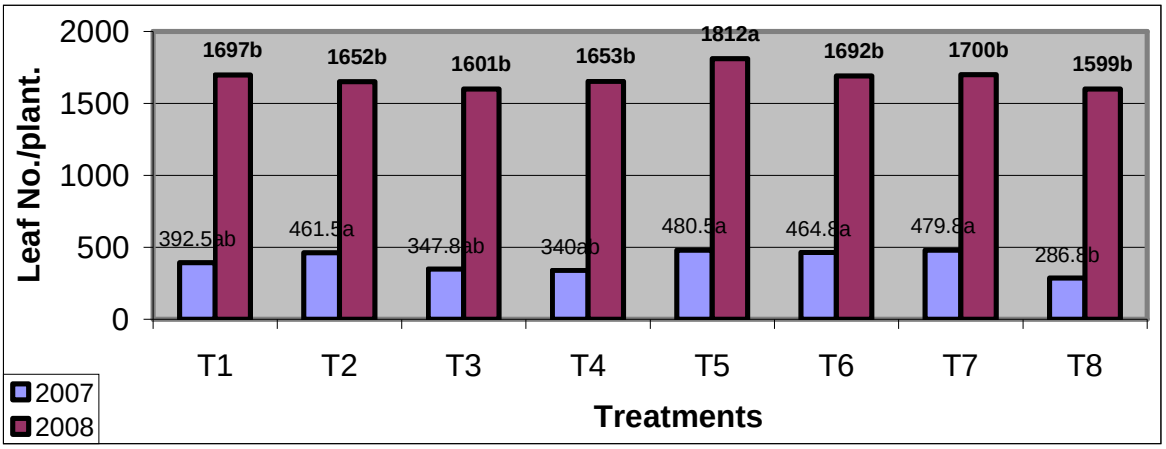


Fig (5): Effect of mineral, organic nitrogen and some other treatments on leaf no. /plant of Picual olive cv. young trees in 2007 and 2008 seasons.

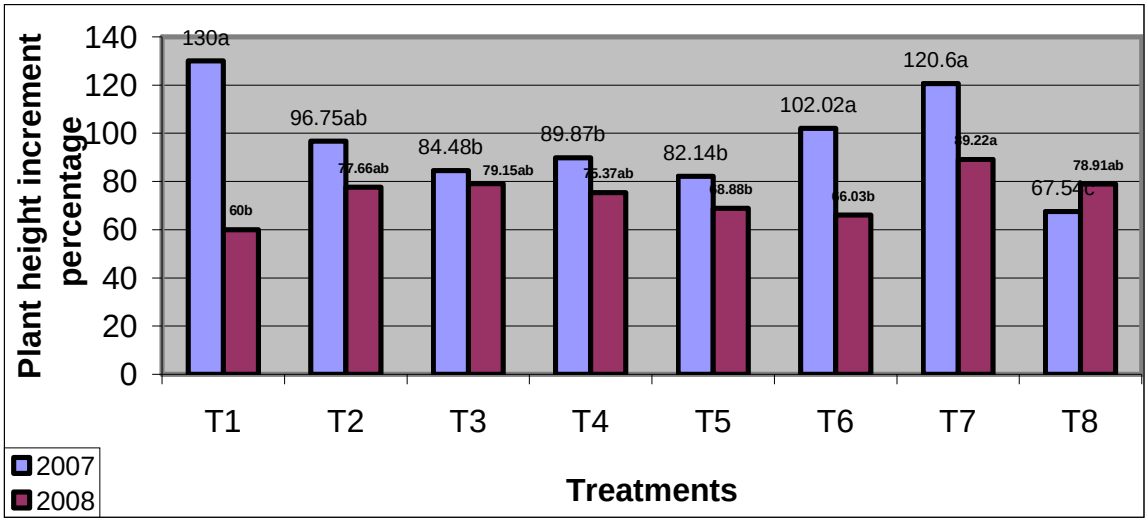


Fig (6): Effect of mineral, organic nitrogen and some other treatments on percentage of plant height increment of Picual olive cv. young trees in 2007 and 2008 seasons.

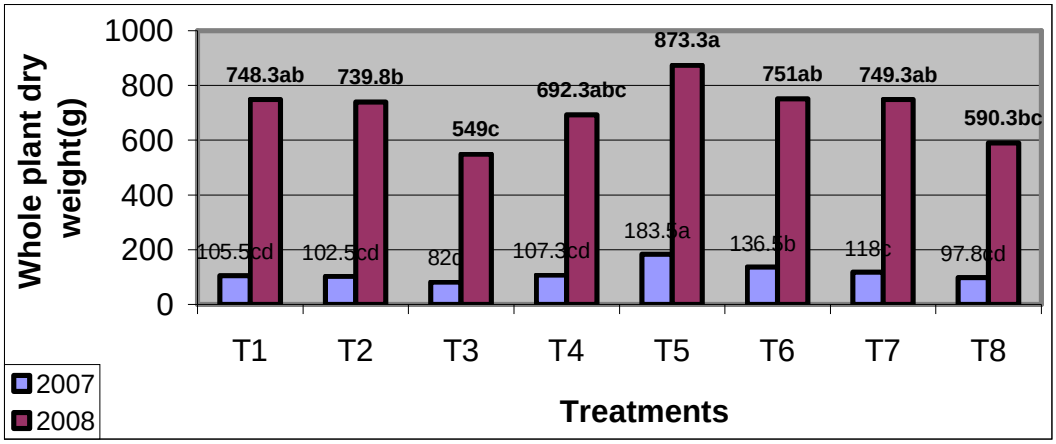


Fig (7): Effect of mineral, organic nitrogen and some other treatments on whole plant dry weight(g) of Picual olive cv. young trees in 2007 and 2008 seasons.

Finally it could be noticed that plant height, shoot number, shoot length, leaves number and stem diameter were not affected by different treatments in both seasons. Meanwhile, whole plants were improved by humic acid treatment compared with control and all other treatments in Picual cv. These results are harmony with those found by Fernández-Escobar *et. al.*(1999) they reported that, foliar application of leonardite extracts(humic substances extracted) under field conditions, stimulated shoot growth of young olive plants. Moreover we can added that, growth parameters were not affected by most treatment may be attributed to low nutritional demand of young olive trees as mentioned by Xiloyannis *et. al.* (2000) they showed that, demand of irrigated olive trees, cultivar Coratina for P and K is minimal during the first four years after planting and can be fulfilled by naturally supplied soils. Low doses of N should be applied through localized fertilization during the year. Moreover Nawaf and Yara (2006) found that, young olive trees benefit from low levels of NPK and N alone and additional fertilizers would not be significant. However, NPK are consider to be essential element for plant growth and development. The 16 g NPK and 32 g N significantly gave the highest shoot and root dry weight, this probably due to nitrogen concentration which increased dry matter accumulation in roots and decreased shoot: root ratio.

References

1. Abdel Fatah H. Gehan; Boshra A. El Sayed and S.M. Shahin (2008):The role of humic acid in reducing the harmful effect of irrigation with saline water on tifway turf.J. Bio. Chem. Environ. Sci...Vol.3 (1):75-89.
2. EmanA. Abd El Moniem and A.S.E. Abd-Allah (2008): Effect of algae extact as foliar spray on vegetative growth, yield and berries quality of Superior grapevines. America Eurasian J. Agic. &Environ.Sci., 4(4):427-433.
3. Fernández-Escobar R.; M. Benlloch; D. Barranco; A. Dueñas and J. A. Gutérrez Gañán (1999): Response of olive trees to foliar application of humic substances extracted from leonardite . Scientia Horticulturae Vol. 66, 3-4, October, Pages 191-200.
4. Hegazy, E. S.; M. R. El-Sonbaty ; Ahmed M. Dorria and T. F. El-Sharnoby (2007): Effect of organic and bio-fertilization on vegetative growth and flowering of Picual olive trees.Wold Journal of Agricultural Science 3(2):210-217.
5. M.A.L.R. (2007): Ministry of Agriculture and Land Reclamation Economic Affairs - Study of Important the Agriculture Statics, Vol.(2)p 353.
6. M.A.L.R. (2007a): Ministry of Agriculture and Land Reclamation.Modern technique for olive cultivation and production.Horticultur Reseasch Institute, Agriculture Research Center bulletin No. 15(in Arabic).
7. Monge, E., J. Val; J.L. Espada; F. Orús and J. Betran (2000): Effects of organic wastes on olive mineral nutrition and its influence on fruit quality. Preliminary results for macronutrients. Acta Horticulturae 512:199-208.
8. Mostafa, E. A. M. and M. S. Abou Raya (2003): Effect of soil application of active dry yeast on growth, yield and fruit qulity of Grand Nain banana cv.. Arab Univ. J. of Agric. Sci. Vol. 12(2):693-704.
9. Nawaf, M. Ferihat and Yara K. Masa`deh (2006): Response of two-year-old tree of four olive cultivars to fertilization. Amrican-Erasian J. Agric. & Environ. Sci., 1 (3): 185-190.
10. Smith D. J. And W. W. Schwabe (1984): Acceleration of early growth of seedlings and rooted cuttings of *Quercus robur* L.. Forestry , 57(2):143-157
11. Snedecor , G. W. and W. G. Cochran (1980) Statistical Methods . 7th Ed.507 pp Iowa State Univ. Press, Ames . Iowa , U.S.A.
12. Xiloyannis C.; G. Celano; A.M. Palese; B. Dichio and V. Nuzzo (2000): Mineral nutrient uptake from the soil in irrigated olive trees, cultivar Coratina, over six years after planting. Acta Horticulturae 586:

7/1/2010

Response of Picual Olive Young Trees to Mineral, Organic Nitrogen Fertilization and Some Other Treatments

M. Abou Rawash[2]; H. El-Wakeel[2]; Laila, F. Hagag[1] ; H. S. A. Hassan[1] and A. Abdel-Galel[1]

1-Pomology Department, National Research Center, Cairo, Egypt
2- Dept. of Hort. Fac. of Agric. Ain Shams Univ., Shobra El-Khiema, Cairo, Egypt

Abstract: This study was carried out through two successive seasons (2007& 2008) on a cultivated Picual olive young trees grown at the Research Station Farm of National Research Center, El Nobarya, El Behera governorate. The investigation aimed to study the effect of applying mineral, organic fertilizers and some other treatments on leaf mineral contents at the first two years of planting. Planting holes were prepared for control plants in the first season only. Each treatment received 100 g actual nitrogen/plant/year as recommended by M.A.R.L. (2007). The following treatments were applied: T1 : control (mineral nitrogen + planting hole preparation), T2(100%mineral nitrogen), T3(100% organic N as cattle manure), T4(50% mineral N + 50% organic N as chicken manure), T5 (100%mineral nitrogen + humic acid as soil application), T6(100% mineral nitrogen + activated dry yeast as soil application), T7 (100%mineral nitrogen + GA_3 spray) and T8 (100% mineral nitrogen + sea algae as soil application).At the end of each season, leaves dry weight per plant, and leaf mineral content were determined and recorded. The obtained results revealed that as follow: Effect of treatment on Leaves dry weight (g) per plant, fifth treatment with humic acid and sixth treatment with activated dry yeast gave the highest significant values in the first season, meanwhile in the second season fourth treatment with 50% cattle manure and fifth treatment with humic acid recorded higher significant values. Leaf nitrogen content revealed that first, fifth, sixth and seventh treatments showed higher significant values respectively than those of other treatments in the first season. In the second season, the first treatment had higher significant leaf nitrogen content compared with most of other treatments. [Journal of American Science. 2010;6(12):180-186]. (ISSN: 1545-1003).

Keywords: Picual Olive; Organic Nitrogen; Treatments

1. Introduction

The Egyptian olive production reached about 507053 tons produced from 110764 feddan and the total area reached about 135692 feddan (according to the statistics of M.A.L.R. (2007).

Xiloyannis *et. al.* (2000) working on mineral nutrient uptake from the soil in irrigated olive trees, cultivar Coratina, over six years after planting they recorded that, the nutrient demand was relatively steady during the different stages of the year. The results showed that demand for P and K is minimal during the first four years after planting and can be fulfilled by naturally supplied soils. Low doses of N should be applied through localized fertilization during the year. Nawaf and Yara (2006) found that, young olive trees benefit from low levels of NPK and N alone and additional fertilizers would not be significant. However, NPK are consider to be essential element for plant growth and development.

The 16 g NPK and 32 g N significantly gave the highest shoot and root dry weight, this probably due to nitrogen concentration which increased dry matter accumulation in roots and decreased shoot: root ratio.

Monge *et. al.*(2000) reported that, organic wastes fertilization did not lead to significant increases in olive mineral leaf concentrations in the first year trial. Hegazy *et. al.*(2007) studied the effect of organic and bio-fertilization on vegetative growth and flowering of Picual olive trees, they recorded that,. N and K contents in leaf increased significantly with applying 100% organic fertilization (poultry manure), but no significant difference was observed on leaf P content in both seasons. The same treatment gave the highest Fe leaf content in both seasons and Mn in the second season, while leaf Zn content increased in second season with using 100% mineral fertilization.

Fernández-Escobar *et. Al* .(1999) mentioned that, Under field conditions, foliar application of leonardite extracts(humic substances extracted) stimulated shoot growth and promoted the accumulation of K, B, Mg, Ca and Fe in leaves. However, when leaf N and leaf K values were below the threshold limit for the sufficiency range, foliar application of humic substances was ineffective to promote accumulation of these nutrients in leaves.

Abdel Fatah *et. al.*(2008) mentioned that, soil drench application of humic acid to Tifway Bermodagrass hybrid improved growth parameters and NPK leaves cotents.

Mostafa and Abou Raya (2003) recorded that, all dry yeast soil application improved growth parameters of Grand Nain banana cv. Compared with control without dry yeast treatment.

Smith and Schwabe (1984) recorded that, top growth of *Quercus robur* could be further accelerated by application of gibberellic acid (GA3) as foliar spray. Eman and Abd-Allah (2008) reported that, progressive increase on percentages of N, P, and K in the Superior grapevine leaves was observed as a results of increasing concentration of algae till 50%.

This investigation aimed to study the effect of mineral and organic nitrogen fertilization sources and some other treatments(humic acid, activated dry yeast, GA3 and sea algae)on leaf mineral contents of Picual young trees at first two years of planting. That to improve and push tree growth through these years.

2. Material and Methods

This study was carried out through two successive seasons (2007& 2008) on Picual cv. young trees in the Expermental research station of National Research Center at El Nobarya, El Behera governorate Egypt. The investigation aimed to study the effect of applying mineral, organic nitrogen fertilizers and some other treatments on vegetative growth characters and leaf mineral contents of young Picual olive cv. trees at the first two years of planting. The soil was characterized by : pH = 8.82, EC =1.11 dS/m, organic matter = 0.31%, CaCO3 =12.8 %, Sand = 63 %,Silt = 13 % and clay = 3%. The soil texture grade was sandy. Drip irrigation system was applied using river Nile water. Planting distance was 5 × 5 meters apart.

In control plots, planting holes were prepared by adding 50 kg cattle manure, 1kg super phosphate, 1/4 kg potassium sulfate and 1/2 kg agricultural sulfur and each treatment received 100 g actual nitrogen/plant/year in each season as recommended by M.A.R.L. (2007a).

The following treatments were applied:

1- Control: recommendation of M.A.R.L. (2007a) (100g actual nitrogen 500 g ammonium sulfate as mineral nitrogen source) + planting holes preparation.
2- Mineral nitrogen only 100 %.
3- Organic nitrogen source 100 % (cattle manure 100g actual nitrogen).
4- Mineral nitrogen source 50 % + organic nitrogen source 50 % (chicken manure).
5- Mineral nitrogen source 100 % + humic acid (monthly doses from March to November each 20 ml/plant).
6- Mineral nitrogen source 100 % + actived dry yeast as drench treatment three times in March, July and October each at 30 g/plant.
7- Mineral nitrogen source 100 % + one spray of GA3 acid at 50 ppm in March.
8- Mineral nitrogen source 50 % + sea algae in March and June each at 50 g/plant.

- Cattle manure analysis was: N = 1.6%, P = 0 46% and K = 0.51%.
- Chicken manure analysis was: N =3.47%, P =0.67% and K = 0.64%.
- Sea algae analysis : N =8%, P = 2%, K = 4%, chelate microelements = 4% and traces of vitamins + amino acids

Ammonium sulfate was divided into five equal doses through growing season. All these treatments were repeated in the second season except holes preparation with control plants only in the first season. The treatments were arranged in randomized complete block design in a simple experiment with four replicates for each treatment and each replicate was represented by one plant. At the end of each season at mid November four plants as replicates for each treatment were removed genteelly with their root system to estimate and record the following data for each cv individually:

1- Leaves dry weight (g) per plant.
2-Leaf mineral content was determined as follow:

The leaves of each young tree at the end of each season were washed several times with tap water then rinsed with distilled water, dried at 70^{o}C in an electric oven, grounded in electric mill and digested according to (Chapman and Prat, 1961). Nitrogen analyses were determined by Micro Kjeldahl method (Jakson, 1967). Phosphorus was determined by the method of (Truog and Meyer, 1929). Potassium was determined by the the flame photometer according to the method of (Brown and Lilleland, 1946). Calcium and magnesium were determined by titration against versenate solution (Chapman and Pratt, 1961). Iron, zinc and manganese were determined by using Atomic Absorption technique. All these macro and micro elements were determined through the two studied seasons. Data obtained throughout this study were statistically analyzed using the analysis of variance method as reported by (Snedecor and Cochran, 1980), and the differences between means were differentiated by using Duncan's range test.

3. Results and Discussions

1- Effect of treatment on Leaves dry weight (g) per plant. Fig. (1) show that, fifth treatment with humic acid and sixth treatment with activated dry yeast gave the highest significant values (46.5 & 43.5), respectively compared with all other treatments in the first season, meanwhile in the second season fourth treatment with 50% cattle manure and fifth treatment with humic acid recorded higher significant values (255.8 &269.3 respectively) compared with most of other treatments.

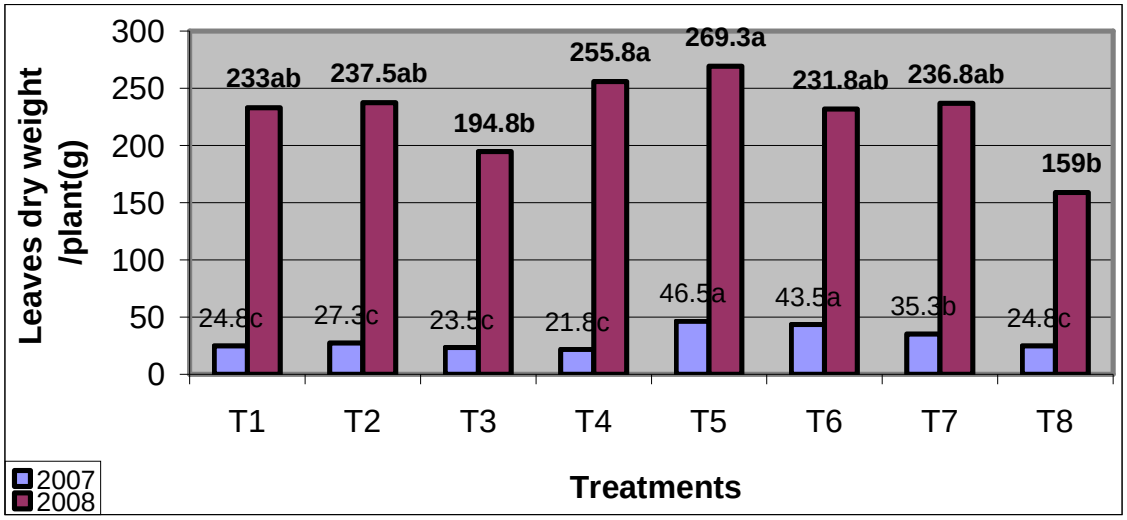


Fig (1): Effect of mineral, organic nitrogen and some other treatments on leaves dry weight/plant(g) of Picual olive cv. young trees in 2007 and 2008 seasons.

2-Effect of treatment on leaf mineral content:

Leaf nitrogen content:

Fig.(2) show that, Leaf nitrogen content revealed that first, fifth, sixth and seventh treatments showed higher significant values (1.51, 1.54, 1.52 & 1.54) respectively than those of other treatments in the first season. In the second season, the first treatment had higher significant leaf nitrogen content (1.54) compared with most of other treatments.

Leaf phosphorus content:

Fig. (3) Show that, Leaf phosphorus content recorded insignificant differences among treatments in both season.

Leaf potassium content:

Fig.(4) show that, Leaf potassium content, differences among treatments lake significances in the first season. In the second season, second treatment with 100% mineral nitrogen gave the highest significant value (0.39) of leaf potassium content compared with all other treatments.

Leaf calcium content:

Fig.(5) show that, Leaf calcium content with the first, fifth, sixth and eighth had higher significant values than those of other treatments in the first season.

In the second season, seventh treatment by GA3 spray showed higher significant leaf calcium content (1.89) compared with most of other treatments.

Leaf magnesium content:

Fig.(6) show that, Leaf magnesium content had lower significant values with the third and eighth treatments (0.17&0.18 respectively), compared with all other treatments in the first season. In the second season, second treatment with 100% mineral nitrogen had the highest significant leaf magnesium content (0.92) compared with all other treatments.

Leaf iron content:

Fig. (7) show that, Leaf iron content differences among treatments lake significance in the first season. In the second season, second treatment with 100% mineral nitrogen recorded higher significant leaf iron content (2373) compared with most of other treatments.

Leaf zinc content:

Fig. (8) show that, Leaf zinc content with the fifth treatment by humic acid recorded highest significant.

In the second season, second treatment with 100% mineral nitrogen showed higher significant leaf zinc content (52) compared with most of other treatments.

Leaf manganese content:

Fig.(9) show that, Leaf manganese content gave insignificant differences among treatments in the first season. In the second season, fifth treatment with humic acid had higher significant leaf manganese content (42.3) compared with all other treatments. Value (40) compared with all other treatments in the first Season.

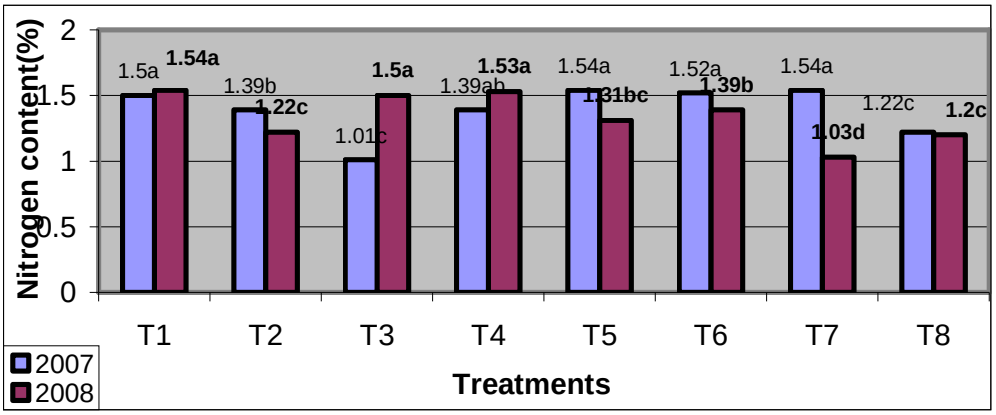


Fig (2): Effect of mineral, organic nitrogen and some other treatments on leaf nitrogen content(%) of Picual olive cv. young trees in 2007 and 2008 seasons.

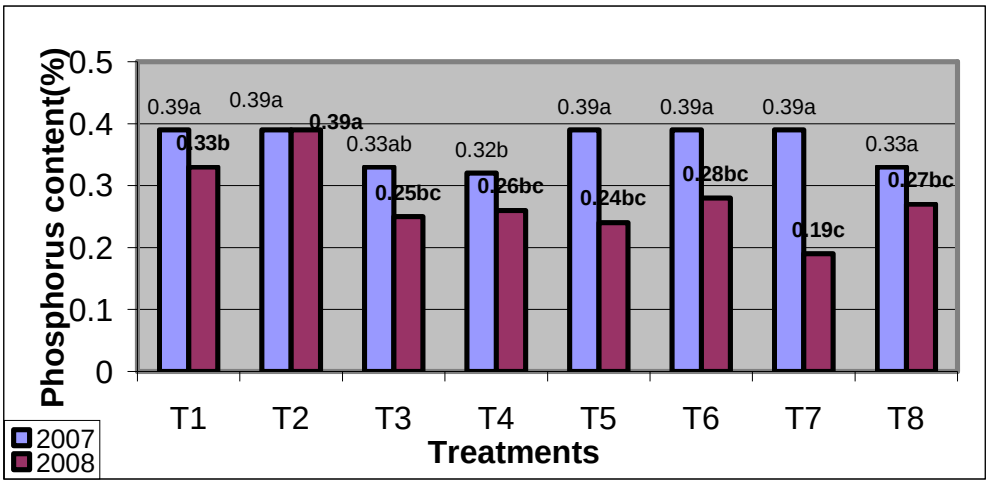


Fig (3): Effect of mineral, organic nitrogen and some other treatments on leaf phosphorus content(%) of Picual olive cv. young trees in 2007 and 2008 seasons

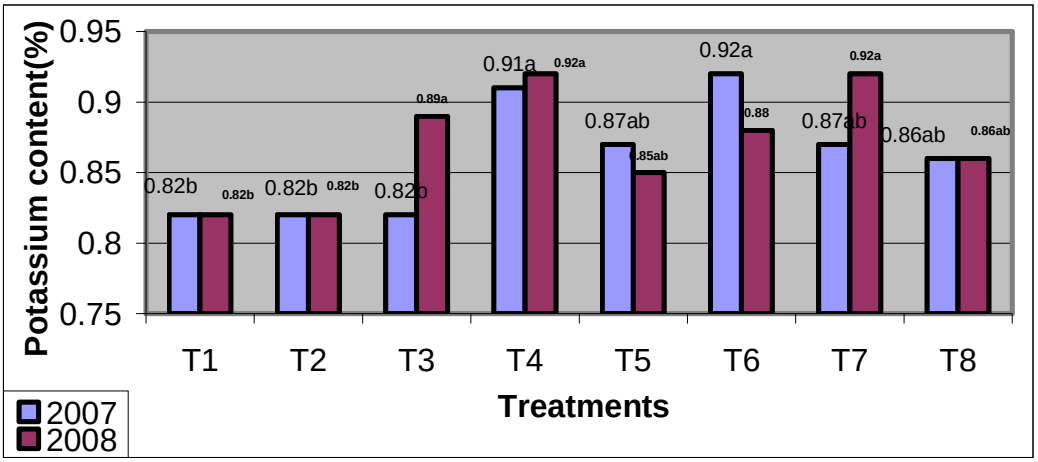


Fig (4): Effect of mineral, organic nitrogen and some other treatments on leaf potassium content(%) of Picual olive cv. young trees in 2007 and 2008 seasons.

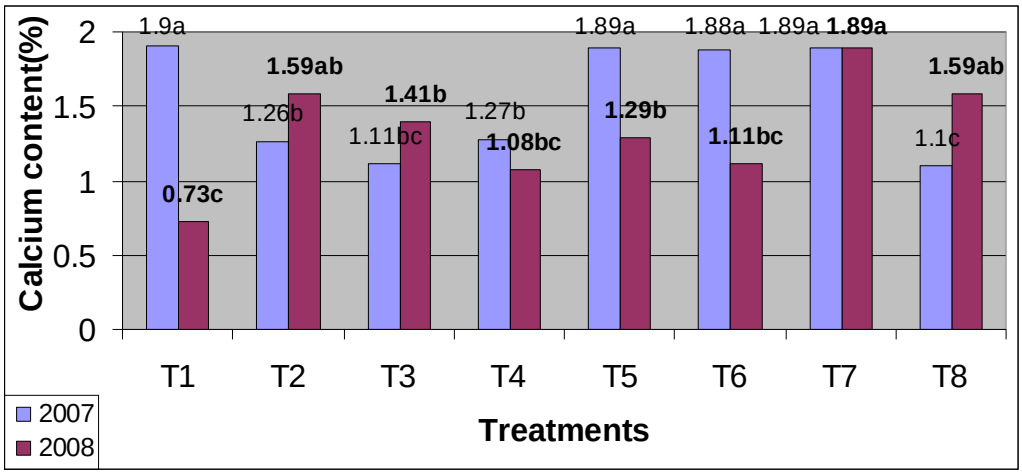


Fig (5): Effect of mineral, organic nitrogen and some other treatments on leaf calcium content(%) of Picual olive cv. young trees in 2007 and 2008 seasons.

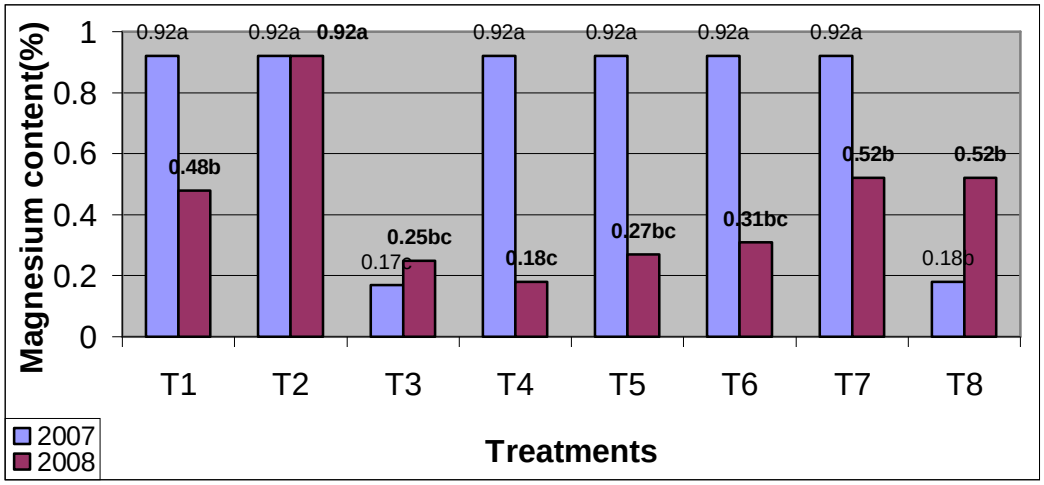


Fig (6): Effect of mineral, organic nitrogen and some other treatments on leaf magnesium content(%) of Picual olive cv. young trees in 2007 and 2008 seasons.

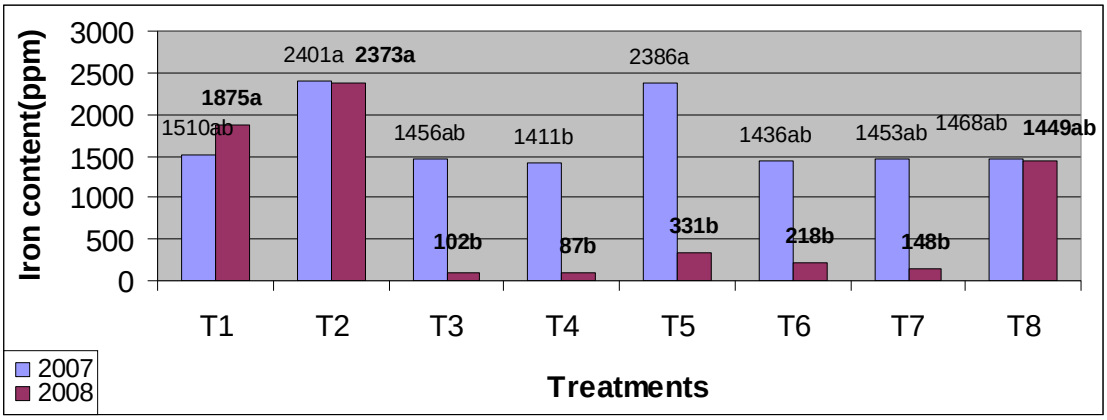


Fig (7): Effect of mineral, organic nitrogen and some other treatments on leaf iron content(ppm) of Picual olive cv. young trees in 2007 and 2008 seasons.

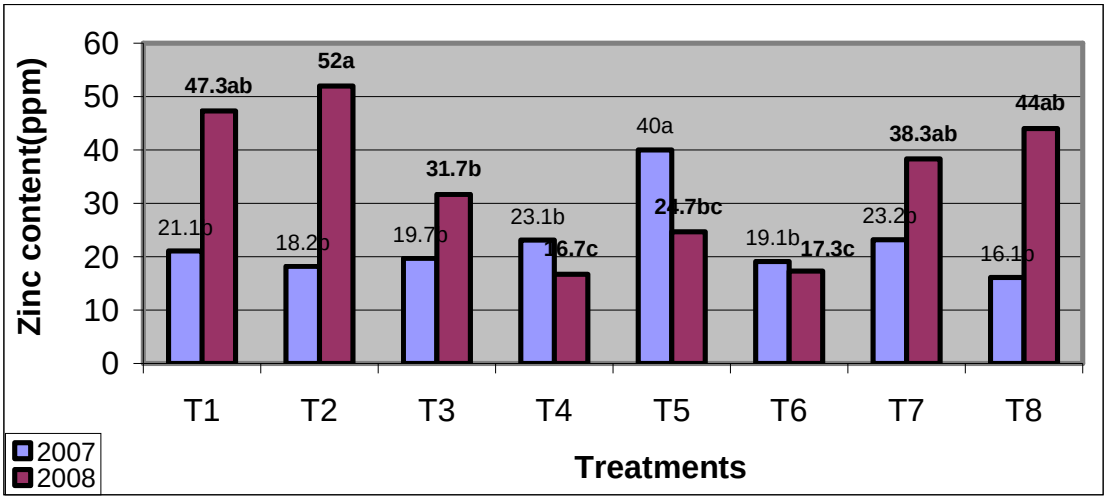


Fig (8): Effect of mineral, organic nitrogen and some other treatments on leaf zinc content(ppm) of Picual olive cv. young trees in 2007 and 2008 seasons

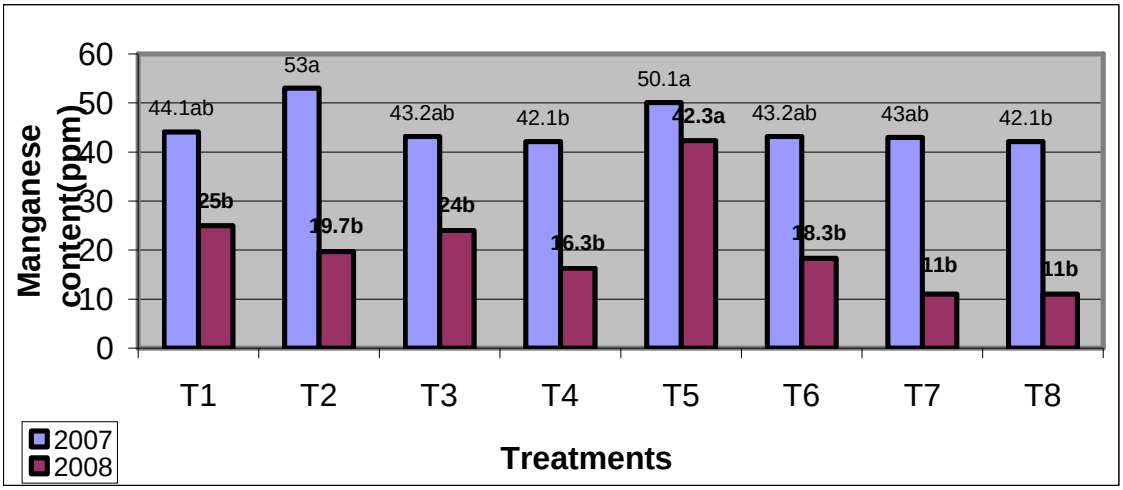


Fig (9): Effect of mineral, organic nitrogen and some other treatments on leaf manganese content(ppm) of Picual olive cv. young trees in 2007 and 2008 seasons.

Finally it could be noticed that, effect of treatment on Leaves dry weight (g) per plant, fifth treatment with humic acid and sixth treatment with activated dry yeast gave the highest significant values in the first season, meanwhile in the second season fourth treatment with 50% cattle manure and fifth treatment with humic acid recorded higher significant values. Leaf nitrogen content revealed that first, fifth, sixth and seventh treatments showed higher significant values, respectively than those of other treatments in the first season. In the second season, the first treatment had higher significant leaf nitrogen content compared with most of other treatments These results are harmony with those found by Fernández-Escobar *et. al.*(1999) they reported that, foliar application of leonardite extracts(humic substances extracted) under field conditions, stimulated plant growth of young olive plants. Moreover we can added that, growth parameters were not affected by most treatment may be attributed to low nutritional demand of young olive trees as mentioned by Xiloyannis *et. al.* (2000) They showed that, demand of irrigated olive trees, cultivar Coratina for P and K is minimal during the first four years after planting and can be fulfilled by naturally supplied soils. Low doses of N should be applied through localized fertilization during the year. Moreover Nawaf and Yara (2006) found that, young olive trees benefit from low levels of NPK and N alone and additional fertilizers would not be significant. However, NPK are consider to be essential element for plant growth and development. The 16 g NPK and 32 g N significantly gave the highest shoot and root dry weight, this probably due

to nitrogen concentration which increased dry matter accumulation in roots and decreased shoot: root ratio. From obtained data P, K, Ca and Mg were higher than their critical levels but nitrogen was lower than it's critical level with most treatments especially in the second season that may be attributed to more vegetative growth in second season than first one which need more demand of nitrogen. These results are contrary with those found by Hegazy *et. al.*(2007) Whose reported that the applying 100% organic fertilization (poultry manure) to Picual olive trees gave the highest Fe leaf content in both seasons and Mn in the second season, while leaf Zn content increased in second season with using 100% mineral fertilization.

References

1. Abdel Fatah H. Gehan; Boshra A. El Sayed and S.M. Shahin (2008): The role of humic acid in reducing the harmful effect of irrigation with saline water on tifway turf.J. Bio. Chem. Environ. Sci..Vol.3(1):75-89.
2. Brown, J. D. and O. Lilleland (1946) Uptake determination of potassium and sodium in plant material and soil extracts by Flame photometry. Proc. Am. Soc., 48:341-346.
3. Chapman , H. D. and P. F. Pratt (1961) Method of Analysis for Soils , Plants and Waters , Univ. California, Div. Agric. Sci. Priced Pub. 4034.
4. EmanA. Abd El Moniem and A.S.E. Abd-Allah (2008): Effect of algae extact as foliar spray on vegetative growth, yield and berries quality of Superior grapevines. America Eurasian J.Agic.& Environ. Sci.,4(4):427-433.
5. Fernández-Escobar R. ; M. Benlloch; D. Barranco; A. Dueñas and J. A. Gutérrez Gañán (1999): Response of olive trees to foliar application of humic substances extracted from leonardite . Scientia Horticulturae Vol. 66, 3-4, October, Pages 191-200.
6. Hegazy, E. S.; M. R. El-Sonbaty ; Ahmed M. Dorria and T. F. El-Sharnoby (2007): Effect of organic and bio-fertilization on vegetative growth and flowering of Picual olive trees.Wold Journal of Agricultural Science 3(2):210-217.
7. Jakson , M. L. (1967) Soil chemical analysis , Makhija Offset Press, New Delhi, India.
8. M.A.L.R. (2007): Ministry of Agriculture and Land Reclamation Economic Affairs - Study of Important the Agriculture Statics, Vol.(2)p 353.
9. M.A.L.R. (2007a): Ministry of Agriculture and Land Reclamation.Modern technique for olive cultivation and production.Horticultur Reseasch Institute, Agriculture Research Center bulletin No. 15(in Arabic).
10. Monge, E., J. Val; J.L. Espada; F. Orús and J. Betran (2000): Effects of organic wastes on olive mineral nutrition and its influence on fruit quality. Preliminary results for macronutrients. Acta Horticulturae 512:199-208.
11. Mostafa, E. A. M. and M. S. Abou Raya (2003): Effect of soil application of active dry yeast on growth, yield and fruit qulity of Grand Nain banana cv.. Arab Univ. J. of Agric. Sci. Vol. 12(2):693-704.
12. Nawaf, M. Ferihat and Yara K. Masa`deh (2006): Response of two-year-old tree of four olive cultivars to fertilization. Amrican-Erasian J. Agric. & Environ. Sci., 1 (3): 185-190.
13. Smith D. J. And W. W. Schwabe (1984): Acceleration of early growth of seedlings and rooted cuttings of *Quercus robur* L.. Forestry , 57(2):143-157
14. Snedecor, G. W. and W. G. Cochran (1980) Statistical Methods. 7th Ed.507 pp Iowa State Univ. Press, Ames . Iowa, U.S.A.
15. Truog, E. and A. H. Meyer (1929) Improvement of the Deniges, coloremetric method for phosphorus and arsenic .Ind. Eng. Chem. Asnal. Ed.1: 136-139.
16. Xiloyannis C.; G. Celano; A.M. Palese; B. Dichio and V. Nuzzo (2000): Mineral nutrient uptake from the soil in irrigated olive trees, cultivar Coratina, over six years after planting. Acta Horticulturae 586:

7/2/2010

Effect of Bud Load on Bud Behavior, Yield, Cluster Characteristics and some Biochemical Contents of the Cane of Crimson Seedless Grapevines

[1]Fawzi M. I. F. [1]Shahin M. F. M. and [2]Kandil E. A.

[1]Pomology Department, National Research Center. [2]Horticulture Research Institute agriculture Research Center, Giza, Egypt

Abstract: This study was conducted through the seasons of 2007 and 2008 to determine the optimum bud loads/ vine for Crimson seedless "grapevines. Eight years old uniform vines were chosen and pruned to six different levels of bud load, namely 78, 91, 104, 117, 130 and 143 buds/ vine. Number of buds was fixed at 13 bud/cane. The results showed that the number of bursted buds was increased significantly by increasing bud load /vine in the two seasons of the study, while the percentage of bursted buds decreased. The bud fertility and fruitfulness were decreased by increasing bud load. Data also indicated that 104 or 117 buds/ vine were more suitable for Cirimson seedless grapevines to produce good yield and fruit quality. On the other hand, 78 or 143 buds/vine was unfavorable science it produced rather compact clusters. Increasing bud load increased number of cluster/vine and yield but reduced cluster weight. Vines pruned to 117 bud/vine gave the greatest cluster weight, length, rachis weight, berry weight, berry firmness, adherence, T.S.S and total sugars. Increasing bud load on the vine significantly increased total carbohydrates and protein contents of the canes during the dormant season. In this respect, vines pruned to 143 bud/vine showed higher percent of both total carbohydrate and protein contents than the other levels of bud load. [Journal of American Science. 2010;6(12):187-194]. (ISSN: 1545-1003).

Key words: Grapevine, winter pruning Crimson seedless, bud load, fruit quality.

1. Introduction:

Grape (Vitis vinifera. L.) is considered on of the most important fruits in the world. "Crimson seedless" is one of the new cultivars which were introduced to Egypt. It is a late - ripening cultivar with medium cluster in size (weight = 0.5kg; length 20cm) conical shaped with shoulders the cluster is well filled to slightly compact, the berries are medium in size 4.0g, 16.6mm in diameter (Ramming et. al., 1995) This cultivar holds significant promises for the Egyptian commercial producers and exporters due to its late maturity date as well as its seedless berries and the crispy texture of berries.

Pruning is considered the most important practice through which grape production can be increased and cluster quality improved. The basal 3-4 buds of the cultivar are less fruitful, so using long fruit canes is important for production normal crop.

Bud load is the most important factor affecting yield and cluster quality as well as vine vigor of Thompson seedless grapevines Morris and Cawthon (1980); Fawzi et at., 1984 ; Marwad et. al., (1993); Omar and Abdel-kawi, 2000; Rubio et al, (2002) on Tempranillo variety and El-Baz et. al; (2002) on Crimson seedless grapevines.

The objective of this study is to determine the optimum bud load per vine for Crimson seedless grape and to study the effect of bud load on bud behavior, cluster quality, yield per vine and total carbohydrates and proteins in the mature canes during the dormant season.

2. Materials and Methods:

This work was carried out in a private vineyard located beside the desert road of Alexandria - Cairo on 8 years - old Crimson seedless grapevines. This study extended for two successive years (2007 and 2008) the vines were grown 1.5 X4.0 meters apart in sandy soil under drip irrigation and trained according to cane pruning under, (Gable) trellis system. At pruning time (at winter) seventy two vines of almost similar vigor were selected and pruning to different bud load levels with fixed the length of canes at 13 bud/cane. The vines received the usual and recommended agriculture practices

The experimental treatments applied were as follows:

T1 - 6 canes X 13 bud/cane = 78 buds
T2- 7 canes X 13 bud /cane = 91 buds
T3- 8 canes X 13 bud /cane = 104 buds
T4- 9 canes X 13 bud /cane = 117 buds
T5- 10 canes X 13 bud /cane = 130 buds
T6- 11 canes X 13 bud /cane = 143 buds

In addition to the renewal spur (2 bud) per each cane. Each treatment contained three replicate of four vines per each. The randomized complete block

design was carried out. The following parameters were investigated for each experimental vine.

Bud behavior:

During the spring of each season number of bursted bud and fruitful buds were counted, then the percentages of bud burst, fertility and fruitfulness were calculated according to Bessis (1960) Bud burst % (number of bursted buds divided by total number of bud, per vine X 100); Bud fertility % (number of cluster per vine divided by total number of bud per vine X 100) and Fruitfulness % (number of fruitful buds dividing by number of bursted buds X 100). Also, numbers of clusters per vine were counted.

Yield / vine:

Average yield / vine was determined an average of number of clusters / vine and average weight of cluster / vine in kilograms at harvesting date (first - October) when average T.S.S % in berry juice reached about 19-20% according to Ramming et al., (1995) and Samra (1998).

Representative samples per each replicate were harvested and taken to laboratory to determine the following characters:

Physical characteristics of clusters:

Average of: cluster weight (g); rachis weight (g); cluster length (cm); number of berries/ cluster; cluster index (Av cluster weight divided by Av. weight of rachis/ cluster); compactness coefficient ratio (Av. number of berries/ cluster divided by Av. cluster length (cm)) according to Winkler (1962).

Physical characteristics of berries:

Samples of 100 berries from each replicate were collected at random to determine an average of berry weight (g); berry index (Av. number of berries presented in 100 gm of cluster); berry firmness and adherence by using push-pull (Dynahometer Model DT 101).

Chemical characteristics of berries:

Berry Juice was extracted and filtered through two Layers of cheese cloth to determine: total soluble solids percentage using a hand refractometer and acidity by titrating 10 ml juice sample against. NaoH (0.1 N).

Acidity was expressed as g tartaric acid/ 100 ml juice according to A.O.A.C. (1980); T.S.S / acid ratio was calculated and total sugar (Smith et al., 1956).

Determination of total carbohydrates and proteins in the canes:

At winter pruning sample of ripened canes were collected and used for determination:

Total carbohydrates were determined according to methods of Pulmmer, (1971). The obtained results of total carbohydrates were presented as g/100 J dry weight.

Total proteins were extracted in solution of 10% S.D.S, 1% mercaptoethanol, 65 ml tris/ Hcl, PH 6.8 as described by Dure & Chlan (1981) and Oster et al., (1981). Protein content was determined spectrophotometrically at 595nm according to Bradford (1976) and results were expressed as g/ 100 g dry weight of the canes.

The obtained data were tabulated and statistically analyzed according to Mead et al. (1993) using the new L.S.D at 5% parameter to compare the differences between various treatments.

3. Results and Discussion:

Bud behaviour:

From table (1) it is clear that the number bursted bud was increased significantly by increasing the number of bud load/ vine in the two seasons 2007 and 2008; The highest value was (102.33 & 103.10) for T6 i.e (143 buds/vine) in the two seasons respectively. On the other hand increasing bud load/vine significantly decreased bud burst percentage.

The highest bud burst percentage was associated with the lower bud load 78 and 91 buds/vine. The same results were obtained by Christensen et al., (1994) and Omar& Abdel - kawi (2000) showed that increasing bud load induced lower percentage of bud burst.

In addition, Ramming et al., (1995) reported that pruned Crimson seedless as a spur pruning produced a lower number of shoot/vine compared with the cane pruning. Thus vines pruned to 10 canes resulted in a significant higher number of shoots from canes than those were 6 or 8 canes/vine. Since vines with 6 canes gave a lower number of shoots/ vine.

As regarded percentages of bud fertility and fruitfulness, it is clear from the same table that percentage of bud fertility and fruitfulness were affected by bud load/ vine in this respect, the highest values were (38.32 & 37.32%) for TI (78 bud) per vine in the two seasons, respectively. Thus, vines pruned to (143 buds)/ vine gave lower bud fertility it recorded (28.83 & 29.48%) at the two seasons 2007 and 2008 respectively.

Concerning the data of fruitfulness vines pruned to (78 buds)/vine were the highest, while, vines pruned to (143 buds)/ vine were the lowest one. The obtained data go in line with those reported by Morris and Couthon (1980) they reported that vines with 30 or 60 buds/ per vine produced a higher estimated fruitfulness than that of 90 buds per vine. Recently Salem et al., (1997) mentioned that when Thompson seedless grapevine were pruned to 72, 84, 96, 108

buds per vine, leaving 96 or 108 buds produced the greatest growth and the lowest percentage of fruitful buds. The low fruitfulness of Crimson seedless grape was due to the high vigor vine, whereas moderated vigor it is usually more fruitful. According to the foregoing results, it could be conclude that Crimson seedless grapevines, which have high growth, get low fruitfulness, whereas the moderated vigor ones are usually more fruitful.

Number of clusters and yield/vine

Data of table (2) clearly indicated that number of cluster/vine were increased significantly as bud load was increased. It is obvious that treatment of (143 buds) / vine gave the highest number of clusters/ vine; it recorded (41.23 & 42.16) in the two seasons, respectively. Whereas leaving (78 buds)/ vine gave a lower significantly number of cluster which recorded (28.33 & 29.11) in both seasons, respectively. Yet, there are no clear differences on the number of clusters that had been obtained by leaving 117 buds or 130 buds/ vine. Similar results ware observed by Omar and Abdel-kawi (2000).

From the same table showed that the yield/ vine was significantly increased by increasing bud load. Moreover, the highest yield/ vine was obtained by those vine pruned to 104 buds & 117 buds/ vine which recorded (13.25 & 13.57kg/ vine) and (15.28 & 16.14kg/ vine in the two seasons, respectively. This increment in vine yield may be attributed increase in both number of clusters/ vine and their weight.

These results in this connection agree with those obtained by Ali, et al., (2000) and Omar & Abdel-kawi (2000) on Thompson seedless grapevines.

Physical characteristics of clusters.

Data in table (3) indicated that increasing the bud load/ vine reduced the average weight of cluster, thus, average cluster weight was least in vines pruned to 143 buds/ vine. Furthermore, leaving 104 buds or 117 buds/vine gave a slight increase in average cluster weight, without significantly differences amonge them. These results agreed with those findings of Omar & Abdel-kawi (2000) who reported that number of bunches per vine increased significantly by increasing the bud load per vine of Thompson seedless, but bunch weight decreased by increasing bud load.

Regarding rachis weight and cluster length data presented in Table (3) indicated that the effect of the different used treatment on rachis weight and cluster length were almost to that of cluster weight.

It is clear from Table (3) generally that the number of berries/ cluster of Crimson seedless grape was found to range between 81.34 – 103.05

These results in this respect are harmony with the findings found of El-Buz et. al., (2002) who that the number of berries/ cluster of Crimson seedless grape was found to range between 98 - 10 7.5. It is also clear from table (3) that clusters compactness values were highest in vine pruned to 91 ar 143 buds/vine. However, vines pruned to 104 as 117 buds/vine had recorded the lowest values of cluster compactness it recorded (3.03 & 3.54) and 3.89 & 3.64) in the two seasons, respectively. This means that bud load of 104 ar 117 buds/vine is stubble for Crimson seedless grape vines to good yield and slight compactness of cluster Moreover, Ramming et al., (1995) and Pommer et al. (1990) mentioned that cluster of Crimson seedless are medium in length and therefore are slight compact.

Concerning cluster index, it is clear from Table (3) that the highest values was found for vines pruned to 78 buds/vine it recorded (38.17 and 38.29) in the two seasons, respectively this increase could be attributed to the increases in fruit setting at this treatment (Marwad et. al., (1993); Rizk et al., 1994; Rizk, 1996 and Ali et al., 2000).

Physical characteristics of berries:

Data of table (4) revealed that the highest values of berry weight was obtained by vines pruned to 117 buds/ vine in the two seasons it is recorded (4-10 & 4.30g), respectively, This increase in berry weight was due to the increase of the cluster weight to the same treatment. (Abdel -Fattah, et al., 1993 and Rizk, (1996) and Ali, et al., 2000).

As for berry index it is clear from table (4) that the highest value was found for vines to 78 buds or 143 buds / vine, Data recorded (28.14 & 27.75 and 28.51 & 28.52) in the two seasons 2007 and 2008, respectively.

Table (4) showed that berry firmness of Crimson seedless grape was higher than berry adherence under different bud loads/ vine. Also, vines pruned to 117 buds / vine inhibited a slight effect on both berry firmness and adherence. No significant differences were noticed in berry firmness and adherence between vines pruned 78 buds were obtained by El-Baz et al., (2002).

Chemical characteristics of berries:

From table (5) it is clear that vines pruned to 117 buds/vine gave the highest significant values concerning T.S.S %, This treatment recorded (22.10 & 23.30%) during 2007 and 2008 seasons, respectively. Vines pruned to 104 buds/vine to the second rank. Vines pruned to 78 buds / vine gave the lower values, in this respect which recorded (19.50& 19.83 %) in the two seasons, respectively. The results agreed with those findings of Omar and Abdel -kawi (2000) who reported that increasing bud loads up to 72 buds/ vine

significantly increased berry T.S.S%, and leaving 48 or 120 buds/ vine recorded lower T.S.S % with no significantly differences with them. Similar results was found by Howell et al., (1991) Abdel - Fattah et al., (1993), Marwad (1993); Rizk, (1996) and Howell & Strieglar (1998) who reported a significant increase in T.S.S with pruning vines to short compared with long cane pruning. On the contrary, Ramming (1995) indicated that T.S.S% was not affected markedly by pruning levels of Crimson seedless. As shown table (5) it is evident that acidity % was increased by increasing bud load/vine. Vines pruned to 143 buds/ vine recorded the highest values of acidity % where it was (0.50 & 0.53%) in the two seasons, respectively. In this respect, El-Baz et al., (2002) found that leaving to 10 canes/ vine gave a slight increment of acidity % in berry juice than above that maintained on leaving 8 canes/ vine but the differences between them were not significant. Yet, vines pruned to 8 canes with 12 nodes produced a lower total acidity % than the other pruning severity of Crimson seedless grapevines. Badr (1997) reported that titratable acidity of crimson seedless was not affect markedly either under cane are spur pruning Also, Ramming et al. (1995) mentioned that, pruning levels gave no clear effect on the titratable acidity of berry jucie of Crimson seedless.

It is clear from Table (5) that the effect bud load /vine on T.S.S/ acid ratio similar to that concerning in total soluble solids.

The same table indicated that total suger decreased by increasing bud load/vine. The least values was found in vines pruned to 143 buds/ vine. It recorded (15.36 and % 15.33) in the two seasons, respectively. However total sugar % was higher in vines pruned to 117 buds / vine which recorded (17.59 and 17.98 %) in the both seasons, respectively. The obtained data are similar to that obtained of Tafazol (1977) who found that a decrease in fruit sugar content by increasing the number of buds / vine.

Total carbohydrates and proteins content in the canes.

Data in table (6) indicated that total carbohydrates content in the cane at dormant period was significantly increased by increasing bud load/vine. In this connection, vines which were pruned to 143 buds /vine appeared to assimilate and store higher carbohydrates content than the other ones pruned to 78 buds/ vine, which was recorded a lower carbohydrates content during the two seasons, of the investigation. This is not strange since this treatment produced a lower number of subsequent shoots compared with the treatment having higher bud load 143 buds / vine which in turn produces higher number of shoot. Our results in this connection agree with those obtained by kliewer (1981) and Gao & Cahoon (1994) who reported that increasing leaves lead to heavy canopy with increase in active photosynthesis and stored carbohydrates in the new canes. Similar results were obtained by Omar & Abdel-kawi (2000) on Thompson seedless grapevines and El-Baz et al; (2002) on Crimson seedless grapevines.

Data of table (6) show the effect of different bud load/ vine on total protein content in the cane of Crimson seedless grape during dormant season. Thus it is appeared to increase by increasing the bud load on the vine. In this respect, vines which were pruned to 78 buds/ vine maintained the lowest content of total protein in their canes during the both seasons of this study. These results in agreement with El-Baz et al; (2002) on Crimson seedless grapevines.

Table (1) Effect of bud load on bud behaviour of "Crimson seedless" grapevines in 2007 and 2008 seasons

Treatments (Bud load per vine)	Number of bud burst		Bursted buds (%)		Fertitity buds (%)		Fruitfulness (%)	
	2007	2008	2007	2008	2007	2008	2007	2008
T1 - (78 bud)	62.31	63.12	79.88	80.92	36.32	37.32	45.47	46.12
T2 - (91 bud)	71.30	72.10	78.35	79.23	33.09	34.32	42.23	43.31
T3 - (104 bud)	80.59	79.30	77.49	76.25	31.98	33.75	41.27	44.26
T4 - (117 bud)	88.33	90.11	79.77	82.05	32.62	33.53	43.20	43.53
T5 - (130 bud)	95.10	101.31	73.15	77.93	30.08	3.079	41.13	40.79
T6 - (143 bud)	102.33	103.10	71.56	72.09	28.83	29.48	40.29	40.89
New L.S.D at 5%	6.3	5.6	2.6	2.5	0.61	0.63	0.86	0.88

Table (2) Effect of bud load on number of cluster per vine and yield of "Crimson seedless" grapevines in 2007 and 2008 seasons

Treatments (Bud load par vine)	Number of cluster/ vine		Yield (kg)/ vine	
	2007	2008	2007	2008
T1 - (78 bud)	28.33	29.11	10.09	10.57
T2 - (91 bud)	30.11	31.23	11.30	12.03
T3 - (104 bud)	33.26	35.10	13.25	13.57
T4 - (117 bud)	38.16	39.23	15.28	16.14
T5 - (130 bud)	39.11	41.33	12.52	13.64
T6 - (143 bud)	41.23	42.16	11.76	12.28
New L.S.D at 5%	0.73	0.92	1.23	1.31

Table (3) Effect of bud load on some cluster characteristics of Crimson seedless grapevines in 2007 and 2008 seasons

Treatments (Bud load per vine)	Cluster weight (g)		Rachis weight (g)		Cluster length (cm)		No. of berries/ cluster		Compactness coefficient		Cluster index	
	2007	2008	2007	2008	2007	2008	2007	2008	2007	2008	2007	2008
T1 - (78 bud)	356.16	363.00	9..33	9.48	23.16	23.51	100.24	100.72	4.33	4.28	38.17	38.29
T2 - (91 bud)	375.33	385.23	11.63	11.16	23.18	23.61	102.16	103.05	4.41	4.65	32.27	34.52
T3 - (104 bud)	398.23	391.26	12.93	13.10	25.18	26.10	101.59	92.28	3.03	3.54	30.79	29.87
T4 - (117 bud)	400.30	411.33	13.16	13.58	25.10	26.31	97.63	95.66	3.89	3.64	30.42	30.29
T5 - (130 bud)	320.16	330.11	12.53	12.93	19.30	18.93	85.45	83.91	4.27	4.33	25.55	25.53
T6 - (143 bud)	285.30	291.16	10.36	10.50	18.33	18.53	81.34	83.08	4.38	4.86	27.54	27.74
New L.S.D at 5%	25.23	26.16	0.81	0.92	2.10	2.51	9.25	8.16	0.16	0.15	2.60	2.70

Table (4) Effect of bud load on some berry characteristics of Crimson seedless grapevines in 2007 and 2008 seasons

Treatments (Bud load per vine)	Berry weight (g)		Berry Index		Berry firmness g/cm		Berry adherence (g)	
	2007	2008	2007	2008	2007	2008	2007	2008
T1 - (78 bud)	3.46	3.51	28.14	27.75	923.10	936.33	699.10	701.33
T2 - (91 bud)	3.56	3.63	27.22	26.75	952.16	958.10	735.33	738.11

T3 - (104 bud)	3.92	4.10	25.50	23.59	954.33	963.18	771.23	778.23
T4 - (117 bud)	4.10	4.30	24.39	23.26	999.30	1003.16	789.16	792.11
T5 - (130 bud)	3.60	3.78	26.39	25.42	968.16	973.11	761.33	750.31
T6 - (143 bud)	3.38	3.35	28.51	28.57	918.31	910.33	700.30	689.33
New L.S.D at 5%	0.23	0.26	2.00	3.3	35.63	36.16	28.33	29.16

Table (5) Effect of bud load on chemical of berries of crimson seedless grapevines in 2007 and 2008 seasons

Treatments (Bud load Per vine)	T.S.S. (%)		Acidity (%)		T.S.S./ acid ratio		Total sugar (%)	
	2007	2008	2007	2008	2007	2008	2007	2008
T1 - (78 bud)	19.50	19.83	0.44	0.45	44.32	44.07	17.10	17.53
T2 - (91 bud)	20.11	20.50	0.44	0.46	45.70	44.57	17.41	17.10
T3 - (104 bud)	21.98	23.10	0.46	0.48	47.78	48.13	17.50	17.90
T4 - (117 bud)	22.10	23.30	0.42	0.45	52.62	51.78	17.59	17.98
T5 - (130 bud)	19.23	19.56	0.48	0.50	40.06	39.12	15.13	16.33
T6 - (143 bud)	19.23	19.50	0.50	0.53	38.46	36.79	15.30	15.33
New L.S.D at 5%	2.23	2.36	0.03	0.05	6.81	7.32	1.31	1.63

Table (6) Effect of bud load on total carbohydrates and proteins in the canes of Crimson seedless grapevines in 2007 and 2008 seasons

Treatments (Bud load per vine)	Total carbohydrates (g/ 100g dry weight)		Total protein (g/ 100g dry weight)	
	2007	2008	2007	2008
T1 - (78 bud)	18.23	18.89	15.30	15.58
T2 - (91 bud)	19.98	20.11	15.90	15.93
T3 - (104 bud)	22.16	23.33	16.30	16.71
T4 - (117 bud)	23.30	24.11	16.33	16.83
T5 - (130 bud)	23.10	24.58	16.90	16.93
T6 - (143 bud)	24.33	25.33	17.10	17.54
New L.S.D at 5%	3.31	4.33	3.19	3.32

Corresponding author
Fawzi M. I. F.
Pomology Department, National Research Center, Giza, Egypt

4. References:

1. A.O.A. C (1980). Association of Official of Analytical Chemist 14th ed. Published by the A.O.A.C.P.O. Box 540, Washington, 4D. C., USA.
2. Abdel-Fattah S.E; Marwad, I. A; and Isis Abdel-shahied Rizk (1993). Effect of bud load and spur length on Roumi Red grapevine I-Weight of pruning and chemical compostion of 1 year - old wood. Zagazig.J. Agric. Res Vol 2.0 No. (6) pp 1889 -1899.
3. Ali, M. A., El-mogy, M.M and Rizk, I. (2000). Effect of cane length on bud behaviour, bunch characteristies, wood ripening and chemical contents of Thompson seedless grapevine Agric, sci., Mansoura Univ. 25 (3), pp1707-1717.
4. Badr, S.A. (1997). Cultural practres for new table varieties selection Research Report for California Table Grape Fresno, California, 13, 1.
5. Bessis, R. (1960). Sur differences Moder D expression quantive Dela fertile chez la vigna pp. 828-882.
6. Bradford, M.M. (1976). A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein dyebinding Analytical Biochemistry pp 72, 248.
7. Christensen, L. P. Leavit, G. M. and Bianch, M. L. (1994). The effects of pruning level and post bud break cane adjustment on Thompson seedless raisin production and quality, Amer. J. Enel and vatic., 45 (2), 141.
8. Dure, I. I. I and Chlan, C. (1981). Developmental bio- chemistry of cotton seed embryogenesis and germination XII. Purification and properties of principle storage protein. Plant physiol pp 68, 180.
9. El- El. T. El- Baz, A.M; Mansour, El- Dengway, El. F. and Samra, B.N. (2002). Influence of pruning seveity on bud behaviour, yield, berry quality and some biochemical contents of the canes of Crimson seedless grapes. Egypt . J. Hort. 29, (1), pp. 39-60.
10. Fawzi, F., Bondok, A.Z. and Ghobrial, G. F. (1984). Effect of cane length on bud behaviour and wood ripening of Thompson seedless grape variety, Annals Agric., Sci., Ain Shams Univ., Cairo 29 (1) 465-474.
11. Gao, Y. and Cahoon, G.A. (1994). Fruit quality, fruit skin colour, and anthocyanin content and composition in Reliance (*Vitis hybrid*) Vitis, 33, (4), pp 205.
12. Howell, G.C; David, P.M.; Charles, E.E. and R.Keith, S. (1991). Influence of training system and pruning severity on yield vine size, and fruit composition of Vignoles grapevines. Vol, 42, No. 3, pp. 191-197 Am. J. Enol. Vitic.
13. Howell, G.S and strieglar, K. (1998). Pruning grapevines in Michigan Hort. Extension Bull; Michigan State Univ. Bull. 25, 1.
14. Kliewer, W.M (1981). Grapevine physiology; How does grapevine make sugar leaflet No. 21231. Cooperative Extension Us Department of Agric, California Univ.
15. Marwad, I. A.; Rizk, N. A and Ibrahim, A. H (1993). Effect of cane length on bud behaviour, Yield, bunch characteristics and wood maturity of Thompson seedless grapevines Egypt. J. Appl. Sci., 8 (12) pp 47-60.
16. Mead, R., Curnow, R. N. and Harted, A. M. (1993). Statistical methods in Agricultural and Experimental Biology. 2nd Ed. Chapman & Hall, London.
17. Morris, J. R and Cawthon, D.L. (1980). Yield and quality response, of Concord grapes to training system and pruning seveity in Arkansas. J. Am. Soc. Hort. Sci. 105: pp 307-310.
18. Omar, A.H and Abdel- kawi, A. (2000). Optimal bud load for Thompson seedless grapevines. J. Agric Sci. Mansoura Univ. 25 (9), pp5769-5777.
19. Oster, V., Kardinal, C., Brghardt, H., Lottspeich, F. and Rudiger, W. (1992). Natural inhibitors of germination and growth. J. plant Physiol. 140, pp 110.
20. Plummer, D. T. (1971). An introduction to practical biochemistry McGraw-Hill Book company (UK) limited. Maidenhead. Berkshire England.
21. Pommer, C.V., Terra, M.M., pires, E.I.P; Passos, I.R.S. and Martins, V.P. (1999). Introduction of the table grape cultivar Fantasy seedless and Crimson seedless in Brazil. Scientia Agric. 56 (1), pp 247.
22. Ramming, D.W., Roland, T. and Badr, S.A. (1995). Crimson seedless: A new late maturity, Red seedless grape. Hort Sci. 30 (7): pp1473 - 1474.
23. Rizk, N.A.; Fizk, I. A, and Grigis, V.H. (1994). Effect of cane length on bud behavior, wood ripening and bunch characteristics of Thompson

seedless grape Egypt. Jour. Appl. Zagazig Univ. Sci, 9 (5) 230-239.

24. Rizk. A. I (1996). Effect of cane length on bud behaviour and bunch characteristics in Thompson seedless grape cultivar. Egypt. J. App Sci; 11 (7): 220-234.
25. Rubio, J.A., Albuquerque M. V., peixy, J.L and yuste . J. (2002). la mejora varietal de la vid, sus consescuencias y beneficios. Vida Rural, pp 149: pp 62-66.
26. Salem, A.T, kilani, A.S and shaker, G.S. (1997). Growth and quality of two grapevine cultivars as affected by pruning severity. Acta Hort. 441.309.
27. Samra, N. R. (1998). Evaluation of table grape cultivars behaviour and training system in Egypt and California. Report for Egypt project of Agricultural Technology Utilization and transfer project (ATUT) pp. 55-58.
28. Smith, F.; M. A. Gilles; j.k Hamilton and Godes, P.A. (1956) .Colorimetric methods for determination of sugar and related substance. Annal., Chem., 28, pp 350-356.
29. Tafazoli, E. (1977). Cane and bud number effect on yield components of non-irrigated grape cv. "Yaghooti. Scientia Horticultura 7 (2): pp133-136 (Hort. Abst., 48 No. 259).
30. WinKler, A. J. (1962). General Viticulture Univ. of California. Press, pp, 135-255, USA.

7/3/2010

The Effect of some Slow Release Nitrogen Fertilizers on Growth, Nutrient Status and Fruiting of "Mit Ghamr" Peach Trees

[1] Kandil, E. A., [2]M. I. F. Fawzi, and [2]M. F. M. Shahin

[1]Hortcultural Research Institute, [2] National Research Center, Dokki, Giza, Egypt.

Abstract: This study was conducted for comparing three slow release N fertilizers namely, urea – formaldehyde, phosphorus – coated urea and sulphur coated- urea and that fast release nitrogen namely (urea) at 500, 750 and 1000g/tree/year for vegetative growth, leaf mineral content, yield and fruit quality of "Mit Ghamr" peach tree grown in a private orchard Aga city Dakahlia Governorate, Egypt, during 2008 and 2009 seasons, were studied. Urea was added at two times at the start of spring growth and after fruit set, while slow – release N fertilizers applied once at the start of spring growth. Results showed that supplying the tree of "Mit Ghamr" peach with the three slow release N fertilizers were superior to the application of the fast one in improving shoot length, leaf area, percentage of leaf N, as well as physical and chemical characteristics of the fruits. Application of sulphur – coated urea (SCU), phosphorus- coated urea (PCU) and urea- formaldehyde in a descending order was very favorable. Generally, "Mit Ghamr" peach trees once with sulphur coated urea at 500-750g/trees/year was the best results on vegetative growth, yield nutritional status of trees and fruit quality. In addition saving nitrogen fertilization cost and reducing nitrate pollution. [Journal of American Science. 2010;6(12):195-201]. (ISSN: 1545-1003).

Keywords: N fertilizers; urea; formaldehyde; phosphorus; sulphur

1. Introduction:

Peach is one of the most important deciduous fruit trees grown in Egypt.

Nitrogen is known to be one of the most major elements for plant nutrition and development science it plays an important role in a constituent of all proteins, nucleic acids and enzymes synthesis (Nijjar, 1985).

Recently, new techniques for fertilization of fruit trees grown under sandy soil were arisen. Out of those, the application of controlled release N fertilizers they were developed mainly to reduce the number of replications per year, minimize the cost of production, improve the efficiency of N used by trees, reactions and the rapid denitrification (Nijjar, 1985, Allen, 1986, Alva, 1992, Scuderi et al, 1993 and Wang & Alua, 1996) The control and continues providing of the trees with their requirements from N can be achieved by using controlled release N fertilizers which are responsible for releasing their own N at a longer period and at the critical date of fruit development.

Previous studies showed that using slow release N fertilizers was preferable than using the fast one in improving growth and nutritional status of the trees. (Alvan & Tucker, 1996, Ahmed et al. 1997, Hammam & Assy, 2000; Wassel et al. 2000; Akl et al. 2002 and Mohamed & Sama Ebeed. 2008).

So, the aim of this investigation was to study the effect of three controlled release N fertilizers urea formaldehyde, phosphorus coated urea and sulphur coated urea compared to fast release (urea) on growth, nutritional status and fruit quality of "Mit Ghamr" peach cultivar trees grown in loamy soil, to find out the best one.

2. Materials and Methods:

During 2008 and 2009 seasons, 18 years old "Mit Ghamr" peach trees (Prunus persica L. Batch) grown in a private orchard, Aga city Dakahlia Governorate, Egypt. The selected and uniform in vigour trees were planted at 5×5 meters apart grown in loamy soil and irrigated via flood irrigation, trained to an open – vase system.

Analysis of the tested soil according to (Wilde et al. 1985) and the data are shown in Table (1).

Table (1): Soil physical and chemical analysis.

Size of particles and their distribution	
Sand %	34.50
Silt %	36.50
Clay %	29.00
Texture	Loamy
pH (1: 2.5 extract)	8.10
E.C. (1: 2.5 extract) (mmhos/1cm)	0.85
O.M%	2.30
Total Co_3%	1.73
Avatilable macronutrients	
Total N %	0.09
Available P (ppm olsen).	12.00
Avaiable K (ppm)	285.00

1. This experiment included the following 12 treatments as follows:
2. Application of urea (46.5% N) at 500g/tree.
3. Application of urea at 750 g/tree.
4. Application of urea 1000g/tree.
5. Application of urea formaldehyde (38.37% N) at 500 g N/tree.
6. Application of urea formaldehyde at 750 g N/tree.
7. Application of urea formaldehyde at 1000 g N/tree.
8. Application of phosphorus-coated urea (37.11% N) at 500g N /tree.
9. Application of phosphorus-coated urea at 750 g N /tree.
10. Application of phosphorus-coated urea at 1000g N /tree.
11. Application of sulphur-coated urea (41% N) at 500g N/ tree.
12. Application of sulphur-coated urea at 750g N/ tree.
13. Application of sulphur-coated urea at 1000g N/ tree.

The experiment was set in a completely randomized block design with three replicates each consisted with two trees.

The three slow release N fertilizers at the prementioned amounts were applied once at the start of spring in circular digs around each tree 50cm apart from trunk and covered with soil, while the fast release N fertilizers (urea) was added twice at spring growth start and after fruit stetting. The treated trees received the basal P and K fertilizers. Other horticultural practices were carried out as usual.

The following parameters were recorded for both seasons:

1- Vegetative growth measurements:

Shoot length (cm), and during mid-August of the two seasons, leaf samples were collected from the middle portion of the current season growth to determine leaf area (cm2) by using a leaf area meter (Model C/203 area meter. CID, INC. USA), and to determined percentage of N, P and K according to (Wilde, et al. 1985).

2-Leaf mineral content:

As follows: samples of leaves were taken, and distilled water washed and oven dried at 70oC till constant weight. Dried samples were pulverized separately and samples of 0.2 (g) each was digested with a mixture of sulphuric acid and hydrogen peroxide, to determine the following:

Total nitrogen percentage was measured by the micro-kjeldahl methods described by Pregl (1945).

Phosphorus percentage was determined calorimetrically according to Murphy & Reily (1962).

Potassium was measured according to Jakson (1973) by flame photometer.

3-Yield per tree: At harvesting time in early June in two seasons yield as Wight in (kg) and number of fruit per tree was recorded.

4-Fruit quality:

Fruit physical and chemical characteristics:

Samples of twenty fruits were taken from each replicate for measuring the following characteristics fruit weight (g), fruit volume (ml) and dimensions (cm); total soluble solids %TSS was measured in fruit juice by hand refractometer, total acidity% in fruit juice was determine as malice acid, and T.S.S./ acidity ratio were calculated. Total sugar contents were estimate according to (A.O.A.C (1985).

Statistical analysis:

The obtained data were statistically analyzed. Means were compared using the New L.S.D at 5% level, according to Snedecor and Cochran (1980).

3. Results and Discussion:

1. Vegetative growth:

It is clear from Table (1) that the application of the slow release N fertilizers, urea formaldehyde (UF), phosphorus – coated urea (PCU) and sulpher - coated urea (SCU) were have high positive effective and significantly improved, shoot length and leaf area of "Mit Ghamr" trees compared to application of fast release N fertilizer (Urea) in 2008 and 2009 seasons. The promotion on vegetative growth traits was associated with increasing the level of N from 500 till 1000g/tree.

Raising N levels from 750 to 1000 g/tree from either fast or slow release fertilizers failed to show any significant increase in these traits.

Moreover, the maximum shoot length and leaf area detected on the trees fertilized with SCU at any level. These results were true in both seasons.

Generally, the improving effects of slow release N fertilizer UF, PCU and SCU on vegetative growth might be attributed to their effect on regulating the release of N according to the plants needed. Also they gave the highest values of residual N in soil due to their low activity index, compared fast release (urea) which gave the lowest values of available N left in the soil (Mikkelsen et al. 1994).

In addition, the role of nitrogen in plants, which increase growth and development of all living tissue, also N considered to be an important constituent of chlorophyll, protoplasm, protein and nucleic acid, so that it resulted in an increase in cell number and cell size with an increase (Said, 1998 and El- Naggar et al. 2002). In addition, the substantially

improved the vegetative growth trails due to sulpher – coated urea may be attributed to acidification resulted from S oxidation that decreasing soil pH that enhanced the solubility of nutrients and increases the activity of micro-organisms. These effects increase the nutrients availability uptake and translocation and increase the vegetative growth (Yousry et al 1984). These results are in harmony with those obtained by Jackson & Davies (1984), Puchades et al. (1985), Koo, (1988), Alva & Tucker (1996), Hegab et al. (1999), Hamman & Assy (2000), Wassel et al. (2000).

2. Leaf mineral content:

It is evident from the data presented in Table (2) that application of N via slow release fertilizers was significantly preferable in increasing leaf N, P, K percentages than application of it via fast release.

In general, results indicated that increasing the level of UF, PCU and SCU were followed by a gradual increase in leaf N percentage. A meaningless, high level of leaf N, P, K percentages was detected by using the highest two levels of each slow release treatments. The best results of leaf N percentage observed at 1000g of SCU, while fast release (Urea) at the same level recorded the lowest value.

The intermediate value was shown at PCU and UF respectively. All the slow release N fertilizers gave the same content of P and K in the leaves. The maximum values of N, P and K were recorded in the tree fertilized with sulpher – coated urea (SCU). These results were true in 2008 and 2009 seasons.

Moreover, using SCU at 500 g/ tree significantly increased the growth and leaf N, P and K percentage compared with other slow release and fast fertilizers. This means that using sulpher coated-urea at 500g/tree was sufficient to improve vegetative growth and tree nutritional status as well as useful in saving N fertilization cost and reducing nitrate pollution. The great reduction in the loss of N and the increase in uptake due to the application of slow realese N fertilizers could explain the reason for their effect in improving the leaf content of N. The vice versa was obtained due the application of the fast fertilizers which was mainly attributed due to great leaching of N from soil via drainage water.

Similar results were obtained by Scuderi et al. (1983), Mquireiro et al. (1984), Marler et al. (1987), Balo et al (1988), Ferguson et al. (1988), Wang & Alva (1996), Hammam & Assy (2000) and Wassel et al. (2000).

3. Yield measurement:

Data in Table (3) showed that "Mit Ghamr" Peach yield component as affected by application of some slow releases fertilizers UF, PCU and SCU at level 500, 750 and 1000 g/tree and fast release (Urea). The treatments showed a significant difference among them in this connection. In general, increasing nitrogen level via either fast or slow release fertilizers resulted in increase yield components. Application of SCU was a considerable effect on increasing fruits number and yield/tree, while, UF and PCU recorded the intermediate values. Moreover "Mit Ghamr" Peach trees that fertilized with 500g/tree of sulphur – coated urea (SCU) recorded the highest values of fruits number and yield /tree compared to other slow release and urea. Therefore, it could be concluded that such treatments was sufficient to get satisfactory increased in yield with good quality and also very useful in saving N fertilization cost and reducing nitrate pollution. These findings are in harmony with those found by Koo, (1986), Koo (1988), Boman (1993), Scuderi, et al. (1993), Wassel et al. (2000) and Akl, et al. (2002).

4. Fruit physical and chemical characteristics.

Data in Table (5) obviously showed that fruit weight and volume were positively affected in response to application of slow release nitrogen fertilizers rather than using urea. The promotion in fruit weight and volume were associated with increasing the level of N applied from 500 till 1000g/tree.

The heaviest fruit were recorded on trees fertilized with 1000g/tree sulphur coated urea. Moreover, raising N levels from 750 to 1000g/tree failed to show any significantly increase in fruit weight and volume.

Also, data in Table (5) indicated that the effect of slow release N fertilizers, UF, PCU and SCU used on fruit length and diameter was almost similar to that concerning in fruit weight and volume.

It is clear from the data in Table (6) that the application of the three slow release N fertilizers increasing the total soluble solids and total sugar while reducing the total acidity compared to via urea at the same levels. The best results with regards to chemical fruit quality showing ascending order by using slow realese N fertilizers , while urea showed the lowest value. These results were true in 2008 and 2009 seasons in "Mit Ghamr" cv.

Generally, "Mit Ghamr" peach trees fertilized with sulphur coated urea at 500-750g/trees/year gave the best results on vegetative growth, yield nutritional status of trees and fruit quality. The stimulation effect of SCU on chemical quality was attributed to its action in enhancing the formation of carbohydrates and advancing ripening Nijar, (1985).

These results are in harmony with those obtained by Wiil and Hahdel 1986; Bomom (1993) Hegab et al. (1999) and Hamman & Asay (2000).

Table (2): Effect of soil application of fast and some slow – realease nitrogen fertilizers on some vegetative growth of "Mit Ghamr" peach trees in 2008 and 2009 seasons.

N source and Level (g/tree)	Shoot length (cm)		Leaf area(cm^2)	
	2008	2009	2008	2009
- U at 500g.	43.30	46.36	24.31	23.63
- U at 750g.	45.50	48.46	25.51	24.43
- U at 1000g.	46.30	49.56	25.81	24.63
- UF at 500g.	52.50	54.45	26.91	25.83
- UF at 750g.	54.70	56.85	27.21	26.78
- UF at 1000g.	55.20	57.35	27.33	26.93
- PCU at 500g.	58.43	61.45	28.43	27.53
- PCU at 750g.	60.03	63.45	28.73	27.63
- PCU at 1000g.	63.33	65.15	28.93	27.83
- SCU at 500g.	66.31	68.45	30.03	28.63
- SCU at 750g.	67.36	70.53	30.23	28.83
- SCU at 1000g.	68.36	71.00	30.46	29.23
- New L. S.D. at 5%	**1.11**	**2.01**	**1.01**	**1.03**

U: Urea; UF: Urea formaldehyde; PCU: Phosphorus-coated Urea ; SCU: Shlphur-coated urea.

Table (3): Effect of soil application of fast and some slow – realease nitrogen fertilizers on N, P and K percentage (dry weight basis) of "Mit Ghamr" peach trees in 2008 and 2009 seasons.

N source and Level (g/tree)	Leaf – N (%)		Leaf-P (%)		Leaf-K (%)	
	2008	2009	2008	2009	2008	2009
- U at 500g.	2.16	2.23	0.20	0.21	1.88	1.91
- U at 750g.	2.23	2.29	0.19	0.20	1.86	1.81
- U at 1000g.	2.24	2.32	0.18	0.19	1.85	1.73
- UF at 500g.	2.31	2.40	0.29	0.26	2.08	2.10
- UF at 750g.	2.38	2.46	0.29	0.26	2.09	2.11
- UF at 1000g.	2.39	2.47	0.30	0.27	2.09	2.10
- PCU at 500g.	2.47	2.53	0.31	0.28	2.08	2.10
- PCU at 750g.	2.54	2.59	0.31	0.29	2.08	2.10
- PCU at 1000g.	2.55	2.60	0.30	0.29	2.08	2.10
- SCU at 500g.	2.66	2.69	0.31	0.29	2.09	2.11
- SCU at 750g.	2.74	2.75	0.31	0.30	2.09	2.11
- SCU at 1000g.	2.75	2.77	0.31	0.30	2.10	2.12
- New L. S.D. at 5%	**0.07**	**0.06**	**0.02**	**0.02**	**0.07**	**0.08**

U: Urea; UF: Urea formaldehyde; PCU: Phosphorus-coated Urea ; SCU: Shlphur-coated urea.

Table (4): Effect of soil application of fast and some slow – realease nitrogen on yield and fruit number in one (kg) of "Mit Ghamr" peach trees in 2008 and 2009 seasons.

N source and Level (g/tree)	Yield/ tree (kg.)		Fruit number in one (kg.)	
	2008	2009	2008	2009
- U at 500g.	25.16	27.33	13.59	13.31
- U at 750g.	26.11	29.11	12.03	11.87
- U at 1000g.	26.53	30.30	11.62	11.45
- UF at 500g.	27.30	31.66	11.09	10.74
- UF at 750g.	28.00	32.33	10.38	10.17
- UF at 1000g.	28.10	32.51	10.07	9.89
- PCU at 500g.	36.11	32.76	9.28	9.15

- PCU at 750g.	36.11	33.10	8.83	8.68
- PCU at 1000g.	36.36	33.56	8.68	8.53
- SCU at 500g.	45.36	46.31	8.18	7.99
- SCU at 750g.	45.31	47.26	7.69	7.73
- SCU at 1000g.	46.00	48.31	7.44	7.35
- New L. S.D. at 5%	**3.36**	**4.28**		

U: Urea; UF: Urea formaldehyde; PCU: Phosphorus-coated Urea; SCU: Shlphur-coated urea.

Table (5): Effect of soil application of fast and some slow - realease nitrogen on some physical characteristics of "Mit Ghamr" peach trees in 2008 and 2009 seasons.

N source and Level (g/tree)	**Fruit weight (g)**		**Fruit volume (mL.)**		**Fruit length (cm)**		**Fruit diameter (cm)**	
	2008	2009	2008	2009	2008	2009	2008	2009
- U at 500g.	73.60	75.11	66.31	70.11	4.90	4.98	4.81	4.93
- U at 750g.	83.16	84.23	77.03	78.31	4.95	5.04	4.87	4.98
- U at 1000g.	86.03	87.30	81.11	81.23	4.99	5.02	4.90	5.02
- UF at 500g.	90.11	93.10	86.33	87.11	5.01	5.14	4.93	5.07
- UF at 750g.	96.31	98.30	90.09	92.13	5.12	5.20	4.92	5.11
- UF at 1000g.	99.30	101.14	93.30	96.14	5.18	5.26	5.03	5.16
- PCU at 500g.	107.71	109.30	101.31	103.30	5.26	5.33	5.17	5.22
- PCU at 750g.	113.23	115.26	106.23	100.10	5.29	5.41	5.20	5.27
- PCU at 1000g.	115.23	117.23	101.26	112.11	5.31	5.46	5.22	5.32
- SCU at 500g.	122.30	125.11	118.81	119.30	5.32	5.51	5.23	5.38
- SCU at 750g.	130.1	129.30	125.30	126.13	5.36	5.56	5.27	5.43
- SCU at 1000g.	134.33	136.11	129.11	131.16	5.36	5.58	5.24	5.46
-New L. S.D. at 5%	**4.1**	**3.3**	**6.31**	**5.53**	**0.28**	**0.33**	**0.29**	**0.36**

U: Urea; UF: Urea formaldehyde; PCU: Phosphorus-coated Urea; SCU: Shlphur-coated urea.

Table (6): Effect of soil application of fast and some slow-realease nitrogen of some chemical characteristics of "Mit Ghamr" peach trees in 2008 and 2009 seasons.

N source and Level (g/tree)	**T.S.S(%)**		**Total acidity (%)**		**T.S.S /acidity**		**Total sugar (%)**	
	2008	2009	2008	2009	2008	2009	2008	2009
- U at 500g.	8.33	9.11	1.23	1.11	6.77	8.21	7.13	7.85
- U at 750g.	8.13	8.00	1.25	1.07	6.50	8.41	7.03	7.55
- U at 1000g.	7.73	9.00	1.39	1.15	5.56	7.83	6.93	7.45
- UF at 500g.	8.83	9.95	1.31	1.19	6.74	8.36	7.53	8.25
- UF at 750g.	8.84	9.96	1.32	1.20	6.68	8.30	7.54	8.05
- UF at 1000g.	8.83	9.94	1.33	1.21	6.64	8.21	7.54	7.95
- PCU at 500g.	9.33	10.11	1.23	1.10	7.59	9.19	8.33	8.75
- PCU at 750g.	9.23	10.10	1.26	1.07	7.33	9.44	8.33	8.75
- PCU at 1000g.	9.26	10.00	1.27	1.06	7.29	9.43	8.32	8.65
- SCU at 500g.	9.63	10.76	1.16	1.03	8.30	10.76	8.63	9.25
- SCU at 750g.	9.53	10.66	1.16	1.04	8.22	10.25	8.63	9.05
- SCU at 1000g.	9.51	10.65	1.17	1.04	8.12	10.65	8.65	9.06
- New L. S.D. at 5%	**0.38**	**0.29**	**0.02**	**0.03**	**0.02**	**0.03**	**0.30**	**0.21**

U: Urea; UF: Urea formaldehyde; PCU: Phosphorus-coated Urea; SCU: Shlphur-coated urea.

Corresponding author
Kandi, E. A. l
Hortcultural Research Institute
Dokki, Giza, Egypt.

References:

1. AKL, A.M.; Ahmed, F.F.; Hegab, M.Y; Abo El-Komsan, E.O; Yaeness- Randa, E. (2002). Effect of different sources, levels and methods of nitrogen applications on growth, leaf mineral content, fruit setting yield and fruit quality of Valencia orange trees. 1- The effect on growth, leaf mineral content and fruit setting. Minia J. of Agric. Res. and Develop. 22 (4): 483-500, Egypt.
2. Allen, S.E. (1986). Slow release nitrogen fertilizers Nitrogen in crop production, 195-206 ASA. CSSA, and ASA, Madison. (Cited from the 2nd scientific conference of Agricultural sciences, Assiut, Oct. 2000 pp. 285-291.
3. Alva, A.K. and Tucker, D. P. H. (1996). Evaluation of a resin coated nitrogen fertilizer for young citrus trees on a deep sand. Proc. Fla. State Hort. Soc. 106: 4-8.
4. Association of Official Agricultural Chemists (1985). Official Methods of Analysis (A.O.A.C) 12th Ed. Pp. 494-500, Benjamin Franklin station Washington, D.C. U.S.A., pp. 444-500.
5. Balo, E., Prileszky, G., Happ, 1., Kaholmi, M. and Vega, L. (1988). Soil improvement and the use of leaf analysis for forecasting nutrient requirements of grapes. Potas Rev. (Subjected 9, 2nd suite, No.6).
6. Boman, B.J. (1993). A comparison of controlled-release to conventional fertilizer on mature March grapefruit. Proc. Florida Sta. Hort. Soc. 106.1.
7. El-Naggar, I.M., El-Madah,M., El-Sobany and El-Tawil, A.Y. (2002). Yield and Yield components of sur flower and some physical and chemical properties of different used soils as affected by organic and mineral fertilization. J. Agric. Mansoura Univ., 27 (11) : 7909-7925.
8. Ferguson, J.J., Crane, J. H. and Olszak, R. (1988). Growth of young Carambola tree using standard and controlled release fertilizers. Proc. Inter – American Soc., Tropica. Hort., 32, 20.
9. Hammam, M. S. and Assy (2000). Productivity of Hindy Bissinara mango trees in response to application of slow release nitrogen fertilizers. The 2nd Sci. Conf. of Agric. Sci., Assuit, 285-291.
10. Hegab, M. Y.; Ali, A. H and El-Dawwey, G.M. (1999). Behaviour of Balady mandarin trees (citrus reticulate L.) grown in sandy soil to some slow release nitrogen fertilizers. Minia J. Agric-Res. & Dev. 19: 81-96.
11. Jackson, L. K. and Davies. F. S. (1984). Mulches and slow release fertilizers in a citrus young tree care program. Proc. Fla. State Hort. Soc. 7:37-39.
12. Jackson, M. L. (1973). Soil and Chemical Analysis. Prentice –Hall. Engle – Wood Cliffs, USA.
13. Koo, R. C. J. (1986). Use of controlled release nitrogen for citrus in a humid region. Citrus culture, 6th Inter. Citrus Congress, Tel, Aviv, Israel, 6-11 March, 2:633-641.
14. Koo, R.G.T. (1988). Use of contoured release nitrogen for citrus. Congress Middle East, Tel Aviv, Israel, 6.11, 2.
15. Maguireiro, A., Climent, M.D., Puchades, R. and Yufera, E.P. (1984). Fertilization of orange trees with sulfur-coated urea. 1- Nitrogen levels in leaves and fruits. Plant and Soil. 80, (2) : 247-254.
16. Marler; T.E.; Ferguson J.J. and Davies, F.S. (1987). Growth of young Hamlin orange trees using standard and controlled release fertilizers. Proc. Fla. State Hort. Sco. 100: 61-64.
17. Mikkelesen, R. L., Williams, H.M., and Behel, A.D. (1994) Nitrogen leaching and plant uptake from controlled release fertilizaers. Fert. Res., 37: 43-50.
18. Murphy, I. and Reily, J. P. (1962). A modified single method for the determination of phosphorus in natural water. Anal. Chem. Acta. 27, 31.
19. Nijjar, G. S. (1985). Nutrition of fruit trees. Mrs Usha Raj Kumar, Kalyani, New Delhi, India, pp. 306-308.
20. Pergl, F. (1945). Quantitative organic Micro Analysis. 4th ed. J. A. Churchill, L.T. D. London.
21. Puchades, R., Moquieira, A., Rudio, J.L. and Tufera.E, P. (1985). Changes in the nitrate and ammonical nitrogen content of a citrus orchard soil fertilized with sulpher-coated urea relationships with foliar nitrogen. Agrochimica 29(1)30.
22. Said, El-A.M. (1998). Contribution of NPK fertilization levels on sunflower productivity. J. Agric Sci. Mansoura Univ. 23 (9): 3601-3610.
23. Scuderi, A.; G. Raciti and Barbagallo, A. (1993). Manuring of citrus, nutritional and yield effects of using slow release nitrogen formulation. Informatore Agric. 39: 40, 2773.
24. Snedecor, G.W. and Cochran, G.W. (1980). Statistical methods. 7th ed. Iowa state Univ. Press, USA.
25. Wang, F.L. and Alva, A.K. (1996). Leaching of nitrogen from slow release urea sources in sandy soils. Soil Scie., Soc. Amer. J. 60:1454-1458.
26. Wassel, A.M.; Ahmed, F.F. and Ebrahiem, T.A. (2000). Nitrogen better management for high

yield and quality of Balady mandarin trees grown in sandy soil. The 2nd Sci. conf. of Agric. Sci., Assuit, 293-300.
27. Wilde, S.A.; R. B. Corey; I.G. Lyer and Voigit, G.K. (1985). Soils and plant Analysis for Tree Culture. 3rd Ed. Oxford, IBH, New Delhi, India, pp. 94-100.
28. Will, N. and Hahndel, R. (1986). Slow release fertilizers with drip irrigation. Gemüse. 22 (9) 365.
29. Yousry, M.,El-Leboudi, A. and Khater, A. (1984). Effect of sulphur and petroleum byproducts on soil characteristics 1- Availability of certain nutrients in a calcareous soil under intermittent leaching. Egypt. J. Soil Sci. 24 (3): 185-194.

Influence of Foliar Application of some Nutrient (Fertifol Misr) and Gibberellic Acid on Fruit Set, Yield, Fruit Quality and Leaf Composition of "Anna" Apple Trees Grown in Sandy Soil

[1]Shahin M. F. M. [1]Fawzi M. I. F. and [2]kandil E. A.
[1]Pomology Department, National Research Center, [2]Horticulture Research Institute agriculture Research Center, Giza, Egypt.

Abstract: The effect of Fertifol Misr (N, P, K, Mg zn, Fe, Mu, Cu, Mo & B) and gibberellic acid on fruit set, drop percentage, yield, fruit quality and leaf chemical composition on "Anna" Apple trees were studied during 2007 and 2008 seasons. Results showed that, fruit set%, drop%,, yield, leaf minerals & chlorophyll contents as well as physical and chemical characters of the fruit were positively effected by single or combined application of Fertifol Misr and gibberellic acid compared to unspraying .There was a slight promotion on such characters with increasing Fertifol Misr concentration from 1.5 – 2.5 g/l. The best results with regard to yield and fruit quality were obtained due to spraying "Anna" apple trees three times with a mixture containing Fertifol Misr at 2.5 g/l and gibberellic acid at 20ppm. [Journal of American Science. 2010;6(12):202-208]. (ISSN: 1545-1003).

Key words: Anna apple, foliar application, nutrients, gibberellic acid.

1. Introduction:

Anna apple (Malus domestica, Borkh) is a low chilling cultivar spreading in many tropic and subtropics area including Egypt. It is considered as one of the most important commercial cultivars planted in Egypt. The adaptation of proper fertilization program is one of the important cultural techniques that greatly promote and enhance the growth and fruit quality. No doubt that the application of soil fertilizers together with foliar feeding solution would probably be of great help in this respect. Amending the apple tree with their requirements from different nutrients via leaves was accompanied by on obvious improvement in both yield and quality of fruit. Many investigations studied the effect of spraying macro and micronutrients an growth, yield and fruit quality. Such as nitrogen, phosphorus, potassium &magnesium (Abd Ela, 1991, Akl, et al., 1993a & 1993b , and Gobara 2001). However zinc (Nijjar 1985 and Kabeel et al., 1998) Cupper and iron (Mohamed et al 1991); boron (Hanson 1991a); manganese (El Shazly, 1999) were highly effective in improving, nutritional status yield and quality of different pear and apple trees.

For instance, Plant growth, flowering fruit quality and yield were improved in apple by gibberellic acid application (El – Fakharany et al. (1995); Makarem & Mokhtar 1996).

The aim of this study was to test the effect of spraying some macro and micronutrient as (Fertifol Misr) and gibberellic acid on fruit set, yield, fruit quality and leaf chemical composition of Anna apple trees.

2. Materials and Methods:

This investigation was carried out during 2007 and 2008 seasons on 12-years old apple trees (Malus domestica, Borkh) budded) on MM 106 rootstock, planted at 3.5x3.5 meters apart and grown in a sandy soil under drip irrigation system in a private orchard located at El-Khatatba, Minufiya governorate, Egypt. Cross pollination was secured by planting Dorset Golden and Ein Shemir as pollinizers which were distributed in the whole orchard.

Soil analysis was carried out according to wiled et. al, (1985) data shown in Table (1). The experiment included six treatments as follows; Control (foliar sprayed with water).

Table (1) Physical and chemical properties of the tested soil.

Character	Volume
Particle size distribution	
Sand %	83.16
Silt %	15.11
clay %	1.73
Texture	Sandy
pH (1:2.5 extract)	7.50
Ec. (mmohs/cm 1:2.5) extract	0.65
Organic Mater %	0.75
Total Ca Co3 %	4.01
Available macro- nutrient	
Total N %	0.058
Available P (ppm,olsen)	5.33
Available K (ppm, ammonium acetate)	178 .06
Soluble cations meal 1, 1:5 soil water extract	
Mg++ ppm	
B (hot water extractable)	1.53
	o.16
DTPA extractable micro nutrieties ppm.	
Fe	1.99
Zn	1.30
Mn	1.00
Cu	2.89

- Foliar spray with Fertifol Misr at 1.5 g/l. This product was purchased from El Delta Company for fertilizers and chemical Al industrial.
- Foliar spray with Fertifol Misr 2.5 g./l .
- Foliar spray with gibberellic acid at 20 ppm.
- Foliar spary with Fertifol Misr at 1.5 g/l + gibberellic acid at 20 ppm.
- Foliar spray with Fertifol Misr at 2.5 g/l + gibberellic acid at 20 ppm.
- Fertifol Misr consists of (25 % N 16% P , 12% K, 0.25% Mg, 300 ppm zn, 1900 ppm Mn, 850 ppm cu, 100 ppm Mo & 200 ppm .

Fifty four healthy tress and nearly uniform in growth vigour and fruiting were selected. All trees had received regularly the same common cultural practices already give to the tree. Selected trees were sprayed 3 times at full bloom, after fruit set (fruit diameters 3 cm) and four weeks after fruit set. Foliar sprays were applied using a hand pressure sprayer. Triton B emulsifier at the rate of o.2% was used as surface to each tree received 8l of spraying solution, each treatment was surrounded with two rows as guard trees. The treatments were arranged in a completely randomized blocks design with three replicates for each treatment and three trees per each replicate. The following parameters were determined in the two seasons of the study.

1- Fruit set and fruit drop percentage.

Two main branches from two direction (east and west) of each tree were chosen and tagged in March of the two experimental seasons the number of flowers was recorded and them set fruits on the selected branches were counted for calculation the percentage of fruit set (divided number of fruits per branch/ Total number of flowers per branch x100).

Pre-harvest fruit drop was calculation by counting the number of dropping fruit from the 4th week of May till the commercial harvesting time under the experimental conditions (3rd week in June), then expressed as a percent from the whole number of fruits existed on the tree at the 4th week of May.

2-Yield per tree.

Yield was pressed in weight (Kg) and number of fruits per tree was recorded at harvest time (3rd week of June).

3-Fruit quality.

Fruit sample consisting of twenty fruits were randomly taken at harvest time from each replicate for the determination of both physical and chemical characteristics.

3-1. Physical characteristics:

Fruit weight (g), fruit length (cm), fruit diameter (cm) and fruit firmness (16/ inch) which was measured by fruit pressure tester on the two opposite sides of the fruit.

3-2.Fruit chemical characteristics

Total soluble solids were determined using a hand refractometer, percentage of titratable acidity in fruit juice was determined according to A.O.AC., (1995), total soluble solid / total acidity ratio were calculated and total sugar in the fruit pulp tissues were also determined by phenol sulfuric method according to (Dubois et al., 1956).

4-Leaf chemical composition.

Samples at twenty leaves from the middle part of the shoots (according to Chuntanaparb and Cummings, 1981) were randomly selected from each replicate (at the 2nd week of June to measure their area (cm2) and to determined their content N%, P %, K %, Mg % and Fe, Zn, Mn, Cu at ppm (according to Wilde et al., 1985). Determinations were carried out on dry weight basis. A leaf portion from each sample was kept fresh for chlorophyll determination, chlorophyll a and b (mg/l) was determined according the methods of Arnon (1949).

Statistical analysis was done according to Mead et.al. (1993) using the new L.S.D. test for comparing between means of different treatments.

3. Results and Discussion:

1-Fruit set and fruit drop percentage

Data presented in Table (2) show clearly that all treatments used significantly increased fruit set percent than the untreated trees during both seasons under this study. The data also indicated that the effect of Fertifol Misr 2.5 g/l + GA3 at 20 ppm application was more pronounced than other used treatments or the control. These results were true in both seasons. These results may be attributed the use of plant hormones, i.e. G A3 could lead to an increase in fruit set for deciduous trees (Makarem and Mokhtar, 1996). Similar results were obtained by Awad and Atawia (1995), Khabeel et al., (1998); Gobara (1998) and El Seging and Khalil (2000).

Regarding fruit drop data reported in Table (2) that spraying Fertifol Misr (N, P, K, Mg., Zn, Fe, Mn, Cu, Mo & B) alone or combined with GA3 was very effective in reducing pre-harvest fruit drop of Anna apple trees compared with the control. The differences between treated and untreated trees was significant. The minimum drop value was presented in Anna apple trees sprayed with Fertifol Misr at 2.5 / L + G A3 at 20 ppm. Similar results were observed in both seasons Zn, Cu, B, K and Fe were responsible

for building and moving carbohydrate from leaves to fruits and encourage the biosynthesis of cellulose which positively streng them the cell wall.

In addition, Zn and B Played an important role in biosyntheses and moving of the natural aux in namely IAA to the pedicels of fruits (Nijjar, 1985) . These results are in harmony with those found by Gill et al (1994) who worked on Zn, Cu, K and Fe.

2-Number of fruit and yield porter.

It is clear from the data in Table (3) that foliar application of Fertifol Misr single or combined with GA3 increased number of fruits per tree compared with the control. Such as increasing effect on fruits number per tree was almost similar to that yield per tree. The same table revealed that trees sprayed with Fertifol Misr at 2.5 g/l plus GA3 at 20 ppm resulted in the highest values of fruit number and yield in both seasons. These results agree with obtained by Bach et.al. (1995) on grapes and Awad & Atwaia (1995) on pears. They all stated that foliar sprays of Fe, Zn and Mn increased the total yield of studied fruit trees. Also Makarem & Mokhtar (1996) and El-Seigny & Khalil (2000) mentioned that foliar spray of GA3 increased the fruit set and fruit weight one.

3-Fruit quality

It is clear from the data in Table (4) that quality of Anna apple trees was positively affected by application of Fertifol Misr (macro and micronutrients) and GA3 either single on in combinations compared with unsprayed. The promotion on fruit quality due to applications of these materials appeared in terms of increasing fruit weight, T.S.S., total sugar content and in decreasing total acidity. The promotion on fruit quality was associated with increasing (Fertifol Misr) concentration. Single application with beneficial in enhancing fruit quality than using GA3 alone. The highest significant value of fruit quality was from trees treated with Fertifol Misr (N, P, K, Mg, Zn, Fe, Mn, Cu, Mo and B) at 2.5g/l plus GA3 at 20 ppm in both seasons. The improving of fruit quality in response to application of nutrients was supported by Ranvir and M'sra (1980) , Saraswathi et al., 1998, Zhao et al., (1999), Ahmed and Morsy, (2001); Younes-Randa (2002) and Fawzi and Abd Al-moneim (2004).Concerning the effect of gibberellic acid on fruit quality was emphasized by the results of Awad and Atawia (1995), Ibrahim et al., (1994), Kabeel et al., (1998) , El Shazly (1999), El Hammady et al., (2000) and El-Seginy and Khalil (2000).

4-Leaf aria and leaf chemical composition.

It is obvious from Table (5) that all tested treatments caused high significantly increased leaf area as compared with the control. Moreover, trees sprayed with Fertifol Misr at 2.5 g/l plus GA3 at 20 ppm was only the treatment that induced the highly significant effect on leaf area. Regarding leaf chlorophyll content data presented in Table (5) show that chlorophyll a and b content showed significant increase due to all tested treatments compared with the control. The same table revealed that trees sprayed with (Fertifol Misr) at 2.5 g/l plus GA3 at 20 ppm resulted in the higher values of leaf chlorophyll content. In general, the observe increase in the concentration of nitrogen, magnesium and iron in the leaves of Anna apple trees sprayed with Fertifol Misr and GA3 might help in interpretation the results obtained. The participation of nitrogen and magnesium in the structure and the integrity of the chlorophyll molecules together play indispensable role with iron in chlorophyll synthesis are well documented by numerous physiologists. Such as Bidwell (1979) and Faust (1989) they reported that over 10-20% of leaf magnesium and more than 80% of the iron content of green leaves are located in the chlorophyll. Concerning leaf macro and micronutrients contents, It is clear from the data in Table (6) that sprays Fertifol Misr (N, P, Mg, Zn, Fe, Mn, Cu, Mo and B) either alone or combined with GA3 significantly improved leaf status of these nutrients compared with control. Improved effect of nutrient, on N, K and Mg in the leaves was confirmed by the results of Abo-Shelbaya (1988), Intrighliolo et. al., (1991), Sheo and Singh (1999) and Lovatt (1999). The reduction in leaf p content might be due to the antagonism between Fe and P (Nawar 1991).

The results regarding the effect of gibberellic acid on leaf mineral contents are in agreement with those obtained by Addicot and Addicot (1982). In addition the increase of plant nutrient status resulted from spraying different solution might by due to quick absorption via leaves and the limited loss of the nutrients when they were sprayed (Marschner, 1995).

The data of the present study also revealed that with respect to sprayed by Fertifol Misr had apparently higher levels of Fe, Zn, Mn and Cu than the control. These results are in line with those reported by Hilail (1993) Awad and Atawia (1995); Kabeel at al., (1998), El Seiginy and Khalil (2000) and El-Shobaky at al., (2001) who worked on deciduous fruit trees.

Table (2): Effect of foliar spray with Fertifol Misr and GA_3 on fruit set and fruit strop percentages of Anna apple trees (2007& 2008 Seasons)

Treatments	Fruit set %		Fruit drop %		No. of fruit/ tree		Yield/ tree Kg.	
	2007	2008	2007	2008	2007	2008	2007	2008
T_1 – Control	15.30	16.90	54.30	51.33	136.30	143.00	10.81	12.07
T_2 – Fertifol Misr at 1.5 g/l	15.80	18.10	51.31	47.11	142.00	151.00	13.25	15.29
T_3 – Fertifol Misr at 2.5 g/l	16.30	18.93	48.30	44.30	148.31	165.51	14.54	18.04
T_4 – GA3 at 20 ppm	17.33	19.50	46.03	41.11	156.16	175.11	15.51	19.29
T_5 – Fertifol Misr at 1.5 g/l + GA3 at 20 ppm	17.83	20.31	41.33	39.16	165.31	180.03	18.35	20.89
T_6 – Fertifol Misr at 2.5 g/l + GA3 at 20 ppm	18.33	21.36	39.12	38.10	171.10	193.31	19.85	23.39
New L. S. D. at 5%	1.80	3.63	6.53	6.73	16.01	28.03	5.06	6.11

Table (3), Effect of foliar spray with Fertifol Misr and GA_3 on physical properties of Anna apple trees (2007& 2008 seasons).

Treatments	Fruit weight (g)		Fruit length (cm)		Fruit diameter (cm)		Fruit firmness ($lb/inch^2$)	
	2007	2008	2007	2008	2007	2008	2007	2008
T_1 – Control	79.31	85.00	4.21	4.41	4.81	4.90	11.62	11.81
T_2 – Fertifol Misr at 1.5 g/l	93.31	101.30	4.32	4.71	4.90	5.30	11.93	12.03
T_3 – Fertifol Misr at 2.5 g/l	98.03	109.00	4.61	4.41	5.30	5.63	12.23	12.43
T_4 – GA3 at 20 ppm	91.30	93.21	4.60	4.85	5.20	5.30	12.11	12.33
T_5 – Fertifol Misr at 1.5 g/l, GA3 at 20 ppm	111.00	116.01	5.10	5.32	5.61	5.83	12.58	12.71
T_6 – Fertifol Misr at 2.5 g/l, GA3 at 20 ppm	116.00	121.01	5.30	5.51	5.831	6.11	12.63	12.85
New L. S. D. at 5%	16.31	16.31	0.19	0.18	0.16	0.23	0.63	0.58

Table (4), Effect of foliar spray with Fertifol Misr and GA_3 on chemical properties of Anna apple trees (2007& 2008 seasons)

Treatments	T.S.S. %		Total acidity %		T.S.S./ acid ratio		Total sugar %	
	2007	2008	2007	2008	2007	2008	2007	2008
T_1 – Control	10.93	11.00	0.56	0.53	19.51	20.75	8.16	8.03
T_2 – Fertifol Misr at 1.5 g/l	11.05	11.43	0.53	0.51	20.85	22.41	8.28	8.31
T_3 – Fertifol Misr at 2.5 g/l	11.53	11.96	0.50	0.48	23.06	24.92	9.03	9.16
T_4 – GA3 at 20 ppm	11.50	12.36	0.46	0.45	25.00	27.47	9.18	9.35
T_5 – Fertifol Misr at 1.5 g/l, GA3 at 20 ppm	11.91	12.43	0.45	0.44	26.47	28.25	9.26	9.53
T_6 – Fertifol Misr at 2.5 g/l, GA3 at 20 ppm	12.36	12.63	0.44	0.42	28.09	30.03	9.31	9.63
New L. S. D. at 5%	0.53	0.73	0.04	0.05	3.31	3.63	0.55	0.75

Table (5), Effect of foliar spray with Fertifol Misr and GA_3 on leaf area and leaf chlorophyll content of Anna apple trees (2007& 2008 seasons)

Treatments	Leaf area cm^2		Leaf chlorophyll content (mg/l)			
			Chl. (a)		Chl. (b)	
	2007	2008	2007	2008	2007	2008
T_1 – Control	48.30	46.38	79.10	75.33	41.30	43.16
T_2 – Fertifol Misr at 1.5 g/l	53.10	51.12	110.30	112.30	58.10	57.30
T_3 – Fertifol Misr at 2.5 g/l	56.30	53.32	118.31	116.23	60.31	62.03
T_4 – GA3 at 20 ppm	56.10	55.12	81.00	79.11	39.11	41.10
T_5 – Fertifol Misr at 1.5 g/l, GA3 at 20 ppm	59.11	58.11	124.11	126.16	63.16	56.10
T_6 – Fertifol Misr at 2.5 g/l, GA3 at 20 ppm	66.18	63.31	126.00	130.10	65.31	69.16
New L. S. D. at 5%	2.10	3.10	10.30	11.26	6.16	7.31

Table (6), Effect of foliar spray with Fertifol Misr and GA_3 on Leaf mineral percentage (2007& 2008 seasons).

Treatments	N %		D %		K %		Mg %		Fe ppm		Zn ppm		Mn ppm		Cu ppm	
	07	08	07	08	07	08	07	08	07	08	07	08	07	08	07	08
T_1 – Control	1.63	1.68	0.23	0.25	1.38	1.36	0.30	0.33	86.00	99.03	21.31	23.12	17.00	18.00	26.00	28.01
T_2 – Fertifol Misr at 1.5 g/l	1.75	1.80	0.20	0.23	1.51	1.48	0.39	0.38	112.00	118.36	31.11	32.31	22.01	23.11	28.00	31.01
T_3 – Fertifol Misr at 2.5 g/l	1.93	1.98	0.18	0.22	1.56	1.53	0.45	0.46	115.33	123.01	36.10	38.10	24.31	26.30	30.00	33.03
T_4 – GA3 at 20 ppm	2.01	2.06	0.17	0.20	1.39	1.37	0.33	0.35	89.31	103.11	34.32	36.10	21.36	23.16	27.00	29.00
T_5 – Fertifol Misr at 1.5 g/l, GA3 at 20 ppm	2.26	2.31	0.18	0.23	1.57	1.54	0.47	0.48	116.03	134.11	39.16	41.16	29.33	30.00	31.00	35.16
T_6 – Fertifol Misr at 2.5 g/l, GA3 at 20 ppm	2.36	2.43	0.19	0.21	1.59	1.56	0.53	0.50	119.31	138.01	41.12	43.18	30.00	32.33	36.00	38.03
New L. S. D. at 5%	2.30	2.41	N.S	N.S	0.09	0.08	0.06	0.05	7.01	8.10	5.01	4.90	4.10	3.00	2.01	2.63

Corresponding author
Shahin M. F. M
[1]Pomology Department, National Research Center Giza, Egypt.

4. References:

1. A.O.A.C. (1995). Official Methods of Analysis (16th ed.) Association of Official Analytical Chemists International, Arlington, Virginia, U.S.A.
2. Abd Elal, A. H. (1991). Effect of soil and foliar application of nitrogen phosphorus and potassium on some vegetative and fruiting characteristics in White Banaty seedless M. Sc. Thesis, Fac. Agric. Minia Univ.
3. Abo-Shelbaya, M. A. M, and Ahmed F.F. (1988). Effect of foliar sprays of urea and micronutrients on improving the productivity of Balady Mandarin cultivar 1- leaf area and composition. Assiut J. of Agric. Sci. Vol 19 (3).
4. Addicott, F. T. and Adicott, A. B. (1982). Abbscission Un. GA Press. Lts. London England P. 30-135.
5. Ahmed, F. F. and Morsy, M.H. (2001). Response of Anna apple trees grown in the new reclaimed and to application of some nutrients and ascorbic acid. The Fifth Arabian Horticulture Conf. Ismailia, Egypt, March 24-27, pp. 27-34,
6. Akl, A. M.; Eid, A. M.; Ahmed F.F. and Abd Elal, A.H. (1993a). The effect of soil and foliar application N, P and K on vegetative and fruiting characteristics in White Banaty seedless grapevines. 11 – Bud behavior, yield and fruit quality. Minaia First Conf. for Hort Crop 2nd and part 432-453.
7. Akl, A.M., Eid, A. M., Ahmed F.F. and Abd Elal, A. H. (1993 b). The effect of soil and foliar application of N, P and K on vegetative and fruiting characteristics in White Banaty seedless grapeving, Vegetative growth and leaf nutrients content. Minia First Conf. for Hort. Crops nd, Part 409-431.
8. Arnon. D. I. (1949). Copper enzymes in isolated choloroplasts, polyphenol oxidase in Beta vulgaris. Plant Physiol. 24:1-15.
9. Awad S. M and Atawia A.R, (1995). Effect of the foliar sprays some micro nutrients on Le Conte pear trees. 1) Tree growth, flowering and leaf mineral contents. Annuals Agric. Sci Cairo 40 (11) 359-397.
10. Awad, S,M and Atawia, A. R. (1995). Effect of the foliar sprays some micro nutrients on Le Conte pear Trees (2) tree fruiting and fruit quality. Annuals Agric. Sci. Cairo, 40 (1): 369-377.
11. Bacha, M. A., Sabbah, S.M. and El-Hammady , M.A. (1995). Effect of foliar application of iron zinc and manganeses on yield, pear quality and leaf mineral composition of Thompson seedless and Roomy Red, grape cultivars. Alex. J. Agric. Res. 40 (3): 3-331.
12. Bidwell R.G.A. (1479). Plant physiology Macmillan publishing Co. Inc. New York, pp. 199-200.

13. Chuntanaparb, N. and Cummings, C. (1981). Seasonal trends in concentration nitrogen, phosphorus, potassium, calcium and magnesium in leaf portions of apple, blue berry, grape and peach J. Am. Coc. Hort. Sci. (6) 933.
14. Dubois, M., Gilles, K. A., Homilton, J. K. Robers, P. A. and Smith F. (1956). Colorimetric methods for determination of sugar and related substances Annal. Chem. 28 (3): 350-458.
15. El- Seginy, Amal M. and Bahan, Khalil, M. (2000). Effect of spraying some nutrients and gibberellic acid on leaf mineral content, fruit characters and yield of Le-Conte pear tree J. Agric, Sci, Mansoura Univ. 25 (6): 3529-3539.
16. El-Fakharany M.M. Wally, A.S. and Makarim, M.M. (1995). Effect of some regulators on Anna apple retained fruits and fruit quality J. Agric. Sc. Mansoura Univ. 20 (10): 4439-4446.
17. El-Hammady A, Abd-El-Hamid N., Saleh N. and Saleh A. (2000). Effect of gibberellic acid and calcium chloride treatments on delaying maturity, quality and storability of "Balady" mandarin, Fruits. Arab Univ. J. Agric. Sci., 3: 755-766.
18. El-Shazly, S.M. (1999) Response of Anna apple trees to foliar sprays of chelated iron, Manganese and zinc. J. Agric. Sci. Mansour Univ., 24(12): 7595-7591.
19. El-Shobaky, M.A., Enas S. Abbas and Hanaa A. El-Helw. (2001) Effect of Micro elements sproy on leaves mineral content, yield and quality and storage ability of Ruby seedless grapes. J. Agric. Sci. Mansoura Univ., 26 (3) , 1721-1733.
20. Evenhuis, B and P.W. De Waard. (1980) Principles and practices in plant analysis. F.A.O. Soil Bull .3 (1): 152-162.
21. Faust, M, (1989). Physiology of temperate zone fruit tree. John Wily and Sons, New York.
22. Fawzi, M.I.F and Eman, Abd El Moneim, A. A. (2004). The effect of foliar application of some trace elements and active dry yeast on vegetative growth, yield, fruit quality and leaf chemical composition of Flame seedless grapevines grown in calcareous soil conditions . Minufiya, J. Agri, Res. Vol. 2 No. 29. 463-478.
23. Gill, G.F., Henriquez , J.A. Vera C.A. and Zoffoli J.P. (1994). Physiological disorders of pears in relation with nutrition, Acta Hort No. 367. 295 (Hort. Abst., 65(5) 3804).
24. Gobara, A. A. Ahmed F.F. and El – Shammaa, M.S. (2001). Effect of varying N. K. and Mg application ratio on productivity of Banaty grapevines. The fifth Arabian Horticulture conf. Ismallia Egypt, March 24-28 pp. 83-90.
25. Gobara, A.A. (1998). Response of Le-Conte Pear trees to folia applications of some nutrients Egypt. J, Hort. 25, No.1,pp. 55-70.
26. Hanson, E. J. (1991a). Sour cherry trees response to foliar boron applications. Hort Sci. 26, 1142.
27. Helail, B.M. (1993). Response of Anna apple trees to foliar sprays with some micronutrnts 1-Tree growth and leaf minerals content. Egypt. J. Appl. Sci. (9) 262-275
28. Intrigliolo, F., Roccuzo, G. and Lacertosa, G. (1991). Foliar diagnosis in Citriculture. Informatore Agraio 55(39): 77-85 Italy.
29. Kabeel, H., Mokhtar, H and Aly, M. M. (1998). Effect of foliar application of different macro – and micro nutrients on yield, fruit quality and leaf mineral composition of L-Conte pear. J Agric. Sci. Mansoura Univ., 23 (7): 3317-3325.
30. Lovatt, C. J. (1999). Timing citrus and avocado foliar nutrient applications to increase fruit set and size. Hort. Technology 9 (4) : 607-612, U.S.A..
31. Makarem, M.M. and Mokhatar, H. (1996). Effect of biozyme and gibberellic acid on fruit set, yield and fruit quality of Anna apple trees J. Agric, Sic. Mansoura Univ. 21(7): 2661-2669.
32. Marschner, H. (1995). Mineral nutrition of higher plants. Second Edition Academic press. London.
33. Mead, R. A.; Currow, R. N. and Harted, A. M. (1993). Statistical Methods in Agricultural and Experimental Biology, 2nd Ed.Chapman,Hall London.10-44.
34. Mohammed, M. A.; Ahmed F. F. and Abdel- Aziz, F. H. (1991). Improving the quality of White Banaty grapes treated with GA3 by some micro and macro nutrients. II – Effect of macronutrients. Minia J. Agric. Res & Dev. 13 (2): 695-705.
35. Nawar, A. (1991). Some physiological and biochemical aspects of leaf iron cholorosis in Anna trees 1- leaf iron fraction mineral composition and activity of peroxidase and catalase enzymes, Alex. J. Agric. Res. 36 (2) 103-117.
36. Nijjar, G. S. (1985). Nutrition of fruit trees Mrs Usho Raj Kumar. Kalyani, New Delhi, pp. 10-20.
37. Ranvir – Singh and Misra, K. K. (1980). Effect of foliar application of micro nutrients on kinnow mandarin Punjat, Hort. & Jour. 20 (3/4) 143-148.
38. Sheo, G. and Singh, I.P. (1999). Eff of foliar application of urea, GA3 and Zn So4 on seedling growth of two citrus species J. of Applied Hort. 1 (1): 51-53 India.
39. Sarawathi, T.; Thangaraj, T., Azhak Manavalan, R., S. and Balakrishnamurthy, G. (1998). Effect of micronutrients on yield and quality of mandarin orange (Citrus reticulata Bianco). South Indian Hort. 46 (36): 128-131.

40. Wild, S.A.; Corey, R. B., lyer J.G. and Vogit, G.k. (1985). Soil and plant Analysis for tree culture. Mohan Primlani, Oxford & IBH. Publishing Co., New Delhi. Pp. 1-192.
41. Youness- Randa (2002). Effect of different sources and methods of nitrogen application on vegetative growth and fruit of valencia orange trees (Citrus sinensis L.) Ph. D. Thesis fac. Agric. Minia Univ.
42. Zhao,x., Sheo H and liao, C. (1999) Boron in soils and with relationship to the fruit quality of Nanfeng orange (Citrus reticutata Blanco in Jianxi Province of China. China Agric. Press: 380-386, China.

6/15/2010

Anti-ulcer Effect of Cinnamon and Chamomile Aqueous Extracts in Rat Models

Amr, A. Rezq* and Maysa, M. Elmallh

Nutrition and Food Science Department, Faculty of Home Economics, Helwan University
*** dr.amr_rezq@yahoo.com**

Abstract: Peptic ulcer disease is a problem of the gastrointestinal tract. Nowadays, drugs are expensive and have many side effects during treatment of any disorders. Therefore, our study aimed to investigate and compare antiulcer effect of cinnamon and chamomile aqueous extracts at doses of 100, 200, 300, 400mg/kg of body weight (b.wt) with antiulcer drug (Zantac ™ Ranitidine). Fifty male rats weighing 160±5g were distributed into ten groups. Group I serves as a positive group. Group II serves as control group (treated with drug). Groups III, IV, V and VI were administered orally the different doses of cinnamon aqueous extract (CIAE). Groups VII, VIII, IX and X were administered orally the different doses of chamomile aqueous extract (CHAE). Values of pH and volume of gastric juice, ulcer area and curative ratio were estimated as well as histopathological examination of stomach. Results revealed that treatment with Zantac and CIAE or CHAE was associated with significant increase in pH values compared to the respective value of the positive group. CHAE was superior to that of CIAE. Oral administration of CIAE or CHAE was associated with significant reduction in the volume of gastric juice compared to positive and control groups. A curative ratio of gastric ulcer was better in rats given CIAE or CHAE over those given Zantac. Furthermore, CHAE was superior over CIAE in its curative ratios of gastric ulcer. Histological study showed necrosis of gastric mucosa associated with congestion of submucosal blood vessels, submucosal edema and hemorrhage in the stomachs of positive rats. The stomachs of group receiving Zantac showed necrosis of gastric mucosa associated with hemorrhage. Whereas, higher dosages of CIAE (300 or 400 mg/kg of b. wt and CHAE dosages i.e., 200, 300 or 400 mg/kg of b.wt were efficient to arrest histopathological changes in the stomachs. In conclusion: results revealed that CHAE and CHAE had potential antiulcer effect, which was superior to the respective effect observed with Zantac. Chamomile extracts were more superior to cinnamon in its protection of the stomach. The antiulcer effect was dose dependent with no adverse effects. [Journal of American Science. 2010;6(12):209-216]. (ISSN: 1545-1003).

Keywords: Chamomile- Cinnamon-Peptic ulcer.

1- Introduction

Peptic ulcer disease is a problem of the gastrointestinal tract characterized by mucosal damage secondary to pepsin and gastric acid secretion. It usually occurs in the stomach and proximal duodenum; less generally, it occurs in the lower esophagus, the distal duodenum, or the jejunum, as in unopposed hypersecretory states such as Zollinger-Ellison syndrome, in hiatus hernias, or in ectopic gastric mucosa (Kalyanakrishnan and Robert, 2007). Some of the causes of these disorders are stress, smoking, nutritional deficiencies and ingestion of non-steroidal anti-inflammatory drugs (Nash et al., 1994; Basil and Howard, 1995). The pathogenesis of gastroduodenal ulcers are influenced by various aggressive and defensive factors, such as acid-pepsin secretion, mucosal barrier, mucus secretion, blood flow, cellular regeneration and endogenous protective agents (prostaglandins and epidermal growth factor) (Salas, 1990).

Nowadays, drugs are expensive and have many side effects during treatment of any disorders. Therefore, the potential of the health promoting and disease preventing properties of plant-derived compounds has received increased attention from researchers in recent years. Plants that have medicinal properties are used to improve symptoms or prevent diseases such as diarrhea, stomach disorder, asthma, hypertension, coughs and other respiratory ailments and urinary tract infections etc.

Cinnamon is a native of Southern Asia and South America. Now it is cultivated in many tropical countries such as China, India, Brazil, Madagascar, Mexico and the Caribbean. Cinnamon (*Cinnamomum cassia*) of the family *Lauracease* is a favorite spice around the world because of its health benefits, flavors and preserves food (Chaudhry and Tariq, 2006). The most favorite chemical constituents of cinnamon are volatile oil (cinnamaldehyde, eugenol, cinnamic acid, and weitherhin), mucilage, diterpenes and proanthocyanidins (Jayaprakasha et al., 2002). Cinnamon possess chemopreventive, antispasmodic, sedative, hypothermic, choleretic, antibacterial, antifungal, antipyretic, antiviral, antiplatelet properties, antiseptic, lipolytic, anesthetic, cytotoxic, anodyne, hypolipidemic, and also stimulate immune system that may be useful adjuncts in helping to reduce the risk of cardiovascular disease and cancer (Cralg, 1999). Medicinally, it is used in the treatment of colic, colds, low vitality, poor appetite, rheumatism, kidney weakness and coldness, fevers,

arthritic angina, and palpitations. It is also used in stimulate of the circulatory system and capillary circulation, spasms, vomiting, controls infections and digestive or stomach complaints related to cold and chills. Cinnamon bark have a potentiating effect on insulin (Khan et al., 1990) and can be useful in the treatment of type 2 diabetes; as well as lowering triglyceride levels and serum cholesterol (Onderoglu et al., 1999; Broadhurst et al., 2000; Khan et al., 2003). Water-based extracts of cinnamon bark might bind endotoxin, thereby protecting against endotoxin-induced organ damage (Azumi et al., 1997), has anti-bacterial effects, clinical trials against *Helicobacter pylori*, associated with gastric ulcer (Martin and Ernst, 2003) and improve symptoms associated with the metabolic syndrome in rodents and humans metabolism and lipid profile (Khan et al., 2003; Kannappan et al., 2006).

Chamomile is one of the most widely used as medicinal plants. It has been included in the pharmacopoeia of 26 countries. Amino acids, polysaccharides, fatty acids, essential oils, mineral elements, flavonoids, and other phenolic compounds are the main constituents of chamomile (McKay and Blumberg, 2006). Chamomile used in modern medicine primarily for their spasmolytic, antiphlogistic, antibacterial properties, and as a multipurpose digestive to treat gastrointestinal disturbances including flatulence, indigestion, diarrhea, anorexia, motion sickness, nausea, and vomiting (Shikov et al., 2008). German chamomile (*Matricaria chamomilla*) is also used to healing wound (Glowania et al., 1997), treat various diseases including diarrhea (de la Motte et al., 1997), and inflammation, cancer (Hernández-Ceruelos et al., 2002). Its extract blocks aggregation of *Helicobacter pylori* and various strains of *Escherichia coli* (Annuk et al., 1999). Chamazulene, alpha-bisabolol, flavonoids, and umbelliferone display antifungal properties against *Trichophyton mentagrophytes* and *Trichophyton rubrum* (Turi et al., 1997). Apigenin, alpha-bisabolol, and the cisspiroethers appear to provide the most significant antispasmodic effects. Other flavonoids and coumarins contribute to smooth muscle relaxation (Achterrath-Tuckermann et al., 1980). The aim of the present study was to investigate and compare the gastroprotective effect of different doses (100, 200, 300 and 400mg/kg of body weight (b.wt.) of cinnamon or chamomile aqueous extracts with antiulcer drug. pH and volume of gastric juice, ulcer area and curative ratio were estimated and a reliable parameter was presented for comparing the data. Histopathological examination of gastric ulcer was achieved.

2. Materials and Methods

2.1 Materials

2.1.1 Herbs

Cinnamon and chamomile were purchased as crude dried material from a local Company for Medicinal Plants and Herbs, Cairo, Egypt.

2.1.2 Animals

Fifty male albino rats, Sprague Dawley strain weighing 160±5g, were obtained from the Laboratory Animal Colony, Helwan, Egypt.

2.1.3 Drugs

Anti-ulcer agent (Zantac ™ (Ranitidine) was obtained in the form of ampoules from Glaxo Smithkline S.A.E., El-Salam City, Egypt.

2.2 Methods:

2.2.1 Preparation of aqueous extract

The aqueous extracts of cinnamon and chamomile were prepared using 10g dried material/100 ml distilled water and boiling for 5 min at 100°C. Then they were filtrated, concentrated at 50 °C under reduced pressure using a Rota vapor. The extracts were kept at -15 °C until it was used in the experiment (Kassi et al., 2004).

2.2.2 Preparation of basal diet

The basal diet (AIN-93M) (Reeves et al., 1993) was formulated to meet recommended nutrients levels for rats as shown in Table (1).

Table (1): Composition of the modified AIN-93M diet.

Ingredient	Content (g/kg)
Casein	140.0
Corn starch	620.69
Sucrose	100.00
Soybean oil	40.0
Fibers	50.0
Mineral mix.	35.0
Vitamin mix.	10.0
L-Cystine	1·8
Choline chloride	2.5
Tert-Butylhydroquinone	0.008

2.2.3 Experimental design

All Animals were fed on the basal diet and water *ad libitum* and they were maintained under healthy conditions of humidity, temperature (20-25°C) and light (12-h light: 12-h dark cycle) for one week before starting the experimental to acclimatization. After acclimatization period, rats were divided into ten groups of equal weight and number (5 rats each). Group (I): kept as positive group and Group (II): service as control group. These two groups fed on the basal diet and given orally saline at volume of 1.0 ml / 100 g b. wt). Groups (III, IV, V and VI) fed on the basal diet and given orally cinnamon aqueous extract (CIAE) by tube feeding for seven days at a dose of 100, 200, 300 and 400 mg/kg b. wt, respectively. Groups (VII, VIII, IX and X): fed on the basal diet and given orally chamomile aqueous extract (CHAE) by tube feeding for seven days at a dose of 100, 200, 300 and 400 mg/kg b. wt, respectively.

At the last day of experimental period (7 days), all rats were starved of food but not of water for 12

hours according to ethanol-induced gastric ulcer protocols. After fasting period, group II (control group) was given (I/P) intraperioteneally Zantac (ranitidine) at a dose 6 mg/100 of body weight, 60 min prior administered ethanol. Groups (I and II) were given orally saline and the other eight groups were given CIAE or CHAE, 120 min prior administered ethanol (Jafri et al., 2001). Then ethanol was administered orally to all groups at 0.5 ml/100g (Hollander et al., 1985).

2.2.4 Gastric ulcer index

The method described by Agrawal et al., (2000) was employed in the present study. In briefly, after 4 hours of administrated ethanol, all rats were sacrificed after using an overdose of diethyl ether and their stomachs removed and washed by saline. The gastric juice was collected in test tube. Then stomachs opened along the greater curvature, washed with saline and examined under dissecting microscope for gastric ulcers. The sum of length for all lesions area for each animal was measured and served as the ulcer index. The curative ratio was calculated for each group using following equation:

Curative ratio (CR) = (LC-LT/LC) x100

LC: The length of gastric ulcer in positive group.

LT: The length of gastric ulcer in treated group.

2.2.5 Determination of gastric juice acidity

Acidity degree (pH) of gastric juice was determined by using pH meter apparatus (HI 9021).

2.2.6 Determination of gastric juice volume

Gastric juices were centrifuged at 500 rpm for 5 minutes, then separated and measured volume by graduated cylinder.

2.2.7 Histopathological examination

The stomachs of the scarified rats were taken and immersed in 10% formalin solution. The fixed specimens were then trimmed, washed and dehydrated in ascending grades of alcohol. Specimens were then cleared in xylol, embedded in paraffin, sectioned at 4-6 microns thickness and stained with Heamtoxylin and Eosin stain for examination of the stomach as described by Carleton, (1979).

2.2.8 Statistical analysis

The obtained results were expressed as Mean ± SE. Data were evaluated statistically using one-way analysis of variance (ANOVA). Significant difference between means was estimated at $p<0.05$ (STATSOFT, 2006).

3. Results

3. 1 pH of gastric juice

Values of pH in rats treated with antiulcer drug and oral administration of CIAE and CHAE at different doses is recorded in Table (2). Results demonstrated that control group given I/P antiulcer drug and groups given orally different doses of CIAE or CHAE (100, 200, 300 or 400mg/kg b.wt) had significant increase in pH value of gastric juice at $p<0.05$ as compared to positive group.

In rats given orally CIAE at doses of 200, 300 and 400mg/kg b.wt, pH values of gastric juice as mean±SE were 7.90±0.19, 8.40±0.37 and 8.60±0.19, respectively and showed significant increase as compared to control group and those given orally 100mg/kg of b. wt CIAE (5.80±0.46 and 6.50±0.45 respectively). In contrast, groups given orally the different doses (100, 200, 300 and 400mg/kg of b.wt.) of CHAE had significant increase in pH values of gastric juice (7.80±0.26, 8.20±0.12, 8.40±0.25 and 8.66±0.19, respectively) compared to control group given I/P anti ulcer drug (5.80±0.46).

The increase in pH values of gastric juice of treated rats with extract was more detectable with increasing the dose. Results revealed that different doses of CHAE caused increase in mean ± SE of pH values than that the similar doses of CIAE.

Table (2): Effect of antiulcer drug and oral administration of CIAE and CHAE on pH value of gastric juice in rats.

Groups		Parameter as Mean ± SE pH of gastric juice	
		Cinnamon	Chamomile
Positive group		c 4.70±0.26	c 4.70±0.26
Control group		b 5.80±0.46	b 5.80±0.46
Treated groups with aqueous extracts at a doses of:	100mg/kg b. wt	b 6.50±0.45	a 7.80±0.26
	200mg/kg b. wt	a 7.90±0.19	a 8.20±0.12
	300mg/kg b. wt	a 8.40±0.37	a 8.40±0.25
	400mg/kg b. wt	a 8.60±0.19	a 8.66±0.19

Different superscript letters in the same column denotes significant differences at $p<0.05$.

3.2 Volume of gastric juice

Volume of gastric juice (cm^3) in rats treated antiulcer drug and oral administration of CIAE and CHAE at different doses is shown in Table (3). Data was obvious that volume of gastric juice (cm^3) as mean±SE of group given I/P antiulcer drug (control group) was not significant decrease (4.30±0.37) at $p<0.05$ as compared to positive group (4.60±0.52). Rats given orally different doses of CIAE or CHAE had significant decrease in volume of gastric juice compared to positive and control rats. Rats given orally CIAE at a dose of 100mg/kg of b.wt. had a significant increase in volume of gastric juice as compared to rats given extracts at doses of 200, 300 and 400mg/kg of b.wt.

Aqueous extract of chamomile at a dose of 400 mg/kg of b.wt caused significant decrease in volume of gastric juice as compared to a dose of 100 mg/kg of b.wt, and there was not significant decrease as compared to treated groups with doses of 200 and 300mg/kg of b.wt. There were not significant differences in volume of gastric juice among groups treated with CHAE at doses of 100, 200 and 300 mg/kg of b.wt.

Tabulated data showed that groups treated with CHAE had the lower volume of gastric juice as mean ± SE (cm^3) compared to those treated with CIAE.

Table (3): Effect of antiulcer drug and oral administration of CIAE and CHAE on the volume of gastric juice (cm^3) in rats.

Groups		Parameter as Mean ± SE	
		Volume of gastric juice (cm^3)	
		Cinnamon	Chamomile
Positive group		a 4.60±0.52	a 4.60±0.52
Control group		a 4.30±0.37	a 4.30±0.37
Treated groups with aqueous extracts at a doses of:	100mg/kg b. wt	b 2.90±0.19	b 2.50±0.16
	200mg/kg b. wt	c 2.00±0.16	bc 1.90±0.19
	300mg/kg b. wt	c 1.70±0.20	bc 1.70±0.12
	400mg/kg b. wt	c 1.60±0.19	c 1.50±0.16

Different superscript letters in the same column denotes significant differences at $p<0.05$.

3.3 Length of gastric ulcer

The length of gastric ulcer (mm) in rats as result of antiulcer drug and oral administration of CIAE or CHAE effect is recorded in Table (4). Tabulated results revealed that the length of gastric ulcer (mm) as mean±SE of treated group with antiulcer drug (control group) was significant decrease (5.90±0.60) at $p<0.05$ compared to untreated group (positive group) (7.40±0.87). Groups given orally different doses of CIAE or CHAE (100, 200, 300 and 400 mg/kg of b.wt) had significant decrease in the length of gastric ulcer at $p<0.05$ as compared to positive and control groups.

Aqueous extract of cinnamon at a dose of 400 mg/kg of b.wt caused significant decrease in the length of gastric ulcer as compared to a dose of 100 mg/kg of b.wt, while there was not significant decrease as compared to doses of 200 and 300 mg/kg of b.wt.

The differences in the length of gastric ulcer in rats given orally CHAE were not significant.

Groups given orally CHAE at different doses had the lower mean±SE values in length of gastric ulcer (mm) as compared to those given orally similar doses of CIAE. The decreases in the length of gastric ulcer (mm) were more detectable with increased doses of CIAE and CHAE.

Table (4): Effect of antiulcer drug and oral administration of CIAE and CHAE on the length of gastric ulcer (mm) in rats.

Groups		Parameter as Mean ± SE	
		Length of gastric ulcer (mm)	
		Cinnamon	Chamomile
Positive group		a 7.40±0.87	a 7.40±0.87
Control group		b 5.90±0.60	b 5.90±0.60
Treated groups with aqueous extracts at a doses of:	100mg/kg b. wt	c 3.20±0.54	c 1.80±0.52
	200mg/kg b. wt	d 1.50±0.16	c 0.88±0.20
	300mg/kg b. wt	d 0.92±0.12	c 0.52±0.01
	400mg/kg b. wt	d 0.70±0.12	c 0.42±0.14

Different superscript letters in the same column denotes significant differences at $p<0.05$.

3.4 Curative ratio

Effect of antiulcer drug and oral administration of CIAE and CHAE at different doses on curative ratio of peptic ulcer in rats is showed in Figure (1). Mean of curative ratio of groups given orally different doses of CIAE or CHAE was higher than that of treated groups with antiulcer drug. Groups treated with CHAE at doses of 100, 200, 300 and 400mg/kg of b.wt had higher mean values of curative ratio (75.68, 88.11, 92.97 and 94.32, respectively) compared to those given orally similar doses of CIAE (56.76, 79.73, 87.57 and 90.54, respectively).

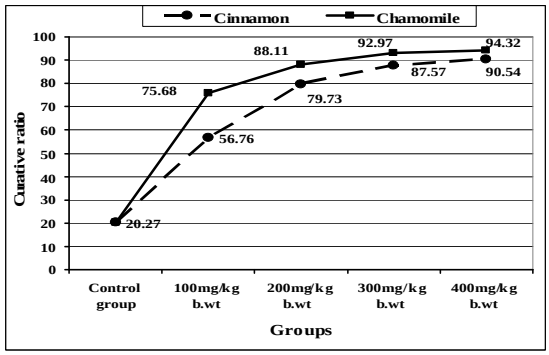


Figure (1): Effect of antiulcer drug and oral administration of CIAE and CHAE on curative ratio of gastric ulcer in rats.

3.5 Histopathological results

Microscopically, stomachs of rats from the positive group showed necrosis of gastric mucosa associated with congestion of submucosal blood

vesselsm, submucosal edema and hemorrhage (Figure 2). Examined stomachs of rats from control group (treated with anti-ulcer drug) revealed necrosis of gastric mucosa associated with hemorrhage as showed in Figure (3).

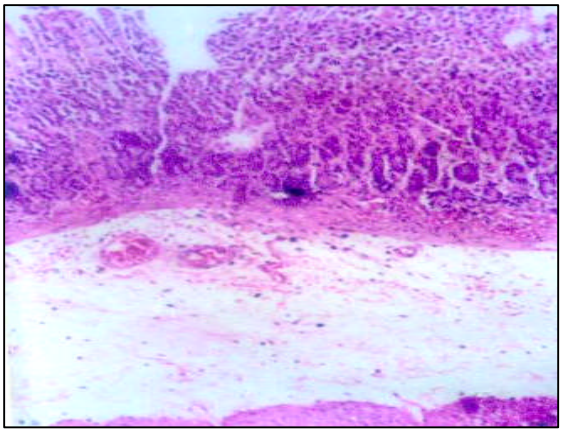


Figure (2): Stomach of positive rat showing necrosis of gastric mucosa, congestion of submucosal blood vessels associated with submucosal edema and hemorrhage (H and E x 100).

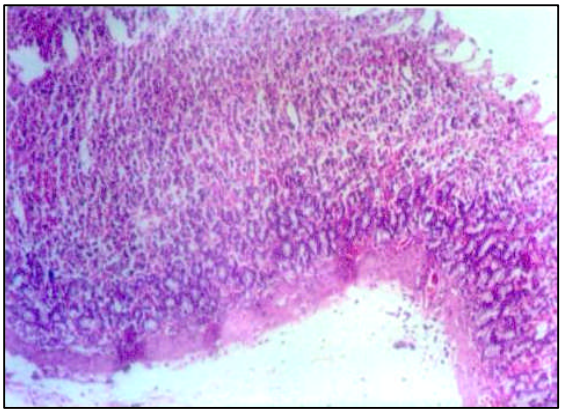


Figure (3): Stomach of control rats showing marked necrosis of gastric mucosa associated with hemorrhage (H and E x 100).

Examined stomachs of rats treated with CIAE at a dose of 100mg/kg of b.wt revealed atrophy of gastric mucosa associated with submucosal edema as showed in Figure (4). Moreover, stomachs of rats from given orally 200mg/kg of b.wt of CIAE showed congestion of submucosal blood vessels associated with edema (Figure 5). Meanwhile, stomachs of rats treated with extract at doses of 300 and 400mg/kg of b.wt showed no histopathological changes (Figure 6).

With regard to the effect of CHAE, histopathological results showed that rats treated with of extract at a dose of 100mg/kg of b.wt showed submucosal leucocytic cells infiltration (Figure 7). Examined sections from treated groups with extracts at doses of 200, 300 and 400mg/kg of b.wt revealed no histopathological changes as showed in Figure (8).

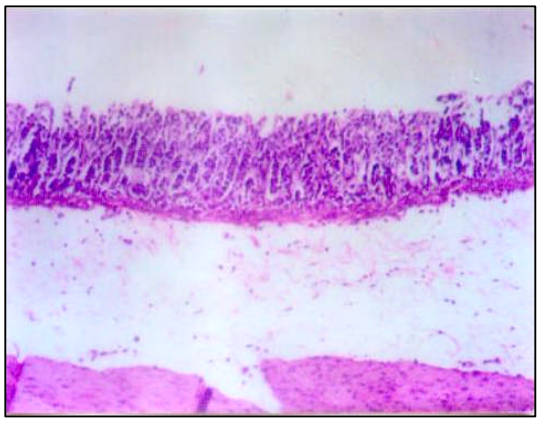


Figure (4): Stomach of rats given orally CIAE at a dose of 100mg/kg of b.wt showing atrophy of gastric mucosa associated with submucosal edema (H and E x 100).

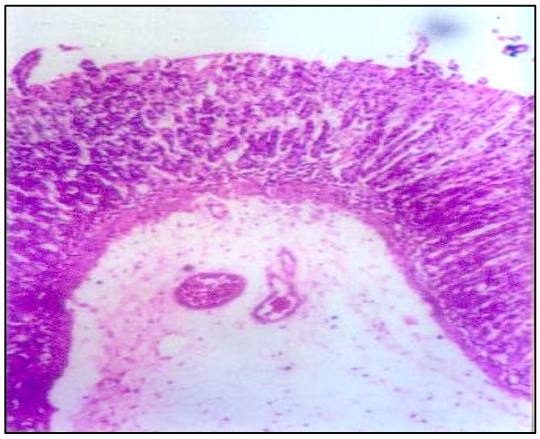


Figure (5): Stomach of rats given orally CIAE a dose of 200mg/kg of b.wt showing congestion of submucosal blood vessels associated with edema (H and E x 100).

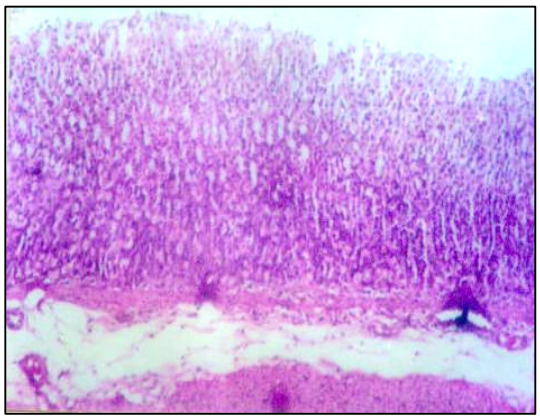


Figure (6): Stomach of rats given orally CIAE at doses of 300 and 400mg/kg of b.wt showing no histological changes (H and E x 100).

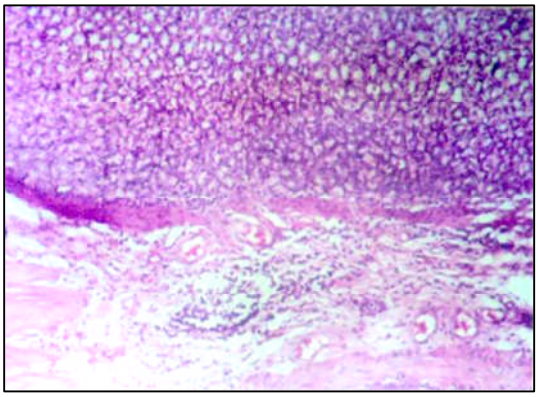


Figure (7): Stomach of rats given orally CHAE at a dose of 100 mg/kg of b.wt showing submucosal leucocytic cells infiltration (H and E x 100).

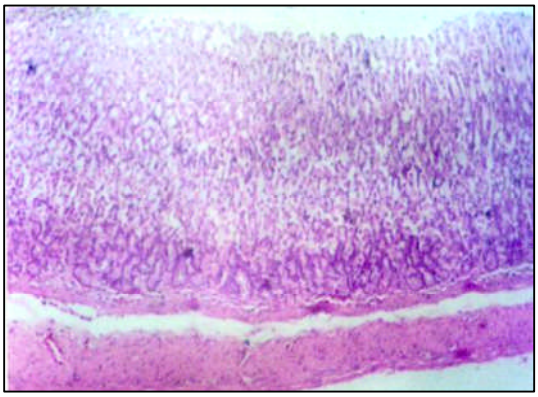


Figure (8): Stomach of rat given orally CHAE at doses of 200, 300 and 400mg/kg of b.wt showing no histological changes (H and E x 100).

4. Discussion

The aim of the present study was to investigate the antiulcer effect of cinnamon and chamomile aqueous extracts and compare them with antiulcer drug as a reference of antiulcer. Our finding showed that cinnamon and chamomile aqueous extracts at the different tested doses (100, 200, 300 and 400 mg/kg of b.wt) had gastroprotective effects on acute experimental gastric ulcer in rats. Antiulcer effect of cinnamon and chamomile aqueous extracts was higher than that of antiulcer drug. Aqueous extract chamomile had much more favorable antiulcer effect, compared to aqueous extract of cinnamon.

Peptic ulcer disease is a problem of the gastrointestinal tract characterized by mucosal damage secondary to pepsin and gastric acid secretion (Kalyanakrishnan and Robert, 2007). Therefore, the major mechanism action of cinnamon or chamomile aqueous extracts as anti-ulcer appears may be due to its effect in the decrease of acid-pepsin secretion and volume of gastric juice, and the promotion of mucosal protection by gastric mucin activity.

In the present study the antiulcer properties of cinnamon, agreed with Akira et al., (1986) who reported that CIAE inhibits gastric secretion and promotes gastric mucosal blood flow. These results confirmed by Tanaka et al., (1989) who demonstrated that cinnamon extract had 3-(2-hydroxyphenyl)-propanoic acid and its O-glucoside that prevent serotonin-induced ulcerogenesis in rats. It inhibits gastric ulcers induced by the ulcerogens such as ethanol, phenylbutazone, and water immersion stress, although it failed to prevent indomethacin-induced ulcers. 3-(2-hydroxyphenyl)-propanoic acid inhibits the secretion of gastric acid and promoted the gastric blood flow. Therefore, the antiulcerogenic effect of this compound is due to the potentiation of defensive factors through the improvement of the circulatory disorder and gastric cytoprotection. Zhu et al., (1993) revealed that water and ether extract of cinnamon had antiulcer effect on four types of experimental gastric ulcer. Aguilar, (1999); Cralg, (1999) also, reported that cinnamon used in the medicine for the treatment of gastric ulcer and digestive or stomach complaints. Recent research demonstrated that cinnamon extract decreased the level of prostaglandin that is associated with gastric ulcers (Jonathan et al., 2008).

On the other hand, the anti-spasmodic and anti-peptic actions of chamomile extract may be due to chamomile flavonoid constituents, apigenin (Achterrath-Tuckermann et al., 1980). In addition to, similar results were observed with alpha-bisabolol and the cis-spiroethers and the small amount of coumarins contribute to smooth muscle relaxation (Holzl et al., 1986). The antiulcer effect of chamomile extract agreed with Mann and Staba, (1986) who reported that chamomile had anti-inflammatory and spasmolytic effects on the stomach and duodenum. Therefore, it is thought to heal ulcers. Previous study reported that chamomile flower extract has a complex effect on the luminal and mucosal environment of the stomach and duodenum. Some of these actions are important in healing the ulcers and others are important in preventing subsequent ulcer relapse. Chamomile flower extract has a direct effect on acid secretion, and increases mucosal resistance against damaging agents such as ethanol and aspirin (Rees, 1992). Recent research revealed that CHAE, singly or combined with other plants have antiulcerogenic activity (Khayyal et al., 2001). Ramos-e-Silva et al., (2006) reported that CHAE had analgesic effect in oral aphtus ulcer.

The anti ulcer action of CHAE may be related to a variety of mineral elements including manganese and magnesium and 1-2% volatile oils including α-bisabolol, α- bisabolol oxides A and B, matricine presented in the chamomile flowers (McKay and Blumberg, 2006). Hwang et al., (2008) demonstrated that chamomilla extract contains many components that may exert antiulcer effects. Phenolic and flavonoids compounds, apigenin, quercetin,

patuletin, luteolin and their glycosides are the major flavonoids present in the flower. The presence of large amounts of cinnamic acid derivatives, frolic and caffeic acid and all of the constituents, may have therapeutic effects. Polysaccharides, amino acids and fatty acids are some of its constituents. Recently, Karbalay-Doust and Noorafshan, (2009) revealed that oral administration of chamomile extract at 400 mg/kg can be effective in preventing gastric ulceration in mice and does not produce toxic effects in doses up to 5000 mg/kg confirmed these results.

5. Conclusion

The present finding concluded that water extracts of cinnamon and chamomile had potential antiulcer effect, which was superior to the respective effect observed with Zantac. Chamomile extracts were more superior to cinnamon in its protection of the stomach. The antiulcer curative ratios were dose dependent with no adverse effects.

Corresponding author
Amr, A. Rezq
Nutrition and Food Science Department, Faculty of Home Economics, Helwan University, Cairo, Egypt.
dr.amr_rezq@yahoo.com

6. References

[1] Achterrath-Tuckermann, U., Kunde, R. and Flaskamp, E., 1980. Pharmacological investigations with compounds of chamomile. V. Investigations on the spasmolytic effect of compounds of chamomile and Kamillosan on the isolated guinea pig ileum. Planta. Med. 39, 38-50.

[2] Agrawal, A., Roo, V., Sairam, K., Joshi, V. and Goel, R., 2000. Effect of piper longgum linn, zingiber officinal is linn and ferule species n gasric ulceration and secretion in rats. Exper. Bio. 38, 994-998.

[3] Aguilar, A., 1999. Plantas Medicinales Del Centro de Mexico. Mexico City: Guias Practicas Mexico Desconocido.

[4] Akira, T., Tanaka, S., Tabata, M., 1986. Pharmacological studies on the antiulcerogenic activity of Chinese cinnamon. Planta. Med. 52, 440- 443.

[5] Annuk H, Hirmo S, Turi E., 1999. Effect on cell surface hydrophobicity and susceptibility of *Helicobacter pylori* to medicinal plant extracts. FEMS Microbiol. Lett. 172, 41- 45.

[6] Azumi, S., Tanimura, A. and Tanamoto, K., 1997. A novel inhibitor of bacterial endotoxin derived from cinnamon bark. Biochem. Biophys. Res .Commun. 234, 506–510.

[7] Basil, M.D. and Howard, M.S., 1995. Clinical gastroenterology. In: Companion Handbook. 4th ed. USA: McGraw-Hill.

[8] Broadhurst, C.L., Polansky, M.M. and Anderson, R.A., 2000. Insulin-like biological activity of culinary and medicinal plant aqueous extracts in vitro. J. Agric. Food Chem. 48(3), 849-852.

[9] Carleton, H., 1979. Histological techniques, 4th Ed, London, Oxford University Press, New York, USA, Toronto.

[10] Chaudhry, N.M. and Tariq, P., 2006. Anti-microbial activity of *Cinnamomum cassla* against diverse microbial flora with its nutritional and medicinal impacts. Pak. J. Bot. 38(1), 169-174.

[11] Cralg, W.J., 1999. Health-promoting properties of common herbs. Amer. J. Clin. Nutr. 70(3), 4915-4995.

[12] de la Motte, S., Bose, O., Reilly, S., Heinisch, M. and Harrison, F., 1997. Double-blind comparison of an apple pectin-chamomile extracts preparation with placebo in children with diarrhea. Arzneimittelforschung. 47, 1247-1249.

[13] Glowania, H.J., Raulin, C. and Swoboda, M., 1997. Effect of chamomile on wound healing – a clinical doubleblind study. Z. Hautkr. 62, 1262-1271.

[14] Hernández-Ceruelos, A.E., Madrigal, B. and de la Cruz, C., 2002. Inhibitory effect of chamomile essential oil on the sister chromatid exchanges induced by daunorubicin and methyl methanesulfonate in mouse bone marrow. Toxicology Letters. 135(5), 103-110.

[15] Hollander, D., Tarnawski, A., Krause, W.J. and Gergely, H., 1985. Protective effect of sucralfate against alcohol-induced gastric mucosal injury in the rat. Gastroenterology. 88, 366-374.

[16] Holzl, J., Ghassemi, N. and Hahn, B., 1986. Preparation of 14C-spiro ethers by chamomile and their use by an investigation of absorption. Planta Med. 52, 553.

[17] Hwang, H.J., Kwon, M.J., Kim, I.H. and Nam, T.J., 2008. The effect of polysaccharide extracted from the marine alga capsosiphon fulvescens on ethanol administration. Food Chem. Toxicol. 46, 2653-2657.

[18] Jafri, M.A., Farah, J.K. and Singh, S., 2001. Evaluation of the gastric antiulcerogenic effect of large cardamom (fruits of Amomum subulatium Roxb). J. Ethnopharmacol. 75, 89-94.

[19] Jayaprakasha, G.K., Raom, L.J. and Sakariah, K.K., 2002. Chemical composition of volatile oil from Cinnamomum zeylanicum buds. Z. Naturforsch. 57(12), 990-993.

[20] Jonathan, C., Marc, L., Yumei, L., Lisa, R. and Randolph, K., 2008. Clinical and biochemical effects of a combination botanical product (ClearGuard™) for allergy: a pilot randomized double-blind placebo-controlled trial. Nutr. J. 7, 20.

[21] Kalyanakrishnan, F. and Robert C., 2007. Peptic ulcer disease. Am. Fam. Physician. 76 (12), 1005-1013.

[22] Kannappan, S., Jayaraman, T., Rajasekar, P., Ravichandran, M.K. and Anuradha, C.V., 2006. Cinnamon bark extract improves glucose metabolism and lipid profile in the fructose-fed rat. J. Singapore Med. 47, 858–863.

[23] Karbalay-Doust, S. and Noorafshan, A., 2009. Antiulcerogenic Effects of Matricaria Chamomilla Extract in Experimental Gastric Ulcer in Mice. Iran. J. Med. Sci. 34(3), 198-203.

[24] Kassi, E., Papoutsi, Z., Fokialakis, N., 2004. Greek plant extracts exhibit selective estrogen receptor modulator (SERM)-like properties. J. Agric. Food Chem. 52, 6956-6961.

[25] Khan, A., Bryden, N.A., Polansky, M.M. and Anderson, R.A., 1990. Insulin potentiating factor and chromium content of selected foods and spices. Biol. Trace Elem. Res. 24(3), 183-188.

[26] Khan, A., Safdar, M., Khan, M.M., Khattak, K.N. and Anderson R.A., 2003. Cinnamon improves glucose and lipids of people with type 2 diabetes. Diabetes Care. 26(12), 3215-3228.

[27] Khayyal, M.T., el-Ghazaly, M.A. and Kenawy, S.A., 2001. Antiulcerogenic effect of some gastrointestinally acting plant extracts and their combination. Arzneimittelforschung. 51, 545-553.

[28] Mann, C. and Staba, E., 1986. The chemistry, pharmacology and commercial formations of chamomile. Herbs Spices and Medicinal plants. 1, 235-280.

[29] Martin, K.W. and Ernst, E., 2003. Herbal medicines for treatment of bacterial infections: a review of controlled clinical trials. J. Antimicrob. Chemother. 51(2), 241-246.

[30] McKay, D.L. and Blumberg, J.B., 2006. A review of thebioactivity and potential health benefits of chamomile tea (Matricaria recutita L). Phytother. Res. 20, 519-530.

[31] Nash, J., Lynn, L. and Deakin, M., 1994. H2-receptor antagonist in peptic ulcer disease. Evidence for a prophylactic use. Drugs. 47, 862-871.

[32] Onderoglu, S., Sozer, S., Erbil, K.M., Ortac, R. and Lermioglu, F., 1999. The evaluation of long-term effects of cinnamon bark and olive leaf on toxicity induced by streptozotocin administration to rats. J. Pharm. Pharmacol. 51(11), 1305-1312.

[33] Ramos-e-Silva, M., Ferreira, A.F., Bibas, R. and Carneiro, S., 2006. Clinical evaluation of fluidextract of Chamomilla recutita for oral aphthae. J. Drugs Dermatol. 5, 612-617.

[34] Rees, W.D., 1992. Prevention of peptic ulcer relapses by sucralfate: mechanisms of action. Scand J. Gastroenterol Suppl. 191, 4-6.

[35] Reeves, P.G., Nielson, F.H. and Fahmy, G.C., 1993. Reports of the American Institute of Nutrition, adhoc-wiling committee on reformulation of the AIN 93. Rodent diet. J. Nutri. 123, 1939-1951.

[36] Salas, C., 1990. Fundamentos fisiopatológicos para el tratamiento de la úlcera péptica. Gen Revista de la Sociedad Venenzolana de Gastroenterología. 44, 180-190.

[37] Shikov, A.N., Pozharitskaya, O.N. and Makarov, V.G., 2008. Antibacterial activity of Chamomilla recutita oil extract against Helicobacter pylori. Phytother. Res. 22, 252-253.

[38] STATSOFT., 2006. Statistica: electronic statistics textbook. Tulsa, Version 6.0.

[39] Tanaka, S., Yoon, Y.H., Fukui, H., Tabata, M., Akira, T., Okano, K., Iwai, M., Iga, Y. and Yokoyama, K., 1989. Antiulcerogenic compounds isolated from Chinese cinnamon. Planta Med. 55(3), 245-248.

[40] Turi, M., Turi, E., Koljalg, S. and Mikelsaar, M., 1997. Influence of aqueous extracts of medicinal plants on surface hydrophobicity of *Escherichia coli* strains of different origin. APMIS. 105, 956-962.

[41] Zhu, Z.P., Zhang, M.F., Shen, Y.Q. and Chen, G.J., 1993. Pharmacological study on spleen-stomach warming and analgesic action of Cinnamomum cassia Presl. Zhongguo Zhong Yao Za Zhi. 18(9), 553-557.

6/12/2010

Botanical Studies On *Phaseolus Vulgaris* L. II-Anatomy Of Vegetative And Reproductive Organs

[1] Rania M. A.Nassar, [2] Mohamed S. Boghdady and [3] Yasser M. Ahmed

1- Department of Agricultural Botany, Faculty of Agriculture, Cairo University, Giza, Egypt.
2- Department of Agricultural Botany and Plant Pathology, Faculty of Agriculture, Zagazig University, Egypt.
3- Department of Vegetable Crops, Faculty of Agriculture, Cairo University, Giza, Egypt.
ranianassar23@yahoo.com

Abstract: The present study is concerned with the histological features of Kidney bean plant. The anatomical structure of different vegetative and reproductive organs was investigated fortnightly throughout the whole growing season. Studied organs included main root, main stem (represented by apical and median internodes), different types of foliage leaves developed on the main stem and on lateral shoot; including lamina and petiole, flower bud, fruit and seed. Histological features of various organs of Kidney bean plant were analyzed microscopically and photomicrographed. [Journal of American Science. 2010;6(12):217-229]. (ISSN: 1545-1003).

Keywords: *Phaseolus vulgaris* L., Kidney bean, Fabaceae, Anatomy, Vegetative organs, Reproductive organs.

1. Introduction

In the first part of this study (Nassar *et al.*, 2010), the authors investigated the morphology of vegetative and reproductive growth of Kidney bean plant (*Phaseolus vulgaris* L.) throughout the consecutive stages of its whole life span. Consequently, in this part of the study, it is aimed to bring to light more information about the anatomical structure of vegetative and reproductive organs of the plant during successive ages of its whole life span in order to complement the phytography study of Kidney bean plant which started in the first part.

Obviously, continued acquisition of new information about different botanical aspects of this species, which is of great interest from the economic point of view, are required.

2. Materials and Methods

The present study was carried out to investigate the anatomical structure of vegetative and reproductive organs of Kidney bean plant (*Phaseolus vulgaris* L.) of the family Fabaceae.

Therefore, a field trial was conducted in the Agricultural Experiments and Researches Station, Faculty of Agriculture, Cairo University, Giza, Egypt during the summer growing season of 2009 to provide the experimental plant materials. The work of microtechnique was carried out at the Laboratory of the Agricultural Botany Department, Faculty of Agriculture, Cairo University during the period from May, 2009 till April, 2010.

The field trial included five replicates, each represented by one plot. The plot was 4×5 m with eight ridges 60 cm apart. Date of cultivation was May 12th, 2009. Seeds of Kidney bean plant cv. Giza 6 were sown in hills spaced 25 cm on one side of the ridge. The plants were thinned to two plants per hill. All field practices were carried out as recommended for the studied crop in the vicinity.

A full microscopical study was carried out to investigate the histology of Kidney bean plant. Samples representing different plant organs were taken periodically, fortnightly, throughout the growing season. The following were investigated:

1. The main root, 1 cm below the hypocotyl.
2. The main stem represented by terminal and median internodes.
3. The mature prophyll and the apical leaflet of the foliage compound leaves number 3, 6 and 9 on the main stem, and of compound leaves representing secondary branches. The petiole of the compound leaf.
4. Flower bud.
5. Mature fruit and seed.
6. Type of stomata was defined using epidermal peals.

Microtechnique procedures given by Nassar and El-Sahhar (1998) were followed. Specimens were killed and fixed for at least 48 hrs in FAA solution, (10 ml formalin, 5 ml glacial acetic acid and

85 ml ethyl alcohol 70%), washed in 50% ethyl alcohol and dehydrated in a series of n-butyl alcohol before embedded in paraffin wax (mp 56-58°C). Transverse sections which were cut on a rotary microtome to a thickness of 20 microns were stained with crystal violet/erythrosin before mounting in Canada balsam. Slides were analyzed microscopically and photomicrographed.

3. Results and Discussion

1- Structure of the main root:

The anatomical structure of Kidney bean root system was investigated through transverse sections of the main root at different stages of plant growth. The tap root of two weeks old seedling, as shown in transverse section (Figure 1), is in primary state of growth. It has an uniseriate epidermis of tabular shaped cells. Cuticle on the outer walls and stomata are absent. Some epidermal cells prolong to form the typically unicellular root hairs. The cortex composed of about 6 layers of thin-walled irregular parenchyma cells with well-developed intercellular space system. The innermost layer of the cortex is the endodermis, an uniseriate zone of small barrel-shaped cells forming a distinct layer surrounding the stele. Next to the endodermis lies a layer of thin-walled parenchyma cells forming the pericycle. The vascular bundle is radial. Xylem and phloem occur in separate patches arranged on alternate radii, intervened by small parenchyma cells. The latter forms the conjunctive tissue. The bundle is tetrarch since four patches of xylem alternate with equal number of phloem patches. Protoxylem vessels occur towards the periphery and metaxylem towards the center, thus showing centripetal mode of differentiation from the procambium. This is the typical exarch xylem of roots. Opposite the protoxylem, pericycle is made up of two or three layers showing the point of origin of lateral roots. The center portion of the stele is occupied by a few compactly arranged thin-walled paranchymatous cells without any inter-cellular spaces forming a very small region of pith.

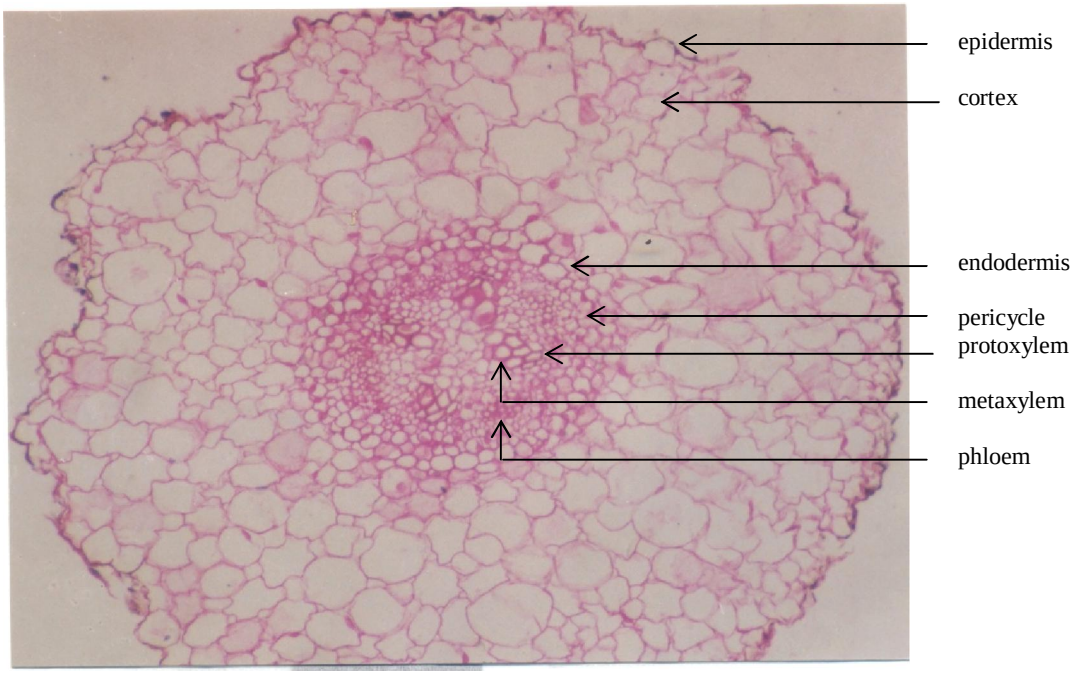


Fig.(1): Transverse section through the main root of *Phaseolus vulgaris* L. plant, aged two weeks, showing its primary structure. (X 144)

At the age of four weeks (Figure 2), the main root is in secondary state of growth, the epidermis as well as the cortex are completely sloughed off and a continuous well defined periderm arising in the pericycle is present. A cambial zone of about four layers is seen and secondary thickening proceeds, the xylem being more in amount than the phloem. The formation of parenchymatous rays from the cambium which was originated in the pericycle opposite to the xylem ridges is clearly obvious in this stage of secondary growth.

The secondary thickening is more prominent as tested plants were six weeks old (Figure 3). At this age, the root comprises mainly of a vascular cylinder surrounded by a periderm. The secondary xylem contains vessels of various diameters which accompanied by fibers and abundant amount of parenchyma cells.

As far as the authors are aware, no detailed study dealing with the anatomical structure of Kidney bean roots was carried out.

2- Structure of the main stem:

a- The apical internode:

The apical internode of the main stem was studied from the anatomical point of view at the age of six weeks to disclose the primary structure of the main stem. The transverse section shown in Figure (4) reveals that the stem surface of Kidney bean plant directly below the shoot apex is strongly ridged and fluted, almost pentagonal in outline. The epidermal cells are nearly square in shape and covered with a thin layer of cuticle. Stomata of paracytic type are present at the same level of the epidermis, each consisting of two guard cells and two accessory ones. Trichomes of nonglandular hairs are observed. The ridges of the stem mainly consist of collenchyma. The cortex at the furrows between the ridges is composed of one or sometimes two layers of collenchyma cells underlying the epidermis followed often by two layers of chlorenchyma and four layers of thin-walled parenchyma cells. A few layers of sclerenchyma forming a nearly continuous band occur next to the last layer of the cortex, it may be called pericycle or perivascular tissue.

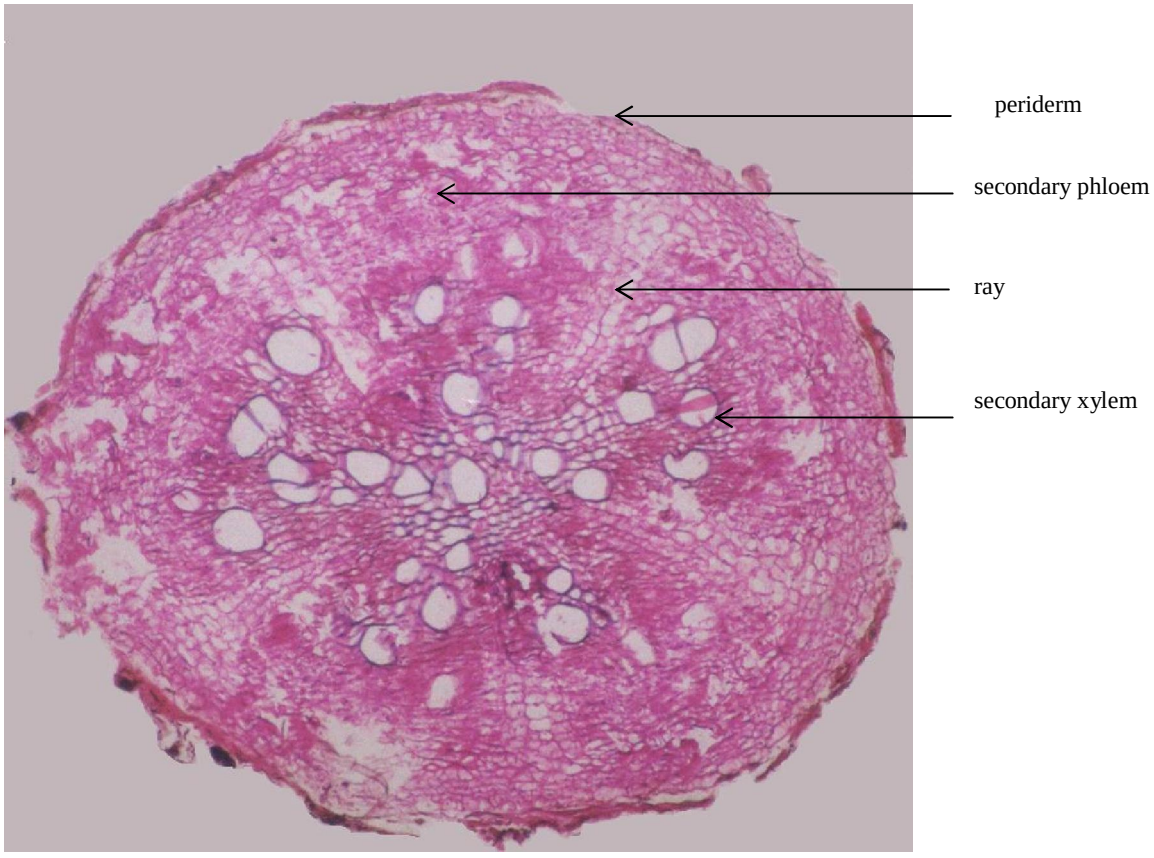


Fig.(2): Transverse section of the main root of *Phaseolus vulgaris* L. plant, aged four weeks, showing its secondary structure. (X 52)

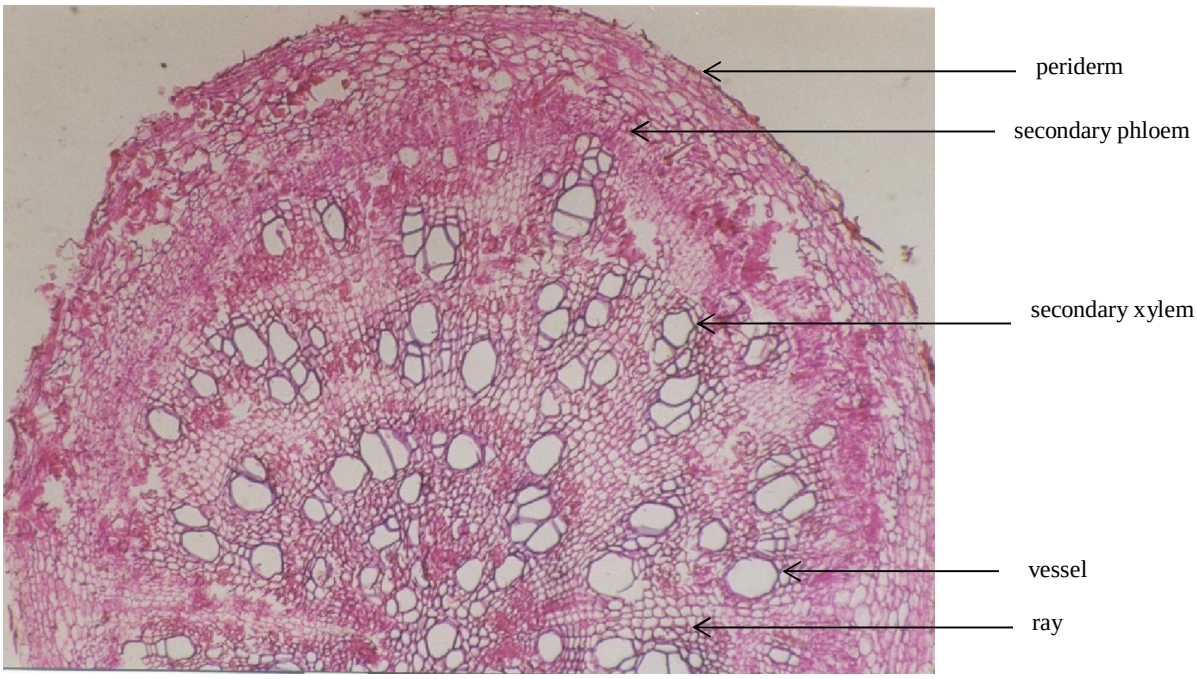


Fig.(3): Transverse section of the main root of *Phaseolus vulgaris* L. plant, aged six weeks, showing an advanced stage of secondary growth. (X 52)

The vascular bundles are almost arranged in a ring, being separated from each other by wide panels of parenchyma tissue which are a part of the ground tissue. There are five major collateral bundles located opposite to the corners. In addition, there are two to four minor collateral bundles between any of two major ones lying opposite to the furrows. The major bundle has 13 to 18 vessels in nearly parallel rows. The minor bundle often comprised of one row contains 3 to 6 vessels.

The pith, which comprises a large portion of the stem core, consists of thin-walled polygonal parenchyma cells which tend to decrease in size towards the periphery.

b- The median internode:

The transverse section through the median internode of the main stem of Kidney bean plant at the age of four weeks is shown in Figure (5). It is obvious that the main stem at its median portion is often ribbed; *i.e.,* polygonal in outline. Worthy to note that the ribs are comparatively smaller in size than those associated with the apical internode and the secondary growth takes place in nearly a continuous cylindrical form.

The epidermis which still having intact cells shows active dilation accompanied by radial division to accommodate with the increase in stem circumference. Also, many of both kinds of cortical cells (collenchyma and parenchyma) show elongation in the tangential direction accompanied sometimes by radial divisions. Stomata are present at the same level of the epidermis. Trichomes are not observed. The cortex composed of about seven layers of which the outer two layers are collenchyma underlying the epidermis and the five rest layers are parenchyma. The innermost layer of the cortex, the starch sheath, is easily recognized. The pericyclic cells show their transformation into fibers abutting on the collateral bundles of the stele. Thus, an incomplete ring of fibrous strands are developed.

The stele consists of 21 collateral bundles arranged in a ring, being separated from one another by two to three rows of well lignified parenchyma cells. The bundles are relatively different in size. There are seven large bundles located opposite to the ridges which being seven in number. There are two intermediate bundles between any of two large ones lying opposite to the furrows. Secondary thickening proceeds and secondary growth takes place in nearly a continuous cylindrical form. The secondary phloem

increases considerably in amount. The secondary xylem has an increased amount of vessels present in nearly radial rows, and the ground tissue where the vessels are embedded is formed of lignified parenchyma cells. The large bundle has 48 to 52 vessels, while the intermediate bundle has 33 to 38 vessels. A complete cambial ring is formed by the continuity of the interfascular cambium with the fascicular one. The primary xylem is recognized abutting on the pith.

The pith consists of polygonal parenchyma cells which tend to decrease in size towards the periphery. Small triangular intercellular spaces are visible.

At the age of 10 weeks, secondary thickening reached its maximum and the stem surface being cylindrical in outline (Figure, 6). The xylem vessels are mostly arranged in radial rows embedded in well lignified parenchyma cells. The vessels are present solitary or in radial groups, each consists of 2-4 vessels. Vascular rays mostly of 2 cells wide are observed and the pith at the center of the section being hollow (destroid).

At the extent of the authors knowledge no detailed studies dealing with the anatomical structure of Kidney bean stem are available.

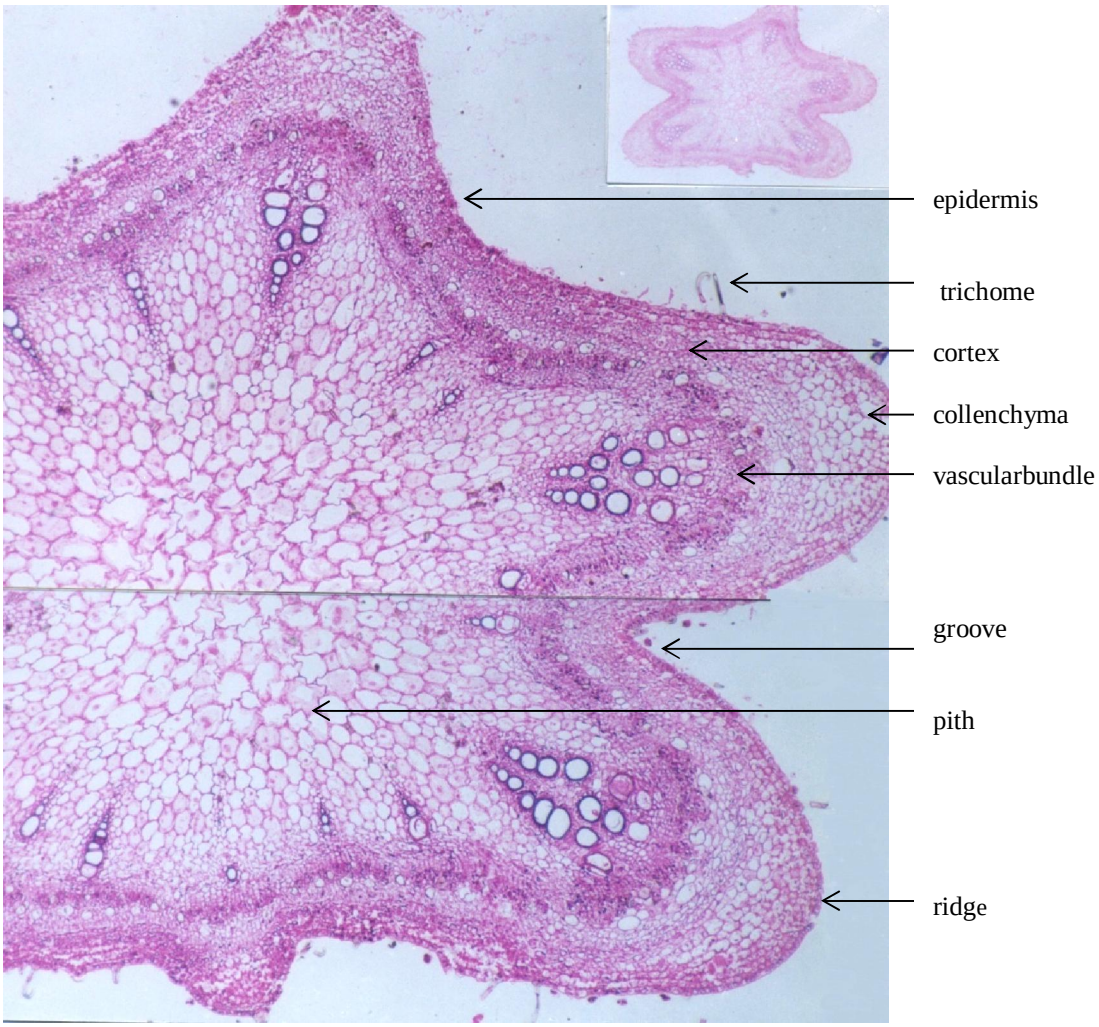


Fig.(4): Transverse section through the apical internode of the main stem of *Phaseolus vulgaris* L. plant at the age of six weeks. (X 52)

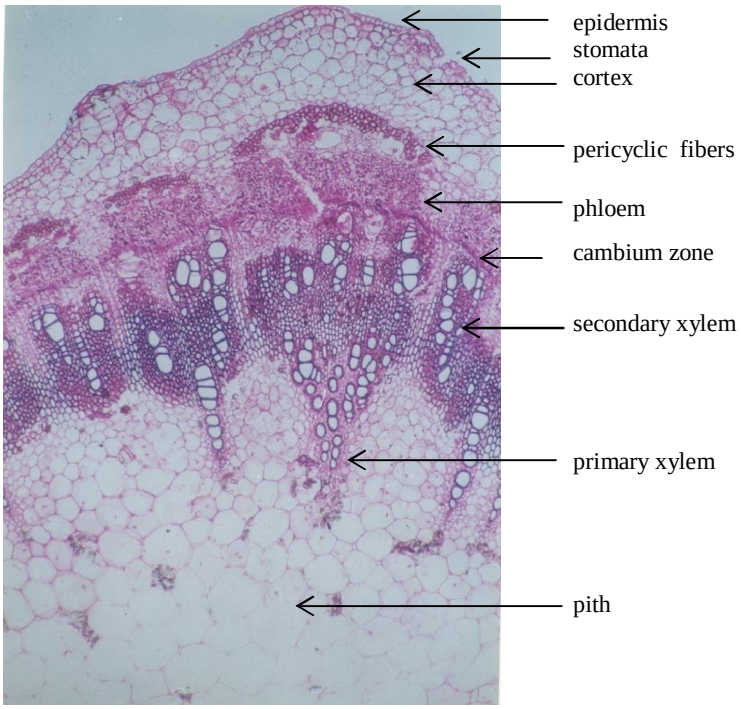


Fig.(5): Transverse section through the median internode of the main stem of *Phaseolus vulgaris* L. plant at the age of four weeks. (X 52)

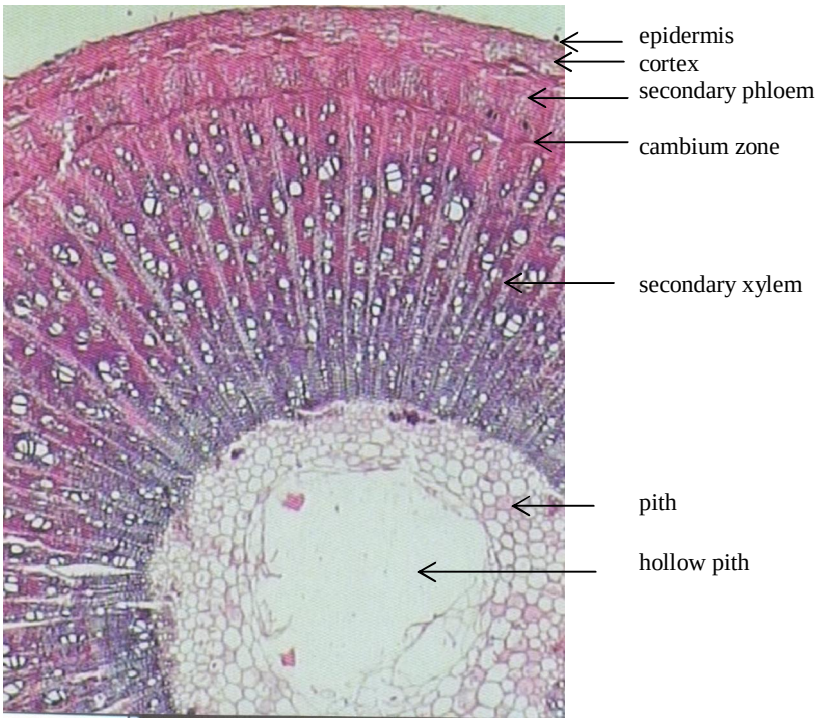


Fig.(6): Transverse section through the median internode of the main stem of *Phaseolus vulgaris* L. plant at the age of ten weeks. (X 52)

3. Structure of the leaf:

a- Leaf blade (lamina):

From the morphological point of view, Kidney bean plant develops two types of leaves as follows:

i- Two simple basal opposite prophylls.
ii- Pinnately trifoliate compound leaves which are alternately arranged on the main stem and the lateral branches.

Therefore, the anatomical structure of leaf blades representing these two different types of leaves were investigated. Transverse sections of mature prophyll as well as of apical leaflet of the compound leaf number 3, 6 and 9 on the main stem and of compound leaves on lateral branches were examined.

It was found that both types of leaves developed by Kidney bean plant almost have the same structure. Generally, they are dorsiventral and composed of three tissue systems as follows:

i- Epidermal tissue system consists of the epidermal layers occurring on the adaxial (upper) and the abaxial (lower) sides.
ii- The ground tissue system, which is known as the mesophyll tissue, is always differentiated into columnar palisade parenchyma on the adaxial side and irregular or isodiametric spongy parenchyma on the abaxial side and this means that the leaves are dorsiventral.
iii- The vascular tissue system is composed of vascular bundles which are usually collateral and form the skeleton of the leaf on which other tissues, the ground tissue, remain inserted. At the midrib, the principle vascular vein develops. In addition, smaller lateral ones constitute the reticulate system of venation.

Transverse section through the median portion of the mature prophyll of Kidney bean plant, two weeks old, (Figure 7) reveals that it consists of two epidermal layers with a mesophyll in between. Both the epidermal layers are uniseriate, composed of nearly compactly arranged rectangular cells with thin rounded cuticularised outer walls. Stomata occur on both sides, being more frequently present on the lower epidermis. Likewise, trichomes of nonglandular hairs, mostly hooked hairs with short basal cells and a large bent terminal cell are present on both surfaces, being more frequently present on the lower epidermis (Figure 7). The mesophyll is differentiated into palisade and spongy cells. The palisade tissue consists of one layer of cells elongate perpendicularly to the surface of the blade being characterized by an abundance of chloroplasts. The palisade tissue occupies ⅓ of the whole thickness of the mesophyll. The spongy tissue occurs towards the lower epidermis and consists of 4 to 5 layers of chlorenchymatous loosely arranged cells with relatively wide intercellular spaces. At the midrib region, both upper and lower epidermis are convex. The bundle in the midrib is the largest in the prophyll and the lateral ones decrease in size towards the margins. The vascular bundle is oriented with the xylem directed toward the adaxial surface and the phloem toward the abaxial one. The vascular bundle of the midvein is embedded in a ground tissue of parenchyma cells with a mass of collenchyma cells underlying the two epidermis. Whereas, the smaller bundles are directly embedded in the mesophyll.

The anatomical structure of leaf blades representing the mature foliage compound leaf of Kidney bean at different stages of plant growth was also investigated. It was found that all investigated leaves have, in general, the same structure. Leaflets are dorsiventral; *i.e.,* the palisade tissue is located on the adaxial side of the blade and the spongy tissue on the abaxial one (Figures 8 and 9). There are two epidermal layers on adaxial and abaxial surfaces of the leaflet. Each is uniseriate, composed of a row of compactly-set tabular cells. The outer walls are cutinised and possess thin cuticle. Stomata occur on both surfaces, being more numerous on the lower epidermis than on the upper one. They are of rubiaceous or paracytic type (Figure 10). The stoma consisting of two guard cells and two accessory ones, subsidiary cells flank the stoma parallel with the long axis of the guard cells. At the midrib region, both the upper and lower epidermis are convex. Trichomes are present on both surfaces, being more numerous on the upper epidermis than on the lower one. They are nonglandular uniseriate with short basal cells accompanied by an elongated terminal cell and most of them being hooked with a large bent terminal cell. The palisade tissue consists of two layers of slender cells of dense plastids, occupying almost one-half of the whole thickness of the mesophyll. The spongy tissue is composed also of 2 to 3 layers of chlorenchymatous cells. There is a mass of collenchymatous cells below the adaxial and abaxial epidermis at the midrib region. Therefore, the included bundle, the principle one, is not directly embedded in the mesophyll as do the smaller ones. The midrib bundle consists of a larger strand opposite to two smaller ones. The midrib is also supported by a fibrous strand, a crescent like cap, abutting on the phloem of its large collateral bundle.

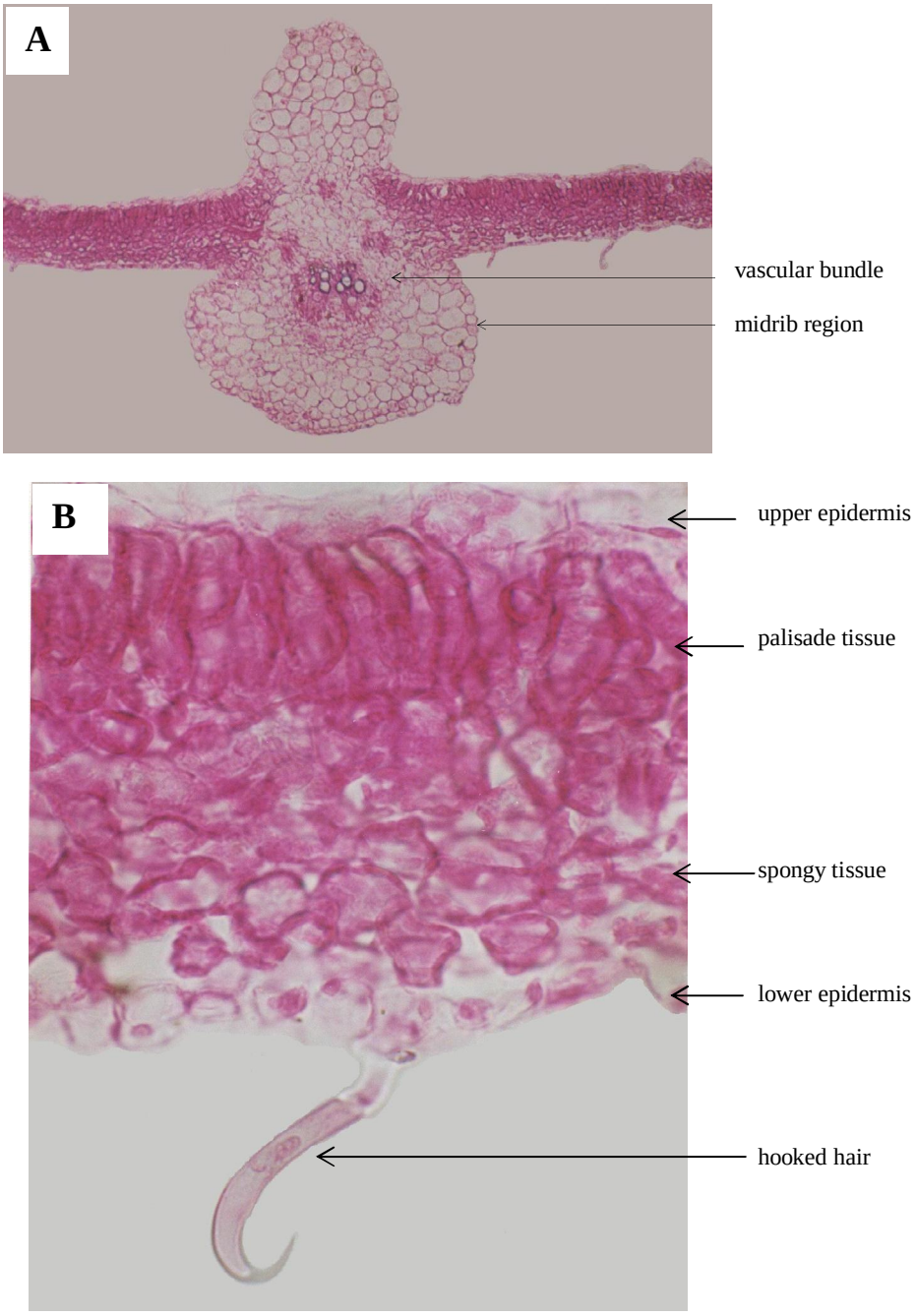


Fig.(7): Transverse section through mature prophyll of *Phaseolus vulgaris* L. plant, two weeks old.

A- Whole section. (X 52)
B- Magnified portion of A. (X 540)

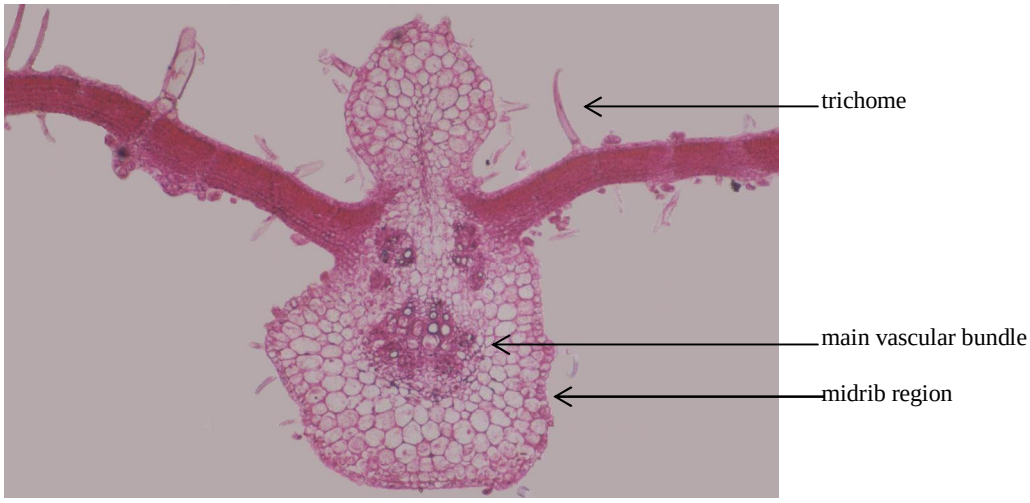


Fig.(8): Transverse section through the apical leaflet of the third foliage compound leaf on the main stem of *Phaseolus vulgaris* L. plant at the age of four weeks. (X 52)

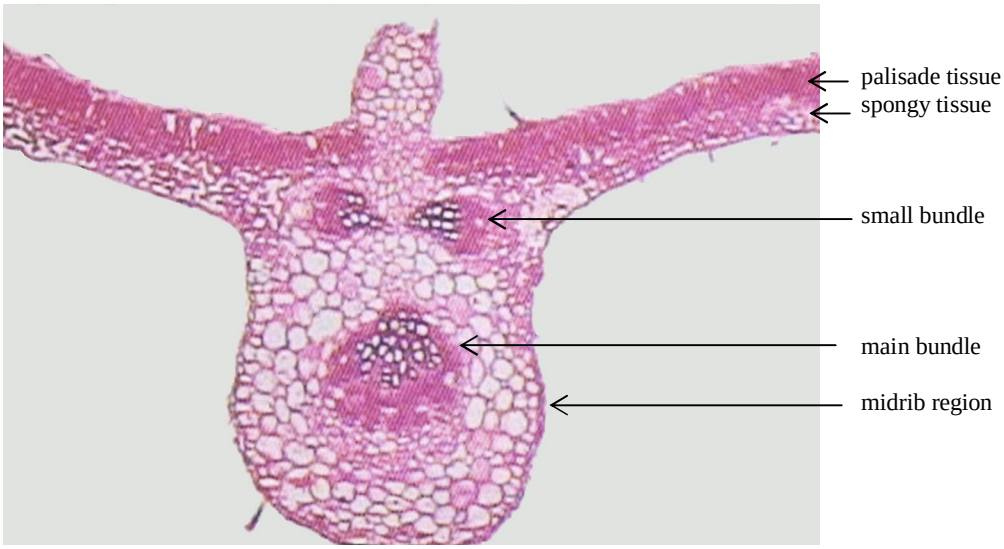


Fig.(9): Transverse section through the apical leaflet of a foliage compound leaf developed on the fourth lateral branch of *Phaseolus vulgaris* L. plant at the age of eight weeks. (X 52)

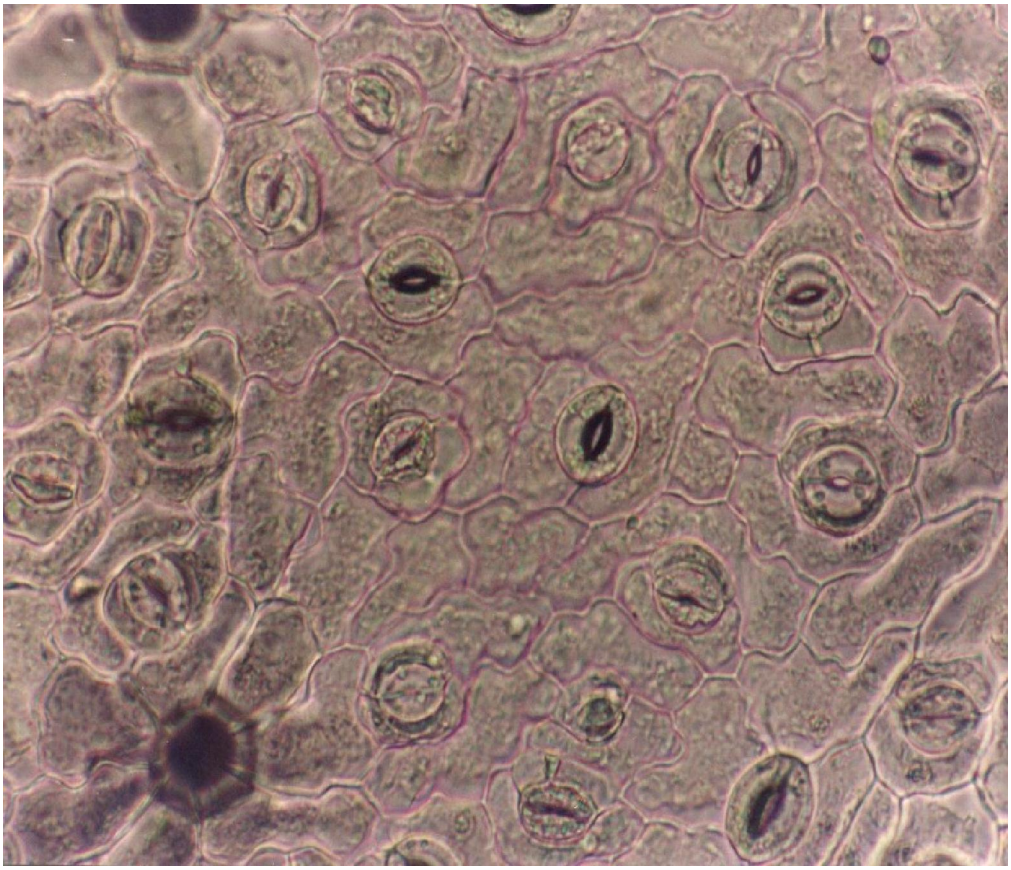


Fig.(10): Epidermal peel showing the paracytic stomata type in leaflet of *Phaseolus vulgaris* L. plant. (X 144)

In this respect, Metcalfe and Chalk (1979) recorded non-glandular hairs in the genus *Phaseolus*. Uniseriate, with a variable number of short basal cells, accompanied by an elongated terminal cell. Hooked hairs, with short basal cells, and a large bent, terminal cell.

b- Leaf petiole:

The petiole of Kidney bean leaf as seen in transverse section (Figure 11) is polygonal in outline (almost pentagonal) with two lateral wings at the corners of the adaxial side. The petiole is bounded by an uniseriate epidermis of nearly square shaped cells. The outer walls of the epidermis are somewhat thickened and covered with a thin layer of cuticle. Stomata and trichomes, similar to those found on the main stem and leaf blades, are present. The ground tissue consists mostly of relatively large parenchyma cells. The angles, beneath the epidermis, consists mainly of collenchyma cells. Inside the collenchyma there is a zone of chlorenchymatous cells ranging from 2 to 3 layers ending at the vascular cylinder. The chlorenchyma cells decidedly smaller than the residual parenchyma cells of the ground tissue.

The vascular tissues are formed of 13 collateral bundles arranged in a ring plus the two accessory small bundles in the ground tissue of the two lateral wings. The bundles are separated from one another by wide areas of ground tissue. The xylem vessels are arranged in radial rows, being in most bundles of one row.

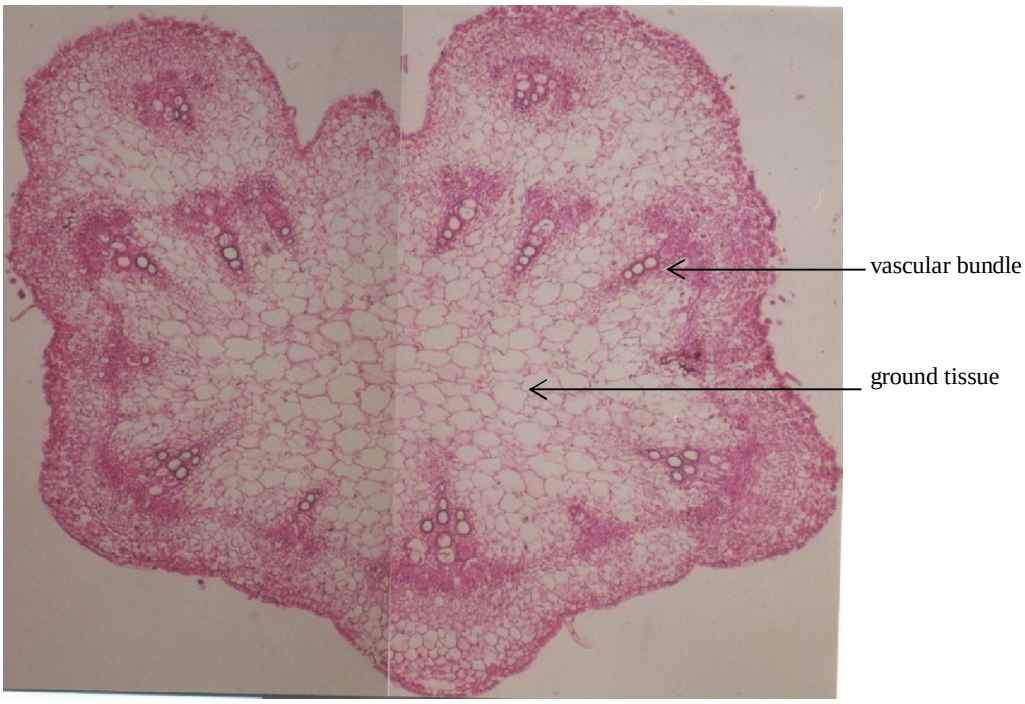


Fig.(11): Transverse section through the petiole of the third foliage compound leaf on the main stem of *Phaseolus vulgaris* L. plant at the age of four weeks. (X 52)

Worthy to note that there are small groups of incompletely differentiated phloem between the bundles and ill differentiated fibrous cap abutting on the phloem of each collateral bundle was observed.

4- Structure of the floral bud:

A transverse section through the floral bud of Kidney bean plant is shown in Figure (12). It is clear that the sepals of the calyx are united and comprised of two epidermal layers and 4-5 layers of ground tissue in between. There are numerous traces which extending through the ground tissue. The corolla is papilionaceous with one posterior petal (the standard), two lateral petals (the wings) and two lower united anterior petals (the keel). Each segment consists of two epidermal layers of nearly square-shaped cells surrounding 2-6 layers of parenchymatous cells forming the mesophyll. Many traces are extending through the mesophyll.

The stamens are ten; each consists of a two-lobed tetrasporangiate anther borne on the filament, a thin stalk with a single vascular bundle. The androecium is diadelphous, since the posterior stamen is free and the other nine stamens are with united filaments from the base to nearly more than half of their length while the anthers are free. The stamens form an open tube enclosing the long ovary. The upper parts of the filaments bend toward the banner.

The gynoecium is composed of a single carple and the ovary is of one locule. Placentation is marginal. The ovary contains two bundles at the placenta side (the dorsal bundles) and a single one at the opposite side (the ventral bundle).

5- Structure of the fruit and the seed:

The fruit of Kidney bean is a simple dehiscent legume which develop from a single carpel, cylindrical, constricted between the seeds, splitting along both sutures at maturity. The legume has two lines of dehiscence; one through the union of the carpel margin and the other along the median vascular bundle.

Transverse section through the mature fruit of Kidney bean is shown in Figure (13). The pericarp, the wall of the matured ovary, consists of three distinct layers; namely, the exocarp, the mesocarp and the endocarp. The exocarp includes the epidermis and a subepidermal layer of 1-2 cells in thickness, both are composed of thick-walled cells. The mesocarp consists of several layers lying next to the exocarp. The component parenchymatous cells of these layers are characterized by their rather thick and slightly lignified walls.

They are tangentially elongated and deeply stained. The mesocarp contains the vascular traces supplying the pod. The endocarp comprises the

remainder of the parenchymatous cells of the fruit wall, the pericarp, and the inner epidermis. The parenchymatous cells are weakly stained being thin-walled and much enlarged in different planes. The inner epidermis though still intact, its constituent cells become weakly outlined and thus could be hardly described.

The seed coat, the integuments of mature ovule, differentiates into a variety of distinct layers. The outermost layer, the epidermis remains uniseriate and develops into the palisade layer characteristic of leguminous seeds. It is composed of macrosclereids with unevenly thickened walls. The cells of the subepidermal layer differentiate into the so-called columnar cells, also termed pillar cells or hourglass cells. The layers beneath the subepidermal layer are of bigger and tangentially elongated parenchyma cells with the innermost layers being largely pressed.

The vascular system is well developed, it is an extension of the vascular bundle from the funiculus to the chlazal region where it branches. Finally, the inner epidermis characterized by very poorly outlined cells, and with rather thick lignified walls. It is worthy to note that, two palisade layers occur in the hilum region. The outer of these is derived from the funiculus and the inner belongs to the seed coat. Moreover, a compact group of cells of unknown role occurs in the hilum region. They are referred to by Esau (1959) to be tracheids. The seed coat envelopes the embryo which consists of two large cotyledons, plumule, nd the radicle. The cotyledons have big thin-walled cells, rich in starch grains of conspicuously large size.

The above description of the seed coat is in agreement with that given by Esau (1959).

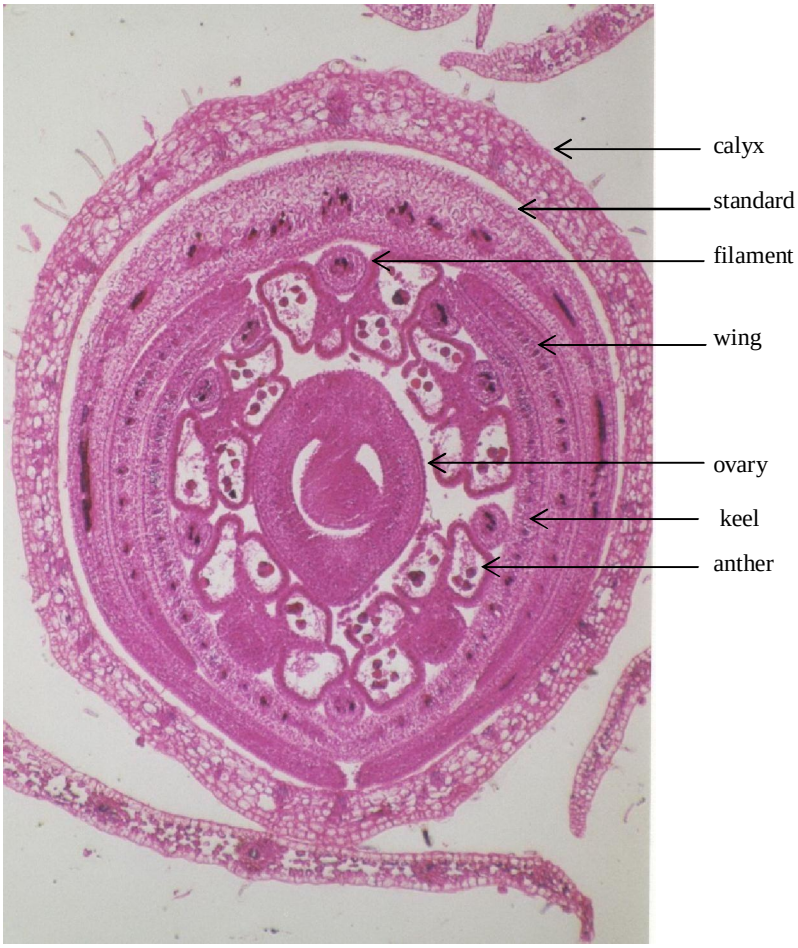


Fig.(12): Transverse section of the floral bud of *Phaseolus vulgaris* L. plant. (X 52)

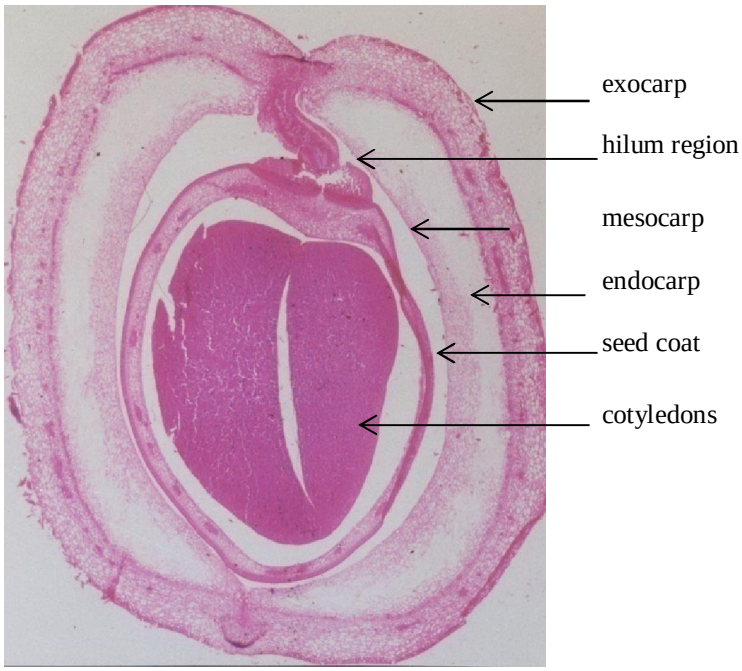


Fig.(13): Transverse section of the mature pod of *Phaseolus vulgaris* L. plant, showing the structure of the fruit and the seed. (X 12)

4- References

1. Esau, K. (1959). Anatomy of Seed Plants. John Wiley and Sons, Inc., N.Y., London, 376 pp.
2. Metcalfe, C.R. and L. Chalk (1979). Anatomy of the Dicotyledons. (Vol. 1). The Clarendon Press, Oxford, p. 502-534.
3. Nassar, M.A. and K.F.El-Sahhar (1998). Botanical Preparations and Microscopy (Microtechnique). Academic Bookshop, Dokki, Giza, Egypt, 219 pp. (In Arabic).
4. Nassar, Rania M.A.; Y.M. Ahmed and M.S.Boghdady (2010). Botanical Studies on *Phaseolus vulgaris* L. I-Morphology of Vegetative and Reproductive Growth. International Journal of Botany, 6 (3): 207-217.

6/28/2010

Efficacy of Intercropping Mango, Mandarin or Egyptian Clover Plants with Date Palm on Soil Properties, Rhizospere Microflora and Quality and Quantity of Date Fruits

[*1]H. F. H. Abouziena, [2]Elham Z. Abd El-Motty, [3]Youssef, R. A. and [4]Sahab, A. F.

[1]Botany Department, [2]Pomology Research Department, [3]Soils and Water Use D Department, [4Plant] Pathology Department, National Research Center, Dokki, Cairo, Egypt

abouzainah@yahoo.com*

Abstract: Intercropping is claimed to be one of the most significant cropping techniques in sustainable agriculture; to its utilization a number of environmental benefits, from promoting land biodiversity to diversifying agricultural outcome. This model integrates low, medium, and tall plants, as well as plants of short, medium, and long life cycles, including trees. Therefore, a study was carried out to evaluate the impact of intercropping mango (*Mangifera indica* L.), Balady mandarin (*Citrus reticulate* Blanco) and Egyptian clover (*Trifolium alexandrinum* L) crops with date palm on soil chemical properties and quality and quantity of date fruits, in comparison with date palm sole. Rhisosphere of palm (pure stand) had a high concentration of N compared to palms intercropped with mango or mandarin. Intercropped mandarin with palms caused a depletion of N from soil by 14.3%, relative to date palm pure stand. High levels of Zn and Mn in soil were recorded in rhisosphere of clover and palms intercropped with mandarin. The effect of intercropping on occurrence and enumeration of microorganisms in the rhizosphere of trees was also studied. The results indicated that the colony count of fungi and bacteria in date palm rhizosphere were fluctuated according to plantation method. Intercropping date palm with mandarin decreased the total fungal count from 21.17 cfu x 10^3g^{-1} in the non-intercropped roots to 16.00 cfu x 10^3g^{-1} (24.4% decrease) in date palm root intercropped with mandarin. While, intercropping date palm with mango and clover increased the total fungal count to 118.32 cfu x 10^3g^{-1} and 52.00 x10^3g^{-1} in date palm root intercropped with mango and clover, respectively. Growing mango or mandarin under date palm resulted in the highest fruit yield/palm. However, intercropping Egyptian clover with date palm caused a significant reduction in fruit diameter. Intercropping mango gave the highest net profit ($8213/ha/yr), followed by the same area intercropped with mandarin ($3992/ha/yr). Evaluation of growing mango, mandarin or Egyptian clover with date palm indicated that growing mango with date palm could be used for combating desertification in sandy soil in arid lands regions and gave the highest net return per unit area. [Journal of American Science. 2010;6(12):230-238]. (ISSN: 1545-1003).

Key words: intercropping, date palm, Egyptian clover, mango, mandarin, fruit

1. Introduction:

In Egypt, date palm ranked the third crop after orange and grape (Agricultural Economic Bulletin, 2005). Date palm trees provide enough space for intercropping even if they are fully grown as they do not cover much area being a very tall tree (Akyurt *et al*. 2002). They reviewed the literature and reported that, it is possible to grow a mixed fruit orchard, such as date intercropped with citrus (Morton 1987 and Anon- 1 2008). Field crops, such as fodders and vegetables may also be grown together with date palms (Anon- 1, 2008; Mahmoudi *et al*. 2008; Shirazi *et al.*, 2008). Intercropping in date palm with suitable crops bring good income and improves the fertility of the soil. During the first few years, intercropping can be practiced with no shortage of irrigation. Intercrops such as gram, peas, mash, moong, moth, senji and lentil can be sown during summer. Intercropping of some vegetables in plantation located near the cities can be practiced if sufficient irrigation and manuring facilities are available. On the other hand, Morton (1987) reported that in mechanized plantations, intercropping is not possible as much as space must be left for the mobile equipment.

In addition, desertification is a worldwide problem that directly affects over 250 million people and a third of the earth's land surface. It is especially concentrated in developing countries. Effectively dealing with desertification will lead to a reduction in global poverty (Millennium Ecosystem Assessment 2005).

The recent report of Egypt indicated that 67.4% of the first class area of the agricultural land was reduced from 1.260 million hectares (in the 2000) to 0.411 million hectares (in 2005). Combating desertification requires an integrated approach. Polyculture is claimed to be one of the most significant cropping techniques in sustainable agriculture, to its utilization a number of environmental benefits, from promoting land biodiversity to diversifying agricultural outcome. This model integrates low, medium, and tall plants, as well as plants of short, medium, and long life cycles, including trees.

Therefore, the tendency for exploitation the land under date palms whether for annual or perennial crops is increasing day by day to better utilization the microclimate and soils under date palm. Little research papers about the impact of intercropping on the date palm were found in the literature, except some reports and fact sheets. Ali *et al*. (1998) reported that the intercropping of legumes, which was found to improve quality and yield, was practiced up to 50%. They add that the inter-cropping of legumes (cowpea and pigeon pea) which was proved to have a positive effect on the soil and consequently dates quality is practiced up to 35%. While 15% of the studied cases practiced the intercropping adversely affects the soil. They also indicated that several crops can be intercropped with date palm utilizing canal irrigation such as alfalfa, okra and tomato that increase the profitability of date palm cultivation when intercropped. While intercropping date palm with other crops is difficult in drip irrigation (Ali *et al*.1998). In the Northern part of the delta and river Nile state date palm is intercropped with wheat, broad beans and fodder. Reyad *et al*. (1997) reported that 70 % of farmers in Umjawasir intercrop alfalfa with date palm. They found that intercropping alfalfa with date palm increases the income of one hectare up to US$ 3085/yr. At the same time, as tomato gained the second most profitable crop that increase the date palm hectare net income to US$ 2740/year. Okra is the third profitable crops when it is intercrop with dates in canal irrigation, which increases the income of one ha up to US$ 1621/yr (ELmakki, 2006). Some farmers growing oil palm intercropping with Cavendish banana (Ong *et al*. 2000).

Date palm is still grown in Egypt at a conventional method and stile cultivated with intercropped crops such as mango, citrus, legume etc. The rhizosphere is the habitat of both bacteria and fungi, which have a negative or positive effect on the growth and development of plants (Kurek and Kobus 1990). Root exudates, which are the main source of amino acids, sugars, vitamins, phenols, organic acids and metal ions, affect the composition of microorganism in the soil, especially in the rhizosphere (Darcy, 1982). Obied (2000) determined mycoflora of date palm associated with some pathological symptoms as *Fusarium moniliforme*, *Mauginiella sp*., *Thiolaviopsis paradoxa*, *Aspergillus sp*. and *Helminthosporium sp.* The distribution and pathogenesis of date palm fungi in Egypt was studied by El-Deeb *et al*. (2008) and in Bahrain by Qaher *et al*. (2005). The fungus *Fusarium oxysporum sp. albedinis* is soilborne; caused Bayoud of date palm (*Phoenix dactylifera* L.), however, the henna bush, when intercropped with date palm, may serve as a symptomless carrier of the fungus (Carpenter and Klotz 1966).

Therefore, a study was carried out to investigate the impact of intercropping mango (*Mangifera indica* L.), Balady mandarin (*Citrus reticulate* Blanco) and Egyptian clover (*Trifolium alexandrinum* L) crops with date palm on the quantity and quality of date fruits. The effect of intercropping on occurrence and enumeration of microorganisms in the rhizosphere of trees was also studied.

2. Material and Methods:

A study was carried out in a 15-yr-old date palm intercropped with mango and mandarin trees (10-yr-old) at a private orchard at Salheia Destricat, Sharkia Governorate, Egypt. The experiments were conducted to evaluate the impact of intercropping mango (*Mangifera indica* L.), Balady mandarin (*Citrus reticulate* Blanco) and Egyptian clover (*Trifolium alexandrinum* L) crops in comparison with date palm sole.

The Intercropping Treatments Were:

1- Date palm (pure stand)
2- Intercropping mango trees with date palm
3- Intercropping mandarin trees with date palm
4- Intercropping Egyptian clover under date palm

Three trees in each treatment were used as a replicate in split-plot Design. Where the date palms occurred in main plot, and the intercropping crops maintained in sub plot. Data recorded: Number of bunches per palm, mean weight of bunch and average yield per palm were recorded.

Physico-chemical characteristics of fruits (length and diameter of fruit, diameter: length ratio, size, percentage of flush weight, average fruit weight and stone criteria (diameter, length, and weight) were studied. Total soluble solids (TSS), total acidity, reducing, non-reducing and total sugars were determined in pulp juice as outlined by A.O.A.C (1995). Tannins content was determined using Indigo carmine indicator after Winton and Winton (1958). For comparison between treatments, approximately net profit ($/ha./yr) was calculated.

Collection of Rhizosphere Samples:

Rhizosphere samples were collected from depth of 15-30 cm adhering very closely to date palm roots. Seven rhizosphere samples from soil of date palm intercropped were obtained from the following treatments:

1. Rhizosphere of non-intercropped date palm (control; pure stand).
2. Rhizosphere of mango (monoculture)
3. Rhizosphere of date palm intercropped with mango
4. Rhizosphere of mango intercropped with date palm
5. Rhizosphere of mandarin (monoculture)
6. Rhizosphere date palm intercropped with mandarin.

7. Rhizosphere date palm intercropped with Egyptian clover

Enumeration of Microorganisms:

To estimate the number of soil microflora, counts were calculated on the basis of serial 10-fold dilutions, in duplicate, using the pour plate method using triplicate samples of 1g soil, and an appropriate dilution (Johnson and Curl, 1972); each value presented here is therefore an average of three individual counts. All petri dishes contained 15ml medium and the plates were incubated at 28-30°C in the dark. Colony-forming unites (CFU) were recorded after 1 week; the average number per gram oven-dry weight of soil was calculated.

For total bacterial flora, soil extract agar medium modified by Mahmoud *et al.*(1964) and Martin's medium (Allen, 1961) for fungi were used.

Microscopic Examination and Identification of Fungal Isolates:

Microscopic examination of mould growth was done by observing the colonial morphology-colour of colony, texture, shape and surface appearance and cultural characteristic- a sexual and sexual reproductive structures like sporangia, conidial head, arthrospores, the vegetative mycelia, septate or non-septate (Gilman, 1957; Nilson et al., 1983 and Barnett and Hunter, 1986). Microscopic examination of the moulds was done by using needle mount method.

A small portion of each colony was picked with sterile needle and teased out in a drop of clean microscopic slide. Slides were prepared likewise, using methylene blue in place of water.

Soil Chemical properties

Electrical conductivity (EC) and pH were measured in a soil 1:10 (w/v) by using a pH meter (WTW Germany, pH 330) and an EC meter (WTW Germany, LF 330), respectively.

To analyze macro and micronutrients in soil (rhisosphere zone), samples were taken from each treatment, and then dried at 70°C. From each sample 0.2 g was digested using 5 cm^3 of the mixture of sulfuric (H_2SO_4) and perchloric ($HCLO_4$) acids (1:1) as described by Peterburgski (1968). Total nitrogen was determined by micro-Kjeldahl method and phosphorus was determined calorimetrically at wavelength 680 nm using spectrophotometer (Spekol) as well as potassium was determined by using Gallen Kamp flame photometer. Micronutrients, i.e., Zn, Fe, Zn and Mn were measured using atomic absorption spectrophotometer Perkin Elmer model 5000 (Cottenie *et al.* 1982).

Statistical Analysis

The obtained data from each season were exposed to the proper statistical analysis of variance according to Gomez and Gomez (1984). The combined analysis of variance for the data of the two seasons was performed after testing the error homogeneity and LSD at 0.05 level of significance was used for the comparison between means.

3. Results and Discussion:

Soil Chemical Properties

Data in Fig.1 indicated that EC, pH and macro and micronutrients were affected by the plant species as well as by the methods of planting i.e. in pure stand or intercropping. Intercropping date palm with mango caused a reduction of EC by 17% (from 0.18 to 0.15 dS/cm), while sowing clover crops and mandarin resulted in increasing the EC value by 61 and 44%, respectively, compared to date palm sole.

Concerning pH value, Data in Fig. (1) indicated that the lowest values of pH were noticed under intercropping date palm with clover or mandarin. Rhisosphere of palm (pure stand) had a high concentration of N compared to palms intercropped with mango or mandarin. This result may be due to that the N not uptake by the palm is uptake by the mandarin or mango trees. Intercropped mandarin with palms caused a depletion of N from soil by 14.3 relative to date palm pure stand (Fig 1). By placing deep-rooted crops intercropping with shallow rooted crops where available N is present in deeper soil layers, nitrogen losses can be reduced (Thorup-Kristensen and Sørensen, 1999). On the other hand sowing clover with palms increased the N availability in soil by 3.6%. The atmospherically-fixed N in mixed swards containing 30-50% clover was estimated as 157kg^{-1}ha yr^{-1} (Kristensen *et al.* 1995).

Phosphorus status in soil was increase under the three intercropping patterns, and the highest level of P in soil was occurred under palm intercropped with clover where it increased by 136.7% than in rhisosphere of palms (sole).Tthis may be that the date palm trees have a high requirement from P nutrient. All intercropping pattern increase the K levels in soil and the highest value was recorded with palm intercropped with mandarin (90% increment). Similar trend was noticed with Fe element (Fig.1). High levels of Zn and Mn in soil were recorded in rhisosphere of clover and palms intercropped with mandarin. We could concluded that the status of nutrients in the soil not affected by the pH value but depends mainly on plant species and intercropping plants (root density, soil microbial activity, root exudates, etc.)

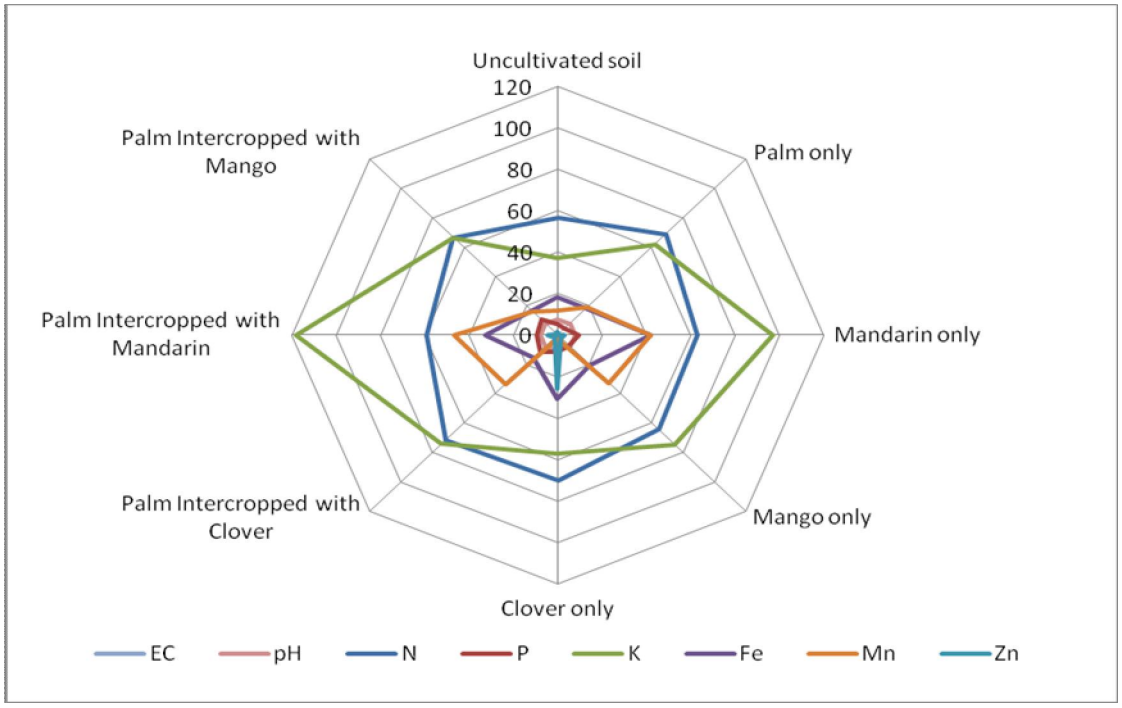


Fig 1: Soil chemical properties as affected by intercropping date palm with clover, mandarin or mango trees.

Rhizosphere Microflora

1-Fungi and Bacteria Occurrence:

Results of a laboratory microbiological analysis of particular rhizosphere samples of date palm showed different numbers of bacteria and fungi (Table, 1). The total number of bacteria in the rhizosphere samples (d.w.) ranged from 56.54 cfu X 10^5 g $^{-1}$to 375.52 cfu x 10^5 g^{-1}. Tyner (1940) reported that the decomposable root debris and root exudates had supplied the microorganisms with available sources of nutrients to grow and proliferate. Similar results were also obtained on date palm rhizosphere by Kurek & Kobus, 1990 and Obied, 2000.

The greatest number of bacteria was observed in the rhizosphere taken from the root of mandarin followed by root of date palm intercropped with mango. One the other hand, the number of bacteria increased from 65.19 cfu x 10^5g^{-1} in the control (non-intercropped palm) to 92.22, 190.82 and 268.15 cfu x 10^5 g^{-1} (41.5%, 192.7 and 311.3% increase) in the roots of date palm intercropped with clover, mandarin and mango, respectively.

The total number of fungi in the rhizosphere of date palm ranged between 16.00 and 128.07 cfu x 10^3g^{-1}. Intercropping date palm with mandarin decreased the total fungal count from 21.17 cfu x 10^3g^{-1} in the non- intercropped roots to 16.00 cfu x 10^3g^{-1} (24.4% decrease) in date palm root intercropped with mandarin. While, intercropping date palm with mango and clover increased the total fungal count to 118.32 cfu x 10^3g^{-1} and 52.00 x10^3 g^{-1}, respectively, compared to date palm sole. On the contrary, Shirazi *et al*., 2008 found that intercropping of date plantations with alfalfa (*Medicago sativa*) and sorghum (*Sorghum vulgaris*) cultivations increased relative humidity in the garden and decreased the disease. They added that alfalfa was more effective than sorghum.

On the other hand, growing clover plants and mango trees under date palms caused increase in the total fungal count by 145.6% and 458.9% respectively. The dynamic increase of the microorganisms in the rhizosphere of date palm intercropped with other cultivations can be explained by the favorable quantitative and qualitative composition of organic compounds provided in the form of root exudates and crop residues. This fact is confirmed by earlier information from the previous investigators (Rovira, 1969 and Funck & Hockenhull, 1984).

2- Frequency and identification of rhizospheric fungi:

The genera and species from the rhizosphere of date palm intercropped with other crops or non- intercropped were isolated and identified (Table, 2). Depending upon their frequency of occurrence genera were grouped as major components and minor components. Major components include most frequently encountered such as *Fusarium sp.*, *Aspergillus niger*, and *Gliocladium sp*. While, minor components include less frequent and sporadic types such as *Alternaria tenuis, Aspergillus sulphoreus, Trichoderma harzianum, harzianum, Rhizoctonia solani* and *Penicillium funiculosum* in descending order.

Fungal species in the rhizosphere of non-intercropped date palm recorded eight fungal species belonging to four genera namely *Aspergillus niger* (30%), *Fusarium sp* (50%), *Mucor mucedo* (10 %) and *Rhizopus nigricans* (10%). On the other hand, some fungi were appeared or disappeared in the rhizosphere of date palm according to the type of intercropped plants. Genearly, the quantitative and qualitative differences in frequent occurrence of fungal genera or species between different treatments were recorded. For example, root of mango intercropped with date palm stimulated the growth *of Phycomycetes* grouping fungi, *Penicillium sp.* and *Rhizoctonia solani* from 0.0 to 7.14%, *Gliocladium sp.* from 0.0 to 4.67% and *T. viride* from 0.0 to 9.52%. While, intercropped mandarin with date palm stimulated the growth of *Fusarium sp* from 50% to 71.43% and *Penicillium sp.* from 0.0 to 21.43 %, but *Mucor mucedo* and *Rhizopus nigricans* disappeared on the roots of date palm (Table.2).

Growing Egyptian clover under date palms led to increased *Gliocladium sp., Penicillium sp.* and *Phycomycetes fungi* from 0.0 to 17.14, 5.72 and 11.43%, respectively. On the other hand, some intercropping mango, mandarin and clover with date palm resulted in reduced or disappearing *A. niger*, *Fusarium sp.*, *R. nigricans* and *Mucor mucedo* that recorded with date palm (non intercropped).

Cultivation of date palm trees intercropped with other crops grow in close proximity to each other and the root exudates may accumulate in the rhizosphere from all sides causing a marked inhibitory or stimulatory effect to microflora in the rhizosphere of date palm. Root exudates are known to either stimulate or inhibit the growth of different species of microorganisms. For example, root exudates of *Crotalaria medicaginea* stimulated the growth of *Penicillium herquei, Aspergillus niger* and *Alternaria humicola* but significantly reduced the growth of *Trichoderma lignorum* (Sulia, 1973). In addition, Hakkou and Bouakka (2004) reported that the spread of the disease is promoted by intercropping, especially by the more water-demanding intercrops.

Table 1: Total bacteria and fungal counts in the rhizosphere of date palm intercropped with some crops.

Intercropped plant	**Bacteria (cfu x $10^5 g^{-1}$)**	**Fungi (cfu x $10^3 g^{-1}$)**
Rhizosphere of palm only (pure stand)	65.19	21.17
Rhizosphere of mango (pure stand)	56.54	31.55
Rhizosphere of palm intercropped with mango	268.15	118.32
Rhizosphere of mango intercropped with palm	136.30	128.07
Rhizosphere of mandarin (pure stand)	375.52	30.16
Rhizosphere of palm intercropped with mandarin	190.82	16.00
Rhizosphere of Palm intercropped with clover	92.22	52.00

Table 2: Fungal genera and species (%) in the rhizosphere of date palm sole or intercropped with (+) other crops

Isolated fungi	Rhizosphere of							Mean
	Palm (Sole)	Mango (Sole)	Mandarin (Sole)	Palm + mango	palm + Mango	Palm + mandarin	Palm + clover	
Alternaria tenuis	-	-	2.38	-	-	-	-	0.34
Aspergillus humicola	-	15.63	-	4.67	7.41	-	-	3.96
A. niger	30.00	6.25	4.77	23.81	24.07	7.14	34.28	18.62
A. sulphoreus	-	6.25	-	-	-	-	-	0.89
Fusarium sp.	50.00	15.63	59.52	19.04	14.81	71.43	22.86	36.18
Gliocladium sp.	-	31.25	9.52	4.67	18.52	-	17.14	11.59
Mucor mucedo	10.00	6.25	9.52	11.90	5.56	-	-	6.18
Penicillium funiculosum	-	3.13	9.52	-	-	-	-	1.81
Penicillium sp.	-	15.63	-	7.14	18.52	21.43	5.72	9.78
Phycomycetes fungi	-	-	-	7.14	-	-	11.43	2.65
Rhizoctonia solani	-	-	-	7.14	-	-	-	1.02
Rhizopus nigricans	10.00	-	4.27	4.76	7.41	-	8.57	4.62
Trichoderma harzianum	-	-	-	-	-	-	-	-
T. viride	-	-	-	9.52	3.70	-	-	1.89

3- Quantity and quality of date fruits:

The fruit quality criteria i.e. fruit weight, fruit diameter and length, pulp thickness, flesh weight, pulp/seed ratio were significantly affected by the intercropping. Monoculture of date palm produced the highest fruit yield/palm, followed by date palm intercropped with mango and mandarin and the lowest fruit yield/palm was obtained from date palms intercropped with clover (Table 3). Data also indicated that no significant differences between sole date palm and that intercropped with mango in number and weight of bunch. This result may be due to that palm tree has a big root system that may extend to 10m from trunk and 3-7m deep or to the water table level. This huge root system makes palm tree resistant to unfavorable conditions (Al-Rawi, 1998).

Concerning intercropping system, growing mango or mandarin under date palm resulted in the highest fruit yield/palm, compared to intercropping with clover. Intercropping Egyptian clover with date palm caused a significant reduction in fruit diameter. Monoculture date palm had the maximum fruit weight (25.8g), which significantly higher than other intercropping system. Intercropping mandarin with date palm caused a significant reduction in fruit diameter, weight and size by 6.2, 6.9 and 5.0%, respectively, compared to sole cropping.

Table 3: Effect of intercropping on date fruits characters(Average of two seasons).

Treatments	Number of bunches /palm	Weight of bunch (kg)	Yield of Palm (kg)	Fruit characters						Stone characters		
				D. (cm)	L. (cm)	D/L	Weight (g)	Size (cm^2)	% of fresh weight	Weight (g)	D. (cm)	L. (cm)
Palm only	9.8	14.86	145.6	2.9	5.2	0.56	25.8	26.50	90.1	2.55	1.1	3.3
Palm with mango	9.4	14.51	136.4	2.7	5.9	0.46	25.7	27.62	91.3	2.23	1.1	3.3
Palm with mandarin	9.4	14.38	135.2	2.7	5.7	0.47	24.2	25.18	90.0	2.43	1.0	3.4
Palm with clover	9.1	13.98	127.2	2.6	6.0	0.43	25.6	26.66	90.8	2.36	0.8	3.5
LSD at 0.05	NS	0.43	7.4	0.3	0.4	0.07	1.1	1.76	0.5	0.16	NS	NS

Abbreviations: D., Diameter; L., Length;

The lowest stone weight (2.23 g) was recorded when date palm intercropped with mango. Intercropping of mango, mandarin or clover had insignificant effect on bunches number/palm and mean weight of bunch as well as on stone diameter and length (Table, 3). These results may be due to that the date palms were planted 7 meters apart, which provides ample space for intercropping. Steiner (1982) reported that the competition for resources between the crops in an intercropping system could be non-competitive, competitive or complementary.

Data in Table 4 indicated that higher and lower total sugars in fruits were obtained from pure stand date palm (85.3%) and date palm intercropped with mandarin, (68.8%). While higher tannins and total soluble solids were obtained from palms intercropped with Egyptian clover and mango, respectively (Table 4).

Data in Table 4 indicated that intercropping mango gave the highest net profit ($8213/ha./yr), followed by the same area (hectare) intercropped with mandarin ($ 3992/ha/yr) which caused increment by 139% and 16 %, over that date palm (pure stand=$3442/ha./yr), respectively. From our results, it could be concluded that intercrops with date palm can be more profitable than growing pure stands.

Similar finding was reported by Elmakki (2006) who stated that intercropping of alfalfa with date palm increases the income of one hectare up to US$ 3085/yr. they added that tomato gained the second most profitable crop that increase the date palm hectare net income to US$2,740. While, okra is the third profitable crops when intercropped with dates in canal irrigation that increases the income of 1 ha up to US$ 1621/yr.

Traditionally, intercropping with other fruit trees (citrus, pomegranates, olives, grapes, guava) or arable crops (alfalfa, barley, beans etc.) is practiced in many of the main production areas. Without the shade provided by the date palms other crops very often cannot grow.

Al-Yahyai (2009) reported that to make the best use of the farm, it was and still a common practice to grow cotton, maize, alfalfa, wheat, vegetables, and fruits between and under the palm trees. Most commonly in the level land is to interplant palms with citrus and alfalfa. According to the FAO paper, conditions in the old world often favor inter-planting. First, the sensitivity of some fruit crops like citrus to harsh conditions, such as the

high temperature, extremely cold or hot dry winds, and the strong sun make the date palm plantation shade the best way of producing high value fruit trees. Second, inter-planted is favored by small poor farmers who own an inherited a small date garden, thus planting some other fruit trees would provide an alternative income source. In addition, growing some of the fruit or fodder crop to be consumed by the household or provide income throughout the year is another perspective of practicing inter-planting in old palm orchards especially at early years of orchard establishment when the return from it is low. These inter-crops also improve nutrition status and the physical properties of the soil.

Table 4: Effect of intercropping on chemical constituents of date fruits and return per hectare/ year (Average of two seasons).

Treatments	Chemical properties						Return ($/ha/yr)				
	Sugars %			Acidity (%)	Tannins (g/100g FW)	Total soluble solids (%)	Date fruits	Second crop	Total	Costs	Net profit
	Total	Reducing	Non reducing								
Date palm	85.3	55.3	30.0	0.35	0.53	15.47	4459	-	4459	1017	3442
Date palm with mango	80.5	50.5	30.0	0.36	0.54	16.30	4177	6664	10841	2628	8213
Palm with mandarin	68.8	48.1	20.7	0.46	0.64	13.63	4140	2352	6492	2500	3992
Date palm with clover	73.3	41.7	31.6	0.46	0.65	12.35	3895	381	4276	1144	3132
LSD at 0.05	3.7	4.5	2.8	0.07	0.08	1.74	360	521	1215	210	472

Abbreviations: yr, year; FW, fresh weight
Return /hectare = number of trees/hectare X average yield in two seasons X mean price ($/ kg)
Income was calculated as: 204 palms x mean yield per palm for date palm X$0.151 , 204 trees X 74 kg X $0.445, for mango, 600 trees X 37 kg X $ 0.107 for mandarin, and for clover 4 cuttings X $ 128.16/ha.

The data in Table 4 showed that intercropping date palm with Egyptian clover resulted in decrement the net profitable per hectare by 9% (from $3442 to $3132). This reduction was attributed to the decrement of bunch number and weight and consequently decreased the total yield/palm. El –Halawany and Shaltout (1993) reported that date palm plantation which is intercropped with alfalfa, vegetables and fruit trees either lake of weed control or have some hand weeding.

4. Conclusion:

Evaluation of growing mango, mandarin or Egyptian clover with date palm indicated that growing mango with date palm as intercropping could be used for combating desertification in the sandy soil and significant cropping techniques in sustainable agriculture. Intercropping is utilized a number of environmental benefits, from promoting land biodiversity to diversifying agricultural outcome. This model integrates low (Egyptian clover), medium (mandarin or mango), and tall plants (date palm), as well as plants of short, medium, and long life cycles, including trees.

Corresponding Authors:
H. F. H. Abouziena
Botany Department, National Research Center, Dokki, Cairo, Egypt
abouzainah@yahoo.com

Acknowledgement

The authors thank Prof. Dr. Ahmad Abd-Elwarth for his facilities and supporting during the experimental period, where this research was conducted in his orchard.

5. References:

1. Agricultural Economic Bulletin. 2005. Ministry of Agriculture and Land Reclamation, A.R.E., Acreage and total production of fruits, pp.177 (in Arabic).
2. Akyurt, M., E. Rehbini, H. Bogis, A.A. Aljinaidi. 2002. A survey of mechanization efforts on date palm crown operations. The 6th Saudi Engineering Conf., KFUPM, Dhahran, December 2002 Vol. 5, 475 - 489.
3. Ali G. A., Karibl A. K., Ibn- Aoof M. 1998. Survey on production, handling, storage and marketing of dates in the northern region. First Inter. Conf. on Date Palms (Al-Ain, UAE, March 8-10, 1998).
4. Allen O. N. 1961. Experiments on soil bacteriology. Burgess Pub. Co. Minneapolis, Minnesota, USA.
5. Al-Rawi A.A.H. 1998.Fertilization of date palm tree (*Phoenix dactylifera* L.) in Iraq. Proc. the 1st Inter. Conf. on date palm, Al-Ain, U.A.E. pp: 320-328.
6. Al Yahyai R. 2009. The Traditional Date Palm Cultivation in Oman: Intercropping. Available from:

http://www.nizwa.net/agr/dates/dates/palm/datepalm3.html [Accessed 14 March 2010].
7. Anon- 1 2008. Pakistan: Stagnant date production. Available from: http://www.freshplaza.com/news_detail.asp?id=29144 [Accessed 14 March 2010].
8. A.O.A.C. 1995. Association of Offical Agricultural Chemists. Official Methods of Analysis. 15th Ed. Published by A.O.A.C. Washington, D.C. (U.S.A).
9. Barnett H.L., Hunter B.B. 1986. Illustrated genera of fungi. 3 ed. Burgess Co. and Minneapolis, Minnesota, USA.
10. Carpenter J.B., Klotz L.J. 1966. Diseases of the date palm. Date Growers Inst. Rep. 43:15-21.
11. Cottenie A., Verloo M., Velghe G., Comerlynk R. 1982. Chemical analysis of plant and soil. Ghent, Belgium, Laboratory of Analytical and Agro-chemistry State University.
12. Darcy A.L. 1982. Study of soya and lens exudates. Kinetics of fenolic compounds, amino acids and sugars in the first days of plant growth. Plant and Soil, 68, 399-403. Available from: http://www.springerlink.com/content/f2235055186781t0/?p=52af615c93fe4812a8355b879c6bf99f&pi=11[Accessed 14 March 2010].
13. El-Deeb H.M., Lashin S.M., Arab Y. A. 2008. Distribution and pathogenesis of date palm fungi in Egypt. Acta Horticultrae, 736: 421-429. Available from: http://www.actahort.org/books/736/736_39.htm [Accessed 14 March 2010].
14. El-Halawany E.F., Shaltout K.H..1993. Weed flora of the date Palm orchards in eastern Saudi Arabia. J. Saudi Arabia Univ.5, Science (1):25-37. Available from: http://repository.ksu.edu.sa/jspui/bitstream/123456789/492/1/Weed%20Flora%20of%20the%20Date%20Palm%20Orchards.pdf [Accessed 14 March 2010].
15. Elmakki O.A.O. 2006. Challenges and Possibilities of Drip and Canal Irrigation in Northern Sudan. Master Sci. Thesis, Norwegian Univ. of Life Sci., Norway
16. Funck J. D., Hockenhull J. 1984. Root exudation, rhizosphere microorganism and disease control. Vaxtskyddsnotiser, 48: 49-54. Available from: http://chaos.bibul.slu.se/sll/slu/vaxtskyddsnotiser/VSN84-3/VSN84-3B.HTM [Accessed 14 March 2010].
17. Gilman J.C. 1957. A manual of soil fungi. Second Ed. The Iowa State Univ. Press, Ames, Iowa, USA, 450pp.
18. Gomez K.A., Gomez A.A. 1984. Statistical Procedure for Agricultural Research. New York: John Wiley and Son.
19. Hakkou A., Bouakka M. 2004. Present state of the Figuig oasis date palm grove and incidence of the Fusarium wilt. Secheresse 15 (2)147-158. Available from: http://www.john-libbey-eurotext.fr/en/revues/agro_biotech/sec/e-docs/ 00/04/04/05/article.phtml [Accessed 14 March 2010].
20. Johnson L.F., Curl E.A. 1972. Methods for research on the ecology of soil- borne plant pathogens. Burgess, Minneapolis.
21. Kristensen E.S., Høgh-Jensen H., Kristensen I. S. 1995. A simple model for estimation of atmospherically-derived nitrogen in grass-clover swards. Biol., Agric. and Hortic., 2, 263-276.
22. Kurek E., Kobus J. 1990. Benificia and harmful effects of rhizosphere microflora on growth and development of plants. Mikrobiol., 24: 103-123. Cited From: AbdElMotty, E. Z., M.F.M.Shahin, M.M.M.Abd El-Migeed and A.F. Sahab. 2009. Comparative Studies of Using Compost Combined with Plant Guard and flespar on the morphological , physiological and rhizosphere microflora of olive seedlings. American-Eurasian J. Agric. & Environ. Sci., 6 (4): 372-382. Available from: http://idosi.org/aejaes/jaes6(4)/1.pdf [Accessed 14 March 2010].
23. Mahmoud S A.Z., Abou El-Fadle M., El-Mofty M. 1964. Studied on the rhizosphere microflora of a desert plants. Folia Microbiologica, 9: 1-8. Available from: http://www.springerlink.com/content/jqw42v35781tx018/ fulltext.pdf?page=1 [Accessed 14 March 2010].
24. Mahmoudi H., Hosseininia G., Azadi H., Fatemi M. 2008. Enhancing date palm processing, marketing and pest control through organic culture. J. of Organic Systems, 3 (2):29-39. Available from: http://www.organic-systems.org/journal/Vol_3(2)/pdf/29-39_Mahmoudi_et_al.pdf [Accessed 14 March 2010].
25. Millennium Ecosystem Assessment 2005. Ecosystems and Human Well-being: Desertification Synthesis. World Resources Institute, Washington, DC.
26. Morton J. 1987. Date *Phoenix Dactylifera* p. 5–11. In: Fruits of warm climates. Julia F. Morton, Miami, FL. Available from: http://www.hort.purdue.edu/newcropmorton/index.html [Accessed 14 March 2010].

27. Nilson P.E., Toussoun T. A, Marasas W.F.O. 1983. *Fusarium* species. Am. Illustrated Manual for identification. Pennsylvania State Univ., Press Park and London, 193pp.
28. Obied M.M.A. 2000. Production and protection of date palms in Sudan. Seventh Arab Congress of Plant Prot., 22-26 October.455-460.
29. Ong K.P., Lee Y.M., Teo L. 2000. Commercial experiences of oil palm intercropping with Cavendish banana. In: Proc. International Planters Conf. 2000 (ed. Pushparajah). Incorporated Soc. of Planters.
30. Peterburgski A.V. 1968. Handbook of Agronomic Chemistry. Kolop Publishing House, Moscow, Russia
31. Rovira A.D. 1969. Plant root exudates. Bot. Rev., 35: 35-57. Available from: http:// www.springerlink.com/content/915681q331 171781/fulltext.pdf?page=1 [Accessed 14 March 2010].
32. Shirazi M.P., Izadi M., Khademi R. 2008. Study the climatical factors effects on bunch fading disorder of date palm in southern Iran and the methods of its control. American-Eurasian J. Agric. & Environ. Sci., 4 (5): 570-574. Available from: http://www.idosi.org/aejaes/jaes4(5)/8.pdf [Accessed 14 March 2010].
33. Steiner K.G. 1982. Intercropping in tropical smallholder agriculture with special reference to West Africa. Deutsche Gesellsaft fur Technische, Zusammenarbeit, Eschborn, Germany
34. Sulia S.B. 1973. Effect of root exudates and extracts on rhizosphere fungi. Plant and Soil, 39: 197-200. Available from: http://www.springerlink.com/content/ t8020802068v7172/ [Accessed 14 March 2010].
35. Thorup-Kristensen, K. and Sørensen, J.N. 1999. Soil Nitrogen Depletion by Vegetable Crops with Variable Root Growth. Acta Agric.Scand., Sect.B, Soil and Plant Sci. 49:92-97.
36. Tyner L. E. 1940. The effect of crop debris on the pathogenicity of cereal root-rotting fungi. Canad. J. Research 18:289-306.
37. Winton A.L., Winton K.B.1958. The analysis of foods. John Wiley and Sones, INC, Lo
.

6/20/2010

Study the Effect of some Metallic Additives on the Physical Properties of the Commercial Pure Aluminum Metal

E.M.Sakr*[1], A.Nassar[2], N.Tawfik[2] and M.Soliman[2]
[1] Physics Department- Faculty of Girls for Arts, Science & Education- Ain Shams University, [2] Physics Division- National Research Center (NRC), Cairo, Egypt.
*Elham.sakr@yahoo.com

Abstract: The aim of the present work is to develop the 6201 alloy, which is the most used for conductor cables by adding different amount of Ce into Al-Mg-Si alloy namely (0.0, 0.024, 0.043, 0.054, 0.133, 0.166 and 0.194 wt% Ce) concentration. Sample alloys were homogenized by annealing at 540° C for various duration in range (½ to 5 hours), followed by water quenching. Tensile tests, hardness, electrical conductivity tests, microstructure characterization in Scanning Electron Microscope (SEM) have all been investigated as-cast and annealing. The results indicate that the alloys with Ce content make a more refined structure of grains and have higher tensile properties especially in range (0.043 to 0.054 wt% Ce) content and also hardly increase resistivity rather than the alloy which is free of cerium. [Journal of American Science. 2010;6(12):239-252]. (ISSN: 1545-1003).

Keywords: Tensile test, hardness, electrical conductivity, microstructure characterization

1. Introduction:

Aluminum and its alloys are characterized by a relatively low density, high electrical and thermal conductivities and a resistance to corrosion [1-3]. Many of these alloys are formed by virtue of high ductility [3], its ductility is maintained even at very low temperatures. The primary limitation of aluminum is its melting temperature (660°C). Mechanical strength of aluminum can be enhanced by cold work and by alloying [1,3]. Principle alloying elements are copper, magnesium, silicon, manganese and zinc. Aluminum is being widely used as a conductor material. The suitable alloy for aluminum conductors belong to Al-Mg-Si system with varied composition. Alloy 6201 is (Al- 0.8 Mg- 0.25 Si) designed for overhead conductor cables because it has excellent strength and good conductivity when suitably treated [4,5,6].

The use of rare – earth metal as a beneficial addition to nonferrous alloys has affected considerable interest in recent years. The rare – earth additions such as cerium have been claimed to led refinement of structure and improvement in mechanical properties. Rare – earth additions were also explored as a modifier to Al-Si alloys [7].

The main aim of the present work is the modification of the commercial aluminum alloy, AA6201 [Al- Mg- Si] to be used as conductor cable in our industries. For that a series of ternary master alloys [Al- Mg- Si] with different amount of cerium were prepared. A study of the effect of Ce content and heat treatment on the microstructure, the mechanical and electrical properties was made.

2. Experimental Technique:

Commercial aluminum of 99.8% purity, magnesium and silicon of high purity were used to produce a ternary alloy (Al- Mg- Si) in our investigations. The alloys were melted in a graphite crucibles open to atmosphere in a resistance furnace held at (700 – 800 ºC). The tested alloys were stirred well with a graphite rod, from time to time and long melting times were used to ensure dissolution, homogenization and uniform distribution of the alloying elements (Si & Mg) in aluminum. Cerium was added in the form of Al- Ce master alloy, the estimated amount of Al-Ce master alloy wrapped in aluminum foil, was plunged into the molten to produce a series of ternary alloys (Al- Mg- Si) with different amounts of Ce. The melt was stirred with a graphite rod then was poured in a steel mould to solidify in atmospheric air. The alloys were provided in the form bar of circular cross section of dimension 3 cm and length of 28 cm. Specimens test cut from the as cast rod with different dimensions for the required measurements before heat treatment.

For the tensile testing and resistivity samples were drawing into wires of 1 mm diameter. Solution treatment is the supersaturated solid solution of alloy structure is produced to take advantages of its precipitation hardening characteristics. Series of samples of each alloy were homogenized at a fixed temperature of 540ºC with annealing time of 30 minutes to 5 hours and thereafter immediately (within 30 seconds) quenched in cold water (at room temperature). The samples are then rinsed and left to dry the surface completely. Tensile test measurements were used as an indicator of mechanical response to heat treatment. The tensile machine used in this investigation is of type, (2wick-1425). All tensile measurements were performed on

wire of 40mm length and 1mm diameter at room temperature. All the specimens had been heat treatment before using.

Hardness measurements were used as an indicator of mechanical response to heat treatment. The sample hardness was measured using Vicker's micro Hardness Tester Shemadzu. All hardness measurements were performed on a block of 5mm thickness at load of 200g and the pressing time is 5 seconds. The sample hardness was measured immediately after heat-treatment.

Microstructures of the as cast samples from each alloy were examined by conventional optical microscopy after mechanical polishing of the specimen followed by etching solution is about 20% hydrofluoric acid in distilled water. The etching was done by immersing the samples into the solution and waiting until a suitable contrast of the grains was obtained. The etched samples were washed, dried and scanned with SEM type. Joel JSM, 5410 to identify the existing phases, their shapes, and size distribution. To obtain further micro structural information, SEM is well suited to identify the existing phases, their shapes, and size distribution. Such investigation was carried on a series of Al- Mg- Si alloys without and with different cerium amounts of 0.024, 0.043, 0.054, 0.133, 0.166 and 0.194 wt% respectively. The tested specimens were solution heat-treated at 540º C for different times ranging from 0.5 hour and the water quenched.

Microstructure of both as-cast and solution heat-treatment alloys were characterized by Scanning Electron Microscopy (SEM) after polishing and etching.

3. Results and Discussion:

The microstructures of the tested alloys were examined by SEM as shown in Fig1, a-g. It can be observed that, the addition of cerium affected the microstructure development in the Al- Mg- Si alloy in various ways. Firstly, for Al- Mg- Si alloy without cerium, the alloy is composed of the primary α matrix and a secondary phase that exists in two kinds of morphologies, i.e. a discontinuous network of coarse particles along grain boundaries Mg_2 Si, and many spherical Si particles that distribute both inside grains and at grain boundaries, as shown in Fig.1, a. Secondly, after adding cerium Fig.1, b, β particles were refined. However, a new kind of distribution characteristics from β precipitate in the alloys with Ce, these particles generally have rod-like shaped and do not have an obvious tendency to distribute in grain [8,9]. The β- phase is found at the grain boundaries as a network of precipitates as observed in Fig.1 b-g. These precipitates depend upon the amount of cerium in the alloys with 0.043, 0.054 and 0.194 wt% {Fig.1, c,d and g}, there are less of spherical particles surrounded by much and continuous rod- shaped precipitates at grain boundaries. While, in alloys with (0.024, 0.133 and 0.166 wt%), there are much discrete spherical particles can be observed either in grains or along grain boundaries, furthermore there are still some rod- shaped precipitates at grain boundaries.

The morphology of all tested alloys (without and with Ce) are changed after the homogenization process as shown in the figures from Fig. 2, a-b to Fig. 8, a-d. The rods like β particles are gradually replaced by a uniform dispersion of new particles. With increasing homogenization time these particles are nucleated and grown. Therefore, the microstructure was found to have a new grains formation. The islands of new formed particles are thickened and spherodized at the expense of the remaining β- phase. At certain annealing time for each alloy, the formation of a homogeneous finely dispersed microstructure occurred. We can conclude that, the suitable time for the concentrations (0.024, 0.043, 0.054 and 0.194 wt% Ce) is 3 hours and it is 0.5 hour for the alloys with the concentrations (0.0, 0.133 and 0.16 wt% Ce). While, further annealing time, (i.e. the number of particles become fewer and bigger caused by coarsening [10].

Tensile testing measurements were carried out for as- received wires of each composition at room temperature as shown in Fig.9. It can be seen that the value of tensile testing for the samples containing Ce addition up to 0.194 wt% is higher compared to the Ce free alloy. The tensile strength and hardness increased from (333 and 70.18 N/mm^2) for the Ce-free alloy to (368.65 and 102.28 N/mm^2) for that containing 0.054 wt% Ce. Further additions up to 0.054 wt% Ce, have little effect on the tensile properties and hardness while, the maximum tensile strength was reduced to (337.436 and 71.27 N/mm^2), when the Ce content was further increased to 0.194 wt %.

Meanwhile, with increasing Ce content in the present alloys, the elongation was decreased compared with the Ce- free alloy. The elongation of alloys with contents 0.043 wt% and 0.194 wt% Ce respectively, were generally lower than that of other alloys. It can be concluded that increasing the Ce content increased the tensile strength and hardness but a slightly decreased in the elongation, compared to the alloy without cerium. This can be accounted due to in Al-Mg-Si alloy, to form the stoichiometric constituent the Mg and Si, which is the primary hardening phase. Any excess of silicon above the required Mg_2Si will contribute significantly to hardening [11].

Distribution of the Mg_2Si inter-metallic around the grain boundaries as was indicated in Fig.1, a in SEM. This kind of distribution of the related inter-metallic phases decreases the mechanical properties of the metal [12].

The addition of cerium improves the mechanical properties, this is ascribed to the grain refining effect during casting as previously shown from SEM micrographs. Also showed the best grain refining which can be obtained when the content of Ce is in the range (0.043-0.054 wt%). The structure refinement is one of the most important methods for improving the strength of alloys [13,14] besides the presence of spheroidal silicon particles and inter-metallic compounds of cerium (Al_2Ce, Al_4Ce,SiCe and $SiCe_4$) [15].

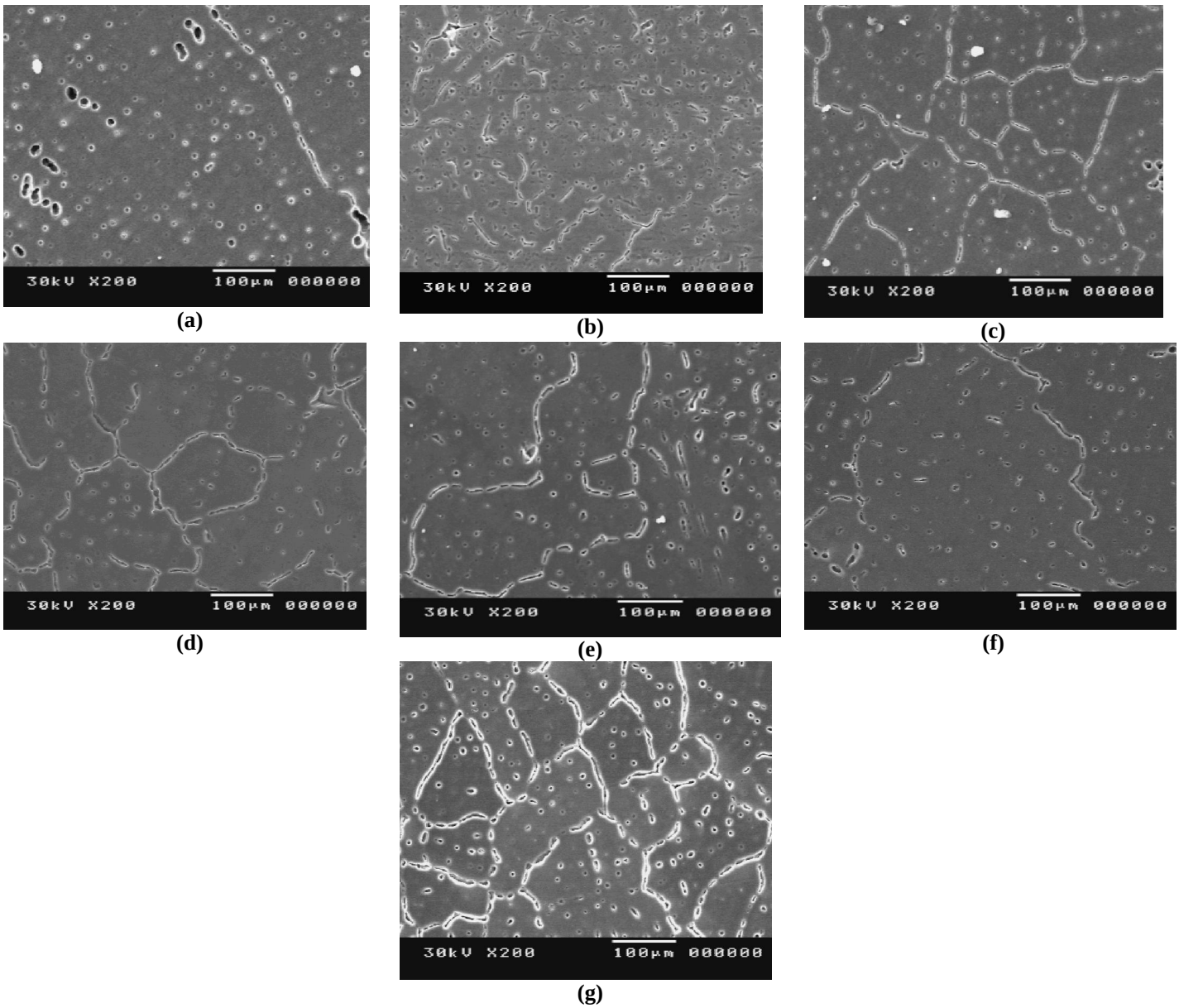
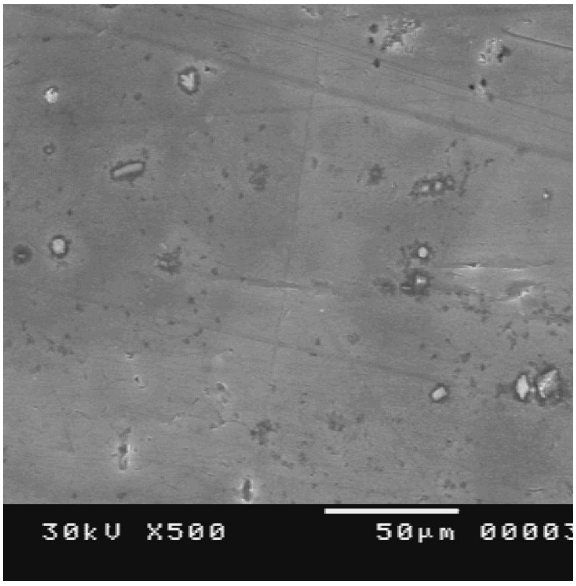
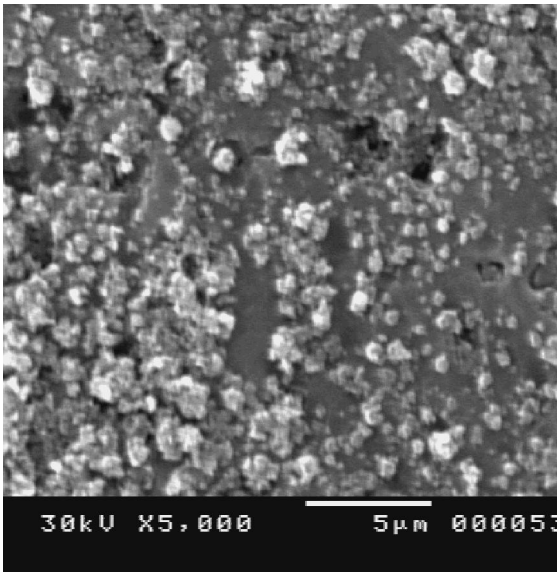


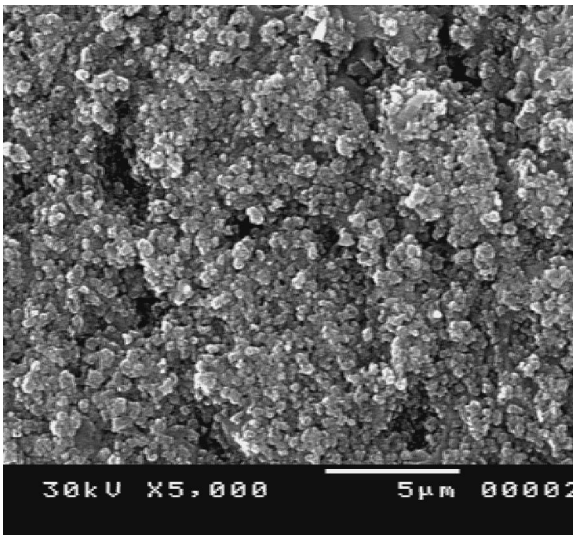
Fig.1a-g: SEM micrographs of the Al-Mg-Si alloy with different Ce concentrations; (a) 0.0 wt%, (b) 0.024 wt%, (c) 0.043 wt%, (d) 0.054 wt%, (e) 0.133 wt%, (f) 0.166 wt% and (g) 0.194 wt %



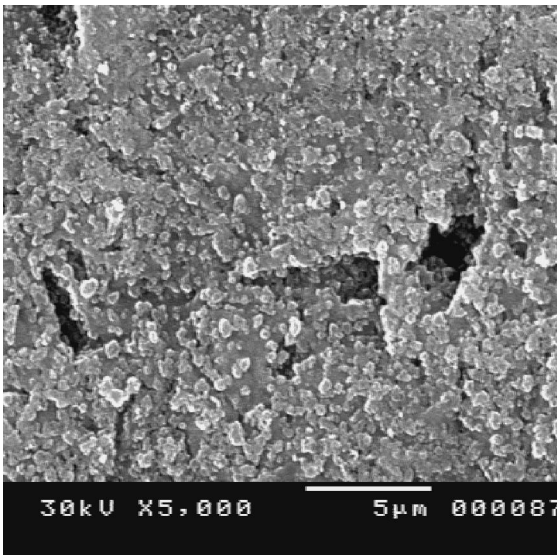
(a) 0.5h



(b) 1h

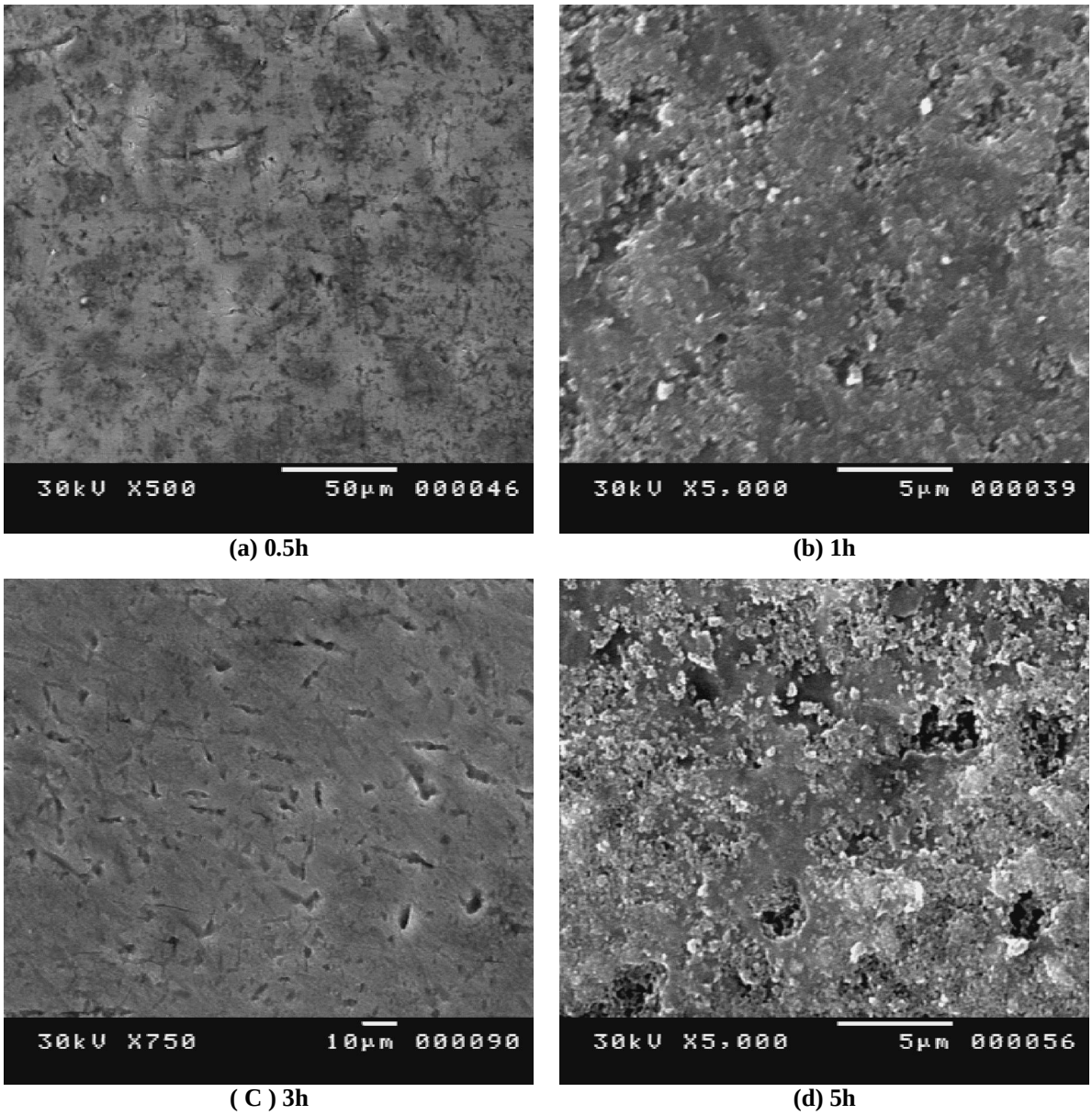


(C) 3h



(d) 5h

Fig.2a-d: SEM micrographs of the Al-Mg-Si alloy without Ce with different homogenization time (a) 0.5h (b) 1h (c) 3h (d) 5h.at 450C^{o}.



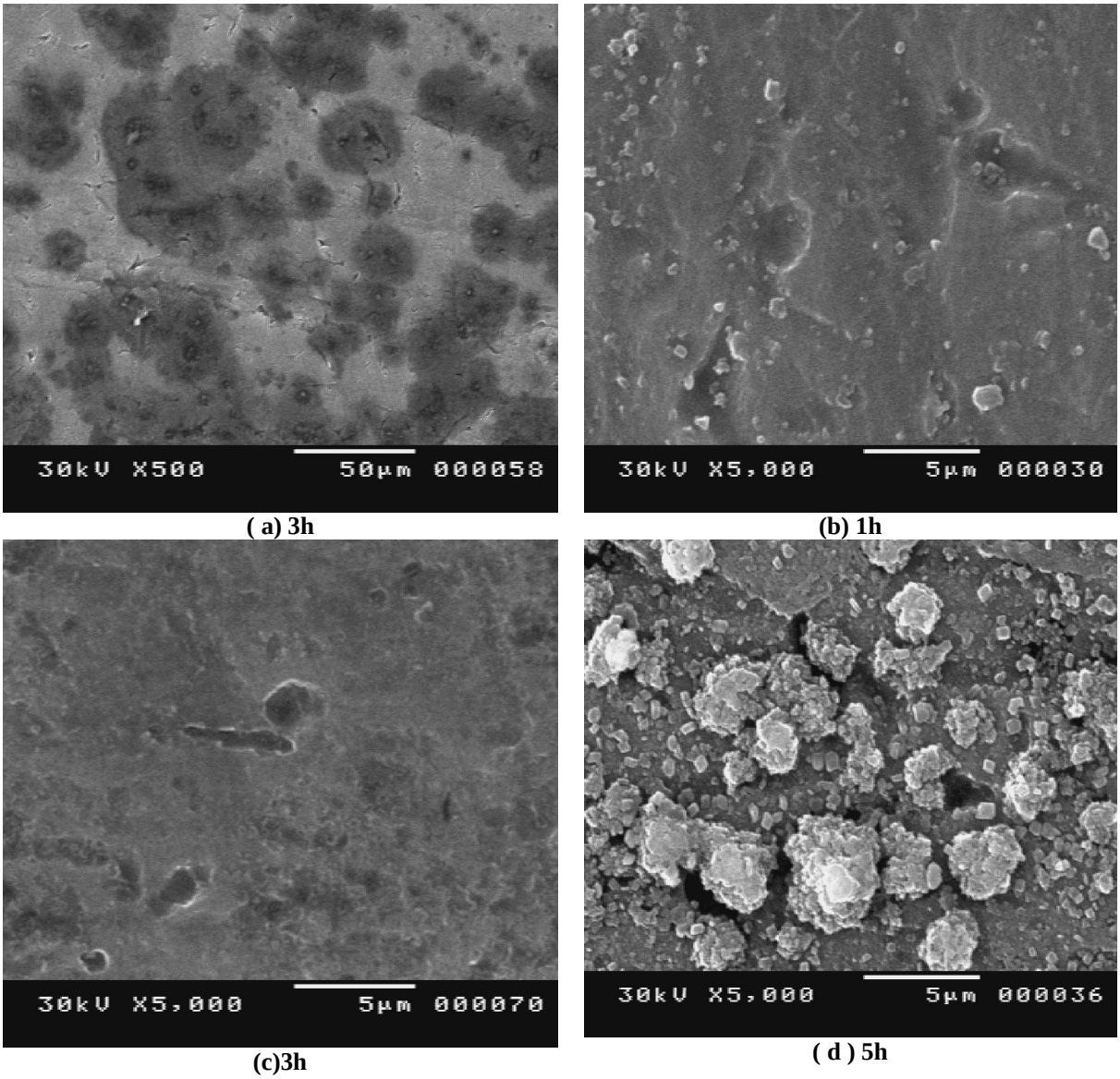
(a) 0.5h **(b) 1h**

(C) 3h **(d) 5h**

Fig.3 a-d: SEM micrographs of the Al-Mg-Si alloy 0.024 wt% Ce with different homogenization time ; (a) 0.5h (b) 1h (c) 3h (d) 5h. at 450^{o}C.

Cerium has low resistivity coefficients and atomic radii that are relatively different from that of aluminum. These characteristics cause solute element to react with crystal defects such as dislocations and grain boundaries and enhance the mechanical properties of the base metal favorably [16]. It has also been reported that Ce reduced the interdendritic spacing of the alloy which can resist the movement of dislocation. In addition, the strengthening effects of Ce atoms segregated at the grain boundary have the contribution of keeping the highest tensile properties. That is, to say, the main reason that makes the segregation of Ce atom at grain boundary increases the sliding resistance of the grain boundary increase the mechanical properties. On the other hand, it is found that an excess of Ce > 0.054 wt% can reduce its useful effect [17].

All the alloys investigated which contain Ce have the highest strength accompanied by low ductility compared to alloy without cerium. The low ductility is due to the addition of cerium, which dissolves in the aluminum matrix and contributes to the formation of insoluble inter-metallic phase is after solidification [18].

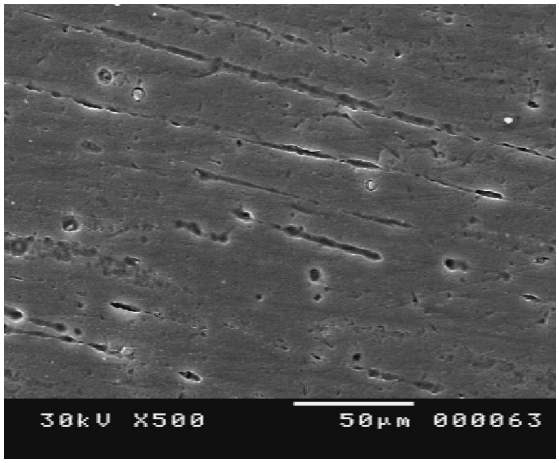


(a) 3h **(b) 1h**

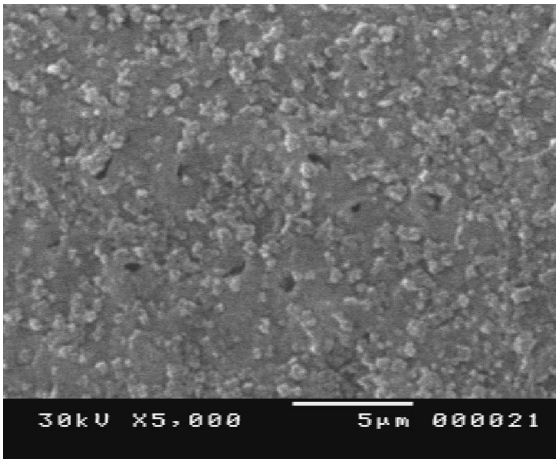
(c)3h **(d) 5h**

Fig. 4a-d: SEM micrographs of the Al-Mg-Si alloy 0.043 wt% Ce with different homogenization time ; (a) 0.5h (b) 1h (c) 3h (d) 5h. at 450^{o}C

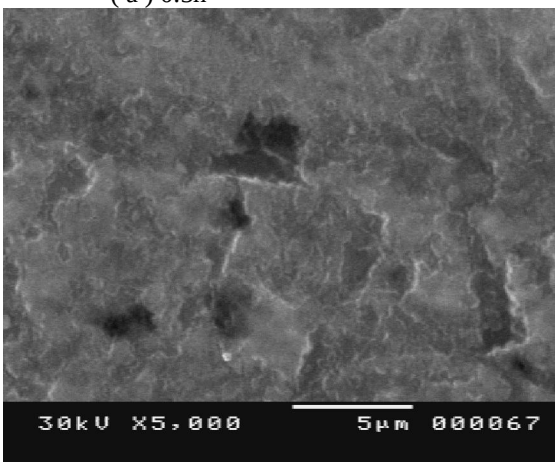
The influences of solutionizing time on the mechanical properties tensile strength, hardness and ductility of the Al-Mg-Si alloy at different Ce content in the range 0.0 wt% - 0.194 wt% are presented in Fig.10, a-c. It can be clearly observed from the figures that, after the homogenization temperature, the changing tendency of tensile testing is different to that at room temperature (unhomogenized alloy). It was observed that solutionizing time causes decreasing on the mechanical strength although the positive influences may be observed on ductility of these alloys depending on the solutionizing time of each composition as seen in Fig.10, a, b, and c. The ductility reaches its maximum value at 3 hours for 0.024, 0.043, 0.054 and 0.194 wt% Ce alloys and at 0.5 hour for (0.0, 0.133 and 0.166 wt% Ce) alloys. Beyond this time the ductility decreases gradually with annealing time as shown in Fig.10, c.



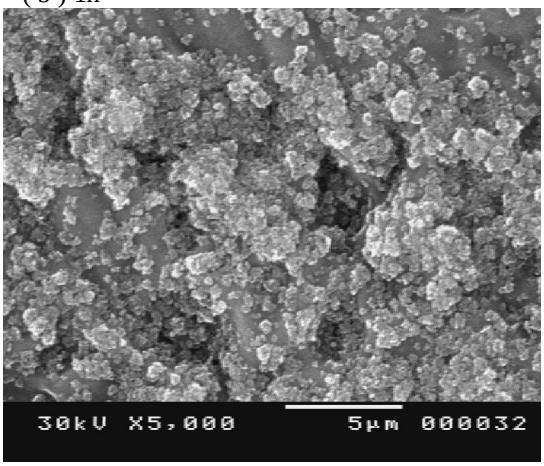
(a) 0.5h



(b) 1h



(c) 3h

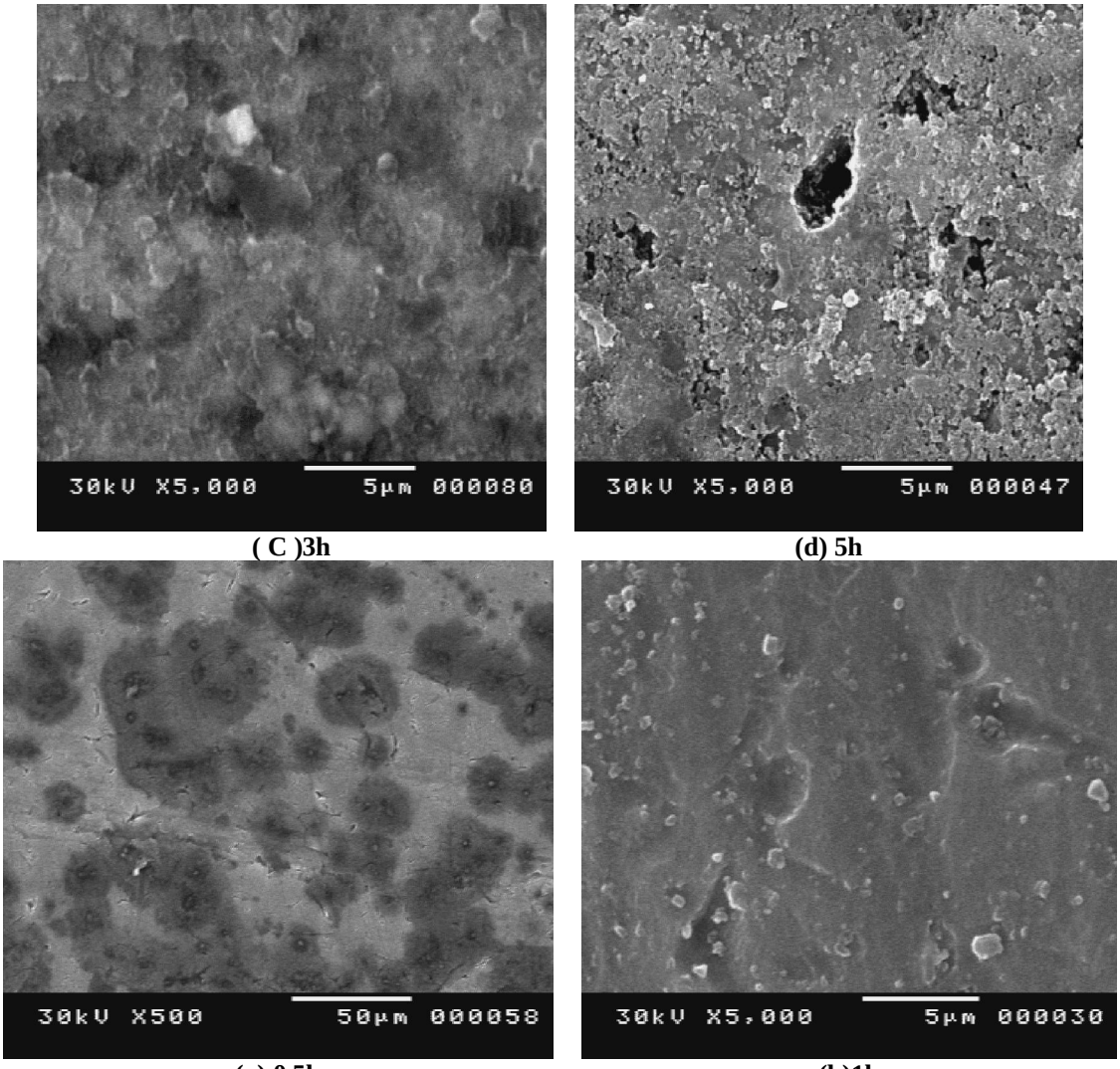


(d) 5h

Fig. 5a-d: SEM micrographs of the Al-Mg-Si alloy 0.054Ce wt% with different homogenization time ; (a) 0.5h (b) 1h (c) 3h (d) 5h. at 450^{o}C

All alloys homogenized and quenched exhibited reduction in both the tensile strength and hardness but the elongation associated with the measuring of homogenized alloys is higher than the unhomogenized . The observed improvement in ductility and the reduction of strength depends on the solutionizing time or soaking time which is applied at the specified heat-treating temperature to provide the chance for the dissolution of undissolved soluble phases and to achieve a homogeneous microstructure [18]. It was found that the mechanical properties change as a function of time as a result of morphology change as shown from SEM photographs. The micro structural development changes for all alloys during the phase transformation of β to α. The rod-shaped particles (β) are gradually replaced by a new uniform dispersion of particles. The solutionizing time, however, is not a constant but affect on particle size, particle geometry, and depend on overall composition [19]. So, it is important to keep the grain size largely unchanged before anneal, because the size of the grains affects the plastic deformation of materials. Shortened anneal time 3 hours for (0.024, 0.043, 0.054 and 0.194 wt% Ce) alloys at 0.5 hour for (0.0, 0.133 and 0.166 wt% Ce) alloys and at annealing temperature 540ºC were efforts to minimize the grain size as in Table (1). Further increasing in the homogenization time, the thickness of the particles increase as a result of coarsening mechanism, which may have a negative effect on the ductility, because the dislocations only flow around particles smaller than a critical size. It is found that the rate of the transformation is affected by the particles size of the alloy; if the particles are spheroidal and smaller, the dislocation may loop around the particles, thereby increase the ductility, and the material is easier to extrude [20].

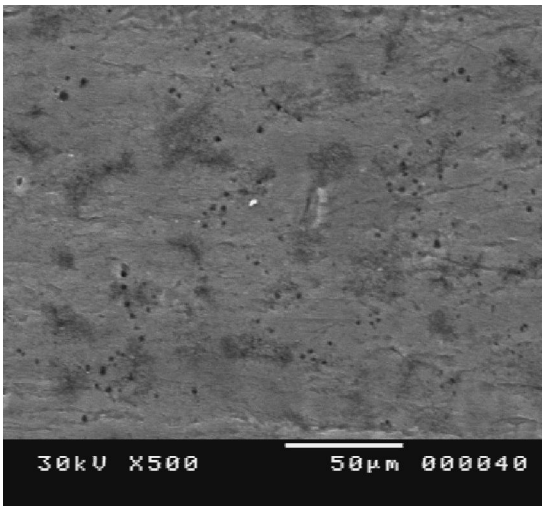
The calculated values of resistivity are summarized in Table (2), it can be observed that the resistivity increases but the variation is low for the unhomogenized samples.



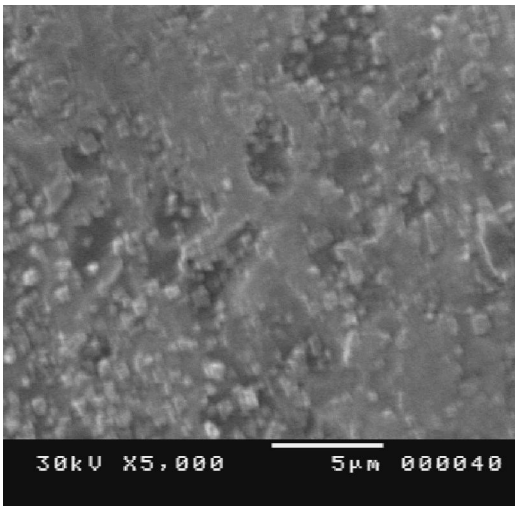
(C)3h **(d) 5h**

(a) 0.5h **(b)1h**

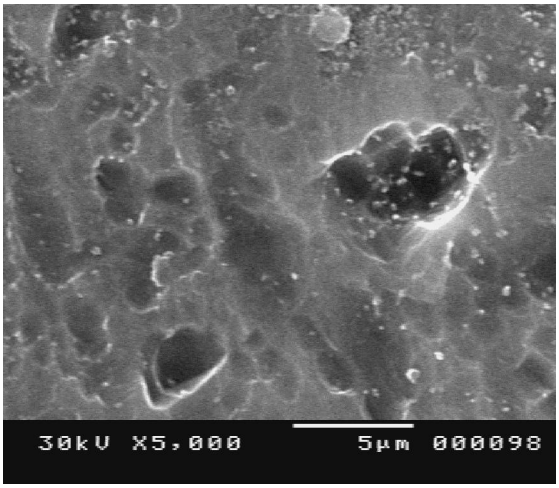
Fig.6 a-d: SEM micrographs of the Al-Mg-Si alloy 0.133 wt% Ce with different homogenization time ; (a) 0.5h (b) 1h (c) 3h (d) 5h. at 450^{o}C



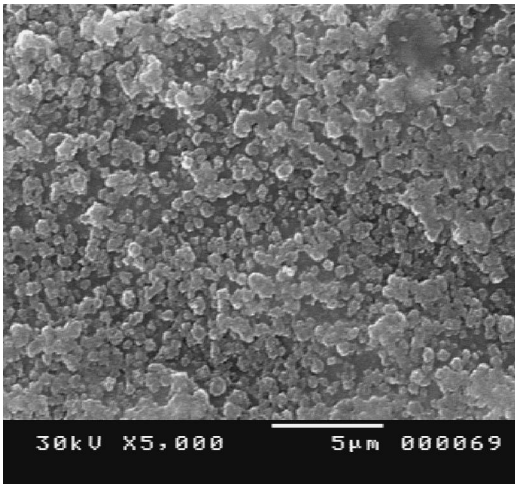
(a) 0.5h



(b) 1h

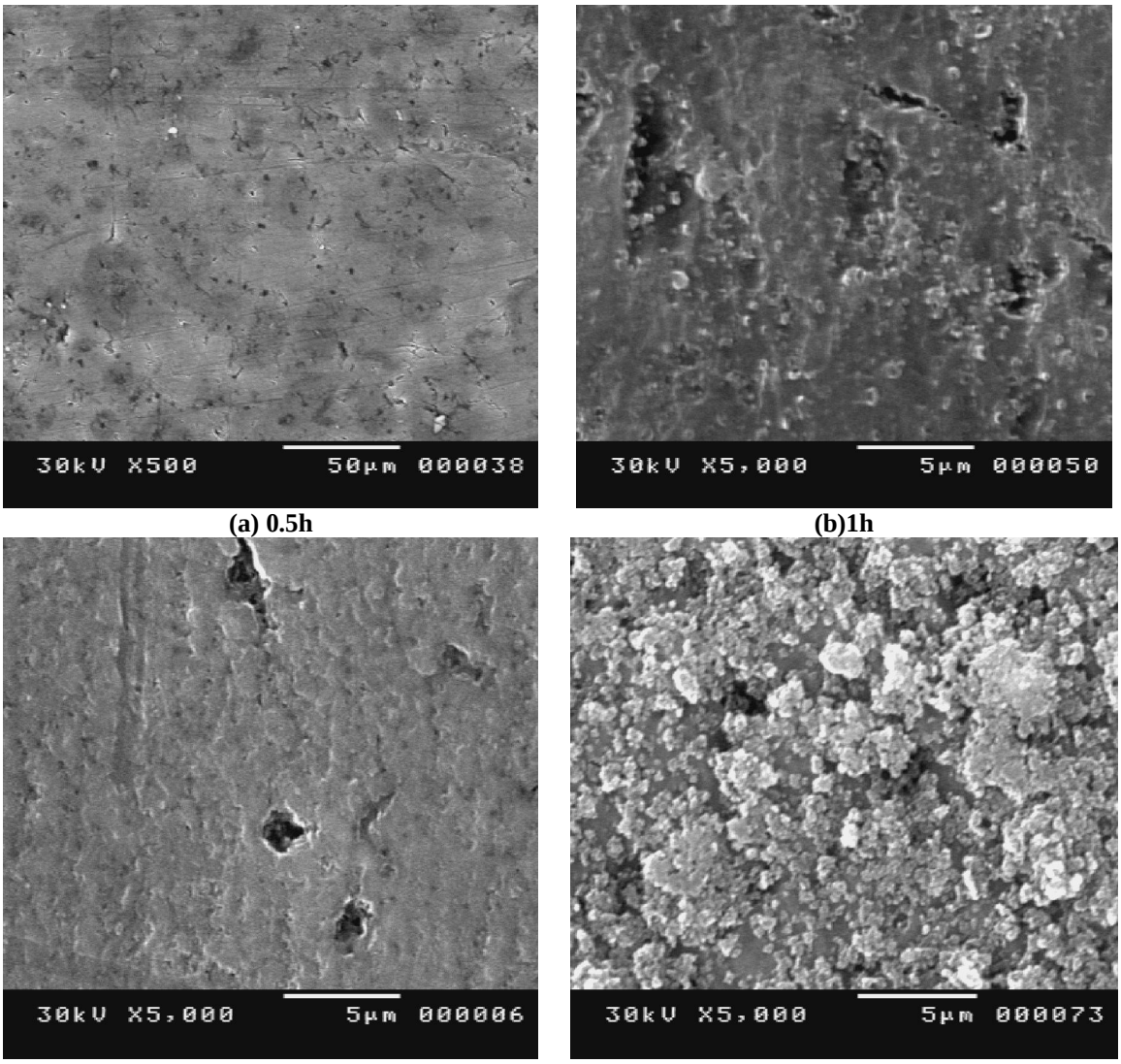


(C) 3h



(d) 5h

Fig.7 a-d: SEM micrographs of the Al-Mg-Si alloy with 0.166 wt% Ce with different homogenization time ; (a) 0.5h (b) 1h (c) 3h (d) 5h. at 450^{o}C.



(a) 0.5h **(b)1h**

(C)3h **(d) 5h**

Fig.8 a-d: SEM micrographs of the Al-Mg-Si alloy with 0.194 wt% Ce with different homogenization time ; (a) 0.5h (b) 1h (c) 3h (d) 5h. at 450^{o}C

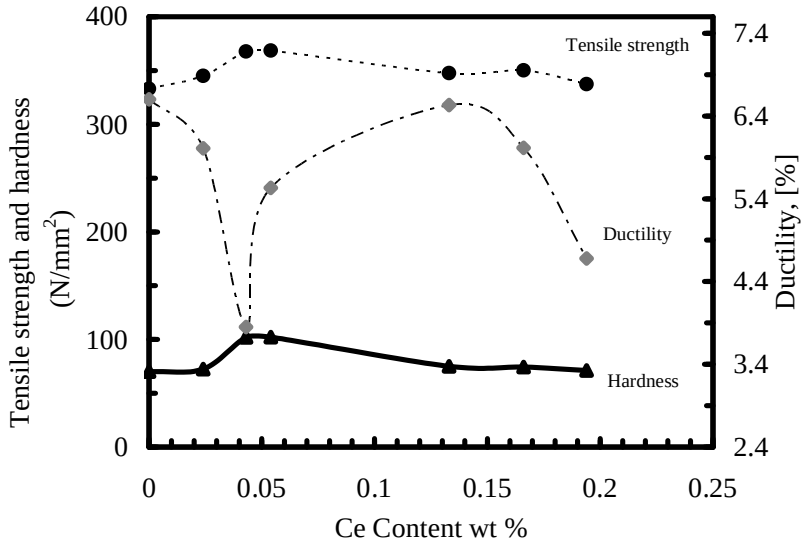


Fig.9: Variation of tensile strength, hardness and ductility as function of cerium content

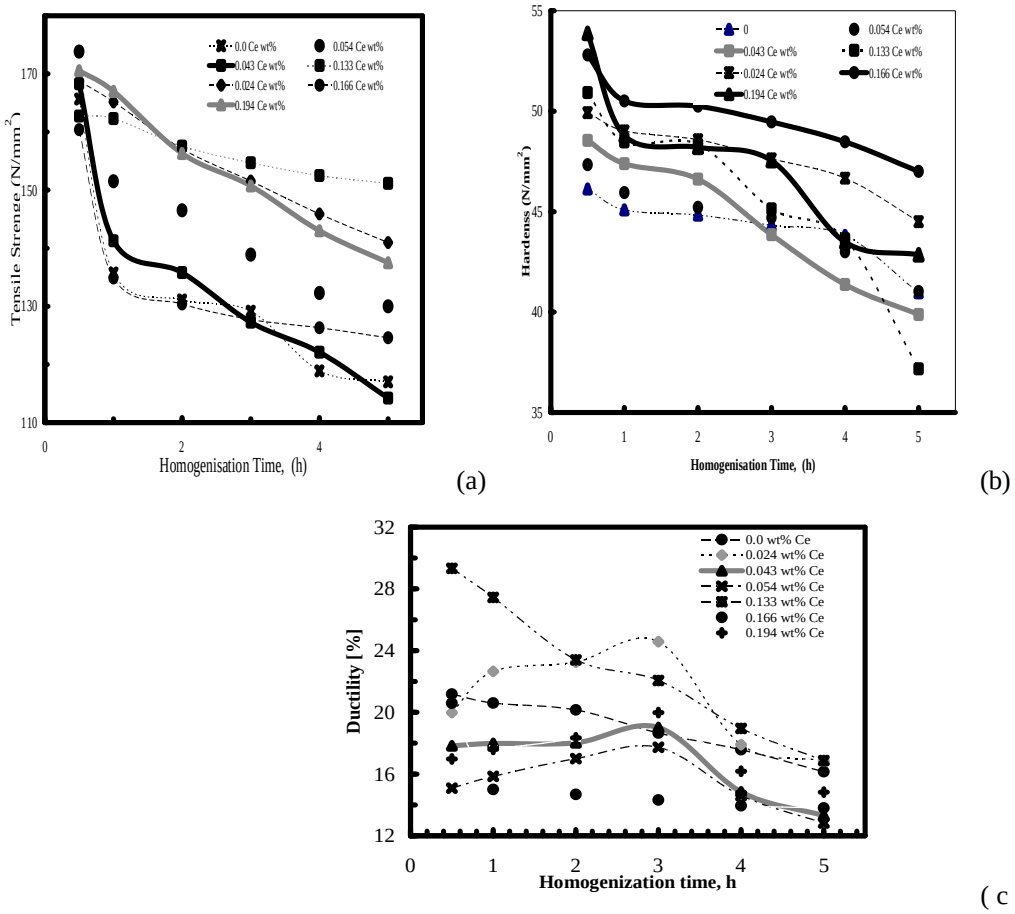


Fig.10 a, b and c: Variation of mechanical properties of Al-Mg-Si alloys; (a) tensile strength, (b) hardness and (c) ductility for different Ce contents as a function solution treatment time at 540^{o}

The increasing in resistivity can be interpreted as; the resistivity is a consequence of disturbances in the atomic periodicity in a crystal structure according to the Block model [21]. These disturbances can be due to atomic vibrations thermal agitation, other electrons, defects in the crystals such as vacancies dislocations or grain boundaries, or substitution of impurity atom in the pure metal lattice sites. The most important of these effects is from the alloying elements in solid solution. The elements such as : Fe, Co, Ni, Sb, La, Ce, Cd, TH, mischmetal "least detrimental to conductivity" forms eutectic phase diagrams at the aluminum-rich end. The eutectic reactions expressed by L à α + β. This is in agreement with the microstructures observed in SEM investigation. The group of elements least detrimental to conductivity form inter-metallic compounds which remain out of solution. The compounds precipitate in the grain boundaries and leave the alpha aluminum almost pure. This helps the alpha to become pure where, in this pure state, it takes the major burden of the electron transport and thus hardly increases the resistivity [22, 23].

Elements in the solution can only be least detrimental to conductivity if they have similar electronic structure to that of aluminum. The rare earth elements, such as cerium have similar electronic configuration, in the solution in an aluminum matrix, produce a very low differential change in the resistivity of aluminum this gives the best bonding with the largest free energy [24]

Table.1-a: Variation of tensile strength, hardness and ductility of Al-Mg-Si alloys for different Ce contents

Ce content (wt%)	Tensile strength	Ductility	Hardness
0	333	6.603	70.18
0.024	345	6.01	72.38
0.043	367.67	3.852	102
0.054	368.65	5.532	102.28
0.133	347.56	6.533	75.133
0.166	350.23	6.015	74.4
0.194	337.436	4.68	71.27

Table1- b:Variation of tensile strength of Al-Mg-Si alloys for different Ce contents as a function of solution treatment time at 540^{o}C

Time	0.0 wt%	0.024 wt%	0.043 wt%	0.054 wt%	0.133 wt%	0.166 wt%	0.194 wt%
0.5	165.666	168.782	168.27	173.828	162.73	160.398	170.55
1	135.74	165.175	141.272	151.51	162.3	134.878	166.988
2	131.1	157.1	135.808	146.513	157.57	130.4	156.293
3	129.16	151.57	127.268	138.883	154.7	127.718	150.72
4	118.9	145.9	122.1	132.28	152.5	126.3	143.01
5	117.015	140.97	114.2	130	151.158	124.58	137.5

Table.1-c: Variation of hardness of Al-Mg-Si alloys for different Ce contents as a function of solution treatment time at 540^{o}C

Time	0.0 wt%	0.024 wt%	0.043 wt %	0.054 wt%	0.133 wt%	0.166 wt%	0.194 wt %
0.5	46.125	49.93	48.543	47.34	50.93	52.8	53.875
1	45.09	49.019	47.386	45.95	48.466	50.507	48.8
2	44.83	48.583	46.614	45.21	48.36	50.25	48.2
3	44.32	47.628	43.85	44.7	45.136	49.467	47.54
4	43.817	46.666	41.37	43	43.657	48.474	43.49
5	40.96	44.5	39.875	41.025	37.17	47	42.85

Also, the solubility is controlled mainly by the ratio of atomic size solvent and solute. If the difference in atomic radii between two elements is less than 15% then there is a large chance of making extended solid solution. In case the difference is greater than 15% solubility is always low [22]. Thus with unfavorable size factor the alloy element will be out of solution generally as a compound. Elements out of solution form small separate particles of low conductivity, but also occupy a very small volume percent of the alloys, and thus have maximum effect on the conductivity. Ce is an example that elements least detrimental to conductivity generally have atomic radii differing widely from that of aluminum.

The low alloy content, the resistivity can be kept reasonably.

For the homogenized samples as seen from Table (2), the general behavior observed is an increasing in resistivity with annealing time. It has been known for a long time that, the resistivity increases nearly linearly with concentration of the alloying elements in solid solution. This explains the increasing in the resistivity at higher homogenization time [26]. The quench itself often produce lattice strain and this is usually considerably increased the electrical resistance [27] as pointed in Table (2).

Table 2: Resistivity ($x10^{-6}\Omega$.cm) for Al-Mg-Si alloys with different Ce content as- received and after solution heat treatment at 540C for different duration time

Conc. wt % / Time .(h)	00.000 Wt%	0.024 wt%	0.043 wt%	0.054 wt%	0.133 wt%	0.166 wt%	0.194 wt%
00	3.49	3.56	3.71	3.46	4.825	3.51	3.50
0.5	3.77	3.63	3.86	3.49	3.72	3.69	3.52
1	3.58	3.69	3.96	3.62	3.51	3.62	3.50
2	3.54	3.84	3.56	3.63	3.90	3.75	3.52
3	3.70	3.53	3.47	3.73	3.49	3.63	3.53
4	3.45	3.71	4.59	3.38	3.72	4.00	3.55
5	3.44	3.83	3.98	4.83	3.76	3.55	3.54

4. Conclusion:

SEM shows that Al- Mg- Si alloy (free Ce) has two kinds of morphologies, Mg_2Si along grain boundaries and round particles that distribute both inside and at grain boundaries. The addition of Ce, a new rod-shaped have an obvious tendency to distribute at grain boundaries and grain refinement the alloys.

All the alloys investigated which contain Ce having the highest strength companied by low ductility compared to alloy free Ce. The improvement of mechanical properties is ascribed to the grain refining. On the other hand, the tensile strength and hardness show a more increase with a suitable Ce addition in the range (0.043 to 0.054 wt%) which have major grain boundaries and the best grain refining, excess of Ce > 0.054 wt% reduce its useful effect. Meanwhile the lowest ductility is due to the formation of insoluble inter-metallic compound in the aluminum matrix.

It is found that Ce form eutectic reaction at the aluminum end of the phase diagram and has electronic structure similar to that of in solution, in an aluminum matrix that due to hardly increases resistivity. Although, the large difference in the atomic radii between Al and Ce but a very low contents are less damaging to conductivity. The quench itself often produces lattice strain and this is usually considerably slightly increased the electrical resistance.

Corresponding author
E.M.Sakr
Physics Department- Faculty of Girls for Arts, Science & Education- Ain Shams University.
*Elham.sakr@yahoo.com

5. References

1- R. E. Smallman, " Modern Physical Metallurgy", Fourth Edition. Butter worths, (1983).
2- John D. Verhoeven, "Fundamentals of Physical Metallurgy", John Wiley & Sons, (1975).
3- Calin D. Marioara, A TEM study of the precipitates no. 6082 Al- Mg- Si Alloy System. PhD Thesis, NTNU, (2000).
4- L.Sd, Properties and Selection of Non-Ferrous Alloys, ASM Metals Handbook, 10th ed, ASM, Metals Park, OH, pp.29-112, (1990).
5- P. Sakaris, H. J. Mc Queen, L. Armberg, et al. (Eds), Aluminum Alloys: Their Physical and Mechanical Properties, ICCA3, NTH – Sinteff, Trondheim, pp. 276-281, (1992).
6- P. Sakaris, H. J. Mc Queen, Q. Meng, Y. Cui, B, Li, in: M. M, Avedersian (Ed.), Advances in Production and Fabrication of Light Metals, CIM, Montreal, pp.605-617, (1992).
7- R. Sharan and S. N. Prasad, Ranchi, India. Light Metal Age, 23-26, (1989).

8- M. H. Mulazimoglu, A. Zaluska, J. E. Gruzleski, F. Paray, Metallurgical and Materials Transactions A, 27A, pp. 929-936, (1996).
9- C. Romming, V. Hansen, J. Gjonnes, Acta Crystallogr. B, B 50, 307, (1994).
10- S. Zajac, B. Hutchinson, A. Johansson and L. O. Gullman, Materials Science and Technology, 10, pp.323, (1994).
11- Sadat Karabay, Muzaffer Zeren and Muharrem Yilmaz, Journal of Materials Processing Technology 135, pp.101 – 108, (2003).
12- Sadat Karabay, Muzaffer Zeren and Muharrem Yilmaz, Journal of Materials Processing Technology 160, pp.138-147, (2005).
13- Liao Hengcheng, Sun Yu, and Sun Guoxiong, Materials Science and Engineering A358, pp. 164-170, (2003).
14- Chen Yuyong, Li Qingehun and Jiang Zhuling, Journal of the Less Common Metals, 110, pp. 175-178, (1985).
15- A. R. Daud, Karen M. C. Wong, Materials Letters 58, pp.2545-2547, (2004).
16- A. Takahashi, L.C. Mcdonald and H. Yasuda Phys. Stat. Sol. 160, 413, (1997).
17- Mingang Zhang, Zhiliang Chang, Junmin Yan and Jin Zhihao, Journal of Materials Processing Technology , 115, pp. 294-297, (2001).
18- H. R. Ammar, C. Moreau, A. M. Samuel, F. A. Samuel and H. W. Doty, Materials Science and Engineering, A489, pp. 426-438, (2008).
19- F. J. Vermolen, C. Vuik, S. Van der Zwaag, Materials Science and Engineering, A347, pp. 265-279, (2003).
20- S. Zajac, Gullman, A. Johansson, B. Bengtsson, Material Science Forum, 217-222, pp. 1193-1198, (1996).
21- S. Klays, (ed) " CRC handbook of electrical resistivities of binary metallic alloys", (CRC, Boca Raton, Fl., pp. 3-102, (1983).
22- R. P. Eliott," Constitution of Binary Alloys, First Supplement", McGraw-Hill Book Co., N.Y., (1965).
23- F. A. Shunk," Constitution of Binary Alloys, Second Supplement" McGraw-Hill Book Co., N.Y., (1969).
24- L. Brewer in "Phase Stability in Metals and Alloys", ed. P. Rudwan, J. Striger, R. I. Jaffe, McGraw-Hill Book Co., N.Y., (1967).
25- M. Hansen, "Constitution of Binary Alloys", McGraw-Hill Book Co., N.Y., (1958).
26- P. Olafsson, R. Sandstorm, A. Karlsson, Journals of Materials Science, 32, pp. 4383-4390, (1979).
27- R. E. Smallman, Modern Physical Metallurgy, 69, pp. 402,302, (1976).

6/25/2010

The Role of some Natural Soil Conditioner and AM Fungi on Growth, Root Density and Distribution, Yield and Quality of Black Monukka Grapevines Grown on Calcareous Soil.

[*1]Mervat, S. Rizk-Alla and [2]Hager, I. Tolba

[1*]Hortcultural Research Institute, [2]Microbiology Dep., Soils, Water & Environment Research Institute, Agricultural Research Center, Giza, Egypt.

Abstract: The current research was carried out during two successive seasons (2007 and 2008) on ten years old Black Monukka grapevines to disclose the role of some natural soil conditioners namely, humic acid (HA), Nile fertile (NF) and AM fungi (AM) in a single application or in combined mixture growth, root density and distribution, yield and quality of Black Monukka grapevines grown under calcareous soil in a private vineyard in Nobaria at Cairo-Alexandria Desert Road; the results showed that all different soil conditioners were effective but the treatment of humic acid at 15 ml/ vine (HA1) + Nile fertile at 200 g/ vine (NF1) + AM fungi gave the best results in comparison with other treatments and control. This treatment enhanced the growth characters namely total leaf area/ vine, shoot diameter and coefficient of wood ripening, total chlorophyll, NPK% of the leaves and total carbohydrates of the canes. Also, the vines of this treatment produced the heighest fibrous root fresh weight, larger number and longest fibrous root. With respect to microbiological activity in the rhizosphere, it was noticed that the best AM infection %, no. of AM spores /g dry soil, total microbial count, phosphatase and dehydrogenase enzymes activity were obtained by the same treatment. From the economic point of view, this treatment was accompanied by the highest yield and best its components namely physical and chemical characteristics of bunches and berries. Under such promising treatment the adverse effects of calcareous soil on growth and production of vines could be overcome. [Journal of American Science. 2010;6(12):253-263]. (ISSN: 1545-1003).

Keywords: grapevine; humic acid (HA); Nile fertile (NF); AM fungi (AM); microbiological activity

1. Introduction:

In Egypt different types of new reclaimed soils particularly calcareous soils have an alkaline reaction. Application of some mineral fertilizers may be ineffective because of the expected problem of its availability, where phosphorus are fixed in insoluble forms and become unavailable to plants. Phosphorus deficiency may be limit plant growth than do nitrogen deficiency (Awad, 1999). In addition, grape tree grown on calcareous soil developed in many instances chlorotic symptoms. This lime induce chlorosis is due it iron (Fe) deficiency whereas, micronutrients e. g. Fe, Mn and Zn are unavailable under condition of high soil pH and $CaCO_3$ content (Clark and Kajiura, 1986).

Humic acids (HA) have been found to a profound effect on not only the biological activity and soil structure, but also on the plant itself. This is due to their positive effect on plant nutrients uptake, increasing the productivity of fruit crops due to the conversion of unavailable minerals into soluble forms that plants can use (El Fakharani, 1999). Agricultural (HA) are reputed to enhance drought tolerance, seed germination and overall plant performance (Morales-Payar and Stall, 2003).

Cheng *et al.,* (1998) reported that, humic acids decrease the loss of soil moisture, enhance water retention, increase the ability rate of wheat leaves for photosynthetic process and also increase root growth in a manner similar to auxins. Where the soil pH is high, correction of Fe, Mn and Zn deficiency can be attempted by adjusting pH with acidifying compounds such as elemental sulphur (Clark and Smith, 1986). Whereas, sulphur is oxidized by soil microorganisms, such as *Thiobacillus* spp. Bacteria which, consider as the most important microorganisms involved in the bioleaching of sulphide compounds to sulphuric acid in amount enough to decrease soil pH, improve availability of most soil nutrients and uptake by plant, enhancing root development and increasing the activity of soil microorganisms (Kassem *et al.,* 1995). Ibrahiem (2003) found that fertilizing the vines by Nile-fertile (263 g/vine) gave the highest yield and best quality of berries of flame seedless grapevines.

Arbuscular mycorrhizal (AM) fungi are ubiquitous soil organisms that can form mutualistic associations with the roots of the majority of vascular plant species (Lindermann, 1988). The establishment of AM association is often beneficial for plant nutrition especially enhance absorption of phosphours and other relatively immobile micronutrients cations, particulary zinc and copper (Marin *et al.,* 2003) also AM fungi produce plant

growth hormones such as auxines, cytokinines and gibberellins (Zhang *et al.*, 2008), increasing water uptake (Graham *et al.*, 1987). AM fungi may also confer tolerance and resistance to various abiotic and biotic stresses to the host plant (Colla *et al.* 2008).

Black monukka is one of the table grape cultivars; ripens in mid July to late Agust, seedless, sweet, crispy, purplish-black coloured, tender skin. The production of small to medium berries and loose bunches are negatively reflected on bunch quality (Harry *et al.*, 1991)

Therefore, the main objective of this study is to evaluate the possible effects of humic acid, Nile fertile, AM fungi and their combination on growth, vine nutritional status, yield/vine and quality of black Monukka grapevines grown in a calcareous soil.

2. Materials and Methods

The current study was carried out during two successive seasons (2007 and 2008) in a private vineyard in Nobaria using 10 years old Black Monukka grapevines. The chosen vines grown in a calcareous soil (Table 1).

Table (1). Main chemical and physical characteristics of the experimental soil.

Particle size distribution%		
Coarse sand		11.75
Fine sand silt		37.10
silt		18.03
Clay		33.12
Texture class		Sandy clay loam
Chemical analysis		
pH(1:2.5 soil water suspension)		8.2
Calcium carbonate (%)		25.8
EC	(dS/m)	1.48
Ca^{++}	(meq/L)	4.13
Mg^{++}	(meq/L)	1.12
Na^{+}	(meq/L)	6.46
K^{+}	(meq/L)	1.43
$CO\ 3^{-}$	(meq/L)	0.00
$HCO3^{-}$	(meq/L)	1.47
Cl^{-}	(meq/L)	6.90
$SO4^{--}$	(meq/L)	4.77
Organic matter	(%)	0.43
Total N	(%)	0.053
Total P	(ppm)	616
Available N	(ppm)	25.9
Available P	(ppm)	4.35

1- Lay out of the experiment:

Spaced between each vine lines at 2X 2.5 meters a part and irrigated by the drip irrigation system, cane-pruned and trellised by the double T shape system. The vines were pruned during the second week of January with bud load of 60 buds/vine. 144 uniform vines were chosen. Each four vines acted as a replicate and each three replicates by one.

All vines were subjected to the normal horticultural practices. Nitrogen (60 kg N/ feddan), potassium (100 Kg K2O/feddan) and phosphorus as phosphoric acid (1 liter/2 weeks) were added. Chelated Fe, Zn and Mn at rats of 200 g, 100 g and 100 g respectively per 600 liter water sprayed two times, once 10 days before anthesis and the other after fruit set (at 3-4 mm berry diameter).

The randomized complete block design was followed the tested treatments that evaluated through the following parameters:

Twelve treatments applied as follows:

- T1- Control (untreated vines).
- T2- P-humex acid at 15 ml/ vine (HA1).
- T3- P-humex acid at 30 ml/ vine (HA2).
- T4- Nile fiertile at 200 g/ vine (NF1).

- T5- Nile fiertile at 300 g/ vine (NF2).
- T6- AM fungi.
- T7- HA1 + AM fungi.
- T8- HA2 + AM fungi.
- T9- NF1 + AM fungi.
- T10- NF2 + AM fungi.
- T11- HA1 + NF1.
- T12- HA1 + NF1+ AM fungi.

Humic acid (HA) content in the liquid organic fertilizer was determined using $BaCl_2$ precipitation method as described by (Fataftah *et al.*, 2001). P-humex acid (HA) contains 25% humic acid, 2% N, 4% P_2O_5, 6% K_2O, 0.2% Fe, 8.9 pH, 45.22 organic matter, 300- 500 meg/ 100g CEC and 1.25 Kg/L density. Humic acid was added at the rate of 15 or 30 ml/vine (according to Ali *et al.*, 2006) on the soil surface after bud buest.

Nile firtile (NF) contains 38% S, some essential elements, (2.7 % N, 3.5% P, 1.2% K, 5% Ca, 2.7% Mg and 1% Fe) and sulphur bacteria, *Thiobacillus* spp. (10^6 CFU/gm). Nile fertile was applied once during winter agricultural management by mixing with soil in the wetting zone adhesive to the root at a recommended rate 200 or 300 g /vine (Ibrahiem, 2003)

AM fungi inoculum: mycorrhizal spores that contained the mixyure of the following genera; *Glomus, Gigaspora* and *Acaulospora* were extracted from Egyptian soil. Extraction and counting of identified mycorrhizal spores were carried out according to the method described by Massoud (2005). The extraeted mycorrhizal spores were mixed with sterilized peat as a carrier (250 spore/gram) and then applied to the soil at a rate of (100 g inocula /m long) so each vine diameter was 2.5 m. so, each vine needed 250 g inoculums.

Growth characters:

Total leaf area /vine (m^2) was determined by multiplying average number of the leaves/ shoot by average leaf area then by the number of shoots per vine. Ten shoots/ vine were labeled to determine the average shoot diameter (mm) and determine the wood ripening coefficient by dividing the length of the mature part of the shoot by total shoot length (Bouard, 1966).

Root system measurements:

Fibrous root density (root fresh weight and total number) was determined in soil samples taken with augar to make a hole of 10 cm in diameter (auger volume = 1153.8 cm^3) and 30 cm depth form four directions at 50 and 100 cm away from vine trunk. Soil sample were taken in late Novamber, average weight per hole was calculated as gm/ hole according to Cahoom *et al.,* (1959). Average length of roots (cm) was determined.

Chemical determinations:

leaf content of total chlorophyll was measured using nondestructive Minolta chlorophyll meter SPAD 502 of the 5^{th} and 6^{th} leaves (Wood *et al.*, 1992) Cane content of total crbohydrates (%) was determined according to (Smith *et al.*, 1956) leaves opposite to the clusters were collected then dried to estimate N, P and K percentages according to (Jackson, 1973).

Yield, clusters and berry characteristics:

Clusters were picked when TSS of each treatment reached about 16- 17% (Tourky *et al.*, 1995). Yield/ vine (kg), cluster weight (g), berry weight (g), berry size (cm^3). Shattering (%) was determined on clusters stored for seven days at room temperature (28 to 32 C^o), Shattering (%) was calculated by dividing weight of Shattering berries by the initial weight of the clusters. T.S.S. (%) was estimated by a hand refracto-meter, total titrable acidity as tartaric acid (%) A.O.A.C. (1985), T.S.S./ acid and total anthocyanin of berry skin (mg/ 100 g fresh weight) was estimated as described by Yildiz and Dikman (1990).

Microbiological parameter:

Arbiscular mycorrhizal infection was determined according to method described by (Massoud, 2005). Spores number after each season was also counted according to (Massoud, 2005). Total microbial count (--- X 10^6 CFU/g soil), dehydrogenase (µg TPF/ g dry soil/ day) and phosphatase enzyme activity(IP /g/ dry soil/ day) were also determined according to the methods described by (Esher and Jensen 1972, Salman 1967 and Drobnikova 1961)

Data obtained were statistically analyzed according to Snedecor and Cochran (1980) using the New L.S.D. test for examining the significant differences between the studied treatments.

3. Results and Discussion:

1- Vegetative growth parameters:

Obtained data in table (2) showed, vegetative growth parameters of Black Monukka cv. as affected by humic acid, Nile fertile and AM fungi and their combinations. The data revealed that, all treatments improved the growth parameters compared to the control. However, it is worthy to notice that, the effect of single application of this soil conditioner was significantly lower compared to the combined application of these materials. However, the highest values of total leaf area/ vine, shoot diameter and coefficient of wood ripening were detected in case of vines treated with (HA1 +NF1+

AM fungi) amounting to 21.8 and 22.5 m^2 for total leaf area/vine, 8.0 and 8.6 mm^2 for shoot diameter and 0.86 and 0.89 for coefficient of wood ripening in both seasons respectively. While, the lowest values were recorded with the control 15.2 and 16.5 m^2 for total leaf area/vine, 6.0 and 5.6 mm^2 for shoot diameter and 0.63 and 0.61 for coefficient of wood ripening in both seasons respectively.

The obtained increasing in vegetative growth parameters recorded with the treatment (HA1 +NF1+ AM fungi) this might be due to the role of humic acid in modifications in the soil-root interface that make the nutrients more available to plants (El Fakharani, 1999) and humic acid improved plant vigour and health (Kassem *et al*., 1995).

Table (2) Effect of soil condition with humic acid, nilefirtile and AM fungi application on some characteristics of vegetative growth of Black Monukka grapevines during 2007 and 2008 seasons.

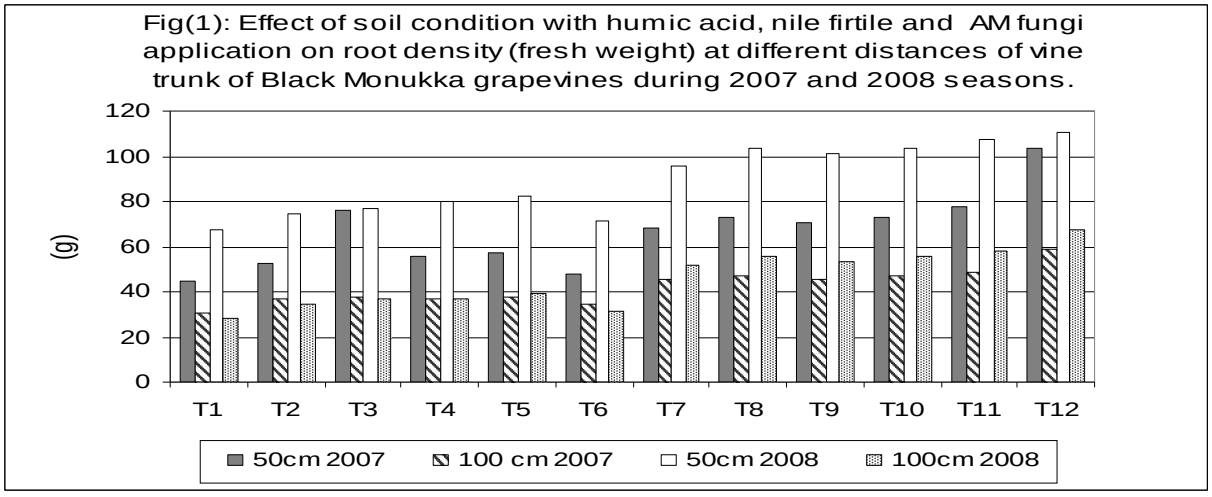
Treatments	Total leaf area/vine (m^2)		Shoot diameter (mm^2)		Coefficient of wood ripening	
	2007	2008	2007	2008	2007	2008
T1	15.2	16.5	6.0	5.6	0.63	0.61
T2	15.5	17.0	6.1	6.1	0.65	0.66
T3	15.8	17.3	6.2	6.4	0.67	0.70
T4	15.6	17.0	6.4	6.1	0.69	0.68
T5	16.4	17.5	6.6	6.4	0.70	0.70
T6	15.5	16.8	6.1	6.0	0.66	0.65
T7	16.6	18.0	6.7	6.7	0.73	0.73
T8	17.0	18.3	6.9	7.0	0.77	0.77
T9	17.5	18.9	7.2	7.4	0.77	0.80
T10	17.9	19.3	7.4	7.7	0.80	0.82
T11	18.6	20.7	7.7	8.1	0.82	0.85
T12	21.8	22.5	8.0	8.6	0.86	0.89
New L.S.D.at 5%	2.8	2.5	0.10	0.30	0.02	0.03

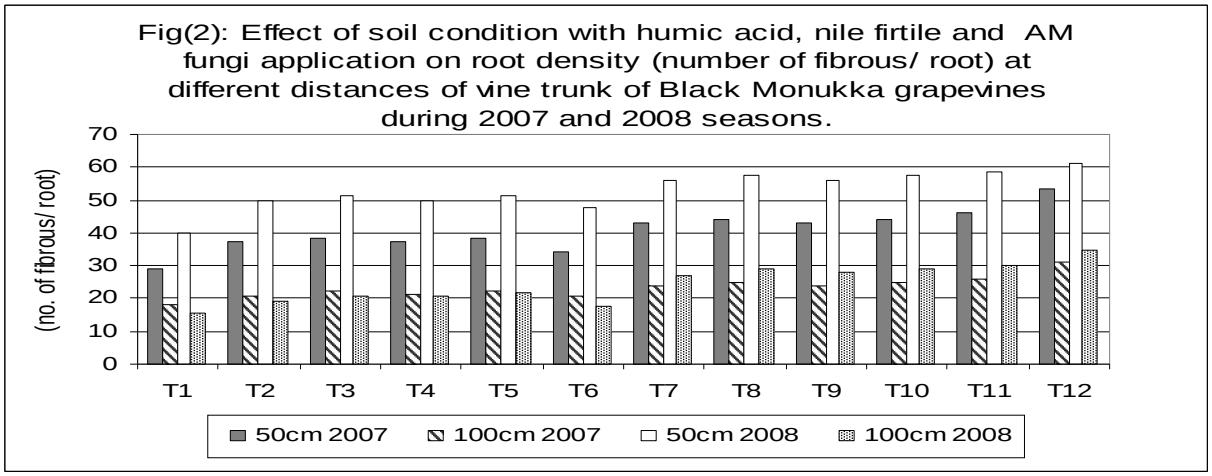
With respect to Nile fertile, it is considered as a soil conditioner, reducing pH, due to acid bacteria that *Thiobacillus* spp. bacteria where, the mechanisms for the growth of *Thiobacillus* spp. bacteria on sulphur is proposed. Initially, the bacteria attach to the sulpher particles and start oxidizing it and the number of attached bacteria increased with time, resulting an increase in the rate of generation of partially simple oxidized sulpher compounds. The sulphur compounds are consumed by plants that contribute on its growth and yield (Bhavaraju *et al.*, 1993). Also increased availability of most nutrients and increasing organic mater (Ibrahiem, 2003) could explain these results with respect to AM fungi that produced enzymes that enhance the respiration efficiency of the root (Zhang *et al.*, 2008) and he also noticed an increase in plant growth due to the improved uptake of elements. However, the colonization with AM fungi gave more repaid growth and increased plant biomass, plant height, leaf area than the non inoculated plants. The findings are in the same line with those of El Shenawy and Fayed (2005), Hussien *et al.*, (2005), Omar (2005) and Ali *et al.*, (2006) who reported that humic acid enhance vegetative growth, Ibrahiem (2003) and Rizh-Alla *et al.*, (2006) who reported that Nile fertile recorded the maximum values of vegetative growth. As for the effect of AM fungi Abd El Wahab *et al.*, (2008) found that dual inoculation with AM fungi increased the vegetative growth parameters.

2- Root system measurements:

a- fresh weight and numbers:

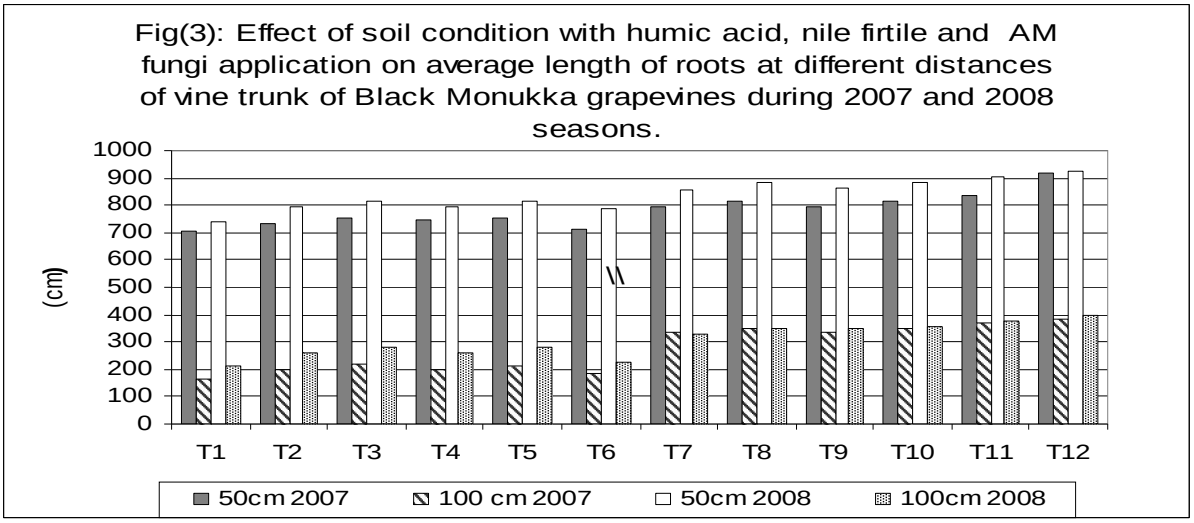
Data of figs (1 and 2) indicate that, vines of applicated with (HA1 +NF1+ AM fungi) produced the highest fibrous roots fresh weight (gm/ hole) at 50 and 100 cm a way from vine trunk and gave highest numbers of fibrous roots in both seasons. On the other hand, untreated vines produced the least fibrous roots fresh weight. While, the other treatments were intermediate.





b- Root distribution:

Distribution of the roots in the soil profile are important to know the volume of wetted root zone, fig (3) show that the treatment (HA1 +NF1+ AM fungi) recorded the longest roots. On the other hand untreated vines had the least values, while the other treatments gave intermediate values in this respect.



Generally, this treatment gave better and larger rooting area in soil. The greatest amount of roots and longest was observed in the 50 cm distance from the vine trunk compared with in the 100 cm distance from the vine trunk.

The obtained root results of this treatment could be attributed to the humic acid has high water-holding and affects the physicochemical properties of soil, which are important in controlling the uptake of nutrients, their retention and counteracting soil acidity (Hartwigsen and Evans, 2000).

The beneficial effect of Nile fertile on increasing the root formation could give an explanation for the present results. These results are in agreement with those obtained by Ahmed *et al.,* (1994) and Ibrahiem (2003).

Vitagliano *et al.,* (1999) showed that, AM fungi increased the growth of rooted cutting of olive cultivar, via an increase in lateral root frequency. It has been recognized that AM fungi symbiosis play role in nutrient cycling in the ecosystem and also protect plants against environmental conditions and stress (Barea and Jeffries, 1995).

3- Chemical determination:

Results presented in Table (3) revealed that total chlorophyll, total carbohydrate content of the canes and percentages of total nitrogen, phosphours and potassium of the leaves were increased significantly by the different treatments. Also, it can be noticed that the application of (HA1 +NF1+ AM fungi) generally resulted in higher values of these parameters as compared to uninoculated vines and the other single application of these materials in the both seasons.

Table (3) Effect of soil condition with humic acid, nile firtile and AM fungi application on total chlorophyll, total charbohydrates, leaf N, P and K % contents of Black Monukka grapevines during 2007 and 2008 seasons.

Treatments	Total chlorophyll (mg/ g F.W)		Total carbohydrates (%)		N%		P%		K%	
	2007	2008	2007	2008	2007	2008	2007	2008	2007	2008
T1	31.5	33.8	23.9	24.1	1.79	2.42	0.30	0.22	1.25	1.35
T2	32.9	34.7	24.9	25.6	1.91	2.53	0.32	0.27	1.35	1.43
T3	34.0	35.5	25.7	26.7	2.00	2.63	0.35	0.31	1.45	1.58
T4	34.7	38.0	26.3	26.8	2.05	2.50	0.33	0.27	1.36	1.55
T5	34.9	38.2	26.8	26.9	2.07	2.63	0.37	0.32	1.46	1.59
T6	32.7	34.6	24.8	25.4	1.88	2.46	0.32	0.28	1.35	1.49
T7	36.0	39.0	27.7	27.6	2.15	2.73	0.40	0.38	1.57	1.67
T8	37.0	39.2	28.5	27.7	2.20	2.8	0.41	0.39	1.64	1.72
T9	37.5	40.0	28.8	27.9	2.27	2.83	0.40	0.41	1.66	1.74
T10	38.5	40.5	29.1	29.1	2.30	2.97	0.42	0.42	1.74	1.81
T11	38.8	41.2	29.6	30.3	2.33	3.15	0.43	0.44	1.84	1.90
T12	40.0	42.0	30.5	31.6	2.43	3.40	0.45	0.48	1.95	2.0
New L.S.D.at 5%	1.0	0.7	0.80	1.10	0.08	0.10	0.02	0.04	0.10	0.09

The obtained results could be interpreted in view of the effect of humic acid and Nile fertile on enhancing the metabolism process of carbohydrates as well as its effect on reducing soil pH which by their turn could be responsible for increasing the availability of nutrients. Also, AM fungi produced enzymes that enhance the respiration of root (Edrees, 1982). AM fungi are able to absorb and translocate elements to host root tissues (Mona, 2001), AM fungi increase nutrient uptake by reducing the distance at which nutrients must diffuse to plant roots (Rhodes and Gerdemann, 1975). AM fungi improved nutrition mode possible by extensive hyphae network. This not only allows the plant to overcome phosphorus depletion from the zone around the root, but also allows it to reach immobile phosphorus that the fungus can solubilize. This phenomenon is most apparent in soils low in phosphorus. (Zarb *et al.,* 1999).

The obtained results are nearly similar to those achieved by several investigators who reported that humic acid enhance these contents El Shenawy and Fayed (2005); Hussien *et al.,* (2005); Omar (2005) and Ali *et al.,* (2006).

As for the effect of Nile fertile, Ibrahiem (2003) and Rizk-Alla et al., (2006) pointed that Nile fertile significantly increased these contents. In addition, some researcher found that AM fungi increased chlorophylls content of leaves El-Sharkawy (1989) and increased carbohydrate content of canes

and leaf mineral content Abd El-Wahab *et al.,* (2008).

4- Yield, cluster weight and some physical characteristics of berries:

Yield and cluster weight in general was significantly increased by all applications compared with control Table (4). The application of (HA1 +NF1+ AM fungi) resulted in the highest values of this estimate (9.0, 9.5 kg/ vine and 422.5, 451.3 g) for both seasons respectively. Whereas, the lowest values were obtained from untreated vines, this recorded (7.91, 8.36 kg/ vine and 383.7, 401.1 g 0for both seasons respectively.

Also, this application gave the best results of physical properties of the berries in terms of increased berry weight, berry size and reduced berry shattering compared to the control. However, it is worthy to note that, the single application of humic acid, Nile fertile or AM fungi were significantly lower compared to the combined application of these natural soil conditioner.

The beneficial effect of these materials on yield, cluster weight and some physical properties of berries could be attributed to its vital role in lowering soil pH. Consequently, vine growth and nutritional status (tables, 2 and 3) are being improved cluster weight, increasing yield/ vine and improved the physical properties of berries.

The obtained results are in agreement with those reported by Zhu and Zhu (2000); Hussien *et al.,* (2005) and Ali *et al.,* (2006) who found that humic acid applications significantly increased berry physical properties. As for the effect of Nile fertile Ibrahiem, (2003) found that Nile fertile improved physical properties of berries. Moreover, Abd El-Wahabe *et al.,* (2008) pointed out that yeast and AM fungi gave the best physical properties of berries.

Table (4): Effect of soil condition with humic acid, nile firtile and AM fungi application on yield and some physical characteristics of berries of Black Monukka grapevines during 2007 and 2008 seasons.

Treatments	Yield/ vine (kg)		Cluster weight (g)		Berry weight (g)		Berry size (cm^3)		Shattering (%)	
	2007	2008	2007	2008	2007	2008	2007	2008	2007	2008
T1	7.91	8.36	383.7	401.4	2.03	2.34	1.89	2.28	24.67	24.95
T2	8.09	8.58	398.6	409.5	2.15	2.43	2.00	2.36	23.37	23.06
T3	8.20	8.72	403.2	415.0	2.26	2.52	2.10	2.41	22.10	21.39
T4	8.14	8.59	400.2	410.3	2.23	2.48	2.07	2.34	23.32	21.40
T5	8.24	8.71	404.6	416.1	2.25	2.50	2.10	2.40	22.06	21.39
T6	8.04	8.47	390.1	406.3	2.14	2.43	1.99	2.34	23.35	23.15
T7	8.39	8.89	409.5	423.5	2.39	2.61	2.21	2.48	20.76	19.59
T8	8.51	9.02	413.5	430.2	2.42	2.70	2.31	2.54	20.70	19.50
T9	8.64	9.13	410.0	436.1	2.44	2.65	2.34	2.59	20.72	19.55
T10	8.75	9.26	415.2	442.0	2.45	2.71	2.36	2.69	20.70	19.50
T11	8.86	9.38	417.7	446.0	2.48	2.80	2.39	2.74	20.69	19.46
T12	9.00	9.50	422.5	451.3	2.60	2.91	2.50	2.80	19.36	17.80
New L.S.D.at 5%	0.12	0.11	4.60	5.10	0.11	0.09	0.10	0.05	1.26	1.66

5- Chemical properties of berries:

Data in table (5) indicated that, there were significant differences between all treatments than control concerning the chemical properties of berries. Moreover, the application of (HA1 +NF1+ AM fungi) improved the chemical quality of berries in terms of increasing the total soluble solids, total soluble solids/ acid ratio and anthocyanin contents of berry skin and reducing the total acidity than the sole application of these materials in the two seasons of the study.

The enhancing of humic acid, Nile fertile and AM fungi on chemical properties of the berries may be ascribed to its role in achieving a good balance between growths and fruiting through nutrients availability in soil which is reflected by its turn on increasing the accumulation of total carbohydrate and resulting the stimulation of ripening. These results are in accordance with those obtained by Li-Nan *et al.,* (1999) Zhu and Zhu (2000) and Ali *et al.,* (2006) who found that humic acid improved the chemical properties of berries. Moreover, Ibrahiem (2003) found that Nile fertile enhancing the chemical properties of berries. As for the effect of AM fungi, Abd El-Wahab *et al.,* (2008) pointed out that AM fungi gave the best chemical properties of berries.

Table (5): Effect of soil condition with humic acid, nile firtile and AM fungi application on chemical characteristics of berries of Black Monukka grapevines during 2007 and 2008 seasons.

Treatments	T.S.S. (%)		Acidity (%)		T.S.S./ Acidity		Anthocyanin (mg/100g F.W.)	
	2007	2008	2007	2008	2007	2008	2007	2008
T1	16.10	15.91	0.72	0.68	22.36	23.40	35.83	37.34
T2	16.27	16.11	0.70	0.67	22.24	24.04	36.24	37.78
T3	16.42	16.31	0.68	0.66	24.15	24.71	36.55	37.90
T4	16.40	16.30	0.69	0.65	23.77	25.08	36.33	37.83
T5	16.43	16.31	0.68	0.65	24.16	25.09	36.58	37.95
T6	16.25	16.11	0.70	0.67	23.21	24.04	35.52	37.46
T7	16.59	16.52	0.65	0.64	25.52	25.81	37.0	38.13
T8	16.61	15.54	0.64	0.63	25.95	26.25	37.2	38.26
T9	16.60	16.57	0.63	0.62	26.35	27.42	37.0	38.15
T10	16.65	17.00	0.62	0.62	26.85	27.42	37.3	38.27
T11	16.70	17.00	0.62	0.62	26.94	27.42	37.5	38.37
T12	16.80	17.2	0.61	0.60	27.54	28.67	37.8	38.5
New L.S.D.at 5%	0.15	0.20	0.02	0.01	0.50	1.10	0.30	0.12

6- Microbiological studies:

Regarding to data in table (6) all treatments caused a significant increase in AM infection % in comparison to control, the best increase was caused by the application of (HA1 +NF1+ AM fungi) in the two seasons of the study, this was recorded 95.33 and 100.00 respectively. This finding indicates that AM fungi may solublize the surrounding weatherable mineral through execration of organic acids such as α-ketoglutraic acid. These organic acids could exert a selective influence on soil microbial communities (Abd El-Wahab *et al.*, 2008).

Moreover, no. of AM spores in soil was significantly increased especially in treatment (HA1 +NF1+ AM fungi) in the two seasons of the study; this was recorded 1022.2 and 1340.0 respectively. These findings are in the same line with those obtained by (Turk et al., 2006) who pointed out that AM fungi colonize plant roots and mainly in the surrounding soil extending the roots depletion zone around the root system and consequently completed its life cycle and obtained plenty of resting spores in soil.

The total microbial count was the same line of increasing especially in the application of (HA1 +NF1+ AM fungi) in the two seasons; this was recorded 81.60 and 93.61 respectively. These results are in agreement with those obtained by (Linderman and Pfleger, 1994) who explained that AM fungi is capable of increasing nutrient content which act as a suitable media for most rhizopheric microorganisms in general. On the other hand, Godeas *et al.*, (1999) explained that the increases in populations of rhizospheric microorganism in roots of most plants are influenced by a combined inculation of microorganism and AM fungi.

Also, phosphatase enzyme activity was significantly increased in all treatments especially in treatment (HA1 +NF1+ AM fungi) in the two seasons of the study, this was recorded 35.33 and 38.00 respectively. These results are in agreement with those obtained by (Hetick, 1989) who found that mycorrhizal hyphae can provide access to insoluble nutrient sources through enzyme activity or some physical or chemical modification of the rhizosphere. The inorganic phosphours compounds can first be hydrolyzed by phosphatase enzyme which mostly originates form plant roots, through the action of fungi and bacteria.

The same increase was observed in dehydrogenase enzyme activity in all treatments in comparison to control where the best increase was caused by the application of (HA1 +NF1+ AM fungi) during the two seasons, this was recorded 113.67and 133.67 respectively. The increase in dehydrogenase enzyme activity was attributed to the intense activity of microflora as a mixture of biomass (Massoud, 2005).

Table (6): Effect of soil condition with humic acid, nilefirtile and AM fungi application on AM infection %, No of AM spores , No of AM spores and Phosphatase enzyme activity and Dehydrogenase activity in rhizospheric zone of Black Monukka grapevines during 2007 and 2008 seasons.

Treatments	AM infection %		No of AM spores /g dry soil.		Total microbial countX106 CFU/gdry soil		Phosphatase enzyme activity(P /g/ dry soil)		Dehydrogenase enzyme activity (µg TPF/ day/ g dry soil).	
	2007	2008	2007	2008	2007	2008	2007	2008	2007	2008
T1	30.00	34.67	27.9	41.3	40.30	41.25	10.23	10.57	40.33	44.33
T2	46.00	51.00	30.4	103.3	44.25	44.29	11.20	12.00	44.67	50.33
T3	60.00	60.00	32.1	111.7	44.56	46.32	15.60	16.87	53.33	55.67
T4	40.00	45.33	25.0	50.3	50.89	50.27	17.27	18.67	53.67	61.67
T5	50.67	55.33	40.0	101.0	55.67	57.41	19.50	22.00	90.33	95.67
T6	70.33	75.00	560.0	640.0	45.32	57.68	24.33	26.33	88.00	93.67
T7	85.33	87.33	708.9	783.3	80.59	87.55	29.00	30.33	87.67	90.33
T8	85.67	87.67	721.1	770.0	75.24	85.42	30.33	30.33	81.00	85.33
T9	70.33	80.67	558.9	633.3	70.26	77.49	25.00	27.67	88.00	89.67
T10	75.00	81.00	613.3	691.7	73.68	80.58	30.33	31.67	95.67	107.00
T11	80.67	81.33	334.4	400.0	80.50	87.22	33.00	34.67	110.33	121.33
T12	95.33	100.00	1022.2	1340.0	81.60	93.61	35.33	38.00	113.67	133.67
New L.S.D.at 5%	8.23	11.47	194.5	219.5	6.7	9.3	5.14	8.97	4.81	5.72

7-Econimical evaluation of the recommended treatment (HA1 +NF1+ AM fungi) compared with control:

Data shown in table (7) clearly indicate that the application of (HA1 +NF1+ AM fungi) gave the maximum net profit compared with the control in both seasons. The very slight raise in the cost of production/ feddan over control for this treatment is economically justifiend in view of the higher price of the yield obtained from this treatment.

Table (7): Some economical data on costs and profit per fed of the recommended treatment (humic acid, nile firtile and AM fungi) compared to control.

Season	Price of humic acid (L.E.)	Price of nile firtile (L.E.)	Price of AM fungi	Labour cost (L.E.)	Total cost (L.E.)	Increase in yield over control per fed (kg)	Price of increase in yield over control (L.E.)	The net profit (L.E.)
2007	160	70	200	50.0	480	763.0	2098.25	1618.25
2008	160	84	-----	50.0	294	798.0	2234.4	1940.4

As a conclusion it could be concluded that, adding humic acid (HA) at 15 cm/ vine plus nile firtile (NF) at 200 g/ vine and AM fungi (AM) this recommended under the calcareous soil having high pH values to obtain the highest yield besides improving the fruit quality and improving microbiological activity.

Corresponding author
Mervat, S. Rizk-Alla
Hortcultural Research Institute
Agricultural Research Center, Giza, Egypt.

References

1. Abd El-Wahab, M. A.; Shaker, S. H. and Massoud, O. N. (2008): Rool of Arbuscular Mycorrhiza (AM) and yeast in improving quality of Black Monukka grapes. J. Agric Sci. Mansoura Univi., 33 (1): 393- 417.
2. Ahmed, F. F.; Mansour, F. M. and EL-Shamaa (1994): How much sulphur in necessary for Thompson seedless grapevines? Minia J. Agric. Res. and Dev. 16 (2): 287- 298.
3. Ali, M. A.; Rafaat, S. S. and El-Shal, S. A. (2006): the role of humic acid in reducing mineral fertilizer rates applied in vineyards. Egypt. J. of Appl. Sci. 21 (11):14- 21.
4. A.O.A.C. (1985): Association of Official Agricultural Chemist Official methods of Analysis. 12th Ed. Washington D.C., U.S.A.
5. Awad, S. M. (1999): Response of "Flame" grape transplants to microrhizal inoculation and phosphorus fertilization. Egypt. J. Hort., 26 (3): 421-433.

6. Barea, J. M. and Jeffries, P. (1995): Arbuscular mycorrhizas in sustainable soil plant system. In. A. Varma and B. Hock (Editors) Mycorriza structure, function. Moleculer Biology and Biochnology, Springer- verag Heidelber pp: 521-560.
7. Bhavaraju, S. R.; Modak, J. M.; Kumar, R. and Gandhi, K. S. (1993): Dissolution of Sulphur Particles by *Thiobacillus ferrooxidans:* Substrate for Unattached Cells. Biotechnology and Bioengineering, Vol. 41, Pp. 612-616.
8. Bouard, J (1966): Recherches physiologiques sur la vigne et en particulier pour l'acutment des sarments. Thesis Sc. Nat Bordeaux-France. Pp. 34.
9. Cahoon, G. A.; Morton, E. S.; Jones, W. W. and Garber, M. J. (1959): Effect of various types of nitrogen fertilizers on root density and distribution as related to water infiltration and fruit yield of Washington navel orange in a long term fertilizer experiment. Proc. Amer., Soc. Hort. Sci., 74: 289- 299.
10. Cheng, F. J.; Yang, D. Q. and Wu, Q. S. (1998): Physiological effects of humic acid on drought resistance of wheat. Chinese J. of Applied Ecology, 6 (4): 363- 367.
11. Clark, C. J. and Kajiura, I. (1986): Nutritional disorders in persimmon. Growing Today. April: 47-50.
12. Clark, C. J. and Smith, G. S. (1986): leaf analysis of persimmon. Growing Today February: 15- 17.
13. Colla, G.; Rouphael, Y.; Cardarelli, M.; Tullio, M.; Rivera, C. M. and Rea, E. (2008): Alleviation of salt stress by arbuscular mycorrhizal in zucchini plants grown at low and high phosphorus concentration. Biol Fertil Soils 44:501–510.
14. Drobnikova, V. (1961): Factors influencing the determination of phosphatase in soil. Folia Microbial. 6: 260- 269.
15. Edrees, M. H. (1982): Studies on growth of citrus seedlings, Ph. thesis, Fac. Agric., Al-Azhar Univ., Cairo, Egypt.
16. El Fakharani, Y. M. (1999): combined effect of P fertilization and organic manuring on barley production in sandy soils. Fayoum J. Agric. Res. and Dev., 13 (2): 81- 97.
17. El-Sharkawy, S. A. (1989): Studies on mycorrhizal responses on citrus plants. M. Sc. thesis, Fac. Agric., Zagazig Univ., Zagazig, Egypt.
18. El-Shenawy, I. E. and Fayed, T. A. (2005): Evaluation of the conventional to organic and bio-fertilizers on 'Crimson Seedless" grapevines in comparison with chemical fertilizers. I- Vegetative growth and nutritional state. Egypt. J. Appl. Sci., 20 (1): 192- 211.
19. Esher, H. and Jensen, V. (1972): Aerobic chemo-organotrophic bacteria of Danish beech forest, OIKOS, 23: 248-260.
20. Fataftah, A. K.; Watia, D. S.; Ains, B. G. and Kotob, S. I. (2001): Comparative evaluation of known liquid humic analysis method. Arctech Inc., Chantilly, V. A. 20165, U.S.A.
21. Graham, J. H.; Syvertsen, J. P. and Smith, M. L. (1987): Water relations of mycorrhizal and phosphorous-fertilized on citrus under droght stress. New Phytologists, 105 (3): 411- 419. Harry, A.; Fred, J. and Elam, P. (1991): Growing quality table grapes in the home garden. University California pp: 1-30.
22. Godeas, A.; Fracchia, S.; Mujica, M. T. and Ocampo, J. A. (1999): influence of soil improverishment on the interaction between Glomus mosseae and saprobe fungi. Mycorrhiza. 9: 185- 189.
23. Harry, A.; Fred, J. and Elam, P. (1991): Growing quality table grapes in the home garden. University California pp: 1-30.
24. Hartwigsew, L. M. and Evans, C. L. (2000): Effect of humic acids on growth and biomass of olive plants. Acta Hort., 453: 75- 80.
25. Hetrick, B. A. D. (1989): Acquisition of phosphors by VA mycorrhizal fungi and the growth responses of their host plants. pp: 205-226. In: L. Boddy, R. Marchant, and D. J. Reid (eds.), Nitrogen, Phosphorus, and Sulphur utilization by fungi. Cambridge University Press, New York, NY, USA.
26. Hussien, A. M.; El Maghraby, T. A.; Sherif, H. M. and El Shal S. A. (2005): Effectof liquid organic fertilization techniques on yield and chemical composition of pear and apricot trees grown in sandy soils at South Tahrir province. Fayoum J. Agric. Res. And Dev., 19 (2): 224-238.
27. Ibrahiem, Alia H. (2003) Effect of sulphur sources the productive efficiency of flame seedless grapevines grown under sandy siol and drip irrigation conditions. Egypt. J. Appl. Sci., 18: 1-4.
28. Jackson, M. L. (1973): Soil chemical analysis. Constable and Company Ltd. London, 175-280.
29. Kassem, A. A.; Kassem, H. A.; Kamal , H. M. and Shalaby, M. M. (1995): the influence of nitrogen fertilizer and/or suluphur application on guava trees grown in alkaline sandy soil. J. Agric. Res., 20 (3): 1203- 1222.
30. Li-Nan, X.; Wang, B. L. and Lu, B. W. (1999): Study of the effect of apple liquid fertilizer on the growth and fruit development of Starkrinson apple variety. China- Fruits, 4: 20-21.

31. Lindermann, R. G. (1988): Mycorrhizal interaction with the rhizosphere microflora: the mycorrhizosphere effect. Phytopathology 78:366–371.
32. Linderman, R. G. and Pfleger, F. L. (1994): General summary, p. 337- 344.In F.L. Pfleger and R. G Linderman,
33. Marin, M.; Mari, A.; Ibrarra, M. and Garacia-Ferriz, L. (2003): Arbuscular mycorrhizal inoculation of micropropagated persimmon plants. J. Hort. Sci. and Biotechnology, 78 (5): 734- 738.
34. Massoud, O. N. (2005): Microbiological and chemical evaluation of compost and its application in organic farming. Ph. D. thesis, department of botany, faculty of sience, El-Menoufia, University.
35. Mona, G. S. (2001): Response of banana and guava plants to some biological and mineral fertilizers. M. Sc. Thesis, Fac. Agric Alexandria Univ. Egypt.
36. Morales-Payar, J. P. and Stall, W. M. (2003): Effect of substrates, boron and humic acid on the growth of papaya transplants. Proc. Fla. State. Hort. Soc. 116: 28-30.
37. Omar, A. L. (2005): Fertilization of Thompson seedless vines with mineral and organic sources of nitrogen. J. Agric. Sci. Mansoura Univ., 30 (12): 7855- 7862.
38. Rizk-Alla, M. S.; Abd El-Wahab, M. A. and Ghobrial G. F. (2006): Alleviating the harmful effects of irrigation water salinity on Thompson seedless grape rootings through the application of nile fertile as a natural soil conditioner. Egypt. J. of Appli. Sci., 21 (6): 25- 31.
39. Rhodes, L. H. and Gerdemann, J. W. (1975): Phosphate uptake zones of mycorrhizal and non-mycorrhizal onion. New phytol. 75: 555-561.
40. Salman, A. C. (1967): Uber die mikrobiello aktivitat and ihr Beziehung Zu Fruchtbartkeits merknmalen. Einiger acherboden under besonderer Berucksichtigurg der dehydrogenase akativitat (TTC-Reduktion). Diss Giesen, ph.H D. Thesis, W. Germany.
41. Smith, F.; Gilles, M. A.; Hamilton, J. K. and Gedess, P. A. (1956): Coloimetric methods for determination of sugar and related substan. Anal. Chem. 28: 350- 356.
42. Snedecor, G. W. and Cochran (1980): Statistical methods. 7th Ed.IOWA State Univ. Press, U. S. A.
43. Tourky, M. N.; El-Shahat, S. S. and Rizk, M. H. (1995): Effect of Dormex on fruit set, quality and storage life of Thompson seedless grapes (Banati grapes). J. Agric. Sci., Mansoura Univ., 20 (12): 5139- 5151.
44. Turk, M. A.; Assaf, T. A.; Hameed, R. M. and Al-Tawha, A. M. (2006): Significance of myccrohizae. World journal of agricultural sciences. 2 (1): 16-20.
45. Vitagliano, C.; Citrnesh, A. S.; Metzidakis, I. T. and Voyiatzis, D. G. (1999): Plant growth of *Olea eupaea* L. as influenced by arbusclar Mycorrhizal fungi. Proceddings of the 3rd international symposium on olive growing. Chania, Crete Greece, 22-26 September 1997, Volume 2, Acta Horticulture. No 474: 357- 361.
46. Wood, C. W.; Reeves, D. W. and Himelrick, D. G. (1992); Relationships between chlorophyll meter readings and leaf chlorophyll concentration. N status and crop yield. A review: Proc. Agro. Sco. N. Z. 23:1-9.
47. Yilidz, F. and Dikman, D. (1990): the extraction of anthocynin from blak grapes and black grape skins. Doga Derigisi, 14 (1): 57-66.
48. Zarb, J., Leifert, C. and Litterick (1999): opportunities and challenges for the use of microbial ineculants in agriculture practice. Proceedings of the sixth international Confernce on Kyusei Nature Farming. 28- 31. October . Pretoria, South, Afric.
49. Zhang, L.; Zhang, J.; Christie P. and Li, X. (2008): Pre-inoculation with Arbuscular mycorrhizal fungi suppresses root knot nematode (*Meloidogyne incognita*) on cucumber (*Cucumis sativus*). Biol Fertil Soils, 45:205–211.
50. Zhu, R. F. and Zhu, R. E. (2000): Effect of (Opal) HA-2002 liquid fertilizers on the growth and fruiting of pears trees. South China Fruits. 29 (2): 43- 47.

6/23/2010

Microbial Bio-Fertilization Approaches to Improve Yield and Quality of Washington Navel Orange and Reducing the Survival of Nematode in the Soil

Abdelaal Shamseldin[1]; Mohamed H. El-Sheikh[2]; H. S. A. Hassan[*3] and Kabeil S.S.[4]
[1]Environmental Biotechnology Department, [2]Horticulture Department at Arid lands Cultivation and Development Institute (ARADI); Mubarak City for Scientific Research and Technology applications, New Borg-Elarab, Alexandria, Egypt, [*3] Pomolgy Department, National Research center, Cairo, Dokki-Egypt. [4]Protien Research Department at Genetic Engineering and Biotechnology Research Institute (GEBRI)
Hsasm2000@yahoo.com

Abstract: To test the ability of microbial strains *Pseudomonas flourescence* strain 843 and *Azospirillum brasilense* strain W24 to improve Washington navel orange fruit quality and to control the persistence of nematode in the soil, strains were applied one time monthly during the period of experiment to trees at two levels 300 ml and 500 ml per tree with 10^{-8} cells ml^{-1}. Bio-fertilizer inoculation with strain *Pseudomonas fluorescence* strain 843 growth promoting rhizobacteria was significantly improve fruit quality as well as increased fruit yield, fruit weight, fruit length, TSS and juice volumes, while inoculation with strain *Azospirillum brasilence* strain W24 increase but not significantly improve fruit quantity and quality of Washington navel orange. Commonly, three types of nematode were detected in the roots including *Tylenchulus Spp*, saprophytic nematode and *Pratylenchulus* while the dominant species was *Tylenchulus semipenetrans*. Generally there is a reduction in the number of nematode with the two examined strains while the addition of *Pseudomonas f.* strain 843 was successfully greater to inhibit the growth of nematode than *Azospirillum b.* strain W24 suggesting that this strain can be use as a bio-fertilizer for promoting citrus growth and bio-control for reducing the distribution and propagation of nematode associated with citrus. Enhancement and maintenance of soil fertility and conservation of the soil's health through bio-fertilizer applications will be a vital role and occupy significant concern for many of researcher in the future as a unique key for sustainable agriculture in developing countries. [Journal of American Science. 2010;6(12):264-271]. (ISSN: 1545-1003).

Key words: Citrus, Bio-fertilizers, *Azospirillum brasilence*, *Pseudomonas fluorescence*, *Tylenchulus semipenetrans* and biological control.

1. Introduction

Citrus (*Citrus spp.*) is one of the most important fruit crops grown in many tropical and subtropical countries. At the moment there is a bout 1.5 million hectares of citrus fruits cultivated for commercial scale in the world yielded nearly 40 million metric tons of oranges, lemons, limes, etc (Anonymous, 2008).

In Egypt, citrus has great attention due to its importance for local consumption or as a main source for foreign currencies by exportation to the European country. The area of citrus cultivated in Egypt was increased rapidly with the reclamation of new desert lands reaches about 35.59 hectare (Anonymous, 2008).

According to the annual report of the ministry of Agriculture in Egypt (2004), the production rate of citrus is mainly low as affected by different factors such as the nitrogen fertilization and soil born diseases like nematode. Nitrogen fertilizer is playing an important role for producing orange fruits due to its accumulative harmful as nitrate or nitrite in fruit juice (Montasser *et al.,* 2003). Thus is giving a special importance for optimizing the nitrogen requirements of citrus through different strategies. One of those strategies is the use of bio-fertilizers. The use of bio-fertilizers in enhancing plant growth and yield has gained momentum in recent years because of higher cost and hazardous effects of chemical fertilizers. Organic and/or bio-fertilizers improved vegetative growth, nutritional status and reduced the residuals of nitrate and nitrite in banana and grape fruits (Gomaa, 1995). Farag, (2006) and Saleh and Ahmed (1988) they noted that organic fertilizers and humic acid significantly decreased nitrogen as nitrate and nitrite content and improved yield and fruit quality of treated vines. Aseri *et al* (2008) found that the use of bio-fertilizers gave a significant improvement of fruits of pomegranate in India as well as enhancing the rhizosphere microbial activity and concentration of various nutrients. Ashokan *et al* (2000) reported a significant enhancement of banana plants as a result of dual inoculation of Arbscular mycorrhizal fungi (AMF) and Azotobacter.

Plant diseases caused by soil borne plant pathogens such as nematode (Duncan, 2005; Abd-

Elgawad *et al*., 2010) are considered major problems in agricultural production throughout the world, reducing yield and quality of crops. The nematode of the citric fruits probably infests more than 50% of the citrus production areas. The losses attributable to this nematode are estimated in the entire world at approximately 10% (Duncan, 2005 and Duncan *et al.*, 1995). There are several species of nematode that known to attack citrus including *Tylenchulus semipenetrans, Belonolaimus longicaudatus, Pratylenchus coffeae* and *P. brachyurus* (Duncan, 2005; Duncan *et al.*, 1995). Controlling of nematode diseases on citrus depends mainly on chemical applications mean while, these chemical substances are always undesirable due to their high cost and their hazard potentials to the environment. Therefore the view of scientists are directed to use bio-control instead of the chemical forms of fungicides or mineral fertilizers that can be a beneficial way to control soil borne plant pathogens and produce the natural clear fruits free from mineral residues (Russo and Berlyn 1990). In this respect, application of bio-fertilizers for controlling soil borne pathogenic fungi has been applied to several plants (Aaltan *et al*., 2003 and Siddiqui *et al*., 2009).

Consequently, the present investigation was outlined to study the effect of inoculation of citrus trees with *Azospirillum brasilense* and *Pseudomonas fluorescence* as growth promoting substances and bio-control on the yield and quantity of Washington navel orange as well as inhibiting the survival of nematode in the soil which causes considerable losses in citrus production in Egypt.

2. Materials and Methods:

2.1 Bacterial strains and growth

Strains of *Azospirillum brasilense* W24 and *Pseudomonas fluorescence* 843 DZM were kindly dedicated by D. Werner, Philipps University of Marburg, Germany. Strains of *Azospirillum* were grown on Doberiner medium but strains of *Pseudomonas* were grown on nutrient broth medium. Strains were grown in liquid medium on a rotary shaker at 30 °C and 120 rpm and the optical density of culture adjusted to 1 at 550 nm, then the culture were added to the trees once per month at a rate of 300 ml or 500 ml per tree.

2.2 Field Experiment:

Experiments were conducted in a silty clay soil in the middle of Delta Nile Valley characterized by high pH (8.9) and salinity level (3.28 mmohs cm^{-1}). The study was carried out in a private orchard at Al–Menoufia Governorate during 2008 and 2009 season on 10 years old Washington Navel orange trees, budded on sour orange (*C. ourantium* L.) rootstock, spaced at 5x5m. Completely block randomize design with four replicates was used for each treatment.

2.3 Collection of samples:

During spring and summer of 2008 and 2009 planted sites representing different habitat types in reclaimed and old parts of El-Menoufia governorate surveyed for the presence of entomopathogenic nematodes (EPN) using a design similar to Mrácek and Webster (1993) samples were taken with a hand trowel to a 20 cm depth and a volume of 1000 cc (cubic mete) per soil sample was collected. Samples were transported to the laboratory in sealed and labeled polyethylene bags within an ice chest.

2.4 Extraction of nematode from the soil and microscopic examination

250 g of the collected soil washed with tape water to remove the residues of roots then precipitated. The precipitate sieved through sieve of 325 mesh three times, then the water removed and the suspension containing nematode transferred to 50 ml beaker. The beaker agitated well then left for one minute for precipitating and 1 ml taken from the suspension and accounted using the 10x lenses of light microscopy and the number of nematode population accounted using this equation.

Number of nematode 250 ml= number of cells per ml x volume of the suspension

2.5 Determination of (N; P and K):

Methods for analyses and determination of (N; P and K):

To determine leaf mineral content, forty leaves were collected in the late of August in each season from tagged non-fruiting and non-flushing spring growth cycle (Jones and Embleton, 1960). Leaf samples were washed with tap water then distilled water several times, dried at 70°C, grinded and digested with percholoric acid to estimate N, P and K contents as percent refers to dry weight according the methods described by Cottenie *et al*. (1982).

2.6 Determination of horticulture aspects:

Fruit quality

At maturity stage, 10 representative fruits were taken from each tree and both the physical and chemical characteristics were determined.

Physical characteristics

Average fruit weight (g) fruit volume (cm3), fruit peel thickness (cm) and fruit firmness (by means Manges Taglor Pressure Tester) were measured. The

fruit length and diameter (cm) were measured by a vernier calliper and the fruit shape index (length/diameter ratio) was calculated.

Rate of fruit weight increase = Fruit weight of inoculated plant- fruit weight of un-inoculated plant÷fruit weight of uninoculated plantx100.

Reduction rate of nematode= Population of nematode in inoculated plant-population of nematode in un-inoculated plant ÷population of nematode in uinoculatedx100.

Chemical characteristics

Juice volume, total soluble solids, acidity content and vitamin C (mg/100 ml juice) were calculated adopting the standard procedures (A. O. A. C., 1990). The total soluble solids (TSS/acid ratio) for each sample were estimated.

Statistical Analysis:

The means and standard deviations of four replicates were estimated and an analysis of variance was carried out using the ANOVA procedure with SPSS software while the comparison of mean effects was based on least significant difference (LSD) multiple-comparison tests. Significant differences were considered at $P < 0.05$.

3. Results and Discussion:

3.1 Effect of bio-fertilizer inoculation on quantity and quality of Washington navel orange fruits

Production of horticultural crops has taken significant increase in the last decade due to development of innovative technologies including integrated nutrient management practices involving bio-fertilizers aiming to reduce the cost of agricultural process and the reduction of environmental pollution that happened due to the extravagant of fertilizers use. These bio-fertilizers include different kinds of microorganisms such as phosphate-solubilizing bacteria (PSBs), symbiotic (*Rhizobium*; *Bradyrhizobium* etc) and non-symbiotic (*Pseudomonas*; *Azospirillum* and *Azotobacter*) N_2-fixing bacteria and arbuscular mycorrhizal fungi (AMF). AMF and *Azospirillum* were found to enhance the growth and production of various vegetable crops (Ghazi, 2006 and Paramaguru *et al*., 1993) while *Azospirillum* and *Azotobacter* were found to increase significantly the production of some fruit plants such as banana and sweet orange (Jeeva *et al*., 1988; Tiwary et al., 1999 and Singh and Sharma, 1993), Besides improving the microbiological activity in the rhizosphere (Kohler *et al*., 2007). Therefore we aimed in our research to study the effect of biofertilizer application on the production of Washington navel orange tree to encourage the clean or green agriculture in order to help small farmers in developing countries such as Egypt to depend on the biofertilizer as a main source to increase the soil fertility in their farming system and to confront the over increase in synthetic fertilizer prizes. Results in Table (1) show the effect of inoculation with bio-fertilizers on fruit characteristics including yield, fruit weight, fruit volume and fruit length. It is clearly shown that inoculation with 500 ml $tree^{-1}$ of strain *Pseudomonas fluorescens* 843 resulted in significant increase of the number of fruit $tree^{-1}$ in the two seasons as well as increasing the yield. The increasing rate in the number of fruits $tree^{-1}$ was 12.3% (231.48-206.18÷206.18x100) over the un-inoculated trees (control) in the first season while the increasing rate was 8.7% in the second season. The fruit weight also increased with the inoculation of this strain by a rate of 49.6% where the increase of fruit weight was 42.9% in the second season. Our results are not only in agreement with those obtained by Abd El-Migeed *et al* (2007) who reported that inoculation of Washington navel orange trees with *Azospirillum lipoferum* as a source of bio-fertilizer improved average fruit weight, and also agree with those published by Aseri *et al*. (2008) who noted that combined inoculation with *Azotobacter chroococcum* and *Glomus mosseae* (AMF) increased the microbial activity in the rhizosphere and yield of Pomegranate.

Bio-fertilizer inoculation with strain of *Pseudomonas fluorescens* had enhanced fruit weight, fruit volume and fruit length by a rate of (33.25% and 31.6%), (4.1% and 9.9%) and (6.2% and 11.7%) for the first and second season on respectively. However non significant results were not observed in fruit diameter and fruit shape index except in fruit diameter in the first season (2008). Results in Table 2 summarize the effect of microbial inoculation on the total soluble salts, acidity, juice volume and vitamin C. As noted from the previous results that strain *Pseudomonas fluorescens* was superior for promoting the TSS, juice volumes in the whole duration of experiment while this strain promoted significantly vitamin C in the second season only. There is no significant results were observed as a result of microbial biofertilization on the fruit shape index (Table 1) and peel thickness (Table 2) as reported by Bassal (2009) who stated that the use of rootstocks were not significantly increase of both the peel thickness and fruit shape. The positive effect of inoculation with strain *Pseudomonas fluorescens* on the quality and quantity of Navel orange may be due synthesis of phytohormones (Xie *et al*., 1986), reduction of membrane potentials of the roots (Bashan and Levanony 1991), synthesis of some enzymes that modulate the level of plant hormones (Glick *et al*. 1998) and solubilizing of inorganic

phosphate (Krasilnikov, 1961). On the other hand, strain *Azospirillum brasilense* was less effective than *Pseudomonas* strain for increasing the fruit quantity (Table 1) and fruit quality (Table 2) although this strain was repeatedly reported as a PGBR in many different publications. The little effect of *Azospirillum brasilense* strain was reported by *Burdman et al. (1997)* who found that high titer (10^{-8} cfu ml^{-1}) like in our case of this strain reduced the shoot and root weight of common bean when it is dual inoculated with *Rhizobium* and *Azospirillum* strain. Perotti and Pidello (1999) stated that the inoculation with *Azospirillum brasilense* strain reduces the activity of urease in the soil and this led to decrease the available nitrogen.

Table (1) Effect of microbial biofertilizers On yield, fruit weight ,fruit volume, fruit length ,fruit diameter and fruit shape index of Washington navel orange trees

Measurement / Treatment	yield				fruit weigh (gm)		fruit volume (cm3)		fruit length (cm)		fruit diameter (cm)		fruit shape index	
	No. of fruit / tree		fruit weigh/tree (kg)		2008	2009	2008	2009	2008	2009	2008	2009	2008	2009
	2008	2009	2008	2009										
Control	206.18	250.30	30.83	36.11	149.50	144.20	208.15	192.63	7.15	6.82	7.08	6.86	1.01	0.99
Azospirillium b. strain W24 (300 ml/tree)	193.05	242.11	28.36	37.40	146.90	154.47	205.47	231.61	7.26	7.85	7.19	7.59	1.01	1.03
Azospirillium b. strain W24 (500 ml/tree)	201.59	238.61	31.10	40.13	154.27	168.18	196.02	200.30	7.02	7.30	7.00	7.22	1.00	1.01
Pseudomonas f. 843 (300 ml/ tree)	210.08	220.43	37.62	43.10	179.07	195.52	215.60	219.08	7.50	7.66	7.35	7.49	1.02	1.02
Pseudomonas f. strain 843 (500 ml tree)	231.48	272.05	46.12	51.62	199.23	189.74	217.10	211.83	7.59	7.73	7.41	7.58	1.02	1.02
L.S.D. at 0.05	18.64	15.18	6.35	8.90	17.03	14.65	8.15	11.40	0.23	0.18	0.09	N.S	N.S	N.S

Azospirillium b.: Azospirillium brasilensce; Pseudomonas f.: Pseudomonas fluorescens strains were dedicated from laboratory of Werner, Philipps University of Marburg, Germany.

Table (2) Effect of microbial biofertilizers on fruit quality of Washington navel orange trees.

Measurement / Treatmen	Peel thickness		TSS %		Acidity		TSS/ Acid %		Juice Volume		Vitamin C (mg /100 ml juice)	
	2008	2009	2008	2009	2008	2009	2008	2009	2008	2009	2008	2009
Control	0.39	0.40	12.80	12.50	1.16	1.19	11.03	10.50	43.20	42.91	41.63	43.08
Azospirillium b. strain W24 (300 ml/tree)	0.38	0.41	12.34	12.59	1.23	1.15	10.03	10.94	43.00	43.58	40.15	43.27
Azospirillium b. strain W24 (500 ml/tree)	0.38	0.40	12.08	12.62	1.30	1.06	9.29	11.90	44.25	45.30	42.60	45.40
Pseudomonas f. 843 (300 ml/ tree)	0.41	0.42	12.87	12.53	1.11	1.10	11.59	11.39	46.18	47.11	42.82	42.85
Pseudomonas f. strain 843 (500 ml tree)	0.42	0.43	12.90	13.02	1.13	1.08	11.41	12.05	46.93	47.15	42.56	46.11
L.S.D. at 0.05	N.S	N.S	0.41	0.32	0.09	N.S	0.93	0.78	1.20	1.53	N.S	1.83

3.2 Effect of bio-fertilizer inoculation on NPK leaf contents of Washington navel orange

Results presented in Table (3) explain the effect of microbial bio-fertilizer on percentage of macro-elements (NPK) in leaves. Both the two examined strains gave significant increase in nitrogen percent, by a rate of (13.7% mean) with strain W24 while this rate was (6.8% mean) with strain 843 in the first year. In the second year the increasing rate was 39.6% and 26.7% with the examined strains on respectively. Strain of *Azospirillum brasillence* gave high nitrogen percent than the pseudomonas strain because this strain is a free living nitrogen fixing bacteria (Perotti, and Pidello, 1999). The P and K contents were also increased due to the application of bio-fertilizer with the two examined strains compared by control.

3.3 Effect of bio-fertilizer inoculation on the reduction of nematode associated with Washington navel orange

Plant-parasitic nematodes cause serious crop losses worldwide and are among the most important agricultural pests (Koenning *et al*., 1999). The management of nematodes is more difficult than that of other pests because nematodes mostly inhabit the soil and usually attack the underground parts of the plants (Stirling, 1991). Although chemical nematocides are effective, easy to apply, and show rapid effects, they have begun to disappear from the market in some developed countries owing to concerns about public health and environmental safety (Schneider *et al*., 2003). The search for novel, environmentally friendly alternatives with which to manage plant-parasitic nematode populations has therefore become increasingly important. The nematode of the citric fruits probably infests more than 50% of the citrus production areas leading to a rate of 10% losses approximately in the entire world (Duncan, 2005 and Duncan *et al.*, 1995). Consequently, one of our main targets was to test the possibility of these strains to reduce citrus infected nematode in the field. Results in Table (4) revealed that there were number of nematode types detected in the roots of Navel orange including *Tylenchulus Spp*, saprophytic nematode and *Tylenchulus semipenetrans* while the dominant species was *Tylenchulus semipenetrans*, therefore results show the reduction in population numbers of *Tylenchulus semipenetrans* nematode as a result of bio-fertilizer inoculation. Generally, the number of infected roots with nematode was increased at low temperature in the winter while it reduced in the summer months due to hot weather. The inoculation with *Pseudomonas fluorescens* strain 843 (500 ml tree^{-1}) did significantly reduce the number of infected roots with nematode after two months by a rate of 56.6% than the control while the reduction rate was 45.5% with the inoculation doze (300 ml tree $^{-1}$). The retardation of the beneficial effect of this strain to reduce the nematode may be due to the time that needed until the strain adapted and multiply in the soil. Several investigators (Shanmugan *et al*. 2002; Aalten *et al*., 2003; Siddiqui *et al*., 2009) have been used strains belong to this species as a bio-control for large numbers of soil borne diseases including nematode. The reduction in nematode infections due to the inoculation with *Pseudomonas fluorescens* strain 843 may be due to decreasing or preventing the deleterious effect of pathogenic microorganisms by produce antibiotics (Sivan and Chet 1992; Mel'nikova *et al*. 2002) or siderophores (Leong 1986), different species of *Pseudomonas* produce *N*-acylhomoserine lactones which is involved in the cell-density dependent control of secondary metabolite and virulence gene expression (Lau *et al*., 2000) or by regulating nematode behavior (Sikora and Hoffmann-Hergarten, 1993), interfering with plant–nematode recognition (Oostendorp and Sikora, 1990), competing for essential nutrients (Oostendorp and Sikora, 1990), promoting plant growth (El-Nagdi and Youssef, 2004), inducing systemic resistance (Hasky-Günther *et al*., 1998) and directly antagonizing by producing of toxins, enzymes and other metabolic products (Siddiqui and Mahmood, 1999). Other authors were reported to produce hydrogen cyanid that kill the eggs of nematode (Aalten *et al*, 2003) or protease enzyme that responsible for inhibiting cell walls (Siddiqui et al, 2005). The work in the future will focus on studying the mode of action of *Pseudomonas fluorescens* strain 843 that helps it to enhance the growth of fruits or decreasing the number of nematode in the soil. This is the first field trial that give promising results for reducing the nematode so that many numbers of farmers ask us to supply them with strains but we need to do the experiments in other area like the north and south of Delta to evaluate this strains correctly before going to the large scale of field application to increase the confidence between researchers and farmers that was missing in the past.

4. Conclusion:

Under Egyptian soil conditions the inoculation of Washington navel orange with *Pseudomonas flourescence* strain 843 was not only highly effective to increase the production of Washington navel orange as well as improving the quality of fruits but also inhibited the survival of nematode in the soil concluding that this strain can be used as bio-fertilizer and bio-control of pathogenic nematode infected citrus trees.

Table (3) Nitrogen, Phosphrus and potassium contents of orange leaves at the end of experiment

First season 2008			
	NPK		
Treatments	**%N**	**%P**	**%K**
Control	1.17	0.11	1.27
Azospirillium b. strain W24 (300 ml/tree)	1.35	0.17	1.45
Azospirillium b. strain W24 (400 ml/tree)	1.30	0.16	1.49
Pseudomonas f. strain 843 (300 ml/tree)	1.27	0.13	1.35
Pseudomonas f. strain 843 (500 ml tree)	1.23	0.15	1.40
L. S. D. 0.05	**0.08**	**0.02**	**0.11**
Second season 2009			
Treatment			
	NPK		
	%N	**%P**	**%K**
Control	1.01	0.13	1.05
Azospirillium b. strain W24 (300 ml/tree)	1.40	0.15	1.50
Azospirillium b. strain W24 (500 ml/tree)	1.42	0.17	1.46
Pseudomonas f. 843 (300 ml/ tree)	1.26	0.13	1.38
Pseudomonas f. 843 (500 ml/ tree)	1.30	0.17	1.33
L.S.D 0.05	**0.12**	**0.03**	**0.24**

Table (4) Number of nematode cells in the soil of experiment for four months

First season					
	Time of sampling				
Treatments	**15/1/2008**	**15/2/2008**	**15/3/2008**	**15/4/2008**	**15/5/2008**
Control	55.3	66.3	78.3	25	35.3
Azospirillium b. strain W24 (200 ml/tree)	67.3	65.7	61.7	25.3	39.3
Azospirillium b. strain W24 (300 ml/tree)	56.7	70.3	65.3	19.3	38
Pseudomonas f. strain 843 (200 ml/tree)	57.6	63.0	42.7	19	16
Pseudomonas f. strain 843 (300 ml tree)	61.7	58.0	34.0	19.7	13.3
L. S. D. 0.05	**N. S.**	**N. S.**	**40**	**4.6**	**9**
Second season					
Treatment	**15/1/2009**	**15/2/2009**	**15/3/2009**	**15/4/2009**	**15/5/2009**
Control	64.0	59.5	56.0	24.3	40.0
Azospirillium b. strain W24 (200 ml/tree)	60.7	67.7	61.7	19.0	30.7
Azospirillium b. strain W24 (300 ml/tree)	55.3	52.3	48.7	12.3	36.3
Pseudomonas f. 843 (200 ml/ tree)	45.0	32.0	26.0	16.3	12.3
Pseudomonas f. 843 (300 ml/ tree)	38.0	29.3	19.7	13.7	8.0
L.S.D 0.05	**15.2**	**24**	**23**	**N. S.**	**18**

Corresponding author
H. S. A. Hassan
Pomolgy Department, National Research center, Cairo, Dokki-Egypt.
Hsans2000@yahoo.com

Acknowledgments
Potentials of D. Werner, at Cell Biology and Applied Botany, Philipps University of Marburg, Germany to support us with standard strains as well as efforts of Nema Fattouah to account nematode population are gratefully acknowledged.

5. References:

1. AOAC. 1990. Official methods of analysis. The association of official analytical chemists. 15th Ed Arlington, West Virginia, USA. Washington D. C.
2. Aalten, P. M., Vitour, D., Blanvillain, D., Gowen, S. R., Sutra L., 2003. Effect of rhizosphere fluorescent *Pseudomonas* strains on plant-parasitic nematodes *Radopholus similis* and *Meloidogyne* spp. Letters in Applied Microbiol. 27: 357-361.
3. Abd El-Migeed, M. M. M., Saleh, M. M. S., Mostafa, E. A. M., 2007. The beneficial effect of

minimizing mineral nitrogen fertilization on Washinton navel orange trees by using organic and biofertilizers. World Journal of Agric. Sci 3: 80-85.
4. Abd-El Gawad, M. M. M., McSorely, R., 2010. Movement of citrus nematode-infested material onto virgin land: detection, current status and solutions with cost-benefit analysis for Egypt. In press.
5. Anonymous 2008. Yearbook of statistics of Ministry of Agriculture. (Agricultural Economical and Statistical Department, Arab Republic of Egypt: Cairo) [In Arabic]**.**
6. Aseri, G. K., Jain, N., Panwar, J., Rao, A.V., Meghwal, P. R., 2008. Bio-fertilizers improve plant growth, fruit yield, nutrition, metabolism and rhizosphere enzyme activities of Pomegranate (*Punica granatum* L.) in Indian Thar Desert. Scientia Horticulturae 117: 130-135.
7. Ashokan, R., Sukhada, M., Lalitha, A., 2000. Bio-fertilizers and bio-pesticides for horticultural crops. Indian Hortic. 45: 44–47.
8. Bashan Y, Levanony, H., 1991. Alterations in membrane potential and in proton efflux in plant roots induced by *Azospirillum brasilense*. Plant Soil 137:99–103.
9. Bassal, M. A., 2009. Growth, yield and fruit quality of 'Marisol' Clementine grown on four rootstocks in Egypt. Scientia Horticulturae 119:132–137.
10. Burdman, S., Kigel, J., Okan, Y., 1997. Effects of *Azospirillum brasilense* on nodulation and growth of common bean (*Phaseolus vulgaris* L.). Soil Biology and Biochem. 29: 923-929.
11. Cottenie A,, Verloo, L., Kiekens, G., Camerlynuck, R., 1982. Chemical analysis of plant and soil. Laboratory of analytical and Agrochemistry State University Belgium.
12. Duncan, L.W., 2005. Nematode parasites of citrus. In: Luc, M. Sikora, R. A. & Bridge, J. (Eds.). Plant Parasitic Nematodes in Subtropical and tropical Agriculture, 2th edition: Wallingford, U.K. CABI Publishing, pp. 593-607.
13. Duncan, L.W., Mashela, P., Ferguson, J., Graham, J., Abou-Setta, M. M., Morshedy, M.M., 1995. Estimation crop loss in orchards with pathches of mature citrus trees infected by *Tylenchulus semipenetrans*. Nematropica 25: 43-51.
14. El-Nagdi W. M. A., Youssef, M. M. A., 2004. Soaking faba bean seed in some bio-agent as prophylactic treatment for controlling *Meloidogyne incognita* root-knot nematode infection. J. Pest Sci. 77: 75–78.
15. Farag, S. G., 2006. Minimizing mineral fertilizers in grapevine farms M. Sc. Thesis institute of environmental studies and research, Ain Shams University, Egypt.
16. Ghazi, N. A. K., 2006. Nursery inoculation of tomato with arbuscular mycorrhizal fungi and subsequent performance under irrigation with sterile water. Sci. Hortic. 109, 1–7.
17. Glick, B. R., Penrose, D. M., Li, J., 1998. A model for the lowering of plant ethylene concentrations by plant growth promoting bacteria. J. Theor. Biol. 190:63–68.
18. Gomaa, A. M. H., 1995. Response of certain vegetable crop to bio-fertilizers. Ph. D. thesis. Fac. of Agric Cairo. Univ. Egypt.
19. Hasky-Günther, K., Hoffmann-Hergarten, S., Sikora, R. A. 1998. Resistance against the potato cyst nematode Globodera pallida systemically induced by the rhizobacteria *Agrobacterium radiobacter* (G12) and *Bacillus sphaericus* (B43). Fund Appl. Nematol. 21: 511–517.
20. Jeeva, S., Kulasekaran, M., Shanmugavelu, K. G., Oblisami, G., 1988. Effect of Azospirillum on growth and development of banana cv. poovan (AAB). South Indian Hortic. 36, 1–4.
21. Jones, W. W., Embleton, T. W., 1960. Leaf analysis nitrogen control programs for oranges. Calif. Citrogr 45: 394-353.
22. Koenning, S. R., Overstreet, C., Noling, J. W., Donald, P. A., Becker, J. O., Fortnum, B. A. 1999. Survey of crop losses in response to phytoparasitic nematodes in the United States for 1994. J. Nematol 31: 587–618.
23. Kohler, J., Caravaca, F., Carrasco, L., Rolden, A., 2007. Interactions between a plant growth-promoting rhizobacterium, an AM fungus and phosphate-solublizing fungus in the rhizosphere of *Lactuca sativa*. Appl. Soil Ecol. 35: 480-487.
24. Krasilnikove, N., 1961. On the role of soil bacteria in plant nutrition. The Journal of General and Appl.Microbiol. 7: 128-144.
25. Laue, B. E.; Jiang, Y.; Chhabra, S. R.; Jacob, S.; Stewart, G. S. A. B., Hardman, A., Downie, J. A., O'Gara, F., Williams, P., 2000. The bio-control strain *Pseudomonas fluorescens* F 113 produces the *Rhizobium* small bacteriocin N- (3-hydroxyl-7- cis tetradecenoyl) homoserine lactone, via HDtS, a putative novel N-acylhomoserine lactone synthase. Microbiology 146: 2469-2480.
26. Leong, J., 1986. Siderophores: their biochemistry and possible role in the biocontrol of plant pathogens. Annu. Rev. Phytopathol 24: 187-208.
27. Mel'nikova, N.N., Bulavenko, L.V., Kurdish, I.K., Titova, L.V. and Ya, K. S., 2002. Formation and function of the legume–*Rhizobium* symbiosis

of Soybean plants while introducing bacterial strains from the Genera *Azotobacter* and *Bacillus*. Appl. Biochm. Microbiol. 38: 368–372.
28. Montasser, A. S., El-Shahat, N., Ghoberial, G. F., El-Wadoud, M. Z., 2003. Residual effect of nitrogen fertilization on leaves and fruits of Thompson seedlees grapes. J. Environ. Sci. 6: 465-484.
29. Mrácek, Z., Webster, J. M., 1993. Survey of Heterorhabditidae and Steinernematidae (*Rhabditida: Nematoda*) in Western Canada. *Journal of Nematology* **25,** 710-717.
30. Oostendorp, M., Sikora, R. A., 1990. In-vitro interrelationships between rhizosphere bacteria and Heterodera schachtii. Rev. Nematol. 13: 269–274.
31. Paramaguru, P., Natarajan, N. S., 1993. Effect of *Azospirillum* on growth and yield of chilli (*Capsicum annuum* L.) growth under semi dry condition. South Indian Hortic. 41: 80–83.
32. Perotti, E. B. and Pidello, A. 1999. Effect of *Azospirillum brasilense* inoculation on urease activity in soil and gamma sterilized soil. Rev. Argent. Microbiol. 31: 36-41.
33. Russo, R.O., Berlyn, G. P., 1990. The use of organic bio stimulants to help low in put sustainable agriculture. J. Sustainable Agric., 1: 19-42.
34. Saleh, E. A., Ahmed, M. A., 1988. Influence of inoculation with *Azotobacter*. *Azospirillum* inoculate on the control of soybean root rot diseases caused by *Fusarium solani f. sp. phaseolina*. Proc 2 Conf. Agric. nd Devlop. Res. Ain shams Univ., pp. 97-115.
35. Schneider, S. M., Rosskopf, E. N., Leesch, J. G., Chellemi, D. O., Bull, C. T., Mazzola, M., 2003. Research on alternatives to methylbromide: pre-plant and post-harvest. Pest Manag. Sci. 59: 814–826.
36. Shanmugam, V., Senthil, N., Raguchander, T., Ramanathan, A., Samiyappan, R., 2002. Interaction of *Pseudomonas fluorescens* with *Rhizobium* for their effect on the management of Peanut root rot. Phytoparasitica 30: 169-176.
37. Siddiqui, I. A,, Haas. D., Heeb, S., 2005. Extracellular protease of *Pseudomonas fluorescens* CHA0, a biocontrol factor with activity against the root-knot nematode *Meloidogyne incognita*. Appl Environ. Microbiol. 71: 5646–5649.
38. Siddiqui, Z. A., Mahmood, I., 1999. Role of bacteria in the management of plant parasitic nematodes: a review. Bioresource Technol 69: 167–179.
39. Siddiqui, Z. A., Qureshi, A., Akhtar, M. S., 2009. Biocontrol of root-knot nematode *Meloidogyne incognita* by *Pseudomonas* and *Bacillus* isolates on *Pisum sativum*. Archives of Phytopathol. Plant Prot. 42: 1154-1164.
40. Sikora, R. A., Hoffmann-Hergarten, S., 1993. Biological control of plant parasitic nematodes with plant-health promoting rhizobacteria. Biologically based technology (umsden PD & Vaugh JL, eds), pp. 166–172. ACS Symposium series, USA.
41. Singh, C., Sharma, B. B., 1993. Leaf nutrient composition of sweet orange as affected by combined use of bio and chemical fertilizers. South Indian Hortic. 41: 131–134.
42. Sivan, A, Chet, I, 1992. Microbial control of plant diseases. In: Mitchell R, editor. Environmental Microbiology. New York: Wiley-Liss, pp. 335–54.
43. Stirling, G. R., 1991. Biological Control of Plant Parasitic Nematode: Progress, Problems and Prospects. CAB International, Wallington, UK.
44. Tiwary, D. K., Hasan, M. A., Chattopadhyay, P. K., 1999. Leaf nutrient and chlorophyll content in banana (Musa AAA) under the influence of Azotobacter and Azospirillum inoculation. Environ. Ecol. 17: 346–350.
45. Xie, H., Pasternak, J. J., Glick, B. R., 1996. Isolation and characterization of mutants of the plant growth-promoting rhizobacterium *Pseudomonas putida* GR12-2 that overproduce indoleacetic acid. Curr Microbiol. 32:67–71.

6/21/2010

Experimental Natural Prints And The Re-Calculated General Equations Of The Electrical Parameters For Buried Bare Pipe -Soil- Earth System With And Without Applying Cathodic Protection System

Dr. Ashraf Abdel Raouf Mohamed Fouad Ahmed
Canadian International College CIC – Egypt
Ashrafahmed9000@yahoo.com

Abstract: The rate of discharge through the stray electrolytic capacitor between the pipe and the remote earth is to be considered as the corrosion current. The electrochemical properties of the soil, which are the soil resistivity of the soil volume, the relative permittivity of the soil layer around the pipe and the chemical properties which could be considered as the pH of the soil film layer around the pipe, are affected directly by the humidity change. When considering the room temperature and by neglecting the effect of CO_2 content in the soil, these values of the electrochemical properties of any soil returns back to its initial conditions after soil dryness to its initial condition. This means that corrosion rate will also be changed during the humidity change around the pipe segment. So, when considering the fact that the pipeline will not be changed or replaced and the surrounding medium around it will not be changed or replaced by another kind of soil, then the behavior of the electrical parameters (stray electrolytic capacitance, stray potential, surface created charge) of the pipe-soil-earth system will act as a print of this combination of this pipe and this soil. This paper recalculates the general form of the equations of the electric parameters and obtains the print curves & constants at natural condition with and without applying cathodic protection system in terms of the electrochemical properties around the pipe. The average error reduced to be less than ± 5%. This will help to study both the corrosion problem and cathodic protection by an electric concept with an electric analogue circuit which is the aim of this study. [Journal of American Science. 2010;6(12):272-283]. (ISSN: 1545-1003).

Keywords: Electrical study of pipe – soil – earth system

1. Introduction:

At humidity equal to zero, the soil medium around pipeline could be considered as a dielectric material which has its relative permittivity. If the humidity is increased, the soil medium is considered to be as an electrolyte associated with a change happened in the values of the relative permittivity, resistivity and pH of the soil. These changes which happened in soil electrochemical properties will continue by increasing the humidity but these values will return back to their original values, or nearby initial values, after the humidity returns back to its initial value. The change of the soil medium between isolation medium and conduction medium according to the percentage of the humidity could be studied electrically. Then, based on the new proposed electrical concept of corrosion which state that: "Due to surrounding medium effect around metal structure buried in the ground, the charge created on metal o.s.a builds up a potential through an electrolytic stray capacitor between metal o.s.a and an imaginary coaxial earthing cylinder " [1][2] , The proposed electric concept of corrosion is depending on the concept of the positive charge created on the outer surface area (o.s.a) of the buried bare pipe line segment due to the building up potential between the pipe and the remote earth. The created charge is dissipated to the surrounding medium (corrosion process) through a stray electrolytic capacitor between the pipe and an imaginary coaxial earthing cylindrical.

In case of coated pipe segment, the dielectric constant of the soil K_S acts with the dielectric constant of the pipe coating material K_C as a coaxial cylindrical capacitor with compound dielectric. As K_C of the coating material is decreased, the total capacitance is decreased (two capacitors are in series) then charge Q = C x V is decreased .That's to say that corrosion process is decreased. If deterioration of coating material occurred, then K_C is increased. That's to say that the total capacitance of the compound dielectric is increased .This means that the corrosion process is increased as the created charge on metal outer surface area Q = C x V will be increased [1][2].

In other words, if two dissimilar electrodes buried in a box which is containing a soil medium, a circulating current will take place between these electrodes due to the difference in electrodes' natural potential while a capacitance in nano farad could be measured between these two dissimilar electrodes (through the soil). The potential difference, the capacitance and the corrosion current between the positive electrode and the negative electrode are

electric quantities. Then, it may be possible to understand the corrosion and cathodic protection by an electrical concept beside the electrochemical and thermodynamic concepts. .

The corrosion may be described electrically (electrons losses) by the equation: $Q = C \times V$, while the rate of discharge dQ/dt is equal to the corrosion current from the +ve electrode to the –ve one [1] [2]. The same concept could be applied on the system of buried pipeline and the surrounding soil medium. The pipeline may be considered the +ve electrode while the remote earth may be considered the –ve electrode. In case of pipe-soil-earth system that it is not subjected to any external interference, if it is possible to find a correlation between the electrical parameters and the electrochemical properties of the soil at different humidity with and without applying cathodic protection system, then the result will be considered as an electrical print or as a data sheet of this pipe-soil-earth system. These electrical prints will be recalculated if any essential values are changed in the pipe-soil-earth system. The importance of these electrical prints are to define both the electrical parameters and the cathodic protection level of any buried pipe segment of the pipe-soil-earth system if the protection current and the electrochemical properties are measured at the pipe segment directly from the field.

Now, as the electrochemical properties of any soil are changed by the change of humidity but returns back to its initial conditions after some time required for soil dryness, we can define a new factor named the soil factor as: "The soil factor (S_f) is the instantaneous value of the electro-chemical properties of the soil based on the electrical properties at Humidity equal to 10% "[1] [2] and is equal to:

$S_f = (1 / K_S)$ pH H log ρ at room temperature (1)

Dimension of $[S_f] = [1 / K_S]$ [pH] [H] [log ρ] = Ω.m %

Where:

S_f = soil factor Ω.m %

K_S = dielectric constant of the soil at H = 10%

pH = power of Hydrogen of the soil

H = humidity of the soil %

ρ = soil resistivity in Ω.m at H = 10%

The importance of the soil factor is that it is combining all parameters which can affect directly on the cathodic protection level or in corrosion process. Such factors which can be obtained by a direct measurements from the field. This means that if it is possible to study the relationship between the soil factor and the electrical parameters of the bare pipe segment, then the print curves and the print constants of the electrical parameters of the pipe-soil-earth system could be obtained at natural condition with and without applying cathodic protection system. The soil factor can be considered to be as the key of many studies based on the new proposed electrical concept of corrosion. For an example, the general equation of the natural stray capacitance between external surface area of bare pipe segment and earth is obtained in terms of the soil factor with an average error ± 30% and its print curves and constants are obtained for pipe-soil system for 10 different soils [3]. Also, the general equation of both the natural stray potential and the natural created charge are obtained in terms of the soil factor with an average error ± 30% and their print curves and constants are obtained for pipe-soil system for 10 different soils [4] [5]. In this paper, we will continue to use the soil factor, to complete such study concerning the natural electrical parameters of pipe-soil-earth system with and without applying cathodic protection system to reduce the average error of the equations of electrical parameters to be less than ± 5%.

2. Case1:

2.1 Pipe – Soil – Earth System Without C.P System

2.1.1 The Experiment

At natural condition system without any influence of any external systems such as cathodic protection systems, pipe crossing.... (only bare pipe + soil + point earth), the experiment is consisting of a system with bare pipe segment (2.1cm diameter, 1mm thickness and 31.1cm length), buried in a soil with humidity equal to 10% and the soil have soil resistivity equal to ρ Ω.m, soil power of hydrogen pH and soil relative permittivity K_S. Table 1 shows the different kind of soils used in this experiment and the range of humidity.

When considering the system pipe-soil-earth for ten different kind of soil as shown in table 1, the test procedures are as follow:

1) Calculate the value of the soil factor according to the eq.1 :
2) Measure both the stray potential $V_{P\text{-}PE}$, the stray capacitance $C_{P\text{-}PE}$ and the correspondent pipe to soil potential $V_{H\text{-}C}$ by using $Cu/CuSO_4$ half cell. Calculate total surface charge $Q = V_{P\text{-}PE} * C_{P\text{-}PE}$
3) Increase the Humidity and calculate the new value of the soil factor.
4) Repeat steps 2 & 3 until humidity around the pipe segment reaches its maximum as shown in table 1.
5) Change this type of soil by another kind of soil and repeat all the steps done before.
6) Repeat again the steps for 10 different kind of soil shown in table 1.

7) Build up the results table.

2.1.1 The Results

If we plot the measured electric quantity (natural stray potential $V_{P\text{-}PE,}$ natural stray capacitance $C_{P\text{-}PE}$ and surface total charge Q) individually as y axis in terms of the correspondent measured soil factor as x axis, we will obtain such following curves as shown in figures 1&2. Except box 4 and box 13, the curves and equations show eight boxes which could be expressed by a 4th degree polynomial equation with an average error equal to zero percent. Table 2 shows the error table for the 10 different soil resistivity.

Table 1: 10 different kind of soil and operating range of H & pH

Kind of soil		ρ_1	ρ_2	ρ_3	ρ_4	ρ_5	ρ_6	ρ_7	ρ_8	ρ_9	ρ_{10}
Box under test		1	4	9	10	13	18	19	24	27	28
Resistivity Ω m		31	37	43	62	125	138	1382	2010	5654	7539
Relative permittivity K_S		138	66	86	43	51	274	22	154	43	355
Humidity %	Start	6	8	5	10	6	5	5	5	5	10
	End	60	85	100	100	100	100	96	80	100	100
pH	Start	7.8	7.3	7.8	7.8	7.3	7.6	7.2	7	7.2	6.7
	End	7	6.5	6	6	5	5	5.3	7	6	7

Table 2: Error table for 10 different kinds of soil and operating range of Humidity

Kind of soil		ρ_1	ρ_2	ρ_3	ρ_4	ρ_5	ρ_6	ρ_7	ρ_8	ρ_9	ρ_{10}
Box under test		1	4	9	10	13	18	19	24	27	28
Polynomial degree		4^{th}	4^{th}	4^{th}	4^{th}	4^{th}	4^{th}	4^{th}	4^{th}	4^{th}	4^{th}
$V_{P\text{-}PE}$ Error %		0	± 30	0	0	0	0	0	0	0	0
$C_{P\text{-}PE}$ Error %		0	± 30	0	0	± 20	0	0	0	0	0
$Q_{TOT.}$ Error %		0	± 50	0	0	± 25	0	0	0	0	0
Humidity %	Start	6	8	5	10	6	5	5	5	5	10
	End	60	85	100	100	100	100	96	80	100	100

2.2 Electrical Parameters Print Curves For Pipe – Soil – Earth Under Test

By considering the measured soil factor as x axis against the measured electrical parameter (stray potential $V_{P\text{-}PE,}$ stray capacitance $C_{P\text{-}PE}$, surface total charge Q) as y axis, the next following print curves were obtained for the pipe - soil - earth systems under test as shown in figures 1&2. The stray capacitance is illustrated by group a, figures 1a & 2a. The stray potential is illustrated by group b, figures 1b & 2b.. The surface total charge is illustrated by group c, figures 1c & 2c.. All points resulting from the experiment could be represented by a trend line for each measured electric quantity and could be represented by a PRINT for each pipe-soil-earth under test, with 4th degree polynomial equation. The average error for all equations is less than ± 5%. The above result is very important. This proves that it may be possible to complete this electrical study of pipe-soil-earth system to find an electric circuit diagram of this combination of pipe segment-soil-earth system which is the real target. This means that, beside the electrochemical and thermodynamic concepts of corrosion, it is possible to have an electric concept of the corrosion process and to convert both the corrosion and cathodic protection problems into an electric problem.

2.3 Stray Potential General Equation For Pipe – Soil – Earth Under Test

For each soil under test and from natural stray potential curves and equations, it can easily observe that the general equation of the natural stray potential from pipe segment to the remote earth during humidity change is a 4th degree polynomial equation which is function of the soil factor, $V_{n\ stray} = f(X = S_f)$. For each soil under test, the general stray potential equation is function of the measured soil factor $V_{n\ stray} = f(X=S_f)$, 4th degree polynomial. The stray potential general equation is given by Eq.2:

$$\mathbf{V_{n\ stray}} = A_{4vn}\mathbf{X^4} + A_{3vn}\mathbf{X^3} + A_{2vn}\mathbf{X^2} + A_{1vn}\mathbf{X} + A_{0vn} \quad (2)$$

Where:

A's: = $A_{(\ )V}$ are the natural stray potential print constants of the pipe soil under test

X = is the value of the soil factor at certain humidity

2.4 Stray Capacitance General Equation For Pipe – Soil – Earth Under Test

For each kind of soil under test and from stray capacitance curves and equations, it can easily observe that the general equation of the stray capacitance, at natural condition without applying cathodic protection, to the remote earth during humidity change is a 4^{th} degree polynomial equation which is function of the soil factor, $C_{n\ stray}$ = f(soil factor **X**)

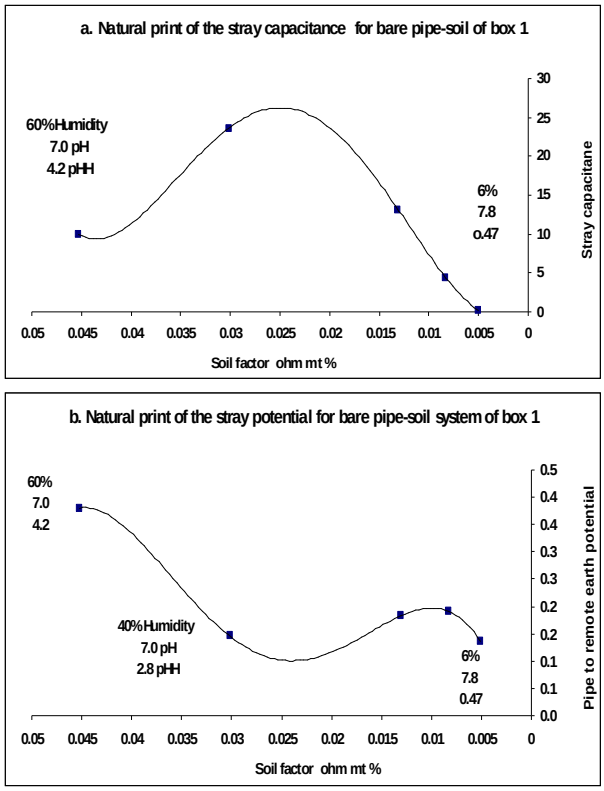
For each soil under test, the general stray capacitance equation is function of the measured soil factor, $C_{\ stray}$ = f(X=S_f), 4^{th} degree polynomial. The stray capacitance general equation is equal to Eq. 3:

$$\mathbf{C}_{\mathbf{n\ stray}} = A_{4cn}\mathbf{X}^4 + A_{3cn}\mathbf{X}^3 + A_{2cn}\mathbf{X}^2 + A_{1cn}\mathbf{X} + A_{0cn} \quad (3)$$

Where:

A's: = $A_{\ ()CN}$ are the stray capacitance print constants of the pipe soil under test

X = is the value of the soil factor at certain humidity



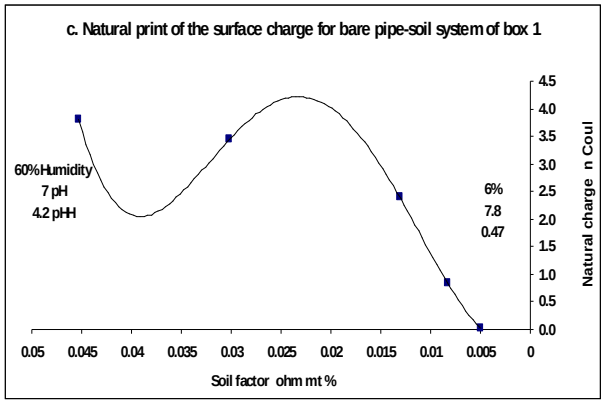


Figure 1: Natural prints of the electrical parameters for buried bare pipe-soil-earth of box 1

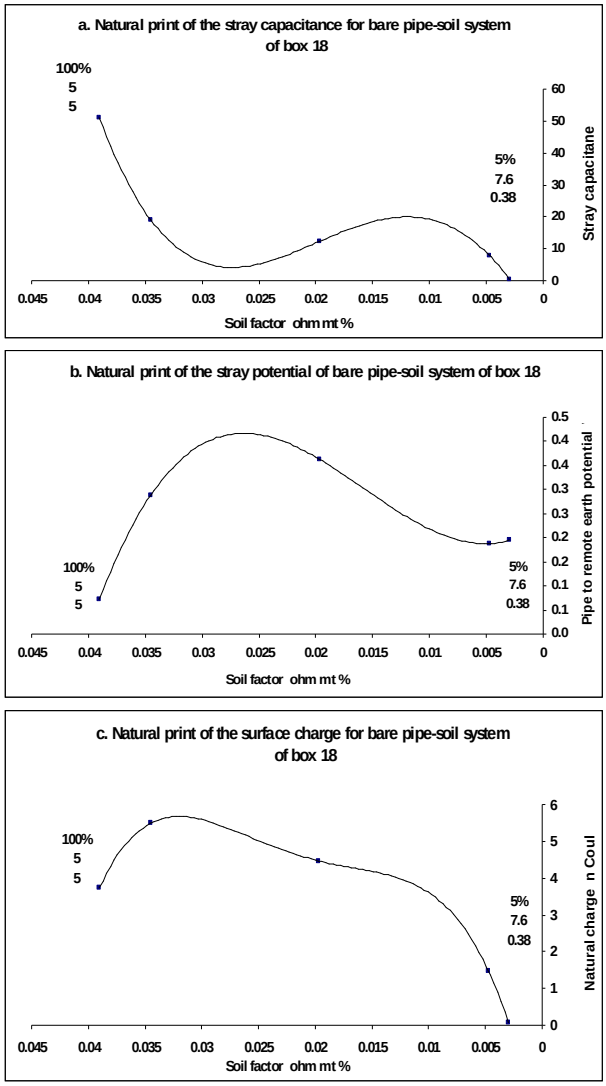


Figure 2: Natural prints of the electrical parameters for buried bare pipe-soil-earth of box 18

2.5 Surface Total Charge General Equation For Pipe – Soil – Earth Under Test

For each kind of soil under test and from the natural surface charge curves and equations, it can easily observe that the general equation of the surface charge, at natural condition without applying cathodic protection, is a 4^{th} degree polynomial equation which is function of the soil factor $Q_N = f$ (X = soil factor). For each soil under test, the general surface natural charge equation is function of the measured soil factor, $Q_N = f(X=S_f)$, 4^{th} degree polynomial. The surface natural charge general equation is equal to Eq. 4:

$$\mathbf{Q}_N = A_{4qn}\mathbf{X}^4 + A_{3qn}\mathbf{X}^3 + A_{2qn}\mathbf{X}^2 + A_{1qn}\mathbf{X} + A_{0qn} \quad (4)$$

Where:

A's: = A ( ) q are the surface natural charge print constants of the pipe - soil under test

X = is the value of the soil factor at certain humidity

2.6 The Electrical Parameter General Equation Of Pipe-Soil-Earth System At Natural Condition Without Applying CP System

We can observe clearly that the general form of any electrical parameter equation is function of the measured soil factor, 4^{th} degree polynomial but the A's print constants are different. From equations 2, 3 and 4, we can easily summarize the natural electrical parameters equations for the pipe-soil-earth under test, without applying cathodic protection system, as follow:

$$\mathbf{C}_{\text{n stray}} = A_{4cn}\mathbf{X}^4 + A_{3cn}\mathbf{X}^3 + A_{2cn}\mathbf{X}^2 + A_{1cn}\mathbf{X} + A_{0cn}$$

$$\mathbf{V}_{\text{n stray}} = A_{4vn}\mathbf{X}^4 + A_{3vn}\mathbf{X}^3 + A_{2vn}\mathbf{X}^2 + A_{1vn}\mathbf{X} + A_{0vn}$$

$$\mathbf{Q}_{\text{n stray}} = A_{4qn}\mathbf{X}^4 + A_{3qn}\mathbf{X}^3 + A_{2qn}\mathbf{X}^2 + A_{1qn}\mathbf{X} + A_{0qn}$$

We can observe clearly that the general form of any electrical parameter equation, at natural condition without applying cathodic protection, is function of the measured soil factor, 4^{th} degree polynomial but the A's PRINT constants are different. Then, the general form of any electrical parameters equation of the pipe-soil-earth system under test at natural condition without applying cathodic protection with the same amount of soil volume will be as shown by the following equation :

Natural electrical parameter of pipe-soil-earth system under test = $A_4\mathbf{X}^4 + A_3\mathbf{X}^3 + A_2\mathbf{X}^2 + A_1\mathbf{X} + A_0$

The A's are obtained from the correspondent print table and **X** is the measured soil factor

The natural electric parameters general equation and PRINT curves show the following important results:

a) Buried pipe in a soil which generates the minimum natural created charge at bare pipe surface at normal steady humidity is defining the most suitable choice of soil to be around the pipe.

b) In case of space or vacuum is the medium which is surrounding the pipeline instead of soil medium, the value of the soil factor will be zero as $K_S = 1$, H = 0% , ρ = infinity. That's to say that the natural created charge will equal to zero (A_O will equal to zero for pipe-vacuum system) which is the ideal case of corrosion prevention.

c) In case of air is the medium which is surrounding the pipeline instead of soil medium, the value of the soil factor will be smaller than that of the soil at same humidity as $K_S \approx 1$ (it is not almost that $K_S \approx 1$ if the humidity exists in air), H = 10% up to 60% or more, pH = 7 & ρ = value according to H%. Consequently, the natural created charge exists in air but with smaller amount than that of soil at same humidity. That's to say that air is most proper surrounding medium for a pipe than soil at same humidity condition.

2.7 Natural Stray Potential Print Constants For Pipe-Soil-Earth Under Test

Now, the natural stray potential print constants of the pipe-soil-earth system under test are A_{4VN}, A_{3VN}, A_{2VN}, A_{1VN} and A_{0VN}. This means that these print values are valid for these pipe-soil systems under test at any time at the correspondent electrochemical properties (soil factor). Table 3 shows result example of the natural stray potential print.

Table 3: PRINT constants of the stray potential for 10 different soil under test

Soil	ρ_1	ρ_2	ρ_3	ρ_4	ρ_5	ρ_6	ρ_7	ρ_8	ρ_9	ρ_{10}
Error	0	±30%	0	0	0	0	0	0	0	0
$\mathbf{A_{4VN}}$	-2.E+06	0	321339	5620	8822.1	0	118.12	140960	0	546856
$\mathbf{A_{3VN}}$	202688	698.68	-78007	-3154.1	-3710.9	-48181	-153.09	-37414	-110.64	-110280
$\mathbf{A_{2VN}}$	-6782.5	-157.71	6025.9	599.26	407.17	2278.7	54.029	3218.8	100.61	7753.7
$\mathbf{A_{1VN}}$	82.539	9.8253	-159.72	-44.958	-3.3452	-19.695	-3.1804	-99.254	-24.97	-215.37
$\mathbf{A_{0VN}}$	-0.133	0.0009	1.377	1.412	0.0954	0.2352	0.1245	0.9629	1.2357	1.8678

2.8 Natural Stray Capacitance Print Constants For Pipe-Soil-Earth Under Test

Now, the stray capacitance print constants of the pipe-soil-earth system under test are A_{4CN}, A_{3CN}, A_{2CN}, A_{1CN} and A_{0CN}. This means that these print values are valid for these pipe-soil systems under test at any time at the correspondent electrochemical properties (the soil factor). Table 4 shows the natural stray capacitance print constants.

Table 4: PRINT constants of natural stray capacitance for 10 different soils under test

Soil	ρ_1	ρ_2	ρ_3	ρ_4	ρ_5	ρ_6	ρ_7	ρ_8	ρ_9	ρ_{10}
Error	0	±30%	0	0	±30%	0	0	0	0	0
A_{4CN}	1.E+08	0	-4.E+06	1.E+06	0	6.E+07	26675	-9.E+06	21770	1.E+07
A_{3CN}	-1.E+07	262166	1.E+06	-620109	-269270	4.E+06	-35297	2.E+06	-16925	-1.E+06
A_{2CN}	322980	-47807	-88692	99288	69267	-379156	13424	-203776	3178	21862
A_{1CN}	-1549	2428	2780	-5207	-1953	6969	-1209	6766	120	874
A_{0CN}	1.27	-24.8	-15.55	84	12.77	-17	31	-58	-6	-6.8

2.9 Surface Natural Charge Print Constants For Pipe-Soil-Earth Under Test

Now, the surface natural charge print constants of the pipe-soil-earth system under test are A_{4q}, A_{3q}, A_{2q}, A_{1q} and A_{0q}. This means that these print values are valid for these pipe-soil systems under test at any time at the correspondent electrochemical properties (soil factor). Table 5 shows surface natural charge print constants.

Table 5: Natural charge PRINT at the pipe surface for 10 different soils under test

Soil	ρ_1	ρ_2	ρ_3	ρ_4	ρ_5	ρ_6	ρ_7	ρ_8	ρ_9	ρ_{10}
Error	0	± 50%	0	0	±25%	0	0	0	0	0
A_{4qn}	3.E+07	0	-919280	526631	0	-4.E+07	9205.4	-376311	-9566.4	-715518
A_{3qn}	-2.E+06	45298	242778	-267958	-26967	4.E+06	-12309	101374	9162.1	157730
A_{2qn}	60696	-8408	-20512	43238	6411	-110112	4771.1	-9536	-2632	-10306
A_{1qn}	-261	432.74	622.04	-2329	-41.27	1486.5	-461.4	360.12	227.38	253
A_{0qn}	0.1	-4.46	-3.368	38.55	-1.36	-3.463	12.625	-3.27	-4.63	-1.168

3. Case 2:

3.1 Pipe – Soil – Earth System With applying Cathodic Protection System

3.1.1The Experiment

At natural condition system without any influence of any external systems such as cathodic protection systems, pipe crossing.... (only bare pipe + soil + impressed current system + point earth), the experiment is consisting of a system with bare pipe segment (2.1cm diameter, 1mm thickness and 31.1cm length), buried in a soil with humidity equal to 10% and the soil have soil resistivity equal to ρ Ω.m, soil power of hydrogen pH and soil relative permittivity K_S. The impressed current system is consisting of a nail as an anode which is connected to the positive terminal of variable d-c source while the negative terminal is connected to the bare pipe segment. Table 1 shows the different kind of soils used in this experiment and the range of humidity.

When considering the system pipe-soil-earth for ten different kind of soil as shown in table 1, the test procedures are as follow:

1) Calculate the value of the soil factor according to the Eq.1 :
2) Measure both the stray potential $V_{P\text{-}PE}$, the stray capacitance $C_{P\text{-}PE}$, the protection current I_P and the correspondent pipe to soil potential $V_{H\text{-}C}$ by using $Cu/CuSO_4$ half cell. Calculate total surface charge $Q = V_{P\text{-}PE} * C_{P\text{-}PE}$
3) Increase the c.p protection current I_P
4) Repeat step 2&3 up to $V_{H\text{-}C}$ = -2 volt
5) Increase the Humidity and calculate the new value of the soil factor.
6) Repeat steps 2, 3&4 until humidity around the pipe segment reaches its maximum as shown in table 1.
7) Change this type of soil by another kind of soil and repeat all the steps done before.
8) Repeat again the steps for 10 different kind of soil as shown in table 1.

9) Build up the results table.

3.1.2 Results

If we plot the measured electric quantity (stray potential $V_{P\text{-}PE,}$ stray capacitance $C_{P\text{-}PE}$, surface total charge Q and protection current I_P) individually as y axis in terms of the correspondent measured soil factor as x axis at the correspondent different levels of pipe to soil potential $V_{H\text{-}C}$ by using $Cu/CuSO_4$ half cell from -0.2 v to -2 volt, we will obtain such following curves as shown in figures 3, 4 & 5. Except box 4 and box 24, the curves and equations of the electrical parameters show eight boxes which could be expressed by a 4^{th} degree polynomial equation with an error equal to zero percent. Table 6 shows the error table for the 10 different soil resistivity under test.

Table 6: Average error table for 10 different kinds of soil and operating range of Humidity

Soil		ρ_1	ρ_2	ρ_3	ρ_4	ρ_5	ρ_6	ρ_7	ρ_8	ρ_9	ρ_{10}
Box under test		1	4	9	10	13	18	19	24	27	28
Polynomial degree		4^{th}	4^{th}	4^{th}	4^{th}	4^{th}	4^{th}	4^{th}	4^{th}	4^{th}	4^{th}
$V_{P\text{-}PE}$ Error %		0	± 30	0	0	0	0	0	± 30	0	0
$C_{P\text{-}PE}$ Error %		0	± 30	0	0	0	0	0	± 30	0	0
$Q_{TOT.}$ Error %		0	± 30	0	0	0	0	0	± 30	0	0
I_P Error %		0	± 30	0	0	0	0	0	± 30	0	0
Humidity %	**Start**	6	8	5	10	6	5	5	5	5	10
	End	60	85	100	100	100	100	96	80	100	100

3.2 Stray Potential For Pipe-Soil-Earth Under Test

From the stray potential PRINT curves and trend lines equations, as shown in figures 3b, 4b and 5b, it can easily observe that the general equation of the stray potential of a cathodically protected bare pipe segment during humidity change under multi level of cathodic protection levels is a 4^{th} degree polynomial equation which is function of the soil factor $V_{Str.}$ = f (X = soil factor). For each soil under test, the general stray potential equation is function of the measured soil factor, $V_{Str.}$ = f(X=S_f), 4^{th} degree polynomial. The stray potential general equation is equal to Eq. 5:

$$\mathbf{V_{Str.}} = A_{4V}\mathbf{X}^4 + A_{3V}\mathbf{X}^3 + A_{2V}\mathbf{X}^2 + A_{1V}\mathbf{X} + A_{0V} \quad (5)$$

Where:
A's: = $A_{(\ )V}$ are the stray potential print constants of the pipe - soil under test
X = is the value of the soil factor at certain humidity

3.3 Stray Capacitance For Pipe-Soil-Earth Under Test

From the stray capacitance PRINT curves and trend lines equations, as shown in figures 3a, 4a and 5a, it can easily observe that the general equation of the stray capacitance of a cathodically protected bare pipe segment during humidity change under multi level of cathodic protection levels is a 4^{th} degree polynomial equation which is function of the soil factor $V_{Str.}$ = f (X = soil factor), the same equation as that of pipe – soil – earth system without applying cathodic protection. For each soil under test, the general stray capacitance equation is function of the measured soil factor,

$C_{Str.}$ = f(X=S_f), 4^{th} degree polynomial. The stray potential general equation is equal to Eq. 6:

$$\mathbf{C_{Str.}} = \mathbf{C}_{\mathbf{n\ stray}} = A_{4cn}\mathbf{X}^4 + A_{3cn}\mathbf{X}^3 + A_{2cn}\mathbf{X}^2 + A_{1cn}\mathbf{X} + A_{0cn} \quad (6)$$

Where:
A's: = $A_{(\ )cn}$ are the stray capacitance print constants of the pipe - soil under test
X = is the value of the soil factor at certain humidity

3.4 Surface Total Charge For Pipe-Soil-Earth Under Test

From the surface total charge PRINT curves and trend lines equations, as shown in figures 3c, 4c and 5c it can easily observe that the general equation of the surface total charge of a cathodically protected bare pipe - segment during humidity change under multi level of cathodic protection levels is a 4^{th} degree polynomial equation which is function of the soil factor $Q_{tot.}$ = f (X = soil factor).
For each soil under test, the general surface total charge equation is function of the measured soil factor, $Q_{tot.}$= f(X=S_f), 4^{th} degree polynomial. The surface total charge general equation is equal to Eq. 7:

$$\mathbf{Q_{tot.}} = A_{4q}\mathbf{X}^4 + A_{3q}\mathbf{X}^3 + A_{2q}\mathbf{X}^2 + A_{1q}\mathbf{X} + A_{0q} \quad (7)$$

Where:

A's: = A ( ) q are the surface total charge print constants of the pipe - soil under test
X = is the value of the soil factor at certain humidity

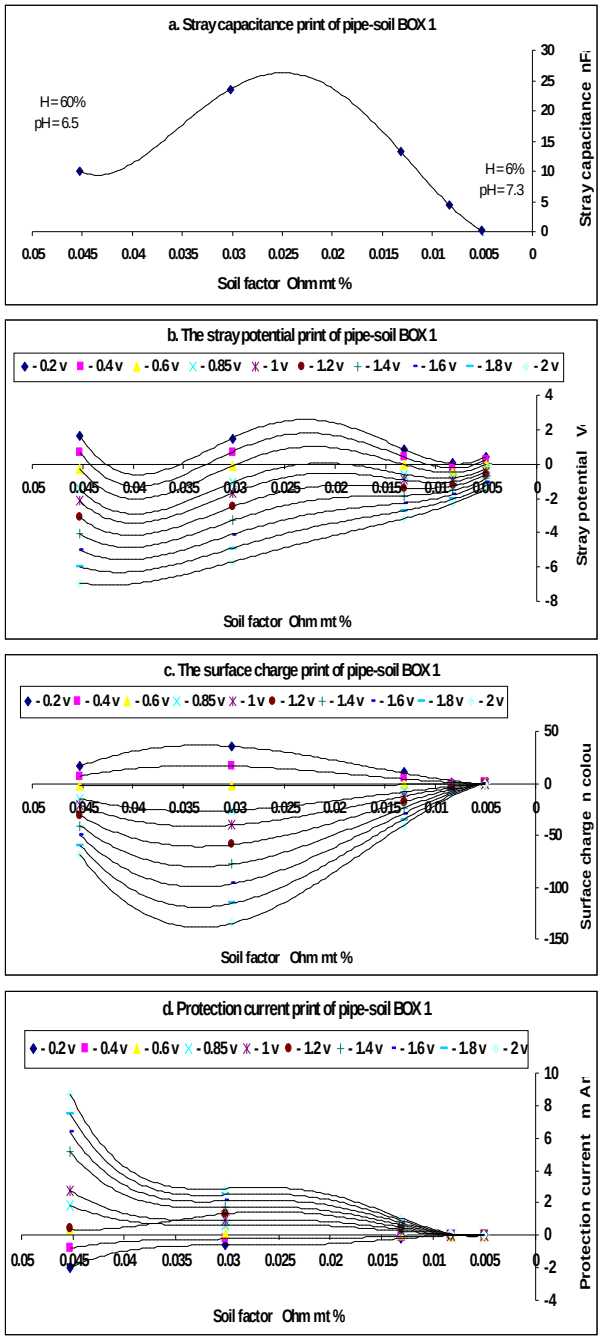


Figure 3: Electrical Parameters PRINT curves of pipe-soil-earth of BOX 1

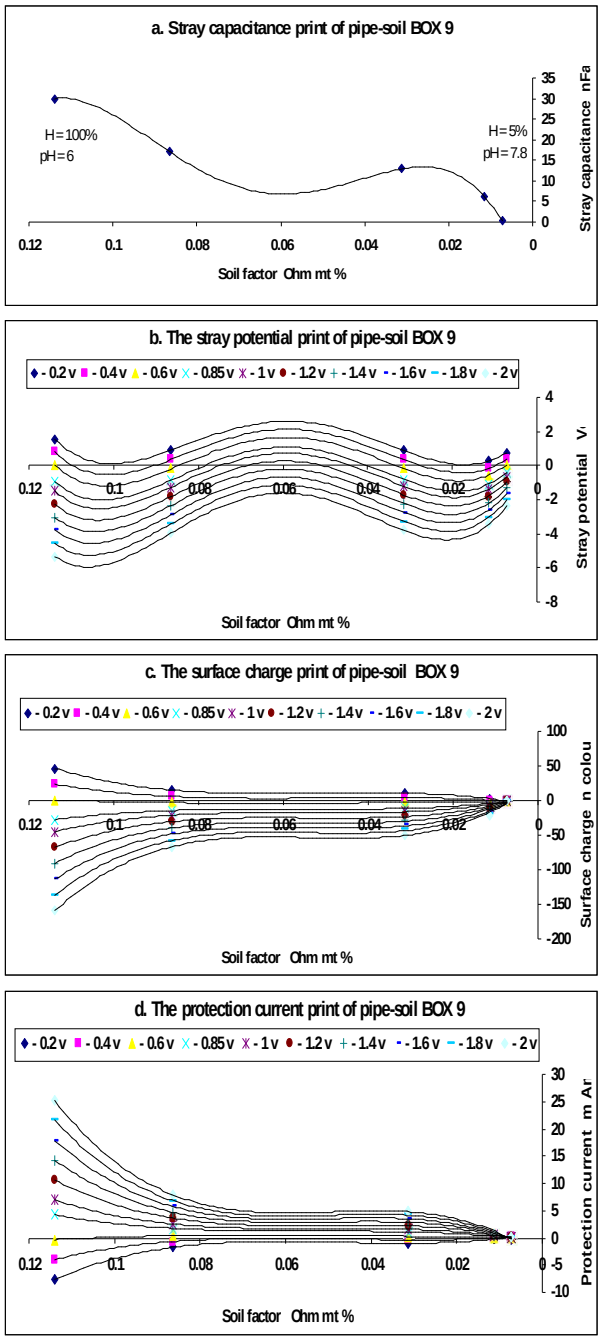


Figure 4: Electrical Parameters PRINT curves of pipe-soil-earth of BOX 9

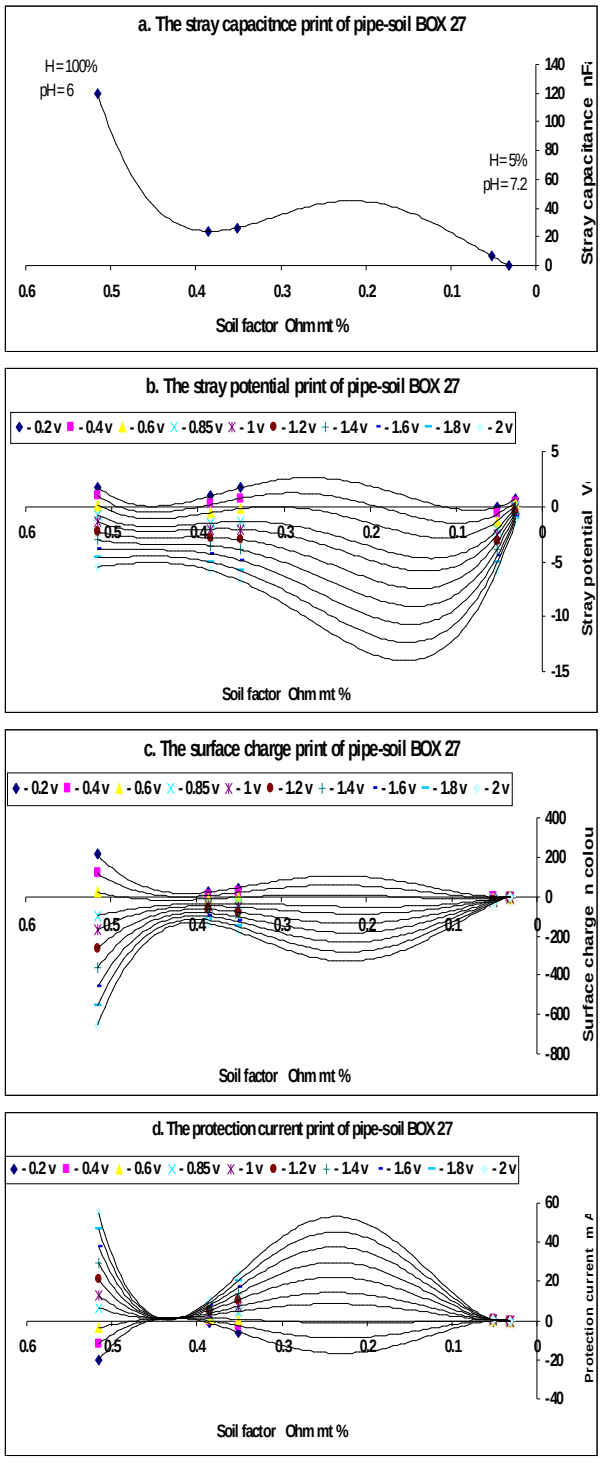


Figure 5: Electrical Parameters PRINT curves of pipe-soil-earth of BOX 27

3.5 Protection Current General Equation For Pipe – Soil – Earth Under Test

Definition: "The ONION curves are the curves of the protection current I_P in terms of the soil factor S_f at different half cell voltage $V_{H.C}$ levels". From the PRINT ONION curves, , as shown in figures 3d, 4d and 5d and trend lines equations, it can easily observe that the general equation of the protection current of a cathodically protected bare pipe segment during humidity change under multi level of cathodic protection levels is a 4th degree polynomial equation which is function of the soil factor, $I_P = f$ (X = soil factor)

For each soil under test, the general protection current equation is function of the measured soil factor $I_P = f(X=S_f)$, 4th degree polynomial. The protection current general equation is equal to Eq. 8:

$$\mathbf{I_P} = A_{4I}\mathbf{X}^4 + A_{3I}\mathbf{X}^3 + A_{2I}\mathbf{X}^2 + A_{1I}\mathbf{X} + A_{0I} \quad (8)$$

Where:
A's: = $A_{(\,)I}$ are the protection current print constants of the pipe soil under test
X = is the value of the soil factor at certain humidity

3.6 The Electrical Parameter General Equation Of Pipe-Soil-Earth System With Applying C.P System

We can observe clearly that the general form of any electrical parameter equation is function of the measured soil factor, 4th degree polynomial but the A's constants are different and are dependant on the pipe to soil potential except the stray capacitance which is independent of the pipe to soil potential . From equations 5, 6, 7 and 8, we can easily summarize the electrical parameters trend lines equations for the pipe-soil-earth under test, with applying c.p system, are in the form as follow:

$$\mathbf{C_{stray}} = A_{4cn}\mathbf{X}^4 + A_{3cn}\mathbf{X}^3 + A_{2cn}\mathbf{X}^2 + A_{1cn}\mathbf{X} + A_{0cn}$$

$$\mathbf{V_{stray}} = A_{4v}\mathbf{X}^4 + A_{3v}\mathbf{X}^3 + A_{2v}\mathbf{X}^2 + A_{1v}\mathbf{X} + A_{0v}$$

$$\mathbf{Q_{tot.}} = A_{4q}\mathbf{X}^4 + A_{3q}\mathbf{X}^3 + A_{2q}\mathbf{X}^2 + A_{1q}\mathbf{X} + A_{0q}$$

$$\mathbf{I_P} = A_{4IP}\mathbf{X}^4 + A_{3IP}\mathbf{X}^3 + A_{2IP}\mathbf{X}^2 + A_{1IP}\mathbf{X} + A_{0IP}$$

Then, the general form of any electrical parameters equation of the pipe-soil-earth system under test, with applying cathodic protection, will be as shown in the following general equation which is the same as that of the system without applying cathodic protection:

$$\mathbf{EP} = A_4\mathbf{X}^4 + A_3\mathbf{X}^3 + A_2\mathbf{X}^2 + A_1\mathbf{X} + A_0$$

The A's are obtained from the print constant tables and the **X** is the measured soil factor.

3.7 Stray Potential Print Constants For Pipe-Soil-Earth Under Test

Now, the stray potential PRINT constants of the pipe-soil-earth systems under test are A_{4V}, A_{3V}, A_{2V},

A_{1V} and A_{0V} at a definite cathodic protection level. This means that these print values are valid for these CP levels for these pipe soil systems under test at any time at the correspondent electrochemical properties (the soil factor). Table 7 shows result example of the stray potential print constants at CP level equal to -0.85 volt.

Table 7: Stray potential finger print constants at pipe to soil potential equal to -0.85 volt

BOX	ρ_1	ρ_2	ρ_3	ρ_4	ρ_5	ρ_6	ρ_7	ρ_8	ρ_9	ρ_{10}
	1	4	9	10	13	18	19	24	27	28
A_{4V}	3.00E+07	0	925051	17354	1149.5	7.00E+07	861.17	0	2228.7	1.00E+06
A_{3V}	-3.00E+06	-1858.9	-223313	-9862.3	-4002.1	-7.00E+06	-1531.8	61.62	-2755.3	-194014
A_{2V}	91780	507.49	16975	1905.4	1569.6	211211	926.56	-232.3	1155.8	12478
A_{1V}	-1023	-37.7	-428	-143	-176.7	-2389.4	-204.8	35.484	-179.3	-321.02
A_{0V}	2.9327	-0.61	1.9	2.6	2.37	4.66	7.154	-2.19	4.49	1.6
Error	0	±30%	0	0	0	0	0	±30%	0	0

3.8 Stray Capacitance Print Constants For Pipe-Soil-earth Under Test

The stray capacitance PRINT constants of the pipe-soil-earth systems under test are A4C, A3C, A2C, A1C and A0C at any cathodic protection level. This means that these print values are valid at any CP levels for these pipe-soil systems under test at any time at the correspondent electrochemical properties (the soil factor). Table 8 shows the stray capacitance print constants at all CP levels.

Table 8: Stray capacitance print constants at any pipe to soil potential

BOX	ρ_1	ρ_2	ρ_3	ρ_4	ρ_5	ρ_6	ρ_7	ρ_8	ρ_9	ρ_{10}
	1	4	9	10	13	18	19	24	27	28
A_{4C}	1E+08	0	-4E+06	1E+06	0	6E+07	26675	-9E+06	21770	1E+07
A_{3C}	-1E+07	262166	1E+06	-620109	-269270	4E+06	-35297	2E+06	-16925	-1E+06
A_{2C}	322980	-47807	-88692	99288	69267	-379156	13424	-203776	3178	21862
A_{1C}	-1549	2428	2781	-5207	-1953	6970	-1209	6766	120.2	874.8
A_{0C}	1.27	-24.8	-15.5	84.15	12.77	-17.1	31.27	-58.51	-6	-6.82
Error	0	±30%	0	0	±30%	0	0	0	0	0

3.9 Surface Total Charge Print Constants For Pipe-Soil Under Test

The surface total charge PRINT constants of the pipe-soil-earth systems under test are A4q, A3q, A2q, A1q and A0q at a definite cathodic protection level. This means that these print values are valid for these CP levels for these pipe soil systems under test at any time at the correspondent electrochemical properties (the soil factor). Table 9 shows result example of the surface total charge print constants at CP level equal to -0.85 volt.

Table 9: Print constants of the surface total charge at pipe to soil potential equal to -0.85 volt

BOX	ρ_1	ρ_2	ρ_3	ρ_4	ρ_5	ρ_6	ρ_7	ρ_8	ρ_9	ρ_{10}
	1	4	9	10	13	18	19	24	27	28
A_{4q}	0	0	6E+06	50596	0	5E+08	-11047	0	-5597	-9E+06
A_{3q}	1E+06	-303338	-1E+06	-36942	57793	-6E+07	12317	-22910	135.97	980353
A_{2q}	-70717	57990	119052	8379	-9785	2E+06	-2698	3326.1	2662	-12884
A_{1q}	-84.36	-3046	-3394	-846	-857	-24242	-460	-120	-881.7	-922.4
A_{0q}	1.71	31.3	18.7	19.6	18.61	55.6	28.1	-6.62	25	7
Error	0	50%	0	0	15%	0	0	50%	0	0

3.10 Protection current Print Constants For Pipe-Soil Under Test

The protection current PRINT constants of the pipe-soil-earth systems under test are A4I, A3I, A2I, A1I and A0I at a definite cathodic protection level. This means that these print values are valid for these CP levels for these pipe-soil systems under test at any time at the correspondent electrochemical properties (the soil factor). Table 10 shows result example of the protection current print constants at CP level equal to -0.85 volt.

Table 10: The PRINT constants of the pipe current at pipe to soil potential equal to -0.85 volt

	ρ_1	ρ_2	ρ_3	ρ_4	ρ_5	ρ_6	ρ_7	ρ_8	ρ_9	ρ_{10}
BOX	1	4	9	10	13	18	19	24	27	28
A_{4I}	7E+06	0	14928	32198	-452755	-1E+07	-206.1	23296	4596.1	4.00E+06
A_{3I}	-636953	-6223	9231	-16158	216364	906336	296.1	1329	-4409	-617139
A_{2I}	17742	1138.7	-1749	2569	-30555	-25814	-118	-596.9	1202	26816
A_{1I}	-148.8	-25.2	99.9	-129	1139	371	12.56	47.3	-66.9	-345.4
A_{0I}	0.4	0.2	-0.62	2	-11.77	-0.88	-0.37	-0.5	1.1	1.34
Error	0	30%	0	0	0	0	0	0	0	0

4. Conclusion:

The behavior of the electrical parameters (stray potential $V_{P\text{-}PE,}$ stray capacitance $C_{P\text{-}PE}$, surface total charge Q and protection current I_P) of the pipe-soil-earth system, during the change of the electrochemical properties of the soil, with and without applying cathodic protection system, could be plotted as an electrical parameter PRINT which will be always valid in all times as the pipe-soil system is maintained and without any external interference. Once the system is changed by replacement another pipe with different dimension and/or the replacement of the soil, there will be another new electrical parameter PRINT for the new pipe-soil-earth system. Also, after completing electrical studies of pipe-soil-earth system in the near future, the buried pipe line segment with soil surrounding medium could be simulated electrically by an electric circuit where the system is subjected to the law: ($Q=C\times V$) between the pipe surface and remote earth. This is where each of circuit electric parameter could be obtained by an equation as a function of the measured electrochemical properties of the soil (soil factor), 4^{th} degree polynomial at room temperature but the A's constants are different for each electric quantity .The constants of each equation (A's) considered to be as a PRINT of such pipe-soil-earth system and valid until pipe and/or soil is changed with of course new print values. For buried bare pipe segments in different kind of soils with and without applying cathodic protection level, the PRINTS of the electrolytic stray capacitor between pipe & earth, the stray potential across the stray capacitance, surface charge and the protection current of the cathodic protection system passed through the pipe segment were obtained in terms of the new parameter, the soil factor. The useful of these PRINTS is to obtain complete electrical data correlated with many cathodic protection levels which help, after complete erection of the pipeline, in defining the c.p level of any pipe line segment through its length by measuring the protection current and calculating the soil factor at the pipe segment from direct field measurements. The average error of the electrical parameters equations reduced to be less than ± 5%. The most important advantage of such prospective electrical analogue circuit of pipe-soil-earth system is the possibility to simulate a complete pipeline – soil system by an electric circuit and to convert the corrosion problem and cathodic protection of the pipeline to an electric problem. In the near future after completing such electrical studies of the pipe-soil-earth systems, this will help in corrosion monitoring and the maintenance of c.p systems.

Acknowledgement:

First and foremost, thanks to GOD the most kind, the most merciful and to whom any success is related.

5. References:

1. Ashraf Abdelraouf M. Fouad Ahmed, A Proposed Systematic Concept to Evaluate the Performance of Cathodic Protection of Buried Pipe Line in the Soil, The Egyptian Society of Chemical Engineers, **TESCE**, Cairo, Egypt, 2003
2. Ashraf Abdelraouf M. Fouad Ahmed, A Proposed Systematic Concept to Evaluate the Performance of Cathodic Protection of Buried Pipe Line in the Soil, International conference "Future Vision and Challenges for Urban

Development", **HBRC** Cairo, Egypt, 2004
3. Ashraf Abdelraouf M. Fouad Ahmed, The General Equation of the Natural Stray Capacitance between External Surface of Bare Pipe Segment and Earth, 25th Annual Conference Corrosion Problems In Industry, **ECS** 2006.
4. Ashraf Abdelraouf M. Fouad Ahmed, The General Equation of the Natural Potential between External Surfaces of Bare Pipe Segment to Earth, 25th Annual Conference Corrosion Problems In Industry, **ECS** 2006.
5. Ashraf Abdelraouf M. Fouad Ahmed, Deduction of The Natural Created Charge General Equation For A Buried Bare Pipe Line In The Soil, 25th Annual Conference Corrosion Problems In Industry, **ECS** 2006.
6. Ashraf Abdelraouf M. Fouad Ahmed, The General Equation and The Print Curves of The Stray Potential of a Cathodically Protected Buried Bare Pipe Segment, Ain- Shams University third international conference on environmental engineering, Ain-Shams University, **ASCEE-3**, 2009.
7. Ashraf Abdelraouf M. Fouad Ahmed, The General Equation and The Print Curves of The Stray Capacitance of a Cathodically Protected Buried Bare Pipe Segment, Ain-Shams University, **ASCEE-3**, 2009.
8. Ashraf Abdelraouf M. Fouad Ahmed, The General Equation and The Print Curves of The Total Surface Charge of a Cathodically Protected Buried Bare Pipe Segment, Ain-Shams University, **ASCEE-3**, 2009.
9. Ashraf Abdelraouf M. Fouad Ahmed, The General Equation and The Print Curves of The Net Current Flow of a Cathodically Protected Buried Bare Pipe Segment, Ain-Shams University, **ASCEE-3**, 2009.

6/25/2010

A Systematic Approach for Mobile Agent Design Based on UML (Class and Sequence Diagrams)

M. S. Al_Kholy, A. R. Khalifa and M. G. Alsaied

Systems and Computer Engineering Department, Faculty of Engineering, Al-Azhar University, Cairo, Egypt

Abstract: Agent researchers are still trying to determine useful ways to represent agents and agent-based systems. So, this paper presents a proposal for a Systematic Approach for Agent Design by using a Unified Modelling Language (UML) diagram. Here we illustrate notions for the behavior of an agent using and extending UML class diagrams. Focus on representing the agent migration from take requests and between other hosts. In this case study, we explain one variant of notation that is the most suitable for given scenario, show that it is easier to design agent applications based on agent UML, by developing software for our case study generated by UML software package. [Journal of American Science. 2010;6(12):284-290]. (ISSN: 1545-1003).

Keywords: Mobile Agent Design, Class Diagram ,Sequence Diagram, UML, A Systematic Approach

1. Introduction:

Since a long time people have been using each other's and sometimes animals as their agents. Developments in information processing technology, computers and their networks, have made it possible to build and use artificial agents. These agents are the advanced tools that people can use to achieve different goals and to solve various problems. The main difference between ordinary tools and agents is that agents can function independently from those who delegated agency to the agents. Now, the most popular approach in artificial intelligence is based on agents. Intelligent agents form a basis for many kinds of advanced software systems that incorporate varying methodologies, diverse sources of domain knowledge, and a variety of data types. The intelligent agent approach has been applied extensively in business applications, and more recently in medical decision support systems [1, 2] as well as ecology [3]. In the general paradigm, the human decision maker is considered to be an agent and is incorporated into the decision process. The overall decision is facilitated by a task manager who assigns subtasks to the appropriate agent and combines conclusions reached by agents to form the final decision. This paper is structured as follows. Section 1 is this introduction. Section 2 gives the concept of agent (definitions). Section 3 represents related work that includes Historical overview and answer the question (why UML?). Section 4 shows the different UML diagrams and their applications for agent-based systems, basically concerning with class diagrams. Section 5 provides a case study with a searcher scenario. Section 6 represents a Class

Diagram for the Case Study. Section 7 concludes the paper.

2. The Concept of an Agent

There are several definitions of intelligent and software agents. Some of the major definitions and descriptions of agents are given as follows:

- Agents are semi-autonomous computer programs that intelligently assist the user with computer applications. This is achieved by employing artificial intelligence techniques to assist users with daily computer tasks, Such as reading electronic mail, maintaining a calendar, and filing information. Thus Agents can learn through example-based reasoning and can improve their performance over time.
- Agents are computational systems that inhabit some complex, dynamic environment, and sense, and thus act autonomously to realize a set of goals or tasks.Agents are software robots that think and act on behalf of a user to carry out tasks. An agent helps meet the growing need for more functional, flexible, personal computing and telecommunications systems. The usage of intelligent agents includes self-contained tasks, operating semi-autonomously, and communication between user and systems resources.
- Agents are software programs that implement user delegation. Agents manage complexity, support user mobility, and lower the entry level for new users. Agents are a design model similar to client-server computing, rather than being strictly a technology, program, or product [4].

- An agent is anything that can be viewed as perceiving its environment through sensors and acting upon that environment through effectors, (Russel and Norvig, [5]).
- Intelligent agents continuously perform three functions: perception of dynamic conditions in the environment; action to affect conditions in the environment; and reasoning to interpret perceptions, solve problems, draw inferences, and determine actions, (Hayes-Roth, [6]).
- Intelligent agents are software entities that carry out some set of operations on behalf of a user or another program, with some degree of independence or autonomy, and in so doing, employ some knowledge or representation of the user's goals or desires [7].
- People, animals, and robots are examples of physical agents. Software agents and Ego in the sense of psychoanalysis are examples of mental agents. The head of a Turing machine (cf., for example, Burgin, [2]) is an example of a structural agent.

3. Related Work

3-1 Historical overview

A considerable number of agent-oriented methodologies and tools are available today, and the agent community is facing the problem of identifying a common vocabulary to support them (for details see the work in [9], on which this section is based). There is a considerable interest in the agent R&D community in methods and tools for analyzing and designing complex agent-based software systems, including various approaches to formal specification (see [10] for a survey). Since 1996, agent-based software engineering has been in the focus of the ATAL Workshop series; it also was the main topic of the 1999 MAAMAW Workshop [11]. Various researchers have developed methodologies for agent design, touching on representational mechanisms, like the GAIA methodology [12] or the extensive program underway at the Free University of Amsterdam on compositional methodologies for requirements [13], design [14], and verification [15]. In [16,17], Kinny et al. propose a modelling technique for BDI agents. The close affinity between design mechanisms employed for agent-based system and those used for object-oriented systems is shared by a number of authors, for example, [18]. In particular, since 2000, the Agent-Oriented Software Engineering Workshop (AOSE) has become the major forum for research carried out on these topics, including new methodologies such as Tropos [19], Prometheus [20], and MESSAGE [21]. Currently, most industrial methodologies are based on the Object Management Group's (OMG) Unified Modelling Language (UML) accompanied by process frameworks such as the Rational Unified Process (RUP), see [22] for details. The Model-Driven Architecture (MDA [23]) from the OMG allows a cascade if code generations from high-level models (platform independent model) via platform dependent models to directly executable code. Another approach for agile software engineering that has been receiving active coverage is Extreme Programming [24].

The UML is a standard modelling language for visualizing, specifying, constructing, and documenting the elements of systems in general, and software systems in particular [25]. UML has a well-defined syntax and semantics. It provides a rich set of graphical artefacts to help in the elicitation and top-down refinement of object-oriented software systems from requirements capture to the deployment of software components.

In UML, systems can be modelled by considering three aspects, the behavioural, the structural and the architectural aspects; each aspect is concerned with both the static and dynamic views of the system. The static view represents a projection onto the static structures of the complete system description. However, the dynamic view represents a projection onto the dynamical behaviour of the system. Finally, views are communicated using a number of diagrams including information emphasizing a particular aspect of the system.

3-2 Why UML

As an OMG standard, UML 2.0 has been considered a "final" standard, as of November 2004 [26]. In other words, many of the errors and inconsistencies of the original submission have been rectified. More than 3000 issues were files and resolved by the UML 2.0 Finalization Task Force. As such, software vendors can begin to build software tools that support the UML 2.0 Superstructure and Infrastructure. In addition, a firmer foundation is now available to adequately support the extensions for agent-based system modelling. The FIPA Modelling Technical Committee [27] and the OMG Agent Special Interest Group are actively working on extending UML for agent-based system modelling. These efforts are primarily supported by the work of more than a dozen software tool vendors.

4. Agent modelling with (UML)

UML is adequate for modelling object-oriented (OO) systems. But UML lacks the capability to readily model and specify agent systems. Unlike [Odell 2001a]'s Agent UML, we feel that every component of the UML must be extended. UML has a long history and is the result of a standardization

effort on different modelling languages (like Entity-Relationship-Diagrams, the Booch-Notation, OMT, OOSE), namely Unified Modelling Language. The most popular versions of UML are UML 1.x, but now UML 2.0 is the upcoming new specification for development of systems. (UML) is a standard modelling language for visualizing (using the standardized graphic UML notations), specifying the static structure, dynamic behaviour and model organization as well as constructing system, by mapping UML to programming environment, generating some code automatically, and documenting every phase of the lifecycle from analysis and design through deployment and maintenance. UML consists of a notation, describing the syntax of the modelling language, a graphical notation, and a meta model describing the semantics of UML, namely the static semantics of UML, but no operational semantics. However, UML defines no software process, since a software process describes the development activities, the dependencies of these activities and how they are applied.

<<Agent>>

<<Host 1>> <<Host 2>>

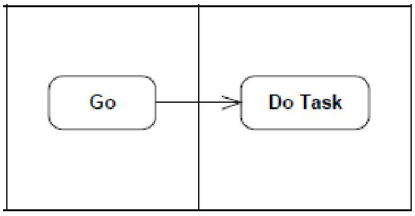


Figure1. Go action in UML

UML 2.0 supports the following diagrams: class, object, component, deployment and composite structure diagrams for modelling the static aspects of the systems and use case, state machine, sequence, activity, interaction overview, timing and communication diagrams for modelling dynamic aspects and packages, models and subsystems for modelling the model management [27]. Figure 1 shows an agent moving from location "host 1" to "host 2" and represented using "Go" activity.

Class diagram

In this section we focus on the first diagram (Class Diagram Figure 2) defined in the Superstructure Specification. We will use this distinction to present the diagram type and how it can be applied for modelling agent-based systems.

A Class Diagram describes on the one side a data model, i.e. collection of declarative (static) model elements, like classes and types, and on the other side their contents and relationships. Moreover the static structure of the system to be developed and all relevant structure dependencies and data types can be modelled with this class diagram [25]. They are applied in various phases of the project, e.g. analysis (conceptual modelling of the domain), design (platform independent description) of the implementation, detailed design (platform specific description) and to bridge the gap to the behavior diagrams. Class diagrams describe classes and interfaces with their attributes and operations, as well as associations between them (including aggregation and composition), but also generalization (a specific kind of inheritance) and dependencies among them. New to UML 2.0 is that attributes have ordering, graphical notations for associations are defined, graphical interface notation are introduced using lollipops, some unification on the notations for e.g. visibility, names and types has been done [26,28]. Moreover attributes have no implicit composition associations and dependencies are completely redefined. Class diagrams are illustrated in Figure 2. An agent model can be defined using class names, inheritance (generalization) of classes and adding name, type, position/role, capabilities and constrains, either directly or via associations. A role hierarchy can be defined using generalization. However, roles cannot be modeled in the necessary detail with any UML 2.0 diagram. Service models can also be done by this diagram type, e.g. defining services with input/output parameters and pre-/post-conditions as classes with attributes and functions (the service interface).

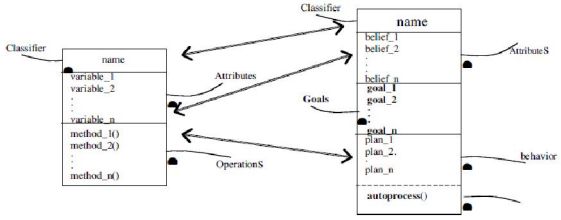


Figure2. Specifying agent behaviour using UML class diagram

5. Case Study: Book Searcher

The case study includes three network nodes: Home, Host 1 (British Library) and Host 2 (Congress Library) Figure 3. On Host 1 and Host 2 resides library agent, which is responsible for providing the books List. The searcher agent is created on the Home node. The input parameter is the item. The Searcher agent migrates from home node to Host1 node and requests library1 agent to give the books list. The library1 agent responds with the whole books list. The searcher extracts the book and migrates to the next node. After visiting all nodes the

Searcher agent migrates back to the Home node and informs the user where it has found the specified item.

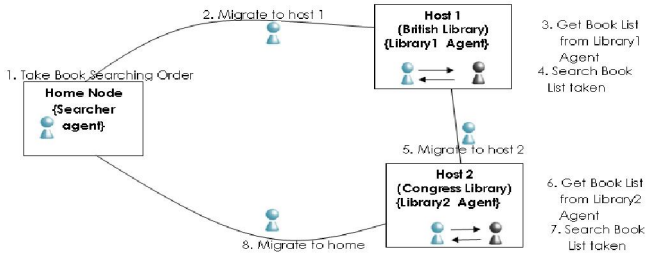


Figure3. Book searcher scenario

The mobile agent one-to-one relationship is the simplest; where the mobile agent (library agent) is placed between two negotiators (user searcher agent and the library) in this case. Similarly, one-to-many and many-to-one relationships; where the mobile agent (library agent) is placed between one negotiator at one side and more than one negotiator at the other side (a user searcher agent and more than one library) in this case.

The user inputs his demand through the Graphical User Interface (GUI) where it is going to be placed as a search_query. The user searcher agent then scans the network in order to build a list of available libraries.

The user searcher agent then takes the search_query and starts the journey by visiting the first library in the list.

Before the user searcher agent can reach the server of the library, it must pass the library's security check. While the user searcher agent enquires about the book needed, a local library agent, residing in the library server, is activated. There will be two scenarios with respect to the library: book found and book not found. The local library agent returns the results to the user searcher agent if the book is found then terminates the communication with the user searcher agent. If the book is not found, then the local library agent informs the user searcher agent that the book wasn't found and then terminates the communication with the user searcher agent. The user searcher agent then follows the itinerary and moves to the next library. Finally, the user searcher agent returns back to the user with the librarie's list where it found the book needed.

6. Sequence Diagram for the Case Study:

Before the user searcher agent can reach the server of the library, it must pass the library's security check. While the user searcher agent enquires about the book needed, a local library agent, residing in the library server, is activated. There will be two scenarios with respect to the library: book found and book not found. The local library agent returns the results to the user searcher agent if the book is found then terminates the communication with the user searcher agent. If the book is not found, then the local library agent informs the user searcher agent that the book wasn't found and then terminates the communication with the user searcher agent. The user searcher agent then follows the itinerary and moves to the next library. Finally, the user searcher agent returns back to the user with the libraries list where it found the book needed figure 4 illustrate Sequence diagram for case study.

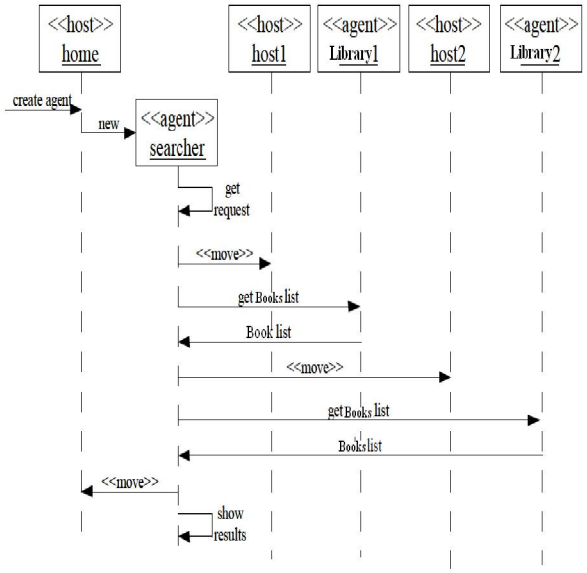


Figure. 4 Agent-Sequence diagrams applied to example

7. Class Diagram for the Case Study

In this section we show how usual UML class diagrams can be used and extended in the framework of agent oriented programming development. We will use the following notation to distinguish between different kinds of agent classes and instances. The first one denotes some agent class, the second some agent class satisfying distinguished roles and the last one defines some agent instance satisfying distinguished roles. The roles can be neglected for agent instances. According to the statement given above what has to be specified for agent classes we specify agents by the agent class diagram.

The usual UML notation can also be used to define such an agent class, but for more understandable reasons we have introduced the above

notation. Using stereotypes, an agent class written as a class diagram can look as shown in Figure.2.

The Class and the Activity diagrams are generated as the static and dynamic aspects of objects by represented the attributes and operations of the object. Figure 5 shows the Class diagram and Activity diagram applied to our example. The Activity diagram shows how to search the information and find the best solution. In the Class diagram, there are four classes for our problem. Each class has attributes and operations, showing their roles as follows:

User_Interface class:

- read_search_query: This method is for reading the search criteria from the user through the GUI of the searcher agent.
- display_results: This method is for displaying the results found.
- trace: This method is for displaying any messages.

Agent Class:

- start_agent: This method is for starting the user searcher agent.
- stop_agent: This method is for stopping the user searcher agent after accomplishing the task.
- terminate: This method is for ending the code.

Agent_Control Class:

- scan_network: This method is for scanning the network to find the libraries servers.
- return_results: This method is for sending the results to the user.
- stop_agent_control: This method is for ending the Agent Control.

Library_Agent Class:

- start_agent: This method is for starting the library agent.
- stop_agent: This method is for stopping the library agent after accomplishing the task.
- find_item: This method is for searching the library server's database for the book needed.
- return_results: This method is for sending the results to the user searcher agent.
- terminate_communication: This method is for ending the communication between the library agent and the user searcher agent.
- inform_termination: This method is for informing the user searcher agent that communication is terminated with the library agent.

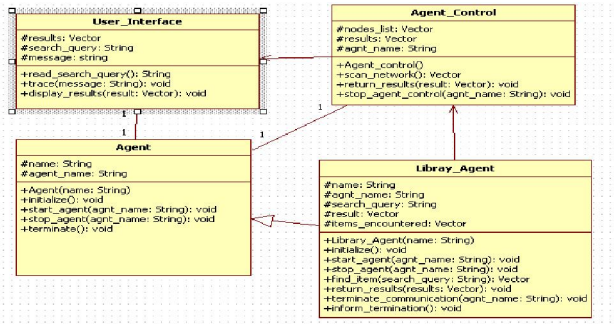


Figure. 5 Agent-class diagrams applied to example

8. Evaluation and Conclusion

This paper presents a Systematic Approach for Agent Design to support the modeling and the implementation of an agent using UML profile which defines a class diagram. From the end user's perspective, the goal is to provide a personal travel assistant, i.e., a software agent that uses information about the users' schedule and provides preferences in order to assist users in travel, including preparation as well as on-trip support. This requires providing ubiquitous access to assistant functions for the user, in the office, at home, and while on trips, using PCs, notebooks, information terminals, PDAs, and mobile phones.

The requirements for artifacts to support the analysis and design became clear, and the material described in this paper has been developed incrementally, driven by these requirements. So far, no empirical tests have been carried out to evaluate the benefits of the Agent UML framework. However, from this paper, we see two advantages as a result: First, they make it easier for users who are familiar with object-oriented software development but new to developing agent systems to understand what multi agent systems are about, and to understand the principles of looking at a system as a society of agents rather than a distributed collection of objects. Second, our estimate is that the time spent for design can be reduced by a minor amount, which grows with the number of agent-based projects. However, we expect that as soon as components are provided to support the implementation based on Agent UML specifications, this will widely enhance the benefit. In our work we use the star UML package to develop software for our case study by generating a code from star UML software package. This software can generate a code by more than one languages such as Java, C++, and others.

As for future work, we are looking forward to implement MA-UML diagrams. Also we plan to the

design and implement of a mobile agent security based on A Systematic Approach for modelling Agent Mobility with other UML Diagrams.

Corresponding Authors
M. S. Al_Kholy
Systems and Computer Engineering Department, Faculty of Engineering, Al-Azhar University, Cairo, Egypt

Acknowledgement

The authors would like to thank Prof. Dr. Mohamed Zaki, for his leadership, continued help, support, and encouragement. We especially are grateful to him for sharing his knowledge, experience, and expertise.

9. References

1. Hsu C., Goldberg H.S.: Knowledge-mediated retrieval of laboratory observations, Proc. JAMIA, v.23:809--813(1999)
2. Lanzola G., Gatti L., Falasconi S., Stefanelli M.: A framework for building cooperative software agents in medical applications, Artificial Intelligence in Medicine, 16, 223--249. (1999)
3. Judson, O.P.: The Rise of the Individual-based model in Ecology, Trends in Ecology and Evolution, 9, 9-14 (1994).
4. Jansen, J.: Using Intelligent Agents to Enhance Search Engine Performance, Firstmonday, No.2/3, http://www.firstmonday.dk (1996)
5. Russel, S.J. and Norvig, P.: Artificial Intelligence: A Modern Approach, Prentice-Hall, Englewood Cliffs, N.J. (1995).
6. Hayes-Roth, B.: An Architecture for Adaptive Intelligent Systems. Artificial Intelligence: Special Issue on Agents and Interactivity, 72, 329-365 (1995).
7. IBM's Intelligent Agent Strategy white paper, http://activist.gpl.ibm.com:81/WhitePaper/ptc2.htm
8. Burgin, M.: Super-recursive Algorithms, Springer, New York/Berlin/Heidelberg (2005).
9. Bauer, B. and Müller, J.P.: Methodologies and Modeling Languages, in: Agent-Based Software Development, Luck M., Ashri R. D'Inverno M. (eds.) Artech House Publishers, Boston, London, 2004.
10. C. A. Iglesias, M. Garijo, and J. C. González. A Survey of Agent-Oriented Methodologies. Proceedings of Fifth International Workshop on Agent Theories, Architectures, and Languages, pages 185-198, University Pierre et Marie Curie, 1998.
11. F. J. Garijo, and M. Boman. Multi-Agent System Engineering. Proceedings of MAAMAW'99. Springer, ed., 1999.
12. M. Wooldridge, N. R. Jennings and D. Kinny. The Gaia Methodology for Agent-Oriented Analysis and Design. International Journal of Autonomous Agents and Multi-Agent Systems, 3, 2000.
13. D. E. Herlea, C. M. Jonker, J. Treur, and N. J. E. Wijngaards. Specification of Behavioural Requirements within Compositional Multi-Agent System Design. Proceedings of Ninth European Workshop on Modelling Autonomous Agents in a Multi-Agent World, pages 8-27, Springer, 1999.
14. F. M. T. Brazier, C. M. Jonkers, and J. Treur. Principles of Compositional Multi-Agent System Development. Proceedings 15th IFIP World Computer Congress, WCC'98, Conference on Information Technology and Knowledge Systems, IT&KNOWS'98, pages 347-360, Chapman and Hall, 1998.
15. C. M. Jonker, and J. Treur. Compositional Verification of Multi-Agent Systems: a Formal Analysis of Pro-activeness and Reactiveness. Proceedings of International Workshop on Compositionality (COMPOS'97), Springer, 1997.
16. D. Kinny, M. Georgeff, and A. Rao. A Methodology and Modelling Technique for Systems of BDI Agents. 7th European Workshop on Modelling Autonomous Agents in a Multi-Agent World (MAAMAW'96)., pages 56-71. Springer, 1996.
17. D. Kinny, and M. Georgeff. Modelling and Design of Multi-Agent Systems. Intelligent Agents III, Springer, 1996.
18. Birgit Burmeister: Models and methodology for agent-oriented analysis and design. In Working Notes of the KI'96 Workshop on Agent-Oriented Programming and Distributed Systems, 1996. DFKI Document D-96-06.
19. A. Th. Schreiber, B. J. Wielinga, J. M. Akkermans, and W. Van de Velde. CommonKADS: A comprehensive methodology for KBS development. Deliverable DM1.2a KADSII/M1/RR/UvA/70/1.1, University of Amsterdam, Netherlands Energy Research Foundation ECN and Free University of Brussels, 1994.
20. Prometheus home page: http://www.cs.rmit.edu.au/agents/SAC/methodology.shtml.
21. MESSAGE web site: http://www.eurescom.de/public/projects/P900-series/p907/

22. Ivar Jacobson, Grady Booch, James Rumbaugh: The Unified Software Development Process, Addison Wesley, 1998.
23. Model-driven Architecture: http://www.omg.org/mda/.
24. Beck, K. Extreme Programming Explained. Addison Wesley, 1999.
25. G. Booch, J. Rumbaugh, I. Jacobson, The Unified Modeling Language User Guide, Addison-Wesley, Reading, MA, 1998.
26. OMG, Unified Modeling Language: version 2.0 (UML 2.0), Final Adopted Specification, 2003, http://www.uml.org/#UML2.0
27. FIPA Modeling TC, FIPA Modeling Area: Deployment and Mobility,2003/05/13, http://www.auml.org/auml/documents/DeploymentMobility.zip
28. Miao Kang, Lan Wang, and Kenji Taguchi, Modelling Mobile Agent Applications in UML 2.0 Activity Diagrams, 2004/04/21, http://www.auml.org/auml/supplements/UML2-AD.pdf

6/21/2010

EFFECT OF VANADIUM TOXICITY IN *CLARIAS LAZERA*

Mona S. Zaki[1]; Nevin E.Sharaf[2] and Mostafa H. Osfor[3]

[1]Head of Department of Hydrobiology, National Research Center, Cairo, Egypt.
[2]Department of Environmental and Occupational Medicine, National, Research Center, Cairo, Egypt
. [3]Department of Nutrition, National, Research Center, Cairo, Egypt
dr_mona_zaki@yahoo.co.uk

Abstract: The effect of dietary carbohydrates and vanadium toxicity on haematalogical profile, blood chemistry and hormonal level was studied in fry *Clarias Lazera*. Fry fish were divided into 3 groups (n=10) and exposed to different doses of vanadium sulfated and carbohydrate. Group1 was served as control, group 2 was fed with carbohydrate and vanadium sulfate (10 mg/ Kg diet ration), group 3 was fed with carbohydrate and vanadium sulfate (15 mg/Kg diet ration). There is a significant decrease in hemoglobin and P.C.V in group (3). There is a significant increase in serum cortisol, cholestrol, AST, ALT, urea, creatinine and alkaline phosphatase in group (3), also there is a significant decrease in serum phosphorous, sodium and potasium in treated fish. There is a significant high level of vanadium content in kidney muscles, heart and spleen in group (3) suggesting toxic effects of vanadium on cat fry fish *Clarious Lazara.* The total viable count of bacteria identified higher in fry fish fed on carbohydrate vandium. Predominate bacteria were identified as *Aeromonas, E. coli, Staph aureus. Pseudomonas, Fluorscences* and *Lacto bacilus* species. We emphasize the finding that increase in carbohydrate concentration causes harmful pathological effects which reduces humoral immune responses and enhances dietary vanadium toxicity. [Journal of American Science. 2010;6(12):291-296]. (ISSN: 1545-1003).

Keywords: Fry *Clarias Lazera,* Vanadium Pollution, Haematological, Biochemical, Clinicopathological, Bacterial count.

1. Introduction

Fry fish plays an important role, not only in human food diets but also in animal and poultry rations. It is a palatable and easily digested food which is rich in vitamins, calcium, phosphorus and iodine. In Egypt, fry fish is considered as a cheap food article if compared with other foods of animal origin. The flesh of healthy fry fish is considered as a marker for the natural aquatic environment.

Vanadium is a rare element found combined in certain minerals and used mainly to produce certain alloys. Most of the vanadium (about 80%) produced is used as ferrovanadium or as a steel additive. Mixed with aluminium in titanium alloys is used in jet engines and high speed air-frames, and steel alloys are used in axles, crankshafts, gears and other critical components. Vanadium oxide (V_2O_5) is used as a catalyst in manufacturing sulfuric acid and in making ceramics. It is added to glass to produce green or blue tint [1].

Vanadium was first discovered in 1971 as a trace element that is essential for normal growth. Since then, vanadium has been found to regulate the activity of various enzymes that induce pronounced changes in metabolic functions.

Vanadium is never found unbound in nature, Vanadium occurs in carbon containing deposits such as crude oil, coal, oil shale and tar sands. Vanadium is abundant in most soils, in variable amounts, especially in areas where chemicals or petrochemicals complex were located, where these areas showed a significant increase in its concentration [2].

Humans may be exposed to excessive vanadium in several situations for example, overconsumption of vanadium-rich foods (e.g: seafood) [3], ingestion of certain dietary regimens specially that of body building, or inhalation of vanadium-rich environmental pollutants in certain occupations including boilermakers and power plant workers, who are often exposed to high levels of vanadium-rich compounds at work.

Because vanadium is vasoactive, individuals exposed to excessive vanadium may develop adverse vascular effects [4] especially pulmonary vascular diseases [5] as well as nanoparticulate of vanadium oxide potentiated vanadium toxicity in human lung cells [6] and Nickel and vanadium rich pollutant dust could be responsible for the respiratory problems reported [7].

Chronic exposure to vanadium pentoxide dust and fumes may cause severe irritation of the eyes, skin, upper respiratory tract, persistent inflammations of the trachea and bronchi, pulmonary edema, and systemic poisoning. Signs and symptoms of overexposure include; conjunctivitis, nasopharyngitis, cough, labored breathing, rapid heart beat, lung changes, chronic bronchitis, skin pallor, greenish-black tongue and an allergic skin rash [1] and [7]

In animals, vanadium causes the inhibition of certain enzymes, which has several neurological effects. Next to the neurological effects vanadium can cause breathing disorders, paralyses and negative effects on the liver and kidneys. Laboratory tests with test animals have shown, that vanadium can cause harm to the reproductive system of male animals, and that it accumulates in the female placenta. Vanadium can be found in fry fishes and many other species. In mussels and crabs vanadium strongly bioaccumulates, which can lead to concentrations of about 10^5 to 10^6 times greater than the concentrations that are found in seawater [8].

In recent years, much attention had been paid to the possible danger of metals poisoning in human as a result of consumption of contaminated fry fishes. So, the present study was carried out to elucidate the impact of vanadium on cat fry fish *Clarias Lazera*. Its haematological, biochemical and hormonal parameters were studied as well as the bacteriological and clinicopathological investigations.

2. Material and Methods

Experimental design

Thirty cat fry fish fry *Clarious Lazera* were used to assess the effect of vanadium sulfate. Fry fish weighing from 15-25g were obtained from Nile River and were kept in glass aquaria supplied with dechlorinated tap water at rate of one liter for each cm of fry fish's body. Fry fish were acclimated to the laboratory conditions for two weeks before the beginning of the experiment, they were fed with a commercial fry fish diet [9], the experiment was determined after 4 weeks. Fry fish were divided into 3 groups (n=10) and exposed to different doses of vanadium sulfated and carbohydrate. Group1 was served as control, group 2 was fed with carbohydrate and vanadium sulfate (10 mg/ Kg diet ration), group 3 was fed with carbohydrate and vanadium sulfate (15 mg/Kg diet ration). Mean of the initial body weight of each examined fry fish was at the beginning of the experiment then after 2-4 weeks of exposure.

Blood samples

Blood samples were collected from the caudal vein after 4 weeks of exposure. Each sample was divided into two parts the first one was heparinized for haematological investigations, while the second was centrifuged at 3000 rpm for 5 minutes to obtain serum for biochemical studies.

Hematological Analysis:

Haematological studies were performed according to Sandnes *et al* 1988 [10], where blood haemologlobin (Hb) and haematocrit (Ht) values were evaluated.

Biochemical Analysis:

The activities of alkaline phosphatase, aspartic aminotransferase (AST) and alanine aminotransferase (ALT) as well as cholesterol, urea and creatinine level were determined according to the method of Varley *et al*. [11] by using commercial kits (Bio Merieus, France).

Total serum protein was estimated according to Drupt [12]. Serum cortisol was analyzed by a Gamma counter using 125 I cortisol radioimmunassay Kit (Baxter Health Care Corporation USA) according to the method described by Pickering and Pottinger [13]. Potassium, Sodium and Phosphorous concentrations were determined by atomic absorption spectrophotometry [11].

Tissue analysis:

Liver, kidney and spleen samples were washed with distilled water then dried in hot air oven, sulphuric acid and hydrogen peroxide were added on samples then heated until the mixture became transparent after performing a wet ash digestion according to the method of Issac and Kerber [14].

Identification of bacteria:

The liver, kidney, spleen, muscle, stomach and gill from each examined fry fish were diluted immediately after sampling in sterile 0.9% saline and 0.1 ml volumes of appropriate dilutions and were spread over the surface of the typtic soy agar (oxoid). The plates were incubated at 22°C and inspected daily for up to 4 weeks.

The isolates were classified and identified according to Stevenson [15] and Quinn *et al*. [16].

The data were evaluated statistically according to Gad and Weil [17].

Water samples

Two water samples were collected from River Nile (Helwan) as well as two water samples

free from any heavy metal pollution El-Kasr El-Eini (control) were analyzed for vanadium concentration by atomic absorption spectrophotometry.

3. Results and Discussion:

Data in table 1 showed that, the vanadium level in Helwan region was clearly higher than the maximum allowable concentration for human consumption as recommended internationally according to WHO (World Health Organization) . Nadal et, al.concluded that the occurrence of vanadium in nature and its use in various industrial processes has increased its inputs in the environment [2]. From the present study it is clear that the low vanadium levels were reported in water samples collected from areas far from industrial discharges, while high vanadium levels in the present study may be due to the collection of samples from areas subjected to industrial pollution.

In table 3 there is a significant decrease in body weight in group 3 (fry fish fed 15 mg vanadium for 4 weeks) than in group 1 (control) and group 2 (fry fish fed 10 mg vanadium), this results agree with that report [18].

The results present in table 6 showed the comparison of cholesterol levels between groups. The level was significantly increased in group 3 (fry fish fed on 15 mg vanadium) than in group 1(control). Hyperchrolestremia might be due to necrotic changes occurring in liver with liberation of cholesterol as a by-product of cell destruction. The present data suggest that impaired liver function lead to increased serum levels of alkaline phosphate, AST and ALT among group 3 (fry fish fed on 15 mg vanadium) and among group 2 (fry fish fed 10 mg vanadium) compared to group1 (control). In this concern Khalaf-Allah concluded that ALT and AST enzymes are good indices for the health status of liver parenchymatous, tissue necrosis is considered as the main source of AST and its increase in the serum of cat fry fish Clarias Lazera declared these necrotic changes [18]. In addition, exposure of fry fish to environmental pollutants might result in stimulation or depression of the enzyme activity depending on the concentration of pollutant and the duration of exposure [19] and [20].

Regarding the effect of vanadium on serum cortisol level in cat fry fish Clarias Lazera, highest level was obtained in group 3 (fry fish fed on 15 mg vanadium) then in group 2 (fry fish fed on 10 mg vanadium) as compared to that obtained in group 1 (control) . The significant increase of cortisol level is probably due to the activation of hypothalamus pituitary internal axis [21].

From the data present in table 6, it is clear that elevation of vanadium level in the diets fed to Clarias Lazera was positively correlated to hemoglobin (Hb) levels and haematocrit (Ht). A marked decrease in the Hb and Ht was recorded after feeding diet containing 15 mg and 10 mg vanadium, respectively. Reduced Hb may reflect metabolic adjustment according to reduced need for oxygen by change in blood pH.

Moyle and Ceeh, Hall and Cliffs recorded, actived acetylcholinesterase of erythrocytes [22], [23]. Further more, Pickering and Dusten concluded that a consistent effect of cortisol was the reduction in the hemoglobin and iron levels as a result of decrease in appetite in rainbow trout fry fish or more likely to be the direct result of catablolic effect of cortisol in the fry fish tissues [24].

The mean phosphorus, sodium and potasium values in the serum of fry fish of group 3 (fry fish fed 15 mg vanadium) were significantly increased respectively than those recorded in the group 1 (control). This retention may be attributing to kidney dysfunction, whereas, the kidney is the normal pass for sodium and potassium. This kidney dysfunction may also explain the increase in serum urea and creatinine especially in group 3, but little known about the mechanisms involved in this association.

The results displayed also in table 6 showed that, there was general decrease in the mean total protein value in serum samples collected from the fry fish of group 3 and 2, respectively. The mean value of these parameters was lower than in group 1. Jagadeesh et al. estimated marked decrease in glycogen in tissues of fresh water fry fish after exposure to vanadium [25].

This experiment showed that the body weight of the examined fry fish was significantly decreased than the initial body weight after 4 weeks of exposure to 15 mg Vanadium. Also, Hilton and Better recorded a significantly reduced growth and increased mortality among the fry fish feeding diets of Vanadium (0,10,100,1000 or 10000 mg/kg) [26]. The increase in muscles and tissue lactic acid (2-12 fold) in association with decrease in pyruvic acid (72% in muscles +26% in liver) reflect a shift towards an anaerobic metabolism of fry fish following long term exposure to vanadium. [26]

Table 4 showed that, the bacterial isolates and counts were increased by feeding the fry fish with CHO and vanadium. The carbohydrates affect immunity and resistance to infection as recorded by Waagbo et al. [9]. Utility of vanadate mimetic protein phosphates inhibitors, to protect fry fish from microorganism [27]. The increase of bacterial count among the fry fish fed on vanadium may be related to the increased level of cortisol which decreases the host immunity.

In the course of experiment, a high concentration of vanadium levels has been found in

kidney, liver, spleen, heart and muscles of cat fry fish Clarias Lazera fed 15 mg vanadium table 5. This suggests that these organs could be useful as a marker for vanadium in the aquatic environment. In this concern, Ray et al. recorded a high concentration of vanadium in kidney, liver and other organs of cat fry fish as the concentration of vanadium in the tissues increased with its concentration in the aquatic environment and exposure time [28]. After exposure of fry fish to increased doses for 4 days, the vanadium content increase in the muscle then increased in all tissues [20], [25] and [26]. The capability of vanadium to be present in fry fish muscles is of particular interest in assessing the exposure of man to environmental vanadium as ingested by food.

Clinicopathological observations:

Abnormal swimming, lighting of the skin, scale loss and haemorrhages, were seen on the external body surface. In addition to congestion of gills, eyes, mouth, liver, kidney, spleen and intestine. This was noticed in fry fish exposed to vanadium sulphate 15 mg (group 3) but not in fry fish exposed to vanadium sulphate 10 mg (group 2)

In conclusion we emphasize that, the reported finding increase of carbohydrate concentrations causes harmful physiological effects, reduces humoral immune responses and enhances dietary vanadium toxicity.

Table 1: Vanadium concentration in water samples collected from two areas in Egypt.

Areas	No	Concentration of Vanadium p.p.m.
Helwan	1	1.04
	2	1.27
Al- Kasr El- Aini	3	0.154
	4	0.163

Table 2: Ingredients and Proximate composition of diets used in the experiment with vanadium supplementation.

Ingredient%	Diet, control	Diet 2	Diet 3
Fish meal	25	25	30
Meat and bone meal	5	5	10
Wheat bran	20	20	20
Skimmed milk	12	12	7
Yeast	10	10	15
Starch	-	10	15
Cod liver oil	2	2	2
Vitamin premix	1	1	1
Vanadium Mg	-	10	15
Crude protein%	40.35	35.95	38.89
Metabolizable energy k cal /kg	2205.4	2551.78	2315.4
Ether extract %	4.29	4.21	2.86
Crude fiber %	4.46	3.73	4.27
Ash %	5.65	6.26	10.25
Lysine %	2.13	1.88	2.29
Methionine %	0.62	0.55	0.613

Mineral and vitamin premix per/Kg of pellet food.

Vit A, 8000 g/u, vit D 900 g/u, vit E 2/u, vit K 4 mg, vit B2 3.6, niacin 20 mg., pyridoxine 0.2 mg, vit B125, Mn 70 mg, Sn 60 mg.

Table 3: Changes in body weight in cat fish fry *(Clarias Lazera)* fed on different levels of dietery carbohydrates in addition to vanadium sulphate.

Groups	Group 1	Group 2	Group 3
Intial body weight g	15±0.15	20±0.16	32±012
After 2 weeks g	18±0.45	30±0.23	38±0.7
After 4 weeks g	14±0.27	22±0.63	38±0.8*

$P < 0.01$

Table 4: Bacterial isolates recovered from the examined fry fish.

No of examined fish 10 /group	Bacterial isolates	**Site of isolation**	**Bacterial count**
Group 3	-Aeromonas sp - E. coli - Staph Aureus - E. coli - Aeromonas - lactobacillus	-Skin -Skin - External surface, Stomach - Gills - Gills, stomach - Gills	2 x10^3 ------ 2x10^2 ------- 1 x 10^3 ---------
Group 2	-Enterbacter sp. -Pseudomonas -Fluroscences -Lactobacillus	-Skin -Spleen, muscles -Stomach -Skin	1 x 10^2 2 x10^3 1 x 10^6 ----------

Table 5: The mean vanadium concentation in the organs of the fry fish mg/g net weight.

groups	muscles	spleen	heart	kidneys	Liver
Group 1	0.18±0.13	0.30±0.82	0.28±0.18	23.15±0.72	1.17±0.59
Group 2	0.17±0.24	0.20±0.70	0.13±0.20	4.0 0±0.83	2.10±0.60
Group 3	0.28±0.27*	0.12± 0.40*	0.17±0.12*	6.00 ± 0.74*	5.21±0.15*

p < 0.01 * Significant

Table 6: Some haematological, biochemical and hormonal parameters in cat fry fish *Clarias Lazera* fed on different levels of dietery carbohydrates in addition to vanadium sulphate.

Groups Parameters	Group 1	Group 2	Group 3
Hemoglobin g/dl	7.5±0.20	7.52±0.14	6.10±0.12*
H.CT %	36.00±0.26	37.4±0.13	30.5±0.20*
Cortisol ng/ml	0.72±0.20	0.83±-0.08	1.30±0.66*
Phosphorous mg/dl	9.5±0.64	9.2±0.27	8.11±0.65*
Soduim M.E.Q	121±1.24	115±0.75	102±0.14*
Potasuim M.E.Q	7.0±0.82	7.03±0.44	6.4+/-0.74*
AlkPhosphatase U/L	21.5±3.2	22±0.60	27±0.70*
AST U/L	124±L40	131±0.85	144±0.25*
ALT U/L	22±0.15	24±0.70	37±0.20*
Cholestrol mg	144±0.25	149±0.13	170±0.54*
Total protein g/dl	9.20±0.75	9.01±0.80	8.01±0.62*
Urea mg/dl	3.3±0.68	3.3±0.78	4.8±0.20*
Creatinine mg/dl	0.77±03	0.73±0.75	0.92±0.42*

P < 0.01 * Significant
n = 30 Fish 10 / group.

References

1. Copyright © 1998-2007 Lenntech Water treatment & air purification HoldingB.V.Rotterdamseweg 402 M 2629 HH Delft, The Netherlands e-mail: info@lenntech.com
2. Nadal M., Schulmacher M., Domingo J.L. 2007: Levels of metals, PCB's PCN's and PAH's in

soils of highly industrialized chemical/petrochemical area, chemosphere, 66, 267-76.
3. Bu- Olayan A.H. and Al-Yakoob S. 1998: Lead, Nickel and Vanadium in sea food an exposure assessment for Kuwait consumers. Sci Environ. 2-3, 81-86.
4. Huang Y.C., Ghio A.J. 2006: Vascular effects of ambient pollutant particles and metals, curr. Vasc. Pharmacol; 4(3): 199-203.
5. Li Z., Carter J.D., Dailey L. A., Huang Y.C.T. 2004: 4-Vanadyl sulfate inhibits NO production via threonine phosphorylation of eNOS, Environ Health Perspect 112:201-206.
6. Worle J.M., Kern K., Schelh C., Helmy A.C., Feldman C. and Krug H.F. 2007: Nanoparticulate vanadium oxide potentiated vanadium toxicity in human lung cells, Environ. Sci. Technol., 41,331-6.
7. Figuero D.A., Rodriquez-Sierra C.J. and Jimenez-velez B.D. 2006: Toxicol & health, 22; 87-99
8. Bu- Olayan A.H. and Subranmanyam M.N.V. 1996: Trace metal in fish from Kuwait coast using microwave acid digestion technique. Environ-Inter., vol. 22,753-758.
9. Waagbo R., Glette J., Sandnes K.and Hemre G.F., 1994: J. Fish Dis., 17, 245.
10. Sandnes K., Lee Q., and Waagabo R., 1988: J. of Fish Biol., 32.129
11. Varley, H., Gwenbek, A. H. and Bell, M. 1980: Practical clinical chemistry, vol. I General I top's commener test, 5th ed. London, William medical books Ltd.
12. Drupt F. 1974: Pharm Biol, 9,77.
13. Pickering A.D. and Pottinger J. 1983: Gen Comp Ender., 49.232
14. Issac R.A. and kerber 1971: Amer Madison. 17
15. Stevenson P. 1987: "Field Guide Systematic Bacteriology". University of Cuelphontaria, Canada pp. 280.
16. Quinn PJ, Carter M.E., Markey B.K. and Carter G.R. 1994: "Clinical Veterinary Microbiology", Wolf Publishing Mosby, Year Book Europe limited.
17. Gad S.C. and Weil C.S. 1986: Statistics for toxicologists. In, Hages A.W. (2nd ed.), "Principles and Methods of toxicology", Raven Press, New York, PP. 273-32.
18. Khalaf-Allah 1998: Screening the effect of water pollution with some pesticides on the immune response in Oreochromis Niloticus fish. Vet. Mid. J. Giza; 46 (4-3); 883-393.
19. Venberg F.G. and Venberg W.B. 1974: Pollutiion and Physiology of marine organisms. Academic Press. New York. pp. 59.
20. Edel J. and E. Sabioni J. 1993: Accumulation and distribution of mussel myailus edulis and the gold fish carassius auratus. Sci.total-Environ. 133 (12), 139-151.
21. Carballo M.J., M.J., Torroba, C.Munoz, D.V.Sanchez, J. D. Tarazona, and J.Dominguez 1992:J. Fish and Shell Fish Immunology. Z, 121
22. Moyle P.B. and Cech J.J. 1982: Blood and Its Circulation in Fish-An Introduction to Ichthyology" (Ed. by, P.B. Moyle, J.H. Cech) pp-52-73 Prentice.
23. Hall I. and Cliffs E. 1982: Aspects and energy response in an Indian catfish Batrachus, Biometais. 11, 95-100.
24. Pickering A.P. and Duston J. J. Fish Biol., 23, 163, (1983).
25. Jagadeesh K.B., Shaffi S.A. and Jeelani S. 1989: Acta Physiologia Hungarica, 74, 43 .
26. Hitton J.W.and Bettgeo W.G. 1988: Aquatitic Toxicology. 12,63 .
27. Evans-Donald L. and Jaso. Friedmann L. 2001: Protection of Teleost fish. Biotclr, Nav. 15, 777.
28. Ray D., Panerjeo S.K. and Chatterjee M. J.1999: Bioaccumulation of Nickel and Vanadium in tissues of Catfish Batracchus. J. Inorg-Biochem 38, 169-173.

6/22/2010

Cubic Nonpolynomial Spline Approach to the Solution of a Second Order Two-Point Boundary Value Problem

[1]W.K. Zahra, [2]F.A. Abd El-Salam, [2]A.A. El-Sabbagh and [2]Z.A. ZAki*

[1]Department of Engineering Mathematics and Physics, Faculty of Engineering, Tanta University, Tanta, Egypt
[2]Department of Engineering Mathematics and Physics, Faculty of Engineering, Benha University, Shoubra, Cairo, Egypt.
Zahmed_2@yahoo.com*

Abstract: Third and fourth order convergent methods based on cubic nonpolynomial spline function at midknotes are presented for the numerical solution of a second order two-point boundary value problem with Neumann conditions. Using this spline function a few consistency relations are derived for computing approximations to the solution of the problem. Convergence analysis of these methods is discussed two numerical examples are given to illustrate practical usefulness of the new methods. [Journal of American Science. 2010;6(12):297-302]. (ISSN: 1545-1003).

Keywords: Cubic nonpolynomial spline; two-point boundary value problem; Neumann boundary conditions.

1. Introduction:

In approximation theory spline functions occupy an important position having a number of applications, especially in the numerical solution of boundary-value problems. We shall consider a numerical solution of the following linear second order two-point boundary value problem, see [5].

$$y^{(2)} + f(x)y = g(x), \quad x \in [a,b] \qquad (1.1)$$

Subject to Neumann boundary conditions:

$$y^{(1)}(a) - A_1 = y^{(1)}(b) - A_2 = 0 \qquad (1.2)$$

Where A_i , $i = 1, 2$ are finite real constants. The functions $f(x)$ and $g(x)$ are continuous on the interval $[a,b]$. The analytical solution of (1.1) subjected to (1.2) cannot be obtained for arbitrary choices of $f(x)$ and $g(x)$.

The numerical analysis literature contains little on the solution of second order two-point boundary value problem (1.1) subjected to Neumann boundary conditions (1.2).while The linear second order two-point boundary value problem (1.1) subjected to Dirichlet boundary conditions solved by different types of spline functions, see [1, 7, 8, 9].

Ramadan et al. [5] solved the problem (1.1) subjected to (1.2) using quadratic polynomial spline, cubic polynomial spline and quadratic nonpolynomial spline at midknotes.

In this paper, we develop cubic nonpolynomial spline at midkontes to get smooth approximations for the solution of the problem (1.1) subjected to Neumann boundary conditions (1.2).

2. Derivation of the method:

We introduce a finite set of grid points x_i by dividing the interval $[a, b]$ into n equal parts.

$x_i = a + ih$, $i = 0, 1, \ldots, n$

$$x_0 = a\ ,\ x_n = b \ \text{ and } \ h = \frac{b-a}{n} \qquad (2.1)$$

Let $y(x)$ be the exact solution of the system (1.1) and (1.2) and S_i be an approximation to $y_i = y(x_i)$ obtained by the spline function $Q_i(x)$ passing through the points (x_i, s_i) and (x_{i+1}, s_{i+1}).

Each nonpolynomial spline segment $Q_i(x)$ has the form.

$$Q_i(x) = a_i \sin k(x - x_i) + b_i \cos k(x - x_i) + c_i(x - x_i) + d_i \ , i = 0,1,\ldots.n-1 \qquad (2.2)$$

Where a_i , b_i , c_i and d_i are constants and k is the frequency of the trigonometric functions which will be used to raise the accuracy of the method and equation (2.2) reduces to cubic polynomial spline function in $[a,b]$when $k \to 0$, Choosing the spline function in this form will enable us to generalize other existing methods by arbitrary choices of the parameters α and β which will be defined later at the end of this section. Thus, our cubic nonpolynomial spline is now defined by the relations:

$$(i)\ S(x) = Q_i(x), x \in [x_i, x_{i+1}], i = 0,1,\ldots\ldots,n-1$$
$$(ii)\ S(x) \in C^{\infty}[a,b] \qquad (2.3)$$

The four coefficients in (2.2) need to be obtained in terms of

$S_{i+1/2}, D_i, M_{i+1/2}, T_i \text{ and } T_{i+1}$ Where

$$\begin{aligned}
&(i)\ Q_i\left(x_{i+1/2}\right) = S_{i+1/2}\\
&(ii)\ Q_i^{(1)}(x_i) = D_i\\
&(iii)\ Q_i^{(2)}\left(x_{i+1/2}\right) = M_{i+1/2}\\
&(iv)\ Q_i^{(3)}(x_i) = \frac{1}{2}\left[T_i + T_{i+1}\right]
\end{aligned} \tag{2.4}$$

We obtain via a straightforward calculation

$$a_i = \frac{-1}{2k^3}\left[T_{i+1} + T_i\right],\quad b_i = \frac{\tan\theta/2}{2k^3}\left[T_{i+1} + T_i\right] - \frac{\sec\theta/2}{k^2} M_{i+1/2}$$

$$C_i = D_i + \frac{1}{2k^2}\left[T_{i+1} + T_i\right],\quad d_i = S_{i+1/2} + \frac{1}{k^2} M_{i+1/2} - \frac{h}{2} D_i - \frac{h}{4k^2}\left[T_{i+1} + T_i\right] \tag{2.5}$$

Where $\theta = kh$ and $i = 0, 1, 2 \ldots n-1$

Now using the continuity conditions (*ii*) and (2.3), that is the continuity of cubic nonpolynomial spline $S(x)$ and its first and second derivates at the point (x_i, s_i), where the two cubics $Q_{i-1}(x)$ and $Q_i(x)$ join, we can have

$$Q_{i-1}^{(m)}(x_i) = Q_i^{(m)}(x_i),\ m = 0, 1, 2 \tag{2.6}$$

Using Eqs. (2.2), (2.4), (2.5) and (2.6) yield the relations:

$$\frac{h}{2}\left[D_i + D_{i-1}\right] = \left(S_{i+1/2} - S_{i-1/2}\right) + \frac{1}{k^2} M_{i+1/2}\left[1 - \sec\theta/2\right] + \frac{1}{k^2} M_{i-1/2}\left[\cos\theta\sec\theta/2 - 1\right] + \left(\frac{\tan\theta/2}{2k^3} - \frac{h}{4k^2}\right)\left(T_{i-1} + 2T_i + T_{i+1}\right) \tag{2.7}$$

$$\frac{h}{2}\left[D_i + D_{i-1}\right] = \frac{h \sin\theta \sec\theta/2}{2k} M_{i-1/2} \tag{2.8}$$

And

$$\frac{\tan\theta/2}{2k^3}\left(T_{i-1} + 2T_i + T_{i+1}\right) = \frac{\sec\theta/2}{k^2} M_{i+1/2} - \frac{\cos\theta\sec\theta/2}{k^2} M_{i-1/2} \tag{2.9}$$

From Eqs. (2.7) – (2.9) we get the following relation:

$$S_{i-3/2} - 2S_{i-1/2} + S_{i+1/2} = h^2\left(\alpha M_{i-3/2} + \beta M_{i-1/2} + \alpha M_{i+1/2}\right), i = 2,3,\ldots,n-1 \tag{2.10}$$

Where

$$\alpha = \frac{\theta - 2\sin\theta/2}{2\theta^2 \sin\theta/2} \quad And \quad \beta = \frac{2\theta\sin^2\theta/2 + 4\sin\theta/2 - \theta(1+\cos\theta)}{2\theta^2 \sin\theta/2}$$

And

$$M_i = -f_i S_i + g_i \ \ with\ \ f_i = f\left(x_i\right) and\ g_i = g\left(x_i\right)$$

The relation (2.10) gives *(n-2)* linear algebraic equations in the *(n)* unknowns $S_{i+1/2}$,i = *0,1,2, ... n-1*, so we need two more equations, one at each end of the range of integration for direct computation of $S_{i+1/2}$. These two equations are deduced by Taylor series and the method of undetermined coefficients. These equations are

$$-hS_0^{(1)} - S_{1/2} + S_{3/2} = h^2\left(w_0 M_{1/2} + w_1 M_{3/2} + w_2 M_{5/2} + w_3 M_{7/2}\right) at\ i = 1 \tag{2.11}$$

And

$$S_{n-3/2} - S_{n-1/2} + hS_n^{(1)} = h^2\left(w_0 M_{n-1/2} + w_1 M_{n-3/2} + w_2 M_{n-5/2} + w_3 M_{n-7/2}\right), at\ i = n \tag{2.12}$$

Where w_i*'s* will be determined later to get the required order of accuracy.

The local truncation errors t_i, *i = 1, 2, ...n* associated with the scheme (2.10) – (2.12) can be obtained as follows, we rewrite the scheme(2.10) – (2.12) in the form

$$-h y_0^{(1)} - y_{1/2} + y_{3/2} = h^2\left(w_0 y_{1/2}^{(2)} + w_1 y_{3/2}^{(2)} + w_2 y_{5/2}^{(2)} + w_3 y_{7/2}^{(2)}\right) + t_1, at\ i = 1 \tag{2.13}$$

$$y_{i-3/2} - 2y_{i-1/2} + y_{i+1/2} = h^2\left(\alpha y_{i-1/2}^{(2)} + \beta y_{i-1/2}^{(2)} + \alpha y_{i+1/2}^{(2)}\right) + t_i, at\ i = 2,3,\ldots,n-1 \tag{2.14}$$

And

$$y_{n-3/2} - y_{n-1/2} + h y_n^{(1)} = h^2\left(w_0 y_{n-1/2}^{(2)} + w_1 y_{n-3/2}^{(2)} + w_2 y_{n-5/2}^{(2)} + w_3 y_{n-7/2}^{(2)}\right) + t_n, at\ i = n \tag{2.15}$$

The terms $y_{i-1/2}$ and $y_{i-1/2}^{(2)}$ …in Eq. (2.14) are expanded around the point x_i using Taylor series and the expressions for t_i, *i = 2, … n-1* can be obtained. Also, expressions for t_i *; i = 1, n* are obtained by expanding Eqs. (2.13) and (2.15) around

the point x_0 and x_n, respectively, using Taylor series and the expressions for t_i ; $i = 1, n$ can be obtained as

$$t_i = \begin{cases} \left[1-(w_0+w_1+w_2+w_3)\right]h^2y_i^{(2)} + \left[\frac{26}{48} - \frac{(w_0+3w_1+5w_2+7w_3)}{2}\right]h^3y_i^{(3)} \\ + \left[\frac{80}{384} - \frac{(w_0+9w_1+25w_2+49w_3)}{8}\right]h^4y_i^{(4)} + \left[\frac{242}{3840} - \frac{(w_0+27w_1+125w_2+343w_3)}{48}\right]h^5y_i^{(5)} \\ + \left[\frac{728}{46080} - \frac{(w_0+81w_1+625w_2+2401w_3)}{384}\right]h^6y_i^{(6)} + O(h^7); i = 1, n \\ (1-2\alpha-\beta)h^2y_i^{(2)} + \left(\alpha + \frac{\beta}{2} - \frac{1}{2}\right)h^3y_i^{(3)} + \left(\frac{5}{24} - \frac{5\alpha}{4} - \frac{\beta}{8}\right)h^4y_i^{(4)} \\ + \left(\frac{-1}{16} + \frac{26\alpha}{48} + \frac{\beta}{48}\right)h^5y_i^{(5)} + \left(\frac{91}{5760} - \frac{82\alpha}{384} - \frac{\beta}{384}\right)h^6y_i^{(6)} + O(h^7); i = 2, \ldots\ldots n-1 \end{cases} \quad (2.16)$$

The scheme (2.10) – (2.12) gives rise to a family of methods of different orders as follows:

For $\alpha = \frac{1}{12}$ *and* $\beta = \frac{10}{12}$

2.1 Third order method

For $(w_0, w_1, w_2, w_3) = (24, -1, 1, 0)/d_1$ where $d_1 = 24$

Then the local truncation errors given by equation (2.16) are

$$t_i = \begin{cases} \frac{-247}{5760}h^5y_i^{(5)} + O\left(h^6\right), \; i = 1, n \\ \frac{-1}{240}h^6y_i^{(6)} + O\left(h^7\right), \; i = 2, 3, \ldots\ldots, n-1 \end{cases} \quad (2.17)$$

2.2 Fourth order method

For $(w_0, w_1, w_2, w_3) = (6007, -981, 981, -247)/d_2$ *where* $d_2 = 5760$

Then the local truncation errors given by equation (2.16) are

$$t_i = \begin{cases} \frac{23}{576}h^6y_i^{(6)} + O\left(h^7\right), \; i = 1, n \\ \frac{-1}{240}h^6y_i^{(6)} + O\left(h^7\right), \; i = 2, 3, \ldots\ldots, n-1 \end{cases} \quad (2.18)$$

Remark

(1) When $\alpha = \frac{1}{8}$ *and* $\beta = \frac{6}{8}$, then the scheme (2.10) is reduced to quadratic polynomial spline in [2, 3].

(2) When $\alpha = \frac{1}{24}$ *and* $\beta = \frac{22}{24}$, then the scheme (2.10) is reduced to cubic polynomial spline in [4]

(3) When $\alpha = \frac{1}{12}$ *and* $\beta = \frac{10}{12}$, we get new scheme that produces numerical results better than both quadratic and cubic polynomial splines in [2, 3, 4].

3. Spline solutions:

The spline solution of (1.1) with the boundary condition (1.2) is based on the linear equations given by (2.10) – (2.12), let

$Y = \left(y_{i+\frac{1}{2}}\right), S = \left(S_{i+\frac{1}{2}}\right), C = (C_i), T = (t_i) E = \left(e_{i+\frac{1}{2}}\right) = Y - S$

Be n-dimensional column vectors, then we can write the standard matrix equations for the nonpolynomial spline method in the form.

(i) $NY = C + T$

(ii) $NS = C$ (3.1)

(iii) $NE = T$

We also have $N = N_0 + h^2BF$; $F = \text{diag}\left(f_{i+\frac{1}{2}}\right)$ (3.2)

The three band symmetric matrix N_0 has the form:

$$N_0 = \begin{pmatrix} -1 & 1 & & & & & \\ 1 & -2 & 1 & & & & \\ & 1 & -2 & 1 & & & \\ & & \ddots & & & & \\ & & & 1 & -2 & 1 & \\ & & & & 1 & -2 & 1 \\ & & & & & 1 & -1 \end{pmatrix} \quad (3.3)$$

The matrix B has the form:

$$B = \begin{pmatrix} w_0 & w_1 & w_2 & w_3 & & & \\ \alpha & \beta & \alpha & & & & \\ & \alpha & \beta & \alpha & & & \\ & & \ddots & & & & \\ & & & \alpha & \beta & \alpha & \\ & & & & \alpha & \beta & \alpha \\ & & & w_3 & w_2 & w_1 & w_0 \end{pmatrix} \quad (3.4)$$

For the vector C, we have

$$C_i = \begin{cases} hA_1 + h^2\left[w_0 g_{1/2} + w_1 g_{3/2} + w_2 g_{5/2} + w_3 g_{7/2}\right], & i = 1 \\ h^2\left[\alpha g_{i-3/2} + \beta g_{i-1/2} + \alpha g_{i+1/2}\right], & i = 2,3,.....,n-1 \\ -hA_2 + h^2\left[w_0 g_{n-1/2} + w_1 g_{n-3/2} + w_2 g_{n-5/2} + w_3 g_{n-7/2}\right], & i = n \end{cases} \quad (3.5)$$

Set $N_0 = M_0 + J_0$ (3.6)

Where

$$M_0 = \begin{pmatrix} -3 & 1 & & & & \\ 1 & -2 & 1 & & & \\ & 1 & -2 & 1 & & \\ & & \ddots & & & \\ & & 1 & -2 & 1 & \\ & & & 1 & -2 & 1 \\ & & & & 1 & -3 \end{pmatrix} \quad (3.7)$$

And

$$J_0 = \begin{pmatrix} 2 & 0 & & & 0 & 0 \\ 0 & 0 & & & & 0 \\ \vdots & & & & & \\ \vdots & & & & & \\ 0 & & & 0 & & \\ & & & & 0 & 0 \\ 0 & 0 & & & 0 & 2 \end{pmatrix} \quad (3.8)$$

4. Convergence analysis

Our main purpose now is to derive a bound on $\|E\|_\infty$. We now turn back to the error equation (*iii*) in (3.1) and rewrite it in the form

$$E = N^{-1}T = \left(M_0 + J_0 + h^2BF\right)^{-1}T = \left(I + M_0^{-1}\left(J_0 + h^2BF\right)\right)^{-1}M_0^{-1}T$$

This implies that

$$\|E\|_\infty = \left\|\left(I + M_0^{-1}\left(J_0 + h^2BF\right)\right)^{-1}\right\|_\infty \left\|M_0^{-1}\right\|_\infty \|T\|_\infty \quad (4.1)$$

In order to derive the bound on $\|E\|_\infty$, the following two lemmas are needed.

Lemma 4.1 ([10]). If G is a square matrix of order n and $\|G\| < 1$, then $(I+G)^{-1}$ exists and

$$\left\|(I+G)^{-1}\right\| < \frac{1}{1 - \|G\|}$$

Lemma 4.2; the matrix $\left(M_0 + J_0 + h^2BF\right)$ is nonsingular if $\|f\| < \dfrac{h^2 - 2w}{h^2 w(\beta + 2\alpha)}$ where

$$w = \frac{1}{8}\left((b-a)^2 + h^2\right)$$

Proof. Since,

$N = \left(M_0 + J_0 + h^2BF\right) = \left(I + M_0^{-1}\left(J_0 + h^2BF\right)\right)M_0$ and the matrix M_0 is nonsingular, so to prove N nonsingular it is sufficient to show $\left(I + M_0^{-1}\left(J_0 + h^2BF\right)\right)$ nonsingular.

Moreover,

$$\|F\|_\infty \le \|f\| = \max_{a \le x \le b} |f(x)| \quad (4.2)$$

$$\left\|M_0^{-1}\right\|_\infty \le \frac{h^{-2}}{8}\left[(b-a)^2 + h^2\right], \text{ see [6]} \quad (4.3)$$

$$\|J_0\|_\infty = 2 \quad (4.4)$$

And $\|B\|_\infty = 2\alpha + \beta$ (4.5)

Also,

$$\left\|M_0^{-1}\left(J_0 + h^2BF\right)\right\|_\infty = \left\|M_0^{-1}\right\|_\infty \cdot \left(\|J_0\|_\infty + h^2\|B\|_\infty\|F\|_\infty\right) \quad (4.6)$$

Therefore, substituting $\|F\|, \left\|M_0^{-1}\right\|, \|J_0\|$ and $\|B\|$ in (4.6) we get

$$\left\|M_0^{-1}\left(J_0 + h^2BF\right)\right\|_\infty \le \frac{h^{-2}}{8}\left((b-a)^2 + h^2\right)\left[2 + h^2(2\alpha + \beta)\|f\|\right] \quad (4.7)$$

Since, $\|f\| < \dfrac{h^2 - 2w}{h^2 w(\beta + 2\alpha)}$ (4.8)

Therefore, Eq. (4.8) leads to

$$\left\|M_0^{-1}\left(J_0 + h^2BF\right)\right\|_\infty \le 1 \quad (4.9)$$

From Lemma 4.1, it shows that the matrix N is nonsingular. Since , $\left\|M_0^{-1}\left(J_0 + h^2BF\right)\right\|_\infty < 1$ so using Lemma (4.1)and Eq. (4.1) follow that

$$\|E\|_\infty \le \frac{\left\|M_0^{-1}\right\|_\infty \|T\|_\infty}{1 - \left\|M_0^{-1}\right\|_\infty \left\|J_0 + h^2BF\right\|_\infty} \quad (4.10)$$

From Eq. (2.17) we have

$$\|T\|_\infty = \frac{247}{5760}h^5 M_5; M_5 = \max_{a \le x \le b} \left|y^{(5)}(x)\right|$$

Then

$$\|E\|_\infty \le \frac{\left\|M_0^{-1}\right\|_\infty \|T\|_\infty}{1 - \left\|M_0^{-1}\right\|_\infty \left\|J_0 + h^2BF\right\|_\infty} \cong O\left(h^3\right) \quad (4.11)$$

Also, from Eq. (2.18) we have

$$\|T\|_\infty = \frac{23}{576}h^6 M_6; M_6 = \max_{a \le x \le b} \left|y^{(6)}(x)\right|$$

Then

$$\|E\|_\infty \le \frac{\|M_0^{-1}\|_\infty \|T\|_\infty}{1 - \|M_0^{-1}\|_\infty \|J_0 + h^2 BF\|_\infty} \cong O(h^4) \tag{4.12}$$

We summarize the above results in the next theorem.

Theorem 4.1

Let $y(x)$ is the exact solution of the continuous boundary value problem (1.1) with the boundary condition (1.2) and let $y_{i+1/2}, i = 0,1,..... n-1$, satisfies the discrete boundary value problem (*ii*) in (3.1). Further, if $e_{i+1/2} = y_{i+1/2} - S_{i+1/2}$ then

1- $\|E\|_\infty \cong O(h^3)$, for third order convergent method

2- $\|E\|_\infty \cong O(h^4)$, for fourth order convergent method

Which are given by (4.11) and (4.12), neglecting all errors due to round off.

5. Numerical examples and discussion:

We now consider two numerical examples illustrating the comparative performance of cubic nonpolynomial spline method (*ii*) in (3.1) over quadratic nonpolynomial spline method and the two polynomial spline methods (quadratic and cubic). All calculations are implemented by MATLAB 7

Example 1

Consider the boundary value problem, see [5]

$$y^{(2)} + y = -1 \tag{5.1}$$

$$y^{(1)}(0) = \frac{1-\cos(1)}{\sin(1)} = -y^{(1)}(1)$$

The analytical solution of (5.1) is

$$y(x) = \cos(x) + \frac{1-\cos(1)}{\sin(1)}\sin(x) - 1 \tag{5.2}$$

Example 2

Consider the boundary value problem, see [5]

$$y^{(2)} + xy = (3 - x - x^2 + x^3)\sin(x) + 4x\cos(x) \tag{5.3}$$

$$y^{(1)}(0) = -1, \; y^{(1)}(1) = 2\sin(1)$$

The analytical solution of (5.3) is

$$y(x) = (x^2 - 1)\sin(x) \tag{5.4}$$

The numerical results of examples 1 and 2 are presented in tables 1 and 2, respectively, for our fourth order method. A comparison between the method (2.10) and the existing methods in Ramadan et al. [5] are provided in tables 3 and 4.

Table 1: Approximate, Exact Solutions and Maximum errors (in absolute value) for Example 1 using our fourth order.

n	S_i (approximated)	y_i (Exact)	*E* (Error)
4	0.13068504600377	0.13060321651340	8.18295–5[a]
8	0.13727099391989	0.13726907762415	1.91630–6
16	0.13893760135665	0.13893757908329	2.22734–8
32	0.00841355938534	0.00841356124929	1.86395–9
64	0.00423742716766	0.00423742736291	1.95255–10
128	0.00212635927486	0.00212635928910	1.42408–11

[a] 8.18295–5 = $8.18295*10^{-5}$

Table 2: Approximate, Exact Solutions and Maximum errors (in absolute value) for Example 2 using our fourth order.

n	S_i (approximated)	y_i (Exact)	*E* (Error)
4	- 0.35932989074946	- 0.35654365069809	2.78624–3
8	- 0.34264531263123	- 0.34258197359850	6.33390–5
16	- 0.29719707294621	- 0.29719640852255	6.64424–7
32	- 0.02582552960734	- 0.02582552960734	6.88566–8
64	- 0.01303052171412	- 0.01303052858255	6.86843–9
128	- 0.00654464520562	- 0.00654464571154	5.05929–10

Table 3: Maximum errors (in absolute value) for Example 1.

n	Our fourth order method	Our third order method	Quadratic nonpoly. [5]	Cubic polyn. [5]	Quadratic polyn. [5]
4	8.18295-5	8.18295-5	1.43181-3	2.85364-3	3.03488-3
8	1.91630-6	8.04854-6	1.75382-4	7.12633-4	7.69627-4
16	2.22734-8	5.91344-7	2.16003-5	1.78109-4	1.93094-4
32	1.86395-9	3.96416-8	2.67705-6	4.45241-5	4.83167-5
64	1.95255-10	2.56011-9	3.33110-7	1.11308-5	1.208186-5
128	1.42408-11	1.62945-10	4.15407-8	2.78270-6	3.02063-6

Table 4: Maximum errors (in absolute value) for Example 2.

n	Our fourth order method	Our third order method	Quadratic nonpoly. [5]	Cubic polyn. [5]	Quadratic polyn. [5]
4	2.78624-3	3.27323-3	2.2425-2	4.62182-2	4.94551-2
8	6.33390-5	3.03799-4	2.66946-3	1.15362-2	1.23088-2
16	6.64424-7	2.17464-5	3.24076-4	2.88302-3	3.08111-3
32	6.88566-8	1.43875-6	3.98761-5	7.20696-4	7.70391-4
64	6.86843-9	9.22972-8	4.94425-6	1.80171-4	1.92590-4
128	5.059294-10	5.84115-9	6.15517-7	4.50424-5	4.79946-5

6. Conclusion:

Two new methods are presented for solving second order two-point boundary value problem with Neumann conditions. These methods are shown to be optimal third and optimal fourth orders which are better than the two polynomial spline methods (quadratic and cubic splines) and quadratic nonpolynomial spline method. Moreover, nonpolynomial spline method has less computational cost over other polynomial spline methods. The obtained numerical results show that the proposed methods maintain a remarkable high accuracy which make them are very encouraging over other existing methods.

Corresponding author
Z.A. ZAki*
Department of Engineering Mathematics and Physics, Faculty of Engineering, Benha University, Shoubra, Cairo, Egypt.
Zahmed_2@yahoo.com

7. References

1. A. Khan, Parametric cubic spline solution of two-point boundary value problems, Applied Mathematics and Computations 154 (2004) 175-182.
2. E.A.Al-Said, Spline solutions for system of second order boundary-value problems, int. J. Comput. Math 62 (1996) 143-154.
3. M.I. Hossam, S.Z. Sakr and W.K. Zahra, Quadratic spline solution to two point boundary value problems, Delta Journal of Science, 27 (2003) 4-14.
4. E.A. Al-Said, The use of cubic splines is the numerical solution of a system of second-order boundary value problems. Comput. Math. Applics 42 (2001) 861-869.
5. M.A. Ramadan, I. F. Lashien, W.K. Zahra, Polynomial and non-polynomial spline approaches to the numerical solution of second-order boundary-value problems, Applied Mathematics and Computations 184 (2007) 476-484.
6. D. Murray, Explicit inverses of Toeplitz and Associated Matrices, Anziam J. 44, 185-215, 2003.
7. W.K. Zahra, Numerical solutions for boundary value problems for ordinary differential equations by using spline functions, M.Sc. thesis, Faculty of Engineering, Tanta University, Egypt, 2004.
8. J.L. Blue, Spline function methods for nonlinear boundary value problems, communications of the ACM (1969).
9. C.V. Raghavarao, Y.V.S.S. Sanyasiraju, S. Suresh, A note on application of cubic splines to two-point boundary value problems, Comput. Math. Applics 27(11) (1994) 45-48.
10. R.A. Usmani, Discrete methods for a boundary value problem with engineering applications, Math. Comput. 32 (144) (1978) 1087-1096.

6/22/2010

The Numerical Solution of Linear Third Order Boundary Value Problems using Nonpolynomial Spline Technique

F.A. Abd El-Salam, A.A. El-Sabbagh and Z.A. ZAki*

Department of Engineering Mathematics and Physics, Faculty of Engineering, Benha University, Shoubra, Cairo, Egypt.
Zahmed_2@yahoo.com*

Abstract: Second and fourth order convergent methods based on Quartic nonpolynomial spline function are presented for the numerical solution of a third order two-point boundary value problem. The proposed approach gives better approximations than existing polynomial spline and finite difference methods and has a lower computational cost. Convergence analysis of the proposed method is discussed; two numerical examples are included to illustrate the efficiency of the method. [Journal of American Science. 2010;6(12):303-309]. (ISSN: 1545-1003).

Keywords: Quartic nonpolynomial spline; third order two-point boundary value problem; convergence analysis, finite difference.

1. Introduction:

Many problems in mathematical and engineering sciences are formulated in boundary value problems for third order differential equations as in physical oceanography and in the frame work of variational inequality theory and in many branches of pure and applied mathematics. For more details show [1, 2]

We shall consider a numerical solution of the following linear third order two-point boundary value problem

$$y^{(3)}+f(x)\,y=g(x),\ x\in[a,b] \qquad (1.1)$$

Subject to the boundary conditions

$$y(a)=k_1,\ y^{(1)}(a)=k_2,\ y^{(1)}(b)=k_3 \qquad (1.2)$$

Where $k_i, i=1,2,3$ are finite real constants, the functions $f(x)$ and $g(x)$ are continuous on the interval $[a, b]$, the analytical solution of (1.1) and (1.2) cannot be obtained for arbitrary choices of $f(x)$ and $g(x)$. The numerical analysis literature contains other methods developed to find approximate solutions of these types of boundary value problems. Al-Said and Noor [3, 4] developed a second order method for solving a system of third order two-point boundary value problems using cubic and quartic polynomial spline functions respectively; Al-Said and Noor [5] have developed a second order finite difference method at midpoints. A.Khan and T.Aziz [6] established and discussed convergent fourth order method for this problem with the change in the boundary conditions $y(a)=k_1,\ y^{(1)}(a)=k_2,\ y(b)=k_3$ using quintic polynomial spline functions.

S.ul.Islam et al. [8] have developed a smooth approximation for solving a system of third order obstacle problem based on nonpolynomial spline which provides bases for our method.

In the present paper, Quartic nonpolynomial spline functions are applied to develop a new numerical method for obtaining smooth approximations to the solution of such third-order differential equation. The method is of order two for arbitrary α and β along with $\alpha+\beta=\frac{1}{2}$ and $\alpha\neq 0$, Which will be defined later at the end of the next section and better results will be obtained for choosing α less than β as we will see from the analysis of the local truncation error. And the method of order four for $\alpha=0$ along with $\alpha+\beta=\frac{1}{2}$, in section 2, we derive the consistency relations and develop the quartic nonpolynomial spline method for solving (1.1) subject to (1.2). In section3 and 4 are devoted for the spline solution and convergence analysis of the method. The numerical experiments are given in section 5.

2. Derivation of the method:

We introduce a finite set of grid points x_i by dividing the interval $[a,b]$ into (n+1) equal subintervals where

$$x_i = a + i\,h,\ i = 0, 1, 2, \ldots\ldots, n, n+1$$
$$x_0 = a,\ x_{n+1} = b \text{ and } h = \frac{b-a}{n+1} \qquad (2.1)$$

Let $y(x)$ be the exact solution of the system (1.1) and (1.2) and S_i be an approximation to $y_i=y(x_i)$ obtained by the spline function $Q_i(x)$ passing through the points (x_i, s_i) and (x_{i+1}, s_{i+1}).

Each quartic nonpolynomial spline segment $Q_i(x)$ has the form:

$$Q_i(x) = a_i \cos k(x - x_i) + b_i \sin k\,(x - x_i) + c_i(x - x_i)^2 + d_i(x - x_i) + e_i \quad , i = 0,1, \ldots \ldots n \qquad (2.2)$$

Where a_i, b_i, c_i, d_i and e_i are constants and k is the frequency of the trigonometric functions which will be used to raise the accuracy of the method and equation (2.2) reduces to quartic polynomial spline function in $[a,b]$when $k \rightarrow 0$, Choosing the spline function in this form will enable us to generalize other existing polynomial spline methods for arbitrary choices of the parameters α and β which will be defined at the end of this section. Thus, this quartic nonpolynomial spline is now defined by the relations:

$$(i)\ S(x) = Q_i(x), x \in [x_i, x_{i+1}], i = 0,1, \ldots \ldots, n$$
$$(ii)\ S(x) \in C^{\infty}[a,b] \qquad (2.3)$$

Following the technique of S.ul.Islam et al. [8] we let:

$$Q_i(x) = S_i \ , Q_i(x_{i+1}) = S_{i+1}$$

$$Q_i^{(1)}(x_i) = D_i \qquad (2.4)$$

$$Q_i^{(3)}(x_i) = T_i, \ Q_i^{(3)}(x_{i+1}) = T_{i+1}$$

For $i = 0,1, \ldots\ldots,n$, to obtain via a straight forward calculations

$$a_i = h^3\left[\frac{T_{i+1} - T_i \cos\theta}{\theta^3 \sin\theta}\right]$$
$$b_i = -h^3\left[\frac{T_i}{\theta^3}\right]$$
$$c_i = \left[\frac{S_{i+1} - S_i}{h^2}\right] - \frac{D_i}{h} - \frac{h}{\theta^2}T_i + \frac{h\,[1-\cos\theta]\,[T_{i+1} + T_i]}{\theta^3 \sin\theta}$$
$$d_i = D_i + \frac{h^2 T_i}{\theta^2}$$
$$e_i = S_i - h^3\left[\frac{T_{i+1} - T_i \cos\theta}{\theta^3 \sin\theta}\right] \qquad (2.5)$$

Where $\theta = kh$, $i = 0, 1, \ldots\ldots, n$

Using the continuity conditions (ii) and (2.3) of the first and second derivatives at the point (x_i, s_i) that is $Q_{i-1}^{(m)}(x_i) = Q_i^{(m)}(x_i), m = 1,2$ (2.6)

Using Eqs. (2.2), (2.4), (2.5) and (2.6) yield the relations:

$$D_i + D_{i-1} = \frac{2}{h}[S_i - S_{i-1}] + \frac{2h^2[1-\cos\theta][T_i + T_{i-1}]}{\theta^3 \sin\theta} - \frac{h^2[T_{i-1} + T_i]}{\theta^2} \qquad (2.7)$$

$$D_i - D_{i-1} = \frac{1}{h}[S_{i-1} - 2S_i + S_{i+1}] + \frac{h^2[1-\cos\theta]}{\theta^3 \sin\theta}[T_{i+1} - T_{i-1}] + \frac{h^2}{\theta^2}[T_{i-1} - T_i] + \frac{h^2 \cos\theta}{\theta \sin\theta}T_i - \frac{h^2}{2\theta \sin\theta}[T_{i+1} + T_{i-1}] \qquad (2.8)$$

Adding Eqs. (2.7) and (2.8) we get

$$D_i = \frac{1}{2h}[S_{i+1} - S_{i-1}] - \frac{h^2}{\theta^2}T_i + \frac{h^2[1-\cos\theta]}{2\,\theta^3 \sin\theta}[T_{i+1} + 2T_i + T_{i-1}] + \frac{h^2 \cos\theta}{2\theta \sin\theta}T_i - \frac{h^2}{4\theta \sin\theta}[T_{i+1} + T_{i-1}] \qquad (2.9)$$

Similarly

$$D_{i-1} = \frac{1}{2h}[S_i - S_{i-2}] - \frac{h^2}{\theta^2}T_{i-1} + \frac{h^3[1-\cos\theta]}{2\,\theta^3 \sin\theta}[T_i + 2T_{i-1} + T_{i-2}] + \frac{h^2 \cos\theta}{2\theta \sin\theta}T_{i-1} - \frac{h^2}{4\theta \sin\theta}[T_i + T_{i-2}] \qquad (2.10)$$

D_i And D_{i-1} are eliminated from equation (2.7) with the help of Eqs. (2.9) and (2.10) to get the following scheme:

$$-S_{i-2} + 3S_{i-1} - 3S_i + S_{i+1} = h^3\left[\alpha(T_{i-2} + T_{i+1}) + \beta(T_{i-1} + T_i)\right], i = 2,3,\ldots,n-1 \qquad (2.11)$$

Where

$$T_i = -f_i S_i + g_i \quad with \ f_i = f(x_i)\ and\ g_i = g(x_i)$$

And

$$\alpha = \left[\frac{1}{2\theta \sin\theta} - \frac{1-\cos\theta}{\theta^3 \sin\theta}\right]$$

$$\beta = \left[\frac{1-2\cos\theta}{2\theta \sin\theta} + \frac{1-\cos\theta}{\theta^3 \sin\theta}\right]$$

The relation (2.11) gives $(n-2)$ linear algebraic equations in the (n) unknowns S_i , $i = 1, 2, \ldots\ldots,n$, so

we need two more equations, one at each end of the range of integration for direct computation of S_i. Here, for our system (1.1) and (1.2) we also derive these two equations by Taylor series and the method of undetermined coefficients, these equations are:

$$-4S_1+S_2=-3S_0-2hS_0^{(1)}+h^3\left(w_0T_0+w_1T_1+w_2T_2+w_3T_3\right) at\ i=1 \tag{2.12}$$

And

$$-3S_{n-2}+8S_{n-1}-5S_n=-2hS_{n+1}^{(1)}+h^3\left(\sigma_0T_n+\sigma_1T_{n-1}+\sigma_2T_{n-2}+\sigma_3T_{n-3}\right), at\ i=n \tag{2.13}$$

Where $w_i's$ and $\sigma_i's$ will be determined later to get the required order of accuracy.

The local truncation errors t_i, $i = 1,2 \ldots n$ associated with the scheme (2.11)– (2.13) can be obtained as follows:

First we rewrite the scheme (2.11) – (2.13) in the form

$$-4y_1+y_2=-3y_0-2hy_0^{(1)}+h^3\left(w_0y_0^{(3)}+w_1y_1^{(3)}+w_2y_2^{(3)}+w_3y_3^{(3)}\right)+t_1, i=1 \tag{2.14}$$

$$-y_{i-2}+3y_{i-1}-3y_i+y_{i+1}=h^3\left[\alpha\left(y_{i-2}^{(3)}+y_{i+1}^{(3)}\right)+\beta\left(y_{i-1}^{(3)}+y_i^{(3)}\right)\right]+t_i, i=2,3,4,\ldots,n-1 \tag{2.15}$$

And

$$-3y_{n-2}+8y_{n-1}-5y_n=-2hy_{n+1}^{(1)}+h^3\left[\sigma_0y_n^{(3)}+\sigma_1y_{n-1}^{(3)}+\sigma_2y_{n-2}^{(3)}+\sigma_3y_{n-3}^{(3)}\right]+t_n, i=n \tag{2.16}$$

The terms $y_{i-2}^{(3)}$, $y_{i+1}^{(3)}$, *etc* in Eq. (2.15) are expanded around the point x_i using Taylor series and the expressions for t_i, $i = 2, \ldots n-1$ can be obtained. Also, expressions for t_i, $i = 1, n$ are obtained by expanding Eqns. (2.14) and (2.16) around the point x_0 and x_n, respectively, using Taylor series and the expressions for t_i, $i = 1, n$ can be obtained as follow:

$$t_i=\begin{cases} h^3y_0^{(3)}\left[\frac{2}{3}-(w_0+w_1+w_2+w_3)\right]+h^4y_0^{(4)}\left[\frac{1}{2}-(w_1+2w_2+3w_3)\right]+h^5y_0^{(5)}\left[\frac{7}{30}-\left(\frac{w_1+4w_2+9w_3}{2}\right)\right] \\ +h^6y_0^{(6)}\left[\frac{1}{12}-\left(\frac{w_1+8w_2+27w_3}{6}\right)\right]+h^7y_0^{(7)}\left[\frac{31}{1260}-\left(\frac{w_1+16w_2+81w_3}{24}\right)\right]+O\left(h^8\right), i=1 \\ \\ h^3y_i^{(3)}[1-(2\alpha-2\beta)]+h^4y_i^{(4)}\left[(\alpha+\beta)-\frac{1}{2}\right]+h^5y_i^{(5)}\left[\frac{1}{4}-\left(\frac{5\alpha+\beta}{2}\right)\right] \\ +h^6y_i^{(6)}\left[\frac{-1}{12}+\left(\frac{7\alpha+\beta}{6}\right)\right]+h^7y_i^{(7)}\left[\frac{1}{40}-\left(\frac{17\alpha+\beta}{24}\right)\right]+O\left(h^8\right), i=2,\ldots n-1 \\ \\ h^3y_n^{(3)}\left[\frac{11}{3}-(\sigma_0+\sigma_1+\sigma_2+\sigma_3)\right]+h^4y_n^{(4)}\left[\frac{-4}{3}+(\sigma_1+2\sigma_2+3\sigma_3)\right]+h^5y_n^{(5)}\left[\frac{49}{60}-\left(\frac{\sigma_1+4\sigma_2+9\sigma_3}{2}\right)\right] \\ +h^6y_n^{(6)}\left[\frac{-43}{180}+\left(\frac{\sigma_1+8\sigma_2+27\sigma_3}{6}\right)\right]+h^7y_n^{(7)}\left[\frac{39}{504}-\left(\frac{\sigma_1+16\sigma_2+81\sigma_3}{24}\right)\right]+O\left(h^8\right), i=n \end{cases} \tag{2.17}$$

The scheme (2.11) – (2.13) gives rise to a family of methods of different orders as follows:

2.1 Second order method

For arbitrary values of α and β along with

$$\alpha+\beta=\frac{1}{2}\ ,\ \alpha\neq 0$$

$$(w_0, w_1, w_2, w_3)=\left(\frac{2}{15}, \frac{7}{12}, \frac{-1}{15}, \frac{1}{60}\right)$$

And

$$(\sigma_0, \sigma_1, \sigma_2, \sigma_3)=\left(\frac{157}{60}, \frac{19}{30}, \frac{11}{20}, \frac{-2}{15}\right)$$

Then the local truncation errors given by equation (2.17)are

$$t_i=\begin{cases} \frac{-29}{2520}h^7y_0^{(7)}+O\left(h^8\right), & i=1 \\ (-2\alpha)h^5y_i^{(5)}+O\left(h^6\right), & i=2,3,\ldots,n-1 \\ \frac{677}{5040}h^7y_n^{(7)}+O\left(h^8\right), & i=n \end{cases} \tag{2.18}$$

So, better results occurred for choosing α less than β whose sum is $\frac{1}{2}$

2.2 Fourth order method

For $\alpha=0$ and $\beta=\frac{1}{2}$

$(w_0, w_1, w_2, w_3)=\left(\frac{2}{15}, \frac{7}{12}, \frac{-1}{15}, \frac{1}{60}\right)$ And

$(\sigma_0, \sigma_1, \sigma_2, \sigma_3)=\left(\frac{157}{60}, \frac{19}{30}, \frac{11}{20}, \frac{-2}{15}\right)$ Then the local truncation errors given by equation (2.17) are

$$t_i = \begin{cases} \frac{-29}{2520} h^7 y_0^{(7)} + O\left(h^8\right), & i = 1 \\ \frac{1}{240} h^7 y_i^{(7)} + O\left(h^8\right), & i = 2, \ldots\ldots, n-1 \\ \frac{677}{5040} h^7 y_n^{(7)} + O\left(h^8\right), & i = n \end{cases} \tag{2.19}$$

Remark

(1) When $\alpha = \frac{1}{12}$ and $\beta = \frac{5}{12}$ then the scheme (2.11) reduces to Al-Said and Noor method based on cubic polynomial spline [3].

(2) When $\alpha = \frac{1}{24}$ and $\beta = \frac{11}{24}$ then the scheme (2.11) reduces to Al-Said and Noor [4], Usmani and Sakai [7] methods based on quartic polynomial spline.

3. Spline solutions:

The spline solution of (1.1) with the boundary condition (1.2) is based on the linear equations given by (2.11) – (2.13).

Let $Y = (y_i)$, $S = (S_i)$, $C = (C_i)$, $T = (T_i)$, $E = (e_i) = Y - S$
Be n-dimensional column vectors, then we can write the standard matrix equations for the nonpolynomial spline method in the form:

$$\begin{aligned} &(i) \quad NY = C + T \\ &(ii) \quad NS = C \\ &(iii) \quad NE = T \end{aligned} \tag{3.1}$$

We also have $N = N_0 + h^3 BF$; $F = \text{diag}\left(f_i\right)$, (3.2)

And the matrices N_0 and B are defined by

$$N_0 = \begin{pmatrix} -4 & 1 & & & & & \\ 3 & -3 & 1 & & & & \\ -1 & 3 & -3 & 1 & & & \\ & -1 & 3 & -3 & 1 & & \\ & & & & & & \\ & & & & -1 & 3 & -3 & 1 \\ & & & & & -3 & 8 & -5 \end{pmatrix} \tag{3.3}$$

The matrix B has the form:

$$B = \begin{pmatrix} w_1 & w_2 & w_3 & & & \\ \beta & \beta & \alpha & & & \\ \alpha & \beta & \beta & \alpha & & \\ & & & & & \\ & & & \alpha & \beta & \beta & \alpha \\ & & & \sigma_3 & \sigma_2 & \sigma_1 & \sigma_0 \end{pmatrix} \tag{3.4}$$

For the vector C,

$$C_i = \begin{cases} -3k_1 - 2hk_2 + h^3\left[w_0(-f_0 k_1 + g_0) + \sum_{J=1}^{J=3} w_J g_J\right], & i = 1 \\ k_1 - h^3 \alpha k_1 f_0 + h^3\left[\alpha(g_0 + g_3) + \beta(g_1 + g_2)\right], & i = 2 \\ h^3\left[\alpha(g_{i-2} + g_{i+1}) + \beta(g_{i-1} + g_i)\right], & i = 3,4,\ldots\ldots,n-1 \\ -2h k_3 + h^3\left[\sum_{J=0}^{J=3} \sigma_J g_{n-J}\right], & i = n \end{cases} \tag{3.5}$$

4. Convergence analysis

Our main purpose now is to derive a bound on $\|E\|_\infty$. We now turn back to the error equation (*iii*) in (3.1) and rewrite it in the form

$$E = N^{-1}T = \left(N_0 + h^3 BF\right)^{-1} T = \left(I + N_0^{-1} h^3 BF\right)^{-1} N_0^{-1} T$$

which implies that:

$$\|E\|_\infty = \left\| I + N_0^{-1} h^3 BF \right\|_\infty \left\| N_0^{-1} \right\|_\infty \|T\|_\infty \tag{4.1}$$

In order to derive the bound on $\|E\|_\infty$, the following two lemmas are needed.

Lemma 4.1, ([9]). If G is a square matrix of order n and $\|G\|_\infty < 1$, then $(I+G)^{-1}$ exists and

$$\left\|(I+G)^{-1}\right\|_\infty < \frac{1}{1 - \|G\|_\infty}$$

Lemma 4.2, the matrix $\left(N_0 + h^3 BF\right)$ is nonsingular, if $\|f\| < \frac{243}{w}$ where:

$$w = 11(b-a)^3\left[2 + \frac{3h^2}{(b-a)^2}\right]$$

Proof. Since,

$N = N_0 + h^3 BF = \left(I + N_0^{-1} h^3 BF\right) N_0$ and the matrix N_0 is nonsingular, so to prove N is

nonsingular it is sufficient to show $\left(I + N_0^{-1}h^3BF\right)$ nonsingular. Moreover,

$$\|F\|_\infty \le \|f\| = \max_{a\le x\le b}|f(x)| \quad (4.2)$$

$$\left\|N_0^{-1}\right\|_\infty \le \frac{2h^{-3}}{81}\left[(b-a)^3 + \frac{3h^2}{2}(b-a)\right], \text{ see [7]} \quad (4.3)$$

$$\|B\|_\infty = \sigma_0 + \sigma_1 + \sigma_2 + \sigma_3 = \frac{11}{3} \quad (4.4)$$

Also, $\left\|N_0^{-1}h^3BF\right\|_\infty = h^3\left\|N_0^{-1}\right\|_\infty \|B\|_\infty \|F\|_\infty$ (4.5)

Therefore, substituting $\|F\|_\infty, \left\|N_0^{-1}\right\|_\infty$ and $\|B\|_\infty$ in (4.5) we get

$$\left\|N_0^{-1}h^3BF\right\|_\infty \le \frac{22}{243}\left[(b-a)^3 + \frac{3h^2(b-a)}{2}\right]\|f\| \quad (4.6)$$

Since, $\|f\| < \frac{243}{w}$ (4.7)

Therefore, Eq. (4.7) leads to

$$\left\|N_0^{-1}h^3BF\right\|_\infty \le 1 \quad (4.8)$$

From Lemma 4.1, it shows that the matrix N is nonsingular. Since $\left\|N_0^{-1}h^3BF\right\|_\infty < 1$, so using Lemma (4.1) and Eq. (4.1) follow that

$$\|E\|_\infty \le \frac{\left\|N_0^{-1}\right\|_\infty \|T\|_\infty}{1 - h^3\left\|N_0^{-1}\right\|\|B\|\|F\|} \quad (4.9)$$

From Eq. (2.18) we have

$$\|T\|_\infty = 2\alpha h^5 M_5; M_5 = \max_{a\le x\le b}\left|y^{(5)}(x)\right|$$

Then

$$\|E\|_\infty \le \frac{\left\|N_0^{-1}\right\|_\infty \|T\|_\infty}{1 - h^3\left\|N_0^{-1}\right\|_\infty \|B\|_\infty \|F\|_\infty} \cong O\left(h^2\right) \quad (4.10)$$

Also, from Eq. (2.19) we have

$$\|T\|_\infty = \frac{677}{5040}h^7 M_7; M_7 = \max_{a\le x\le b}\left|y^{(7)}(x)\right|$$

Then

$$\|E\|_\infty \le \frac{\left\|N_0^{-1}\right\|_\infty \cdot \|T\|_\infty}{1 - h^3\left\|N_0^{-1}\right\|_\infty \|B\|_\infty \|F\|_\infty} \cong O\left(h^4\right) \quad (4.11)$$

We summarize the above results in the next theorem.

Theorem 4.1

Let $y(x)$ be the exact solution of the continuous boundary value problem (1.1) with the boundary condition (1.2) and let $y_i, i = 1,2,.....n$, satisfy the discrete boundary value problem (*ii*) in (3.1), further, if $e_i = y_i - S_i$ then

1- $\|E\|_\infty \cong O\left(h^2\right)$, for second order convergent method

2- $\|E\|_\infty \cong O\left(h^4\right)$, for fourth order convergent method

Which are given by (4.10) and (4.11), neglecting all errors due to round off.

5. Numerical examples and discussion:

In this section we illustrate the numerical Techniques discussed in the previous sections by the following two boundary value problems of (1.1) and (1.2), in order to illustrate the comparative Performance of our method (*ii*) in (3.1) over other existing methods. All calculations are implemented by MATLAB 7 .

Example 1:

Consider the boundary value problem

$$y^{(3)} - x\,y = (x^3 - 2x^2 - 5x - 3)e^x \quad (5.1)$$

$$y(0) = 0,\ y^{(1)}(0) = 1,\ y^{(1)}(1) = -e$$

The analytical solution of (5.1) is

$$y(x) = x(1 - x)e^x$$

Example 2

Consider the boundary value problem

$$y^{(3)} + y = (x-4)\sin x + (1-x)\cos x \quad (5.2)$$

$$y(0) = 0,\ y^{(1)}(0) = -1,\ y^{(1)}(1) = \sin(1)$$

The analytical solution of (5.2) is

$$y(x) = (x - 1)\sin x$$

The numerical results for our fourth and second orders are summarized in tables 1-4 and compared with the other existing polynomial splines and finite difference methods.

Table 1: ***The observed maximum absolute errors for Example 1***

h	*Fourth order method* $\alpha = 0 \ , \ \beta = \frac{1}{2}$	*Second order method* $\alpha = \frac{1}{128} \ , \ \beta = \frac{1}{2} - \alpha$
$\frac{1}{16}$	5.2992 – 7	1.5540 – 4 [a]
$\frac{1}{32}$	2.6127 – 8	4.1551 – 5
$\frac{1}{64}$	1.4999 – 9	1.0575 – 5
$\frac{1}{128}$	8.9762 – 11	2.6562 – 6

[a] 1.5540 – 4 = $1.5540*10^{-4}$

Table 2: ***The observed maximum absolute errors for Example 1***

h	**Our fourth order method** $\alpha = 0, \beta = \frac{1}{2}$	**Islam et al. [8]**	**Al-Said and Noor [5]**	**Al-Said and Noor [4]**	**Al-Said and Noor [3]**
$\frac{1}{16}$	5.2992-7	2.1974-5	8.1224-4	8.3597-4	1.6861-3
$\frac{1}{32}$	2.6127-8	1.6192-6	2.1812-4	2.2207-4	4.4510-4
$\frac{1}{64}$	1.4999-9	1.1006-7	5.5859-5	5.6432-5	1.1293-4
$\frac{1}{128}$	8.9762-11	7.1764-9	1.4091-5	1.4168-5	2.8340-5

Table 3: ***The observed maximum absolute errors for Example 2***

h	*Fourth order method* $\alpha = 0 \ , \ \beta = \frac{1}{2}$	*Second order method* $\alpha = \frac{1}{128} \ , \ \beta = \frac{1}{2} - \alpha$
$\frac{1}{16}$	2.3819 – 8	9.0774 – 6
$\frac{1}{32}$	1.1184 – 9	2.4289 – 6
$\frac{1}{64}$	6.3020 – 11	6.1842 – 7
$\frac{1}{128}$	3.7640 – 12	1.5534 – 7

Table 4: ***The observed maximum absolute errors for Example 2***

h	**Our fourth order method** $\alpha = 0, \beta = \frac{1}{2}$	**Islam et al. [8]**	**Al-Said and Noor [5]**	**Al-Said and Noor [4]**	**Al-Said and Noor [3]**
$\frac{1}{16}$	2.3819–8	9.2517–7	4.5978–5	4.8237–5	9.7501–5
$\frac{1}{32}$	1.1184–9	6.8079–8	1.2530–5	1.2948–5	2.5965–5
$\frac{1}{64}$	6.3020–11	4.5822–9	3.2356–6	3.2980–6	6.6004–6
$\frac{1}{128}$	3.7640–12	2.9515–10	8.1999–7	8.284–7	1.6573–6

It is verified from the Tables 1- 4 that on reducing the step size from h to $\frac{h}{2}$ the maximum error $\|E\|$ is approximately reduced by a factor $\frac{1}{2^p}$, where p is the order of the method which confirms that our method is a second and fourth orders convergent as predicted in section 4.

6. Conclusion:

Two new methods are presented for solving third order two-point boundary value problem using quartic nonpolynomial spline functions. These methods are shown to be optimal second and optimal fourth orders which have better accuracy compared with Al-Said and Noor [3-5] and S.ul.Islam et al [8]. The obtained numerical results show that the proposed methods maintain a very remarkable high accuracy which make them are very encouraging for dealing with the solution of two-point boundary value problems.

Corresponding author
Z.A. ZAki*
Department of Engineering Mathematics and Physics, Faculty of Engineering, Benha University, Shoubra, Cairo, Egypt.
Zahmed_2@yahoo.com

7. References

1. M.A. Noor, General variational inequalities, Applied mathematics letters 1(1988) 119-122.
2. M.A. Noor, Variational inequalities in physical oceanography, ocean wave engineering, Edited by M. Rahman, computational mechanics publications, Southampton, England, (1994)201- 226.
3. E.A. Al-Said, M.A. Noor, Cubic splines methods for a system of third order boundary-value problems, applied mathematics and computation 142 (2003)195-204.
4. M.A. Noor, E.A. Al-Said, Quartic spline solution of the third-order obstacle problems, applied mathematics and computation 153 (2004)307-316.
5. E.A. Al-Said, M.A. Noor, Numerical solutions of third-order system of boundary value problems, applied mathematics and computation 190(2007) 332- 338.
6. A.Khan, T. Aziz, The numerical solution of third order boundary value problems using quintic splines, applied mathematics and computation 137 (2003)253- 260.
7. K.A. Usmani, M. Sakai, Quartic spline solutions for two point boundary problems involving third order differential equations, J. Math phys. Sci. 18 (1984)365- 380.
8. S.ul.Islam, M.A. Khan, I.A. Tirmizi, E.H. Twizell, Nonpolynomial spline approach to the solution of a system of third-order boundary value problems, applied mathematics and computation 168(2005)152- 163.
9. R.A. Usmani, Discrete methods for a boundary value problem with engineering applications, math. Comput. 144(1978) 1087- 1096.

6/22/2010

The Numerical Solution of Linear Fourth Order Boundary Value Problems using Nonpolynomial Spline Technique

F.A. Abd El-Salam and Z.A. ZAki*

Department of Engineering Mathematics and Physics, Faculty of Engineering, Benha University, Shoubra, Cairo, Egypt.
*Zahmed_2@yahoo.com

Abstract: In this paper we develop a class of accurate methods based on quartic nonpolynomial spline function at midknots for the numerical solution of a fourth order two point boundary value problems associated with plate deflection theory. Using this spline function a few consistency relations are derived for computing approximations to the solution of the problem. Existing second and fourth order finite difference and spline functions based methods developed at midknots become special cases of the new approach. Convergence analysis of the proposed method is discussed. Two numerical examples are included to illustrate the practical usefulness of our method. [Journal of American Science. 2010;6(12):310-316]. (ISSN: 1545-1003).

Keywords: Quartic nonpolynomial spline; two point boundary value problem; plate deflection theory; convergence analysis.

1. Introduction:

It is well known that the elastic beam is one of the most used elements in structures of aircrafts, buildings, ships and bridges. Beam deflection under certain load can be modeled by a fourth order two point Boundary value problems. We consider the problem of bending a rectangular simply supported beam of length L resting on an elastic foundation the vertical deflection U of the beam satisfies the system:

$$U^{(4)} + \left(Z/D\right)U = D^{-1}q(x) \quad (1.1)$$

$$U(0) = U(L) = U^{(2)}(0) = U^{(2)}(L) = 0 \quad (1.2)$$

Where D is the flexural rigidity of the beam, Z is the spring constant of the elastic foundation, and the load $q(x)$ acts vertically downwards per unit length of the beam. The details of the mechanical interpretation are given in [1]. Mathematically the system (1.1) and (1.2) belongs to a general class of boundary value problems of the form

$$y^{(4)} + f(x)\, y = \mathrm{g}(x) \quad , \quad x \in [a, b] \quad (1.3)$$

Subject to the boundary conditions

$$y(a) = A_1 \ , y(b) = A_2 \ , y^{(2)}(a) = B_1 \ , y^{(2)}(b) = B_2 \quad (1.4)$$

Where $f(x)$ and $\mathrm{g}(x)$ are continuous on $[a, b]$ and A_i, B_i $(i = 1,2)$ are finite real arbitrary constants. The analytical solution of (1.3) subject to (1.4) cannot be obtained for arbitrary choices of $f(x)$ and $\mathrm{g}(x)$. The numerical analysis literature contains other methods developed to find an approximate solution of this problem using spline functions and finite difference.

Usmani [2], Usmani and Warsi [3] solved linear fourth order two point boundary value problems using quartic, quinitic and sextic polynomial spline functions. Al-Said et al. [4,5] solved fourth order obstacle problems using cubic and quartic spline functions, respectively. Usmani [6] solved this problem with the boundary conditions involving first derivatives using quintic and sextic polynomial spline functions. Also, Rashidinia and Golbabaee [7] and Siddiqi and Ghazala [8] solved the preceding problem using quintic spline functions. VanDaele et al. [9] solved the above boundary value problem with the boundary conditions involving first derivatives using nonpolynomial spline function. Zhu [10] introduced optimal quartic spline collocation methods for the numerical solution of this problem based on perturbation technique which gives rise to two optimal quartic spline one step and three step collocation methods.

Al-Said et al. [11] developed a fourth order finite difference method for the system (1.3) and (1.4). Ramadan et al. [12, 13] solved this problem using quintic nonpolynomial spline function. The aim of this paper is to construct a new spline method based on a nonpolynomial spline function that has a polynomial part and a trigonometric part to develop numerical methods for obtaining smooth approximations for the solution of the system (1.3) and (1.4); the paper is organized as follows: in section 2, we present the derivation of our method. The method is formulated in a matrix form in section 3. Convergence analysis for second, fourth and six order methods is established in section 4. Numerical results are presented to illustrate the applicability and accuracy in section 5. Finally, in section 6, the results of the proposed methods are concluded to illustrate their practical usefulness and accuracy.

2. Derivation of the method:

We introduce a finite set of grid points x_i by dividing the interval $[a,b]$ into n equal parts.

$$x_i = a + ih \ , \ i = 0,1, \dots \dots, n$$
$$x_0 = a \ \ , x_n = b \ and \ h = \frac{b-a}{n} \qquad (2.1)$$

Let $y(x)$ be the exact solution of the system (1.3) and (1.4) and s_i be an approximation to $y_i = y(x_i)$ obtained by the spline function $Q_i(x)$ passing through the points (x_i, s_i) and (x_{i+1}, s_{i+1}).

Each nonpolynomial spline segment $Q_i(x)$ has the form:

$$Q_i(x) = a_i \sin k\,(x - x_i) + b_i \cos k\,(x - x_i) + c_i\,(x - x_i)^2 + d_i\,(x - x_i) + e_i \qquad (2.2)$$

$i = 0,1,2, \dots \dots, n-1$,where a_i ,b_i ,c_i d_i, and e_i are constants and k is the frequency of the trigonometric functions which will be used to raise the accuracy of the method and Eq. (2.2) reduces to quartic polynomial spline function in $[a,b]$ when $k \to 0$ choosing the spline function in this form will enable us to generalize other existing methods by arbitrary choices of the parameters α , $\beta$and γ which will be defined later in the end of this section. Thus, our quartic nonpolynomial spline is now defined by the relations:

$$(i)\ S(x) = Q_i(x)\ ,\ x \in [x_i, x_{i+1}], i = 0,1, \dots n-1$$
$$(ii)\ S(x) \in C^{\infty}[a,b] \qquad (2.3)$$

First, we develop expressions for the five coefficients of (2.2) in terms of $S_{i+1/2}, D_i, M_{i+1/2}\ and\ T_i, W_{i+1/2}$, where

$$(i)\ Q_i\left(x_{i+1/2}\right) = S_{i+1/2}, Q_i^{(1)}(x_i) = D_i$$
$$(ii)\ Q_i^{(2)}\left(x_{i+1/2}\right) = M_{i+1/2} \qquad (2.4)$$
$$(iii)\ Q_i^{(3)}(x_i) = T_i \ \ , \ \ Q_i^{(4)}\left(x_{i+1/2}\right) = W_{i+1/2}$$

We obtain via a straight forward calculation the following expressions:

$$a_i = \frac{-T_i}{k^3} \ \ ; \ \ b_i = \frac{T_i}{k^3}\tan(\theta/2) + \frac{1}{k^4\cos(\theta/2)}\,W_{i+1/2}$$
$$c_i = \frac{1}{2}\,M_{i+1/2} + \frac{1}{2k^2}\,W_{i+1/2} \ \ ; \ \ d_i = D_i + \frac{T_i}{k^3} \qquad (2.5)$$
$$e_i = S_{i+1/2} - \frac{h}{2}D_i - \frac{h^2}{8}M_{i+1/2} - \frac{h}{2k^2}T_i - \left[\frac{1}{k^4} + \frac{h^2}{8k^2}\right]W_{i+1/2}$$

Where $\theta = kh\ and\ i = 0,1,2, \dots \dots, n-1$ Now using the continuity (ii) in (2.3) that is the continuity of quartic nonpolynomial spline $S(x)$ and its derivatives up to order three are involved at the point (x_i,s_i) where the two quartics $Q_{i-1}(x)$ and $Q_i(x)$ join. Thus, $Q_{i-1}^{(m)}(x_i) = Q_i^{(m)}(x_i)$, $m = 0,1,2\ and\ 3$ which on using Eqs. (2.4) and (2.5) yield the following consistency relations:

$$\frac{h}{2}[D_i + D_{i-1}] = \left(S_{i+1/2} - S_{i-1/2}\right) - \frac{h^2}{8}\left[M_{i+1/2} + 3M_{i-1/2}\right] + \left[\frac{\tan\theta/2}{k^3} - \frac{h}{2k^2}\right][T_i + T_{i-1}] + \left[\frac{1}{k^4\cos(\theta/2)} - \frac{1}{k^4} - \frac{h^2}{8k^2}\right]W_{i+1/2} + \left[\frac{1}{k^4} - \frac{\cos(\theta)}{k^4\cos(\theta/2)} - \frac{3h^2}{8k^2}\right]W_{i-1/2} \qquad (2.6)$$

$$\frac{h}{2}[D_i - D_{i-1}] = \frac{h^2}{2}M_{i-1/2} + \left[\frac{h^2}{2k^2} - \frac{h\sin(\theta/2)}{k^3}\right]W_{i-1/2} \qquad (2.7)$$

$$\frac{\tan(\theta/2)}{k}\,[T_i + T_{i-1}] = \left[M_{i+1/2} - M_{i-1/2}\right] + \left[\frac{-1+\cos(\theta/2)}{k^2\cos(\theta/2)}\right]W_{i+1/2} + \left[\frac{\cos\theta - \cos(\theta/2)}{k^2\cos(\theta/2)}\right]W_{i-1/2} \qquad (2.8)$$

$$\frac{\tan(\theta/2)}{k}\,[T_i - T_{i-1}] = \frac{2\sin^2(\theta/2)}{k^2\cos(\theta/2)}W_{i-1/2} \qquad (2.9)$$

Adding Eqs. (2.6) and (2.7) then use equation (2.8), it follows that

$$hD_i = \left(S_{i+1/2} - S_{i-1/2}\right) + \left[\frac{1}{k^2} - \frac{h}{2k\tan(\theta/2)} - \frac{h^2}{8}\right]\left(M_{i+1/2} - M_{i-1/2}\right) + \left[\frac{h^2}{8k^2} + \frac{h}{2k^3\tan(\theta/2)} - \frac{h}{2k^3\sin(\theta/2)}\right]\left(W_{i-1/2} - W_{i+1/2}\right) \qquad (2.10)$$

Adding Eqs. (2.8) and (2.9), it follows that

$$T_i = \frac{k}{2\tan(\theta/2)}\left(M_{i+1/2} - M_{i-1/2}\right) + \left(\frac{1}{2k\sin\theta/2} - \frac{1}{2k\,tan\theta/2}\right)\left(W_{i-1/2} - W_{i+1/2}\right) \qquad (2.11)$$

Eliminating $T's$ from Eqs. (2.9) and (2.11), it follows that:

$$\left(\frac{1}{2k\,tan\theta/2} - \frac{1}{2k\,sin\theta/2}\right)\left(W_{i-3/2} + W_{i+1/2}\right) + \left(\frac{1}{k\sin\theta/2} - \frac{1}{k\,tan\theta/2} - \frac{2\sin\theta/2}{k}\right)W_{i-1/2} = \left(\frac{k}{2tan\theta/2}\right)\left(-M_{i+1/2} + 2M_{i-1/2} - M_{i-3/2}\right) \qquad (2.12)$$

Eliminating $D's$ from Eqs. (2.7) and (2.10) then use equation (2.12) it follows that:

$$h^2 M_{i-1/2} = \left(S_{i-3/2} - 2S_{i-1/2} + S_{i+1/2}\right) + \left(\frac{1}{k^4 cos^{\theta}/_2} - \frac{h^2}{8k^2 cos^{\theta}/_2} - \frac{1}{k^4}\right)\left(W_{i-3/2} + W_{i+1/2}\right)$$

$$+\left(\frac{2+4\tan^{\theta}/_2 sin\,^{\theta}/_2}{k^4} + \frac{h^2}{4k^2 cos^{\theta}/_2} - \frac{h^2 sin^{\theta}/_2\, tan^{\theta}/_2}{2k^2} - \frac{2}{k^4 cos^{\theta}/_2} - \frac{h^2}{k^2}\right) W_{i-1/2} \quad (2.13)$$

Eliminating $M's$ from the Eqs. (2.12) and (2.13), it follows that:

$$S_{i-5/2} - 4S_{i-3/2} + 6S_{i-1/2} - 4S_{i+1/2} + S_{i+3/2} = h^4\left[\alpha\left(W_{i-5/2} + W_{i+3/2}\right) + \beta\left(W_{i-3/2} + W_{i+1/2}\right) + \gamma W_{i-1/2}\right], i = 3,.,n-2 \quad (2.14)$$

Where $W_i = f_i S_i + g_i$, with $f_i = f(x_i)$ and $g_i = g(x_i)$,

$$\alpha = \frac{-8\,tan^{\theta}/_2 + \theta^2 tan^{\theta}/_2 + 8\,sin^{\theta}/_2}{8\,\theta^4\,sin^{\theta}/_2}$$

$$\beta = \frac{8\ + 3\,\theta^2 - 16\,cos^{\theta}/_2 + (8-\theta^2)\cos\theta}{4\,\theta^4\,cos^{\theta}/_2}$$

$$\gamma = \frac{-8\ + \theta^2 + 24\,cos\left(^{\theta}/_2\right) + (16+6\,\theta^2)\cos\theta}{4\,\theta^4\,cos^{\theta}/_2}$$

If $k \to 0$ that is $\theta \to 0, (\alpha, \beta, \gamma) \to \left(\frac{1}{384}, \frac{76}{384}, \frac{230}{384}\right)$ So that the relation (2.14) reduce to quartic polynomial spline relation [2].

Eq. (2.14) gives (n-4) linear algebraic equations in the (n) unknowns $S_{i+1/2}, i = 0,1,2,\ldots\ldots,n-1$, so we need four more equations, two at each end of the range of integration for direct computation of $S_{i+1/2}$. These four equations are deduced by Taylor series along with the method of undetermined coefficients.

$$10S_{1/2} - 5\,S_{3/2} + S_{5/2} = 6\,S_0 - \frac{5}{4}\,h^2 S_0^{(2)} + h^4\left[\alpha_0 S_0^{(4)} + \sum_{j=1}^{5}\alpha_J S_{J-(1/2)}^{(4)}\right], for\ i = 1 \quad (2.15)$$

$$-5S_{1/2} + 6\,S_{3/2} - 4S_{5/2} + S_{7/2} = -2\,S_0 - \frac{h^2}{4}\,S_0^{(2)} + h^4\left[\sum_{J=1}^{6}\beta_J S_{J-(1/2)}^{(4)}\right], for\ i = 2 \quad (2.16)$$

$$S_{n-7/2} - 4\,S_{n-5/2} + 6S_{n-3/2} - 5S_{n-1/2} = -2\,S_n - \frac{h^2}{4}\,S_n^{(2)} + h^4\left[\sum_{J=1}^{6}\beta_{7-J} S_{n+J-(13/2)}^{(4)}\right], for\ i = n-1 \quad (2.17)$$

$$S_{n-5/2} - 5\,S_{n-3/2} + 10S_{n-1/2} = 6S_n - \frac{5}{4}\,h^2 S_n^{(2)} + h^4\left[\alpha_0 S_n^{(4)} + \sum_{J=1}^{5}\alpha_{6-J} S_{n+J-(11/2)}^{(4)}\right], for\ i = n \quad (2.18)$$

The local truncation errors $t_i, i = 1,2,\ldots\ldots,n$ associated with the scheme (2.14 – 2.18) can be obtained as follows: first we rewrite the scheme (2.14 – 2.18) in the form:

$$10y_{1/2} - 5\,y_{3/2} + y_{5/2} = 6\,y_0 - \frac{5}{4}\,h^2 y_0^{(2)} + h^4\left[\alpha_0 y_0^{(4)} + \sum_{J=1}^{5}\alpha_J y_{J-(1/2)}^{(4)}\right] + t_1;\ at\ i = 1 \quad (2.19)$$

$$-5y_{1/2} + 6\,y_{3/2} - 4y_{5/2} + y_{7/2} = -2\,y_0 - \frac{h^2}{4}\,y_0^{(2)} + h^4\left[\sum_{J=1}^{6}\beta_J y_{J-(1/2)}^{(4)}\right] + t_2;\ at\ i = 2 \quad (2.20)$$

$$y_{i-5/2} - 4y_{i-3/2} + 6y_{i-1/2} - 4y_{i+1/2} + y_{i+3/2} = \left[\alpha\left(y_{i-5/2}^{(4)} + y_{i+3/2}^{(4)}\right) + \beta\left(y_{i-3/2}^{(4)} + y_{i+1/2}^{(4)}\right) + \gamma\, y_{i-1/2}^{(4)}\right] + t_i;\ at\ i = 3,4,\ldots\ldots,n-2 \quad (2.21)$$

$$y_{n-7/2} - 4\,y_{n-5/2} + 6y_{n-3/2} - 5y_{n-1/2} = -2\,y_n - \frac{h^2}{4}\,y_n^{(2)} + h^4\left[\sum_{J=1}^{6}\beta_{7-J}\,y_{n+J-(13/2)}^{(4)}\right] + t_{n-1};\ at\ i = n-1 \quad (2.22)$$

$$y_{n-5/2} - 5\,y_{n-3/2} + 10y_{n-1/2} = 6y_n - \frac{5}{4}\,h^2 y_n^{(2)} + h^4[\alpha_0 y_n^{(4)} + \sum_{J=1}^{5}\alpha_{6-J}\,y_{n+J-(11/2)}^{(4)}] + t_n;\ at\ i = n \quad (2.23)$$

The terms $y_{i-1/2}\ and\ y_{i-1/2}^{(4)}, \ldots\ldots\ldots$ in Eq. (2.21) are expanded around the point x_i using Taylor series and the expressions for $t_i, i = 3,4,\ldots,n-2$ can be obtained. Also, expressions for $t_i, i = 1,2,n-1,n$ are obtained in a similar manner by expanding around $x_0, for\ i = 1,2$ and around $x_n, for\ i = n-1,n$, The local truncation errors $t_i, i = 3,4,\ldots,n-2$ associated with the scheme (2.14) are

$$t_i = \left(1-(2\alpha+2\beta+\gamma)\right)h^4\, y_i^{(4)} + \frac{1}{2}(-1+2\alpha+2\beta+\gamma)h^5\, y_i^{(5)} + \frac{1}{24}\left(7-3(34\alpha+10\beta+\gamma)\right)h^6\, y_i^{(6)} + \frac{1}{48}(-5+98\alpha+26\beta+\gamma)h^7 y_i^{(7)} + \frac{1}{1920}\left(69-5(706\alpha+82\beta+\gamma)\right)h^8 y_i^{(8)} + \frac{1}{11520}(-115+84646\alpha+726\beta+3\gamma)\,h^9 y_i^{(9)} + \frac{1}{967680}\left(2497-21(16354\alpha+730\beta+\gamma)\right)h^{10}\, y_i^{(10)} + O(h^{11}),\quad at\ i=3,..,n-2 \tag{2.24}$$

The scheme (2.14 – 2.18) gives rise to a class of methods of different orders as follows:

(I) Second order method

For any choice of arbitrary α and β with $\gamma = 1-2(\alpha+\beta)$

And $(\alpha_0,\alpha_1,\alpha_2,\alpha_3,\alpha_4,\alpha_5) = \left(\frac{-77}{192}, \frac{192}{192}, 0,0,0,0\right)$

$(\beta_1,\beta_2,\beta_3,\beta_4,\beta_5,\beta_6) = \left(\frac{-3}{384}, \frac{385}{384}, 0,0,0,0\right)$

Then the local truncation errors for $(\alpha,\beta,\gamma) = \left(\frac{3}{200}, \frac{30}{200}, \frac{134}{200}\right)$ are

$$t_i = \begin{cases} \frac{623}{4608}\, h^6\, y_i^{(6)} + O(h^7)\ ;\ i=1,n \\ \frac{1897}{11520}\, h^6\, y_i^{(6)} + O(h^7)\ ;\ i=2,n-1 \\ \frac{-13}{300}\, h^6\, y_i^{(6)} + O(h^7)\ ;\ i=3,\dots\dots,n-2 \end{cases} \tag{2.25}$$

(II) Fourth order method

For any choice of arbitrary α with $\beta = \frac{1-24\alpha}{6}$ and $\gamma = 1-2(\alpha+\beta)$

And

$$(\alpha_0,\alpha_1,\alpha_2,\alpha_3,\alpha_4,\alpha_5) = \left(\frac{-108}{5760}, \frac{2410}{5760}, \frac{1195}{5760}, \frac{-47}{5760}, 0, 0\right)$$

$$(\beta_1,\beta_2,\beta_3,\beta_4,\beta_5,\beta_6) = \left(\frac{3575}{23040}, \frac{15629}{23040}, \frac{3677}{23040}, \frac{39}{23040}, 0, 0\right)$$

Then the local truncation errors for $(\alpha,\beta,\gamma) = \left(\frac{-7}{4850}, \frac{2509}{14550}, \frac{4787}{7275}\right)$ are

$$t_i = \begin{cases} \frac{9587}{3096576}\, h^8\, y_i^{(8)} + O(h^9)\ ;\ i=1,n \\ \frac{-44761}{15482880}\, h^8\, y_i^{(8)} + O(h^9)\ ;\ i=2,n-1 \\ \frac{19}{349200}\, h^8\, y_i^{(8)} + O(h^9)\ ;\ i=3,\dots\dots,n-2 \end{cases} \tag{2.26}$$

(III) Six order method

For $(\alpha,\beta,\gamma) = \left(\frac{-1}{720}, \frac{124}{720}, \frac{474}{720}\right)$ and $(\alpha_0,\alpha_1,\alpha_2,\alpha_3,\alpha_4,\alpha_5)=$

$$\left(\frac{-622}{589935}, \frac{856}{2265}, \frac{358}{1416}, \frac{-790}{18878}, \frac{354}{28317}, \frac{-395}{226534}\right)$$

$(\beta_1,\beta_2,\beta_3,\beta_4,\beta_5,\beta_6) =$

$$\left(\frac{108}{719}, \frac{253}{360}, \frac{458}{3995}, \frac{661}{239720}, \frac{-120}{7192}, \frac{661}{239720}\right)$$

Then the local truncation errors are

$$t_i = \begin{cases} \frac{833}{902891}\, h^{10}\, y_i^{(10)} + O(h^{11})\ ;\ i=1,n \\ \frac{-3307}{1538523}\, h^{10}\, y_i^{(10)} + O(h^{11})\ ;\ i=2,n-1 \\ \frac{1}{3024}\, h^{10}\, y_i^{(10)} + O(h^{11})\ ;\ i=3,\dots\dots,n-2 \end{cases} \tag{2.27}$$

Remark:

(i) When $\alpha = \frac{1}{384}, \beta = \frac{76}{384}$ and $\gamma = \frac{230}{384}$ then the scheme (2.14) is reduced to Usmani method based on quartic polynomial spline [2].

(ii) When $\alpha = 0, \beta = \frac{62526}{375156}$ and $\gamma = \frac{250104}{375156}$ then the scheme (2.14) is reduced to Al-Said and Noor based on finite difference method [11].

(iii) When $\alpha = 0, \beta = \frac{1}{24}$ and $\gamma = \frac{22}{24}$ then the scheme (2.14) is reduced to Al-Said and Noor based on cubic polynomial spline method [5].

3. Spline solutions:

The spline solution of (1.3) with the boundary condition (1.4) is based on the linear equations given by (2.14 – 2.18) , Let $Y = \left(y_{i+1/2}\right), S = \left(s_{i+1/2}\right), C = (c_i), T = (t_i), E = e_{i+1/2} = Y - S$ be n-dimensional column vectors, then we can write the standard matrix equations in the form:

$$(i)NY = C+T$$
$$(ii)NS = C \tag{3.1}$$
$$(iii)NE = T$$

We also have $N = N_0 + h^4 BF, F = diag\ \left(f_{i+1/2}\right)$

(3.2)

Where

$$N_0 = \begin{bmatrix} 10 & -5 & 1 & & & & \\ -5 & 6 & -4 & 1 & & & \\ 1 & -4 & 6 & -4 & 1 & & \\ & & 1 & -4 & 6 & -4 & 1 \\ & & & 1 & -4 & 6 & -5 \\ & & & & 1 & -5 & 10 \end{bmatrix} \quad (3.3)$$

The matrix B has the form

$$B = \begin{bmatrix} \alpha_1 & \alpha_2 & \alpha_3 & \alpha_4 & \alpha_5 & & \\ \beta_1 & \beta_2 & \beta_3 & \beta_4 & \beta_5 & \beta_6 & \\ \alpha & \beta & \gamma & \beta & \alpha & & \\ & & & & & & \\ & & \alpha & \beta & \gamma & \beta & \alpha \\ & \beta_6 & \beta_5 & \beta_4 & \beta_3 & \beta_2 & \beta_1 \\ & & \alpha_5 & \alpha_4 & \alpha_3 & \alpha_2 & \alpha_1 \end{bmatrix} \quad (3.4)$$

For the vector C, we have

$$C_i = \begin{cases} 6A_1 - \frac{5}{4}h^2\beta_1 + h^4\left[\alpha_0(-A_1 f_0 + g_0) + \sum_{J=1}^{5}\alpha_J g_{J-(1/2)}\right], & i=1 \\ -2A_1 - \frac{h^2}{4}\beta_1 + h^4\left[\sum_{J=1}^{6}\beta_J g_{J-(1/2)}\right], & i=2 \\ h^4\left[\alpha\left(g_{i-5/2}+g_{i+3/2}\right)+\beta\left(g_{i-3/2}+g_{i+1/2}\right)+\gamma\left(g_{i-1/2}\right)\right], & i=3,.....,n-2 \\ -2A_2 - \frac{h^2}{4}\beta_2 + h^4\left[\sum_{J=1}^{6}\beta_{7-J}\ g_{n+J-(13/2)}\right], & i=n-1 \\ 6A_2 - \frac{5}{4}h^2\beta_2 + h^4\left[\alpha_0(-A_2 f_n + g_n) + \sum_{J=1}^{5}\alpha_J g_{n+J-(11/2)}\right], & i=n \end{cases} \quad (3.5)$$

4. Convergence analysis

Our main purpose now is to derive a bound on $\|E\|$ where $\|\ .\ \|$ represents the infinity norm. Now we turn back to the error equation (iii) in (3.1) and rewrite it in the form

$$E = N^{-1}T = (N_0 + h^4BF)^{-1}T$$
$$= (I - N_0^{-1}h^4BF)^{-1}\ N_0^{-1}\ T$$

We get

$$\|E\| \le \frac{\|N_0^{-1}\|\ \|T\|}{1-h^4\|N_0^{-1}\|\ \|B\|\ \|F\|} \quad (4.1)$$

Provided that $h^4\|N_0^{-1}\|\ \|B\|\ \|F\| < 1$

It was shown in [2] that N_0 is nonsingular and its inverse satisfies the inequality

$$\|N_0^{-1}\| \le \frac{5(b-a)^4+10(b-a)^2\ h^2+9h^4}{384\ h^4} = O(h^{-4}) \quad (4.2)$$

Lemma 4.1. The matrix N given by (3.2) is nonsingular, provided that $\|B\|\ |f(x)|\ \tilde{w} < 1$ (4.3)

Where $\tilde{w} = \frac{5(b-a)^4+10(b-a)^2\ h^2+9h^4}{384}$ and $\|B\|$ is a finite number; the proof of this lemma follows from the following statement [14]; if G is a square matrix of order n and $\|G\| < 1$ then $(I+G)^{-1}$ exists and $\|(I+G)^{-1}\| < \frac{1}{1-\|G\|}$

As a consequence of lemma 4.1, the discrete boundary value problem has a unique solution if $\|B\|\ |f(x)|\ \tilde{w} < 1$.

Now from Eqs. (2.25 – 2.27) we investigate the following three cases:

Case (*i*): second order convergent method

We have from Eq (2.25)

$$\|T\| = \frac{1897}{11520}\ h^6\ M_6, M_6 = max_{a\le x\le b}\left|y^{(6)}(x)\right| \quad (4.4)$$

And then it follows that:

$$\|E\| \le \frac{1897\tilde{w}M_6\ h^2}{11520[1-\tilde{w}\ \|B\|\ |f(x)|]} = G_2\ h^2\ \cong O\ (h^2) \quad (4.5)$$

Where $G_2 = \frac{1897\tilde{w}M_6}{11520[1-\tilde{w}\ \|B\|\ |f(x)|]}$

Case (*ii*) : fourth order convergent method

We have from Eq (2.26)

$$\|T\| = \frac{9587}{3096576}\ h^8\ M_8, M_8 = max_{a\le x\le b}\left|y^{(8)}(x)\right| \quad (4.6)$$

And then it follows that:

$$\|E\| \le \frac{9587\ \tilde{w}M_8\ h^4}{3096576[1-\tilde{w}\ \|B\|\ |f(x)|]} = G_4\ h^4\ \cong O\ (h^4) \quad (4.7)$$

Where $G_4 = \frac{9587\ \tilde{w}\ M_8}{3096576[1-\tilde{w}\ \|B\|\ |f(x)|]}$

Case (*iii*): six order convergent method

We have from Eq (2.27)

$$\|T\| = \frac{3307}{1538523}\ h^{10}\ M_{10}, M_{10} = max_{a\le x\le b}\left|y^{(10)}(x)\right| \quad (4.8)$$

And then it follows that:

$$\|E\| \le \frac{3307\ \tilde{w}M_{10}\ h^6}{1538523[1-\tilde{w}\ \|B\|\ |f(x)|]} = G_6\ h^6\ \cong O\ (h^6) \quad (4.9)$$

Where $G_6 = \frac{3307\ \tilde{w}\ M_{10}}{1538523[1-\tilde{w}\ \|B\|\ |f(x)|]}$

We summarize the above results in the next theorem

Theorem 4.1
Let $y(x)$ be the exact solution of the continuous boundary value problem (1.3) with the boundary condition (1.4) and let $y_{i+1/2}, i = 0, 1, \ldots, n-1,$ satisfy the discrete boundary value problem (*ii*) in (3.1). Further, if $e_{i+1/2} = y_{i+1/2} - S_{i+1/2}$ then

(1) $\|E\| \cong O(h^2)$, is a second order method which is given by (4.5).
(2) $\|E\| \cong O(h^4)$, is a fourth order method which is given by (4.7).
(3) $\|E\| \cong O(h^6)$, is a six order method which is given by (4.9).

5. Numerical examples and discussion:

We now consider two numerical examples to illustrate the comparative performance of our method (*ii*) in (3.1) over other existing methods. All calculations are implemented by MATLAB 7 .

Example 1. Consider the boundary value problem

$$y^{(4)} - y = -4(2x\cos(x) + 3\sin(x)) \quad (5.1)$$
$$y(0) = y(1) = 0, y^{(2)}(0) = 0, y^{(2)}(1) = 2\,sin(1) + 4\cos(1)$$

The analytical solution of (5.1) is

$$y(x) = (x^2 - 1)\sin x \quad (5.2)$$

The numerical results for our second, fourth and six orders are summarized in Table 1.

*Table1: The observed maximum errors for Example*1

h	Six order method	Fourth order method	Second order method
$\frac{1}{8}$	5.07 – 10	3.77 – 8	5.10- 5 [a]
$\frac{1}{16}$	7.81 – 12	3.18 – 10	2.97 – 5
$\frac{1}{32}$	1.02 – 13	9.11 – 12	9.92 – 6

[a] $5.10 - 5 = 5.10 \times 10^{-5}$

Example 2. Consider the boundary value problem

$$y^{(4)} + xy = -(8 + 7x + x^3)e^x \quad (5.3)$$
$$y(0) = y(1) = 0, y^{(2)}(0) = 0, y^{(2)}(1) = -4e$$

The analytical solution of (5.3) is

$$y(x) = x(1 - x)e^x \quad (5.4)$$

Table 2: The observed maximum errors for example 2

h	Six order method	Fourth order method	Second order method
$\frac{1}{8}$	1.48 – 9	1.08 – 7	1.91 – 4
$\frac{1}{16}$	2.22 – 11	1.13 – 9	6.98 – 5
$\frac{1}{32}$	5.79 – 13	2.92 – 11	2.54 – 5

Table 3: The observed maximum errors for example 2

h	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$
Our six order method	1.48 – 9	2.22 – 11	5.79 – 13
Ramadan et al. [13]	1.76 – 8	2.98 – 10	4.75 – 12
Ramadan et al. [12]	1.91 – 7	3.12 – 9	5.02 – 11
Al-Said and Noor [11]	2.86 – 7	2.27 – 8	1.49 – 9
Zhu [10]	6.90 – 7	1.30 – 8	2.20 – 10
Usmani and Warsi [3]	1.26 – 6	7.87 – 8	4.91 – 9
Al-Said et al. [5]	5.69 – 4	1.47 – 4	3.71 – 5
Usmani [2]	4.24 – 4	1.08 – 4	2.70 – 5
Usmani and Warsi [3]	8.67 – 4	2.16 – 4	5.40 – 5
Al-Said and Noor [4]	1.62 – 3	6.39 – 4	5.88 – 5

The numerical results for our six, fourth and second ordered methods are summarized in Tables 1-3 and compared with other existing methods. The results in Table 3 clearly show superiority over the

existing methods and also confirm that on halving the step size h , the $\|E\|$ is approximately reduced by a factor of $\frac{1}{2^p}$ where p is the order of the numerical method.The proposed quartic nonpolynomial spline method generalizes other existing methods through arbitrary choices of $\alpha, \beta \; and \; \gamma$ where we get six, fourth and second ordered methods.

6. Conclusion:

Three new methods are presented for solving fourth order two point boundary value problem using quartic nonpolynomial spline. These methods are shown to be optimal second, fourth and six ordered methods which are better than other existing methods [2-5, 10-13]. Introduction of the parameter k in the trigonometric part of the nonpolynomial spline function of the present methods improves the accuracy of the schemes which is evident from the numerical results given in tables 1-3, and these results show that the proposed methods maintain a very remarkable high accuracy which make them are very encouraging for dealing with the solution of two point boundary value problems.

Corresponding author
Z.A. ZAki*
Department of Engineering Mathematics and Physics, Faculty of Engineering, Benha University, Shoubra, Cairo, Egypt.
Zahmed_2@yahoo.com

7. References

1. E.L. Reiss, A.J. Callegari, D.S. Ahluwalia, Ordinary differential equation with Applications, Holt, Rinehart and Winston, New York, 1976.
2. R.A. Usmani, The use of quartic splines in the numerical solution of a fourth order boundary problems, Journal of Computational and Applied Mathematics, 44 (1992), 187 – 199.
3. R.A. Usmani, S.A. Warsi, Smooth spline solutions for boundary value problems in plate deflection theory, Computers and Mathematics with Applications, 6(1980), 205 – 211.
4. E.A. Al-Said, M.A. Noor, Quartic spline method for solving fourth order obstacle boundary value problems, Journal of Computational and applied Mathematics, 143 (2002), 107 – 116.
5. E.A. Al-Said, M.A. Noor, T.M. Rassias, Cubic splines method for solving fourth order obstacle boundary value problems, Applied Mathematics and Computations, 174 (2006), 180 – 187.
6. R.A. Usmani, Smooth spline approximations for the solution of a boundary value problem with engineering applications, Journal of Computational and Applied Mathematics, 6 (1980), 93 – 98.
7. J. Rashidinia, A. Golbabaee, Covergence of numerical solution of a fourth order boundary value problem, Applied Mathematics and Computation, 171(2005), 1296 – 1305.
8. S.S. Siddiqi, G. Akram, Solution of the system of fourth order boundary value problems using non polynomial spline technique, Applied Mathematics and Computation, 185(2007), 128 – 135.
9. M. VanDaele, G. Vanden Beghe, H. De Meyer, A smooth approximation for the solution of a fourth order boundary value problem based on nonpolynomial splines, Journal of Computational and Applied Mathematics, 51(1994), 383 – 394.
10. Y. Zhu, Quartic spline collocation methods for fourth order two point boundary value problems, Msc Thesis, Department of Computer Science, Univesity of Toronto, 2001.
11. E.A. Al-Said, M.A. Noor, Finite difference method for solving fourth order obstacle problems, Int. J. Comput. Math. 81(2004), 741 – 748.
12. M.A. Ramadan, I.F. Lashien, W.K. Zahra, Quintic nonpolynomial spline solutions for fourth order two point boundary value problems. Communications in Nonlinear Science and Numerical Simulations, 14(2009), 1105 – 1114.
13. M.A. Ramadan, I.F. Lashien, W.K. Zahra, High order accuracy nonpolynomial spline solutions for $2\mu th$ order two point boundary value problems, Applied Mathematics and Computation 204(2008), 920 – 927.
14. R.A. Usmani, Discrete methods for a boundary value problem with Engineering applications, Math. Comput. 32(1978), 1087 – 1096.

6/29/2010

Lambda, the pyrethroid insecticide as a mutagenic agent in both somatic and germ cells.

Abdel Aziz K.B. and Abdel Rahem H.M.

Cell Biology Department, National Research Center, Cairo, Egypt.
k.badrakhan@yahoo.com

Abstract: Cytogenetic evaluations of pyrethroid insecticide cyhalothrin (lambda) were investigated in mice *in vivo* by recording chromosomal aberrations in bone marrow cells and in primary spermatocytes. Cyhalothrin (lambda) insecticide was orally administrated with 2, 2.5, 5 mg/kg b.wt. (1/10, 1/8, 1/4 LD50 doses respectively) for repeated treatment. Cyhalothrin (lambda) was found to produce a significant structural and numerical chromosomal damage after subacute treatment in both bone marrow cells and primary spermatocytes. This effect was dose and time-dependent. For studying sperm abnormalities, mice were orally treated with the highest dose, 1/4 LD50. Cyhalothrin (lambda) insecticide was found to induce a significant increase in the percentage of sperm abnormalities which was mainly in the head. The present study clearly indicates that Cyhalothrin (lambda) insecticide is genotoxic to the different kinds of cells analyzed. Accordingly, much more care should be taken during the use of these pesticides. [Journal of American Science. 2010;6(12):317-326]. (ISSN: 1545-1003).

Keywords: Pyrethroid insecticides; Lambda-cyhalothrin; chromosomal aberrations; Sperm abnormalities; genotoxicity.

1. Introduction

Problems associated with pesticide hazards to man and the environment are not confined only to the developing countries. Developed nations have already suffered these problems, and still facing some problems in certain locations. For many reasons, the severity of pesticide hazard is much pronounced in Third World Countries. The misuse of pesticides by concerned individuals, in addition to lack of or weak national controlling plans is behind the outbreak of adverse effects in developing countries (Mansour, 2004). The indiscriminate use of pesticides and herbicides to increase crop productivity has aroused a great concern among the environmental and health scientists due to their adverse effects in both targets as well as non-target species. Although substantial information is available regarding their environmental and ecological impact, not much is known in regard to its toxicity in the mammalian system (Patel *et al*., 2007).Pyrethroids (also known as synthetic pyrethroids) are neurotoxic insecticides widely used to control agricultural and domestic insect pests, chemically similar to pyrethrins found in natural pyrethrum extracted from the flowers of chrysanthemum, known for centuries for their insecticidal activity, but are often more toxic to insects, as well as to mammals, and last longer in the environment than pyrethrins (WHO, 2005, 2003).

Apart from their use in agriculture, pyrethroids play an important role in public health programmes. Globally, more than 520 tonnes of active ingredient of pyrethroids is annually used in vector control programmes alone (Zaim & Jambulingam, 2004). The general population is potentially chronically exposed to pyrethroids through food consumption (Fortina *et al*., 2008). All these compounds are widely distributed in the market and used continuously in our houses. Thus, risks of these pesticides surround us and our sons daily (Kaya *et al.*, 2010). Probabilities of their accumulation in the human bodies are increasingly elevated. Cyhalothrin (lambda), a synthetic pyrethroid type II insecticide, is widely used in Egypt valued for its broad-spectrum control a wide range of pests in variety of applications such as the protection of cotton, cereals, hops, ornamentals and vegetables as well as in public health application against insect, ticks and flies which may act as disease vectors. So, the aim of the present study is to evaluate *in vivo* the genotoxicity of the most commonly used pyrethroid insecticide Cyhalothrin (lambda) in Egypt. The cytogenetic evaluations were conducted on male laboratory mice on three levels: Bone marrow cells as a model of mitotic chromosome abnormalities, Spermatocytes as a model for meiotic chromosome abnormalities and Sperm count and morphological abnormalities.

2. Material and Methods

In this work adult male swiss albino mice (mas muscles) weighing from 25 to 30 gm were used.

Cyhalothrin (lambda) 5%EC was chosen to be detected as a broad spectrum synthetic pyrethroid insecticide, used in Egypt. The insecticide under test was administered orally at three different doses 5, 2.5 and 2 mg/kg (bodyweight). To study the effect on both somatic and germ cells, six groups of mice in each case were used each contained six animals. The first and the second groups were treated with the high dose (5mg/kg) for 2 and 4 months respectively. The third and the fourth ones with the median dose (2.5 mg/kg), the fifth and sixth ones with the low dose(2mg/kg),in addition to untreated group which acted as control. Mice were sacrificed 24 hours after the last injection. Each one of these doses was studied as a sub-acute treatment, the dose was given once a week for two different times 2 and 4 months for both somatic and germ studies. Chromosomes were prepared from bone marrow cells to study the abnormalities in somatic cells according to the method of (Yosida and Amona 1965). Chromosomes from germ cells (spermatocytes) were prepared according to (Brewen and Preston 1978) method. Slides were prepared and stained, then so good metaphase spreads for each animal were examined for recording abnormalities in both somatic and germ cells. In the same time sperm morphology was examined for recoding morphological abnormalities using the method of (Wyrobek and Bruce, 1978). Mice were orally administrated with the insecticide (5mg/kg), Cyclophosamide as a positive control (20mg/kg), and a negative control group was used (non-treated) for this experiment. Finally statistical analysis was carried out using analysis of variance (ANOVA) according to (Snedcor and Cochran 1980) and least significant difference (LSD) test according to (Steel and Torrie, 1981).

3. Results

Cytogenetic effect on somatic cells:

As listed in (table I), the observed types of structural and neumerical abnormalities showed highly significance, even in the low dose (2mg/kg) which showed statistically significant over the control after the two times used 2 and 4 months. In the case of median dose, the significance level was over control and the low dose in most types of structural and numerical aberrations (table1). The high dose effect showed statistically significance at a level of $P \leq 0.05$ over the control and the other two treatments for the numerical aberrations (hypoploidy and hyperploids), the increased significance was compared to control and low dose treatment but no significance over the median dose treatment was observed (table1). Also the effect of time was listed in the same table (2 and 4 months) which showed that the increase in the duration of oral treatment increased significantly in all types of structural and numerical aberrations except in the case of centromeric attenuation, which was statistically decreased as the time of duration increased (table1) Also, the same table demonstrated the interaction between doses and times which showed that after the sub acute treatments for 2 months, there was a gradual increase in the number of cells with structural and numerical aberrations as the dose increased which demonstrated high significant increase in the total cells with aberrations as the dose increased (table1). After the sub acute treatment for 4 months, the abnormal cells showed obvious increase as the dose increased.

Cytogenetic effect on germ cells:

The cytogenetic effect of insecticide under test on germ cells was listed in (table2) which illustrated this effect in the following: there was a statistical significance increase in both structural and numerical aberrations over control even with the low dose (2mg/kg) except the cells with polyploidy which showed non- significant comparing to the control (table2). In the case of median dose, the significant level also was over the low dose. In the case of high dose the statistical analysis demonstrated high significance over control and the other two treatments except a chain aberration which showed insignificant when compared with median dose treatment (table2). The effect of time of treatment showed, structural aberrations increased significantly as the time of treatment increased but numerical aberrations showed slight significant decrease as the duration time increased. The genotoxic effect of the insecticide under test showed, gradual increase in number of abnormal germ cells as the duration time of treatment increased, 4 months were more than 2 months. Also, total cells with aberrations showed a dose- dependent increase. I.e. increasing the dose increased the total number of abnormal cells significantly over control (Table 2).

Cytogenetic effect on sperm morphology:

(Table 3 and fig1) illustrated the harmful effects of cyhalothrin, the insecticide under test on sperm morphology and sperm count. The results showed that sperm counts were significantly high decreased after treatment with the insecticide than positive and negative controls. The Cyhalothrin (lambda) with highest tested dose (5mg/kg) and

Cyclophosphamide (20mg/kg) produced a higher rate of aberrations than the negative control. Cyhalothrin (lambda) under test induced a highly significant increase over the control group in many types of sperm abnormalities which include: amorphous, without hook, banana, small and big for head abnormalities, also for tail abnormalities include coild tail and two tails, which increased over the control. Cyhalothrin (lambda) also showed a statistically significant increase when compared with Cyclophosphamide treatment in different types of sperm abnormalities. In contrast, it showed statistically non-significant for heads without hook, small head abnormalities and tail abnormalities.

Fig. (1): Sperm morphology of treated mouse.

(a) Normal sperm morphology of control mouse.
(b) Sperm of treated mouse showing head without hook.
(c) Sperm of treated mouse showing big head.
(d) Sperm of treated mouse showing small head.
(e) Sperm of treated mouse showing banana shaped head.
(f) Sperm of treated mouse showing amorphous head.
(g) Sperm of treated mouse showing two tails.
(h) Sperm of treated mouse showing coiled tail.

Table (1): Effect of dose, time and their interaction on bone marrow structural and numerical aberrations.

	Structural aberration (M ± Sd)									Numerical aberration (M ±Sd)						Total Aberrations (M±Sd) (54 ± Sd)
Treatments	Chromated Gap	Chromos. Gap	Chromated Break	Chromos. Break	Centric fusion	End to end	Deletion	Fragment	Total	Centrom. Attune.	Hypoploid	hyperploids	Polyploidy	Endomitosis	Total	
Control	5.83 b ± 0.55	0.50 c ± 0.21	1.33 c ± 0.64	0.33c ± 0.01	1.83 d ± 0.94	2.17 d ± 0.55	0.83 d ± 0.19	0.00 c ± 0.00	12.83 d ± 3.18	4.50 c± 0.57	2.67 c ± 0.39	0.00 d ± 0.00	0.00 b ± 0.00	3.00 b ± 0.99	10.17 d ± 1.89	23.00 d ± 4.27
1/10 LD_{50}	17..33 a ± 4.14	3.17 b ± 2.10	3.17 b± 0.19	0.50 bc ± 0.18	4.00 c± 1.48	4.67 b ± 0.78	5.00 c ± 2.05	0.33 c ± 0.37	3 8.17 c± 11.67	10.17 b± 0.77	6.67 b ± 0.78	0.33 c ± 0.07	0.50 b ± 0.55	0.83 c ± 0.19	18.50 c ± 1.99	56.67 c ± 10.73
1/8 LD_{50}	18.83 a ± 3.60	7. 00 a ± 2.83	6.17 a± 2.91	0.67 b ± 0.83	6.17 b ± 1.66	4.00 c ± 0.39	6.50 b± 1.75	1.50 b ± 0.97	50.83 b ± 13.53	10.33 b± 1.22	8.67 a ± 0.59	1.83 a ± 1.28	1.33 a ± 1.79	4.83 b ± 3.45	27.00 b ± 4.84	77.84 b ± 17.88
1/4 LD_{50}	18.83 a ± 3.60	7.17a± 3.08	5.67a ± 1.60	2.17 a ± 1.28	9.34 a ± 0.84	5.00 a ± 0.14	7.83 a ± 2.54	4.17 a ± 1.18	60.17 a ± 11.36	13.43 a ± 0.87	8.83 a ± 2.10	0.67 b ± 0.73	0.50 b± 0.55	8.83 a ± 2.47	32.17 a ± 2.24	92.33a ± 12.46
LSD at a 0.05	1.96	1.35	1.23	0.28	0.39	0.24	0.99	0.56	5.85	0.63	0.57	0.07	0.71	2.05	2.61	6.21
2. m	13.25 b ± 5.22	2.75 b ± 2.21	3.17 b ± 1.59	0.42 b ± 0.38	4.25 b ± 2.99	3.75 b ± 1.32	3.67 b ± 2.11	1.00 b ± 1.45	32.25 b ± 16.27	10.00 a ± 3.86	6.42 b ± 2.59	0.58 b ± 0.52	0.25 b ± 0.45	3.42 b ± 3.3	20.67 b ± 8.75	52.92 b ± 24.65
4 .m	17.17 a ± 6.72	6.17 a ± 3.96	5.00 a± 3.01	1.42 a ± 1.25	6.42 a ± 2.86	4.17 a± 1.13	6.42 a ± 3.58	2.00 a ± 2.08	48.75 a ± 22.02	9.17 b ± 2.89	7.00 a ± 3.01	0.83 a ± 1.32	0.92 a± 1.34	5.33 a ± 3.86	23.25 a ± 9.45	72.00 a ± 30.88
LSD at a 0.05	1.39	0.95	0.873	0.197	0.27	0.167	0.69	0.39	4.14	0.45	0.40	0.05	0.50	1.45	1.84	4.39
Control	5.33 e ± 0.04	0.33 c ± 0.17	1.00 f ±	0.33 de ± 0.02	1.00 g ± 0.01	1.67 e ± 0.01	0.67 f ± 0.04	0.00 d ± 0.00	10.33 e ± 2.34	4.00 f ± 0.26	2.33 e ± 0.17	0.00 e ± 0.00	0.00 b ± 0.00	2.67 b ± 1.04	9.00 d ± 1.67	19.33 0 ± 1.04
1/10 LD_{50} x2m	13.67 d ± 1.09	1.33 c ± 0.64	3.00 de ±	0.33 de ± 0.02	2.67 f ± 0.22	4.00 c ± 0.26	3.33 e ± 1.04	0.00 d ± 0.00	28.33 d ± 4.81	10.67 c ± 0.66	7.33 c ± 0.33	0.33 d ± 0.11	0.00 b ± 0.00	0.67 b ± 0.04	19.00 c ± 2.76	47.33 d ± 2.43
1/8 LD_{50} x2 m	16.00 cd ± 1.98	4.67 b ± 1.04	3.67 cd ±	0.00 e ± 0.00	4.67 e ± 0.28	4.33 b ± 0.21	5.00 d ± 0.54	0.67 d ± 0.04	39.00 c ± 5.48	11.33c ± 0.43	9.00 b ± 0.34	0.67 c ± 0.04	0.00 b ± 0.00	2.00 b ± 0.50	23.00 b ± 2.29	62.00 c ± 6.32
1/4 LD_{50} x2m	18.00 bc ± 2.28	4.67 b ± 1.64	5.00 be ±	1.00 bc ± 0.10	8.67 b± 0.40	5.00 a ± 0.20	5.67 cc ± 0.82	3.33 b ± 0.43	51.33 b ± 6.29	14.00 a ± 0.35	7.00 c ± 0.29	1.33 b ± 0.07	1.00 b ± 0.10	8.33 a ± 2.34	31.67 a ± 2.66	83.00 b ± 8.65
1/10 LD_{50} x4m	21.00 a ± 1.10	5.00 b ± 0.74	3.33 cde ± 0.01	0.67 cd ± 0.04	5.33 d± 0.33	5.33 a± 0.33	6.67 b ± 1.07	0.67 d ± 0.04	48.00 b ± 5 .22	9.67 d ± 0.55	6.00 d± 0.26	0.33 d ± 0.02	1.00 b ± 0.10	1.00 b ± 0.10	18.00 c ± 1.26	66.00 c ± 4.56
1/8 LD_{50} x4m	21.67 a ± 2.09	9.33 a ± 1.64	8.67 a ±	1.33 b ± 0.64	7.67 c ± 0.15	3.67 c ± 0.16	8.00 b ± 0.79	2.33 c ± 0.51	62.67 a ± 2 .74	9.33 d ± 0.74	8.33 b ± 0.66	3.00 a ± 0.10	2.67 a ± 1.64	7.67 a ± 2.34	31.00 a ± 2.29	93.67 a ± 2.66
1/4 LD_{50} x4m	19.7 ab ± 2.15	9.67 a ± 1.51	6.33 b	3.33 a ± 0.07	10.00 a ± 0.52	5.00 a ± 0.10	10.00 a ± 1.18	5.00 a ± 1.10	69.00 a ± 7.01	12.67 b ± 0.66	10.67 a ± 0.94	0.00 e ± 0.00	0.00 b ± 0.00	9.33 a ± 3.01	32.67 a ± 2.17	101.67 a ± 7.21
LSD at a 0.05	2.78	1.90	1.75	0.395	0.545	0.33	1.39	0.79	8.28	0.89	0.80	0.095	1.01	2.89	3.69	8.78

Statistical analysis of results were done according to least significant difference (LSD) test Different letters (a, b, c, d, e) within each column means the degree or significance at 0.05 level.

(M ± Sd): M is the mean value, Sd: Standard deviation.

Table (2): Effect of dose, time and their interaction on structural aberration and numerical aberrations of Spermatocytes.

Treatments	Structural aberration (M ± Sd)						Numerical aberration (M ±Sd)				Total aberrations (M± Sd)
	Autosomal univalent	X-Y univalent	Chain	Fragment	Ring	Total	Hypoploidy	Hyperploidy	Polyploidy	Total	
Control	3.167 d± 0.93	2.334 c± 1.01	0.000 ± 0.00	0.000 d± 0.00	0.000 d± 0.00	5.500 d± 1.44	2.833 d± 0.55	0.000 c± 0.00	2.500 c± 0.55	5.334 d± 1.00	10.834 d± 1.32
1/10 LD_{50}	9.833 c± 1.89	9.167 b± 2.56	3.333 b± 0.03	0.334 c± 0.37	0.334 c± 0.37	23.000 c± 5.28	8.000 c± 0.67	0.000 c± 0.00	1.667 c± 0.76	8.833 c± 1.28	31.833 c± 4.76
1/8 LD_{50}	15.000 b± 3.07	10.000 b ± 1.44	4.334 a± 0.40	0.833 b± 0.55	0.500 b± 0.21	30.667 b± 5.02	10.333 b± 0.97	0.834 b ± 0.19	4.167 b± 2.52	15.333 b± 2.88	46.000 b± 2.81
1/4 LD_{50}	17.167 a± 1.65	11.834a± 2.56	4.500 a± 0.37	2.000 a± 1.14	0.834 a± 0.18	36.333 a± 5.43	12.334 a± 1.73	2.167 a± 0.55	6.334 a± 2.32	20.833 a± 3.17	57.167 a± 2.89
LSD at a 0.05	1.31	1.29	0.25	0.21	0.08	2.47	1.02	0.04	1.20	1.99	1.79
2 M 4 M	9.917 b± 6.37	6.917 b± 3.27	2.917 b± 1.82	0.333 b± 0.44	0.334 b± 0.35	20.417 b± 10.53	8.583± 4.38	0.583 b± 0.71	4.500 a± 3.07	13.667 a± 7.62	34.083 b± 17.76
4 M	12.667 a± 5.13	9.750 a± 4.53	3.167 a± 1.99	1.250 a± 1.18	0.500 a± 0.40	27.333 a± 13.99	8.167± 3.21	0.917 a± 1.14	2.833 b± 1.31	11.500 b± 5.14	38.833 a± 18.55
LSD at a 0.05	0.93	0.92	0.18	0.15	0.06	1.75	N.S	0.03	0.85	1.408	1.26
Control x2 M	3.000 e± 1.02	2.000 e± 1.08	0.000 d± 0.00	0.000 f± 0.00	0.000 d± 0.00	5.000 e± 1.10	2.333 d± 0.02	0.000 e± 0.00	2.667 c± 0.03	5.000 e± 1.03	10.000 g± 1.08
Control x4 M	3.333 e± 1.03	2.667 e ± 1.03	0.000 d± 0.00	0.000 f± 0.00	0.000 d± 0.00	6.000 e± 1.79	3.333 d± 0.03	0.000 e± 0.00	2.333 c± 0.82	5.667 e± 1.06	11.667 g± 1.06
1/10 LD50 x2 M	8.333 d± 1.06	7.000 d± 1.07	3.333 c± 0.03	0.000 f± 0.00	0.000 d± 0.00	18.667 d± 1.63	8.000 c± 1.03	0.000 e± 0.00	1.000 c± 0.10	9.000 d± 1.67	27.667 f± 1.66
1/10 LD50 x4 M	11.333 c± 1.03	11.333 b± 1.06	3.333 c± 0.03	0.667 d± 0.03	0.667 b± 0.03	27.333 c± 3.27	8.000 c± 0.26	0.000 e± 0.00	2.333 c± 0.34	8.667 d± 1.09	36.000 e± 1.35
1/8 1.1)50x2	12.333 c± 1.06	9.000 c± 1.05	4.000 b± 0.27	0.333 e± 0.04	0.667 b± 0.03	26.333 c± 1.51	10.333 b± 1.03	0.667 d± 0.03	6.333 ab± 1.34	17.333 b± 2.42	43.667 d± 1.44
1/8 1.1)50 x4 M	17.667 ab ± 1.06	11.000 b± 1.03	4.667 a± 0.03	1.333 b± 0.03	0.333 c± 0.17	35.000 b± 2.10	10.333 b± 1.12	1.000 c± 0.07	2.000 c± 0.10	13.333 c± 1.68	48.333 c± 1.16
1/4 LD50 x2 M	16.000 b± 1.27	9.667 bc± 1.06	4.333 ab± 0.51	1.000 c ± 0.21	0.667 b± 0.03	31.667 b± 0.82	13.667 a± 1.03	1.667 b± 0.03	8.000 a± 1.83	23.333 a± 2.01	55.000 b± 1.74
1/4 1.1)50 x4 M	18.333 a± 1.06	14.000 a± 1.09	4.667 a± 0.03	3.000 a± 0.44	1.000 a± 0.03	41.000 a± 2.76	11.000 b± 1.03	2.667 a± 0.03	4.667 b± 1.33	18.333 b± 1.51	59.333 a± 1.93
LSD at a 0.05	1.86	1.84	0.35	0.30	0.11	3.49	1.45	0.06	1.69	2.815	2.52

Statistical analysis of results was done according to least significant difference (LSD) test. Different letters (a, b, c, d, e) within each column means the degree of significance at 0.05 level. (M ± Sd): M is the mean value, Sd: Stadndard deviation.

Table (3): Effect of control + ve, control - ve and 1/4 LD50 on sperm morphology.

		Head Abnormalities (M ± Sd)						Tail Abnormalities (M ± Sd)			Total aberrations (M ± Sd)	Total count of sperm $\times 10^6$ (M ± Sd)
Treatments	Normal	Without hook	Big head	Small head	Banana	Amorphous	Total head	Two tail	Coiled tail	Total tail		
-VE	95.4 a ± 0.86	0.96c± 0.26	0.48c± 0.30	1.28 b ± 0.50	0.68c± 0.41	1.16 b ± 0.26	4.6c± 0.864	0.00 b ± 0.00	0.00b± 10.00	0.00 b± 0.00	4.56c ± 0.86	5.93 a ±0.31
1/4LD50	73.7 c± 3.85	3.52 b± 0.89	4.12 a ± 1.24	4.96 a ± 1.77	4.36 a ± 0.98	8.56 a ± 4.89	25.5 a± 3.651	0.44 a± 0.30	0.32 a± 0.46	0.76 a± 0.52	26.28 a ± 3.85	2.11 c ±0.31
+ VE	78.9 b ± 1 78	6.08 a ± 0 39	2.08 b ± 0.23	5.24 a ± 0.61	2.48 b ± 1.11	4.48 b ± 0.30	20.4 b± 1.705	0.56 a± 0.22	0.20 a± 0.14	0.76 a± 0.22	21.12 b± 1.78	3.55 b± 0.33
LSD at a 0.05	3.443	0.800	1.030	1.544	1.226	3.903	3.279	0.167	0.292	0.447	3.443	0.427

Statistical analyses of results were done according to least significant difference (LSD) test. Different letters (a, h, c, d, e) within each column means the degree of significance at 0.05 level. (NI f Sd): M is the mean value, Sd: Stadndard deviation.

4. Discussions

The use of pesticides in food production cannot be avoided especially in developing over populated countries. On the other hand, the application of such chemicals is a major risk due to the danger of being genotoxic and/or carcinogeneic and the complete danger is transmitting the mutations to the next generations. Raynauda *et al.* (2008) suggested that carcinogenesis is a multistep process involving multiple mutations and chromosomal aberrations. Also, Au *et al*. (1990) hypothesized that chromosomal aberrations were in the background of carcinogenesis and that the determination of their incidence was an important parameter for the effect of various agents on the health status of mammals and man. Awa (1983) detected a positive correlation between the risk of genetic diseases in populations and the level of cytogenetic damage. The short-term tests for genotoxicity are widely used increasing the potential hazards of such chemicals. In this chapter the results are discussed in an attempt to shed more light on the genotoxicity of Cyhalothrin (lambda) 5% EC (the active substance:λ-Cyhalothrin, a type II synthetic pyrethroid insecticide), which is widely used in Egypt.

Effect of insecticide on somatic cell abnormalities:

As the results showed, Cyhalothrin (lambda) insecticide caused an increase in many types of chromosomal aberrations. Gaps, breaks, deletions, centric fusions, fragments; hypoploidy, centromeric attenuations, polyploidy and endomitosis were the main types of aberrations, which were dose-and time-dependent. The observed significant chromosomal aberration after treatment with Cyhalothrin (lambda) insecticide in the present study are in keeping with the results obtained from the experiments performed using formlated lambda-cyhalothrin (Campana, 2003; Celik *et al*., 2003, 2005,Naravaneni and Jamil, 2005; Georgieva, 2006 and El-Demerdash, 2007). Similar types of aberrations were also detected in mammals under the effects of pyrethroids (Giri *et al*. 2002, Gabbianelli *et al*. 2004, El Khatib *et al* 2006, Farag *et al* 2001, 2007 and Quan et al 2010). The presence of gaps and breaks are both indicators of genetic damage as other types of aberrations (Koller, 1973, Anderson & Richardson 1981 and Brogger, 1982 and). Concerning numerical aberrations, according to (Pati and Bhunya 1989) the induction of aneuploidy and polyploidy may be a result of mitotic arrest due to disturbance of spindle which causes a malsegration. Aneuploidies are the most serious and frequent chromosomal defect in humans. Numerical chromosomal abnormalities are associated with congenital defect (Griffin, 1996) and are critical in both the early initiation stages and the progression of a wide array of malignant tumors (parry *et al*., 2002) Adose-and-time dependent decrease in mitotic index was observed following Cyhalothrin (lambda) insecticide administration. Similar findings were obtained by El-Khatib *et al*. (2006) and Farag *et al*. (2007) who found significant decrease in the mitotic activity after treatment with cypermethrin (a type II synthetic pyrethroid insecticide) and permethrin (a type I synthetic pyrethroid insecticide). The observed

dose and time-dependent depression of mitotic activity in the present study may be attributed to the cumulative and cytotoxic effects of Cyhalothrin (lambda) insecticide. Thus in the present study, it has been found that cyhalothrin (lambda) insecticide has the ability to induce chromosomal abnormalities in bone marrow cells which could be an indicator that it may induce chromosomal abnormality in spermatocytes.

Effect on Germinal cells (meiotic) abnormalities:

It is particularly relevant to study the genotoxic effects of pesticides in germinal cells because this is the only system in which transmissible genetic damage from one generation to another takes place (Brewen and prestone, 1978 and William and Hsu, 1980). In the present study, the insecticide under test induced chromosomal abnormality in mice spermatocytes. The abnormalities were in the form of autosomal and X-Y univalent, chain, fragment aneuploidy and polyploidy. Up to our knowledge, there are not any previous study reports regarding the genotoxicity of Cyhalothrin (lambda) insecticide on spermatocytes of animal. Abnormalities in spermatocytes can result in producing abnormal sperms which can affect the fertility (Ibeh *et al*., 1994) Alterations in the testis after the insecticide treatment in mice could be due to the direct effect of the substance under test on the testis or indirect effect through the reduction in serum testosterone concentration which is very important for its function (Verma and Nair, 2001). (Muthuviveganandavel *et al*. 2008 and Wang *et al.* 2009), found that cypermethrin one of pyrithroid insecticides used in Egypt increased the malondialdehyde (MDA) content of testis of male rats. Additional to chromosomal abnormalities, a reduction of meiotic division after cyhalothrin (lambda) treatment was also observed. This could be explained by two reasons: the first is the ability of the insecticide to reduce DNA synthesis, which subsequently affect cell division (Sotomayor *et al*., 2003). The second reason is the toxic effect of the substance under test which affects the rate of cell division (Verma and Nair. 2001). The present study revealed that somatic cell chromosomes were more sensitive to the induced aberrations by cyhalothrin (lambda) pesticide than the germ cell chromosomes. Similar findings were also recorded in mammalian cells (Abd El-Aziz and El Ashmawy, 1989, Abd El-Aziz; El Nahass, 1990, Abd El Aziz *et al*., 1993;Farag *et al*., 2001;). Russel(1978) explained that the germ cells are protected from any exposure of chemicals in blood stream by gonadal barriers, which reduce the risk of exposure on germ cells compared to somatic cells.

Effect on sperm morphology:

In the present study cyhalothrin (lambda) insecticide was found to induce abnormalities in sperm shape either in head or tails. The sperm head deformities affect the size of nuclear mass (DNA content) (Wyrobek & Bruce, 1978 and Wyrobek *et al*., 1984) whereas the tail deformity gives an impression of limitation of sperm movement which causes reduction of fertility in human and experimental animals (Lancranijan *et al*., 1975). A statistically significant increase in the number of abnormal sperms occurred after treatment with the high dose (5mg/kg) of cyhalothrin (lambda) insecticide compared with the control. This increase was mainly in the head rather than in the tail.

The reduction in sperm count and motility after the insectide cyhalothrin (lambda) treatment means, increase incidence of sperm abnormalities and subsequently reduction in fertility according to the regardation of (Agnes and Akborsha, 2003). Previous studies reveld that exposure to pesticides has been associated with reproductive dysfunction, such as spontaneous abortions, infant prematurity, congenital malformations, reduced fertility, sperm mortality decrease and hyperploidy/polyploidy in the spermatozoa of exposed men (Oliva *et al*., 2001, Recio *et al*., 2001, Crisosotomo and Molina, 2002;; Chauhan *and* Gupta 2005, qua *et al.,* 2008 and Nada et al., 2010). The positive correlation between cytogenetic damage and sperm abnormality in the present study was also reported by (Bernardini *et al*., 1998 and Xia *et al*., 2004).

In conclusion, cyhalothrin (lambda) insecticide was found to be genotoxic on both somatic and germinal cells; which was dose and time dependent, also the statistical analysis revealed that cyhalothrin (lambda) insecticide is more genotoxic on bone marrow cells than germinal cells. It was shown that cyhalothrin (lambda) insecticide has a damaging effect on the sperm morphology, which may be the cause for infertility and abnormal embryos. Thus care must be taken when handling this insecticide. Educating people about pesticide safety is important and encourage them to buy fresh and processed organic foods thorough system of administration has been put in place to control the advertisement, sale, storage, supply and use of pesticides.

References

1. Abd El-Aziz, K. B. (1993): pirimphos-methyl as a mutagenic agent in mice and their embryos. Egypt J. App. Sci., 8:77-82.
2. Abd El-Aziz, K. B.; El-Nahass, E. and Halima S.A. (1993): Tamaron-induced sperm shape abnormalities and chromosomal aberrations in mouse. J. Egypt. Ger. Soc. Zool., 12:99-113.
3. Abd El-Aziz, K. B. and El-Nahass, E. (1990): Genotoxic effect of coca extract on somatic and germ cells of mice. Egypt. J. Agric. Res. & Dev., Col. 11, No.2, 591-604.
4. Abd El-Aziz, K. B. and El Ashmawy, S. H. (1989): Cytogenetic effects of praziquantel on bone marrow and soermatogonia of mice. Mini. J. Agric. Res. &Dev., Col. 11, no.2, 591-604.
5. Agnes, V.F. and Akbarsha, M.A. (2003): Spermatotoxic effect of Aflatoxin B. in the albino mouse. Food chem. toxicol. 41:119-130.
6. Anderson, D. and Reichardoson, C. R. (1981): Issues relevant to the assessment of chemically induced chromosome damage in vivo and their relationship to chemical mutagenesis. Mutat. Res., 90(30, 261-272.
7. Au W. W.; Cantelli-forti, G.; Hrelia, P. and Legator, M.s. (1990): Cytogenetic assays in genotoxic studies, somatic cell effects of benzene and germinal cell effects of dibromochloropropane. Teratogenesis, Carcinogenesis and Mutagenesis, 10 (2): 125-34.
8. Awa A. A (1983): In: Radiation Induced Chromosome Damage in Man. Liss, New York.
9. Bernardini, L.; Borini, A.; Preti, S.; Conte, N.; Flamigni, C.; Capitanio, G. L. and Venturini, P. L. (1998): study of aneuploidy in normal and abnormal germ cells from semen of fertile and infertile men. Hum Report. 13: 3406-3413.
10. Brewen, J. C. and Preston, R. J. (1978): Analysis of chromosome aberrations in mammalian germ cells, in: A. Hollaender and F.J. dde serres (EDS), Chemical mutagens, Vol, 5, Plenum, New York, P, 127-150.
11. Brogger, A. (1982): the chromatid gap-a useful parameter in genotoxicology. Cytogenet., Cell Genet., 33:14-19.
12. Campana mA, Panzeri AM, MMoreno VJ and Dulout FN (2003): Micronuclei indction in Rana catesbeiana tadpoles by the pyrethroid insecticide lambda-cyhalothrin. Genet. Mol. Biol., 26: 1-8.
13. Celik, A.; Mazmanci, B.; camlica, Y.; Askin, A. and Comelekoglu U. (2003): Cytogenetic effects of lambda-cyhalothrin on Wistar rat bone marrow. Mutat. Res., 5: 539(1-2):91-97.
14. Celik, A.; Mazmanci, B.; camlica, Y.; Askin, A. and Comelekoglu U. (2005): Evaluation of cytogenetic effects of lambda-cyhalothrin on Wistar rat bone marrow by gavage administration. Ecotoxicology and Environmental Safety, 61 (1): 128-133.
15. Chauhan, L.K and Gupta, S.K. (2005): Combined cytogenetic and ultrastructural effects of substitute urea herbicides and synthetic pyrethroid insecticide on the root meristem cells of Allium cepa. Pesticide Biochemistry and physiology, 82: 27-35.
16. Crisostomo, L. and Molina, V.V. (2002): Pregnancy outcomes among farming households of Nueva Ecija with conventional pesticide use versus integrated pest management. Int. J. Occup. Environ. Health, 8: 232-242.
17. El-Demerdash, F.M. (2007): Lambda-cyhalothrin-induced changes in oxidative stress biomarkers in rabbit erythrocytes and alleviation effect of some antioxidants. Toxicol. In Vitro. 21: 392-397.
18. El-Khatib, E. N.; Abd El-Aziz, M.; Badr, Y. and Kamal, N. (2006): In vivo genotoxicity of the synthetic pyrethroid pesticide'cypermethrin' in rat liver cells by comet assay. Toxicology Letters, 164S; S1-S324.
19. Farag, I. M.; Abdel Aziz, K. B. and Al-Zahaby, A.S. (2001): Cytogenetical and Ultrastrurctural changes in somatic and germ cells mice due to the effect of sumithion and permethrin. Bull. NRC. Egypt, 26: 343-356.
20. Farag, A. T.; Godaa, N. F.; Shaaban, N. A. and Mansee, A. H. (2007): Effects of oral exposure of synthetic pytethroid, cypermethrin on the behavior of F1- progeny in mice. Reproductive Toxicology, 23:560-567.
21. Fortina, M.; Boucharda, M.; Carrier, G. and Dumas, P. (2008): Biological monitoring of exposure to pyrethrins and pyrethroids in a metropolitan population of the province of quebec, Canada. Environmental Research, 107(3): 343-350.
22. Gabbianelli, R.; Facioni, G.; Cantalamessa, F. and Nasuti, C. (2004): Lymphocyte DNA damage in rats exposed to pyrethroids: effect of supplementation with vitamins E and C. Toxicology, 203: 17-26.
23. Georgieva, SV. (2006): Investigation of the cytogenetic effect of the insecticide karate on rabbit peripheral blood lymphocytes. Trakia Joournal of Sciences, 4: 34-38.

24. Giri, S.; Giri, A.; Sharma, G. D. and Prasad, S. B. (2002): Fenvalerate-induced chromosome aberrations and sister chromatid exchanges in the bone marrow cells of mice in vivo. Mutation Reseach, 520: 125-132.
25. Griffin D. K. (1996): The incidence, origin, and etiology of aneuploidy. Int. Rev. Cytol., 167:263-296.
26. Ibeh, I. N.; Uraih, N. and Ogonar, J. I. (1994): Dietry exposure to aflatoxin in human infertility in Benin City Nigeria- International of fertility and Mnopausal studies. 39:208-214.
27. Kaya,B.; Demir,E.; Kocaoğlu,S.; Çetin,H. and Marcos R. (*2010):* In vivo genotoxicity testing of four synthetic pyrethroids with combinations piperonyl butoxide in the Drosophila SMART assay Toxicology Letters, Volume 196, Supplement1,17:S315.
28. Kuller, P. C. (1973): incidence cancer in subjects with congenital and acquired chromosome aberrations. Res. Prog. Med. (Roma), 54: 383-386.
29. Lancranijan, I. Papescu, H. I. Gavanescu, O. K; Lapsch, I. and Serbacnescu, M. (1975): Reproductive ability of workmen occupationally exposed to lead. Arch. Environ. Health, 30:396-401.
30. Mansour, S. A. (2004): pesticide exposure-Egyptian scence. Toxicology, 198: 91-115.
31. Muthuviveganandavela, V.; Muthuramana, P.; Muthua S. and Srikumar, K. (2008): A study on low dose cypermethrin induced histopathology, lipid peroxidation and marker enzyme changes in male rat. Pesticide Biochemistry and Physiology, 91: 12-16.
32. Nada M.H. ; Al-Hamdani, H.N. and Yajurvedi *(2010*): Cypermethrin reversibly alters sperm count without altering fertility in mice *Ecotoxicology and Environmental Safety*, *In* Press, Corrected Proof, Available online 1 May
33. Naravaneni, and Jamil, K. (2005): Evaluation of cytogenetic effects of Lambda-acyhalothrin on Human Lymphocytes. J. Biochem. Molecular Toxicology. 19: 304-310.
34. Oliva, A.; Spira, A. and Multigner, L. (2001): Contribution of environmental factors to the risk of male infertility. Hum. Reprod., 16: 1768-1776.
35. Parry, j. M.; Al Obaidly, A.; Al Walhaib, M.; Kayani, M.; Nabeel, T.; Strefford, J. and Parry, E.M. (2002): Spontaneous and induced aneuploidy, considerations which may influence chromosome malesgregation. Mutat. Res., 504: 119-129.
36. Patel, S.; Pandey, A. K.; Bajpayee, M.; Parmar, D. and Dhawan, A. (2007): In vitro induction of cytotoxicity and DNA strand breaks in CHO cells exposed to cypermethrin, pendimethalin and dichlorvos. Toxicology in Vitro, 21, 8: 1409-141.
37. Pati, P.C. and Bhuniya, S. P., (1989): Cytogenetic effects of fenvalerate in mammalians in vivo test system. Mutat. Res., 222: 149-154.
38. Qua, J. H.; Honga, X.; Chena, J.F.; Wanga, Y. B.; Suna, H.; Xua, X. L.; Songa, L.; Wang, S. A. and Wang X. (2008): Fenvalerate inhibits progesterone production through cAMP-dependent signal pathway, Toxicology Letters, 176: 31-39.
39. Quan, Z.; Cui, W.; Liwei, S.; Ling, L. and Meirong, Z. (*2010):* Cytotoxicity of lambda-cyhalothrin on the macrophage cell line RAW 264.7 *Journal of Environmental Sciences, Volume 22, Issue 3: 428-432.*
40. Raynauda, C. M; Sabatier, L.; Philipotb, O.; Olaussen, K. A. and Soria, J.C. (2008): Telomere lengh, telomeric proteins and genomic instability during the multistep carcinogenic process. Critical reviews in Oncology/Hematology, 66 (2): 99-117.
41. Recio, R.; Robbins, W. a.; Borja-aburto, V.; moran-Martinez, J.; Froines, J. R.; Hernandez, R. M. and Cebrian, M. e. (2001): Organophosphorus pesticide exposure increases the frequency of sperm sex null aneuploidy. Environ. Health Perspect., 109: 1237-1240.
42. Russel, L. B. (1978): Somatic cells as indicators of germinal mutations in the mouse. Environ. Health Prespect., 24: 113-116.
43. Snedecor, G. W. and Cochran, W. G. (1980): Statistical Methods 6th Ed. The Iowa State Univ. press, Amer.Iowa, USA, 593.
44. Sotomayor, R. E.; Wahington, M.; Ng-ugen, L.; Myang anyi, R.; hinton, D. M. and Chou, m. (2003): Effect to intermittent exposure to Aflatoxin B., on DNA and RNA adduct formation in rat liver: Dose-Response and Termoral pattern. Toxicological Science, 73:329-338.
45. Steel, R. G. and Torrie, J. H. (1981): Principles and procedures of statistics: A Biometrical Approach. 2nd Ed. McGraw-Hill Book Co., New York, NY.
46. Verma, R. J. and Nair, A. (2001): Vitamin E ameliorates aflatoxin-induced biochemical changes in the testis of mice. Asian J. Androl., 3: 305-309.
47. Wang,X.-Z. ; Liu,S.-S. ; Sun,Y. ; Wu, J.-Y. ; Zhou, Y.-L. and Zhang J.-H (2009): Beta-

cypermethrin impairs reproductive function in male mice by inducing oxidative stressTheriogenology, *Volume 72, Issue 5, 15 : 599-611*

48. Who (2005): Safety of pyrethroids for public health use. Geneva, World Health Organization (WHO/CDS/WHOPES/GCDPP/2005.10).
49. WHO (2003): Toxicological profile for pyrethrins and pyrethroids. World Health Organization.
50. William, w. and hsu, T. C. (1980): The genotoxic effects of Adriamycin in somatic and germinal cells of the mouse. Mutation Res., 79:351-361.
51. Wyrobek, A. J. and Bruce, W. R. (1978): The induction of sperm shape-abnormalities in mice and humans. Chemical Mutagens, 5:257-285.
52. Wyrobek, A. J.; Watchmarker, G. and Gordon, L. (1984): Sperm morphology testing in mice in (Handbook of mutagenicity test procedures). Elsevier Science Publishers BV, second edition.
53. Xia, Y., Bian, Q., Xu, L., Cheng, S., Song, L., Liu, J., Wu, W, Wang, S. and Wang, X., (2004): Genotoxic effect on human spermatozoa among pesticide factory workers exposed to fenvalerate. Toxicology, 203: 49-60.
54. Yosida, T. H. and Amano, K. (1965): autosomal polymorphism in laboratory bred and wild Norway rats, Rattus norvegicus, found in misima. Chromosoma, 16: 658-667.
55. Zaim, M. and Jambulingam, P. (2004): Global insecticide use for vector-borne disease control, 2nd ed. Geneva, World Health rganization (WHO/CDS/WHOPES/GCDPP/2004.9).

6/2/2010

Chlorophyll-a dynamics in relation to environmental parameters in a tropical lagoon

[1]P. C. Onuoha, [2]D.I. Nwankwo and [3]Vyverman, W.

[1]Department of Fisheries and Marine Biology, Federal College of Fisheries and Marine Technology, Bar-beach Victoria Island, Lagos Nigeria. E-mail- hydro_vision@yahoo.com
[2]Department of Marine Sciences University of Lagos, Akoka, Lagos, Nigeria
[3]Protistology and Aquatic Ecology Research Laboratory, University of Ghent, Belgium

Abstract: The chlorophyll-*a* dynamics and environmental factors of the Ologe lagoon, Lagos were investigated for 2 years (Feb., 2002 – Jan., 2004). The environmental indices reflected seasonal changes related to rainfall distributive pattern and tidal seawater incursion. Air temperature (27-34 ^{0}C), surface water temperature (25-32^{o}C), transparency (24-76cm), total dissolved solids (48-294mg/l), salinity (0-0.5‰), conductivity (83-631µS/cm), pH (5.8-8.1), total alkalinity (42-162mg/l), biochemical oxygen demand (0-28mg/l), chemical oxygen demand (6-39mg/l), total hardness (62-342mg/l), cations, and heavy metals recorded increasing values in the dry season than the wet months, while dissolved oxygen (7-12.7mg/l), total suspended solids (7-378mg/l), nitrate-nitrogen (0.02-1.02mg/l), phosphate-phosphorus (0.03-1.79mg/l) and silicate (2.05-9.54mg/l) had higher values in the wet season than the dry season. Estimation of phytoplankton biomass by chlorophyll-*a* concentration ranged from 0.1 to 64.5ug/l with mean value of 16.99ug/l. Values for chlorophyll-*a* were higher in the dry than wet season for the lagoon. Analysis, using Pearson correlation co-efficient recorded positive relationship between chlorophyll-a values and air temperature, surface water temperature, salinity, conductivity, total dissolved solids, pH, transparency, biochemical oxygen demand, chemical oxygen demand, alkalinity, total hardness and cations. Analysis using ANOVA showed significant differences in the sample means of physico-chemical parameters of effluent discharge station (OL4) and the other stations within the lagoon at 5% level of probability. Recorded chlorophyll-*a* values placed the Ologe lagoon between the mesotrophic and eutrophic status. It is suggested that increasing tidal influence associated with reduction in rain events may have encouraged elevated salinities and created conditions for the development of more algal cells, hence higher chlorophyll *a* records. [Journal of American Science 2010;6(12):327-337]. (ISSN: 1545-1003).

Keywords: Chlorophyll-a, environmental factors, mesotrophic, eutrophic, Ologe

Introduction

Lagoons are ecologically and economically important aquatic ecosystems in South-western Nigeria. They provide natural food resources rich in protein which includes an array of fish and fisheries. They are also important in water transportation, energy generation, exploitation and exploration of some mineral resources including sand (FAO, 1969; Kirk and Lauder, 2000; Onyema *et al.,* 2003, 2007; Chukwu and Nwankwo, 2004; Onyema, 2008a). Lagoons also inadvertently serve as sinks for the disposal of both domestic, municipal and industrial wastes in the region. There are nine lagoons in South-western Nigeria namely: Yewa, Ologe, Badagry, Iyagbe, Lagos, Kuramo**,** Epe, Lekki and Mahin lagoons from the west to the east (FAO, 1969, Webb, 1958a; Nwankwo, 2004b; Onyema, 2008).

Furthermore, chlorophyll *a* is an essential plant and concentrations of it could be used to reflect algal biomass and hence, level of primary production. Chlorophyll *a* can be an effective measure of trophic status (Lee, 1999). However, elevated chlorophyll *a* concentrations often indicate poor water quality and low levels often suggest good conditions (Ogamba *et al.,* 2004). According to Lee (1999), higher phytoplankton biomass would directly reflect in higher level of chlorophyll *a* in such regions method to determine the amount of plant materials present in a water sample is to filter out the phytoplankton, count the cells and multiply the number counted by the average mass per individual cell from a sample (Sverdrup *et. al.,* 2006).

The immense ecological significance of phytoplankton diversity studies especially in relation to aquatic trophic relationships cannot be understated (Smith, 1950; Lee, 1999; Nwankwo, 1984, 2004a). Coastal areas are generally more productive than the open oceans because rivers and land run-offs supply nutrients along coasts and adjoining estuarine systems. With regard to the annual rates of global primary production and productivity, Lagos offshore falls under the high productivity category (=300 gC/m) (Sverdrup *el al.,* 2006).

Determination of primary production in the Lagos

Lagoon has primarily been by biomass estimation using cells number of phytoplankton (Nwankwo, 2004). With regard to chlorophyll *a* in Nigeria, there exist a report by Kadiri (1993) on the Ipkoba reservoir in Benin and another by Ogamba *et al.*, (2004) on chlorophyll *a* levels and variations in the Niger Delta region. Hence studies in Nigeria suing chlorophyll *a* method are limited.

At present, there is no report on any of the nine lagoons of South-western Nigeria with regard to the chlorophyll *a* method of estimation. The aim of this study was to investigate the seasonality in chlorophyll *a* concentration and relate findings to environmental factors in the Ologe lagoon.

Material and Methods

Description of Study Site

The Ologe lagoon (Fig 1) is located in Lagos State, Nigeria and is one of the nine lagoons in South-western Nigeria (Webb, 1958; Nwankwo, 2004b). It is pressumbaly the smallest of the lagoons in South Western Nigeria with a surface area of 9.4km^2, and lies at the distal end of Badagry creek between longitudes 6° 26'N to 6° 30'N and latitudes 3° 01'E to 3° 07'E. The main body of the lagoon lies within Badagry Local Government Area and it opens up to the Atlantic ocean via the Lagos Harbour and Dahomey in the Republic of Benin. The major source of water are River Owo with a source in a town called Toto Owo where River Ore and Illo form a confluent with River Oponu in Ogun State (Akanni, 1992). Seventeen stations were chosen for sampling within the lagoon. The lagoon is shallow at most points and is open all year round via the Lagos habour to the sea (Hill and Webb, 1958; Sandison, 1966; Sandison and Hill, 1966). Like all parts of South-western Nigeria, the Ologe lagoon is exposed to two distinct seasons namely the wet (May – October) and the dry (November – April) (Nwankwo, 2004b). Like all parts of South-western Nigeria, the Ologe lagoon is exposed to two distinct seasons namely the wet (May – October) and the dry season (November – April) (Nwankwo, 2004b; Sandison and Hill, 1966). The harmmattan, a short season of dry, dusty North-East Trade winds experienced sometimes between November and January in the region reducing visibility and lowering assemblages is the common macrofloral assemblages especially in areas with reduced anthropogenic influence. The lagoon deposits are varied, and are reflected in the pattern and type of vegetation in the region. Most parts of the Ologe lagoon are colonized by recognizable riparian dense swamp rainforest community dominated by raphia palms especially *Raphia hookeri, Elaeis guineensis, Acrotiscum aureum* and *Cocos nucifera* (Akinsoji *et al.*, 2002).Very few mangrove communities are recognizable around the Badagry creek end. Notable fauna of the area includes amphipods, Oligochaetes, few polychaetes, isopods, barnacles, oysters, periwinkle, nematodes, fiddler crabs, crabs, among others (Sandison and Hill, 1966; Onyema, 2008b).

Collection of samples

Collection of water samples

Twelve sampling stations were selected to cover the lagoon area and for the collection of sample. Table 1 shows the G.P.S location, names and number of sampling stations. Monthly surface water sample was collected for twenty-four consecutive months for physico-chemical characteristics analysis using 500ml plastic containers with screw caps. Collection of samples from the stations was always between 10 and 15hr each time. Water samples were collected just a few centimeters below the water surface at each of the twelve stations. The plastic containers was then labeled appropriately and transported to the laboratory immediately after collection for further analysis. Water samples for dissolved oxygen was collected also in 50cl bottles and fixed on site with white and black ampoules.

The Pearson correlation Co-efficient matrix (Ogeibu, 2005) for the relationship between the different environmental parameters and chlorophyll *a* were obtained using SPSS 4.0.

Results

The minimum and maximum values obtained for the estimates of environmental factors, their means and standard deviation are presents in Table 3. Also in Table 3 is whether each parameter recorded higher values in the wet or dry season for the two (2) years of study. Fig. 2 showed seasonal variations in some environmental factors at four selected stations each at the Ologe lagoon from Feb., 2002 to Jan., 2004. Stations represented were selected based on their importance as confluence points and areas exposed to possible anthropogenic stresses or not.

Air temperature values ranged between 27^{o}C to 33.5^{o}C among all the sampled stations within the study period. The highest air temperature (33.5^{o}C) was recorded at station OL1 (Idolowu) in March 2002, while the lowest was recorded at station OL14 (center of lagoon between Otto-Ijaniki and OL6) in August the same year. The lowest surface water temperature estimated was 25.2^{o}C (August, 2002), the highest value obtained was 31.8^{o}C (March, 2002. The highest transparency value (76cm) was recorded at station OL8 (between Ibiye and Obele) in March

2002, while the lowest values (24cm) was recorded at stations OL16 and OLI0 (Asepe Mushin) in the months of August and September 2003. Total dissolved solids ranged between 48 to 294mg/l, with the lowest value recorded in station OL5 (confluence between Owo River and Ologe lagoon) in September 2003. The highest total dissolved solids value (294mg/l) value was recorded at station OL1 (Idolowu) in March 2002. Total suspended solids valued ranged between 10 to 378mg/l,10 (OL3-Otto jetty in March 2003) and 378mg/l (OL10-Asepe Mushin in September 2003). Rainfall volumes showed both monthly changes and varied from one year to the next. In the first year the highest rainfall volume was recorded: highest rainfall volume (372.1 mm) was recorded in June 2002 and the lowest rainfall volume (41 mm) was in February 2002. In the second year the highest rainfall as in highest rainfall volume (383 mm) was recorded in July 2004 and the least (0.6 mm) was in December 2003.

Hydrogen ion concentration (pH) values ranged between 5.8 (station OL4-Owo River point effluent discharge in the month of July 2002) to 8.16 (station OL11 in the month of March 2002). throughout the sampling period. Whereas the lowest conductivity estimated was 83 µS/cm and recorded in OL5 (confluence between Owo River and ologe lagoon) in September 2003, the highest value obtained was621 µS/cm recorded in station OL11 (Gbanko) in March 2002, Salinity values ranged between 0.00 (station OL4 (point of effluent discharge), and 0.5 ‰ at station OL11 (Gbanko) in March, 2002. Alkalinity values were between 42 (station OL4 -Owo river- point of effluent discharge in September 2003) to 162 mg/l (station OL1 (Idolowu) in April 2002).

Dissolved Oxygen values ranged between 7 (station OL6 in March 2003) to 12.7mg/l (station OL14 -centre of Ologe lagoon between Otto-Ijaniki and OL6 in the month of June 2002). Biological Oxygen Demand values ranged between 3 (stations OL12 (Ajido) and OL13 (between Ajido and Idolowu) in September and October 2003 respectively) to 28mg/l (station OL3 (Otto jetty) in April 2002). Nitrate-nitrogen values were between (0.01 station OL4 (Owo river- point of effluent discharge) in January 2003) to 1.02mg/l (station OL3 (Otto jetty) in June 2003). Phosphate-phosphorus recorded between 0.03 (station OL17 in the month of March 2002) to 1.79mg/l (station OL10 in the month of June 2003). Silica values fell between 2.05 (station OL15 (between Ibiye and Idolowu) in March 2002) and 9.54mg/l (OL11 (Gbanko) in the month of May 2003). Calcium levels were between 34mg/l (station OL6 and OL8 (between Ibiye and Obele) in September and October, 2003 respectively) to 228mg/l (station OL1 (Idolowu) in April 2002). Magnesium estimates were between 10(at station OL8 (between Ibiye and Obele) in September 2003) and 76mg/l(station OL1 (Idolowu) in the month of April 2002). Copper values was between 23.1(station OL8 (between Ibiye and Obele) in July 2003) to 56.9µg/l(station OL11 (Gbanko) in the month of March 2002).Iron levels ranged between106(station OL17 in the month of September 2002) and 987µg/l(station OL4 (point of effluent discharge) in the month of March 2002) Zinc values ranged between 2.62 station OL7 (Ibiye) in August 2003) and 30.88µg/l(station OL4 (Owo river end receiving effluent) in the month of March 2002).

Fig 3 showed the spatio-temporal variations in chlorophyll-*a* values at the different stations in the Ologe lagoon from Feb., 2002 to Jan., 2004. Chlorophyll-*a* values ranged between 0.1 to 64.5ug/l. The highest concentration of chlorophyll-a (64.5µg/l) was recorded at station OL1 (Idolowu) in the month of December 2002 while the lowest value (0.1 µg/l) was observed at station OL4 (point of effluent discharge) and OL5 (confluence between Owo River and Ologe lagoon) in the month of June 2003. Mean values for this parameter were comparatively higher during the dry season than the wet season among all sampling stations during the study period.

Analysis with correlation co-efficient matrix (table 4), showed that chlorophyll-a concentration had positive relationship with the following parameters; air temperature, water temperature, chemical oxygen demand, chemical oxygen demand , pH, salinity, conductivity, alkalinity, Nitrate-nitrogen, Phosphate-phosphorous, silicate, total dissolved solids, transparency, total hardness, calcium, potassium, sodium, magnesium, copper, iron, chromium and zinc, while negative association were observed with dissolved oxygen and total suspended solids.

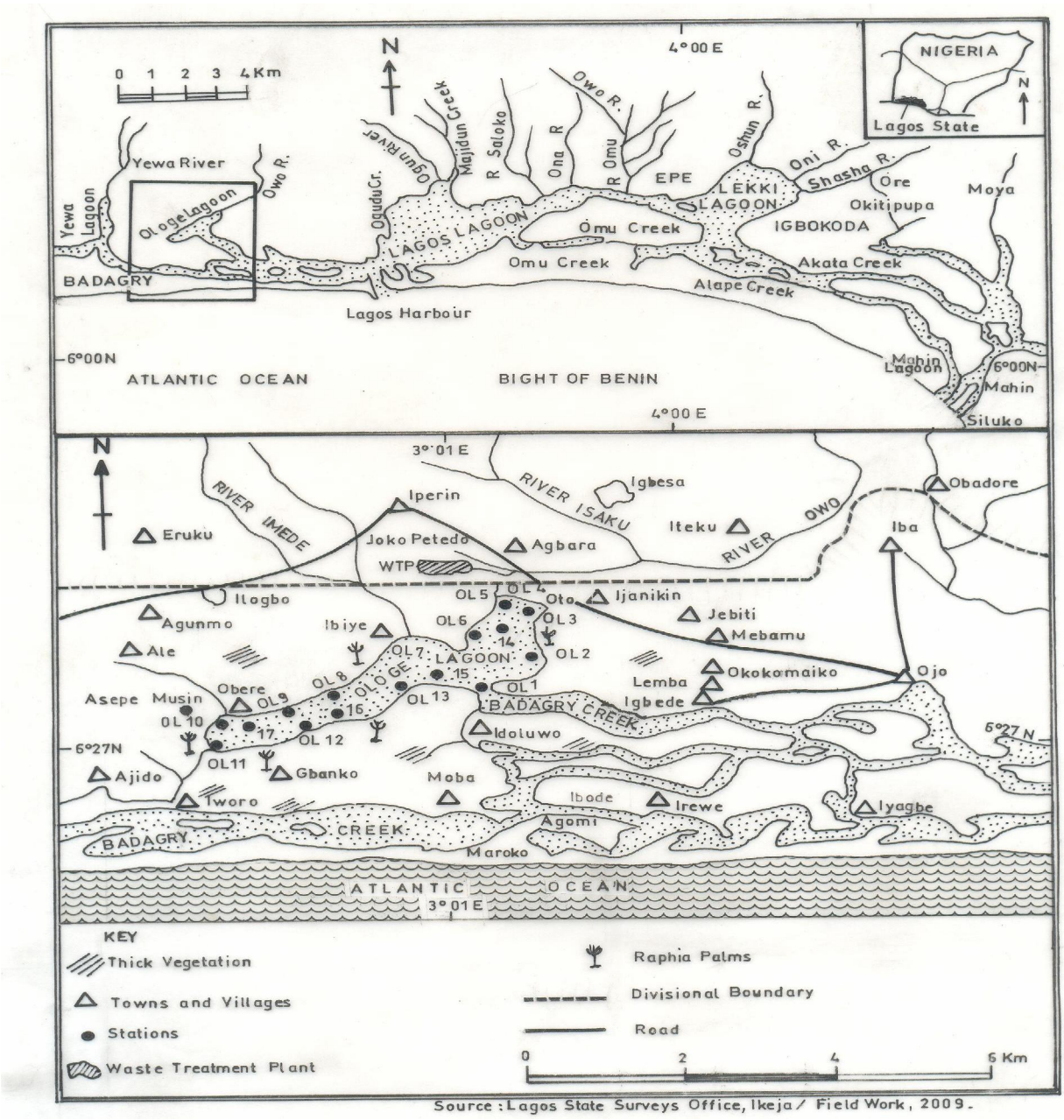


Fig. 1: Parts of Ologe lagoon Showing Sampling Stations.

Table 1: G.P.S. locations and station names of sampled areas in the Ologe lagoon

Station No.	Station name	G.P.S. locations
Station OL1	Idolowu	Latitude 6º28'.3 N, Longitude 3º05'.3 E
Station OL2	Between Idolowu and Otto-jetty	Latitude 6º28'.6 N, Longitude 3º05'.5 E
Station OL3	Otto-jetty	Latitude 6º29'.0 N, Longitude 3º06'.0 E
Station OL4	Point of effluent discharge	Latitude 6 º29'.5 N, Longitude 3 º06'.0 E
Station 0L5	Confluence of Owo River and Ologe lagoon	Latitude 6º30'.0 N, Longitude 3 º06'.1 E
Station OL6	Between station 5 and Ibiye	Latitude 6 º29'.3 N, Longitude 3 º06'.0 E
Station OL7	Ibiye	Latitude 6 º29'.0 N, Longitude 3 º05'.6 E
Station OL8	Between Ibiye and Obele	Latitude 6 º29'.2 N, Longitude 3 º06'.9 E
Station OL9	Obele	Latitude 6 º28'.2 N, Longitude 3 º05'.7 E
Station OL10	Asepe Mushin	Latitude 6 º28'.6 N, Longitude 3 º06'.0 E
Station OL11	Gbanko	Latitude 6 º28'.0 N, Longitude 3 º05'.8 E
Station OL14	Centre of Ologe lagoon between otto-jetty and station 6	Latitude 6o ,30'.5 N Longitude 3o 06'.4 E
Station OL15	Centre of Ologe lagoon between Ibiye and Idolowu	Latitude 6o ,30'.2 N Longitude 3o 06'.6 E
Station OL16	Centre of Ologe lagoon between Obele and Ajido	Latitude 6o ,29'.5 N Longitude 3o 06'.0 E
Station OL17	Centre of Ologe lagoon between Asepe Mushin and Gbanko	Latitude 6o ,28'.7 N Longitude 3o 06'.7 E

Table 2: Summary of environmental factors and method/device used for their estimation.

	Parameter/Unit	Method/Device	Reference
1.	Air temperature (^{o} C)	Horiba U-10	
2.	Water temperature (^{o} C)	Horiba U-10	
3.	Transparency (cm)	Secchi disc method	Onyema 2008
4.	Depth (cm)	Graduated pole	Brown 1998
5.	Rainfall (mm)	Acquired from NIMET, Oshodi, Lagos	
6.	Total Dissolved Solids (mgL^{-1})	Cole Palmer TDS meter	
7.	Total Suspended Solids (mgL^{-1})	Gravimetric method	APHA(1998)
8.	Total hardness (mgL^{-1})	Titrimetric method	APHA(1998)
9.	pH	Electrometric / Cole Parmer Testr3	
10.	Conductivity (µS/cm)	Philip PW9505 Conductivity meter	
11.	Salinity ($‰$)	HANNA Instrument	APHA(1998)
12.	Alkalinity (mgL^{-1})	Titration method	APHA(1998)
13.	Dissolved oxygen (mgL^{-1})	Titration method	APHA(1998)
14.	Biological oxygen demand (mgL^{-1})	Incubation and Titration	APHA(1998)
15.	Chemical oxygen demand (mgL^{-1})	Titration method	APHA(1998)
16.	Nitrate – nitrogen (mgL^{-1})	Colorimetric method	APHA(1998)
17.	Phosphate – phosphorus (mgL^{-1})	Colorimetric method	APHA(1998)
18.	Silica (mgL^{-1})	Colorimetric (DR2010)	APHA(1998)
19.	Sodium (mgl-1)	Flame Photometer	APHA(1998)
20.	Potassium (mgl-1)	Flame Photometer	APHA(1998)
21.	Calcium (mgL^{-1})	Titrimetric method	APHA(1998)
22.	Magnesium (mgL^{-1})	Titrimetric method	APHA(1998)
23.	Copper (mgL^{-1})	Atomic Absorption Spectrophotometer Perkin Elmer 5000 AAS	Perkin Elmer Application (2002)
24.	Iron (mgL^{-1})	Atomic absorption Spectrophotometer Perkin Elmer 5000 AAS	Perkin Elmer Application (2002)

25.	Zinc (mgL^{-1})	Atomic Absorption Spectrophotometer Perkin Elmer 5000 AAS	Perkin Elmer Application (2002)
26	Chromium (mgl^{-1})	Atomic Absorption Spectrophotometer Perkin Elmer 5000 AAS	Perkin Elmer Application (2002)
27.			Chlorophyll *a* (µg/l) Colorimetric Method APHA(1998)

Table 3: A summary of the minimum, maximum and mean / standard deviation estimate values for environmental factors from the Ologe lagoon (February, 2002 – December, 2004).

	Parameter/ Unit	Minimum value	Maximum value	Mean value ± S.D.	Higher values reported in t In the---
1	Air temperature (oC)	27	34	31.10 ± 0.22	Dry season
2	Water temperature (oC)	25.2	31.8	29.01 ± 0.47	Dry season
3	Transparency (cm)	24	76	51.54 ± 5.65	Dry season
4	Depth (m)	3.2	7	4.4	Wet season
5	Total Dissolved Solids (mgl-1)	48	294	139.23 ± 17.89	Dry season
6	Total Suspended Solids (mgl-1)	7	378	184.36 ± 14.90	Wet season
7	Rainfall (mm)	0.6	383	137.37	Wet season
8	Total hardness (mgl-1)	62	342	146.38 ± 26.52	Dry season
9	pH	5.8	8.1	6.92 ± 0.14	Dry season
10	Conductivity (µS/cm)	83	621	256.59 ± 36.65	Dry season
11	Salinity (‰)	0.0	0.5	0.10 ± 0.03	Dry season
12	Alkalinity (mgl-1)	42	162	100.20 ± 9.37	Dry season
13	Dissolved oxygen (mgl-1)	7	12.7	9.08 ± 0.42	Wet season
14	Biological oxygen demand (mgl-1)	0	28	13.11 ± 1.79	Dry season
15	Chemical oxygen demand (mgl-1)	6	39	21.34 ± 2.52	Dry season
16	Nitrate – nitrogen (mgl-1)	0.02	1.02	0.44 ± 0.08	Wet season
17	Phosphate – phosphorus (mgl-1)	0.03	1.79	0.80 ± 0.10	Wet season
18	Silica (mgl-1)	2.05	9.54	5.07 ± 0.45	Wet season
19	Sodium (mgl-1)	2.6	22.7	30.82 ± 6.13	Dry season
20	Potassium (mgl-1)	0.1	7.6	8.71 ± 1.78	Dry season
21	Calcium (mgl-1)	34	227	91.27 ± 17.89	Dry season
22	Magnesium (mgl-1)	0.01	7.6	2.64 ± 0.62	Dry season
23	Copper (mgl-1)	0.02	0.06	0.03 ± 0.001	Dry season
24	Iron (mgl-1)	0.12	0.99	0.35 ± 0.04	Dry season
25	Zinc (mgl-1)	0.002	0.03	0.01 ± 0.001	Dry season
26	Chromium (mgl-1)	0.001	0.04	0.02 ± 0.002	Dry season
27	Chlorophyll – a (µg/L)	0.1	64.5	16.99 ± 7.83	Dry season

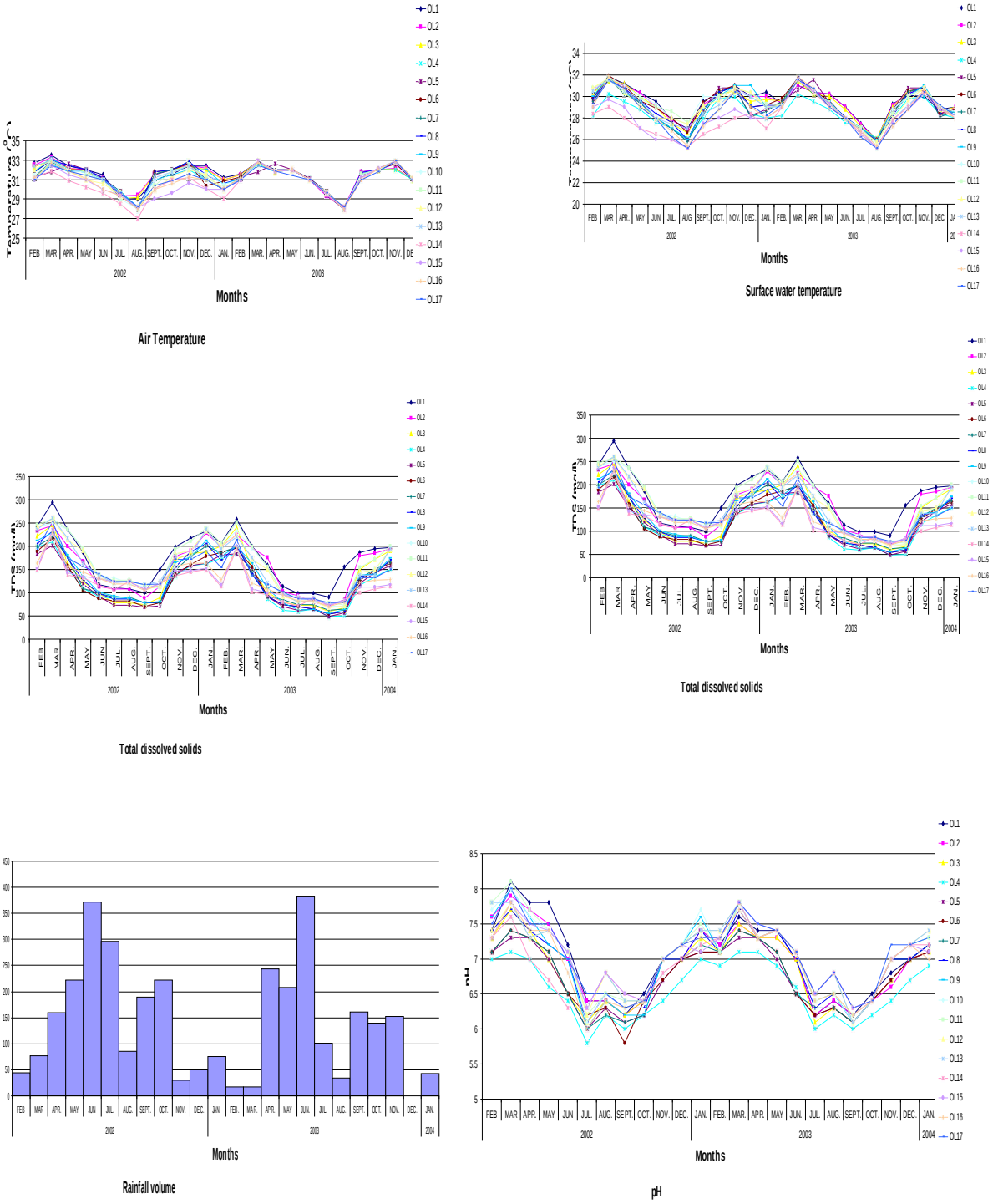


Fig. 2: Seasonal variations in some environmental factors at the Ologe lagoon, Lagos (Feb.2002-Jan.2004).

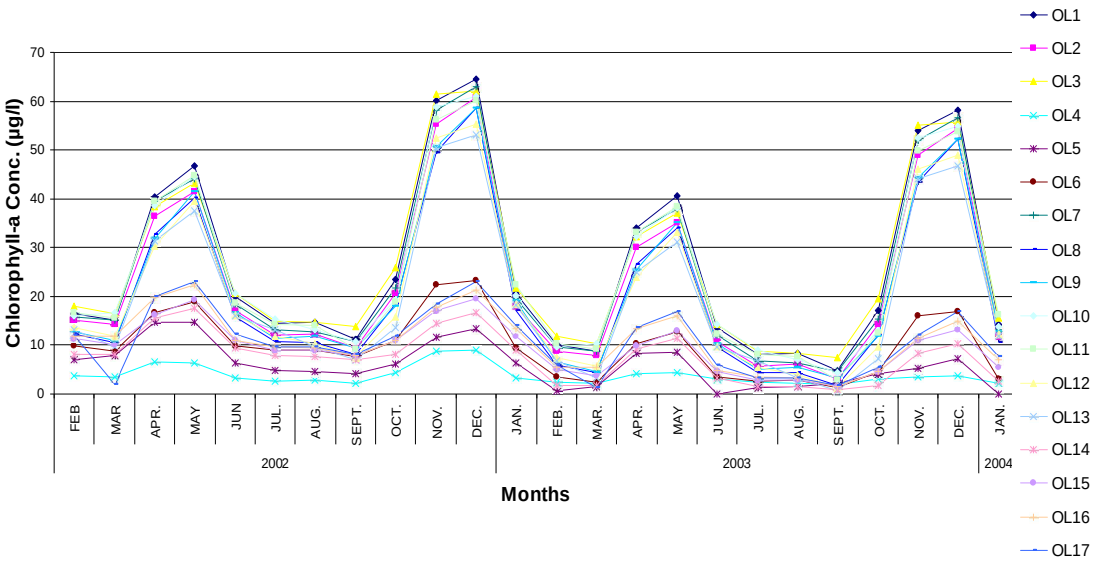


Fig 3: Spatio-temporal variations in Chlorophyll-*a* concentrations at the different stations in the Ologe lagoon, (Feb., 2002 – Jan., 2004

Table (4): Pearson Correlation Co-efficient Matrix of Environmental Characteristics at Ologe Lagoon, Lagos (Feb.2002-Jan.2004)

	AIR TEMP	WATER TEMP	DO	COD	BOD	PH	SALINITY	CONDUCTIVITY	ALKALINITY	NO3-N	PO4-P	SIO2	TDS	TSS	TS	TRANSPARENCY	TOTAL HARDNES	CHLOROPHYLL-a	Ca	K	Na	Mg	Cu	Fe	Cr	Zn
AIR TEMP	1																									
WATER TEMP	0,95	1																								
DO	-0,36	-0,46	1																							
COD	0,39	0,52	-0,71	1																						
BOD	0,33	0,50	-0,42	0,91	1																					
PH	0,51	0,64	-0,75	0,91	0,84	1																				
SALINITY	0,51	0,69	-0,72	0,79	0,74	0,87	1																			
CONDUCTIVITY	0,50	0,68	-0,74	0,81	0,76	0,90	0,99	1																		
ALKALINITY	0,42	0,59	-0,30	0,78	0,93	0,77	0,67	0,68	1																	
NO3-N	-0,30	-0,51	0,37	-0,54	-0,63	-0,58	-0,83	-0,79	-0,54	1																
PO4-P	-0,45	-0,60	0,78	-0,61	-0,60	-0,71	-0,89	-0,86	-0,54	0,98	1															
SIO2	-0,08	-0,26	-0,02	-0,08	-0,28	-0,19	-0,55	-0,51	-0,31	0,76	0,75	1														
TDS	0,46	0,66	-0,58	0,79	0,84	0,88	0,96	0,97	0,81	-0,83	-0,86	-0,59	1													
TSS	-0,51	-0,70	0,73	-0,83	-0,79	-0,91	-0,97	-0,98	-0,75	0,77	0,86	0,50	-0,97	1												
TS	-0,52	-0,69	0,82	-0,82	-0,70	-0,89	-0,93	-0,94	-0,67	0,68	0,84	0,39	-0,89	0,98	1											
TRANSPARENCY	0,52	0,70	-0,59	0,73	0,75	0,87	0,93	0,93	0,77	-0,75	-0,82	-0,58	0,96	-0,96	-0,92	1										
TOTAL HARDNES	0,40	0,57	-0,42	0,88	0,95	0,76	0,69	0,69	0,90	-0,59	-0,54	-0,19	0,75	-0,71	-0,65	0,64	1									
CHLOROPHYLL-a	0,36	0,38	-0,11	0,15	0,24	0,32	0,18	0,20	0,48	0,08	0,04	0,07	0,29	-0,29	-0,28	0,34	0,21	1								
Ca	0,41	0,56	-0,52	0,92	0,93	0,78	0,70	0,70	0,86	-0,57	-0,52	-0,13	0,73	-0,73	-0,69	0,63	0,99	0,17	1							

K	0,35	0,52	-0,25	0,80	0,95	0,69	0,63	0,63	0,94	-0,61	-0,52	-0,28	0,74	-0,67	-0,56	0,64	0,97	0,25	0,93	1						
Na	0,39	0,55	-0,44	0,89	0,95	0,75	0,68	0,68	0,89	-0,59	-0,52	-0,18	0,74	-0,71	-0,65	0,63	0,99	0,18	0,99	0,97	1					
Mg	0,42	0,57	-0,51	0,92	0,94	0,79	0,69	0,70	0,87	-0,55	-0,51	-0,11	0,73	-0,73	-0,68	0,63	0,99	0,20	1,00	0,94	0,99	1				
Cu	0,53	0,70	-0,71	0,93	0,88	0,91	0,91	0,91	0,79	-0,76	-0,80	-0,32	0,90	-0,92	-0,90	0,85	0,88	0,14	0,90	0,81	0,88	0,9	1			
Fe	0,47	0,63	-0,69	0,92	0,86	0,87	0,91	0,91	0,72	-0,79	-0,81	-0,36	0,88	-0,89	-0,85	0,81	0,85	0,01	0,88	0,79	0,86	0,87	0,98	1		
Cr	0,42	0,61	-0,68	0,88	0,85	0,87	0,95	0,95	0,74	-0,86	-0,90	-0,49	0,94	-0,95	-0,90	0,89	0,80	0,09	0,81	0,75	0,80	0,8	0,97	0,97	1	
Zn	0,50	0,70	-0,62	0,78	0,79	0,84	0,98	0,97	0,76	-0,84	-0,88	-0,62	0,98	-0,97	-0,92	0,94	0,75	0,25	0,74	0,72	0,74	0,7	0,91	0,90	0,95	1

Discussion

The characteristics of environmental factors from this study shows clearly that the Ologe lagoon experiences environmental gradients likened to a tropical estuarine aquatic environments from year to year (Hill and Webb, 1958; Webb, 1960; Sandison and Hill, 1966; Kjerfve, 1994; Kirk and Lauder, 2000). Furthermore environmental factors of the lagoon exhibited seasonal changes that were closely related to the distributive pattern of rainfall of the region. For instance during the wet season, reduced levels for air and water temperatures, transparency, salinity, pH, total dissolved solids, conductivity, chloride, total hardness, sulphate, calcium, magnesium, total dissolved solids and alkalinity were recorded. Conversely, in the dry season the values for these parameters increased. Reduced rain events and its associated input of floodwaters from rivers, creeks, adjoining wetlands and the effect of tidal seawater incursion probably lead to this trend of environmental gradients. Reduced phytoplankton densities as reflected in chlorophyll *a* values in the wet season may be linked to the low water clarity which reduces the amount of light getting to planktonic algal component for photosynthesis. Higher chlorophyll *a* values recorded in the dry season is a pointer to improved water clarity at this time which probably allowed greater light penetration. According to Suzuki *et al.* (2002), low chlorophyll *a* values reflecting limited phytoplankton growth in an investigation of a Mexican lagoon were associated to dark water which reduced light penetration into the lagoon considerably.

Pearson correlation co-efficient showed positive correlation between chlorophyll *a* values, salinity, total dissolved solids, alkalinity, pH, conductivity, and total hardness values among others. The flushing of planktonic algal forms towards the sea through Badagry creek during the rains by flood waters and hence dilution, could also account for the low chlorophyll *a* values (phytoplankton densities) recorded at such times. The range of chlorophyll *a* values for the Ologe lagoon was between o.1 and 55µg/l i.e between the mesotrophic and eutrophic productivity status (Suzuki *et al.*, 2002, APHA, 1998). Furthermore, Ogamba *et al.*, (2004) reported a chlorophyll *a* range of 0.15 – 37.4µg/l for the wet season and 0.10 and 40.28µg/l for the dry season in the Elechi creek in the Niger delta. Kadiri (1993) also reported a range of 4.20 – 35.20 mgm-3 for chlorophyll *a* for the Ikpoba reservoir in Benin. Kadiri (1993) reported on the seasonal changes in the chlorophyll *a* situation of a shallow reservoir in Benin, Nigeria.

Higher cloud cover situations attributed to the rainy season have been noted to impair chlorophyll *a* estimates (Kadiri, 1993) and phytoplankton biomass (Nwankwo, 1988) in some parts of the country. On the other hand, increases in insolation usually noted in the dry season likely encourage higher productivity, as recorded for this study. Furthermore, Onyema *et al.* (2003, 2007), are of the view that higher insolation, increased hydrological stability and marine situation are important encouraging factors for primary production in the Lagos lagoon. According to Kadiri (1993) seasonal fluctuation in abundance of phytoplankton is influenced by changes in the physical and chemical properties of the water which themselves can be dependent on rainfall. Similarly, rainfall and salinity are known to regulate the occurrence and distribution of biota in the Lagos lagoon and its associated creeks (Nwankwo and Onyema, 2003; Nwankwo, 2004).

Besides the ample availability of nutrients in this region, values for chlorophyll *a* were comparatively low especially in the peak of wet season which likely indicated limited phytoplankton production. Similarly, Dissolved oxygen levels throughout the period of study were high, though comparatively higher in the wet season than the dry season as result heavy influx of freshwater from adjoing rivers and creeks and lower temperature. It is possible that higher primary productivity which resulted in higher chlorophyll *a* values during the dry season, did not revealed a similar trend in dissolved oxygen values, as a result of masking of high dissolved oxygen by higher biochemical oxygen demand and higher temperature during this period . It could therefore be inferred that variations in chlorophyll-*a* values from this study were largely determined by the trend and continuum of

environmental characteristics of the lagoon which varies seasonally.

Correspondence to:
Dr Onuoha Chuks Paul
Department of Fisheries and Marine Biology, Federal College of Fisheries and Marine Technology, Bar-beach Victoria Island, Lagos Nigeria
Mobile Phone: 234-8023011048
E-mail- hydro_vision@yahoo.com

References.

1. American Public Health Association. (1998): Standard Methods for the Examination of Water and Waste Water. 20th ed. APHA New York. 1270pp.
2. Akinsoji, A., John, A. and Adekanye, M. (2002): Aquatic macrophytes of three selected sites in Lagos, Southwest Nigeria. Journal of Science, Technology Research and Development. 2(1): 9 – 15.
3. Brown, C.A. (1998): Distribution and population dynamics of an estuarine population of *Aloidis trigona* Hinds (Bivalvia). Acta. Hydrobiol. 40(4): 227 – 237.
4. Brown, C.A. and Oyenekan, J.A. (1998). Temporal variability in the structure of benthic macrofauna communities of the Lagos lagoon and harbour, Nigeria. Pol. Arch. Hydrobiol. 45(1): 45 – 54.
5. Chukwu, L. O. and Nwankwo, D. I. (2004): The impact of land based pollution on the hydro-chemistry and macrobenthic community of a tropical West African Creek. The Ekologia. 2 (1-2): 1 – 9.
6. F.A.O. (1969): Fisheries Survey in the Western and Mid-Western Regions of Nigeria. FAO/Sf: 74/NIR 6. 142pp.
7. Kjerfve, B. (Ed.) 1994: Coastal lagoon processes. Elsevier Oceanography Series 60. Elsevier, Amsterdam. pp. 577
8. Kirk, R.M. and Lauder, G.A. (2000): Significant coastal lagoon systems in the South Island, New Zealand. Coastal processes and lagoon mouth closure. *Science for conservation.*146**:** 47 p
9. Kadiri, M.O. (1999): Phytoplankton distribution in some coastal waters of Nigeria. *Nigeria Journal of Botany.***12** (1): 51 – 62.
10. Lee, R.E. (1999): Phycology. Cambridge University Press, New York. 614pp.
11. Nwankwo, D.I. (1984): Seasonal changes of phytoplankton of Lagos lagoon and the adjacent sea in relation to environmental factors. Ph.D. Thesis, University of Lagos. 447pp.
12. Nwankwo, D.I. (2004b). The Microalgae: Our indispensable allies in aquatic monitoring and biodiversity sustainability. University of Lagos Press. Inaugural lecture seris. 44pp.
13. Ogamba, E.N. Chindah, A.C., Ekweozor, I.K.E. and Onwuteaka, J.N. (2004): Water quality of phytoplankton distribution in Elechi creek complex of the Niger delta. Journal of Nigerian Environmental Society. 2(2): 121 – 130.
14. Ogbeibu, A.E. (2005): *Biostatistics*: A practical approach to reseach and data handling. Mindex Publishing Company limited, Benin city, Nigeria.264pp.
15. Onyema, I.C. (2008a): Phytoplankton biomass and diversity at the Iyagbe lagoon Lagos, Nigeria University of Lagos, Akoka. Department of Marine Sciences. 266pp.
16. Onyema, I.C. (2008b): A checklist of phytoplankton species of the Iyagbe lagoon, Lagos. Journal of Fisheries and Aquatic Sciences. **3**(3): 167 – 175.
17. Onyema, I.C. and Nwankwo, D.I. (2006): The epipelic assemblage of a polluted estuarine creek in Lagos, Nigeria. Pollution Research. **25** (3): 459 – 468.
18. Onyema, I.C., Otudeko, O.G. and Nwankwo, D.I. (2003): The distribution and composition of plankton around a sewage disposal site at Iddo, Nigeria. Journal of Scientific Research Development.**7:** 11-26.
19. Onyema, 1.C., Okpara, C.U., Ogbebor, C.I. Otudeko, O. and Nwankwo, D.I. (2007): Comparative studies of the water chemistry characteristics and temporal plankton variations at two polluted sites along the Lagos lagoon, Nigeria. Ecology, Environment and Conservation. 13: 1 – 12.
20. Sandison, E.E. (1966). The effect of salinity fluctuation on the life of *Balanus pallidus strusburi* (Darwin) in Lagos Habour, Nigeria. Journal of Animal Ecology. 35: 365 – 378.
21. Sandison, E.E. and Hill, M.B. (1966): The distribution of *Balanus pallidus* Strusburi (Darwin), Gryphaea gasar (Adanson) Dautzenbergi, *Mercierella enigmatica* farvel and *Hydroides uncinata* (Philippi) in relation to salinity in Lagos Harbour and adjacent creek. Journal of Animal Ecology. **38**: 235-258.
22. Suzuki, M.S., Figueiredo, R.O., Castro, S.C., Silva, C.F., Pereira, E.A., Silva, J.A. and Aragon, G.T. (2002): Sand bar opening in a castal lagoon (Iquipari) in the Northern region of Rio De Janeiro state: Hydrological and hydrochemical changes. Braz. J. Biol., 62 (1): 51 – 62.
23. Sverdrup, K. a., Duxbury, A. B., and Duxbury, A. C. (2006): Fundamentals of Oceanography

5th ed. New York: McGraw Hill Companies, Inc. 342. pp.
24. Webb, J.E. (1958a): The Ecology of Lagos lagoon. 1: The lagoons of the Guinea Coast. Philosophical. Transaction Royal Society London. Ser B: 241-283.
25. Webb, J.E. (1958b): The life history of *Branchiostoma nigeriense* Webb. Philosophical Transaction Royal Society London. Ser B:335-354.
26. Webb, J.E. (1960): Biology in the tropics. Nature. London. 188(4151): 617 - 619.
27. Webb, J.E. and Hill, M.B. (1958): On the Reactions of *Branchiostoma nigerence* Webb to its environment. Philosophical Transactions of the Royal Society of London. Ser B. 241: 355-391.

6/1/2010

Effect of Mineral, Organic Nitrogen Fertilization and some other Treatments on Vegetative Growth of Kalamata Olive Young Trees.

Hassan, H. S. A[*1]; Laila, F. Hagag[1]; H. El-Wakeel[2]; M. Abou Rawash[2] and A. Abdel-Galel[1]

[1]Pomology Department, National Research Center, [2] Dept. of Hort. Fac. of Agric. Ain Shams Univ., Shobra El-Khiema, Cairo, Egypt
[*]Hsasm2000@yahoo.com

Abstract: This study was carried out through two successive seasons (2007& 2008) on Klamata olive young trees grown at the Research Station Farm of National Research Center, El Nobarya, El Behera governorate. The investigation aimed to study the effect of applying mineral, organic fertilizers and some other treatments on growth parameters at the first two years of planting. Planting holes were prepared for control plants in the first season only. Each treatment received 100 g actual nitrogen/plant/year as recommended by M.A.R.L. (2007). The following treatments were applied: T1 : control (mineral nitrogen + planting hole preparation), T2(100%mineral nitrogen), T3(100% organic N as cattle manure), T4(50% mineral N + 50% organic N as chicken manure), T5 (100%mineral nitrogen + humic acid as soil application), T6(100% mineral nitrogen + activated dry yeast as soil application), T7 (100%mineral nitrogen + GA_3 spray) and T8 (100% mineral nitrogen + sea algae as soil application).At the end of each season, plant height, stem diameter, lateral shoot number, lateral shoot length, leaves numbers per plant, percentage of plant height increment, whole plant dry weight were determined and recorded. The obtained results revealed that plant height, shoots number, shoot length, leaves number and stem diameter were not affected by different treatments. However the fifth treatment with humic acid and seventh treatment with GA_3 spray gave highest significant values of leaf numbers per plant compared with all other treatments in the first season, but in the second one, the differences among treatments lake significance. As for Whole plant dry weight, no significant differences among treatments could be noticed in both seasons. [Journal of American Science 2010;6(12):338-343]. (ISSN: 1545-1003).

Keywords: Klamata olive; mineral fertilizer; organic fertilizer; growth parameter; plant

1. Introduction:

The Egyptian olive production reached about 507053 tons produced from 110764 feddan and the total area reached about 135692 feddan (according to the statistics of M.A.L.R. (2007).

Nawaf and Yara (2006) found that, young olive trees benefit from low levels of NPK and N alone and additional fertilizers would not be significant. However, NPK are consider to be essential element for plant growth and development. The 16 g NPK and 32 g N significantly gave the highest shoot and root dry weight, this probably due to nitrogen concentration which increased dry matter accumulation in roots and decreased shoot: root ratio.

Monge *et. al.*(2000) reported that, organic wastes fertilization did not lead to significant increases in olive mineral leaf concentrations in the first year trial. Hegazy *et. al*.(2007) studied the effect of organic and bio-fertilization on vegetative growth and flowering of Picual olive trees, they recorded that, the highest values of the studied growth characters were obtained with 100% organic fertilization (poultry manure).

Fernández-Escobar *et. al.*(1999) mentioned that, foliar application of leonardite extracts(humic substances extracted) to young olive plants stimulated shoot growth when they were growing without the addition of mineral elements to the irrigation water, but did not promote growth when applied to plants watered with a nutrient solution, although growth of fertilized plants was greater than that of unfertilized ones. Under field conditions, foliar application of leonardite extracts stimulated shoot growth and promoted the accumulation of K, B, Mg, Ca and Fe in leaves. Abdel Fatah *et. al.*(2008) mentioned that, soil drench application of humic acid to Tifway Bermodagrass hybrid improved growth parameters and NPK leaves cotents.

Mostafa and Abou Raya (2003) recorded that, all dry yeast soil application improved growth parameters of Grand Nain banana cv. Compared with control without dry yeast treatment.

Sidahmed (1987) working on eight-month-old seedlings of sour orange were sprayed with 0, 25, 50, 75, 100 and 200 ppm of the water soluble salt of gibberellic acid at 15-day intervals.Data collected two months later revealed that GA_3 significantly increased ($P < 0.05$) seedling height by internodal expansion and that the seedling height was positively

correlated with GA_3 level. The percentage increase in height was 13.95, 18.68, 19.63, 25.90, 31.80 and 38.80 respectively for 0, 25, 50, 75, 100 and 200 ppm GA_3 treatments. Smith and Schwabe (1984) recorded that, top growth of *Quercus robur* could be further accelerated by application of gibberellic acid (GA3) as foliar spray. Sheo (1999) mentioned that, the seedling growth of Karun Jamir (*C. aurantium*) and Cleopatra mandarin (*C. reshni*) was significantly increased by spraying with urea and GA_3 alone or in combination. However, $ZnSO_4$ alone did not have any significant effect on growth. Seedling growth of both the species was greater with the combinations 0.5% urea + 50 ppm GA_3 + 0.2% ZnSO4 and 0.5% urea + 50 ppm GA_3. The results indicated that the seedling growth of Karun Jamir and Cleopatra mandarin can be increased considerably by two sprays of 0.5% urea + 50 ppm GA_3 in the 3rd and 8th months after sowing.

This investigation aimed to study the effect of mineral and organic nitrogen fertilization sources and some other treatments (humic acid, activated dry yeast, GA_3 and sea algae) on growth parameters of Kalamata young trees at first two years of planting.

2. Materials and Methods:

This study was carried out through two successive seasons (2007& 2008) on Kalamata olive young trees grown at the Expermental research station of National Research Center at El Nobarya, El Behera governorate Egypt. The investigation aimed to study the effect of applying mineral, organic nitrogen fertilizers and some other treatments on vegetative growth characters of young Kalamata olive trees at the first two years of planting. The soil was characterized by: pH = 8.82, EC =1.11 dS/m, organic matter = 0.31%, $CaCO_3$ =12.8 %, Sand = 63 %,Silt = 13 % and clay = 3%. The soil texture grade was sandy. Drip irrigation system was applied using river Nile water. Planting distance was 5 × 5 meters apart.

The following treatments were applied:

1- Control: recommendation of M.A.R.L. (2007a) (100g actual nitrogen 500 g ammonium sulfate as mineral nitrogen source) + planting holes preparation.
2- Mineral nitrogen only 100 %.
3- Organic nitrogen source 100 % (cattle manure 100g actual nitrogen).
4- Mineral nitrogen source 50 % + organic nitrogen source 50 % (chicken manure).
5- Mineral nitrogen source 100 % + humic acid (monthly doses from March to November each 20 ml/plant).
6- Mineral nitrogen source 100 % + actived dry yeast as drench treatment three times in March, July and October each at 30 g/plant.
7- Mineral nitrogen source 100 % + one spray of GA_3 acid at 50 ppm in March.
8- Mineral nitrogen source 50 % + sea algae in March and June each at 50 g/plant.

Cattle manure analysis was: N = 1.6%, P = 0 46% and K = 0.51%.
Chicken manure analysis was: N =3.47%, P =0.67% and K = 0.64%.
Sea algae analysis: N =8%, P = 2%, K = 4%, chelate microelements = 4% and traces of vitamins + amino acids.

Ammonium sulfate was divided into five equal doses through growing season. All these treatments were repeated in the second season except holes preparation with control plants only in the first season. The treatments were arranged in randomized complete block design in a simple experiment with four replicates for each treatment and each replicate was represented by one plant. At the end of each season at mid November four plants as replicates for each treatment were removed genteelly with their root system to estimate and record the following data for each cv individually:

1- Plant height (cm).
2- Stem diameter (cm) was measured at 5cm above the grafting zone.
3- Lateral shoot length average (cm).
4- Leaf number per plant.
5- Lateral shoot number per plant.
6-Percentage of plant height increment
7- Whole plant dry weight (g).

Data obtained throughout this study were statistically analyzed using the analysis of variance method as reported by (Snedecor and Cochran, 1980) and the differences between means were differentiated by using Duncan's range test.

3. Results and Discussion:

Effect of treatments on growth characters:

In control plots, planting holes were prepared by adding 50 kg c

Fig.(1) show that, Insignificant differences among treatment were found in both seasons in values of plant height But one can notice that the second treatment (100% mineral nitrogen) gave the highest value in the first season(38.8 cm) and the lowest value was obtained by humic acid and sea algae treatments (35 cm &35 cm). In the second season the sixth treatment recorded the highest value (78.3 cm) and the lowest values were shown by the forth treatment with 50% chicken manure and seventh treatment with GA_3 (77 cm &77 cm).

Stem diameter fig.(2) show that, data showed insignificant differences among treatments in

both seasons . In addition all treatments recorded the same value in the second season (1.5 cm).

Lateral shoot number per plant, fig.(3) show that, insignificant differences among treatments in both seasons. However, the first treatment (control) recorded the lowest value in first season (3.5) compared with other treatments. In the second season sixth treatment with yeast showed the lowest value (7.3).

Lateral shoot length fig.(4) show that, Insignificant differences among treatments in both seasons were recorded. But one can notice that, the sixth treatment with yeast gave the highest value in both seasons (18.3 cm &68.7 cm respectivly).

On the other hand, fig (5) showed that the fifth treatment with humic acid and the seventh treatment with GA3 spray gave the highest significant values of leaf number fig.(5) show that, per plant compared with all other treatments in the first season, but in the second season, the differences among treatments lake significance.

Percentage of plant height increment fig.(6) show that, The seventh and the eighth treatments gave lower significant values than other treatments in the first season .But in the second season differences among treatments lake significant .

Whole plant dry weight fig.(7) show that, result indicated that treat.5(100% mineral N+HA) showed the significantly greatest value (80g) compared with treat. 8 (50%mineral N +sea algae)(34g) in the first season. Differences among other studied treatments were insignificant. In the second season, no significant differences could be detected, although the eighth treatment showed the lowest value (144.30 g.).

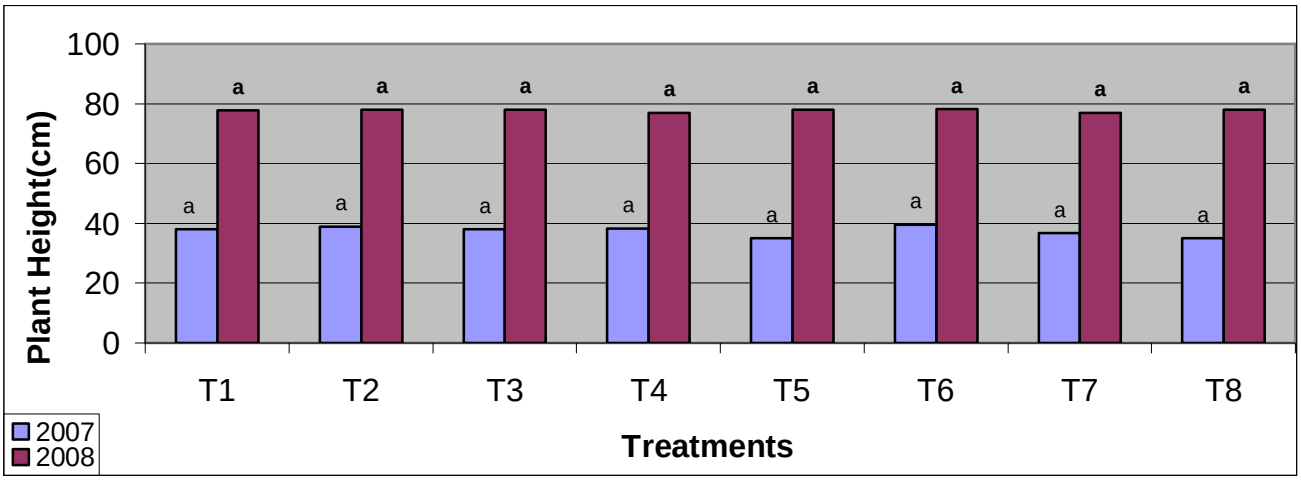


Fig. (1): Effect of mineral, organic nitrogen and some other treatments on plant height(cm) of Klamata olive cv. young trees in 2007 and 2008 seasons.

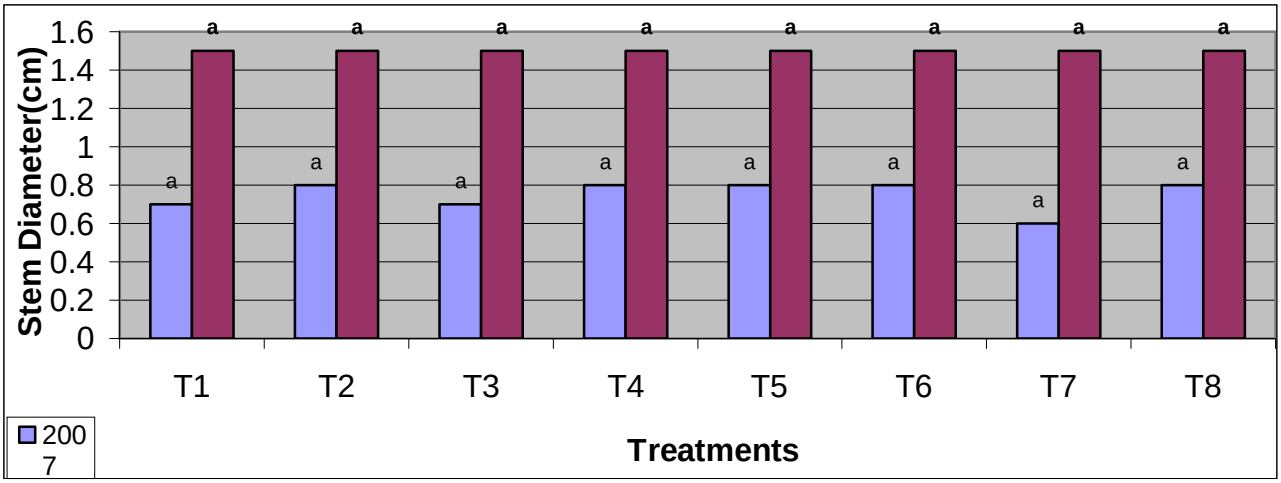


Fig. (2): Effect of mineral, organic nitrogen and some other treatments on stem diameter(cm) of Klamata olive cv. young trees in 2007 and 2008 seasons.

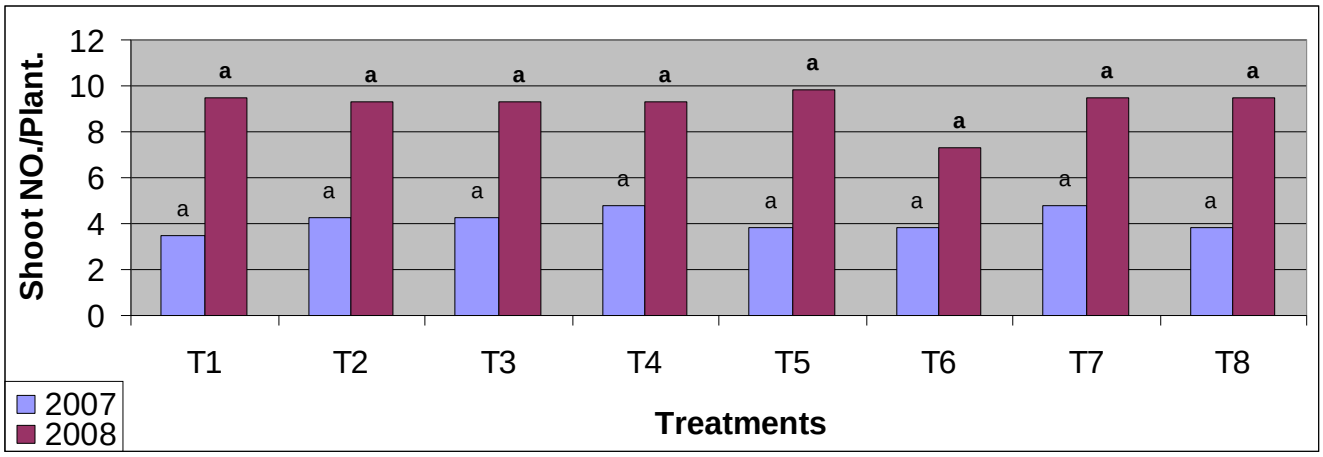


Fig. (3): Effect of mineral, organic nitrogen and some other treatments on shoot No./plant of Klamata olive cv. young trees in 2007 and 2008 seasons.

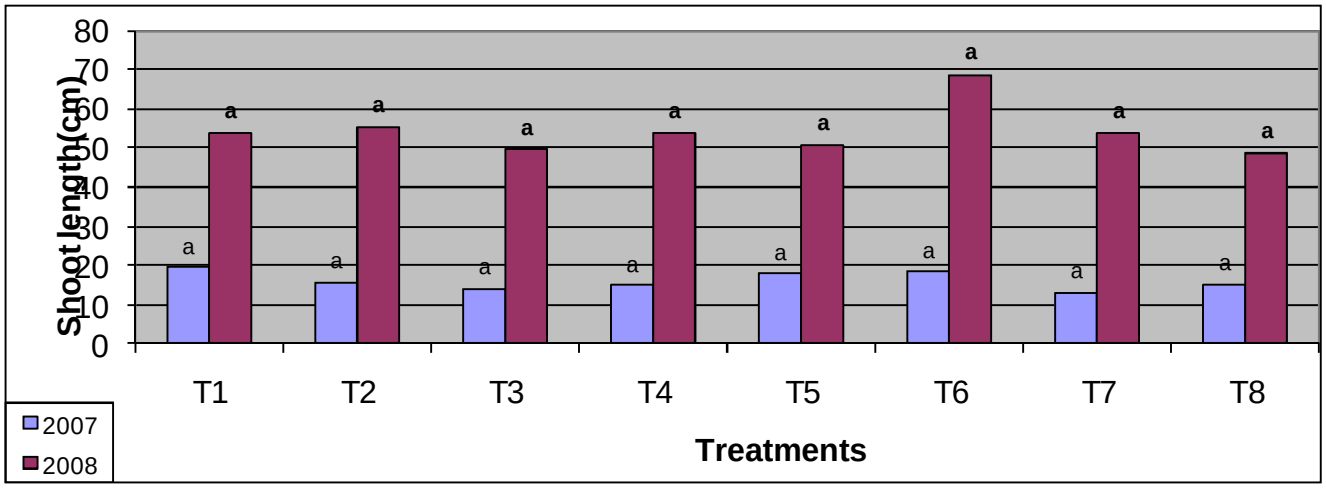


Fig. (4): Effect of mineral, organic nitrogen and some other treatments on average shoot length(cm) of Klamata olive cv. young trees in 2007 and 2008 seasons.

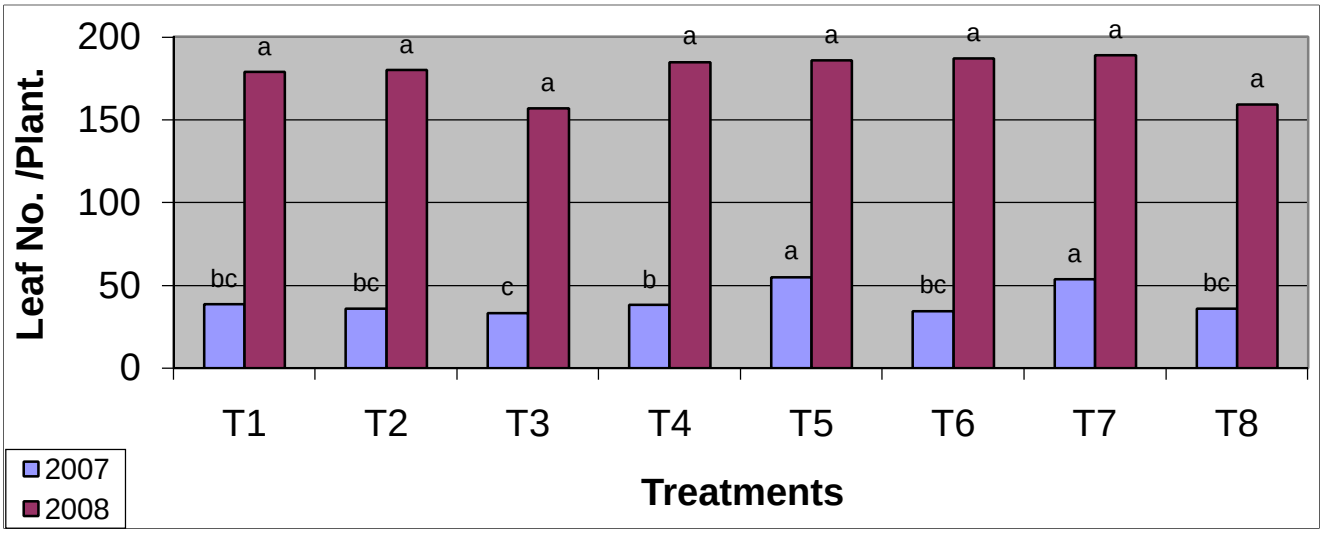


Fig. (5): Effect of mineral, organic nitrogen and some other treatments on leaf No. /plant of Klamata olive cv. young trees in 2007 and 2008 seasons.

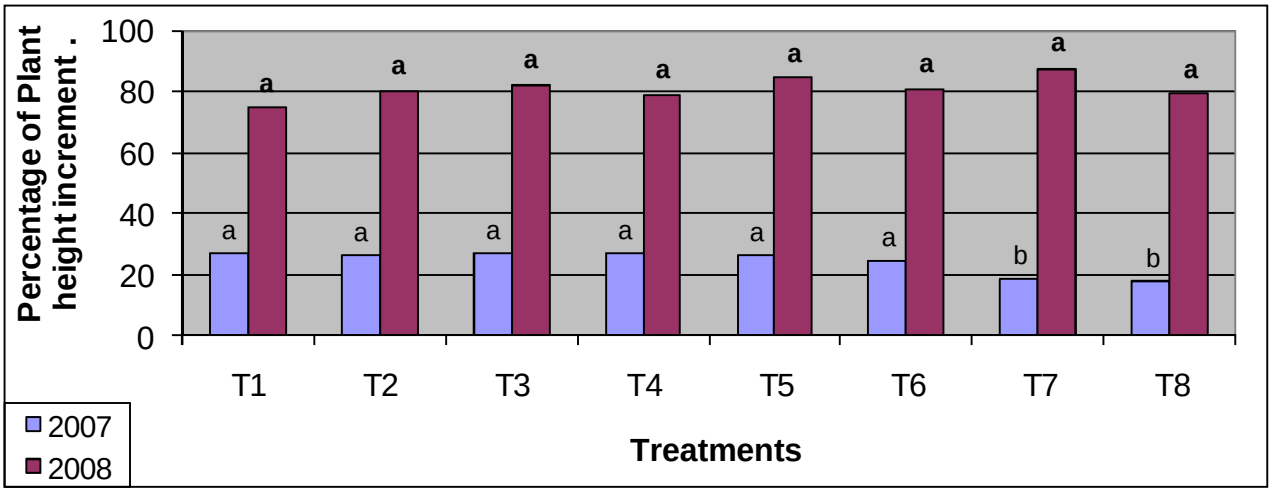


Fig. (6): Effect of mineral, organic nitrogen and some other treatments on percentage of plant height increment of Klamata olive cv. young trees in 2007 and 2008 seasons

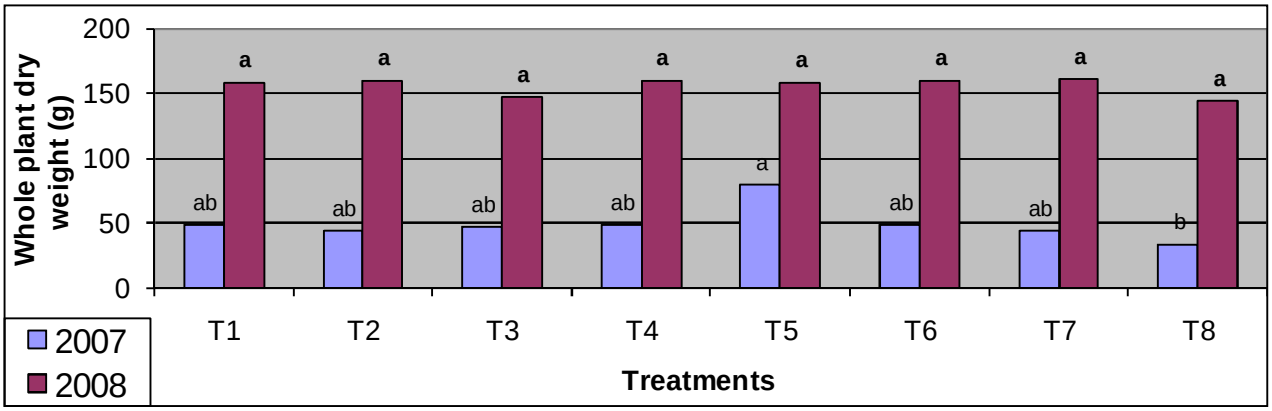


Fig. (7): Effect of mineral, organic nitrogen and some other treatments on whole plant dry weight (g) of Klamata olive cv. young trees in 2007 and 2008 seasons.

Finally it could be noticed that plant height, shoot number, shoot length, leaves number and stem diameter were not affected by different treatments Insignificant differences among treatment in both seasons in values of plant height, , stem diameter lateral shoot number per plant and lateral shoot length average. Meanwhile, the fifth treatment with humic acid and seventh treatment with GA_3 spray gave highest significant values of leaf number per plant compared with all other treatments in the first season, but in the second season, the differences among treatments lake significance. Concerning the whole plant dry weight, no significant differences among treatments could be noticed in both seasons. These results are in harmony with those found by Fernández-Escobar *et. al.*(1999), they reported that, foliar application of leonardite extracts(humic substances extracted) under field conditions, stimulated shoot growth of young olive plants. Moreover we can added that, growth parameters were not affected by most treatments, this may be attributed to low nutritional demand of young olive trees as mentioned by Xiloyannis *et. al.* (2000) they showed that, demand of irrigated olive trees, cultivar Coratina for P and K is minimal during the first four years after planting and can be fulfilled by naturally supplied soils. Low doses of N should be applied through localized fertilization during the year. Moreover, Nawaf and Yara (2006) found that, young olive trees benefit from low levels of NPK and N alone and additional fertilizers would not be significant. However, NPK are consider to be essential element for plant growth and development. The 16 g NPK and 32 g N significantly gave the highest shoot and root dry weight, this probably due to nitrogen concentration which increased dry matter accumulation in roots and decreased shoot: root ratio.

Corresponding author
H. S. A. Hassan
Pomolgy Department, National Research center, Cairo, Dokki-Egypt.
Hsans2000@yahoo.com

4. References:

1. Abdel Fatah H. Gehan; Boshra A. El Sayed and S.M. Shahin (2008): The role of humic acid in reducing the harmful effect of irrigation with saline water on tifway turf.J. Bio. Chem. Environ. Sci.Vol.3 (1):75-89.
2. Fernández-Escobar R.; M. Benlloch; D. Barranco; A. Dueñas and J. A. Gutérrez Gañán (1999): Response of olive trees to foliar application of humic substances extracted from leonardite. Scientia Horticulturae
Vol. 66, 3-4, October, Pages 191-200.
3. Hegazy, E. S.; M. R. El-Sonbaty ; Ahmed M. Dorria and T. F. El-Sharnoby (2007): Effect of organic and bio-fertilization on vegetative growth and flowering of Picual olive trees.Wold Journal of Agricultural Science 3(2):210-217.
4. M.A.L.R. (2007): Ministry of Agriculture and Land Reclamation Economic Affairs - Study of Important the Agriculture Statics, Vol.(2)p 353.
5. M.A.L.R. (2007a): Ministry of Agriculture and Land Reclamation.Modern technique for olive cultivation and production. Horticultur Reseasch Institute, Agriculture Research Center bulletin No. 15(in Arabic).
6. Monge, E., J. Val; J.L. Espada; F. Orús and J. Betran (2000): Effects of organic wastes on olive mineral nutrition and its influence on fruit quality. Preliminary results for macronutrients. Acta Horticulturae 512:199-208.
7. Mostafa, E. A. M. and M. S. Abou Raya (2003): Effect of soil application of active dry yeast on growth, yield and fruit qulity of Grand Nain banana cv.. Arab Univ. J. of Agric. Sci. Vol. 12(2):693-704.
8. Nawaf, M. Ferihat and Yara K. Masa`deh (2006): Response of two-year-old tree of four olive cultivars to fertilization. Amrican-Erasian J. Agric. & Environ. Sci., 1 (3): 185-190.
9. Sheo, G. S. I. P. (1999): Effect of foliar application of urea, GA3 and ZnSO4 on seedling growth of two citrus species. J. Appl. Hort., 1(1):51-53.
10. Sidahmed O.A. (1987): Effects of different levels of gibberellic acid (GA_3) on growth of sour orange (*C.Aurantium* L.) Seedlings. Acta Horticulturae 84: V Africa Symposium on Horticultural Crops.
11. Smith D. J. And W. W. Schwabe (1984): Acceleration of early growth of seedlings and rooted cuttings of *Quercus robur* L.. Forestry , 57(2):143-157
12. Snedecor, G. W. and W. G. Cochran (1980) Statistical Methods. 7th Ed.507 pp Iowa State Univ. Press, Ames. Iowa, U.S.A. .
13. Xiloyannis C.; G. Celano; A.M. Palese; B. Dichio and V. Nuzzo (2000): Mineral nutrient uptake from the soil in irrigated olive trees, cultivar Coratina, over six years after planting. Acta Horticulturae 586: IV International Symposium on Olive Growing.

6/6/2010

The Proposed Electric Circuit Diagram Of The Buried Bare Pipe Segment-Soil - Earth System With And Without Applying Cathodic Protection System

Dr. Ashraf Abdel Raouf Mohamed Fouad Ahmed

Canadian International College CIC – Egypt
Ashrafahmed9000@yahoo.com

Abstract: Based on proposed electrical concept of corrosion process, it is possible to simulate buried bare pipe segment with the surrounding soil medium by an electric circuit where the circuit electric quantities are function of the electrochemical properties of the soil as 4th degree polynomial equations. The equivalent cylindrical electrolytic capacitor between the pipe and the remote earth and the potential across it, verifies the equation that charge Q = C × V at natural condition with & without applying cathodic protection system. The created positive charges consists with an equivalent negative charge (electrons losses) a charged stray electrolytic capacitor between the pipe and the earth through thin film soil layer around the pipe as cylindrical capacitor. The amounts of these charges are depending on the electrochemical properties of the soil which are surrounding the pipe segment, the length of the pipe segment and its diameter. The rate of discharge (equivalent to capacitor self discharge) is to be considered as the corrosion current. That's beside the facts deduced before that all electrical parameters prints & equations are function of the electrochemical properties of soil medium around the pipe at different cathodic protection levels. The error of these new equations of the electrical parameters reduced to be less than ± 5%. This will help to study both the corrosion problem and cathodic protection for a complete pipeline by an electric concept with an electric analogue circuit which is the aim of this study. This will help, in the future, in the choice of pipeline route, pipeline cathodic protection design and cathodic protection maintenance process for the pipe line along its route, however long it is. [Journal of American Science 2010;6(12):344-354]. (ISSN: 1545-1003).

Keywords: Electrical study of pipe – soil – earth system

1. Introduction

At humidity equal to zero, the soil medium around pipeline could be considered as a dielectric material which has its relative permittivity. If the humidity is increased, the soil medium is considered to be as an electrolyte associated with a change happened in the values of the relative permittivity, resistivity and pH of the soil. This change happened in the electrochemical properties of the soil will continue by increasing the humidity but these values will return back to their original values, or nearby initial values, after the humidity returns back to it's initial value. This nature of the soil medium between isolation medium and conduction medium according to the percentage of the humidity could be studied electrically.

In other words, if two dissimilar electrodes buried in a box which is containing a soil medium, a corrosion current will take place between these electrodes due to the difference in electrodes' natural potential while a capacitance in nano farad could be measured between these dissimilar electrodes (through the soil). The potential difference, the capacitance and the corrosion current between the positive and negative electrodes are electric quantities. Then, it may be possible to understand the corrosion and cathodic protection by an electrical concept beside the electrochemical and thermodynamic concepts. Now the corrosion may be described electrically by the equation: (Q = C × V), while the rate of discharge dQ/dt is equal to the corrosion current from the +ve electrode to the –ve one [1] [2]. The same concept could be applied on the system of buried pipeline and the surrounding soil medium. The pipeline may be considered the +ve electrode while the remote earth may be considered the –ve electrode. In case of pipe-soil-earth system which is not subjected to any external interference, it is possible to find a correlation between the electrical parameters and the electrochemical properties of the soil at different humidity and at many cathodic protection levels with the results to be considered as an electrical parameters print or as a data sheet of this pipe-soil-earth system [6] [7] [8] [9] [10]. These electrical prints will be recalculated if the pipe and/or soil are changed. The importance of these electrical prints are not only to help to deduce the proposed electrical circuit of the combination pipe-soil-earth system but also to define both the electrical parameters and the cathodic protection level of any buried pipe segment if the protection current and the electrochemical properties are measured at the pipe segment directly from the field.

2. Literature Review

The only way in which atoms of the metal may detach themselves from the surface and enter the solution is in the form of positively charged ions. In electrochemical concept of corrosion process, for buried metal pipe in the ground, the following equations describe the corrosion process:

At the anode

Fe (solid) → Fe^{++} (ion) + $2e^{-}$ (electrons)

At the cathode

$2H^{+}$ (hydrogen ions) + $2e^{-}$ → H_2 (gas)
Or $2H^{+}$ + ½ O_2 (air) + $2e^{-}$ → H_2O
Or O_2 + 2 H_2O + $4e^{-}$ → 4 OH^{-} (hydroxyl ion)

Then,

Fe^{++} + 2$(OH)^{-}$ → Fe $(OH)_2$
Fe^{+++} + 3$(OH)^{-}$ → Fe $(OH)_3$

In the proposed electrical concept of the corrosion process of bare pipe-soil system, the anodic reaction and the surrounding soil around the pipe may be represented, electrically, by formation of a charged electrolytic stray capacitor as shown in Fig.1 and Fig.2a [1] [2]. The electrolytic capacitor is consisting of pipe segment as the positive electrode, thin film of soil layer as the dielectric material of the capacitor and an imaginary co-axial earthing cylinder as the negative electrode. This is while for coating pipe-soil system, the stray capacitor may be considered as cylindrical capacitor with compound dielectric materials (coating of the pipe + thin film soil layer) as shown in Fig.2b [1] [2]. The formation of Fe $(OH)_2$ & Fe $(OH)_3$ in the electrochemical concept may be understood electrically by the positive charge discharged through the electrolytic stray capacitor to the imaginary co-axial earthing cylinder of radius r_3 (self discharge of the capacitor). The rate of discharge is equal to the stray corrosion current (equivalent to electron loss).

In other words, the cathodic reaction at the imaginary co-axial earthing cylinder (the negative electrode) and formation of hydroxyl ion (OH^{-}), detach the positive ion from metal surface and form ferric oxides. Electrically, it may be understood as the discharge of the positive charge from the positive electrode to the imaginary grounded negative electrode through thin film of soil layer as shown in Fig.1. The above clarification could be summarized as follow:

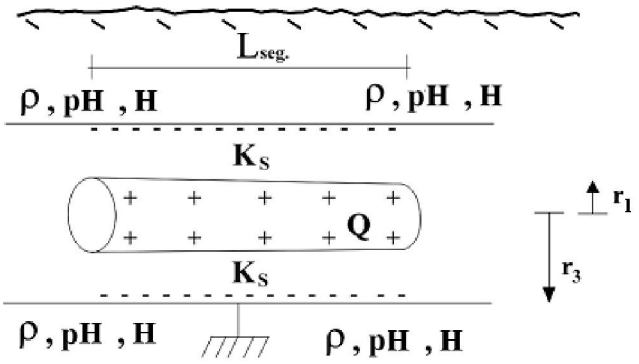


Figure 1: Proposed electrical concept of bare pipe segment with soil medium

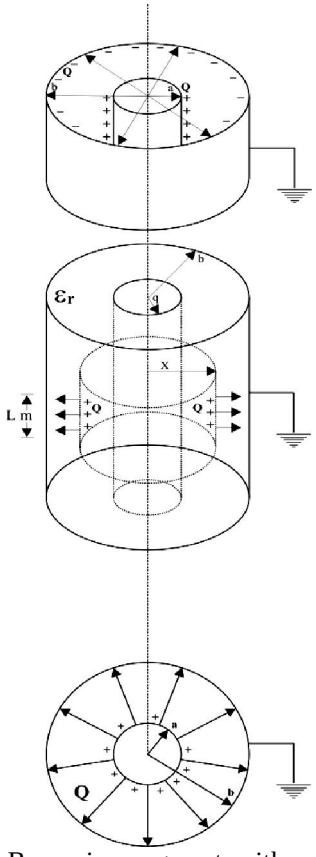


Figure 2a: Bare pipe segment with an imaginary Coaxial earthing cylinder form a charged electrolytic capacitor

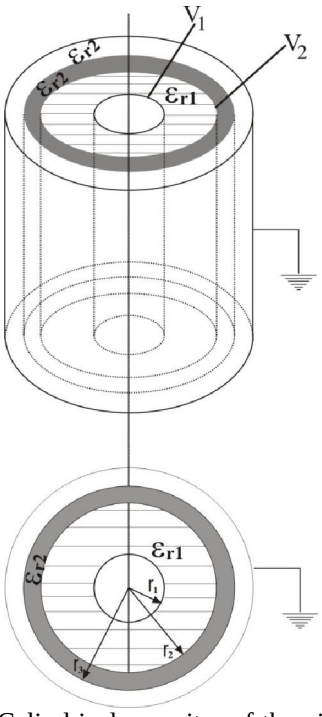


Figure 2b: Cylindrical capacitor of the pipe segment and the compound dielectric of coating material and soil layer

The electric concept of corrosion for pipe-soil-earth system may be written by the following proposed concept:

"Due to surrounding medium effect around metal structure buried in the ground, the charge created on metal outer surface area (o.s.a) builds up a potential through a stray electrolytic capacitor between metal o.s.a and an imaginary coaxial earthing cylinder where rate of discharge is equal to the corrosion current "[1][2].

The dielectric constant of the soil layer around the pipe K_S acts with the dielectric constant of the pipe coating material K_C as a coaxial cylindrical capacitor with compound dielectric. As K_C of the coating material is decreased, the total capacitance value is decreased (two capacitors are in series) then charge is decreased .That's to say, the corrosion process (electrons losses) is decreased. If deterioration of coating material occurred, then K_C is increased i.e. total capacitance value of the compound dielectric is increased .That's to say that corrosion process (electrons losses) is increased as the created charge on metal outer surface area *Q* will be increased . This paper deduces the equivalent electrical circuit of bare pipe segment-soil-earth system with and without applying cathodic protection system.

3. The Soil Factor

As the values of the electrochemical properties of any soil medium are changed by the change of humidity, they return back to their initial conditions, or nearby the initial values, after some time, when humidity returns back to its initial value. Then we can define a new factor named the soil factor as:

"The soil factor is the instantaneous or present value of the electro-chemical properties of the soil based on the electrical properties at Humidity equal to 10% "[1] [2]

The soil factor is equal to:

$S_f = (1 / Ks)\ pH\ H \log \rho$ at room temperature (1)

Dimension of $[S_f] = [1 / K_S]\ [pH]\ [H]\ [\log \rho] = \Omega.m\ \%$

Where:

S_f = soil factor

Ks = Dielectric constant of the soil at H = 10%
(a reference value of this property)

pH = power of Hydrogen of the soil

ρ = Soil resistivity in Ω.m. at H = 10%
(a reference value of this property)

H = Humidity of the soil %

Fig. 3 shows the range of the soil factor S_f and the range of humidity for ten soils under test.

The importance of this new parameter, the soil factor, is that it is combining all parameters which can affect directly on the cathodic protection level or in corrosion process (the effect of temperature and CO_2 could be added in future studies). Such parameters which can be obtained by a direct measurement from the field only one time then use the humidity in the soil factor calculations. This means that if it is possible to study the relationship between the soil factor and the electrical parameters of the pipe-soil system at natural condition with and without applying cathodic protection system, then the electrical parameters PRINTS of the pipe-soil-earth system could be obtained.

The soil factor can be considered to be as the main key of many studies based on the proposed electrical concept of corrosion. For an example, the general equation of the natural stray capacitance, without applying CP, between external surface area of bare pipe segment and earth is obtained in terms of the soil factor with an average error less than ± 5% and its print curves are obtained for pipe-soil-earth system for 10 different soils [3][10]. Also, the general equation of both the natural stray potential and the natural surface charge, without applying CP,

are obtained in terms of the soil factor with an average error less than ± 5% and their print curves are obtained for pipe-soil-earth system for 10 different soils [4] [5] [10]. Also, by the use of this new parameter, the soil factor, it is possible to find a correlation between the electrical parameters and the electrochemical properties of the soil, with applying CP, at different humidity and at many cathodic protection levels with the results to be considered as an electrical parameters print or as a data sheet of this pipe-soil-earth system [6] [7] [8] [9] [10].

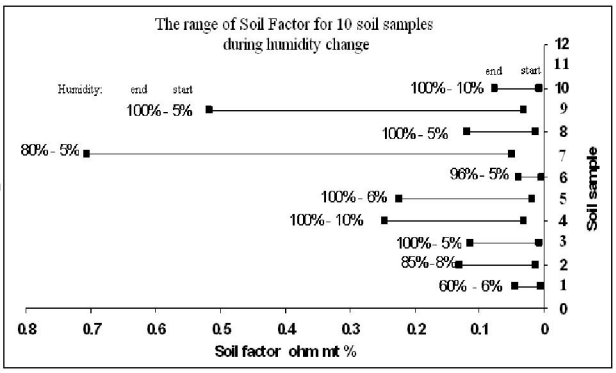


Figure 3: The range of the soil factor & humidity range for the soils under test

4. Case 1:

4.1 Proposed Electrical Analogue Circuit of The Pipe Segment – Soil - Earth System Without CP System [1] [2]

In a corrosion process, the metal pipe could be considered as a current source (stray corrosion current) to the surrounding medium (stray capacitor to the remote earth). The electrical analogue circuit of the pipe line segment with the surrounding medium effect could be represented as a current source connected in series with the stray capacitance between metal o.s.a and the remote earth. Consequently, the corrosion process could be electrically simulated for both bare pipe and at bad condition of coating material as shown in Fig.4.

The general electrical analogue circuit of pipe segment-soil-earth system will consists of current source in series with an equivalent impedance $Z_{eq.}$ correlated to humidity (R_{eq} in parallel with C_{eq}) which is connected from pipe to the remote earth as shown in Fig.5. The importance of this proposed electrical circuit is that it converts both the corrosion and/or cathodic protection process into an electric problem. As we obtained before the electric parameters C, V and Q of the bare pipe segment-soil-earth system in terms of the electrochemical properties of the soil i.e. the general equations of the natural stray capacitance [3],[10], the natural stray potential [4][10], the surface natural created charge[5][10]. All electrical parameters are deduced in terms of the soil factor, 4th degree polynomial equations with an average error less than ± 5%. In this paper, we will continue and use the results obtained before to deduce the electric analogue circuit of the natural condition pipe-soil-earth system without applying cathodic protection system.

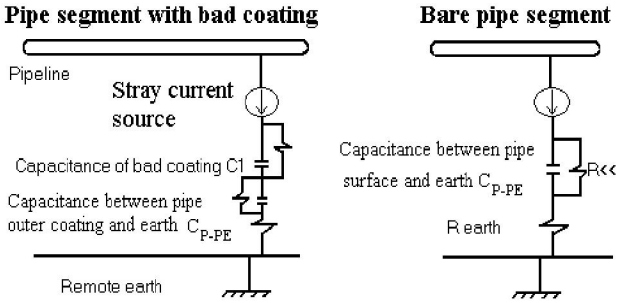


Figure 4: Proposed electrical analogue circuit of the pipe segment – soil system at the corrosion process

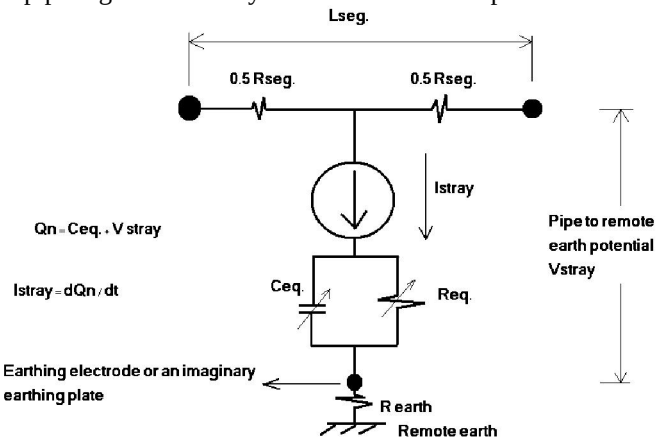


Figure 5: The proposed general electrical analogue circuit of pipe-soil system

4.2 Calculations Of The Electrical Parameters Of The Pipe-Soil-Earth system Without Applying CP System

Now, to obtain the pipe segment natural electric parameters without applying cathodic protection system, we have for the pipe-soil-earth system the following results:

4.2.1 General Equation Of The Natural Stray Electrolytic Capacitor [3],[10]

This is equal to Eq. 2:

$$\mathbf{C}_{\mathbf{n\ stray}} = A_{4cn}\,\mathbf{X^4} + A_{3cn}\,\mathbf{X^3} + A_{2cn}\,\mathbf{X^2} + A_{1cn}\,\mathbf{X} + A_{0cn} \quad (2)$$

Where:

A's = $A_{(\ )CN}$ are the stray capacitance PRINT constants of the pipe soil under test

X = instantaneous value of the electrochemical value of the soil, the soil factor

4.2.2 General Equation Of The Natural Stray Potential [4],[10]

This is equal to Eq. 3:

$$\mathbf{V_{n\ stray}} = A_{4vn}\,\mathbf{X^4} + A_{3vn}\,\mathbf{X^3} + A_{2vn}\,\mathbf{X^2} + A_{1vn}\,\mathbf{X} + A_{0vn} \quad (3)$$

Where:

A's: = $A_{(\ )V}$ are the natural stray potential print constants of the pipe soil under test

X = instantaneous value of the electrochemical value of the soil, the soil factor

4.2.3 General Equation Of The Natural Surface Charge [5],[10]

This is equal to Eq. 4:

$$\mathbf{Q_N} = A_{4qn}\,\mathbf{X^4} + A_{3qn}\,\mathbf{X^3} + A_{2qn}\,\mathbf{X^2} + A_{1qn}\,\mathbf{X} + A_{0qn} \quad (4)$$

Where:

A's: = $A_{(\ )qn}$ are the surface natural charge print constants of the pipe - soil under test

X = instantaneous value of the electrochemical value of the soil, the soil factor

4.2.4 General Equation Of The Protection Current:

As we consider the natural condition without applying cathodic protection system, then the rectifier output will equal to zero.

$$I_P = 0 \quad (5)$$

4.2.5 The Earthing Resistance

The earthing resistance R_E could be easily measured from the field by the use of earth tester.

$$R_E = \text{Measured from the field} \quad (6)$$

4.2.6 The Pipe Segment resistance

The resistance of the pipe segment will equal to:

$$R_{SEG.} = (¼\ \pi\ (D_O^2 - D_I^2) \times L_{SEG.} \times \sigma_{IRON}) \quad (7)$$

Where:
D_O = Outer diameter of the pipe segment
D_I = Inner diameter of the pipe segment
L_{SEG} = Length of the pipe segment
σ_{IRON} = Iron specific resistance (pipe material)

4.2.7 The Natural Stray Corrosion Current Calculation

As per Eq. 4:

$$\mathbf{Q_N} = A_{4qn}\,\mathbf{X^4} + A_{3qn}\,\mathbf{X^3} + A_{2qn}\,\mathbf{X^2} + A_{1qn}\,\mathbf{X} + A_{0qn}$$

Then, rate of discharge $\mathbf{dQ_N/dt}$ will equal to the corrosion current

$$\mathbf{dQ_n/dt} = \dot{\mathbf{X}}\,[(\,4A_{4qn}\,\mathbf{X^3} + 3A_{3qn}\,\mathbf{X^2} + 2A_{2qn}\,\mathbf{X}\,) + A_{1qn} \quad (8)$$

As **X** = soil factor as per Eq.1, applying the **X** value in Eq. 8,
Then $\mathbf{I_{STRAY}}$:

$$\mathbf{dQ_n/dt} = \dot{\mathbf{X}}\,[4A_{4qn}((1/K_S)\log\rho)^3_{H=10\%}\,\mathbf{(pH.H)^3} + 3A_{3qn}((1/K_S)\log\rho)^2_{H=10\%}\,\mathbf{(pH.H)^2} + 2A_{2qn}((1/K_S)\log\rho)_{H=10\%}\,\mathbf{(pH.H)} + A_{1qn}]$$

Now, for bare pipe segment-soil-earth system under test, without applying any c.p system, without any external interference, at room temperature, with soil volume under test and by neglecting CO_2 effect, the natural corrosion current from pipe surface to the surrounding medium could be obtained from an electrical concept of the corrosion and will equal to third order polynomial equation function of measured humidity and pH of the soil as shown in equation 9.

Natural corrosion current of the bare pipe segment to the surrounding medium I_{STRAY}

$$\mathbf{I_{STRAY}} = \dot{\mathbf{X}}[B_{3qn}\mathbf{(pH.H)^3} + B_{2qn}\mathbf{(pH.H)^2} + B_{1qn}\mathbf{(pH.H)} + A_{1qn}\,\mathbf{]} \quad (9)$$

Where:
pH.H = Variable quantity equal to (pH *Humidity) measured around the pipe segment
$\dot{\mathbf{X}}$ = Rate of soil factor change by time dx/dt = $d(S_f)/dt$
B_{3qn} = Constant print equal to $4A_{4qn}((1/K_S)\log\rho)^3_{at\ H=10\%}$
B_{2qn} = Constant print equal to $3A_{3qn}((1/K_S)\log\rho)^2_{at\ H=10\%}$
B_{1qn} = Constant print equal to $2A_{2qn}((1/K_S)\log\rho)_{at\ H=10\%}$
A_{1qn} = Constant print from natural charge equation
A_{2qn} = Constant print from natural charge equation
A_{3qn} = Constant print from natural charge equation
A_{4qn} = Constant print from natural charge equation
K_S = Constant equal to the dielectric constant of the soil at H = 10%
ρ = Constant equal to the soil resistivity in Ω.m at H = 10%

Referring to the proposed electrical circuit of bare pipe segment-soil-earth system in figures 5, the stray current source may be represented by equation 9. Then, the final proposed electric circuit of such system may be as the circuit diagram shows in figure 6 taking into account that the pipe-soil-earth system is without applying cathodic protection system and without any external interference i.e. natural condition.

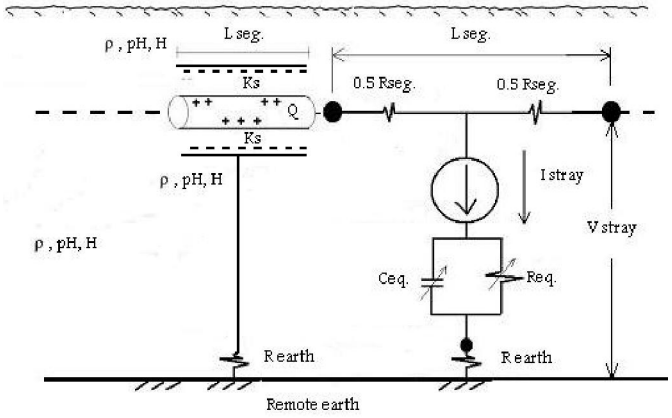


Figure 6: Final proposed electric circuit diagram of bare pipe segment-soil-earth system at natural condition without applying CP system

5. Case 2:

5.1 Proposed Electrical Analogue Circuit Of The Pipe Segment – Soil - Earth System With CP System [1] [2]

In case of cathodic protection process, the protection current either it is greater than the stray current (net current will flow through the pipe) or less than the stray current (net current will flow through the stray capacitor to the remote earth). The c.p level of the pipe line segment could be determined if the protection current before and after the pipe line segment is measured by using the proposed voltage drop canister pigged with an intelligent pig tool [1] [2]. For well coated pipe line segment, the electrical analogue circuit is as in Fig.7a. Also, Fig.7b shows the electric analogue circuit of the pipe segment which is cathodically protected by galvanic system or impressed current system Fig.7c.

The general electrical analogue circuit of pipe segment-soil-earth system will consist of current source in series with an equivalent impedance $Z_{eq.}$ correlated to humidity (R_{eq} in parallel with C_{eq}) which is connected from pipe to the remote earth as shown in figure 8. The importance of this proposed electrical circuit is that it converts both the corrosion and/or cathodic protection process into an electric problem. As we obtained before the electric parameters C, V and Q of the bare pipe segment-soil-earth system in terms of the electrochemical properties of the soil i.e. the general equations of the stray capacitance at many CP levels [6][10], the stray potential at many CP levels [7],[10], the surface total charge at many CP levels [8],[10] and finally the amount of the protection current at many CP levels [9],[10]. All electrical parameters deduced in terms of the soil factor, 4th degree polynomial equations with an average error less than ± 5%. In this paper, we will continue and use the results obtained before to deduce the electric analogue circuit of the natural condition pipe-soil-earth system with applying cathodic protection system.

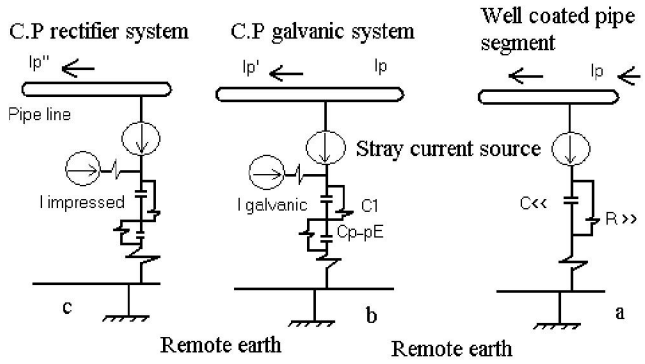


Figure 7: Proposed electrical analogue circuit of the pipe segment-soil system at the cathodic protection process (a) By well coating material (b) By galvanic system (c) By using impressed current system

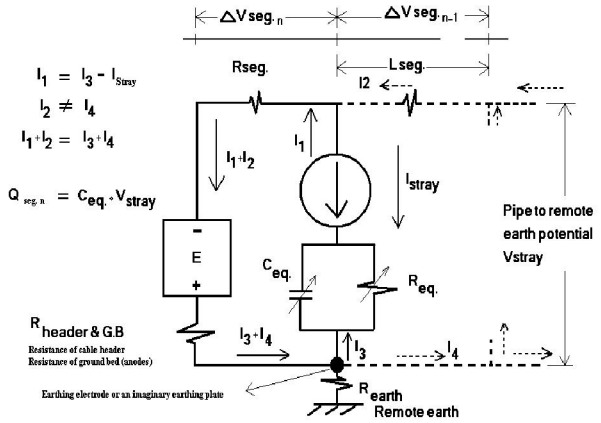


Figure 8: The proposed general electrical analogue circuit of pipe-soil system

5.2 Calculations of The Electrical Parameters Of The Pipe-Soil-Earth system With Applying CP System

Now, to obtain the pipe segment natural electric parameters with applying cathodic protection system, we have for the pipe-soil-earth system the following results:

5.2.1 General Equation Of The Electrolytic Capacitance [6][10]

This is equal to Eq. 10:

$$\mathbf{C_{stray}} = A_{4C}\,\mathbf{X^4} + A_{3C}\,\mathbf{X^3} + A_{2C}\,\mathbf{X^2} + A_{1C}\,\mathbf{X} + A_{0C} \quad (10)$$

Where:
A's: = $A_{(\ )C}$ are the stray capacitance print constants of the pipe soil under test
X = instantaneous value of the electrochemical value of the soil, the soil factor

5.2.2 General Equation Of The Stray Potential [7][11] This is equal to Eq. 11:

$$\mathbf{V_{stray}} = A_{4V}\,\mathbf{X^4} + A_{3V}\,\mathbf{X^3} + A_{2V}\,\mathbf{X^2} + A_{1V}\,\mathbf{X} + A_{0V} \quad (11)$$

Where:
A's = $A_{(\ )V}$ are the natural stray potential print constants of the pipe soil under test
X = instantaneous value of the electrochemical value of the soil, the soil factor

5.2.3 General Equation Of The Surface Charge [9],[10]
This is equal to Eq. 12:

$$\mathbf{Q} = A_{4q}\,\mathbf{X^4} + A_{3q}\,\mathbf{X^3} + A_{2q}\,\mathbf{X^2} + A_{1q}\,\mathbf{X} + A_{0q} \quad (12)$$

Where:
A's: = $A_{(\ )q}$ are the surface charge print constants of the pipe - soil under test
X = instantaneous value of the electrochemical value of the soil, the soil factor

5.2.4 General Equation Of The Protection Current Flow To The Pipe Segment (I_3) [10]
This is equal to Eq. 13:

$$\mathbf{I_3 = I_P} = A_{4I}\,\mathbf{X^4} + A_{3I}\,\mathbf{X^3} + A_{2I}\,\mathbf{X^2} + A_{1I}\,\mathbf{X} + A_{0I} \quad (13)$$

Where:
A's: = $A_{(\ )I}$ are the protection current print constants of the pipe soil under test
X = instantaneous value of the electrochemical value of the soil, the soil factor

5.2.5 Natural Stray Corrosion Current I_{STRAY} [10]
This is equal to Eq. 14:

$$\mathbf{dQ_n/dt} = \dot{\mathbf{X}}\,[\,B_{3qn}\mathbf{(pH.H)^3} + B_{2qn}\mathbf{(pH.H)^2} + B_{1qn}\mathbf{(pH.H)} + A_{1qn}\,] \quad (14)$$

Where:
pH.H = Variable quantity equal to (pH* Humidity) measured around the pipe segment
$\dot{\mathbf{X}}$ = Rate of soil factor change by time dx/dt = $d(S_f)/dt$
B_{3qn} = Constant print equal to $4A_{4qn}((1/Ks)\log\rho)^3_{at\ H=10\%}$
B_{2qn} = Constant print equal to $3A_{3qn}((1/Ks)\log\rho)^2_{at\ H=10\%}$
B_{1qn} = Constant print equal to $2A_{2qn}((1/Ks)\log\rho)_{at\ H=10\%}$
A_{1qn} = Constant print from natural charge equation
A_{2qn} = Constant print from natural charge equation
A_{3qn} = Constant print from natural charge equation
A_{4qn} = Constant print from natural charge equation
Ks = Constant equal to the dielectric constant of the soil at H = 10%
ρ = Constant equal to the soil resistivity in Ω.m at H = 10%

5.2.6 Earthing Resistance

The earthing resistance RE could be easily measured from the field by the use of earth tester.

R_E = Measured from the field
(15)

5.2.7 Pipe Segment Resistance

The resistance of the pipe segment will equal to:

$$R_{SEG.} = (¼\,\pi\,(D_O^2 - D_I^2) * L_{SEG.} * \sigma_{IRON}) \quad (16)$$

Where:
D_O = Outer diameter of the pipe segment
D_I = Inner diameter of the pipe segment
L_{SEG} = Length of the pipe segment
σ_{IRON} = Iron specific resistance (pipe material)

5.2.8 Calculation Of The Net Current Flow Through The Pipe Segment ($I_1 + I_2$) [9]

5.2.8.1Calculation of Pipe Segment Flow Current

As total surface charge is equal to;

$$\mathbf{Q_{surface}} = A_{4q}\,\mathbf{X^4} + A_{3q}\,\mathbf{X^3} + A_{2q}\,\mathbf{X^2} + A_{1q}\,\mathbf{X} + A_{0q}$$

Then, the pipe segment flow current, ($I_1 + I_2$) components as shown in Fig.8 is equal to:
$dQ_{surface}/dt$

$$\mathbf{dQ_{surface}/dt} = \dot{X} \; [\; 4A_{4q}\,\mathbf{X^3} + 3A_{3q}\,\mathbf{X^2} + 2A_{2q}\,\mathbf{X} + A_{1q} \;]$$

From Eq. 1, applying the value of the soil factor as $X = S_f = (1 / K_S)\ pH\ H \log \rho$
Then:

$$\mathbf{dQ_{surface}/dt} = \dot{X}\,[\; 4A_{4q}\,((1 / Ks) \log \rho)^3_{at\ H = 10\%}\mathbf{(pH.H)^3} + 3A_{3q}\,((1 / Ks) \log \rho)^2_{at\ H = 10\%}\mathbf{(pH.H)^2} + 2A_{2q}\,((1 / Ks) \log \rho)_{at\ H = 10\%}\mathbf{(pH.H)} + A_{1q}\;]$$

$$\mathbf{dQ_{surface}/dt} = \dot{X}\,[\; C_{3q}\mathbf{(pH.H)^3} + C_{2q}\mathbf{(pH.H)^2} + C_{1q}\mathbf{(pH.H)} + A_{1q}\;] \quad (17)$$

Now, for bare pipe segment-soil-earth system under test which applying c.p system, without any external interference, at room temperature, with soil volume under test and by neglecting CO_2 effect, the flow current of the pipe segment-soil under test could be obtained from an electrical concept of the corrosion and will equal to third order polynomial equation function of the measured (pH*humidity) as shown in equation 18.

Flow current of the pipe segment under test

$$\mathbf{I_1 + I_2} = \dot{X}\,[C_{3q}\mathbf{(pH.H)^3} + C_{2q}\mathbf{(pH.H)^2} + C_{1q}\mathbf{(pH.H)} + A_{1q}\;] \quad \mathbf{(18)}$$

Where:
pH.H = Variable quantity equal to (pH*Humidity) measured around the pipe segment
$\dot{X}$ = Rate of soil factor change by time $dx/dt = d(S_f)/dt$
C_{3q} = Constant print equal to $4A_{4q}\,((1/Ks) \log \rho)^3_{at\ H=10\%}$
C_{2q} = Constant print equal to $3A_{3q}\,((1/Ks) \log \rho)^2_{at\ H=10\%}$
C_{1q} = Constant print equal to $2A_{2q}\,((1/Ks) \log \rho)_{at\ H=10\%}$
A_{1q} = Constant print from surface charge equation
A_{2q} = Constant print from surface charge equation
A_{3q} = Constant print from surface charge equation
A_{4q} = Constant print from surface charge equation
Ks = Constant equal to the dielectric constant of the soil at H = 10%
ρ = Constant equal to the soil resistivity in Ω.m at H = 10%

5.2.8.1Calculation of the Current Reaches the Pipe Segment from d-c Source (I_3)

We have: Total surface charge $\mathbf{Q_{surface}}$ = charge supplied by cathodic protection $\mathbf{Q_{C.P}}$ – natural charge

i.e $\mathbf{Q_{surface} = Q_{C.P} - Q_n}$

Then:

$$\mathbf{dQ_{CP}/dt} = \mathbf{dQ_{surface}/dt} + \mathbf{dQ_n/dt}$$

As:

$$\mathbf{dQ_n/dt} = \dot{X}\,[\; B_{3qn}\mathbf{(pH.H)^3} + B_{2qn}\mathbf{(pH.H)^2} + B_{1qn}\mathbf{(pH.H)} + A_{1qn}\;] = \mathbf{I_{stray}}$$
from Eq.(9)

$$\mathbf{dQ_{surface}/dt} = \dot{X}\,[\; C_{3q}\mathbf{(pH.H)^3} + C_{2q}\mathbf{(pH.H)^2} + C_{1q}\mathbf{(pH.H)} + A_{1q}\;] = \mathbf{I_1 + I_2}$$
from Eq.(17)

$$\mathbf{I_1} = \mathbf{I_3} - \mathbf{I_{stray}}$$

$$\mathbf{I_{DC}} = \mathbf{I_3} + \mathbf{I_4}$$

In our case of pipe segment study, $I_4 = 0$ & $I_2 = 0$
Then $\mathbf{I_{DC}}$ will equal to:

$$\mathbf{dQ_{CP}/dt} = \dot{X}\,[\; (B_{3qn}+C_{3q})\,\mathbf{(pH.H)^3} + (B_{2qn}+C_{2q})\,\mathbf{(pH.H)^2} + (B_{1qn}+C_{1q})\,\mathbf{(pH.H)} + (A_{1qn} + A_{1q})\;]$$

$$\mathbf{dQ_{CP}/dt} = \dot{X}\,[\; D_3\mathbf{(pH.H)^3} + D_2\mathbf{(pH.H)^2} + D_1\mathbf{(pH.H)} + D_0\;]$$

Now, for bare pipe segment-soil-earth system under test which applying c.p system, without any external interference, at room temperature, with soil volume under test and by neglecting CO_2 effect, the amount of rectifier current reaches the pipe – soil system under test could be obtained from an electrical concept of the corrosion and will equal to third order polynomial equation function of the measured (pH*humidity) as shown in Eq.19.

Amount of rectifier current reaches the pipe segment under test

$$\mathbf{I_{DC}} = \dot{X}\,[D_3\mathbf{(pH.H)^3} + D_2\mathbf{(pH.H)^2} + D_1\mathbf{(pH.H)} + D_0] \quad (19)$$

Where:
pH.H = Variable quantity equal to (pH* Humidity) measured around the pipe segment
$\dot{X}$ = Rate of soil factor change by time $dx/dt = d(S_f)/dt$
D_3 = Constant equal to $(B_{3qn}+C_{3q}) = 4(A_{4q} + A_{4qn})\,((1/Ks) \log \rho)^3_{at\ H=10\%}$

D_2 = Constant equal to $(B_{2qn}+C_{2q}) = 3(A_{3q} + A_{2qn})$ $((1/Ks) \log \rho)^2$ at H=10%

D_1 = Constant equal to $(B_{1qn}+C_{1q}) = 2(A_{2q} + A_{2qn})$ $((1/Ks) \log \rho)$ at H=10%

D_0 = Constant equal to $(A_{1qn} + A_{1q})$

Ks = Constant equal to the dielectric constant of the soil at H = 10%

ρ = Constant equal to the soil resistivity in Ω.m at H = 10%

Referring to the proposed electrical circuit of the bare pipe segment-soil-earth system in figure 8, all current values are now determined for the pipe segment-soil-earth system under test. Also the values of the equivalent stray electrolytic capacitor, the potential across it, the natural stray current source from the pipe segment, pipe segment net current flow and finally the amount of DC current share for the pipe segment are determined. Then, the final proposed electric circuit of such system may be as the electrical circuit diagram shown in figure 9a & 9b.

The electrical parameters of the bare pipe segment-soil-earth system under test with applying CP system are as follow:

$$C_{eq.} = C_{stray} = A_{4cn}\mathbf{X^4} + A_{3cn}\mathbf{X^3} + A_{2cn}\mathbf{X^2} + A_{1cn}\mathbf{X} + A_{0cn}$$

$$V_{stray} = A_{4v}\mathbf{X^4} + A_{3v}\mathbf{X^3} + A_{2v}\mathbf{X^2} + A_{1v}\mathbf{X} + A_{0v}$$

$$I_{stray} = \dot{\mathbf{X}} [B_{3qn}(\mathbf{pH.H})^3 + B_{2qn}(\mathbf{pH.H})^2 + B_{1qn}(\mathbf{pH.H}) + A_{1qn}]$$

$$I_1 = \dot{\mathbf{X}} [C_{3q}(\mathbf{pH.H})^3 + C_{2q}(\mathbf{pH.H})^2 + C_{1q}(\mathbf{pH.H}) + A_{1q}]$$

$$I_{DC} = I_3 = \dot{\mathbf{X}} [D_3(\mathbf{pH.H})^3 + D_2(\mathbf{pH.H})^2 + D_1(\mathbf{pH.H}) + D_0]$$

$I_2 = I_4 = 0$ for pipe segment under test

Where:

A's, B's, C's and D's: are the PRINT constants of the bare pipe-soil-earth system

X : instantaneous value of the electrochemical value of the soil, the soil factor (Eq.1)

$\dot{\mathbf{X}}$ = Rate of soil factor change by time d**x**/dt = $d(S_f)/dt$

H: is the measured humidity

pH: is the measured power of hydrogen

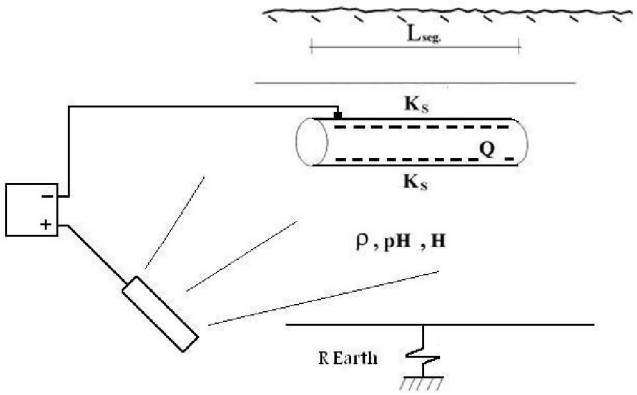


Figure 9a: shematic diagram of buried bare pipe segment with applying cathodic protection

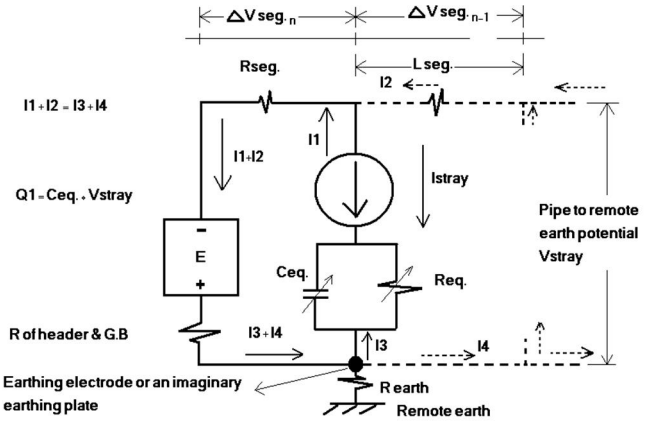


Figure 9b: The final proposed electric circuit diagram of bare pipe segment – soil – earth system with cathodic protection system

6. Circular ΔV PIG Idea: [1][2]

This is a new idea of the voltage drop technique to measure the protection current I_P passed through the buried pipeline. By considering a pipe line with total length L m, if such length is divided into segments with length L m./segment

Then: Total length L = segment length L_{Seg} x number of segments n

Electrically, the pipe line could be considered as: total resistance = segment resistance × n as shown in Fig.10.

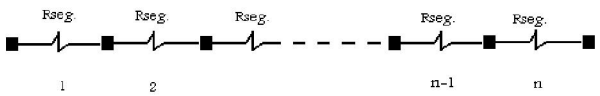


Figure 10: Electrical analogue resistance of the total pipe line length

Now if the voltage between points a & b of the segment is measured, as shown in figure 11, then the

instantaneous measured protection current will equal to:

$$Ip = \frac{\Delta V}{R_{seg.}}$$

That means that an additional circular voltage drop canister could be added with the available intelligent pig to measure the protection current I_P. Figure 11 shows such canister, and in the meantime by using GPS technology to determine the segment position.

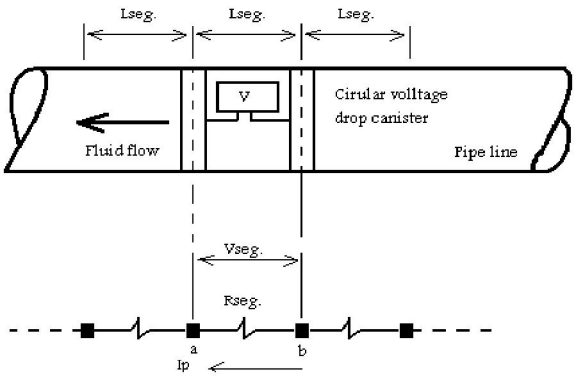


Figure 11: Idea of voltage drop canister to be pigged with the fluid through the pipeline

By the use of this voltage drop canister which pigged with the intelligent pig and by the use of GPS system, each segment flow current I_P could be measured. Then by measuring the humidity around this pipe segment, the soil factor could be determined. Finally, from the ONION curves obtained before [9] (which correlate I_P, S_F and $V_{H.C}$), the equivalent pipe to soil potential of this buried pipe segment could be determined without the need of test point and without the need of $Cu/CuSO_4$ half cell. The most important result is that: the pipe to soil potential of any buried pipeline could be obtained segmental along its route without the need of any test points.

7. Conclusion

The behavior of the electrical parameters of the pipe-soil-earth system during the change of the electrochemical properties of the soil could be plotted in electrical parameters PRINT which will be always valid in all times as the pipe-soil system is maintained and without any external interference. Once the system is changed by replacement another pipe with different dimension and/or the replacement of the soil, there will be another new electrical parameters PRINT for the new pipe-soil-earth system. Also, the buried pipe line segment with soil surrounding medium could be simulated electrically by an electric circuit where the system is subjected to the law: (charge = capacitance × volt) between the pipe surface and the remote earth. This is where each of circuit electric parameter could be obtained by an equation as a function of the measured electrochemical properties of the soil (soil factor), 4th degree polynomial at room temperature but the A's constants are different for each electric quantity .The constants of each equation (A's) considered to be as a PRINT of such pipe-soil-earth system and valid until pipe and/or soil is changed with of course new print values. For buried bare pipe segments in different kind of soils at different cathodic protection level, the PRINTS of the electrolytic stray capacitor between pipe & earth, the stray potential across the stray capacitance, surface charge and the protection current of the cathodic protection system passed through the pipe segment were obtained in terms of the new parameter, the soil factor. The useful of these prints is to obtain complete electrical data correlated with many cathodic protection levels which help, after complete erection of the pipeline, in defining the c.p level (pipe to soil potential) of any pipe line segment through it's length by measuring the protection current and calculating the soil factor at the pipe segment from direct field measurements. Not only has that but also to define the most suitable route of the pipe line, before the erection process, which generates the minimum surface charge. The error of electric parameters equations reduced to be less than ± 5%. The most important advantage of such electrical analogue circuit of pipe segment-soil-earth system is the possibility to simulate a complete pipeline – soil system by an electric circuit and to convert both the corrosion and cathodic protection problems of the pipeline to an electric problem. This will help in corrosion monitoring and the maintenance of c.p systems. The most important result is that: the pipe to soil potential of any buried pipeline could be obtained segmental along its route without the need of any test points. This is by the use of the new electric concept of pipe-soil-earth system.

Acknowledgement

First and foremost, thanks to GOD the most kind, the most merciful and to whom any success is related.

8. References:

1. Ashraf Abdelraouf M. Fouad Ahmed, A Proposed Systematic Concept to Evaluate the Performance of Cathodic Protection of Buried Pipe Line in the Soil, The Egyptian Society of Chemical Engineers, **TESCE**, Cairo, Egypt, 2003
2. Ashraf Abdelraouf M. Fouad Ahmed, A Proposed Systematic Concept to Evaluate the Performance of Cathodic Protection of Buried Pipe Line in the Soil, International conference "Future Vision and Challenges for Urban Development", **HBRC** Cairo, Egypt, 2004
3. Ashraf Abdelraouf M. Fouad Ahmed, The General Equation of the Natural Stray Capacitance between External Surface of Bare Pipe Segment and Earth, 25th Annual Conference Corrosion Problems In Industry, Egyptian Corrosion Society, **ECS** 2006.
4. Ashraf Abdelraouf M. Fouad Ahmed, The General Equation of the Natural Potential between External Surfaces of Bare Pipe Segment to Earth, 25th Annual Conference Corrosion Problems In Industry, **ECS** 2006.
5. Ashraf Abdelraouf M. Fouad Ahmed, Deduction of The Natural Created Charge General Equation For A Buried Bare Pipe Line In The Soil, 25th Annual Conference Corrosion Problems In Industry, ECS 2006.
6. Ashraf Abdelraouf M. Fouad Ahmed, The General Equation and The Print Curves of The Stray Potential of a Cathodically Protected Buried Bare Pipe Segment, Ain- Shams University third international conference on environmental engineering , **ASCEE-3**, 2009.
7. Ashraf Abdelraouf M. Fouad Ahmed, The General Equation and The Print Curves of The Stray Capacitance of a Cathodically Protected Buried Bare Pipe Segment, Ain- Shams University third international conference on environmental engineering, **ASCEE-3**, 2009.
8. Ashraf Abdelraouf M. Fouad Ahmed, The General Equation and The Print Curves of The Total Surface Charge of a Cathodically Protected Buried Bare Pipe Segment, Ain-Shams University third international conference on environmental engineering, **ASCEE-3**, 2009.
9. Ashraf Abdelraouf M. Fouad Ahmed, The General Equation and The Print Curves of The Net Current Flow of a Cathodically Protected Buried Bare Pipe Segment, Ain-Shams University, ASCEE-**3**, 2009.
10. Ashraf Abdelraouf M. Fouad Ahmed, Experimental Natural Prints And The Re-calculated General Equations Of The Electrical Parameters For Buried bare Pipe-Soil-Earth System With And Without Applying Cathodic Protection System, Under publication (J. American Science).

6/6/2010

Molecular Markers for New Promising Drought Tolerant Lines of Rice under Drought Stress *via* RAPD-PCR and ISSR Markers

Youssef; M. A., Mansour A. and Solliman S. S

Genetics Department, Faculty of Agriculture, Zagazig University, Zagazig, Egypt, bakr2000us@yahoo.com

Abstract: Random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) and inter simple sequence repeats (ISSRs) markers were performed to detect the genetic diversity among 6 new rice lines and 4 cultivars with different responses to drought tolerance and establish specific DNA markers associated with drought tolerance. Among 16 RAPD primers tested, only 5 produced bands polymorphic between lines with an average of 5.2 bands per primer (ranging from approximately 252 to 1232 bp) and 73.02 % were polymorphic. Among the tested ISSR primers, only five amplified polymorphic ISSR loci with an average number of 4.4 bands per primer (ranging from approximately 80 to 813 bp) and the mean percentage of ISSR polymorphism was 90.91. Based on band polymorphisms generated by RAPD-PCR and ISSR after using the primers, the highest similarity value (0.93) was found between P-5-3-b line and P-5-3-a line and the lowest value (0.44) was found between P-5-3-b line and Giza 172. The dendrogram separated all cultivars and new lines into two clusters and indicated that the cross of tolerant line (P-5-3-b) and susceptible cultivar (Giza 172) is suggested as the most suitable cross for drought tolerance analysis studies as they have the lowest similarity value (0.44) and also grouped in distinct cluster. Since two fragments of about (315 and 505 bp) were visualized using HP15 primer in the genomic DNA of the drought tolerant lines while were absent in the sensitive cultivars, they can be considered as positive drought tolerant markers. [Journal of American Science 2010;6(12):355-363]. (ISSN: 1545-1003).

Key words: RAPD-PCR, ISSRs, rice, drought stress, dendogram.

1. Introduction:

Rice (*Oryza sativa*), one of the important food crops, is grown on 154 million hectares world-wide in a wide range of environments and about 45% of the world's rice is cultivated in rainfed ecosystems (Nazari and Pakniyat, 2008). These areas often experience severe water deficits due to low and uneven rainfall distribution patterns and yields are largely reduced by drought. Drought stress is a serious limiting factor to rice production and yield stability in rainfed areas and 18 million tons of rice valued at US$ 3600 millions is lost annually to drought (Ribaut and Poland, 1999). Development of drought resistant cultivars will considerably improve rainfed rice production. However, little progress has been made in improving the genetic potential of rice for drought resistance because lack of phenotyping facilities to precisely screen large germplasm for drought resistance, inherent variation in the field and only one experimentally droughted crop per year (Ribaut *et al*., 1997).

In Egypt, the cultivated varieties require large amount of water irrigation (16500 m3/ha).The available amount of irrigation water from River Nile is not only limited (55.5 million m3/year) but liable to decrease year after year due to competition between the agriculture, industry and human consumption in the fixed amount of water from River Nile, in addition to the competition between groove countries of River Nile. As well as about 15.20% from rice areas was suffering a decreasing of yield due to short of water (Mahasson *et. al*, 1999). Accordingly, the future of rice cultivation in Egypt depends upon breeding for drought resistance because the cultivated varieties (lowland) require large amount of water and susceptibility of water deficits. The first trial for breeding drought tolerance in Egypt initiated at 1986 by ours obtaining on rice breeding for drought stress project (NARP No. 329). Investigations at this project included on genetical and physiological studies on the drought tolerance related characters as well as grain yield and its components (Soliman 1993a, Soliman 1993b). The effect of drought stress on disease infections and quality characters susceptible and drought tolerance genotypes were studied also (Abou-zaid *et. al*, 1993**,** Wafaa *et. al*, 1998). In the same time the breeding was done and we obtained on new promising drought tolerant lines of rice. These lines were evaluated for grain yield and its attributes as well as quality characters (Tables 1 and 2).cultivation of new drought tolerant lines in the large scale at sandy soil (light) by using microjectsprinkler irrigation as well as cultivation of its lines under heavy soil on large scale (Ghazi and Soliman, 2008). The findings of these experiments confirmed that the importance of these lines for solving of rice cultivation in Egypt and gave higher grain yield under drought stress than under normal irrigation (traditional method).

Molecular tools facilitate the identification of genomic locations linked to traits of interest and help

in indirect selection of such complex traits without the need for difficult phenotypic measurements. In the last few decades, new DNA molecular markers, based on the PCR technique, such as random amplified polymorphic DNA (RAPD; Williams *et al*. 1990) and inter simple sequence repeats (ISSRs; Zietkiewicz *et al*. 1994), among others, have become excellent tools for plant breeders (Lima-Brito *et al*. 2006). When there is insufficient information about the genome sequence of a wild species, or there are economic constraints, one of the most adequate marker systems is RAPD amplification (Lima-Brito *et al*. 2006). This technique gives fast results but also has limitations, such as dependence on the genetic background, low reproducibility, and level of polymorphism obtained (Zietkiewicz *et al*. 1994; Godwin *et al*. 1997 and Fern´andez *et al*. 2002). In contrast to RAPD amplification, the ISSR markers are more feasible and reproducible (Godwin *et al*. 1997), and the distribution of ISSRs in the eukaryotic genome makes them highly informative (Tautz and Renz 1984). They are also highly polymorphic and their use is cost effective, requiring no prior information of the sequence (Bornet *et al*. 2002). In cereals, ISSR markers have been used to study genetic diversity and phylogenetic relationships (Kantety *et al*. 1995; Matos *et al*. 2001 and Fern´andez *et al*. 2002), for gene mapping (Kojima *et al*. 1998), for gene tagging in molecular assisted selection (Akagi *et al*. 1996 and Kaushik *et al*. 2003), and for DNA fingerprinting (Carvalho *et al*. 2005).

The objectives of this study were to use RAPD-PCR and ISSR markers to assess genetic diversity and identification of 6 new rice lines and 4 cultivars with different responses to drought tolerance by comparison of local cultivation and establish specific DNA markers associated with drought tolerance using RAPD-PCR and ISSR markers to assessed of breeders to selection drought tolerant genotypes on the molecular level at the laboratory only subsequently acceleration and facilitates of drought breeding programs.

2. Materials and Methods

This work was carried out in Molecular Genetics Lab. Genetic Dept., Fac. of Agric., Zagazig Univ.

Plant materials:

Six new drought tolerant lines and four sensitive cultivars were used in this study under drought stress (Table 3).

RAPD and ISSR amplification

Total genomic DNA was extracted from young leaves by the CTAB (cethyltrimethylammonium bromide) method followed by an RNase-A treatment (Sigma, St. Louis, MO; R-4875) for 30 min at 37°C.

Primers:

A set of sixteen 10-mer oligonucleotides was analyzed for RAPD-PCR and a total of twenty primers were tested for ISSR. Based on the accurate amplified bands profiles and the produced polymorphic patterns of DNA fingerprinting selected five different primers were chosen for RAPD-PCR and another five primers for ISSR (Table 4).

RAPD- PCR reactions

The RAPD amplification reactions were carried out in 50 µl containing 20 ng/µl of template DNA, 10× buffer (NH4)2SO4; Fermentas, St. Leon-Rot, Germany), 2.5mM MgCl2 (Fermentas), 2.5mM dNTPs, 0.25 µM primer and 1 Unit *Taq* DNA polymerase (Fermentas). The RAPD amplifications occurred under the following conditions: an initial denaturation step at 94◦C for 7 min and 30 cycles at 94◦C for 1 min, 35◦C for 1 min and 72◦C for 2 min; the final elongation step was at 72◦C for 6 min.

ISSR- PCR reactions

The ISSR amplification reactions were carried out in 25µl per tube, containing 2µl DNA (20 ng), 1 unit of *Taq* DNA polymerase enzyme, 2µl 10X buffer, 2 µl $MgCl_2$ (25 mM), 2µl $dNTP_s$ (2.5 mM of each), 2 µl primer (10 pmol) and 14.8µl H_2O. The following conditions were used for ISSR amplifications: an initial denaturation step of 94◦C for 5 min, followed by 45 cycles of denaturation at 94◦C for 30 s, a primer annealing step at 52◦C for 45 s, and an extension at 72◦C for 2 min; then a final extension was carried out at 72◦C for 5 min. The annealing temperature varied according to the melting temperature of each primer.

Both RAPD and ISSR amplification reactions were carried out on a Perkin-Elmer Gene Amp PCR system (model 2400), and each reaction was repeated twice.

Band analysis:

The reaction products were analyzed by electrophoresis on 1.4% agarose gels, stained with ethidium bromide, and photographed under UV transilluminator by digital camera with UV filter adaptor. The synthetic DNA, ladder 100 bp (Pharmacia) was employed as molecular markers for bands molecular weight. Each amplified band profile was defined by the presence or absence of bands at particular positions on the gel. Profiles were considered different when at least one polymorphic band was identified. Fragments were scored as 1 if

present or 0 if absent based on standard marker using GelAnalyzer 3 (Egygene) software. Pairwise combinations, genetic similarity and genetic distances were estimated following Lynch (1990 and 1991). The computer package SPSS was used to construct a dendrogram based on the matrix of distance using Unweighted Pair Group Method with Arithmetic averages (UPGMA) (Sneath and Sokal 1973).

3. Results:

RAPD and ISSR analysis

The total number of amplified fragments, number of monomorphic fragments, number of polymorphic fragments and percentage of polymorphism obtained per each RAPD and ISSR primer are shown in table (5). Among the 16 RAPD primers tested, only 5 produced bands polymorphic between lines. An average of 5.2 bands per primer was amplified (ranging from approximately 252 to 1232 bp) and 73.02 % were polymorphic. The oligonucleotide OPA-05 and OPA-11 presented the highest percentage of RAPD polymorphism (100 %; table 5). OPB-10 oligonucleotides, presented one unique band (665 bp) to Giza177 cultivar and OPD-07 oligonucleotides, presented one unique band (589 bp) to Giza 159 cultivar while, OPA-11 oligonucleotides, presented two unique bands (252 and 292 bp) to P-2-1-2-1 line and P-58-1-2 line respectively (Table 6).

Among the tested ISSR primers, only five amplified polymorphic ISSR loci. An average number of 4.4 bands per primer were amplified (ranging from approximately 80 to 813 bp) and the mean percentage of ISSR polymorphism was 90.91 (table 5). The oligonucleotide HP12 amplified the highest number of ISSR loci (6 bands) but primers HP9, HP12 and HP14 gave the highest percentage of polymorphism (100 %; table 5). HP12 oligonucleotides, presented two unique bands (519 and 773 bp) to P-5-3-b line while, HP14 and HP15 oligonucleotides, presented one unique band (783 and 453 bp) respectively to P-72-11-1-1 line (Table 7).

The patterns obtained with HP15 oligonucleotide for lines suggested that this primer has the ability to produce drought tolerant markers. Since two fragments of about (315 and 505 bp) were visualized using HP15 oligonucleotide in the genomic DNA of the drought tolerant lines while were absent in the sensitive lines, they can be considered as positive drought tolerant markers (Table 7 and Figure 1).

Phylogenetic relationship among new drought tolerant lines and local cultivars (Susceptible) based on amplified RAPD-PCR and ISSR fragments (bands):

The similarity coefficient values among all cultivars and new lines based on band polymorphisms generated by RAPD-PCR and ISSR after using the primers are presented in Table (8). The highest similarity value (0.93) was found between P-5-3-b line and P-5-3-a line and the lowest value (0.44) was found between P-5-3-b line and Giza 172.

The dendrogram of genetic distances among all tested genotypes based on band polymorphisms generated by RAPD-PCR and ISSR after using the primers is shown in (Fig. 2). The dendrogram separated all cultivars and new lines into two clusters. First cluster divided into two subclusters, first subcluster included P-58-1-2, P-5-3-b, P-5-3-a and second subcluster formed a separate subcluster with P-58-1-2-1. Second cluster was further divided into two subclusters. Among the two subclusters, first subcluster formed a separate subcluster with P-2-1-2-1, Sakha 104 and Giza177and second subcluster included Giza172, Giza159 and P-72-11-1-1.

Table (1): The average mean of quality characters and days to heading for new promising drought tolerant lines and local varieties of rice under drought stress.

	Day heading	Protein content	Milling characters			
			Brown rice	Brain and germ	Hask	White rice
P-58-1-2	96.0	9.81	68.0	9.9	32.0	58.1
P-5-3-b	83.0	10.43	66.5	10.3	33.5	56.3
P-5-3-a	78.0	10.22	65.3	10.1	34.7	55.2
58-1-2-1	95.0	9.60	67.5	10.2	32.5	57.3
P-2-1-2-1	82.0	8.34	79.10	8.0	21.0	71.0
Sakha 104	93.0	6.50	64.5	9.0	35.5	55.5
G-177	85.0	6.20	64.0	8.7	36.0	55.3
G-172	102.0	6.60	67.0	8.6	33.0	58.4
G-159	105	6.10	65.0	8.8	35.0	56.2
P-72-11-1-1	38.0	7.93	64.4	8.2	35.6	56.2
L.S.D 0.05	6.5	1.5	4.5	1.5	3.5	5.5

Table (2): The average mean of grain yield per plant and its attributes for new promising drought tolerant lines and local varieties of rice under drought stress.

	Culm length	Panacle length	No. of tillers/plant	100 Grain weight	Grain yield/plant
P-58-1-2	124.0	27.0	11.0	4.2	33.5
P-5-3-b	90.2	24.0	22.0	1.95	40.4
P-5-3-a	78.0	23.3	13.0	2.1	25.8
58-1-2-1	120.0	27.2	10.5	3.95	32.6
P-2-1-2-1	80.5	24.8	20.5	2.15	42.2
Sakha 104	110.5	22.0	8.5	1.92	26.2
G-177	115.5	22.5	7.6	1.98	24.5
G-172	120.0	21.0	6.8	1.85	20.6
G-159	120.0	20.0	7.0	1.78	19.5
P-72-11-1-1	79.2	24.4	19.4	1.8	40.6
L.S.D 0.05	16.366	3.793	7.714	0.533	11.57

Table (3): The Six new drought tolerant lines and the four sensitive cultivars which used in this study and its pedigree.

1	Cultivar Code	Cultivar Name	Code of tolerance	Pedigree
1	P-58-1-2	New line	Drought Tolerant	Selected line from (Gisa 159 x IET 1444)
2	P-5-3-b	New line	Drought Tolerant	Selected line from IR 4786-13-2-1 after treated by EMS 0.5%
3	P-5-3-a	New line	Drought Tolerant	Selected line from IR 4786-13-2-1 after treated by EMS 0.5%
4	P-58-1-2-1	New line	Drought Tolerant	Selected line from (Gisa 159 x IET 1444)
5	P-2-1-2-1	New line	Drought Tolerant	Selected line from (arbida x bluebell)
6	Sakha 104	Cultivar	Sensitive	Local modern Egyptian cultivar, salt tolerance
7	Giza177	Cultivar	Sensitive	Local modern Egyptian cultivar, salt tolerance
8	Giza 172	Cultivar	Sensitive	Local Egyptian variety
9	Giza 159	Cultivar	Sensitive	Local Egyptian variety
10	P-72-11-1-1	New line	Drought Tolerant	Selected line from Moroerkan after treated by γ 25 Rad

Table (4): Sequence and operon codes of the RAPD and ISSR primers used to detection of variation in different new drought tolerant lines and local varieties

Primer codes	Sequence (5^{-} to 3^{-})
RAPD	
OPA-05	AGG GGT CTT G
OPA-11	CAA TCG CCG T
OPB-10	CTG CTG GGA C
OPC-02	GTG AGG CGT C
OPD-07	TTG GCA CGG G
ISSR	
HB9	(GT)6GG
HB12	(CAC)3GC
HB13	(GAG)3GC
HB14	(CTC)3GC
HB15	(GTG)3GC

Table (5): Number of monomorphic fragments, polymorphic fragments and percentage of polymorphism obtained per each RAPD and ISSR primer for all cultivars and new lines

Primers	Range of fragment sizes (bp)	Total No. of fragments	Monomorphic fragments	Polymorphic fragments	Polymorphism %
RAPD					
OPA-05	276-788	5	0	5	100
OPA-11	252-339	5	0	5	100
OPB-10	524-1232	4	1	3	75
OPC-02	414-896	8	3	5	62.5
OPD-07	474-1004	4	3	1	25
Total	252-1232	26	7	19	73.02
Average		5.2	1.4	3.8	
ISSR					
HP9	79-181	3	0	3	100
HP12	343-813	6	0	6	100
HP13	506-748	3	1	2	66.67
HP14	287-783	5	0	5	100
HP15	251-505	5	1	4	80
Total	79-813	22	2	20	90.91
Average		4.4	0.4	4	

Table (6): RAPD Primers, molecular weight (bp), monomorphic, polymorphic and unique bands for 6 new promising drought tolerant lines and 4 local cultivars of rice.

Primer	M.W (pb)	P-58-1-2	P-5-3-b	P-5-3-a	P-58-1-2-1	P-2-1-2-1	Sakha 104	Giza177	Giza 172	Giza 159	P-72-11-1-1	Polmorphism
OPA-05	788	0	0	0	0	1	1	1	1	1	1	Polymorphic
	676	1	1	1	0	0	0	0	0	0	0	Polymorphic
	633	0	0	0	0	0	0	0	1	1	1	Polymorphic
	309	0	0	0	0	1	1	1	1	1	1	Polymorphic
	276	1	1	1	0	0	0	0	0	0	0	Polymorphic
OPA-11	339	0	0	0	0	1	1	1	1	0	0	Polymorphic
	317	0	0	0	0	0	0	0	0	1	1	Polymorphic
	313	0	1	1	1	0	0	0	0	0	0	Polymorphic
	292	1	0	0	0	0	0	0	0	0	0	Unique
	252	0	0	0	0	1	0	0	0	0	0	Unique
OPB-10	1232	0	0	0	1	0	0	0	0	1	0	Polymorphic
	881	0	1	1	1	1	0	1	0	1	1	Polymorphic
	665	0	0	0	0	0	0	1	0	0	0	Unique
	524	1	1	1	1	1	1	1	1	1	1	Monomorphic
OPC-02	896	1	1	1	1	1	1	1	1	1	1	Monomorphic
	786	0	0	0	0	0	0	0	1	1	1	Polymorphic
	744	1	1	1	1	1	1	1	0	0	0	Polymorphic
	706	0	0	0	0	0	0	0	1	1	0	Polymorphic
	682	0	1	0	1	0	0	0	0	0	0	Polymorphic
	629	0	1	0	1	0	0	0	1	1	0	Polymorphic
	560	1	1	1	1	1	1	1	1	1	1	Monomorphic
	414	1	1	1	1	1	1	1	1	1	1	Monomorphic
OPD-07	1004	1	1	1	1	1	1	1	1	1	1	Monomorphic
	741	1	1	1	1	1	1	1	1	1	1	Monomorphic
	589	0	0	0	0	0	0	0	0	1	0	Unique
	474	1	1	1	1	1	1	1	1	1	1	Monomorphic
Total		11	14	12	13	13	11	13	14	17	13	

Table (7): ISSR Primers, molecular weight (bp), monomorphic, polymorphic and unique bands for 6 new promising drought tolerant lines and 4 local cultivars of rice.

Primer	M.W (pb)	P-58-1-2	P-5-3-b	P-5-3-a	P-58-1-2-1	P-2-1-2-1	Sakha 104	Giza177	Giza 172	Giza 159	P-72-11-1-1	Polymorphism
HP9	181	0	1	1	0	0	0	0	0	0	0	Polymorphic
	160	0	0	0	0	0	0	1	1	0	0	Polymorphic
	79	1	0	0	0	0	0	0	1	0	0	Polymorphic
HP12	813	0	0	0	1	1	1	1	1	1	1	Polymorphic
	773	0	1	0	0	0	0	0	0	0	0	Unique
	621	0	0	0	0	0	1	1	1	0	0	Polymorphic
	519	0	1	0	0	0	0	0	0	0	0	Unique
	512	0	0	0	1	1	1	1	1	1	1	Polymorphic
	343	0	0	0	1	1	1	1	1	1	1	Polymorphic
HP13	748	1	1	1	1	1	1	1	1	1	1	Monomorphic
	566	0	0	0	1	1	1	0	1	1	1	Polymorphic
	506	0	0	0	1	1	1	0	1	1	1	Polymorphic
HP14	783	0	0	0	0	0	0	0	0	0	1	Unique
	665	0	0	0	0	1	0	0	0	0	1	Polymorphic
	432	1	1	1	1	1	0	0	1	1	1	Polymorphic
	355	0	1	1	0	0	0	0	0	0	0	Polymorphic
	287	0	1	1	1	1	0	0	1	1	1	Polymorphic
HP15	505	1	1	1	1	0	0	0	0	0	1	Positive molecular marker
	454	0	0	0	0	0	0	0	0	0	1	Unique
	401	1	1	1	1	0	1	0	1	1	0	Polymorphic
	313	1	1	1	1	0	0	0	0	0	1	Positive molecular marker
	251	1	1	1	1	1	1	1	1	1	1	Monomorphic
Total		7	11	9	12	10	9	7	13	10	14	

Table (8): The similarity coefficient values among all cultivars and new lines based on band polymorphisms generated by RAPD-PCR and ISSR after using the primers

Cultivar	P-5-3-b	P-5-3-a	P-58-1-2-1	P-2-1-2-1	Sakha 104	Giza177	Giza 172	Giza 159	P-72-11-1-1
P-58-1-2	0.79	0.86	0.67	0.58	0.65	0.58	0.54	0.47	0.53
P-5-3-b		**0.93**	0.74	0.55	0.51	0.51	**0.44**	0.47	0.49
P-5-3-a			0.74	0.61	0.58	0.58	0.47	0.51	0.56
P-58-1-2-1				0.74	0.71	0.63	0.63	0.74	0.69
P-2-1-2-1					0.86	0.82	0.75	0.75	0.81
Sakha 104						0.86	0.82	0.72	0.70
Giza177							0.72	0.65	0.67
Giza 172								0.82	0.74
Giza 159									0.81

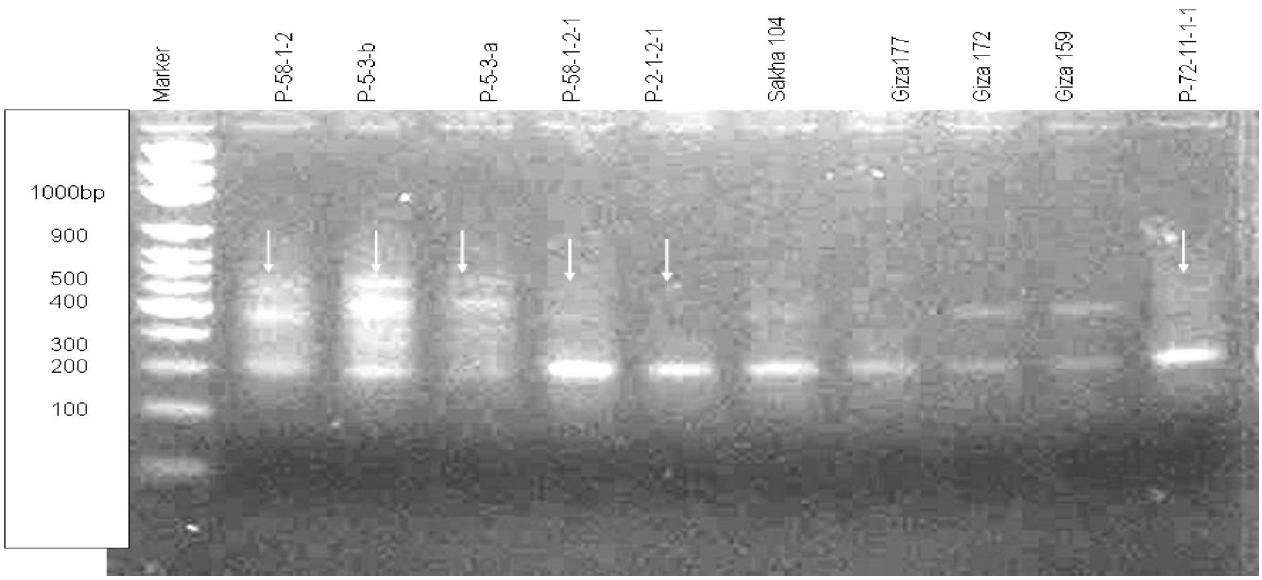


Fig. (1): Results of ISSR amplification based on the use of HP15 primer in the six new drought tolerant lines and the four sensitive cultivars

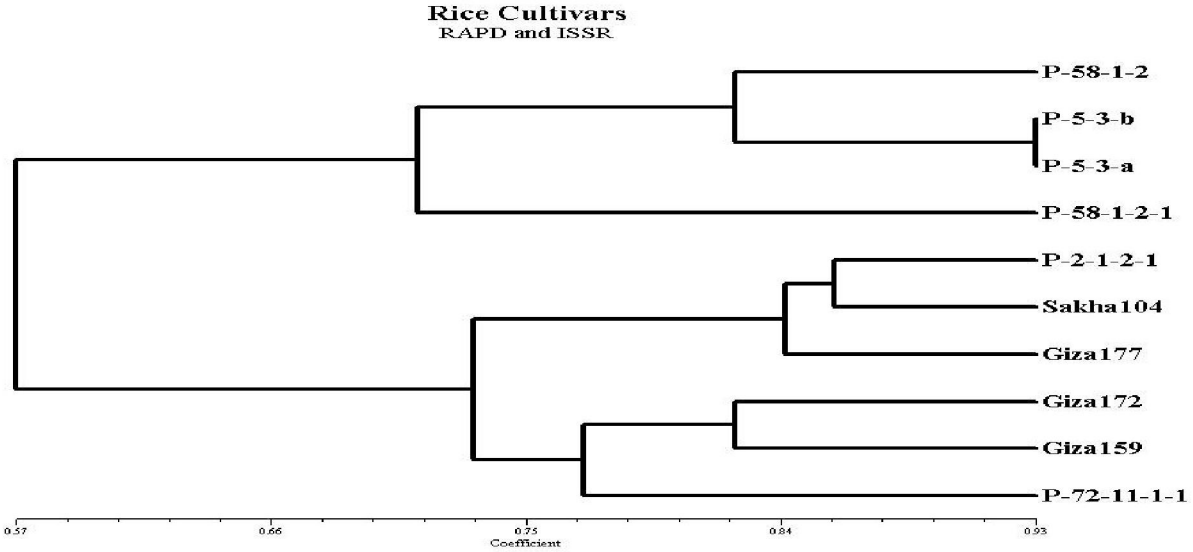


Fig. (2): The dendrogram of genetic distances among all tested genotypes based on band polymorphisms generated by RAPD-PCR and ISSR after using the primers.

4. Discussion

Molecular characterization revealed 73.02 % polymorphism of RAPD markers and 90.91 % polymorphism of ISSR markers between lines (table 5). The difference is perhaps explained by the difference in the DNA segments targeted by the two methods, and is consistent with some previous studies which reported that ISSR markers are more polymorphic than RAPD markers (Zietkiewicz et al. 1994; Godwin *et al*. 1997 and Nagaoka and Ogihara 1997). Fern´andez et al. (2002) used RAPD and ISSR markers for DNA fingerprinting, because they provide a quick, reliable and highly informative system and can also be used to establish genetic relationships. Although in our work the ISSR markers showed higher percentage of polymorphism than RAPD markers, we believe that both could be useful for DNA and genomic fingerprinting.

Regarding to fingerprinting of new drought tolerant lines with comparison of local cultivars (drought susceptible), four unique bands were detected between genotypes at RAPD-PCR. The first unique band posses 292 bp length with OPA-11

primer and it was found in P-58-1-2 line (drought tolerant), the second unique band (252 bp) with OPA-11 distinguish P-2-1-2-1 line (drought tolerant). The third and fourth unique bands, i.e., 665 bp with OPB-10 and 589 bp at OPD-O7, they identified of Gisa 174 and Giza 159, respectively. While, ISSR results gave four unique bands also but for drought tolerant lines. The first and second unique bands identified P-5-3-b line; these bands were 773 bp and 519 bp with HP-12 primer. As well as, P-72-11-1-1 line posse two unique bands (783 bp with HP-13 primer and 454 bp with HP-15 primer). These results determine the fingerprinting of very important drought tolerant lines because they interred into confirmed experiments in season 2010 under seed production and possess height yield under drought stress.

There was close relationship between some of the cultivars and new lines used in this study, presumably they might have been collected from similar locations or these cultivars and new lines may have been derived from the same pedigree. The high similarity between P-5-3-b line and P-5-3-a line indicating, that these lines are closely related because they were developed from the same genotype (IR 4786-13-2-1).

If there is possibility of several crosses, two patents should be crossed in order they have the QTLs involved in drought tolerance. On this basis, the cross of tolerant line (P-5-3-b) and susceptible cultivar (Giza 172) is suggested as the most suitable cross for drought tolerance analysis studies as they have the lowest similarity value (0.44) and also grouped in distinct cluster.

Nagaoka and Ogihara (1997) have reported that the ISSR primers produced several times more information than RAPD markers in wheat.

Fern´andez *et al*. (2002) have studied 16 barley cultivars form different countries and they have found high similarity index by ISSRs than by RAPDs. It may be due to highly polymorphic, abundant nature of the microsattelites due to slippage in DNA eplication.

Galvan *et al*. (2003) concluded that ISSR would be a better tool than RAPD for phylogenetic studies.

Through RAPD and ISSR techniques, which are relatively cheap and require small quantities of DNA, it was possible to identify one primer (HP15) from ISSR that generated polymorphic bands in tolerant and non-tolerant lines (315 and 505 bp).These bands can be considered as potential markers to identify drought tolerant lines or may even be more useful when converted into a simple-sequence PCR-based marker that can be used for large-scale drought tolerance screening of cultivars. On the other hand, RAPD-PCR did not detect positive drought marker. Present results suggest that one characteristic is not good predictor of genetic marker.

Polymorphic bands were determined drought tolerance in rice at present study confidence as a very important results because it is the first molecular markers for drought tolerance in Egyptian rice genotypes. These molecular markers could be assessing to acceleration of detection to drought tolerant genotypes on the bases of molecular markers at laboratory conditions only with comparison to field screening, which is very difficult and less accuracy.

Pakniyat *et al*. (2004) introduced markers linked to salt tolerance in cultivated and wild barley using RAPD-PCR. Pakniyat and Tavakol (2007) found markers related to drought tolerance in bread wheat genotypes using these markers. Also Nazari and Pakniyat (2008) found markers associated with drought tolerance in wild and cultivated barley genotypes using RAPD markers.

Corresponding author
Youssef; M. A.,
Genetics Department, Faculty of Agriculture, Zagazig University, Zagazig, Egypt
bakr2000us@yahoo.com

5. References

1. Abou-Zaid, M. F., Soliman S. S. and Koriem A. M. (1993).Reaction of some new rice promising lines to rice blast (*Pyricularia oryzae*).Egypt. J . Appl. Sci.(5)922-936.
2. Akagi H., Yokozeki Y., Inagaki A., Nakamura A. and Fujimura T.(1996). A co-dominant DNA marker closely linked to the rice nuclear restorer gene, Rf-1, identified with inter-SSR fingerprinting. Genome 39, 1205–1209.
3. Bornet B. C., Muller F. P. and Branchard M. (2002). Highly informative nature of inter simple sequence repeat (ISSR) sequences amplified using tri- and tetra-nucleotide primers from DNA of cauliflower (Brassica oleracea var. 'botrytus' L.). Genome 45, 890–896.
4. Carvalho A., Matos M., Lima-Brito J., Guedes-Pinto H. and Benito C. (2005). DNA fingerprint of F1 interspecific hybrids from the Triticeae tribe using ISSRs. Euphytica 143, 93–99.
5. Fern´andez M. E., Figueiras A. M. and Benito C. (2002). The use of ISSR and RAPD markers for detecting DNA polymorphism, genotype identification and genetic diversity among barley cultivars with known origin. Theor. Appl. Genet. 104, 845–851.
6. Galvana, M. Z.; Bornet, B.; Balatti, P. A. and Branchard, M. (2003). Inter simple sequence repeat (ISSR) marker as a tool for the assessment of both genetic diversity and gene pool origin in

common bean (Phaseolus vulgaris L.). Euphytica. 132, no. 3, p. 297-301.
7. Ghazy, A. I. and Soliman S. S. (2008). Cultivation of drought tolerant rice lines under micro jet sprinkler irrigation system. Egypt. J. of sci, 23(1) 1-15.
8. Godwin I. D., Aitken E. A. B. and Smith L. W. (1997). Application of inter simple sequence repeat (ISSR) markers to plant genetics. Electrophoresis 18, 1524–1528.
9. Kantety R. V., Zeng X. P., Bennetzen J. L. and Zehr B. E. (1995). Assessment of genetic diversity in Dent and Popcorn (Zea mays L.) inbred lines using inter-simple sequence repeat (ISSR) amplification. Mol. Breed. 1, 365–373.
10. Kaushik A., Saini N., Jain S., Rana P., Singh R. K. and Jain R. K. (2003). Genetic analysis of a CSR10 (indica) × Taraori Basmati F3 population segregating for salt tolerance using ISSR markers. Euphytica 134, 231–238.
11. Kojima T., Nagaoka T., Noda K. and Ogihara Y. (1998). Genetic linkage map of ISSR and RAPD markers in Einkorn wheat in relation to that of RFLP markers. Theor. Appl. Genet. 96, 37–45.
12. Lima-Brito J., Carvalho A., Martin A., Heslop-Harrison J. S. and Guedes-Pinto H. (2006). Morphological, yield, cytological and molecular characterization of a breed wheat × tritorderm F1 hybrid. J. Genet. 85, 123–131
13. Lynch M. (1990). The similarly index and DNA fingerprinting. Molecular Biology and Evolution, 7: 478-484.
14. Lynch M. (1991). Analysis of population genetic structure by DNA fingerprinting. In: DNA Fingerprinting Approaches and Applications (ed. by T. Burke, G. Dolf, A.J. Je،reys & R.Wolf), pp.113-126. Springer Verlag, Basel.
15. Mahasson, S. Sayed-Ahmed, Soliman,S.S. and A. F. Abd-El- Maksood (1999). Genetic analysis for some milling quality characters in rice under drought conditions. Zagazig J. Agric. Res. 26 (6) 101-110.
16. Matos M., Pinto-Carnide O. and Benito C. (2001). Phylogenetic relationships among Portuguese rye based on isozyme, RAPD and ISSR markers. Hereditas 134, 229–236.
17. Nagaoka T. and Ogihara Y. (1997). Applicability of inter-simple sequence repeat polymorphisms in wheat for use as DNA markers in comparison to RFLP and RAPD markers. Theor. Appl. Genet. 94, 597–602.
18. Nazari N., and Pakniyat, H. (2008). Genetic diversity of wild and cultivated barley genotypes under drought stress using RAPD markers. Biotechnology 7 (4): 745-750.
19. Pakniyat, H. and Tavakol, E. (2007). RAPD markers associated with drought tolerance in bread wheat (*Triticum aestivum* L.). Pak. Biol. Sci.,10: 3237-3239.
20. Pakniyat, H., Namayandeh A. and Forster B. P. (2004). Identification of RAPD markers linked to salt tolerance in cultivated and wild barley. Proceedings of the 9th International Barley Gentics Symposium, June 20-26, Brono. Czech Republic, 191-194.
21. Ribaut, J. M. and Poland D. (1999). Molecular Approaches for the Genetic Improvement of Cereals for Stable Production in Water Limited Environments. In: A Strategic Planning Workshop .. CIMMYT, El Batan, Mexico.
22. Ribaut, J. M., Jiang C. , Gonzalez-de-Leon D., Edmeades G. O. and D. A. Hoisington, (1997). Identification of quantitative trait loci under drought conditions in tropical maize. 2. Yield components and marker-assisted selection strategies. Theor. Appl. Genet., 94: 887-896.
23. Sneath P. H. A. and Sokal R. R. (1973). Numerical Taxonomy.W.H. Freeman, San Francisco, USA.
24. Soliman,S.S. (1993a).Genetic behavior of grain yield and its attributes under normal and drought conditions in rice. Egypt. J .Appl. Sci. ,8 (7) 827-841.
25. Soliman,S.S. (1993b). Gene action and combining ability of yield components under normal and drought conditions in rice. Egypt. J .Appl. Sci. ,8(7) 878-895.
26. Tautz D. and Renz M. (1984). Simple sequences are ubiquitous repetitive components of eukaryotic genomes. Nucl. Acids Res. 12, 4127–4137.
27. Waffa, M. El-Attar, Amer M. M. A., Al-Gaby A. M. and Soliman S. S. (1998). Effect of drought on some rice biochemical constituents. Annals of Agric. Sci. Moshtoher, 36 (2): 915-924.
28. Williams J. G. K., Kubelik A. R., Livak K. J., Rafalski J. A. and Tingey S. V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucl. Acids Res. 18, 6531– 6535.
29. Zietkiewicz E., Rafalski A. and Labuda D. (1994). Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics 20, 176–183.

6/5/2010

Genetic Evaluation and Molecular Markers for Heat Tolerance in Tomato (*Lycopersicon esculentum* Mill.)

M.A. Kamel*[2], S.S.Soliman[1], A.E. Mandour[1] and Mahassen S. S. Ahmed[1]

[1]Genetics Department, Fac. Agric., Zagazig University, Egypt, [2] Samtrade trials station, Samtrade, Samir Fahmy Group, Egypt

*kamel_moh77@yahoo.com

Abstract: Genetic evaluation was performed on twenty three genotypes of tomato (*Lycopersicon esculentum* Mill.) under high temperature at summer season to determine the variation between them for heat tolerance. Heat tolerance related criteria, i.e., pollen viability, fruit setting, osmotic pressure and fruit yield per plant. LSSS1, Homestead 24, Black Russian Plum, Super Marmand and Money Maker possess more tolerance of heat. In contrast, Super Stain B, Castle Rock, Cherokee Purple, Moskvich and Nicholevna Pink were more susceptible of heat. The pollen grain viability and fruit setting criteria consider as suitable morphological markers for heat tolerance than other heat tolerant related criteria as osmotic pressure. Heritability was high and moderately whereas, the genetic improvement of new strains could be done. From previous evaluation, Lsss1 as tolerant line and Super Strain B as sensitive cultivar of heat tolerance was crossed for study of molecular markers related to heat tolerance by using bulk segregant analysis (BSA). Crossing was carried out between these two genotypes to obtain the F_1 seeds which were left for selfing to obtain the F_2 seeds. The two selected genotypes, their F_1 and F_2 plants were evaluated for their response to heat stress by recording some heat stress related traits. Bulk of the two extremely F_2 plants (most tolerant and most sensitive F_2 groups), the two contrasting parents and their F_1, were used to develop some molecular genetic markers associated with heat tolerance in tomato by using ten RAPD and six ISSR primers. two RAPD markers (with molecular sizes of 100 bp for primers A16 and 500 bp for primer Z13)and one ISSR marker(with molecular size of 650 bp) were considered as reliable markers for heat tolerance as well as susceptible genotypes possessed eight RAPD markers (with molecular sizes 500 and 1500 bp for primer C02, 1750 and750 bp for primer C03, 2400 bp for primer C05, 550 bp for primer C08, 400 bp for primer C14 and 650 bp for primer C15). [Journal of American Science 2010;6(12):364-374]. (ISSN: 1545-1003).

Keywords: Tomato, Heat stress, Heat related traits, Molecular markers, RAPD-PCR, ISSR-PCR. Bulked segregant analysis (BSA), Marker assisted selection (MAS).

1. Introduction:

Heat tolerance is generally defined as the ability of the plant to grow and produce economic yield under high temperatures. However, while some researchers believe that night temperatures are major limiting factors others have argued that day and night temperatures do not affect the plant independently and that the diurnal means temperature is a bitter predictor of plant response to high temperature with day temperature having a secondary role (Peet and Willits, 1998).

Heat stress due to high ambient temperatures to a serious threat to crop production worldwide (Hall, 2001). Gaseous amissions due to human activities are substantially adding to the existing concentrations of greenhouse gases, particularly CO_2, methane, chlorofluorocarbons and nitrous oxides.

Different global circulation models predict that greenhouse gases will gradually increase world´s average ambient temperature. According to a report of the Intergovernmental panel on Climatic Change (IPCC), global mean temperature will rise 0.3C° per decade (Jones *et al*., 1999) reaching to approximately 1 and 3C ° above the present value by years 2025 and 2100, respectively, and leading to global warming. Rising temperatures may lead to altered geographical distribution and growing season of agricultural crops by allowing the threshold temperature for the start of the season and crop maturity to reach earlier (Porter, 2005).

Tomato (Lycopersicon esculentum Mill.) belongs to the family Solanaceae and is an important vegetable crop all over the world. Tomatoes, although originating from elevated regions of the Andes, can be adapted to various conditions. In the last years, interest through the cultivation of this produce has steadily increased in tropical and subtropical zones. Therefore, tomatoes from these regions should be resistant or tolerant to heat, while the most common problem is the abortion of flowers due to high temperatures.

A threshold temperature refers to a value of daily mean temperature at which a detectable reduction in growth begins upper under lower

developmental threshold temperature have been determined for many plant species through controlled laboratory and field experiments. In tomato, for example, when the ambient temperature exceeds 35 C°, its seed germination, seedling and vegetative growth, flowering, fruit set and fruit ripening are adversely affected (Miller *et al*., 2001). The criteria, which more affecting than the other for heat stress are, pollen grain viability, osmotic pressure, fruit setting and fruit yield (Saeed *et al*. 2007, Abdul-Baki 1991, Peter *et al*. 2002, Adul-Baki and John 1995, Firon *et al*., 2006).

Traditional breeding methods provide little information on the chromosomal regions controlling these complex traits, the simultaneous effects of each chromosomal region on other traits (epistasis, pleiotropy,or linkage), or the genetic basis of such yield related traits (dominance or over-dominance) (Semel *et al*. 2006). If based only on phenotype analyses, selection by traditional breeding methods is difficult under conditions of large genotype–environment interactions. There is no reliable field screening technique that can be used year after year or generation after generation.

Molecular markers can be used not only for estimating the genetic diversity of germplasm collections but also for distinguishing genotypes within population. Kantety *et al*.,(1995) showed that ISSR technology was able to detect differences between the closely related inbred lines of corn. Thin ISSR should be very useful for studying tomato genotypes.

One approach to facilitate the selection and breeding of polygenic traits is to identify genetic markers linked to the traits of interest. DNA markers have facilitated quantitative trait locus (QTL) mapping studies in segregated populations, and showed that certain genomic regions derived from wild germplasm have the potential to improve fruit-related traits (Gur and Zamir 2004). The application of molecular markers in plant breeding programs facilitates the improvement of many crop species (Williams *et al*.,1990). The detection of RAPD markers on the genomic map of different field crop is beneficial to improve breeding programs of these crops. It offers the simplest and fastest method for detecting a great number of genomic markers in less period of time (Edwards *et al*., 1992). Michelmore *et al*. (1991) developed the bulked segregant analysis of F_2 plants as a simpler alternative technique to isogenic line analysis where the highest and lowest extremes of the F_2 population are bulked for the development of RAPD and SSR molecular markers needed for QTL-assisted selection. ISSR markers have recently found to be highly variable, require less investment in time, money and labor than other methods, and have the ability to be inherited (Wolfe and Liston, 1998).

Therefore the present study aimed to genetic evaluation of twenty three introduced local lines and cultivars for heat tolerance, as well as trial to discovery of some molecular genetic markers associated with heat stress (RAPD and ISSR markers) using bulk segregant analysis (BSA) to be used in marker assisted selection (MAS) program and to develop a database which will enable the utilization of genetic markers as selection tools to improve crop characterization.

2. Materials and Methods

1- Evaluation of genotypes for heat tolerance

1.1. Materials

Twenty cultivars and three lines of tomato were used for this study Table (1). These genotypes was evaluated for heat tolerance during late summer season 2007 at experimental farm of the Faculty of Agriculture, Zagazig University. The seeds of twenty cultivars were kindly obtained from Horticulture Department, and three lines were selected previous study at Genetic Department, Faculty of agriculture, Zagazig University, Egypt.

1.2. Methods:

Seeds of genotypes were sown on may 15th, 2007 in nursery in multi-pot transplant trays field with a mixture of peat-moss and vermiculite (1:1, v/v) medium. After one month from sowing, transplants were transferred to the filed. The mean monthly air temperature in the cultivated area during the growing season are indicated in Table (2).

1.3.Measured characters

- osmotic pressure:

The osmotic pressure was estimated from transforming the total soluble solids (TSS) to osmotic pressure as air pressure (bar) and multiplied by the factor (1.013) to represent osmotic pressure (bar). The total soluble solids was determined as refraction index using zeiss refractometer after 90 days from transplanting at room temperature (morgan,1977).

- Percentage of fruit set per plant was determined as the total number of fruit divided by the total flower number on clusters 2-6 of plant.
- Pollen viability by staining of pollen grains with acetocarmine dye and estimation of pollen viability according to the method followed by (Moreira and Gurgel 1941).

Total yield (kg/plant.) was estimated from total weight of harvested fruits from each plant.

Table 1 : Name , source and characterization of tomato genotypes.

NAME	source	Characterization
MANITOBA	Roguelands seeds, UK	Determinate, red fruit color, fruit weight 90 g
MARION RED	Roguelands seeds, UK	Determinate, red fruit color, fruit weight 85 g
MOSKVICH	Roguelands seeds, UK	Determinate, red fruit color, fruit weight 110 g
BLACK RUSSIAN PLUM	Roguelands seeds, UK	Indeterminate, orange fruit color, fruit weight 25 g
CHEROKEE PURPLE	Roguelands seeds, UK	Semi-determinate, red fruit color, fruit weight 185 g
HOMESTEAD 24	Roguelands seeds, UK	Determinate, red fruit color, fruit weight 195 g
KAZAHK SCHALAVIJE	Roguelands seeds, UK	Semi-determinate, red fruit color, fruit weight 225 g
PLUM LEMON	Roguelands seeds, UK	Indeterminate, yellow fruit color, fruit weight 50 g
NICHOLEVNA PINK	Roguelands seeds, UK	Determinate, red fruit color, fruit weight 150 g
WALTER RED	Roguelands seeds, UK	Determinate, red fruit color, fruit weight 125 g
SUPER STRAIN B	Sun seed, USA	Determinate, red fruit color, fruit weight 140 g
CASTLE ROCK	Castle s, USA	Determinate, red fruit color, fruit weight 125 g
SUBER MARMAND	Daehnfeldt, Holland	Semi-determinate, red fruit color, fruit weight 110 g
MONEY MAKER	Yates, New Zealand Ltd	Indeterminate, red fruit color, fruit weight 40 g
FALCON	Antakya seed, Turkey	Determinate, red fruit color, fruit weight 65 g
ALEDO	Clause, France	Determinate, red fruit color, fruit weight 55 g
RED STAR	Sun seed, USA	Determinate, red fruit color, fruit weight 150 g
PETO 86	Peto Seed, USA	Determinate, red fruit color, fruit weight 85 g
SUPER QUEEN	Sun seed, USA	Determinate, red fruit color, fruit weight 125 g
VF145-B52	Commercial Egypt	Determinate, red fruit color, fruit weight 125 g
LSSS1	New developed strain*	Determinate, red fruit color, fruit weight 85 g
LSSS2	New developed strain*	Determinate, red fruit color, fruit weight 110 g
LSSS3	New developed strain*	Determinate, red fruit color, fruit weight 165 g

* by Soliman, S.S. and Kamel, M.A., Genetics Dept., Zagazig Univ.

Table 2: The mean monthly air temperatures in the cultivated area during the growing season 2007 and 2009.

Month	Temperature (C^{o})			
	2007		2009.	
	Max.	Min.	Max.	Min.
June	48	20	40	20
July	45	17	40	19
August	45	19	39	19
September	40	16	35	16

1.4. Statistical analysis

Collected data were analyzed using the statistical software SPSS version 9.0. One-way analysis of variance (ANOVA) was used to determine differences among genotypes. Relationships between variable characters were estimated as correlation coefficient. Heritability in broad sense were done as follows:

$$h^2 \text{ (in broad sense)} = \frac{\text{genotypic variance}}{\text{genotypic variance + environmental variance}} \text{ X } 100$$

2. Bulk Segregant analysis (BSA)

2.1. Materials

Two genotypes of tomato namely; Lsss1 line (heat tolerant) and Super Strain B (heat sensitive) were chosen after evaluation for heat tolerance of twenty three genotypes in above experiment. The seeds of these genotypes were kindly obtained from Horticulture Department, Faculty of Agriculture, Zagazig Universtiy, Egypt.

2.2. Methods

2.2.1. Sand culture experiment

The two selected genotypes (Lsss1 and Super Strain B) were grown in field and crossed to obtain the F_1 seeds. Some of the F_1 seeds were transplanting in the field and selfed to obtain the F_2 seeds.

Seeds of genotypes, i.e., two parents, F_1'S and F_2 generations were sown on may 15th, 2009 in nursery in multi-pot transplant trays field with a mixture of peat-moss and vermiculite (1:1, v/v) medium. After one month from sowing, transplants were transferred to the filed. The mean monthly air temperatures in the cultivated area during the growing season are indicated in Table (2).

Data were recorded for ten plants for each genotype of the following related traits; Percentage of fruit set, Pollen viability and total yield per plant. The F_2 plants represented by 200 plants were classified into groups according to their behavior under heat stress.

Samples of the two parents, their F_1 and the two extreme groups of F_2 individuals (the most ten plants heat tolerant and the most ten plants heat sensitive) were taken for further molecular analysis.

2.3. Molecular genetic studies

2.3.1 Genomic DNA extraction.

DNeasy™ Plant Mini Kit (Qiagen Inc., Cat. No. 69104) was used for DNA isolation as described in the manufacturer manual from plant samples (the two parents; F_1 and the two extreme groups of F_2 plants) using bulked segregant analysis (BSA) technique.

2.3.2. RAPD-PCR analysis

PCR reactions were performed according to Williams *et al.*(1990) using ten 10-mer primers (Operon Technology, USA) table (3).

Amplification reactions were performed in a volume of 25 µl containing 1X Reaction buffer, 2 mM MgCl2, 0.2mM dNTPS, 0.2 mM primers, 25 ng of genomic DNA, and 1 units of Taq DNA polymerase.

Amplification was performed on a top quality thermal cycler programmed for 45 cycles of 1 minute at 94°, 1 minute at 36°, and 2 minutes at 72°. Amplification products were analyzed by electrophoresis in 1.4% agarose gels and detected by staining with ethidium bromide.

2.3.3. ISSR-PCR analysis

ISSR-PCR reactions were conducted according to Sharma *et al.* (1995) using six primers Table (3).

Amplification reactions were performed in a volume of 25 µl containing 1X Reaction buffer, 1.5 mM MgCl2, 0.2mM dNTPS, 50 pmol primers, 25 ng of genomic DNA, and 1 units of Taq DNA polymerase.

PCR amplification was performed in a hybrid Cycler programmed to fulfill 35 cycles after an initial denaturation cycle for 4 min at 94° C . Each cycle consisted of denaturation step at 94° C for 45 sec, an annealing step at 50° C for 30 sec. and an elongation step at 72° C for 2 min and 30 sec. The primer extension segment was extended to 5 min at 72° C in the final cycle. Agarose gel (1.4 %) electrophoresis was used for separating the PCR products.

2.3.4. Desitometric scanning

All bands resulting from RAPD and ISSR gels were detected on an UV-transilluminator filter. All gels were photographed under UV light with Polaroid film 667 and scanned with Bio-Rad video densitometer Model 620 at waveltngth of 577. Software data analysis for Bio-Rad Model 620 USA densitometer and computer were used.

Table (3): list of primers RAPD and ISSR

No.	Primer	Sequence (5' to 3')
RAPD		
1	A16	5'-AGCCAGCGAA-3'
2	B01	5'-GTTTCGCTCC-3'
3	C02	5'-GTGAGGCGTC-3'
4	C03	5'-GGGGGTCTTT-3'
5	C05	5'-GATGACCGCC-3'
6	C08	5'-TGGACCGGTG-3'
7	C14	5'-TGCGTGCTTG-3'
8	C15	5'-GACGGATCAG-3'
9	C19	5'-GTTGCCAGCC-3'
10	Z13	5'-GACTAAGCCC-3'
ISSR		
1	844A	5' (CT)8AC 3'
2	844B	5'(CT)8GC 3'
3	17899B	5'(CA)6AC 3'
4	HB08	5'(GA)6GG 3'
5	HB10	5'(GA)6CC 3'
6	HB13	5'(GAG)3GC 3'

3. Results and Discussion:

1. Genetic evaluation of heat tolerance related traits.

1.1 Genetic variation and heritability of tomato heat tolerance

Highly significant differences between studied genotypes were recorded for osmotic pressure, pollen viability, fruit setting and total yield per plant, as well as high heritability in broad-sense (h^2_{bs}) for these traits (Table 4). These results confirmed that genetic improvements were done of heat tolerance through related characters of it. These results are similar to those obtained by Grilli *et al.*(2003), where the fruit set of various tomato genotypes at high temperatures had about 89.5 % broad sense heritability, thus suggesting that the selection of individuals based on characteristic evaluated can be efficient.

Table 4: The mean squares of four studied characters of twenty three genotypes in tomato.

S.V.	d.f.	**Osmotic pressure**	**Pollen viability**	**Fruit setting**	**Total yield/plant**
Replicate	2	0.547101	0.461449	0.58971	0.009275
Genotypes	22	6.868248**	1095.465**	1139.682**	4.171357**
Error	44	0.399374	4.603874	3.581528	0.025033
h^2		0.8438	0.9875	0.9910	0.9787
C.V %		8.50	4.20	4.00	8.80

h^2_{bs} = Broad sense heritability cv = Coefficient of variability

1.2 Mean performance for studied traits of twenty three genotypes in tomato.

The mean performance for four studied characters of twenty three genotypes (Table 5) confirmed the wide difference between genotypes under study for heat tolerance related characters. Higher values of four traits were recorded for Black Russian Plum, Homstead 24, Super Marmand, Mony Maker, LSSS1 and LSSS3 than the other genotypes suggested as a donor for heat tolerance by using of them in hybridization, with remark to some more tolerant cultivars possess small fruit size as Black Russian Plum and Mony Maker. In contrast several heat susceptible genotypes were recorded, i.e., Moskvich, Nicholevna Pink, Super Strain B, Castle Rock. In addition, the most of studied genotypes consider as intermediate heat tolerance as Manitoba, Marion Red, Khazahk Schalavije, Plum Lemon, Walter Red, Falcon, Aledo, Red Star, Peto 86, Super Queen, VF145-B52 and LSSS2. From these results may be concluded that the high tolerant genotypes possess high values for four studied traits, and vise versa, susceptible heat genotypes possess low values for four studied traits. No obvious trend were recorded in intermediate heat tolerant genotypes for four studied traits. Comparable study was done by Saeed *et al*.(2007) who suggested that genotype, which will produce better yield under high temperature conditions, would be heat tolerant. The value of high broad sense heritability (0.9715) that showed that about 90% of the variation observed was genetically determined. Abdul-Baki (1991) who assessed fruit yield of tolerant and sensitive tomato lines and cultivars in field under high temperature condition. The heat tolerant lines produced higher fruit yield than heat sensitive cultivars. Peter *et al*. (2002) who reported that high temperatures fruit set (heat tolerance) was a critical trait of tomato. In the same trend for pollen grain viability, Adul-Baki and John (1995) demonstrated that using pollen viability as a selection criterion for high temperature tolerance was genotype effect as well as Firon *et al*., (2006) reported that heat stress caused a reduction in number of pollen grains in heat – sensitive cultivars, caused reduced fruit set. In heat tolerant cultivars, however, number and quality of pollen grains, number of fruits were less affected by high temperatures.

1.3 Relationship between four studied criteria related to heat tolerance in tomato.

Correlation coefficient (r) for four characters in tomato are shown in (Table 6). Osmotic pressure was positively and significantly correlated to pollen viability (r = 0.46), fruit set (r = 0.48) and yield (r = 0.54). positive correlation was also observed between pollen viability and both of fruit set and yield (r = 0.95 and 0.77 respectively). As well as correlation between fruit set and yield were positive and highly significant (r = 0.74). These results confirmed that, increased yield under heat stress might be obtained by breeding genotypes that were high in osmotic pressure, pollen viability or fruit set under heat stress. These results agreed with Weaver and Timm (1989) whose reported a positive correlation between heat tolerance and pollen viability maintenance after briefly exposing flowering tomato plants in the greenhouse to high temperatures.

2. Bulk Segregant Analysis (BSA)

2.1 Responses of the F_2 plants

F_2 plants presented by 200 individuals were classified into groups according to their behavior under high temperature stress. The first group refers to the best growing F_2 plants and the last group refers to the worst ones under high temperature stress. The F_2 plants were arranged in descending order according to their frequency, so plants with high frequency in group one were chosen as the most tolerant F_2 plants. While the plants in the last group were taken to represent the most sensitive F_2 plants.

According to these classifications, ten F_2 plants were taken to represent the most tolerant and the most sensitive ones to high temperature stress for each trait as shown in Table (7).

These twenty plants were used for bulked segregant analysis to obtain molecular ($RAPD_S$ and $ISSR_S$) markers linked with high temperature stress.

2.2 Molecular genetic markers for heat tolerance.

2. 2.1 RAPD molecular markers

DNA isolated from the two contrasting parents, LSSS1 as a heat tolerant parent and Super Strain B as a heat sensitive parent, their subsequent F_1 and DNA bulks of the tolerant and sensitive groups of F_2 segregating population were tested against ten preselected primers.

All primers gave polymorphisms with the studied genotypes, while 8 primers developed molecular markers for heat and sensitive tolerance as shown in table(8). Primers A16 and Z13 exhibited 2 positive molecular markers which were found only in the tolerant parent (LSSS1), F_1 and the tolerant F_2 bulk with molecular sizes of 100 bp for primers A16 and 500 bp for primer Z13, while there were absent in the sensitive parent (Super S train B) and the sensitive F_2 bulk Fig(1). On the other hand, primers C02, C03, C05, C08, C14 and C15 exhibited eight molecular markers which were found only in the sensitive F_2 bulk with molecular sizes 500 and 1500 bp for primer C02, 1750 and750 bp for primer C03, 2400 bp for primer C05, 550 bp for primer C08, 400 bp for primer C14 and 650 bp for primer C15 Fig(2).

Table 5: Mean performance and least significant difference (LSD) of four studied characters of twenty three genotypes in tomato under heat stress at summer season, 2007.

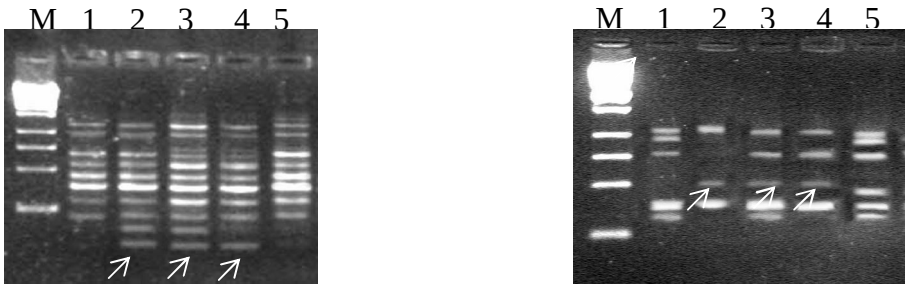
Name	Osmotic pressure	Pollen viability %	Fruit setting %	Total yield/plant (Kg)
MANITOBA	6.78	53.7	40.5	1.3
MARION RED	7.345	41.8	35.2	1.1
MOSKVICH	7.91	32.4	29.3	0.6
BLACK RUSSIAN PLUM	11.3	75.7	68.1	3.5
CHEROKEE PURPLE	7.345	22.0	23.7	1.0
HOMESTEAD 24	9.605	76.8	88.5	3.2
KAZAHK SCHALAVIJE	6.78	44.4	33.7	1.5
PLUM LEMON	6.215	62.7	61.2	2.1
NICHOLEVNA PINK	6.215	17.3	14.8	0.7
WALTER RED	6.78	42.6	48.2	1.4
SUPER STRAIN B	6.215	20.7	19.3	0.5
CASTLE ROCK	9.605	24.2	16.3	0.6
SUBER MARMAND	9.04	77.6	63.1	4.1
MONEY MAKER	10.17	77.5	74.7	3.8
FALCON	10.735	68.6	66.3	1.8
ALEDO	6.78	57.1	48.2	1.1
RED STAR	10.735	42.3	40.4	1.2
PETO 86	6.78	51.0	43.1	0.9
SUPER QUEEN	8.0	43.0	47.7	1.2
VF145-B52	7.571	71.2	67.2	1.1
LSSS1	10.735	61.6	58.1	3.3
LSSS2	10.17	49.2	46.0	1.4
LSSS3	9.04	59.0	53.1	3.8
L.S.D 5%	1.07	3.63	3.20	0.27
L.S.D 1%	1.45	4.94	4.36	0.36

Table 6: Correlation coefficient (r) among different related characters to heat tolerance in tomato.

Trait	Osmotic pressure	Pollen viability	Fruit set	Yield/plant
Osmotic pressure	1			
Pollen viability	0.46*	1		
Fruit set	0.48*	0.95**	1	
Yield/plant	0.54**	0.77**	0.74**	1

Table (7): The most tolerant and the most sensitive F_2 plants according to some heat tolerance related traits.

	Plant no.	Fruit setting (%)	Pollen viability (%)	Yield/plant (Kg)
Most tolerant	156	68.5	70.4	4.1
	189	67.9	70.1	3.9
	45	66.7	69.3	3.8
	78	66.6	69.6	3.5
	7	66.4	69.5	3.7
	16	65.8	68.4	3.5
	122	65.8	68.2	3.3
	82	64.3	66.9	3.1
	95	64.2	66.7	3.0
	110	64.1	66.4	3.0
Most sensitive	33	18.5	20.4	0.4
	64	18.4	20.6	0.4
	151	18.4	20.3	0.4
	154	18.1	19.9	0.4
	12	17.9	18.7	0.3
	69	17.8	18.2	0.3
	130	17.6	18.5	0.3
	173	17.2	18.0	0.3
	54	17.1	18.1	0.3
	28	16.8	17.8	0.2



(a) **(b)**

Fig. 1: Amplification patterns using RAPD primers to identify the a) A16and b) Z13 markers linked to resistance alleles. Lane M 1-kb molecular- weight ladder,1) susceptible parent Super Strain B, 2) tolerant parent LSSS1, 3) F_1, 4) tolerant F_2 bulk, 5) susceptible F_2 bulk.

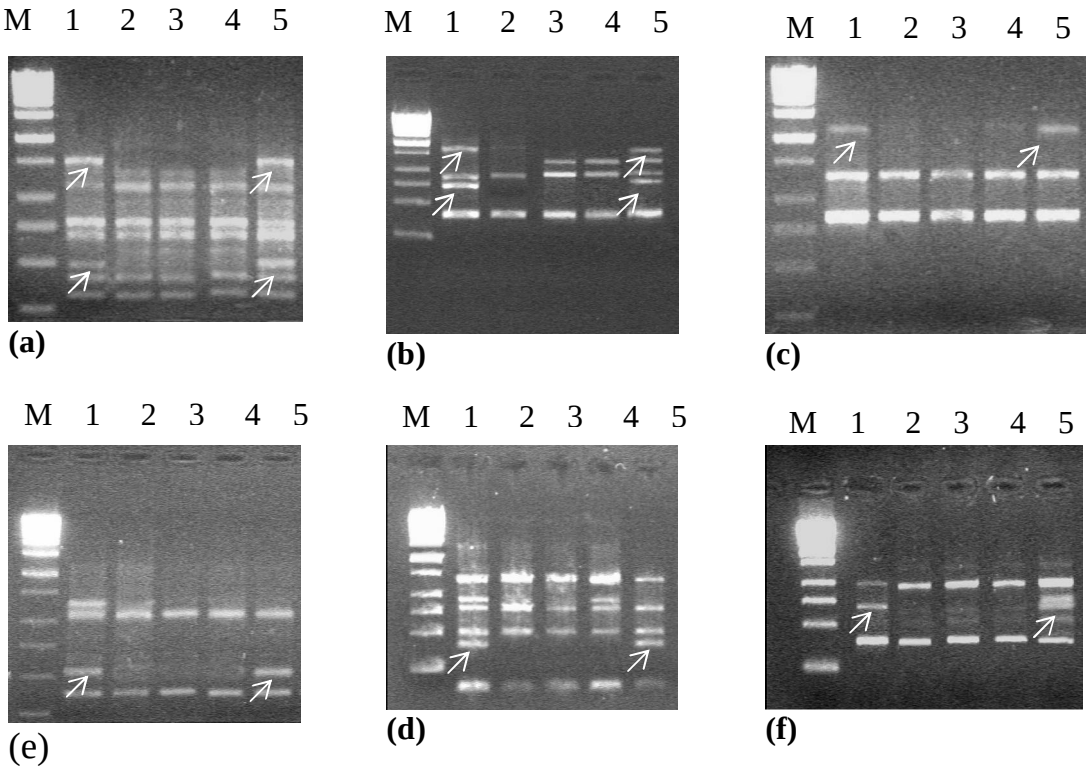


Fig. 2: Amplification patterns using RAPD primers to identify the a) C02, b) C03, c) C05, d) C08, e)C14 and f) C15 markers linked to resistance alleles. Lane M 1-kb molecular- weight ladder, 1) susceptible parent Super Strain B, 2) tolerant parent LSSS1, 3) F_1, 4) tolerant F_2 bulk, 5) susceptible F_2 bulk.

These two positive and eight negative RAPD markers could be considered as reliable markers for heat tolerance in tomato. These results agreed with (Zhang *et al*. 1994 and Mackay and Caligari 2000) whose reported that RAPD analysis combined with BSA has been used to screen for markers linked to genes of interest. Moreover Lin *et al*. (2006) identified random amplified polymorphic DNA (RAPD) markers linked to heat tolerance traits in tomatoes under heat stress by using the bulked segregant analysis. In addition, bulked segregant analysis was used to identify RAPD markers linked to the Sw-5 gene for resistance to tomato spotted wilt virus (TSWV) in tomato (Chague *et al*. 1997).

Table 8: RAPD-PCR polymorphic bands of eight primers linked to heat and sensitive tolerance with the two parents, their subsequent F_1 and two bulks of F_2.

Primer name	PBN	M.W (bp)	SP	TP	F_1	Tb	Sb	MT
A16	1	2000	0	0	1	0	1	-
	4	750	0	0	1	0	0	-
	11	150	0	1	1	0	0	-
	12	100	0	1	1	1	0	P
Z13	1	1200	1	1	0	0	0	-
	2	950	1	0	1	1	1	-
	3	850	0	0	0	0	1	-
	4	775	1	0	1	1	1	-
	5	500	0	1	1	1	1	P
	6	450	0	0	0	0	1	-
	8	350	1	0	1	0	1	-
C02	1	1500	1	0	0	0	1	N
	5	500	1	0	0	0	1	N
C03	1	1750	1	0	0	0	1	N
	2	1250	0	0	1	1	1	-
	4	750	1	0	0	0	1	N
C05	1	2400	1	0	0	0	1	N
C08	1	1300	1	0	0	0	0	-
	3	550	1	0	0	0	1	N
C14	2	900	1	0	0	1	0	-
	5	400	1	0	0	0	1	N
C15	2	750	0	0	0	0	1	-
	3	650	1	0	0	0	1	N
	4	550	0	0	0	0	1	-

PBN: polymorphic band number Tb: tolerant bulk P: positive
SP: sensitive parent Sb: sensitive bulk N: negative
TP: tolerant parent MT: marker type

2.2.2 ISSR molecular markers

DNA isolated from the two contrasting parents, LSSS1 as a heat tolerant parent and Super Strain B as a heat sensitive parent, their subsequent F_1 and DNA bulks of the tolerant and sensitive groups of F_2 segregating population were tested against six preselected primers.

All primers gave polymorphisms among the studied genotypes, while only one primer developed molecular markers for heat tolerance Table(9). Primer 844A showed one positive molecular marker which were found only in the tolerant parent (LSSS1), F_1 and the tolerant F_2 bulk with molecular size of 650 bp Fig. (3).

Our results were in harmony with those of Lin *et al*. (2010) who used the 160 F_2 tomato plants segregating population to identification of ISSR markers linked to fruit related traits in the tomato subjected to high temperatures. ISSR were useful for finding markers associated with major and minor genes controlling agronomical important traits in wheat (Ammiraju *et al*., 2001). Also several ISSR markers had been found to be tightly linked to the gene that determined the ratio of fructose to glucose in mature tomato fruits (Levin *et al*., 2000).

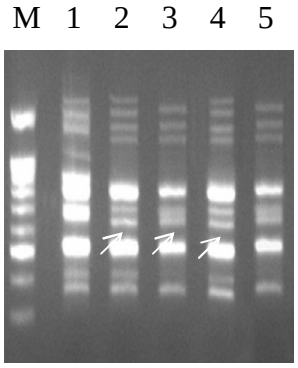


Fig. 3: Amplification patterns using ISSR primers to identify the 844A markers linked to resistance alleles. Lane M 100 bp molecular- weight ladder, 1 susceptible parent Super Strain B, 2 tolerant parent LSSS1, 3 F_1, 4 tolerant F_2 bulk, 5 susceptible F_2 bulk.

Table 9: ISSR-PCR polymorphic bands of one primers linked to heat tolerance with the two parents, their subsequent F_1 and two bulks of F_2.

Primer name	PBN	M.W (bp)	SP	TP	F_1	Tb	Sb	MT
844A	1	1250	1	1	0	1	0	-
	4	950	0	1	1	1	1	-
	5	925	1	0	0	0	0	-
	8	650	0	1	1	1	0	P
	10	450	1	1	0	1	0	-

PBN: polymorphic band number Tb: tolerant bulk P: positive
SP: sensitive parent Sb: sensitive bulk
TP: tolerant parent MT: marker type

These results confirmed that the possibility for breeding of new Egyptian lines and hybrids possess high tolerance of heat and simultaneously high fruit yield for cultivation of summer season, which the temperature up to 40°C in about two months July and August per year.

The present discovered molecular markers for heat tolerance and sensitively in Egyptian cultivars will be acceleration of breeding program for development of new lines and subsequently new hybrids having more tolerance to heat and high fruit yield at the summer season.

The highly significant correlation between four heat tolerance related characters under study by using discovered molecular markers for pollen grain viability, osmotic pressure, fruit set and fruit yield per plant and recent study trial to determine the relationships between new molecular markers and quantitative trait loci (QTL) from database and subsequently, helpful to determine the controlling genes for heat tolerance, as well as the study by Lin *et al*. (2010) , which to determine the quantitative trait loci influencing fruit- related characteristics of tomato grown in high temperature.

In conclusion, our goal was to find RAPD and ISSR markers linked to heat tolerance genes in order to use them in marker-assisted breeding programs. BSA allowed us to rapidly find marker linked to heat tolerance. The results showed that only two RAPD and one ISSR markers were linked to heat tolerance. Thus, BSA allowed us to directly target the gene, as demonstrated by Michelmore et al.(1991). The level of polymorphism detected in molecular marker followed by using marker-assisted selection (MAS) has been proven to be good alternative method of the agronomic selection, where it provides plant breeders with environmental- independent genetic markers for certain economic traits.

Corresponding author
M.A. Kamel
Samtrade trials station, Samtrade, Samir Fahmy Group, Egypt
kamel_moh77@yahoo.com

Acknowledgements

This work is co-funded by Research Support Fund, Zagazig University. Utilization and molecular markers for heat stress in tomato project, Principal investigator Prof. Dr. Said S. Soliman.

The authors would like to thank chairman of the board of Samir Fahmy group (Eng. Samir Fahmy) and all employee of the company for assistance in this work

4. References:

1. Abdul-Baki A. 1991. Tolerance of tomato cultivars and selected germplasm to heat stress. J. Amer. Soc. Hort. Sci. 116: 1113-1116.
2. Abdul-Baki A., John R. 1995. Pollen viability and fruit set of tomato genotypes under optimum high temperature regimes. Hortscience 30: 115-117.
3. Ammiraju J.S.A., Dohlakia B.B., Santra D.K., Singh H., Lagu M.D, Tamhankar S.A., Dhaliwal H.S., Rao V.S.. Gupta V.S., Ranjekar P.K. 2001. Identification of inter simple sequence repeat (ISSR) markers associated with seed size in wheat. Theor. Appl. Genet. 102: 726-732.
4. Changue V., Mercier J.C., Guenard M. 1997. Identification of RAPD markers linked to a locus involved in quantitive resistance to TYLCV in tomato by bulked segregant analysis. Theor. Appl. Genet. 95: 671-677.
5. Edwards, M.D., Helntijaris T.T., Wright S., Stuber C.W. 1992. Molecular marker facilitated investigation of quantitative trait loci in maize. 4. analysis based on genome saturation with isozyme and restriction fragment length polymorphism markers. Theor. Appl. Genet. 83: 765-774.
6. Firon, N., Shaked R., Peet M.M., Pharr D.M., Zamski E., Rosenfeld K., Althan L., Pressman E.2006. Pollen grains of heat tolerant tomato cultivars retain higher carbohydrate concentration under heat stress conditions. Scientia Horti. 109: 212-217.

7. Grilli G.V., Braz L.T., Perecin D., Oliveria J.A. 2003. Genetic control of fruit-setting percentage of tomatoes tolerant to high-temperatures. Acta Hort. 607: 179-184.
8. Gur A., Zamir D. 2004. Unused natural variation can lift yield barriers in plant breeding. PLoS Biol 2:1610–1615.
9. Hall, A.E., 2001. Crop Responses to Environment.CRCPress LLC, Boca Raton, Florida.
10. Kantety R.V., Zeng X., Bennetzen J.L., Zehr B.E., 1995. Assessment of genetic diversity in dent and popcorn (*Zea mays* L.) inbred lines using inter-simple sequence repeat (ISSR) amplification, Mol. Breed. 1 : 365-373.
11. Levin I., Giboa N., Yeselson E., Shen S., Schaffer A.A. 2000. Fgr, a major locus that modulates the fructose to glucose ratio in mature tomato fruits. Theor. Appl. Genet. 102: 885-891.
12. Lin K.H., Lo H.F., Leo S.P., Kuo C.G., Chen J.T., Yeh W.L. 2006. RAPD markers for the identification of yield traits in tomatoes under heat stress via bulked segregant analysis. Hereditas 143: 142-154.
13. Lin K.H., Yeh W.L. Chen H.M., Lo H.F. 2010. Quantitative trait loci influencing fruit-related characteristics of tomato grown in high-temperature conditions. Euphytica 13 Feb. (online).
14. Jones, P.D., New, M., Parker, D.E., Mortin, S., Rigor, I.G., 1999. Surface area temperature and its change over the past 150 years. Rev. Geophys. 37,173–199.
15. Mackay I.J., Caligari P.D.S. 2000. Efficiencies of F_2 and backcross generations for bulked segregant analysis using dominant markers. Crop Sci. 40: 626-630.
16. Michelmore R.W., Paran I., Kessel R.V. 1991. Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. Proc. Natl. Acad. Sci. USA 88: 9829-9832.
17. Miller, P., Lanier, W., Brandt, S., 2001. Using Growing Degree Days to Predict Plant Stages. Ag/Extension Communications Coordinator, Communications Services, Montana State University-Bozeman, Bozeman, MO.
18. Moreira, s. and J.J.A. Gurgel, 1941. Pollen fertility and its correlation with number of seeds in species and form genus citrus. Borgantia, San Paulo, 1: 667-711.
19. Morgan J.M. 1977. Differences in osmoregulation between wheat genotypes. Nature 270: 234-235.
20. Peet, M.M., Willits, D.H., 1998. The effect of night temperature on greenhouse grown tomato yields in warm climate. Agric. Forest Meteorol. 92, 191–202.
21. Peter M. Hanson, Jen-tzu C., George K. 2002. Gene action and heritability of high-temperature fruit set in tomato line CL 5915. Hortscience 37: 172-175.
22. Porter, J.R., 2005. Rising temperatures are likely to reduce crop yields. Nature 436, 174.
23. Saeed A., Hayat K., Khan A.A., Jqbal S. 2007. Heat tolerance studies in tomato (*Lycopersicon esculantum* Maill.). Int. J. Agri. Biol., 9(4): 649-652.
24. Semel Y., Nissenbaum J., Menda N., Zinder M., Krieger U., Issman N. 2006. Overdominant quantitative trait loci for yield and fitness in tomato. Proc Natl Acad Sci USA 103:12 981–12986.
25. Sharama P.C., Huttel B., Winters P., Khahi G., Gardner R.C., Weising K. 1995. The potential of microsatalites for hybridization and polymerase chain reaction based DNA fingerprinting of Chick-pea and related species. Electrophoresis 16: 1755-1761.
26. Weaver M.L., Timm H. 1989. Screening tomato for high temperature tolerance through pollen viability testes. Hortscience 24: 493-495.
27. Williams J.G.K., Kubelik A. R., Livak K.J., Rafalski J.A., Tingey S.V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucl. Acids Res., 18: 6531-6535.
28. Wolfe A.D., Liston A. 1998. RAPD markers. Pp.43-86 in: Soltis D.E., Solties P.S., Doyle J.J. (eds.). Molecular Systematics of Plants II: DNA sequencing. Boston.
29. Zhang Q., Shen B.Z., Dai B.Z. 1994. Using bulked extremes and recessive class to map genes for photoperiod- sensitive genetic make sterility in rice. Proc. Natl. Acad. Sci. USA 91: 8675-8679.

6/2/2010

The Risk of Primary Open Angle Glaucoma and Glutathione S-Transferase M1 and T1 Polymorphism among Egyptians

Hoiyda A Abdel Rasool[1], Shahira Riad Nowier[2], Moataz Gheith[3], Ahmed T.S. Saif [4] and Somaia Ismail[5]

Dept. of Clinical and Chemical Pathology, Fayoum University[1], Dept. of Genetics, Research Institute of Ophthalmology[2], Dept. Ophthalmology, Research Institute of Ophthalmology[3], Dept. Ophthalmology, Fayoum University[4] Dept. Medical genetics, National Research Center[5], Cairo, Egypt

Abstract: Purpose: Glaucoma, the second leading cause of blindness, is characterized by changes in the optic disc and visual field defects. The elevated intraocular pressure was considered the prime factor responsible for the glaucomatous optic neuropathy involving death of retinal ganglion cells and their axons. Extensive investigations into the pathophysiology of glaucoma now reveal the role of multiple factors in the development of retinal ganglion cell death. Genetic factors and oxidative damage have been shown to have a role in the development of primary open angle glaucoma (POAG). Glutathione S-transferases (GSTs) are a family of enzymes that inactivate xenobiotics and endogenous end products formed as secondary metabolites during oxidative stress. In humans, GSTT1 and GSTM1 deletion genotypes are associated with a variety of pathologic processes including certain ophthalmologic diseases. The aim of this study was to determine the effects of genetic polymorphisms of glutathione S transferase GSTM1 and GSTT1 on the risk of POAG in an Egyptian population. Methods: We compared the prevalence of GSTT1 and GSTM1 deletion genotypes, which were determined by multiplex polymerase chain reaction, in 32 patients with primary open angle glaucoma to 16 age, sex, and ethnically matched controls. Results: The GSTM1 positive genotype had an increased risk of developing POAG ($p< 0.05$, OR 4.681, 95% CI 1.190 – 18.412). The risk of glaucoma also increased significantly in subjects with a combination of GSTM1 positive and GSTT1 null genotypes ($p< 0.05$, OR 4.700, 95% CI 0.959 – 23.033). Conclusion: The GSTM1 positive genotype or the combination of both GSTM1 positive and GSTT1 null genotypes may be associated with the increased risk of development of POAG in the Egyptian population. The overall results indicate a possible variable association between various GSTT1 and GSTM1 genotypes and primary open angle glaucoma. Decreased GST function might interfere with the metabolism of oxidative intermediates and exacerbate the direct or indirect damaging effects of oxidative stress on the optic nerve. It is possible that these GST polymorphisms may be risk factors for primary open angle glaucoma [Journal of American Science 2010;6(12):375-381]. (ISSN: 1545-1003).

Keywords: Glaucoma; optic disc and visual field defects; primary open angle glaucoma (POAG); Glutathione S-transferases (GSTs)

1. Introduction

Glaucoma is the most common optic neuropathic process affecting human and the second most common cause of blindness worldwide (1).

It is a disease in which progressive loss of retinal ganglion cells is characterized by a recognizable pattern of both visual function loss and optic nerve head pallor and excavation. If, untreated, the natural course is towards blindness, or at least significant visual loss disability (2).

Primary open angle glaucoma (POAG), which affects almost 2% of the world population, accounts for most of the glaucoma cases. Although the pathophysiology of POAG is not precisely known, its causes are clearly multifactorial. It is a result of multiple interactive genetic and environmental effects. Although the most prominent known risk factor for developing POAG is elevated intraocular pressure, there are also other suspected risk factors such as positive family history, age, hypertension and diabetes. Its prevalence increases with age. It is well known that POAG is an age-related disorder. There is a general consensus that cumulative oxidative damage is responsible for aging, and may, therefore, play an important role in the pathogenesis of an age-related disorder such as glaucoma. Oxidant stress and antioxidant systems are potentially important for ocular tissues. Exposure to light by photosensitizing mechanisms may lead to the formation of reactive oxygen species. Many of the ocular tissues regenerate slowly, causing an increase in the risk for an accumulation of oxidant-inflicted damage in the tissue components. The damage caused by xenobiotics and oxidants can result in a number of molecular changes that contribute to the development of glaucoma, cataract, and other age related diseases. Therefore the eye must posses efficient reducing systems, as well as, detoxification enzymes such as

catalase, superoxide dismutase, glutathione peroxidase and glutathione S-transferase (GST) for protection from oxidative damage. The ocular ciliary epithelium expresses genes coding for GST and other enzymes involved in the glutathione cycle, such as glutathione peroxidase. Several epidemiological studies suggested that individual susceptibility to several disorders, including eye diseases might be connected with the GST system (2).

The glutathione S-transferases (GSTs) are a family of enzymes consisting of numerous cytosolic, mitochondrial, and microsomal proteins capable of multiple reactions with endogenous and xenobiotic substrates. They catalyze the conjugation of reduced glutathione to electrophilic centers via the sulfhydryl group on a wide variety of substrates. GSTs bind toxins, function as transport proteins, detoxify endogenous compounds such as peroxidized lipids, and inactivate endogenous end products formed as secondary metabolites during oxidative stress (1).

The GST isoenzymes expressed in human tissues comprise the alpha, mu, pi, theta, kappa, sigma, zeta and omega gene families. As many GST genes are polymorphic, there has been considerable interest in determining whether particular allelic variants are associated with altered risk (or outcome) of a variety of pathologies including cancers, cardiovascular diseases and respiratory diseases. Of these classes of GSTs, five (GSTM1, GSTM3, GSTT1, GSTP1 and GSTZ1) have been shown to be polymorphically distributed. Five mu-class genes (GSTM1–GSTM5) are situated on chromosome 1.11 Polymorphisms identified in GSTM1 are GSTM1*0, GSTM1*A and GSTM1*B. GSTM1*0 is deleted, and homozygotes (GSTM1 null genotype) express no protein. GSTM1*A and GSTM1*B differ by a single base, and thecatalytic effectiveness of the enzymes encoded by these alleles is similar. There are two theta-class genes, GSTT1 and GSTT2, located on chromosome 22.6 GSTT1 is represented by two alleles: a functional or wild allele (GSTT1*1), and a nonfunctional or null allele (GSTT1*0). Studies have shown that the GSTT1*0 allele corresponds to a total or partial deletion of the gene, causing a deficiency in enzymatic activity (3).

Because of the role of GSTs in inactivating endogenous end products formed as secondary metabolites during oxidative stress, we decided to compare the distribution of *GSTM1* and *GSTT1* polymorphisms in Egyptian patients with POAG, compared to the distribution in matching healthy controls so as to explore the possible association between different GST variants and the incidence of POAG.

2. Subjects and Methods

Patient and Control Selection:

This case–control study was comprised of 48 subjects; thirty two patients with POAG and sixteen disease-free controls. The studied subjects were recruited from the Research Institute of Ophthalmology and Fayoum University Teaching Hospital in the period from January 2009 to January 2010. A complete examination was done to detect other abnormalities, a full medical history was taken and a thorough pedigree analysis was conducted to determine consanguinity and other affected family members. An informed consent was obtained from all subjects after explanation of the nature of the study.

The sixteen age-matched healthy volunteers were selected as control group; they were non smokers and had neither diabetes nor any systemic illness. They had no family or personal history of glaucoma. They had clinical healthy appearing optic discs as demonstrated by indirect ophthalmoscope with a cup-to-disc ratio of 0.3 or lower, and glaucoma hemifield test (GHT) within normal limits. Mean intraocular pressure (IOP) level of the controls was 13.1 ± 3.0 mmHg (range 10 and 21 mmHg).

Diagnosis of POAG required all of the following: open angle: intraocular pressure higher than 21 mmHg ; characteristic optic changes (e.g., vertical cup -to-disc ratio higher than 0.6); thin or notched neuroretinal rim or disc hemorrhage ; and characteristic visual field changes. The mean IOP level was 24.2 ± 2.1 mm Hg (range 22 –28 mm Hg) at the time of diagnosis. Cup-to-disc ratios were between 0.6 and 0.9. Patients with a history of eye surgery before the diagnosis of glaucoma, or with evidence of secondary glaucoma, such as exfoliation, pigment dispersion or uveitis, were excluded. The patients with POAG who met the inclusion criteria were selected consecutively

Statistical Analysis:

Age of the patient and the control group was compared with student's *t* test. The chi-square test was applied to compare differences in gender between patients and controls. All values were represented as mean ± S.D. GSTT1 and GSTM1 genotypes were classified as either null (homozygous deletion) or non-deleted. Odds ratio (OR) with 95% confidence limits calculated by logistic regression was used to analyze the occurrence of frequencies of the GSTM1 and GSTT1 genotypes. P-values were two-tailed and a value of < 0.05 was considered statistically significant. All analyses were performed using SPSS v. 11.5 statistical analysis software.

Specimen Collection:

Two ml venous blood was collected by venapuncture in a tube containing ethylenediamine tetraacetate (EDTA) as an anticoagulant for DNA extraction.

Method:

Genomic DNA was extracted from peripheral venous blood using a salting out protocol, as described by Miller et al., 1988 (4). GSTM1 and GSTT1 genetic polymorphisms were evaluated using multiplex polymerase chain reaction (PCR) technique. The PCR primers were synthesized according to Arand et al., 1996, (5). Primers for GSTM1 were 5' – GAA CTC CCT GAA AAG CTAA AGC and 5' GTT GGG CTC AAA TAT ACG GTG G and for GSTT1 were 5' – TTC CTT ACT GGT CCT CAC ATC TC and 5' – TCA CCG GACAT GGC CAG CA. The ß – globin locus was used as an internal control to avoid false-negative readings. Primers for ß – globin were 5' – CAA CTT CAT CCA CGT TCA CC and 5' – GAA GAG CCA AGG ACA GGT AC. PCR reaction was carried out in a total volume of 25 ul containing 10 pmol of each primer, 2.5 mmol / L of MgCl2, 0.2 mmol/L of each deoxynucleotide triphosphate, 1 unit of Taq polymerase. And 100 ng of genomic DNA. Amplification was performed by initial denaturation at 94 ^{0} C for 5 minutes, followed by 30 cycles at 94 ^{0} C for 1 minute, 64 ^{0} C for 1 minute and 72 ^{0} C for 1 minute and a final extension of 72 ^{0} C for 7 minutes. The amplified products were identified by electrophoresis in a 1.5% agarose gel and stained with 0.5 ug/ml ethidium bromide. The product lengths were 215 bp, 480 bp, and 268 bp for GSTM1, GSTT1 and ß – globin, respectively. Absence of PCR product for GSTM1 or GSTT1 in the presence of the ß – globin band was indicative of a null genotype for GSTM1 or GSTT1. Individuals with one or two copies of the relevant gene were classified as a positive genotype and individuals with homozygous deletions as a null genotype.

3. Results:

Table (1) shows the demographic data for POAG patients and the control group. The mean age of the control group was 47.30 ± 11.60 years, 7 of them (43.5 %) were males and 9 of them (56.5 %) were females. The mean age of the POAG group was 51.03 ± 14.68 years, 15 of them (47.5 %) were males and 17 of them (53.5 %) were females. The groups were not statistically different with respect to age and gender ($p > 0.05$).

Table (2) shows the GST genotype distribution among all POAG patients and the control group. The frequencies of GSTT1 and GSTM1 – null genotypes were 25 % and 31.5 % respectively in the POAG patients. The proportion of GSTT1 null genotypes was higher in the POAG patients as compared to controls but with no significant difference (25% versus 6.25%) (OR: 0.183, 95% CI: 0.02–1.683). The proportion of GSTM1 null genotypes was higher in the control group as compared to the POAG group (62.5% versus 31.5%), $p<0.05$. The GSTM1 present genotype had an increased risk of developing POAG (OR: 4.681, 95% CI: 1.190-18.412).

Table 3 shows the association between GST genotype profile and the development of POAG. The data suggested a trend of decreasing risk of POAG with the combination of GSTM1 null genotype and GSTT1 positive genotype. (p: <0.05, OR: 4.700, 95% CI: 0.959-23.033).

Table (1) Demographic Data of the Study Groups

Study Groups	Control Group	POAG Group
Number of Subjects	16	32
Sex Male, n (%) Female, n (%)	 7/16 (43.5%) 9/16 (56.5%)	 15/32 (47.5 %) 17/32 (53.5%)
Age (years) Mean ± SD	 47.30 ± 11.60	 51.03 ± 14.68
Hypertension, n (%)	----	5/32 (17.5%)
Diabetes, n (%)	----	11/32 (34.5%)
Smoker, n (%)	----	10/32 (31.5%)
Consanguinity, n (%)	----	11/32 (34.5%)
Family history, n (%)	----	5/32 (17.5%)

POAG = primary open angle glaucoma

Table (2) Glutathione S Transferase (GST) Genotypes and the Risk of Developing Primary Open Angle Glaucoma (POAG).

Genotype	Control Group (N = 16)	POAG (N= 32)	OR	95% CI	P value
GSTM1[b]					
Present, n (%)	6 (37.5%)	22 (67%)	1.0	Reference	< 0.05
Null, n (%)	10 (62.5%)	10 (31.5%)	4.681	1.190 – 18.412	
GSTT1[b]					
Present, n (%)	15 (93.75%)	24 (75%)	1.0	Reference	NS
Null, n (%)	1 (6.25%)	8 (25%)	0.183	0.02 – 1.683	

OR: odds ratio CI: Confidence interval from binary logistic regression
[b]: Carriers of at least one intact allele are used as reference
POAG: primary open angle glaucoma

Table (3) Association between GST Genotype Profile and the Development of POAG

Genotype Combination						
GSTM1	GSTT1	Control Group (N =16)	POAG Group (N = 32)	OR	95 %CI	P value
Present	Present	5 (31.25%)	14 (43.5%)	1	Reference	
Present	Null	1 (6.25%)	8 (25%)	0.014	0.00 – 986.944	NS
Null	Present	10 (62.5%)	10 (31.5%)	4.700	0.959 – 23.033	< 0.05

OR: odds ratio CI: Confidence interval from binary logistic regression
POAG: primary open angle glaucoma

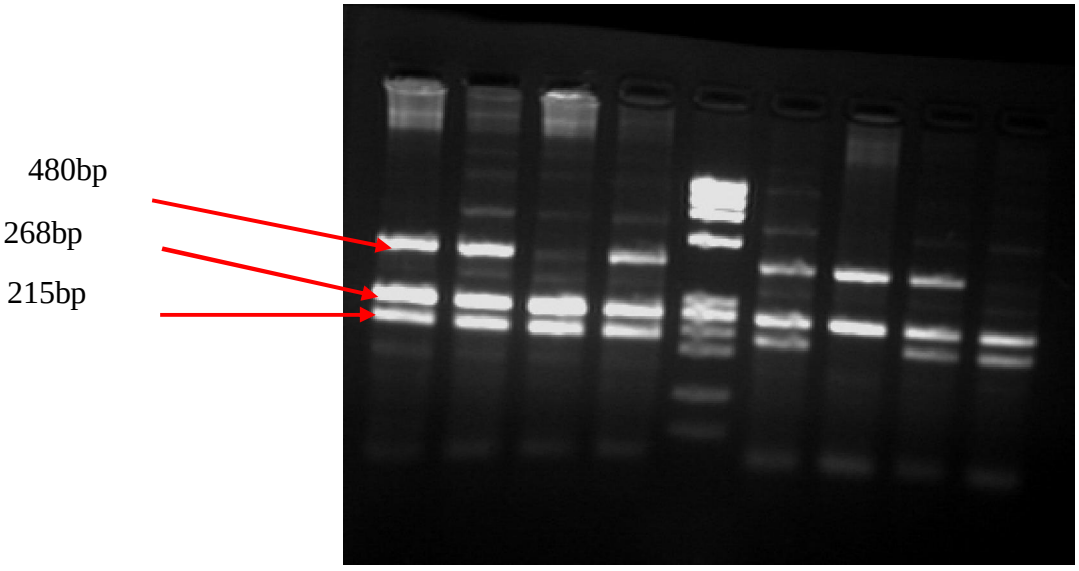
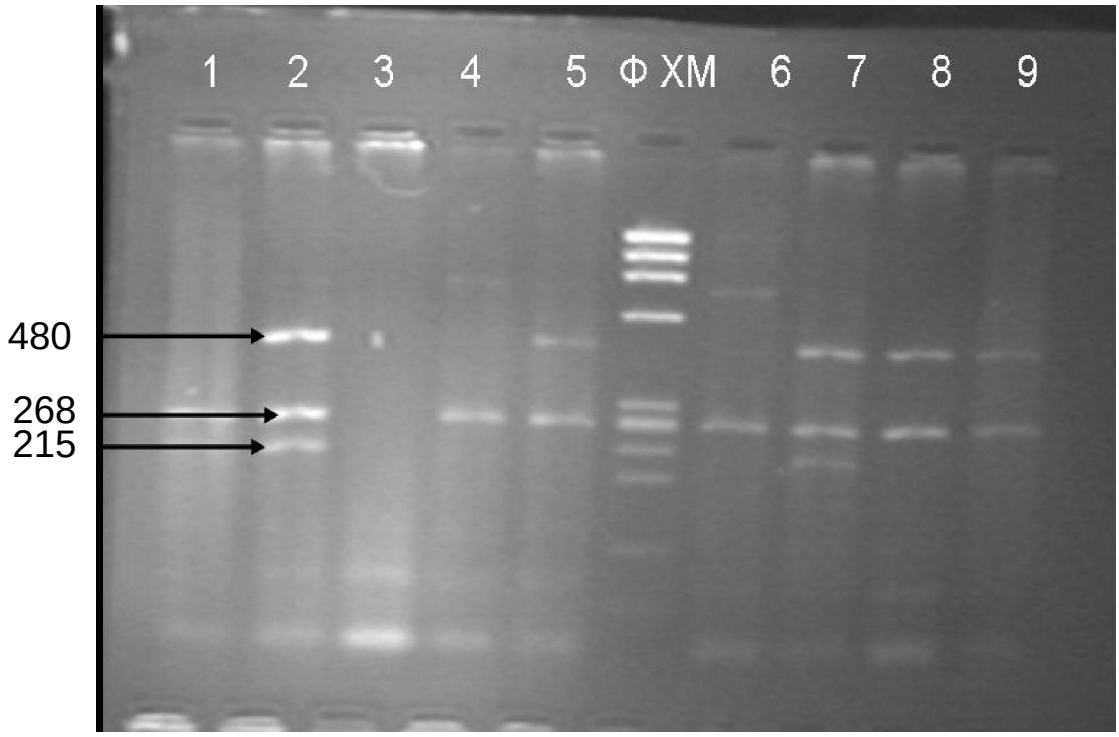


Figure (1): Amplified PCR products of the GSTT1 and GSTM1 gene polymorphism in the patients with primary open angle glaucoma (POAG). The product lengths were 215bp for GSTM1, 480bp for GSTT1 and 268bp for Beta globin. Lanes (1,2,4,5&7) heterozygous for GSTT1and GSTM1. Lanes (3&8) were homozygous deletion for GSTT1. Lane 6 was homozygous deletion for GSTM1 and then lane M was ØX marker.



Fig(2) 1.5% Agarose gel stained with ethidium bromide illustrating different band sizes in the control group Lane 1 homozygous deletion for GSTT1, Lanes (2 & 7) heterozygous for GSTT1 and GSTM1. Lanes (8&9) homozygous deletion for GSTM1.In Lane 3 there was no band due to failure in DNA extraction or in the PCR process.

4. Discussion:

The basic cause of glaucoma is largely unknown. First degree relatives of glaucoma cases have 8–10 times increased risk of developing the disease, making genetic predisposition a strong risk factor (2). Most genetic polymorphisms do not cause a recognizable change in the organism in which they occur. However, some either cause a disease or alter disease susceptibility. A large number of studies had attempted to show links between disease susceptibility and GST polymorphic variants. In addition, some studies have focused on the risk of association between the GST polymorphisms and ocular diseases including cataract, senile macular degeneration and glaucoma. In this study, we aimed to determine the effects of genetic polymorphisms of GSTM1 and GSTT1 on the risk of POAG in an Egyptian population.

As the pathogenic role of ROS in glaucoma has been suggested by many studies, cellular defense mechanisms alleviating the toxic manifestations of oxidative insult must have an important role in protection against the development of glaucoma. As GST enzymes are one of the important families of enzymes against oxidative stress, their genetic polymorphisms may alter the critical function of the enzymes in protecting against electrophiles and the products of oxidative stress in glaucoma (3).

The results presented in this study imply that defects in GST activity may well be risk factors for developing POAG. The exact mechanisms by which this occurs are not clear, which is not surprising given that the exact mechanisms of GST activity have yet to be elucidated.

Glaucoma patients in this study met strict criteria for POAG. Controls in this study were well matched to patients for age, sex and ethnicity.

In our study the GSTM1 positive genotype was significantly more common in the POAG group compared to the control group which shows a correlation between the GSTM1 positive genotype and the incidence of POAG. The results of our study are in concordance with a previous study by Unal et al, 2007 (3) in the Turkish population who found that GSTM1 positive genotype was a risk factor for developing POAG. Juronen et al, 2000 (6), who were the first to examine the possible association between the polymorphic GST genotypes and adult-onset POAG in an Estonian population, also found a similar relationship between the GSTM1 genotype and the incidence of POAG. They found that the

frequency of GSTM1 positive individuals was significantly higher in the glaucoma group compared with the control group. They suggested that the GSTM1 positive phenotype might be a genetic risk factor for the development of POAG. The same results were reported in the study by Khaled et al., 2008 (1) among an Arab population. We believe that several factors might explain the association between the GSTM1 positive genotype and POAG. Although GST enzymes catalyse detoxification reactions, they also take part in reactions that result in toxic products, which may cause structural changes in the proteins present in the trabecular meshwork and aqueous humor. This can lead to aggregation or modification of the proteins in the trabecular meshwork and promote the development of POAG (6). In addition, subjects with the GSTM1 null genotype have been shown to express fewer GST mu-class enzymes than subjects with the GSTM1 positive genotype (7 & 8). This may selectively cause stimulation of other non-toxic end products producing biotransformation enzyme systems to detoxify the substrates that were originally detoxified by the GST enzymes. Further evidence for involvement of GSTM in glaucoma comes from studies on autoimmunity. Yang et al., 2001 (9) showed that GST antigen was found in 52% of cases with glaucoma and in 20% of controls. The patients had significantly higher titres of anti-GST antibody compared with controls. Furthermore, the related retinal antigen belonged to the GST mu class (9). Thus, it may be hypothesized that people who express GSTM1 are at increased risk of developing autoantibodies against this protein, which is connected to an increased risk of developing glaucoma.

Contrary to our results Izzotti et al, 2004 (10) reported that POAG was associated with the GSTM1 null genotype in an Italian population. In addition, in another study by Yildirim et al., 2005 (2), the GSTM1 null genotype has been found to be associated with an increased incidence of POAG in a Turkish population. Another study by Jansson et al., 2003 (11) reported that there was no evidence of association between GSTM1 polymorphism and glaucoma in the Swedish population.

In our study the frequency of the GSTT1 null genotype was not statistically different between the POAG cases and the controls. The results of our study supports the study of Yildrim et al., 2005 (2) and the study by Izzotti et al., 2003 (10). Our results are contrary to Unal et al., 2007 (3) and Khaled et al., 2008 (1) where they reported that GSTT1 null genotype was significantly associated with POAG.

The combination of GSTM1 null genotype and GSTT1 positive genotype in our study showed a 4.7 fold decreased risk of glaucoma. It has been already suggested that combination of the GST polymorphisms rather than individual polymorphism make humans more susceptible to genotoxic insults (12).

Many factors might account for the difference in results between similar studies. Firstly, it may reflect the differences in the ethnic, genetic and environmental background of the populations studied. For instance, GSTT1 deficiency is less frequent than GSTM1 deficiency, but in both cases the frequency in the population varies between different ethnic groups (13). There may be differences even in the same population because of genetic and environmental factors. Secondly, the differences in the number of subjects studied in genetic researches may also lead to different outcomes. Thirdly, methodological issues should also be considered. For example, Jansson et al, 2003 (11), who reported that there was no evidence of association between GSTM1 and glaucoma in the Swedish population, used two methods for genotyping: multiplex PCR and pyrosequencing. In contrast, Juronen et al., 2000 (6), performed their analysis using only ELISA. The GST genes are located in complex genomic regions that could be affected by copy number variation and rearrangements, so different genotyping methods could give different results.

The present study suggests that the GSTM1 positive genotype may be a genetic risk factor for the development of POAG. The combination of GSTM1 null genotype and GSTT1 positive genotype decreased the risk of POAG. It has already been suggested that the combination of the GST polymorphisms rather than individual polymorphisms makes humans more susceptible to genotoxic insults.

In conclusion, this study is only one in a series of case-control studies of the possible association between glaucoma and GST. Some find evidence of GST positive genotypes being predisposing to glaucoma and others that GST positive genotypes being protective from glaucoma. These results imply that further studies of the precise mechanisms by which genetic polymorphism of metabolizing enzymes influences the nature history of glaucoma development are merited.

Corresponding author
Hoiyda A Abdel Rasool
Dept. of Clinical and Chemical Pathology, Fayoum University', Fayoum, Egypt

5. References:

1. Khaled K. Abu-Amero, Morales J., Gamal H. Mohamed, Mazen N. Osman and Thomas M. bosley: Glutathione S-transferase M1 and T1

polymorphisms in Arab glaucoma patients. *Molecular Vision* 2008; 14:425-430
2. Yildirim O., Aras N. Ates, Tamer L., Oz O., Yilmaz A., Atik U., Camdeviren H.: May glutathione S-Transferase M1 positive genotype afford protection against primary open-angle glaucoma? Graefe's Arch Clin Exp Ophthalmol, 2005, 243; 327 – 333.
3. Unal M. nal, Gu¨ven M., Devranog˘k. lu, O¨ zaydın A., Batar B. , Tamc¸N. elik, Erog˘ E. lu Go¨rgu¨n, Uc¸ar, D., Sarıcı A.: Glutathione transferase M1 and T1 genetic polymorphisms are related to the risk of primary open-angle glaucoma: a study in a Turkish population. Br J Ophthalmol 2007;91:527–530
4. Miller SA, Dykes DD and Polesky HF: (1988): A simple salting out procedure for extracting DNA from human cells, Nucleic acids Research. Vol 16, Number 3, p. 1215.
5. Arand M, Muhlbauer R, Hengstler J et al, A multiplex polymerase chain reaction protocol for the simultaneous analysis of the glutathione S-transferase GSTM1 and GSTT1 polymorphisms. Anal Biochem. 1996; 236: 184 – 186.
6. Joronen E., Tasa G., Veromann S., Parts L., Tiddla A., Pugles R., Panov A., Soovere L., Koka K. and Mikelssar A.V.: Polymorphic glutathione S-transferase M1 is a risk factor of primary open angle glaucoma among Estonians. Exp. Eye Res. 2000: 71; 447 – 452.
7. Anttila S, Luostarinen L, Hirvonen A, et al. Pulmonary expression of glutathione S-transferase M3 in lung cancer patients: association with GSTM1 polymorphism, smoking, and asbestos exposure. Cancer Res 1995;55:3305–9.
8. Nakajima T, Elovaara E, Anttila S, et al. Expression and polymorphism of glutathione S-transferase in human lungs: risk factors in smoking-related lung cancer. Carcinogenesis 1995;16:707–11.
9. Yang J, Tezel G, Patil RV, et al. Serum autoantibody against glutathione Stransferase in patients with glaucoma. Invest Ophthalmol Vis Sci 2001;42:1273–6.
10. Izzotti A, Bagnis A, Sacca SC. The role of oxidative stress in glaucoma. Mutat Res 2006;612:105–14.
11. Jansson M, Rada A, Tomic L, et al. Analysis of the glutathione S-transferase M1 gene using pyrosequencing and multiplex PCR—no evidence of association to glaucoma. Exp Eye Res 2003;77:239–43.
12. Tars K, Larsson AK, Shokeer A, et al. Structural basis of the suppressed catalytic activity of wild-type human glutathione transferase T1-1 compared to its W234R mutant. J Mol Biol 2006; 355:96–105.
13. Johansson AS, Mannervik B. Interindividual variability of glutathione transferase expression. In: Pacifici GM, Pelkonen O, eds. Interindividual variability of glutathione transferase expression. London: Taylor & Francis, 2001:460–519.

6/1/2010

Determination of milk urea nitrogen for the Egyptian cattle fed the summer and winter diets.

Ahlam El Shewy; Sobhy Kholif; Tarek Morsy
Dairy Sci. Dept. National Research Center, Dokki, Giza, Egypt.
ahlam58aa@yahoo.com

Abstract: Milk urea nitrogen (MUN) equilibrates with and is proportion to blood urea nitrogen. So, it is an excellent indicator of urea nitrogen status in dairy cows. The objective of this study was to determine the MUN during the summer (with a temperature range of 35-40°C) and winter (with a temperature range of 18-22°C) seasons. Forty hetero- parity lactating cattle twenty of each cows and buffaloes, at different stages of lactation were used to collect milk samples. All animals received the diet consisting of concentrate, fodder, and rice straw as 2:1:1 on DM basis. The fodder was berseem(*Trifolium alexandrium*) and rayana corn(*Zea mays mexicana*) in the winter and summer, respectively. The dietary crude protein was 11.38 and 8.97 % and the dietary gross energy was 3.86 and 3.83 Mcal/kg DM for the winter and summer diets, respectively. The results indicated, milk protein content was 3.06 and 3.18 % and MUN was 24.57 and 28.00 mg/dl for cows, while milk protein was 3.96 and 2.67 % and MUN was 19.60 and 28.03 mg/dl for buffaloes during the winter and summer seasons, respectively .This study revealed that the heat- summer significantly (P<.05) increased MUN of lactating buffaloes and this phenomenon needs further studies. [Journal of American Science 2010;6(12):382-384]. (ISSN: 1545-1003).

Keywords: dietary protein, cow, buffaloes, milk urea nitrogen.

1. Introduction

Milk urea nitrogen is the major single contributor to milk non – protein – nitrogen. Milk urea is derived primarily from blood urea which is produced from excess ruminal ammonia and amino acids catabolism in the liver (De Peters and Ferguson, 1992). In the mammary gland, urea diffuses into and out of the mammary gland cells, equilibrating with urea in the blood. Because of this process, milk urea nitrogen (MUN) equilibrates with and is proportional to blood urea nitrogen. This equilibration allows MUN to be an excellent indicator of urea nitrogen status in dairy cows (Roseler et al.1993)

Several studies have shown an association between MUN and fertility reporting that increasing MUN levels appear to be negatively related to dairy cow fertility and are associated with a lower risk of detectable pregnancy at herd checks (Butler et al .1996) and Rajala – Schultz et al . (2001) .

MUN concentrations provided a reliable estimate of dietary crude protein content, and provided an accurate prediction of urinary – nitrogen – excretion (Ciszuk and Gebregziabher, 1994, Kohn, et al. 1997, Jonker, et al. 1998, Froidmont, et al. 2003, Nousiamen, et al. 2004 and Huhtanen, et al .2008) .

Concerning the factors affecting the MUN in cattle, it could be reported that dietary concentrations of crude protein and the value of ruminal protein balance (PBV) were the main nutritional factors influencing MUN. It is of interest to define PBV by the difference between the ruminal degradable protein (RDP) supply and microbial requirements of RDP (Nousiamen, et al .2004 and Huhtanen et al .2008).

The purpose of this study was to determine the MUN concentrations for lactating cattle fed the summer and winter diets in Egypt.

2. Material and Methods

Forty hetero –parity lactating cattle, twenty of each cows and buffaloes, at different stages of lactation were used to collect milk samples during the winter and summer months, in the Atfih village of the Helwan governorate.

The day of winter months (January and February) was with a temperature range of 18-22°C, while it of the summer months (June and July) was with a temperature range of 35-40°C. All animals received the same diet consisting of concentrate, green fodder, and rice straw at 50:25:25 (on DM basis), respectively. The green fodder was berseem (*Trifolium alexandrium*) and rayana corn *(Zea mays mexicana*) for the winter and summer, respectively .Drinking water was available *ad. lib.* The chemical composition of feed ingredients is shown in Table 1. The analytical methods were performed according to A.O.AC., 1995.

All animals were milked individually twice daily .For each animal ,milk yield was daily recorded .Milk samples were taken biweekly and frozen at -18°C until analysis for the milk protein and urea by the infra red spectro photometry (Foss 120 Milko Scan, Foss Electric, Hillered, Denmark) according to A.O.A.C., 1995.

The dietary crude protein content, dietary gross energy, and the average of the daily milk yield are shown in Table 2. The gross energy was calculated by the equation suggested by Blaxter, 1968, as follows:
Gross energy (Mcal/ Kg DM)= 4.15 CF+ 5.65 CP + 9.40 EE + 4.15 NFE.
Dietary CF,CP,EE,and NFE were calculated on the basis of contents illustrated in Table 1.

Analysis of variance was conducted according to Snedecor and Cochran, (1982). Since, four replications of a 2 ×2 factorial arrangement of groups were used . The applied model was:

$y_{ijk} = \mu + A_i + D_j + W_k + (AD)_{ij} + E_{ijk}$

whereas, y_{ijk} is an observation, μ is a population mean, A_i is effect of animal type (cow or buffalo), D_j is an effect of diet (summer of winter diet), W_k is an effect of sampling time (biweekly), $(AD)_{ij}$ is an interaction effect between animal and diet, and E_{ijk} is a residual error. The differences among means were tested using Tukey test according to the same reference.

3. Results

Data of Table 2 clearly indicated that the CP content of the summer diet (8.97 %) decreased by 20 % of that for the winter diet (11.38 %). It is a reliable result attributing to having the winter fodder (berseem) CP (13.8 %) as three times of that of the summer fodder (rayana corn) (4.15 %).

Also, it could be reported that both diets contained equal calories. The winter diet had 3.86 Mcal/kg DM, and the summer diet had 3.83 Mcal/kg DM.

Milk protein and milk urea nitrogen

Data of Table (3) indicated that milk of lactating Baladi cows contained 3.06 and 3.18 g protein / 100 ml milk for cows fed the winter and summer diets, respectively. The data showed, also, the average of milk protein content was 3.96 and 2.67 % for buffaloes fed berseem and rayana corn during the winter and summer, respectively. Statistically, non significant difference (P > .05) was noticed between the cows averages, while a high significant variance (P<.05) was detected between the buffaloes means.

Obviously, the values of milk urea nitrogen (MUN) have taken the same trend of milk protein contents for lactating Baladi cows during the winter and summer (24.57 vs. 28.00 mg/ dl, respectively) .However, there was a high significant difference between the MUN averages (19.60 and 28.03 mg/ dl milk) of lactating buffaloes during the winter and summer, respectively.

Table 1. The chemical composition of feed ingredients

Ingredients	DM %	Contents on DM basis %				
		CF	CP	EE	NFE	Ash
Concentrate[1]	93.49	15.23	14.10	04.08	53.14	13.45
Berseem	18.00	27.40	13.80	02.60	42.04	14.16
Rayana corn	23.26	25.47	04.15	03.42	52.82	14.14
Rice straw	91.80	34.20	03.50	01.40	52.47	08.43

1Concentrate feed mixture

Table 2. Dietary CP ,Dietary gross energy ,and milk yield

Item	Diets			
	winter		summer	
Dietary CP %	11.38		08.97	
Gross energy Mcal/Kg DM	03.86		03.83	
Item	Cattle			
	Cows		Buffaloes	
	winter	summer	Winter	Summer
Milk yield Kg/h/d	05.55	05.21	06.90	5.7

Table 3. Milk protein and milk urea nitrogen of lactating cattle fed winter and summer diets .

Item	Cows Diet		Buffaloes Diet		SE
	winter	summer	winter	summer	
Milk protein content %	3.06^{cb}	3.18^{ab}	3.96^{a}	2.67^{d}	0.47
Average %	3.12		3.31		0.095
Milk urea nitrogen (mg/dl)	24.57^{cb}	28.00^{ab}	19.60^{d}	28.03^{a}	3.45
Average (mg/dl)	26.28		23.82		1.23

a,b,c,d means in the same row with different superscripts differed significantly at (P ≤ 0.05) .

4. Discussion

First of the all, it could be reported that buffaloes not cows exposed to heat – stress when an ambient temperature ranged from 28 to 44°C (Ross Cockrill, 1974). In the present work, the summer was with a temperature range of 35-40°C.

The significance increasing of MUN for buffaloes may possibly be related to a change in the acid – base balance caused by the heat- stress. Also, MUN increasing in buffalo milk can reflect adverse effects on the buffalo fertility and on the nitrogen emission during the hot summer in Egypt.

5. Conclusion

The heat – summer had an adverse effect only on the lactating buffaloes not cows, whereas, their MUN significantly increased ($P \leq 0.05$) by ≈ 40% in the summer comparing with that in the winter.

6. Comment

In our opinion an additional research is needed to explain why MUN level was high for buffaloes in the summer and to decrease this level - may be - by the DCAD tool or by the urease inhibitors tool . DCAD means the dietary- cation – anion – difference .

References :

1. A.O.A.C**.** Official Methods of Analysis 16th ed .Association of Analytical Chemists , Washington , DC . 1995
2. Blaxter K.L**.** The Energy Metabolism Ruminants, 2nd ed., Charles Thomas, Publisher, Springfield, Illinois,USA**.** 1968.
3. Butler W.R ., Calaman J . J., Beam S.W .. Plasma and milk urea nitrogen in relation to pregnancy rate in lactating dairy cattle . J.Anim .Sci 1996; 74 :858 -865
4. Ciszuk A.U ., Gebregziabher T**.**. Milk urea as an estimate of nitrogen of urine nitrogen of dairy cows and goats . Acta. Agric. Scand. 1994; 44: 87 – 95.
5. De peters E.J., Ferguson J.D., Non protein nitrogen and protein distribution in the milk of cows . J . Dairy Sci. 1992; 75 :3192 .
6. Froidmont E., Bartiaux – Thill N., Frand X , .Decruyenaere V; Van Reusel A ., Fabry J.. Milk urea concentration as a tool to evaluate protein / energy balance of dairy cows feeding. Dairy Sci.. Abstract 2003 ; Vol.65. No.2.page :125 (776) .
7. Huhtanen P, Nousiainen J. I . Rinne M . , Kytola k, khalili H .Utilization and partition of dietary nitrogen in dairy cows fed grass silage – based diets . J .Dairy Sci. 2008**;** 91 : 3589-3599
8. Jonker J. S . , Kohan R .A, Erdman R.A. Using milk urea nitrogen to predict nitrogen excretion and utilization efficiency in lactating dairy cows .J. Dairy Sci. 1998; 81 : 2681-2692 .
9. Kohn R.A., Jonker J.S., Erdman R.E., High J. Milk urea nitrogen: theory and practice. Pages: 83-90 in Proc. Maryland Nutr . Conf. Feed Manuf ., Boltimore . Univ . Maryland College Park 1997.
10. Nousiamen J., Shingfield K.J., Huhtanen P., Evaluation of milk urea concentration as a diagnostic of protein feeding .J. Dairy Sci. 2004; 87: 386398 .
11. Rajala **–** Schultz P.J. , Saville W . W . A., Frazer Nations G.S., Wittum T.E. Association between milk urea nitrogen and fertility in Ohio dairy cows. J.Dairy Sci. 2001; 84 :482-489
12. Roseler D.k . , Ferguson J. D., Sniffen C.J. , Herrema J. Dietary protein degradability effect on plasma and milk urea nitrogen and milk non protein nitrogen in Holstein cows. .J. Dairy Sci. 1993; 76 : 525-534.
13. Ross Cockrill W. The husbandry and health of the domestic buffalo .Food And Agriculture Organization of The United Nations Rome ,1974**.**
14. Snedecor G.W., Cochran. W. G. Statistical Methods. 7th ed . Iowa State Univ . Press Ames Iowa. USA. 1982 .

6/3/2010

In Vitro Propagation of *Tylophora indica*-Influence of Explanting Season, Growth Regulator Synergy, Culture Passage and Planting Substrate

Sulekha Rani, J S Rana*

Department of Bio & Nano Technology, Guru Jambheshwar University Of Science & Technology
Hisar, Haryana (India) -125001, jogenderrana@yahoo.co.uk

Abstract: An efficient protocol for rapid clonal propagation of an endangered medicinal plant, *Tylophora indica* (Burm. *f.*) Merrill through *in vitro* culture is described. High frequency bud break (85%) and multiple shoot formation were induced from nodal segments explanted between September through November and cultured on MS medium supplemented with 2.0mg/l BAP. Although callus- free multiple shoot formation was a function of cytokinin activity alone, faster bud break coupled with enhanced frequency of shoot development (95%) and internode elongation were dependent on the synergistic effect of GA_3(0.2mg/l). By repeated sub culturing of nodal segments harvested from the newly formed axenic shoots, prolific shoot cultures, free of proximal callusing, showing a high frequency multiplication rate were established within three months. The percentage shoot multiplication as well as the number of shoots per node attained the highest values (100%, 7 shoots/node) during the first two culture passages; beyond this there was a gradual decline in shoot bud differentiation. Rooting of the excised shoots from secondary or subsequent cultures was best induced on ½ strength MS medium containing 0.5 mg/l IBA. Vermi-compost was the most suitable planting substrate for hardening and its use ensured high frequency survival (96%) of regenerated plantlets prior to outdoor transfer. Regenerated plants get established in pots containing garden soil followed by their transfer to natural soil under full sun. The *in vitro* regenerated plants were uniform and identical in growth characteristics and morphology to the donor plants. [Journal of American Science 2010;6(12):385-392]. (ISSN: 1545-1003).

Keywords:*Tylophora indica,* medicinal plant, micropropagation, culture media, explants, growth regulators

1. Introduction

Biotechnology has provided several unconventional techniques for crop improvement and its use is revolutionizing traditional plant breeding methods. Plant propagation *via* organogenesis is one of the techniques used in plant tissue culture to obtain large number of plants, irrespective of season and with conservation of space and time. Organogenesis has been reported in many species and factors influencing it has also been studied intensively (Koroch *et al*., 2002; Thao *et al*., 2003; Yemets *et al*., 2003).

Tylophora indica (Burm *f.*) Merrill (Asclepiadaceae) is a threatened medicinal climber shrub native to the plain and hill forests of eastern and southern India up to an altitude of 900 m. The plant has been traditionally exploited by tribes in certain regions of India for the treatment of various ailments (Anonymous, *1976*). The plant contains several phenanthroindolizidine alkaloids (Gellert, *1982*) and pharmacological investigations have confirmed the anti-asthmatic effects of its leaf extracts (Shivpuri *et al*., *1972*). The major alkaloid present—tylophorine—has been reported to have immunosuppressive, anti-inflammatory (Gopalakrishnan *et a*l., *1980*) and anti-tumor (Donaldson *et al*., *1968*) properties. The powdered leaves, stems and roots also contain other minor alkaloids (Rao and Wilson, 1971) including tylophorinine, cryptopleurine, antofine and ficuseptine C which are pharmacologically active and anticancer tylophorinidine has also been isolated from the roots of three-year old plant (Mulchandani *et al*., 1971). Thus the plant is in great demand for the production of traditional and modern medicines.

Owing to large scale and uncontrolled exploitation of this natural resource in order to meet its ever-increasing demand in the pharmaceutical companies, wild stock of this plant species has been markedly depleted over past few years. Unfortunately, efforts for its replenishment by conventional cultivation have been handicapped, because it is not amenable to vegetative propagation through cuttings, and propagation through seeds would result in variation. Therefore, the application of a reliable, *in vitro* clonal propagation system would provide an alternative method of propagation to meet the pharmaceutical needs and for effective conservation of this precious plant species. It is advantageous for conservation of germplasm and multiplication of best genotypes with high alkaloid contents.

Although micro propagation of *T. indica* by axillary shoot induction and adventitious shoot production (Sharma and Chandel, *1992;* Faisal *et al.*,

2007) and callus-mediated somatic embryogenesis from leaf (Jayanthi and Mandal, *2001;* Chandrasekhar *et al*., 2006) and inter nodal (Thomas, *2006*) explants have been previously reported but these studies were of preliminary nature and more studies and further refinements of techniques were absolutely necessary. Hence in the present investigation we evaluate various factors influencing *in vitro* axillary shoot proliferation and resulted in an efficient and reproducible procedure for rapid clonal multiplication of this pharmaceutically important plant species.

2. Material and Methods

2.1 Plant material and sterilization

During different month's healthy nodal explant (0.4-0.6 cm) were collected from plants of *T. indica* from 5-year-old plants grown in the medicinal plant garden of CCS HAU, Hisar (Haryana, India). The explants were initially soaked in 5% (v/v) liquid detergent (Teepol, Reckitt and Colman, India) for 10 min, then washed under running tap water for 30 min and rinsed in distilled water. The explants were surface sterilized with 0.1% (w/v) $HgCl_2$ for 10 min and thoroughly rinsed four or five times with sterile double distilled water.

2.2 Culture media and conditions

The basal culture medium used for the present study was MS (Murashige and Skoog, 1962) medium supplement with 100mg/l (w/v) myo-inositol and 3% (w/v) sucrose. To this medium was further added 0.25-5.0 mg/l BAP(6-Benzylaminopurine) or Kin (Kinetin) and 0.1-0.5 mg/l GA_3 (Gibberellic acid)) was tested either individually or in combination with BAP or KIN. The pH of the media was adjusted to 5.8±0.1 before gelling it with 0.8% (w/v) agar (Merck, India) and dispensed in 25-ml aliquots into 200 ml screw capped glass jars (Excel glasses Ltd. Allepey, India) or 150 ml Erlenmeyer flasks (Borosil, India) prior to autoclaving at 121°C for 20 min. The surface disinfected explants were implanted horizontally on the culture medium (5 explants per jar or flask). Cultures in all experiments were incubated in a growth chamber maintained at 24±1°C and 60-65% relative humidity under a 16/8-h (light/dark) photoperiod with light supplied by cool-white fluorescent tubes (Philips, India) at an intensity of 48 µmol m^{-2} s^{-1}.

2.3 Multiplication of shoots

Primary shoots formed *in vitro* were isolated and sectioned into one-node pieces after removing the leaves. These nodal segments, each containing the axillary bud, were cultured on MS medium fortified with 2.0 mg/l BAP+0.2mg/l GA_3 for further multiplication. Subsequent subcultures were in the same medium at a periodic interval of 4 weeks.

2.4 Rooting of shoots

For root induction, the shoots (ca. 4-5 cm high) with three to five leaves were harvested from secondary cultures and transferred to ½ strength MS medium containing 2% (w/v) sucrose and 0.8% (w/v) agar. The medium were further supplemented with 0.5-2.0 mg/l IAA (Indole-3-Acetic Acid), NAA (α-Napthaleneacetic Acid) or IBA (Indole Butyric Acid). Data were recorded on the percentage of rooting, the mean number of roots per shoot and the root length after four weeks of transfer onto the rooting medium.

2.5 Acclimatization and transfer of plantlets to soil

Plantlets with well developed roots were removed from the culture medium and after washing the roots gently under running tap water; the plantlets were transferred to plastic pots containing autoclaved garden soil or artificial soil or vermi-compost moistened with autoclaved tap water. Two different types of artificial soil were examined viz. vermiculite and soilrite mix. The potted plantlets were maintained inside the plant growth chamber set at 25±1°C, 80–85% relative humidity under a 16/8-h (light/dark) photoperiod with light supplied by cool-white fluorescent tubes (Philips, India) at an intensity of 50 µmol m^{-2} s^{-1}. After 30 days the plantlets were transplanted to earthenware pots containing garden soil and kept under shade in green house for another 2 weeks before transferring to outdoors under full sun.

3. Results

3.1 Shoot Proliferation

Bud breakage and development of shoots from nodal explants was a function of cytokinin activity. The morphogenic responses of nodal explants collected during September through November to various concentrations of cytokinins are shown in Table 1. There was no sign of bud break even after 30 days on MS basal medium without any growth regulator supplement. Of the two cytokinins tested BAP was more effective than Kin in inducing bud break as well as multiple shoot formation. There was a linear correlation between the increase in concentration of BAP up to the optimum level (2.0 mg/l) and percentage shoot development. The number of shoots per explant also increased with increments in BAP concentrations up to the optimum level (2.0 mg/l). MS containing 2.0 mg/l BAP induced bud break in ca. 84% of the nodal explants. The explants cultured on the medium showed their first response by an initial enlargement of the existing axillary bud following bud break within 10-12 days. From each developing bud a single shoot elongated within 12-15 days. These shoots attained a height averaging 4.20 cm in 30 days bearing 4-6 leaves (Table 1). All

regenerated shoots were free from callus formation at their proximal ends. The percentage bud break and induction declined with the increase in BAP concentration beyond the optimal level (2.0 mg/l, Table 1). Above 2.0 mg/l, BAP caused a suppression of sprouting. Multiple shoots were induced from each sprouted bud at higher concentrations of BAP (5.0 mg/l), but these failed to elongate (Table 1). On the other hand, when MS containing a singular supplement of GA_3 was used, the sprouting period was lengthened, but percentage bud break and shoots/explant were substantially reduced. A medium of only 14-16% of the axillary buds sprouted within 18-20 days of culture on MS with an optimal concentration of GA_3 (0.2 mg/l) alone. Only one or two shoots were formed which attained a height averaging 4.5 cm within 30 days of culture. Concentrations of GA_3
higher than 0.2 mg/l had no promotive influence on percentage shoot development and on shoot number per explant. However, there was a progressive increase in shoot length at increasing GA_3 concentrations. A combination of optimal concentration of BAP (2.0 mg/l) and GA_3 (0.2 mg/l) in the culture medium not only induced a faster bud break (within 7 days) but also enhanced the frequency of bud break (ca. 95%). In addition a BAP-GA_3 coupling had a synergistic effect on multiple shoot formation (Fig. 1A) as well as on internode elongation (6.5 cm high shoots after 30 days, Table 1). The synergistic effect was also noticed in a combination of Kin (2.0 mg/l) and GA_3 (0.3 mg/l), but with respect to percentage shoot development, shoot number and shoot length it was inferior to BAP- GA_3 coupling.

3.2 Influence of Explanting Season on Culture Establishment

The shoot proliferation was greatly influenced by the month of the year during which the explants had been collected. Highest frequency bud break (95%) coupled with maximum number of shoots formed (four to five shoots/explant) occurred with nodal explants excised between September through November representing autumn season in India. Other explanting periods were comparatively less suitable (Table 2), the period between December to February (winter season) being the least responsive.

3.3 Nodal Multiplication of Axenic Shoots

Nodal segments excised from the primary, *in vitro* raised shoots cultured on MS containing 2.0 mg/l BAP plus 0.2 mg/l GA_3 produced six to eight shoots per node within 4 wk. By repeated subculturing of nodal segments from the newly formed axenic shoots harvested from each culture passage at every 4 week interval, prolific shoot cultures free from basal callusing was established within three months. The percentage shoot multiplication as well as the number of shoots per node attained the highest values (100%, 7 shoots/node) during the first two culture passages, beyond which there was a gradual decline in shoot bud differentiation (Fig.2).

3.4 Rooting of regenerated shoots

The *in vitro* regenerated shoots were transferred to ½ MS medium with or without auxins. Root formation from the basal cut portion of the shoots was observed one week after transfer to the rooting medium and rooting frequency gradually increased over time & reached a maximum after four weeks of culture. The presence of an auxin (IAA, IBA or NAA) at a low concentration in ½ MS medium was found to be more effective for rooting (Fig.1B) and the best rooting was achieved in the medium fortified with 0.5 mg/l IBA; fairly good shoot numbers (4.55 ± 0.40) and root lengths per shoot (5.25 ± 0.08) were obtained (Table 3).

3.5 Evaluation of Planting Substrates for Acclimatization Prior to Outdoor Transfer

Plantlets with five to six leaves and well developed roots were successfully hardened off in the environmentally controlled growth chamber in the selected planting substrates for 5 wk and eventually established in natural soil. Of the four different types of planting substrates used, the highest survival rates for the micropropagated plants were achieved in vermi-compost (96%, Fig.1C), followed in decreasing order by that in soilrite mix (82%), vermiculite (68%) and garden soil (66%) (Table 4). All plants survived following transfer from the vermi-compost to natural soil (Fig. 1D). There was no detectable variation among the potted plants with respect to morphological and growth characteristics.

Table 1. Morphogenic Response of Nodal Explants of *T. indica* to Different Concentrations of Cytokinins (KIN, BAP) and/or GA_3

Growth Regulator(mg/l)	Shoot Development (%)	Shoot Number/Explant	Shoot Length (cm)
0.0	-	-	-
KIN			
0.25	28.86 ± 1.76	1.80 ± 0.00	1.38 ± 0.07
0.5	43.52 ± 1.32	1.12 ± 0.12	1.82 ± 004
1.0	57.96 ± 1.45	1.56 ±0.13	1.98 ± 0.03

2.0	61.86 ± 1.74	1.66 ± 014	2.86 ± 0.06
5.0	41.55 ± 1.46	1.35 ± 0.06	0.55 ± 0.04
BAP			
0.25	46.86 ± 1.36	1.21 ± 1.76	1.86 ± 0.11
0.5	67.52 ± 1.22	1.85 ± 1.32	2.88 ± 0.16
1.0	76.96 ± 1.35	3.66 ± 1.45	2.20 ± 0.17
2.0	84.86 ± 1.74	3.98 ± 1.64	4.10 ± 0.09
5.0	41.55 ± 1.46	1.55 ± 1.40	0.55 ± 0.10
GA_3			
0.1	14.50 ± 1.70	1.12 ± 1.45	4.0 ± 0.16
0.2	16.00 ± 0.24	1.18 ± 0.74	4.5 ± 1.00
0.3	12.90 ± 1.38	1.12 ±0.40	5.00 ± 0.45
0.4	10.86 ± 1.94	1.10 ± 0.24	6.10 ±0.74
0.5	10.00 ± 1.13	1.00± 0.13	6.55 ± 0.46
KIN + GA_3			
2.0 + 0.2	68.56 ± 1.76	2.66 ± 0.76	4.46 ± 0.78
2.0 + 0.3	74.52 ± 1.32	2.90 ± 0.30	5.52 ±0.22
2.0 + 0.4	72.90 ± 1.47	2.95 ±0.12	5.96 ± 0.43
BAP + GA_3			
2.0 + 0.2	95.00 ± 1.70	4.86 ± 1.76	6.80 ± 0.70
2.0 + 0.3	80.52 ± 1.82	4.50 ± 1.32	6.10 ± 0.35
2.0 + 0.4	77.96 ± 1.65	4.00 ± 0.45	6.00 ± 0.40

Data presented as the mean value ± standard error after 30 days of culture from four independent experiments each with 20 replicates.

Table 2. Influence of Explanting Period on Culture Establishment of *T. indica* on MS supplemented with BAP (2.0 mg/l) and GA_3 (0.2 mg/l)

Months of Collection	% Bud Break	Shoot Number/Explant
December – February	58.48 ± 1.15	2.30 ± 0.11
March – May	67.55 ± 2.40	2.25 ± 0.08
June – August	79.20 ± 2.35	4.10 ± 0.19
September - November	95.74 ± 3.19	4.50 ± 0.20

Data presented as the mean value ± standard error after 30 days of culture from four independent experiments each with 20 replicates.

Table 3. Effect of Auxins on root formation of *in vitro* formed shoots of *T. indica* in half-strength MS medium.

Auxins (mg/l)			Rooting (%)	Mean number of roots/shoot	Mean root length (cm)
IAA[1]	IBA	NAA			
–	–	–	70	2.70 ± 0.19	2.50 ± 0.30
0.5	–	–	70	3.65 ± 0.30	3.00 ± 0.25
1.0	–	–	50	1.90± 0.25	2.75 ± 0.20
2.0	–	–	20	1.48 ± 0.15	2.30 ± 0.10
–	0.5	–	90	4.55 ± 0.40	5.25 ± 0.08
–	1.0	–	65	2.20 ± 0.35	4.80 ± 0.19
–	2.0	–	28	1.74 ± 0.19	3.10 ± 0.17
–	–	0.5	79	3.75 ± 0.60	2.49 ± 0.25
–	–	1.0	54	2.40 ± 0.21	2.00 ± 0.11
–	–	2.0	19	1.70 ± 0.25	1.98 ± 0.10

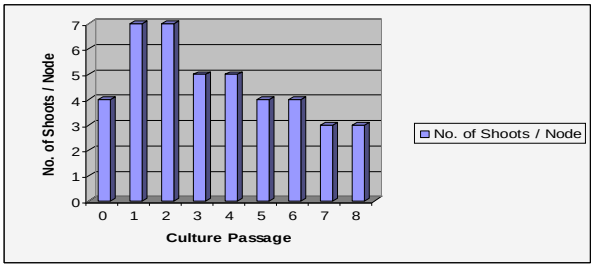
Data presented as the mean value ± standard error of roots formed after 30 days of culture from four independent experiments each with 20 replicates.

Table 4. Evaluation of Different Planting Substrates for Acclimatization of *In Vitro* Shoots

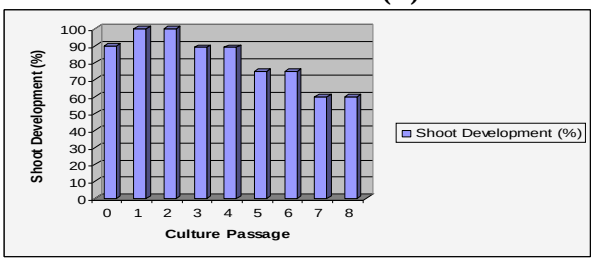
Planting Substrate	Number of Plants Transferred	Number of Plants Survived	Survival (%)	Shoot Height (cm)	Leaves/Plantlet	Root Length (cm)
Vermiculite	50	34	68	6.65 ± 1.19	6 ± 3	8.70 ± 0.12
Soilrite mix	50	41	82	12.65 ± 1.30	8 ± 1	8.60 ± 0.30
Vermi-compost	50	48	96	15.90± 1.15	10± 2	9.60± 0.25
Garden soil	50	33	64	8.48 ± 1.15	9 ± 1	6.40 ± 0.12

Data presented as the mean value ± standard error after 30 days of culture on planting substrates.

Figure 1. A-D.
A - Multiple Shoot formation from Nodal Explants of *T. indica* on MS + 2.0 mg/l BAP + 0.2 mg/l GA_3.
B – Rooted Shoot of *T. indica* on ½ MS + IBA (0.5mg/l)
C – A regenerated *T. indica* plant acclimatized on Vermi-compost.
D – *T. indica* plant established on natural soil.



(A)



(B)

Figure 2. A-B: Secondary shoot multiplication using nodal segments from primary -shoot cultures through successive culture passage (4 week) on MS + 2.0 mg/l BAP + 0.2 mg/l GA_3.

4. Discussions

Nodal segments containing axillary buds have quiescent or active meristems depending upon the physiological stage of the plant. These buds have the potential to develop into complete plantlets. In nature, these buds remain dormant for a specific period depending on the growth pattern of the plant. However, using tissue culture techniques, the rate of shoot multiplication can be greatly enhanced by performing axillary bud culture in a nutrient medium containing suitable combinations and concentrations of growth regulators.

In the present study, the stimulatory effect of a singular supplement of BAP on bud break and multiple shoot formation in *T.indica* was similar to that reported earlier in other medicinal species including *Chlorophytum borivilianum* (Purohit *et al*., 1994) and *Ocimum spp.* (Pattnaik & Chand, 1996 and Sahoo *et al*., 1997). Our observations on the suppression of sprouting at higher BAP concentrations were in accordance with those of Pattnaik & Chand (1996) in *O. americanum* and *O. sanctum*. In another species of *Ocimum viz. O. basilicum*, although the nodal segments required the presence of BAP at 1.0 mg/l at the initial stage of bud break their further growth and proliferation demanded transfer to a medium containing BAP at a relatively low concentration (0.25 mg/l) (Sahoo *et al*. ,1997).

Singular supplement of GA_3 at an optimal concentration (0.2 mg/l) had promotive influence on shoot development but in *Plumbago indica* (Nitsch & Nitsch, 1967) and *Duboisia myoporoides* (Kukreja & Mathur, 1985), GA_3 has been shown to suppress shoot bud differentiation. Thus the role of GA_3 in shoot induction and development in medicinal plant species remains controversial.

The synergistic effect of BA in combination with an auxin has been demonstrated in many medicinal plants from the Asclepiadaceae family, such as *Gymnema sylvestre* (Reddy *et al*., *1998*), *Holostemma annulare* (Sudha *et al*., *1998*), *Hemidesmus indicus* (Sreekumar *et al*., *2000*), *Holostemma ada-kodien* (Martin ,*2002*), *Leptadenia reticulata* (Arya *et al., 2003*), and *Ceropegia candelabrum* (Beena *et al., 2003*). In accordance with these reports, the present work studied the effect of low concentration of cytokinin (BAP and Kin) in combination with GA_3 on shoot induction efficiency and BAP- GA_3 coupling had shown a noticeable synergistic influence on multiple shoot formation. The promotive effect of GA_3 in combination with BAP on shoot bud induction was also reported earlier in other perennial medicinal herbs including *O. americanum* and *O. sanctum* (Pattnaik & Chand, 1996 and Sahoo *et al*., 1997) and *Tridax procumbens* (Sahoo & Chand, 1998).

Fluctuations in environmental factors in different seasons had a definite effect on shoot bud differentiation from explanted nodal segments in *T. indica* as similarly shown in other medicinal herbs including *Ocimum* species (Pattnaik & Chand 1996).

The multiplication rate of the shoot cultures derived from the nodal explants of the primary shoots was dependent on the number of the subsequent culture passages. The gradual decline in the frequency of shoot development and the number of the shoots per node was also reported earlier for *Tridax procumbens* (Sahoo & Chand, 1998), is indicative of a gradual loss of the morphogenic potential with advance in culture.

To optimize the rooting response of plantlets raised *in vitro*, different auxins(IAA,IBA and NAA) were tested at various concentrations. IBA was found to induce a strong rooting response. The success of IBA in promoting efficient root induction has been reported earlier in other species by Sreekumar *et a*l., *2000* ; Fracaro and Echeverrigaray, *2001;* Martin, *2002*; Beena *et al*,. 2003; Faisal *et al*., 2006 and also in *Tylophora indica* by Faisal *et al*., 2007.

The period of transition after transfer from lab to land is the most critical step in the tissue culture techniques as during the process of *in vitro* regeneration in the lab, plants are subjected to heterotrophic mode of nutrition and there is lack of adaptation or exposure to the outside environment. So, during the period of hardening care is taken over the physical (temperature, light intensities, relative humidity, air current, atmospheric CO_2) and other factors (mineral nutrition, pH etc.) employed. Another

important factor during acclimatization is the type of potting material used. In the present study, Vermi-compost was found to be most suitable planting substrate for hardening and its use ensured high frequency survival (96%) of regenerated plantlets prior to outdoor transfer.

In conclusion, the present investigation has resulted in a protocol, which could be used for ex situ conservation and true to type mass propagation of this shrub of immense pharmaceutical relevance.

Acknowledgements:

We are thankful to the University Grants Commission, New Delhi, India for award of Junior Research Fellowship (Engineering &Technology) in the subject of Biotechnology for pursuing the present work.

***Corresponding Author:**
Prof. J. S Rana
Department of Bio & Nano Technology
Guru Jambheshwar University Of Science & Technology
Hisar, Haryana (India) -125001
Email: jogenderrana@yahoo.co.uk
Telephone No.: 0091-9996200256
Fax No.: 0091-1662-276025

References

1. Anonymous. Wealth of India, Raw materials. CSIR, New Delhi, 1976; 398–399.
2. Arya V, Shekhawat NS, Singh RP. Micropropagation of *Leptadenia reticulata*—a medicinal plant. In Vitro Cellular & Developmental Biology- Plant 2003; 39: 180–185.
3. Beena MR, Martin KP, Kirti PB, Hariharan M. Rapid *in vitro* propagation of medicinally important *Ceropegia candelabrum*. Plant Cell.,Tissue & Organ Culture 2003;2: 285–289.
4. Chandrasekhar T, Hussian MT, Gopal GR, Rao JVS. Somatic embryogenesis of *Tylophora indica* (Burm. f.) Merril. An important medicinal plant. International Journal of Applied Sciences & Engineering 2006; 4: 33–40.
5. Donaldson GR, Atkinson MR, Murray AW. Inhibition of protein synthesis in Ehrlich ascites-tumor cells by the phenanthrene alkaloids tylophorine, tylocrebrine and cryptopleurine. Biochem Biophys Res Commun 1968; 1: 104–109.
6. Faisal M, Ahmed Naseem, Mohammad Anis. An efficient micropropogation system for *Tylophora indica*: an endangered, medicinally important plant. Plant Biotechnology Reports 2007; 1(3): 55-161.
7. Faisal M, Siddique I, Anis M. In vitro rapid regeneration of plantlets from nodal explants of *Mucuna pruriens*: a valuable medicinal plant. Annals of Applied Biology 2006; 48: 1–6.
8. Fracaro F, Echeverrigaray, S. Micropropagation of *Cunila galioides*, a popular medicinal plant of south Brazil. Plant Cell, Tissue & Organ Culture 2001; 64: 1–4.
9. Gellert E. The indolizidine alkaloids. Journal of Natural Products 1982; 45: 50–73.
10. Gopalakrishnan C, Shankaranarayan D, Nazimudeen SK, Kameswaran L. Effect of tylophorine, a major alkaloid of *Tylophora indica*, on immunopathological and inflammatory reactions. Indian Journal of Medical Research 1980; 71: 940–948.
11. Jayanthi M, Mandal PK. Plant regeneration through somatic embryogenesis and RAPD analysis of regenerated plantlets in *Tylophora indica* (Burm. f.) Merrill. In Vitro Cellular & Developmental Biology- Plant 2001;37:576–580.
12. Koroch A, Juliani HR, Kapteyn J, Simon, JE. In vitro regeneration of *Echinacea purpurea* from leaf explants. Plant Cell, Tissue & Organ Culture 2002; 69:79–83.
13. Kukreja AK, Mathur AK. Tissue culture studies in *Dubosia myoporoides*. Planta Medica 1985; 2: 93–96.
14. Martin KP. Rapid propagation of *Holostemma ada-kodien* Schult, a rare medicinal plant, through axillary bud multiplication and indirect organogenesis. Plant Cell Reports 2002; 21: 112–117.
15. Mulchandani NB, Iyer SS, Badheka LP. Structure of tylophorinidine: a new potential antitumor alkaloid from *Tylophora indica*. Chem Ind 1971; 19:505–506.
16. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 1962; 15: 473–497.
17. Nitsch C, Nitsch JP. The induction of flowering in vitro in stem segments of *Plumbago indica*. 1. The production of vegetative buds. Planta 1967; 72: 355-370.
18. Pattnaik SK, Chand PK. In vitro propagation of the medicinal herb *Ocimum americanum* L. Syn. *O. canumsims* (hoary basil) and *Ocimum sanctum* (holy basil). Plant Cell Reports 1996;15:846–850.
19. Purohit SD, Dave A, Kukda G. Micropropagation of Safed musli (*Chlorophytum borivilianum*), a rare medicinal herb. Plant Cell, Tissue & Organ Culture 1994; 39: 93–96.
20. Rao KV, Wilson RA, Cummings B. Alkaloids of *Tylophora*- New alkaloids of *Tylophora indica*

(Burm) Merrill and *Tylophora dalzellii* Hook.f. Journal of Pharmaceutical Science 1971; 60(11): 1725-1726.
21. Reddy PS, Gopal GR, Sita, GL. In vitro multiplication of Gymnema sylvestre: an important medicinal plant. Current Science 1998; 75:843–845.
22. Sahoo Y, Chand PK. In vitro multiplication of a medicinal herb *Tridax procumbens*: Influence of explanting season, growth regulator synergy, culture passage and planting substrate. Phytomorphology 1998; 48:195-205.
23. Sahoo Y, Pattnaik SK, Chand PK. In vitro clonal propagation of an aromatic medicinal herb *Ocimum basilicum* L (Sweet basil) by axillary shoots proliferation. In Vitro Cellular & Developmental. Biology –Plant 1997;33:293-296.
24. Sharma N, Chandel KPS. Effects of ascorbic acid on axillary shoot induction in *Tylophora indica* (Burm. F) Merrill. Plant Cell, Tissue & Organ Culture 1992; 29: 109-113.
25. Shivpuri DN, Singhal SC, Prakash D. Treatment of asthma with an alcoholic extract of *Tylophora indica:* a crossover, double-blind study. Annals of Allergy 1972; 30: 407–412.
26. Sreekumar S, Seeni S, Pushpangadan P. Micropropagation of *Hemidesmus indicus* for cultivation and production of 2-hydroxy 4-methyl benzaldehyde. Plant Cell, Tissue & Organ Culture 2000; 62: 211–218.
27. Sudha GC, Krishnan PN, Pushpangadan P. In vitro propagation of *Holostemma annulare*, a rare medicinal plant. In Vitro Cellular & Developmental Biology- Plant 1998; 33: 57–63.
28. Thao NTP, Ozaki Y, Okubo H. Callus induction and plantlet regeneration in ornamental *Alocasia micholiziana*. Plant Cell, Tissue & Organ Culture 2003; 73: 285-289.
29. Thomas TD. Effect of sugars, gibberellic acid and abscisic acid on somatic embryogenesis in *Tylophora indica* (Burm. F.) Merrill. Chinese Journal of Biotechnology 2006; 22(3): 465-471.
30. Yemets AI, Klimkina IA, Tarassenko IV, Blume YB. Efficient callus formation and plantlet regeneration of goosegrass (*Eleusine indica*). Plant Cell Reports 2003; 21: 503-510.

7/7/2010

Kinetic and Electrolytic Conductivity of C.I. Acid Orange 15 and C.I. Acid Red 97 dyes in Different Media

A.M. Gamal

Chemistry Department, Faculty of Science, Al-Azhar University (Girls), Nasr City, Cairo, Egypt

Abstract: The electrolytic conductivities for C.I. Acid Orange 15 and C.I. Acid Red 97 have been studied. The electrolytic conductivities have been analyzed by Deby HUckel-Onsager theory, The degree of ionization, α, Van't Hoffs factor, i, and thickness of ionic atmosphere, X^{-1}. were calculated. Each value diminishes with increasing dye concentration. The dye anion transport number, t, mobility's, μ were also computed at infinite dilution. The results provide evidence for the presence of interionic attraction and association. Furthermore the kinetics of two acid dyes has been studied using spectrophotometric and conductimetric methods. The former study was carried out at 28°C at different percentage of solvents. The results revealed that the reaction rate was govemed by a pseudo-first order. [Journal of American Science 2010;6(12):393-399]. (ISSN: 1545-1003).

Keywords : C.I. Acid Orange 15, C.I. Acid Red 97, solvents, electrolytic conductivity, spectrophotometry, kinetics.

1. Introduction

Since the environmental pollutions are increasing day-by-day, the need to reduce the imparities particularly in wastewaters of textile industry is important.

Effluents from the dyeing industry contain highly colored species, such wastes are not only aesthetically displeasing but also hinder light penetration and may in consequence disturbiological processes in water bodies. Dyes are toxic to some organisms and hence harmful to aquatic animals. The expanded uses of azo dyes have shown that some of them are highly carcinogenic. Therefore, removal of dyes before disposal of wastewater is necessary [1,2].

Acid dyes are so called as normally applied from acidic dye liquor. The good sunlight resistance of acid dyes wool textile materials is due to the electron stability of the acid dyes chromophore. This enables the acid dye molecule to resist the photochemical degradation which the ultraviolet rays of sunlight might cause [3]. Some of the dyes were found to have a remarkable mutaganicity, bearing similarities with some antibiotics [4].

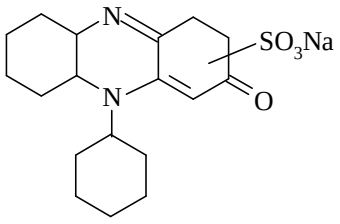
Also azo dye is an interesting and commercially important class compounds that are used for many years as acid-base and redox indicators, stains for bacteriological and histological investigations[5]. Conductivity measurements arc recommended for studying the behavior of dyes in aqueous solution, thus the variation of conductance with concentration in dilute solution provide powerful support for the presence of interionic attraction[6-9]. The mobility of dyes were investigated because its importance in nature and inverious technologies such as adsorption and measurement the drift velocity which can be used to obtain an expression for the ionic current density flowing through a solutions[10]. Mobility has proved to be an important property for providing an insight into the size, fractional coefficients [11] and it is successfully utilized for the control of precipitation.

Kinetic and calorimetric studies have been carried out to determine rate constants for the hydrolysis for some acid dyes at different media. The hydrolysis reactions were carried out in hemogenous media using acetone, dioxane the calorimetric data have been obtained using a sensitive rating-period calorimeter. The results indicate that, irrespective of whether the rate constants k are dependent on percentage of solvents. Taking into consideration both the kinetic and condutimetric data, it is suggested that, for the hydrolysis reaction [4].

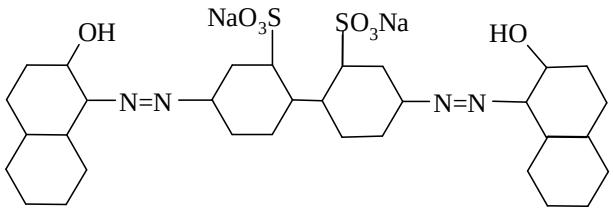
2. Experimental

Reagents

C.I. Acid Orange 15 and C.I. Acid Red 97 were provided by CIBA-GEIGY (Seitzerland), purified by recrystalization twice from 30% ethanol-water mixture and then dried under vacuum at 30°C. Solutions of different concentrations of the dye were prepared by dilution with triply distilled water from stock dye solution (1.0×10^{-2}M).



Mol. wt: 375.65 g/mol C.I. Acid Orange 15



Mol. wt.: 698:.04 g/mol, C.I. Acid Red 97, Fig. (1)

The organic solvents used were dioxiane and acetone being of analytical grade.

Apparatus and techniques

A perkin-Elmer lambda 3A. double beam spectrophotometer digital was used. It is a reading and recording instrument operating at the wave length range 190-900 nm (made in Germany).

The conductivity-meter type jenway 4010/REVC (made in United Kingdom) laboratory model with digital, supplied with dipping type conductivity cell with platinized electrodes was used. The cell constant 1.02 and the instrument were provided with a direct reading scale expressed in Siemens (ohm^{-1}).

The equivalent conductance and physical parameters such as, mobility, transport number and thickness of ionic atmosphere were described according to Robinson and Yadav[12].

Calculation:

In spectrophotometer measurements the kinetic study was carried out by mixing with the dye solution appropriate quantities with solvents. The absorbance was then recorded at the maximum wavelength ($\lambda_{max.}$ 410nm for C.I. Acid Orange 15 and $\lambda_{max.}$ 520 nm for C.I. Acid Red 97) every 2 mints till attaining equilibrium (constant reading) over a period of 120 mints. All the measurements were performed at 28°C (room temperature). The solubility of dye under different conditions was checked during 48 hours. The variation of the dye concentration with time at different percentage of solvents with varying polarity was found to fit a straight line of a pseudo-first order rate constant. From slope of these plots, the rate constant of the hydrolysis reaction was calculated using the relation[13].

$$k = \frac{1}{t} \ln \frac{a}{a-x} \qquad (1)$$

Where "k" is rate constant, "x" is the amount of the reactant used up after time "t" and "a" is the initial concentration of the reactant.

The conductimetric measurements the kinetic study of hydrolysis were carried out under the above conditions using time t_1 and t_2, for time intervals, L_1 and L_2 for the respective conductance, one for equation the (1) relation[13].

$$k = \frac{1}{(t_2 - t_1)} \ln \frac{a - L_1}{a - L_2} \qquad (2)$$

If (t_2-t_1) is a constant time interval, then

$$\frac{a - L_1}{a - L_2} = C \qquad (3)$$

where C is constant, rearranging eq. (3) are gets.

$L_1 = CL_2 - a(C-1)$ (4)

Thus, if L_1 is plotted against L_2, a straight line should be obtained with slope C. The logarithm of this value multiplied by $2.303/\Delta t$ gives k.

3. Results and Discussion:

I- Conductimetric study[14]

1-Specific Conductance

The specific conductance of aqueous solution for the mono sulphonic azo dye, C.I Acid Orange 15 at various concentration ranging from 1.0 - 9.0 x 10^{-4}g.eq.dm^{-3} and disulphonic azo dye C.I Acid Red 97 covering the concentration range 1.0-9.0x10^{-4} g.eq. dm^{-3} at different percentage of solvents were measured and collected in Figs (*2,3).* The values of the specific conductance for C.I. Acid Red 97 were high, which may be attributed to the presence of the disulphonic groups attached to the dye with high charge and the presence of more counter ions surrounded the ionisable groups. The plot of the specific conductance, as a function of the concentration for the two dyes at different percentage of solvents Figs (2,3) indicate that in very dilute solutions, specific conductance, increases sharply with increasing dye concentration. "This behaviour may be attributed to the ionization of dyes into sodium ion and dye anions.

$$RNa \longrightarrow R' + Na^+ \qquad (5)$$

$$R(Na)_2 \longrightarrow R' + 2Na^+ \qquad (6)$$

Due to the mutual affinity of ions of different signs surrounding the dye anion. A slight increase in, specific conductance, for monosulphonic and disulphonic azo dyes re-spectively may be attributed to an increase in entropy which arises from the melting of ice-berg" structure around the polar molecules.

Experimental data show that the specific conductance; decreases as the percentage of solvents increase Figs (2,3). This may be explained in terms of the increase in the effective degree of ionization with different percentage of solvents, or it may lower the restricting potential

for migration on raising the percentage of solvents. The specific conductance values can be represented by excellent straight line over the entire different percentage of solvents for each concentration

2-Equivalent conductance, A

The equivalent conductance; A for the investigated dyes in aqueous solutions at different percentage of solvents Figs (4,5) diminishes with increasing dye concentration which either due to the influence of the ionic atmosphere salvation of ions or decrease in mobility and partial dissociation of dyes[15]. The relation between equivalent conductance and square root of concentration is shown in Figs (4,5).

This behaviour may be due to the increase in viscosity [16] and degree of dye aggregates or hindering in the movement of the contour ions. Estimation of, A (equivalent conductance at infinite dilution) where a definite charged ion depends only on its nature and the inter ionic effect disappears. These done by extrapolation to zero concentration The .values of A at different percentage of solvents for the two dyes are to be found in Tables (1,2). These values increase with increasing percentage of solvents represents a high mobility of ions due to the increase in thermal energy and breaking of high number of hydrogen bond. The vibrational, rotational and translation energy also vary with percentage of solvents [17]. The limiting equivalent conductance of an ionic dye molecule λ_o^- is obtained by subtracting the limiting equivalent conductance of sodium ion, λ_o^+ at different percentage of solvents from the conductance at infinite dilution. The acquired values of λ_o at different percentage of solvents are given in Tables (1,2)

3-Van't Hoffs factors i, and thickens of ionic atmosphere,X^{-1}.

Van't Hoffs factors, i Tables (3,4) were computed from the relation i =α (v-l)+l where v is the number of ions and α degree of ionization at different concentrations and different percentage of solvents were estimated as discussed previously. The data in Tables (3,4) indicate those values of Van't Hoffs factors, i decrease with increasing dye concentration indicating incomplete ionization and repulsion between ionic species. This may be due to the decrease in thickness of ionic atmosphere, X^{-1} (Deby length).

To confirm this explanation calculation of, X^{-1} was obtained. The results in Tables (3,4) denoted that the values of X^{-1} decreases as the concentration of the dye increases, which confirms that dye molecules interact with each other and prefer to be in the aggregate form[18].

4- Mobility of dye anion and transport number.

Mobility μ_o is a most important characteristic of ions reflecting their specific participation in electrical conductance of an electrolyte. The mobility's of anionic dye molecule μo at different % of solvents were obtained from the relation μo = λo/zF. Tables (1,2) indicate that mobilities of C.I Acid Orange 15 anions are higher than C.I Red 97 since it has smaller molecular weight than the latter. Further, the lower mobility of C.I Acid Red 97 may be due to the presence of donor and acceptor function groups [19]. This is attributed to the disaggregation and change in the intermolecular bonding. The value of the transport numbers were calculating according to Yadav[12] and recorded in Tables (1,2). The fraction of the total current carried by dye anion is higher than the sodium ion and the values are almost equal at all solvents.

II- Spectrophotometric studies

1- Effect of concentration

The UV-visible spectra of solutions for C.I. Acid Orange 15 and C.I. Acid Red 97 in water were recorded at 28°C within the wavelength range 600 to 350 nm at different concentrations (1.0-9.0x10^{-4}g.eq. dm^{-3}). The spectra showed increase in absorbance on increasing the dye concentrations.

2- Effect of solvent

The spectra of C.I. Acid Orange 15 and C.I. Acid Red 97 in presence of solvents of varying polarities of dioxane (2.7), aetone (17.9). they dye exhibit similar band in the blue region in absence and presence of solvents. A slight-red shift was notices with different solvents polarity, which may be due to solute-solvent interactions.

3- Kinetic study

The kinetic data of the hydrolysis of dye in presence solvents, fitted pseudo-first order behaviour. This was accomplished by a computer program designed for linear and non linear least squares method, correlation coefficient (r=0.99- 1). The rate constant of hydrolysis k_{abs}., calculated from the slopes of straight lines are recorded in Table (5, 6) results revealed an increase in the rate constant with the increase of the water content of a given solvent system. This may be probably due to the increase of the free hydroxyl ion concentration. Since, it is known that the proton in water has the structure H^+ $(H_2O)_4$ may be left free OH^- in the medium[20].

Table (1): The equivalent conductance, A the limiting equivalent conductance λ_o, dye anion mobility, transport number t, and thickness of ionic atmosphere X^{-1} at infinite dilution in absence and presence of different percentage of solvents for C.I. Acid Orange 15.

Mole% of solvents	A $\Omega^{-1}cm^2\ mol^{-1}$	λ_o $\Omega^{-1}cm^2\ mol^{-1}$	μ_o $cmV^{-1}S^{-1}10^3$	X_o^{-1} $m10^5$	t_o^-	t_o^+
H_2o	265.810	185.810	1.925	2.401	0.699	0.301
Aceton						
10	252.232	178.646	1.851	2.383	0.709	0.291
30	241.016	172.513	1.788	2.364	0.716	0.284
50	220.132	160.732	1.666	2.344	0.723	0.277
Dioxane						
10	208.304	150.845	1.563	2.326	0.724	0.276
30	182.210	132.145	1.370	2.292	0.725	0.275
50	170.740	126.240	1.308	2.286	0.731	0.269

Table (2): The equivalent conductance, A, the limiting equivalent conductance λ_o, dye anion mobility, transport number t, and thickness of ionic atmosphere X^{-1} at infinite dilution in absence and presence of different percentage of solvents for C.I. Acid Red 97.

Mole% of solvents	A $\Omega^{-1}cm^2\ mol^{-1}$	λ_o $\Omega^{-1}cm^2\ mol^{-1}$	μ_o $cmV^{-1}S^{-1}10^3$	X_o^{-1} $m10^5$	t_o^-	t_o^+
H_2o	233.306	169.982	1.644	2.090	0.721	0.279
Acetone						
10	222.400	154.22	1.572	2.000	0.746	0.254
30	210.106	136.17	1.390	1.965	0.763	0.237
50	192.720	129.70	1.227	1.818	0.790	0.210
Dioxane						
10	200.64	146.82	1.368	1.870	0.768	0.232
30	185.951	120.21	1.165	1.724	0.792	0.208
50	164.310	109.73	1.090	1.560	0.801	0.199

Table (3) : Vant Hoff's factors i, and thickness of ionic atmosphere X^{-1} for C.I, Acid Orange 15 at different concentrations of dye and different percentage of solvents.

$C\times10^{-4}$	H_2O		Acetone						Dioxane					
			10%		30%		50%		10%		30%		50%	
	i	X^{-1} $m10^5$	i	X^{-1} $m10^5$	i	X^{-1} $m10^5$	i	X^{-1} $m10^5$	i	X^{-1} $m10^5$	i	X^{-1} $m10^5$	i	X^{-1} $m10^5$
1.0	3.109	8.517	2.790	8.324	2.462	8.211	2.211	2.104	2.091	8.00	1.860	7.856	1.800	7.520
2.0	2.798	7.726	2.624	7.460	2.319	7.270	2.100	7.109	1.940	7.00	1.814	6.801	1.770	6.407
3.0	2.520	7.094	2.602	7.00	2.278	6.814	2.000	6.640	1.876	6.424	1.793	6.260	1.735	6.000
4.0	2.280	6.520	2.314	6.314	2.260	6.100	1.856	6.000	1.800	5.851	1.740	5.514	1.700	5.126
5.0	2.240	6.100	2.201	5.926	7.961	5.720	1.773	5.416	1.716	5.226	1.700	5.090	1.656	5.001
6.0	2.009	5.456	2.010	5.120	1.972	5.010	1.951	4.801	1.900	4.511	1.863	4.210	1.772	4.010
7.0	1.840	4.981	1.792	4.811	1.716	4.514	1.660	4.322	1.612	4.106	1.470	4.00	1.401	3.892
8.0	1.730	4.611	1.715	4.210	1.600	4.026	1.542	3.815	1.502	3.460	1.426	3.172	1.373	3.060
9.0	1.640	4.300	1.610	4.001	1.541	3.810	1.470	3.414	1.414	3.001	1.380	2.817	1.306	2.516

Table (4) : Vant Hoff's factors i, and thickness of ionic atmosphere X^{-1} for C.I, Acid Red 97 at different concentrations of dye and different percentage of solvents .

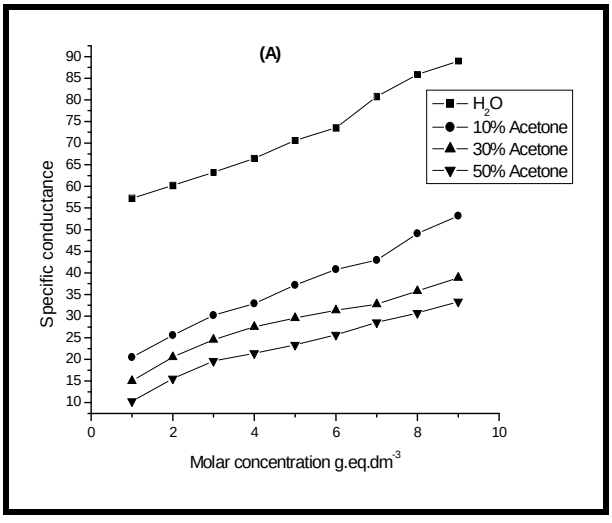
$C\times10^{-4}$	H_2o		Acetone						Dioxane					
			10%		30%		50%		10%		30%		50%	
	I	X^{-1} $m10^5$	i	X^{-1} $m10^5$	i	X^{-1} $m10^5$	i	X^{-1} $m10^5$	i	X^{-1} $m10^5$	i	X^{-1} $m10^5$	i	X^{-1} $m10^5$
1.0	2.926	8.290	2.407	8.010	2.190	7.826	2.000	7.561	1.846	7.280	1.605	7.100	1.314	6.740
2.0	2.773	6.114	2.226	6.002	2.001	5.910	1.810	5.200	1.690	5.050	1.470	4.672	1.276	4.421
3.0	2.590	6.000	2.185	5.860	1.824	5.614	1.562	4.736	1.442	4.329	1.236	4.103	1.090	3.919
4.0	2.260	5.842	2.000	5.460	1.673	5.110	1.336	4.401	1.227	4.100	1.009	3.808	0.985	3.617
5.0	2.090	5.316	1.896	5.096	1.404	4.367	1.227	4.004	1.101	3.870	0.974	3.526	0.874	3.270
6.0	1.960	5.070	1.636	4.854	1.286	4.109	1.090	3.860	0.919	3.566	0.860	3.229	0.800	3.009
7.0	1.814	4.690	1.470	4.246	1.109	3.816	0.960	3.472	0.896	3.219	0.801	3.001	0.764	2.860
8.0	1.653	4.185	1.196	4.60	1.000	3.360	0.924	3.099	0.800	2.809	0.762	2.624	0.716	2.472
9.0	1.375	4.001	0.986	3.870	0.886	3.118	0.817	2.822	0.750	2.636	0.709	2.339	0.640	2.126

Table (5): Rate constant for C.I. Acid Orange 15 in different percentage of solvents at 28°C.

Mole % of solvents	K $min^{-1} \times 10^4$	
	Cound.	Spectral
H_2O	18.264	20.201
Acetone		
10	11.060	15.731
30	9.725	12.000
50	7.851	9.246
Dioxane		
10	15.606	19.926
30	12.501	16.011
50	10267	13.000

Table (6): Rate constant for C.I. Red 97 in different percentage of solvents at 28°C.

Mole % of solvents	K $min^{-1} \times 10^4$	
	Cound.	Spectral
H_2O	23.650	27.443
Acetone		
10	16.781	20.185
30	13.446	16.704
50	10.219	13.227
Dioxane		
10	19.849	24.368
30	16.908	19.806
50	13.753	15.630



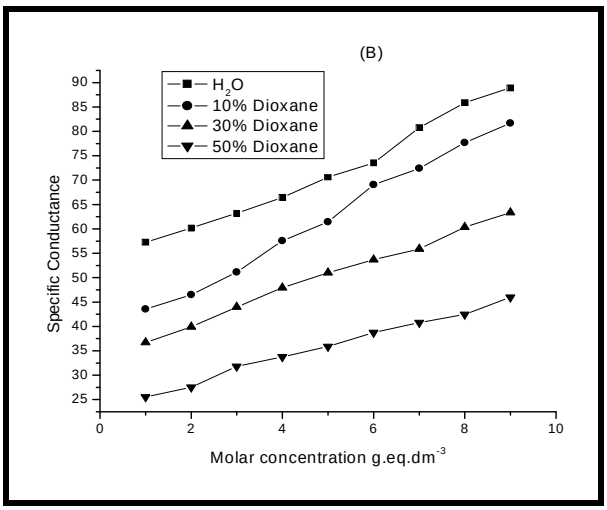
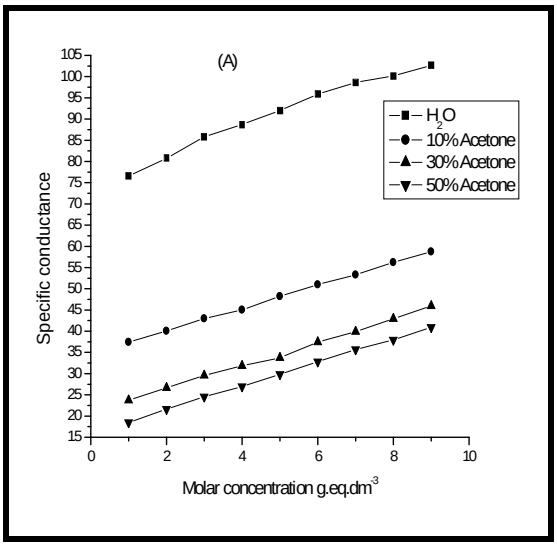


Fig (2a,b): The relation between specific conductance and the molar concentration of C.I. Acid orange 15 in absence and presence of solvents

.



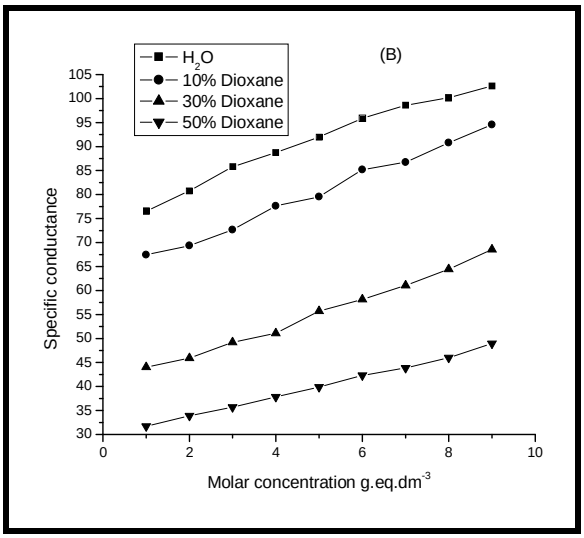
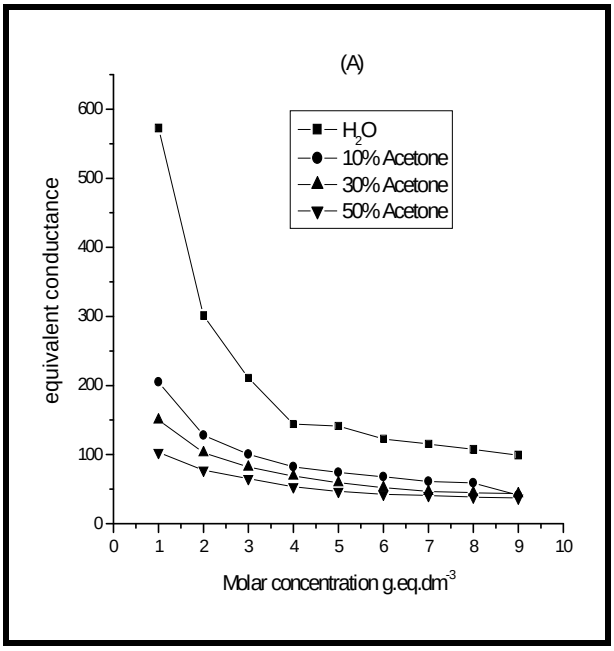


Fig (3a,b,): The relation between specific conductance and the molar concentration of C.I. Acid Red 97 in absence and presence of solvents



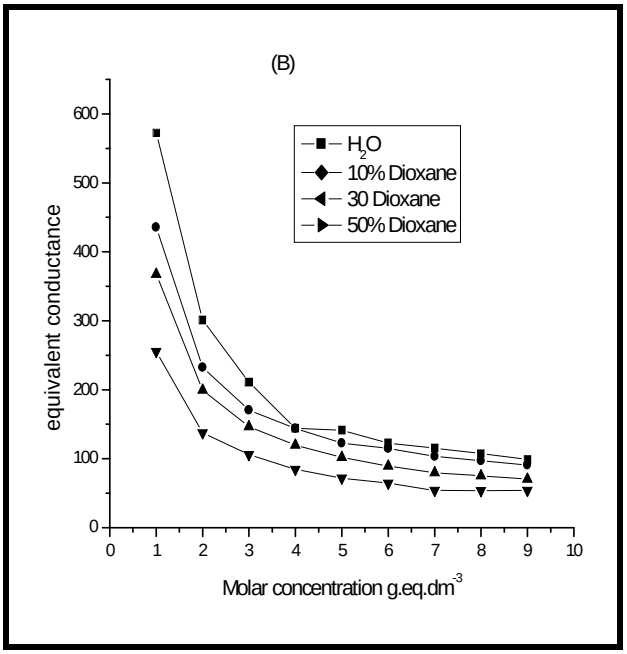
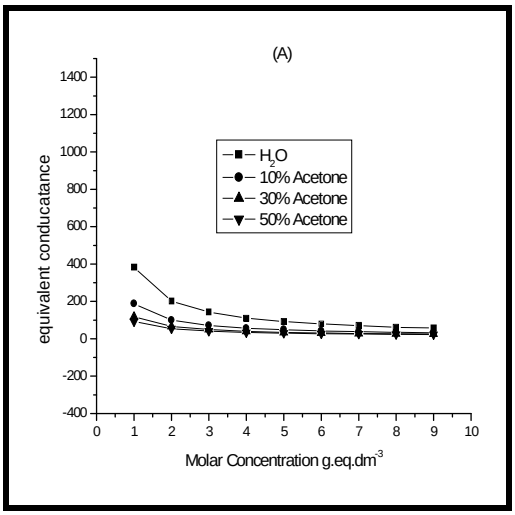


Fig (4a,b): The relation between equivalent conductance and the molar concentration of C.I, Acid Orange 15 in absence and presence of solvents.



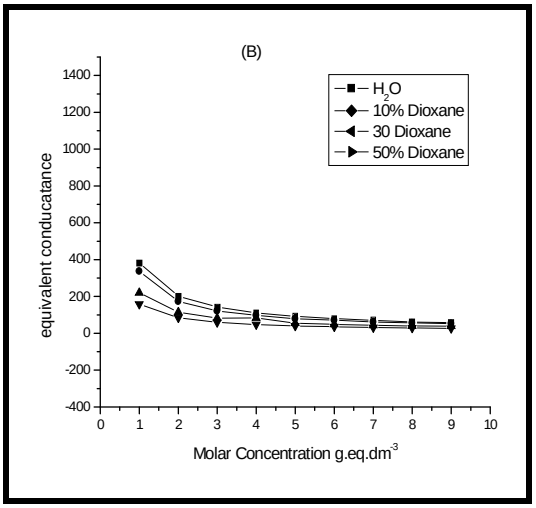


Fig (5a,b) : The relation between equivalent conductance and the molar concentration of C.I., Acid Red 97 in absence and presence of solvents.

4.Conclusions

- The specific conductance increase with concentration of dye increase and decrease in presence of solvent.
- The equivalent conductance decrease with concentration of dye increase and decrease in presence of solvents.
- The dye anion mobility and transport number decrease on solvents than H_2O and in monosulphonic large than disulphonic.
- The Van Hoff's factors i, and thickness of ionic atmosphere decrease on solvents than H_2O and in monosulphonic large than disulphonic .
- The rate constant obeys pesudo first-order reaction in kinetic and conductimentric.

Corresponding author
A.M. Gamal
e-mail:Dr_Azza-au@yahoo.com
Chemistry Department, Faculty of Science, Al-Azhar University (Girls), Nasr City, Cairo, Egypt

5. References

1. Sheiha S. Ashour, Journal of Saudi Chemical Society (2010) 14, 47-53.
2. Monika Wawrzkiewicz, Zbigniew Hubicki, Chemical Engineering Journal (2010) 157, 29–34.
3. Abdel-Aal, A.Y.Gamal, A.M. and Abofarah, S.A. Mans. Sci. Bull. (Achem.) Vol. 30 (1), June, (2003).
4. Arsheen Moiz, M. Aleem Ahmed, Naheed Kausar, Kamran Ahmed, Munnaza Sohail Journal of Saudi Chemical Society (2010) 14, 69-76.
5. Mostafa O.I, Abdel-Aal A.Y and El-Bayaa A.A, J. Chin. Chem. Soc., 42(3) 507(1995).
6. El-Sabbagii, I.A; Sallam, Ii.B; I'l-I'as.S, M.M; Mousa, E.A and Maiimoui), A.A; The Trans Of The Egyptian Soc. Chem. Engineers, 17(1), 132(1991).
7. Tawarah, K.M. and Wazwaz, A.A; J. Dyes and Pigm. 2 1(2) 97 (1993).
8. Hui, Lu and Leaist, D. G; J. Solution Chem., 19 (4) 391 (1990).
9. Hidetaka, Oka and Akira Kimura, J. S.D .C, 111,311(1995).
10. Laurier, L. Schramm; Shmuel Yariv; Dipak. K. Ghosh, and Laren G. Hepier; Can. J. Chem. 75; 1868 (1997).
11. Meenakshi Singh, Anilkumar, Shirley Easo, and Prasad, B.B; J. Can. Chem. 75; 414 (1997).
12. Yadav M.S; Electrochemistry, First Edition Darya Ganj. New Delahi 141 and 183 (1990).
13. Abd El-Aal A.Y. and El-Baya A.A. Al-Azhar Bull. Sci. Vol. 11, N. 1, pp. 197-205, (2000)
14. El-Baya A.A. Al-Azhar Bull. Sci. Vol. 11, N. 1, pp. 207-218, (2000).
15. Mehrotrotra K.N, Kirti Tandom And Rawat M.K; J. Indian Chem. Soc., 67, 719(1990).
16. Austen Angell, O; Imrie Corrie, T. and Ingram Malcolm, D; J. Polymer International, 41, (1) 9 (1998).
17. Shwara, J. Bhat And B1ndu, P. J. Indian Chem., Soc. 72, 783 (1995).
18. Suresh Das, And Kamatprashant, V; J. Phys. Cheni.103, 209 (1999).
19. Tameev Alek R; Kozlov Aleksey A. and Vannikov Anatoly V; J. Polymer International 47(2) 198 (1998).
20. Madhava M. Rao, Rmana D.K.; Seshaiah K. Wang M.C.; Chang Gien S.W. Journal of Hazardous 166, 1006-1013 (2009).

6/1/2010

Adsorption of Cadmium (II) and Mercury (II) onto Natural Adsorbent Rice Husk Ash (RHA) from Aqueous Solutions: Study in Single and Binary System

A.G. El-Said, N.A. Badawy, and S.E. Garamon

Chemistry Department. Faculty of Science, Al-Azhar University (Girls), Nasr City, Cairo, Egypt
asrargoma@yahoo.com

Abstract: The present study deals with the competitive adsorption of cadmium (Cd(II)) and mercury (Hg(II)) ions onto rice husk ash (RHA) from single component and binary systems. Equilibrium adsorption is affected by the initial pH (pH_0) of the solution. The $pH_0 \approx 6.0$ is found to be the optimum for the individual removal of Cd(II) and Hg(II) ions by RHA. The pH of the system, however, increases during the initial sorption process for about 60 min and, thereafter, it remains constant. The equilibrium adsorption data were obtained at different initial concentrations (C_0 = 10–100 mg/l), 6 h contact time, 25 °C temperature, RHA dosage of 10 g/l at pH_0 6. The single ion equilibrium adsorption data were fitted to the non-competitive Langmuir and Freundlich isotherm models. The Freundlich models represent the equilibrium data better than the Langmuir model in the studied initial metal concentration range (10–100 mg/l). The adsorption capacity of Cd(II) is higher than that for Hg(II) for the binary metal solutions and is in agreement with the single-component adsorption data. The equilibrium metal removal decreases with increasing concentrations of the other metal ion and the combined action of Hg(II) and Cd(II) ions on RHA is generally found to be antagonistic. Equilibrium isotherms for the binary adsorption of Cd(II) and Hg(II) ions onto RHA have been analyzed by using Langmuir and Freundlich models.. Desorption with various solvents showed that the nitric acid is the best solvent; the maximum elution being about 28.41 % for Cd(II) and about 31.53 for Hg(II).
[A.G. El-Said, N.A. Badawy, and S.E. Garamon. **Adsorption of Cadmium (II) and Mercury (II) onto Natural Adsorbent Rice Husk Ash (RHA) from Aqueous Solutions: Study in Single and Binary System.** Journal of American Science 2010;6(12):400-409]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Mercury (II); Cadmium(II); Binary adsorption; Rice husk ash (RHA); Simultaneous metal removal; Adsorption isotherms.

1. Introduction:

Most of the metals are known to be toxic and half of these, including cadmium, chromium, copper, lead, mercury, nickel, selenium, silver and zinc, are released into the environment in quantities that pose a risk to human health [1].

Heavy metal contamination exist in aqueous waste streams from diverse industries such as metal plating, manufacturing, batteries, as well as agricultural sources where fertilizers and fungicidal sprays are intensively used. Cu, Zn, Hg, and Cd are harmful wastes produced by industry that pose a risk of contamination groundwater and other water resources. Heavy metals are not biodegradable and tend to accumulate in living organisms, causing various diseases and disorders [2-11]. For example, Cadmium causes serious renal damage, anemia, hypertension and itai-itai [2].

The reduction of the pollutant to an acceptable level is necessary when toxic metals are present in aquatic system [12]. Adsorption and ion exchange processes are the most useful methods to remove them. These methods explore the availability of different kinds of adsorbents associated with convenient procedures for obtaining high efficiency [13-14]. A large number of different adsorbent materials containing a variety of attached chemical functional groups have been reported for this purpose. For instance, activated carbon is the most popular material; however, its high cost restricts its large-scale use [15, 16].

In recent years, special attention has been focused on the use of natural adsorbents as an alternative to replace the conventional adsorbents, based on both the environmental and the economical points of view [15, 16]. Natural materials that are available in large quantities, or certain waste products from industrial or agricultural operations, may have potential as inexpensive sorbents. Due to their low cost, when these materials the end of their lifetime, they can be disposed of without expensive regeneration. The abundance and availability of agricultural by-products make them good sources of raw materials for natural sorbents. Rice husk is used as a fuel by a number of industries to produce steam, thus, conversing both energy and resources. During the burning of rice husk, the residue ash, called rice husk ash (RHA) is collected from the dust collection

device attached upstream to the stacks of rice husk – fired boilers and furnaces. RHA has good adsorptive properties and has been used previously for the adsorptive removal of metal ions [17] and dye [18], and filtration of arsenic from water [19]. RHA, obtained from heating rice husk at 300 C, has been shown to adsorb more gold – thiourea than the conventionally used activated carbon [20].

The main techniques used to remove heavy metal ions from aqueous streams include ion-exchange chromatography, reverse – osmosis, Chemical precipitation and adsorption. Adsorption as a waste water treatment process has aroused considerable interest during recent years [21].

Much of the work on the adsorption of heavy metal ions by various kinds of adsorbents has focused on the uptake of single metal ions. Since industrial effluents can contain several metals, it is necessary to study the simultaneous sorption of two or more metal ions. Since industrial effluent can contain several or more metal ions and also to quantify the interactive affect of one metal ion on the other. Thus, the study of adsorption of heavy metal ions from binary and ternary system is important. No information is available in literature for the simultaneous removal of mercury (II) and cadmium (II) ions by rice husk ash.

In this paper the work aims to study the feasibility of using RHA as an adsorbent for the single and binary removal of Hg (II) and Cd (II) metal ions from aqueous solution, study the effect of initial pH, determine the applicability of adsorption isotherm models (e.g. Langmiur and Freundlich) for single component, and gather experimental data on adsorption equilibrium for the binary system containing Hg (II) and Cd (II) ions.

2. Materials and Methods

2.1. Adsorbent and its characterization

The rice husk used was obtained from agriculture land in kaluobia governmental, Egypt. The rice husk were crushed and sieved with 30-mesh siever. Then, the husks were thoroughly washed distilled water to remove all dirt and were dried at 100 °C till constant weight. The dried husks were stored in desiccator until used. The rice husk ash (RHA) obtained from burning of rice husk in electrical oven at 700 °C for 1 hour. The rice husk ash was sieved [with Retsch Prufsieb / sieve, Type (ASTM), practical size was found to be 40% (0.18mm), 44 %(0.355mm) and 16% (0.855mm)] and stored with a desiccators until used. The particular chemical and physical properties are shown previous show the x-ray diffraction analysis, which indicates that the RHA mainly consist of amorphous materials [22].

2.2. Adsorbates

All the chemical used in the study were of analytical reagent (AR) grade, cadmium sulphate octahydrate ($3CdSO_4.8H_2O$), mercury chloride ($HgCl_2$), NaOH and HCl. Stock solution having concentrations of 1000mg/l of Cd(II) and Hg(II) were prepared by dissolving exact amount of $CdSO_4.8H_2O$ and $HgCl_2$ in double-distilled water (DDW), respectively.

2.3. Batch adsorption studies

For each experimental run, 100 ml aqueous solution of known concentration of Cd (II) and Hg (II) for the single and binary mixture of these components was taken in 250 ml conical flask containing fixed amount of RHA. These flasks were agitated at a constant shaking rate of 150 rpm in a temperature controlled orbital shaker(VOR TEX MVOR-03, Spain) maintained at 30°C .The initial pH (pH_0) of the adsorbate solution was adjusted using 1 N (36.5 g/l) HCl or 1 N (40 g/l) NaOH aqueous solution without any further adjustment during the sorption process. The samples were withdrawn from the flasks at different time intervals to check whether equilibrium has been attained. The sample were centrifuged using research centrifuge (Hettich, Germany, EBA-20 Baujahr E xin) at 5000 rpm for 5 min and the supernatant liquid was analyzed for residual concentration of metal ions using inductively coupled plasma .

2.4. Effect of initial pH (pH_0)

The effect of (pH_0) on the sorption was studied by adjusting the (pH_0) in the range of 2.0-8.0. In these experiments, the RHA loading was kept at 10 g/l of solution containing 100 mg/l each of Cd (II) and Hg (II) at 30 °C. The contact time (t) was kept as 6 hr for Cd (II) and Hg (II), since equilibrium was found to have been attained in 6 hr contact time for Cd (II) and Hg (II).

2.5. Adsorption isotherm experiments

For single metal-ion-RHA systems, initial metal ion concentration was varied from 10 to 100 mg/l. In binary metal ion mixture RHA systems, for each initial concentration of Cd(II) and Hg(II) solution : viz., 10,20,30,50 and 100 mg/l, the Cd(II) or Hg(II) concentration was varied in range of 10-100 mg/l (viz., 10, 20, 30, 50 and 100 mg/l). In all cases, the (pH_0) of solution was maintained at 6.0 for Cd (II) and Hg (II).

2.6. Desorption studies

For batch desorption experiments, a series of 250 ml Erlenmeyer flask containing 100 ml of DDW or aqueous solution of HCl, H_2SO_4, HNO_3 and

CH_3COOH of know concentration were conducted with metal-loaded RHA (1 g) at 30 °C the mixtures were agitated at 150 rpm for 6 hr in the orbital shaker. Thereafter, the mixture was centrifuged and the supernatant was analyzed for metal ion released into the solvent.

2.7. Analysis of cadmium (II)and mercury (II)

The concentration of Cd (II) and Hg (II) and in the sample was determined by Inductively Coupled Plasma, (ICAP), 6500 DUO, Thermo Scientific England. 1000 mg/l multi-element certified standard solution, Merck, Germany was used as stock solution for instrument standardization.

Percentage metal ion Removal,

$$(\%) = 100\ (Ci - Ce)/Ci \quad (1)$$

where Ci and Ce are the initial and equilibrium metal ion concentrations, respectively.

Amount of adsorbed metal ions per g of solid,

$$qe(mg/g) = V(Ci - Ce)/m \quad (2)$$

where, Ci is the initial metal concentration in solution (mg/l) V is the volume (l) and m is the mass of adsorbent (mg)[23].

3. Results and Discussion:

3.1 Effect of initial pH (pH_o)

The pH of the solution affects on the surface charge of the adsorbents as well as the degree of ionization and speciation of different pollutants [24]. It is known that metal species [M(II)= Cd(II), Hg(II)] are present in deionized water in the form of M^{+2}, $M(OH)_2$, M(OH) (S), etc.[25].

It is obvious that the adsorption of M (II) must be higher in alkaline solution. But at higher pH, the precipitation as $M(OH)_2$ (S) plays the main role in removing the M (II) ions. Therefore, all the experiments were conducted at $pH \leq 8.0$.

The influence of the pH of metal ion solution on the extent of adsorption of Cd (II), and Hg (II) ions onto RHA is shown in Fig. 1. The adsorption of metal ions increases with an increase in pH. Up to pH 4, the increase in adsorption is gradual, which however, increase drastically at $pH > 4$. At higher $pH \geq 6.0$, Cd (II) and Hg (II) ions adsorption become nearly constant. During the initial stage of the sorption process up to 30 min., the pH of the solution was found to rise sharply. Thereafter, the pH of the solution remained invariant with time.

. Fig. 1. Shows the final pH values (pH_f) as a function of pH,the pH_f values are higher than the pH_o values for $pH < 7.5$ for Co =100 mg/l. Although the metal removal increases sharply with an increase in pH_o, the pH_f values were almost constant for $6 \leq pH_o \leq 7.5$. The pH_f values, are therefore, considered to indicate the equilibrium pH values [26]. Surface charge developed at low pH_o is not favorable to adsorption of the RHA. Besides, a higher concentration of H^+ in the solution competes with Cd (II) and Hg (II). However, it was found that with pH_o 6.0, the pH_o of the solution rises sharply and stabilizes at pH 7.2 and 7.6 for Hg (II) and Cd (II) respectively. Therefore, it may be concluded that the H^+ in the solution competes with Hg (II) and Cd (II) for the adsorption sites of RHA at pH_o 6.0. Also, the rise in pH for Cd-RHA system is lower than that of Hg-RHA system. It means that the amount of H^+ adsorption is more than that of Cd (II) and Hg (II) ions. The lower adsorption value observed for Hg (II) it can also be presumably due to the stronger interaction of Hg (II) with Cl^- species such as $HgCl^+$ and $HgCl_2$ in solution phase while for Cd (II) this interaction is negligible. The effectiveness of the process can be expressed by the quantity adsorbed (q_e (mg/g)) versus pH_o plot for the cations involved, as represented in Fig. 2.

The metallic ion (Cd) could be suffering hydrolysis, starting at pH higher than 6, forming Cd $(OH)^+$ species, which promotes a reduction of the adsorption capacity, due to the diminution of the formal charge of the metallic ion. In case of Hg (II) in solution, these ions are extensively hydrolyzed and the specie formed is HgO, and require acidification to prevent the formation of poly nuclear hydroxyl-bridged species or the precipitation of basic salts [27]. This specie does not present electrostatic interaction with active site of the RHA.

3.2 Effect of adsorbent dosage (m)

The effect of adsorbent dosage on the uptake of cadmium (II) and mercury (II) ions onto rice husk ash (RHA) was studied and is shown in Fig. 3. This figure reveals that the removal of metal ions increases with an increase in the adsorbent dosage from 1 to 10 g/l. The removal remains unchanged above 10 g/l of rice husk ash; dosage can be attributed to the larger availability of greater surface area and more adsorption sites. At $m < 7$ g/l, the adsorbent surface becomes saturated with metal ions and the residual metal ion concentration in the solution is large. At $m > 7$ g/l, the incremental metal ions removal becomes very low, and at m = 10 g/l, the removal efficiency becomes almost constant [23]. Maximum removal of metal ions at C_o = 100 mg/l was found to be (53.68 %) for Cd (II) and (35.06 %) for Hg (II).

3.3 Effect of initial metal ion concentration (C_o)

There are various possible interaction effects between different species in solution and, in particular, potential interactions on the surface depending on the adsorption mechanism. Factors that

affect the binding sites (e.g. functional groups, structures, surface properties, etc.), the properties of the adsorbates (e.g. concentration, ionic nature or standard redox potential, etc.) and the solution chemistry (e.g. pH, ionic strength, etc.). In the context of adsorption, a number of properties have been suggested for use in the ordering of affinity rank, including ionic radius and solubility product constant as hydroxides [28], puling electro negativity and standard reduction potential [29], first hydrolysis constant [30] . The properties may play an important role in metal ion adsorbent interaction, but can only partly explain high- or low-adsorption capacities.

The atomic weight of Hg(II) is highest as compared to Cd (II), and the ionic radius of Hg(II) is highest (1.02 A^o) as compared to Cd(II) (0.96 A^o). Both the tested adsorbent showed higher adsorption capacity for Cd (II) ions as compared to Hg (II).

However, the adsorption order is found to be in the order of increasing molecular weight and ionic radius, e.g. Cd(II) > Hg(II)..

The increase in C_o also enhances the interaction between the metal ions in the aqueous phase and the adsorbents. Therefore, an increase in the C_o of the metal ions enhances the adsorption uptake of Cd (II) and Hg (II) and ions onto RHA as shown in Fig 4.

3.4 Effect of contact time (t)

At pH 6, aqueous metal ion solution with C_o = 100 mg/l were kept in contact with the RHA for 24 hr. The residual concentrations at 6 hr. Contact time were found to be higher by a maximum of ≈ 1 % than those obtained after 24 hr. Contact time, a steady state approximation was assumed and equilibrium situation was accepted. Accordingly all the batch experiments were conducted with a contact time of 6 hr. under vigorous shaking conditions. Fig. 5. Show the effect of (t) on the uptake of Cd (II), and Hg (II) ions from aqueous metal ion solutions. The rate of metal ion removal is found to be very rapid during the initial 15 min and there fore, the rate of metal ion removal decreases. No significant change in metal ion removal is observed after about 120 min. During the initial stage of sorption, a large number of vacant surface sites are available for adsorption. After laps of some time, the remaining vacant surface sites are difficult to be occupied due to repulsive forces between the solute molecules adsorbed on the solid surface and the bulk phase. Besides, the metal ions are adsorbed into the meso-pores that get almost saturated with metal ions during the initial stage of adsorption. Thereafter, the metal ions have to traverse farther and deeper into the pores encountering much larger resistance. This results in the slowing down of the adsorption during the later period of adsorption. Da Fonseca et al; [31] have reported 12 hr. Equilibrium contact time for removal of Cd (II) by vermiculiteat.

3.5. Single and binary adsorption of cadmium (II), and mercury (II) ions

Binary adsorption studies are particularly important for assessing the degree of interference posed by common metal ions in adsorptive treatment of wastewaters.

The equilibrium uptakes and the adsorption yield obtained for single component (Cd (II) and Hg (II)) solution at pH_o are shown in Fig (6,7). As seen from the figures, an increase in the initial metal concentration up to 100 mg/l increases the equilibrium uptake and decreases the adsorption yield of both the components. When the initial ion concentration increase from 10 to 100 mg/l, the loading capacity of RHA increases from 0.875 to 5.316 mg/g for Cd (II)and from 0.807 to 3.401 for Hg (II). The initial concentration provides the necessary driving forces to over come the resistance to the mass transfer of Cd (II) and Hg (II) ions between the aqueous and the RHA. The increase in initial concentration also enhances the interaction between the metal ions in the aqueous phase and the RHA [32]. Therefore, an increase in initial concentration of metal ions enhances the adsorption uptake of the Cd (II) and Hg (II) ions. Also, it is observed that the adsorption capacity of RHA for Cd (II) is greater than that for Hg (II). Binary component adsorption systems were prepared by solubilizing a combination of either Cd (II)-Hg (II), with presence of each metal [Cd (II)/ Hg (II)]. Binary adsorption of metal ions was conducted with the same operating conditions as for mono-component adsorption in terms of volume (100 ml.), RHA weight (1 g.), agitation time (2 hr.) and agitation rate (150 rpm). Initial individual concentration of three metal ions in binary system ranged from 10 to 100 mg/l.

The simultaneous adsorption of Cd (II) and Hg (II) ions from binary mixtures also investigated at pH_o 6.0 . In the first stage of adsorption studies, while initial Hg (II) concentration was changed from 0 to 100 mg/l, at each initial Cd (II) ion concentration of 0, 10, 20, 30, 50, and 100 mg/l. The non –linear adsorption isotherms of Hg (II) ions in the absence and presence of increasing concentration of Cd (II) ions are shown in Fig 7. It is seen that the equilibrium Hg(II) uptake increase with an increase in the initial Hg(II) concentration up to 100 mg/l at all Cd(II) ion concentration. The equilibrium uptake of Hg (II) decrease continuously with increasing Cd (II) ion concentration. The individual and total adsorption equilibrium uptakes and yields of Hg (II) and Cd (II) ions on RHA as obtained at different Hg (II)

concentration in the absence of Cd (II) or the presence of Cd (II) ions with increasing concentration are also listed in Table 1.

In general, an increasing in Cd (II) ion concentration decreases the individual adsorption yield of Hg (II) and total adsorption yield for each experiment run. The results also show that the equilibrium uptake of Hg (II) ion decreases with increasing initial Cd (II) ion concentration. At 100 mg/l initial Hg (II) concentration, without Cd (II) ions and in the presence of 100 mg/l Cd (II) ion concentration, adsorbed Hg (II) quantities at equilibrium are found to be 3.401 and 1.806 mg/g, respectively. Fig. (6) depicts the variations in the uptake of Cd (II) at equilibrium with increasing initial Cd (II) concentration (from 0 to 100 mg/l) at a constant initial Hg (II) concentration (10-100 mg/l) at pH_o 6.0. Similar adsorption pattern are observed both in the individual –Cd(II) ion and binary Cd(II)-Hg(II) ion systems; Cd(II) ion equilibrium uptake increase with an increase in the initial Cd(II) concentration up to 100 mg/l. Increase in Hg(II) concentration decrease the equilibrium uptake of Cd(II) ions. With no Hg (II) present in the solution, equilibrium uptake of Cd (II) ion is found to be 5.316 mg/g at 100 mg/l initial Cd (II) ion concentration. When the Hg (II) concentration is kept at 100 mg/l at the same initial Cd (II) ion concentration, the equilibrium Cd (II) uptake decreases to 2.248 mg/g.

In general, multi-component adsorbates – adsorbents generally exhibit three possible types of behavior:

- Synergism (the effect of the mixture is greater than that the single components in the mixture).
- Antagonism (the effect of the mixture is less than that of each of the components in the mixture).
- Non-interaction (the mixture has no effect on the adsorption of each of the adsorbates in the mixture), [33].

The combined effect of the binary mixture of Cd (II) and Hg (II) seems to be antagonistic. To analyze the antagonistic sorption behavior of the two components, the adsorption yield of single and binary component systems were also compared. The experimental equilibrium sorption data obtained for the single component and the binary systems indicate that the adsorption capacity of RHA for Hg (II) is, in general, less than that of Cd (II). Kandah et al, Singh and Yenkie, Flogeac et al, and Sharma et al, has reported similar results using waste ship manure waste, granular activated carbon columns, franc soil and shelled moringa oleifera seeds, respectively, as adsorbents [34-37].

3.6. Single-component adsorption isotherm models

The individual Langmiur and Freundlish adsorption isotherm parameters [23] for Cd(II) and Hg(II) at pH_o 6.0 obtained from the fitting of experimental data are listed in Table (2) along with the regression coefficients, R^2 . The R^2 values are closer to unity for the Freundlish models in comparison to that for Langmiur model. Therefore, the equilibrium adsorption data of Cd(II) and Hg (II) –RHA can be represented more appropriately by the Freundlish models in the studied concentration range.

Langmiur: $q_e = q_m K_L C_e / 1 + K_L C_e$ (3)

Freundlich: $qe = K_F C_e^{1/n}$ (4)

The data in Table (2) also indicate that the amount of Cd(II) ions per unit weight of RHA for the complete monolayer surface coverage was higher than that of Hg(II). A large value of K_L also implies the strong affinity of Cd(II) ions to RHA. K_F and n, the single-component Freundlish constants, indicate the adsorption capacity and adsorption intensity, respectively.. It is found from Table (2) that the RHA shows greater heterogeneity for Cd(II) than that for Hg(II) ions. Since $1/n < 1$; both the Cd (II) and Hg (II) ions are favorably adsorbed by RHA. The magnitude of K_F also showed the higher uptake of Cd(II) that than of Hg(II) ions by RHA at pH_o 6.0. The comparison of the single component experimental equilibrium adsorption. Uptake and the predicted uptake (q_e) from the Langmiur and Freundlich models for Cd(II) and Hg(II) onto RHA at pH_o 6.0 are presented in Table (3) .

3.7. Binary- component adsorption isotherm models

Adsorption behaviors of metal ions in binary system have been modeled using Langmiur and Freundlich equation as presented in Table (3). As depicted in the Figures (6,7) for each metal ion, co-adsorption induces a decrease in equilibrium adsorption capacity but the percentage depends on co-metal ion present in the system . Adsorption behavior of metal ions in binary system was modeled using Langmiur and Freundlich isotherm equation as predicted in mono-component system. Values of Langmiur and Freundlich parameter for each metal ion of binary system, that is, Cd(II) + Hg(II), and their respective determination coefficient R^2 value are presented in Table (4).

3.8. Desorption study and disposal of spent RHA

The regeneration of the adsorbent and/or disposal of the adsorbate-loaded adsorbent (or spent adsorbent) is very important. For the desorption experiments, several solvents (acids and bases) have been used. Batch desorption experiments were carried out and the desorption efficiencies are compared in Fig 8 .Acetic acid showed the maximum desorption efficiency of 17.53 % for cadmium (II)

and 24.58 % for mercury (II). On the other hand, mineral acids, HCl, H_2SO_4, and HNO_3 showed almost equal but higher recovery efficiency (≈ 31.5 %) for Hg (II). However, for Cd (II), H_2SO_4 proved to be the best. Hydrogen ions released from the acids replace metal cations on the RHA. Overall, any of the mineral acids can be selected as the optimal eluting agent for the system studied, however, the desorption efficiency is not very significant. Several authors have demonstrated that the sorption process of trace metals is not completely reversible. Several explanations have been proposed for such observations, including diffusion of trace metals within oxide particles or into micro-pores [38-40], precipitation [41], incorporation of metals into oxides [42], and re-adsorption [43]. For the present study, it seems that the chemisorptive adsorption of metal ions onto RHA hinders the desorption of metal ions from the spent RHA.

Table 1. Comparison of individual and total adsorption equilibrium uptake and yields found at different mercury (II) concentrations at varying concentration of cadmium(II) ions onto rice hush ash.

C_o,Hg	C_o,Cd	C_e,Hg	C_e,Cd	q_e,Hg	q_e,Cd	AdHg%	AdCd%	AdTot%
0	10	0	1.25	0	0.875	0	87.50	87.50
0	20	0	3.97	0	1.603	0	80.15	80.15
0	30	0	8.04	0	2.196	0	73.20	73.20
0	50	0	17.10	0	3.290	0	65.80	65.80
0	100	0	46.84	0	5.316	0	53.16	53.16
10	0	1.93	0	0.807	0	80.7	0	80.70
10	10	3.45	2.26	0.655	0.774	65.5	77.40	71.47
10	20	4.58	6.93	0.542	1.307	54.2	65.34	59.77
10	30	5.26	12.65	0.474	1.735	47.4	57.83	52.62
10	50	5.79	27.12	0.421	2.288	42.1	45.76	43.93
10	100	6.42	55.34	0.358	4.466	35.8	44.66	40.24
20	0	7.13	0	1.287	0	64.2	0	64.20
20	10	8.52	3.57	1.148	0.643	57.4	64.30	60.85
20	20	11.34	7.87	0.866	1.213	43.3	60.65	51.98
20	30	11.66	15.01	0.804	1.499	40.2	49.97	45.08
20	50	13.52	28.23	0.648	2.177	32.4	43.54	37.97
20	100	14.66	69.41	0.547	3.059	27.35	30.59	28.97
30	0	12.55	0	1.745	0	58.1	0	58.10
30	10	15.56	4.62	1.414	0.539	47.13	53.80	50.46
30	20	16.54	9.89	1.346	1.011	44.867	50.55	47.71
30	30	19.23	16.73	1.077	1.327	35.5	44.23	40.06
30	50	19.95	30.05	1.005	1.995	33.5	39.90	36.70
30	100	21.26	72.42	0.874	2.758	29.13	27.58	28.35
50	0	29.59	0	2.410	0	48.04	0	48.04
50	10	36.23	4.92	2.045	0.508	40.9	50.80	45.85
50	20	38.59	10.83	1.940	0.917	38.8	45.85	42.33
50	30	40.06	18.02	1.770	1.198	35.4	39.93	37.35
50	50	41.58	31.24	1.530	1.876	30.6	37.52	34.06
50	100	43.22	74.26	1.178	2.574	23.56	25.74	24.65
100	0	65.99	0	3.401	0	34.01	0	34.01
100	10	69.23	5.37	3.077	0.463	30.77	46.30	38.54
100	20	70.56	12.03	2.944	0.797	29.67	39.85	34.65
100	30	76.33	19.78	2.367	1.022	23.67	34.07	28.87
100	50	79.54	34.10	2.046	1.590	20.46	31.80	26.13
100	100	81.94	77.50	1.806	2.248	18.06	22.48	20.27

Table 2. Isotherm parameters values for the removal of cadmium (II) and mercury (II) in mono-component system by rice husk ash.

Adsorbate	K_L (l/mg)	q_m (mg/g)	R^2
Langmuir constants			
Cadmium(II)	0.077	6.57	0.965
Mercury(II)	0.073	4.00	0.977
Adsorbate	K_F ((mg/g) / (mg/l)$^{1/n}$)	1/n	R^2
Freundlich constants			
Cadmium(II)	0.789	0.49	0.999
Mercury(II)	0.599	0.42	0.996

Table 3. Comparison of the experimental and calculated qe values evaluated from the mono-component Langmiur and Freundlich models for the individual adsorption of cadmium (II) and mercury (II) to rice husk ash.

C_o (mg/l)	C_e (mg/l)	$q_{e,exp}$ (mg/g)	$q_{e,calc}$ (mg/g)	
Cadmium (II)			*Langmiur*	*Freundlich*
10	01.25	0.875	0.577	0.880
20	03.97	1.603	1.538	1.570
30	08.04	2.196	2.510	2.228
50	17.10	3.290	3.730	3.240
100	46.84	5.320	5.140	5.340
Mercury (II)				
10	01.93	0.807	0.493	0.787
20	07.20	1.284	1.370	1.360
30	12.55	1.745	1.912	1.720
50	25.98	2.402	2.620	2.323
100	65.99	3.401	3.313	3.420

Table 4. Modeling the result of adsorption isotherm of binary system.

			Langmiur			*Freundlich*		
			Q_m	K_L	R^2	K_F	1/n	R^2
C_o (mg/l)	**Binary metal system**	**Metal ion**						
10	Cd +Hg	Cd	4.96	0.049	0.904	0.49	0.51	0.999
		Hg	4.02	0.043	0.966	0.36	0.51	0.995
20	Cd +Hg	Cd	3.88	0.049	0.989	0.37	0.52	0.979
		Hg	4.58	0.025	0.975	0.20	0.64	0.989
30	Cd +Hg	Cd	3.87	0.034	0.996	0.42	0.56	0.985
		Hg	3.26	0.026	0.974	0.17	0.63	0.979
50	Cd +Hg	Cd	3.73	0.029	0.989	0.21	0.60	0.969
		Hg	3.26	0.022	0.946	0.14	0.64	0.969
100	Cd +Hg	Cd	3.32	0.026	0.981	0.18	0.65	0.999
		Hg	2.88	0.019	0.954	0.11	0.60	0.989

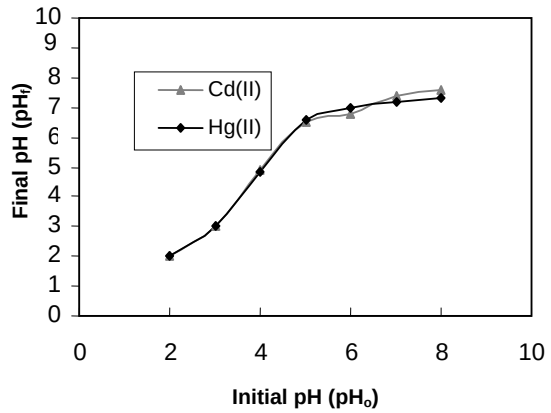


Fig. 1. The variation in equilibrium system pH_f with pH_o during the sorption of Cd(II) and Hg(II) onto rice husk ash

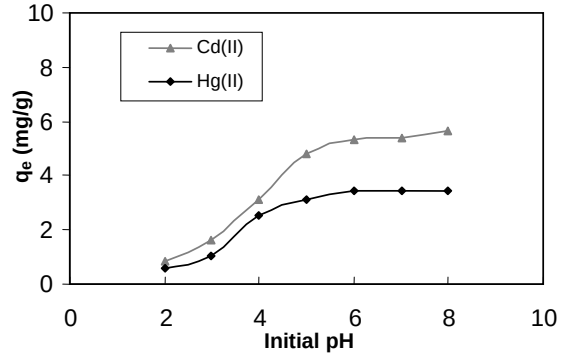


Fig. 2. Effect of pH_o on the removal of cadmium (II) and mercury (II) ions for mono-component adsorbate aqueous solution by rice husk ash. T= 25 ^oC, t = 6 hr, C_o = 100 mg/l, and m = 10g/l.

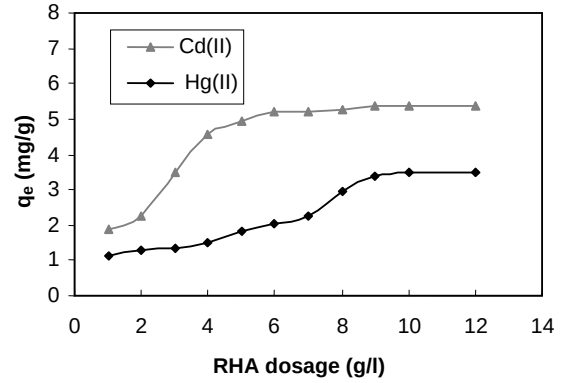


Fig. 3. Effect of rice husk ash (RHA) dosage on the removal of cadmium (II) and mercury (II). T= 25 ^oC, t = 6 hr and C_o = 100 mg/l.

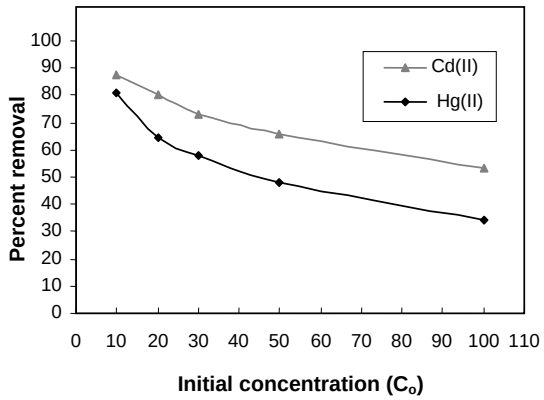


Fig. 4. Effect of the initial metal ion concentration on the removal of cadmium (II) and mercury (II) by rice husk ash. T= 25 ^oC, t = 6 hr, pH = 6, and m = 10 g/l.

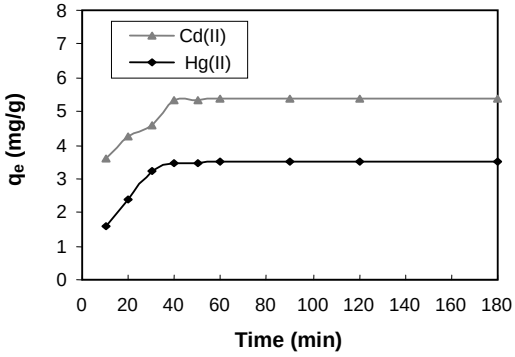


Fig. 5. Effect of contact time on the adsorption of cadmium (II) and mercury (II) by rice husk ash. T= 25 ^oC, t = 6 hr, C_o = 100 mg/l, and m = 10 g/l

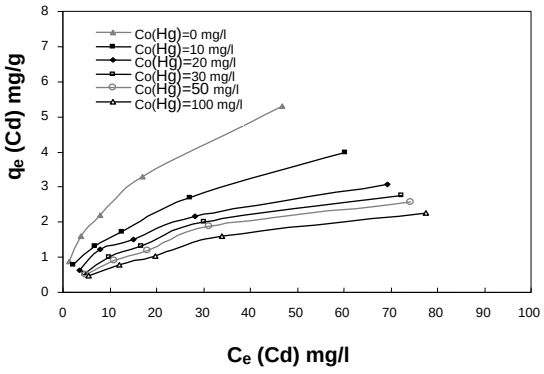


Fig. 6.Comparison of the equilibrium adsorption of cadmium (II) ion at varying concentrations of mercury (II) ion. pH_o = 6, t = 6 hr, T = 25 ^oC, C_o[Cd(II)] = 10-100 mg/l, and rice husk ash dosage = 10 g/l.

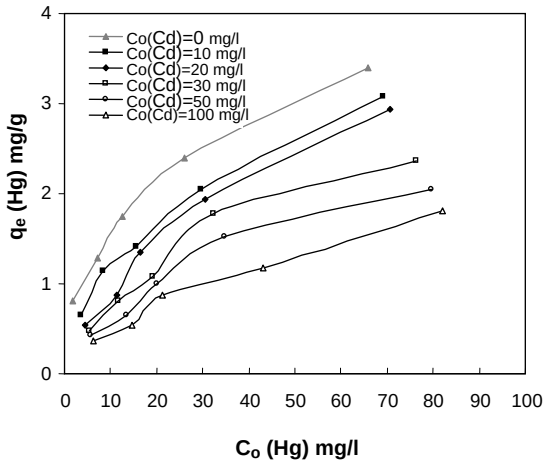


Fig. 7. Comparison of the equilibrium adsorption of mercury (II) ion at varying concentrations of cadmium (II) ion. pH_o = 6, t = 6 hr, T = 25 ^oC, C_o[Hg(II)] = 10-100 mg/l, and rice husk ash dosage = 10 g/l.

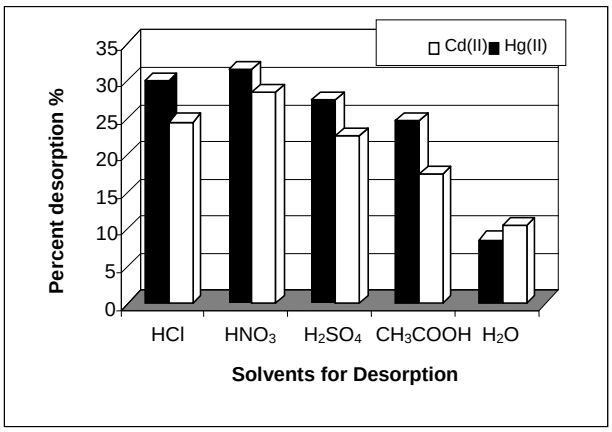


Fig. 8. Desorption of metal ions from metal loaded rice husk ash by solvents. T = 25 ^oC, t = 6 hr, C_o (solvent concentration) = 0.1N, and m = 10 g/l.

4. Conclusion

The present study shows that the rice husk ash is an effective adsorbent for the removal of Cd(II) and Hg(II) metal ions from aqueous solution. Maximum sorption for both mercury (II) and cadmium (II) metal ions was found to occur at pH_o 6.0. Higher percentage of metal ion removal was possible provided that the initial adsorbate concentration in the solution was low. Frendlich isotherm show very good fits with experimental single component adsorption equilibrium data. The. affinity of rice husk ash for cadmium (II) ions was greater than that for mercury (II), for both single component and the binary solutions under similar experimental conditions**.**

Corresponding author:
A.G. El-Said Chemistry Department. Faculty of Science, Al-Azhar University (Girls), Nasr City, Cairo, Egypt

References

1. M.P. Ireland, in: H.K. Dillon, M.H. Ho (Eds.), 1991. Biological Monitoring of Heavy Metals, Wiley, New York.
2. L.M. Friberg, G.F. Piscaor, T. Nordberg,(1974). Cadmium in the environmental, 2 nd ed., CRC Press, Cleveland, OH.
3. Y. Guo, j.Qi, S.Yang, K.Yu, Z. Wang, H. Xu, (2002). Rice husk as a potentially low-cost biosorbent for heavy metal and dye removal: an overview, Mat. Chem. Phys. 78, 132-137.
4. M. Albert, C. Demesmay, J.L. Rocca, (1995). Analysis of organic and nonorganic arsenious or selenious compound by capillary electrophoresis, Fresenius J. Anal. Chem. 351, 126-432.
5. Y. Nuhoglu, E. Malkoc, A.Guurses, N. Canpolat, (2002). The removal of Cu(II) aqueous solutions by Ulothrix zonata, Bioresour. Technol. 85, 331-333.
6. W.S.W.Ngah, C.S. Endud, R.Mayanar, (2002). Removal of copper (II) ions from aqueous solution onto chitosan and cross-linked chitosan beads, React. Funct. Polymer. 50, 181-190.
7. W.Y. Baik, J.H.Bae, K.W. Cho, W.W.Hartmeier, (2002). Biosorption of heavy metals using whole mold mycelia and parts thereof, Bioresour. Technol. 81, 167-170.
8. Toxicological profile for Chromium, (2000). US Department of health services public health service agency of toxic substances and disease registry, September.
9. S.E. Manaham (2000). Environmental Chemistry, 7th ed., CRC Press, Boca Raton.FI.
10. M.H. Yu, (2005). Environmental toxicity – Biological and Health Effects of Pollutants, 2nd ed., CRC Press, Boca Raton.FI.
11. K.A. Matis, A.I. Zouboulis, N.K. Lazaridis, (2003). Heavy metals remove by biosorption and flotation, Water Air Soil Pollut. 3, 143-151.
12. V.K. Gupta, C.K. Jain, I. Ali, M. Sharma, V.K. Saini, (2003). Removal of cadmium and nickel from wastewater using bagasse fly ash- a suger industry waste, Water Res. 37, 4038-4044.
13. C.E. Harland, (1994) Ion exchange theory and practices, 2nd ed., Royal society of chemistry. Cambridge, United Kingdom, 258.
14. D.O. Cooney, (1999). Adsorption Design for Wastewater Treatment, Lewis Puplication, Boca Raton, FI, 189.

15. S. Bable, T.A. Kurniawan, (2003). Low-cost adsorbent for heavy metals uptake from contaminated water; a review J. Hazard. Mater. 97, 219-243.
16. S.E. Bailey, T.I.Olin, M. Bricka, D. Adrian, (1999). Areview of potentially low-cost sorbents for heavy metals, Water Res. 33, 2469-2479.
17. V. C. Srivastava , I. D. Mall , I. M. Mishra, (2006). "Characterization of misoporous rice husk ash (RHA) and adsorption kinetics of metal ions from aqueous solution onto RHA, J. Hazard. Mater. B 134, 257-267.
18. V. Mane, I. D. Mall, V. C. Srivastava, (2007). Kinetic and equilibrium isotherm studies for the adsorptive removal of Brilliant Green dye from aqueous solution by rice husk ash, J. Environ. Manage, 84(4), 390-400,
19. J. C. Saha, K. Diksit, M. Bandyopadhyay, (2001). "Comparative Studies for selection of Technologies for Arsenic Removal from Drinking Water". BUET-UNU International Workshop on Technologies for Arsenic Removal from Drinking Water, Bangladesh.
20. W. Nakbanpote, P. Thiravetyan, C. Kalambaheti, (2000). Preconcentration of gold by rice husk ash, Miner. Eng., 13(4), 391-400.
21. Q. Feng, Q. Lin, F. Gong, S. Sugita, M. Shoya, (2004). Adsorption of lead and mercury buy rice husk ash, J. Colloid Interf. Sci., 278, 1-8.
22. N. Bouzoubaa, B. Fournier, (2001). Concert Incorporating Rice Husk Ash: compressive strength and chloride – ion penetrability, International center for sustainable development of cement and concrete (ICON), CANMET, Natural Resources Canada, Ottawa, Canada.
23. V. C. Srivastava, I. D. Mall, I. M. Mishra, (2008). Removal of cadmium (II) and zinc (II) metal ions from binary aqueous solution by rice husk ash, Colloids and Surface A: Physicochem. Eng. Aspects, 312, 172-184.
24. H. A. Elliott, C. P. Huang, (1981). Adsorption characteristic of some Cu (II) complexes on alumina silicates, Water Res., 15, 849-854.
25. V. L. Snoeyink, D. Jenkins (1980). Water chemistry, Johnwiley and sons, New York.
26. J.S.Kwan, S. T. Yun, S.O. Kim, B. M. I. Hutcheon, (2005). Chemosphere, 60(10), 1416.
27. N. N. Greenwood, A. Earnshaw, (1997). Chemistry of the elements, 2nd ed., Butterworth-Heinemann, Oxford, 1217.
28. A. Saeed, M. Iqbal, M.W. Akhtar, (2005). Removal and recovery of lead (II) from single and multimetal (Cd, Cu, Ni, Zn) solution by crop milling waste (black gram husk), J. Hazard.Mater. B117, 65-73.
29. G. McKay, J.F. Porter, (1997). Equilibrium parameter for the sorption of copper, cadmium and zinc ion onto peat, J. Chem. Technol. Biotechnology, 69(3), 309-320.
30. F. Pagnaneli, A. Esposito, L.Toro, F.Veglio, (2003). Metal speciation and ph effect on Pb, Cu, Zn, and Cd biosorption onto Sphaerotilus natans: Langmiur – type empirical model, Water Res., 37(3), 627-633.
31. M.G.ds Fonseca, M.M.de Oliveira, L.N.H. Arakaki, (2006). J. Hazard. Mater. 137, 288.
32. I.D. Mall, V.C. Srivastava, G.V.A. Kumar, I.M. Mishra, (2006). Colloid Surf. A: Physicochem. Eng. Asp. 278(1-3), 175 .
33. V.C. Srivastava, I.D. Mall, I.M. Mishra, (2006). Sep. Sci. Technol. 41, 2685.
34. M. Kandah, F.A. Ul Rub, N. Al- Dabaibeh, (2002). Competitive sorption of coppernickel and copper–cadmium binary on ship manure waste, Eng. Life Sci. 2, 237–243.
35. S. Singh, M.K.N. Yenkie, (2004). Competitive adsorption of some hazardous organic pollutants from their binary and ternary solutions onto granular activated carbon columns, J. Water, Air, Soil Pollut. 156275–286.
36. K. Flogeac, E. Guillon, M. Aplincourt, (2007). Competitive sorption of metal ions onto a north-eastern France soil. Isotherms and XAFS studies, Geoderma 139, 180–189.
37. P. Sharma, P. Kumari, M.M. Srivastava, S. Srivastava, (2007). Ternary biosorption studies of Cd(II), Cr(III) and Ni (II) on shelled Moringa oleifera seeds, Bioresour. Technol. 98, 474–4772.
38. g.w. Brummer, J. Gerth, K.G. Tiller, (1988). J. Soil Sci., 39, 37.
39. C.A. Backes, R.G. McLaren, A.W. Rate, R.S. Swift, (1995). 59, 778.
40. C.W. Gray, R.G. McLaren, A.H.C. Roberts, L.M. Condron, (1998). J. Soil Res. 36, 199.
41. H. Farrah, W.F. Pickering, (1978). Water Air Soil Pollut., 9, 491.
42. C.C. Ainsworth, J.L. Pilon, P.L. Gassman, W.G. Van Der Sluys, (1994). Soil Sci. Soc. Am. J., 58, 1615.
43. A.P. Davis, M. Upadhyaya, (1996). Water Res., 30, 1894.

7/6/2010

Impact of Gibberellic Acid Enhancing Treatments on Shortening Time to Budding of Citrus Nursery Stocks

HODA, M.MOHAMED; ABD EL-RAHMAN, G.F. and ABD EL-RAHEEM, M.E.

Horticultural institute, Agricultural Research Center, GIza, Egypt.

Abstract: Screen house experiment was conducted to study the application of gibberellic acid (GA_3) at different concentrations on budding shortening time of Volkamer lemon (*C.Volkameriana* Ten & Pasq) and Sour orange (*C.aurantium L.)* rootstocks in two seasons (2008-2009). Shortening the period to reach suitable diameter for budding seedling would benefit nurserymen by reducing various production inputs and their costs. The results indicated that, the highest success rate of suitable seedlings for budding was in mid-July. This time led to shortening the period for budding about 8 months, whereas, resulting seedlings could be budded because their stem diameter reached of a pencil size (5.4 mm) or larger. Also, this study revealed that, Volkamer lemon rootstock was superior as compared to sour orange rootstock in terms of vegetative growth, root distribution, leaf mineral content and percent of suitable seedlings for budding, while leaves of sour orange contained higher chlorophyll and total carbohydrate. It could be recommended to use T_5 (Soaked seeds and seedling treated with GA_3 at 200 ppm) for giving the best vegetative growth and suitable seedlings for budding in mid July. [Journal of American Science 2010;6(12):410-422]. (ISSN: 1545-1003).

Keywords: Screen house; gibberellic acid (GA_3); lemon; vegetative growth

1. Introduction

Citrus seedlings are normally used as rootstocks for the more desirable varieties; Citrus seed germination is usually slow and erratic. A number of reasons can contributed to the slow germination of citrus seeds, e.g. presence of growth inhibitors and physical resistance of seed coat to radical protrusion (Cohen, 1956). There is considerable evidence that gibberellins may promote the germination of various seeds in different ways. Several workers reported that gibberellic acid (GA) increases either germination rates (i.e. the rapidity of germination) for instance in Sweet orange (Burns and Coggins, 1969), Cleopatra mandarin and Sour orange (Rawash, *et al*, 1980). Also, a concentration of 500 ppm has been reported as having improved the germination of sweet lime (*C.Limettoides Tan.*) (Achituv and Mendal, 1973) , as well as 1000 ppm that of Sweet orange (Burns and Coggins, 1969). Moreover, 250ppm of GA, improve germination (Though not significant) on Trifoliate orange rootstock (Suzuki and Konakahara, 1985). The time required to grow citrus seedlings to a suitable size for budding may be as long as 1 or 2 years, therefore, shortening this time is considered very important.

Application of gibberellic acid (GA) to plants influences on growth vigor. Increased plant height (Misra, *et al*, 1982) on Malta common seedlings (*Citrus Sinensis*), (Suzuki and Konakahara, 1985) on Trifoliate orange seedlings, (Mehouochi, *et al*, 1996) on Carrizo Citrange rootstock , internode length, (Monselise and Halevy, 1962) and (Eshghi and Tafazoli, 2007) on citrus seedlings and stem diameter (Ismael and Young, 1982) on Sour orange seedlings have been reported.

Also, spray of GA_3 on citrus seedlings decreased chlorophyll content of leaves on Sweet lime (Monselise and Halevy, 1962), moreover, (Mauk, *et al*, 2004) showed that spray of Sour orange and Trifoliate orange seedlings with both BA and GA_3 decreased chlorophyll (a,b) . Concerning the effect of spray GA_3 on citrus seedlings on root system, (Monselise and Halevy, 1962) indicated that dry weight of leaves and roots were decreased on sweet lime seedlings sprayed with gibberellic acid, also, GA_3 decreased root tip width and reduced all parameters related to radial expansion (Tadeo, *et al.*, 1997).

Regarding the effect of GA_3 on total carbohydrates of leaves, the action of GA_3 in stimulating growth is mediated by an accumulation of sugars in shoots and consequently an increase in carbon supply (Mehouachi *et al.*, 1996) on "Carrizo" Citrange rootstock seedlings, (Miyamoto, *et al*, 1993) on Pea seedlings and (Mostafa and Baninasab, 2008) on two almond rootstock seedling (*Prunus amygdaluw and P.Webbii).* Respecting, the effect of application of GA_3 on leaf mineral content, (Monge *et al*, 1994) studied that foliar sprays of 1000 mg L-1 GA_3 to adult peach trees and found that leaves had a significantly lower concentration of N and Ca and Mn and slightly greater concentration of K.

This study aims to assess effect GA3 at different concentrations on budding shortening period

of some citrus rootstocks (Sour orange and Volkamer lemon) to be benefit for nurserymen by reducing various production inputs and their costs.

2. Materials and Methods

The present study was carried out during 2007/2008 and 2008/2009 seasons to investigate the effect of different concentrations of gibberellins on budding shortening period to of two citrus rootstocks e.g. Sour orange (*C.aurantium)* and Volkamer lemon (*C.Volkameriana*) in Screen house in the experimental farm of the Horticulture Research Institute, Giza, Egypt.

Mature fruits of citrus rootstocks were collected. Freshly extracted seeds were shade dried and treated with Rizolex-T ® 50 % WP as a fungside and stored in 5°C tell planting time (mid April), some seeds (300 seeds for rootstock each) were soaked in GA_3 at 750 ppm for 24 h. before planting and other untreated (control). The time limit for germination was after 23 days from planting for Volkamer lemon and after 30 days from planting for sour orange. At the end of September for each season (2008 and 2009) experimental seedling rootstocks were individually planted in plastic black bags (17 x 30 cm) filled with (25% peat-moss +75% sandy soil) in the screen house and were routinely irrigated whenever it is needed. Moreover, ammonium sulfate (20.6 %) solution (1.0 gm / L) was added weekly as liquid fertilizer with tap water. Also Greenzit* (* Ciba- Geigy , Basel , Switzerland, a foliar nutrient solution) was sprayed fortnightly at 1 ml / L were applied to all seedlings under study.

All seedlings were topping (cut their stem top about 5 cm) when stem length reached in about 55 cm and stem diameter >3.00 mm. ,and all lateral shoots removal when they were growing (Suckering process) .Some seedlings were foliar spray of GA_3 at 200 ppm and other at 400 ppm after one month from transplanting in plastic bags. In foliar spray treatments, each treatment contained a wetting agent (0.1% triton B) and was applied by spraying each seedling to run-off. Seedlings were budded with "Valencia" orange (*Citrus Sinensis* L. Osbeck) using T-budding method at a height of 30-35 cm above soil surface in the pot at three time intervals (mid June, mid July and mid August) according for stem diameter of seedlings in both seasons of the study.

Treatments: Treatments were carried out for 2 rootstocks (Sour orange, Volkamer lemon) under study as follow:

1- T_1 - Control (untreated seeds and seedlings by GA_3).
2- T_2 - Seedlings were treated by GA_3 at 200 ppm from untreated seeds.
3- T_3 - Seedlings were treated by GA_3 at 400 ppm from untreated seeds.
4- T_4 - Seedlings were untreated and soaked seeds by gibberellins (750 ppm).
5- T_5 - Seedlings were treated by GA_3 at 200 ppm and soaked seeds by gibberellins (750 ppm).
6- T_6 - Seedlings were treated by GA_3 at 400 ppm and soaked seeds by gibberellins (750 ppm).

The GA_3 source was Berelex 10% w/w powder formulation, a trademark of imperial Chemical Industries Pic Frenhurst, Haselemer Surney, England.

Measurements:

1- Germination percentage: Percent germination of two citrus rootstock seeds after pre-plant soaking of gibberellins was count and measured for two studied seasons.

2- Vegetative growth parameters: It has been carried out at the end of every season for both rootstock and scion.

a. Rootstock measures: Stem length, leaf numbers, leaf area and stem diameter. Stem length was estimated from the soil surface to the end of the growing point. All leaves on each seedling were numbered and measured. Leaf area was measured (cm^2) according to (Singh and Snyder, 1984). Stem diameter was measured at 30-35 cm above the soil surface. Rootstock measures were taken just before budding time.

b. Scion measures: Stem length, leaf number, leaf area and shoot number. All shoots on each scion were numbered and measured.

3- Total root dry weight (gm). The seedlings from each treatment were dissected at the end of study. The planting media was carefully removed from the plastic black bag then roots were washed thoroughly with tap water. Total roots were oven dried at 70° for 48 hr. and total root dry weight were recorded.

4- Leaf chlorophyll a, b (μ/cm^2): Leaf samples from two citrus seedling rootstocks were washed three times with tap water, and then washed again with distilled water, and it was determined according to (Moran and Porath, 1980) method and then total chlorophyll was calculated.

5- Leaf total carbohydrates (%): Total carbohydrates content of two citrus seedling rootstocks were determined as percent of dry weight according to (Dubois *et al.*, 1956).

6- Leaves mineral content: Leaf samples six months age from two citrus seedling rootstocks were individually collected. These samples were washed several times with distilled water and then dried at 70°c for dry matter estimation. Dried samples has

been milled for determine leaf content of N, P, K, Fe, Zn and Mn .

a. Total nitrogen (%): was determined in 0.2 g of dried substance of the leaves as percentages using microkjeldahl method according to (Pregl, 1945); (Chapman and Pratt, 1978).

b. Phosphorus (%): was determined as percentages colourimetrically using stannous chloride- sulfuric acid method according to (Troug and Meyer, 1939).

c. Potassium (%): was determined as percentages using the flame photometric method according to (Brown and Lilliland, 1966).

d. Iron, Zinc and Manganese (ppm): were determined as PPM Using Atomic Absorption according to (Carter, 1993).

7- Percent of suitable seedlings amenable for budding: Percent of suitable seedlings amenable for budding were count and measured at three time intervals (mid June, mid July and mid August).

Statistical analysis of the data: The experiment was designed in a completely randomized block design and the study comprised six treatments for each rootstock and each treatment was presented by three replicates (20 seedlings per replicate).The obtained data of both seasons were subjected to analysis of variance according to (Clarke and Kempson, 1997) and the means were differentiated using Duncan multiple range test at 5% level (Duncan, 1955).

3. Results and Discussion:

1- Percent Germination of two citrus rootstock seeds. It is clear from Table (1) that, there were no significant differences effect in final percentage germination for Volkamer lemon and Sour orange rootstock seeds. It is also noticed that, the soaked seeds by gibberellins (at 750 ppm) improved germination but not significant. These results might be attributed to that there is no dormancy in citrus seeds which make a problem for germination (Schneider, 1968). Similar results were reported by (Suzuki and Konakahara, 1985) who found that, the application of GA_3 at 250 ppm did not affect in the final percentage of germination on Trifoliate orange rootstock. Also, (Soetisna *et al.*, 1985) reported that GA_3 has little or no effect on lemon seed germination. Moreover the same results were found by (Muhammad, *et al.*, 2002) on some citrus species.

Table (1). Percent germination of two citrus rootstock seeds after pre-plant soaking of gibberellins.

Treatments(T)	Season, 2008			Season, 2009		
	Germination (%)					
	Rootstocks(R)			Rootstocks(R)		
	SO	VO	Mean (T)	SO	VO	Mean (T)
Control(untreated seeds and seedlings by GA_3)	80.57 a	82.52 a	81.54 a	80.25 a	82.21 a	81.23 a
Soaked seeds by gibberellins (750 ppm)	83.21 a	85.34 a	84.28 a	81.18 a	83.37 a	82.28 a
Means (R)	81.89 a	83.93 a		80.72 a	82.79 a	

Mean separation within columns by Duncan's multiple range test, 5% level. Values that don't share the same letter are significantly different

2- Vegetative growth of rootstocks.

Data concerning vegetative growth of the two studied rootstock seedlings, i.e. Sour orange (SO) and Volkamer lemon (VO) as affected by foliar GA_3 indicated that, there were significant differences between all treatments in the two seasons of study.

a. Stem length (cm): Data in Table (2a) showed that, the maximum values of stem length were produced by Volkamer lemon (122.51 and 124.51 cm) with T6 treatment followed by Volkamer lemon (115.44 and 117.44 cm) with T5 treatment, while, the lowest significant values were with Sour orange (40.31 and 42.31 cm) under control treatment followed by (67.53 and 65.97 cm) under T2 treatment (Seedlings were treated by GA_3 at 200 ppm) in the first and second seasons, respectively.

b. Leaf number: Data presented in Table (2a) showed that, GA_3 application did not influence number of leaves and there were no significant differences between all treatments and rootstocks during the two studied seasons (2008 and 2009).

c. Leaf area (cm^2): Data in Table (2b) showed that, GA_3 application decreased leaf area, whereas, Sour orange recorded the greatest average of leaf area (35.51 and 33.59 cm^2) under control treatment. Meanwhile, the lowest vigorous were belonged to Volkamer lemon (25.75 and 23.75 cm^2) with T6, but the other treatments gave the intermediate values for the first and second seasons respectively, Table (2).

d. Stem diameter (mm): Data tabulated in Table (2b) showed that, GA_3 application increased stem diameter whereas; the higher significant values for stem diameter were belonged to Volkamer lemon (7.22 and 7.55 mm) with T6 followed by Volkamer lemon (6.68 and 7.50 mm) under T3 (seedlings were treated by GA_3 at 400 ppm), while the lower significant values for stem diameter were belonged to Sour orange (4.53 and 4.00 mm) under control treatment. Meanwhile, the other treatments gave the intermediate values in this regard in the two seasons under study.

Table (2a). Effect of foliar spray with gibberellic acid (GA_3) on some vegetative growth parameters of two citrus rootstock seedlings in 2008 and 2009 seasons.

Treatments(T)	Season, 2008			Season, 2009		
	Stem length (cm)					
	Rootstocks(R)			Rootstocks(R)		
	SO	VO	Mean (T)	SO	VO	Mean (T)
Control (untreated seeds and seedlings by GA_3) - (T_1)	40.31 h	65.97 g	53.14 f	42.31 h	70.42 fg	56.36 f
Seedlings were treated by GA_3 at 200 ppm - (T_2)	67.53 g	70.42 fg	68.97 e	65.97 g	80.32 def	73.15 e
Seedlings were treated by GA_3 at 400 ppm – (T_3)	70.63 fg	95.25 cd	82.94 d	72.63 efg	90.38 cd	81.51 d
Seedlings were untreated and soaked seeds by gibberellins - (T_4).	75.40 ef	100.46 c	87.93 c	76.40 efg	105.46 b	90.93 c
Seedlings were treated by GA_3 at 200 ppm and soaked seeds by gibberellins - (T5).	80.32 e	115.44 b	97.88 b	82.32 de	117.44 a	99.88 b
Seedlings were treated by GA_3 at 400 ppm and soaked seeds by gibberellins – (T_6).	90.38 d	122.51 a	106.4 a	95.38 bc	124.51 a	109.94 a
Means (R)	70.76 b	95.01 a		72.50 b	98.09 a	
Leaf number						
Control (untreated seeds and seedlings by GA_3) - (T_1)	34.36 ab	38.38 ab	36.37 a	36.34 a	40.78 a	38.56 a
Seedlings were treated by GA_3 at 200 ppm - (T_2)	33.51 ab	36.44 ab	34.98 a	34.60 a	39.62 a	37.11 a
Seedlings were treated by GA_3 at 400 ppm – (T_3)	35.43 ab	39.78 a	37.61 a	36.38 a	37.90 a	37.02 a
Seedlings were untreated and soaked seeds by gibberellins - (T_4).	32.17 b	34.50 ab	33.33 a	34.60 a	38.31 a	36.46 a
Seedlings were treated by GA_3 at 200 ppm and soaked seeds by gibberellins - (T5).	35.90 ab	34.55 ab	35.23 a	35.60 a	36.24 a	35.92 a
Seedlings were treated by GA_3 at 400 ppm and soaked seeds by gibberellins – (T_6).	34.60 ab	35.44 ab	35.02 a	37.78 a	36.36 a	37.07 a
Means (R)	34.33 a	36.51 a		35.84 a	38.20 a	

Mean separation within columns by Duncan's multiple range test, 5% level. Values that don't share the same letter are significantly different

Table (2b). Effect of foliar spray with gibberellic acid (GA_3) on some vegetative growth parameters of two citrus rootstock seedlings in 2008 and 2009 seasons.

Treatments(T)	Season, 2008			Season, 2009		
	Leaf area (cm^2)					
	Rootstocks(R)			Rootstocks(R)		
	SO	VO	Mean (T)	SO	VO	Mean (T)
Control (untreated seeds and seedlings by GA_3) - (T_1)	35.51 a	30.20 abc	32.85 a	33.59 a	28.57 abcd	31.08 a
Seedlings were treated by GA_3 at 200 ppm - (T_2)	32.20 abc	28.57 bc	30.39 ab	30.57 abc	26.88 bcd	28.73 ab
Seedlings were treated by GA_3 at 400 ppm – (T_3)	31.39 abc	26.44 c	28.92 ab	29.44 abcd	25.75 bcd	27.59 ab
Seedlings were untreated and soaked seeds by gibberellins - (T_4).	33.59 ab	29.39 abc	31.49 ab	31.39 ab	27.44 abcd	29.42 ab
Seedlings were treated by GA_3 at 200 ppm and soaked seeds by gibberellins - (T5).	30.57 abc	27.88 bc	29.23 ab	28.44 abcd	24.36 cd	26.40 ab
Seedlings were treated by GA_3 at 400 ppm and soaked seeds by gibberellins – (T_6).	29.44 abc	25.75 c	27.59 b	27.75 abcd	23.75 d	25.75 b
Means (R)	32.12 a	28.04 b		30.20 a	26.13 b	
Stem diameter (mm)						
Control (untreated seeds and seedlings by GA_3) - (T_1)	4.53 e	4.76 de	4.65 b	4.00 d	5.00 cd	4.53 d
Seedlings were treated by GA_3 at 200 ppm - (T_2)	5.90 abcde	5.34 bcde	5.62 a	5.50 bc	6.34 abc	5.92 bc
Seedlings were treated by GA_3 at 400 ppm – (T_3)	5.33 bcde	6.68 ab	6.01 a	5.91 bc	7.50 a	6.71 ab
Seedlings were untreated and soaked seeds by gibberellins - (T_4).	4.93 cde	6.50 abc	5.72 a	5.00 cd	5.10 bcd	5.05 cd
Seedlings were treated by GA_3 at 200 ppm and soaked seeds by gibberellins - (T5).	6.18 abcd	6.44 abc	6.13 a	6.11 abc	6.35 abc	6.23 ab
Seedlings were treated by GA_3 at 400 ppm and soaked seeds by gibberellins – (T_6).	5.00 cde	7.22 a	6.11 a	6.50 ab	7.55 a	7.03 a
Means (R)	5.31 b	6.16 a		5.50 b	6.31 a	

Mean separation within columns by Duncan's multiple range test, 5% level. Values that don't share the same letter are significantly different

3- Vegetative growth of Valencia orange scion.

Data concerning the vegetative growth of Valencia orange scion on the two studied rootstocks, i.e. Sour orange (SO) and Volkamer lemon (VO) as affected by foliar spray of GA_3 are presented in Table (3a&b).

a. Stem length (cm): Data in Table (3a) indicated that, application of GA_3 increased stem length of Valencia orange scion, whereas, stem length of Valencia orange scion on Volkamer lemon with T6 had higher significant values (30.35 and 36.26 cm) while, scion on Volkamer lemon under control

treatment had lower values (20.44 and 25.41 cm). Meanwhile, the other treatments scored the intermediate values in this regard for 2008 and 2009 seasons, respectively Table (3a).

Table (3 a).Effect of foliar spray with gibberellic acid(GA_3) on some vegetative growth parameters of Valencia orange scion budded on two citrus rootstock seedlings in 2008 and 2009 seasons.

Treatments(T)	Season, 2008			Season, 2009		
	Stem length (cm)					
	Rootstocks(R)			Rootstocks(R)		
	SO	VO	Mean (T)	SO	VO	Mean (T)
Control (untreated seeds and seedlings by GA_3) - (T_1)	21.68 ab	20.44 b	21.06 b	26.40 b	25.41 b	25.91 b
Seedlings were treated by GA_3 at 200 ppm - (T_2)	23.52 ab	24.32 ab	23.92 ab	27.53 b	27.43 b	27.48 b
Seedlings were treated by GA_3 at 400 ppm – (T_3)	26.55 ab	28.32 ab	27.44 ab	28.55 ab	31.66 ab	30.10 ab
Seedlings were untreated and soaked seeds by gibberellins - (T_4).	22.59 ab	21.31 ab	21.95 b	27.27 ab	28.39 ab	28.06 ab
Seedlings were treated by GA_3 at 200 ppm and soaked seeds by gibberellins - (T5).	25.50 ab	28.29 ab	26.90 ab	29.69 ab	33.26 ab	31.48 ab
Seedlings were treated by GA_3 at 400 ppm and soaked seeds by gibberellins – (T_6).	27.55 ab	30.35 a	28.95 a	31.47 ab	36.26 a	33.87 a
Means (R)	24.57 a	25.51 a		28.56 a	30.40 a	
Leaf number						
Control (untreated seeds and seedlings by GA_3) - (T_1)	24.51 a	23.54 a	24.03 a	27.43 a	28.50 a	27.96 a
Seedlings were treated by GA_3 at 200 ppm - (T_2)	23.57 a	24.54 a	24.05 a	26.55 a	27.38 a	26.97 a
Seedlings were treated by GA_3 at 400 ppm – (T_3)	24.62 a	23.31 a	23.97 a	27.40 a	29.23 a	28.32 a
Seedlings were untreated and soaked seeds by gibberellins - (T_4).	25.47 a	25.57 a	25.52 a	26.50 a	26.27 a	26.39 a
Seedlings were treated by GA_3 at 200 ppm and soaked seeds by gibberellins - (T5).	24.42 a	26.64 a	25.53 a	28.32 a	27.22 a	27.77 a
Seedlings were treated by GA_3 at 400 ppm and soaked seeds by gibberellins – (T_6).	25.47 a	24.53 a	25.00 a	26.69 a	28.07 a	27.38 a
Means (R)	24.68 a	24.69 a		27.15 a	27.78 a	

Mean separation within columns by Duncan's multiple range test, 5% level. Values that don't share the same letter are significantly different

b. Leaf number: Data tabulated in Table (3a) indicated that, GA_3 application did not show any distinctive effect on leaf number of Valencia orange scion during 2008 and 2009 seasons.

c. Leaf area (cm^2): Data presented in Table (3b) showed that, leaf area of Valencia orange scion decreased by increasing GA_3 concentrations, whereas, the highest significant values was in scion on Sour orange under control treatment (26.59 and 27.62 cm^2) while, the lowest values was in scion on Volkamer lemon (17.58 and 19.38 cm^2) with T6.

d. Shoot number: Data presented in Table (3b) showed that, shoot number of Valencia orange scion increased by increasing GA_3 concentration. However, scion on Volkamer lemon with T6 scored the greatest values(3.07 and 3.30) followed in descending order by scion on Sour orange with T5 (Seedlings were treated by GA_3 at 200 ppm and soaked seeds by gibberellins.) (2.43 and 2.50) while, scion on Sour orange under control treatment gave the lowest values (1.67 and 2.00). On the other hand, the other treatments had the intermediate values for this regard in 2008 and 2009 seasons, respectively.

Table (3b). Effect of foliar spray with gibberellic acid (GA_3) on some vegetative growth parameters of Valencia orange scion budded on two citrus rootstock seedlings in 2008 and 2009 seasons.

Treatments(T)	Season, 2008			Season, 2009		
	Leaf area (cm^2)					
	Rootstocks(R)			Rootstocks(R)		
	SO	VO	Mean (T)	SO	VO	Mean (T)
Control (untreated seeds and seedlings by GA_3) - (T_1)	26.59 a	24.40 a	25.49 a	27.62 a	26.36 abc	26.99 a
Seedlings were treated by GA_3 at 200 ppm - (T_2)	24.42 a	22.53 a	23.47 a	26.54 ab	25.37 abc	25.96 ab
Seedlings were treated by GA_3 at 400 ppm – (T_3)	22.30 a	20.52 a	21.41 a	24.30 abc	24.16 abc	24.23 abc
Seedlings were untreated and soaked seeds by gibberellins - (T_4).	24.58 a	22.52 a	23.55 a	26.48 abc	25.29 abc	25.89 ab
Seedlings were treated by GA_3 at 200 ppm and soaked seeds by gibberellins - (T5).	22.59 a	20.61 a	21.60 a	22.29 abc	21.26 abc	21.77 bc
Seedlings were treated by GA_3 at 400 ppm and soaked seeds by gibberellins – (T_6).	20.63 a	17.58 a	19.10 a	20.39 bc	19.38 c	19.89 c
Means (R)	23.52 a	21.36 a		24.60 a	23.64 a	
Shoot number						

Control (untreated seeds and seedlings by GA_3) - (T_1)	1.67 d	1.83 cd	1.75 d	2.00 c	2.50 abc	2.25 b
Seedlings were treated by GA_3 at 200 ppm - (T_2)	1.80 cd	2.15 bcd	1.97 cd	2.17 bc	2.67 abc	2.42 ab
Seedlings were treated by GA_3 at 400 ppm – (T_3)	2.10 bcd	2.53 abc	2.32 bc	2.50 abc	3.00 ab	2.75 ab
Seedlings were untreated and soaked seeds by gibberellins - (T_4).	1.91 bcd	2.33 abcd	2.12 bcd	2.15 bc	2.60 abc	2.38 ab
Seedlings were treated by GA_3 at 200 ppm and soaked seeds by gibberellins - (T5).	2.43 abc	2.63 ab	2.53 ab	2.50 abc	3.30 a	2.90 ab
Seedlings were treated by GA_3 at 400 ppm and soaked seeds by gibberellins – (T_6).	2.65 ab	3.07 a	2.86 a	2.75 abc	3.30 a	3.06 a
Means (R)	2.09 b	2.42 a		2.35 b	2.89 a	

Mean separation within columns by Duncan's multiple range test, 5% level. Values that don't share the same letter are significantly different

Generally, the above results clarified that increasing concentrations of GA_3 as shown in T6 (Seedlings were treated by GA_3 at 400 ppm and soaked seeds by gibberellins 750 ppm) progressively increased stem length, shoot number and stem diameter, did not influence number of leaves and decreased leaf area. These results are in harmony with (Suzuki and Konakahara, 1985) they reported that the application of GA_3 on Trifoliate orange increased plant height. Also, the same results were found by (Mehouochi, *et al*., 1996) on Carrizo Citrange rootstock.

Moreover, (Ismael and Young, 1982) indicated that, Sour orange seedlings treated by GA_3 increased stem diameter. Furthermore, the same trend was observed by (Monselise and Halevy, 1962) who found that, spraying of GA_3 on sweet lime seedlings decreased leaf area.

Also, our results indicated that Volkamer lemon was superior for giving the highest values for vegetative growth as compared with Sour orange rootstock. These results are in line with those obtained by (Dawood, 1996 and Mohamed-Hoda, 2007). They mentioned that, Volkamer lemon is suitable citrus rootstock for most citrus scion varieties for their vigorous growth.

4- Total root dry weight (gm.).

Data presented in Table (4) showed the effect of foliar spray GA_3 on total root dry weight of Sour orange (SO) and Volkamer lemon (VO) rootstock seedlings in 2008 and 2009 seasons and indicated that, total root dry weights were decreased over all GA_3 concentrations. However, Volkamer lemon under control treatment had the highest significant values (8.50 and 9.50 gm), while, Sour orange produced the lowest significant values (3.79 and 5.17 gm) with T6 (Seedlings were treated by GA_3 at 400 ppm and soaked seeds by gibberellins) and the other treatments gave in between significant values for total root dry weight in 2008 and 2009 seasons, respectively. Table(4).

Table (4) Effect of foliar spray with gibberellic acid GA_3 on total root dry weight of two citrus rootstock seedlings in 2008 and 2009 seasons.

Treatments(T)	Season, 2008			Season, 2009		
	Total root dry weight (gm)					
	Rootstocks(R)			Rootstocks(R)		
	SO	VO	Mean (T)	SO	VO	Mean (T)
Control (untreated seeds and seedlings by GA_3) - (T_1)	6.37 abcd	8.50 a	7.44 a	7.34 ab	9.50 a	8.42 a
Seedlings were treated by GA_3 at 200 ppm - (T_2)	5.86 bcde	8.06 a	6.96 ab	7.00 ab	8.12 ab	7.56 ab
Seedlings were treated by GA_3 at 400 ppm – (T_3)	4.58 de	7.61 ab	6.26 abc	6.67 ab	7.14 ab	6.90 ab
Seedlings were untreated and soaked seeds by gibberellins - (T_4).	5.07 cde	8.03 ab	6.55 abc	6.81 ab	7.67 ab	7.24 ab
Seedlings were treated by GA_3 at 200 ppm and soaked seeds by gibberellins - (T5).	4.03 e	7.06 abc	5.57 bc	5.90 b	7.05 ab	6.48 ab
Seedlings were treated by GA_3 at 400 ppm and soaked seeds by gibberellins – (T_6).	3.79 e	6.56 abc	5.18 c	5.17 b	6.43 ab	5.80 b
Means (R)	4.96 b	7.69 a		6.48 b	7.65 a	

Mean separation within columns by Duncan's multiple range test, 5% level. Values that don't share the same letter are significantly different

Generally, it could be concluded that, foliar spray GA_3 of Sour orange and Volkamer lemon rootstocks reduced total root dry weight and there were differences between rootstocks in this response.

These results are in line with those reported by (Monselise and Halevy, 1962) who studied the effect of foliar spray of gibberellins (GA_3) at concentrations ranging between (50 and 1600 ppm) for sweet lime seedlings and found that dry weights of roots were decreased for all concentrations.

Also, (Tadeo *et al*., 1997) indicated that GA_3 decreased root tip width and reduced all parameters related to radial expansion.

Furthermore, the effect of GA_3 on root growth is indirect, by means of its effect on the growth of the aerial part, because of the action exerted by GA_3 on cell elongation (Tanimoto, 1990).

5- Leaf chlorophyll content (μ/cm^2): Data presented in Table (5) showed the effect of GA_3 application on chlorophyll (a,b) of two citrus rootstocks (Sour orange and Volkamer lemon) in 2008 and 2009 seasons, data indicated that chlorophyll content of leaves decreased by increasing GA_3 concentrations.

Table (5) Effect of foliar spray with gibberellic acid (GA_3) on Leaf chlorophyll (a & b) contents of two citrus rootstock seedlings in 2008 and 2009 seasons.

Treatments(T)	Season, 2008			Season, 2009		
	Chlorophyll a (μ/cm^2)					
	Rootstocks(R)			Rootstocks(R)		
	SO	VO	Mean (T)	SO	VO	Mean (T)
Control (untreated seeds and seedlings by GA_3) - (T_1)	91.37 a	84.50 ab	87.93 a	86.71 a	78.38 ab	82.55 a
Seedlings were treated by GA_3 at 200 ppm - (T_2)	87.40 ab	80.54 abc	83.97 a	83.45 ab	75.39 abc	79.42 a
Seedlings were treated by GA_3 at 400 ppm – (T_3)	82.41 abc	75.37 abc	78.89 ab	79.37 ab	71.42 abc	75.39 ab
Seedlings were untreated and soaked seeds by gibberellins - (T_4).	85.50 ab	78.62 abc	82.06 ab	80.30 ab	70.21 bc	75.26 ab
Seedlings were treated by GA_3 at 200 ppm and soaked seeds by gibberellins - (T5).	80.46 abc	73.17 bc	76.82 ab	76.35 abc	69.74 bc	73.04 ab
Seedlings were treated by GA_3 at 400 ppm and soaked seeds by gibberellins – (T_6).	75.55 abc	65.45 c	70.00 b	70.78 abc	60.67 c	65.72 b
Means (R)	83.78 a	76.28 b		79.49 a	70.97 b	
Chlorophyll b (μ/cm^2)						
Control (untreated seeds and seedlings by GA_3) - (T_1)	33.72 a	27.66 abc	30.69 a	31.51 a	27.52 abc	29.52 a
Seedlings were treated by GA_3 at 200 ppm - (T_2)	30.53 ab	25.47 abcd	28.00 ab	29.52 ab	25.53 abc	27.52 ab
Seedlings were treated by GA_3 at 400 ppm – (T_3)	28.62 abc	22.48 bcd	25.55 ab	26.49 abc	21.43 bc	23.96 abc
Seedlings were untreated and soaked seeds by gibberellins - (T_4).	29.85 ab	24.48 bcd	27.16 ab	27.65 abc	24.59 abc	26.21 ab
Seedlings were treated by GA_3 at 200 ppm and soaked seeds by gibberellins - (T5).	26.87 abcd	20.59 cd	23.73 b	23.54 abc	20.54 bc	22.04 bc
Seedlings were treated by GA_3 at 400 ppm and soaked seeds by gibberellins – (T_6).	25.75abcd	18.52 d	22.13 b	20.83 bc	18.52 c	19.67 c
Means (R)	29.22 a	23.20 b		26.59 a	23.02 b	

Mean separation within columns by Duncan's multiple range test, 5% level. Values that don't share the same letter are significantly different

It is also clear that Sour orange under control treatment gave the greatest averages for chlorophyll (a, b), While the lowest averages were belonged to Volkamer lemon with T6, on the other hand, the other treatments gave the intermediate values for the first and second seasons, respectively, Table (5).Our results showed that, reduction of chlorophylls (a) and (b) as a result of foliar spray of gibberellins GA_3 on leaves of citrus rootstock seedlings. These results are in harmony with those obtained by (Monselise and Halevy 1962) who reported that chlorophyll content was decreased when sweat lime seedlings treated by GA_3. Moreover, (Mauk *et al.*, 1987) found that GA_3 enhanced chlorophylls (a, b) but sharply reduced on Trifoliate orange and Sour orange rootstocks. Similar results were reported by (Monge, *et al.*, 1994), they studied the effect of spray of 1000 mg/L^{-1} GA_3 on adult peach trees {*Prunus persica (L.) Batsch}* and found that GA_3 significantly reduced the concentrations of chlorophylls (a, b).On the other hand, our results indicated that Sour orange rootstock had higher leaf chlorophyll content as compared with Volkamer lemon; these results are in line with the conclusion of (Mohamed-Hoda, 2007).

6- Leaf total carbohydrates (%):

The results were given in Table (6) showed the leaf total carbohydrates of two citrus rootstock seedlings as influenced by foliar spray of GA_3 in 2008 and 2009 seasons.

Data indicated that, GA_3 application increased leaf total carbohydrates, whereas, Sour orange with T6 scored the highest significant values for leaf total carbohydrates (35.72 and 37.76 %).While, Volkamer lemon under control treatment had the lowest values (20.59 and 22.27 %), meanwhile, the other treatments scored in between values of leaf total carbohydrate for the first and second seasons, Table (5).

Generally, it seems from the foregoing results that GA_3 application increased vegetative growth and leaf total carbohydrates, also topping and suckering processes led to carbohydrate accumulation in rootstock stem. These finding agree with those obtained by (Miyamoto *et al.*, 1993) who reported that, there is positively correlation between

vegetative growth and carbohydrate accumulation in leaves, whereas, seedling growth is enhanced by trans located sucrose, also, GA_3 promoted growth may be mediated by accumulation of soluble sugars, starch and cell wall polysaccharides. Also, (Mehouachi, *et al*, 1996) reported that GA_3 stimulated growth and synthesis and turnover of sugars, increasing carbon supply in shoots, furthermore GA_3 shifted the assimilates to the shoot which probably resulted in increased shoot growth and development in Carrizo Citrange rootstock. Moreover, (Mostafa and Baninasab, 2008) studied the effect of GA_3 on carbohydrate accumulation in shoots and roots of two almond rootstock seedlings and found that high level of soluble sugars and starch in the shoot and root were observed when GA_3 application on both rootstocks. On the other hand, our results indicated that Volkamer lemon scored the lowest significant values for leaf total carbohydrates as compared with Sour orange rootstock this decrease in carbohydrate values could be attributed to active vegetative growth which consumes higher amounts of carbohydrates.

Similar pattern of response was found by (Dawood, *et al*., 2002) who reported that, leaves of scions on Volkamer lemon and Rough lemon had lower carbohydrate level. However, trees on Sour orange like Troyer Citrange rootstock recorded intermediate values in this respect. These conclusions agree with those obtained by (Abdel-Kader and Hayat, 1989) and (Mohamed- Hoda, 2007).

Table (6) Effect of foliar spray with gibberellic acid (GA_3) on Leaf total carbohydrates (%) contents of two citrus rootstock seedlings in 2008 and 2009 seasons

Treatments(T)	Season, 2008			Season, 2009		
	Total carbohydrates (%)					
	Rootstocks(R)			Rootstocks(R)		
	SO	VO	Mean (T)	SO	VO	Mean (T)
Control (untreated seeds and seedlings by GA_3) - (T_1)	24.54 bc	20.59 c	22.57 c	26.62 bcd	22.27 d	24.45 c
Seedlings were treated by GA_3 at 200 ppm - (T_2)	27.62 abc	24.62 bc	26.12 bc	29.45 abcd	25.45 cd	27.45 bc
Seedlings were treated by GA_3 at 400 ppm – (T_3)	30.67 ab	27.63 abc	29.15 ab	32.66 abc	29.68 abcd	31.17 ab
Seedlings were untreated and soaked seeds by gibberellins - (T_4).	26.56 bc	22.65 bc	24.61 bc	28.70 abcd	24.40 cd	26.55 bc
Seedlings were treated by GA_3 at 200 ppm and soaked seeds by gibberellins - (T5).	31.34 ab	28.40 abc	29.87 ab	33.61 abc	31.75 abc	32.68 ab
Seedlings were treated by GA_3 at 400 ppm and soaked seeds by gibberellins – (T_6).	35.72 a	31.57 ab	33.65 a	37.76 a	35.60 ab	36.68 a
Means (R)	29.41 a	25.91 b		31.47 a	28.19 b	

Mean separation within columns by Duncan's multiple range test, 5% level. Values that don't share the same letter are significantly different

7- Leaf mineral content:

a. Macro element (N, P and K). It is clear from Table (7a) that, the effect of GA_3 application on two citrus rootstock seedlings did not show any distinctive effect on leaf Nitrogen and Phosphorus contents during 2008 and 2009 seasons. It is also noticed that, leaves of Volkamer lemon with T5 had higher of (K) content (0.96 and 0.99 %) followed in descending order by Volkamer lemon with T2 (0.90 and 0.91%) and Sour orange with T5 (0.90, 0.90 %) while, Sour orange under control treatment gave the lowest values (0.64 and 0.63 %) in this regard. Anyhow, the differences between the different rootstocks and treatments in this regard were so high to be significant. Table (7a)

Table (7a).Effect of foliar spray with gibberellic acid GA_3 on some macro element of two citrus rootstock seedlings in 2008 and 2009 seasons.

Treatments(T)	Season, 2008			Season, 2009		
	N (%)					
	Rootstocks(R)			Rootstocks(R)		
	SO	VO	Mean (T)	SO	VO	Mean (T)
Control (untreated seeds and seedlings by GA_3) - (T_1)	2.38 a	2.35 a	2.37 a	2.59 a	2.40 a	2.50 a
Seedlings were treated by GA_3 at 200 ppm - (T_2)	2.45 a	2.40 a	2.43 a	2.53 a	2.50 a	2.52 a
Seedlings were treated by GA_3 at 400 ppm – (T_3)	2.50 a	2.48 a	2.49 a	2.50 a	2.45 a	2.48 a
Seedlings were untreated and soaked seeds by gibberellins - (T_4).	2.55 a	2.51 a	2.53 a	2.53 a	2.48 a	2.51 a
Seedlings were treated by GA_3 at 200 ppm and soaked seeds by gibberellins - (T5).	2.60 a	2.57 a	2.59 a	2.56 a	2.54 a	2.55 a
Seedlings were treated by GA_3 at 400 ppm and soaked seeds by gibberellins – (T_6).	2.64 a	2.60 a	2.62 a	2.67 a	2.36 a	2.52 a
Means (R)	2.52 a	2.49 a		2.56 a	2.46	
P (%)						
Control (untreated seeds and seedlings by GA_3) - (T_1)	0.125 a	0.128 a	0.127 a	0.126 a	0.129 a	0.128 a

Seedlings were treated by GA_3 at 200 ppm - (T_2)	0.150 a	0.145 a	0.147 a	0.148 a	0.145 a	0.147 a
Seedlings were treated by GA_3 at 400 ppm – (T_3)	0.144 a	0.133 a	0.139 a	0.142 a	0.134 a	0.138 a
Seedlings were untreated and soaked seeds by gibberellins - (T_4).	0.130 a	0.140 a	0.135 a	0.132 a	0.140 a	0.136 a
Seedlings were treated by GA_3 at 200 ppm and soaked seeds by gibberellins - (T5).	0.155 a	0.150 a	0.135 a	0.154 a	0.149 a	0.152 a
Seedlings were treated by GA_3 at 400 ppm and soaked seeds by gibberellins – (T_6).	0.139 a	0.123 a	0.131 a	0.138 a	0.122 a	0.130 a
Means (R)	0.141 a	0.137 a		0.140 a	0.137 a	
K (%)						
Control (untreated seeds and seedlings by GA_3) - (T_1)	0.640 f	0.740 def	0.69 d	0.63 e	0.740 de	0.71 c
Seedlings were treated by GA_3 at 200 ppm - (T_2)	0.854 b	0.903 ab	0.88 a	0.853 bc	0.913 ab	0.88 a
Seedlings were treated by GA_3 at 400 ppm – (T_3)	0.804 bcd	0.800 bcd	0.80 b	0.802 cd	0.800 cd	0.80 b
Seedlings were untreated and soaked seeds by gibberellins - (T_4).	0.690 ef	0.844 bc	0.77 bc	0.703 e	0.863 bc	0.78 b
Seedlings were treated by GA_3 at 200 ppm and soaked seeds by gibberellins - (T5).	0.895 ab	0.955 a	0.93 a	0.901 ab	0.990 a	0.95 a
Seedlings were treated by GA_3 at 400 ppm and soaked seeds by gibberellins – (T_6).	0.750 cde	0.690 ef	0.72 cd	0.741 de	0.680 e	0.71 c
Means (R)	0.772 b	0.822 a		0.778 b	0.831 a	

Mean separation within columns by Duncan's multiple range test, 5% level. Values that don't share the same letter are significantly different

b. Micro elements

Iron (Fe ppm). The concentration of iron (Fe) in leaf tissues of Volkamer lemon and Sour orange seedlings in response toGA_3 application were presented in Table (7b). It was cleared that, Fe content of leaves was decreased by increasing GA_3 concentrations. Also, it is noticed that leaves of Sour orange had higher concentrations of (Fe) (80.20 and 85.27 ppm) than Volkamer lemon (63.63 and 63.18 ppm). Regarding the interaction between rootstocks and treatments data also revealed that, Sour orange under control treatment gave the highest significant values (108.4 and 105.00 ppm). Meanwhile, Volkamer lemon with T6 produced the lowest significant values (40.66 and 43.54 ppm) in this respect and the differences between different rootstocks and treatments were so high to be significant in the first and second seasons, Table (7b). Zinc (Zn ppm). Data in Table (7b) showed that, the higher values for leaf content were on Sour orange with T5 (82.30 and 80.20 ppm) and the lower values were on Volkamer with T6 (65.80 and 60.70 ppm), Besides, leaves of other treatments seedlings scored in between values of zinc content in two seasons under study.

Manganese (Mn ppm). Data presented in Table (7b) indicated that, leaves of Sour orange with T6 gave the highest significant values for leaf content (167.3 ppm) in 2008 season and with T4 (172.4 ppm) in 2009 season. Meanwhile, the lowest significant values were belonged for Volkamer lemon with T3 (102.7 ppm) in the first season and on T6 (103.7 ppm) in the second season. Anyhow, the differences between different rootstocks and treatments were so high to be significant in both seasons, respectively. Table (7b).

Generally, our results of in the present study indicated that there were no significant differences between leaf N and P contents. While, leaf K content was increasing as a result for spray of GA_3. These results are in harmony with those obtained by (Monge, *et al*., 1994); they studied effect of spray of GA_3 to adult peach trees and found that the concentrations of P, Mg and K were unaffected. For micronutrient our data revealed that leaf Fe content was decreased by increasing GA_3 concentrations, and there were positively correlation between leaf Fe concentrations and leaf chlorophyll content. Also, there were significant differences between all treatments for leaf Zn and Mn. Several researches have reported on antagonism between Fe and Mn, which could lead to Fe chlorosis (Bindra, 1980) and (Casero and Carpena, 1987) probably due to a substitution of Fe by Mn in the biosynthesis of chlorophyll (Clairmont, *et al.*, 1986). Anyhow, using standard values of citrus seedling leaves mineral nutrient concentration (Chapman, 1960) the nutritional status of our citrus seedlings was good except for Fe, which was slightly above normal levels and increasing GA_3 concentrations.

Also, our results indicated that leaves of Volkamer lemon scored the highest significant values for K content and leaves of Sour orange recorded the highest significant values for Fe content, while, there were no significant between rootstocks for other element contents. This is in agreement with (Abou-Rawash, *et al.*, 1995) and (Eid, *et al.*, 2000); they mentioned that citrus rootstocks varied in their uptake of nutrients, since some rootstocks such as Volkamer lemon can absorb more macronutrient.

8- Percent of suitable seedlings amenable forbudding.

Data tabulated in Table (8) showed the average percent of suitable seedlings amenable for budding of Sour orange and Volkamer lemon which influenced by foliar spray of GA_3 and were taken at

three time intervals (mid June, mid July and mid August) during 2008 and 2009 seasons.

At mid June. Data in Table (8) showed that, Volkamer lemon seedlings under T2, T3, T4 and T5 gave the highest significant values (62.65, 62.60, 63.61 and 64.58 %) with no significant between them, while the lowest percent was in Sour orange with control treatment (45.53 and 47.53 %) in two seasons under study.

It is noticed that, Sour orange with T3 (Seedlings were treated by GA_3 at 400 ppm) recorded the highest percent of suitable seedlings amenable for budding (62.46 and 60.62 %), while the lowest value was in Sour orange under control treatment (45.31 and 47.53 %) respectively in both seasons 2008 and 2009.

At mid July. Data of Table (8) indicated that increasing rate of suitable seedlings amenable for budding as compared with the obtained results from the previous time (mid June) because in this time, stem diameter of resulting seedlings reached of a pencil size or larger. Data also showed that, Volkamer lemon with T5 had the highest averages (97.25 and 98.17%) and Sour orange (90.65 and 93.38 %) followed by Volkamer lemon with T2 (94.68 and 95.30%) and Sour orange (86.60 and 87.72 %), while the lowest significant values were on Sour orange (71.63 and 74.47%) under control treatment for the first and second seasons, respectively. Table(8).

At mid August. Data tabulated in Table (8) showed that decreasing rate of percent of suitable seedlings amenable for budding compared with the previous time (mid-July). It is also clear that, Sour orange had the highest values (75.78 and 71.75 %) under T_3 in both seasons. But, Volkamer lemon gave the most vigorous values under T3 (76.96%) in the first season, and (72.65%) under T5 in the second season. Meanwhile, Sour orange and Volkamer lemon under T6 produced the lowest values (65.47 and 59.58 %) and (60.78 and 58.52%) for two seasons, respectively. Anyhow, the differences between all treatments were so high to be significant in 2008 and 2009, seasons.

From the above results it could be concluded that, there is positively correlation between the percent of suitable seedlings amenable for budding and vigorous growth, stem diameter and leaf mineral content of the rootstocks. These results can be attributed to the vigorous growth of Volkamer lemon rootstock which possessed the highest values for stem diameter and leaf mineral content. These results are in line with those obtained with (Mohamed-Hoda, 2007) who studied the behavior of Valencia orange buddlings grafted on
some citrus rootstocks growing in various soils and found that the highest percent of suitable seedlings amenable for budding were obtained with Volkamer lemon followed by Sour orange, while Troyer Citrange had the lowest values in this respect.

Moreover, our results reported that, the highest success rate of suitable seedlings amenable for budding was in mid-July for two seasons. This time led to shortening the period for budding in citrus seedlings about 8 months, whereas, resulting seedlings could be budded because their stem diameter reached of a pencil size (5.4 mm or larger). Shortening this time would benefit nurserymen by reducing various production inputs and their costs.

Table (7b). Effect of foliar spray with gibberellic acid GA_3 on some micro element of two citrus rootstock seedlings in 2008 and 2009 seasons.

Treatments(T)	Season, 2008			Season, 2009		
	Fe (ppm)					
	Rootstocks(R)			Rootstocks(R)		
	SO	VO	Mean (T)	SO	VO	Mean (T)
Control (untreated seeds and seedlings by GA_3) - (T_1)	108.4 a	95.48 a	102.0 a	105.5 a	89.52 bc	97.50 a
Seedlings were treated by GA_3 at 200 ppm - (T_2)	75.45 b	60.60 cde	68.03 bc	85.47 bc	58.98 ef	72.22 b
Seedlings were treated by GA_3 at 400 ppm – (T_3)	76.64 b	59.03 de	67.83 bc	80.50 bcd	55.65 fg	68.08 bc
Seedlings were untreated and soaked seeds by gibberellins - (T_4).	80.74 b	70.62 bcd	75.68 b	93.74 ab	85.72 bc	89.73 a
Seedlings were treated by GA_3 at 200 ppm and soaked seeds by gibberellins - (T5).	73.32 bc	55.38 e	64.35 c	75.75 cd	45.66 fg	60.71 cd
Seedlings were treated by GA_3 at 400 ppm and soaked seeds by gibberellins – (T_6).	66.60 bcde	40.66 f	53.63 d	70.68 de	43.54 g	57.11 d
Means (R)	80.20 a	63.63 b		85.27 a	63.18 b	
Zn (ppm)						
Control (untreated seeds and seedlings by GA_3) - (T_1)	64.99 b	68.70 ab	66.85 b	62.40 cd	66.73 abcd	64.57 b
Seedlings were treated by GA_3 at 200 ppm - (T_2)	73.53 ab	75.30 ab	74.42 ab	75.80 abc	75.33 abc	75.57 a
Seedlings were treated by GA_3 at 400 ppm – (T_3)	73.90 ab	70.90 ab	72.40 ab	72.20 abcd	69.80 abcd	71.00 ab
Seedlings were untreated and soaked seeds by gibberellins - (T_4).	69.80 ab	73.33 ab	71.57 ab	69.30 abcd	73.40 abcd	71.35 ab
Seedlings were treated by GA_3 at 200 ppm and soaked	82.30 a	80.50 a	81.40 a	80.20 a	78.80 ab	79.50 a

seeds by gibberellins - (T5).						
Seedlings were treated by GA_3 at 400 ppm and soaked seeds by gibberellins – (T_6).	69.40 ab	65.80 b	67.60 b	64.80 bcd	60.70 d	62.75 b
Means (R)	72.32 a	72.42 a		70.78 a	70.79 a	
Mn (ppm)						
Control (untreated seeds and seedlings by GA_3) - (T_1)	156.40 ab	120.60 de	138.50 b	124.40 de	129.70 de	127.10 cd
Seedlings were treated by GA_3 at 200 ppm - (T_2)	110.50 ef	130.70 cd	120.60 c	140.60 cd	140.70 cd	140.60 b
Seedlings were treated by GA_3 at 400 ppm – (T_3)	140.40 bc	102.70 f	121.50 c	119.40 ef	160.60 ab	140.00 bc
Seedlings were untreated and soaked seeds by gibberellins - (T_4).	152.70 ab	150.70 ab	151.70 a	172.40 a	153.60 bc	163.00 a
Seedlings were treated by GA_3 at 200 ppm and soaked seeds by gibberellins - (T5).	132.40 cd	140.60 bc	136.50 b	120.50 ef	125.60 de	123.00 d
Seedlings were treated by GA_3 at 400 ppm and soaked seeds by gibberellins – (T_6).	167.30 a	144.60 bc	156.00 a	163.60 ab	103.70 f	133.70 bc
Means (R)	143.30 a	131.60 b		140.20 a	135.60 a	

Mean separation within columns by Duncan's multiple range test, 5% level. Values that don't share the same letter are significantly different

Table (8).Effect of foliar spray with gibberellic acid GA_3 on percent of suitable seedlings amenable for budding of two citrus rootstock seedlings in 2008 and 2009 seasons.

Treatments(T)	Season, 2008			Season, 2009		
	Mid June					
	Rootstocks(R)			Rootstocks(R)		
	SO	VO	Mean (T)	SO	VO	Mean (T)
Control (untreated seeds and seedlings by GA_3) - (T_1)	45.31 c	60.48 ab	52.90 b	47.53 c	58.40 abc	52.96 a
Seedlings were treated by GA_3 at 200 ppm - (T_2)	60.45 ab	62.65 a	61.55 a	58.55 abc	59.52 ab	59.03 a
Seedlings were treated by GA_3 at 400 ppm – (T_3)	62.46 ab	62.60 a	62.53 a	60.62 a	58.56 abc	59.59 a
Seedlings were untreated and soaked seeds by gibberellins - (T_4).	50.56 bc	63.61 a	57.09 ab	48.50 bc	57.45 abc	52.98 a
Seedlings were treated by GA_3 at 200 ppm and soaked seeds by gibberellins - (T5).	60.59 ab	64.58 a	62.59 a	56.21 abc	58.37 abc	57.29 a
Seedlings were treated by GA_3 at 400 ppm and soaked seeds by gibberellins – (T_6).	58.53 ab	55.65 abc	57.09 ab	54.51 abc	53.50 abc	54.01 a
Means (R)	56.32 b	61.60 a		54.32 a	57.63 a	
Mid July						
Control (untreated seeds and seedlings by GA_3) - (T_1)	71.63 f	82.61 cd	77.12 d	74.47 f	85.61 cde	80.04 c
Seedlings were treated by GA_3 at 200 ppm - (T_2)	86.60 bc	94.68 a	90.64 a	87.72 bcd	95.30 ab	91.51 ab
Seedlings were treated by GA_3 at 400 ppm – (T_3)	82.68 cd	87.48 bc	85.08 b	84.42 cde	88.34 bcd	86.38 bc
Seedlings were untreated and soaked seeds by gibberellins - (T_4).	74.38 ef	90.61 ab	82.50 bc	77.82 ef	91.58 abc	84.70 c
Seedlings were treated by GA_3 at 200 ppm and soaked seeds by gibberellins - (T5).	90.65 ab	97.25 a	93.95 a	93.38 abc	98.17 a	95.78 a
Seedlings were treated by GA_3 at 400 ppm and soaked seeds by gibberellins – (T_6).	78.58 de	78.33 de	78.96 cd	80.30 def	80.55 def	80.42 c
Means (R)	80.76 b	88.66 a		83.02 b	89.93 a	
Mid August						
Control (untreated seeds and seedlings by GA_3) - (T_1)	65.75 bc	70.54 abc	68.15 bc	63.57 abc	65.38 abc	64.47 ab
Seedlings were treated by GA_3 at 200 ppm - (T_2)	73.71 ab	75.56 ab	74.63 ab	69.61 abc	70.44 abc	70.03 a
Seedlings were treated by GA_3 at 400 ppm – (T_3)	75.78 ab	76.96 a	76.37 a	71.75 ab	70.55 abc	71.15 a
Seedlings were untreated and soaked seeds by gibberellins - (T_4).	70.84 abc	72.29 ab	71.57 ab	68.46 abc	64.13 abc	66.29 ab
Seedlings were treated by GA_3 at 200 ppm and soaked seeds by gibberellins - (T5).	75.36 ab	75.69 ab	75.52 ab	70.49 abc	72.65 a	71.57 a
Seedlings were treated by GA_3 at 400 ppm and soaked seeds by gibberellins – (T_6).	65.47 bc	60.78 c	63.13 c	59.58 bc	58.52 c	59.05 b
Means (R)	71.15 a	71.97 a		67.25 a	66.94 a	

Mean separation within columns by Duncan's multiple range test, 5% level. Values that don't share the same letter are significantly different

Corresponding author
HODA, M.MOHAMED
Horticultural institute, Agricultural Research Center, GIza, Egypt.

4. References

1. Abdel-Kader and Hayat, M. (1989). Physiological and anatomical studies on Washington navel orange and Clementine mandarin trees budded on

some local and introduced citrus rootstocks. Ph.D. Desertation, Fac. of Agric., Alex. Univ. U.A.R.
2. Abou-Rawash, M.; El-Hammady, A.M.; Abou-Aziz, A.; Abd El-Hamid, N. and Abd El-Moneim, E. (1995). Growth and mineral composition of four citrus rootstock seedlings grown under two different soil types, Annals of Agric. Sci. Ain Shams Univ. 40 (1): 307-325, Egypt.
3. Achituv, M. and Mendal, K. (1973). Effect of certain treatment on the germination of sweet lime (*Citrus limettioides Tan.*) Seed. PI. Prop., 19: 15-20.
4. Bindra, A.S (1980). Iron chlorosis in horticultural and field crops. Annu. Rev. Plant Sci. 2: 221-312.
5. Brown, J.D. and Lilliland, O. (1966) Rapid determination of potassium and sodium in plan material and soil extracts by Flame-photometry. J.Amer. Soc. Hort. Sci., 48: 341-346.
6. Burns, R.M. and Coggins, Jr.e.w/ (1969). Sweet orange germination and growth aided by water and gibberellin seed soak. Calif.Agric. Dec. Pp.18-19.
7. Carter, M.R. (1993). Soil sampling and methods of analysis, Canada Soc., Soil. Sci. Lewis, London, Tokyo.
8. Casero, T. and Carpena, O. (1987). Relaciones nutritivas en melocotonera "Sundanell". Inv. Agrar Prod Port Veg. 2: 19-30.
9. Chapman, H.D. (1960). Leaf and soil analysis of citrus orchards. Calif. Agric. Expt. Sta. Manual, 25: 35-53.
10. Chapman, H.D. and Pratt, P.F. (1978). Methods of analysis for soils, plants and water. Univ. of Calif. Div. Agric. Sci, Priced publication 4034.
11. Clairmont, K.; Hagar, W. and Davies, E. (1986). Manganese toxicity to chlorophyll synthesis in tobacco callus. Plant Physiol. 80:291-293.
12. Clarke,G.M. and Kempson,R.E.(1997) Introduction to the design and analysis of experiments. Arnold, a member of the Holder Headline Group,1st Edt.,London,UK.
13. Cohen, A. (1956). Studies on the viability of citrus seeds and certain properties of their seed coats. Bull, Res. Council Israel Sec. D. 5: 200-209.
14. Dawood, D.S.A.; El-Sammak, A.F.; Mohamed, E.M. and El-Sayed- Somaia, A. (2002). Evaluation of 3-citrus varieties budded on 5-citrus rootstocks grown on slightly saline alkaline soil at "Sakha, Kafr El-Sheikh Governorate (1) vegetative growth root density and distribution and some organic substances. Egypt. J. Agric. Res. 80 (1): 309-323.
15. Dawood, S.A. (1996). Evaluation of vegetative growth and nutrient composition of nine citrus rootstocks under North Delta Environmental conditions, 1st Egypt, Hung. Hort. Conf., (1) 171-181.
16. Dubois, M.; Gilles, K.A.; Hamilton, J.K.; Robers, P.A. and Smith, F. (1956). Calorimetric method for determination of sugars and related substances. Analytical chemistry 28 (3): 350-356.
17. Duncan, D.B. (1955) Multiple range and multiple F-Test Biometrics,11:1-42.
18. Eid, A.F.M.; Mohamed , M.A.; Hegab, M.Y. and Yehia, M.A.(2000). Effect of some citrus rootstocks and NPK fertilization on growth and leaf chemical composition of Valencia and Washington Nave orange seedlings, 2. The effect on the leaf chemical comosition. Minufiya J.Agric. Res. 25 (4): 1085-1097, Egypt.
19. Eshghi, S. and Tafazoli, E. (2007). Reduction of growth period prior to budding in lime (*citrus aurantifolia*) seedlings and budtake by use of plant growth regulators and pinching. J. Sci. & Technol. Agric. & Natur. Resour., Vol.10, No.(4) (A) Winter, Isf. Univ. Technol., Isf. Iran.
20. Ismael, A.M. and Young, M.J. (1982). Influence of gibberellic acid and effectiveness of several carriers on growth of Sour orange. (*Citrus aurantium L.*) seedings. Hort. Sci. 17 (4): 673-674.
21. Mauk, C.S.; Bausher, M.G. and yelenosky, G. (1987). Physiological effects and temperature and growth regulators on foliar chlorophyll, soluble protein, and cold hardiness in citrus. Plant growth regulation, Vol.(5) No.(2), 141-154.
22. Mehouachi. F.R. Tadeo; Zaragoza, S.; Primo-Millo, E. and Talon, M. (1996). Effect of gibberellic acid and paclobutrazol on growth and carbohydrate accumulation in shoots and roots of citrus rootstock seedlings .Journal of Horticultural Science.71 (5) 747-754
23. Misra,R.S.; Singh, S.B and Awasthi, D.N. (1982). Effect of plant growth regulators and ascorbic acid on germination and growth of Malta common seedlings [*Citrus sinensis* Osbeek] in Garhwal Hills Progressive Horticulture, 14, 165-168 (C.F Hort, Abst., 1984, 54, No.8 :5797.
24. Miyamoto, K.; Ueda, J. and Kamisaka, S. (1993). Gibberellin-enhanced sugar accumulation in growing subhooks of etiolated *Pisum Sativum* Seedlings. Effects of gibberellic acid, indoleacetic acid and cycloheximide on invertase activity, sugar accumulation and growth Physiologia plantarum, 88, 301-306.
25. Mohamed-Hoda, M.A. (2007). Behavior of Valencia orange budlings grafted on some citrus rootstocks produced *in vitro* and grown in various soils. Ph.D Thesis in pomology, Fac. Agric., Cairo Univ., A.R.E.

26. Monge, E.; Aguirre, R. and Blanco, E.E. (1994). Application of paclobutrazol and GA_3 to adult peach trees: Effects on nutritional status and photosynthetic pigments. J. Plant Growth. Reg. Vol. (13) No. (1), 15-19.
27. Monselise,S.P and Halevy,A.H.(1962). Effect of gibberelline and AMO. 1618 on growth, dry-matter accumulation, chlorophyll content and peroxidase activity of citrus seedlings. Amer. J. Bot.49:405-412.
28. Moran, R. and Porath, D. (1980). Chlorophyll determination in intact tissues using N,N-dimethyl formamide. Plant Physiol. 65: 478-479.
29. Mostafa, M. and Baninasab, B. (2005). Effects of plant growth regulators on growth and carbohydrate accumulation in shoots and roots of two almond rootstock seedlings. Fruits Issue (6), (63), 363-370.
30. Muhammad, M.K.; Muhammad, U.S; Rashid, w. and Muhammad, A.A. (2002) . Role of gibberellic acid GA_3 on citrus seed germination and study of some morphological characteristics, Pak 1 Agric. Sci. Vol. 39 (2).
31. Pregl, F. (1945) Quantitative organic micro-analysis, 4^{th} ed J.A. Churchill, Ltd, London.
32. Rawash, M.A.; Montaser, A.; Habib, S.S.; Nabawy, S.E. and Mahmoud, N. (1980). Germination of some citrus as affected by soaking in growth regulators, water washing and sowing date. Res. Bull. Faculty Agric. Ain Shams Univ. 1299: 1-10.
33. Schneider, H. (1968). The anatomy of citrus In: the citrus industry (Eds.) Rather, W.L., I. Batchelor and H. Webber, Vol.II: pp. 1-85 Univ. Of Calif., Berkeley, USA.
34. Singh, S.F and Snyder, G.H. (1984). Leaf area index and dry biomass. Taro-Agron. J.76: 350-353.
35. Soetisna, L.J.; King, M.W. and Roberts, E.H. (1985). Germination test recommendations for estimating the viability of moist or dry seeds of lemon (*Citrus Limon*) and lime (*C.aurantifolia*) Seed Sci. & Tech. 13:87 IIO.
36. Suzuki, T.and Konakahara, M. (1985).Improvement of growing methods for citrus nursery stock. I. Promotion of seed germination and growth of Trifoliate orange rootstocks. Bulletin of the Shizuoka prefectural citrus Experiment Station, No. 21,9-18, (C.F Hort. Abst., 1987, Vol.57, No.4: 2958.
37. Tadeo, F.R.; Gomez-Cadenas, A.; Ben-Cheikh, W.; Primo-Millo, E. and Talon, M.(1997). Gibberellin-ethylene interaction controls radial expansion in citrus roots. Planta, 202: 370-378.
38. Tanimoto, E. (1990). Gibberellin requirement for the normal growth of roots. In: Takahashi, N.; Phinney, B.O. and MacMillan, J. (Ed), Gibberelines. New York: Springer- Verlag, Cap.22, p.229-240.
39. Troug, E. and Meyer, A.H. (1939) Improvement in deiness colorimetric method for phosphorous and arsenic. Ind. Eng. Chem. Anal., Ed. I: 136-159.

6/1/2010

Prognostic Impact of Elevated Serum Hyulronic Acid, Ferritin and Interleukin-6 in Patients with Acute Myeloid Leukemia

Nabila Abd El Maksoud,[1] Halla M. Ragab[1], Maha M. Abd El Latif[2] and Sh. Abdalla[3]
[1]Department of Biochemistry, Genetic Engineering and Biotechnology Division, [2]Department of research and applications of supplementary medicine, National Research Centre, and [3]Department of Clinical Pathology, El-Sahel Teaching Hospital, Cairo, Egypt .

Absract

Background: Acute myeloid leukemia (AML) is a clonal disease of hematopoiesis with poor clinical outcome despite recent improvements in chemotherapy and stem cell transplantation regimens. It is the most common acute leukemia in adults. Hyaluronic acid, ferritin and Interleukin-6 are involved in the pathogenesis of acute myeloid leukemia , but their prognostic significance in these diseases is unknown. In the current study, the authors assessed the serum levels of these parameters in different stages of the disease to predict their prognostic value, which might therefore represent interesting target for immunotherapy in patients with different hematological malignancies.
Methods: Serum levels of. hyaluronic acid, ferritin and Interleukin-6 were measured using a commercially available sandwich Enzyme Linked Immune Sorbent Assay (ELISA) kit in patients with AML who were attending for treatment at National Cancer Institute, Cairo University from September 2006 through January 2009.
Results: Newly diagnosed and relapsed patients with AML had significantly higher serum levels of hyaluronic acid, ferritin and Interleukin-6 compared with both control group and leukemic patients in remission stage. Serum levels of hyaluronic acid, ferritin and interleukin–6 in patients with AML (at diagnosis and at relapse) correlated inversely with the hemoglobin concentration. While their serum levels correlated positively with both total leukocyte count and with the % of blast cells in bone marrow in patients with AML

Conclusions: It could be concluded that serum levels of hyaluronic acid, ferritin and Interleukin-6 can be used as prognostic markers at diagnosis of adult AML and it could be used as follow up parameters for early detection of relapse. Furthermore, they might represent interesting target for immunotherapy in patients with different hematological malignancies. [Journal of American Science 2010;6(12):423-432]. (ISSN: 1545-1003).

Keywords: Acute myeloid leukemia (AML), Hyaluronic acid (HA), Ferritin (Fe), Interleukin–6 (IL-6).

1. Introduction:

Acute myeloid leukemia is a hematological disease characterized by the clonal proliferation of undifferentiated myeloid progenitor cells. AML is the most common variant of acute leukemia occurring in adults, comprising approximately 80 to 85% of cases of acute leukemia diagnosed in individuals greater than 20 years of age. Most of the patients with AML achieve a complete hematological remission by chemotherapeutical regimes. However, the long-term prognosis for all AML patients is rather poor with a 5-year overall survival of only 20-25% depending on the individual risk profile and the treatment option chosen. This clinical outcome suggests that the majority of the patients in complete hematological remission have minimal residual disease, subsequently leading to relapse. Obviously the leukemia-bearing host is immunologically tolerant to the remaining leukemia cells and therefore fails to eradicate the disease **(Li *et al.*, 2003).**

Hyaluronic acid (HA) is a nonsulfated glycosaminoglycan (molecular weight of 10^6 dalton). It is the only glycosaminoglycan that is not attached to any protein core. It is a component of tissue matrix and tissue fluids, it maintains the cartilage integrity and osmotic balance. Also it regulates cell adhesion, migration and proliferation. It is produced mainly by fibroblasts and other specialized connective tissue cells. It plays a structural role as part of the connective tissue matrix (proteoglycan) and participates in various cell-to-cell interactions. Synovial hyaluronic acid passes into plasma via the lymphatic system, and is quickly removed from the blood by a receptor dependent mechanism in sinusoidal epithelial cells of the liver and by the enzymatic action of hyaluronidase . Thus, hyaluronan is a glucosaminoglycan synthesized by the mesenchymal cells and

degraded by hepatic sinusoidal endothelial cells by a specific receptor-mediated process **(Plevris *at al.* 2000).**

It has been reported that HA is synthesized mostly by tumor stromal fibroblasts and that tumor cells activate the fibroblasts to synthesize a high levels of hyaluronic acid .The concentration of HA is elevated in several carcinomas (e.g., lung, breast, colon, Wilms' tumor **(Delpech *et al.*, 1997).** More importantly, it was shown that the HA concentration is elevated in the urine of bladder cancer patients and serves as a diagnostic marker for detecting bladder tumor regardless of its grade **(Lokeshwar *et al.*, 1997).**

Hyaluronan (HA) has been reported to bind specifically and with high affinity to various cell types and to directly modify cell behaviour. It was demonstrated that both high molecular weight molecules (HAH) and HA-derived oligosaccharides were efficient at triggering terminal differentiation of acute myeloid leukemia (AML) blasts, in vitro **(Marie *et al.*, 2004).**

Iron-storage compounds in the body include hemoglobin, hemosiderin, myoglobulin and the cytochromes **(Adams, 1998).** In most tissues, ferritin is a major iron storage protein with a molecular weight of 45 kd. Each molecule contains as many as 4000 iron atoms. Ferritin represents 25% of the total iron found in the body. High concentrations of ferritin are found in the cytoplasm of the reticuloendothelial system, the liver, spleen and bone marrow. Methods previously used to measure iron in such tissues are invasive, cause patient trauma and lack adequate sensitivity. Ferritin levels in serum have been used to evaluate clinical conditions not related to iron storage, including inflammation, chronic liver disease and malignancy **(Casaril *et al.*, 2000).** In normal conditions ferritin is mainly expressed in red cell precursors and reticuloendothelial cells, and this is in keeping with the peculiar role of these cells in iron metabolism. Abnormal cell ferritin contents can be observed in both iron overload and malignancy. Elevated serum ferritin might indicate the presence of malignant disease . It was reported that serum ferritin was elevated in breast carcinoma, different urologic malignancies, and acute and chronic leukemia and in M1 and M2 myeloid leukemia **(Ulbrich *et al.*, 2003, Ahlawat *et al.*, 1994 and Aulbert *et al.*, 1991).**

Cytokines are involved in the pathogenesis of acute myeloid leukemia (AML) and high-risk myelodysplastic syndromes (MDS), but their prognostic significance in these diseases is unknown **(Tsimberidou *et al.*, 2008)**.

Interleukin-6 (IL-6) is a pleiotropic cytokine produced by a variety of cell types, including fibroblasts, endothelial cells, monocytes, normal hematopoietic cells, and lymphocytes. Serum IL-6 levels have been correlated with an increased risk for development of lymphoma in patients with AIDS and in renal transplant recipients. Serum IL-6 levels are increased in diffuse large cell lymphomas, associated with adverse prognostic features, and predictive of a poor failure-free and overall survival in multivariate analysis. Interestingly, serum IL-6 levels may also be elevated and correlate with poor prognostic features and an inferior outcome in Hodgkin disease, indolent non-Hodgkin lymphomas (NHLs), renal cell carcinoma, prostatic cancer, ovarian cancer, and multiple myeloma (**Kawano *et al.*, 1988)**.

2. Aim of the work:

The present study was planned to estimate the serum levels of hyaluronic acid, ferritin and interleukin-6 in patients with acute myeloid leukemia in different stages of the disease and to assess their prognostic value.

3. Subjects and Methods:

The subjects of this study were (115) divided into two groups:

Group A: Patients group consisted of 80 with AML attending the National Cancer Institute, Cairo University. They were 48 males and 32 females. They included 32 patients (group A I*)*, at diagnosis before starting therapy with age range between 25 and 56 years (mean 44.2 ± 8.6). 28 patients in remission (group A II), but still under therapy with age range between 29 – 58 years (mean 47.5 ± 9.3). and 20 patients (group A III),with bone marrow relapse with age range between 35 – 58 years (mean 48.3 ± 9.5).

Group B: (Control group) 35 healthy volunteers were used for comparison with AML patients, 20 males and 15 females with age range between 32 - 57 years (mean 45.7 ± 4.7). They were free from any acute or chronic disease (e.g., Liver dysfunction, diabetes, infection, etc.) at the time of samples withdrawal.

3.1. Methods

All patients included in this study were subjected to:

1- Full medical history with particular stress on age at diagnosis, sex, bone aches, neurological complications (for evidence of CNS involvement).

2 -Thorough clinical examination was done with special emphasis on the weight, height, temperature, lymph node enlargement,

organomegaly, the presence of any mass as well as central nervous system and chest examination.

3 -Investigations included:

- Complete hemogram (using Automated Coulter Counter T-660).
- Examination of bone marrow aspiration and/or biopsy for morphology, FAB classification and immunophentyping done for the patients.
- Examination of CSF for evidence of CNS involvement.
- Radiological investigations included chest X-ray, bone survey, ultra-sonic and computed tomography scanning to chest and abdomen, and others as indicated.

3.2. Laboratory investigations:

All patients were subjected to :

3.2.1. Determination of serum Hyaluronic Acid: Serum samples were analyzed for Hyaluronic Acid (HA) using a commercially available sandwich Enzyme Linked Immune Sorbent Assay (ELISA) kit obtained from Corgenix, inc., USA **(Chichibu *et al.*,1989).** HA levels in patients and control samples were determined against a reference curve. Each standard and sample was assayed in duplicate.

3.2.2. Determination of serum Ferritin: Samples were analyzed for Ferritin using a commercially available sandwich ELISA kit obtained from DRG International inc., USA **(White *et al.*, 1986).** Each standard and sample was assayed in duplicate.

3.2.3. Determination of serum IL-6: Analysis was performed using commercially available kit (IL-6 ELISA Kit), Diaclone Research, (URS), - France. The minimum detectable dose of IL-6 is less than 2 pg/ml. Intra and Inter - Assay coefficients of variation of the assay were 0.83-3.86% and 1.89-5.84% **(Robak *et al.*, 1999)**.

4. Statistical analysis:

Data were analyzed with standard program of SPSS, Echo Soft corporation, USA, 1995 statistical package. Student t test was applied to the data conforming to normal distribution. For non-parametric data Mann Whitney U test was applied. Correlation coefficient (r) was used to determine the relationships between different quantitative values. For all tests a probability <0.05 was considered significant (**Saunders and Trapp 1995)**.

5. Results:

The results of this study are demonstrated in the tables (1,2, and 3) and figures (1,2, and 3).

Table (1) shows descriptive data of the patients with acute myeloid leukemia and control groups. Newly diagnosed and relapsed AML patients had statistically significant lower hemoglobin and platelet count compared to control group ($p< 0.001$). TLC was statistically high among newly diagnosed and relapsed AML patients compared to control group and patients with AML at remission stage ($p < 0.001$). There was no significant difference between AML patients in remission and control group.

The results showed that newly diagnosed and relapsed patients with AML had significant high serum levels of hyaluronic acid, ferritin and interleukin–6 compared to both control group and leukemic patients in remission stage (Table 2).

There was no significant difference in serum levels of hyaluronic acid, ferritin and interleukin–6 between newly diagnosed and relapsed AML patients, as well as between leukemic patients in remission stage and control group, also there was no significant difference as regards FAB morphological classification and immunophenotype .

Serum levels of hyaluronic acid, ferritin and interleukin–6 in patients with AML (at diagnosis and at relapse) correlated inversely with the hemoglobin concentration ($p < 0.05$). While their serum levels correlated positively with both total leukocyte count (TLC) ($p < 0.05$) and with the % of blast cells in bone marrow in patients with AML.

Table (1): Descriptive data of patients with Acute Myeloid Leukemia and control group.

Diagnosis	Age years	Hb gm%	WBCs $X10^9/L$	Plt $X10^9/L$	FAB Classification (n)
Group A I (n= 32)					M2 = (10)
At diagnosis					M3 = (14)
Range	25-56	4.2-11.4	14-112	121-196	M4 = (8)
Mean	44.2	8.1	36.6	72.4	
SD	8.6	2.4	30.1	59.9	
Group A II (n = 28)					
In remission					
Range	29-58	8.5-13.3	1.9-8	74-320	M2 = (8)
Mean	47.5	10.2	5.8	196.6	M3 = (11)
SD	9.3	1.7	1.9	2.32	M4 = (9)
Group A III (n =20)					
In relapse					
Range	35–58	4.2–11.1	15–52	28–304	M2 = (6)
Mean	48.3	7.43	24.2	115.8	M3 = (7)
SD	9.5	1.9	8.4	94.1	M4 = (7)
Group B (n = 35)					
Controls					
Range	32–57	10–13	4.2–9.7	181–340	
Mean	45.7	11.4	6.5	272	
SD	4.7	0.93	1.7	66.2	

n : number of patients Hb : hemoglobin Plt : platelet count

WBCs : white blood count SD : standard deviation

Table (2): Serum levels of of Hyaluronic Acid, Ferritin, Interleukin 6 in the different studied groups of Acute Myeloid Leukemia and control group..

	Hyaluronic Acid (ng/ml)	Ferritin (ng/ml)	Interleukin – 6 pg/ml
AML group			
Group A I (n= 32)			
At diagnosis			
Range	65.62-286.87	617.25-1875.35	0 - 64.23
Median	184.33** ■■	1400.25** ■■	24.83** ■■
Group A II (n = 28)			
In remission			
Range	13.75-60.62	36.3 - 530.23	1.34 -18.34
Median	33.65	99.34	8.24
Group A III (n =20)			
In relapse			
Range	70.73-286.22	749.25 -1896.54	3.41 - 65.12
Median	188.98* *■■	1431.45** ■■	19.32** ■■
Group B (n = 35)			
Controls			
Range	9.37-55.26	8.86 - 98.23	0 -18.23
Median	23.18	36.69	7.14

* compared to control group (group B)

■ compared to leukemic patients at remission (group A II)

**■■ = highly significant p < 0.0001

Table (3): Correlation between Hyaluronic acid, Ferritin, Interleukin–6 and Hemoglobin concentration, Platelets count, White blood cells count and Blast cells in patients with AML at diagnosis and in relapse .

	Hyaluronic acid	Ferritin	Interleukin 6
Hb	r = -0.69**	r = - 0.44 *	r = - 665**
Platelets	r = -0.41	r = - 0.39	r =-0.40
White blood cells	r = 0.49*	r = 0.69 **	r = 0.45*
Blast cells	r = 0.52*	r = 0.79 **	r = 0.49*
Hyaluronic acid		r = 0.076	r = - 0.26
Ferritin			r = - 0.089

r = correlation coefficient
* significant $p < 0.05$
** highly significant $p < 0.01$

Fig. (1) : Serum levels of Hyaluronic Acid (ng/ml) in the different studied groups of Acute Myeloid Leukemia.

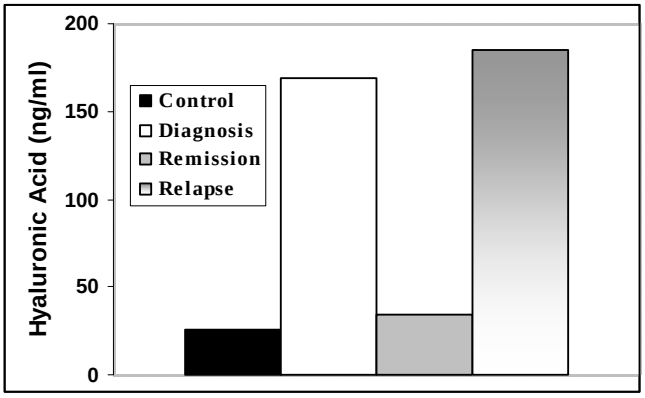


Fig. (2) : Serum levels of Ferritin (ng/ml) in the different studied groups of Acute Myeloid Leukemia.

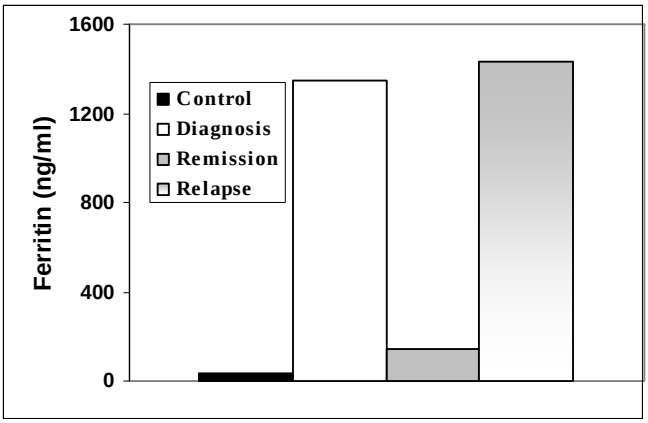
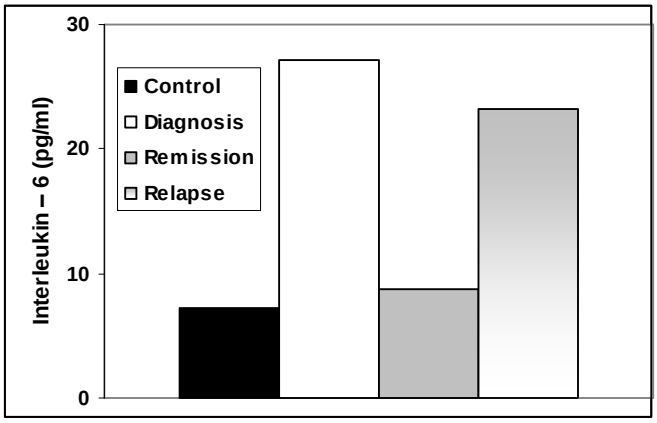


Fig. (3) : Serum levels of Interleukin – 6 (pg/ml) in the different studied groups of Acute Myeloid Leukemia



6. Discussion :

Acute myeloid leukemia (AML) is the most common acute leukemia in adults. With intensive induction therapy, most patients younger than 60 years achieve complete remission. However, even if these younger patients were treated intensively, more than 50% will relapse. Clinical results of patients older than 60 years are more unfavorable. Therefore, in all patients with AML, the overall survival is still low **(Tamm *et. al,* 2007).**

In our study, we demonstrated that adults with AML group (A) have higher plasma levels of hyaluronic acid, ferritin and Interleukin 6 when compared to control group. As regard Serum levels of hyaluronic acid, this study used an HABP based sandwich ELISA to show that serum HA level is raised in patients with AML in newly diagnosed and relapsed patients. In remission, serum levels of hyaluronic acid concentration decreased to the normal range. Hyaluronan (HA) has been reported to bind specifically and with high affinity to various cell types and to directly modify cell behaviour.

In a previous report **Courel *et. al,* 2004** demonstrated that both high molecular weight molecules (HAH) and HA-derived oligosaccharides were efficient at triggering terminal differentiation of acute myeloid leukemia (AML) blasts, in vitro. There have been a few reports of raised serum levels of HA in advanced cancer; **Delpech *et. al,* (1985)**. These studies confirm that a raised serum HA may accompany malignant disease. As yet, the mechanism of increased serum levels of HA in cancer is unclear. However, there are some clues from the case reports. Greatly increased levels of serum HA have been observed in Wilms' tumour **(Wu *et. al,* 1984; and Bracey *et. al,* 1987)** and neuroblastoma **(Pusch *et. al,* 2010).** In a few patients the rise of serum HA was associated with increased viscosity.

Our results agree with **Sanada *et. al,* 1999** who reported that levels of serum hyaluronic acid (HA) in adult T-cell leukemia (ATL) patients moved in parallel with the clinical activity of their disease. A hyaluronan-rich environment often correlate with tumor progression and may be there is one mechanism for the invasive behavior of malignancies. Eradication of hyaluronan by hyaluronidase administration could reduce tumor aggressiveness and would provide,therefore, a new anti-cancer strategy **(Adamia *et. al,* 2005).**

Giannopoulos *et. al,* 2009 reported that receptor for hyaluronic acid-mediated motility expression appears to be of prognostic value, as well as may reflect the proliferative capacity of chronic lymphocytic leukemia cells, and might therefore represent interesting target for immunotherapy in patients with different hematological malignancies **(Greiner *et. al,* 2010).**

In our study serum ferritin concentrations in patients with AML were found to increase in newly diagnosed and relapsed patients. This agree with findings of **Worwood *et. al,* 1974** who reported that the increased capacity for ferritin synthesis shown by myelogenous cells from patients with AML suggests that these cells themselves are the source of the increased amount of circulating protein. While patients during remission, the serum ferritin concentration decreased but still higher than the normal range. **White *et. al,* 1974** have shown that the increase in cirulating ferritin during chemotherapy could be due to an increased release from damaged leukaemic cells and this increase in serum ferritin

concentration was not correlated with the amount of blood transfused or the degree of liver damage .

Similar results were obtained by **Garcia-Manero in (2008)**and confirm the previous study of serum ferritin concentrations in acute leukaemia**.** It shows that serum ferritin is a marker of acute phase reactions and iron storage. In addition, hematologic malignancies are associated with elevated serum ferritin levels. There are a number of factors which are likely to contribute to raised serum ferritin concentrations in leukaemia: (1) Most of the patients are anaemic and have increased amounts of storage ferritin which are reflected by increased serum ferritin concentrations. (2) Increased synthesis of ferritin in the large mass of leukaemic cells is also reflected in high serum ferritin concentrations. In the earlier study, there was a significant correlation between leucocyte and serum ferritin concentration. (3) Abnormal release of ferritin from damaged cells is another possible cause of high serum ferritin concentrations **(Cragg *et. al,* 1977).**

Data from 90 patients with a variety of hematologic malignant neoplasms were studied by **Patel *et. al,* 1980** to determine the relation between changes in serum ferritin concentration and the clinical status of the patients. Patients with Hodgkin's disease, non-Hodgkin's lymphoma, multiple myeloma, blastic crisis of chronic myelocytic leukemia, acute myeloblastic leukemia and acute lymphoblastic leukemia were found to have significantly elevated serum ferritin levels. The serum ferritin level reflects acute phase reactions and is usually associated with iron storage. Iron overload increases the susceptibility to organ damage and the risk for infection **(Miceli *et. al,* 2006).** Other recent studies have suggested that ferritin is a surrogate for advanced disease and has an impact on relapse, because elevated serum ferritin predicts overall survival (OS) and relapse-free survival following autologous stem cell transplantation for lymphomas **(Armand *et. al,* 2007, Mahindra *et. al,* 2008 and Moo-Kon Song *et. al,* 2009) .**

However, **Patel *et. al,*** in their study in **(1980)** showed that patients with hematologic malignancies have significantly higher serum ferritin levels. Another study showed that the intensity of the 99 mTc-MIBI scan correlates with the serum ferritin level as a marker of disease activity **(Alexandrakis *et. al,* 2001). Papadaki *et. al,*** in their study in **(1997)** found that elevated serum sIL-6R levels were related to the growth of myeloma cells and that the concentration was an indicator of disease activity; the sIL-6R level was correlated with the serum ferritin concentrations as a marker of disease activity. However, the low serum ferritin concentrations found in patients with AML in remission suggest that ferritin concentration may be a useful index for the prediction of relapse and as a prognostic sign.

Several cytokines have been shown to promote the growth of malignant cells in vitro and are therefore believed to contribute to the aggressiveness of the disease **(Tsimberidou *et. al,* 2008).** In our study increased serum IL 6 concentrations were found in newly diagnosed and relapsed patients with AML. In remission, serum IL 6 concentration fall within the normal range. Several studies by **Lauta, 2003, Sohara *et. al,* 2005,and Hong *et. al,* 2007** reported that IL-6 is a pleiotropic cytokine with many ascribed effects including stimulation of acute phase reactants, immune regulation, angiogenesis, and osteoclast activation, and it originates from a multitude of cell types, including mononuclear phagocytes, vascular endothelial cells, fibroblasts, hepatocytes, B-cell lymphomas, and the neoplastic plasma cells of multiple myeloma. It appears to serve as a stimulatory factor in multiple myeloma; produced by both the malignant cells and bone marrow stromal cells**(Lauta, 2003).**

Our results agreed with **Tsimberidou *et. al,* 2008** who concluded that interleukin-6 (IL-6) may play a relevant role in the pathogenesis of several hematologic malignancies. IL-6 has diverse effects on the growth of AML blasts, including stimulation and maintenance of their growth through the IL-6/ IL-6 receptor signaling system. Also our results agreed with **Thomas *et. al,* 1997** who reported that AML relapse is suggested to result from treatment failure due to leukemic cells being resistant to chemotherapy and/or escaping immune surveillance. Due to the association of IL-6 expression with disease progression reported in previous studies, and it is conceivable that IL-6 may play an active role in relapse of ALL by supporting chemoresistance and inhibition of immunocompetent cells. Also they concluded that serum levels of IL-6 are a powerful prognostic factor in diffuse large cell lymphoma and chronic lymphocytic leukemia.

In the current study serum levels of hyaluronic acid, ferritin and interleukin–6 in patients with AML (at diagnosis and at relapse) correlated inversely with the hemoglobin concentration ($p < 0.05$). While their serum levels correlated positively with both total leukocyte count (TLC) ($p < 0.05$)

and with the % of blast cells in bone marrow in patients with AML. So, these parameters may be useful and sensitive tumour markers.

Our results agree with **Sanada *et. al,* 1999** who reported that levels of serum hyaluronic acid (HA) in adult T-cell leukemia (ATL) patients moved in parallel with the clinical activity of their disease. **Papadaki *et. al,* 1997** concluded that the sIL-6R level was correlated with the serum ferritin concentrations as a marker of disease activity. Also they reported that low serum ferritin concentrations found in patients with AML in remission suggest that ferritin concentration may be also a useful index for the prediction of relapse and as a prognostic sign. This also agree with **Tsimberidou *et. al,* 2008** who found that IL-6 correlated inversely with Hb % and positively with the absolute number of circulating myeloblasts and the proportion of bone marrow myeloblasts in patients with high risk AML. Also **Preti *et. al,* 1997 and Fayad *et. al,* 2001** reported that serum levels of IL-6 are a powerful prognostic factor in diffuse large cell lymphoma and chronic lymphocytic leukemia. Also multiple studies have been done to explain the role for elevated IL-6 levels at diagnosis as a marker of poor prognosis in various cancers including multiple myeloma **(Lauta, 2003),** malignant melanoma **(Mouawad *et. al,* 2002 and Soubrane *et. al,* 2005)**, non-Hodgkin's lymphoma **(El-Far *et. al,* 2004 and Pedersen *et. al,* 2005)**, prostate cancer **(George *et. al,* 2005)**, squamous cell carcinoma of the head and neck **(Riedel *et. al,* 2005)**, and various sarcomas **(Rutkowski *et. al,* 2002).**

In conclusion, our data suggest that serum levels of hyaluronic acid, ferritin and interleukin–6 can be used as prognostic serum markers at diagnosis of adult acute myeloid leukemia and it could be used as follow up parameters for early detection of relapse.Understanding their roles may therefore represent interesting target for designing new therapeutic strategies for patients with different hematological malignancies.

Corresponding author:

Halla M. Ragab
*Department of Biochemistry, Genetic Engineering and Biotechnology Division, National Research Centre, Dokki, Cairo, Egypt.
E-mail: hmragab@yahoo.com

References:

1) Adamia S., Maxwell C. and Pilarski L. Hyaluronan and hyaluronan synthases: potential therapeutic targets in cancer. Curr Drug Targets Cardiovasc Haematol Disord.; 2005, 5(1):3-14.
2) Adams P. Iron overload in viral and alcoholic liver disease. J. Hepatol. ;1998, 28: 19- 20.
3) Ahlawat S., Bhargava M., Arya LS., Kochupillai V. and Kumar R. Role of serum ferritin in assessment of disease activity in acute & chronic leukemia. Indian J Med Res.; 1994, 100: 66-69.
4) Alexandrakis M., Kyriakou D., Passam F., Koukouraki S. and Karkavitsas N. Value of Tc-99m sestamibi scintigraphy in the detection of bone lesions in multiple myeloma: comparison with Tc-99m methylene diphosphonate. Ann Hematol; 2001,80:349–353.
5) Armand P., Kim H. and Cutler C. Prognostic impact of elevated pretransplantation serum ferritin in patients undergoing myeloablative stem cell transplantation. Blood; 2007;109:4586–4588.
6) Aulbert E., Fromm H. and Hornemann H. Ferritin in acute leukemia. Serum ferritin concentration as a nonspecific tumor marker for M1 and M2 myeloid leukemia. Med Klin (Munich); 1991, 15, 86(6): 297-304.
7) Bracey A., WU A., Aceves J., Chow T., Carlile S. and Hoots W. Platelet dysfunction associated with Wilms' tumour and hyaluronic acid. Am. J. Hematol.; 1987, 24, 247 - 254.
8) Cragg S., Jacobs A., Parry D., Wagstaff M. and Worwood M. Isoferritins in acute leukaemia. J. Cancer; 1977,35, 635- 640.
9) Casaril M., Stanzial A. and Tognella P. Role of iron load on fibrogenesis in chronic hepatitis C. Hepatogastroenterology; 2000, 47(31): 220- 225.
10) Chichibu K., Matsuura T. and Shichijo S. Assay of serum hyaluronic acid in clinical application. Clin Chimica Acta; 1989,181: 317-324.
11) Courel M., Maingonnat C Bertrand P., Chauzy C., Smadja-Joffe F. and Delpech B. Biodistribution of injected iritiated hyaluronic acid in mice: A comparison between macromolecules and hyaluronic acid-derived oligosaccharides. In Vivo; 2004, 18:181-188.
12) Delpech B., Girard N., Bertrand P., Courel M., Chauzy C. and Delpech A. Hyaluronan: fundamental principles and

applications in cancer. J. Intern. Med.; 1997, 242: 41-48.
13) Delpech, B., Bertrand P. and Maingonnat C. Immunoenzymoassay of the hyaluronic acid-hyaluronectin interaction. Application to the detection of hyaluronic acid in serum of normal subjects and cancer patients. Anal. Biochem.; 1985, 149, 555 - 560.
14) El-Far M., Fouda M., Yahya R. and El-Baz H. Serum IL-10 and IL-6 levels at diagnosis as independent predictors of outcome in non-Hodgkin's lymphoma. J Physiol Biochem; 2004, 60, 253–258.
15) Fayad L., Keating M. and Reuben J. Interleukin-6 and interleukin-10 levels in chronic lymphocytic leukemia: correlation with phenotypic characteristics and outcome. Blood; 2001, 97, 256-263.
16) Giannopoulos K., Mertens D., Bühler A., Barth T. , Idler I., Möller P. and Kröber A. The candidate immunotherapeutical target, the receptor for hyaluronic acid-mediated motility, is associated with proliferation and shows prognostic value in B-cell chronic lymphocytic leukemiaRHAMM as immunotarget and prognostic marker in CLL Leukemia; 2009, 23, 519-527.
17) Garcia G., Shan J., Faderl S., Cortes J., Ravandi F., Borthakur G., Wierda W., Pierce S., Estey E., Liu J., Huang X. and Kantarjian H. A prognostic score for patients with lower risk myelodysplastic syndrome; 2008, 22(3),538-43.
18) George D., Halabi S. and Shepard T. The prognostic significance of plasma interleukin-6 levels in patients with metastatic hormone-refractory prostate cancer: results from cancer and leukemia group B 9480. Clin Cancer Res; 2005,11,1815–1820.
19) Hong D., Angelo L. and Kurzrock R. Interleukin-6 and its receptor in cancer: implications for Translational Therapeutics. Cancer; 2007,110,1911–1928.
20) Jochen G., Anita S., Krzysztof G., Markus T., Marlies Götz G., Hartmut D.and Michael S. High dose RHAMM-R3 peptide vaccination for patients with acute myeloid leukemia (AML), myelodysplastic syndrome (MDS) and multiple myeloma (MM) Haematologica; 2010, 10.3324- 3326.
21) Kawano M., Hirano T., Matsuda T., Taga T., Horii Y., Iwato K., Asaoku H., Tang B., Tanabe 0., Tanaka H., Kuramoto A. and Kishimoto, T. Autocrine generation and requirement of BSF-2 / IL-6 for human multiple myelomas. Nature; 1988, 332, 83 - 85.
22) Lauta V. A review of the cytokine network in multiple myeloma: diagnostic, prognostic, and therapeutic implications. Cancer; 2003, 97, 2440–2452.
23) Li L., Anita S., Peter R., Jochen G., Mark R., Bianca V., Martin B., Markus V., Markus W., Hartmut D. and Michael S. Reconstitution of CD40 and CD80 in dendritic cells generated from blasts of patients with acute myeloid leukemia. Cancer Immunity; 2003; 3, 8 – 20.
24) Lokeshwar V., Öbek C., Soloway M. and Block N. Tumor-associated hyaluronic acid: a new sensitive and specific urine marker for bladder cancer. Cancer Res.; 1997, 57: 773-777.
25) Mahindra A., Bolwell B. and Sobecks R. Elevated ferritin is associated with relapse after autologous hematopoietic stem cell transplantation for lymphoma. Biol Blood Marrow Transplant; 2008,14:1239–1244.
26) Marie N., Catherine M., Philippe B., Florence S. and Bertrand D. Biodistribution of Injected Tritiated Hyaluronic Acid in Mice: A Comparison Between Macromolecules and Hyaluronic Acid-derived Oligosaccharides. In vivo ; 2004; 18 (2), 181-188.
27) Miceli M., Dong L. and Grazziutti M. Iron overload is a major risk factor for severe infection after autologous stem cell transplantation: a study of 367 myeloma patients. Bone Marrow Transplant; 2006,37:857–864.
28) Moo-Kon S., Joo-Seop C., Young M., HoJin S., Young J. and Goon-Jae C. Elevation of serum ferritin is associated with the outcome of patients with newly diagnosed multiple myeloma. Korean J Intern Med.; 2009, 24(4): 368–373.
29) Mouawad R., Rixe O., Meric J., Khayat D., Soubrane C. Serum interleukin-6 concentrations as predictive factor of time to progression in metastatic malignant melanoma patients treated by biochemotherapy: a retrospective study. Cytokines Cell Mol Ther; 2002, 7, 151–156.
30) Papadaki H., Kyriakou D., Foudoulakis A., Markidou F., Alexandrakis M. and Eliopoulos G. Serum levels of soluble IL-6 receptor in multiple myeloma as indicator of disease activity. Acta Haematol; 1997, 97, 191–195.
31) Patel A., Shah P,. Vohra R., Hart W. and Shah J. Serum ferritin levels in hematologic malignant neoplasms. Arch Pathol Lab Med; 1980,104:509–512.

32) Pedersen L., Klausen T., Davidsen U. and Johnsen H. Early changes in serum IL-6 and VEGF levels predict clinical outcome following first-line therapy in aggressive non-Hodgkin's lymphoma. Ann Hematol; 2005, 84, 510–516.

33) Plevris J., Haydon G., Dawkes R., Ludlum C., Harrison D. and Hayes P. Serum hyaluronan, a non-invasive test for diagnosing liver cirrhosis. Eur J Gastroenterol Hepatol ; 2000; 12(10): 1121-1127.

34) Preti H., Cabanillas F.,Talpaz M.,Tucker S., Seymour F. and Kurzrock R. Prognostic value of serum interleukin-6 in diffuse large-cell lymphoma. Ann Intern Med; 1997, 127, 186-194.

35) Pusch A., Boeckenhoff A., Glaser T., Kaminski T., Kirfel G., Hans M., Steinfarz B., Swandulla D., Kubitscheck U., Gieselmann V., Brüstle O. and Kappler J. CD44 and hyaluronan promote invasive growth of B35 neuroblastoma cells into the brain. Biochim Biophys Acta; 2010,1803(2):261-274.

36) Riedel F., Zaiss I., Herzog D., Gotte K., Naim R. and Hormann K. Serum levels of interleukin-6 in patients with primary head and neck squamous cell carcinoma. Anticancer Res; 2005, 25, 2761–2765.

37) Robak T., Wierzbowska A., Blasinska-Morawiec M., Korycka A., and Blonski J. Serum levels of IL-6 type cytokines and soluble IL-6 receptors in active B-cell chronic lymphocytic leukemia and in cladribine induced remission. Mediators Inflamm, 1999; 8(6): 277-286.

38) Rutkowski P., Kaminska J., Kowalska M., Ruka W. and Steffen J. Cytokine serum levels in soft tissue sarcoma patients: correlations with clinico-pathological features and prognosis. Int J Cancer; 2002, 100, 463–471.

39) Sanada I., Kawano F., Tsukamoto A., Kiyokawa T. Adult T-cell leukemia with elevated serum hyaluronic acid levels paralleling disease activity ; 1999, 40(1):51-54.

40) Saunders and Trapp M. Basic and clinical biostatistics. Second edition. Appleton and Lang.; 1995, 2: 85-90.

41) Tamm I. Richter S. and Oltersdorf D. High expression levels of x-linked inhibitor of apoptosis protein and survivin correlate with poor overall survival in childhood de novo acute myeloid leukemia. Clin Cancer Res 2004;10:3737–3744.

42) Sohara Y., Shimada H., Minkin C., Erdreich A., Nolta A. and DeClerck Y. Bone marrow mesenchymal stem cells provide an alternate pathway of osteoclast activation and bone destruction by cancer cells. Cancer Res; 2005, 65,1129–1135.

43) Soubrane C., Rixe O., Meric J., Khayat D. and Mouawad R. Pretreatment serum interleukin-6 concentration as a prognostic factor of overall survival in metastatic malignant melanoma patients treated with biochemotherapy: a retrospective study. Melanoma Res; 2005, 15, 199–204.

44) Thomas X., Hirschauer C. and Troncy J. Serum Interleukin-6 levels in adult acute myelogenous leukemia: relationship with disease characteristics and outcome. Leukemia and Lymphoma;1997, 24 (30), 291–300.

45) Tsimberidou A., Estey E., Wen S., Pierce S., Kantarjian H., Albitar M. and Kurzrock R. The prognostic significance of cytokine levels in newly diagnosed acute myeloid leukemia and high-risk myelodysplastic syndromes. Cancer ; 2008; 113(7):1605-1613.

46) Ulbrich E., Lebrecht A., Schneider I., Ludwig E., Koelbl H. and Hefler L.. Serum parameters of iron metabolism in patients with breast cancer. Anticancer Res.; 2003,23 (6): 5107-5115.

47) White D., Kramer D., Johnson,G., Dick F. and Hamilton H. Enzyme Immunoassay for the Quantitative Determination of Human Ferritin . Am Clin. Path; 1986, 198, 72: 346-352.

48) White G. , Worwood M., Parry D. and Jacobs A. Ferritin Synthesis in Normal and Leukaemic Leucocytes. Nature, Lond.; 1974, 250, 584- 590.

49) Worwood M., Summers M., Miller F., Jacobs A. and Whittaker, J. Ferritin in Blood Cells from Normal Subjects and Patients with Leukaemia. Br. J. Haematol.; 1974, 28, 27.

50) Wu A., Parker O. and Ford L. Hyperviscosity caused by hyaluronic acid in the serum in a case of Wilms' tumour. Clin. Chem.; 1984, 30, 914 - 920.

25 / 7 / 2010

The Effect of Green, Roasted and Decaffeinated Coffee on Serum Glucose, Insulin and Serum Lipid Profile in Diabetic Experimental Animals

Eman A.Sadeek[*1] , Hala, A. Abd El;-Rahman[2] and Waffa, Sh. Ali[3]

[1]Department of Biochemistry & Nutrition -Women's College –Ain –Shams University. [2] Food Tech. Res. Ins. Agric. Res. Center. [3]College of Home Economics, Helwan University. Cairo, Egypt
[*]dr_emansadeek@yahoo.com

Abstract: Aim of the work: Assessing the Effect of green, roasted and decaffeinated coffee on serum glucose, insulin and serum lipid profile in diabetic rat models. Methods: Design of the Study: Thirty female wistar rats weighing 124.5 ±5.41g (mean ±S.D) were divided into 5 groups. The first group served as a control and consumed a standard diet according to (AIN – 93). The other 4 groups were injected intraperitoneally with 105 mg / kg body weight of alloxan . One group was kept without further treatment and served as a positive diabetic control. Groups 3, 4, 5 consumed 5% green, roasted and decaffeinated coffee in drinking water, respectively. The feeding trial continued for four weeks. At the end of the experiments, the animals were sacrificed, blood samples were collected, and the liver, kidney, spleen and heart were separated, washed, dried and weighed. Laboratory investigations Consisted of serum glucose, insulin, calcium, phosphorus and complete lipid profile was determined to test the magnitude of antioxidant potential green, roasted and decaffeinated coffee. Results: The present study show a significant difference ($p < 0.05$) in body weight gain and food intake between all treatment groups , with non significant difference in water intake , relative weight of organs including liver , kidney , spleen and heart . the study also shows significant elevation ($p < 0.05$) in serum glucose and insulin in diabetic control group as compared to normal control group. This indicates uncontrolled hyperglycemia in alloxan diabetic rats. While consumption of green, roasted and decaffeinated coffee resulted in a decrease in serum glucose and insulin ($p < 0.05$) .There is a significant decrease ($p < 0.05$) in serum calcium and serum phosphorus in groups 3,4 and 5 fed green, roasted and decaffeinated coffee respectively indicating an association between coffee consumption and bone health. our results also shows that alloxan injection produced a significant increase($p < 0.05$) in serum total- cholesterol(TC); triacylglycerol (TAG); LDL-C ; VLDL-C and in LDL\ HDL ratio and TC \ HDL ratio however a significant decrease ($p < 0.05$) in serum HDL-C is observed ; In diabetic rats compared to normal control .green, roasted and decaffeinated coffee resulted in a significant decrease ($p < 0.05$) in triacylglycerol (TAG); LDL-C ; VLDL-C and in LDL\ HDL ratio and TC \ HDL ratio .on the other hand a significant increase ($p < 0.05$) in serum HDL-C is observed in green, roasted and decaffeinated coffee groups compared to diabetic rats compared to normal control with the highest value for green coffee .Non significant effect on serum total- cholesterol(TC) reported in this study . Conclusion: The observed improvement in glucose, insulin profile, triacylglycerol and HDL-C confirm the potent biological action of green, roasted and decaffeinated coffee and suggest that chlorogenic acid (a component in coffee) might have an antagonistic effect on glucose transport. Suggesting a novel function of coffee on lowering the risk factors of diabetes and delaying the progress of diabetes complications as well.
[Eman A.Sadeek, Hala, A. Abd El;-Rahman and Waffa, Sh. Ali. The Effect of Green, Roasted and Decaffeinated Coffee on Serum Glucose, Insulin and Serum Lipid Profile in Diabetic Experimental Animals. Journal of American Science 2010;6(12):433-441]. (ISSN: 1545-1003).

Keywords: Green, roasted, decaffeinated coffee, glucose, insulin and lipid profile.

1. Introduction:

Type 2 diabetes is a chronic disease associated with high rates of morbidity and premature mortality(Nathan , 1993)1 An alarming increase in the prevalence of type 2 diabetes is expected,(Wild et al ., 2004) and the need for preventive action is widely acknowledged. While increased physical activity and restriction of energy intake can substantially reduce the incidence of type 2 diabetes (Tuomilehto et al., 2001 Knowler et al., 2002) , insight into the role of other lifestyle factors may contribute to additional prevention strategies for type 2 diabetes.

Coffee is considered one of the most popular beverages consumed in the world due to its pleasant flavor and pharmacological properties(DÓREA and COSTA ., 2005). Prospective and epidemiologic studies of green and especially of roasted coffee consumption has been carried out to investigate its biological effects on lipids, blood pressure and

glycaemia(CORTI et al ., 2002 ; DAGLIA et al ., 2000 and ROBINSON et al. ,2004) . Scientific evidences have demonstrated that green and regular coffee beverages present high antioxidant properties in vivo and in vitro (KARAKAWA, 2004 and SOMOZA et al. ., 2003). Few recent studies have indicated that soluble extracts of green coffee were effective against the high blood pressure in mice(SUZUKI, A. et al. ,2000) and in human(KOZUM. et al. , 2005 and OCHIAI, . et al. 2004). It is possible that its antihypertensive action be related to vasoreactive factors produced and released from the vascular endothelium (OCHIAI, R. et al. 2004).

The roasting process causes a loss of water from the green bean and degradation of many of the compounds including the antioxidant polyphenols; however, there is very little difference in total antioxidants between the different roasts of a bean (Daglia et al., 2000).

There are three main methods of coffee preparation; boiled unfiltered coffee, filtered coffee, and decaffeinated coffee, the latter primarily consumed as instant coffee.

There are over a thousand compounds, many formed during the roasting process,which produce the unique taste and smell of coffee(Parliament et al ., 2005). However, from the point of view of concentration in coffee, prior detection of the parent compound or metabolites in the body, and physiological effects, there are essentially only three ingredients that are important; caffeine, the diterpene alcohols cafestol and kahweol, and chlorogenic acid and other polyphenols. In specialty coffees consumed outside the home the range is 18–80 mg/cup and decaffeinated coffees averaged 5 mg/cup(McCusker et al ., 2003). Coffee is an important source of caffeine; it provides 71% of the caffeine in the US diet (Frary et al., 2005). The diterpenoid alcohols are the oils in coffee and their concentration depends on the how the coffee is prepared. Filtered coffee has less than 0.1 mg/100 ml, i.e. essentially none, and unfiltered coffee can have between 0.2 and 18 mg/100 ml depending on the method.

High consumption of unfiltered types of coffee, such as French press and boiled coffee has been shown to increase low-density-lipoprotein-cholesterol concentrations. In addition, limiting caffeinated coffee intake during pregnancy seems a prudent choice. However, evidence has been accumulating that frequent consumption of coffee may reduce risk of type 2 diabetes and liver cancer(van Dam ., 2008).

Higher habitual coffee consumption was associated with higher insulin sensitivity (Arnlov et al ., 2004) and a lower risk for type 2 diabetes(van Dam et al ., 2002 ; Rosengren et al ., 2004 ; Salazar-Martinez et al ., 2004 ; Tuomilehto et al ., 2004 and Carlsson et al ., 2004) in diverse populations. In contrast, short-term metabolic studies showed that caffeine intake can acutely lower insulin sensitivity (Keijzers et al ., 2002 and Thong et al ., 2002) and increase glucose concentrations(Mougios et al ., 2003 and Lane et al ., 2004)

Tunnicliffe and Shearer, 2008 found that Coffee consumption may also mediate levels of gut peptides (glucose-dependent insulinotropic polypeptide and glucagon-like peptide-1), hormones intimately involved in the regulation of satiety and insulin secretion. Finally, coffee may have prebiotic-like properties, altering gut flora and ultimately digestion.

It has been reported that Coffee intake might have an antiatherogenic property by increasing of ATP-binding cassette transporter (ABC)G1 and scavenger receptor class B type I (SR-BI) expression and enhancing HDL-mediated cholesterol efflux from the macrophages via its plasma phenolic acids(Uto-Kondo et al ., 2010).

2. Materials and methods

Materials:

Chemicals:

All chemicals including alloxan were fine grade, chemicals purchased from local distributer (Sigma chemical) Cairo.Egypt.

Green, roasted and decaffeinated coffee where purchased from a local market ,Cairo, Egypt and was added to drinking water at a concentration of 5 g% after following preparation : 5g of green , roasted and decaffeinated coffee dissolved in 100 ml boiled water for 10 minutes .

The basal standard diet was prepared in accordance with AIN-93 formulation (Revees et al., 1993).

Composition of diet (g/100g)

Corn starch 62.07;casein 14 ;sucrose 10 ;cellulose 5 ;corn oil 4 ;salt mixture 3.5 ; vitamin mixture 1;L-cystine0.18; choline bitratrate 0.25 and tert.butylhydroxy quinine 0.008.

Animals

In the present study 30 female rats of wistar strain weighing (124.50 ±5.41 g) obtained from Institute of Ophthalmology(Cairo, Egypt) were used in this study . The rats were maintained under standard laboratory conditions in an air conditioned room and housed in stainless steel cages one per cage at temperature 22±3 °C and relative humidity 30-70 %. The animal diet was given ad libitum . Animals were acclimatized for one week prior to experiment.

Thirty rats were divided into 6 groups each of 6 rats.

Group 1(G1): Served as normal control and received standard diet.

Group 2(G2): Diabetic control group. Green, roasted and decaffeinated coffee

Group 3(G3): Diabetic group which received 5 % green coffee in drinking water.

Group 4(G4): Diabetic group which received 5 % roasted coffee in drinking water.

Group 5(G5 Diabetic group which received 5 % decaffeinated coffee in drinking water.

The experiment lasted for 4 weeks.

Assays:

At the end of experimental period, all rats were fasted overnight and then anesthetized by ether and sacrificed. Blood was collected and allowed to clot; serum was separated by centrifugation at 3000 rpm for 15 minutes serum was then transferred into properly labeled sterile vials and stored at -20° C till the performance of Laboratory analysis.

liver , kidney and spleen and heart were excised, rinsed in chilled saline solution and then blotted on filter paper ,weighed separately to calculate the relative weight.

$$\text{The relative weight of organ} = \frac{\text{absolute weight of organ}}{\text{Final body weight of rat}} \times 100$$

Serum was used for determination of seum glucose according to Barham and Trinder , (1972) . Serum insulin was determined according to Vuppugalla et al., (2003). Serum total cholesterol was assayed by the method of Richmond , (1973) , serum triacylglycerol according to Fossati and Prencipe , (1982) , serum HDL by the method of Steele et al ., (1976) while serum LDL-cholesterol by the use of the equation of Friedewald et al ., (1972) .

Statistical analysis:

Statistical analysis: were performed using SPSS for Windows 10.0(SPSS Inc,Chicago.IL.USA). Data were expressed as mean ± S.D. One way analysis of variance (ANOVA) at ($p < 0.05$) was used to compare mean values of continuous variable in cases and control.

3. Results

The present study show a significant difference ($p < 0.05$) in body weight gain and food intake between all treatment groups, with non significant difference in water intake, relative weight of organs including liver , kidney , spleen and heart .these data suggesting that green, roasted and decaffeinated coffee did not influence the relative organ weight and caused the reduction in food intake and gain weight in diabetic rats as compared to normal control group.(Table 1).

Table 2 shows significant elevation ($p < 0.05$) in serum glucose and insulin in diabetic control group as compared to normal control group at the end of experiment. This indicates uncontrolled hyperglycemia in alloxan diabetic rats. While consumption of green, roasted and decaffeinated coffee resulted in a decrease in serum glucose and insulin ($p < 0.05$) with the lowest glucose value observed with green coffee and with decaffeinated coffee for insulin.

There is a significant decrease ($p < 0.05$) in serum calcium and serum phosphorus in groups 3,4 and 5 fed green and roasted coffee respectively indicating an association between caffeine consumption and bone health.(Table 3)

Table (4) shows that alloxan injection produced a significant increase($p < 0.05$) in serum total- cholesterol(TC);triacylglycerol(TAG); LDL-C ; VLDL-C and in LDL\ HDL ratio and TC \ HDL ratio however a significant decrease ($p < 0.05$) in serum HDL-C is observed ; In diabetic rats compared to normal control .

Green, roasted and decaffeinated coffee resulted in a significant decrease ($p < 0.05$) in triacylglycerol(TAG); LDL-C ; VLDL-C and in LDL\ HDL ratio and TC \ HDL ratio .on the other hand a significant increase ($p < 0.05$) in serum HDL-C is observed in green, roasted and decaffeinated coffee groups compared to diabetic rats with the highest value for green coffee .Non significant effect on serum total- cholesterol(TC) reported in this study .

4. Discussion:

In this study the observed decrease in body weight is in agreement with animal studies and the prospective epidemiologic studies on weight loss (Muroyama et al ., 2003 and van Dam et al., 2006) suggest that long-term caffeine and coffee consumption could decrease body weight in humans.

Shimod et al., (2006) showed that consumption of green coffee bean extract (GCBE) for 14 days caused a suppressive effect on weight gain and visceral fat accumulation in mice. GCBE contains 10% caffeine and 27% chlorogenic acid as the principal constituents, and these constituents showed a tendency to suppress body weight gain and visceral fat accumulation. Thus, these constituents are suggested to be partially involved in the suppressive effect of GCBE on body weight gain and visceral fat accumulation. Caffeine is known to be a lipolytic compound. On the other hand, the effect of

Table (1): Effect of green, roasted and decaffeinated coffee on weight gain , food intake and water intake/day and relative weights of different organs (liver , kidney& spleen and heart) In diabetic rats (Mean ± S.D.).

Groups / Parameters	Group (1) Normal control	Group (2) Diabetic	Group (3) Diabetic + Green coffee	Group (4) Diabetic + Roasted coffee	Group (5) Diabetic + Decaffeinated coffee
Weight gain(g)	45.00 ± 4.98	a 35.83 ± 2.99	a 36.00 ± 4.98	a 36.17 ± 3.60	a 36.50 ± 2.81
Food intake(g\day)	18.10 ± 0.85	17.82 ±0.96	a,b 15.75 ± 0.62	a,b 15.67 ± 1.18	a,b 15.05 ± 0.80
Water intake (ml\day)	14.25 ± 1.44	14.67 ± 1.13	14.42 ± 1.06	14.83 ± 1.37	14.42 ± 1.66
Relative weight of liver (g%)	2.62 ± 0.33	2.63 ± 0.19	2.61 ± 0.11	2.43 ± 0.29	2.58 ± 0.25
Relative weight of kidney (g%)	0.57 ± 0.08	0.53 ± 0.11	0.59 ± 0.09	0.57 ± 0.09	0.64 ± 0.05
Relative weight of spleen (g%)	0.16 ± 0.03	0.15 ± 0.04	0.17 ± 0.03	0.16 ± 0.02	0.16 ± 0.03
Relative weight of herat (g%)	0.24 ± 0.03	0.25 ± 0.04	0.25 ± 0.05	0.26 ± 0.03	0.24 ± 0.05

Significant difference (P < 0.05): (a) compared to group 1, (b): to group 2, (c): to group 3, (d): to group 4, (e): to group 5.

Table (2) :): Effect of green, roasted and decaffeinated coffee on serum glucose and insulin In diabetic rats (Mean ± S.D.).

Groups / Parameters	**Group (1) Normal control**	**Group (2) Diabetic**	**Group (3) Diabetic + Green coffee**	**Group (4) Diabetic + Roasted coffee**	**Group (5) Diabetic + Decaffeinated coffee**
Glucose (mg\dl)	96.40 ± 0.42	a 183.03 ± 2.18	b 97.77 ± 1.06	a,b,c,e 101.40 ± 0.72	a,b,d 98.58 ± 1.35
Insulin (µ/ml)	35.67 ± 0.43	a 42.18 ± 1.71	b 36.33 ± 0.64	a,b 37.37 ± 0.84	b 36.13 ± 1.18

Significant difference (P < 0.05): (a) compared to group 1, (b): to group 2, (c): to group 3, (d): to group 4, (e): to group 5.

Table (3) :): Effect of green, roasted and decaffeinated coffee on serum calcium and phosphurus In diabetic rats (Mean ± S.D.).

Groups / Parameters	**Group (1) Normal control**	**Group (2) Diabetic**	**Group (3) Diabetic + Green coffee**	**Group (4) Diabetic + Roasted coffee**	**Group (5) Diabetic + Decaffeinated coffee**
Calcium (mg\dl)	7.75 ± 0.23	7.48 ± 0.29	a,b,e 6.53 ± 0.22	a,b,e 6.57 ± 0.18	c,d 7.43 ± 0.46
phosphorus (mg\dl)	3.53 ± 0.15	3.62 ± 0.22	a,b 2.75 ± 0.15	a,b 2.74 ± 0.13	c,d 3.29 ± 0.47

Significant difference (P < 0.05): (a) compared to group 1, (b): to group 2, (c): to group 3, (d): to group 4, (e): to group5.

Table (4): Effect of green, roasted and decaffeinated coffee on serum total- cholesterol(TC); triacylglycerol(TAG); LDL-C; HDL-C;VLDL-C and on LDL\HDL ratio and TC \HDL ratio In diabetic rats (Mean ± S.D.).

Groups / Parameters	**Group (1) Normal control**	**Group (2) Diabetic**	**Group (3) Diabetic + Green coffee**	**Group (4) Diabetic + Roasted coffee**	**Group (5) Diabetic + Decaffeinated coffee**
(TC ,mg/dl)	91.75 ± 0.73	a 132.65 ± 0.70	a 131.78 ± 1.15	a 132.50 ± 0.75	a 131.83 ± 0.98

(TAG,mg/dl)	79.30 ± 0.89	a 161.95 ± 1.59	a,b 146.75 ± 1.36	a,b 145.77 ± 1.84	a,b 146.53 ± 1.79
(HDL-C ,mg/dl)	28.95 ± 0.69	a 19.37 ± 0.79	a,b 27.13 ± 0.63	a,b,c 26.13 ± 0.89	a,b 26.75 ± 0.58
(LDL-C,mg/dl)	46.94 ± 1.17	a 80.89 ± 1.21	a,b 75.30 ± 1.14	a,b,c 77.21 ± 0.95	a,b 75.78 ± 1.54
(VLDL-C, mg/dl)	15.86 ± 0.17	a 32.39 ± 0.32	a,b 29.35 ± 0.27	a,b 29.15 ± 0.37	a,b 29.31 ± 0.36
LDLl HDL ratio	1.62 ± 0.07	a 4.18 ± 0.22	a,b 2.78 ± 0.082	a,b,c 2.96 ± 0.13	a,b 2.83 ± 0.11
TC l HDL ratio	3.17 ± 0.09	a 6.86 ± 0.28	a,b 4.86 ± 0.097	a,b,c 5.07 ± 0.16	a,b 4.93 ± 0.13

Significant difference ($P < 0.05$): (a) compared to group 1, (b): to group 2, (c): to group 3, (d): to group 4, (e): to group 5.

chlorogenic acid on body weight gain has not yet been established.

Elevated serum glucose and insulin in diabetic control group as compared to normal control group confirm uncontrolled hyperglycemia, whereas green, roasted and decaffeinated coffee decreased serum glucose and insulin ($p < 0.05$) with the lowest glucose value observed with green coffee and with decaffeinated coffee for insulin.

These results are with the line of van Dam., (2008) who found that frequent consumption of coffee may reduce risk of type 2 diabetes and liver cancer.

Several plausible mechanisms for a beneficial effect of coffee on glucose metabolism exist. Coffee has been shown to be a major contributor to the total in vitro antioxidant capacity of the diet (Pulido et al ., 2003) which may be relevant as oxidative stress can contribute to the development of type 2 diabetes. Coffee is the major source of the phenol chlorogenic acid.(Clifford 2000) Intake of chlorogenic acid has been shown to reduce glucose concentrations in rats(Andrade-Cetto and Wiedenfeld ., 2001 and Rodriguez de Sotillo and Hadley 2002 and intake of quinides, degradation products of chlorogenic acids, increased insulin sensitivity in rats.(Shearer et al ., 2003) Chlorogenic acid contributes to the antioxidant effects of coffee, (Clifford 2000) may reduce hepatic glucose output through inhibition of glucose-6-phosphatase, (Arion et al ., 1997)and may improve tissue mineral distribution through its action as a metal chelator. (Rodriguez de Sotillo and Hadley 2002). In addition, chlorogenic acid acts as a competitive inhibitor of glucose absorption in the intestine. (Clifford 2000)Indeed, decaffeinated coffee seemed to delay intestinal absorption of glucose and increased glucagon-like peptide-1 concentrations in an intervention study in humans.(Johnston et al ., 2003) Glucagon-like peptide-1 is well known for its beneficial effects on glucose-induced insulin secretion and insulin action.(Drucker 1998) This effect may explain the observation that higher coffee consumption was associated with lower postload, rather than fasting, glucose concentrations.(Yamaji et al ., 2004 and)

Caffeine ingestion can acutely reduce glucose storage, but beneficial effects of caffeine on lipid oxidation and uncoupling protein-3 expression have also been suggested.(Yoshioka et al ., 2004) In US studies, decaffeinated coffee consumption was inversely associated with risk of type 2 diabetes.(Salazar-Martinez et al., 2004) In addition, in a Japanese study, the inverse association with hyperglycemia was stronger for coffee than for caffeine.(Isogawa et al ., 2003) These observations suggest that coffee components other than caffeine may have beneficial effects on risk of type 2 diabetes. Coffee also contains substantial amounts of magnesium, which has been linked to better insulin sensitivity and insulin secretion.(de Valk 1999) However, adjustment for magnesium intake did not explain the association between coffee consumption and risk of type 2 diabetes(Salazar-Martinez et al., 2004)

As the beneficial effects of coffee consumption exist for both decaffeinated and caffeinated coffee, a component of coffee other than caffeine must be responsible. Tunnicliffe and Shearer2008 reported that, being plant-derived; coffee contains many beneficial compounds found in fruits and vegetables, including antioxidants. In fact, coffee is the largest source of dietary antioxidants in industrialized nations. When green coffee is roasted at high temperatures, Maillard reactions create a number of unique compounds. Roasting causes a portion of the antioxidant, chlorogenic acid, to be transformed into quinides.

Decreased serum insulin in this study is in agreement with The decreased insulin secretion reported by Tianying et al., (2005) is consistent with

the increased insulin sensitivity observed by Arnlov et al.,(2004). In contrast, Arnlov et al., 2004 did not observe a decrease in insulin secretion as assessed by early insulin response under glucose stimulation. However, C-peptide has a longer half-life than insulin and thus may better represent insulin secretion than insulin levels do (Chen et al., 1999).The independent association between decaffeinated coffee and C-peptide indicates active ingredients other than caffeine. Antioxidants may improve insulin sensitivity Bruce et al., 2003 (in type 2 diabetes and decrease insulin levels in rats (Thirunavukkarasu., 2004).

Tianying et al., (2005) concluded caffeinated and decaffeinated coffee consumption might prove to be an effective strategy for reducing insulin resistance, especially in overweight women.

Oka, 2007 demonstrated that the prophylactic effects of coffee on diabetes involve pleiotropy of plural components in accordance to the degree of the roasting. A new concept of nutritional blended coffee may be important to optimize the prophylactic effects of coffee on lowering the risk factors of diabetes and delaying the progress of diabetes complications as well.

On the other hand Contrary to our study Kempf et al., 2010 demonstrated that coffee consumption led to an increase in coffee-derived compounds, mainly serum caffeine, chlorogenic acid, and caffeic acid metabolites. , Whereas no changes were seen for markers of glucose metabolism in an oral-glucose-tolerance test.

On the other hand Robinson et al ., 2004 found evidence of a non significant caffeine-induced increase in insulin secretion in men with type 2 diabetes, and Petrie et al ., 2004 found no increase in such insulin secretion in obese men.

In this study the significant decrease in serum calcium and serum phosphorus in groups 3, 4 and 5 fed green, roasted coffee respectively is in agreement with the finding of Barrett-Connor et al., (1994) who reported that caffeinated coffee intake equivalent to two cups per day is associated with decreased bone density in older women who do not drink milk on a daily basis.

Also are in agreement with those of Rapuri, et al ., (2001) who reported that Intakes of caffeine in amounts >300 mg/d (≈514 g, or 18 oz, brewed coffee) accelerate bone loss at the spine in elderly postmenopausal women. They found a significant negative correlation between caffeine intake and calcium intake and suggested that high caffeine consumption per se has a negative effect on bone mineral density (BMD), which may be further accentuated by low calcium intakes. However, they could not gain insight into the mechanism of how caffeine exerts its negative effect because we found no significant changes in any of the biochemical indexes measured.

The decrease in serum calcium may be due to the effect of coffee consumption which caused an increase in endogenous fecal calcium and urinary calcium excretion.

our results on the other hand disagree with those of Sakamoto et al ., (2001) reported that strongly indicates that coffee does not stimulate bone loss in rats. They clarify the relationship between coffee consumption and bone metabolism using male Wistar rats. assigned to three treatment groups including a control-diet group , a 0.62% coffee-diet group, and a 1.36% coffee-diet group. They indicated no significant differences in body weight change, serum and urinary biochemical markers of bone metabolism, and bone histomorphometry were found between the coffee-diet groups and the control-diet group, except that urinary phosphorus excretion after 140 days of both coffee diets was significantly increased compared with controls ($p < 0.05$). In addition, the coffee diets were not associated with differences in tumor necrosis factor-α and interleukin-6, which have been implicated in the pathogenesis of bone loss together with interleukin-1β.

Green, roasted and decaffeinated coffee resulted in a significant decrease ($p < 0.05$) in triacylglycerol (TAG); LDL-C; VLDL-C and in LDL\ HDL ratio and TC \ HDL ratio. on the other hand a significant increase ($p < 0.05$) in serum HDL-C is observed in green, roasted and decaffeinated coffee groups compared to diabetic rats compared to normal control with the highest value for green coffee ,with non significant effect on serum total- cholesterol(TC) reported in this study .

Our results are in agreement with those of Kempf et al., 2010 who reported that coffee consumption led to an increase in coffee-derived compounds, mainly serum caffeine, chlorogenic acid, and caffeic acid metabolites. Significant changes were also observed for serum concentrations of interleukin-18, 8-isoprostane, and adiponectin (8 compared with 0 cups coffee/d). Serum concentrations of total cholesterol, HDL cholesterol, and apolipoprotein A-I increased significantly by r, whereas the ratios of LDL to HDL cholesterol and of apolipoprotein B to apolipoprotein A-I decreased significantly by 8% and 9%, respectively (8 compared with 0 cups coffee/d), this indicate that coffee consumption appears to have beneficial effects on subclinical inflammation and HDL cholesterol.

In accordance to our study Shimod et al.,(2006) reported that serum and hepatic TG levels were lowered with intravenous administration of chlorogenic acid in Zucker fa/fa rats. However, the TG level in the adipose tissue was not lowered. Therefore, chlorogenic acid is suspected to be effective on hepatic TG, and not adipose TG. Chlorogenic acid is also a dietary polyphenolic compound with antioxidative activity. Thus, it is suggested that caffeine, chlorogenic acid and other polyphenolic compounds in GCBE act synergistically to suppress body weight gain and visceral fat accumulation in mice.

Uto-Kondo et al. (2010) hypothesized that coffee may enhance reverse cholesterol transport (RCT) as the antiatherogenic properties of high-density lipoprotein (HDL). Caffeic acid and ferulic acid, the major phenolic acids of coffee, enhanced cholesterol efflux from THP-1 macrophages mediated by HDL, but not apoA-I. Furthermore, .they concluded that Coffee intake might have an antiatherogenic property by increasing of ATP-binding cassette transporter (ABC)G1 and scavenger receptor class B type I (SR-BI) expression and enhancing HDL-mediated cholesterol efflux from the macrophages via its plasma phenolic acids.

Lee ., 2009 demonstrated that coffee may guard against Alzheimer's disease and other forms of dementia and somehow soften the blow of a heart attack.

Ozercana et al. (2006) found that lipid peroxidation products that increased in the plasma and liver tissue of the CCl4 group decreased by (instant coffee) IC administration. There was an increase in the measured antioxidant parameters, which were total antioxidant capacity (TAOC), sulphydryl (SH) and ceruloplasmin levels. They concluded that IC had a protective role in acute liver injury induced by CCl4, but did not affect steatosis.Lopez-Garcia et al., (2006) reported that there is no evidence that coffee consumption increases the risk of CHD.

Our results on the other hand disagree with the fnding of Rodrigues and Klein. (2006) who found that Caffeine is the most widely consumed psychostimulant drug in the world that mostly is consumed in the form of coffee. They examined the effects of caffeine intake, both alone and via coffee consumption, on key blood markers of CVD risk: lipoproteins (cholesterol, triglycerides), fibrinogen (a biomarker of blood clotting) and C-reactive protein (CRP; a biomarker of inflammation). They indicated a strong relationship between boiled, unfiltered coffee consumption and elevated cholesterol levels. Also disagree with those of Ricketts et al. (1993) who suggest that caffeine consumption is associated with increased serum cholesterol and/or low density lipoprotein cholesterol. They confirmed that when consumption of caffeine reaches 200 mg or more total cholesterol significantly increased in males. Low density lipoprotein cholesterol concentrations were somewhat increased in males who consumed 200 mg or more. In women, triglyceride levels significantly increased when dietary caffeine intake was 200 mg or higher. Dietary caffeine intake may be a factor to consider when evaluating serum lipid levels.

5. Conclusion

The observed improvement in glucose, insulin profile, triacylglycerol and HDL-C confirm the potent biological action of green, roasted and decaffeinated coffee and suggest that chlorogenic acid (a component in coffee) might have an antagonistic effect on glucose transport. Suggesting a novel function of coffee on lowering the risk factors of diabetes and delaying the progress of diabetes complications as well.

Corresponding author
Eman A.Sadeek
Department of Biochemistry & Nutrition -Women's College –Ain –Shams University, Cairo, Egypt
*dr_emansadeek@yahoo.com

6. References:

1. Andrade-Cetto A, Wiedenfeld H. (2001): Hypoglycemic effect of Cecropia obtusifolia on streptozotocin diabetic rats. J Ethnopharmacol.; 78:145-149.
2. Arion WJ, Canfield WK, Ramos FC, et al. (1997):Chlorogenic acid and hydroxynitrobenzaldehyde: new inhibitors of hepatic glucose 6-phosphatase. Arch Biochem Biophys.
3. Arnlov J, Vessby B, Riserus U (2004): Coffee consumption and insulin sensitivity. JAMA 291:1199–1201,
4. Barham,D. and Trinder ,P. (1972): An important colour reagent for the determination of blood glucose by the oxidase system .Analyst 97: 142-145.
5. Barrett-Connor, E.; Chang, C.C. and Edelstein, S.L. (1994): Coffee-Associated Osteoporosis Offset by Daily Milk Consumption. JAMA. ; 271(4):280-283.
6. Bruce CR, Carey AL, Hawley JA, Febbraio MA (2003): Intramuscular heat shock protein 72 and heme oxygenase-1 mRNA are reduced in patients with type 2 diabetes: evidence that insulin resistance is associated with a disturbed antioxidant defense mechanism. Diabetes 52:2338–2345.

7. Carlsson S, Hammar N, Grill V, Kaprio J(2004): Coffee consumption and risk of type 2 diabetes in Finnish twins (Letter). Int J Epidemiol 33:616–617.
8. Chen CH, Tsai ST, Chou P(1999) : Correlation of fasting serum C-peptide and insulin with markers of metabolic syndrome-X in a homogenous Chinese population with normal glucose tolerance. Int J Cardiol 68:179–186.
9. Clifford MN. (2000): Chlorogenic acid and other cinnamates—nature, occurrence, dietary burden, absorption and metabolism. J Sci Food Agric.80:1033-1043.
10. CORTI, R. et al. (2002): Coffee acutely increases sympathetic nerve activity and blood pressure independently of caffeine content. Circulation,. 106 : 2935-2936,
11. Daglia M, Papetti A, Gregotti C, Berte F, Gazzani G. (2000) : In vitro antioxidant
12. and ex vivo protective activities of green and roasted coffee. J Agric Food Chem 48:1449–54.
13. De Valk HW. (1999): Magnesium in diabetes mellitus. Neth J Med.54:139-146.
14. DÓREA, J. G.; COSTA, T. H. N.(2005) : Is coffee functional food? Br. J. Nutr. 93, :p.773-782,.
15. Drucker DJ. (1998): Glucagon-like peptides. Diabetes.47:159-169.
16. Fossati, P. and Prencipe, L. (1982): Serum triglycerides determined colorimetrically with an enzyme that produces hydrogen peroxide. Clin. Chem. 28:2077.-2080.
17. Frary CD, Johnson RK, Wang MQ. (2005) : Food sources and intakes of caffeine
18. in the diets of persons in the United States. J Am Diet Assoc;105:110–3
19. Friedewald, W.T.; Levy, R.J. and Fredrickson, D.S. (1972): Estimation of the concentration of low density lipoprotein cholesterol in plasma without the use of preparative ultracentrifuge.Clin.Chem.18:499.
20. Isogawa A, Noda M, Takahashi Y, Kadowaki T, Tsugane S.(2003): Coffee consumption and risk of type 2 diabetes mellitus. Lancet. 361:703-704.
21. Johnston KL, Clifford MN, Morgan LM.(2003) : Coffee acutely modifies gastrointestinal hormone secretion and glucose tolerance in humans: glycemic effects of chlorogenic acid and caffeine. Am J Clin Nutr.78:728-733.
22. KARAKAWA, S.(2004) : Bioavailability of phenolic compounds Crit. Rev. Food Sci. Nutr., 44, :. 453-464,
23. Keijzers GB, De Galan BE, Tack CJ, Smits P (2002): Caffeine can decrease insulin sensitivity in humans. Diabetes Care 25:364–369,
24. Kempf K, Herder C, Erlund I, Kolb H, Martin S, Carstensen M, Koenig W, Sundvall J, Bidel S, Kuha S, Jaakko T.(2010) : Effects of coffee consumption on subclinical inflammation and other risk factors for type 2 diabetes: a clinical trial. Am J Clin Nutr. 91(4):950-7.
25. Knowler WC, Barrett-Connor E, Fowler SE, et al.(2002): Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. N Engl J Med.346:393-403.
26. KOZUMA, K. et al. (2005): Antihypertensive effect of green coffee bean extract on mildly hypertensive subjects .Hypert. Res., 28: 711-718, 2005.
27. Lane JD, Barkauskas CE, Surwit RS, Feinglos MN (2004): Caffeine impairs glucose metabolism in type 2 diabetes (Brief Report). Diabetes Care 27:2047–2048.
28. Lee TH. (2009) : Good news for coffee addicts . Har Bus Rev.87(6):22, 112
29. Lopez-Garcia E, van Dam RM, Willett WC, Rimm EB, Manson JE, Stampfer MJ, Rexrode KM, Hu FB.(2006) : Coffee consumption and coronary heart disease in men and women: a prospective cohort study. J Fam Pract. 2006 Sep; 55(9):757-8.
30. McCusker RR, Goldberger BA, Cone EJ. (2003): Caffeine content of specialty coffees. J Anal Toxicol; 27:520–2.
31. Mougios V, Ring S, Petridou A, Nikolaidis MG (2003): Duration of coffee- and exercise-induced changes in the fatty acid profile of human serum. J Appl Physiol 94:476–484.
32. Muroyama K, Murosaki S, Yamamoto Y, Odaka H, Chung HC, Miyoshi M.(2003) : Anti-obesity effects of a mixture of thiamin, arginine,caffeine, and citric acid in non-insulin dependent diabetic KK mice.Nutr Sci Vitaminol (Tokyo) ,49:56–63.
33. Nathan DM.(1993) : Long-term complications of diabetes mellitus. N Engl J Med.; 328:1676-1685.
34. OCHIAI, R. et al. (2004.): Green coffee extract improves human vasoreactivity. Hyperten. Res., 27:.731-737,
35. Oka K. (2007): Pharmacological bases of coffee nutrients for diabetes prevention, Yakugaku Zasshi.127(11):1825-36.
36. Ozercana,I.H. Daglia,A.F.; Ustundagb,B.; Ozercana,M.R.; Bahcecioglu c,I.H. ; Çelikd,H. ; Yalnizc,M.; Poyrazoglu cO.K. and Ataseven,H.(2006) : Does instant coffee_ prevent acute liver injury induced by carbon tetrachloride (CCl4)? Hepatology Research. 35(3): 163-168.
37. Parliament TH, Stahl HB. What makes the coffee smell so good? (2005): Chem Tech: 38–47.
38. Petrie HJ, Chown SE, Belfie LM, et al (2004): Caffeine ingestion increases the insulin response to an oral-glucose-tolerance test in obese men before and after weight loss. Am J Clin Nutr; 80:22– 8.
39. Pulido R, Hernandez-Garcia M, Saura-Calixto F(2003) : Contribution of beverages to the intake of lipophilic and hydrophilic antioxidants in the Spanish diet. Eur J Clin Nutr.57:1275-1282.
40. Rapuri, P.B. ; Gallagher, C.J. ,;Kinyamu H K and Ryschon , L K.(2001) : Caffeine intake increases the rate of bone loss in elderly women and interacts with vitamin D receptor genotypes . Am.J. of Clin. Nut., 74(5) : 694-700.
41. Reeves, P.G.; Nielsen, F.H. and Fahey, G.C. (1993): Ain-93Purified diets for laboratory rodents. J.Nutr.123:1939.
42. Richmond, W. (1973): Determination of seum total cholesterol. Clin.Chem .19:1350.
43. Ricketts, C.D. Forrester,F. and Brevard,P.B. (1993) : Effect of dietary caffeine intake on serum lipid levels in healthy adults. Nutr.Res. 13, (6,): 639-647

44. Robinson LE, Savani S, Battram DS, McLaren DH, Sathasivam P, Graham TE. (2004): Caffeine ingestion before andoral glucose tolerance test impairs blood glucose management in men with type 2 diabetes. J Nutr; 134:2528 –33.
45. Rodrigues I.M. and Klein L.C.(2006) : Boiled or filtered coffee? Effects of coffee and caffeine on cholesterol, fibrinogen and C-reactive protein. Toxicol rev.25(1):55-69
46. Rodriguez de Sotillo DV, Hadley M. (2002): Chlorogenic acid modifies plasma and liver concentrations of: cholesterol, triacylglycerol, and minerals in (fa/fa) Zucker rats. J Nutr Biochem.13:717-726.
47. Rosengren A, Dotevall A, Wilhelmsen L, Thelle D, Johansson S(2004) : Coffee and incidence of diabetes in Swedish women: a prospective 18-year follow-up study. J Intern Med 255:89–95.
48. Sakamoto,W.; Nishihira, J.;Fujie K., Iizuka,T.,; Handa, H. Ozaki , M. and Yukawa, S.(2001) :Effect of coffee consumption on bone metabolism Bone , 28, (3): 332-336
49. Salazar-Martinez E, Willett WC, Ascherio A, et al. (2004): Coffee consumption and risk for type 2 diabetes mellitus. Ann Intern Med.140:1-8.
50. Shearer J, Farah A, de Paulis T, et al.(2003) : Quinides of roasted coffee enhance insulin action in conscious rats. J Nutr.133:3529-3532.
51. Shimoda, H.; Seki, E and Aitani ,M.(2006) : Inhibitory effect of green coffee bean extract on fat accumulation and body weight gain in mice.BMC Complementary and Alternative Medicine, 6:9doi:10.1186/1472-6882-6-9
52. SOMOZA, V. et al.(2003.) : Activity-guided identifi cation of a chemopreventive compound in coffee beverage using in vitro and in vivo techniques. J. Agric. Food Chem., 51 : 6861-6869,
53. Steele, B.W.; Kochler, D.F.and Azar, M.M (1976): Enzymatic determination of cholesterol in hight-density lipoprotein fraction prepared by precipitation technique. Clin.Chem.22:98-101.
54. SUZUKI, A. et al.(2000) : Short and long term effects of ferulic acid on blood pressure in spontaneous hypertensive rats. Am. J. Hypert., 15,: 351-357.
55. Svilaas A, Sakhi AK, Andersen LF, et al.(2004) : Intakes of antioxidants in coffee, wine, and vegetables are correlated with plasma carotenoids in humans. J Nutr.134:562-567.
56. Thirunavukkarasu V, Anuradha CV (2004) : Influence of alpha-lipoic acid on lipid peroxidation and antioxidant defence system in blood of insulin-resistant rats. Diabetes Obes Metab 6:200–207.
57. Thong FS, Derave W, Kiens B, Graham TE, Urso B, Wojtaszewski JFP, Hansen BF, Richter EA(2002) : Caffeine-induced impairment of insulin action but not insulin signaling in human skeletal muscle is reduced by exercise. Diabetes 51:583–590.
58. Thong FS, Graham TE. (2002) : Caffeine-induced impairment of glucose toleranceis abolished by beta-adrenergic receptor blockade in humans, J Appl Physiol;92:2347–52.
59. Tianying, Wu, Willett W. C. ; Hankinson, S. E. and Giovannucci, E. (2005):Caffeinated Coffee, Decaffeinated Coffee, and Caffeine in Relation to Plasma C-Peptide Levels, a Marker of Insulin Secretion, in U.S. Women .Diabetes Care. 28 (6):1390-1396
60. Tunnicliffe JM, Shearer J.(2008) : Coffee, glucose homeostasis, and insulin resistance: physiological mechanisms and mediators. Appl.Physiol.Nut.Met. 33(6):1290-300.
61. Tuomilehto J, Hu G, Bidel S, Lindstrom J, Jousilahti P(2004) : Coffee consumption and risk of type 2 diabetes mellitus among middle-aged Finnish men and women. JAMA 291:1213–1219.
62. Tuomilehto J, Lindstrom J, Eriksson JG, et al.(2001) : Prevention of type 2 diabetes mellitus by changes in lifestyle among subjects with impaired glucose tolerance. N Engl J Med.344:1343-1350.
63. Uto-Kondo H, Ayaori M, Ogura M, Nakaya K, Ito M, Suzuki A, Takiguchi S, Yakushiji E, Terao Y, Ozasa H, Hisada T, Sasaki M, Ohsuzu F, Ikewaki K. (2010): Coffee consumption enhances high-density lipoprotein-mediated cholesterol efflux in macrophages. Circ Res. 106(4):627-9.
64. Van Dam RM, Feskens EJ(2002) : Coffee consumption and risk of type 2 diabetes mellitus. Lancet 360:1477–1478.
65. Van Dam RM, Willett WC, Manson JE, Hu FB. (2006): Coffee, caffeine, and risk of type 2 diabetes: a prospective cohort study in younger and middle-aged U.S. women. Diabetes Care; 29:398–403.
66. Van Dam RM.(2008) : Coffee consumption and risk of type 2 diabetes, cardiovascular diseses and cancer. Appl.Physiol.Nut.Met ;33(6):1269-83
67. Vuppugalla R, Agarwal V, Khan MA.(2003) : A simple HPLC method for the simultaneous analysis of insulin and ovomucoid. Pharmazie. 58(11): 793-795.
68. Wild S, Roglic G, Green A, Sicree R, King H. (2004) : Globalprevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care. 7:1047-1053.
69. Yamaji T, Mizoue T, Tabata S, et al.(. 2004): Coffee consumption and glucose tolerance status in middle-aged Japanese men. Diabetologia.47:2145-2151.
70. Yoshioka K, Kogure A, Yoshida T, Yoshikawa T. (2003): Coffee consumption and risk of type 2 diabetes mellitus. Lancet.361:703.

9/1/2010

Combined at-admission estimation of plasma gelsolin and injury severity score could predict the outcome of multiple trauma patients

Adel F. Al-Kholy [1], Mosad M Odah [1], Jehan Sabry [2], Ehab El-Shahat [3], Ehab Said [3]

[1] Department of Medical Biochemistry, Faculty of Medicine, Benha University
[2] Department of Clinical Pathology, Faculty of Medicine, Benha University
[3] Department of Anesthesia & ICU, Faculty of Medicine, Benha University
adeladel59@yahoo.com

Abstract: To estimate plasma gelsolin levels in multiple trauma patients and its predictability for their outcome in relation to clinical data. The study included 70 multiple trauma patients and 20 healthy adult controls for blood donation as control group for the plasma level of gelsolin. All patients underwent history taking, time elapsed since trauma inflection and amount of external bleeding if present. Clinical evaluation included both Acute Physiology and Chronic Health Evaluation II (APACHE II) and Injury Severity Scores (ISS). Patients were evaluated daily throughout their ICU or hospital stay for the development of secondary morbidities and/or mortality. Venous blood samples were obtained at 12 hours after ICU admission for ELISA estimation of plasma gelsolin level. During hospital stay, 20 patients (28.6%) developed secondary morbidities and 8 patients (11.4%) died. Mean plasma gelsolin levels were significantly lower in patients compared to control levels with significantly lower levels in non-survivors compared to controls and survivors. Development of secondary morbidities showed a positive significant correlation with at admission ISS score and a negative significant correlation with plasma gelsolin. Survival rate showed positive significant correlation with plasma gelsolin level and negative significant correlation with both time since trauma inflection and ISS score. ROC curve analysis, defined prolonged time since trauma inflection as the significant sensitive predictor for both morbidity and mortality, while plasma gelsolin level was significant specific predictor for development of secondary morbidity and combined with ISS score were significant specific predictors for mortality. Conclusion: At admission plasma gelsolin level is a specific independent marker for prediction of the development of secondary morbidities that may progress to endanger patients' life and time since trauma inflection was found to be significant sensitive parameter for the patients' survival irrespective of development of these morbidities.

[Adel F. Al-Kholy, Mosad M Odah, Jehan Sabry, Ehab El-Shahat, Ehab Said**. Combined at-admission estimation of plasma gelsolin and injury severity score could predict the outcome of multiple trauma patients**. Journal of American Science 2010;6(12):442-447]. (ISSN: 1545-1003).

Keywords: Gelsolin, Trauma, Sepsis, Morbidity, Mortality

1. Introduction

Most patients with severe traumatic injury have a prolonged stay in the intensive care unit, the outcome being a long-term disability or death, with a minority of patients achieving a functionally independent outcome and despite the therapeutic advances; certain patients remain at high risk of infection and the attendant morbidity and mortality. Several clinical studies, (Somasundar et al., 2004 and Jeremitsky et al., 2005) tried to identify certain prognostic factors that may influence outcome; however within certain limitations. Moreover, the development of adverse secondary events including development of adult respiratory distress, systemic inflammatory response syndrome, or multiple organ dysfunction syndromes may mislead the clinician during scoring and disturb the primary score and its predictability (Calfee et al., 2007).

This diagnostic dilemma pointed to the need for an early predictor for these secondary events prior to its commencement; Aslar et al., (2006) found APACHE II score and the arterial lactate level are the most important determinants of clinical outcome in critically injured patients and a correlation exits between lactate and APACHE II and between lactate and base deficit. Cunningham et al., (2006) found the admission lipopoly-saccharide-binding protein (LBP) concentration was significantly greater in non-survivors than in survivors, but after controlling for age and ISS, the admission LBP concentration did not predict death. Meisner et al., (2006) found that in patients with multiple trauma, procalcitonin level provides more information than the C-reactive protein level since only moderate amounts of procalcitonin is induced, and higher concentrations correlate with more severe trauma and a higher frequency of

various complications, including sepsis and infection.

Gelsolin is a protein found in both the cytosol and the plasma that has been reported to function primarily as a part of the actin-scavenging system with Gc globulin (vitamin D-binding protein) (Sun et al., 1999). Cellular disruption or necrosis releases cellular actin, both globular and filamentous fractions, into the circulation which results in capillary plug formation with subsequent tissue ischemia. The plasma actin scavenging system functions to cleave the filaments, cap the ends, preventing repolymerization and assist in the plasma clearance of filamentous actin. Additionally, gelsolin is known for its ability to bind lipids such as lipopolysaccharide, lysophosphatidic acid and phosphotidylinositol (Goetzl et al., 2000 and Karliner et al., 2001) and may modulate the inflammatory system. Experimentally, animals subjected to 40% total body-surface area burns demonstrated attenuated acute lung injury with the infusion of recombinant gelsolin to normal plasma levels (Rothenbach et al., 2004). Therefore, the present study aimed to estimate plasma gelsolin levels in multiple trauma patients and its predictability for their outcome in relation to clinical data.

2. Patients and Methods

The present study was conducted since Jan 2006 till June 2009 at Departments of Anesthesia & ICU and Clinical Pathology, Benha University Hospital in conjunction with Medical Biochemistry Department, Faculty of Medicine, Benha University. After approval of the study protocol by the Local Ethical Committee, the study was designed to include 70 trauma patients, irrespective of the anatomical site of trauma, required management and admission to surgical ICU. All patients arrived to the emergency unit dying or gasping was excluded of the study. The study was also designed to enroll 20 healthy adult controls for blood donation as control group for the plasma level of gelsolin.

All patients underwent full history taking with special regard time elapsed since trauma inflection, loss of consciousness and time elapsed till its regaining, amount of external bleeding if present. Clinical evaluation included both Acute Physiology and Chronic Health Evaluation II (APACHE II), (Rowan et al., 1993) and Injury Severity Scores (ISS), (Copes et al., 1988) determination.

All patients received first aid management at emergency unit, and when indicated surgical interference was carried upon, patients were admitted to ICU either immediately for conservative treatment or for postoperative follow-up for either discharge from the ICU or the development of additional morbidity or mortality.

Patients were evaluated daily for the development of septic morbidities including systemic inflammatory response syndrome (SIRS), sepsis, severe sepsis or septic shock. The frequency of development and outcome of these complications was recorded either at ICU or at ward after discharge from ICU.

Venous blood samples were obtained at 12 hours after hospital admission and were collected in EDTA containing tube to prevent clotting and plasma was separated by centrifugation and then separated and stored at -80^{o}C till ELISA (Uscn Life Science Inc. Wuhan, USA) estimation of plasma gelsolin level, (Smith et al., 1987).

Obtained data were presented as mean±SD, ranges, numbers and ratios. Results were analyzed using Chi-square test and Wilcoxon Signed Ranks Test. Sensitivity & specificity of evaluated parameters as predictors for patients' outcome were evaluated using the receiver operating characteristic (ROC) curve analysis judged by the area under the curve (AUC) and Logestic Regression analysis (Stepwise Method). Statistical analysis was conducted using the SPSS (Version 10, 2002) for Windows statistical package. P value <0.05 was considered statistically significant.

3. Results

The study included 70 patients who had successful resuscitation so as to be fully examined and managed and all were immediately admitted to the surgical ICU. During hospital stay, 11 patients (15.7%) developed adult respiratory distress syndrome (ARDS), 5 patients (7.1%) had sepsis and 4 patients (5.7%) had multiple organ failure (MOF) with a total secondary morbidity rate of 28.6%. At end of one month follow-up, 62 patients (88.6%) were survivors and 8 patients died with a total mortality rate of 11.4%. Three of non-survivors developed septic shock that could not respond to conservative treatment; 4 patients with MOF could not withstand and one patient with ARDS developed acute respiratory failure and died 3-days later. Detailed baseline characteristics and clinical data of the study

population, stratified as survivors or non-survivors, are presented in Table 1 and showed non-significant difference between survivors and non-survivors concerning these data apart from a significantly higher ISS, (Z=2.524, p=0.012) and APACHE II (Z=2.035, p=0.042) scores in non-survivor compared to survivors.

Table (1): Patients' characteristics and clinical data determined at admission

		Survivors	Non-survivors
Number		62 (88.6%)	8 (11.4%)
Age (years)		33.7±9	38±13.4
Gender; M:F		50:12	5:3
Time since trauma (min)		52.5±21.3 (20-100)	77.5±19.3 (45-100)
Scene external bleeding		18 (29%)	5 (62.5%)
Loss of Conscious-ness	Number	10 (16.2%)	3 (27.5%)
	Duration	6±3.5 (4-10)	4.7±2.5 (1-9)
ISS score data		20.3±9.2 (11-59)	45±13.9 (29-66)*
APACHE II score		11.3±4.5 (7-15)	13.9±1.7 (8-19)*

Data are presented as mean±SD, ratios & numbers; ranges & percentages are in parenthesis
ISS score: injury severity score
*: significant versus survivors

Figure 1 shows that mean plasma gelsolin levels were significantly lower (Z=3.576, p<0.001) in patients (127.7±34; range: 45.6-192.4 ng/ml) compared to control levels, (196.4±27.6; range: 134.5-246.5 ng/ml) and were significantly lower in patients categorized according to survival compared to control levels with significantly lower (Z=2.521, p=0.012) levels in non-survivors (84.4±32.2; range: 45.6-145.2 ng/ml) compared to survivors (133.3±30.2; range: 59-192.4 ng/ml).

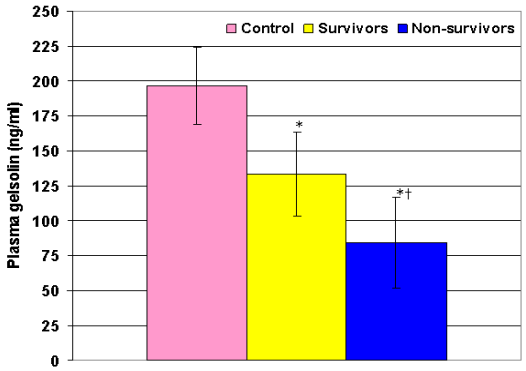


Figure 1: Mean (±SD) plasma gelsolin levels estimated in patients categorized according to the survival compared to control levels
*: significant versus control †: significant versus survivors

Patients developed secondary morbidities showed a significantly higher (Z=2.576, p=0.01) ISS score with a significantly (Z=3.246, p=0.001) lower plasma gelsolin levels and were presented after significantly (Z=2.577, p=0.01) longer time since trauma inflection compared to those passed smoothly without development of secondary morbidities. Patients' age and body mass index showed non-significant difference between both patients' groups, (Table 2).

Table (2): Levels of evaluated parameters in studied patients categorized according to the development of secondary morbidities

	Secondary Morbidities	
	No (n=50)	Present (n=20)
Age	33.3±8.8	36.5±11.2
BMI	28.9±3.9	29.7±3.6
ISS score	19.1±7.3	33.2±16.8*
[a]Time	50.9±20.4	66.5±23.8*
P. Gelsolin	136.4±29.2	106.2±36.3*

Data are presented as mean±SD
Time since trauma affliction (minutes)
P. Gelsolin: plasma gelsolin levels
*: significant versus no secondary morbidities

Regression analysis defined at admission decreased plasma gelsolin as a significant (p<0.001) predictor for the development of secondary morbidities, while long time since trauma inflection and at admission high ISS score were the significant predictors for mortality, (p<0.001 & =0.011, respectively). Using ROC curve analysis, as shown in figures 2 and 3, defined prolonged time since trauma inflection as the significant sensitive predictor for both morbidity, (AUC=0.338, p=0.035) and mortality,

(AUC=0.075, p<0.001), while at admission plasma gelsolin levels were the most significant specific predictor for development of secondary morbidity, (AUC=0.506, p<0.001). On the other hand, at admission, ISS score and plasma gelsolin were the significant specific predictors for mortality, (AUC=0.787 & 0.871, p=0.009 & 0.001, respectively).

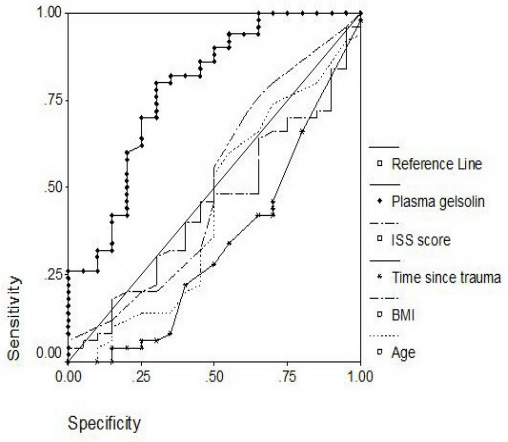
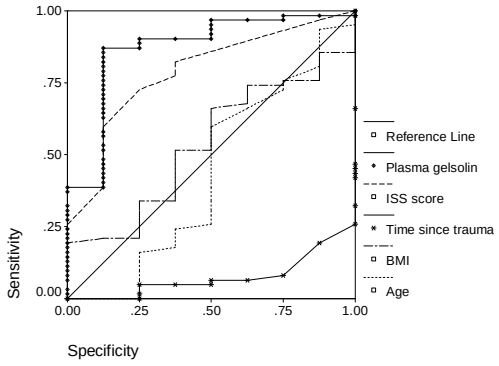


Figure (2): Specificity of evaluated parameters for prediction of secondary morbidities development



4. Discussion

Prediction of outcome, either as regards morbidity or mortality, of multiple trauma patients still a diagnostic dilemma. Twenty of the studied 70 multiple trauma patients developed secondary morbidities with a frequency of 28.6% and 8 patients could not withstand and died with a mortality rate of 11.4%. There was a non-significant difference between survivors and non-survivors as regard the constitutional parameters, and the number had external bleeding. However, non-survivors had significantly higher APACHE II and ISS scores with significantly longer time since trauma inflection.

These data illustrate a fact that trauma itself and its sequalae, and body systems and regions affected impose a high effect on the outcome, in support of this assumption there was a positive significant correlation between the determined ISS score and the survival of trauma patients and the frequency of development of secondary morbidities, however, the correlation was stronger with survival and this was assured using ROC curve analysis that defined ISS score as one of specific predictors of survival not for development of secondary morbidities.

Prolonged time since inflection of trauma till patient arrival to the hospital was found to be a highly sensitive predictor for both mortality and development of secondary morbidities; such parameter indicated bad transport systems for trauma patients, lack of road ambulance first aid facilities and experiences of health care providers, and lack of general knowledge about first aid till arrival of ambulance among population.

Considering survival is a first target for multiple trauma patients, post-traumatic mortalities could not be a secondary to trauma itself but to development of secondary mortalities. Eight patients died among this series all of them had secondary mortalities; 4 had MOF, 3 developed septic complications progressed to septic shock and one had ARDS progressed to acute respiratory failure and patient died. These findings point to the necessity for early prediction of such morbidities; plasma gelsolin, the plasma parameter evaluated through the present study, was found significantly depleted in non-survivors compared to both controls and survivors and in survivors compared to control. These findings illustrate the impact of trauma itself on plasma gelsolin level, irrespective of the outcome and its depletion is a bad finding that predicts the development of secondary morbidities and the possibility of its progression to endanger patients' survival. In support of this assumption, there was a negative significant correlation between plasma gelsolin levels and both morbidities and mortality and regression analysis defined it as a significantly specific predictor for morbidities; a result that assured using ROC curve analysis.

These findings supported that previously documented in literature, Dahl et al., (1999) found gelsolin level on admission was

reduced significantly in the trauma patients compared with normal controls, but they found no correlation between admission levels of gelsolin and ISS or survival. Thereafter, Lee et al., (2006) found low plasma gelsolin levels were associated with increased risk of death occurring in the ICU and could predicted longer ICU stay, prolonged ventilator dependence, and increased overall in-hospital mortality. Also, Wang et al., (2008) found the admission gelsolin levels were significantly decreased in severe sepsis compared with non-septic critically ill patients and healthy control individuals and survivors of severe sepsis exhibited substantial recovery of their depressed plasma gelsolin levels, whereas gelsolin levels in non-survivors remained at or below their depleted admission levels and concluded that plasma gelsolin may be a valuable marker for severe sepsis and recovery of depleted plasma gelsolin levels correlated with clinical improvement.

Gelsolin is one of actin-scavenging proteins that counteract the pathophysiological consequences of actin leaked into the circulation from dying cells, but the capacity of this defense system can be overwhelmed by massive tissue injury. Various studies tried to explore the pathogenesis of gelsolin depletion after trauma Löfberg et al., (1998) measured the serum gelsolin levels in five patients after rhabdomyolysis and observed a tendency of serum gelsolin to increase during the study period of 11 days with no intracellular gelsolin found in the serum, although it is abundant in muscle, and the destruction was severe as judged by other parameters and concluded that serum gelsolin thus behaves differently in rhabdomyolysis than after acute tissue damage in other organs, such as liver necrosis and adult respiratory distress syndrome.

Mounzer et al., (1999) failed to attribute gelsolin depletion to generalized protein loss due to trauma as they reported no correlation between post-traumatic hemoglobin values and plasma gelsolin concentrations but found plasma levels of albumin correlated with plasma gelsolin, however, the extent of plasma gelsolin depletion was much greater than that of albumin and concluded that plasma gelsolin depletion was specific and not a result of generalized plasma protein loss. On contrary, Lee et al., (2006) found plasma albumin depletion of no prognostic value but documented the predictability of depleted gelsolin for development of secondary morbidities, despite the absence of correlation with the development of ARDS, a contradictory finding to both Mounzer et al., (1999) and the current study.

These data point to a fact that depletion of plasma gelsolin is independent of extent of body protein loss, hemoglobin loss, or sole muscle injury but is dependent on injury severity of other organs and tissues especially in patients liable to secondary morbidities. It could be concluded that at admission estimation of plasma gelsolin is a specific independent marker for prediction of the development of secondary morbidities that may progress to endanger patients' life and time since trauma inflection was found to be significant sensitive parameter for the patients' survival irrespective of development of these morbidities. However, wider scale studies were advocated to correlate the predictability of estimation of plasma gelsolin versus inflammatory markers.

Acknowledgements:

Authors are grateful to Faculty of Medicine, Benha University for financial support to carry out this work.

Corresponding Author:
Dr. Adel Al-Kholy
Department of Medical Biochemistry
Faculty of Medicine, Benha University
Benha, Kaluobia, Egypt
E-mail: adeladel59@yahoo.com

References

1. Somasundar PS, Mucha P, McFadden DW. The evolving management of blunt hepatic trauma in a rural setting. Am Surg. 2004; 70(1):45-8.
2. Jeremitsky E, Omert LA, Dunham CM, Wilberger J, Rodriguez A. The impact of hyperglycemia on patients with severe brain injury. J Trauma. 2005; 58(1): 47-50.
3. Calfee CS, Eisner MD, Ware LB, Thompson BT, Parsons PE, Wheeler AP, Korpak A, Matthay MA. Acute Respiratory Distress Syndrome Network, National Heart, Lung, and Blood Institute: Trauma-associated lung injury differs clinically and biologically from acute lung injury due to other clinical disorders. Crit Care Med. 2007; 35(10):2243-50.
4. Aslar AK, Kuzu MA, Elhan AH, Tanik A, Hengirmen S. Admission lactate level and the APACHE II score are the most useful predictors of prognosis following torso trauma. Injury. 2004; 35(8):746-52.

5. Cunningham SC, Malone DL, Bochicchio GV, Genuit T, Keledjian K, Tracy JK, Napolitano LM. Serum lipopolysaccharide-binding protein concentrations in trauma victims. Surg Infect (Larchmt). 2006; 7(3):251-61.
6. Meisner M, Adina H, Schmidt J. Correlation of procalcitonin and C-reactive protein to inflammation, complications, and outcome during the intensive care unit course of multiple-trauma patients. Crit Care. 2006; 10(1):R1.
7. Sun HQ, Yamamoto M, Mejillano M, Yin HL. Gelsolin, a Multifunctional Actin Regulatory Protein. J Biol Chem. 1999; 274:33179–82.
8. Goetzl EJ, Lee H, Azuma T, Stossel TP, Turck CW, Karliner JS. Gelsolin Binding and Cellular Presentation of Lysophosphatidic Acid. J Biol Chem 2000; 275: 14573–78.
9. Karliner JS, Honbo N, Summers K, Gray MO, Goetzl EJ. The Lysophospholipids Sphingosine-1-Phosphate and Lysophosphatidic Acid Enhance Survival during Hypoxia in Neonatal Rat Cardiac Myocytes. J Mol Cell Card. 2001; 33:1713–7.
10. Rothenbach PA, Dahl B, Schwartz JJ, O'Keefe GE, Yamamoto M, Lee WM, Horton JW, Yin HL, Turnage RH. Recombinant plasma gelsolin infusion attenuates burn-induced pulmonary microvascular dysfunction. J Appl Phys. 2004; 96:25 – 31.
11. Rowan KM, Kerr JH, Major E, et al. Intensive Care Society's APACHE II study in Britain and Ireland: I. Variations in case mix of adult admissions to general intensive care units and impact on outcome. BMJ, 1993; 307:972–7.
12. Copes WS, Champion HR, Sacco WJ: The Injury Severity Score revisited. J Trauma, 1988; 28(1): 69-77.
13. Smith DB, Janmey PA, Herbert TJ, et al. Quantitative measurement of plasma gelsolin and its incorporation into fibrin clots. J Lab Clin Med. 1987; 110:189 –195.
14. Dahl B, Schiødt FV, Ott P, Gvozdenovic R, Yin HL, Lee WM. Plasma gelsolin is reduced in trauma patients. Shock. 1999; 12(2):102-4.
15. Lee PS, Drager LR, Stossel TP, Moore FD, Rogers SO. Relationship of plasma gelsolin levels to outcomes in critically ill surgical patients. Ann Surg. 2006; 243(3):399-403.
16. Wang H, Cheng B, Chen Q, Wu S, Lv C, Xie G, Jin Y, Fang X. Time course of plasma gelsolin concentrations during severe sepsis in critically ill surgical patients. Crit Care. 2008; 12(4):R106.
17. Löfberg M, Paunio T, Tähtelä R, Kiuru S, Somer H: Serum gelsolin and rhabdomyolysis. J Neurol Sci. 1998; 157(2):187-90.
18. Mounzer KC, Moncure M, Smith YR, Dinubile MJ. Relationship of admission plasma gelsolin levels to clinical outcomes in patients after major trauma. Am J Respir Crit Care Med. 1999; 160(5 Pt 1):1673-81.

10/2/2010

Serum levels of placental growth factor and retinol-binding protein-4 in pregnancy-induced hypertensive women

Adel F. Al-Kholy [1], Mamdouh Z. Abadier [1], Ebrahem M. Rageh [2], Hany El-Kallaf [3]

[1] Department of Medical Biochemistry, Faculty of Medicine, Benha University
[2] Department of Clinical Pathology, Faculty of Medicine, Benha University
[3] Department of Obstetrics & Gynecology, Faculty of Medicine, Benha University
adeladel59@yahoo.com

Abstract: To investigate the relationship between clinical parameters of pre-eclampsia (PE) and serum levels of Retinol binding protein4 (RBP4) and Placental growth factor (PlGF). Patients and Methods: The study included 90 pregnant women categorized as Group I: Control group (n= 20), included pregnant women who continued their pregnancy without development of PE manifestations, Group II: included patients had Mild PE (n=56) and group III included patients had Severe PE (n=14). After clinical evaluation and ultrasonographic examination, samples of maternal peripheral blood were obtained either at time of diagnosis of PE in groups II and III or at time of delivery in control group for ELISA estimation of serum RBP4 and PlGF. Results: PE patients had significantly lower serum PlGF, but significantly higher serum RBP4 levels when compared to the corresponding levels of the control group. Serum levels of PlGF showed negative correlation with systolic and diastolic blood pressures (SBP and DBP) and extent of proteinuria, but showed positive significant correlation with birth weight, while serum levels of RBP4 showed positive significant correlation with DBP, extent of proteinuria and patients' body weight measures. Conclusions: RBP4 and PlGF were associated with the development and severity of PE.
[Adel F. Al-Kholy, Mamdouh Z. Abadier, Ebrahem M. Rageh, Hany El-Kallaf. **Serum levels of placental growth factor and retinol-binding protein-4 in pregnancy-induced hypertensive women**. Journal of American Science 2010;6(12):448-455]. (ISSN: 1545-1003).

Keywords: Pre-eclampsia, Placental growth factor, Retinol-binding protein

1. Introduction

Pregnancy is a physiological situation where major changes in energy homeostasis occur to meet the nutrient demands of fetal growth. The energy needs are met by increased food intake and/or mobilization of stored fuels and decreased sensitivity of maternal tissues to insulin. This change in insulin sensitivity allows for decreased glucose utilization and a shift to fat metabolism by maternal peripheral tissues and increased availability to the feto-placental unit. The pregnant state parallels the insulin-resistant states of obesity and type-2 diabetes, which are also characterized by insulin resistance, and can become manifested as gestational diabetes in humans (Herrera, 2000, Di Cianni et al., 2003).

Pre-eclampsia is the major cause of maternal and fetal morbidity and mortality, involving 15% to 20% of pregnancies in developed countries and even more in less developed parts of the world. Superficial placentation driven by immune maladaptation, with subsequently reduced concentrations of angiogenic growth factors and increased placental debris in the maternal circulation, are likely responsible (Dechend and Luft, 2008).

Retinol (vitamin A) bound to its specific transport protein, retinol-binding protein (RBP), is the predominant (95% or more) retinoid form in the fasting circulation. Postprandially, retinyl ester packaged in chylomicrons and chylomicron remnants can constitute a large percentage of the total retinoid present in the circulation. As RBP is the sole specific transport protein for retinol, it has been proposed to play an important role in the delivery of retinoid from mother to fetus. However, the mechanisms and the physiology of maternal-fetal vitamin A transfer are not fully understood (Soprano and Blaner, 1994, Sapin et al., 1998).

Dysregulation of maternal circulating adipokines has been implicated in several "great obstetrical syndromes" including pre-eclampsia, small-for-gestational age, neonate and fetal death. It has been suggested that adipokines provide a molecular link between metabolic derangements and inflammatory response in complicated pregnancies. Retinol binding protein 4, a novel adipokine, plays a role in obesity-related disorders, as well as in the regulation of the immune response (Vaisbuch et al., 2009). Solini et al., (2009) determined serum RBP4,

leptin, adiponectin, and resistin levels in hypertensive and normotensive lean non-pregnant women with normal glucose tolerance and found serum RBP4 levels are increased in naive hypertensive women and correlated with the degree of intima-media thickness

Placental growth factor is a pregnancy-specific hormone that has been proposed to play a role in trophoblast invasion and fetal growth, as well as maternal adaptation to pregnancy (Lacroix et al., 2005). PlGF demonstrates somatotrophic, lactogenic and lipolytic properties similar to pituitary growth hormone, although its growth-promoting activity surpasses its other functions. Syncytiotrophoblast and extravillous cytotrophoblast express PlGF mRNA and protein. This hormone is secreted in a non-pulsatile fashion and can be detected in maternal blood at as early as 5 weeks of gestation (Chellakooty et al., 2004) and increases throughout pregnancy until term (Chellakooty et al., 2002), at which time PlGF concentration has been observed to either plateau or slightly decrease (Chellakooty et al., 2004).

Central to the pathogenesis of PE is shallow placentation with abnormal maternal-placental vascular development. Shallow placentation causes release of endothelial deranging factors to the maternal circulation. Among these placenta-derived factors are the antiangiogenic proteins such as soluble fms-like tyrosine kinase receptor (sFlt1) which binds and reduces the free circulating levels of the proangiogenic factors such as vascular endothelial growth factor (VEGF) and placental growth factor (PlGF). sFlt thereby blunts the beneficial effects of these proangiogenic factors on maternal endothelium, with consequent maternal hypertension and proteinuria (Redman and Sargent, 2005, Maynard et al., 2003, Troisi et al., 2008).

The aim of the present study is to evaluate the association between the levels of serum RBP4 and PIGF and the development of PIH or its severity.

2. Patients and Methods

Pre-eclampsia was diagnosed by the presence of gestational hypertension beginning after the 20th week of pregnancy with an absolute blood pressure ≥140 mmHg systolic and/or 90 mmHg diastolic on at least two occasions, 4 hours apart, and proteinuria (one dipstick measurement ≥2+ on a voided random urine sample). Severe PE was defined as severe hypertension (diastolic blood pressure ≥ 110 mmHg) plus mild proteinuria (2+ protein by dipstick measurement) or mild hypertension plus severe proteinuria (≥ 3+ protein by dipstick measurement). Mild PE was defined as mild hypertension with diastolic blood pressure <110 mmHg plus mild proteinuria (≥ 2+ protein by dipstick measurement). Patients with eclampsia were also classified in the severe pre-eclampsia category (Gifford et al., 2000).

This study was conducted at Benha University hospital in conjunction with Medical Biochemistry Department, Benha Faculty of Medicine and included 90 pregnant women, signed a fully informed written consent and categorized as Group I: Control group (n= 20), included pregnant women who continued their pregnancy without development of PE manifestations, group II: included patients had Mild PE (n=56) and group III included patients had Severe PE (n=14). Exclusion criteria included multiple gestation and preexisting medical conditions such as diabetes, chronic hypertension, and renal diseases.

At time of enrollment in the study, all women underwent full history taking, general and abdominal examination to determine a baseline arterial blood pressure and body mass index (BMI). Ultrasonographic examination was conducted to confirm the gestational age, and to exclude the presence of fetal congenital abnormalities. Body mass index was calculated according to the equation: BMI = [(Weight)/ (Height) ^{2}]. A person with a BMI of ≥30 are considered obese and morbidity rise sharply when the BMI is >30 kg/m^{2} (Vella et al., 2003).

Throughout the period since baseline data collection, all women were examined weekly for the progress of pregnancy and fetal wellbeing, the extent of hypertension and occurrence of other complications. Urine analysis was performed for the presence of urinary tract infection and the degree of proteinuria.

Samples of maternal peripheral blood were obtained either at time of diagnosis of pre-eclampsia in hypertensive group or at time of delivery in control group. Collected maternal blood samples were allowed to clot then serum was separated by centrifugation at 2000 rpm for 10 min. Serum was removed, placed in pyrogen-free Eppendorf tubes and stored at -80°C until ELISA assayed (within one month) for estimation of serum RBP4 (AdipoGen Inc., Seoul, Korea), (Lewis et al., 2007) and PlGF (RayBiotech, USA) concentrations, (Nemzek et al., 2001).

Statistical analysis: obtained data were presented as mean±SD, ranges, numbers and ratios. Results were analyzed using one-way ANOVA test and Chi-square test. Statistical analysis was conducted using the SPSS (Version 10, 2002) for Windows statistical package. P value <0.05 was considered statistically significant.

.

3. Results

The study was extended till enrollment of 70 PE pregnant women; Table (1) shows that mean age of enrolled PE patients was 27.9±3; range with a mean BMI of 31.2±1.8 kg/m^2. At time of diagnosis, mean SBP was 150.3±5.9 mmHg and mean DBP was 100.6±6.4 mmHg. All patients were proteinuric with a mean level of 1.8±0.7; range by dipstick measurement. Mean gestational age at time of enrollment was 30.9±3.8 weeks. The study also included a control group consisted of 20 pregnant women of cross-matched age and gestational age.

Table (1): Characteristics of the entire study population at time of enrollment

		Control group (n=20)	PE group (n=70)
Age (years)		28.3±2.4 (25-33)	27.9±3 (22-33)
Weight (Kg)		80.5±2.9 (75-85)	82.9±2.9 (79-90)
Height (cm)		164.7±4.9 (158-172)	163±3.5 (156-173)
BMI (kg/m^2)		29.7±2.1 (27.3-33.6)	31.2±1.8 (27.4-36.1)
Nulli:multipara		13:7	43:27
Gestational age (wk) at time of enrollment		29.7±3.6 (24-36)	30.9±3.8 (23-37)
Blood pressure	SBP	122±4.7 (115-130)	150.3±5.9 (140-165)*
	DBP	79.3±5.2 (70-85)	100.6±6.4 (90-118)*
‡Level of protein in urine		0.7±0.5 (0 to +1)	1.8±0.7 (+1 to +4)*

Data are presented as mean±SD, ranges are in parenthesis
BMI: Body mass index
SBP: systolic blood pressure
DBP: diastolic blood pressure
*: significant difference versus control group
‡: Level of protein in urine as judged by dipstick measurement and expressed as number of + marks

Table (2) shows that 14 women had severe pre-eclampsia with a mean SBP and DBP of 154.8±8.5 mmHg and 107.9±7.4 mmHg, respectively and the mean proteinuria level of 2.7±0.8 by dipstick measurement. While the other 56 women had mild pre-eclampsia with a mean SBP and DBP of 149.2±4.4 mmHg and 98.8±4.7 mmHg, respectively and mean proteinuria level of 1.6±0.5 by dipstick measurement. Patients developed severe PE had significantly higher mean blood pressure measures and significantly higher level of protein in urine compared to those had mild PE. However, other evaluated parameters showed non-significant difference between severe and mild PE patients.

Table (2): Clinical characteristics of pre-eclamptic patients

		Mild PE (n=56)	Severe PE (n=14)
Age (years)		27.8±3.2 (23-33)	27.9±3 (22-33)
Weight (Kg		82.9±3.2 (79-87)	82.8±2.8 (79-90)
Height (cm)		164±2.8 (159-169)	162.7±3.7 (156-172)
BMI (kg/m^2)		30.9±1.3 (29-33.2)	31.3±1.9 (27.4-36.1)
Nulli: multipara		35:21	8:6
Gestational age (wk) at time of enrollment		30.7±4.2 (24-36)	30.9±3.8 (23-37)
Blood pressure	SBP	149.2±4.4 (140-155)	154.8±8.5 (142-165)*
	DBP	98.8±4.7 (90-106)	107.9±7.4 (95-118)*
‡Level of protein in urine		1.6±0.5 (+1 to +2)	2.7±0.8 (+2 to+4)*

Data are presented as mean±SD, ranges are in parenthesis
BMI: Body mass index
SBP: systolic blood pressure
DBP: diastolic blood pressure
*: significant difference versus control group
‡: Level of protein in urine as judged by dipstick measurement and expressed as number of + marks

Table (3) shows that among the studied 90 women, 56 women (62.2%) had vaginal delivery, while 34 women (37.8%) had cesarean section (CS). Women developed PE had significantly higher, (X^2=3.853, p<0.05) frequency of CS compared to control group (41.4% versus 25%, respectively) and women developed severe PE had significantly higher, (X^2=4.962, p<0.01) frequency of CS compared to control group (57.1% versus 25%, respectively). Gestational age at time of delivery was significantly shorter in PE group compared to control group and in those developed severe PE compared to those had mild PE. Moreover,

birth weight was significantly (P<0.01) lower in PE group compared to control group and in those developed severe PE compared to those had mild PE.

Table (3): Delivery data of the study population

		Control (n=20)	Mild PE (n=56)	Severe PE (n=14)
‡GA (weeks)		39.7±1.3 (38-42)	35.4±1.3 (34-38)	33.3±1.1† (32-35)
Gestational weight (gm)		3.31±0.5 (2.5-4.2)	2.8±0.62 (1.6-3.9)	2.44±0.55† (1.6-3.35)
Delivery mode	CS	5 (25%)	21 (37.5%)	8 (57.1%)
	Vaginal	15 (75%)	35 (62.5%)	6 (42.9%)

Data are presented as mean±SD & numbers, ranges & percentages are in parenthesis
CS: cesarean section
‡Gestational age at time of delivery
*: significant difference versus control
†: significant difference versus mild PE

Table (4) shows significantly lower PlGF and higher RBP4 serum levels in PE women compared to control women and in women had severe PE compared to both controls and those had mild PE with significant difference between mild pre-eclamptic and controls.

Table (4): Mean (±SD) levels of serum PlGF and RBP4 in control and PE groups

		Serum PlGF (µg/ml)	Serum RBP4 (ng/ml)
Control group (n=20)		346.7±113.6 (221.5-578.6)	20.7±8.6 (9-45)
PE group	Mild (n=56)	242.3±84.3* (103.7-395.4)	60.1±18.4* (29-105)
	Severe (n=14)	183±42.1*† (122.4-250.4)	104.9±17*† (72-127)
	Total (n=70)	230.4±81* (103.7-395.4)	69.1±25.5* (29-127)

Data are presented as mean±SD, ranges are in parenthesis
*: significant difference versus control
†: significant difference versus mild pre-eclampsia

Figure (1) shows a significantly lower PlGF serum levels in PE women compared to control women, with significantly lower levels in women had severe PE compared to controls and those had mild PE and between patients had mild PE and controls. On contrary, figure (2) shows a significantly higher RBP4 serum levels in PE women compared to control women with significantly higher levels in women had severe PE compared to controls and those had mild PE and between patients had mild PE and controls.

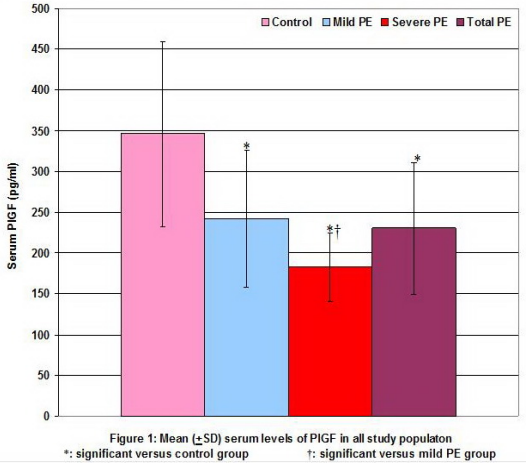


Figure 1: Mean (±SD) serum levels of PlGF in all study populaton
*: significant versus control group †: significant versus mild PE group

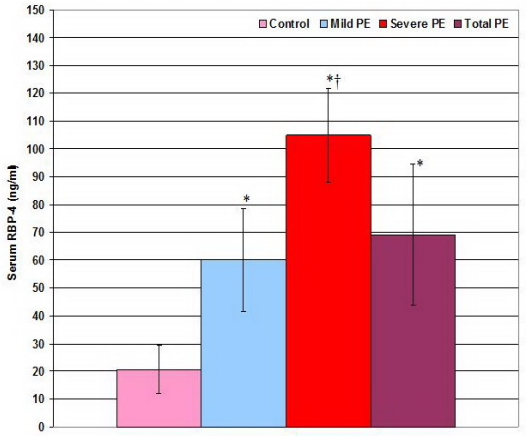


Figure 2: Mean (±SD) serum levels of RBP-4 in all study populaton
*: significant versus control group †: significant versus mild PE group

Serum levels of PlGF showed negative significant correlation with the severity of PE manifested as SBP (-0.429, p<0.001), DBP (-0.276, p=0.021) and the extent of proteinuria (-0.236, p<0.001), while serum levels of RBP4 showed positive significant correlation with as DBP (0.509, p<0.001) and extent of proteinuria (0.417, p<0.001). Serum levels of PlGF showed positive significant correlation with birth weight (0.312, p=0.009). On contrary, such correlation was non-significant with serum RBP4 that showed positive significant correlation with body weight (0.246, p=0.040) and mass index (0.318, p=0.007). Moreover, there was a

negative non-significant correlation between serum levels of PlGF and RBP-4.

4. Discussion

Depending on both DBP and extent of proteinuria, 14 cases had developed severe PE and 56 cases had mild PE. All cases developed PE, irrespective of its severity, showed significantly higher serum levels of RBP4 and lower PlGF compared to levels estimated in control group and in patients who had developed severe PE compared to those had mild PE. These findings pointed to an association between both RBP4 and PIGF serum levels and the development of PE. In support of such assumption, there was a significant correlation between serum levels of both parameters and estimated DBP and extent of proteinuria defined at time of inclusion in the study.

As regards serum RBP4, the obtained results go hand in hand with Inoue et al., (2009) who found that serum RBP4 levels were increased in pregnant women with PIH compared with normal pregnancies. However, Stepan et al., (2009) found that the mean maternal serum RBP4 concentrations were not significantly different in PE as compared with controls. This discrepancy could be attributed to small sized sample of PE women (n=16) studied by Stepan et al., (2009). In support our results, Vaisbuch et al., (2009) found that the maternal plasma RBP4 concentration was higher among patients with PE than in those with a normal pregnancy, and in patients with preterm PE (<37 weeks) than those with either term PE or normal pregnancy.

The obtained data suggested a participation of RBP4; an adipocytokine; in the pathogenesis and/or modulation of the PIH process and pointed to a role exerted by the adipose tissue as an endocrine organ. In support of such assumption, the current study reported a positive significant correlation between serum RBP4 and body weight and body mass index which coincided with that reported by Broch et al. (2009) who found that circulating RBP4 levels positively correlate with waist circumference, one of the obesity-related parameters.

The role played by RBP4 in pathogenesis and/or modulation of PIH could be attributed to concomitant insulin resistance that occurs during pregnancy and several previous studies documented such attribution between RBP4 and insulin resistance; Yang et al. (2005) demonstrated that adipose tissue-specific (Glut4)knockout mice have increased serum levels of RBP4 and downregulation of GLUT4 in adipose tissue is an important feature of insulin resistance. Clinical studies also reported that RBP4 is positively related to insulin resistance especially in obese (Graham et al., 2006) patients with impaired glucose tolerance (Cho et al., 2006) and type-2 diabetics (Cheng et al., 2009).

Insulin has important endothelial-dependent vasodilator actions mediated by nitric oxide (NO) via phosphatidylinositol 3-kinase (PI 3-kinase)–dependent activation of endothelial NO synthase (Zeng et al., 2000), but interestingly, insulin also has vasoconstrictor actions mediated by mitogen-activated protein kinase (MAPK)-dependent endothelial secretion of endothelin-1 (ET-1) (Cardillo et al., 1999). Insulin resistance is characterized by selective impairment in PI 3-kinase–dependent signaling pathways regulating metabolic actions of insulin in skeletal muscle with intact MAPK signaling pathways (Cusi et al., 2000) and is accompanied by compensatory hyperinsulinemia that serves to overcome impairment in PI 3-kinase signaling to maintain euglycemia. However, this hyperinsulinemia is predicted to overdrive unaffected MAPK signaling that may promote pathological actions of insulin, including increased secretion of ET-1, increased expression of vascular adhesion molecules, proliferation of vascular smooth muscle, increased expression of proinflammatory cytokines and activation of cation pumps (Potenza et al., 2005). These factors may shift the balance between vasodilator and vasoconstrictor actions of insulin and result in predisposition to hypertension in insulin-resistant states (Sowers, 2004).

As another explanation for association between RBP4 and PIH, Cabré et al., (2007) found that plasma RBP4 concentration might be a biomarker of nephropathy and cardiovascular disease in type 2 diabetic subjects and Frey et al., (2009) also demonstrated that there is a strong correlation between kidney function and RBP4 isoforms; thus serum RBP4 may reflect the degree of renal affection in these pre-eclamptic women and in support of this, the our study reported a positive significant correlation between serum RBP4 and extent of proteinuria.

Our results also showed that serum PlGF levels were significantly lower in PE group especially patients who had severe PE, a fact indicating abnormal placentation process as PlGF; an angiogentic factor; is physiologically essential for embryogenesis and development.

Such decrease of serum PlGF levels was manifested as increased frequency of small-for-gestational age (SGA) newborns with a negative significant correlation between serum PlGF and birth weight. These findings go hand in hand with multiple previous studies which reported significantly lower serum PlGF in association with low birth weight in pre-eclamptic patients in comparison to control group (Shibata et al., 2005, Espinoza et al., 2007, Romero et al., 2008).

Osol et al., (2008) experimentally reported that PlGF is a potent vasodilator of several vessel types in both humans and rats. Its potency and mechanism vary with physiological state and vessel location and are mediated solely by the vascular endothelial growth factor receptor-1 (VEGFR-1) subtype and gestational changes in the uterine circulation. The authors added that this might suggest that PIGF may play a role in modulating uterine vascular remodeling and blood flow during the pregnant state.

Erez et al., (2008) documented that changes in the maternal plasma concentrations of soluble form of endoglin (s-Eng), sVEGFR-1, PlGF or their ratios between the first and second trimesters of pregnancy confer an increased risk to deliver an SGA neonate and/or develop PE. Thus the effect imposed by decreased serum PlGF and development or maintenance of PE could be attributed to the loss of the vasodilator effect of PlGF. In support of this attribution, the current study reported a negative significant correlation between serum PlGF and both SBP and DBP.

In conclusion: both RBP4 and PlGF were strongly associated with the development and/or severity of PE. However, such association might need wider scale studies to be confirmed.

Acknowledgements:

Authors are grateful to Faculty of Medicine, Benha University for financial support to carry out this work.

Corresponding Author:
Dr. Adel Al-Kholy
Department of Medical Biochemistry
Faculty of Medicine, Benha University
Benha, Kaluobia, Egypt
E-mail: adeladel59@yahoo.com

References

1. Herrera E. Metabolic adaptations in pregnancy and their implications for the availability of substrates to the fetus. European Journal of Clinical Nutrition. 2000; 54 S47–S51.
2. Di Cianni G, Miccoli R, Volpe L et al. Intermediate metabolism in normal pregnancy and in gestational diabetes. Diabetes/Metabolism Research and Reviews. 2003; 19 259–70.
3. Dechend R, Luft FC. Are we getting closer to a Nobel Prize for unraveling pre-eclampsia? Curr Cardiol Rep. 2008; 10(6):440-7.
4. Soprano DR, Blaner WS. Plasma retinol-binding protein. In: The Retinoids, Biology, Chemistry and Medicine, edited by Sporn MB, Roberts AB, and Goodman DS. New York: Raven. 1994, p. 257–82.
5. Sapin V, Begue RJ, Dastugue B et al. Retinoids and mouse placentation. Trophoblast Res. 1998; 12: 57–76.
6. Vaisbuch E, Romero R, Mazaki-Tovi S et al. Retinol binding protein 4 - a novel association with early-onset preeclampsia. J Perinat Med.; Epub 2009 Sep 30.
7. Solini A, Santini E, Madec S et al. Retinol-binding protein-4 in women with untreated essential hypertension. Am J Hypertens. 2009; 22(9):1001-6.
8. Lacroix MC, Guibourdenche J, Fournier T et al. Stimulation of human trophoblast invasion by placental growth hormone. Endocrinology. 2005; 146:2434–44.
9. Chellakooty M, Vangsgaard K, Larsen T et al. A longitudinal study of intrauterine growth and the placental growth hormone (GH)-insulin-like growth factor I axis in maternal circulation: association between placental GH and fetal growth. J Clin Endocrinol Metab. 2004; 89:384–91.
10. Chellakooty M, Skibsted L, Skouby SO et al. Longitudinal study of serum placental GH in 455 normal pregnancies: correlation to gestational age, fetal gender, and weight. J Clin Endocrinol Metab. 2002; 87:2734–9.
11. Redman CW, Sargent IL. Latest advances in understanding preeclampsia. Science. 2005; 308:1592–4.
12. Maynard SE, Min JY, Merchan J, et al. Excess placental soluble fms-like tyrosine kinase I (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. J Clin Invest. 2003; 111:649–58.
13. Troisi R, Braekke K, Harsem NK et al. Blood pressure augmentation and maternal circulating concentrations of angiogenic factors at delivery in pre-eclamptic and

uncomplicated pregnancies. Am J Obstet Gynecol. 2008; 199(6):653.e1-10.
14. Gifford RW, August PA, Cunningham FG et al. Report of the national high blood pressure working group on research on hypertension in pregnancy. Am. J Obstet. Gynecol., 2000; 183:S1–22.
15. Vella M, Galloway DJ. Laparoscopic adjustable gastric banding for severe obesity. Obestet. Surg. 2003; 13(4): 642-8.
16. Lewis JG, Shand BI, Frampton CM, Elder PA. An ELISA for plasma retinol-binding protein using monoclonal and polyclonal antibodies: plasma variation in normal and insulin-resistant subjects. Clin Biochem. 2007; 40(11): 828-34.
17. Nemzek JA, Siddiqui J, Remick DG. Development and optimization of cytokine ELISAs using commercial antibody pairs. J Immunol. Methods, 2001; 255:149–57.
18. Inoue S, Takamoto N, Akahori Y et al. Elevated level of serum retinol-binding protein 4 in pregnancy-induced hypertension. J Obstet Gynaecol Res. 2009; 35(2):293-300.
19. Stepan H, Ebert T, Schrey S et al. Preliminary report: Serum levels of retinol-binding protein 4 in preeclampsia. Metabolism. 2009; 58(3):275-7.
20. Broch M, Auguet MT, Ramírez R et al. Parallel downregulation of retinol-binding protein-4 and adiponectin expression in subcutaneous adipose tissue of non-morbidly obese subjects. Eur J Endocrinol. 2009; 161(1):87-94.
21. Yang Q, Graham TE, Mody N et al. Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes. Nature. 2005; 436: 356-62.
22. Graham TE, Yang Q, Bluher M et al. Retinol-binding protein 4 and insulin resistance in lean, obese, and diabetic subjects. N Engl J Med. 2006; 354:2552–63.
23. Cho YM, Youn BS, Lee H et al. Plasma retinol-binding protein-4 concentrations are elevated in human subjects with impaired glucose tolerance and type-2 diabetes. Diabetes Care. 2006; 29:2457–61.
24. Cheng X, Zhang H. Serum retinal-binding protein 4 is positively related to insulin resistance in Chinese subjects with type 2 diabetes. Diabetes Res Clin Pract. 2009; 84(1): 58-60.
25. Zeng G, Nystrom FH, Ravichandran LV et al. Roles for insulin receptor, PI 3-kinase, and Akt in insulin signaling pathways related to production of nitric oxide in human vascular endothelial cells. Circulation. 2000; 101:1539 –45.
26. Cardillo C, Nambi SS, Kilcoyne CM et al. Insulin stimulates both endothelin and nitric oxide activity in the human forearm. Circulation. 1999; 100:820–5.
27. Cusi K, Maezono K, Osman A et al. Insulin resistance differentially affects the PI 3-kinase- and MAP kinase-mediated signaling in human muscle. J Clin Invest. 2000; 105:311–20.
28. Potenza MA, Marasciulo FL, Chieppa DM GS et al. Insulin resistance in spontaneously hypertensive rats is associated with endothelial dysfunction characterized by imbalance between NO and ET-1 production. Am J Physiol Heart Circ Physiol. 2005; 289:H813–H822.
29. Sowers JR. Insulin resistance and hypertension. Am J Physiol Heart Circ Physiol. 2004; 286: H1597–H1602.
30. Cabré A, Lázaro I, Girona J et al. Retinol-binding protein 4 as a plasma biomarker of renal dysfunction and cardiovascular disease in type 2 diabetes. J Intern Med., 2007; 262(4):496-503.
31. Frey SK, Henze A, Nagl B et al. Effect of renal replacement therapy on retinol-binding protein 4 isoforms. Clin Chim Acta. 2009; 401(1-2):46-50.
32. Shibata E, Rajakumar A, Powers RW et al. Soluble fms-like tyrosine kinase 1 is increased in preeclampsia but not in normotensive pregnancies with small-for-gestational-age neonates: relationship to circulating placental growth factor. J Clin Endocrinol Metab. 2005; 90(8):4895-903.
33. Espinoza J, Romero R, Nien JK et al. Identification of patients at risk for early onset and/or severe preeclampsia with the use of uterine artery Doppler velocimetry and placental growth factor. Am J Obstet Gynecol. 2007; 196(4):326.e1-13.
34. Romero R, Nien JK, Espinoza J et al. A longitudinal study of angiogenic (placental growth factor) and anti-angiogenic (soluble endoglin and soluble vascular endothelial growth factor receptor-1) factors in normal pregnancy and patients destined to develop preeclampsia and deliver a small for gestational age neonate. J Matern Fetal Neonatal Med. 2008; 21(1):9-23.
35. Osol G, Celia G, Gokina N et al. Placental growth factor is a potent vasodilator of rat

and human resistance arteries. Am J Physiol Heart Circ Physiol. 2008; 294(3):H1381-7.
36. Erez O, Romero R, Espinoza J et al. The change in concentrations of angiogenic and anti-angiogenic factors in maternal plasma between the first and second trimesters in risk assessment for the subsequent development of preeclampsia and small-for-gestational age. J Matern Fetal Neonatal Med. 2008; 21(5):279-87.

10/2/2010

Soil Plant Nutrients and Maize Performance as Influenced by Oilpalm Bunch Ash plus NPK Fertilizer

Ojeniyi, S. O.[1], Awanlemhen, B. E.[2] and Adejoro, S. A.[1*]

[1.]Department of Crop, Soil and Pest Management, Federal University of Technology, PMB 704 Akure, Nigeria.
[2.]Nigeria Institute for Oilpalm Research, Benin City, Nigeria.
solomonadejoro@yahoo.com

ABSTRACT: The work investigated the effects of combined application of oilpalm bunch ash (OPBA) with NPK fertilizer (NPK) on soil and plant nutrient content and maize performance at two sites in southern Nigeria. Six treatments: control, OPBA at 4 t/ha, NPK (15-15-15) at 300 kg/ha, 75% NPK + 25% OPBA, 50% NPK + 50% OPBA, 25% NPK + 75% OPBA were applied to maize at Nigeria Institute for Oilpalm Research (NIFOR) Benin and Ekiadolor in rainforest zone of Nigeria. Relative to control, other treatments increased soil organic matter (OM), N, P, K, Ca, Mg and pH, and plant nutrients content, growth and cob yield. The effects were generally significant except in case of OPBA alone. The NPK, 75% NPK + 25% OPBA and 50% NPK + 50% OPBA gave significantly high and similar values of the parameters. The treatments increased cob yield by 20 – 22%, OPBA most increased soil pH and K.
[Ojeniyi, S. O, Awanlemhen, B. E, Adejoro, S. A. Soil Plant Nutrients and Maize Performance as Influenced by Oilpalm Bunch Ash plus NPK Fertilizer. Journal of American Science 2010;6(12):456-460]. (ISSN: 1545-1003).

Key words: oilpalm bunch ash; nutrient; maize

INTRODUCTION

Maize is staple food for an estimated fifty percent of human population in Subsahara Africa (SSA) being a source of carbohydrates, protein, iron, vitamin B and minerals. However its production is hampered by inadequate soil fertility, high cost and scarcity of the recommended NPK fertilizer. Hence the area devoted to maize continues to reduce and maize farmers shifted to more resistant crops like sorghum and millet. In Nigeria output per hectare of maize grain stand at 1.31, which is 25 percent of world total output (RMRDC, 2004), use of N fertilizers and declining soil fertility are problems for maize production in SSA. Also the present high recommended rates of NPK fertilizer (NPK) are unaffordable and do not give corresponding high yields (Uyovbisere *et al*, 2000). Therefore, there is renewed interest in the use of organic wastes as source of nutrients. Those wastes that have been found useful include animal wastes, plant wastes, ash, and agro-industrial wastes. They were found to improve soil fertility, reduce soil acidity and improve crop yield. Types of ash derived from plants also produce similar effects on maize (Nottidge *et al*, 2005a, 2005b, 2006, 2007; Ayeni *et al*, 2008; Ayeni *et al*, 2009). The wastes can be used alone or fortified with inorganic fertilizers.

The oilpalm bunch ash (OPBA) has not received adequate research attention in maize production, although study by Ojeniyi *et al* (2006) found that OPBA supplied organic matter (OM) N, P, Ca and Mg to soil and maize and increased its yield by 26% when used at 4 t/ha. It was also found to increase nutrient supply to cassava and its yield significantly (Ezekiek *et al*, 2009a, 2009b,). The OPBA results from incineration of oilpalm bunch waste. The bunch waste is generated at about 850 kg/ha on yearly basis in oilpalm plantations in Nigeria. According to Omoti *et al* (1989) there is great potential of reducing fertilizers bills by recycling empty oilpalm bunch waste. Particularly combined application of OPBA and NPK fertilizer in maize production has not received research attention. In the tropics integrated application of organic and inorganic wastes is the sustainable method of maintaining soil fertility and productivity. Hence the objective of this work is to find suitable rates for combined application of OPBA and NPK fertilizer in maize production. The effect on soil and crop nutrient content, growth and yield of maize will also be determined in relation to sole use of fertilizer and OPBA

MATERIALS AND METHODS

Field Experiment

Two trials were conducted at Nigerian Institute for Oilpalm Research (NIFOR) Benin (06^o 33' N, 05^o 37' E) and Ekiadolor (06^o 34' N, 05^o 38' E) in rainforest zone of Southern Nigeria. The sites were manually cleared. Ridges were made at 1 m interval and maize seedlings planted at 40 cm interval on ridges were thinned to one plant per stand.

There were six treatments applied to maize. They were: (1) control, (2) 100% oilpalm bunch ash (OPBA) at 4 t/ha, (3) 100% NPK (15-15-15) fertilizer (NPK) at 300 kg/ha, (4) 75% NPK + 25% OPBA, (5)

50% NPK + 50% OPBA, and (6) 25% NPK + 75% OPBA. Treatments were replicated three times using a randomized complete block design, and were applied three weeks after planting in ring form. NPK and OPBA were mixed. There were 75 plants per each of 15 plots in each site. Weeding was done once.

Ten plants were selected per plot. Ear leaf length and width were determined 9 weeks after treatment, and leaf area was calculated by multiplying the product of the two parameters by 0.65 (Saxena and Suigh, 1965). Plant height and stem growth (at 10 cm height) were determined. At 80 days after treatment, harvest was done and cob weight was determined.

Leaf Analysis

Ear leaf samples collected per plot were oven-dried at 90 ^{o}C 24 hr, milled and ashed for 6 hr. at 500 ^{o}C. Nutrients were extracted using nitric perchloric acid mixture (Tel, 1984) and N was determined by microkjeldahl approach. The P in extract was determined using molybdenum blue colorimetry and read on spectrophotometer. The K was determined on flame photometer, and Ca and Mg by EDTA titration.

Soil Analysis

Composite soil samples collected after land clearing and soil samples collected at harvest from treatment plots were air-dried, ground and sieved using 2 mm sieve mesh. They were chemically analysed as described by Tel (1984). Organic matter was determined by wet oxidation method through chromic acid digestion. Nitrogen was determined by microkjeldahl approach; P was extracted by Bray-P1 solution and determined using the spectrophotometric method. Exchangeable K, Ca and Mg were extracted using ammonium acetate; K was determined using flame photometer, and Ca and Mg by EDTA titration method. Soil pH in ratio 1:2 water suspension was determined using a glass electrode.

Statistical analysis was performed using analysis of variance, and means separated using Fischer's least significance test at 5% level of probability.

RESULTS AND DISCUSSION

Data on soil properties at NIFOR and Ekiadolor sites are shown in Table 1. The soils are sandy, slightly acidic, low in organic matter (OM), total N, available P, exchangeable K and Ca.

Table 1: Soil properties at NIFOR and Ekiadolor

Properties	NIFOR	Ekiadolor
Sand %	92.3	93.8
Silt %	3.7	2.1
Clay %	4.0	4.1
pH (water)	5.3	5.5
Organic matter %	1.40	1.52
Total N %	0.04	0.07
Available P mg/kg	8.6	7.1
Exchangeable K c mol/kg	0.13	0.10
Exchangeable Ca c mol/kg	0.95	1.02
Exchangeable Mg c mol/kg	0.60	0.72

OPBA, NPK and their combined use at reduced levels increased soil OM, N, P, K, Ca and Mg at both sites (Tables 2 and 3). The increases were significant in case of NPK and its combined use with OPBA. However, OPBA gave highest values of soil pH and K, and had significant effect on these parameters. NPK gave highest values of soil N, P at NIFOR and P, Ca and Mg at Ekiadolor. The increases in soil nutrients due to NPK and OPBA can be adduced to increased soil OM which might be due to enhanced microbial activity. The OM is a natural source of nutrients and cation exchange. The OPBA due to its composition is also able to release macronutrients thereby increasing soil fertility and crop nutrient uptake. Analysis of OPBA as given by Ojeniyi *et al* (2006) was 1.60% OM, 0.19% N, 0.13% P, 29.8% K, 7.95% Ca and 3.2% Mg. Hence because of its high content of cations (K, Ca, Mg), OPBA was able to give highest soil pH and K. Therefore, OPBA had liming effect, and it is an effective source of K. In Ghana cocoa pod ash was used as source of K for maize (Adu-Dapach *et al*, 1994).

Table 2: Soil nutrients content as influenced by oilpalm bunch ash (OPBA) and NPK fertilizer (NPK) at NIFOR

Treatment	pH	OM %	N %	P mg/kg	K cmol/kg	Ca cmol/kg	Mg cmol/kg	H^+	ECEC cmol/kg
Control	5.5	1.45	0.05	9.6	0.14	1.06	0.44	0.17	1.85
OPBA 100% (4 t/ha)	6.5	1.61	0.07	22.6	0.44	3.01	0.93	0.10	4.42
NPK 100% (300 kg/ha)	5.9	2.89	0.18	38.5	0.22	7.68	1.52	0.17	10.58
75% NPK + 25% OPBA	6.4	3.80	0.17	35.3	0.24	7.07	1.65	0.13	9.39
50% NPK + 50% OPBA	6.0	2.68	0.14	32.4	0.32	5.56	1.44	0.13	7.55
25% NPK + 75% OPBA	6.2	2.38	0.12	28.4	0.38	3.73	1.57	0.13	6.23
LSD (0.05)	0.4	0.48	0.03	4.4	0.07	1.22	NS	NS	0.80

NS = Not significant

Table 3: Soil nutrient content as influenced by oilpalm bunch ash (OPBA) and NPK fertilizer at Ekiadolor
NS = Not significant

Treatment	pH	OM %	N %	P mg/kg	K cmol/kg	Ca cmol/kg	Mg cmol/kg	H^+	ECEC cmol/kg
Control	5.3	1.39	0.08	14.0	0.11	1.22	0.37	0.47	1.69
OPBA 100% (4 t/ha)	6.2	1.45	0.09	21.7	0.36	2.69	0.69	0.13	4.20
NPK 100% (300 kg/ha)	5.7	2.82	0.16	39.9	0.19	7.88	1.46	0.17	10.09
75% NPK + 25% OPBA	5.9	3.34	0.16	33.7	0.20	6.27	1.37	0.20	8.27
50% NPK + 50% OPBA	6.1	2.48	0.14	30.3	0.25	5.10	1.30	0.13	6.87
25% NPK + 75% OPBA	6.0	2.24	0.12	26.2	0.32	3.63	1.35	0.17	5.78
LSD (0.05)	0.40	0.38	0.03	5.8	0.04	1.30	NS	NS	1.56

Increases in plant nutrient contents given by OPBA were not significant (Tables 4 and 5). Infact tissue N was reduced relative to control by OPBA at Ekiadolor. Combined application of OPBA and NPK and NPK alone significantly increased tissue N, P, K, Ca and Mg (Tables 3 and 4). The treatments generally gave higher tissue N, P, K, Mg and Ca. Treatments 75% NPK + 25% OPBA, 50% NPK + 50% OPBA and NPK had similar values of plant N, P, K, Ca and Mg. Among the combined treatments, 25% NPK + 75% OPBA generally had least plant nutrients content. This might be due to its highest OPBA concentration. However, it gave higher nutrients content than the control and OPBA alone. It is suggested that addition of NPK to OPBA enhanced nutrients availability. This is attributable to enhanced mineralization of nutrients in OPBA.

Increased availability of nutrients in soil and maize crop led to enhanced growth and cob yield (Table 6). This affirms the importance of N, P, K, Ca and Mg to maize performance. Generally OPBA, NPK and their combinations at reduced levels significantly increased plant height, leaf area and cob weight. Treatments NPK, 75% NPK + 25% OPBA and 50% NPK + 50% OPBA with similar and highest values of soil and plant nutrients content also had highest and similar values of growth and yield parameters. OPBA which had least values of soil and plant nutrients compared with NPK and its combined applications with

Table 4: Maize nutrients content as influenced by oilpalm bunch ash (OPBA) and NPK fertilizer (NPK) at NIFOR (%)

Treatment	N	P	K	Ca	Mg
Control	0.84	0.23	0.71	0.77	0.07
OPBA 100% (4 t/ha)	0.92	0.26	0.75	0.78	0.09
NPK 100% (300 kg/ha)	1.24	0.30	0.79	1.02	0.15
75% NPK + 25% OPBA	1.31	0.30	0.78	1.14	0.13
50% NPK + 50% OPBA	1.41	0.28	0.75	0.99	0.12
25% NPK + 75% OPBA	1.38	0.28	0.73	0.83	0.12
LSD (0.05)	0.15	0.03	NS	0.18	0.03

NS = Not significant

Table 5: Maize nutrients content as influenced by oilpalm bunch ash (OPBA) and NPK fertilizer (NPK) at Ekiadolor (%)

Treatment	N	P	K	Ca	Mg
Control	0.81	0.24	0.69	0.73	0.10
OPBA 100% (4 t/ha)	0.56	0.26	0.73	0.78	0.09
NPK 100% (300 kg/ha)	1.25	0.34	0.89	1.05	0.13
75% NPK + 25% OPBA	1.26	0.29	0.69	1.11	0.12
50% NPK + 50% OPBA	1.34	0.26	0.72	0.90	0.10
25% NPK + 75% OPBA	1.31	0.22	0.70	0.84	0.11
LSD (0.05)	0.09	NS	0.08	0.17	0.02

NS = Not significant

OPBA had least values of growth and yield parameters which were not significantly different from that of control. Therefore, OPBA is less suitable for maize production compared with its combination with NPK fertilizer. Compared with control, OPBA, NPK, 75% NPK + 25% OPBA, 50% NPK + 50% OPBA, and 25% NPK + 75% OPBA increased cob weight by 11, 22, 20, 22 and 16% respectively. Therefore NPK, 75% NPK + 25% OPBA, and 50% NPK + 50% OPBA are equally suitable for maize production. Instead of the recommended 300 kg/ha NPK fertilizer, OPBA could be combined with NPK fertilizer as 215 kg NPK/ha + 1t/ha OPBA, or 150 kg NPK/ha + 2 t/ha OPBA for maize production without loss of yield, soil and plant nutrient content.

Table 6: Growth and yield of maize as influenced by oilpalm bunch ash (OPBA) and NPK fertilizer (NPK)

Treatment	Plant height (cm)		Leaf area (cm^2)		Cob weight (g)	
	Site 1	Site 2	Site 1	Site 2	Site 1	Site 2
Control	135.3	127.5	366	428	333	301
OPBA 100% (4 t/ha)	168.5	157.7	440	495	357	345
NPK 100% (300 kg/ha)	182.0	170.0	530	597	395	376
75% NPK + 25% OPBA	177.4	164.3	507	572	387	374
50% NPK + 50% OPBA	182.6	166.7	501	624	398	373
25% NPK + 75% OPBA	176.3	167.7	494	585	383	352
LSD (0.05)	4.1	5.1	25.8	41.9	35.8	18.9

Site 1 = NIFOR, Site 2 = Ekiadolor

The findings from this work align with the observation of Ezekiel et al, (2009a, 2009b) who noted that sole and amended forms of OPBA had beneficial effects on soil chemical properties. Ojeniyi *et al* (2006, 2009), Ezekiel *et al* (2009a, 2009b) also found that OPBA used alone increased nutrient availability, controlled soil acidity and increased yield of maize and cassava.

CONCLUSION

Oilpalm bunch ash (OPBA) is suitable for reducing soil acidity and supplying macronutrients

especially potassium. It could be combined with NPK fertilizer at 25% + 75% or 50% + 50% OPBA + NPK in order to reduce expenditure on NPK and reduce soil acidity without loss in soil fertility, nutrient availability and maize yield.

REFERENCES

1. Adu-Dapach, H.K., Cobbina, J. and Asare, E.O. Effect of cocoa pod ash on the growth of maize. J. Agric. Sci., 1994: 122: 31-33.
2. Ayeni, L. S.; Adetunji, M. T.; Ojeniyi, S. O.; Ewulo, B. S. and Adeyemo, A. J. Comparative and cumulative effect of cocoa pod husk ash and poultry manure on soil and maize nutrients contents and yield. American-Euroasian Journal of Sustainable Agriculture, 2008: 2(1), 92 – 97.
3. Ayeni, L. S.; Adetunji, M. T. and Ojeniyi, S. O. Comparative nutrients release from cocoa pod ash, poultry manure and NPK 20:10:10 fertilizer and their combinations – incubation study. Nigerian Journal of Soil Science, 2008: 18, 114 – 123.
4. Ayeni, L. S.; Adetunji, M. T. and Ojeniyi, S. O. Integrated application of NPK fertilizer, cocoa pod ash and poultry manure: effect on maize performance, plant and soil nutrient content. International Journal of Pure and Applied Science, 2009: 2(2), 34 – 41.
   and root yield of seeet and bitter cassava. Nigerian Journal of Soil Science, 2009a: 19: 1 – 5.
5. Ezekiel, P. O.; Ojeniyi, S. O.; Asawalam, D. O. and Awo, A. O. Root growth, dry root yield and NPK content of cassava as influenced by oilpalm bunch ash on ultisols of southeast Nigeria. Nigerian Journal of Soil Science, 2009b:19, 6 – 10.
6. Nottidge, D. O.; Ojeniyi, S. O. and Asawalam, D. O. Comparative effect of plant residue and NPK fertilizer on nutrient status and yield of maize in a humid ultisol. Nigerian Journal of Soil Science, 2005a: 15, 1 – 8.
7. Nottidge, D. O.; Ojeniyi, S. O. and Asawalam, D. O. Comparative effect of plant residues and NPK fertilizer on soil properties in a humid ultisol. Nigerian Journal of Soil Science, 2005b: 15, 9 – 13.
8. Nottidge, D. O.; Ojeniyi, S. O. and Asawalam, D. O. Effect of different levels of wood ash on soil chemical properties in an acid ultisol of southeast Nigeria. Nigerian Journal of Soil Science, 2006: 16, 109 – 114.
9. Nottidge, D. O.; Ojeniyi, S. O. and Asawalam, D. O. Effect of different levels of wood ash on nutrients content of maize and grain yield in an acid ultisol of southeast Nigeria. Nigerian Journal of Soil Science, 2007: 17, 98 – 103.
10. Ojeniyi, S. O.; Ezekiel, P. O.; Asawalam, D. O.; Ano, A. O.; Odedina, S. A. and Odedina, A. J. Root growth and NPK status of cassava as influenced by oilpalm bunch ash. African Journal of Biotechnology, 2009:8 (18), 4407-4412.
11. Ojeniyi, S. O.; Adesoye, S. O.; Awodun, M. A. and Odedina, S. A. Effect of oilpalm bunch ash refuse ash on soil and plant nutrient composition and yield of maize. Proceedings 30th Annual Conference of Soil Science Society of Nigeria. University of Agriculture, Makurdi, 2006: pp. 177 – 182.
12. Omoti, U.; Obatolu, C. R. and Fagbenro, J. A. Complementary use of organic and inorganic fertilizer for tree and forest crops. First National Organic Fertilizer Seminar, Kaduna. March 26 – 28. FMA/NFC, 1991.
13. RMRDC. Report on survey of agro raw material in Nigeria. Raw Materials Research and Development Council. pp. 171, October 2004.
14. Saxena, M. C. and Singh, Y. A note on leaf area estimation on intact maize leaves. Indian Journal of Agronomy, 1965: 10: 437 – 439.
15. Tel, D. A. Soil and plant analysis. Department of Land Resources Science, University of Gnelph, Ontario, Canada, 1984: pp. 277.
16. Uyovbisere, E. O.; Chude, V. O. and Bationo, A. Promising nutrients ratios in fertilizer formulatio: the need for a review of recommendations. Nigerian Journal of Soil Research, 2000: 1, 29 – 34.

26/10/2010

The Protective Effect of White Ginseng against Biochemical and Pathological Changes Induced by Aflatoxins in Rats

Abdel- Fattah, Sh. M.[*1]; Sanad, M.I[2].; Safaa,M.A[2] and Ragaa F. F.Ghanem
[1]Department of Food Toxins and Contaminants, Dokki, Cairo, Egypt.
[2]Department of Biochemistry, Faculty of Agriculture, Mansoura University, Mansoura, Egypt.
shaabanmostafa@yahoo.com[*]

Abstract: The objective of the present study was to explore modification in toxico-pathological responses of rats toward aflatoxins (AF) in the presence of white ginseng. The dietary supplementation with white ginseng (WG) at levels of of 0.0, 1 and 2 % (W/W) of the composition of daily rations, on the performance and toxicity of female Albino rats received aflatoxins-contaminated diets (1.011 mg/kg ration, of dry matter basis), were successively examined for six weeks, as attempt to prevent or minimize the negative probabilities due to ingesting aflatoxin(s) contaminated food. Thirty native apparently healthy female Albino rats with average weight of 100± 3.4 gm., were put under observation for two weeks, then they were divided into five equal groups of six rats each according to their live body weight for performing feeding trials. An exposure study extended for two different stages was conducted using female Albino rats. The 1st stage (pre-treatment) was suggested to compare the performance of animal groupings under the normal conditions before receiving any treatment, either level of contamination(s) or dosage(s) of additive, such stage extended for 2 weeks. The 2nd stage (treatment), the animals received different levels of aflatoxin(s) and the food additive (white ginseng), such stage extended for 4 weeks. Rats treated with AF-contaminated diet alone showed depression, decrease in feed intake, body weight and loose feces. The activities of serum ALT, AST enzymes, which are reflecting liver function, were obviously affected during exposure to aflatoxins, but such levels came back to normal as the level of the WG in the ration increased. Serum urea and creatinine concentrations had also severed and such severe effects came back to moderate when receiving the proposed additive. Livers exhibited fatty change, necrosis and newly formed bile ducts. Lesions in kidney included tubular necrosis and pink homogeneous tubular casts. Rats fed white ginseng only had no significant differences compared to the negative control group (fed on a sole diet without any additives). A concurrent treatment of AF with white ginseng indicated a potentiation of the animal performance reflected by decreased severity of clinical signs and inccreased body weight gains. The studied food additive minimized and reduced significantly the deterioratsion of such performance which obviously observed in animal grouping received AF-contaminated diet. Female rats were responding to contaminated diets and to the food additive as well. Thus, our data strongly suggested that deleterious effects of AF could be overcome or, at least, significantly were diminished by WG. Moreover, this plant by itself did not show any toxic effects.
[Abdel- Fattah, Sh. M.; Sanad, M.I; Safaa,M.A and Ragaa F. F.Ghanem. **The Protective Effect of White Ginseng against Biochemical and Pathological Changes Induced by Aflatoxins in Rats**. Journal of American Science 2010;6(12):461-472]. (ISSN: 1545-1003).

Keywords: Ginseng; Aflatoxins; Histopathological changes; Food additives.

1. Introduction:

Both human and animal health has been dramatically affected in outbreaks of acute mycotoxicosis, but these tragic events may be only a part of the cost to society in terms of impaired health and productivity from the ingestion of sub-clinical levels of aflatoxins (WHO, 1981; Saad, 1993 and Moos, 2002). Aflatoxins B1, B2, G1 and G2 were occurred on different foodstuffs when exposed to certain strains of Aspergillus flavus and Aspergillus parasiticus (Wogan, 1977 and Casado et al., 2001). AFB_1 is the most abundant and toxic form of all naturally occurring aflatoxins. AFB_1 represents 75% of all aflatoxins found in contaminated food and feeds. Many reports stated the harmful effects of aflatoxins on wide variety of animals and human; depression of growth and production (Saad, 1993), immunosuppression (yeong–Hsiang, 2001), liver disorders (Guerre, et al., 1996 and Anong and Suparats, 2006), abnormalities of enzyme picture (Patrick et al., 2002 and Tulayakul et al., 2005). However, such harmful effects seem to be difficult to overcome since aflatoxicosis gave no reaction response for any available treatment of drugs and / or antibiotics (Abdelhamid, 1990 and saad, 1993). The metabolisms of aflatoxins are due mainly to sex and specie differences besides the mitochondrial enzymatic reaction. AFB1 is first metabolized mainly by the cytochrome P-450 enzyme (CYP450) system found in the microsome. This metabolism will produce a variety of metabolites such as AFB_1-epoxide and hydroxylated metabolites (AFM1, AFP1, AFQ1, AFB 2α and aflatoxicol). (Tylayakul et al., 2005 and Anong 2006). The rate of metabolism, the repeativeness of exposure, the dosage and type of

mycotoxin(s) were reported as limiting factors affect the type of toxic action of mycotoxicosis (Saad, 1994; Jia-Sheng and John, 1999; Hussein and Jeffrey, 2001).

Natural substances that can prevent AFB1 toxicity would be helpful to human and animal health with minimal cost in foods and feed. Traditional medicinal plants were used by some authors for their antifungal, anti-aflatoxiGEnic and antioxidant activity (Joseph et al., 2005; Kumar et al., 2007).

Ginseng, a traditional medicinal herb in Asia, has become internationally popular in recent years. The traditional source of ginseng root has been Asian ginseng (Panax ginseng C.A. Meyer) but American ginseng (Panax quinquefolium L.), a plant native to North America, is now also cultivated and used in many countries. While the full pharmaceutical activity of ginseng is due to a ranGE of compounds, the triterpene saponins, known as ginsenosides, are widely considered to be the most important components contributing to the multiple medicinal properties of both Asian and American ginseng. The importance of the saponins in Asian ginseng has been known for about 100 years and, with the advent of modern chromatographic techniques, about 30 triterpene saponin glycosides, designated as neutral ginsenosides, have been identified (Sticher, 1998). The presence of neutral ginsenosides in American ginseng was first demonstrated by Ando et al., (1971) with later studies confirming that a range of neutral ginsenosides exists in this species similar to the previously studied Asian ginseng (Panax ginseng). The two major groups of ginsenosides are the Rb and Rg groups, which have 20 (S) protopanaxadiol and 20 (S) protopanaxatriol, respectively, as the sapogenines. Rb group includes the ginsenosides Rb1, Rb2, Rc and Rd, while Rg group includes the Re, Rf and Rg1 ones as the main compounds. Among all these ginsenosides, Rb1 and Rg1 are the most effective compounds (Tanaka and Kasai, 1984).

In previous works, we reported that Korean Panax ginseng has a protective role against many toxicants (Mannaa et al., 2006; Khalil et al., 2008; El-Kady et al., 2006). However, the components of ginseng that might bring about the decreased cancer risk remain unknown.

The aim of the present work is to investigate the effects of AF on lethality, on some haematological and biochemical parameter changes and on histological damages in the absence or presence of ginseng in order to appreciate the potential protective effects of this medicinal plant against AF- toxicities. The effects of this herb alone were also evaluated.

2. Materials and methods

2.1- Experimental design.

Depending on our previous results (Abdel-Fattah, 2002; Abdel-Fattah and Abdel-Salam, 2004 and Abu-Seif, *et al.,* 2009), concerning the antimicrobial effects of herbs and medicinal plants, this study was achieved. Sixty female Albino rats were used in this study. Animals were divided into six groups as shown in Table (1). All groups were exposed to the main two stages of the experiment period as follows, the first 2 weeks were the pre-treatment period, followed by 4 weeks of treatment. Both the 1st and 2nd groups were fed on sound rations, free from aflatoxins, but the 1st one had no additive to act as “negative control”, while the 2nd group fed on the same sound ration plus the studied additive at concentration equivalent to 0.2 % (w/w). The other three groups ingested aflatoxin(s) contaminated diets containing 225 mg B1, 30 mg B2, 70 mg G1 and 10 mg G2 /kg . The 3rd group exposed to contaminated diets only without any addition, but the 4th and 5th groups fed on aflatoxin(s) contaminated diets, plus the proposed additive (WG) at 1 and 2 % w/w. Feed intake and body weight gain were recorded daily. At the end of the treatment period all animals were fasted for 12 h, then blood samples were collected from the retroorbital venous plexus under diethyl ether anesthesia. Sera were separated using cooling centrifugation and stored at - 20 oC until analysis. After the collection of blood samples, animals were killed and samples of the liver and kidny of each animal were dissected.

Quantitative measurement of serum biochemical parameters included ALT, AST, urea and creatinine, were determined using a commercial kits.

2.2. Experimental animals

One-month old Female white Albino rats weighting 100–110 g (purchased from animal house of National Research Centre, Cairo, Egypt) were maintained on standard lab diet (protein: 160.4; fat: 36.3; fibre: 41 g/kg and metabolizable energy 12.08 MJ), and housed in a room free from any source of chemical contamination, artificially illuminated and thermally controlled, at the Animal House Lab., National Research Centre, Dokki, Cairo, Egypt. After an acclimatization period of 2 weeks, the animals were divided into five groups (6 rats/group) and housed in filter-top polycarbonate cages. All animals were received humane care in compliance with the guidelines of the Animal Care and Use Committee of the National Research Centre, Dokki, Cairo, Egypt.

2.3- Organisms. *Aspergillus parasiticus* (*A.parasiticus*) NRRL 2999 was obtained as lyophilized preparation from the Mycotoxin lab. National Research Center, Dokki, Giza, Egypt.

2.4- Plant material:

American white ginseng was purchased from an Egyptian local market (Harraz Co., Cairo, Egypt).

2.5- Aflatoxins standards and chemicals:

All standards of Aflatoxins (B1, B1, G1,G2, and B2 α) were purchased from sigma company, USA. All Chemicals and solvents used were of ACS grade. Thin layer TLC aluminum plates recoated with 0.25 mm silicagel 60 (Merk).

2.6- Preparation of aflatoxin(s)- artificially contaminated ingredient.

A balanced ration of growing rats with 14 % crude protein and 3100 kg calorie were purchased and artificially infected (in vitro) with a certain strain of *Aspergillus parasiticus* (NRRL-2999) which identified as an aflatoxin(s) producing strain. The inoculated substrates were incubated at 28 °c for 18 days (Shotwell et al., 1966). Qualitative and quantitative assay for the presence of aflatoxins in the contaminated substrate has been caried out using HPLC (Agilent Technologies, Waldbronn, Germany) as recommended by AOAC (1990). The AFs within the contaminated material consisted of 225 mg B1, 30 mg B2, 70 mg G1 and 10 mg G2 /kg. The contaminated material was incorporated into the basal diet in the ratio 15.55. % of the daily ration, to provide the desired level of 1.011 mg of total AFs or 0.7 mg AFB1/Kg diet.

2.7- Sampling

2.7.1- Blood samples:

Blood samples were taken weekly from the jugular vein prior to the morning feeding, at the following times: 0, 6, 13, 20 and 27 days of treatment period. Blood samples were placed on ice, allowed to clot and after centrifugation, serum was separated and frozen at -20 °C until it was analyzed for AFB 1, AFB2α and aflatoxicol.

2.7.2- Sampling of feces and urine

During the 3rd wk of the experiment, total feces and urine of animals were collected twice daily over a 7-days period. Urine was collected from an indwelling cyatic catheter, which was also placed 1-day before the toxin administration. Feces were collected in fecal bags. Following the collection period, total samples of urine and feces from each animal were homogenized, and aliquot samples were stored at -20°C until further analysis. Sampling and methods of analyses were adopted as recommended by (AOAC, 1990).

2.8- Histological study:

At the end the treatment stage, all animals were slaughtered, liver and kidney were dissected out, Organs were fixed in 10% neutral buffer formalin and processed for histopathological examination using routine paraffin embedding technique. Sections of 5 mm thickness stained with hematoxylin and eosin (H&E) were examined for morphological alterations and morphometric measurements. The slides were examined under 400 imagnification using an optical microscope (Carl Zeiss, Germany) as reported by Pearse (1979) and Sheehan and Harapcbak (1980).

2.9- Analytical methods:

2.9.1- Aflatoxins analysis:

Analysis of aflatoxins.

Aflatoxin(s) in feed and feces samples were extracted by B.F. method as described in AOAC (2000). The AFB1, its metabolites B2α and aflatoxicol were analyzed in urine, and serum samples according to method of Richarda and Lyona (1986).

Visualization and quantitation of aflatoxin B1, aflatoxicol and aflatoxin B2α.

AFB_1, aflatoxicol and AFB2α, were located by UV exposure. Extracts were dissolved in soul chloform and vortex, 20µl aliquot and 10µ of the standards were stopped on TLC plates and developed in dark room with chloroform : actone (90:10). After drying the spots were examined with U.V at a wave length of 365 nm. For the quantitative determination of the compounds, the silica plates were developed in solvent system (chloroform: actone, 90:10) and scanned in a Vitatron LTD 100 densitometer equipped with a mercury lamp (excitation at 366 nm and emission at 460 mm). The recorded areas of the spots were compared with standards of the respective compounds.

2.9.2- Serum biochemical parameters determination:

Blood was collected from the jugular vein of rats anesthetized with ether vapors on the days, 0, 14 and 28 of treatment period. Serum separated from clotted blood was stored at -20 ^{o}C for estimation of different biochemical parameters which included, total aflatoxins (AOAC method, 1990), ALT (Randox UK, catalogue no. AL 484), AST (Randox UK, Catalogue no. TR 1697) creatinine (Randox UK, catalogue no. CR 523), and urea (Randox UK, catalogue no. CH 280).

2.10- Statistical analysis

The data was subjected to the analysis of variance test. Different group means were compared by Duncan's multiple range test using a computer statistical package ($p \leq 0.05$) (Duncan, 1955).

3. Results and Discussion:

(1) Effects of ingesting aflatoxins and the WG additive on the performance of treated animals:

The selected doses of aflatoxin were literature based (Gelderblom et al., 2002) however; the selected

dose of WG was based on previous work (Abdel-Wahhab and Ahmed, 2004, Saad and Abdel-Fattah, 2008). The levels of aflatoxin B_1 selected in the present study were derived from the reported LD50 of both substances. Aflatoxin B_1 LD50 through oral route for rats has been reported as 5 mg/kg b. wt. (Betina, 1984). To produce a clinical aflatoxicosis, 1/10 LD50 (1 mg/kg b. wt.) was selected for daily doses.

In the current study, we evaluated the protective effects of WG against AFB_1 initiated and promoted hepatic carcinogenesis in rat model. The effect of different treatments on body weight gain of rats is depicted in Table (1). During the pre-treatment stage the average of body weight and body weight gain of both five groups show almostly similar values and ranges.

Rats fed with AF at level 1mg/kg diet in the present study exhibited a significant decrease in feed intake and body weights, the negative and positive control groups showed almostly the same values obtained from groups 4 and 5 which fed on high level of contamination simultaneously with the additive (WG) at concentrations of 1 and 2%.. Reduced weight gains have been observed in rats following an exposure of AF-contaminated diets (Saad and Abdel-Fattah, 2008).

Our results indicated that ingestion aflatoxin-contaminated diets resulted in a significant decrease in food intake and consequently the body weight gain was also reduced. Similar decrease in food consumption and body weight was reported in rats fed AFB_1-contaminated diet (Mayura et al., 1998, Abdel-Wahhab and Aly, 2003;, El-Nekeety et al., 2007, Saad and Abdel-Fattah, 2008). The reduced feed intake may indicate protein catabolism, thereby contributing to the observed kidney injury and causing impaired glomerular filtration (Tessari et al., 2006). On the other hand, the decrease in body weight in the animals treated with the aflatoxins alone may be due to the effects of these aflatoxins on the balance between orexigenic and anorexigenic circuits that regulate the homeostatic loop of body weight regulation, leading to cachexia (Rastog et al., 2001). In this regards, Abdel-Wahhab et al. (2006) reported that rats treated with AFB_1 showed a significant decrease in leptin. Low leptin concentration is usually associated with the high levels of cortisol and IL-6 which toGEther act to influence the feeding response, causing weight loss in patients with pancreatic cancer (Barber et al., 2004). This correlation may explain the recorded decrease in body weight inanimals inGEsted AFB1 and FB. Since leptin and its receptor are the key players in the regulation of energy balance and body weight control (Yuan et al., 2004; Abdel-Wahhab et al., 2006). Moreover, food intake was improved when the combined treatments of toxin plus WG was applied. Whereas, significant differences were still found between the control or WG alone groups and the other treatment groups.

Mnay authors stated that ingesting aflatoxin(s) contaminated food leads to negative effects on both animal and human performance (WHO, 1981, Park, 1983 and Saad, 1993; Abdel-Fattah, et al., 2006; Saad and Abdel-Fattah, 2008). It's worthy to mention that the group 2 which ingested the studied additive WG and sound diets showed non significant ($P<0.5$) effects compared with the negative control and the groups 4 and 5 which ingested the high level of contamination and the food additive at 1 and 2%. These findings are in agreement with those reported by Casado *et al.,* (2001) on rats and Biing-Hui *et al.,* (2002) working on swine.

Table 1. Means ± SE and comparison of body weight changes in all groups during experiment period (6 weeks).

Groups	Means ± SE					
	At zero time	After one week	After two weeks	After three weeks	After four weeks	After six weeks
Group 1	89.0±1.52^{Aa}	117.3±3.23^{Ab}	154.8±2.53^{Bc}	166.4±2.53^{Bc}	177.6±2.15^{Bc}	191.3±5.01^{Cd}
Group 2	84.0±1.81^{Aa}	106.2±3.68^{Ab}	136.4±3.05^{Ac}	145.64±2.87^{Ad}	159.1±3.23^{Ae}	165.6±2.67^{Be}
Group 3	84.0±1.80^{Aa}	99.5±3.30^{Ab}	132.6±3.4^{Ac}	145.5±4.28^{Ad}	154.2±3.30^{Ad}	151.6±3.58^{Ad}
Group 4	88.0±2.06^{Aa}	105.6±3.45^{Ab}	134.4±2.84^{Ac}	149.0±3.54^{Ad}	150.5±4.47^{Ad}	143.3±3.01^{Ad}
Group **5**	87.0±65^{Aa}	105.4±4.28^{Ab}	134.06±2.06^{Ac}	140.11±3.62^{Ac}	147.4±4.14^{Ad}	148.4±3.66^{Ad}
LSDp≤0.05	12.57					

N.B.: 1- The same capital litters in columns denotes no significant difference between treatments in the same period at ($p \leq 0.05$) and vise versa. But the differences in small litters in raws denotes significant difference between periods in the same treatment at ($p \leq 0.05$) and vise versa. Day (0): Beginning of the experiment. Day (42): End of the experiment

2- Some biochemical analysis of rat serum (liver and kidney function tests) as affected by dietary aflatoxin and / or WG treatment.

Data for selected serum constituents are presented, from the beginning and the end of the treatment period, in Table (2). There is no a consistent pattern shown in the metabolic indicators as influenced by the AF and / or the WG food additive additive. The average(s) of the transaminases (ALT and AST) level and both urea

and creatinine concentrations showed the normal picture during the pre-treatment stage with no differences between groups (Table 2). During the treatment stage, the groups 1 and 2 showed constant level of both ALT and AST enzymes activities, while serum activities of ALT and AST of animals fed dietary AF only (group 3) had elevated ($p \leq 0.05$) compared with those fed sound rations with or without WG additive. Similarly, urea and creatinine concentrations were higher ($p \leq 0.05$) at the end of the treatment period for group which fed AF-contaminated diet only. On the other hand, the level(s) of both ALT and AST activities were not significantly affected during treatment in the 4th and the 5th groups, leading to suggest the positive effect of studied additive on the liver function when the animals exposed to high level of aflatoxin(s) contamination.

Tracing the level(s) of transaminase ALT and AST in both animals of the 2nd group which received the additive only without any level of contamination, it could be easily noted that no changes were obtained during the two successive stages of the study. The obtained data were in accordance with those reported by Yeong-Hsiang *et al*. (2001), working on ducklings, Biing-Hui *et al*. (2002) on swine and Anong and Suparats (2006) on broilers.

High serum levels of AST and ALT are usually indicative of liver damage in animals (Lind et al. 1989) and humans (Gil et al. 1988; Hassal et al. 1990; Rati et al., 1991). It is of interest to mention that in animals fed diets contaminated with toxicants, the serum levels of these enzymes increased after liver damage because of increased membrane permeability or because of liver cell necrosis and cytosol leakage into the serum (Abdel-Wahhab, *et al.,* 2002 ; Saad and Abdel-Fattah, 2008 and Ozer *et al.,* 2008).

The intermediary metabolites produced during biotransformation of aflatoxins are held responsible for hepatotoxicity and the increase of serum activities of liver enzymes. They may cause cellular damage by covalent binding to cellular components such as enzymes, nucleic acids and proteins or by another mechanism. Damage of cellular components may play an important role in death of liver cells (Lind et al. 1989; and Teppema et al. 2002), hence, ALT and AST may be released to serum levels of these enzymes would increase.

Serum enzyme activities of AST and ALT are generally elevated in aflatoxicosis and are indicative for changes in the hepatic tissues and biliary system (Abdel-Wahhab et al., 2002), whereas increased levels of urea and creatinine may indicate protein catabolism and/or renal dysfunction (Abdel-Wahhab and Aly, 2003, 2005). Moreover, El-Nekeety et al. (2007) and Abdel-Wahhab et al. (2002) reported that rats fed FB-contaminated diet showed a significant increase in serum transaminases which indicated a necrosis in the liver tissue. These results clearly indicated that AFB_1 have stressful effects on the hepatic and renal tissues, consistent with those reported in the literature of mycotoxicosis (Sherif et al., 2009).

The results of our study (Table 2) are in agreement with the reports of another studies performed using antioxidants and hepatotoxic substances (). Alterations in different serum biochemical parameters observed in the present study are in agreement with the previous reports (Durak et al. 1996; Huang et al. 1996; Naziroglu 1999; Manna et al., 2004; Abdel-Wahhab and Aly, 2003, 2005). Adding of WG during aflatoxins treatment succeeded to improve ALT, AST, activities and a significant improvement was also found in urea and creatinine concentrations.

Several studies on the mechanisms of aflatoxins induced liver injury have demonstrated that glutathione plays an important role in the detoxification of the reactive and toxic metabolites of these aflatoxins, and the liver necrosis begins when the glutathione stores are almost exhausted (Dilkin et al., (2003); Abdel-Wahhab and Aly, 2003, 2005). In the same regards, Kim et al., (1997) reported that ginseng has a potent protective action against CCL4-induced toxicity and it showed inhibitory effect on cytochrome P450-associated monoxygenase activities. Therefore, it is suggested that the protective effect of WG is attributed to its free radical scavenging activity (Abdel-Wahhab and Ahmed, 2004; Mannaa et al., 2006). Generally, these results indicated that WG have protective effects against liver injury induced by aflatoxins and it plays a role in increasing the antioxidant status as well as lowering the oxidative damage of nucleic acids in the body (Abdel-Wahhab and Ahmed, 2004; Mannaa et al., 2006). Furthermore, Yun et al. (1987) reported that prolonged administration of Korean red ginseng (KRG) extract resulted in substantial suppression of pulmonary tumorigenesis induced by such chemical carcinogens as aflatoxin B_1. It was reported that the non-saponin components of red ginseng (RG) suppressed the harmful effects of free oxygen radicals (O2, H2O2, and OH2), which exercise an important role in tissue degeneration (Kim et al., 1997). Moreover, Zhang et al., (1996) showed that hydroxyl radical formed by the Fenton reaction were completely inhibited by ginseng extract. This antioxidant effect of ginseng may be responsible for its wide pharmacological actions in clinical practice by a free radical reaction-inhibition mechanism.

Therefore, the protective effects of WG may be related to the antioxidant properties consequently decreased risk for most cancers including carcinomas of the esophagus, stomach, colon, pancreas, lung and liver (Matsuda et al., 1986; Jeong et al., 1997; Dayan and Paine, 2001). Recently, Li et al., (2008) postulated that Ginsenoside Rg1, cinnamic acid, and tanshinone IIA isolated from ginseng could serve as protective agents in cancer prevention and treatment.

Treatment of the intoxicated rats with WG resulted in significant improvement in kidney function as indicated by the marked decrease in serum urea and creatinine levels. These results were in conformity with those reported by Yokozawa et al., (1994) who demonstrated that WG and its active component, saponin, could significantly reduce the blood urea nitrogen and creatinine levels in the blood of nephrectomized rats. Other studies asserted the nephroprotective effect of Korean ginseng saponin against cisplatin-nephrotoxicity (Liu and Zhou 2000; Abdel-Wahhab and Ahmed, 2004). These authors suggested that Korean ginseng saponin reduced cisplatin-induced cytosolic free [Ca2+] ions overload and formation of DNA interstrand cross-link and DNA–protein cross-link.

Furthermore, Yokozawa and Liu (2000) demonstrated that ginsenoside could decrease the severity of renal injury induced by cisplatin. These authors suggested that decreased level of urea in serum in rats given WG reflected the protective action of ginsenoside against the renal dysfunction.

Our results may lead us to suggest that there is a significant liver and kidny dysfunction in the AF-treated groups, 1.011mg of AF/kg diet was sufficient to impair performance and cause liver and kidny damage in female Albino rats, adding of WG during the mycotxins treatment (groups 4 and 5) resulted in a significant improvement in ALT and AST activity as well as urea and creatinine concentrations. It was suggested that WG displays a pronounced hepatoprotective effect, assessed through the transaminases (ALT, AST) activities following hepatotoxicity in rats treated with AF-contaminated diets.

Table 2. Means ± SE and comparison of enzymes levels and biochemical parameters in all groups during the AF and WG treatments for 4 weeks.

Items / Groups	Means ± SE							
	*AST (IU·L^{-1})		ALT (IU·L^{-1})		Urea (mg·dl−1)		Creatinine (mg·dl−1)	
	Day (0)	Day (28)	Day (0)	Day (28)	Day (0)	Day (28)	Day (0)	Day (28)
	20							
Group 1	20.7±0.73^{Aa}	22.8±1.12^{Aa}	13.3±0.58^{Aa}	14.3±0.80^{Aa}	42.06±2.43^{A}	48.8±2.33^{Aa}	37.7±2.45^{Aa}	39.8±3.37^{Aa}
Group 2	21.01±1.08^{Aa}	27.07±0.69^{Aa}	12.9±0.48^{Aa}	17.02±1.05^{Aa}	44.07±2.83^{A}	45.6±2.85^{Aa}	38.7±2.94^{Aa}	43.6±3.01^{Aa}
Group 3	20.0±0.68^{Aa}	85.4±2.98^{Cb}	13.3±0.42^{Aa}	49.01±1.90^{Cb}	44.2±1.649^{A}	96.4±5.43^{Cb}	40.2±3.16^{Aa}	109.9±5.19^{Cb}
Group 4	20.03±1.07^{Aa}	76.27±2.85^{Cb}	13.3±0.29^{Aa}	32.4±1.136^{Bb}	43.3±2.28^{A}	77.00±3.16^{Bb}	37.12±2.22^{Aa}	91.1±4.21^{Bb}
Group **5**	19.6±0.76^{Aa}	40.8±1.46^{Bb}	13.1±0.36^{Aa}	35.5±1.21^{Bb}	46.96±2.08^{A}	66.5±3.03^{Bb}	40.9±1.49^{Aa}	80.30±3.31^{Bb}
LSD p≤0.05	10.03		7.5		9.67		11.53	

N.B.: 1- The same capital litters in columns denotes no significant difference between treatments in the same period at ($p \leq 0.05$) and vise versa. But the differences in small litters in raws denotes significant difference between periods in the same treatment and the same parameter, at ($p \leq 0.05$) and vise versa.

2- [a] AST = aspartate aminotransferase; and ALT = alanine aminotrasferase..

Day (0): Beginning of the experiment Day (28): End of the experiment

(3) The histopathological changes in different studied groups:

The biochemical results reported in the current study were confirmed by the histopathological study of the liver and kidny.

Liver: The results obtained from studies with slices prepared from the livers of AF-treated rats indicate that there is some disturbance in protein synthesis throughout the course of the poisoning. One may speculate whether such an inhibition could play a role in the cellular necrosis. Rats of control groups (Groups 1, 2) did not show any histopathological alteration in liver tissue (Fig. 1). In groups fed AF-contaminated diets, hepatocytes were swollen and had cytoplasmic vacuoles at the end of experiment. In group 3, vacuolar degeneration of some hepatocytes and local areas of necrotic hepatocytes was found and hepatocytes had small roughly rounded multiple vacuoles in the cytoplasm. These changes became more intense and extensive accompanied by cellular swelling and disorganized hepatic cord pattern. A portal areas showed newly formed bile ducts and foci of cellular necrosis in the parenchyma. Aggregates of cells having round to oval vesicular nuclei and indistinct cytoplasm were

present in the parenchyma. Rats of groups 4 and 5, in which animals treated with WG at levels 1 and 2%, respictively, showed microscopic lesions slight to those of group 3. However, lesions in group 4 given the low level of WG were more severe than those of group fed AF and 1 % WG.

The present results revealed that WG had no harmful effects on liver tissues. However, the liver of the animals in the aflatoxins-treated groups showed severe histopathological changes typical to those reported in the literature. In this concern, Gelderblom et al. (2002), Abdel-Wahhab et al. (2002) and El-Nekeety et al. (2007) stated that treatment with FB_1 resulted in hepatotoxicity, apoptosis and inhibitory effect on cell proliferation, interferes with normal growth related processes and hence the disruption of normal liver homeostasis. Moreover, AFB_1 treatment induced a severe cytotoxicity and inhibition of hepatocytes cell proliferation (Neal and Cabral, 1980; Mayura et al., 1998; Abdel- Wahhab et al., 1998, 2002, 2007).

In the current study, the sequential treatment with AFB_1 resulted in cirrhotic livers with numerous regenerative and dysplastic nodules encircled extensively by ballooning and fatty degeneration cells. Therefore, the sequential treatment of AFB_1 extensively enhance the susceptibility of the liver to the toxicity and the induction of dysplastic nodules.

Kidneys: Kidneys of the groups given WG and AF together also showed changes not similar to those observed in groups given AF alone (Fig. 2). In group 3, there were severe and diffuse degenerative changes of the renal tubular cells, haemorrhage and congested blood vessels and pyknotic nuclei of epithelium of proximal and distal convoluted tubules were observed. Distal tubules had coagulated material in their lumens. Degeneration and necrosis of tubular epithelial cells those of collecting ducts were present. Histopathological lesions induced by AF in liver and kidneys of rats in the present study were similar to those reported by other workers (Hussain et al., 2009; Saad and Abdel-Fattah, 2008; Abdel-Wahab et al., 2007).

Our results suggested that a potentiation of the toxic response was evident by increased severity of clinical signs, increased activities of some liver enzymes, decreased body weights of rats administered AF. Alterations in serum biochemical and pathological parameters employed in the present study could detect some modification in toxic responses of rats administrated the two agents (AF plus WG) concurrently suggesting that these parameters were compromised by concurrent exposure to AF and WG. Since both the levels of AF in the present study produced toxic changes as evident by all the parameters used, it is possible that the modulation of the toxic response by AF might have been masked by the alterations induced by WG dose levels.

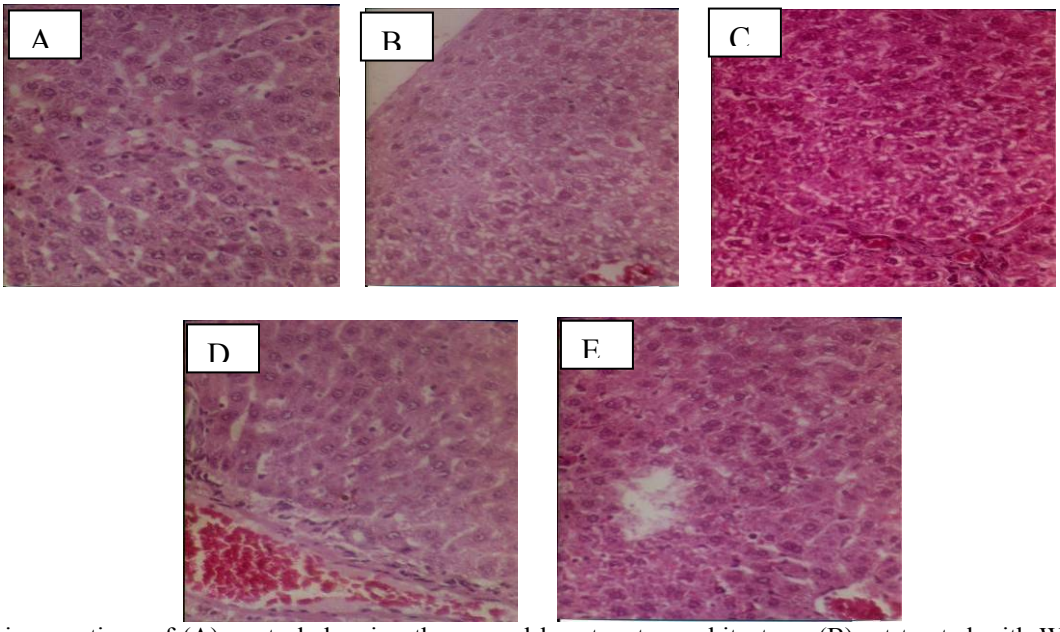


Fig. 1. Liver sections of (A) control showing the normal hepatocytes architecture, (B) rat treated with WG alone showing normal hepatocytes architecture, (C) rat liver treated with AF alone showing vacuolar degeneration of some hepatocytes and local areas of necrotic hepatocytes, (D) rat treated with WG at level of 1 % of daily ration (W/W) during AF treatment, showing congestion of blood vessels with degenerative changes of hepatocytes; (E) rat treated with WG at level of 2 % of daily ration (W/W) during AF treatment, showing slight vacuolar and other

degenerative changes of the hepatocytes with local necrotic area, marked decrease in damaged area and the hepatocytes restoring their normal structure (H&E) (X400).

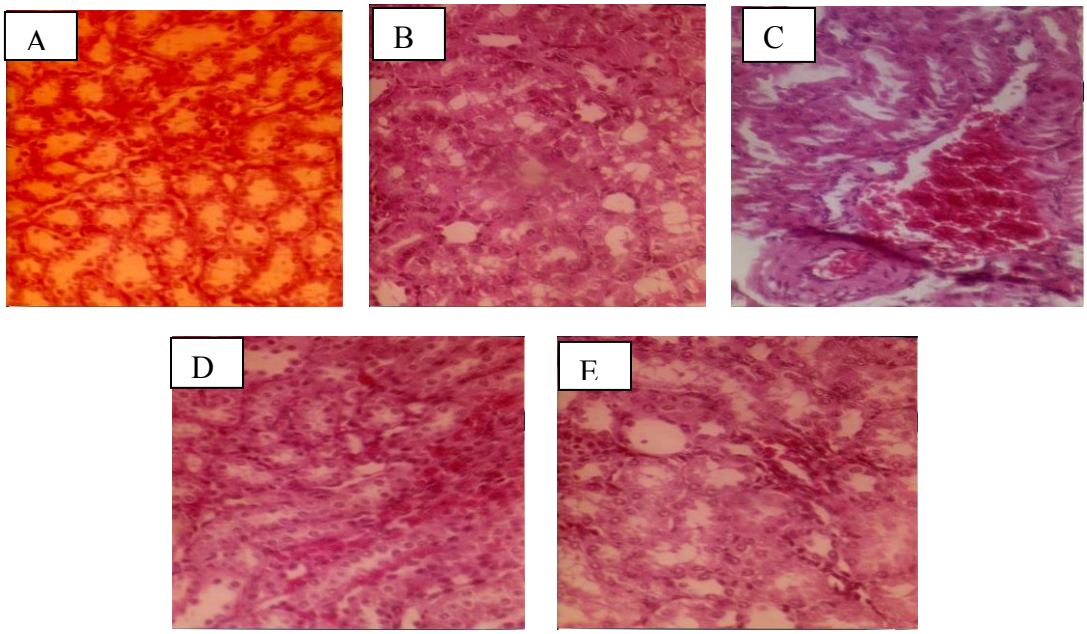


Fig. 2. Kidney sections of (A) control showing the normal renal tubules; (B) rat treated with WG alone showing normal normal renal tubules; (C) rat liver treated with AF alone illustrating severe and diffuse degenerative changes of the renal tubular cells, haemorrhage and congested blood vessels; (D) rat treated with WG at level of 1 % of daily ration (W/W) during AF treatment demonstrating severe and diffuse necrotic changes of the renal tubules; (E) rat treated with WG at level of 2 % of daily ration (W/W) during AF treatment demonstrating diffuse areas of necrosis of the renal tubules, vacuolar degeneration of some renal tubular cells (H&E) (X400).

4- Proportional urinary and fecal excretion of aflatoxib B_1, and its corresponding metabolites ($AFB_{2\alpha}$, aflatoxicol), for rats fed AFs-contaminated diets with or without WG.

Data in Table (3) represent the proportional excretions of AFB_1 and its metabolites ($AFB_{2\alpha}$ and aflatoxicol) via feces and urine. AFB_1 and $AFB_{2\alpha}$ concentrations in feces and urine varied according to dietary treatment. The cumulative excretion of $AFB_{2\alpha}$ is expressed in terms of AFB_1 equivalents. Results shown in Table (3) clearly indicate that treatment with WG in groups treated with dietary aflatoxin, had affected the route of AF excretion and metabolism where major the most of the excreted AFB_1 was found as the metabolite $AFB_{2\alpha}$ in the urine (41.13 to 59.35%), whereas approximately 5.11 to 8.53% was found as $AFB_{2\alpha}$ in feces. In contrast, fecal and urinary excretion of $AFB_{2\alpha}$ in the group fed dietary aflatoxin only was very low (6.17 and 8.19%, respectively). No aflatoxicol amount was detected neither in feces nor urine samples tested. These results indicate that the major excretory route was found to be the urine (accounting for 15.22% to 67.49% of the tatal AFs-excretion forms, whereas less than 10% of these forms were excreted in the feces. Treatment with WG improved the AF excretion via feces and urine in identifiable forms, mainly $AFB_{2\alpha}$ and unchanged AFB_1 without any detectable amount of aflatoxicol. In this respect, our results were in contrast with those observed by Richarda and Lyona (1986) in pigs.

Fecal excretion of $AFB_{2\alpha}$ reduced significantly by the WG-treated groups, this might be explained by a more pronounced renal elimination, which in turn might result in lower biliary secretion of $AFB_{2\alpha}$ in these groups. Our reslts were in the same trend with those observed by (Bennett *et al.,* 1981), who found that the lower toxicities of AFB_1 and $AFB_{2\alpha}$ in mammals are mainly as a result of a faster rate of clearance via urine and feces compared with that of AFB_1. Our results indicated that AFB_1 metabolites are cleared at a much faster rate than AFB_1. Hence, the rate of AFB_1 biotransformation represents the main mechanism through which detoxification occurs.

It's worthy to report that data obtained from the analysed samples of excreta (faces + urine) of the different studied groups showed no traces of aflatoxicol. This finding might be due to the

biotransformation of the ingested contaminants, aflatoxin B1, B2, G1 and G2 to other metabolites and /or the uncompetitiveness of Table (3).

Table (3) Proportional urinary (ng/mL) and fecal excretions (ng/gm) of aflatoxin B_1, and its corresponding metabolites ($AFB_{2\alpha}$, aflatoxicol), of female Albino rats fed aflatoxin (s)-contaminated diets with or without WG for 4 weeks.

Item		Groups										LSD (p≤0.05)
		Group 1		Group 2		Group 3		Group 4		Group 5		
		M+SD	%	M+SD	%	M+SD	%	M+SD	%	M+SD	%	
Feces	AFB1	0 ± 0^{A}	0.0	0 ± 0^{A}	0.0	165 ± 9.78^{B}	0.49	210 ± 13.4^{C}	.0.63	297 ± 18.7^{D}	0.89	31.4
	AFB2α	0 ± 0^{A}	0.0	0 ± 0^{A}	0.0	1005 ± 32057^{C}	6.17	1703 ± 64.36^{B}	5.11	2843 ± 54.3^{D}	8.53	46.5
	Aflatoxicol	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-
Urine	AFB1	0 ± 0^{A}	0.0	0 ± 0^{A}	0.0	9380 ± 84.5^{D}	28.14	3363 ± 113^{C}	10.09	2713 ± 56.5^{B}	8.14	85.2
	AFB2α	0 ± 0^{A}	0.0	0 ± 0^{A}	0.0	2730 ± 22.15^{B}	8.19	13710 ± 53.6^{C}	41.13	19783 ± 110^{D}	59.35	119.5
	Aflatoxicol	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-
Total recovery, %		0.0		0.0		42.99		56.96		76.91		-

N.B: 1- The same capital litters in columns denotes no significant difference between treatments in the same raw at ($p \leq 0.05$) and vice versa.

2- Excretion of $AFB_{2\alpha}$ expressed in terms of AFB_1 equivalents as calculated from the molecular weight of AFB_1 /molecular weight of $AFB_{2\alpha}$ (412/430) x mg of $AFB_{2\alpha}$.

In conclusion, we determined that aflatoxin could increase the liver enzyme levels and affect some hematological parameters. Increase in these parameters may occur due to peroxidation reactions, arising in aflatoxin biotransformation, and these reactions may inflict oxidative injury to cellular components. Administration of WG to rats received AFB_1-contaminated diet, resulted in a significant improvement in all biochemical parameters as well as improvement histopathological picture of the liver and kidny in different experimental groups.. In the light of these results, WG was found to induce the potent protective action in rats may play a role in the prevention of hepatic cellular injury produced by aflatoxins.

Acknowledgment

The authors would like to acknowledge Dr. Alaa El-Dein Helal; Animal Health Inst., Cairo, Egypt; for his sincere help and hard efforts to finish this work.

Corresponding author
Abdel- Fattah, Sh. M.
Department of Food Toxins and Contaminants, Dokki, Cairo, Egypt.
shaabanmostafa@yahoo.com*

4. References:

1. Abdel-Fattah, Sh.M. and Abdel-Salam, A. M. Anti-microbial activities of yoghurt containing licorice extract against mycotoxigenic fungi and mycotoxin formation, Bulletin of the National Nut.Inst. of the Arab Republic of Egypt, 2004, 25: 22-43.
2. Abdel-Fattah, Sh.M. The efficacy of liccorice extract on both mould growth and aflalatoxin production. J Agric., sci., mansoura univ., 2002, 27 : (12), 2855 – 2868.
3. Abdel-Fattah, Sh.M.; Abo Sree, Y.H.; El-Deeb. M.K.; El-Bealawy, A.M. and Helal,A.D. Toxicological studies on leucaena meal with two different dietary supplementation, on growth pwerformance and appreciable residues of its toxic constituents (Mimosine and 3,4- DHP) in milk of Egyptian goats. Journal of the Egyptian Vet. Med.Association, 2006, 66 (2): 83-99.
4. Abdelhamid, A. M**.** Occurrence of mycotoxins in various Egyptian feeds. J. Arch. Anim. Nut., Berlin, 1990,40(7):647-664.
5. Abdel-Wahhab, M.A., Abdel-Galil, M.M., Hassan, A.M., Hassan, N.H., Nada, S.A., Saeed, A., El-Sayed, M.M. Zizyphus spina-christi extract protects against aflatoxin B1-intitiated hepatic carcinogenicity. Afr. J. Trad., 2007, CAM 4 (3), 248–256.
6. Abdel-Wahhab, M.A., Ahmed, H.H. Protective effects of Korean Panax ginseng against chromium VI toxicity and free radical generation in rats. J. Ginseng Res., 2004, 28, 11–17.

7. Abdel-Wahhab, M.A., Ahmed, H.H., Hagazi, M.M. Prevention of aflatoxin B1-initiated hepatotoxicity in rat by marine algae extracts. J. Appl. Toxicol., 2006, 26 (3), 229–238.
8. Abdel-Wahhab, M.A., Aly, S.E. Antioxidants and radical scavenging properties of vegetable extracts in rats fed aflatoxin-contaminated diet. J. Agric. Food Chem., 2003, 51, 2409–2414.
9. Abdel-Wahhab, M.A., Aly, S.E.,. Antioxidant property of Nagilia sativa (Black cumin) and Syzygium aromaticum (Clove) in rats during aflatoxicosis. J. Appl. Toxicol., 2005, 25, 218–223.
10. Abdel-Wahhab, M.A., Nada, S.A., Farag, I.M., Abbas, N.F., Amra, H.A., Potential protective effect of HSCAS and bentonite against dietary aflatoxicosis in rat: with special reference to chromosomal aberrations. Nat. Toxins, 1998, 6, 211–218.
11. Abdel-Wahhab, M.A., Nada, S.A., Khalil, F.A. Physiological and toxicological responses in rats fed aflatoxin-contaminated diet with or without sorbent materials. Anim. Feed Sci. Technol., 2002, 10740, 1–11.
12. Abu-Seif, Feryala A.; Abdel-Fattah, Sh,M.; Abo Sreia, Y.H.; Shaaban, H.A.; Ramadan,M., Manal. Antifungal properties of some medicinal plants against undesirable and mycotoxin-producing fungi. J. Agric. Mansuora Univ., 2009, 34(3): 1745-1756.
13. Ando, T., Tanaka, O., & Shibata, S. Chemical studies on the oriental plant drugs. XXV. Comparative studies on the saponins and sapogenins of ginseng and related crude drugs. Svoyakugaku Zasshi, 1971, 25, 28–32.
14. Anong, B. and Suparat, K. Effect of dietary calcium propionate on performance, hepatic enzyme activities and aflatoxin residues in broilers fed a diet containing low level of aflatoxin B_1. J. Toxican, 2006, 47: 41-46.
15. Anong, B. and Suparat, K. Effect of dietary calcium propionate on performance, hepatic enzyme activities and aflatoxin residues in broilers fed a diet containing low level of aflatoxin B_1. J. Toxican, 2006, 47: 41-46.
16. AOAC Association of Official Analytical Chemistis, official methods of analysis. 13th Ed, 1990. Benjamin Franklin st., Washington Dc 20044,.
17. AOAC,. Official Method of Analysis. 2000, No. 990.33: Natural Toxins, 17th ed., vol. 2. Association of Official Analytical Chemist, Gaithersburg, MD, USA, pp. 20–22.
18. Bennett, Richard A.; Essigmann, J. M.; and Wogan, G. N. Excretion of an Aflatoxin-Guanine Adduct in the Urine of Aflatoxin B,-treated Rats. Cancer Research. 1981; 41: 650-654,.
19. Betina, VMycotoxins. In: Development in Food Science, vol. 8. Elsevier, Amsterdam, 1984. p. 111.
20. Biing-Hui, L., Feng-Yih, Y., Ming-Huan, C. and Yi-Ling, Y. The effects of mycotoxins, fumonsin B and aflatoxin B_1 on primary swine alveolar macrophages. J. of Toxical & Appl. Pharm., 2002, 180: 197-204.
21. Casado, J., Theumer, M., Masih, D., Chulze, S. and Rubinstein, H. Experimental subchronic mycotoxicosis in mice. J. Food and Chem. Toxicol., 2001, 39: 579-586.
22. Dayan, A.D., Paine, A.J. Mechanisms of chromium toxicity, carcinogenicity and allergenicity: review of the literature from 1985 to 2000. Hum. Exp. Toxicol., 2001, 20, 439–451.
23. Dilkin, P., Zorzete, P., Mallmann, C.A., Gomes, J.D.F., Utiyama, C.E., Oetting, L.L., Corrêa, B., Toxicological effects of chronic low doses of aflatoxin B1 and fumonisin B1-containing Fusarium moniliforme culture material in weaned piglets. Food Chem. Toxicol., 2003, 41 (10), 1345–1353.
24. Duncan, D.B. Multiple Ranges and Multiple F .test. Biometrics. 1955; 11: 10
25. El-Kady, A.A., Hosain, A.M., Park, M.H., Kim, Y.T., Park, H.H., Abdel-Wahhab, M.A., Efficacy of Panax ginseng extract standardized with ginsenosides Rg3 against oxidative stress of PCBs in rats. In: Proceeding of the Ninth International Symposium on Ginseng, Korea, 2006, 25–28. pp 599–615.
26. El-Nekeety, A.A., El-Kholy, W., Abbas, N.F., Ebaid, A., Amra, H.A., Abdel-Wahhab, M.A. Efficacy of royal jelly against the oxidative stress of fumonisin in rats. Toxicon, 2007, 50, 256–269.
27. Gelderblom, W.C.A., Marasas, W.F.O., Lebepe-Mazur, S., Swanevelder, S., Vessey, C.J., de la M Hall, P.,. Interaction of fumonisin B1 and aflatoxin B1 in a shorttermcarcinogenesis model in rat liver. Toxicology, 2002, 171, 161–173.
28. Gil F., Fiserova-Bergerova V., Altman N. H. Hepatic protection from chemical injury by isofluorane. Anesth. Analg., 1988, 67, 860-867

29. Guerre, P., Eckhoutte, C., Larrieu,G., Burgat, V. and Galtier, P. Dose-related effect of aflatoxin B_1 on liver drug metabolizing enzymes in rabbits. J. Toxicol., 1996, 108: 39-84.
30. Hassal E., Israel D. M., Gunesakaran T. M. Halothane hepatitis in children. J. Pediatr. Gastroenterol. Nutr., 1990, 11: 553—557
31. Huang Z. S., Wang C. H., Yip P. K., Yang C. Y., Lee T. K.: In hypercholesterolemia, lower peripheral monocyte count is unique among the major predictors of atherosclerosis. Arterioscler. Thromb. Vasc. Biol., 1996, 16, 256—261
32. Hussain S.; Zargham, K. M.; Khan, A; Javed, I. and Rafique A. M. Toxico-pathological effects in rats induced by concurrent exposure to aflatoxin and cypermethrin Toxicon, 2009,53 :33–41.
33. Hussein, S. and Jeffrey, M. Toxicity, metabolism, and impact of mycotoxins on humans and animals. J. Toxicol., 2001, 167: 101-134.
34. Jeong, T.C., Kim, H.J., Park, J.I., Ha, C.S., Kim, S.I., Rho, J.K.,. Protective effects of red ginseng saponins against carbon tetrachloride-induced hepatotoxicity in Albino rats. Planta Med., 1997, 63, 136–140.
35. Jia-Sheng, W. and John, D. DNA damages by mycotoxins. J. Mutation Res., 1999, 424: 167-181.
36. Joseph, G.S., Jayaprakasha, G.K., Selvi, A.T., Jena, B.S., Sakariah, K.K.,. Antiaflatoxigenic and antioxidant activities of Garcinia extracts. Int. J. Food Microbiol, 2005,101, 153–160.
37. Khalil, W.K.B., Hassan, A.M., Ahmed, K.A., Park, M.H., Kim, Y., Park, H.H., Abdel- Wahhab, M.A., Protective effects of Panax ginseng extract standardized with ginsenoside Rg3 against EDTA-induced toxicity in male rats. Arch. Toxicol., 2008, 82 (3), 183–195.
38. Kim, H.J., Chun, Y.J., Park, J.D., Kim, S.I., Roh, J.K., Jeong, T.C. Protection of rat liver microsomes against carbon tetrachloride-induced lipid peroxidation byred ginseng saponin through cytochrome P450 inhibition. Planta Med., 1997, 63, 415–418.
39. Kumar, R., Mishra, A.K., Dubey, N.K., Tripathi, Y.B. Evaluation of Chenopodium ambrosioides oil as a potential source of antifungal, antiaflatoxigenic and antioxidant activity. Int. J. Food Microbiol., 2007, 115, 159.
40. Li, Q., Shi, S., Liu, Q., Tang, J., Song, J., Liang, Y. Anticancer effects of ginsenoside Rg1, cinnamic acid, and tanshinone in osteosarcoma MG-63 cells: Nuclear matrix down regulation and cytoplasmic trafficking of nucleophosmin. Int. J. Biochem. Cell Biol., 2008, 40 (9), 1918–1929.
41. Lind R. C., Gandolfi A. J., Hall P. M. The role of oxidative biotransformation of halothane in the guinea pig model of halothane-associated hepatotoxicity. Anesthesiology, 1989, 70, 649—653
42. Liu, S.J., Zhou, S.W., 2000. Panax notoginseng saponins attenuated cisplatin-induced nephrotoxicity. Acta Pharmacol. Sin. 21 (3), 257–265.
43. Mannaa, F., Abdel-Wahhab, M.A., Ahmed, H.H., Park, M.H., Protective role of Panax ginseng extract standardized with ginsenoside Rg3 against acrylamideinduced neurotoxicity in rats. J. Appl. Toxicol., 2006, 26: 198–206.
44. Matsuda, H., Namba, K., Fukuda, S., Tani, T., Kubo, M., 1986. Pharmacological study on Panax ginseng C.A. Meyer. IV. Effects of red ginseng on experimental disseminated intravascular coagulation. (3). Effect of ginsenoside-Ro on the blood coagulative and fibrinolytic system. Chem. Pharm. Bull. (Tokyo) 34 (5), 2100–2104.
45. Mayura, K., Abdel-Wahhab, M.A., McKenzie, K.S., Sarr, A.B., Edwards, J.F., Naguib, K., Phillips, T.D., Prevention of maternal and developmental toxicity in rats via dietary inclusion of common aflatoxin sorbents: potential for hidden risks. Toxicol. Sci., 1998, 41:175–182.
46. Moos, O. M. (2002). Risk assessment for Aflatoxins in foodstuffs. J. Int. Biodet. &Biodeg.50:137-142.
47. Naziroglu M. (1999): Protective role of intraperitoneally administered vitamin E and selenium in rats anesthetized with enflurane. Biol. Trace Elem. Res. 69, 199—209
48. Neal, G.E., Cabral, J.R.P., 1980. Effect of partial hepatectomy on the response of rat liver to aflatoxin B1. Cancer Res. 40, 4739–4743.
49. Ozer, J.; Ratner, M.; Shaw, M.; Bailey, W. and Schomaker, S. The current state of serum biomarkers of hepatotoxicity. Toxicology. 2008; 245:194-205.
50. Park, D. (1983). Mycotoxins – an overview. Proc. of the Int. Conf. on Mycotoxins, Egypt.

51. Patrick, J., Terry, R., Van Vleet, J., Hall, R. and Colombe, Jr. (2002). Biochemical factors underlying the age related sensitivity of turkeys to aflatoxins B1. J. Comp. Biochem. and Phys. Part C , 132: 193-201.
52. Pearse, A. G. (1979). Histochemistry – Theoretical and applied, vol. 2, Publ. Churchil Livingstone, London.
53. Rastog, R., Srivastava, A.K., Rastogi, A.K., 2001. Biochemical changes induced in liver and serum of aflatoxin B1-treated male Wister rats: preventive effect of picroliv. Pharmacol. Toxicol. 88, 53–58.
54. Rati, E.R., Shantha, T., Ramesh,p, H.P., 1991. Effect of long term feeding and withdrawal of AF B1 and ochratoxin A on kidney cell transformation in Albino rats. Indian J. Exp. Biol. 29, 813–817.
55. Richarda, J. L. and Lyona R. L. Aflatoxins and Their Detection in Animal Tissues and Fluids Journal of Toxicology: Toxin Reviews. 1986; 5 (2): 197 – 215.
56. Saad, M. M. (1993). Preliminary study on formulation and application of aflatoxins(s) – antidote. J. Vet. Med., Giza, 41 (1): 33-37.
57. Saad, M. M. (1994). The effects of aflatoxins antidote on metabolism and reduction of aflatoxin B_1 by livers of six monogastric species. Bull. of Nut. Inst., 14(1):116-126 , Egypt.
58. Saad, M. M. M. and Abdel-Fattah, Sh.M. A food additive formula to minimize the negative effects due to ingesting aflatoxin(s) contaminated food. J. of Saudi Society for Food and Nutrition. 2008; 3 (1): 17-31.
59. Sheehan, D. and Harapcbak,B.(1980). Theory and practice of histotechnology. 2nd Ed. Mosbly Co., London, USA.
60. Sherif, S.O., Salama, E.E., Abdel-Wahhab, M.A., 2009. Mycotoxins and child health: the need for health risk assessment. (Review) Int. J. Hyg. Environ. Health 212, 347–368.
61. Shotwell, O.L., Hesseltine, C.W., Stubblefield, R.D., Sorenson, W.G. Production of aflatoxin on rice. Appl. Microbiol., 1966, 14, 425–428.
62. Sticher, O. (1998). Biochemical, pharmaceutical and medical perspectives of ginseng. In R. B. Larry & D. Lawson (Eds.), Phytomedicines of Europe, chemistry and biological activity (pp. 221–240). Washington, DC: American Chemical Society.
63. Tanaka, O., and Kasai, R., 1984. Saponins of ginseng and related plants. In: Herz, W., Grisebach, H., Kirby, G.W., Tamm, Ch. (Eds.), Progress in the Chemistry of Organic Natural Products, Vol. 46. Springer- Verlag, Berlin, pp. 1–76.
64. Teppema L. J., Nieuwenhuijs D., Sarton E., Romberg R., Olievier N. C., Ward D. S., Dahan A. Antioxidants prevent depression of the acute hypoxic ventilatory response by subanaesthetic halothane in men. J. Physiol. (London), 2002, 544: 931—938.
65. Tessari, E.N., Oliveira, C.A., Cardoso, A.L., Ledoux, D.R., Rottinghaus, G.E., Effects of aflatoxin B1 and fumonisin B1 on body weight, antibody titres and histology of broiler chicks. Br. Poult. Sci, 2006, 47 (3): 357–364.
66. Tulayakul, P. , Sakuda, S. , Dong, K. and Kumagai, S. (2005). Comparative activities of glutathione-s-transferase and dialdhyde reductase towards aflatoxin B_1 in livers of experimental and farm animals. J. Toxic. 46:204-209.
67. WHO (1981). Environmental health criteria for mycotoxins, World Health Organization vol.11.WHO, Geneva.
68. Wogan, G. N. (1977). Mode of action of aflatoxins. C.F. Mycotoxins in human and animal health. Publ. Pathotox. Inc. USA.
69. Yeong-Hsiang, C. , Tian-Fuh, S. , Victor, F. and Bao-Ji, C. (2001). Effects of aflatoxin and carotenoids on growth performance and immune response in ducklings. J. Comp. Biochem. and Phys. , part c, 128:19-26.
70. Yokozawa, T., Zhou, J.J., Hattori, M., Inaba, S., Okada, T., Oura, H., 1994. Effects of ginseng in nephrectomized rats. Biol. Pharm. Bull. 17 (11), 1485–1489.

10/5/2010

Akhond Khorasani's Viewpoints towards the Modern Concepts of Freedom and Justice

Alireza Soroush[1] , Sarvinder Kaur Sandhu[2] , Hamed Alaei[3]

[1] Department of Government and Civilization Studies, Faculty of Human Ecology, Universiti Putra Malaysia,43300 Serdang, Selangor Darul Ehsan, Malaysia, Email: soroush_alireza@yahoo.ca

[2] Department of Government and Civilization Studies, Faculty of Human Ecology, Universiti Putra Malaysia,43300 Serdang, Selangor Darul Ehsan, Malaysia, Email: sarvinder@putra.upm.edu.my

[3]Graduate School of Management, Universiti Putra Malaysia,43300 Serdang, Selangor Darul Ehsan, Malaysia, Email: hamedala@yahoo.com

Abstract: This article seeks to understand the mentality of Akhond Khorasani, the leader of the Iranian constitutional revolution (1905-1911) regarding the political and social concepts such as freedom and justice. In the Iranian society, concepts such as freedom and justice were always affected by various kinds of understanding and comprehension. These concepts were never interpreted based on their original and true meanings which are essentially the principles of democracy. In other words, the Iranian society was faced some problems and difficulties in absorbing these concepts and it seems even nowadays these concepts do not possess their true meaning in the political social culture of Iran and everybody explain them based on their own personal assumptions and subjectivity. It is for this reason that Iran has not had much of a practical experience from the existence of these concepts and achieving such and experience needs more time. Understanding the opinions of Akhond Khorasani (the revolution's leader) vis-à-vis these concepts can be an indication of the formation of democracy's pillar in Iran and also an indication of how the clergy faced these concepts, understand them and what practical ways they used to realize them. The theoretical framework of this article is based on the modernity theory. In essence, modernity comprises the theoretical aspects of the entire social, political, economical and cultural issues and guide human societies through the passageway of tradition to the modern world. The methodology used in this study is the unobtrusive research methodology, since this is a qualitative and historical research. The content analysis method which is one of the methods used in qualitative and historical researchers has also been implemented in this research.

[Alireza Soroush, Sarvinder Kaur Sandhu, Hamed Alaei. Akhond Khorasani's Viewpoints Towards Modern Concepts such as Freedom and Justice. Journal of American Science 2010;6(12):473-479]. (ISSN: 1545-1003).

Keywords: Iran; politics; modernity; freedom; justice; democracy

1. Introduction

One of the most prominent transitions in political history of Iran was the Constitutional Revolution *(Mashruteh)* which changed the political and social structures of Iran. Modern and traditional attitudes, social and political philosophy of thinkers and theologians (as subjective conditions), livelihood and economical conditions, social classes (as objective conditions) as well as a kind of exercising of authority by governors were the most important reasons which resulted in Constitution Revolution and the consequent social and political changes.

2. Freedom

Freedom is very general concept and has been defined in a number of ways. An absolute definition of freedom is impossible because of the relative character of the concept. The word freedom does not have any clear or definite meaning. The encyclopedia Britannica defines it *"As a state of freedom, especially opposed to political subjection, imprisonment or slavery"* (Jahanbakhsh, 1997)

Freedom has been regarded as a negative concept in the sense that its existence requires the absence of something that might be considered as restraint, limit or compulsion. The possession of freedom is indispensable for any individual. It gives a sense of identity and character to a person. Moreover, it is freedom that allows and individual to be present in a society and think of him\herself as part of it. A free human being is someone who is aware of all his\her rights and receives them accordingly and in order to be aware of those rights he\she is educated.

On the other side, the society does not spoil those rights and gives to every individual his\her entitled rights.

Furthermore a free human being is someone who has the right to choose and is able to, along with civil freedoms, possess a suitable carrier and in accordance to it, have sufficient income and live a satisfied life. He\she can receive education so that by which his\her practical and theoretical abilities are ameliorated. The individual can also complain if his\her rights are not respected and can be sure that complain will be paid attention to justly. In a free society individuals have the option to freely participate in among all others political, religious, cultural, and artistic activities or not participate all together. Moreover they can have any religion and any political tendency and not be accountable because of them. They are allowed to have freedom of speech and state their religious and political believes freely and not have any constraint.In order to further clarify the subject matter, it should be mentioned that human beings are not free to commit any actions they want and do not always have the right to do as they wish. If it was so, there would be little difference between humans and animals. (Zargarinejad, 2008)

Freedom should not be mistaken with being self will. Freedom only makes sense when the individual does not hurt him\herself or others and it is law that constraints freedom in such away. In other words the individuals are free when they can benefit from all of their abilities in order to reach their entitled rights in a society and at the same time not inflict any hurt or harm to their fellow human beings.. The limitations imposed on freedom can be different in any country. And thus every society shapes these limitations are the laws of a country. It is the law that specifies how freedoms are given and how individuals can benefit from them. Each individual must be educated with law in order to benefit from true freedom. It is law that defines how individuals should conduct themselves in a society. (Zargarinejad, 2008)

Laws are usually written by governments which intend to ratify them in a way as to limit freedom. Nonetheless people have the right to protest against rules and laws and to modify them this symbolizes the prevalent of freedom over law.

3. Freedom in Iran during the constitution revolution

The concept of freedom preoccupied the writings and concerns of many constitutionalists during this period. What was freedom and when and how could it restricted and contained, was perhaps the most debatable issue during the first phase of the constitutional movement (1906-1908) (Minuchehr, 1998).

In spite of all the debates and discussions, not all constitutionalists could provide a single, homogenous understanding of this term, which could mean that freedom, actually represented multiple meanings for every distinct group. The sudden opening up of public sphere, perhaps accounts for the plurality of visions and ideas during this period, which included the multiple understanding of the term freedom. As the public sphere emerged as a social, cultural and political sphere for the open expression of ideas, with the publication of various newspapers and formation of numerous associations, the limits of freedom became a main issue for the government and the individuals. Specially, the sudden unrestricted freedom to express anti governmental sentiments started to make the shah and his people quite uncomfortable, creating a "freedom crisis." In recognition of this unbridled stage of freedom, Fereydoun Adamiyat, the renowned qajar historian, named his book, "the First parliament and the crisis of Freedom." This point to the fact that the public sphere of 1906-1908 encountered a major crisis with regard to freedom. (Minuchehr, 1998)

It was in such an unrestricted atmosphere that intellectuals and constitutionalists began to define the meaning of freedom.

4. Justice

Justice is a beautiful concept which has always been sought by the humankind. The sense of seeking justice is one of the prominent and clear characteristic of any human being and many of the historical evolution and social changes were formed with the motivation of implementing justice. The account of justice is described by john Rawles as fairness. The principals of justice formed in order to characterize the moral foundation up on which a political government is constructed are defined. The hypothetical model of human nature theorized by Rawles. He supports his model by using his theory of justice. These principals state that humans are in need of freedom and liberty so that their interests are pursued provided that others are not harmed by them. Contentment is realized by the people when they are able to freely follow their interests in a society that is supportive. Rawles argues that "every citizen deserves the same opportunities to succeed as every other citizen", because of this argument Rawles is

considered to be a liberal political philosophy. (Choptiany, 1973)

Justice is a political concept it can be defined as the connection between politics and ethnics, i.e. it is true justice that politics become ethical. Both politics and ethnics are governed by the concept of justice which is itself a complex subject. Justice means the balance between rights and obligation. The concept of justice as a balance corresponds with the conception of justice being on rights, when we speak of rights, we speak of obligations. A person is considered to be just when that person is someone who forms a balance between rights and obligations through the fulfillment of both in a justified manner. (Soroush, 2007)

According to Plato, justice is defined as *"every citizen performing one social service in the state for which his nature is best adopted." (Soroush, 2007)*

The principals of religious faith in addition to law and politics seem to be the roots of social justice values. The aforementioned themes are all inter linked in various ways and in different culture they play out in different ways. By examining writings from the mean branches of Christianity, Judaism and Islam references to social justice and its values can be found, all stating that before God every person is equal to the other and that all the people must behave respectfully towards each other. (Mayer, 2007)

5. Justice at the time of the Iranian constitutional revolution

Up until the constitutional revolution in Iran, the concept of justice had no meaning. The country was administered and managed based on self – wellness and there did not exist a place where judges can judge between the people so that the oppressors and the oppressed are differentiated. According to Kasravi's opinion at that time, in Iran, there was no department of justice (Adliye) where judgment takes place justly between the able and disables the rich and the poor. (Kasravi, 2006)

However, Kasravi continues by saying that people at that time were not greatly in need of a justice the parliament, since they were less inclined to oppress or commit crime against each other and if there was a case it would had been resolved by the clergies or the elders but then again sometimes the people and their properties were violated by the countries and or those people close to power and it was then that the need for a courthouse that did not exist in Iran was felt. (Kasravi, 2006)

In the early days of the revolution when the leaders of the clergy took refuge in Shah Abdolazim (in Shahreray, a city in south of Tehran), as a sign of protest they proclaimed their request to the Shah. One of the most important of those requests was the establishment of a house of justice (such as the justice department today).

Shah (Mozaffaredin Shah) accepted all the demands of the clergies through a letter to his prime minister (Ainoddoleh) and wrote a separate letter regarding the establishment of a house of justice as the clergies important request and in that letter he emphasized on it. An abstract of that letter had presented below to his Excellency, the Prime Minister (Atabak Azam), as we said several times establishing a governmental house of justice for implementing the laws of the Shariah and the well – being of the people is more essential than any other important goal. We clearly determine that for the execution of Islamic laws in the whole of the nation, the house of justice must be soon established and there must not be any discrimination between different strata of the people, and to take sides any pay heed to somebody in appropriately is absolutely forbidden. (Kasravi, 2006)

The discussion about the existence of justice in the society and a democratic modern courthouse that can be accessible for complaining against the oppressor had started exactly before the writing of this letter by Mozaffaredin Shah. Kasravi writes that the government was forced to make a law to accommodate to request of the clergy regarding the establishment of a house of justice and this was the first step towards the presence of law in the country and thus constitutionalism. He continues by saying that the clergies return to the city (Tehran) with pride and honor and then they were not satisfied with only a house of justice and thus made their next request apparent which was the establishment of a parliament. (Kasravi, 2006)

What is certain is that a despotic way of thought is against law and parliament. With this kind of thinking, Ainoddoleh (prime minister) disregarded the demands of the clergy and even the Shah's letter and resisted against the idea of establishing the house of justice. Ayatollah Tabatabaei, one of Tehran's prominent clergies, made speech after the letter of the Shah was not paid attention to for four mounts. He said:

"there is a cure for every malady and the cure for self – wellness is council. Whether it takes a year or ten years we want justice and the place for

realizing it. We want a parliament where the Shah and the beggar are treated equally." (Kasravi, 2006)

6. Freedom in the view of Akhond

Reaching freedom is a constant struggle and an effort by social movements pursuing it as a holy objective. The concept of freedom does not mean unrestraint, libertinage and having a laissez – fair and unbound society. In its modern sense, freedom is limitable and it is the law of each country that indicates those limitations. In reality when social – political freedom receives its reason for existence from law and it is determined what situations and conditions encompass freedom, it is then that democracy is formed. Freedom is more inclined towards democracy rather than despotism, however it is the constitution law that determents the measure and degree of this inclination. As Habermas pointed out that all citizens must be free and equal with respect to constitutional law, it is these freedoms equality that guarantees a government's legitimacy.

Akhond Khorasani views freedom as being the opposite of despotism. He considers nation as free when the government respects the rights of the people and does not transform them in to obedient slaves by oppression and tyranny. This is how he describes freedom:

"the freedom of each nation which is based on constitution consists of the government not dominating by means of intractable and self – willed orders, in addition to a lack of obstacles in the way of realizing the legitimate right of the nation. In other words, the will of a nation does not lack any capability any in comparison to the will of the government. The reality of the freedom is the government and the nation not violating laws that are in accordance with religion. These are the laws that preserve the interests of Muslims and establish order in the society and are barriers against enemies of the nation." (kadivar, 2006)

The Akhond considers freedom as a divine blessing and a right of all humans, and further states that where there is no freedom, there is bondage. He indicates that the nation must make the effort of liberating itself from the self – willed decision of the government and not obeying oppressive officials. (kadivar, 2006)

The Akhond reminds the modern and lawful definition of freedom to associations and the media and tells them to respect each other and make their efforts towards the accomplishment of their scientific and practical matters, in addition to accepting all of the parliament's decisions and not interfering in any of them moreover, he states that newspapers are free to educate the people regarding the fundamentals of religiousness and civilization. Also he expects the newspapers to be active in growing the cultural civility and the morality of the nation. However, he reminds them that shirking this duty means violating the limits of the Sharia and the laws of the nation. (kadivar, 2006). Freedom is a concept not given to the people by dictator governments, thus for the people to experience freedom with such governments there must be struggle and conflict. Akhond Khorasani believes one of the constitutional government's objectives to be gaining the experience of freedom. He requested intellectuals residing to spend time make the necessary effort for the realization of freedom in Iran so that Iranians also could experience and understand freedom and progress in an announcement directed at the French people, he subtly reminds them of their struggle in attaining freedom and requests that they help Iran to realizing it as well.

"I request the noble French to remember the golden pages of their history when they fought for freedom and assist Iran in reaching freedom from lawlessness and tyranny." (kadivar, 2006)

Habermas fundamentally believes in freedom for a society, in such a way that he considers a society to be ideal when it encompasses freedom and justice. Such a society will be able to achieve social – moral amelioration for which modern science and knowledge are effective and modernity lays the groundwork for the establishment of such condition, in other words, it is through modernity that freedom is realized. Moreover, he believes that freedom, especially religious freedom, controls the level of confliction a society and prevents imbalance between the powerful and powerless groups, therefore, freedom is essential for a civil society.

Habermas is placed exactly on the same path where Akhond Khorasani is on for achieving freedom and as the Akhond believes individuals to be free to the extent defined by constitutional law prepares the conditions for such freedom to happen. The important issue that both are sensitive to is that the government gains legitimacy through freedom supported by law and conferring it to social group, the press and political parties. Otherwise a tyranny will take place. As Habermas considers all parties to be free, the Akhond too believes all social groups are free to elect parliament representatives as well. The Akhond does agree with freedom on the basis of law and the Islamic faith and considers it essential for the society. Moreover he believes it to be the cause of a

government's legitimacy and considers it the opposition to a despotic government in addition to being the necessary precedent to a constitutionalist government.

It is clear that freedom leading to anarchy and disorder and a situation where everybody does as he or she wishes in a society is not anybody's intention of freedom. Nonetheless those freedoms specified on the bases of a country's laws and according to be respected and possess a democratically form, where by the press can be published in such a way that they are allowed to criticize the establishment without any fear and moreover political parties can be active with the objective of creating awareness for the people and no belief, whether it be political or religious, is not imposed and finally can be their own decision makers, in their private lives individuals and followers of the law in their social lives.

Constitutionalism sought to establish freedom of thought, equality of individual's rights, and the governance of people on the people so that it leaves the destiny of the land and the nation to the hands in the people themselves and that the nation be placed in a situation where it is free to pass laws based on morality and the society's interests. (Malekzadeh, 1984)

7. Justice in Akhond's viewpoint

The ultimate goal of any society is attaining justice for the purpose of living a better life. The justice mentioned by Plato in his utopian society where every citizen serves it on the basis of his or her on specialty, and the justice defined by Habermas as the people's participation in the public sphere where its constitution leads to preservation of justice and freedom in the society, and finally the statement by Akhond Khorasani that says in order to diffuse justice both the government and the nation must act on the basis of laws that are according to Islam and they must not violate each other's rights, all three viewpoint consider justice as the ultimate goal and believes that all social – social political activities are only justify when they are in the path of reaching justice, otherwise the society will progress in the wrong direction caused by injustice.

In Akhond's eyes, justice is the objective of constitutionalism and he considers its reality based on which the constitutionalist movement was formed. The point he emphasize on is that we demand constitutionalism for the establishment of justice and progress and the renewal of the history of civilization. (kadivar, 2006)

In a letter to Ahmad Shah, he writes:

"struggle for the expansion of true justice and equality in such a way that the Shah himself is equal to the weakest individuals of the nation in terms of rights. Whenever the Shah is resolute and determined on this path and makes the necessary efforts for the implementation of this duty, undoubtedly the enemies will be in despair and the foundation of justice will strengthen." (Kafaei, 1980)

Justice was a true cause of Akhond Khorasani and equality of all people of his purpose, in such a way that in his view even the clergy are equal to the people and they all should benefit from equal rights. For instance some clergies demanded that he issues order an appointing five of Tehran's clergies to lifetime parliamental seats and that this becomes one of the constitutions laws. Hearing this, the Akhond became enraged and said:

"What a futile statement this is, the dignity and honor of this parliament is in equality. Nobody is to be preferred over somebody else otherwise this will lead to conflict and destruction." (Kafaei, 1980)

The Akhond furthermore considers the action of taking refuge to the Shah Abdolazim Shrine (a city in south of Tehran) by some of Tehrani's clergy, a movement on the path to justice and requests that the people accompany them who raised the banner of seeking justice for the purpose of its diffusion and the removal of oppression and took refuge in the cold winter. (kadivar, 2006)

Habermas believes that all the laws and decrease of the courts must be equal for all citizens; otherwise, equality will only be a verbal concept. In a similar manner, Akhond Khorasani writes to Ahmad Shah emphasizing that one must be determined in implementing justice and is realization must not be only the subject of conversations but must put to action. Akhond Khorasani held a modern an at the same time practical view of achieving justice in the society by means of the formation and existence of a parliament. In responding to a question posed regarding parliament, he said:

"During the two – year period since the formation of the parliament, not even one tenth of the oppression imposed on the people during the time of despotism took place, and if it did, take place it was on behalf of ill – wishers not the parliament." (kadivar, 2006)

He did not sanction offending any individual, be it Muslim or non-Muslim, so that in

this way the grate civilization of Iran is known to other countries. Likewise, similar to the Akhond, habermas considers all citizens to have equal share of citizenship rights from which they can benefit without the government pressuring the due to the political – religious standpoint. In conclusion governing essentially means taking actions in direction of justice. Both Akhond Khorasani and Habermas emphasize governing justly, which is to say governing without violating others' rights and the equality of all people in a society in front of the law can guaranty the legitimacy of a government; otherwise, it will not take long for it to collapse.

An economical justice that has a practical aspect to it and is not only rhetoric was one of the Akhond's plans for reforming Iran. He found training of the work force and acquiring knowledge in a variety of specialized technological and industrial fields to be crucial. A skilled and professional work force, in his opinion, can locally produce the needed goods and thus reduce the level of Iran's dependence on other countries and moreover is a propellant for eradicating poverty and expanding economical justice among the people. To emphasize his views, in a letter to Ahmad Shah, Khorasani brought up Japan and its emperor Mikado as examples, stating that *"the emperor of Japan knew that the key and the way to the nations progress is the people not needing foreign imported goods and it is due to this policy that Japan attained remarkable levels of progress and development. To whatever extent you choose such an admirable path and cause the nations progress, you will reach a society where poverty is a radiated and its people are self – sufficient. This principle causes the nations advancement and true independence and eventually the expansion of justice."* (kadivar, 2006)

Furthermore, he requests from the Shah for the ground work of learning and diffusing modern sciences and industries to be laid and he believe that the pinnacle of the countries glory depends of acquiring them and that Iranians have always had the necessary talent and potential in the fields of sciences and industry. He proclaimed that these abilities have been that manifested trough out history and that the poverty and backwardness of the Iranian society at the time are due to neglecting this very historical fact and not pursuing the learning of modern industries and sciences. Utilizing this two important factors (modern sciences and industry), he believes, results in the revitalization and rejuvenation of Iran. Not paying attention to these two important issues consequently results in higher dependence on foreign goods and with it more poverty and destitution becomes apparent among the people.

The emphasize of the Akhond on the accordance of ratified parliament laws on the Islamic laws manifests itself when he claimed that when the social laws passed by the parliament are not in contradiction with the Islamic Shariah laws, these two together give the Islamic nation the benefit of increased justice. Islamic is a form of rule that insist on justice and thus is a suitable framework for the parliament's representatives to notify social and political laws and regulations inside it. So, that all the citizenship rights for both Muslims and non Muslims of the society are respected and nobody endues operation and disrespect. Moreover, if the case arias where, and individual's rights are violated, in accordance to laws obtain from the Islamic Shariah and the parliament, that individual would be able to complain to a court of justice and redeem his or her rights.

In Akhond's view Islam is like a container from which social laws are taken by the parliament representatives. Although these laws do not represent absolute justice but they are so very near it. It is for this reason that politics gets situated next to religion and the conformity of these two has the benefits of revealing the function of religion while deepening the behavior of politics for the people and making it more acceptable by them.

8. Conclusion

Iran is a religious country and the effect of religion has penetrated all the country's social and political structures. Today, Iran has been named an Islamic republic meaning a totally religious form of government administered and managed by the clergy. The mentality of Akhond Khorasani is essentially the same mentality of those clergy who today believe in the governmental and political principals on the basis of Islamic principles and also similar to the Akhond believe that not only Islam does not contradict modern political concepts if placed next to each other, the ability of establishing a free and equal society based on constitutional law can be attained. However, today this stratum of the clergy is not able to directory be involved in the society's administration and the opposite way of thinking that is currently in power believes in the contradiction between Islam and modern concepts and the impossibility of the accordance of democracy and Islam. Thus in reality the lack of mutual understanding regarding freedom, justice, is still ongoing.

References

1. Choptiany, L. A Critique on John Rawls's Principles of justice. Ethics. 1973.

2. Jahanbakhsh, F. Islam, Democracy and Religious Modernism in Iran (1953-1997) from Bazargan to Soroush. McGill university Montreal. 1997.

3. Kadivar, m. Political Manifesto (Vol. 1). Kavir, Tehran, Iran. 2006.

4. Kafaei, A. M. The Death of the Light. Zavvar. Tehran, Iran. 1980.

5. Kasravi, A. History of Iran's Constitutionalim. Negah. Tehran, Iran. 2006.

6. Malekzadeh, M. The history of Iran's constitutional revolution (Vol. 2). Elmi. Tehran, Iran. 1984.

7. Mayer, S. E. Social Justice. Effective Communities, LLC. 2007.

8. Minuchehr, P. The Constitutional Movement Within a Global Perspective. Columbia University. 1998.

9. Soroush, A. The Beauty of Justice. CSD Bulletin. 2007;14.

10. Zargarinejad, G. Disquisition of Constitutionalism. Research Institute of Human Science. Tehran, Iran. 2008.

10/26/2010

Chemical Composition and Potential Application of *Spirulina platensis* Biomass

Aly, M. S[*1] and Amber. S., Gad[2]♀

[1]Agriculture microbiology Dept, [2]Chemistry of Natural and Microbial products Dept., NRC, Cairo, Egypt.
[*]mohamed_saad_1@hotmail.com

Abstract: Submerged batch cultures, Semi -pilot scale cultivations and Outdoor biomass production were performed to increase *Spirulina platensis* biomass which is naturally grown in El Khadra lake water body. Comparing *Chlorella vulgaris* and *Spirulina platensis* showed higher protein contents of *Spirulina* as it reached 64 % (w/w) so, it may be used in agriculture as a nitrogen biofertilizer and as an animal and fish growth promoter. Bio-chemical analysis of *Spirulina* biomass showed presence of 17 amino acids, 10% (w/w) carbohydrates, 8 %(w/w) fibers and 8 %(w/w) lipids. *The biomass of Spirulina* contained 0.04 ppm Mg, 0.3 ppm Ca, 0.16 ppm Mn, .0.8 ppm Fe, 0.16 ppm Zn, 11.3 ppm Na, 0.003 ppm Se and 5.6 ppm K. It also contained 1 ppm Cu, 0.04 ppm Hg, 0.03 ppm Ni, 0.9 ppm Cr, 0.1 ppm Cd, and 0.6 ppm Co.
[Aly, M. S and Amber. S., Gad. **Chemical Composition and Potential Application of *Spirulina platensis* Biomass**. Journal of American Science 2010;6(12):480-488]. (ISSN: 1545-1003).

Key words: *Spirulina platensi,,* El Khadra lake, biofertilizer..

1. Introduction:

The mixotrophic culture might be used as an alternative to conventional photoautotrophic mass culture systems for production of high value pharmaceuticals by *Spirulina platensis*. Chen (1996) satisfactorily cultured *S. platensis* in a mixotrophic mode by using a mix of different sources of energy and carbon ; glucose at high cell biomass densities 10.24 (g/ L) for the production of phycocyanin. Under mixotrophic conditions, some microalgae are known to grow rapidly and to have a higher growth rate than under photoautotrophic conditions, Samejima and Myers (1958). The algae usually not only contain nearly every required vitamin and mineral, but also have the effect of increasing oxygen while reducing nitrogen and carbon. Lee *et al.* (1996) cultured *Chlorella sorokiniana* mixotrophically in an outdoor enclosed tubular photobioreactor reaching an optimum biomass productivity of 10.2 (g /L/ d) during the day and 5.9(g /L/ d)during the night using an initial glucose concentration of 0.1 M. The daily volumetric productivity of photosynthetic *Chlorella* cultures in a similar tubular photobioreactor was about 3 times lower, Lee and Low (1992). These values are the highest reported for outdoor conditions, Ogbonna *et al*. (1997). Chen and Zhang (1997) stated that growing *S. platensi* under mixotrophic conditions reported a productivity 2.4-fold higher than of photoautotrophic cultures. Mixotrophic cultivation is an alternative to photoautotrophic production of biomass. The growth rates and biomass concentrations increase, apparently due to a synergistic effect of light and the organic substrate, Cid *et al.* (1992). Mixotrophic cultivation is a dual limiting process in which low light intensities or low organic carbon substrate concentrations may limit cell growth; while high light intensities or high carbon substrate concentrations may inhibit cell growth, Chen (1996). The cellular concentrations of the photosynthetic products depend on the relative heterotrophic and photoautotrophic growth rates. At high cell concentrations, light becomes limiting and the contribution of the photoautotrophic growth to the overall growth rate is very low. Under this condition, both the protein and the chlorophyll contents of the cells are much lower than of those of the autotrophic cultures, Ogbonna *et al.* (1997).Heterotrophic growth of microalgae could eliminate the requirements for light, and thus may offer the possibility of greatly increasing the algal cell concentration and productivity on a large-scale; however, only a few industrial heterotrophic processes have been attempted to date**,** Chen and Johns (1995). This is probably because limited number of available heterotrophic algal species; increased potential of contamination by bacteria; inhibition of growth by organic substrates at low concentrations; and the inability to produce some light-induced products, such as pigments ,Chen (1996). Photosynthetic microorganisms are able to synthesis organic C, N, and P which allow treating wastewater and simultaneously producing useful biomass when intensive algal culture is employed in outdoor ponds as tertiary for the removal of waste residual compounds, Kim**,** *et.al* (2000). *S.platensis* cultivation is usually preformed in open ponds so that the solar energy is used to fix inorganic carbon. Since heterotrophic metabolism is faster than autotrophic, simple carbon source is used to sustain growth. However, heterotrophic metabolism tends to suppress

the photosynthetic activity, therefore a mixed heterotrophic and autotrophic (mixotrophic) culture could be preferable, Lee *et al*. (1992 and 1996).

The high content protein indicates relatively good amino acid profile and low metal content enabled the use of algal biomass as feed supplement. The use of micro- algae in industry encourage the development of better cultivation system in order to optimize the production of algae rich in active substances such as vitamins protein, amino acids,fatty acids and trace elements . This study aimed to evaluate the amino acid composition of corkscrew-shaped filament *S. platensis* microalgae grown in El Khafra lake water. This seaweed is also characterized by fast growth, (dividing three times a day) , Furthermore, the *Spirulina* has advantages over other seaweeds by having the pleasant taste and thus it is used in the preparations of *Spirulina* capsules or in foods such as beverages and pastes. *Spirulina* causes no problems for digestion and no toxicity to humans, in contrary to occur to other seaweeds such as *Chlorella* and *Scenedesmus*. *Spirulina* is also known for its antioxidant and hypocholesterolemic actions**;**Parikh *et al*. (2001); Mao *et al*.(2005) **;** Colla,. *et al. (* 2008)and Muthuraman *et al.* (2009*).*

2. Materials and Methods:

Micro-organisms:

Spirulina platensis and *Chlorella vulgaris* isolates used in this study were obtained from El-Khadra lake at Wadi El Natroun, Egypt, characterized by extreme conditions of pH 10.5 and salt concentration of 0.55 M,,(Aly ,2000).

Maintenance stock media:

Zarrouk's synthetic solid medium (Zarrouk, 1966), amended with 2% (w/v) agar was used for maintaining *S.platensis* N-8 medium; Vonshak(1986)used to maintain the unicellular green algae *Chlorella vulgaris.*

Inoculums preparation:

Spirulina stock cultures were propagated in 50 ml Zarrouk's synthetic broth medium at 30±2 °C under continuous illumination with fluorescent lights for seven days in 500 mL Erlenmeyer flasks; Colla *et al.* (2004). The inocula concentrations were determined by spectrophotometer using a standard curve. a calibrated at 560 nm ;Volkman(2008). Inoculation conditions of *C.vulgaris* were performed according to Mandalam and Polsson and (1998).

Batch culture condition.

The *S. platensis* batch culture was incubated_at agitation of 160 rpm, temperature, 30ºC and luminosity 5000 lux in 250 mL Erlenmeyer flasks containing 50 ml of both basal Zarrouk medium, Zarrouk, (1966), and El Khadra lake water. Fermentations media were sterilized at 120°C for 20 min. The stock cultures were transferred to outdoors 130-L photobioreactor earlier 24 hours after inoculation. Cultivation conditions of *C. vulgaris* were performed according to Mandalam and Polsson and (1998).

Semi -pilot scale condition:

The bioreactor was inoculated by the previously prepared locally isolated *S.platensis* and *C. vulgaris* with initial biomass concentration of 0.16 g/l, These cyanophytes were used to inoculate Zarrouk's synthetic media ,Zarrouk, (1966) ; N-8 medium ,Vonshak (1986) and El Khadra water respectively. Cultures were incubated at 30°C with 5000 lux/cm^2 and pH 10.5 for 7 days . Zarrouk's medium contained (g/L):$NaHCO_3$, 16.8; K_2HPO_4, 0.5; $NaNO_3$, 2.5; K_2SO_4, 1.0; NaCl, 1.0; $MgSO_4 \cdot 7H2O$, 0.2; $CaCl_2$, 0.04; $FeSO4 \cdot 7H_2O$, 0.01; EDTA, 0.08; H_3BO_3, 2.86 mg/L; $MnCl_2 \cdot 4H_2O$, 1.81 mg/L; $ZnSO_4 \cdot 7H_2O$, 220 µg/L; $CuSO_4 \cdot 5H_2O$, 79 µg/L; MoO_3 , 15 µg/L; and Na_2MoO_4, 21 µg/L; Cases *et al*. (2001)). All the reagents used were of analytical grade, obtained from Merck Chemical Co. (Darmstadt, Germany). Aeration was conducted using air pump according to Costa *et al.*, (2002), and was incubated for 7 days at 30°C under illumination with fluorescent lamps (5000 lux) with 12h light /dark photoperiod according to Vonshak *et al.,* (1982).

It was supplied with flow rate of 0.03% CO_2. (100ml /min) in a 3L batch fermentor of 2.5L working volume with air. Cultivation conditions of *C.vulgaris* were performed according to Mandalam and Polsson and (1998) .After seven days , the biomass was filtered through a 20-µm membrane, thoroughly washed with distilled water, and stored after freeze drying.

Outdoor *Spirulina* biomass production*:*

2 L Erlenmeyer flasks, containing 1.8 L of Zarrouk's nutritive medium (Zarrouk, 1966) were used as starter inocula. *S. platensis* were grown in water outdoor mass culture 130 L photobioreactor, Colla *et al*. (2004).

Harvesting the biomass

At the end of seven days, the algal biomass was aseptically filtered and washed with distilled water to remove the salts from the algal surface then, lyophilized and stored at 4-5◦C °until need according to ,Colla *et al*.(2004) or dried at 50° C. The dried *S.*

platensis was powdered and stored in plastic container till used.

Preparation of *Spirulina* aquous extract (SAE):

The air dried *S.platensis* cells (5 g) was mixed under cooling conditions with sterile dist. water. The slurry was filtered through 50 mesh polyester rope and centrifuged at 5000 rpm for 10min. The clear supernatant was taken and made up to 100 ml then, sterilized through Millipore membrane 0.25µ.This sterilized *Spirulina* aqueous extract (SAE) represents the stock sources used for chemical analysis.

Analytical procedures.

pH of El Khadra lake was determined directly in the field with scan 2 pH meter .

Determination of the protein content of the cultivated material according to Lowry *et al.* (1951).

Determination of amino acids The amino acid profile was analyzed in algal protein precipitate after HCl hydrolysis using LC3000 amino acid analyzer with a flow rate , 0.2 ml/min , pressure for buffer, 0-50 bar, pressure for reagent ninhydrin 0-150 bar, column temp. 50°C.

Total carbohydrates were determined as glucose according to Dubois *et al*. (1956).

Fibers contents of *S. platensis* were determined according to the standard method of A.O.A.C. (1980).

Lipids were determined according to Folsh *et al.* (1975) method using extraction mixture of methanol – chloroform mixture (1:1,v/v) at 28ºC for 24 hrs in darkness followed by filtration. The extract was mixed thoroughly with half volume of 0.9% NaCl solution and the organic phase containing fatty acids was separated. The solvent was evaporated under nitrogen and the lipid content was gravimetrically estimated.

Heavy metals and minerals were analyzed by atomic absorption spectrophotometer varian A220, NRC, Cairo.

Spirulina semi-pilot scale production studies:

100 mg axenic culture prepared from powder of the local *S.platensis*; 15 mg PVP (polyvinylpyrrolidone) ; 10mg magnesium stearate and 300 mg avicel .For all formulations with (5-20,w/w) PVP the mixture was compressed using single punch tablet machine , hardness tester, disintegration apparatus and Roche Frabilator Erweka (GmbH. Frankfurt) in the machine setting were adjusted to produce *S.platensis* pellets having approximately the same hardness and weight .

3. Results and Discussion:

Protein content of *S. platensis* and *C.vulgaris* grown in El Khadra Lake and on basal synthetic media.

Fig(1) shows that the bluish-green seaweed *Spirulina* is characterized by its high protein content (64.0,and 58,0)% on dry weight basis when grown in Zarrouks, and El Khadra lake water ,respectively compared to *C. vulgaris* grown in N-8 medium Vonshak (1986)and El Khadra lake water .So, further studies will be focused on *Spirulina* .As a cyanobacterium, *S. platensis* does not contain a heterocyst necessary for nitrogen fixation. Thus, it must absorb nitrate from the media, and cultivation requires substantial inputs of soluble nitrogen. Exclusive use of sodium nitrate is essential for biomass production. *S. platensis* grown on basal medium because of its high solubile nitrate.

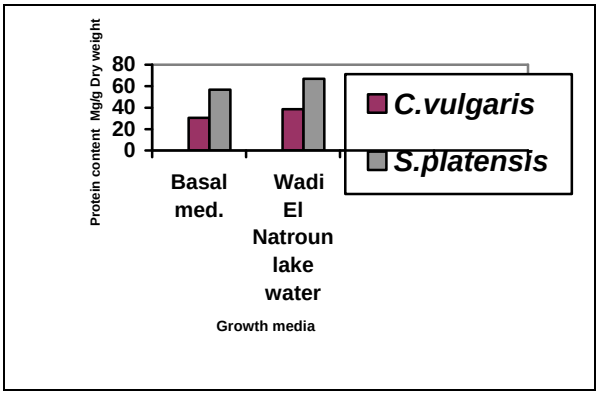


Fig.(1)Protein content of *Chlorella* and *Spirulina* grown in basal media and Waadi El Natroun Khadra lake body water.

The Biochemical analysis of *S.platensis*

Nitrate assimilation involves its uptake and sequential reduction by nitrate reductase (NR) and nitrite reductase (NiR) into ammonium ions, which are then incorporated into amino acids mainly by the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway. In higher plants, these enzymes are induced by nitrate and regulated by light, hormones, sugars and carbon and nitrogen metabolites, Raghuram *et al,* (2006). The genes involved in these reactions have been cloned from many plants and mutants and transgenic lines are also available,Lochab *et al.* (2007). *Spirulina* contains at least 10-fold higher protein than rice, indicating a correspondingly high capacity for nitrate utilization,(Goto *et al.* 1998,;Kronzucker *et al.* 2000;Ali *et al.*(2007). However, its biochemical basis is not known ,Vonshak (1997), except nitrate induction by NR and its inhibition by nitrite and

ammonium ions ,Jha *et al*., (2007), Characterization of NiR (Yabuki *et al*., 1985; Ali *et al.* 2008), and regulation of nitrate assimilation by calcium and phosphate were reported ,Singh and Singh (2000). Wong and Chan (1980) found that the protein content of sewage grown *Spirulina* was 45.6 %and fat was 14.7% of the total dry weight

Results obtained in table (1) revealed the *Spirulina* high crude protein content which amounted 64.00(% w/w) on Zarrouk medium. Crude fat was 8% (w/w); 10%(w/w) total carbohydrate content;8%(w/w) fiber .Wong and Chan(1990)revealed that *C.vulgaris* and *S.bijuga* have a fiber content of 5-8%(w/w)and 10%(w/w) minerals. The protein malnutrition is a public health problem that has affected a large proportion of the world population for many years, especially in the developing countries indicates tremendous importance of this alga in nutritional, industrial and environmental biotechnology, Vonshak (1997). However, it is not clear how the organism steers its nitrogen metabolism to produce so much protein. This alga is a nitrate-utilizing, non-nitrogen fixing and photosynthetic organism. The nitrate-utilizing ability of *Spirulina* has been exploited in the decontamination of nitrate-polluted waters and effluents (Kim *et a*l., 2000; Lodi *et al*., 2003).

Amino acid profile of *S. platensis*

Seventeen amino acids were detected in *S. platensis* grown on Zarrouks growth media as table (2) shows that *S. platensis* contains wide spectrum of amino acids . Glutamic acid was the most common amino acid of the dry matter of *S. platensis* followed by aspartic acid. Isoleucine was the most abundant essential amino acid as indicated in table (2).The phenyl aalanine is also present in comparatively high doses, therefore, people with phenylketonuria should avoid *Spirulina*.

The high protein and amino acid content of the algae grown on agriculture drainage water could be attributed to the availability of essential elements in quite high amounts as well as the tendency of algae for bioaccumulation and incorporation of these element into their macromolecules .El Adel *et al*. (2003) reported that salinity ,inorganic N and P of agriculture drainage water resulted in higher protein content in *Spirulina* especially due to the lack of organelles and intracellular transport constraints. The contents of proline and sulfur containing amino acid :methionine and cystine of the algae is low remarkably. The results reported that amino acids aspartic, serine, alanine, leucine, and glycine collectively amounted 50% of the detected amino acid content while methionine, cystine, tyrosine, and histidine collectively amounted less than 20 %.

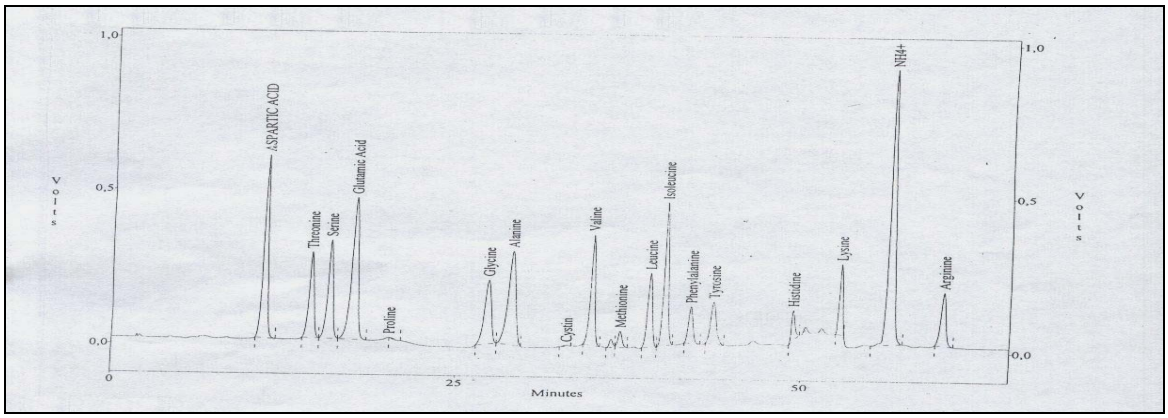
The amino acid profile of *S. platensis* biomass is a presented in Fig (2).

Table(1). The Biochemical analysis El Khada lake of *S. platensis* biomass grown in Zarrouks basal medium.

Component	Concentration (%w/w)
Crude protein	64.00
Fat (lipids)	8.00
Total carbohydrate	10.00
Fiber	8.00
Minerals	10.00

Table (2).Combined amino acids in the biomass of *S .platensis* grown on Zarrouk's basal medium .

Eessential Amino acid	(µg/mg) DW.	Non essential Amino acid	(ug/mg)DW.
TTheorinnine	16.068	Alanine	29.564
PPhenylalanine	11.537	Serine	16.109
A Arginine	23.359	Tyrosine	16.765
Histidine	7.147	Proline	8.767
Luecine	19.485	Glycine	
Isoluecine	37.325	Glutamic acid	52.159
Lysine	13.460	Aspartic acid	38.186
Valine	22.394	Cystine	1.1
Methionine	4.840		



Fig(2)-HPLC- chart amino acid profile of combined amino acids of. *S.platensis.*
(Amino acids in order from left to right)Aspartic-Theorinnine- Serine- Glutamic acid- Proline- Glycine- Alanine- Cystine Valine – Methionine-Luecine- Isoluecine- Phenylalanine- Tyrosine-Histidine- Lysine.

Minerals in *S. platensis*

Table (3) shows that, *Spirulina* might concentrate ions found in its environment. The natural El khadra lake waters *Spirulina* are so saturated with minerals originating from ancient soils and mountains while *Spirulina* favors to live in this aquatic environment no other plants can live there. Because *Spirulina* prefer to thrives in such alkaline waters, it incorporates and synthesizes many minerals and derivative compounds into their cells. The absorbed minerals are transformed into natural organic forms by *Spirulina.* Minerals become chelated with amino acids and are become more easily assimilated by the body .The biomass of *S. platensis* contained 0.04 ppm Mg, 0.3ppm Ca, 0.16 ppm Mn,0.8 ppm Fe,0.16 ppm Zn,11.3 ppm Na,0.003pppse,and 5.6pppK. Potassium is a crucial mineral that regulates body electrolyte balance, Beck (2000). Its deficiency can cause heart arrest, hypertension, adrenal exhaustion and muscular collapse. Calcium is especially important to bone and dental health and also in neural transmissions to the muscles; Aloia, *et al.(*1996). Zinc is the pivot point of over thirty vital enzymatic reactions, with profound effects on mental health, skin tone, prostate function and healing capacity**, Gonzalez**(2009). Magnesium deficiency can lead to spasmodic muscle disorders, including cardiac irregularities. This element helps in assimilation of C, B vitamins and proteins; Sawka and Montain(2000). Manganese at concentration of 0.16 (ppm) activates enzyme systems, along with zinc. It promotes the activity of neurotransmitter acetylcholine, and helps stabilize blood sugar ,Takeda(2003). Selenium was originally believed to be a toxic heavy metal, but now known to be necessary for health. It retards aging, harmful oxidation and free radical formation, reduces and improves cardiac efficiency. Selenium is an essential trace mineral that functions as an antioxidant and promotes a healthy immune system; Cases *et al.* (2001), and Tsavachidou *et al*. (2009). Iron is required in remarkably small amounts .It promotes formation of hemoglobin, the oxygen-carrying blood pigment found in healthy red blood cells. Iron deficiency is most common among women in their reproductive years, Steinberg (2001).

Table (3). The minerals content of *S. Platensis* .

Mineral	Conc. (ppm)	Mineral	Conc. (ppm)
Mg^{+2}	0.04	Zn^{+2}	0.16
Ca^{+2}	9.3	Na^{+}	11.3
Mn^{+2}	0.16	Se^{+4}	0.003
Fe^{+2}	0.8	K^{+}	5.9

Heavy metals *in S. platensis:*

The UN-FAO recognizes *Spirulina* as a potential weapon against malnutrition for the third world and has sponsored safety studies since the early 1980. Table (4) shows the heavy metals content of *Spirulina*. Phytoremediation potential of *S. platensis:* resemble *Chlorella* which known to bind to the heavy metals .Biosorption and toxicity probably most concern is *Spirulina's* ability to absorb and concentrate heavy metals such as lead and mercury if they are present in its environment The biomass of *S. platensis* contained 1.00 ppm Cu, 0.04 ppm Hg, 0.03 ppm Ni,0.90 ppm Cr,0.10 ppm Cd,and 0.6 ppm Co.. However, *Spirulina*-associated hepatotoxicity and reactions from heavy metal contamination are possible. *Spirulina* is considered nontoxic to humans at usual levels of consumption .This justify the use of alga biomass in nutrition if necessary; however, information is limited as insufficient clinical data to guide dosing of *Spirulina* for therapeutic effect.

Table (4).The Heavy metal content of *S. Platensis*

Heavy metal	Conc. (ppm)	Heavy metal	Conc. (ppm)	Heavy meta	Conc (ppm)
Cu^{+2}	1.00	Hg^{+2}	0.04	Ni^{+3}	0.03
Cr^{+2}	0.901	Cd^{+2}	0.10	Co^{+3}	0.60

Therapeutic *Spirulina* pellets:

Because of the high cost of extraction of polyunsaturated fatty acids and other constituents from *Spirulina* ,it seems that the best way is to use *Spirulina* by direct consumption as a nutritional supplement,Kapoor and Mehta (1993), *Spirulina* can be used either as a food supplement or taken in capsule form.Capsules appeare to be the preferred form at present, Colla *et al*. (2004).

Table (5) shows that 5 % (w/w) poly vinyl pyrrylidone (PVP) is the most suitable for capsules formation. *Spirulina* is sold as a feed additive for aquaculture and as a dietary supplement It has a long history of use as food and it is the nature's richest and most complete source of organic nutrition. The concentrated nutritional profile of *Spirulina* occurs naturally, so it is ideal for those preferring a whole food supplement to artificial nutrient sources. *Spirulina* has a unique blend of nutrients that no single source can provide. It has been labeled as a powerful food, rich in proteins, carbohydrates, polyunsaturated fatty acids, sterols and some more vital elements like calcium, iron, zinc, magnesium, manganese and selenium. It is a natural source of vitamin B12, vitamin E, ascorbic acid, tocopherols and whole spectrum of natural mixed carotene and xanthophylls phyto-pigments (Chamorro *et al*., 1996; Pinero Estrada *et al* .2001; Chamorro *et al.,* 2002, Islk *et al.,* 2006, and Al Attat 2010).*Spirulina* is also used as a feed ingredient for pigmentation of ornamental fish, especially gold fish and fancy red carp. *The spirulina* is reported as a potent anti-cancer (Ismail *et al.,* 2009), hypocholesterolemic and hypolipidemic (Colla *et al.,* 2008), antidiabetic (Muthuraman *et al.*, 2009) as well as for health improvement (Annapurna *et al.,* 1991; Cingi *et al.* 2008, and Al Attar, 2010).

Despite considerable progress in medical therapy, there is no satisfactory drug to treat kidney stones. So, capsules could be used for antilithiatic activity of *Spirulina* . In addition, the incidence of kidney stones has been increased in most societies in the last five decades, especially in association with economic development. In spite of tremendous advances in the field of medicine, there is no truly satisfactory drug for the treatment of nephrolithiasis. Recently, there is increasing evidence that many healthy natural food and medicinal herbal and supplements have the potential to become valuable complementary therapy in the treatment of various renal disorders and in the protection against iatrogenic nephrotoxicity. The administration of *Spirulina* solution to rats with ethylene glycol induced nephrolithiasis reduced and prevented the growth of kidney stones, renal and hepatic impairment can be concluded that the food supplementation with *Spirulina* has a beneficial effect on nephrolithiasis induced by ethylene glycol, Al-Attar(2010). The *Spirulina* components which are responsible for these therapeutic properties are thought to be compounds with antioxidant abilities such as polyunsaturated fatty acids, phycocyanin and phenolics ,Colla *et al*. (2004),and Islk *et al.(*2006).

Table (5).Physical characteristics of *S. platensis* pellets formulation.

PVP conc.(%)	Weight mg(±CV)	Thickness(±SD)	Friability(%)	Hardness(kg)	Disintegration time(min)
5	300±1.70	0.321±0.80	1.05	6.43	0.22
10	338±1.90	0.351±0.80	0.99	5.92	1.73
15	278±0.50	0.333±1.21	0.06	5.77	2.76
20	302±1.75	0.322±1.53	0.05	6.99	6.28

Corresponding author
Amber. S., Gad
Chemistry of Natural and Microbial products Dept. NRC, Cairo, Egypt.

5. References:

1. Ali, A.; Sivakami, S., and Raghuram, N. (2007a). Effect of nitrate, nitrite, ammonium, glutamate, glutamine and 2- oxoglutarate on the RNA levels and enzyme activities of nitrate reductase and nitrite reductase in rice. Physiol. Mol. Biol. Plants, 13 (1): 17-25.
2. Ali, A.; Sivakami, S., and Raghuram, N. (2007b). Regulation of activity and transcript levels of NR in rice (*Oryza sativa):* Roles of protein kinase and G-protein. Plant Sci. ,172: 406-413.
3. Ali,A.; Kuljeet, J.; Sandhu ,S. ,and Raghuram, N. (2008). *Spirulina* nitrate-assimilating enzymes (NR, NiR, GS) have higher specific activities and are more stable than those of rice.Physiol. Mol. Biol. Plants; 14(3) : 179-182.
4. Aloia, JF, Vaswani, A, Yeh, JK, *et al*. (1994); Calcium supplementation with and without hormone replacement therapy to prevent postmenopausal bone loss. Ann Intern Med 120:97.
5. Aly, M.S. (2000). Ecological studies on phytoplankton in closed lakes at Wadi El-Natrun, Egypt J. Phycol., 1:2000.
6. Annapurna, V.; Shah N.; Bhaskaran P.; Bamji, S.M., and V. Reddy, (1991). Bioavailability of *Spirulina* carotenes in preschool children. J. Clin. Biochem. Nutr., 10: 145-151.
7. A.O.A.C.(1980).Analysis of the association of official Agricultural chemists ,13th ed.Washington,D.C. USA.
8. Atef M. Al-Attar(2010).Antilithiatic Influence of *Spirulina* on Ethylene Glycol-Induced Nephrolithiasis in Male Rats. American J. of Biochem. and Biotechnol., 6 (1): 25-31.
9. Beck, L.H.(200). "The aging kidney. Defending a delicate balance of fluid and electrolytes." Geriatrics 55, 4 (26-28, 31-32.).
10. Cases, J;. Vacchina,, V. ;Napolitano, A.; Caporiccio ,B; Besançon P.; Lobinski R. ; and Rouanet, JM.(2001*).* Selenium from Selenium-Rich Spirulina Is Less Bioavailable than Selenium from Sodium Selenite and Selenomethionine in Selenium-Deficient Rats, J. of Nutrition. 131:2343-2350.
11. Chamorro,G.;Salazar,M.;AraijomK.G.;dis Santos, C.P. and Ceballos, G. et al (2002). Update on the pharmacology of *Spirulina (Anthrospira*) an unconventional food. Arch. Latinoam. Nutr., 52:232-240.
12. Chamorro,G.; Salazar, M.; Favila, L.,and Bourges, H.(1996); Pharmacology and toxicology of *Spirulina*. Rev.Invest Clin. 48:389-399.
13. Chen, F. (1996). High cell density culture of microalgae in heterotrophic growth. Trends Biotechnol. , 14: 421–426.
14. Chen F,and Johns ,MR. (1995) .A strategy for high density culture of heterotrophic microalgae with inhibitory substrates. J. Appl. Phycol.,7: 43–46.
15. Chen, F., and Zhang, Y. (1997). High cell density mixotrophic culture of *Spirulina platensis* on glucose for phycocyanin production using a fed-batch system. Enz. Microb. Technol. 20: 221–224...
16. Cid, A.; Abalde ,J.,and Concepcion, H. (1992). High yield mixotrophic cultures of the marine microalga *Tetraselmis suecica* Butcher. J. appl. Phycol., 4: 31–3.
17. Cingi, C., Conk-Dalay, M., Cakli, H., & Bal, C. (2008). "The effects of *Spirulina* on allergic rhinitis". Eur. Archives of Oto-Rhino-Larynol. 265 (10): 1219–1223.
18. Colla, L. M.; Bertolin T. E., and Costa ,J. A.V. (2004).Fatty Acids profile of *Spirulina platensis* grown under different temperatures and nitrogen concentrations Z. Naturforsch. 59c, 55D59.
19. *Colla, L.M., Muccillo-Baisch, A.M. , and Costa, J.A.V. (2008).* Spirulina platensis *effects on the levels of total cholesterol, HDL and triacylglycerols* in rabbits fed with a hypercholesterolemic diet. Braz Arch. Biol. Technol.51 (2) .405-411.
20. Costa J. A. V., Colla L. M., Filho P. D., Kabke K., and Weber A. (2002*),* Modelling of *Spirulina platensis* supplementations on the lipids from the cyanobacteria growth. Comparative effects of exogenous fatty acid in fresh water using response surface method-ology. World J. Microbiol. Biotechnol. 18, 603D607
21. Dubois B.; Gilles KA; Hamilton, JK,and Rebers, PA. (1965); Colorimetric method for determination of sugar and related substances. Anal Chem .,28:350-6.
22. El Adel, H.M.; Dawah,A.M., and Ibrahim ,N.A,(2003). Use of wastewater for growing microalgae of high content of combined amino acids N.Egypt.J. Microbiol..,5:198-205
23. Folsh,J.;Lees,M. ,and Sloane-Stanley, G.H. (1975). A simple method for isolation and purification of total lipids from animal tissue.J.Biol. Chem. 226-497.

24. Gonzalez A, Peters U, Lampe JW, and White, E.(2009). Zinc intake from supplements and diet and prostate cancer. Nutr Cancer.; 61(2):206-15.
25. Goto, S.; Akagawa, T.; Kojima, S.; Hayakawa, T. and Yamaya,T. (1998). Organization and structure of NADH dependent glutamate synthase gene from rice plants. Biochem. Biophys. Acta ,1387: 298- 308.
26. Işık, O. ; Hızarcı, L. ; Sayın, S. ; Gökpınar, Ş.; Durmaz, Y.,and Göksan,T. (2006).The Effect of the Environmental Factors on the Vitamin C (Ascorbic Acid), E (Alpha-tocopherol), β-carotene Contents and the Fatty Acid Composition of *Spirulina platensis*. . Cilt,23, sayi/issue (3-4):257-261.
27. Ismail, M.F., D.A. Ali, A. Fernando, M.E. Abdraboh and R.L. Gaur *et al.,* (2009). Chemoprevention of rat liver toxicity and carcinogenesis by *Spirulina.* Int. J. Biol. Sci., 5: 377-387.
28. Jha, P.; Ali, A., and Raghuram, N. (2007). Nitrate induction of nitrate reductase and its inhibition by nitrite and ammonium ions in *Spirulina plantensis.* Physiol. Mol. Biol. Plants, 13 (2): 163-16.
29. Kapoor R, and Mehta, U.(1993) . Utilization of beta-carotene from *Spirulina platensis* by rats. Plant Foods Hum Nutr: 43(1):1-7.
30. Kim, M.H., Chung, W.T., Lee, M.K., Lee, J.Y., Ohh, S.J.,
31. Lee, J.H., Park, D.H., Kim, D.J. and Lee, H.Y. (2000). Kinetics of removing nitrogenous and phosphorous compounds from swine waste by growth of microalga,*Spirulina platensis*. J. Microbiol. Biotechnol. ,10 (4): 455-461.
32. Kronzucker, H.J., Glass, A.D.M., Siddiqi, M.Y. and Kirk,
33. G.J.D. (2000). Comparative kinetic analysis of ammonium and nitrate acquisition by tropical lowland rice: implications for rice cultivation and yield potential. , New Phytol. 145: 471-476.
34. Lee, YK.,and Low, CS. (1992). Productivity of outdoor algal cultures in enclosed tubular photobioreactor. ,Biotechnol. Bioeng. , 40: 1119–1122.
35. Lee, YK. Ding, SY. ; Hoe, CH.,and Low ,CS. (1996) .Mixotrophic growth of *Chlorella sorokiniana* in outdoor enclosed photobioreactor. J. Appl. Phycol. ,8: 163–169.
36. Lochab, S.; Pathak, R.R., and Raghuram, N. (2007). Molecular approaches for enhancement of nitrogen use efficiency in plants. In: Agricultural Nitrogen use & its Environmental Implications (Eds. Abrol, Y.P., Raghuram, N. and Sachdev, M.S.) IK International, Delhi, pp. 327-350.
37. Lodi, A., Binaghi, L., Solisio, C., Converti, A. and Del Borghi, M. (2003). Nitrate and phosphate removal by *Spirulina platensis*. J. Ind. Microbiol. Biotechnol. 30: 656-660.
38. Lowry, OH. Rosbrough, NJ.; Farr, AL., and Randall, RJ. (1951).Protein measurement with the Folin-Phenol reagents. J. Biol. Chem. 193: 265.
39. Mao, TK.; Water, JV.; Gershwin, ME. *et al* (2005). Effects of a *Spirulina*-based dietary supplement on cytokine production from allergic rhinitis patients. J Med Food. 8:27-30.
40. Mandalam,R.K. and Palsson,B.O.(1989).Elemental balancing of biomass and medium composition enhances growth capability in high density C.vulgaris .Biotech.Bioeng.59(5):605-611.
41. Muthuraman, P.; Senthilkumar, R. and. Srikumar ,K.(2009). Alterations in beta-islets of Langerhans in alloxan-induced diabetic rats by marine *Spirulina platensis*.,J. Enz. Inhib. Med. Chem., 24: 1253-1256.
42. Ogbonna,JC.;Masui, H., and Tanaka, H. (1997). Sequential heterotrophic/autotrophic cultivation – An efficient method of producing *Chlorella* biomass for health food and animal feed. J. appl.Phycol. ,9: 359–366.
43. Parikh, P.; Mani ,U.,and Iyer, U. (2001). Role of *Spirulina* in the control of glycemia and lipidemia in type 2 *Diabetes mellitus*., J. Med. Food, 4: 193-199.
44. Pinero-estrasa,JE.; Bermejo Bescos,P. ,and Villar Del FrencoA.M. (2002).Antioxidant acyiivity of different fractions of Spirulina *platensis* protean extract.Farmaco,56:497-500.
45. Raghuram, N.; Pathak, R.R., and Sharma, P. (2006). Signalling and the molecular aspects of N- Use-Efficiency in higher plants. In: Biotechnological approaches to improve nitrogen use efficiency in plants (Eds. Singh, R. P. and
46. Samejima, H., and Myers,J. (1958) .On the heterotrophic growth of *Chlorella pyrenoidosa*. J. gen. Microbiol.,18: 107–117.
47. Sawka, M. N., and Montain., S. J. (2000).Fluid and electrolyte supplementation for exercise heat stress." American Journal of Clinical Nutrition 72, 2 suppl: 564S-572
48. Singh, D.P. and Singh, N. (2000). Calcium and phosphateregulation of nitrogen metabolism in the cyanobacterium*Spirulina platensis* under high light stress. Curr. Microbiol., 41: 368-363.
49. Steinberg, M.H. (2001). Disorders of Hemoglobin: Genetics, Pathophysiology, and Clinical Management. Cambridge University Press. ISBN 0-521-63266-8.

50. Takeda, A.(2003). Manganese Action in Brain Function. Brain Res Rev. Jan;41(1):79-87.
51. Tsavachidou, D; McDonnell, T.J., Wen S, et al. (2009). "Selenium and vitamin E: cell type- and intervention-specific tissue effects in prostate cancer". Journal of the National Cancer Institute 101 (5): 306–20.
52. Volkman, H.; ImianovskyIm, U.; Oliveira ,J. L.B.,and Sant'Anna ,E.S. (2008) .Cultivation of *Arthrospira (Spirulina) platensis* in desalinator wastewater and salinated synthetic medium: protein content and amino-acid profile Braz. J. Microbiol. .39 (1)/ São Paulo.
53. Vonshak, A., Abeliovich, A., Boussiba, S., Arad, S. and Richmond, A., (1982). Production of *Spirulina* Biomass: Effects of Environmental Factors and Population Density, Biomass, vol. 2, pp. 175-185.
54. Vonshak, A.(1986).Laboratory tevhnologies for cultivation of microalgae.PP: 117-145.In A.Richmond (ed).Handbook of microalgaal mass culture.CRC.Press.Baco.Raton.FL.
55. Vonshak, A. (1997). *Spirulina platensis (Arthrospira):*Physiology, Cell-biol., and Biotechnol. Taylor and Francis, London.
56. Wong, P.K., and Chan, K.Y. (1980).Algal single cell protein production from sweage effluent with high salinity.Experientia; 36:1065-1066.
57. Wong, P.K., and Chan, K.Y. (1990). Growth and value of *Chlorella S. alina* grown in highly saline sewage effluent. A gric ecosystem and Environment, 30: 235-250.
58. Yabuki, Y.; Mori, E., and Tamura, G. (1985). Nitrite reductase in the cyanobacterium *Spirulina platensis*; Agric. Biol. Chem.,49: 3061- 3062.
59. Zarrouk C. (1966*), Contribution a` l'e´tude d'une* cyano phyce´e. Influence de divers facteurs physiques et chimiques sur la croissance et la photosynthe`se de*. Spirulina maxima.* Ph. D. Thesis, Universite´.

10/26/2010

Microbial load as Pollution Indicator in Water of El-Khadra Lake at Wadi El-Natroun, Egypt

Ali, M. S. [1]and Osman, G. A. [2]
[1]Agriculture Microbiology Department, National Research Centre, Cairo, Egypt. [2]Bacteriology Lab., Water Pollution Research Department, National Research Center, Cairo, Egypt.
*mohamed_saad_1@hotmail.com gamalosmanali2005@yahoo.com

Abstract: Occurrence and survival of some classical bacterial indicators, (salmonellae group, total staphylococci and *Pseudomonas spp.)* in water samples at surface and one meter depth of El-Khadra lake have been studied as well as, cyanobacteria and fish lagoons were included for comparison. The results showed that, fecal streptococci and *Pseudomonas spp.* are not present in surface and deep lake water samples respectively, while other bacteria tested are presented. Similarly, salmonellae group and fecal coliform were absent in all water samples from the fish lagoon and the deep lake samples. In addition, the high and low log average counts of total viable bacteria incubated at 37 ºC for 24 hours were 7.5 and 3.4 /100m in cyanobacteria lagoon and surface lake water samples respectively. On the other hand, the high log average counts of total viable bacterial incubated at 22 ºC for 48 hours was 7.3 /100m in cyanobacteria lagoon, while the low recorded 3.67 /100m in surface water samples. The statistical analysis (log average) showed that, some factors such as human activity, sun ray and sedimentation as well as biological activity play role on the bacterial distribution in all water samples tested.
[Ali, M. S. and Osman, G. A. **Microbial load as Pollution Indicator in Water of El-Khadra Lake at Wadi El-Natroun, Egypt**. Journal of American Science 2010;6(12):489-496]. (ISSN: 1545-1003).

Key words: Lake water, Classical bacterial indicators, Salmonellae group, Total staphylococci and *Pseudomonas spp.*

1. Introduction:

Water must be safe and free of risk factors. Risk factors related to water pollution, can be divided into two basic categories: chemical and biological pollutants. Both categories derive from human activity which inevitably tends to modify water composition, with respect to its original state in nature (Grabow, 1996 and Payment *et al*, 1997). The lake water sources must be protected from contamination by human and animal wastes which contains a variety of bacterial, viral, protozoan pathogens and helminthes parasites.

The heterotrophic plate count (HPC), gives a valuable indication of general microbiological quality of water. The test is widely used to monitor the water pollution, as well as the deterioration of water quality during storage and distribution (Reasoner *et al*, 1989; Grabow, 1996 and WHO, 2001).

Generally, the European Union regulation require that the HPC be assessed at two recovery temperatures: 22 ºC for 72 hrs and 37 ºC for 24 hrs. The 37 ºC plate count was believed to give an indication of fast-growing bacteria more likely to be related to pathogenic types and 22 ºC. Plate count was used for enumeration of characteristic water bacteria that tend to develop slowly (Ramalho *et al,* 2001).

Coliform group that have a wide distribution in the environment and are not specific to faecal material. *Enterobacter* (*Aerobacter*) *aerogenes* and *Enterobacter cloacae* are frequently found on various types of vegetation in soil and in polluted water. All of these coliforms group, may be found in sewage and in polluted water environment (Edeberg *et al,* 1990). The value of selected indicators for assessment of faecal pollution as well as the distinction of faecal pollution of human and animal origin has been investigated (Jagals *et al,* 1995).

Several Studies for the survival of faecal coliforms are numerous (Dawe & Penrose, 1978 and LaBelle *et al*, 1980). Most investigations have involved either soil or marine environments and have concentrated only on reduction in bacterial numbers over time. Studies in recent years have frequently revealed much higher numbers of indicator and pathogenic bacteria in sediments than in overlying waters. Apparently, higher concentrations of indicator and pathogenic bacteria in the sediments are due to a combination of sedimentation, sorption (which provides protection from bacteriophage and microbial toxicants (Weiss, 1951 and Roper& Marshall, 1978), and the phenomenon of extended survival in sediments (Goyal and Adams, 1984). The water quality testing criteria in use at present do not take into account sediments as a potential reservoir of pathogens. The higher numbers of pathogens occurring in sediments, along with increasing usage of recreational waters, creates a potential health hazard from resuspension and subsequent ingestion (Matson *et al*, 1978 and Grimes, 1980). Thus, there is a need to obtain additional information on the survival of indicator and pathogenic bacteria in sediments and the factors which contribute to their survival.

Enterococci have also been related to human disease becoming firmly established as major nosocomial pathogens (McDonald *et al*, 1997). In addition, *Enterococcus* and *Streptococcus* have been proposed as indicators of faecal contamination in water because of their high abundance in feces and their long survival in the environment. Although the ratio of faecal coliforms to faecal streptococci have been ruled out as indicator (Pourcher *et al*, 1991 & Olajide, 2010). With respect to the relative proportions of faecal coliforms and faecal streptococci, faecal streptococci species profiles whether these characteristics can be used to distinguish between human and animal effluent (Sinton and Donnison, 1994).

The presence of *Pseudomonas aeruginosa* in water indicates that the source has become polluted either by organic material or contamination. De Victorica and Galvan (2001) reported that *Pseudomonas aeruginosa* is used as indicator of health risk association with drinking water.

Pseudomonas aeruginosa is considered as opportunistic bacteria expresses virulence factors which are related to serious infections in human especially in immuno-compromised individuals and special precautions may be required to limit the exposure of these susceptible populations (Warburton 2000). It is also, widespread in natural and industrial environments and is able to grow in water (Leclerc and Da Costa, 1998).

Staphylococci have been recently investigated as possible indicator for pollution of swimming pool waters and other aquatic environment (De Araujo *et al*, 1990 and WHO, 2003). Because the staphylococci had a higher resistance to the chlorine level in pool waters than coliforms and streptococci, they were isolated when coliforms and streptococci were absent (Favero *et al*, 1964 and Antai, 1987).

The enumeration of either total staphylococci or specific *Staphylococcus aureus* appears to provide a useful index for the water quality level of the aquatic sources. Staphylococci are salt tolerant which survive in the marine environment (Gabutti *et al*, 2000 and Kamel, 2005).

Salmonellae grow at temperatures ranging from 7 to 48°C, at pH 4–8, and at water activities above 0.93 (Baird-Parker 1990). Salmonellae are capable of prolonged survival in faecal materials, in slurry, or on pasture (Wray & Sotka, 1977). The fact that salmonellae are able to survive and readily multiply in the environment is an important factor in the transmission and spread of salmonellosis. Examples quoted by Williams (1984) illustrate this: salmonellae will live for 28 months in naturally infected avian faeces; *S. heidelberg* was recovered from contaminated poultry litter, grit, feed, and dust held for extended periods at room temperature (the poultry litter was positive at 7 months); *S. thompson* survived 4–5 weeks in old poultry litter and 8–20 weeks in new litter; and *S. typhimurium* survived in urban garden soil in England for at least 280 days (WHO, 2003).

The specific objectives of this study are to describe the relative association of classical bacterial indicators as well as some pathogenic bacteria in Lake water samples during winter 2010 at Wadi El-Natron, Egypt and in lagoons for growing cyanobacteria and fish.

2. Materials and methods

Sample collection. Lake water samples were collected monthly during winter season 2010, from El-Khadra lake at Wadi El-Natron, Egypt. Water samples were collected in 1 liter sterile glass bottles and then transferred from the sites to the lab in ice box. Water sampling was taken at 0, 30 and 100 cm deep from El-Khadra lake. Water samples were taken also from cyanobacteria lagoon and fish lagoon to assess the pollution load.

Microbial load of water samples

Collected water samples were analyzed for total viable microbial count and total count of different bacterial indicators using the poured plates and the most probable number (MPN) technique, respectively.

Poured plates technique (APHA, 2005).

Method for decimal dilution of water samples was used for determination of total bacterial load, on nutrient agar (APHA, 2005). The plates were incubated for 1-2 days for fast growing bacteria at 37°C and 2-3 days at 22°C for characteristic water bacteria (APHA, 2005).

The most probable number technique [MPN] (APHA, 2005).

The most probable number technique was carried out for estimation of some microbial indicators in the tested water samples using special presumptive and confirmed tests for each indicator. During presumptive test, 5ml of each appropriate three decimal dilutions of raw water samples were used to inoculate five tubes (20 x 1.5cm^2) each containing 5ml of proper medium (single strength), and the tubes were incubated at 37°C for 48 hours. The positive presumptive tubes were used to inoculate the confirmed test which detected the bacterial indicators as following:-

- Total coliform; Lauryl tryptose broth medium was used for presumptive test. The positive tubes which showed gas and acid were used to inoculate brilliant

green lactose bile broth medium (BGB), as a confirmed test. The production of gas and acid was recorded as positive confirmed test for total coliforms (APHA, 2005).

- Faecal coliform estimation was carried out by inoculation in the EC broth tubes from positive BGB broth medium tubes, then incubated at 44.5°C for 24 hours (APHA, 2005). The positive tubes containing gas production were used to detect the count per 100 ml sample (MPN index / 100ml) and streak the eosin methylen blue agar medium (EMB) plates, then incubated at 37°C for 24 hours. Metallic sheen colonies considered as a positive confirmed results for *E. coli* presence (APHA 2005).
- Faecal streptococci; Azide dextrose broth was used as presumptive test without fermentation tubes. The positive tubes were turbid, (APHA, 2005), then used to inoculate ethyl violt azide broth medium, (EVA) as a confirmed test. The positive results were turbid after incubation at 37°C for 48 hours (Gerhardt, *et al*, 1981). The positive tubes were used for the confirmed test which detected by streaking on m-Enterococci medium (APHA, 2005).

Salmonellae groups was counted from inoculated buffer peptone water (British Standard Institute, 2002) tubes (incubated at 37°C for 24 hours) which used as MPN technique (Morinigo *et al*, 1992). One loop from these tubes were streaked on the plates of bismuth sulphate agar as a confirmed test. After incubation at 37°C for 48 hours, typical black colonies with or without metallic sheen and blackening extended beyond the colonies considered as confirmed positive results for the presence of salmonellae.

Staphylococcus sp was determined by streaking one loop from buffer peptone water tubes on the surface of mannitol salt agar plates, then incubated at 37°C for 24 hours. The growing colonies had yellow zones, flat and 1.2 mm diameters (APHA, 2005).

Pseudomonas sp. was detected in the lake water samples using asparagine broth medium as a presumptive test. The positive tubes produced a greenish fluorescent color after exposing to long-wave ultraviolet light were used to streak the surface of acetamide agar slants as confirmed test. a positive confirmed tubes with the purple color indicated high pH value after incubation at 37°C for 24 hours (APHA,2005).

3. Results and Discussion

The bacteriological quality of the collected water samples was evaluated by monitoring of the total bacterial counts, fecal bacterial indicators and some pathogenic bacteria in five different water body sites at Wadi El-Natron, Egypt.

The results in table (1) show that; generally the high log average counts of total viable bacterial counts were increased at 37°C and 22°C in water samples taken at 100 cm depth of the lake than that at 0 and 30 cm depth. Whereas, the high log average counts recorded 6.47 cfu at 22°C the low log average was 2.9 cfu at 37°C / 100ml in the water samples taken at 100 cm. These results agree with Mansour and Sidky, (2003) where they found total viable bacterial counts in Qarun lake water samples and Rayan lake water $5.8x10^6$ and $8.1x10^5$ respectively. Also Ali, *et. al.* (2008) counted average of the total bacteria at 37°C and they found that the counts reached 2.1 x 10^6/ 100ml in lake water sample. This may be due to the effect the sunlight (UV ray), organic substances, biological activity and sedimentation (Olayemi, 1993). Phillip *et al*, (1988) suggest that the decline in lake water of bacteria that are resistant to starvation may be a result of protozoan grazing and that the extent of growth of introduced species may be limited by the supply of available carbon and sometimes of nitrogen and phosphorus, and by predation by indigenous protozoa. In addition, Olayemi (1993) found that sun light was shown to cause sublethel injury to some bacteria from10^5 to 10 for *St. bovis* and 10^6 to10^5 for *E.coli* 1n 14 days. On the other hand, he noticed more decrease in the counts of these bacteria with exposure to UV irradiation from 10^5 to 10^2 and from 10^4 to not detected for *St. bovis* and *E.coli* respectively.

In 100 cm deep water samples of this lake the faecal streptococci were recorded log average 1.43 cfu / 100ml, while it was absent in the surface (0 cm) and sub-surface (30 cm) water samples. On the other hand, total coliforms were detected, with the high and low log average count 1.57 and 1.3 cfu / 100ml respectively. Moreover, faecal coliforms was present in surface and sub-surface water samples while they were absent in the bottom water samples. The average log numbers of fecal coliforms was 0.43 cfu / 100ml, these results conflicted with U.S. Environmental Protection Agency (2003). Ola et al, (2006) confirmed our investigation where they found that *E. coli* levels in three sites out of studied 11 sites of Michigan lake shore was less than the recommended U.S. Environmental Protection Agency (2003) limits, 235 cfu/100 ml while the others ranging from 0 to 6,900 cfu/100 ml. in addition, the authors suggested that *E. coli* and enterococci survival and growth in the direct sun light. Moreover, the ability of *E. coli* to survive for several days in aquatic sediment in situ suggests that faecal coliforms in water may not always indicate recent faecal contamination of that water but rather reuses pension

of viable sediment-bound bacteria Lalibertet and Grimes, (1982).

Table (2) shows the counts of all types of bacteria tested and it shows that the bacteria were excesses in water samples which collected from cyanobacteria and fish lagoons than lake in water samples. The bacterial count log average in water samples from cyanobacteria lagoon at 22°C, 37°C showed that total coliforms, fecal coliforms and fecal streptococci were 7.37, 7.5, 3.3, 2.13 and 2.13 / 100ml respectively. With regarded to the water samples collected from fish lagoon recorded 4.47, 4.3, 1.97, 1.13 and 1.43 cfu / 100ml for total bacterial counts at 22°C, 37°C, total coliforms, fecal coliforms and fecal streptococci respectively.

Table (1): The average log of the viable count of the total bacterial load as well as classical bacterial indicators in the tested water samples during winter season of 2010 at El-Khadra Lake, Wadi El-Natron, Egypt.

**Site of water samples	Date	Samples number	Log number of colony forming unit (cfu) / 100 ml				
			Total bacterial count at:-		Bacterial indicators *(MPN-index)		
			22 °C	37 °C	TC	FC	FS
Surface lake (0 cm)	12/1/2010	1	3.5	2.9	1.3	0	0
	9/2/2010	2	3.7	3.6	1.7	1.3	0
	9/3/2010	3	3.8	3.7	1.7	0	0
		Average	3.67	3.4	1.57	0.43	0
Sub-surface lake (30 cm)	12/1/2010	1	3.6	3.8	1.8	0	0
	9/2/2010	2	3.9	5.2	3.5	1.3	0
	9/3/2010	3	3.8	5.3	1.3	0	0
		Average	3.77	4.67	2.2	0.43	0
One meter deep of lake (100 cm)	12/1/2010	1	6.9	4.8	1.3	0	1.3
	9/2/2010	2	6.1	5.1	1.3	0	1.3
	9/3/2010	3	6.4	5.3	1.3	0	1.7
		Average	6.47	5.07	1.3	0	1.43

Note:
* TC: Total coliforms FC: Faecal coliforms FS: Faecal streptococci

Table (2): The average log off the viable count of the total bacterial load in water of El-Khadra Lake at Wadi El-Natroun, Egypt used in growing cyanobacteria and fish outside the water body of the lagoon.

Site of water samples	Date	Samples number	Log number of colony forming unit (cfu) / 100 ml				
			Total bacterial count at:-		Bacterial indicators (MPN-index)		
			22 °C	37 °C	TC	FC	FS
Cyanobacteria lagoon	12/1/2010	1	7.1	7.5	3.5	2.2	2.1
	9/2/2010	2	7.4	7.3	3.2	2.1	2.2
	9/3/2010	3	7.6	7.7	3.2	2.1	2.1
		Average	7.37	7.5	3.3	2.13	2.13
Fish lagoon	12/1/2010	1	4.3	4.7	1.7	0	1.3
	9/2/2010	2	4.5	4.8	2.1	1.7	1.3
	9/3/2010	3	4.6	4.5	2.1	1.7	1.7
		Average	4.47	4.3	1.97	1.13	1.43

This phenomenon may be due to the nutrients availability for growth of the bacteria. Niemi and Niemi (1991) and Putheti & Leburu (2009) reported that domestic and industrial wastewater, agriculture waste environment are sources of faecal bacterial to rivers. In these lagoons the water was found to be contaminated with pathogenic microorganisms, some of which originate

from animal wastes (Ho & Tam, 1998). Contamination of water sources by wastewater can pose a health risk due to the presence of pathogenic microorganism agents in water used for recreation, drinking and fishing Olajide, (2010). In Uruguay (Laguna de Rocha) piccini (2006) found that bacterial counts (4.3 x 10^5cfu / ml) were higher average three times in the brackish southern part of the lagoon than the freshwater, in addition they suggested that this may be a consequence of better growth conditions. Moreover, they reported that the lagoon receives domestic waste from a small town through its main tributary

Table (3) demonstrates the occurrence of some pathogenic bacteria (Salmonellae groups, *Staphylococcus sp.* and *Pseudomonas sp.*) in lake water samples [except at 100 cm depth of the lake for *Pseudomonas sp* and Salmonellae groups in fish lagoon water samples (table 4)]. The water samples from the lake water showed that Salmonellae groups were decreased and the log average count recorded 2.37, 2.17 and 1.57 cfu / 100ml for the surface, the surface, sub-surface and the bottom respectively.

Table (4) shows the occurrence of the same pathogenic group in the lake water transported to special tanks to grow cyanobacteria and fish. Nearly the log counts of Salmonellae groups were stable in all water samples collected from cyanobacteria lagoon while absent in fish lagoon. The log average counts of *Staphylococcus sp* were 1.7 / 100ml in cyanobacteria lagoon. Pe´rez *et al,* (2004) reported that protozoa can be grazing bacteria where they found to reduce the survival of another pathogenic bacterium, *Vibrio cholerae*, in brackish waters (Christoffersen, 2004).

Table (3): The log average viable count of some pathogenic bacteria in lake water samples during winter 2010 at Wadi El-Natron, Egypt.

* Site of water samples	Samples number	Date	Number of cell forming unit (cfu) / 100 ml		
			Salmonellae group	Total staphylococci	*Psudomonas spp.*
Surface lake	1	12/1/2010	2.3	4.4	2.4
	2	9/2/2010	2.6	4.1	1.9
	3	9/3/2010	2.2	4.6	2.6
	Average		2.37	4.37	2.3
Sub-surface lake (30 cm)	1	12/1/2010	2.1	4.6	1.3
	2	9/2/2010	2.2	4.5	1.7
	3	9/3/2010	2.2	4.4	1.3
	Average		2.17	4.5	1.43
One meter depth	1	12/1/2010	1.3	4.5	0
	2	9/2/2010	1.7	4.5	0
	3	9/3/2010	1.7	5.1	0
	Average		1.57	4.7	0

The results show that *Pseudomonas sp.* were absent in one meter deep of lake water samples while detected in others. This may be due to the competition between the microbes in aquatic environmente. Schallenberg *et al,* (2005) demonstrated that *Daphnia carinata* (40 cell /liter) were grazing type bacteria (*Campylobacter jejuni*) where it declined 2 orders (from 10^7 to 10^5/ ml) of magnitude in day.

The log counts of Salmonellae groups were stable in all water samples collected from cyanobacteria lagoon while decline in fish lagoon. These results conflict those of Khatun1, et al 2007, who found the Salmonellae groups (in the average of 3.3 x 10^6 cfu / 100ml.) in swamp water sample used in growing the fish during November 2001 to October 2002. On the other hand, these results agree with those of Sangu, et al (1985) who reported that the total population of bacterial species specially *Escherichia coli, Aerobacter aerogenes, Staphylococcus aureus*, *Pseudomonas sp.* and *Micrococcus sp*. were considerably high in Keetham Lake (India) water sample.

In this investigation, the count log average of *Pseudomonas* sp. Recorded high values being 3.6/100ml in water samples collected from fish lagoon (table 4). On the other hand, the results show the log counts of *Staphylococcus* sp. were from 1.7 to 4.7/100ml which detected in water samples from cyanobacteria lagoon and one meter deep of the lake (table 3&4).

The *Pseudomonas sp.* Were absent in one meter deep of lake water samples while detected in others (table 3). These results agree with Ahmed and Naim, (2006) where they found that *Flavobacterium* sp., *Micrococcus* sp., *Streptococcus* sp., *Burkholderia*

glumae and *Pasteurella* sp. were present in some seasons of the year as well as *Pseudomonas fluorescens* and *Salmonella* sp. were present only in winter, whereas *Pasteurella pneumotropica* was found only in summer. In addition, results agree with Naim and Ahmed, (2004) where they found that. *Flavobacterium* sp. and *Pseudomonas* spp. were dominant only in the winter. This may be due to ambient seasonal temperature variation could account for some of the bacterial population variation.

The counts and the type of bacteria in the fish lagoon indicated special variation which might be due to the intestinal flora of fish is characterized by both the day-to-day and individual-to-individual variations (Sugita, 1990).

It is necessary to assess the microbial pollution load which is likely related to the chemical composition of the water in lake throughout the seasonal variations to which the lake water is exposed. This particularly important when the lake water is used for production of special biomaterials for potential commercial uses.

Table (4): The log average viable count of some pathogenic bacteria in water of El-Khadra Lake at Wadi El-Natron, Egypt used in growing cyanobacteria and fish outside the water body of the lagoon.

Site of water samples	Samples number	Date	Number of cell forming unit (cfu) / 100 ml		
			Salmonellae group	Salmonellae group	Salmonellae group
Cyanobacteria lagoon	1	12/1/2010	1.3	1.7	3.1
	2	9/2/2010	1.3	1.3	1.9
	3	9/3/2010	1.3	2.1	2.2
	Average		1.3	1.7	2.4
Fish lagoon	1	12/1/2010	0	4.1	3.2
	2	9/2/2010	0	4.2	3.5
	3	9/3/2010	0	4.4	4.1
	Average		0	4.23	3.6

Corresponding author
Ali, M. S. [1]
Osman, G. A. [2]
[1]Agriculture Microbiology Department, National Research Centre, Cairo, Egypt.
[2]Bacteriology Lab., Water Pollution Research Department, National Research Center, Cairo, Egypt.
*mohamed_saad_1@hotmail.com
gamalosmanali2005@yahoo.com

4. References:

1. Ahmed, H.and Naim, M. (2006). Seasonal changes in bacterial flora of fish pond sediments in Saudi Arabia. J. Appl. Aquacult. 18: pp. 35 – 45.
2. Ali, F. K.; El-Shafai, S. A.; Samhan, F. A. and Khalil, W. K. (2008). Effect of water pollution on expression of immune response genes of *Solea aegyptiaca* in Lake Qarun. Afri. J. Biotechno. 7: pp. 1418- 1425.
3. Antai, S. P. (1987). Incidence of *Staphylococcus aureus*, coliforms and antibiotic-resistant strains of *Escherichia coli* in rural water supplies in Port Harcourt. J. Appl. Bacteriol., 62: 371 – 375.
4. APHA [American Public Health Association], (2005). Standard Methods for the Examination of Water and Wastewater 21th ed. APHA, Inc. Washington, D C.
5. Baird-Parker, A. C. (1990). Food borne salmonellosis. The Lancet, 339: pp. 1231 – 1235.
6. British standard Institute (BSI), (2002). BSEN ISO 6579 incorporating corrigendum. No. 1. Microbiology of food and Animal Feeding Stuffs-horizontal method for the detection of salmonella spp. London: BSI
7. Christoffersen, K.; Ahl, T. and Nybroe, O. (2004). Grazing of nonindigenous bacteria by nano-sized protozoa in a natural coastal system. J. Microbial Ecol. 30: pp. 67 - 78.
8. Dawe, L. L., and Penrose, W. R. (1978). Bactericidal" property of seawater: death or

debilitation?. J. Appl. Environ. Microbiol., 35: pp. 829-833.
9. De Araujo, M. A.; Guimaraes, V. F.; Mendonca-Hagler, L. C. and Hagler, A. N. (1990). *Staphylococcus aureus* and faecal streptococci in fresh and marine waters of Rio De Janeiro, Brazil. Rev. Microbiol., 21(2): 141–147.
10. De Victoria, J. and Galvan, M. (2001). *Pseudomonas aeruginosa* as an indicator of health risk in water for human consumption. Wat. Sci. & Tech., 43(12): 48– 52.
11. Edberg, S. C.; Allen, M. J.; Smith, D B. and Kriz , N. J. (1990). Enumeration of total coliforms and *Escherichia coli* from Source Water by the Defined Substrate Technology. Appl. Environ. Microbiol., 56(2): 366 – 369.
12. Favero, M. S.; Drake, C. H. and Randall, G. B. (1964). Use of staphylococci as indicators of swimming pool pollution. Pub. Hlth. Rep., 79: 61 – 70.
13. Gabutti, G.; De Donno, A.; Bagordo, F. and Montagna, T. (2000). Comparative survival of faecal and human contaminants and use of *Staphylococcus aureus* as an effective indicator of human pollution. Mar. Poll. Bulletin, 40(8): 697-700
14. Gerhardt, P.; Murray, R. G.; Costilow, N. R.; Nester, W. E.; Wood, A. W.; Krig, R. N. and Phillips, B. G. (1981). American Society for Microbiology Manual of Methods for General Bacteriology, Washington, D.C.
15. Goyal, S. M., and Adams, W. N. (1984). Drug-resistant bacteria in continental shelf sediments. J. Appl. Environ. Microbiol., 48: 861- 862.
16. Grabow, W. O. K. (1996). Waterborne diseases: Update on water quality assessment and control water SA., 22: 193 – 202.
17. Grimes, D. J. (1980). Bacteriological water quality effects of hydraulically dredging contaminated upper Mississippi River bottom sediment. J. Appl. Environ. Microbiol., 39: 782 - 789.
18. Ho, B. S. W. and Tam, T. Y. (1998). Giardia and Cryptosporidium in sewage contaminated river waters. Wat. Rec., 32: 2860 – 2864.
19. Jagals, P.; Grabow, W. O. K. and De-Villiers J. C. (1995). Evaluation of indicators for assessment of human and animal faecal pollution of surface runoff. Wat. Sci. Techol. 31 (5-6): 235 – 241.
20. Kamel, M. M. (2005). Evaluation of various selective and modified media for recovery of *Staphylococcus aureus* from aquatic environments. Egypt. J. Appl. Sci., 20(8A): 11 – 20.
21. Khatun1, H.; Afza1, R.; Hossain1, M.; Hussain, M.; Khan1, A.; Rahman1, M. and Ashrafi N.(2007). Load of *Aeromonas salmonicida* in swamp water and it's effect on tilapia (*Oreochromis mossambicus*). Bio-Sci. 15: 165 - 168.
22. LaBelle, R. L.; Gerba, C. P.; Goyal, S. M. J.; Melnick, L.; Cech, I. and Bogdan, G. F. (1980). Relationships between environmental factors, bacterial indicators, and the occurrence of enteric viruses in estuarine sediments. J. Appl. Environ. Microbiol., 39: pp. 588 - 596.
23. Leclerc, H. and Da Costa, M.S. (1998). The microbiology of natural mineral waters. In Technology of Bottled Water (ed. D.A.G. Senior and P. Ashurst), pp. 223–274, Academic Press, Sheffield.
24. Lalibertet, P. and Grimes, D. (1982). Survival of *Escherichia coli* in Lake Bottom sediment. J. Appl. Environ. Microbiol., 43: pp. 623 - 628.
25. Mansour, S. A. and Sidky, M. M. (2003). Ecotoxicological Studies. 6. The first comparative study between Lake Qarun and Wadi El-Rayan wetland (Egypt), with respect to contamination of their major components. Food Chem. 82: pp. 181 – 189.
26. Matson, E. A., Hornor, S. G. and Buck, J. D. (1978). Pollution indicators and other microorganisms in river sediment. J. Wat. Pollut. Cont. Fed., 50: 13 - 19.
27. McDonald, C. L.; Kuehnert, M. J.; Tenover, F. C. and Jarvis, W. R. (1997). Vancomycin-resistant enterococci outside the health-care setting: prevalence, sources, and public health implications. J. Emerg. Infect. Dis., 3: 311–317.
28. Morinigo, M. A.; Muňoz, M. A.; Cornax, R. Martinez-Manzanares, E. and Borrego, J. J. (1992). Presence of indicators and Salmonella in natural waters affected by outfall wastewater discharges. Wat. Sci. Technol., 25: 1 – 8
29. Naim ,M. and Ahmed, H. (2004). Seasonal variation of bacterial flora in ponds in Saudi Arabia used for tilapia aquaculture. J. Appl. Aquacult. 16: pp. 53 – 61.
30. Niemi, R.M. and Niemi, J. S. (1991). Bacterial pollution of waters in pristine and agricultural lands. J. Environ. Qual. 20: 620 – 627.
31. Ola A. Olapade, Morgan M. Depas, Erika T. Jensen, and Sandra L. McLellan (2006). Microbial communities and fecal indicator bacteria associated with *Cladophora* mats on beach sites along Lake Michigan shores. J. Appl. Environ. Microbiol., 72: pp. 1932–1938.
32. Olajide A. A. (2010). Characterization of surface and ground water with reference to microbiological study in Akungba-Akoko, Ondo

State. J. Microbiology and Antimicrobials Vol. 2(1). pp. 11- 127.
33. Olayemi, A. B. (1993). Survival of *Escherichia coli* and some pathogenic bacteria in pond water exposed to solar and UV-irradiations. Biosci. Res. Commun., 5: pp. 11 – 127.
34. Pe´rez, M., Macek, M. and Galva´n, M. (2004). Do protozoa control the elimination of *Vibrio cholerae* in brackish water?. Int. Rev. Hydrobiol., 89: 215 – 227
35. Payment P.; Siemiatycki J.; Richardson L.; Renaud G. and Prevost M. (1997). A prospective epidermislogical study of gastrointestinal health effects due to the consumption of drinking water Int J. Environ. Hlth. Res., 7: 5 – 31.
36. Phillip, R.; John, P. and Martin, A. (1988). Factors affecting the survival and growth of bacteria introduced into lake water. Arch. of Microbiol., 150: 320 - 325.
37. Piccini, C.; Conde, D.; Alonso, C.; Sommaruga, R. and Pernthaler, J. (2006). Blooms of single bacterial species in a coastal lagoon of the southwestern Atlantic Ocean. J. Appl. Environ. Microbiol., 72: pp. 623-628.
38. Pourcher A. M.; Devriese, L. A.; Hernaders, J. F. and Delattre, J. M. (1991). Enumeration by a miniaturized method of *Escherichia coli, Streptococcus bovies* and Enterococcus indicates of origin of faecal pollution of waters. J. Appl. Bacteriol., 70: 525 – 530.
39. Putheti R. and Leburu R. (2009). Role of probiotics and their influence in different physico-chemical and microbiological studies of water – a case study. Intern. J. Fisher Aquacult. 1(1): 1- 4
40. Ramalho, R.; Cunha, J.; Teixeira, P. and Gibbs, P. A. (2001). Improved methods for enumeration of heterotrophic bacteria in bottled mineral waters. J. of Methods 44: 97 - 103.
41. Reasoner, D. G.; Blannon, J. C.; Geldreich, E. E. and Barnick, J. (1989). Nonphotosynthetic pigmented bacteria in a potable water treatment and distribution system. Appl. Environ. Microbiol., 55(4): 912-921.
42. Roper, M. M., and Marshall, K. C. (1978). Effects of salinity on sedimentation and of particulates on survival on bacteria in estuarine habitats. J. Geomicrobiol., 1: 103 - 116.
43. Sangu, R.; Sharma, K; Pathak, P and Sengar, S. (1985). Characteristics of water of Keetham Lake. J. Environ. and Ecol. 3: pp. 386 - 389.
44. Schallenberg, M.; Bremer, P.; Henkel, S.; Launhardt,A. and Burns, C. (2005). Survival of *Campylobacter jejuni* in water: effect of grazing by the freshwater crustacean *Daphnia carinata* (Cladocera) J. Appl. Environ. Microbiol., 71: pp. 5085 - 5088.
45. Sinton L.W. and Donnison A. M. (1994). Characterization of faecal streptococci from some New Zealand effluents and receiving waters. New Zealand J. Mar. & Freshwater Res., 28: 145–158.
46. Sugita, H; Miyajima, C ; Kobiki, Y and Deguchi, Y.(1990). The daily fluctuation and inter-individual variation of the faecal flora of carp, Cyprinus carpio L. J. Fish Biol. 36: pp. 103 - 105.
47. U.S. Environmental Protection Agency (2003). Bacterial water quality standards for recreational waters. EPA-823-R-03-008. U.S. Environmental Protection Agency Office of Water, Washington, D.C.
48. Warburton, D. W. (2000). Microbiology for screening bottled water for the presence of indicator and pathogenic bacteria. Intern. J. Food Microbiol., 17: 3 – 12.
49. Weiss, C. M. (1951). Adsorption of *Escherichia coli* on river and estuarine silts. Sew. Ind. Wast. 23: 227 - 237.
50. WHO [World Health Organization] (2001). Water Quality: Guidelines, Standards and Health. Edited by Lorna Fewtrell and Jamie Bartram. Published by IWA Publishing, London, UK. ISBN-900222-280 World Health Organization.
51. WHO [World Health Organization] (2003). Heterotrophic Plate Counts and Drinking-water Safety. Edited by J. Bartram, J. Cotruvo, M. Exner, C. Fricker, A. Glasmacher. Published by IWA Publishing, London, UK. ISBN: 1 84339 025 6.
52. Williams, J. E. (1984). Paratyphoid infections. In Diseases of Poultry, 8th edn (ed. M.S. Hofstad), chapter 3, Iowa State Univ. Press, Ames, IA.
53. Wray, C. and Sojka, W. J. (1977). Reviews of the progress of dairy science: bovine salmonellosis. J. Dairy Res.

10/7/2010

Effect of Different Rates of Cobalt on some Macro-Micronutrients and Heavy Metals Contents in Lettuce under Different Types of Recently Reclamation Soils

Abdel Fattah. M. S and Khaled. S.M.

Plant Nutrition Dept. National Research centre-Cairo, Egypt.

Abstract: The main objective of this research work is to assess the influence of cobalt element addition on the uptake of some macronutrients (N, P and K) and some heavy metals (Cd, Ni and pb) in two different reclaimed soils. The first soil was sandy from (Abu- Rwash) region, the second soil was calcareous from (El Noboria) region. Cobalt was added with different rates (10, 15 and 20) ppm after plantation stage. Nitrogen was added by rate 100 ppm N at form amonium nitrate NH_4NO_3. Moreover, Dihydrogen potassium phosphate H_2KPO_4 at rate 200 ppm as source of phosphours and potassium was added at the same time. Lettuce plant of class (lactuca sativa var capitata). The obtained results can be summarized as follows: In sandy soil a positive connection between rates of cobalt and (N,P,K) contents, negative contact was found between cobalt concentrations and heavy metals contents (Cd, Ni, pb). Dry weight gave a positive contact with cobalt treatments, all differences were significantly to each of chlorophyll concentration and all trace elements contents except Mn were a positive relationship with cobalt treatments. All differences between treatments were significantly. In calcareous soil negative contact was found between rates of cobalt and nitrogen, while potassium a positive contact was found with phosphorus, concerning the heavy metals (Cd, Ni, pb) contents, positive contact was found with rates of cobalt. All this connections were significantly. Dry weight gave a negative connection with cobalt treatments but not significantly. Chlorophyll concentration and trace elements contents were in a positive relationship with cobalt treatments. All differences between treatments were significantly. Dry weight gave a negative connection with cobalt treatments but notsignificantly. Chlorophyll concentration and trace elements contents were in a positive relationship with cobalt treatments. All differences between treatments were significantly.

[Abdel Fattah. M. S and Khaled.S.M. **Effect of Different Rates of Cobalt on some Macro-Micronutrients and Heavy Metals Contents in Lettuce under Different Types of Recently Reclamation Soils**. Journal of American Science 2010;6(12):497-502]. (ISSN: 1545-1003).

Key words: Cobalt – lettuce plant – Sandy- Calcareous soil- Macronutrients – Heavy metals – Trace elements - Chlorophyll- Dry weight.

1. Introduction:

The need to determine the best practices for land use in regions with rigorous climates makes it necessary to recognize that any type of human use of land involves a chang in the natural ecosystems or their replacement by different artifical biological systems. The basis of decision making with respect to land use should be to maintain control of the anticipated transformation and to apply ecologicl principles so that the adverse effects on the environment will be minimum despite of introduction of permanent human use of the land resource.

The modern ecological term "ecosystem" describe show the biological community, functions as unit with extremely complex interactions. When crop growing and livestock raising are introduced and developed into highly specialized forms of land use, biological systems are created which differ greatly from natural ecosystems.

Nutrient availability in the soil differs in many aspects. Factors of major importance for the mineral nutrition of plants are root induced changes in rhizosphere, pH and amount and composition of root exudates (Uren and Reiseauer, 1988). The proximity, extent, and pattern of contact between soil and root are important factors in the absorption of heavy metals which are tightly bound to the soil colloids (Merckx et al., 1986). Many investigators reported a relationship between solubility of heavy metals in soil and soil pH. It is well established that concentration of most of heavy metals increases to various degree with the decrease in soil pH (smis, 1986 and förstner, 1988). Root – induced changes in the rhizosphere are important factors for the metal dynamics in this zone. However, little attention has been paid to the extent to which plant roots effect the ability and distribution of heavy metals (CO) in the vicinity of the roots.

In this work, it was found that applied Co at a different rate on some mineral and heavy metals contents in lettuce plant in different reclamation soils (sandy and calcareous).

2. Materials and methods

A pot experiment was conducted to study the availability of some macronutrients namely, nitrogen, phosphorus and potassium and some heavy metals as cadmium, nickel and lead contents in lettuce plants of class (lactuca sativa var capitata) as affected by the addition of cobalt element at different rates (10, 15 and 20) Ug/g soil and two different types of reclamation soils, sandy soil of (Abu-Rawash region) and calcareous soil (El Nobaria region). Table (1) indicat to some physical and chemical properties. Pot contents as 5 kg soil. Nitrogen was added at form ammonium nitrate NH_4No_3 at a rate 100ppm nitrogen, dihydrogen potassium phosphate H_2Kpo_4 as sources potassium and phosphours by rate 200 ppm was added.

All the fertilizers were added at one dose after plantation stage.

Three levels of cobalt were added as $CoSo_4.7H_2O$ at (10,15 and 20) ppm, urifornly mixed with soil. The moisture content of the pot was maintained at 80% of the water holding capacity along tim of the experiment. Plants were harvested on 50 day.

Plant samples were reweighed and dried to constant weight at 80°C in a ventilated oven. Dried samples material were analysed for total nitrogen a chieved determined by distillation using microkjeldahel distillation (Jackson 1985). Phosphours, potassium, heavy metals (Cd, Ni, pb) and tracelements as according (Jackson 1985) .

Chlorophyll concentration in the fresh leaf was determined according to (licheloem and weil burn, 1983).

Table (1): Som physcial and chemical properties of the studied soils

Site	Texture	pH 1:2.5	EC Dsm⁻¹ 1:5	Cations meq/L				Ppm	Anions meq/l			%	Heavy metalsmg μ g/g	Cations meq/L					
				Ca	Mg	Na	K	Fe	$SO_4^=$	HCO_3^-	Cl^-	CaCo3	OM	Available			Total		
														Cd	Ni	Cd	Ni	Cd	Ni
Abu-Rawash	Sandy	7.92	0.185	6.12	36.0	9.35	4.20	153	5.32	7.31	123	0.40	0.26	0.02	0.18	0.22	0.12	1.06	2.11
Nobaria	Calcareous	8.32	0.200	8.02	24.0	67.5	4.02	236	7.53	9.51	95	15.8	0.19	0.01	0.20	0.25	0.15	1.09	1.85

3. Results and Discussion

Sandy Soil:

The data presented in table (2) reveal that some macronutrients (N,p and k) contents in lettuce plant in different types of reclamation soils as affected by different rates of cobalt (10,15 and 20) ppm. N,P and K percentages contents in sandy soil was in positive relations with rates of cabalt as cobalt concentration increasing follow up (N,P,K) percentages contents in lettuce plant increased. Head values of (N,P,K) registered with rate of cobalt 20 ppm while among value came with rate of cobalt 15 ppm, the least values of (N,P,K) with cobalt concentration 10 ppm. Cobalt maybe enhance to rise representation of nutrients to plant as it important role in constitution of Co-enzymes and some hormons. (Youssef 1997).

It has been reported that cobalt increased the total dry matter and yield of pigeon pea and peanut (shehata 1989) found that cobalt significantly increased the percentage of stomatal closure and increase dramatically ABA which play a central role in some hormon of iso plant. (Anter and nadia 1999). Revealed that cobalt applicat ion[reduced transpi-ration rate and leaf water potential specially at low cobalt concentrations Abscisic Acid in contrary to both Auxine and Gibberelins was promotively affected by cobolt application. Root xylem and phloem were beneficially affected by cobalt specially at water deficit condition. Of what previously mention show that cobalt stimulate to the nutrients plant uptake.

Table (2): Macronutrients contents as percentage (%) in lettuce plant as affected by different rates of cobalt and soil types.

Cobalt treatment (ppm)	Sandy soil		
	N	P	K
Control	2.52	0.79	0.16
10	2.11	0.31	0.18
15	2.80	0.46	0.19
20	3.64	0.65	0.21
L.S.D 0.05	0.23	0.028	0.017
Calcareous soil			
Control	2.24	0.69	0.22
10	4.85	0.79	0.22
15	4.09	0.91	0.14
20	2.67	1.19	0.11
L.S.D 0.05	0.32	0.062	0.013

Calcareous soil:

Table (2) Concerning calcareous soil as lettuce plant uptake some macronutrients (N, P and K) as affected by different rates of cobalt show that a negative relation between (N,K) uptake and cobalt concentrations as increasing of cobalt rates decrease of (N, K) content in lettuce plant.

Head values of nitrogen and potassium (N and K) uptake were with cobalt rate 10 ppm and among values with 15 ppm, the least values of (N and K) content with 20ppm of cobalt rate, may be due to some properties of the studied calcareous soil such as rising of calcium carbonate rate thus rising rate of sodium cations and soil pH this is to lead to little of exchangeable sites. All differences between the treatments were significantly.

Phasphorus contents was in a positive relation with cobalt rates as it increasing phosphorus contents increase. Head value of P contents with 20 ppm of cobalt rate, among value with 15 ppm and the least value of P contents with 10 ppm of cobalt rate, it seems that the cobalt element stimulation to phosphorus uptake despite the phosphorus problems in calcareous soils such as the fixation it to make at form unavailable. All differences between the treatments were significantly (youssef et al 2001) show that cobalt addition by rate 40 ppm to cause decreasing soil pH almost 2 units in the rhizosphere compared to that of bulk soil, due to soluble and available phosphours increased in calcareous soil.

Date in table (3) showed that some heavy metals content (Cd, Ni and pb) in lettuce plant as affected by different rates addition of cobalt (10, 15 and 20) ppm and soil types (sandy and calcareous).

Sandy soil:

The data presented in table (3) reveal that entity negative relation between cobalt rates and (Cd, Ni and pb) content , head values of heavy metals were with rate of cobalt 10 then 15 ppm differences between them were slightly can be negligible.

The least values of heavy metals content with 20 ppm of cobalt rate. That's meaning , cobalt inhibition effect on heavy metals content especially with the rising of cobalt rates in sandy soil.

(Zhang, M; and zixia, ke 2009) showed that in the polluted soils by heavy metals may be enhance entity of cobalt element with the fertilization in the little levels of heavy metals contents.

Calcareous soil:

Head values of some heavy metals (Cd, Ni, pb) were with 20 ppm of cobalt rate, among values with 15 ppm of cobalt concentration, the least values was with 10 ppm of cobalt rate.

This results meaning entity positive contact between cobalt rates and heavy metals content in lettuce plant. May be due to properties of calcareous soils such rise of soil pH, calcium carbonate highly rate and sodium cations rising concentration in soil studied. All the differences between the treatments were significantly.

Table (3): Heavy metals contents in lettuce plant (ppm) as affected by different rates of cobalt and soil type.

Cobalt treatment (ppm)	Sandy soil		
	Cd	Ni	Pb
Control	10	141	653
10	34.2	230	420
15	33.3	229	415
20	14.6	150	133
L.S.D 0.05	1.48	13.7	184
Calcareous soil			
Control	42.5	269	446
10	17.5	117	173
15	40.0	231	376
20	45.0	278	455
L.S.D 0.05	1.57	11.9	16.2

Table (4) Dry weight, chlorophyll and micronutrients content in lettuce plant as affected by different rates of cobalt and different types of soils.

Cobalt Treatments (ppm)	Sandy Soil					
	Dry. W	Chlorophyll	Ppm			
Ppm	Gm	Mg/g	Fe	Mn	Zn	Co
Control	10.3	0.22	85	110	18.9	17.5
10	15.5	0.35	126	135	22.3	31.6
15	17.8	0.78	175	117	29.7	46.5
20	25.8	1.1	215	118	35.2	67.3
L.S.D. 0.05	2.04	0.15	12.3	10.9	2.69	10.2
Calcareous soil						
Control	11.8	0.32	114	146	15.4	25.3
10	21.4	0.65	208	158	19.7	34.7
15	18.7	0.94	203	173	25.8	59.8
20	17.3	1.21	255	167	30.7	66.3
L.S.D. 0.05	2.03	0.20	10.8	10.7	2.18	11.6

Sandy soil:

Data in table (4) shows that a positive contact between for each iron, manganese zinc and cobalt in lettuce plant uptake and dry weight (gm) where as its increased with increasing of this micronutrients with increasing of cobalt treatments in a sandy soil where Fe uptake with a rate comparison with control (48, 106 and 153) % with cobalt treatments increasing respectively. May be cobalt nutrient stimulate Fe, zin and co.

Behavior Mn was in contrary relation with dry weight to lettuce plant. May be Mn at form unavailable for uptake in sandy soil or it competition with Fe on granulars surface in sandy soil where, as a little surface specific specially.

Chlorophylle concentration to be have same of relationships with cobalt treatments and it gave similar results with (Fe, Mn, Zn and Co) contents in lettuce plant. My be that find a positive effect between dry weight and chlorophylle concentration in lettuce plant.

Calcareous soil:

It was found opposite contact between cobalt treatments and dry weight but the differences were negligible may be cause soil pH rising which due to limited hinder to cobalt available. Positive contact between cobalt treatments and all nutrients conten in lettuce plant under study (Fe, Mn, Zn and Co, all the differences between nutrients content and cobalt tseatments were significantly, where that (Fe) absorption percentage comparison control (82, 78, 123) % respectively.

Chlorophyll concentration in lettuce plant was at a positive contact with cobalt treatments where as percentage comparison control (103, 194, 278) respectively.May be due to increasing of (Fe) in plant.

(Perez, et al, 2008) revealed that cobalt remarkably increased fresh and dry weight, of shoots and roots. The amounts of dry matter of tomato plants were the highest when Co was spotted in 1mm compartment compared with that of other soil compartments. The influence of Co placements on plant growth was most pronounced in roots than shoots. It has been reported that low concentration of Co had favorable effect on plant growth.(Takahashi and satio, 2007) found that the trace elements were increased under cobalt treatments wich to follow increasing chlorophyll concentration in tomato plant (Sposito, 2003) got a general conclusion that activities of the micromutrients in solution rather than concentrations per shops would be more meaning ful.

In this study.

The reduction in trace elements extractability was accompanied by Co placements.

4. Summary and Conclusion:

In sandy soil cobalt treatments gave a positive effect with most of the previously results at all cobalt concentrations, therefore were recommended to using it with example this soils.

In Calcareous soil cobalt element gave a negative effect with high concentrations, while it gave a positive effect with low concentrations, therefore were recommended to using it where not more cobalt concentration of (10 ppm) in example this soils.

Corresponding author
Abdel Fattah. M. S
Plant Nutrition Dept. National Research centre-Cairo, Egypt

5. References:

1. Anter, F' and Nadia, G. (1999). Cobalt absorption in relation to plant water balance J. plant. Nutr 40 : 251 – 262.
2. Forstner, U. (1988). Analysis and prognosis of metal mobility in soil and wastes. Klwere Acad, pp. 1-10.
3. Jakson, M.L. (1985). Soil chemical analysis prentice – Hall, Inc Englewood cliffis, N. J.
4. Lichetoemthaler, H.K and weilburn, A.R. (1983). determination of total coroteniods and chlorophy a and b of leaf extracts in different solvents Biochem. Soc. Tans., 11: 591-592.
5. Merckx, R.; sinnaeve, J. and cremers, A. (1986). Plant – induced changes in the rhizosphere of maize and wheat. II- Complexation of cobalt, zinc and managenese in rhizosphere of maize and wheat. Plant and soil 96.95.
6. Perez, M.D; Murcia, R Moral and Moreno, J. A. (2008). I: cobalt induced stress in soil less lettuce cultivation II Effect on nutrient evalution Acta-Horticulturae (501): 289-292.
7. Shehata, N.G (1989). Effect of cobalt on the growth and mineral composition of plant. M.Sc thesis, Fac. Agric, Ain shams univ., Egypt.
8. Smis J.T. (1986). Soil pH effect on the distribution and plant availability of manganese, copper and zinc. Soil Sci. Soc Am. J. 50.
9. Sposito, G. (2003). The chemical forms of trace metals in soils. In : Applied, Environment Geochemical, 1. Thornton (Ed.) A cad. Press, London, pp: 123-170.
10. Takahashi, k; and saito, Y. (2007). studies on micronutrients and effect it on chlorophyll formation in agricultural land in tomato plant. Japan, Bull, shikoku, Agri. Expt. Station., 83: 29.
11. Uren, N.C. and Reiseauer, H.M. (1988). The role of root exudates in nutrient acquisition. Adv. plant nutr. 3.79.

12. Youssef, R. A. (1997). Studies on nickl and mangaese dynamic in the rhizosphere of wheat. Soil Sci. plant nutr, 43, 1021.

13. Youssef, R.A.; Gad, N; Anter, F. (2001). Studies on the behavior of cobalt in the rhizosphere of tomato seedlings. I changes in pH in relation to Co distribution across the rhizosphere. Egypt J. soil. Sci. 41 (1-2), 123.

14. Zhang, M; and zixia ke. (2009). Heavy metals, phosphorus and some other elements in urban soils of Hangzhou city, china. Zhejiang university pedosphere; 22 (2) :180-187.

10/5/2010

Phenotypic Stability Analysis, Heritability and Protein Patterns of snake Cucumber Genotypes.

AbdEl-Salam,M.M.M[1].; I.S. El-Demardash[*2], and A.H.Hussein[1]

[1]Dep. of vegetable – Hort. Res. Inst., Agric.Res. Center, Giza, Egypt. [2]National Research Center, Genetic Section, Giza, Egypt.
[*]lola_El-Demardash@yahoo.com

Abstract: Stability analysis was carried out for six traits in snakecucumber by growing 5 genotypes (1,2,3,4,5) collected from different regions of Egypt (Assiut,Ismialia, El-kalyoubia, Domiat and Fayoom) respectively, in 3 years at El-kassaseen region, Ismailia. Genotypes × environment interaction was significant for all studied traits; the linear component of genotype × environment interaction was significant for number of fruits plant, yield / Fadden and fruit shape index. Environments (linear) were significant for yield / plant, yield / Fadden, fruit diameter and fruit shape index . The linear regression on environmental means (bi) close to unite with significant for genotypes (2,3,5,) for number of fruits / plant and (3,4,5,) for fruit diameter . Broad sense heritability was high for number of fruits / plant, yield / plant, fruit length and fruit shape index, but it was moderate for yield / Fadden and fruit diameter. The figure genotypes showed different patterns in presence of bands, the maximum number of band (6) in genotype (2) and the minimum number (3) was present in genotype (6), there are non resemblance between any genotypes, each genotype was characterized by a unique Finger print, except genotype (2) was monomorphic .
[**AbdEl-Salam,M.M.M; I.S. El-Demardash, and A.H.Hussein. Phenotypic Stability Analysis, Heritability and Protein Patterns of snake Cucumber Genotypes**. Journal of American Science 2010;6(12):503-507]. (ISSN: 1545-1003).

Keywords: Phenotypic Stability; Analysis; Heritability; Protein; snake; Cucumber; Genotype

1. Introduction:

Snakecucumber (Cucumis melo var.flexuosus Naud.) belongs to cucurbits family, preferred to the consumer, it is rich in A, B and C vitamins and contents of iron, calcium, phosphors and zink elements, moreover, it has medical benefits. In spite of, it does not exist on the agriculture map of Ismailia, as other members of its family (melon, squash, watermelon, cucumber). Snakecucumber can be a replacing option for cucumber; its cultivation has been reduced significantly in the late summer season and has been planted under plastic tunnels in the November month or under a greenhouse. The study aimed at the introduction of snakecucumber variety grown in Ismailia, with choice of the most suitable ways to improving of the important snakecucumber genetic traits under conditions of the region. Rare researches on snakecucumber breeding were done if compared with melon. Stability parameters for yield components were described by Gill and Kumar (1989) on watermelon, Yhia (1999) evaluated some of snakecucumber ecotypes for three years under Assiut conditions. Parmer and Lal (2005) and Singh and Lal (2005) studied the genetic variability and heritability for yield traits on muskmelon.

2. Materials and methods

Five genotypes of snake cucumber were collected from different regions of Egypt (Table.1).

Table 1: Serial number of genotypes and its sources

Sources	Serial number of genotypes
Assiut	**1**
Ismailia	**2**
Kalyoubia	**3**
Domiat	**4**
Fayoom	**5**

The genotypes were tested at El –Kasaseen research station, Ismailia, during summer seasons of 2005, 2006 and 2007 using a complete randomized block design with 3 replications . Each experimental plot was 15 m long, 150 cm wide and 50 cm a part between hills, all agricultural practices were carried out, by equal and optimum quantities to each plant. Observations were recorded for number of fruits / plant, yield / plant, yield /Fadden, fruit length, fruit diameter and fruit shape index. Stability analysis was carried out following Eberhart and Russell (1966). Heritability in the broad sense was estimated for the former traits, as illustrated by Collins et.al. (1987) according to the following formula,

$H\% = \delta^2 g / (\delta^2 g + \delta^2 m) \times 100$

Coefficient of variability values were estimated depends on phenotypic (P.C.V) and genotypic (G.C.V) variances using the next equations:

$$P.C.V = \sqrt{Vph / \bar{x}} \times 100$$

$$G.C.V = \sqrt{Vg / \bar{x}} \times 100$$

Whereas $\sqrt{Vph}$ = Phenotypic standard deviation.

$\sqrt{Vg}$ = Genotypic standard deviation.

$\bar{x}$ = Genotypes means.

Electrophoresis studies:

Protein electrophoresis

This investigation was carried out at the laboratory of Genetic Department, National Research center. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according the method of Leammli (1970). After being modified by Studier (1973) the youngest fully expanded leaf samples were taken from each of the four genotypes, (2) Ismailia, (3) Kalyoubia, (4) Domiat and (5) Fayoom. Samples of 0.5 gram of each genotype with 5 ml. of buffer was homogenized, then they centrifuged for 15 minutes at 15000 rpm. Supernatants containing water soluble protein to eppindorf tubes. Incubation and agitation were carried out at room temperature until bands appeared in clear background then the gel was washed with distilled water then gel was photographed Yamamato *et.al* (1982) .

3. Results and Discussion

Differences among genotypes were significant for all the traits, except fruit diameter, indicating the presence of considerable genotypic variation in the germplasm material for these traits (Table 1). The significant mean squares due to environment (years) for all traits suggested that environment (years) considerably influenced on the genotypic performance. The interactions between genotypes and environments for all traits were significant indicating that genotypes behaved differently under different years, this result was in accordance with Gill and Kumar (1989), with respect to yield/plant and number of fruits/plant. Significant mean square due to environments (linear) for yield/plant, yield/Fadden, fruit diameter and fruit shape index indicating the differences between 3 years (environment) and their considerable influence on these traits. The higher linear component of G × E than non-linear component for number of fruits/plant indicates the possibility of production of genotypes in different environments, the mean square due to environment +(G × E)was significant for number of fruits/plant, fruit diameter and fruit shape index. It shows that there was considerable interaction of genotypes with environmental condition in different years. The regression analysis (Table 3)shows that genotypes (2,3,5) had high mean performance for number of fruits/plant with significant regression close to unity (b=1.2,1.4,1.4 respectively), indicating their suitability for all environments. The relatively high values of regression coefficients in genotype 2 (b=3.7) and genotype 4 (b=4.04) with high mean performance for yield/Fadden, reflected the suitability of these genotypes to favorable conditions, like high fertility, timely sowing and good management practices. The S2di values were significant for all genotypes classifying them as unstable for yield/plant, genotype 5 has highest yield/Fadden (8.0 tones) and its bi value is less than 1.0 (b=0.59), revealing its adaptability to unfavorable or poor environmental and management condition. The crooking of snakecucumber fruits that is caused by their excessive length is causing marketing problems. Consequently, that gives importance to the studding of fruit length, diameter and shape index traits. All genotypes under studying were selected on the basis of moderate length. The genotype 4 gives a regularity performance for fruit length, diameter and shape index traits with regression close to unity (b=1.05, 1.1 and 0.99 respectively) and least deviation from regression S2di. The genotypes 2 and 4 could be considered most stable for yield/Fadden; also these genotypes have high yield/Fadden (7.9 and 7.5 tones, respectively). As reported by Perkins and Jinks (1968) and Finlay (1971) the stability like any other character is a heritable trait, thus these two genotypes can be judiciously used in snakecucumber breeding programs as a source of genes for stability and high productivity.

The values of genotypic, phenotypic and error variance, heritability, genotypic (G.C.V) and phenotypic (P.C.V) coefficients of variation are presented in Table (4). For all the studied traits, the genotypic and phenotypic estimated variance appeared large, in comparison with the estimated values of error variance, such a result seemed to indicate that the number of replicates used in the evaluation experiment of these genotypes were adequate to give a better estimation for the error variance. Heritability percentage in the broad sense was found moderate values for number of fruits/plant, yield/Fadden and fruit diameter as appears in Table (3). Accordingly, it might be stated that phenotypic selection for these traits would be reasonably effective. The higher estimated heritability values for yield/plant, fruit length and fruit shape index indicating that phenotypic selection for these traits could be highly efficient. These results were in harmony with those obtained by Parmar and Lal (2005) and Singh and Lal (2005).

The estimations of genotypic (G.C.V) and phenotypic (P.C.V) coefficients of variation exhibited small differences between genotypic and phenotypic coefficient of variation for yield/plant, fruit length and fruit shape index, revealing that environmental effects

were not great importance on these traits. These results were assured by heritability values

Protein electrophoresis:

The four genotypes showed different patterns in presence of bands (Fig 1 and Table 5), the maximum number of band (6) in(Ismailia 2) and the minimum number (3) was present in (Fayoom5)and (Kalyoubia). However, there are non resemblance between any genotypes each genotype was characterized by a unique fingerprint except for genotype (Ismailia2) was monomorphic. At the same time there was a marker band (5) for some genotypes such as band 2 at 49 KD for genotype (Fayoom 5), band 5 at M.W 39 KD for genotype (Domiat4). These results were in agreement with Jurgen and Helmut (1978) who confirmed that SDS- protein page was a highly successful technique in genotype identification Staub *et al*. (1983) reported that electrophresis employed as enzymatic for studying breeding material in the genus and taxonomy of cucumber.In relation to number and intensity of bands, genotypes (Ismailia 2) and (Domiat4) had the same number groups of bands with more intensity than other genotypes (Kalyoubia 3 and Fayoom 5).From the previous results, it could be deduced that the variation in banding patterns between four genotypes; showed different behavior for genotypes under Ismailia conditions, whereas genotypes (Ismailia 2 and Domiat 4) were more adaptation, and confirm that these four genotypes are genotipically and evolutionary different.

Table (2) Estimations of mean squares of six traits in snakecucumber.

Mean squares							
Source of variation	d.f	Number of fruits / plant	Total yield /plant (kg)	Total yield /Fadden (ton)	Fruit length (cm)	Fruit diameter (cm)	Fruit shape index
Environmental (years)	2	66.3**	0.33**	0.67*	119.7**	9.45**	8.9**
Genotypes	4	2.9**	0.14**	0.53*	13.3**	0.04	1.5**
Genotypes × Environments	8	3.61*	0.13**	1.14**	10.67**	0.097*	1.7**
Environment + (Genotype × environment)	10	5.38**	0.04	0.33	10.74	0.661**	1.03**
Environmental (linear)	1	1.1	0.84**	0.91*	1.0	1.01**	1.0**
Genotype×Environmental(linear)	4	12.87**	0.009	0.613*	1.53	0.028	2.32**
Pooled deviation	5	.24	0.098	0.09	4.44	0.02	0.01
Pooled error	30	1.4	0.003	0.16	1.3	0.06	0.4

P*≤ 0.05, P**≤ 0.01

Table (3) Estimation of stability parameters for six traits in snakecucumber

Genotypes	Number of fruits/plant			Total yield/plant (kg)			Total yield/Fadden (ton)			Fruit length (cm)			Fruit diameter (cm)			Fruit shape index		
	X	(bi)	S^2di	X	(bi)	S^2di	X	(bi)	S^2di	X	(bi)	S^2di	X	(bi)	S^2di	X	(bi)	S^2di
1	4.4	0.6**	-0.5	1.6	1.04	0.05**	7.5	-2.2	-0.1	20.9	1.38*	1.14	3.5	0.96**	-.01	6.0	0.95**	-.05
2	4.7	1.2**	-0.5	1.5	0.64	0.40**	7.9	3.7*	-.01	18.8	1.2	12.4**	3.5	0.79**	-.02	5.4	-.51**	-.09
3	5.1	1.4**	0.1	1.7	1.36	0.03**	7.6	-0.4	-0.1	21.61	0.65	5.7*	3.5	1.02**	-.01	6.4	1.83**	0.01
4	4.0	0.36*	-0.2	1.6	-.88	0.12**	7.5	4.04**	0.1	20.0	1.05	0.52	3.7	1.1**	0.03	5.6	.99**	-.08
5	5.5	1.4**	0.7	1.8	1.5	0.24**	8.0	0.59	0.12	19.0	0.72	0.33	3.4	1.13**	0.0	5.8	1.85**	-.12

X=Mean, bi=Regression coefficient, S^2di=Deviation from regression P*≤0.05, p**≤0.01

1=Assiut, 2=Ismailia, 3=Kalyoubia, 4=Domiat, 5=Fayoo

Table (4) Genotypic (δ2 g), Phenotypic (δ2ph) and error variances (δ2e) , Heritability (H%) in the broad sense and Genotypic(G.C.V) and Phenotypic(P.C.V) coefficients of variation estimates for six traits in snakecucumber.

Traits	Genotypic variation(δ^2g)	Phenotypic variation(δ^2ph)	Error variation(δ^2e)	Heritability (H%)	(G.C.V)	(P.C.V)
Number of fruits/plant	2.9	4.26	1.4	68.08	35.9	43.7
Total yield/plant	0.14	0.184	0.003	76.09	23.4	24.0
Total yield/Fadden	0.53	0.933	0.21	56.81	9.5	11.2
Fruit length	13.3	17.0	1.3	78.24	18.2	19.1
Fruit diameter	0.04	0.079	0.06	50.63	5.7	9.0
Fruit shape index	1.5	2.11	0.4	71.09	21.1	23.8

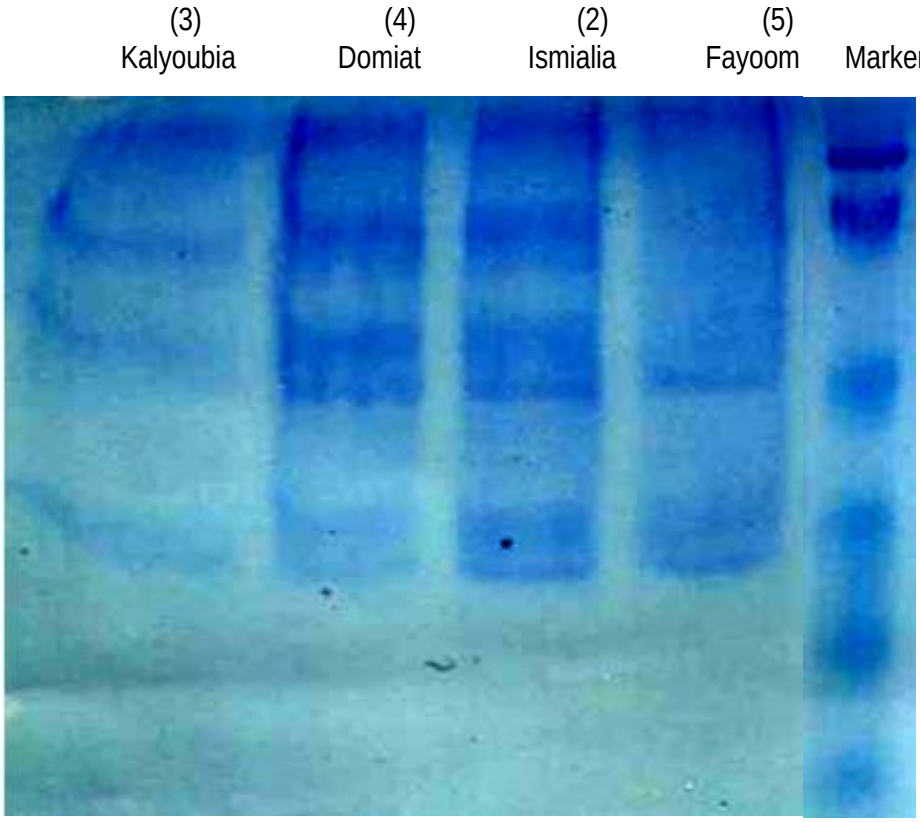


Fig1: Gel photographed of Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS. PAGE) of snakecucumber genotypes

Table (5) Analysis of bands for gel photographed of Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS. PAGE) of snakecucumber genotypes

No. of Band	MW	Genotypes			
		5	2	4	3
1	50	0	1	1	0
2	49	0	1	1	1
3	46	0	1	1	1
4	40	1	1	1	0
5	39	1	1	1	0
6	36	1	1	0	1
Total		3	6	5	3

Corresponding author
I.S.El-Demrdash
National Research Center, Genetic Section, Giza, Egypt.
lola_El-Demardash@yahoo.com

4. References:

1. Collins, W.W.; L.G.Wilson; S.Arrendell and L.F.Dicky (1987). Genotype×environment interaction in sweet potato yield and quality factors. J. Amer. Soc. Hort. Sci. 112 (3): 579 – 589.
2. Eberhart, S.A. and W.A.Russell (1966). Stability parameters for comparing varieties. Crop Science 6 : 36 – 40.
3. Finlay, K.W. (1971). Breeding for yield in barley. Proceedings of international barley genetic symposium 2 : 345 – 388.
4. Gill, B.S. and J.C.Kumar (1989). Phenotypic stability in watermelon. Indian journal of agricultural sciences 59 (3) : 145 – 148.
5. Jurgen,F. and K.L. Helmut (1978) plant micro body proteins European journal of Biochemistry volume 92 issue 1 , December (1) , : 35 – 43
6. Laemmli,U.K. (1970) . Cleavage of structural prteins during the assembly of the head of bacteriophage T4. Nature. 227 : 680 – 685 .
7. Parmar, A.M. and T.Lal (2005). Variability studies in melon. Research on crops 6 (2) : 314 – 317 Hisar, India.
8. Perkins, J.M. and J.L. Jinks (1968). Environmental and genotype environmental components of variability. □. Multiple lines and crosses. Heredity, London. 23: 339 – 356.
9. Singh, G. and T. Lal (2005). Genetic variability, heritability and genetic advance for yield and its contributing traits in muskmelon. Journal of research. Punjab agricultural Univ. India 42 (2): 168 – 174.
10. Staub,J.E.; R.S. Kupper, and T.C. wehner (1983) preliminary of isozyme polymorphism in cucumes cucurbit genetics cooperative report 6 : 32–34 (article 16).
11. Studier,F.W. (1973). Analysis of bacteriophage T1 early RNAs and proteins of slab gels .J. Mol. Bio., 79: 237 – 248
12. Yamamato, K.O.; Maitani and Ando (1982). Karyotypic and isozymatic polymorphism in species of the section faba (Genus vicia). Tech. Bull- Fac. Agric. Kagawa Univ., 34:1-10
13. Yahi, A.M. (1999). The performance of some snakecucumber ecotypes under Assiut conditions. M.Sc. Thesis, Fac. Agric. Assiut Univ., Egypt.

10/11/2010

Bio-removal of nitrogen from wastewaters-A review

Gaber Z. Breisha[1], Josef Winter[2]

[1.] Department of Agricultural Microbiology, Faculty of Agriculture, Minia University, Minia, Egypt
[2.] Institut für Ingenieurbiologie und Biotechnologie des Abwasser, Universität Karlsruhe, Germany
gaberbresha@yahoo.com, Josef.Winter@iba.uka.de

Abstract: If the present large volumes of nitrogen-containing wastewater of domestic and industrial origin are discharged into the environment without proper treatment, they lead to extensive soil and water pollution. Proper elimination of pollutants from these effluents is essential in industrialized countries and is becoming increasingly important from an environmental and human health point of view in developing and emerging countries. Beside the conventional nitrogen removal process (lithoautotrophic nitrification and denitrification), novel and cost-effective biological nitrogen elimination processes have been developed, including simultaneous nitrification and denitrification, anaerobic ammonium oxidation (Anammox), and its combined system (completely autotrophic nitrogen removal over nitrite, Canon). This review summarizes the recent studies dealing with agricultural, domestic and industrial wastewaters regarding their nitrogen content. Traditional and novel biological nitrogen elimination technologies are reviewed. Furthermore, recent studies dealing with temperature, dissolved oxygen, nitrate concentration, salinity, pH or the free ammonia concentration as factors affecting the nitrogen removal efficiency have also been incorporated.
[Gaber Z. Breisha, Josef Winter. Bio-removal of nitrogen from wastewaters-A review. Journal of American Science 2010;6(12):508-528]. (ISSN: 1545-1003).

Keywords: wastewaters; nitrogen removal; salinity; free ammonia; temperature; dissolved oxygen

1. Introduction

Although access to safe drinking water has improved steadily and substantially over the last decades in almost every part of the world [1, 2], in the developing countries 90% of all wastewater still goes untreated into local rivers and streams [3] and thus limits safe fresh water supply. Some 50 countries, with roughly a third of the world's population, also suffer from medium or high water stress, at least during the rain season and 17 of these extract more water annually than is recharged through their natural water cycles [4]**.** The increasing water demand not only affects surface freshwater bodies like rivers and lakes, but it also degrades groundwater resources. Due to more groundwater extraction than recharge it is expected that for instance the soil surface of Jakarta/Indonesia will settle 0.4-0.6 m until 2020 [The Jakarta Post, 25. 08.2009] and periodic flooding of many city parts during the rain season will be the consequence. Eutrophication**,** associated with discharge of nitrogen compounds or nitrogen compounds-containing wastewater into freshwater has become a severe water pollution problem in many countries [5]. The water quality is deteriorated and potential hazards to human or animal health e.g. by toxic algal blooms are consequences. The presence of excess nitrogen in the environment has caused serious alterations of the natural nutrient cycle between the living world and the soil, water, and atmosphere [6]. Excess discharge of nitrate as a fertilizer but also as one the most common water and groundwater pollutants causes serious problems including cancer, blue-baby disease in new-born infants and methaemoglobinaemia [7]. However there are many other pollutants in water such as e.g. antibiotics, X-ray contrasting agents, health care residues or sugar derivatives in industrial wastes or wastewater that are also potential toxicants. In recent years, a number of studies have focused on carbon, nitrogen and phosphate removal from domestic, agricultural and industrial wastewaters. The objectives of this review are a) to identify nitrogen pollutants concentrations in domestic, agricultural and industrial wastewaters, b) to compile the latest achievements of technologies developed for the removal of nitrogen from these wastewaters and c) to clarify the effect of temperature, dissolved oxygen, nitrate concentration, salinity, pH and free ammonia concentrations as factors that influence the nitrogen removal efficiency.

2. Nitrogen -containing wastewaters

2.1. Nitrogen in agricultural wastewater

In recent years, several papers have addressed the recovery of nitrogen compounds from agricultural manures. The concentration of nitrogen compounds varies according to the origin of the respective manure. Poultry manure in a farm near Istanbul, Turkey, for instance, contained 1580 mg l^{-1}

total Kjeldahl nitrogen and 1318 mg l^{-1} NH_4^+-N [8]. Livestock species, their type and age, the nature of their feed and how it is fed, whether or not the livestock are housed, weather and climate, all contribute to wastewater composition, volume and its rate of production [9]. The variation of ammonium-N concentrations (mg l^{-1}) in livestock wastewaters in south County Waterford, Ireland, was measured for almost a decade (Table 1). There were high annual variations of ammonia and even higher variations within single years [10].

Table 1. Ammonium concentration (mg l^{-1}) in livestock wastewater includes discharges from eight cattle/dairy farmyard and one sheep yard [10].

Year	Mean	SD[1]	N	SEM	%CV	Min	Max
2001	41.30	74.29	46	10.95	179.89	179.89	480.00
2002	85.95	193.28	6	78.91	224.87	5.88	470.00
2003	93.42	226.07	92	23.57	241.99	0.10	1900.00
2004	67.45	96.09	120	8.77	142.47	0.19	654.00
2005	51.80	64.64	193	4.65	124.78	0.03	613.90
2006	36.76	42.39	77	4.83	115.31	0.27	180.44
2007	32.50	35.38	80	3.96	108.85	0.07	185.70
2008	47.05	48.25	62	6.13	102.57	0.00	262.12
2009	43.86	43.38	14	11.59	98.91	4.85	152.27

[1]SD = standard deviation; N= sample size (l); SEM = standard error of the mean; %CV = coefficient of variation; Min. = minimum; Max. = maximum.

Excessive amounts of chemical nitrogen fertilizers are applied in agriculture in many parts of the world under a broad spectrum of climatic conditions. Humid weather conditions may cause nitrate leaching, leading to pollution of surface and ground water resources. The consequences are eutrophication of surface waters and contamination of groundwater with nitrate. Such raw water sources should no longer be used as sources of potable water without treatment [11-13]. The horizontal subsurface drainage system, in addition to controlling water table and leaching out harmful dissolved salts from the drained soil profile, may also cause losses of various forms of nitrogen through the drainage effluent [14]. Such nitrogen losses, besides wasting a part of the applied fertilizer, are also likely to cause environmental degradation that will be detrimental to aquatic life, plants, and animals. Nitrogen leaching through subsurface drainage systems has been studied under different irrigation and fertilizer management regimes for semi-arid, arid and humid climates [15-17]. Gheysari et al. [18] studied NO_3-N leaching from a soil depth of 30 cm under different nitrogen fertilizer levels and different irrigation systems. The estimated leached NO_3-N ranged from 3.1 kg ha^{-1} at no N application and deficit irrigation to 40.8 Kg ha^{-1} at fertilization level of 142 kg N ha^{-1} and full irrigation, which would be the minimum annual N-requirement for corn in European countries.

2.2. Nitrogen in domestic wastewater

Effluent of domestic wastewater treatment plants contains high concentrations of inorganic nitrogen that may lead to eutrophication of the receiving water bodies [19, 20]. In Rajasthan, India the raw sewage received at the activated sludge plant has a BOD of 600–800 mg l^{-1} and a NH_4^+-N concentration of 80–110 mg l^{-1} during summer when water shortage was acute [21]. Ammonia and eventually nitrate in the effluent caused eutrophication. The disposal of domestic wastewater in areas not served by sewer systems is almost exclusively by use of septic tanks and seepage fields. Effluents from septic tanks generally contain high concentrations of ammonia. Zeng et al. [22] found that the ammonia concentration in real domestic wastewater from one septic tank in China was 54-74 mg l^{-1}. A similar finding was reported by Guo et al. [23]. The effluent of septic tanks is usually discharged to aerobic seepage fields, where ammonia and organic nitrogen are transformed to nitrate, which may be trickling into the groundwater [24]. Table (2) summarizes the values of total Kjeldahl nitrogen (TKN) and ammonia nitrogen (AN) found in domestic wastewaters in different locations during the recent years.

2.3. Nitrogen in agro-industrial and industrial wastewaters

The concentration of nitrogen compounds in some industrial wastewaters is tremendously higher than what is found in agricultural and domestic wastewater. Ammonia and nitrate are the most

problematic nitrogen compounds in this sort of wastewater. Ammonia in industrial wastewater is normally eliminated by nitrification which is achieved by the complete oxidation of ammonia. Thus, nitrate removal from these types of industrial wastewater is an inevitable step in treatment. Different industrial and agro-industrial wastewaters are reported to contain more than 200 mg l^{-1} NO_3^--N [25, 26] and some contain even higher nitrate levels. For instance, the wastewater from glasshouses contained 325 mg l^{-1} NO_3^--N [27]. Several other industries generate wastewater with varying amounts of nitrate, being 222 mg l^{-1} in a tannery wastewater of Pisa. Italy, [28], 2320 mg l^{-1} in wastewater from the cochineal insects processing to produce natural carmine used principally as a coloring agent in cosmetics, beverages and products with low pH [29], 3600 mg l^{-1} generated from an initiating explosive factory in China [30] and 4000-6000 mg l^{-1} produced during the frosting process of bottles in a winery [31]. In Egypt the El-Nasr Pharmaceutical and Chemical Company, South-East of Cairo, discharges both industrial (6000 m^3 d^{-1}) and municipal wastewater (128 m^3 d^{-1}) into a nearby evaporation pond without any treatment. The generated raw wastewater is characterized by high values of ammonium (about 300 mg l^{-1}) [32].

Table 2. Concentrations of TKN and AN in different domestic wastewaters.

Location	Description	TKN (mg l^{-1})	AN (mg l^{-1})	Reference
Belgium	Collected domestic wastewater samples for a period of 450 days.	40	24 ±11	[183]
Australia	Weekly collected domestic wastewater samples after on-site primary edimentation and predenitrification treating.	43	-	[184]
China	Domestic wastewater derived mainly from restaurants and dormitories.	70	40	[185]
Nigeria	Samples collected from a septic tank.	17	13	[186]
China	Samples collected from a septic tank.	85	79	[187]

TKN = total Kjeldahl nitrogen.
AN = ammonia nitrogen.

3. Processes for N-removal

3.1. Nitrification/denitrification

Biological autotrophic nitrification followed by heterotrophic denitrification has long been applied in municipal wastewater treatment. For application of nitrification-denitrification in industrial wastewater treatment, however, things become more complicated because the characteristics of wastewaters vary case by case and sometimes even day by day. Wastewater from antibiotics production, for instance, usually contains large amounts of fermentation products, some residual antibiotic activity, and a high concentration of ammonia. These wastewaters and some fermentation byproducts may not be easily utilized by denitrifiers as electron donors, and the residual antibiotics have a toxic effect on microorganisms [33].

Under strict aerobic conditions, complete nitrification is carried out in two sequential oxidative stages: ammonia is first converted to nitrite by ammonia-oxidizing bacteria:

$$NH_4^+ + 1.5O_2 \rightarrow NO_2^- + H_2O + 2H^+ \quad (1)$$

Then the nitrite is further converted to nitrate by nitrite-oxidizing bacteria:

$$NO_2^- + 0.5O_2 \rightarrow NO3^- \quad (2)$$

Each oxidative stage is performed by different bacterial genera which use ammonia or nitrite as an energy sources and molecular oxygen as electron acceptor, while carbon dioxide is used as a carbon source. The most commonly recognized genus of bacteria that carries out ammonia oxidation is *Nitrosomonas.* However, *Nitrosococcus*, *Nitrosopira*, *Nitrosovibrio* and *Nitrosolobus* are also able to oxidize ammonium to nitrite. These ammonia oxidizers are genetically diverse, but related to each other, and can be found in the beta subdivision of the *Proteobacteria*. For nitrite oxidation several genera such as *Nitrospira*, *Nitrospina*, *Nitrococcus*, and *Nitrocystis* are known to be involved. However, the most famous nitrite oxidizing genus is *Nitrobacter*,

which genetically is closely related within the alpha subdivision of the *Proteobacteria* [34]. The complete nitrification, as seen during wastewater treatment can be expressed as follow:

$$NH_4^+ + 2O_2 \rightarrow NO_3^- + H_2O + 2H^+ \quad (3)$$

In a subsequent process denitrification is generally performed by heterotrophic denitrifyers under anoxic conditions. The oxidized nitrogen compounds (NO_2^- and NO_3^-) are reduced to gaseous nitrogen by heterotrophic microorganisms that use nitrite and/or nitrate instead of oxygen as electron acceptors and organic matter as a carbon and energy source. Denitrifiers are common among the Gram-negative bacteria such as *Pseudomonas*, *Alcaligenes*, *Paracoccus*, and *Thiobacillus*. Some Gram-positive bacteria (such as *Bacillus*) and a few halophilic archaeal microorganisms (e.g. *Haloferax denitrificans*) are able to denitrify [35, 36]. Unlike some contaminants which are in need for a certain microbe to be treated, denitrifying bacteria are ubiquitous in nature [37] and numerous researchers cultivated them using mixed cultures taken from wastewater treatment plants as seeds. There has been a huge interest towards microbial removal of nitrate as the most environmentally friendly and cost-effective method, although biological denitrification may be slow, particularly for industrial wastewaters that contain high concentrations of nitrate [38]. The process of heterotrophic denitrification in environmental biotechnology is accomplished with a variety of electron donors and carbon sources. Both liquid and solid forms of organic carbon sources are conventionally used although the aqueous type is more common for treatment of water and wastewater. Among liquid carbon sources, the most common ones are methanol, ethanol [39, 40] and acetic acid which have been used for wastewater denitrification as well as in full-scale plants of drinking water treatment [41]. A combined carbon source using methanol and acetic acid was found to be superior in nitrogen removal and additional benefits of this mixed carbon source included the excellent sludge settling properties compared to the use of methanol or acetic acid alone [42]. Park et al. [43] treated waste plant material either physically or biologically to produce several organic carbon rich liquors for use in denitrification experiments. The choice of substrate depends on a number of considerations such as costs, capacity and configuration of reactors and on the post-treatment process of the denitrified water. The theoretical methanol requirement for nitrate is 2.47 mg CH_3OH per mg NO_3-N as indicated in equation (4) [44].

$$NO_3^- + 1.08CH_3OH + 0.24H_2CO_3 \rightarrow 0.056C_5H_7O_2N + 0.47N_2 + 1.68H_2O + HCO_3^- \quad (4)$$

Later studies tried to speed up biological denitrification by applying different process strategies through which a better contact of the nitrate in the water with microorganisms was maintained, such as packed beds [45], rotating biological contactors [46] and fiber-based biofilm reactor [47]. Efforts are still ongoing and some novelties in combination of biological and other methods, such as membrane biofilm reactors (MBR), were manifested [48].

3.2. Simultaneous nitrification and denitrification (SND)

The SND process starts with a partial nitrification of NH_4^+ to nitrite and subsequently continues with a direct reduction of nitrite to N_2 gas [49, 50]. In SND nitrification and denitrification occur concurrently in the same reactor vessel under identical operating condition. If successful, this process could reduce the relatively large reactor volumes and energy costs for recirculation that are required for a separated aerobic and anoxic system. Several types of treatment units have been proposed in which SND can be realized [51]. Zhang et al. [52] introduced a flexible biofilm reactor having adjustable aerobic, buffer and anoxic zones with liquid circulation being dependent on the aeration flow rate. Both studies were successful in proving the possibility of nitrification and denitrification in one reactor. Successful SND experiments were also carried out by Walters et al. [53] who used a biofilm airlift suspension reactor with biodegradable carrier material. Investigation of Fux et al. [54] of the shortened nitrogen removal pathway via nitrite revealed a high reduction of the COD demand for denitrification, a high rate of denitrification, low biomass yield during anaerobic growth and no apparent nitrite toxicity effects for the microorganisms in the reactor. SND is also effective in maintaining a neutral pH level in the reactor, without the addition of an acid or base. This is important since the optimal pH for the nitrifying and denitrifying bacteria lies between 7 and 8.5 [55]. Further, Ma et al. [56] constructed a bench-scale continuous flow system, consisting to remove nitrogen and carbon simultaneously from terramycin crystallization mother solution (TCMS). Approximately 82% of the chemical oxygen demand (COD) and 81% total nitrogen were removed by the system when tap water diluted TCMS was continuously fed (dilution ratio, 1:4). Sulfide which was produced during anaerobic hydrolysis was used as part of electron donors for denitrification in the anoxic reactor.

Polymeric beads, in which a nitrifier and a denitrifier were co-immobilized, were used to remove

nitrogen in a single step. Uemoto and Saiki [57] investigated a novel immobilized-cell bioreactor containing packed gel envelopes capable of simultaneous nitrification and denitrification. The packed gel envelopes consisted of two polymeric gel plates with an internal space between them for injecting the electron donor for denitrification. An ammonia oxidizer, namely, *Nitrosomonas europaea*, and a denitrifier, namely, *Paracoccus denitrificans*, were coimmobilized in the plate gel. The immobilized *N. europaea* oxidized ammonia to nitrite on the outer surface of the plate that was in aerobic contact with the wastewater containing ammonia; the immobilized *P. denitrificans* reduced nitrite to nitrogen gas on the inside of the plate that was in anaerobic contact with the electron donor. This system did not require an additional aerobic step because the electron donors were not supplied to the wastewater directly but to the internal space of the gel plate. This resulted in an increase of the utilization efficiency of the electron donor for the denitrification process and a decrease in the quantity of surplus sludge. In another attempt, a bioreactor system with 30 packed gel envelopes was installed in a thermal power plant for the removal of nitrogen from ammonia-containing desulfurization wastewater. Each envelope consisted of double-sided plate gels containing *Nitrosomonas europaea* and *Paracoccus denitrificans* cells with an internal space in between for injecting an electron donor. The envelope could remove ammonia from wastewater in a single step. During continuous wastewater treatment with the bioreactor system 95.0% removal of the total nitrogen was obtained. The total nitrogen concentration in the outlet was below 9 mg l^{-1}. Since the bioreactor system could use the electron donor effectively, it was not necessary to use an additional aerobic tank to remove the electron donor and a settling tank to segregate the surplus sludge containing bacteria from wastewater [58].

3.3. Autotrophic denitrification

The heterotrophic denitrification rate was strongly dependent on the type of carbon source, the concentration of the carbon source and the C/N ratio [59]. This could vary for different microorganisms, water streams, and environmental conditions [60]. In contrast, autotrophic denitrifiers utilized inorganic carbon substrates (carbon dioxide or bicarbonate) as a sole source of carbon. Some advantages of autotrophic over heterotrophic denitrification are; evasion of the poisoning effect of some organic carbon, low biomass build-up and less sludge production which results in reduction of reactor clogging and easier post-treatment [61]. Since some wastewaters have a very low concentration of biodegradable organic materials, autotrophic denitrification, which utilizes CO_2 from water as carbon source requires addition of an electron donor substrate. Extensive studies have been carried out on elemental sulfur [62-66] and H_2 [67-69] as electron donors for autotrophic denitrification systems. Under typical aquifer conditions, iron sulphide (pyrite) is typically expected to be the electron donor [70] for denitrification assisted by *Thiobacillus denitrificans*:

$$5\ FeS_2 + 14\ NO_3^- + 4\ H^+ \rightarrow 7\ N_2 + 10\ SO_4^{2-} + 5\ Fe^{2+} + 2\ H_2O \quad (5)$$

In the sulfur-limestone autotrophic denitrification (SLAD) process element sulfur is used as electron donor and limestone is used to adjust the pH, but an increase of the sulfate concentration and hardness limits its application. Hydrogen gas would be an ideal electron donor for biological autotrophic denitrification. It has, however, a poor solubility in water. A biofilm-electrode reactor as a combined electrochemical and biological reactor was developed by Sakakibara et al. [71] and improved by Prosnansky et al. [72] to solve these problems. In this system, autotrophic denitrifying microorganisms are immobilized on the surface of the cathode and hydrogen gas as an electron donor is produced by electrolysis of water. Combining this bioelectrochemical and sulfur autotrophic denitrification system for water denitrification was proposed by Wang and Qu [73] and applied at large scale by Wan et al. [66]. In such a process, sulfur and hydrogen autotrophic bacteria were integrated for the following reasons: the H^+ generated during denitrification with sulfur could be consumed by the bioelectrochemical denitrification with hydrogen to achieve neutralization, thus the limestone added into the SLAD system could be left away and the hardness increase could be avoided; the sulfate concentration of the effluent could be controlled by the nitrogen load of the autotrophic sulfur denitrification process, and would be lower than in the SLAD process. In general *Thiobacillus denitrificans* and *Thiomicrospira denitrificans* are the two most commonly reported autotrophic denitrifiers [74]. Because in nature these bacteria are likely to encounter autotrophic and heterotrophic conditions, it is of considerable interest that their nitrate removal characteristics under mixotrophic conditions are determined.

3.4. Anaerobic ammonium oxidation (Anammox) process

Anaerobic ammonium oxidation (anammox) has received special attention since its discovery, because it is an efficient biological alternative to conventional nitrogen removal from wastewaters. Under anaerobic conditions, ammonium is oxidized

to nitrogen gas with nitrite as the electron acceptor (Eq. 6) and carbon dioxide is used for growth of the anammox microorganisms involved. In comparison to traditional nitrification–denitrification process, this autotrophic process consumes 100% less biodegradable organic carbon and at least 50% less oxygen [75] and has, therefore, lower operating cost.

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2\,H_2O \quad (6)$$

Anammox needs ammonium and nitrite in a ratio of roughly one to one. For sludge digester effluents, this ratio can be achieved without control, because these effluents contain bicarbonate as the counter ion for ammonium. When half of the ammonium is converted, the alkalinity of the water is depleted leading to a drop in pH and preventing further nitrification (Equation Eq. (7) [76]:

$$NH_4^+ + HCO_3^- + 0.75\,O_2 \rightarrow 0.5\,NH_4^+ + 0.5\,NO_2^- + CO_2 + 1.5\,H_2O \quad (7)$$

If the Anammox process is combined with a preceding nitrification step, only part of the ammonium needs to be nitrified to nitrite, while the Anammox process combines the remaining ammonium with the nitrite to yield dinitrogen gas. This will reduce the oxygen demand in the nitrification reactor and reduce costs. The biomass yield is very low, and consequently, little sludge is produced. This is another factor that contributes to substantially lower operation costs of Anammox compared to the conventional denitrification process. However, the low biomass yield also necessitates an efficient system for sludge retention, and long start-up times are required to obtain a sufficient biomass concentration [77].

The possible metabolic pathways for anaerobic ammonium oxidation are shown in Fig. 1 [78]. Using ^{15}N-labelling the experiments showed that the electron acceptor nitrite is reduced to hydroxylamine and that hydroxylamine somehow reacts with the electron donor ammonium, leading to the ultimate production of dinitrogen gas. In batch experiments with excess hydroxylamine and ammonium, a transient accumulation of hydrazine was observed, indicating that hydrazine is the intermediate of this final step. Jetten et al. [77] postulated that the oxidation of hydrazine to dinitrogen gas generates the electrons for the initial reduction of nitrite to hydroxylamine. It is well known that occurrence of free hydrazine in microbial nitrogen metabolism is rare, if not unique [79].

The anaerobic ammonium oxidizing bacteria (AnAOB) are autotrophic members of the *Brocadiales*, belonging to the phylum *Planctomycetes*, which is one of the major distinct divisions of the bacteria. Currently, five genera of AnAOB have been reported: *Candidatus brocadia*, *Candidatus kuenenia*, *Candidatus scalindua*, *Candidatus anammoxoglobus*, and *Candidatus jettenia*. However the most common AnAOB are "*Brocadia anammoxidans*" [80] and "*Kuenenia stuttgartiensis*" [81]. These two bacteria are very similar. They have the same overall structure and also produce hydrazine from exogenously supplied hydroxylamine. The high Anammox activity is detectable for both bacteria in a pH range between 6.4 and 8.3 and a temperature between 20 °C and 43 °C [82]. The optimum pH and temperature of the two organisms are very similar. These bacteria have a highly unusual physiology, in that they live by consuming ammonia in the absence of oxygen. Furthermore, these metabolically versatile bacteria are, for example, capable of oxidizing short chain fatty acids with nitrate [83], co-oxidizing propionate and ammonium in the presence of nitrite and nitrate [84], and performing dissimilatory nitrate reduction to ammonium [85]. Anammox is highly exergonic and linked to the energy metabolism of the organisms involved. In addition, Anammox bacteria were recently shown to be able to tolerate higher O_2 concentrations than originally established by Strous et al. [86] being metabolically active at oxygen concentrations of up to ~13 µmol O_2 l^{-1} [87]. Altogether, these results have important ecological and biogeochemical implications, since they extend the metabolic and environmental spectra of these bacteria.

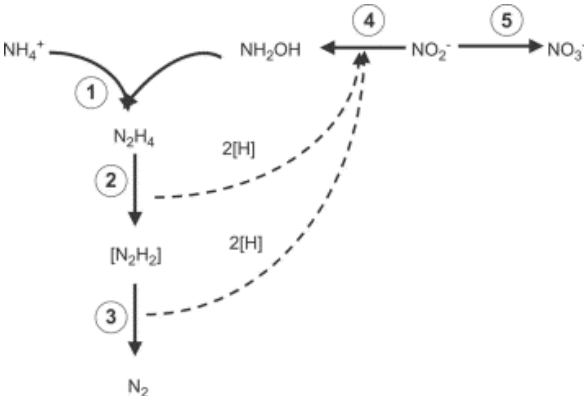


Figure 1. Metabolic pathways for anaerobic ammonium oxidation

The Anammox process is suitable for wastewater with low C:N ratios. At C:N ratios above 1, the Anammox bacteria are no longer able to compete with heterotrophic denitrifying bacteria . The organic loading rate was found to affect the Anammox process performance, but the exact inhibitory levels still remain unclear [88, 89]. An organic matter concentration above 300 mg COD l^{-1} was found to inactivate Anammox communities in a

UASB reactor fed with fat milk as organic matter source [90]. Concentrations of 50 mM of acetate resulted in 70% inhibition of the Anammox process [91]. Therefore it is necessary to clearly establish the COD levels inhibiting the Anammox process.

The Anammox process has also been maintained easily in a gas lift reactor achieving nitrogen removal rates of up to 8.9 kg $N/m^3 \cdot day$. This removal rate was 20 times higher compared to the removal rates previously achieved in the laboratory [92]. Ammonia removal via Anammox has been developed for the treatment of many different wastewaters with low organic matter content (below 1700 mg COD l^{-1}), such as water from the secondary clarifier of a municipal wastewater treatment plant in a down flow biofilter [93], nitrous organic wastewater in ASBR reactors and landfill leachate in a continuous reactor [94]. Only a few studies have investigated the possibility of using the Anammox process for ammonia removal from animal waste treatment water, which is indeed a residue with high organic matter and nitrogen content [95]. However, there is still a big gap regarding the effect of different pre-treatments (reducing organic and ammonia loads) of the wastewater streams on Anammox process performance. Up to 98.5 ± 0.8% of ammonia was removed from a diluted partially oxidized pig manure effluent (121 mg COD l^{-1}) using the Anammox process under different organic loadings in a semi-continuous UASB reactor. Mass balance clearly showed that an increase in organic loading (from 121 mg COD l^{-1} to 290 mg COD l^{-1} negatively affected the Anammox process and facilitated heterotrophic denitrification [5].

3.5. Partial nitrification/Anammox

The increases in the operating costs of wastewater treatment systems is challenged by a novel microbial process, combining the Anammox reaction with partial nitrification in one reactor, entitled CANON (completely autotrophic nitrogen removal over nitrite) [96]. This combination of the preceding partial nitrification and the subsequent anaerobic ammonium oxidation is regarded as a promising new method of removing nitrogen from wastewater with a low C/N ratio and a large quantity of ammonium [97]. Compared to the conventional nitrification and denitrification process, more than 50% [98] or 62.5% [99] less oxygen demand and the non-requirement of organic carbon addition, in the combined partial nitrification/Anammox process offer considerable cost savings. The combination of partial nitrification and Anammox is based on the fact that nitrite is an intermediary compound in both. Therefore, it will be convenient and economical to achieve 50% partial nitrification up to a condition wherein one-half of ammonia is converted to nitrite and the other half is not, followed by the anammox to ensure total nitrogen removal throughout an autotrophic process. [100, 101]. In the CANON systems, *Nitrosomonas*-like aerobic ammonium-oxidizing bacteria and *Planctomycete*-like Anammox bacteria perform two sequential reactions simultaneously under oxygen limited conditions [102, 103]. The nitrifiers oxidize ammonium to nitrite, consume oxygen and so create anoxic conditions needed by the Anammox bacteria. The produced nitrite is utilized with the remainder of the ammonium by Anammox bacteria and converted into dinitrogen gas [104]. Equations number (8), (9) and (10) represent these reactions:

$$2NO_3^- + 12H^+ + 10\ e^- = N_2 + 6H_2O \quad (8)$$

$$NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- + 0.13H^+ \rightarrow 0.066\ CH_2O_{0.5}\ N_{0.15} + 1.02\ N_2 + 0.26\ NO_3^- + 2.03\ H_2O \quad (9)$$

$$NH_4^+ + 0.85\ O_2 \rightarrow 0.44\ N_2 + 0.11\ NO_3^- + 1.43\ H_2O + 0.14\ H^+ \quad (10)$$

Typically in the CANON process the *Nitrosomonas*-like ammonium-oxidizing bacteria are active in the outer aerobic region of both biofilm and aggregates, while Anammox bacteria are active in the inner anoxic region. This way the Anammox bacteria are protected from oxygen, which is consumed in the outer aerobic region. Oxygen would inhibit the Anammox activity [86]. The cooperation of these two groups of ammonium-oxidizing bacteria results in completely autotrophic nitrogen removal under oxygen limited conditions in one single reactor.

The CANON process has quite sensitive operational characteristics for dissolved oxygen, the nitrogen-surface load, biofilm thickness and temperature, etc. [105]. The oxygen-mass transfer efficiency from gas to the liquid phase and effective biomass retention are considered two key rate-limited factors for the operation of a CANON system [92]. Moreover, the growth rate of autotrophic ammonium oxidizing bacteria is lower than that of heterotrophic bacteria, with which they have to compete for oxygen. Without long retention times the suspended nitrifiers will be easily washed out of the reactor. The biomass concentration is increased by recirculation of the sludge after sedimentation, but limited by the efficiency of the sedimentation vessel. Besides, the ammonia oxidation rate is strongly influenced by the nature of nitrifying cultures and a variety of environmental factors, including substrate concentration, dissolved oxygen, temperature and pH. To overcome these problems and to promote the oxygen-mass transfer in a high biomass retention reactor configuration, immobilization techniques can

be used. This is an important challenge in order to scale up CANON systems from laboratory to industrial application [106, 107]. Immobilization is an efficient method to prevent biomass from being washed out and allows hyperconcentrated cultures. This can lead to relatively small reactors and provide some protection from adverse temperatures and toxic shocks, which would help in maintaining year-round treatment [108]. Immobilized biomass can be divided into "naturally" attached biomass (biofilm) and "artificially" immobilized biomass. Biofilms have been widely applied in wastewater treatment. However, some particles can become anaerobic in the centre and settle to the reactor floor. Membrane-aerated biofilm reactor (MABR) represent a new technology for aerobic wastewater treatment, in which hydrophobic, gas-permeable membranes are used for bubbleless oxygen transfer [109, 110]. Membrane aeration is advantageous because gas transfer efficiencies are much higher than conventional bubble diffusers. In an MABR, the microporous membranes play two roles: the oxygen gas supplemental material and the carrier for bacterial immobilization [111]. The oxygen on the lumen side of the membrane is transported through the pores of the membrane wall without the formation of bubbles and utilized by microorganisms in the membrane attached biofilm. Extremely high oxygen-mass transfer efficiency can be achieved [112, 113]. Recent research has demonstrated that thick membrane-aerated biofilms can simultaneously provide favorable conditions for both nitrification (near the membrane) and denitrification (near the biofilm-liquid boundary) within a single biofilm [114]. Gong et al. [107] developed a novel MABR, equipped with non-woven fabrics support around the microporous carbon tube membrane, and investigated its feasibility and process performance of the CANON-type single-stage autotrophic nitrogen removal to treat the synthetic ammonium-rich wastewater like anaerobic sludge liquids. This reactor allowed air to be supplied through the microporous carbon tube wall to the biofilm that was supported by non-woven fabrics. The partial nitrification and consumption of dissolved oxygen occurred in the inner layer and Anammox in the anoxic outer layer of the non-woven fabrics, thus realizing autotrophic nitrogen removal in a single reactor. This study demonstrated that MABR was a very suitable experimental set-up for the operation of the single-stage autotrophic nitrogen removal process.

One of the most common techniques for artificially immobilization is gel entrapment. Both natural and synthetic polymers can be used as the immobilization support, but it must fulfill various requirements, such as photo-transparency, non-toxicity, retention of cellular viability, and stability in the culture medium. This immobilization technique is commonly used to immobilize a pure strain of bacteria because the mechanisms of pure strains are more easy to understand [115, 116]. Nevertheless, the immobilization of activated sludge has also been reported [117, 118]. Compared to pure strains of bacteria, immobilization of activated sludge could remove multiple pollutants due to the biodiversity of the activated sludge. Yan et al. [106] studied the characteristics of the partial nitrification and degradation of organics with an immobilized biomass in treating ammonium-rich organic wastewater. It serves as a first step in the Anammox process with partial denitrification via nitrite. They used four materials, i.e. sodium carboxymethylcellulose, sodium alginate, polyvinyl alcohol and sodium alginate, and chitosan for entrapping the biomass. Sodium alginate was selected as the best entrapment support after comparing partial nitrification rates and the adsorption efficiency.

4. Factors affecting nitrogen removal efficiency

4.1. Effect of temperature

The temperature range of 22–37 °C gave best results in terms of maximum nitrogen and carbon removal from a shrimp aquaculture wastewater [119], but denitrification processes will normally occur in the range 2–50 °C [120] and possibly beyond, where bacteria have evolved to cope with specific environmental conditions. Groundwater temperatures are typically around 10 °C (in northern Europe), with the exception of shallow groundwaters impacted by extreme surface temperatures. Reaction rates are typically assumed to double for every 10 °C increase in temperature (i.e. Arrhenius rate law). Elefsiniotis and Li [121] investigated the role of temperature within the range of 10–30 °C on biological denitrification using synthetically produced volatile fatty acids as carbon sources. Their results confirmed that a temperature change from 10 to 20 °C exerted a greater effect on both the specific denitrification and carbon consumption rates than a further temperature increase from 20 to 30 °C, which was also evident in the corresponding temperature coefficient values. At a given temperature, the specific denitrification rate appeared to depend on the initial nitrogen concentration, while the specific carbon consumption rate was a function of the initial carbon content. The nitrogen removal capabilities of the denitrification process, when treating sanitary landfill leachate containing an ammonia concentration of over 2200 mg N/l^{-1}, were investigated at operating temperatures down to 10°C. When the operating temperature was decreased from 20 to 17°C, an approximate 15%

decrease in denitrification was immediately experienced, with no noticeable effect on nitrification. With the temperature of 14°C, aerobic wasting was also stopped and methanol (carbon source for denitrification) loading was progressively decreased to match actual denitrification requirements. At 10°C, the system suffered major nitrification and denitrification inhibition. Changes in operating parameters, such as a decrease in influent ammonia and methanol loading, as well as an increase in ambient temperatures, from 10 to 15°C, did not significantly improve the overall system performance, within a reasonable time frame. Changes in the rate of denitrification with seasonal temperature variations may be masked by variations in the rate of organic carbon flux. For example, freeze–thaw cycles increase the flux of carbon to the unsaturated zone and can create anaerobic micro-environments in which denitrification can be established [122].

Present reports show that high temperature of 28–38 °C is favorable for nitrogen removal via nitrite due to the fact that the specific growth rate of AOB is higher than that of NOB [123]. The reports about the effect of temperature on nitritation can be grouped into two classes: (1) achievement and maintenance of nitritation at high temperatures of 28–35 °C [124], and (2) start-up of nitritation at high temperatures and a gradually decline of the temperatures. Nitritation was maintained at room or low temperatures [125]. Some researches also proved that nitritation start-up could be promoted and accelerated at high temperatures [126]. However, the temperatures of real domestic wastewater (usually at 10–25 °C), especially in winter, cannot reach the optimal temperature of 30 °C for nitrogen removal via nitrite. In the temperature range of 10–20 °C, a high nitrite accumulation rate can hardly be maintained due to the fact that the specific growth rate of NOB is higher than that of AOB [127]. Therefore, a relatively low wastewater temperature such as in domestic wastewater is the major obstacle for achievement and full-scale application of nitrogen removal via nitrite. However Zeng et al. [22] achieved nitritation at a temperature of 19 ± 1 °C by controlling the dissolved oxygen (DO) concentration and pH. The dominance of ammonia oxidizing bacteria (AOB) was enhanced through the combination of a low DO concentrations (<1.0 mg/l) and a preset short-cycle control of the aeration time. Nitritation was successfully established with a NO_2^--N/NO_x^--N ratio over 95%.

Several authors [128,129] found that the optimum temperature for the operation of the Anammox process was around 30–40 °C. Perhaps for this reason, most of the works where this process was applied were carried out at temperature values higher than 30 °C [130]. Recently, Cema et al. [131] proved that a rotating biological contactor (RBC) with an established Anammox process could be successfully operated at temperatures around 20 °C. Similar results were reported by other workers [132, 133] who operated an anaerobic biological filtrate reactor (ABF) which treated 8.1 g N $(l\ d)^{-1}$. Moreover, several works done with marine Anammox samples reported measurable activities at low temperatures. Rysgaard et al. [134] working with sediments of the east and west coasts of Greenland, observed Anammox activity between −2 and 30 °C, the optimum temperature being 12 °C. Similar results were found by Dalsgaard and Thamdrup [135] working with marine sediments from the Skagerrak (Baltic-North Sea). These results indicate that the application of the Anammox process must not be restricted to effluents with temperatures around 30 °C. Therefore, Dosta et al. [136] evaluated the effects of moderately low temperatures on the stability of this process. First, the short-term effects of temperature on the Anammox biomass were studied using batch tests and the maximum activity was found at 35–40 °C. Activity tests done at 45 °C showed an irreversible loss of the activity due to biomass lysis. Temperatures from 30 to 15 °C were used to determine long-term effects. The system was successfully operated at 18 °C but when the temperature was decreased to 15 °C, nitrite started to accumulate and the system lost its stability. On the other hand, some authors reported that the denitrification rate showed only a rather weak dependence on the temperature, the rate at 3°C being approximately 55% of that at 15°C. The maximum denitrification rate obtained at 15°C was 2.7 g NO_x^--N m^{-2} carrier d^{-1}. The maximum denitrification rate at 3°C during an 8-day period was found to be constant [137].

4.2. Effect of dissolved oxygen

Denitrifiers are facultative bacteria that energetically prefer oxygen over nitrate as the terminal electron acceptor. Denitrifying bacteria use nitrogen oxides as terminal electron acceptors most rapidly in the absence of oxygen. Thus, the dissolved oxygen (DO) concentration has an important influence on the success of the nitrogen removal process. A high DO plays a crucial role in nitrification and has a negative influence on biological denitrification. DO can inhibit denitrification because oxygen functions as the electron acceptor for microorganisms over nitrate and aerobic conditions repress enzymes involved in denitrification [138]. Although high DO concentrations are necessary to enhance the activity

of nitrifying bacteria in the biofilm reactor, denitrification is inhibited by oxygen. Lowering the aeration rate i.e. operating the wastewater treatment at low DO concentrations is a possible measure to control the inhibitory effect of DO on denitrification [139]. The negative effects of high DO concentrations on the denitrification process depended on the carbon source. Denitrification with alcohols such as ethanol and methanol was less affected by DO than with sucrose. The development of a biofilm was also influenced by the DO concentration as excess O_2 caused reduced biofilm growth. Biofilms that developed in presence of oxygen revealed a smaller bacterial density and a smaller atio of denitrifying versus nitrate reducing bacteria, which led to an unfavorable inorganic nitrogen removal and the presence of nitrite in the treated water. All these effects were more pronounced when sucrose was used as carbon source [140].

Until now, a significant amount of research has focused on the partial nitrification and SND achieved by low DO [141]. Using low DO, Blackburne et al. [142] achieved partial nitrification to nitrite in a lab-scale continuous-flow reactor treating synthetic wastewater containing ammonium as the sole energy source. Ma et al. [143] showed a clear correlation between nitrite accumulation and low DO levels in a continuously run pilot plant. For nitrogen removal via nitrite with real wastewater the nitrite pathway in a continuous-flow system has not been fully demonstrated previously [144]. However, Ma et al. [145] established the nitrite pathway in a pilot-scale continuous pre-denitrification plant (V = 300 L) treating domestic wastewater by controlling the DO concentration at 0.4–0.7 mg/l. It was demonstrated that the nitrite pathway could be repeatedly and reliably achieved, with over 95% of the oxidized nitrogen compounds at the end of the aerobic zone being nitrite. The nitrite pathway improved the total nitrogen removal by about 20% in comparison to the nitrate pathway, and also reduced aeration costs by 24%. Moreover, the short-term effect of DO on biological nitrogen removal has been discussed in many studies using batch test [146, 147]. With the exception of the report of Guo et al. [148], limited reports are available on comparisons of partial nitrification performance under different DO for long-term operation. It is still doubtful whether high a DO level would destroy the stable and high nitrite accumulation ratio built by low DO or other operational factors. It is also not very clear whether high DO would cause the recovery of NOB after long time operation. Guo et al. [148] found that the average efficiencies of SND in a high DO (above 3 mg/l on average) and a low DO (0.4–0.8 mg/l) reactor were 7.7% and 44.9%, and the specific SND rates were 0.20 and 0.83 mg N/(mg MLSS h), respectively. Low DO did not produce sludge with poorer settling properties but attained lower turbidities of the effluent than high DO. AOB were the dominant nitrifying bacteria and NOB did not be recovered in spite of exposing nitrifying sludge to high DO.

4.3. Effect of nitrate concentration

Excess nitrate concentrations affect the denitrification process by inhibiting the formation of N_2 gas and causing the denitrification process to terminate with the formation of N_2O [149]. A small number of research studies has been published to date on the denitrification of wastewater containing nitrate at concentrations higher than 600 mg NO_3-N l^{-1} [150]. Biological denitrification of high nitrate concentration in wastewater is a slow process. To increase the rate of denitrification, parameters such as pH, temperature, COD/NO_3^--N and biomass concentration of the process must be optimized. Acclimatization of sludge to nitrate wastewater is one of the methods used to develop the suitable consortium to treat high strength nitrate wastewater. Sludge, generally consists of different types of bacteria, broadly divided into two categories viz; nitrate tolerant and nitrate intolerant bacteria. Nitrate tolerant bacteria include nitrate respirators (capable of reducing nitrate to nitrite) and true denitrifiers (capable of reducing nitrate to nitrogen). The growth rate of nitrate tolerant and nitrate intolerant bacteria varies depending upon nitrate concentrations. At high nitrate concentration, the population of nitrate tolerant bacteria multiplies faster than that of nitrate intolerant bacteria. Thus, acclimatization is essentially a process of manipulating differential growth rates of two types of bacteria to obtain a desired population balance by subjecting them at controlled nitrate concentration. To acclimatize the sludge for treating high nitrate wastewater, it is subjected to high nitrate concentrations in which nitrate tolerant bacteria outgrow nitrate intolerant bacteria [153].

4.4. Effect of salinity

The effect of high salt concentration on nitrification and denitrification has been previously investigated [154, 155]. Seawater has been used as an alternative water source for toilet flushing in some arid areas such as Hong Kong and some other coastal cities, resulting in a high salt content in the sewage [156, 157]. Salinity levels have a definite impact on the microbial community structure in the wastewater and may affect the nitrification and denitrification process [158] and ultimately the performance of

wastewater treatment systems. High salt concentrations in wastewater induce salt stress to the microbial flora, resulting in the inhibition of many enzymes, decreasing cell activity and eventually leading to plasmolysis [159]. It was reported in these studies that nitrification and denitrification activities were sustained by gradual acclimatization of freshwater sludge to high salt conditions. Halophilic denitrifying bacteria were isolated from the long-term acclimated sludge, and higher denitrification performances were demonstrated when the long-term acclimated sludge was used as inoculums [160]. Furthermore, Furukawa et al. [161] reported that nitrifying sludge taken from a night soil treatment plant employing a sea-water dilution in the summer season could adapt more smoothly to high salt condition than sludge from freshwater.

Rene et al. [162] investigated the effect of different COD/N ratio (3–6) and salt concentrations (up to 3.2%) on organics and nitrogen removal efficiencies in fish market wastewater under different operating schedules. Different combinations of the COD/N ratio and salinity showed a negligible effect on organics removal, while they affected nitrification and denitrification efficiency to a larger extent. However, salt inhibition can be reduced significantly after long time acclimatization of the biomass. The treatment showed high COD (>80%) and nitrogen (>40%) removal efficiencies despite of high loading rates and COD/N fluctuations, which is due to the acclimatization of the biomass within the SBR. Using a sequencing batch reactor for the treatment of shrimp aquaculture wastewater, the results of Fontenot et al. [107] indicated that the salinity of 28–40 parts per thousand, produced best results in terms of maximum nitrogen and carbon removal from the wastewater.

Since the partial nitritation–Anammox process was successfully applied to the treatment of sewage sludge digester liquor, it opened doors for application to many kinds of wastewater treatment such as industrial wastewater, livestock wastewater, and landfill leachate. However, these wastewaters contain high concentrations of salts which have been considered as an inhibition factor in the biological nitrogen removal process [163]. However, marine Anammox bacteria belonging to the genus *Scalindua* have been detected in natural surroundings [164] and recently Nakajima et al. [165] enriched them from an enclosed coastal sea in Japan using a continuous culture system. These results suggested that Anammox bacteria, inherently preferring high concentration of salts and living in the high salt habitats, would be enriched in the cultivations and would be available for industrial application. On the other hand, there is an inconsistent experiment. Kartal et al. [166] adapted *Candidatus Kuenenia stuttgartiensis* and *Candidatus Scalindua wagneri* to a salt concentration of 30 g l^{-1}. Although this would be the culture conditions suitable for growth of *Candidatus scalindua*, they reported that the major Anammox bacteria after the acclimation were *Candidatus kuenenia stuttgartiensis* enriched from freshwater. Because Kartal et al. [166] used the seed sludge containing marine Anammox bacteria besides a freshwater Anammox bacterium, the result that major Anammox bacteria at high salt conditions were freshwater Anammox species is an open question. In addition, Kartal et al. [166] focused on only the population of Anammox bacteria species without the evaluation of the coexistent bacteria community. The effect of high salt concentration on the Anammox treatment was investigated by Liu et al. [167] to establish an acclimation strategy under high salt concentration conditions. An Anammox fixed-bed reactor with non-woven biomass carrier was used and the salt concentration was gradually increased from 2.5 g l^{-1} to 33 g l^{-1}. The Anammox reactor revealed a stable nitrogen removal rate (NRR) of 1.7 kg-N m^3 d^{-1} for 65 days under a salt concentration of 30 g L^{-1}. However, the NRR sharply declined at a salt concentration of greater than 30 g l^{-1}.

4.5. Effect of pH

The pH range preferred by heterotrophic denitrifiers is between 5.95 and 7.9 [168], although the optimal pH level in an anoxic/oxic membrane bioreactor with over 99.9% of nitrate removal and without accumulation of nitrite was 7.5-8.5 [30]. The pH values outside this range may hinder the denitrification process, but the optimal pH is site-specific because of the effects of acclimation and adaptation to the microbial ecosystem. Strongly acidic environments (pH < 5) inhibit denitrification and tend to arrest the denitrification chain with the formation of nitrite or N_2O [120]. In well-buffered calcareous aquifers, such acidification is unlikely [169]. *Halomonas campisalis* (ATCC 700597) however was shown to completely reduce nitrate at 125 g/L NaCl and pH 9 in brine produced from regeneration of ion exchange resins with NaCl, containing a high concentration of nitrate that was difficult to remove using standard biological, physical, or chemical technologies [26]. On the other hand, experiments of nitrate removal from high salinity wastewater are usually carried out without controlling the pH because denitrification from high salinity wastewater favors high pH levels [170]. Heterotrophic denitrification itself can increase the pH because it causes a release of hydroxyl ions and raises alkalinity. Each mg of nitrate-N reduced to N_2

causes an alkalinity increase of 3.57 mg $CaCO_3$ according to the following stoichiometry:

$2NO_3^- + 12\ H^+ + 10\ e^- = N_2 + 6\ H_2O$ [171]

Contrary to heterotrophic denitrification, autotrophic denitrification consumes alkalinity and, in addition, generates high concentrations of sulfate. High sulfate concentrations do not pose an undue problem in coastal areas, where treated wastewater can be discharged directly to the sea, which has a natural sulfate concentration of 2.7 g l^{-1}. Alkalinity of 3.91 g (as $CaCO_3$) will be consumed for reducing 1 g of NO_3^--N to nitrogen gas. Previous research showed that the optimum pH for growth of *Thiobacillus denitrificans* (*T. denitrificans*) cultures was between 6.8 and 8.2, approaching zero at pH 5.5 [172] and with a maximum efficiency at 8.4. Increasing the pH above 8.6 caused a significant decrease in the nitrate removal rate and a dramatic increase in nitrite accumulation [173]. Therefore, alkalinity may have to be supplied to the autotrophic denitrification system to control the pH. The most effective and commonly used alkalinity source in research is $NaHCO_3$. For wastewater of low alkalinity, large amounts of $NaHCO_3$ are required to maintain the autotrophic denitrification process. An alternative and cheaper alkalinity source in conjunction with elemental sulfur particles is granular limestone. If the initial alkalinity of the wastewater is insufficient for complete denitrification, limestone can supply effective buffering capacity [174]. Furthermore Jha and Bose [175] evaluated the suitability of pyrite (FeS_2) as an in situ buffering agent for arresting pH increase during metallic iron assisted hydrogenotrophic denitrification by microorganisms that reduce nitrate to nitrogen gas by utilizing hydrogen as energy source. Pyrite is considered promising for this purpose because it is a mineral which is unstable under moderately reducing, i.e., anoxic conditions, where such denitrification takes place, and therefore is expected to consume hydroxide ions produced due to hydrogenotrophic denitrification reactions and get oxidized to ferrous hydroxide $Fe(OH)_2$. Experimental evaluation of the buffering efficiency of pyrite showed that it was effective in arresting a pH increase associated with denitrification in both, batch systems and during flow through reactive porous media. Furthermore, addition of pyrite had no demonstrable toxic effect on the denitrifying microorganisms, though elevated sulfate concentration was seen in the effluent after denitrification.

4.6. Effect of free ammonia concentration

Traditionally, accumulation of nitrite resulting from higher activities of AOB than NOB is considered undesirable in biological wastewater treatment systems. Factors such as pH, temperature, and the concentrations of DO, CO_2 and heavy metals were all found to influence the nitrite build-up [30]. However, one of the main causes is believed to be the inhibitory effects of free ammonia (FA) [176]. Anthonisen et al. [177] observed that both ammonium and nitrite oxidations are inhibited by FA; inhibition of nitrite oxidation by *Nitrobacter* began at a concentration of 0.1–1.0 mg FA/l, while ammonium oxidation by *Nitrosomonas* became inhibited at 10–150 mg FA l^{-1}, allowing selective inhibition of nitrite oxidation at a range of FA concentrations of 1.0–10 mg/l. Supporting this observation, Bae et al. [178] reported that nitrite accumulation occurred at an initial FA concentration of around 4.7 mg/l, giving a high NO_2/NO_X ratio (up to 77%) in a batch reactor. Chung et al. [179] accomplished a long-term accumulation of nitrite in a continuous-flow reactor by maintaining the FA concentration in the reactor around 20 mg/l. Chung et al. [180], however, found that a FA concentration of 5–10 mg/l was most efficient in inhibiting nitrite oxidation without slowing down the rate of ammonium oxidation. To have appropriate kinetic expressions for both ammonium oxidation and nitrite oxidation under inhibition, Park and Bae [181] studied inhibition of ammonium oxidation and nitrite oxidation by FA using three different sludges. An uncompetitive inhibition model fitted the experimental data well when the reactions were under FA inhibition. The estimates of the inhibition constant (K_I) were 46 μM for nitrite oxidation and 290–1600 μM for ammonium oxidation. The much smaller values of K_I for nitrite oxidation reflected the susceptibility of that reaction to inhibition by FA, which could lead to accumulation of nitrite during nitrification. Such studies revealed the impact of FA on the respiration of NOB. Little information was gained with regard to the impact of FA on the growth of NOB. However Vadivelu et al. [182] indicated that FA has a limited inhibitory effect on the respiratory capability of *Nitrobacter*. While the real mechanisms remain to be identified, the study of Vadivelu et al. [182] indicates that the FA inhibition of *Nitrobacter* is likely much more serious than suggested by previous studies where the presence of inorganic carbon (or the equivalent nitrite oxidation rate) was used as the sole measure of the inhibitory effects.

Acknowledgements:

Gaber Zayed Breisha would like to thank Alexander von Humboldt Foundation, Bonn, Germany, for the financial support. He also thanks the stuff of Institut für Ingenieurbiologie und Biotechnologie des Abwasser, Universität Karlsruhe

for supporting him during his stay in Karlruhe, Germany.

Corresponding Author:
Dr. Gaber Z. Breisha
Department of Agricultural Microbiology
Faculty of Agriculture
Minia University, Minia, Egypt
E-mail: geetakh@gmail.com

References

1. Baroni L, Cenci L, Tettamanti M, Berati M. Evaluating the environmental impact of various dietary patterns combined with different food production systems. Eur J Clin Nutr. 61(2007) 279–286.
2. Lomborg B. The Skeptical Environmentalist. Cambridge University Press; 2001. P. 22.
3. UNEP International Environment (2002) Environmentally Sound Technology for Wastewater and Stormwater Management: An International Source Book. IWA Publishing.
4. Ravindranath N, Jayant H, Sathaye A. Climate Change and Developing Countries. Springer; 2002.
5. Molinuevo B, García MC, Karakashev D, Angelidaki I. Anammox for ammonia removal from pig manure effluents: Effect of organic matter content on process performance. Bioresour Technol. 100 (2009) 2171-2175.
6. Obaja D, Macé S, Costa J, Sans C, Mata-Alvarez J. Nitrification, denitrification and biological phosphorus removal in piggery wastewater using a sequencing batch reactor. Bioresour Technol. 87**(**2003) 103–111.
7. Höring H, Chapman D. Nitrates and Nitrites in Drinking Water. World Health Organization Drinking Water Series, IWA Publishing, London; 2004.
8. Yetilmezsoy K, Sapci-Zengin S. Recovery of ammonium nitrogen from the effluent of UASB treating poultry manure wastewater by MAP precipitation as a slow release fertilizer. J Haza Mater. 166 (2009) 260-269.
9. Knight R, Payne V, Borer RE, Clarke RA, Pries JH. Constructed wetlands for livestock wastewater management. Ecol Eng. 15 (2000) 41–55.
10. Harrington R, McInnes R. Integrated Constructed Wetlands (ICW) for livestock wastewater management. Bioresource Technology. 100 (2009) 5498-5505.
11. Asadi ME, Clemente RS, Gupta AD, Loof R, Hansen GK. Impacts of fertigation via sprinkler irrigation on nitrate leaching and corn yield in an acid–sulphate soil in Thailand. Agric Water Manage. 52 (2002) 197–213.
12. Jalali M. Nitrates leaching from agricultural land in Hamadan, western Iran. Agric Ecosyst Environ 110 (2005) 210–218.
13. Barton L, Colmer TD. Irrigation and fertilizer strategies for minimizing nitrogen leaching from turfgrass. Agric Water Manage. 80 (2006) 160–175.
14. Singh M, Bhattacharya AK, Nair TVR, Singh AK. Nitrogen loss through subsurface drainage effluent in coastal rice field from India. Agric Water Manage. 52 (2001) 249-260.
15. Tamini T, Mermoud A Water and nitrogen dynamics under irrigation onion in a semi-arid area. Irrig Drain. 51 (2002) 77–86.
16. Darwish T, Atallah T, Hajhasan S, Chranek A. Management of nitrogen by fertigation of potato in Lebanon. Nutr Cycl Agroecosyst. 67 (2003) 1–11.
17. Rajput TBS, Patel N Water and nitrate movement in drip-irrigated onion under fertigation and irrigation treatments. Agric Water Manage. 79 (2006) 293–311.
18. Gheysari M, Mirlatifi SM, Homaee M, Esmaeil Asadi M, Gerrit Hoogenboom G. Nitrate leaching in a silage maize field under different irrigation and nitrogen fertilizer rates. Agric Water Manag. 96 (2009) 946-954.
19. Mallick N. Biotechnological potential of immobilized algae for wastewater N, P and metal removal: a review. Biometals. 15 (2002) 377–390.
20. [20] de-Bashan LE, Hernandez JP, Morey T Bashan Y. Microalgae growth-promoting bacteria as “helpers” for microalgae: a novel approach for removing ammonium and phosphorus from municipal wastewater. Water Res. 38 (2004) 466–474.
21. Gupta AB, Gupta SK. Simultaneous carbon and nitrogen removal from high strength domestic wastewater in an aerobic RBC biofilm. Water Res 35 (2001) 1714-1722.
22. Zeng W, Zhang Y, Li L, Peng Y, Wang S. Control and optimization of nitrifying communities for nitritation from domestic wastewater at room temperatures. Enzym Microb Technol. 45 (2009) 226-232.

23. Guo JH, Peng YZ, Wang SY, Zheng YN, Huang HJ, Ge SJ. Effective and robust partial nitrification to nitrite by real-time aeration duration control in an SBR treating domestic wastewater. Process Biochem. 44 (2009) 979-985.
24. Walker WG, Bouma J, Keeney DR, Olcott PG, Nitrogen transformation during subsurface disposal of septic tank effluent in sands II Groundwater quality. J Environ Qual. 2 (1992) 521–525.
25. Zayed G, Winter J. Removal of organic pollutants and of nitrate from wastewater from dairy industry by denitrification. Appl Microbiol Biotechnol 49 (1998) 469–474.
26. [26] Peyton BM, Mormile MR, Petersen JN. Nitrate Reduction with *Halomonas campisalis:* Kinetics of Denitrification at pH 9 and 12.5% NaCl. Water Res. 35 (2001) 4237-4242.
27. Park JBK, Craggs RJ, Sukias JPS. Removal of nitrate and phosphorus from hydroponic wastewater using a hybrid denitrification filter (HDF). Bioresour Technol. 100 (2009) 3175-3179.
28. Munz G, Gori R, Cammilli L, Lubello C. Characterization of tannery wastewater and biomass in a membrane bioreactor using respirometric analysis. Bioresour Technol. 99 (2008) 8612-8618.
29. Chimenos JM, Fernández AI, Villalba G, Segarra M, rruticoechea A, Artaza B, Espiell F. Removal of ammonium and phosphates from wastewater resulting from the process of cochineal extraction using MgO-containing by-product. Water Res. 37 (2003) 1601-1607.
30. Shen J, He R, Han W, Sun X, Li J, Wang L. Biological denitrification of high-nitrate wastewater in a modified anoxic/oxic-membrane bioreactor (A/O-MBR). J Haz Mater 172 (2009) 595-600.
31. Carrera J, Vicent T, Lafuente J. Effect of influent COD/N ratio on biological nitrogen removal (BNR) from high-strength ammonium industrial wastewater. Process Biochem. 39 (2004) 2035-2041.
32. Badawy MI, Wahaab RA, El-Kalliny AS. Fenton-biological treatment processes for the removal of some pharmaceuticals from industrial wastewater. J Hazard Mater. 167 (2009) 567-574.
33. Watkinson AJ, Murby EJ, Costanzo SD. Removal of antibiotics in conventional and advanced wastewater treatment: implications for environmental discharge and wastewater recycling. Water Res. 41(2007) 4164–4176.
34. Rittmann BE, McCarty PL. Environmental biotechnology: principles and applications 10020, McGraw-Hill, New York, NY; 2001.
35. Kim JK, Park KJ, Cho KS, Nam S, Park T, Bajpai R. Aerobic nitrification–denitrification by heterotrophic *Bacillus* strains. Biores Technol. 6 (2005) 1897-1906.
36. Cyplik P, Grajek W, Marecik R, Kroliczak P. Effect of macro/micro nutrients and carbon source over the denitrification rate of *Haloferax denitrificans* archaeon. Enzyme Microbial Technol. 40 (2007) 212-220.
37. Szekeres S, Kiss I, Bejerano TT, Soares MIM. Hydrogen-dependent denitrification in a two-reactor bio-electrochemical system. Water Res 35 (2001) 715–719.
38. Foglar L, Briski F, Sipos L Vukovic M. High nitrate removal from synthetic wastewater with the mixed bacterial culture. Biores Technol 96 (2005) 879–888.
39. Gomez MA, Galvez JM, Hontoria E, Gonzalez-Lopez J. Influence of concentration on biofilm bacterial composition from a denitrifying submerged filter used for contaminated groundwater. J Biosci Bioeng. 95 (2003) 245–251.
40. Osaka T, Shirotani K, Yoshie S, Tsuneda S. Effects of carbon source on denitrification efficiency and microbial community structure in a saline wastewater treatment process. Water Res. 42 (2008) 3709-3718.
41. Feleke Z, Sakakibara Y. A bio-electrochemical reactor coupled with adsorber for the removal of nitrate and inhibitory pesticide. Water Res. 36 (2002) 3092–3102.
42. Cho E, Molof A. Effect of sequentially combining methanol and acetic acid on the performance of biological nitrogen and phosphorus removal. J Environ Manage. 73 (2004) 183-187.
43. [43] Park JBK, Craggs RJ, Sukias JPS. Treatment of hydroponic wastewater by denitrification filters using plant burnings as the organic carbon source. Bioresour Technol. 99 (2008) 2711-2716.
44. Young-Ho Ahn. Sustainable nitrogen elimination biotechnologies: A review. Process Biochem . 41 (2006) 1709-1721.
45. Daniel LM, Pozzi E, Foresti E, Fabio Alexandre Chinalia A. Removal of ammonium via simultaneous nitrification–denitrification nitrite-shortcut in a single

packed-bed batch reactor. Bioresour Technol. 100 (2009) 1100-1107.
46. Teixeira P, Oliveira R. Denitrification in a closed rotating biological contactor: effect of disk submergence. Process Biochem. 37 (2001) 345-349.
47. Wang Q, Feng C, Zhao Y, Hao C. Denitrification of nitrate contaminated groundwater with a fiber-based biofilm reactor. Bioresour Technol. 100 (2009) 2223-2227.
48. Terada A, Yamamoto T, Igarashi R, Tsuneda S, Hirata Feasibility of a membrane-aerated biofilm reactor to achieve controllable nitrification. Biochem Eng J. 28 (2006) 123–130.
49. Jenicek P, Svehla P, Zabranska J, Dohanyos M. Factors affecting nitrogen removal by nitritation/denitritation. Water Sci Technol. 49 (2004) 73–79
50. Lai E, Senkpiel S, Solley D, Keller J. Nitrogen removal of high strength wastewater via nitritation/denitritation using a sequencing batch reactor. Water Sci Technol 50 (2004) 27–33.
51. Guo H, Zhou J, Su J, Zhang Z. Integration of nitrification and denitrification in airlift bioreactor. Biochem Engin J. 23 (2005) 57–62.
52. Zhang X, Zhou J, Guo H, Qu Y, Liu G, Zhao L. Nitrogen removal performance in a novel combined biofilm reactor. Process Biochem; 42(2007) 620–626.
53. Walters E, Hille A, He M, Ochmann C, Horn H. Simultaneous nitrification/denitrification in a biofilm airlift suspension (BAS) reactor with biodegradable carrier material. Water Res. 43 (2009) 4461-4468 .
54. Fux C, Velten S, Carozzi V, Solley D Keller J. Efficient and stable nitritation and denitritation of ammonium-rich sludge dewatering liquor using an SBR with continuous loading. Water Res. 40 (2006) 2765–2775.
55. Chang YJ, Tseng SK. A novel double-membrane system for simultaneous nitrification and denitrification in a single tank. Lett Appl Microbiol. 28 **(**1999) 453–456.
56. Ma Y, Peng Y, Wang S, Yuan Z Wang X. Achieving nitrogen removal via nitrite in a pilot-scale continuous pre-denitrification plant. Water Res. 43(2009) 563-572.
57. Uemoto H, Saiki H. Nitrogen removal reactor using packed gel envelopes containing *Nitrosomonas europaea* and *Paracoccus denitrificans.* Biotechnol Bioeng. 67 (2000) 80–86.
58. Morita M, Uemoto H, Watanabe A. Nitrogen-removal bioreactor capable of simultaneous nitrification and denitrification for application to industrial wastewater treatment. Biochem Eng J. 41(2008) 59-66.
59. Galvez JM, Gomez MA, Hontoria E, Gonzalez-Lopez J. Influence of hydraulic loading and air flowrate on urban wastewater nitrogen removal with a submerged fixed-film reactor. J Hazard Mater. 101(2003) 219–229.
60. Chiu YC, Chung MS. Determination of optimal COD/nitrate ratio for biological denitrification. Int Biodeterior Biodegrad. 51 (2003) 43–49.
61. Rijn JV, Tal Y, Schreier HJ. Denitrification in recirculating systems: theory and applications. Aquacul Eng. 34 (2006) 364–376.
62. Broers HP. Nitrate reduction and pyrite oxidation in the Netherlands. In: Razowska-Jaworek L, Sadurski A Editors, Nitrates in Groundwater, International Association of Hydrogeologists Selected Papers 5, Balkema, Leiden; 2004.
63. Moon HS, Ahn KH, Lee S, Nam K, Kim JY. Use of autotrophic sulfur-oxidizers to remove nitrate from bank filtrate in a permeable reactive barrier system. Environ Pollut. 129 (2004) 499–507.
64. Zeng H, Zhang TC. Evaluation of kinetic parameters of a sulfur-limestone autotrophic denitrification biofilm process. Water Res. 39 (2005) 4941–4952.
65. Sierra-Alvarez R, Beristain-Cardoso R, Salazar M, Gómez J, Razo-Flores E, Field JA. Chemolithotrophic denitrification with elemental sulfur for groundwater treatment. Water Res 41(. (2005) 1253–1262.
66. [66] Wan D, Liu H, Qu J, Lei P, Xiao S, Hou Y. Using the combined bioelectrochemical and sulfur autotrophic denitrification system for groundwater denitrification. Bioresour Technol. 100 (2009) 142-148.
67. Mansell BO, Schroeder ED. Hydrogenotrophic denitrification in a microporous membrane bioreactor. Water Res. 36 (2002) 4683–4690.
68. Biswas S, Bose P. Zero-valent iron-assisted autotrophic denitrification. J Environ Eng. 131(2005) 1212–1220.

69. Rezania B, Oleszkiewicz JA, Cicek N. Hydrogen-dependent denitrification of water in an anaerobic submerged membrane bioreactor coupled with a novel hydrogen delivery system. Water Res. 41(2007) 1074–1080.
70. Pauwels H, Foucher J, Kloppmann W. Denitrification and mixing in a schist aquifer: influence on water chemistry and isotopes. Chem Geol. 168 (2002) 307-324.
71. Sakakibara Y, Kuroda M. Electric prompting and control of denitrification. Biotechnol Bioeng. 42 (1993) 535–537.
72. [72] Prosnansky M, Sakakibara Y, Kuroda M. High-rate denitrification and SS rejection by biofilm-electrode reactor (BER) combined with microfiltration. Water Res. 36 (2002) 4801–4810.
73. Wang HY, Qu JH. Combined bioelectrochemical and sulfur autotrophic denitrification for drinking water treatment. Water Res. 37 (2003) 3767–3775.
74. Brettar I, Labrenz M, Flavier S, Botel J, Kuosa H, Christen R, Hofle MG. Identification of a *Thiomicrospira denitrificans*-like *Epsilonproteobacterium* as a catalyst for autotrophic denitrification in the Central Baltic Sea. Appl Environ Microbiol. 72 (2006) 1364–1372.
75. Tal JEM, Watts J, Schreier HJ. Anaerobic ammonium-oxidizing (Anammox) bacteria and associated activity in fixed-film biofilters of a marine recirculating aquaculture system. Appl Environ Microbiol 72: (2006) 2896–2904.
76. Jetten MSM, Wagner M, Fuerst J, van Loosdrecht M, Kuenen G, Marc Strous M. Microbiology and application of the anaerobic ammonium oxidation (‘anammox’) process. Curr Opin Biotechnol. 12 (2001) 283-288.
77. Jetten MSM, Strous M, van de Pas-Schoonen KT, Schalk J van Dongen L, van de Graaf, AA, Logemann S, Muyzer G, van Loosdrecht MCM, Kuenen JG. The anaerobic oxidation of ammonium. FEMS Microbiol Rev. 22 (1999) 421–437.
78. van de Graaf AA, de Bruijn P, Robertson LA, Jetten MSM, Kuenen JG. Metabolic pathway of anaerobic ammonium oxidation on the basis of N-15 studies in a fluidized bed reactor. Microbiol. 143 (1997) 2415–2421.
79. Schalk J. A study of the metabolic pathway of anaerobic ammonium oxidation (PhD Thesis). Technology University, Delft; 2000.
80. Strous M, Fuerst JA, Kramer EHM, Logemann S, Muyzer G, van de Pas-Schoonen KT. Missing lithotroph identified as new *Planctomycete*. Nature**.** 400 (1999) 446–449
81. Schmid M, Twachtmann U, Klein M, Strous M, Juretschko S, Jetten MSM. Molecular evidence for genus level diversity of bacteria capable of catalyzing anaerobic ammonia oxidation. Syst Appl Microbiol. 23 (2000) 93–106.
82. Egli K, Fanger U. Alvarezz PJJ, Siegrist H, van der Meer JR, Zehnder AJB. Enrichment and characterization of an anammox bacterium from a rotating biological contactor treating ammonium rich leachate. Arch Microbiol. 175 (2001) 198–207.
83. Güven D, Dapena A, Kartal B, Schmid MC, Maas B, van de Pas-Schoonen K, Sozen S, Mendez R, Op de Camp HJM, Jetten MSM, Strous M, Schmidt I. Propionate oxidation by and methanol inhibition of anaerobic ammonium oxidizing bacteria. Appl Environ Microbiol. 71(2005) 1066–1071.
84. Kartal B, Kuypers MMM, Lavik G, Schalk J, Op den Camp HJM, Jetten MSM, Strous M. Anammox bacteria disguised as denitrifiers: nitrate reduction to dinitrogen gas via nitrite and ammonium. Environ Microbiol. 9 (2007) 635–642.
85. Kartal B, Rattray J, van Niftrik L, van de Vossenberg J, Schmid M, Webb RI, Schouten S, Fuerst JA, Damste JS, Jetten MSM, Strous M. Candidatus “Anammoxoglobus propionicus” gen. nov., sp. nov., a new propionate oxidizing species of anaerobic ammonium oxidizing bacteria. Syste Appl Microbiol. 30 (2007) 39–49.
86. Strous M, van Gerven E, Zheng P, Kuenen JG, Jetten, MSM. Ammonium removal from concentrated waste streams with the anaerobic ammonium oxidation (ANAMMOX) process in different reactor configurations. Water Res. 31(1997) 1955–1962.
87. Jensen, M.M., Kuypers, M.M.M, Lavik, G. and Thamdrup, B. Rates and regulation of anaerobic ammonium oxidation and denitrification in the Black Sea. Limnology and Oceanography 53 (2008) 23–36.
88. Sabumon PC. Anaerobic ammonia removal in presence of organic matter: A novel route. J Haz Mater. 149 (2007) 49–59.

89. Wang J, Kang J. The characteristics of anaerobic ammonia oxidation (ANAEROBIC AMMONIA REMOVAL) by granular sludge from an EGSB reactor. Process Biochem. 40 (2005) 1973–1978.
90. Chamchoi N, Nitisoravut S, Schmidt JE. Inactivation of ANAMMOX communities under concurrent operation of anaerobic ammonium oxidation and denitrification. Biores Technol. 99 (2008) 3331–3336.
91. Dapena-Mora A, Fernández J, Campos JL, Mosquera-Corral A, Méndez R, Jetten MSM. Evaluation of activity and inhibition effects on Anammox process and batch tests based on the nitrogen gas production. Enz Microb Technol. 40 (2007) 859–865.
92. Sliekers AO, Third KA, Abma W, Kuenen JG, Jetten MSM. CANON and Anammox in a gas lift reactor. FEMS Microbiol Lett. 218 (2003) 339–344.
93. Li J, Xiong B, Zhang S, Yang H, Zhang J. Anaerobic: ammonium oxidation for advanced municipal wastewater treatment is it feasible?. J Environ Sci. 17 (2005) 1022–1024.
94. Liang Z, Liu J. Landfill leachate treatment with a novel process: Anaerobic ammonium oxidation (Anammox) combined with soil infiltration system. J Haz Mater. 151(2008) 202–212.
95. Waki M, Tokutomi T, Yokoyama H, Tanaka Y. Nitrogen removal from animal waste treatment water by anammox enrichment. Bioresour Technol. 98 (2007) 2775–2780.
96. Third KA, Olav Sliekers A, Kuenen JG, Jetten MSM. The CANON system (completely autotrophic nitrogen-removal over nitrite) under ammonium limitation: interaction and competition between three groups of bacteria. Syst Appl Microbiol. 24 **(**2001) 588–596.
97. Ciudad G, Rubilar O, Muñoz P, Ruiz G, Chamy R, Vergara C, Jeison D. Partial nitrification of high ammonia concentration wastewater as a part of a shortcut biological nitrogen removal process. Process Biochem. 40 (2005) 1715–1719.
98. Fux C, Boehler M, Huber P, Brunner I, Siegrist H. Biological treatment of ammonium-rich wastewater by partial nitritation and subsequent anaerobic ammonium oxidation (Anammox) in a pilot plant. J Biotechnol. 9 (2002) 295–306.
99. Feng Y, Tseng S, Hsia T, Ho C Chou W. Partial nitrification of ammonium-rich wastewater as pretreatment for anaerobic ammonium oxidation (Anammox) using membrane aeration bioreactor. J of Biosci Bioengin. 104 (2007) 182-187.
100. Khin T, Annachhatre AP. Novel microbial nitrogen removal processes. Biotechnol Adv. 22 (2004) 519–532.
101. Yamamoto T, Takaki K, Koyama T, Furukawa K. Long-term stability of partial nitration of swine wastewater digester liquor and its subsequent treatment by Anammox, Bioresour Technol. 99 (2008) 6419–6425.
102. Hao XD, van Loosdrecht MCM, Heijnen JJ. Model-based evaluation of kinetic, biofilm and process parameters in a one-reactor ammonium removal (CANON) process. Biotechnol Bioeng. 77 (2002) 266–277.
103. Sliekers AO, Derwort N, Gomez JLC, Strous M, Kuenen JG, Jetten MSM. Completely autotrophic nitrogen removal over nitrite in one single reactor. Water Res. 36 (2002) 2475–2482.
104. Nielsen M, Bollmann A, Sliekers O, Jetten M, Schmid M, Strous M, Schmidt I, Larsen LH, Nielsen LP, Revsbech NP. Kinetics, diffusional limitation and microscale distribution of chemistry and organisms in a CANON reactor. FEMS Microbiol Ecol. 51 (2005) 247–256.
105. Hao XD, van Loosdrecht MCM. Model-based evaluation of COD influence on a partial nitrification-Anammox biofilm (CANON) process. Water Sci Technol. 49 (2004) 83–90.
106. Yan Y, Hu YY. Partial nitrification to nitrite for treating ammonium-rich organic wastewater by immobilized biomass system. Bioresour Technol. 100 (2009) 2341-2347.
107. Gong Z, Yang F, Liu S, Bao H, Hu S, Furukawa K. Feasibility of a membrane-aerated biofilm reactor to achieve single-stage autotrophic nitrogen removal based on Anammox. Chemosphere 69 (2007) 776-784.
108. Morita M, Kudo N, Uemoto H, Watanabe A, Shinozaki H. Protective effect of immobilized ammonia oxidizers and phenol-degrading bacteria on nitrification in ammonia- and phenol containing wastewater. Eng Life Sci. 7 (2007) 587–592.
109. Casey E, Glennon B, Hamer G. Review of membrane aerated biofilm reactors. Resour Conserv Recy. 27 (1999) 203–215.
110. Lapara TM, Cole AC, Shanahan JW Semmens MJ. The effects of organic carbon, ammoniacal-nitrogen, and oxygen partial

pressure on the stratification of membrane-aerated biofilms. J Ind Microbiol Biotechnol. 33 (2006) 315–323.
111. Brindle K, Stephenson T. The application of membrane biological reactors for the treatment of wastewaters. Biotechnol Bioeng. 49 (1996) 601–610.
112. Brindle K, Stephenson T, Semmens MJ. Nitrification and oxygen utilisation in a membrane aeration bioreactor. J Membr Sci. 144 (1998) 197–209.
113. Casey E, Glennon B, Hamer G. Oxygen mass transfer characteristics in a membrane-aerated biofilm reactor. Biotechnol Bioeng. 62 (1999) 183–192.
114. Cole AC, Semmens MJ, LaPara TM. Stratification of activity and bacterial community structure in biofilms grown on membranes transferring oxygen. Appl Environ Microb. 70 (2004) 1982–1989.
115. Fierro S, Sánchez-Saavedra MP, Copalcúa C. Nitrate and phosphate removal by chitosan immobilized scenedesmus. Bioresour Technol. 99 (2008) 1274–1279.
116. Hill BC, Khan E. A comparative study of immobilized nitrifying and co-immobilized nitrifying and denitrifying bacteria for ammonia removal from sludge digester supernatant. Water Air Soil Poll 195 (2008) 23–33.
117. Rostron WM, Stuckey DC, Young AA. Nitrification of high strength ammonia wastewaters: comparative study of immobilisation media. Water Res. 35 (2001) 1169–1178.
118. Isaka K, Sumino T, Tsuneda S. High nitrogen removal performance at moderately low temperature utilizing anaerobic ammonium oxidation reactions. J Biosci Bioeng. 103 (2007) 486–490.
119. Fontenot Q, Bonvillain C, Kilgen M, Boopathy R. Effects of temperature, salinity, and carbon: nitrogen ratio on sequencing batch reactor treating shrimp aquaculture wastewater. Bioresour Technol. 98 (2007) 1700-1703.
120. [120] Brady NC, Weil RR The Nature and Properties of Soils, 13th edn, Prentice Hall, New Jersey, 2002.
121. Elefsiniotis P, Li D. The effect of temperature and carbon source on denitrification using volatile fatty acids. Biochem Engin J. 28 (2006) 148-155.
122. Cannavo P, Richaume A, Lafolie F. Fate of nitrogen and carbon in the vadose zone: in situ and laboratory measurements of seasonal variations in aerobic respiratory and denitrifying activities. Soil Biol Biochem. 36 (2004) 463–478.
123. Brouwer M, van Loosdrecht MCM, Heijnen JJ. One reactor system for ammonium removal via nitrite. STOWA report 96-01, STOWA, Utrecht, The Netherlands, 1996.
124. Karakashev D, Schmidt JE, Angelidaki I. Innovative process scheme for removal of organic matter, phosphorus and nitrogen from pig manure. Water Res. 42 (2008) 4083–4090.
125. Peng YZ, Yang Q, Liu XH, Zeng W, Mino T, Satoh H. Achieve nitrogen removal via nitrite form municipal wastewater at low temperatures using real-time control to optimize nitrifying communities. Environ Sci Technol. 41 (2007) 8159–8164.
126. Zeng, W, Peng YZ, Wang SY, Peng CY. Process control of an alternating aerobic-anoxic sequencing batch reactor for nitrogen removal via nitrite. Chem Eng Technol. 31 (2008) 582–587.
127. van Dongen U, Jetten MSM, van Loosdrecht MCM. The SHARON-ANAMMOX process for treatment of ammonium rich wastewater. Water Sci Technol. 44 (2001) 153–160.
128. Strous M, Kuenen JG, Jetten MSM. Key physiology of anaerobic ammonium oxidation. Appl Environ Microbiol. 65 (1999) 3248–3250.
129. Toh SK, Webb RI, Ashbolt NJ. Enrichment of autotrophic anaerobic ammonium-oxidizing consortia from various wastewaters. Microb Ecol. 43 (2002) 154–167.
130. Imajo U, Tokutomi T, Furukawa K. Granulation of Anammox microorganisms in up-flow reactors. Water Sci Technol. 49 (2004) 155–163.
131. Cema G, Wiszniowski J, Żabczyński S, Zabłocka-Godlewska E, Raszka A, Surmacz-Górska J. Biological nitrogen removal from landfill leachate by deammonification assisted by heterotrophic denitrification in a rotating biological contactor (RBC). Water Sci Technol. 55 (2007) 35–42.
132. Vázquez-Padín J, Fernádez I, Figueroa M, Mosquera-Corral A, Campos J, Méndez R. Applications of Anammox based processes to treat anaerobic digester supernatant at room temperature. Bioresour Technol. 100 (2009) 2988-2994.
133. Isaka K, Yoshie S, Sumino T, Inamori Y, Tsuneda S. Nitrification of landfill leachate

using immobilized nitrifying bacteria at low temperatures. Biochem Eng J. 37 (2007) 49–55.
134. Rysgaard S, Glud RN, Risgaard-Petersen N, Dalsgaard T. Denitrification and Anammox activity in Arctic marine sediments. Limnol Oceanogr 49 (2004) 1493–1502.
135. Dalsgaard T, Thamdrup B. Factors controlling anaerobic ammonium oxidation with nitrite in marine sediments. Appl Environ Microbiol. 68 (2002) 3802–3808.
136. Dosta J, Fernández I, Vázquez-Padín JR, Mosquera-Corral A, Campos JL, Mata-Álvarez J, Méndez R. Short- and long-term effects of temperature on the Anammox process. J of Hazard Mater. 154 (2008) 688-693.
137. Welander U, Mattiasson B. Denitrification at low temperatures using a suspended carrier biofilm process. Water Res. 37 (2003) 2394-2398.
138. Zumft WG. Cell biology and molecular basis of denitrification. Microbiol Mol Biol Rev. 61(1997) 533–616.
139. Tan TW, Ng HY. Influence of mixed liquor recycle ratio and dissolved oxygen on performance of pre-denitrification submerged membrane bioreactors. Water Res. 42 (2008) 1122-1132.
140. Gómez MA, Hontoria E, González-López J. Effect of dissolved oxygen concentration on nitrate removal from groundwater using a denitrifying submerged filter. J Hazard Mater. 90 (2002) 267-278.
141. Aslan V, Miller L, Dahab M. Ammonium oxidation via nitrite accumulation under limited oxygen concentration in sequencing batch reactors. Bioresour Technol. 100 (2009) 659–664.
142. Blackburne R, Yuan ZG, Keller J. Partial nitrification to nitrite using low dissolved oxygen concentration as the main selection factor. Biodegrad. 19 (2008) 303–312.
143. Ma Y, Peng Y, Wang S. Nitritation by real-time control in a pilot-scale pre-denitrification plant treating domestic wastewater. *Proceedings (DVD)* of the 4th International Conference on Marine Waste Water Disposal and Marine Environment *(MWWD 2006), Antalya, Turkey, November 06–10* (2006) ISBN 9944-5566-2-9
144. Yuan Z, Oehmen A, Peng YZ, Ma Y, Keller J. Sludge population optimisation in biological nutrient removal wastewater treatment systems through on-line process control: a review. Rev Environ Sci. Biotechnol. 45(2008) 29-38.
145. Ma WL, Zhang Y, Qi R, Wang, V, Liang, C Z, Yang M. Performance of a successive hydrolysis, denitrification and nitrification system for simultaneous removal of COD and nitrogen from terramycin production wastewater. Biochem Eng J. 45 (2009) 30-34.
146. Ciudad G, Rubilar O. Munoz P, Ruiz G, Chamy R, Vergara C, Jeison D. Partial nitrification of high ammonia concentration wastewater as a part of a shortcut biological nitrogen removal process. Process Biochem. 40 (2005) 1715–1719.
147. Park HD, Noguera DR. Evaluating the effect of dissolved oxygen on ammonia-oxidizing bacterial communities in activated sludge. Water Res. 38 (2004) 3275–3286.
148. Guo J, Peng Y, Wang S, Zheng Y, Huang H, Wang Z. Long-term effect of dissolved oxygen on partial nitrification performance and microbial community structure. Bioresour Technol. 100 (2009) 2796-2802.
149. Blackmer AM, Bremner, JM. Inhibitory effect of nitrate on reduction of N_2O to N_2 by soil micro-organisms. Soil Biol Biochem. 10 (1978) 187–191.
150. Austerman-Haun U, Meyer H, Seyfried CF, Rosenwinkel KH. Full scale experiences with anaerobic/aerobic treatment plants in the food and beverage industry. Water Sci Technol. 40 (1999) 305–312.
151. Cyplik P, Grajek W, Marecik R, Króliczak P, Dembczyński R. Application of a membrane bioreactor to denitrification of brine. Desalination. 207 (2007) 134–143.
152. Nair RR, Dhamole PB, Lele SS, D'Souza SF. Biological denitrification of high strength nitrate waste using preadapted denitrifying sludge. Chemosphere 67(2007) 1612–1617.
153. Dhamole PB, Nair RR, D'Souza SF, Lele SS. Denitrification of high strength nitrate waste. Bioresour Technol. 98 (2007) 247-252.
154. Campos JL, Mosquera-Corral A, Sanchez M, Mendez R, Lema JM. Nitrification in saline wastewater with high ammonia concentration in an activated sludge unit. Water Res. 36 (2002) 2555–2560.
155. Moussa MS, Sumanasekera DU, Ibrahim SH, Lubberding HJ, Hooijmans CM, Gijzen HJ, van Loosdrecht MCM. Long term effects of salt on activity, population structure and floc characteristics in enriched

bacterial cultures of nitrifiers. Water Res. 40 (2006) 1377–1388.
156. Wu Y, Tam NFY, Wong MH. Effects of salinity on treatment of municipal wastewater by constructed mangrove wetland microcosms Marine Pollu Bullet. 57 (2008) 727-734.
157. Sudarno S, Winter J, Gallert C. Nitrification in fixed-bed reactors treating saline wasterwater. J Appl Microbiol Biotechnol. 41(2009) 137–146.
158. Colt J, Tomasso JR. Hatchery water supply and treatment. In: Wedemeyer GA Editor, Fish Hatchery Management (second ed.), American Fisheries Society, Bethesda, Maryland, 2001; p 91.
159. Uygur A. Specific nutrient removal rates in saline wastewater treatment using sequencing batch reactor, Process Biochem. 41(2006) 61–66.
160. Yoshie S, Ogawa T, Makino H, Hirosawa H, Tsuneda S, Hirata ACharacteristics of bacteria showing high denitrification activity in saline wastewater Lett Appl Microbiol. 42 (2006) 277–283.
161. Furukawa K, Ike A, Fujita M. Preparation of marine nitrifying sludge. J Ferment Bioeng. 76 (1993) 134–139.
162. Rene ER, Kim SJ, Park HS. Effect of COD/N ratio and salinity on the performance of sequencing batch reactors. Bioresour Technol. 99 (2008) 839-846.
163. van der Star WRL, Abma WR, Blommers D, Mulder JW, Tokutomi T, Strous M, Picioreanu C, van Loosdrecht MCM. Startup reactors for anoxic ammonium oxidation: experiences from the first full-scale anammox reactor in Rotterdam. Water Res. 41(2007) 4149–4163.
164. Schmid MC, Risgaard-Petersen N, van de Vossenberg J, Kuypers MMM, Lavik G, Petersen J, Hulth S, Thamdrup B, Canfield D, Dalsgaard T, Rysgaard S, Sejr MK, Strous M, den Camp HJMO, Jetten MSM. Anaerobic ammonium-oxidizing bacteria in marine environments: widespread occurrence but low diversity. Environ Microbiol. 9 (2007) 1476–1484.
165. Nakajima J, Sakka M, Kimura T, Furukawa K Sakka K. Enrichment of anammox bacteria from marine environment for the construction of a bioremediation reactor, Appl Microbiol Biotechnol. 77(2008) 1159–1166.
166. Kartal B, Koleva M, Arsov R, van der Star W, Jetten MSM, Strous M. Adaption of a freshwater anammox population to high salinity wastewater. J Biotechnol. 126 (2006) 546–553.
167. Liu C, Yamamoto T, Nishiyama T, Fujii T, Furukawa K. Effect of salt concentration in anammox treatment using non woven biomass carrier. J Biosci Bioeng 107 (2009) 519-523.
168. Salem Z, Lebik H, Cherafa WK, Allia K. Valorisation of olive pits using biological denitrification. Desalination 204 (2007) 72-78.
169. Amirbahman A, Schönenberger R, Johnson CA, Sigg L. Aqueous- and solid-phase biogeochemistry of a calcareous aquifer system downgradient from a municipal solid waste landfill (Winterthur, Switzerland). Environ Sci Technol. 32 (1998) 1933–1940.
170. Hwang C, Wu WM, Gentry TJ, Carley J, Carroll SL, Schadt C, Watson D, Jardine PM, Zhou J, Hickey RF, Criddle CS, Fields MW. Change in bacterial community structure correlate with initial operating conditions of a field-scale denitrifying fluidized bed reactor. Appl Microbiol Biotechnol. 71(2006) 748–760.
171. van Rijn J, Yossi Tal Y, Schreier HJ. Denitrification in recirculating systems: Theory and applications. Aquacultu Eng. 34 (2006) 364-376.
172. Koenig A, Liu LH. Microbial aspects of autotrophic denitrification of wastewaters. In: Matsuo T, Hanaki K, Takizawa S, Satoh H Editors, Advances in Water and Wastewater Treatment Technology: Molecular Technology, Nutrient Removal, Sludge Reduction and Environmental Health, Elsevier Science, Amsterdam. 2001; p 217.
173. Lee K, Rittmann B. Effects of pH and precipitation on autohydrogenotrophic denitrification using the hollow-fiber membrane-biofilm reactor. Water Res. 37 (2003) 1551-1556.
174. Koenig A, Liu LH. Use of limestone for pH control in autotrophic denitrification: continuous flow experiments in pilot-scale packed bed reactors. J Biotechnol. 99 (2002) 161-171.
175. Jha J, Bose P. Use of pyrite for pH control during hydrogenotrophic denitrification using metallic iron as the ultimate electron donor. Chemosphere 61(2005) 1020-1031.
176. Philips S, Laanbroek HJ, Verstraete W. Origin causes and effects of increased nitrite

concentrations in aquatic environments. Environ Sci Biotechnol. 1(2002) 115–141.
177. Anthonisen AC, Loehr RC, Prakasam TBS, Srinath EG. Inhibition of nitrication by ammonia and nitrous acid. J Water Pollut Control Fed. 48 (1976) 835–852.
178. Bae W, Baek S, Chung J, Lee Y. Optimal operational factors for nitrite accumulation in batch reactors. Biodegrad. 12 (2001) 359–366.
179. Chung J, Bae W, Lee YW, Rittmann BE. Shortcut biological nitrogen removal in hybrid biofilm/suspended growth reactors. Process Biochem. 42 (2007) 320–328.
180. Chung J, Shim H, Park S, Kim SJ Bae W. Optimization of free ammonia concentration for nitrite accumulation in shortcut biological nitrogen removal process. Bioprocess Biosyst Engin. 28 (2006) 275–282.
181. Park S, Bae W. Modeling kinetics of ammonium oxidation and nitrite oxidation under simultaneous inhibition by free ammonia and free nitrous acid. Process Biochem. 44 (2009) 631-640.
182. Vadivelu V, Keller J, Yuan Z. Effect of free ammonia on the respiration and growth processes of an enriched *Nitrobacter* culture. Water Res. 41(2007) 826-834.
183. Aiyuk S, Amoako J, Raskin L, van Haandel A, Verstraete W. Removal of carbon and nutrients from domestic wastewater using a low investment, integrated treatment concept. Water Res. 38 (2004) 3031-3042.
184. Blackburne R, Yuan Z, Keller J. Demonstration of nitrogen removal via nitrite in a sequencing batch reactor treating domestic wastewater. Water Res. 42 (2007) 2166-2176.
185. Feng H, Hu L, Mahmood Q, Qiu C, Fang C, Shen D Anaerobic domestic wastewater treatment with bamboo carrier anaerobic baffled reactor. Intern Biodeterior Biodegrad. 62 (2008) 232-238.
186. Oladoja N, Ademoroti CMA The use of fortified soil-clay as on-site system for domestic wastewater purification. Water Res. 40 (2006) 613-620.
187. Wu C, Chen Z, Liu X, Yongzhen Peng Y. Nitrification–denitrification via nitrite in SBR using real-time control strategy when treating domestic wastewater. Biochem Eng J. 36 (2007) 87-92.

10/16/2010

Comparison of Electrostatic and Spinning-discs Spray Nozzles on Wheat Weeds Control

Ali Esehaghbeygi[1], Ali Tadayyon[2], Shahin Besharati[2]

[1]Department of Agricultural Engineering, [2]Department of Agronomy and Plant Breeding
College of Agriculture, Shahrekord University, Shahrekord, Iran, 115
esehaghbeygi@cc.iut.ac.ir

Abstract: Electrostatic spraying is the method that is noted for improving the spraying efficiency and droplet deposition. The efficacy of electrostatic charge and spinning-discs spraying were assessed for the application of 2, 4-D to control weeds in irrigated wheat. Sprayer nozzle performance was evaluated in terms of wheat grain yield (*Ghods* variety), weed shoot biomass, and wheat residual (straw) at the research farm of Shahrekord University in 2007 and 2008. The results indicated that electrostatic spraying gave better weed control. Spray penetration through dense weeds enhanced with electrostatic charging. The spinning disc nozzle decreased water use and so was cheaper to operate, but it did not significantly improve herbicide efficacy, especially in dense canopies compared with the electrostatic charge.

[Ali Esehaghbeygi, Ali Tadayyon, Shahin Besharati. **Comparison of Electrostatic and Spinning-discs Spray Nozzles on Wheat Weeds Control.** Journal of American Science 2010;6(12):529-533]. (ISSN: 1545-1003). http://www.americanscience.org

Keywords: electrostatic, spinning disc, wheat, weed

1. Introduction

As summarized in the foregoing review, there have been significant advances in the research and development of electrostatic-spraying technology for beneficial agricultural and biological applications throughout the 20th century (Law, 2001). Hislop (1987) concluded that different application methods and droplet size spectra produced by hydraulic nozzles make relatively little difference in spray partitioning between different parts of the canopy and that canopy density or growth stage is of much greater importance. Coarser sprays are recommended to enhance canopy penetration, although optimum spray quality for penetration is probably specific to the canopy architecture (Spillman, 1984). When spraying into a no-till canopy, droplet interception by the stubble should be minimized and capture by the target weed maximized. These two goals may not be reconcilable because sedimentation and impact on stubble and weeds may be governed by the same criteria. Additionally, because canopies differ in texture, morphology, orientation, and depth, generalization is difficult (Bache and Johnstone, 1992). Grain yield losses due to weed competition in the wheat crop are estimated to be 25% (Montazeri *et al.*, 2005). The importance of better herbicide application equipment has been reported by Shaw (1982) for integrated weed control management. Such equipment could decrease chemical and water application per unit area. A spinning disc nozzle is suggested as a tool to reach such objectives. Uremis *et al.* (2004) stated that spinning disc nozzles with a reduced spray volume did not improve weed control and gave inadequate weed control with reduced dosage of herbicide. Spinning disc nozzles are recommended for both weed and insect control to meet the goals of integrated pest management systems. Although integrated weed management has been used for over a decade, weed management practices still need to be improved to achieve its goals. Based on Sikkema *et al.* (2008) study, the optimum nozzle type, water carrier volume, and spray pressure is herbicide and weed species-specific.

Diverse crop rotations, competitive cultivars, higher crop seed rates, reduced row spacing, specific fertilizer placement, and cover crops have been identified as integral components of competitive cropping systems (Blackshaw *et al.*, 2006). Electrostatic charging of agricultural sprays has several demonstrated advantages over conventional application methods, the most significant being more spray deposition on the target plants and less deposition on the ground (Bailey, 1988). Physical characteristics of charged sprays, such as their predisposition to deposit in the upper regions of the crop canopy (Morton, 1982), contribute to erratic pest control and have required that canopy penetration are an important part of the evaluation of such technology. Because most electrostatic charging has been done on naturally sedimenting sprays, the use of hydraulic pressure or air assistance has been suggested as a means of

enhancing the penetration characteristics of such sprays (Hislop, 1988). The combination of 45 kV electrostatic charge and 50 cm nozzle spacing produced maximum spray deposition on weeds and increase in deposition compared to the uncharged controls (Wolf *et al.,* 2000). Use of electrostatic sprayers has been studied for agricultural spraying (Kirk *et al.,* 2001, Kang *et al.,* 2004). Deposition of charged sprays on leaf abaxial (underside) and adaxial (upper) surfaces as influenced by the spray charging voltage, application speed, target height, and orientation parameters was studied in the laboratory by Maski and Durairaj (2010). Results showed that electrostatically charged spray improved the underside (abaxial) and overall deposition. Electrostatic spraying of pesticides was not successfully commercialized because of the higher cost of equipment and the relatively small coverage, especially on cereals. The latter was due to less penetration in to the crop canopy although the charge on small droplets was effective, which increased deposition and reduced downwind drift (Allen *et al.,* 1983; Lake and Marchant, 1984). The aim of this study was to investigate the effectiveness of different herbicide application methods of electrostatic charge and spinning-discs under natural weed flora in the irrigated wheat field of Shahrekord University region, Iran.

2. Materials and Methods

An air-assisted electrostatic induction spray charging system for water-based liquids designed at the Agricultural Research Engineering of Jehade-Isfahan, Iran was used (Fig. 1). That required a high velocity air flow (30 m s^{-1}) within the spraying head assembly to keep the charging electrode (8 kV) from accumulating water and then earthing the electrode. Earthed electrodes close to the spray had absorbent tubes along their lower edges and a suction system to recover spray liquid attracted to the electrodes.

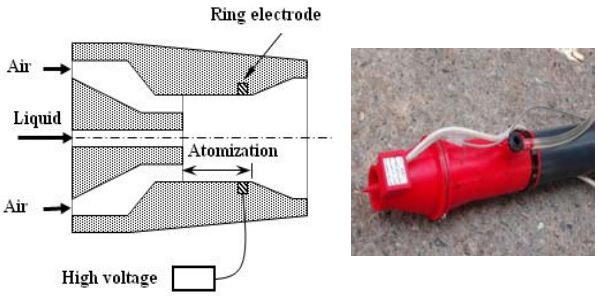


Figure 1. Electrostatic induction spray head

The charging nozzle consisted of a circular electrode and fluid jets. The high-voltage generator supplied high electric potential to the nozzle's electrode. The discharge rate of spray liquid was constant. A regulated quantity of spray liquid to the nozzle's individual fluid jets was supplied via spray liquid distributor. Two field experiments were carried out on irrigated wheat (*Triticum aestivum* L.) at the research farm of Shahrekord University in 2007 and 2008 to investigate the efficiency of different sprayer's nozzles on weed control in wheat grain yield, *Ghods* variety. A low volume spinning disc with the disc speed of 2000 rpm, *HERBI-4* (Micron Sprayers Ltd., UK) and electrostatic charge sprayer Jehade-Isfahan, Iran were used to spraying 2, 4-D at tillering stage of wheat to control broadleaved weed in cultivated wheat. Plot size measured 30×30 m, separated to be a distance of 5 m. Seedbed preparation was accomplished based on common local practices. Wheat population was 400 plants m^{-2}. The spray head was kept about 200 mm above the ground or weed foliage. The effective rate of 0.7 kg ha^{-1} 2, 4-D manufacturer's recommended dose, was used in all treatments. Sprayers were operated at a speed of 0.75 m s^{-1} at air temperatures of 20-25 ^{0}C and a relative humidity of around 36%. The wind speed at 2 m above the ground level was measured at 1-2 m s^{-1} using a direct reading cup anemometer, and the temperature and relative humidity were measured by a psychrometer whirled in the shade. Micron sprayers had a gravity feed reservoir and were powered with 6-V DC batteries. Weed population was measured separately for each quadrate to be counting the number of weeds and shoot weed biomass. Wheat grain yield were measured at maturity stage.

Water sensitive papers coated with Bromo Phenol Blue (30×100 mm) were used to determine drop density and size when the herbicide was applied. The water sensitive papers were evaluated using standard cards in WINDIAS software, Delta-T devices LTD, UK. Weeds shoot biomass was measured for each replicate of spray applications. The wheat yield was measured at crop maturity by hand harvesting the plots. The yields were adjusted to 13-14% moisture content. At harvesting time, all weed species were cut separately from soil surface and weighed. The effectiveness for herbicide on wheat crop was evaluated to be measuring wheat grain yield and weed shoot biomass. ANOVA from RCBD design was used for all data analyses with five replications. All the data met the assumptions of normality, so transformations of the data were not necessary. Significant mean values were tested with LSD at $P<0.05$.

3. Results and Discussion

The greater weed density and more variation of weed species were observed in the second year compared to the first year of the experiment (Table 1). *Bromus sp., Convolvulus arvensis, Galium sp.* were the most common weeds in the first year of the experiment and *Geranium sp., Descurainia sophia*, and *Bromus sp.* were the most infested weeds in the second years of the experiment. According to the analysis of variance, spraying herbicide on the wheat grain yield was significantly affected (P<0.05), but no significant differences were observed as a result of sprayer nozzles. The lowest yield was obtained with control treatment that had no spraying (Table 2).

Table 1. Weed composition of cultivated wheat in two years of the experiment

Year 2007	Year 2008	
Bromus sp.	*Vaccaria sp.*	*Descurainia sophia*
Convolvulus arvensis	*Anchusa sp.*	*Cirsium arvense*
Erodium sp.	*Cenesio vulgaris*	*Solanum nigrum*
Galium sp.	*Thlaspi arvense*	*Taraxacum officinale*
Centaurea cyanus	*Chenopodium album*	*Bromus sp.*
Cynodon dactylon	*Lactuca scariola*	*Geranium sp.*
Vicia villosa	*Cynodon dactylon*	*Vicia villosa*
Vicia sativa	*Centaurea cyanus*	*Convolvulus arvensis*

Table 2. Wheat grain yield and component yield (straw) of wheat in two years of 1 m^2 quadrate

	Spinning-disc	Electrostatic charge	Control
Wheat yield, g m^{-2}			
2007	419±61.4 ab	422.3±65.8 a	285.2±77.8 c
2008	358.3±90.1 abc	380.2±91.3 ab	281.1±92.8 c
Straw, g m^{-2}			
2007	371.6±122.5 bc	312.1±110.4 b	549±151.3 ab
2008	506.1±84.1 abc	455.5± 98.4 a	545±137.1 ab

[a-c] Different letters in the pair rows shows significant difference, LSD 5%.
[±] Estimates standard deviation based on a sample in 5 replications.

It would be imprudent to extrapolate results from the present study to other species or herbicide mixtures because the demonstrated deposit size effect is likely dependent on the properties of the active ingredient or the adjuvant included in the mixture. Some spray components may have phytotoxic effects at high concentrations per unit leaf area, which may become important with large, no spreading deposit sizes (Wolf *et al.*, 1992). Previous research showed that the deposition was substantially influenced by factors such as charging voltage, application speed, plant target height, and target orientation (Maski and Durairaj 2010). Chemical weed control reduced weed competition in wheat, thereby giving the crop a better growing environment for enhanced growth and development. The results for weed control with spinning disc nozzles varied from poor to acceptable control when used in combination with herbicides or other agents compared with conventional nozzles (Walker 1986; Mohan and Nelson 1982; Scoresby and Nalewaja 1982). In our study, no significant differences were observed among different spraying methods. Due to more competition, grain wheat yield generally was lower in 2008 compared to in 2007. The varied relationship between the density of weeds and crop yield can be explained partially with the different environmental conditions during the growing season prevailing in two years. Weed dry matter production was the least in 2008 and the most in 2007 (Table 3). These results are in agreement with those reported in Mason *et al.* (1998). The plants may not have been conductive to electric charges and may, therefore, not have been a preferred ground for the charged spray compared to other weeds.

Table 3. Weeds biomass and weeds number in cultivated wheat in two years of 1 m^2 quadrate

	Spinning-disc	Electrostatic charge	Control
Weed dry matter, g m^{-2}			
2007	69.9 abc	61.5 b	105.1 a
2008	35.3 cd	41.5 cd	85.2 ab
Number of weeds			
2007	242 ab	235 a	277.4 abc
2008	67.6 de	86.1 cde	97.2 cde

[a-e] Different letters in rows shows significant difference, LSD 5%.
[±] Estimates standard deviation based on a sample in 5 replications.

Improved deposition, distribution, and penetration of charged spray into the plant canopy considerably increase the biological efficacy (Hislop, 1988). It seems that the effectiveness for electrostatic spraying was higher in dense weed populations in the first year. The trends may be due to the fluctuation in

the environmental conditions of the experimental site. Spinning disc dispense the spray solution horizontally rather than downward as do the electrostatic sprayer. Therefore, gravity is the major force moving the droplets into the plant canopy. Possibly, smaller VMDs with spinning disc nozzles in the warm and windy conditions caused the inefficiency of herbicides in weed control. Buhler and Burnside (1987) speculated that increased weed control at larger droplet sizes may be due to greater canopy penetration of the herbicide solution. Increasing droplet frequency should increase the number of droplets penetrating the crop canopy. Droplet diameter could have effect on changing the efficacy of herbicides when applied with nozzles. Knoche (1994) reported that decreasing droplet size generally caused an increase in the performance of foliage to which herbicides had been applied, whereas decreasing carrier volume mainly caused a decrease in the performance. Droplets with small diameters can be affected with environmental conditions such as wind and temperature with drifting without reaching the target leaf surface. *HERBI-4* with 2000 rpm disc speed had a bigger VMD and had more effect, but electrostatic sprayer had lower uniformity and used more water. Pearson *et al.* (1981) found that spinning disc nozzles gave better results with 250 µm, VMD than smaller VMDs. Factors such as target (leaf) height from nozzle, target position on the plant, and plant species significantly influenced the depositional efficiency of electrostatic spraying (Sopp and Palmer, 1990). Derksen *et al.* (1991) reported that the low volume electrostatic sprayer performed better than the high volume sprayer while using only 1/25th of the carrier volume and treating the plants in one-third of the time.

4. Conclusions

Electrostatic forces on small droplets are more prominent than the gravitational forces and therefore, electrostatic charging of spray droplets can provide an improved deposition with reduced drift. The spinning disc nozzle had more droplet uniformity, but it did not significantly improve herbicide efficacy in dense canopy compared with the electrostatic charging sprayer. Spinning disc sprayers decreased water use and so was cheaper to operate, but did not improve herbicide efficacy. Spray penetration through dense weeds enhanced with electrostatic charging. If the problem of poor redistribution and poor retention of coarse sprays can be addressed, then such sprays may provide an opportunity for increasing spray penetration through a weed stubble canopy. Further work is required to identify the relative capture efficiencies of weed stubbles for a variety of sprays so that both spray penetration and retention on weeds can be optimized.

Acknowledgements

The authors would like to thank the research assistant of Shahrekord University for providing the laboratory facilities and financial support for this project.

Corresponding Author:
Dr. Ali Esehaghbeygi
Department of Agricultural Engineering, College of Agriculture, Shahrekord University, Shahrekord, Iran, 115
E-mail: esehaghbeygi@cc.iut.ac.ir

References

1. Allen JG, Austin DJ, Butt DJ, Swathi AAJ, Warman TM. Experience with a hand-held ULV charged drop sprayer on fruit. In: Proceedings, 10th International Congress of Plant Protection, Brighton, UK, November 20-25, 1983:501:(2).
2. Bache DH, Johnstone DR. Microclimate and Spray Dispersion. 1st ed. London: Ellis Horwood Ltd. 1992:239 p.
3. Bailey AG. Electrostatic Spraying of Liquids. Taunton, Great Britain: Research Studies Press Ltd. 1988:197 p.
4. Blackshaw RE, O'donovan JT, Harker KN, Clayton GW, Stougaard RN. Reduced herbicide doses in field crops: A review. Weed Biol Manag 2006:6, 10-17.
5. Buhler DD, Burnside OC. Effects of application variables on glyphosate phytotoxicty. Weed Technol. 1987:1, 14-17.
6. Derksen RC, Sagi T, Sanderson J. Green house liquid applicator performance evaluations. ASAE Paper 1991: No. 91-1026.
7. Hislop EC. Can we define and achieve optimum pesticide deposits? Asp. Appl. Biol. 1987:14, 153-172.
8. Hislop EC. Electrostatic ground-rig spraying: an overview. Weed Technol. 1988:2:94-105.
9. Hoffmann WC, Farooq M, Walker TW, Fritz B, Szumlas D, Quinn B, Bernier U, Hogsette J, Lan Y, Huang Y, Smith VL, Robinson CA. Canopy penetration and deposition of barrier sprays from electrostatic and conventional sprayers. J. Am. Mosquito Control Assoc. 2009:25(3): 323-331.
10. Kang TG, Lee DH, Lee CS, Kim SH, Lee GI, Choi WK, No SY. Spray and depositional characteristics of electrostatic nozzles for orchard sprayers. St. Joseph, MI Am. Society of Ag. Eng. (ASAE). ASAE Paper 2004:No. 041005.

11. Kirk IW, Hoffmann WC, Carlton JB. Aerial electrostatic spray system performance. Trans ASAE 2001:44:1089-1092.
12. Knoche M. Effect of droplet size and carrier volume on performance of foliage-applied herbicides. Crop Prot. 1994:13(3):163-178.
13. Lake JR, Marchant JA. Wind tunnel experiment and a mathematical model of electrostatic spray deposition in barley. J. Agri. Eng. Res. 1984:30(2):185-195.
14. Law SE. Agricultural electrostatic spray application: a review of significant research and development during the 20th century. J Electrost. 2001:51-52:25-42.
15. Maski D, Durairaj D. Effects of charging voltage, application speed, target height, and orientation upon charged spray deposition on leaf abaxial and adaxial surfaces. Crop Prot. 2010:29, 134-141.
16. Mason JM, Matthews GA, Wright DJ. Appraisal of spinning disc technology for the application of entomopathogenic nematodes. Crop Prot. 1998:17(5):453-461.
17. Micron sprayers Ltd. Battery powered, hand-held agricultural spraying machine, *HERBI-4*. Bromyard Industrial Estate, Bromyard, Herefordshire, UK.
18. Mohan RG, Nelson JE. On-farm comparative herbicide performance with conventional and controlled droplet applicators. Proceedings of the North Central Weed Control Conference, Indianapolis, 1982:Vol.37, USA, p. 15.
19. Montazeri M, Zand E, Baghestani MA. Weeds and their Control in Wheat Fields of Iran, first ed. Agricultural Research and Education Organization Press, Tehran, Iran. 2005 (in Persian with English abstract).
20. Morton N. The Electrodyn sprayer: first studies of spray coverage in cotton. Crop Prot. 1982:1:27-54.
21. Pearson SC, Bode LE, Butler BJ. Characteristics of controlled droplet applicators. Proceedings of the North Central Weed Control Conference 1981:Vol.36 USA, pp. 1-2.
22. Scoresby JR, Nalewaja JD. The controlled droplet applicator for herbicide application. Proceedings of the North Central Weed Control Conference 1982:Vol. 37, Indianapolis, USA.
23. Shaw WC. Integrated weed management systems technology for pest management. Weed Sci. 1982:30, 3-12.
24. Sikkema PH, Brown L, Shropshire C, Spieser H, Soltani N. Flat fan and air induction nozzles affect soybean herbicide efficacy. Weed Biol. Manage. 2008:8, 31-38.
25. Sopp PL, Palmer A. Deposition patterns and biological effectiveness of spray deposits on pot plants, applied by the ulvafan and three prototype electrostatic sprayers. Crop Prot. 1990:4, 295-302.
26. Spillman JJ. Spray impaction, retention and adhesion: an introduction to basic characteristics. Pestic. Sci. 1984:15:97-106.
27. Uremis I, Bayat A, Uludag A, Bozdogan N, Aksoy E, Soysal A, Gonen O. Studies on different herbicide application methods in second-crop maize fields. Crop Prot. 2004:23, 1137-1144.
28. Walker SR. Comparison of controlled droplet and conventional application of post emergence herbicides. Queensland J. Agric. Anim. Sci. 1986:43(2):135-139.
29. Wolf TM, Caldwell BC, McIntyre GI, Hsiao AI. Effect of droplet size and herbicide concentration on absorption and translocation of 14C-2, 4-D in oriental mustard (Sysimbrium orientale). Weed Sci. 1992:40, 568-575.
30. Wolf TM, Harrison SK, Hall FR, Cooper J. Optimizing post emergence herbicide deposition and efficacy through application variables in no-till systems. Weed Sci. 2000:48, 761-768.

7/21/2010

The Influences of work support and family support on Work-Family Conflict (W-FC) Among Married Female Nurses in Shiraz-Iran

Hajar Namayandeh[1], Siti Nor Yaacob [1], Rumaya Juhari [1]

[1.] Faculty of Human Ecology, University Putra Malaysia
h_nemayandeh@yahoo.com

Abstract: The present study highlights the significance of work support (supervisor and coworker support) on work-family conflict. Furthermore, this paper also examines the effects of family support (husband and family members/relatives support) on work-family conflict. This study examines 198 married female nurses in Shiraz-Iran. The findings revealed that low support received from husband, family members/relatives and supervisor might increase perceived conflict between work and family. Unlike previous studies, the finding also indicates that there is no significant relationship between the respondents' support from co-worker with work- family conflict, which may be explained by the specific cultural context in Iran. Implications are discussed and recommendations are made regarding future researches in this area.
[Hajar Namayandeh, Siti Nor Yaacob, Rumaya Juhari. **The Influences of work support and family support on Work- Family Conflict (W-FC) Among Married Female Nurses in Shiraz-Iran**. Journal of American Science 2010;6(12):534-540]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Work- family conflict, Work support, Family support

1. Introduction:

The rise in women's participation in the workforce has introduced new challenges for most families (Davidson & Burke, 2004). Families in which both husband and wife work, are more likely to create even more important conflicts between work and family (Livingston & Judge, 2008). In such families employed women have higher combined pressures from work and family responsibilities that lead them to experience work-family conflict (Pleck, 1977; Rexroat & Shehan, 1987). Based on this situation, scientists dedicated many investigations on this issue. However, according to Karimi (2008), more than 80% of investigations in this domain have been carried out in developed western society and very few comprehensive studies have been conducted in developing countries such as Iran. The results of the studies in developed countries are not necessarily interpretable in developing countries due to different cultural beliefs and practices (Grzywacz et al., 2007; Aryee 1992).

The participation of women in the workplace has increased in Iran. Statistics from 1956 to 1986 showed that about 9% of women in Iran were employed (Moayedi, 1994). The reported statistics for 2004 further revealed that 79.5% of women were employed (Statistical Bureau of Iran, 2004). Although women are working out of the home in Iran today, the structure of traditional way of thought about domestic works does not change much. Iranian women who already have a traditional role as housewives have to assume additional responsibility as an employee out of the home (Rastegarkhaled, 2004).

Nurses, like other female employees are faced with the demands of work and home responsibilities as their main daily tasks. According to Ministry of Health, nurses in Iran are mainly women. As of 2009, it was reported that 79.5% of Iranian nurses are female (ISNA, 2009). Nurses basically have to work in critical situation that includes dealing with high workloads and time pressures, number of hours worked, shift work, death and life situation, and stressful and demanding responsibilities. These situations have been denoted as the major issues in the nature of nurses' job (Adibhajbagheri, Mehnosh & Fazlallah, 2004).

Working outside and inside the house has formed two of the most central domains in women's life, each containing its own duties. According to Pleck (1977) work and family are two fundamental and interdependent systems for dual-career live that inconsistency in any one system may consequently influence the other one as well. This construct

represents conflict, namely, work interference with family (WIF). Accordingly, work-family conflict (W-FC) can be defined as the amount of conflict an individual experiences between her job or career and home-life (Mirrashidi, 1999).

The present study focuses on the role of social support in two dominant spheres of our lives, work and family. Studies of social support within the family and work could be relevant to the system theory. Both work and family could be classified into two subsystems. In the current investigation, the subsystem of work includes supervisor and co-workers support and subsystem of the family includes husband and family members/relatives support. Each of these subsystems might emotionally and/or instrumentally support the individual. This may suggests that support from each subsystem may influence other system. The present study aimed to consider the relationships between work support (supervisor and co-workers) and family support (husband and family members/relatives) with W-FC among nurses in the developing country with specific culture like Iran.

2. Social Support

Social support involves the exchange of resources between at least two persons, with the aim of helping the person who receives the support (House, 1981). For employed individuals, source of social support can be from work and family context. Social support can also be discussed in terms of emotional support (love, empathy, trust, concern) and instrumental support (time, money, goods, and services) (House, 1981). Further, two dimensions of social support sources have been identified: work support and family support. Both work and family setting may provide individuals with emotional and instrumental support.

3. Work support and W-FC

Social support at work can be derived from supervisor and co-worker. Previous studies successfully demonstrated relationship between social support and work interference with family. According to Yildirim and Aycan (2008) supervisory support included both instrumental and emotional supports. Instrumental support implied providing assistance and advice whereas emotional support referred to supervisor's emphatic understanding and sensitivity to work-family issue. Yildirim and Aycan found that social support could be best conceptualized as the main effect directly influencing work-family conflict among nurses in Turkey. Ahmad (1997) investigated the relationship between perceived support from supervisors, co-workers, and spouse, family and friends and work-family conflict among 239 married female production operators. The results showed that about two third of the operators experienced moderate to high intensities of conflict. In general, the operators received less support from their supervisors and they stated that if they received support from their supervisors, they may experienced less conflict.

In a different study, Thomas and Ganster (1995) examined the effects of supportive supervisors on work-family conflict. The respondents of study were 398 health professionals who had children aged 16 years or younger. The finding by Thomas and Ganster indicated a direct positive effect of supportive supervisors on employee perceptions of control over work and family matters. Control perceptions, in turn, were associated with lower levels of work-family conflict. The research study by Rastegarkhaled (2004) also found significant relationship between support from work and family with work-family conflict. The finding suggested that supportive supervisor provide more support for employee and this may reduce the conflict experienced by employees in their occupation and family environment.

Prominent among the sources of support from work is co-worker support. Michael et al. (2004) investigated on work-family conflict and social support received by the respondents from work colleagues and family members. Their research showed that colleague support reduced work interference with family sphere. Furthermore, Payamibosari (1995) concluded that support from co-worker is effective in reducing the work-family conflict of nurses in one of the hospital in Tehran. In contrast, the study by Kirrane and Buckley (2004) indicated that support of co-workers and supervisors at the workplace did not influence work-family conflict among 170 Irish working cohorts. The insignificant relationship may be due to the belief that after having a young child (6 years of age), the spouse-partner instrumental support becomes more important in determining W-FC.

4. Family Support and W-FC

Social support can be derived from husband and the family members/relatives. Past studies showed that family support functions to ameliorate work-family conflict. The study by Kim and Ling

(2001) indicated that if men provide greater support in terms of household chores and childcare, work-family conflict would not be a major problem for working women. However, this study indicated that spouse support plays an important role in reducing work-family conflict. Spouse emotional support has the greatest influence in reducing the level of work-family conflict among Singapore women. In another study, Aryee (1992) examined the impact of some antecedents of work and family domain variables on three types of work-family conflict (job-spouse, job-parent, and job-homemaker) among 354 married professional women from dual-career families in Singapore. Results indicated that spouse support reduces work-family conflict for these professional women.

Likewise, Thomas and Ganster (1995) reported that emotional support from one's partner in a dual earner relationship reduces the negative effects of work on family life among 398 health professionals who had children aged 16 years or younger at home. The results of this study suggested that organizations could take steps that can increase employees' control over family responsibilities and that this control might help employees better manage conflicting demands of work and family life. According to Chee (1997) respondents did not experience much work-family conflict primarily because they received a lot of support from their spouses. The above findings contradict the study by Aryee et al. (1999). Aryee et al. examined the relationship between role stressors, interrole conflict, and wellbeing and the moderating influences of spousal support and coping behaviours. The respondents were 243 Hong Kong Chinese parents from dual-earner families. The results of the study revealed that spousal support was unrelated to work-family conflict.

Another important source of family support is support from family members/relatives. According to an investigation by Rastegarkhaled (2004), family support for women could lead to lower level of work-family conflict experience among working women. Michael et al. (2004) investigated work-family conflict in relationship to the role of social support from work colleagues and family members. Their result indicated that family support was significantly correlated with work-family conflict. Those with higher family support tend to experience lower W-FC. The study by Carlson and Perrewé (1999) examined the role of social support in work-family conflict. The respondents of study were 403 from a department of a state government in the southeast. Their result indicated that family support was negatively associated with work-family conflict. Support from one's partner in a dual earner relationship reduces the negative effects of work on family life.

5. Methodology

The main objective of this study is to determine the relationship between work support (supervisor and co-workers), family support **(**husband and family members/relatives) and W-FC, among married female nurses in Shiraz-Iran. The population of the study consists of N = 647 married female nurses in 13 public hospitals in Shiraz. There were four criterias established for the selection of the population of this research; firstly, married female nurses who work for public hospital; secondly, the married female nurses who live together with their husbands; thirdly, nurses who have a minimum of 6 months job experience and have at least one child.

The method of data collection used was self-administered questionnaire and the study is correlation in nature. The respondents were selected by using simple random sampling technique. Initially, the identified and eligible sample size for the study was 323 respondents. Out of the total questionnaire distributed to the eligible respondents, only198 (61.30%) questionnaires were returned. Pilot study was conducted before the actual data collection to assess the adequacy of the questionnaire. In the present study, the Cronbach's Alpha for all measures used is mostly more than 0.70 indicating that the instruments are reliable to be used.

6. Measures

6.1 Work Family Conflict (W-FC)

Work-family conflict was measured by using work-family conflict and family-work conflict scale developed by Netemeyer, Boles, and McMurrian (1996). The scale consisted of 2 subscales: W-FC and F-WC. Each subscales consisted of 5 items. In this study W-FC subscale was used. Responses were obtained using a seven point Likert type scale where 1= strongly disagree, 2= disagree, 3= slightly disagree, 4= neutral, 5= slightly agree, 6= agree and 7= strongly agree. A sample item from this scale is "The amount of time my job takes up makes it difficult to fulfill family responsibilities". The scale scores range from 5 to 35, with high score indicates a high level of perceived

conflict between work and family and low score will indicate a low level of perceived conflict between work and family. The Cronbach's Alpha estimate in the present study for work-family conflict scale is .86.

6.2 Social Support: Work Support, Family Support

Social support from the work and from family was measured by using social support scale developed by Caplan, Cobb, French Jr, Harrison, and Pinneau Jr (1975) with minor adaptation. The scale consisted 4 items related to emotional support (the extent to which the sources of support will help, how easy to talk to the sources of support about your personal problems) and instrumental support (sources of support include things easier and can be relied on). This measure has been widely used and has remained one of the most established scales used to measure social support in a job (Lim, 1996). One new item was added to respondents' satisfaction with the support received from sources of support. Altogether, 5 items was used to measure support from:

1. Husband
2. Family members / relatives
3. Supervisor
4. Co-workers

The item were answered on a 4 point likert-type scale option from 0 = not at all, 1 = a little, 2 = somewhat and 3 = very much for the four items, while the score for additional item range from 0 = very dissatisfied, 1 = dissatisfied, 2 = satisfied and 3 = very satisfied. The scores for each source of support range from 0 to 15. High scores show that respondents perceived high support from each source of social support. The Cronbach's Alpha estimates for social support in the present study are husband (.89), family members/relatives (.88), co-worker (.83) and supervisor (.83).

7. Results and Discussion

7.1 Descriptive Statistics

Table 1 presents descriptive data that includes mean, standard deviations, minimum and maximum scores of all variables of the study. The finding of this study shows that most of the participants (56.1%) are below 36.50 years old. More than half of the respondents (56.1%) have been married for less than 11.68 years. Moreover, more than half of the respondents (56.6%) have less than 12.49 years job experience.

Figure 1 presents categories of the scores for all variables of the study. The finding shows that most of the respondents received high support from husbands and family members/relatives while large proportion of respondents received moderate and low level of supervisors and co-worker support. Respondents' total scores in W-FC are illustrated in Figure 1. A large proportion of the respondents perceived moderate and high level of work-family conflict. The remaining respondents have low level of work-family conflict.

Table 1: Descriptive Statistics

Variables	Min	Max	Mean	SD
Age (Years)	23	59	36.50	7.15
Job Experience	1	30	12.49	6.86
Duration of Marriage	1.90	40	11.68	7.55
Work-family conflict	8.00	35.00	24.36	6.51
Support from husband	0.00	15.00	9.42	4.10
Family members/relatives' support	0.00	15.00	9.00	4.20
Co-worker' support	0.00	15.00	6.53	3.55
Supervisor' support	0.00	15.00	5.89	3.79

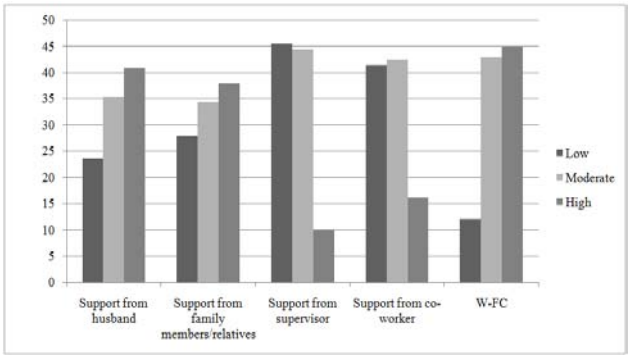


Figure 1: Categories of the scores of variables

7.2 Hypotheses Testing

Ho1: *There is no significant relationship between respondents' support from supervisor and W-FC.*

The finding from Pearson Correlation analyses showed a significant negative relationship between the respondents' support from supervisor with W-FC ($r = -.143$, $p \leq .05$). Hence Ho1 was rejected. The finding indicates that decreasing support from supervisor is associated with increasing W-FC. The finding is consistent with findings of past studies by Yildirim and Aycan (2008), Rastegarkhaled (2004) and Ahmad (1997), which have successfully recognized that supervisory support

could be best conceptualized as having effect on W-FC. Considering the available evidence, it appears that support from supervisor in work will influence respondents' life with family. Married female nurses require supervisors to provide enabling conditions at the hospital to minimize work interference with family responsibilities.

Ho2: *There is no significant relationship between respondents' support from co-worker and W-FC.*

The finding indicated that there was no significant relationship between the respondents' support from co-worker with W-FC ($r = .071$, $p>.05$). Hence Ho4 was failed to be rejected. This direction of relationship is consistent with finding of past study by Kirrane and Buckley (2004) that reported co-workers did not influence W-FC among 170 Irish working adults. In contrast, Payamibosari (1995) concluded that support from co-worker is the more effective support reducing the W-FC of nurses in one of the hospital in Tehran. Furthermore, Michael, Brough, and Kalliath (2004) showed that colleague support could reduce the consequences of work interference with family in some situations. It should be mentioned that despite the lack of significant relationship between the respondents' support from co-worker with W-FC there was discrepancy in regard to two variables. One possible explanation for this result may be due to difficult conditions of work in hospital, support from co-worker is not common among nurses in these hospitals. According to Adibhajbagheri, Mehnosh and Fazlallah (2004) feeling of lack cohesion among nurses create an atmosphere of lack of support and mutual trust between them.

Ho3: *There is no significant relationship between respondents' support from husband and W-FC.*

The finding showed a significant negative relationship between the respondents' support from husband and W-FC ($r = -.183$, $p\leq.05$). Hence Ho3 was rejected. The finding indicates that increasing support from husband is associated with decreasing W-FC. This direction of relationship is consistent with finding of past studies by Aryee (1992) and Kim and Ling (2001) which found that spouse support reduces W-FC for Singapore women. Another studies by Greenhaus and Beutell (1985), Chee (1997) and Thomas and Ganster (1995) also mentioned that social support from supportive spouse is a factor that can reduce W-FC. Considering the available evidence, it appears that respondents' support from husband has consistently been shown to be the most significant antecedent of W-FC. Based on this finding, it would be of interest to elucidate that support from husband in family would influence work. Respondents need the husband support to provide enabling conditions at home to minimize the interference of work with family responsibilities.

Ho4: *There is no significant relationship between respondents' support from family members/relatives and W-FC.*

The finding from Pearson Correlation analyses showed a significant negative relationship between the respondents' support from family members/relatives and W-FC ($r = -.198$, $p\leq.01$). Hence Ho1 was rejected. The finding indicates that increasing support from family members/relatives is associated with decreasing W-FC. This direction of relationship is consistent with findings of past studies by Michael, Brough, and Kalliath (2004) and Carlson and Perrewé (1999) who found that respondents who received higher family support experienced less conflict between work and family. The present finding also support Rastegarkhaled's (2004) study which concluded that family support reduce W-FC. Considering the available evidence, it appears that respondents' support from family members/relatives has consistently been shown to be the most significant antecedent of W-FC. Based on this finding, support from family members/relatives in family will influence work. Respondents' need the family members/relatives support to provide enabling conditions at home to minimize the interference of work with family responsibilities.

8. Conclusion and implication

In conclusion, results from the present study suggest that low support received from husband, family members/relatives and supervisor might increase perceived conflict between work and family. Establishing and reinforcing family support policies that include emotional family support and sharing of household chores can be effective in balancing cohesion and adaptability among family members. Consequently, balance in cohesion and adaptability in family could affect work positively and hence reduce W-FC. In addition, according to Adibhajbagheri, Mehnosh, and Fazlallah (2004), difficult work condition in hospital has increased feelings a lack of support and cohesion among nurses in Iran.

Therefore, administering work support policies (e.g. child care and elder care services, flexible working schedules and welfare of nurses) may result in higher work satisfaction and motivation among nurses; and ultimately culminated a sense of cohesion and closeness among them. Hence, these policies might help working women manage the demands from work and family domain, and consequently reduces the W-FC.

9. Recommendations for Future Study

There are several recommendations and limitations that have been identified throughout this study, which may direct future studies. Primarily, having a low response rate and relying on one city for data collection limit the generalizability of the findings. Also, findings cannot be attributed to nurses of private hospitals. Accordingly, the results are not generalizable to the entire nurse population in Iran. Assessing the variables of the model across gender would contribute to the generalizability of the results. This study also needs to be replicated with a more heterogeneous population such as other ethnic groups, religions, occupational variations and different cultural values. More studies of this nature should be conducted especially among women in Middle Eastern countries. Finally, this study used a self-report measure (questionnaire). Thus, future researches can use combined methods of data collection to strengthen and enrich the findings.

References

1. Adibhajbagheri, M., Mehnosh, S., & Fazlallah, A. (2004). Qualitative research on the concept of professional nursing competencies. *faze, 83*(29).
2. Ahmad, A. (1997). Work-Family Conflict and Social Support: A Study of Female Secretaries in Malaysia. *Pertanika J. Soc. Sci. & Hum, 5*(2), 93-101.
3. Aryee, S. (1992). Antecedents and outcomes of work-family conflict among married professional women: Evidence from Singapore. *Human Relations, 45*(8), 813.
4. Aryee, S., Luk, V., Leung, A., & Lo, S. (1999). Role stressors, interrole conflict, and well-being: The moderating influence of spousal support and coping behaviors among employed parents in Hong Kong. *Journal of Vocational Behavior, 54*(2), 259-278.
5. Caplan, R. D., Cobb, S., French Jr, J. R. P., Harrison, R. V., & Pinneau Jr, S. R. (1975). Job demands and worker health (HEW Publication No. NIOSH 75-160). *Washington, DC: US Government Printing Office*.
6. Carlson, D. S., & Perrewé, P. L. (1999). The role of social support in the stressor-strain relationship: An examination of work-family conflict. *Journal of Management, 25*(4), 513.
7. Chee, W. Y. (1997). *Interrole conflict and social support a study of married women nurses.* University Putra Malaysia, Serdang.
8. Davidson, M. J., & Burke, R. J. (2004). *Women in Management Worldwide: Progress and Prospects*. Ashgate, UK.
9. Greenhaus, J. H., & Beutell, N. J. (1985). Sources of conflict between work and family roles. *Academy of management review*, 76-88.
10. Grzywacz, J. G., Arcury, T. A., Márin, A., Carrillo, L., Burke, B., Coates, M. L., et al. (2007). Work-family conflict: Experiences and health implications among immigrant Latinos. *Journal of Applied Psychology, 92*(4), 1119-1130.
11. ISNA, H. I. S. N. A. (2009). Department of Health Nursing Office News from http://modirnurse.blogfa.com/.
12. Karimi, L. (2008). Do Female and Male Employees in Iran Experience Similar Work-Family Interference, Job, and Life Satisfaction? *Journal of Family Issues, 30*, 124-142.
13. Karimi, L., & Nouri, A. (2009). Do work demands and resources predict work-to-family conflict and facilitation? A study of Iranian male employees. *Journal of Family and Economic Issues, 30*(2), 193-202.
14. Kim, J. L. S., & Ling, C. S. (2001). Work-family conflict of women entrepreneurs in Singapore. *Women in Management review, 16*(5), 204-221.
15. Kirrane, M., & Buckley, F. (2004). The influence of support relationships on work-family conflict: differentiating emotional from instrumental support. *Equal Opportunities International, 23*(1/2), 78-96.
16. Lim, V. K. G. (1996). Job insecurity and its outcomes: Moderating effects of work-based and nonwork-based social support. *Human Relations, 49*(2), 171.
17. Livingston, B. A., & Judge, T. A. (2008). Emotional responses to work-family conflict: An examination of gender role orientation among working men and women. *Journal of Applied Psychology, 93*(1), 207-215.

18. Michael, P. O. D., Brough, P., & Kalliath, T. J. (2004). Work/family conflict, psychological well-being, satisfaction and social support: a longitudinal study in New Zealand. *Equal Opportunities International, 23*.
19. Mirrashidi, T. (1999). *Integrating work and family: Stress, social support and well-being among ethnically diverse working women.* Unpublished Ph.D., California School of Professional Psychology - Los Angeles, United States -- California.
20. Moayedi, M. (1994). Statistical Glance at Women's Employment in Iran. from http://www.parstimes.com/women/glance_womens_employment.html.
21. Netemeyer, R. G., Boles, J. S., & McMurrian, R. (1996). Development and validation of work-family conflict and family-work conflict scales. *Journal of Applied Psychology, 81*(4), 400-410.
22. Payamibosari, M. (1995). University of Medical Sciences Tehran.
23. Pleck, J. H. (1977). The work-family role system. *Social Problems*, 417-427.
24. Rastegarkhaled, A. (2004). The investigate relationship between work and family conflict and social factors *Do mah name daneshgah Shahed, 11*(4).
25. Rexroat, C., & Shehan, C. (1987). The family life cycle and spouses' time in housework. *Journal of Marriage and the Family, 49*(4), 737-750.
26. Thomas, L. T., & Ganster, D. C. (1995). Impact of family-supportive work variables on work-family conflict and strain: A control perspective. *Journal of Applied Psychology, 80*(1), 6-15.
27. Yildirim, D., & Aycan, Z. (2008). Nurses' work demands and work-family conflict: A questionnaire survey. *International Journal of Nursing Studies, 45*(9), 1366-1378.

9/10/2010

Effect of using pectin on lead toxicity

Dalia, M. El-Nahal

Special Food and Nutrition Dep., Food Technology Research Institute, Agric. Res. Center, Giza, Egypt
daliaelnahal@hotmail.com

ABSTRACT: Lead has many undesired effects on humans and animals, including neurological, behavioral, respiratory, visual, growth retardation, hematological immunological, renal, hepatic. **The aim of** the present study was to investigate the alterations in biochemical parameters in serum and blood due to lead retention in blood, organs and estimating the role of low and high esterified pectin in alleviating the negative effects of lead. **Material and Methods**: Sixty male *albino* rats which were divided into ten groups (6 rats for each). The first group (was fed on basal diet ;normal control). Groups 1,2 and 3 [which were fed on basal diet and administrated lead acetate (LA) daily once a time for 30 days by gavages at three different concentrations 61.94, 30.97 and 15.49 mg /Kg bw (1/4, 1/8, and 1/16 of lead acetate LD_{50};positive control]. Groups 4,5 and 6 [were fed on basal diet containing 10% low esterified pectin (LEP, DE 31%) and administered the same LA doses]. Groups 7,8 and 9 [were fed on basal diet containing 10% high esterified pectin (HEP, DE 73.5%) with the administration of the same LA doses]. **Results** obtained showed that LA significantly induced a decrease in body weight, serum total protein, albumin, globulin, total billirubin, direct billirubin, indirect billirubin, RBCs and WBCs counts, blood haemoglobin (Hb), heamatocrite values (PVC), serum triidothyronine (T3)and thyroxin (T4) levels. In the contrary, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AlP), gamma glutamyl transferase (γ-GT) activities, serum urea, uric acid and creatinine were significantly increased in positive control rat groups. Additionally, treatment of rats with LA led to a considerable increase in accumulation of the metal in the blood, liver, kidney, brain, heart and bones compared with the normal group. LEP and HLP significantly decreased the effect of LA on the tested parameters and level of lead in different organs. Histopathological examination clearly indicated that LEP or HEP eliminated from the harmful effect of LA on liver, kidney and brain tissues. **In conclusion**, LEP and HLP have beneficial effects which could be able to antagonize lead toxicity. Moreover, LEP was contributed to fast elimination of the lead acetate to blood, organs and bones, whereas HEP removed lesser amount of lead. It could be recommended that LEP has a good effect to bind material of lead and should be incorporated into human food to reduce the hazards toxicity of lead pollution of food and water.

[Dalia, M. El-Nahal. **Effect of using pectin on lead toxicity Dalia, M. El-Nahal.** Journal of American Science 2010;6(12):541-554]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: Lead toxicity, esterified pectin, Histopathological examination

1-INTRODUCTION

Lead is a pervasive and persistent environmental pollutant that can be detected in almost all phases of environment and biological systems. Lead dispersion in ambient air, in many foods, in drinking water, and in dust, Khotimchenko *et al,* (2007) and especially in the 20th century, because of the industrial applications, Landrigan *et al.*, (2000). Although lead is one of the most useful metals, it is also one of the most toxic, Shotyk and Le Roux (2005). It was indicated that lead can cause neurological, hematological, gastrointestinal, reproductive, circulatory, and immunological pathologies, Hao *et al.*,(2002); Patrick, (2006), reproductive dysfunctions ,Marchlewicz *et al.*, (1993). Moreover, lead acetate and lead phosphate are listed as carcinogens.Inorganic lead compounds were classified as 2B chemicals by IARC, Pulido and Parrish, (2003). It was reported that lead increased the level of lipid per-oxidation,Upasani *et al.*, (2001). It can lead to inhibition of the activities of antioxidant enzymes, including glutathione peroxidase, catalase and superoxide dismutase, Silbergeld *et al.*, (2000). Furthermore, generation of reactive oxygen species (ROS), stimulation of lipid peroxidation and depletion of antioxidant reserves which was postulated to be major contributors to lead-exposure related diseases ,Patrick, (2006).

Pectin is the ionic plant polysaccharides widely used in food industry because of their gelling and thickening properties, Thakur *et al*, (1997). Pectin is a group of complex polysaccharides that contain 1,4-linked-d-galacturonic acid , Willats *et al*, (2001). There are three major pectic polysaccharides: homogalacturonans, rhamnogalacturonans-I, and rhamnogalacturonans-II. Natural pectins are highly esterified and contain more than 50% of esterified carboxyl groups, whereas LE pectins can be prepared Ridley *et al, (*2001). The number of esterified carboxyl groups determines the degree of

esterification, which is one of the important chemical parameters of pectins ,Schols and Voragen (1996).

It was shown that some physiological effects, for example, interactions with bile acids or drugs, may depend on the pectin structure, Dongowski *et al*, (1997).

The goal of this study was to determinate the relationship between structure of the pectin compounds with a high degree of esterification (HE pectin), and low esterified pectin (LE pectin) and their effects on lead absorption, retention, and removal in laboratory rats.

2-MATERIALS AND METHODS

2.1.Materials:

2.1.1.Chemicals and kits:

1. Pure lead acetate was purchased from Sigma Chemical (St. Louis, Mo). All other chemicals were of the highest available quality.

2. Genu® citrus pectin type 105 rapid set has high degree of estrification (DE) 73.5% and partially esterified Genu® citrus pectin type LM 104 As (31%DE) were obtained from Copenhagen pectin, Lille Skenved, Denmark.

3. Nitric acid 69% analar BDH and perchloric acid 70% Aldrich.

4. Biomerieux kits were obtained from Randox Laboratories Ltd., Diamond Road, Crumlin Co., Antrim, United Kingdom, BT294QY.

2.1.2. Experimental animals:

-Sixty male *albino* rats weighing 100 ± 5 g were obtained and housed in Food Technology Research Institute, Agricultural Research Center, Giza, Egypt. The rats were kept under normal health laboratory conditions and fed on basal diet for one week. Water and basal diet were provided *ad libitum.* The basal diet consisted of protein (casein) 20%, cellulose 10 %, salt mixture 4%, vitamin mixture 1%, corn oil 10% and corn starch 55% according to (Pell *et al.* 1992). The adaptation period was one week.

-Rats were weighted twice weekly, total feed intake of each rat was weighted and feed conversion efficiency (gain weight of rat/ total feed intake, g) was calculated.

-Animals were weighted and randomly divided into 10 groups each one containing six rats, as follows:

Normal control: fed on basal diet.

Group 1: Positive control 1, fed on basal diet and administered 1/4 LD_{50} of LA.

Group 2: Positive control 2, fed on basal diet and administered 1/8 LD_{50} of LA.

Group 3: Positive control 3, fed on basal diet and administered 1/16 LD_{50} of LA.

Group 4: Fed on basal diet containing10% low esterified pectin (LEP) and administered 1/4 LD_{50} of LA.

Group 5: Fed on basal diet containing 10% low esterified pectin (LEP) and administered 1/8 LD_{50} of LA.

Group 6: Fed basal diet containing 10% low esterified pectin (LEP) and administered 1/16 LD_{50} of LA.

Group 7: Fed basal diet containing 10% high esterified pectin (HEP) and administered 1/4 LD_{50} of LA.

Group 8: Fed basal diet containing 10% high esterified pectin (HEP) and administered 1/8 LD_{50} of LA.

Group 9: Fed basal diet containing 10% high esterified pectin (HEP) and administered 1/16 LD_{50} of LA.

2.2. Methods:

2.2.1. Low and high esterified pectin:

2.2.1.1. Determination of galactouronic acid: The pectin content of low and high esterified pectins were determined as galactouronic acid by high performance liquid chromatography as described by Hicks *et al.,* (1985) with modification: 200 mg of low and high esterified pectin, dispersed in 1 ml of iced-cold 80% sulfuric acid, was allowed to set at 25 ºC for 18 hours. The sample was then diluted to 13 ml, sealed in a vail, and placed in a boiling water bath for 5 hours. The resulting dark solution was neutralized with solid calcium carbonate, filtered (0.2 µm) and injected into the chromatography.

Galactouronic acid was identified by a Hewlett Packard HP 1050 High performance liquid chromatography (HPLC) equipped with refractive index 1047 HP, Column compartment was set at 85 ºC, degaser and autosmpler. The chromatograph was fitted with Bio Rad HPX-87-C model (30cm× 7.8m id.) Isocratic elution system was used by deionized water at the flow rate 0.8ml/min.

2.2.2.1. Determination of degree of esterification: Methoxyl content was determined by the measurement of methanol liberated on saponification of the pectin according to the method described by Speirs *et al.,* (1980) as follows: 2 g of tested high and low pectins were homogenized with 150 ml water; the samples were saponified by the addition of 20 ml of 1M NaOH then allowed to stand for 30 min. at room temperature. The alkali was then neutralized by the addition of an equivalent amount of 0.5M HCl. The resulting acid mixture was transferred to 250ml volumetric flask and made up to the volume with distilled water. The contents of the flask were centrifuged at 12000 rpm for 20 min. the supernatant

was decanted off and retained until analysis of methanol released by using gas chromatography (Knuth, *et al.,* 1984 and litchman and Upton, 1972).

Gas chromatography condition: Analysis was performed on Hewlett- Packard Model 5890 gas chromatography equipped with flame ionization detector. The instrument was also equipped with HP-1 column (cross-lanked methyl silicone) 30 m x0.53 mmx 0.88 µm film thickness. Detector and injection port temperature was 250 ºC for each, nitrogen (15ml/min) was used as a carrier gas and hydrogen (15 ml/min and air 240ml/min was used for the flame operation. On-column injections of 1.0µl were used for all samples and standards. Columns were pre-conditioned with a nitrogen flow (15 ml/min) for 1 hr. at 30 ºC, then programmed at 4 ºC/min to 150 and held for 15 min. The reactive area under the peaks obtained from the chromatogram may be used to calculate the methoxyl content of the sample using the following relationship.

Methoxyl content (W/W)% = $1.211A_1 / (A_2 \times W)$. When: A_1 = area under the peak of sample. A_2 = area under the peak of standard. W = weight of sample (g).

Degree of esterification (DE) = (methoxyl content × 612%)/ galactouronic acid%.

2.2.2. Biochemical assay:

Blood samples were collected from the animals. Heparin was used as an anticoagulant. Serum was kept frozen at –20 ºC for biochemical assayas following:

-Alanine aminotransferase (ALT; EC 2.6.1.2) and aspartate aminotransferase (AST; EC 2.6.1.1) activities were assayed by the method of Bergmeyer and Harder (1986).

-Alkaline phosphatase (Al P; EC 3.1.3.1) activity was measured at 405 nm using the method of Varley *et al* (1980).

- Gamma -GT was measured according to the method described by Szasz (1960).

-Serum total protein (TP) was analyzed using the method of Lowry *et al* (1951).

- Albumin concentrations were determined by the method of Doumas *et al* (1977)

- Globulin concentrations were determined by difference (TP - albumin).

-Serum total billirubin and direct billirubin was measured using the method of Walters and Gerade (1970). Indirect billirubin was calculated by difference between Serum total billirubin and direct billirubin. - Creatinine, urea and uric acid were determined by using the methods described by Larsen (1972), Patton and Crouch (1977) and Caraway (1955).

-Total Triiodothyronine (T3) and Total Thyroxin (T4) hormones were estimated in serum samples using DIMA GmbH Diagnostics kits, Goettingen, Germany according to the method described by Young *et al,* (1975) and Sterling (1975), respectively

2.2.2. Hematological evaluation:

Leucoytes count (WBC_s), red blood cell (RBC_s), blood haemoglobin (Hg) and haematocrite value (PVC) estimated by Wintrobe (1967); Dacie and Lewis (1975); Leong *et al* (2003) and Burch and Siegel (1971),respectively.

2.2.3. Measurement of lead concentration:

2.2.3.1. In the blood;

Approximately 1 ml of blood was digested with nitric and perchloric acid mixture (Kolmer *et al.*, 1951). Two to three blank samples were run simultaneously with each batch of the digestion where bio-sample was substituted by de-ionized distilled water. The equal quantity of acid mixture was added to the blank, standard and test digestion tubes during low heat digestion.

2.2.3.2. In organs and feces;

At the end of the experimental period, organs (liver, kidney, brain, heart and bones) were isolated and feces were collected and stored at -20ºC for wet digestion and analysis of lead content.

To accurately weight of tissue and feces in 125 ml Erlenmeyer flask, add glass beads and 25ml deionized water, add 10 ml 1:2 (equal volumes) mixture of concentrated HNO_3 and $HClO_4$. Boil the sample until the solution is clear ,Parker *et al*, (1967). Transfer the solution quantitatively to 100 ml volumetric flask. Dilute to volume with deionized water and mix well.

The lead concentrate in blood, organs and feces was estimated using a Perkin Elmer Plasma 400, Emission spectrometer, Koirtyohann (1994). The average reading of the blanks was first subtracted from that of standard and test samples and then calculation was made for their lead concentration in µg/dl of blood and µg/g of wet tissue and feces.

2.2.4. Histopathological studies:

Tissue specimens from rat's liver, kidney and brain were fixed in 10 % neutral buffered formalin solution. The fixed specimens were trimmed, washed and dehydrated in ascending grades of alcohol, cleaned in xylene, embedded in paraffin then sectioned (4-6 micron) and stained with hemato-xylene and eosin according to Bancroft *et al* (1996). The degree of injury was estimated using an ordinal scale modified from Palaa and Charbonneau (1994).

2.2.5. Statistical analysis:

Each parameter was analyzed separately by using one-way analysis of variance (ANOVA). For determining differences between groups, the Duncan test was used. All *p* values of <0.05were considered to be significant.

3. RESULTS

As a majority of polysaccharides, pectins are heterogeneous compounds regarding structure, molecular weight, and physicochemical properties. These parameters of pectins vary from one fruit species to another and also during the different developmental stages of the fruit (Chang *et al*, 1994) as well as in the process of chemical and enzymatic modifications.

The galactouronic acid concentration in high and low esterified pectin were 75.72 and 78.53%, respectively. The assay showed degree of esterification to be approximately 73.5 and 31% respectively in high and low esterified pectin respectively**.**

3.1. Growth of rats:

Data in Table (1) show changes in the weight and the feed conversion efficiency (FCE) of the tested rat groups. Significant differences between groups in weight gain were noticed. The highest accumulated weight gain was recorded by G9 and normal control which fed 10% HEP + 1/16 LD_{50} of Pb and normal control, respectively. However, the lowest accumulated weight gain was observed in positive control rat groups (G1, G2 and G3). No significant difference appeared for feed intake of all tested rat groups. At the same time, feed conversion efficiency (FCE) was also decreased by administered oral doses of lead acetate without fed HEP or LEP (G1, G2 and G3).

Data presented in Table (3), show significant differences among tested rat groups in their serum total protein, albumin, globulin, total billirubin, direct billirubin and indirect billirubin at the end of experimental period. Positive rat groups (G1, G2 and G3) had significant decrease compared with treated rat groups and normal control. There were no significant differences observed between the tested rat groups in A/G ratio.

3.2. Biochemical assay:

3.2.1. 1.Liver function

The level of serum ALT, AST, ALP and γ-GT activities are presented in Table (2). Significant increases were observed in these enzymes activity in rat groups administered lead acetate doses without fed HEP or LEP. There was friary difference between normal control, G6 (fed 10% LEP + 1/16 LD_{50} of Pb) and G9 (fed 10% HEP + 1/16 LD_{50} of Pb) at the end of experimental period in serum ALT and AST activities. Moreover, No significant difference was observed in serum ALP and γ- GT activities among tested rat groups expect positive control rat groups (G1, G2 and G3) at the end of experimental period.

Table (1): Weight gain, feed intake and feed efficiency ratio of rats fed on 10 % LEP or HEP and administrated different doses of lead acetate

Rat groups	Initial weight (g.)	Final weight (g.)	Weight gain (g.)	Feed intake (g.)	Feed efficiency ratio
Normal control	100.23 ± 6.91^{a}	204.16 ±	103.92 ±	375.76 ±	0.280 ±
G1 (1/4 LD_{50} of Pb)	101.51 ±	170.83 ± 5.18^{b}	69.32 ± 3.72^{d}	330.23 ±	0.210 ±
G2 (1/8 LD_{50} of Pb)	99.72 ±	169.71 ± 3.46^{b}	70.67 ± 3.89^{d}	316.19 ±	0.223 ±
G3 (1/16 LD_{50} of Pb)	99.23 ±	173.45 ± 6.27^{b}	74.22 ± 2.98^{d}	323.49 ±	0.230 ± 0.010 ^{cd}
G4 (fed 10% LEP +1/4 LD_{50} of Pb)	100.58 ±	191.02 ± 16.09^{ab}	90.44 ± 7.01^{bc}	366.50 ±	0.247 ± 0.006 ^{cd}
G5 (fed 10% LEP +1/8 LD_{50} of Pb)	101.84 ±	203.20 ±	101.53 ± 3.48 ^{ab}	381.03 ±	0.267 ± 0.006 ^{ab}
G6 (fed 10% LEP +1/16 LD_{50} of Pb)	100.49 ±	202.49 ±	101.91 ± 6.79 ^{ab}	378.20 ±	0.270 ± 0.010 ^{ab}
G7 (fed 10% HEP +1/4 LD_{50} of Pb)	100.49 ±	188.45 ± 1.74^{ab}	88.05 ± 6.74^{c}	345.24 ±	0.240 ± 0.010 ^{de}
G8 (fed 10% HEP +1/8 LD_{50} of Pb)	100.71 ±	196.39 ±	95.68 ± 5.52^{bc}	372.97 ±	0.257 ± 0.006 ^{bc}
G9 (fed 10% HEP +1/16 LD_{50} of Pb)	100.86 ±	206.26 ±	115.53 ±	400.74 ±	0.263 ± 0.005 ^{ab}
L.S.D.	11.6405	15.6354	9.2249	10.0508	0.0132

Each value represents the mean ± SE. The mean values with different superscript alphabets indicate significant differences ($P \leq 0.05$) using LSD test.

Table (2): Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), Alkaline phosphatase (ALP) andgamma glutamyl transferase (Gamma GT) of rats fed on 10% LEP or HEP and administrated different doses of lead acetate

Rat groups	ALT		AST		ALP		Gamma GT	
	Zero time	End	Zero time	End	Zero time	nd	Zero time	End
Normal control	39.20 ± 1.24 ^{af}	41.26 ± 2.31^{a}	82.34 ± 3.77^{a}	84.66 ± 4.35^{a}	144.33 ± 5.75^{a}	152.34 ± 8.47^{a}	4.62 ±	4.30 ±
G1 (1/4 LD_{50} of Pb)	39.01 ± 2.83 ^{af}	61.65 ±	80.25 ± 5.09^{a}	128.97 ±	152.80 ± 9.50^{a}	263.02 ±	4.41 ±	6.14 ± 0.81^{b}
G2 (1/8 LD_{50} of Pb)	37.63 ±	62.12 ±	80.84 ± 2.60^{a}	128.83 ±	145.02 ± 8.06^{a}	257.75 ±	4.23 ±	5.70 ± 0.28^{b}
G3 (1/16 LD_{50} of Pb)	37.93 ± 1.56 ^{af}	58.73 ±	79.74 ± 2.83^{a}	124.10 ±	143.86 ± 8.04^{a}	217.93 ± 8.31 ^{b}	4.67 ±	5.73 ± 0.23^{b}
G4 (fed 10% LEP+ 1/4 LD_{50} of Pb)	37.77 ± 1.14 ^{af}	48.48 ± 4.33 ^{bc}	81.14 ± 2.74^{a}	94.00 ± 5.09^{b}	151.16 ± 4.89^{a}	149.78 ± 6.85 ^{c}	4.53 ±	4.46 ±
G5 (fed 10% LEP+ 1/8 LD_{50} of Pb)	39.82 ± 1.70 ^{af}	43.26 ± 2.19 ^{bc}	80.83 ± 3.31^{a}	93.32 ± 2.89^{b}	150.74 ± 9.30^{a}	152.10 ± 10.29 ^{c}	4.29 ±	4.51 ±
G6 (fed 10% LEP+ 1/16 LD_{50} of Pb)	39.67 ± 2.39 ^{af}	40.07 ± 1.66 ^{af}	80.09 ± 4.21^{a}	80.61 ± 5.76^{c}	154.66 ± 7.10^{a}	144.30 ± 11.09 ^{c}	4.60 ±	4.04 ±
G7 (fed 10% HEP+ 1/4 LD_{50} of Pb)	37.16 ±	52.16 ± 3.57^{b}	79.74 ± 3.11^{a}	100.10 ± 6.46^{b}	149.43 ± 9.68^{a}	158.50 ± 7.29 ^{c}	4.35 ±	4.38 ±
G8 (fed 10% HEP+ 1/8 LD_{50} of Pb)	38.28 ±	44.28 ± 1.34 ^{cd}	80.37 ± 3.93^{a}	94.89 ± 4.27^{b}	151.37 ± 10.22^{a}	149.92 ± 4.10 ^{c}	4.11 ±	4.27 ±
G9 (fed 10% HEP+ 1/16 LD_{50} of Pb)	39.43 ±	40.53 ± 2.44 ^{af}	79.79 ± 4.30^{a}	85.03 ± 3.20^{c}	151.84 ± 6.49^{a}	143.43 ± 3.78 ^{c}	4.22 ±	4.40 ±
L.S.D.	4.6228		8.1085		13.1888		0.9308	

Each value represents the mean ± SE. The mean values with different superscript alphabets
indicate significant differences ($P \leq 0.05$) using LSD test.

3.2.2. **Kidney function:**

The level of serum urea, uric acid and creatinine are showed in Table (4). There was slight significant difference among the rat groups at zero time and rat groups fed 10 % LEP or HEP and administered different doses of lead acetate at the end of experimental period.

Table (3): Serum total protein (TP), albumin, globulin, A/G ratio, total, direct and indirect bilirubin of rats fed on 10 % LEP or HEP and administrated different doses of lead acetate

Rat groups	Total protein (g/dl)		Albumin (g/dl)		Globulin (g/dl)		A/G ratio		Total bilirubin (mg/dl)		Direct bilirubin (mg/dl)		Indirect bilirubin (mg/dl)	
	Zero time	End	Zero time	End	Zero time	End	Zero time	End	Zero time	End	Zero time	End	Zero time	End
Normal control	6.76 ± 0.39^{bc}	5.77 ± 0.29^{cd}	4.2 ±	4.02 ±	2.74 ±	2.35 ±	1.45 ± 0.1^{ab}	1.49 ± 0.29^{ab}	0.231 ± 0.011^{bc}	0.253 ± 0.006^{b}	0.162 ± 0.01^{cd}	0.167 ± 0.008^{bc}	0.070 ±	0.086 ±
G1 (1/4 LD_{50} of Pb)	6.96 ±	4.48 ± 0.58^{g}	4.23 ±	2.61 ± 0.19^{g}	2.95 ±	1.87 ± 0.23^{g}	1.56 ± 0.06^{ab}	1.4 ± 0.15^{ab}	0.245 ± 0.013^{a}	0.515 ±	0.147 ± 0.002^{ab}	0.288 ±	0.098 ± 0.007^{ab}	0.201 ±
G2 (1/8 LD_{50} of Pb)	7.03 ±	4.92 ± 0.18^{ef}	4.34 ±	2.95 ± 0.14^{fg}	2.86 ±	1.87 ± 0.51^{g}	1.49 ± 0.25^{ab}	1.79 ± 0.35^{ab}	0.234 ± 0.003^{a}	0.485 ± 0.006^{ab}	0.144 ± 0.01^{ab}	0.279 ± 0.019^{cd}	0.089 ± 0.006^{ab}	0.2 ±
G3 (1/16 LD_{50} of Pb)	6.98 ±	5.48 ± 0.49^{de}	4.18 ±	3.04 ± 0.21^{ef}	2.89 ±	2.44 ± 0.26^{fg}	1.45 ± 0.05^{ab}	1.36 ± 0.27^{ab}	0.254 ± 0.013^{a}	0.495 ± 0.013^{ab}	0.152 ± 0.006^{ab}	0.252 ± 0.008^{b}	0.078 ± 0.006^{cd}	0.2 ±
G4 (fed10% LEP+1/4 LD_{50} of Pb)	6.97 ±	5.68 ± 0.58^{cd}	4.34 ±	3.34 ± 0.19^{cd}	2.95 ±	2.22 ± 0.38^{gh}	1.56 ± 0.06^{ab}	1.65 ± 0.51^{ab}	0.236 ± 0.004^{a}	0.264 ± 0.025^{cd}	0.152 ± 0.006^{ab}	0.162 ± 0.007^{de}	0.083 ± 0.002^{ab}	0.101 ± 0.01^{ab}
G5 (fed10% LEP+1/8 LD_{50} of Pb)	6.97 ±	5.03 ± 0.16^{ef}	4.26 ±	3.05 ± 0.18^{ef}	2.94 ±	2.29 ± 0.54^{fg}	1.45 ± 0.25^{ab}	1.51 ± 0.23^{ab}	0.236 ± 0.02^{a}	0.255 ± 0.008^{cd}	0.135 ± 0.004^{ab}	0.153 ± 0.009^{de}	0.083 ± 0.002^{ab}	0.101 ± 0.01^{ab}
G6 (fed10% LEP+1/16 LD_{50} of Pb)	6.95 ±	5.97 ±	4.28 ±	4.04 ±	2.95 ±	2.52 ±	1.37 ± 0.04^{ab}	1.41 ± 0.15^{ab}	0.233 ± 0.01^{a}	0.245 ± 0.013^{ab}	0.147 ± 0.001^{ab}	0.161 ± 0.003^{de}	0.084 ± 0.01^{ab}	0.104 ± 0.01^{ab}
G7 (fed10% HEP+1/4 LD_{50} of Pb)	6.99 ±	5.52 ± 0.58^{de}	4.19 ±	3.45 ± 0.1^{de}	2.79 ±	2.15 ± 0.29^{gh}	1.47 ± 0.17^{ab}	1.72 ±	0.229 ± 0.01^{a}	0.267 ± 0.004^{cd}	0.154 ± 0.006^{ab}	0.169 ± 0.01^{de}	0.077 ± 0.002^{ab}	0.1 ± 0.02^{ab}
G8 (fed10% HEP+1/8 LD_{50} of Pb)	7.04 ±	5.72 ± 0.34^{cd}	4.31 ±	3.45 ± 0.2^{de}	2.89 ±	2.64 ±	1.42 ± 0.25^{ab}	1.48 ± 0.29^{ab}	0.231 ± 0.01^{a}	0.267 ± 0.005^{cd}	0.135 ± 0.01^{ab}	0.153 ± 0.011^{de}	0.078 ± 0.002^{cd}	0.114 ± 0.01^{ab}
G9 (fed10% HEP+1/16 LD_{50} of Pb)	6.91 ±	5.52 ± 0.58^{de}	4.21 ±	3.31 ± 0.21^{ef}	2.89 ±	2.61 ±	1.41 ± 0.11^{ab}	1.50 ± 0.14^{ab}	0.228 ± 0.01^{a}	0.247 ± 0.003^{ab}	0.152 ± 0.006^{ab}	0.144 ± 0.012^{de}	0.066 ± 0.002^{ab}	0.104 ± 0.01^{ab}
L.S.D.	0.5562		0.5255		0.6753		0.3284		0.0402		0.0179		0.01934	

Each value represents the mean ± SE. The mean values with different superscript alphabets indicate significant differences ($P \leq 0.05$) using LSD test.

3.2.3. **Level of thyroid hormones, triidothyronine (T3) and thyroxin (T4):**

The effect of feeding on LEP or HEP on serum T3 and T4 levels of rats administrated different doses of lead acetate were studied and the results are shown in Table (6). Normal control rats group had significantly the highest serum T3 and T4 levels. Meanwhile, group 1 (administered 1/4 LD_{50} of Pb) had significantly the lowest serum T3 and T4 levels.

3.3. Hematological evaluation:

Hematological parameters observed in the normal control and experimental rat groups are shown in Table (5).The RBCs and WBCs counts, blood haemoglobin (Hb) and haematocrite values (PVC) significantly decreased in positive control rat groups compared to normal control group at the end of experimental period. However, these parameters significantly increased in rat groups fed on 10% LEP or HEP. It could be noticed that, RBCs and WBCs counts in rat groups fed with LEP were higher than others being fed with HLP in the same concentration of lead acetate. There was a fairly significant difference among all treated rat groups and normal control rats group in Hb. Meanwhile, there was no significant differences in heamatocrite values (PVC) between all treated rat groups and normal control rats group expect G7 (fed on 10 % HEP and administrated 1/4 LD_{50} of lead.

Table (4): Serum urea, uric acid and creatinene of rats fed on10 % LEP or HEP and administrated different doses of lead acetate

Rat groups	Urea (mg/dl)		Uric acid (mg/dl)		Creatinine (mg/dl)	
	Zero time	End	Zero time	End	Zero time	End
Normal control	22.48 ± 1.37^{b}	21.34 ± 1.45^{b}	1.35 ± 0.11^{b}	1.39 ± 0.11^{b}	1.55 ± 0.04^{a}	1.59 ± 0.08^{a}
G1 (1/4 LD_{50} of Pb)	22.37 ± 0.79^{b}	44.98 ±	1.34 ± 0.14^{b}	2.11 ±	1.59 ± 0.08^{a}	2.69 ±
G2 (1/8 LD_{50} of Pb)	21.22 ± 1.49^{b}	44.20 ±	1.29 ± 0.19^{b}	2.06 ±	1.54 ± 0.06^{a}	2.49 ± 0.23^{b}
G3 (1/16 LD_{50} of Pb)	21.06 ± 1.46^{b}	40.93 ±	1.43 ± 0.10^{b}	2.03 ±	1.55 ± 0.05^{a}	2.26 ± 0.13^{c}
G4 (fed10% LEP+1/4 LD_{50} of Pb)	20.40 ± 3.31^{bc}	23.42 ± 1.52^{b}	1.41 ± 0.09^{b}	1.34 ± 0.09^{b}	1.59 ± 0.06^{a}	1.75 ± 0.09^{d}
G5 (fed10% LEP+1/8 LD_{50} of Pb)	21.95 ± 0.29^{b}	22.25 ± 1.78^{b}	1.38 ± 0.09^{b}	1.32 ± 0.15^{b}	1.57 ± 0.06^{a}	1.59 ± 0.10^{a}
G6 (fed10% LEP+1/16 LD_{50} of Pb)	20.59 ± 1.72^{b}	21.56 ± 0.87^{b}	1.44 ± 0.06^{b}	1.41 ± 0.08^{b}	1.57 ± 0.04^{a}	1.68 ± 0.04^{d}
G7 (fed10% HEP+1/4 LD_{50} of Pb)	21.28 ± 1.38^{b}	23.49 ± 1.70^{b}	1.45 ± 0.07^{b}	1.39 ± 0.22^{b}	1.63 ± 0.07^{a}	1.86 ± 0.06^{d}
G8 (fed10% HEP+1/8 LD_{50} of Pb)	20.24 ± 1.19^{bc}	22.59 ± 1.00^{b}	1.44 ± 0.14^{b}	1.42 ± 0.03^{b}	1.52 ± 0.05^{ef}	1.62 ± 0.02^{a}
G9 (fed10% HEP+1/416 LD_{50} of Pb)	21.47 ± 1.23^{c}	20.96 ± 1.60^{b}	1.37 ± 0.08^{b}	1.41 ± 0.14^{b}	1.50 ± 0.02^{ef}	1.66 ± 0.01^{de}
L.S.D.	5.0473		0.2762		0.1671	

Each value represents the mean ± SE. The mean values with different superscript alphabets indicate significant differences ($P \leq 0.05$) using LSD test.

Table (6): Serum Total Triiodothyronine (T3) and Total Thyroxin(T4) hormones of rats fed on 10 % LEP or HEP and administrated different doses of lead acetate

Rat groups	T3 (ng/ml)		T4 (nmol/L)	
	Zero time	End	Zero time	End
Normal control	1.74 ± 0.19^{cd}	2.56 ±	42.68 ± 1.84^{bc}	57.84 ±
G1 (1/4 LD_{50} of Pb)	1.78 ± 0.15^{cd}	0.90 ±	42.23 ± 3.87^{bc}	19.39 ± 2.15^{h}
G2 (1/8 LD_{50} of Pb)	1.87 ± 0.16^{cd}	1.15 ±	42.07 ± 2.91^{bc}	25.21 ±
G3 (1/16 LD_{50} of Pb)	1.81 ± 0.11^{cd}	1.55 ± 0.22^{e}	41.33 ± 3.38^{bc}	29.74 ± 1.91^{fg}
G4 (fed 10% LEP+ 1/4 LD_{50} of Pb)	1.94 ± 0.12^{bc}	1.83 ± 0.02^{cd}	40.98 ± 3.41^{cd}	35.21 ± 0.98^{ef}
G5 (fed 10% LEP+ 1/8 LD_{50} of Pb)	1.95 ± 0.26^{bc}	2.17 ± 0.11^{b}	42.24 ± 2.24^{bc}	46.00 ± 1.20^{b}
G6 (fed 10% LEP+ 1/16 LD_{50} of Pb)	1.84 ± 0.06^{cd}	2.42 ±	41.98 ± 2.91^{bc}	53.96 ±
G7 (fed 10% HEP+ 1/4 LD_{50} of Pb)	1.83 ± 0.07^{cd}	1.72 ± 0.08^{de}	40.32 ± 3.75^{cd}	34.21 ± 2.36^{ef}
G8 (fed 10% HEP+ 1/8 LD_{50} of Pb)	1.85 ± 0.10^{cd}	1.95 ± 0.07^{bc}	40.38 ± 4.30^{cd}	36.46 ± 2.37^{de}
G9 (fed 10% HEP+ 1/16 LD_{50} of Pb)	1.93 ± 0.09^{bc}	2.01 ± 0.23^{bc}	41.83 ± 1.17^{bc}	42.99 ± 2.94^{b}
L.S.D.	0.2354		5.3577	

Each value represents the mean ± SE. The mean values with different superscript alphabets indicate significant differences ($P \leq 0.05$) using LSD test.

Table (5): Blood leukocytes count (WBC_s), red blood cell (RBC_s), blood haemoglobin (Hg) and heamatocrite value (PVC) of rats fed on 10 % LEP or HEP and administrated different doses of lead acetate

Rat groups	WBC_s (N x 10^3/ul)		RBC_s (N x 10^6/ul)		Hg (g/dl)		PVC %	
	Zero time	End	Zero time	End	Zero time	End	Zero time	End
Normal control	14.33 ± 0.81 ^{bc}	14.81 ±	9.69 ± 0.47 ^{cd}	11.02 ±	13.99 ± 0.94 ^{bc}	14.68 ± 0.95 ^{bc}	49.47 ±	51.07 ±
G1 (1/4 LD_{50} of Pb)	14.86 ±	7.18 ± 0.42 ^{h}	9.62 ± 0.67 ^{cd}	7.18 ±	14.23 ± 0.28 ^{bc}	9.72 ± 0.56 ^{e}	49.89 ±	37.01 ± 1.98 ^{e}
G2 (1/8 LD_{50} of Pb)	14.72 ± 0.89 ^{ab}	8.52 ±	9.46 ± 0.55 ^{cd}	7.57 ± 0.49 ^{fg}	14.52 ± 0.51 ^{bc}	10.82 ± 0.51 ^{d}	49.34 ±	36.46 ± 3.53 ^{e}
G3 (1/16 LD_{50} of Pb)	13.93 ± 1.44 ^{bc}	8.93 ±	10.25 ± 0.77 ^{ab}	8.34 ± 0.64 ^{ef}	14.28 ± 0.55 ^{bc}	11.02 ± 0.60 ^{d}	49.46 ±	41.48 ± 5.71 ^{c}
G4 (fed 10% LEP + 1/4 LD_{50} of Pb)	14.34 ± 0.40 ^{bc}	11.76 ± 0.71 ^{ef}	9.85 ± 0.29 ^{cd}	10.11 ± 0.35 ^{ab}	14.58 ± 0.23 ^{bc}	14.60 ± 0.46 ^{bc}	48.17 ±	48.31 ±
G5 (fed 10% LEP + 1/8 LD_{50} of Pb)	14.15 ± 0.47 ^{bc}	12.59 ± 0.57 ^{de}	9.78 ± 0.48 ^{cd}	10.20 ± 0.38 ^{ab}	14.30 ± 0.54 ^{bc}	14.84 ± 0.29 ^{bc}	47.80 ±	47.98 ±
G6 (fed 10% LEP + 1/16 LD_{50} of Pb)	14.17 ± 0.21 ^{bc}	14.15 ± 1.03 ^{bc}	9.57 ± 0.34 ^{cd}	10.62 ± 0.34 ^{ab}	14.42 ± 0.45 ^{bc}	15.35 ±	48.59 ±	48.61 ±
G7 (fed 10% HEP + 1/4 LD_{50} of Pb)	14.27 ± 0.53 ^{bc}	10.67 ±	9.89 ± 0.81 ^{cd}	8.91 ± 0.40 ^{de}	14.23 ± 0.52 ^{bc}	13.71 ± 0.44 ^{c}	48.05 ±	45.23 ± 2.47 ^{b}
G8 (fed 10% HEP + 1/8 LD_{50} of Pb)	13.23 ± 0.65 ^{bc}	11.54 ± 0.57 ^{ef}	9.77 ± 0.34 ^{cd}	9.91 ± 0.24 ^{cd}	14.89 ± 0.68 ^{bc}	14.41 ± 0.79 ^{bc}	49.16 ±	47.93 ±
G9 (fed 10% HEP + 1/16 LD_{50} of Pb)	12.97 ± 0.34 ^{cd}	12.61 ± 0.24 ^{de}	9.95 ± 0.82 ^{cd}	9.89 ± 0.58 ^{cd}	14.49 ± 0.37 ^{bc}	15.19 ± 0.47 ^{ab}	49.30 ±	49.08 ±
L.S.D.	1.2892		0.9435		1.0976		3.8799	

Each value represents the mean ± SE. The mean values with different superscript alphabets indicate significant differences ($P \leq 0.05$) using LSD test.

3.4.Concentration of lead retention in blood, organs and feces:

The blood lead level of 0.35 lg/ml is the critical level of poisoning, but the deaths commence at 1.0 lg/ml (Radostits *et al*, 2000). Sometimes, blood lead level above this critical level may not be manifested by characteristic clinical signs in animals (Koh and Babidge, 1986), particularly in chronically exposed cases. Moreover, uptake by bone mineral is a function of the metal's plasma concentration, its affinity for the bone mineral, and its effect on the extracellular matrix. It is also a function of the degree of mineralization of the skeleton.

Treating the rats with Pb in the form of acetate led to a considerable increase of accumulation of the metal in the blood, liver, kidney, brain, heart and bones compared with the normal group. Data in Table (7) showed that significant increase was observed in all rat groups administered lead acetate. At rat groups fed on LEP or HEP, there was decrease in lead concentration of blood, organs and bones. It could be noticed that, the efficiency of LEP on removal lead from tested blood and organs were higher than HEP in the presence of the same doses of lead acetate. Also, there was no significant difference in the lead concentration of heart compared to normal control group and G6 (fed on 10 % LEP and administrated 1/16 LD_{50} of lead. In all groups of animals fed on LEP or HEP, there was significant increase lead concentration in feces compared to the positive control rat groups (G1, G2 and G3) indicating continuous lead elimination through the digestive tract. LEP was helped to increase the amount of lead being excreted with feces. Amount of lead in feces of rats fed on HEP was significant decrease from that of rats fed on LEP in different doses of lead.

In this study, the use of the pectin substances in animals preliminary exposed to high doses of lead acetate contributed to fast elimination of the metal from the organs, in particular, the bones. Low esterified pectin prevented lead absorption in the intestine, resulting in slowed tissue retention of lead, whereas HE pectin removed lesser amount of lead. These results were confirmed by enhanced concentration on lead in feces of rats treated with LEP and HEP.

Table (7): Lead content of blood, liver, kidney, brain, heart, bone and feces of rats fed on 10 % LEP or HEP and administrated different doses of lead acetate

Rat groups	Blood (µg/dl)	Liver (µg/g)	Kidney (µg/g)	Brain (µg/g)	Heart (µg/g)	Bone (µg/g)	Feces (µg/g)
Normal control	4.74 ±	0.50 ±	2.05 ±	0.49 ± 0.07 ^{gh}	0.45 ±	3.28 ±	1.12 ±
G1 (1/4 LD_{50} of Pb)	60.33 ±	23.75 ± 1.61 ^{b}	49.28 ± 2.99 ^{b}	3.40 ±	2.77 ± 0.11 ^{f}	61.97 ± 2.81 ^{f}	60.10 ± 2.46 ^{c}
G2 (1/8 LD_{50} of Pb)	35.16 ± 2.30 ^{b}	13.41 ± 0.74 ^{c}	29.54 ± 1.76 ^{b}	1.96 ± 0.10 ^{c}	1.66 ± 0.12 ^{c}	35.99 ± 1.64 ^{b}	35.79 ±
G3 (1/16 LD_{50} of Pb)	20.67 ± 1.53 ^{d}	7.75 ± 0.64 ^{ef}	19.33 ± 1.51 ^{d}	1.16 ± 0.04 ^{d}	0.97 ± 0.09 ^{e}	21.82 ± 1.47 ^{c}	16.53 ± 0.82 ^{h}
G4 (fed 10% LEP+ 1/4 LD_{50} of Pb)	19.79 ± 1.62 ^{d}	10.35 ± 0.62 ^{d}	18.56 ± 0.81 ^{d}	1.04 ± 0.12 ^{de}	1.52 ± 0.10 ^{c}	25.17 ± 1.13 ^{c}	75.11 ± 3.49 ^{a}
G5 (fed 10% LEP+ 1/8 LD_{50} of Pb)	11.28 ± 0.62 ^{e}	6.36 ± 0.21 ^{f}	10.87 ±	0.66 ± 0.03 ^{fg}	0.91 ± 0.06 ^{e}	14.38 ± 1.81 ^{d}	47.76 ± 1.51 ^{d}
G6 (fed 10% LEP+ 1/16 LD_{50} of Pb)	6.36 ±	3.06 ±	5.91 ±	0.40 ± 0.07 ^{h}	0.52 ±	7.52 ± 0.50 ^{e}	23.10 ±
G7 (fed 10% HEP+ 1/4 LD_{50} of Pb)	31.28 ± 1.34 ^{c}	14.92 ± 0.76 ^{b}	24.28 ± 1.74 ^{c}	2.68 ± 0.21 ^{b}	2.11 ± 0.14 ^{b}	40.03 ± 4.04 ^{b}	69.94 ± 2.38 ^{b}
G8 (fed 10% HEP+ 1/8 LD_{50} of Pb)	18.41 ± 1.09 ^{d}	9.02 ± 0.16 ^{de}	15.03 ± 1.26 ^{e}	1.76 ± 0.14 ^{c}	1.28 ± 0.11 ^{d}	23.99 ± 1.51 ^{c}	40.17 ± 2.02 ^{e}
G9 (fed 10% HEP+ 1/16 LD_{50} of Pb)	11.71 ± 0.88 ^{e}	5.32 ±	9.78 ±	0.82 ± 0.07 ^{ef}	0.97 ± 0.10 ^{e}	15.31 ± 1.01 ^{d}	18.93 ± 1.22 ^{h}
L.S.D.	3.4494	1.4653	2.9341	0.2447	0.2001	4.0132	3.8275

Each value represents the mean ± SE. The mean values with different superscript alphabets indicate significant differences ($P \leq 0.05$) using LSD test.

3.5.Histopathological studies:

3.5.1. Liver:

Histopathological changes of rats administered different doses of lead acetate with or without LEP or HEP are presented in Fig (1), where, liver samples of normal rats group showed normal

hepatic lobule. Liver of rats administered ¼ LD_{50} of LA (positive control 1) showed congestion of hepatoportal blood vessel and edema in the portal tract. Moreover, liver of rats administered 1/8 and 1/16 LD_{50} of LA (positive control 2 and 3) appeared focal hepatic hemorrhage and vacuolar degeneration of hepatocytes. Additionally, liver of rats administered ¼ LD_{50} of LA + fed on 10% of LHP clearly slight hydropic degeneration of some heptocytes. There was vacuolar degeneration of some hepatocytes of rats liver administered ¼ LD_{50} of LA + fed on 10% of HHP. No histopathological changes were observed in rats liver administered 1/8 LD_{50} or 1/16 LD_{50} of LA + fed on 10% of LHP or HHP.

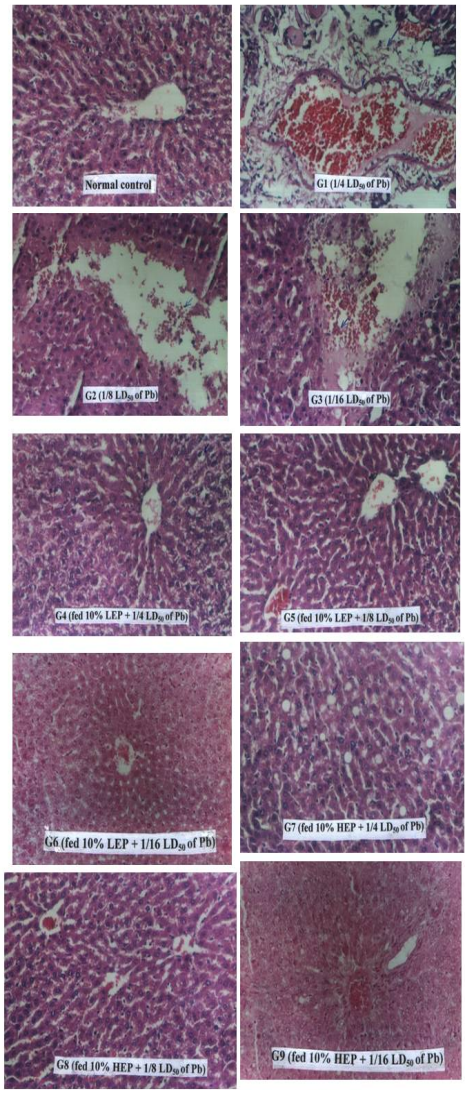


Fig. 1. Histopathological examination of rats liver fed on 10 % LEP or HEP and administrated different doses of lead acetate

3.5.2. Kidney:

Concerning kidney of normal control rats group, it showed the normal histology of renal parenchyma, Fig (2). Kidney samples of group positive control 1 appeared congestion of renal blood vessel and swelling of epithelial liming some renal tubules. Furthermore, kidney of rats administered 1/8 LD_{50} of LA (positive control 2) observed marked dilation and congestion of renal blood vessel. Additionally, there were vacuolation of epithebal lining renal tubules and thickening of the glomerular basement membrane of rats kidney administered 1/16 LD_{50} of LA (positive control 3). All the tested rat groups administered different doses of LA +fed on 10% of LHP or HHP showed no histopathological changes of kidney samples. It could be observed that LHP or HHP had the same effect to protect the kidney tissues from the harmful of lead acetate.

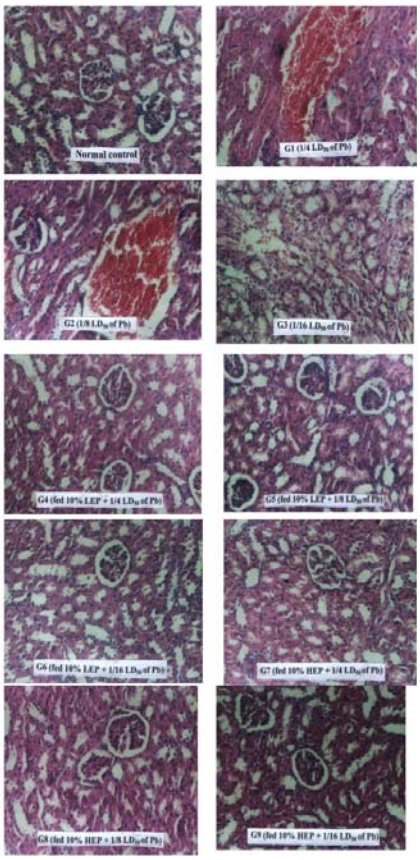


Fig. 2. Histopathological examination of rats kidney fed on 10 % LEP or HEP and administrated different doses of lead acetate

3.5.3.Brain:

Regarding the brain samples of normal control rats group, there was normal histological structure, Fig (3). In contrast, brain of rats from positive control 1 showed neuronophagia of

degenerated neurons. Moreover, there were pyknosis of neurons, focal gliosis and congestion of cerebral blood vessel in brain samples of positive control 2 and 3. The feeding on LHP had protected effect of brain tissues. There was no histopathological changes of brain rat groups fed LEP. Brain of rats administered ¼ LD_{50} of LA + fed on 10% of HHP showed neuronophagia of pyknotic neurons. However, cellular edema was the only change observed in brain of rats administered 1/8 LD_{50} of LA + fed on 10% of HHP. Some examined sections of brain rats administered 1/16 LD_{50} of LA + fed on 10% of HHP showed apparent normal structure.

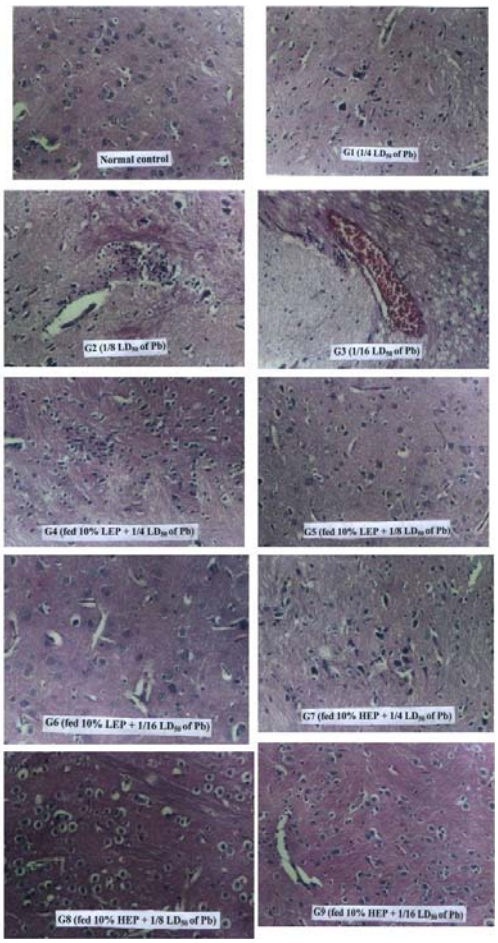


Fig. 3. Histopathological examination of rats fed on 10 % of LEP or HEP and administrated different doses of lead acetate 4.DISCUSION

Lead is a common environmental occupational toxic heavy metal, is known to have direct and indirect effects on biological systems and cells . It is an ubiquitous toxic agent, and its toxicity remains an important public health problem because of the great amount of sources in any household and environment .Its toxicity affecting the nervous system, blood, and blood-forming organs, kidneys, and gastrointestinal tract, Paoliello and De Capitani,(2005).

Our results revealed that the lowest accumulated rat weight gain was observed in positive control rat groups. In addition, there were no significant difference appeared for feed intake of all tested rat groups. At the same time, feed conversion efficiency (FCE) was also decreased by administered oral doses of lead acetate without fed HEP or LEP .This is in accordance to Kang *et al.* (2004) ; Berrahal *et al* (2007);El Nekeety *et al* (2009) ; who reported that mean body weight of the animals treated with LA was significantly lower than that of the other groups. With other organisms also, Hiraga *et al* (2008) exposed young chickens to five lead shot and found that rate of growth was slightly lower in the Pb-exposed group from day 6.

Liver is considered to be the principal target organ for lead toxicity, Abdel-Wahhab *et al.*,(2007).To test for liver function in the present study, results of indicated that HEP or LEP had positive effect on level of serum ALT, AST, ALP and γ- GT activities which were raised by different doses of lead acetate especially at the lowest doses of LA. These results agreed with results obtained by many investigators who studied the effect of lead on liver function; Othman *et al, (*1998); Sivaprasad *et al* (2003) ;Shalan *et al.* (2005) . On the contrary, experiments conducted by Pande *et al*,(2001);Singh *et al.*, (1994) in rats receiving oral administration of lead acetate they reported a significant decrease in the activities of these two enzymes compared to the controls. Studies conducted by Jarrar and Mahmoud (2000); Jevtovic-Stoimenov *et al,(* 2003); Shalan *et al.*, (2005) showed significant increase in the γ- GT activity after a period of Pb exposure for the rats.

Studies in humans have indicated an increase in the activity of these enzymes. Nehru and Kaushal (1993) ;Goswami and Bhattacharya (2000) .Serum total proteins and albumin level, normally used to assess the ability of synthetic function of the liver is well understood and documented ,Adeyemi *et al* (2009). Billirubin is regarded as a member of the antioxidant family, even though it is known to have toxic effects at high concentrations ,Hagymàsi *et al.*, (2003) . It has been regarded for many years as cytotoxic, mainly because of its association with neonate jaundice and its possibility of provoking irreversible brain damage at high concentrations ,Tomaro and Batlle,(2002).

Our results reported that serum billirubin of test rat was found to be about two folds that of control while the serum albumin concentration of test rat was about half that of the control. This result agreed with experiments conducted in rats receiving

lead acetate reported similar decrease in the serum total protein and albumin levels ,Jevtovic-Stoimenov *et al,(* 2003) ,Swarup *et al(,*2007). The decreased level of protein may indicate protein catabolism dysfunction ,Abdel-Wahhab *et al.*,(2008). Moreover, Berrahal *et al* (2007); Adeyemi *et al* (2009) noted a significant increase in the billirubin level (*p* the group of rats treated with Pb compared to control, while serum globulin and albumin concentrations of serum of test rat was significantly lower ($p < 0.05$) than those of control.

Urea and uric acid are the principal waste products of protein catabolism. They are synthesized in the liver from ammonia produced as a result of the de-amination of amino acids ,Young, (1972). Creatinine is the major waste product of creatine metabolism by muscle. In the kidney, it is filtered by the glomerulus and activity excreted by the tubules. Moreover, free creatinine appears in the blood serum ,Baker *et al*, (1979).

There was increase in serum urea, uric acid and creatinine appeared in positive rat groups of our results. Which situation agreed with results of Abdel-Wahhab *et al.* (2007); El Nekeety *et al* (2009); Adeyemi *et al* (2009) studies who illustrated that the exposed to lead increase levels of serum urea may indicate kidney dysfunction than normal control. Scientists explained cause of diseases of the kidney for two consequences; The first is failure to retain substances such as protein, amino acids, sugar, water and ions. The second consequence is failure to excrete substances such as urea, creatinine and the waste products. The presence of lead might have caused impairment of the brush border epithelial cells and making them impermeable to urea and creatinine thereby causing their elevated levels in the blood. The over all effect of this may be impaired kidney function, Oloyede *et al,(*2003).

Serum T3 and T4 levels are considered valuable indicators of thyroid function in animals ,Bruker, (1998); Chaurasia and Kar, (1998). It was clearly from our results that LEP and HEP had a positive effect to keep the level of T3 and T4 in serum nearly within normal especially in group that fed on 10% LEP and administered 1/16 LD_{50} of Pb. In this concern, Wade *et al* (2002) cited that altered serum thyroxine (T4), triiodothyronine (T3) and histomorphology of thyroid gland were recorded following subchronic exposure to a complex mixture of 16 organochlorine chemicals, lead and cadmium in male rats for 70 days. Decreased serum thyroxine (T4) and triiodothyronine(T3) level was also noticed by Pratima *et al,(*1997)in fresh water fish (*Clarias batrachus*) after subchronic exposure to lead and cadmium. Chaurasia and Kar, (1998) noticed that in chicken exposed to dietary lead at 1 mg/kg bodyweight for 30 days ,a significant reduction in T3 level and T4 in their serum . Contradictory reports were also available in human subject on the level of circulating thyroid hormones after lead exposure ,Schantz and Widholm, (2001). In addition, Dundar *et al* (2006) mentioned that long-term low-level lead exposure may lead to reduced T4 level without significant changes in T3 level in adolescents even at low Pb-Blood levels.

The hematological system is the major target of low level lead exposure ,Warren *et al.*, (1998). Therefore, lead exposure induces severe oxidative damage in RBCs by inhibiting heme , hemoglobin synthesis and changing erythrocyte morphology and survival , as a result from direct interaction of lead with RBCs membranes, inducing lipid peroxidation , Leggett (1993) ;Sandhir *et al.*,(1994).

From our results ,it could be noticed that, RBCs and WBCs counts in rat groups fed with LEP were higher than others being fed with HLP in the same concentration of lead acetate. Also, there was a fairly significant difference among all treated rat groups and normal control rats group in Hb. Meanwhile, there was no significant differences in heamatocrite values (PVC) between all treated rat groups and normal control rats group expect G7 (fed on 10 % HEP and administrated 1/4 LD_{50} of lead. In concerning that, Simsek *et al* (2009) found that RBCs and WBCs counts, Hb, PCV and values significantly decreased in rats group exposed to lead acetate compared to the control group. Also, Ancheva *et al* (2003) illustrated that lead cause damage to the erythrocyte membrane resulting in hemolysis or a decrease of blood iron level which may be the cause of decreased concentration of haemoglobine and hematocrit value.Chronic oral lead administration cause the development of hypochromic anemia and hemolytic anemia.

Treating the rats with Pb in the form of acetate led to a considerable increase of accumulation of the metal in the blood, liver, kidney, brain, heart and bones compared with the normal group. O'Flaherty (1991) in accordance, showed that the average Pb concentration in bone of rats administered lead acetate rapidly increased. Timchalk *et al* (2006) exposed the rats to oral gavages doses of 1, 10, or 100 mg Pb-acetate/kg/day daily. They cited that Pb-acetate when administered by oral gavages weres rapidly absorbed, since peak blood Pb concentrations were attained within 30 min to 1 h post-dosing and the Pb rapidly redistributed (within 5-days post-treatment) from the blood into the bone compartment based on the substantial decrease in WBcs and RBC Pb concentration, and the concurrent increase in bone Pb following repeated exposure at all dose levels. Moreover, Adeyemi *et al* (2009) found that possible

damage to the tissues of rats placed on water contaminated with lead (0.015 lg/l). The heart and liver were the last sensitive tissues to lead retention.In addition, all groups of tested animals in the present study, fed on LEP or HEP, showed a significant increase lead concentration in feces compared to the positive control rat groups indicating continuous lead elimination through the digestive tract. In this situation, lead concentration in serum and organs was reduced after pectin administration. Dongowski *et al,(*1999) stated that application of native pectin as well as oligogalacturonic acids increased lead elimination through blood and organs. And addition of rhamnogalacturonic parts of pectin into the lead-enriched diet in rats contributed to slow absorption of lead in rats and enhanced lead excretion with feces during the period of the experiment. Moreover, the structural parameters of pectin influence by its microbial degradation in the intestinal tract. Also, enzymes from intestinal microorganisms involved in pectin fermentation include pectatelyase, polygalacturonase and pectinesterase (Dongowski and Lorenz, 1998 and Tierny *et al*, 1994). Additionally, Serguschenko *et al* (2004) proved that pectin exerts high metal binding activity regarding bivalent metal ions. Drawing of absorption isotherms showed that pectin possesses pronounced affinity to lead ions in comparison to other bivalent metals, and some types of natural pectins may be more effective than chelating agents currently used to manage lead toxicity. In some cases, orally administered pectins contributed to increased retention of heavy metal in tissues (Rose and Quarterman,1987).

Many studies illustrated the effect of degree of methylation on the degradation of pectin in the intestinal tract of rats. Dongowski *et al* (2002) found that Low-methoxyl pectin was fermented faster than high-methoxyl pectins in *vivo* and in *vitro*. Kim *et al* (1978) illustrated that high esterified pectins require large amounts of sugar and low pH values for gel formation. While low- ester pectins form gel with or without sugar in the presence of divalent cations. So that the quantity of metal bound to pectin is determined by the number of free carboxyl groups ,Serguschenko *et al.,* (2007). This could enhance the concentration of lead acetate in feces of rats treated with low esterified pectin than high esterified pectin. Moreover, Kartel *et al* (1999) illustrated that high esterified pectin is characterized by a major part of carboxyl group in galacturonan pattern to be occupied with methyl radicals preventing interaction with metal. Therefore, the lowest binding activity of lead was registered in animals treated with different doses of high pectin sample. Also, using high doses of lead acetate and pectins contributed to a considerably rapid elimination of the lead from the rat body. Fast elimination of metal from tissues as a result of treatment with pectin substances led to redistribution of lead in the body of the animals. This could be confirmed by several fold increases of the metal contents in the liver and heart. This phenomenon is sometimes called “rebound” effect ,Gerhardsson *et al*, (1999).

Through our histological study, it was noticed that there was vacuolar degeneration of some hepatocytes of rats liver administered ¼ LD_{50} of LA + fed on 10% of HHP. There were no histopathological changes observed in rats liver administered 1/8 LD_{50} or 1/16 LD_{50} of LA + fed on 10% of LHP or HHP.

The histopathological examination of the liver tissue of the animals treated with lead showed that, lead(Pb)-induce DNA damage (Fracasso *et al.* 2002; Danadevi *et al.,* 2003 and Xu *et al.* 2003). On the other hand, Shalana *et al.* (2005) found that lead reduced hepatic total RNA content indicating a lower rate of hepatic protein synthesis. Furthermore, El-Zayat *et al.* (1996) reported a decrease in hepatic total protein content in response to lead intoxication. These authors attributed that to a decreased utilization of free amino acids for protein synthesis. In another report, Pagliara *et al* (2003) showed that lead-induced liver hyperplasia followed by apoptosis mediated by oxidative stress in kupffer cells. It could be observed that LHP or HHP had the same effect to protect the kidney tissues from the harmful effect of lead acetate.

Many investigates illustrated that the exposed of experimental rats to lead induced significant histopathological changes of kidney tissues ,Patra *et al.*, (2001) and El-Sokkary *et al*,(2005).

brain of rats from positive control , showed neuronophagia of degenerated neurons. Moreover, there were pyknosis of neurons, focal congestion of cerebral blood vessel in brain samples of positive control. The feeding on LHP had protected effect of brain tissues. There was no histopathological changes of brain rat groups fed LEP. Results clearly showed that LA had a harmful and stressful influence on the hepatic, renal and brain tissues consistent with those reported in the literature ,Nehru and Kaushal,(1993) and Singh *et al.,* (1994).

In conclusion, many chelating agents are currently used to manage lead toxicity. At the same time, the most common, however, are nonspecific and have some adverse effects in humans such as induction of misbalance of essential microelements . Because pectins are both specific and effective in complexion with lead, these compounds may be considered as nutritional products that could be used to decrease lead intestinal absorption, prevention of

lead accumulation, and amelioration of lead toxicity. In the present investigation, LE pectin was contributed to fast elimination of the lead acetate to blood, organs and bones, whereas HE pectin removed lesser amount of lead. However, additional studies in rats and humans are required before developing the pectins as preventive or curative agents of lead exposure and toxicity in humans.

5. Refrences

1. **Abdel-Wahhab, M.A.; M.M. Abdel-Galil; A.M. Hassan; N.H. Hassan; S.A. Nada; A. Saeed and M.M. El Sayed (2007).**
2. Zizyphus spina-christi extract protects against aflatoxin B1-intitiated hepatic carcinogenicity. Afr. J. Trad. CAM 4 (3): 248–256.
3. **Abdel-Wahhab, M.A.; S.H. Abdel-Azim and A.A. El-Nekeety (2008).** Inula crithmoides extract protect against ochratoxin A-induced oxidative stress, clastogenic and mutagenic alterations in male rats. Toxicon 52 (4): 566–573.
4. **Adeyemi, O; J.O. Ajayi; A.M. Olajuyin; O.B. Oloyede; A.T. Oladiji; O.M. Oluba; O. Adeyemi; I.A. Ololade and E.A. Adebayo (2009).** Toxicological evaluation of the effect of water contaminated with lead, phenol and benzene on liver, kidney and colon of *albino* rats. Food and Chemical Toxicology 47: 885–887.
5. **Ancheva, M.T.; R. Metcheva and S. Teodorova (2003).** Bioaccumulation and damaging action of polymetal industrial dust on laboratory mice *Mus musculus* alba. II. Genetic, cell, and metabolic disturbances. Environ. Res. 92: 152–160.
6. **Bae, D.S.; C. Gennings; W. H. Carter; J. Raymond; S. H. Yang and J. A. Campain (2001).** Toxicological interactions among arsenic, cadmium, chromium, and lead in human keratinocytes. Toxicological Sci. 63: 132–142.
7. **Baker, H.J., Lindsey, J.R. and Weisbroth, S.H. (1979).** The laboratory rats. Biological and Diseases (volume 1). 1 st ed., Academic Press, Harcourt Brace Jovanovicn, New York.
8. **Bancroft, J. D.; A. Stevans and D.R. Turner (1996).** Theory and practice of histological techniques. 4 th. Ed. Churchill Livinigstone, Edinburgh, London, Melbourne, New York.
9. **Bergmeyer, H. U. and M. Harder (1986).** A colorimeteric methods of determination of serum glutamic oxaloacetic and glutamic pyruvic transaminase. Clin. Biochem. 24: 28 – 34.
10. **Berrahal, A.A.; A. Nehdi; N. Hajjaji; N. Gharbi and S. El-Fazâa (2007).** Antioxidant enzymes activities and bilirubin level in adult rat treated with lead. C. R. Biologies 330:581–588.
11. **Burch, H.B. and A.L. Siegel (1971).** Improved method for measurement of 6-a-levulinic acid dehydratase activity of human erythrocytes. Clin. Chem., 17(10): 103.
12. **Caraway , W.T. (1955).** Determination of uric acid in serum by a carbonate method. Amer. J. of Clin. Pathology 2: 840-845.
13. **Chang, K.; N. Dhurandhar; X. You and A. Miyamoto (1994).**Cultivar/location and processing methods affect yield and quality of sunflower pectin. J Food Sci. 59:602-605.
14. **Chaurasia, S.S. and A. Kar (1998).** Lead induced oxidative damage to the membrane associated type-I iodothyronine-monodeiodinase activity in chicken liver homogenate. Fresenius Envir. Bulletin 7:209–215.
15. **Cosgrove, D.J. (1997).** Assembly and enlargement of the primary cell wall in plants. Annu. Rev. Cell Dev. Biol. 13:171–201.
16. **Dacie, J.V. and S. M. Lewis (1975).** Practical hematology. A collaborative analysis of an improved produce.
17. **Danadevi, K.; R. Rozati; B. Saleha-Banu; P. Hanumanth-Rao and P. Grover (2003).** DNA damage in workers exposed to lead using comet assay. Toxicology 187 (2–3):183–193.
18. **Dongowski G. and A. Lorenz (1998).** Unsaturated oligogalacturonic acids are generated by in vitro treatment of pectin with human fecal flora. Carbohydr. Res. 314: 237–244.
19. **Dongowski G.; A. Lorenz and J. Proll (2002).** The degree of methylation influences the degradation of pectin in the intestinal tract of rats and in v*itro*. J. Nutr. 132: 1935–1944.
20. **Dongowski G; B. Schnorrenberger; M. Platzer; M. Schwarz and R. Neubert (1997).** Interactions between food components and drugs. Part 5: Effect of acetylation and amidation of pectins on the interaction with drugs. Int. J Pharm.158:99-107.
21. **Dongowski G.; E. Walzel; B. Ozierensk; C. Stark; J. Kroll and A. Lorenz (1999).** Effects of pectin and oligogalacturonic acids on excretion and incorporation of lead in subchronic lead exposed rats. In: Hartemink R, editor. Non-digestible oligosaccharides: healthy food for the colon. Wageningen: Graduate School VLAG; p. 151.
22. **Doumas, B.T.; W.A. Watson and H.G. Biggs (1977).** Albumin standards and the measurement of serum albumin with bromocresol green. Clin. Chem. Acta. 31:87–96.
23. **Dundara, B.; F.O. ktema; M. K. Arslana; N Delibas; B. Baykalc; C. Arsland, M. Gultepee; I. E. Ilhan (2006).** The effect of long-term low-dose lead exposure on thyroid function in adolescents. Environmental Research 101: 140–145.
24. **El-Nekeety, A.A.; A. A. El-Kady; M.S. Soliman; N. S. Hassan and M. A. Abdel-Wahhab (2009).** Protective effect of Aquilegia vulgaris (L.) against lead acetate-induced oxidative stress in rats. Food and Chemical Toxicology 47: 2209–2215.
25. **El-Sokkary, G. H.; G. H. Abdel-Rahman and E. S. Kamel (2005).** Melatonin protects against lead-induced hepatic and renal toxicity in male rats. Toxicology 213: 25–33.

26. **El-Zayat, E.M.; N.A. El-Ymany; and Z.H. Kamel (1996).** Combined supplementation of zinc and vitamin C as protective agents against lead toxicity in growing male *albino* rats. 1. Liver functions. J. Egypt Ger. Soc. Zool. 20 (A): 115–139.
27. **Fracasso, M.E.; L. Perbellini ; S. Solda ; G. Talamini and P. Franceschetti (2002).** Lead induced DNA strand breaks in lymphocytes of exposed workers: role of reactive oxygen species and protein kinase C. Mutat. Res. 515 (1–2): 159–169.
28. **Gee, J.M.; W. Lee-Finglas; G.W. Wortley and I. T. Johnson (1996).** Fermentable carbohydrates elevate plasma enteroglucagon but high viscosity is also necessary to stimulate small bowel mucosal cell proliferation in rats. J. Nutr. 126: 373-379.
29. **Gerhardsson, L; J. Borjesson; S. Mattsson; A. Schutz and S. Skerfving (1999).** Chelated lead in relation to lead in bone and ALAD genotype. Environ. Res. 80:389-98.
30. **Goswami, K. and B. Bhattacharya (2000).** Lead as an environmental pollutant: its effect on hepatic functions, J. Vivekananda Inst. Med. Sci. 23 (1) (2000) 13–17.
31. **Gurer, H.; R., Neal; P. Yang; S. Oztezcan and N. Ercal (1999).** Captopril as an antioxidant in lead-exposed Fischer 344 rats. Hum. Exp. Toxicol. 18: 27–32.
32. **Hagymàsi, K.; I. Kocsis; G. Lengyel; P. Sipos; J. Fehér and A. Blàzovics (2003).** Further evidence of altered redox status of hyperbilirubinemic patients: role of bilirubin in Gilbert syndrome. Acta. Biol. Szeged. 47 (1–4): 131–134.
33. **Hicks, K.B.; C.P. Lim and M. J. Haas (1985).** Analysis of uronic and aldonic acids, their lactones and related compounds by high performance liquid chromatography on cation-exchange resins. J. Chromatography, 319: 159-171
34. **Hiraga,T.; K. Ohyama; A. Hashigaya; T. Ishikawa; W. Muramoto; H. Kitagawa; N.Mizuno and H. Teraoka (2008).** Lead exposure induces pycnosis and enucleation of peripheral erythrocytes in the domestic fowl. The Veterinary Journal 178:109–114.
35. **Jarrar, B.M. and Z.N. Mahmoud (2000).** Histochemical demonstration of changes in the activity of hepatic phosphatases induced by experimental lead poisoning in male white rats (*Rattus norvegicus*). Toxicol. Ind. Health 16: 7–15.
36. **Jevtovic-Stoimenov, T.; G. Kocic; D. Pavlovic; I. Stojanovic; T. Cvetkovic and D. Sokolovic (2003).** Effects of captopril on membrane-associated enzymes in leadinduced hepatotoxicity in rats. Acta Fac. Med. Naiss 20 (3): 183–188.
37. **Kang, J.K.; D. Sul; J.K. Kang; S. Nam; H. Kim and E. Lee (2004).** Effects of lead exposure on the expression of phospholipid hydroperoxidase glutathione peroxidase mRNA in the rat brain. Toxicol. Sci. 82: 228–236.
38. **Kartel, M.T.; L.A. Kupchik and B.K. Veisov (1999).** Evaluation of pectin binding of heavy metal ions in aqueous solutions. Chemosphere 38: 2591-2596.
39. **Khotimchenko, M; V. Kovalev and Y. Khotimchenko (2007).** Equilibrium studies of sorption of lead(II) ions by different pectin compounds. J. of Hazardous Materials 149: 693–699.
40. **Kim, W.J.; V.N.M. Rao and C.J.B. Smit (1978).** Effect of chemical composition on compressive mechanical properties of low ester pectin gels. J. Food Sci., 43: 572-575.
41. **Knuth, M.L. and M.D. Hoglmnd (1984**). Quantitative analysis of 68 polar compounds from ten chemical classes by direct aqueous injection gas chromatography. J. of Chromatography, 285: 153-160.
42. **Koh, T.S. and P.J. Babidge (1986).** A comparison of blood lead levels in dogs from a lead-mining, lead-smelting, urban and rural island environment.Australian Veterinary J. 63: 282–285.
43. **Koirtyohann, S.R. (1994).** Precise determination of isotopic ratios for some biologically significant elements by inductively coupled plasma mass spectroscopy. Spectrochimm Acta, 49B. Nos 12-14, p 1305-1311.
44. **Kolmer, J.A.; E. H. Spaudling and H.W. Robinson (1951).** Approved Laboratory Techniques. Appleton Century Crafts, New York, pp. 1090–1091 (C.F. Research in Veterinary Science 82: 16–21).
45. **Landrigan, P.J.; P. Boffetta and P. Apostoli (2000).** The reproductive toxicity and carcinogenicity of lead: a critical review. Am. J. Ind. Med. 38, 231–243.
46. **Larsen, K. (1972).** Creatinine assay by a reaction kinetic principle. Clin. Chem. Acta 41: 209-213.
47. **Leggett, R.W. (1993).** An age-specific kinetic model of lead metabolismin humans. Environ. Health Perspect 101:598–616.
48. **Leong W.I.; C.L. Bowlus; J. Tallkvist; and B. Lonnerdal (2003).** DMT1 and FPN1 expression during infancy: developmental regulation of iron absorption. Am J Physiol Gastrointest Liver Physiol 285:G1153–1161.
49. **Litchman, M. A., and R. P. Upton (1972).** Gas chromatographic determination of residual methanol in food activities. Analytical Chemistry, 44(8): 1495-1497.
50. **Lowry O.H.; N.J. Rosebrough; A.L. Farr and R. J. Randall (1951).** Protein measurement with the Folin Phenol Reagent. J Bio. Chem. 193:269–275.
51. **Marchlewicz, M.; M. Protasouicki; L. Rozewicka; M. Piasecka and M. Laszczynska (1993).** Effect of long-term exposure to lead on testis and epididymis in rats. Folia-Histochem. Cytobiol. 31 (2):55–62.
52. **Nehru, B. and S. Kaushal (1993).** Alteration in the hepatic enzymes following experimental lead poisoning. Biol. Trace Elem. Res. 38:27–34.
53. **O'Flaherty, E.J., (1991).** Physiologically baed models for bone-seeking elements. II. Kinetics of lead disposition in rats. Toxicol. Appl. Pharmacol. 111: 299–312.
54. **Oloyede, O.B.; O. Adeyemi; T.O. Sunmonu and A.A. Bakare (2003).** The effect of polluted water on selected rat liver enzymes. NISEB J. 3 (3):91–97.

55. **Othman, A.I. and M.A. El Missiry (1998).** Role of selenium against lead toxicity in male rats. J. Biochem. Mol. Toxicol. 12: 345–349.
56. **Pagliara, P.; A. Chionna; E.C. Carla ; S. Caforiao and L. Dini (2003).** Lead nitrate and gadolinium chloride administration modify hepatocytes cell surfaces. Cell Tiss. Res. 312 (1): 41–48.
57. **Palaa, G. L. and M. Charbonneau (1994).** Detection and evaluation of chemically induced liver injury: In Hayes, A. W. (ed.) Principles and methods of toxicology. 3 rd. Ed., Raven press, New York.
58. **Pande, M.; A. Mehta; B. Pant and S.J.S. Flora (2001).** Combined administration of a chelating agent and an antioxidant in the prevention and treatment of acute lead intoxication in rats. Environ. Toxicol. Pharmacol. 9: 173–184.
59. **Paoliello, M.M. and E.M. De Capitani (2005).** Environmental contamination and human exposure to lead in Brazil. Rev Environ Contam. Toxicol.184:59-96.
60. **Parker, M.M.; F.L. Humoller and D.J. Mahler (1967).** Determination of copper, zinc and other trace elements in biological material. Clin. Chem., 13: 40.
61. **Patra, R.C.; D., Swarup, S.K. and Dwivedi (2001).** Antioxidant effects of a-tocopherol, ascorbic acid and L-methionine on lead induced oxidative stress to the liver, kidney and brain in rats. Toxicology 162: 81–88.
62. **Patrick, L. (2006).** Lead toxicity part II: the role of free radical damage and the use of antioxidants in the pathology and treatment of lead toxicity. Altern. Med. Rev.11: 114–127.
63. **Patton, C. J. and S. R. Crouc (1977).** Spectrophotometric and kinetic investigation of the bertha lot reaction for the determination of ammonia. Anal. Chem. 49: 464.
64. **Pell, J. D.; J.M. Gee; G.M. Wortley and I.T. Johnson (1992).** Dietary corn oil and guar gum stimulate intestinal crypt cell pro liferation in rats by independent but potentially synergistic mechanisms. J. Nutr. 122: 2447-2456.
65. **Pratima, G.; S.S. Chaurasia; K. Anand; P.K. Maiti; P. Gupta and A. Kar (1997).** Influence of cadmium on thyroid hormone concentrations and lipid peroxidation in a fresh water fish, *Clarias batrachus*. Fresenius Envir. Bulletin 6: 355–358.
66. **Pulido, M.D. and A.R. Parrish (2003).** Metal-induced apoptosis: mechanisms. Mutat. Res. 533: 227–241.
67. **Radostits, O.M.; D.C. Blood; C.C. Gay and H.W. Hinchcliff (2000).** Veterinary Medicine. A Text Book of Diseases of Cattle, Sheep, Pigs, Goats and Horses. WB Saunders, London, pp. 1241–1250.
68. **Ridley, B.L.; M.A. O'Neill and D. Mohnen (2001).** Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. Phytochemistry 57: 929-967.
69. **Rose, H.E. and J. Quarterman (1987).** Dietary fibers and heavy metal retention in the rat. Environ. Res.42:166-175.
70. **Sandhir R.; D. Julka and K.D. Gill (1994).** Lipoperoxidative damage on lead exposure in rat brain and its implications on membrane bound enzymes. Pharmacol. Toxicol. 74:66–71.
71. **Schantz, S.L. and J.J. Widholm (2001).** Cognitive effects of endocrine disrupting chemicals in animals. Environmental Health Perspectives 109: 1197–1206.
72. **Schols, H.A. and A.G.J. Voragen (1996).** Complex pectins: structure elucidation using enzymes. In: Visser, J.and Voragen A.G.J. (Eds): Pectins and pectinases, Elsevier Science, Amsterdam, pp. 3-19.
73. **Serguschenko, I.S.; V.V. Kovalev; V.E. Bednyak and YS. A. Khotimchenko (2004).** Comparative evaluation of metal-binding activity of low-esterified pectin from seagrass Zostera marina and other sorbents. Russ. J. Mar. Biol.30:83-85.
74. **Serguschenko, I.S.; E. Kolenchenko and M. Khoti Mchenco (2007).** Low esterified pectin accelerates removal of lead ions in rats. Nutrition Research 27: 633-639.
75. **Shalana, M.G.; M.S. Mostafa; M.M. Hassouna; S.E. El-Nabi and A. El- Refaie (2005).** Amelioration of lead toxicity on rat liver with vitamin C and silymarin supplements. Toxicology 206 (1): 1–15.
76. **Shotyk, W. and G. Le Roux (2005).** Biogeochemistry and cycling of lead. Met. Ions Biol.Syst. 43: 239–275.
77. **Silbergeld, E.K.; M. Waalkes and Rice, J.M. (2000).** Lead as a carcinogen: experimental evidence and mechanisms of action. Am. J. Ind. Med. 38: 316–323.
78. **Simsek, N.; A. Karadeniz; Y. Kalkan; O. N. Keles and B. Unal (2009).** *Spirulina platensis* feeding inhibited the anemia- and leucopenia-induced lead and cadmium in rats. J. of Hazardous Materials 164: 1304–1309.
79. **Singh, B.; D. Dhawan; B. Nehru; M.L. Garg; P.C. Mangal; B. Chand and P.N. Trehan (1994).** Impact of lead pollution on the status of other trace metals in blood and alterations in hepatic functions. Biol. Trace Elem. Res. 40: 21–29.
80. **Sivaprasad, R.; M. Nagaraj, and P.Varalakshmi (2003).** Combined efficacies of lipoic acid and meso-2 3-dimercaptosuccinic acid on lead-induced erythrocytemembrane lipid peroxidation and antioxidant status in rats, Hum. Exp. Toxicol. 22 (4)183–192.
81. **Speirs C.I.; G.C.Blackwood and J.R. Mitchell, J.R. (1980).** Potential use of fruit wastecontaining in vivo de-esterified pectin as a thichender in canned products. J. Sci. Food Agric., 32: 1287-1294.
82. **Sterling, L. (1975).** Diagnosis and Treatment of Thyroid Disease. Cleveland CRC Press, P.19 –51.
83. **Swarup, D.; V.P. Ram Naresh; M. Varshney; P. Balagangatharathilagar; D. Kumar; D. Nandi and R.C. Patra (2007).** Changes in plasma hormones profile and liver function in cows naturally exposed to lead and cadmium around different industrial areas. Research in Veterinary Science 82:16–21.
84. **Szasz, G. (1969).** Determination of γ- L-Y-Glutamyl transferase (γ -GT). Clin.Chem.,22:124 -136.
85. **Tahiri. M ; P. Pellerin; JC. Tressol ; T. Doco ; D. Pepin and Y. Rayssiguier (2000).**The rhamnogalacturonan-II dimer decreases intestinal

absorption and tissue accumulation of lead in rats. J Nutr. 130:249-53.

86. **Thakur B.R.; R.K. Singh and A.K. Handa (1997).** Chemistry and uses of pectin. A review. Crit. Rev. Food Sci Nutr 1997;37:47-73.
87. **Tierny, Y.; M. Bechet ; J.C. Joncquiert ; H.C. Dubourguler and J.B. Guillaume (1994).** Molecular cloning and expression in *Escherichia coli* of genes encoding pectate lyase and pectin methylesterase activities from *Bacteroides thetaiotaomicron*. J. Appl. Bacteriol. 75: 592–602.
88. **Timchalk, C.; Y. Lin; K.K. Weitz; H. Wu; R.A. Gies; D.A. Moore and W. Yantasee (2006).** Disposition of lead (Pb) in saliva and blood of Sprague-Dawley rats following a single or repeated oral exposure to Pb-acetate. Toxicology 222: 86–94.
89. **Tomaro, M.L. and A.M.C. Batlle (2002).** Bilirubin: its role in cytoprotection against oxidative stress, Int. J. Biochem. Cell Biol. 34: 216–220.
90. **Upasani, C.D.; A. Khera and R. Balaraman (2001).** Effect of lead with Vitamins E, C, or Spirulina on malondialdehyde: conjugated dienes and hydroperoxides in rats.Ind. J. Exp. Biol. 39 (1):70–74.
91. **Varley, H.; A. Gewenlock and M. Bell (1980).** Practical Clinical Biochemistry. Vol. 1. 5th edn, pp. 741, 897. London: William Heinemen Medical. Books, Ltd.
92. **Wade, M.G.; S., Parent; K.W. Finnson; W. Foster; E. Younglai; A.McMahon; D.G. Cyr and C. Hughese (2002).** Thyroid toxicity due to subchronic exposure to a complex mixture of 16 organochlorines, lead and cadmium. Toxicological Sci. 67: 207–218.
93. **Walters, M. and H. Gerade (1972).** Ultramicromethod for the determination of conjugated and total bilirubin in serum or plasma. Microchem J. 15:231.
94. **Warren M.J.; J.B. Cooper; S.P. Wood and P.M. Shoolingin-Jordan (1998).** Lead poisoning, haem synthesis and 5-aminolaevulinic acid dehydratase. Trends Biochem Sci 23:217–21.
95. **Willats, W.G.; L. McCartney, W. Mackie and J.P. Knox (2001).** Pectin: cell biology and prospects for functional analysis. Plant Mol. Biol. 47:9–27.
96. **Wintrobe, M.M. (1967).** The Wintrobe Erythrocytic Indexes. Clinical Haematology.6 th ed., Lea & Febiger, Phila. P: 414 – 419.
97. **Xu, D.X.; H.M. Shen; Q.X. Ahu and C.N. Chua (2003).** The associations among semen quality, oxidative DNA damage in human spermatozoa and concentrations of cadmium, lead and selenium in seminal plasma. Mutat. Res. 534 (1–2): 155–163.
98. **Young, D.C. (1972)**. Effects of drugs on clinical laboratory tests. Clin. Chem. 18: 430D – 432D.
99. **Young, D.C.; L.C. Pestaner, L.C. and U. Giberman (1975).** Effects of drugs on clinical laboratory tests. Clinical Chemistry 21: 3660.

8/5/2010

Chemopreventive effect of celecoxib and expression of cyclooxygenase-2, Casapase-3 and AGNOR on chemically- induced rat submandibular salivary gland neoplasm.

Mohamed Zayed

Lecturer, Oral Biology, Oral Histopathology Department, Misr International University (MIU).
dr_zayed2@yahoo.com

Abstract: BACKGROUND: Cyclooxygenase-2 (COX)-2 inhibitor (Celecoxib) is a non-steroidal anti-inflammatory drug (NSAIDs) and over-expression of COX-2 protein and mRNA has been reported in various cancer tissues. Therefore, it has been suggested that COX-2 is related to carcinogenesis. **METHODS:** Twenty five albino rats were used .They were divided into 3 groups; group I (normal control) and group II and III which was delivered 4-NQ in the drinking water .Meanwhile group III was given 1500 ppm celecoxib. Submandibular salivary glands were obtained after 32 weeks. Immuno-histochemical staining for COX-2 was performed to determine the COX-2 level and Caspase-3 immuno-expression was done for detection of apoptosis and silver nitrate staining of nucleolar organizer regions (AgNORs) was done for estimating the proliferating cells. The data were analyzed using Student's independent t-test and one-way analysis of variance (ANOVA). **RESULTS:** The group II and III showed pathological evidence of cancer. COX-2 immuno-staining was stronger in group II than in Group III. Caspase-3 immuno-reaction was statistically highly significant in group III ($p<0.05$) .Meanwhile proliferation estimated by AgNOR nuclear count was statistically highly significant group II ($p<0.05$). **CONCLUSION:** The COX-2 expression was increased in group II (untreated group) than group III. Administration of celecoxib demonstrated the chemo-preventive potential against the carcinogenesis through induction of apoptosis and suppression of tumor growth and proliferation.

[Mohamed Zayed. Chemopreventive effect of celecoxib and expression of cyclooxygenase-2, Casapase-3 and AGNOR on chemically- induced rat submandibular salivary gland neoplasm. Journal of American Science 2010;6(12):555-363]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: Oral cancer, Cyclooxygenase-2, submandibular salivary gland

11. Introduction

Oral cancer is one of 10 most frequent cancers in the world. Squamous cell carcinoma (SCC) is the most common malignant tumor of the oral cavity, accounting for over 90% of the malignant neoplasms in this region. Furthermore; recent epidemiologic data have indicated that the incidence of oral cancer is increasing, Nishimura et al. (2004). Despite a better understanding of the disease and the advent of modern technology and rationally targeted drugs, the incidence and cure rate of cancer have not improved, Bharat and Shishir (2006). Therefore, new molecular targets are needed for the prevention and treatment of oral cancer.Chemoprevention is to use of pharmacological or natural agents to prevent, suppress, interrupt, or reverse the process of carcinogenesis Garay & Engstrom (1999). Regular use of aspirin has been shown to lower the risk of colon cancer in man, Thun et al. (1991) and also Reddy et al. 1993; Rao et al. 1995 added that NSAIDs can suppress colon carcinogenesis induced by azoxymethane in rats ,Schreinemaches & Everson (1994); Harris et al. (1996). They demonstrated that there are significant reductions of breast cancer risk with the use of NSAIDs. In this field Lin and Nelson (2003) added that the experimental and epidemiologic studies have demonstrated that the NSAIDs are effective in the prevention of human cancers.

It is known that NSAIDs decrease prostanoid synthesis through the inhibition of cyclo-oxygenase (COX) activity, Van (1971). There are two different isoforms of COX, COX-1 and COX-2. COX-1 is constitutively expressed in most normal tissues to maintain stable physiological conditions such as cytoprotection in the stomach, vasodilatation in the kidney, and production of the pro-aggregatory prostanoid thromboxane by platelets Vane, (1994). Where as COX-2 is transiently induced by pro-inflammatory cytokines and growth factors of epithelial cells involved in inflammation and mitogenesis, Herschman (1996) and its patho-physiological role has been primarily connected to PG production in response to inflammation ,O'Banion et al. (1992); Crofford et al. (1994).

COX-2 has been recently reported to be up regulated and over-expressed in various of human malignancies, such as colon, lung, stomach esophagus, pancreas, endocervix, urinary bladder, prostate and skin and breast cancers, and also in squamous cell carcinoma of the head and neck Leong *et al.* (*1996*)*;* Wilson et al. (1998); Chan et

al. (1999); Tucker et al. (1999); *Stolina* et al. (2000); Yoshimura et al. (2000) and can be modulated by a variety of cytokines, hormones and tumor promoters Parrett et al. (1997;Wolff et al. (1998). In addition COX-1 and COX-2 have been detected in rat mammary gland tumors induced by various carcinogens, including DMBA,Robertson et. al. (1998), N-nitrosomethyl urea,Hamid et al. (1999) and 2-amino-1-methyl-6-henylimidazo[4,5-b] pyridine ,Nakatsug et al. (2000). Moreover, COX-2 has been implicated in the development of colon cancer and may play a role in promoting invasion, metastasis, and angiogenesis in established tumors ,Dubois et al. (1998) and Tsujii et al.,(1998).

Conventional NSAIDs such as aspirin, sulindac and indomethacin block both COX-1 and COX-2, resulting in unwanted side effects including gastritis and gastric ulceration. Therefore, when NSAIDs are used over long period as chemo-preventive agent, a selective COX-2 inhibitor must be used ,Jang et al. (2002). Celecoxib is new NSIADs that inhibits COX-2 and has significant anti-inflammatory and analgesic properties ,Seibert et al. (1994). Harris et al. (2000) reported that the celecoxib shows inhibitory effects on the development of rodent mammary cancer.

In this study, we examined the COX-2 expression in rodent sub-mandibular salivary gland neoplasm induced by 4-NQ and the chemo-preventive effect of celecoxib, a selective COX-2 inhibitor, for tumor development with correlation to its induction of apoptosis and proliferation.

2. MATERIALS AND METHODS

This study was performed in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals, the National Institute of Health Guide for the Care and Use of Laboratory Animals, and the Animal Welfare Act (A 3775-01).

2.1. Materials:

2.1.1. Celecoxib and an animal model

Twenty five adult male albino rats weighing (150-200 gm) were selected and divided into three groups.

The rats were watched for 1 week before the study. Three rats were allocated to each cage and were fed standard laboratory chow and water and libitum. The animals were housed and caged separately in the animals' house of the Faculty of Medicine, Cairo University.

Celecoxib (Celebrex) was manufactured and supplied by Pfizer Egypt S.A.E under Authority of G. D. Searle &CO.USA.

The rats have been then randomly divided into 3 groups: (I) control – 5 rats; (II & III) 10 rats for each.

The weight of all hamsters was recorded prior to the medication and subsequently on the end of this study.

2.2. Methods:

2.2.1. Induction of cancer

Carcinogenesis was induced in the animals of both group **II** and group **III** in which the carcinogen 4-NQ was obtained as a powder (Sigma, St. Louis, MO, A, cat. # N8141) and dissolved in the drinking water for rats of both groups to a final concentration of 0.02 g/l (20ppm). The prepared drinking water was changed once a week and the rats were allowed access to the drinking water at all times during the experiment is given in the drinking water in addition to the standard laboratory chow *diet.*

2.2.2. Chemoprevention and follow-up

Celecoxib was given to group III only from the beginning of the experiment after addition of one capsule, 100 mg into fine powder which was added to the laboratory chow of the rats every day. Clinically, physicians usually use daily 200 mg or 400 mg Celecoxib for treatment of rheumatoid arthritis or other diseases, and both doses have been approved by FDA. There is no specific guidance and dose for its application in animal model. However, currently 1,500 p.p.m probably is the most common dose to be used in rodent models. Kawamori et al. (1998) reported their findings to establish a therapeutic blood level of Celecoxib for chemoprevention in such models. The study identified daily administration of 1,500 p.p.m as an ideal dose to significantly suppress the colonic aberrant crypt foci formation. These findings served as the basis for our dose selected for this study. Rats were allowed access to the drinking water at all times during the experiment. At the 20th week of the experiment, two rats from each group were sacrificed and analyzed for precancerous and cancerous lesions. The study period was extended for thirty two weeks. After that all the remaining rats were sacrificed and the submandibular salivary glands were dissected.

Salivary glands biopsy specimens were taken for histological examination. The specimens were fixed in 10% formalin, processed in a standard manner, and stained with hematoxylin and eosin and examined under light microscope.

2.2.3. Immuno-histochemical analysis:

For immuno-histochemical staining, paraffin embedded tissues, sectioned at 4 μm and collected at

serial sections on positive charged slides (SuperFrost Plus-Menzel GmbH) were deparaffinized and dehydrated. Antigen retrieval was performed by boiling the slides in 10Mm citrate buffer, pH 6.0 for 20 minutes in a domestic microwave. Slides were left to cool for 30 minutes at room temperature. Sections were incubated in 3% hydrogen peroxide for 20 minutes. Novocastra protein block (RE7102 Novocastra, UK) was applied for 10 minutes after which the slides were incubated with the primary monoclonal Mouse anti body COX-2 [(CX-294) Dako,Glostrup, Denimark] for COX-2 immunostaining and while the other slides with the primary rabbit monoclonal antibody: anti-caspase 3 for caspase-3 immunostaining [(CPP32) Ab-4 Thermo Fisher Scientific, USA] diluted 1:100; for 30 min. at room temperature in a humidified chamber. After rinsing twice with TBS (Tris Buffered Saline, Amresco-USA), sections were treated with biotinylated secondary antibody (RE7103 Novocastra, UK) then labeled with streptavidin-biotin kit (RE110-k Novocastra, UK). The sections were then incubated in 3,3'diaminobenzidine (RE7190-k Novocastra, UK) for 5 minutes and counterstained with Mayer's hematoxylin (RE7107 Novocastra, UK).

2*.2.4. Silver nucleolar organizer regions (AgNOR) staining technique*:

Equal proportions of 50% silver nitrate soln. and gelatin soln. were mixed immediately before use. Sections were dewaxed in xylene and hydrated through ethanols to water then the slides were rinsed in distilled water. The Sections were then incubated in freshly prepared AgNOR working solution for 45 minutes at room temperature. After that the slides were washed in distilled water for one minute, then dehydrated, cleared and mounted in non aqueous mounting medium.

***2*.2.5. Cox-2, Caspase-3 and AgNOR staining assessment:**

The histological sections were examined using light microscope to assess the prevalence of positive ones. For Cox-2 and caspase-3 positive cytoplasmic immuno-expression, the percentage of positive cells was measured in the form of an area and area percent inside a standard measuring frame of area 11434.9 micrometer^2 per 10 fields using a magnification (x200), using image analysis software (Leica-Qwin) system. In addition, the number and area percent of AgNOR positive dots were counted per 10 fields using the image analysis. The nuclei that were overlapped or those with indiscernible AgNORs were excluded.

2.2.6.Statistical analysis: Quantitative data of the image analyzer were statistically evaluated and presented as means and standard deviation (SD) values. Student's t-test was used to compare mean values of Cox-2 and caspase-3 immuno-expression related parameters in group II & III; however the ANOVA (analysis of variance) test was used to compare the mean values of AgNOR obtained data between the two experimental groups. The significance level was set at $P \leq 0.05$.

3. Results:

3.1.H&E examinations revealed the rodent submandibular salivary glands showed signs of dysplasia and malignancy after administration of 4-NQ which appeared sever in group II while in group III (treated by celecoxib) appeared to be less affected.

In Group II the serous acini showed dramatic changes ranged from shrinkage of the lobules to total atrophy. As the serous cells demonstrated disruption of its characteristic acinar arrangement, with appearance of isolated acinar cells and significant shrinking of the acinar cells and necrosis were termed as ‘‘abnormal acini’’ the acinar cell become disrupted, distorted and detached from adjacent acinar cells. (Fig. 1A). The nuclei were hyperchromatic and showed pleomorphism (Fig. 1A) the intralobular Ducts that demonstrated considerable cellular hyperplasia and atypia with extravasation of red blood cells (fig. 1A), while inter lobular duct showed hyperplasia where as in other areas revealed atrophy and necrosis (Fig. 1C). While mucous acini were more resistant to effect of carcinogenic agent (Fig. 1B).

In group III, by administration of celecoxib the evidence of cancer appeared to be reduced or cured. The serous cells were arranged in abnormal acinar arrangement (Fig. 2A). The acinar cells became smaller in size. In other area showed loss their acinar arrangement (Fig. 2B). The nuclei showed piknosis and hyperchromatism (Fig. 2B). The duct did not show significant changes but show extravasations of RBC. (Fig. 2A).

3.2.COX-2 immunostaining:

The group II showed strong positive immunoreactivity in serous acini more than group I &III (Fig. 3A). While the mucous acini show positive peri-nuclear immunoreactions (Fig. 3B). The duct revealed strong cytoplasmic immuno-expression (Fig. 3 B). While in group III showed occasionally positive immunoreactions in serous acini and intralobular duct (Fig. 3C).

3.3. Caspase-3 immunostaining:

Occasional immunoreactivity was observed for caspase-3 in group II (4- NQ treated group) less than group I [Fig.3A]. On the other hand, sectionsof the serous acini of group III revealed a higher immuno-expression of caspase-3 (Fig.4B) immunostaining in duct cells (original magnification, x 400).

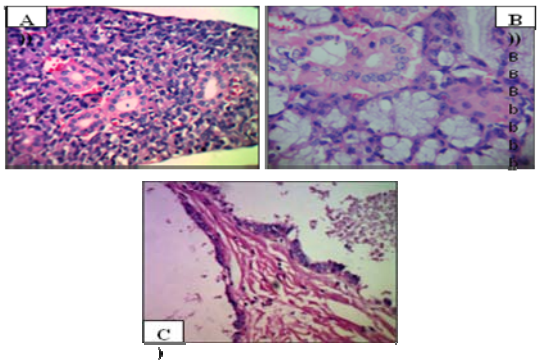


Fig. 1: Photomicrographs of serous and mucous glands of group II (A) show disruption and loss of the characteristic acinar arrangement, appearance ‘‘Abnormal serous acini’’ (ABS) of isolated acinar cells, and significant shrinking of the acinar cells (H&E. original magnification, x 200). (B) Mucous acini retain their normal morphology (H&E original magnification, x 400). (C) The interlobular duct shows in some areas hyperplasia and in other areas shows disruption, loss of continuity and atrophy arrows (H&E. original magnification, x 200).

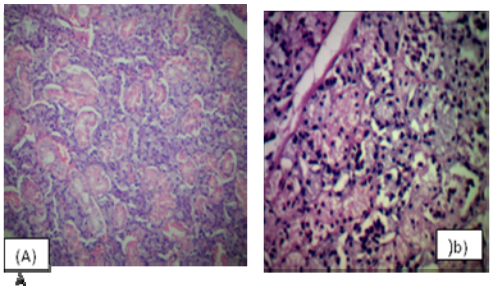


Fig.2: Photomicrographs of group III demonstrate (A). smaller serous acini and become atrophied. The duct appeared less effected and show lesser degree of extravasation piknotic and hyper chromatic. (H&E. original magnification, x 400).

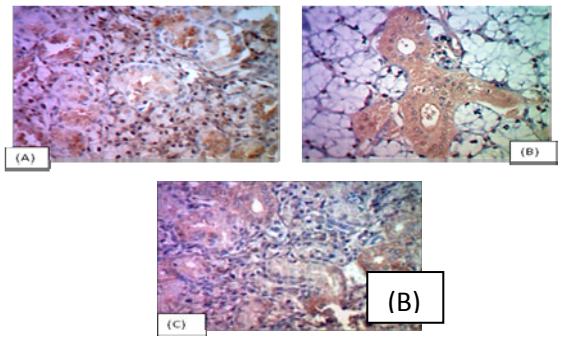


Fig. 3: photomicrographs of (A) group II show strong cox-2 immuno-expression in serous acini (original magnification, x 200). (B) A mucous acini show Cox-2 positive peri-nuclear immunoreaction & strong cytoplasmic immunostaining in ducts. (Original magnification, x 200). (C) Group III showing few positive cox-2 immuno-expression in acinar cell with positive immunoreactions in ducts (original magnification, x 400). of RBC. (H&E. original magnification, x 200). (B) The nuclei become.

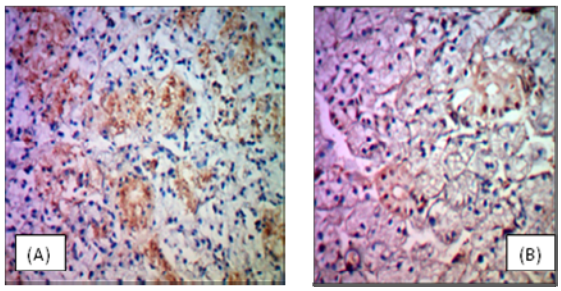


Fig. 4: photomicrographs of caspase 3 immunostaining (A) group II show negative immunoreaction with few positive cells (original magnification, x 400). (B) While in group III demonstrate higher positive immunoreactions in acinar cells with positive

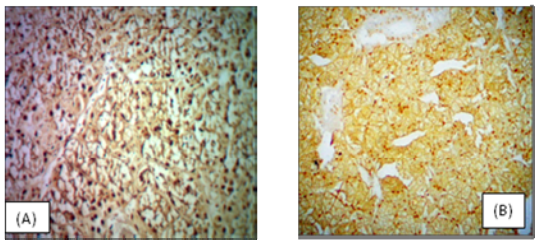


Fig.5: (A) Silver stained section of group II showing numerous silver stained nuclei in of serous cell (original magnification, x400). (B) Group III stained with silver showing less number of AgNOR dots in serous acini than group II (original magnification, x400)

3.4. AgNOR staining:

AgNOR were strictly located within the nucleus and were distinctly stained in black, being visible as dots. Silver stained dots were great in group II sections, almost all of the nuclei were stained (Fig.5a), meanwhile group III revealed lesser silver stained nuclei, restricted to acinar cell (Fig.5b)

3.5. Statistical analysis:

The results of the student's t-test revealed a non significant area percentage of the immunoreaction of COX-2 ($p>0.01$) between the group II & group III (table 1& fig. 6).

Table (1): Comparing the area percentage of COX-2 positive cells in group II and group III

Statistical profile	Group II	Group III
Mean± SD	110.6±0.8	108±1.0
Student's t-test	1.9	
P-value	0.084391	

** Significant at $P \leq 0.05$*

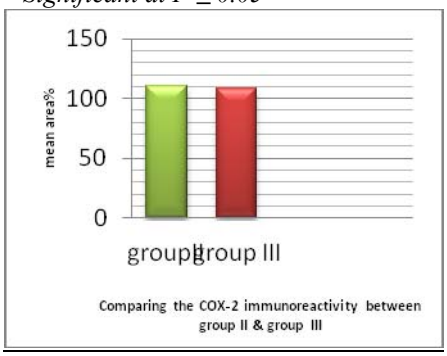


Fig.6: Bar chart illustrating the difference in area percentage of COX-2

And a highly significant area percentage of the immunoreaction of caspase-3 ($p<0.01$) between the two group II & group III (table 2& fig. 7).

Table (2): Comparing the area percentage of caspase-3 positive cells in group II and group III

Statistical profile	Group II	Group III
Mean±SD	114.2±1.3	179.8±0.78
Student's t-test	46.5939	
P-value	<0.001*	

** Significant at $P \leq 0.05$*

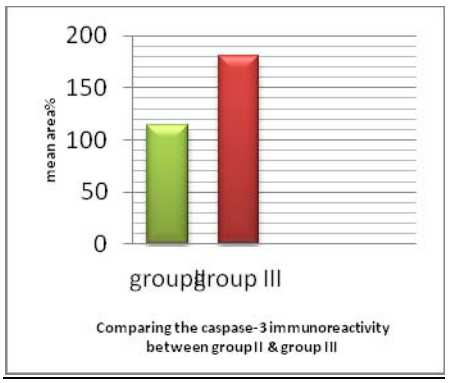


Fig.7: Bar chart illustrating the difference in area percentage of caspase-3 immunoreactivity in group II & group III

In addition, the ANOVA test showed a highly significant area percentage of the positive AgNOR dots ($p<0.01$) between group II & group III (table 3& fig.8).

Table (3): Comparing the area percentage of the AgNOR positive nuclei in group II and group III immunoreactivity in the group II & group III

Statistical Profile	Group II	Group III
Mean±SD	8.5±0.66	16±1.6
F- value	4.23259	
P- value	<0.001*	

- **Significant at $P \leq 0.05$*

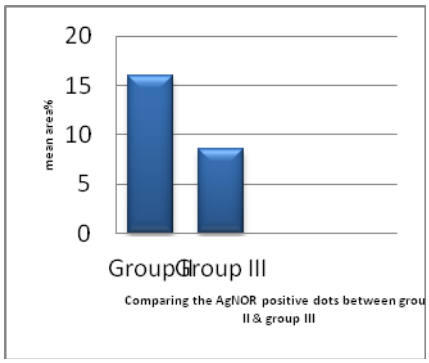


Fig.8: Bar chart illustrating the difference in the area percentage of the positive AgNOR dots in the groups II &III.

4. Discussion:

In the present study, we investigated the chemo-preventive effects of celecoxib in chemically induced 4-NQ cancer in submandibular rodent

salivary gland. We found that nearly all rats of (group II & III) that received 4-NQ suffered from developing salivary gland cancers 19/20 cases. This result is in accordance with Tang et al. (2004) as they demonstrated that murine 4-NQ induced oral and esophageal carcinogenesis model simulates many aspects of human oral cavity and esophageal carcinogenesis. They added that the availability of this mouse model should permit analysis of oral cavity and esophageal cancer development in various mutant and transgenic mouse strains. This model will also allow testing of cancer chemo-preventive drugs in various transgenic mouse strains COX-2 immuno-expression in group II was up regulated than in group III (treated by Celecoxib). These results are in accordance with Hwang et al. (1998); Mohammed et al. (1999); Kulkarni et al. (2001).They found that COX-2 was over expressed in various malignant tumor, such as cervical cancer, breast, skin also in squamous cell carcinoma of the head and neck. In this field, Nishimura et al. (2004) as they demonstrated that COX-2 was known to be over expressed in a variety of human pre-malignant and malignant lesions including oral ones. They also found that the administration of celecoxib produced inhibitory effects against oral SCC development both at the initiation phase ,during promotion and progression phases of carcinogenesis. The inhibition of COX-2 production led to marked lymphocytic infiltration of the tumor and reduced tumor growth, Stolina et al., (2000)

In this regard, Sawaoka et al., (1998); Chan et al., (1999) cleared that the mechanism(s) by which NSAIDs inhibit tumor growth was not clearly understood, but it could involve blockage of COXs, which suppress PGs production and might affect cell proliferation, apoptosis and immune response. And also, Tsujii et al.,(1998) added that there were evidences in colon cancer cells suggested that excessive PG production due to COX-2 over-expression played a role in tumor growth and spread. While, Lin and Nelson (2003) explained in more detail that COX-2 may be involved in carcinogenesis via 2 distinct mechanisms: (1). DNA damage and (2). PG-mediated effects. Reactions mediated by COX-2 form reactive oxygen species that could directly induce the oxidation of DNA or instigate the bioactivation of carcinogens. Prostaglandin E2, a byproduct of COX-2-mediated arachidonic acid metabolism, exhibits several biologic actions that have been shown to promote tumorigenesis and tumor progression. These actions include increased cell proliferation, promotion of angiogenesis, and the elevated expression of the antiapoptotic protein Bcl-2. In addition, PGE2 decreases natural killer cell activity and alters immune surveillance.

Caspase are fundamental component of the mammalian apoptotic machinery. Caspase 3 is a prototypical enzyme that becomes activated during apoptosis in a wide variety of tissues, Woo et al., (1998). The quantification of immuno-expression of caspase 3 might constitute a good method for measuring apoptotic activity in prostate cancer,Santamaria et al. (2005). In our study the immuno-expression of Caspase in group II was weak or negative while in group III the positive cell was abundant denoting the apoptosis process was markedly noticed. This result are in accordance with Pasricha et al., (1995) as they demonstrated that one of the most striking events in which PGs have been implicated in tumorigenesis is the inhibition of apoptosis in the colonic epithelium of familial adenomatous polyposis patients.. More directly, Tsujii and DuBois (1995) showed that rat intestinal cells increasing COX-2 expression by gene transfer became resistant to butyrate induced apoptosis, which can be overcome by addition of the non-specific COX inhibitor sulindac sulfide. In this field, Gupta and Dubois (2001) reported that the studies in colonic cancer showed that induction of COX-2 was associated with inhibition of apoptosis increased in angiogenesis, and metastatic potential. Also, Hashitani et al. (2003) reported that celecoxib induced apoptosis in cultured head and neck cancer cell lines to a significantly greater degree than sulindac Nucleolar organizer regions (NORs) are the sites of ribosomal RNA which reflects protein synthesis. AgNOR dots are the visualized structures of NORs that can be selectively stained by a silver colloid technique and can be visualized as black dots under the transmission microscope,Uno et al. (1998). According to Derenzini and Trerè (1994), the higher the number of NORs, the lower is the duration of the cell cycle and the higher is the velocity of cell proliferation. Such relationship makes the quantative analysis of NORs an excellent indicator of the proliferation activity of the cells and a valuable diagnostic tool, since it enables the differentiation of benign from malignant cells and even predicts the prognosis of different types of cancer ,Trerè et al. (1991). The results of this work showed that group II showed a higher number of AgNOR dots than group III. Chatterjee et al. (1997) demonstrated that the number of AgNORs rises with increasing the proliferative activity of cells, thus the number of AgNOR dots in malignant lesions is higher than normal or benign lesions. Moreover, Reddy et al. (1996) found that 1500 ppm celecoxib inhibits aberrant crypt foci multiplicity by 40–49% without

gross changes in the intestines. In this field, Wang, (2005) added that the over-expression of Cox-2 enhances cell proliferation, inhibits apoptosis and increases metastatic potential, thereby contributing to carcinogenesis. Eslami et al. (2006) concluded that there is an increase in the number of AgNOR dots with the advancement of malignancy.

Consequently, the findings of the present work suggested that celecoxib might exert a chemo-preventive effect on rodent salivary gland tumor through suppression PG production and inhibition of tumor growth and proliferation of cells and induction of apoptosis. In this regard, Ning et al. (2005) added Celecoxib is a highly selective inhibitor of COX-2, with less toxicity than traditional COX inhibitors. It may be used for reversing or stopping oral carcinogenesis at an early stage of disease.

In conclusion, our present results clearly indicated COX-2 protein to be increasingly expressed in the malignant salivary gland and provided the evidence that celecoxib, possessed the chemo-preventive potential by delayed onset of tumor development, retarded tumor growth and inducing apoptosis. From these findings, it was indicated that celecoxib could serve as a potent chemo-preventive agent with low toxicity against oral carcinogenesis. In addition, further studies are recommended on determining the mechanism of anticancer activity of celecoxib and its active components against oral cancers.

5. References

-Bharat B. and Shishir S.: Molecular targets of dietary agents for prevention and therapy of cancer. biochemical pharmacology 2006; 7: 1397– 421

-Chan, G., J. O. Boyle, E. K. Yang, F. Zhang, P. G. Sacks, J. P. Shah, D. Edelstein, R. A. Soslow, A. T. Koki, B. M. and Woerner, et al.: Cyclooxygenase-2 expression is up-regulated in squamous cell carcinoma of the head and neck. Cancer Res. 1999; 59:991-8.

-Chatterjee, R; Mukhopadhyay, D; Chakraborty, RN; Mitra, RB. Evaluation of Argyrophilic Nucleolar Organizer Regions (AgNORs) in relation to human papilloma virus infection and Cytokinetics. J Oral Pathol Med 1997; 26: 310-4.

-Crofford LJ, Wilder RL, Ristimaki AP, et al.: Cyclooxygenase-1 and 2 expression in rheumatoid synovial tissues: effect of interleukin-1b, phorbol ester, and corticosteroids. J Clin Invest 1994; 93: 1095–101.

-- Derenzini M, Trerè D. AgNOR proteins as a parameter of the rapidity of cell proliferation. Zentralbl Pathol. 1994; 140:7-10.

-Dubois R. N., Abramson S. B., Crofford L., Gupta R. A., Simon L. S., Van De Putte L. B. and Lipsky P. E.: Cyclooxygenase in biology and disease. FASEB J. 1998; 12:1063-72.

-Eslami, B; Rahimi, H; Rahimi, F; Khiavi, M; Ebadifar, A. Diagnostic value of silver nitrate staining for nucleolar organizer regions in selected head and neck tumors. Journal of Cancer Research and Therapeutics 2006; 2: 129-131.

-Garay C.A. and Engstrom P.F.: chemoprevention of colorectal cancer: dietary and pharmacologic approaches. Oncology 1999; 13: 89-98.

-Gupta RA and Dubois RN.: Colorectal cancer prevention and treatment by inhibition of cyclooxygenase-2. *Nat. Rev. Cancer* 2001; 1: 11–21.

-Harris R.E., Alshafie G.A., Abou-Issa H.M., and Seibert K.: Chemoprevention of breast cancer in rats by celecoxib, a *Cyclooxygenase* 2 inhibitor. Cancer Res. 2000; 60: 2101-3.

-Harris R.E., Namboodiri K.K. and Farrar W.B.: non-steroidal anti-inflammatory drugs and breast cancer. Epidemiology. 1996; 7: 203-5.

-Hashitani S, Urade M, Nishimura N, et al. Apoptosis induction and enhancement of cyto-toxicity of anticancer drugs by celecoxib, a selective cyclooxygenase-2 inhibitor, in human head and neck carcinoma cell lines. Int J Oncol. 2003; 23: 665–72.

-Hamid R., Singh J., Reddy B. And Cohen L.: inhibition by dietary menhaden oil of cyclooxygenase-1 and 2 in N-nitrosomethylurea-induced rat mammary mammarytumours. Int. J. Oncol. 1999;14: 523-8.

-Herschman H.R.: Prostaglandin synthase 2. Biochim. Biophys. Acta 1996; 1299: 125-40.

-Hwang d., Scollard D., Byrne J. and Levine E.: Expression of cyclooxygenase-1 and cyclooxygenase-2 in human breast cancer. J. Nat. Cancer Inst. 1998; 90: 455-60.

-Jang T.J., Jung H.G., Jung K. H. and Kuo M.: chemopreventive effect of celecoxib and expression of cyclooxygenase-1 and cyclooooxygenase-2 on

chemically- induced rat mammary tumors. Int. J. Exp. Path. 2002; 83: 173-82.

-Kulkarni S, Rader JS, Zhang F, et al.: Cyclooxygenase-2 expression is over-expressed in human cervical cancer. Clin Cancer Res 2001; 7: 429–34.

-Kawamori T., Rao C.V., Seibert K., And Reddy B.S.: Chemoprevention activity of celecoxib, a specific cyclooxygenase-2 inhibitor, against colon carcinogenesis. Cancer Res. 1998; 58: 409-12.

-Leong J, Hughes-Fulfold M, Rakhlin N, Habib A, Maclouf J. and Goldyne ME.: Cyclooxygenase-2 in human and mouse skin and cultured human keratinocytes: association of COX-2 expression with human keratinocyte
-differentiation. Exp. Cell. Res. 1996; 224: 79–87.

Lin DW and Nelson PS.: The role of cyclooxygenase-2 inhibition for the prevention and treatment of prostate carcinoma. Clin Prostate Cancer. 2003 Sep; 2(2): 119-26.

-Mohammed SI, Knapp DW, Bostwick DG, et al. Expression of cyclooxygenase-2 (COX-2) in human invasive transitional cell carcinoma (TCC) of the urinary bladder. Cancer Res. 1999; 59: 5647–50.

--Nakatsug S., Ohta T., Kawamori T. et al.: chemoprevention by nimelsulide, a selective cyclooxygenase-2 inhibitor, of 2-amino-1-methyl-6-phenylimidazo [4, 5-b] pyridine (PhIP)-induced mammary gland carcinogenesis in rats. Jap. J. Cancer Res. 2000; 91: 886-92.

-Ning L, Sandeep S, Su W, Mingzhu F, Peng W, Zheng S, Chung SY, Xiaoxin Ch.: Overexpression of 5-Lipoxygenase and Cyclooxygenase 2 in Hamster and Human Oral Cancer and Chemopreventive Effects of Zileuton and Celecoxib. Clinical Cancer March 1; 2005 11:2089-96.

-Nishimura N., Urade M., Hashitani S., Noguchi K., Manno Y., Takaoka K. and Sakura K.: Increased expression of cyclo-oxygenase (COX)-2 in DMBA-induced hamster cheek pouch carcinogenesis and chemopreventive effect of a selective COX-2 inhibitor celecoxib. J. Oral Pathol. Med. 2004; 33: 614–21

-O'Banion MK, Winn VD and Young DA. cDNA cloning and functional activity of a glucocorticoid-regulated inflammatory cyclooxygenase. Proc Natl Acad Sci USA 1992; 89: 4888–92.

-Parrett M.L., Harris R.E., Joarder F.S., Ross M.S., Clausen K.P. and Robertson F.M.: *cyclooxygenase*-2 gene expression in human breast cancer. Int. J. Oncol. 1997; 10: 503-7.

-Pasricha PJ, Bedi A, O'Connor K, et al. The effects of sulindac on colorectal proliferation and apoptosis in familial adenomatous polyposis. Gastroenterology 1995;109: 994–8.

-Rao C.V., Rivenson A., Simi B. et al.: Chemoprevention of colon carcinogenesis by Sulindac, nonsteriodal anti-inflammatory agent. Cancer Res. 1995; 55: 1464-72.

-Reddy B. S., Rao C.V., Rivenson A. and Kelloff G.: inhibitory effect of aspirin on azoxymethane-*induced colon carcinogenesis in F344 rats. Carcinogenesis. 1993; 14: 1493-7.*

-Reddy BS, Rao CV, Seibert K. Evaluation of cyclo-oxygenase- 2 inhibitor for potential chemo-preventive properties in colon carcinogenesis. Cancer Res 1996; 56: 4566–9.

-Robertson F.M., Parrett M.L., Joarder F.S. et al.: Ibuprofen-induced inhibition of cyclo-oxygenase isoform gene expression and regression of rat mammary carcinoma. Cancer Lett. 1998; 122: 165-75.

- Santamaria L., Martin R., Gomez V., Ingelmo I., Lopez C. And Revestido R.: stereologic estimation of Ki-67, Caspase 3, and GSTP1 positive cells in prostate lesions. Image Anal Stereol. 2005; 24: 77-84.
-Sawaoka H, Kawano S, Tsuji S, Tsuji M, Gunawan ES, Takei Y, Nagano K, Hori M. Cyclooxygenase-2 inhibitors suppress the growth of gastric cancer xenografts via induction of apoptosis in nude mice. Am J Physiol. 1998;247: G1061–G1067.

-Schreinemachers D.M. and Everson R.B.: Aspirin use and lung, colon, and breast cancer incidence in a prospective study. Epidemiology. 1994; 5: 138-46.

- Seibert K., Zhang Y., Leahy K. et al.: Pharmacological and biochemical demonstration of the role of *Cyclooxygenase* 2 in inflammation and pain. Proc. Nat. Acas. Sci. 1994; 91: 12013-7.

-**Stolina M., Sharma S., Ying L., Dohadwala M., Gardner B., Luo J., Zhu L., Kronenberg M., Miller P., Portanova J., Lee J. and Dubinet S.**: Specific Inhibition of Cyclo-oxygenase 2 Restores Anttumor Reactivity by Altering the Balance of IL-10 and IL-12 Synthesis *The Journal of Immunology*, 2000; 164: 361-70.

-**Tang, X., Knudsen, B., Bemis, D., Tickoo, S. and Gudas, L.:** Oral Cavity and Esophageal Carcinogenesis Modeled in Carcinogen-Treated Mice. Clinical Cancer Research 2004; 10: 301–13.

- **Trerè D, Farabegoli F, Cancellieri A, Ceccarelli C, Eusebi V, Derenzini M.** AgNOR area in interphase nuclei of human tumours correlates with the proliferative activity evaluated by bromo-deoxyuridine labeling and Ki-67 immunostaining. J Pathol. 1991; 165: 53-9.

-**Thun M. J., Namboodiri M.M., Calle E.E.,** Flanders W.D. and Heath C.W.: Aspirin use and reduced risk of fatal colon cancer. N. Eng. J. Med. 1991; 325: 1593-6.

-**Tsujii M. and DuBois RN**. Alterations in cellular adhesion and apoptosis in epithelial cells over-expressing prostaglandin endoperoxide synthase 2. Cell 1995; 83: 493–501.

-**Tsujii, M., Kawano, S. Tsuji, H. Sawaoka, M., Hori, R. and DuBois R.:** Cyclo-oxygenase regulates angiogenesis induced by colon cancer cells. Cell 1998; 93:705-14.

-**Tucker O. N., Dannenberg A. J., Yang E. K., Zhang F. L., Teng, J. M., Daly R. A., Soslow, J. L., Masferrer, B. M., Woerner,** A. T., Koki, 3rd. and Fahey. T. J.: Cyclooxygenase-2 expression is up-regulated in human pancreatic cancer. Cancer Res. 1999; 59: 987-94.

-**Wang Z.** The role of COX-2 in oral cancer development, and chemoprevention/treatment of oral cancer by selective COX-2 inhibitors. Current Pharmaceutical Design 2005; 11: 1771–1777.

-**Wilson, K., Ramanujam, S. and Meltzer D.:** Increased expression of inducible nitric oxide synthase and cyclooxygenase-2 in Barrett's esophagus and associated adenocarcinomas. Cancer Res. 1998; 58: 2929-37.

-**Wolff H., Saukkonen K, Anttila S, Karjalainen A, Vainio H and Ristimaki A.:** Expression of cyclooxygenase-2 in human lung carcinoma. Cancer Res. 1998; 58: 4997–5001.

-**Woo M., Hakem R., Soengas M., Duncan G., Shahinian A., Kagi D., Hakem A., McCurrach M., Khoo W., et al**.: Essential contribution of Caspase 3/CPP32 to apoptosis and its associated nuclear changes. Genes Dev. 1998; 12: 806-19.

-**Uno, T; Hashimoto, S; Shimono, M.:** A study of the proliferative activity of the long junctional epithelium using argyrophilic nucleolar organizer region (AgNORs) staining. J Periodont Res 1998; 33: 298-309

-**Van J.R.:** Inhibition of prostaglandin synthesis as a mechanism of action for aspirin- like drugs. Nature New Biol. 1971; 231: 232-5.

-**Vane J.:** Towards a better aspirin. Nature 1994; 367: 215–8.

-**Yoshimura R, Sano H, Masuda C, et al.:** Expression of cyclooxygenase-2 in prostate carcinoma. Cancer 2000; 89: 589–96.

8/9/2010

The Outcomes of Concomitant Radiation Therapy plus Capecitabine for Refractory Locally Advanced Breast Cancer Patients Pre-Treated with Anthracycline Based Regimens

Fatma Zakaria Hussen; Hanan Shawky Gamal El-Deen*; Amr Abd- El Aziz Ghanam; Samar Galal U and Omnia Abd –El-Fatah G.
Clinical Oncology Department, Faculty of Medicine, Tanta University Hospital, Tanta, Egypt
hannshawky@yahoo.com*

Abstract: Purpose: Anthracycline based chemotherapy is the first line treatment for most of patients with locally advanced breast cancer (LABC). However, some patients fail to respond to these regimens and no established second line treatment. Effective treatments options for patients with LABC resistant to anthracyclins based regimens are limited. We have conducted a phase II trial of capecitabine concomitant with radiation therapy to assess the safety, tolerability and efficacy of this regimen as a second line for down staging those inoperable patients with LABC. Patients and methods: Between February 2008 and September 2009, 27 patients with infiltrating ductal carcinoma, locally advanced breast cancer , who were refractory to first line anthracycline based regimens were planed to receive radiation therapy (50Gy/25f) and concomitant capecitabine (850 mg/m^2) twice daily for 14 days every 3 weeks, at Clinical Oncology Department , Faculty Of Medicine, Tanta University Hospital. All patients were assessed for objective response rate (ORR), progression –free survival (PFS), overall survival (OS), safety and tolerability. Results: Eighty five percent of patients (23 out of 27) became operable. The remaining four patients didn't undergo surgery because of progressive disease. Objective response rates (ORR) including those with complete clinical response 0.0% and partial clinical response in 10 (37%) patients. A complete pathological response for primary tumor and axillary lymph nodes was seen in 1 patient (3.7%). Pathologically negative axillary lymph nodes were seen in 5 patients (18.5%). The median follow up period was 16 months (range 6-26 months), the median PFS for all patients was 10 months (range 2-22 months), the one –year PFS was 29%. The median OS was not reached, the mean OS was 20.8 months (95% CI 17.78 - 23.84) and the two-year OS rate was 69.5%. Positive significant correlations were observed for PFS in patients with age ≥ 45 years , postmenopausal , +ve estrogen receptors (ER), +ve progesterone receptors (PR), -ve human epidermal growth factor receptors (HER-2), non triple negative patients, patients with ER/PR positive tumors, non inflammatory breast cancer (IBC) patients and those with axillary lymph node ratio (ALNR) <50%. There were no grade 3 or 4 adverse events with study protocol. Conclusion: The results of this phase II trial prove that concomitant capecitabine and radiation therapy is safe and effective in down staging of inoperable locally advanced breast cancer patients resistant to primary anthracycline based regimens. We are ongoing trial to use capecitabine as a maintenance monotherapy in patients with advanced breast cancer.

[Fatma Zakaria Hussen; Hanan Shawky Gamal El-Deen; Amr Abd- El Aziz Ghanam; Samar Galal U and Omnia Abd –El-Fatah G. The Outcomes of Concomitant Radiation Therapy plus Capecitabine for Refractory Locally Advanced Breast Cancer Patients Pre-Treated with Anthracycline Based Regimens. Journal of American Science 2010;6(12):564-574]. (ISSN: 1545-1003). http://www.americanscience.org.

Key Words: Locally advanced breast cancer, radiosensitizing agents, neoadjuvant treatment, capecitabine.

1. Introduction:

Locally advanced breast cancer (LABC) and inflammatory breast cancer (IBC) refer to a heterogeneous group of breast cancer without evidence of distant metastases (M0) and represents only 2% to 5% of all breast cancer in The United States[1]. Data from a population based registry in Tanta cancer center, Gharbia, Egypt, demonstrated that about 58% of breast cancer patients presented with a disease that extended to the loco-regional lymph nodes[2]. Patients with these cancers include those with, operable disease at presentation (clinical stage T3 N1), inoperable disease at presentation (clinical stage T4 and / or N2-3), and inflammatory breast cancer (clinical stage T4d N0-3) according to the 6th edition of the AJCC Cancer Staging Manual[3].

Locally advanced breast cancer was either presented as operable disease or inoperable disease. The current standard treatment for all patients with inoperable breast cancer is to proceed with neoadjuvant chemotherapy as the initial therapy. Approximately 80% to 90% of patients with advanced breast cancer showed partial or complete clinical response to neoadjuvant chemotherapy, and

most patients who presented with inoperable breast cancer became candidates for surgery[4,5].

First-line anthracycline–based, neoadjuvant chemotherapy is often effective, however, about 30% of the patients failed to respond to this regimen and to date there is no established second-line treatment[6].

Capecitabine (Xeloda, Hoffmann- La Roche, Basel, Switzerland) is a highly effective oral fluoropyrimidine that generates 5-fluorouracil (5-Fu) preferentially in tumor tissues through a three-step enzymatic process. The final step in the generation of 5-Fu from capecitabine is catalyzed by thymidine phosphorylase, an enzyme which is expressed at up to five times higher concentrations in tumor compared with healthy tissue[7,8].

We have studied the concomitant use of radiation therapy and capecitabine, to investigate the toxicity and efficacy of this regimen as a second-line neoadjuvant treatment in locally advanced breast cancer patients pre-treated with anthracycline based regimens.

2. Materials and methods

Patients

Between February 2008 and September 2009, twenty seven women more than 18 years old, with ECOG performance status of up to 2, had histologically confirmed diagnosis of infiltrating ductal carcinoma of the breast, at Clinical Oncology Department, Faculty of Medicine, Tanta University. All patients had locally advanced breast cancer (stage IIB, III, T3, T4 or N2) with measurable disease, which remained inoperable after primary anthracycline based chemotherapy.

Patients were ineligible for this study if they had metastases to distant sites, a white-cell count <4,000 per mm^3, an absolute neutrophil count (ANC) <1,800 per mm^3, a platelet count <100,000 per mm^3, a serum creatinine >1.5 mg/dL, a creatinine clearance of <50 ml/min (0.84 ml per second). Patients with non-malignant systemic disease that precluded them from receiving study therapy (eg, active infection, renal impairment, any clinically significant cardiac arrhythmia, or congestive heart failure) or patients who were pregnant or have dementia, altered mental status, or any psychiatric condition that would prohibit the understanding or rendering of informed consent were not eligible.

All were considered inoperable because they had either extensive edema of the skin, inflammatory breast cancer, fixation of the tumor to the chest wall, or involved axillary lymph nodes larger than 2.5 cm or fixed to the skin or deep structure. All patients received primary chemotherapy that included anthracycline, either, in the form of, FAC, AC, or TA. In patients found to be inoperable, staging was repeated and those without metastatic disease entered the study. All patients signed an informed consent.

Study design:

Eligible patients received irradiation to the whole breast through opposed parallel fields and also to the draining lymph node through direct fields. The total radiation dose was 50Gy given in 5 weeks (200 cGy/fraction/ day). Concomitant chemotherapy with capecitabine 850 mg/m2 was given orally twice daily for 14 days and repeated every 3 weeks during radiation therapy. After the end of radiation therapy by 3 weeks, patients were re-evaluated, if down staging was achieved and surgical interference became possible, patients were prepared for modified radical mastectomy. The median interval between the completion of radiotherapy and the date of surgery was 1.3 months (range 1-4 months). After mastectomy, hormone receptor- positive patients were assigned to receive hormonal therapy.

All patients followed for toxicity, response, PFS and OS with follow up period ranged from 6 to 24 months.

Evaluation:

Clinical response:

A complete physical examination was performed before each cycle of chemotherapy and before surgery. The product of the 2 greatest perpendicular diameters of the breast tumor was calculated. Objective response was defined as complete if there was disappearance of the known disease. Partial response was considered to be a ≥50% decrease in tumor area (calculated by multiplying the longest diameter by the greatest perpendicular diameter). Progressive disease was defined as a greater than 25% increase in the size of the target lesion or the appearance of any new lesion. Stable disease was defined as, a bi-dimensionally measurable decrease of less than 50% or increase of less than 25% in the sum of the products of the largest perpendicular diameters of the measurable lesion

Toxicity:

Acute toxicity was evaluated after each dose of concomitant chemotherapy and radiation therapy and classified according to the NCI common toxicity criteria version 2.0[9].

Pathological response:

Pathological response was assessed postoperatively. The surgical specimens were evaluated for pathologic tumor response by hematoxylin and eosin staining. A pathologic complete response (PCR) was defined as the absence of invasive carcinoma in the breast, along with

absence of any involved axillary lymph nodes. When residual tumor was present in the breast, it was estimated as ≤ 1cm (microscopic residual {MiR}, near-complete response) or > 1 cm (macroscopic residual {MaR})[10].

Immunohistochemistry was performed on formalin-fixed paraffin-embedded tissues from the diagnostic biopsies for estrogen, progesterone and human epidermal growth factor receptors (Her-2).

Statistical analysis:

Objective response rates were the primary end point; secondary end points were progression – free survival, overall survival and safety profile. SPSS package (version 12.0) was used for data analysis. Mean and standard deviation were estimates of quantitative data. Chi-square and Fischer exact were tests of proportion independence. Overall survival which calculated from time of study entry until death or last follow- up and progression free survival were assessed according to the Kaplan – Meier method. Progression –free survival was compared by the Kaplan –Meier method with statistical significance assessed by the Log -rank test. All P values were two-tailed; a value of ≤ 0.05 was considered significant.

3. Results

Patients' populations:

Twenty-Seven patients were recruited in the study with pathologically proven locally advanced breast cancer. The base line characteristics were listed in table (1), with the mean age 45.4± 8.0 years old years (range; 36-69), 16 patients (59.3%) were premenopausal and 11 patients (40.7%) were postmenopausal. Twelve patients (44.4%) had positive estrogen receptors (ER), while 44.4% of the patients had positive progesterone (PR) receptors and 10 patients (37%) had positive ER and PR. Six patients (22.2%) had positive human epidermal growth factor receptor (Her-2), six patients (22.2%) had triple negative hormonal receptors and four patients had inflammatory breast cancer (IBC).

Impact of concomitant capecitabine and radiation therapy on response:

Twenty three patients (85%) achieved good clinical response and became operable. Ten patients (37%) had partial clinical response, 13 patients (48.1%) had stable disease and 4 patients had progressive disease, (Table 2).

As regard pathological response, one patient (3.7%) achieved complete pathologic response, four patients (14.8%) had a near complete response ≤ 1cm and 18 patients (66.7%) had a residual primary tumor > one centimeter (Table 2). A completely, negative axillary lymph nodes was observed in 5 patients (18.5%). Median clinical tumor size before treatment was 143 cm^2 (range 36-272) and it was reduced to 36 cm^2 range (6-288) {p < 0.0001} (95% CI 54.6 – 97.5) with reduction rate (75%) after treatment. After surgery median pathologic residual tumor size was 12 cm^2 (range 4–48 cm^2).

The median number of dissected lymph nodes was 20 (range; 8 – 25) and the median number of involved lymph nodes was 7 (range; 0 – 24). The median follow-up period was 16 months (range 6-26 months). The median duration of response was 10 months (range 2-22 months) (95% CI SE 0.64 (8.7 - 11.25)). There were no significant correlation between clinical response rates and menopausal status (p=0.25), estrogen receptor status (p=0.19), progesterone receptor status (p=0.21), ER/PR positive receptors status (p=0.28), her-2- receptor expression (p=1.0). Only, triple negative patients had significant correlation with poorest clinical response (P=0.04) (Table 3).

Impact of treatment on survival:

At the time of this analysis, 8 patients had died. The median duration of follow-up was 16 months, (range 6-26 months). On the basis of Kaplan- Meier estimates, the median OS for all patients with LABC was not reached while the mean OS was 20.81 months (95% confidence interval, 17.78-23.84) and the two-year OS rate was 69.5%, (Fig, 1).The median PFS was 10 months (95% confidence interval, 8.75-11.25) (Fig, 2). The eighteen months PFS was 11%.

We analyzed the median PFS in relation to different prognostic factors, including age; in patients aged < 45 years old, the PFS was 0% at 18 months, while in patients aged > 45 years old, the 18 months PFS was 23% (P=0.0176). As regard menopausal status; in premenopausal patients, the 18 months PFS was 0% in comparison to 27% for postmenopausal patients (P=0.02), (Table 4).

In correlation of PFS to hormonal receptor status, we found that the 18 months PFS for ER –ve status was 0%, while it was 33% for ER +ve status (P=0.001). For PR status, PFS were 0% and 33% for –ve and +ve progesterone receptors respectively (P=0.014), (Table 4).

Among patients who did not have +ve Her-2-neu receptors at presentation, PFS was significantly better. Eighteen months PFS rate was 19% for patients without Her-2-neu receptors expression but dropped to 0% for patients with Her-2-neu receptors over-expression (P=0.0178), (Fig, 3). In triple negative patients the 18 months PFS were 0% while it was 14% for non triple negative patients (P=0.0987), (Fig, 4), (Table 4).

As regard to ER/PR +ve patients, the 18months PFS was 30% for ER/PR +ve patients, while it was 0% for non ER/PR +ve patients (P=0.002), (Fig, 5).

Among patients without IBC, the PFS was significantly better than patients with IBC. The 18 months PFS rate were 13% versus 0.0% respectively with no patient alive at 18 months with IBC (p = < 0.001), (Table 4).

The ratio between positive lymph nodes and total excised axillary lymph nodes (ALNR) were reported as <50% and ≥50% for all operable patients in this study, 18 months PFS rates was 21.4% versus 0.0% respectively, (P=0.0076), (Fig 6), (Table 4).

Toxicity profile:

All the 27 patients were assessable for toxicity according to the NCI common toxicity criteria version 2.0[9]. The treatment regimen was well tolerated with no grade 3 or 4 events. Hand-foot syndrome was not observed. Non-Hematological toxicities were observed in 12 patients (44%) (GI in 32% and GII in 12.4%). Hematological toxicities were observed in one patient (3.7%) in the form of GI anemia which didn't required hospitalization or treatment interruption. After 6 months of surgery, 20 patients were re-examined where lymphedema and functional restriction (GI) were present in 3 patients (11.1%), (Table 5).

Table (1): Pre-treatment patients' and tumor characteristics of the 27 patients with LABC.

Characteristic	No. patients (%)
Age (years)	
Mean	45.4years
Range	(36-69)
< 45 years	14 (51.9%)
> 45 years	13 (48.1%)
Stage	
IIB	1 (3.7%)
IIIA	4 (14.8%)
IIIB	18 (66.7%)
IIIC	4 (14.8%)
ER	
+ve	12 (44.4%)
-ve	15 (55.6%)
PR	
+ve	12 (44.4%)
-ve	15 (55.6%)
Her-2-neu	
+ve	6 (22.2%)
-ve	21 (77.8%)
ER/PR +ve	
Yes	10 (37%)
No	17 (63%)
Triple –ve	
Yes	6 (22.2%)
No	21 (77.8%)
Menopausal status	
Pre	16 (59.3%)
Post	11 (40.7%)
Previous chemotherapy	
AC	4 (14.8%)
FAC	21 (77.8%)
AT	2 (7.4%)
Operability	
Yes	23 (85.2%)
No	4 (14.8%)
Initial tumor clinical size	
(median, range)	143cm^2 (36-272)
Lymph nodes dissected	
(median, range)	20 (8-25)
Involved lymph node	
(median, range)	7 (0-24)

ER estrogen receptors PR progesterone receptors. AC adriamycin + cyclophosphomide
FAC 5-flouorauracil +adriamycin + cyclophosphamide AT adriamycin + taxanes.

Table (2): Evaluation of response to treatment among all patients

Response	No. (%)	
Clinical response		
PR	10	(37%)
PD	4	(14.8%)
SD	13	(48.1%)
Pathological response		
CR	1	(3.7)
≤ 1cm	4	(14.8%)
> 1cm	18	(66.7%)
NE	4	(14.8%)

CR complete response ; PR partial response; SD stable disease; PD progressive disease; NE not evaluable.

Table (3): Correlation of clinical response after concomitant capecitabine and radiation therapy to different prognostic factors

Variables	Clinical response						Total %		P-value
	PR		PD		SD				
Menopausal status									
Pre	5	31.3%	4	25%	7	43.8%	16	100%	
Post	5	45.4%	0	0%	6	54.5%	11	100%	0.25
Total %	10	37%	4	14.8%	13	48%	27	100%	
ER									
-ve	4	26.7%	4	26.7%	7	46.7%	15	100%	
+ve	6	50.0%	0	0%	6	50%	12	100%	0.19
Total %	10	37%	4	14.8%	13	48%	27	100%	
PR									
-ve	5	33.3%	4	26%	6	40.0%	15	100%	
+ve	5	41.7%	0	0%	7	58.3%	12	100%	0.21
Total %	10	37%	4	14.8%	13	48%	27	100%	
Her2-neu									1.0
-ve	8	38.1%	3	14.3%	10	47.6%	21	100%	
+ve	2	33.3%	1	16.7%	3	50%	6	100%	
Total %	10	37%	4	14.8%	13	48%	27	100%	
Triple negative									
Yes	1	16.7%	3	50%	2	33.3%	6	100%	
No	9	42.9%	1	4.8%	11	52.4%	21	100%	0.04
Total %	10	37%	4	14.8%	13	48%	27	100%	
ER/PR +ve									
Yes	5	50%	0	0%	5	50%	10	100%	
No	5	29.4%	4	23.5%	8	47.1%	17	100%	0.28
Total %	10	37%	4	14.8%	13	48%	27	100%	

Table (4): Correlation between different variables and progression-free survival in locally advanced breast cancer patients.

Variables	Progression free survival		95% CI	P-value
	12 months	18 months		
Age				
< 45	14%	0%	(5.69-12.31)	0.0176
≥ 45	46%	23%	(7.48-14.52)	
Menopausal status				
Pre	18%	0%	(8.09-9.91)	0.02
Post	45%	27%	(7.76-14-24)	
ER				
-ve	6%	0%	5.63-10.37)	0.0001
+ve	58%	33%	(8.98-19.02)	
PR				
-ve	13%	0%	(5.42-12.58)	0.0143
+ve	50%	33%	(6.47-15.53)	
Her-2-neu				
-ve	38%	19%	(9.55-12.45)	0.0178
+ve	0.0%	0.0%	(0 – 0)	
Triple negative				
Yes	16%	0%	(0.40-7.60)	0.0987
No	33%	14%	(8.52-11.48)	
ER/PR + ve				
Yes	60%	30%	(9.45-18.55)	0.0020
No	11%	0%	(0.0 – 0.0)	
IBC				
Yes	0%	0%	(0.0 – 0.0)	0.0000
No	34%	13%	(8.43-11.57)	
ALNR				
<50%	50%	21.4%	(8.07-13.93)	0.0076
≥ 50%	11%	0.0%	(6.56-11.44)	

ALNR: The axillary lymph node ratio
IBC: inflammatory breast cancer

Table (5): Adverse events in 27 patients with LABC

Toxicity		No (%)	
Non-Hematologic GI Toxicities	No	19	(70.4%)
	Nausea	4	(14.8%)
	Nausea/vomiting	2	(7.4%)
	Mucositis	2	(7.4%)
Non-Hematologic GII Toxicities	No	23	(85.2%)
	Nausea	1	(3.7%)
	Nausea+vomiting	2	(7.4%)
	Mucositis	1	(3.7%)
Hematologic GI Toxicities	No	26	(96.3%)
	Anemia	1	(3.7%)

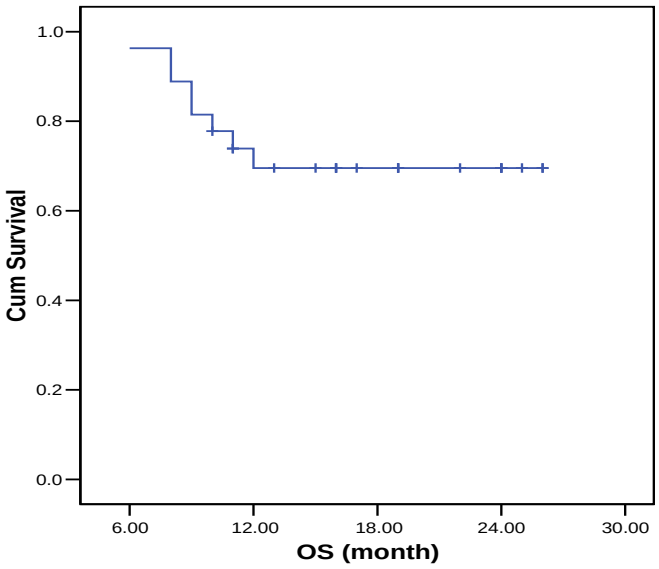


Figure 1. Overall survival for all patients

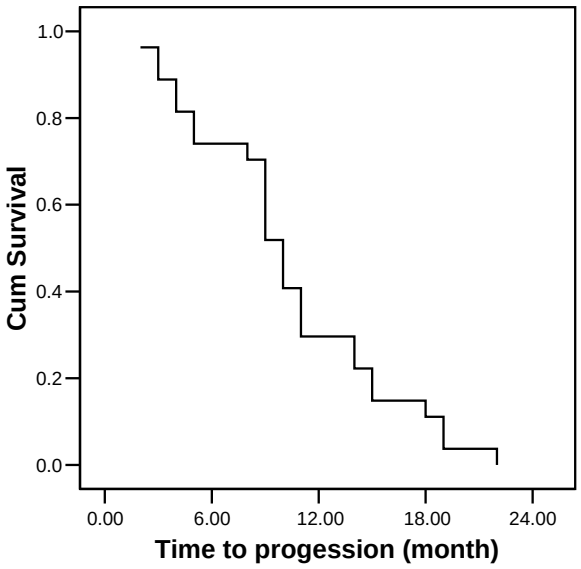


Figure 2. Progression free survival for all patients

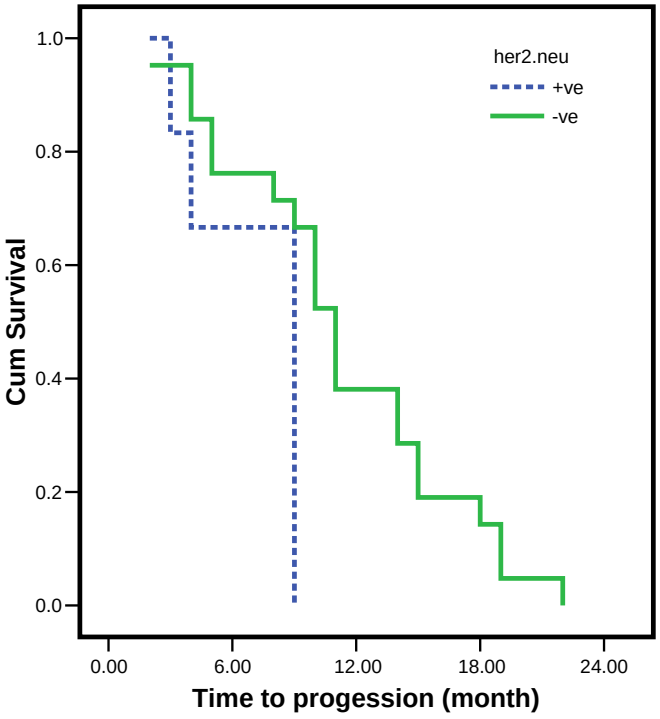


Figure 3. Progression free survival according to Her2neu receptors status

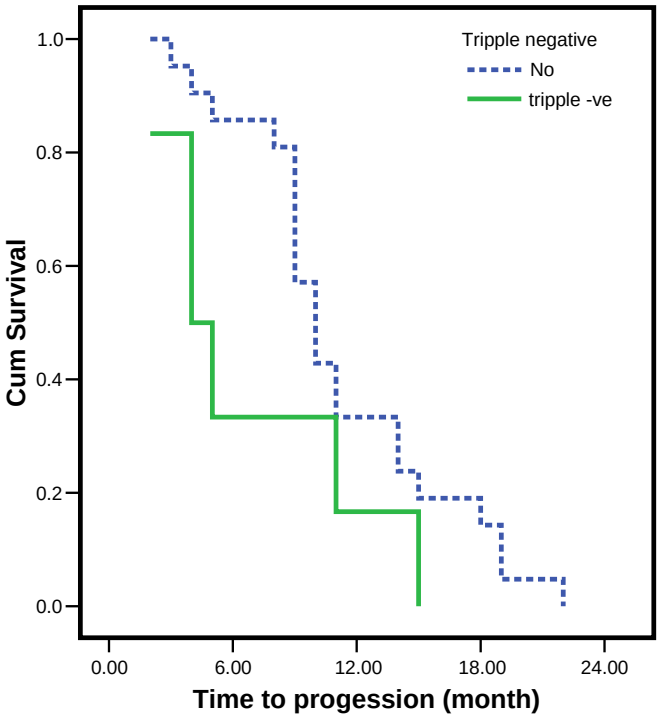


Figure 4. Progression free survival for triple negative patients versus non triple negative patients

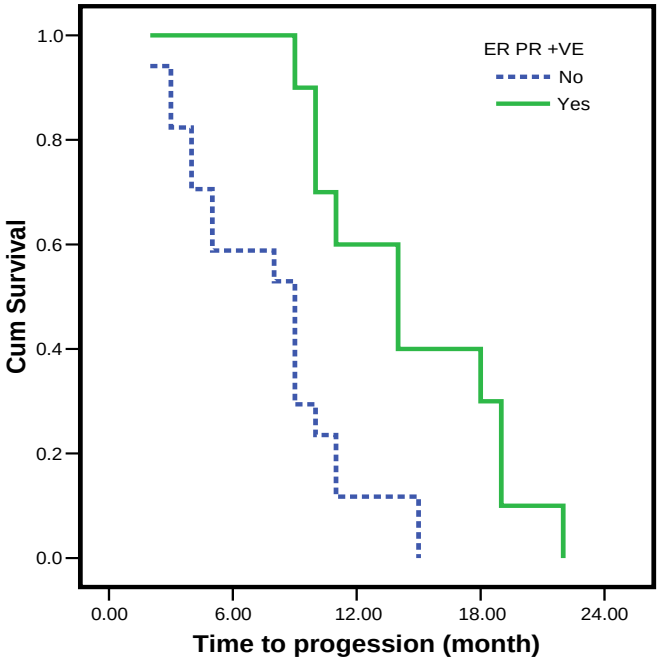


Figure 5. Progression free survival for ER/PR positive patients versus non ER/PR positive patients

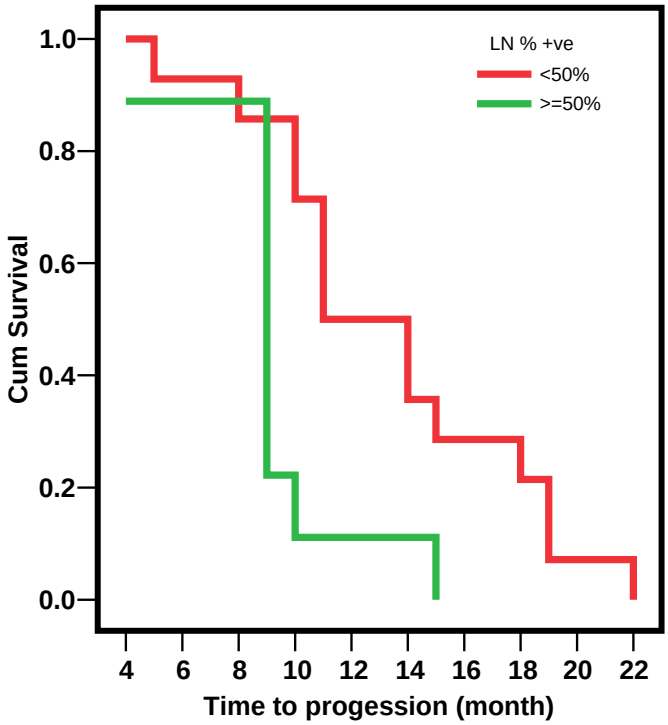


Figure 6. Progression free survival for patients with axillary lymph node ratio (ALNR) < 50% versus patients with axillary lymph node ratio (ALNR) ≥ 50%

4. Discussion:

This pilot study evaluates the efficacy and safety of the concomitant capecitabine and radiation therapy as second line treatment in locally advanced breast cancer patients pre-treated with anthracycline based regimens, at Tanta University Hospital, Clinical Oncology Department.

In this study, the operability rate was 85% (23/27 patients), the objective clinical response (PR) was 37% (10/27 patients), one patient with complete pathologic response (3.7%) and a near-complete pathologic response was seen in 4 patients (14.8%), while, 18 patients (66.7%) had a pathologic residual tumor more than 1cm. Pathologically, negative axillary lymph nodes were observed in 5 patients (18.5%). There was improvement for response with concomitant capecitabine and radiation therapy irrespective to menopausal status, ER, PR, and Her-2 receptors status. There was 75% reduction in initial tumor clinical size after treatment with capecitabine plus radiation therapy. Progression free survival (PFS) at 18 months for all patients was 11% with statistical significant correlation with age ≥ 45 years, postmenopausal status, +ve ER, +ve PR, –ve Her-2 neu receptors, non IBC and sector of patients with ALNR <50%. In our results, the median OS was not reached, while the mean survival was 20.8 months (limited to 26 months), 2-year overall survival was 69.5%. Minor adverse events which didn't need hospitalization or interruption of treatment were observed.

Several randomized trials in patients with LABC show that various chemotherapeutic regimens given pre-operatively result in a spectrum of objective response rates ranging from 10 to 66%[11,12] with variable rates of pathologic response ranging from 3.5% to 34%[11,12,13].

In many other trials where more than 90% of patients with LABC were pretreated with anthracycline, the mean response rate to cisplatinum\ vinorelbine regimen was 46% (25% to 74%)[14,15,16,17]. In another study carried out by Ali et al[18], used cisplatinum /vinorelbine on 13 patients with LABC, One patient achieved a complete clinical and radiological response 1/13, and 11 patients had a partial response for over all response rate of 92% but no pathologic complete response was documented[18].

Capecitabine is an oral drug, thereby avoiding the need for intravenous line and for hospital admission. Capecitabine seems to improve the survival of patients with advanced breast cancer

either as a single agent or in combination with docetaxel[19], and has shown promising activity in a phase II study in the neoadjuvant setting [20].

Vinorelbine – fluoropyrimidine has demonstrated to be an active combination for advanced breast cancer (LABC and MBC). Two multicentric phase II trials conducted to assess the efficacy and safety of vinorelbine- capecitabine combination (NavCap) and N+C followed by docetaxel (L) as sequential block regimen (Next) using the same selection criteria. Between April 2001 and September 2003, 73 consecutive patients were enrolled into these two trials (31 patients in NavCap and 42 patients in Next). Objective response rate were 68% and 75% respectively, median PFS were 10.8 months and 12.6 ms respectively, finally median survival was 30.4 months for NavCap and median survival was not reached in Next trial [21].

Our results revealed 85% of patients became operable, 37% ORR, 10ms (range 2-22) for median PFS and median survival was not reached with overall survival at 2 year (69.5%). The differences with our results may be due to smaller number of patients, less aggressive chemotherapeutic agent and poor prognostic features of our patients who were refractory to first line anthracycline based regimen in the neoadjuvant setting.

All those chemotherapeutic agents in the neoadjuvant setting were associated with higher rate of toxicities ; in vinorelbine / cisplatinum trial the adverse events were represented as follow, GII neutropenia in 10%, febrile neutropenia in 3% necessating hospital admission, GIII nephrotoxicity in 3%, GIII vomiting in 4%, and GII anemia in 8%[18]. With NavCap study, GIII neutropeina was 13%, GIII asthenia was 7%, and GIII Hand foot syndrome was 3%. For Next study, GIII neutropenia was reported in 15%, febrile neutropenia was recorded in 10%, while, asthenia occurred in 15%, and hand foot syndrome was seen in 2.5%. The combination of capecitabine / docetaxel had a high incidence of grade 3 adverse events (primarly hand foot syndrome)[22].

Another study carried out by Thomas et al[23] examined ixabepilone (40 mg/m^2 intravenously on day 1 of a 21-day cycle) plus capecitabine (2.000 mg/m^2 orally on days1 through 14 days of a 21-day cycle versus capecitabine alone (2500 mg/m^2 on same schedule) in advanced breast cancer patients, drug toxicity led to treatment discontinuation for 18% patients in combined arm and for 7% of patients in capecitabine group due to hand-foot syndrome, leucopenia and neutropenia. This study demonstrated superior PFS and OS after the addition of a second agent to capecitabine in patients resistant to anthracycline and taxanes, irrespective to Her-2 receptors expression[23].

Our results were in consistent with that reported in a land mark series of 38 patients from M.D Anderson Cancer Center treated with preoperative radiation therapy in refractory locally advanced breast cancer, in which 32 patients (84%) were able to undergo mastectomy. After completion of treatment, only 3 patients (9%) achieved a complete pathologic response. Completely negative lymph nodes were observed in 8 patients, (27%). The patients studied are quite different, tumor size was smaller before treatment in the radiation alone study and patients with inflammatory breast cancer were excluded. The rate of postoperative complications was highest in those who received radiation dose more than 54 Gys (70% versus 9%). The authors concluded that, despite the poor prognosis, radiation therapy alone improved the prognosis in these patients, however, in view of the high morbidity, they suggested that novel treatment strategies such as radiation therapy combined with radiosensitizing agents should be examined [24].

Another study by Gaui et al[25] examined the use of cisplatinum and 5-Fu continuous infusion as a radiation sensitizer for LABC. From January 1994 to February 1998, 58 inoperable patients who had anthracycline refractory LABC were treated with cisplatinum 25 mg/m^2 in bolus and 5-Fu 1gm/m^2 continuous infusion for 4 days, on days l and 28. Simultaneous radiation with 45 Gy was applied to the breast. Fifty seven patients (98%) became operable. After surgery a complete pathologic response in both the primary breast tumor and in the axillary lymph nodes was observed in 3 patients (5%), over all survival in 60 months was 27%[25].

The Brazilian investigators had conducted many studies to assess the role of radiation therapy in patients with LABC. One of them retrospectively examined the results of treatment with radiation therapy alone given to 38 patients with locally advanced infiltrating ductal carcinoma who were inoperable after first line chemotherapy from July 2000 to November 2002. After radiation therapy, only 23 patients (60%) were considered operable and underwent a mastectomy. Two patients (9%) achieved a pathologic complete response, one with a complete absence of residual and the other with microscopic disease. Only 2 patients were classified as a pathologic no response. The 3-year OS was 44%, and the 3-year failure free survival was 10% [26].

At 2007, the Brazilian investigators at Rio de Janeiro conducted a phase II trial evaluating the role of capecitabine as a radiosensitizer for patients with LABC, rendered 82% (23/28) of the patients operable, 38.8% reduction in the median clinical

tumor size after treatment. After surgery, a complete pathologic response was seen in 1 patients (4.3%), 3 patients (13%) achieved a near-complete pathologic response (less than 1cm), with minimal adverse effects[6].

As regard to the prognostic factors, our results showed that, Her-2 status had no effect on ORR in LABC which were comparable with the results of Chae et al[30]. ORR showed only significant correlation with non triple negative patients. Significant correlations of PFS were seen with , age > 45 years, post menopausal status , ER+ve, PR+ve patients with no IBC as reported in many other reports[18,31,32], while, ALNR <50% was in agreement with the report published by Hatoum et al[33].

Radiation therapy has been demonstrated to act synergistically with capecitabine in human tumor xenagrafts[29]. In our study, the concomitant capecitabine and radiation therapy has shown synergistic anti-tumor activity. This regimen can be given with an acceptable cost and tolerable toxicity with no need for extra supportive measures. Therefore, there is a need for addition of capecitabine to radiation therapy as a radiosensitizer, to act as a new treatment for hormone and chemotherapy-resistant, locally advanced breast cancer patients as reported in many other reports[27,28].

In conclusion, our results have confirmed the favorable safety profile of capecitabine that makes it specially suited for use in this group of patients with poor prognostic features with LABC. Capecitabine plus radiation therapy seems active and is feasible as secondary neoadjuvant therapy, in locally advanced breast cancer patients. We are ongoing trial to use capecitabine as maintenance monotherapy in patients with advanced breast cancer.

Corresponding author:
Hanan Shawky Gamal El-Deen
Clinical Oncology Department, Faculty of Medicine,
Tanta University, Egypt
hannshawky@yahoo.com

5. References:

1. Burstein HJ, Harris JR, Morrow M. Cancer of the Breast. In: Principles and Practice of Oncology, Ed.: De Vita VT, Hellman S, and Rosenberg SA, 8th edition, Ch. 43-2, p 1644 Publish. Lippincott Williams and Wilkins, 2008.
2. Ibrahim AS, Abdel-Bar I, Hussein H, et al. Cancer profile in Gharbia-Egypt, methodology and results (1999-December 2002). Gharbia based Cancer Registry, part (3): 43-4, 2007.
3. Greene FL, Page DL, Fleming ID, et al. eds. AJCC Cancer Staging Manual, 6th ed. New York: Springer-Verlag, 2002.
4. Fisher B, Brown A, Mamounas E ,et al. Effect of preoperative chemotherapy on local-regional disease in women with operable breast cancer: Findings from National Surgical Adjuvant Breast and Bowel Project B-18. J. Clin Oncol ; 15:2483-93,1997.
5. Kuerer HM, Newman LA, Smith TL, et al. Clinical course of breast cancer patients with complete pathologic primary tumor and axillary lymph node response to doxorubicin-based neoadjuvant chemotheapy J Clin Oncol ; 17:460-9, 1999.
6. Marie DF, Gilberto A, Roberto A, et al.: A phase II study of second line neoadjuvant chemotherapy with capecitabine and radiation therapy for anthracycline resistant locally advanced breast cancer. Am J Clin Oncol; 30:78-81, 2007.
7. Miwa M, Ura M, Nishida M, et al. Design of a novel oral fluoropyrimidine carbamate, capecitabine, which generates 5-fluorouracil selectively in tumors by enzymes concentrated in human liver and cancer tissue. Eur J Cancer; 34: 1274-81, 1998.
8. Schuller J, Cassidy J, Dumont E, et al. Preferential activation of capecitabine in tumor following oral administration to colorectal cancer patients. Cancer Chemother Pharmacol ; 45:291-7, 2000.
9. National Cancer Institute: cancer therapy evaluation program: common toxicity criteria. Available at http://ctep.cancer.gov/reporting/CTC-3test.html. August 1999. accessed December, 2005.
10. Stearns V, Singh B, Tsangaris T, et al. A prospective randomized pilot study to evaluate predictors of response in serial core biopsies to single agent neoadjuvant doxorubicin or paclitaxel for patients with locally advanced breast cancer. Clin Cancer Res. 9:124-33, 2003.
11. Gardin G, Rosso R, Campora E, et al. Locally advanced non-metastatic breast cancer: analysis of prognostic factors in 125 patients homogeneously treated with a combined modality approach. Eur J Cancer. 31A: 1428-33, 1995.
12. Smith IE, Walsh G, Jones A, et al. High complete remission rates with primary neoadjuvant infusional chemotherapy for large early breast cancer. J Clin Oncol.; 13: 424-9, 1995.
13. Smith IC, Heys SD, Hutcheon AW, et al. Neoadjuvant chemotherapy in breast cancer: significantly enhanced response with docetaxel. J Clin Oncol.; 20: 1456-66, 2002.

14. Shamseddine AI, Taher A, Dabaja B, et al. Combination cisplatin- vinorelbine for relapsed and chemotherapy –pretreated metastatic breast cancer. Am J Clin Oncol.; 22: 298-302, 1999.
15. Ray-Coquard I, Biron P, Bachelot T, et al. Vinorelbine and cisplatin (CIVIC regimen) for the treatment of metastatic breast carcinoma after failure of anthracycline- and/or paclitaxel-containing regimens. Cancer; 82:134-40, 1998.
16. Gunel N, Akcali Z, Yamac D, et al. Cisplatin plus vinorelbine as a salvage regimen in refractory breast cancer.Tumor; 86:283-5, 2000.
17. Vassilomanolakis M, Koumakis G, Barbounis V, et al. Vinorelbine and cisplatin in metastatic breast cancer patients previously treated with anthracyclines. Ann Oncol.; 11:1155-60, 2000.
18. Ali S, Mohammed K, Aref C, et al. A clinical phase II study of cisplatinum and vinorelbine (PVn) in Advanced Breast cancer (ABC). Am J Clin Oncol; 28 (4): 393 –8, 2005.
19. Shaughnessy J, Miles D, Vukelja S, et al. Superior survival with capecitabine plus docetaxel combination therapy in anthracycline-pre-treated patients with advanced breast cancer: phase IIItrial results. J Clin Oncol.; 20: 2812-23, 2002.
20. Lebowitz PF, eng-Wong J, Swain SM, et al. A phase II trial of neoadjuvant docetaxel and capecitabine for locally advanced breast cancer. Clin Cancer Res.; 10: 6764-9, 2004.
21. Ghosn M, Chahine G, Kattan J, et al. Vinorelbine / capecitabine combination in advanced breast cancer (ABC); long term results of two multicentric phase II trials. J Clin Oncol; 2005 ASCO Annual Meeting Proceedings, 23, (16S), Part I of II: 673; 2005.
22. Leonard R, Shaughnessy O, Vukelja S, et al. Detailed analysis of a randomized phase III trial: can the tolerability of capecitabine plus docetaxel be improved without compromising its survival advantage? European Society for Medical oncology, 2006. Available at http://academic.research.microsoft.com/Paper/11505728.aspx
23. Thomas ES, Gomez HL , Li RK, et al. Ixabepilone plus capecitabine for metastatic breast cancer progressing after anthracycline and taxane treatment. J Clin Oncol.; 25(33): 5210-17, 2007.
24. Huang E, Buzdar AU, Buhholz TA, et al. Locoregional treatment outcomes for inoperable anthracycline-resistant breast cancer. Int J Radiat Oncol Biol Phys.; 53: 1225-33, 2002.
25. Gaui MF, Salgado R, Spector N,et al. Second-line neoadjuvant chemotherapy with cisplatin / 5-fluorouracil and radiotherapy for locally advanced breast cancer refractory to anthracyclines: evaluation after a long-term follow up. Proc Am Soc Clin Oncol.; 23:27, (Abstract 602), 2004.
26. Schwartsmann G. Breast cancer in Sourth America: challenges to improve early detection and medical management of a public health problem. J Clin Oncol.; 19: 118-24, 2001.
27. Blum JL, Jones SE, Buzdar AU, et al. Multicenter phase II study of capecitabine in paclitaxel-refractory metastatic breast cancer. J Clin Oncol; 17: 485-98, 1999.
28. Fumoleau P, Largillier R, Clippe C, et al. Multicentre, phase II study evaluating capecitabine monotherapy in patients with anthracycline and taxane-pretreated metastatic breast cancer. Eur J Cancer; 40:536-42, 2004.
29. Sawada N, Ishikawa T, Sekiguchi F, et al. X-ray irradiation induces thymidine phosphorylase and enhances the efficacy of capecitabine (Xeloda) in human cancer xenografts. Clin Cancer Res.; 5: 2948-53, 1999.
30. Chae BJ, Bae JS, Lee A, et al . P53 as a prognostic factor in triple negative breast cancer.Jpn J Clin Oncol.;39 (4): 217-24, 2009.
31. Staaf J, Ringner M, Vallon-Chirstersson J, et al. Identification of subtypes in human epidermal growth factor receptor 2—positive breast cancer reveals a gene signature prognostic of outcome. J Clin Oncol.; 28 (11): 1813-20, 2010.
32. Geyer CE, Forster J, Lindquist D, et al. Lapatinib plus capecitabine for HER2-positive advanced breast cancer. N Engl J Med.; 355: 2733-43, 2006.
33. Hatoum HA, Jamali FR, El-saghir NS, et al. Ratio between positive lymph nods and total excised axillary lymph nodes as an independent prognostic facor for overall survival in patients with non-metastatic lymph node-positive breast cancer. Ann Surg oncol; 16 (12): 3388-95, 2009.

9/4/2010

Modulation of ochratoxin-induced oxidative stress, genotoxicity and spermatotoxic alterations by *Lactobacillus rhamnosus* GG in male Albino mice

Farag, I.M. [a]; Abdel-Aziz, K.B.[a]; Nada, S.A[c]; Tawfek, N.S.[b]; Farouk, T.[b] and Darwish, H.R.[a]

[a] Cell Biology Dept., National Research Centre, , Cairo , Egypt.

, [b] Zoology Dept., faculty of Science, Al-Mania Uni., Egypt.

[c] Pharmacology Dept. National Research Centre, , Cairo , Egypt

Abstract: The mycotoxin ochratoxin A (OTA) is a widespread contaminant in human food and animal feed. It is a carcinogenic, genotoxic, teratogenic, immunotoxic, and hepatonephrotoxic agent. Therefore, the present study was designed to assess the possible protective effect of *Lactobacillus rhamnosus* GG (LGG) against OTA-induced toxicity in mice. Four groups of 30 mice each were used: control group, LGG-treated group (1×10^{10} CFU), OTA-treated group (1.8 mg/kg b.w.) and a group of mice given LGG two hours before OTA gavage. The levels of malondialdehyde (MDA), glutathione (GSH) and superoxide dismutase (SOD) activity were measured in of liver and kidney. Bone marrow micronucleus test and chromosomal aberrations in spermatocytes, as well as mitotic and meiotic activities were performed to assess the genotoxicity; besides sperm parameters were evaluated. Results showed that OTA significantly decreased the body weight. OTA significantly elevated the tissue levels of MDA, whereas the levels of GSH as well as SOD activity were significantly decreased in both liver and kidney. OTA increased statistically the frequencies of MNPCEs in bone marrow and structural and numerical aberrations in spermatocytes. In addition, mitotic and meiotic activities of somatic and germ cells were declined significantly. Also, OTA caused a high significant reduction in cauda epididymal sperm count, sperm motility and increased sperm abnormalities, as compared to control. In mice received LGG before OTA gavage, a significant amelioration in LPO in liver and kidney, by increasing the contents of GSH and SOD activity, have been occurred. Cytogenetic analyses revealed that LGG administration before OTA gavage significantly reduced frequencies of MNPCEs in bone marrow and chromosomal aberrations in spermatocytes, and recovered mitotic and meiotic activities as well. Moreover, gavage LGG before OTA intoxication caused significant recovery in all sperm parameters studied. In conclusion, LGG was found to be safe and successful agent counteracting the oxidative stress and protected against the genotoxicity induced by OTA, in addition to reduction in spermatotoxic alterations.

[Farag, I.M.; Abdel-Aziz, K.B.; Nada, S.A; Tawfek, N.S.; Farouk, T. and Darwish, H.R. **Modulation of ochratoxin-induced oxidative stress, genotoxicity and spermatotoxic alterations by *Lactobacillus rhamnosus* GG in male Albino mice**. Journal of American Science 2010;6(12):575-587]. (ISSN: 1545-1003).

Key words: ochratoxin A, *Lactobacillus rhamnosus*, oxidative stress, micronucleus, spermatocytes, sperm

1 Introduction

Ochratoxin A (OTA) is a ubiquitous secondary metabolite produced by a number of mold genera, including *Penicillium* and *Aspergillus* (Castella et al., 2002; Pardo et al., 2005). OTA is found in a wide range of foodstuffs including wheat, corn, oats, beans, nuts and coffee beans, and in dry foods such soybeans, garbanzo beans, nuts and dried fruit, also in grapes, coffee, and pork (Bennet and Klich, 2003; Sage et al. 2004). OTA is hepatonephrotoxic, neurotoxic, immunosuppressive, cytotoxic, genotoxic, mutagenic, teratogenic, and carcinogenic agent (IARC, 1993; IPCS 2001). OTA was classified by the International Agency for Research on Cancer, as a possible human carcinogen (2B) (IARC, 1993). Its toxicity has been associated with inhibition of protein synthesis, DNA and RNA synthesis, mitochondrial dysfunction, formation of DNA adducts, disruption of calcium homeostasis, and the generation of reactive oxygen species (Gekle et al., 2005; Ringot et al., 2006; Marin-Kuan et al., 2006; Rached et al., 2007). Chronic exposure to OTA in humans found to be associated with a high incidence of progressive nephropathy and urinary tract tumors (Pfohl-Leszkowicz and Manderville, 2007). Schwartz (2002) hypothesized that OTA is a causative agent for testicular cancer. OTA induced lipid peroxidation (LPO), formation of reactive oxygen species (ROS) and consequent oxidative DNA damage, and decreasing intracellular reduced glutathione (GSH) level. Schaaf et al. (2002) observed an elevation in ROS levels, depletion of

GSH levels and an increase in oxidative DNA damage in rat proximal tubular cells and in LLC-PK1 cells treated by OTA. Furthermore, *in vitro* gene expression data obtained in HK-2 cells suggested an induction of mitochondrial ROS production due to OTA exposure. LPO levels were significantly increased in rat serum and in liver and kidney exposed to OTA, whereas, the glutathione level and antioxidant enzyme activities (SOD, CAT, GPX and GR) were significantly decreased (Meki and Hussein, 2001). Moreover, gene expression data showed that OTA alters a battery of genes, in F344 rat's kidney, that are involved in antioxidant defense and detoxification (Marin-Kuan et al., 2006); these results suggested a reduction of antioxidant defence as mechanism of OTA nephrocarcinogenicity (Cavin et al., 2007). Furthermore, DNA-oxidative damage was detected in HK-2 cells, at cytotoxic concentrations (Arbillaga et al., 2007). Also, oxidative damage to DNA was detected in target (kidney) and non-target (liver) tissues in male F344 rats (Kamp et al., 2005; Mally et al., 2005). Chromosomal aberrations have been induced by OTA *in vivo* in mouse cells (Bose and Sinha, 1994) and *in vitro* in human-derived hepatoma cells (Ehrlich et al., 2002). Dose dependent increases in the frequency of DNA single-strand breaks and alkali-labile sites, as measured by the Comet assay, and in micronuclei frequency were obtained in primary kidney cells from both male rats and human of both genders treated with OTA (Robbiano et al., 2004). OTA induced micronuclei (MN), in a dose-dependent manner, in Syrian hamster fibroblasts (Dopp et al., 1999), in human hepatic (HepG2) cells (Ehrlich et al., 2002) and in V79 Chinese hamster fibroblast cells and in primary cultures of porcine urothelial bladder epithelial cells (Föllmann, et al., 2007). OTA induced, also, MN in cytokinesis-blocked lymphocytes and led to a clear decrease in the percentage of binucleated cells in human lymphocytes (Dönmez-Altuntas et al., 2003). It also induce DNA-ploidy in kidney after chronic exposure (Brown, et al., 2007), and causes increase in endoreduplicated cells (Mosesso, et al., 2008). OTA inhibits the catalytic activity of topoisomerase II and may interfere with chromosome distribution during cell division (Cosimi, et al., 2009). Concerning reproductive toxicity, OTA was found to be a reproductive toxicant and prolong exposure to it caused a significant decrease in sperm count and increased abnormalities in sperm morphology (Bose and Sinha, 1994; Biró et al., 2003). In a recent study, male albino mice were treated orally with OTA (50 and 100 μg/day) for 45 days; alterations in various reproductive parameters were observed (sperm count, sperm motility, sperm viability and fertility rate), in a dose-dependent way (Chakraborty and Verma, 2009).

Currently there is considerable interest in the potential antigenotoxic and anti-carcinogenic effects of probiotics. Lactic acid producing bacteria (LAB), particularly *lactobacilli* and *bifidobacteria* are considered as the most probable agents responsible for these effects. Probiotics have been proved to exert health-promoting influences in human and animals (Ouwehand et al., 2002; Saxelin et al., 2005). *Lactobacillus rhamnosus GG (LGG)* is one of the best-studied probiotic bacteria in clinical trials for treating and/or preventing several intestinal disorders, including inflammatory bowel diseases and diarrhea (Yan and Polk, 2002, 2006). Furthermore *LGG (*ATCC 53013*)* efficiently binds, *in vitro,* several mycotoxins, including aflatoxin B1 and aflatoxin M1 (Pierides et al., 2000; El-Nezami et al., 2002). It had been reported that some strains of lactobacillus could protect against toxins contained in foods such as heterocyclic aromatic amines, polycyclic aromatic hydrocarbons, mycotoxins and reactive oxygen species (Knasmüller et al., 2001; Stidl et al., 2007). Probiotic *lactobacilli* demonstrated antimutagenic activity against 4-nitroquinoline-1-oxide and N-methyl-NV-nitro-N-nitrosoguanidine by microbial and mammalian cell-based tests (Caldini et al., 2005). Protection against *in vivo* genotoxicity had been observed after co-administration of LAB with N-methyl-N-nitro-N-nitrosoguanidine ((Pool-Zobel et al., 1993). It had been claimed that lactic acid bacteria which are contained in fermented foods and are part of the intestinal microflora may protect human against colon cancer (Wollowski et al., 2001; Rafter, 2004; McGarr et al., 2005).

Strategies for minimizing the possible deleterious effects resulting from human and animals exposure to genotoxic and/or carcinogenic agents in our environment are of utmost need. The aim of the present study was to evaluate the *in vivo* antioxidant, antigenotoxic and antispermatotoxic effects of lactic acid bacteria (*Lactobacillus rhamnosus GG)* against the well-known mycotoxin ochratoxin A in male albino mice.

2-MATERIALS AND METHODS

2.1.Materials:.

2.1.1.Chemicals, reagents, and reagent kits,

Were purchased from Riedel-de Haën, Germany and Biodiagnostic, Cairo, Egypt. Ochratoxin A was obtained from Food Toxicology and Contaminants Dept., National Research Centre, Egypt as a crude mycotoxin.

2.1.2. Bacterial strain and culture preparation:

Lactobacillus rhamnosus strain GG (ATCC 53013) was a kind gift provided by Food Toxicology and Contaminants Dept., National Research Center, Egypt as lyophilized powder and stored at -80°C. LGG cultures were prepared according to the procedure of El-Nezami et al. (2002). In which, bacterial cultures of LGG were obtained by incubating 0.1 g of lyophilized bacteria in 10 ml of deMan-Rogosa-Sharpe (MRS) broth under aerobic conditions at 37°C for 24 h. The number of lactic acid bacteria cells was enumerated by serial dilution in peptone water (0.1 % w/v) and plate counts on deMan-Rogosa-Sharpe agar (MRSA) medium.

2.2.Methods:

2.2.1 Experimental Animals:

Male Swiss Albino mice (*Mus musculus*) three months old weighing 25-30 grams were obtained from the animal house colony, National Research Centre (Giza, Egypt). The animals were maintained on standard casein diet and water *ad libitum* and housed individually in a temperature-controlled and artificially illuminated room free from any source of chemical contamination.

2.2.2.. Experimental design

Mice were randomly divided into four groups each consisting of 30 mice, each group was divided into three subgroups (10 mice for each). Animals were treated orally for successive 7 days as follows: (1) untreated control given $NaHCO_3$ and MRS broth daily, (2) treated with OTA (1.8 mg/kg b.w.) in 0.4ml $NaHCO_3$, (3) treated with LGG (1×10^{10} CFU) in MRS broth and (4) treated with the LGG (1×10^{10} CFU) 2 hours before OTA gavage (1.8 mg/kg b.w.). On the 8th day of the study, the 1st subgroup was killed and femoral bones were removed, stripped and cleaned from extraneous tissues. Also, liver and kidney samples were dissected out and washed immediately with ice-cold saline to remove as much blood as possible, and then stored immediately at -80º C until analysis. On the 15th day of the study, the 2nd subgroup was killed and both testes removed and washed in warm citrate saline. At the end of the experiment (35th day), cauda epididymis, of the 3rd subgroup, were quickly isolated, blotted free of blood and utilized for the analysis of various reproductive parameters.

2.2.3.. Body weight

Mice were weighed at the beginning of the study, at the 8th day, 15th day and the 35th day from the beginning of the study. The percentage of weight gain or loss was then calculated.

2.2.4.. Biochemical analyses

2.2.4.1. Measurement of lipid peroxidation:

Liver and kidney tissues were homogenized individually in 20 mm Tris–HCl (pH 7.4). Homogenates were centrifuged at 6000 g for 30 min. MDA levels in the supernatants were determined using a spectrophotometric assay kit according to the manufacturer's instructions. Briefly, thiobarbituric acid (TBA) reacts with malondialdehyde (MDA) in acidic medium at temperature of 95°C for 30 min to form thiobarbituric acid reactive product the absorbance of the resultant pink product can be measured at 534 nm (Ohkawa et al., 1979). The lipid peroxidation values are expressed as nm MDA/mg tissue.

2.2.4.2. Reduced Glutathione (GSH) content

GSH levels were measured using a spectrophotometric assay kit according to the manufacturer's instructions. 5,5` dithiobis-2-nitrobenzoic acid (DTNB) is reduced by glutathione (GSH) to produce a yellow compound .The reduced chromogen directly proportional to GSH concentration and its absorbance can be measured at 405 nm (Beutler et al., 1963). GSH values are expressed as mmol/g tissue.

2.2.4.3. Superoxide dismutase (SOD) activity

Liver and kidney homogenates were prepared in cold Tris–HCl (5 mmol/L, containing 2 mmol/L EDTA, pH 7.4) using a homogenizer. The unbroken cells and cell debris were removed by centrifugation at 10,000g for 10 min at 4º C. The supernatant was used immediately for the assays for SOD. 100µl of supernatants were added to 2.8 ml tris HCL buffer containing 25µl pyrogallol and 20µl catalase (Marklund and Marklund, 1974). The activities of all of these enzymes were determined. The SOD activities were expressed as units per mg of tissue.

2.2.5 Micronucleus test

Bone marrow slides were prepared according to the method described by (Krishna and Hayashi, 2000). The bone marrow was washed with 1 ml of fetal calf serum and then smeared on clean slides. The slides were left to air dry and then fixed in methanol for 5 min, followed by staining in May-Grünwald-Giemsa for 5 minutes then washed in distilled water and mounted.

For each animal, 2000 polychromatic erythrocytes (PCEs) were examined for the presence of micronuclei. In order to evaluate bone marrow cytotoxicity, we scored 1000 erythrocytes per animal and the rate of polychromatic erythrocytes (PE) relative to the number of normochromatic erythrocytes was calculated.

2.2.5. Chromosomal aberrations examination

Metaphases for analysis of chromosome aberrations in spermatocytes were prepared according to the method of Evans et al. (1964) and recommendations by Russo (2000) were considered. Structural aberrations analysis was studied in metaphase I (MI): MI with only 20 bivalents was scored; the presence of univalents, chromosome breaks, fragments and chain or ring multivalents, which are classified as reciprocal translocations were considered. For aneuploidy assay, metaphase II (MII) was studied: MII with 18<n>22 chromosomes were recorded, and polyploidy was considered as 2n, 3n or 4n. Fifty metaphase spreads were analyzed per animal. For Meiotic activity of spermatocytes; meiotic index was calculated as the frequency of MII/MI, normal ratio should be equal 2.

2.2.6.. Sperm parameters:

Sperm parameters were prepared and analyzed according to the protocols of Wyrobek and Bruce (1975).

2.2.6.1. *Collection of Epididymal sperm*:

Epididymal sperm were collected by cutting the cauda epididymis and perfusing the cauda with normal saline (0.9%) at 37◦ C. The epididymal perfusate was centrifuged at 225×g for 10 min. The pellet was re-suspended in 1.0 ml of normal saline. An aliquot of sperm suspension was used for the sperm examination.

2.2.6.2. Epididymal sperm counts and sperm motility:

Epididymal sperm counts and evaluation of the motility were performed visually using counting chamber. The count was repeated three times for each sample to minimize error, and calculated as 10^6 per sperm dilution. Sperm motility was determined by counting both motile and non-motile sperms in at least 16 separate and randomly selected fields. These results were expressed as percent motility.

2.2.6.3. Epididymal sperm morphology

A drop of sperm suspension was smeared onto a slide, left to dry; then stained with Eosin A, the slides were washed in water and air dried again. The smears were microscopically analyzed at a magnification of ×1000 for observation of abnormalities.

2.2.7. Statistical analysis

Statistical analyses were performed by one-way ANOVA followed by Tuckey's test or by Two-way ANOVA followed by Bonferroni's test comparing all groups. Analysis was conducted with GraphPad Prism software V.5.0.3 (Inc., San Diego, CA; USA).

3. Results

3.1. Change in body weight:

The current results indicated that no mortalities were recorded among any treated groups; no specific symptoms occurred within all groups. At the 8th day of the study, OTA-treated mice showed a significant reduction in body weight gain in comparison with the control group at $p<0.001$ (Fig. 1). Furthermore, at the 15th day, OTA-treated mice showed significant weight loss which was significant (6.10 % at $p< 0.001$), this weight loss reached 9.74 % at the 35th day and it was highly significant compared to all other groups at $p < 0.001$. On the other hand, mice treated with LGG before OTA showed an insignificant decrease in body weight gain compared with the control ($p<0.05$). This group showed a very significant weight recovery when compared with OTA group for all time points. Mice given LGG alone showed an insignificant increase in body weight gain compared with control at $p<0.05$.

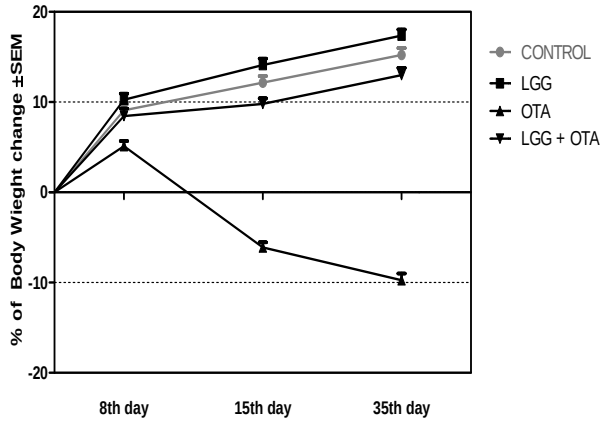


Figure 1: Effects of LGG on ochratoxin-induced mice body weight change.

3.2. Biochemical study

3.2.1. Effect on MDA levels

In OTA-treated mice, MDA level showed a high significant increase ($p<0.01$) in liver and kidney tissues as compared to that of control (table 1). LGG gavage before OTA-intoxication normalized MDA levels in liver tissues to that of control group, which was non significant in comparison with control, while it was still significantly higher in case of kidney tissue compared to control group ($p<0.01$). Whereas, the reduction in MDA in both liver and kidney in this group was statistically significant at $p<0.01$, when compared to the OTA group. In mice

receiving LGG alone, no significant differences were found in MDA level in kidney tissue when compared with control, while data showed a significant decrease in liver MDA level when compared with control at $p < 0.01$.

Table 1: Effects of LGG on MDA, GSH levels and SOD activity in liver and kidney of mice treated with OTA.

Experimental Groups	Parameters					
	MDA		GSH		SOD	
	LIVER	KIDNEY	LIVER	KIDNEY	LIVER	KIDNEY
Control (Broth/NaH CO_3)	339.0± 12.8^{a}	258.0± 6.60^{a}	13.1± 0.40^{a}	17.3 ± 0.31^{a}	29.1± 1.76^{a}	70.7± 3.11^{a}
Ochratoxin (1.8 mg/kg b.w.)	751.0± 15.0^{c}	798.0± 13.3^{c}	4.28± 0.39^{c}	8.12± 0.62^{c}	15.9± 1.56^{c}	26.8± 2.82^{c}
LGG (1×10^{10})	273.0± 8.70^{b}	214.0± 5.80^{a}	13.9± 0.28^{a}	19.4± 0.54 a	37.6± 1.22^{b}	78.4± 2.66^{a}
LGG plus Ochratoxin	352.0± 15.4^{a}	390.0± 14.3^{b}	10.6± 0.46^{b}	14.5± 0.37 b	27.6± 1.10^{a}	53.2± 1.85^{b}

Means with different superscript letters (a, b, c) are -significantly different ($P < 0.01$).
-All data are expressed as means ± SEM.

3.2.2 Effect on the reduced glutathione (GSH level)

Reduced glutathione content in both liver and kidney decreased significantly in OTA-treated group as compared to the control or LGG groups at $p < 0.01$. Mice received LGG before OTA intoxication showed a significant increase in GSH level when compared with the OTA-treated group at $p<0.01$. This increase was significantly below the GSH level of control and LGG groups at $p<0.01$. Mice given LGG alone exhibited increase in GSH content as compared to control, which was insignificant in liver and significant in kidney tissue at $p < 0.01$.

3.2.3 Effect on Superoxide dismutase (SOD) activity

OTA administered group showed high significant decrease in superoxide dismutase activity (in both liver and kidney) as compared to other groups ($p < 0.01$). However, the activity of SOD in the group received LGG before OTA was significantly increased as compared to the OTA-treated group in liver and kidney tissues (Table 1) at $p < 0.01$. This enhancement reached the value of controls in both liver and kidney, which was statistically nonsignificant ($p<0.01$) compared to control. Mice received LGG alone showed an insignificant increase in SOD activity in kidney tissue, while it was statistically significant in liver tissue, as compared with the control group at $p < 0.01$.

3.3. Cytogenetic studies:

3.3.1. Effects of LGG on OTA genotoxicity in bone marrow cells

Results for the MNPE rate are indicated in Table 2, mice treated with OTA showed a high significant increase in MNPCEs (with mean value of 32.8 at $P< 0.01$) when compared with other groups; where insignificant differences can be seen between the control and the LGG treated mice (a mean of 3.0 and of 2.4 per 2000 MNPCEs at $P< 0.05$, respectively). Mice received LGG before OTA gavage revealed a significant reduction in MN value (mean 11.0) with respect to the OTA-treated group, but this reduction was not enough to lower the MN to the basal level of control group, where it remained significant high. The mitotic index values were shown in table 2. OTA intoxication caused a high significant reduction in mitotic division compared with control ($p<0.01$). Whereas, administration of LGG before OTA treatment restored the division ability of bone marrow cells close to that of control, which was statistically significant when compared to the OTA-treated group at $p<0.01$. LGG given group exhibited no statistically differences in mitotic division, when compared to control or LGG plus OTA groups at $p<0.01$.

Table (2): Mean values of the frequencies of Micronucleated polychromatic erythrocytes (MNPCEs) in bone marrow cells of mice administred ochratoxin and/or LGG.

	Untreated Control	OTA	LGG	LGG plus OTA
MNPCEs/2000 cells	3.0± 0.32^{a}	32.8± 1.65^{c}	2.4± 0.24^{a}	11.0± 0.71^{b}
% PCE/NCE	0.49± 0.005^{ab}	0.32± 0.011^{c}	0.52± 0.010^{b}	0.46± 0.10^{a}

Means with different superscript letters (a, b, c) are -significantly different ($P < 0.001$).
- All data are expressed as means ± SEM.

3.3.2. Effects of LGG on OTA genotoxicity in germ cells (spermatocytes MI, MII)

The results of our study revealed that oral treatment with ochratoxin induced structural and numerical chromosomal aberrations in germ cells of

male mice (Table 3). The administration of OTA caused a high increase in X-Y univalents and autosomal univalent which was highly significant at p<0.01 compared with control or LGG groups. The total structure aberrations (x-y and autosomal univalents) increased significantly (P < 0.01) in OTA-treated mice compared to other groups. In mice given LGG before OTA, structural aberrations were decreased significantly compared to the OTA-treated animals at p < 0.01, but still revealed a significant difference compared with the control group. The LGG only treated group showed no significant differences in structure aberrations in respect to the control. Periploidy, polyploidy and the total numerical aberrations observed in spermatocytes were significant elevated (p<0.1) in the OTA treated group compared to all other groups. The LGG plus OTA group showed a significant reduction in numerical aberrations compared to the OTA-treated group and no significant differences compared to the control and LGG groups, except for the total numerical aberration when compared with the LGG group. Meanwhile, the LGG only treated group showed no significant differences in numerical aberrations in respect to the control.

The meiotic index (Table 3) revealed a significant meiotic delay in mice treated with OTA with respect to all other groups (p < 0.01). In the LGG and LGG plus OTA groups, there were no significant differences observed compared with them or the control group.

Table 3: Mean values of different types of chromosomal aberrations in spermatocyte of male mice OTA-treated with or without LGG

Experimental Groups	Structural aberrations (MI)			Numerical aberrations (MII)			Meiotic Index
	X-Y univalents	Autosomal univalents	Total	Aeuploidy	Polyploidy	Total	
Untreated Control	1.2 ± 0.20^{ab}	1.0 ± 0.32^{a}	2.20 ± 0.32^{ab}	$0.8\pm.37^{a}$	1.40 ± 0.25^{a}	2.40 ± 0.25^{ab}	1.99 ± 0.028^{a}
OTA	5.8 ± 0.37^{c}	6.0 ± 0.71^{b}	11.80 ± 0.37^{d}	4.80 ± 0.37^{b}	6.40 ± 0.51^{b}	11.60 ± 0.68^{c}	1.22 ± 0.028^{b}
LGG	0.8 ± 0.20^{b}	0.60 ± 0.25^{a}	1.40 ± 0.40^{b}	0.60 ± 0.26^{a}	1.20 ± 0.20^{a}	2.00 ± 0.31^{b}	2.08 ± 0.053^{a}
LGG plus OTA	2.4 ± 0.26^{a}	1.8 ± 0.20^{a}	4.20 ± 0.37^{c}	1.40 ± 0.25^{a}	2.20 ± 0.20^{a}	3.60 ± 0.25^{a}	1.87 ± 0.072^{a}

Means with different superscript letters (a, b, c, d) are significantly different (P <0.05).

-All data are expressed as means ± SEM.

3.4. Spermatological parameters:

3.4.1. Sperm count and motility

Data on sperm concentration, motility and morphology are presented in Table 4. There was a highly significant change in the sperm concentration of mice treated with OTA showing drastic reduction in sperm concentration (6.8×10^6). Administration of LGG before OTA intoxication caused a significant increase in sperm count respect to the OTA-treated group (18.4×10^6); this increase was significantly below that of control and LGG groups at p < 0.01. In mice given LGG alone, a significant increase in sperm count was observed compared to the control at p<0.05.

The motility of the sperm was affected dramatically in OTA-intoxicated mice which was 26.0 %, this reduction was statistically highly significant at p <0.01. In LGG plus OTA group, there was a significant enhancement in sperm concentration (63.8%) when compared to the OTA-treated group. Again, this increase is still below the basal count of the control. LGG group showed no significant increase in sperm count in respect to that of the control. LGG group showed non-significant enhancement in sperm concentration, compared with control at p<0.05.

3.4.2. Sperm Morphology:

OTA induced a high significant increase in sperm abnormalities in comparing with control at p<0.001 (Table 4). The various head abnormalities were existed, specially head without hook, unusual head shapes, big head and decapitation (Plate 1 B, D). The mid-piece abnormalities consisted of hair-pin, folded, and disrupted neck (Plate 1 E). The tail abnormalities essentially consisted of angular and bi- or coiled tail (Plate 1 F, J). In OTA-treated mice, 10.6 % of sperm head was detached from the flagellum, which was significant compared to control at p<0.01. In addition, OTA caused a fairly high percentage of sperm (13.4 %) that had sticky flagellum (agglutination), where several sperms remained fused in various numbers over short to long distances (Plate 1H, 1G), it was statistically significant at p<0.01.The retention of cytoplasmic droplet (CD) by the cauda epididymal sperm of control as well as OTA-treated mice was observed (Plate1 K). The retention of CD by the cauda epididymal sperm was 10.6% in control mice whereas it was 44.6 % in the OTA-treated mice, this difference was statistically highly significant. In mice receiving LGG before OTA intoxication, different sperm abnormalities significantly reduced in comparing with OTA-treated group, this enhancement showed significant differences with respect with either control or LGG groups at p <

0.05. Meanwhile, LGG group, mice showed a significant reduction in CD retention when compared with the control group ($p<0.01$).

Table 4: Effect of LGG on ochratoxin-induced changes in sperm parameters in male mice.

Experimental Groups	Sperm Count ($\times 10^6$)	% Sperm Motility	Sperm Morphology %						
			Head Abnormality	Mid-piece Abnormality	Tail Abnormality	Decapitation	Agglutination	Total Abnormality	Cytoplasmic Droplets
Untreated Control	22.6 ± 1.18^a	85.0 ± 1.58^a	2.8 ± 0.24^a	3.40 ± 0.24^a	2.40 ± 0.25^a	3.20 ± 3.7^a	0.00 ± 0.00^a	12.2 ± 0.74^a	10.6 ± 0.50^a
OTA	8.6 ± 0.98^c	26.0 ± 1.87^c	24.2 ± 1.16^d	19.6 ± 0.68^d	11. 0 ± 0.70^d	10.6 ± 0.75^d	13.4 ± 0.81^d	78.8 ± 1.28^d	44.6 ± 1.03^d
LGG	26.8 ± 1.21^a	89.0 ± 1.00^a	2.4 ± 0.25^a	2.80 ± 0.37^a	2.20 ± 0.20^a	3.2 ± 0.37^a	0.00 ± 0.00^a	10.6 ± 0.51^a	5.0 ± 0.32^b
LGG then OTA	18.4 ± 0.71^b	63.8 ± 1.87^b	10.0 ± 0.71^c	7.60 ± 0.40^c	3.80 ± 0.37^a	8.0 ± 0.55^c	5.20 ± 0.58^c	34.6 ± 1.10^c	19.6 ± 0.93^c

Means with different superscript letters (a, b, c, d) are significantly different ($P < 0.01$).

- All data are expressed as means ± SEM.
-

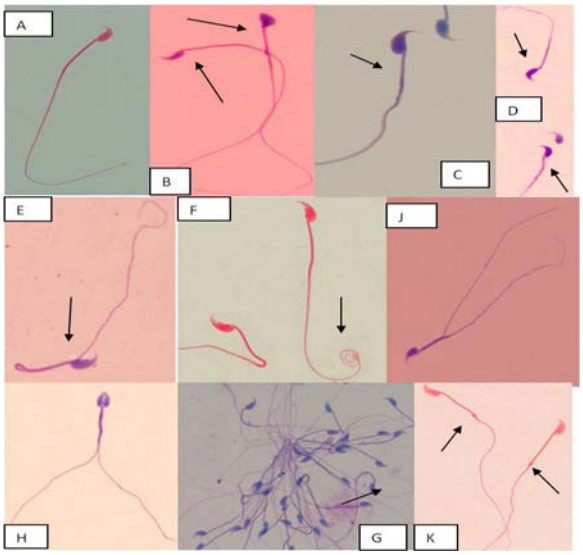


Plate 1: Giemsa-Eosin-stained sperm of mouse. (A) Control mouse. The various abnormalities of the sperm of OTA-treated mice (B–D) abnormal head shape; e.g, amorphous head, big head, banana-like; (E), hair-pin; (F-J) tail angulation bi- and coiled tails; (G), sperm agglutination ; (K), sperm retained CD

4. Discussion

Chronic exposure to OTA is believed to be an important factor for several diseases in human and animals, including renal tumors (Abdel-Wahhab et al., 2005; Brown et al., 2007). Therefore, many intervention strategies are set to prevent or alleviate OTA-induced disorders; probiotics are one of them, but still needed to be evaluated as protective agents for human health. In current study, the role of LGG on the OTA-toxicity was investigated in male albino mice. The present results clearly indicated that OTA treatment resulted in a significant reduction in body weight gain. These results are consistent with study in rats where the reduction in body weight was attributed to OTA and not to reduced food intake (Abdel-Wahhab et al., 2005). In the present study, this reduction in body weight may explained by the ability of OTA to generate free radicals (Pfohl-Leszkowicz et al., 1993), which may lead to DNA breakage, inhibition of protein biosynthesis and gluconeogenesis, lipid peroxidation, disruption of oxidative phosphorylation in mitochondria, inhibition of blood clotting and apoptosis (Hohler, 1998; Petzinger and Ziegler, 2000). LGG gavage alone resulted in insignificant increase in body weight gain; on the other hand, the pretreatment with LGG before OTA intoxication resulted in a significant improvement in body weight gain, comparable with the intoxicated group. This might be attributed to the alleviation of overall oxidative status, improved antioxidant defenses and decrease in genotoxic and cytotoxic effects (Bruno-Barcena et al., 2004; Klinder et al., 2004; Chen et al., 2005; Koller et al., 2008).

The results of the experiment showed that OTA treatment elevated MDA concentration in liver and kidney tissues, which indicated to the increase of lipid peroxidation. MDA is an end product of lipid peroxidation, and this may be considered a late biomarker of oxidative stress and cellular damage (Vaca et al., 1998). The results of this study confirm and extend previous data which have demonstrated that OTA induces a significant increase in LPO in liver and kidney induced by OTA as increasing in malondialdehyde (MDA) production (Petrik et al., 2003; Abdel-Wahhab et al., 2005, 2008). Previously, Gautier et al. (2001) stated that OTA did evoke oxidative stress, which might contribute, at least in part, to OTA renal toxicity and carcinogenicity in rats during long-term exposure. To assess the balance of reactive oxygen species (ROS) production in liver and kidney, levels of non-enzymatic antioxidants GSH and enzymatic antioxidant (SOD) activity were measured. Current results showed that OTA caused significant decrease in the levels of GSH, along with decrease in the activity of SOD; this decrease indicated the cell damage of liver and kidney tissues (Doorten et al., 2004). Soyöz et al. (2004) stated that intracellular GSH status appears to be a sensitive

indicator of cell's overall health and its ability to toxic challenges, whereas, the decrease in SOD activity will increase the level of superoxide radicals, leading to an increase in oxidative stress enhancing early cell death, probably by apoptotic mechanisms. Our finding of decrease in the activities of SOD and GSH corroborates with that of previous studies (Ozcelik et al., 2004; Abdel-Wahhab et al., 2008). The results of the experiment with LGG showed that treatment with LGG before OTA gavage ameliorated the biochemical parameters in liver and kidney, where MDA level decreased and SOD activity increased, along with an increase in GSH contents. Many *in vitro* studies, reported that LAB strains possess antioxidant properties and inactivate ROS via enzymatic mechanisms, e.g. by a coupled NADH oxidase/ peroxidase system, superoxide dismutase and catalase (Kullisaar et al., 2003; Bruno-Barcena et al., 2004; Lee et al., 2005). *Bifidobacterium longum ATCC 15708* and to a lesser *extent L. acidophilus ATCC 4356* inhibited linoleic acid peroxidation and scavenged free radicals (Lin and Chang, 2000). Also, it was found in human and animal studies that some LAB strains, which inactivate ROS, decrease biochemical parameters of oxidative stress (Han, 2004; Songisepp et al., 2005; Han et al., 2006). In a recent study, Koller et al. (2008) investigated the prevention of oxidative DNA damage in human derived colon (HT29) cells by 55 strains of lactic acid bacteria, they indicated that the reduction of oxidative damage was only seen with viable bacteria but not with heat inactivated cells and that it took place when the colon cells were separated from the LAB by permeable filter membranes indicating that the bacteria release ROS protective factors into the medium.

Considering the genotoxicity, the present study indicated that OTA caused high significant increase in DNA damage and cytotoxic effects in both cell types; we have investigated the OTA genotoxicity by induction of micronuclei (MN) in somatic cells (bone marrow), as an endpoint suitable to detect both aneugenic and clastogenic effects and chromosomal aberrations in germ cells (spermatocytes). The present data revealed that OTA induced a very high significant increase in MN in bone marrow cells, and increased structural and numerical aberrations in spermatocytes. Moreover, mitotic and meiotic activities had declined in a significant way. These findings are in agreement with the previous studies; OTA induced micronuclei in ovine seminal vesicle cells (Degen, 1997), in Syrian hamster fibroblasts (Dopp et al., 1999) and in human hepatic (HepG2) cells (Ehrlich et al., 2002). Significant dose-dependent increases in the frequency of micronucleated cells were also obtained in primary kidney cells from both male rats and humans of both genders with OTA (Robbiano et al., 2004). A statistical increase of structural chromosomal aberrations and sister chromatid exchanges associated with a decrease of the mitotic index was observed in bovine lymphocytes (Lioi et al., 2004). *In vivo*, oxidative damage to DNA was detected in target (kidney) and non-target (liver) tissues in male F344 rats (Kamp et al., 2005; Mally et al., 2005). Also, OTA induced structural and numerical chromosomal aberrations in bone marrow and germ cells of male mice (Ezz El-Arab et al., 2006). As shown in table (2, 3), data showed that OTA reduced the mitotic and meiotic ability significantly in somatic and germ cells; these results are in agreement with many studies which had demonstrated that even at very low concentration, ochratoxin was able to induce apoptosis in kidney cells in rats (Soyoz et al., 2004), and inhibit cell cycle progression by arresting cells at G2/M phase (Palma, et al., 2007). In addition OTA modulates key regulators of chromosome segregation and progression through mitosis (Adler et al., 2009). OTA found to inhibit the catalytic activity of topoisomerase II and might interfere with chromosome distribution during cell division (Cosimi, et al., 2009). Pfohl-Leszkowicz et al. (1993) reported that the ability of OTA to generate free radicals and to enhance lipid peroxidation has been linked to the genotoxicity expressed by DNA adduct. Free radicals may lead to DNA breakage, inhibition of protein biosynthesis and gluconeogenesis, lipid peroxidation, disruption of oxidative phosphorylation in mitochondria, inhibition of blood dotting and apoptosis (Petzinger and Ziegler, 2000). Pfohl-Leszkowicz and Manderville (2007) proposed that OTA genotoxicity might be caused by direct (covalent DNA adduction) and indirect (oxidative DNA damage) mechanisms of action. Gautier et al. (2001) and Baldi et al. (2004) reported that oxidative stress is an important factor in OTA cytotoxicity. Arbillaga et al. (2006) suggest that oxidative stress precedes cytotoxicity and genotoxicity and plays an important role regarding the mechanisms involved in OTA nephrotoxicity and carcinogenicity. Also, El Golli-Bennour et al. (2010) found that OTA induced genotoxic and cytotoxic effects in cultured monkey kidney Vero cells. On the other hand, results showed that the administration of LGG before OTA-intoxication reduced the OTA-induced genotoxicity (somatic and germ cells by around three folds) and cytotoxicity in both cell types. These data are consistent with other experimental studies which had evidenced the ability of *lactobacilli* and *bifidobacteria* to decrease the genotoxic activity of some chemical compounds (Tavan et al., 2002;

Burns and Rowland, 2004; Caldini et al., 2008). Also, our results revealed that LGG gavage before OTA treatment, enhanced the mitotic and meiotic activities of bone marrow cells and spermatocytes in OTA-treated mice to nearly to the basal level of control animals; this finding is going along with the mechanistic studies by Yan et al. (2007) who found that LGG prevent cytokine-induced apoptosis in intestinal epithelial cells through secreting two soluble proteins (p75 and p40).

Regarding the reproductive toxicity, the present study clearly indicated that oral administration of OTA caused adverse effects on male reproductive parameters in mice (Table 4). In OTA-treated mice cauda epididymal sperm count was reduced significantly (6.8 $x10^6$), along with a decrease in motility (26.0%) and a dramatically increase in sperm abnormalities (78.8 %). These findings are in agreement with some authors who reported similar kind of observations in different animals emphasizing ochratoxin as a reproductive toxicant; OTA induced chromosomal abnormalities and a decrease in spermatogenic numbers in mice (Bose and Sinha, 1994), decreased motility and longevity of breeding boar semen (Solti et al.,1999), impaired spermatogenesis and caused accumulation of premeiotic germinal cells (Fenske and Fink-Gremmels, 1990), inhibited testosterone secretion in isolated testicular interstitial cells of gerbils in *in vitro* condition (Gharbi et al., 1993). In human, sperm motility reduction might be due to mitochondrial disruption and/ or an increase in lipid peroxidation (Lodish et al., 2003). The lipid peroxidation of unsaturated fatty acids in sperm membranes is one of the most important effects from ROS-induced cell damage (Hsieh et al., 2006), and might impair sperm motility (Saradha et al., 2006; Hsieh et al., 2006). Chitra et al. (2003) observed that increased levels of lipid peroxidation caused the reduction of sperm count and viability. Moreover, the results of the present study showed that OTA intoxication rendered a significantly higher percentage of the cauda epididymal sperm (44.6 %) to retain cytoplasmic droplets (CD) than in the control mice. The residual cytoplasm contains high concentration of certain cytoplasmic enzymes (G_6PDH, SOD), and are also a source of ROS (Gomez et al., 1996). ROS damages phosphatides of cell membrane by peroxidized metabolites of fatty acids, whereby damaging the sperm function and morphology (Alvarez et al., 1987). Our data substantiated these claims where midpiece and tail of OTA-treated mice showed a very high percentage of malformation (hair-pin, disruption, folding, tail angulation, tail coiling), which might caused by peroxidation of cell components and disrupted the cytoskeletal proteins (44.0 %). The sticky flagellum (13.4 %) observed in this study might formed by fusing of two or more spermatozoa, where two or more axonemes are in a common cytoplasm ((Agnes and Akbarsha, 2003). These data reflected the aberrant spermatogenesis and/ or spermiogenesis caused by OTA treatment.

On the other hand, pre-treatment with LGG significantly mitigates OTA-induced alterations in reproductive parameters in mice, where sperm count elevated to reach 18.4 x 10^6 and the motility recovered to 63.8 %. Also, sperm morphology showed a significant enhancement (44.2 %). Moreover, LGG reduced CD retention by about 2.3 folds with respect to the OTA-treated group, which might due to the reduction in ROS.

Some authors attributed the protective effect of these bacteria to different mechanisms such as binding of OTA *in vitro* (Del Prete et al., 2007; Fuchs et al., 2008; Mateo et al., 2010). Previous work with more than 250 strains of lactic acid bacteria showed that *Lactobacillus rhamnosus* strains, LGG and LC705 were the most efficient strains in binding a range of mycotoxins, including aflatoxins (El-Nezami et al., 1998). Piotrowska and Zakowska (2005) verified that *L. acidophilus* and *L. rhamnosus* caused OTA reductions of 70% and 87% of 1 mg OTA/L after five days at 37 °C, and that significant levels of the OTA were present in the centrifuged bacteria cells. Gratz et al. (2006) suggested that LGG treatment reduced the hepatotoxic effects caused by a high dose of AFB_1, by increasing the excretion of orally dosed aflatoxin via the fecal route and suggested that LGG was able to retain additional AFB_1 and AFM_1 inside the intestinal lumens of rats. Nevertheless, Fuchs et al. (2008) consider that metabolism may also be involved, where viable cells of *L. acidophilus* removed OTA more efficiently than unviable. In addition, LAB found to cause reduction of the formation of secondary bile acids (Mirasoli et al., 2002) and enhancement of the immune system (Wallace et al., 2003; Schultz et al., 2003; Bengmark and Martindale, 2005).

In conclusion, the overall data indicate that the LGG have a broad range of biomodulatory properties; alleviates the OTA-oxidative stress (by decreasing in LPO and enhancing the activity of antioxidant enzymes and glutathione content) and protects against OTA-genotoxicity; as well as mitigates the spermatotoxic effects induced by OTA. However, further studies are needed to better understand the *in vivo* possible mechanism(s) by which LGG may reduce OTA toxicity.

5. References

1. **Abdel-Wahhab, M. A., Abdel-Azim, S. H. and El-Nekeety, A. A. (2008):** Inula crithmoides extract protects against ochratoxin A-induced oxidative stress, clastogenic and mutagenic alterations in male rats. Toxicon, 52: 566–573.
2. **Abdel-Wahhab, M.A., Abdel-Galil, M.M. and Ellithey, M.M. (2005):** Melatonin counteracts oxidative stress in rats fed ochratoxin A-contaminated diet. J. Pineal Res., 38: 130–135.
3. **Adler, M., Müller, K., Rached, E., Dekant, W. and Mally, A. (2009):** Modulation of key regulators of mitosis linked to chromosomal instability is an early event in ochratoxin A carcinogenicity. Carcinogenesis, 30 (4): 711–719.
4. **Agnes, V.F. and Akbarsha, M.A. (2003):** Spermatotoxic effect of aflatoxin B1 in albino mouse. Food and Chemical Toxicology, 41: 119–130.
5. **Alvarez, J.G., Touchstone, J.C., Blascol, J.C. and Storey B.T. (1987):** Spontanous lipid peroxidation and hydrogen peroxide and superoxide in human spermatozoa: superoxide dismutase as major enzyme protectant against oxygen toxicity. J. Androl., 18: 338- 348.
6. **Arbillaga, L., Azqueta, A., Ezpeleta, O. and de Cerain, A.D. (2007):** Oxidative DNA damage induced by ochratoxin A in the HK-2 human kidney cell line: evidence of the relationship with cytotoxicity. Mutagenesis, 22: 35–42.
7. Baldi, A., Losio, M.N., Cheli, F., Rebucci, R., Sangalli, L., Fusi, E., Bertasi, B., Pavoni, E.
8. **Carli, S.and Politis, I. (2004):** Evaluation of the protective effects of alpha-tocopherol and retinol against ochratoxin A cytotoxicity. Br. J. Nutr., 91: 507–512.
9. **Bengmark, S. and Martindale, R. (2005):** Prebiotics and synbiotics in clinical medicine. Nutr. Clin. Pract., 20: 244–261.
10. **Bennet, J.W. and Klich, M. (2003**): Mycotoxins. Clinical Microbiolgy Reviews, 16:497-498.
11. Beutler, E., Dubon O.B. and Kelly, M. (1963): Improved method for the determination of blood glutathione. J. Lab. Clin. Med., 61:882–888.
12. **Biro, K., Barna-Vetro, I., Pecsi, T., Szabo, E., Winkler, G., Fink-Gremmels, J. and Solti, L. (2003)**: Evaluation of spermatological parameters in ochratoxin A-challenged boars. Theriogenology, 60: 199-207.
13. **Bose, S. and Sinha, S.P. (1994):** Modulation of ochratoxin-produced genotoxicity in mice by vitamin C. Food Chem. Toxicol., 32: 533–537.
14. Brown, A.L., Odell, E.W. and Mantle, P.G. (2007): DNA ploidy distribution in renal tumours induced in male rats by dietary ochratoxin A. Exp. Toxicol. Pathol., 59: 85–95.
15. **Bruno-Barcena, J.M., Andrus, J.M., Libby, S.L., Klaenhammer, T.R. and Hassan, H.M., (2004):** Expression of a heterologous manganese superoxide dismutase gene in intestinal lactobacilli provides protection against hydrogen peroxide toxicity. Appl. Environ. Microbiol., 70: 4702–4710.
16. **Burns, A.J.R and Rowland, L.R. (2004**): Anti-carcinogenicity of probiotics and prebiotics on faecal water-induced DNA damage in human colon adenocarcinoma cells. *Mutat. Res.*, 551: 233-243.
17. **Caldini, G., Trotta, F., Villarini, M., Moretti, M., Pasquini, R., Scassellati-Sforzolini, G. and Cenci, G. (2005):** Screening of potential lactobacilli antigenotoxicity by microbial and mammalian cell-based tests. Int. J. Food Microbiol., 102: 37–47.
18. **Castella, G., Larsen, T.O., Cabanes, J., Schmidt, H,, Alboresi, A., Niessen, Farber, L.P. and Geisen, R.(2002)** Molecular characterization of ochratoxin A producing strains of the genus Penicillium. Syst. Appl. Microbiol., 25:74–83.
19. **Cavin, C., Delatour, T., Marin-Kuan, M., Holzhauser, D., Higgins, L., Bezencon, C., Guignard, G., Junod, S., Piguet, D., Richoz-Payot, J., Gremaux, E., Hayes, J.D., Nestler, S., Mantle, P. and Schilter, B. (2007):** Reduction in antioxidant defences may contribute to ochratoxin A toxicity and carcinogenicity. Toxicol. Sci., 96 (1): 30–39.
20. **Chakraborty, D. and Verma, R. (2009):** Spermatotoxic effect of ochratoxin and its amelioration by *emblica officinalis* aqueous extract Acta Poloniae Pharmaceutica. Drug Research, 66 (6): 689-695.
21. **Chen, C.C., Louie, S., Shi, H.N. and Walker, W.A., (2005**): Preinoculation with the probiotic Lactobacillus acidophilus early in life effectively inhibits murine Citrobacter rodentium colitis. Pediatr. Res., 58:1185–1191.
22. **Chitra, K.C., Latchoumycandane, C. and Mathur, P.P. (2003):** Induction of oxidative stress by bisphenol A in the epididymal sperm of rats. Toxicology, 185: 119–127.
23. **Cosimi, S., Orta, L., Mateos, S. and Felipe Cortes, F. (2009):** The mycotoxin ochratoxin A inhibits DNA topoisomerase II and induces polyploidy in cultured CHO cells Toxicology in Vitro 23: 1110–1115.
24. **Degen, G.H. (1997):** Induction of micronuclei with ochratoxin A in ovine seminal vesicle cell cultures. Archives in Toxicology, 71:365-371.
25. **Del Prete, V., Rodríguez, H., Carrascosa, A. V., De Las Rivas, A., García-Moruno, E., and Muñoz, R. (2007):** In vitro removal of ochratoxin A by wine lactic acid bacteria. Journal of Food Protection, 9: 2155–2160.
26. **Dönmez-Altuntas, H., Hamurcu, Z., Imamoglu, N., and Liman, B.C. (2003**): Effects of ochratoxin A on micronucleus frequency in human lymphocytes. Nahrung, 47(1):33.
27. D**oorten, S.Y., Bull, S., Van Der Doelen, M.A. and Fink-Gremmels, J. (2004):** Metabolism-mediated cytotoxicity of ochratoxin A. Toxicol. In Vitro, 18 (3): 271–277.
28. **Dopp, E., Muller, J., Hahnel, C., and Schiffmann, D. (1999):** Induction of genotoxic effects and modulation of the intracellular calcium level in Syrian hamster embryo (SHE) fibroblasts caused by ochratoxin A. Food and Chemical Toxicology, 37(7):713-721.
29. **Ehrlich, V., Darroudi, F., Uhl, M., Steinkellner, H., Gann, M., Majer, B.J., Eisenbauer, M., and Knasmüller, S. (2002):** Genotoxic effects of

ochratoxin A in human-derived hepatoma (HepG2) cells. Food and Chemical Toxicology, 40:1085.

30. **El Golli-Bennour, E., Kouidhi, B., Bouslimi, A., Abid-Essefi, S. Hassen, W. and Bacha, H. (2010):** Cytotoxicity and Genotoxicity Induced by Aflatoxin B1, Ochratoxin A, and Their Combination in Cultured Vero Cells. J Biochem Molecular Toxicology, 24: 1, 42-50.
31. **El-Nezami, H., Kankaanpää, P., Salminen, S., and Ahokas, J. (1998):** Ability of dairy strains of lactic acid bacteria to bind a common food carcinogen, aflatoxin B1. Food and Chemical Toxicology, 36: 321–326.
32. **El-Nezami, H., N. Polychronaki, S. Salminen, and H. Mykkänen. (2002):** Binding rather than metabolism may explain the interaction of two food grade Lactobacillus strains with zearalenone and its derivative b-zearalenol. Appl. Environ. Microbiol., 68:3545–3549.
33. **Evans, E.P. Breckon, G. and Ford, C.E. (1964):** An air-drying method for meiotic preparations for mammalian testes. Cytogenetics, 3: 289–294.
34. **Ezz El-Arab, A., Girgis, S., Hegazy, E. and Abd El-Khalek, A. (2006):** Effect of dietary honey on intestinal microflora and toxicity of mycotoxins in mice. BMC Complementary and Alternative Medicine, 6:6.
35. **Fenske, M. and Fink-Gremmels, J. (1990**): Effects of fungal metabolites on testosterone secretion *in vitro*. Arch Toxicol., 64: 72–75.
36. F**öllmann, W., Behm, C. and Degen, G.H. (2007):** Induction of micronuclei by ochratoxin A is a sensitive parameter of its genotoxicity in cultured cells. Mycotoxin Research, 23 (2): 101-109.
37. **Fuchs, S., Sontag, G., Stidl, R., Ehrlich, V., Kundi, M., and Knasmüller, S. (2008):** Detoxification of patulin and ochratoxin A, two abundant mycotoxins, by lactic acid bacteria. Food and Chemical Toxicology, 46: 1398–1407.
38. **Gautier, J., Richoz, J., Welti, D.H., Markovic, J., Gremaud, E., Guengerich, F.P., and Turesky, R.J. (2001):** Metabolism of ochratoxin A: Absence of formation of genotoxic derivatives by human and rat enzymes. *Chem. Res. Toxicol.*, 14 (1): 34.
39. **Gekle, M., Sauvant, C. and Schwerdt, G. (2005):** Ochratoxin A at nanomolar concentrations: a signal modulator in renal cells. Mol. Nutr. Food Res., 49:118–130.
40. **Gharbi, A., Trillon, O., Betbeder, A.M., Counord, J., Gauret, M.F., Pfohl-Leszkowicz, A., Dirheimer, G. and Creppy, E.E. (1993):** Some effects of ochratoxin A, a mycotoxin contaminating feeds and food, on rat testis. Toxicology, 83: 9–18.
41. **Gomez, E., Buckingham, D.W., Brindle, J., Lanzafame, F., Irvine, D.S., Aitken, R.J. (1996):** Development of an image analysis system to monitor the retention of residual cytoplasm by human spermatozoa: correlation with biochemical markers of the cytoplasmic space, oxidative stress, and sperm function. J Androl., 17: 276-87.
42. **Gratz, S., Täubel,M., Juvonen, R.O., Viluksela, M., Turner, P.C., Mykkänen, H., El- Nezami, H. (2006)**: Lactobacillus rhamnosus strain GG modulates intestinal absorption, fecal excretion, and toxicity of aflatoxin B1 in rats. Appl. Environ. Microbiol., 72:7398–7400.
43. **Han, W. (2004):** Improvement of an experimental colitis in rats by treatment with lactic acid bacteria producing superoxide dismutase. Am. Gastroenterol. Associat., 577.
44. **Han, W., Mercenier, A., Ait-Belgnaoui, A., Pavan, S., Lamine, F., Van, S., Ii, Kleerebezem, M., Salvador-Cartier, C., Hisbergues, M., Bueno, L., Theodorou, V., Fioramonti, J., (2006):** Improvement of an experimental colitis in rats by lactic acid bacteria producing superoxide dismutase. Inflamm. Bowel. Dis., 12: 1044–1052.
45. **Hohler, D. (1998):** Ochratoxin A in food and feed: occurrence, legislation and mode of action. Z. Ernahrungswiss, 37: 2–12.
46. **Hsieh, Y.Y., Chang, C.C. and Lin, C.S. (2006):** Seminal malondialdehyde concentration but not glutathione peroxidase activity is negatively correlated with seminal concentration and motility. Int. J. Biol. Sci., 2: 23–29.
47. **IARC (1993)**: Ochratoxin A In: IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Some Naturally Occurring Substances: Food Items and Constituents, Hetrocyclic Aromatic Amines and Mycotoxins. Vol. 56, Lyon: IARC, 489-521.
48. **IPCS (International Programme on Chemical Safety) (2001):** Safety Evaluation of Certain Mycotoxins in Food, WHO Food Additives Series 47. World Health Organization, Geneva.
49. **Kamp, H.G., Eisenbrand, G., Janzowski, C., Kiossev, J., Latendresse, J.R., Schlatter, J. and Turesky, R.J., (2005**): Ochratoxin A induces oxidative DNA damage in liver and kidney after oral dosing to rats. Mol. Nutr. Food Res., 49: 1160–1167.
50. **Klinder, A., Forster, A., Caderni, G., Femia, A.P. and Pool-Zobel, B.L., (2004):** Fecal water genotoxicity is predictive of tumor-preventive activities by inulin-like oligofructoses, probiotics (Lactobacillus rhamnosus and Bifidobacterium lactis), and their synbiotic combination. Nutr. Cancer, 49: 144–155.
51. **Knasmüller, S., Steinknéllner, H., Hirschl, A.M., Rabot, S., Nobis, E.C. and Kassle, F. (2001)**: Impact of bacteria in dairy products and of intestinal microflora on the genotoxic and carcinogenic effects of heterocyclic aromatic amines. Mut. Res., 480: 129–138.
52. **Koller, V. J., Marian, B., Stidl, R., Nersesyan, A.,Winter, H., Simic´, T., Sontag, G., and Knasmüller, S. (2008):** Impact of lactic acid bacteria on oxidative DNA damage in human derived colon cells. Food and Chemical Toxicology, 46: 1221–1229.
53. **Krishna, G. and Hayashi, M. (2000**): *In vivo* rodent micronucleus assay: protocol, conduct and data interpretation. Mutation Res., 455: 155–166.
54. **Kullisaar, T., Songisepp, E., Mikelsaar, M., Zilmer, K., Vihalemm, T., Zilmer, M., (2003):** Antioxidative probiotic fermented goats' milk decreases oxidative

stress-mediated atherogenicity in human subjects. Br. J. Nutr., 90: 449–456.

55. **Lee, J., Hwang, K.T., Heo, M.S., Lee, J.H., Park, K.Y. (2005):** Resistance of Lactobacillus plantarum KCTC 3099 from Kimchi to oxidative stress. J. Med. Food, 8: 299–304.
56. **Lin, M.Y. and Chang, F.Y. (2000)**: Antioxidative effect of intestinal bacteria Bifidobacterium longumATCC15708 and Lactobacillus acidophilus ATCC 4356. Dig. Dis. Sci., 45:1617–1622.
57. **Lioi, M.B., Santoro, A., Barbieri, R., Salzano, S. and Ursini, M.V. (2004**): Ochratoxin A and zearalenone: a comparative study on genotoxic effects and cell death induced in bovine lymphocytes. Mutation Res., 557:19–27.
58. **Lodish, H., Berk A., Kaiser, C.A., Kreiger M., Scott, M.P., Zipursky, S.L. and Derreli J. (2003):** Molecular Cell Biology. Scientific American Books, Freeman and Company, New York 5.
59. **Mally, A., Pepe, G., Ravoori, S., Fiore, M., Gupta, R., Dekant, W. and Mosesso, P. (2005):** Ochratoxin A causes DNA damage and cytogenetic effects but no DNA adducts in rats. Chem. Res. Toxicol., 18: 1253–1261.
60. **Marin-Kuan, M., Nestler, S., Verguet, C., Bezencon, C., Piguet, D., Mansourian, R., Holzwarth, J., Grigorov, M., Delatour, T., Mantle, P., Cavin, C. and Schilter, B. (2006**): A toxicogenomics approach to identify new plausible epigenetic mechanisms of ochratoxin A carcinogenicity in rat. Toxicol. Sci., 89 (1): 120–134.
61. **Marklund, S., and Marklund, G., (1974)**: Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur. J. Biochem., 47: 469–474.
62. **Mcgarr, S.E., Ridlon, J.M. and Hylemon, P.B., (2005):** Diet, anaerobic bacterial metabolism, and colon cancer: a review of the literature. J. Clin. Gastroenterol., 39: 98–109.
63. **Meki, A. and Hussein, A. (2001):** Melatonin reduces oxidative stress induced by ochratoxin A in rat liver and kidney. Comparative Biochemistry and Physiology Part *C*, 130: 305.
64. **Mateo, E.M. Medina, A., Mateo, F., Francisco M. Valle-Algarra, Pardo, I. Jiménez, M. (2010):** Ochratoxin A removal in synthetic media by living and heat-inactivated cells of Oenococcus oeni isolated from wines. Food Control, 21: 23–28.
65. **Mirasoli, M., Roda, A., Montagnani, M., Azzaroli, F. and Roda, E. (2002):** Cholic acid metabolism in human fecal cultures during diet supplementation with Lactobacillus rhamnosus GG. Minerva. Gastroenterol. Dietol., 48: 45–49.
66. **Mosesso, P., Cinelli, S., Pinero, J., Belacima, R. and Pepe, G. (2008):** In vitro cytogenetic results supporting a DNA nonreactive mechanism for ochratoxin A, potentially relevant for its carcinogenicity. Chem. Res. Toxicol., 21: 1235–1243.
67. **Ohkawa, H., Ohishi, N. and Yagi, K., (1979):** Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical Biochemistry, 95: 351–358.
68. **Ouwehand, A.C., Salminen, S. and Isolauri, E. (2002):** Probiotics: an overview of beneficial effects. Antonie Van Leeuwenhoek, 82: 279-89.
69. **Ozcelik, N., Soyoz, M. and Kilink, I. (2004):** Effects of ochratoxin A on oxidative damage in rat kidney: protective role of melatonin. J. Appl. Toxicol., 24: 211–215.
70. **Palma, N., Cinelli, S., Sapora, O.,Wilson, S. H. and Dogliotti, E. (2007):** Ochratoxin A-induced mutagenesis in mammalian cells is consistent with the production of oxidative stress. Chem. Res. Toxicol., 20: 1031 –1037.
71. **Pardo, E., Marín, S., Ramos, A.J. and Sanchis, V. (2004**): Occurrence of ochratoxigenic fungi and ochratoxin a in green coffee from different origins. Food Science and Technology International, 10: 45-49.
72. **Petrik, J., Zanic-Grubisic, T., Barisic, K., Pepeljnjak, S., Radic, B., Ferencic, Z., and Cepelak, I. (2003)**: Apoptosis and oxidative stress induced by ochratoxin A in rat kidney. Archives in Toxicology, 77:685.
73. **Petzinger E. and Ziegler, K. (2000**): Ochratoxin A from a toxicological perspective. J. Vet. Pharmacol. Therap., 23: 91.98.
74. **Pfohl-Leszkowicz, A. and Manderville, R.A. (2007**): Ochratoxin A: an overview on toxicity and carcinogenicity in animals and humans. Mol Nutr Food Res., 51:61–99.
75. **Pfohl-Leszkowicz, A., Grosse, Y., Kane, A., Gharbi, A., Baudrimont, I., Obrecht, S., Creppy, E.E. and Dirheimer, G. (1993**): Is the oxidative pathway implicated in the genotoxicity of ochratoxin A? In: Creppy, E.E., Castegnaro, M., Dirheimer, G. (Eds.):, Human Ochratoxicosis and its Pathologies, vol. 231 Colloque INSERM/John Libbey Eurotext Ltd., 177–187.
76. **Pierides M., El-Nezami H., Peltonen K., Salminen S. and Ahokas J., (2000**): Ability of dairy strains of lactic acid bacteria to bind aflatoxin M1 in a food model. J. Food Prot., 63; 645-650.
77. **Piotrowska, M., and Zakowska, Z. (2005):** The elimination of ochratoxin A by lactic acid bacteria strains. Polish Journal of Microbiology, 4: 279–286.
78. **Pool-Zobel, B.L., Munzner, R.and Holzapfel, W.H. (1993):** Antigenotoxic properties of lactic acid bacteria in the S. typhimurium mutagenicity assay. Nutr. Cancer, 20: 261–270.
79. **Rached, E., Hard, G.C., Blumbach, K., Weber, K., Draheim, R., Lutz, W.K., Ozden, S., Steger, U., Dekant, W. and Mally, A. (2007):** Ochratoxin A: 13-week oral toxicity and cell proliferation in male F344/n rats. Toxicol. Sci., 97; 288–298.
80. **Rafter, J. (2004**): The effects of probiotics on colon cancer development. Nutr. Res. Rev., 17: 277–284.
81. **Ringot, D., Chango, A., Schneider, Y. and Larondelle, Y. (2006**): Toxicokinetics and toxicodynamics of ochratoxin A, an update. Chem. Biol. Interact., 159: 18 –46.
82. **Robbiano L., Baroni, D., Carrozzino, R., Mereto, E. and Brambilla, G. (2004)**: DNA damage and

micronuclei induced in rat and human kidney cells by six chemicals carcinogenic to the rat kidney. Toxicology, 204: 187 –195.
83. **Russo, A. (2000)**: *In vivo* cytogenetics: Mammalian germ cells. Mutation Res., 455: 167–189.
84. **Sage, L., Garon, D. and Seigle-Murandi, F. (2004):** Fungal microflora and ochratoxin risk in French vineyards. J. Agric Food Chem., 52: 5764–5768.
85. **Saradha, B. and Mathur, P.P. (2006):** Induction of oxidative stress by lindane in epididymis of adult male rats. Environ. Toxicol., Pharmacol., 22: 90–96.
86. **Saxelin, M., Tynkkynen, S., Mattila-Sandholm, T. and De Vos, W.M. (2005):** Probiotic and other functional microbes: from markets to mechanisms. Curr. Opin. Biotechnol., 16: 204–211.
87. **Schaaf, G.J., Nijmeijer, S.M., Maas, R.F., Roestenberg, P., de Groene, E.M. and Fink-Gremmels, J. (2002**): The role of oxidative stress in the ochratoxin A-mediated toxicity in proximal tubular cells. Biochim. Biophys. Acta, 1588 (2): 149–158.
88. **Schultz, M., Linde, H.J., Lehn, N., Zimmermann, K., Grossmann, J., Falk, W. and Scholmerich, J., (2003)**: Immunomodulatory consequences of oral administration of Lactobacillus rhamnosus strain GG in healthy volunteers. J. Dairy Res., 70: 165–173.
89. **Schwartz, G.G. (2002**): Hypothesis: Does ochratoxin A cause testicular cancer? Cancer Causes and Control, 13: 91-100.
90. **Solti, L., Pecs, T., Betro-Barna, I., Szasz, F., Biro, K. and Azabo, E. (1999):** Analysis of serum and seminal plasma after feeding ochratxoin A with breeding boars. Anim Reprod Sci., 28: 123–132.
91. **Songisepp, E., Kals, J., Kullisaar, T., Mandar, R., Hutt, P., Zilmer, M. and Mikelsaar, M., (2005):** Evaluation of the functional efficacy of an antioxidative probiotic in healthy volunteers. Nutr. J., 4: 22.
92. **Soyöz, M., Ozcelik, N., Kilinc, I. and Altantas I. (2004)**: The effects of ochratoxin A on lipid peroxidation and antioxidant enzymes: a protective role of melatonin. Cell Biol Toxico., 20:213–219.
93. **Stidl, R., Fuchs, S., Koller, V., Marian, B., Sontag, G., Ehrlich, V. and Knasmueller, S. (2007):** DNA-protective properties of lactic acid bacteria. In: Durackova, Z., Slamenova, D. (Eds.), Synthetic and Natural Compounds in Cancer Therapy and Prevention. Bratislava, Slovakia.
94. **Tavan, E., Cayuela, C., Antoine, J.M., Trugnan, G., Chaugier, C. and Cassand, P. (2002**): Effects of dairy products on heterocyclic aromatic amine-induced rat colon carcinogenesis. Carcinogenesis, 23; 477–483.
95. **Vaca, C.E., Wilhelm, J., Hartwig, A. and Harms-Ringdahl, M. (1998):** Interaction of lipid peroxidation products with DNA: A review. Mutat. Res., 195: 137–149.
96. **Wallace, T.D., Bradley, S., Buckley, N.D. and Green-Johnson, J.M. (2003):** Interactions of lactic acid bacteria with human intestinal epithelial cells: effects on cytokine production. J. Food Prot. 66:466–472.
97. **Wollowski, I., Rechkemmer, G. and Pool-Zobel, B.L. (2001**): Protective role of probiotics and prebiotics in colon cancer. Am. J. Clin. Nutr., 73: 451S–455S.
98. **Wyrobek, A. J. and Bruce, W. R. (1975):** Chemical induction of sperm abnormalities in mice Proc. Nat. Acad. Sci. USA, 72 (11): 4425-4429.
99. **Yan, F. and Polk, D.B. (2002**): Probiotic bacterium prevents cytokine-induced apoptosis in intestinal epithelial cells. J. Biol .Chem., 277:50959–50965.
100. **Yan, F., and Polk, D.B. (2006):** Probiotics as functional food in the treatment of diarrhea. Curr. Opin. Nutr. Metab. Care, 9:717–721.

Corresponding Authors:
1- faragimdiab@yahoo.com
2- somaianada@yahoo.com

9/6/2010

Study of Risk Factors Involved in the Progression of Non Alcoholic Fatty Liver Disease in Egyptian Patients

Elsayed A. Wasfy[1], Nadia M. Elwan[*1], Shreif L. Bayomi[2], Thanaa F. El- Sheikh[3], Sahar A. El-yamani[1] and Boshra E. Talha[1]

Tropical Medicine[1], Bathology[2] and Biochemistry[3] Departments, Tanta University, Tanta, Egypt
*nadiaelwan@yahoo.com

Abstract: Nonalcoholic fatty liver disease (NAFLD) includes hepatic steatosis, nonalcoholic steatohepatitis (NASH), fibrosis, and cirrhosis .NAFLD has also the potential to progress to hepatocellular carcinoma (HCC) or liver failure. NAFLD is linked to obesity, insulin resistance, hyperlipidaemia and genetic factors. Our objective was to study the risk factors that involved in the progression of non alcoholic fatty liver disease. Subjects and methods: Thirty-three patients and ten healthy controls were included in our study. Patients were classified into 3 groups. Group I included12 patients with simple liver steatosis. Group II included 11 patients with NASH. Group III included 10 patients with cirrhosis most probably a late sequel of NASH. Results: BMI, fasting blood glucose, insulin and HOMA-IR were significantly higher in patients with fatty liver, NASH and cirrhosis, also, NASH patients showed a significant high serum triglycerides and ALT. All previous parameters were significantly increased with the increased severity of histopathological score in patients with fatty liver and NASH. Serum AST levels and AST / ALT ratio were significantly increased in NASH and cirrhotic patients as compared to patients with steatosis alone and controls. Mitochondrial ATP levels in patients with fatty liver and NASH showed a statistically significant decrease. Also patients with NASH showed a statistically significant decrease when compared to patients with fatty liver. Finally, patients with fatty liver and NASH showed a significant decrease in mitochondrial ATP with increased BMI and histopathological score. Conclusion: Increased BMI, hyperglycemia, hypertriglyceridaemia, insulin resistance and depletion of mitochondrial ATP in hepatocytes can be considered risk factors involved in the development and progression of fatty liver to NASH and cirrhosis.
[Elsayed A. Wasfy, Nadia M. Elwan, Shreif L. Bayomi, Thanaa F. El- Sheikh, Sahar A. El-yamani and Boshra E. Talha. Study of Risk Factors Involved in the Progression of Non Alcoholic Fatty Liver Disease in Egyptian Patients. Journal of American Science 2010;6(12):588-596]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: BMI, insulin resistance, mitochondrial ATP, NAFLD

1. Introduction:

Nonalcoholic fatty liver disease (NAFLD) is a significant health problem and affects 70 million adults in the United States. NAFLD occurs in up to 53% of obese children [(1)]

NAFLD is defined as an excess of fat in the liver in which at least 5% of hepatocytes display lipid droplets that exceed 5% - 10% of liver weight in patients who do not consume significant amounts of alcohol. [(2)]

Non alcoholic fatty liver disease refers to a spectrum of diseases of the liver ranging from steatosis (i.e., fatty infiltration of the liver), nonalcoholic steatohepatitis (NASH) (i.e., steatosis with inflammation and hepatocyte necrosis) to cirrhosis [(3).] Nonalcoholic steatohepatitis (NASH) is defined as necroinflammatory disorder with fatty infiltration of hepatocytes, this term is employed when steatohepatitis occurs in individuals whose alcohol consumption is nil or negligible i.e less than 20g ethanol/day in women and less than 40g in men.[(4).]

Nonalcoholic fatty liver disease, NAFLD is now considered a metabolic pathway to advanced liver disease; cirrhosis and hepatocellular carcinoma but hepatic steatosis without concomitant inflammation or fibrosis usually considered a benign condition.[(5)]

Two types of NASH exist: primary NASH (which is associated with metabolic syndrome–related conditions, such as insulin resistance, obesity, type -2 diabetes, and hyperlipidemia) and secondary NASH (which occurs after obesity-related intestinal surgery, rapid weight loss in the obese, total parenteral nutrition, treatment with drugs such as amiodarone or corticosteroids, lipodystrophy, or Wilson's disease). Many aspects of the disease are common to both presentations.[(6)]

Hepatic steatosis can be reversible or progress to NASH depending on the cessation or persistence of the underlying provocative cause respectively.[(7)]

The first step of the pathophysiology of nonalcoholic steatohepatitis is the lipid accumulation in the liver causing steatosis. This increases the sensitivity of the liver to injury, inflammation and fibrosis.[8]

The second step involves the cytokines and other factors causing oxidative stress and lipid peroxidation, in time leading to steatohepatitis.[9]

Our objective were studying the risk factors such as obesity, insulin resistance, type -2 diabetes, hyperlipidemia and mitochondrial ATP, that involved in the progression of non alcoholic fatty liver disease.

2. Subjects and methods:

All patients were selected from inpatient and outpatient clinics of Tropical Medicine and Infectious Diseases Department and Surgery Department of Tanta University Hospital in the period from August 2004 to January 2008.

This study was conducted on thirty-three patients and ten healthy controls, subjects were divided into the following groups:-

***Group I*:** Included (12) patients with steatosis.

Group II: Included (11) patients with NASH.

Group III: Included (10) patients with cirrhosis most probably a late sequel of NASH;

Diagnosis of this group was based on history of fatty liver and absence of history of any chronic liver disease, drugs causing NAFLD, abdominal ultrasonography, negative viral markers for HBV and HCV and negative laboratory tests of autoimmune hepatitis, primary biliary cirrhosis, Wilson's disease, alcoholic liver diseases, and haemochromatosis.

Group IV: Included (10) normal healthy individuals as a control group.

All patients were subjected to full history and clinical examination, body mass index (BMI), was calculated by the weight in kilograms divided by the square of the height in meters. (BMI; kg/m2), ultrasonography, urine and stool analysis, biochemical tests including complete blood count, lipid profile, liver function tests. Two-hour a 75-g oral glucose tolerance test (OGTT) was done for patients not known to have diabetes mellitus and measurement of level of fasting insulin and glucose in the blood during the test, insulin resistance was calculated using the homeostasis assessment model (HOMA-IR)) on the basis of fasting glucose and fasting insulin. HOMA-IR was calculated using the following equation: -

[Fasting glucose (mg/dl) x fasting insulin (µU/ml)] /405.

A HOMA-IR greater than 2.0 is considered to indicate the presence of insulin resistance.

Serum in sulin level was measured using kit IMMULITE 2000 Insulin which is a solid-phase, two-site chemiluminescent immunometric assay.

Serological tests for HBV; HBsAg and HBcAb and HCV; HCV Ab.

liver biopsy was done for group I and group II. Liver biopsies of controls were obtained intraoperatively from surgery department from individuals admitted for cholecystectomy. Each liver biopsy was examined for steatosis, inflammation, fibrosis and mitochondrial ATP level was estimated.

Different histological parameters were evaluated including steatosis, lobular inflammation, ballooning degeneration, and pericellular fibrosis, portal/septal fibrosis.

Histological grading of steatosis was done according to kleiner et al. (2005)[10]:

The main histological features commonly described in NALFD/NASH including:- Steatosis,inflammation (portal and lobular), hepatocyte ballooning and fibrosis .

Histological criteria of NASH were based on steatosis (≥5 % of lobular hepatocytes affected) and two of the following three: lobular inflammation, ballooning degeneration, and pericellular fibrosis.

The main histological features were scored according to the scoring system for NAFLD, recently developed by Kleiner et al.(2005)[10]; NAS-II (NASH activity score) (NASH clinical research network revision).

NASH activity score; is defined as the sum of the scores for steatosis (0–3), lobular inflammation (0 - 3) and ballooning degeneration (0–2).

Scores therefore ranged from 0 to 8. Cases with NAS- II of 0 to 2 were considered not diagnostic of NASH.

Finally, ATP concentration of mitochondrial suspension was measured by luminometer using commercial kits.

3. Results

BMI, fasting blood glucose and insulin and HOMA-IR were significantly higher in patients with fatty liver, NASH and cirrhosis (Fig,1,2,3,4) also, NASH patients showed a significant high serum triglycerides (Table,1) and ALT. histological examination of group I and group II is illustrated in (Table 2, 3)

According to score of Kleiner, et al, (2005)[10]; NAS-II (NASH activity score) (NASH clinical research network revision), the results were as follows:

Group I: 41.7% of patients had S1; steatosis

< 33%, 50% had S2; steatosis 33%-66% and 8.3% had S3; steatosis > 66%. All fatty infiltration was macrovesicular except one patient had mixed macrovesicular and microvesicular.

Group II: 18.2% of patients had score 3, 54.5% of patients had score 5, 18.2% of patients had score7 and 9.1% of patients had score 8.

Fibrosis was scored as 0 (no fibrosis) in 18.2% of patients, 1 (Perisinusoidal fibrosis) in 27.3% of patients, 2 (Perisinusoidal and portal / periportal fibrosis) in 18.2% of patients and 3 (Bridging fibrosis) in 36.3% of patients

All previous parameters were significantly increased with increase severity of histopathological score in patients with fatty liver and NASH. (Table,4,5).

Serum AST levels and AST / ALT ratio were significantly increased in NASH and cirrhotic patients as compared to patients with steatosis alone and controls.

Mitochondrial ATP levels in patients with fatty liver and NASH showed a statistically significant decrease. Also patients with NASH showed a statistically significant decrease when compared to patients with fatty liver (Table 6). Finally, patients with fatty liver and NASH showed a significant decrease in mitochondrial ATP with increase BMI and histopathological score. (Table 7).

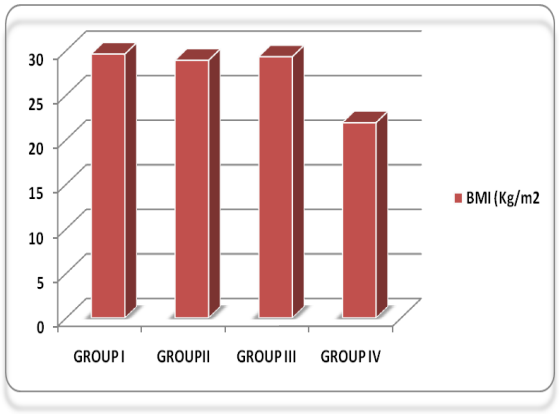


Fig. (1) : BMI (kg/m^2) of the studied groups.

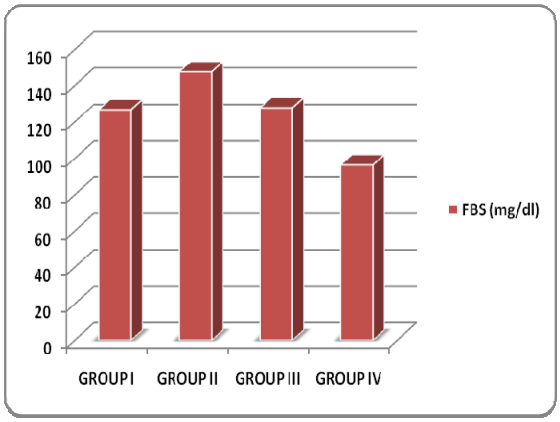


Fig.(2): Fasting blood glucose (mg/dl) in the studied groups

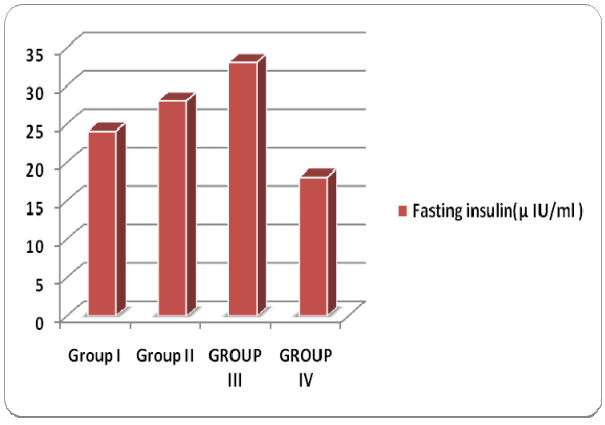


Fig. (3): Fasting blood insulin (µ IU/ ml) in the studied groups

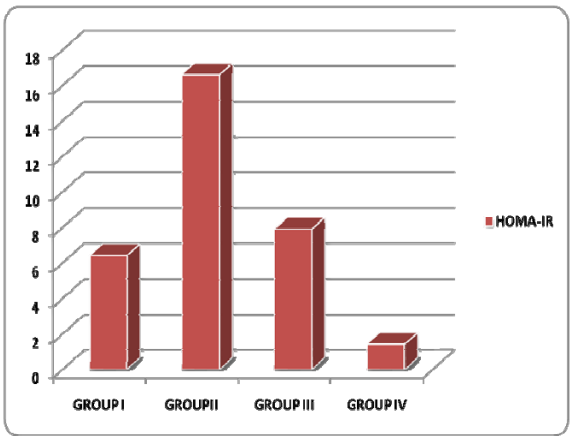


Fig. (4): HOMA-IR in the studied groups

Table (1): Results of lipid profile in the studied groups

	Group I (n=12)		Group II (n=11)		Group III (n=10)		Control Group (n=10)		P value
	Range	M±SD	range	M±SD	range	M±SD	range	M±SD	
Cholesterol (mg/dl)	147-196	160.5± 27.4	133-201	163.9 ± 27.2	106-188	161.4± 23.6	117-148	164.8± 21.5	> 0.05
HDL (mg/dl)	43-51	45.58± 3.15	48-52	46.18 ± 4.85	40-53	49.9 ± 5.74	43-64	50.5± 6.4	> 0.05
Triglyceride (mg/dl)	126-240	201± 18.43	138-261	*214± 43.9	145-257	148.4± 18.75	134-150	141.6± 8.7	<0.05 a >0.05

a: GII vs GIII, GIV. P is significant < 0.05 * *significant* .

Table (2) : Histopathological score in group I and group II

	Histopathological score in group I (n=12)					
	0	1	2	3	-	-
Number of patients	0	5	6	1	-	-
%	0	41.7%	50%	8.3%	-	-
	Histopathological score in group II(n=11)					
	3	4	5	6	7	8
Number of patients	2	0	6	0	2	1
%	18.2%	0	54.5%	0	18.2%	9.1%

Table (3): Liver fibrosis score in group II

	Score of liver fibrosis				
	0	1	2	3	4
Number of patients	2	3	2	4	0
%	18.2%	27.3%	18.2%	36.3%	0

0: None
1 : Perisinusoidal or periportal
2: Perisinusoidal and portal / periportal 3: Bridging fibrosis
4: cirrhosis

Table (4): Correlation between body mass index (BMI) (kg/m^2) and histopathological score of group I and group II

	BMI (kg/m^2)			
	Group I (n= 12)		Group II (n= 11)	
	r	P	r	P
NAS-II (NASH activity score)	0.619	0.0308*	0.653	0.026*
Fibrosis score	-	-	0.717	0.011*

Table (5): Correlation between fasting blood glucose; (mg/dl), fasting blood insulin (µIU/L) &HOMA-IR and histopathological and fibrosis scores of group I and group II

	Histopathological score (NAS-II)				*Fibrosis score*	
	Group I (n=12)		Group II (n=11)		Group II (n=11)	
	r	P	r	P	r	P
Fasting blood glucose (mg/dl)	0.741	0.004*	0.754	0.005*	0.554	0.07
Fasting blood insulin (µIU/L)	0.574	0.043*	0.674	0.02*	0.578	0.055
HOMA -IR	0.853	0.0004*	0.761	0.0065*	0.595	0.043*

*significant NAS-II: (NASH activity score-II)

Table (6): Results of mitochondrial ATP (n mol /mg protein) in group I, group II and group IV

	Group I (n=12)	Group II (n=11)	Control group (n=10)
Range	13.7-21.8	14.3-18.1	19.2-23.2
Mean ±SD	17.76 ± 2.25*	14.25 ± 1.49*	22.14 ± 1.67*
Significance	*S(P-value < 0.05) a,b		

ATP: Adenosine triphosphate
**significant a: GI,GII vs IV b: GII vs G I*

Table (7): Correlation between mitochondrial ATP (n mol /mg protein) and BMI in patients of Group I & Group II

	Mitochondrial ATP (n mol /mg protein)			
	Group I (n=12)		Group II (n=11)	
	r	P	r	P
BMI (kg/m^2)	**-0.634**	**0.024***	**-0.141**	**0.013***

4. Discussion:

In our study, patients with fatty liver, NASH and cirrhosis showed a significant higher BMI as compared to control group. This finding agrees with Wanless and Lentz (1990) [(11)], Abhasnee Sobhonslidsuk et al, (2007) [(12)] and Fabbrini E et al,(2009[(13)] who reported that, obesity was found in 40%-100% of fatty liver. Fatty liver has been documented in up to 70% to 80% of obese individuals, also Clark and Diehl (2003) [(14)], who reported that patients with steatosis and NASH had elevated BMI.

Patients with NASH and steatosis alone showed increased serum triglycerides with significant high serum triglycerides in NASH patients as compared to control group and patients with cirrhosis.

Our findings were in agreement with Ender Sern et al, (2002) [(15)] and Joong-Won; et al, (2007) [(16)] who reported that serum triglyceride did not show significant differences between steatosis and NASH groups and triglyceride, was significantly associated with NASH.

Fasting blood glucose increased in patients with fatty liver, NASH and cirrhosis and showed a significant high level in patients with NASH as compared to control group. This finding agreed with James and Day, et al ,(1998) [(17)] who stated that up to one third of patients have diabetes or fasting hyperglycemia at the time of diagnosis of NASH .
Bookman et al, (2006) [(18)] reported that fasting serum glucose showed significant increase in NASH patients. This finding is supported by data showing the profibrogenic role of hyperglycemia in experimental animals. (Paradis, et al, 2001). [(19)]

Fasting blood insulin showed statistically significant increase in all patients as compared to control group. Our findings were in agreement with Bookmanm, et al (2006) [(18)] who reported that the patients with steatosis and NASH had high levels of fasting blood insulin. This hyperinsulinaemia basically results from compensatory hypersecretion by beta-cells in fatty liver and NASH patients and from reduced insulin breakdown in liver as a result of cirrhosis. (Marchesini, et al, 2003). [(20)]

Insulin resistance was significantly increased across all patients as compared to control group, and the difference between NASH patients and fatty liver alone was statistically significant. These findings are consistent with many published studies done by Marchesini, et al (2003)[(20)] Bookman, et al (2006)[(18)], they stated that Insulin resistance was higher in both NASH and fatty liver patients than healthy controls and subjects with NASH had more severe insulin resistance when compared to those with simple fatty liver.

The patients with steatosis and NASH had varying degrees of steatosis, parenchymal inflammation and fibrosis. A significant positive correlation had been found between BMI and histopathological score in patients with fatty liver alone and histopathological and fibrosis score of patients with NASH.

This finding agreed with Abhasnee Sobhonslidsuk, et al, (2007) [(21)]; they reported increased BMI was positively correlated with the grades of parenchymal inflammation and stages of fibrosis in patients with NASH. Other study by Luyckx, et al (2000) [(22)] concluded that, the grade of steatosis correlates with the severity of obesity and obesity also correlates with the stages of fibrosis in NASH.

The fasting serum glucose and insulin level in patients with fatty liver and NASH were positively correlated with the histopathological score. These results were in agreement with Ender Sern, et al, (2002)[(23)] who found that serum insulin levels correlated with severity of steatosis and inflammation in NASH patients, Klein, et al, (2004) [(24)] who reported that hyperinsulinemia was positively correlated with severity of histopathology of fatty liver and NASH and Pierre, et al, (2007) [(25)] who stated that hyperglycemia was strongly associated with the presence of NASH.

The fasting serum glucose and insulin level in our study were not significantly correlated with the fibrosis score of NASH patients. These results were in agreement with Bookmanm, et al (2006) [18] who reported that fasting blood glucose levels were not significantly correlated with any stage of fibrosis and Chow, et al, (2007) [26] who stated that high serum glucose was not significantly correlated with hepatic fibrosis in NASH.

Our work, showed significant positive correlation between HOMA-IR levels and histopathological and fibrosis scores in patients with fatty liver and NASH respectively.

This finding was in accordance with many different studies. Bookman (2006) [18] reported a significant positive correlation between liver histopathology in patients with fatty liver alone and NASH and insulin resistance, also Rector, et al, (2008) [27] who mentioned that insulin resistance associated with the exacerbation of NAFLD.

Our results agreed with Cortez-Pinto, et al (1999) [28] who found that; a greater association of severity of histopathology with triglyceride values in NASH. These findings could suggest that hypertriglyceridemia may be considered as a risk factor for development of steatosis and progression to NASH.

Mitochondrdial ATP levels in patients with fatty liver and NASH were significantly decreased when compared to the control group. Also patients with NASH showed a statistically significant decrease when compared to patients with fatty liver. These results were in accordance with many different studies. Cortez-Pinto, et al, (1999) [28] demonstrated that patients with NASH have decreased mitochondrial ATP levels and Fan, et al, (2005)[29] reported that, mitochondrial ATP levels were significantly reduced in rats with NAFLD compared with the control group. Finally, Serviddio, et al, (2008) [30] reported that, the mitochondrial ATP content was significantly lower in NASH livers in a rodent model.

This may be explained by mitochondrial injury as a one cause of reduced hepatocellular ATP stores in NASH. This is supported by Dominique Pessayre, et al, (2002) [31] that, identified crystalline structures of uncertain composition coupled with the mitochondrial matrix in patients with NASH, these ultrastructural mitochondrial lesions, decreased activity of respiratory chain complexes, and impaired ability to synthesize ATP. These data were in agreement with our work as they reported that mitochondrial ATP levels negatively correlated with severity of obesity in NAFLD.

These findings could be explained by the following, in obesity hepatocytes induce uncoupling protein-2 (UCP-2) mRNA and protein expression. Thus, when confronted with an abundant substrate supply i.e free fatty acids (FFA), hepatocytes activate pathways that are not efficiently coupled to ATP synthesis i.e uncoupling of oxidative phosphorylation (Skulachev, et al, 1996)[32]. Additionally, there is growing evidence that uncoupling protein -2 (UCP-2) may be a tumor necrosis factor- α (TNF- α) inducible gene that involved in pathogenesis of NASH. (Gimeno, et al, 1997).[33]

In our study, an insignificant negative correlation was found between mitochondrial ATP levels in fatty liver and NASH patients and fasting serum glucose, fasting serum insulin and HOMA-IR. i.e mitochondrial ATP was decreasing with increasing these parameters but the relation was not statistically significant. This could be supported to some extent with significant correlation between these parameters and obesity that significantly associated with hepatic ATP depletion. (Fan, et al, 2005).[29]

Patients with fatty liver and NASH showed a significant negative correlation between mitochondrial ATP levels and histopathological score. This finding may be explained by ATP depletion that may predispose to hepatocellular injury because ATP is critical for maintaining cellular integrity (Chavin, et al, 1999)[34] and the patients had factors lead to increased free fatty acids and reactive oxygen species (ROS) oxidize accumulated unsaturated fatty acids, causing lipid peroxidation, which releases reactive aldehyde that increase hepatic fibrogenesis in two ways. First, these lipid peroxidation products enhance the hepatic production of transforming growth factor -β1 (TGF- β1), which activates hepatic stellate cells into collagen-secreting myofibroblasts. Second, lipid peroxidation products also directly enhance collagen production by hepatic stellate cells.

ROS also increase the synthesis of several cytokines in the liver, particularly TNF- α, which can cause both apoptosis and necrosis .Finally, ROS-associated lipid peroxidation and cytokines may be involved in the inflammatory cell infiltrate, because, lipid peroxidation products, TGF-β1, and interleukin-8 are chemoattractants for neutrophils. (Pessayre, et al, 2001).[35]

These data suggest that, mitochondarial dysfunction and decreased mitochondrial ATP levels are involved in progression of simple fatty liver to NASH and cirrhosis. This is supported by, the fact that ATP is critical for maintaining cellular integrity so its depletion may predispose to hepatocellular injury and necrosis.

5. Conclusion:

Nonalcoholic fatty liver disease, NAFLD is now considered a metabolic pathway to advanced liver disease; cirrhosis and hepatocellular carcinoma. Increased BMI, hyperglycemia, hypertriglyceridaemia, insulin resistance and depletion of mitochondrial ATP in hepatocytes can be considered risk factors involved in the development and progression of fatty liver to NASH and cirrhosis.

Corresponding author
Nadia M. Elwan
Tropical Medicine Department, Tanta University, Tanta, Egypt
*nadiaelwan@yahoo.com

6. References:

1. Tiniakos DG, Vos MBand Brunt EM. (2010) .Nonalcoholic Fatty liver disease: pathology and pathogenesis. Annu Rev Pathol. ; 5:145-71
2. Marchesini G, Bugianesi E, Forlani G, et al, (2003). Nonalcoholic fatty liver, steatohepatitis, and the metabolic syndrome. Hepatology; 37(4): 917–23.
3. Charlton M. (2004). Nonalcoholic fatty liver disease: a review of current understanding and future impact. Clin. Gastroenterol. Hepatol.; 2:1048–58.
4. Neuschwander-Tetri BA, Brunt EM, Wehmeier KR. Et al. (2003). Improved nonalcoholic steatohepatitis after 48 weeks of treatment with the PPAR-gamma lig and rosiglitazone. Hepatology; 38: 1008-17.
5. Ratziu V, Gira lP, Charlotte F, et al., (2000). Liver fibrosis in overweight patients. Gastroen-terology 118:1117–1123.
6. Chitturi S, Abeygunasekera S, Farrell A, et al., (2002). NASH and insuline Res.istance: insulin hypersecretion and specific association with the insulin Res.istance syndrome. Hepatology; 35: 373-9.
7. Bellentani S, Saccoccio G, Masutti F, et al., (2000). Prevalence of risk factors for hepatic steatosis in Northern Italy. Ann .Intern Med.; 132:112 -7.
8. Day CP and James OF. (1998). Steatohepatitis: a tale of two "hits"? Gastroenterology; 114: 842-845.
9. Yongzhong W., Scott R., John PT., et al, (2008). Nonalcoholic fatty liver disease and mitochondrial dysfunction World J. Gastroenterol ; January 14; 14(2): 193-199.
10. Kleiner DE, Brunt EM, Van Natta M, et al, (2005). Nonalcoholic Steatohepatitis Clinical Research Network. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology, 41: 1313-21.
11. Wanless IR and Lentz J. (1990) . Fatty liver hepatitis (steatohepatitis) and obesity : an autopsy study with analysis of risk factors. Hepa-tology 12:1106–1110.
12. Abhasnee S., Sutipong J. and Ammarin T., (2007). Visceral fat and insulin Res.istance as predictors of non-alcoholic steatohepatitis, World JGastroenterol July 14; 13 (26): 3614 – 3618.
13. Fabbrini E, Sullivan Sand Klein S.(2009). Obesity and nonalcoholic fatty liver disease: Biochemical, metabolic, and clinical implications. Hepatology. Sep 9.
14. Clark JM and Diehl AM. (2003): Nonalcoholic fatty liver disease: an underrecognized cause of cryptogenic cirrhosis. JAMA; 289:3000-4.
15. Ender SERİN, Hikmet AKKIZ, Figen DORAN, et al, (2002). Obesity and related parameters of non-alcoholic steatohepatitis;The Turkish Journal of Gastroenterology 13(2): 71-77.
16. Joong-Won P.; Gyu J.; Sang Jk., et al., (2007). Predictors Reflecting the Pathological Severity of Non-alcoholic Fatty Liver Disease: Comprehensive Study of Clinical and munohistochemical Findings in Younger Asian Patients, Journal of Gastroenterology and Hepatology.; 25: 325-334.
17. James OF. and Day CP. (1998). Nonalcoholic steatohepatitis (NASH): a disease of emerging identity and importance. J. Hepatol ; 29: 495-501.
18. Bookman ID; J. Pham; M. Guindi; et al., (2006).Distinguishing Nonalcoholic Steatohepatitis from Fatty Liver: Serum-free Fatty Acids, Insulin Resistance, and Serum Lipoproteins- Liver International. Jun; 43(4): 12-23
19. Paradis V, Perlemuter G, Bonvoust F,et al, (2001). High glucose and hyperinsulinemia stimulate connective tissue growth factor expression: a potential mechanism involved in progRes.sion to fibrosis in nonalcoholic steatohepatitis. Hepatology ; 34: 738-744
20. Marchesini G, Bugianesi E, Forlani G, et al, (2003). Nonalcoholic fatty liver, steatohepatitis, and the metabolic syndrome. Hepatology; 37(4): 917–23
21. Abhasnee S., Sutipong J. and Ammarin T., (2007). Visceral fat and insulin Res.istance as predictors of non-alcoholic steatohepatitis, World JGastroenterol July 14; 13 (26): 3614 – 3618.
22. Luyckx FH, Lefebvre PJ, Scheen AJ. et al, (2000). Nonalcoholic steatohepatitis: association with obesity and insuline resistance . Diabetes Metab; 26: 98-106.

23. Ender Serin, Hikmet Akkiz, Figen Doran, et al, (2002). Obesity and related parameters of non-alcoholic steatohepatitis;The Turkish Journal of Gastroenterology 13(2): 71-77.
24. Klein S, Fontana L, Young V L, et al. (2004). Absence of an effect of liposuction on insulin action and risk factors for coronary heart disease. NEJM ; 350(25): 2549–57.
25. Pierre M. Gholam,; Louis Flancbaum,; Jason T. Machan, et al, (2007). Nonalcoholic Fatty Liver Disease in Severely Obese Subjects; The American Journal of Gastroenterology;(6), 521-528
26. Chow WC, Tai ES, Lian SC, et al, (2007). Significant non-alcoholic fatty liver disease is found in non-diabetic, pre-obese Chinese in Singapore. Singapore Med J. Aug ; 48(8):752-7.
27. Rector RS, Thyfault JP, Wei Y, et al, (2008). Non-alcoholic fatty liver disease and the metabolic syndrome: An update. World J. Gastroenterol. Jan 14; 14(2):185-92.
28. Cortez-Pinto H., John Chatham, V. P. Chacko et al, (1999). Alterations in Liver ATP Homeostasis in Human Nonalcoholic Steatohepatitis JAMA. ;282:1659-1664.
29. Fan JG , Ding XD , Wang GL ,et al, (2005). ExpRes.sion of uncoupling protein 2 and its relationship to the content of adenosine triphosphate in the nonalcoholic fatty livers of rats fed a high-fat diet; Zhonghua Gan Zang Bing Za Zhi, 13(5): 374-7.
30. Serviddio G, Bellanti F, Tamborra R,et al, (2008). Alterations of hepatic ATP homeostasis and respiratory chain during development of non-alcoholic steatohepatitis in a rodent model. Eur J. Clin Invest. Apr;38(4):245-52
31. Dominique P., Abdellah M., and Bernard F.(2002).Nonalcoholic Steatosis and Steatohepatitis V. Mitochondrial dysfunction in steatohepatitis. Am J. Physiol Gastrointest Liver Physiol 282: G193-G199.
32. Skulachev, V. P. (1996). Isulin resistance and diabetes mellitus; Q. Rev. Biophys. 29,169-202.
33. Gimeno, R. E., Dembski, M., Weng, X., et al, (1997). Diabetes mellitus ,Nature, 335, 773-80.
34. Chavin K, Yang SQ, Lin HZ, et al, (1999). Obesity induces expRes.sion of uncoupling protein-2 in hepatocytes and promotes liver ATP depletion. J. Biol Chem. 274:5692-5700
35. Pessayre D, Berson A, Fromenty B, et al., (2001). Mitochondria in steatohepatitis. Sem Liver Dis, 21, 57-69.

9/21/2010

Optimization of Cadmium, Zinc and Copper biosorption in an aqueous solution by *Saccharomyces cerevisiae*

[1]Salem M. Hamza [2*]Hanan F. Ahmed [3] Ehab A. M. [2]Mohammad F. M.
[1] Faculty of Science-Chemistry Department. El-Menoufia University, El-Menoufia, Egypt
[2] Food Technology Res. Institute, Agric. Res. Center, Giza, Egypt.
[3] Food Science Department, Faculty of Agriculture. Cairo University, Cairo, Egypt
*hanan_fawzy29@yahoo.com

Abstract: Optimization of Cd (II), Zn (II) and Cu (II) biosorption from contaminated water were performed as function of parameters (pH, contact time, initial metal ions concentration and yeast dose). The experimental results showed that the highest equilibrium adsorption capacity at the optimum pH were 8.5 for Cd (II) and 6 for Zn (II) and 6 for Cu (II). Optimum pH values were carried out to evaluate other parameters. Results demonstrate that removal efficiency increased with increased contact time for the three metal ions. Results indicated that removal efficiency increased with increased yeast dose up to 2 g/ 100ml, and removal efficiency decreased with increased yeast dose from 2.2 g/100ml to 4 g/100ml. The results also showed that increasing removal efficiency from 1 to 20 mg/L concentration for the three metal ions and the removal efficiency decreasing with increasing initial concentration from 25 to 50 mg/L. It is evident that the highest removal efficiency for Cd (II) ion compared to Zn (II) and Cu (II). This study revealed that use of baker's yeast is suitable for removal of these ions from contaminated water in order Cd > Zn > Cu at these conditions. The negative values of the standard free energy change (ΔG°) indicate spontaneous nature of the process. Competitive biosorption of (Zn and Cu) ions was investigated in terms of sorption quantity. The amount of Cu metal ion adsorbed onto unit weight of biosorbent (q_e) decreased with increasing the competing metal ion (Zn), in contrast, the amount of Zn ion adsorbed onto unit weight of yeast has been increased with increasing the competing metal ion (Cu). The binding capacity for Zn (II) is more than for Cu (II). Ion exchange is probably one of the main mechanism during adsorptive process.
[Salem M. Hamza, Hanan F. Ahmed Ehab A. M., Mohammad F. M. Optimization of Cadmium, Zinc and Copper biosorption in an aqueous solution by *Saccharomyces cerevisiae.* Journal of American Science 2010;6(12):597-604]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Optimization; Cadmium; Zinc; Copper; biosorption; *Saccharomyces cerevisiae*

1. Introduction:

Biosorption can be defined as the removal of metal or metalloid species compounds and particulates from solution by biological material Gadd, (1993). Heavy metal pollution has become one of the most serious environmental problems today. Biosorption, using biomaterials such as bacteria, fungi, yeast and algae is regarded as a cost-effective biotechnology for the treatment of high volume and low concentration complex wastewater containing heavy metals in the order of 1 to100 mg/L. Veglio and Beolchini, (1997). Some strong toxic metal ions, such as Hg and Cd (II), are very toxic even in lower concentration of 0.001–0.1 mg/L Alkorta *et al.,* (2004).

There is always a possibility that metals will be taken up by plants such that they end up in the human food chain. Metal studies have shown that various oxidation states of metals are toxic to humans and also that they are associated with several types of diseases Atwood *et al.,* (2000). In order to facilitate the analysis of metal ions in aquatic environments, analytical methodologies that address aspects of sampling; sample clean-up and analyte enrichment are necessary. If possible, such methodologies should be simple, robust and inexpensive as well as address limitations associated with collecting samples from remote sampling sites. Biological substrates including the baker's yeast, *Saccharomyces cerevisiae* has been shown to have good sorption characteristics for several Heavy metals Volesky and May-Phillips (1995). As will as it's abundance of yeast as a by-product of fermentation processes Blackwell *et al*., (1995). For biosorption of heavy metal ions, pH is one of the most important environmental factors. The biosorptive capacity of metal cations increases with increasing pH of the sorption system, but not in a liner relationship. Esposito *et al*., (2002). Yeast cells of *S. cerevisiae* are able to remove heavy metals from wastewaters between pH 5 and 9 Wang, (2009).

The biosorption capacity and the removal efficiency of metal ions by *S. cerevisiae* became higher with prolonging the contact time Wang, (2006). The uptake rate of the metal ion will be increased with increasing the initial concentration if the amount of biomass is kept unchanged Wang,

(2002). Evidenced that the use of a large excess of yeast biomass comparatively to metals allowed an efficient removal of metals from real and synthetic effluents, reducing the amount of metal in solution to the limit of discharge of wastewater in natural waters Machado *et al*.. (2010)

Although copper and zinc are essential trace elements, high levels can cause harmful health effects. Copper is also toxic to a variety of aquatic organisms, even at very low concentration Sheng *et al*., (2004).

Cadmium, Zinc and Copper ions were chosen for biosorption studies with regard to their wide use in industry. Zinc and Copper are very widely used metal in our daily life, but like any other heavy metal it is potentially toxic for all the living organisms. Volesky, (1990). Cadmium can contaminate the environment from anthropogenic sources as well as natural geochemical processes. It can accumulate along the food chain and is not amenable to biological degradation Dursun, (2003).

This investigation aimed to use baker's yeast for adsorption of some metal ions from contaminated aqueous solutions, In addition, to study the influence of the uptake of Cd (II), Zn (II) and Cu (II) by *S. cerevisiae* in different adsorptive conditions and to compare the biosorption behavior of a single-metal system and two-system.

2. Materials and methods

Baker's yeast

The baker's yeast employed in this study was DSM-70460 strain of *Saccharomyces cerevisiae* used in baking industry. It is supplied in the form of dry matter and it's supplied from Deutsche Sammlung Von Mikroorganismen und Zellkulturen GmbH (Germany).

Optimized of pH

In this study, three sets of experiments were conducted to study the effect of pH. The tested solutions 5 mg/L metal ions and yeast dose 0.2 gm/100 ml were adjusted at pH 3, 4.5, 6, 7, 8.5, 9.5 and 11 using 0.1 M HCl or 0.1 M NaOH. All pH measurements were carried out with a HANNA digital pH meter HANNA Instruments and left for 30 min. to reach the equilibrium.

Analytical methods

Samples were analyzed for Cd (II), Zn (II) and Cu (II) using Perkin Elmer (3300) Atomic Absorption-Flame Absorption Spectrophotometer, a Calhodeon Ltd. Hollow Cathode Lamp as light source for Cd (II), Zn (II) and Cu (II) and mixture of air-C_2H_2 as the flame atomizer gas. A standard calibration curves were prepared using Cd (II), Zn (II) and Cu (II) solutions of known concentration in the range 1-100 mg/L.

Evaluation of the effect of various parameters on uptake of metal ions at optimized pH for three metals:

1 - Biosorption experiments

The experiments were conducted with Cd (II), Zn (II) and Cu (II) aqueous solution of initial concentration from 1 to 50 mg/L, yeast dose from 0.2 to 4.0 g/100ml, were mixed at pH range from 3 to 11 and Shaken at a constant speed of 120 RPM in a shaking at temperature 298 K for 15 to 120 min. The samples were centrifuged at 2000 rpm for 15 min. (Hermale Z 200A) the supernated were analyzed and the experiment were carried out at least twice the values used in calculations were mostly the arithmetic average of the experiment data.

2 - Sorption studies

Cd (II), Zn (II) and Cu (II) uptake were calculated by two equations according to Cojocaru *et al.,* (2009).

3 - Effect of contact time on biosorption

Samples were withdrawn at 15, 30, 60, 90 and 120 min. intervals during the biosorption experiments and analyzed for Cd (II), Zn (II) and Cu (II). This factor that influence time was examined in 5 mg/L metal ions and 0.2 g of yeast dose /100 ml.

4 - Effect of yeast dose on biosorption

This factor that influence yeast dose were examined in 5 mg/L for 30 min. yeast dose of 0.2, 0.4, 0.8, 1.0, 1.2, 1.6, 2.0, 2.4, 2.8, 3.2, 3.6 and 4.0 gm were added to test solution taken in series of 250 ml Erlenmeyer flasks with 100 ml tested solution and biosorption experiments were carried out.

5 - Effect of initial metal ions concentrations on biosorption

This factor that influence initial metal ions were examined in 0.2 g/100 ml for 30 min. Test solutions containing 1, 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 mg/L for $CdSo_4$, $ZnSo_4$and $CuSo_4$ were subjected to biosorption. The salt was chosen a sulphate since sulphate ions exist in most wastewater and mine drainage and thus simulating the potential application of biosorption for Cd (II), Zn (II) and Cu (II) ions removal.

6 - Competitive adsorption in binary metal system

In this group of experiments, competitive adsorption of Zn (II) and Cu (II) ions from their binary solutions was investigated by following a similar procedure as described above. These studies

were performed at a initial pH of 6 at 298 K. Experiments of competitive adsorption of Zn (II) and Cu (II) included two parts: (i) the competitive adsorption of Zn (II) and Cu (II) in the total metal concentration was fixed (5 mg/L); (ii) in a serious of two metal ions solution, the initial concentration of Zn (II) was fixed to 5 mg/L, where as, concentration of Cu (II) were varied from 0 to 4 mg/L. In another binary system, initial concentration of Cu (II) was constant in 5 mg/L, and concentrations of Zn (II) were varied from 0 to 4 mg/L.

3. Results and Discussion:

The biosorption has attracted the attention as low-cost treatment technology for the removal of heavy metals from wastewaters.

Optimized of pH

The charge of the adsorbate and adsorbent often depends on the pH. The adsorption of Cd (II), Zn (II) and Cu (II) as a function of pH were measured. As shown in Table (1) there were increases in biosorption uptake equilibrium adsorption capacity (q_e) of Zn (II) and Cu (II) with increasing pH from 3 to 6 for both metal ions. On the other hand maximum Cd (II) uptake observed at pH 8.5. The lowest metal uptake values were determined at pH 3 for three metal ions. At pH values above the isoelectric point, there is a net negative charge on the cell wall components, which will be promate reaction with metal ions. As the pH is lowered, however, the overall surface charge on the cells will be positive, which will inhibit the approach of positively charged metal ions Goksungur *et al*., (2005).

Table (1): The optimization initial of solution pH on biosorption for Cd (II), Zn(II) and Cu (II) at 5 mg/L metal ions and yeast dose 0.2 (gm) after 30 min and 25 °C.

	pH	C_o mg/L	C_e mg/L	q_e mg/g	q_{max} mg/g	Y %
Cd	3	4.99	3.67	0.660	1.32	26.45
	4.5	4.93	2.79	1.070	2.14	43.41
	6	4.97	2.6	1.185	2.37	47.68
	7	4.90	1.69	1.605	3.21	65.51
	8.5	4.95	0.22	2.365	4.73	95.55
	9.5	4.91	0.53	2.190	4.38	89.20
	11	4.98	0.92	2.030	4.06	81.16
Zn	3	4.95	2.633	1.1555	2.317	46.66
	4.5	4.89	2.60	1.145	2.29	46.83
	6	4.96	1.498	1.731	3.462	69.80
	7	4.90	2.14	1.380	2.76	56.32
	8.5	4.88	2.878	1.005	2.01	41.18
	9.5	4.91	3.10	0.905	1.81	36.86
	11	4.92	3.20	0.860	1.72	34.95
Cu	3	4.98	3.957	0.515	1.033	20.89
	4.5	4.91	2.859	1.0205	2.041	41.54
	6	4.90	2.206	1.347	2.694	54.98
	7	4.96	2.968	1.0063	2.0025	40.32
	8.5	4.92	3.16	0.880	1.76	35.77
	9.5	4.90	3.323	0.7905	1.58	32.24
	11	4.95	3.46	0.746	1.49	30.10

C_o : initial metal concentrations (mg /L) C_e : final metal concentrations (mg /L)
q_e : equilibrium adsorption capacity (mg /g) q_{max} : amount of bioaccumulated ions in yeast (mg/g)
Y : removal efficiency yeast (%)

Similar results were detected by Volesky (1990) who found that the optimal pH value is 5 – 9 for Cu (II) biosorption by *S. cerevisiae*. Also Mapolelo and Torto, (2004) proved that the optimal pH values are greater than 5 for Cu^{2+} and Zn^{2+}. In this study maximum Cd (II) uptake was observed only at alkaline pH 8.5.

However; Mullen *et al*., (1992) have reported an increased trend in biosorption of Cd (II) by *A. niger* to the tune of 2.9 times when pH was increased from 4 to 7. Also Parvathi *et al*., (2007) found that biosorption of Mn (II) increased with rise in pH, and maximum manganese uptakes were observed at initial pH 9 by *S. cerevisiae*.

Generally, an increase of pH causes deprotonation of metal ions binding sites exposed by cellular surface. However, a decrease of pH causes competition between protons and positively charged metal ions. However these rules concern only cations .Since biosorption is reversible process, decreasing pH would result in deprotonation. This property is used in regeneration of biosorbents Naja and Volesky, (2010).

Effect of time:

As shown in Fig. (1); It was found that the equilibrium adsorption capacity increased as the contact time increased. The biosorption of Cd (II), Zn (II) and Cu (II) on yeast was rapid for first 15 min. and equilibrium was nearly reached after 90 min. Results revealed that the highest removal efficiency Cd (II) occurred after 15 min., then the increased removal efficiency occurred slowly. This trend emphasizes that sorption times have an important effect on recovery efficiency, which decreases with increase biosorbent contact time with metal solution. Metal accumulation inside the cell may resulted from bioaccumulation, slow metabolic dependent removal mechanism, or by simple metal diffusion Gaad, (1990).

Tavares *et al*., (1995) mentioned that, biosorption kinetics with an initial rapid metal uptake followed by slow uptake was observed, this kinetic model has been accepted for various biosorbents such as bacteria and fungi (yeast) under similar operation conditions. A similar trend was observed for the uptake of three metal ions.

The data shown in Fig. (1) indicate that selected sorption time for biosorption capacity was 120 min., this sorption period equilibrium adsorption capacity reached maximum at 90 min. for Zn (II) and Cu (II). The highest Cd (II) biosorption capacity was observed compared with Zn (II) and Cu (II), the explanation based on it's most noxious valency, may readily participate in cell metabolism Salem, (1994).

Goyal *et al*., (2003) reported that the uptake of metal ions by microorganisms in batch systems has been shown to occur in two stages: an initial rapid stage (passive uptake), followed by much slower process (active uptake). The first stage is physical adsorption or ion exchange at the surface of the biomass, which is biosorption. The biosorption equilibrium occurs at the end of rapid physical adsorption stage (first-stage). Adsorption isotherm equation is frequently used to represent this equilibrium. The same behavior was observed by Han *et al.,* (2006a) equilibrium time for Zn (II) and Cu (II).

Özer *et al.,* (1999) found that the adsorption equilibrium of Cd (II) on *S. leibleinii* was established in 15 – 20 min. and the adsorbed amount did not change with time.

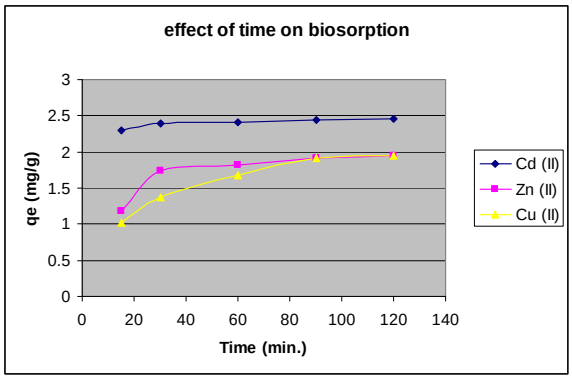


Fig. (1) Effect of time on biosorption for Cd (II), Zn (II) and Cu (II) at 5 mg/L and yeast dose 0.2 (gm) at 25^{o}C.

Effect of yeast dosage

The effect of yeast dosage on removal efficiency (Y) % showed in Fig. (2). Results showed that increasing metal removal as increased yeast up to 2 g/ 100 ml solution. As expected (Fig. 2), the uptake of Cd (II) was much more sensitive to increase amount of yeast in solution compared to that Zn (II) and Cu (II). The increase in metal uptake with yeast dosage confirms the increase in binding sites for metal ions. On the other hand, it was observed that the metal uptake decreased with increasing the amount of yeast dose from 2.2 to 4.0 g/ 100 ml. Decreased removal efficiency may be due to aggregation of yeast. Al-Asheh and Duvnjak, (1995) reported that higher uptake at lower biomass dosage could be due to metal ions and biosorbent ratio, which decrease upon an increase in biomass dosage due to of high biomass dosage resulted aggregates of biomass and may cause interference between binding sites at higher biomass dosage or insufficiently of metal ions in the solution with respect to available binding sites. It is likely that protons will then combine with metal ions for the ligands and thereby decrease the interaction of metal ions with the cell components Saĝ and Kutsal, (1996). It should be mentioned that the cadmium ion adsorption capacity decreased with increase of biosorbent dosage Vasudevan *et al.,* (2002). Their theory is similar to that advanced by Zou *et al*. (2006) to account for the cell surface remaining unsaturated at higher biosorbent dosage.Uslu and Tanyol, (2006) found that the initial adsorption rates of Cu (II) decreased with increased biosorbent concentration. Fig. (2) shows that the uptake of Zn (II) reached a maximum at 2 g/ 100 ml yeast dosage. These data probably

indicate that low uptake values reflect a sluggish adsorption process. Wilhalmi and Duncan, (1996) reported that the Zn (II) is preferable that the adsorption process on the membrane take places over short period of time to avoid interalisation of metal ions which would result in lower ions would result in lower desorption and enrichment of metal ions.

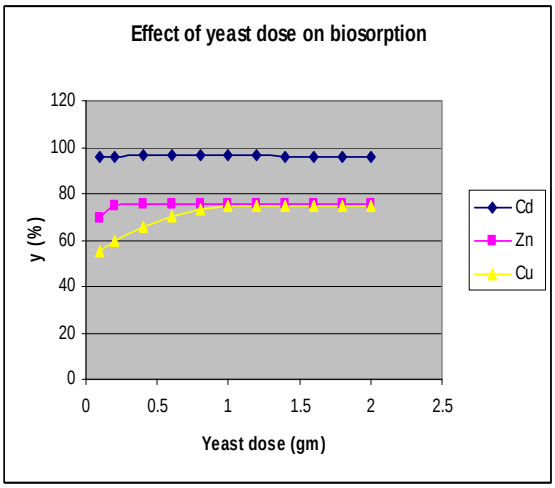


Fig. (2) Effect of yeast dosage on biosorption for Cd (II), Zn (II) and Cu (II) at 5 mg/L after 30 min and 25 oC.

Effect of initial concentration ions:

As shown in Fig. (3); Removal efficiency increased with increasing the metal ions concentration from1 to 20 mg/L in three metal ions. On the other hand, after 20 mg/L of Cd (II), Zn (II) and Cu (II), ions decreasing uptake for three metal ions. As increase initial concentration from 25 to 50 mg/L decreasing removal efficiency from 84.80, 66.14 and 45.65 to 77.52, 38.44 and 33.52 for Cd (II), Zn (II) and Cu (II), respectively. This could be attributed to the strong effect of initial concentration, which is very high comparing with the relatively low sorption capacity of biosorbent. This agrees with Cojocaru *et al*., (2009) who found that increase in density of the negative charge on the cell surface, causing proton removal from solution, thereby decreasing biosorption capacity of Cu (II) because of competitively adsorption of protons. A similar observation was reported by Ferraz and Teixeirain, (1999) who suggested an increase of electrostatic interaction at high biomass concentration inhibited metal biosorption. When the biomass concentration is low, metal ions in the solution would not only be adsorbed to the surface of the biomass, but also enter into intracellular part through facilitating the concentration gradient of metal ion Wang, (2002).

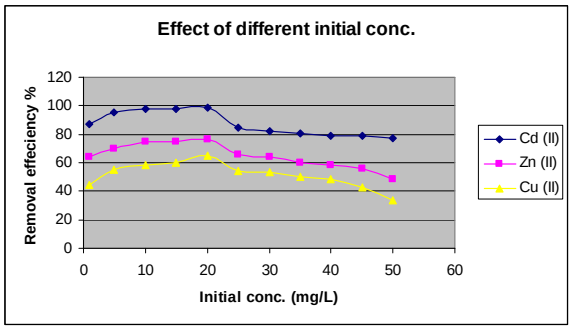


Fig. (3) Removal efficiencyof Cd (II), Zn (II) and Cu (II) at different initial concentrations.

Adsorption equilibrium

Adsorption isotherms show the distribution of solute between the liquid and solid phases equilibrium conditions. Many different isotherm models have been proposed for the adsorption of solutes in a liquid solution onto a solid surface. Langmuir model is probably the most popular isotherm models due to its simplicity and its good agreement with experimental data. The Langmuir model, the saturated monolayer isotherm, can be described by the linear form Langmuir, (1916). Although the Langmuir constant q_{max} is dependent on experimental conditions such as solution pH and temperature, it is a good measure for comparing different sorbents for the same (metal) sorbate. Another important factor in evaluating sorbent performance is the initial gradient of the adsorption isotherm, since it indicates the sorbent affinity at low metal concentrations. In the Langmuir equation, this initial gradient corresponds to the affinity constant (b). A high value of the affinity constant is thus desirable Sheng *et al*., (2004). The equilibrium established between the adsorbed metal ions (q_e) and that remained free in the solution (C_e) was also represented by the Freundlich adsorption isotherm, the linear equation as following Freundlich, (1906).The equilibrium data of (Cd), (Zn) and (Cu) ions adsorption by baker's yeast obtained at 25 ^oC (298 K) was applied to Langmuir and Freundlich models. The relative parameters and correlation coefficients (R^2) were listed in Table (2). The mono-component Langmuir constant, q_{max}, represents the monolayer saturation at equilibrium or the total capacity of yeast for (Cd), (Zn) and (Cu) ions. From table (2), the maximum loading capacities of yeast was obtained as 4.545 mg/ g for (Cd) 0.6676 mg/ g for (Zn) and 0.4833 mg/ g for (Cu). So the ability of Cd (II) adsorption on yeast is biggest than Zn (II) and Cu (II). From Table (2), the values of k_f showed easy uptake of metal ions with high adsorption capacity of baker's yeast. As $0.1 < 1/n < 1.0$, indicating that (Cd)

and (Zn) ions are favorably adsorbed by yeast at temp 25 °C. studied Aksu , (2002). The Gibbes free energy of biosorption determined Han *et al.,* (2005). The free energy changes for Cd (II), Zn (II) and Cu (II) sorption onto yeast were determined at 25 °C and at optimum pH, are -7.60, -2.075 and -0.495 and kJmol^{-1}, respectively. The negative values of ΔG^{o} valid at the feasibility of the biosorption process Cd (II), Zn (II) and Cu (II) the spontaneity of biosorption.

Effect of coexistence ions on the biosorption of zinc and copper ions on baker's yeast:-

Table (2): Constants of Langmuir and Freundich isotherms for Cadmium, Zinc and Copper biosorption by *S.cerevisiae* at optimum pH and 25 °C.

Metals	pH	Langmuir			Freundich		
		B L/mg	qmax mg / g	R2	1/n	kf mg/L	R2
Cd	8.5	4.545	2.368	0.981	0.322	1.62	0.810
Zn	6	0.6676	1.731	0.952	0.497	0.384	0.793
Cu	6	0.4833	1.347	0.999	0.982	0.524	0.999

1 - The effect on biosorption of Cu ion with the presence of Zinc ion in the solution

As shown in Fig (4 down curve); with increasing Zn (II) concentration the biosorption quantity of Cu (II) decreased. Also, the adsorptive quantity of Cu (II) decreased with the increasing Zn (II) initial concentration. The biosorption quantity of copper ion decreased from 1.374 mg/g to 1.132 mg/g. Thus it can be seen that the existence of Zn (II) has greater effect on the biosorption of copper on yeast. When the Zinc ion existence in the solution it will be adsorbed by yeast and compete to the copper ions and it will hold some activated sites on yeast, hence the adsorption capacity of copper will decrease. So when the ions of Zn (II) existence it will be a disadvantage condition to the biosorption of copper ion by yeast. Furthermore, the concentration of Zn (II) is more, the effect is serious. The biosorption capacity of one metal ion is interfered and reduced by co-ions, including other metal ions and anions presenting in solution, however the gross uptake capacity of all metals in solutions remains almost unchangeable Wang, (2006).

2 - The effect on biosorption of Zn ion with the presence of Copper ion in the solution

As shown in Fig. (4 upper curve); with increasing the copper concentration, the biosorption quantity of Zn ion increased. Also, the adsorption quantity of Zn (II) increased with the increasing of Cu (II) concentration. The biosorption quantity (q_e) of Zn (II) increased from 1.731 to 1.905 mg/g when the concentration of Cu (II) ranged from o to 4 mg/L. As the copper ions existence in solution, similarly, it will be adsorbed by yeast and compete to the Zn ions. So where the ions of copper existence, it will be advantage condition to the biosorption of Zn ion on yeast, Furthermore, the concentration is higher, the effect is also serious. The decrease of metal uptake in competitive conditions was thought to be a response to increased competition between same charged species for binding sites of the yeast cells Goksungur *et al.*, (2005).

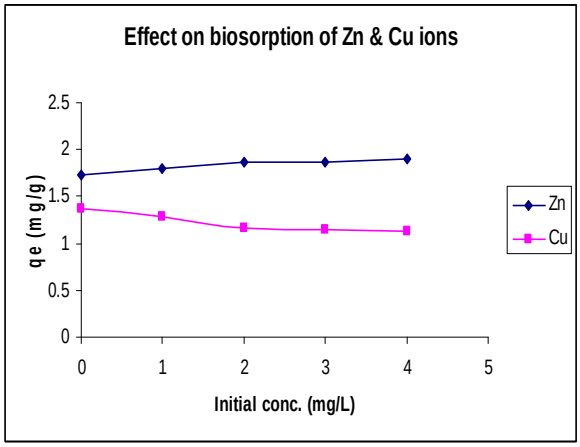


Fig. (4) The effect on biosorption of zinc when copper in existence (upper curve) and the effect on biosorption of copper when zinc in existence (down curve).

3 - The competitive adsorption of Cu (II) and Zn (II) in the total concentration fixed

As shown in Fig (5); it was observed that when increased the concentration of copper, reduced the concentration of zinc, the uptake increased for Zn (II) while decreasing for Cu (II) when there was two metal ion existence, the value of q_e about zinc was bigger than copper, so the binding ability for zinc is strong than copper. The baker's yeast can be used to remove copper and zinc ions in solution when there was two metal ions coexistence. Above all, competitive biosorption of two metals was investigated in terms of sorption capacity and found that the biosorption capacity of biomass decreased with increasing the competing metal ion concentration. Han *et al.*, (2006b) found that the amount of one metal ion adsorbed onto unit weight of

biosorbent decreased with increasing competing metal ion concentration, but from our investigation showed in Fig. (5) it can be concluded that the total capacity for adsorbing copper ion decreased while zinc ions increased.

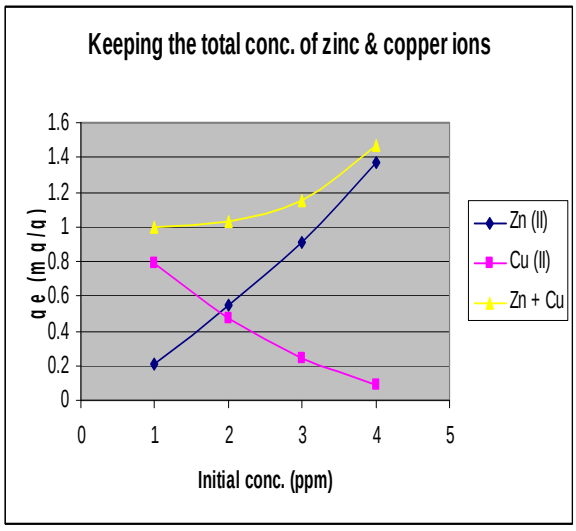


Fig. (5) Keeping the total concentration of zinc and copper ions fixed, the equilibrium level of zinc and copper ions onto baker's yeast.

4. Conclusion:

The analysis of our results shows the possibility used of baker's yeast in dried form for remove Cd, Zn, Cu ions from contaminated aqueous solutions .Results obtained for the removal of initial metal ions concentration in an aqueous solution using *Saccharomyces cerevisiae* showed a decrease of biosorption efficiency at low and high value initial pH. The removes percentage for Cd (II),is the highest removal efficiency in optimal condition (pH=8.5)compared with Zn (II) and Cu (II) (pH=6). The degree of removal of this hazards ions were depend on the pH, contact time and initial concentration of the contaminated water.

Competitive biosorption of two metals (Zn & Cu) ions was investigated in terms of sorption capacity and found that the biosorption capacity for Cu ion decreased with Zn metal ion concentration.

Corresponding author
Hanan F. Ahmed
Food Science Department, Faculty of Agriculture. Cairo University, Cairo, Egypt
hanan_fawzy29@yahoo.com

5. References:

1. Al-Asheh, S. and Duvnjak, Z. (1995): Adsorption of copper and chromium by *Aspergillus carbonarius*. Biotechnol. Prog., 11:638–642.
2. Alkorta, I., Hernández- Allica Becerril, J. M., Amezaga, I., Albizu, I. and Garbisu C. (2004): Recent findings on the phytoremediation of soils contaminated with environmentally toxic heavy metals and metalloids such as zinc, cadmium, lead, and arsenic. Rev Environ Sci Biotechnol., 3: 71–90.
3. Aksu, Z. (2002): Determination of the equilibrium, kinetic and thermodynamic parameters of the batch biosorption of lead (II) ions onto *Chlorella vulgaris*, Process Biochem.38: 89–99.
4. Atwood, C.S., Scarpa, R.C., Huang, X.D., Moir, R.D., Jones, W.D., Fairlie, D.P., Tanzi, R.E. and Bush, A.I. (2000): Characterization of copper interactions with Alzaheimer AB peptides-identification of an attomolar affinity copper binding site AB-42 J. Neurochem, 75:1219-1233.
5. Blackwell, K.J., Singleton, I. and Tobin, J.M. (1995): Metal cation uptake by yeast: a review. Appl. Microbiol. Biotechnol. 43: 579-584.
6. Cojocaru, C., Diaconu, M., Cretescu, I., Savic, J. and Vasic,V. (2009): Biosorptionof copper (II) ions from aqua solutions using dried yeast biomass. Colloids and Surfaces a Physicochemical and Engineering Aspects , 335:181–188.
7. Dursun, A.Y. (2003): The effect of pH on the equilibrium of heavy metal biosorption by *Aspergillus niger*. Fresenius Environ Bull., 12: 1315–22.
8. Esposito, A., Pagnanelli, F., Veglio, F. (2002): pH related equilibria models for biosorption in single metal systems. Chem. Eng. Sci., 57:307–13.
9. Freundlich, H. M. F. (1906): Uber die adsorption in lasungen. Z. Phys. Chem., 57:385–470.
10. Ferraz, A. I. and Teixeira, J. A. (1999): The use of flocculating brewer's yeast for Cr (III) and Pb (II) removal from residual wastewaters. Bioprocess. Eng., 21: 431–437.
11. Gaad, G. M. (1990): Fungi and yeast for metal accumulation, in microbial mineral recovery, in: H.L. Lehrlich, C.L. Brierley (Eds.), Environmental Biotechnology, McGraw-Hill, pp. 249-275, Chapter 11.
12. Gadd, G. M. (1993): Interactions of fungi with toxic metals. Phytologist., 124 : 25–60.
13. Goksungur, Y., Uren, S. and Guvenc, U. (2005): Biosorption of cadmium and lead ions by ethanol treated waste baker's yeast biomass, Bioresour. Technol., 96:103–109.
14. Goyal, N., Jain, S. C. and Banerjee, U. C. (2003): Comparative studies on the microbial adsorption of heavy metals. Adv Environ Res., 7: 311–9.
15. Han, R. P., Zhang, J. H., Zou, W. H., Shi, J. and Liu H.M. (2005): Equilibrium biosorption isotherm for lead ion on chaff, J . Hazard. Mater, B125: 266–271.
16. Han, R., Li, H., Li, Y., Zhang, J., Xiao. H. and Shi, J. (2006 b): Biosorption of copper and lead ions by waste beer yeast. J. Hazard. Mater., 137:1569–1576.

17. Han, R., Zhang, J., Zou, W., Xiao, H., Shi, J. and Liu, H. (2006 a): Biosorption of copper (II) and lead (II) from aqueous solution by chan in a fixed – bed column. J. Hazard. Mater., 133(1–3): 262–268.
18. Langmuir, I. (1916): The constitution and fundamental properties of solids and liquids, J. Am. Chem. Soc., 38:2221–2295.
19. Manuela, D., Eduardo, V. S. and Helena M.V.M.S. (2010): Removal of heavy metals using a brewer's yeast strain of *Saccharomyces cerevisiae*: Chemical speciation as a tool in the prediction and improving of treatment efficiency of real electroplating effluents Journal of Hazardous Materials 180: 347–353.
20. Mapolelo, M. and Torto, N. (2004): Trace enrichment of metal ions in aquatic environments by *Saccharomyces cerevisiae*. Talanta., 64:39–47.
21. Mullen, M. D., Wolf, D. C., Beveridge, T. J. and Bailey, G. W. (1992): Sorption of heavy metals by the soil fungi *Aspergillus niger* and *Mucor rouxii*. Soil Biol Biochem 24:129–135
22. Naja, G. M. and Volesky, B. (2010): Treatment of Metal-Bearing Effluents: Removal and Recovery. In: Wang L.K., Chen J.P., Hung Y.T., Shammas N.K., editors. Handbook on Heavy Metals in the Environment. Boca Raton, F.L.: Taylor & Francis and CRC Press.
23. Özer, A., Özer, D. and Ekiz, H.I. (1999): Application of Freundlish and Langmuir modles to multistage purification process to remove heavy metal ions by using *Schizomeris leibleinii*, process., Biochem. 34: 919-927.
24. Parvathi, K., Nagendran, R. and Nareshkumar, R. (2007): Lead biosorption onto waste beer yeast by-product, a means to decontaminate effluent generated by battery manufacturing industry, Electronic J. Biotechnol., 10 : 92–105.
25. Saĝ, Y. and Kutsal, T. (1996): The selective biosorption of chromium (VI) and copper (II) ions from binary metal mixtures by *R. arrhizus* . Process Biochem., 31: 561–572.
26. Salem, F. (1994): The role of chroumium as an essential nutrient, in: S. Katz, H. Salem (Eds.), The Biological and Environmental Chemistry of Chromium, VCH Publishers, New York, pp. 121-152, Chapter 56.
27. Sheng P.X., Ting Y.P., Chen J.P. and Hong L., (2004): Sorption of lead, copper, cadmium, zinc and nickel by marine algal biomass: characterization of biosorptive capacity and investigation of mechanisms, J. Colloid Interface Sci. 275:131–141.
28. Tavares, M. T., Martins, C. and Neto, P. (1995): Biotreatment of Cr (VI) effluents., in: A.K. Sengupta (Ed.) Hazardous and industrial Wastes, Technomics Publishing Co.. pp. 223-232.
29. Uslu, G. and Tanyol, M. (2006): Equilibrium and thermo dynamic parameters of single and binary mixture biosorption of lead (II) and copper (II) ions onto *Pseudomonas putida* : effect of temperature , J. Hazard. Mater., 135: 87–93.
30. Zou, W. H., Han, R. P., Chen, Z. Z., Shi, J. and Liu, H. M. (2006): Characterization and properties of manganese oxide coated zeolite (MOCZ) as adsorbent for removal of copper(II) and lead (II) ions from solution . J. Chem. Eng. Data., 51:534–541.
31. Vasudevan, P., Padmavathy, V. and Dhingra, S. C. (2002): Biosorption of monovalent and divalent ions on bakers yeast. Bioresour.Technol. , .82: 285–289.
32. Veglio, F. and Beolchini, F. (1997): Removal of metals by biosorption: a review. Hydrometallurgy, 44:301–16.
33. Volesky, B. (1990): Biosorption and biosorbents. In: Volesky B, editor. Biosorption of Heavy Metals. Florida: CRC press; p.3–5.
34. Volesky, B. and May-Phillips, H. A. (1995): Biosorption of heavy metals by by *Saccharomyces cerevisiae* Appl. Microbiol. Biotechnol., 42: 797-806 .
35. Wang, J. L. (2002): Immobilization techniques for biocatalysts and water Pollution control. Beijing: Science Press; [in Chinese].
36. Wang, J. and Li, Y. (2006): Chemical reduction /oxidation ,in : Wang, L.K. Pereira, N.C. Hung Y. (Eds.), Advanced Physicochemical Treatment Processes, Handbook of Environmental Engineering, vol. 4, Humana Press, p.485, cap 15.
37. Wang, J.L. and Chen, C. (2009): Biosorbents for heavy metals removal and their future, Biotechnol. Adv.27:195–226.
38. Wilhelmi, B. S. and Duncan, J. R. (1996): Reusability of immobilised *Saccharomyces cerevisiae* with successive adsorption–desorption cycle Biotechnology Letters., 18 : 531-536.

9/9/2010

Neuro Fuzzy Modeling Scheme for the Prediction of Air Pollution

Tharwat E. Alhanafy[*1], Fareed Zaghlool[1], and Abdou Saad El Din Moustafa [3]

[1]Computer and System Engineering Department, Al-Azhar University, Cairo, Egypt
[2]Director General, Arab Co. for Engineering & Systems Consultations (AEC) Certified Lead Auditor.
dr. s_ewiss@yahoo.com inf@aecegypt.org

Abstract: The techniques of artificial intelligence based in fuzzy logic and neural networks are frequently applied together. The reasons to combine these two paradigms come out of the difficulties and inherent limitations of each isolated paradigm. Hybrid of Artificial Neural Networks (ANN) and Fuzzy Inference Systems (FIS) have attracted the growing interest of researchers in various scientific and engineering areas due to the growing need of adaptive intelligent systems to solve the real world problems. ANN learns from scratch by adjusting the interconnections between layers. FIS is a popular computing framework based on the concept of fuzzy set theory, fuzzy if-then rules, and fuzzy reasoning. The structure of the model is based on three-layered neural fuzzy architecture with back propagation learning algorithm. The main objective of this paper is two folds. The first objective is to develop Fuzzy controller, scheme for the prediction of the changing for the NO2 or SO2, over urban zones based on the measurement of NO2 or SO2 over defined industrial sources. The second objective is to develop a neural net, NN; scheme for the prediction of O3 based on NO2 and SO2 measurements.
[Tharwat E. Alhanafy, Fareed Zaghlool, and Abdou Saad El Din Moustafa. Neuro Fuzzy Modeling Scheme for the Prediction of Air Pollution. Journal of American Science 2010;6(12):605-616]. (ISSN: 1545-1003).

Keywords: Neuro Fuzzy Modeling Scheme for the Prediction of Air Pollution

1. Introduction:

The modern techniques of artificial intelligence have found application in almost all the fields of the human knowledge. However, a great emphasis is given to the accurate sciences areas; perhaps the biggest expression of the success of these techniques is in engineering field. These two techniques neural Networks and fuzzy logic are many times applied together for solving engineering problems where the classic techniques do not supply an easy and accurate solution. The neuro-fuzzy term was born by the fusing of these two techniques. As each researcher combines these two tools in different way, then, some confusion was created on the exact meaning of this term. Still there is no absolute consensus but in general, the neuro-fuzzy term means a type of system characterized for a similar structure of a fuzzy controller where the fuzzy sets and rules are adjusted using neural networks tuning techniques in an iterative way with data vectors (input and output system data). Such systems show two distinct ways of behavior. In a first phase, called learning phase, it behaves like neural networks that learns its internal parameters off-line. Later, in the execution phase, it behaves like a fuzzy logic system. Separately, each one of these techniques possesses advantages and disadvantages that, when mixed Together, theirs cooperage provides better results than the ones achieved with the use of each isolated technique.

The advantages of a combination of ANN and FIS are obvious. There are several approaches to integrate ANN and FIS and very often it depends on the application. We broadly classify the integration of ANN and FIS into three categories namely concurrent model, cooperative model and fully fused model. This paper starts with a discussion of the features of each model and generalizes the advantages and deficiencies of each model. We further focus the review on the different types of fused neuro-fuzzy systems and citing the advantages and disadvantages of each model.. In fact, this model consists of if then rules with fuzzy antecedents and mathematical functions in the consequent part. The task of system identification is to determine both the non-linear parameters of the antecedents and the linear parameters of the rules consequent.

Air pollution is the introduction of chemicals, particulate matter, or biological materials that cause harm or discomfort to humans or other living organisms, or ,damages the natural environment into the atmosphere.

The atmosphere is a complex dynamic natural gaseous system that is essential to support life on planet Earth. Stratospheric ozone depletion due to air pollution has long been recognized as a threat to human health as well as to the Earth's ecosystems.
Indoor air pollution and urban air quality are listed as two of the world's worst pollution problems in the 2008 Blacksmith Institute World's Worst Polluted Places report.[1]

An air pollutant is known as a substance in the air that can cause harm to humans and the environment. Pollutants can be in the form of solid particles, liquid droplets, or gases. In addition, they may be natural or man-made.[2]

Pollutants can be classified as either primary or secondary. Usually, primary pollutants are substances directly emitted from a process, such as ash from a volcanic eruption, the carbon monoxide gas from a motor vehicle exhaust or sulfur dioxide released from factories.

Secondary pollutants are not emitted directly. Rather, they form in the air when primary pollutants react or interact. An important example of a secondary pollutant is ground level ozone — one of the many secondary pollutants that make up photochemical smog.

Air pollution has become an exceedingly inescapable part of urban living. The presence of pollutants is reported to cause adverse effects on human health as well as damage to structures [1, 2, 3]. Air quality in Cairo City is an important public concern. Average daily emissions of primary pollutants, such as hydrocarbons, nitrogen oxides, carbon monoxide, and others are among the largest in the world. Private and public transportation as well as industrial activities contribute the most to these emissions. When primary pollutants are exposed to sunshine, they undergo chemical reactions and yield a wide variety of secondary pollutants, Ozone, O3, being the most important one. Besides the health problems this molecule may cause, ozone is considered as an indicator of air quality in urban atmospheres [1, 2]

Modeling of urban air pollution is an important facet of pollution control and abatement [1, 2, 3]. Models explain the occurrence, intensity, and movement of pollutants in order to predict pollutant levels at locations away from defined sources. Air pollution prediction is inherently a difficult problem for conventional and stochastic modeling methods due to its intrinsic dynamic, random, and nonlinear nature. In this paper, however; a sophisticated modeling scheme for the prediction of air pollution (nitrogen dioxide NO2, sulpher dioxide SO2 and ozone O3) using neural nets is proposed. Neural network modeling scheme provides an efficient computational tool for mapping input-output or cause-effect relationships and establish an intelligent what if scenarios based on robust learning mechanisms. The proposed prediction schemes have been applied to study the effect of industrial and traffic areas: Tabbin, Shoubra, Fum elkhalieg, Gomhorya and Kulaly on urban areas: Maadi and Giza.

2. Problem Formulation

The prediction problem has been formulated as follows:

(a) For given measured readings of NO2 and SO2 emissions at measured values of temperature, wind speed, and wind direction in industrial and dense traffic areas; what will be the predicted emission values of NO2 and SO2 at urban areas?

(b) For given measured readings of NO2 and SO2 emissions at measured values of temperature, wind speed, and wind direction in industrial and dense traffic areas; what will be the predicted emission values of O3 at urban areas?

Due to the complex relation between inputs and outputs, neural net stands as a reliable mapping tool for this application. The proposed neural net first prediction scheme takes industrial area readings (NO2 or SO2 level, temperature T, winds speed WS and wind direction WD) as input values and computes NO2 or SO2 estimates for urban areas. The second prediction scheme computes estimates of O3 levels as output values based on NO2, SO2, temperature, wind speed, wind direction input values. The neural net schemes are reconfigured to provide category or class (safe, acceptable, not acceptable, and dangerous) for output (NO2 or SO2 or O3) levels.

The neural net forecasting scheme works in two sequential modes of operation [4, 5, 6, 7]. The first mode is learning under supervision, and the second mode is autonomous operation and testing.

3- Fuzzy Systems

Fuzzy systems propose a mathematic calculus to translate the subjective human knowledge of the real processes. This is a way to manipulate practical knowledge with some level of uncertainty. The fuzzy sets theory was initiated by Lofti Zadeh [16], in 1965. The behavior of such systems is described through a set of fuzzy rules, like:

IF <premise> THEN <consequent>

that uses linguistics variables with symbolic terms. Each term represents a fuzzy set. The terms of the input space (typically 5-7 for each linguistic variable) compose the fuzzy partition. The fuzzy inference mechanism consists of three stages: in the first stage, the values of the numerical inputs are mapped by a function according to a degree of compatibility of the respective fuzzy sets, this operation can be called fuzzyfication. In the second stage, the fuzzy system processes the rules in accordance with the firing strengths of the inputs. In the third stage, the resultant fuzzy values are transformed again into numerical values; this operation can be called defuzzyfication. Essentially, this procedure makes possible the use fuzzy categories in representation of words and

abstracts ideas of the human beings in the description of the decision taking procedure. The advantages of the fuzzy systems are: capacity to represent inherent uncertainties of the human knowledge with linguistic variables; simple interaction of the expert of the domain with the engineer designer of the system; easy interpretation of the results, because of the natural rules representation; easy extension of the base of knowledge through the addition of new rules; robustness in relation of the possible disturbances in the system. And its disadvantages are: incapable to generalize, or either, it only answers to what is written in its rule base; not robust in relation the topological changes of the system, such changes would demand alterations in the rule base; depends on the existence of a expert to determine the inference logical rules;

4 Neural Networks

The neural networks try to shape the biological functions of the human brain. This leads to the idealization of the neurons as discrete units of distributed processing. Its local or global connections inside of a net also are idealized, thus leading to the capacity of the nervous system in assimilating, learning or to foresee reactions or decisions to be taken. W. S. McCulloch, W. Pits, described the first Neural Network model and F. Rosenblatt (Perceptron) and B. Widrow (Adaline) develop the first training algorithm. The main characteristic of the neural networks is the fact that these structures can learn with examples (training vectors, input and output samples of the system). The neural networks modifies its internal structure and the weights of the connections between its artificial neurons to make the mapping, with a level of acceptable error for the application, of the relation input/output that represent the behavior of the modeled system. The advantages of the neural networks are: learning capacity; generalization capacity; robustness in relation to disturbances. And its disadvantages are: impossible interpretation of the functionality; difficulty in determining the number of layers and number of neurons.

5 Neuro Fuzzy Systems

Since the moment that fuzzy systems become popular in industrial application, the community perceived that the development of a fuzzy system with good performance is not an easy task. The problem of finding membership functions and appropriate rules is frequently a tiring process of attempt and error. This lead to the idea of applying learning algorithms to the fuzzy systems. The neural networks, that have efficient learning algorithms, had been presented as an alternative to automate or to support the development of tuning fuzzy systems. The first studies of the neuro-fuzzy systems date of the beginning of the 90's decade, with Jang, Lin and Lee in 1991, Berenji in 1992 and Nauck from 1993, etc. The majority of the first applications were in process control. Gradually, its application spread for all the areas of the knowledge like, data analysis, data classification, imperfections detection and support to decision-making, etc. Neural networks and fuzzy systems can be combined to join its advantages and to cure its individual illness. Neural networks introduce its computational characteristics of learning in the fuzzy systems and receive from them the interpretation and clarity of systems representation. Thus, the disadvantages of the fuzzy systems are compensated by the capacities of the neural networks. These techniques are complementary, which justifies its use together.

5 Models of fuzzy neural systems

In response to linguistic statements, the fuzzy interface block provides an input vector to a multi-layer neural network [15]. The neural network can be adapted (trained) to yield desired command outputs or decisions as shown in Fig. (1). Fig. (2) shows the second model of fuzzy neural system. Fig (3) shows the SimuLink Model of fuzzy Logic Controller

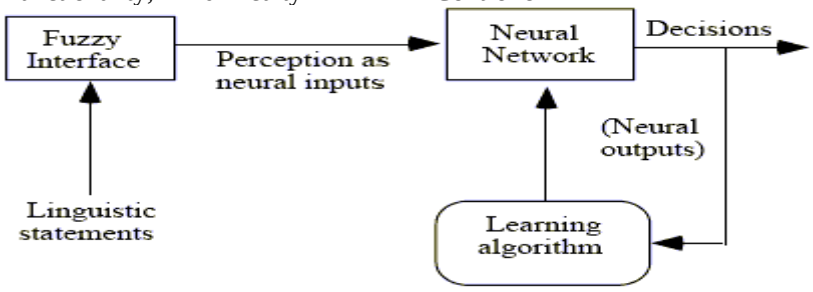


Fig. (1) First Model of Fuzzy Neural Systems

• A multi-layered neural network drives the fuzzy inference mechanism.

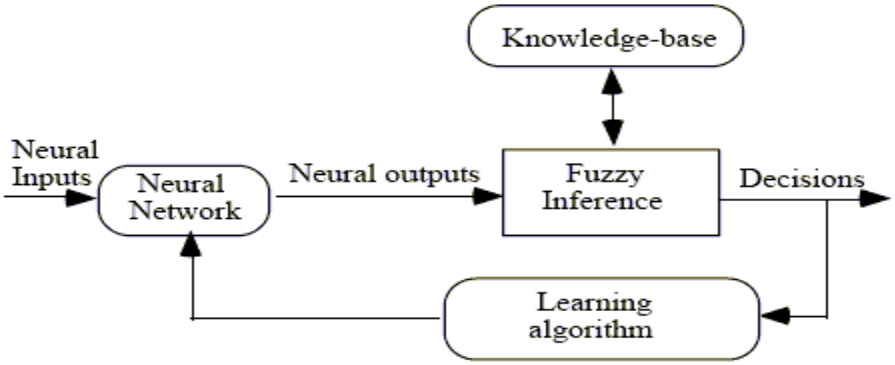


Fig. (2) Second model of fuzzy neural system

In this paper we are using the First Model of Fuzzy Neural Systems. The structure of Fuzzy Model is presented in Fig (11). The initial membership function is shown in Fig (12, 13, 14) for inputs. Fig (17) Membership from inputs to outputs flow of rule base. The system response SO2, NO2, O3 are shown in Fig(18, 19, 20). Fig(21, 22, 23) show the three dimensional of SO2, NO2, O3. The simplification rule base used in the implementation as follow.

Rule Base

1-If (error is small) and (c_of_error is Small) then (So2 is Small)(No2 is Small)(O3 is Small)

2-If (error is Medium) and (c_of_error is Medium) then (So2 is Medium)(No2 is Medium)(O3 is Medium)

3-If (error is small) and (c_of_error is big) then (So2 is Big)(No2 is Big)(O3 is Big)

4-If (error is Big) and (c_of_error is Big) then (So2 is Big)(No2 is Big)(O3 is Big)

5-If (error is small) and (c_of_error is Small) then (So2 is Small)(No2 is Small)(O3 is Small)

6-If (error is Big) and (c_of_error is Small) then (So2 is Small)(No2 is Small)(O3 is Small)

7-If (error is Big) and (c_of_error is Medium) then (So2 is Medium)(No2 is Medium)(O3 is Medium)

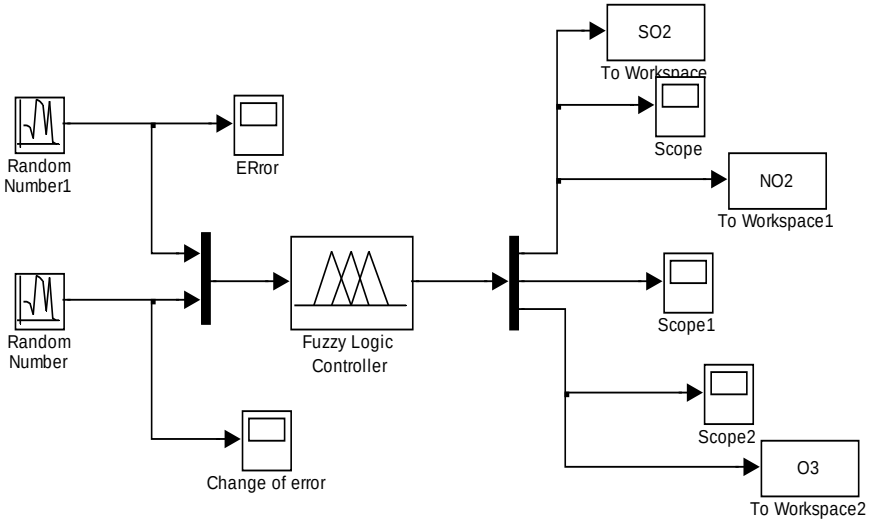


Fig (3) SimuLink Model of fuzzy Logic Controller

6. Data preparation

Recorded Data for the amount of NO2, SO2, and O3 in air have been obtained from Egyptian environmental affairs Authority (EEAA) in the form of average value per month for the years 1998, 1999 for the following areas:

(One) Industrial areas: Tabbin and Shoubra. (b) Traffic areas: Fum elkhalieg, Gomhorya, and Kulaly. © Urban areas: Maadi and Giza.

Normally distributed emission data have been generated using given mean values, and assuming variance values. Available data lie mainly only in the first two classes or categories. In order to completely perform the learning or training phase of the classifier, data samples for the second two classes have been generated within the limits of each class.

Data of temperature, wind speed, and wind direction have been obtained from weather Forecasting Authority for the years 1998, 1999. Data of temperature has been provided in the form of: (minimum, maximum, and average) temperature values (in degree centigrade) per month. Wind speed has been provided as average value in knots per month. Wind directions have been provided in the form of a table with rows representing twelve dominant wind direction sectors, columns representing range of dominant wind speed values, and cell value representing time duration of specific wind speed range within a specific wind direction sector. Based on theses available statistically abstracted data, thirty (assuming one reading/day) normally distributed temperature values and thirty normally distributed wind speed values have been generated, see Fig.4 and Fig.5. Thirty wind direction values have also been generated based on relative time duration ratio.

7. Neural Networks Modeling Schemes

Neural network is based on computer simulation of activities of human brain; neural network performs modeling without defined mathematical relation between variables. Neural network has two distinct learning techniques unsupervised Learning and supervised Learning.

The proposed prediction schemes use three-layered neural nets with supervised back propagation learning algorithm [4, 5, 6, 7]. The first neural net for the prediction of O3 level is shown in Fig.6. The input layer has five nodes (NO2, SO2, WS, WD, T), the middle hidden layer has (on the average) 15 nodes, and the output layer has one complex node (O3). The second neural has the same architecture as the first neural net, but with four input nodes (NO2 or SO2, WS, WD, T). The output node provides either NO2 or SO2 level based on the input feature vector first element value (NO2 or SO2). Neural nets are also reconfigured to have four nodes in the output with only one node is firing at a time representing the category or class (safe S , acceptable A, not acceptable NA, dangerous D) of output O3 level in the first neural net, and NO2 or SO2 category in the second neural net, see Fig.7.

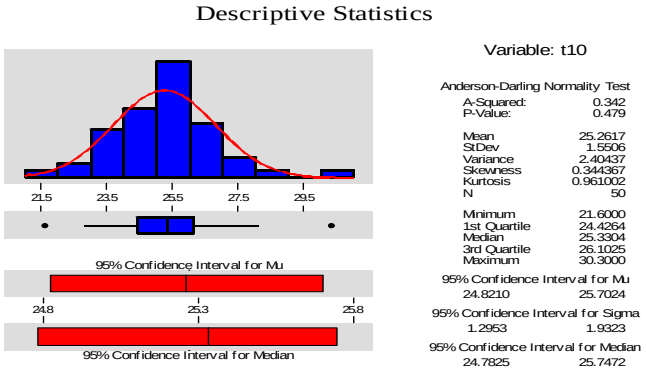


Fig.4. Descriptive statistic of generated data of Oct., temperature

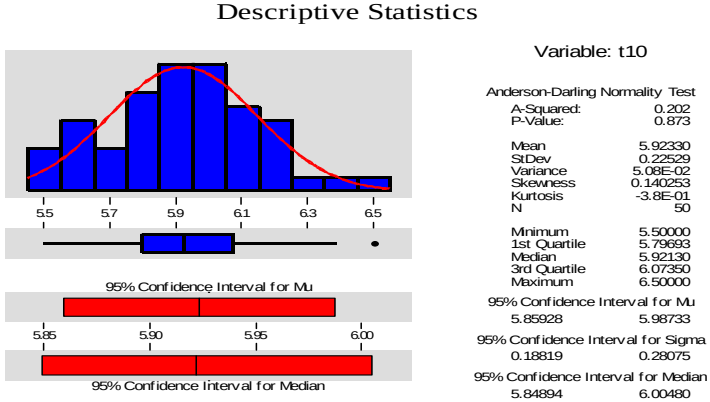


Fig.5.Descriptive statistic of generated data of Oct., wind speed

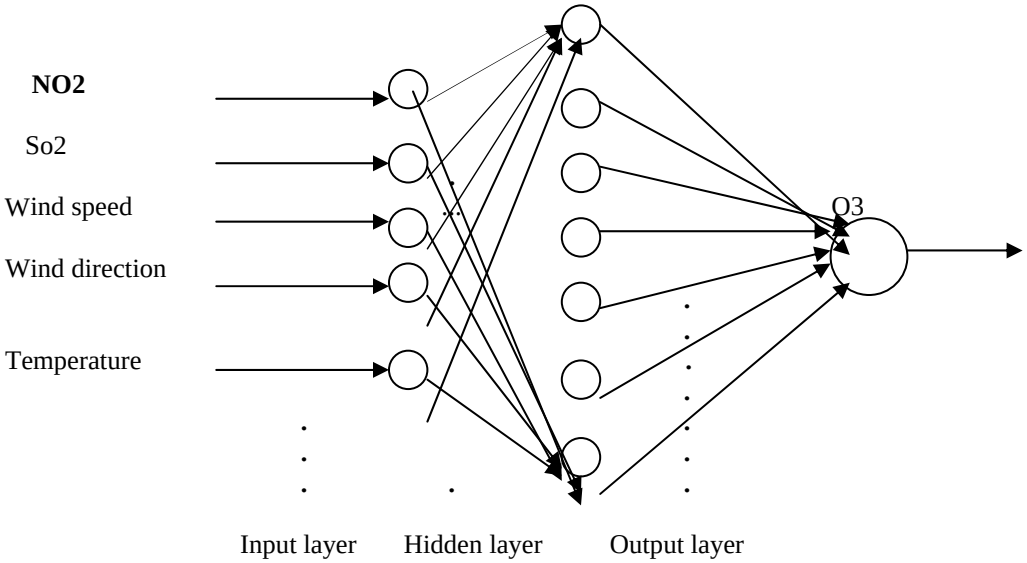


Fig.6. Neural net model for ozone prediction: output, based on measured (NO2, SO2, wind speed and direction, temperature): input

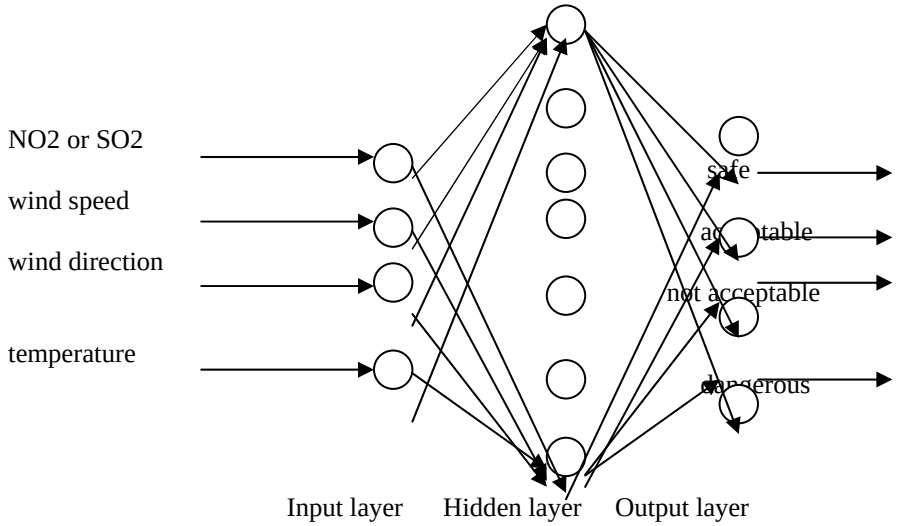


Fig.7. Neural net classification scheme for catogorizing (on four classes) NO2 or SO2 levels on urban areas: output, based on measured level values of (NO2 or SO2, wind speed, wind direction, temperature) on industrial areas : input.

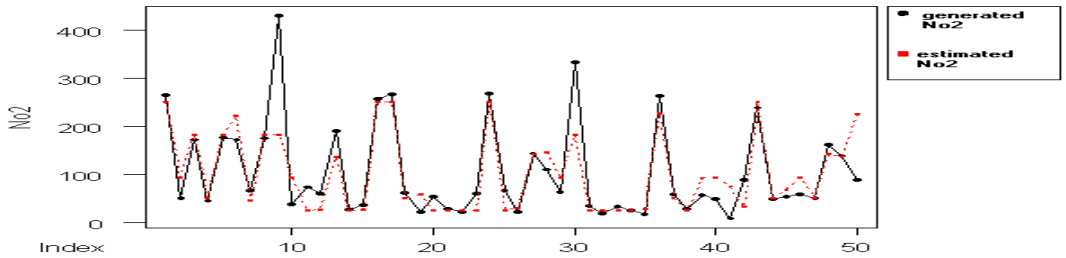


Fig.8. Graph of No2: measured (solid line) and predicted (dotted line)

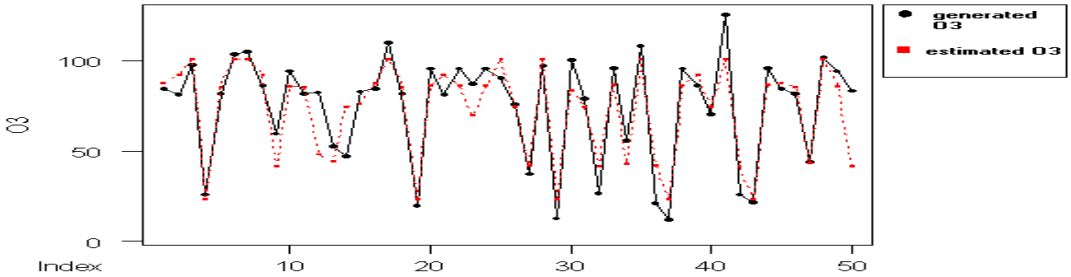


Fig.9 Graph of So2: measured (solid line) and predicted (dotted line)

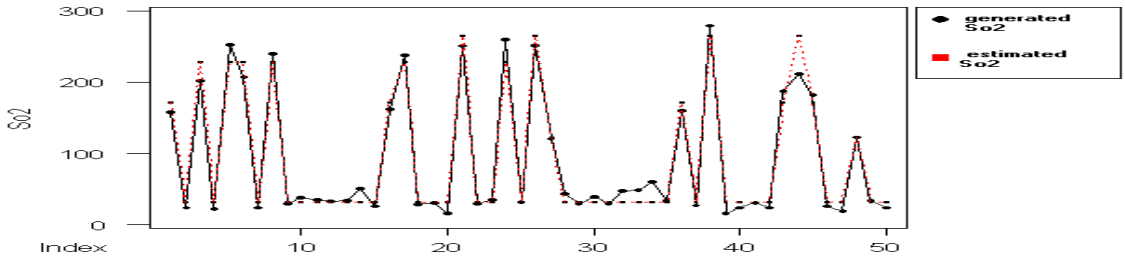


Fig.10 Graph of O3: measured (solid line), and predicted (dotted line)

6. Results and Performance Evaluation

Emissions of NO2 or SO2 on urban area can be categorized as shown in table1**.** The neural net schemes have been set as follows: train data set: 85 %, validation data set: 5%, and test data: 10% where data order is set to be random.

Results of NO2, SO2, and O3 classification nets are summarized in performance tables 2, 3, and 4, where diagonal data represent correct class and off-diagonal represent misclassify data. Sample of the results of neural net prediction schemes for NO2, SO2, O3 are shown in figures 8, 9, and 10. The performance of the prediction scheme is evaluated in terms of mean squared error MSE as recorded in table 5, where the first column provides the range of reading values for NO2, SO2 or O3.

Table1.Range and categories of NO2 and SO2 emissions

Category	Range	
	NO2/SO2	O3
Safe (S)	0-100	0-30
Acceptable (A)	101-150	31-50
Not acceptable (NA)	151-200	50-100
Dangerous (D)	>200	>100

Table 2. NO2 classifier performance table

Year	1998				1998 and 1999				1999			
Class / categ.	S	A	NA	D	S	A	NA	D	S	A	NA	D
S	86	8	0	0	108	6	0	0	165	0	0	0
A	14	30	0	0	13	23	0	0	1	0	0	0
NA	1	8	0	0	0	13	0	0	0	0	0	0
D	0	8	0	0	0	3	0	0	0	0	0	0
% correctrecog	77.33336 %				78.915665 %				99.397591 %			

Table 3. SO2 classifier performance table

Year	1998				1998 and 1999				1999			
Class / categ.	S	A	NA	D	S	A	NA	D	S	A	NA	D
S	43	0	0	0	96	1	0	0	64	0	0	0
A	4	5	0	1	2	4	3	0	0	0	0	1
NA	0	3	0	1	0	3	18	2	0	0	4	6
D	0	1	0	2	0	2	1	33	0	0	0	30
correc recog.	83.3 %				91.5 %				93.3%			

Table 4. O3 classifier performance table

	Safe	Accept	Not Accept	Dangerous
Safe	10	1	0	0
Accept	2	0	2	0
Not Accept	1	0	48	0
Dangerous	0	0	10	0

Average percentage of correct recognition for O3 classification scheme is 80 %

Table 5. Performance table for prediction neural net schemes.

	Rang	1998	1999	1998and 1999
NO2	10-400	20.53	7.726	16.84
SO2	10-290	15.45	6.89	13.486
O3	20-170	8.505	----	------

7. Conclusion

This paper presented proposed fuzzy neural schemes for forecasting and classifying of NO2; SO2 emissions over urban areas based on measured emissions over industrial areas. The scheme also provides predictions of O3 emissions based on NO2 and SO2 measurements. The performance of the proposed scheme is evaluated in terms of average percentage of correct recognition and mean squared error value, however the accuracy of the performance is limited to the available data. In other words some of the data are provided in terms of mean value per month like NO2, SO2, O3 emissions, other data are either provided in terms of range of values like wind directions, or minimum and maximum values per month like temperature. Data have been generated from normal distributions with available provided mean, variance (or proposed), and range parameters. However, correlation of specific day data (temperature, wind speed, wind direction, NO2 or SO2 or O3 measurement) is not guaranteed since day data are statistically generated assuming one measurement per day. System performance could be more accurate and more reliable if detailed true daily-recorded data are used.

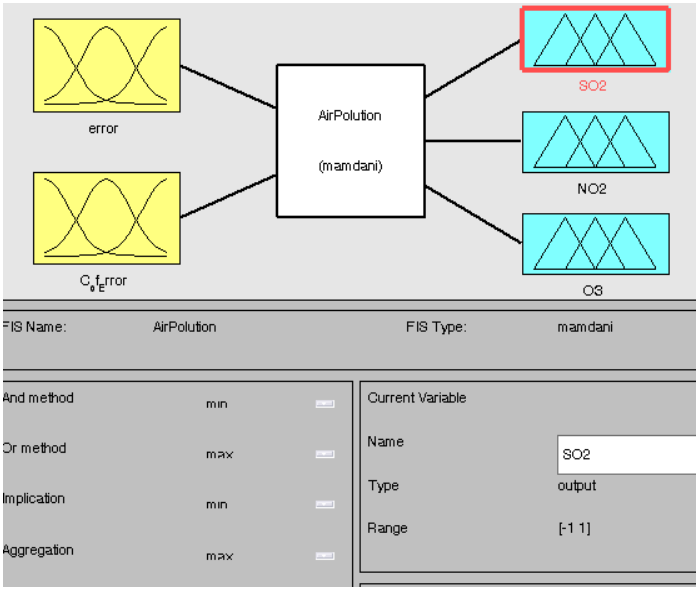


Fig (11) Fuzzy Model

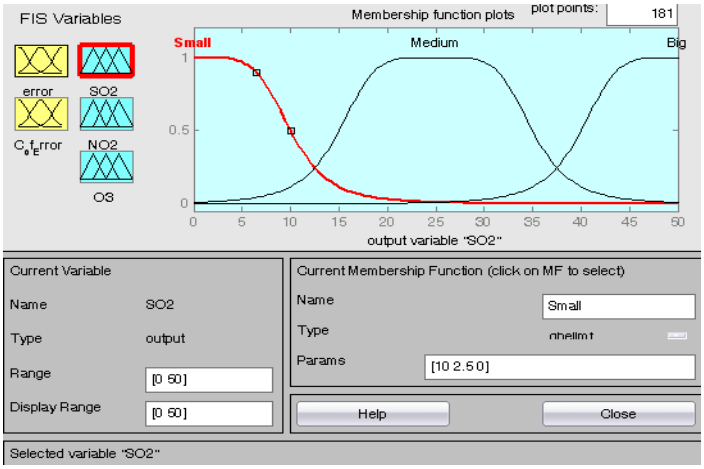


Fig (12) Membership Function For SO2

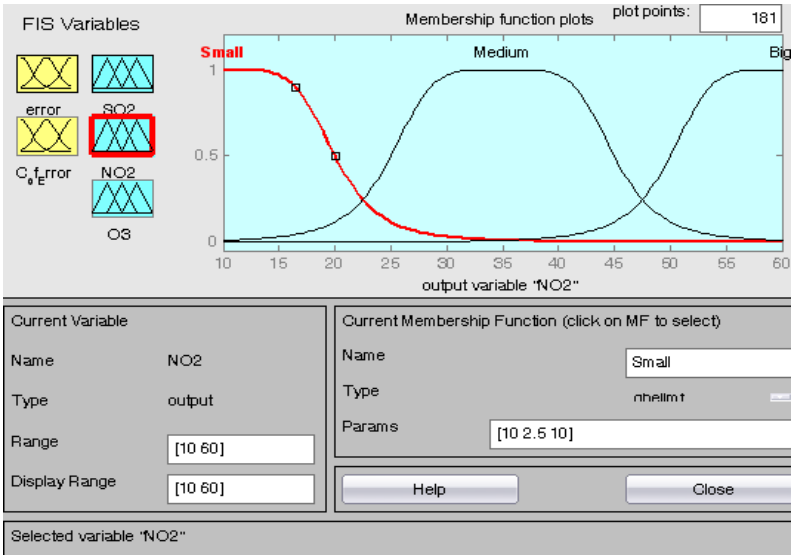


Fig (13)Membership Function For NO2

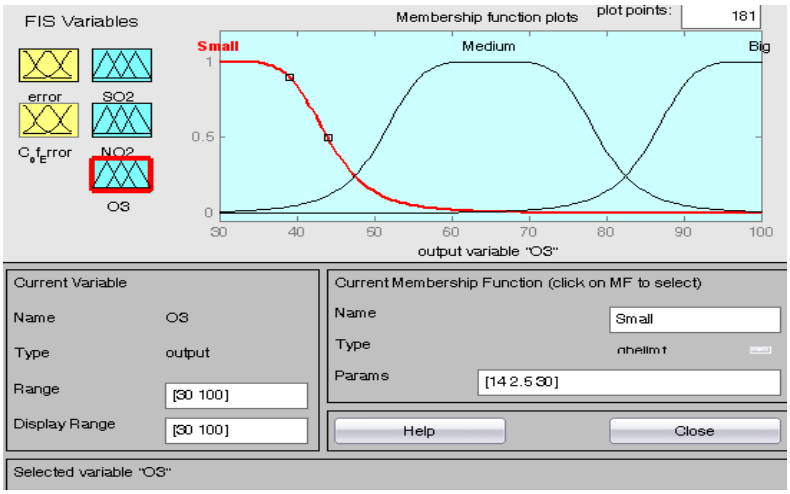


Fig (14) Membership Function For O3

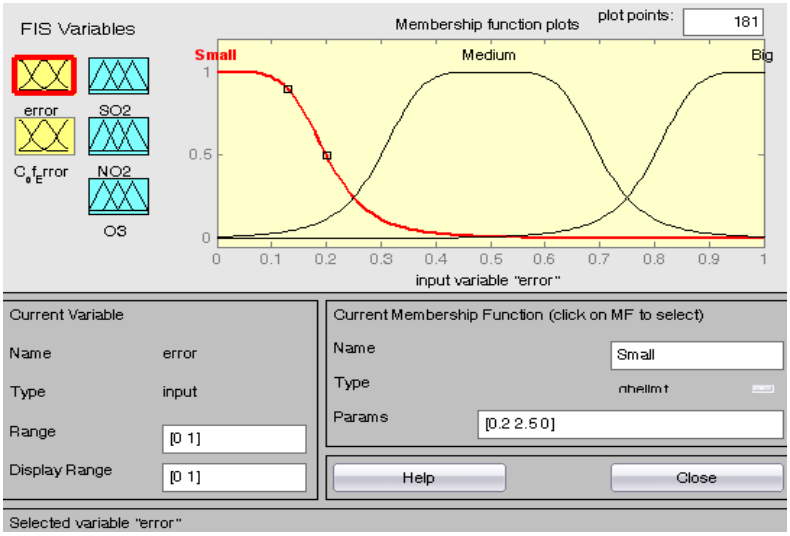


Fig (15) Membership Function for Error

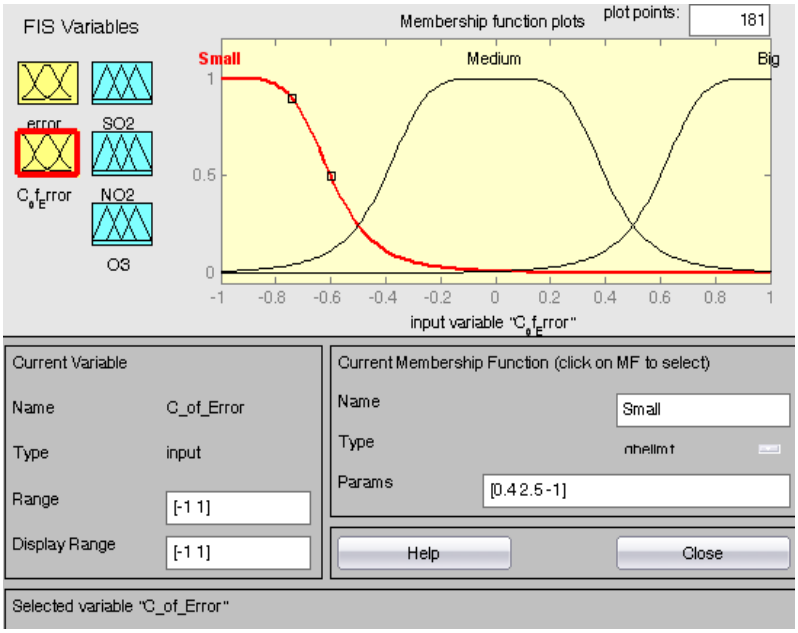


Fig (16) Membership Function For Change of Error

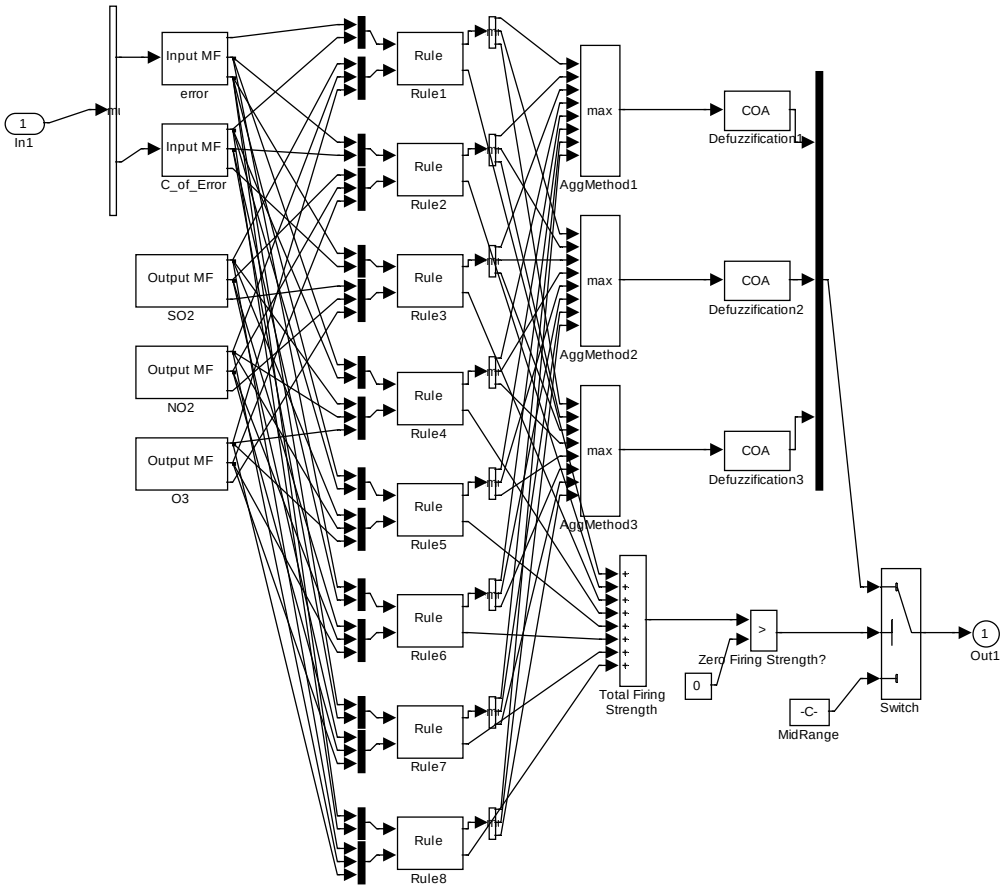


Fig (17) Membership from inputs to outputs flow of rule base

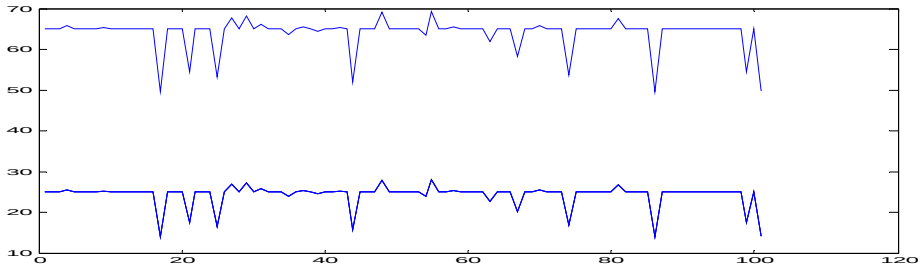


Fig (18) System Response of So2

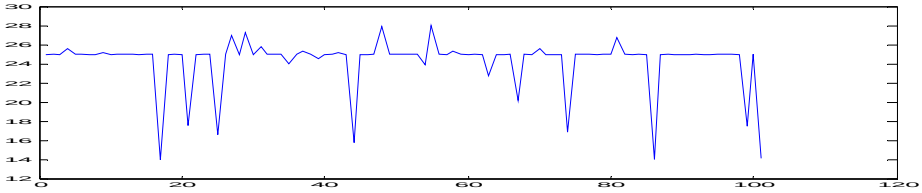


Fig (19) System Response of No2

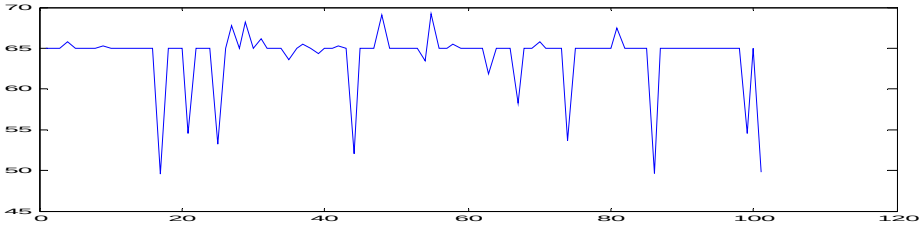


Fig (20) System Response of O3

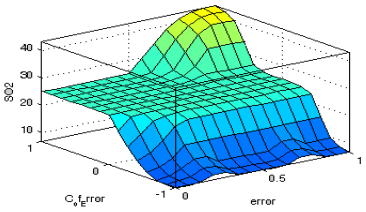
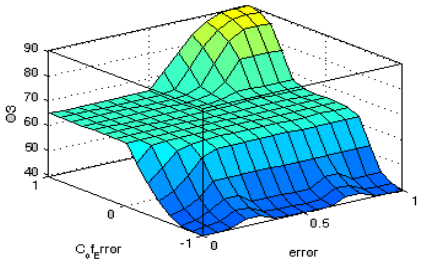


Fig (21) System Response of So2



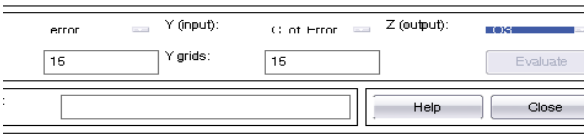


Fig (23) System Response of O3

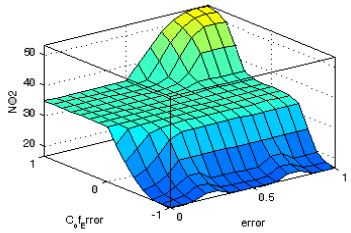


Fig (22) System Response of No2

Corresponding author
Tharwat E. Alhanafy
Computer and System Engineering Department, Al-Azhar University, Cairo, Egypt
Dr. s_ewiss@yahoo.com

8. References:

1. A. Abraham and Baikunth Nath, " Hybrid Intelligent Systems: A Review of a decade of Research", School of Computing and Information Technology, Faculty of Information Technology , Monash University, Australia, Technical Report Series, 5/2000, 2000, pp. 1-55.
2. H. R. Berenji and P. Khedkar, "Learning and Tuning Fuzzy Logic Controllers through Reinforcements", IEEE Transactions on Neural Networks, 1992, Vol. 3, pp. 724-740.
3. E. Czogala and J. Leski, "Neuro-Fuzzy Intelligent Systems, Studies in Fuzziness and Soft Computing", Springer Verlag, Germany, 2000.
4. M. Figueiredo and F. Gomide; "Design of Fuzzy Systems Using Neuro-Fuzzy Networks", IEEE Transactions on Neural Networks, 1999, Vol. 10, no. 4, pp.815-827.
5. R. Jang, "Neuro-Fuzzy Modelling: Architectures, Analysis and Applications", PhD Thesis, University of California, Berkley, July 1992.
6. F. C. Juang, T. Chin Lin, "An On-Line Self Constructing Neural Fuzzy Inference Network and its applications", IEEE Transactions on Fuzzy Systems, 1998, Vol. 6, pp. 12-32.
7. N. Kasabov e Qun Song, "Dynamic Evolving Fuzzy Neural Networks with 'm-out-of-n' Activation Nodes for On-Line Adaptive Systems", Technical Report TR99/04, Departement of Information Science, University of Otago, 1999.
8. B. Kosko, "Neural Networks and Fuzzy Systems: A Dynamical System Approach to Machine Intelligence", Prentice Hall, Englewood Cliffs, New Jersey, 1992.
9. [9] T. C. Lin, C. S. Lee, "Neural Network Based Fuzzy Logic Control and Decision System", IEEE Transactions on Computers, 1991, Vol. 40, no. 12, pp. 1320-1336.
10. [D. Nauck, F. Klawon; R. Kruse, "Foundations of Neuro-Fuzzy Systems", J. Wiley & Sons, 1997.
11. J.C. Ruiz-Suarez, O.A. Mayora, R. Smith-perez, L.G. Ruiz-Suarez, "A Neural Network-based Prediction Model of Ozone For Mexico City", Proceeding of air Pollution Theory and Simulation Conference 2000, pp.393-400.
12. Faizal A. Hasham, Stephen J. Stanley, Warren B. Kindzierski, "Modelling Of Urban Air Pollution In The Edmonton Strathcona industrial Area Using Artificial Neural Networks", Proceeding of air Pollution Theory and Simulation Conference 2000, pp.246-255.
13. M. Boznar, P. Mlakar, "Neural Networks- A New Mathematical Tool For Air Pollution Modelling", Proceeding of air Pollution Theory and Simulation Conference 2000, pp.259-266.
14. A.M.ELramsisi, Osama S. Elshehry, "A Fractal-Based Approach For Radar Target Recognition Using Neural Networks," Proceedings of the first International Conference on Electrical Engineering,ICEENG, Military Technical College, Cairo,Egypt, 24-26 March, 1998, pp. 383-390.
15. Mohamed Zaki, Abdallah EL-Ramsisi, Rostom Omran, " A Soft Computing Approach For Accurate Recognition of Occluded Shapes," journal of intelligent and fuzzy systems, 9(2000) 85-99 ISSN 1064-1246, IOS press
16. A.M. ELramsisi, W.I. Khedr, " A Computer Security Scheme for Dynamic User Identification, and Intruder Detection Using Neural Network Behavior-based Classifier," Proceedings of the nineth International Conference on Computer Theory and Applications ICCTA'99, Alexandaria, Egypt, August 28-30, 1999, pp. 13-22.
17. A.M.ELramsisi, Osama S. Elshehry, "Three-View Target Recognition Using Neural Nets With Fractal Features and Moments Invariants In The Presence Of Noise," Proceedings of the sixth International Conference on Artificial Intelligence Applications, ICAIA' 98 Cairo, Egypt, Feb. 18-23, 1998, pp. 183-195.

9/26/2010

Ester Phosphate of Discarded Palm Oil from Potato Chip Factories as Fat-Liquoring Agent

Mohamed G. Megahed[1] and El-Shahat H. A. Nashy[2*]

[1]Department of Fats and Oils, National Research Centre, Dokki, Cairo, Egypt.
[2]Department of Chemistry of Tanning Materials and Leather Technology, National Research Centre, Dokki, Cairo, Egypt.
[*]nashy_eha@yahoo.com & dr_mgmegahed@hotmail.com

Abstract

In Egypt most potato chip factories used palm oil for frying. The quantity of palm oil resulting from frying processes as discarded represents more than half of the total other oils used in the Egyptian food factories. Discarded palm oil resulting from frying processes was preliminary treated by purification and bleaching as well as characterized via its physico-chemical properties and fatty acids composition. Therefore, this work was devoted to explore the application of the discarded palm oil in leather industry as fat-liquoring agent.

Fat-liquors help to prevent the loosening of leather grain and ugly appearance of chrome tanned leather after drying. In addition, fat-liquoring process improves leather characters such as soft handle, full, flexibility, and pliability as well as enhancement its mechanical properties. The study involved preparation of discarded palm fat-liquor via phosphoration process. The importance of the prepared fat-liquor is due to their environmentally friendly nature, relatively safe utilization by human being, in addition to their economical feasibility. The fat-liquored leather led to an improvement in the mechanical properties of the leather e.g. tensile strength, elongation at break and tear strength. In addition a great enhancement in the texture of the treated leather by discarded palm fat-liquor as indicated from the scanning electron microscope (SEM).

[M. G. Megahed and El-Shahat H. A. Nashy. Ester Phosphate of Discarded Palm Oil from Potato Chip Factories as Fat-Liquoring Agent. Journal of American Science 2010;6(12):617-626]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Discarded Palm Oil, Fatty Acids, Fat-liquor, Phosphoration, Chrome Tanned Leather, FT-IR, HLB, Strength Properties, Scanning Electron Microscope, Frying wastes.

1. Introduction

In the leather industry, hides and skins proceed via various chemical and mechanical processes to produce finished leather. Chrome tannage has been the state of the art, it accounts over 80% of the activity in the tanning process [1] and used for the production of various types of leather [2]. But chrome-tanned leather when dried out becomes bony, hard and thus will not be suitable for use in most purposes, besides its color becomes dark and gains a disagreeable appearance. This means that, as water is removed during the drying stage, cohesion of the fibers takes place resulting in hard intractable leather which is difficult to rehydrate [3]. Thus, incorporation of fatty matter into leather through fat-liquoring process will decrease the effect of air oxidation and improves leather full and soft handle, flexibility, pliability and at the same time enhance its mechanical properties [4-6]. Therefore, introducing a lubricant into the leather keeps the fibers apart during drying and reduces the frictional forces within the fiber weaves thus allowing the fibers to move laterally over each other.

The palm oil used essentially for frying can be blended with various plant oils in different proportions to obtain liquid oil [7]. Most discarded edible oils (include palm oil & palm kernel oil) and fats are of immense importance for industrial applications as surfactant and soap manufacture [8-12]. Therefore, this work was devoted to explore the application of the discarded palm oil in leather industry as fat-liquoring agent.

The potato chip factories, in Egypt, use palm oil for frying of potato chips which are considerably popular as food in Egypt. Potato chip factories produce a large quantity of discarded palm oil. The large quantity of these oils resulting from frying processes causes pollution of environment. In nearly all countries on the earth the protection of the environment has become increasingly a very serious concern. As a result, we can utilize these quantities of wasted oils to obtain useful product.

Therefore, this work was devoted to study the phosphoration of purified discarded palm oil as well as explore its application for further use as leather fat-liquoring. Evaluation of the resulting chrome leather fat-liquored will be taken into consideration. This will add an economical value to this waste and at the same time prevent its environmental pollution. For this purpose a commercially available discarded palm oil (by-product) was used as a starting material.

2. Materials and methods

Materials

- Palm oil samples before and after frying were collected from some Egyptian potato chip factories.
- Chemicals used for different analysis of oil were supplied by international companies (Merk, Germany and BDH, England).
- Ortho- Phosphoric acid (98.5%) was pure chemical grade.
- Local commercial full grain chrome tanned leather was used for the present investigation from Radio Tannery, Cairo, Egypt.

Note: All chemical additives doses were calculated on the basis of leather weight (w/w).

Methods

Analysis of palm Oil

a- Purification of discarded palm oil

Discarded palm oil was heated to 90 ± 5°C and washed with a hot brine solution [5% Na Cl, w/v]. The ratio of discarded oil and brine solution was 10: 1 (v/v). The hot mixture [discarded oil and brine solution] was stirred using a stirrer at a speed of 60 rpm for 60 min. After that, the mixture was left to rest for 8 hrs in order to separate the mixture into two phases. The bottom phase [brine solution +impurities] was separated by siphoning. Sodium sulfite anhydrous was added to catch the traces of moisture from discarded palm oil, which was then filtered through filter paper (Whatman no. 1), Girgis [13]. The color was measured using Lovibond tintometer, model E, using 5.25 inch cell, and impurities of discarded palm oil before and after purification were determined according to the methods described in the A.O.C.S., 1997 [14]; while the odor was evaluated according to the method reported by Shyam [15].

b- Bleaching of purified discarded palm oil

Purified discarded palm oil was heated to 70 ± 5°C and bleached with a 2% H_2O_2 solution (v/v). The sample was stirred using a stirrer at a speed of 80 rpm for 30 min while maintaining a temperature of 70 ± 5°C. The discarded palm oil was filtered through filter paper [Whatman no. 1], Girgis [13], and the color was measured.

c- Physical and chemical characteristics

Palm oil and discarded palm oil were analyzed for its physical and chemical characteristics according to the American Oil Chemists Society Methods, (AOCS, 1998) [16]. Refractive index, acid value, peroxide value, saponification value, iodine value and unsaponifiable matters were measured. The ester value was estimated by subtracting the acid value from saponification value (SV-AV).

d- Fatty acid composition

Fried palm Fatty acid methyl esters were prepared by IUPAC standard methods, 1987 [17], and according to A.O.C.S. method [16]. Determination of fatty acid composition was performed as described by Mitruke [18] using Hewlett Packard HP 5890 series II gas chromatography, equipped with flame ionization detector (FID), operated under the following conditions:

Detector, flame ionization (FID); column, capillary, 30.0 m x 530 µm, 1.0 µm thickness, polyethylene glycol phase (INNO Wax); N_2 with flow rate, 15 ml per min with average velocity 89 cm/s (8.2 psi); H_2 flow rate, 30 ml per min; air flow rate, 300 ml per min; split ratio, 8:1, split flow, 120 ml per min; gas saver, 20 ml per min. Detector temperature, 280ºC; column temperature, 240ºC; injection temperature, 280ºC. Temperature programming starting from 100ºC to reach a maximum of 240ºC was used for eluting the fatty acid methyl esters. The identification of peaks was made as compared with chromatograms of standard fatty acids methyl esters (Sigma, USA).

Phosphoration processes

- phosphoration of the oil (100 g) were carried out in a three necked flask fitted with a stirrer, a thermometer and also with an inlet for the addition of the reagents. Phosphoric acid (H_3PO_4, 30% of the oil weight) was added drop wise at interval times with slow stirring while maintaining the temperature below 30°C during the addition. The overall reaction time was 3 hours.
- The phosphated oil was washed by 10% sodium chloride at ambient temperature and neutralized with ≈ 30% sodium hydroxide with agitation for 30-40 min.
- The phosphated products so obtained were analyzed according to official methods [19, 20]. The product was prepared at a concentration of about 60-70% prior (pH≈7) to its uses as a fat-liquor.

FT-IR Analysis

The change in functional groups of oils were studied using FT-IR analysis, it was performed using Mattson 5000 FTIR, USA spectrophotometer with resolution 4 cm^{-1}.

Hydrophile-Lipophile Balance (HLB)

Hydrophile-lipophile balance of emulsifiers was calculated using the following equation of Griffin [21]:

HLB = 20 (1 — S/A)

Where:

S= (Saponification value of the phosphated fat-liquor).

A = (acid value of the total fatty acids of the original sample).

Fat-Liquoring Process

The leather pieces were first washed with water for about 15 minutes and water drained off. Then neutralization process was carried out using 1% sodium formate and running the drum for 15 minutes. Thereafter, 0.5 % sodium bicarbonate was added and the drum was run for further 10 min. The leather pieces gave a greenish blue color with bromo cresol green throughout the whole thickness (pH 5.0- 5.3). The neutralized leather pieces were washed with water and dyed with acid dye, 5 % for 30 minutes. Then, 6% fat-emulsion was added to the dyeing bath at room temperature. After complete addition of the fat liquor, the drum was run for 40 minutes. The leather pieces were washed with water for about 10 minutes, removed from the drum, sammed, set out and left to dry in air through hanging up at room temperature. The dried leather pieces were used for investigation.

Mechanical Measurements

Fat-liquored leather samples were cut with special steel press knives from the position parallel to the backbone and about 5 cm away from it as specified in the Egyptian Standard Methods, ES-123 [22].

Dumbbell shaped specimens 50 mm length and 4 mm (neck width) were prepared according to ES-123 [22] for mechanical properties measurements using Rauenstein-Tensile force up to 500 Kg. The measured data are the average of four transverse and longitudinal measurements. The cross-head speed was controlled at 50 mm/min and the tests were done at room temperature (25°C).

Tensile Strength

Tensile Strength is calculated in kg/cm^2 from the load required to rupture the test specimen under tension (breaking load) divided by the area of the original cross section of the same specimen.

$$\text{Tensile strength (kg / cm}^2\text{)} = \frac{\text{Breaking load (kg)} \times 100}{\text{Thickness (cm)} \times \text{width (cm)}}$$

Elongation (at break)

The reading on the scale of the machine at the instant when the rupture occurred was taken to calculate the percentage of elongation.

$$\text{Percentage of elongation, \%} = \frac{\text{Increase of length (mm)} \times 100}{\text{Length of specimen, middle part (mm)}}$$

Stitch tear (single hole)

A hole (1×10 mm) was punched on the long axis of the leather specimens. The stitch tear was then calculated from the load required to tear the leather specimen from a steel rod passing through the hole of the specimen.

$$\text{Stitch tear (kg / cm)} = \frac{\text{Breaking load (kg)} \times 10}{\text{Mean thickness of leather sample (mm)}}$$

Scanning Electron Microscope

Specimens of experimental and control were prepared as circular samples (10 mm) and then subjected to sputter coating of gold ions to prepare a conducting medium (sputter coater-Edwards-Model S -150 A, Eng). A Jeol scanning microscope (Japan) JSM-T20 was used for the microscopic study.

3. Results and Discussion:

Purification of Discarded Oil

The effect of washing with brine solution and bleaching with H_2O_2 on the color, impurities and odor of the discarded palm oil are illustrated in Table (1).

Table (1): Effect of washing and bleaching on the color, impurities and odor of the discarded palm oil

Discarded palm oil	Lovibond color	Impurities, (%)	Odor
Before washing	36.0 Y+ 12.7 R+ 1.2 B*	1.4	Like odor to potato frying
After washing (Before bleaching)	36.0 Y+ 12.2 R+ 1.0 B*	0.8	Slight odor to potato frying
After bleaching	35.0 Y+ 6.6 R + 0.5 B*	0.7	Slight odor to potato frying

*** Where: Y= Yellow, R = Red, B = Blue**

Table (1) reveals that, washing with brine solution induces some improvements of the color, odor and impurity levels. Also, it is noticed that addition of H_2O_2 improves the color due to its action as oxidizing agent which reduces the color level of

the oil pigments. These results are in good accordance with previous results reported by Woollatt [23] and Girgis [13]. The improvements may stem from the mutual solubility of the impurities isolated at the bottom [13,24].

Physical and Chemical Properties

Physical and chemical characteristics of palm oil before and after frying have been extensively investigated. The obtained results are shown in Table (2).

Table (2): Physico- chemical properties of palm oil before and after frying.

Properties	Palm oil	
	Before frying	After frying
Refractive index at 40 ºC	1.4570	1.4597
Saponification value, mg KOH / g oil	199.2	199.8
Iodine value, mg I_2 / g oil	49.10	40.5
Acid value, mg KOH / g oil	0.2	1.1
Peroxide value, m Eq/Kg	1.3	6.5
Unsaponifiable matter, (%)	0.3	0.7
Ester value (S.V- A.V), mg KOH /g oil	199.0	198.7

It was noticed from table (2) that, the saponification and ester values of both palm oil before and after frying are almost the same (199.2 , 199.0 and 199.8, 198.7 mg KOH/g oil) respectively. While iodine value before frying had a higher value (49.1 mg I_2/g oil) compared with after frying (40.5 mg I_2 / g oil) [8,13].

It is also clearly seen that, the acid, peroxide values and unsaponifiable matter of palm oil increased after frying (1.1mg/g, 6.5 mEq/kg and 0.7%) compared with palm oil before frying (0.2 mg/g, 1.3 mEq/kg and 0.3%) respectively. These results are similar to those obtained by many investigators [13, 25, 26].

Fatty acids composition

Fatty acids composition of palm oil before and after frying is illustrated in Table (3).

Table: (3) Fatty acids composition of palm oil before and after frying.

Fatty acids (%)	Palm oil	
	Before frying	After frying
Saturated fatty acids:		
Myristic ($C_{14:0}$)	1.82	3.22
Palmitic ($C_{16:0}$)	43.96	45.20
Stearic ($C_{18:0}$)	4.87	7.41
Total:	**50.65**	**59.83**
Unsaturated fatty acids:		
Oleic ($C_{18:1}$)	39.50	36.29
Linoleic ($C_{18:2}$)	9.40	7.57
Linolenic ($C_{18:3}$)	0.45	0.31
Total:	**49.35**	**40.17**

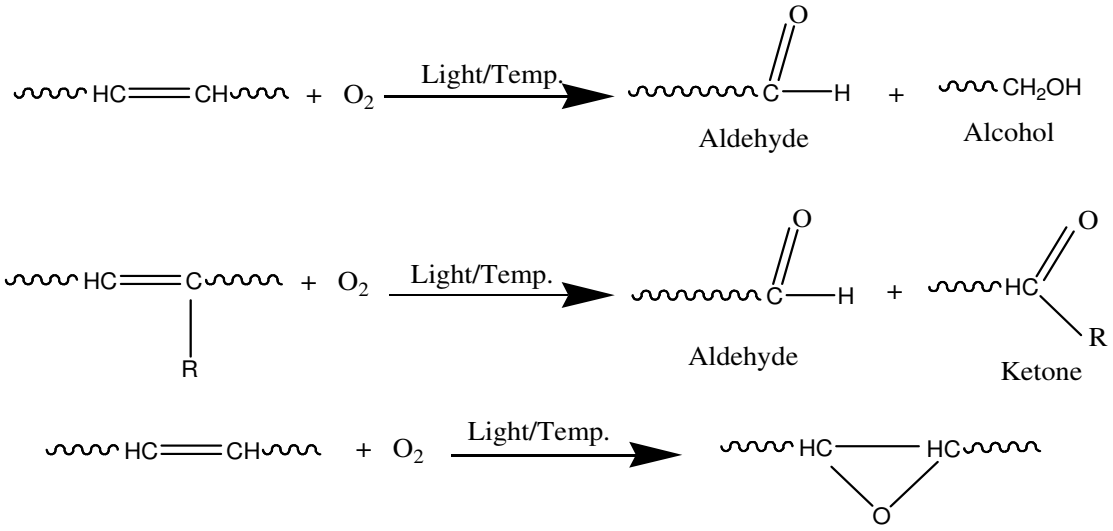
It can be seen from Table (3) that, the saturated fatty acids contents of palm oil before and after frying are (50.65% and 59.83%) and unsaturated fatty acids are (49.35% and 40.17%). Also, it is noticed that, the ratio of total unsaturated fatty acids of palm oil is almost equal to the total saturated fatty acids (1:1.03), while this ratio is different after frying (1:1.49). Oleic acid constitutes more than 80.0% and 90.0% of the unsaturated fatty acids of palm oil and discarded palm oil respectively, while palmitic acids

show more than 43.0% and 45.0% of the total fatty acids, respectively. On the other hand linoleic acid of palm oil has slightly higher value (9.40%) compared with discarded palm oil (7.57%).

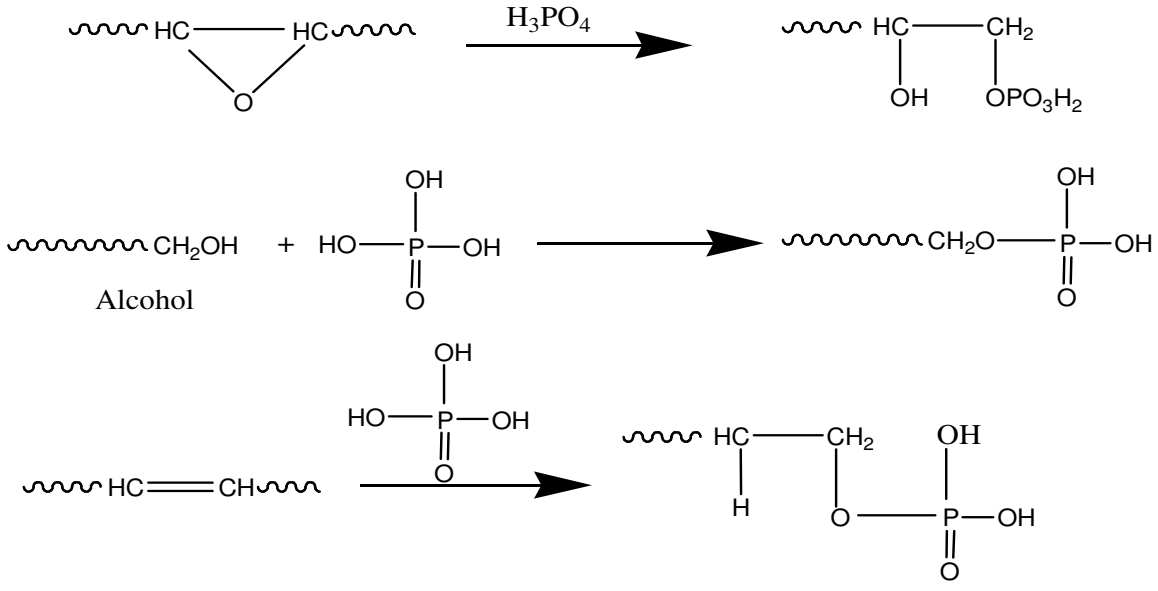
These results are in a good accordance with those reported by Helmy & Megahed [8], Girgis [13], Tyagi & Vasishtha [26], and Megahed [27] whos found that during frying, a progressive decrease in unsaturation was observed in the oil by the determination of iodine value. This decrease in unsaturation could be due to the destruction of double bonds by oxidation, Tables (2,3).

Phosphoration Reaction

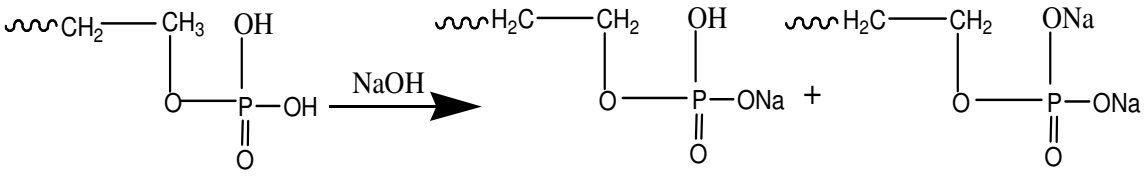
The discarded palm oil is oxidized by air under high temperature resulting in the scission of double bonds leading to increase the reactive centers of the fried oil which easily reacts with H_3PO_4, as illustrated in schemes (1-3). This is confirmed by decreasing the percent of unsaturation fatty acids and iodine value as shown in tables (2,3) and appearance of carbonyl groups in FT-IR spectra, figure (1).



Scheme (1): Oxidation reactions and possible products



Scheme (2): Phosphoration reactions and ester-phosphate products



Scheme (3): Neutralization of Phosphated products

It can be seen from schemes that, the hydrophilicity of the Phosphated product increased due to the formation of polar groups (hydroxyl and ester phosphate groups) as confirmed by FT-IR, Figure (1).

FT-IR Analysis

Figure 1 illustrates the spectrum of palm oil, discarded palm oil and phosphate palm oil samples with the FT-IR/ Absorbance in the range of 4000 to 500 Cm^{-1}. The spectrum of palm oil before frying shows characteristics absorption band associated with common oil. The stretching and bending absorption peaks at 3004 and 723 cm^{-1} are given by olefinic (cis = CH). The strong absorption peaks at around at 2900 to 2850 cm^{-1} are assigned to CH_3 and CH_2 asymmetric stretching vibration. Also, the spectra show stretching absorption bands at 1652 cm^{-1} and 1467 cm^{-1} which correspond to non-conjugated (cis C= C) bond and C-H scissoring respectively, Guillen and Che Man [28, 29]. The spectra of discarded palm oil show stretching absorption bands at 1745 cm^{-1}, 1172 cm^{-1} and 3527 cm^{-1} corresponding to C=O, C-O and OH groups respectively. The appearance of bands at 2720 cm^{-1} indicates the presence of C-H aldehydic group.

While the spectrum of phosphate discarded palm oil shows characteristic absorption peaks at 1090 cm^{-1} corresponding to sym. dihydrogen phosphate and at 1035 cm^{-1} corresponding to asym. groups. It can also be seen from Figure (1) that, a strong broad band appeared at 865 cm^{-1} and (3527 & 3475) cm^{-1} which corresponds to the mono hydrogen phosphate and hydroxyl groups respectively.

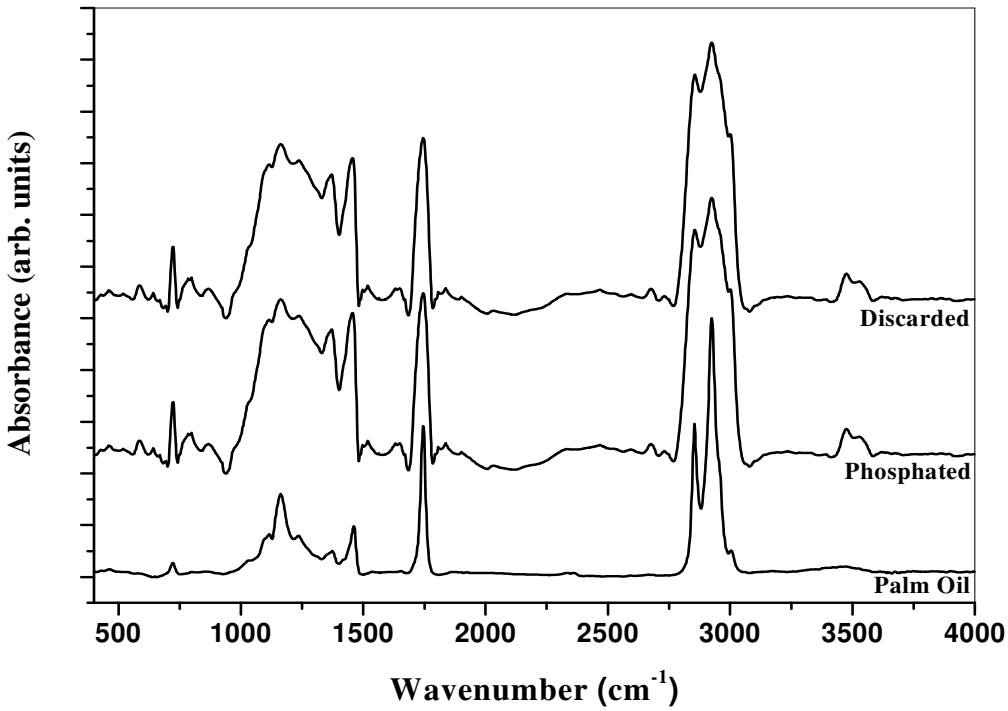


Figure (1): FT-IR Spectra of Palm oil, discarded palm oil and phosphated discarded palm oil.

HLB of the Prepared Fat-liquor

The HLB is an expression of the relative simultaneous attraction of surfactant for water and/or for oil (or for the two phases). The HLB of surfactant (Phosphoated oil) determines the emulsion types that tend to be formed, Griffin [21].

As previously mentioned, fat-liquoring is one of an important step in leather manufacturing, as it is intended to lubricate the leather fibers resulting in softness, pliability, flexibility, full and stretchy depending upon its final utility. Fat liquor helps to prevent both loosening of the leather grain and ugly appearance after drying and at the same time, improving the mechanical properties of leather. Thus, the fat separated from the emulsion is deposited inside the leather and fixed to the leather fibers. Therefore, the stability of fat-liquor in its water emulsion is an important factor in fat-liquoring of leather. i.e., if the fat-liquor is unstable, it cannot give a proper fat-liquoring effect. So that, for the application of the prepared Phosphated fat-liquor in leather treatment, it's necessary to evaluate the stabilization of the fat-liquor emulsion by Hydrophile-Lipophile Balance (HLB).

Therefore, the prepared Phosphated fat-liquor has HLB value of 11.06 i.e., it forms a "O/W" emulsion type, and simply dispersible in water. This means that a fineness of emulsion is formed since Phosphated ester or acids as well as the non-Phosphated portion of the ester present in the fat-liquor are emulsifiable. So that the prepared fat-liquor can form a stable emulsion and transfers from the aqueous bath to the leather and penetrates in it. The fat molecules and the fiber undergo physical bonding. However, the physically bonded fat molecules might undergo some chemical reactions with its surroundings [30].

Mechanical Properties of Fat-liquored Leather

The fat-liquoring process was carried out on neutralized leather using about 5% per 100 g leather. The mechanical tests include the measurement of the tensile strength, elongation at break and stitch tear. The mechanical properties have been given the greatest consideration on the evaluation of fat liquored leather, because, it gives an indication of fiber lubricity. The mechanical properties were evaluated according to Egyptian standard specification of leather [31].

It is found from figures (3,4) that, elongation at break and stitch tear are higher than that one of famous fat-liquor TRUPONOL® PEW2, (Phosphoric esters of synthetic fatting substance, pH ≈7) used in Egyptian tanneries, but tensile strength figure (2) is relatively high.

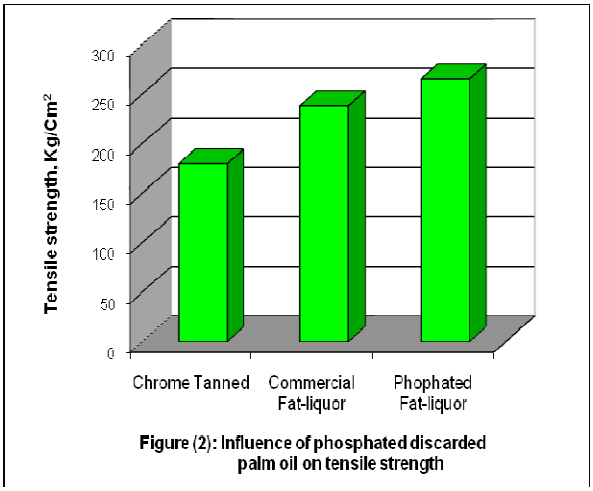


Figure (2): Influence of phosphated discarded palm oil on tensile strength

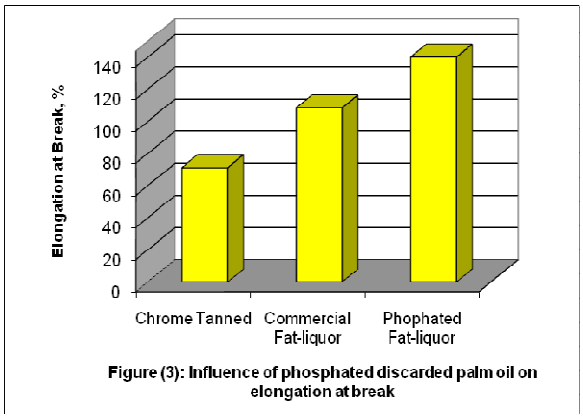


Figure (3): Influence of phosphated discarded palm oil on elongation at break

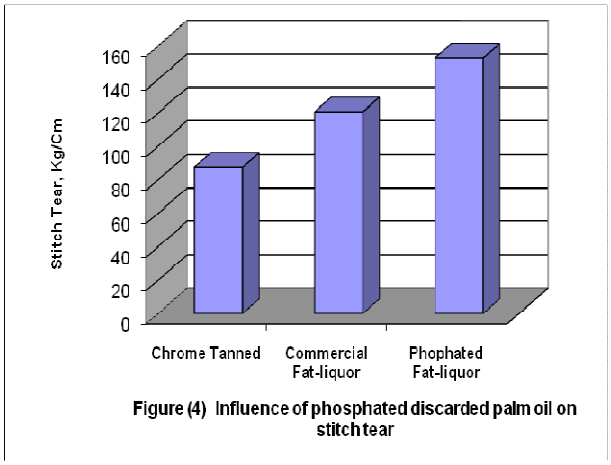


Figure (4) Influence of phosphated discarded palm oil on stitch tear

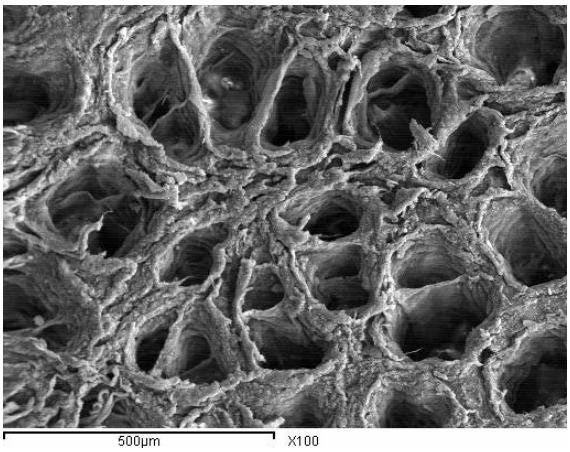
The improvement in the mechanical properties of the treated leather is due to good lubrication of the fibers as shown in SEMicrograph. The phosphated portion of oil (hydrophilic fatty matter) during liquoring of chrome tanned leather is chemically bound to the leather fibers, i.e. interacts with active centers in the collagen molecules of

leather fibers, while the emulsified portion (hydrophobic portions) is mainly located between the fiber bundles. Because the prepared fat-liquor contains hydrophilic and at the same time hydrophobic portions can penetrated into the leather fibers with the prepared fat-liquor because of its good penetration power and the stability of its emulsion (HLB concept).

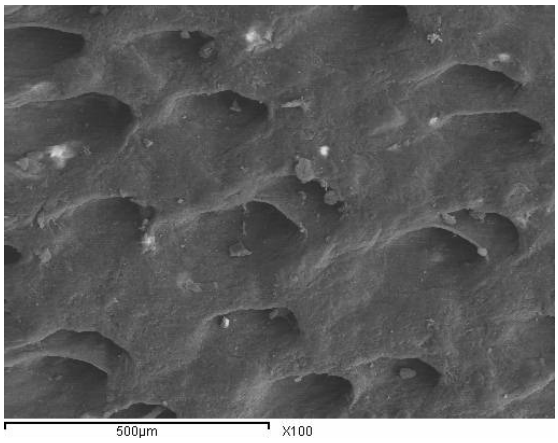
In general, the physical properties which are recorded above demonstrates to a far extent the suitability of the prepared phosphated local fried palm oil for fat-liquoring of chrome tanned leather, where by values which lie more or less within the limits of the Egyptian standard specifications, are attained.

Scanning Electron Microscopy (SEM)

SEM looks deeply into hide fiber structure and shows the effect of fat liquor on fiber and grain surface. SEM of the grain surface (x 100) of the fat liquored leather exhibits a soft grain without any fatty-spew; Figure (5-a & b). Also, the cross-section (x 500) of leather fiber before and after fat liquoring showed a significant lubrication of fiber bundles; Figure (6-a & b).

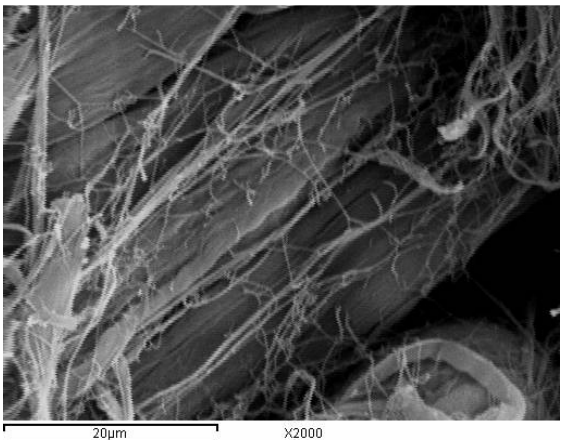


a) Chrome tanned leather

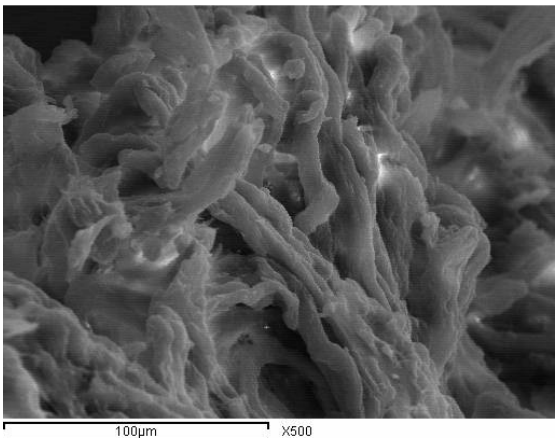


b) Fat-liquored leather.

Figure 5: SE-Micrograph for fiber bundles (X500).



a) Chrome tanned leather.



b) Fat-liquored leather.

Figure 6: SE-Micrograph for grain surface (X100).

Conclusion

In conclusion, it can be stated that this study on fat-liquoring of chrome tanned leather proved that:

1- The utilization of phosphated discarded palm oil (by product) as fat-liquoring agent.
2- An Improvement the texture and strength properties of leather fat-liquored using Phosphated discarded palm oil.
3- Substituting successfully part of the imported leather fat-liquoring agents.

Corresponding author
El-Shahat H. A. Nashy
Department of Chemistry of Tanning Materials and Leather Technology, National
*nashy-eha@yahoo.com

Received: September, 2010
Accepted: November, 2010

References

1. Hauber, C. and Germann, H.P. (1999) Investigation on a possible formation and avoidance of chromate in leather. *World leather*, October, 73-80.
2. Wachsmann, H. (2001) Retannage or Combination Process. *World leather*, April, 64-65.
3. Burgess, D. (1993) General aspects of fat-liquoring: An introduction to the application and chemistry of fat liquoring. J. soc. Leather Techno. & chemists, 78: 39-43.
4. Kronick, P.L. (Haverford PA) (1998) Use of Polymerizable Oil for Leather Fat liquor. U.S. Patent 5, 853, 427.
5. Alexander, K.T.W., Convington, A.D., Stosic, R.G. (1993) The Production of Soft Leather: Part3. Measuring Softness. J. Am. Leather Chem. Assoc 88: 254-269.
6. Heidemann E. (1993) Fundamentals of leather manufacturing, E. Roether KG, Chapter 15.
7. Tan, B. K. and Flingoh, C.H. (1981) Olein and stearin from Malaysian palm oil: Chemical and physical characteristics. PORIM Technology, ISSN, No. 4, 0127-0257.
8. Helmy, H. E. and Megahed, M.G. (2010) Utilization of olein oil resulting from frying processes in soap manufacture. J. Food and Dairy Sci., Mansoura Univ., Vol. 1 (6): 367-373.
9. Kunton, A. and Kifli, H. (1998) Properties of soap derived from distilled palm stearin and palm kernel fatty acids. J. of Surfactants and Detecrgents, 1 (3): 329.
10. Jaya, G. Kuntom, A and Cheak, K. (2000) Quality bar soaps from palm based soap noodles. Palm oil Technical Bulletin (MPOB) publication, 6 (6) 2-3.
11. MPOB (2007) Palm Kernel Products – Characteristics and Applications. MPOB Bangi.
12. Derek, N. G. (2007) Growth of Oleo chemicals in China. Paper presented in Oils and Fats International China (OFIC). Guangzhou. 11-13, Sept.
13. Girgis, A.Y. (2004) The utilization of discarded oil from potato chip factories in toilet soap making. Grasas Y Aceites (Sevilla, Spain), 55(3): 264-272.
14. A.O.C.S. (1993 and 1997). Official and Tentative Methods for the American Oil Chemist's Society. 6 and 7th ed. American Oil Chemist's Society, Champaign, U.S.A.
15. Shyam, G. (1991) Chemistry, chemical and physical properties and raw materials, in Soap technology for the 1990's, Chapter 2, p. 70-88. S. Luis (Ed.), Am. Oil Chem. Soc., U.S.A.
16. Davied Firestone (1998) Official Methods and Recommended Practices of the American Oil Chemists Society, Fifth Edition includes all changes 1993-1997, Edition Analytical methods, Champaigen-Illionois, March.
17. IUPAC (1987). International Union of Pure and Applied Chemistry. Standard Methods for the Analysis of Oils and Fats and derivatives, 7th revised and enlarged edn., edited by C. Paquot and A. Haut Fenne, Black Scientific, London.
18. Mitruke, B. N. (1984) Gas Chromatographic Application in Microbiology and Medicine, John Wiley and Sons, Inc., New York.
19. Official Methods Analysis of Association of Official Analytical Chemists, (1990) 15th Ed., Published by the Association of Official Analytical Chemists, Nineteenth Street, Suite 210, Arlington, Virginia, U.S.A.
20. William Horwitz (1970) Official Methods of Analysis 10th ed. Chapter 28, AOAC, International.
21. Griffin W. C. (1965) Emulsions in: Encyclopedia of chemical technology, 8: 117-154, Inter Science Publishers, New York, London, Sdeny.
22. Egyptian Standard Specifications (1986) Physical methods of leather, E.S 123.
23. Woollatt, E. (1985) The manufacture of soaps, other detergents and glycerine, p. 42-46. Ellis Horwood Limited, Chester, England.
24. Donald, B. and Halloran, K. (1991) Washing, refining, bleaching, deodorization and

hydrogenation, in Soap technology for the 1990's, Chapter 3, p. 99. S. Luis, (Ed.) Am. Oil Chem. Soc., U.S.A.

25. Izaki, Y., Yoshikawa, S. and Ochiyama, N. (1984) Effect of ingestion of thermally oxidized frying oil on peroxidative criteria in rats. Lipids, 19, 324-331.
26. Tyagi, V.K. and Vasishtha, A.K. (1996) Changes in the characteristics and composition of oils during deep-fat frying. J. Am. Oil Chem. Soc., 73 (4), 499-506.
27. Megahed, M.G. (2001) Microwave roasting of peanuts: Effects on oil characteristics and composition. Nahrung 45: 255-257.
28- Guillen, M.D. and Cabo, N. (1997) Infrared spectroscopy in the study of edible oils and fats. J. Sci. Food and Agriculture, 75: 1-11.
29- Che Man Y. B. and Setiowaty G. (1999) Multivariate calibration of Fourier transform infrared spectra in determing iodine value of palm oil products. Food Chemistry 67: 193-198.
30- Puntener, A. (1996) The influence of fat-liquors on the light fastness of dyed leather. J. Am. Leather Chem. Assoc., 91, 126-135
31- Egyptian Standard Specifications (1986) Physical tests of leather. No.11.

10/2/2010

Reuse of Industrial Materials in Buildings to Activate their Application in Egypt

Nermin Mokhtar Mohamed
Civil and Architectural Department, Engineering division, National research centre, Egypt
Nermin_farag@yahoo.com

Abstract: Increasingly stringent rules and regulations on construction and demolition waste, diminishing landfill space and depletion of natural resources are all reasons for the push for industrial byproduct materials recovery. In Egypt, Industrial byproduct materials are generated in large volumes every day that are potentially usable materials, and must be disposed of. The main goal of this paper is to change the way Egyptians' think about waste—to see the value of a used material as a product or commodity, not as a waste, and encourage the use and recycling of these rich, largely untapped resources. Positive economic rewards and environmental results are moving our partners toward more waste reduction and materials management. This paper summarizes the proposed Egyptian industrial materials waste management guidelines to reuse in building, which cover: (1) Identify the parties involved and the distribution of responsibilities; (2) Complementarily of roles of parties(owner, engineer, designer, and contractor) involved in the process of re-use to remove the causes that hinder the management of such material in Egypt; and (3) Participation of the Parties to the proposed project to achieve sustainable development fields at the actual application of the project.
[Nermin Mokhtar Mohamed. **Reuse of Industrial Materials in Buildings to Activate their Application in Egypt.** Journal of American Science 2010;6(12):627-639]. (ISSN: 1545-1003). http://www.americanscience.org.

KEYWORD: reuse –industrial byproduct materials, waste management, sustainability, Egypt.

1. Introduction:

The world is becoming increasingly conscious of the environmental implications not only of production processes but also of products discarded after use. The recycling of waste materials as a means of tackling the solid waste problem is attracting growing interest.

This is the problem of solid waste currently producing and increase with increasing Census world population of the most serious problems and will remain polluted environment for long periods of up to thousands of years which cause environmental damage and health problems.

In Egypt, the traditional method to get rid of Industrial wastes is send to landfill as waste. Industrial wastes are more damaging to environment, and public health, In addition, Construction and demolition (C&D) materials led to urban pollution image around, as well as the economic burden and the cost of waste transportation.

In pursuit of sustainable development principles, which aims to rely on recycling waste came importance of research on alternatives, Salvaging materials for reuse can be both an economical and environmentally sound alternate to waste stream disposal, and it also saves energy and environmental impacts of producing new products from virgin materials that help communities be sustainable in infrastructure renovation, construction, and maintenance.

With the pursuit of Egypt now to the sustainable development of areas and provide jobs for young graduates, In urgent need for alternative construction materials with rising cost of wood universal waste and demolition and construction that represent a high proportion of solid waste, Find the possibility of reuse industrial materials in building that Re-cycling building materials is an essential part of ecological sustainability.

And therefore need to find research benefit from industrial materials in the possibility of exploitation as an economic environment friendly housing in Egypt organization management process participation and funding for projects on physical development, to activate application construction for environmental development, economic, social and physical in areas

Despite the government attempts to impose penalties or fines or recourse to academic studies and research centers to develop solutions for waste management but it is still a deficiency in complete control of the problem in Egypt.

Research aims to propose a comprehensive and integrated approach to establishing industrial materials and its activation in Egypt by identifying the parties involved and their respective roles for the protection of areas affected by the effects of disposal opening alternatives to creating markets for reuse

industrial materials in physical development, and to conserve energy and preserve natural resources.

The main objective of research can be achieved through several sub-objectives, namely: monitor problems resulting from industrial materials pollution in Egypt, current status assessment and study of causes that hinder the proper management of residues in Egypt, finance and operations planning part of the parties involved to upgrade the project such areas, Identify the areas of development environmental, economic, social, physical and role of parties involved in achieving these areas

2. 1- Sustainable

Sustainable development meets the needs of the present without compromising the ability of future generations to meet their own needs. Basically, it's another term for "green" or "environmentally friendly".

Implementing sustainable projects means achieving an ecologically, socially and economically acceptable future[i].

By taking into consideration all economical aspects and the effects on people, and the environment during the planning and development phases, minimize the use of energy and resources to protect the environment, and increase the efficiency of all projects[ii].

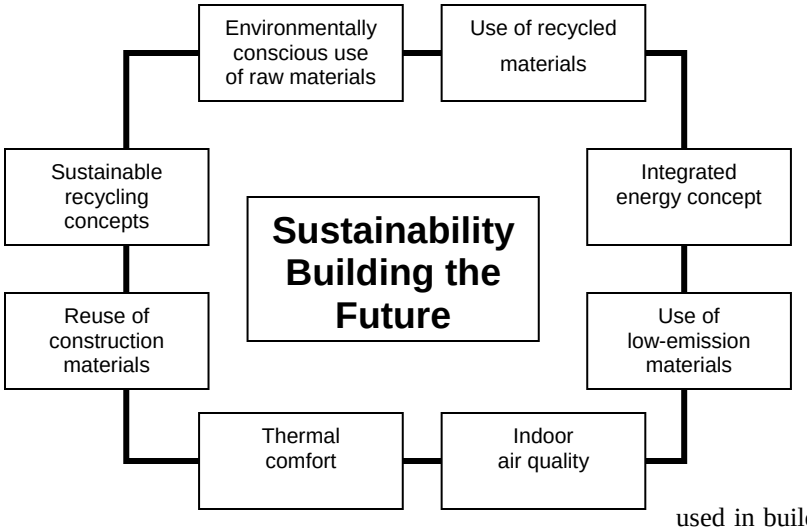


Fig. (1)Aspects of the Sustainability

1-1 Choosing sustainable materials

It can be difficult to assess exactly how sustainable a product is and which materials are preferable to others[iii]. There are some tools that can help you to choose the building materials, table (1).

There are many considerations that should be taken into account when choosing building materials. Since many different definitions exist concerning what constitutes an environmentally friendly or green material, this study use the following set of terms as factors in determining environmentally preferred materials and products[iv].

By-product

Unused or waste material from one manufacturing or energy-producing process that can be used in another manufacturing or energy-producing process [v]

Agricultural by-product: Unused or waste material from farming operations, several of which can be used in building products such as strawboard panels, etc.

Industrial by-product: Unused or waste material from power plants or manufacturing operations, several of which can be used in building products, e.g. fly ash concrete, etc.

2- Industrial materials

Industrial materials recycling, referred to as beneficial use, means reusing or recycling byproduct materials generated from industrial processes. These materials can be used as substitutions for raw materials in the manufacture of consumer products, roads, bridges, buildings, and other construction projects. Thousands of manufacturing and industrial processes and electric utility generators create hundreds of millions of tons of nonhazardous industrial materials that are often wasted[vi].

2-1 Examples of practical recycling applications

Nonhazardous industrial materials, such as coal ash, foundry sand, construction and demolition materials are valuable products of industrial

processes. Each material may be recycled in a variety of diverse applications, table (2). These materials have many of the same chemical and physical properties as the virgin materials they replace - they can even improve the quality of a product[vii].

3- Advantages and disadvantages of the process of recycling waste

The process of recycling has some of the advantages and disadvantages[viii], Table (3).

Table (1): Evaluation tools to choose the building materials

Evaluation tools	**Life-cycle assessment**	This means considering the impact during the extraction of the raw materials, manufacture, transport, handling, installation, the lifetime of its use, recycling and disposal.
	Embodied energy	The total amount of energy that is needed to produce, transport it to site and install it. For building products, it is commonly measured in Mega Joules (MJ) per unit of product.
	Renewable resources	These are resources that will be replenished with time; they include plant and animal products such as timber, paper, cork, wool and leather.
	Sustainable resources	Sustainable resources are the products of cyclic closed systems that do not require outside inputs, and do not generate waste.
	Local resources	Locally sourced products need less energy for transport and they support your local economy.
	Toxicity	Some materials are relatively harmless for humans, but their production might cause habitat destruction or release toxins into the environment. Toxic materials can also be a problem for installers or when they are disposed of at the end of their life cycle.
	Quality	The expected lifetime of the building is short; it makes little sense to use very durable materials.
	Re-use and recycling	Using second-hand or recycled materials is another option for reducing resource use.
	Uncertainty	Materials that have been tested for a long time in your local conditions are a safer choice than new materials or those which have not been proven locally.

Table (2): The properties of industrial materials and recycling applications in building

industrial materials	The properties and the problem	recycling applications in building
Cement dust produced during the cement industry	Produced from the burning and grinding of raw materials used in the manufacture of cement, contains a high proportion of the components of the cement but in different proportions, and fails to plant one of the factories of the Egyptian cement per day at least 300 tons in the Mediterranean.	It has been used in many engineering applications, including: Partially replace cement in some industries of construction materials such as bricks, tiles, the cement industry, glass, rubber, sewage treatment, the foundation layer for roads.
Steel slag	It is a byproduct of iron and steel industry and contains a high proportion of the components of the cement, but in different proportions. The amount of (steel slag) from iron and steel sector about a million tons annually, which is a national problem as well as emissions generated from the accumulation of slag	It is used in many engineering applications, including: as heap in concrete works of traditional and light production, types of cement (Ferro-cement - high iron slag - high resistance to sulfate).
Foundry Sands	It is sand that is used to make molds and cores in the metal casting process. Although generally recycled many times internally by the foundries, about 3-4 million tons of foundry sand is discarded each year. The recycling of nonhazardous, spent foundry sand can save energy, reduce the need to mine virgin materials, and may reduce costs for both producers and end users.	the spent foundry sands is used As partial replacement for fine aggregate in asphalt mixtures; in Portland cement concrete; As source material for the manufacture of Portland cement; and As a sand used in masonry mortar mixes, And in the other applications
Coal combustion	CCPs include the following materials:	Fly ash can be used as a replacement for

products	Fly Ash; Ash; Boiler; Flue Gas Desulphurization Material (FGD); and Other types of material such as fluidized bed combustion ash, and scrubber residues The characteristics and physical properties of CCPs vary. In general, the size, shape, and chemical composition of these materials determines their beneficial reuse as a component of building materials or as a replacement to other virgin materials such as sand, gravel, or gypsum.	the Portland cement that binds traditional concrete mixes. The manufacture of Portland cement requires large inputs of energy, and it is estimated that its manufacture constitutes about 8% of all carbon dioxide emissions from human sources. Approximately 75% of the fly ash produced annually is disposed of in landfills, which makes incorporating it into concrete a resource-efficient alternative.
Pulp and paper byproducts	Two significant byproducts from the paper industry are WWTP residuals and boiler ash	There are numerous examples of other uses Building board/fixture, Brick or concrete additive, Glass or lightweight aggregate
Construction and demolition (C&D) materials[ix]	It consists of the debris generated during the construction, renovation, and demolition of buildings, roads, and bridges. C&D materials often contain bulky, heavy materials, such as concrete, wood, metals, glass, and salvaged building components[x]. In Egypt, the daily quantity of construction and demolition (C&D) waste has been estimated as 10,000 tones. That is equivalent to one third of the total daily municipal solid wastes generated per day in Egypt[xi].	It can make a number of products (solid cement bricks, hollow bricks, paving blocks) using the broken bricks and broken ceramics. As possible, get a light concrete using broken bricks as an alternative partial or total ruins of the great used in industry. You can also use the surplus concrete and rubble after rounding heap for the production of concrete suitable for the various structural elements.

Table (3) Advantages and disadvantages of the process of recycling waste

disadvantages of recycling waste	Advantages of recycling waste
- Some materials are generally more difficult to recycle - other materials are dangerous or require more energy inputs to be recycled - The durability of some materials can be extended if they properly protected and maintained while in use - Environmentally preferable materials may be more expensive or difficult to locate - Determining a product's environmental preferability can be a complex process for which no tools exist - Prepare the materials may need more time - Need efficient labors	- Conserves energy and reduces greenhouse gas emissions by decreasing the demand for products made from energy intensive manufacturing processes - reduce the volume of materials which are sent to landfill as waste to achieve the continued development - save the embodied energy content - Preserves our natural resources by decreasing the demand for virgin materials - Saves money by decreasing disposal costs for the generator and decreasing materials costs for end users. - Local employment creation - Reuse of old buildings and use of recycled materials.

4- Building Applications for Industrial Materials

The beneficial use of industrial materials that were previously considered wastes has been expanding with a number of applications gaining market and regulator acceptance. Environmental and economic benefits derived from the recycling and beneficial use of industrial materials are becoming more evident:

- Conserving energy and reducing greenhouse gas emissions by decreasing the demand for products made from energy-intensive manufacturing processes;
- Preserving natural resources by decreasing the demand for virgin materials - recycled materials have many of the same properties as the virgin material they replace, and may improve the quality of the products in which they are used;

- Decreasing the economic and environmental burdens of disposal, as well as reducing the cost of material for end users.

This diagram illustrates a variety of common building applications for industrial materials. Note that the availability of specific industrial materials can vary regionally, fig. (2)[xii].

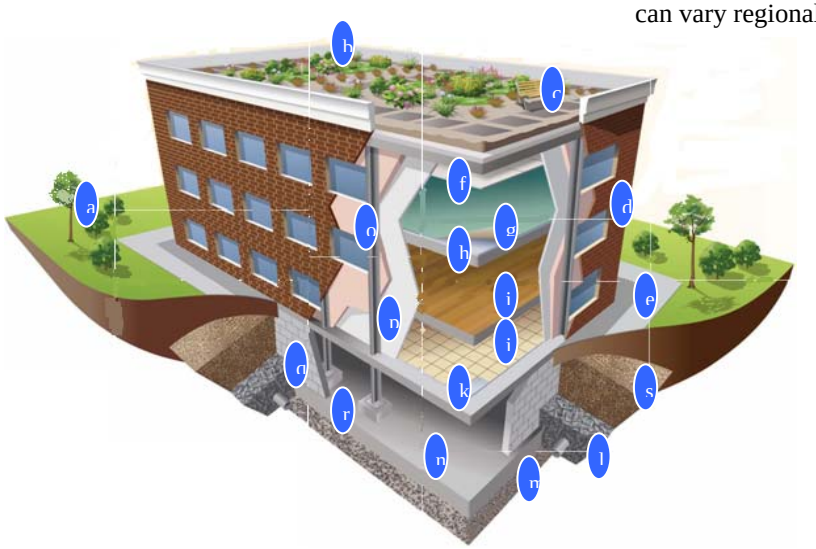


Fig. (2) Building Applications for Industrial Materials

Table (4) : a variety of common building applications for industrial materials

(a,b) Green Roofs & Landscaping	Green roofs are roofs covered with plants; they reduce storm runoff and provide insulation. Scrap tires can be used to make rubber tile for walkways. Bottom ash can be used as bedding material. Clean wood, recycled gypsum wallboard, and cardboard can be ground and used as soil amendments in both green roofs and landscaping applications.
(c) Landscape Furniture	Benches can be made with plastic lumber containing fly ash or with recycled C&D wood.
(d) Building Facing Material	Manufactured stone, which is concrete mixed with aggregates, is commonly used as building facing materials. fly ash can be used in the production of manufactured stone
(e) Sidewalks	Industrial materials can be used to make concrete sidewalks, and used tires can be recycled to create rubberized sidewalks. Asphalt concrete sidewalks can be made with recycled asphalt pavement and recycled asphalt shingles.
(f) Ceiling Tile	Ceiling tile can contain flue gas desulfurization (FGD) gypsum (a material resulting from burning coal to produce electricity), fly ash, recycled gypsum wallboard, or air-cooled blast furnace slag.
(g) Flooring	Industrial materials can be used in various flooring applications. (h) Carpet backing: Used tires, fly ash, or recycled carpet. (i) Wood flooring: Salvaged lumber or recycled wood. (j) Flooring tile: Fly ash, blast furnace slag. (k) Tile underpayment: Fly ash.
(l) Backfill (Foundation Support)	Backfill surrounds the building foundation, supporting it and providing drainage. Scrap tires provide superior drainage, insulation, and wall pressure relief. Blast furnace slag and recycled concrete also can be used for drainage.
(m) Foundation Structural Fill	Structural fill is an engineered fill that is constructed in layers and compacted to a desired density. Coal fly ash, bottom ash, slag, and spent foundry sand can all be used as structural fill. Concrete can be crushed and used onsite as structural fill.
(n) Poured Concrete	Concrete, which is composed of cement, aggregate, and water, is used in a

Foundation	wide array of building applications. Industrial materials can be recycled in cement and concrete in many ways. Here are a few examples: • Fly ash and ground granulated blast furnace slag can be used as partial cement replacements. Using these materials can produce stronger, longer-lasting concrete. • Portland cement itself can be made with fly ash, FGD gypsum, foundry sand, recycled gypsum wallboard, blast furnace, and steel slag. • Concrete aggregates can include bottom ash, foundry sand, crushed concrete, and blast furnace slag.
(o) Insulation	Air-cooled blast furnace slag can be used to produce mineral or rock wool insulation (also known as slag wool insulation).
(p)Drywall/Wallboard	FGD gypsum and recycled gypsum wall board can be used to manufacture drywall.
(q) Mortars, Grouts, Stucco	Mortars, grouts, and stucco contain aggregate (sand), binder, and water. Fly ash, foundry sand, silica fume, and slag cement can all be used as partial cement replacements.
(r) Masonry Blocks	Masonry blocks are made from cement and aggregate. Slag cement, fly ash, or silica fume can substitute partially for cement. Bottom ash, blast furnace slag, and recycled concrete aggregate can substitute for newly mined materials.
(s) Base Material	Spent foundry sand can be used in place of natural soil as base material for the building site. Recycled concrete is also commonly used as base material.

5-Case study

Some of the experiences of the World created by using industrial materials reused, table (5)

Case study	Location & Building Specs	Design	Materials Reused or Recycled	Positive Community Impacts
The Lazarus Building[xiii] is one of the most significant green rehabilitation projects in Columbus.  *Lazarus Before Redevelopment*  *Lazarus After Redevelopment*	 Renovation of a 600,000 square foot  commercial building in downtown Columbus, Ohio.	-Reducing, reusing, and recycling materials during renovation and construction -Using recycled-content products and materials in construction -Cost savings and environmental benefits -Environmental awards and recognition -Local community revitalization	Developers retained over 75 percent of the original structure, significantly reducing the amount of materials needed for the project. The renovation employed -Coal fly ash in concrete; -Recycled glass and tile flooring containing up to 100 percent recycled materials; -Carpets containing recycled nylon; -Restroom partitions containing 100 percent post-consumer recycled plastic; -Drywall containing at least 96 percent recycled materials, including flue gas desulphurization gypsum; and -Building siding containing 60 percent recycled metal.	-cost savings and avoided local community impacts from trucks hauling debris away. - The project created local jobs, improving the local economy. -a showcase of innovative ways to reduce waste.

Case study	Location & Building Specs	Design	Recycled	Positive Community Impacts
EPA's new building[xiv]  **ARCHITECTURAL SCALE MODEL**  **The exterior environment**  **The interior environment**	200,000 square foot EPA Regional Headquarters office building in downtown Kansas City, Kansas.  	-Many building features contribute to exceptional energy efficiency - The building's operations and maintenance environmentally friendly. -building products and materials are more environmentally sensitive, or "greener"	-Utilizing Fly Ash in concrete design -The aluminum mullions and trim on the windows, sheer wall, sunscreens, cable trays and skylight are all constructed from recycled aluminum. -In restrooms, the floors and walls were constructed with ceramic tile made from over 70% post-industrial recycled waste glass. -The Shaw carpet is made of 25% recycled material - The wood wall base in the atrium is 100% recyclable. - The ceiling tile is made from 93%-recycled slag and the grid system from light gauge steel made from 67% recycled material.	-the reduction of smog was considered when selecting building materials. In order to help reduce the contribution of VOCs into the atmosphere. -a showcase of innovative ways to reduce waste.

6- Waste Management in Egypt

Waste Management is a component of sustainable development, which seeks to reduce the impact of human activities on public health and the environment and development.

The industrial development in Egypt is the main engine of economic growth, where is the advantage of good Waste and exhaust for different industries, one of indicators of progress in the United. Industrial waste are serious environmental problem and that the lack of local industries based on Exploitation of the waste in other industries, making it a burden and a waste of environmental resources used In production processes, fig. (3). In fact, it can be exploited to become the waste materials with high economic value.

To sustainable improve waste and materials management in Egypt[xv] will focus on the active involvement of industrial waste generators in the reduction of waste volumes at source, and will also encourage private sector participation in solid waste management services, particularly collection and recycling.

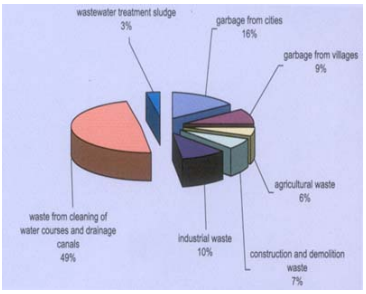


Fig. (3) The proportion of solid waste in Egypt

6-1 About a method to organize and manage the re-use of industrial materials in construction in Egypt:

This part comes to address the negative aspects which appear in the overlapping of roles and responsibilities of the parties involved in the project and overcome the causes that impede the management of re-use of industrial materials in Egypt to achieve urban development[xvi].

6-1-1 Identify the parties involved and the distribution of responsibilities

Parties to the proposed approach are different bodies responsible for policy development re-use of industrial materials in construction.

They are the government, institutions of technical advisory, executive agencies (investors and a group of capital and local governance), popular participation (beneficiaries), research centers, contractors and specialists (architects). In order to integrate the curriculum leading to activation of the application re-use of industrial materials in construction in Egypt should be considered interested parties and their respective roles to achieve the restructuring of the curriculum by ideal integration of limbs.

(I) the role of government

The success of the reuse industrial materials in building depends heavily on local government engagement and action. Their role is large and vital[xvii].

- Coordinate and facilitate partnerships to implement the reuse materials action plan.
- Lead by example in government
- Provide incentives that encourage green design, construction, and deconstruction and begin removing disincentives
- Expand capacity and markets for reusing and recycling construction and demolition materials.
- Increase awareness, knowledge, and access to reuse industrial materials
- Encourage innovative product design
- To encourage beneficiaries to participate in these projects beneficial to the environment
- Amend laws and existing environmental legislation

(Ii) The role of NGOs non-governmental organizations:

- Implementation of development projects in the environment and recycling waste.
- Training of local technical staff
- Coordination between the professional societies and funding for the implementation of various development programs.
- Galvanize the efforts of various parties (government, individuals, and investors) with the coordination between them.
- Support salvaged materials collection centers.
- Subsidize warehouse space to support the collection and distribution of salvaged materials.
- Create incentives for deconstruction, recycling, and the use of salvaged or recycled materials into construction procurement contracts.

(III) The role of professional engineers[xviii]

Specialists are planners, architects, economists, social, the most significant roles played by professionals in the following:

- Create new buildings that save energy and water, use fewer material resources, and create less waste.
- The design appropriate to the building to suit the Egyptian environment, according to the needs of the population and location
- Created propose a method suitable for the Egyptian environment and the work of drawings.
- Technical guidance and training courses for users and individuals to create technical staff can participate in all phases of the project.
- building designers have a responsibility to specify preferred materials and methods of construction which are suited to recycling

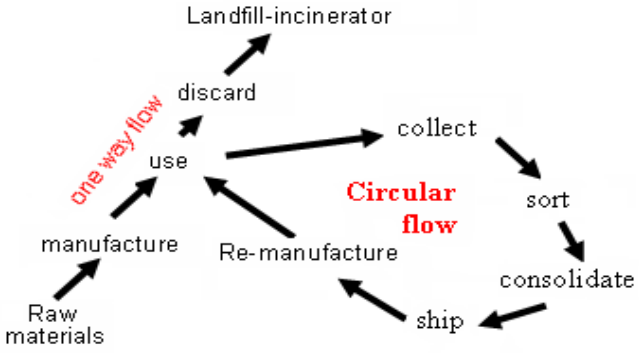


Fig. (4): Circular and linear flows of materials

(IV) Contractor` Role

- Design and plan to Prevent Waste, and develop a Construction Waste Management Plan
- Survey the Site Before Demolition or Deconstruction, Plan for Recyclable Materials
- Identify Reusable or Salvageable Items, all materials should be examined using a precautionary approach to eliminate possible toxicity or future regulatory constraints to their use and disposition.
- Select Salvage Removal Alternatives
- Estimate the Costs and Savings
- Prevent Contamination
- Separating the components will facilitate adaptation and reduce the complexity of deconstruction
- Building contractors need to exercise care during demolition, and should be prepared to re-use suitable materials on projects.


Fig. (5) Reuse –recycle can occur onsite and offsite [xix]

(V) The role of research centers

- Examine the adequacy of recycled materials used with the Egyptian environment.
- Insurance system building against fire, moisture and insects, and the work of all the tests required, and improve the implementation of this new technology.
- Study ways to improve the properties of the construction of the building using this new technology.
- Employment training to create this type of installations.
- the use of certain programs to achieve energy efficiency within enterprises, and may remove energy consumption to the minimum (Zero Energy)
- Monitoring of constraints and variables that occur in the region and draw conclusions to contribute to approve the project with the environment and the needs of the user, the study of the potential physical and technical implementation, and improve the economic viability of the project.

(Vi) The role of Group Capital (investors and businessmen, banks, real estate specialist)

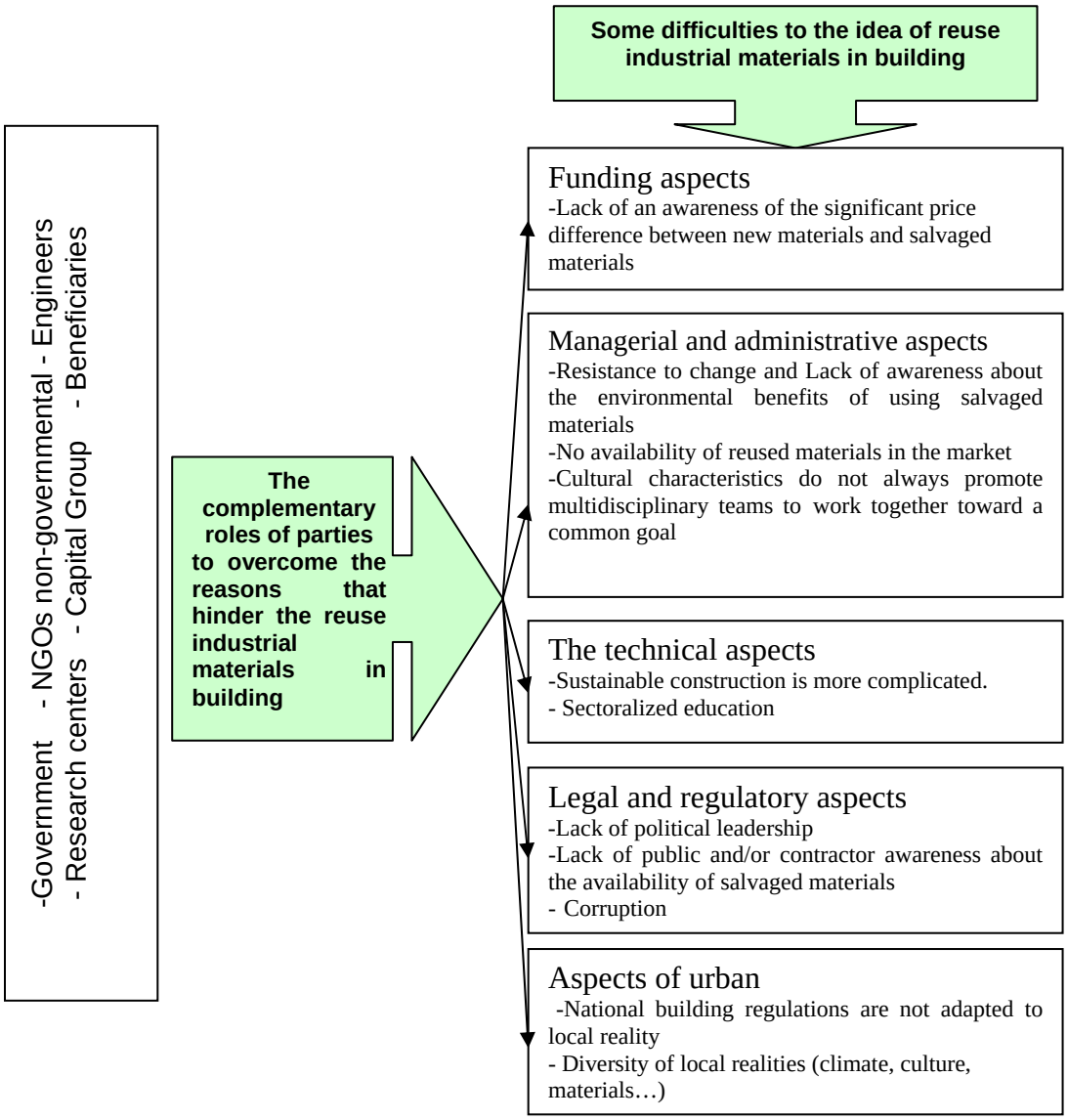
- Develop and/or fund training programs. Subsidize training costs for participants.
- receipt of the site, and provide the necessary potential for re-use of building materials to be used
- coordination between manufacturers and specialists to bring in industrial materials and re-use in construction
- Follow-up after implementation of changes in operations and maintenance

(Vii) The role of beneficiaries / individuals the user is the basis of urban development which is capable of maintaining the physical output, and can be user participation in several areas.

- participate in the implementation of self-help
- participate in the maintenance and improve the local environment
- Participating home improvement loans to upgrade facilities and infrastructure and public services.
- Owners should insist on the use of recycled materials… in the interest of ecologically sustainable development.

6-1-2 Complementarily of roles of parties involved in the process of re-use to remove the causes that hinder the management of such material in Egypt, figure (6).

(a) between industry and regulators to increase the understanding of the beneficial use of industrial materials and regulatory programs; (b) among state regulators to share information and experiences on beneficial use regulations and determination processes; (c) among industries to share experiences on successful reuse and recycling, and to assess the potential to utilize each others' materials, and (d) among researchers, nonprofit representatives, industry, and regulators to share information and concerns regarding risk assessment, beneficial use determinations, environmental safety, and new research.



Fig(6) overcome the reasons that hinder the reuse industrial materials in building in Egypt

6-1-3 Participation of the Parties to the proposed project to achieve sustainable development fields at the actual application of the project.

Integration of the roles of the participants in the process of re-use of industrial materials in construction is conducive to the development of environmental, economic, physical and social**,** figure(7).

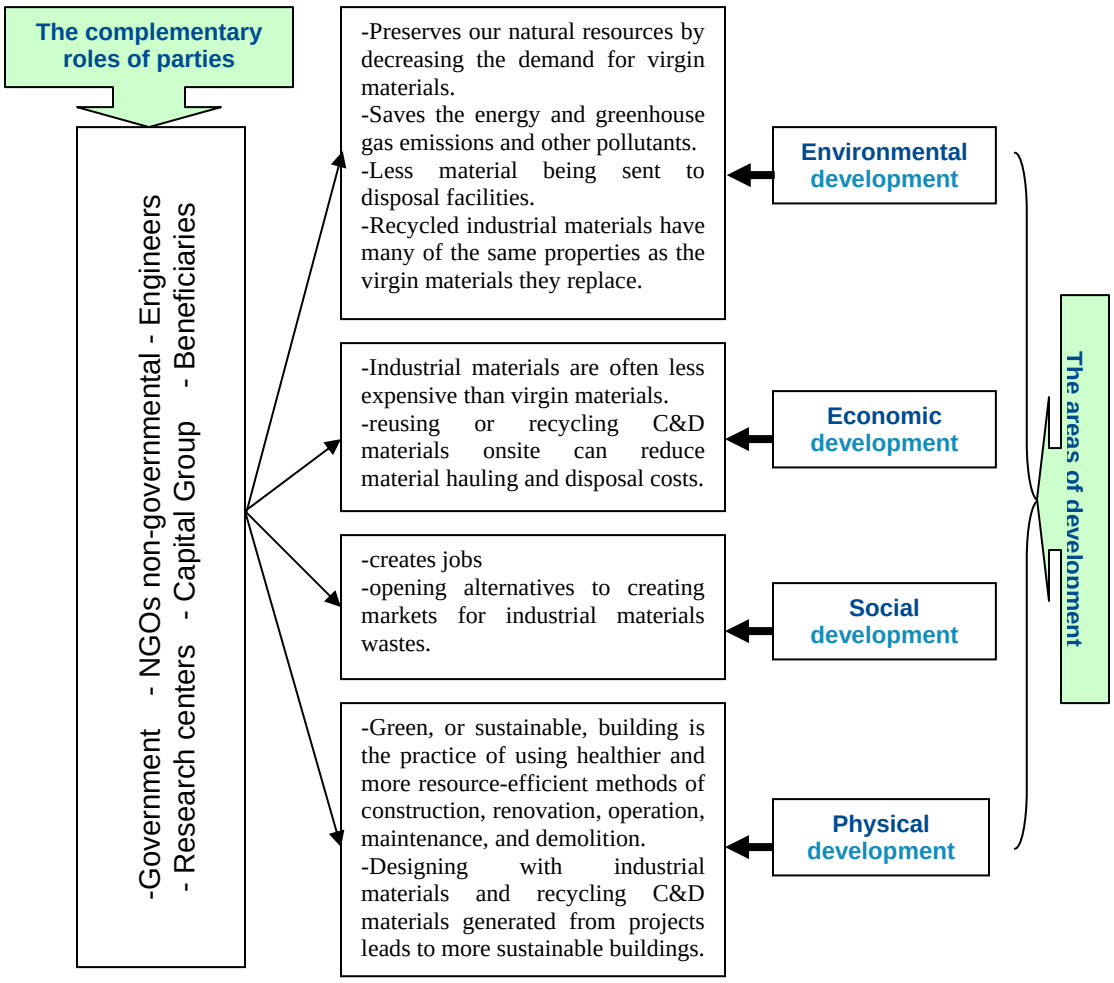


Fig (7) increase the effectiveness of the parties involved in the process of reuse industrial materials in building in various facets of development (environmental, economic, social and physical) for sustainable development

7- CONCLUSIONS

Based on the data collected from the literature su rvey, it is revealed that the production of industrial materials waste is escalating both on the international and national scales. Furthermore, environmental, safety, visual and technical related problems generated from these wastes has severely added to the long-term negative impacts of these wastes on the surrounding environment.

The essence of the recommended guidelines in this paper is to offer systematic procedures that could help in minimizing the magnitude of the industrial materials waste problem in Egypt. Therefore the disposal option can be avoided by the implementation of reuse them in building.

Waste reduction opportunities begin with the earliest choices made in the building process, including architectural design and material selection.

Effectively balancing resource-efficient design concepts requires the attention of skilled and environmentally conscious building professionals. These concepts include waste prevention, durability, and recyclables.

And has been monitoring some of the negatives facing the potential to activate the application of construction materials industry and are summarized as follows:

(1) Limited NGO non-governmental organizations concerned with the field of urban development in general and re-use of industrial materials in particular.

(2) Does not represent low-income housing a sufficient degree of urbanization, culture and enable them to participate in such environmental projects in an effective manner.

(3) Difficulty of maintaining such environmental projects for low-income.

(4) Building design re-use of industrial materials zero energy consumption in Egypt still needs some time.

5. Non-participation of specialists sometimes leads to delays in implementation and increase the cost and other obstacles that may face the project when actual implementation.

8- Recommendations

(1) Increased awareness, acceptance and proactive government policies are critical in order to continue the upward trend of recycling and reusing materials whenever possible

(2) More political support is required to enforce the implementation of waste management scheme in the construction/building field, collect industrial material wastes under the direct supervision of authorities. Imposing a special tax levied on wastes when exceeding a certain level determined by the government.

(3) It is also recommended to extend research on the area of recycling and reusing techniques of industrial materials in building to induct feasibility studies, including cost/benefit and payback period analysis for each technique. The research should survey the Egyptian market and seek the potential possibility of using waste as raw materials in factories. This research should integrate both the construction industry and the manufacturing industry to bridge the gap between the two disciplines.

(4) Overcoming these challenges may require advocacy work to strengthen policies and incentives to reduce construction and demolition waste, intensive education and marketing to expand the demand for reused building materials, as well as smart partnerships and inventory management to keep the right mix of reused materials in stock to meet local demand.

Corresponding author
Nermin Mokhtar Mohamed
Civil and Architectural Department, Engineering division, National research centre, Egypt
Nermin_farag@yahoo.com

9. References:

1. Halliday,Sandy," Sustainable Construction", Elsevier, 2008.
2. Mendler, S., Odell, W., The HOK Guidebook to Sustainable Design, New York, NY: John Wiley & Sons, Inc., 2000.
3. Ross Spiegel, Dru Meadows ," Green Building Materials", John Wiley&sons, 1999.
4. Giamberdine, R., et al, Guiding Principles of Sustainable Design, Denver, CO: The National Park Service, 1993
5. Jon Nunan," The Complete Guide to Alternative Home Building Materials & Methods", Atlantic publishing group, Inc., 2010.
6. Managing nonhazardous solid waste, http://www.epa.gov/osw/inforesources/pubs/orientat/rom2.pdf
7. International Conference on Non-conventional Materials and Technologies "Materials & Technologies for Sustainable Infrastructure Systems"21-23 September, 2010 Cairo, Egypt
8. Pinto,T. P., and Agopayan, V. (1994). "Construction wastes as raw materials for low – cost construction products" Sustainable construction (Proc. 1st Conf. Of CIB TG 16), J.Kibert,ed., Ctr. For Constr. and Envir., Gainesville, Fla., 335-342.
9. Webster, M., The Use of Salvaged Structural Materials in New Construction, The Austin Papers, Brattleboro, VT: Building Green, Inc., 2002
10. Al-Ansary," Sustainable Guidelines for Managing Demolition Waste in Egypt", International RILEM Conference on the Use of Recycled Materials in Building and Structures,2004.
11. 1) Elliott, S., 'Don't Waste Time', *International Construction* (March 2000) 15-17.
12. Using Recycled Industrial Materials in Buildings
13. http://www.epa.gov/epawaste/conserve/rrr/imr/pdfs/recy-bldg.pdf
14. Green renovation brings a community back to life: The Lazarus Building. Available At http://www.epa.gov/epawaste/conserve/rrr/imr/pdfs/lazarus.pdf (2010)

15. Building Materials Available At http://www.epa.gov/region07/p2/offtheshelf/offshelfpage16.htm (2010)
16. Al-Ansary, M.S., 'Recommending Egyptian Guidelines for Managing Construction and Demolition (C&D) waste', MSc. Thesis, The American University in Cairo (AUC), Egypt,1999.
17. UN Documents Cooperation Circles: Our Common Future, Chapter 2: Towards Sustainable Development. http://www.un-documents.net/ocf-02.htm
18. Egyptian Environmental Law No.4 for year 1994 and its Executive Regulations, 'Promulgating a law concerning Environment', 1st Edn (Al Matba El Ameraya, Egypt, 1994)
19. Otto, K.N., Wood, K.L., Product Design: Techniques in Reverse Engineering and New Product Development, Upper Saddle, NY: Prentice Hall, 2001
20. EPA Industrial Materials Recycling—www.epa.gov/industrialmaterials.

10/2/2010

Mitochonderial Cytochrome C Oxidase Subunit 1 (*cox 1*) Gene Sequence of the *Hymenolepis* Species.

Omnia M. Kandil*, Mona S. Mahmoud, Nesreen A.T. Allam, Amira H. El Namaky

Parasitology and Animal Diseases Department, National Research Center, Dokki, Giza, Egypt,
kandil_om@yahoo.com*

Abstract: In the current study, Mitochondrial Cytochrome *c* oxidase gene especially codons within subunit 1 (*cox1*) of *H. diminuta and H. nana* Egyptian isolates from two stages (adult worms and eggs) and hosts origin (human and rat) were amplified, sequenced and aligned. PCR products were approximately 700 bp, 702 bp and 715 bp of *H. nana* rat isolates, *H. diminuta* rat isolates, and *H. nana* human isolates, respectively. Moreover, despite their host susceptibility differences they all gathered in one cluster with three genbank published isolates of *H. nana*; AB033412.1, AB494472.1 and AY121842.1), forming one clade with 100% similarity, which was non significantly decreased on internal nodes. In addition, clearly far away from *H. diminuta* published sequence AB033412.1 who's assumed to be genetically closely related to Egyptian *H. diminuta* than all other *H. nana* isolates. Both Egyptian murine isolates of Hymenolipidid; *H. diminuta* and *H. nana*, were closer to each other than being to *H. nana* of human origin. The annotated sequences of Egyptian isolates were deposited in GenBank under the following accession numbers; *H. diminuta* (GU433102), *H. nana* rat isolate (GU433103), and *H. nana* human isolate (GU433104). Finally, the development of effective control strategies will only be possible if complete understanding of the epidemiology of infection is elucidated.

[Omnia M. Kandil, Mona S. Mahmoud, Nesreen A.T. Allam, Amira H. El Namaky. Mitochonderial Cytochrome C Oxidase Subunit 1 (*cox 1*) Gene Sequence of the *Hymenolepis* Species. Journal of American Science 2010;6(12):640-647]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Hymenolepidid, Phylogeny, Cytochrome *c* oxidase subunit 1 gene (*cox1*), Sequencing.

1. Introduction:

Hymenolepis nana and *H. diminuta* are the most common cestodes in humans, mice, domestic and wild rats (Macko and Hanzelova 2008). It is believed that infections with *Hymenolipis* spp., in general, may have been under diagnosed due to sporadic egg shedding (Thompson et al. 2001; Raether and Hänel 2003). Since isolates of *H. nana* infecting humans and rodents are morphologically identical, the only way they can be reliably distinguished is comparing the parasite in each host using molecular techniques (Macnish et al. 2002a, b).

Mitochondrial (mt) genomes are small (usually less than 20000 bp), circular, and maternally inherited (Boore 1999). In addition to high copy-number per cell which has made them attractive and more tractable targets for characterization, population genetic and phylogenetic studies (Hu et al. 2004; McManus et al. 2004). Regions within the mitochondrial DNA (*mt*DNA) have been proven useful in biology, epidemiology and diagnosis of several parasitic infections of human and veterinary importance (Ngarmamonpirat et al. 2005; Ando et al. 2006). Methods used to obtain data from flatworm *mt* genomes have included DNA sequencing, restriction fragment length polymorphism (RFLP) analysis and single-strand conformation polymorphism (PCR-SSCP) (Boore and Brown 1998; Avise, 2000). Intraspecific sequence variation in coding portions (genes) of the *mt* genomes seems to range from small to moderate, especially when compared with interspecific variation that have demonstrated the deep separations among strains of same species (Littlewood et al. 2008).

Complete or near-complete *mt*DNA sequences are available for 12 species of parasitic flatworms; six cestodes including *Taenia crassiceps* (Le et al. 2000), *Echinococcus multilocularis* (Nakao et al. 2000) and *Hymenolepis diminuta* (von Nickisch-Rosenegk et al. 2001). Cytochrome c oxidase (COX) is a 13-subunit protein complex located on the inner mitochondrial membrane that catalyzes electron transfer, proton translocation processes, production of up to 95% of the energy of eukaryotic living cells (Saraste 1999; Johnston 2006), thus directly influence metabolic performance. *mt cox* sub unit 1 is the most highly conserved among 3 genes coding for cytochrome oxidase, therefore has been employed in several phylogenetic studies (Traversa et al. 2007).

DNA sequencing of informative regions within the gene encoding for the COX1 protein have emphasized specific comparative aspects without yet making a detailed genome description but revealed data for basic and applied potential differential studies on *Hymenolopis* spp. determining host

specificity and transmission patterns (Macnish et al. 2003). Therefore, allow more appropriate approach for control of endemic infestations in Egypt, particular where rodent's population is above control limits and hygienic measures are not strictly applied. Furthermore, for diagnostic purposes since using techniques able to overcome inherent limits of the classical approaches (Constantine 2003; Thompson et al. 2004). Epidemiologically, despite this infection is a hand-to-mouth rote that in general not very pathogenic, however it is extremely difficult to be controlled (Littlewood et al. 2008). Till now education in hygiene is probably the only practical way to reduce the incidence in addition to rodent's eradication (Behera et al. 2008). The genotyping of *Hymenolepis* isolates in different hosts will help in determine host specificity and transmission patterns and thus allow more appropriate approach to control infections in endemic communities. From a public health perspective, a better understanding of the transmission dynamics of a parasite species previously believed to be infective only to rodents will be required to answer questions about the potential for transfer of this parasite to humans and/or animals.

Since control of parasitic disease is dependent on the rapid and accurate detection of causative agents this necessitated traditional techniques being complemented by molecular tools that provide predictive data on genetic variation in and among parasites (Thompson et al. 2004). Thus the present work aims is to characterize, for the first time, partial sequences of *cox1* genes of *H. diminuta* and *H. nana* Egyptian isolates to promote basic knowledge on their *mt*DNA composition, to assess the sequence variation level within local Hymenolepidid from different sources, different developmental stages (adult worms and eggs) and hosts origin (human and rat), and to discuss the potential benefits of such molecular information as record sheets for ecological, epidemiological, transmission and host-parasite interaction and as diagnostic approach of infection in Egypt.

2. Materials and methods

Parasites Samples:

H nana eggs were obtained from infected humans in Endemic Diseases institute. Approximately 2000 *H. nana* eggs were inoculated into 5-week-old male white mice (Movsesyan et al. 2008). Adult worms were dissected from the small intestine approximately 14 days post-inoculation (Tanowitz et al., 2001). *H. diminuta* worms were obtained from naturally infected *norvegicus* rat from Abu Rawash, Giza, Egypt. Rats were killed by cervical dislocation and entire small intestine was removed from gut. The worms and eggs washed repeatedly in phosphate buffered saline (PBS) and stored at -80 ^{o}C until used for DNA extraction.

Isolation of DNA from Adult Worms and Eggs

Templates DNA were purified from *H. nana* and *H. diminuta* using QIAmp tissue purification kit (Qiagen, Hilden, Germany) according to manufacturer's instructions (Macnish et al. 2002a). DNA was eluted in 200 µl Tris-EDTA (TE) buffer and 1 µl of the extract was added to the polymerase chain reaction mix. Single adult worm and/or eggs for each isolate were used for DNA extraction.

Oligonucleotide Primers Design

Entire *mt* genomes of the following species were aligned *Hymenolepis diminuta* (accession number AB033412.1), *Taenia crassiceps* (accession number NC_002547), *T. solium* (accession number NC_004022), *T. asiatica* (accession number NC_004826), *Echinococcus granulosus* (accession number NC_008075), and *E. multilocularis* (accession number NC_000928); and annotated sequence of *Hymenolepis nana* (accession number AF314223.1) (Nakoo et al. 2000, 2002; von Nickisch-Rosenegk et al. 2001). It was not deemed necessary to include all the available sequences from *Taenia* or *Echinococcus* as conservation of alignable positions between genera and being > 30% GC was more important for PCR primer design. PCR primers pair designed *cox1*-F 5`-ACTTCATTGCTTTTGCTTTTTAGA-3` and *cox1*-R 5`-TGCTGTCATAAATGAACCAACAGT-3` were synthesized by Metabion International AG (Martinsried/Deutschland).

PCR Amplification Protocol

Fragments of the mitochondrial cytochrome *c* oxidase subunit 1 gene were amplified using designed primers and each PCR mix was prepared in 50 µl total volume with 1 µl of template (50 ng), 10 pMoles of each primer, 45 µl of Ready TaqMix Complete (Mater Mix, AllianceBio, USA), and nuclease free water (Qiagen, Germany) to complete the total volume of the reactions. PCRs were performed in a PTC-100™ Thermal Cycler (MJ Research Inc., USA) using the following cycling protocol: initial denaturation at 95^{0}C for 3 min and then 40 cycles of 94^{0}C for 1 min 50 sec, 58^{0}C for 1 min 30 sec, and 72^{0}C for 1 min. Final extension was carried out at 72^{0}C for 7 min. A reagent blank was run as control in every PCR procedure. Positive results by PCR were retested on two further occasions several days later to examine the reproducibility of PCR. Amplified products from the PCRs were electrophorised on 1.5% agarose gels

(Bioshop Canada, Burlington, Ontario, Canada) stained with ethidium bromide (0.5 µg/ml) (Bioshop Canada) (Sambrook et al. 1989). A 100 bp ladder (Jena Bioscince, GmbH, Germany) was loaded in each gel then photographed under UV light with gel documentation system.

Sequencing of *cox1* Gene Products

PCR-product of each isolates were purified with QIAquick-spin PCR purification kit (Qiagen, Germany) then directly sequenced from both directions using ABI Prism™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystem, FosterCity, California) according to manufacturer's instructions on a 3130XL Genetic Analyzer (Applied Biosystems). At least two independent PCR products were used for sequencing per isolate.

Sequences Analysis

The resulting aligned output was manually adjusted (Lee et al. 2007). Sequences corresponding to the PCR amplification primers were excluded prior to multiple sequence alignment and phylogenetic analysis. The confirmed sequences were then deposited in the EMBL/GenBank Data Libraries of the NCBI. In order to improve the homology statements out group included *Taenia saginata* (AB465239.1), *T. solium* (AY211880.1), *T. multiceps* (GQ228818.1), *Echinococcus granulosus* (AF314223.1), *E. multilocularis* (AF314223.1) and *Spirometra erinaceieuropaei* (AB374543.1), as well as all annotated sequences of *Hymenolepis diminuta* (AB033412.1) and *Hymenolepis nana* (AF314223.1, AY121842.1, AB494471.1, AB494472.1, AB033412.1, AF314223.1) by Basic Local Alignment Search Tool (nBLAST) (www.ncbi.nih.gov/BLAST/) in the NCBI database (National Center for Biotechnology Information, NIH, Bethesda, Maryland, USA) (Tatusova and Madden 1999). The alignment gaps were treated as missing data. Phylogeny of Egyptian *Hymenolipis nana* and *H. diminuta* human and rat isolates based on *coxI* gene partial sequences and multiple alignment analysis were performed with CLUSTAL W computer program (Thompson e al. 1994).

Phylogeny Construction

The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees and node reliability in which the associated taxa clustered together in the bootstrap test is shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkand and Pauling 1965) and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 99 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007). Neighbor-Joining and UPGMA methods were used to calculate the evolutionary relationship of the Egyptian isolates with genbank references strains (Saitou and Nei 1987).

3. Results

PCR Products of *mt cox1* Gene

PCR products were amplified from *mt* genomes using synthesized primers set. Across the alignment of *mt* genomes few regions were suitably conserved to allow primer design. The PCR products were approximately 700 bp, 702 bp and 715 bp for *mt cox1* gene of *H. nana* rat isolates, *H. diminuta* rat isolates, and *H. nana* human isolates, respectively, (Figure 1).

Sequences Analysis

Variation occurred in terms of sequence length and nucleotide differences and gaps (nucleotide insertions, deletions, and substitutions), but not G+C percentage where the overall numbers did not differ between amplified fragments; A (23%), C (10%), G (22%) and T (45%). Where Nucleotide alterations were found to be variable and several nucleotide insertions, deletions and substitutions were detected with gaps in the same or different positions (Figure 2).

Phylogeny Construction

Similar topologies were observed in the Egyptian isolates with genbank references strains. Optimal phylogenetic tree with the sum of branch length = 1.91459848 is shown (Figure 3). Egyptian species were genetically distinct from other species used in this study that are phylogenetically relating to Hymenolipidid. In addition, despite their host susceptibility differences they all gathered in one cluster with three genbank published isolates of *H. nana*; AB033412.1 (gi|6045204), AB494472.1 (gi|2262378), and AY121842.1 (gi|2221354), forming one clade with 100% similarity, which was non significantly decreased on internal nodes. Moreover, obviously far away from *H. diminuta* published sequence AB033412.1 (gi|1399136) who's assumed to be quite genetically closely related to Egyptian *H. diminuta* than all other *H. nana* isolates.

Both Egyptian murine isolates of Hymenolipidid; *H. diminuta* and *H. nana*, were closer to each other than being to *H. nana* of human origin.

GenBank accession numbers of Egyptian amplicons

The annotated sequences of Egyptian isolates were then deposited in the GenBank of NCBI under the following accession numbers; *H. diminuta* (GU433102), *H. nana* rat isolate (GU433103), and *H. nana* human isolate (GU433104).

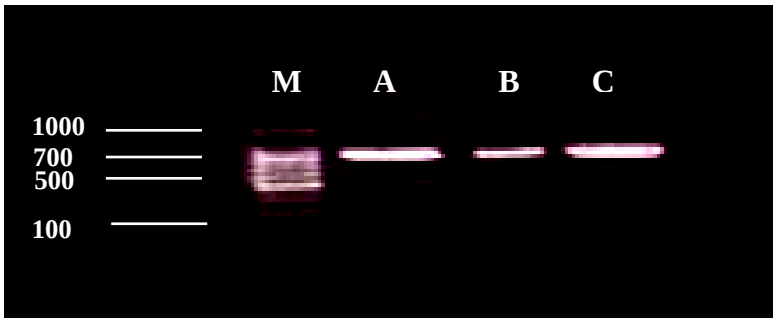


Fig. 1 PCR products of *mt cox1* gene amplified by specified primers pair from Egyptian isolates of (A) *Hymenolipis diminuta* 703 bp fragment, (B) *H. nana* rat isolate 699 bp fragment, (C) *H. nana* human isolate 715 bp fragment, and (M) 100 bp DNA Ladder.

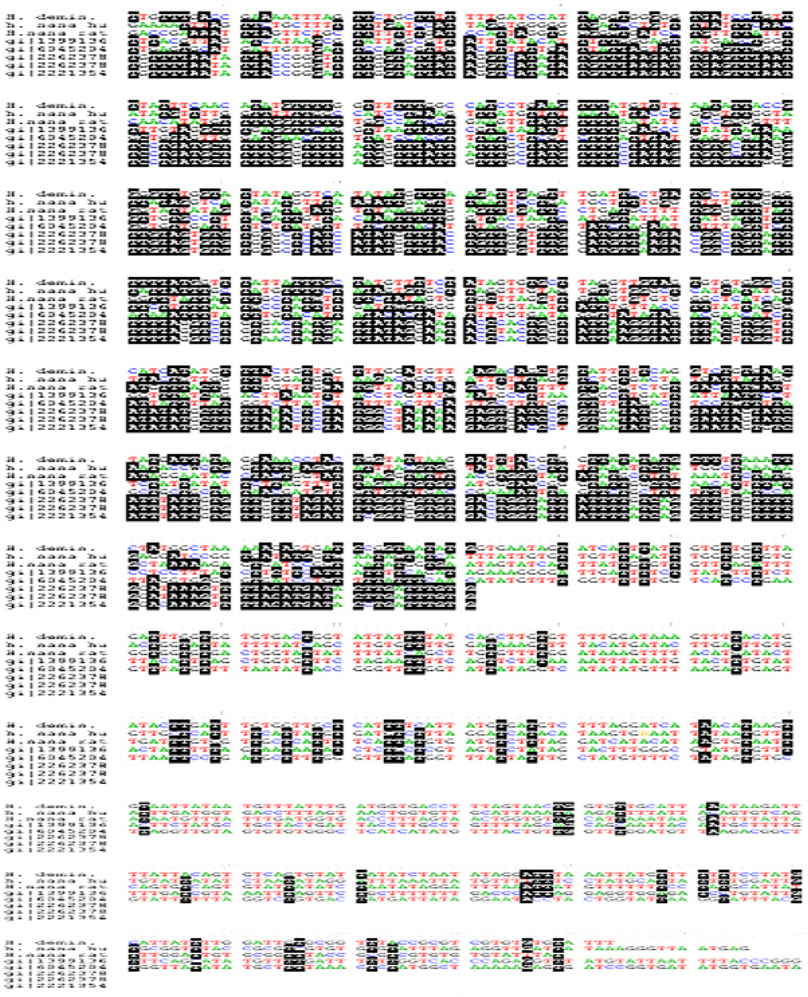


Fig. 2 Nucleotides multiple alignment of partial *mt cox1* gene sequences of Egyptian *H. diminuta*, *H. nana* human isolate, *H. nana* rat isolate, and reference gi|13991366: *H. diminuta*, gi|226237884: *H. nana* isolat: HnanaMon, gi|22213549:*H. nana*, gi|6045204: *H. nana*, gi|14009612: *Echinococcus granulosus* genotype 1, gi|15042575: *Echinococcus equinus* , gi|193884329: *Spirometra erinaceieuropaei*, gi|239997751: *Taenia multiceps*, gi|28856111: *Taenia solium*, and gi|260162222: *Taenia saginata*, isolate: TsagT017KANTH. Black columns represent identical nucleotide sequences between aligned isolates.

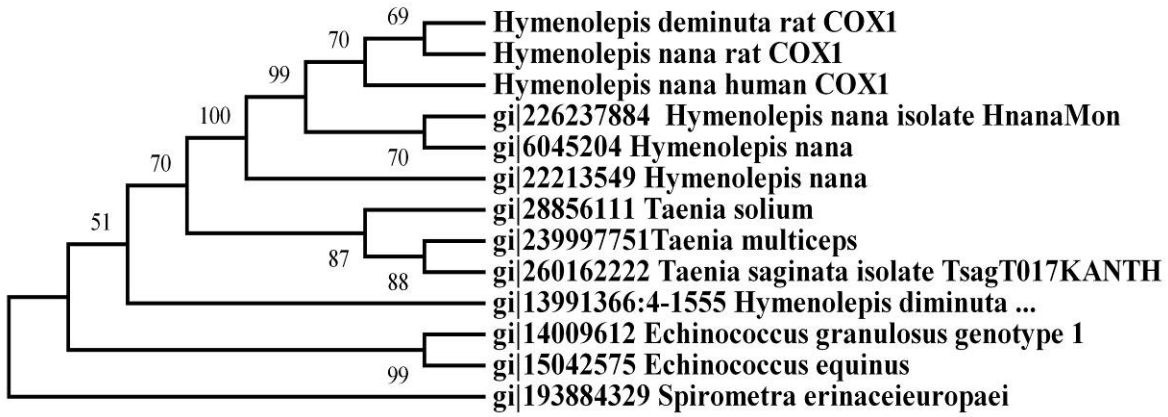


Fig. 3 Rooted phylogenetic tree based on amino acids sequences of in silico translated partial *mt cox1* gene sequences of Egyptian *H. diminuta, H. nana* human isolate, *H. nana* rat isolate and reference gi|13991366: *H. diminuta*, gi|226237884: *H. nana* isolate: HnanaMon, gi|22213549: *H. nana*, gi|6045204: *H. nana*, gi|14009612: *Echinococcus granulosus* genotype 1, gi|15042575: *Echinococcus equinus*, gi|193884329: *Spirometra erinaceieuropaei*, gi|239997751: *Taenia multiceps*, gi|28856111: *Taenia solium*, and gi|260162222: *Taenia saginata*, isolate: TsagT017KANTH. Similar topologies were developed when both Neighbor-Joining and UPGMA methods were applied (MEGA4 software).

4. Discussion:

Mitochonderia play a central role in metabolism, apoptosis, disease, and aging (Le et al. 2002). They are the site of oxidative phosphorylation, essential for the production of ATP, as well as a variety of other biochemical functions. Within these subcellular organelles is a genome, separate from the nuclear chromatin, referred to as mitochondrial DNA (mtDNA), very commonly used in studies of molecular phylogenetics (Avise 2000). Flatworm mitochondrial genomes have a number of distinct features including all genes are coded on the same strand (von Nickisch-Rosenegk et al. 2001), utilize a unique mitochondrial genetic code (Boore 1999) and truncated stop codons have also been found among a number of genes (Nakao et al. 2000, 2003).

In the current study the earliest genbank records of *cox1* gene of Egyptian *Hymenolepis* spp. are declared. So far, only a few is known about the relative divergence rates of mitochondrial DNA in *hymenolipis species* especially Egyptian isolates (Vilas et al. 2005), hence only one species belonging to *H. diminuta* is completely sequenced and published in genbank (Littlewood et al. 2008). However, PCR technology and DNA sequencing techniques permit the identification of species, strains, and populations from any stage in their life history to distinguish among morphologically similar parasites (Boore 1999).

Egyptian species were genetically distinct from other species used in this study that are phylogenetically relating to Hymenolipidid. Both Egyptian murine isolates of Hymenolipidid; *H. diminuta* and *H. nana*, were closer to each other than being to *H. nana* of human origin. WHO annual reports maintained the traditional host specificity of hymenolepidid till few years ago (Lee et al. 2007). Unfortunately, the unexpected discovery of a mixed infection with specie which is known to infect rodent as definitive host in surveyed individuals (Thompson et al. 2001; Macnish et al. 2003) as well as in dogs living in the same locality as their infected owners declared the public health impact of new infections, and meditating urgent thorough understanding of the epidemiology of these parasites (Jenkins and Andrew 1993; Thompson et al. 1993; Macnish et al. 2003). Since such deviation in patency of infection was not previously recorded thus highlights the growing importance of using molecular techniques in both the detection and characterization of parasite species in human and animals' populations especially between morphologically similar species (Okamoto et al. 1997; Nakao et al. 2000; von Nickisch-Rosenegk et al. 2001; Macnish et al. 2003).

In a comparison of genetic makeup, our result suggests that *cox1* gene is generally conserved by each isolate nucleotide sequence analysis. Despite of variation occurred in terms of sequence length and nucleotide differences and gaps (nucleotide insertions, deletions, and substitutions), but not G+C percentage where the overall numbers did not differ between amplified fragments mostly triggered by a variety of hosts' biological conditions (Macnish et al. 2002a, b). Such data showed consistent patterns with other researcher groups in this regard. They reported that mitochondrial DNA sequences of the Platyhelminthes accumulate nucleotide substitutions at a much higher rate than sequences in comparisons

of genetic distances (Littlewood and Bray 2001; Vilas et al. 2005). Base substitutions and additions are characterized by high T content which can, at times, represent poly-T structures. In addition, this may be a consequence of frame-shift mutations or premature stop codons, however, protein-coding genes of the *mt*DNA are error-checked by translating the nucleotide sequences (Benasson et al. 2001). Specific substitution rates include metabolic rates and body mass, generation time, differential fixation of slightly deleterious mutations, DNA repair mechanisms, and nucleotide composition (Vilas et al. 2005).

According to the inferred topology of amino acids phylogeny of Egyptian hymenolipidid, hosts effect on the evolutionary relationship between isolates was clear despite their intra species differences which agree with Johnston (2006). This could explain the closer relation of *Hymenolipis* spp. (*H. nana* and *H. diminuta*) collected from rat to be arranged in one cluster despite the disparities in host species and morphology which is in continence with Littlewood et al. (2008). These results agree with previous reports supported variant biological features of *H. diminuta* that are not always identical between isolates is built on genetic background (Okamoto et al. 1997). However, there results are conflicting with both the characteristic cryptic species of *H. nana* (Macnish et al. 2002a, b), and Schmidt classification where *H. nana* should be closer to *H. microstoma* than *H. diminuta* (Schmidt 1986). These observations that were revealed from the present study which should not be applied unambiguously to host-parasite associations since it does not take into consideration other factors related to the ecology of the hosts and the dynamics of the host-parasite assemblages (Johnston 2006). However, it should highlight the danger of triggering changes in genetic interspecificity subsequently definitive host susceptibility. Since, *mt cox1* resultant phylogenetic tree did not support the current hypotheses on the basis of morphological evidence for the separation of species (Littlewood et al. 2008).

In conclusion, molecular protocol developed in this study will provide the tools for achieving supplementary comprehensive epidemiological portrait of infection in Egypt. Consequently, should be applied on much broader scale in screening for *Hymenolipis* spp. infections. Sufficient clarification of evolutionary relationship of *Hymenolipis* spp. by other ribosomal DNA content, and complete *mt* genome sequencing and its genes arrangement are essentials. These data will ultimately aid investigations on dynamics of morphological and developmental evolution, as well as the biology of parasitism.

Corresponding author
Omnia M. Kandil
Parasitology and Animal Diseases Department, National Research Center, Dokki, Giza, Egypt, kandil_om@yahoo.com*

5. References:

1. Ando K, Tsunemori M, Akahane H, Tesana S, Hasegawa H, Chinzei Y (2006) Comparative study on DNA sequences of ribosomal DNA and cytochrome *c* oxidase subunit 1 of mitochondrial DNA among five species of gnathostomes. J Helminthol 80:7-13
2. Avise JC (2000) *Phylogeography: The history and formation of species*, Harvard University Press.
3. Behera B, Mirdha BR, Makharia GK, Bhatnagar S, Dattagupta S, Samantaray JC (2008) Parasites in patients with malabsorption syndrome: A clinical study in children and adults. Dig Dis Sci 53:672–679
4. Benasson D, Zhang DX, Hart DL, Hewitt GM (2001) Mitochondrial pseudogenes: evolution's misplaced witnesses. Trends Ecol Evol 16:314-321
5. Boore JL (1999) Animal mitochondrial genomes. Nucleic Acids Res 27:1767–1780
6. Boore, JL, Brown WM (1998) Big trees from little genomes: Mitochondrial gene order as a phylogenetic tool. Curr Opin Genet Dev 8:668–674
7. Constantine CC (2003) Importance and pitfalls of molecular analysis to parasite epidemiology. Trends Parasitol 19:346-348.
8. Felsenstein J (1985) Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783-791
9. Hu M, Chilton NB, Gasser RB (2004) The mitochondrial genomics of parasitic nematodes of socio-economic importance: recent progress, and implications for population genetics and systematics. Adv Parasitol 56:133-212
10. Jenkins DJ, Andrew PL (1993) Intestinal parasites in dogs from an Aboriginal community in New South Wales. Aust Vet J 70:115–116
11. Johnston DA (2006) Genomes and genomics of parasitic flatworms. In: Maule AG, Marks NJ (Eds.) *Parasitic flatworms*: *molecular biology*, *biochemistry*, *immunology and physiology*. CAB International, Wallingford, pp 37-80
12. Le TH, Blair D, Agatsuma T, Humair PF, Campbell NJ, Iwagami M, Littlewood DT, Peacock B, Johnston DA, Bartley J, Rollinson D, Herniou EA, Zarlenga DS, McManus DP (2000) Phylogenies inferred from mitochondrial gene

orders—a cautionary tale from the parasitic flatworms. Mol Biol Evol 17:1123–1125
13. Le TH, Blair D, McManus DP (2002) Mitochondrial genomes of parasitic flatworms. Trends Parasitol 18:206–213
14. Lee S-U, Chung H-C, Huh S (2007) Molecular phylogeny of parasitic Platyhelminthes based on sequences of partial 28S rDNA D1 and mitochondrial cytochrome c oxidase subunit I. Korean J Parasitol 45(3):181-189
15. Littlewood DTJ, Bray RA (2001) Interrelationships of the Platyhelminthes. Taylor and Francis, London, UK.
16. Littlewood DTJ, Waeschenbach A, Nikolov PN (2008) In search of mitochondrial markers for resolving the phylogeny of cyclophyllidean tapeworms (Platyhelminthes, Cestoda)– a test study with Davaineidae. Acta Parasitol 53(2):133–144
17. Macko JK, Hanzelova V (2008) New books. Helminthologia 45(4):211
18. Macnish MG, Morgan UM, Behnke JM, Thompson RCA (2002a) Failure to infect laboratory rodents with humans isolates of *Rodentolepis* (*Hymenolepis*) nana. J Helminthol 76:37–43
19. Macnish MG, Morgan-Ryan UM, Monis PT, Behnke JM, Thompson RCA (2002b) A molecular phylogeny of nuclear and mitochondrial sequences in *Hymenolepis nana* (Cestoda) supports the existence of a cryptic species. Parasitol 125:567–575
20. Macnish MG, Ryan UM, Behnke JM, Thompson RCA (2003) Detection of the rodent tapeworm *Rodentolepis* (*Hymenolepis*) *microstoma* in humans. A new zoonosis? Int J Parasitol 33:1079–1085
21. McManus DP, Le TH, Blair D (2004) Genomics of parasitic flatworms. Int J Parasitol 34:153–158
22. Movsesyan SO, Jivanyan KA, Chubaryan FA, Malczewski A, Terenina NB, Petrossyan R, Ter-Oganyan KS (2008) Experimental hymenolepiasis of rats: preliminary data on histopathological changes of visceral organs. Acta Parasitol 53(2):193–196
23. Nakao M, Sako Y, Ito A (2003) The mitochondrial genome of the tapeworm *Taenia solium*: A finding of the abbreviated stop codon U. J. Parasitol 89:633–635
24. Nakao M, Sako Y, Yokoyama N, Fukunaga M, Ito A (2000) Mitochonderial genetic code in cestodes. Mol Bioch Parasitol 111:415-424
25. Nakao M, Yokoyama N, Sako Y, Fukunaga M, Ito A (2002) The complete mitochondrial DNA sequence of the cestode *Echinococcus multilocularis* (Cyclophyllidea: Taeniidae). Mitochondrion 1:497–509
26. Ngarmamonpirat C, Waikagul J, Petmitr S, Dekumyoy P, Rojekittikhun W, Anantapruti MT (2005) Analysis of sequence variation in *Gnathostoma spinigerum* mitochondrial DNA by single-strand conformation polymorphism analysis and DNA sequence. Int J Parasitol 54:65-68
27. Okamoto M, Agatsuma T, Kurosawa T, Ito A (1997) Phylogenetic relationships of three hymenolepidid species inferred from nuclear ribosomal and mitochondrial DNA sequences. Parasitol 115:661-666
28. Raether WH, Hänel H (2003) Epidemiology, clinical manifestation and diagnosis of zoonotic cestode infections: An update. Parasitol Res 91:412-438
29. Saitou N, Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406-425
30. Sambrook J, Fritscgh EF, Meniates A (1989) Molecular cloning: A loboratory manual. Cold Spring Harbor Laboratory Press, New York.
31. Saraste M (1999) Oxidative Phosphorylation at the fin de siècle. Science 283(5407):1488-1493
32. Schmidt GD (1986) CRC handbook of Tapeworm identification. CRC Press, Boca Raton.
33. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596-1599
34. Tanowitz HB, Louis M, Weiss MD, Wittner M (2001) Tapeworms. Curr infect dis rep., 3: 77-84.
35. Tatusova, T.A. and Madden, T.L. (1999): BLAST 2 Sequences, a new tool for comparing protein and nucleotide sequences. FEMS Microbiol Lett 174(2):247-250. Erratum in: FEMS Microbiol Lett 1999, 177(1):187-188
36. Thompson RCA, Meloni BP, Hopkins RM, Deplazes P, Reynoldson JA (1993) Observations on the endo- and ectoparasites affecting dogs and cats in Aboriginal communities in the north-west of Western Australia. Aust Vet J 70:268–269
37. Thompson RCA, Reynoldson JA, Garrow SC, McCarthy JS, Behnke JM (2001) Towards the eradication of hookworm in an isolated Australian community. Lancet North Am Ed 357:770–771
38. Thompson RCA, Zarlenga DS, La Rosa G, Pozio E, Rosenthal B, Bandi C, Mortarino M, Casiraghi M, Genchi C, Gasser RB, Hu M, Chilton NB, Matthews JB, Hodgkinson JE (2004) Molecular systematics and diagnosis. Vet Parasitol 125:69-92
39. Traversa D, Costanzo F, Iorio R, Aroch I, Lavy E (2007) Mitochoderial cytochrome *c* oxidase

subunit 1 (*cox1*) gene sequence of *Spirocerca lupi* (Nematoda, Spirurida): Avenues for implications. Vet Parasitol 146:263-270
40. Vilas R, Criscione CD, Blouin MS (2005) A comparison between mitochondrial DNA and the ribosomal internal transcribed regions in prospecting for cryptic species of Platyhelminth parasites. Parasitol 131:839-846
41. von Nickisch-Rosenegk M, Brown WM, Boore JL (2001) Complete sequence of the mitochondrial genome of the tapeworm *Hymenolepis diminuta*: Gene arrangements indicate that Platyhelminths are Eutrochozoan. Mol Biol Evol 18(5):721–730
42. Zuckerkandl E, Pauling L (1965) Evolutionary divergence and convergence in proteins. In: Bryson V, Vogel HJ (Eds) Evolving Genes and Proteins, Academic Press, New York, pp 97-166.

10/8/2010

Synthesis and Characterization of Poly (Acrylamide - co - Acrylic acid) Hydrogel Containing Silver Nanoparticles for Antimicrobial Applications

Fatma S. Aggor [1] ; Enas M. Ahmed[1]*, A.T. El-Aref [2] and M. A. Asem[3]

[1] Department of Chemical Engineering & Pilot Plant [2] Department of Pre-treatments and Finishing.
[3] Department of Chemistry of Natural and Microbial Products, National Research Center, Dokki, Cairo, Egypt
*elarefenas123@yahoo.com

Abstract: Acrylamide was copolymerized with acrylic acid at different ratios using potassium persulphate initiation system in presence of a crosslinking agent and different doses of silver nitrate to yield hydrogels containing silver nanoparticles upon post treatment with sodium hydroxide. Swelling capacity and kinetics of swelling of these hydrogels were studied. Size and distribution of the nanoparticles and their dependence on acrylamide / acrylic acid ratios as well as on the dose of silver nitrate were also studied using Transmission Electron Microscopy (TEM). Furthermore, the antimicrobial and antifungal activities of the hydrogels in correlation with TEM results were reported. Hydrogels samples having relatively large number of Ag nanoparticles and widely distributed smaller particle size inhibit bacterial and fungal growth.
[Fatma S. Aggor; Enas M. Ahmed, A.T. El-Aref and M. A. Asem. **Synthesis and Characterization of Poly (Acrylamide - co - Acrylic acid) Hydrogel Containing Silver Nanoparticles for Antimicrobial Applications.** Journal of American Science 2010;6(12):648-656]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: hydrogel; silver nanoparticles; kinetic study; antimicrobial activity.

1. Introduction:

By definition, hydrogels represent polymeric networks capable of absorbing large quantities of water, but remain insoluble due to chemical or physical crosslinks between individual polymeric chains [1]. Recently, there is a great deal of interest concerning the production of nanoparticles in the hydrogel networks since they have enormous valuable applications in bio-related fields [2]. Indeed the design and development of nanoparticles and nanostructural materials have opened a new era for constructing well designed nanostructures that have been considered as a novel class of materials for catalytic, optical, electronic and biomedical application.

Previous studies concerned with metal nanoparticles especially those of silver, gold and copper were found to exhibit resistance to microorganisms [3, 4]. It was also reported that among these nanoparticles, nanosilver displayed acceptable antimicrobial properties [5, 6]. Nanosilver particles were introduced to a wide range of medical applications and water purifying systems [7, 8]. Silver nanoparticles proved to be non-toxic and eco-friendly antibacterial agents. The problem of their weak binding properties was overcome via preparation of stabilized nanoparticles in a polymer, notably hydrogel networks where the nanoparticles were embedded therein [9, 10].

Gels of importance are of polymeric nature due to the possibilities they offer to design systems with well defined and controlled properties. Silver and silver ions have long been known to have strong inhibitory and anti bacterial effects as well as a broad spectrum of antimicrobial activities [11]. But silver is expensive and the antibiotic introduction in the last century made it out of use. In recent years, however, the nanoscale techniques were developed for producing silver nanoparticles which may assist the medical use resurgence of silver, especially in applications where fighting germs is a concern.

Silver nanoparticles, which have a high specific surface area and a high fraction of surface atoms, have attracted the attention of the industry because of their unique characteristics, high efficiency and antimicrobial activity, even at low concentrations [12, 13]. The exact antimicrobial mechanism of silver is not well established; however, it was reported that the free silver ion is the active agent, combining the thiol (SH) groups, which leads to the protein inactivation [14]. There is evidence that antibacterial potency of silver is directly proportional to the concentration of silver ions in the medium [15–16].

Polymer nanocomposite containing metal nanoparticles can be prepared by several methods. Methods used for preparation comprise mechanical mixing of a polymer with metal nanoparticles, the in situ polymerization of a monomer in the presence of metal nanoparticles and the in situ reduction of metal salts or in a polymer [17]. Copolymeric hydrogel networks are composed of two or more different monomer species with at least one hydrophilic component, arranged in a random, block or alternating configuration along the chain of the

polymer network. The copolymeric hydrogel networks are generally covalently or ionically crosslinked structures, which are not water soluble.

In the present work, in situ synthesis of silver nanoparticles within swollen hydrogel networks is undertaken. Thus, copolymer gels were prepared by simultaneous polymerization of acrylamide (AAm) and acrylic acid (AAc) using potassium persulphate (KPS) as free radical initiator and N,N`-methylenebisacrylamide (MBA) as crosslinking agent in presence of silver nitrate. Swelling behavior and swelling kinetics of the so obtained hydrogels were evaluated. Distribution of the silver nanoparticles through their characterization within the hydrogels using TEM was studied. Also studied were the antibacterial and antifungal activities of the hydrogels under investigation.

2. Experimental

2.1. Materials

Acrylamide (AAm) and acrylic acid (AAc) in the monomeric form were provided by Sisco Research Lab. PVT. LTD, India; while N,N`-methylenebisacrylamide (MBA), crosslinking agent, potassium persulphate (KPS), initiator and silver nitrate (AgNO3) were supplied by Sigma – Aldrich, Inc. These reagents were used as laboratory grade chemicals.

Escherichia coli NRRL B-210 (Gram -ve bacteria), *Bacillus subtilis* NRRL B-543 and *Staphylococcus aureus* (Gram +ve bacteria), *Candida albicans* NRRL Y-477 (Fungi) were obtained from Northern Utilisation Research and Development Division, U.S. Department of Agricultural Peoria, Illinois, USA.

2.2. Procedures

2.2.1. Preparation of Ag nanoparticles

Hydrogel loaded with silver nanoparticles were prepared according to the procedure described elsewhere [17]. The hydrogel disks were prepared by free radical aqueous copolymerization of AAm and AAc in presence of MBA as crosslinker and KPS for initiating the polymerization system. A weighed amount of $AgNO_3$ was dissolved in double distilled water; desired amounts of AAm/AAc and MBA were added and the final volume was made 10 ml. A certain amount of KPS was dissolved and the whole reaction mixture was transferred to the test tube and then heated gently up to 70°C for 30 minutes. The test tube was broken and the resulting hydrogels were cut into slices of same thickness and then washed thoroughly with double distilled water. The hydrogel was then added to an aqueous solution of NaOH (5 wt %), and kept overnight till complete reduction of Ag^+ ions as indicated by faint yellow color of the colloidal Ag nanoparticles within the hydrogel network, washed several times in double distilled water and kept therein till complete swelling.

Blank experiment (omitting Ag^+ ions) and Ag/PAAm/AAc hydrogel prepared using different molar ratios of AAm/ Ag^+ ions were also performed. The prepared hydrogels were air-dried followed by vacuum drying and kept for characterization. Table I contains the monomers ratios and silver nitrate doses used throughout nanosilver hydrogel composite preparation.

Table I: Monomers ratio and silver nitrate dose.

Sample	AAm/AAc Ratio (g/g)	Silver nitrate dose (g/g monomer mixture)
HG_o (Blank)	50/50	------
HG_1	100/0	0.01
HG_2	70/30	0.01
HG_3	50/50	0.01
HG_4	30/70	0.01
HG_5	0/100	0.01
HG_6	50/50	0.02
HG_7	50/50	0.03
HG_8	50/50	0.04

1.3. Characterization

2.3.1. Swelling studies

The swelling characteristics of the prepared hydrogel were measured through gravimetric analysis [18]. The dried samples were placed in 50 ml of distilled water at room temperature (25 ± 1 ^{0} C) and taken from water at regular time intervals. The surface water on the swollen hydrogel was removed by soft pressing the sample between the folds of a filter paper; an increase in weight was recorded. The swelling equilibrium of the gels was determined as follows: gels were dried for a day at room temperature and were then dried in vacuum at 60°C. After the weight of the dried samples was determined, the samples were equilibrated in distilled water for 3 days at room temperature and then weighed again. The swelling ratio (S) and equilibrium swelling ratio (S_{eq}) were determined and calculated by application in the following equations [17]:

$$S(g/g) = \frac{W_t - W_o}{W_o} \quad (1)$$

$$S_{eq}(g/g) = \frac{W_{eq} - W_o}{W_o} \quad (2)$$

Where, W_o, W_t, and W_{eq} are the weights of the

samples in the dry state, the swollen state at a certain time, and the completely (equilibrium) swollen state, respectively.

2.3.2. Antimicrobial assay

The prepared hydrogel samples were screened in vitro for their antimicrobial activities against *Escherichia coli* NRRL B-210 (Gram -ve bacteria), *Bacillus subtilis* NRRL B-543 and *Staphylococcus aureus* (Gram +ve bacteria), *Candida albicans* NRRL Y-477 (Fungi).

The agar diffusion method [19] was used for this purpose. Bacteria and fungi were maintained on nutrient agar and Czapek's-Dox agar media, respectively. The assay medium flasks containing 50 ml of nutrient agar for bacteria and Czapek's-Dox agar medium for fungi respectively were allowed to reach 40-50^{o}c to be incubated with 0.5ml of the test organism cell suspension.

The flasks were mixed well and poured each into a Petri dish (15 x 2 cm) and allowed to solidify. After solidification, holes (6 mm diameter) were made in the agar plate by the aid of a sterile cork poorer. The prepared hydrogel samples were cut to small pieces and allowed to swallow using sterile distilled water then left on agar surface. The Petri dishes were kept at 5°C for 1 hour to allow diffusion of the samples through the agar medium and retard the growth of the test organism. Plates were incubated at 30 ^{o}C for 24 hours for bacteria and 72 h of incubation at 28 ^{o}C for fungi. The diameter of the resulted inhibition zone was measured in mm [19].

2.3.3. Transmission Electron Microscopy (TEM)

Images for the blank hydrogel and nanosilver hydrogel composites were recorded using a JEOL JEM-1230 electron microscope operating at an acceleration voltage of 100 kV. Specimens for TEM were prepared by the placement of a swollen sample of hydrogel on a 400-mesh copper grid followed by evaporation of excess water in air under ambient conditions (25 ± 1^{o}C).

3. Results and Discussion

The concept of producing nanoparticles in the networks of hydrogel systems was recognized as an important approach due to its direct applicability in various biomedical applications. That is why, a number of composite systems were evaluated [20]. The P(AAm-co-AAc)/silver nanoparticles hydrogels prepared as described above were investigated. In accordance with previous reports [17] the free network spaces between hydrogel networks reserve and stabilize nanoparticles. Moreover, the in situ reduction of metal ions and the stabilization of particles can be verified by the following: (a) metal ions are attached to reactive sites of hydrogel networks, and larger amounts of these ions are embedded in the network spaces of hydrogels; (b) the reduction process proceeds under the action of sodium hydroxide; and (c) the resulting particles are well stabilized through the hydrogel network interspaces**.**

3.1. Swelling behaviour

Swelling experiments were carried out with a view of evaluation the swelling capacity of the hydrogels under investigation in distilled water. Results of these experiments indicated that the increase in weight of the swollen hydrogels is directly related to the duration of swelling. The swelling behaviour observed could be associated with the absorption mechanism, which, in turn, is determined by the diffusion process.

Figure 1 illustrates the effect of AAm /AAc ratio in presence of silver nanoparticles on the swelling characteristics of the prepared hydrogel. It is evident that the swelling ratio increases sharply upon prolongation of swelling time up to ca 60 minutes then levels off. It was also observed that the swellability of the prepared hydrogel increases at higher concentrations of Polyacrylic acid (PAAc) ratio in the matrix. This is expected since abundance of the hydrophilic groups of AAc causes an improvement in the swelling characteristics of the hydrogel prepared under these conditions.

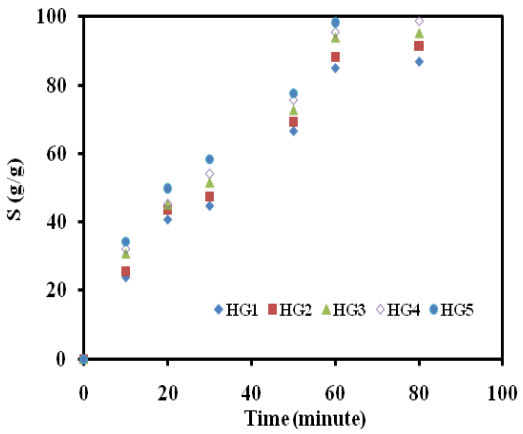


Figure 1: Effect of variation in AAm/AAc ratio on the swelling behavior of P(AAm-co-AAc)/silver nanoparticles hydrogel at different swelling durations

3.2. Kinetic study of swelling

The swelling kinetics of the prepared hydrogel was undertaken with view of clarifying the controlling mechanism of the swelling processes. This was visualized through kinetic models. The

latter were used to examine the results concerning swelling efficiency of the prepared hydrogels at different durations derived from the experimental work. A simple kinetic analysis represented by a second-order equation was applied in this study using the following relationship [17]:

$$\frac{dS}{dt} = k_s (S_{eq} - S)^2 \qquad (3)$$

Where:

k_S is the swelling rate constant and,
S_{eq} is the degree of swelling at the state of equilibrium.

After integration, when the initial conditions S = 0 at t = 0 and S = S at t = t, are applicable, equation (3) is modified to be as follows:

$$\frac{t}{S} = A + Bt \qquad (4)$$

Where

$A = 1/k_S.S_{eq}^2$ is the reciprocal of the rate of swellin at the initial state $[(dS/dt)_o]$ of the hydrogel, $B = 1/S_{eq}$ is the inverse of the maximum or equilibrium swelling and, k_S represents the swelling rate constant.

The kinetic models were examined by plotting **t/S** vs. **t** for the HG_1, HG_2, HG_3, HG_4 and HG_5. Figure 2 shows the linear regression of the swelling curves obtained by means of Equation 4 for the P(AAm –co-AAc)/ silver nanoparticles hydrogels in question. The initial swelling rate (r), the swelling rate constant (k_S) and the values of theoretical equilibrium swelling $(S_{eq})_{max}$ of all hydrogels were calculated from the slope and the intersection of the lines. The results are presented in Table II.

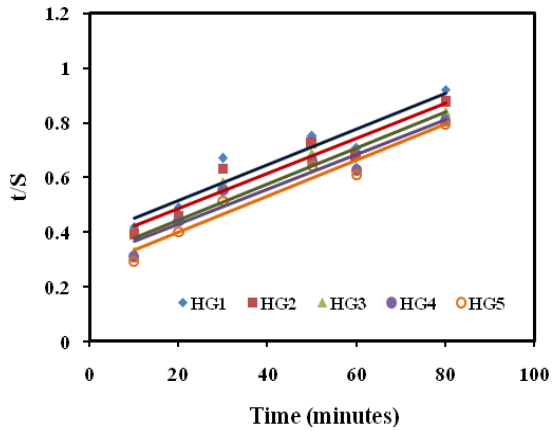


Figure 2: Swelling kinetic relations of P(AAm-co-AAc)/silver nanoparticles hydrogels at different ratios of AAm/AAc.

When a hydrogel is brought into contact with water, water diffuses and the hydrogel swells. Diffusion involves migration of water into pre-existing or dynamically formed spaces between hydrogel chains. Swelling of the hydrogel involves larger scale segmental motion resulting, ultimately, in an increased distance of separation between hydrogel chains. Analysis of the mechanisms of water diffusion in swellable polymeric systems is of prime importance due to applications of swellable polymers in the biomedical, pharmaceutical, environmental, and agricultural engineering fields. The swelling mechanism of the samples was determined by applying in the following equation **[17]:**

$$S\ (g/g) = \frac{[W_t - W_0]}{W_0} = Kt^n$$

Where

K is the swelling constant and
n is the swelling exponent calculated from the slopes of the lines of Ln(S) – Ln(t) plots.

For cylindrical shapes, n = 0.45-0.50 and corresponds to Fickian diffusion, whereas $0.50 < n < 1{:}0$ indicates that diffusion is non-Fickian. This equation is applied to the initial stages of swelling and plots of Ln(S) versus Ln (t) yield straight lines. For the hydrogels, Ln(S) versus Ln (t) plots was drawn using the kinetics of swelling and some representative results are shown in Figure 3. The swelling exponents ***n*** were calculated from the slopes of the lines and are listed in Table II. The values of the diffusional exponent range are generally between 0.5405 and 0.6387. Hence, the diffusion of water into P(AAm –co-AAc)/silver nanoparticles hydrogels had a non-Fickian character. The value of ***n*** higher than 0.5 indicating diffusion of water to the interior of all the hydrogels, follows an anomalous mechanism. The anomalous behaviour of the hydrogel is due to the regularity of the chain and strong interaction via the formation of hydrogen bonding, leading to a compact structure which would prove the anomalous aspects of diffusion even for a molecule as small as water [17].

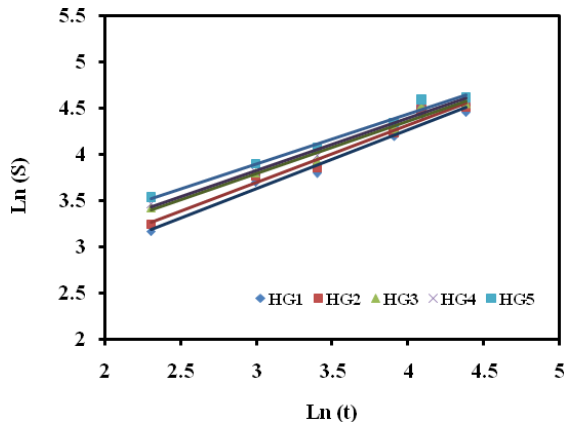


Figure 3: Swelling kinetic relations of P(AAm-co-AAc)/silver nanoparticles hydrogel at different ratios of AAm/AAc.

Non-Fickian diffusion processes have been thoroughly investigated [**21-24**]. Depending on the relative rates of chain relaxation and diffusion, non-Fickian diffusion has been classified into: "Case II transport" and "Anomalous transport" (Figure 4). Case II transport is dominated when the diffusion is very rapid compared to relaxation ($R_{diff} >> R_{relax}$), with relaxation occurring at an observable rate. Here, the rate of mass uptake is directly proportional to time. The anomalous transport is observed when the diffusion and relaxation rates are comparable ($R_{diff} \approx R_{relax}$).

Since most polymers swell when they are in contact with certain solvents, Fick's laws should be applied with modified boundary conditions and/or a generalized diffusion coefficient to address the non-Fickian behavior. An explanation of the swelling mechanism of the prepared hydrogels through their non-fickian characteristics can be illustrated by the scheme represented in Figure 4 **[25].**

Table II: Some Swelling Parameters of P (AAm-co-AAc)/silver Nanoparticles Hydrogel.

Swelling parameter	AAm / AAc Ratio				
	100/0	**70/30**	**50/50**	**30/70**	**0/100**
$S_{exp.}$	86.9	91.4	95.4	98.9	100.4
S_{eq}	153.8	156.3	151.5	156.25	151.52
K_s*10^4	1.0922	1.1422	1.3997	1.3585	1.6290
r	2.59	2.79	3.21	3.32	3.74
n	0.6387	0.6226	0.5698	0.5695	0.5405

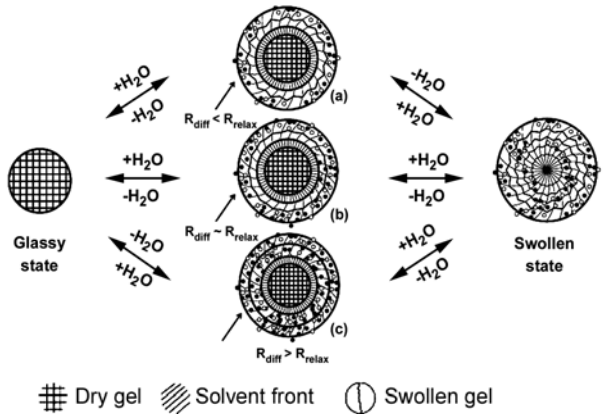


Figure 4: The mechanisms of Case II and anomalous diffusion.

3.2. Antimicrobial study

A recent study indicated that the bactericidal effect of silver nanoparticles mostly depends on the size of particles where silver nanoparticles having smaller sizes are more efficient and concluded that 1-10 nm have a direct interaction with bacteria [26]. The antibacterial activity is a manifestation of the release of silver nanoparticles from the Ag/PAAm-co-AAc hydrogel. Silver nanoparticles exhibit relatively large surface area, thus increasing their contact with bacteria or fungi. Silver nanoparticles show powerful bactericidal activity by binding with microbial DNA, thereby preventing bacterial replication.

P(AAm-co-AAc) hydrogel (HG_o) showed no inhibition zone. Results of the antimicrobial activity test against *Escherichia coli* (Gram -ve bacteria) and *Bacillus subtilis* (Gram +ve bacteria) showed that HG_3, HG_6 and HG_8 have an antibacterial activity while the HG_o were generally inefficient. Table III represents the antimicrobial activity of the prepared hydrogels with different doses of silver nitrate upon

the selected bacteria and fungi, while figure 5 shows the growth of bacteria in a Petri dish containing hydrogels with different silver doses.

Table III: In vitro antimicrobial activity by agar method diffusion of tested materials

Sample in vials	*Candida albicans*	*Escherichia coli*	*Staphylococcus aureus*	*Bacillus subtilis*
HG_o	-	-	-	--
HG_3	+	+	+	+
HG_6	+	+	+	+
HG_8	+	++	++	++

+ve, zone of inhibition 10 mm or less

++ve, zone of inhibition 20 mm or less

-ve, no inhibition.

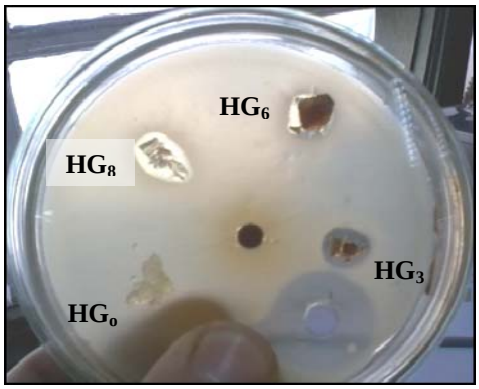


Figure 5: photograph showing the effect of nanocomposite upon bacterial activity

The same holds true for experiments carried out in parallel lines with fungi where silver nanoparticles derived from the prepared hydrogels show antifungal activity against *Candida albicans* as compared with the inactive blank sample HG_o.

The inhibition area follows the order: $HG_8 > HG_6 > HG_3$ as shown in Figure 9.This behaviour is expected where abundance and small sizes of silver nanoparticles (1-12 nm) in the prepared hydrogels are responsible for inhibition of bacterial and fungal growth.

3.3. Transmission Electron Microscopy

The size and morphology of silver nanoparticles formed on the P(AAm-co-AAc) hydrogel were determined through TEM imaging. Figure 6 represents a micrograph of a blank sample of, HG_o, of P(AAm-co-AAc) hydrogel in absence of silver nanoparticles.

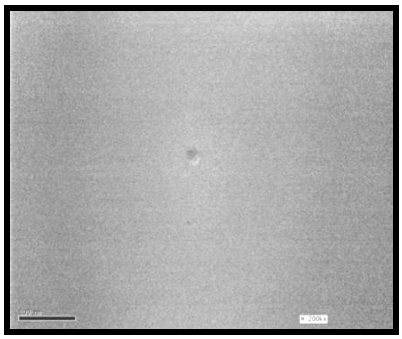


Figure 6: **TEM micrograph of P(AAM-co-AAc) hydrogel**

Figures 7-A, 8-A and 9-A represent images of micrographs for P(AAm-co-AAc) hydrogel/silver nanoparticles; referred here as HG_3, HG_6 and HG_8 using 0.01, 0.02 and 0.04 g silver nitrate respectively in preparation of these hydrogels. TEM micrographs revealed varieties in distribution of silver nanoparticles.

Careful examination of the images showed the silver nanoparticles with different variable sizes as well as smaller polydispersed particles. It should be noted, however, that the majority of the silver nanoparticles were scattered, a few of them showing aggregates indicating stabilization of the nanoparticles. The results represented by TEM images concluded that the particle size of individual nanoparticles seem to be 1-12 nm, whereas majority of silver nanoparticles exhibit smaller sizes.

Histograms of size distribution derived from TEM images for hydrogels HG_3, HG_6 and HG_8 are shown in figures 7-B, 8-B and 9-B. A detailed correlation between particle size and size distribution for each of these hydrogel systems is given below:

Figure 7-B shows the size range of silver nanoparticles formed on hydrogel; HG_3 prepared in presence of 0.01g silver nitrate. Sizes of these nanoparticles lie within the range 1-7 nm. TEM micrographs revealed also that a size range of 3-4 nm is prevailing. Size distribution of silver nanoparticles embedded in hydrogel HG_6 prepared under the action of 0.02 g silver nitrate as shown in Figure 8-B was found to be in the range of 1–12 nm. Nanoparticles having sizes ranging from 6-12 nm constitute the largest percentage within the specimen examined. Moreover, increasing the concentration of silver nitrate up to 0.04 g causes a considerable increase in the amount of silver nanoparticles with smaller sizes; 1-3 nm with some particles sized 4-5 nm and a low percentage of nanoparticles having sizes within the range of 6-12 nm. This is rather clarified by the data of the histogram represented in Figure 9-B.

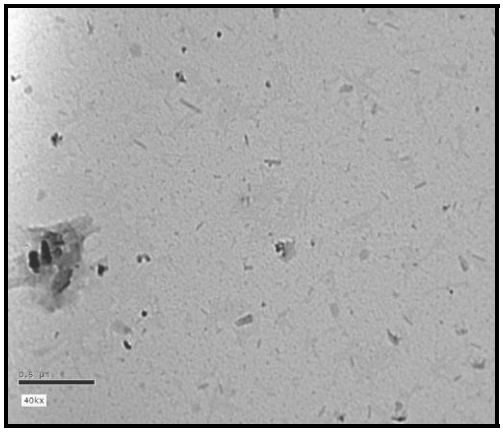


Figure 7-A: TEM micrograph of P(AAM-co-AAc)/ silver nanoparticles hydrogel prepared using 0.01g $AgNO_3$

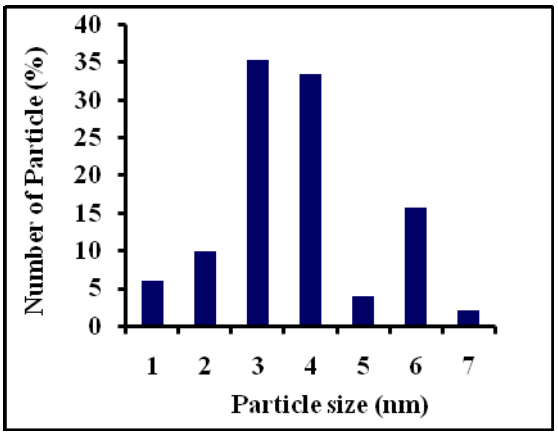


Figure 7-B: Histogram illustrating size distribution of silver nanoparticles via TEM micrograph of hydrogel prepared using 0.01g $AgNO_3$

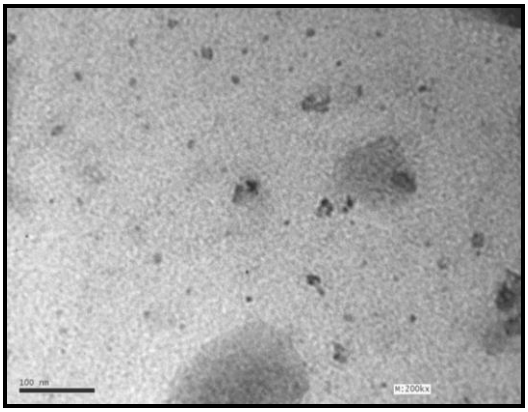


Figure 8-A: TEM micrograph of P (AAM-co-AAc)/ silver nanoparticles hydrogel prepared using 0.02g $AgNO_3$

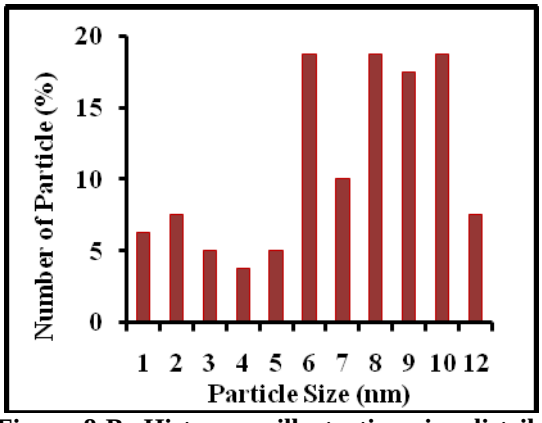


Figure 8-B: Histogram illustrating size distribution of silver nanoparticles via TEM micrograph of hydrogel prepared using 0.02 g $AgNO_3$

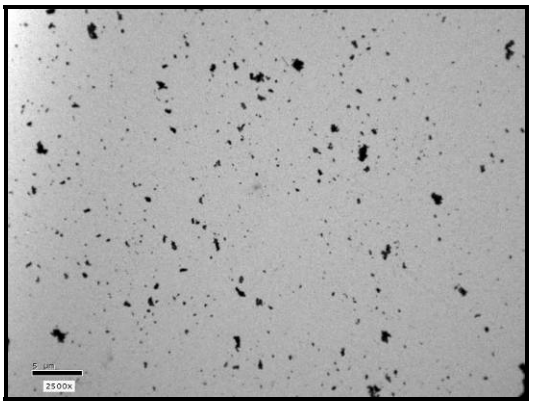


Figure 9-A: TEM micrograph of P (AAM-co-AAc)/ silver nanoparticles hydrogel prepared using 0.04g $AgNO_3$

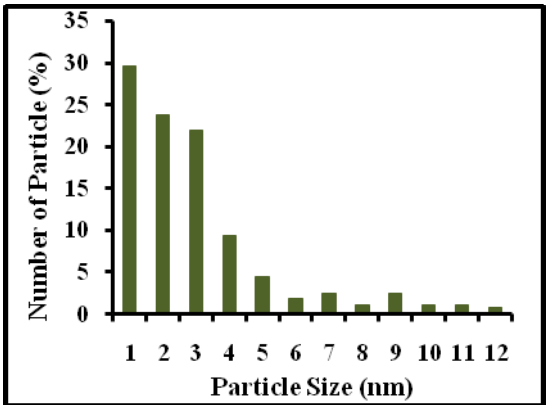


Figure 9-B: Histogram illustrating size distribution of silver nanoparticles via TEM micrograph of hydrogel prepared using 0.04 g $AgNO_3$

3.4. Antimicrobial activity and TEM micrographs

Correlation of the data of antimicrobial activity with those of TEM for hydrogels under investigation, conceive that both size and number of silver nanoparticles play an important role in the inhibition of bacterial and fungal growth. This can be exemplified by the study on sample HG_3 as shown by Figures 7-A and 7-B and Table III where low number and small size of silver nanoparticles resulted in a small inhibition zone, i.e. poor antimicrobial activity. Small sizes and larger number of silver nanoparticles observed with HG_6 sample, as shown in Figures 8-A and 8-B and Table III caused a slight improvement in bacterial and fungal inhibition.

On the other hand, hydrogel sample HG_8 exhibited a comparatively larger number of silver nanoparticles having widely distributed smaller particle sizes as illustrated by Figures 9-A and 9-B. The efficiency of this hydrogel sample to inhibit bacterial and fungal growth revealed a wide and distinct inhibition zone. This was confirmed by the data given in Table III.

It can be concluded that the results of TEM imaging of the prepared hydrogels are in accordance with those arrived at from the antimicrobial activities of these hydrogels.

4. Conclusion

P(AAm-co-AAc) hydrogels containing silver nanoparticles were successfully prepared via a free radical copolymerization, in presence of silver nitrate at varying doses along with concurrent, crosslinking and reduction of Ag^+ ions to develop silver nanoparticles in the hydrogel matrix. The swelling capacities of the resulting hydrogels were evaluated. Swelling was found to be directly related to swelling duration and AAm/AAc ratio used in the polymerisation process. Kinetic studies of swelling concluded that the diffusion of these hydrogels was of the non-Fickian type.The hydrogel products were also characterized for size distribution of silver nanoparticles through examination of micrographs and histograms obtained using TEM. Antimicrobial activity of these hydrogels was also investigated. Correlation of TEM results with those of the hydrogel capability to inhibit bacterial and fungal growth was made and confirmed that small sizes and abundance of silver nanoparticles determine the antimicrobial activity of the hydrogel.

Corresponding author
Enas M. Ahmed[1]
Department of Chemical Engineering & Pilot Plant
National Research Center, Dokki, Cairo, Egypt
elarefenas123@yahoo.com

5. References:

1. Kashyap, N., Kumar, N. and Kumar, M. Hydrogels for Pharmaceutica and Biomedical Applications, Critical Review in Therapeutic Drug Carrier Systems, 22(2005), p.107.
2. V. Thomas, Murali Mohan Yallapu, B. Sreedhar, S.K. Bajpai; A versatile strategy to fabricate hydrogel–silver nanocomposites and investigation of their antimicrobial activity, Journal of Colloid and Interface Science 315 (2007), p.389.
3. Q.L. Feng, F.Z. Cui, T.N. Kim, J.W. Kim, Ag-substituted hydroxyapatite coatings with both antimicrobial effects and biocompatibility J. Mater. Sci. Lett. 18 (1999), p. 559.
4. Lee, H.J., Yeo, S.Y., and Jeong, S.H., Antibacterial Effect of Nanosized Silver Colloidal Solution on Textile Fabrics, *J. Mater. Sci*., 38(*2003*), p.*2199*.
5. I. Margaret, S.L. Lui, V.K.M. Poon, I. Lung, A. Burd, Antimicrobial activities of silver dressings: an in vitro comparison, J. Med. Microbiol, 55 (2006), p.59.
6. John J. Curtin and Rodney M. Donlan, Using Bacteriophages To Reduce Formation of Catheter-Associated Biofilms by Staphylococcus epidermidis Antimicrob. Agents Chemother. 50 (2006), p.1268.
7. P. Jain, T. Pradeep, Biotechnology and Bioengineering Potential of silver nanoparticle-coated polyurethane foam as an antibacterial water filter, Prashant Jain and T.Pradeep, 90 (2005), p.59.
8. J. Zuhuang, patent number CN 1387700, 2003; M. Chen, S. Chen, patent number CN 1355335, 2002.
9. C.Wang, N.T. Flynn, R. Langer, Morphologically Well-defined Gold Nanoparticles Embedded in Thermo-Responsive Hydrogel Matrices Mater. Res. Soc. Symp. Proc. 820 (2004), R2.2.1.
10. Y. Murali. Mohan, T. Premkumar, K.J. Lee, K.E., Fabrication of Silver Nanoparticles in Hydrogel Networks, Geckeler, Macromol. Rapid Commun. 27 (2006), p.1346.
11. Grier, N, "Silver and Its Compounds," In: Disinfection, Sterilization and Preservation, edited by S. Block, Lea & Febiger, Philadelphia, (1983), p. 375.
12. M. Kowshik, S. Ashtaputre, S. Kharrazi, W. Vogel, J. Urban, SK. Kulkarni, KM. Paknikar: Extracellular synthesis of silver

nanoparticles by a silver-tolerant yeast strain MKY3. Nanotechnology 14 (95) (2003) p. 100.
13. N. Duran, P.D. Marcato, O.L. Alves, G. Souza, Mechanistic aspects of biosynthesis of silver nanoparticles by several Fusarium oxysporum strains. Journal of Nanotechnology 3 (2005), p.8.
14. Lehninger Principles of Biochemistry, 3rd ed.; By David L. Nelson and Michael M. Cox. Worth Publishers: New York, 2000. ISBN 1-57259-9316. 1255 pp.
15. A.B. Lansdown, Silver in health care: antimicrobial effects and safety in use Current Problems in Dermatology 33 (17) (2006), p.34.
16. A. Chakravarti, S. Gangodawila, M.J. Long, N.S. Morris, A.R. Blacklock, D.J. Stickler, An Electrified Catheter to Resist Encrustation by Proteus Mirabilis Biofilm. Journal of Urology 174 (1129) (2005), p. 32.
17. Enas M. Ahmed, Fatma S. Aggor. Swelling kinetic study and characterisatio of crosslinked Hydrogel containing Silver Nanoparticles. J. Appl. Polym. Sci. 117(2010), p.2168.
18.]Tuncer Caykara, Simin kipper, Gokhan Demirel,. Thermosensitive poly(N-isopropylacrylamide-co-acrylamide) hydrogels: synthesis, swelling and interaction with ionic surfactants, European polymer. J. 42(2005), p.348.
19. R. Cruickshank, J.P. Duguid, B.P. Marion, R.H.A. Swain, Medicinal Microbiology, 12th ed., vol. II, Churchill Livingstone, London,(1975), p. 196.
20. Bajpai, S.K., Mohan, Y. M., Bajpai, M., Tankhiwale, R., & Thomas,V. Synthesis of polymer stabilized silver and gold nanostructures. Journal of nanoscience and nanotechnology, 7(2007), p. 2994.
21. Liu Q, Wang X, Kee DD, Mass transport through swelling membranes, *Int J Eng Sci*, 43(2005), p.1464.
22. Vrentas JS, Vrentas CM, Steady viscoelastic diffusion, *J Appl Polym Sci*, 88**(**2003), p.3256.
23. Afif AE, Grme M, Non-Fickian mass transport in polymers, *J Rheol*, 46 (2002), p. 591.
24. Rajagopa KR, Diffusion through polymeric solids undergoing large deformations, *Mater Sci Tech*, 19(2003), p.1175.
25. Rossi G, Mazich KA, Kinetics of swelling for a cross-linked elastomer or gel in the presence of a good solvent, *Phys Rev A*, 44 (1991), p.4793.
26. Morones, J.R., Elechiguerra, J.L., Camacho, A., Holt, K., Kouri, J.B., Ramírez, J.P., & Yacaman M.J. The bactericidal effect of silver nanoparticles. Nanotechnology, 16(2005), p.2346.

7/1/2010

Novel Switching H_2/H_∞ Control: Combination of Dwell Time Switching Signal and Multiple Lyapunov Function

Fatemeh Jamshidi [1], Mohammad Taghi Hamidi Beheshti [1]

[1] Communication and Control Lab, School of Computer and Electrical Engineering, Tarbiat Modares University, Tehran, Iran
Fjamshidi59@yahoo.com, mbehesht@modares.ac.ir

Abstract: In this paper, a switching strategy is employed to solve the H_2/H_∞ multi objective controller design. Two controllers are designed to meet the H_2 and H_∞ performance specifications. Linear matrix inequalities are used in the controller design process. New switching signal is defined which is the combination of dwell time switching signal and multiple Lyapunov function such that stability of closed loop system is guaranteed as well as desired performance. Simulation results show that proposed switching strategy improves the performance of the controller and reduces the conservation in comparison with the common H_2/H_∞ controller.
[Fatemeh Jamshidi, Mohammad Taghi Hamidi Beheshti. Novel Switching H_2/H_∞ Control: Combination of Dwell Time Switching Signal and Multiple Lyapunov Function. Journal of American Science 2010;6(12):657-663]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Asymptotical Stability, Dwell time, H_2/H_∞ control, Multiple Lyapunov function, Switching signal.

1. Introduction

There has been increasing interest in hybrid control in recent years, due to its potential to overcome limitations of adaptive control and benefits in controlling of systems that cannot achieve the desired performance by a single controller. Indeed, hybrid control scheme provides an effective mechanism when facing large modelling uncertainty and highly complex systems. Even for simple linear time invariant systems, controllers switching can be utilized in improving the performance (Sun, 2005, Feuer, 1997, and McClamroch, 2000). To date, Morse, Hespanha and Liberzon have established a theoretical backbone for hybrid controllers (Morse, 1997, Hespanha, 1999, and Liberzon, 2003). By now, stabilizing a continuous system via hybrid output feedback has attracted a number of authors, such as (Santarelli, 2008) where a comparison between the responses of the switching controller and two other forms of LTI control have been made. An experimental assessment of controller switching with state and control magnitude constraints is carried out in Kogiso, 2004. In Zheng, 2006, the multi-objective robust control of an induction motor with tracking and disturbance rejection specifications is proposed via switching. In Essounbouli, 2006, DeCarlo, 1988, and Jamshidi, 2010 controller switching has been proposed to improve the trade-offs in design multi objectives.

Supervisory control employs logic-based switching for adaptation, instead of continuous tuning of parameters as in conventional adaptive control. This type of switching-based supervisory control scheme consists of the following subsystems: a plant to be controlled, a bank of controllers, and a switching logic. Dwell-time method is representative of the trajectory independent switching logic for supervisory control (see Yoon, 2007 and its references). On the other hand, Lyapunov functions are employed in such trajectory dependent switching methods as in Yoon, 2007.

In an actual engineering control problem, different contradictory requirements must be satisfied such as attenuation of various types of disturbances, set point tracking, bounds on the signal peaks, and robustness to changing conditions and plant uncertainties. The synthesis problems with a combination of performances are known as multi objective control. General multi objective control problems are difficult and remain mostly open up to date. The usual approach for the general multi objective control problem is to find a controller transfer matrix for all objective designs and to use the same Lyapunov matrix for the separate design specifications. Though meeting all the objectives of a control application is desirable, the design of a single multi-objective controller is a trade-off among competitive problems such as disturbance rejection, tracking, regulation, constraints of the signals…. So a single controller may be restrictive (Boyd, 1991, Scherer, 1997, and Khargonekar 1991).

The mixed H_2/H_∞ control is an important robust control method and has been studied by many researchers. The mixed H_2/H_∞ control is concerned with the design of a controller that minimizes the H2 performance of the system with respect to some

inputs while guarantees certain worst case H_∞ performance with respect to other inputs. In engineering applications, the mixed H_2/H_∞ control is more attractive than the sole H_∞ control since the H_∞ control is a worst-case design which tends to be conservative whereas the mixed H_2/H_∞ minimizes the average performance with a guaranteed worst-case performance.

Here, the H_2/H_∞ control problem of complex systems is treated using switching controller. That is, the desired plant behavior is achieved by switching between pre-designed controllers, each to meet a set of relevant specifications. Our aim is using switching controller to reduce the conservatism of the controller synthesis and the resultant performance degradation; therefore, we apply the concept of multiple controllers and utilize the switching signal to orchestrate the switching among pre-designed controllers to improve performance of closed-loop system.

In this paper, we present a new switching logic. At every time instant, we search for a controller corresponding to the best performance. We then decide whether to switch to that controller or not by comparing the value of Lyapunov function at the previous switching instant to this controller with its prospective value that would result from the switching; if a certain inequality condition is satisfied, switching is allowed. We then further employ a dwell-time algorithm together. We show that asymptotic convergence is ensured by the proposed switching control scheme resulting from the combination of the Lyapunov-function-based switching and the dwell-time switching.

This paper is organized as follows: Section 2 presents the system definition, and the controllers used in this paper. The problem of synthesis switching signal is described in Section 3. A simple illustrative example is presented in Section 4. Section 5 contains some concluding remarks.

2. Problem Statement

This paper presents a controller design strategy for fulfilling H_2/H_∞ control objectives resulting in no compromise between distinctive specifications. Instead of considering all of the objectives in a single controller, switching between these controllers in the timely manner is proposed to meet the desired performance. In this case, we can obtain our multi-criterion goal without introducing conservatism to the problem, as each specification or a set of relevant objectives are assumed to be accomplished by a single controller without considering any contradictory objective in the design procedure. As switching can cause instability, a new switching strategy is introduced that lets switching based on either dwell time switching or multiple Lyapunov function.

The state-space realization of the LTI plant, P, for which H_2 and H_∞ output-feedback controller is designed is as follows:

$$P:\begin{cases}\dot{x} = Ax + B_u u_C + B_w w \\ z = C_z x + D_{zu} u_C + D_{zw} w \\ y = C_y x + D_{yw} w\end{cases} \quad (1)$$

$x \in R^{n_x}$ is state, $w \in R^{n_w}$ is the exogenous input signal (noise, disturbance and reference input), $u_C \in R^{n_u}$ is the control input, $y \in R^{n_y}$ is the measured output and $z \in R^{n_z}$ is the output to be regulated and defines the performance objectives of the closed loop system. The assumptions of H_2 and H_∞ controls are true. The diagram of the closed loop system is depicted in Figure 1.

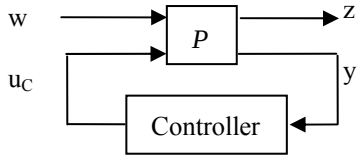


Figure 1. The Standard Diagram for H_2 and H_∞ Controls

The standard H_2/H_∞ performance criterion is considered: The objective is synthesis of the switching controller that internally stabilizes the closed loop system and minimize $\left\| T_{w_2 \to z_2} \right\|_2$ while $\left\| T_{w_\infty \to z_\infty} \right\|_\infty < \gamma$. $w_i = R_i^{-1} w$, $i \in I = \{H_2, H_\infty\}$ is the exogenous input and $z_i = L_i z$ is the controlled output (Scherer, 1997). The available design methods in addition to convex optimization problem defined by different Linear Matrix Inequalities (LMI) for H_2 and H_∞ closed-loop specifications given in Scherer, 1997 is used to find transfer matrices of the controllers.

Figure 2. illustrates the closed loop configuration used in this context, where u denotes the control input, y_P the process output, r a bounded reference signal (set point), d unknown but bounded input disturbance, and n unknown but bounded measurement noise. The process is a LTI system with strictly proper transfer matrix $H_P(s)$. $\{A_P, B_P, C_P\}$ is a minimal realization for $H_P(s)$. For the sake of conformity of the closed loop configuration in Figure 2. and the closed loop block diagram in Figure 1., the dashed line in Figure 2. is

considered as P in Figure 1., external inputs $\begin{bmatrix} r \\ d \\ n \end{bmatrix}$ in Figure 2. forms w in Figure 1.

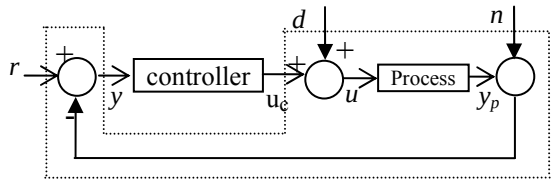


Figure 2. The Closed Loop Configuration

3. Purposed Switching Signal

We present a switching logic using the Multiple Lyapunov functions in Liberzon, 2003. The proposed algorithm is referred to as S_{MLF} and is given as follows: (Algorithm S_{MLF})

1- Initialize $\sigma(t)$ and Δt

2- Find the best controller: i := arg Best Controller

3. If $\sigma(t-\Delta t) \neq i$, and

$$V_i(t_i) \geq V_i(t) \qquad (2)$$

$V_i(.)$ is the Lyapunov function of the closed loop system with i th controller, t_i is the last time that switching to i th controller occurred, then let $\sigma(t^+) = i$ and $t_i = t$.

4. $t = t + \Delta t$ and go to step 2.

Switching takes place in S_{MLF} when two conditions are met: firstly there should be a better controller leading to the better performance, and secondly the inequality in (2) should hold to grantee the stability according to the following Lemma.

Lemma. 1. (Liberzon, 2003): Let $\dot{x} = f_i(x), i \in I$ be a finite family of globally asymptotically stable systems, and let $V_i, i \in I$ be a family of corresponding radially unbounded Lyapunov functions. Suppose that there exists a family of positive definite continuous functions $W_i, i \in I$ with the property that for every pair of switching times (t_p, t_q), $p < q$ such that $\sigma(t_p) = \sigma(t_q) = i \in I$ and ($\sigma(t_k) \neq$ i for $t_p < t_k < t_q$, we have $V_i(x(t_q)) - V_i(x(t_p)) \leq -W_i(x(t_p))$. Then the switched system is globally asymptotically stable.

In other words, switching is not allowed even when there is a better controller, if use of this new controller violates the condition given in (2). Checking the two conditions implies that both stability and performance is considered in S_{MLF} . Using this algorithm, switching system is asymptotically stable, the state variables are bounded and converge to zero, and all the signals remain bounded.

A dwell time, τ_D , is a lower bound for the difference between two consecutive switching instants; switching is allowed after waiting for the dwell time. Here we combine the dwell-time algorithm with switching logic proposed above. A switching logic using the dwell time switching is referred to as S_D which is time dependent and trajectory independent.

Lemma. 2. (Liberzon, 2003): The switched system $\dot{x} = A_i x$ is asymptotically stable if the time interval between consecutive switching instant between their asymptotical stable subsystems is not smaller than $\tau_D \geq \sup_{i \in I} \left(\frac{a_i}{\lambda_i} \right)$. Where $\left\| e^{A_i t} \right\| \leq e^{a_i - \lambda_i t}$ and $a_i = \log \left(\frac{\sigma_{\max}(M_i)}{\sigma_{\min}(M_i)} \right)$, M_i is the modal matrix (i.e. the matrix with eigenvectors as its columns) of the stable matrix A_i and $\sigma_{\max}(M_i)$ and $\sigma_{\min}(M_i)$ are the maximum and minimum singular values of M_i , respectively. The positive scalar λ_i is simply the absolute value of the real part of the eigen values A_i nearest to the imaginary axis (stability degree of stable matrix A_i).

As there are two switching logics involved, we use two subscripts for switching times to clarify which logic causes the switching; let $t_{p,q}$ denote the switching instant which is due to the q th switching by S_D in a row after the p th switching by the S_{MLF} . $t_{p,0}$ denotes the p th switching instant due to switching by S_{MLF} .

If there exists other controller with better performance but the condition (2) for S_{MLF} does not hold for $\bar{t} \in (t_{p,q} \quad t_{p,q} + \tau_D]$, then S_D forces switching to take place, leading to $t_{p,q+1} = t_{p,q} + \tau_D$. If the condition in (2) holds for some $\bar{t} \in (t_{p,q} \quad t_{p,q} + \tau_D)$ then switching results from S_{MLF} , leading to $t_{p+1,0} = \bar{t}$.

The two switching logics S_{MLF} and S_D are employed together in the proposed switching control scheme; as a result, switching takes place whichever logic allows without destroying stability as is shown

below. The proposed switching logic is referred to as $S_{MLF} \bigcup S_D$, and is depicted in Figure 3.

Plant with each controller forms a linear subsystem of switching signal. Suppose that at time instant $t_{p,0}$ switching to i th subsystem has occurred by S_{MLF} logic. The subsystems that switchings to them have occurred in the time interval $\left[t_{p,0} \quad t_{p+1,0}\right)$ by S_D logic can be considered as a subsystem of switching system with zero input nonlinear dynamic $\dot{x} = f_i(x)$ which is asymptotically stable according to Lemma 2.

As a result, the asymptotically stable linear subsystems, A_i, under switching logic $S_{MLF} \bigcup S_D$ is a combination of the asymptotically stable linear subsystems, A_i, and the asymptotically stable non linear subsystems, f_i, under S_{MLF} switching logic. If all the linear subsystems that switch together consecutively by S_D switching logic, and their switching start from i th subsystem are considered as one nonlinear subsystem, the number of subsystems is bounded. And according to Lemma 1 are asymptotically stable.

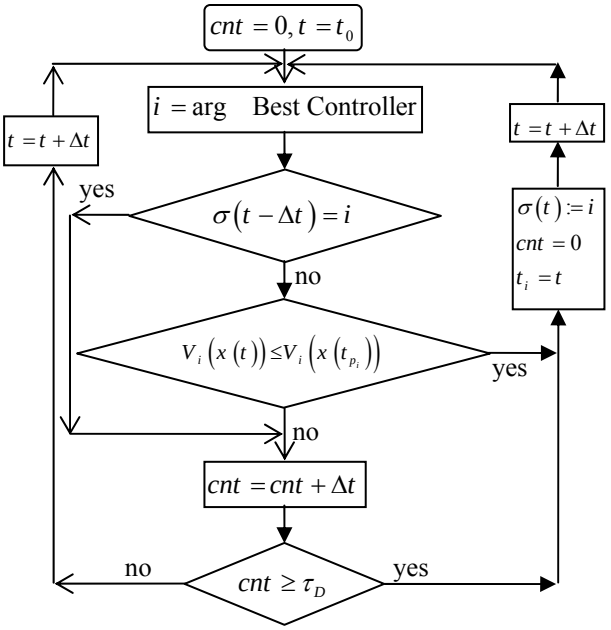


Figure 3. The Proposed Switching Logic $S_{MLF} \bigcup S_D$

4. Example

In this section, we briefly illustrate the enhancement of multi objective control performance via switching. The proposed approach is applied to the dynamic model of the roll angle of an aircraft taken from Vegte, 1994, and Hespanha, 2002:

$$H_P(s) = \frac{-1000}{s(s+0.875)(s+50)} \tag{3}$$

In this example, it is considered that white measurement noise with a large variance is injected in the time intervals $t \in [18 \quad 40]$, $t \in [73 \quad 93]$ and $t \in [128 \quad 148]$. In the presence of the measurement noise, noise rejection and the slower response are the objectives, while in the absence of the measurement noise a fast response and good tracking should be considered in the design procedure. This design problem is a multi-objective problem with conflicting criteria, because if a controller has low closed-loop bandwidth and is therefore not very sensitive to noise, it will exhibit a slow response and if a controller has high bandwidth and is therefore fast, it will be very sensitive to noise.

Following realization is considered for $H_P(s)$:

$$\dot{x} = \underbrace{\begin{bmatrix} -50.875 & -43.75 & 0 \\ 1 & 0 & 0 \\ 0 & -1000 & 0 \end{bmatrix}}_{A_P} x + \underbrace{\begin{bmatrix} 1 \\ 0 \\ 0 \end{bmatrix}}_{B_P} u$$

$$y_P = \underbrace{\begin{bmatrix} 0 & 0 & 1 \end{bmatrix}}_{C_P} x$$

It is easy to find that $\dot{y}_P = \begin{bmatrix} 0 & -1000 & 0 \end{bmatrix} x$.

Since $u = u_C + d$, $w = \begin{bmatrix} r \\ d \\ n \end{bmatrix}$ and $y = r - n - y_P$:

$$\dot{x} = \underbrace{\begin{bmatrix} -50.875 & -43.75 & 0 \\ 1 & 0 & 0 \\ 0 & -1000 & 0 \end{bmatrix}}_{A} x + \underbrace{\begin{bmatrix} 1 \\ 0 \\ 0 \end{bmatrix}}_{B_u} u_C + \underbrace{\begin{bmatrix} 0 & 1 & 0 \\ 0 & 0 & 0 \\ 0 & 0 & 0 \end{bmatrix}}_{B_w} w$$

$$y = \underbrace{\begin{bmatrix} 0 & 0 & -1 \end{bmatrix}}_{C_y} x + \underbrace{\begin{bmatrix} 1 & 0 & -1 \end{bmatrix}}_{D_{yw}} w$$

To have a good measurement noise rejection the controller, K_{H_2}, with more robust performance regarding measurement noise is designed using LQG/LQR. The regulator gains are computed by minimizing the cost $J = E\left\{\lim_{t\to\infty} \frac{1}{T} \int_0^T \left(y_P^2(t) + \dot{y}_P^2(t) + 100 u_C^2(t)\right) dt\right\}$. The design of the optimal LQG gain was done assuming that the load disturbance, d, and the measurement noise, n, were uncorrelated white noise processes with $E\left(d(t)d(\tau)\right) = \delta(t-\tau)$ and $E\left(n(t)n(\tau)\right) = \mu\delta(t-\tau)$, where $\mu = 10^{-1}$. It is easy to show that

$$J = E\left\{\lim_{t\to\infty}\frac{1}{T}\int_0^T \left(z_2(t)^T\ z_2(t)\right)dt\right\} = \left\|T_{w_2\to z_2}\right\|_2^2,$$

where $z_2 = \begin{bmatrix} y & \dot{y} & \rho u_C \end{bmatrix}$ and $w_2 = \begin{bmatrix} d \\ n \\ \frac{}{\sqrt{\mu}} \end{bmatrix}$, in other words

$$z_2 = \begin{bmatrix} y_P \\ \dot{y}_P \\ \rho u_C \end{bmatrix} = \underbrace{\begin{bmatrix} 0 & 0 & 1 \\ 0 & -1000 & 0 \\ 0 & 0 & 0 \end{bmatrix}}_{C_{z_2}} x + \underbrace{\begin{bmatrix} 0 \\ 0 \\ \rho \end{bmatrix}}_{D_{z_2 u}} u_C$$

. It is clear that, $B_{w_2} = \begin{bmatrix} 1 & 0 \\ 0 & 0 \\ 0 & 0 \end{bmatrix}$ and $D_{z_2 w_2} = \begin{bmatrix} 0 & -\sqrt{\mu} \end{bmatrix}$.

To get a relatively good tracking and to prevent the extra increasing of the value of input signal, the controller, K_{H_∞}, is designed by minimizing $\left\|T_{w_\infty\to z_\infty}\right\|_\infty$ where $w_\infty = r$ and:

$$z_\infty = \begin{bmatrix} y \\ u_C \end{bmatrix} = \underbrace{\begin{bmatrix} 0 & 0 & -1 \\ 0 & 0 & 0 \end{bmatrix}}_{C_{z_\infty}} x + \underbrace{\begin{bmatrix} 0 \\ 1 \end{bmatrix}}_{D_{z_\infty u}} u_C + \underbrace{\begin{bmatrix} 1 & 0 & -1 \\ 0 & 0 & 0 \end{bmatrix}}_{D_{z_\infty w}} w,$$

$$z_\infty = \begin{bmatrix} y \\ u_C \end{bmatrix} = \underbrace{\begin{bmatrix} 0 & 0 & -1 \\ 0 & 0 & 0 \end{bmatrix}}_{C_{z_\infty}} x + \underbrace{\begin{bmatrix} 0 \\ 1 \end{bmatrix}}_{D_{z_\infty u}} u_C + \underbrace{\begin{bmatrix} 1 \\ 0 \end{bmatrix}}_{D_{z_\infty w_\infty}} w_\infty$$

In Figure 4, the left plots show the closed-loop response of controller K_{H_∞} and the right plots show the closed-loop response of controller K_{H_2} to a square set point. It can be seen from this Figure that the controller K_{H_∞} exhibits a faster response but is more sensitive to measurement noise. The top plots show the output, y_P, and the bottom plots the tracking error, y.

The left plots in Figure 5 show the closed-loop response of the switching controller, $K_{switching}$, to square reference. This structure inherits the fast performance of K_{H_∞} in normal cases, and a good noise rejection of K_{H_2} in the presence of the white measurement noise.

Using Lemma 2 the minimum time interval between consecutive switching between controllers K_{H_2} and K_{H_∞} is equal to $\tau_D = 22.9083s$. $V_{K_2}(t)$ and $V_{K_\infty}(t)$ denote the Lyapunov functions of closed loop system with controller K_{H_2} and K_{H_∞} at time instant t, respectively.

In time interval $[0\ \ 18]$ controller K_{H_∞} and in time interval $[18\ \ 38]$ controller K_{H_2} are in the loop. Since $V_{K_\infty}(0) > V_{K_\infty}(38)$, according to algorithm we can switch to controller K_{H_∞}. At $t = 73$, because of the time interval between consecutive switching is $73 - 38 > 22.9083$, according to Figure 3. without checking inequality (2) we switch to controller K_{H_2}. Since $V_{K_\infty}(38) < V_{K_\infty}(93)$, according to Figure 3, we check the constraint (2) until $\tau_D = 22.9083s$ after previous switching. Any time that the constraint is satisfied, we can switch to controller K_{H_∞}. Since during this time interval the constraint is not satisfied switching to controller is occurred at $t = 73 + \tau_D$. At $t = 128$ similar to $t = 73$ we switch to controller K_{H_2}. Since $V_{K_\infty}(93) < V_{K_\infty}(148)$, according to Figure 3, we check the constraint (2) until $\tau_D = 22.9083s$ after previous switching. Any time that the constraint is satisfied, we can switch to controller K_{H_∞}. At $t = 148.03$ the constraint is satisfied and switching to controller K_{H_∞} is occurred.

The right plots show the closed-loop response of the common multi objective controller K_{H_2/H_∞} that minimize $\left\|T_{w_2\to z_2}\right\|_2$, subject to $\left\|T_{r\to z_\infty}\right\|_\infty < \gamma$ (a single controller that considers both design objectives concurrently). The top plots show the output, y_P, and the bottom plots the tracking error, y.

The comparison illustrates the conservatism reduction by means of the switching controller in comparison with the performance of the common multi objective controller. As depicted in this Figure, it is apparent that we have approached meeting both design objectives better using the switching controller. It can be seen, from Table 1. that the switching controller minimizes the 2- norm and ∞-norm of tracking error, y, in comparison with other controllers.

Table 1. The Performance of the Controllers

	K_{H_2}	K_{H_∞}	K_{H_2/H_∞}	$K_{switching}$
$\sup_n \frac{\|y\|_\infty}{\|n\|_2}$	3.658	3.8836	4.7811	3.6575
$\sup_n \frac{\|y\|_2}{\|n\|_2}$	84.162	83.2744	100.334	82.0997

5. Conclusion

In this paper, a switching H_2/H_∞ controller is developed. This structure allows us to take the benefits of both H_2 and H_∞ controllers and to efficiently eliminate their disadvantages. The transients caused by switching may result in instability which is avoided by appropriate choice of switching signal. Purposed new switching signal is the combination of dwell time switching and multiple Lyapunov function. The simulations illustrate that a switching controller scheme inherits characteristics of all the controllers in the time intervals they have been in the loop, and diminish the performance degradation caused by considering all the control objectives in the design of a unique controller.

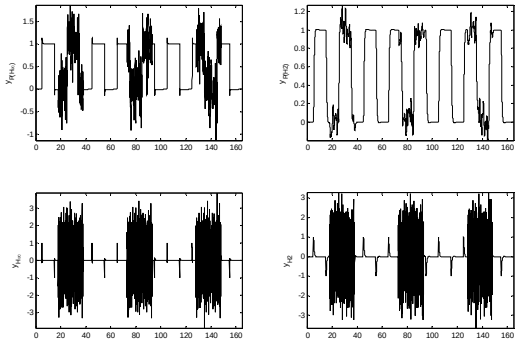


Figure 4. The Closed Loop Response of K_{H_∞} and K_{H_2}.

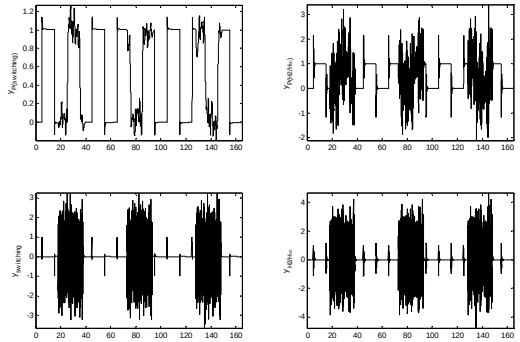


Figure 5. The Closed Loop Response of Supervisory Based Switching Controller, $K_{switching}$, and Common Multi Objective Controller, K_{H_2/H_∞}.

Corresponding Author:
Dr. Mohammad Taghi Hamidi Beheshti
Department of Computer and Electrical Engineering
Tarbiat Modares University
Tehran, Iran
E-mail: mbehesht@modares.ac.ir

References

1. Sun Z, Ge SS. Analysis and synthesis of switched linear control systems. Automatica. 2005;41:181-195.
2. Feuer A, Goodween GC, Saldago M. Potential benefits of hybrid control for linear time invariant plants. Proceedings of American Control Conference. New Mexico. 1997:2790-2794.
3. McClamroch NH, Kalmanovsky I. Performance benefits of hybrid control design for linear and nonlinear systems. Proceedings of IEEE, 2000:1083-1096.
4. Morse AS. Control using logic-based switching. Springer-Verlag New York, Secaucus, New Jersy, USA, 1997.
5. Hespanha JP. Stabilization of nonholonomic integrators via logic-based switching, Automatica. 1999;35:385-393.
6. Liberzon D. Switching in systems and control. Systems and control: Foundations and applications. MA, Birkh, Boston, 2003.
7. Santarelli KR, Dahleh MA. Comparison of a switching controller to two LTI controllers for a class of LTI plants. Proceedings of American Control Conference. Seattle, Washington. 2008:4640-4646.
8. Kogiso K, Hirata K. Controller switching strategies for constrained mechanical systems with application to the remote control over networks. Proceedings of IEEE International Conference on Control Applications. 2004:480-484.
9. Zheng K, Lee AH, Bentsman J, Krein PT. High performance robust linear controller synthesis for an induction motor using a multi-objective hybrid control strategy. Nonlinear Analysis. 2006;65:2061-2081.
10. Essounbouli N, Manamanni N, Hamzaoui A, Zaytoon J. Synthesis of switching controllers: A fuzzy supervisor approach. Nonlinear Analysis. 2006;65:1689–1704.
11. DeCarlo RA, Zak SH, Matthews GP. Variable structure control of non-linear multivariable systems: A tutorial. Proceedings of the Decision and control Conference. 1988:212–232.
12. Jamshidi F, Beheshti MTH, Fakharian A. Fuzzy supervisor approach on logic based switching H_2/H_∞. Proceedings of the Institution of Mechanical Engineers, Part I, Journal of Systems and Control Engineering. 2010;11-19.
13. Yoon TW, Kim JS, Morse AS. Supervisory control using a new control relevant switching. Automatica. 2007;43:1791-1798.
14. Boyd SP, Barratt CH. Linear Controller Design: Limits of Performance. Prentice-Hall, New Jersey, 1991.

15. Scherer C, Gahinet P, Chilali M. Multi objective output feedback control via LMI optimization. IEEE Transactions on Automatic Control. 1997; 42(7):896- 911.
16. Khargonekar PP, Rotea MA. Mixed H_2/H_∞ control: a convex optimization approach. IEEE Transactions on Automatic Control. 1991;39:824-837.
17. Vegte JV. Feedback Control Systems. Prentice Hall, New Jersey, 1994.
18. Hespanha JP, Morse AS. Switching between stabilizing controllers. Automatica. 2002;38:1905-1917.

10/10/2010

Investigation of Groundwater quality for Domestic and Irrigation purposes around Gubrunde and Environs, northeastern Nigeria

[1]Arabi, Suleiman Abdullahi; [1]Funtua, Idris Isa; [2]Alagbe, Solomon Ayodele; [2]Zabosrki, Peter; and [1]Dewu, Bala Bello Muhammad

[1]Centre for Energy Research and Training, Ahmadu Bello University, Zaria
[2]Department of Geology, Ahmadu Bello University, Zaria-Nigeria
Email: arabisuleiman@gmail.com

Abstract: Fourteen groundwater samples were collected from boreholes, springs and hand dug wells in and around Gubrunde in Borno State north-eastern Nigeria to investigate its quality for domestic and irrigation uses. The area investigated falls within longitude 11° 35' - 12° 05' and latitude 10° 10' - 10° 31'. The samples were analyzed using Atomic Absorption Spectrometer (AAS), multi-analyte photometer and Flame photometer while interpretation of the results was carried out with RockWare Aq•QA software, a spreadsheet for water analysis. Six of the samples investigated are of NaCl water type while fourteen were CaCl water types. Sodium Adsorption Ratio (SAR) values recorded ranges from 0.80 – 2.84, Exchangeable Sodium Ratio (ESR) 0.33 – 1.78, Magnesium hazard (MH) 5.19 – 47.9, Residual Sodium Carbonate (RSC) 0.00, Hardness 0.65 – 221.48 and Total Dissolved Solid (TDS) ranges from 130 – 407308mg/l. Twelve of the samples analyzed had medium Salinity Hazard (SH), and one each for high and low Salinity Hazard (SH), respectively. The variation in chemical composition of groundwater in the study area may be due to leaching of terrestrial salts, extensive use of chemical fertilizers and ion exchange between water and the host rock. The result of samples analyzed indicates that all the samples are undersaturated in calcite and aragonite, while most of the major anion and cations falls within World Health Organization and Nigeria Industrial Standard for Drinking water Values. Nine samples had NO_3 values ranging from 53 – 106mg/l exceeding the 50mg/l standards. NO_3 values exceeding 50mg/l has the tendency of causing asphyxia to infants less than three months old. A plot of SO_4, HCO_3 and Cl indicates that the groundwater samples are from intermediate water category (neither fresh nor old). Generally, the groundwater quality is fairly suitable for agricultural uses and suitable for domestic utilization.[Arabi, Suleiman Abdullahi; Funtua, Idris Isa; Alagbe, Solomon Ayodele; Zabosrki, Peter; and Dewu, Bala Bello Muhammad. Investigation of Groundwater quality for Domestic and Irrigation purposes around Gubrunde and Environs, northeastern Nigeria, Journal of American Science 2010; 6(12):664-672]. (ISSN: 1545-1003).

Keywords: Adverse effects; Sodium Adsorption Ratio; Exchangeable Sodium Ratio, asphyxia

1. Introduction

Urbanization, population increase, dewatering of aquifers for irrigation and extensive use of chemical fertilizers are some of the factors that have direct effects on quantity and quality of groundwater resources especially in arid and semi arid region of northern Nigeria. Hydrochemical data has the potential uses for tracing the origin and history of water. Globally, the quantity and quality of groundwater reserves is diminishing day by day. Therefore, any study that can aid in identifying new sources or threats to groundwater is desirous not only around the study area but anywhere. There is no life without water, therefore, it is essential to safeguard the future of our water resources by studying its past and present both quantitatively and qualitatively. This Study of hydrochemistry of groundwater from Gubrunde and environs utilizes the results of the chemical analysis of water samples from different sources around the study area. The locations sampled falls between longitude 11° 35' - 12° 05' and latitude 10° 10' - 10° 31' in Borno State, north-eastern Nigeria, covering an area of about 2250km^2 (Fig. 1). Gubrunde village is central in the Geology of Nigeria because it is one of the three Uranium mineralization areas in north-eastern Nigeria. It can be accessed through Dadin-kowa from Gombe State and/or through Biu or Guyuk from Adamawa State. he Geology of the area (Fig. 2) is made of the tertiary basalt of the Biu plateau on the north, sedimentary sequence of Bryel and Zange grabens on the north-west and south-east, respectively. The area is underlain by the crystalline basement of north-eastern Nigeria.

Groundwater in the crystalline basement rock is confined to pockets and patches of weathered rock and to fractures. Wells usually encounter water at shallow depths but yields are often low and subject to

seasonal fluctuations. Boreholes are usually sited on basement along drainage lines where overburden is often thickest. On the basalt capped plateau, the weathered zone near some of the larger streams is up to 9m thick and yields moderate amounts of water, usually 2 to 3 liter per second (Nur, et al.).

The composition of water changes through reactions with the environment and the natural chemistry can have an important bearing on anything living that utilizes this resources including human beings, livestock and even plants, so a detailed analysis of major, minor and trace constituents of groundwater is very important. Fourteen groundwater samples were collected from boreholes, springs and hand dug wells from the study area (Fig. 1) using standard sample collection procedures and analyzed for major, minor and trace constituents at the Center for Energy Research and Training, Ahmadu Bello University, Zaria and The Regional Groundwater Laboratory, Gombe using an Atomic Absorption Spectrophotometer, multi-analyte photometer and Flame Photometer and the data was interpreted with the help of RockWare Aq•QA software, a spreadsheet for water analysis to determine its suitability for use in culinary and agricultural purposes.

Six of the samples investigated are of NaCl water type while fourteen were CaCl water types. Sodium Adsorption Ratio (SAR) recorded values ranges from 0.80 – 2.84, Exchangeable Sodium Ratio (ESR) 0.33 – 1.78, Magnesium hazard (MH) 5.19 – 47.9, Residual Sodium Carbonate (RSC) 0.00, Hardness 0.65 – 221.48 and Total Dissolved Solid (TDS) ranges from 130 – 407308mg/l. Twelve of the samples analyzed had medium Salinity Hazard (SH), and one each for high and low Salinity Hazard (SH), respectively. For water with high salinity hazard, adverse effect is expected on crops, medium salinity hazard has detrimental effects only on crop that are sensitive to salinity while low salinity is suitable for all crops. The variation in chemical composition of groundwater in the study area may be due to leaching of terrestrial salts, extensive use of chemical fertilizers and ion exchange between water and the host rock.

The investigation will serve as an avenue to update groundwater data bank of the study area for those whose responsibility is the provision of safe drinking water to the entire populace around the area. It will also help in planning agricultural practices in advising the farmers on choosing the appropriate crops to be cultivated around the study area.

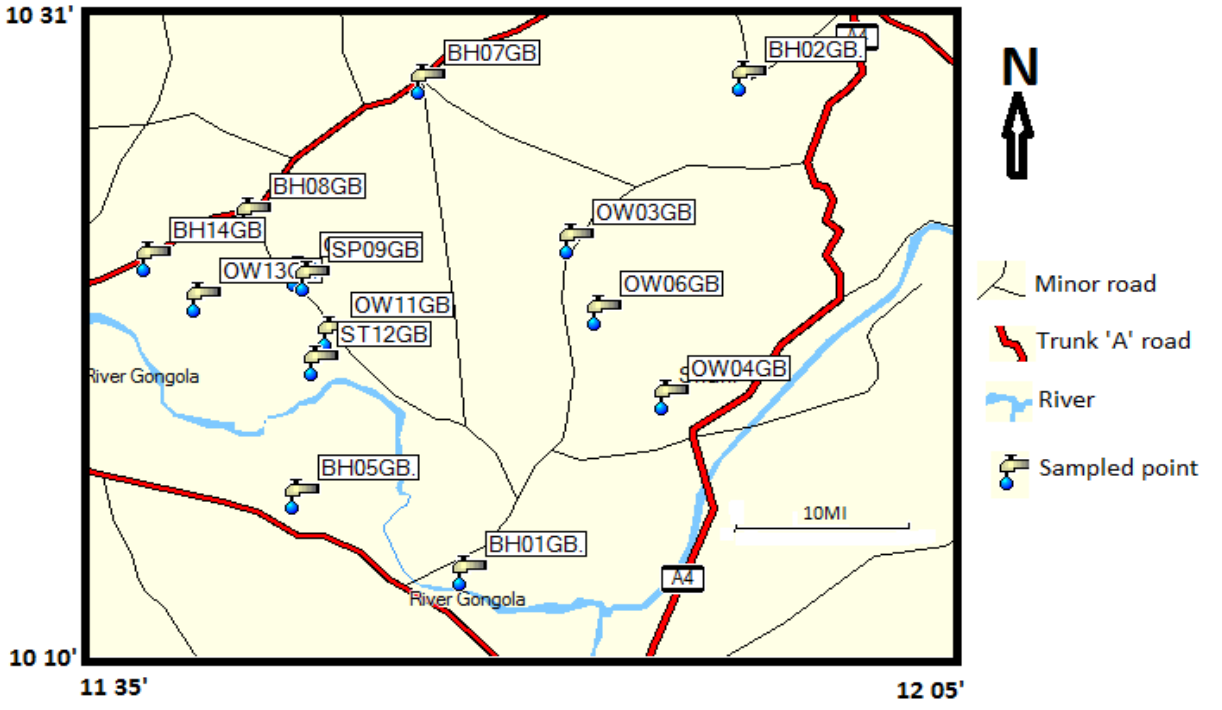


Figure 1: Location map of the study area showing sampled points (MapSource, 2006)

T

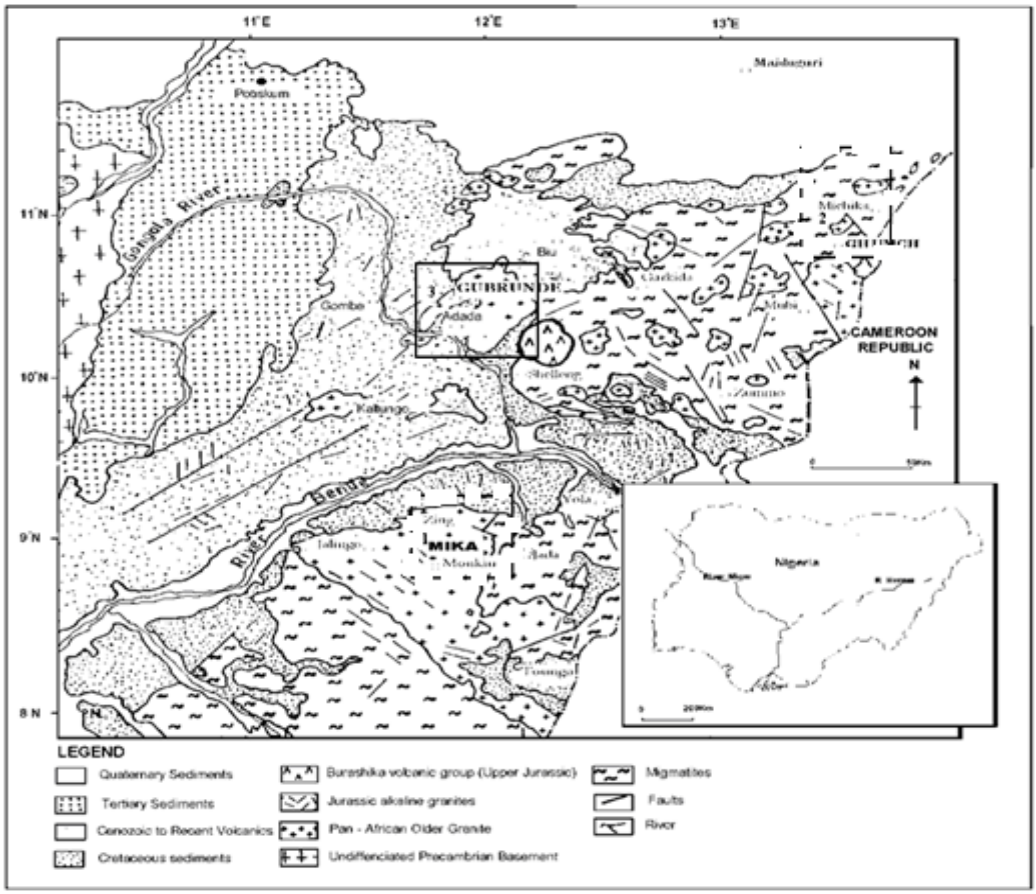


Fig.2: Geologic map of northeastern Nigeria showing the study area (Funtua, 1992)

2. Materials and Method

The water samples were collected in April, 2009 from boreholes, springs and hand dug wells with the aid of environmental sampler in order to have representative sample free from contamination from sampling tools. After each sample is collected, an in-situ measurement was made for conductivity, pH, TDS and temperature using Sension Platinum Series portable pH and Conductivity meter (HACH made). Also measured at the field are coordinates, elevation and static water level of each of the locations sampled (Table 1) using GPS and a deep meter. Samples were then stored in a plastic container after acidification with nitric acid before transporting them to the laboratory. The analysis of Si, K, O_2 and P were carried out using V2000 multi-analyte photometer, Na and K were carried out with a CORNING FLAME PHOTOMETER 410 after calibrating it with the analyte standard while the remaining analyte were carried out with BUCK SCIENTIFIC 210 VGP ATOMIC ABSORPTION SPECTROPHOTOMETER. The results obtained were then interpreted using RockWare (2006) Aq•QA spreadsheet for water analysis.

3. Results

The results of measurements obtained in-situ is presented in Table1, these include pH, conductivity, TDS, static water levels, coordinates of sampled locations, temperature and elevation of each point. Results of analysis of major and minor elements, determined water types, Sodium Adsorption Ratio (SAR), Exchangeable Sodium Ratio (ESR), Magnesium hazard (MH), Residual Sodium Carbonate (RSC), and Total Dissolved Solid (TDS) is presented in Table 2.

A graph of SO_4, HCO_3 and Cl distribution in groundwater samples is also presented in Figure 3
.

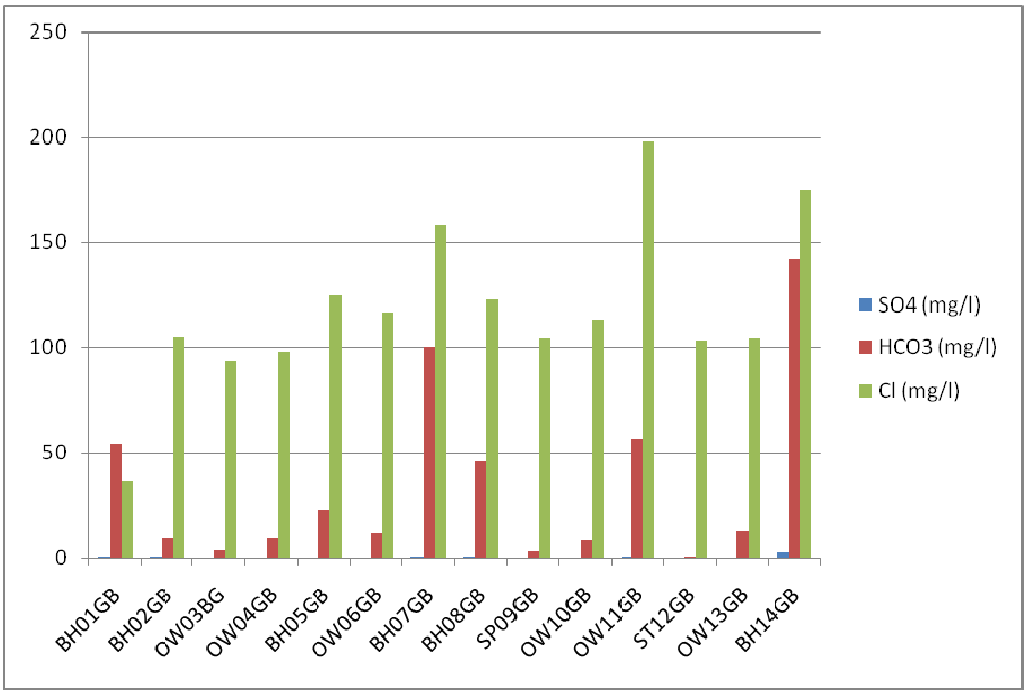


Figure 3: A graph of SO_4, HCO_3 and Cl in groundwater samples from Gubrunde and environs

Table 1: Parameters measured in-situ during field work

S/N	SAMPLE ID	COORDINATES LATITUDE	LONGITUDE	ELEV (m)	S.W.L (m)	H-HEAD (m)	PH	COND. (µs/cm)	TEMPT. (˚C)	TDS (mg/L)
1	BH01GB	09°44.176´	11°59.862´	169.47	5.00	164.47	6.55	384.00	31.20	185.30
2	BH02GB	10°36.750´	12°11.475´	757.73	10.79	746.94	6.92	1,271.00	28.20	628.00
3	OW03BG	10°21.645´	11°58.216´	312.12	2.38	309.74	5.68	71.80	28.50	33.80
4	OW04GB	10°13.370´	12°03.141´	259.69	3.54	256.15	6.59	197.10	30.00	94.30
5	BH05GB	09°33.746´	12°00.463´	188.37	6.25	182.12	7.24	779.00	32.40	380.00
6	OW06GB	10°17.853´	11°59.704´	368.81	5.33	363.47	7.45	281.00	31.20	135.00
7	BH07GB	10°30.167´	11°50.628´	350.83	11.89	338.94	6.81	1,155.00	31.30	569.00
8	BH08GB	10°23.045´	11°41.760´	306.02	25.91	280.11	6.85	699.00	31.00	340.00
9	SP09GB	10°19.688´	11°44.793´	294.13	0	294.13	6.87	142.10	28.50	67.70
10	OW10GB	10°19.934´	11°44.315´	295.96	2.50	293.46	6.39	684.00	29.10	333.00
11	OW11GB	10°16.679´	11°45.933´	254.81	11.03	243.78	6.59	1,206.00	29.50	595.00
12	ST12GB	10°15.199´	11°42.219´	261.21	0	261.21	6.15	101.70	29.40	48.20
13	OW13GB	10°18.571´	11°39.2025´	299.92	6.89	293.04	6.54	220.00	29.80	105.30
14	BH14GB	10°20.710´	11°36.656´	241.10	10.67	230.43	6.58	3,250.00	30.70	1,667.00

ELEV = elevation, S.W.L =static water level, H-HEAD = hydraulic head, COND = conductivity, TEMP = temperature and TDS = total dissolved solid

Table 2: List of major, minor elements and some fluid property parameters as determined in water samples from Gubrunde and environs

S/N	SAMPLE ID	Na (mg/l)	K (mg/l)	O_2 (mg/l)	Fe (mg/l)	NO_3 (mg/l)	Mg (mg/l)	P (mg/l)	Si (mg/l)	Ca (mg/l)	SO_4 (mg/l)	HCO_3 (mg/l)	Cl (mg/l)	Water type	SAR	ESR	Salinity
1	BH01GB	16.3±0.02	10.6±0.51	6.24±0.80	0.14±0.02	27.2±2.01	3.74±0.21	0.02±0.001	28±0.20	6.70±0.05	0.54±0.01	54±0.21	37±0.21	Na-Cl	1.25	1.10	low
2	BH02GB	36.4±0.12	16.8±0.12	6.0±0.01	0.23±0.21	53±1.3	5.26±0.62	0.05±0.004	29.4±0.13	10.70±0.03	0.50±0.01	9.8±0.05	104.7±0.71	Na-Cl	2.27	1.64	Medium
3	OW03BG	29.7±0.022	12.5±0.33	7.73±0.62	0.74±0.12	25±0.2	4.57±0.51	0.05±0.001	33.6±0.25	10.7±0.27	0.01±0.001	3.60±0.01	93.4±0.25	Na-Cl	1.91	1.42	Medium
4	OW04GB	17.7±0.14	3.4±0.23	6.77±0.62	0.81±0.15	34±3.5	3.05±0.17	0.15±0.011	31.7±0.65	32±0.32	0.12±0.001	9.8±0.21	98. ±0.51	Ca-Cl	0.80	0.42	Medium
5	BH05GB	52±0.11	16.2±0.71	6.03±0.32	0.13±0.12	65±0.95	3.79±0.21	0.02±0.01	28.9±0.32	19.16±0.04	0.26±0.01	22.96±0.31	125±0.32	Na-Cl	2.84	1.78	Medium
6	OW06GB	36.4±0.25	2.9±0.52	6.55±0.53	0.59±0.23	76±1.9	3.49±0.51	0.04±0.001	29.4±0.52	29.8±0.06	0.14±0.001	11.84±0.18	117±0.66	Na-Cl	1.68	0.90	Medium
7	BH07GB	32.9±0.21	6.4±0.25	6.14±0.53.	0.04±0.25	47±2.1	4.06±0.90	0.01±0.002	33.1±0.51	79.6±0.21	0.8±0.002	99.68±0.54	158±0.85	Ca-Cl	0.97	0.33	Medium
8	BH08GB	36.4±0.32	2.0±0.11	5.84±0.32	0.41±0.32	21.2±0.52	3.84±0.55	0.01±0.003	20.9±0.54	41.71±0.62	0.31±0.01	45.55±0.31	123±0.32	Ca-Cl	1.44	0.66	Medium
9	SP09GB	28.3±0.57	1.3±0.23	6.03±0.52	3.01±0.25	63.8±0.25	2.64±0.01	0.04±0.002	40.6±0.61	27±0.51	0.01±0.001	2.86±0.62	104.3±0.61	Ca-Cl	1.39	0.79	Medium
10	OW10GB	33.5±0.52	26.1±0.08	6.47±0.51	0.06±0.51	72.01±2.1	3.76±0.54	0.08±0.002	27.9±0.51	14.7±0.08	0.09±0.01	8.72±0.21	113.2±0.34	Na-Cl	2.02	1.40	Medium
11	OW11GB	35.2±0.21	23.6±0.21	6.44±0.11	0.13±0.31	56.3±1.3	4.1±0.23	0.12±0.005	24.7±0.41	75±0.21	0.82±0.001	56.28±0.51	197.6±0.61	Ca-Cl	1.07	0.38	Medium
12	ST12GB	16.7±0.21	1.1±0.62	6.19±0.61	2.28±0.63	76.3±1.2	2.04±0.001	0.05±0.001	25.7±0.25	36.4±0.61	0.01±0.001	0.40±0.01	103.2±0.21	Ca-Cl	0.73	0.37	Medium
13	OW13GB	21.9±0.32	13.2±0.28	6.05±0.22	4.1±0.11	62±.23	2.5±0.11	0.17±0.01	34.5±0.31	27.6±0.05	0.21±0.01	13.16±0.31	104.5±0.61	Ca-Cl	1.07	0.60	Medium
14	BH14GB	93.8±0.02	46.4±0.01	5.9±0.32	0.18±0.01	106.5±1.2	2.8±0.21	0.21±0.001	48.9±0.61	84.3±0.11	2.75±0.03	142.6±0.71	174.4±0.61	Ca-Cl	2.14	0.72	High

SAR = sodium Adsorption Ratio,
ESR = Exchangeable Sodium Ratio

4. Discussion

The results of chemical analysis of groundwater in the study area are discussed in the following order:

a) Water types and major and minor constituents
b) Sodium Adsorption Ratio (SAR)
c) Mineral Saturation (MS)
d) Hardness
e) Residual Sodium Carbonate (RSC)
f) Total Dissolved Solid (TDS)
g) Hydrochemical data
   - Piper diagram
   - Schoeller diagram.

a. Water type

The analysis of water samples allowed the categorization of water from the study area in to two water types with formulae Ca-Cl and Na-Cl indicating Calcium and Sodium water types.

Calcium is the most abundant of alkaline earth metal and a major constituent of vast common rock minerals. Sources of calcium (Ca^{2+}) in water include calcite, aragonite, dolomite, gypsum, anhydrite, fluorite, plagioclase, pyroxene and amphibole (Brian, et al. 1980). From health point of view, the content of calcium in groundwater is unimportant. Its concentration in natural waters is typically <15mg/l. concentration of calcium in water samples analyzed ranges from 6.7 – 84.33mg/l.

Sources of sodium are halite, sea spray, some silicate and rare minerals such as plagioclase, plagioclase variety of albite and nepheline. Most sodium results from natural ion exchange. Sodium and potassium are common constituents of natural waters with sodium being more prevalent than potassium. From health point of view, potassium is unimportant but sodium can have negative effects on people with heart disease. Sodium hydrogen carbonate mineral waters are important for treatment of gastric and biliary tract diseases. WHO(2008) and the NIS(2007) Nigeria Standard for Drinking water Quality has set limit for sodium in drinking water at 200mg/l. The highest value recorded for sodium in the samples analyzed is 93.8mg/l. Except for nitrate which exceeded the limited of 50mg/l in nine of the samples analyzed all the other parameters measured (Table 2) complied with the set standard. Nitrate levels exceeded 50mg/l in drinking water having the potential of causing cyanosis, and asphyxia (blue baby syndrome) in infants less than 3 months.

Based on the HCO_3, SO_4 and Cl recorded in the samples and plotted on bar graph (Fig. 3), the water samples analyzed belong to the intermediate water category (not fresh nor old) because $Cl > HCO_3 > SO_4$.

b) Sodium Adsorption Ratio (SAR)

Sodium Adsorption Ratio (SAR) is an estimate of the degree to which sodium will be adsorbed by the soil. It is used to evaluate the suitability of water for irrigation. High value of SAR means that sodium in the water may replace calcium and magnesium ions in the soil, potentially causing damage to the soil structure (Lloyd, 1985). SAR is calculated from the formula;

$$SAR = \frac{[Na^+]}{\sqrt{\frac{[Ca^{2+}] + [Mg^{2+}]}{2}}}$$

Most of the analyzed groundwater samples are medium sodium waters meaning that the water is most suitable when used on coarse-textured or organic soil with good permeability and plants with good salt tolerance. The sodium hazard is a function of both SAR and Salinity. Salinity hazard dividing points are 250, 750 and 2250µohms, resulting in four categories as given in Table 3 with corresponding µohms values.

.

Table 3: Sodium and Salinity control values (Wilcox, 1955)

	Salinity status	**Sodium status**
<250 µohms	Low salinity water	Low sodium water
250 -750 µohms	Medium salinity water	Medium sodium water
750 -2250 µohms	High salinity water	High sodium water
>2250 µohms	Very high salinity water	Very high sodium water

c) Mineral Saturation (MS)

The minerals calcite and aragonite have the same chemical composition ($CaCO_3$), but different chemical structures. The saturation index of these minerals is given as;

SI = log Q/K = log Q – log K,

where Q is the ion activity product and K the equilibrium constant and this tells whether they are;

1. supersaturated (Saturation Index > 1)
2. saturated (Saturation Index = 0) or
3. under-saturated (Saturation Index < 0)

All the samples analyzed are under-saturated in both calcite and aragonite with saturation values ranging from -4.34 to -0.71 for calcite and -4.89 to 0.88 for aragonite.

d) Hardness

Hardness is the sum of Ca^{2+} and Mg^{2+} concentrations expressed in terms of mg/l of calcium carbonate:

Hardness = 2.5 Ca (mg/l) + 4.1 Mg(mg/l) (Fournier,1981)

Calcium and magnesium form an insoluble residue with soap. The degree of hardness in water is commonly based on the classification listed in Table 5(Sawyer and McCarty, 1967).

Table 5: Classification of water hardness (Sawyer and Mc Carty, 1967)

Hardness range (mg/l of $CaCO_3$)	**Water classification**
0 – 75	Soft
75 – 150	Moderately hard
150 – 300	Hard
>300	Very hard

Most of the groundwater samples analyzed had hardness value ranging from 0.65 – 74.5mg/l except sample BH07GB and BH14GB which have hardness value of 163.07 and 221.48, respectively. Hardness value below 75mg/l indicate that the samples analyzed are soft water indicating that twelve of the fourteen water samples analyzed are soft water while the remaining two are hard water. These samples also

recorded the highest conductivity values of 664 and 962µs/cm, respectively. So, most of the samples analyzed passed the hardness limit set by WHO, 2008 and NIS, 2007 Nigeria Standard for Drinking Water Quality except samples BH07GB and BH14GB.

e) Residual Sodium Carbonate (RSC)

Residual Sodium Carbonate (RSC) value considers the bicarbonate content of the water. High concentration of bicarbonate leads to an increases in pH value of water that causes dissolution of organic matter. An increase in RSC value leads also to precipitate calcium and magnesium that can cause an increase in sodium content in the soil. The high concentration of bicarbonate ion in irrigation water leads to its toxicity and affects the mineral nutrition of plants.

According to Eaton's classification, water with RSC greater than +2.5epm is considered unsuitable for irrigation. The water with RSC of +1.25 to +2.5 is considered as marginal and those with a value less than +1.25 are safe for irrigation purpose. All the water samples analyzed had RSC values of less than 1.25 suggesting that the water can be used for irrigation purpose.

f) Total Dissolved Solid (TDS)

Increase in dissolved solids in irrigation water affects soil efficiency and growth and yield of plants. For long term irrigation under average conditions, the total dissolved solids should not exceed 2000mg/l. High increase in water salinity increases salts amount in soil and leads to salinization problem. Classification of water according to TDS values (Wilcox, 1955) is given in Table 4.

Table 4: Classification of irrigation water based on TDS value (Wilcox, 1955)

TDS (mg/l)	Status
200–500	Best quality water
1000–2000	Water involving Hazard
3000-7000	Used for irrigation only with leaching and perfect drainage

The highest TDS value recorded in the examined groundwater samples is 670mg/l and the lowest is 130mg/l which indicates that base on TDS categorization, the water in the area studied is good for irrigation purpose.

g) Graphical presentation of data

Piper diagram is a combination of anions and cations triangle that lies on a common baseline. It divides waters into basic types according to their placement near the four corners of the diamond. Water that plots at the top of the diamond is high in Ca^{2+} + Mg^{2+} and HCO_3^- and is the region of waters with temporary hardness. Waters plotted at the lower corner of the diamond is composed primarily of Na^+ + K^+ and HCO_3^- + CO_3^{2-}. The plot according to this arrangement is presented in Figure 4 where three classes of combinations were obtained. The Schoeller diagram represents the combination of major and minor constituents of groundwater in the study area in a diagram (Fig. 5) and the result obtained indicates that Cl is dominant and SO_4 as the least in the following order: Cl, Na + K, Ca, Mg, HCO_3,+ Co_2 then SO_4.

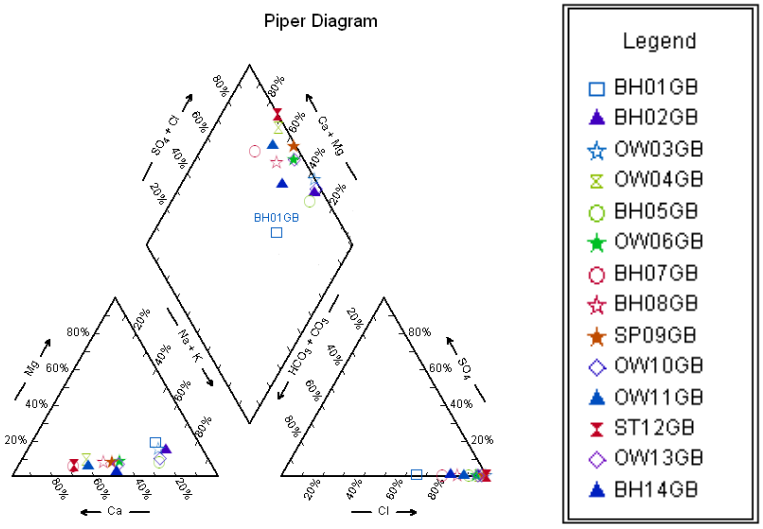


Figure 4: Piper diagram for water samples from Gubrunde and environs

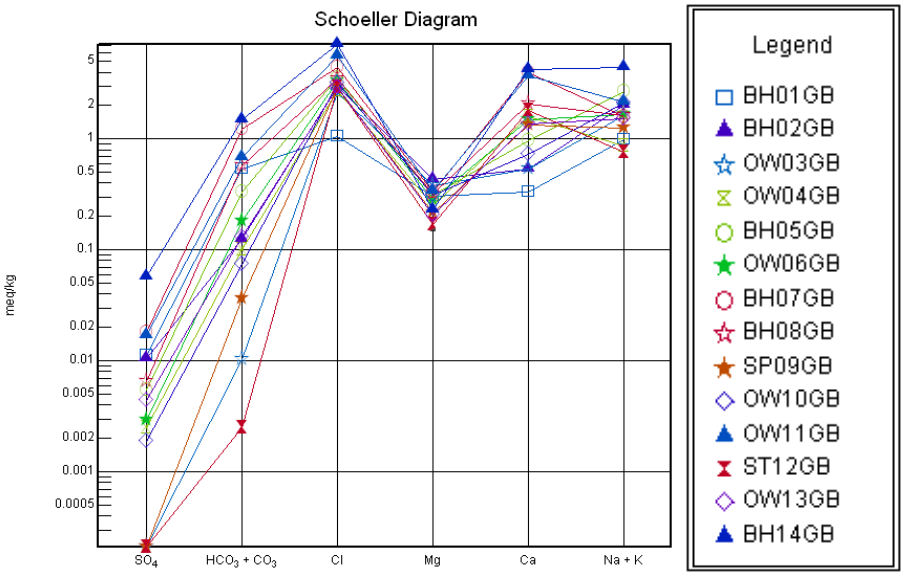


Figure 5: Schoeller diagram for water samples from Gubrunde and environs

5. Conclusions

The results of analysis and interpretation of groundwater from Gubrunde and environs for domestic and irrigation purpose indicates that samples BH14GB is not suitable for irrigation while sample BH01GB being most suitable, the remaining twelve samples is most suitable on coarse-textured or organic soil with good permeability and plants with good salt tolerance. For domestic uses and based on WHO, 2008 and NIS, 2007 Nigeria Standards, the water is good for drinking and other culinary purposes except the high concentration recorded for nitrate which can cause asphyxia in infants less than three months. Based on the findings, the groundwater in the area studied is fairly suitable for agricultural purpose and suitable for use in homes.

Acknowledgement:

The authors wish to acknowledge the immense contributions towards this work from the Center for Energy Research and Training (CERT), Ahmadu Bello University (ABU), Zaria, The Petroleum Technology Development Fund (PTDF), The International Atomic Energy Agency (IAEA) for providing the required logistics, equipment and facilities used in the field and laboratory. We also wish to acknowledge the contributions of Attahiru Alhassan Jaoji and the driver Malam Iliya Musa both of the Centre for Energy Research and Training, Ahmadu Bello University, Zaria for their assistance during sample collection.

Corresponding author:
Arabi Suleiman Abdullahi
Centre for Energy Research and Training,
Ahmadu Bello University, Zaria
P.M.B. 1014 ABU Zaria
Zaria – Nigeria
www.cert-abu.net
email: arabisuleiman@gmail.com
Tel: +2348062773153

References

1. Brian, J. S and Stephen C. P. Physical Geology. Fourthedition, Wiley, 2006.
2. Fournier, R.O. Application of water Geochemistry to Geothermal exploration and reservoir engineering *in L. Rybach and L.J.P. Muffler, eds., Geothermal Systems, Principles and Case Histories, Wiley, New York*, 1981. p. 109-143.
3. Funtua, I.I. Geology and Geochemistry of Uranium Mineralization in Mika, north-eastern Nigeria, *PhD thesis Department of Geology, Ahmadu Bello University, Zaria Nigeria.* , 1992
4. Lloyd, J.W. and Heathcote, J.A. Natural Inorganic Hydrochemistry in Relation to Groundwater. Oxford Press Oxford, 1985.296 pp.
5. MapSource. Trip waypoint manager V4.4.00, Garmin limited. Version 6.11.6, 2006.

6. Nigeria industrial Standard,NIS:554. Nigeria Drinking Water Quality Standard: 2007. ICS 13.060.20. *Approved by the Standard Organization of Nigeria,* ,2007. *Pp30*
7. Nur, A and Kujir, A. S. Hydro-Geoelectrical study in the northeastern part of Adamawa State, Nigeria. *Journal of environmental Hydrology. Vol 14(19).* 2006.
8. RockWare. Spreadsheet software for water analysis. *Prairie city computing, inc. Aq•QA Application* 1.1.1 [1.1.5.1] (Unicode Release) 07/22/2006
9. Sawyer, C. N., and Mc Carty, P. L. Chemistry for Sanitary Engineers, and classification of naturally soft and naturally hard waters to sources and hardness of their water supplies., J. Hyg. 1967
10. Wilcox, L. V. Classification and Use of irrigation Waters, *U.S.A. Salinity lab. Circulation. No. 969,* 1955.
11. World Health Organization. Guidelines for Drinking Water Quality. *Third edition incorporating the first and second addenda, Vol.1.Recommendation,NCW classifications WA675,* 2008.

10/14/2010

Effect of the Type of Aggregate on the Properties of Alumina Refractory Concrete

S.A. Ghonaim[1], H.B.G. Ghazal[2], and M.F. Abadir[*3]

[1] Egyptian Organization for Standardization and Quality, [2]The High Institute of Engineering, Shorouk
[3]Chemical Engineering Department, Faculty of Engineering, Cairo University, Cairo, Egypt.
[*]magdi.abadir@yahoo.com

Abstract: Low cement refractory concrete samples were prepared by mixing cement (containing 50% alumina) in percentages ranging from 10 to 20% with some aggregates and the necessary amount of water. Two types of refractory aggregate were used: Bauxite containing 81% alumina and grog containing 52% alumina. Four particle sizes of each aggregate were used. The cast samples were left in their moulds for 24 hours in a 100% relative humidity cabinet. The de–molded specimens were left in an open air until their moisture content reaches 3–6%, then kept in a drying oven at (110 ± 5) ºC until reaching constant weight. They were then tested for phase constitution, water absorption, bulk density, apparent porosity and cold crushing strength (after 28 days curing). It was found that bauxite based samples gave better results than those prepared with grog. It was also found using statistical analysis that the percent cement used affects all properties much more than does the particle size of aggregate.
[S.A. Ghonaim, H.B.G. Ghazal, and M.F. Abadir. Effect of the Type of Aggregate on the Properties of Alumina Refractory Concrete. Journal of American Science 2010;6(12):673-684]. (ISSN: 1545-1003).

Key Words: Refractory concrete – Alumina – Grog – Sodium citrate – Bauxite

1. Introduction:

The analysis of the composition, the structure, and the properties of conventional refractory concretes shows that their refractory properties are governed by the filler contained in them. The binder component (bonding agent) of the concretes imparts the strength required during transportation and erection; this strength is attained after setting and drying. During subsequent heating up to the temperatures preceding sintering, irreversible destructive processes occur, as a rule, in the binder. In view of the fact that the binder (along with the finely milled additives or aggregates) forms a continuous matrix phase in the structure of the concrete, the thermo–mechanical characteristics of the material are adversely affected. Therefore, in order to improve the existing refractory concretes and to create new concretes, it is necessary to decrease the content of the conventional binders (e.g., high–alumina cements) in them to the minimum possible extent or to produce them without introducing any conventional binders [1, 2, 3, 4].

Thermally stable aggregates combined with a bonding agent are the principal ingredients of a monolithic refractory. These raw materials are available both naturally and artificially. Raw materials available in nature unavoidably vary slightly in their compositions. However it is important to take advantage of the characteristics of these natural minerals that cannot be developed artificially rather than to avoid their use due to variations of chemical composition.

Unlike natural raw materials, artificial raw materials allow adjustment of chemical composition as well as their mineral constituents, and it is possible to get a uniform quality.

One common type of aggregate is bauxite, a raw material for alumina containing about 60% alumina. When calcined, the alumina level is usually raised to above 85%. Bauxite for refractories is calcined in a rotary kiln to make a stable product. Calcined bauxite contains corundum as its principal component, mullite and a small glassy phase.

On the other hand, grog is an artificial aggregate usually obtained from crushed defective refractory bricks. Its alumina content depends on that of the original bricks. It usually ranges from 40 to 80%.

Other types of aggregate include diaspore ($A1_2O_3.\ H_2O$), corundum ($A1_2O_3$), magnesia (MgO), zirconia (ZrO_2), etc…

In the present paper, the physico–mechanical properties of refractory concrete samples prepared from bauxite and grog with varying amounts of cement and varying particle size of aggregate have been studied.

2. Experimental

2.1 Raw Materials:

The raw materials used are:

1- Refractory cement having 50% alumina was obtained from Lafarge Cement Company.

2- Calcined bauxite was obtained from the Alexandria Company for Refractories with an alumina content exceeding 80%.
3- Grog was obtained from previously fired defective bricks that were crushed, ground and screened.

2.2 X–Ray Fluorescence Analysis (XRF):

X–ray fluorescence spectrometry (XRF) was employed for the elemental analysis of the starting materials. The analysis was run on a AXIOS, panalytical 2005, Wave length Dispersive (WD–XRF) Sequential Spectrometer.

2.3 X–Ray Diffraction (XRD):

X–Ray diffraction was used for the qualitative analysis of the phases present in the tested samples.

For X–Ray diffraction study of bauxite and grog analysis, the aliquots for bulk mineral analysis were finely ground (–200 mesh), and analyzed by a BRUKUR D8 ADVANCE COMPUTERIZED X–Ray Diffractometer apparatus with mono – chromatized Cu Kα radiation, operated at 40 kV and 40 mA.

2.4 Particle Size Distribution of Aggregate:

In order to determine the grain size distribution, the procedure described by ASTM D 422/2007[5] was used. The standard sieves method was applied using screen apertures ranging from 6.68 mm (3 mesh) down to 74 μm (200 mesh).

2.5 Preparation of Specimens:

Forty dry mixtures of different formulations for both bauxite and grog at various percentages cement (20%, 17.5%, 15%, 12.5%, 10%) by weight were kneaded with an adequate amount of water, which was determined for each batch according to the standard "good ball in hand test [6]". The mixed batches were then cast into cubes of 50 mm side length using a vibrating table at a frequency of 50 Hz for 4 minutes. The cast samples were left in their moulds for 24 hours in a 100% relative humidity cabinet. The hydrated samples were then demolded. The specimens were left in an open air until their moisture content reaches 3–6%, then put in the drying oven at (110 ± 5) ºC until reached a constant weight. They were then tested for water absorption, bulk density, apparent porosity and cold crushing strength.

2.6 Apparent Porosity, Water Absorption, and Bulk Density:

These properties were determined according to the ASTM Standards C 20/2007 [7]. For each test, the average measurements of five specimens at least were considered.

The five specimens for each test were weighed for their dry weight (D). The specimens were then immersed in water and boiled for 2 h without contact with the heated bottom of the container. They were cooled down to the room temperature while still completely immersed in water. The weight (S) of each specimen was determined after boiling and while suspended in water. The saturated weight (W) was also determined.

Apparent porosity (P) was calculated from the following formula:

$$P,\% = \frac{W - D}{V} \times 100 \quad (1)$$

Water absorption (A) was calculated from the following formula:

$$A,\% = \frac{W - D}{D} \times 100 \quad (2)$$

While bulk density (ρ_B) was calculated from the following formula**:**

$$\rho_B = \frac{D}{V} \times 100 \quad (3)$$

Where:
P = apparent porosity, (%);
W = weight of the specimen as saturated with water, (g);
D = dry weight, (g);
S = weight of the specimen as suspended in water, (g);
V = volume of specimen = W –S, (cm^3);
A = water absorption, (%);
ρ_B = bulk density, (g/cm^3).

2.7 Cold Crushing Strength:

This was carried out on three specimens representing each mix composition after curing for 28 days. Each specimen was placed between two plates of the compression strength tester. This was followed by the application of an axial uniform load. The load at which a crack appears on the sample was noted. The strength was calculated according to BS EN Standards 993–5/2000 [8]:

$$C.C.S\,(\sigma_c) = \frac{W}{a} \quad (4)$$

Where:

σ_c = cold crushing strength, (MPa);

W = total maximum load at visible failure, (N);

a = average of gross area of the two faces, (mm^2).

3. Results and Discussion:

the elemental chemical analysis of the employed refractory cement, bauxite and grog samples.

Table (1): Chemical Analysis of Materials Used

Constituents (wt. %)	Cement	Bauxite Sample	Grog Sample
SiO_2	5.5	9.264	26.640
TiO_2	——	1.451	3.740
Al_2O_3	52.95	81.291	51.929
$Fe_2O_3^{tot.}$	2.5	1.816	2.994
MgO	Traces	0.372	0.510
CaO	38.05	0.435	1.215
Na_2O	< 0.1%	0.066	1.418
K_2O	< 0.1%	0.174	4.901
P_2O_5	——	0.542	1.056
SO_3	——	0.020	0.875
Cr_2O_3	——	0.120	0.069
Co_3O_4	——	0.084	0.039
Ga_2O_3	——	0.023	0.014
SrO	Traces	0.16	0.106
Y_2O_3	——	0.021	0.020
ZrO_2	——	0.272	0.190
Nb_2O_5, La_2O_3 ,CeO_2, Nd_2O_3, ThO_2	——	< 0.1%	< 0.1%
WO_3	——	0.237	——
PbO	Traces	1.6	0.011
Cl	——	0.022	4.183
L.O.I	——	1.811	——
Total	**≈100**	**≈100**	**≈100**

3.1 Chemical Analysis of Raw Materials:

Table (1) shows the XRF results related to

3.2 XRD of Raw Materials:

Figures (1) and (2) show the XRD patterns of bauxite and grog, respectively. Calcined bauxite consisted exclusively of corundum (Al_2O_3) and mullite ($3Al_2O_3.2SiO_2$). This is expected from the phase equilibrium diagram: Al_2O_3 – SiO_2 for compositions containing > 80% alumina [9]. On the other hand, the XRD pattern of grog (Fig. 3) shows beside the expected phases of mullite and quartz, some non–equilibrium phases of corundum and cristobalite. Halite is also present as an impurity.

Grog and Bauxite were screened to the required size fractions using standard sieves ranging from 3 mesh (6.680 mm) down to 200 mesh (0.074 mm). The mean particle size of a fraction passing through a certain sieve and retained over the next was

taken as the arithmetic average of the two openings. This way, the following mean sizes were used: 4.699 mm, 2.794 mm, 1.651 mm, 1.168mm, 0.991mm, 0.295 mm, 0.175 mm, 0.147 mm, and 0.074 mm.

Figure (3) shows the cumulative screen analyses for grog and bauxite used in the present investigation.

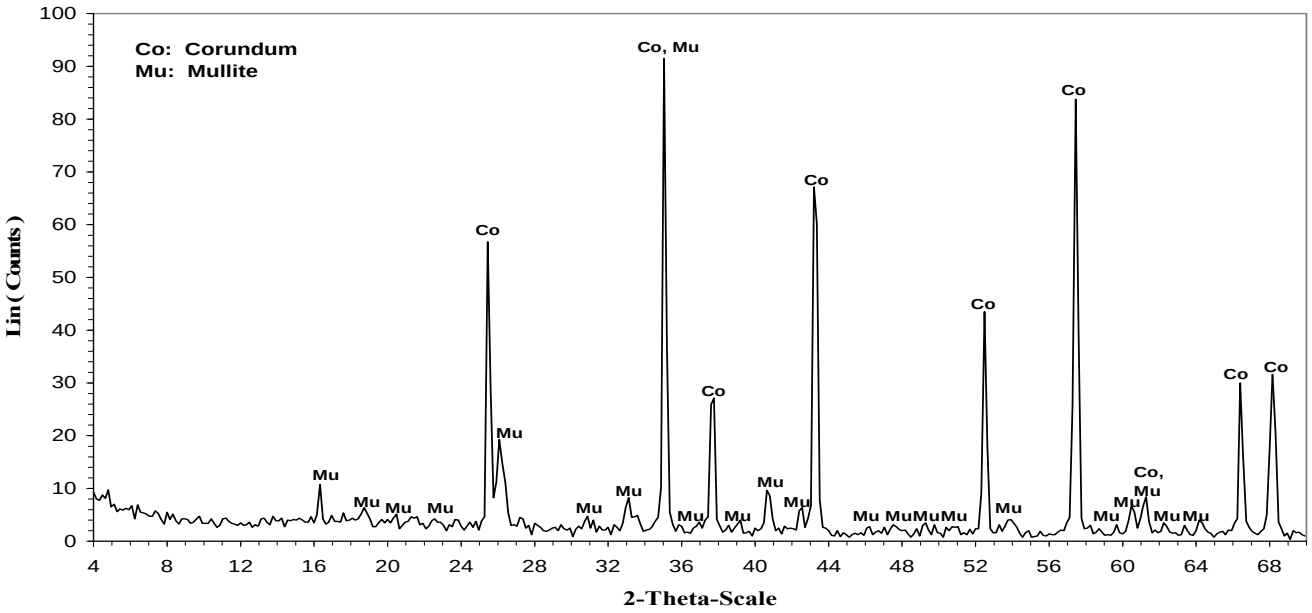


Fig. (1): XRD Pattern of Calcined Bauxite

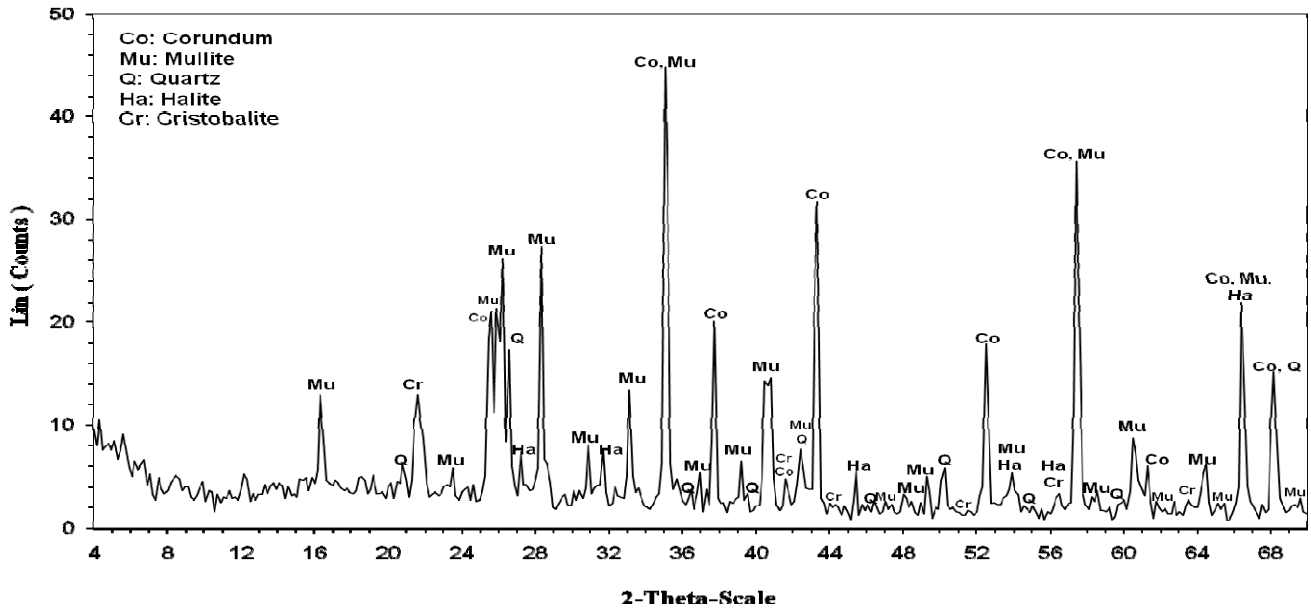


Fig. (2): XRD Pattern of Grog

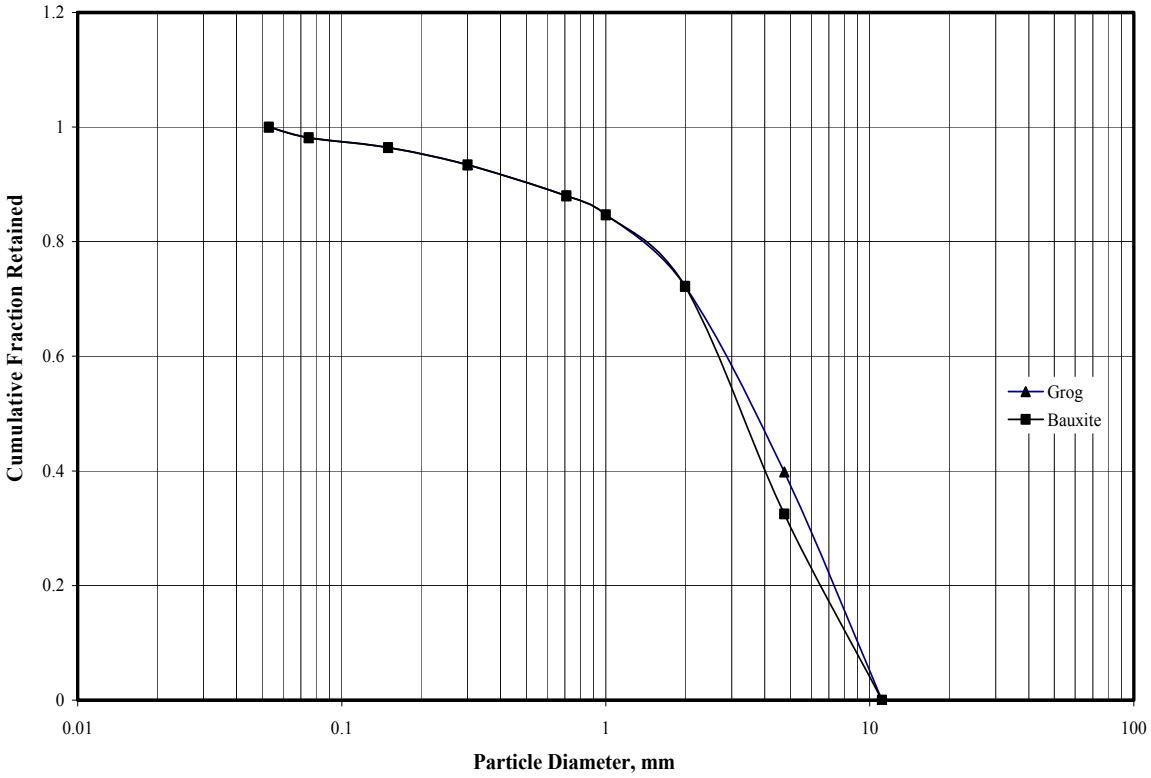


Fig. (3): Particle Size Distribution of Aggregates

3.4 Mean Particle Size of Aggregates:

In order to assess the effect of particle size of the aggregates employed (bauxite and grog,) on the workability, physical and mechanical properties of refractory concrete paste, four different particle size mixes were used. Each is a combination of three different particle size ranges in order to maximize compactness of samples. Table (2) shows the four mixes together with the average particle diameter of each as calculated using the method suggested by McCabe et al [10].

Table (2): Mean Particle Size of Aggregate Formulation Used

	% Weight			
	0–1 mm	**1–3 mm**	**3–5 mm**	**Mean Particle Size (mm)**
A	10	75	15	1.63
B	25	60	15	1.19
C	40	45	15	0.94
D	55	30	15	0.78

3.5 Effect of Mean Particle Size on Water Consumption:

During mixing, the ratio of water added certainly affects technological properties of the final product considerably [2]. Also, when the amount of coarse particles in the particle size distributions increases, the surface area of the particles decreases and less water is required. The amount of consumed water as function of the mean particle size of bauxite and grog are shown in figures (4) and (5), respectively.

The results show that the amount of water added increases with the decrease of both particle size in the aggregate mixture and with the amount of cement. It is also seen that water consumption for samples containing grog is higher than for those containing bauxite. These values range from 7.5 to 9.1% in case of bauxite based formulations against 9.5 to 10.9% for grog–based formulations. This is presumably due to the presence of much more open pores in grog than in bauxite particles. To assess this, the apparent porosity of samples of both types of particles was determined. And found to be 6.7% and 16.5% for bauxite and grog particles, respectively.

Using the excel DATA ANALYSIS module, it was possible to establish correlation tables in both cases to show the relative influence of both cement percent and particle size on the percent of required water. The results are given in tables (3) and (4).

The results given in tables (3) and (4) suggest the following:

First, the relation between the percent water added and percent cement used is an increasing relation. On the other hand the negative sign

associated with the effect of particle size means an inverse relation between the percent water added and particle size.

Second, in the case of using bauxite, the effect of varying particle size on the percent water added is higher than that of varying the cement ratio. On the other hand, on using grog, the two variables have comparable effects.

Table (3): Correlation Table for Water Added in Bauxite Based Mixes

	% Cement	Particle Size	% Water
% Cement	1		
Particle Size	0	1	
% Water	0.642372	− 0.7489	1

Table (4): Correlation Table for Water Added in Grog Based Mixes

	% Cement	Particle Size	% Water
% Cement	1		
Particle Size	0	1	
% Water	0.696767	− 0.68928	1

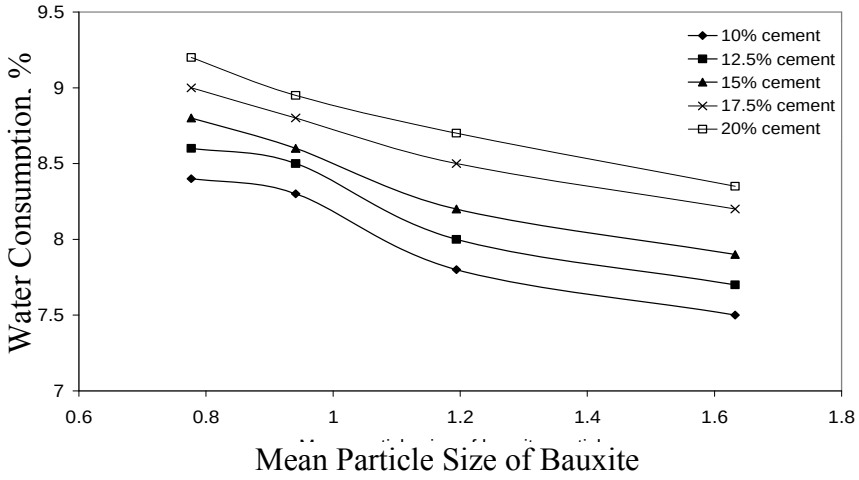


Fig. (4): Effect of Mean Particle Size of Bauxite Aggregates Containing Different Cement Percents on Water Consumption for Mixes

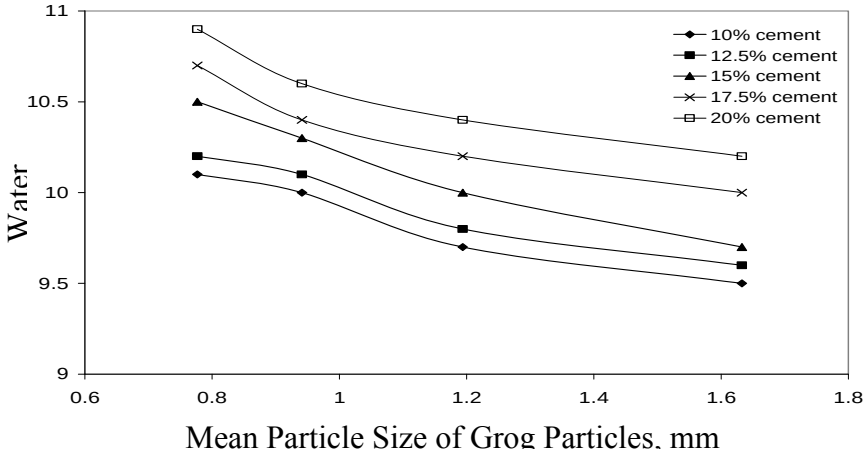


Fig. (5): Effect of Mean Particle Size of Grog Aggregates Containing Different Cement Percents on Water Consumption for Mixes

3.6 Effect of Mean Particle Size on Water Absorption:

Figures (6) and (7) show the relations between the percent water absorption of cast cubes and the mean particle size of either bauxite or grog, respectively.

The results suggest that the percent water absorption appreciably decreases with the increase of cement content. However, as the cement content exceeds 15%, its effect on water absorption diminished. This is expected since higher cement content enhances the closure of available pores.

Of interest is the difference between the water absorption values observed in either cases. It is clear from Fig. (6) that the values of water absorption in case of bauxite based formulations range from about 4.8% to 9.5% depending on the cement level and particle size. In case of using grog, the range of water absorption is 5.3 – 10.5% showing that the use of grog has lead to higher water absorption presumably due to increased open porosity of such samples.

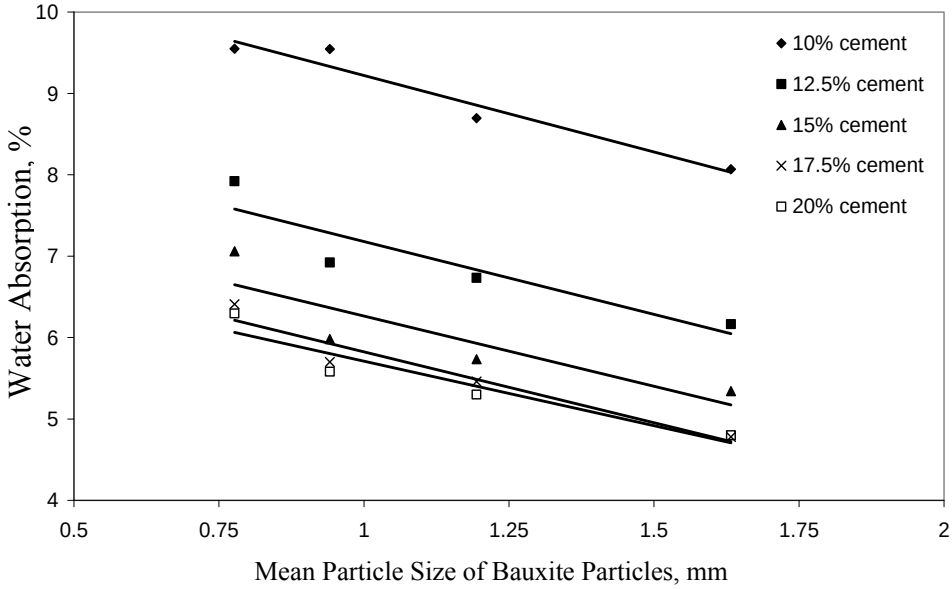


Fig. (6): Effect of Mean Particle Size of Bauxite Aggregates Containing Different Cement Percents on Percent Water Absorption for Mixes

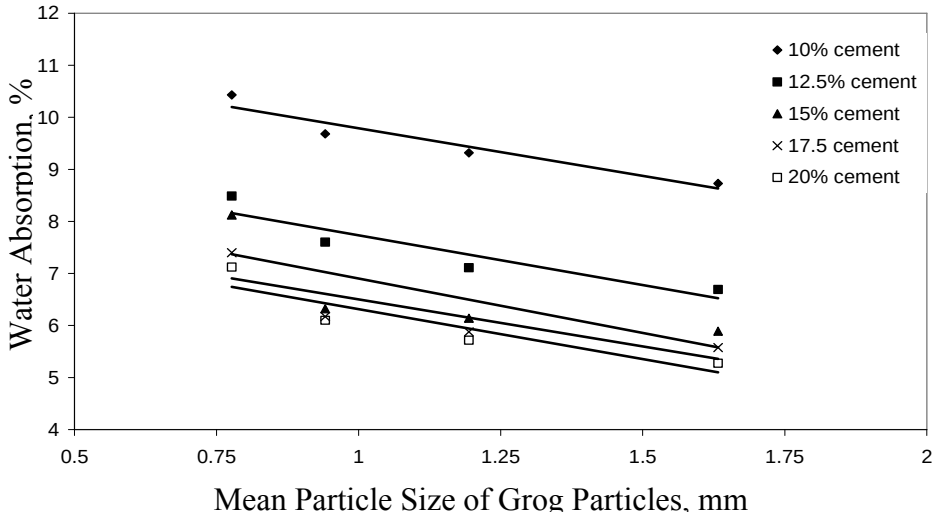


Fig. (7): Effect of Mean Particle Size of Grog Aggregates Containing Different Cement Percents on Percent Water Absorption for Mixes

Also these figures indicate that the percent water absorption is only slightly affected by the increase in particle size. To assess this, the excel DATA ANALYSIS module was used to establish correlation tables which show the relative influence of percent cement and particle size on the percent water absorption in both cases. The results are listed in tables (5) and (6).

Table (5): Correlation Table for Percent Water Absorption for Bauxite Based Mixes

	% Cement	Particle Size	% W.A.
% Cement	1		
Particle Size	0	1	
% W.A.	− 0.82455	− 0.39599	1

Table (6): Correlation Table for Percent Water Absorption for Grog Based Mixes

	% Cement	Particle Size	% W.A.
% Cement	1		
Particle Size	0	1	
% W.A.	− 0.79392	− 0.42302	1

Tables (5) and (6) show that both variables affect negatively the percent water absorption whereas the effect of the variation of percent cement is almost as twice as that of fineness.

3.7 Effect of mean particle Size on Apparent Porosity:

Figures (8) and (9) show the relations between apparent porosity and the mean particle size for formulations containing bauxite and grog. It can be seen that the apparent porosity decreases with the increase of cement content. This is expected since higher cement content enhances the closure of available pores. Also these figures show that the apparent porosity is negatively affected by the increase of particle size.

Using the excel DATA ANALYSIS module, it is possible to establish correlation tables in both cases to show the relative influence of percent cement and particle size on the apparent porosity. Although such tables are not shown, their result indicates that both variables affect negatively the apparent porosity whereas the effect of the variation of percent cement on porosity is almost as twice as that of fineness.

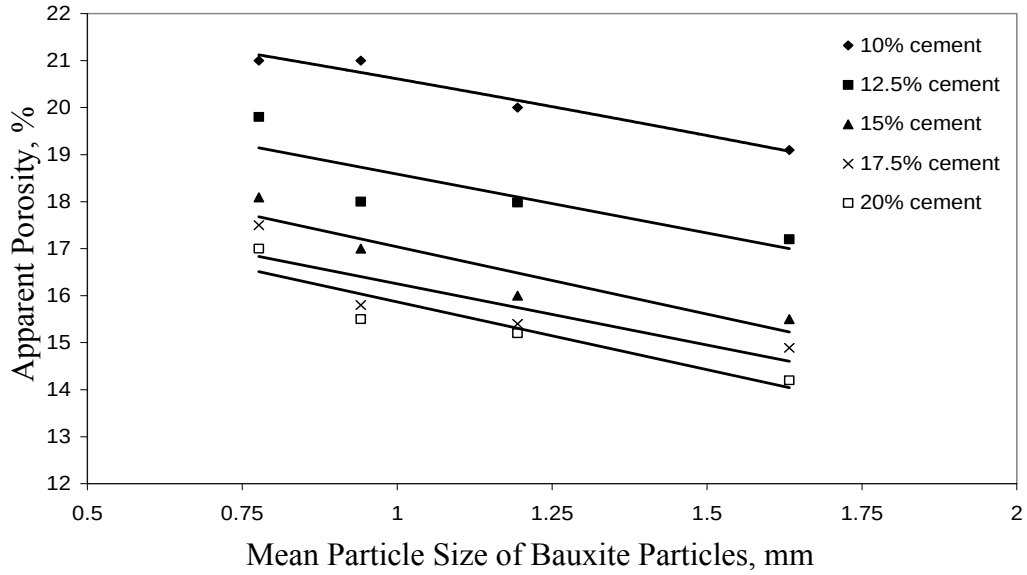


Fig. (8): Effect Mean Particle Size of Bauxite Aggregates Containing Different Cement Percents on Percent Apparent Porosity for Mixes

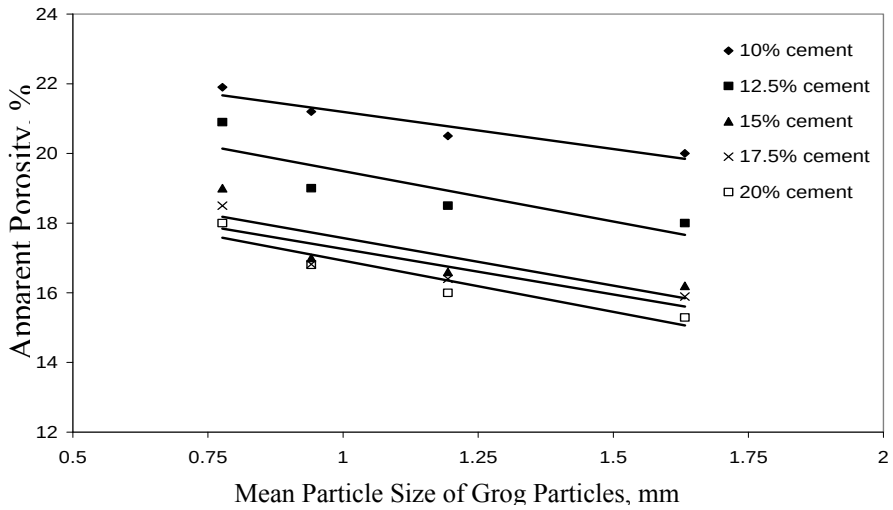


Fig. (9): Effect Mean Particle Size of Grog Aggregates Containing Different Cement Percents on Percent Apparent Porosity for Mixes

3.8 Effect of Particle Size Distribution on Bulk Density:

Figures (10) and (11) show the relations between bulk density and the mean particle size for formulations containing bauxite and grog.

The results indicate that the bulk density increases with the increase in cement content. This is expected since higher cement contents decrease the porosity. Also these figures show that the bulk density is slightly increased with the increase of particle size.

On the other hand, for the same particle size formulation, the values of bulk density of the samples containing bauxite are higher than of those containing grog. For example, at mean particle size = 1.63 mm, the bulk density of samples containing bauxite ranged from 2.37 to 2.96 g/cm^3, depending on the amount of cement added compared with 2.3 to 2.9 g/cm^3 for grog containing samples.

Also, due to the irregular shape of the grog particles , the packing efficiency of a body containing grog is less than that of bauxite. This assists the increased density in the case of using bauxite.

Correlation tables were established to show the relative effect of variations in cement content and particle size for the samples containing either bauxite or grog. These tables show that both cement content and higher particle size favour higher bulk density although the effect of cement variation on bulk density is more pronounced than that of particle size.

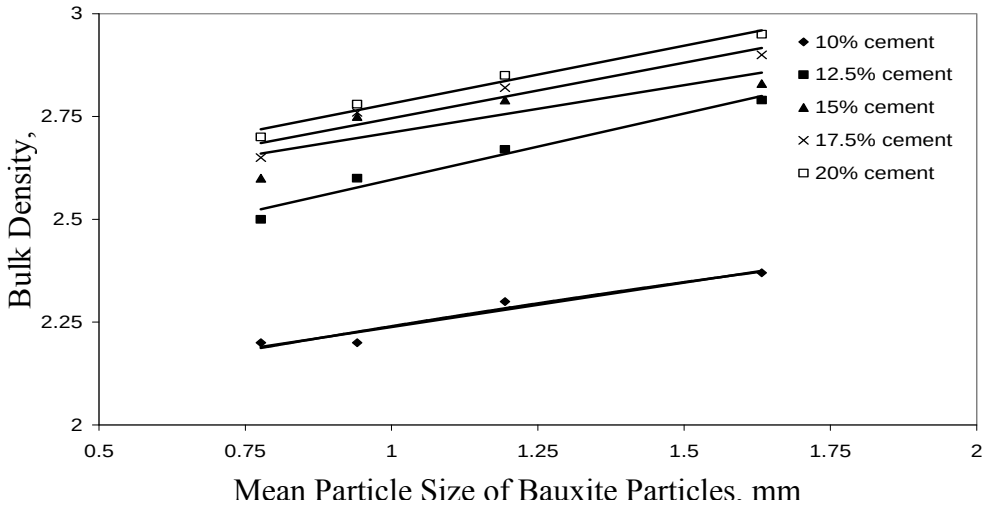


Fig. (10): Effect of Mean Particle Size of Bauxite Aggregates Containing Different Cement Percents on the Bulk Density for Mixes

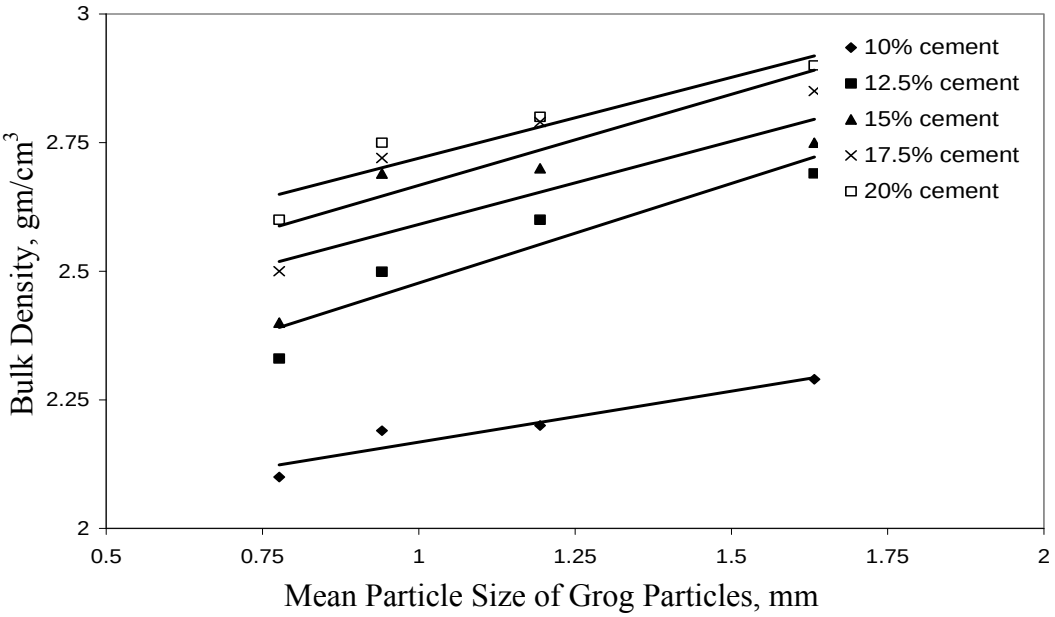


Fig. (11): Effect of Mean Particle Size of Grog Aggregates Containing Different Cement Percents on the Bulk Density for Mixes

3.9 Effect of Particle Size Distribution on Cold Crushing Strength:

Figures (12) and (13) show the relations between the 28 days – cold crushing strength and the mean particle used for different cement contents (bauxite and grog).

It could be seen that the compressive strength increases with the increase in cement content. The effect of mean particle size is more complicated. All curves seem to follow the same pattern: First, the cold crushing strength increases up to a mean particle size of about 0.95 mm then degreases with further increase in particle size. This mean particle size corresponds to formulation C in which the fine particles (D < 1 mm) constitute 40% of the mix whereas the coarse portion represents the remaining 60% (Table 2). Such recipe approaches a state of a minimum total porosity [11] and maximum compaction of the mix leading to a maximum value in C.C.S.

The values of crushing strength were higher for the mixes containing bauxite than for those containing grog: In the former the C.C.S. ranged between 34 and 39 MPa, compared with 32 to 37 MPa in the latter case.

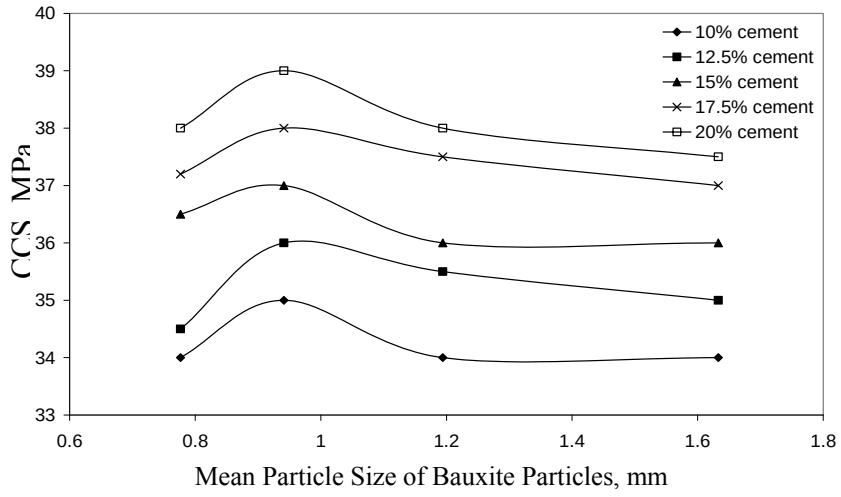


Fig. (12): Effect of Mean Particle Size of Bauxite Aggregates Containing Different Cement Percents on the Cold Crushing Strength of Mixes

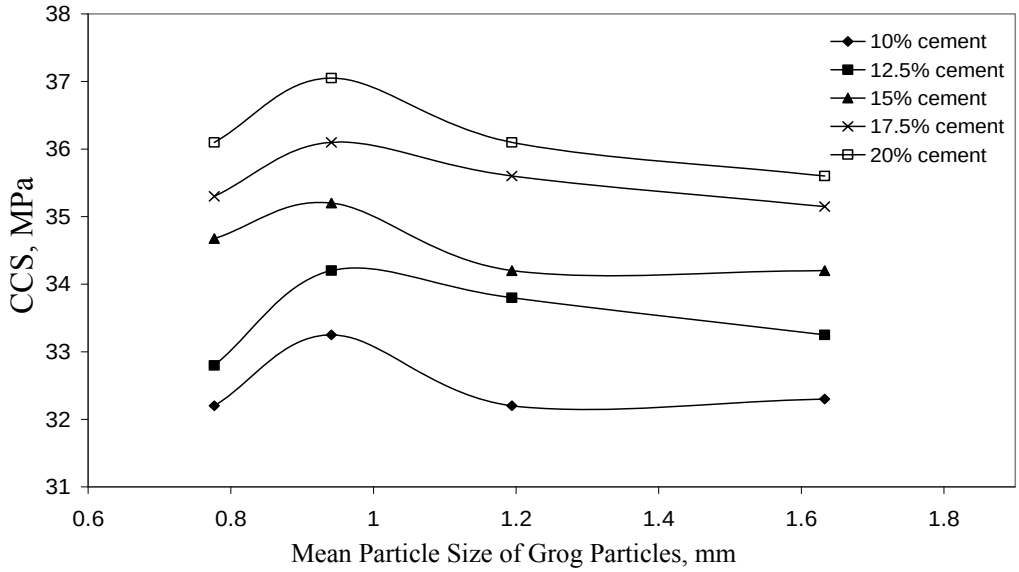


Fig. (13): Effect of Mean Particle Size of Grog Aggregates Containing Different Cement Percents on the Cold Crushing Strength of Mixes

Tables (7) and (8) describe the relative effect of the variation in cement content and mean particle size on the variation in CCS for both types of samples. These tables show that, in both cases, the variation of cement content plays a much higher role than that of particle size in assessing variations in CCS.

Table (7): Correlation Table for CCS for Bauxite Based Mixes

	% Cement	Particle Size	CCS
% Cement	1		
Particle Size	0	1	
CCS	0.945608	− 0.12905	1

Table (8): Correlation Table for CCS for Grog Based Mixes

	% Cement	Particle Size	CCS
% Cement	1		
Particle Size	0	1	
CCS	0.943331	− 0.12633	1

4. Conclusions:

Samples of refractory concrete wereprepared using between 10 to 20% of cement containing 50% alumina and two types of aggregates: calcined bauxite (~ 81% Al_2O_3) and grog containing 52% alumina. These were graded to yield four portions of different mean particle size.

The following results could be deduced:

- Increasing the amount of cement added lead to higher water / solid ratio, lower water absorption lower porosity and higher bulk density.
-
-
- Using coarser aggregate resulted in a reduction in water used for mixing, lower water absorption, lower porosity and a higher bulk density.
- The effect of variation in cement content on the aforementioned properties is generally higher than that of variation of particle size.
- A higher cement content favored higher cold crushing strength and a maximum value was obtained at a mean aggregate particle size of about 0.95 mm corresponding to a state of maximum compactness.
- Better results were generally obtained on using bauxite aggregate rather than grog presumably due to their lower intrinsic porosity.

Corresponding author
M.F. Abadir
Chemical Engineering Department, Faculty of Engineering, Cairo University, Cairo, Egypt.
magdi.abadir@yahoo.com

5. References:

1. Hommer H., Wutz K., Von Seyerl J., "The Effect of Polycarboxylate Ethers as Deflocculants in Castables", 49th International Colloquium on Refractories, Aachen, Proceedings, 2006, p.p. 93−96.
2. Assis G., Parr C., Faryda and Michel Auvray J., "The Impact of Different Additive Systems Upon the Properties of a high Purity Low Cement Castable", Technical Paper, Published in Refractories World Forum, 1st of August, 2009, p.p. 58−62.
3. Sahu N.K., "Study Of Erosion Resistance Of Ulcc Based Precast With Indigenous High Alumina Cement", M.S. Thesis, Department of Ceramic Engineering National Institute of Technology Rourkela, 2009.
4. Angelescu N.G., Muthu K.U., Ionita G.G., Nicolae A.N.A, and Nicolae M.A., "Theoretical Fundaments and Applications about Special Additives", Valahia University Targoviste and Metallurgical Research Institute Bucharest, Romania, ICCBT 2008 - A - (14), 2008, p.p.165−176.
5. ASTM Standards D 422/2007, "Method for Particle–Size Analysis of Soils", Annual book of American Society for Testing of Material (ASTM), U.S.A., 2008, Vol. 4.08.
6. ASTM Standards C 1446/2007, "Standard Test Method for Measuring Consistency of Self−Flowing Castable Refractories", Annual book of American Society for Testing of Material (ASTM), U.S.A., 2008, Vol. 15.01.
7. ASTM Standards C 20/2007, "Standard Test Methods for Apparent Porosity, Water Absorption, Apparent Specific Gravity, and Bulk Density of Burned Refractory Brick and Shapes by Boiling Water", Annual book of American Society for Testing of Material (ASTM), U.S.A., 2008, Vol. 15.4.
8. BS EN Standards 993−5/2000, "Methods of test for Dense Shaped Refractory Products – Part (5): Determination of Cold Crushing Strength", British Standard, 2000.
9. Aramaki S., and Roy R., "Mullite–Corundum Boundary in the Systems $MgO–Al_2O_3–SiO_2$ and $CaO–Al_2O_3–SiO_2$", J. Am. Ceram. Soc., 42, 1959, pp. 644–645.
10. McCabe W., Smith J.C., and Hariot P., "Unit Operations of Chemical Engineering", McGraw Hill, 7th Edition, 2005.
11. Richerson D., "Modern Ceramic Engineering", Taylor and Francis, 2nd Edition, 2006.

10/10/2010

Osteoporosis in Diabetic Children

Enas R. Abdel Hameed[*1], Hisham W. Badr[1], Azza A.Abdallah [1], Wagdi M. Hanna[1]and Nehal Salah[2]

[1]Child Health Department and [2]Clinical Pathology department, National Research Center, Cairo, Egypt
[*]enas_raafat@hotmail.com

Abstract: Background: Osteoporosis is a disease characterized by low bone mass and deterioration of bone structure that causes bone fragility and increases the risk of fracture. . Children and adolescents with type 1 (insulin-dependent) diabetes mellitus (T1DM) show several impairment of bone metabolism and structure, resulting in a higher risk of decreased bone mass and its related complications later in life. Objective: to analyze whether bone mineral density (BMD) with bone status are influenced in children with T1DM and evaluate their relationships with clinical status, age and duration. Patients and Methods: Forty cases (age 7.5 $\pm$ 3.4 and duration of disease 3.7 $\pm$ 2.5 years) were studied. BMD expressed as Z-score was measured at neck of femur and Lumbar spines ($L_2 - L_4$) using dual energy x-ray absorptiometry (DEXA) for 15 cases. Urinary excretion of deoxypyridinoline (DPD) was measured by radio immunoassay and was corrected by creatinine (Cr). Serum levels of osteocalcin, osteoprotegrin, procollagen and rankle – markers of bone formation and resorption were measured. They were matched by age and sex for another 40 normal children as control. Results: there was a significant decrease in serum level of osteocalcin in 12 of our patients,all cases showed significant increase in serum rankle with significant difference $P < 0.05$ compared to control. Mean values of procollagen showed no significant difference compared to controls. As regard DPD mean values of cases showed a significant increase compared to control. BMD – expressed as Z-score-by DEXA revealed 10 cases with mild degree osteopenia, while the other 5 cases showed moderate degree. Conclusion: pediatric patients with T1DM appear to constitute a population at risk of developing osteopenia. Age-optimizing of metabolic control in growing diabetic children may prevent osteoporosis in later life.
[Enas R. Abdel Hameed, Hisham W. Badr, Azza A.Abdallah, Wagdi M. Hanna and Nehal Salah. **Osteoporosis in Diabetic Children.** Journal of American Science 2010;6(12):685-690]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Osteoporosis- Type 1 Diabetes Mellitus-Dual energy x-ray absorptiometry (DEXA), Bone Mass density – Osteopenia.

1. Introduction:

There are several different forms of idiopathic osteoporosis that can affect both children and adolescents (Sone,.2010). Juvenile osteoporosis affects previously healthy children between the ages of 8-14 (Heap et al., 2004).

The disease almost always goes into remission around the time of puberty with a resumption of normal bone growth at that time, (Ingberg et al., 2004). Patient with mild or moderate forms of the disease may be left with a curvature of the spine and short stature, but those with a more severe form may be incapacitated for life (Alonso et al,2010 and Gooch et al., 2000).

Diabetic osteoporosis is increasingly recognized as a significant comorbidity of type 1 diabetes mellitus (Saha et al, 2009 and Galluzzi et al,2005). . Alterations of the nuclear factor-kappa B ligand (RANKL)/osteoprotegerin (OPG) system have been implicated in several metabolic bone diseases characterized by increased osteoclast differentiation and activation and enhanced bone resorption (Carnevale et al,2004) Data uniformly support the concept that new bone formation as well as bone microarchitectural integrity are altered in the diabetic state, leading to an increased risk for fragility fracture and inadequate bone regeneration following injury, (Stagi et al,2010 and He et al., 2004).

The osteopenia associated with diabetes appears to be associated with a decreased bone turnover associated with impaired osteoclastic maturation and function (Brandao et al,2007) . This is reflected in a decrease in serum markers of bone formation, such as osteocalcin. Bone resorption and formation are usually tightly coupled, (Heilman et al,2009 and Gunczler et al., 2001).

Insulinopenia as occurs in type 1 diabetes is associated with several deleterious consequences for skeletal health. Skeletal defects that are observed in conjunction with T1DM include : 1) diminished linear bone growth during the pubertal growth spurt, 2) decreased adult bone density, 3) an increased risk for adult osteoporosis, 4) increased risk of fragility fracture, and 5) poor bone healing and regeneration characteristics,(Saha et al,2009 and Campos et al., 2000).

Aim of the study : To analyze whether bone mineral density (BMD) with bone status are

influenced in children with type 1 diabetes mellitus (T1DM) and evaluate their relationships with clinical status, age and duration.

2. Subjects and Methods:

Forty patients with type 1 Diabetes Mellitus – from endocrinology clinic, Children Hospital, Cairo University – were included in this study. Mean age was (7.5 ± 3.4 years) and mean duration of the disease (3.7 ± 2.5 years). They were matched by age and sex for another 40 normal children as control.

All the above cases were subjected to:

Full history taking for the clinical status, all investigations were done for diagnosis and full details about scheme of treatment.

Special clinical files were done for all the studied cases with all their clinical data.*Blood samples were collected. Separated sea was froozen at –20.

Serum levels of osteocalcin were measured by host-ELISA kit prepared by Bio Source Europe S.A. Serum procollagen Type 1 was detected by Enzyme Immunoassay. Osteoprotegrin was measured by Biovendor Human Osteoprotegrin, while serum level of RANKL was detected by Biomedica Gruppe

Enzyme Immunoassay.

BMD was done by DEXA manifested on the femur bone and Lumbar spines (L_2- L_4) expressed as Z-score.

Statistical methods:

All the above data were collected and statistically tested by analysis of variance or students t-test. Correlations were studied by simple pearsons coefficient. Significance was defined as $P < 0.05$.

3. Results

Our study revealed significant decrease in osteocalcin level (range 2.1 – 5 ng/ml) in 28 of our T1DM cases compared to 12 cases showed normal range of (5-25), with mean values of osteocalcion 5.7 ± 6 for cases against 9.5 ± 7 ng/ml for control with statistical significant difference $P < 0.05$ (Table 1 and Fig. 1).

Positive correlation ($r = 0.6$) was recorded between osteocalcin level and age of the patients in years (Table 2 and Fig. 2).

Forty cases of the study, showed increase in procollagen type 1 level (mean 352.4 ±18.3) compared to control (mean 339.2 ± 19.2) but without significant differene $P > 0.05$. Table (1).

Levels of osteoprotegnin were markedly decreased in all the cases (3.1 ± 6.9) against (7.9 ± 5.4) for controls with $P < 0.05$ (Table 1 and Fig.1). A positive correlation ($r = 0.5$) was detected between osteoprotegrin level and duration of the disease (Table 3 and Fig. 3).

As regard Rankl levels, the cases record significant increase in all the cases (mean 78420.9 ± 16.2) compared to control (15977.2 ± 25.1) with $P < 0.05$ Table (1).

Urinary levels of DPD, revealed marked increase in all the cases (mean 68.65 ± 8.2) compared to control (36.11 ± 1.2) with statistical significant difference $P < 0.05$ Table (1).

The BMD- expressed as Z score – was measured in 15 cases only at the femur and lumbar spines (L_2 – L_4). Our results showed 10 cases with mild form of osteopenia (-1: -1.5) and the other 5 cases with moderate form of osteopenia (-1.5: -2).

Table (1) : Mean Values of some Laboratory Finding of Patients Versus control:

	Osteocalcin ng/ml	Procollagen ng/ml	Rankl Pmol/L	Osteoprotegrin Pmol/L	Deoxypyridin (DPD) n mol DPD/ n mol Cr
Patient (n=40) mean ± SD	5.7 ± 6	352.4 ± 18.3	78420.9 ± 16.2	3.1 ± 6.9	68.65 ± 8.2
Control (n=40) mean ± SD	9.5 ± 7	339.2 ± 19.2	15977.2 ± 25.1	7.9 ± 5.4	36.11 ± 1.2
P-value	P < 0.05	P > 0.05	P < 0.05	P < 0.05	P < 0.05

Statistical sig. difference p<0.0

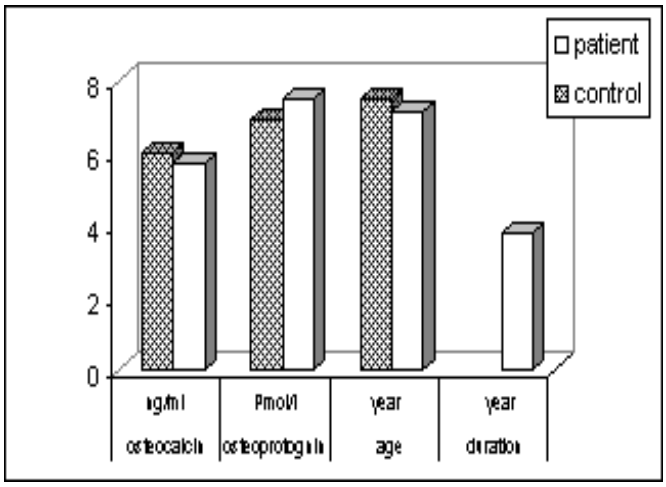
Table 2 : Relation between Osteocalcin and age of the patients.

	Osteocalcin (ng/ml)	Age of patients (year)
Patients (n = 40) Mean ± SD	5.7 ± 6.2	7.50 ± 3.4
r	+ 0.6	

Table 3 : Correlation between Osteoprotegrin and duration of disease.

	Osteoprotegrin (Pmol /L)	Duration of Disease (year)
Patients (n = 40) Mean ± SD	7.9 ± 5.4	3.7 ± 2.5
r	+ 0.f	

Statistical sig. difference p<0.0



Fig, 1: Mean values of some laboratory finding of patients versus controls.

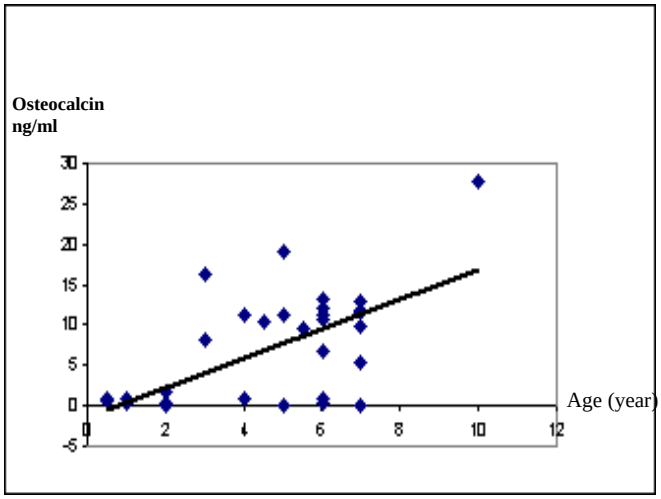


Fig. 2 :Relation between Osteocalcin and age of the patients

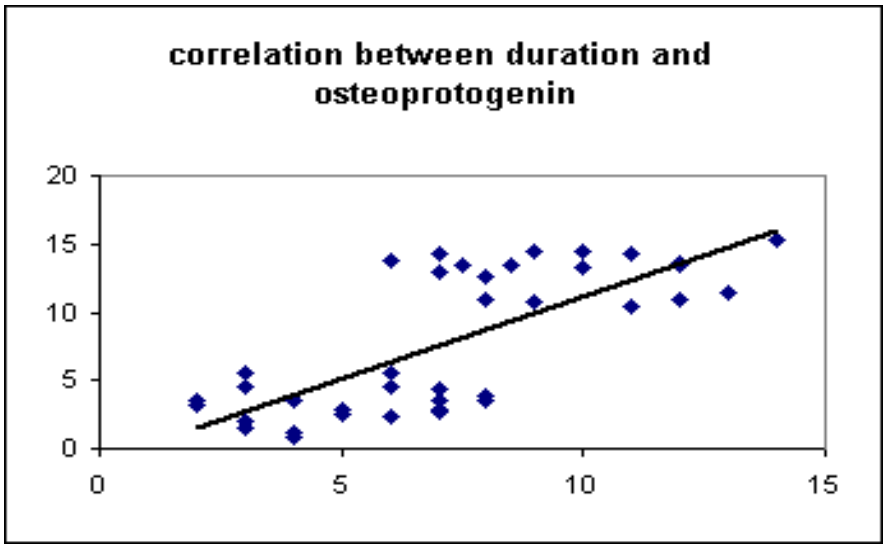


Fig.3 Correlation between osteoprotegrin and duration of disease.

4. Discussion:

Osteoporosis can vary broadly and may involve more than one disorder. Some young patients with osteoporosis may have a primary defect in the regulation of bone cell function, resulting in depressed bone formation, increased bone resorption or both,(Alonso et al,2010 and Heap et al., 2004).

The result of this study on T1DM patients revealed significant decrease in serum values of osteocalcin and osteoprotegrin, (2.1 – 5 ng/mL, 62 ng/ml, 3.1 – 6.9 pmol/L respectively). These findings indicate active depression of bone formation during diabetic illness.

Mean while, our data showed increase mean values of serum Rankl (0.3 – 0.4 pmol/L) and urine values of DPD (600-852 pmol/L). Both indicate increase bone resorption.

These finding indicate that, early onset of diabetes, in particular, is associated with reduced bone density, and patients with type 1 diabetes show evidence of low bone mass following adolescence. Our data fit the same founded by, Geurs, et al., in (2003)and Heilman et al., (2009) who stated that, osteopenia with diabetes appears to be associated with a decrease bone turnover and impaired osteoblastic maturation and function. This is reflected in a decrease in serum markers of bone formation. Bone resorption and formation are usually tightly coupled, (Melhus et al., 2003).

Positive correlation between osteoprotegrin and duration of the disease (r = 0.5), could be explained by the fact that, all bone metabolic changes which occure in early childhood of type 1 diabetic patients will be gradually corrected with long duration of the disease, these results fit the same of Ingberg et al., (2004) and Gilluzzi et al., (2oo5) who suggested that the impact of T1DM on skeletal health may by especially pertinent during adolescence.

The patients in the study were all between age of 5 and 16 and had been receiving treatment for diabetes for at least five years in the endocrinology clinic at Abu-reach hospital. Bone mineral density and bone mineral content measurements were taken for neck of femur and lumbar spine (L_2-L_4) by dual energy X-ray absorptiometry (DEXA). Our result revealed ten cases with mild form of osteopenia (Z-score - 1: -1.5) while only 5 cases were of moderate osteopenia (Z score - 1.5: -2). Careful clinical history taking revealed that, those 15 cases of T1DM were with uncontrolled diabetes with in adequate doses of insulin. This can be explained by findings of, Hou et al.,(1993) vankuijk,(2010)and Kemink et al.(2000), who stated that insulin is an anabolic agent in bone. It can preserve and increase bone density and bone strength, presumably through direct and /or indirect effects on bone formation.

The results of this work demonstrated that osteopenia and osteoporosis are frequent complications of T1DM. This fit the results of Heilman et al, (2009) and Heap et al., (2004). It is relevant therefore, that many studies confirm that T1DM is associated with decreased bone density, (Lopez et al., 2001 and Rozadilla et al., 2003) and a state of low bone turnover, (Thrailkill, 2004).

The osteopenia founded in some of our T1DM population, can be explained by numerous factors which may contribute to the development of osteoporosis over the life time of those diabetic children, 1) insufficient skeletal mineralization during critical periods of bone mass accrual; 2) increased urinary calcium excretion coupled with diminished calcium absorption, leading to chronic clacium deficiency, 3) life long effects of chronic hyperglycemia on osteoblast function; 4) detrimental effects of accumulated glycated end products on bone formation; 5) insulinopenia.

Kemink et al. (2000), suggested that, although insulin as an anabolic agent can preserve and increase bone strength through its effects on bone formation, the persistence of fracture risk in certain hyperinsulinemic states, however, under scores the multifactorial nature of the effects of diabetes on bone and may suggest a threshold for insulin in promoting healthy bone. Multiple confounding variables may have independent negative impacts upon bone mineral acquisition in T1DM and ultimately, on peak bone mass, (Rozadilla et al., 2003).

5. Conclusion:

Pediatric patients with type 1 Diabetes Mellitus appear to constitute a population at risk of osteoporosis in adulthood. Poor metabolic control may expose those patients at adolescents to the risk of osteopenia. So, optimization of metabolic control in growing diabetic children may prevent osteoporosis in later life.

5. Recommendations:

Type 1 diabetes does appear to be a significant risk factor for osteoporosis. Currently we recommend that patients with type 1 diabetes be monitored more carefully than persons without diabetes. Those patients should be encouraged to consume a diet high in both calcium (at least 1200 mg/day) and vitamin D (400-600 Iu/day). It appears that intensive insulin therapy and a stable body weight in patients with type 1 diabetes are important in preventing bone loss.

Corresponding author
Enas R. Abdel Hameed
Child Health Department, National Research Center, Cairo, Egypt
enas_raafat@hotmail.com

7. References:

1. Alonso Franch M, Redondo Del Río MP, Suárez Cortina L; [Nutrition and bone health in children] En Nombre del Comité de Nutrición de la Asociación Española de Pediatría.An Pediatr (Barc). 2010 Jan;72(1):80.e1-11
2. Brandao FR, Vicente EJ, Daltro CH, Sacramento M, Moreira A, Adan L.
3. Bone metabolism is linked to disease duration and metabolic control in type 1 diabetes mellitus. Diabetes Res Clin Pract. 2007 Dec;78(3):334-9.
4. Campos- MM; Lopez – PJ; Escobar- JF… et al.: Intensive insulin therapy and bone mineral density in type 1 Diabetes Mellitus. Osteoporosis Int.2000 (11): 455 – 59.
5. Carnevale V, Romagnoli E & D'Erasmo E. Skeletal involvement in patients with diabetes mellitus. *Diabetes Metabolism Research and Reviews.* 2004 (20) 196–204.[Web of Science]
6. Follak-N; Kloting-I; Wolf-E and Merck-H. : Delayed remodeling in the early period of fracture in diabetic patients. Histopathol .2004 (19): 473 – 486.
7. Galluzzi F, Stagi S, Salti R, Toni S, Piscitelli E, Simonini G, Falcini F, Chiarelli F. Osteoprotegerin serum levels in children with type 1 diabetes: a potential modulating role in bone status. Eur J Endocrinol. 2005 Dec;153(6):879-85.
8. Geurs-NC; Lewis- CE and Jeffcoat- MK.: Osteoporosis and Periodontal disease Progression. Periodontal.2003 (32): 105-10.
9. Gooch- HL; Hale- JE; Fujioka-H; Balian-G… et al.: Alterations of Cartilage and collagen expression during fracture in Diabetes Mellitus. Connect Tissue Res.2000 (410): 81 – 91.
10. Gunczler-P; Lanes-R; Paoli-M; Martinis-R.: Decreased bone mineral density and bone formation markers shortly after diagnosis of TIDM.
11. J Pediatre Endocrinol Metab.2001 (14): 525-28.
12. Hanley- DA; Brown-PA, Tenenhouse-A… et al.: Associations among disease conditions, bone mineral denisty and vertebral deformities: cross sectional results from the Canadian Multicentre Osteoporosis study. J. Bone Miner.2003 (18) 784-90.
13. Heap-J; Murray-MA; Miller-SG and Mayor-LJ.: Alterations in bone characteristics associated with glycemic control in adolescents with type 1 – Diabetes Mellitus. J Pediatre.2004 (144): 56-62.
14. He- H; Liu-R; Desta- T; Leone – C and Graves-DT.: Diabetes causes decrease osteclastogenesis, reduced bone formation in bacteria stimulated bone loss. Endocrinology.2004 (145): 447-52.
15. Heilman K, Zilmer M, Zilmer K, Tillmann V. Lower bone mineral density in children with type 1 diabetes is associated with poor glycemic control and higher serum ICAM-1 and urinary isoprostane levels. J Bone Miner Metab. 2009;27(5):598-604. Epub 2009 Apr 17.
16. Hofbauer LC & Schoppet M. Clinical implications of the osteoprotegerin/RANKL/RANK system for bone and vascular diseases. JAMA 2004 (292) 490–495
17. Hou-JC, Zernicke-R and Barnard-R.: Effects of sever diabetes and insulin on femoral neck. J. Orthop.1993 (11): 263-71.
18. Ingberg- CM; Plamer- M; Aman- J and Bern-C.: Body Composition and bone mineral denisty in T1DM. J. Int Med.2004 (255): 3.92-98.
19. Kemink- S; Hermns- C and Smals- G.: Osteopenia in insulin dependant DM. J. Endocr. Inv.2000 (23): 295 –303.
20. Lopez-P; Pastor- M; Pardo-M; Luna-J…. et al.: Bone mineral density at clinical diagnosis of adult-onset type 1 diabetes Mellitus. Endocr Pract.2001 (7): 346-51.
21. Melhus-H; Michaelsson- K and Vessby – B.: Serum bone metabolite and risk of Fracture. N Engl J. Med.2003 Jan (23); 348 (4): 287 – 94.
22. Rozadilla-A; Nolla- M; Fiter- J and Soler-J.: Bone Mineral density in patinet of T1DM, Joint Bone Spine.2003 (67): 215-18.
23. Saha MT, Sievänen H, Salo MK, Tulokas S, Saha HH. Bone mass and structure in adolescents with type 1 diabetes compared to healthy peers. Osteoporos Int. 2009 Aug;20(8):1401-6
24. Salvatoni A, Mancassola G, Biasoli R, Cardani R, Salvatore S, Broggini M, NespoliL. Bone mineral density in diabetic children and adolescents: a follow-up study.Bone. 2004 May;34(5):900-4.

25. Sone T, Onaru K. [Bone and joint diseases in children. Abnormal bone mineral density in children] Clin Calcium. 2010 Jun;20(6):859-65
26. Stagi S, Masi L, Capannini S, Cimaz R, Tonini G, Matucci-Cerinic M, de Martino M, Falcini F. Cross-sectional and longitudinal evaluation of bone mass in children and young adults with juvenile idiopathic arthritis: the role of bone mass determinants in a large cohort of patients. J Rheumatol. 2010 Sep;37(9):1935-43.
27. Thrailkill- K.M.: Diabetes care for adolescents. In: Diabetes in women, edited by Reece EA, Coustan DR, and Gabbe SG. Philadelphia, PA: Lippincott Williams & Willkins, 2004.
28. Valerio G, del Puente A, Esposito-del Puente A, Buono P, Mozzillo E, Franzese A. The lumbar bone mineral density is affected by long-term poor metabolic control in adolescents with type 1 diabetes mellitus. Horm Res. 2002;58(6):266-72
29. VanKuijk C. Pediatric bone densitometry. Radiol Clin North Am. 2010 May;48(3):623-7.

10/5/2010

Studies on Antimicrobial and Antioxidant Efficiency of Some Essential Oils in Minced Beef

Amany, M. Salem*, Reham, A. Amin and Gehan, S. A. Afifi

Food Control Department, Fac. Vet. Med., Benha University, Benha, Egypt
dr_amany40@yahoo.com*

Abstract: In this study, the antioxidant and antibacterial effect of garlic (G), thyme (T) and lemon grass (L) oils were investigated in refrigerated minced beef. It is noticed that, all essential oils used had considerable effectiveness in decreasing aerobic plate count (APC), *Enterobacteriaceae* count, Coliform count and *Staphylococci count*, as well as chemical indices as pH, total volatile nitrogen (TVN) and thiobarbituric acid (TBA). Sensory analysis indicated significant advantages in using lemon grass and thyme oils in refrigerated minced beef. In addition, a highly significant differences ($P<0.05$) between the different oils were noticed. Also, results indicated that the bacterial counts, pH, TVN and TBA values decrease as the concentration of the oil increases since the concentration (1.5%) gives the best effectiveness. The antioxidant and antibacterial activities of the added essential oils followed the order lemon grass oil > thyme oil > garlic oil. The treated minced beef samples extend the shelf life of the treated samples more than the control samples by 6 days. In conclusion, lemon grass, thyme and garlic oils can play an important role as antioxidant and antibacterial agents in refrigerated minced beef, but lemon grass oil is the best one.

[Amany, M. Salem, Reham, A. Amin and Gehan, S. A. Afifi. Studies on Antimicrobial and Antioxidant Efficiency of Some Essential Oils in Minced Beef. Journal of American Science 2010;6(12):691-700]. (ISSN: 1545-1003).

Keywords: Antimicrobial; Antioxidant; Efficiency; Oil; Beef

1. Introduction:

Meat and its products have experienced increasing popularity and become widely spread all over the world. The appearance of food is one of the major determinants of its appeal to consumers and consequently, sales of the product. However, during storage, quality attributes of the products deteriorate due to lipid oxidation and bacterial growth which are the main factors that determine food quality loss and shelf life reduction. Lipid oxidation leads to the degradation of lipids and proteins which, in turn, contribute to the reduction in nutritional quality as well as deterioration in flavor, color and texture of displayed meat products (Aguirrezábal *et al.*, 2000), while bacterial contamination can precipitate major public health hazard and economic loss in terms of food poisoning and meat spoilage (Fernández –López *et al.*, 2005).

Although several synthetic food additives have been widely used in the meat industry to extend food shelf life, inhibit lipid oxidation and delay or inhibit the growth of pathogenic microorganisms, the trend is to decrease their use because of the growing concern among consumers about such chemical additives. Consequently, search for natural additives, especially of plant origin, has notably increased in recent years indicating that the application of natural food additives possessing both antioxidant and antimicrobial activities may be useful for maintaining meat quality, extending shelf life and preventing economic loss (Yin and Cheng, 2003 and Mielnik *et al.*, 2008).

Essential oils are regarded as natural alternatives of chemical preservatives and their use in food meets the demands of consumers for mildly processed or natural products, since in modern food industries, mild processes are applied in order to obtain safe products which have a natural or "green" image (Burt, 2004). However, the practical application of essential oils is limited because of flavor considerations, as well as, their effectiveness is moderate due to their interaction with food ingredients and structure (Skandamis *et al.*, 2001). Essential oils of herbs and their components, products from the secondary metabolism of plants, have many applications in ethno-medicine, food flavoring and preservation as well as the fragrance and pharmaceutical industries (Fabian *et al.*, 2006).

Garlic is one of the most commonly used ingredients as a flavor enhancement for sausage. In addition, garlic is appreciated for its medical properties. Garlic has a wide spectrum of actions, not only antibacterial, antifungal and antiprotozoal, but also it has beneficial effects on the cardiovascular and immune systems (Harris *et al.*, 2001). During the last decade, the antimicrobial activity of garlic and garlic derived organosulfur compounds was widely investigated against both food spoilage bacteria and food borne pathogens (Leuschner and Ielsch, 2003).

Thyme is commonly used in foods mainly for its flavor and aroma. Also, thymol, which is found in thyme, has been commercially available as part of mouthwash for more than hundred years. Besides, it is active against *E. coli* and *St. aureus* and spoilage flora in meat products (Solomakos *et al.,* 2008), completely stops the growth of fungi at low concentrations (Soliman and Badeaa, 2002) and inhibits the aflatoxin production (Chiasson *et al.,* 2004), so it has a role as pharmaceutical and preservative (Hammer *et al.,* 1999).

Lemon grass is a rich source of citral, which is used in perfumery and pharmaceutical industries, and bioactive compounds (flavonides and vitamin C). The natural flavonides are also attracting more and more attention not only due to their antioxidant properties, but as anti-carcinogenic and anti-inflammatory agents because of their lipid anti-peroxidation effects (Martin *et al.,* 2002).

Moreover, some researchers reported that there is a relationship between the chemical structures of the most abundant compounds in plants and their above mentioned functional properties (Deans and Svoboda, 1990).

The objective of the present study was to investigate the antioxidant as well as the antimicrobial effectiveness of three essential oils (garlic, thyme & lemon grass oils) at various concentrations on the quality of fresh minced beef during refrigerated storage (4ºC).

2. Materials and methods

A grand total of thirty random samples of fresh minced beef were collected from different butcher shops in Kaluobyia governorate. The samples were taken and transferred directly to the laboratory under complete aseptic conditions without undue delay. The samples were divided into untreated (control) and treated samples. The treated samples were homogenized with garlic, thyme and lemon grass oils in 0.5%, 1% and 1.5% concentrations for each oil. Each sample was packed in polyethylene bag, labeled and stored at 4 ºC. Each sample was analysed promptly at 3 days intervals during storage as follows:

1. Sensory examination:

It was carried out according to Pearson and Tauber (1984).

2. Chemical examination includes:

2.1. PH values were carried out according the technique recommended by ISO (1979).

2.2. Total volatile nitrogen (TVN) was done according the technique recommended by FAO (1980).

2.3. Thiobarbituric acid (TBA) was carried out according the technique recommended by Vyncke (1970).

3. Microbiological examination:

3.1. Determination of aerobic plate count (APC) which was performed according to ICMSF (1996).

3.2. Determination of total coliforms count which was done according to APHA (1985).

3.3 Enumeration and identification of *Enterobacteriaceae* which were carried out according to ICMSF (1996).

3.4. Determination of total *Staphylococci* count which was performed according to Oxoid (1986).

4. Statistical Analysis:

ANOVA was carried out on data of the sensory, chemical and microbiological evaluations. Data are expressed as mean + SE (Gomez and Gomez, 1984).

3. Results and Discussion:

It is obvious from results obtained in table (1) that the sensory properties of different treated minced beef samples during cold storage (4ºC) were enhanced by increasing the concentrations of oils compared to the untreated (control) samples at zero, 3rd and 6th day of the storage period. Generally, samples containing 1.5% lemon grass oil, thyme and garlic oils, respectively demonstrated the highest enhancement of sensory attributes, while the samples treated with 0.5% garlic oil demonstrated the lowest enhancement. The direct addition of essential oils to food may alter the sensory characteristics of food (Seydim and Sarikus, 2006). Nearly similar results were obtained by El-Desouky *et al.,* (2006) and Mielnik *et al.,* (2008).

Lipid oxidation and other degradation reactions lead to the formation of low molecular compounds, which contribute to the sensory profile. Hydroperoxides and secondary oxidation products can react with protein and amino acids during processing and storage period affecting the flavor, odour and texture of meat products (Frankel, 1998).

The differences in pH mean values between different treated and untreated samples were significant during storage at 4ºC (table 3). The results showed a significant ($P<0.05$) increase in pH mean values in different treatments during storage by different rates. The highest incremental rates (pH values) were found in the untreated (control) samples. The samples treated with 1.5% and 1% lemon grass oil, showed the highest significant ($P<0.05$) effect on

pH lowering its values than those of untreated samples, followed by samples treated with 1.5%, 1% and 0.5% thyme oil, respectively, and finally the samples treated with 1.5%, 1% and 0.5% garlic oil, respectively, till the end of the storage period. There was significant ($P<0.05$) increase in pH mean values of all untreated and treated samples with garlic, thyme and lemon grass oils at all concentrations at the 6th day of the storage period. This may be due to the activation effect of microbial load which may cause protein hydrolysis with the appearances of alkyl groups (Yassin - Nessrien, 2003).

The mean values of TVN are summarized in table (3) estimating the degree of meat deterioration during the storage period (zero, 3rd and 6th day). As the storage period at 4^{o}C increased, the TVN values increased as shown in table (3) for all minced meat samples with different rates depending on the nature of treatments. This may be attributed to the breakdown of proteins as a result of activity of microbial strains and proteolytic enzymes (Yassin - Nessrien, 2003). EOS (2005) stated that 20 mg TVN/ 100 gm raw samples indicates the spoilage of minced meat. The highest rate of increase of TVN values was recorded in control samples. The treatments with 1.5% lemon grass, thyme and garlic oils, respectively, were more effective in delaying the rate of TVN increase during the subsequent cold storage. This may be attributed to the role of such oils on microbial population and bacterial growth as antimicrobial agents (Sacchetti *et al.,* 2005).

The evaluation of TBA mean values of control and treated samples during storage at 4^{o}C are shown in table (3). The highest incremental rate was recorded in the untreated (control) samples, while the lowest significant incremental rate was recorded in samples treated with 1.5% lemon grass oil, followed by samples treated with 1% of the same oil. The incremental pattern in TBA values for all the stored samples with advancing the chilling storage time may be due to the auto-oxidation of meat lipids, bacteriological and/or oxidative rancidity. TBA value is routinely used as an index of lipid oxidation in meat products in store (Raharjo and Sofos, 1993) and the rancid flavor is initially detected in meat products between TBA values of 0.5 and 2.0 (Gray and Pearson, 1987).

Thus, it has been reported that thyme oil may act as a high scavenger of radicals involved in lipid peroxidation protecting lipids from oxidation during cold storage as discussed by Kulisic *et al.* (2005) and Bozin *et al.* (2006), while garlic oil posses effective antioxidant activity (Jackson *et al.,* 2002) which is mainly attributed to a variety of sulphur-containing compounds and their precursors (Song *et al.,* 2004). In the same field, Fernández – López *et al.* (2005) found that about 50% of the rancidity of meat products can be controlled by the citrus preparations (e.g. lemon grass oil) with significant advantages in acceptability and aroma in rancidity-susceptible meat products. This antioxidant activity has been mainly attributed to flavonoids and ascorbic acid in citrus fruits (hesperidin, neohesperidin and eriocitrin) (Schwarz *et al.,* 2001). All of these polyphenolic compounds have the ability to act as antioxidants by a free radical scavenging mechanism and also through their known ability to chelate transition metals (inactivation of iron ions) (Martin *et al.,* 2002).

In the past few years, a variety of plant materials containing phenolic compounds have been to be effective antioxidants in model systems. Since ancient times, herbs and spices have been added to food to improve sensory properties and prolong shelf life. Among the main objections against the use of spices as antioxidants, is the characteristic flavor which they give to the meat products. However, this could be turned towards a positive new exciting sensory sensation. The acceptability of the taste of highly spiced food is transmitted both culturally and genetically, and the countries with hotter climate use spices more frequently and at much higher levels than countries with cooler climates. Essential oils rich in polyphenols exhibit antioxidative activities as they scavenge free radicals, similar to synthetic phenolic antioxidants (Cuvelier *et al.,* 1996 and Billing and Sherman, 1998).

The mean values of total aerobic counts (APC) of different untreated and treated minced beef samples during cold storage were shown in table (4). The control samples showed the highest APC counts comparing to others containing lemon grass, thyme or garlic oils with different concentrations (table 4). The relatively high initial counts of control samples may be attributed to the grinding process, which compounds the problem by introducing the pathogens into the interior of the meat and contributes to the increase of total viable counts of meat (Nychas *et al.,* 1991 and Mead and Griffin, 1998). APC counts were gradually increased during cold storage for all samples with different ratios depending on the concentration of oil. The incremental pattern in APC

can be arranged in a descending order as follows: samples treated with garlic, thyme and finally lemon grass oil at 0.5%, 1% & 1.5% concentration levels, respectively. In general, as the concentration of oil decreased, APC increased as discussed by Marino *et al.* (2001).

As shown in table (4), it could be observed that the control samples had the highest counts of *Enterobacteriaceae* and coliform at any time of cold storage compared to other treatments. It is clear that lemon grass, thyme and garlic oils at concentration 1.5% have strong effects against the growth of *Enterobacteriaceae* and coliform, and as the concentration of these essential oils increases, the counts of *Enterobacteriacea*e and coliform reduce especially at the 3rd and 6th days of cold storage.

Data presented in table (5) showed staphylococci counts of different treated and untreated minced beef samples during cold storage. As demonstrated by the different treatments, the treated samples with lemon grass, thyme and garlic oils at concentration 1.5% respectively showed the lowest counts in this parameter at zero, 3rd and 6th days of cold storage.

Table (6) summarized the initial microflora of chilled minced beef samples related to *Enterobacteriaceae*. The contribution of this group to the final flora depends on the type and the concentration of the used essential oil. It needs to be stressed that the rate of growth, lag phase and the final incidence of enteric bacteria were affected by the addition of essential oils (table 6). During the cold storage of minced beef, *Enterobacteriaceae* count reached the highest level with the 6th day of the storage period. *Proteus vulgaris* being the most dominant (66.67%) followed by *Citrobacter freundii* (55.56%) and then *Enterobacter aerogenes* (44.45%) in control samples. The addition of essential oils reduces the growth of *C. diversus, C. freundii, E. aerogenes, K. pneumoniae, P. mirabilis & P. vulagaris* and suppressed the growth of *E. cloacae* & *Serratia liquefaciens* completely. The addition of lemon grass oil showed pronounced inhibition of *C.diversus, E. cloacae, K. pneumoniae, Proteus mirabilis* & *Serratia liquefaciens* and reduced the incidence of *C. freundii* (11.11%), *E. aerogenes* (11.11%) & *P. vulgaris* (22.2%) when compared to the control and the other treated samples with thyme and garlic oils.

Nearly similar results were obtained by Yassin-Nessrien and Abou-Taleb (2007) and Gutierrez *et al.* (2008).

The essential oils will result in immediate reduction of bacterial population (Seydim and Sarikus, 2006) and might be more effective against food borne pathogens and spoilage bacteria when applied directly on foods ready to be used, containing a high protein level at acidic pH, as well as, lower levels of fat or carbohydrates (Gutierrez *et al.*, 2008).

The antimicrobial activity of thyme oil has been thoroughly investigated (Ozcan *et al.*, 2006 and Mielnik *et al.*, 2008) and found to be active against food borne and spoilage flora (Solomakos *et al.*, 2008). This significant rate of antibacterial activities is mostly attributable to the phenolic compounds (cavracrol) and to the hydrocarbons which can be bactericidal or bacteriostatic depending on their effective concentration (Bozin *et al.*, 2006 and Yassin – Nessrien and Abou – Taleb, 2007).

Also garlic oil provides antimicrobial benefits (Sallam *et al.*, 2004), where garlic oil is rich in organosulfur compounds and their precursors (allicin, dially sulfide & diallyl trisulfides) (Ankri and Mirelman, 1999) inhibiting the growth of a lot of pathogens as APC, *E. coli* & *S. aureus* by reacting with their cystine, inactivating the thio-containing enzymes or affecting the metabolism of lipids (Song *et al.*, 2004) and subsequently, extending the shelf life of the product, so the garlic extracts are potentially useful in preserving meat products (Pranoto *et al.*, 2005).

Moreover, lemon grass oil was observed to possess high antimicrobial activity, so it can be used as a way of combating the growth of common causes of food poisoning (Fisher and Phillips, 2006). In the same field, Adegoke and Odesola (1996) added that some bacteria associates with food spoilage / intoxication were inhibited by the essential oil of lemon grass. Lemon grass oil was found to be effective at all concentrations (Chahal *et al.*, 2007), where it is composed of three main components, the alpha and beta-citral components which elicit antibacterial action on Gram-positive and Gram-negative organisms, while the third component is mycrene which provided enhanced activities when mixed with either of the two main previously identified components (Onawunmi *et al.*, 1984)

As an overall conclusion, garlic, thyme and lemon grass oils showed general enhancement in sensory, chemical and microbial attributes due to the

action of these oils in retarding oxidation as well as microbial population in the fresh minced meat during cold storage at 4ºC and the action of these oils is concentration dependent. Also we found that lemon grass oil is the most effective at all concentrations especially at 1.5% than thyme and garlic oils, respectively. The low effectiveness of garlic oil in comparison with thyme & lemon grass oils could be attributed to the losses of volatile sulfur compounds, which have high biological activity, during distillation, and also due to the nature of garlic oil itself, which is volatile and hydrophobic (Sallam *et al.*, 2004).

Therefore, it is suggested that garlic, thyme and lemon grass oils can be used as natural meat preservatives with both antioxidants and antimicrobial activities against food borne pathogens and spoilage organisms, and therefore may be useful in maintaining the meat quality, extending shelf- life of meat products, preventing economic loss and providing the consumer with food containing natural additives, which might be seen more healthful than those of synthetic origin. Further research is necessary to explore the efficiency and palatability of suitable concentrations of natural oils in meat industry.

Table (1): Sensory evaluation of the untreated (control) and treated samples of minced beef during cold storage at 4ºC

Samples	Zero day	3rd day	6th day
Control	Excellent	Very poor	Very very poor
Garlic oil			
0.5%	Excellent	Fair with the presence of garlic odour	Very very poor
1%	Excellent	Fair with the presence of garlic odour	Very poor
1.5 %	Excellent	Fair with the presence of garlic odour	Very poor
Thyme oil			
0.5%	Excellent	Fair with the presence of thyme odour	Very poor
1%	Excellent	Fair with the presence of thyme odour	Very poor
1.5 %	Excellent	Medium with the presence of thyme odour	Fair with the presence of thyme odour
Lemon grass oil			
0.5%	Excellent	Excellent with the presence of lemon odour	Very good with the presence of lemon odour
1%	Excellent	Excellent with the presence of lemon odour	Very good with the presence of lemon odour
1.5 %	Excellent	Excellent with the presence of lemon odour	Very good with the presence of lemon odour

Table (2): Score System for Sensory Evaluation (Pearson and Tauber, 1984)

Score System	
Points	Quality
9	Excellent
8	Very very good
7	Very good
6	Good
5	Medium
4	Fair
3	Poor
2	Very poor
1	Very very poor

(3): Mean values of chemical induces of the examined untreated (control) and treated samples of minced beef during cold storage at 4ºC

Samples	pH			TVN			TBA		
	Zero day	3rd day	6th day	Zero day	3rd day	6th day	Zero day	3rd day	6th day
Control*	5.71± 0.03	6.76 ± 0.02	7.05 ± 0.04	6.33 ± 0.21	44.29 ± 0.41	63.68 ± 1. 17	0.04 ± 0.01	0.42 ± 0.02	0.58 ± 0.01
Garlic oil 0.5 %	5.71± 0.03	6.34 ± 0.03	6.86 ± 0.03	6.33 ± 0.21	26.06 ± 0.32	41.58 ± 0.87	0.04 ± 0.01	0.26 ± 0.01	0.44 ± 0.02
1 %	5.71± 0.03	6.23 ± 0.01	6.73 ± 0.05	6.33 ± 0.21	22.81 ± 0.22	36.25 ± 0.45	0.04 ± 0.01	0.21 ± 0.01	0.38 ± 0.02
1.5 %	5.71± 0.03	6.19 ± 0.02	6.67 ± 0.02	6.33 ± 0.21	20.34 ± 0.75	29.89 ± 0.33	0.04 ± 0.01	0.20 ± 0.01	0.36 ± 0.03
Thyme oil 0.5 %	5.71± 0.03	6.17 ± 0.02	6.78 ± 0.04	6.33 ± 0.21	20.72 ± 0.31	35.56 ± 0.61	0.04 ± 0.01	0.22 ± 0.03	0.38 ± 0.03
1%	5.71± 0.03	6.09 ± 0.01	6.61 ± 0.02	6.33 ± 0.21	18.64 ± 0.22	31.25 ± 0.43	0.04 ± 0.01	0.19 ± 0.01	0.36 ± 0.02
1.5 %	5.71± 0.03	6.02 ± 0.03	6.37 ± 0.03	6.33 ± 0.21	16.81 ± 0.17	28.51 ± 0.37	0.04 ± 0.01	0.18 ± 0.02	0.30 ± 0.02
Lemon grass oil 0.5%	5.71± 0.03	6.09 ± 0.02	6.57 ± 0.03	6.33 ± 0.21	18.41 ± 0.30	32.90 ± 0.51	0.04 ± 0.01	0.19 ± 0.02	0.34 ± 0.02
1%	5.71± 0.03	6.00 ± 0.01	6.33 ± 0.02	6.33 ± 0.21	16.77 ± 0.21	27.36 ± 0.38	0.04 ± 0.01	0.16 ± 0.01	0.29 ± 0.01

+ ($P < 0.05$)

TVN 20 mg / 100 gm raw minced beef (EOS, 2005)

TBA 0.9 mg Melanoaldehyde / kg raw minced beef (EOS, 2005)

Table (4): Mean values of APC and *Enterobacteriaceae* count of the examined untreated (control) and treated samples of minced beef during cold storage at 4ºC

Samples	*APC*			*Enterobacteriaceae* count		
	Zero day	3rd day	6th day	Zero day	3rd day	6th day
Control*	$5.61x10^5 \pm 0.92x10^5$	$6.29x10^7 \pm 1.22x10^7$	$3.08x10^9 \pm 0.62x10^5$	$8.79x10^3 \pm 2.25x10^3$	$6.81x10^5 \pm 1.06x10^5$	$9.25x10^6 \pm 2.89x10^6$
Garlic oil						
0.5 %	$5.61x10^5 \pm 0.92x10^5$	$1.15x10^7 \pm 0.31x10^7$	$9.94x10^8 \pm 2.75x10^8$	$8.79x10^3 \pm 2.25x10^3$	$3.66x10^5 \pm 0.57x10^5$	$5.81x10^6 \pm 0.92x10^6$
1 %	$5.61x10^5 \pm 0.92x10^5$	$8.52x10^6 \pm 2.16x10^6$	$7.27x10^8 \pm 1.53x10^8$	$8.79x10^3 \pm 2.25x10^3$	$1.03x10^5 \pm 0.16x10^5$	$3.78x10^6 \pm 0.64x10^6$
1.5 %	$5.61x10^5 \pm 0.92x10^5$	$7.36x10^6 \pm 1.46x10^6$	$7.01x10^8 \pm 1.29x10^8$	$8.79x10^3 \pm 2.25x10^3$	$7.56x10^4 \pm 1.83x10^4$	$8.49x10^5 \pm 2.38x10^5$
Thyme oil						
0.5 %	$5.61x10^5 \pm 0.92x10^5$	$3.58x10^7 \pm 0.62x10^7$	$7.92x10^8 \pm 1.80x10^8$	$8.79x10^3 \pm 2.25x10^3$	$2.07x10^5 \pm 0.35x10^5$	$3.58x10^6 \pm 0.76x10^6$
1%	$5.61x10^5 \pm 0.92x10^5$	$8.13x10^6 \pm 2.40x10^6$	$3.67x10^8 \pm 0.75x10^8$	$8.79x10^3 \pm 2.25x10^3$	$6.95x10^4 \pm 1.10x10^4$	$1.14x10^6 \pm 0.30x10^6$
1.5 %	$5.61x10^5 \pm 0.92x10^5$	$4.21x10^6 \pm 0.72x10^6$	$8.39x10^7 \pm 2.14x10^7$	$8.79x10^3 \pm 2.25x10^3$	$5.12x10^4 \pm 0.78x10^4$	$5.63x10^5 \pm 0.87x10^5$
Lemon grass oil						
0.5%	$5.61x10^5 \pm 0.92x10^5$	$7.93x10^6 \pm 2.05x10^6$	$4.73x10^8 \pm 0.82x10^8$	$8.79x10^3 \pm 2.25x10^3$	$8.14x10^4 \pm 2.36x10^4$	$8.73x10^5 \pm 2.21x10^5$
1%	$5.61x10^5 \pm 0.92x10^5$	$2.45x10^6 \pm 0.37x10^6$	$1.96x10^7 \pm 0..38x10^7$	$8.79x10^3 \pm 2.25x10^3$	$2.75x10^4 \pm 0.49x10^4$	$4.09x10^5 \pm 0.75x10^5$
1.5 %	$5.61x10^5 \pm 0.92x10^5$	$1.70x10^6 \pm 0.24x10^6$	$9.01x10^4 \pm 3.12x10^6$	$8.79x10^3 \pm 2.25x10^3$	$1.18x10^4 \pm 0.22x10^4$	$8.15x10^4 \pm 2.51x10^4$

*** Significant differences as a result of oil treatments (P< 0.05)**

Table (5): Mean values of coliform and Staphylococci counts of the examined untreated (control) and treated samples of minced beef during cold storage at 4ºC

Samples	Coliform count			*Staphylococci* count		
	Zero day	3rd day	6th day	Zero day	3rd day	6th day
Control*	$5.12x10^3 \pm 0.95x10^3$	$4.27x10^5 \pm 0.81x10^5$	$6.49x10^6 \pm 1.24x10^6$	$2.37x\ 10^4 \pm 0.55x10^4$	$9.96x10^5 \pm 3.01x10^5$	$8.73x10^6 \pm 2.58x10^6$
Garlic oil 0.5 %	$5.12x10^3 \pm 0.95x10^3$	$1.53x10^5 \pm 0.46x10^5$	$3.05x10^6 \pm 0.68x10^6$	$2.37x\ 10^4 \pm 0.55x10^4$	$7.31x10^5 \pm 1.69x10^5$	$5.43x10^6 \pm 1.17x10^6$
1 %	$5.12x10^3 \pm 0.95x10^3$	$8.19x10^4 \pm 1.98x10^4$	$1.18x10^6 \pm 0.27x10^6$	$2.37x\ 10^4 \pm 0.55x10^4$	$2.48x10^5 \pm 0.85x10^5$	$2.18x10^6 \pm 0.49x10^6$
1.5 %	$5.12x10^3 \pm 0.95x10^3$	$5.67x10^4 \pm 1.01x10^4$	$5.39x10^5 \pm 0.97x10^5$	$2.37x\ 10^4 \pm 0.55x10^4$	$1.15x10^5 \pm 0.42x10^5$	$6.92x10^5 \pm 1.56x10^5$
Thyme oil 0.5 %	$5.12x10^3 \pm 0.95x10^3$	$9.78x10^4 \pm 3.04x10^4$	$1.27x10^6 \pm 0.33x10^6$	$2.37x\ 10^4 \pm 0.55x10^4$	$4.87x10^5 \pm 0.96x10^5$	$2.51x10^6 \pm 0.64x10^6$
1%	$5.12x10^3 \pm 0.95x10^3$	$4.93x10^4 \pm 0.82x10^4$	$8.41x10^5 \pm 1.99x10^5$	$2.37x\ 10^4 \pm 0.55x10^4$	$1.09x10^5 \pm 0.34x10^5$	$6.03x10^5 \pm 1.41x10^5$
1.5 %	$5.12x10^3 \pm 0.95x10^3$	$2.46x10^4 \pm 0.53x10^4$	$3.89x10^5 \pm 0.77x10^5$	$2.37x\ 10^4 \pm 0.55x10^4$	$7.65x10^4 \pm 2.12x10^4$	$1.24x10^5 \pm 0.38x10^5$
Lemon grass oil 0.5%	$5.12x10^3 \pm 0.95x10^3$	$6.48x10^4 \pm 1.31x10^4$	$6.92x10^5 \pm 1.49x10^5$	$2.37x\ 10^4 \pm 0.55x10^4$	$1.19x10^5 \pm 0.27x10^5$	$7.82x10^5 \pm 1.53x10^5$
1%	$5.12x10^3 \pm 0.95x10^3$	$9.22x10^3 \pm 2.71x10^3$	$9.85x10^4 \pm 2.63x10^4$	$2.37x\ 10^4 \pm 0.55x10^4$	$6.58x10^4 \pm 1.75x10^4$	$3.45x10^5 \pm 0.71x10^5$
1.5 %	$5.12x10^3 \pm 0.95x10^3$	$8.52x10^3 \pm 2.39x10^3$	$6.01x10^4 \pm 1.26x10^4$	$2.37x\ 10^4 \pm 0.55x10^4$	$2.91x10^4 \pm 0.63x10^4$	$8.16x10^4 \pm 2.33x10^4$

* Significant differences as a result of oil treatments ($P < 0.05$)

Table (6): Incidence of enteric bacteria isolated from untreated (control) and treated samples of minced beef during cold storage at 4ºC

Group / Identified *Enterobacteriaceae*	Control		Garlic oil treated group		Thyme oil treated group		Lemon grass oil treated group	
	No.	%	No.	%	No.	%	No.	%
Citrobacter diversus	3	33.33	1	11.11	1	11.11	–	–
Citrobacter freundii	5	55.56	2	22.22	1	11.11	1	11.11
Enterobacter aerogenes	4	44.45	2	22.22	2	22.22	1	11.11
Enterobacter cloacae	1	11.11	–	–	–	–	–	–
Klebsiella pneumoniae	3	33.33	1	11.11	1	11.11	–	–
Proteus mirabilis	2	22.22	1	11.11	–	–	–	–
Proteus vulgaris	6	66.67	3	33.33	2	22.22	2	22.22
Serratia liquefaciens	2	22.22	–	–	–	–	–	–

Corresponding author
Amany, M. Salem
Food Control Department, Fac. Vet. Med., Benha University, Benha, Egypt
dr_amany40@yahoo.com*

4. References:

1. Adegoke, G. O. and Odesola, B. A. (1996): Storage of maize and cowpea and inhibition of microbial agents of biodeterioration using the powder and essential oil of lemon grass. International Biodeterioration & Biodegradation J. 81- 84.
2. Aguirrezábal, M. M.; Mateo, J.; Dominguez, M. C. and Zumalacarregui, J. M. (2000): The effect of paprika, garlic and salt on rancidity in dry sausages. J. Meat Sci. 54: 77-81.
3. Ankri, S. and Mirelman, D. (1999): Antimicrobial properties of allicin from garlic. Microbes and Infection, 1: 125- 129.
4. APHA (American Public Heath Association) (1985): Standard methods for the examination of dairy products. 15th Ed., New York, USA.
5. Billing, J. and Sherman, P. W. (1998): Antimicrobial functions of spices: Why some like it hot. The Quarterly Review of Biology, 73: 3- 49.
6. Bozin, B.; Mimica- Dukie, N.; Simin, N. and Anackov, G. (2006): Characterization of the volatile composition of essential oils of some Lamiaceae spices and the antimicrobial and antioxidant activities of the entire oils. J. Agric. Food Chem. 54: 1822 – 1828.
7. Burt, S. (2004): Essential oils: their antibacterial properties and potential applications in foods. A review. Int. J. Food Microbiol. 94: 223 – 253.
8. Chahal, K. K.; Kumari, M.; Joia, B. S. and Chhabra, B. R. (2007): Chemistry and potential of lemon grass oil as stored grain protectant. Society of pesticide Science India, New Delhi, India. Pesticide Research J. 19 (2): 141- 144.
9. Chiasson, F.; Borsa, J.; Ouattara, B. and Lacroix, M. (2004): Radiosensitization of *Escherichia coli* and *Salmonella typhi* in ground beef. J. Food Protec. 67: 1157 – 1162.
10. Cuvelier, M. E.; Richard, H. and Berset, C. (1996): Antioxidative and phenolic composition of pilot-plant and commercial extracts of sage and rosemary. American Oil Chemists Society J. 73: 645- 652.
11. Deans, S. G. and Svoboda, K. P. (1990): The antimicrobial properties of marjoram volatile oil. Flavour and Fragrance J. 5: 187- 190.
12. El-Desouky, A. L.; Bahlol, H. E. M. and Sharoba, A. M. A. (2006): Effect of some essential oils and preservatives on the growth of *E. coli* O_{157}: H_7 and quality of refrigerated minced meat. Annuals of Agric. Sci. Moshtohor, 44 (4): 1675- 1695.
13. EOS (Egyptian Organization for Standardization and Quality) (2005): Egyptian Standard, ES.
14. Fabian, D.; Sabol M.; Domaracka, K. and Bujank Ova, D. (2006): Essential oils, their antimicrobial activity against *E. coli* and effect on intestinal cell viability. Toxicology in vitro J. 20: 1453- 1445.
15. FAO (Food and Agriculture Organization of the United Nations) (1980): Manual of food quality control, 3- commodities. United Nations, Rome.
16. Fernàndez – López, J.; Zhi, N.; Aleson- Carbonell, L.; Perez- Alvarez, J. A. and Kuri, V. (2005): Antioxidant and antibacterial activities of natural extracts: Application in beef meat balls. J. Meat Sci. 69: 371- 380.
17. Fisher, K. and Phillips, C. A. (2006): The effect of lemon, orange and bergamot essential oils and their components on the survival of *Campylobacter jejuni*, *Escherichia coli* O_{157}:H_7, *Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus aureus* in vitro and in food systems. J. Appl. Microbiol. 101 (6): 1232- 1240.
18. Frankel, E. N. (1998): Foods. In lipid oxidation volume 10 in the Oily Press Lipid Library, 187- 226. Glasgow, Scotland: Bell, Bain Ltd.
19. Gomez, K. A. and Gomez, A. A. (1984): Statistical procedures for agriculture research. John Wiliy and Sons Editor Inc. USA (2Ed.), Chapter 3: 129-184.
20. Gray, J. I. and Pearson, A. M. (1987): Rancidity and warmed- over flavour. In: Pearson, A.M.; Duston, T.R., editors. Advances in meat research 3. NY. USA: Van Nostrand Company. 221- 269.
21. Gutierrez, J.; Barry- Ryan, C. and Bourke, P. (2008): The antimicrobial efficacy of plant essential oil combinations and with food ingredients. Int. J. Food Microbiol. 124 (1): 91-97.
22. Hammer, K. A.; Carson, C. F. and Riley, T. V. (1999): Antimicrobial activity of essential oils and other plant extracts. J. Appl. Microbiol. 86: 985 – 990.
23. Harris, J. C.; Cottrell, S. L.; Plummer, S. L. and Lloyd, D. (2001): Antimicrobial properties of garlic. J. Appl. Microbiol. and Biotechnol. 57: 282- 286.
24. ICMSF (International Commission on Microbiological Specifications for Foods) (1996): Microorganisms in foods. Black Academic and Professional, London.
25. ISO (1979): International Standards, ISO- 2917, Meat and meat products: Measurement of pH.
26. Jackson, R.; McNeil, B.; Taylor, C.; Holl, G.; Ruff, D. and Gwebu, E. T. (2002): Effect of aged garlic extract on casepase- 3 activity, in vitro. Nutritional Neuroscience, 5: 287- 290.
27. Kulisic, T.; Radonic, A. and Milos, M. (2005): Antioxidant properties of thyme and wild thyme essential oils Italian J. Food Sci. 17: 315- 324.
28. Leuschner, R. G. K. and Ielsch, V. (2003): Antimicrobial effects of garlic, clore and red hot chilli on *Listeria monocytogenes* in broth model systems and soft cheese. Int. J. of Food Sci. and Nut. 54: 127-133.

29. Löliger, J. (1991): The use of antioxidants in foods. In: I. O. Aruona and K. Halliwell (Eds.), Free radicals and food additives (pp. 121-131). London: Taylor and Francis.
30. Marino, M.; Bersani, C. and Comi, G. (2001): Impedance measurements to study the antimicrobial activity of essential oils from *Lamiaceae* and *Compositae.* Int. J. Food Microbiol. 67: 187-195.
31. Martin, F. R.; Frutos, M. J.; Pérez – Alvarez, J. A.; Martinez – Sánchez, F. and Tel Rio, J. A. (2002): Flavonoids as neutraceutical: structural related antioxidant properties and their role on ascorbic acid preservation. In: Atta- Ur- Rahman (Ed.), Studies in natural products chemistry. 26: 324-389. Amestrdam: Elsevier Science.
32. Mead, P. S. and Griffin, P. G. (1998): *Escherichia coli* O_{157}:H_7. The Lancet vol. 352, October 10.
33. Mielnik, M. B.; Signe, S.; Bjrg, E. and Grete, S. (2008): By-products from herbs essential oil production as ingredient in marinade for turkey thighs. LWT. 41: 93- 100.
34. Nychas, G. J.; Robinson, A. and Board, R. G. (1991): Microbiological and physico-chemical evaluation of ground beef from retail shops. Fleischwirtschaft, 71: 1057- 1059.
35. Onawunmi, G. O.; Yisak, W. A. and Ogunlan, E. O. (1984): Antibacterial constituents in the essential oil of *Cymbopogon citratus*. Ethnopharmacol J. 12 (3): 279- 286.
36. Oxoid (1986): Culture media, ingredients and other laboratory services. 7th Ed. Published by Oxoid, Limited, London.
37. Ozcan, M. M.; Sagdic, O. and Ozakan, G. (2006): Inhibitory effects of spice essential oils on the growth of *Bacillus species*. Med. Food J. 9 (3): 418-421.
38. Pearson, M. A. and Tauber, W. F. (1984): Processed meat. 2nd Ed., pp: 93. AVI Pub. Com., Inc. West port, Connection.
39. Pranoto, Y.; Salokhe, V. M. and Rakshit, S. K. (2005): Physical and antibacterial properties of alginate based edible film incorporated with garlic oil. Food Research International. 38 (3): 267- 272.
40. Raharjo, S. and Sofos, J. N. (1993): Methodology for measuring malonaldhyde as a product of lipid peroxidation in muscle tissues. J. Meat Sci. 35: 145-169.
41. Sacchetti, G.; Maietti, S.; Muzzoli. M.; Scaglianti, S.; Manfredini, Radice, M. and Brni, R. (2005): Comparative evaluation of eleven essential oils of different origin as functional antioxidants, antiradicals and antimicrobials in foods. J. Food Chem. 91: 621- 632.
42. Sallam, K. I.; Ishioroshi, M. and Samejima, K. (2004): Antioxidant and antibacterial effects of garlic in chicken sausage. Lebenson Wiss Technol. J. 37 (8) 849-855.
43. Schwarz, K.; Bertelsen, G.; Nissen, L. R.; Gardner, P. T.; Heinonen, M. I. and Hopia, A. (2001): Investigation of plant extractsfor the protection of processed foods against lipid oxidation. Comparison of antioxidant assays based on radical scavenging, lipid oxidation and analysis of the principal antioxidant compounds. European Food Research Technology 212: 319-328.
44. Seydim, A. C. and Sarikus, G. (2006): Antimicrobial activity of whey protein based edible films incorporated with oregano, rosemary and garlic essential oils. Food Res. Int. 39: 639-644.
45. Skandamis, P. N. and Nychas, G. J. E. (2001): Effect of oregano essential oil on microbiological and physico-chemical attributes of minced meat stored in air and modified atmospheres. J. Appl. Microbiol. 91: 1011- 1022.
46. Skandamis, P.; Koutsoumanis, K.; Fasseas, K. and Nychas, G. J. E. (2001): Inhibition of oregano essential oil and EDTA on *Escherichia coli* O_{157}:H_7. Italian J. Food Sci. 13 (1): 65- 75.
47. Soliman, K. M. and Badeaa, R. I. (2002): Effect of oil extracted from some medicinal plants on different mycotoxigenic fungi. Food and Chem. Toxicol. 40: 1669 – 1675.
48. Solomakos, N.; Govaris, A.; Koidis, P. and Botsoglou, N. (2008): The antimicrobial effect of thyme essential oil, nisin, and their combination against *Listeria monocytogenes* in minced beef during refrigerated storage. J. Food Microbiol. 25 (1): 120- 127.
49. Song WeicGo; Li Baoju and Lui Kai Qi (2004): Advances in research on chemical components in garlic (*Allium sativum*) and mechanism of their inhibitory reaction with pathogens in plants. Acta Harticulturae Sinica. Chinese Society for Harticultural Science, Beijing, China, 31 (2): 263 – 268.
50. Vyncke, W. (1970): Direct determination of the thiobarbituric acid value in trichloroacetic acid extracts of fish as a measure of oxidative rancidity. Fetteseifen Astrichmitted, 2: 1084- 1094.
51. Yassin -Nessrien, M. N. (2003): Effect of storage conditions on the quality parameters of differently treated fish. Ph. D. Thesis, Fac. Agric. Ain Shams, Univ. Cairo. Egypt.
52. Yassin -Nessrien, M. N. and Abou - Taleb, M. (2007): Antioxidant and antimicrobial effects of Marjoram and thyme in coated refrigerated semifried mull et fish fillets. World J. Dairy & Food Sci. 2 (1): 1-9.
53. Yin, M. C. and Cheng, W. S. (2003): Antioxidant and antimicrobial effect of four garlic-derived organosulfur compounds in ground beef. J. Meat Sci. 63: 23- 28.

10/9/2010

Recent Techniques used for Isolation and Characterization of *Staphylococcus Aureus* from Mastitic Cows.

[1]El-Seedy,F.R [2] El-Shabrawy, M; [2]Hakim, A. S; [2*]Dorgham,S.M. [2]Ata, S. Nagwa; [2]Bakry, M.A and [2]Osman,N.M.N.

[1]Bact. Mycol. and Immuno. Dept. Vet. Med. Beni-Suef University, Beni-Suef, Egypt
[2]Micro. and Immuno. Dept. National Research Centre, Cairo, Egypt
Sohad_dorgham@yahoo.com

Abstract: A total of 152 cows was examined in this study for Staphylococcus species, it was found that 44.3% of cows and were clinically mastitic whereas 14.5% were subclinically mastitic respectively. The identification of Staphylococcus species revealed that *S. aureus, S. epidermidis, S. intermedius* and *S. hyicus* for cows were (17.2%, 7.5%, 3.9% and 1.6%) respectively. *Staphylococcus aureus* isolates were confirmed after biochemical identification by API test. The study of virulence factors of total *S. aureus* isolates from mastitic cows revealed that lipase, fibrinolysin, DNase and protein A production were presented as percentage 67.3, 74.0, 85.6 and 84.6 respectively. The antibiotic sensitivity for *S. aureus* revealed that 96.2% of cow isolates were methicillin sensitive which considered the drug of choice for these isolates. The study also included the identification of *S. aureus* enterotoxins using set-RPLA and multiplex PCR. The incidence of enterotoxins C,A,B and D by set-RPLA were 36.5%, 14.4%, 10.6% and 2.9% respectively. Meanwhile the results of multiplex PCR were 7 isolates as enterotoxin C,4 isolates as enterotoxin E and one isolate for each A,B, and D respectively. The identification of MRSA of cow's isolates using PCR revealed that 3 isolates out of 5 isolates were positive.
[El-Seedy,F.R, El-Shabrawy, M; Hakim, A. S; Dorgham,S.M.m Ata, S. Nagwa; Bakry, M.A and Osman,N.M.N. Recent Techniques used for Isolation and Characterization of *Staphylococcus Aureus* from Mastitic Cows. Journal of American Science 2010;6(12):701-708]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: *Staphylococcus aureus*; mastitis; methicillin sensitive; set- RPLA, multiplex PCR.

1. Introduction:

Mastitis is the most common infectious disease affecting the dairy cows and remains the most economically important disease of dairy industries around the world. (Khan *et al.,* 1998).

Milk and its products can harbor a variety of microorganisms and can be important sources of food borne pathogens. The presence of food borne pathogens in milk is due to direct contact with contaminated sources in the dairy farm environment or with excretions from the udder of infected animals (Oliver *et al.,* 2005).

A wide variety of bacteria can be involved, but the most common mastitis pathogen is *Staphylococcus aureus*, *S. aureus* is a major pathogen of bovine mastitis worldwide. Despite implementing intensive control measures, it is difficult to eradicate the intramammary infections caused by this pathogen and it remains a substantial economic problem. (Salmon, 2002). *Staphylococcus aureus* produces a broad spectrum of surface components (proteins and capsular polysaccharide) and exotoxins, they are virulence factors involved in the pathogenesis of bovine mastitis as these toxins and products are injurious to milk producing cells of the mammary gland, impair glands and immune defense mechanisms, while they are capable to reside intracellular contributes the ability of *S. aureus* to establish a chronic infection that can persist for the life of the animal(Taverna *et al*., 2007). Enterotoxigenic *S. aureus* in raw milk posses a potential health hazard to consumers and the identification of such strains should be used as a part of analysis of milk and milk products (Zouharova and Rysanek, 2008).

Because of the organisms propensity to acquire antimicrobial resistance, whereas most infections can be treated or prophylacted with antibiotic; antimicrobial resistance of *S. aureus* especially methicillin resistant *S. aureus* (MRSA) continues to be a problem for clinicians worldwide justifies their recognition as a "New Emerging Pathogen" (Shittu and Lin, 2006).

So the present study was conducted to evaluate the recent techniques for isolation and characterization of antibiotic resistant staphylococci (*S. aureus*) from mastitic animals in correlation to its virulent factors.

2. Materials and methods

Samples

Six hundred and eight milk samples were collected from udder quarters of examined cows, 388 were collected from 97 clinically mastitic cows

which had clinical signs of abnormal secretions of mammary glands containing clots or flakes, with udders showing swelling and hardness and 220 from apparently healthy cows detected by palpation of udder and were subjected to California Mastitis Test (CMT) to detect subclinical mastitis.

Isolation of Staphylococci:

The mastitic milk samples were activated by incubation for 18-24 hours at 37°C, then milk samples were centrifuged at 3000 rpm for 20 minutes and the cream and supernatant fluids were discarded, the sediments were streaked onto the surface of the following media: Nutrient agar, Blood agar medium, Mannitol Salt agar, Baird Parker agar and Vogel Johnson agar. The inoculated plates were incubated for 24-48 hours at 37°C, after which they were examined for colony characters, cellular morphology and the purity of the culture. The suspected colonies were identified according to Collee *et al*. (1996) and Quinn *et al*. (2002).

Staphylococci latex agglutination test:

Staphylococci were tested using dry spot kit and colonies from previous media Fresh culture grown overnight 18-36 hours incubation were used. A positive result showed agglutination of the latex particles occurs within 20 seconds. This indicates the presence of *S.aureus*.

Identification of *S. aureus* isolates using API system:

The organism was sub cultured onto Columbia blood agar at 37°C for 18-24 hours. Single well-isolated colony (young culture) from blood agar inoculated into API staph medium to make homogeneous bacterial suspension with a turbidity equivalent to McFarland tube No. 0.5 and this suspension used immediately after preparation. Identification is obtained with the numerical profile on the result sheet, the tests are separated into groups of 3 and a value 1, 2 or 4 is indicated for each. By adding together the values corresponding to positive reactions within each group, a 7-digit profile number is obtained for the 20 tests of API strip.

Antimicrobial sensitivity test of *S. aureus* isolates:

Using disk diffusion method which applied according to (Finegold and Martin, 1982).

Detection of staphylococcal enterotoxins by SET-RPLA kit:

The clear culture supernatant fluids were tested serologically by reversed passive latex agglutination technique using Oxoid SET-RPLA [A Kit for detection of Staphylococcal enterotoxins A, B, C and D] (Shingaki *et al*., 1981).

Extraction of DNA from the Staphylococcal isolates according to Sritharan and Barker (1991):

Extraction of DNA from the Staphylococcal isolates by Hexadechyl trimethyl ammonium bromide (CTAB) according to Sambrook *et al.* (1989):

Multiplex polymerase chain reaction (multiplex PCR) according to Becker *et al*. (1998):

All reactions were carried out in a final volume of 50 µl in micro application tubes (PCR tubes). The reaction mixture consists of 5 µl of the extracted DNA template from the bacterial cultures, 5 µl of 10x PCR buffer, (75 M Tris Hcl PH9.0, 2mM MgCl2, 50 mM Kcl , 20 mM$(NH4)_2So_4$),1 µl dNTPS (40µM),1µl(1U Ampli Taq DNA Polymerase) and 1µl from the forward and reverse primers of (SAEA F-SAEA R), (SAEB F-SAEB R), (GSECR.1-GSECR.2), (GSEDR.1-GSEDR.2) and (GSEER.1-GSEER.2). All primers were used together and volume of the reaction mixture was completed to 50 µl using DDW. 40 µl paraffin oil wax was added and the thermal cycler was adjusted as following program: initial denaturation at 92˚C for 5 minutes followed by 35 cycles of denaturation at 92˚C for 1 minute, annealing step at 52˚C for 1 minute and extension at 72˚C for 1 minute. A final extension step was done at 72˚C for 10 minutes. The PCR products were stored in the thermal cycler at 4˚C until they were collected.

Amplification of mec A gene from DNA of *Staphylococcus aureus* isolates according to Riffon *et al.* (2001):

Each reaction was performed in a final volume of 25 µl in PCR tubes (ependorff). Each reaction contained mixture consists of 3µl of the extracted DNA template from the bacterial cultures plus 20 µl of ready to used master mix and 1 µl from the forward and reverse primer of MecAR1-MecAR2. At the surface of the tube, 40 µl paraffin oil was added to avoid evaporation of the reaction mixture and the thermal cycle was adjusted as following program: initial denaturation at 94˚C for 4 minutes followed by 30 cycles of denaturation at 94˚C for 45 seconds, annealing step at 58˚C for 1 minute and extension at 72˚C for 2 minute. A final extension step was done at 72˚C for 10 minutes. The PCR products were stored in the thermal cycler at 4˚C until they were collected.

The PCR products were electrophoresed in 1.5% agarose gel using Tris-acetate EDTA buffer. The gel containing separated DNA was stained with ethidium bromide. Standard marker containing known fragments of DNA either 100 bp or 250 bp ladders was used.

Table (1): The primers used for PCR

Primer	Sequence(5¯- 3¯)	Product size(bp)
SAEA-F	CCTTTGGAAACGGTTAAAACG	127
SAEA-R	TCTGAACCTTCCCATCAAAAC	
SAEB-F	TCGCATCAAACTGACAAACG	477
SAEB-R	GCAGGTACTCTATATAGTGCC	
GSECR-1	AGATGAAGTAGTTGATGTGTATGG	451
GSECR-2	CACACTTTTAGAATCAACCG	
GSEDR-1	CCAATAATAGGAGAAAATAAAAG	278
GSEDR-2	ATTGGTATTTTTTTTCGTTC	
GSEER-1	AGGTTTTTTCACAGGTCATCC	209
GSEER-2	CTTTTTTTCTTCGGTCAATC	
Mec AR1	GTGGAATTGGCCAATACAGG	1339
Mec AR2	TGAGTTCTGCAGTACCGGAT	

3. Results and Discussion:

From the results presented in table (2) examination of 388 quarter milk samples collected from 97 clinically mastitic cows by bacteriological examination revealed positive results in 232 (44.3%) of them while the examination of 136 quarter milk samples collected from 34 subclinically mastitic cows by CMT revealed positive results in 76 (14.5%) of them as shown in table ().These results are nearly similar to those mentioned by Bakken (1981) and Kossaibat *et al.*, (1998). El –Rashidy *et al.*, (1990) recorded that the incidence of subclinical mastitis was 26.08% and Seddek *et al.*, (1999) 7.1% to 29% among cows.

It is clear from table (3) The affection in two quarters is higher than the other quarter's affection in clinically and subclinically mastitic cows with an incidence of 34.0% and 38.2% respectively. In clinically mastitic cows three quarters affection are 27.8% followed by one quarter affection 21.6% then four quarter affection 16.5% meanwhile in subclinically mastitic cows one quarter affection is 26.5% followed by three quarter affection 20.6 % then four quarter affection 14.7 % . Concerning quarter involvement in mastitic cows, the rate of involvement in one and two quarters were relatively higher in subclinical mastitis (26.5% and 38.2%) than in clinical mastitis (21.6% and 34%) while the affection in the three and four quarters were higher in clinical mastitis (27.8% and 16.5%) than in subclinical mastitis (20.6% and 14.7%), whereas Bansal *et al.*,(1990) found that 64% of the lactating cows were infected in one quarter, 25% in two quarters, 5% in three quarters and 0% in all four quarters respectively, the variation in the quarter involvement maybe due to the differences in the defense reaction among quarters of the same animal (Dopfer *et al.*, 1999).

Table (2): Incidence of mastitis among the examined milk samples of cows.

Healthy state of the udder	Examined cows	Examined quarter	Negative quarter milk samples		Positive quartermilk samples	
			No.	%	No.	%
Clinical mastitis	97	388	156	29.8	232	44.3
Subclinical mastitis	34	136	60	11.5	76	14.5
Total	131	524	216	41.2	308	58.8

Table (3): The distribution of infected quarters in clinically and subclinical mastitic cows:

Number of affected quarters	clinically mastitic cows		subclinically mastitic cows	
	No.	**%**	**No.**	**%**
One quarter	21	21.6	9	26.5
Two quarters	33	34	13	38.2
Three quarters	27	27.8	7	20.6
Four quarters	16	16.5	5	14.7
Total	97	100	34	100

Table (4) illustrated the bacteriological examination of 232 milk samples and 76 milk samples collected from clinical and subclinical mastitis in cows respectively. It was found that only 71 and 22 were positive milk samples for staphylococcal species with an incidence of 30.6% and 28.9% respectively. In clinical mastitis the percentage of *S. aureus* isolates were (17.7%) as major pathogen followed by *S. epidermidis* (6.9%), *S. intermedius* (4.3%) and the lowest incidence was *S. hyicus* (1.7%). On other hand, in subclinical mastitis the incidence of *S. aureus* was (15.8%), *S. epidermidis* was (9.2%), *S. intermedius* was (2.6 %) and *S. hyicus* was (1.3%). These results showed that *Staphylococcus aureus* was the most microorganisms incriminated as cause of clinical and subclinical mastitis, as it represented 17.7% and 15.8% of the total bacterial isolates from examined quarter milk of cows respectively. In agreement with this result Esmat and Bader (1996) and Dego *et al.,* (2002) recorded that *S .aureus* was the most prevalent bacterial agent associated with mastitis in cows.

Table (5) illustrated the tested virulence factors of the *S. aureus* isolates in the present study, 88 out of 104 *S. aureus* isolates (84.6%) showed positive SpA by agglutination test. This observation was in agreement with that mentioned by Rosenberg *et al*, (2000) and Farage (2008). Moreover, the association of virulence genes and clinical mastitis proved the role of spa gene as risk factor (Zecconi *et al.* ,2005). Also the polymorphism of spa gene was confirmed to be scientifically associated with inflammatory response and growth rate (Zecconi *et al.* ,2006). (Kalorey *et al*., 2007).

In the present work all isolates of *S.aureus* were subjected for detection of clumping factor and capsular polysaccharide using dry spot kit (staphtect plus) (Oxoid).It is a latex slide agglutination test for differentiation of *S.aureus* than other staphylococci.
Concerning lipase activity on egg yolk agar medium, only 34 *S. aureus* strains were negative to this test with an incidence of 32.7%. On the other hand 70 strains out of 104 *S. aureus* isolates had lipase activity with percentage of 67.3. These results goes parallel to that recorded by Leung *et al*.,(1993) and Annemuller and Zschock

Table (4): Prevalence of Staphylococcus species isolated from clinically and subclinically mastitic milk samples in cows (1999).

Source of milk samples	No. of examined milk samples	Staphylococcus species								Total number of isolates	%
		S. aureus		*S. epidermidis*		*S. intermedius*		*S. hyicus*			
		No.	%	No.	%	No.	%	No.	%		
Clinical mastitis	232	41	17.7	16	6.9	10	4.3	4	1.7	71	30.6
Subclinical mastitis	76	12	15.8	7	9.2	2	2.6	1	1.3	22	28.9
Total	308	53	17.2	23	7.5	12	3.9	5	1.6	93	30.2

(100%) then subclinically mastitic cows (66.7%).

Table (5): Incidence of virulent factors in *S. aureus* isolates from cows.

Source of isolates	Clinical mastitic cows				Sub Clinical mastitic cows			
No of samples	41				12			
	positive		negative		positive		negative	
	No	%	No	%	No	%	No	%
Lipase activity	29	70.7	12	29.3	7	58.3	5	41.7
Fibrinolysine activity	33	80.5	8	19.5	9	75	3	25
DNase	41	100	-	-	8	66.7	4	33.3
SPA	37	90.2	4	9.8	10	83.3	2	16.7

In the present investigation high sensitivity was recorded to methicillin (96.2%) among the examined *S. aureus* isolates in cows followed by gentamycin (90.6%) and amoxicillin ,clavulinic acid and enrofloxacin (84.9% each) then ciprofloxacin (83.0%) and rifampicin (79.2%). Meanwhile 71.7% of the examined *S. aureus* isolates were resistant to streptomycin, 64.2% to penicillin and 54.7% to oxytetracycline. These results agreed to large extent with the finding of Pengov (1996) and Bhalerao *et al*., (2000).

Table (6): Antibacterial sensitivity test of *S. aureus* isolates from milk samples of cows with clinical and subclinical mastitis.

Antimicrobial agent	µg/disc	Sensitive		intermediate		resistant	
		No.	%	No.	%	No.	%
Ampicillin	10	26	49.0	16	30.2	11	20.8
Amoxycillin	25	32	60.4	9	16.9	12	22.6
Amoxycillin +Clavulinic acid	20+10	45	84.9	2	3.8	6	11.3
Penicillin-G	10 unit	15	28.3	4	7.5	34	64.2
Ciprofloxacin	10	44	83.0	5	9.4	4	7.5
Enrofloxacin	15	45	84.9	3	5.7	5	9.4
Gentamycin	10	48	90.6	3	5.7	2	3.8
Clindamycin	20	35	66.0	5	9.4	13	24.5
Neomycin	30	31	58.5	7	13.2	15	28.3
Streptomycin	10	11	20.8	4	7.5	38	71.7
Rifampicin	30	42	79.2	3	5.7	8	15.1
Cloxacillin	1	36	67.9	5	9.4	12	22.6
Methicilline	5	51	96.2	-	-	2	3.8
Oxytetracycline	30	14	26.4	10	18.9	29	54.7
Sulphamethoxazole-trimethoprim	23.75+ 1.25	20	37.7	12	22.6	21	39.6

The present study detected toxigenic strains in *S. aureus* isolates using commercial available kits, reverse passive latex agglutination test (RPLA). Results obtained showed high incidence of type C enterotoxin followed by type A then type B and type D. These results are in agreement with that mentioned by Jorgensen *et al.*, (2005) who found SEC was the most common enterotoxin detected in *S. aureus* isolates from bovine mastitis. In addition to that mentioned by Soriano *et al.*,(2002) and Badia (2004) who found that obtained results showed high incidence of type C (22 - 41.5%) followed by enterotoxin A, enterotoxin B and enterotoxin D whose numbers of isolates were 7 (13.2%), 5(9.4%) and 3 (5.7%) respectively. Detection of staphylococcal enterotoxins is decisive for confirmation of an outbreak and determination of the enterotoxigenicity of the strains. Since the recognition of their antigenicity, large number of serological methods for detection of enterotoxins in food and culture media has been proposed (Dacunha *et al.*, 2007). From our point of view the distribution of infection in the udder tissues may be related to the role played by toxins, this observation was in accordance to that mentioned by Hillerton and Walton (1991).

Table (7): Prevalence of toxigenic *S. aureus* isolates using RPLA test:

Source of *S. aureus*	No. of *S. aureus* isolates	Toxigenic isolates		Types of toxins							
				A		B		C		D	
		No.	%	No.	%	No.	%	No.	%	No.	%
Cows	53	37	69.8	7	13.2	5	9.4	22	41.5	3	5.7
Buffaloes	51	30	58.8	8	15.7	6	11.8	16	31.4	-	-
Total	104	67	64.4	15	14.4	11	10.6	38	36.5	3	2.9

Detection of toxigenic strains in *S. aureus* isolates using multiplex polymerase chain reaction technique (multiplex PCR). Total number of 12 isolates previously tested by using RPLA and the results were confirmed using multiplex PCR as recent technique. Results obtained showed that 100% agreement between the two tests RPLA and multiplex PCR. Our findings also agree with that of Zouharova and Rysanek(2008) who found that the results of both methods were identical concerning SEB and SED. It was concluded that detection of SEs by multiplex PCR was a useful additional tool to support identification of enterotoxigenic strains. Photo (1) showed the analysis of the results obtained by SET-RPLA method for the productivity of classical enterotoxins A-D and the results obtained by PCR for the presence of sea-sed genes revealed the correlation between each other (Lawrynowicz-Paciorek *et al.*, 2007).

To amplify the mecA gene from the extracted DNA of the previously selected *S. aureus* isolates with MR1-MR2 primers which amplify 1339

bp fragment of mecA gene were used. Results presented in photo (2) revealed that positive amplification of the 1339 bp fragment of mecA gene from the extracted DNA of 3 *S. aureus* isolates out of 5 examined samples. These 5 results of antibiogram of such five isolates were "3 strains methicillin-resistant while 2 strains were sensitive" which indicated that PCR technique could detect the mecA gene in the mecA resistant. This finding was supported by Riffon *et al*., (2001)

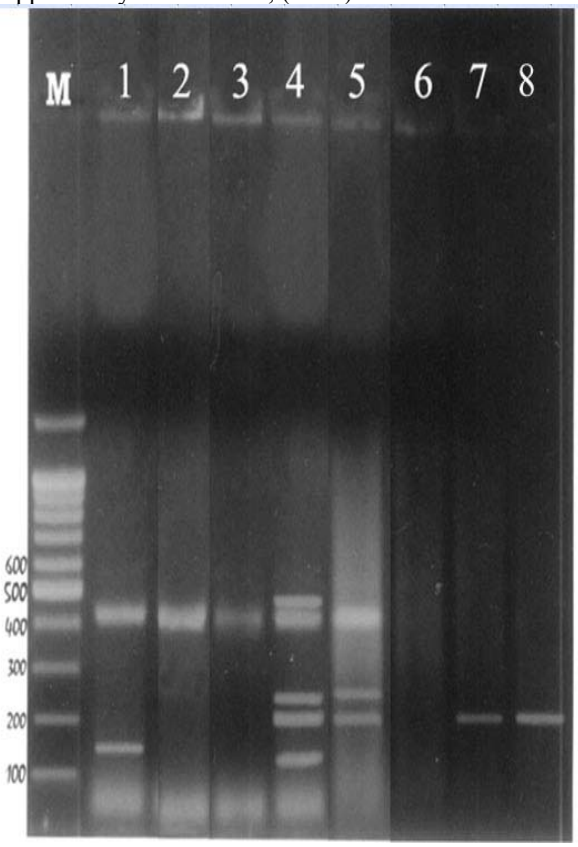


Photo (1): Agarose gel electrophoresis showing the result of multiplex PCR for detection of enterotoxin genes from *S. aureus*

M: The DNA molecular weight marker (100bp ladder)
Lane (1): positive amplification of 127 bp for enterotoxin A and 451bp for enterotoxin C in mastitic cows
Lane (2): positive amplification of 451 bp for enterotoxin C in mastitic cows
Lane (3): positive amplification of 451 bp for enterotoxin C in mastitic cows
Lane (4): positive control
Lane (5): positive amplification of 209 bp for enterotoxin E and 278 bp for enterotoxin D and 451bp for enterotoxin C in mastitic cows
Lane (6): no amplification in mastitic cows
Lane (7): positive amplification of 209 bp for enterotoxin E in mastitic cows.
Lane (8): positive amplification of 209 bp for enterotoxin E in mastitic cows.

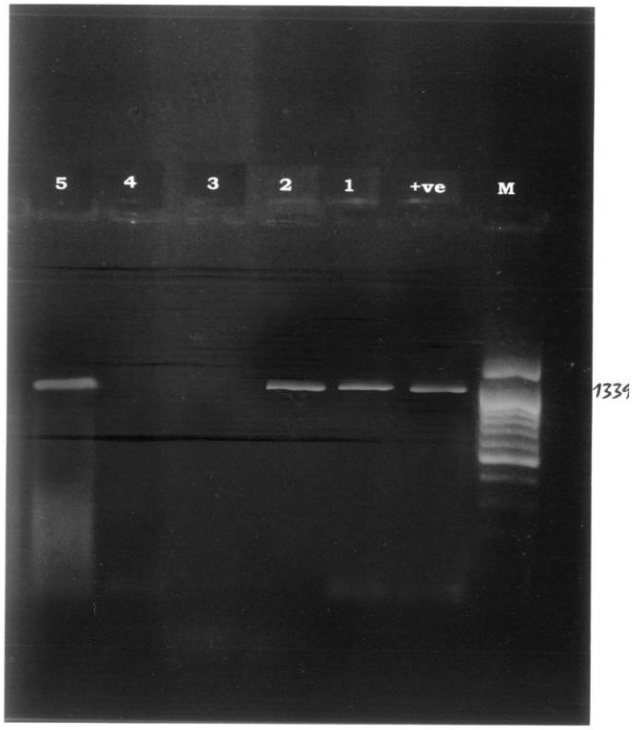


Photo (2): Agarose gel electrophoresis showing amplification of the 1339 bp fragment of mecAgene

M: The DNA molecular weight marker (100bp ladder)
+ve: positive control
Lane (1 & 2): methicillin resistant mastitic cows
Lane (3): methicillin sensitive mastitic cows.

Corresponding author
Dorgham,S.M
Micro. and Immuno. Dept. National Research Centre, Cairo, Egypt

4. References:

1. Annemuller, T. and Zchock, M. (1999): Genotyping of *S.aureus* isolated from bovine mastitis. Vet. Microbiol., 69: 217-224.
2. Badia, S.E.(2004): Studies on microbial causes of mastitis in buffaloes.
3. Thesis Ph.D. Fac. Vet. Med. Cairo University
4. Bakken, G. (1981): Subclinical mastitis in Norwegian dairy cows. Prevalence rate and epidemiological assessments. Acta Agric Scand., 31: 273-286.
5. Bansal, B.K.; Singh, K. B.; and Randhawa, S.S. (1990): Incidence of clinical mastitis in dairy animals. Indian J. Dairy Sci., 43 (3): 355-358.

6. Bhalerao ,D.; Jogadish, S.; Keskar, D. V.; Dangore, A. D. and Sharma, L. (2000): Antibiogram and treatment of bovine subclinical mastitis. Indian Vet. J., 77(3): 244-246.
7. Becker, K.; Roth, R. and Peter, G. (1998): Rapid and specific detection of toxigenic *S.aureus* using multiplex PCR. J.Clin. Microbiol., 36: 2548-2553.
8. Collee, J.G.; Fraser, A.G.; Marmion, B.P. and Simmons, A. (1996): Practical medical Microbiology. 14th edition Churchill, Livingstone, New York.
9. Dacuncha Mde,L.; Calsolari, R.A. and Junior, J.P.(2007): Detection of enterotoxins and toxic syndrome toxin-1 genes in *Staphylococcus* by PCR and RPLA assay. Microbiol. Immun., 51(4): 381-390.
10. Dego ,O. K.; Dijk,J.E.Van; Nederbragt, H. and Van-Dijk-J.E.(2002): Factors involved in the early pathogenesis of bovine *Staphylococcus aureus* mastitis with emphasis on bacterial adhesion and invasion.///
11. Dopfer, D.; Barkema,H. W.; Lam,T. J. and Gaastra, W. (1999): Recurrent clinical mastitis caused by *Escherichia coli* in dairy cows. J.Dairy Sci., 82(1): 80-85.
12. Elgabry, A.E.(2006): Characterization of *S. aureus* from different sources with special reference to protein A. A Thesis M. V. Sc. (Microbiology), Fac. Vet. Med. Cairo University.
13. El-Rashidy, A.A.; Garhi,M.M. and Metias,K.N.(1990): Incidence of bovine subclinical mastitis in individual cases belonging to Egyptian Farms in Giza Province. 4th Sci. Cong., Fac. Vet. Med., Assiut University, 3: 789.
14. Esmat, M. and Bader, A. (1996): Some studies on mastitis meteritis agalcti syndrome in cows. Vet. Med. J. Giza., 44(2): 303-309.
15. Farage, S. Randa(2008): Properties of enterotoxigenic *S. aureus* isolated from mastitic cattle and buffaloes. A Thesis M .V.Sc.(Microbiology), Fac. of Vet. Med. Cairo University.
16. Finegold, S.M. and Martin, W.J. (1982): Baily and Scott' Diagnostic Microbiology 6th Ed., The C.V. Mosby Company, St.Louis, Torento, London.
17. Hillerton, J.E. and Walton, A.W. (1991): Identification of subclinical mastitis with a hand-held electricaxal conductivity meter. Vet. Rec., 128: 513-515.
18. Jorgensen, H.; Mork, T.; Hogasen, H.R. and Rorvik,L.M. (2005): Enterotoxigenic *S. aureus* strains in bulk milk in Norway. J. Appl. Microbiol., 99(1): 158-166.
19. Kalorey, D.R.; Shanmugam, Y.; Kurkure,N.V.; Chousalkar,K.K. and Barbuddhe, S.B.(2007): PCR based detection of genes encoding virulence determinants in *S. aureus* from bovine clinical mastitis cases. J. Vet. Sci., 8(2):151-154.
20. Khan,M.A.; Kim, C.H.; Kakoma, I. and Baek, B.K. (1998): Detection of *Staphylococcus aureus* in milk by use of polymerase chain reaction analysis. Am.Vet.Res., (59): 807-813.
21. Kossaibat, M.A.; Hovi, m. and Esslemant, R.J.(1998): Incidence of clinical mastitis in dairy herds in England. Vet. Rec., 143(24): 649-653.
22. Lawrynowicz-Paciorek, M.; Kochman, M.; Piekarska, K.; Grochowska, A. and Windyga, B.(2007): The distribution of enterotoxin and enterotoxin-like genes in *S. aureus* strains. Int. J. Food Microbiol., 117(3): 319-323.
23. Leung, D.Y.M.; Fulton,D.R.; Kotzin,B.L. and Schlievert, P.M. (1993): Toxic shock syndrome toxin- secreting *Staphylococcus aureus* in Kawasaki syndrome. Lancet 342: 1385-1388.
24. National Committees For Clinical Laboratory Standard (2002): Performance standards for antimicrobial disk susceptibility tests, document M100-S12, Pennsylvania.
25. Oliver, S. P.; Jayarao, B.M. and Almeida, R. A.(2005): Food borne pathogens in milk and the dairy farm environment: Food Safety and Public Health Implications. Food borne path. Dis., 2(2):115-129.
26. Pengov,S.B. (1996):Use of cleaved amplified polymorphic sequences to distinguish strains of *Staphylococcus epidermis.* J.Clin. Microbiol., 34: 2680-2865.
27. Quinn, P.J.; Markey, B. k.; Carter, M. E.; Donnelly, W. J. C., Leonard, F. C. and Maguire, D. (2002): Veterinary Microbiology and Microbial Diseases 1st published Blackwell Science 1td.
28. Riffon, R.; Sayasisth,K.; Khalil,H.; Dubereuil, P.; Droletr,M. and Lagace, J.(2001): Development of a rapid and sensitive test for identification of major

pathogens in bovine mastitis by PCR. J. Clin. Microbiol. , 39:2584-2586.
29. Rosenberg, J.B.;Love,B. and Patterson,D.L.(2000): Bacterial cure and somatic cell count response of dairy cows with a positive CMT at calving to therapy with cephapirin Sodium. Veterinary therapeutics 3(4): 381-386
30. Salmon, S. A. (2002): Use of antimicrobial susceptibility data to assist in determining the best therapy for clinical mastitis. Page 36 in proc. NMC 41st Annu Mtg., Orlando, FL. National Mastitis Council Madison, WI.
31. Sambrook,J.; Fritsch, E.F. and Maniates, T. (1989): Molecular cloning. A laboratory manual, 2nd edition cold spring Harbor Laboratory Press. New York.
32. Seddek, S.R.; Abdelkader, H.A. and AbdelHafeen, M.M.(1999): Bacteriological studies of subclinical mastitis in Friesian cattle in Assiut Governorate. Assiut Vet. Med. J., 42(83): 77-88.
33. Shingaki, M.; Igarashi,H. and Sakai, S. (1981): Study on reverse passive latex agglutination test for detection of staphylococcal enterotoxins A, B, and C. Annu. Rep. Tokyo, Metrop.Res.Lab. Public Health., 32(1): 128-131.
34. Shittu, A.O. and Lin, J.(2006): Antimicrobial susceptibility patterns and characterization of clinical isolates of *S. aureus* in KwaZulu-Natal Province, South Africa. BMC Infect. Dis., 6(1): 125.
35. Soriano , J.M.; Font, G.; Rico, H.; Molta, J. C. and Manes, J. (2002): Incidence of enterotoxigenic staphylococci and their toxins in food. J. Food Prot., 65(5):857-860.
36. Sritharan, V. and Barker, R.H. (1991): A simple method for diagnosis of mycobacterium tuberculosis infections in clinical samples sing PCR. Mol. Cell Probes, 5: 385-395.
37. Taverna, F.; Negri, A.; Piccinini, R.; Zecconi, A.; Nonnis, S.; Ronchi, S. and Tedeschi,G.(2007): Characterization of cell wall associated proteins of *S. aureus* isolated from bovine mastitis. Vet. Microbiol., 119(2-4):240-247.
38. Wann, E.R. and Dassay,B.; Forier,J.M. and Foster, T.J.(1999): Genetic analysis of the cap 5 locus of *S. aureus* . FEMS Microbiol.Lett., 170:97-103.
39. Zecconi ,A.; Binda, E.; Borromeo,V. and Piccinini, R.(2005): Relationship between some *S. aureus* pathogenic factors and growth rates and somatic cell counts. J. Dairy Res., 72(2): 203-208.
40. Zecconi ,A.; Cesaris, L.; Liandris, E.; Dapra, V. and Piccinini, R. (2006): Role of several *S. aureus* virulence factors on the inflammatory response in bovine mammary gland. Microbiol. Path., 40(4): 177-183.
41. Zouharova, M. and Rysanek, D.(2008): Multiplex PCR and RPLA identification of *S. aureus* enterotoxigenic strains from Bulk Tank milk. Zoonoses Public Health.

10/15/2010

Biosynthesis and Characterization of *Aspergillus Niger* AUMC 4301 Tannase.

M. Z. El-Fouly[1]; Z. El-Awamry[2]; Azza A.M. Shahin[1]; Heba A. El-Bialy[1]; E. Naeem[2] and Ghadeer E. El-Saeed[1]*

[1] National Center for Radiation Research and Technology (NCRRT), Radiation Microbiology Department, Atomic Energy Authority, Cairo, Egypt. [2] Faculty of Science, Azhar University, Cairo, Egypt.

Abstract: A study on biosynthesis and characterization of an extracellular tannase from *Aspergillus niger* AUMC 4301 was carried out. *A. niger* AUMC 4301 was selected out of one hundred and thirty fungal isolates have the ability to grow in the presence of tannic acid. Maximum enzyme synthesis under solid state fermentation was attained in the presence of 3% tannic acid and 0.2% ammonium nitrate after five days incubation at 30°C. Effect of different carbon and nitrogen sources on tannase formation was also investigated. Crude tannase had maximum activity at pH 4.8, 60°C and 20 min as a function of reaction time. The catalytic action of biosynthesized tannase was directly proportional to the amount of enzyme in the reaction mixture. Using tannic acid as substrate, the K_m value for tannase was 2.50 mM. Gallic acid was shown to be a competitive inhibitor to tannase and the inhibition constant (K_i) was 1.35 mM. Effect of EDTA and some metal salts on enzyme activity was also studied.
[M. Z. El-Fouly; Z. El-Awamry; Azza A.M. Shahin; Heba A. El-Bialy; E. Naeem and Ghadeer E. El-Saeed. **Biosynthesis and Characterization of *Aspergillus Niger* AUMC 4301 Tannase.** Journal of American Science 2010;6(12):709-721]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: *Aspergillus niger*, tannase, tannins, gallic acid, solid state fermentation

1. Introduction:

Tannase (tannin acyl hydrolse, E.C.3.1.1.20) catalyses the hydrolysis of ester and depside bonds of hydrolysable tannins as tannic acid, methygallate, ethylgallate, n-propylgallate and isoamylgallate releasing glucose and gallic acid (Barthomeuf *et al*., 1994). Tannase can be obtained from plant, animal and microbial sources. Industrially, the most important source for tannase production is microbial way because of its stability (Bhat *et al*., 1998). Over the past decade, a few bacterial species have been reported to produce tannase (Ayed and Hamdi, 2002 and Manjit *et al*., 2009) whereas the most reports are fungal origin including *A. aculeatus*, *A. aureus*, *A. flavus*, *A. foetidus*, *A. japonicas*, *A. niger*, *A. oryzae* (Batra and Saxena, 2005 and Purohit *et al*., 2006), *Aureobasidium pullulans* (Banerjee and Pati, 2005), *Paecilomyces variotii* (Mahendran *et al*., 2006), *Penicillium chrysogenum* (Batra and Saxena, 2005), *Penicillium variable*, (Sharma *et al*., 2008) and *Rhizopus oryzae* (Purohit *et al*., 2006).

Nowadays, untraditional fruit juices (pomegranate, cranberry, raspberry, etc.) have been acclaimed for their health benefits, in particular, for its disease-fighting antioxidant potential. The presence of high tannin content in these fruits is responsible for haze results from protein–polyphenol interaction, tannase applied to remove haze and improve color, bitterness, and astringency of the juice upon storage (Rout and Banerjee, 2006). Tannase is extensively used in the production of instant tea by solubilization of tea cream and in the manufacture of coffee-flavored soft drinks (Lu *et al*., 2009). Tannase also participates in the preparation of animal feeding (Nuero and Reyes, 2002) and in leather industry (Orlita, 2004). Tannase can be potentially used for the degradation of tannins present in the effluents of tanneries, which represent serious environmental problems (Van de-Lagemaat and Pyle, 2001). As well as, tannase is used in the treatment of waste water containing polyphenolic compounds such as tannic acids and as an analytical probe for determining the structures of naturally occurring gallic acid esters (Mukherjee and Banerjee, 2006).

Other important application of tannase is the production of gallic acid and propylgallate (Kar *et al*., 2002). Propylgallate is considered as food antioxidant and used in the food industry and some dyestuffs (Sharma and Gupta, 2003). Gallic acid possesses a wide range of biological activities, such as antioxidant, antibacterial, antiviral, analgesic. It also shows cytotoxic activity against cancer cells, without harming normal cells (Beniwal and Chhokar, 2010). Gallic acid is used in the pharmaceutical industry for the synthesis of antibacterial drugs and in the food industry as substrate for the chemical synthesis of food preservatives such as pyrogallol and gallates. It is also used as an ingredient of developer in photography and printing inks.

Since, *Aspergillus niger* is an officially approved microorganism in France for enzyme

production in the food industry and is also classified as 'generally regarded as safe' (GRAS) by the US Food and Drug Administration (Barthomeuf *et al*., 1994), this study will concern on emphasis to produce tannase using local strain of *A. niger* AUMC 4301 through two strategies; optimization of cultivation conditions of *A. niger* AUMC 4301 and optimization of kinetic behavior of produced tannase by optimization of reaction mixture parameters.

2. Materials and methods

2.1. Chemicals

All chemicals used were analytical grades (Adwic, Egypt); Tannic and gallic acids were purchased from Mumbai, India.

2.2. Microorganisms

One hundred and thirty fungal isolates were screened for their ability to grow in the presence of tannic acid in a preliminary experiment. Fungal isolates succeeded to grow in the presence of tannic acid were tested for tannase production individually (data not shown). The most active tannase producer was selected and identified as *Aspergillus niger* AUMC 4301 at Mycological Center, Faculty of Science, Assuit University, Egypt.

2.3. Fermentation process

Aspergillus niger was grown on Czapek-Doxs agar medium for one week at 30°C to enhance spore formation. Spores were collected under aseptic condition using Tween 80 (2.9%). The prepared spore suspension was adjusted to 10^7 spores/ml. Three milliliters of prepared spore suspension were inoculated into 250 ml Erlenmeyer flasks containing 10 g of wheat bran, 3 g tannic acid and supplemented with 10 ml of mineral medium. Mineral medium containing (g/L) K_2HPO_4 1.0, NH_4NO_3 2.0, $MgSO_4.7H_2O$ 2.0 and $CaCl_2.2H_2O$ 0.002 was adjusted at pH 5.7. The inoculated flasks containing media were incubated at 30°C for 5 days.

2.4. Crude extracellular tannase extraction

Tannase was extracted from the fermented medium of the cultures cultivated under SSF conditions by adding 80 of 20 mM acetate buffer (pH 5.0). Flasks were shaken for 1 hr at 200 rpm to extract crude enyme. The buffer containing enzyme was filtered twice through cloth filter and Whatman filter paper. Then, the filtrate was used as crude extracellular tannase.

2.5. Tannase assay

Tannase activity was determined spectrophotometrically using tannic acid as a substrate according to the protocol of Mondal *et al.* (2001a) but with some modifications. The crude extracellular tannase (1ml) was incubated with 1ml of 4µmole standard tannic acid (substrate); in 0.2M acetate buffer (pH 5.4). After 20 min incubation in a water bath at 60°C, the reaction was terminated by icing. Tannase activity was determined spectro-photometrically at 530 nm using 1ml of ferric chloride reagent (0.13M). The enzyme activity was calculated from the difference in absorbance at zero time (beginning of the reaction) and after enzymatic reaction. The unknown amounts of tannic acid were determined from a standard curve prepared by the same procedure (Data not shown). One unit of tannase activity was defined as the amount of tannic acid hydrolyzed by 1ml of enzyme per minute of reaction. The protein concentration of crude extracellular tannase was determined by Lowry *et al*., (1951) after dialyzed against distilled water for 24 hr using dialysis bag (Medicell International Ltd.) to avoid interfered reactions with phenolic compounds. The specific activity of tannase was calculated according to amount of consumed tannic acid and protein content. Specific activity = Activity (U)/Protein concentration (mg).

2.6. Gallic acid assay

Gallic aid was determined spectrophotometrically according to the method of (Pinto *et al*., 2006).

2.7. Factors affecting tannase activity of A. niger AUMC 4301

Tannase was maximized through two stages of optimization; physiological optimization of tannase producer (*A. niger* AUMC 4301**)** and kinetic optimization of tannase itself. All experiments were studied separately by one way experimental design system (ANOVA) and the results were analyzed by Duncan test to detect significant differences among the treatments with a probability of 5% using SAS software package version 6.12.

To achieve physiological optimization; the effect of different incubation periods (2, 3. 4. 5, 6, 7) days, different incubation temperature degrees (5, 20, 30, 40) C° and various tannic acid concentrations (1.5, 3, 6, 9, 12) g/l were investigated on tannase activity of *A. niger*. As well as, the tannase activity of *A. niger* AUMC 4301 was determined at different fermentation states (solid state fermentation and submerged fermentation either under agitated or static conditions). For submerged fermentation, the wheat bran was removed from constituents of cultivation medium. In another experiment, tannic acid was removed from constituents of medium in individual experiment and replaced by different carbon sources (fructose, glucose, glycerol, mannitol,

starch, sucrose and xylose). In addition, ten different nitrogen sources were chosen to determine their efficiency to support tannase activity. They were L-alanine, L-cystein, L-lysine, L-ornithine and L-serine, as organic nitrogen sources and ammonium chloride, ammonium sulfate, potassium nitrate and sodium nitrate as inorganic nitrogen sources as well as ammonium nitrate was served as control. Ammonium nitrate concentration in cultivation medium was increased from 0 to 5 gl^{-1} in order to determine the best nitrogen source concentration that achieved the maximum tannase activity.

To optimize kinetic parameters of tannase itself; the reaction was carried out as a function of time (5-60) min, the reaction mixture was incubated at different temperatures (30, 40, 50, 60, 70, 80)°C and the concentration of tannic acid in the reaction mixture was changed from one to ten micromole. The optimum pH that achieved that maximum tannase activity was estimated using sodium phosphate buffer with different pH values (3.6, 4.2, 4.8, 5.4, 5.6). The ratio between substrate (Tannic acid) and enzyme (Tannase) in the reaction mixture was studied and K_m value of substrate was determined. Thermal stability of crude extracellular tannase at 60 and 70 °C and the effect of gallic acid, inhibitors as EDTA and metal ions such as calcium, cobalt, cupper, iron, magnesium, manganese and zinc addition into reaction mixture were also investigated.

3. 3. Results and Discussion

In general, the degrading enzymes of aromatic compounds are of considerable interest for bioremediation and biodegradation of organic waste products. Tannase hydrolyses ester bond of tannins to produce glucose and gallic acid. It is also utilized in a number of industrial applications including the manufacture of instant tea, beverages and gallic acid.

3.1. Optimization of cultivation conditions

3.1.1. Rate of gallic acid formation from tannic acid by tannase of A. niger AUMC 4301

The present experiment aims to demonstrate the rate of gallic acid formation from tannic acid by tannase of *A. niger* AUMC 4301. Figure (1) illustrated that tannic acid was hydrolyzed by the action of tannase yielding gallic acid and the increase in the amount of formed gallic acid was associated with a decrease in the amount of added tannic acid i.e the amount of consumed tannic acid after 30 minutes was almost equivalent to that of the formed gallic acid on equimolar basis.

3.1.2. Production of tannase by *A. niger* AUMC 4301 using different fermentation states.

Three states of fermentation; solid state, submerged states either shaking or static state were designed to compare their intensifying effect on tannase productivity by *A. niger*. Results recorded in table (1) indicated that tannase activity and accordingly specific activity of *A. niger* AUMC 4301 incubated under solid state fermentation (3.37±0.172 U/mg protein) was higher than those obtained with cultures grown under static (0.014±0.002 U/mg protein) and shaking condition (0.04±0.009 U/mg protein). In this concern, Lekha and Lonsane (1994) reported that titers of extracellular tannase produced by *A. niger* PKL 104 in solid state fermentation (SSF) were 2.5 and 4.8 times higher and required only about half of the fermentation time in comparison with those in the same medium in submerged and liquid surface fermentation, respectively.

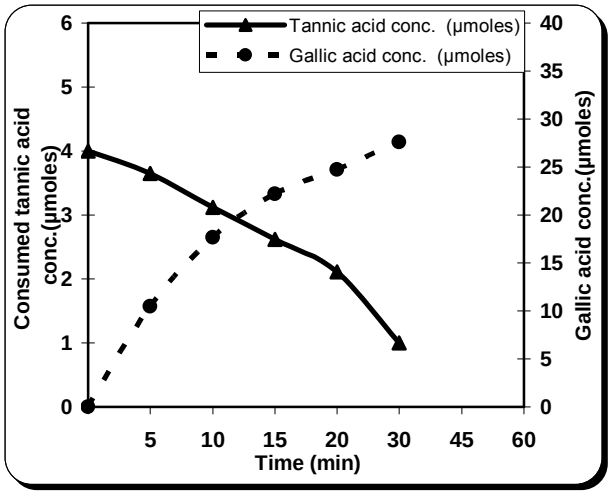


Fig. (1): Rate of gallic acid formation from tannic acid by tannase of *A. niger* AUMC 4301

Other studies were conducted to evaluate tannase production in submerged and solid state cultures (Belmares *et al*., 2004 and Marco *et al*., 2009). These studies proved that tannase yield by *Aspergillus* under SSF conditions was higher than that produced under submerged conditions. On the other hand, Srivastava and Kar (2009) stated that extracellular tannase of *A. niger* ITCC 6514.07 was produced optimally under submerged fermentation conditions. The favorability of tannase production using solid state fermentation in the present study could be explained by the positive effect of SSF on the production process itself and/or on the enzyme producer (*A. niger* AUMC 4301). The presence of support material as rice straw in SSF could be

considerable as additional source of carbon and energy (Sabu *et al*., 2005).

Table (1): Production of tannase by *A. niger* AUMC 4301 using different fermentation states.

Fermentation state	Specific activity (U/mg protein)
Solid state	3.37±0.172[B*]
shaking state	0.038±0.009[B]
Static state	0.014±0.002[A]

*Different symbols means there is a significant difference

3.1.3. Effect of different incubation periods on the production of *A. niger* AUMC 4301

Tannase activity of the fungus under investigation was determined at different incubation periods ranging from 2-7 days. Figure (2) showed that the ability of *A. niger* AUMC 4301 to produce tannase didn't start before 2 days. The lag phase was followed by the exponential phase during third and fourth days where the specific activity of tannase was increased gradually (1.76±0.038 and 2.98±0.082 U/mg protein) and maximized at the fifth day of incubation (3.30±0.11 U/mg protein). Many authors mentioned different incubation periods ranged from (72hr to 120hr) that achieved maximum tannase, this variation depends on the specificity of producers and state of fermentation **(Sabu *et al.*, 2005; Banerjee and Pati, 2007; Rodrigues *et al.*, 2008** and **Enemuor and Odibo, 2009)**. After that, the specific activity of the produced tannase was significantly decreased (R^2=0.994). The decrease in tannase yield with prolonged incubation could be explained by shift in the reaction equilibrium due to accumulation of end product (gallic acid) in the fermentation media **(Kar *et al.*, 1999)** or accumulation of toxic metabolites in the fermentation medium due to fungal metabolism and tannic acid degradation that leads to fungal cell autolysis or enzyme denaturation **(Gautam *et al.*, 2002)**.

3.1.4. Influence of incubation temperatures on the production of *A. niger* AUMC 4301 tannase.

Biological processes generally only occur within a relatively narrow range of temperature (Sabu *et al.*, 2005). The present experiment aims to determine the optimal temperature for tannase formation by *A. niger* AUMC 4301 when grown at various degrees of temperatures ranging from 5 - 40°C. Figure (3) show the optimal temperature for enzyme production was 30°C and a slight decrease in enzyme specific activity was observed at 20 and 40°C where it recorded 2.69±0.062 and 2.34±0.09 U/mg protein in the same order. So, tannase production by local strain of *A. niger* AUMC 4301 can be evaluated at room temperature in our nation without additional efforts or costs. Similar temperature optima was reported for tannases of *A. niger* ATCC 16620 (Sabu *et al*., 2005) and *A. oryzae* (Rodrigues *et al*., 2008) while a somewhat different temperature optimum was recorded for tannase production by *A. fumigates* MA (25°C) (Manjit *et al*., 2008) and for *Trichoderma. viride* tannase (45°C) (Lokeswari *et al*., 2010).

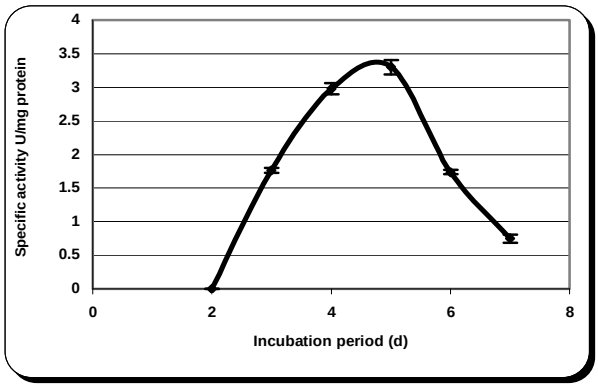


Fig. (2): Effect of different incubation periods on the production of *A. niger* AUMC 4301

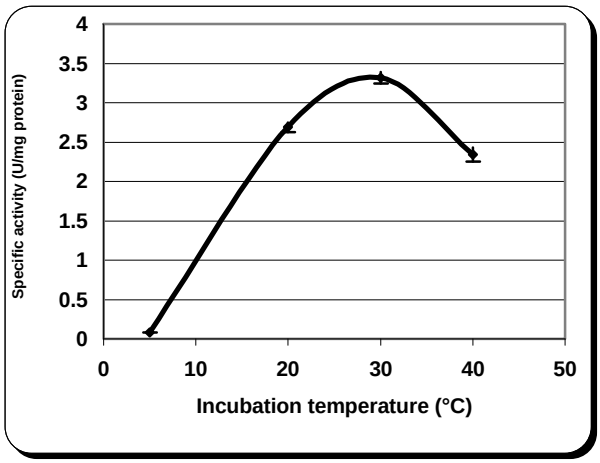


Fig. (3): Influence of different incubation temperatures on the production of *A. niger* AUMC 4301 tannase.

4.1.5. Effect of different carbon sources on the production of *A. niger* AUMC 4301 tannase.

All heterotrophic microorganisms need suitable carbon source to grow and positively react with surrounding environment to produce valuable compounds such as enzymes. Since tannase is an

inducible enzyme, in the following experiment, different carbon sources instead of tannic acid were tested for tannase enhancement by the selected fungal strain *A. niger* AUMC 4301. The tested carbon sources were added to the fermentation medium at the same concentration of tannic acid. Table (2) recorded that none of the tested carbon sources stimulated enzyme formation as compared with tannic acid-grown cultures (control). Glucose caused about 50% repression in enzyme production (Specific activity was 1.68±0.013 U/mg protein), whereas mannitol completely suppressed it. Fructose, glycerol, starch, sucrose and xylose resulted in great decrease in the enzyme activity in comparison with control; they supported specific activity about 0.5 U/mg protein. Comparable studies dealing with the induction of microbial tannase synthesis by tannic acid were recorded by many workers (Banerjee *et al*., 2001, Banerjee *et al*., 2007 and Paranthaman *et al*., 2009). It is worthy to mention that tannase of *A. japonicus* (Bradoo *et al*., 1996) was produced constitutively on simple and complex sugar substrates but activity was doubled in the presence of tannic acid as the sole carbon source. The suppression effect of readily metabolized sugars was also reported by (Lekha and Lonsane, 1997, Mondal *et al*., 2001b and Manjit *et al*. (2009). On the other hand, Van de Lagemaat and Pyle (2005) reported that if carbon source present in the media will be exhausted easily and rapidly, this may lead to the partial induction of tannase. Sabu *et al*. (2005) stated that glucose and other readily metabolized carbon source reduce the lag period required for tannase synthesis and production.

Table (2): Effect of different carbon sources on the production of *A. niger* AUMC 4301 tannase.

Carbon sources	Specific activity (U/mg protein)
Tannic acid(control)	3.35±0.081^{A*}
Fructose	0.56±0.022^{C}
Glucose	1.68±0.134^{B}
Glycerol	0.43±0.058^{CD}
Mannitol	0.03±0.044^{E}
Starch	0.32±0.022^{D}
Sucrose	0.38±0.020^{CD}
Xylose	0.52±0.032^{CD}

*Different symbols means there is a significant difference.

3.1.6. Synthesis of tannase as a function of tannic acid concentration in the culture medium of *A. niger* AUMC 4301.

From the preceding experiment, it is evident that tannic acid was the most suitable carbon source for tannase induction by the experimental fungus. It was then necessary to test the effect of tannic acid concentration (Ranging from 1.5 to 12 %) in the medium on the enzyme yield. The data illustrated in figure (4) show the optimum concentration of tannic acid for *A. niger* AUMC 4301 tannase was 3%, thereafter, the enzyme activity decreased by elevating tannic acid concentration in the medium and almost disappeared at 12%. Banerjee and Pati (2007) noticed the decrease of *A. pullulans* DBS66 tannase at higher tannic acid concentration, they explained this finding on the basis that tannic acid in higher concentration makes an irreversible reaction with surface proteins of the organism, thereby both growth and enzyme production may be reduced. Similarly, Seth and Chand, (2000) found that an over increase in tannic acid concentration induced decrease in tannase activity by deposition of gallic acid on the cell surface.

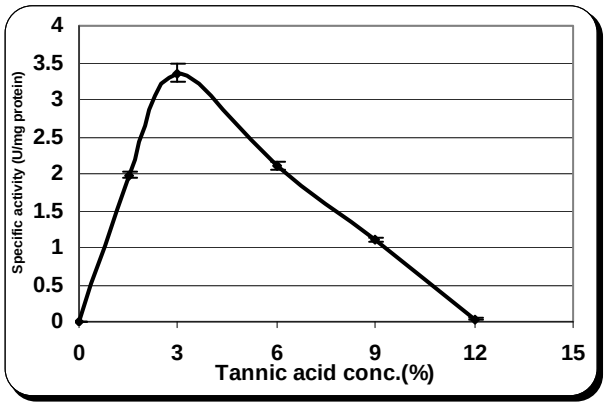


Fig. (4): Effect of different tannic acid concentrations on the production of *A. niger* AUMC 4301 tannase.

3.1.7. Effect of different nitrogen sources on tannase production by *A. niger* AUMC 4301.

Tannase or any other enzyme production depends mainly on the availability of both carbon and nitrogen sources in the medium. Both have regulatory effects on enzyme synthesis (Patel *et al.*, 2005). The nitrogen source (NH_4NO_3 2gm/l) presented in the basal medium was replaced by equivalent amounts of nine different nitrogen sources in dry weight basis. It is obvious from the results cited in table (3) that none of the tested nitrogen sources enhanced the synthesis of *A. niger* AUMC 4301 tannase over that of ammonium nitrate–grown cultures (control). Tannase activity reduced significantly by nearly 90% when L-lysine, L-ornithine or L-serine were used as a nitrogen source

in the culture media (R^2=0.975), whereas ammonium sulfate suppressed it completely. These results suggested the production of tannase enzyme is highly affected by the nature of used nitrogen source. The inhibition of tannase by ammonium sulfate could be due to the toxicity of sulfate ion itself on fungal growth. In addition, organic nitrogen sources can be react with tannic acid forming a complex precipitate, this complex structure inhibit fungal consumption of both carbon and nitrogen source; so it affect greatly on the fungal growth (Kumar *et al*., 2007).

Table (3): Effect of different nitrogen sources on the production of *A. niger* AUMC 4301 tannase.

Nitrogen sources	Specific activity (U/mg protein)
Ammonium nitrate (control)	3.25±0.102^{A*}
L-Alanine	1.22±0.031^{B}
Ammonium chloride	1.25±0.078^{B}
Ammonium sulfate	0.02±0.009^{F}
L-Cysteine	0.70±0.102^{D}
L-Lysine	0.40±0.042^{E}
L-Ornithine	0.34±0.038^{E}
Potassium nitrate	0.99±0.061^{C}
L-Serine	0.28±0.015^{E}
Sodium nitrate	0.83±0.079^{CD}

*Different symbols means there is a significant difference.

3.1.8. Dependence of tannase formation by *A. niger* AUMC 4301 on ammonium nitrate concentration.

As shown in table (3) ammonium nitrate was the most favorable nitrogen source for production of *A. niger* AUMC 4301 tannase. So, it is interesting to study the effect of ammonium nitrate concentration on tannase activity. Data presented in figure (5) demonstrated that the specific activity of *A. niger* AUMC 4301 tannase was increased with increasing ammonium nitrate concentration up to 0.2% in culture medium. After that, tannase activity decreased rapidly with increasing ammonium nitrate concentration, the specific activity of tannase recorded 1.41±0.038, 1.14±0.033 and 0.56±0.035 U/mg protein at ammonium nitrate concentrations 3, 4, and 5 g/l respectively. In spite of ammonium nitrate is readily utilizable nitrogen that stimulates the synthesis of proteins (Djekrif-Dakhmouche *et al*., 2006), high concentration of ammonium nitrate caused tannase denaturation by changing the protein tertiary structure.

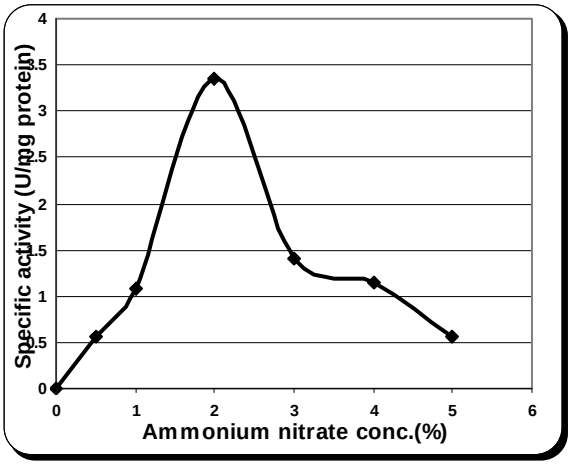


Fig. (5): Effect of different ammonium nitrate concentrations on the production of *A. niger* AUMC 4301 tannase.

3.2. Some kinetics and properties of A. niger AUMC 4301.

Any enzyme is a protein in nature that functions as a catalyst. The role of enzymes is to speed up the rate of chemical reactions without undergoing any permanent changes itself. In our case, extracellular tannase of selected *A. niger* AUMC 4301 strain was mixed with tannic acid (the substrate) and the conditions that maximize tannase activity and consequently tannic acid degradation in the reaction mixture were tested individually. They were reaction time, reaction pH, reaction temperature, enzyme concentration, substrate concentration and addition of metal ions, inhibitors and gallic acid.

3.2.1. Effect of reaction time on the activity of tannase from *A. niger* AUMC 4301.

An experiment was conducted to determine tannase activity as a function of reaction time. The reaction mixture was incubated at 60°C, and samples were withdrawn periodically for a period of 60 min and assayed for tannase activity. Results obtained are graphically presented in figure (6). The reaction was found to be more or less linear with time up to 30 min. Further increase in the reaction time resulted in a sharp decrease in enzyme activity; the percentage of decrease was nearly 70% after 60 min. So, it could be concluded that the rate of the reaction catalyzed by *A. niger* AUMC 4301 tannase couldn't proceed to completion. This may be attributed to the accumulation of gallic acid, one of the products of the reaction, which retard the reaction rate. Similarly,

Sabu *et al.* (2005) reported that the tannase activity by *Aspergillus niger* ATCC16620 (3.9 U/ml) was maximized and stabilized at 15 and 20 min of reaction time and it decreased with further increase in reaction time. **Mukherjee and Banerjee (2006)** reported that an increase up to 5 min was observed in tannase activity of a co-culture of *R. oryzae* and *A. foetidus* followed by a decrease thereafter and the curve started leveling off. The difference in reported reaction time could be attributed to microorganisms employed.

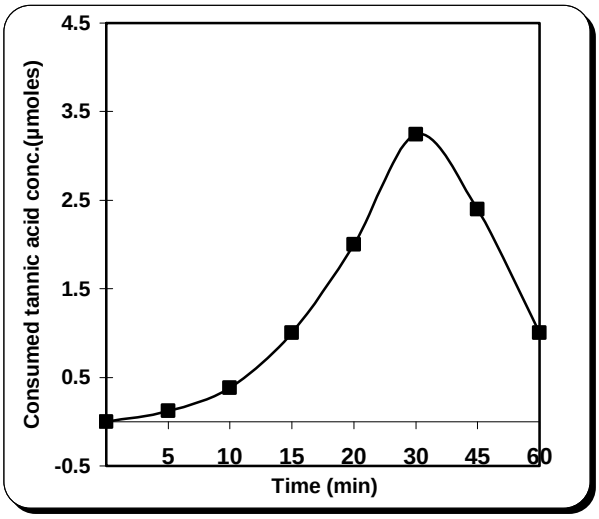


Fig. (6): Effect of reaction time on the activity of *A. niger* AUMC 4301 tannase.

3.2.2. Dependence of tannase activity of *A. niger* AUMC 4301on pH.

Different pH values (3.6, 4.2, 4.8, 5.4 and 5.6) were chosen to investigate the influence of pH on the catalytic activity of tannase. Figure (7) demonstrated the relationship, between pH and activity of *A. niger* AUMC 4301 tannase. Maximal enzyme activity was obtained at pH 4.8 where the amount of consumed tannic acid reached 2.93 µmol. By increasing the pH value above 4.8, a gradual decrease in enzyme activity was recorded. This pH optimum is more or less similar to that reported for the enzyme from *A. aculeatus* DBF6 (Banerjee *et al*., 2001), *A. awamori nakazawa* (Mahapatra *et al*., 2005), *A. foetidus* and *R. oryzae* (Mukherjee and Banerjee, 2006), and *P. variable* (Sharma *et al*., 2008). The pH value for optimal tannase activity of many strains of *A. niger* previously studied was about 6 (Anwar *et al*., 2009; Marco *et al*., 2009 and Srivastava and Kar, 2009). Tannase was active at acidic pH and activity decreased as the pH approached the alkaline range. Any change in pH affects the protein structure and a decline in enzyme activity beyond the optimum pH could be due to enzyme inactivation or its instability (Mahapatra *et al*., 2005).

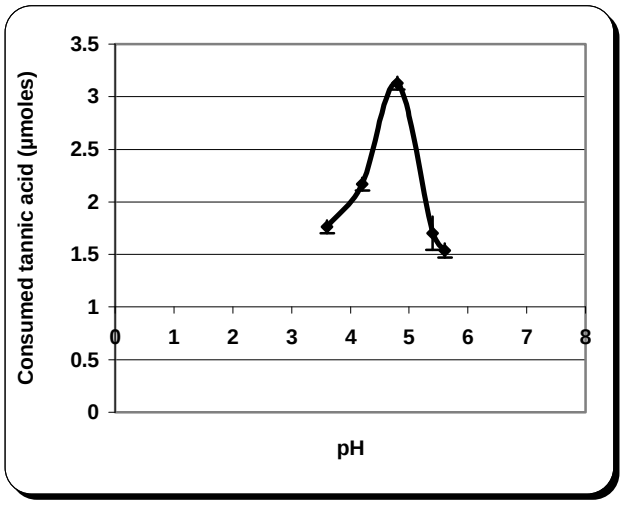


Fig. (7): Effect of reaction pH on the activity of *A. niger* AUMC 4301tannase.

3.2.3. Effect of temperature on tannase activity of *A. niger* AUMC 4301.

An experiment was conducted to find out the degree of temperature at which optimum activity of *A. niger* AUMC 4301 tannase could be achieved. A series of identical reaction mixtures were made and each was incubated at a different temperature. The range of temperatures used was from 30°C to 80°C. Figure (8) showed that maximal enzyme activity was achieved at 60°C (Consumed tannic acid was 3.3 µmol). The *A. niger* AUMC 4301 tannase activity at 70°C was about 50 % of that obtained at 60°C. Presumably the enzyme was denaturated at 70°C, an indication of its thermolability. The amount of consumed tannic acid declined to 1.68±0.13 and 0.98±0.074 at 70°C and 80°C respectively. Our data are in accordance with those reported for the tannase activity of *A. niger* van Teighem (Sharma *et al*., 1999) and *A. niger* GHI (Marco *et al*., 2009) in having an optimal temperature at 60°C whereas the activity of *A. niger* Aa-20 tannase was found to be maximal at 60 to 70°C (Ramirez-Coronel *et al*., 2003). These results indicated that elevating temperature to certain limit has positive effect on tannase activity; this could be attributed to increase in the kinetic energy of the substrate and enzyme molecules or/and increase the reaction rate with elevating temperatures. Beyond the optimum level of temperature, the internal energy of the molecules including translational, vibrational and rotational energy of the molecules increased, some of the weak bonds determining the three-dimensional shape of the active proteins break leading to thermal denaturation

of the tannase protein causing its inactivation. Temperatures above the optimum value also affect the protein ionization state, and the solubility of species in solution, which thus resulted in a reduction in enzyme activity **(Mukherjee and Banerjee, 2006)**.

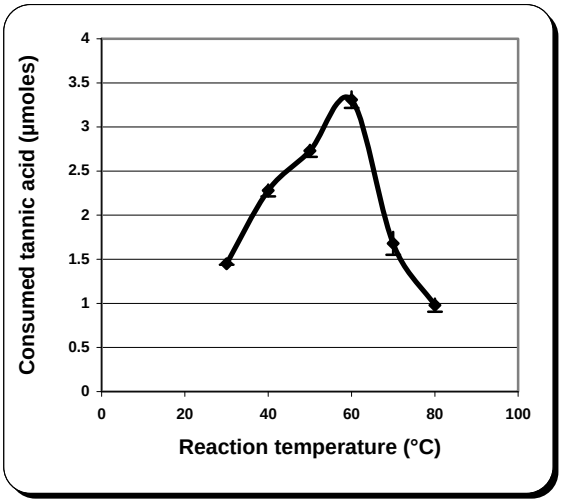


Fig. (8): Effect of reaction temperature on the activity of *A. niger* AUMC 4301tannase.

3.2.4. Heat inactivation kinetics of tannase from *A. niger* AUMC 4301.

Another experiment was designed to test the stability of tannase activity of *A. niger* AUMC 4301 in acetate buffer (pH 4.8) during incubation the enzyme (in absence of tannic acid) at either 60°C or 70°C for different time intervals. It is evident from the results represented in figure (9), a total loss of tannase activity occurred when the enzyme was incubated at 70°C for 5 min. However, exposing the enzyme to 60°C for 5 min and 20 min resulted in about 20% and 60% loss of its activity, respectively. These results indicate that *A. niger* AUMC 4301 tannase is thermolabile. Although the optimum temperature for the tannase activity was 60°C, yet it was inactivated when incubated at the same temperature, in absence of the substrate. This indicates that presence of the substrate in the reaction mixture protects the catalytic site from heat inactivation. Similarly, tannases of *A. niger* LCF8 (Barthomeuf *et al*., 1994) and *A. niger* GHI (Marco *et al*., 2009), were found to be thermolabile. Alternatively, Battestin and Macedo (2007) stated that the crude tannase of *P. variotii* was thermo-stable where it retained 96% and 99% residual activity at 20 and 70°C respectively.

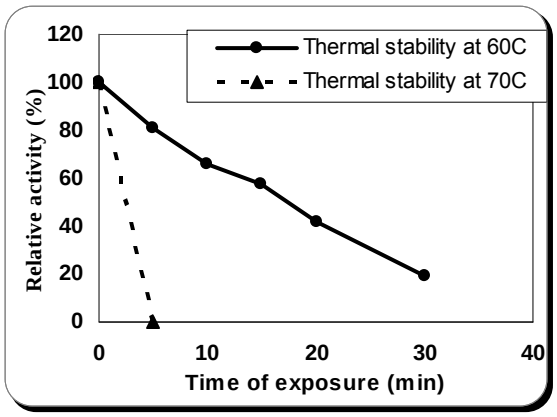


Fig. (9): Heat inactivation kinetics of *A. niger* AUMC 4301tannase.

3.2. 5. Tannase activity as a function of enzyme concentration

An experiment was designed to prove that the rate of catalytic activity of *A. niger* AUMC 4301 tannase is dependent on the amount of enzyme in the reaction mixture. Several reaction mixtures were set up which contained the same amount of tannic acid in acetate buffer at pH 4.8, but had varying amounts of the enzyme. Figure (10) revealed the relationship between tannase activity and protein concentration, the results indicate that the extent of catalytic action is directly proportional to the concentration of the enzyme (R= 0.9961). This could be explained by increasing enzyme concentration to certain limit, increase the availability of more active sites free on the enzyme and increase the incidence of substrate molecules to react with them.

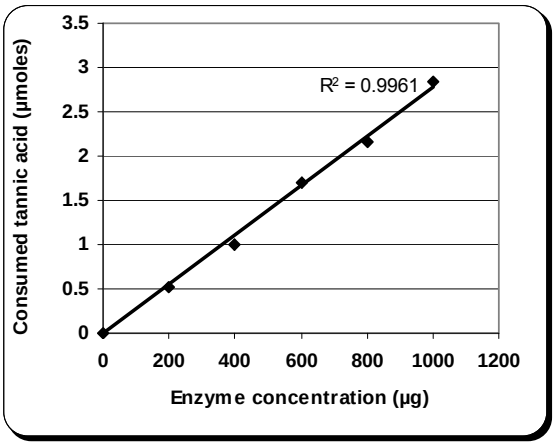


Fig. (10): Tannase activity of *A. niger* AUMC 4301as a function of enzyme concentration.

3.2.6. Determination of the apparent K_m value of *A. niger* AUMC 4301 tannase for tannic acid.

Determination of the apparent K_m (Michaelis constant) value of *A. niger* AUMC 4301 tannase was achieved through a study relating substrate concentration to the velocity of the reaction. Different concentrations of tannic acid were incubated with the same amounts of enzyme protein in acetate buffer (pH 4.8) at 60°C for 20 min. Figure (11) illustrates the effect of tannic acid concentrations on tannase activity of *A. niger* AUMC 4301. Figure (12) represents a Lineweaver Burk plot (Lineweaver and Burk, 1934) of the reciprocal of initial velocities and tannic acid concentrations. From this plot the apparent K_m value of the enzyme was calculated and found to be 2.5 mM. It is clear also from the results seen in Fig. (11) that the enzyme activity was increased with increasing concentrations of tannic acid up to 2.5mM. Further increase in tannic acid concentrations resulted in a significant decrease in enzyme activity ($P>0.0001$). It could be suggested from such finding that gallic acid as a product of the reaction catalyzed by tannase may have an inhibitory effect on enzyme activity. However, tannase of *P. variable* exhibited a much higher K_m value (32 mM) for tannic acid (Sharma *et al*., 2008). On the contrary, lower K_m values for tannic acid were reported for tannases produced by both of *R. oryzae* and *A. foetidus* (Mukherjee and Banerjee, 2006), *A. niger* GHI (Marco *et al*., 2009) and *A. niger* HAYATI (Anwar *et al.,* 2009).

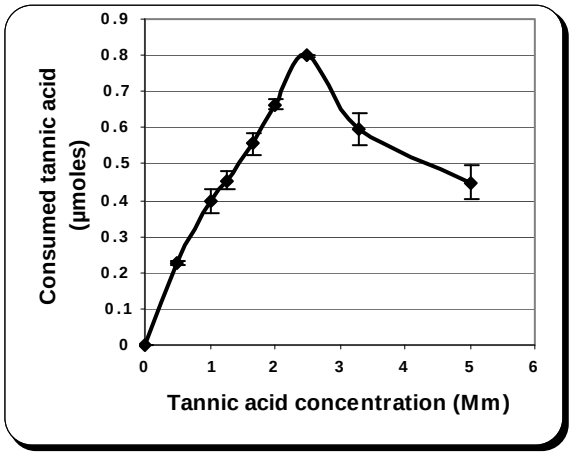
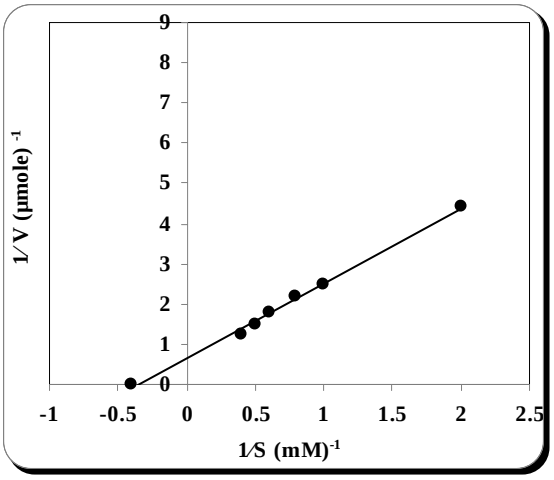


Fig. (11): Determination of the apparent K_m value of *A. niger* AUMC 4301 tannase for tannic acid.



-1/ K_m = - 0.4 mM **K_m value = 2.5 mM**

Fig. (12): Lineweaver-Burk plot of the reciprocals of initial velocities and tannic acid concentration.

3.2.7. Influence of gallic acid on the activity of *A. niger* AUMC 4301 tannase.

Results of tannase activity as function of reaction time and substrate concentration revealed that gallic acid may be an inhibitor to the activity of *A. niger* AUMC 4301 tannase. Thus, another experiment was designed to investigate the effect of gallic acid on the tannase activity. This was carried out by adding gallic acid at a concentration of 5 mM to the reaction mixtures containing increasing concentrations of tannic acid (substrate) and measuring the rates of the reaction catalyzed by the enzyme. This was compared with analogous rates obtained without gallic acid addition. Data obtained are graphically presented in figures (13&14). These results indicated that gallic acid is a competitive inhibitor to *A. niger* AUMC 4301 tannase especially at high substrate concentrations. The K_i (inhibition constant) was calculated and found to be 1.35 mM. Gallic acid itself acts as competitive inhibitor (Kar *et al*., 1999; Kar and Banerjee, 2000). Competitive inhibitors are substances; usually structurally related to substrate; that is able to combine with the enzyme at the same site as the substrate are competitive. Inhibitor and substrate therefore compete for the same site forming enzyme-substrate (ES) and enzyme-inhibitor (EI) complexes, respectively; ESI complexes are not produced. These unfavorable complexes (EI) greatly affect tannase activity. This was clearly appeared by decreasing Michaelis constant (K_i) in the presence of inhibitor compared to absence of it (K_m).

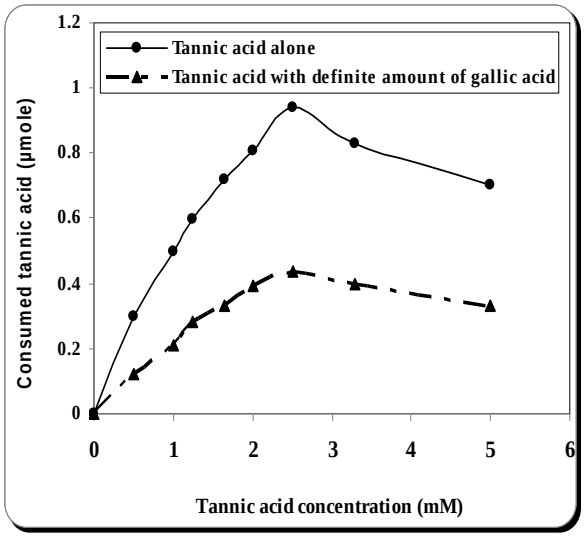
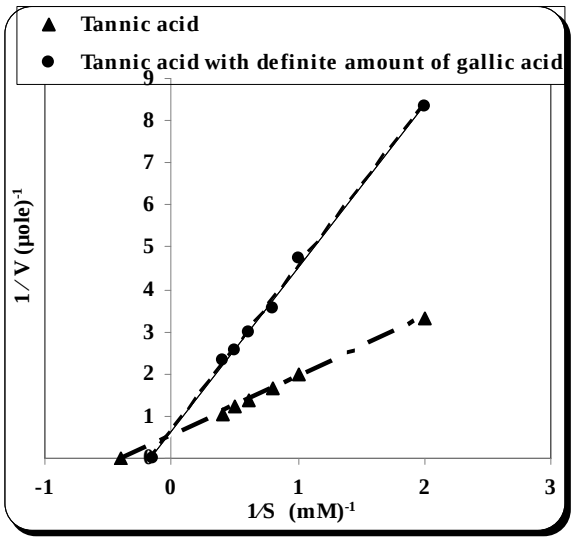


Fig. (13): Influence of gallic acid on the activity of *A. niger* AUMC 4301 tannase.



K_m = 2.5 mM. K_p = 1.35 mM .

Fig. (14): Determination of the inhibition type exerted by gallic acid on tannase activity of *A. niger* AUMC 4301.

3.2.9. Effect of EDTA and some metal salts on enzyme activity of *A. niger* AUMC 4301 tannase.

Many enzymes require metal ion activators for expressing their absolute catalytic activity. Since achieving maximum catalytic activity during enzymatic reactions is very important industrially, the effect of metal ion on tannase activity was studied in the following experiment. The results illustrated by figure (15) revealed that all the tested metal ions reduce tannase activity with different degrees. Hence, Fe^{++}, Mn^{++}, Cu^{++}, Zn^{++} and Ca^{++} showed inhibitory effect in a decreasing order on enzyme activity (Ranged from 90% to 20%), Co^{++} and Mn^{++} caused a least decrease in enzyme activity (About 6%). Since inhibition studies provide an insight into the nature of the enzyme and its cofactor requirements, in this experiment the effect of EDTA as a metal chelating agent, on the activity of *A. niger* AUMC 4301 tannase was also investigated. Addition of EDTA to the reaction mixture didn't inhibit tannase activity; this confirmed the negative effect of studied metal ions on the tannase under investigation. The effect of metal ions was studied in many previous reviews, some of them revealed the positive effect of metal ions on tannase activity while others attained the negative effect. Similar to our results, tannase obtained from *A. niger* MTCC 2425 was found to be inhibited by Ca^{+2} (Bhardwaj *et al*., 2003). Sabu *et al*. (2005) also studied effect of metal ions on tannase from *A. niger* ATCC 16620 and found that the addition of metal ions like Zn^{+2}, Mn^{+2}, Cu^{+2}, Ca^{+2}, Mg^{+2}, and Fe^{+2} inhibited the enzyme activity. At the same trend, Kasieczka-Burnecka *et al*. (2007) have reported inhibitory effect of Zn^{+2}, Cu, $^{+2}$ K^{+}, Cd^{+2}, Ag^{+} , Fe^{+3}, Mn^{+2}, Co^{+2}, Hg^{+2}, Pb^{+2}, and Sn^{+2} on tannase from *Verticillium sp*. Decrease in tannase activity in the presence of divalent cations could be due to nonspecific binding or aggregation of the enzyme.

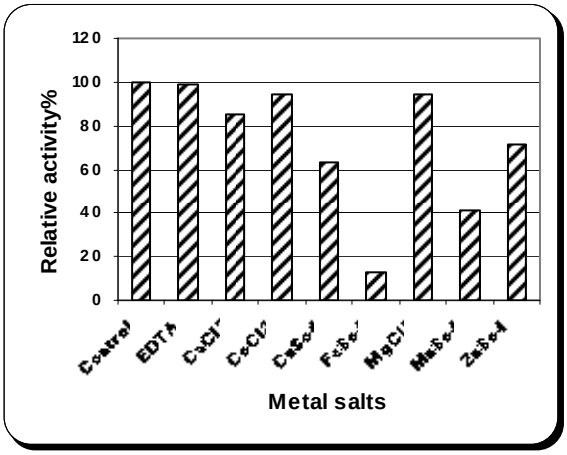


Fig. (15): Effect of some metal salts and ethylene diamine tetra acetate (EDTA) on the activity of *A. niger* AUMC 4301 tannase.

4. Conclusion

The results revealed in this study show that *A. niger* AUMC 4301 represents a valuable source of an economically attractive tannase with potential for application in various industries. The major

physicochemical and kinetic properties of the crude tannase were identified with special emphasis on the effect of substrate concentration, final product (Gallic acid) and metal ions on the enzyme activity. Other research work is in progress toward production of tannase utilizing agricultural wastes instead of tannic acid and improving the tannase productivity by *A. niger* AUMC 4301 using gamma radiation.

Corresponding author
Heba A. El-Bialy
National Center for Radiation Research and Technology (NCRRT), Radiation Microbiology Department, Atomic Energy Authority, Cairo, Egypt

5. References:

1- Anwar, Y.A.S.; Artika, I.M. and Danuri, H. (2009). Fractionation and Characterization of tannin Acyl Hydrolase from *Aspergillus niger* HAYATI Journal of Biosciences, 16 (3): 95-99.
2- Ayed, L. and Hamdi, M. (2002). Culture conditions of tannase production by *Lactobacillus plantarum.* Biotechnology Letters 24: 1763-1765.
3- Bajpai, B.; Banerjee, T. and Patil, S. (1999). Gallotannin hydrolysis by immobilized fungal mycelia in a packed bed bioreactor. Indian J. Exp. Biol. 37: 94-97.
4- Banerjee, D.; Mondal, K.C. and Pati, B.R. (2001). Production and characterization of extracellular and intracellular tannase from newly isolated *Aspergillus aculeatus* DBF 9. J. Basic Microbiol. 41: 313-318.
5- Banerjee, D.; Mondal, K.C. and Pati, B.R. (2007).Tannase production by *Aspergillus aculeatus* DBF9 through solid state fermentation. Acta. Microbiol. Immunol. Hung. 54(2):159-166.
6- Banerjee, D. and Pati, B.R. (2007). Optimization of tannase production by *Aureobasidium pullulans* DBS 66. J Microbiol Biotechnol. 17(6):1049-1053.
7- Banerjee, R.; Mukherjee, G. and Patra, K.C. (2005). Microbial transformation of tannin-rich substrate to gallic acid through co-culture method. Bioresour. Technol. 96: 949-953.
8- Barthomeuf, C.; Regerat F. and Pourrat, H. (1994). Production, purification and characterization of a tannase from *Aspergillus niger* LCF 8. J. Ferment. Bioeng. 77: 320-323.
9- Batra, A. and Saxena, R.K. (2005). Potential tannase producers from the genera *Aspergillus* and *Penicillium*. Process Biochem. 40: 1553-1557.
10- Battestin, V. and Macedo, G.A. (2007). Tannase production by *Paecilomyces variotii*. Bioresour. Technol. 98: 1832-1837.
11- Belmares, R.; Contreras-Esquivel, J.C.; Rodríguez-Herrera, R.; Ramírez-Coronel, A.; Aguilar, C.N. (2004). Microbial production of tannase: an enzyme with potential use in food industry. Lebensmit. Wiss. und-Technol. 37(8): 857-864.
12- Beniwal, V. and Chhokar, V. (2010). Statistical Optimization of Culture Conditions for Tannase Production by *Aspergillus awamori* MTCC 9299 under Submerged Fermentation. Asian J. of Biotechnol. 2(1): 46-52.
13- Bhardwaj, R.; Bhat, T.K. and Singh, B. (2003). Purification and characterization of tannin acyl hydrolase from A. niger MTCC-2425. J Basic Microbiol. 43:449–61.
14- Bhat, T.K.; Singh, B.; Sharma, O.P. (1998). Microbial degradation of tannins -A current perspective. Biodegradation, 9: 343-357.
15- Bradoo, S.; Gupta, R.; Saxena, R.K. (1996). Screening of extracellular tannase-producing fungi: development of a rapid and simple plate assay. J. Gen. Appl. Microbiol. 42: 325-329.
16- Djekrif-Dakhmouche, S.; Gheribi-Aoulmi, Z.; Meraihi, Z.; Bennamoun, L. (2006). Application of a statistical design to the optimization of culture medium for α-amylase production by *Aspergillus niger* ATCC 16,404 grown on orange waste powder. J. Food Eng. 73: 190-197.
17- Enemuor, S.C. and Odibo, F.J.C. (2009). Culture conditions for the production of a tannase of *Aspergillus tamarii* IMI388810 (B). African J. of Biotechnol. 8 (11): 2554-2557.
18- Gautam, P.; Sabu, A.; Pandey, A.; Szakacs, G.; Soccol, C.R. (2002). Microbial production of extracellular phytase using polystyrene as inert solid support. Bioresour. Technol. 83: 229-233.
19- Kar, B. and Banerjee, R. (2000). Biosynthesis of tannin acyl hydrolase from tannin-rich forest residue under different fermentation conditions. J. Ind. Microbiol. Biotech. 25: 29-38.
20- Kar, B.; Banerjee, R. and Bhattacharyya, B.C. (1999). Microbial production of gallic acid modified solid state fermentation. J. Ind. Microbiol. Biotechnol. 23: 173-177.
21- Kar, B.; Banerjee, R. and Bhattacharyya, B.C. (2002). Optimization of physicochemical parameters for gallic acid production by evolutionary operation-factorial design technique. Process Biochem. 37: 1395-1401.
22- Kasieczka-Burnecka, M..; Karina, K.; Kalinowska, H.; Knap, M. and Turkiewicz, M. (2007). Purification and characterization of two

cold-adapted extracellular tannin acyl hydrolases from an Antarctic strain Verticillium sp. P9. Appl. Microbiol. and Biotechnol. 77:77–89.
23- Kumar, R.; Sharma, J. and Singh, R. (2007). Production of tannase from *Aspergillus ruber* under solid-state fermentation using Jamun (*Syzygium cumini*) leaves. Microbiol. Res. 162(4): 384-390.
24- Lekha, P.K. and Lonsane, B.K. (1994). Comparative titres, location and properties of tannin acyl hydrolase produced by *Aspergillus niger* PKL 104 in solid state, liquid surface and submerged fermentations. Process Biochem. 29: 497-503.
25- Lekha, P.K.; Lonsane, B.K. (1997). Production and application of tannin acyl hydrolase: state of the art. Adv. Appl. Microbiol. 44: 215-260.
26- Lineweaver, H. and Burk, D. (1934). The determination of enzyme dissociation constants. J. Am. Chem. Soc. 56: 658- 666.
27- Lokeswari, N.; JayaRaju, K.; Pola, S. and Bobbarala, V. (2010). Tannin acyl hydrolase from *Trichoderma viride***.** International Journal of Chemical and Analytical Science, 1(5): 106-109.
28- Lowry, O.H.; Rosebrough, N.J.; Farr, A.L. and Randall, R.J. (1951). Protein measurement with the Folin-phenol reagent. J. Biol. Chem. 193: 265-275.
29- Lu, M-J; Chu, S-C; Yan, L. and Chen, C. (2009). Effect of tannase treatment on protein–tannin aggregation and sensory attributes of green tea infusion LWT - Food Science and Technology. 42 (1): 338-342.
30- Mahapatra, K.; Nanda, R.K.; Bag, S.S.; Banerjee, R.; Pandey, A. and Szakacs, G. (2005). Purification, characterization and some studies on secondary structure of tannase from *Aspergillus awamori nakazawa*. Process Biochem. 40: 3251-3254.
31- Mahendran, B.; Raman, N. and Kim, D.J. (2006). Purification and characterization of tannase from *Paecilomyces variotii*: hydrolysis of tannic acid using immobilized tannase. Appl Microbiol Biotechnol. 70(4): 444-450.
32- Manjit, S.K.; Anita, Y.; Aggarwal, N.K.; Kumar, K. and Kumar, A. (2008). Tannase production by *Aspergillus fumigatus* MA under solid-state fermentation. World J. Microbiol. Biotechnol. 24: 3023-3030.
33- Manjit, S.K.; Anita. Y.; Krishan, S.K.; Aggarwal, N.K.; Gupta, R. and Gautam S.K. (2009). Optimization of cultural conditions for tannase production by *Pseudomonas aeruginosa* IIIB 8914 under submerged fermentation. World Journal of Microbiology and Biotechnology, 26(4): 599-605.
34- Marco, M-G; Luis, V.R; Erika, L.R.; Jacqueline, R.; Mario, A. C-H; Raúl, R.; Juan, C. and Cristóbal, N.A. (2009). Anovel tannase from the xerophilic fungus *Aspergillus niger* GH1. J. Microbiol. Biotechnol. 1: 1-10.
35- Mondal, K.C.; Banerjee, D.; Jana, M. and Pati, B.R. (2001a). Colorimetric assay for determination of tannin acyl hydrolase (E.C. 3.1.1.20) activity. Anal Biochem 295:168-171.
36- Mondal, K.C.D.; Banerjee, D.; Banerjee, R. and Pati, B.R. (2001b). Production and characterization of tannase from *Bacillus cereus* KBR9. J. Gen. Appl. Microbiol. 47: 263-267.
37- Mukherjee, G.; Banerjee, R. (2006). Effects of temperature, pH and additives on the activity of tannase produced by a co-culture of *Rhizopus oryzae* and *Aspergillus foetidus*. World J. Microbiol. Biotechnol. 22: 207–212.
38- Nuero, O. M. and Reyes, F. (2002). Enzymes for animal feeding from *Penicillium chrysogenum* mycelial wastes from penicillin manufacture. Letters in Appl. Microbiol., 34(6): 413–416.
39- Orlita, A. (2004). Microbial biodeterioration of leather and its control: a review. International Biodeterioration & Biodegradation. 53 (3): 157-163.
40- Paranthaman, R.; Vidyalakshmi, R.; Murugesh, S. and Singaravadivel, K. (2009). Optimization of Various Culture Media for Tannase Production in Submerged Fermentation by *Aspergillus flavus***.** Advances in Biological Research 3 (1-2): 34-39.
41- Patel, R.; Dodia, M. and Singh, S.P. (2005). Extracellular alkaline protease from a newly isolated haloalkaliphilic *Bacillus* sp.: production and optimization. Process Biochem. 40: 3569-3575.
42- Pinto, G.A.S.; Couri, S. and Gonçalves, E.B. (2006). Replacement of methanol by ethanol on gallic acid determination by rhodanine and its impacts on the tannase assay. Eletronic J. Environ. Agric. Food Chem. 5: 1560-1568.
43- Purohit, J.S.; Dutta, J.R.; Nanda, R.K; and Banerjee, R. (2006). Strain improvement for tannase production from co-culture of *Aspergillus foetidus* and *Rhizopus oryzae***.** Bioresource Technol. 97(6): 795-801.
44- Ramirez-Coronel, M.A.; Viniegra-González, G.; Darvill, A. and Augur, C. (2003). A novel tannase from *Aspergillus niger* with beta-glucosidase activity. Microbiol. 149:2941–2946.
45- Rodrigues, T.R.S.; Pinto, G.A.S. and Gonçalves, L.R.B. (2008). Effects of inoculum concentration, temperature and carbon sources

on tannase production during solid state fermentation of cashew apple bagasse. Biotechnol. and Bioprocess Eng. 13: 571-576

46- Rout, S. and Banerjee, R. (2006). Production of tannase under solid state fermentation and its application in fruit juice debittering. Indian Journal of Biotechnology.5: 346-350.

47- Sabu, A.; Pandey, A.; Daud, M.J. and Szakacs, G. (2005). Tamarind seed powder and palm kernel cake: two novel agro residues for the production of tannase under solid state fermentation by *Aspergillus niger* ATCC 16620. Bioresour. Technol. 96(11): 1223-1228.

48- Seth, M. and Chand, S. (2000). Biosynthesis of tannase and hydrolysis of tannins to gallic acid by *Aspergillus awamori* – optimization ofprocess parameters. *Proc. Biochem.* 36: 39–44.

49- Sharma, S.; Agarwal, L. and Saxena, R.K. (2008). Purification, immobilization and characterization of tannase from *Penicillium variable*. Biores Technol., 99: 2244-2251.

50- Sharma, S.; Bhat, T.K. and Dawra, R.K. (1999). Isolation, purification and properties of tannase from *A. niger* van Teighem. World J Microbiol. 15:673–637.

51- Sharma, S. and Gupta, M.N. (2003). Synthesis of antioxidant propyl gallate using tannase from *Aspergillus niger* van Teighem in nonaqueous media. Bioorganic and Medicinal Chemistry Letters, 13(3): 395-397.

52- Srivastava, A. and Kar, R. (2009). Characterization and Application of tannase prodced by *Aspergillus niger* ITCC 6514.07 .07 on pomegranate rind. Brazilian J. of Microbiol. 40: 782-789.

53- Van-de Lagemaat, J. and Pyle, D.L. (2001). Solid-state fermentation and bioremediation: development of a continuous process for the production of fungal tannase. Chem. Eng. J. 84: 115-123.

54- Van de-Lagemaat, J.; Pyle, D.L. (2005). Modelling the uptake and growth kinetics of *Penicillium glabrum* in a tannic acid-containing solid-state fermentation for tannase production Process Biochemistry. 40(5): 1773-1782.

10/10/2010

Growth, Yield and Fruit Quality of Sweet Pepper Plants (*Capsicum annuum* L.) as Affected by Potassium Fertilization

El-Bassiony, A.M.*; Z.F. Fawzy; E.H. Abd El-Samad and G.S. Riad

Vegetables Crop Research Dept., National Research Centre, Dokki, Cairo, Egypt
*mohsin2eg@yahoo.com

Abstract: Two field experiments were conducted during the two successive summer seasons of 2009 and 2010 at the Experimental Farm of the National Research Centre in El-Nobaria region, Behira Governorate, to investigate the response of sweet pepper plants cv. California wonder to different rates of potassium fertilization (50, 100 and 200 kg/fed.) as potassium sulfate in addition to foliar application by potassium oxide (2 and 4 cm/L) and potassium humate (4 gm/L) as a stimulative dose. Potassium foliar applications were made 3 times in a 15 days interval with the same doses during the growing period (30, 45 and 60 days after transplanting). The highest potassium fertilization rate (200 kg/fed.) gave the tallest sweet pepper plants, the highest number of leaves and branches per plants and the highest fresh and dry weights of leaves as well as the highest total yield. Also, the obtained results reported that the fruit measurements expressed as fruit length, average fruit weight and vitamin C content, as well as leaves chemical composition (N, P, K and total chlorophyll) were increased with increasing potassium fertilization rate. On the other hand, spraying sweet pepper plants with potassium humate at rate of 4 gm/L markedly increased vegetative growth, yield, fruit quality and chemical composition. The favorable effects of the potassium on the growth, total yield and fruit parameters were obtained when sweet pepper plants fertilized with 200 kg/fed. potassium sulfate plus foliar application of potassium humate 4 gm/L followed statistically by 200 Kg/fed. potassium sulfate with foliar application of either 2 or 4 gm/L potassium oxide with no significant differenced between them but both of them were significantly higher than control.

[El-Bassiony, A.M.; Z.F. Fawzy; E.H. Abd El-Samad and G.S. Riad. Growth, Yield and Fruit Quality of Sweet Pepper Plants (*Capsicum annuum* L.) as Affected by Potassium Fertilization. Journal of American Science, 2010; 6(12): 722-729]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Potassium stimulative dose, Potassium humate, Potassium oxide, Foliar spraying, Vegetative growth, Total yield, Fruit quality, Chemical composition.

1. Introduction:

Sweet pepper (*Capsicum annuum* L) is a member of the solanaceous fruity vegetables group. It is one of the most important, popular and favorite vegetable crops cultivated in Egypt for local consumption and exportation, it is commonly called "filfil akhdar", where "filfil" means pepper and "akhdar" means green. It covers a production area of 42,136 ha in year 2007 that yielded 684,640 tons according to Ministry of Agriculture Statistics.

Plant high yields, depend on many factors, the most important factor amongst them is plant nutrition. The nutrients should be provided through suitable sources on adequate amounts and forms in a right time to ensure that plants have adequate amounts of the nutrients required for high yields. There are two ways of application nutrients, first through plant roots and it is the main way and the second through foliar application (additional way). The interest in foliar fertilizers arose due to the multiple advantages of foliar application methods. It is also recognized that supplementary foliar fertilization during crop growth can improve the mineral status of plants and increase the crop yield and quality (Kolota and Osinska, 2001).

Although, potassium is not a constituent of any plant structures or compounds, but it plays a part in many important regulatory roles in the plant, i.e. osmo-regulation process, regulation of plant stomata and water use, translocation of sugars and formation of carbohydrates, energy status of the plant, the regulation of enzyme activities, protein synthesis and many other processes needed to sustain plant growth and reproduction (Hsiao and Läuchli, 1986). It is a highly mobile element in the plant and has a specific phenomenon, it is called luxury consumption. In addition, it plays a very important role in plant tolerance of biotic and abiotic stresses (Marschner, 1995). Potassium is also known as the quality nutrient because of its important effects on quality factors (Imas and Bansal, 1999 and Lester *et al.*, 2006). With the exception of nitrogen, potassium is required by plants in much greater amounts than all the other soil-supplied nutrients (Tisdale *et al.*, 1985).

Nowadays, in Egypt, potassium fertilizers became a highly expensive ones of input factors in

production processes (ton ~ 1250 $), so many farmers minimizing the used amount to the minimum dose. In addition to use any other newly and cheapest potassium sources through foliar application as a stimulative dose to overcome such problem and to maximize their net return to cover the additional cost of this K fertilizer source.

Increasing plant vegetative growth, yield as well as fruit quality and chemical composition due to increasing potassium fertilization levels have been reported by many workers on different crops El-Masry (2000), Nassar *et al.* (2001) and Fawzy *et al.* (2005) on sweet pepper, Chen Zhen De *et al.* (1996) and Fawzy *et al.* (2007) on eggplant, Nanadal *et al.* (1998), Al-Karaki (2000) and Gupta and Sengar (2000) on tomato and Lester *et al.*, (2006) on muskmelon. Potassium humate can be used as a non-expensive source for potassium and it could be used as soil dressing, drenching or foliar applications. It was already subjected to many studies in various areas of agriculture. The mechanism of humate material in promoting plant growth is not completely known. It was reported by many researchers that K-humate application increased the plant growth, nutrient uptake and plant yield and quality as well (Böhme and Thi Lua, 1997; Padem and Ocal, 1999; Hoang and Böhme, 2001; Türkmen *et al.*, 2005; Zaky *et al.*, 2006 and Karakurt *et al.*, 2009).

Soil and/or foliar K-humate applications might successfully be used to obtain higher fruit yield and can significantly enhance pepper fruit quality as demonstrated by Arancon *et al.* (2006). Also, El-Bassiony (2006) and Fawzy *et al.* (2007) found that spraying potassium oxide as a stimulative dose led to the highest plant growth (plant length, number of leaves/plant, and fresh weight of leaves) as well as the highest yield and quality of onion and aubergine plants, respectively. In the same regard, Lester *et al.* (2006) concluded that soil K with foliar K applications during muskmelon fruit development and maturation improved fruit quality by increasing sugar content, ascorbic acid, and ß-carotene levels.

The main objective of this study was to investigate the effect of different potassium fertilization rates in addition to foliar spraying of potassium oxide or K-humate as stimulative doses on the vegetative growth parameters, total fruit yield and its physical and chemical constituents of sweet pepper plants.

2. Materials and methods

Sweet pepper seeds cv. "California wonder" were sown in 209 cell foam trays filled with peat moss:vermiculite (1:1 v/v) media. Afterwards, trays were kept in the greenhouse at the Experimental Station of the National Research Centre in El-Nobaria region, Behira Governorate, and cared by regular practices for seedlings production in greenhouse. After 8 weeks, uniform pepper seedlings were transplanted into the field on the second week of March in both seasons of 2009 and 2010. Seedlings were planted on ridges of 100 cm width, 8 m length on both sides of ridge and 50 cm apart. Each experimental plot included 4 ridges with a net area of 32 m^2. The physical properties and chemical analysis of the experimental soil are presented in Table (1). All agricultural practices were carried out according to the recommendations of Ministry of Agriculture for sweet pepper production in El-Nobaria region.

Potassium sulfate fertilizer (50% K_2O and 18% S, Tessenderlo Co., Belgium) was applied as a fertigation application through drip irrigation system with rates of 50, 100 and 200 kg/fed. Regarding potassium stimulative doses, sweet pepper plants were foliar sprayed for three times in a 15 days interval with the same doses of potassium oxide (2 and 4 ml/L, 37.5% K_2O, Kafr El Zayat Pesticides & Chemicals Co., Egypt), potassium humate (4 gm/L, Power Humus, 12% K_2O, Humintech Co., Germany) and with tap water as control treatment, during the growing period at 30, 45 and 60 days after transplanting. Few drops of wetting agent (Sticky, AGRICO International Co., Egypt) were added to spraying solution.

Experimental design and statistical analysis

The experiment was arranged in a split plot design with four replicates, where potassium fertilization rates were arranged randomly within the main 3 plots, while stimulative doses of potassium foliar spraying treatments plus control treatment were distributed in the sub-plots. The obtained data were statistically analyzed and means separation were done using LSD test according to the method described by Gomez and Gomez (1984).

Table (1): Physical properties and chemical analysis of the experimental soil.

Physical properties					
Sand	Clay	Silt	Texture	F.C.%	W.P.%
90.08	9.26	0.66	Sandy	16.57	5.25

Chemical analysis							
E.C. mmohs/cm	pH	meq./L					
		Ca	Mg	Na	K	HCO_3	Cl
1.7	8.2	7.02	0.527	0.982	0.310	1.3	0.566

Measured parameters:

Five plants were chosen randomly from each sub-plot at 90 days after transplanting date and transferred to laboratory to record the following data:

1- Vegetative growth characters

1- Plant height (cm).
2- Number of leaves/plant.
3- Number of branches/plant.
4- Fresh and dry weights of leaves (gm).

2- Fruit yield and quality

At harvesting time, samples of green pepper fruits were randomly harvested from each sub-plot to measure fruit length, fruit diameter and average fruit weight. In addition, total weight of fruits in each treatment were recorded by harvesting pepper fruits twice weekly and then the total yield as ton/fed. was calculated.

3- Chemical content

Fruit samples were randomly taken at harvesting time to determine vitamin C content in the fruit as mg/100gm fresh weight according to method described by A.O.A.C. (1990). Also, total chlorophyll content in fully expanded leaves was measured as SPAD units using Minolta SPAD-501 chlorophyll Meter (Minolta Co. Ltd., Japan). Leaf samples were oven dried at 68^{o}C for 72 hours, then fine grinded and used to determine ion contents on a dry weight basis. Total nitrogen and phosphorus contents were determined using Kieldahl method and colorimetric method using spectrophotometer (SPECTRONIC 20D, Milton Roy Co. Ltd., USA), respectively, according to the procedure described by Cottenie (1980). Potassium content was measured using flame photometer method (JENWAY, PFP-7, ELE Instrument Co. Ltd., UK) as described by Chapman and Pratt (1982).

3. Results and Discussion:

Vegetative growth characters

There were significant increases of all vegetative growth characters with increasing potassium fertilization rates from 50 to 200 kg/fed. except for number of branches per plant in the second season only, where no significant effect was realized (Table 2). In general, the highest values of plant height, number of leaves, number of branches per plant and fresh and dry weights of leaves of sweet pepper plants were recorded by plants which received 200 kg/fed. potassium sulfate, while the lowest values were recorded by plants received 50 kg/fed. These findings were true in both seasons. These results may be due to the role of potassium element in metabolism and many processes needed to sustain and promotion plant vegetative growth and development. Moreover, many studies proved that K plays a major role in many physiological and biochemical processes such as cell division and elongation and metabolism of carbohydrates and protein compounds (Hsiao and Läuchli, 1986 and Marschner, 1995). The obtained results are in harmony with those of El-Masry (2000), Nassar *et al.* (2001) and Fawzy *et al.* (2005) on sweet pepper, Fawzy *et al.* (2007) on eggplant, Al-Karaki (2000) and Gupta and Sengar (2000) on tomato and Lester *et al.* (2006) on muskmelon.

Concerning the foliar spraying of potassium as a stimulative dose (Table 2), there were significant increases in all vegetative growth parameters with using potassium as foliar application in both seasons, except for number of branches per plant in the second season only. The highest values of plant growth characters expressed as plant height, number of leaves, number of branches per plant and fresh and dry weights of leaves were recorded by using 4 gm/L of K-humate followed by 4 ml/L of potassium oxide when compared with control treatment. The above findings were completely similar in both seasons.

These results are in agreement with those of Hoang and Böhme (2001); Türkmen *et al.* (2005); Zaky *et al.* (2006) and Karakurt *et al.* (2009). They reported that K-humate application led to increasing and improving plant growth parameters. In addition, Böhme and Thi Lua (1997) reported that K-humate had beneficial effects on nutrient uptake by plants and was particularly important for the transport and availability of micro nutrients needed for optimal plant growth and development. In the same respect,

spraying potassium oxide led to the highest values of plant growth characters (plant length, number of leaves/plant and fresh weight of leaves) as reported by El-Bassiony (2006) on onion and Fawzy *et al.* (2007) on eggplant.

Regarding the interaction effect, there were significant differences in all measured vegetative parameters except for number of branches per plant in both seasons. Generally, it could be stated that the highest plant growth characters were recorded by using 200 kg/fed. potassium sulfate with foliar application of K-humate (4 gm/L) followed by using 200 kg/fed. potassium sulfate with foliar application of potassium oxide (4 ml/L) and these findings were true in both experimental seasons.

Fruit yield and quality

Data presented in Table (3) clearly demonstrated that there were significant differences in the total yield and all fruit quality parameters except for the average of fruit diameter. The highest total yield of sweet pepper plants was produced by using 200 kg/fed. potassium sulfate. Concerning, fruit quality measurements (fruit length and average fruit weight), the obtained results concluded that there were significant increases in sweet pepper fruit parameters with increasing potassium levels from 50 to 200 kg/fed. While, potassium sulfate fertilization had no significant effect on pepper fruit diameter. These findings were true in both experimental seasons.

Table (2): Effect of different potassium levels and potassium foliar application on vegetative growth parameters of pepper plant in seasons of 2009 and 2010.

Characters		2009					2010				
		Plant length (cm)	Leaf number	Branch number	Leaves fresh weight (g)	Leaves dry weight (g)	Plant length (cm)	Leaf number	Branch number	Leaves fresh weight (g)	Leaves dry weight (g)
Treatments		**Effect of potassium level**									
50 kg/ fed.		37.27	158.5	6.81	77.42	18.58	39.14	180.25	8.81	85.59	20.12
100 kg/fed.		41.57	182.5	8.44	95.00	26.47	47.90	203.75	10.25	100.89	26.61
200 kg/fed.		43.88	191.0	9.13	106.01	28.76	49.66	212.50	9.75	110.95	29.92
LSD at 5%		**1.55**	**8.77**	**1.45**	**6.18**	**1.32**	**1.36**	**6.56**	**NS**	**7.87**	**2.08**
		Effect of foliar application									
Control		36.65	165.67	6.58	81.94	21.63	39.89	177.67	7.08	86.31	22.29
2 cm/L K_2O		37.74	167.33	7.75	87.90	23.46	42.23	189.33	9.00	92.52	24.12
4 cm/L K_2O		42.71	184.67	8.75	90.94	24.97	46.46	200.00	10.67	94.57	26.50
4 gm/L K-humate		46.52	191.67	9.42	110.45	28.35	53.67	228.33	11.67	123.17	29.27
LSD at 5%		**4.32**	**9.47**	**1.15**	**3.24**	**1.02**	**2.14**	**9.42**	**NS**	**3.69**	**1.73**
		Effect of the interaction									
50 kg/fed.	Control	35.54	150	6.25	74.67	16.32	31.67	162	7.50	77.85	17.33
	2 cm/L K_2O	34.35	147	6.50	74.98	17.03	34.50	175	8.00	79.38	18.15
	4 cm/L K_2O	37.50	158	7.00	76.01	18.35	39.22	182	9.25	83.67	21.32
	4 gm/L K-humate	41.67	179	7.50	84.01	22.63	51.15	202	10.50	101.44	23.67
100 kg/fed.	Control	36.80	172	6.50	85.68	23.12	41.50	194	6.50	90.14	23.42
	2 cm/L K_2O	36.33	173	8.25	94.04	26.22	45.45	195	10.50	95.73	25.36
	4 cm/L K_2O	46.50	187	9.50	93.57	27.46	51.67	197	12.25	93.98	27.77
	4 gm/L K-humate	46.65	198	9.50	106.71	29.09	52.98	229	11.75	123.71	29.89
200 kg/fed.	Control	37.60	175	7.00	85.47	25.45	46.50	177	7.25	90.94	26.13
	2 cm/L K_2O	42.55	182	8.50	94.69	27.14	46.75	198	8.50	102.44	28.86
	4 cm/L K_2O	44.13	209	9.75	103.25	29.11	48.50	221	10.50	106.06	30.42
	4 gm/L K-humate	51.23	198	11.25	140.62	33.34	56.88	254	12.75	144.36	34.26
LSD at 5%		**2.35**	**11.16**	**NS**	**10.65**	**3.82**	**2.13**	**10.43**	**NS**	**5.72**	**2.46**

The obtained results might be due to the role of potassium in fruit quality, where it is known as the quality nutrient because of its important effects on fruit quality parameters (Imas and Bansal, 1999 and Lester *et al.*, 2006). Also, the obtained results were in accordance with those obtained by El-Masry (2000), Ni-Wu *et al.* (2001), Ruchi-Sood and Sharma (2004) and Fawzy *et al.* (2005) on pepper plants, Fawzy *et al.* (2007) on eggplant and Al-Karaki (2000) and Gupta and Sengar (2000) on tomato. They concluded that increasing potassium fertilization levels could be used to improve or enhance plant yield and fruit quality as well.

Regarding foliar application of potassium oxide or potassium humate, foliar application treatments had a significant effect on total yield and fruit quality of sweet pepper plants except for fruit diameter in both seasons. The highest values of fruit yield and quality parameters were obtained when sweet pepper plants sprayed with K-humate (4 gm/L) followed by potassium oxide (4 ml/L).

The obtained results are in agreement with Karakurt *et al.* (2009) who showed that humic acid had no significant effect on fruit length or diameter. Also, they demonstrated that humic acid applications might successfully be used to obtain higher fruit yield and can significantly enhance fruit quality in organically grown pepper. Moreover, Arancon *et al.* (2006) reported that pepper plants treated with humic acid significantly produced more fruits and flowers than untreated plants. In addition, Padem and Ocal (1999) demonstrated that increasing K-humate application dose led to a significant increase in fruit weight and total yield. In the same regard, El-Bassiony (2006) and Fawzy *et al.* (2007) summarized that spraying potassium oxide resulted in the highest yield and quality of onion and aubergine plants, respectively.

Concerning factors interaction, there was no significant interaction effect on fruit diameter in both seasons. Whereas, there were significant interaction effects on total yield, fruit weight and fruit length in both seasons. Generally, it could be mentioned that the best results were obtained when potassium sulfate was used at rate of 200 kg/fed. plus K-humate (4 gm/L) followed by using potassium sulfate at rate of 200 kg/fed. plus potassium oxide (4 ml/L) as a foliar application.

Table (3): Effect of different potassium levels and potassium foliar application on fruit yield and quality of pepper plant in seasons of 2009 and 2010.

Characters		2009				2010			
		Fruit length (cm)	**Fruit diameter (cm)**	**Fruit weight (g)**	**Total yield ton/fed.**	**Fruit length (cm)**	**Fruit diameter (cm)**	**Fruit weight (g)**	**Total yield ton/fed.**
Treatments		**Effect of potassium level**							
50 kg/fed.		7.65	5.75	66.43	7.15	8.23	6.20	67.87	7.07
100 kg/fed.		9.63	6.05	82.65	7.64	10.18	6.43	83.85	7.68
200 kg/fed.		9.85	6.63	103.22	8.59	10.40	7.08	108.30	8.64
LSD at 5%		**1.03**	**NS**	**7.34**	**0.09**	**0.15**	**NS**	**13.66**	**0.27**
		Effect of foliar application							
Control		7.27	5.13	65.89	7.10	7.50	5.67	66.83	7.09
2 cm/L K_2O		8.50	6.13	73.74	7.47	8.97	6.30	75.67	7.62
4 cm/L K_2O		9.50	6.33	93.11	8.14	10.30	6.93	95.95	8.03
4 gm/L K-humate		10.90	6.97	103.66	8.45	11.63	7.37	108.24	8.45
LSD at 5%		**1.10**	**NS**	**5.53**	**0.06**	**1.18**	**NS**	**7.54**	**0.17**
		Effect of the interaction							
50 kg/fed.	Control	6.3	4.8	49.17	6.32	6.4	5.1	50.67	6.35
	2 cm/L K_2O	7.5	6.2	55.25	6.84	8.2	6.4	58.00	6.92
	4 cm/L K_2O	7.3	5.9	77.36	7.63	8.5	6.7	78.23	7.08
	4 gm/L K-humate	9.5	6.1	83.93	7.82	9.8	6.6	84.59	7.93
100 kg/fed.	Control	8.3	5.4	71.17	7.12	8.8	5.7	71.25	6.97
	2 cm/L K_2O	9.1	6.1	69.30	7.25	9.6	6.3	75.70	7.37
	4 cm/L K_2O	9.5	6.2	93.14	7.85	9.8	6.9	93.87	8.15
	4 gm/L K-humate	11.6	6.5	96.97	8.32	12.5	6.8	94.57	8.24
200 kg/fed.	Control	7.2	5.2	77.32	7.87	7.3	6.2	78.58	7.96
	2 cm/L K_2O	8.9	6.1	96.68	8.32	9.1	6.2	93.30	8.57
	4 cm/L K_2O	11.7	6.9	108.82	8.95	12.6	7.2	115.76	8.86
	4 gm/L K-humate	11.6	8.3	130.07	9.22	12.6	8.7	145.55	9.17
LSD at 5%		**1.45**	**NS**	**18.75**	**0.36**	**1.35**	**NS**	**22.14**	**0.56**

Chemical content

Increasing potassium fertilization rates from 50 to 200 kg/fed. significantly increased all chemical composition, i.e. total chlorophyll, N, P and K in leaves and vitamin C content in fruits. In general, the highest and lowest values of measured chemical composition of sweet pepper plants were recorded by plants which received 200 and 50 kg/fed. potassium sulfate, respectively, in both seasons as shown in Table (4). These results may be due to the role of potassium in plant metabolism and many important regulatory processes in the plant. Also, it could be increased mineral uptake by plants (Hsiao and Läuchli, 1986 and Marschner, 1995).

The obtained results are in accordance with Nassar *et al.* (2001) and Fawzy *et al.* (2005) on pepper, Fawzy *et al.* (2007) on eggplant, Al-Karaki (2000) and Gupta and Sengar (2000) on tomato. They proposed that potassium fertilization levels significantly affect fruit quality parameters and plant chemical composition. In the same regard, Lester *et al.* (2006) concluded that potassium fertilization with potassium foliar applications during muskmelon fruit development and maturation improved fruit quality by increasing sugar content, ascorbic acid, and ß-carotene levels.

Table (4): Effect of different potassium levels and potassium foliar application on chemical composition of pepper plant in seasons of 2009 and 2010.

Characters		2009					2010				
		Vitamin C	Total chlorophyll	N%	P%	K%	Vitamin C	Total chlorophyll	N%	P%	K%
Treatments		**Effect of potassium level**									
50 kg/fed.		69.48	48.36	1.38	0.52	1.87	71.45	49.83	1.41	0.52	1.96
100 kg/fed.		79.01	53.25	1.56	0.62	2.20	81.81	53.08	1.57	0.63	2.23
200 kg/fed.		94.76	55.38	1.72	0.69	2.47	100.03	57.74	1.73	0.71	2.51
LSD at 5%		**4.25**	**1.12**	**0.05**	**0.04**	**0.09**	**7.75**	**1.55**	**0.12**	**0.05**	**0.11**
		Effect of foliar application									
Control		73.23	45.80	1.37	0.52	1.86	75.58	49.64	1.40	0.54	1.92
2 cm/L K_2O		77.90	52.95	1.46	0.57	2.09	80.24	52.30	1.51	0.58	2.12
4 cm/L K_2O		82.47	54.60	1.64	0.64	2.32	87.57	54.39	1.59	0.65	2.38
4 gm/L K-humate		90.74	55.97	1.73	0.71	2.46	94.32	57.88	1.77	0.71	2.53
LSD at 5%		**2.17**	**1.16**	**0.04**	**0.06**	**0.03**	**5.13**	**1.23**	**0.07**	**NS**	**0.12**
		Effect of the interaction									
50 kg/fed.	Control	65.13	41.50	1.23	0.44	1.65	68.22	45.70	1.35	0.45	1.73
	2 cm/L K_2O	68.12	48.25	1.34	0.51	1.74	70.09	49.20	1.37	0.49	1.86
	4 cm/L K_2O	71.15	51.30	1.41	0.52	1.95	71.72	51.30	1.38	0.56	2.03
	4 gm/L K-humate	73.52	52.40	1.52	0.61	2.15	75.76	53.13	1.52	0.59	2.23
100 kg/fed.	Control	72.52	47.90	1.36	0.51	1.92	73.76	49.73	1.35	0.54	1.95
	2 cm/L K_2O	75.25	53.10	1.47	0.59	2.16	77.95	51.60	1.55	0.59	2.23
	4 cm/L K_2O	81.11	55.50	1.67	0.64	2.35	84.76	53.67	1.63	0.67	2.33
	4 gm/L K-humate	87.36	56.50	1.72	0.73	2.37	90.75	57.33	1.75	0.71	2.42
200 kg/fed.	Control	82.23	48.00	1.52	0.61	2.02	84.76	53.50	1.49	0.63	2.09
	2 cm/L K_2O	90.32	57.50	1.56	0.62	2.37	92.67	56.09	1.62	0.67	2.26
	4 cm/L K_2O	95.15	57.00	1.83	0.75	2.65	106.23	58.20	1.75	0.73	2.77
	4 gm/L K-humate	111.35	59.00	1.95	0.79	2.85	116.44	63.17	2.05	0.82	2.93
LSD at 5%		**15.42**	**6.13**	**NS**	**NS**	**0.11**	**8.64**	**3.15**	**NS**	**NS**	**0.09**

Potassium not only increased fruit yields but also improved fruit quality by increasing dry matter and vitamin C contents, as well as increasing sugar content and titratable acidity levels of tomato as reported by Economakis and Daskalaki (2003).

Concerning foliar application of potassium oxide with rates of 2 and 4 ml/L or potassium humate with 4 gm/L as stimulative doses, it is of interest to note that they had a positive significant effect on chemical composition of sweet pepper plants except for phosphorus percentage in the second season only. Data presented in Table (4) indicated that the highest values of vitamin C content in fruits and total chlorophyll in leaves, as well as the highest percentages of N, P and K in pepper leaves were recorded when pepper plants sprayed by K-humate (4 gm/L) followed by potassium oxide (4 ml/L) treatments when compared with control treatment in both seasons.

The obtained results are in harmony with Hoang and Böhme (2001), Zaky *et al.* (2006) and Karakurt *et al.* (2009). Also, Böhme and Thi Lua (1997) showed that K-humate had beneficial effects on nutrient uptake and was particularly important for the transport and availability of micro nutrients. In addition, foliar spraying of potassium as a stimulative dose had a significant effect on N and K percentages in dry leaf tissues and significantly increased fruit quality in eggplant as reported by Fawzy *et al.* (2007). Also, Padem and Ocal (1999) and Lester *et al.* (2006) demonstrated that potassium applications can improve fruit quality, i.e. firmness, sugar content and vitamin C content in processing tomato and muskmelon fruits.

The interaction effect between potassium fertilization rates and potassium foliar application had a significant effect on vitamin C content in fruits, total chlorophyll and potassium percentage in leaves. While, no significant effects were realized on nitrogen and phosphorus percentages. These results are similar in both seasons. From data presented in Table (4) it could be clearly summarized that the highest values of vitamin C content in sweet pepper fruits, total chlorophyll and potassium percentage in sweet pepper leaves were recorded when sweet pepper plants received 200 kg/fed. potassium sulfate as fertilization plus 4 gm/L of K-humate as foliar application followed by plants that received 200 kg/fed. potassium sulfate plus 4 gm/L of potassium oxide.

4. Conclusion:

From the above mention results it could be concluded that foliar application of potassium humate (4 gm/L) or potassium oxide (4 ml/L) as a stimulative dose could be successfully used in addition to fertilization application of potassium sulfate with rate of 200 kg/fed. to obtain the highest vegetative growth parameters, total fruit yield, and significantly enhanced fruit quality and chemical composition of sweet pepper plants.

Corresponding author
El-Bassiony, A.M.
Vegetables Crop Research Dept., National Research Centre, Dokki, Cairo, Egypt.
mohsin2eg@yahoo.com

5. References:

1. Al-Karaki, G.N. 2000. Growth, sodium, and potassium uptake and translocation in salt stressed tomato. J. Plant Nutrition, 23(3): 369-379.
2. A.O.A.C. 1990. Official Methods of Analysis. 15th ed., Association of Official Analytical Chemists, Washington, D.C., USA.
3. Arancon, N.Q.; C.A. Edwards; S. Lee and R. Byrne 2006. Effects of humic acids from vermicomposts on plant growth. European J. Soil Bio., 42(Suppl.1): S65-S69.
4. Böhme, M. and H. Thi Lua 1997. Influence of mineral and organic treatments in the rizosphere on the growth of tomato plants. Acta Hort., 450: 161-168.
5. Chapman, H.D. and P.F. Pratt 1982. Methods of Plant Analysis, I. Methods of Analysis for Soil, Plant and Water. Chapman Publishers, Riverside, California, USA.
6. Chen Zhen De; Huang Jun Jie and Cai Kui 1996. Studies on fertilizer application levels of seedling stage of eggplant raised with mixed media. China Vegetables, 4: 16-18.
7. Cottenie, A.L. 1980. Soils and plant testing as a basis of fertilizer recommendation. FAO Soil Bull., No. 3812.
8. Economakis, C. and A. Daskalaki 2003. Effect of potassium nutrition on yield and quality of tomato plants grown with nutrient film technique under sodium chloride saline conditions. Acta Hort., 609: 337-339.
9. El-Bassiony, A.M. 2006. Effect of potassium fertilization on growth, yield and quality of onion plants. J. Appl. Sci. Res., 2(10): 780-785.
10. El-Masry, T.A. 2000. Growth, yield and fruit quality response in sweet pepper to varying rates of potassium fertilization and different concentrations of paclobutrazol

foliar application. Annals Agric. Sci., Moshtohor, 38(2): 1147-1157.
11. Fawzy, Z.F.; A.G. Behairy and S.A. Shehata 2005. Effect of potassium fertilizer on growth and yield of sweet pepper plants (*Capsicum annuum*, L.). Egypt. J. Agric. Res., 2(2): 599-610.
12. Fawzy, Z.F.; M.A. El-Nemr and S.A. Saleh 2007. Influence of levels and methods of potassium fertilizer application on growth and yield of eggplant. J. Appl. Sci. Res., 3(1): 42-49.
13. Gomez, K.A. and A.A. Gomez 1984. Statistical Procedures for Agriculture Research. 2nd ed. Willey Inter Science Publisher, pp. 357-423.
14. Gupta, C.R. and S.S. Sengar 2000. Response of tomato (*Lycopersicon esculentum* Mill.) to nitrogen and potassium fertilization in acidic soil of Bastar. Veg. Sci., 27(1): 94-95.
15. Hoang, L. and M. Böhme 2001. Influence of humic acid on the growth of tomato in hydroponic systems. Acta Hort., 548: 451-458.
16. Hsiao, C. and A. Läuchli 1986. Role of potassium in plant-water relation. In: Advances in plant nutrition 2nd ed., pp. 281-312., Tinker and A. Läuchli (eds.). Praeger, New York.
17. Imas, P. and S.K. Bansal 1999. Potassium and integrated nutrient management in potato. Presented at the global conference on potato, Dec., 6-11, New Delhi, India.
18. Karakurt, Y.; H. Unlu and H. Padem 2009. The influence of foliar and soil fertilization of humic acid on yield and quality of pepper. Acta Agric. Scandinavica, 59(3): 233-237.
19. Kolota, E. and M. Osinska 2001. Efficiency of foliar nutrition of field vegetables grown at different nitrogen rates. Acta Hort., 563: 87-91.
20. Lester, G.E.; J.L. Jifon and D.J. Makus 2006. Supplemental foliar potassium applications with or without a surfactant can enhance netted muskmelon quality. HortSci., 41(3): 741-744.
21. Marschner, H. 1995. Mineral nutrition of higher plants. 2nd ed. Academic. Press. London. New York.
22. Nanadal, J.K.; Ramesh-Vasist and U.C. Pandey 1998. Effect of phosphorus and potassium on growth yield and quality of tomato. J. Potassium Research, 14(1/4): 44-49.
23. Nassar, H.H.; M.A. Barakat; T.A. El-Masry and A.S. Osman 2001. Effect of potassium fertilization and paclobutrazol foliar application on vegetative growth and chemical composition of sweet pepper. Egypt. J. Hort., 28(1): 113-129.
24. Ni-Wu, Z.; S. Liang-Jian and R. Hardter 2001. Yield and quality responses of selected solanaceous vegetable crops to potassium fertilization. Pedosphere, 11(3): 251-255.
25. Padem, H. and A. Ocal 1999. Effects of humic acid applications on yield and some characteristics of processing tomato. Acta Hort., 487: 159-164.
26. Ruchi-Sood and S.K. Sharma 2004. Growth and yield of bell pepper (*Capsicum annuum* var. Grossum) as influenced by micronutrient sprays. Indian J. Agric. Sci., 74(10): 557-559.
27. Tisdale, S.L.; W.L. Nelson and J.D. Beaton 1985. Soil and fertilizer potassium. In: Soil Fertility and Fertilizers 4th ed., S.L. Tisdale, W.L. Nelson, and J.D. Beaton (eds.), pp. 249-291, MacMillan Publ. Co., New York.
28. Türkmen, Ö.; S. Demir; S. Sensoy and A. Dursun 2005. Effects of arbuscular mycorrhizal fungus and humic acid on the seedling development and nutrient content of pepper grown under saline soil conditions. J. Bio. Sci., 5(5): 568-574.
29. Zaky, M.H.; O.A.H. El-Zeiny and M.E. Ahmed 2006. Effects of humic acid on growth and productivity of bean plants grown under plastic low tunnels and open field. Egypt. J. Appl. Sci., 21(4B): 582-596.

10/8/2010

How Do University Students Spend Their Time On Facebook? An Exploratory Study

Alimohammad Aghazamani
Faculty of Technology and Science, Karlstad University, Karlstad, Sweden
Aghazamani99@yahoo.com

Abstract: Despite major productive uses of Internet technology in today's digital world, users prefer to spend much more time on social networking sites (SNSs) like Facebook. The objective of this study is to determine student motives for using Facebook. A close-ended questionnaire was administered to 595 University students who were recognized as users of the site at Karlstad University in Sweden. Male users spend more time on the site than female users during both weekdays (p-value=0.9238) and weekends (p-value=0.9953). The survey showed that undergraduate students login more times per day than graduate students (p-value=0.2138). In addition, friendship was named the most favorite activity among male users (p-value=0.8883) and also among undergraduate students comparing with graduate students (p-value=0.2045). If users were asked to pay a membership fee to use the site, the results showed that male users (p-value=0.9991) and undergraduate students (p-value=0.9884) were more likely to pay the charge than other groups (females and graduate students). It is apparent that using Facebook can be seen as an important part of daily life among University students and its phenomenon spread out inevitably.
[Alimohammad Aghazamani**. How Do University Students Spend Their Time On Facebook? An Exploratory Study.** Journal of American Science 2010;6(12):730-735]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Internet; Facebook; Global village; Social networking

1. Introduction

Among the variety of online tools now available for communication, social networking sites (SNSs) are one of the most recent and significant tools for connecting people throughout the world. The online world has already proven to impact numerous aspects of the human life including commerce, education, and health (Jalalian M., 2010, Jalalian M. 2008). These virtual spaces, the new building blocks of today's Internet, provide not only the power to access distributed and heterogeneous information, but also interact with millions of people all over the world. SNSs are virtual spaces that allow individuals to create personal profiles - visible to other users- to establish connections and join an online social network. These websites enable computer-mediated communication (CMC) between people. Social networking has been the tool that brings people together in just a click of a mouse (Raskin, 2006).

It seems that SNSs such as Facebook are changing the nature of social relationships (Body & Elisson, 2007). In fact, virtual spaces like Facebook and MySpace (www.myspace.com) offer today's online users a discursive space to experience "communal affiliation around shared experiences" (Mitra, 1997). SNSs users join cyber-communities to overcome geographical distance as well.

Facebook was established in February 2004 by Mark Zuckerberg, a former Harvard University student. The website has more than 500 million active users worldwide while about 70 percent of its users are outside the United States (Facebook, 2010).

Facebook offers its services in more than 75 languages (five times more than its nearest competitor, MySpace) and because of its popularity among Internet users, the number of users has increased considerably. The study on which this research is based examined the amount of time spent on Facebook and factors that persuade university students to fulfill their needs and desires through Facebook. Information was disclosed through paper-based interviews. The investigation assessed gender, education, and age differences in the disclosure of personal preferences and amount of time spent on Facebook. Respondents were students at Karlstad University in Sweden. The survey provided interviews among 595 students during November and December 2009.

The paper begins by discussing our hypothesis and research questions that were asked in the questionnaire. Next, we describe the methodology used to empirically investigate the hypotheses, followed by our analysis and discussion of the results. Finally, we discuss the implications and limitations of this research and suggestions for future research. A major contribution of this study lies in understanding the relationship among individual differences (gender and education), amount of time spent on Facebook, and individual preferences in Facebook usage among university students.

2. Material and Methods

Twelve hypotheses (Hs) regarding the subject of survey were defined and research on the basis of close-ended questions was designed. Investigations were conducted by questionnaire using six questions, with each question offering two to four proposed answers (PA). The questionnaire was developed using a variety of research questionnaires and the results of prior studies. For instance, question No. 4 addresses student motives (gratification) for using Facebook and other questions examine how a student's individual differences in gender and education are related to their use of Facebook. Table 1 illustrates the structure of the questionnaire.

The hypotheses of the survey were defined as follows:

H1: Facebook male users spend more time on Facebook during weekdays than female users.

H2: Undergraduate students use Facebook more during weekdays than graduate students.

H3: Most of Facebook male users spend more time on Facebook during weekends than females.

H4: Undergraduate students use Facebook more than graduate students during weekends.

H5: Most of Facebook male users login more than once a day than female users.

H6: More undergraduate students login to Facebook each day than graduate students.

H7: Friendship is the most favorite activity among male users on Facebook.

H8: Friendship is the most favorite activity among undergraduate users.

H9: Most of Facebook male users like to connect in the morning – more than female users do.

H10: More undergraduate students use Facebook in the morning than graduate students.

H11: Facebook male users are more favorably disposed top paying a fee to connect to the site than females.

H12: Undergraduate Facebook users are more favorably disposed to paying a fee to connect to the site than graduate students.

The city selected for this study is Karlstad, the capital of Värmland County in Sweden. The authors chose Karlstad University, which has approximately 12,000 students with diverse undergraduate and graduate majors. The survey was conducted on the basis of paper interviews that were held in November and December 2009.The data in this study was obtained using a survey questionnaire completed by undergraduate and master's students at Karlstad University. The University was chosen because of its wide range of Swedish and international students in an academic environment where there are many students who are familiar to Facebook. Students were interested in participating in the survey about Facebook. They were asked to comment on readability; understand the questions, and complete the questionnaire in a small period of time. The results indicate that the questions were clear and understandable and required about 15-20 minutes to complete. The goals of these questions were to consider how differences in education and gender are affected by Facebook use among University students.

Table 1. Questionnaire employed in the study

Gender: ( )Male ( ) Female
Do you currently have a Facebook account? ( )Yes ( ) No If you answered ''Yes" to above question, please continue to item No. 1. If No, thank you for your time. If you connect to Facebook every day (Except weekend), answer the question below:
RQ1. How much time do you spend on Facebook during the day? AA1. a. Maximum 5 minutes b. Maximum one hour c. more than one hour
RQ2. How much time do you spend on Facebook during the weekend? AA2. a. Not at all b. Maximum 5 minutes c. Maximum one hour d. more than one hour
RQ3. How many times do you connect to Facebook during the day? AA3. a. Only one time b. more than one time
RQ4. What is your first preference for using Facebook usually? AA4. a. Chat b. Posts and comments c. Friendship d. Other
RQ5. What times do you typically connect to Facebook? AA5. a. 8-12 b. 12-17 c. after 17
RQ6. If you had to pay a charge to login to Facebook, how would you be willing to pay per month? AA6. a. Maximum 50 Kr b. More than 50 Kr c. No charge

To collect the data, we used a questionnaire that was distributed to 750 students and 595 (79.3 percent) answered the research questions regarding their Facebook usage. Responses were voluntary and those who never had a Facebook account did not participate in the survey. We asked students to answer questions and respond to one of the alternatives for each question. After capturing data from students in order to consider our hypothesis, we used Statistical Package for Social Science (SPSS) to analyze the questionnaires. Mean, standard deviation, and coefficient of variance for each question and

group of questions were calculated to determine the order of importance of research factors. ANOVA statistical analysis (t-test) was used to assess the validity of attitudes of male and female students at different education levels toward using Facebook.

3. Results

Table 2 illustrates the frequency distributions of the survey based on gender and education while Table 3 illustrates the separate results. The results showed that 57.6 percent of respondents were male and 42.4 percent were female. Most students were undergraduates. The total results, separated by survey's questionnaire, are presented in Table 2. To consider 12 hypotheses based on p-value test, we used Table 3 to evaluate our hypothesis.

TABLE 2. Frequency Distributions in the study sample

Percentage of the sample (n=595)	
Variable	Frequency (%)
Gender	
Male	342 (57.6)
Female	253 (42.4)
Education	
Undergraduate	434 (72.9)
Graduate	161 (27.1)

Ho is assumed as a null hypothesis. P is the ratio of Facebook users. P has a different meaning with respect to the subject of any hypothesis. For instance, in hypothesis No. 1, P male is the ratio of Facebook male users who spend more time on the site than female users. Results of p-value tests are shown below:

H1. Accepted
H_0: $P_{male} = P_{female}$
H_a: $P_{male} \neq P_{female}$
P-value= 0.9238> 0.05

H2.Accepted
H_0: $P_{Undergraduate} = P_{Graduale}$
H_a: $P_{Undergraduate} \neq P_{Graduale}$
P-value=1 >0.05

H3.Accepted
H_0: $P_{male} \geq P_{female}$
H_a: $P_{male} < P_{female}$
P-value=0.9953 > 0.05

H4.Accepted
H_0: $P_{Undergraduate} = P_{Graduale}$
H_a: $P_{Undergraduate} \neq P_{Graduale}$
P-value=0.3421> 0.05

H5.Accepted
H_0: $P_{male} \geq P_{female}$
H_a: $P_{male} < P_{female}$
P-value=0.9813> 0.05

H6.Rejected
H_0: $P_{Undergraduate} = P_{Graduale}$
H_a: $P_{Undergraduate} \neq P_{Graduale}$
P-value=0.2138> 0.05

H7. Accepted
H_0: $P_{Undergraduate} = P_{Graduale}$
H_a: $P_{Undergraduate} \neq P_{Graduale}$
P-value=0.8883> 0.05

H8. Accepted
H_0: $P_{Undergraduate} = P_{Graduale}$
H_a: $P_{Undergraduate} \neq P_{Graduale}$
P-value=0.2045>0.05

H9. Accepted
H_0: $P_{Undergraduate} = P_{Graduale}$
H_a: $P_{Undergraduate} \neq P_{Graduale}$
P-value=0.3923> 0.05

H10. Accepted
H_0: $P_{Undergraduate} = P_{Graduale}$
H_a: $P_{Undergraduate} \neq P_{Graduale}$
P-value=0.9755> 0.05

H11. Rejected
H_0: $P_{male} \geq P_{female}$
H_a: $P_{male} < P_{female}$
P-value=0.9991>0.05

H12. Accepted
H_0: $P_{Undergraduate} = P_{Graduale}$
H_a: $P_{Undergraduate} \neq P_{Graduale}$
P-value= 0.9884>0.05

TABLE 3. Frequency of survey results based on gender and education

		Education		Gender	
		Undergraduate	Graduate	Male	Female
How much time do you spend on Facebook during the day?	Seldom.	0	4	4	0
	Less than 5 minutes.	25	17	24	18
	5 to 30 minutes.	48	31	41	38
	More than one hour.	350	90	245	195
How much time do you spend on Facebook on the weekend?	Seldom.	213	45	133	125
	Less than 5 minutes.	27	12	16	23
	5 to 30 minutes.	44	40	54	30
	More than one hour.	146	61	134	73
How many times do you connect to Facebook per day? (Except weekends)	Only once.	47	24	30	41
	More than once .	367	114	277	204
	Less than daily.	9	1	6	4
What is your first preference for using Facebook usually?	Chat.	103	24	56	71
	Posts and comments.	140	49	121	68
	Friendship.	176	75	143	108
	Other.	14	12	21	5
What time do you typically connect to Facebook?	8-12.	62	23	45	40
	12-17.	77	33	68	42
	After 17.	292	105	229	168
	Sometimes.	0	0	0	0
If you had to pay a charge to login to Facebook, how much would you be willing to pay per month?	Up to 50 Kr.	88	62	103	47
	50 to 200 Kr.	122	34	93	63
	No charge.	221	65	146	140
How does Facebook affect your social relationships in the real world?	It increases my social relations completely.	94	42	99	37
	It decreases my social relations in completely.	163	49	107	105
	It causes negative and positive effects.	74	40	66	48
	I have never thought about that.	100	28	67	61

4. Discussions

The aim of the study was to examine how university students who deal with SNSs, particularly Facebook.com, spend their time on the site and what their motivations are. In accordance with different gender, education levels, and preferences in using Facebook, results revealed that male Facebook users spend more time on Facebook during the weekday (p-value=0.9238>0.05) than female users. In addition, males spend more time on the site on weekends (p-value=0.9953>0.05) than female users. The number of site logins among graduate students appears to be less than undergraduate students (P-value=0.2138>0.05). Moreover, most male respondents chose friendship as their most favorite activity (p-value=0.8883>0.05) instead of other activities (chat, postings, and comments). Also, friendship was recognized as the most popular activity for undergraduate students compared to graduate students (p-value=0.2045>0.05). One question related to the user's willingness to pay a membership fee to connect the site. The result of p-value test found that male users (p-value=0.9991>0.05) and undergraduate students (p-value=0.9884>0.05) were more interested in paying a charge than other groups (female and graduate students).

The results showed that the vast majority of respondents in our survey use Facebook. One explanation for this popularity is that SNS usage among Internet users has increased dramatically in

today's information-driven societies in Western countries.

Numerous articles outline the significant relationship between SNS and psychological subjects. Some believe that motivations that determine how young people use online social networks are psychological ones, and they are strongly related to the human needs of communication, socialization, being an active part of a group, or maintaining long-distance friendships (Baltaretu & Balaban, 2010). Moreover, there has been significant discussion about the relationship between online media and offline social activities and interpersonal interaction (DiMaggio, Hargittai, Neuman & Robinson, 2001). Thus, the findings show that Facebook usage among Swedish university students seems to be an important part of their everyday life but can present some challenges in their social relationships.

Some researchers have suggested that greater Internet use results in smaller social circles, less communication at home, and loneliness (Kraut et al., 1998). Spending more time on the Internet and particularly in SNSs can create an addiction to the Internet. Internet addiction is a prevalent problem affecting individuals throughout the world and is recognized as an artifact stage of Internet adoption within a society (Zhang & Amos, 2008). The author believes that there are some limitations to this research.

Like any research, the investigation has many limitations that should be considered before generalizing the results to other contexts and recommending future research. First, the survey only sought opinions of students from just one university. However, every university is different. Therefore, norms and preferences of Facebook users and time usage on this SNS might vary from one university to another or from one part of the country to another.

The next limitation of this study involves the lack of facilities and human resources to fully examine the topic of spending time on SNS. Due to these constraints, only 595 students were surveyed. The data comes from students at Karlstad University in Sweden. It is problematic to generalize these findings to the entire worldwide population of SNS members who have diverse backgrounds and different levels of education with different nationalities, attitudes, preferences, and expectations.

Third, the participants were students at one university in Sweden. In this case, the choice of students was appropriate since they are the primary target of one SNS used as a frame of reference in this study. It seems that SNS users are have a wide range of education and interests. Accordingly, to increase the validation of these results and generalize the study's findings, future research should examine other sample groups in different age groups such as high school students, homeowners, and so on.

In addition, the study focused on participants who were studying in Nordic countries. Culture, stage of economic development, and political–economic traditions in Sweden surely influenced the preferences of Facebook users in this country.

The number of Facebook users has increased dramatically in today's global village. Not surprisingly, the site is an important part of daily life among students and its phenomenon has spread out steadily.

Therefore, further investigation is needed to learn if there are specific cultural and nationality differences among Facebook users that could influence generalizability. However, it must be kept in mind that many methodological differences exist among these studies, each of which uses different estimation techniques, question formats, and/or vehicles of payment. Some supplementary issues that should be considered in future research include users' reasons and expectations for using social networking sites such as Facebook. Is it to keep in touch with personal friends or to meet new friends? The content and use of social networking should certainly be a topic of discussion in any managerial aspect such as human resource management (HRM), informal learning within organization, and so on. Consequently, these aspects can be used to either approve or reject our findings.

Acknowledgements:

The author would like to acknowledge Dr. Mehdi Semati, associate professor of communication at Eastern Illinois University (USA) for his helpful comments.

Corresponding Author:
Alimohammad Aghazamani
Duettgatan 3, 65636, Karlstad, Sweden
Phone: +46.720.417131
E-mail: Aghazamani99@yahoo.com

References

1. Baltaretu, C., Balaban, C (2010) Motivation in Using Social Network Sites by Romanian Students. A Qualitative Approach, Journal of Media Research, pp. 67-74
2. Boyd, D., & Ellison, N. (2007). Social network sites: Definition, history, and scholarship. Journal of Computer-Mediated Communication, 13(1), article 11. http://jcmc.indiana.edu/vol13/issue1/boyd.ellison.html

3. DiMaggio, P., Hargittai, E., Neuman, W. R., & Robinson, J. P. (2001). Social implications of the Internet. Annual Review of Sociology, 27, 307–336.
4. Facebook (2010) Statistics, [Electronic] Available: http://www.FACEBOOK.com/press/info.php?statistics [2010.09.05]
5. Jalalian M., Latiff AL., Syed Hassan ST., Hanachi P., and Othman M. (2008). Application of Internet and Information Technology in Recruitment of Safe Blood Donors. Journal of applied sciences, Volume 8, Number 24, p4684-4688.
6. Jalalian M., Latiff L., Syed Hassan ST., Hanachi P., and Othman M. Development of a questionnaire for assessing factors predicting blood donation among university students: A pilot study. Southeast Asian journal of tropical medicine and public health. 2010; 41(3):660-666
7. Kraut, R., Patterson, M., Lundmark, V., Kiesler, S., Mukopadhyay, T., & Scherlis, W. (1998). Internet paradox: A social technology that reduces social involvement and psychological well-being. American Psychologist, 53(9), 1017–1032.
8. Mitra, A. (1997). Diasporic Web Sites: Ingroup and Out-group Discourse. Critical Studies in Mass Communication, 14, 158-182.
9. Raskin,R (2006) FACEBOOK(TM) faces its future , Young Consumers; 2006 Quarter 1, Vol. 7 Issue 2, p56-58
10. Zhang, L, Amos, C (2008), a Comparative Study of Internet Addiction between the United States and China, CYBERPSYCHOLOGY & BEHAVIOR Volume 11, Number 6

10/10/2010

Succession Planning In Iranian Governmental Agencies

Masoud Porkiani[1], Malikeh Beheshtifar[2], Mahmood Nekoie-Moghadam[3]

[1.] Assistant Professor, Management Department, Islamic Azad University, Kerman Branch, Kerman, Iran
[2.] Member of scientific board, Management Department, Islamic AZAD University, Rafsanjan Branch, Rafsanjan, Iran
[3.] Associate Professor, Management Department, Kerman University of Medical Sciences, Kerman, Iran
m.beheshtifar@yahoo.com

Abstract: It is becoming increasingly challenging for organizations to obtain qualified and talented staff. Succession planning is often introduced as a way to attract and employ such staff. Succession planning is a process of recruitment and development of employees for vital roles within the organization. Implementation of succession planning is central to certain organizational requirements. This research surveyed organizational requirements in Iranian governmental agencies and their relation to the implementation of succession planning. This study used descriptive methods with correlation. The statistical population consisted of two groups, experts and managers of Iranian governmental agencies, and data was collected using three questionnaires. The findings of this study demonstrated a meaningful relationship between organizational requirements such as managers' commitment, organizational culture, organizational readiness, and managers' competencies with the implementation of succession planning. By considering these organizational requirements in their management practices, managers are more likely to be successful in recruiting, evaluating, training and developing talent as dimensions of the implementation of succession planning.

[Masoud Porkiani, Malikeh Beheshtifar, Mahmood Nekoie-Moghadam. **Succession Planning In Iranian Governmental Agencies.** Journal of American Science 2010;6(12):736-741]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: succession planning, requirements, managers' commitment, organizational readiness, competencies

1. Introduction

In the modern, globally-competitive environment, the need for substantial changes and creative work is always felt by organizations. This has forced the modern organizations to consider new working methods when encountering less stability and rapid change in their sectors. However, human resources departments should not only solve specific problems, but also must ensure the success of the organizations in facing potential challenges of the future. One of these challenges is the demand for qualified and talented staff. In order to attract and employ such staff, succession planning (SP) is introduced. SP is vital for day-to-day decision-making in an organization and is considered an important tool for developing individuals (Huang, 2001). This system is also one of the most important mechanisms available for ensuring that an organization can train its employees to provide its future workforce.

SP can be defined as an attempt at proper planning regarding the number and quality of managers and staff with key skills in order to compensate retirement, death, serious illness or promotion, and any new circumstance that may be created in the future plans of the organization (Sambrook, 2005). Likewise, SP is a component of human resource planning that is consistent with the overall strategy of the organization (Beaver and Hutchings, 2004) and is a studied as an intentional program for which the organization and staff must be prepared for future vacant posts (Nuttall, *et al*., 2007). Xavier (2007) shows that it is necessary for managers to venture future managers' development.

Leaders rarely consider the results of their leadership, and after leaving the organization, they realize that the majority of their job was futile, because they were unable to find a qualified successor (Blackaby and Blackaby, 2001), while each post in the organization is required to have an identified successor (Ndwandwe, 2007). Some organizations seek qualified individuals to replace the leadership, but often these eligible employees are replaced for posts which are not related to their skills or career goals (Khurana, 2002). A recent study of Fortune 1000 companies shows that only 64 percent of these companies apply SP processes, and only 50 percent believe in its effectiveness (Wider & Enders, 2004). According to Schramn (2005), the latest forecast by the Society for Human Resources Management (SHRM) shows that few organizations implement management succession plans. Another study shows that, nearly two-thirds of executive managers in the world's largest agencies believe that

they will leave the organization in the next 10 years. Among these managers, 39 percent said they had a substitute for themselves in their mind, and 45 percent of these managers had not set any successor (Jusko, 2005). Alternatively, in some governmental agencies, human resource professionals avoid SP because they worried that the merit system principles of their organization would not be executed (Green, 2000). Although the implementation of SP is simple in theory, in practice it may prove difficult. Besson and Haddadj (2003) believe that implementation of SP processes seems to be difficult for two reasons. First, senior executive do not want to predict successors because choosing a successor means accepting one's resignation. Second, senior executives often prefer to choose his or her own successor from the members of his or her family. Murphy (2006) proposes these administrative problems, as well—underestimating the talent in the organization, failure to create educational opportunities and improvement, lack of managers' sense of responsibility for SP, and attention to SP in high levels of the organization.

Investing in existing staff and improving their skills (Seymour, 2008) through SP can be beneficial for an organization, as SP can increase the ability of the staff to respond to environmental changes (Fairburn, 2008). Additionally it creates incentives in the workforce and considers organizational processes such as recruitment, maintenance, training and development, and the performance management (Hammer, 2004). According to Rothwell (2005) the first step to succession planning is recognition of the organization's problems. However, SP is not considered a serious issue for many organizations. Managers have been reluctant to invest in and support SP because it seems that the workforce of the country is relatively stable and the government feels that any retirement crisis that may occur is in the far future. The most important causes of the failure SP for many organizations can be found in the disability of the program to maintain the necessary support from the organizational culture for example, looking at the program and neglecting the need for comprehensive attitude (Abolala'ei and Ghafari, 2007). Additionally, the findings of Iran's administrators database by the Management and Planning Organization in 2003 show that 52 percent of the country's managers had diploma and associate degree qualifications, and many senior managers have not participated in quality management training. The knowledge and skills of the managers and their managerial experience does not often correspondent with their responsibilities. In government agencies and industrial firms, the number of senior managers who began their career after completing an undergraduate degree and have reached high positions by demonstrating their performance capabilities is very low. Alternatively, the lack of suitable replacements for key organizational posts, i.e. managers and supervisors, at times of emergency such as retirement or promotion of managers is among the many problems faced by organizations in Iran. Most organizations are facing serious problems in filling management posts from among capable and qualified individuals. Lack of a rational and efficient system to promote everyone based on merit and performance and the lack of knowledge among many Iranian directors about the SP system and its implementation results are a main source of difficulties encountered by Iranian governmental organizations (Asmarian, 2004).

Figure 1. A model for SP

The second step is recognition of organizational requirements before the implementation of SP (Rothwell, 2005). Many organizations fail due to a lack of requirements as a main factor. The requirements that any organization must consider before implementing SP include management commitment to SP systems (Rothwell, 2005; Abolala'ei and Ghafari, 2007); participative and supportive organizational culture for the SP systems (Tropiano, 2004; Groves, 2007); preparedness of the organization for the SP system (Rothwell, 2005; Abolala'ei and Ghafari, 2007); capabilities of managers for the SP system (Brooks

and Henderson, 2005; Horton and Duggan, 2005; Lavinga, 2005). After recognition of these organizational requirements, implementation of SP is important. By studying the SP models, it was found that implementation of SP consists of three components—evaluation of present resources (Rothwell, 2005; Wider and Enders, 2004; Brooks & Henderson, 2005); Training and developing talented individuals (Rothwell, 2005; Curlin, 2009; Horton and Duggan, 2005; Groves, 2007; Brooks and Henderson, 2005; Steeves and Ross, 2003), and recruitment of talented individuals (Rothwell, 2006; Groves, 2007). Ultimately, a model for SP is designed (figure 1).

Successful SP in Iranian governmental organizations could be important. For example, SP will lead to the codification of capability models, identification of training needs, promotion of a competence culture, development of staff skills, and improvement of their capabilities. Additionally, providing a management database and detection of management talent within the organization through scientific method can help the organization's authorities to select qualified people for key posts more accurately and avoid common mistakes of manager selection. At the same time, the qualified individuals could reach higher levels of organizational hierarchy outside the normal path of career progress. This research will consider the status of organizational requirements and SP implementation in the public sector. Therefore, seeks to answer the following questions:

1. What is the status of organizational requirements in Iranian governmental agencies?
2. What is the status of SP implementation in Iranian governmental agencies?
3. What is the relationship between organizational requirements and SP implementation in Iranian governmental agencies?

2. Material and Methods

This study was uses the descriptive method with correlation. It uses the Delphi method to design the early model and the questionnaire. The statistical population consists of two groups; the first group consists of 30 experts chosen by selective sampling throughout the country. The second group consists of all managers of executive organizations chosen from five provinces of the 30 provinces in Iran by selective sampling. In each province, there are 72 governmental organizations from which was chosen a senior manager and a middle manager. It is choosing these 720 managers, seven organizations did not cooperate. The sample size is then 65 organizations and 670 managers. Ultimately, 628 managers filled in the questionnaires.

The collection of data is conducted using three questionnaires. The first is used to design an introductory model by experts, which is accepted. The second and third questionnaires evaluate organizational requirements and SP implementation, respectively. The validity of the organizational requirements questionnaire and the SP implementation questionnaire are 0.884 and 0.865, and the reliability of these two questionnaires are 0.881 and 0.884. SPSS is used analyze these data.

3. Results

The demographic characteristics are categorized as follows—626 managers among of 628 marked their ages. 21 managers (3.4 percent) were 30 years old and under; 228 managers (36.4 percent) were between 31 and 40 years old; 377 managers (60.2 percent) were 40 years old or more. Of the 628 managers interviewed, 622 marked their education level. Fifteen managers (2.5 percent) had diploma degree; 318 (51.1 percent) had a Bachelor's degree; 219 managers (35.2 percent) had a Master's degree; and 70 managers (11.3 percent) had a PhD degree.

The Sign Test is used to survey the status of organizational requirements and SP implementation. Managers were asked their opinion of the status of organizational requirements and SP implementation, and the median was evaluated to be more than 3 (rather good). The median of managers' opinions about manager commitment, organizational culture, organizational readiness, managers' competencies (as organizational requirements), evaluation of present resources; training and developing talents; and recruitment of talents (as SP implementation) was evaluated to be more than 3 (rather good).

Table 1. Result of Corelational analysis.

	Variables	Correlation coefficient	P Value
Organizational requirements	SP implementation	0.609	0.000
	Evaluation of individuals	0.515	0.000
	Training and developing talents	0.612	0.000
	Recruitment of talents	0.502	0.000

By using the Spearman Test, the correlation coefficient calculates the P Value with sample size n=628 was less than 0.05; this shows a meaningful

relationship between organizational requirements and SP implementation. Other results show a meaningful relationship between manager commitment, organizational culture, organizational readiness and manager competencies with SP implementation (Table1).

4. Discussions

Results show a meaningful relationship between organizational requirements and SP implementation. Rothwell (2005) emphasizes that the basis of SP implementation is recognition of organizational requirements before implementation. A study of Ibrahim, *et al*. (2004) shows elements that are vital for SP, including management competencies and skills, and successor's commitment to the organization. Many studies such as Rothwell (2005), Horton and Duggan (2005), Tropiano (2004), Murray (2007), and Steeves ajnd Ross (2003) show these relationships. Therefore, organizational requirements are emphasized as an urgent prerequisite in SP implementation. Therefore, it is prospected with the existence of organizational requirements that managers perform better in the evaluation of present resources, and training, development and recruitment of talent. Management commitment as a secondary variable is a force by which a person participate in the activities related to a specific aim (Shahnawaz and Juyal, 2006); so it is needed to have committed individuals for obtaining the success of an organization (Martin, 2008). Many studies confirm the positive effects of manager commitment. Committed individuals can recognize organization goals and values better, and they have stronger tendency for dependency to the organization (Nehmeh, 2009). Huang (2001) shows that SP cannot improve HR performance, but manager commitment to SP is an initial requirement for success. Therefore, it is prospected that manager commitment to SP is caused by desirable SP implementation.

Organizational culture as another variable is a main factor to the success of the leader (Walseth, 2009), and it plays a basic role in SP implementation (Ndubisi, 2008). Tan (2009) also shows how SP processes are influenced by culture. Organizational culture, especially a culture of collaboration and support, are essential for SP implementation (Tropiano, 2004). As a collaboration culture plays a key role in the learning process, a supportive culture can affect continuous commitment (Song and Kim, 2009). A collaboration culture is caused by continuous investment in increasing employees' skills, and persuading cooperation for development. A supportive culture supports talents in new roles. Therefore, a prospected collaboration culture and a supportive culture are influenced by desirable SP implementation. Organizational readiness is another variable that stresses the preparation of all departments of an organization (World Health Organization, 2009). Although there is not any particular frame to SP, a proper starting point for SP implementation is organizational readiness and pursuit of activities. Therefore, organizations should evaluate their readiness (Ritchie, 2005), as it is necessary to ensure plan success. Organizational readiness such as prospecting management needs, an active HR department, a tendency to merit, and retaining organization from politic pressures is needed.

Manager competencies, as a final variable, are employed as basis for all SP activities (Herr, 2007). Additionally, competency models are considered the base of SP implementation. These models provide a plan to make present and future required competencies (Rothwell, 2005). Surveys show that the competency format can be targeted toward different goals in HRM, such as management development, career planning, SP, and performance management (Wickramasinghe and De Zoyza, 2009). Alternatively, competencies provide a common language in HR areas such as selection, development, and SP (Berge, *et al*. 2002). Manager competencies such as conceptual skills, personality factors, decision making skills, and communication skills should be considered in organizations.

Other results show that dimensions like evaluation of human resources; training and developing talents, recruitment of talents to implement SP should be required. In the evaluation of human resources, managers can use performance management and evaluation centers. To develop talent, managers must pay attention to career policies in regards to the necessity of talented forces, to forming partnerships in training plans, and to appointments and promotions in regard to training and growth. Additionally, when training talents, managers should notice training plans and their feedback. When recruiting talents, it is necessary for managers to identify present employees and talents of outside organizations for forming the talent pool. Meanwhile, managers should select young and apt forces for increasing management reserves, and consider recruitment tests for identifying talents in the organization. Ultimately, SP is a strategic plan and a powerful tool not only for reinforcing and developing talent but also for turning a failing organization on a positive path. Identifying organizational requirements and implementing SP are complex tasks that require ongoing attention and sufficient resources. The organizations should spend tremendous resources and time in designing skills

and competencies to recruit, train and evaluate talent. Therefore, the revival and continuous updating of organizational plans is a good start for SP.

It is suggested that organizations' middle managers, senior managers and human resource managers place SP among their main tasks. It is necessary to design a native competencies model to use within governmental organizations. It is important to identify the educational needs of managers at different levels. Iranian organizations should consider activities such as equipping human resource departments, emphasizing the constancy of senior management teams, and utilizing educated and experienced managers to ensure readiness. Organizations should construct data banks for managers to detect management talent within the organization. Investment for developing future managers and establishment of an institute for developing young managers is necessary. It is also suggested to conduct empirical research to examine the realistic results.

Acknowledgements:

The authors would like to acknowledge the department of research, Kerman Branch of Islamic Azad University for their support and contribution to this study

Malikeh Beheshtifar, Management Department,
Islamic Azad University, Rafsanjan Branch,
Rafsanjan, Iran. Email: m.beheshtifar@yahoo.com ,
Tel. +98.9133430417

Corresponding Author:
Malikeh Beheshtifar
Management Department
Islamic Azad University, Rafsanjan Branch
Rafsanjan, Iran
Tel. +98.9133430417
E-mail: m.beheshtifar@yahoo.com

References

1. Abolalai B, Ghafari A (2008). Future managers: talent plans and succession planning. Organization of industrial management. 2(3):23-24.
2. Asmarian A. (2004). Evaluation Center and Succession planning Management. Tadbir Journal. 145: 24-25.
3. Beaver G, Hutchings K (2004). The big business of strategic HRM in SMEs in Stewart, HRD in Small Organisations. Routledge Research Studies in HRD Series. Routledge. London. 81
4. Berge Z, Verneil M, Berge N, Davis N. & Smith D (2002). The Increasing Scope of Training and Development Competency. Benchmarking: A International Journal. 9(1): 57.
5. Besson D, Haddadj S (2003). Dysfunctions in owner-manager succession process in family firms and how a SEAM intervener-researcher can address them, Journal of organizational change management. 16(1): 83.
6. Blackaby H, Blackaby R (2001). Spiritual leadership. TN: broadman and holman. 279.
7. Brooks v, Henderson TL (2005). Georgia's flexible succession planning model: growing tomorrow's leaders today. Georgia merit system. 12.
8. Curlin T (2009). You Can't Do Succession Planning in the Public Sector …Or Can You?. Human Resources Business Unit. City of Charlotte. Human Resources Key Business:4
9. Fairburn LK (2008). Succession planning: OD consulting, facilitation, training and succession planning. Make things happen limited:2. www. Make Things Happen.net.
10. Green ME (2000). Beware and prepare: the government workforce of the future. Public personnel management. 29(4): 435.
11. Groves KS (2007). Integrating leadership development and succession planning best practices. Journal of management development. 26(3): 244.
12. Hammer B (2004). Succession planning: a critical issue for all businesses. Reprinted from The Alcott Adviso.2.
13. Herr Niki (2007). Avilar Releases Competency Model to Support Integrated Talent Management Solutions. Avilar, Inc.
14. Horton DS, Duggan BP (2005). Succession planning: embracing the future.Nova scotia business journal. June: 2.
15. Huang TC (2001). Succession management systems and human resource outcomes. International journal of manpower, 22(8): 736.
16. Ibrahim AB, Soufani K, Poutziouris P, Lam J (2004). Qualities of An Effective Successor: the Role of Education and Training. Education & Training. 46(8/9):477.
17. Jusko J (2005). Unplanned future. Industry week. 254(3):1. Retrieved May 5, 2005 from ProQuest.
18. Khurana R (2002). Searching for a corporate savior: the irrational quest for charismatic CEOs. Princeton. NJ: Princeton:1.
19. Lavigna B (2006). Preparing the Next Generation of Leaders. Government Finance Review. 22(1): 8.
20. Martin S (2008). Relational and economic antecedents of organizational commitment. Personnel Review. 37(6):590.

21. Murphy TH (2006). Succession planning and legal considerations. ABA labor and employment law section equal employment opportunity committee national conference on EEO law. March 22-25: 4-5.
22. Murray A (2007). Growing Your Own: Developing Leaders through Succession Planning. Liber Quarterly. 17 :¾. http://liber.library.uu.nl/
23. Ndubisi N (2008). Impact of Personal Culture on Sophisticated Succession Planning by SMEs on Malaysia. Proceedings of the Allied Academies. 15(2):187.
24. Ndwandwe E (2007). Succession planning is about taking risks in potential Ceo. 6(7): 1.
25. Nehmeh R (2009). What is Organizational Commitment, SMC Working Papare. Swiss Management Center. ISSN 1662- 761x: 2.
26. Nuttall I, Falkner T, Roberts V (2007). Succession planning: whatever you call it, do it. AACRAO. Boston. Massachusetts. March 2: 10.
27. Ritchie Ch (2005). Who Will Lead Tomorrow's Workforce? The Status of Succession Planning in North Carolina Municipalities. Paper Presented at the Capstone Conference. Univ of N.C. at Chapel Hill Apr. 8: 7.
28. Rothwell WJ (2005). Effective succession planning: ensuring leadership continuity and building talent from within. New York. NY: American management association: 34
29. Rothwell WJ (2006). Ten Key Steps to Effective Succession Planning. Rothwell & Associates Inc: 5. http://www.halogensoftware.com
30. Sambrook S (2005). Exploring succession planning in small, growing firms. Journal of small business and enterprise development. 12(4): 580.
31. Schramn J (2005). Learning curves. HR magazine. 50(2): 144.
32. Seymour S (2008). Boost your business value with succession planning. Human resource management international digest. 16(4): 4.
33. Song Ji, Kim H (2009). "The Integrative Structure of Employee Commitment. Leadership & Organization Development Journal. 30(3): 244.
34. Steeves R, Ross B (2003). Using Succession Planning To Transform Organizations. The Centre for Exceptional Leadership Inc: 4.
35. Tropiano M (2004). Effective succession planning. Defense AT&L: May-June: 50.
36. Walseth T (2009). The Case for Growing our Own. The Online Journal of Academic Leadership. 7(1). Feb 20.
37. Wickramasinghe V, De Zoyza N (2009). A comparative analysis of managerial competency needs across areas of functional specialization. Journal of Management Development. 28(4): 345.
38. Wider C, Enders G (2004). Succession management: a case study in diversity and change. Academic stream: human resource management. United states Postal Service. May: 3.
39. World Health Organization (2009). Whole-of-Society, Pandemic Readiness, WHO guidelines for pandemic preparedness and response in the non-health sector. Geneva. July: 6.
40. Xavier S (2007). Developing emerging leaders: a new solution to an old problem. Business Strategy Series.8(5): 343..

11/05/2010

Identification Of Fungi Prevalent On Environmental Labour Ward Of General Hospital Umuguma And Umezuruike Hospital Labourward

Ijioma B. C. Ph.D[2], Nwachukwu C. U. Ph.D[2], Akobundu, C.[1]

Department of Biology, Alvan Ikoku Federal College of Education, Owerri, Imo State, Nigeria[2].
Department of Microbiology, Federal University of Technology, Owerri, Imo State, Nigeria[1]
nwachukwucu2005@yahoo.co.uk

Abstract: There has been an increase in the frequency of fungal infections over the past decade. Nosocomial transmission of fungal pathogens and the recognition of resistance to antifungal agents pose a significant problem. This study identified the fungi species prevalent in the labour ward of the general hospital Umuguma and Umezuruike Hospital, Owerri Imo State Nigeria. Fungi are eukaryotic cells and therefore more complex than bacteria. The data available shows that Mucor Species and Rhizopus Species are the predominate species found in both hospitals in decreasing order. Fungal infection are often severe, rapidly progressive and difficult to diagnose or treat, therefore a thorough appreciation and understanding of fungi infections, including diagnostic and therapeutic modalities are needed among clinicians and microbiologists to provide a better patient care.
[Ijioma B. C. Ph.D, Nwachukwu C. U. Ph.D, Akobundu, C. **Identification Of Fungi Prevalent On Environmental Labour Ward Of General Hospital Umuguma And Umezuruike Hospital Labourward.** Journal of American Science 2010;6(12):742-746]. (ISSN: 1545-1003). http://www.americanscience.org.

Key Words: Nosocomial infections, fungi, Mucor, Rhizopus, Penicillin, Hospital, etc.

Introduction

Advances in the medical and surgical therapy over the past two decades have changed the type of patients cared for all over the world. Newer technologies and therapies such a bone marrow or solid-organ transplants and chemotherapeutic agents have become common at many medical centres resulting in many immuno-compromised individuals. Also, care in specialized units and the use of invasive monitoring devices, patient's nutrition, broad-spectrum anti-microbial agents and assisted ventilation have helped to treat patients suffering from previously devastating or fatal diseases and have provided life to premature neonates previously thought to be nonviable.

However, these successes have also resulted in complications in the severely ill and immuno-compromised patients who are highly susceptible to nosocomial infections caused by organisms such as fungi that were previously considered to be of low virulence or "non-pathogenic" (Bodey, 1988). Fungi infections in these patients are often severe, rapidly progressive and difficult to diagnose or treat (Edwards, 1991). In the mid -1980s, many institutions, including cancer research, university and community hospitals reported that fungi were becoming common pathogens in nosocomial infections (Harvey and Myers, 1987). In addition, National Nosocomial Infections Surveillance (NNIS) system reported a steady increase in the rate of nosocomial fungal infections, from 2.0 to 3.8 per 1,000 discharges (Beck-Sague and Jarvis, 1993). The modes of transmission vary and include environmental spread through air, carriage on the hands of hospital personnel and contamination of medical products or devices before (ie intrinsic contamination) or after (ie extrinsic contamination) shipment to hospitals. Understanding the pathophysiology of pathogenic fungi is critical in determining the cause of an outbreak (Benneti *et al*, 1995).

Nosocomial infection could be defined as infections which are as a result of treatment in a hospital or treatment in a healthcare service unit, but secondary to the patient's original condition (Andereoli *et al*, 1997; Nester *et al*, 2004) that is hospital acquired. These infections can also appear within 30 days after discharge, it may be rampant among people who are sick and whose immune systems are in a weakened state, host – flora equilibrium imbalance caused by prolonged intake of antibiotics, the pathogen could be exogenous or endogenous (Andreoli *et al*, 1997). Opportunistic mycoses are equally pressing problems, and occurs primarily in immuno copromised patients (particularly those with malignancies and AIDS) and in patients who have undergone major surgery, bone marrow or solid organ transplantation or who have been severely burned. *Candida albacores*, *Aspergillus* spp. and *zygomycetes* spp. (Bross *et al*, 1989) have been implicated in mycoses infection. *Aspergillus fumigatus*, *A. flavus* and A. *terreus* have become a common cause of nosocomial infection in highly immunocompromised patients such as those with haematologic malignancy undergoing bone marrow or solid organ transplantation or receiving corticosteroid therapy (Anaissie and Bodey, 1989). *Trichosporon bergelii*, *Acremonium* Spp., *Candida* Spp. (*C. albicans* is by far the most common Candida Spp causing infections in humans),

Fusarium Spp., *M. Pachydermatis, Paecilomyces* Spp. have all been implicate in one nosocomila infection or the other (Rowsey *et al*, 1979; Chang *et al*, 2007). Some studies have identified common risk factors for patients developing fungal infections in labour ward, some due to the presence of immunosuppressant or a combination of factors (Karabinis *et al*, 1988). The health care environment can become highly contaminated with nosocomial pathogens that are able to survive for long periods of time like weeks or even months, on bed rails, telephones, call buttons, taps, door handles, mattresses, chairs, floors and on surfaces frequently touched by hand, long enough to be transmitted to cause infection as well as in the air and in dust (Johnson and Conly, 2006). Sometimes health care personnel may be a carrier of a pathogen such as *Candida* Spp. or *Aspergillus* Spp. which they pass on the patient during care providing (Andreoli *et al* 1997; Nester et al, 2004).

Materials And Methods

Preparation Of Media, Chemicals And Reagents

Samples collected were from floor, bed rail, mackintosh, wall and mattress etc. sterilized swab stick moistened with sterile peptone water was used to swab the wall, floor, mackintosh, mattress, bed rail and baby bed in the labour ward while the air was sampled by exposing Sabourand dextrose agar (SDA) plate for 2hrs. The samples collected using the swab stick was inoculated onto the already prepared SDA plates dried in the oven at 40^{o}C and incubated for 72hrs (3 days) at room temperature. Identification using microscopic examination was done by wet preparation using lactophenol cotton blue stain and then examined under the microscope. Cultural and morphological characteristics were also used for the identification. The size, shape, speculation and pigmentation were seen with a magnifying lens, identity of the isolates were further confirmed microscopically with reference to Barnett Hunter (1987). The prominent fungi isolated includes Aspergillus, Mucor, Penicillum, Rhizopus species (yeast) and Candida.

Results: Same sample used for the two hospitals

TABLE 1: *Aspergillus niger*

Sample Code	Colony Code	Gross morphology macroculture	Microscopic Appearance	Most Probable Identity
Uf	Ufi	Black spores with white condiospores. It grows in colonies or clusters	Septate hyphae conidia occur on large radiating heads. condiospores arises from a segment of mycelium called a foot cell.	Aspergillus niger
Um	Um	Black spores with white condiospores.	Aseptate unbranched stipes with swollen vesvlerhizoids are borrn derectly on the vescile.	Aspergillus niger
Gw	Gwi	Black spore with white condiospore in zones. Conidia head radiate and tends to split	Conidiosphores stipes smooth walled; Conidia borne on the stigma	Aspergillis Niger
	GBB_2	Black spores with white condiospores in zones conidia head radiate and tends to split.	Conidiosphores stipes smooth walled, Conidia borne on the stigma.	*Aspergillus niger*

TABLE 2: *Candida* Species

Sample Code	Colony Code	Macroculture	Microscopic Appearance	Most Probable Identity
	GMT_2	2mm whitish colonies with short-hair like spite around the periphery irregular	Large gram positive oval budding yeast cells with short strands of pseudomycellium	Candida species
	UF_2	About 2mm whitish colonies with short hair like spite around the periphery and irregular shape.	Large gram positive oval budding yeast cells with short strands of pseudomycellium	Candida species

TABLE 3: *Penicillium* Species

Sample Code	Colony Code	Macroculture	Microscopic Appearance	Most Probable Identity
	UIR_2	Olive green spores with white periphery.	Septate hyphae conidiophores with smooth stripe and branched.	Penicillium species
	GW_2	Olive green spores with white periphery.	Septate hyphae conidiophores with smooth stripe and branched.	Penicillium species.
	GBR	Olive green spores with white periphery.	Septate hyphae conidiophores with smooth stripe.	Penicillium species.

TABLE 4: *Rhizopus* Species

Sample Code	Colony Code	macroculture	Microscopic Appearance	Most Probable Identity
UBB	UBB	White filamentous hyphae bearing black spores.	Sporangia are globose with slightly rough walled stolen opposite the branched rhizoids.	Rhizopus species
	UMT	White filamentous hyphae bearing black spores.	Sporangia are globose with slightly rough walled stolen opposite the branched rhizoids.	Rhizopus species
	GA_2	White filamentous hyphae bearing black spores.	Sporangia are globose with slightly rough walled stolen opposite the branched rhizoids.	Rhizopus species

TABLE 5: *Mucor* Spp

Sample Code	Colony Code	macroculture	Microscopic Appearance	Most Probable Identity
GF	GF	Black spores with white periphery	Colony composed of both tall and short sporangia which are branched in conopodia fashion.	Species
GBB	GBB_1	Greyish cotton hyphae raised from plates.	Non septate hyphac, sporangiospores symbolically branched with long and short criminate branches	mucor Species
	GA_1	Black spores with white periphery	Colony composed of both tall and short sporangia which are branched in conopodia fashion	mucor Species
UBR	UBR	Greyish green cotton hyphae raised from plates.	Non septate hyphae, sporangiospores symbolically branched with long and short criminate branches	mucor Mucor Ciricinelloides
ULR	ULR_1	woolly like hyphae spreading on the surface of the plate.	Colony composed of both tall and short sporangia which are branched in conopodia fashion.	Species
GMT	GMT	Olive green spores with white periphery	Colony composed of both tall and short sporangia which are branched in conopodia fashion.	Species
	ULW	Olive green spores with white periphery	Colony composed of both tall and short sporangia which are branched in conopodia fashion.	Species

NOTES:
UMT: Represents Umezuruike Hospital mackintosh
GBR: Represents General hospital Umuguma Bedrail
GBF: Represents General hospital Umuguma floor
GW: Represents General hospital Umuguma wall
GM: Represents general hospital Umuguma mattress
GBB: Represents General hospital Umuguma baby bed
GA: Represents General hospital Umuguma Air

DISCUSSION

The effects of fungi in labour wards cannot be under estimated, the result obtained showed that *Aspergillus* Spp have become a common cause of nosocomial fungal infection in highly immuno compromised patients such as those with rheumatologic malignancy undergoing bone corticosteroid therapy. *A. funigatus* are tolerant at temperature up to 50^{o}C, colonies grow rapidly and are white and velvety at first but soon become green, yellowish or black and powdery as the conidia are formed. *Aspergillus* Spp. infections usually occur in the lungs, people breathing clouds of conidia from the air of granaries, barns and silos, spores simply germinate in the lungs and form fungus balls. In the more invasive form, *Aspergillus* Spp. produces a necrotic pneumonia and disseminates to the brain, heart, skin and a wide range of other organs systemic aspergillosis usually occurs in very ill hospitalized patients with a poor prognosis (Anaissie and Bodey, 1989). Candida Spp the prevailing opportunistic pathogens of humans are the yeasts of Candida which are extremely wide spread yeast, it is the major cause of Candisiasis (also called Candidosis or moniliasis). *Candida albicans* occurs as normal flora in the oral clarity, genitalia, large intestine or skin of 20% humans. Although Candidacies is usually endogenous and not contagious, it can be spread in nurseries or through surgery, child birth, sexual contact and it account for nearly 80% of nosocomial fungal infections and 80% of nosoconial infections, 30% of deaths from nosocomial infections in general; *Candida albicans* causes local infections of the mouth vagina, skin and lungs. It can also disseminate to internal organs. The mucous membranes most frequently involved are the oral cavity and vagina (Bodey, 1988). The rhizopus species are extremely abundant saprobic fungi found in soil, water and food. Their large prolific sporangia release multitudes on humans and usually do little harm beside spoiling foods and rotten of fruits and vegetables. But an increasing number of critically ill patients are contracting a disease called zygomycosis which can cause a patchy infection of the nail bed like tineaungium involved in infection of the lungs of tubercular or highly immunosuppressed patients and occasionally infect the eyes, toe, nails and burned nails. (Rowsey *et al*, 1979). Mucor species can be differentiated from moulds of genera absidia, Rhizomes and Rhizomucor by the shape and insertion of the columnella and the lack of rhizoids. Some mucor species produce chlamydospores. The species have become a common cause of nosocomial fungal infection in imminocompromised patients such as renal failure, diabetes mellitus, receipt of antimicrobial agents, severe underlying disease and exposure to hospital construction activity. Penicillum Spp have been considered as an important causative agents of extrinsic bronchial asthma, they can penetrate the living of the intestine and invade the liver, lungs and skin. Untreated cases with extensive damage to the organs experience a high death rate.

From this study it has become pertinent to note that nosocomial fungal infection are becoming more prominent. There is an increase in the number of immunocompromised patients and patients receiving a broader range of antimicrobial agents in hospitals today compared with previous years. Consequently infections due to previously obscure fungi are being seen more commonly in hospitalized patients. Although diagnostic and therapeutic modalities for some fungi are improving such as those used for Candidiasis or aspergillosis, there is still much to learn about many of the other fungi discussed. Standards for susceptibility testing are currently being developed and should help guide clinicians and hospital epidemiologists in the management of nosocomial fungal infections. Continued epidemiologic and laboratory research is needed to better characterize these pathogens, allowing for improved diagnostic and therapeutic strategies in future. Sensitivity of organisms to antibiotics in use should be checked, needles and sharp objects should be discarded in rigid, puncture proof container without contact using bare hands or replacement of needle caps. Linen and solid reusable items should be placed in protective bags to prevent leaking and further contamination.

REFERENCES

1. Anaissie, E. and Bodey, G. P. (1989). Nosocomial fungal infections; old problems and new challenges infect. *Dis.Chin North Am* **3:** 867-882.
2. Andreoli, T. E., Bennett, T. C., Carpenter, C. and plum, F. (1997). Cecil Essentials of Medicine, 2nd ed. Phladelphia; W.B. Saunders Co., Pp. 110.
3. Beck-Sague, C. M. and Jarvis, W. R. (1993). The national Nosocomial infections surveillance system. Secular trends in the epidemiology of Nosocomial infections surveillance system. Secular trends in the epidemiology of Nosocomial fungal infection in the United States, 1980-1990. *J. Inject. Dis.* **167:** 1247-1251.
4. Benneti, S. N., Villarino, D. M., Perrotta, D. R., Burwen, S. F., Welbel, D. A., Pegues, L. (1995). Post operative infections traced to extrinsic contamination of an intravenous anaesthetic. *N. Engl. J. Med.* **333:** 147-154.

5. Bodey, G. P. (1988). The emergency of fungi as major hospital pathogens. *J. Hosp. infect.* **2** (a); 411- 426.
6. Bross, J., Talbot, G. H. G., Maislin, G., Hurwhitz, S. and Strom B. L. (1989). Risk factors for nosocomial candidemia; a case-control study. *Am. J. Med.* **87:** 614-620.
7. Chang, P.C Huang, L.M. and Lin H.C. (2007). Control of an outbreak of pandrug resistaint Atinotobacter Baumann colonization and infection in a neonatal intensive care vent. *Infect. Control Hosp. Epidemiol* **28(4):** 423-429.
8. Edwards, T. E. (1991). Invasive candida infections Evolution of a fungal pathogen. *N. Engl. J. med.* **324:** 1060-1062.
9. Harvay, R. L. and Myers, J. P. (1987). Neocolonial fungemia in a large community teaching hospital. *Arch. Intern. Med.* **147:** 2117-2120.
10. Johnson, B. L. and Conly, J. (2006). *Clostriduim difficile;* The evolving story. *Canadian J. infect Dis. And Medical Microbiol.* **18(6):** 341-345.
11. Karabinis, A., Leclerg, B., Tancrede, C., Baume, D. and Andremont, A. (1988). Risk factors for candidemia in cancer patients; a case control study. *J. Clin. Microbiol.* **26:** 429-432.
12. Nester, E.W., Anderson, D. G. and Roberts, C. E. (2004). Microbrobgy; A Human perspective, 4th ed. McGraw HIV; New york U. S. A. Pp. 499.
13. Rowsey, J. J., Acers, T. E., Smith, D. L., Mohr, J. A., Newsom, D. L. and Rodriguez, J. (1979).
a. *Fusaruim oxysporum* endophathalmitis. *Arch. Opthalmol.* **977:** 103-105.

10/26/2010

A modified Algorithm to Model Highly Nonlinear System

Tharwat O. S. Hanafy

Al_Azhar University,Faculty of Engineering, Systems and Computers Department
s_ewiss@yahoo.com

Abstract: In this paper, the Fusion of neural and fuzzy Systems will be investigated. Membership Function Generation and its mapping to Neural Network are introduced. An adaptive network fuzzy inference system (ANFIS) is introduced, and Multiple Inputs /Outputs Systems (Extended ANFIS Algorithm) is implemented. A Modification algorithm of ANFIS, Coupling of ANFIS called coactive neuro fuzzy system (CANFIS), is introduced and implemented using Matlab. The software of the modified algorithm of MIMO model identification is built. To test the validity of the modified algorithm ANFIS (CANFIS algorithm), an example is simulated from the numerical equation. The result of modified algorithm (CANFIS) showed a conformance with the simulated example and the root mean square (RMSE) is very small.
[Tharwat O. S. Hanafy. A modified Algorithm to Model Highly Nonlinear System. Journal of American Science 2010;6(12):747-759]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: A modified Algorithm to Model Highly Nonlinear System

1. Introduction:

Fuzzy logic was first developed by Zadeh [1] in the mid-1960s for representing uncertain and imprecise knowledge. It provides an approximate but effective means of describing the behavior of systems that are too complex, ill-defined, or not easily analyzed mathematically. Fuzzy variables are processed using a system called a fuzzy logic controller. It involves fuzzification, fuzzy inference, and defuzzification[2]. An adaptive network fuzzy inference system is investigated. In this paper we will introduce two different learning algorithms. Also, Multiple Inputs /Outputs Systems (Extended ANFIS Algorithm) is implemented using two methods. Modification algorithm, Coupling of ANFIS is called coactive neuro fuzzy system (CANFIS), is introduced and implemented using Matlab. Also, software for modification algorithm for MIMO is built. To test the validity of the modified algorithm ANFIS (CANFIS) algorithm, an example is simulated from the numerical equation[3]. This paper organized seven sections. The first section is introduction. The structure of neuro fuzzy systems is introduced in section 2. The generating fuzzy rules are investigated in section three. Two different ANFIS learning algorithm presented in section four. The modified algorithm (CANFIS) is introduced in section five. Section six introduced conclusion.

2. Neural Fuzzy Systems

In order to process fuzzy rules by neural networks, it is necessary to modify the standard neural network structure appropriately [2]. Since fuzzy systems are universal approximators, it is expected that their equivalent neural network representations will posses the same property. The reason to represent a fuzzy system in terms of neural network is to utilize the learning capability of neural networks to improve performance, such as adaptation of fuzzy systems. Thus, the training algorithm in the modified neural networks should be examined.

2.1 Membership Function Generation

A generalized bell membership function, commonly referred to as bell MF, is characterized by three parameters namely a, b, c.

$$\mu_{Ai}(x_1) = \exp\{-[(\frac{x - c_i}{a_i})^2]^{b_i}\}, \qquad (1)$$

A desired, generalized bell membership function can be obtained with the proper selection of the parameters a, b, c. The parameters a and c represent the width and the center of the bell function, and b represents the slopes at the crossover points. Various other membership functions such as triangular, trapezoidal, Gaussian, and sigmoidal can be used in the formulation of membership functions [3]. The triangular and trapezoidal membership functions, due to their simplicity and computational efficiency, are used extensively in the formulation of membership functions (MF) consist. The Gaussian, the generalized bell function, and the sigmoidal membership functions are smooth and nonlinear functions and are increasingly popular for specifying fuzzy sets. The generalized bell function has one parameter more than the Gaussian membership functions, resulting in an extra degree of freedom to adjust the steepness at the crossover points [4].

The reason to represent a fuzzy system in terms of a neural network is to utilize the learning

capability of neural networks to improve performance, such as adaptation of fuzzy systems. To convert Membership Function from FIS structure to construct the same membership using NN layer. The parameter of membership equals to weights and bias in neural network. Fig. (2) Shows the representation of parameters for membership function using NN. Let the bell MF from eq. (1)

Where $a(n) = e^{-n^2}$ Transfer Function (TF) (2)

Let

$$b = 1$$

$$n = \frac{x-c}{a} = \frac{1}{a}x - \frac{c}{a}, \quad \because n = wx + bias \qquad (3)$$

$$w = \frac{1}{a}, \quad b\text{ias} = -\frac{c}{a}$$

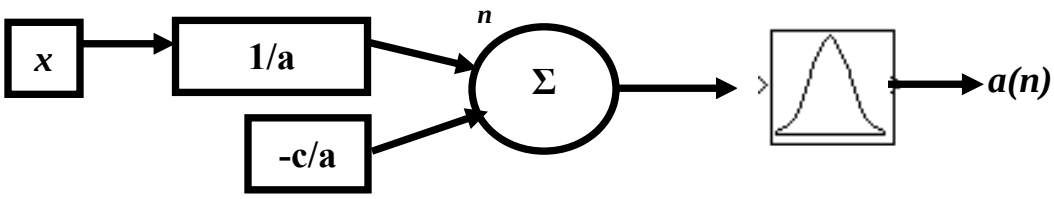


Fig. (2) Representations of membership function using NN

2.2 Adaptive network fuzzy inference systems

To illustrate the use of neural networks for fuzzy inference, we present some successful adaptive neural network fuzzy inference systems, along with training algorithm known as ANFIS. These structures, also known as adaptive neuro fuzzy inference systems, were proposed by Jang [6]. It should be noted that similar structures were also proposed independently by Jang [6]. These structures are useful for control and for many other applications. To fix the ideas, consider the problem of graphically representing the way fuzzy control is achieved in the Sugeno-Takagi model. For a simple example, consider a fuzzy rule base consisting of only two rules [7]:

R_1: if x_1 is A_1 and x_2 is B_1 then $y=f_1(x)$
R_2: if x_1 is A_2 and x_2 is B_2 then $y=f_2(x)$
(4)

where A_i and B_i are fuzzy sets and

$$f_1(x) = a_0^1 + a_1^1 x_1 + a_2^1 x_2$$

$$f_2(x) = a_0^2 + a_1^2 x_1 + a_2^2 x_2$$

Recall that when numerical input x= (x_1, x_2) is presented, the inference mechanism will produce the numerical output [8]

$$y^* = \frac{A_1(x_1)B_1(x_2)f_1(x) + A_2(x_1)B_2(x_2)f_2(x)}{A_1(x_1)B_1(x_2) + A_2(x_1)B_2(x_2)} \quad \textbf{(5)}$$

The ANFIS model suggested by Takagi and Sugeno represents a mathematical tool, which is used to build an ANFIS model of a system. ANFIS model of a non-linear system implication rule contains fuzzy variables with unimodal membership functions. Since such membership functions are linguistically understandable, the fuzzy variables are also called linguistic variables. Takagi and Sugeno's fuzzy model approximates a nonlinear system with a combination of several linear systems by decomposing the input space into several subspaces and representing the input/output relationship, in each subspace, with a linear equation.

3 Generating Fuzzy Rules

Linguistic labels in our natural language convey useful information in human control strategies as well as in other cognitive decision processes. The fuzzy set theory approach to modelling this type of information is based on this thesis that each linguistic label can be represented as a fuzzy subset of an appropriate set U, expressing the semantics of the label. While this seems quit reasonable from a modelling point of view, the concern in applications is determining the membership function of a label. This is related to the more general and more difficult problem of determining rules. There are several approaches to answer this concern. Rules and membership functions can be given by experts, either in a subjective manner or by using some statistical sampling methods. When experts are not available, but instead, numerical experimental data are at hand, it is possible to use neural networks as a solution to the problem of rule and membership function determination. With ANFIS, the structure of the rules and the types of the membership functions are specified in advance, and the parameters of the membership functions are learned from the data [11]. However, rules and membership functions can also be determined by using methods that do not presuppose a rule structure. Both the extraction of rules and the determination of membership functions can be implemented by some

kid of clustering. Clustering using neural networks belongs to the domain of unsupervised learning that relies on input data and no corresponding outputs. As in conventional clustering, the goal is to group data points that are similar to each other in some way- that is forming clusters. Given a set of crisp input-output tuples, or training data (x_i, y_i), $i=1,\ldots n$, fuzzy clustering techniques are applied to the input data to determine a collection of fuzzy clusters. Each fuzzy cluster represents one fuzzy "IF….then…"rule, where the fuzzy membership functions in the rule are obtained by projecting the cluster to input and output spaces [2, 6, 9].

In the forward pass of the hybrid learning algorithm, node outputs go forward until layer 4 and the consequent parameters are identified by the least squares method outlined above. In backward pass, the signals that propagate backwards are the error signals and the premise parameters are updated by the gradient descent method.

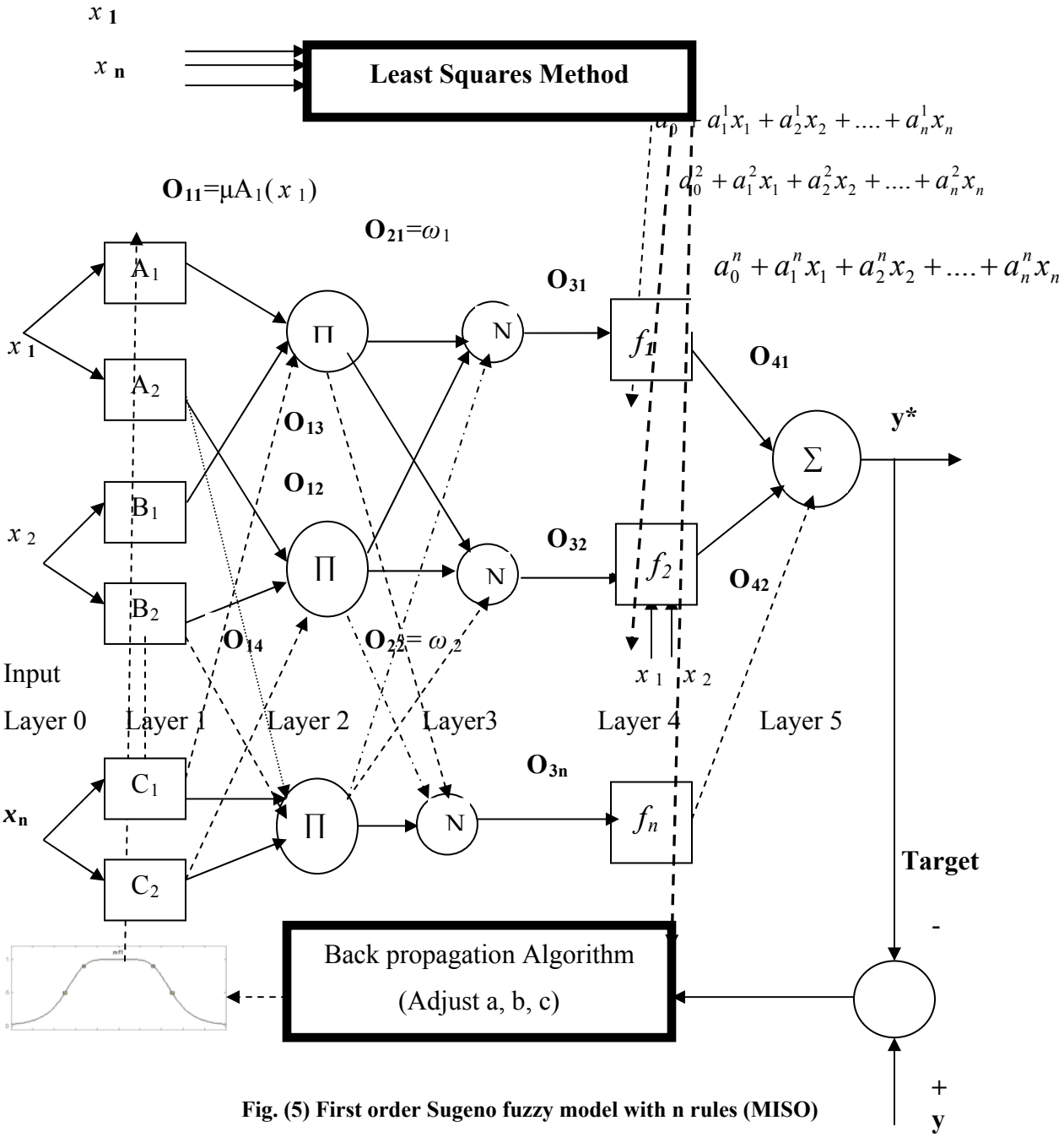


Fig. (5) First order Sugeno fuzzy model with n rules (MISO)

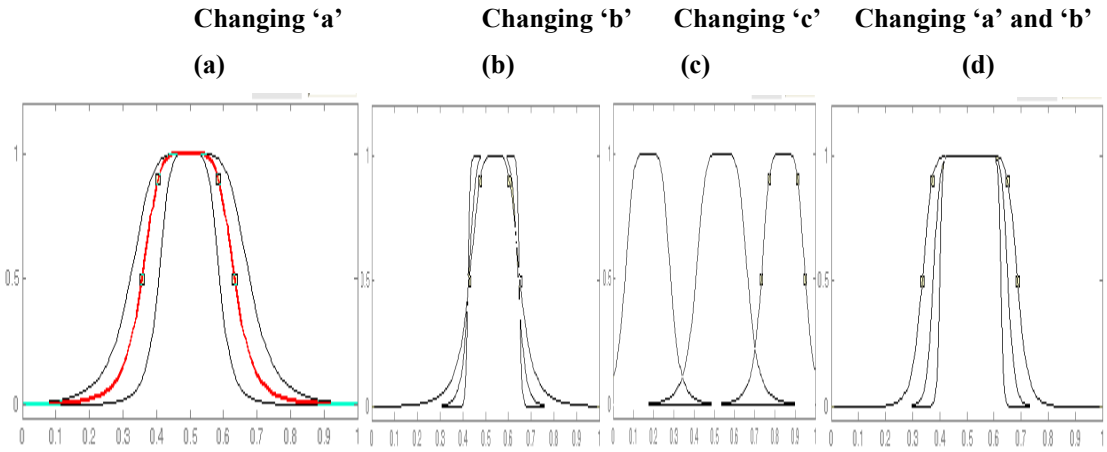


Fig.(6) The effects of changing parameters in Bell MFs: (a)Changing 'a' (b)Changing 'b' (c) Changing 'c' (d) Changing 'a' and 'b'

3.1 Input Space Partitioning

Now it should be clear that the spirit of fuzzy inference systems resembles that of "divide and conquer"- the antecedent of fuzzy rule defines local fuzzy region, while the consequent describes the behavior within the region via various constituents [24]. The consequent constituent can be a consequent MF, a constant value (Zero-order Sugeno Model), or a linear equation (first order Sugeno Model). Different consequent constituents result in different fuzzy inference systems, but their antecedents are always the same. Therefore, the following discussion of methods of partitioning input spaces to form the antecedent of fuzzy rules is applicable to all three types of fuzzy inferences [24].

1- Grid partitioning 2- Tree partitioning3- Scatter partitioning

3.2 Data Clustering Algorithms

Clustering algorithms are used extensively not only to organize and categorize data, but are also useful for data compression and model construction. Two different clustering algorithms are introduced [14]. The first algorithm introduces the most representative of line clustering techniques frequently used in conjunction with radial basis function networks and fuzzy modeling: subtractive clustering. Second, introduces the most representative on line clustering techniques frequently called evolving clustering methods (ECM). The advantages of this method are

1-Creating new cluster through training.

2-New fuzzy rules are created and updated during the operation of the system (through learning). Clustering partitions a data set into several groups such that the similarity within a group is larger than the among groups [15].

Clustering techniques are used in conjunction with radial basis function networks or fuzzy modeling primarily to determine initial locations for radial basis functions or fuzzy if-then rules. For this purpose, clustering techniques are validated on the basis of the following assumptions [16]:

Assumption 1:- States that the target system to be modeled is a smooth input outputs; mapping this is generally true for real world systems.

Assumption 2:- requires the data set to conform to some specific type of distribution; however, this is not always true. Therefore, clustering techniques used for structure identification in neural or fuzzy modeling are highly heuristic, and finding a data set to which clustering techniques cannot be applied is not uncommon.

4 ANFIS learning algorithm

In this section we introduce two different learning algorithms [26].

4.1 The First Learning Algorithm (Hybrid – Learning Algorithm)

From the ANFIS architecture in Fig. (5) We observe that when the values of the premise parameters are fixed, the overall output can be expressed as a linear combination of the consequent parameters [1].. Therefore, the formulas are usually referred to as kalman filter algorithm. In the forward pass of the hybrid learning algorithm, node outputs go forward until layer 4 and the consequent parameters are identified by the least squares method outlined above. In backward pass, the signals that propagate backwards are the error signals and the premise parameters are updated by the gradient descent method [16]**.** Table 3.2 parameters update during the forward and backward passes in the hybrid

learning procedure for ANFIS [12].

Table 2. Two passes in the hybrid learning procedure for ANFIS

Signal flow direction	forward pass	backward pass
Consequent parameters	Least-Squares estimator	Fixed
Premise Parameters	Fixed	Gradient descent method
Signals	Node outputs	Error signals

4.2 The Second Learning Algorithm (ANFIS)

The representation in the preceding section of a neural network is simply a graphical display of the computation steps in the sugeno-Tackagi procedure. In order for this representation to be more useful in implementing the model, one needs to equip it with an efficient learning algorithm [16]. In conventional neural networks, the back propagation algorithm is used to learn, or adjust, weights, on connecting arrows between neurons from input-output training samples. In the ANFIS structure, the parameters of the premises and consequents play the role of weights. Specifically, that is, the shape is specified and the function is determined by a finite number of parameters, these parameters are called **premise parameters**, whereas the parameters a_i, b_i, c_i, i=1, 2 in the "then" part of the rules are referred to as **consequent parameters**. The ANFIS learning algorithm consists of adjusting the above set of parameters from sample data $((x_1^k, x_2^k), y^k)$, k=1,…,N.

It is important to keep in mind that when we develop a set of prototype fuzzy rules, we are in fact representing possibly nonlinear input-output relationships [16]. The effectiveness of fuzzy models representing nonlinear input-output relationships depends on the membership functions, is an important issue in fuzzy modelling [16]. This tuning task can be viewed as an optimization problem; neural network offer a possibility to solve this problem [3]. In order to train a fuzzy neural network, we need a set of training data in the form of input – output tuples, and a specification of the rules, including a preliminary definition of the corresponding membership functions [4]. A standard approach is to assume a certain shape for the membership functions depend on parameters that can be learned by the neural network. We describe one method for learning membership functions of the antecedent and consequent parts of fuzzy "If….then…" rules. Suppose an unknown function, to be realized by a fuzzy inference system is known only through the training set [16]

$\{(x^1, y^1),\ldots,(x^k, y^k)\}$ (22)

where $x^k = ((x_1^k,\ldots,x_n^k) \in R.$ and, $y^k \in R$. To model the unknown function, we use fuzzy "If….then…" rules R_i, $i=1,\ldots., m,$ of the following type

R_i : If x_1^k *is* A_i^1 and … and x_n^k is A_i^n then

$$y = \sum_{j=1}^{n} z_i^j x_j^k + z_i \quad (23)$$

where A_i^j are fuzzy membership functions and z_i^j are real numbers.

5 Multiple Input /Output Systems (Extended ANFIS Algorithm)

One way to get multiple outputs is to place, as many ANFIS models side by side as there are required outputs. In this MANFIS (multiple ANFIS) no modifiable parameters set of fuzzy a rule, which makes it difficult to realize possible certain correlation between outputs [24]. An additional concern resides in the number of adjustable parameters, which drastically increases as outputs increase. This approach is represented in Fig. (7) and Fig. (8). another way of generating multiple outputs is to maintain the same antecedents of fuzzy rules among multiple CANFIS models. In short, fuzzy rules are constructed with shared membership values to express correlations between outputs. In this thesis we investigate the Second approach CANFIS to produce multiple outputs. This model is illustrated in Fig.(9).

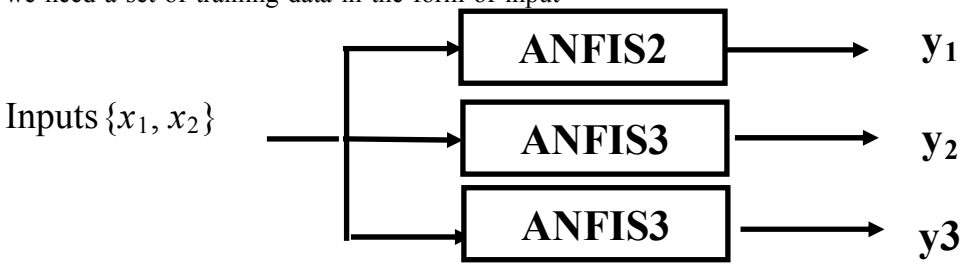


Fig (7). MANFIS Structure Decoupling MANFIS Structure

In this work we convert Fuzzy inference system structure to neural network structure, such as, membership functions for input variables, membership functions for outputs variables, Rules base.

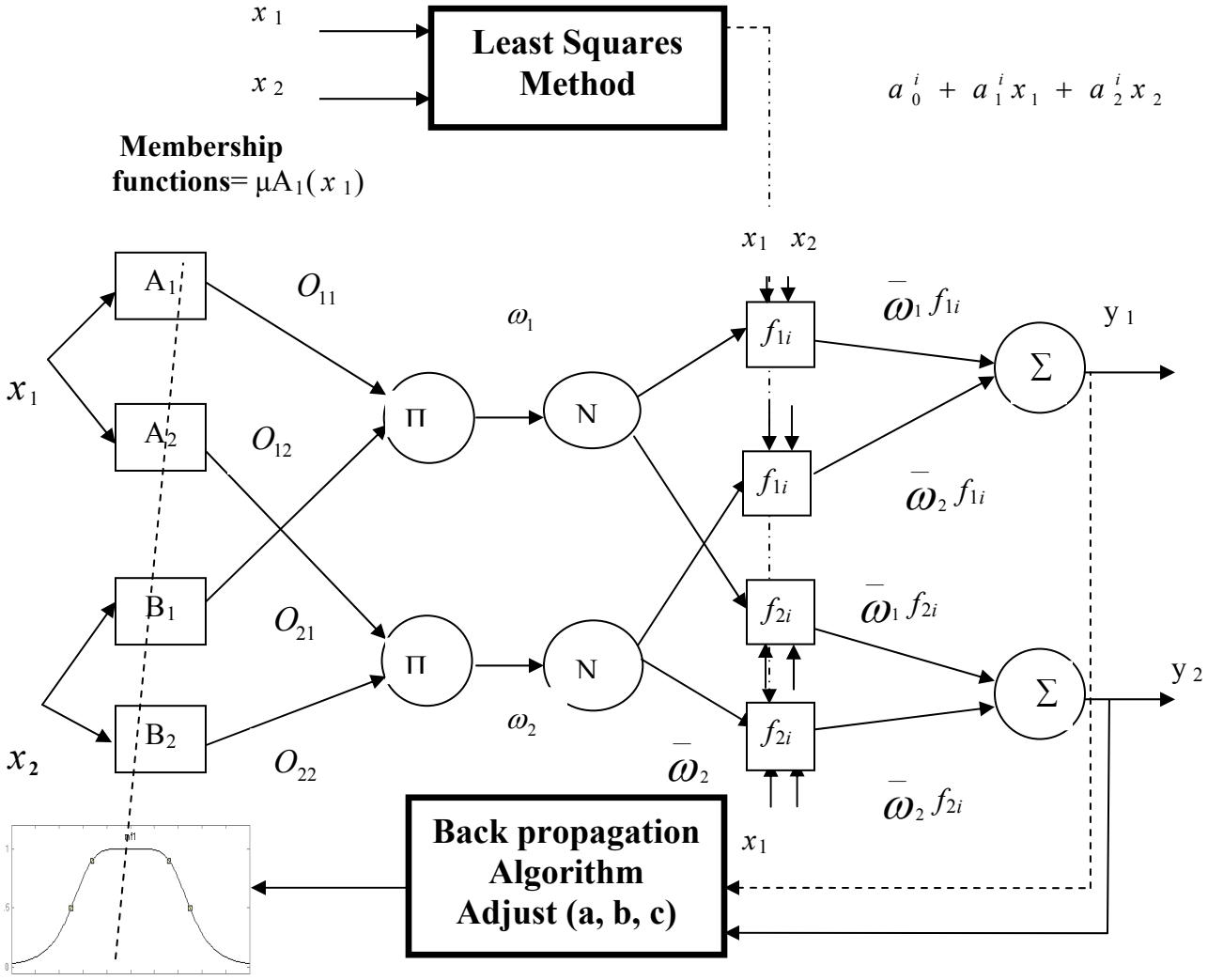


Fig. (8) Two outputs CANFIS Architecture with two rules per outputs

After conversion of FIS structure to NN, we train fuzzy neural network (FNN) and compute weights of each layer, weights of inputs, and weights of outputs to generate model. Then the weights are transformed to membership function parameters to construct FIS structure. The layer structure of CANFIS is defined as follows [2, 24, 26].

Layer 1: Every node *i* in this layer is a square node with a node function

$$O_{1,i} = \mu_{Ai}(x_1), \quad \text{For } i=1, 2, \quad \text{or}$$

$$O_{1,i} = \mu_{B\,i-2}(x_2) \quad \text{For } i=3, 4 \qquad (33)$$

where x_1 (or x_2) is the input to node i, and A_i or(B_{i-2})is a linguistic label, such as (small, large, etc.) associated with this node. In other words, O_i^1 is the membership grade of a fuzzy set A=(=A_1, A_2, B_1 or B_2) and it specifies the degree to which the given x_1 or x_2 satisfies the quantifier A. Here the membership function for A can be any appropriate parameterized membership function such as the generalized bell function:

$$\mu A_i(x_1) = \frac{1}{1+\left|\frac{x-c_i}{a_i}\right|^{2b}} \quad (34)$$

Or

$$\mu A_i(x_1) = \exp\{-[(\frac{x-c_i}{a_i})^2]^{b_i}\}, \quad (35)$$

where $\{a_i, b_i, c_i\}$ is the parameter set. As the values of these parameters change, the bell shaped functions vary accordingly, thus exhibiting various forms of membership functions for fuzzy set A. In fact, any continuous and piecewise differentiable functions, such as commonly used trapezoidal or triangular-shaped membership functions, are also qualified candidates for node functions in this layer. In this thesis we convert equation (35) to NN structure. Parameters in this layer are referred as premise parameters.

Layer 2: Every node in this layer is a circle node labeled π, which multiplies the incoming, signals and sends the product out. For instance,

$$\omega_i = \mu A_i(x_1) \times \mu B_i(x_2) \qquad i = 1,2. \quad (36)$$

Each node output represents the firing strength of a rule. (In fact, other T-norm operator that performs generalized AND can be used as the node function in this layer).

Layer 3: Every node in this layer is a circle node labeled N. the *i*-th node calculates the ratio of the *i*-th rule's firing strength to the sum of all rules' firing strengths:

$$\varpi_i = \frac{\omega_i}{\omega_1+\omega_2}, \quad i=1, 2 \quad (37)$$

For convenience, outputs of this layer will be called normalized firing strengths.

Layer 4: Every node *i* in this layer is a square node with a node function

$$o_i^4 = \varpi_i f_i = \varpi_i(a_0^1 + a_1^1 x_1 + a_2^1), \quad (38)$$

Where ϖ_i is the output of layer 3, and (a_{0i}, a_{1i}, a_{2i}) is the parameter set. Parameters in this layer will be referred to as consequent parameters. $f_{ji} = a_0^i + a_1^i x_1 + a_2^i x_2$, *i=1,2,..n* where *n* is number of rule, *j* number of *output*

Layer 5: The single node in this layer is a circle node labeled $\sum$ that computes the overall output as the summation of all incoming signals, i.e.,

$$o_j^5 = overall\text{output}j = \sum_i \varpi_i f_{ji} = \frac{\sum \omega_i f_{ji}}{\sum_i \omega_i}$$

where *j=1, 2, 3* (39)

Thus we have constructed an adaptive network, which is functionally equivalent to a type-3 fuzzy inference system as shown in Fig. (5) from chapter 2. Fig. (10) Shows the NN layers corresponding to FIS of multiple inputs three outputs system.

The structure of NN layer for MIMO (2 i/p-3 o/p) Simulink Model of Fuzzy Neural Network is introduced. We add layer 1 for distribution. We use layer 1 to distribute inputs; one input per one membership function. Also, we add sub layer in the consequent parameter for layer 5 to compute multiple outputs. Layer 6 is the overall outputs. Table 3.2 Present the comparison between ANFIS and CANFIS

Table 3. Comparison between ANFIS and CANFIS

	Layer 1	Layer 2	Layer 3	Layer 4	Layer 5	Layer 6
ANFIS	------	Convert Crisp To member ship value	Product	Normalization	Adaptive node	Over all outputs
CANFIS	Distribution	Convert Crisp To membership value	Product	Normalization	Add sub Layer for rule sharing	Over all outputs

6 Procedure of Neuro fuzzy System Identification

In this section we introduce the procedure to construct Neuro fuzzy model. Fig. (13) Presents the preprocessing data Using Subtractive clustering Data. The Flow chart of learning ANFIS model is introduced in Fig. (12).

7- Testing of the Modified Algorithm (CANFIS**)**

To test the validity of the modified algorithm ANFIS (CANFIS) algorithm, an example is simulated from the numerical equation. The result of modified algorithm (CANFIS) showed a conformance with the simulated example and the root mean square (RMSE) is in the range of 0.00248735. This section presents the simulation results of the proposed CANFIS with off line learning. In this example, CANFIS is used to model highly nonlinear functions and the result is discussed as follows. Modeling (2) two inputs (3) three outputs nonlinear functions, in this example we consider using CANFIS to model a nonlinear sin-cos equations.

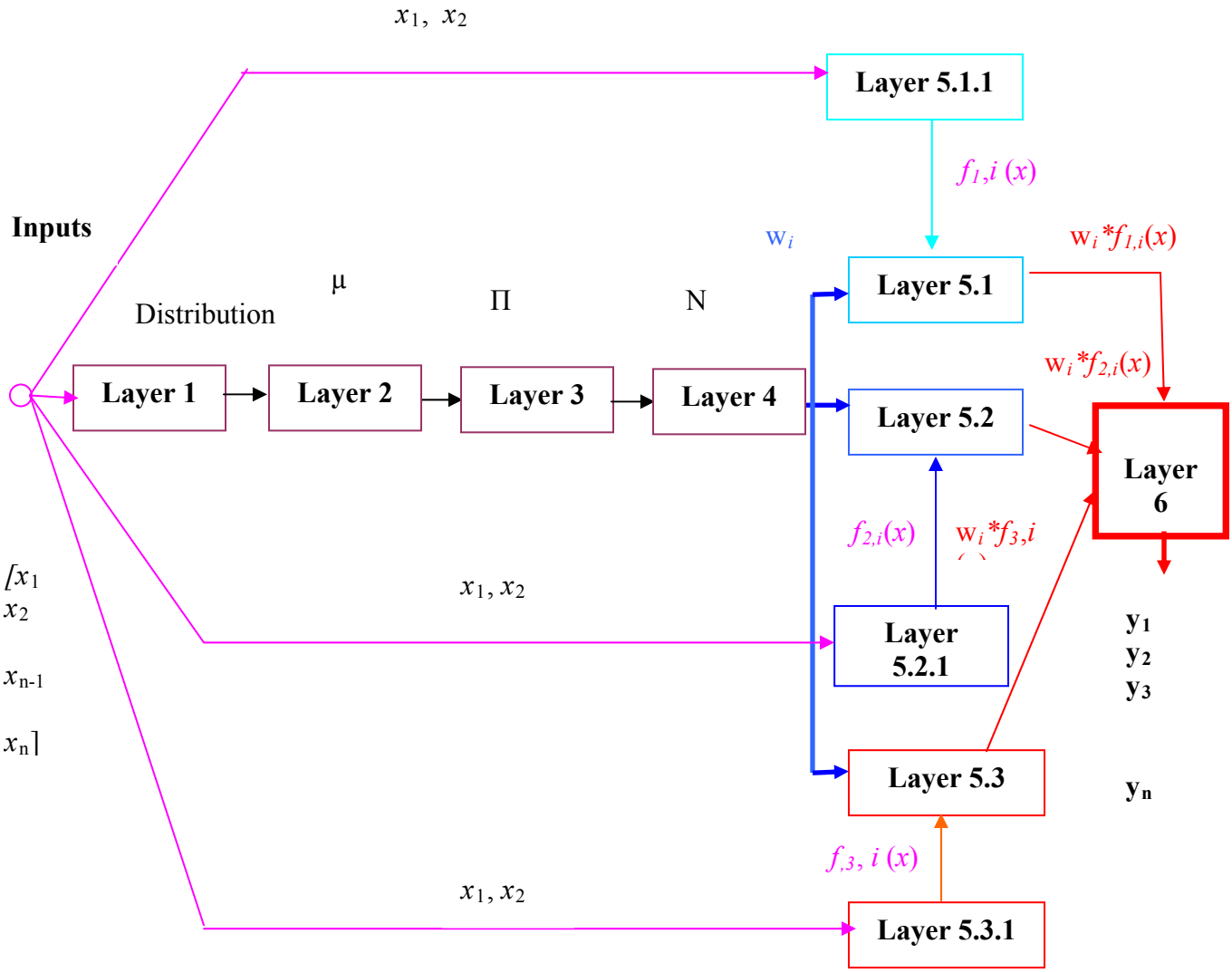


Fig. (10) NN Implementation to FIS

The following three equations are presented to model 2 two inputs (3) three outputs using CANFIS. Fig. (14) Shows the relationship between inputs and outputs before training (above figure) after training (below figure) for first output. Fig. (15) Shows the relationship between inputs and outputs before training (above figure) after training (below figure) for second output. Fig. (16) Shows the relationship between inputs and outputs before training (above figure) after training (below figure) for third output where x_1, x_2=sample number, period time=0.01 second.

$$y_1 = (\sin(\frac{x_1 * \pi}{180}))' * \sin(\frac{x_2 * \pi}{180}); \qquad (40)$$

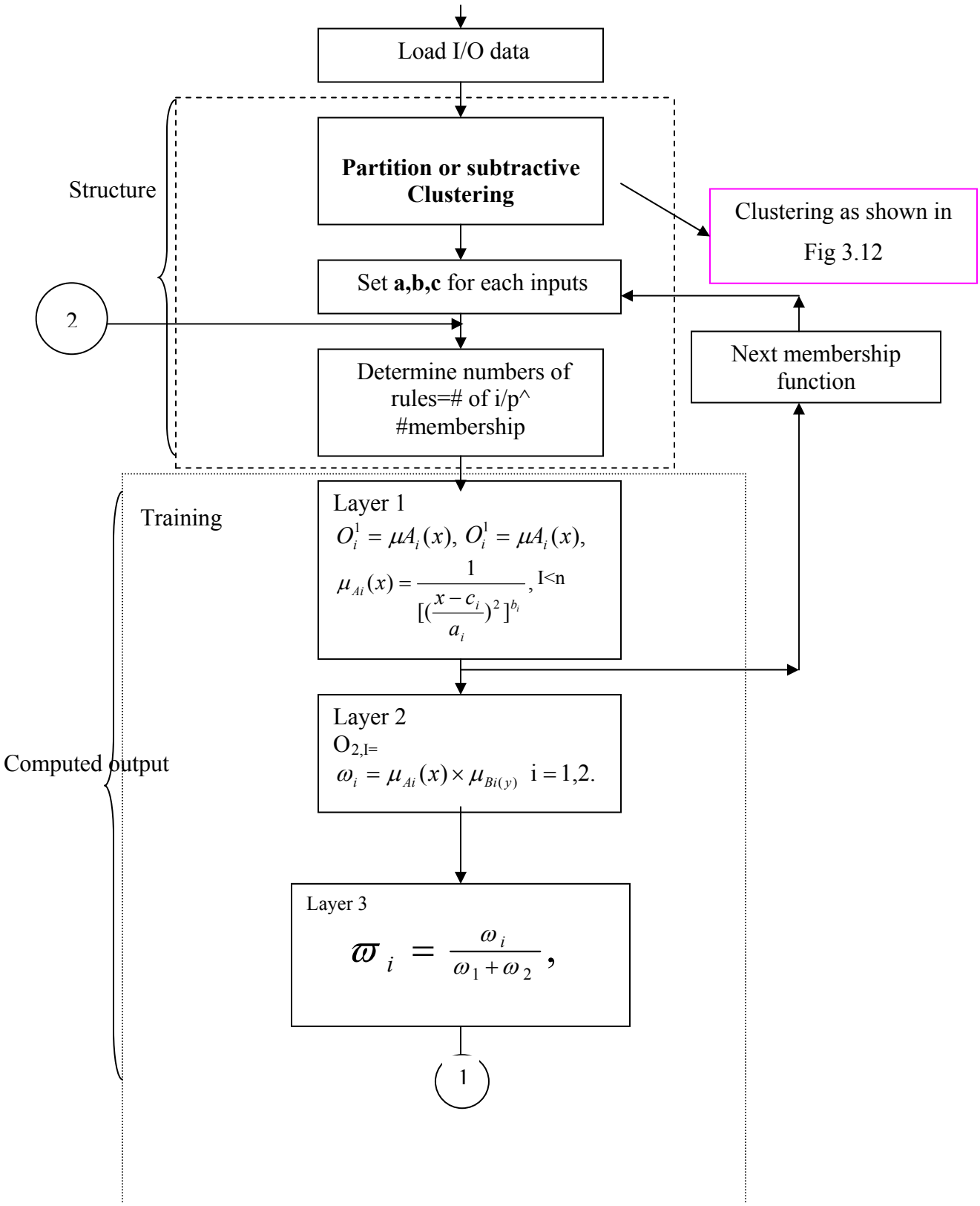
$$y_2 = (\cos(\frac{x_1 * \pi}{180}))' * \cos(\frac{x_2 * \pi}{180}); \qquad (41)$$

$$y_3 = (\sin(\frac{x_1 * \pi}{180}))' * \cos(\frac{x_2 * \pi}{180}); \qquad (42)$$

$e_i = y_j - \hat{y}_j$, The root mean square error for model is given by

$$\text{RMSE} = (\frac{1}{N}\sum_{1}^{n} e_i^{\,2})^{\frac{1}{2}} \qquad i=1,2,.....N,\ j=1,\ 2,\ 3 \quad (43)$$

Where N is number of samples, y_j is the output of numerical equation; $\hat{y}_j$ is the actual output of CANFIS Model. Mean square error of modified algorithm is equal to (MSE) =0.00248735, after execution of program (TestFIS) which is found in the CD of programs. The RMSE is computed from equation (43). This result shows very good performance of the modified algorithm.


Load I/O data
Structure
Partition or subtractive Clustering
Clustering as shown in Fig 3.12
Set a,b,c for each inputs
2
Next membership function
Determine numbers of rules=# of i/p^ #membership
Training
Layer 1
$O_i^1 = \mu A_i(x),\ O_i^1 = \mu A_i(x),$
$\mu_{Ai}(x) = \frac{1}{[(\frac{x-c_i}{a_i})^2]^{b_i}}$, I<n
Layer 2
$O_{2,I=}$
$\omega_i = \mu_{Ai}(x) \times \mu_{Bi(y)}$ i = 1,2.
Computed output
Layer 3
$\varpi_i = \frac{\omega_i}{\omega_1 + \omega_2}$,
1

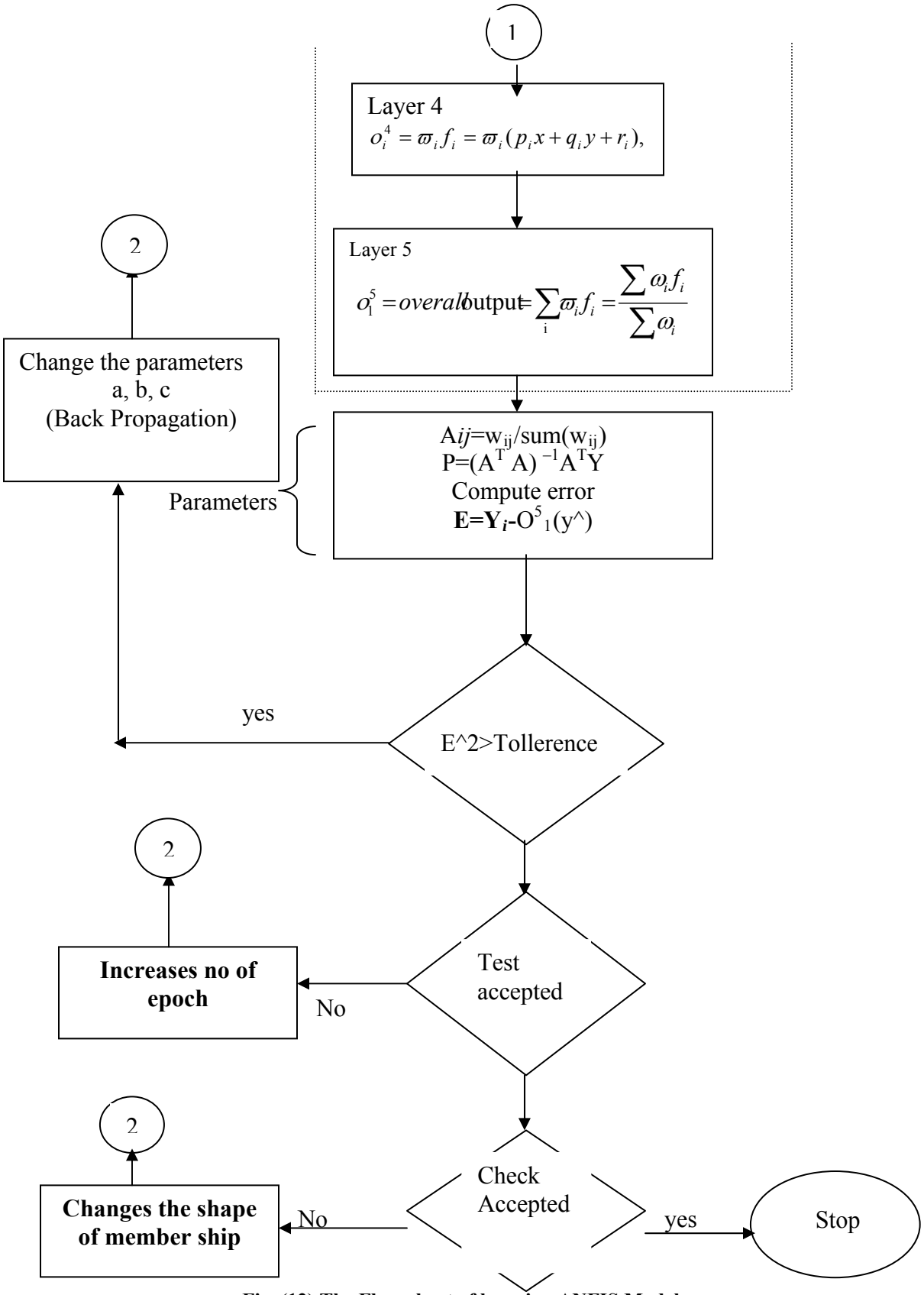


Fig. (12) The Flow chart of learning ANFIS Model

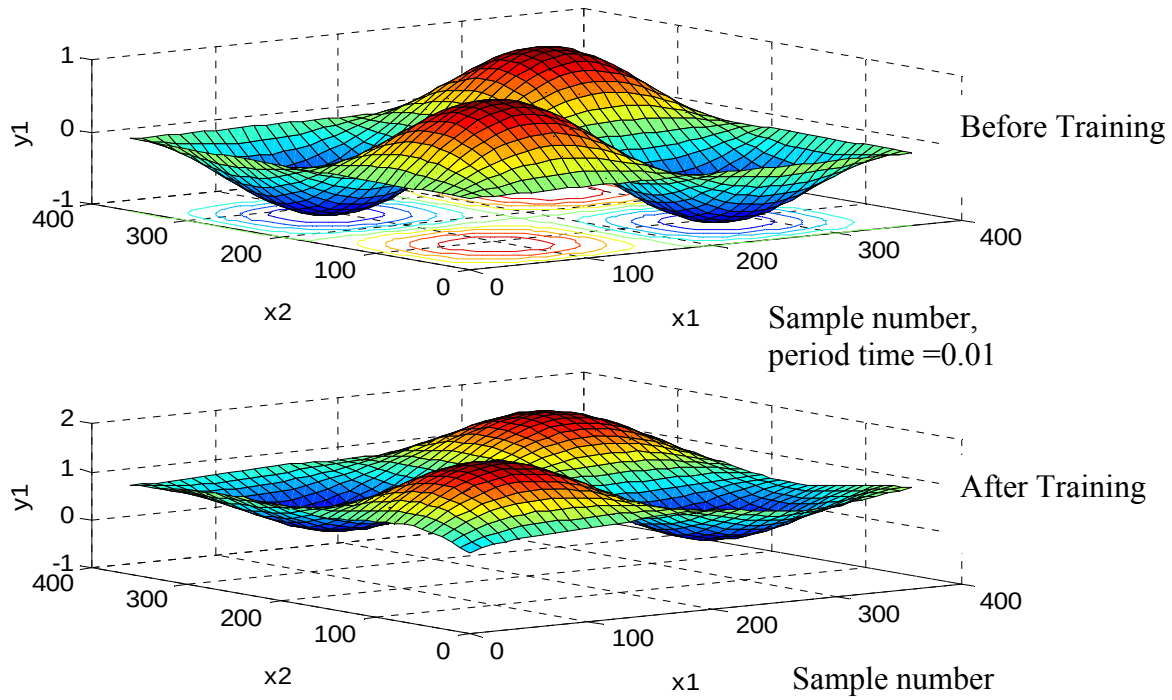


Fig. (14) System Response of CANFIS after Training for 2 inputs three outputs for first output

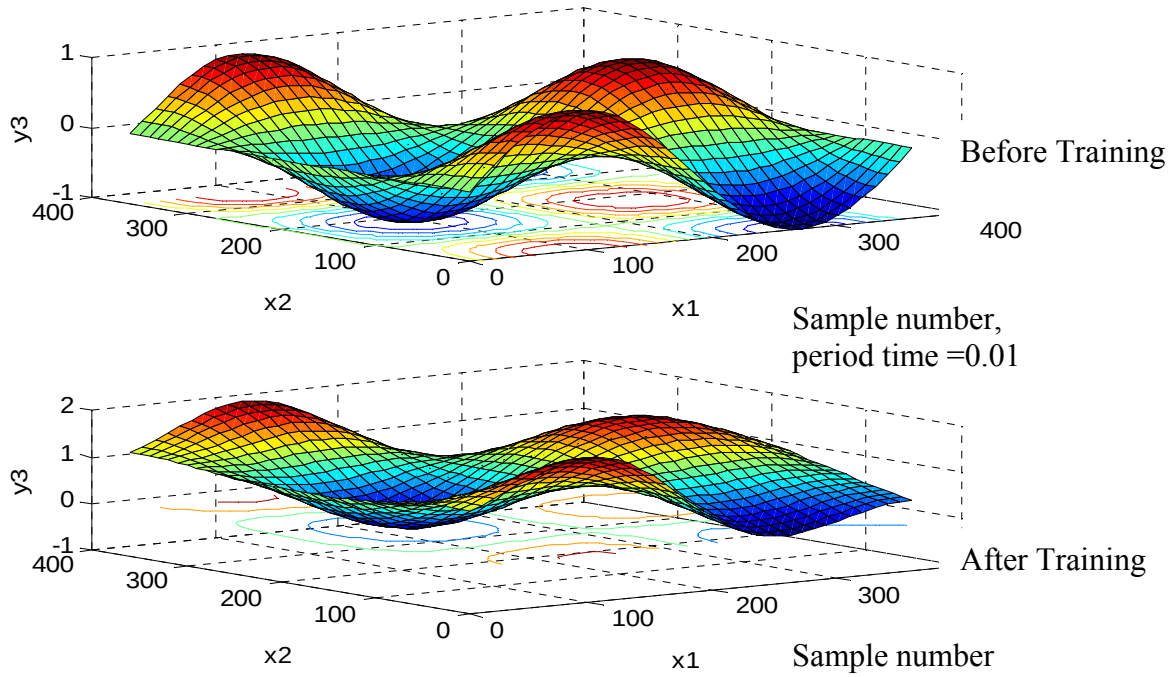


Fig. (16) System Response of CANFIS after Training for 2 inputs three outputs for third output

8 Conclusions

In this paper, the principles of neuro fuzzy Systems have been investigated. Membership Function Generation and its mapping to Neural Network are introduced. An adaptive network fuzzy inference system (ANFIS) is introduced, and Multiple Inputs /Outputs Systems (Extended ANFIS Algorithm) is implemented. A Modification algorithm of ANFIS, Coupling of ANFIS called coactive neuro fuzzy system (CANFIS), is introduced and implemented using Matlab. The software of the modified algorithm of MIMO model identification is built.

To test the validity of the modified algorithm ANFIS (CANFIS algorithm), an example is simulated from the numerical equation. The result of modified algorithm (CANFIS) showed a conformance with the simulated example and the root mean square (RMSE) is in the range of 0.00248735.

Corresponding author
Dr. Eng Tharwat O. S. Hanafy
Al_Azhar University, Faculty of Engineering, Systems and Computers Eng. Department, Cairo, Egypt
s_ewiss@yahoo.com

References

1. Hossein Salehfar, Nagy B., Jun Huang, "Systematic Approach to Linguistic Fuzzy Modeling Based on Input-Output Data ", proceeding of the 2000 winter Simulation Conference.
2. Jang J.-S. R. "ANFIS: Adaptive Network-based Fuzzy Inference System", IEEE Transactions on Systems, Man and Cybernetics, (1993), 23 (3), 665-685.
3. E. Kim, M. Park, S. Ji, and M. Park, "A new approach to fuzzy modeling", IEEE Trans. on Fuzzy Syst., vol. 5, no. 3, pp.328-337, 1997.
4. Amine Tabelsi, Frederic, "Identification of Nonlinear Multivariable Systems by Adaptive Fuzzy Takagi-Sugeno Model", International Journal of Computational Cognition,September 2004, page 137-153.
5. Azeem M. F., Hanmandlu M. and Ahmad N. "Generalization of adaptive neuro-fuzzy inference system", IEEE Transactions on Neural Networks, 11(6), 1332-1346. 2000
6. Kim J. and Kabasov N. "Adaptive Neuro-Fuzzy inference systems and their application to nonlinear dynamical systems", IEEE Transactions on Neural Networks, (1999), 12 (9), 1301-1319.
7. Lin C.-T., "A neural fuzzy control scheme with structure and parameter learning", Fuzzy Sets and Systems, (1995), 70, 183-212.
8. M. Sugeno and T. Yasukawa, "A fuzzy-logic-based approach to qualitative modeling", IEEE Trans. on Fuzzy Systems, vol. 1, no. 1, pp. 7-31, 1993
9. Mackey M. C. and Glass L. "Oscillation and chaos in physiological control systems", Science, 1997, 287-289.
10. Mamdani, E.H. "applications of fuzzy algorithms for control simple dynamic plants, Proc. of the IEE, (1984), 121, 1585-1588.
11. Nauck D. and Kruse R., "Neuro-fuzzy systems for function approximation", Fuzzy Sets and Systems, 1999, 101, 261-271.
12. Pal K. and N. R. Pal. "A neuro-fuzzy system for infringing", International Journal of Intelligent and Fuzzy Systems, 1999, 14(11), 1155-1182.
13. Ann L Casebeer and Marja J Verhoef, " Combining Qualitative and Quantitative Research Methods: Considering the Possibilities for Enhancing the Study of Chronic Diseases", Volume 18, No.3 -1997
14. Paiva R. P., "Neuro-Fuzzy Identification: Interpretability Issues", MSc Thesis, Department of Informatics Engineering, Faculty of Sciences and Technology, University of Coimbra, Portugal, 1999.
15. Paiva R. P., Dourado A. and Duarte B. "Applying subtractive clustering for neuro-fuzzy modeling of a bleaching plant", Proceedings of the European Control Conference -1999, ECC'99, CD-ROM.
16. Jyh-Shing, Roger Jang "Neuro Fuzzy Modeling and Control", proceeding of the IEEE, March 1995.
17. R. Fuller, "Introduction to Neuro-Fuzzy Systems, Advances in Soft Computing Series", Springer-Verlag, Berlin, 1999.
18. Chiu S. L. "Fuzzy model identification based on cluster estimation", Journal of Intelligent and Fuzzy Systems, 1994, 2 (3), 267-278
19. Dave R. N. and Krish napuram R. "Robust clustering methods: a unified view", IEEE Transactions on Fuzzy Systems, 1997, Vol. 5 No. 2, 270-293.
20. Robert Shorten "On The Interpretation And Identification of Dynamic Takgi-Sugeno Fuzzy Models". IEEE Trans. on Fuzzy Systems, Vol. 8, No 3, Jun 2000.
21. R. Babuska J.A. Roubos H.B. Verbruggen, "Identification of MIMO systems by input -output TS fuzzy models" Delft University of Technology, Department of Electrical Engineering, Control Laboratory-Mekelweg, 2004, P.O. Box 5031, 2600 GA Delft, The Netherlands.
22. Carmona, P. Castro, J.L. Zurita, J.M, "FRIWE: Fuzzy Rule Identification with Exceptions", IEEE

Transactions on Fuzzy Systems, Publication Date: Feb. 2004 on page(s):140-151Volume: 12.
23. Yu, W. Li, X., "Fuzzy Identification Using Fuzzy Neural Networks With Stable Learning Algorithms", IEEE Transactions on Fuzzy Systems, June 2004 On page(s): 411-420 Volume: 12, Issue:3, ISSN: 1063-6706.
24. Jang R, Sun C T and Mizutani E., "Neuro-fuzzy and soft computing: A computational Approach to Learning and Machine Intelligence", Prentice Hall NJ, 1997.
25. Robert Babuska, "Neuro fuzzy methods for modeling and identification", Delft university of technology, faculty of information technology and systems, Recent advances in intelligent Paradigms and applications, Springer- 2002.
26. Hung T. R. Prasad, Carol L. "First Course in Fuzzy and Neural Control", @2003 by Chapman & Hall/CRC.
27. Ho Jae Lee, Hagbae Kim, Young Hoon Joo, Wook Chang, Jin Bae Park, "A new intelligent digital redesign for T-S fuzzy systems: global approach" IEEE Transactions on Fuzzy Systems" April 2004 On page(s): 274 284,Volume:12,Issue:2ISSN: 1063-6706.
28. Jyh-Shung, Roger Jang, 1995, "Neuro Fuzzy Modeling and Control", proceeding of the IEEE, March 1995.

10/10/2010

In vitro and *in vivo* Activity of some Antibiotics against Staphylococcal Biofilm and Planktonic Cells Isolated from Diabetic Foot Infections.

A. Abd El-Aziz[1], T. El-Banna[1], A. Abo-Kamar[1], A. Ghazal[2], and R. AboZahra[*3]

[1]Microbiology Department, Faculty of Pharmacy, Tanta University, Tanta, Egypt
[2]Microbiology Department, Medical Research Institute, Alexandria University, Alexandria, Egypt.
[3]Microbiology Department, Faculty of Pharmacy, Pharos University, Alexandria, Egypt
[*]rania_abozahra@yahoo.com

Abstract: The diabetic foot syndrome is clearly one of the most important complications of diabetes and is the most common cause of hospitalization among diabetic patients. *Staphylococcus aureus* is found to be the commonest pathogen present in diabetic foot infections. The aim of the present study is to determine activities of three kinds of antibiotics against Staphylococcal biofilm and planktonic cultures *in vitro*, and to indicate the difference in wound healing between staphylococcal planktonic and biofilm stage of colonization *in vivo* by using diabetic rat models. Biofilm forming staphylococci were identified by using the modified microtiter plate method. And the effect of different concentrations of several antibiotics (including ciprofloxacin, gentamycin and amoxicillin-clavulanic acid) on eight isolates was determined. The result showed that out of 86 Staphylococcal isolates, eight strains were found to be strong biofilm forming. It was found that the preformed biofilm was very difficult to remove with most isolates even with multiples of the MIC and that the biofilm MBC reached 46 times the planktonic MBC in some isolates. This was also noticed in case of the diabetic foot infection of the rat model, as the treatment was more efficient when it started immediately after infection, before the formation of the biofilm, as the bacterial infection was eliminated within 3-4 days, while it could not be completely eliminated when treatment started after the biofilm formation. This was also observed from the rate of healing and confirmed by histological examination.

[**A. Abd El-Aziz, T. El-Banna, A. Abo-Kamar, A. Ghazal, and R. AboZahra**. *In vitro* and *in vivo* Activity of some Antibiotics against Staphylococcal Biofilm and Planktonic Cells Isolated from Diabetic Foot Infections. Journal of American Science 2010;6(12):760-770]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: *In vitro- in vivo-* diabetic foot- staphylococcus- biofilm

1. Introduction:

The diabetic foot syndrome is clearly one of the most important complications of diabetes and is the most common cause of hospitalization among diabetic patients (Wrobel *et al.*, 2001), people with diabetes are 25 times more likely to have a leg amputated than those without the condition, according to the International Diabetes Federation (Lau *et al*., 2008). The combination of peripheral neuropathy, peripheral arterial disease and infections would result in unhealing ulcers, gangrene and amputation (Aulivola *et al*., 2004). Kandemir *et al*. (2007) found that the most frequent etiological agent for diabetic foot infections was staphylococci. In tissues removed from patients with recurrent staphylococcal infections, the cells are frequently organized in confluent colonies with a biofilm-like appearance. The pathogenesis of staphylococcal infection begins with primary bacterial adhesion and colonization of the host tissues (Morikawa *et al*., 2005). Once a biofilm has been established, it is a major concern for clinicians in the treatment of infectious disease because of the resistance to a wide range of antibiotics (Amorena *et al*., 1999). Therefore, a better understanding of bacterial biofilms is needed, and this may ultimately result in development of novel therapeutics for the prevention and treatment of wound infections (Davis *et al*., 2006).

Biofilm formation occurs upon initial rapid attachment of staphylococci to the surface, followed by multilayered cellular proliferation and intercellular adhesion in an extracellular polysaccharide matrix excreted by the bacteria (Gotz, 2002). Cell adhesion between staphylococci is mediated by polysaccharide intercellular adhesin (PIA), a linear homopolymer of ß-1,6-linked *N*-acetylglucosamine residues (Arciola *et al*., 2001).

Biofilm infections are difficult to treat due to their inherent antibiotic resistance. Once staphylococcal biofilm has formed on damaged tissue, it is difficult to disrupt. Most antimicrobial therapies for biofilms have largely proven unsuccessful. The mechanism of biofilm-associated

antibiotic resistance is uncertain and likely multifactorial. A number of factors have been postulated, including binding of antibiotic to the slime, poor penetration of antibiotic into the biofilm, slow growth rate of organisms in the biofilm, high bacterial density, and changes in gene expression in biofilm bacteria. Bacteria released from biofilms retain susceptibility to antibiotics characteristic of free-growing bacteria rather than biofilms, implying that the mechanism of resistance is not genetic change (Saginur *et al*., 2006).

The objective of this study was to determine the effect of different antibiotics on biofilm removal, also to compare the planktonic and biofilm MBC of staphylococcal isolates retrieved from diabetic ulcers to these antibiotics, and then to study the effect of planktonic and biofilm stage of colonization on an *in vivo* diabetic rat model.

2. Materials and methods

Bacterial strains: In this study 140 clinical swab form foot ulcers of diabetic inpatients in the university hospital (Alexandria, Egypt) were collected. Out of 197 isolate 86 isolates were identified as Gram positive bacteria (Staphylococi). Identification of the staphylococcal strains was done by Gram staining, catalase, coagulase tests, and cultivation on mannitol salt agar, further confirmation was carried out by using the API Staph system. Out of the 86 staphylococci isolated 62 (70.4%) were *Staphylococcus aureus* and 25 (29.6%) were Coagulase negative staphylococci (CONS).

Quantification of biofilm formation: This was done by using the modified microtiter plate method (Stepanovic *et al*., 2000), where the strains were grown in TSB supplemented with 2.5% glucose (Christensen *et al*., 1985). All strains were categorized as non, weakly, moderately, or strongly adherent, based upon the ODs of bacterial films as described by Stepanovic *et al*. (2000).

Antimicrobial agents used: Gentamycin (CN) (Alexandria Co., Egypt), Ciprofloxacin (CIP) (Amriya, Egypt), and Amoxicillin-Clavulanic acid (AMC) (GlaxoWellcome) were used.

MIC and MBC determination: Susceptibility testing to each drug was performed on planktonic cultures using the two-fold dilution method according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2003). MICs were performed in 96-well microplates (Greiner, Wemmel, Belgium) and results were recorded after incubation at 35 °C for 18 h. For MBC determination, 10µl aliquots were removed from the wells after incubation and spread onto Mueller–Hinton agar in Petri dishes and incubated overnight at 35 °C (Tre-Hardy *et al*., 2008).

Effect of antimicrobial drugs on pre-formed biofilms:

Biofilms were allowed to form as described above. The content of the wells was then aspirated and the wells were washed and fresh TSB containing two fold serial dilution of the antimicrobial agent was added to the wells as described above. The plate was then incubated at 35^{o}C for 24h. The content of the wells was aspirated then washed and stained by using crystal violet as described above. The optical densities were determined after elution as before. Biofilm persistence in the presence of antimicrobial agents was calculated using the following formula (Tre-Hardy *et al.*, 2008).

Percentage of biofilm persistence = $[(A_{590x} - A_{590\text{negative control}})/ (A_{590\text{ positive control}} - A_{590\text{negative control}})] \times 100$, where x corresponds to the antimicrobial agent used.

Animal model

Alloxan-induced diabetes model:

Diabetes was induced by a single intraperitoneal injection of 130 mg/kg monohydrated alloxan (Sigma, St. Louis, MO, USA) dissolved in sterile 0.9% saline. Rats were made to fast prior to alloxan administration. After 12 h, a 10% glucose solution was offered to the animals to prevent hypoglycemia (Diniz *et al*., 2008). After 72 h, blood samples were collected from the eye of the animals for evaluation of plasma glucose levels by the glucose-oxidase enzymatic colorimetric method (Biomedical Systems, Spain). Animals presenting glucose levels above 300 mg/dL were included in the diabetic group.

Diabetic foot ulcer study:

The adult diabetic rats (>300 mg/dl) were used for wound induction. On the day of wound induction (defined as day 0) each rat was anesthetized with an intraperitoneal injection of 50 mg/kg thiopental sodium. A rectangular pattern was marked on the dorsal surface of the foot using a flexible transparent plastic template, and then a layer of skin in full thickness with standard area of 2 mm × 5 mm was removed as shown in Figure 1. The initial wound size was measured on day 1 (Lau *et al*., 2009).

Treatment groups:

A total of 16 rats were used in this study, they were divided into control and treatment groups, each group was composed of three rats and one rat was sacrificed on day 2 for the histological studies to confirm the formation of the bacterial biofilm within the wound bed. The treatment groups included two groups, the first received ciprofloxacin and the second received amoxicillin clavulanic acid. Half the

number of rats in each group started the treatment 15 minutes after inoculation (representing the planktonic stage), the other half started the treatment after 48 hours (representing the colonized bacteria forming the biofilm). Antimicrobial agents were administered orally by gavages. This experiment was made once with the *S. aureus* isolate (25S), and another time with the *S. epidermides* (78S).

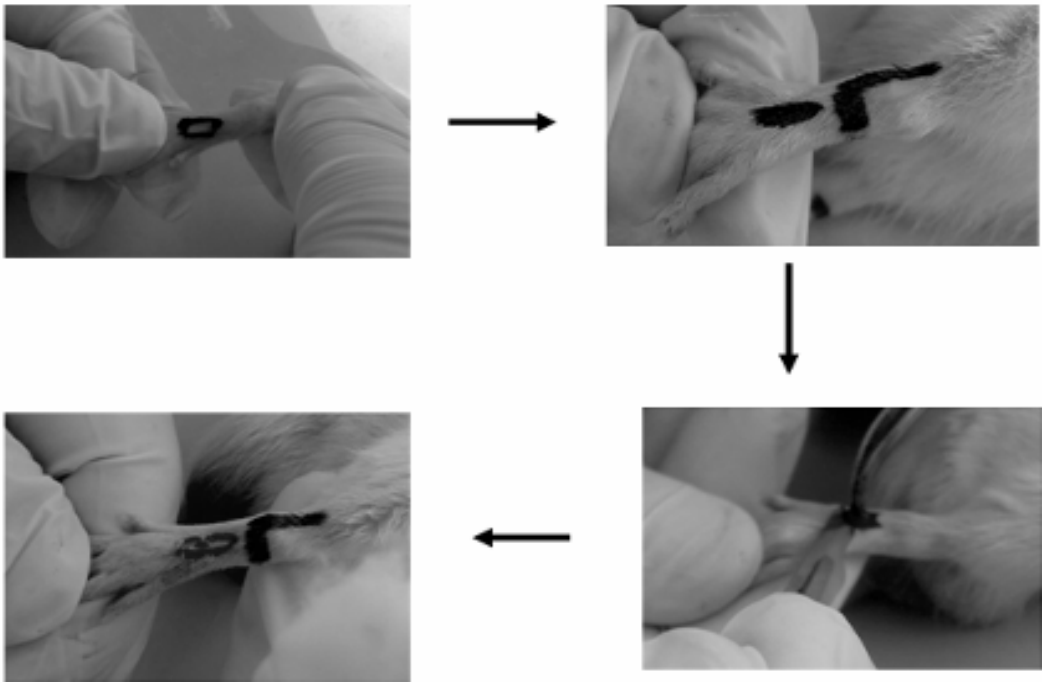


Figure 1: Wound formation in the dorsal surface of the rat hind paw.

Preparation of bacterial inoculums:

Overnight cultures of the staphylococcal isolate were used to inoculate the wounds. The wounds representing the planktonic stage of bacterial colonization started the antimicrobial treatment 15min. after inoculation, while the wounds representing the biofilm-associated cells were inoculated with the initial inoculum and left for 48 hours to allow wounds to be colonized (Davis *et al.,* 2008a)

Sampling and Wound area measurement:

In this study we selected the one-point sampling method because of the relatively small size of wounds that can be induced in rats and to better avoid contamination with peri-wound flora. In this method the swab cultures were taken from the center of each surgical wound by rotating the swab three times clockwise. Ulcer digital photographs and swabs were taken on days 1, 3, 5 and 9. Ulcer area measurement were made by using the digital photographs of the wounds perpendicular to a metric reference scale (Sullivan *et al.,* 2004), which allowed us to enlarge the photograph in order to increase the accuracy of wound area measurement and the photographs were processed by using Microsoft Word where the wound area was traced and measured on the scale present in the same photograph as shown in Figure 2.

Antimicrobial therapy:

Antimicrobial doses were chosen to approximate, in the rat, the serum or tissue AUCs achieved in humans. The doses were 350/87.5 mg/kg and 200 mg/kg for amoxicillin/clavulanic acid and ciprofloxacin respectively (Berry *et al*., 2000). Rats receive the antimicrobial treatment twice daily for 7 days (Cagni *et al*., 1995).

Histological examination:

Biopsy specimens were obtained 48 hours after inoculation and colonization and at the end of the treatment for evaluation with light microscope. Specimens were placed in 10% formaldehyde and stained with hematoxylin and eosin and with Gram crystal violet (Kugelberg *etal.,* 2005)

3. Results

Quantification of biofilm formation:

The experiment performed was carried out to measure the degree of adherence and subsequent biofilm formation of all staphylococcal isolates. Out of the 86 staphylococcal isolates 4 (4.65%) were non-adherent, 35 (40.7%) were weakly adherent, 39 (45.35%) were moderately adherent and 8(9.3%) were strongly adherent. The eight strong biofilm forming strains (16S, 17S, 18S, 25S, 45S, 78S, 90S and 108S) were selected for further investigations. Three of which (16S, 25S and 45S) were *S. aureus*, while 17S, 18S, 78S, 90S and 108S were CONS.

MIC and MBC determination: MICs and MBCs recorded for the tested antimicrobial agents against the eight staphylococcal isolates are summarised in Table1.The biofilm MBCs were found to be much higher than the planktonic MBCs. These results are shown in Figure 3.

In case of ciprofloxacin, the biofilm MBC was 2 to 512 times higher than the planktonic MBC. Also, the biofilm MBC for gentamycin was 2 to 256 times higher than that of the planktonic culture. Whereas, the biofilm MBC in case of amoxicillin-clavulanic acid was 4 to 64 times higher than that of the planktonic culture.

Variable behavior in biofilm formation for the 8 staphylococcal isolates was observed in biofilm and planktonic cultures; no fixed increase was noticed for all strains with the different antibiotics used.

Detachment of established staphylococcal biofilms after antibiotics exposure:

The effect of MIC and its multiples on the pre-formed biofilm was tested in order to determine the biofilm removing capacity of the studied antibiotics. It was found that ciprofloxacin showed relatively the best results in biofilm detachment as it removed from 40-80% of an already formed biofilm in five (62.5%) of tested isolates. However, amoxicillin-clavulanic acid showed the lowest ability for biofilm detachment. On the other hand, two strains (25S, 45S) showed increase in the biofilm formation by the addition of antibiotics (Figure 4).

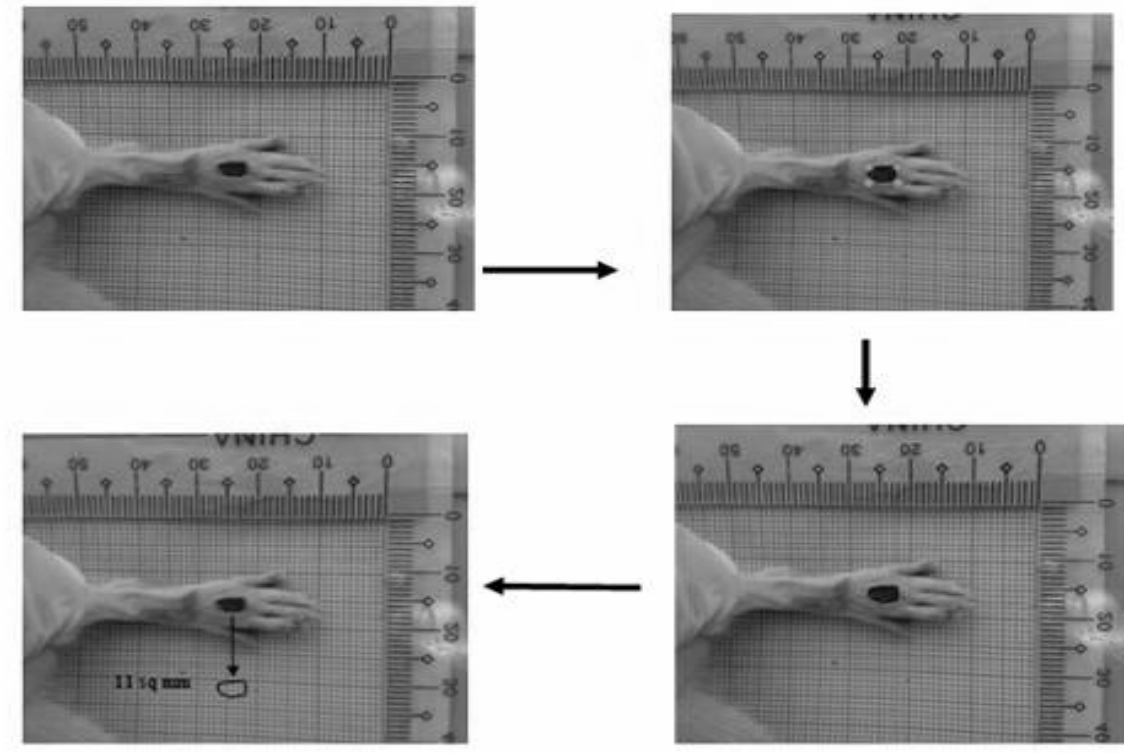


Figure 2: Wound area measurement in the diabetic rat model.

Table 1: MIC and MBC values for the tested antibiotics against the 8 staphylococcal isolates. The equivalent MIC breakpoints for *staphylococcus* spp. (CSLI 2005) were R: ≥4, S:≤ 1 for ciprofloxacin; R: ≥8, S:≤ 4 for gentamycin and R: ≥ 8/4, S: 4/2 for amoxicillin-clavulanic acid.

Strain	Ciprofloxacin		Gentamycin		Amoxicillin-clavulanic acid	
	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)
16S	0.5	1	2	4	8	32
25S	0.5	1	4	8	8	32
45S	128	512	128	256	64	256
18S	32	128	128	256	32	32
17S	8	8	64	128	8	8
78S	0.5	0.5	0.25	0.5	0.25	0.25
108S	0.125	0.5	1	2	0.25	1
90S	8	16	128	128	8	32

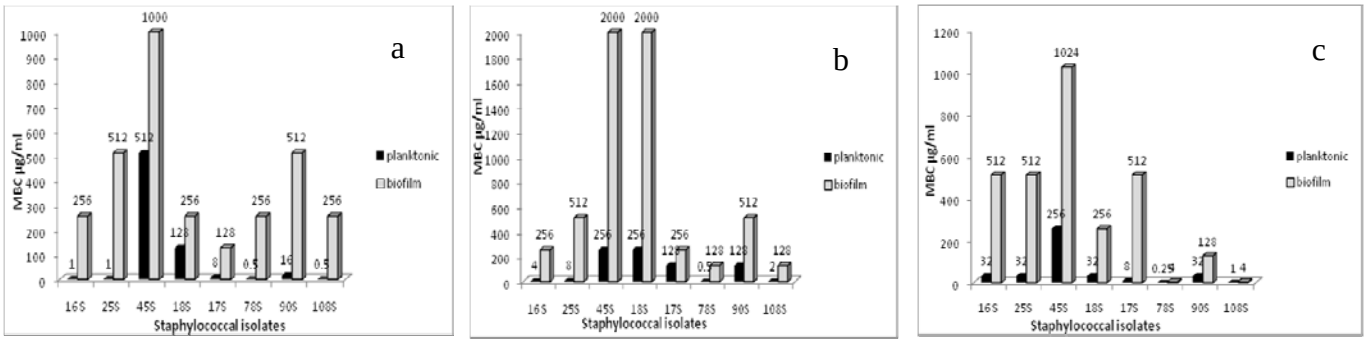


Figure 3: Comparison between planktonic and biofilm MBC of ciprofloxacin (a), gentamycin (b), and amoxicillin-clavulanic acid (c) for the 8 staphylococcal isolates.

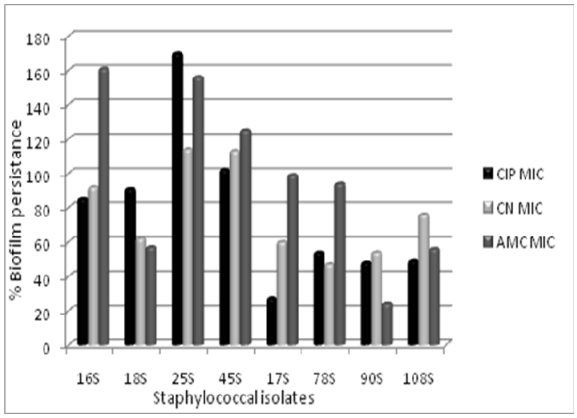


Figure 4: Effect of the MIC of each antibiotic on the removal of a pre-formed biofilm

Animal model:

Wound area measurement:

The wound healing effect of ciprofloxacin and amoxicillin clavulanic acid in case of planktonic stage of bacterial infection was compared with that after biofilm formation on days 1, 3, 5 and 9 in the diabetic rat foot infection model.

In case of the *S. aureus* isolate 25S, a trend of greater reduction of the ulcer area was observed in case of planktonic bacterial colonization treated with the antibiotic than that after the biofilm formation, where the average ulcer area in the control untreated groups decreased from 9mm^2 (100%) on day 1 to 3 mm^2 (33.33%) on day 9, whereas that treated with ciprofloxacin decreased from 13 mm^2 (100%) on day 1 to complete healing on day 9 (Figure 9), and that of the group treated with amoxicillin-clavulanic acid decreased from 14 mm^2 (100%) on day 1 to 1 mm^2 (7%) on day 9 .

Similar results were obtained in case of the wound inoculation with the isolate 78S, where the wound area started with 9, 18and 17 mm^2 (100%) on day 1 and was reduced to 3 mm^2 (33.33%), 1 mm^2 (5%) and 1 mm^2 (5.8%) in case of the control, ciprofloxacin and amoxicillin clavulanic acid treatment groups respectively.

It was noticed from the wound swab samples taken that complete eradication of the staphylococcal infection was achieved after four days in case of the isolate 25S when treated with either of the antibiotics, and three days for the isolate 78S in case of both

antibiotics. Whereas, after the formation of a biofilm it was difficult to completely eradicate the bacterial infection throughout the nine days of the experiment.

Histopathological studies:

Histopathological examination of rat skin sections of different treatment groups was made. Figures 6, 7 shows a wound made 48 hours before the examination, with the bacteria forming biofilm, this section was stained with H&E (Figure 6), and with Gram Stain (Figure 7) to visualize the Gram positive bacteria. Figure 8 showed complete healing and recovery from bacteria which was achieved with the ciprofloxacin treatment in case of the planktonic cells infection with the *S. aureus* isolate 25S. Whereas treatment after the biofilm formation with amoxicillin-clavulanic acid showed the persistence of bacterial infection with bacteria found around the hair follicles (Figure 9).

4. Discussion:

Biofilms, products of bacterial adherence, are structured communities of bacterial cells enclosed in a self-produced exopolysaccharide matrix and adherent to an inert or living surface. Establishment of a biofilm is the prelude to the development of various chronic, intractable infections, such as biomaterial-associated infections and pulmonary infection in patients with cystic fibrosis (Bonaventura *et al.*, 2004). Despite various efforts, treatment of an infection after biofilm is established is frequently futile because of the reduced susceptibility of biofilm to antibiotics. At least three mechanisms have been proposed to account for recalcitrance of biofilms to antimicrobial agents: (i) failure of the antimicrobial to penetrate the biofilm, (ii) slow growth and the stress response, and (iii) induction of a biofilm phenotype (Bonaventura *et al.*, 2004).

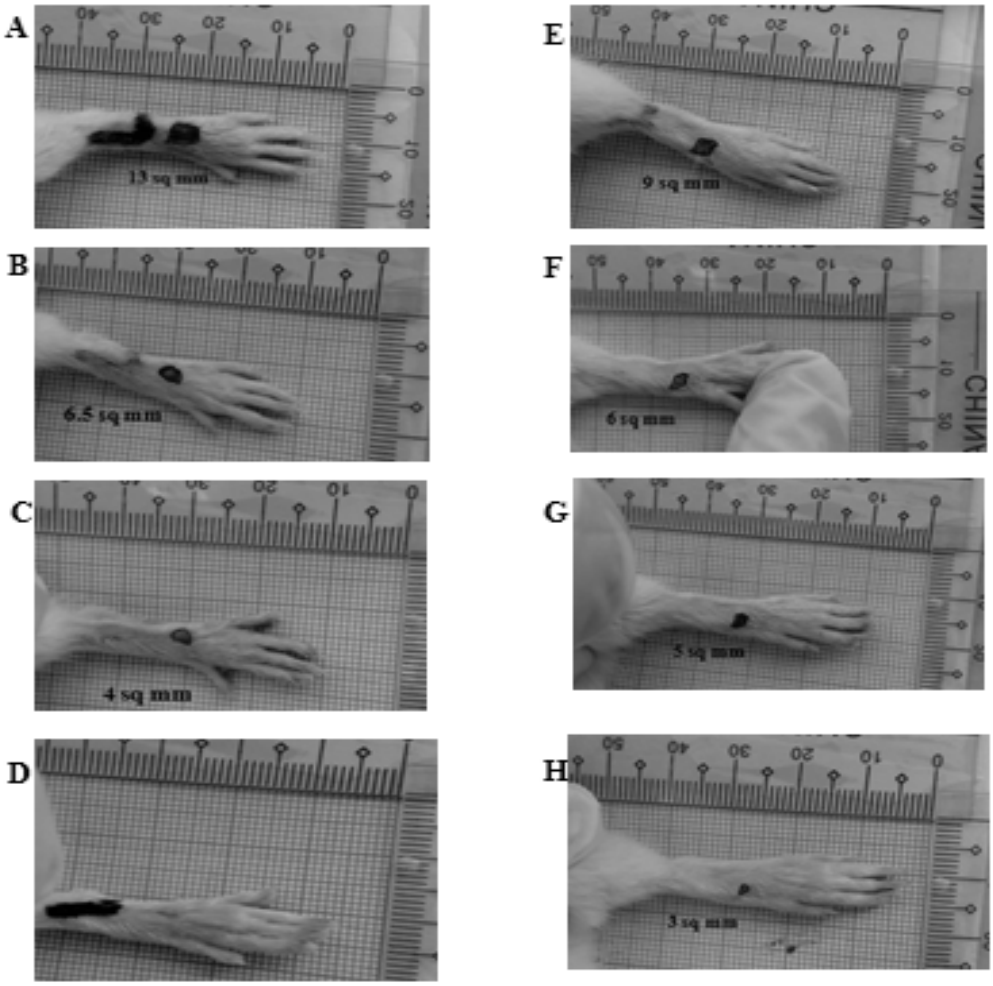


Figure 5: Effect of the treatment with ciprofloxacin on planktonic infection (on the left), compared to the control (untreated) (on the right) on wound healing of diabetic rat foot infection with *S. aureus* 25S.

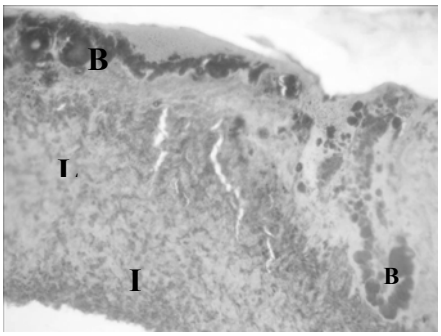


Figure 6: H & E stained section in diabetic rat skin 48 hours after wound formation and infection at X400 magnification using light microscopy. In the wound bed there are circular basophilic granular structures, which are multiple bacteria joined together living in biofilm-like structure (B) in the wound bed in the epidermis while the dermis shows lytic necrosis (L) and mononuclear cellular infiltrate (I). There is also hyalinizatrion and vaculation of the epithelial cells, and pathological changes in the epidermal cells was observed with mononuclear cellular infiltrate, hemorrhagic areas.

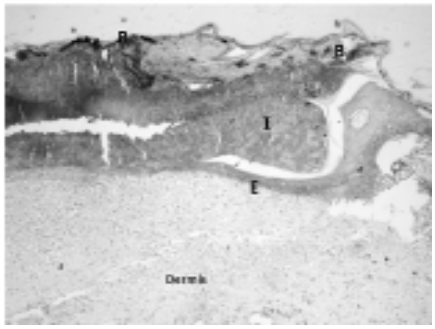


Figure 7: Section of 48 hours untreated wound stained with Gram stain showing Gram positive bacteria (B), cellular infiltrate (I), and reduced epidermis (E) (X100).

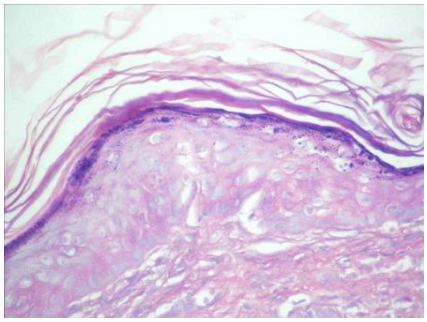


Figure 8: High power view of the epithelium showing normal skin, where complete healing was achieved in case of treatment of *S. aureus* 25S infection as planktonic cells with ciprofloxacin. (H&E stain X400)

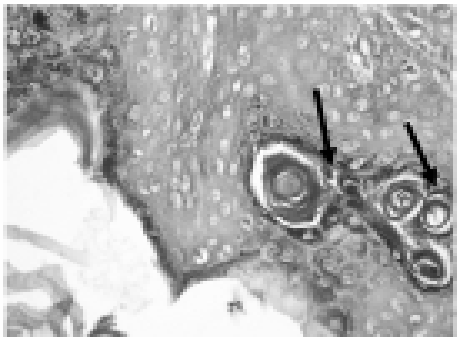


Figure 9: Another section in rat skin showing bacteria around transversely cut hair follicles with mononuclear inflammatory infiltrate. (H&E stain X400)

Microtiter plate systems for quantifying adherence and biofilm formation were used in this study, these techniques have been investigated with many different organisms and stains (Christensen *et al*., 1985) and they have been widely used because they are simple, reproducible, and quantitative methods. However, the staining measurements reflect the total amount of biofilm (sessile cells plus exopolysaccharide matrix) but do not give any information about its viability (Griffis *et al*., 2009). Thus, in this study, cell viability was also measured by determination of biofilm MBC, which was found to be very high (512 × planktonic MBC) in case of the isolates 25S, 78S and 108S when tested with ciprofloxacin. However, in other cases the difference between biofilm and planktonic MBC is much less (2 × planktonic MBC) in case of the isolate 18S when tested with ciprofloxacin also. These results was also noticed by other investigators who found that 4× MBC of the antibiotics used is not sufficient for killing *S. aureus* in biofilm (Amorena, *et al*., 1999). Also *Pseudomonas aeruginosa* in biofilm showed a 4-fold greater resistance against ciprofloxacin and gentamycin compared with free-living forms (Agarwal *et al*., 2005).

In the present study, the effects of several antibiotics on Staphylococcal adherence were tested. The studied antibiotics were chosen for several reasons. Amoxicillin-clavulanic acid was tested because it is frequently used in the therapy of Staphylococcal infections. Quinolone (ciprofloxacin) was chosen because of their interesting activity against gram-negative (Baskin *et al*., 2002) and gram-positive (Wilcox *et al*., 1991) bacterial biofilms. Currently there are no antibiotics on the market specifically indicated for the prevention of diabetic foot infections. Furthermore, diabetic ulcers are often associated with vascular disease and restricted peripheral blood flow, which may render

systemically acting antibiotics less effective. By achieving very high localized concentrations of antibiotic, gentamycin may be used to overcome these concerns if used locally**.**

In case of the preformed biofilm, ciprofloxacin showed the best results in biofilm removal. Concentrations ranging from 0.5-2 MIC caused removal of 50% of an already formed biofilm in five (62.5 %) of the tested isolates, followed by gentamycin where the MIC caused the removal of 50% of an already formed biofilm in only four (50%) of the tested isolates. However, amoxicillin-clavulanic acid showed the least results in biofilm removal (Figure 4). In addition, the effect of MIC of the tested antibiotics on a preformed biofilm showed that there is no fixed pattern for the effect of antibiotics on this preformed biofilm but it was noticed that the bacterial behavior in response to the different antibiotics applied is strain dependant, which requires further investigations. These results suggest that ciprofloxacin is able to penetrate staphylococcal biofilm with a strain-specific efficiency, as suggested by the high strain-to-strain variation, probably due to chemical and physical heterogeneity of staphylococcal biofilms. This difficulty in biofim removal with antibiotics alone led Krespi YP *et al*. (2010) to use ciprofloxacin in combination with different types of lasers to remove more than 80% of Staphylococcal biofilm cells. Other investigators used other approaches to treat biofilm-associated staphylococcal infections such as cell-wall degrading enzymes (Son J *et al*. 2009).

From this study we can conclude that once biofilms are formed they are very difficult to be removed even by applying multiples of the MIC of the tested antibiotics. And it was found that there is a high strain-to-strain variation in the behavior towards the tested antibiotics, so it is advised to study each strain separately.

The majority of skin pathogenic and non pathogenic biofilm research is performed *in vitro*. Although *in vitro* assays have several advantages, including lower cost, and the ability to control the number of bacteria, they do not take into consideration the effect of wound fluid, growth factors, proteases, and antimicrobial peptides (Davis *et al.*, 2008a). In this study the rat model used was anticipated to help clarify the role of bacterial biofilms in diabetic wound infection and healing.

In *in vivo* studies of ulcer healing, surgical ulcers were usually induced on the chest or back of a rat or a mouse. With the aim of mimicking more closely the clinical condition of diabetic foot ulcers, the ulcers were created on the feet of alloxan-diabetic rats. The animal model was probably suitable for *in vivo* studies of ulcers in response to different medications. In this study, the diabetic rat foot ulcer model was created by removal of full-thickness skin. This artificially created ulcer is different from the ulcer in the human diabetic foot which usually results from local pressure. Human diabetic foot ulcers usually extend beyond the surface boundaries and this subcutaneous extension does not occur in this animal model (Lau *et al*., 2008).

The authors previously demonstrated that pathogenic wound bacteria *in vitro* can form a mature biofilm within 10 hours. Results of an *in vivo* study have shown that occluding wounds following inoculation with pathogenic bacteria facilitates the development of biofilms after 24 hours. The authors believe that biofilms are likely to be present in chronic wounds and additional strategies to disturb or eliminate them are sorely needed (Davis *et al*., 2008b).

Studies made by Dr. Akiyama's group demonstrate biofilm-like structures in a mouse wound model and one in human skin. Using confocal laser scanning microscopy they showed colonies of bacteria surrounded by glycocalyx. These studies firmly established that biofilm-like structures are present in the murine wound infection model (Akiyama *et al*., 2004)

The management of chronic wounds such as those found in diabetic feet or leg ulcers is placing an increasing burden on health service systems (Krouskop, 2002). Chronic wounds, however, have an unfortunate tendency of healing very slowly. Any measurement technique used for the purpose of establishing healing progress therefore has to be very precise in order to capture small changes in a wound's dimensions.

The 'gold standard' for area measurement is the practice of tracing the perimeter of a wound through a double layer of a flexible transparent sheet material (Keast, 2004). While the layer in contact with the wounds is discarded, the upper layer with the tracing is then measured by either placing it on metric graph paper, planimeters or by a second round of tracing using a digitizing tablet (Thawer, 2002). This contact making method can be painful to the patient and may risk infection. In practice the use of unsuitably thick or exhausted marker pens and the high degree of dexterity required by the clinician performing the tracing significantly reduce the theoretical precision of the method and errors up to 25% have been reported (Lagan, 2000).

Photography has the advantage of avoiding contact with the patient (Smith, 1992; Stacey, 1991). Wound pictures are usually measured on a computer screen where they can also be enlarged so that the tracing process is freed from time restrictions and physical demands. Although area measurements

produced this way tend to be more precise than those obtained by transparency tracings the method depends on the photography skills of the clinician who has to combine a frame-filling wound picture together with a reference scale in a single image taken perpendicular to the wound site (Solomon, 1995).

Digital photography was the way used in the wound area measurement in this study, where the wound and the metric reference scale were included in the same picture as shown in Figure (2). This gave us the advantage of avoiding direct contact with the wound and allowed us to enlarge the wound picture together with scale on the computer screen in order to increase the accuracy of wound area measurement.

In this study bacterial biofilm colonization in an animal model was obtained, where the wounds were inoculated with the selected isolate and left untreated, swabs were taken to ensure the presence of the bacteria in the wound of the control group through out the study period. This was also made by Davis *et al*. (2008a) who observed colonies of bacteria firmly attached to and on the wound surface, encased in an amorphous substance; they also observed the polymorphnuclear cells on the surface of the biofilm, but not within the biofilm. Histologically, bacterial biofilms are tissue-like structures resembling multicellular eukaryotic tissues. According to the histology text book edited by Leon Weiss (1988) a tissue is a multicellular aggregate of similar cells. In line with this definition bacterial biofilm can be described as a "bacterial tissue" within the host tissue.

In order to show a physiological difference between the planktonic and biofilm bacteria *in vivo*, we studied the efficacy of two antibiotics on two bacterial isolates in the rat model. The treatment with ciprofloxacin or amoxicillin-clavulanic acid was extremely effective in eradicating the organisms that were not attached to the wound surface (3-4 days). However, after allowing the biofilm formation, this treatment was not able to eradicate the staphylococcal infection at such a short time because the biofilm bacteria were more persistent, fulfilling one of the criteria for biofilm-associated diseases, which is reduced susceptibility. These results were also demonstrated by Davis *et al*. (2008a) who studied the effect of two topical antibiotics on *S. aureus* infected wound healing, where the antibiotics were not able to eradicate bacteria that were allowed to form biofilm.

The concordance of *in vitro* and *in vivo* findings observed in the staphylococcal isolates tested using this biofilm test methodology is encouraging. This concordance suggests that the test could be useful in clinical practice and extensive studies especially needed to eradicate the increasingly frequent chronic staphylococcal infections and in particular those associated with the diabetic foot ulcers.

The use of an *in vivo* model in this study supports the hypothesis that staphylococcal biofilm are more difficult to eradicate and may play an important role in acute and chronic diabetic foot infections. We have provided evidence that biofilms play a role in diabetic foot infections. A new paradigm has arisen; it is not sufficient to think of bacteria in infections as unicellular independent pathogens, rather it is now important to understand that they form multicellular tissue like structures (Davis *et al.*, 2008a).

Corresponding author
R. AboZahra
Microbiology Department, Faculty of Pharmacy, Pharos University, Alexandria, Egypt
rania_abozahra@yahoo.com

5. References:

1. Agarwal, G., Kapil, A., Kabra, S. K., Das, B. K., Dwivedi, S. N. 2005. In vitro efficacy of ciprofloxacin and gentamicin against a biofilm of Pseudomonas aeruginosa and its free-living forms. The National Medical Journal of India. 18(4): 184-186.
2. Akiyama, H., Oono, T., Saito, M, and Iwatsuki, K. 2004. Assessment of cadexomer iodine against *Staphylococcus aureus* biofilm in vivo and in vitro using confocal laser scanning microscopy. J Dermatol. 31: 529-34.
3. Amorena, B., Gracia, E., Monzon, M., Leiva, J., Oteiza, C., Pérez, M., Alabart, J., and Hernández-Yago, J. 1999. Antibiotic susceptibility assay for *Staphylococcus aureus* in biofilms developed in vitro. J Antimicrob Chemothr. 44:43-55
4. Arciola, C. R., Baldassarri, L, and Montanaro, L. 2001. Presence of icaA and icaD Genes and slime production in a collection of staphylococcal strains from catheter-associated infections. J. Clin. Microbiol. 39: 2151-2156.
5. Aulivola, B., Hile, C. N., Hamdan, A. D., Sheahan, M. G., Veralid, J. R., Skillman, J. J., Campbell, D. R., Scovel, S. D., Logerfo, F. W., and Pomposelli, F. B. 2004. Major lower extremity amputation: outcome of a modern series. Arch Surg. 139: 395-399.
6. Baskin, H., Dogan, Y., Bahar, I. H., and Yulug, N. 2002. Effect of subminimal inhibitory concentrations of three fluoroquinolones on adherence of uropathogenic strains of *Escherichia coli*. Int. J. Antimicrob. Agents 19:79–82.
7. Berry, V., Page, R., Satterfield, J., Singley, C., Straub, R., and Woodnutt, G. 2000. Comparative efficacy of gemifloxacin in experimental models

of polynephritis and wound infection. J Antimicrob Chemother. 45: 87-93.
8. Bonaventura, G. D., Spedicato, I., D'Antonio, D., Robuffo, I., and Piccolomini, R. 2004. Biofilm Formation by *Stenotrophomonas maltophilia*: Modulation by Quinolones, Trimethoprim-Sulfamethoxazole, and Ceftazidime. Antimicrob. Agents Chemother. 48:151-160.
9. Cagni, A., Chuard, C., Vaudaux, P. E., Schrenzel, J., and Lew, D. P. 1995. Comparison of Sparfloxacin, Temafloxacin, and Ciprofloxacin for Prophylaxis and treatment of experimental foreign-body infection by methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. 39: 1655-1660.
10. Christensen, G. D., Simpson, W. A., Younger, J. J., Baddour, L. M., Barrett, F. F., Melton, D. M., and Beachey, E. H. 1985. Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. J. Clin. Microbiol. 22:996-1006.
11. Clinical and Laboratory Standards Institute (CLSI) (2003). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. 6th ed. Approved standard. M7-A6. Wayne, PA: CLSI.
12. Davis, C., Martiner, L., and Kirsner, R. 2006. The diabetic foot: The importance of biofilms and wound bed preparation. Current Diabetes Reports. 6: 439-445.
13. Davis, S. C., Ricotti, C., Cazzaniga, A., Welsh, E., Eaglstein, W. H., and Mertz, P. M. 2008a. Microscopic and physiologic evidence for biofilm-associated wound colonization in vivo. Wound Rep Reg. 16:23-29
14. Davis, C. S., and Mertz, P. M. 2008b.Determining the effect of an Oak Bark Formulation on Methicillin-resistant Staphylococcus aureus and wound healing in porcine wound models.
15. Diniz, S. F., Amorim, F. P. L. G., Cavalcante-Neto, F. F., Bocca, A. L., Batista, A. C., Simm, G. E. P. M., and Silva, T. A. 2008. Alloxan-induced diabetes delays repair in a rat model of closed tibial fracture. Braz. J. Med. Biol. Res., 41(5): 373-379.
16. Gotz, F. 2002. *Staphylococcus* and biofilms. Mol. Microbiol. 43:1367-1378.
17. Griffis, C. D., Metcalfe, S., Bowling, F. L., Boulton, A. J. M., and Armstrong, D. G. 2009. The use of gentamicin-impregnated foam in the management of Diabetic foot infections: a promising delivery system. Expert opinion on drug delivery. 6 (6): 639-642.
18. Kandemir, O., Akbay, E., Sahin, E., Milcan, A., and Gen, R. 2007. Risk factors of infection of the diabetic foot with multi-antibiotic resistant microorganisms. J. infect. 54: 439-445.
19. Keast, D. H., Bowering, C. K., Evans, A. W., Mackean, G. L., Burrows, C., and D'Souza, L. 2004. A proposed assessment framework for developing best practice recommendations for wound assessment Wound Repair and Regeneration. MEASURE. 12, s1-s17.
20. Krespi, Y. P., Kizhner, V., Nistico, L., Hall-Stoodley, L., Stoodley, P. 2010. Laser disruption and killing of methicillin-resistant *Staphylococcus aureus*. In Press.
21. Krouskop, T. A., Baker, R., and Wilson, M. S., 2002. A non contact wound measurement system, J Rehabil Res Dev. 39: 337-346.
22. Kugelberg, E., Norstrom, T., Petersen, T. K., Petersen, T. K., Duvold, T. 2005. Establishment of a superfacial skin infection model in mice by using *Staphylococcus aureus* and *Streptococcus pyogens*. Antimicorb. Agents Chemother. 49: 3435-3441.
23. Lagan, K. M., Dusoir, A. E., McDonough, S. M., and Baxter, G. D. 2000. Wound measurement: the comparative reliability of direct versus photographic tracings analyzed by planimetry versus digitizing techniques Arch Phys Med Rehabil, 81, 1110-1116.
24. Lau, T.W., Sahota, D.S., Lau, C.H., Chan, C.M., Lam, F.C., Ho, Y.Y., Fung, K.P., Lau, C.B.S., Leung, P.C. 2008. An in vivo Investigation on the Wound-Healing Effect of two Medicinal Herbs Using an Animal Model with Foot Ulcer Eur Surg Res. 41:15-23
25. Lau, T. W., Lam, F. F. Y., Lau, K. M., Chan, Y. W., Lee, K.M., Sahota, D. S., Ho, Y. Y., Fung, K. P., Leung, P. C., and Lau, C. B. S. 2009. Pharmacological investigation on the wound healing effects of Radix Rehmanniae in an animal model of diabetic foot ulcer. Jo urnal of Ethnopharmacology. 123: 155-162.
26. Morikawa, K., Nonaka, M., Yoshikawa, Y., and Torii, I. 2005. Synergistic effect of fosfamycin and arbekacin on a methicillin-resistant *Staphylococcus aureus*-induced biofilm in a rat model. Int. J antimicrob agents. 25(1): 44-50.
27. Saginur, R., St Denis M., Ferris, W., Aaron, S. D., Chan, F., Lee, C., and Ramotar, K. 2006. Multiple combination bactericidal testing of staphylococcal biofilms from implant-associated infections. Antimicrob. Agents Chemother. 50: 55-61.
28. Smith, D. J., Bhat, S., and Bulgrin, J. P. 1992. Video image analysis of wound repair. Wounds. 4: 6-15.
29. Solomon, C., Munro, A. R., Van Rij, A. M., and Christie, R. 1995. The use of video image analysis

for the measurement of venous ulcers. Br J Dermatol. 133: 565-570.
30. Son, J., Lee, S., Jun, S. Y., Yoon, S. J., Kang, S. H., Paik, H. R., Kang, J. O., and Choi, Y. 2009. Antibacterial and biofilm removal activity of podoviridae *Staphylococcus aureus* bacteriophage SAP-2 and a derived recombinant cell-wall-degrading enzyme. Appl. Microbiol. Biotechnol. 86 (5): 1439-1449.
31. Stacey, M. C., Burnand, K. G., Layer, G. T., Pattison, M., and Browse, N. L. 1991. Measurement of the healing of venous ulcers. Aust N Z J Surg. 61: 844-848
32. Stepanovic, S., Vukovic, D., Dakic, I., Savic, B., and Svabic-Vlahovic, M. 2000. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. J. of Microbiol. Methods. 40: 175-179
33. Sullivan, P. K., Conner-Kerr TA, Hamilton H, Smith EP, Tefertiller, C., and Webb, A. 2004. Assessment of Wound Bioburden Development in a Rat Acute Wound Model: Quantitative Swab versus Tissue Biopsy.
34. Thawer, H. A., Houghton, P. E., Woodbury, M. G., Keast, D., and Campbell, K. 2002. A comparison of computer-assisted and manual wound size measurement, Ostomy Wound Management, 48, 46-53
35. Tre-Hardy, M., Vanderbist F, Traore, H., and Devleeschouwer, M. J. 2008. In vitro activity of antibiotic combinations against *Pseudomonas aeruginosa* biofilm and planktonic cultures. Int. J. Antimicrob. Agents. 31:329-336.
36. Weiss, L., (ed.). 1988. Cell and tissue biology: a textbook of histology. 6th edn. Urban and Schwarzenberg. 115pp.
37. Wilcox, M. H., Finch, R. G., Smith, D. G. E., Williams, P., and Denyer, S. P. 1991. Effects of carbon dioxide and sub-lethal levels of antibiotics on adherence of coagulase-negative staphylococci to polystyrene and silicone rubber. J. Antimicrob. Chemother. 27:577–587.
38. Wrobel, J. S., Mayfield, J. A., and Reiber, G. E. 2001. Geographic variation of lower-extremity major amputation in individuals with and without diabetes in the Medicare population. Diabetes care. 24: 860-864.

10/11/2010

Women Participation in Agro-allied Small and Medium Scale Enterprise and Its Impact on Poverty Alleviation in Oyo State Nigeria

Fajimi F.O and Omonona B.T

Department of Agricultural Economics, University of Ibadan, Ibadan, Nigeria
ffovivid@yahoo.com

Abstract: This study examined the impact of women participation in agro-allied small and medium scale enterprises (SME) on poverty alleviation. Data were collected using the multistage sampling technique from 119 respondents in the study area made up of 59 participants and 60 non-participants. Data generated were analysed using descriptive statistics, FGT – weighted poverty measures and Probit regression analysis. Results from the study showed that the non-participants have the highest poverty level (51%), while the participants have poverty level of (17%) and the non-participants contribute greatly to whole group poverty. The estimated probit regression analysis showed that marital status, household size and women status in the family are poverty enhancing while educational status participation in Small and Medium Enterprises, income and monogamous family type are poverty reducing. Hence participation in agro-allied Small and Medium Enterprises is antidote to reducing poverty among women.

[Fajimi F.O, Omonona B.T. **Women Participation in Agro-allied Small and Medium Scale Enterprise and Its Impact on Poverty Alleviation in Oyo State Nigeria**. Journal of American Science 2010;6(12):771-780]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Poverty, SMEs, Agro-allied, Women, Participation

1. Introduction

Poverty in Nigeria has been described as "widespread and severe" (World Bank, 1996). In spite of the country's vast resources it is known with low GDP per capita, high unemployment rate, low industrial utilization capacity, high birth rate and agricultural dependent (Jhingan, 2005). Poverty is basically a situation of deprivation experienced by human beings. Burkey (1993) defined poverty in terms of the absence of basic needs which includes clean air and water, adequate food and balanced diet, emotional and physical security and culturally and climatically, appropriate clothing and shelter. Poverty is often described as a threat to economic and social stability and is defined as a condition of being in want of something. It is also characterized by malnutrition, illiteracy, disease, high infant mortality and low life expectancy at birth. Poverty is not only a state of existence but also a process with many dimensions and complexities (Khan, 2000). Table 1 shows the trends in poverty level in Nigeria between 1980 and 1996. The incidence of poverty increased sharply both between 1980 and 1985 and between 1992 and 1996. The 27.2% for 1980 translated to 17.7 million persons whereas there were 34.7 million poor persons in 1985. As at 1996, two out of every three Nigerians were below the poverty line (Yusuf, 2002).

Table 1: Trends in poverty level: 1980-1996 (in %)

Year	Poverty Level (%)	Estimated population (million)	Population in poverty (million)
1980	28.1	65	18.3
1985	46.3	75	34.7
1992	42.7	91.5	39.1
1996	65.6	102.3	67.1

Source: FOS, 1999

In an attempt to deal with the problem of poverty through poverty alleviation programme in an agrarian country like Nigeria, knowledge of poverty profile is essential. It has been empirically established that low productivity in agriculture is the cause of high incidence of poverty in Nigeria (World bank, 1996). This is obvious as agriculture is the mainstay of Nigeria's economy contributing about 42% to total GDP and employing about 77% of the working population. It therefore imperative that any policy measure aimed at alleviating poverty must take agriculture and rural development into consideration.

The Federal Office Statistic/World Bank in their analysis of the poverty trend in Nigeria noted that poor families are in higher proportion in farming household that are mainly in the rural areas (Adeolu & Taiwo, 2004). Poverty has been described to have a predominant 'female face' in both developing and industrialized countries (Odejide, 1997). Neo Leen (1996) reports that more than 564 million women live in absolute poverty and revealed the following about the state of women in the world.

1. Women own ten percent of the world poverty and hold one percent of chief executive position worldwide.
2. Women comprise two-thirds of people who cannot read or write.
3. Women are 70 percent of the world's absolute poor.
4. Women and children comprise 75 percent of the displaced people in ecologically fragile zones.

Women are regarded as the world's poor because the majority of the 1.5 billion people living on 1 dollar a day or less are women and earn an average slightly more than 50 percent of what men earn (UNDP, 2000). Women understand poverty in different ways such as; a situation of deprivation, inability to measure up to certain expectations related to basic needs and inability to create resources for recreation and holidays also, Nigerian women are affected with poverty and have long duration in poverty because they often have too many children spaced too closed together to the detriment of their health and productivity (Ijaiya, 2000, Adepoju, 2001). The incidence of poverty among Nigerian women has been progressively increasing. It has increase from 26.9% in 1980 to 58.5% in 1996 and in the past two decades women have constantly been put under the pressure of retrenchment, belt-tightening fiscal policies to boost the deteriorating economic activities/conditions more than men and has forced them to share the preserved role of the provider with men or in some cases assume the entire responsibility (FOS, 1999, Adepoju, 2001). The core source of the entire gender differential in poverty is that women relative to men are more vulnerable because of the socio-cultural framework of human society. The socio-cultural beliefs are the limiting factors, which limits the opportunities and capabilities of women, and make them resource less and powerless individuals (Ijaiya, 2000). Poverty reduction is the supreme goal of development policy. The incidence, depth, and severity of poverty should be known by the policy makers so as to reduce poverty. The paper estimates poverty measures using the gap and squared gap poverty measures as well as the headcount ratio; Easterly (2000) and Ravallion (1997) limit their analysis to the headcount measure. The uses of depth and severity measures of poverty is important as these two additional measures of poverty and, hence complement the poverty– spread picture painted by the headcount ratio. Given the importance of small and medium scale enterprises as discussed, the main objective of this study is to determine the impact of women participation on the poverty status of women. This becomes imperative in Nigeria due to dearth of literature on women's participation in small and medium scale enterprises on women poverty status. The working hypothesis is that women's participation in agro-allied small and medium scale enterprises will significantly affect the poverty status of the women.

2. CONCEPTUAL FRAMEWORK

The definition of small and medium scale enterprises differ from one country to another. It has been defined against various parameters such as the number of workers employed, volume of output or sales, the value of assets or capital employed, and the type of energy used. Some definitions are based on whether the owner of the enterprises works alongside with the workers, the degree of sophistication in management, and whether or not an enterprises lies in the "formal" section. SMIES and NERFUND (2004) define SMES as an enterprise with an asset base not exceeding N200,000,000.00 excluding land and working capital with staff strength of not less than 10 and not more than 300. A cursory glance at the structure of SMES in Nigeria reveals that 50% are engage in distributive trade, 10% in manufacturing, 30% in agriculture and the rest 10% in services. Obitayo (2000) stated that globally, the small and medium scale enterprises are noted for their immense contributions to development process and as engine of economic growth. They are promoted as a critical segment of the manufacturing sub-sector, effective strategy for tackling unemployment, diversifying output and achieving trade and balance of payment, given their nature and characteristics with respect to quick adaptation of technologies, manageable number of workers and reduced capital intensiveness. Nnanna (2001) stated that small and medium scale enterprises needs funds to bring together the other factors of production- land, labour and capital for production to take place. Sule (1986) stated that, it is evidence around the world that small and medium scale enterprises provide an effective means of stimulating indigenous entrepreneurship, enhancing greater employment opportunities per unit of capital invested and aiding the development of local technology. Nnanna (2003) acknowledged that, small and medium scale enterprises are considered generally as the bedrock of the industrial

development of any country. The small and medium scale enterprises (SMES) have been generally acknowledged as the bedrock of the industrial development of any country (Yerima et.al, 2007).

The dynamic role of small and medium scale enterprises in developing countries have been highly emphasized. These enterprises have been identified as the means through which the rapid industrialization, job creation, poverty alleviation and other development goals of these countries can be realized. The changing role of small and medium scale enterprises in developing countries as an engine through which the growth objectives of developing countries can be achieved has long been recognized. It is estimated that SMEs employ 22% of the adult population in developing countries. These enterprises have been recognized as the engines through which the growth objectives of developing countries can be achieved. They are potential sources of employment and income in many developing countries. (Daniels and Ngirira, 1992; Daniels and Fisseha, 1992). Women's participation in small scale enterprises are alternative systems of production operating on the principle of human economy, in that their goal is to contribute to the education and health of the family with minimum emphasis on profit. This is based on the rationale that a greater proportion of women's income compared to that of men goes to meet family needs. The micro-enterprise activities provide opportunities for women to develop the skills in decision-making, problem solving and information-seeking. According to Jariah and Laily (1999), a rural enterprise project has the potential of providing an avenue for the rural women not only to improve their socio-economic wellbeing, but more so to increase their entrepreneurial abilities and personal empowerment. Over the past twenty years there has been a due recognition of women's potential contribution which has resulted in a major shift towards women as a key target group for programmes using small and medium scale enterprises development as a way to achieve wider poverty reduction (ILO, Gender Briefs Series No.3). Tanko (1995) observed that women play a pivotal role in alleviating poverty through productive work that they are engaged in outside their home. Although increasing women's participation in small and medium scale enterprise is among the developmental goals and targets to reduce poverty, improved family health and empower women's economic status. Therefore, this concept is very important in the study of women participation in agro-allied small and medium scale enterprises and its impact on poverty.

Hence, women participation in small and medium scale enterprises is expected to be positively associated with reduced poverty through increased cash income and opportunities for women to develop their entrepreneurial skills. The rest of the paper is divided into three: section three discusses the methodology while section four is on results and discussion. The last section concludes the paper.

3. Research Methodology

Area of Study

The study was conducted in Oyo state of Nigeria, the state is located in the southwest region of Nigeria and lies between latitude 7^0 and 9^0 30' North of the equator and between longitude 2.5^0 and 5^0 east of the prime meridian. The state is made up of 33 Local Government Areas. 5 Local Government Area were chosen for the study.

Sampling Procedure

Multistage sampling technique was employed for selecting the representative women. The first stage was random selection of five local government areas from the Ibadan/Ibarapa zone of the state. The second stage was sampling of villages/areas based on the list of community development project of the State Ministry of Women Affairs and University Village Association (UNIVA). The third stage involved the use of systematic random sampling to obtain the required women, by choosing every fifth housing unit in which four women were randomly selected for the interview. A total of 150 women were sampled but only 119 were used for the final analysis. Table 2 shows the distribution 119 women whose questionnaires were used for the analysis, while table 3 shows the distribution of Small and Medium Scale Enterprises engaged in by the women as obtained from the study.

Table 2: Distribution of Sampled Women

Location/LGA	SME Participant	SME Non-participant
Akinyele	15	15
Egbeda	12	12
Ibadan North East	7	8
Iddo	14	14
Ona-Ara	11	11
Total	59	60

Source: Field Survey, 2005.

Table 3: Distribution of Women Participants by the types of Small and Medium Scale Enterprises

Type of SME	Frequency	Percentage
Oil palm processing	32	54.1
Cassava processing	10	16.9
Tie and dye	8	13.6
Soap making	6	10.2
Livestock management	3	5.1
Total	59	100

Source: Field Survey, 2005.

Analytical Techniques

The analytical techniques used in the analysis of the data include Foster, Greer and Thorbecke's (FGT) weighted poverty index and the Probit Regression Model.

The FGT weighted poverty measure, otherwise called the Pα measure is used to obtain the incidence, depth and severity of poverty. The FGT measure is mathematically given as:

$$P_\alpha = \frac{1}{n_i}\sum_{i=1}^{q_i}\left[\frac{z - Y_{ij}}{z}\right]^\alpha \qquad 1$$

where Pαi is the weighted poverty index for the ith subgroup; ni is the total number of households in subgroup i, qi is the number of the ith subgroup households in poverty; Yi is the per capita expenditure (PCE) of women j in sub group i; Z is the poverty line and α is the degree of aversion.

When α = 0, it gives the incidence of poverty; α = 1 gives the depth of poverty and α = 2 gives the severity of poverty.

The contribution (Ci) of each sub-group's weighted poverty measure to the whole group's poverty measure was determined as follows:

$$C_i = \frac{n_i P_{\alpha i}}{n P_\alpha} \qquad 2$$

Where ni is the total number of women in the subgroup i, Pαi is weighted poverty for the subgroup i, pα is the whole group poverty index.

The poverty line for the study was obtained by converting the nationally determined poverty line of ₦395/annum in 1985 constant prices to the 2005 prices. This was done by multiplying the derived raising factor of 66.2361 by the nationally obtained poverty line of ₦395 per annum at constant 1985 prices by FOS (1999). This value indicate by how much 1985 poverty line must be raised to give 2005 poverty line and this was then divided by 12 to obtain the monthly poverty line per capita for the study.

The correlates of poverty are isolated using a Probit model in which a dichotomous variable representing whether or not a household is poor is regressed on a set of supposedly exogenous explanatory variables.

The probit regression model hypothesizing the determinants of poverty and ascertaining the effects of certain factors (especially SME participation among women) is stated below as:

$$Y = \beta_0 + \sum_{i=1}^{n}\beta_i X_i \qquad 3$$

The dependent variable Y is of a binary nature (poverty status of women), which takes 1 and 0, 1 if poor, 0 if non-poor. The independent variables (X) are as follows:

X_1 - Age of the respondents (in years)

X_2 - Marital status (Dummy D = 1, if married, 0 if otherwise)

X_3 - Household type (Dummy, D =1 if monogamous, 0 if otherwise)

X_4 - Household size

X_5 - Educational status (Number of years spent in school)

X_6 - Women status in family (Dummy, D =1 if household head, 0 if otherwise)

X_7 - Participation Dummy (D = 1 if participant, 0 if non-participation).

X_8 - Income (Naira)

4. Results and Discussion

The results in table 1 shows the average age for the participants stood at 48 years while that of the non-participants is 43 years, also the two groups has higher proportion of married women of 76% and 66% respectively. The mean year of schooling for the two groups is very close of about 6 years and 7 years while the average number of family size of about 5 persons is common to the two groups. The participants has a higher proportion of women who have polygamous household type of 61% and 50% for the non-participants also the status of women within the family also follow the same manner of 34% for the participants and 18% for the non-participants.

Table 4: Socio-Economic Variables of the Representative Women.

Variables	Participants (P)	Non-Participants (NP)
Age of representative women(mean)	48.28	43.10
Marital status (% married)	66	76
Educational Status (mean)	5.9	6.9
Household size (mean)	5	5
Household Type (% polygamous)	61	50
Women status (%household head)	34	18.3
Income(mean monthly)	11,912.44	6,500.00

Source: Field survey, 2005

Poverty Profile of Sampled Women

The poverty status of the sampled women was decomposed based on factors such as age, marital status and educational status of the women. Others are household size, household type, women status within the family and household expenditure.

Women's Household expenditure

Table 5 below shows that the non-participants have higher poverty incidence, depth, and severity than the participants and contributes about (75%) to the whole group. Therefore from the table, one can conclude that poverty is more prevalent among the non – participants than the participants judging from the poverty incidence, depth and severity. This may be due to the higher mean per capita expenditure than the non-participants.

Table 5: Poverty Profile by Women Household Expenditure

Participants index	P_0	P_1	P_2	Contribution to		
				P_0	P_1	P_2
Participants	0.169	0.021	0.002	0.243	0.142	0.066
Non-participants	0.516	0.124	0.030	0.755	0.856	1.008
All	0.3445	0.64	0.012			

Source: Field survey, 2005.

Marital Status of Sampled Women

Table 6 showed that the married women are poorer for the participants and non – participants and also contributes higher proportion to the whole group poverty level. The higher poverty level among the married may be due to the joint effect of increased household size and dependency ratio.

Table 6: Poverty Profile by Marital Status of Women

Participants marital status	P_0	P_1	P_2	Contribution to P_0	Contribution to P_1	Contribution to P_2
Married	0.118	0.049	0.021	0.169	0.337	0.694
Single	0.051	0.021	0.008	0.073	0.142	0.264
Non–participants						
Married	0.400	0.096	0.023	0.585	0.663	0.773
Single	0.117	0.048	0.006	178	0.130	0.199

Source: Field survey, 2005.

Age of the Sampled Women

Table 7: Poverty Profile by Age

Participants Age group (years)	P_0	P_1	P_2	Contribution to P_0	Contribution to P_1	Contribution to P_2
26-35	0.00	0.00	0.00	0.00	0.00	0.00
36-45	0.033	0.004	0.001	0.047	0.027	0.016
46-55	0.067	0.008	0.001	0.096	0.054	0.036
56-65	0.067	0.008	0.001	0.096	0.054	0.036
Non-participants						
26-35	0.050	0.012	0.001	0.073	0.085	0.033
36-45	0.216	0.052	0.012	0.316	0.035	0.403
46-55	0.183	0.044	0.010	0.267	0.303	0.336
56-65	0.060	0.024	0.003	0.087	0.165	0.100

Source: Field Survey, 2005

Four age groups were used to profile poverty among the women. These are 26 – 35 years, 36 – 45 years, 46 – 55 years and 56 – 65 years. The table below shows that poverty incidence is higher for ages 36 – 45 (21%) and 46 - 55 years (18%) in the non-participants group than the participant that has 3% and 6% respectively. For the participant there was non-poor in ages between 26 – 35 years while 5% were poor for the non – participant in this age group. For ages between 56 – 65 years the participants and non – participants have the same proportion of 6% poor, the respective contribution follows the same pattern. The participants belonging to poor while the non-participants are poorer so also the other age groups such as 36-45, 46-55 and 50-65.

Household Size of Women

The level of poverty experienced by any household is directly related to the number of the members of the household.

Table 8: Poverty Profile by Household Size

Participants Household size	P_0	P_1	P_2	Contribution to P_0	Contribution to P_1	Contribution to P_2
4-6	0.016	0.006	0.002	0.023	0.041	0.066
7-9	0.152	0.063	0.026	0.218	0.427	6.859
Non-participants						
4-6	0.35	0.084	0.02	0.023	0.041	0.066
7-9	0.16	0.040	0.002	0.241	0.278	0.324

Source: Field survey, 2005.

The table above shows that while 2 percent of those households with 4-6 members are poor, 15 percent is poor in households with between 7 and 9 members for the participants while for the non-participants 35 percent of those households with 4-6 members are poor, 16 percent is poor in households with between 7 and 9 members, though non participants have large family size but are less poor than those with smaller household size, this may be due to the dependency ratio.

Household Type of Sampled Women

Table 9: Poverty Profile by Household Type

Participants	P_0	P_1	P_2	Contribution		
				P_0	P_1	P_2
Monogamous	0.067	0.028	0.011	0.096	0.190	0.388
Polygamous	0.101	0.043	0.017	0.145	0.292	0.586
Non-participants						
Monogamous	0.333	0.080	0.019	0.487	0.552	0.638
Polygamous	0.183	0.044	0.011	0.267	0.303	0.369

Source: Field survey, 2005.

Table 9 revealed that (33%) of women from a monogamous household are poor and poorer than their counterparts in polygamous household for the non-participants while it is vice-versa for the participants in which the polygamous households are poorer than the monogamous household.
From their respective contribution to poverty, monogamous household contributes more than the polygamous household for the non-participants and polygamous contributes more than monogamous household for the participants.

Educational Status of Sampled Women

Table 10 below shows that participants across the four education groups have less than 10% poverty incidence no formal education (5%) primary school (6%), secondary school (3%) and tertiary (1%) while for the non – participants have more than (10%) poverty incidence with the secondary school (18%) the poorest among the groups no formal education (16%), primary school (15%) and tertiary (1%). Therefore, the women in the non-participants group are poorer in terms of education than the participants and contribute more to poverty of whole group.

Table 10: Poverty Profile by Educational Status.

Participants Educational status	P_0	P_1	P_2	Contribution to		
				P_0	P_1	P_2
Non-formal education	0.050	0.021	0.008	0.072	0.142	0.264
Primary school	0.067	0.028	0.011	0.169	0.190	0.388
Secondary school	0.034	0.014	0.005	0.048	0.095	0.165
Tertiary	0.016	0.006	0.002	0.023	0.040	0.066
Non-Participants						
No formal education	0.166	0.040	0.009	0.242	0.278	0.324
Primary school	0.150	0.036	0.008	0.219	0.248	0.292
Secondary school	0.183	0.044	0.011	0.267	0.303	0.369
Tertiary	0.016	0.004	0.001	0.025	0.002	0.033

Source: Field survey, 2005.

Women Status within the Family

Table 11 showed both the participants and non-participant women who are not the household heads are poorer than their counterparts, who are the household heads in their respective household for the participants and non-participants, so also their contribution to poverty.

Table 11: Poverty Profile by Women Status

Participants Women status	P_0	P_1	P_2	Contribution to P_0	 P_1	 P_2
Household head Non-household head	0.067 0.101	0.028 0.043	0.011 0.017	0.169 0.145	0.190 0.292	0.388 0.56
Non-participants						
Household head Non-household head	0.116 0.40	0.027 0.096	0.006 0.023	0.169 0.585	0.186 0.663	.0201 0.773

Source: Field survey, 2005.

Determinants of Poverty and Impact of SMEs on Poverty

As indicated in the methodology, the determinants of poverty among women and effect of participation of women in agro-allied SMEs were examined. The results are indicated in Table 12. The correlation coefficient (Bo) is estimated at 5.3133 and it represents the independent poverty depth among the women in the study area, the model is statistically significant at the 1% critical level, this show that the model has a good fit to the data. Only 1 of the explanatory variables was not statistically significant of all the 8 variables. The variables that are significant includes marital status, household type (polygamous), household size, educational status (number of years spent in school) status of the woman in the family (household head or non-household head), participation in SME (yes or no) and income. The coefficient of each variable is related to the independent poverty depth as follows: The coefficient of the intercept of dummy of the marital status of the women is 0.774. This indicates that the autonomous poverty level of the women will be increased by 0.774 to become 6.089 while that of unmarried women will remain as 5.3133. This may be connected to the fact that married women have a larger household size than the unmarried which will decrease the per capita income.

Household type also has a significant effect on the poverty level of the sampled women. The coefficient of the variable is –0.628. Therefore woman with a monogamous household have a reduced poverty level by 0.628 to 4.685 while that of a polygamous household is 5.313. This is so because polygamous household have a larger household size. For the household size which has a coefficient of 2.119. This means that a unit increase in household size will increase the poverty of the household by 2.119 because larger household size will reduces per capita income. For educational status the poverty level of the women will be decreased by 0.3823, while for the status of women as either household head or not the poverty level of women will be increased by 2.2319 if there is a unit change in the status. Participation of women in SMEs has a coefficient of –3.199. This indicate that the poverty depth of the women will be reduced by –3.199 to 2.114 if there is unit increase in participation by women so also the income will reduce the poverty depth by 0.0004528 if there is a unit increase in income. Therefore variables which are positively correlated with the poverty level indicates that a unit increase in such will increase the poverty depth of women household and are poverty enhancing variables or determinants, while those variables that are negatively associated indicates that a unit decrease in such variable will decrease the poverty depth of the women household and are poverty reducing variables.

Table 12: Results of Regression Analysis

Variables	Coefficients	Standard Error	t-Value
Constant	5.3133	1.8287	2.9050
Age	0.0459	0.0362	0.1270
Marital status	0.7736*	0.3086	2.5070
Household type	-0.6280***	0.3591	-1.7490
Household size	2.1189*	0.4215	5.0270
Educational status	-0.3829***	0.2177	-1.7590
Women status in family	2.2320***	0.8592	2.5980
Participation index	-3.1992***	1.1475	-2.7880
Income	-0.0005*	0.00002	-1.7270

*** Denotes significance at 1%, ** at 5% and * at 1%

5. Policy Recommendations

1. Education has been seen as a major tool for human capital development which transcends into national development. Therefore the process of acquiring good education should be made available because participation of women will be limited to their level of education also with the adoption of new technology for the expansion of the small and medium scale enterprise activities. Also poverty decreases as the level of education increases therefore women should be given the opportunities to education.
2. Women should be sensitized the more on the awareness of birth control, because poverty increases with the number of people in the households. If this is done, it will reduce the ratio of child depending on the parents.
3. Participation in the agro-allied small and medium scale enterprise should be encouraged among the women folk either as a full time or part-time employment because income generated from the agro-allied small and medium scale enterprises serves as empowering tool. There should be enlightenment campaigns and programmes on the benefits and importance of women participation in SMEs as a poverty alleviation strategy.

Corresponding Author:
Fajimi Funke Olayemi
Department of Agricultural Economics
University of Ibadan
Ibadan, Nigeria
E-mail: **ffovivid@yahoo.com**

References

1. Adeolu, B.A. and Taiwo, A. The Impact of National Fadama Facility in Alleviating Rural Poverty and Enhancing Agricultural Development in South-Western Nigeria. *Journal of Social Science,* 2004 *9*(3): 157-161.
2. Adepoju, A. 'Feminization of Poverty in Nigeria cities'. Africa Population Studies (Supplement A to Volume 19) 2001:141-153.
3. Burkey Stan People First: A Guide to Self–Reliant Participatory Rural Development. Zed Books, London.1993
4. Daniels, L. and Fasseha. Micro and Small Scale Enterprises in Botswana: Results of a Nationwide Survey. Gemini Technical Report, 1(3) 1992.
5. Daniels, L. and Ngwira, A. Results of a Nationwide Survey on Micro, Small and Medium Enterprises in Malawi. Gemini technical Report, 53. (1993).
6. Easterly, W. The Effects of IMF and World Bank Programs on Poverty. World Bank Mimeo. 2000
7. Federal Office of Statistics Poverty Profile for Nigeria: A Statistical Analysis of 1996/97 National Consumer Survey. Federal Officer of Statistics, Nigeria April 1999
8. Foster, J., Greer, J. and Thorbecke, E: A Class of Decomposable Poverty Measures *Economietrica,* 1984 52**:** 761-66
9. Ijaiya G.T "Feminization of Poverty in Nigeria: A Case Study of Households in Ilorin Metropolis" African Journal of Business and Economic Research 2000, 1(2):141-148.
10. ILO Globalisation and Gender Briefs Series No 3.
11. Jariah, M. and Laily, P. Economic Empowerment of Rural women through Micro-Enterprise. In Maimunah, I. & Aminah, A. (Eds.) *Women and Work: Challenges in Industrialising Nations.* London: Asean Academic Press, 1999:133-152.

12. Jhingan, M.L. Advanced Economic Theory (Micro and Macro economics, 12th Revised and Enlarged Edition, Vrinda Publications (P) Ltd, India. 2005
13. Khan, M.H. "Rural Poverty in Developing Countries" Finance and Development, December 2000, Washington: IMF. 2000
14. National Economic Reconstruction Fund (NERFUND). Interim Report 1(12).1999
15. Nnanna, O.J. Financing Small and Medium Scale Businesses under the new CBN Directives and its likely Impact on Industries Growth of the Nigeria Economy, CBN, 25(3). 2001
16. Nnanna, O.J. The Role of Central Bank of Nigeria in Enterprises Financing. CBN, 27(1). 2003
17. Obitayo, K.M. Creating an enabling environment for small scale industries, CBN, 25(3). 2001
18. Odejide A.F C "Breaking the vicious circle of poverty among women in Developing countries: The case for Microcredit". Proceeding of the Nigerian Economic Society Annual conference on poverty Alleviation in Nigeria. Ibadan NES 367-381. 1997
19. Sule, E.K. Small scale industries in Nigeria concepts: Appraisal of government policies and suggestion solutions to identified problems CBN Economic and Financial Review, 24(4). 1986
20. Tanko N.M "Development Programs and the Effective Participation of Women in agriculture." Report of a Research Study Sponsored by Winrock International. 1993
21. World Bank (1996). "Nigeria: Poverty in the Most of Plant. The Challenge of Growth with Inclusion". A World Bank Poverty Assessment, Washington, D.C. World Bank.
22. Yerima Wilson Musa and David M. Danjuma. Small and Medium Scale Enterprises: A Veritable Tool for Sustainable Job Creation in Nigeria. Journal of Business and Public Policy 2007. 1(4): 1-25.

25/02/2010

Coag-flocculation studies of *Moringa oleifera* coagulant (MOC) in brewery effluent: Nephelometric approach.

***Menkiti Matthew .C. and Onukwuli Okechukwu .D.**

Department of Chemical Engineering, Nnamdi Azikiwe University, Awka, Nigeria.
*Corresponding author
Department of Chemical Engineering, Nnamdi Azikiwe University, Awka, Nigeria.
E-mail: cmenkiti@yahoo.com; Telephone: +234 8037441882

ABSTRACT: The coag-flocculation behavior of MOC in respect of pH variation in brewery effluent has been investigated at room temperature using various dosages of unblended MOC. Coag-flocculation parameters such as order of reaction α, rate constants (K and K_s), coagulation period $\tau_{1/2}$ e.t.c were determined. Turbidity measurement was carried out using the single angle (90°) nephelometric standard jar test while MOC processing was based on work reported by Ghebremichael. Microsoft excel package was employed in the evaluation of simulated parameter K_s. The maximum MOC performance are recorded at K of 6.6667 x $10^{-4}m^3$/kg.s, dosage of 0.4kg/m^3, pH of 4 and $\tau_{1/2}$ of 289.2614s while the minimum are recorded at K of 1.3333 x $10^{-4}m^3$/kg.s, dosage of 0.5kg/m^3, pH of 2 and $\tau_{1/2}$of 1446.6419s. The least value of E (%) recorded after 30 minutes is > 78%, thus confirming MOC as effective coag-flocculant. In general, the parameter obtained lie within the range of previous works, confirming that the theory of perikinetics holds for coag-flocculation of brewery effluent using MOC at the conditions of the experiment.

[Menkiti Matthew. C. and Onukwuli Okechukwu. D. **Coag-flocculation studies of *Moringa oleifera* coagulant (MOC) in brewery effluent; Nephelometric approach**. Journal of American Science 2010;6(12):788-806]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: *Moringa oleifera;* coag-flocculation; coal effluent; kinetics; coagulation

INTRODUCTION

The use of renewable resources of plant origin in local community for the removal of colloidal and non colloidal turbidity in waste water is not a new phenomenon. This phenomenon highlights the concept of coagulation / flocculation (cog-flocculation),a routine water treatment procedure that stimulate the formation of flocs arising from the destabilization of charged colloids(Ma et al,2001; Diterlizzi, 1994; Edzwald, 1987; O Melia, 1978). Among the factors that can influence the aggregation process are temperature, pH, effluent quality, stirring time e.t.c (Jin,2005).

Readily, coag-flocculation has been achieved via inorganic and organic synthetic substances such as alum, $FeCl_3$, polyamine e.t.c. However, the coag-flocculation performance of these aggregating agents are well researched and documented with inadequate attention especially in the aspect of kinetics given to the study of coag-flocculation behavior of the plant and animal derivatives. To this end, focus is hereby given to the study of coag-flocculation performance of plant origin, *Moringa oleifera* seed.

Moringa oleifera is a small, fast growing drought deciduous tree commonly found in the tropics such as Eastern Nigeria. The seed kernels of *Moringa olifera* contain significant quantities of positively charged water soluble protein which bind with the negatively charged particles in water to promote floc formation (Schwarz, 2000). *Moringa oleifera* are edible, non toxic, biodegradable and biocompatible substances. Previous results from its coagulation performance highlight prospects for renewable organic aggregating agent with extensive application in large scale water treatment technology.

Against this backdrop, this work embraces coag-flocculation kinetics and performance of MOC under varying pH of brewery effluent and MOC dosages, using single angle (90°) and simulated multi angle light scattering (nephelometry) techniques. Thus if well harnessed and developed, MOC can be an alternative to or be used in conjunction with the conventional coagulants. Ultimately, the post usage handling and health challenges posed by the inorganic coagulant can be reduced.

THEORETICAL PRINCIPLES AND MODEL DEVELOPMENT

For a uniformly coag-flocculating equilibrium phase with negligible influence of external force (Hunter, 1993):

$$\mu_i = \overline{G}_i = \left[\frac{\partial G}{\partial n_i}\right]_{P,T,n} = \text{a constant} \quad \ldots.1$$

And

$$f_d = \frac{-K_B T}{C_i}\frac{dC_i}{dx} \quad \ldots2$$

Where G is the total Gibbs free energy

n_i is the number of moles of component i

μ_i *is* chemical potential

C_i is concentration

x is diffusion distance

f_d is viscous drag force.

But from Ficks law

$$D' = \frac{-f_d}{B}\frac{C}{\left(dC/dx\right)} \quad \ldots3$$

Where D' is diffusion coefficient

B is friction factor

Comparing equation 2 and 3 generates Einstein's equation:

$$D' = \frac{K_B T}{B} \quad \ldots4$$

For similar phase, the rate of successful collision between particles sizes i and j (mass concentration/Time) to form particle of size k is (Thomas et al, 1999):

$N_{ij} = \varepsilon_p \beta (i,j) n_i n_j$ …5

where

ε_p = collision efficiency

β (i, j) = collision factor between particles of size i and j

$n_i n_j$ = particle concentration for particles of size i and j, respectively.

Assuming monodisperse, no break up and bi particle collision, the general model for perikinetic coag-flocculation is given as (Swift and Friedlander, 1964; Von Smoluchowski, 1917):

$$\frac{dn_k}{dt} = \frac{1}{2}\sum_{i+j=k}\beta(i,j)n_i n_j - \sum_{i=1}^{\infty}\beta(i,k)n_i n_k \quad \ldots6$$

where $\frac{dn_k}{dt}$ is the rate of change of concentration of particle of size k (concentration / time).

β is a function of the coag-flocculation transport mechanism.

The appropriate value of β for Brownian transport is given as(Von Smoluchowski,1917):

$$\beta_{BR} = \frac{8}{3}\varepsilon_p \frac{k_B T}{\eta} \quad \ldots7$$

Where K_B is Boltzmann's constant (J/K)

T is Absolute temperature (K)

The generic aggregation rate of particles (during coagulation / flocculation) can be derived by the combination of equations 6 and 7 to yield:

$$-\frac{dN_t}{dt} = KN_t^{\alpha} \quad \ldots8$$

Where N_t is total particle concentration at time t, $N_t = \sum n_k$ (mass / volume)

Meanwhile $K = \frac{1}{2}\beta_{BR}$ …9

K is the menkonu coag-flocculation rate constant

α is the order of coag-flocculation process

Also, $\beta_{BR} = 2\varepsilon_p K_R$ …10

Combining equations 8, 9 and 10 produce:

$$-\frac{dN_t}{dt} = \varepsilon_p K_R N_t^{\alpha} \quad \text{….11}$$

Where K_R is the Von smoluchowski rate constant for rapid coagulation (Van Zanten, 1992)

However $K_R = 8\pi a D'$ ….12

$$R_p = 2a \quad \text{….13}$$

Where a is particle radius.

From Einstein's equation: $D' = K_B T / B$

From Stoke's equation : $B = 6\pi\eta a$ ….14

where η is the viscosity of the coag-flocculating fluid

Combining equations 11 to14 gives:

$$-\frac{dN_t}{dt} = \frac{4}{3}\varepsilon_p \frac{K_B T}{\eta} N_t^{\alpha} \quad \text{…15}$$

Comparing equations 8 and 15 show:

$$K = \frac{4}{3}\varepsilon_p \frac{K_B T}{\eta} \quad \text{...16}$$

For perikinetic aggregation, α theoretically equals 2 as would be shown below (Fridkhsberg,1984; Hunter,1993):

From Fick's law,

$$J_f = D' 4\pi R_p^{\,2} \frac{dN_t}{dR_p} \quad \text{..17}$$

Integrating equation 17 at initial conditions $N_t = 0$, $R = 2a$:

$$\frac{J_f}{D' 4\pi} \int_0^{R_p} \frac{dR_p}{R_p^{\,2}} = \int_{N_0}^{N_t} dN_t \quad \text{...18}$$

Thus $J_f = 8\pi D' a N_0$ …19

For central particle of same size undergoing Brownian motion, the initial rate of rapid coag-flocculation is:

$$-\frac{dN_t}{dt} = J_f \cdot \varepsilon_p \cdot N_0 \quad \text{…20}$$

$$= \frac{4}{3}\varepsilon_p \frac{K_B T}{\eta} \cdot N_0^2 \quad \text{...21}$$

$$\equiv \frac{4}{3}\varepsilon_p \frac{K_B T}{\eta} N_t^2 \text{ at } t > 0$$

Hence, from equation 21, $\alpha = 2$

For $\alpha = 2$; equivalence of equation 8 yields:

$$\frac{dN}{dt} = -KN^2$$

Hence:

$$\int_{N_0}^{N} \frac{dN}{N^2} = -K \int_0^t dt \quad \text{…22}$$

Thus $\frac{1}{N} = Kt + {}^{1}\!/_{N_0}$ ...23

Plot of $\left({}^{1}\!/_{N}\right)$ Vs t produces a slope of K and intercept of ${}^{1}\!/_{N_0}$.

For the evaluation of coagulation period ($\tau_{1/2}$), from Equation 23:

$$N = \frac{N_0}{\left[1 + \frac{t}{\left({}^{1}\!/_{N_0 K}\right)}\right]} \quad \ldots 24$$

Where $\tau = \left[{}^{1}\!/_{N_0 K}\right]$ …25

Hence:

$$N = \frac{N_0}{1 + (t/\tau)} \quad \ldots 26$$

When $t = \tau$, equation 26 becomes

$$N = {}^{N_0}\!/_{2} \quad \ldots 27$$

Therefore as $N_0 \rightarrow 0.5N_0; \tau \rightarrow \tau_{1/2}$

Hence $\tau_{1/2} = {}^{1}\!/_{(0.5N_0 K)}$ ...28

For Brownian (perikinetic) aggregation at early stages (t≤30 minutes), equation 6 can be solved exactly, resulting in the generic expression

$$\frac{N_{m(t)}}{N_0} = \frac{\left[{}^{t}\!/_{\tau'}\right]^{m-1}}{\left[1 + {}^{t}\!/_{\tau'}\right]^{m+1}} \quad \ldots 29$$

Where $\tau' = 2\tau$

Hence, for singlets *(m=1)*

$$N_1 = N_0\left[\frac{1}{\left(1 + {}^{t}\!/_{\tau'}\right)^2}\right] \quad \ldots 30$$

For doublets *(m=2)*

$$N_2 = N_0\left[\frac{\left({}^{t}\!/_{\tau'}\right)}{\left(1 + {}^{t}\!/_{\tau'}\right)^3}\right] \quad \ldots 31$$

For triplets *(m=3)*

$$N_3 = N_0\left[\frac{\left({}^{t}\!/_{\tau'}\right)^2}{\left(1 + {}^{t}\!/_{\tau'}\right)^4}\right] \quad \ldots 32$$

Also for the coag-flocculating phase and multi angle nephelometry, the intensity of light scattered from suspension of monodispersed phase is described as (Van Zanten,1992):

$$I(q,\mathrm{T}) = I(q,0)\left[1+2\sum_{m=2}^{\infty} C_m(\mathrm{T})A_m(q)\right] \quad \ldots.33$$

Where I (q, *T*) is the intensity of light scattered by the initially unaggregated suspension ;
$T = \mathrm{t}/\tau'$ (dimensionless time)
q is the scattering wave vector

$$q = \left(\frac{4\pi}{\lambda_0}\right) n_0 \sin(0.5\theta) \quad \ldots34$$

Where λ_0 is the wave length of the laser incident light in Vacuum, *($\lambda_0 = 2\pi a / 6$)*
n_0 is the refractive index of the suspending medium
θ is the scattering angle
A_m is the form factor for an aggregate consisting of m primary particles.
a is radius of particles sphere.
If the coagulating medium obeys the Rayleigh-Gans-Debye (RGD) approximations, then

$$A_m(q) = \sum_{i}^{m}\sum_{j>1}^{m} \frac{\sin qr_i}{qr_{ij}} \quad \ldots35$$

Where r_{ij} is the centre-to-centre separation of primary particles *i* and *j* in the given m-fold aggregate. The summation accounts for all pairs of particle centers in the aggregate.

The expression for the scattered intensity in view of many possible configurations arising from larger aggregates is:

$$I(q,\mathrm{T}) = I(q,0)\left[1+2\frac{\sin qd_0}{qd_0}\frac{\mathrm{T}}{(1+\mathrm{T})^3}+2\sum_{m=3}^{\infty} C_m(\mathrm{T})A_m(q)\right] \quad \ldots36$$

The form factors are given by an average of all contributing structures where d_0 is hard core interaction diameter of singlets. Differentiating equation 36 as t→0 yields:

$$\frac{I}{I(q,0)}\left(\frac{dI(q,t)}{dt}\right)_{t\to 0} = \frac{d}{dt}\left[1+2\frac{\sin qd_0}{qd_0}\frac{\mathrm{T}}{(1+\mathrm{T})^3}+2\sum_{m=3}^{\infty} C_m(\mathrm{T})A_m(q)\right] \quad \ldots37$$

$$\frac{I}{I(q,0)}\left(\frac{dI(q,t)}{dt}\right)_{t\to 0} = \left[\beta_{BR}N_0\frac{\sin qd_0}{qd_0}\right] \quad \ldots38$$

Using simulated version of equation 38, K_S (simulated *K*) can easily be determined at several scattering angles. A plot of $\frac{I}{I(q,0)}\left(\frac{dI(q,t)}{dt}\right)_{t\to 0} Vs \frac{\sin qd_0}{qd_0}$ gives a slope of $N_0\beta_{BR}$ from where $(K_S)_{t\to 0}$ could be determined.

MATERIALS AND METHOD

The sample of *Moringa olifera* seeds was sourced from Agulu Town, Anambra state Nigeria and processed to MOC based on the work reported by Ghebremichael (2004).

The jar test was conducted based on standard Bench scale Nephelometric method (single angle procedure) for the examination of water and waste water (WST,2005; AWWA,2005) using model WZS-185 MC Turbidimeter, APPNo 688644A Gulenhamp magnetic stirrer and mettler Toledo Delta 320 pH meter. For the simulation of multi angle nephelometry, excel package was used while d_0 = 0.8875μm and n_o were sourced from ENSWC (2008) and simple refractometric measurment respectively.

RESULTS AND DISCUSSION

Coag-flocculation parameters

The values of coag-flocculation parameters are presented in tables 1 to 6.At a general level, the values of R^2 for the results presented are quite satisfactory, confirming the theory of perikinetics that shown α=2 .Meanwhile, it is evident that α relates inversely with K.Thus, for a higher α to be obtained, a lower K is a necessary condition (Fridkhsberg, 1984). This is readily amplified in tables 1 to 5.For K (=0.5β_{BR}), there is no regular observed trend, as the pH increases from 2 to 10 for tables 1 to 5.However, it should be observed that the highest values if K are recorded at pH of 4.This explains why lowest $\tau_{1/2}$are also recorded at pH of 4.This is amply demonstrated in figs 5 to 9 where best coag-flocculation were

recorded for pH of 4.Also,observations from tables 1 to 5 indicate that the values of K experiences minimal variation for 0.1kg/m^3 to 0.5kg/m^3 at a given pH. This clearly emphasizes that coag-flocculation is more sensitive to changes in pH than that of dosages. It can be argued that at given pH, a similar mechanism controls the process. This can account for the little variation in K at a given pH.

In theoretical bases, $\tau_{1/2}$,ε_p , and K_R are coagulation effectiveness factors, understood to be accounting for the coagulation efficiency, before flocculation sets in. In view of the particle distribution plots (Figs 17 and 18); the values of $\tau_{1/2}$ obtained are satisfactory, though millisecond has been reported in system where minimal Zeta potential exists (Hunter, 1993). For a case of minimal variation in K_R, ε_p relates directly to $2K=\beta_{BR}$. Hence ,high ε_p results in high kinetic energy to overcome the Zeta potential either by double layer compression or colloid destabilization.

Meanwhile, K_S, the simulated version of K for varying pH and dosages are presented in table 6.It is an attempt to assess the variation between K (from single angle) and K_S (from simulated multi angle) in view of the angular dependence of K_S .The agreement between K and K_S is satisfactory. Note that K and K_S were obtained from equations 23 and 38 respectively where the slope of equation 38 is $\beta_{BR}N_0$.Representative results are presented in (figs 1 and 2) and (Figs 3 and 4) for equation 23 and 38 respectively.

The discrepancies observed in the results presented in tables 1 to 6 are due to unattainable assumption that mixing of particles and MOC throughout the dispersion is 100% efficient before any aggregation occurs (Yates etal,2001). Another account is the interplay between Van der waal forces and the hydrodynamic interactions which alters the theoretically predicted parameter values by a factor ±2 .Other additional short range forces do also contribute (Holthof,etal,1997; Holthof, etal,1996).

SP (=N in kg/m^3) vs. time plots.

Plots of SP vs. time are presented in figs 5 to 9; with initial SP of 10.3691kg/m^3 .Generally, for the plots, the SP decreases with time. This is expected because during coag-flocculation, particles aggregate into large flocs, settling under gravity and progressively decreasing the intensity of light scattered. The rapid settling of SP is very apparent in figs 5 to 9 in view of the initial value of SP. This is in consonance with the theory of rapid coag-flocculation. The best coag-flocculation was recorded at pH of 4 supporting lowest and highest values of $\tau_{1/2}$ and K respectively recorded in the study.

However, the least $\tau_{1/2}$ recorded is = 289.2614 at pH of 4.This is high in view that milliseconds have been recorded. This can be adduced to low initial SP present in the brew effluent. Secondly, the resulting flocs may be of less density such that there may exist disproportionality between the rate of coagulation and rate of settling of the flocs formed.

Efficiency E (%) vs. time

E (%) vs. time plots are presented in figs 10 to 14 .The E (%) indicates the effectiveness of ABC to remove SP from the effluent. The least E (%) recorded after 30 minutes of coag-flocculation is > 78% in spite of low value of initial SP .This confirms MOC as a plant derived aggregating agent. This collaborates with the general real life experience where in some cases, 90% particle removal are routinely achieved in minutes during water treatments. Observed that in general, the best efficiency is recorded at pH of 4.

Plots of E (%) vs. pH

This is presented in fig 15.it indicates the performance of various doses of MOC at varying pH. Maximum efficiency are recorded at pH of 4 followed by pH of 10.Low doses of 0.1kg/m^3 and 0.2kg/m^3 exhibit high E (%) at pH of 2 while high doses of 0.4kg/m^3 and 0.5kg/m^3 perform maximally at pH of 4 and 10.

Plot of E (%) vs. Dosages (kg/m^3)

This is presented in fig 16.The optimum dosages are recorded for pH of 4 at 0.4kg/m^3 though performances for pH of 10 at 0.4kg/m^3 are satisfactory. It confirms the earlier observation that pH of 4 provides optimum coag-flocculation properties for MOC .The minimum performance was recorded for pH of 2 at 0.5kg/m^3 and 0.4kg/m^3

Time evolution of the cluster size distribution

These are graphically presented in figs 17 and 18.They represent the time evolution for singlets , doublets and triplets obtained for $\tau_{1/2}$ = 289.2614s and $\tau_{1/2}$ = 1446.642s .Both figs 17 and 18 exhibit similar trend , an indication of a process controlled by similar mechanism. The curves represent a case in which the values of N_3 and $\sum N_i$ are close such that their variation with time is near same. Also, similar trend with respect to N_2 and N_1 is obtained .The curves indicate that there exists a wide margin of difference in concentration of SP between the pair of (N_3 and $\sum N_i$) and (N_2 and N_1).The implication is the existence of high shear force and resistance to collision .This is clearly demonstrated by the values of $\tau_{1/2}$ which are high. This is an indication of high Zeta potential associated with the process.

CONCLUSION

The potential of MOC as an effective natural organic coag-flocculants applicable in large scale water treatment has been confirmed. The conformity

of the values of the obtained parameters with perikinetics theory in conjunction with the establishment of common parameter β_{BR} for both single (90°) and multi angle light scattering theories present a novelty in this work. These indicate a step further in what had already be developed in the coag-flocculation study.

The optimum dosage, pH and $\tau_{1/2}$ recorded are 0.4kg/m^3, 4 and 289.2614s respectively. The degree of agreement between K and K_S emphasizes that both single and multi angle light scattering techniques can be used to study coag-flocculation .This authenticates the validity of Von Smoluchowski theory. Overall, the results obtained are in line with previous works (Jin,2005; Van Zanten and Elimelech,1992;Holthof *et al*,1996;Menkiti *et al*,2009; Menkiti *et al*,2010).

NOMENCLATURE

K :α^{th} order coag-flocculation constant
β_{BR}: Collision factor for Brownian Transport
ε_p: Collision Efficiency
$\tau_{1/2}$: Coagulation Period / Half life
E: Coag-flocculation Efficiency
R^2: Coefficient of Determination
α: Coag-flocculation reaction order
$-r$: Coag-flocculation reaction rate
$(SP)_0^c$: Computed initial suspended particle (kg/m^3)
J_f:Flux
f_d: Drag force
K_S : K value obtained from simulation.
MOC: *Moringa olifera* coagulant
d_o : Hard core interaction diameter of the primary particle.
a: Radius of the primary particle

Table 1: Coag-flocculation Functional parameters for varying pH and constant dosage of 0.1kg/m^3 MOC

Parameter	pH=2	pH=4	pH=6	pH=8	pH=10
α	2	2	2	2	2
R^2	0.966	0.993	0.939	0.996	0.992
K (m^3/kg.s)	3.333 x 10^{-4}	5.001 x 10^{-4}	3.333 x 10^{-4}	1.667 x 10^{-4}	1.500 x 10^{-4}
β_{Br} (m^3/kg.s)	6.667 x 10^{-4}	1.000x10^{-3}	6.667x10^{-4}	3.333x10^{-4}	3.000x10^{-4}
K_R (m^3/s)	1.129x10^{-16}	1.698x10^{-16}	1.441x10^{-16}	1.698x10^{-16}	1.698x10^{-16}
ε_p (kg^{-1})	5.903x10^{12}	5.892x10^{12}	4.627x10^{12}	1.964x10^{12}	1.767x10^{12}
$\tau_{1/2}(s)$	578.648	385.762	578.648	1157.261	128.872
$(SP)_0^c$ (kg/m^3)	5	2.5	3.333	3.333	2.500
$(N_p)_0^c$ (m-^{3})	3.011 x 10^{27}	1.506 x 10^{27}	2.007 x 10^{27}	2.007 x 10^{27}	1.506 x 10^{27}
$-r$	3.333x10^{-4} c^2	5.001x10^{-4} c^2	3.333x10^{-4} c^2	1.667x10^{-4} c^2	1.500x10^{-4} c^2

Table 2: Coag-flocculation Functional parameters for varying pH and constant dosage of 0.2kg/m^3 MOC

Parameter	pH=2	pH=4	pH=6	pH=8	pH=10
α	2	2	2	2	2
R^2	0.964	0.972	0.942	0.988	0.999
K (m^3/kg.s)	3.333 x 10^{-4}	6.667 x 10^{-4}	1.667 x 10^{-4}	3.333 x 10^{-4}	1.667 x 10^{-4}
β_{Br} (m^3/kg.s)	6.666 x 10^{-4}	1.333x10^{-3}	3.333x10^{-4}	6.667x10^{-4}	3.333x10^{-4}
K_R (m^3/s)	1.143x10^{-16}	1.625x10^{-16}	1.639x10^{-16}	1.626x10^{-16}	1.625x10^{-16}
ε_p (kg^{-1})	5.830x10^{12}	8.203x10^{12}	2.034x10^{12}	4.101x10^{12}	2.051x10^{12}
$\tau_{1/2}(s)$	578.648	289.320	1157.261	578.648	1157.261
$(SP)_0^c$ (kg/m^3)	3.333	10.00	2.500	3.333	2.500
$(N_p)_0^c$ (m-^{3})	2.007 x 10^{27}	6.022 x 10^{27}	1.506 x 10^{27}	2.007 x 10^{27}	2.007 x 10^{27}
$-r$	3.333x10^{-4} c^2	6.667x10^{-4}c^2	1.667x10^{-4} c^2	3.333x10^{-4} c^2	1.667x10^{-4} c^2

Table3: Coag-flocculation Functional parameters for varying pH and constant dosage of 0.3kg/m^3 MOC

Parameter	pH=2	pH=4	pH=6	pH=8	pH=10
α	2	2	2	2	2
R^2	0.975	0.992	0.992	0.952	0.945
K (m^3/kg.s)	3.333 x 10^{-4}	5.001 x 10^{-4}	5.001 x 10^{-4}	3.333 x 10^{-4}	3.333 x 10^{-4}
β_{Br} (m^3/kg.s)	6.667 x 10^{-4}	1.000x10^{-3}	1.000x10^{-3}	6.667x10^{-4}	6.667x10^{-4}
K_R (m^3/s)	1.154x10^{-16}	1.315x10^{-16}	1.307x10^{-16}	1.333x10^{-16}	1.242x10^{-16}
ε_p (kg^{-1})	5.779x10^{12}	7.608x10^{12}	7.653x10^{12}	5.000x10^{12}	5.368x10^{12}
$\tau_{½}(s)$	578.648	385.762	385.762	578.648	578.648
$(SP)_0^c$ (kg/m^3)	5	3.333	3.333	2.500	2.00
$(N_p)_0^c$ (m-^{3})	3.011 x 10^{27}	2.007 x 10^{27}	2.007 x 10^{27}	1.506 x 10^{27}	1.204 x 10^{27}
$-r$	3.333x10^{-4} c^2	5.001x10^{-4} c^2	5.001x10^{-4} c^2	3.333x10^{-4} c^2	3.333x10^{-4} c^2

Table4: Coag-flocculation Functional parameters for varying pH and constant dosage of 0.4kg/m^3 MOC

Parameter	pH=2	pH=4	pH=6	pH=8	pH=10
α	2	2	2	2	2
R^2	0.986	0.857	0.952	0.895	0.998
K (m^3/kg.s)	1.667 x 10^{-4}	6.667 x 10^{-4}	6.667 x 10^{-4}	3.333 x 10^{-4}	6.667 x 10^{-4}
β_{Br} (m^3/kg.s)	3.333x10^{-4}	1.333x10^{-3}	1.333x10^{-3}	6.667x10^{-4}	1.333x10^{-3}
K_R (m^3/s)	1.165x10^{-16}	1.231x10^{-16}	1.260x10^{-16}	1.235x10^{-16}	1.242x10^{-16}
ε_p (kg^{-1})	2.862x10^{12}	1.083x10^{13}	1.059x10^{13}	5.399x10^{12}	1.073x10^{13}
$\tau_{½}(s)$	1157.261	289.320	289.320	578.648	289.320
$(SP)_0^c$ (kg/m^3)	5.000	10.00	5.000	2.000	3.333
$(N_p)_0^c$ (m-^{3})	3.011 x 10^{27}	6.022 x 10^{27}	3.011 x 10^{27}	1.204 x 10^{27}	2.007 x 10^{27}
$-r$	1.667x10^{-4}c^2	6.667x10^{-4}c^2	6.667x10^{-4} c^2	3.333x10^{-4} c^2	6.667x10^{-4} c^2

Table5: Coag-flocculation Functional parameters for varying pH and constant dosage of 0.5kg/m^3 MOC

Parameter	pH=2	pH=4	pH=6	pH=8	pH=10
α	2	2	2	2	2
R^2	0.975	0.981	0.955	0.897	0.866
K (m^3/kg.s)	1.333 x 10^{-4}	5.000 x 10^{-4}	3.333 x 10^{-4}	3.333 x 10^{-4}	3.333 x 10^{-4}
β_{Br} (m^3/kg.s)	2.667 x 10^{-4}	1.000x10^{-3}	6.667x10^{-4}	6.667x10^{-4}	6.667x10^{-4}
K_R (m^3/s)	1.107x10^{-16}	1.420x10^{-16}	1.542x10^{-16}	1.408x10^{-16}	1.461x10^{-16}
ε_p (kg^{-1})	2.409x10^{12}	7.042x10^{12}	4.323x10^{12}	4.735x10^{12}	4.564x10^{12}
$\tau_{½}(s)$	1446.642	385.762	578.648	578.648	578.648
$(SP)_0^c$ (kg/m^3)	5	5	2.500	2.00	2.00
$(N_p)_0^c$ (m-^{3})	3.011 x 10^{27}	3.011 x 10^{27}	1.506 x 10^{27}	1.204 x 10^{27}	1.204 x 10^{27}
$-r$	1.333x10^{-4} c^2	5.000x10^{-4} c^2	3.333x10^{-4} c^2	3.333x10^{-4} c^2	3.333x10^{-4} c^2

Table 6: Representative values of K (Experimental) and K_s (Simulated) at varying dosage and pH

pH	Dosage(kg/m^3)	N_0(Particles / m^3)	d_0(µm)	K(m^3/kg.s)	K_S(m^3/kg.s)
2	0.4	3.011 x 10^{27}	0.8875	1.667 x 10^{-4}	1.661 x 10^{-4}
4	0.4	2.007x 10^{27}	0.8875	6.667 x 10^{-4}	6.642 x 10^{-4}
6	0.4	2.007x 10^{27}	0.8875	6.667 x 10^{-4}	6.642 x 10^{-4}
8	0.4	1.506 x 10^{27}	0.8875	3.333 x 10^{-4}	3.321 x 10^{-4}
10	0.4	1.204 x 10^{27}	0.8875	6.667 x 10^{-4}	7.473 x 10^{-4}
4	0.1	1.506 x 10^{27}	0.8875	5.001 x 10^{-4}	6.642 x 10^{-4}
4	0.2	6.022 x 10^{27}	0.8875	6.667 x 10^{-4}	6.642 x 10^{-4}
4	0.3	2.007 x 10^{27}	0.8875	5.001 x 10^{-4}	4.982 x 10^{-4}
4	0.4	6.022 x 10^{27}	0.8875	6.667 x 10^{-4}	6.642 x 10^{-4}
4	0.5	3.011 x 10^{27}	0.8875	5.001 x 10^{-4}	4.982 x 10^{-4}

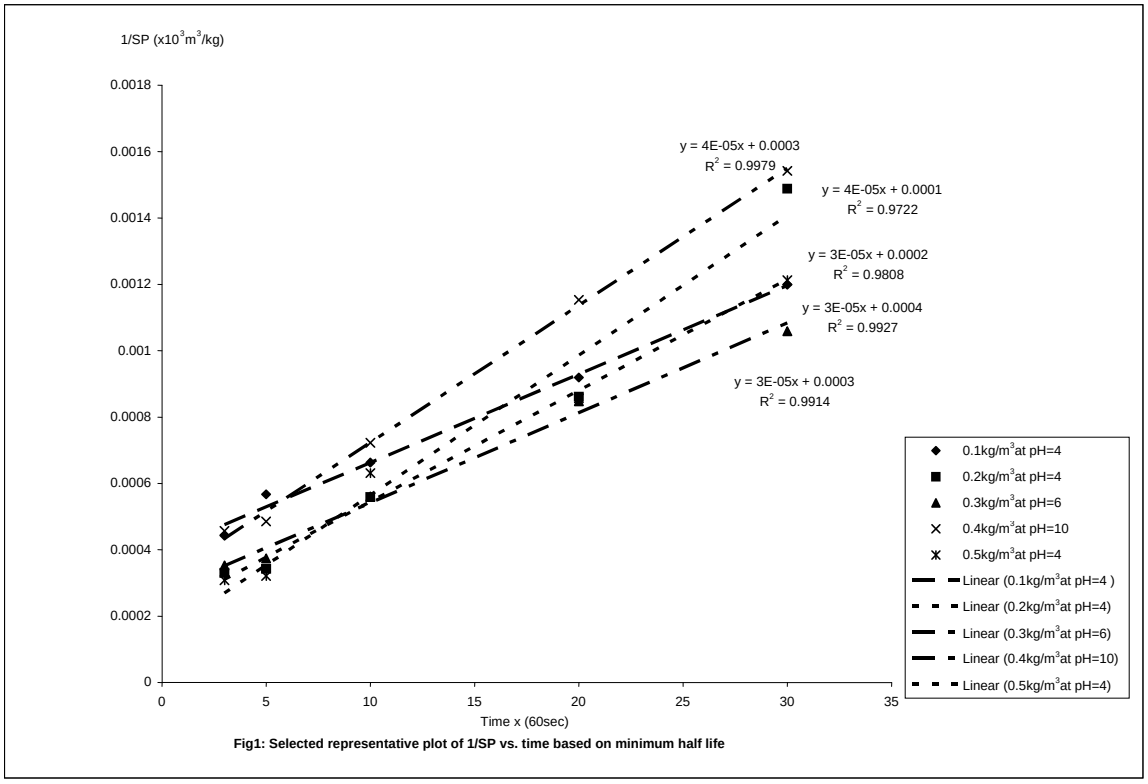


Fig1: Selected representative plot of 1/SP vs. time based on minimum half life

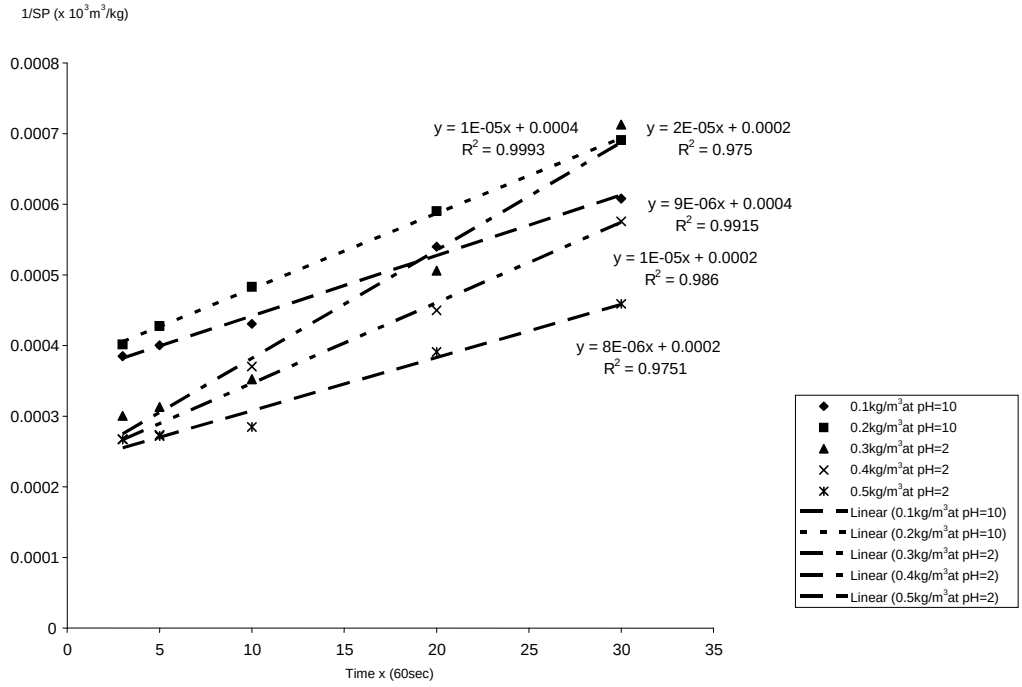


Fig 2: Selected representative plot of 1/SP vs. time based on maximum half life

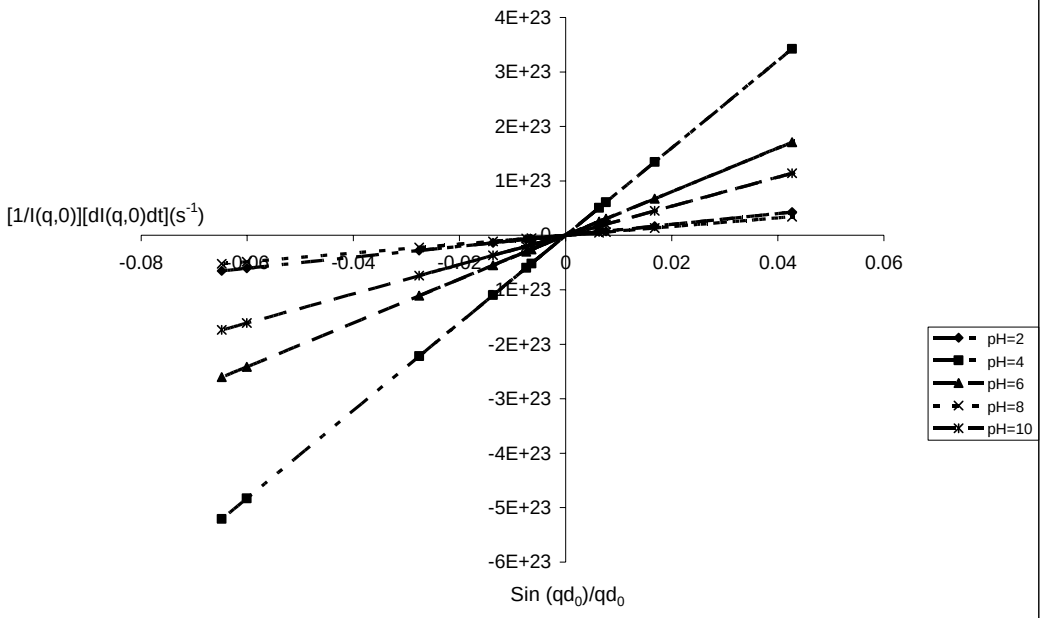


Fig 3:Initial Intensity changes vs. interference factor at 0.3kg/m^3 MOC

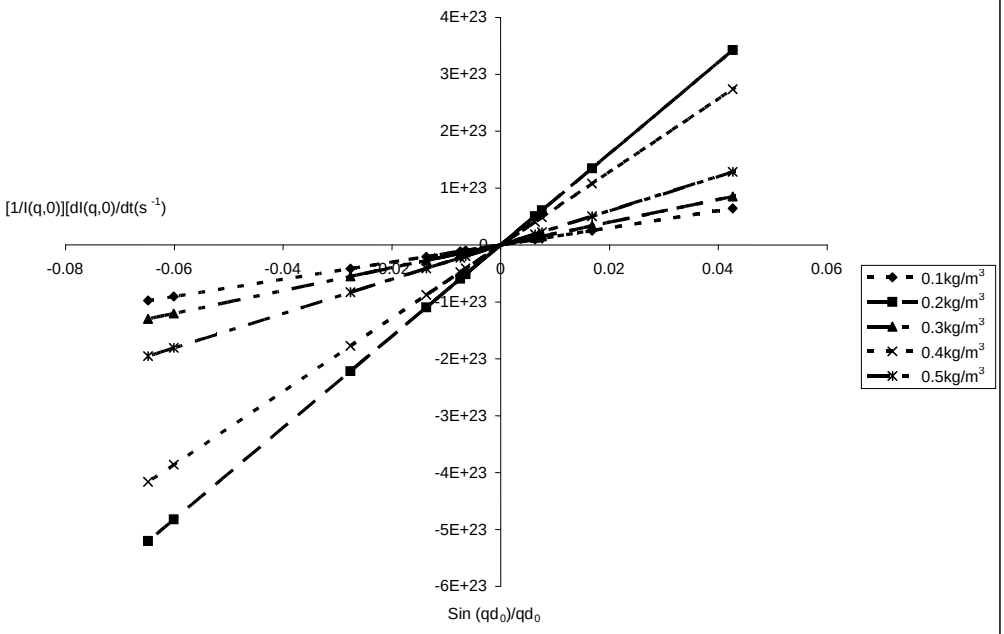


Fig 4:Initial Intensity changes vs. interference factor for MOC at pH=4

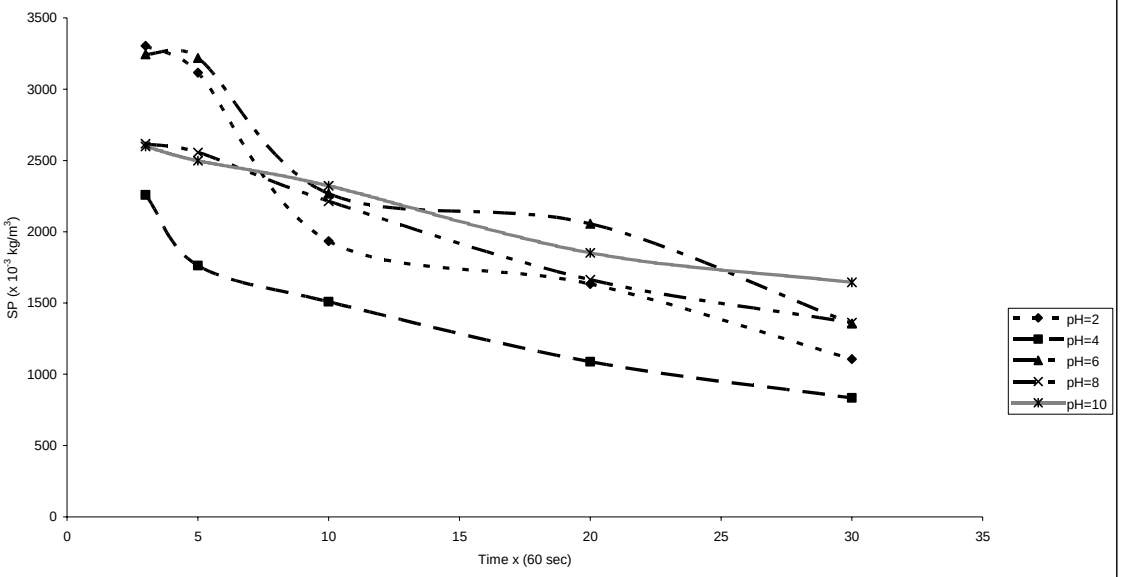


Fig 5:Plot of SP vs. coag-flocculation time for 0.1kg/m^3 MOC at varying pH

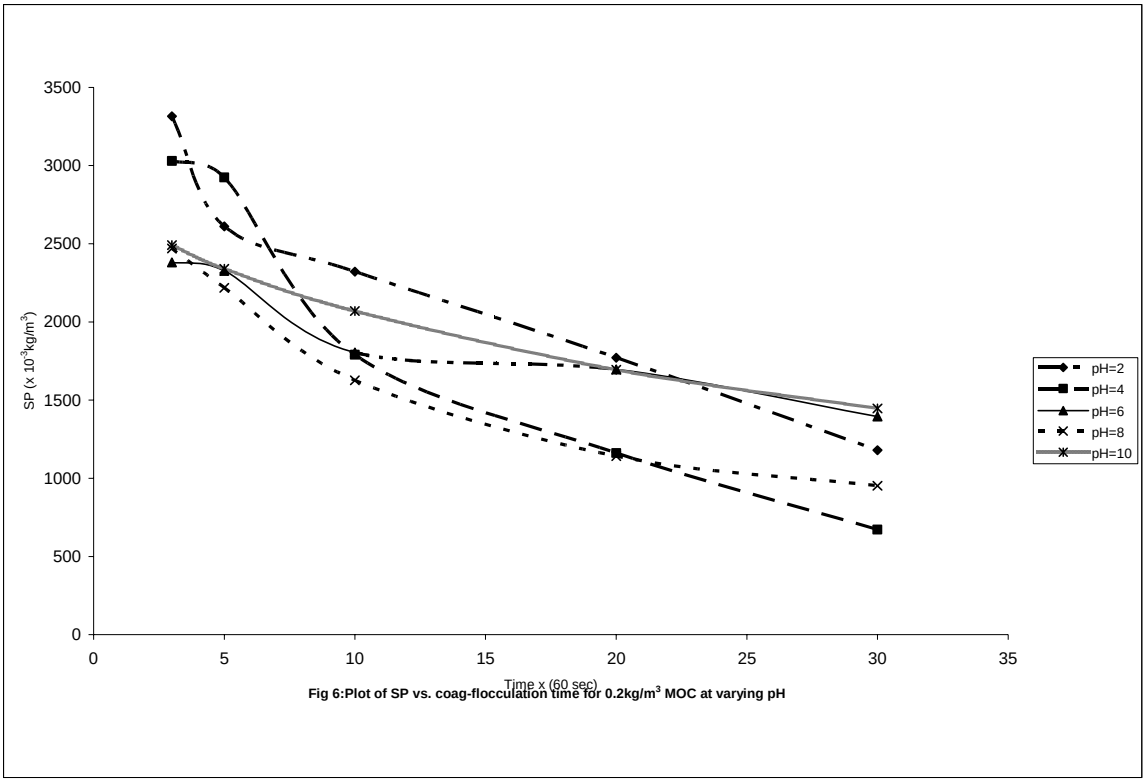


Fig 6:Plot of SP vs. coag-flocculation time for 0.2kg/m^3 MOC at varying pH

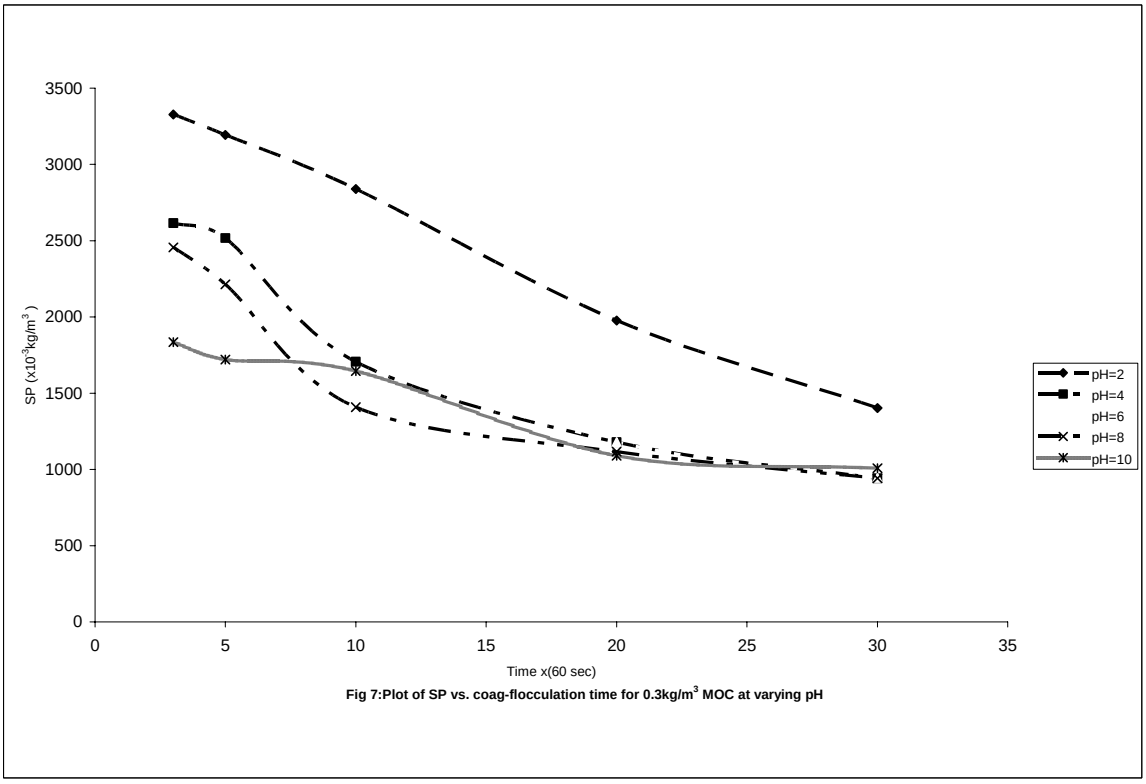


Fig 7:Plot of SP vs. coag-flocculation time for 0.3kg/m^3 MOC at varying pH

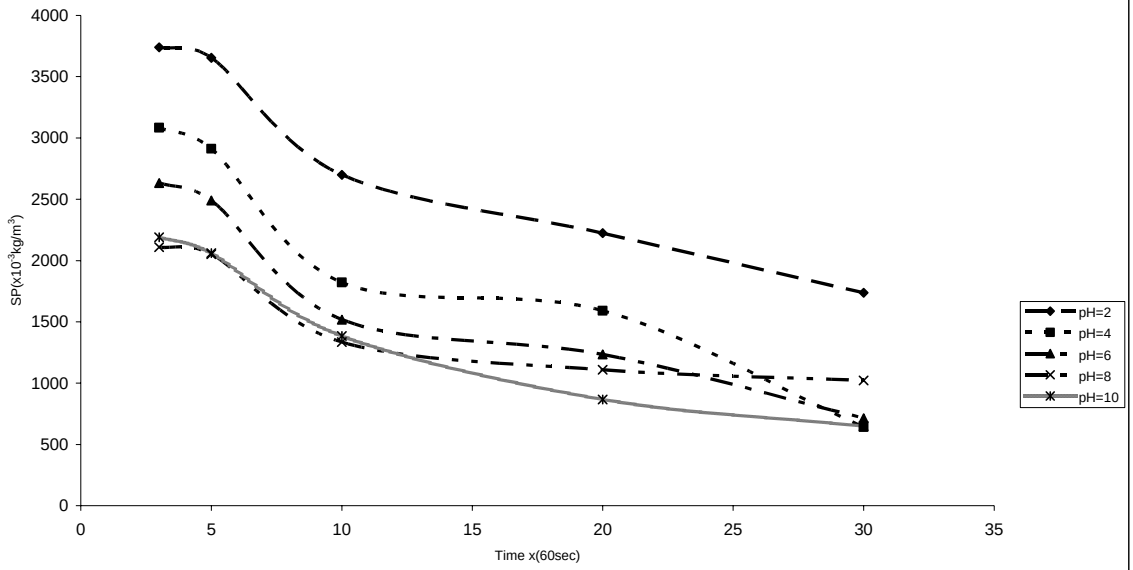


Fig 8:plot of Sp vs. coag-flocculation time for 0.4kg//m^3 MOC at varyinf pH

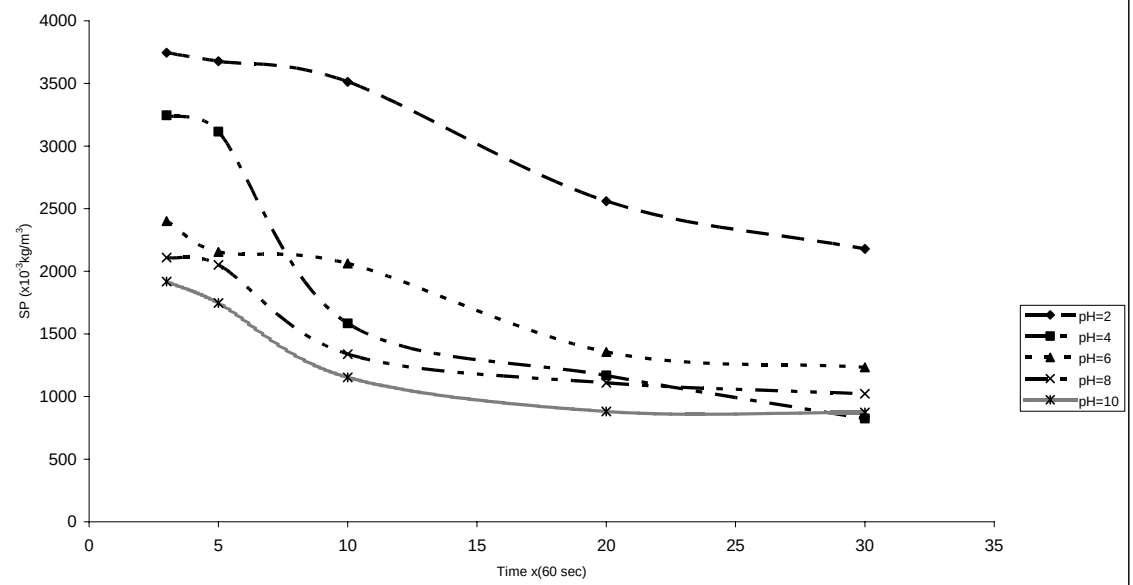


Fig 9:Plot of SP vs. coag-flocculation time for 0.5kg/m^3 MOC at varying pH

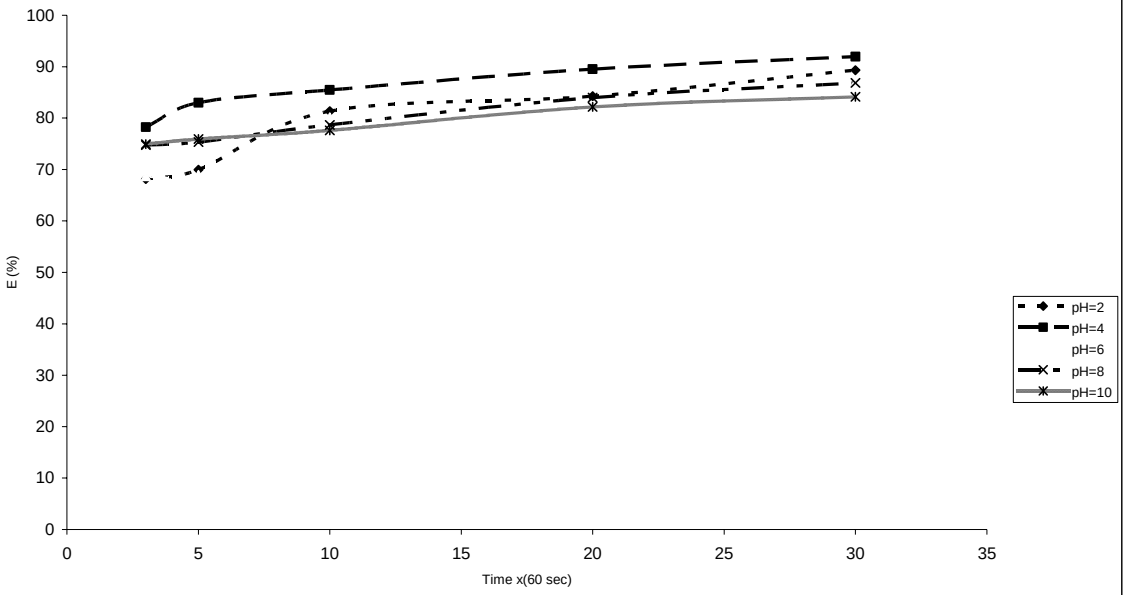


Fig 10:Plot of E vs. coag-flocculation time at varying pH and constant dosage of 0.1kg/m^3

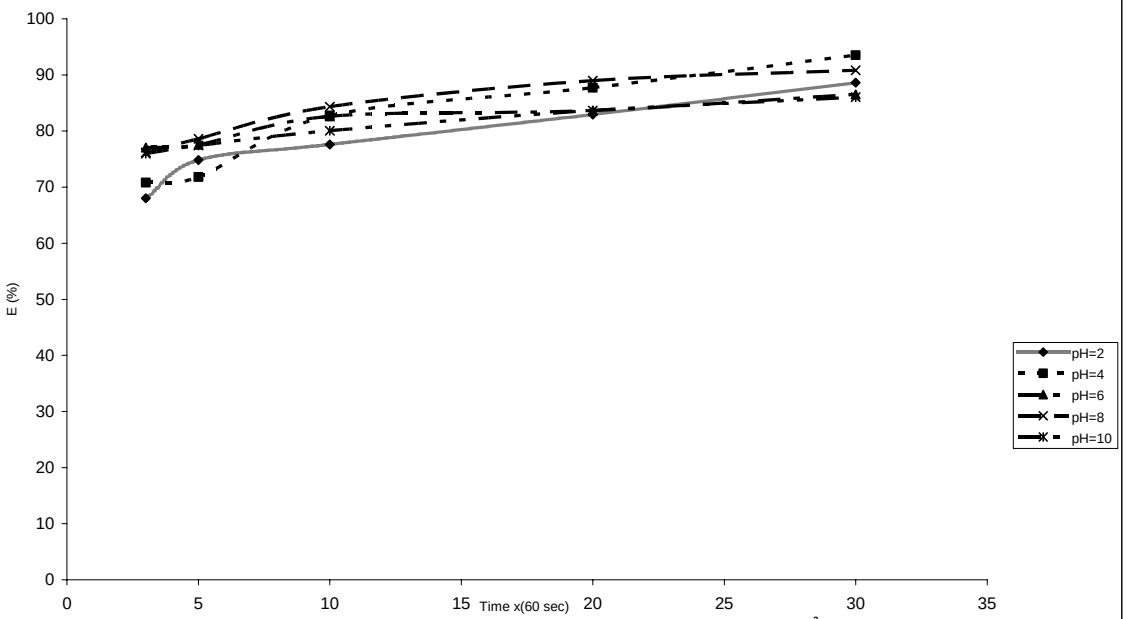


Fig 11:Plot of E vs. coag-flocculation time at varying pH and constant dosage of 0.2kg/m^3

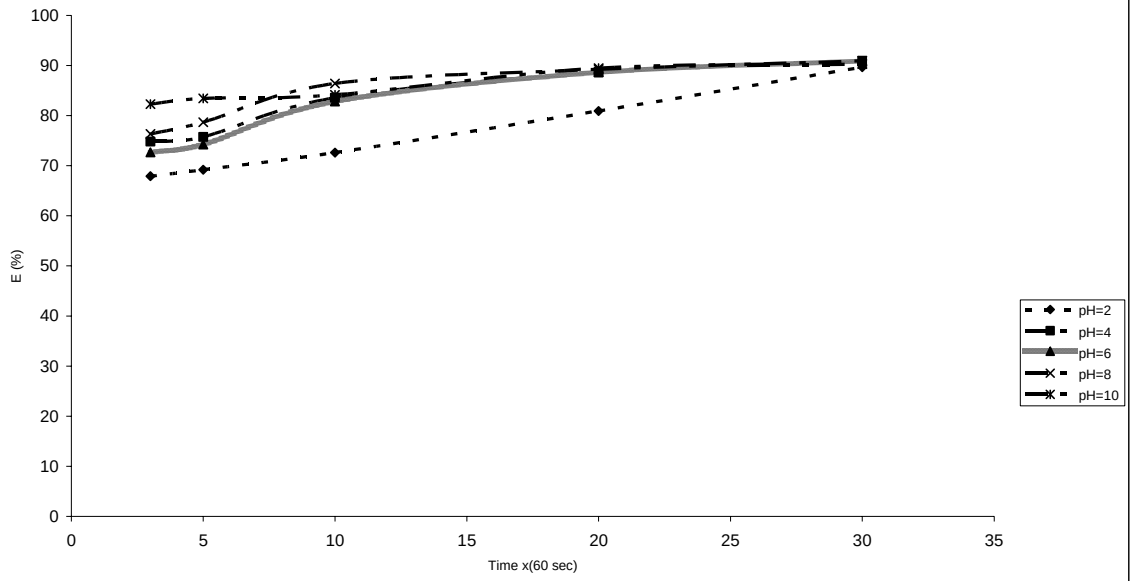


Fig 12: plot of E vs. coag-flocculation time at varying pH and constant dosage of 0.3kg/m^3

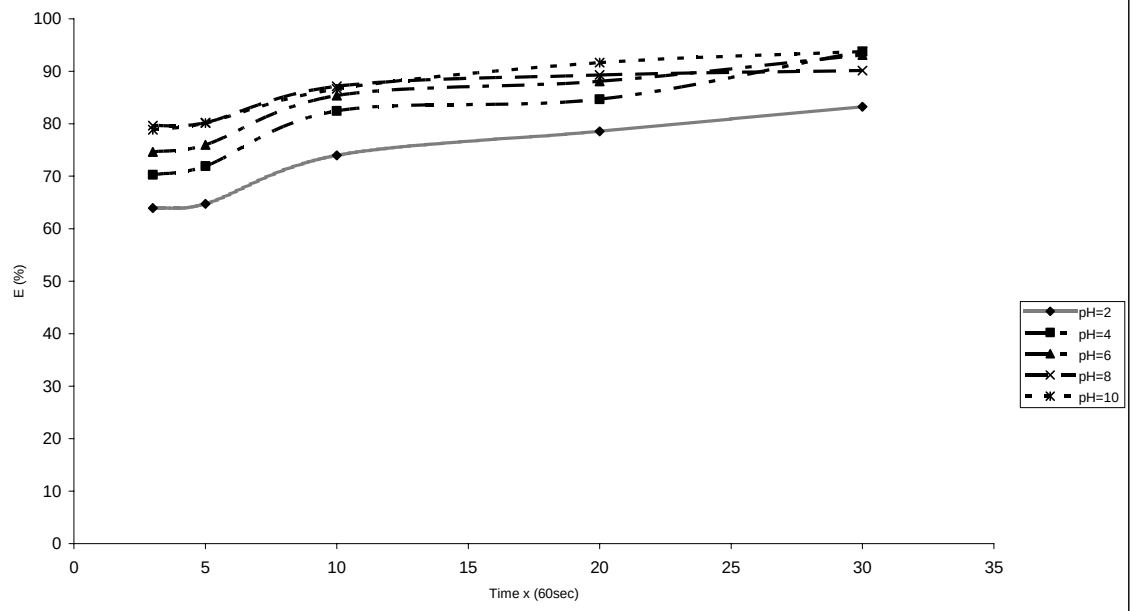


Fig 13: Plot of E vs. coag-flocculation time at varying pH and constant dosage of 0.4kg/m^3

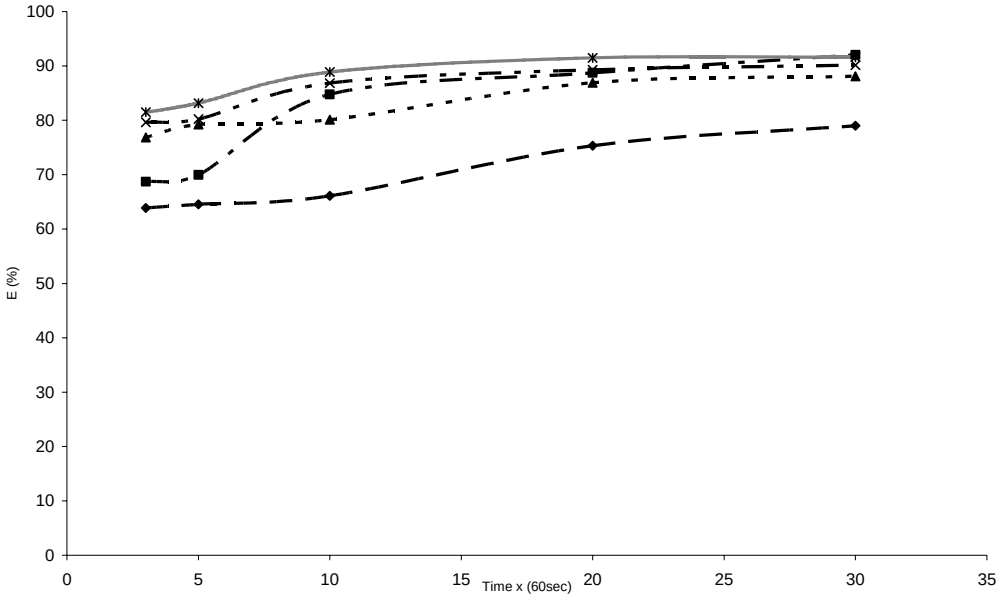


Fig 14:plot of E vs. coag-floccualtion time at varying pH and constant dosage of 0.5kg/m^3

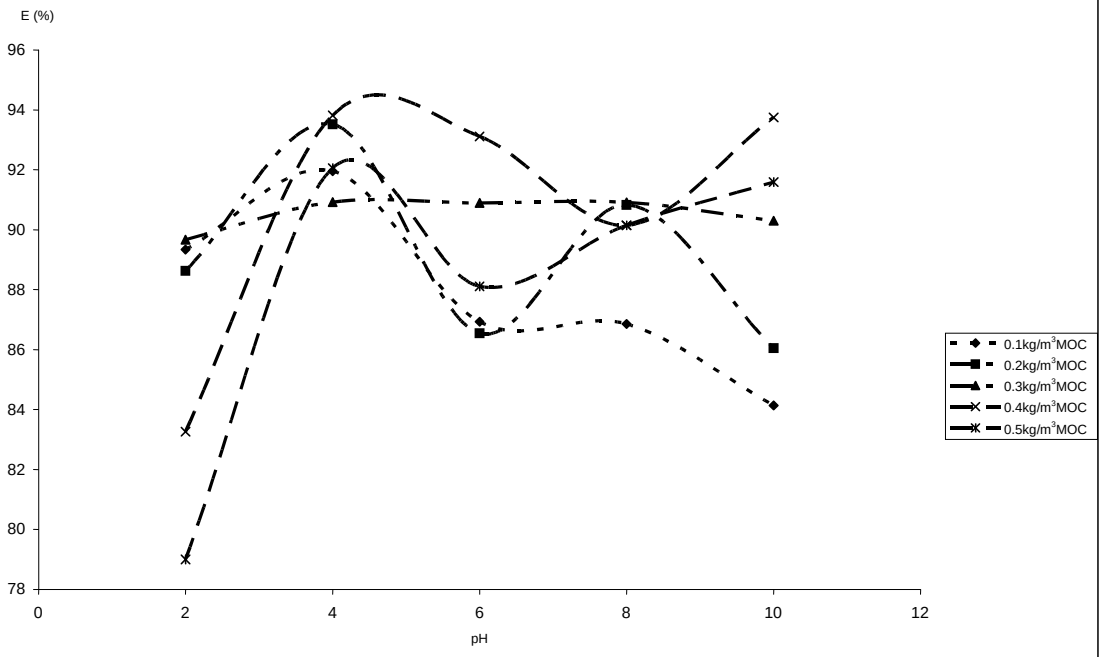


Fig15: Plot of E vs. pH at 30 mins for varying dosages

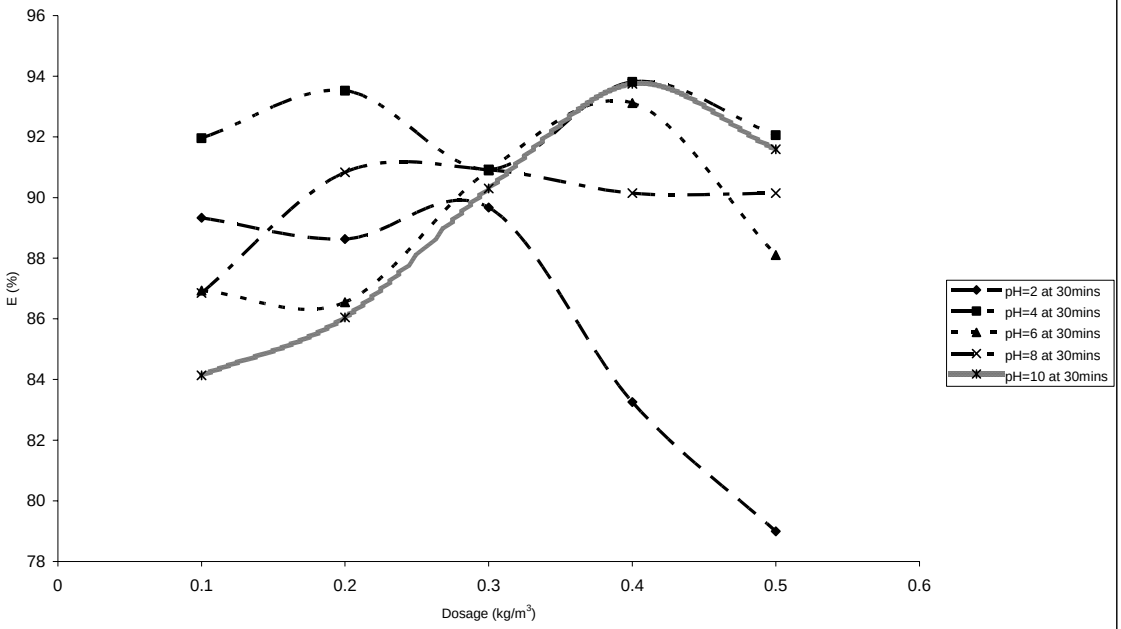


Fig16:Plot of E vs. Dosage at varying pH and constant time

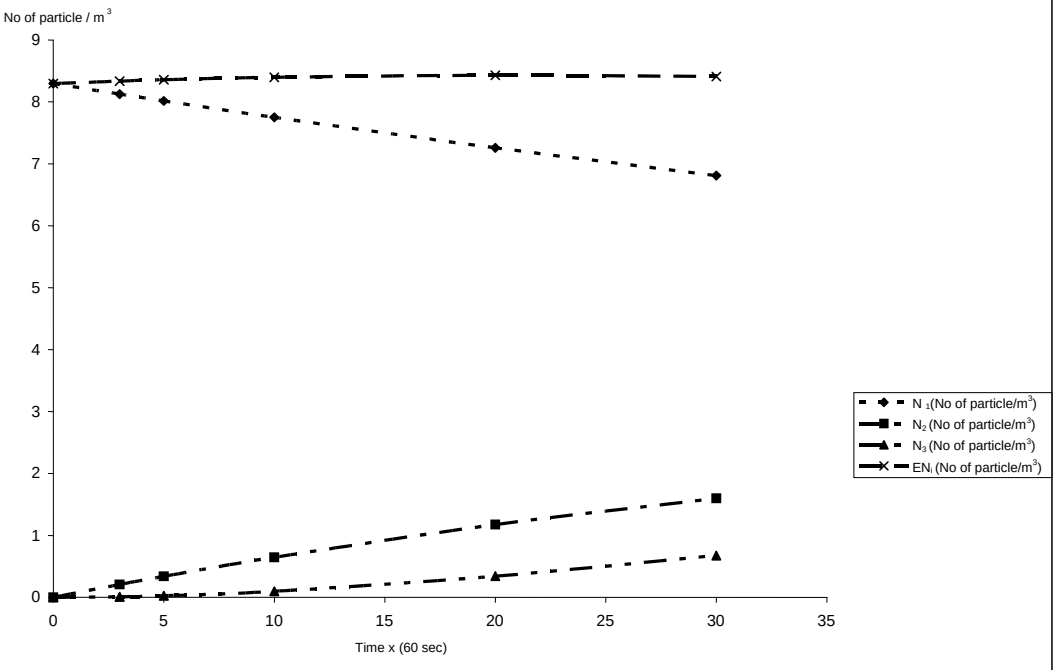


Fig 17: Time evolution of the cluster size distribution for half life of 289.261s

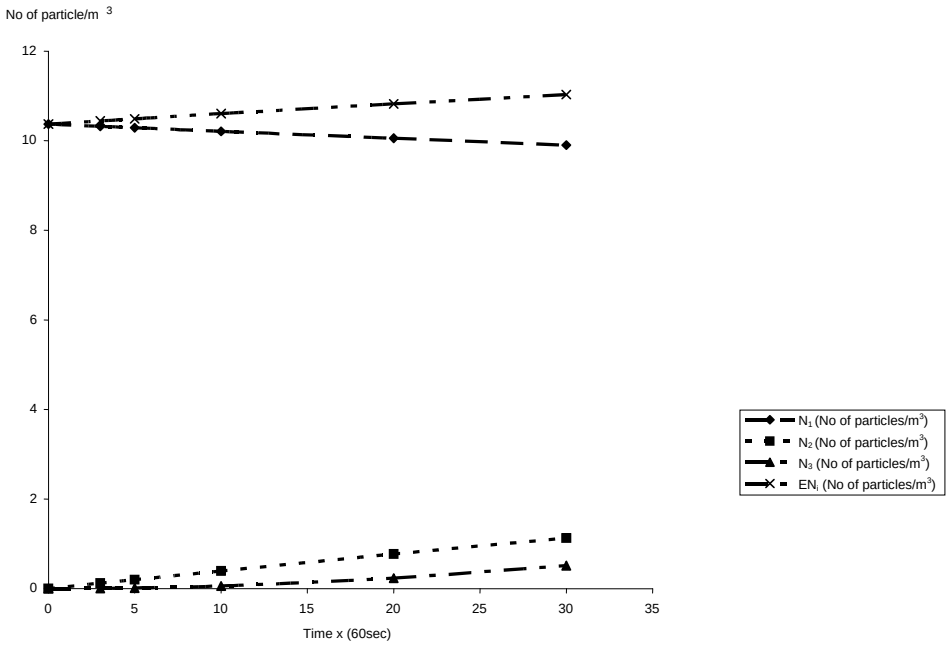


Fig 18: Time evolution of the cluster size distribution for half life of 1446.642s

Correspondence to:
Menkiti Matthew Chukwudi
Department of Chemical Engineering,
Nnamdi Azikiwe University Awka Nigeria.
E-mail: cmenkiti@yahoo.com
Phone no: +234 8037441882

REFERENCE

1. AWWA; American Water Works Association, *Standard Methods for the Examination of Water and Waste water Effluent*, New York, U.S.A. 2005.
2. Diterlizzi S.D; *Introduction to Coagulation and Flocculation of Waste water*, Environmental System Project, U.S.A 1994.
3. Edzwald, J.K; *Coagulation – sedimentation filtration process for removing organic substances in drinking and waste water.* Noyes Data Corporation, Park Bridge, New Jersey. 1987.
4. ENSWC;Quality control unit, Enugu State Water corporation, Enugu, Enugu State Nigeria 2008.
5. Fridkhsberg, D.A; *A course in Colloid Chemistry;* Mir Publishers Moscow, Russia. 1984:266-268
6. Ghebremichael K.; Moringa seed and pumice as alternative natural materials for drinking water,TRITA-LWR PHD 1013 Ph.D. Thesis. KTH land and water Resources Engineering. 2004.
7. Holthof, H., Egelhaaf, S.U, Borkovec, M., Schurtenberger P and Sticher, H; Coagulation Rate Measurement of Colloidal Particles by Simultaneous Static and Dynamic Light Scattering, *Langmuir* 1996:**12:**5541.
8. Holthof, H., Schmitt, A., Ferńandez-Barbero, Borkovec. M., Cabrerizo-Vilehez., Schurtenberger, P and Hidalgo-Alvarez, R, Measurement of Absolute Coagulation Rate Constants for Colloidal Particles: Comparison of Single and Multiparticle Light Scattering Techniques; *Journal of Colloid and Interface Science*. 1997:**192:**463-470.
9. Hunter, R. J; *Introduction to Modern Colloid Science*, 4th Oxford University Press, New York: 1993: 33-38; 289-290.
10. Jin, Y; *Use of high Resolution Photographic Technique for studying Coagulation/flocculation in water treatment*, M.Sc thesis, University of Saskatchewan, Saskatoon, Canada. 2005:22-29.
11. Ma J.J; Li G; Chen, G.R; Xu, G.O and Cai G.Q; Enhanced coagulation of surface waters with high organic content by permanganate per oxidation. *Water science and Technology: Water Supply* 2001: **1**(1):51-61.

12. Menkiti, M.C, Nnaji, P.C, Onukwuli, O.D; Coag-flocculation kinetics and functional parameters response of Periwinkle shell coagulant (PSC) to pH variation in organic rich coal effluent medium; *Nature and Science* 2009: **7**(6) pp 1-18.
13. Menkiti, M.C, Nnaji. P.C, Nwoye,C.I, Onukwuli, O.D; Coag-flocculation kinetics and functional parameters response of Mucuna seed coagulant to pH variation in organic rich coal effluent medium, *Journal of minerals and materials characterization and engineering*, 2010:**9** (2) pp13-27.
14. O'Meila C.R; Coagulation in waste water treatment: The scientific basis of flocculation (NATO Advanced study Inst. Series E, Appl .Sc. No 27) in: Ives, K.J (Ed). Sijtholf and Noordhoff, Alpenan den Rijn, Netherlands. 1978: pp: 219-268.
15. Schwarz .D; Water clarification using *Moringa olifera*, Technical information W1e, Gate information service Germany 2000: pp 1-4.
16. Swift, DL and Friedlander S.K. The coagulation of hydrolysis by Brownian motion and laminar shear flow. *Journal of Colloid Science* 1964:**19:**621.
17. Thomas, D.N; Judd, S.J and Fawcett N; Flocculation modeling: A review. *Water Resources* 1999:**33**(7):1579-1592.
18. Van Zanten, J.H., and Elimelech M.; Determination of Rate constants by multi angle light scattering; *Journal of colloid and interface* 1992: **154** (1): 621.
19. Von Smoluchowski, M; Versucheiner Mathematischen Theorie der Koagulations Kinetic Kolloider Lousungen. *Z. Phys. Chem*. 1917:**92**:129-168.
20. Water Specialist Technology (WST); *About Coagulation and Flocculation:* Information Bulletins, U.S.A.2003.
21. Yates, P; Yan,Y; Jamson G J and Biggs. S; Heteroaggregation of particle system: Aggregation Mechanisms and aggregate structure determination; *6th World Congress of Chemical Engineering*, Melbourne,Australia, 23rd -27th September.1-10:2001.

9/9/2010

Association between inflammation and the risk of cardiovascular disorders in atherogenic male rats: Role of virgin and refined olive oil

Azza M. EL Wakf[*1]; Hamdy A. Ebraheem[1]; Hanaa A. Serag[1]; Hanaa A. Hassan[1]; Hussein S. Gumaih[2]

[1]Faculty of Science, Mansoura University, Mansoura, Egypt,
[2]Faculty of Education, Sana'a University, Sana'a, Yemen
[*]dr_azzaelwakf@yahoo.com

Abstract: The aim of the present study was to determine changes in inflammatory markers, lipid profile and vascular wall integrity, (monitored as nitric oxide levels) in the male rats with experimental atherosclerosis. Also, to evaluate the role of two olive oils (virgin and refined) in these changes. Experimental atherosclerosis was induced by feeding rats normolipidemic diet (NLD) supplemented with (4% cholesterol, 1% cholic acid and 0.5% thiouracil, w/w) for three months. Feeding atherogenic diet (AD) exhibited marked elevation in serum total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), very low density lipoprotein cholesterol (VLDL) and triglycerides (TG), along with decreased high density lipoprotein cholesterol (HDL-C). Besides, an elevation in serum level of the two inflammatory markers, tumor necrosis factor-α (TNF-α) and fibrinogen was demonstrated with a lowered nitric oxide (NO) levels in both aorta and cardiac tissues, indicating impaired vessel wall integrity and development of cardiovascular disorders in response to hyperlipidemia and enhanced inflammation. Subsequently, marked elevations in total leucocytes and other inflammatory mediators, including monocytes and lymphocytes have been recorded in the atherogenic diet fed rats. In addition, a significant reduction in erythrocytes count, hemoglobin (Hb) content and other hematologic indices was demonstrated, indicating further signs of inflammation. However, administration of olive oil (OO) [(in particular virgin olive oil (VOO)] to atherogenic rats exhibited improved inflammatory status, lipid profile and NO levels. Therefore, VOO might be a good candidate to replace other fats in the functional food for retarding atherosclerosis and risk of cardiovascular disorders.
[Azza M. EL Wakf; Hamdy A. Ebraheem; Hanaa A. Serag; Hanaa A. Hassan; Hussein S. Gumaih. **Association between inflammation and the risk of cardiovascular disorders in atherogenic male rats: Role of virgin and refined olive oil**. Journal of American Science 2010;6(12):807-817]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: Atherogenic diet, inflammatory markers, nitric oxide, vascular wall injury

1. Introduction:

Atherosclerosis is a slow and complex disease that represents one of the most prevalent causes of death world wide (Jaffer *et al.,* 2006). It is an arterial disease in which fatty substances, cellular wastes, and other elements build up in the inner lining of the artery. This build is called plaque that can grow large enough to significantly reduce blood flow throw the vessel (Earnest e*t al.,* 2005). Within the arterial wall, many processes act in a seemingly concerted manner to initiate the formation of lesions that result in occlusion of blood flow (Ross 1999). These processes include injury to the endothelium and retention of lipoproteins within the arterial wall, (Brown *et al.,* 2004) which, in turn leads to the formation of foam cells and the development of atherosclerosis (Hansson, 2009).

In broad outline, atherosclerosis can be considered to be a form of chronic inflammation resulting from interaction between lipoproteins, monocyte-derived macrophages, T cells, inflammatory cytokines and the normal cellular elements of the arterial wall. This inflammatory process can ultimately lead to the development of complex lesions, or plaques, that protrude into the arterial lumen (Glass and Witztum, 2001). It is now recognized that atherosclerosis is an inflammatory disease of the arterial wall that underlies many of the common causes of cardiovascular diseases (CVD) (Zhang, 2009).

Dietary, oils may affect vascular wall integrity and consequently the process of atherosclerosis and its related cardiac complications (Sekalska *et al.,* 2007). Among edible oils, olive oil (OO) is the only one produced from a fresh fruit and can be consumed just as obtained from the olive, like fruit juices. It is a pure natural product requiring and is becoming increasingly present in food as the healthiest alternative to other oils (Perona *et al.,* 2005). Olive oil is extensively used by people in the

Mediterranean region that has been associated with reduced incidence of CVD in these people (Nagaraju & Belur, 2008). This may be the result of its high content of monounsaturated fatty acids (MUFAs) mainly oleic acid and minor biologically active components, such as phenolic compounds (PhCs) (Miles *et al.,* 2005).

The present study was carried out to evaluate the effect of prolonged intake of OO on the risk of cardiovascular disorders in male rats with experimental atherosclerosis, in terms of selected inflammatory markers and lipid parameters, as well as NO levels. Two olive oils; refined olive oil (ROO) and VOO (identical in their fatty acids composition, but different in phenolics content) were used to compare their effects.

2. Materials and methods

Experimental animals:

This study was performed on male albino rats of Wistar strain, initially weighing 105±5 g. Rats were obtained from the Institute of Ophthalmic Disease Research, Cairo. They were housed in stainless steel cages at a well ventilated animal house. Rats were permitted adequate standard diet and given water *ad libitum* for one week of adaptation period prior to the experimental work.

Research design:

Rats were randomly divided into six groups of five animals each. The first one was the control group received NLD without any supplementation (The Nutrient Requirements of Laboratory Animals, 1995). The second and third groups were fed NLD supplemented with 10% ROO or VOO, respectively (El-Seweidy *et al.,* 2005). The fourth group received AD (NLD supplemented with 4% cholesterol, 1% cholic acid and 0.5% thiouracil, w/w) (Parmer and Kar, 2007), while the fifth and sixth groups received AD supplemented with ROO (10%) or VOO (10%), as described in 2nd and 3rd groups. All animal groups were maintained on their respective diets for duration of three months.

Samples collection:

At the end of the study period, overnight fasted rats were sacrificed under ether anesthesia. From each rat, two blood samples were collected. The first blood sample was taken on EDTA as anticoagulant for determination of hematological parameters. The second blood sample was collected with no additives to obtain serum by centrifugation at 3000 r.p.m for 10 minutes for biochemical analysis. Immediately after collecting blood, the heart and aorta from each rat were removed, and placed into clean and dry tubes. The tissues were then homogenized using a homogenizer surrounded with an ice jacket and the homogenate was kept frozen at $-20C^0$ until being analyzed.

Biochemical analysis:

Serum concentrations of glucose, TC, HDL, and TG were determined calorimetrically using Spectrophotometer with Kits from Spinreact Co., according to the manufacture instructions, as described by McCleary and Codd (1991); Zoppi and Fellini (1976); Wahlefeld (1974); Naito (1984), respectively. LDL-C and VLDL:-C, as well as atherogenic index (AI) was calculated according the following equation:

LDL-C = TC – HDL-C – TG/5 (Ahmedi *et al;* 2008). VLDL-C = TG/5 (Satheesh and Pari, 2008).

AI = (VLDL-C + LDL-C) / HDL-C (Pandya *et al.,* 2006).

The concentration of nitric oxide (NO) was measured by determination of total nitrate and nitrite concentrations according to the method of Green *et al.* (1982).

Inflammatory and hematologic analysis:

Serum TNF-α was measured, using ELIZA technique, that is a solid phase enzyme amplified sensitivity immunoassay, according to Aggarwal (1985). Quantitative determination of fibrinogen in serum was carried out using Multifibren – U – Kit, as described by Cooper and Douglas (1991).

Red blood cells (RBCs), platelets (Plts) and total white blood cells (WBCs) count was measured by hemocytometer neubauer slide, while differential count of WBCs was determined by Leishman's stained blood film (Harvey, 2001). Whole blood Hb was measured colorimetrically by Randox kits according to Haemat (1967). Hematocrit (Hct) value was determined using hematocrit capillary tubes. The percent of Hct was determined by the use of microscopically reader (Alwan *et al.,* 2009). Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated using the formulae mentioned by Dacie and Lewis (2001).

Statistical analysis:

All data are represented as means ± SE. One-way analysis of variance ANOVA was used to analyze the results. The level of statistical significance at $P < 0.05$ was performed by Least Significant Difference (LSD) test (SPSS Inc. 1999).

3. Results

The present study investigates the impact of two types of olive oil (OO) [(refined olive oil (ROO)

and virgin olive oil (VOO)] on biochemical, inflammatory and hematological changes developed in male rats with experimentally induced atherosclerosis. The statistical evaluation of obtained data were calculated for the atherogenic group according to the normolipidemic diet (NLD) group and for OO+AD treated groups according to both the atherogenic and the NLD groups (no comparison between the two groups OO+AD and ROO, VOO groups were carried out).

In the present study, administration of ROO or VOO to normal rats did not produce significant changes in all measured parameters if compared to NLD group (Table 1, 2, 3, 4). On the other hand, administration of atherogenic diet exhibited elevated serum lipids (TC, TG and LDL-C), along with decreased HDL-C concentrations. Besides, serum glucose level was increased in the atherogenic animals, if compared to NLD group (Table 1). Meanwhile, the levels of NO in both aorta and cardiac tissues showed significant reduction (Table 2), along with marked elevation in the serum levels of the two inflammatory markers, TNF-α and fibrinogen (Table 3). Additionally, Table 4 showed elevations of Plts count, as well as WBCs, monocytes and lymphocytes, concomitantly with a reduction in RBCs count, Hb content and other indices (Hct%, MCV, MCH and MCHC) in the atherogenic rats, in comparison with NLD group. On the other hand, feeding rats an atherogenic diet supplemented with ROO or VOO caused an ameliorative effect on all these parameters, as evidenced by restoration of the changed lipid profile, inflammatory markers, NO level and hematologic indices near to normal values. However, the greatest effect was recorded with VOO administration.

Table (1): Effect of refined or virgin olive oil on serum lipid profile and glucose level in atherogenic diet fed rats.

Groups	NLD group	ROO group	VOO group	AD group	ROO+AD group	VOO+AD group
TC (mg/dl)	189.5±3.62	189.1±3.00	182.2±2.51	382.1±9.57^{a}	247.0±5.74^{ab}	204.6±2.96^{abc}
TG (mg/dl)	125±0.43	121.6±2.25	118.9±1.41	183.6±3.37^{a}	163.2±3.47^{ab}	128.5±1.08^{bc}
LDL-C(mg/dl)	96.9±2.22	96.5±1.12	89.5±2.01	316.3±8.14^{a}	179.6±4.59^{ab}	121.1±2.08^{abc}
VLDL-C(mg/dl)	25.00±0.09	24.31±0.45	23.78±0.23	36.72±0.67^{a}	32.64±0.69^{ab}	25.70±0.21^{bc}
AI	1.80±0.03	1.77±0.07	1.65±0.04	12.12^{a}±0.29	6.10±0.31^{ab}	2.54±0.02^{abc}
HDL-C (mg/dl)	67. 7±1.54	68.3±2.70	68.9±1.71	29.1±1.10^{a}	34. 8±1.68^{ab}	57.7±0.98^{abc}
Glucose (mg/dl)	106.7±1.88	105±3.08	104.3±1.09	125.3±3.28^{a}	121.0±1.09^{a}	111.3±1.18^{bc}
ANOVA	P < 0.05					

Values are means ± SE for 5 animals in each group.
a: significant compared with the control group, b: significant compared with the atherogenic group and c: significant compared with the refined olive oil group.
Normolipidemic diet (NLD), refined olive oil (ROO), virgin olive oil (VOO), atherogenic diet (AD), refined olive oil + atherogenic diet (ROO+AD) and virgin olive oil + atherogenic diet (VOO+AD).

Table 2: Effect of refined or virgin olive oil on aorta and cardiac nitric oxide (NO) level in atherogenic diet fed rats

Groups	NLD group	ROO group	VOO group	AD group	ROO+AD group	VOO+AD group
Aorta NO (µmol/g)	1.6±0.02	1.6±0.04	1.7±0.02	0.9±0.07^{a}	1.0±0.06^{a}	1.5±0.04^{bc}
Heart NO (µmol/g)	1.1±0.16	1.2±0.18	1.3±0.19	0.5±0.10^{a}	0.9±0.08^{ab}	1.0±0.10^{b}
ANOVA	P < 0.05					

Values are means ± SE for 5 animals in each group.
a: significant compared with the control group, b: significant compared with the atherogenic group and c: significant compared with the refined olive oil group.
Normolipidemic diet (NLD), refined olive oil (ROO), virgin olive oil (VOO), atherogenic diet (AD), refined olive oil + atherogenic diet (ROO+AD) and virgin olive oil + atherogenic diet (VOO+AD).

Table 3: Effect of refined or virgin olive oil on serum level of tumor necrosis factor- α (TNF-α and fibrinogen in atherogenic diet fed rats

Groups	NLD group	ROO group	VOO group	AD group	ROO+AD group	VOO+AD group
TNF-α (pg/mg)	7.4±0.13	7.2±0.12	6.7 ±0.17	11.0 ±0.45[a]	9.6±0.20[ab]	7.6±0.15[bc]
Fibrinogen (mg/dL)	223.6±0.93	223.2 ±1.96	222.2±1.36	389.0 ±5.00[a]	333.0 ±3.48[ab]	234.6±2.27[abc]
ANOVA	P < 0.05					

Values are means ± SE for 5 animals in each group.
a: significant compared with the control group, b: significant compared with the atherogenic group and c: significant compared with the refined olive oil group.
Normolipidemic diet (NLD), refined olive oil (ROO), virgin olive oil (VOO), atherogenic diet (AD), refined olive oil + atherogenic diet (ROO+AD) and virgin olive oil + atherogenic diet (VOO+AD).

Table 4: Effect of refined or virgin olive oil on different hematologic parameters in atherogenic diet fed rats

Groups	NLD group	ROO group	VOO group	AD group	ROO+AD group	VOO+AD group
WBCs ($X10^3/\mu L$)	5.2±0.17	5.1±0.16	5.0±0.34	6.5±0.34[a]	6.1±0.62	5.2±0.40[bc]
Lymphocytes%	42.2±1.10	43±2.10	40±0.60	55.6±1.96[a]	47.4±1.12[ab]	43.2±1.77[bc]
Monocytes %	5.60±0.37	5.60±0.25	5.40±0.25	6.8±0.58[a]	6.0±0.45	5.8±0.58[b]
PLts ($X10^3/\mu L$)	228.6±5.09	229.6±6.55	227.0±6.61	387.8±15.92[a]	268.8±8.72[ab]	231.0±9.56[bc]
RBCs ($X10^6/\mu L$)	5.5±0.09	5.8±0.26	5.9±0.22	4.2±0.27[a]	4.2±0.07[ab]	5.1±0.20[bc]
Hb(g/dL)	13.3±0.66	12.7±0.24	13.4±1.00	9.0±0.40[a]	10.3±0.37[a]	10.5±0.17[ab]
Hct%	41.8±1.59	40.7±0.57	41.2±1.30	27.2±1.65[a]	27.9±0.79[a]	39.8±1.70[bc]
MCV (*f*L)	76.6±4.23	75.7±2.67	76.6±1.45	57.9±1.66[a]	70.8±2.56[b]	76.0±1.36[b]
MCH (pg/ml)	22.4±1.14	21.3±0.66	21.8±0.25	19.7±0.51[a]	20.5±0.78[a]	20.8±0.59[b]
MCHC%	31.2±0.99	31.0±0.32	32.4±1.36	26.3±2.29[a]	28.5±1.48[a]	30.8±1.11[b]
ANOVA	P < 0.05					

Values are means ± SE for 5 animals in each group.
a: significant compared with the control group, b: significant compared with the atherogenic group and c: significant compared with the refined olive oil group.
Normolipidemic diet (NLD), refined olive oil (ROO), virgin olive oil (VOO), atherogenic diet (AD), refined olive oil + atherogenic diet (ROO+AD) and virgin olive oil + atherogenic diet (VOO+AD).

4. Discussion

Atherosclerosis is generally assumed to develop following prolonged exposure of the vascular wall to elevated levels of cholesterol and glucose and also to several inflammatory and hematological changes. However, due to special anatomical position of the vascular endothelium (between circulation and vascular wall) it is a primary target for these risk factors, which in turn causes endothelial dysfunction. Dysfunction of vascular endothelium appears to be a key event in initiating, progression and complications of atherosclerosis (Vallance & Chan, 2001). One of the principal mechanisms underlying endothelial dysfunction is through increased generation of reactive oxygen species (ROS) with consequent oxidative stress (OS) (Hassanabad *et al.,* 2010). Lipid metabolic abnormalities are considered the main cause for increased OS under atherogenic state (Rizzo *et al.,* 2009). However, hyperglycemia (occurred as a result of insulin resistance) associated with lipid abnormalities seemed to be also responsible (Ferroni *et al.,* 2006). In this regard, the present study has shown that feeding rats an atherogenic diet induced elevations in serum levels of glucose and lipids (TC, LDL-C and TG), which may reflect increased generation of ROS that contribute to atherosclerosis development.

Increased ROS is suggested to play a role in atherosclerosis by inducing endothelial dysfunction characterized by impaired production of NO (Balakumar *et al.,* 2009). ROS including O_2^- can react with NO to form peroxynitrite ($OONO^-$), thus inactivating NO and directly decreased endothelium synthesis of NO (Perona *et al.,* 2006). Consistent with this, it was reported that patients with developing atherosclerosis have reduced NO bioavailability in both coronary and peripheral vasculature (Barbato & Tzeng, 2004). Additionally, functional NO has been demonstrated to decrease in plasma and tissues of experimental atherogenic conditions (Deepa and Varalakshmi, 2004). In the

present study, observed finding of decreased aorta and cardiac NO levels following atherogenic diet feeding could therefore indicate development of atherosclerosis with increased risk of CVD.

Nitric oxide normally functions to maintain vascular homeostasis through a number of physiologic processes. One prevalent action involves the activation of soluble guanyl cyclase, which then produces cyclic guanisine monophosphate (cGMP), being responsible for vasorelaxation (Dhir and Kulkarni, 2007). Nitric oxide can also act directly on calcium dependent potassium channels, leading to relaxation of vascular smooth muscles (SMCs) (Costa & Assreuy, 2005). Moreover, the vasoprotective effect of NO includes promotion of endothelial proliferation and protection of endothelial cells (ECs) from apoptosis and adherence of inflammatory cells (Hengartner, 2000) thus serves to limit endothelial inflammation. Based on this, the reduction in NO, as in case of atherosclerosis has suggested to promote pro-inflammatory endothelial response (Napoli *et al.,* 2006). It involves enhanced vulnerability of vascular wall to circulating leukocytes and other inflammatory mediators, leading to atherosclerotic vascular injury (Naseem, 2005). It is now clear that inflammatory mediators are intimately involved in atherogenic process (Libby, 2002), thereby demonstrating atherosclerosis as an inflammatory disease (Burger-Kentischer *et al.,* 2006).

Excessive inflammation leads to patho-logical situation that including leucocytes migration into the inflammatory site. During vascular atherosclerosis, leucocytes and ECs are the major cellular players of the inflammatory reactions, while numerous mediators are involved in augmenting the inflammatory process (Zakynthinos & Pappa, 2009). It was explained that ECs coordinate the recruitment of inflammatory leucocytes to sites of vascular injury that consequently result in production and release of inflammatory cytokines, which are large family of small chemoattractant proteins (Reape & Groot, 1999). Among these, TNF-α is recognized to have a major role in inflammation, apoptosis, cell proliferation and stimulating synthesis of other cytokines, which further augment expression of cell adhesion molecules, such as intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin (Hennig & Toborek, 2001). Circulating leucocytes (in particular monocytes) are attracted by these molecules and adhered to endothelium, from which they migrate to the subendothelial space. Once within the vascular wall, monocytes differentiate into macrophages, which express scavenger receptors, allowing them to scavenge oxidized LDL-C (Ox-LDL-C) (Fuhrman *et al.,* 2008). LDL-C can be oxidized by ECs, SMCs and macrophages present in the arterial wall and the formed Ox-LDL-C may directly damage the endothelium and contribute to atheroma plaque formation, through increased adherence and migration of monocytes and lymphocytes (predominantly T-lymphocytes) into the vascular wall (Quehenberger, 2005). T lymphocytes recognize specially antigens that are produced in plaques including Ox-LDL-C (McLaren and Ramji, 2008). In this regard, the study of Kentischer *et al.* (2002) demonstrated that monocytes, macrophages and T-lymphocytes are critical cells characterizing all stages of atherosclerosis. Once stimulated they increase production of several pro-inflammatory cytokines, such as TNF-α, which are important in initiating the expression of variety of genes that in turn promote cell adhesion and other processes required for progression of atherosclerosis (Zhang *et al.,* 2007). Further evidence that atherosclerosis is linked to inflammation is provided by the present observation that feeding rats on atherogenic diet induced marked elevation in several inflammatory markers, including total leucocytes, monocytes and lymphocytes, as well as serum level of the pro-inflammatory and pro-atherogenic TNF-α. In other studies, the interactions between these inflammatory mediators promote SMCs migration and proliferation which in turn leads to atheroma plaque rupture with subsequent adherence and aggregation of platelets, leading to progression of atherosclerotic injury (Chon *et al.,* 2006).

Platelets are circulating cells that play a key role in hemostasis system (Ni & Freedman, 2003). By adhering to damaged blood vessels PLts become activated, which in turn release PLts derived growth factor that promote plaque growth contributing to progression of atherosclerotic disease (Crowther, 2005). Besides, activated PLts play a role in atherosclerosis through providing other hemostatic factors, including fibrinogen (Ni and Freedman, 2003). This latter effect has already been demonstrated in the present study, as evidenced from the raised fibrinogen levels in parallel to the increased PLts count following atherogenic diet feeding. Raised plasma fibrinogen is associated with development of atherosclerosis and is considered a risk factor for CVD (Sato *et al.,* 2000). Several reasons for the association between fibrinogen and the risk of CVD have been suggested. They include the effect on plasma viscosity, coagulation, fibrinolysis (Junker *et al.,* 1998), promotion of vascular SMCs proliferation (Rauch *et al.,* 2007) and also the induction of PLts aggregation (Willoughby *et al.,* 2002). In addition, recent studies indicated that fibrinogen accumulation in the vessel wall may

contribute to the early vascular inflammation (Heffron *et al.,* 2009). Other studies showed that fibrinogen induced production of the systemic inflammatory marker C-reactive protein (CRP) in SMCs, both in messenger ribonucleic acid (m-RNA) and protein levels (Guo *et al.,* 2009), suggesting that fibrinogen possesses a pro-inflammatory properties which is related to atherosclerosis development. In support, Guo *et al.,* (2009) reported that macrophages accumulation in the arterial wall may be associated with increased plasma concentrations of both fibrinogen and CRP, two markers of inflammation thought to be early signs of atherosclerosis and CVD. Therefore, increased fibrinogen level as detected in this study may indicate enhanced inflammatory status under present atherogenic condition.

Apart from the role of the two hemostatic factors, fibrinogen and PLts in atherosclerosis there is evidence that RBCs may also serve as a potential partner in this process, through activation of the vascular endothelium leading to vascular inflammation and atherosclerosis (Blum, 2009). A number of changes can affect RBCs and thereby lead to development and progression of atherosclerosis. Researchers have indicated that RBCs are particularly exposed to oxidative hazards because of their specific role as oxygen carriers (Manna *et al.,* 1999). Under normal physiologic conditions, there is a steady state balance between the production of ROS and their destruction by the endogenous antioxidant defense system (Takenaka *et al.,* 1991). However, if ROS are over produced, as in atherosclerosis, this can damage both plasma membrane and cytosolic components, leading to oxidative hemolysis of RBCs and decreased survival of oxidized RBCs in circulation (Manna *et al.,* 1999). Therefore, the possibility of RBCs oxidative modification appears to be an explanation for the reduction in RBCs count and other hematologic indices (Hb, Hct, MCV, MCH and MCHC) observed under present atherogenic condition.

As a consequence of oxidative modification, drastic changes in RBCs morphology occur, causing RBCs activation thereby, microvesicles of RBCs are formed (Leopold & Loscalzo, 2009). Formation of membrane vesicles is associated with loss of plasma membrane asymmetry, consequently, RBCs shed microvesicles from their main body, and circulating levels of microvesicles are augmented, as in case of most CVD (Blum, 2009). Microvesicles activate ECs, attract leucocytes and enhance their adherence to ECs, as well as induce production of pro-inflammatory cytokines and upregulation of ICAM-1 involved in the recruitment of monocytes into the arterial wall, thereby promoting the whole cascade of inflammatory events, leading to evolution of atherosclerosis with increased risk of CVD (Kaperonis *et al.,* 2006).

In contrast to these multiple atherogenic events, there is compelling evidence that nutrition can affect genesis of atherosclerosis by modulating functional properties of vascular ECs. In particular the lipid environment of the vascular endothelium may profoundly influence the inflammatory response during atherosclerosis and thereby confer an overall beneficial effect (Ringseis & Eder, 2010). With the discovery of the relationship between dietary fat and atherosclerosis; olive oil rich diets have been acclaimed for its protective effects on several of changes associated with atherosclerosis and heart disease (Acın *et al.,* 2007). This was in accordance with the present observations, where feeding rats on atherogenic diet supplemented with VOO or ROO has shown lowered atherogenic hazards. This effect was observed mainly in terms of modulating a number of inflammatory markers. Besides, a reduction in the hyperlipidemic status, concomitant with increased NO levels were recorded. The present data showed also that VOO was more effective than refined ROO, where the maximal protection was observed with VOO administration.

Evidence from other studies indicated that each of VOO or ROO consists mainly of monounsaturated fatty acids (MUFAs), with minor constituents, like phenolic compounds (PhCs) that contribute to the stability of the oil and exhibit wide range of biological activities (Ruano *et al.* 2005). From previous studies, it was indicated that PhCs are influenced by procedures of OO extraction and that PhCs are lost when OO is refined (Mir´o-Casas *et al.,* 2001). Therefore, VOO obtained by physical procedures has high amounts of PhCs. Actually, both VOO and ROO contain similar proportions of MUFAs; however the total PhCs concentration is greater in VOO, which may contribute to the more beneficial properties of VOO compared to ROO, as observed in this study.

In some studies the healthy effects of OO on cardiovascular risk factors have been attributed to its high content of MUFAs, such as oleic acid (Massimo *et al.,* 2009). In fact, MUFAs are suggested to be effective in improving serum lipid profile, through a decrease in TC, LDL-C and TGs and increase in HDL-C (Moreno & Mitjavila, 2003). Besides, OO has been shown to lower blood glucose levels (Tahvonen *et al.,* 2005), which together with improved blood lipids (as shown in this study) may reflect some protection against development of atherosclerosis.

Other studies suggested that nutrients, including MUFAs may modulate atherosclerosis by

affecting vascular endothelium (Covas, 2007), through increasing the amount of oleic acid in the arterial wall and displacing saturated fatty acids (SFAs), while leaving polyunsaturated fatty acid (PUFAs). This change in vessel wall composition is favorable for three reasons: (1) Saturated fatty acids are atherogenic and favor platelet aggregation through decreasing prostacyclin and increasing thromboxan production. They can thus be considered prothrombotic substances. (2) Polyunsaturated fatty acids reduce platelet activity and prothrombotic capacity of the arterial wall. (3) The accumulation of MUFAs in the arterial wall decreases the expression of several endothelial adhesion molecules involved in the selective monocytes recruitment in the arterial wall (Perona *et al.,* 2006). Thus, oleic acid may contribute to prevention of atherosclerosis, mainly through a protective effect against atherogenic vascular wall lesions.

Despite the beneficial effects attributed to oleic acid, it is very likely that other minor components with antioxidant and anti-inflammatory properties, such as PhCs could be responsible for OO effects observed in this study and in other experimental trials. Oleuropin and its derivative hydroxytyrosol have the strongest radical scavenging properties among all olive oil PhCs (Oram *et al.,* 2004). Massaro *et al.* (2002) hypothesized that these components may exert direct vascular athero-protective effects by inhibiting endothelial dysfunction through quenching ROS and reversing the imbalance between increased OS and impaired antioxidant status. Association between OS and impaired endothelial function, characterized by reduced NO bioavailability have been demonstrated in humans and experimental animal models of atherosclerosis (Moraes *et al.,* 2007). Endothelial dysfunction could be reversed in atherogenic patients by administration of agents capable of scavenging ROS (Jackson *et al.,* 1998), such as OO and its PhCs (Covas, 2007). In this regard, it was reported that oleuropein and hydroxytyrosol are potent scavengers of ROS and O_2^- in neutrophils, which might prevent formation of the powerful oxidant peroxynitite ($OONO^-$) leading to an increase in NO levels, being linked to endothelial improvement and inhibition of atherosclerosis (Briante *et al.,* 2001). In support, the present data showed increased NO levels in both aorta and cardiac tissues in rats fed atherogenic diet supplemented with OO.

Other mechanisms have been suggested to explain beneficial effects of olive oil PhCs. They include: (1) Antioxidant protection of LDL-C from oxidative modification, either directly or with interaction with endogenous antioxidants likes vitamin E (Rajasekhar *et al.,* 2004). (2) Reduction of microphage uptake of Ox-LDL-C (Rosenblat *et al.,* 2008). (3) Increase in size of LDL-C particles that become less pro-atherogenic (Perona *et al.,* 2006). Apart from protecting LDL-C from oxidation, PhCs exhibited also some beneficial effects on PLts (Singh *et al*., 2006), fibrinogen and erythrocytes, as consequence of its antioxidant properties (Covas, 2007). PhCs from olive leaf extract significantly inhibited PLts aggregation *in vitro,* possibly through their H_2O_2 scavenging properties, which may offer a degree of protection from thrombosis and other CVD risk factors (Singh *et al.*, 2006). Moreover, OO with different phenolic contents were found to reduce fibrinogen levels, but increase RBCs count (Huang & Sumpio 2009), as well as Hb concentration, MCHC and Hct (Ashour *et al.,* 2007). Similar observations were also demonstrated with the present study, where feeding rats an atherogenic diet supplemented with OO showed a lowering effect on both PLts and fibrinogen levels, concomitant with elevations in RBCs count and other hematological indices (Hb, Hct, MCV, MCH, and MCHC), thereby suggesting a potent antiatherogenic role of OO.

Other studies in humans and animals evidenced that PhCs from OO have demonstrated anti-inflammatory effects, through inhibiting a number of inflammatory mediators released by endothelial cells (Ros *et al.,* 2004). De La Puerta *et al.* (1999) studied a range of VOO phenolics (oleuropein glycoside, caffiec acid and tyrosol) in rat peritoneal leucocytes, indicating inhibited production of leukotriene B_4 (LTB_4), which is a chemoattractant and activator of neutrophiles. Moreover, Miles *et al.* (2005) found a very strong effect of oleuropein glycocide on production of cytokines, IL-6 and TNF-α. Also, monocyte adhesion to ECs can be modulated by VOO- PhCs, through inhibiting mRNA expression of several adhesion molecules, including VCAM-1 (Carluccio *et al.,* 2003). This occurs as a result of interfering with activation of the most important transcription factor controlling endothelial activation, nuclear factor kB (NFkB) (Perona *et al.,* 2007). In this respect the present study showed marked reduction in total leucocytes, monocytes and lymphocytes, as well as the pro-inflammatory marker TNF-α in rats fed atherogenic diet supplemented with OO. The out come of these effects in all, is indicative of lowered inflammatory tendency in response to OO intake, which in turn may decrease the risk of atherosclerosis and cardiovascular disorders.

In conclusion, the results of this study illustrated the role of inflammatory changes as an etiology of atherosclerosis and further indicated the ability of OO (in particular VOO) to favorably influence these changes. Therefore, VOO may be

useful in designing dietary strategies to minimize development of atherosclerosis and related cardiovascular disorders in humans.

Corresponding author
Azza M. EL Wakf
Faculty of Science, Mansoura University, Mansoura, Egypt,
dr_azzaelwakf@yahoo.com

5. References:

1. Acín, S.; Navarro, M. A.; Osada, J. and Ruiz-Gutiérrez, V. (2007): Olive oil preparation determines the atherosclerotic protection in apolipoprotein E knockout mice. *J. Nutr. Biochem.* 18(6):418-424.
2. Aggarawal, B. B. (1985): Human tumor necrosis factor production, purification and characterization. *J. Biol. Chem.* 260. 2345-2354.
3. Ahmedi, S. A.; Boroumand, M. A.; Moghddam, K. G.; Tajik P.and Dibaj, S. M. (2008): The impact of low serum triglyceride on LDL-C estimation. *Archives Iranian Med.* 11 (3): 318 – 321.
4. Alwan, S. F.; Hadi, A. A. and Shokr, A. E. (2009) Alterations in hematological parameters of fresh water fish, Tilapia zillii, Exposed to Aluminum. *J. Sci. Applic.,* 3 (1) 12-19.
5. Ashour, A. A.; Yassin, M. M.; Abu aasi, N. M. and Ali, R. M. (2007): Blood, serum glucose and renal parameters in lead-loaded albino rats and treatment with some chelating agents and natural oils. *Turk. J Biol.,* 31: 25-34.
6. Balakumar, P.; Chakkarwar, V. A.; Krishan, P. and Singh, M. (2009): Vascular endothelial dysfunction: A tug of war in diabetic nephropathy. *Biomed. Pharmacol.*, (63): 171-179.
7. Barbato, J. E. and Tzeng, E. (2004): Nitric oxide and arterial disease *J. Vascul. Surgery,* 40:1 187-193.
8. Blum, A. (2009): The possible role of red blood cell microvesicles in atherosclerosis. *Euro J. Inter. Med.* 20: 101–105.
9. Briante, R.; La Cara, F.; Tonziello, M. P.; Febbraio, F. and Nucci, R. (2001): Antioxidant activity of the main bioactive derivatives from oleuropein hydrolysis by hyperthermophilic beta-glycosidase, *J. Agric. Food Chem.*, 49 (7): 3198–3203.
10. Brown, M. D.; Jin, L.; Jien, M.; Matsumoto, A. H.; Helm, G. A.; Lusis, A. J.; Frank, S. and Shi, W. (2004): Lipid retention in the arterial wall of two mouse strains with different atherosclerosis susceptibility. *J. Lipid Res.,* 45: 1155-1161.
11. Burger-Kentischer, A.; Gobel, H.; Kleemann, R.; Zernecke, A.; Bucala, R.; Leng, L.; Finkelmeier, D.; Geiger, G.; Ihling, C. and Bernhagen, J. (2006): Reduction of the aortic inflammatory response in spontaneous atherosclerosis by blockade of macrophage migration inhibitory factor (MIF). *Atheroscler.,* 184 28–38.
12. Carluccio, M. A.; Siculella, L.; Ancora, M. A.; Massaro, M.; Scoditti, E. and Storelli, C. (2003): Olive oil and red wine antioxidant polyphenols inhibit endothelial activation: antiatherogenic properties of Mediterranean diet phytochemicals. *Arterioscler. Thromb. Vascul. Biol.*, 23(4): 622–629.
13. Chon, H.; Verhaar, M. C.; Koomans, H. A.; Joles, J. A. and Braam, B. (2006): Role of circulating karyocytes in the initiation and progression of atherosclerosis. *Hypertension,* 47: 803-810.
14. Cooper, J. and Douglas, A. S. (1991): Fibrinogen level as a predictor or mortality on survivors of myocardial infarction. *Fibrinolysis* 5: 105-108.
15. Costa, R. and Assreuy, J. (2005): Multiple potassium channels mediate nitric oxide-induced inhibition of rat vascular smooth muscle cell proliferation. *Nitric Oxide*, 13:145–151.
16. Covas, M. (2007): Olive oil and the cardiovascular system *Pharmacol. Res.,* 55 (3):175-186.
17. Crowther, M. A. (2005): Pathogenesis of Atherosclerosis. *Am. Soc. Hematol. Educ. Program.*, 436-441.
18. Dacie, J. V. and S. M. Lewis, (2001): Practical Hematology 9th ed. Churchill Livingstone, London, 633.
19. Deepa, P. R. and Varalakshmi, P. (2004): Protective effects of certoparin sodium, a low molecular weight heparin derivative, in experimental atherosclerosis. *Clini. Chem. Act.*, 339 (1-2): 105-115.
20. De La Puerta, R.; Ruiz Gutierrez, V. and Hoult, J. R. (1999): Inhibition of leukocyte 5-lipoxygenase by phenolics from virgin olive oil. *Biochem. Pharmacol.,* 57:445–449.
21. Dhir, A. and Kulkarni, S. K. (2007): Involvement of L-arginine–nitric oxide–cyclic guanosine monophosphate pathway in the antidepressant-like effect of venlafaxine in mice. *Prog. Neuro-Psychopharmacol. Biol. Psychol.*, 31: 921–925.
22. Earnest, C. P.; Jordan, A. N.; Safir, M. Weaver, E. and Church, T. (2005): Cholesterol-lowering effects of bovine serum immunoglobulin in participants with mild hypercholesterolemia. *Am. J. Clinic. Nutr.*, 814: 792-798.
23. El Seweidy, M. M. El-Swefy, S. E. Abdallah, F. R. Hashem, R. M. (2005): Dietary fatty acid unsaturation levels, lipoprotein oxidation and circulating chemokine in experimentally induced

atherosclerotic rats. *J. Pharm. Pharma.*, 57 (11): 1467.
24. Ferroni, P.; Basili, S. Paoletti, V. and Dav, G.(2006): Endothelial dysfunction and oxidative stress in arterial hypertension. *Nutr. Metabol. Cardiovas. Dis*., (16): 222-233.
25. Fuhrman, B.; Partoush, A.; Volkova, N. and Aviram, M. (2008): Ox-LDL induces monocyte-to-macrophage differentiation in vivo:Possible role for the macrophage colony stimulating factor receptor (M-CSF-R). *Atheroscler.,* 196: 598–607.
26. Glass, C. K. Witztum, J. L. (2001): Atherosclerosis: The road ahead. *Cell,* 104:503–16.
27. Green, L. C. Wagner, D. A. Glogowski, J. Skipper, P. L. Wishnok, J. S. and Tannenbaum, S. R. (1982): Analysis of nitrate, nitrite, and [N]nitrate in biological fluids. *Analy. Biochem*., 126 (1): 131-138.
28. Guo, F.; Liu, J.; Wang, C.; Liu, N. and Lu, P. (2009): Fibrinogen, fibrin, and FDP induce C-reactive protein generation in rat vascular smooth muscle cells: Pro-inflammatory effect on atherosclerosis. *Bioch. Biophys. Res. Comm.,* 390: 942–946.
29. Haemat, B. J. (1967): International committee for standarization in heamatology, 13: 71-72
30. Hansson, G. K. (2009): Atherosclerosis-An immune disease *Atheroscler*. 202: 2–10.
31. Harvey, J. W. (2001): Atlas of veterinary hematology. WB Saunders Company, Philadelphia, 41.
32. Hassanabad, Z. F.; Chan, C. B. and Furman, B. L. (2010): Reactive oxygen species and endothelial function in diabetes. *Euro. J. Pharmacol*., 636: 8–17.
33. Heffron, S. P.; Parastatidis, I.; Cuchel, M.; Wolfe, M. L.; Tadesse, M. G.; Ischiropoulos, M. H.; Rader, D. J. and Reilly, M. P. (2009): Inflammation induces fibrinogen nitration in experimental human endotoxemia. *Free Radic. Biol. Med.,* 47: 1140–1146.
34. Hengartner, M.O., (2000): The biochemistry of apoptosis. *Nature* 407, 770–776.
35. Hennig, B. and Toborek, M. (2001): Nutrition and endothelial cell function: implications in atherosclerosis. Nutr. Res., 21 (1-2): 279-293.
36. Huang, C. L and Sumpio, B. E. (2009): Olive Oil, the Mediterranean Diet, and Cardiovascular Health *J. Am. Coll. Surg*., 207(3): 407-416.
37. Jackson, T. S.; Xu, A.; Vita, J. A. and Keaney, J. (1998): Ascorbate prevents the interaction of superoxide and nitric oxide only at very high physiological concentrations. *Circul. Res*., 83(9): 916– 22.
38. Jaffer, F. A.; Libby, P. & Weissleder, R. (2006) Molecular and cellular imaging of atherosclerosis: Emerging applications. *J. Am. Coll. Cardiol.,* 47:1328–1338.
39. Junker, R.; Heinrich, J.; Ulbrich, H. Schulte, H. and Assmann, G. (1998): Relationship between plasma viscosity and the severity of coronary heart disease. *Arterioscler. Thromb. Vascul. Biol.,* 18: 870-875.
40. Kaperonis, E. A.; Liapis, C. D.; Kakisis, J. D. Dimitroulis, D. and Papavassiliou, V.G. (2006): Inflammation and Atherosclerosis . *Euro. J. Vasc. Endovasc. Surg.,* 31: 386–393.
41. Kentischer, A.; Goebel, H.; Seiler, R.; Fraedrich, G.; Schaefer, H. E.; Dimmeler, S.; Kleemann, R.; Bernhagen, J. and Ihling, C. (2002): Expression of Macrophage Migration Inhibitory Factor in Different Stages of Human Atherosclerosis. . *Circulation,* 105:1561-1566.
42. Leopold, J. A. and Loscalzo, J. (2009): Oxidative risk for atherothrombotic cardiovascular disease. *Free Radical Biol. Med.,* 47: 1673–1706.
43. Libby, P. (2002): Inflammation in atherosclerosis. *Nature,* 420: 868–874.
44. Manna, C.; Galletti, P.; Cucciolla, V.; Montedoro, G. and V. Zappia, (1999): Olive oil hydroxytyrosol protects human erythrocytes against oxidative damages. *J. Nutr. Biochem*., 10:159 –165.
45. Massaro, M.; Basta, G.; Lazzerini, G.; Carluccio, M. A.; Bosetti, F. and Solaini, G. (2002): Quenching of intracellular ROS generation as a mechanism for oleate-induced reduction of endothelial activation and early atherogenesis. *Thromb. Haemost*., 88 (2): 335– 44.
46. Massimo, C.; Lucio, T.; Jesٍs, M.; Giovann, L. and Giuseppe, C. (2009): Extra virgin olive oil and oleic acid *Nutr. Clin. Diet, Hosp.,* 29 (3):12-24.
47. McLaren, J. E. and Ramji, D. P. (2008): Interferon gamma: A master regulator of atherosclerosis. CGFR., 517: 1- 11.
48. Mcleary, B. V. and Codd, R. (1991): Measurments (1-3); (1-4)-ß- glucan in barely and aots: a streamlined enzymic procedure *J. Sci. Food Agric.,* 55: 303.
49. Miles, E. A.; Zoubouli, P. and Calder, P. C. (2005): Differential anti-inflammatory effects of phenolic compounds from extra virgin olive oil identified in human whole blood cultures. *Nutr., 21*(3):389– 94.
50. Mir´o-Casas, E.; Farr´e, A. M. and Covas, M. I. (2001): Capillary gas chromatography–mass spectrometry quantitative determination of hydroxytyrosol and tyrosol in human urine after olive oil intake. *Anal. Biochem*., 294:63–72.

51. Moraes, C.; Camargo, E. C.; Anyunus, E.; Nucci, G. and Zanesco, A. (2007): Reactivity of mesenteric and aortic rings from trained rats fed with high caloric diet. *Comp. Biochem. Physiol.,* 147: 788-792.
52. Moreno, J. J. and Mitjavila, M. T. (2003): The degree of unsaturation of dietary fatty acids and the development of atherosclerosis. *J. Nutr .Biochem.,* 14 (4): 182-195.
53. Nagaraju, A. and Belur, L. R. (2008): Rats fed blended oils containing coconut oil with groundnut oil or olive oil showed an enhanced activity of hepatic antioxidant enzymes and a reduction in LDL oxidation. F. Chem., 108: 950–957.
54. Naito, H. K. (1984): high density lipoprotein cholesterol *Clin. Chem. C. V. Mosby Co. st. Louis. Toronto* 1207-1213.
55. Napoli, C.; de Nigris, F.; Williams-Ignarro, S.; Pignalosa, O.; Sica, V. and Ignarro, L. J. (2006): Nitric oxide and atherosclerosis. *Nitric Oxide,* 15 (14): 265-279.
56. Naseem, K. M. (2005): The role of nitric oxide in cardiovascular diseases. *Molec. Asp. Med.,* 26: 33–65.
57. Ni, H. and Freedman, J. (2003): Platelets in hemostasis and thrombosis: role of integrins and their ligands. *Transfu. Apher. Sci.,* 28: 257–264.
58. Nutrient requirements of laboratory animals (1995): *Fourth revised edition* National Research Council.
59. Oram, J. F. and Bornfeldt, K. E. (2004): Direct effects of long-chain non-esterified fatty acids on vascular cells and their relevance to macrovascular complications of diabetes. *Front Biosci* 9:1240– 53.
60. Pandya, N.; Santani, D. and Jain, S. (2006): ntioxidant activity of ezetimibe in hyper-cholesterolemic rats. Ind. J., 38. (3): 205-206.
61. Parmar, H. S. and Kar, A. (2007): Atherogenic diet induced diabetes mellitus: Involvement of thyroid hormones. *Euro. J. Pharmacol.* 570 (1-3): 244-248.
62. Perona, J. S.; Rodriguez, R. and Ruiz-Gutierrez, V. (2005): Effects of oleic acid rich oils on aorta lipids and lipoprotein lipase activity of spontaneously hypertensive rats. J. Agric. Food Chem., 53: 7330–7336.
63. Perona, J. S.; Cabello-Moruno, R. and Ruiz-Gutierrez, V. (2006): The role of virgin olive oil components in the modulation of endothelial function. J. Nutr. Biochem., 17 (7): 429-445.
64. Perona, J. S.; Cabello-Moruno, R. and Ruiz-Gutierrez, V. (2007): Modulation of the effects of chylomicron remnants on endothelial function by minor dietary lipid components. *Biochem. Soci. Trans*., 35 (3): 446-450
65. Puerta, R.; Gutierrez, V. and Hoult, J. R. (1999): Inhibition of leukocyte 5- lipoxygenase by phenolics from virgin olive oil. *Biochem Pharmacol* 57(4):445–449.
66. Quehenberger, O. (2005): Molecular mechanisms regulating monocyte recruitment in atherosclerosis. *J. Lipid Res.*, 46: 1582-1590.
67. Rajasekhar , D. ; Roa,P.S. Latheef, .; Saibaba K and Suberamanyam , G (2004) : association of serum antioxidants and risk of coronary heart diease in south indian population.*Indian J. Med. Sci*., 58(11) :465 – 427.
68. Rauch, B. H.; Müschenborn, B.; Braun, M.; Weber, A. and Schrör, K. (2007): ICAM-1 and p38 MAPK mediate fibrinogen-induced migration of human vascular smooth muscle cells. *Euro. J. Pharmacol.,* 577: 54–57.
69. Reape T. J. and Groot, P. H. (1999): Chemokines and atherosclerosis. *Atheroscler.,*. 147:213–225.
70. Ren, M.; Rajendran, R.; Ning, P.; Huat, B. K.; Nam, O. C.; Watt, F.; Jenner, A. and Halliwell, B. (2006): Zinc supplementation de-creases the development of atherosclerosis in rabbits. Free Rad. Biol. Med., 41: 222–225.
71. Ringseis, R.and Eder, K. (2010): Fatty acids and signaling in endothelial cells prostaglandins, *Leuko. Essen. Fatty Acids,* 82:189–198
72. Rizzo, M.; Stelvuljevic, J.; K.; Berneis, K.; Spinas, G.; Rini, G. B.; Ivanovic, Z. J. Kalimanovska, V.; and Vekic, J. (2009): Atherogenic dyslipidemia and oxidative stress: a new look. *Transla. Res.,* 153 (5): 217-223.
73. Ros., E.; Nunez, I.; Perez-Heras, A.; Serra, M.; Gilabert, R. and Casals, E. (2004): A walnut diet improves endothelial function in hypercholesterolemic subjects: a randomized crossover trial, *Circul*, 109 (13):1609–1614.
74. Rosenblat, M.; Volkova, N.; Coleman, R.; Almagor, Y. and Aviram, M. (2008): Antiatherogenicity of extra virgin olive oil and its enrichment with green tea polyphenols in the atherosclerotic apolipoprotein-E-deficient mice: enhanced macrophage cholesterol efflux. *J. Nutr. Biochem*., 19: 514–523.
75. Ross, R. (1999): atherosclerosis, an inflammatory disease (review article), *N. Engl. J. Med* 340: 115–126.
76. Ruano, J.; Lopez-Miranda, J. Fuentes, F. (2005): Phenolic content of virgin olive oil improves ischemic reactive hyperemia in hypercholesterolemic patients. *J. Am. Coll. Cardiol.,* 46:1864–8.
77. Satheesh, M. and Pari. L. (2008): Effect of pterostilbene on lipids and lipid profiles in

streptozotocin–nicotinamide induced type 2 diabetes mellitus. J. Appl. Biomed., 6: 31–37.
78. Sato, S.; Nakamura, M.; Iida, M.; Naito, Y.; Kitamura, A.; Okamura, T.; Nakagawa, Y.; Imano, H.; Kiyama, M.; Iso, H.; Shimamoto, T. and Komachi, Y. (2000): Plasma Fibrinogen and Coronary Heart Disease in Urban Japanese. *Am. J. Epidemiol.,* 152: 5 420-423.
79. Sekalska, B.; Ciechanowicz, A.; Dolegowska, B. and Naruszewicz, M. (2007): Effect of ibuprofen on the development of fat-induced atherosclerosis in New Zealand rabbits. *J. Exper. Anim. Sci.*, 43 (4): 283-299.
80. Singh, I.; Mok, M.; Christensen, A.; Turner, A. H. and John, A. Hawley, (2006): The effects of polyphenols in olive leaves on platelet function. *Nutr. Metabol. Cardiovascul. Dis.,* 18: 127-132.
81. SPSS 10 (1999): "SPSS for windows release 10.0.1 27 Oct 1999" Standard version, Copyright SPSS Inc.
82. Tahvonen, R. L.; Schwab, U. S.; Linderborg, K. M.; Mykk7nen, H. M. Kallio, H. P. (2005): Black currant seed oil and fish oil supplements differ in their effects on fatty acid profiles of plasma lipids, and concentrations of serum total and lipoprotein lipids, plasma glucose and insulin. *J. Nutr. Biochem.*, 16: 353–359.
83. Takenaka, Y., Miki, M., Yasuda, H., and Mino, M. (1991). The effect of a-tocopherol as an antioxidant on the oxidation of membrane protein thiols induced by free radicals generated in different sites. *Arch. Biochem. Biophys.,* 285, 344–350.
84. Tanner, F. C. (1991): Endothelium, lipoproteins and atherosclerotic vascular disease. Vascul. Med., 2 (2): 161-176.
85. Wahlefeld, A. W. (1974): Determination of triglycerides. *In methods of enzymatic analysis*, 5: 1831-1835.
86. Vallance, P. and Chan, N. (2001): Endothelial function and nitric oxide: clinical relevance. *Heart*, 85: 342–350.
87. Willoughby, S.; Holmes, A. and Loscalzo, J. (2002): Platelets and cardiovascular disease. *Euro. J. Cardiovascul. Nurs.*, 1: 273–288
88. Zakynthinos, E. and Pappa, N. (2009): Inflammatory biomarkers in coronary artery disease. *J. Cardiol.*, 53: 317—333.
89. Zhang, G. and Zhang, W. (2009): Heart rate, lifespan, and mortality risk. *Ageing Res. Rev*. 8: 52–60.
90. Zhang, L.; Peppel, K.; Sivashanmugam, P.; Orman, E.; Brian, L.d; Exum, S. T. and Freedman, N. J.(2007): Expression of Tumor Necrosis Factor Receptor-1 in Arterial Wall Cells Promotes Atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.,* 27(5): 1087–1094.
91. Zollner, N. and Kirsch, K. (1962): Über die quantitative Bestimmung von Lipoiden (Mikromethode) mittels der vielen natürlichen Lipoiden (allen bekannten Plasmalipoiden) gemeinsamen Sulfo-phosphovanillin Reaktion. *Exp. Med*., 135: 545.
92. Zoppi, F. and Fellini, D. (1976): Estimation of total cholesterol. *Clin. Chem.*, 22: 960.

10/6/2010

Response of Wheat to Magnesium and Copper Foliar Feeding under Sandy Soil Condition

EL-Metwally, A.E.[1]; F.E. Abdalla[2]; A.M. El-Saady[2]; S.A. Safina[1] and Sara S. EI-Sawy[2]

[1.] Agronomy Dept., Fac. of Agric., Cairo University, Cairo Egypt
[2]. Fertilization Technology Dept., National Research Centre (NRC), Dokki – Cairo Egypt

Abstract: Two field experiments were conducted during the winter seasons of 2007/2008 and 2008/2009 at Ismailia Experimental Station, Agriculture Research Center, Ismailia Governorate, to study the influence of foliar feeding with magnesium (Mg), copper (Cu) either as single nutrient or in combination on growth of wheat (*Triticum aestivum* L.) cv. Sakha 94. Nine treatments were applied: two levels of Mg, two levels of Cu and four combined treatment (Mg + Cu), in addition to control treatment. Results showed that the highest positive significant effect on flag leaf area, chlorophyll contents and dry matter/m^2 were achieved by spraying the highest Mg level + the highest Cu level (6.72 kg Mg + 1.68 kg Cu/fed.) Results also, showed that most of both macro and micronutrients content increased markedly due to the same previous treatment.

[EL-Metwally, A.E.; F.E. Abdalla; A.M. El-Saady; S.A. Safina and Sara S. EI-Sawy. **Response of Wheat to Magnesium and Copper Foliar Feeding under Sandy Soil Condition**. Journal of American Science 2010;6(12):818-823]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Wheat, *Triticum aestivum* L., Magnesium, Copper and Sandy soil.

1. Introduction:

Wheat (*Triticum aestivum L.*) is the most important cereal crop in the world. Wheat ranks as the first among all cultivated cereals in Egypt. Its cultivated area reached to about 1.2 million hectare in year 2008, produced about 7.9 million tons (FAO, 2008). However, this production did not meet consumption party due to crop leakage for other misused and as well as the over growing population and hence, consumption. It is clear that there is a great shortage in the production of wheat in Egypt. This reflects the size of the problem that shows the need of increasing both of vertical and horizontal expansion in old alluvial soils and new desert area. Sandy soils predominate in most newly cultivated area. These soils suffer from very low soil fertility level and as well very low water holding and nutrients retention capacity.

Nutrients play a very important role in chemical, biochemical, physiological, metabolic, geochemical, biogeochemical, and enzymatic processes. Magnesium has major physiological and molecular roles in plants, such as being a component of the chlorophyll molecule, a cofactor for many enzymatic processes associated with phosphorylation, dephosphorylation, and the hydrolysis of various compounds, and as a structural stabilizer for various nucleotides. Studies indicate that 15 to 30% of the total magnesium in plants is associated with the chlorophyll molecule (Marschner 1995).

Copper is an essential micronutrient that requires for the functioning of more than 30 enzymes, all of which are either redox catalysts (e.g., cytochrome oxidase and nitrate reductase) or dioxygen carriers (e.g., haemocyanin). El-Magid *et al*. (2000) recorded that, spraying wheat plant with Fe, Zn, Mn or B increased shoot height, while Cu had little effect on this parameter. Spraying such nutrients increased the number of tillers per plant and shoot weight. It was found also that spraying Cu and B reduced N content, while spraying Zn, Mn, B, Fe and Cu increased wheat plant P and K contents. Ziaeian and Malakouti (2001) reported that copper as well as the other micronutrient application led to significantly increase in their concentration and uptake in grain and flag leaf. Such nutrient application increased significantly grain protein content. Shaaban (2002) showed that foliar fertilizer feeding containing 5.2 % Mn, 0.65 % Zn and 0.65 % Cu increased the concentration of Mg, Ca, Fe, Mn, Zn and Cu in the leaves of wheat.

El-Maghraby (2004) found that the soaking of wheat grains in $FeSO_4$, $MnSO_4$, $ZnSO_4$ and $CuSO_4$ had highly significant effects on the uptake of N, K, Fe, Mn, Zn and Cu in straw. The treatments had highly significant effects on the uptake of N, P, K, Fe, Mn and Zn by grains**.** Korzeniowska (2008) found that wheat plants after Cu application showed higher N concentration than control plants. Moreover, high correlation between Cu and N concentration in wheat shoots were obtained.

The purpose of this work is to study the response of wheat cultivar Sakha 94 to magnesium and copper levels either alone or in combination to induce the highest values of growth parameters as well as nutritional status of plants under sandy soil condition.

2. Materials and methods

Two field experiments were carried out at Ismailia Experimental Station, Agric. Res. Center, Ismailia Governorate, during 2007/2008 and 2008/2009 winter seasons to study the influence of magnesium, copper and either alone or in combinations on growth and nutritional of wheat cv. Sakha 94. The experimental design was randomize complete block with six replicates nine treatments.

Treatments:

The experiment contained nine foliar spray treatments as follows:

1- Control (water spray) 2- 3.36 kg Mg/feddan
3- 6.72 kg Mg/feddan 4- 0.84 kg Cu/feddan
4- 68 kg Cu/feddan
5- .36 kg Mg + 0.84 kg Cu/feddan
6- 3.36 kg Mg + 1.68 kg Cu/feddan
7- 6.72 kg Mg + 0.84 kg Cu/feddan
8- .72 kg Mg + 1.68 kg Cu/feddan

Wheat plants were sprayed with the aforementioned treatment two times, the first was 45 and the second was 60 days after planting. The sprayed solution volume was 350 and 400 L/fed. in the first and second spray, respectively. Soil was ploughed using a chisel plough and divided into experimental units, 2.0 m long and 3.0 m wide. Every plot contained 15 rows each of 20 cm width. Wheat grains were sown on November 22 th and 13th in 2007/2008 and 2008/2009 seasons; respectively at the rate of 60 kg/feddan by hand drilling in rows.

Soil Analysis:

Representative soil samples were taken after soil preparation and before fertilization from the experimental sites (0-30 cm depth) for physico-chemical characteristics (Table 1).

The samples were air-dried and passed through 2mm sieve pores. Soil fractions were determined using the hydrometer method (Bauyoucos, 1954). E.C. and pH were determined in soil/water extract (1:2.5) according to Jackson (1973). $CaCO_3$ was determined using the calcimeter method according to Black (1965). Organic matter was determined using the potassium dichromate method according to Walkely and Black (1934). Soil phosphorus was extracted using sodium bicarbonate (Olsen *et al*., 1954). Potassium, sodium and magnesium were extracted using ammonium acetate (Jackson, 1973). Iron, manganese, zinc and copper were extracted using DTPA-solution (Lindsay and Norvell, 1978).

Table (1): Soil Physico-chemical characteristics (0 – 30 cm) in 2007/2008 and 2008/2009 seasons.

Characteristics	2007/2008	2008/2009
Physical Properties		
Sand (%)	88.4	90
Silt (%)	4.0	3.2
Clay (%)	7.6	6.8
Texture	Sand	Sand
E.C dS/m	0.20	0.35
pH	8.95	9.15
Chemical Properties		
$CaCO_3$ %	1.20	1.84
Organic Matter %	0.54	0.09
Exchangeable macronutrients (mg/100g soil)		
P	0.36	0.62
K	7.6	4.24
Na	18.0	41.4
Ca	240	308
Mg	9.0	3.64
Determined micronutrients (ppm)		
Fe	3.7	3.83
Mn	2.8	1.13
Zn	0.22	0.15
Cu	0.1	0.25

Nitrogen, phosphorus and potassium were added at rate of 106 kg N/fed, 37 kg P_2O_5/fed., and 24 kg K_2O/fed., respectively. Nitrogen was applied as ammonium sulfate (20.6 % N) in three equal splits (at planting, 30 and 50 days after sowing) in both seasons. Phosphorus was applied as a single super phosphate (15.5 % P_2O_5) during soil preparation. Potassium was applied as Potassium sulphate (50 % K_2O) at 30 days after sowing. The whole experimental plots were also sprayed with mixed iron, manganese and zinc in EDTA form two times (45 and 60 days after planting) at rate of 0.5 g/L. from each nutrient.

Plants were irrigated at 6 days interval using sprinkler system. Weeds were controlled by hoeing.

Plant samples:

Wheat shoots at 75 days after the second spray, were taken to determine the dry weight (g/m^2). The samples were washed with tap water (0.01 N HCl) and bi-distilled water, then oven dried at 70º C for 24 hours and ground. The ground material was dry-ashed in a muffle furnace at 550º C for 6 hours. The residue then suspended in 0.3 N HCl.

Recorded Data:

A sample of 25 x 25 cm2/plot was randomly taken at 75 days after sowing to determine dry weight

per plant, shoot macro and micronutrients concentration. Flag leaf area (cm^2) per plant was also calculated using formula (Length x maximum width x 0.79) according to Voldeng and Simpson (1967).

Nutrients Determination:

Total nitrogen was determined in the dried plant shoots based on Micro-Kjeldahl method (Ma and Zauzage, 1942). Phosphorus was photometrical determined using the molybdate-vanadate (Jackson, 1973). Potassium, Ca, Fe, Mn, Zn and Cu were determined according to Chapman and Pratt (1978). Dr. Lang -M8D Flame-photometer was used for K and Ca, while Atomic Absorption Spectrophotometer (Perkin-Elmer 100 B) was used for Fe, Mn, Zn and Cu.

Statistical analysis:

Collected data were subjected to the proper statistical analysis with the method described by Snedecor and Cochoran (1967). Since the data in both seasons took similar trends and variances were homogeneous according to Bartllets test, the combined analysis of both seasons' data was done. LSD test at 5 % level was used for comparing the numerical averages according to Waller and Duncan (1969).

3. Results and Discussion:

Experimental soil presentation:

Data in Table (1) showed that the experiment soil was sand in texture, very high alkalinily in reaction, had low content of macro and micronutrients. The soil was poor in organic matter, without any salinity problems. Soil evaluation was according to Ankerman and Large, 1974.

Effect of magnesium and copper foliar application on wheat growth:

Data in Table 2 showed that all magnesium and/or copper foliar application significantly affected wheat growth parameters. (i.e. flag leaf area, chlorophyll and total dry weight /m^2 at 70 days after sowing.

The increment ranged between 19-31% in flag leaf area, 7-29% in chlorophyll content and 22-88% in total dry weight, over control treatment. The highest increment recorded for the aforementioned parameters was due to spraying plants with the treatment contains the high level of both magnesium and copper (i.e. 6.72 kg Mg + 1.68 kg Cu/fed).

Positive effect of magnesium and copper foliar application on studied wheat growth parameters can be attributed to the important function of copper in plant metabolism since copper participates in photosynthesis and chloroplast development (Amberger, 1974). Since, magnesium is the central atom in the chlorophyll molecule. This makes it essential for photosynthesis. It also plays other critical roles in plant growth (Marschner, 1995). The same trend was found with Mg application on twelve sorghum genotypes (Tan *et al.*, 1992). El-Magid (2000) reported that application of Cu at 0.1% with other micronutrients increased the shoot wheat plants. Kumar *et al.* (2009) found in a pot experiment that production of wheat dry matter enhanced with increasing Cu levels and reached to the maximum at 1.5 mg kg^{-1}.

Table (2): Effect of magnesium and copper on growth characteristics of wheat plants at 70 days after sowing (combined of 2007/2008 and 2008/2009 seasons).

Treatment	Growth characteristic		
	Flag leaf area (cm^2)	Chlorophyll (mg/g)	Total dry weight(g/m^2) (gm / m^2)
0 Cu + 0 Mg	19.23	2.22	463.4
3.36 kg Mg /fed.	22.85	2.38	567.3
6.72 kg Mg /fed.	24.00	2.52	604.6
0.84 kg Cu /fed.	23.25	2.55	577.3
1.68 kg Cu /fed.	24.23	2.60	632.0
3.36 kg Mg + 0.84 kg Cu /fed.	24.42	2.57	686.4
3.36 kg Mg + 1.68 kg Cu /fed.	24.53	2.70	766.8
6.72 kg Mg + 0.84 kg Cu /fed.	24.07	2.78	810.4
6.72 kg Mg + 1.68 kg Cu /fed.	25.13	2.85	871.8
LSD at 0.05	1.78	0.15	40. 5

Macronutrients Status:

Concentration of macronutrients in wheat shoots as affected by different treatments of magnesium and/or copper are shown in Table 4. It is obvious that nitrogen concentration was significantly increased as a response to application of magnesium and copperas compared with control.

Table (3): Effect of magnesium and copper on macronutrient contents (%) of wheat shoots at 70 days after sowing (combined of 2007/2008 and 2008/2009 seasons).

Treatment	Macronutrient (%)				
	(N)	(P)	(K)	(Mg)	(Ca)
0 Cu + 0 Mg	1.73	0.21	2.70	0.14	0.32
3.36 kg Mg /fed.	2.49	0.24	3.32	0.15	0.35
6.72 kg Mg /fed.	2.70	0.28	3.94	0.17	0.44
0.84 kg Cu /fed.	2.55	0.26	3.67	0.14	0.41
1.68 kg Cu /fed.	2.71	0.26	3.95	0.14	0.45
3.36 kg Mg + 0.84 kg Cu /fed.	2.40	0.29	3.26	0.16	0.50
3.36 kg Mg + 1.68 kg Cu /fed.	2.68	0.29	3.57	0.16	0.50
6.72 kg Mg + 0.84 kg Cu /fed.	2.85	0.29	3.47	0.18	0.51
6.72 kg Mg + 1.68 kg Cu /fed.	3.06	0.30	3.84	0.18	0.51
LSD at 0.05	0.07	0.01	0.35	0.01	0.06

Phosphorus concentration determined in wheat shoots significantly increased with foliar application of 6.72 kg Mg + 1.68 kg Cu/fed.

Concerning potassium content, it was found that either spraying plants with the highest level of magnesium or the highest level of copper or their combination gave the highest content, 3.94, 3.95, and 3.84, respectively. Magnesium has major physiological and molecular roles in plants, such as being a component of the chlorophyll molecule, a cofactor for many enzymatic processes associated with phosphorylation, dephosphorylation, and the hydrolysis of various compounds, and as a structural stabilizer for various nucleotides. Studies indicated that 15 to 30% of the total magnesium in plants is associated with the chlorophyll molecule (Marschner 1995). These results are in agreement with the results of El-Magid (2000), Brohi *et al*. (2000), Shaaban (2002), El-Maghraby (2004) and Korzeniowska (2008).

Micronutrients Status:

Micronutrient concentrations in wheat shoots were differed significantly with different treatments (Table 4).

Table (4): Effect of magnesium and copper on micronutrient contents (%) of wheat shoots at 70 days after sowing (combined of 2007/2008 and 2008/2009 seasons).

Treatment	Micronutrient (ppm)			
	(Fe)	(Zn)	(Mn)	(Cu)
Control (0 Cu+0 Mg)	114.0	31.2	18.0	5.2
3.36 kg Mg /fed.	139.6	38.4	21.2	6.0
6.72 kg Mg /fed.	153.0	40.4	25.3	6.3
0.84 kg Cu /fed.	135.4	38.6	23.5	10.6
1.68 kg Cu /fed.	155.7	40.8	24.8	17.1
3.36 kg Mg + 0.84 kg Cu /fed.	156.9	41.9	25.2	12.1
3.36 kg Mg + 1.68 kg Cu /fed.	163.8	48.2	25.7	19.3
6.72 kg Mg + 0.84 kg Cu /fed.	164.5	48.0	26.3	12.5
6.72 kg Mg + 1.68 kg Cu /fed.	176.5	50.0	27.0	18.9
LSD at 0.05	7.6	6.7	2.4	1.5

It is obvious that the highest Fe, Mn, Zn and Cu concentrations were obtained by spraying wheat plants with 6.72 kg Mg + 1.68 kg Cu/fed. Sicene, the fertilizer contains relatively higher concentrations of nutrients than other treatments which mostly absorbed by the plant shoots and raise their concentrations in the shoot tissues (Kassab et al. 2004). The same trend was found by Brohi et al. (2000) who determined the effect of Mg fertilization on the rice yield grown on artificial siltation soil and N, P, K, Fe, Cu, Zn and Mn contents. The uptake of all nutrients in straw was increased with Mg

treatment. El-Maghraby (2004) found that the soaking of wheat grains in Fe (FeSO4), Mn (MnSO4), Zn (ZnSO4) and Cu (CuSO4) had highly significant effects on the uptake of micronutrients (Fe, Mn, Zn and Cu) by straw.

4. Conclusions

It could be concluded that:

1. Magnesium and copper fertilization is very important for the growth of winter wheat plants under sandy soil condition.
2. Combined treatments (Mg + Cu) under experiment conditions could improve growth and nutritional status of wheat.
3. Spraying wheat plants with 6.72 kg Mg + 1.68 kg Cu/fed. Is highly recommended to achieve maximum growth and nutritional status value.

Acknowledgements:

This work was carried out as a part of the activities of the Egypto-German Project" Micronutrients and other Plant Nutrition Problems in Egypt" conducted by National Research Centre, Cairo (Coordinator: Prof. Dr. M.M. El-Fouly) and the Institute of Plant Nutrition, Technical University of Munich (Prof. Dr. A. Amberger).

Corresponding author

EL-Metwally, A.E

Agronomy Dept., Fac. of Agric., Cairo University, Cairo, Egypt

5. References:

1. Amberger, A. (1974). Micronutrients, dynamics in the soil and function in plant metabolism. IV. Copper. Proc. Egypt Botany Society. Workshop 1, Cairo. pp 113-120.
2. Ankerman D. and Large L. (1974). Soil and Plant Analysis, Technical Bulletin, A&L Agricultural Laboratories Inc. USA, 82 pp.
3. Black, C.A. (1965). Methods of soil analysis. 1st Ed., American Society Agronomy, Madison, WI., USA.
4. Brohi, A.R; M.R. Karaman; M.T. Topbas; A. Aktas and E. Savasli (2000). Effect of potassium and magnesium fertilization on yield and nutrient content of rice crop grown on artificial siltation soil. Turk Journal Agriculture, 24: 429-435.
5. Bouyoucos, H.H. (1954). A recalibration of the hydrometer for making mechanical analysis of soils. Agronomy Journal, 43: 434.
6. Chapman, H.D. and P.F. Pratt (1978). Methods of Analysis for Soils, Plants and Water, University California, Department Agriculture Science USA, 320 P.
7. El-Magid, A.A.A.; R. E. Knany and H. G. A. El-Fotoh (2000). Effect of foliar application of some micronutrients on wheat yield and quality. Annals of Agricultural Science Cairo, 1(Special): 301-313.
8. El-Maghraby, T.A. (2004). Effect of wheat grain soaking in some micronutrient solutions on crop production under rainfall condition. Egyptian Journal of Soil Science, 44 (3): 429-440.
9. FAO, (2008) Food and Agriculture Organization of the United Nation, Economic and Social Department.
10. Jackson, M.I. (1973). Soil Chemical Analysis. Prentice Hall of India, New Delhi.
11. Kassab, O.M.; H.A.E. Zeiny and M.M. Ibrahim (2004). Effect of water deficit and micronutrients foliar application on the productivity of wheat plants. Minufiya Journal Agriculture Research, 29: 925-932.
12. Korzeniowska, J. (2008). Winter wheat response to copper application under different cultivation technologies and nitrogen fertilization. Ecological Chemistry and Engineering, 15(1, 2):81-88.
13. Kumar, R.; N.K. Mehrotra; B.D. Nautiyal; P. Kumar and P.K. Singh (2009). Effect of copper on growth, yield and concentration of Fe, Mn, Zn and Cu in wheat plants (*Triticum aestivum L.*). Journal of Environmental Biology, 30(4): 485-488.
14. Lindsay, W.L. and W.A. Norvell (1978). Development of a DTPA micronutrients soil test. Soil Science Society American Journal, 42: 421-428.
15. Marschner, H. (1995). Mineral Nutrition of Higher Plants. 2nd ed. New York: Academic Press, Harcourt Brace Jovanovich, Published 674 P.
16. Ma, T.S. and C. Zauzage (1942). Micro-kjeldahl determination of nitrogen, a new indicator and improved rapid method. Industry Engineering Chemistry Annals Ed., 14: 280-286.
17. Olsen, S.R.; S.V. Colu; F.S. Watanabe and L. A. Dean (1954). Estimation of available phosphorus in soil by extraction with bicarbonate. U. S. Dept. Circ. 939.
18. Shaaban, M.M. (2002). Effect of trace-nutrient foliar fertilizer on nutrient balance, growth, yield and yield components of two cereal crops. Pakistan Journal of Biological Sciences, 7(4): 770-774.
19. Snedecor, G. W. and Cochran, W. G. (1967). Statistical Methods, 6 th Edition The Iowa State College, Ames Iowa, U. S. A.
20. Tan, K.; W.G. Keltjens and G. R. Findenegg (1992). Acid soil damage in sorghum genotypes:

Role of magnesium deficiency and root impairment. Plant and Soil 139: 149-155.
21. Voldeng, H.D. and G.M. Simpson (1967). Leaf area as an indicator of potential grain yield in wheat. Canadian Journal Plant Science 47: 359-365.
22. Walkely, A. and I.A. Black (1934). An examination of the degtjareff method for determining soil organic matter, and a proposed modification of the chromic acid titration method. Soil Science 37:29-38.
23. Waller, R.A. and D.B. Duncan (1969). A bays rule for the symmetric multiple comparisons problem. American Stat. Associated Journal December. 1485.
24. Ziaeian, A.H. and M.J. Malakouti (2001). Effects of Fe, Mn, Zn and Cu fertilization on the yield and grain quality of wheat in the calcareous soils of Iran. Plant Nutrition - Food security and sustainability of agro-ecosystem, 92: 840-841.

10/2/2010

Effect of yeast (*Saccharomyces cerevisiae*) on reduction of aflatoxicosis, enhancement of growth performance and expression of neural and gonadal genes in Japanese quail

Mariam G. Eshak[1], Wagdy K.B. Khalil[1], Eman M. Hegazy[2], Ibrahim M. Farag[1], M. Fadel[3] and Farid K.R. Stino[4]

[1]Cell Biology Department, National Research Centre, Dokki, Giza, Egypt.
[2]Food Toxicology and Contaminants Department, National Research Centre, Dokki, Giza, Egypt.
[3]Microbial Chemistry Department, National Research Centre, Dokki, Giza, Egypt.
[4]Faculty of Agriculture, Cairo University, Egypt.
mgergis@yahoo.com; wagdykh@yahoo.com

Abstract: The present investigation was designed to evaluate the role of yeast, *Saccharomyces cerevisiae* (SC) in the reduction of aflatoxicosis induced by aflatoxin B_1 (AFB_1) in Japanese quail. Sixty male quail were used and distributed into six groups. The first group received basal diet. The other five groups received the basal diet plus 0.5 mg AFB_1/kg diet. Four of them received increasing levels of SC (0.5, 1.0, 2.0 and 2.5 gm/kg diet, respectively). All groups received their prospective diets for 35 days. The birds were weighed weekly to determine body weight (BW) and body weight gain (BWG). The results showed that addition of the SC to AFB_1-containing diet significantly reduced the adverse affect of AFB_1 on quail BW and BWG. The concentrations of AFB_1 had been lowered in the breast muscle and liver samples of quail fed diet containing AFB_1 plus SC than those found in such quail organs of AFB_1 group. The expression levels of neural and gonadal genes were significantly up-regulated in quail fed diet containing AFB_1 plus high levels of SC compared to those of AFB_1 group. It could be concluded that SC supplementation to quail diets suppressed the aflatoxicosis in quail tissues leading to improvement of growth performances and enhancement of expression levels of neural and gonadal genes. Thus, the use of HPLC and gene expression analysis might contribute in detecting aflatoxin contamination in the poultry industry in Egypt.
[Mariam G. Eshak, Wagdy K.B. Khalil, Eman M. Hegazy, Ibrahim M. Farag, M. Fadel and Farid K.R. Stino. **Effect of yeast (*Saccharomyces cerevisiae*) on reduction of aflatoxicosis, enhancement of growth performance and expression of neural and gonadal genes in Japanese quail**. Journal of American Science 2010;6(12):824-838]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: Quail; body weight; growth rate; yeast; aflatoxin B_1; sqRT-PCR; gene expression.

1. Introduction:

Aflatoxins are a group of closely related, biologically active mycotoxins which are produced by storage fungi during growth on a number of foods and feed materials (Abo *et al.*, 1995 and Oliveria *et al.*, 2002). Aspergilla are the most common fungal species that can produce aflatoxins (AFs) in food and feedstuffs (Oliveria *et al.*, 2002 and Abousadi *et al.*, 2007). Among the different types of AFs produced, AFB_1 is the most prevalent and potent and is often found in high concentrations in cereal grains and peanut meal, which comprises between 50 and 60 percent of many poultry diets (Stanley *et al.*, 1993; Miazzo *et al.*, 2000; Parlat *et al.*, 2001 and Gowda *et al.*, 2004). Aflatoxicosis due to AFB_1 in poultry, causes listlessness, anorexia with lowered growth rate, immunosupression, decreased body weight gain, poor feed utilization, reduced egg production and increased mortality (Oguz and Kurtoglu, 2000; Oliveria *et al.*, 2002 and Abousadi *et al.*, 2007).

Removing AF from contaminated food and feedstuffs remains a major problem and there is a great demand for effective decontamination technology. These procedures have focused on degrading, destroying, inactivating or removing AF by physical (heat, irradiation), chemical (ammoniation, sulphites, hypochlorides, ozone), nutritional (vitamins, minerals) or biological (bacteria, yeast) methods (Stanley *et al.*, 1993; El-Nezami *et al.*, 2000; Raju and Devegowda, 2000; Galvano *et al.*, 2001; Abousadi *et al.*, 2007 and Tovar-Ramirez *et al.*, 2010). A successful detoxification process must be economically capable of eliminating all traces of a toxin without having harmful residues and must not impair the nutritional quality of the commodity (Leeson *et al.*, 1995 and Kubena *et al.*, 1998). One approach of many to this problem is the use of the yeast *Saccharomyces cerevisiae* (SC) and its cell wall component (manner oligosaccharide) for minimizing the adverse effects of AF in poultry on the basis of biological

degradation (Parlat *et al.*, 2001).This approach will not only benefit human health but also result in increased profit and productivity of poultry (Galvano *et al.*, 2001). The inclusion of SC (1gm/kg) to the AFB_1-containing diet provided significant improvements on the adverse effects of AFB_1 (5 mg/kg) in broiler chicks fed for 28 days (Stanely *et al.*, 1993). Also, Raju and Devegowda (2000) extracted mannan oligosccharide, which was believed to be responsible for the beneficial effect against AFB_1 from the wall of SC. They added (1gm/kg) to AFB_1-containnated feed and reported significant amelioration on the adverse effect of AFB_1 (0.3mg/kg) in broiler fed for 35 days. Moreover, Abousadi *et al.* (2007) observed that the addition of SC (0.2%) to AFB_1-containing diet significantly improved the adverse effect of AFB_1 (125 ppb) on growth performances in broiler chicks fed for 21 days. In quail, Parlat *et al.*, (2001) reported that the supplementation of SC (1gm/kg) to the AF-containing diet significantly reduced the deleterious effect of AFB_1 (2.5mg/kg diet) on body weight and body weight gain in birds fed for 35 days (Parlat *et al.*, 2001)

The present study was designed to investigate the role of SC in reducing aflatoxicosis caused by AFB_1 in Japanese quail. Therefore, the measurements of growth performances and the concentrations of AFB_1 in some quail organs were determined. Moreover, the expression levels of some neural(Glycerinaldehyde-3-phosphate ehydrogenase, GAPDH, neural cell adhesion molecule, NCAM, and Cadherin-2, CDH2) and gonadal (Cytochrome P450 cholesterol side chain cleavage, P450scc) genes were studied.

2. Materials and Methods

Birds and diet:

Sixty unvaccinated 14 days old male Japanese quail chicks were obtained from the quail project, Faculty of Agriculture, Cairo University. The quail were divided into six groups. Each group of 10 quail, were weighted and placed in a heated wooden brooder (battery cage). The birds received 24 h of light per day (continues light) for the duration of the experiment. They also received *ad lib*., water and a commercial growing quail ration (basal diet) containing 24% protein and 2,900 Kcal ME/Kg diet. The diet also contained all the required amino acids, vitamins and minerals according to the recommendations of the National Research Council (NRC, 1994), without adding antibiotics, coccidiostats or growth promoters. All animals received humane care in compliance with the guidelines of the Animal Care and Use Committee of Faculty of Agriculture, Cairo University, Egypt.

Aflatoxin B_1 (AFB_1):

AFB_1 used in this experiment was purchased from Sigma (Buchs, Switzerland). Twenty five milligrams of pure crystalline of AFB_1 were dissolved in chloroform. The chloroform solution was placed in a flask, and kept in a water bath at 60°C until complete evaporation of the solvent. Then AFB_1 was dissolved in 500 ml sterile maize oil. This solution was then added to 50 kg basal diet and homogenized to obtain the required final levels of AFB_1 which was 0.5mg of AFB_1/kg diet according to procedure of Soares and Rodrigues-Amaya (1989).

The appropriate experimental doses of AFB_1 that affect quail organs were determined previously by Sinsek *et al.* (2007). These appropriate doses ranged from 0.5 to 6.0 mg of AFB_1/kg diet.

Saccharomyces cerevisiae (SC):

SC was obtained from Microbial Chemistry Department, National Research Centre, Giza, Egypt. The SC culture was supplied at four rates, 0.5 gm, 1.0 gm, 2.0 gm and 2.5 gm/kg of feed. The live yeast was added to the appropriate diets mixed in cooking grade soybean oil. The soybean oil-SC suspension was added to the feed in the final mix as the last step in mixing, such as would occur commercially according to the procedure of Parlat *et al.* (2001). The AFB_1 was incorporated into the mixed feed before SC was added.

Experimental design:

Six groups of quail were supplemented with six dietary treatments as follows; (1) control, basal diet; (2) basal diet plus 0.5 mg of AFB_1/kg diet; (3) basal diet plus 0.5 mg of AFB_1 plus 0.5 gm of SC/kg diet; (4) basal diet plus 0.5 mg of AFB_1 plus 1.0 gm of SC /kg diet (5) basal diet plus 0.5 mg of AFB_1 and 2.0 gm of SC/kg diet (6) basal diet plus 0.5 mg of AFB_1 plus 2.5 gm of SC /kg diet.

Performance parameters:

a) Growth performance

The trial period was carried out for 5 weeks. During the experiment, the birds were weighed weekly to determine their body weight (BW) and body weight gain (BWG) at 14, 21, 28, 35, 42 and 49 days of age after fasting for 8 hours, to the nearest gram using a digital scale. The cumulative body weight gains were calculated by subtracting W2-W1.

After the end of the experiment, all birds were euthanized. Liver, brain, testis and breast muscle were collected, in order to (i) determine the aflatoxin concentrations in breast muscle and liver

samples by using HPLC analysis and (ii) to evaluate the expression levels of some neural and gonadal genes by using sqRT-PCR method.

b) Analysis of toxin residues in quail organs:

Samples of liver and breast muscle were collected, squashed, homogenized and extracted by using acetonitrile-water solution (85/15) (v/v). The sample extraction was filtered and diluted (5ml) with 95 ml of phosphate buffer saline (PBS). The filtrated solution was applied onto the immuno-affinity column (Alfa BG. 1003, VICAM). The column was rinsed twice with 10 ml of deionized water and the toxin was eluted from the column with 1.0 ml of acetonitrile-methanol mixture (3+2). The column was subsequently washed with 1ml of deionized water and the washing was combined with the acetonitrile-methanol elute. AFB_1 in the acetonitrile-methanol-water mixture was determined by an HPLC method using a Lich rospher 100 PR-18 ECO Pack column (5µm, 25 x 4.6 mm i.d., Merek, Portugal), with post-column derivatization involving bromination with pyridinum hydrobroide perbromide (PBPB, Sigma P-3179, Quimica S.A., Spain) and fluorescence detection (Merck Hitachi, excitation and emission wavelengths were 360 nm and 420 nm, respectively). The mobile phase was water-acetonitrile-methanol solution (6/2/3) (v/v/v) and flow rates were 1.00 ml/min for mobile phase and 0.30 ml/min for the PBPB reagent (Martins *et al.*, 2008).

c) Gene expression Analysis:

1. Extraction of total RNA

Liver, brain and testis tissues of quail of all groups were used individually to extract total RNA using TRIzol® Reagent (cat#15596-026, Invitrogen, Germany). Total RNA of each tissue was treated individually with 1 U of RQ1 RNAse-free DNAse (Invitrogen, Germany) to digest DNA residues, re-suspended in DEPC-treated water and photospectrometrically quantified at A_{260}. Purity of total RNA was assessed by the 260/280 nm ratio (between 1.8 and 2.1). Additionally, integrity was assured with ethidium bromide-stain analysis of 28S and 18S bands by formaldehyde-containing agarose gel electrophoresis. Aliquots were used immediately for reverse transcription (RT), otherwise stored at -80°C.

2. Synthesis of the cDNA using reverse transcription (RT) reaction

The complete Poly(A)$^+$ RNA isolated from quail tissues was reverse transcribed into cDNA in a total volume of 20 µl using RevertAid^{TM} First Strand cDNA Synthesis Kit (MBI Fermentas, Germany). An amount of total RNA (5µg) was used with a reaction mixture, termed as master mix (MM). The MM was consisted of 50 mM $MgCl_2$, 5x reverse transcription (RT) buffer (50 mM KCl; 10 mM Tris-HCl; pH 8.3), 10 mM of each dNTP, 50 µM oligo-dT primer, 20 U ribonuclease inhibitor (50 kDa recombinant enzyme to inhibit RNase activity) and 50 U M-MuLV reverse transcriptase. The mixture of each sample was centrifuged for 30 sec at 1000 g and transferred to the thermocycler (Biometra GmbH, Göttingen, Germany). The RT reaction was carried out at 25°C for 10 min, followed by 1 h at 42°C, and finished with a denaturation step at 99°C for 5 min (Khalil *et al.*, 2008a,b). Afterwards the reaction tubes containing RT preparations were flash-cooled in an ice chamber until being used for DNA amplification through sqRT-PCR.

3. Semi Quantitative Real Time-Polymerase Chain Reaction (sqRT-PCR)

An iQ5-BIO-RAD Cycler (Cepheid. USA) was used to determine the quail cDNA copy number. PCR reactions were set up in 25 µL reaction mixtures containing 12.5 µL 1× SYBR® Premix Ex TaqTM (TaKaRa, Biotech. Co. Ltd.), 0.5 µL 0.2 µM sense primer, 0.5 µL 0.2 µM antisense primer, 6.5 µL distilled water, and 5 µL of cDNA template. The reaction program was allocated to 3 steps. First step was at 95.0°C for 3 min. Second step consisted of 40 cycles in which each cycle divided to 3 steps: (a) at 95.0°C for 15 sec; (b) at 55.0°C for 30 sec; and (c) at 72.0°C for 30 sec. The third step consisted of 71 cycles which started at 60.0°C and then increased about 0.5°C every 10 sec up to 95.0°C. At the end of each sqRT-PCR a melting curve analysis was performed at 95.0°C to check the quality of the used primers. Each experiment included a distilled water control.

The quantitative values of RT-PCR of Glyceraldehyde Phosphate dehydrogenase (GAPDH-F: 5′-GGT GAA AGT CGG AGT CCA-3′, GAPDH-R: 5′-TTC TGT GTG GCT GTG ATG -3′, (SQUITTI *et al.*, 1999); N-cadherin (N-cadherin-F: 5′-GAT GTC AAT GAC AAT CCT CC-3′, N-cadherin-R: 5′-CAT CCT AGT TGC GTC TTC AAA G -3′, (Squitti *et al.*, 1999); Neural cell adhesion molecule (NCAM-F: 5′-GCC TGA AAC CTG AGA CAA C-3′, NCAM -R: 5′- CTT ACG AAC TGG CTG TGT TC -3′, (Squitti *et al.*, 1999); and Cytochrome P450 cholesterol side chain cleavage (P450scc-F: 5′- ACA GCA GTT CAT CGA CGC CG -3′, P450scc-R: 5′-AAG GAG GCT GAA GAG GAT G -3′, (KANDA *et al.*, 2000) genes were normalized on the bases of β-actin (β-actin-F: 5′- TGT GAT GGT GGG AAT GGG TCA G -3′, B-actin-R: 5′- TTT GAT GTC

ACG CAC GAT TTC C -3′, (Hwang *et al.*, 2009) expression.

The selected genes are responsible for different functions during cell differentiations. Where, GAPDH is a gene coding an enzyme that catalyzes the sixth step of glycolysis and thus serves to break down glucose for energy and carbon molecules. NCAD is a gene coding a protein that has been implicated as having a role in cluster of differentiations. Also, NCAM is a gene coding a hemophilic binding glycoprotein expressed on the surface of neurons, ganglia, skeletal muscle and natural killer cells. P450scc is a gene coding a mitochondrial enzyme associated with the conversion of cholesterol to pregnenolone.

At the end of each sqRT-PCR a melting curve analysis was performed at 95.0°C to check the quality of the used primers.

4. Calculation of Gene Expression

First the amplification efficiency (Ef) was calculated from the slope of the standard curve using the following formulae (BIO-RAD, 2006):

$Ef = 10^{-1/slope}$

Efficiency (%) = (Ef – 1) x 100

The relative quantification of the target to the reference was determined by using the ΔC_T method if E for the target (GH, IGF-1) and the reference primers (β-Actin) are the same (Bio-Rad, 2006)

$Ratio_{(reference/\ target\ gene)} = Ef^{C_T(reference)} - {}^{C_T(target)}$

Statistical analysis:

Body weight (BW), body weight gain (BWG) and gene expression data were analyzed as a one-way analysis of variance using the General Linear Model, SAS software (SAS INSTITUTE, 2004). Weight data were reported as least square means (LSM) ± standard errors (SEM). Gene expression data are expressed as means ± SEM. Mean values were separated, when significance is present, using Duncan′s Multiple Range Test (Duncan, 1955). Significance level was set at 5%.

3. Results

Effect of AFB_1 and yeast on growth performances:

Data presented in Table (1) showed the effect of dietary treatments on body weight (BW). Feeding AFB_1 alone suppressed the BW from the 3rd week (BW3) to week 7th week (BW7) compared to control. This deleterious effect of AFB_1 on BW was significant ($P \leq 0.05$) in the 6th week and the 7th week. The addition of (SC) at levels of 0.5-2.5 gm/kg to AFB_1-containing diets had ameliorated the adverse effect on BW. However, it was only significant during the 6th and 7th week of age.

Feeding AFB_1 alone also suppressed the BWG (Table 2) from the first week onwards and increased progressively until the end of the experimental period compared to controls. This suppression in weight gain was significant ($P \leq 0.05$) from the 6th to the 7th week of age and highly significant ($P \leq 0.01$) from 2nd to the 7th week of age (overall). The addition of SC to AFB_1 -containing diets improved the adverse effect of AFB_1 on BWG. This improvement was significant ($P \leq 0.05$) in group fed diet containing AFB_1 plus SC at 2.5 gm/kg diet level for the overall period.

Toxin analysis in some quail organs by using HPLC method:

As shown in Table (3), the concentrations of AFB_1 were higher in breast muscle (Figure 1[a-f]) and liver (Figure 2[a-f]) samples of quail fed diet containing AFB_1 than those found in samples of quail fed basal diet alone (control). The liver samples had the highest concentration of AFB_1. On the other hand, the amount of AFB_1 were reduced in breast muscle and liver samples that were collected from quail fed diets containing AFB_1 plus SC compared to those observed in samples of quail fed diets containing AFB_1 alone. The concentrations of AFB_1 in breast muscle and liver samples of quail fed diets containing AFB_1 plus high levels of SC (2.5 gm/kg) had the lowest amount of AFB_1 followed by the group that was fed diet containing AFB_1 plus 2.0 gm SC/kg diet.

Gene expression analysis:

Determination of the linear range of PCR amplification

The optimum values for the oligonucleotide primer concentrations were performed by using quail first strand cDNA as a template. The relationship between the CT value and the logarithm of the dilution factor of cDNA was evaluated under conditions that optimized the amplification of the target genes (GAPDH, N-cadherin, NCAM and P450scc). This evaluation was shown to be linear with a correlation coefficient > 0.99.

Gene expression of GAPDH, N-cadherin, NCAM and P450scc genes

The present results (Figures 3-5) revealed a significant ($p \leq 0.01$) decrease of gene expression levels of GAPDH, N-cadherin and NCAM genes in quail fed diet containing AFB_1 compared with those of the control group.

In contrary, the expression levels of GAPDH, N- cadherin and NCAM genes in quail fed diet containing AFB_1 plus low levels of SC (0.5 gm or 1.0 gm SC/kg diet) were higher than those found in quail fed diet containing AFB_1 alone. However,

these differences were not statistically significant. On the other hand, the expression levels of GAPDH, N-cadherin and NCAM genes in the brain and liver samples collected from quail fed diet containing AFB_1 plus high levels of SC (2.0 gm or 2.5 gm SC/kg diet) were significantly ($p \leq 0.01$) higher than those observed in quail fed diet containing AFB_1 alone.

Table (1): Effect of different levels of *Sccharomyces cerevisiae* on body weight (BW) of Japanese quail fed diets containing aflatoxin B_1 from 14 to 49 days of age.

Treatments	BW2 LSM±SEM	BW3 LSM±SEM	BW4 LSM±SEM	BW5 LSM±SEM	BW6 LSM±SEM	BW7 LSM±SEM
BD (C)	49±2.3	102±3.7	161±4.5	207±5.5	241±7.2 ^{a}	268±10.3^{a}
BD + T	50±2.8	96±5.5	149±7.1	191±6.7	215±6.6 ^{b}	224±5.3 ^{c}
BD + T + SC_1	51±2.9	88±5.3	147±5.1	196±5.8	221±6.8 ^{ab}	228±8.1 ^{bc}
BD + T + SC_2	48±2.7	100±5.2	154±8.5	193±7.8	222±9.8 ^{ab}	232±9.4 ^{bc}
BD + T + SC_3	50±1.4	96±4.7	155±6.5	202±8.9	225±10.4 ^{ab}	232±10.6 ^{bc}
BD + T + SC_4	48±2.4	97±4.4	157±4.1	208±4.9	238±6.4 ^{ab}	253±7.2 ^{ab}

a-c means, within age group, followed by different superscripts, differ significantly ($P \leq 0.05$); BD (C) = Basal diet (control); T = AFB_1 (0.5mg/kg diet); SC_1 = 0.5 gm SC/kg diet; SC_2 = 1.0 gm SC /kg diet; SC_3 = 2.0 gm SC/kg diet; SC_4 = 2.5 gm SC /kg diet.

Table (2): Effect of different levels of *Sccharomyces cerevisiae* on body weight gain (BWG) of Japanese quail fed diet containing aflatoxin B_1 from 14 to 49 days of age.

Treatments	BWG2 LSM±SEM 2wks-3wks	BWG3 LSM±SEM 3wks-4wks	BWG4 LSM±SEM 4wks-5wks	BWG5 LSM±SEM 5wks-6wks	BWG6 LSM±SEM 6wks-7wks	BWG7 LSM±SEM 2wks-7wks
BD (C)	53±2.7^{a}	59±1.5	45±2.5^{ab}	34±5.3	26±6.0 ^{a}	218±10.3^{a}
BD + T	46±3.7^{ab}	53±3.0	43±3.0^{ab}	23±2.6	9±3.0 ^{b}	174±5.4 ^{c}
BD + T + SC_1	37±4.3^{b}	59±2.3	50±3.0^{a}	24±3.0	7±3.2 ^{b}	177±8.2 ^{c}
BD + T + SC_2	52±5.0^{a}	54±6.0	40±9.6^{b}	29±4.0	10±3.4 ^{b}	189±7.6 ^{bc}
BD + T + SC_3	48±4.0^{ab}	60±3.1	47±3.1^{ab}	24±3.7	6±2.6 ^{b}	183±9.7 ^{bc}
BD + T + SC_4	48±2.5^{ab}	59±3.6	51±2.7^{a}	31±2.5	15±2.7 ^{b}	203±6.8 ^{ab}

a-c means, within age group, followed by different superscripts, differ significantly ($P \leq 0.05$); BD (C) = Basal diet (control); T = AFB_1 (0.5mg/kg diet); SC_1 = 0.5 gm SC/kg diet; SC_2 = 1.0 gm SC /kg diet; SC_3 = 2.0 gm SC/kg diet; SC_4 = 2.5 gm SC /kg diet.

Table (3): Concentrations of AFB_1 in breast muscle and liver samples of quail fed different diets.

Quail Diets or treatments	Aflatoxin B_1 ppb µg/kg	
	Breast muscle	Liver
BD (C)	0.43	0.07
BD + T	4.37	8.64
BD + T + SC_1	4.01	5.93
BD + T + SC_2	2.19	2.74
BD + T + SC_3	1.37	1.95
BD + T + SC_4	0.86	0.94

BD (C) = Basal diet (control); T = AFB_1 (0.5mg/kg diet); SC_1 = 0.5 gm SC/kg diet; SC_2 = 1.0 gm SC /kg diet; SC_3 = 2.0 gm SC/kg diet; SC_4 = 2.5 gm SC /kg diet.

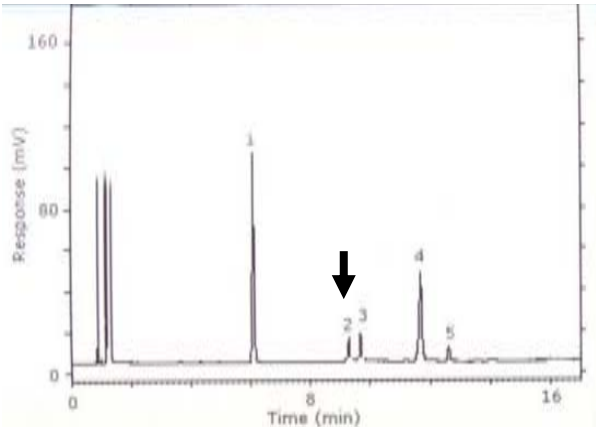


Fig. (a). Concentration of AFB_1 (0.43/ppb) in control group

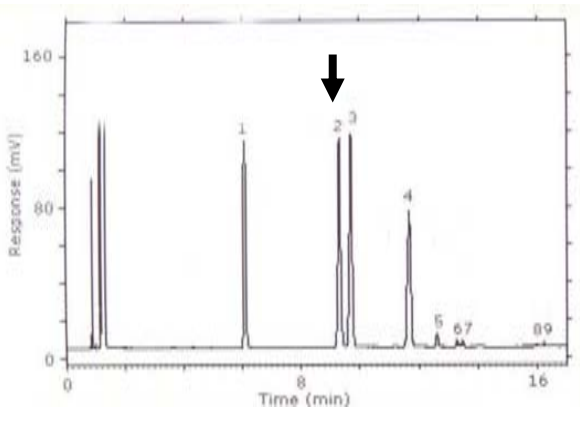


Fig. (b). Concentration of AFB_1 (4.37 ppb) in quail fed diet containing AFB_1 (0.5 mg/kg)

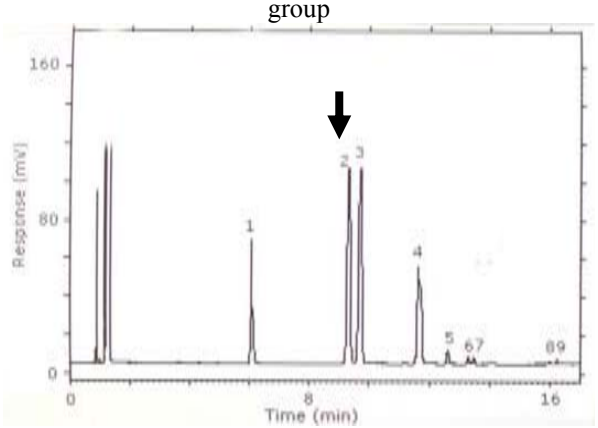


Fig. (c). Concentration of AFB_1 (4.01 ppb) in quail fed diet containing AFB_1 plus 0.5 gm SC/kg.

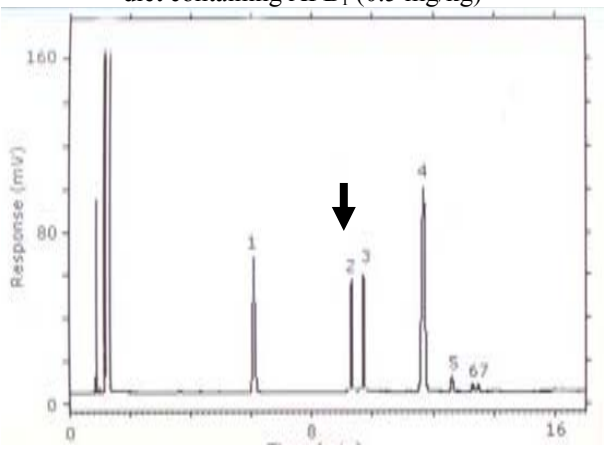


Fig. (d). Concentration of AFB_1 (2.19 ppb) in quail fed diet containing AFB_1 plus 1.0 gm SC/kg.

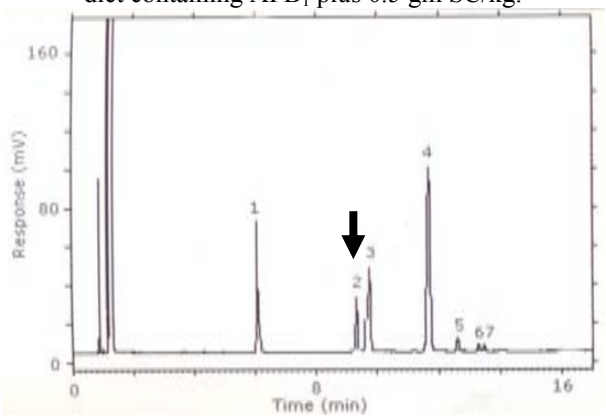


Fig. (e). Concentration of AFB_1 (1.37 ppb) in quail fed diet containing AFB_1 plus 2.0 gm SC/kg.

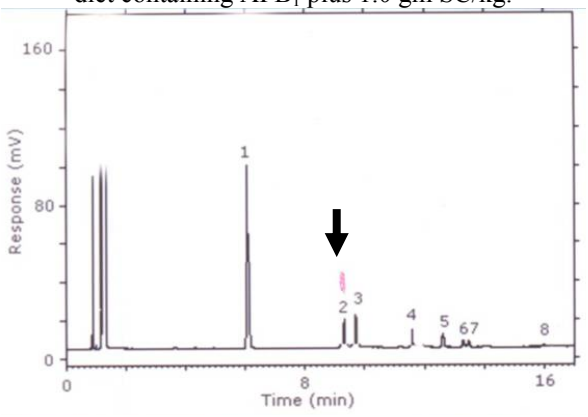


Fig. (f). Concentration of AFB_1 (0.86 ppb) in quail fed diet containing AFB_1 plus 2.5 gm SC/kg.

Fig. 1: Concentrations of AFB_1 as confirmed by HPLC method in quail breast muscle.

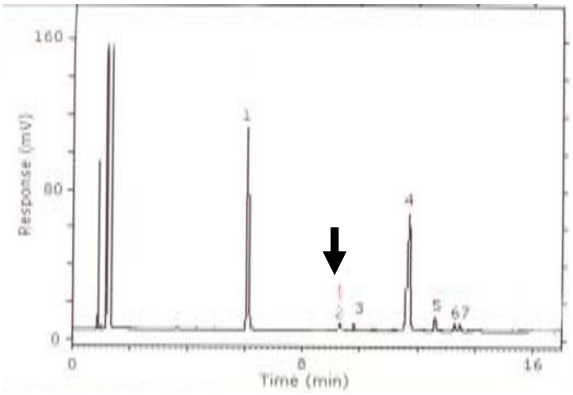


Fig. (a). Concentration of AFB_1 (0.071 ppb) in quail control group

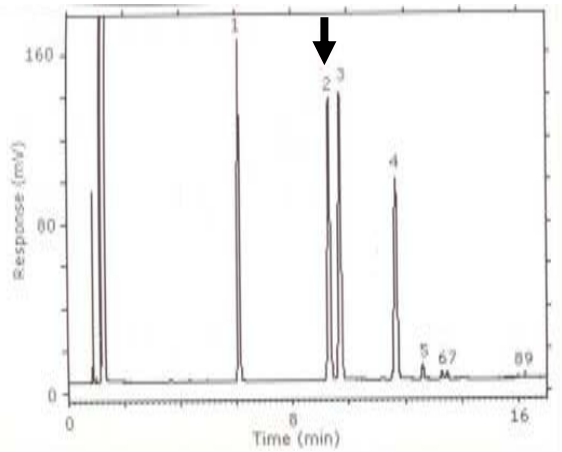


Fig. (b). Concentration of AFB_1 (8.639 ppb) in quail fed diet containing AFB_1 (0.5 mg/kg)

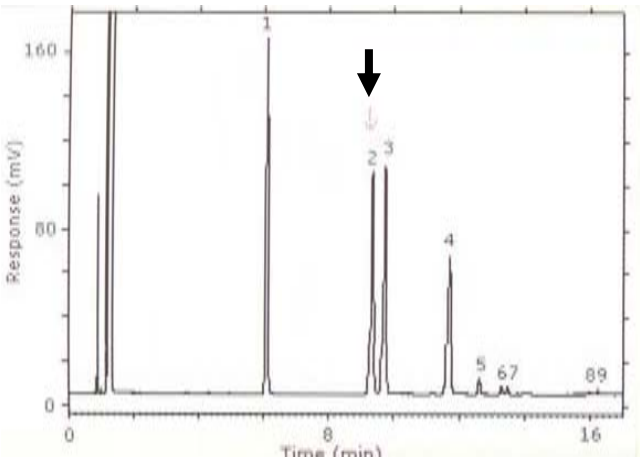


Fig. (c). Concentration of AFB_1 (5.93ppb) in quail fed diet containing AFB_1 plus 0.5 gm SC/kg.

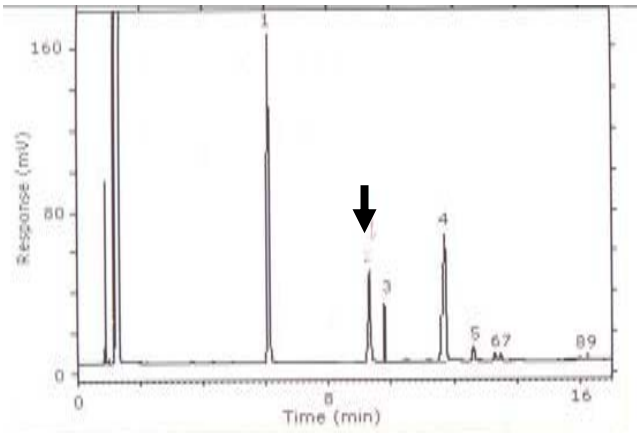


Fig. (d). Concentration of AFB_1 (2.74 ppb) in quail fed diet containing AFB_1 plus 1.0 gm SC/kg.

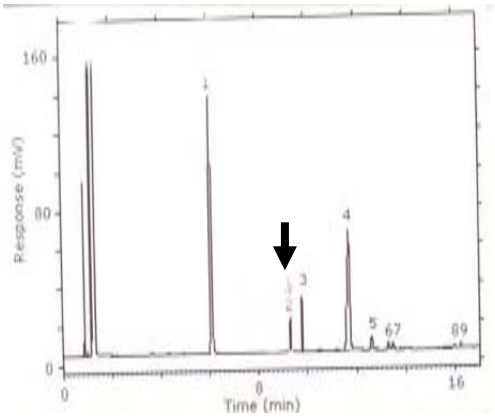


Fig. (e). Concentration of AFB_1 (1.95 ppb) in quail fed diet containing AFB_1 plus 2.0 gm SC/kg.

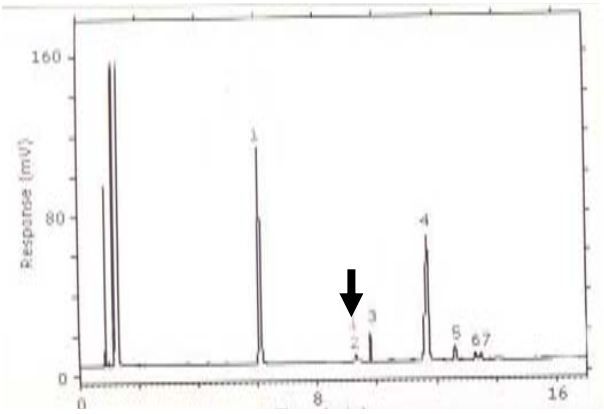


Fig. (f). Concentration of AFB_1 (0.935 ppb) in quail fed diet containing AFB_1 plus 2.5 gm SC/kg.

Fig. 2: Concentrations of AFB_1 as confirmed by HPLC method in quail liver.

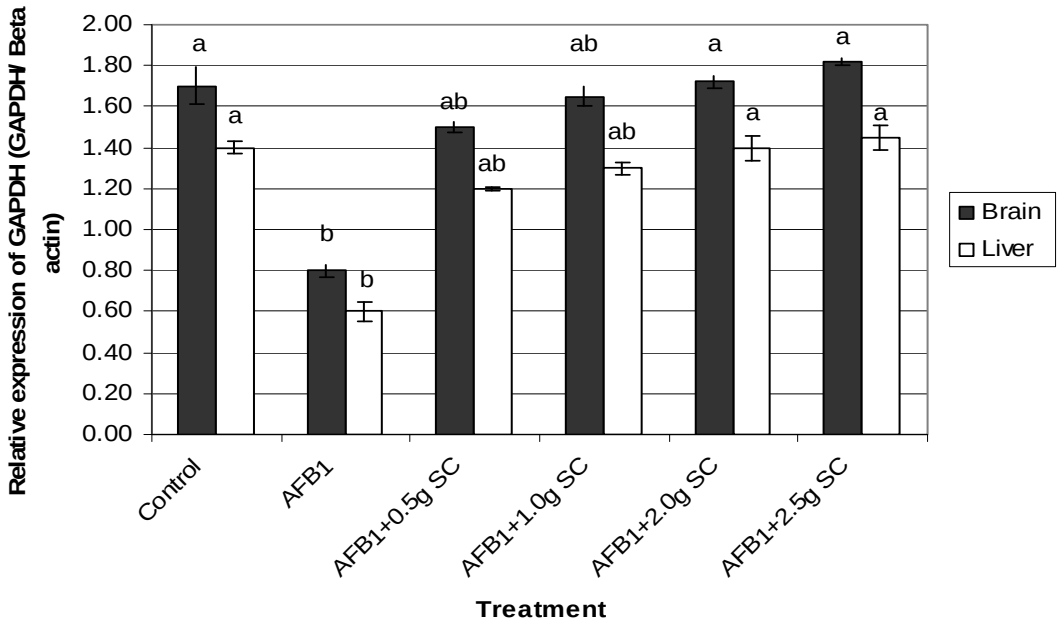


Fig. 3: Semi-quantitative Real Time-PCR analysis of GAPDH-mRNAs in brain and liver tissues collected from male quail (n=10) fed standard diet containing AFB_1 alone or AFB_1 combined with different concentrations of SC. Means with different letters, within tissue, differ significantly ($p \leq 0.05$).

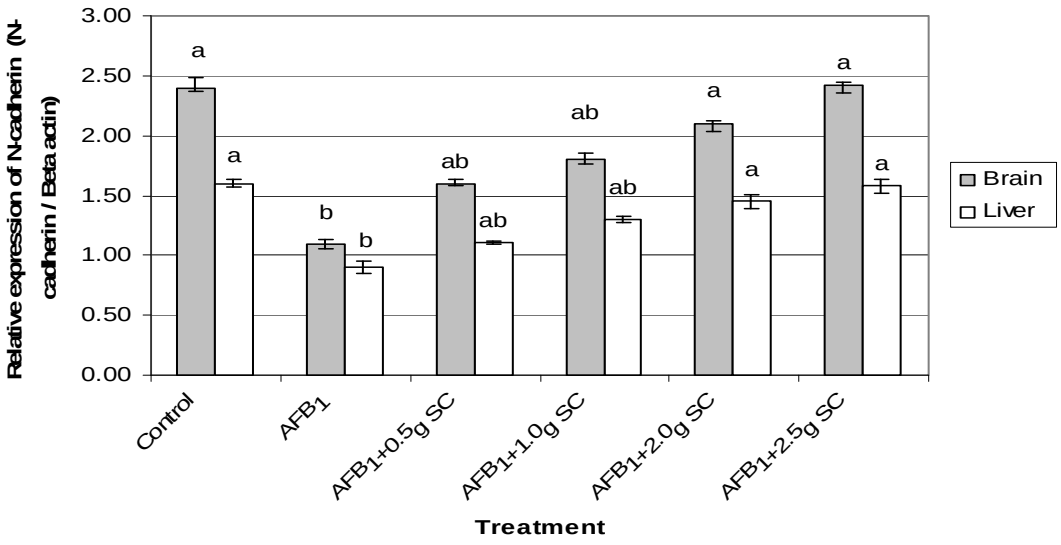


Fig. 4: Semi-quantitative Real Time-PCR analysis of N-cadherin-mRNAs in brain and liver tissues collected from male quail (n=10) fed standard diet containing AFB_1 alone or AFB_1 combined with different concentrations of SC. Means with different letters, within tissue, differ significantly ($p \leq 0.05$).

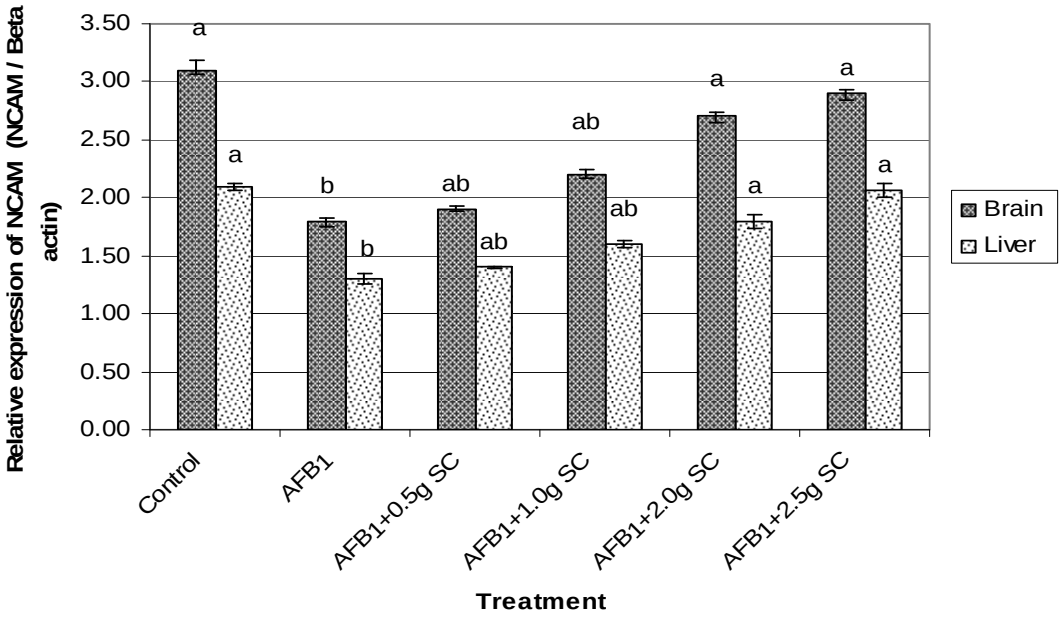


Fig. 5: Semi-quantitative Real Time-PCR analysis of NCAM-mRNAs in brain and liver tissues collected from male quail (n=10) fed standard diet containing AFB_1 alone or AFB_1 combined with different concentrations of SC. Means with different letters, within tissue, differ significantly ($p \leq 0.05$).

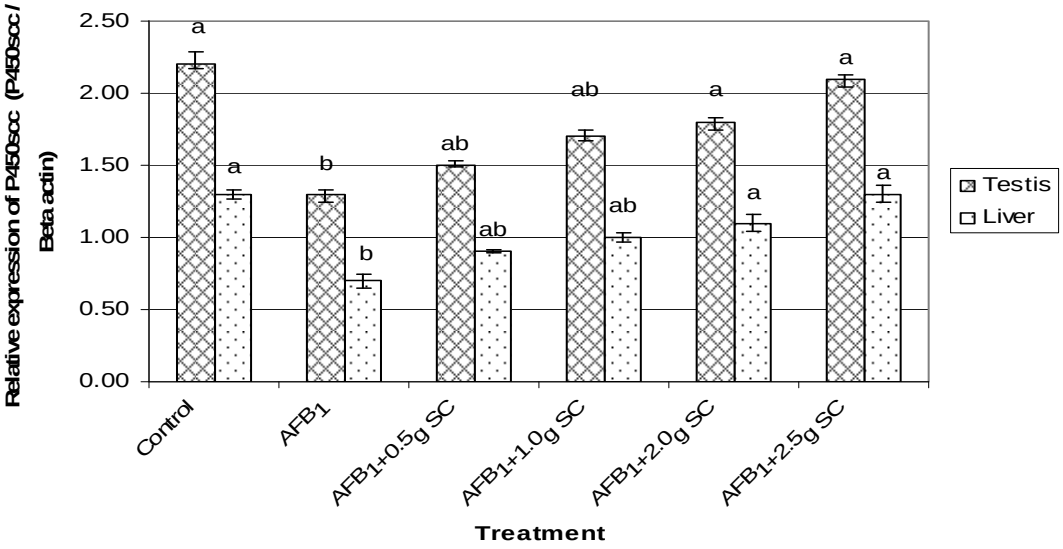


Fig. 6: Semi-quantitative Real Time-PCR analysis of P450scc-mRNAs in testis and liver tissues collected from male quail (n=10) fed standard diet containing AFB_1 alone or AFB_1 combined with different concentrations of SC. Means with different letters, within tissue, differ significantly ($p \leq 0.05$).

4. Discussion:

Regarding to P450scc gene, the present results (Figure 6) revealed that the expression level of this gene in testis and liver samples of quail fed diet containing AFB_1 was significantly ($p \leq 0.05$) low compared to those of the control group.

The expression level of P450scc gene in quail fed diet containing AFB_1 plus low level of SC (0.5 gm or 1.0 gm SC/kg diet) were higher than those detected in quail fed diet containing AFB_1 alone. However, these increases were not statistically significant. On the other hand, the gene expression level of P450scc in quail fed diet containing AFB_1 plus high levels of SC (2.0 gm or 2.5 gm SC/kg diet) were significantly higher ($p \leq 0.05$) compared with those found in quail fed diet containing AFB_1 alone.

In the present study, the quail consumed AFB_1 (0.5 mg/kg) containing diet showed poor body weight (BW) and body weight gain (BWG). These deleterious effects of AFB_1 on BW were statistically significant at 6 weeks ($P \leq 0.05$) and 7 weeks ($P \leq 0.01$) of age compared to control. Also the suppression of BWG was statistically significant ($P \leq 0.05$) from 6 to 7 weeks of age and highly significant ($P \leq 0.01$) from 2 to 7 weeks of age (overall). These results agree with other research on experimental aflatoxicosis in quail (Parlat *et al.*, 1999; Celik *et al.*, 2001 and Denli *et al.*, 2003); in broiler (Kubena *et al.*, 1998 and Celik *et al.*, 2001); and other poultry species (Kubena *et al.*, 1991 and Stewart *et al.*, 1998). These adverse effects of AFB_1 on BW and BWG may be due to anorexia, listlessness, inhibition of protein synthesis and lipogenesis (Campbell *et al.*, 1983; Oguz and Kurtoglu, 2000; Oguz *et al.*, 2000a,b and Parlat *et al.*, 2001). Moreover, Campbell *et al.* (1983) reported that the AF- contaminated feed decreased the activities of several enzymes, which are important to the digestion of carbohydrates, proteins, lipids and nucleic acid in broiler chicks. Also, Boden and Jensen (1985) stated that toxic effect induced by AF could have disrupted the activity of the digestive enzymes and the absorption of essential nutrients.

On the other hand, the present results showed that the addition of SC to AFB_1–containing diet significantly improved the adverse effect of AFB_1 on BW and BWG in quail. These findings were similar to that reported in quail by Parlat *et al.* (2001). They found that the addition of SC (1.0 gm/kg) to the AFB_1-containing diet, significantly elevated the adverse effects of AFB_1 (2.5 mg/kg diet) on BW and BWG in quail fed for 35 days. Our results were also supported by the study of Stanley *et al.* (1993) on broiler chicks, who observed significant amelioration of the adverse effects of AFB_1 (0.5 mg /kg) on performance in broilers fed for 28 days. Moreover, Abousadi *et al.* (2007) reported that the addition of SC (0.2%) to AFB_1-containing diet significantly decreased the adverse effect of AFB_1 (125 ppb) on BW and BWG in broiler chicks fed for 21 days.

The role of SC on the detoxification were attributed to its ability to produce biological enzymes that interacts with the AF molecules (Stanley *et al.*, 1993) and other growth promoting effects (Raju and Devegowda, 2000). It was also reported that yeast has been known to alter stress in animals by providing a source of vitamins, enzymes and growth protein for reducing stress, to enhance the biological value of nitrogen compounds along the digestive tract (Stanley *et al.*, 1993). Moreover the additional benefits of SC which were observed in the present study may be due to stimulation of the immune response (Savage *et al.*, 1996), alteration of intestinal microbial environment (Newman, 1994) and producing enzymes for gut micro flora to enhance the nutrients bioavailability (Stanley *et al.*, 1993; Raju and Devegowda, 2000; Parlat *et al.*, 2001 and Abousadi *et al.*, 2007).

The mode of action of yeast or its constituents (antioxidant compounds) against mutagenic or toxic effect of AFB_1 in animal cells (*in vivo*) may be due to binding with the mutagens or inhibition of activation of enzymes of cytochrome system-mediated N-hydroxylation with consequently enhancement of liver and kidney functions and reduction of abnormalities of genetic materials (Wang *et al.*, 2004 and Devaraj *et al.*, 2008).

As known, the liver is considered the principal target organ for Aflatoxins (Heathcote and Hibbert, 1978 and Phillips *et al.*, 1995). From the present results it was observed that the concentrations of AFB_1 were higher in liver samples than those found in breast muscle samples. These results are similar to those reported by Bintvihok *et al.* (2002) who found that the levels of AFB_1 and its metabolites, including acid-hydrolysable metabolites, were much higher (about 10-fold) in liver than those observed in muscle cells in all species of treated domestic fowls, with such toxin. Our findings were also supported by those results reported by Howard and Eaton (1990); Eaton and Groopman (1994); Cullen and Newberne (1994) and Smele and Curier (2001) who observed that the liver is considered the main organ in which the AFB_1 are metabolized by enzymes of cytochrome P450 group and converted to many metabolic product such aflatoxins Q_1, P_1 and M_1 and also Aflatoxin 8, 9 epoxide.

The present results showed that the concentrations of AFB_1 have been reduced in liver and breast muscle samples that were obtained from quail groups receiving AFB_1+SC than those found in

the liver and breast muscle samples from quail receiving AFB_1 alone. As discussed above, the SC or its constituents (some of the carotenoids and vitamins) have the ability for detoxification of AFB_1 through interacting or binding with AFB_1 molecule (Stanley *et al.*, 1993 and Raju and Devegowda, 2000). Also, Gradelet *et al.* (1998) reported that carotenoids exert their protective effect through the deviation of AFB_1 metabolism towards detoxification pathways in rats. In quail, Denli *et al.* (2003) observed that the dietary vitamin A reduced the toxic effects of AFB_1 so yeast addition caused less toxicity in the liver and kidney than the AFB_1 group.

The present results revealed that the gene expression of neural cell adhesion molecule (GAPDH, N-cadherin- and NCAM) genes were down-regulated in the quail fed diet containing AFB_1. Such changes of gene expression of neural genes are characteristic due to the AFB_1 according to Kreutzberg (1995). The present findings are in agreement with those reported by Ahmed and Singh (1984) in chickens and by Ibegwuonu (1983) and Llewellyna *et al.* (1988) in rats. Those authors found that the concentrations of RNA in the nervous tissues were depressed by AFB_1 treatment. The breakdown of gene expression of neural genes which was observed in the present study may be due to retrograde signals from the nervous terminal as a result of AFB_1 treatment (Pershon *et al.*, 1989; Pahl and Baeuerle, 1996 and O′Neill and Kaltschmidt, 1998).

The present results also showed that the expression level of P450scc gene was down-regulated in quail fed diet containing AFB_1. These findings are supported by Macé *et al.* (1997) who found that treatment with AFB_1 induced DNA adduct formation and P^{53} mutations in CYP450 in human liver cell lines. Epidemiological studies by Bressac *et al.* (1991); Harris (1996) and Soini *et al.* (1996) showed a positive association between AFB_1 intake and a hot-spot guanine to thymine transversion mutation at the third base in the codon 249 of the P^{53} tumor suppressor gene. Moreover, some *in vitro* studies have provided indirect evidence for a mutative role of aflatoxin exposure in p^{53} mutagenesis by showing that AFB_1 causes primarily G-T transversions in bacteria (Foster *et al.*, 1993) or human cells (Trottier *et al.*, 1992 and Cariello *et al.*, 1994). Aflatoxins have been found to be a potent mutagenic food component (Hall *et al.*, 1988). This component is metabolized by the mixed function oxidase system to a number of hydroxylated metabolites and to aflatoxin 8, 9 epoxide which binds to DNA forming covalent adducts (Bushy and Wogan, 1984). The DNA adduct formation causes genomic instability including gene expression of animal genes (Soini *et al.*, 1996; Harris, 1996; Macé *et al.*, 1997; Huang and Kolodner, 2005 and Guardiola *et al.*, 2008).

On the other hand, the present results showed that SC was able to prevent the genetic alterations induced by AFB_1 in the quail tissues. Where, the mRNA concentrations were significantly increased in AFB_1 + SC groups compared to AFB_1 group in quail brain, liver and testis. To our knowledge, there is no available information about the use of SC as protective agents on gene expression of animal genes against the toxic effect of mycotoxins. However, some studies reported that the yeast cells contain high amounts of carotenoids, vitamins, minerals, and essential amino acids as well as the presence of B-D glucans on the cell wall of yeasts (Hussein *et al.*, 1996; Vetvicka, 2001; Brown and Gordon, 2003). These constituents are considered as antioxidant agents, that interrupts the free radical-initial chain reaction of oxidation or scavenge and disable free radicals (ROS) and reduced DNA-oxidative damage (Vasankari *et al.*, 1997; Sener *et al.*, 2007 and Oliveria, *et al.*, 2009) leading to genomic stability including gene expression of animal genes (Vetvicka, 2001; Brown and Gordon, 2003 and Van Breda *et al.*, 2005).

Furthermore, in a previous study, Park *et al.* (2000) identified five peroxiredoxins in the SC that were named Tsa1 (CTPX1), Tas2, AhP1, Dot5 and Prx1, of which Tsa1 possesses the most potent ability to scavenge H_2 O_2. Also, Huang and Koshland, (2003) reported that the Tsa1 is the most potent protector of genomic stability and prevents a broad spectrum of mutations.

In Conclusion; *Sccharomyces cerevisiae* yeast has the ability to reduce the toxic effect of AFB_1 in quail. It was also apparent that the higher the inclusion rates of SC in the diet of quail (2.5 mg/kg) the more the effective it is. This was apparent from the BW, BWG data and the level of AFB_1 in the different quail tissues and the gene expression data.

Corresponding authors
Mariam Gergis Eshak, PhD*
Researcher of Animal Genetics
Wagdy K. B. Khalil, PhD*
Associate Professor of Molecular Genetics
*Cell Biology Department,
Genetic Engineering and Biotechnology Division,
National Research Centre (NRC)
El.Tahrir Street, 12622 Dokki, Giza, EGYPT
Fax: +202-33370931

References:

1. Abo-Norag, M., T. S. Edrington, L.F. Kubena, R.B. Harvey (1995): Influence of a hydrated

sodium calcium aluminosilicate and virginiamycin on aflatoxicosis in broiler chicks. *Poultry Science* 74: 626-632.
2. Abousadi, A. M., E. Rowghani, M. Ebrahimi Honarmand (2007): The efficacy of various additives to reduce the toxicity of aflatoxin B1 in broiler chicks. *Iranian Journal of Veterinary Research* 8: 144-150.
3. Ahmed, N., U. S. Singh (1984): Effect of aflatoxin B1 on brain serotonin and catecholamines in chickens. *Toxicology Letters* 21: 365-367.
4. Bintvihok, A.S., k. D. I. Thiengnin, S. Kumagal (2002): Residues of aflatoxins in the liver, muscle and eggs of domestic fowls. *Journal of Veterinary Medical Science* 64: 1037-1039.
5. Bio-Rad Laboratories, Inc. (2006) Real-Time PCR applications guide. *Bulletin* 5279, pp.101.
6. Boden, S., L. Jensen (1985): The effect of marginal levels of calcium, fish meal, torulas yeast and alfalfa meal on feed intake, hepatic lipid accumulation, plasma estradiol and egg shell quality among laying hens. *Poultry Science* 64: 937-946.
7. Bressac, B., M. Kew, J. Wands, M. Ozturk (1991): Selective G to T mutations of p53 gene in hepatocellular carcinoma from Southern Africa. *Nature* 350: 429-431.
8. Brown, G. D., S. Gordon (2003): Fungalbetaglucons and mammalian immunity. *Immunity* 19: 311-315.
9. Bushy, W. F., G. N. Wogan (1984): Aflatoxins. In: Chemical carcinogens. Second edition, Volume 2, ACS Monograph 182, C.E. Searle, editor, American Chemical Society, *Washington DC*, Chapter 16, pp: 945-1136.
10. Campbell, M. l., J. D. May, w. E. Huff, J. A. Doevr (1983): Evaluation of young broiler chicken during aflatoxicosis and ochratoxicosis. *Poultry Science* 62, 2138-2144.
11. Cariello, N. F., l. Cui, T. R. Skopek (1994): In vitro mutational spectrum of aflatoxin B1 in the human hypoxanthine guanine phosphoribosyl-transferase gene. *Cancer Research* 54: 4436-4441.
12. Celik, K., M. Denli, M. Erturk, O. Ozturkcan, F. Doran (2001): Evaluation of dry yeast (Saccharomyces cerevisiae) compounds in the feed to reduce aflatoxin B1 (AFB1) residues and toxicity to Japanese quails (*Coturnix coturnix japonica*). *Journal of Applied Animal Research* 20:245-250.
13. Cullen, J.M., P.M. Newberne (1994): Acute hepatotoxicity of aflatoxins. In: Toxicology of Aflatoxins. J. D. Groopman, ed., pp: 43-70.
14. Denli, M., K. Celik, F. Okan (2003): Effects of vitamin A supplementary in the feed to reduce toxic effects of aflatoxin B1 on Japanese quails (Coturnix cotrunix japonica). *International Journal of Poultry Science* 2: 147-177.
15. Devaraj, S., S. Mathur, A. Basu, H. H. Aung, V. T. Vasu, S. Meyers, I. Jialal (2008): A dose-response study on the effects of purified lycopene supplementation on biomarkers of oxidative stress. *Journal of the American College of Nutrition* 27: 267-273.
16. Duncan, D. B. (1955): Multiple range and multiple F tests. *Biometrics* 11: 1-42.
17. Eaton, D.L., J. D. Groopman (1994): The Toxicology of the aflatoxins. In: Human Health, Veterinary, and Agricultural Significance. Academic Press, *London, UK*., pp: 49-87.
18. El-Nezami, H., H. Mykkänen, P. Kankaanpä, S. Salminen, J. Ahokas, (2000): Ability of lactobacillus and propionobacterium strains to remove aflatoxin B1 from chicken duodenum. *Journal of Food Protection* 63: 549-552.
19. Foster, P.L., E. Eisenstadt, J. H. Miller (1993): Base substitution mutations induced by metabolically activated aflatoxin B1 *in vitro*. *Proceedings of the National Academy of Sciences* 80: 2695-2698.
20. Galvano, F., A. Piva, A. Ritieni, G. Galvano (2001): Dietary strategies to counteract the effects of mycotoxins: a review. *Journal of Food Protection* 64: 120-131.
21. Gowda, N. K. S., V. Malathi, R. V. Suganthi (2004): Screening for aflatoxin and the effect of moisture, duration of storage and form of feed on fungal growth and toxin production in livestock feeds. *Animal Nutrition* and *Feed Technology* 3: 45-51.
22. Gradelet, S., A. M. Le Bon, R. Berges, M. Suschetet, P. Astorg (1998); Dietary carotenoids inhibit aflatoxin B1 – induced liver preneoplastic foci and DNA damage in the rat: role of the modulation of aflatoxin B1 metabolism. *Carcinogenesis* 19: 403-411.
23. Guardiola, F., B. Ricard, C. Rafel, T. Alba, A. Tric (2008): Determination of hydroperoxides in food sand biological samples by the ferrous oxidation-xylenol orange method: a review of the factors that influence the method's performance. *Analytical Biochemistry* 377: 1-15.
24. Hall, J.C., S. C. Jori, J. Arrowman (1988): Food associated intoxicants. Nutrition & Food. *Science*, 12: 1-43.
25. Harris, C.C. (1996): The 1995 Walter Hubert lecture-molecular epidemiology of human

cancer: insights from the mutational analysis of the p53 tumor-suppressor gene. *British Journal of Cancer,* 73: 261-269.
26. Heathcote, J. C., J. R. Hibert (1978): Pathological effects. In: Aflatoxins: Chemical and Biological Aspects. Elsevier Scientific Publishing Company, *New York*, pp: 83-111.
27. Howard, S. R., d. l. Eaton (1990): Species susceptibility to aflatoxin B1 carcinogenesis. *Cancer Research* 50: 615-620.
28. Huang, D., D. Koshland (2003): Chromosome integrity in Saccharomyces cerevisiae: the interplay of DNA replication initiation factors, elongation factors, and origins. *Genes & Development* 17: 1741-1754.
29. Huang, M. E., R. D. Kolodner, (2005): A biological network in Saccharomyces cerevisiae prevents the deleterious effects of endogenous oxidative DNA damage. *Molecular Cell* 17: 709-720.
30. Hussein, A. S., A. H. Cantor, A. J. Peascatore, T. H. Johnson (1996): Effect of dietary protein and energy levels on pullet development. Department of Animal Sciences, University of Kentucky, Lexington, *Kentucky*, pp: 40215-40546.
31. Hwang, H. S., K. J. Han, Y. H. Ryu, E. J. Yang, Y. S. Kim, S. Y. Jeong, Y. S. Lee, M. S. Lee, S. T. Koo, S. M. Choi (2009). Protective effects of electroacupuncture on acetylsalicylic acid-induced acute gastritis in rats. *World J Gastroenterol* 15: 973-977.
32. Ibegwuonu, F.I. (1983): The neurotoxicity of aflatoxin B1 in the rat. *Toxicology* 28: 247-259.
33. Kanda, I., Y. Akazome, O. Ogasawara, T. Mori (2000): Expression of cytochrome P450 cholesterol side chain cleavage and 3beta-hydroxysteroid dehydrogenase during embryogenesis in chicken adrenal glands and gonads. *General and Comparative Endocrinology* 118: 96-104.
34. Khalil, W. K. B., T. Stadtlander, G. Francis, K. Becker, U. Focken (2008a): Effect of feed supplementation with different saponin fractions from Trigonella foenumgraecum on growth and expression of genes encoding GH and IGF-l in carp, *Cyprinus carpio*. *Proceedings of the Society of Nutrition Physiology*, 17: 32.
35. Khalil, W. K. B., T. Stadtlander, G. Francis, U. Focken, K. Becker, B. Levavi-Sivan, Z. Kerem, H. Dweik, M. Qutob, S. Abu-Lafi (2008b): Saponin fractions from *Trigonella foenum-graecum* in the diet affect growth and expression of GH and IGF-1 encoding genes in tilapia (*Oreochromis niloticus*). *Comparative Biochemistry and Physiology Abstracts/ Part A* 151: 512–513.
36. Kreutzberg G. W. (1995): Reaction of neuronal cell body to axonal damage. In: The axon. S. G. Waxman, J. D. Kocsis and P. K. Stys (eds.), Oxford University Press, *New York*, pp: 335–374.
37. Kubena, L. F., R. B. Harvery, R. H. Balitey, S. A. Buckley, G. E. Rottinghaus (1998): Effects of a hydrated sodium calcium aluminosilicate (T-BindTM) on mycotoxicosis in young broiler chikens. *Poultry Science* 77: 1502-1509.
38. Kubena, L. F., R. B. Harvey, A. G. Yersin, M. H. Ellisalde, D. A. Witzel, L. E. Giroir, T. D. Phillips, H. D., Petersen (1991): Effect of hydrated sodium calcium aluminosilicate on growing turkey poults during aflatoxicosis. *Poultry Science* 70: 1823-1830.
39. Leeson, S., G. Dizy, J. D. Summers (1995): Aflatoxins. In: Poultry metabolic disorders and mycotoxins. S. Leeson; G. Diaz and J.D. Summers (Eds.). Guelph, Ontario, University Books, pp: 248-279.
40. Llewellyna, G.C., W. V. Dashekb, T. D. Kimbroughc, C. E. O'Reard, L. B. Weekleye (1988): Aflatoxin B1 affects feed consumption in male rats through an action on the central nervous system. *International Biodeterioration* 24: 417-425.
41. Macé, K., F. Aguilar, J. S. Wang, P. Vautavers, M. Gómez-Lechón, F. J. Gonzalez, J. Croopman, C. C. Harris, M. A. Pfeifer (1997): Aflatoxin B1-induced DNA adduct formation and P53 mutations in CYP450-expressing human liver cell lines. *Carcinogenesis* 18: 1291-1297.
42. Martins, M.H., I. Almeida, M. Marques, F. Bernardo (2008): Interaction of wild Strains of Aspergillua with Aspergillus parasites ATCC 15517 and aflatoxin production. *International Journal of Molecular Sciences* 9: 394-400.
43. Miazzo, R., C. A. Rosa, E. C. DeQueiroz Carvalho, C. Magnoli, S. M. Chiaccnieva, C. Palocio, M. Saenz, A. Kikot, E. Basaldella, A. Dalcero (2000): Efficacy of synthetic zeolite to reduce the toxicity of aflatoxin in broiler chicks. *Poultry Science* 79: 1-6.
44. NRC. (1994): Nutrient Requirements of Poultry. 9th rev. National Academy Press, Washington, DC.
45. Newman, K. E. (1994): Mannan oligosaccharide, natural polymers with significant impact on the gastrointestinal microflora and immune system. In: Biotechnology in the feed industry. T.P. Lyons

and K.A. Jaques (eds.). *Kentucky*: Alltech Inc. pp: 167-174.
46. O'Neill, L. A. J., C. Kaltschmidt (1998): NF-KB: a crucial transcription factor for glial and neuronal cell function. *Trends in Neurosciences* 20: 252–258.
47. Oguz, H., V. Kurtoglu (2000): Effect of clinoptiloite on fattening performance of broiler chickens during experimental aflatoxicosis. *British Poultry Science* 41: 512-517.
48. Oguz, H., T. Kecel, Y. O. Birdane, F. Onder, V. Kurtoglu (2000a): Effect of clinoptilolite on serum biochemical and hematological characters of broiler chickens during experimental aflatoxicosis. *Research in Veterinary Science* 69: 89 – 93.
49. Oguz, H., V. Kurtoglu, B. Coskun (2000b). Preventive efficacy of clinoptilolite in broilers during chronic aflatoxin (50 and 100 ppb) exposure. *Research in Veterinary Science* 69:197-201.
50. Oliveria, C. A. F., J. F. Rosmaninho, P. butkeraitis, B. Corréa, T. A. Reis, J. L. Guerra, R. Albuquerque, M. E. G. Moro (2002): Effect of low levels of dietary aflatoxin B1 on laying Japanese quail. *Poultry Science* 81: 976-980.
51. Oliveria, S.A., K. Okoshi, A. P. Lima–Leopoldo, A. S. Leopoldo, D. H. Campos, P. F. Martinez, M. P. Okoshi, C. R. Padovani, M. D. Pai-Silva, A. C. Cicogna (2009): Nutritional and cardiovascular profiles of normotensive and hypertensive rats kept on a high fat diet. *Arquivos Brasileiros de Cardiologia* 93: 526-533.
52. Pahl, L., P. A. Baeuerle (1996); Control of gene expression by proteolysis. *Current Opinion in Cell Biology* 8: 340–347.
53. Park, S. G., M. K. Cha, W. Jeong, I. H. Kim, (2000); Distinct Physiological functions of thiol peroxidase isoenzymes in Saccharomyces cerevisiae. *Journal of Biological Chemistry* 275: 5723-5732.
54. Parlat, S. S., M. H. Ozcan, H. Oguz (2001): Biological Suppression of aflatoxicosis in Japanese quail (*Coturnix coturnix japonica*) by dietary addition of yeast (Saccharomyces cerevisiae). *Research in Veterinary Science* 71: 207-211.
55. Pershon, E., G. E. Pollemberg, M. Schachner (1989): Immunoelectron-microscopic localization of the 180 KD component of the neural cell adhesion molecule NCAM in postsynaptic membranes. *Journal of Comparative Neurology* 288: 92–100.
56. Phillips, T. D., A. B. Sarr, P. G. Grant (1995): Selective chemisorption and detoxification of Aflatoxins by phyllosilicate clay. *Natural Toxins* 3: 204-213.
57. Raju, M. V., G. Devegowda (2000); Influence of esterified glucomannan on performance and organ morphology, serum biochemistry and hematology in broilers exposed to individual and combined mycotoxicosis (aflatoxin, ochratoxin and T-2 toxin). *British Poultry Science* 41: 640-650.
58. SAS Institute Inc. (2004): SAS/STAT®9.1 User's Guide, Cary, NC: SAS Institute Inc.
59. Savage, T. F., P. F. Cotter, E. I. Zakrzewska (1996): The effect of feeding mannan oligosaccharide on immunoglobulins, plasma IgG and bile IgA of Wordstad MW male turkeys. *Poultry Science* 75: 53-64.
60. Sener, G., H. Aksoy, O. Sehili, M. Yuksel, C. Aral, N. Gedik, S. Cetrinel, B. C. Yegen (2007): Erdosteine prevents colonic inflammation through its antioxidant and free radical scavenging activities. *Digestive Diseases and Sciences* 52: 2122-2132.
61. Sinsek, N., L. Ergun, E. Rgun, B. Alabay, D. Essiz (2007): The effects of experimental aflatoxicosis on the exocrine pancreas in quails (*Coturnix coturnix japonica*). *Archive of Toxicology* 81: 583-588.
62. Smele, M. E., S. S. Curier (2001): The chemistry and biology of aflatoxin B1. *Carcinogenesis* 22: 535-545.
63. Soares, L. M. V., D. B. Rodrigues–Amaya (1989): Survey of alfatoxins, ochratoxins A Zearalenone and sterigmatocystin in some Brazilian foods by using multitoxin thin layer chromatographic method. *Journal of the Association of Official Analytical Chemists* 72: 22-26.
64. Soini, Y., S. C. Chia, W. P. Bennett (1996): An aflatoxin-associated mutational hotspot at condon 249 in the p53 tumor suppressor gene occurs in hepatocellular carcinomas from Mexico. *Carcinogenesis* 17:1007-1012.
65. Squitti, R., M. E. De Stefano, D. Edgar, G. Toschi (1999): Effects of axotomy on the expression and ultra structural localization of N-cadherin and neural cell adhesion molecule in the quail ciliary ganglion: An in vivo model of neuroplasticity. *Neuroscience* 91: 707-722.
66. Stanley, V. G., R. Ojo, S. Moldesenbet, D. H. Hutchinson, L. P. Kubena (1993); The use of Saccharomyces cerevisiae to suppress the effects of aflatoxicosis in broiler chicks. *Poultry Science* 12, 1867-1872.
67. Stewart, S., X. Kal-Lu, W. F. Huff, L. F. Kubena, R. B. Harvey, H. A. Dunsford (1998): Aflatoxin exposure produces serum

alphafoetoprotein elevations and marked oval cell proliferation in young male Pekin duckling. Pathology 30: 34-39.
68. Tovar-Ramirez, D., D. Mazurais, J. F. Gatesoupe, P. Quazuguel, C. L. Cahu, J. L. Zambonino-Infante (2010): Dietary probiotic live yeast modulates antioxidant enzyme activities and gene expression of sea bass (*Dicentrachus labrax*) larvae. *Aquaculture* 300: 142-147.
69. Trottier, Y., W. I. Walthe, A. Anderson (1992): Kinds of mutations induced by aflatoxing B1 in a shuttle vector replicating in human cells transiently expressing cytochrome P4501 A2 cDNA. *Mol. Carcinogenesis* 6: 140-147.
70. Van Breda, S.G., E. Van Agen, S. Van Sanden, T. Burzykowski, J. C. Kleinjans, J. H. Van Delft (2005): Vegetables affect the expression of genes involved in carcinogenic and anticarcinogenic processes in the lungs of female C57B1/6 mice. *Journal of Nutrition* 135: 2546-2552.
71. Vasankari, T. J., U. M. Kujala, T. M. Vasankari, T. Vuorimaa, M. Ahotupa (1997): Increased serum and low-density-lipoprotein antioxidant potential after antioxidant supplementation in endurance athletes. *American Journal of Clinical Nutrition* 65: 1052-1056.
72. Vetvicka, V., (2001): B-glucans as immunomodulators. *JANA* 3: 31-34.
73. Wang, S., E. A. Konorev, S. Kotamraju, J. Joseph, S. Kalivendi, B. Kalyanaraman (2004): Doxorubicin induce apotosis in normal and tumor cells via distinctly different mechanisms. Intermediacy of H2O2 and p53- dependent pathways. *Journal of Biological Chemistry* 279: 25535-25543.

10/2/2010

Perceived Family-Supportive Work Culture, Affective Commitment and Turnover Intention of Employees

Aminah Ahmad, Zoharah Omar

Department of Professional Development and Continuing Education
Faculty of f Educational Studies, Universiti Putra Malaysia
43400 Serdang, Selangor, Malaysia
aminah@ace.upm.edu.my

Abstract: The objective of this research is to examine the role of perceived family-supportive work culture in reducing turnover intention of employees and the mediating role of affective commitment in the relationship between perceived family-supportive work culture and turnover intention. The subjects in this study constituted 693 employees from 20 private service organizations in the Klang Valley, Malaysia. Results of multiple regression analyses indicate that perceived family-supportive work culture is positively related to turnover intention of employees and employees' affective commitment mediate the relationship between perceived family-supportive work culture and turnover intention. The results imply the need for employers to understand how employees view the family-friendly programs in terms of the support provided and the values they place on the programs as captured in perceived family-supportive work culture. Positive perceptions would help reduce turnover intention as well the affective commitment of employees.
[Aminah Ahmad, Zoharah Omar. Perceived Family-Supportive Work Culture, Affective Commitment and Turnover Intention of Employees. Journal of American Science 2010;6(12):839-846]. (ISSN: 1545-1003).
http://www.americanscience.org.

Keywords: Perceived family-supportive work culture; turnover intention; affective commitment

1. Introduction

Employee turnover is a persistent problem in organizations since organizations have to deal with costs due to termination, advertising, recruitment, selection, and hiring (Abbasi and Hollman, 2008). Employee turnover may jeopardize an organization's strategic plans to achieve its objectives (Abasi and Hollman, 2008). When an organization loses its critical employees, there might be several outcomes including a reduction in the overall level of innovation and quality of customer service (Denvir and McMahon, 2002; Miller, 2010). According to Garino and Martin (2005), organizations suffer the loss of job-specific skills and disruption in production, and incur the costs of hiring and training new workers when employees leave the organization.

For many years, researchers have tried to understand the major determinants of turnover intention and some managerial implications (Tuzun, 2007) especially with the rapidly changing demography of the workforce including the increase of women workers. Such a change results in more couples having to juggle work and family roles (Aminah and Zoharah, 2008; Perrewe´, Treadway and Hall, 2003) and hence it is expected that organizations be more sensitive to employees' family responsibilities and needs outside the workplace.

Consequently, contemporary public and private organizations are increasingly seeking initiatives to help employees balance their work and family responsibilities that may lead to positive organizational outcomes. One of the initiatives is to offer benefits that are family-supportive. Thompson, Beauvais, and Lyness (1999) introduced the concept of work-family culture which refers to the shared assumptions, beliefs, and values regarding the extent to which an organization supports and values the work-family integration of employees. This concept includes three dimensions namely, managerial support for work-family balance, career consequences associated with utilizing work-family benefits, and organizational time expectations that may interfere with family responsibilities. A supportive work–family culture increases the likelihood that employees will feel comfortable using family-friendly benefits like flextime, as they are less likely to worry about possible negative career consequences (Thompson et al., 1999). Another similar concept, organizational family support, refers to the organization's interest in helping employees achieve work-life balance and it encompasses work-family policies and practices offered by an organization (Allen, 2001).

Previous research have shown that employees who find that their organizations are less responsive to their family needs would be less committed to organizations and hence may leave the organizations (Haar and Spell, 2004; Rothbard, Phillips and Dumas, 2005; Wang and Walumbwa, 2007). In addition, research have also shown that affective organizational commitment, which refers to employee's emotional attachment to, identification with, and involvement in the organization (Meyer and Allen, 1991), is the strongest predictor of organizationally desired outcomes such as employee retention (Allen et al., 2003; Meyer and Smith, 2000; Rhoades et al., 2001) as compared to other forms of commitment, namely normative and continuance commitment. An employee who is affectively committed strongly identifies with the goals of the organization and desires to remain as part of the organization (Meyer and Allen, 1993) and are least likely to leave the organization (Thompson and Prottas, 2005; Gaan, 2008).

Despite the acknowledgement of the importance of workplace culture and studies examining employees' perceptions towards family-supportive culture, there is a need to examine the role of perceived workplace culture in reducing negative outcomes such as turnover intention and increasing organizational commitment since previous studies, with the exception of the work discussed earlier, have focused more on benefits offered rather than how comfortable employees feel using the family-friendly benefits and the values they place on the benefits as captured in perceived family-supportive work culture. The purpose of this study is to examine the role of perceived family-supportive work culture in reducing turnover intention and the mediating role of affective commitment. This study draws upon the social exchange theory (Blau, 1964) to examine relationships between these variables. The social exchange theory recognizes conditions under which individuals feel obligated to reciprocate when they personally benefit from another's actions (Lambert, 2000). The social exchange theory can be used to explain the relationships between family-supportive work culture and employees' commitment to organization and turnover intention. Besides this theory, the results of previous research also offer the bases for the formulation of hypotheses.

2. Literature Review

2.1 Perceived family-supportive work culture and affective organizational commitment

Thompson et al. (2004) found that employees who perceive that their supervisors and organization are family-supportive are more committed affectively to their organization. According to Gibson and Tremble (2006), employees' affective organizational commitment is derived from their perceptions of the extent to which the employer is committed to and supportive of them. Assistance in balancing the demands of work and family life is a promising intervention for improving employee experiences and increasing retention in the organization. Haar and Spell (2004) examined the relationship between the perceived value of work-family practices among employees and their affective commitment and found a significant relationship. Similar findings were obtained by Muse et al. (2008). Thus empirical evidence seems to support the relationship between perceived family-supportive work culture and affective commitment, and the following hypothesis was tested.

H1: There is a positive relationship between perceived family-supportive work culture and affective commitment.

2.2 Perceived family-supportive work culture and turnover intention

Availability of organizational family support (family benefits and alternative schedules) and informal organizational support (work-family culture, supervisor support, and coworker support) have been suggested as a means to reduce employees turnover intention (Gaan, 2008). Previous research indicates that employees who perceive that their organization support them in integrating between work and family responsibilities will have less intention to leave the organization (Allen, 2001).

According Pasewark and Viator (2006), flexible work arrangement, which is part of the work-family support, seem to be effective in reducing turnover intention. Earlier, Thompson and Prottas (2005) investigated the relationships among informal organizational support (work–family culture, supervisor support, and coworker support) and turnover intention. They found that the informal organizational support was associated with reduced turnover intention. Recently, Yanadoria and Katob (2010) examined the effects of work-family support at the workplace in Japanese firms and found statistically significant associations between work-family support and female employee turnover in Japan. Previous research findings seem to support the relationship between work-family support at work place and turnover intention and the following hypothesis was tested.

H2: There is a negative correlation between family-supportive work culture and turnover intention

2.3 Affective commitment and turnover intention

Affective organizational commitment refers to employee's emotional attachment to, identification with, and involvement in the organization (Meyer and Allen, 1991). Lack of organizational affective commitment has detrimental effects including increase in turnover rate and turnover intention (Baotham et al., 2010). As evident by several studies, affective commitment is negatively correlated with employee turnover intention (Ali and Baloch, 2009; Addae and Parboteeah, 2008; Yeoh et al., 2010). In other words, employees who are more committed to their organizations are less likely to leave the organization. Since employees' affective commitment could be a predictor of turnover intention, the following hypothesis was tested:

H3: There is a negative correlation between affective commitment and turnover intention.

2.4 Affective commitment as a mediator in the relationship between family supportive work culture and turnover intention

Besides examining the magnitude of the perceived family-supportive work culture and turnover linkage, this present study also examined the mediating role played by affective commitment in this relationship. Based on the literature review as has been discussed earlier, perceived family-supportive work culture is related to affective commitment and the latter is also related to turnover intention. Drawing upon the social exchange theory (Blau, 1964) positive employee outcomes (e.g. organizational commitment and employee retention) could be achieved in response to benefits provided by organizations. Based on previous findings and the social exchange theory, the following hypothesis is formulated:

H4: Affective commitment mediates the relationship between perceived family-supportive work culture and turnover intention

3. Materials and methods

3.1 Sample

A total of 693 employees from 20 private service organizations in the Klang Valley, Malaysia, participated in this study. Only organizations with a minimum of 100 employees were included in this study since larger organizations are more likely to provide support in the form of family-friendly policies such as flexible work and child care arrangements than smaller organizations, while smaller organizations will adhere to basic requirements such as leave arrangement and medical coverage (Wood et al., 2003; Dulk et al., 2005). According to Poelmans et al. (2003), the provision of such support depends on several factors including organizational size. Forty employees from three categories, namely (1) managerial and executive, (2) supervisory and technical and (3) clerical and other support staff from each organization, 10 from the first category, 10 from the second category and 20 from the third category.

3.2 Measurement

3.2.1 Perceived Family-supportive work culture

Perceived family-supportive work culture was measured using 18 items from the work-family culture scale developed by Thompson et al. (1999). Three dimensions of work-family culture were measured, namely managerial support, career consequences and organizational time demand. For each support scale, items were measured on a 5-point Likert scale that ranged from (1) strongly disagree to (5) strongly agree; high scores represented more managerial support, career consequences and organizational time demand. Examples of items are: "The higher management in this organization encourages supervisors to be sensitive to employees' family and personal needs" and "In this organization, employees are encouraged to strike a balance between their work and family lives". The reliability coefficient of the scale was. 92.

3.2.2 Affective organizational commitment

Affective organizational commitment was measured using 8 items from Allen and Meyer (1990). The employees were requested to respond using five-point scaled response options ranging from strongly disagree (1) to strongly agree (5). Examples of items include "I feel a strong sense of belonging to my organization" and "I feel emotionally attached to this company". The reliability coefficient of the scale was .87.

3.2.3 Turnover intention

Turnover intention was measured using 6 items, two items were adopted from the instrument developed by Scott, Bishop and Chen (2003), three by Lee and Mowday (1987) and one from Seashore, Lawler, Mirvis, and Cammann (1983). The subjects of this study were requested to respond using five-point scaled response options ranging from very unlikely (1) to very likely (5). Examples of items are: "I often think of quitting my current job" and "I am actively looking for a job with another company". The reliability coefficient (alpha) of this scale was 0.92.

3.3 Statistical analysis

Descriptive statistics were calculated to describe the main characteristic of the respondents. Correlation coefficients were computed to examine the relationships among family-supportive work culture, affective commitment and turnover intention. A series of regression analyses was employed to test the hypotheses of the study. Baron and Kenny (1986) recommended the use of a series of regression models to test mediational hypotheses. First, regressing the mediator on the independent variable; second, regressing the dependent variable on the mediator; third, regressing the independent variables on the dependent variables and fourth, regressing the dependent variable on both the independent variable and the mediator. The following are the four conditions for establishing mediation: (1) The independent variable significantly affects the dependent variable; (2) The independent variable significantly affects the mediator; (3) The mediator significantly affects the dependent variable; (4) The effect of the independent variable on the dependent variable shrinks upon the addition of the mediator to the model. If the independent variable does not affect the dependent variable upon regressing the dependent variable on both the independent variable and the mediator, then full mediation is established. If otherwise, the test supports partial mediation.

4. Results

The respondents' age ranged from 18-57 years (M = 32.35, SD = 8.56). About two-fifths of respondent belonged to the 26-33 age group. There were about equal proportions of females (50.5%) and males (49.5%). Managerial and executive staff constituted 32.2% of the total respondents, supervisors and technical 16.2%, and clerical and other support staff 51.6% (Table 1).

The mean score for perceived family-supportive work culture was 3.25 (SD = 0.43), affective commitment 3.57 (SD = 0.70) and turnover intention 2.60 (SD = 0.82) (Table 2). Correlational analyses results revealed that perceived family-supportive work culture was positively related to affective commitment ($r = 0.31$, $p<0.01$) and negatively related to turnover intention ($r = -0.28$, $p<0.01$) (Table 2). Affective commitment was negatively related to turnover intention ($r = -0.49$, $p<0.01$) (Table 2). The results show that an increase in employees' scores of perceived family-supportive work culture leads to an increase in affective commitment and a decrease in turnover intention. An increase in affective commitment leads to a decrease in turnover intention. These results support H1, H2 and H3.

Table 1. Characteristics of respondents

Characteristics	Frequency	%	Mean	SD
Age (years) (n = 680)			32.35	8.56
≤25 years old	154	22.2		
26 - 33 years old	281	40.5		
34 - 41 years old	131	18.9		
42 - 49 years old	75	10.8		
≥ 55 years old	39	5.6		
Gender (n = 693)				
Male	343	49.5		
Female	350	50.5		
Job category (n = 684)				
Managerial and executive	220	32.2		
Supervisory and technical	111	16.2		
Clerical and other support staff	353	51.6		

Table 2. Means, standard deviations and intercorrelations of the variables

	Variable	1	2	3	Mean	SD
1	Turnover intention	1.00			2.60	0.82
2	Affective Commitment	-0.49**	1.00		3.57	0.70
3	Perceived family-supportive culture	-0.28**	0.31**	1.00	3.25	0.43

** <0.01

Table 3 presents the results of the regression analyses (N = 693) testing whether the relationship between perceived family-supportive work culture and turnover intention is mediated by affective commitment.

Step 1: Effect of perceived family-supportive work culture and turnover intention (Fig.1a) is statistically significant ($\beta=-0.28$, $p<.01$), satisfying step 1 of Baron and Kenny's method.

Step 2: Statistically significant effect of perceived family-supportive work culture on affective commitment ($\beta = 0.31$, $p<0.01$) (Fig. 1b), meets the stipulation of this step.

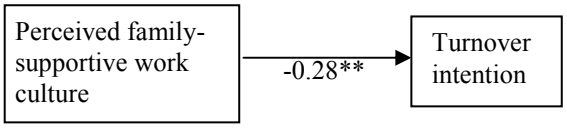
Step 3: The effect of affective commitment on turnover intention is statistically significant (β=-0.44, p<0.01). This relationship is independent of the association between perceived family-supportive work culture and turnover intention.

Step 4: The effect of perceived family-supportive work culture on turnover intention shrinks upon the addition of affective commitment (the mediator) to the model (bottom of Fig. 1b), (β=-0.15, p<0.01) and this is consistent with mediation. Since the perceived family-supportive work culture does affect the turnover intention upon regressing the turnover intention on both perceived family-supportive work culture and on affective commitment, then partial mediation is established. Hence, the results support H4.

Table 3. Regression Analysis

Step	Independent Variable	Dependent Variable	β	R2
1	Perceived family-supportive culture	Affective commitment	.31**	
2	Affective commitment	Turnover intention	-.49**	
3	Perceived family-supportive culture	Turnover intention	-.28**	
4	Perceived family-supportive culture Affective commitment	Turnover intention	-.15** -.44**	.26

(1a)



(1b)

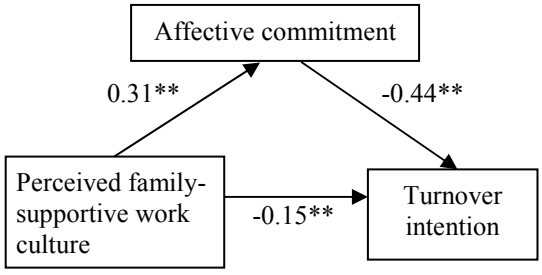


Figure 1.Regression analysis results.
(1a) portrays the simple model of perceived family-supportive work culture, affective commitment and turnover intention.
1(b) depicts the full model that includes affective commitment as the mediator.

Table 3 shows the results of regression analyses. The regression analysis between perceived family-supportive work culture and turnover intention has an R^2 of 0.08. Adding affective commitment to the model increases the value of R^2 to 0.26. Thus the change in R^2 associated with adding affective commitment is 0.18. The inclusion of affective commitment in the model accounts for an additional 18% of the variance in turnover intention.

5. Discussion

The findings that perceived family-supportive work culture is a significant and negative predictor of turnover intention have also been reported by Thompson and Prottas (2005) and the social support-commitment relationship has also been reported by other researchers (Gaan, 2008; Pasewark and Viator, 2006). The findings of this study show that employees who perceive that their organizations are supportive of their family needs are less likely to leave the organizations. With regard to perceived family-supportive work culture and its association with affective commitment, this study found that an increase in perceived support by managers or organizations led to an increase in affective commitment. These findings are consistent with the findings of Allen (2001) and Haar and Spell (2004). Similar findings have also been reported by O'Neill et al. (2009). In other words, employees who receive support to manage their work and family lives are more committed to their organizations and are more likely to stay in their organizations.

The significant relationship between affective commitment and turnover intention is consistent with the results reported by Baotham et al. (2010) whereby individuals with higher levels of affective commitment tended to report lower levels of intention to leave. Similar findings have been reported by Addae and Parboteeah (2008), Ali and Baloch (2009) and Yeoh et al. (2010).

With regard to the mediating effect of affective commitment in the relationship between perceived family-supportive work culture and turnover intention, the results show that employees with more positive perceptions of organizational support tend to experience higher levels of affective commitment and this would in turn decrease their levels of intention to leave the organization. Theoretically, the findings have shown that the social exchange theory (Blau, 1964) offer a theoretical guide to understanding the outcomes of perceived family–supportive work culture whereby positive employee outcomes (e.g. organizational commitment and employee retention) could be achieved in response to benefits provided by organizations.

The findings of this study have important implications for organizations. The findings demonstrate that employees' perception of organizations' family-supportive culture is an important factor that is related to employees' affective organizational commitment and turnover intention. Given that turnover is a serious problem since it involves costs due to termination, advertising, recruitment, selection, and hiring (Abbasi and Hollman, 2008), identifying factors that that could further explain turnover is an important attempt. Employers should also look into the possibility of developing family-friendly practices that are sensitive to employee family needs to assist employees in managing work and family roles such that employees will not be hesitant in associating themselves with such practices since employers will be perceived as supportive. With this supportive perception there is a greater tendency for employees to be more committed to the organization and an increased likelihood to remain in the organization.

The implication for future research is that, when exploring the influence of family-supportive work-life programs or practices on attitudes (e.g. commitment and turnover), it is meaningful to explore how employees view the work-life programs or practices besides examining the practices or the offering of programs. Simply offering work-life programs does not necessarily mean that employees find the organization supportive of their work-life needs (Thompson et al., 1999).

Several limitations of this study should be noted. First, a significant limitation of the present investigation is the sample size that was utilized. The results reported here may only be generalized to employees working in private service organizations located in Klang Valley, Malaysia. Caution must be exercised in generalizing the findings from this sample to employees in other organizations such as manufacturing organizations. There is also a need for future researchers to examine the work culture perceptions and its effects on employees' organizational commitment and turnover intention in other industries such as the manufacturing industry which is another important industry in Malaysia. Second, the inferences drawn from this study are limited by self-report data and cross-sectional characteristics of the data.

6. Conclusion

We could conclude that perception of family-supportive work culture is an important antecedent of turnover intention and affective commitment is a mediator in this supportive culture-turnover relationship. An employee who perceives that there exists family-supportive culture in an organization, characterized by high responsiveness to work-family issues, seems to be more likely to remain in the organization and have a greater sense of affective commitment to the organization.

Acknowledgement

Authors are grateful to the Ministry of Science, Technology and Innovation, Malaysia for providing financial support to conduct this research.

Corresponding Author

Aminah Ahmad, Ph.D.
Department of Professional Development and Continuing Education,
Faculty of f Educational Studies,
Universiti Putra Malaysia
43400 Serdang, Selangor, Malaysia
E-mail: aminah@ace.upm.edu.my

References

1. Abbasi SM, Hollman KW, Hayes RD. Bad Bosses and How Not To Be One. Information Management Journal. 2008;42(1): 52-56.
2. Addae HM, Parboteeah KP. Role Stressors and Organizational Commitment: Public Sector Employment in St Lucia. International Journal of Manpower. 2008;29(6):727-743.
3. Ali N, Baloch QB. Predictors of Organizational Commitment and Turnover Intention of Medical Representatives (An Empirical Evidence of Pakistani Companies). Journal of Managerial Sciences. 2009;3(2): 263-273.
4. Allen TD. Family-Supportive Work Environments: The Role of Organizational Perceptions. Journal of Vocational Behavior. 2001;58(3):414–435.
5. Allen NJ, Meyer JP. The Measurement and Antecedents of Affective, Continuance and Normative Commitment to the Organization. Journal of Occupational Psychology. 1990; 63: 1-18.
6. Allen DG, Shore LM, Griffith RW. The Role of Perceived Organizational Support and Supportive Human Resource Practices in the Turnover Process. Journal of Management. 2003; 29:99-118.
7. Aminah Ahmad, Zoharah Omar. Gender Differences in Work-Family Conflict and Family-Friendly Employment Policy Practices. The International Journal of the Humanities. 2008; 6(3):15-26.
8. Baotham S, Hongkhuntod W, and Rattanajun S. The Effects of Job Satisfaction and Organizational Commitment on Voluntary Turnover Intention of Thai Employees In The New University. Review of Business Research. 2010;10(1):73-82.

9. Baron RM, Kenny DA. The Moderator-Mediator Variable Distinction in Social Psychological Research: Conceptual, Strategic, and Statistical Considerations. Journal of Personality and Social Psychology. 1986;51:1173-1182.
10. Blau PM. Exchange and Power in Social Life. New York: Wiley. 1964.
11. Denvir A, McMahon F. Labour Turnover in London Hotels and the Cost Effectiveness of Preventative Measures. International Journal of Hospitality Management.2002;11(2):143-54.
12. Dulk L den, Peper A, Doorne-Huiskes A van. Work and Family Life in Europe: Employment Patterns of Working Parents across Welfare States. In Peper, A., Doorne-Huiskes, A. van., and Dulk, L.den. (Eds.), Flexible Working and Organizational Change. The Integration of Work and Personal life. 2005. (pp.13-38). Cheltenham: Edward Elgar.
13. Gaan N. Stress, Social Support, Job Attitudes and Job Outcome Across Gender. The Icfai University Journal of Organizational Behavior. 2008;52:34-44.
14. Garino G, Martin C. The Impact of Labour Turnover: Theory and Evidence from UK Micro-Data. Discussion Papers in Economics 05/10, Department of Economics, University of Leicester. 2005.
15. Gibson JL, Tremble TR. Influences of Work-Life Support of Officers' Organizational Commitment and Negative Work-Family Spillover. Personnel Performance and Training Technology. 2006;270:1-23.
16. Haar JM, Spell CS. Programme Knowledge and Value of Work-Family Practice and Organizational Commitment. International Journal of Human Resource Management**.** 2004;15(6):1040-1055.
17. Lambert SJ. Added benefits: The Link Between Work-Life Benefits and Organizational Citizenship Behavior. Academy of Management Journal. 2000;43(5):801-815.
18. Lee TW, Mowday RT. Voluntarily Leaving an Organization : An Empirical Investigation of Steers and Mowday's Model of Turnover. Academy of Management Journal. 1987;30(3):721-743.
19. Meyer JP, Allen NJ. A Tree-component Conceptualization of Organizational Commitment. Human Resource Management Review. 1991;1:61-89.
20. Meyer JP. Smith CA. HRM Practices and Organizational Commitment: Test of a Mediation Model. Canadian Journal of Administration Sciences. 2000;17:319-331.
21. Miller Y. 2010. Labour Turnover and its Impact on Customer Service and Success of Restaurants in London. Retrieved on July 6, 2010 from http://www.articleblast.com/
22. Muse L, Harris SG, Giles WF, Field HS. Work-life Benefits and Positive Organizational Behavior: is there a Connection? Journal of Organizational Behavior. 2008;29:171–192.
23. O'Neill JW, Harrison MM, Cleveland J, Almeida D, Stawski R. Crouter AC. Work–family Climate, Organizational Commitment, and Turnover: Multilevel Contagion Effects of Leaders. Journal of Vocational Behavior. 2009;74(1):18-29.
24. Perrewe´ PL, Treadway DC, Hall AT. The Work and Family Interface: Conflict, Family-friendly Policies, and Employee Well-being, in Health and Safety in Organizations: A Multilevel Perspective, eds. D.A. Hoffman and L.E. Tetrick, San Francisco, CA: Jossey-Bass, 2003. pp. 285–315.
25. Pasewark WR. Viator RE. Sources of Work-Family Conflict in the Accounting Profession. Behavioral Research in Accounting. 2006;18:147–165.
26. Poelmans SAY, N. Chinchilla P. Cardona. The Adoption of Family-friendly HRM Policies: Competing for Scarce Resources in Labor Market. International Journal of Manpower. 2003. 24:128-147.
27. Rhoades S, Eisenberger R, Armeli S. Affective Commitment: The Contribution of Perceived Organizational Support. Journal of Applied Psychology. 2001;86:825-836.
28. Rothbard NP, Phillips KW, Dumas TL. Managing Multiple Roles: Work-Family Policies and Individuals' Desires for Segmentation. Organization Science. 2005;16(3):243-258.
29. Seashore SI, Lawler EE, Mirvis PH, Cammann C. Assessing Organizational Change - A Guide to Methods, Measure and Practices New York: John Wiley and Sons. 1983.
30. Scott D, Bishop JW, Chen X. An Examination of the Relationship of Employee Involvement with Job Satisfaction, Employee Cooperation, and Intention to Quit in US Invested Enterprise in China. The International Journal of Organizational Analysis. 2003;11:3-19.
31. Thompson CA, Beauvais LL, Lyness KS. When Work-family Benefits are not Enough: The Influence of Work-family Culture on Benefit Utilization, Organizational Attachment, and Work-family Conflict. Journal of Vocational Behavior. 1999; 54:392–415.
32. Thompson CA, Jahn EW, Kopelman RE, Prottas DJ. Perceived Organizational Family Support: A

Longitudinal and Multilevel Analysis. Journal of Managerial Issues. 2004;16:545–565.
33. Thompson CA, Prottas DJ. Relationships among Organizational Family Support, Job Autonomy, Perceived Control, and Employee well-being. Journal of Occupational Health Psychology. 2005;11(1):100-118
34. Tuzun IK. Antecedents of Turnover Intention toward a Service Provider. The Business Review. 2007;8:128-135.
35. Wang P, Walumbwa FO. Family-friendly Programs, Organizational Commitment, and Work Withdrawal: The Moderating Role of Transformational Leadership. Personnel Psychology. 2007;60(2):397-427.
36. Wood S, De Menezes L, Lasaosa A. Family-friendly Management in Great Britain: Testing Various Perspectives. Ind. Relat. 2003;42:221-50.
37. Yanadoria Y, Katob T. Work and Family Practices in Japanese Firms: Their Scope, Nature and Impact on Employee Turnover. The International Journal of Human Resource Management. 2010;20(2):439–456.
38. Yeoh SF, Lim C-L, Syuhaily Osman. An Exploratory Study on Turnover Intention among Private Sector Employees. International Journal of Business and Management. 2010;5(8):57-64.

11/10/2010

Synbiotic Tarhana as a functional food

***Shreef G N Gabrial, ** Ahmed H Zaghloul, ***Abd El-Rahman M Khalaf-Allah, ***Nagwa M El-Shimi, *Rasha S Mohamed and *Gamal N Gabrial**

* Food Science and Nutrition Department, National Research Centre, Dokki, Cairo, Egypt.
**Dairy Science Department, National Research Centre, Dokki, Cairo, Egypt.
***Food Science Department, Faculty of Agriculture, Cairo University, Giza, Egypt.
snoub_2000@yahoo.com

Abstract: In the present study formulated synbiotic tarhana (Turkish fermented cereal food) was produced as a functional food from the fermentation of wheat flour, some spices [salt, pepper, dill and sweet marjoram (Organum majorana)], some vegetables [tomato (Lycoprsicum esculentum), pepper (Capsicum annum) and onion (Allium cepa)], and synbiotic yoghurt which prepared with prebiotic (inulin and lactose each 3%) and different concentrations of the probiotic culture (0.5, 1.5, 3, 4.5% DVS-ABT2 containing Streptococcus thermophilus, Lactobacillus acidophilus and Bifidobacterium bifidum). After fermentation (3 days), tarhana dough was dried in the sun. The effect of the fermentation (0, 1, 2 and 3 days) and the probiotic culture concentration on the chemical composition and the probiotic population of the wet tarhana were evaluated. The effect of the probiotic culture concentration on the chemical composition, the probiotic population and the sensory attribute of dried tarhana were evaluated. Also the effect of dried tarhana (prepared from yoghurt which was fermented by 4.5% probiotic culture) on the plasma lipid profile of human subjects was studied. The results showed that the pH value decreased while the acidity increased, acetaldehyde and diacetyl values increased during the fermentation period and by increasing the probiotic culture concentration of the wet and the dried tarhana. Neither the fermentation nor the concentration of the probiotic culture of wet and dried tarhana affected the crude protein, ether extract, crude fibre, and ash values. The numbers of probiotic bacteria increased until the second day of fermentation. However, in the following day, with an increase of the acid content their number decreased. Generally the increasing of the probiotic culture concentration increased the numbers of probiotic bacteria of the wet and dried tarhana. Also the concentration of the probiotic culture didn't affect the sensory attributes of dried tarhana. Subjects supplemented with dried tarhana showed significant reduction in total plasma cholesterol, low density lipoproteins (LDL-C) and triglycerides, while high density lipoprotein (HDL-C) increased.
[Shreef G N Gabrial, Ahmed H Zaghloul, Abd El-Rahman M Khalaf-Allah, Nagwa M El-Shimi, Rasha S Mohamed and Gamal N Gabrial. **Synbiotic Tarhana as a functional food**. Journal of American Science 2010;6(12):847-857]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: Tarhana, functional food, fermented food, probiotic, synbiotic yoghurt, serum lipids.

1. Introduction

Fermentation as an old and economical method of producing and preserving food, it is carried out to enhance flavor, aroma, shelf-life, texture, nutritional value and other pleasant and appealing properties of foods (Steinkraus, 2002). It is possible to obtain probiotic foods from several matrices, including both fermented and non-fermented products (Rivera-Espinoza and Gallardo-Navarro 2010). Tarhana is a traditional fermented milk-cereal mixture containing lactic acid bacteria with probiotic properties. Tarhana has been considered as one of the oldest probiotic foods (Ozdemir *et al*. 2007).

Tarhana is a popular traditional Turkish fermented wheat food produced both commercially and in homes. It is mainly used in the form of a thick and creamy soup consumed at lunch or dinner and is easily digested (Bilgicli and Elgun 2005). Tarhana is prepared by mixing wheat flour, yoghurt, yeast and a variety of cooked vegetables and spices (tomatoes, onions, salt, mint, paprika) followed by fermentation for one to seven days (Daglioglu 2000). Lactic acid bacteria and the yeast are responsible for the acid formation during fermentation and the leavening effect. The dough at fermentation is called as wet tarhana. Afterwards, the dough is dried in the sun or by dryer as a lamp, nugget or thin layers to obtain dry tarhana. Also the tarhana is locally consumed as snack after being dried as thin layer or nugget, not to be ground. Since there is no standard production method, nutritional properties of tarhana strictly depend on ingredients and their ratios in the recipe (Erbas *et al*. 2006).

Tarhana is a good source of minerals, organic acids and free amino acids which make it healthy for children, the elderly and medical patients. In addition, it is a good source of vitamins such as

thiamine, riboflavin and vitamin B12 (Ibanoglu *et al*. 1995). Ascorbic acid, niacin, pantothenic and folic acid are also present (Ekinci, 2005, Ekinci and Kadakal 2005). Lacticacid bacteria (LAB) from yoghurt also aid in absorption of nutrients, which would otherwise, be indigestible or poorly digestible. (Farnworth, 2003).

Fermentation of tarhana dough is generally carried out using yoghurt bacteria, such as Lactobacillus bulgaricus, Streptococcus thermophilus and Baker's yeast (Saccharomyces cerevisiae) (Bilgicli and Ibanoglu 2007). This is similar to other natural systems (e.g. Kefir grains) in which associations of LAB and yeasts are used in food fermentation (Gobbetti 1998). The fermentations occur simultaneously during this aspect of production (Bilgicli et al. 2006). Yeast fermentation proceeds through the Embden-Meyerhof pathway (EMP), in which glucose is transformed into ethanol (via piruvate and acetaldehyde), carbon dioxide, and traces of other acids and carbonyl compounds (Gobbetti 1998, Gelinas and McKinnon 2000). According to Mugula et al. (2003) a combined culture of yeasts and lactobacilli cause a more significant decrease in pH (increase in acidity), than with the use of single cultures in the fermented millet.

The present study was initiated to produce synbiotic tarhana and evaluate it as a functional food. The effect of fermentation time (0, 1, 2 and 3 days) and starter concentrations (0.5, 1.5, 3 and 4.5%) on the chemical composition and the probiotic bacterial counts of wet tarhana were evaluated. Also the effect of starter concentrations (0.5, 1.5, 3 and 4.5%) on the sensory attributes, chemical composition and the probiotic bacterial counts of dried tarhana were evaluated. The hypocholesterolemic effect of dried tarhana on human subjects was studied.

2. Material and Methods

Tarhana ingredients: Vegetables [Tomato (Lycopersicum Esculentum), Green Pepper (Capsicum Annum), Chicory (Cicohorium Intybus) and Onion (Allium Cepa)], Cereals [Wheat (Triticum oestivum)], Spices [salt, pepper, dill and sweet marjoram (Origanum majorana)], Yeast (Saccharomyces cervisiae, press form) were purchased from the local market, Cairo, Egypt. Lactulose syrup (52.40% lactulose, 4.3% lactulose and 2.5 galactose) was obtained from the Egyptian International Industries Company (EIPICO), Cairo, Egypt and Spray dried skim milk (low heat) was obtained from Dina for Agriculture Investments, Egypt.

Probiotic Culture: DVS-ABT2 (containing Streptococcus thermophilus, Lactobacillus acidophilus and Bifidobacterium bifidum) were obtained from Chr. Hansen's Lab., Copenhagen Denmark. M17 agar, MRS-Salccin agar, violet red bile agar (VRBA), potato dextrose agar, and MRS agar. All media were purchased from Oxoid LTD, London.

Preparation of Chicory Water-Soluble Extract

Chicory Water-Soluble Extract was prepared according to the methods described by Kim and Shin (1998) as follows: 10g dried chicory plant was dissolved into 200 ml distillated water, soaked for 24 hours under refrigeration, heated at 70^{o}C for 15 min, then filtered. Chicory extract was then added to the synbiotic yoghurt.

Preparation of synbiotic yoghurt

Milk samples were standardized by adding skim milk powder to achieve 16% total solids content, pasteurized (15 min. at 85^{o}C) and cooled to 40^{o}C. Chicory extract and lactulose syrup (3% each) were individually added to milk samples, then inoculated with different concentrations (0.5, 1.5, 3, 4.5%) of the DVS-ABT2 culture, then milk was dispensed into pasteurized plastic cups (100 ml), capped, incubated (5 hours at 44^{o}C) cooled and stored in the refrigerator at 5^{o}C to prepare synbiotic tarhana.

Preparation of synbiotic tarhana

The ingredients of tarhana are presented in Table (1). Production method of tarhana is presented in fig. (1). All ingredients were prepared (cleaned, peeled and cut), then mixed, blended, pasteurized (30 min. at 65^{o}C) and cooled at 25^{o}C, whole flour, salt, synbiotic yoghurt (with different concentrations of the probiotic culture) and Baker's yeast were added to the mixture, then kneaded to form tarhana dough. The dough was fermented (3 days at 25^{o}C) in an incubator. The samples were withdrawn at time intervals (0, 1, 2 and 3 days) for chemical analysis and microbial analysis. Also tarhana samples (fermented for 3 days) were dried in the sun, filled in small packages and stored. The dried tarhana were subject to chemical, microbial and sensory evaluation and it was also used in human studies to evaluate its hypolipidemic effect.

Chemical analysis

pH value, total acidity, crude proteins, ether extract, crude fiber and ash were determined according to AOAC (2000). Acetaldehyde was estimated as described by Lees and Jaco (1969). Diacetyl was determined according to Lees and Jaco (1970).

Microbial analysis

Bifidobacterium bifidum was enumerated according to Dave and Shah (1969) using the modified MRS agar supplemented with 0.05% L. cysteine-HCl. The plates were anaerobically incubated at 37°C for 48 hours using anaerogen sheets.

Lactobacillus acidophilus count was estimated according to Dave and Shah (1969) on MRS-salccin agar. Incubation was carried out at 37°C for 48 hours.

Streptococcus thermophilus count was estimated according to Terzaghi and Sandine, (1975) using M17 agar. Incubation was carried out at 25°C for 48 hours.

Moulds were enumerated according to standard methods for examination of dairy products (APHA, 1994). Incubation was carried out at 25°C for 4 – 5 days.

Coliform group bacteria were enumerated according to standard methods for examination of dairy products (APHA, 1994) using violet red bile agar (VRBA). Incubation was carried out at 37°C for 48 hours.

Sensory evaluations

Synbiotic tarhana samples were organoleptically evaluated by 10 panelists from the staff members of food science and nutrition department of the National Research Center, Dokki, Cairo, Egypt. The panelists evaluated the samples using a five point Hedonic scale (5 = Liked Extremely to 1 = Unacceptable) adopted from (Iwe, 2000). All samples were evaluated for appearance, taste and general acceptability. The samples were filled in small white porcelain bowl (150 ml) and they were coded with numbers and served to the panelists at random.

Human experiment

Fifteen hyperlipidemic volunteers aged between 40 and 55 years old were studied, all were in good general health, with no history of cardiovascular or gallbladder disease, non of the volunteers were taking any medications. They were given their regular diet which was daily supplemented with 200g of synbiotic tarhana (prepared from yoghurt which was inoculated by 4.5% probiotic culture) for 45 days.

Blood sampling

Blood samples were collected from each volunteer, before supplementation and at the end of the experimental period, after overnight fasting and withdrawn through heparinized tubes for serum. The blood was allowed to clot at room temperature for one hour, and then the serum was separated by centrifugation at 3000 rpm for 15 minutes, clear serum was divided into aliquots and stored at 20°C until analyzed.

Biochemical analysis

Blood lipids were estimated according to the following methods, total cholesterol (Allain et al., 1974), total triglycerides (Fossati and Prencipe 1982), high density lipoproteins (Lopes-Virella et al., 1977), and low density lipoproteins (Friedewald et al., 1977).

3. Results

Evaluation of tarhana dough

Changes in some of the chemical components of tarhana dough samples [prepared with yoghurt and inoculated by different concentrations (0.5, 1.5, 3 and 4.5%) of the probiotic culture (DVS-ABT2)] were studied in relation to different fermentation time. Table 2 shows that for all tested tarhana dough samples, fermentation time had no effect on crude fiber, ether extract and ash. However, fermentation time had an effect on pH value and acidity of tarhana dough. The acidity content of all studied tarhana dough samples increased by increasing the fermentation time and consequently the pH values decreased until the end of fermentation course. Also increasing of probiotic culture concentrations from 0.5 to 4.5% decreased the pH values and increased the acidity content of the resultant tarhana dough samples. The pH value decreased from 5.47, 4.89, 4.62 and 4.57 to 4.89, 4.09, 4.08 and 3.92 while the acidity increased from 3.9, 5.0, 6.8 and 7.7 to 7.4, 9.7, 10.2 and 13.6 respectively during the fermentation time when a probiotic culture inoculation of 0.5, 1.5, 3 and 4.5% respectively was added.

Acetaldehyde and diacetyl contents of tarhana dough samples increased during fermentation. Also they increased with increasing of the probiotic culture concentrations in tarhana dough as shown in Figure 2 and 3. The effect on fermentation time on microbial counts of tarhana dough samples are presented in Table 3. Results indicate that the microbial counts numbers increased by increasing the fermentation time reaching the highest level at the second day of fermentation, then these decreases by increasing that period to record the lowest level at the end of fermentation (3 days). It was found that increasing the probiotic culture concentrations 0.5, 1.5, 3 and 4.5% (samples A, B, C and D) increased the numbers of probiotic bacteria as shown in Table 3. The highest population of probiotic bacteria from tarhana dough samples that contained 0.5, 1.5, 3 and 4.5% probiotic culture was recorded at the second day of fermentation being 8.5 x 10^7, 5.9 x

10^9. 9.7 x 10^{10} and 9.9 x 10^{10} (cfu/g) for *L. acidophilus*, 7.2 x 10^7, 6.4 x 10^9, 6.1 x 10^{10} and 7.6 x 10^9 (cfu/g) for *S. thermophilus* and 6.4 x 10^7, 8.6 x 10^8, 8.2 x 10^9 and 9.0 x 10^9 (cfu/g) for *B. bifidum*. Also, the results indicate that all tarhana dough samples were free from coliform and mold during the fermentation period, indicating no contamination occurred from the environment or the row materials.

Sensory characteristics (flavor, body and texture and appearance) of dried tarhana samples (A, B, C and D) prepared with different concentrations of probiotic culture (0.5, 1.5, 3 and 4.5%) were evaluated as shown in Table 4. The obtained results show that the sensory evaluation properties of dried tarhana had good scores and were acceptable for all the samples which contained different concentrations of probiotic culture.

Chemical composition of dried tarhana samples prepared by yoghurt inoculated with different concentrations of probiotic culture (0.5, 1.5, 3 and 4.5%) is presented in Table 5. Results indicate that all dried samples (A, B, C and D) had a protein content that ranged between (19.87-19.88%). All dried samples (A, B, C and D) had a fiber and ash content that ranged between (3.80-3.82%) and (9.93-9.95%) respectively. All dried samples (A, B, C and D) had an ether extract that ranged between (2.90-2.92%). Concerning pH value and acidity content of dried tarhana sample results Table 5 disclose that the acidity increased and pH value decreased by increasing the concentration of the probiotic culture. The highest acidity (13.4) and the lowest pH value (3.9) were recorded for dried tarhana sample D (containing 4.5% probiotic culture). Acetaldehyde and diacetyl contents of the dried tarhana are shown in figure 4. Acetaldehyde and diacetyl contents of the dried tarhana samples increased by increasing probiotic culture concentration. The highest content of Acetaldehyde (0.65 µmol/ml) and diacetyl (0.55 µmol/ml) were obtained for tarhana samples contained 4.5% probiotic culture, while the lowest contents were obtained for sample having 0.5% probiotic culture being 0.46 and 0.31 µmol/ml consecutively.

Effect of sun drying on microbial population of tarhana samples (A, B, C, and D) is shown in Table 6, data presented disclose that all dried tarhana samples recorded a sharp decrease in probiotic bacterial counts after drying compared to the corresponding values at the end of fermentation (day three) as shown in Table 3.

Hypolipidemic effect

Table 7 shows the changes in total cholesterol, total triglycerides, low and high density lipoprotein of the subjects that consumed dried synbiotic tarhana for 45 days (prepared from yoghurt which was inoculated by 4.5% probiotic culture). Results show a significant hypocholesterolemic effect where the mean of the serum cholesterol concentration was (222.0 ± 5.2) at the start of experiment then decreased to (202.6 ± 8.5) at the end of the experiment, triglyceride level showed a highly significant reduction from (179.8 ± 5.4) at the start of experiment to (169.0 ± 5.5) at the end of the experiment, high-density lipoprotein cholesterol was significantly raised from (50.1 ± 1.0) to (57.8 ± 0.9) at the end of the experiment. As for the low-density lipoprotein cholesterol there was no significant change with a value of (92.7 ± 0.7) at the start of experiment to (81.9 ± 0.5) at the end of the experiment.

Table 1: Synbiotic Tarhana Ingredients (% w/w)

Ingredients	% w/w
Whole wheat flour	35
Synbiotic Yoghurt	25
Fresh onions	12
Fresh tomato	10
Fresh red pepper	6
Green pepper	4
Baker's yeast	4
Salt	2
Dill powder	1
Sweet marjoram	1

Table 2: Changing in pH value, acidity, crude protein, crude fibre, ether extract and ash of tarhana dough samples prepared with yoghurt fermented by different concentrations of DVS-ABT2 culture during fermentation.

Tarhana samples	Fermentation time (days)	pH	Acidity	Crude protein	Ether Extract	Crude Fibre	Ash
				(g/100g)			
A	0	5.47	3.9	19.88	2.90	3.80	9.94
	1	5.24	4.2	19.88	2.91	3.81	9.93
	2	4.90	7.4	19.87	2.91	3.81	9.94
	3	4.89	7.4	19.88	2.92	3.80	9.91
B	0	4.89	5.0	19.89	2.91	3.81	9.95
	1	4.67	6.9	19.90	2.92	3.80	9.97
	2	4.11	9.5	19.87	2.92	3.81	9.93
	3	4.09	9.7	19.88	2.91	3.81	9.94
C	0	4.62	6.8	19.89	2.91	3.82	9.96
	1	4.44	6.5	19.90	2.90	3.83	9.94
	2	4.10	10.0	19.88	2.93	3.82	9.95
	3	4.08	10.2	19.87	2.91	3.81	9.95
D	0	4.57	7.7	19.89	2.91	3.80	9.91
	1	4.36	9.2	19.89	2.90	3.82	9.94
	2	4.94	13.5	19.87	2.91	3.81	9.95
	3	3.92	13.6	19.88	2.93	3.81	9.93

(A): prepared using yoghurt incubation with 0.5% probiotic culture. (B): prepared using yoghurt incubation with 1.5% probiotic culture. (C): prepared using yoghurt incubation with 3% probiotic culture. (D): prepared using yoghurt incubation with 4.5% probiotic culture.

Table 3: Effect of fermentation time on microbial counts (cfu/g) of tarhana dough prepared with yoghurt fermented by different concentrations of DVS-ABT2 culture.

Tarhana samples	Fermentation time (days)	Microbial counts (cfu/g)				
		L. acidophilus	S. thermophilus	B. bifidum	Molds	Coliform
A	0	9.3×10^4	8.7×10^4	2.2×10^4	ND	ND
	1	7.9×10^5	6.7×10^5	5.3×10^5	ND	ND
	2	8.5×10^7	7.2×10^7	6.4×10^7	ND	ND
	3	4.0×10^6	6.9×10^5	5.0×10^6	ND	ND
B	0	5.9×10^7	7.2×10^7	8.3×10^6	ND	ND
	1	6.1×10^8	3.9×10^8	5.9×10^7	ND	ND
	2	5.9×10^9	6.4×10^9	8.6×10^8	ND	ND
	3	7.5×10^8	6.6×10^8	9.2×10^7	ND	ND
C	0	8.0×10^8	5.5×10^8	3.8×10^7	ND	ND
	1	2.9×10^9	2.5×10^9	1.1×10^8	ND	ND
	2	9.7×10^{10}	6.1×10^{10}	8.2×10^9	ND	ND
	3	8.4×10^9	6.9×10^9	8.0×10^8	ND	ND
D	0	8.9×10^8	7.3×10^9	8.8×10^7	ND	ND
	1	5.3×10^9	6.1×10^9	3.6×10^8	ND	ND
	2	9.9×10^{10}	7.6×10^{10}	9.0×10^9	ND	ND
	3	7.0×10^9	6.5×10^9	9.1×10^8	ND	ND

(A): Prepared using yoghurt incubation with 0.5% probiotic culture. (B): Prepared using yoghurt incubation with 1.5% probiotic culture. (C): Prepared using yoghurt incubation with 3% probiotic culture. (D): Prepared using yoghurt incubation with 4.5% probiotic culture.
ND = Not Detected

Table 4: sensory attributes of dried tarhana prepared with yoghurt fermented by different concentrations of DVS-ABT2 culture.

Tarhana samples	flavor	body and texture	appearance	Total (15)
A	4.5	4.6	3.5	12.6
B	4.6	4.6	4.0	13.2
C	4.5	4.5	4.0	13
D	4.5	4.6	3.5	12.6

Each value represents the mean of ten panel's degree
(A): Prepared using yoghurt incubation with 0.5% probiotic culture. (B): Prepared using yoghurt incubation with 1.5% probiotic culture. (C): Prepared using yoghurt incubation with 3% probiotic culture. (D): Prepared using yoghurt incubation with 4.5% probiotic culture.

Table 5: chemical composition of dried tarhana prepared with yoghurt fermented by different concentrations of DVS-ABT2 culture.

Components	Tarhana samples			
	A	B	C	D
pH	4.90	4.08	4.10	3.94
Acidity	7.5	9.5	10.4	13.4
Crude protein(g/100g)	19.87	19.88	19.87	19.89
Ether extract (g/100g)	2.92	2.92	2.91	2.90
Crude fiber (g/100g)	3.81	3.82	3.82	3.80
Ash (g/100g)	9.93	9.94	9.98	9.93

(A): Prepared using yoghurt incubation with 0.5% probiotic culture. (B): Prepared using yoghurt incubation with 1.5% probiotic culture. (C): Prepared using yoghurt incubation with 3% probiotic culture. (D): Prepared using yoghurt incubation with 4.5% probiotic culture.

Table 6: Microbial counts of dried tarhana samples prepared with yoghurt fermented by different concentrations of DVS-ABT2 culture.

Microorganisms (CFU/g)	Tarhana samples			
	A	B	C	D
L. acidophilus	5.0×10^2	6.2×10^3	4.0×10^4	7.4×10^4
S. thermophilus	9.2×10^2	4.0×10^3	3.6×10^4	7.9×10^4
B. bifidum	3.4×10^2	5.1×10^3	4.1×10^4	8.8×10^4
Molds	ND	ND	ND	ND
Coliform	ND	ND	ND	ND

(A): Prepared using yoghurt incubation with 0.5% probiotic culture. (B): Prepared using yoghurt incubation with 1.5% probiotic culture. (C): Prepared using yoghurt incubation with 3% probiotic culture. (D): Prepared using yoghurt incubation with 4.5% probiotic culture.
ND = Not Detected

Table 7: Plasma lipid profile of experimental group before and after 45 days of dietary supplement. [1]

Parameters	Before (Mean±SE)	After (Mean±SE)
Cholesterol (mg/dl)	222.0 ± 5.2	202.6 ± 8.5*
TGs (mg/dl)	179.8 ± 5.4	169.0 ± 5.5**
HDL-Ch (mg/dl)	50.1 ± 1.0	57.8 ± 0.9**
LDL-Ch (mg/dl)	92.7 ± 0.7	81.9 ± 0.5

[1] Supplement by dried synbiotic tarhana (prepared from yoghurt which was inoculated by 4.5% probiotic culture), n=15, *= significant $p<0.05$ **= high significant $p<0.01$
TGs = Triglycerides, HDL-Ch = high-density lipoprotein cholesterol , LDL-Ch = low-density lipoprotein cholesterol.

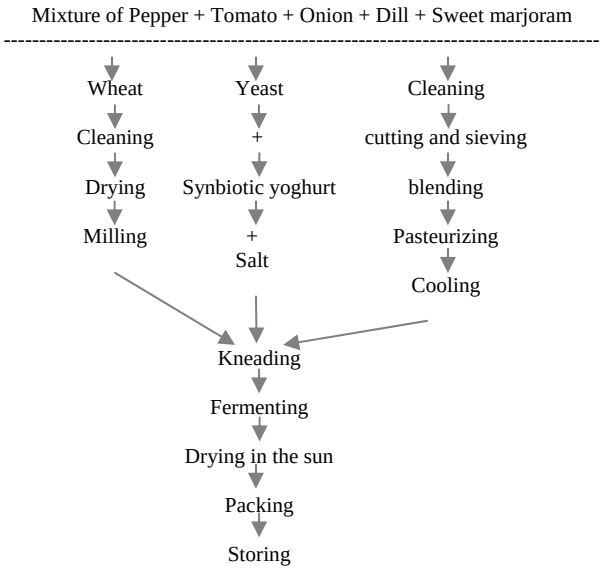


Figure 1: Flow chart for the preparation of tarhana.

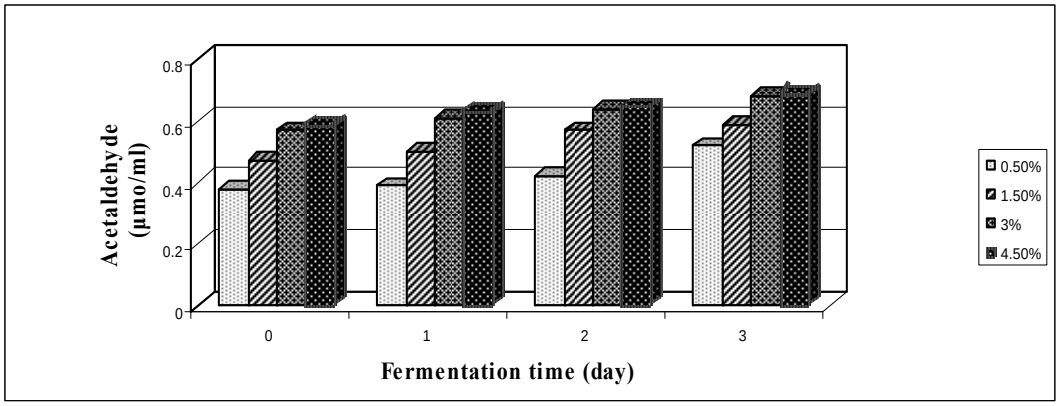


Figure 2: Effect of fermentation time on acetaldehyde contents (µmo/ml) of tarhana dough prepared with yoghurt fermented by different concentration (0.5, 1.5, 3 and 4.5%) of DVS-ABT2 culture.

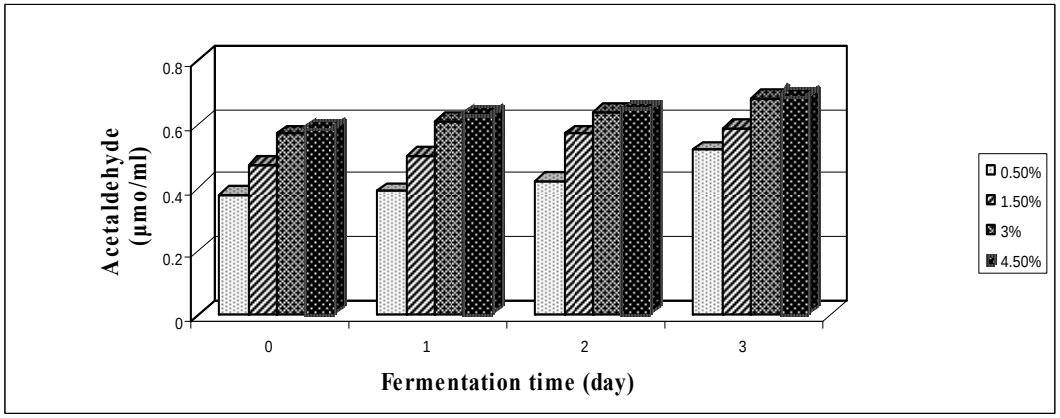


Figure 3: Effect of fermentation time on diacetyl contents (µmo/ml) of tarhana dough prepared with yohgurt fermented by different concentration (0.5, 1.5, 3 and 4.5%) of DVS-ABT2 culture.

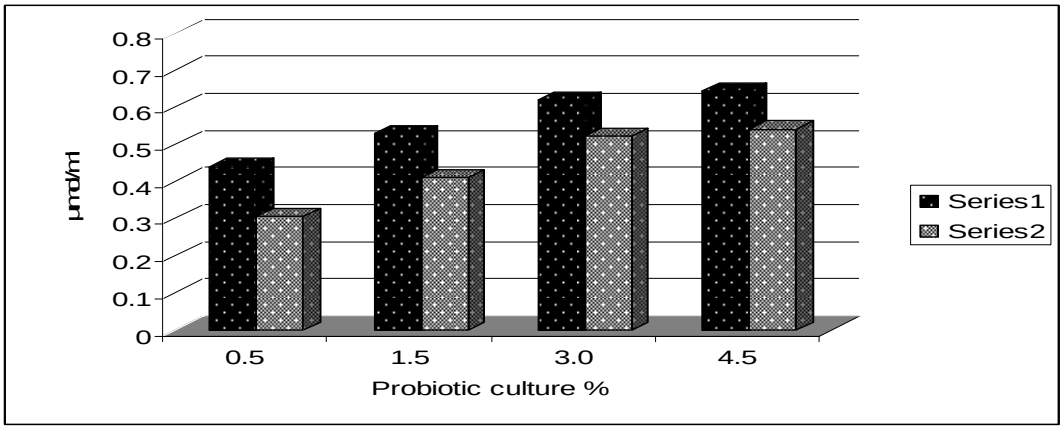


Figure 4: Acetaldehyde and diacetyl contents (µmol/ml) of the dried tarhana prepared with yoghurt fermented by different concentrations of DVS-ABT2 culture.

4. Discussion:

In the present study, changes in some of the chemical components of tarhana dough samples were studied in relation to different fermentation time. Results show that for all tested tarhana dough samples, fermentation time had no effect on crude fiber, ether extract and ash. This is in the agreement with the results obtained by Erbas *et al*. (2005), who found out that fermentation had no significant effect on dry matter, crude protein, ether extract and ash.

Data obtained revealed that the acidity content of all studied tarhana dough samples increased by increasing the fermentation time and consequently the pH values decreased until the end of fermentation course. The increase in the acidity followed by a decrease in pH value may be due to the formation of organic acids from fermentation of sugars mostly by probiotic bacteria. Such findings coincide with those reported by Ibanoglu *et al*. (1995) and Erbas *et al*. (2005), who demonstrated that the acidity of tarhana dough increased and the pH decreased during fermentation.

Acetaldehyde and diacetyl are two important aromatic compound. Acetaldehyde and diacetyl contents of the tarhana dough samples increased with increasing of the probiotic culture concentrations in tarhana dough. Acetaldehyde content is attributed to probiotic bacteria (Erbas *et al*. 2005).

Results indicate that the microbial counts numbers increased by increasing the fermentation time reaching the highest level at the second day of fermentation, then it decreases by increasing that period to record the lowest level at the end of fermentation (3 days). These results are in accordance with those obtained by Daglioglu *et al*. (2002) using Turkish tarhana. The results of bacterial counts are close to those reported by Capela *et al*. (2006), who, reported that the viability of probiotic organisms in yoghurt that had been inoculated with 4% was more than in yoghurt that had been inoculated with 2% before and after fermentation. Ibanoglu *et al*. (1999) found that the increasing of yoghurt amount from 500g to 1000g in tarhana during the fermentation increased the population of probiotic bacteria of tarhana.

Sun drying is a slower but a more common and economical approach for traditional tarhana production. As for the drying process the critical moisture value is 13 – 15% for the inhibition of undesirable microbial growth in dry recipes produced from wheat flour (Bozkurt and gurbuz, 2008). The moisture content of tarhana is low, that it can be stored for 2 or 3 years without deterioration (Ibanoglu et al. 1999, Tarakc et al. 2004).

The results of the sensory analysis show that the use of yeast in the tarhana formula had a positive effect on the sensory properties. This shows that yoghurt bacteria and yeast together produce lactic acid, ethyl alcohol, carbon dioxide, and other fermentation products, which give tarhana its characteristic taste and flavour (Koca et al. 2002).

The protein content of the dried samples was higher than that obtained by Daglioglu (2000), Kose and Cagndi (2002) who reported that the crude protein content of dried tarhana were between 14.5 – 16 %. The latter author added that dried tarhana is a good source of protein. This can be explained by the differences in the tarhana formulas produced in different regions of Turkey.

The fiber and ash content in the present study were higher than that obtained by Daglioglu (2000), Erbas *et al*. (2006) who reported that the crude fiber and ash content of dried tarhana were 1% and 6.2%. The ether extract content was less than that obtained by Daglioglu (2000), Erbas *et al*. (2006) who reported that the ether extract content of dried tarhana was 5.4%. This can be explained by the

differences in the tarhana formulas produced in different regions of Turkey.

Drying treatments can slightly affect the increase in the pH and the decrease in acidity contents of the tarhana samples. The acetaldehyde and diacetyl contents of the dried tarhana may be due to the formation of aromatic components which is attributed to the presence of probiotic bacteria as reported by Erbas et al. (2005).

The sharp decrease in probiotic bacterial counts after drying coincide with the results reported by Daglioglu et al. (2002) who observed a sharp drop in L.A.B. population of tarhana samples after conventional drying. Also Erbas *et al*. (2005) attributed the decrease of L.A.B. population to the low water content of dried tarhana samples.

In the present study it was found that increasing the probiotic culture concentrations increased the number of probiotic bacteria of dried tarhana. These results are in agreement with those reported by Capela *et al*. (2006) who, disclosed that the viability of probiotic organisms in freeze-dried yoghurt was increased by increasing the inoculums volume from 2 to 4 %.

Hypolipidemic effect

The relationship between atherosclerotic cardiovascular disease and nutrition is very important. Many functional foods have been found to be potentially beneficial in the prevention and treatment of cardiovascular disease. (Anderson 2003).

The hypocholesterolemic effect of dried tarhana may be due to its content of probiotic bacteria and prebiotic inulin because it is soluble in water and not hydrolyzed by human digestive enzymes, it is expected to behave like a soluble fiber and to have a hypolipidemic effect (Kim and Shin 1998), wheat flour which as explained by Illman et al. 1993 lowers plasma cholesterol and increases cecal steroids relative to whole wheat flour, wheat bran and wheat pollard in rats. Also wheat is among cereals containing high concentrations of ß-glucan which is known to have a cholesterol lowering effect (Newman et al. 1989, Mc Intoch et al. 1991). Vegetables such as onion have hypocholesterolemic effect by inhibiting hepatic cholesterol biosynthesis (Gupta and Porter 2001, Singh and Porter, 2006) , Lycopene from tomatoe led to reduction of serum total cholesterol (Agarwal and Rao 1998) and green pepper which prevents arteriosclerosis and lower cholesterol (Mezzetti et al. 1995).

5. Conclusion

Fermentation process is an important stage for the development of sensory profile of tarhana. Fermentation and increasing probiotic culture concentrations decreased the pH values and increased acidity, acetaldehyde and diacetyl values while neither the fermentation nor the concentrations of the probiotic culture affected crude protein, ether extract, crude fibre and ash values of wet and dried tarhana.

The increasing of the probiotic culture concentration from 0.5 to 3% ensured probiotic bacteria population of wet and dried tarhana at satisfactory level while increasing the probiotic culture concentration from 3 to 4.5% slightly increased probiotic bacteria population. Generally drying process decreased the viability of probiotic bacteria as drying decreased the water activity. So it has a poor medium for pathogens and spoilage organisms.

Since tarhana is a good source of B vitamins, minerals, organic acids, and free amino acids, and since it is a product of L.A.B. and yeast fermentation, it may be considered a functional and probiotic food with hypolipidemic effect.

Corresponding Author:
Shreef G.N. Gabrial
Food Science and Nutrition Department,
National Research Centre (NRC), Dokki, Cairo 12311, Egypt
E-mail: snoub_2000@yahoo.com

References

1. AOAC, Official Methods of Analysis. The Association of Official Agricultural Chem., 17th ed., U.S.A. (2000).
2. Agarwal, S and Rao, AV, Tomato lycopene and low density lipoprotein oxidation: a human dietary intervention study. *Lipids* 33:981- 4 (1998).
3. Allain CC, Poon LS, Chon CSG, Richmond U, Fu PC, Enzymatic determination of total serum cholesterol. Clin Chem 20:470–475 (1974).
4. American Public Health Association (APHA), Standard methods of the examination of dairy products. 16th ed., Washington, U.S.A. (1994).
5. Anderson, JW, Whole grains protect against atherosclerotic cardiovascular disease. *Proc.Nutr.Soc.* 62(1):135-142 (2003).
6. Bilgicli, N and Elgun, A, Changes in some physical and nutritional properties of tarhana, a Turkish fermented cereal food, added various phytase sources. *Food Science and Technology International*, 11(5):383-389 (2005).
7. Bilgicli, N and Ibanoglu, S, Effect of wheat germ and wheat bran on the fermentation activity, phytic acid content and colour or tarhana, a wheat flour- yoghurt mixture. *Journal of Food Engineering*, 78(2):681 – 686 (2007).

8. Bilgicli N, Elgun A, Herken EN, Turker S, Ertas N. and Ibanoglu, S, Effect of wheat and sensory quality of tarhana, a fermented wheat flour-yoghurt germ/bran addition on the chemical, nutritional product. *Journal of Food Engineering*, 77(3):680 – 686 (2006).
9. Bozkurt O and Gurbuz O, Comparison of lactic acid contents between dried and frozen tarhana. *Food Chemistry* 108:198 – 204 (2008).
10. Capela P, Hay TKC and Shah NP, Effect of cryoprotectants, preboitic and microencapsulatin on survival of probiotic organisms in yoghurt and freez-dried yoghurt. *Food Research International* 39:203 – 211 (2006).
11. Cavallero A, Empilli S, Brighenti F and Stanca AM, High (1-3, 1-4)-b-glucan barley fractions in bread making and their effect on human glycemic response. *Journal of Cereal Science* 36:59-66 (2002).
12. Daglioglu O, Tarhana as a traditional Turkish fermented cereal food. Its recipe, production and composition. *Die Nahrung* 44(2):85- 88 (2000).
13. Daglioglu O, Arici M, Konyali M and Gumus T, Effects of tarhana fermentation and drying methods on the fate of Escherichia coli O157:H7 and Staphylococcus aureus. *European Food Research and Technology* 215(6):515-519 (2002).
14. Dave RI, and Shah NP, Evaluation of media for selective enumeration of Streptococcus thermophilus, Lactobaccilus deblrueckii ssp. Bulgaricus, Lactobaccilus acidophilus and Bifidobacterium. *J. Dairy. Sci.* 79:1592 (1996).
15. Davidson MH, Maki KC and Synecki C, Evaluation of the influence of dietary inulin on serum lipids in adults with hypercholesterolemia. *Nutrition* 18: 503-17 (1998).
16. Ekinci R, The effect of fermentation and drying on the water-soluble vitamin content of tarhana, a traditional Turkish cereal food. *Food Chemistry* 90(2):127-132(2005).
17. Ekinci R and Kadakal C, Determination of seven water-soluble vitamins in tarhana, a traditional Turkish cereal food, by high-performance liquid chromatography. *Acta Chromatographica* 15:289-297 (2005).
18. Erbas M, Certel M and Uslu MK, Microbiological and chemical properties of tarhana during fermentation and storage as wet-sensorial properties of tarhana soup. *Swiss Society of Food Science and Technology* 38:409-416 (2005).
19. Erbas M, Uslu MK, Erbas MO and Certel M, Effects of fermentation and storage on the organic and fatty acid contents of tarhana, a Turkish fermented cereal food. *Journal of Food Composition and Analysis* 19:294-301(2006).
20. Farnworth ER, Handbook of fermented functional foods. Boca Raton, FL: CRC Press (2003).
21. Fossati P and Prencipe I, Serum triglyceride determintation colorimetrically with an enzyme that produce hydrogen peroxide. *Clin. Chem.* 28:2077 (1982).
22. Friedewald WT, Levy RI andFredrickson DS, Estimation of the concentration of low density lipoprotein cholesterol in plasma without the use of the preparative ultracentrifuge. Clin Chem 18:499–502, (1972).
23. Gelinas P and McKinnon C, Fermentation and microbiological processes in cereal foods. In Kulp K & Ponte JJ (Eds.). Handbook of cereal science and technology. New York: Marcel Dekker, Inc. vol. 99:741-754(2000).
24. Gobbetti M, The sour dough microflora: interactions of lactic acid bacteria and yeasts. *Trends in food science and technology* 9(7),267-274 (1998).
25. Gupta N and Porter TD, Garlic and garlic-derived compounds inhibit human squalene mono-oxygenase. *The Journal of Nutrition* 131:1662-1667 (2001).
26. Hayta M, Alpaslan M and Baysar A, Effect of drying methods on functional properties of Tarhana: a wheat flour-yoghurt mixture. *Journal of Food Science* 67(2):740-744 (2002).
27. Ibanoglu S, Ainsworth P, Wilson G and Hayes GD, The effect of fermentation conditions on the nutrients and acceptability of tarhana. *Food Chemistry* 53(2):143-147 (1995).
28. Ibanoglu S, Ibanoglu E and Ainsworth P, Effect of different ingredients on the fermentation activity in tarhana. *Food Chemistry* 64(1):103-106 (1999).
29. Illman R J, Storer G B and Topping D L, White wheat flour lowers plasma cholesterol and increases cecal steroids relative to whole wheat flour, wheat bran and wheat pollard in rats. *J. Nutr.* 123: 1094–1100 (1993).
30. Iwe MO, Sensory methods and analysis. *Regional Com., Aba, Nig.* 86-89 (2000).
31. Jacobs DR Jr, Marquart L, Slavin J and Kushi LH, Whole-grain intake and cancer: an expanded review and meta-analysis. *Nutrition and Cancer* 30(2):85-96 (1998).
32. Kim M and Shin HK The water-soluble extract of chicory influences serum and liver lipid concentrations, cecal short-chain fatty acid concentrations and fecal lipid excretion in rats. *The Journal of Nutrition* 128:1731-1736 (1998).

33. Koca AF, Yazici F, Anil M, Utilization of soy yoghurt in tarhana production. *European Food Research and Technology* 215: 293–297(2002).
34. Kose E and Cagindi OS An investigation into the use of different flours in tarhana. *Int. J. Food Sci. Tech.* 37(2):219-222 (2002).
35. Lees GJ and Jaco GR, Method for the estimation of acetaldehyde in cultured dairy products. Aust. J. Dairy Tech. 24:181 (1969).
36. Lees GJ and Jaco GR, The estimation of diacetyl in cultured in the presence of other carbonyl compounds. *J. Dairy Res.* 37:129 (1970).
37. Lopes-Virella MF, Stone P, Ellis S and Coldwell JA, Cholesterol determinations in high density liproproteins separated by three methods. Clin Chem 23:882–884, (1977).
38. McIntoch GH, Whyte J, McArther R and Nestel PJ, Barley and wheat foods: influence on plasma cholesterol concentrations in hypercholesterolemic men. *Am J Clin Nutr* 53(5):1205-1209 (1991).
39. Mensah P, Fermentation the key to food safety assurance in Africa. *Food Control* 8:271-278(1997).
40. Mezzetti A, Lapenna D, Piedromenico SD, Cala.ore AM, Constantini F, Riario-Sforza G, Imbastaro T, Neri M and Cuccurullo F, Vitamin E, C and lipid peroxidation in plasma and arterial tissue of smokers and non smokers, *Atherosclerosis* 112:91-99 (1995).
41. Mugula JK, Narvhus JA and Sorhaug T, Use of starter cultures of lactic acid bacteria and yeasts in the preparation of togwa, a Tanzanian fermented food. *International Journal of Food Microbiology*, 83(3):307-318 (2003).
42. Newman RK, Lewis SE, Newman CW, Boik RJ and Ramage RT, Hypocholesterolemic effect of barley foods on healthy men. *Nutritional Reports International* 39:749-760 (1989).
43. Nout MJR and Motarjemi Y, Assessment of fermentation as a household technology for improving food safety: a joint FAO/WHO workshop. *Food Control* 8:221-226 (1997).
44. Ozdemir S, Gocemen D and Kumral Y, A traditional Turkish fermented cereal food: Tarhana. *Food Rev. Int.* 23: 107-121 (2007).
45. Rivera-Espinoza Y and Gallardo-Navarro Y, Non dairy probiotic products. *Food Microbiology* 27:1-11 (2010).
46. Singh DK and Porter TD, Inhibition of sterol 4 alpha-methyl oxidase is the principal mechanism by which garlic decreases cholesterol synthesis. *Journal of Nutrition* 136:759-764 (2006).
47. Steinkraus KH, Fermentations in world food processing. Comprehensive Reviews in Food Science and Food Safety 1:23-30 (2002).
48. Tarakc Z, Dogan IS and Koca AF, A traditional fermented Turkish soup, tarhana, formulated with corn flour and whey. International Journal of Food Science and Technology 39:455-458 (2004).
49. Terzaghi BE and Sandine WE, Improved medium for lactic Streptococci and their bacterophages. Appl. Microbial 29:807 (1975).

11/1/2010

Response of Wheat to Different Rates and Ratios of Organic Residues on Yield and Chemical Composition under Two Types of Soil

Yassen, A.A*; Khaled, S.M and Sahar, M. Zaghloul

Plant nutrition Dept., National Research Centre, Dokki, Giza, Egypt.
*azimyassen@yahoo.com

Abstract: Two field experiments were conducted in two successive seasons (2007-2008 and 2008-2009) at Atta, Giza –Governorate and Nubaria region to study the effect of different rates and ratios of organic residues (Farmyard manure and filter mud) on yield and chemical composition of wheat under two types of soils (sandy and Calcareous soil). Results showed that, application of farmyard manure and filter mud residue gave a significant increase in grain and straw weight, total yield, crop index, harvest index, curd protein, N, P and K compared to the control treatment. Data also, indicated that significant increase grain, straw and total yield in sandy soil compared with calcareous soil under study in all treatments. On the other hand, the addition of organic materials (Farmyard manure and filter mud) were effective either individual or mixed with other. The pronounced increase in grain and straw weight, N, P and K content and uptake was noticed when farmyard manure was combined with filter mud at the rate of 2% compared with 1% of organic residues.

[Yassen, A.A; Khaled, S.M and Sahar, M. Zaghloul. Response of Wheat to Different Rates and Ratios of Organic Residues on Yield and Chemical Composition under Two Types of Soil. Journal of American Science 2010;6(12):858-864]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: wheat plant - organic residues –yield –N, P, K

1. Introduction:

Wheat is considered one of the most important and strategically crops in Egypt, but its area produced only about 30% of the domestic needs. There are several ways for increasing wheat production; one of them is the appropriate application of organic residues, especially in the newly reclaimed areas. Most of the newly reclaimed areas in the deserts of Egypt are sandy soils, which have certain problems in their cultivation. Sandy soils are very poor in their organic matter contents as well as their primitive fertility. On the other hand, organic materials such as crop residues, farmyard manure, industrial wastes (filter mud), etc., are available in abundance and reach tremendous amounts every day. Organic matter is a key component of the soil because it carries out many functions in agro-ecosystem. Organic manure is commonly applied to the soil to hence improve their physical, chemical and biological properties of many soils (Jimenez *et al.*, 2002 Nardi *et al.*, 2004, Weil and Magdoff, 2004 and Celik, *et al.*, 2004).

Fliessbach *et al.*, (2000) suggested that, organic manure application increased the transfer elements between the solid phase and soil solution in addition to higher microbial activity. They also, reported that organic soil management improved the soil structure by increasing soil aggregate, thus reducing the risk of soil erosion and promoted the development of the earth condition for plant. The activity of soil microorganisms was higher in the organic farming system, which helped the nutrient uptake to be faster.

Thind *et al.*, (2002) found that, significant increase in N uptake by maize and wheat was observed with continuous application of organic manures.

In wheat plant Sing *et al.*, (2002); Sushila and Gajendra (2002); Nehra and Hooda (2002); Thangavel and Prabakaran (2003); Tawfik and Gomaa(2005), and Zeidan *et al.*, (2005), found that farmyard manure application significantly enhanced the yield and N, P and K uptake of wheat. Sieling et *al.*, (2006) found that the former N treatments (pig slurry) enhanced grain yield and total N uptake of wheat compared with the former unfertilized control, Yaduvanshi and Sharma (2008), found that application farmyard manure with chemical amendment increased wheat yield and N, P and K uptake in grain yield.

Gong et al., (2009) and Enke Liu et al., (2010) indicated that, long-term additions of organic manure have the most beneficial effects on grain yield of wheat and maize.

In Egypt, a tremendous mass of filter mud as byproducts obtained from the clarification of cane juice in sugar industries. These waste residues present a problem for disposal; therefore, it was through useful to use residues as an organic source. Sugar can filter mud contain a considerable amount of plant

nutrients, mainly nitrogen (Arafat 1994). Sugar can filter mud is a good source of available N when applied to soil and its application cane reduce the amount of fertilizer nitrogen required for optimum crop yield and play a role in decreasing the pollution effect of excessive N mineral fertilizer in soil (Arafat *et al.,* 1997 and Yassen *et al.,* (2002).

The current investigation was carried out to study the effect of different sources and ratios of organic residues on yield and chemical composition of wheat under two types of soil.

2. Materials and methods

Two field experiments were conducted in two successive seasons (2007-2008 and 2008-2009) at Atta, Giza Governorate and Nubaria, Behaira Governorate to study the effect of different rates and ratios of organic residues on yield and chemical composition of wheat under two types of soil. Some soil physical and chemical characteristics of the studied soil are recorded in table (1).

The experimental design included 12 treatments which were as follows:

1- Control (without fertilizer)
2- NPK (recommended does 100: 50:50 kg /fed)
3 - FY (1 %)
4 - FY (2 %)
5 - FM (1 %)
6 - FM (2 %)
7- FY: FM (1%) 1: 1
8 - FY: FM (1%) 2: 1
9 - FY: FM (1%) 1: 2
10- FY: FM (2%) 1: 1
11 - FY: FM (2%) 2:
12 - FY: FM (2%) 1: 2

Table (1) some characteristics of soil under investigated

Characteristics	Sandy soil	Calcareous soil
pH 1.2.5	8.25	8.09
EC 1:5	0.15	3.99
CaCO3	1.73	18.43
Organic matter %	0.31	0.48
Available N ppm	33.0	24.0
Available P ppm	11.0	9.00
Available K ppm	4.00	2.00
Mechanical analysis		
Sand %	82.52	78.09
Silt %	10.68	3.49
Clay %	6.8	18.42

The design of each experiment was a complete randomized block system in three replicate. The area of the experimental plot was 10 m^2. The organic materials (Farmyard manure and filter mud) were thoroughly mixed with 0 – 30 cm of the surface soil layer before sowing, (Table2).

Table (2) some properties of farmyard manure (FY) and filter mud (FM)

characteristics	pH	EC dSm^{-1}	Organic matter %	Organic carbon %	Total (%)			Available micro.Nutrient (ppm)		
					N	P	K	Fe	Zn	Mn
Farmyard manure	7.97	2.6	66.68	38.76	1.78	0.31	0.89	412	138	281
filter mud	8.82	0.72	69.14	40.20	2.37	1.48	0.49	1854	121	253

Basal dose of 50 kg P_2O_5 fed^{-1} and 50 kg K_2O fed^{-1} in the form of Superphosphate (15.5%) and potassium sulphate (48% P_2O_5) was added before transplanting, the recommended does of nitrogen was 100 kg N/ fed.

Wheat seeds (Triticum *a estivum L,*) c.v Gemaza 9 were sown in the chosen soil on the last of November for both seasons. The grains were broadcasted on the soil at the rate of 60 kg/fed. At the maturity stage, the plants were harvested and separated into grains and straw. Production was recorded and prepared for analysis. Samples were digested with the acid mixture. Total nitrogen, phosphorus and potassium were determined according to the method described Cottonie et al., (1982).

Statistical analysis of all results was conducted using (NLSD) according to Gomez and Gomez (1984) and the combined analysis of the two seasons was calculated according to the method of Steel and Torrie (1980). The physical and chemical properties of the soil were determined according to Chapman and Pratt (1961)

3. Results and Discussion:

Effect of organic residue on wheat production

The data in table (3) represent that, the wheat production under different rates and ratios of organic

residues with the two types of soil of wheat. The addition of farmyard manure and filter mud residue within all tested rates resulted in a significant increase in grain, straw weight and total yield compared to the control treatment, and consequently, the biological yield of wheat plant. These results are in a good harmony with Yassen *et al.,* (2002) and Zeidan *et al.,* (2005), Yaduvanshi and Sharma (2008). They found that the addition of farmyard manure or/and filter mud had a beneficial effect on grain and straw of wheat plant.

Data also, indicated that application of filter mud decreased grain and straw yield compared to farmyard manure with the two types of soil. This phenomenon may be due to high C/N ratio of filter mud (Arafat *et al.,* 1997).

Concerning the effect of farmyard manure and filter mud, data showed that significant increase grain, straw and total yield in sandy soil compared with calcareous soil with all treatments. In the same time, data showed that, applying farmyard manure and filter mud as a soul at a rate of 2% were more effective in producing grain and straw than application farmyard manure and filter mud at a rate of 1% for both soil. It could be concluded that increasing organic matter to wheat plants induced more grain and straw yield. This may be due to the ability of organic manure to support the growth plants with micro and macro nutrients need for their growth. Similar Results were obtained by Barzegar *et al.,* (2002). Results also, indicated that application of the farmyard manure and filter mud ratio at (1: 1); (2: 1) and (1:2) in both rate at 1% and 2% improve grain, straw weight and biological yield compared with application farmyard manure and filter mud alone in both soil. Farmyard manure addition combined with filter mud may correct the final C/N ratio mixture in order to obtain a preferable condition for enhancing the mineralization of the organic N.

It was worthy to mention that data obtained FY: FM (1:2) had a beneficial and pronounced effect on yield production than other treatments. Data also, observed that application FY and FM increased crop index and harvest index compared the control under types of soil. It has been noticed that farmyard manure combined with filter mud at a ratio (1:1) were markedly increased in the crop index and harvest index in sandy soil while increased CI and HI at a ratio (2:1) in calcareous soil at rate 1%.

Table (3) effect of different rates and ratios of organic residue on grain and straw total yield of wheat plant under two types of soil (Average two seasons)

treatments	Sandy soil					Calcareous soil				
	Grain ten/fed	Straw ten/fed	Total ten/fed	CI	HI	Grain ten/fed	Straw ten/fed	Total ten/fed	CI	HI
control	0.625	1.663	2.288	0.38	27.73	0.544	1.430	1.974	0.38	27.25
NPK	2.161	3.929	6.090	0.55	35.48	1.892	3.282	5.174	0.58	36.57
FY 1 %	1.950	3.860	5.910	0.53	32.99	1.634	2.833	4.467	0.58	36.58
FY 2%	2.127	4.769	6.896	0.45	30.84	1.854	3.054	4.908	0.61	37.78
FM 1 %	1.747	3.073	4.820	0.56	36.24	1.522	2.673	4.195	0.57	36.28
FM 2%	1.815	3.279	5.093	0.55	35.64	1.678	2.895	4.573	0.58	36.69
FY : FM	1%									
1 : 1	2.808	3.431	6.239	0.82	45.00	1.830	2.982	4.812	0.61	38.03
2 : 1	2.597	3.617	6.241	0.72	41.50	2.091	2.987	5.078	0.70	41.17
1 : 2	2.236	4.242	6.478	0.53	34.51	1.793	3.550	5.343	0.51	33.55
FY : FM	2%									
1 : 1	2.330	3.674	6.003	0.63	38.81	1.687	2.901	4.588	0.58	36.77
2 : 1	2.154	4.094	6.048	0.48	35.61	2.084	3.834	5.918	0.54	35.21
1 : 2	2.225	4.259	6.479	0.52	34.26	1.723	3.952	5.675	0.43	30.36
L.S.D 0.05	0.15	0.23	0.36	0.04	2.33	0.11	0.20	0.30	0.03	2.18

FY: farmyard manure FM: filter mud CI: crop index = grain / straw x 100
HI: harvest index = grain /total yield x100

Chemical composition

The N concentration and uptake of nitrogen in wheat plant grown in sandy and calcareous soil treated with different rates and ratios of farmyard manure and filter mud are recorded in table (4). Data indicated that, all treatments tended to increase nitrogen concentration and uptake in grain and straw as compared with the control treatment. The increasing of N concentration and its uptake with organic matter application may be attributed to the mineralization of organic minerals and slow release of minerals in an available form, from organic manure and may be due to the effect of several organic acids, produced during manure decomposition. These results are in a good agreement with that obtained Zeidan *et al.,* (2005)

Sieling et *al.*, (2006) they stated applying farmyard manure to the soil increased content and uptake by grain and straw due to the beneficial effect of organic matter for improving the nutritional statue, particularly nitrogen.

It is interested to mention that, nitrogen concentration in grain and straw yield with respect to a ratio; rate and type of organic residue (FY and FM) were very clear. Taking the nitrogen uptake into consideration, data in the same table showed that, N uptake increased in sandy soil than calcareous soil. This increase seems to be due to the increase in dry matter formation.

With respect to the effect of farmyard manure and filter mud at different rates of 1% and 2 % and ratios (1:1), (2:1), and (1:2) combined with filter mud data declared that, applying the two sources with each other, gave the higher increase in total N content for both grain and straw compared to farmyard manure or filter mud applied alone. The same trend was observed in N uptake. The pronounced increase in N content and uptake was noticed when farmyard manure was combined with filter mud at a rate of 2%.

On the other hand, data in the same table indicated that, protein content increased when the two organic residues combined with each other as compared to farmyard manure or/ and filter mud alone, the highest value observed at 2%. These finding are harmony with those obtained by Eghbal et al.,(2004) and Mohammed (2004)

Data recorded in tables (5 and 6) illustrate that, effect of different organic residue farmyard manure either alone or in with mixed filter mud and different rates and ratios on phosphorus and potassium content and uptake, data showed the obvious increase for different as compare with control. Similar Suggestions was also reported by Barzegar *et al.*,(2002). In contrast, the addition of organic material was effective either individual or mixed with other.

Data indicated that, increased phosphorus and potassium concentration and uptake in grain and straw as compared with the control treatment. These results are in a good agreement that obtained by Nehra and Hooda (2002) and Thangavel and Prabakaran (2003)

Phosphorus and potassium content in wheat plant (grain and straw) increased in sandy soil as compared with calcareous soil, due to the differences in its physiochemical properties. Concerning P and K uptake data illustrated that, the uptake was higher in the sandy soil as compared to the calcareous soil for both P and K.

Data also, indicated that P and K content and uptake in both grain and straw increased continuously with increasing farmyard manure and filter mud rate applied from 1% and 2%. This indicates that, due to the increase in P and K farmyard manure and filter mud amended soil enhanced microbial activates, which increase nutrient availability and their uptake and increasing root distribution. These results are in a good agreement that obtained by Yaduvanshi and Sharma (2008)

Table (4) effect of different rates and ratios of organic residue on N content % and uptake kg/ fed. Protein % content of wheat plant under two types of soil (Average two seasons)

treatments	Sandy soil					Calcareous soil				
	Grain			straw		Grain			straw	
	N %	Uptake Kg / fed	Protein %	N %	Uptake Kg / fed	N %	Uptake Kg /fed	Protein %	N %	Uptake Kg /fed
control	098	3.85	5.64	0.26	4.32	0.88	4.79	5.06	0.22	3.15
NPK	1.55	33.50	8.91	0.70	27.50	1.46	27.62	8.40	0.45	14.77
FY 1 %	1.55	30.23	8.91	0.64	24.70	1.27	20.75	7.30	0.49	13.88
FY 2%	1.45	30.84	8.34	0.58	27.66	1.32	24.47	7.59	0.50	15.27
FM 1 %	1.10	19.22	6.33	0.39	11.98	0.99	15.07	5.69	0.28	7.48
FM 2%	1.26	22.87	7.25	0.45	14.78	1.11	18.63	6.38	0.33	9.55
FY : FM	1%									
1 : 1	1.64	46.05	9.43	0.61	20.93	1.55	28.37	8.91	0.48	14.31
2 : 1	1.56	40.51	8.97	0.66	23.87	1.49	31.16	8.57	0.52	15.53
1 : 2	1.61	36.00	9.26	0.56	23.76	1.61	28.86	9.26	0.55	19.53
FY : FM	2%									
1 : 1	1.71	39.84	9.83	0.65	23.88	1.57	26.48	9.03	0.50	14.50
2 : 1	1.75	45.41	10.06	0.68	27.84	1.72	35.84	9.89	0.52	19.94
1 : 2	1.67	37.34	9.60	0.61	25.98	1.58	27.22	9.09	0.49	19.36
L.S.D 0.05	0.09	2.75	0.45	0.04	1.57	0.08	1.83	0.51	0.03	1.14

Table (5) effect of different rates and ratios of organic residue on P content % and uptake kg/ fed. of wheat plant under two types of soil (Average two seasons)

treatments	Sandy soil				Calcareous soil			
	Grain		straw		Grain		straw	
	P %	Uptake Kg / fed	P %	Uptake Kg / fed	N %	Uptake Kg /fed	N %	Uptake Kg /fed
control	0.25	1.56	0.11	1.83	0.19	1.03	0.09	1.29
NPK	0.40	8.64	0.15	5.89	0.32	6.05	0.11	3.61
FY 1 %	0.32	6.24	0.14	5.40	0.28	4.57	0.13	3.68
FY 2%	0.35	7.44	0.15	7.15	0.32	5.93	0.15	4.58
FM 1 %	0.35	6.11	0.15	4.60	0.34	5.17	0.11	2.94
FM 2%	0.38	6.90	0.16	5.25	0.36	6.04	0.13	3.76
FY : FM	1%							
1 : 1	0.39	10.95	0.16	5.49	.35	6.40	0.12	3.57
2 : 1	0.41	10.64	0.18	6.51	0.37	7.74	0.14	4.18
1 : 2	0.44	9.84	0.19	8.06	0.42	7.53	0.15	5.33
FY : FM	2%							
1 : 1	0.41	9.55	0.16	5.88	0.37	6.24	0.11	3.19
2 : 1	0.45	9.69	0.20	8.19	0.41	8.54	0.15	5.75
1 : 2	0.48	10.68	0.22	9.37	0.48	8.28	0.16	6.32
L.S.D 0.05	0.02	0.24	0.01	0.44	0.02	0.22	0.01	0.31

Table (5) effect of different rates and ratios of organic residue on K content % and uptake kg/ fed. of wheat plant under two types of soil (Average two seasons)

treatments	Sandy soil				Calcareous soil			
	Grain		straw		Grain		straw	
	K %	Uptake Kg / fed	K %	Uptake Kg / fed	K %	Uptake Kg /fed	K %	Uptake Kg /fed
control	0.46	2.88	1.42	23.62	0.41	2.23	1.33	19.02
NPK	0.65	14.05	2.88	113.16	0.52	9.83	2.45	80.41
FY 1 %	0.71	13.84	2.09	80.67	0.50	8.17	2.19	62.04
FY 2%	0.74	15.73	1.91	91.09	0.55	10.20	2.82	86.12
FM 1 %	0.59	10.31	2.17	66.68	0.49	7.46	2.34	62.55
FM 2%	0.69	12.52	2.32	76.07	0.50	8.39	2.27	65.72
FY : FM	1%							
1 : 1	0.68	19.09	2.74	94.01	0.64	11.71	2.67	79.62
2 : 1	0.87	22.59	2.59	93.60	0.76	15.89	2.44	72.88
1 : 2	0.67	14.98	2.84	120.47	0.61	10.93	2.55	90.53
FY : FM	2%							
1 : 1	0.71	16.54	2.55	93.68	0.56	9.44	2.33	67.59
2 : 1	0.67	14.43	2.64	108.08	0.62	12.92	2.78	106.83
1 : 2	0.72	16.01	2.64	112.44	0.72	12.41	2.53	99.98
L.S.D 0.05	0.04	1.03	0.15	6.28	0.03	0.70	0.14	5.41

azimyassen@yahoo.com

Corresponding author
Yassen, A. A.
Plant nutrition Dept., National Research Centre,
Dokki, Giza, Egypt.

4. References:

1. Arafat, S.M. (1994) Evaluations of sugarcane filter mud on improving soil characteristics and watermelon Yield. Egypt, J. Appl. Sci., 9 (9):287-295.
2. Arafat, S.M.; H. El- Aila and A. Algli (1997). Utilization of sugar cane filter mud to minimize nitrogen fertilizers for sorghum growth. J. Agric. Sci. Mansoura Univ., 22(4): 1267 -1276.
3. Barzegar, A.R.;A.Yousefi and A. Daryashenas (2002)the effect of addition of different amounts and types of organic materials on soil physical properties and yield of wheat . Plant and soil 247: 295 – 310.
4. Celik, I.; I. Ortas and S. Kilic, (2004) Effects of compost, mycorrhiza, manure and fertilizer on some physical properties of a Chromoxerert soil. Soil Till. Res. 78, 59 –67.
5. Chapman, H.D. and P.F. Pratt, (1961) Methods of Analysis for Soils, plant and water. Div.of Agric. Sci. Univ. of Calif, pp309
6. Cottenie A, M.; Verloo, L.; Kiekens, G.Velgh; and R.Camerlynch (1982). Chemical Analysis of Plants and Soils. Lab. Anal. Agrochem, State Univ Ghent, Belgium 63.
7. Eghbal,B ;D.Ginting; J.A.Gilley,(2004) Residual effects of manure and compost applications on corn production and soil properties.Agron.J.96:442- 447
8. Enke Liu; Changrong Yan; Xurong Mei; Wenqing He; So Hwat Bing ;Linping Ding; Qin Liu ; Shuang Liu and Tinglu Fan(2010) Long-term effect of chemical fertilizer, straw, and manure on soil chemical and biological properties in northwest China .Geoderma 158 173–180
9. Fliessbach, A.; P. Mader; D. Dubois; and L. Gunst (2000). Results from a 21 years old field trial. Organic farming Enhance Soil Fertility and Biodiviersity Fi Bl Dossier N1, 15, pp19.
10. Gomez K.A. and A.A. Gomez (1984) Statistical Procedures for Agriculture Research " 2td (ed) John Wiley and Sons Inc. New York.
11. Gong, W.; X.Yan; J.Wang; T.Hu; Y. Gong (2009) Long-term manure and fertilizer effects on soil organic matter fractions and microbes under a wheat–maize cropping system in northern China. Geoderma 149, 318–324
12. Jimenez, M.P.; A.M. Horra, L. Pruzzo and R.M. Palma, (2002), Soil quality: a new index based on microbiological and biochemical parameter, Biology and Fertility of Soils 35 pp. 302–306.
13. Mohammed,S.S.(2004) assessment of the relative effectiveness for some organic materials conjucted with mineral nitrogen on a newly cultivated soil Egypt. J. Appl. Sci.19 (3)298 – 310.
14. Nardi, S.; F.Morari; A.Berti; M.Tosoni and L. Giardini (2004) Soil organic matter properties after 40 years of different use of organic and minerals fertilizers. Eur.J.Agronomy21,357–367.
15. Nehra,A.S. and I.S. Hooda, (2002) Influence of integrated use of organic manure and inorganic fertilizer on wheat yields and soil properties. Research on Corp. 3(1):11 -16.
16. Sieling ,K.; T.Brase and V.Svib(2006) Residual effects of different N fertilizer treatments on growth N uptake and yield of oil seed rape, wheat and barley . Europ. J. Agronomy 25:40–48
17. Sing,V.;K. Sing; R.V.Singh and S. Singh (2002) Nutrient uptake and yield of wheat as influenced by iron and farmyard manure application in an alluvial soil. Annals of Aric., 23(1) :4 -7.
18. Steel, R.G.D.and J.H.Torrie, 1980. Principles and Procedures of statistics .Mcgrow-hill book Co.,Inc., Newyork ,Toronto, London.
19. Sushila,R. and G. Gajendra (2002) Influence of farmyard manure, nitrogen and biofertilizer on growth attributed and yield of wheat (triticum aestivum) under limited water supply .Indian J. of Agronomy. 45 (3): 590 – 595
20. Tawfik, M.M. and A.M.Gomaa(2005) Effect of organic and biofertilizeron growth and yield of wheat plants. Egypt. J. Agric. Res., 2(2): 711 - 725.
21. Thangavel, P. andJ. Prabakaran, (2003) Exploring the possibility of crop production in magnetite mine spoils with amendments. Advances in Plant Sci.16 :155 -160.
22. Thind,S.S ; Manmohan Singh; A.S. Sidhu; I.M. Chhibba and M.Singh (2002) Influence of continuous application of organic manures and nitrogen fertilizer on crop yield, N-uptake and nutrient status under maize-wheat rotation. Journal of Research, Punjab Agricultural University. 39: 3: 357-361
23. Weil, R.R. and F. Magdoff, (2004) Significance of soil organic matter to soil quality and health. In: F. Magdoff and R.R. Weil, Editors, Soil Organic Matter in Sustainable Agriculture, CRC Press, Boca Raton, FL, USA, pp. 1–43.
24. Yaduvanshi, N.P.S. and D.R. Sharma (2008)Tillage and residual organic manures/chemical amendment effects on soil organic matter and yield of wheat under sodic water irrigation. Soil & Tillage Research 98 11–16.
25. Yassen A.A, Arafat S.M. and Sahar, M. Zaghloul (2002) Maximizing use of vinasse and filter mud as by-products of sugar can on wheat production. J. of Agric. Sci. Mansoura Univ., 27(11) 7865 – 7873.

26. Zeidan, M.S.; M. Hozayn and M.F.El- Krammany (2005) Effect of different organic fertilizer sources and levels on growth and yield of wheat (Triticum *a estivum L,*) in sandy soil Egypt. J. Agric. Res., 2(2): 643.

11/2/2010

Radioprotective activity of L- Carnitine and α -Lipoic acid against whole body γ- irradiation in rats

Sally S. Alam[1*], Aziza M. Hassan[1], Nermine K. El Halawaney[1], Dalia E. El-Nashar[2], Mona G. Abd El-Azeem[2]

[1]Cell Biology Department, National Research Center, 12622 Dokki, Giza, Egypt.
[2]Health Radiation Research, National Center for Radiation Research and Technology (NCRRT). Atomic Energy Authority, Cairo, Egypt.
dr_Sallyalam@yahoo.com

Abstract: The present study was designed to investigate the radioprotective efficacy of naturally occurring antioxidants, L - carnitine (LC) and α -Lipoic acid (LA) on radiation-induced bone marrow and liver damages in a rat model. The cellular changes were estimated by evaluation the expression of antioxidant enzymes, superoxide dismutase (SOD) and glutathione peroxidase (GPx), genes using RT-PCR and DNA damage in bone marrow and liver cells. The histopathological and ultra structural changes were also determined. To evaluate the effects of the above antioxidants, adult rats were treated with LC (300 mg/kg b wt) and LA (150 mg/kg b wt) after exposure to whole-body γ-rays (6 Gy) for 10 days, or treated with LC & LA for 7 consecutive days and one hour after the last administration, animals irradiated a single dose of whole-body γ-rays (6 Gy) and received again LC & LA in same dose for 10 days. The obtained data revealed that γ –irradiation significantly decreases the expression of SOD and GPx genes and increases DNA fragmentation in liver cells as well as the incident of micronuclei in bone marrow cells. In addition, different histological and ultra structural alterations in the liver of irradiated animals were recorded. These alterations were varied from hemorrhage, congestion in blood vessels, pyknosis and necrosis as well as complete degenerated area in the liver electron micrographs recorded swollen mitochondria, fragmented endoplasmic reticulum, distorted nuclei and cell membrane. Treatment with LC & LA post-exposure to radiation attenuated most of these changes. Whereas pre- and post- treatment with LC & LA to γ-irradiation normalized the expression of the antioxidant genes enzymes, decreased the DNA fragmentation and micronuclei formation with a normal restoration of histopathologial and ultra structure liver architecture. Thus, our results suggested that pre-treatment with LC & LA offers protection against γ-irradiation induced cellular damage.
[Sally S. Alam, Aziza M. Hassan, Nermine K. El Halawaney, Dalia E. El-Nashar, Mona G. Abd El-Azeem. Radioprotective activity of L- Carnitine and α -Lipoic acid against whole body γ- irradiation in rats. Journal of American Science 2010; 6(12):865-879]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: L-Carnitine, α-Lipoic acid, γ-irradiation, DNA fragmentation, Antioxidant gene expression, Ultra structure, Histopathology.

1. Introduction

Ionizing radiation (IR) is an important environmental risk factor for various cancers and also a major therapeutic agent for cancer treatment. Radiation toxicity occurs either by direct attack on the genetic material and/or by generating reactive oxygen species (ROS) by radiolysis of water. It also attenuates the endogenous antioxidant enzymes which are considered as first line defense mechanism to maintain redox balance and normal biochemical processes (Parihar *et al.,* 2007). Consequently, the organs become more susceptible to the deleterious effects of ROS that attack various cellular components including DNA, RNA, proteins and membrane lipids, thereby leading to significant cellular damage (Tominaga *et al.,* 2004).

The potential application of radioprotective chemicals in the event of planned exposures or radiation accidents/incidents has been investigated from the beginning of the nuclear era (Weiss and Simic, 1988). It has also been considered possible that radiation therapy for cancer patients could be improved by the use of radioprotectors to protect normal tissue. They include sulfhydryl compounds, antioxidants, plant extracts, immunomodulators, and other agents (Nair *et al.,* 2001).

α-Lipoic acid (LA), a naturally occurring sulphydryl compound found in virtually all plants and animal species that functions as a coenzyme in pyruvate dehydrogenase and α-ketoglutarate dehydrogenase mitochondrial reactions, leading to the production of cellular energy. In human body LA

is rapidly reduced to dihydrolipoic acid (DHLA) after intake into the cellular system (Packer *et al.,* 1995). Both, LA and DHLA are effective against conditions in which oxidative stress has a role (Packer *et al.,* 1999). LA, which is a universal antioxidant functions both in aqueous and membrane phases (Kagan *et al.,* 1992). LA has been shown to quench free radicals, prevent singlet oxygen-induced DNA damage, exhibit chelating activity, reduce lipid peroxidation and protein damage through the redox regeneration of other antioxidants such as vitamins C and E, and by increasing intracellular glutathione (Moini *et al.,* 2002; Biewenga *et al.,* 1997). It shows beneficial effects in oxidative stress conditions because of its synergistic action with other antioxidants (Suzuki *et al.,* 1993).

L-carnitine (LC) is a natural compound known as vitamin B_T (γ-trimethyl amino butyrate) widely distributed in the body. It is mainly required in the transport of activated fatty acids from the cytosol into the mitochondrial matrix for subsequent β-oxidation, and in the transfer of the products of peroxisomal β-oxidation to the mitochondria for utilization in energy generation process (Ramsay *et al.,* 2001; Santoro *et al.,* 2005). It has been shown that LC has a scavenger effect on ROS and a stabilizing effect on damaged cell membranes (Fritz and Arrigoni-Martelli, 1993). Carnitine can also act as a chelator by decreasing the concentration of cytosolic iron that plays a very important role in free radical chemistry (Reznick *et al.,* 1992). LC has a capacity to enhance non-enzymatic antioxidants, such as vitamin E (Arockia Rani and Panneerselvam, 2001).

Several studies demonstrated that both LA and LC protected intact tissues against injurious effects of cancer treatments such as radiotherapy or chemotherapy, without an inhibitory effect against their therapeutic effects (Chang *et al.,* 2002; Pisano *et al.,* 2003; Selvakumar *et al.,* 2006; Prahalathan *et al.,* 2006). Previous study has shown that the combined effect of both these drugs improves mitochondrial enzyme activities (Savitha and Panneerselvam, 2006). Muthuswamy *et al.* (2006) found that co-administration of L-carnitine and DL-α-lipoic acid mitigates ROS-induced oxidative damage to macromolecules such as lipids, proteins and DNA in brain of aged rat.

The aim of the present study was to investigate the radioprotective role of L-carnitine and α-Lipoic acid against γ-irradiation-induced liver and bone marrow damage in rat model.

2. Materials and methods

2.1. Source of chemicals

L-Carnitine and R-α-lipoic acid were a kind gift from EVA Pharma for pharmaceuticals and medical appliances, Egypt. All other chemicals used were of the highest analytical grade.

2.2. Irradiation

Whole-body Gamma - irradiation was performed at the National Centre for Radiation Research and Technology (NCRRT), Cairo, Egypt, using a Gamma Cell-40 biological irradiator. Animals were irradiated at an acute single dose level of 6 Gy delivered at a dose rate of 0.72 Gy/ min.

2.3. Animals

Female albino rats of Wistar strain weighing 150-200 g was obtained from the animals house Colony, National Research Centre, Dokki, Giza, Egypt. The animals were housed in large spacious cages and given food and water *ad libitum*. The animals were well ventilated with a 12-h light/dark cycle, throughout the experimental period.

2.4. Experimental design

A total of (50) rat was randomly divided into five groups (10 animals per group).

Group I: control group, animals were received normal saline (0.5 ml/150 g b wt.) for 10 consecutive days.

Group II: LC&LA group, animals were received LC & LA for 10 consecutive days.

Group III: IR group, rats received the same dose of normal saline as the first group and on day seven, animals were irradiated with a single dose of whole-body γ-rays (6 Gy) and left for 10 days.

Group IV: IR + (LC&LA) group, animals were irradiated with a single dose of whole-body γ-rays then after one hour they administered LC & LA for 10 days.

Group V: (LC&LA) + IR group, animals received LC & LA for 7 consecutive days, then after one hour of the last administration, animals irradiated a single dose of whole-body γ-rays (6 Gy) and received again LC & LA in same dose for 10 days.

L-Carnitine (300 mg/ (kg b wt) day) (Kumaran *et al.,* 2005) was dissolved in 0.89% physiological saline and R -α- lipoic acid (150 mg/ (kg b wt) day)

(Savitha *et al.,* 2005) was dissolved in 0.5% of KOH in physiological saline and administered orally. Animals were killed by cervical decapitation and a part of liver was immediately frozen and stored at - 80°C for RNA extraction and DNA fragmentation analysis. Other part of liver was collected for the histopathological and ultra structural examination.

2.5. Molecular genetics assays

2.5.1. RNA isolation and Reverse transcription (RT)

Total RNA was isolated from liver tissues using Trizol reagent (Invitrogen, Paisley, UK). RNA samples were subjected to DNase1 treatment to remove genomic DNA contamination in the presence of RNase inhibitor. The purity and integrity of the total RNA was determined by spectrophotometry and agarose gel electrophoresis (Sambrook *et al.,* 1989). The first-strand cDNA was prepared from the 5 µg of total RNA using Fermentas kits (Sigma, St. Louis, MO) as per the manufacturer's instructions. The RT-program used was: 60 min at 42^{0}C (cDNA synthesis); 5 min at 94^{0}C (denaturation). Afterwards the reaction tubes containing RT preparations were ash-cooled in an ice chamber until used for DNA amplification through polymerase chain reaction (PCR) (Brun *et al.,* 2006).

2.5.2. Polymerase chain reaction (PCR)

The first-strand cDNA from different rat samples was used as the template for amplification by the PCR with the following pairs of specific primers (from5′to3′):

Cu-ZnSOD forward: GCAGAAGGCAAGCGGTGAAC,
Cu-ZnSOD reverse: TAGCAGGACAGCAGATGAGT,
GPx forward: CTCTCCGCGGTGGCACAGT,
GPx reverse: CCACCACCGGGTCGGACATAC,

that are taken from the literature (Limaye *et al.,* 2003). β-actin, a house-keeping gene, was used for normalizing mRNA levels of the target genes. The PCR cycling parameters were one cycle of 94 ^{0}C for 5min, 35 cycles of 94 ^{0}C for 30 s, 60 ^{0}C (Cu-Zn SOD and GPx gene) for 30 s, 70 ^{0}C for 40 s, and 72 ^{0}C for 5 min. The PCR products were electrophoresed onto ethidium bromide stained a 2.0% agarose gels. The ethidium bromide-stained gel bands were scanned and the signal intensities were quantified by the computerized Gel-Pro program.

2.6. Quantification of fragmented DNA by diphenylamine (DPA)

To measure hepatic DNA fragmentation by spectrophotometry, a portion of the liver was homogenized in hypotonic lysis buffer (0.2% Triton X-100, 10 mM Tris, 1 mM EDTA, pH 8.0) and centrifuged for 15 min at 10,000 rpm to separate intact chromatin in the pellet from fragmented/damaged DNA in the supernatant. Pellets were resuspended in 0.5 N perchloric acid and 5.5 N perchloric acid was added to supernatant fractions to final concentration of 0.5 N. Samples were heated at 90 ^{0}C for 15 min and centrifuged at 10,000 rpm for 10 min to remove proteins. Supernatant fractions were reacted with diphenylamine (DPA) reagent [0.088 M DPA, 98% (v/v) glacial acetic acid, 1.5% (v/v) conc. H_2SO_4 and 0.5% of 1.6% acetaldehyde solution] and the samples were kept at room temperature for 20 h Paradones *et al.* (1993). Absorbance was measured at 600 nm using a UV-double beam spectrophotometer (Shimadzu 160A).The percentage of DNA fragmentation was taken as the ratio of DNA in the supernatant to total amount of DNA in pellet and supernatant.

2.7. Agarose gel electrophoresis for DNA fragmentation

Agarose gel electrophoresis was carried out for the analysis of DNA fragmentation by the method of Yokozawa and Dong (2001). The DNA samples (1 µg) were electrophoresed on 1.4% agarose gel using TBE buffer at 40 V for 5 h. Then the gel was stained with ethidium bromide and viewed under UV transilluminator and photographed.

2.8. Micronucleus assay

Immediately after the animals were sacrificed, bone marrow was collected from each animal for the micronucleus assay as described by Schmid (1975). In brief, the femurs were dissected and washed with 1ml of fetal calf serum, smeared on clean and dry slide, fixed with absolute methanol for 10 min and stained with 5% (v/v) Giemsa stain diluted in phosphate buffer. One thousand polychromatic erythrocytes (PCEs) were analyzed per animal to ascertain the frequency of micronuclei and the micronucleated cells in the bone marrow of each rat in the different treatment groups. The ratio of PCEs to normochromatic erythrocyte (NCEs) was calculated for the determination of the cytotoxicity in bone marrow.

2.9. Histopathological study

After animals dissections, liver were removed immediately and fixed in 10% normal saline and neutral buffered formalin for 7 days, then the tissues were washed and dehydrated in ascending grades of ethyl alcohol cleared in benzene and impregnated in paraffin for 1.5 h in the oven at 55^{0}C. Serial section, 5 µm were cut and stained with

Haematoxylin & Eosin (H&E) as described by Bancrofet and Stevens (1977).

2.10. Electron Microscopic Study

For electron microscopic examination, portion of liver were cut into small pieces, fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) and post in 1% osmium tetroxide in 0.3 M cacodylate buffer. The specimens were then dehydrated and embedded in Epson 812 (Hayat, 1973). Ultrathin sections were cut and stained according to Reynold (1963). Sections were examined with Joel 100.cx a transmission electron microscope at NCRRT.

2.11. Statistical analysis

Data were analyzed using One-way analysis of variance (ANOVA) using the SPSS 11 program, followed by Post-Hoc test for multiple comparisons. The data were expressed as means ± standard error of the mean. Differences were considered significant at $P<0.05$.

3. Results

3.1. Evaluation of gene expression

Bands produced from amplifying cDNA of GPx, SOD and the house keeping gene β-actin as a control were analyzed and the results of gene expression was based on quantifying the signal intensities in each band. Results were expressed as the ratio between maximum optical density (max OD) for each band of the target amplification product and the corresponding max OD of β-actin. Expression of GPx and SOD mRNA in liver of the different groups of rats are summarized in Figs. (1 and 2). The results show that there was a significant decrease in the expression level of the examined genes in the γ-irradiated group of rats as compared to the other groups. However, treatment with LC & LA resulted in a significant increase in the expression level of GPx and SOD mRNA ($P < 0.05$) versus the control group. As well as treatment of LC & LA prior to γ-irradiation exposure significantly ($P < 0.05$) up-regulated the expression of both genes. Moreover, pre- and post- treatment with LC & LA to γ-irradiation normalized the expression levels of these genes relative to the control level.

3.2. Analysis of DNA Fragmentation

Results of DNA fragmentation are shown in Table (1). γ-irradiated animals produced 33.01 % fragmented DNA in hepatic tissue, whereas control group shows a negligible 6.5 % fragmented DNA, which are associated significantly ($P < 0.05$). Supplementation with LC & LA significantly brought down the levels of DNA damage that reached 22.41 % for post – treated group, and 13.33% in pre- and post-treated group. In addition, the pattern of DNA fragmentation elicited by γ-irradiation showed the characteristic DNA ladder which is significantly restored in the groups treated with LC & LA (Fig.3).

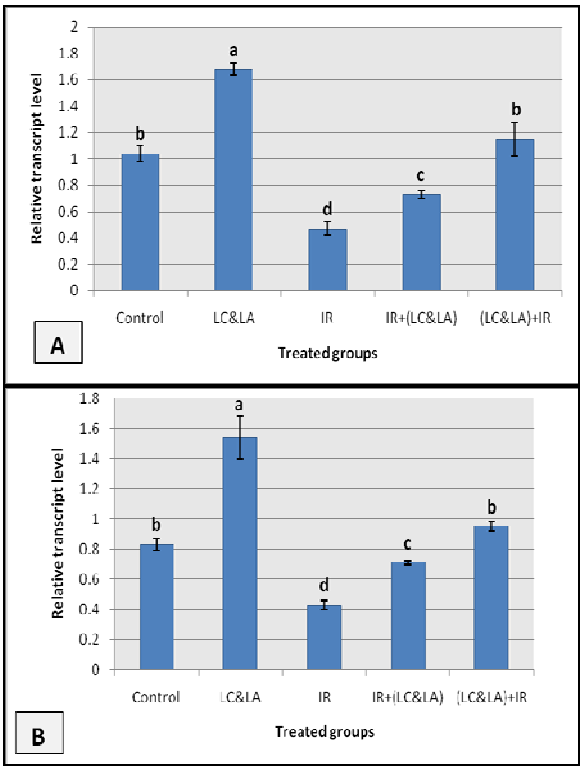


Fig. 1. RNA expression of SOD (A) and GPx (B), in the liver of control and treated rats. The results depicted are normalized to levels of β-actin gene. Data are mean ± S.E. of ratios of intensity for each gene divided by that for β-actin.

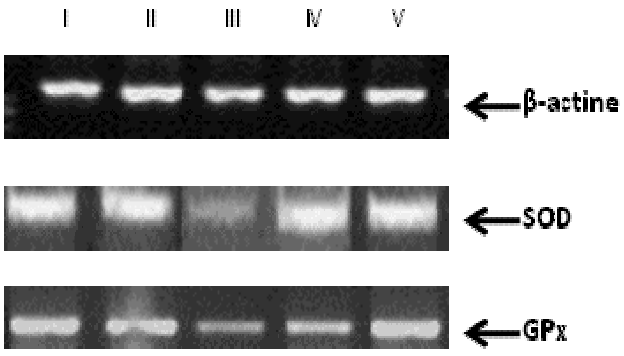


Fig.2. Effects of LC & LA on transcript product of hepatic antioxidant genes in γ-irradiated rats. Agarose gel electrophoresis of SOD, GPx and β-actin RT-PCR products of different groups. Group I: Control, Group II: LC&LA, Group III: IR, Group IV: IR + (LC&LA) and Group V: (LC&LA) + IR.

Table 1. Effects of LC & LA on the percentage of DNA fragmentation in liver of rats exposed to γ- irradiation.

Treated Groups	Percent DNA fragmentation Mean ±S.E.	Change %
Control	6.5±0.64[d]	-
LC&LA	6.0±0.40[d]	0.5
IR	33.01±3.04[a]	26.5
IR+(LC&LA)	22.41±2.22[b]	15.91
(LC&LA)+IR	13.33±1.30[c]	6.83

Within each column, means superscript with different letter are significantly different (*P< 0.05*).

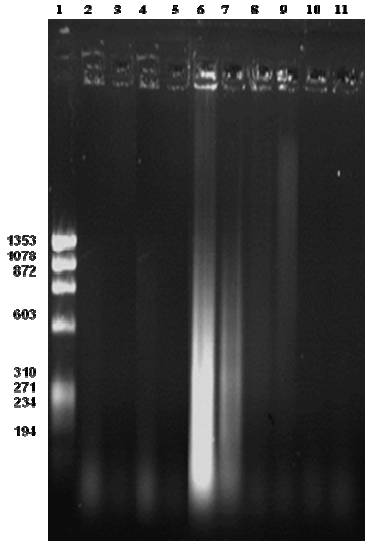


Fig.3. Agarose gel electrophoretic pattern of DNA isolated from liver tissue of control and treated rats. Lane 1: phi x marker; Lane 2-3: control group; Lane 4-5: LC&LA group; Lane 6-7: IR group; Lane 8-9: IR + (LC&LA) group; Lane 10-11: (LC&LA) + IR group.

3.3. Effects of LC and LA on γ-irradiation–induced micronuclei formation

The cytogenetic damage induced by γ-irradiation as well as the antimutagenic effects of treatment with LC and LA were investigated in bone marrow of female rats utilizing micronucleus assay. Table (2) showed that the frequencies of micronucleated polychromatic erythrocytes (Mn-PCEs) as resulted from administration of LC & LA was within the accepted spontaneous range for control. Exposure to γ-irradiation induced a significant increase in the frequencies of Mn-PCEs (26.6 ± 1.44) as compared to the control group (4.2 ± 0.58). Pre - and post- treatments with LC & LA to γ-irradiation was found to decrease the Mn-PCEs (7.0 ± 0.45) significantly when compared to the post – treated group (12.4 ± 0.81). Moreover, the inhibition percentages reached (63.4%) in post-treated group meanwhile, a maximum inhibition (87.5%) was shown in pre- and post- treated group (Fig. 4 A and B). In spite of this reduction, the frequencies of Mn-PCEs were significantly higher than the control group. Our results clearly indicated that animals exposed to γ-irradiation showed severe bone marrow cytoxicity as indicated by the reduction in PCEs percentage and the PCEs/NCEs ratio as compared to the control group. The treatment with LC & LA resulted in an increase in PCEs percentage and the PCEs/NCEs ratio when compared with γ-irradiated rats.

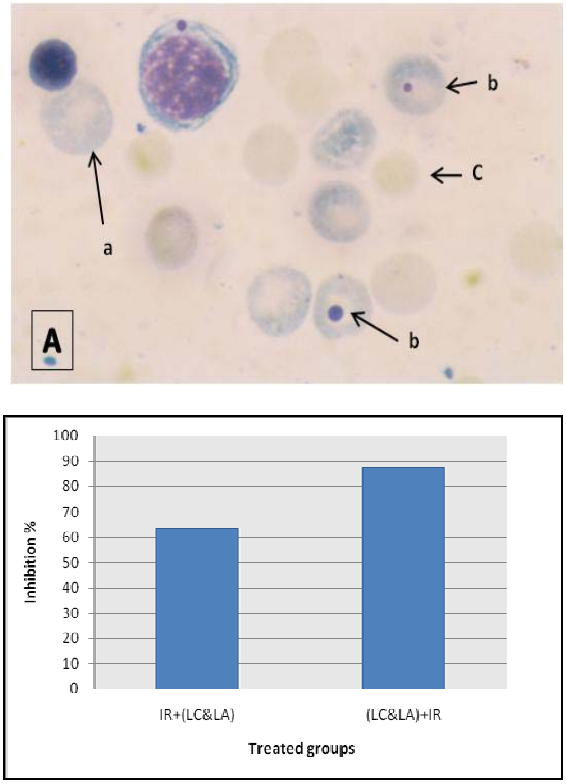


Fig.4. (A) Showing (a) polychromatic erythrocyte (PCE), (b) micronucleated polychromatic erythrocyte (Mn- PCE), and (c) normo chromatic erythrocyte (NCE). **(B)** Histogram showing inhibition of micronuclei incidence – induced by γ-irradiated by LC&LA.

Table 2. Effects of LC&LA on the frequency of Mn-PCEs and PCEs in the bone marrow of rats exposed to γ- irradiation.

Treated Groups	No of Mn-PCEs/5000PCEs (Mean ± S.E.)	PCEs %	NCEs %	PCEs/NCEs ratio (Mean ± S.E.)
Control	21 4.2 ± 0.58^{d}	51	49.0	1.04 ± 0.05^{b}
LC&LA	11 2.20 ± 0.37^{e}	59.8	40.2	1.49 ± 0.08^{a}
IR	133 26.6 ± 1.44^{a}	39.6	60.4	0.65 ± 0.03^{e}
IR+(LC&LA)	62 12.4 ± 0.81^{b}	45.6	54.4	0.84 ± 0.02^{d}
(LC&LA)+IR	35 7.0 ± 0.45^{c}	48.8	51.6	0.94 ± 0.02^{C}

Within each column, means superscript with different letter are significantly different ($P< 0.05$).

3.4. Histopathological evaluation

Examination of H & E stained liver sections obtained from control rats showed a normal lobular architecture with central veins and radiating hepatic cords are separated by narrow blood sinusoids lined with two types of cells, endothelial (with thin, small, rod-like nucleus) and van-kupffer cells (containing oval dense nuclei), the hepatocytes are large spherical cells with spherical nuclei and prominent deeply stained nucleoli (Fig.5 A). The liver sections of rats treated with LC and LP illustrating the hepatic architecture shows no abnormalities. The hepatocytes reveal the same appearance as the normal ones. Also, the hepatic, sinusoids and kupffer cells show no abnormalities (Fig.5 B). However, liver sections of γ-irradiated rats showed radiolesions in the form of dilatation and congestion in blood vessels and appearance of inflammatory cells. The hepatocytes showed vacuolated cytoplasm, dilated sinusoidal spaces, large number of binucleated hepatocytes cells and necrotic cells along with many pyknotic nuclei (Fig. 5 C, D and E). In Fig. (5 F, G and H) liver of rat in group post- treated with LC&LA showed hepatocytes were found in focal hepatic hemorrhage and binucleated hepatocytes wider sinusoidal space, vacuolar degeneration of some hepatocytes, dilated blood vessel were noticed. Nevertheless, other hepatocytes were abnormal with either pyknotic or karyolitic nuclei with noticeable increase of endothelial and of kupffer cells within the tissue. Whereas photomicrograph of liver in pre- and post-treated with LC&LA to γ-irradiation showed lesser damage when compare to post-treated one. Liver sections of these rats almost have normal hepatic architecture as well as mild cytoplasmic vacuolation, binucleated cells were evident in photomicrograph, hemorrhage disappeared, slight dilated blood vessels and dilated blood sinusoids were noticed (Fig. 5 I).

3.5. Ultra structural evaluation

The ultra structural of a normal hepatocyte obtained from control and LC&LA is presented in Fig. (6 A and B). Electron micrographs showed large rounded euchromatic nuclei with prominent nucleoli. The cellular organelles were uniformly distributed throughout the cytoplasm, where numerous mitochondria, cisternae of rough endoplasmic reticulum and electron dense cytoplasm. Electron micrographs showed ultra structural changes in different hepatocyte components after exposure to γ-irradiated revealed focal degeneration of cytoplasmic matrix accompanied by ill defined cytoplasmic organelles, degeneration hepatocytes with lysis cytoplasm matrix, degeneration nuclei with wrinkled nuclear membrane (Fig. 6 C and E). Swelling mitochondria with rupture of its cristae. Also, fragmentation of the rough endoplasmic reticulum (Fig. 6 D). In addition, the blood sinusoids exhibited prominent dilatation with abnormal lymphocytes and also contain numerous R. B. Cs. (Fig. 6 C, E and F). Also, dilated bile canal with abnormal microvillus (Fig. 6 F). Mitosis of the nuclei appears in Fig. (6 A).

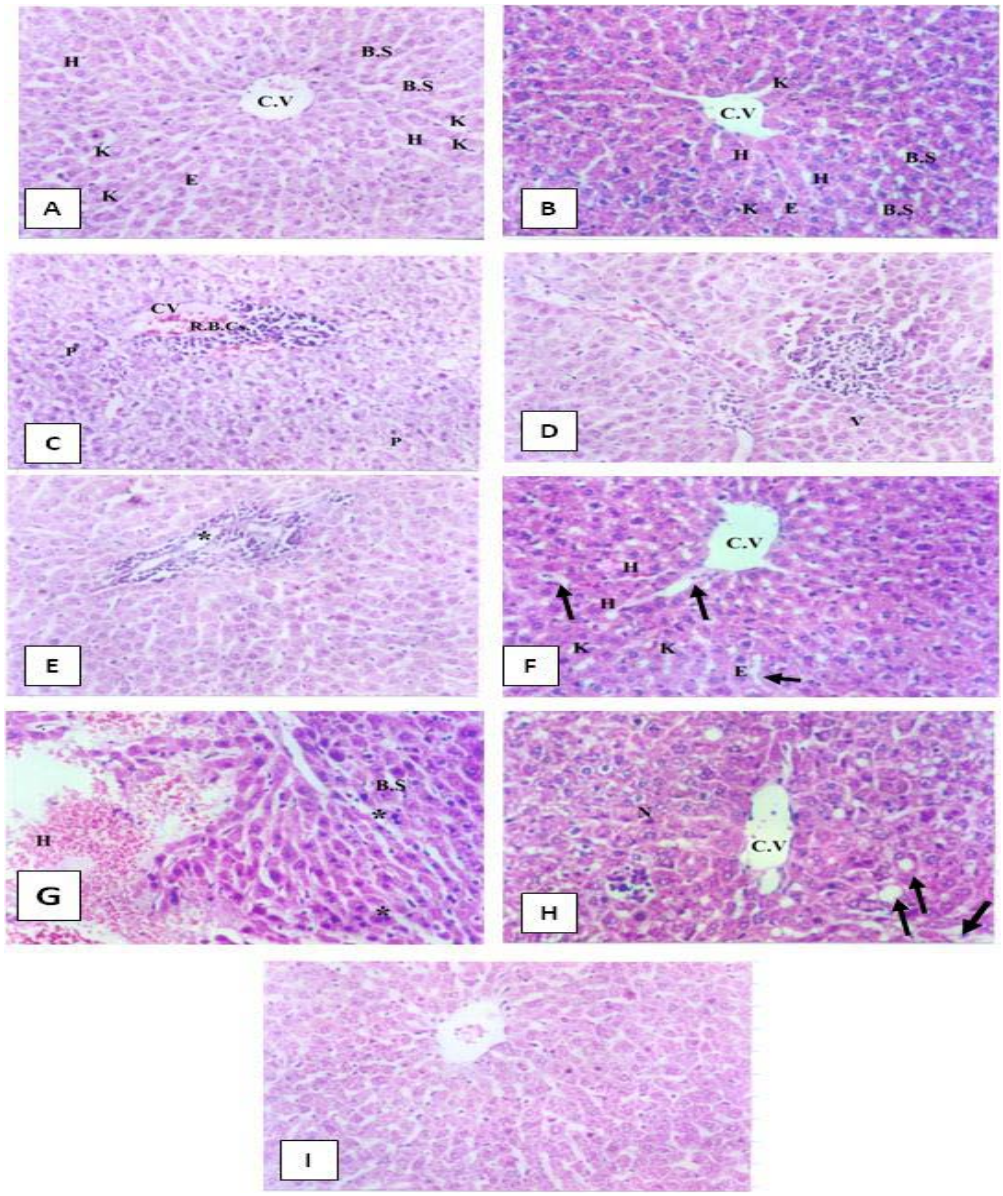


Fig. 5. (A) (Group I-control) Showing normal histological structure of rat liver; hepatocytes (H.), central vein (CV), blood sinusoid (BS), endothelial cell (E) and Kupffer cell (K). **(B) (GroupII-LC&LA)** Showing normal architecture of hepatic cells; the hepatocytes (H). The blood sinusoids (BS) are lined by a layer of endothelial cells (E) as well as Kupffer cell (K). **(C) (GroupIII-IR)** Showing central vein (CV) appears congested with blood cells (RBCs), the central vein (CV); is located at the center of the hepatic lobules. phyknosis in the hepatocytes nuclei (P) and vacuolated hepatocytes cytoplasm, cellular edema and binucleated hepatocytes, necrotic cells portal infiltration with leucocytes. **(D) (GroupIII-IR)** Showing focal area of hepatic necrosis associated with leucocytic cells infiltration and vacuolated hepatocytes cytoplasm (V) binucleated hepatocytes, dilated sinusoidal spaces. **(E) (GroupIII-IR)** Showing portal infiltration with leucocyte (*) **(F) (Group IV- IR+(LC&LA))** Showing dilated blood sinusoids (thick arrow), dilated central vein (CV) normal arrangement of the hepatic cords with invasion of endothelial cells (E) and kupffer cells (K)and haemorrhage (H). **(G) (Group IV- IR+(LC&LA))** Showing focal hepatic hemorrhage (H) and binucleated hepatocytes (*) wider sinusoidal space (B.S). **(H) (Group IV-IR+(LC&LA))** Showing vacuolar degeneration (arrow) of some hepatocytes, dilated central vein (CV), dilated blood sinusoids (thick arrows) and necrosis (N). **(I) (Group V-(LC&LA)+IR)** Showing the arranged hepatic architecture, dissociation of giant hepatocytes with parenchymatous islands. (H & E stain, X -200).

However, electron micrograph of liver from post–treated group revealed that LC & LA minimized most the injuries noticed in the hepatocytes of those rats exposed to γ-irradiated. Improvement in the nucleus and its nuclear membrane and chromatin, normal mitochondria, regeneration in the endoplasmic reticulum mean while, mild irregularity in nuclear membrane and fragmented endoplasmic reticulum were still found in different hepatocytes (Fig. 7 A, B and C). Ultra structurally, the hepatocyte sections of pre- and post-treated group (Figs. 7 D, E, F and G) revealed normal appearance of the liver cell membrane. The hepatocytes showing complete regeneration in the nucleus, nuclear membrane regular appear. Regeneration in the endoplasmic reticulum, the bile canal demonstrated in normal shape normal mitochondria in between rough endoplasmic reticulum.

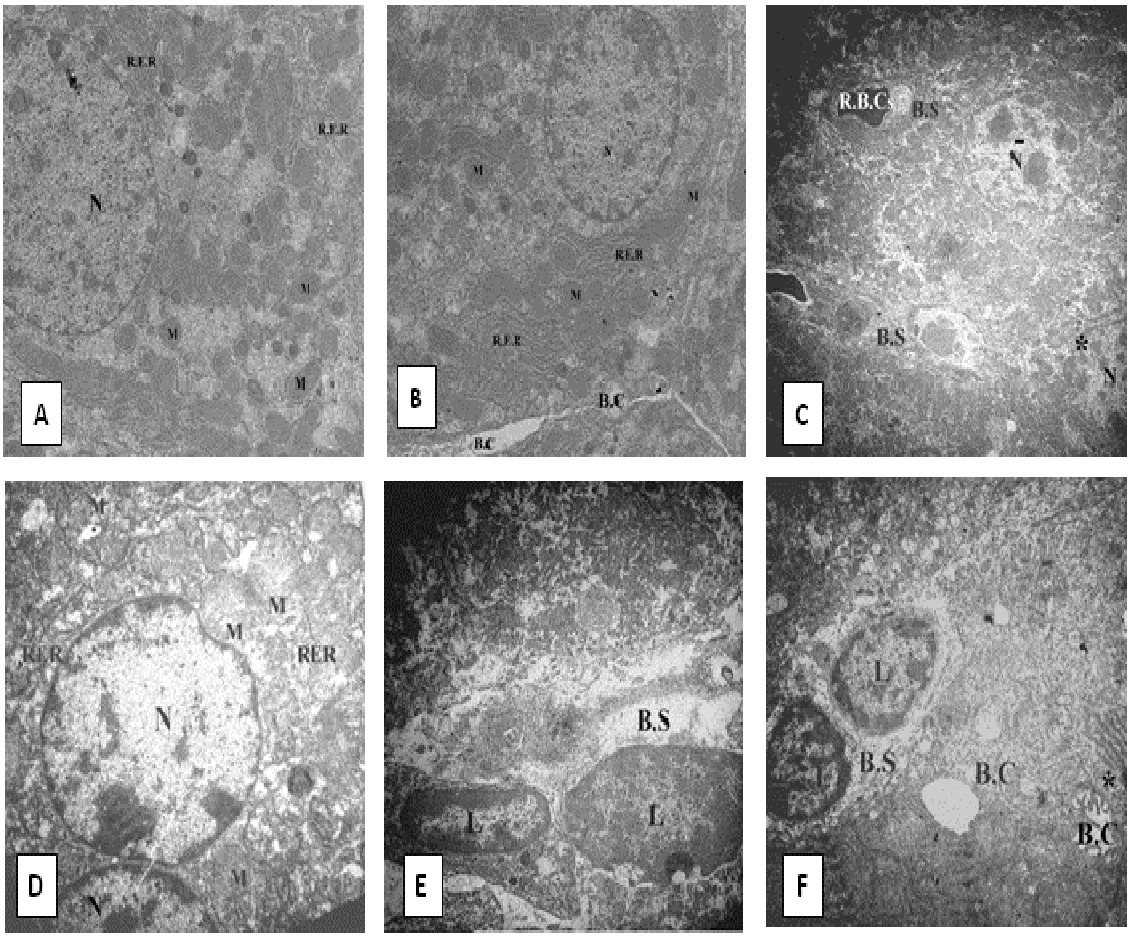


Fig. 6. Electron micrograph of hepatocyte section of albino rat **(A) (Group I-control)** Showing normal nucleus (N), normal variable sized mitochondria (M), and rough endoplasmic reticulum R. E. R. (X-8000). **(B) (GroupII-LC&LA)** Showing nearly normal ultrastructure, normal nucleus (N), normal mitochondria (M) R.E.R and normal bile duct (BC) and its canal (BC) (X-8000). **(C) (GroupIII-IR)** Showing degeneration hepatocytes with lysis cytoplasm matrix, degenerated nuclei (N) with wrinkled nuclear membrane (*) and ill defined cytoplasmic organelles, another showing mitosis (N), dilated B.S which contain, numerous R.B.Cs. (X-4000). **(D) (GroupIII-IR)** Showing irregularity in nuclear membrane (arrow), swollen mitochondria and rupture in its cristae (M), fragmentation of R.E.R. and binucleated (N) cells. (X -12000). **(E)(GroupIII-IR)** Showing degeneration hepatocytes with lyses and ill defined cytoplasmic organelles and dilated blood sinusoids (B.S) with abnormal lymphocytes (L) (X-100.000). **(F) (GroupIII-IR)** Showing: dilated bile canal (B.C.) with abnormal microvilli (*), also dilated blood sinusoids (B.S) with abnormal lymphocytes (L) (X -10.000).

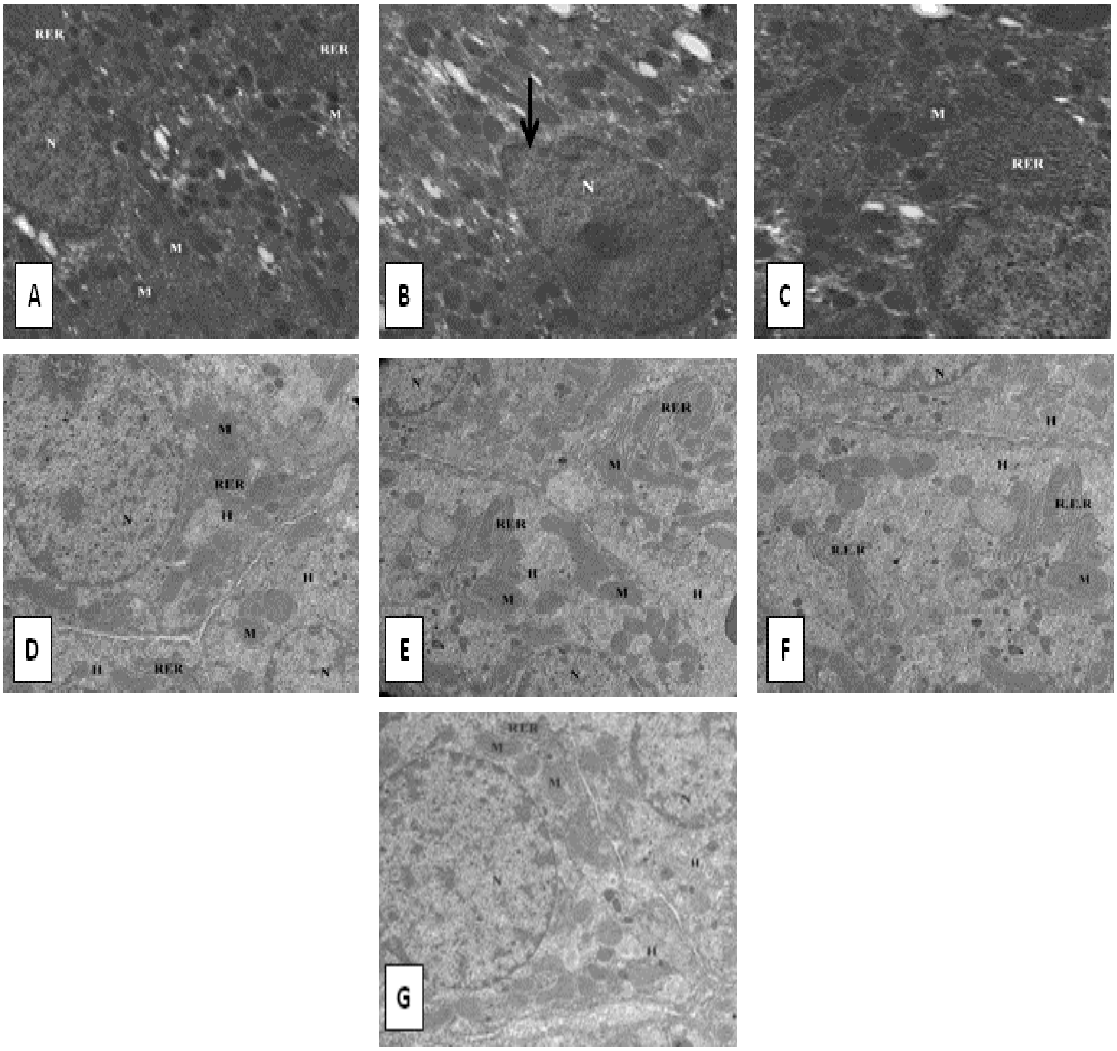


Fig. 7. Electron micrograph of hepatocyte section of albino rat: **(A) (Group IV- IR+(LC&LA))** Showing regeneration in the hepatocyte, notice: improvement in the nucleus (N), nuclear membrane, regeneration in the rough endoplasmic reticulum (RER), improvement of mitochondria (M) (X-8000). **(B) (Group IV- IR+ (LC&LA))** Showing regeneration in the hepatocyte, improvement in nucleus (N), nuclear chromatin and neucleolus and irregularity in nuclear membrane (arrow) (X 8000). **(C) (Group IV-IR+ (LC&LA))** Showing regeneration in the hepatocyte, improvement in the nucleus and its chromatin, regular nuclear membrane, regeneration in the rough endoplasmic reticulum (RER) and improvement of mitochondria (X -13300). **(D) (Group V-(LC&LA)+IR)** Showing complete regeneration in the hepatocyte with nucleus (N), regular nuclear membrane, regeneration in the rough endoplasmic reticulum (R.E.R) normal mitochondria in between R.E.R, and an intact cell membrane between 3 hepatocytes (H) (X-8000). **(E) (Group V-(LC&LA)+IR)** Showing bile canal can be demonstrated B. C. Showing complete regeneration in the hepatocyte with nucleus (N), regular nuclear membrane, regeneration in the rough endoplasmic reticulum (R.E.R) normal mitochondria in between R.E.R, and an intact cell membrane between 2 hepatocyte (H) (X -6400).**(F) (Group V-(LC&LA)+IR)** Showing complete regeneration in the hepatocyte with nucleus (N), regular nuclear membrane, regeneration in the rough endoplasmic reticulum (R.E.R) normal mitochondria in between R.E.R, and an intact cell membrane between 2 hepatocytes (H) (X -8080). **(G) (Group V-(LC&LA)+IR)** Showing complete regeneration in the hepatocyte with nucleus (N), regular nuclear membrane, regeneration in the rough endoplasmic reticulum (R.E.R) normal mitochondria (M) in between R.E.R, and an intact cell membrane between 2 hepatocytes (H) (X- 8080).

4. Discussion

The deleterious effects of ionizing radiation in the biological systems are mainly mediated through the generation of ROS, a process called oxidative stress, in a variety of cells as a result of water radiolysis (Kamat *et al.,* 2000). To control the flux of ROS, aerobic cells have developed their own defense system, the antioxidant system, which includes the superoxide dismutase (SOD), the first line of defense against oxygen-derived free radicals, catalysis the dismutation of superoxide anion into H_2O_2. Glutathione peroxidase (GPx) reduces lipidic or nonlipidic hydroperoxides as well as H_2O_2 (Taysi *et al.,* 2002). Antioxidant enzymes are regulated by multiple factors. The oxidative status of the cell is the primary factor regulating gene expression and activity of these enzymes (Rodriguez *et al.,* 2004). Both, endogenous (Nicotera *et al.,* 1989) and exogenous (Yoo *et al.,* 1999) agents act as oxidants and alter cellular oxidative equilibrium and, consequently, antioxidant enzyme gene expression.

In the present study, the expression of GPx and SOD was down-regulatated at the liver of γ-irradiated animals. It is reported that translocation of redox-sensitive transcription factors into the nucleus is influenced by oxidative stress (Sen and Packer, 1996). Besides, elevation of oxidative stress is known to cause destabilization of mRNAs (Mayo *et al.,* 2002). Therefore, the reduction in the mRNA of SOD and GPx in γ-irradiated rats could be either due to the oxidation of transcription factors or due to the decrease in the half lives of mRNAs. In agreement with our results, Mansour *et al.* (2008) recorded a significant decrease in the activities of SOD and GPx in hepatic tissues after whole body γ-irradiation. Ushakova *et al.* (1999) reported that radiation inhibited the expression of SOD gene in splenocytes from control mice.

The down-regulated mRNA of the hepatic GPx and SOD in γ-irradiated rats is alleviated by antioxidant treatments, suggesting the transcriptional control by LC & LA. In additions pre- and post-treatments with LC & LA significantly increased the levels of mRNA expression of hepatic antioxidant enzymes in irradiated rats than the post-treated group. These results are in agreement with that found by Miguel-Carrasco *et al.* (2010) who reported that L-carnitine treatment was able to reverse the down-regulation of cardiac GPx and SOD mRNA expression observed in hypertensive rats, increasing values to the levels observed in control, normotensive animals. Mansour (2006) reported that LC could increase the endogenous antioxidant defense mechanism in rats and thereby protect the animals from radiation-induced organ toxicity. Kocer *et al.* (2007) found that LC protected the lenses of rats against the damage produced by γ- irradiation by increasing the activity of the SOD enzyme and by scavenging free radicals generated by ionizing radiation. In another study, Davis *et al.* (2009) found that LA treatment restored antioxidant levels, reduced cell injury and protected cells against irradiation-induced cytotoxicity. Yang *et al.* (2008) determined that LA ingestion up-regulated the expression of genes related to free-radical scavenger enzymes of mice fed with high fat diet. LA also enhances the decrease of SOD activity and this may be related to its direct scavenger effect (Sundaram *et al*., 2006).

Numerous studies have demonstrated that exposure of mammalian cells to ionizing radiation induces several types of damage to DNA when the capacity of enzymatic and nonenzymatic antioxidant system is inadequate (Ames *et al.,* 1993). They induce a variety of lesions in DNA, including double and single-strand breaks, base and sugar damage, as well as DNA–DNA and DNA–protein cross-links (Barker *et al.,* 2005). Our results clearly showed that γ-irradiation, induces DNA damage in rat liver that was detected by the elevation the percentage of DNA fragmentation and the visualization of DNA ladders upon gel electrophoresis in treated animals. In addition, an increase of micronuclei in bone marrow cells was demonstrated in γ-irradiated rat. The induction of micronuclei was accompanied by cytotoxicity that evidenced through the significant reduction in the number of PCE/1000 cells. This reduction could be a consequence of a direct cytotoxicity or due to the micronuclei formation itself or to heavy DNA damages leading to cell death or apoptosis (Zorgui *et al.,* 2009). An increase in the DNA damage after γ-irradiation has been observed in different studies (Hosseinimehr *et al.,* 2003; Mansour *et al.,* 2008). It has long been known that the damaging effects of ionizing radiation on cellular DNA are brought about by both direct and indirect mechanisms. Direct action produces disruption of chemical bonds in the molecular structure of DNA, while indirect effects result from highly reactive free radicals such as •OH and •H produced during the radiolysis of water, and their subsequent interaction with cellular DNA (Pons and Sullivan, 1994).

Treatment with LC & LA in combination with γ-irradiation was markedly efficient and significantly inhibited the micronucleated PCE in bone marrow cells that reached 63.4% in post-treated and 87.5% in pre- and post- treated groups. Moreover, LC & LA treatment decreases the percentage of DNA fragmentation induced in liver cells as evidenced by the reduction of DNA fragments in agarose gel electrophoresis. The

observed protective effect of LC & LA maybe due to the direct protection against DNA damage by scavenging free radicals generated by ionizing radiation before they induce damage to the genetic material, i.e. the extent of primary damage in cellular DNA may be significantly reduced (Sundaram *et al.,* 2006; Kocer *et al.,* 2007). Secondly, LC & LA may indirectly alter the final level of DNA damage by enhancing the activity of DNA repairing enzyme, poly (ADP-ribosyl) polymerase, a nuclear protein that is intimately linked with the occurrence of DNA strand breaks and also other related repair mechanisms (Boerighter *et al.,* 1993; Szabodos *et al.,* 1999), so that the damage DNA is repaired more rapidly in irradiated cells pre-treated with LC & LA. Other studies have also observed that the LC & LA maintains thiol-containing compound and improves the glutathione redox status, which could protect DNA repairing enzymes, thereby preventing DNA strand break (Savitha and Panneerselvam, 2006). LC & LA treatment was also shown to decrease the levels of oxidative stress mediated DNA damage during aging probably by decreasing the oxidant production and by improving the antioxidant status in aged rats (Savitha and Panneerselvam, 2007). These results are in complete agreement with our reports on the protective effect of the LC & LA against DNA damage induced by ionizing radiation.

The histopathological architecture of the liver in γ-irradiated groups showed radiolesions in various form in liver that included congestion and dilatation in blood vessels, haemorrhage, vacuolation, lymphocyte infiltrations and necrobiotic changes in the hepatocytes. Concomitantly, the ultra structural examination for the same group showed different changes in the hepatocytes of treated rats. These changes were represented as swelling and ruptured mitochondria, fragmentation of the endoplasmic reticulum and nuclear damage with wrinkled nuclear membrane. Aforesaid alterations in the liver tissue showed similarity and conforms those recorded by several authors, with various experimental animals exposed to radiation (Nasr *et al.,* 1993; Zavodnik *et al.,* 2003; Mansour *et al.,* 2008). These histopathological and ultra structural changes in rats liver treated with irradiation were attributed to the direct harmful effect of irradiation on the biological system or indirect effect of free radicals liberated in the body after irradiation (Hagn, 1989).

In the current study, LC & LA were histopathologically and ultra structurally proven to be radioprotective agents by reducing the severity of radiation-induced liver damage. Most of the histopathological lesions observed in γ-irradiated group were disappeared to a large extent; where the normal architecture of the liver was restored in the pre- and post-treated group. whereas, slight dilated blood vessels and blood sinusoids were still found, in photomicrograph of liver sections in post- treated group, hepatocytes were found in focal hepatic hemorrhage and binucleated hepatocytes wider sinusoidal space, vacuolar degeneration of some hepatocytes, dilated blood vessel were noticed. Also, other hepatocyts were abnormal with either pyknotic or karyolitic nuclei. Meanwhile, the results of electron microscopy showed that post - treatment with LC & LA minimized most of the injuries noticed in the hepatocytes of rats exposed to γ-irradiation. While, a complete regeneration in the nucleus, nuclear membrane, endoplasmic reticulum and mitochondrial shape were detected in rats treated with LC & LA prior and post exposure to γ-irradiation.

In the previous studies, Altas *et al.* (2006) found that LC was able to ameliorate radiation-induced cochlear histopathologic damage in guinea pigs. Also, Sezen *et al.* (2008) histopathologically demonstrated that LC has a radioprotective feature by reducing the severity of radiation-induced brain damage. Ucuncu *et al.* (2006) reported that LC had radioprotective properties by delaying the starting day and reducing the severity of radiation-induced oral mucositis. L-carnitine is an endogenous mitochondrial membrane compound (Ueda *et al.,* 2002), these data support the idea that L-carnitine-mediated cytoprotection is due, in part, to inhibition of the mitochondrial apoptotic pathway. Moreover, LC has been shown to act as an important anti-apoptotic mediator (Moretti *et al.,* 2002). Ishii *et al.* (2000) reported that acetyl carnitine has shown to attenuate DNA fragmentation and nuclear condensation in cultured neurons promoting neuronal survival. Similarly, Sayed-Ahmed *et al.* (2004) reported that acetyl carnitine has a protective effect against Bleomycin-induced oxidative stress and energy depletion in lung tissues in rats, through its free radical scavenging properties with the consequent improvement in mitochondrial function and ATP production. Recently, Dadhania *et al.* (2010) have reported that LA pretreatment ameliorates MTX-induced intestinal toxicity in rat as evident from the protection against oxidative stress, decrease in DNA damage and protection of cellular morphology. Similarly, LA pretreatment affected cell death by decreasing the number of apoptotic and necrotic cells induced in cyclophosphamide treated rat (Selvakumar *et al.,* 2006). It has also been demonstrated that LA and DHLA both inhibit the apoptosis of rat thymocytes after exposure to either methylprednisolone or etoposide and this inhibition

was manifested at an early stage in the apoptosis as cell shrinkage, chromatin fragmentation (Bustamante *et al.,* 1995).

In the present study, we chose to co-supplement α-lipoic acid, an antioxidant, with L-carnitine to decrease the damaging effects in rat exposed to irradiation. Previous studies have shown an improvement in mitochondrial functions upon supplementation with these two mitochondrial metabolites (Hagen *et al.,* 2002). Moreover, Savitha and Panneerselvam (2006) have also shown that this feeding regimen improves the activities of mitochondrial enzymes and respiration, thereby improving the ATP levels during aging. Alie *et al*. (2008) demonstrated that feeding LA and acetyl carnitine ameliorated age-associated mitochondrial ultrastructural decay.

Treatment with LC & LA could protect against radiation damage by up-regulation the gene expression of the antioxidant enzymes (GPx and SOD), that is in turn may lead to inhibition of DNA fragmentation in liver cells, micronuclei incidence in PCE (bone marrow cells), and normal restoration of histopathologial and ultra structure liver architecture. It is interesting that being injected prior to irradiation, LC and LA could prevent the development of oxidative attack initiated by irradiation suggesting that LC and LA exhibit an antioxidant activity. Thus we can concluded that LC & LA may protect against the damage produced by radiation by preventing oxidative stress by scavenging ROS directly and increasing those of free-radical scavenger enzymes genes expression indirectly.

*** Correspondence to:**
Dr. Sally S. Alam
Cell Biology Department,
National Research Center,
E-mail: dr_sallyalam@yahoo.com
Tel.office:+202-33335978
Mobile: +20104359631
Fax.work:+202-33370931

References

1. Alie, G., Liu, J., Shenk, J.C. ,Fischbach. K, Pacheco, G.J., Chen, S.G., Obrenovich, M.E., Ward, W.F., Richardson, A.G., Smith, M.A., Gasimov, E., Perry, G. and Ames, B.N. (2008). Neuronal mitochondrial amelioration by feeding acetyl-L-carnitine and lipoic acid to aged rats. J Cell Mol Med.10: l582–4934.
2. Altas, E., Ertekin, M.V., Gundogdu, C. and Demirci, E. (2006). L-Carnitine reduces cochlear damage induced by gamma irradiation in Guinea pigs. Ann Clin Lab Sci. 36:312–318.
3. Ames, B. N., Shigenaga, M. K. and Hagen, T. M. (1993). Oxidants, antioxidants, and the degenerative diseases of aging. Proc. Natl. Acad. Sci. U S A. 90:7915–7922.
4. Arockia Rani, P.J. and Panneerselvam, C. (2001). Carnitine as a free radical scavenger in ageing. Exp Gerentol 36:1713–1726.
5. Bancrofet, J. and Stevens, A. (1977). Theory and practice of histological techniques. Chuchil Livingstone Edinburgh-London and New York, USA, p. 98.
6. Barker, S., Weinfeld, M., Zheng, J. and Li Murray, D. (2005). Identification of mammalian proteins cross-linked to DNA by ionizing radiation. J Biol Chem. 280(40):33826–33838.
7. Biewenga, G.P., Haenen, G.R. and Bast, A. (1997). The pharmacology of the antioxidant lipoic acid, Gen. Pharmacol. 29 (3): 315–331.
8. Boerighter, M.E., Franceschi, C., Arrigone-Martelli, E., Wei, J.Y., Vijg, J. (1993). The effect of L-carnitine and acetyl-L-carnitine on the disappearance of DNA single-strand breaks in human peripheral blood lymphocytes. Carcinogenesis 14: 2131–2136.
9. Brun, M.E., Gasca,S.,Girard,C., Bouton, K., De Massy,B. and De Sario, A. (2006). Characterization and expression analysis during embryo development of the mouse ortholog of MLL3, Gene 371: 25–33.
10. Bustamante, J., Slater, A.F., Orrenius, S. (1995). Antioxidant inhibition of thymocyte apoptosis by dihydrolipoic acid. Free Radic. Biol. Med. 19: 339–347.
11. Chang, B., Nishikawa, M., Sato. E., Utsumi. K. and Inoue, M. (2002). L-Carnitine inhibits cisplatin-induced injury of the kidney and small intestine. Arch Biochem Biophys. 405: 55–64.
12. Dadhania, V.P., Tripathi, D.N., Vikram, A., Ramaraob, P. and Jena, G.B. (2010). Intervention of α-lipoic acid ameliorates methotrexate-induced oxidative stress and genotoxicity: A study in rat intestine Chemico-Biological Interactions183: 85–97.
13. Davis, G.D., Masilamoni, J.G., Arul, V., Kumar, M.S., Baraneedharan, U., Paul, S.F., Sakthivelu, I.V., Jesudason, E.P. and Jayakumar, R. (2009). Radioprotective effect of DL-α-lipoic acid on mice skin fibroblasts. Cell Biol Toxicol. Aug;25(4):331-40.
14. Fritz, I.B. and Arrigoni-Martelli, E. (1993). Sites of action of carnitine and its derivatives on the cardiovascular system: interactions with membranes. Trends Pharmacol Sci 14:355–360.
15. Hagen, T.M., Liu, J., Lykkesfeldt, J., Wehr, C.M., Ingersoll, R.T., Vinarsky, V., Bartholomew, J.C. and Ames, B.N. (2002). Feeding acetyl-l-carnitine and lipoic acid to old rats significantly improves metabolic function while decreasing oxidative stress. Proc Natl Acad Sci. 99(4):1870–1875.

16. Hagn, V. (1989). Biochemical aspects of radiation biology. Experientia. 45.7.
17. Hayat, M.A. (1973). Basic technique for transmission electron microscopy. New York, Harcaurt Brace, Jonamovich.
18. Hosseinimehr, S.J., Tavakoli, H., Pourheidari, G.R., Sobhani, A. and Shafiee, A. (2003). Radioprotective effects of citrus extract against gamma irradiation in mouse bone marrow cells. J Radiat Res.44: 237–241.
19. Ishii, T., Shimpo, Y., Matsuoka, Y. and Kinoshita, K. (2000). Antiapoptotic effect of acetyl-L-carnitine and L-carnitine in primary cultured neurons. Jpn. J. Pharmacol. 83: 11–24.
20. Kagan,V.E., Shvedova, A., Serbinova, E., Khan, S., Swanson, C., Powell,R. and Packer, L. (1992). Dihydrolipoic acid—a universal antioxidant both in the membrane and in the aqueous phase. Biochem. Pharmacol. 44: 1637–1649.
21. Kamat, J.P., Boloor, K.K., Devasagayam, T.P.A. and Venkatachalam, S.R. (2000). Antioxidant properties of Asparagus racemosus against damage induced by gamma radiation in rat liver mitochondria, J. Ethnopharmacol. 71: 425–435.
22. Kocer, I., Taysi, S., Ertekin, M.V., Karslioglu, I., Gepdiremen, A., Sezen ,O. and Serifoglu, K. (2007). The effect of L-carnitine in the prevention of ionizing radiation-induced cataracts: a rat model. Graefes Arch Clin Exp Ophthalmol. 245: 588–597.
23. Kumaran, S., Subathra, M., Balu, M. and Panneerselvam, C. (2005). Supplementation of L-carnitine improves mitochondrial enzymes in heart and skeletal muscle of aged rats. Exp. Aging Res. 31: 55–67.
24. Limaye,PV., Raghuram, N. and Sivakami, S. (2003). Oxidative stress and gene expression of antioxidant enzymes in the renal cortex of streptozotocin induced diabetic rats. Mol Cell Biochem. 243: 147–152.
25. Mansour, H.H. (2006). Protective role of carnitine ester against radiation-induced oxidative stress in rats Pharmacological Research 54: 165–171.
26. Mansour, H.H., Hafez, H.F., Fahmy, N.M. and Hanafi, N. (2008). Protective effect of N-acetyLCysteine against radiation induced DNA damage and hepatic toxicity in rats. Biochemical pharmacology 75: 773– 780.
27. Mayo, J.C., Sainz, R.M., Antoli, I., Herrera, F., Martin, V. and Rodriguez, C. (2002). Melatonin regulation of antioxidant enzyme gene expression, Cell. Mol.Life Sci.59: 1706-1713.
28. Miguel-Carrasco, J.L., Monserrat, M.T., Mate, A. and Vázquez, C.M. (2010). Comparative effects of captopril and L-carnitine on blood pressure and antioxidant enzyme gene expression in the heart of spontaneously hypertensive rats. European Journal of Pharmacology, on line.
29. Moini, H., Packer, L. and Saris, N.E. (2002). Antioxidant and prooxidant activities of α -lipoic acid and dihydrolipoic acid, Toxicol. Appl. Pharmacol. 182: 84–90.
30. Moretti, S., Famularo, G., Marcellini, S., Boschini, A., Santini, G., Trinchieri, V., Lucci, L., Alesse, E. and De Simone, C. (2002). L-carnitine reduces lymphocyte apoptosis and oxidant stress in HIV-1-infected subjects treated with zidovudine and didanosine. Antioxid Redox Signal. 4:391–403.
31. Muthuswamy, A.D.,Vedagiri, K., Ganesan, M. and Chinnakannu, P. (2006). Oxidative stress-mediated macromolecular damage and dwindle in antioxidant status in aged rat brain regions: Role of l-carnitine and dl- α -lipoic acid Clinica Chimica Acta. 368: 84 – 92.
32. Nair, C.K.K., Parida, D.K. and Nomura, T. (2001). Radioprotectors in radiotherapy. J Radiat Res. 42: 21–37.
33. Nasr, S. A., Wahba, S. R., Roushdy, H. M. and Abou-Nour, S.A. (1993). Misonidazole sensitization of radiation induced ultrastructural alterations in the liver of rat. J. Egypt. Ger. Soc. Zool., 10 (c): 29.
34. Nicotera, T. M., Notaro J. and Notaro, S. (1989).Elevated superoxide dismutase in Bloom's syndrome: a genetic condition of oxidative stress. Cancer Res. 49:5239– 5243.
35. Packer, L., Witt, E.H. and Tritschler, H.J. (1995). α -Lipoic acid as a biological antioxidant, Free Radic. Biol. Med. 19: 227–250.
36. Packer, L.,Witt, E.H. and Tritschler, H.J. (1999). Alpha-lipoic as biological antioxidant. A review. Free Radic. Biol. Med. 19: 227–250.
37. Paradones, C.E., Illera, V.A., Peckham, D., Stunz, L.L. and Ashman, R.F. (1993). Regulation of apoptosis in vitro in mature spleen T cell. J. of Immunol.151, 3521.
38. Parihar, V.K., Dhawan, J., Kumar, S., Manjula, S.N., Subramanian, G., Unnikrishnan, M.K. and Mallikarjuna, Rao, C. (2007). Free radical scavenging and radioprotective activity of dehydrozingerone against whole body gamma irradiation in Swiss albino mice. Chem Biol Interact.170: 49–58.
39. Pisano,C., Pratesi,G., Laccabue, D., Zumino, F., Lo Giudice, P., Bellocci, A., Pacifici, L., Camerini, B., Vesci, L., Castorina, M.,Cocuzza, S., Tredici, G., Marmiroli, P., Nicolini, G., Galbiati, S., Calvani, M., Carminati, P. and Cavalletti, G. (2003). Paclitaxel and ciplastin induced neurotoxicity: a protective role of acetyl-l-carnitine. Clin. Cancer Res. 9: 5756–5767.

40. Pons, P. and Sullivan, J.B. (1994). Radiation and Radioactive Emergencies, in: B. John, Sullivan, R. Garry, Krieger (Eds.), Hazardous Materials Toxicology, Clinical Principles of Environmental Health, 2nd ed., Williams and Wilkins, A Waverly Company, pp. 441–450, Chapter 40.
41. Prahalathan, C., Selvakumar, E., Varalakshmi, P., Kumarasamy,P. and Saravanan, R.(2006). Salubrious effects of lipoic acid against adriamycin-induced clastogenesis and apoptosis in Wistar rat bone marrow cells. Toxicology 222: 225–232.
42. Ramsay, R.R., Gandour, R.D. and van der Leij, F.R. (2001). Molecular enzymology of carnitine transfer and transport, Biochim. Biophys. Acta. 1546: 21–43.
43. Reynold, E.S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. 17: 208-212.
44. Reznick ,A.Z., Kagan, V.E., Ramsey, R., Tsuchiya, M., Khwaja, S., Serbinova, E.A .and Packer, L. (1992). Antiradical effects in L-propionyl carnitine protection of the heart against ischemia-reperfusion injury: the possible role of iron chelation. Arch Biochem Biophys 296:394–401.
45. Rodriguez, C., Mayo, J. C., Sainz, R. M., Antolin, I., Herrera, F., Martin,V. and Reiter, R.J. (2004). Regulation of antioxidant enzymes: a significant role for melatonin: a minireview. J Pineal Res. 36: 1 – 9.
46. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). Molecular Cloning. A Laboratory. Cold Spring Harbor Laboratory Press, New York.
47. Santoro ,A, Brigida Lioi, M., Monfregola, J., Salzano, S., Barbieri, R. and Ursini, M.V. (2005). L-Carnitine protects mammalian cells from chromosome aberrations but not from inhibition of cell proliferation induced by hydrogen peroxide Mutat. Res. 587: 16–25.
48. Savitha, S. and Panneerselvam , C. (2007). Mitigation of age-dependent oxidative damage to DNA in rat heart by carnitine and lipoic acid. Mech Ageing Dev. 128(2): 206-212.
49. Savitha, S. and Panneerselvam, C. (2006). L-Carnitine and α -Lipoic acid improve age associated decline in mitochondrial respiratory chain activity in rat heart muscle. J. Gerontol. A Biol. Sci. Med. Sci. 61: 650–659.
50. Savitha, S., Tamilselvan, J., Anusuyadevi, M. and Panneerselvam, C. (2005). Oxidative stress on mitochondrial antioxidant defense system in the aging process: Role of dl- α -lipoic acid and l-carnitine. Clinica Chimica Acta 355: 173–180.
51. Sayed-Ahmed, M.M., Mansour, H.H., Gharib, O.A. and Hafez, H.F. (2004). Acetyll- carnitine modulates Bleomycin-induced oxidative stress and energy depletion in lung tissues. J Egypt Nat Cancer Inst 16(4): 237–243.
52. Schmid, W. (1975). The micronucleus test. Mutat Res. 33, 39.
53. Selvakumar, E., Prahalathan, C., Varalakshmi, P., Kumarasamyb, P. and Saravanan, R. (2006). Modification of cyclophosphamide - induced clastogenesis and apoptosis in rats by α -lipoic acid Mutat Res. 606: 85–91.
54. Sen, C.K. and Packer, L. (1996). Antioxidant and redox regulation of gene transcription, FASEB J. 10: 709-720.
55. Sezen, O., Ertekin, M.V., Demircan, M., Karslıoğlu, I., Erdoğan, F., Koçer I., Çalık I.and Gepdiremen A. (2008). Vitamin E and L-carnitine, separately or in combination, in the prevention of radiation-induced brain and retinal damages. Neurosurg Rev. 31:205–213.
56. Sundaram, K. and Panneerselvam, K.S. (2006). Oxidative stress and DNA single strand breaks in skeletal muscle of aged rats: role of carnitine and lipoic acid. Biogerontology 7:111–118.
57. Suzuki, Y.J., Tsuchiya, M. and Packer, L. (1993). Antioxidant activities of dihydrolipoic acid and its structural homologues. Free Radic. Res. Commun. 18: 115–122.
58. Szabodos, E., Fischer, G.M., Gallyas, F., Kispal, Jr., G. and Sumegi, B. (1999). Enhancement of ADP-ribosylation and its diminution by lipoate after ischemia-reperfusion in perfused rat heart. Free Radic. Biol. Med. 27: 1103–1113.
59. Taysi, S., Polat, F., Gul, M., Sari, R.A. and Bakan, E. (2002). Lipid peroxidation, some extracellular antioxidants and antioxidant enzymes in serum of patients with rheumatoid arthritis. Rheumatol Int. 21: 200–204.
60. Tominaga, H., Kodama, S., Matsuda, N., Suzuki, K. and Watanabe, M. (2004). Involvement of reactive oxygen species (ROS) in the induction of genetic instability by radiation. J Radiat Res. 45(2):181–188.
61. Ucuncu, H., Ertekin, M.V., Yoruk, O., Sezen, O., Ozkan, A., Erdogan, F., Kiziltunc, A. and Gundogdu, C. (2006). Vitamin E and L-carnitine, separately or in combination, in the prevention of radiation induced oral mucositis and myelosuppression: a controlled study in a rat model. J Radiat Res. 47: 91–102.
62. Ueda, S., Masutani, H., Nakamura, H., Tanaka, T., Ueno, M. and Yodoi, J. (2002). Redox control of cell death. Antioxid Redox Signal. 4(3):405–414.
63. Ushakova, T., Melkonyan, H., Nikonova, L., Afanasyev,V., Gaziev, A. I., Mudrik, N., Bradbury, R. and Gogvadze, V. (1999). Modification of gene expression by dietary antioxidants in radiation-induced apoptosis of amice splenocytes. Free Radical Biology & Medicine. (26): 887–891.
64. Weiss, J.F. and Simic, M.G. (1988). Perspectives in Radioprotection. Pharmacol. Ther. 39, 1/414.

65. Yang, R., Shi, Y., Li, W. and Yue P. (2008). Effect of lipoic acid on gene expression related to oxidative stress, lipid and glucose metabolism of mice fed with high fat diet. Wei Sheng Yan Jiu. Sep; 37(5): 560-562, 565. Chinese.
66. Yokozawa, T. and Dong, E. (2001). Role of ginsenoside-Rd in cisplatin-induced renal injury: special reference to DNA fragmentation. Nephron 9: 433– 438.
67. Yoo, H. Y., Chang, M. S. and Rho, H. M. (1999).The activation of the rat copper/zinc superoxide dismutase gene by hydrogen peroxide through the hydrogen peroxide-responsive element and by parquet and heat shock through the same heat shock element. J. Biol. Chem. 23: 887– 892.
68. Zavodnik, L. B., Kravchuk, R., Artsukevich, A., Chumachenko, S., Sheibak, M., Ovchinnikov, V. and Buko, V. (2003). Dynamics of structural changes in rats liver after single dose of gamma-irradiation. Radiats Biol Radioecol.43(6): 618-624.
69. Zorgui, L., Ayed-Boussema, I., Ayed, Y., Bacha, H. and Hassen, W. (2009).The antigenotoxic activities of cactus (Opuntia ficus-indica) cladodes against the mycotoxin zearalenone in Balb/c mice: Prevention of micronuclei, chromosome aberrations and DNA fragmentation. Food and Chem Toxicol. 47: 662–667.

22/11/2010

Fresh Water (The Nile And Its Branches) As One Of The Ways For The Development Of Fish Protein Sources In Egypt

Saber Mostafa Mohamed, Mahmoud Khalifa Ahmed and l. A. E l Karyony

National Institute of Oceangraphic & fishries (NIoF) Alexandria
saber_mostafa0@yahoo.com

Abstract: This study aimed to identify the freshwater fisheries in Egypt in terms of its evolution of fish production, the economic significance of the geographical distribution of the fish with identifying the seasonal productivity and measuring the impact of effort done on the fish production with emphasis on ways of development of those fisheries. The study had been adopted to achieve its goals on both economic analysis and descriptive statistical. The most important results were as follows: The fish production increased from river Nile fisheries from 57.8 thousand tons in 1995 to 79.7 thousand tons in 2008, after interest in the development of this source to provide fry tilapia and carp used in the development of water bodies.The study has been identified on the most important species and their relative importance, which represents about 72.3% of the average fish production during the study period, estimated at approximately 87500 tons. The tilapia, catfish, carp, and bayad are the most important varieties of high production are estimated the relative importance of 32.5%, 17.8%, 12.7%, and 9.8% respectively. While, the order comes after that in the arrangement, Nile perch, Shelan, (unicornfish) Albesaria, Nile lebeo, Eel, and barbal with an estimated relative importance of 3.9%, 3.6%, 3.3%, 2.1%, and 0.8%, respectively. The 96% of the annual variability production is due to changes in the productivity of varieties perch, catfish and tilapia. The middel Delta region (Desouk, Kafr El-Zayat, Menouf, Qanatier, and Benha) of the most important productive areas for fish in the River Nile, where a production of about 39.1%, followed by the region of the Nile Valley, which includes (Cairo, Giza, Fayoum, Beni Suef, Minya, and Assiut) represents 26.1%, while the production of Aswan region, represent 16.3%, which include (Sohag, Qena, Aswan).With regard to the employment and fishing boats, has decreased from 16400 boats in 1990 to about 11800 boats in 2008. While, the employment of fishing has decreased at high rates, which dropped from 51.5 thousand fishermen in 1990 to about 7.9 thousand fishermen in 2008, mostly working through the primitive ways, which have lacked in the safety manner. Furthermore, the number of boats licensed reflects the non reality where, the manual boat needed two or more person to complete the various operations on the boat, which indicates an increase in employment of fishing, non-licensed in those fisheries. The average production of the boat has increased with an average annual increase of productivity of 0.28 kg, while the average annual increase of productivity of a fisherman about 0.72 kg per year. However, the number and the productivity of boats are affected by 98% due to the annual changes of the production. Regarding, the examining of seasonal productivity and using seasonality index after excluding the effect of the general trend shows that, production is more than the overall average in the months of May, June, August and December. Whilst, the production is lower than of the overall average in the rest of the year months. Now there are a lot of efforts for the development of freshwater fisheries, through a variety of development programs (i.e. protect fisheries from pollution, fishery Seed supply, determine the appropriate fishing effort, and re-evaluate the characteristics and working methods of fishing). The targeted development plans to increase fish production through the overall development and coordination between the various parties to prevent the pollution of water resources and expansion in the construction and clearing waterways of plants, and re-stocking, especially carp fish, the Nile fish varieties, which became extinct with the quiet water stream, and made use of fish production.

[Saber Mostafa Mohamed, Mahmoud Khalifa Ahmed and l. A. E l Karyony. Fresh Water (The Nile And Its Branches) As One Of The Ways For The Development Of Fish Protein Sources In Egypt. Journal of American Science 2010;6(12):880-888]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Fresh water; Fish protein; tilapia, catfish, carp.

1. Introduction

At the moment, the problem of providing food is one the largest and most difficult problems of development in developing countries, especially in Africa. During the past years, a number of Arabs and African countries plans to address the problem of providing food and focused on the exploitation of fishery resources that constitute reserves important to meet the needs of the animal protein. In addition to, create a fundamental basis for the manufacture of fish products and their derivatives. Especially, the countries which have bodies, and waterways large as the Arab Republic of Egypt, which could take advantage of this wealth and make up the shortfall in

animal production, which contain beaches on capture fishery area is estimated by 13.9 million acres, which constitute, and include more than 200 varieties of different fish, 71 species of molluscs and about 14 types of cephalopods to other shrimp, crustaceans and other items, in addition to the varieties of freshwater. The importance of freshwater fisheries (the river Nile and its branches) in being the lifeblood of Egypt, which extends for a distance of about 1600 km, and is divided into two branches in the Delta region, and forks from small branches and channels cover a large area, and empty its waters in Mediterranean sea. Due to the fresh water utmost importance for all agricultural activities and industrial water needs of the Egyptian, as well as being one of the resource Fish Egyptians, which represents production of about 7.5% of the total fish production, the Egyptian in 2008, although the area is estimated at 178 thousand acres represent about 1.3% of the total area of fishery Egyptian according to the evaluation of the General Authority for Fish Resources Development in 2008. [1]

The increase in the fisheries is considered a statistical increase rather than substantive, due to expansion in the scope of registration and registration by the traditional fishing sector so that it includes additional landing sites.

Research problem

The main problem of fish production in Egypt is the inability of fish production, to fulfill the needs of consumer, although the contribution of all sources of production, including fresh water (the Nile and its branches), which exposed many of the changes that have affected the production capacity and hence ecological balance and Chemical composition, varieties and the disappearance of some fish species as a result of these changes as well as the lower contribution of this source in the production, so the strategy for the development of freshwater fisheries (the Nile and its branches) is considered one of the most important axes of fish production in Egypt.

Objective of the study

The aims of this research are to study the Egyptian freshwater fisheries in terms of (1) the development of fish production, (2) the economic importance of these fisheries, (3) the geographical distribution of fish varieties, (4) seasonal productivity of freshwater fisheries, (5) measuring the impact of efforts done on fish production, and (6) ways of development of those fisheries.

2. Material and Methods

Data Source

The study Adopted in achieving its goals on descriptive and quantitative manners in the interpretation and description of economic variables, using the simple statistical methods such as simple percentages and averages, as well as the use of a linear model to estimate rates of production of freshwater fish, in addition to the regression model to measure Fish varieties and effort aspirated on the total production.

Methods

The study relied on two main sources of data; the secondary data are derived from published annual statistical bulletins issued by the Ministry of Agriculture and Land Reclamation represented in the records of the General Authority for Fish Resources Development as well as some references, thesis and scientific research on the subject of study.

3. Results

Economic importance of fish production and development of freshwater aquaculture (the Nile and its branches)

The economic importance of these aquacultures is concerning with productivity, employment, and fishing fleet, as well as the highly productive fish varieties. In spite of the misguided area compared to Egyptian aquacultures area where production is estimated at 37.9 thousand tons in 1990 represents representing 12.8% of the total fish production, including Egyptian equivalent to about 159.1 million pounds representing 9.3% of the total income of fish. Identifying the effectiveness of the five years plans strategy's of the General Authority for Fish Resources Development [2] toward the development of freshwater aquaculture, has been shown that, production was estimated at 67.9 thousand tons in 1995, representing 16.7% value of 305.4 million pounds, which represents 14.4% of the value of fish production, increasing to an estimated 80.3 thousand ton in 2000, accounting for about 11.1% with an estimated value of 483.4 million pounds, which represents about 8.5% of the value of fish production in the same year, while estimated at 83800 tons in 2005 represents 9.4% of the total fish production Egyptian in the same year, worth estimated 559.7 million pounds represent 7.2% of the value of fish production for the same year declined due to changes to about 79.7 thousand tons, representing 7.5% a value of 654.2 million pounds, which represents 9.5% of the value of fish production in 2008. This contribution is a little for the Egyptian fish production and needs attention for the development as shown, relatively low contribution in production in spite of the increase in production, as

well as the low percentage of the successive production values.

Studying the evolution of the production of freshwater aquaculture through the study period was characterized by fluctuations between high and low, when it was estimated at 57.9 thousand tons in 1995 increased until it reached a peak in 2002 to an estimated 120.8 thousand tons, then dropped to 83 800 tons in 2005 and then increased again to an estimated 104 thousand tons in 2006. These aquacultures constitute an important source for the population in the Central Delta and the Nile Valley in reliance on consumption of fresh fish, representing the central area for production also the amount of production based upon the fry and fingerlings used in the development of water bodies in previous years.

With regard to fishing vessels operating in these fisheries has been shown that it is estimated at 16.4 thousand in 1990, mostly boats of the third degree, which is on the decrease until estimated at 11.8 thousand boats in 2008. The employment of fishing has decreased at high rates, as estimated at 51.5 thousand fishermen in 1990 decreased by up to an estimated 7.9 thousand fishermen in 2008, mostly working through the primitive manner which has lacks in safety procedures for the fisherman and production and, hence, the number of boats licensed reflects the non reality where, the manual boat needed two or more person to complete the various operations on the boat, which indicates an increase in employment of fishing, non-licensed in those fisheries.

Study the general time trend for the production of freshwater aquaculture of their respective areas to identify the development and production growth during the period (1995 - 2008). This reflects the impact of Productivity policies for the development and growth of fish production from natural sources.

Form the data, it has been shown from the following equation that fish production from freshwater increasing annually by 31.7 thousand tons. Significantly, the statistical estimate in accordance with the coefficient of determination (R^2) is 36% of the changes to the annual production of fresh water due to factors related to time.

As indicated by the following formula:

$$P^e = 63385.4 + 31743.18\ T_e$$
$$(6.205) \qquad (2.645)\ *$$
$$R^2 = 0.36 \qquad F = 217.3\ *$$

Where:
P^e = the amount of fish production from freshwater per Ton

T_e= time in years 1, 2, 3, ….., 14
* Significant at level of 0.01

The relative importance of the most important fish species

There are many different species and varieties of fish as well as the quantity of production areas as a result of the impact of natural (i.e. the inventory diversity of flora and fauna located in rural water), climatic (i.e. temperature, light and movement of water) and surrounding biological factors. The influence of these factors on quality and quantity of fish has been identified the most important fish species of freshwater aquaculture in accordance with the fishing of different species during the period (1995 - 2008).

Table (1) shows, the most important varieties and their relative importance, which represents about 72.3% of the average fish production during the study period, estimated at 87500 tons. The tilapia, catfish, carp and bayad are the most important varieties of high production, estimated at 32.5%, 17.8%, 12.7%, and 9.8%, respectively and then comes in the arrangement Nile perch, Shelan, Albesaria, lebeo, eel and barbal with a relative importance of 3.9%, 3.6%, 3.3%, 2.1%, and 0.8% respectively. Studying the impact of fish species on the fish production from freshwater aquaculture, shows that the best models estimated is the double logarithmic as the following equation:
Where, the fish production of fresh water identified as a dependent variable (y) on one hand and the production of varieties, on the other hand, in the form of an independent and is in the tilapia (x1), Aqraamit (x2), Schall (x3), perch (x4), bayad (x5), eel (x6), barbal (x7), lebeo (x8), Albesaria (x9) and carp (x10).

$$\text{Log } y^8{}_n = \log 2.686 + 0.143 \log X4 + 0.440 \log X2 + 0.323 \log X1 \text{ (equation 1)}$$
$$(1.847) \quad (0.143)^* \quad (6.747)^* \quad (2.551)^*$$
$$R^2 = 0.96 \qquad F = 61.25$$

Where:
$\mathbf{y^8{}_n}$ = quantitative estimates for the total production of fresh water
X4 = annual production of fish Nile perch.
X 2 = annual production of fish, catfish.
X 1 = annual production of tilapia.
Xn = variable time in years 1, 2, 3 ....., 14.

* Significant at 0.01 level

It is clear from equation (1), change of 10% in the production of Nile perch (x 4) leads to the change OK in the total production is estimated at

10.4%, (2) change of 10% in the production of catfish (x 2) leads to a change approved in the total production estimated at 4.4%, (3) change of 10% in the production of tilapia (x1) lead to a change in the total production estimated at 3.2%, (4) 96% of the changes in annual production of freshwater aquaculture due to annual changes in fish species, according to the coefficient of determination (R^2), and (5) proved to be significant at the level of a probabilistic model better than 0.001.

Table (1) the development of fish production per ton, classified as freshwater fisheries during the period (1995-2008).

Collected and calculated from:
- Central Agency for Public Mobilization and Statistics, Bulletin of fish production, Oaadadmokhtlvp, period (1995-2008).
- Ministry of Agriculture and Land Reclamation, the General Authority for the development of fisheries, fish production statistics (1995-2008).

Statement	Tilapia	Aqraamit	Chillan	Peel Perch	Perch	Eel	Barbal	Lebeo	Bassaria	Carp	Other	Total
1995	21914	10755	1799	840	5390	274	-	1632	908	349	14012	57873
1996	25504	11310	1715	795	5826	193	-	1441	875	534	16210	64403
1997	26402	12317	5902	787	273	1553	-	1386	935	3603	12377	65535
1998	26566	11613	5723	1223	201	1581	887	1230	3559	218	15451	68252
1999	27260	10951	1777	1248	5665	327	58	783	158	1592	14161	63980
2000	30885	14486	2563	1671	8395	377	15	1217	1731	11142	7839	80321
2001	22955	23215	3923	3745	15472	269	449	1716	3657	14550	19936	109887
2002	33854	25439	5494	4265	19026	475	389	1608	4843	1648	23811	120852
2003	28881	25158	5985	7481	16437	514	479	2237	4305	18054	8769	118300
2004	36290	12992	1843	8453	10228	466	860	2562	2036	23712	5558	105000
2005	27874	13422	1454	2917	7900	350	636	2111	1745	22317	3077	83803
2006	34187	15532	2038	5939	11952	2105	779	3501	4205	19435	5303	104976
2007	30198	15295	1843	4536	6668	976	920	2359	6881	21629	6405	97710
2008	24256	14685	1903	3982	5647	411	629	1908	4431	16911	4925	79688
%	32.5	17.8	3.6	3.9	9.8	0.8	0.5	2.1	3.3	12.7	12.9	100

The relative importance of the most productive areas (the geographical distribution of Nile fish)

To study the relative importance of the production of fish from fresh water (the Nile and its branches), the production areas were divided according to the General Authority for Fish Resources Development, which divided production areas into six areas as follows: the Damietta, includes (Damietta, Mansoura, and Zagazig), Western Region includes (Behaira), the meddle Delta includes (Desouk, Kafr El-Zayat, Menouf, Qanatir, and Benha), the eastern region includes (Al-Salam Canal, Ismailia Canal area), the Nile Valley includes (Cairo, Giza, Fayoum, Beni Suef, Minya, and Assiut area) and Aswan includes (Sohag, Qena, and Aswan).

Table (2) shows, the relative importance of fish production to the average period (1995-2008), it was found that, the meddle Delta is the most important areas of production, producing about 39.4% of the production of fresh water at an average production estimated at 34,183 tons. While, the Nile Valley comes in the second ranked, which estimated at 26% of the fresh water production at an average production estimated at 22,553 tons, whilst the Aswan region comes at a third ranked, producing about 15.3% of the production of fresh water at an average production estimated at 13,325 tons, whereas the Damietta region occupies the fourth ranked, where it produces about 14.4% of the average production of fresh water production estimated at 12,466 tons, while the eastern region came in fifth ranked, where it produces about 2.8% of the average production of fresh water production estimated at 2427 tons, while the western region occupies rank where the final output is estimated at 1.3% of the average production of fresh water production is estimated at 1094 tons, during the study period (1995-2008). From the abovementioned discussion clearly, the productions of fish freshwater are concentrated mainly in the central Delta.

Table (2) the ratio of the important areas of fish production in the River Nile during the period (1995 - 2008).

Statement	Damietta region	Western region	West delta region	Eastern region	Nile valley region	Aswan region	Grand Total
1995	10232	-	33686	-	6169	7389	57872
1996	10270	-	35219	-	7584	9100	64403
1997	10984	-	33736	1267	9294	10254	65535
1998	12811	-	32334	1593	12861	10121	68252
1999	12943	-	21344	2000	16004	11251	63981
2000	13776	-	31377	3194	17236	13728	80321
2001	13840	2288	43583	2951	30632	16593	109887
2002	15515	2636	41151	3378	38525	19647	120852
2003	15298	3165	33620	3266	41649	21302	118300
2004	12970	2601	27984	4159	31539	25747	105000
2005	12946	2105	30667	3783	27509	5793	83803
2006	12816	1243	38055	2305	31727	18830	100476
2007	9954	745	41683	3535	26142	15651	97710
2008	10173	528	34127	2553	18866	1143	79688
General average	12466	1094	34183	2427	22552	13325	86863
%	14.4	1.3	39.4	2.8	26	15.3	100

Collected and calculated from
- Central Agency for Public Mobilization and Statistics, Bulletin of fish production, different issues, period (1995-2008).
- Ministry of Agriculture and Land Reclamation, the General Authority for the development of fisheries, fish production statistics (1995-2008).

Statement	Production per ton												General average
	January	February	March	April	May	June	July	August	September	October	November	December	
Tilapia	2531	2310	2511	2694	2680	2773	2314	2658	2589	2480	2344	2275	30159
Aqraamit	1975	1812	1798	1777	1732	1665	1652	1645	1702	1708	1646	1671	20783
Chillan	1217	1155	1157	1197	1325	1315	1263	1184	1179	1171	1161	1061	14385
Peel Perch	332	321	332	333	360	355	369	348	348	345	336	325	4104
Perch	703	618	667	718	750	773	715	727	675	633	634	626	8239
Eel	493	533	385	399	375	377	456	418	438	468	402	423	5167
Barbal	234	211	209	206	192	194	226	224	230	189	203	188	2506
Lebeo	152	148	166	166	142	138	138	152	136	159	156	162	1815
Bassaria	72	80	91	102	87	79	91	94	51	59	52	49	907
Carp	96	88	70	66	59	50	64	43	40	43	48	48	715
Other	442	436	423	439	435	431	432	438	422	421	402	389	5110
Total	8247	7712	7809	8097	8137	8150	7720	7931	7810	7676	7384	7217	93890

Table (3) Monthly averages for the fish varieties of fresh water during the period (1995-2008)

Source calculated and collected: collected and calculated from:
- Central Agency for Public Mobilization and Statistics, Bulletin of fish production, Oaadadmokhtlvp, period (1990-2004).
- Ministry of Agriculture and Land Reclamation, the General Authority for the development of fisheries, fish production statistics (1990-2004).

Seasonal production (seasonal ratios after excluding the effect of the general trend)

Seasonal fish production means, all changes in fish production, with systematic successive periods of time less than twelve months, also known as seasonal concentration of production in certain periods of the year or decline or lack in the remaining months of the year, leading to changes in price. These fluctuations are attributable to a number of influences, which is mostly systematic. Study the seasonal production is useful to identify seasonal periods of low or increase production to avoid the effect's changes in productivity for the product and the consumer as well as policy planners [4].

Studying the seasonal fluctuations of fish production to the most important fish species's aquaculture of the Nile during the study period, as shown in Table (4) shows, a fluctuating seasonal pattern, where the production rise and decline from the annual average year, estimated at 7829 tons during the period from January until December, where reach the maximum in January, 8247 tons, then reduced to a minimum during December, to estimated 7214 tones, due to the multiplicity of varieties of fish from River Nile aquaculture and the different patterns of seasonal products to another, which requires analysis of the fluctuations of the most important species for production, especially tilapia, catfish, bayad and carp (Table 4).

The results have been shown that, the seasonal fluctuations of fish tilapia rise and fall as well as the fluctuation of seasonal monthly production during the study period, with reduced production from the total average monthly of the year about 30.2 thousand tons during the period from January to December, culminating in June by seasonal amounted to about 9.2% and then production to fall below during december, as the percentage of seasonal 5%. As it turns out seasonal fluctuations of catfish production, which reduced production from the average monthly year of about 14.4 thousand tons during the period of January to December, where production diminished to below during February, the seasonal rate reached about 8%, while peaked in June by seasonal amounted to about 9.1%.

As for the carp is characterized by a pattern of clearly seasonal and determined by two periods, firstly, start from January to April, where more than production during that period than the average annual year, estimated at 20.8 thousand tons, reached its maximum in January, with average seasonal amounted to 9.5% and then secondly, start from May to December, it is characterized by a decrease of production from the total average annual, reached the lowest level in August by seasonal amounted to 7.9%.

Regarding to the bayad fish, it has been shown, the increase in production for the general average of 8.2 thousand tons during the period from January to December. The production fell to below during the month of February, it reached about 7.5%, while peaked in june by seasonal reached about 9.4 %.

Table (4) Seasonal productivity of the fish Nile

Statement	Average Monthly	Directional values	Average Monthly	Seasonal Guide
January	8247	8166.7	101.0	99.1
February	7711	8105.4	95.1	93.3
March	7809	8044.1	97.1	95.2
April	8098	7982.8	101.4	99.5
May	8138	7921.5	102.7	100.8
June	8150	7860.2	103.7	101.7
July	7717	7798.9	98.9	97.1
August	7995	7737.6	103.3	101.4
September	7809	7676.3	101.7	99.8
October	7676	7615	100.8	98.9
November	7385	7553.7	97.8	95.9
December	7214	6032.0	119.6	117.3
Total	93949	92494.2	1223.2	1200
Average	7829			

$y^n = 8227 - 61.3\ x_n$

(56.4) (- 3.1)* $R^2 = 0.69$ $F = 9.57^*$

Where: y^n= Monthly average fish production from freshwater per ton

x_n = time per months (January .. December)

* Significant at 0.01 level of significance

The decrease in the winter months to drop the fish to the depths as a result of cold, which is difficult to access fishing by the methods used, and explain the increased production of some species in the summer months due to the temperatures rise and the growth of phytoplankton close to the surface of the water, leading the fish to rise and thus increase the chances of fishing. Examining the seasonal productivity of fish production after excluding the impact of general direction and in accordance with the table (3) shows that, the production is more than the overall average in the months of May, June, August and December, down from the average year in the remaining months of the year Fig (1)

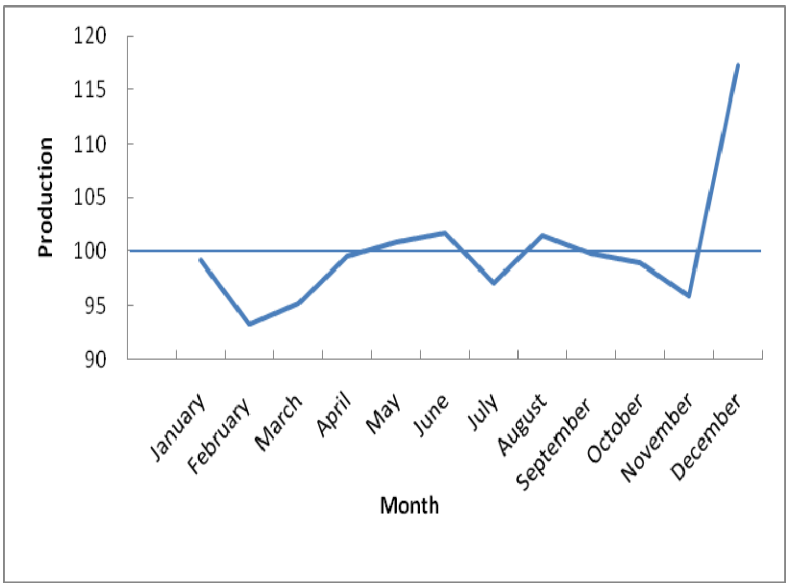


Figure (1) Guide to the interposed seasonal production of fresh water during the period (1995-2008)

The impact of fishing efforts on production: -

The number of workers, number of fishing boats and size of equipment affect largely on the quantity and quality of fish caught in addition to the productivity of the boat and fisherman,

The outcome of production is low, due to the disproportionate between the numbers of boats and the size of fish stocks available for fishing. It is clear from Table (5), the average number of boats operating in fresh water was about 15182 boats, whereas, the average productivity of the boat was about 5.1 tons and the average number of fishermen about 28491 while, the average productivity of a fisherman about 4.8 tons.

Studying the fishing effort on production, according to a model of multiple regression showed that the best models estimated [5] is the linear model in its following were identified as production of fresh water as the dependent variable (y) on the one hand, and the preparation of boats, on the one hand, as an independent variable (x1) and the average productivity of the boat (x 2) and the preparation of the fishermen (x 3) and the average productivity of a fisherman (x 4)

$$Y^8n = -86355.2 + 5.58x_1 + 15216.2\ x_2$$
$$(-10.59) \quad (12.86)^* \quad (26.57)^*$$
$$R^2 = 0.98 \qquad F=363.6$$

Where=

Y^8n = estimated quantity of total production of fresh water

x_1 = number of vessels operating fishing in the fresh water.

x_2 = average productivity of the boats

x_n = variable time in years 1,2,3 ....., 14.

* Significant at 0.01 level of significance

It is clear from the previous model that (1) Change one unit in the number of boats (x1), offset by corresponding change in the total production by 5.6 units, (2) change the amount of one unit in the average productivity of the boat (x2) counteract with change in the total production by (15216.2), (3) the independent variables explain 98% of the annual changes in the total production, and (4) proved to be

significant at the level of a probabilistic model better than 0.001.

Table (5) the average productivity of the boat and fisherman from freshwater fisheries (the Nile and its branches)

Years	Production(ton)	Number of boats (boats/ boat)	Productivity of the boat	Number of fishermen	Productivity of fishermen (ton)
1990	37882	16353	2.317	51543	0.735
1991	41268	16614	2.484	50771	0.813
1992	39623	14233	2.784	44763	0.885
1993	49897	14230	3.506	44760	1.115
1994	57512	18025	3.191	58728	0.979
1995	57873	13701	4.224	42398	1.365
1996	64403	19360	3.327	42230	1.525
1997	65535	13728	4.774	42197	1.553
1998	68252	12094	5.643	30371	2.247
1999	63980	14308	4.472	23772	3.398
2000	80321	16757	4.793	17287	4.646
2001	109887	18036	6.093	14518	7.569
2002	120852	18039	6.699	11748	10.287
2003	118300	18360	6.443	14182	8.342
2004	105000	14725	7.131	10382	10.114
2005	83803	12399	6.759	10333	8.110
2006	104976	13914	7.545	10617	9.888
2007	97710	11806	8.276	12777	7.647
2008	79688	11773	6.769	7950	10.02

Collected and calculated from:

- Central Agency for Public Mobilization and Statistics, Bulletin of fish production, Oaadadmokhtlvp, period (1995-2008).
- Ministry of Agriculture and Land Reclamation, the General Authority for the development of fisheries, fish production statistics (1995-2008).

Ways to the development of freshwater fisheries (the Nile and its branches)

Five years plans for economic development, aimed to increase fish production from freshwater sources [6] can only be achieved through the comprehensive development of this source and coordination between the various parties to prevent the pollution of water resources and expansion in the construction and clearing waterways of plants, with re-stocking fingerlings, especially carp, fish Nile, which became extinct with the quiet power, and benefit from the research production of fry fish friendly environment away from pollution, and attention to guidance fish, especially in the area of development for the indirect fish farming in the rice fields, as well as improve the efficiency of freshwater aquaculture as being one of the renewable natural resources, which may dry up if used at rates that exceed natural regeneration, and therefore requires an amendment radical in the methods and systems and the use of freshwater fisheries by avoiding fishing small fish in the seasons of natural spawning with requiring fishermen to use nets legal with mesh wide, and to prevent over fishing, and modifying the licensing boats and fishermen, with the adoption of research on the dates of spawning of different items and regions so as to regulate fishing, and reliance on electronic record for statistical quantities produced so that data can be modified depending on the circumstances available and follow-up of production. Attention to raising the efficiency of workers with the fishery sector in freshwater fisheries through training in the field of limited interest in fish and abide by the laws and regulations of hunting, with regard to fishing boats can be equipped to suit the possibilities of fishing, with attention to fry fish collection sites used in providing those fisheries.

References

1- Central Agency for Public Mobilization and Statistics, Bulletin of fish production, Oaadadmokhtlvp, period (1995-2008).
2- Ministry of Agriculture and Land Reclamation, General Authority for the development of fisheries, fish production statistics (1995-2008).
3- Ministry of Agriculture and Land Reclamation (2008). General Authority for Fish Resources Development 'device and Follow-up, Cairo.

4- The General Authority for Fish Resources Development, the role of aquaculture in the strategy of the General Authority for Fish Resources Development to the Year - 2012 aradina.kenanaonline.com/topics/57220/links/4747.
5- Jalal Abdel Fattah Navigator (2002). Entrance to study the economic market, tools Thalpelep, demand and supply, Center for Translation and copyright of King Faisal University.
6- Mohammed Kamel Rehan, (1971). An analytical study of the resources used and the output in the manufacture of grinding wheat, Ph.D. thesis, Department of Agricultural Economics, University of Ain Shams.

11/5/2010

Role of lactic acid bacteria as a biopreservative agent of Talbina

Amal S. Hathout*, Soher E. Aly

Toxicology & Food Contaminant Dept., Food Industries & Nutrition Division, National Research Centre, Dokki 12622, Cairo, Egypt
amal_hathout@hotmail.com

Abstract: Talbina is a mixture of barley flour and milk. The aim of this study is to evaluate the role of probiotic bacteria (*L. gasseri*, *L. reuteri*) compared to yoghurt starter bacteria (*S. thermophilus* and *L. delbreukii* sub sp. *bulgaricus*) as a biopreservative agent of Talbina samples. Shelf life of refrigerated Talbina processed by lower count (ratio 1:3 LAB : Talbina) of *L. gasseri* or *L. reuteri* increased and reached over 21 days at 6±2°C, compared to yoghurt starter bacteria which ranged between 6 and 14 days depending on the type of milk used. Storage temperatures are considered the main factors for biopreservation action of lactic acid bacteria (LAB). Increasing storage temperature to 12±2°C increased total fungal count and greatly changed fungal profile. It could be concluded that the potential of LAB to inhibit the growth of common food spoiling fungi opens up new perspectives for the bio-preservation of food products.
[Amal S. Hathout, Soher E. Aly. **Role of lactic acid bacteria as a biopreservative agent of Talbina**. Journal of American Science 2010; 6 (12):889-898]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: lactic acid bacteria; Talbina; barley flour; fungi; bio-preservation

1. Introduction

Bio-preservation has gained increasing attention as natural means for controlling the shelf life and safety of food products. The application of bioprotective cultures to ensure the hygienic quality is a promising tool although, it should be considered only as an additional measure to good manufacturing, processing, and storage and distribution practices (Holzapfel et al., 1995). LAB have shown a major potential for use in bio-preservation because of safety for human consumption (GRAS status) and the prevalent microflora during storage in many foods (Vignolo et al., 2008). LAB can produce a wide range of antimicrobial metabolites, i.e. organic acids, diacetyl, acetoin, hydrogen peroxide and bacteriocins. These antimicrobial activities can contribute in the microbiological safety by controlling the growth of other microorganisms, and inhibition of pathogenic bacteria such as *Listeria monocytogenes*, *Staphylococcus* sp. and *Clostridium* sp. (Stiles 1996; Caplice and Fitzgerald, 1999)

The nutritious and therapeutic benefits of probiotic microorganisms have been most extensively investigated in dairy products such as milk, yogurt (Khalil and Mansour, 1998), and cheese (Ong et al., 2006; Meyer et al., 2007). Vasiljevic and Shah (2008) added that probiotic bacteria have been exploited extensively by the dairy industry as a tool for the development of novel functional products. Probiotics have been also incorporated in edible spreads (Charteris et al., 2002); meat (Arihara et al., 1998); Ras cheese (Abdalla et al., 2008); and beef burger (Mohsen et al., 2009).

In recent years there has been a growing research interest for the utilization of barley in a wide range of food applications (Bilgi and Çelik, 2004) as it can be processed into a number of palatable, nutritious food products. Barley flour is suitable for a wide range of food applications, and it is used in a wide range of traditional Arabic, Kurdish, Persian, and Turkish foodstuffs including *kashkak*, *kashk* and *murri*. In Saudi Arabia, barley soup is traditionally eaten during Ramadan (Long, 2005). Historically, barley has been an important food source in northern and Eastern Europe and Asia (Newman and Newman, 2006).

Talbina is a popular traditional food product in the Arab world prepared by mixing barley flour and milk and cooking for 10 to 15 minutes. In Islam, Talbina was prescribed for seven diseases (Hadith), these include grief, high cholesterol levels, heart disease, treatment of cancer, effects of aging, diabetes and hypertension. Recently Miller et al. (2000) confirmed these facts, whereas Majchrzak et al. (2004) added that barley contains a number of antioxidants that can reduce the incidence of chronic diseases, including cancer. Because dairy products provide the ideal food system for delivery of probiotic bacteria (Shehata et al., 2004), therefore, the objectives of this study are to evaluate the effect of probiotic bacteria compared to yoghurt starter bacteria on the shelf life of Talbina produced and on fungal profile during refrigerated storage period. The viability of LAB was also evaluated.

2. Material and Methods

2.1. Materials

Raw fresh cow milk having 12.26% TS, 3.51% fat, 3.42% protein, 0.17% titratable acidity and pH 6.50 was obtained from a private farm in Calyoubia Governorate, whereas UHT (Ultra Heated treatment) milk as well as barley flour were obtained from supermarkets. Raw milk was pasteurized at 72°C for 15 s before using in the processing of Talbina.

2.2. Cultures

Lactobacillus delbreukii sub sp *bulgaricus* and *S. thermophilus* were obtained from Chr. Hansen's lab. Denmark, while the probiotic bacteria *L. gasseri* LA39 and *L. reuteri* LA6 were kindly donated by Dr. T. Saito, Faculty of Biological Resource Science, Tohoku University, Japan.

2.3. Organism preparation

De Man Rogosa Sharpe (MRS) broth (Difco Labs., Detroit, MI, USA) was used for propagation of the LAB. For each culture, MRS broth was inoculated at 1% using a freshly prepared culture of the desired strain of LAB and incubated at 37°C. Fermented milk was prepared by inoculating the colonies in reconstituted 10% no-fat dry milk (NFDM) until coagulation.

2.4. Preparation of Talbina

Talbina samples were prepared by mixing milk (UHT milk, fresh cow milk) and barley flour (10%) and cooking for 10 minutes. After cooling LAB>10^7 CFU/g was added to the mixture. The LAB added to the Talbina was divided into two different concentrations: Lower count (1:3 LAB: Talbina) and higher count (1:1 LAB: Talbina).

Different treatments (T) used in this study were as follows:-

T 1: *L. gasseri*, lower count, UHT milk
T2: *L. gasseri*, higher count, UHT milk
T3: *L. gasseri*, lower count, fresh milk
T4: *L. gasseri*, higher count, fresh milk
T5: *L. reuteri*, lower count, UHT milk
T6: *L. reuteri*, higher count, UHT milk
T7: *L. reuteri*, lower count, fresh milk
T8: *L. reuteri*, higher count, fresh milk
T9:Yoghurt starter bacteria, lower count, UHT milk
T10:Yoghurt starter bacteria, higher count, UHT milk
T11:Yoghurt starter bacteria, lower count, fresh milk
T12:Yoghurt starter bacteria, higher count, fresh milk
T13: Control Talbina, UHT milk
T14: Control Talbina, fresh milk

All Talbina samples were distributed in plastic cups (100g each) and stored at 6±2 and 12±2°C for 21 days. Samples were prepared in three replicates.

2.5. Microbiological analysis

Samples were prepared for microbiological examination according to ICMSF (1996). Samples were examined for total fungal count (CFU/g); according to American Public Health Association (APHA, 1992). Isolated fungi were identified according to Nelson et al., (1983). Viable cells of Lactobacilli in Probiotic Talbina were determined on MRS agar (Dave and Shah, 1996).

2.6. Titratable acidity and pH

Samples were analyzed during storage for titratable acidity (%) according to the method of AOAC (1990). Samples were also analyzed during storage for pH value using a wireless Mess-Stab 656 pH meter (Knick, Berlin, Germany).

2.7. Statistical analysis

Statistical analysis was performed using SPSS statistical program for windows (Version 16) (SPSS Inc., Chicago, IL, and USA). Standard deviation of mean was used to describe data, and a Student's T-test was used to evaluate the significant differences between control and Talbina samples. P value was considered significant if less than 0.05 at 95%.

3. Results

3.1. Microbiological analysis

Talbina is a new traditional food processed from barley flour, milk and LAB. A specific factor for Talbina shelf life is to prevent fungal growth, therefore, it is necessary to discuss the role of different factors affecting fungal occurrence during refrigeration storage.

3.1.1. Total fungal count

Fungal counts in processed Talbina samples were demonstrated in Figure (1). Lower counts of fungi were isolated from Talbina samples stored at both 6±2 and 12±2°C compared to control. Fungi were not observed in Talbina samples (Figure 1) processed by UHT milk with lower LAB count (ratio 1:3) of *L. gasseri* or *L. reuteri* during the storage period (21 days) at 6±2°C revealing significant difference to control samples. On the other hand, the period reached to 14 days for samples processed by yoghurt starter bacteria. Using higher LAB count (ratio 1:1), (Figure 1a, c) decreased the shelf life period to 14 and 7 days for samples processed by *L.*

gasseri and *L. reuteri* respectively. Increasing storage temperatures to 12±2°C lead to higher fungal count (Figure 1) in processed Talbina.

3.1.2. Occurrence and distribution of fungi

Data in Table (1) showed that increasing the storage temperature to 12±2°C caused a great change in fungal profile, where *Aspergillus* species were the most dominant fungi, whereas *Penicillium* species were the most dominant on those stored at 6±2°C. In addition, *Fusarium* species appeared only on Talbina processed by fresh milk. This variation of fungal species may be due to the difference of optimum temperature for each genus of fungal growth.

The occurrence of *A. parasiticus*, *A. flavus, A. niger* and *A. carbonarius* (Table 2) differed according to the type of milk used. During storage at 6±2°C, *A. flavus* was the most existing fungi followed by *A. niger* and *A. carbonarius* respectively in Talbina samples processed by raw milk. Meanwhile in Talbina samples processed by UHT milk, *A. niger* was the dominant species followed by *A. carbonarius* and *A. parasiticus* respectively. On the other hand, *A. parasiticus* was the most prevailing fungi in both Talbina samples processed by either fresh milk or UHT milk at 12±2°C.

3.1.3. Lactic acid bacteria viable count

Results in Table (3) showed that Talbina processed by either low or high viable count of *L. gasseri* or *L. reuteri* were over 10^7 CFU/g (7.00 $\log_{10}$ CFU/g). Data also indicated that Lactobacilli increased during the development of the storage period at 6±2°C. After 7 days of storage viable count recorded 7.681 and 7.301 $\log_{10}$ CFU/g for *L. gasseri* and *L. reuteri* respectively for the lower starting count (1:3). The viable counts continuously increased during storage period as shown in Table (3).

3.2. Chemical analysis

3.2.1. Titratable acidity (%)

Samples with higher count of bacteria, UHT milk and stored at 6±2°C (Figure 2) showed higher titratable acidity (1.14%) for *L. gasseri* samples at zero time compared with those processed by lower count of bacteria (0.69%). These findings were recorded for all treatments; this may be due to metabolites produced by LAB, which were proportional to the bacterial count in fermented milk.

Figure (2a, b) also revealed that Talbina processed by yoghurt starter bacteria and UHT milk showed the highest titratable acidity (0.96%) followed by *L. gasseri* (0.69%) and *L. reuteri* (0.64%) respectively for samples stored at 6±2°C at zero time. Samples stored at 12±2°C showed that increasing titratable acidity was also recorded for samples processed by fresh milk (Figure 2d) compared to those processed by UHT milk (Figure 2c).

3.2.2. pH value

Table (4) showed that the pH value of control Talbina was similar to that of milk, and that there was no great change in the pH value during storage period. The unchanged pH value of control Talbina during storage is evidence that Talbina is processed under sanitation conditions. On the other hand, the pH values ranged from 5.44 to 5.25 and from 5.11 to 5.00 for *L. gasseri* Talbina processed by UHT milk and fresh milk respectively during storage period at 6±2°C revealing significant difference to control samples. Furthermore, *L. gasseri* Talbina samples had higher pH values than those of *L. reuteri* followed by yoghurt starter. These findings are related to the change in titratable acidity (%).

3.3. Shelf life

Figure (3) revealed that *L. gasseri* and *L. reuteri* Talbina samples stored at 6±2°C showed long shelf life followed by yoghurt Talbina. All samples stored at 12±2°C showed a lower shelf life, which reached up to 7 days (data no shown). Furthermore, the UHT milk (Figure 3) had a long shelf life in all samples compared to fresh milk. Shelf life means that samples are without any unfavourable changes in flavour, taste, appearance and no fungal growth observed, low titratable acidity, high pH values .Results also indicated that probiotic bacteria had a positive effect on the shelf life of samples compared to either control or samples processed by yoghurt starter bacteria. On the other hand, lower bacterial count for both *L. gasseri* and *L. reuteri* showed the best results such as long shelf life (21 days) compared to those of higher bacterial count (Figure 3).

4. Discussion

LAB are considered to be important components of the microbiota, playing a large variety of health-promoting functions. Strains belonging to the *Lactobacillus* genera have traditionally been used as probiotics and added as functional components to various food products. Talbina samples processed by fresh milk were highly contaminated by fungi compared to those processed by UHT milk. These results are in agreement with Brenier-Pinchart et al. (2006) who surveyed different products for fungal contamination and found that UHT milk is not contaminated by filamentous fungi, since UHT milk is heated to 135°C for a couple of seconds to kill harmful bacteria that may be present. In addition UHT milk is sometimes called 'long-life milk,' and it is slightly different from fresh milk and has an extra

treatment that enables it to be stored at room temperature (as long as it is unopened) for extended periods. Meanwhile the heat treatment for fresh milk (pasteurization or boiling) may not be enough to ensure the safety of the fresh milk against microorganisms. The effect of heat treatment of milk was reported by Ismail et al. (2004) who indicated that heat treatment might reduce the curd tension by partial precipitation of calcium salts and changes in protein. On the other hand, local fresh milk may be contaminated with antibiotic residues or preservative agents that can affect the growth of LAB in dairy product (Saneez et al., 1995). In an effort to overcome this problem for home or Simi pilot scale industries, the possibility is considered to replace totally or partially fresh milk in dairy product by UHT milk, which is considered as a preferable consumer's health milk.

The storage conditions, especially temperature, represent an important factor affecting the microbiological quality of foods and feeds, and the improper storage temperature may prolong survival of the microorganisms or even enhance their multiplication (Zmysłowska and Lewandowska 2000). In agreement, Collombo et al. (1992) reported that low temperature was more effective for prolonged cheese storage than high temperature. Therefore, it could be demonstrated that storage temperature is considered the first factor affecting the shelf life of Talbina, thus refrigerating temperature is important to control spoilage or fungal growth of probiotic Talbina.

Our results stated that *L. gasseri* followed by *L. reuteri* were able to delay and/or decrease fungal contamination in Talbina compared to control These findings may be due to the production of the bacteriocins gassericin A and reutericin 6 from *L. gasseri* LA39 and *L. reuteri* LA6 respectively (Kawai et al., 2004). The bacteriocin gassericin A showed the highest antimicrobial activity against gram-positive food borne pathogenic bacteria, *Listeria monocytogenes*, and *Staphylococcus aureus* (Kawai et al., 2006). Arakawa (2008) added that this bacteriocin can be produced in natural media such as milk and milk based media. Arakawa et al. (2009) explained that bacteriocins (gassericin A, reutericin 6) might act on the cytoplasmic membrane of the target cell and cause death of the cell by efflux of potassium ion.

Previously AbdAlla et al. (2008) found that two probiotic bacterial strains (*L. reuteri* and *L. casei*) inhibited the fungal growth of *A. parasiticus* on Ras cheese during 90 days of storage. Recently Mohsen et al., (2009) reported that the use of LAB completely inhibited the pathogenic microorganisms and total fungal count in beef burger during 45 days of storage at 4°C.

Various media have been studied as carriers for probiotic bacteria, such as cheese (Bergamini et al., 2005), and yoghurts (Vinderola et al., 2000), but there is no data for Talbina as probiotic vehicles. Our results stated that when Lactobacilli were inoculated in Talbina, it sustained a cell count at recommended concentrations of probiotic in food (10^7 or 10^8 CFU/g) (Reid, 2001). These results are in good harmony with those recorded by Ishibashi and Shimamura (1993) who reported that food containing such bacteria should contain at least 10^7 live microorganisms per g or per ml at the time of consumption, in order to benefit the consumer. On the other hand, the count of LAB were still in the limit up to day 14 of storage period as recommended by Svensson (1999) who reported that probiotic cultures should be able to withstand food processing and storage conditions encountered during the manufacture of functional foods under industrial conditions.

Results showed that titratable acidity (%) was higher for yoghurt starter bacteria followed by *L. reuteri* and *L. gasseri* respectively in descending order, whereas control samples showed the lowest titratable acidity and no remarkable change during storage. It was also noticed that titratable acidity increased sharply during storage period. Results are in synchronization with those reported by Joshi and Sharma (2009) who revealed that titratable acidity increased with the advancement of fermentation period up to 16 days, meanwhile El Owni and Hamed (2009) added that the titratable acidity of cheese samples stored at room temperature was higher in comparison with those stored at refrigerator temperature. Our results were confirmed by Rivera-Espinoza and Gallardo-Navarro (2010) who indicated that *L. delbreukii* produced significantly more titratable acidity expressed as lactic acid than *L. casei*, and that *L. delbreukii* was capable of surviving at low pH and high acidity. These findings were discussed previously by Carr et al., (2002) who stated that LAB encompass a heterogeneous group of microorganisms, which have a common metabolic property that produces lactic acid from the fermentation of carbohydrates. The decrease in pH of Talbina during storage was related to titratable acidity (%). It is also necessary to know that pH measures free hydrogen ion concentration, thus it is a more direct measurement, as it circumvents "apparent acidity" and is usually less subject to error or misinterpretation than titratable acidity measurement. This may have been due to the production of lactic and organic acids by LAB, which had an effect on lowering pH value as reported by Kuipers et al.

(2000) and Shah (2007). Thus, the pH value of the product was in the range of 6.4 to 4.5, which is the best pH for encouraging lactic acid bacterial growth as reported by Rivera-Espinoza and Gallardo-Navarro, (2010). Therefore, pH value plays an important role for the microbiological growth affecting the shelf life of the products.

Our investigation revealed that LAB play an important role as a preservative agent depending on the type of bacteria and its count during application leading to the increase in shelf life of the Talbina. These findings were confirmed by Shah (2007) who reported that LAB produce some components, which have a bacteriocidal and bacteriostatic effect, and therefore, lead to the delaying and/or disappearance of fungal growth in samples during storage period.

Results also revealed that storage temperature is the most important factor affecting the preserving action of LAB in the product, which is higher at 6±2°C, followed by the type of LAB used depending on the preserving action of bacteriocin. The third factor is the count of bacteria that produce metabolites parallel with the count of bacteria such lactic acid, and organic acid related to titratable acidity during storage.

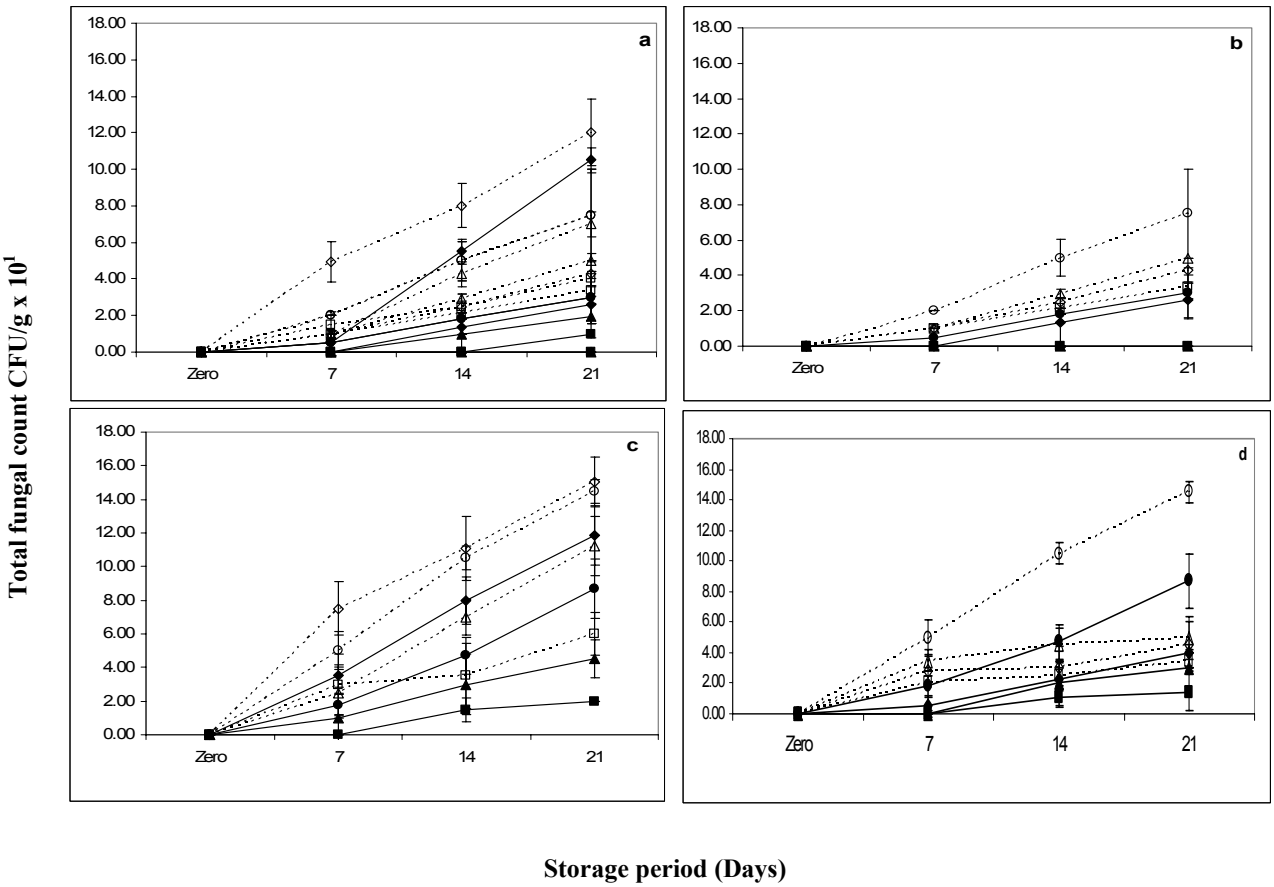


Fig.1. Changes in total fungal count CFU/g in Talbina

a)UHT milk and higher count of LAB, b)UHT milk and lower counts of LAB, c)Fresh milk and higher counts of LAB, d) Fresh milk and lower counts of LAB during storage at 6±2°C ___ and 12±2°C ---. Data are Mean ± SD. Results revealed no significant difference. ● Control at 6±2°C, ○ Control at 12±2°C, ■ *L. gasseri* at 6±2°C, □ *L. gasseri* at 12±2°C, ▲ *L. reuteri* at 6±2°C, △ *L. reuteri* at 12±2°C, ◆ Yoghurt starter at 6±2°C, ◇Yoghurt starter at 12±2°C

Table 1
Occurrence and distribution of fungi in samples of Talbina during storage

Fungi	6±2°C				12±2°C			
	UHT milk		Raw milk		UHT milk		Raw milk	
	CFU/g	%	CFU/g	%	CFU/g	%	CFU/g	%
Aspergillus	10	45.45	22	59.46	27	84.37	39	82.98
Penicillium	11	50.00	10	27.03	2	6.25	3	6.38
Fusarium	0	0.00	4	10.81	0	0.00	1	2.13
Rhizopus	1	4.55	1	2.7	3	9.37	4	8.51
Total	22	100	37	100	32	100	47	100

Table 2
Occurrence and distribution of *Aspergillus* species in samples of Talbina during storage

Aspergillus species	UHT milk				Raw milk			
	6±2°C		12±2°C		6±2°C		12±2°C	
	CFU/g	%	CFU/g	%	CFU/g	%	CFU/g	%
A. parasiticus	2	20	11	40.74	3	13.64	16	41.03
A. flavus	0	0	4	14.81	8	36.36	8	20.51
A. niger	5	50	7	25.93	6	27.27	10	25.64
A. carbonarius	3	30	5	18.52	5	22.73	5	12.82
Total	10	100	27	100	22	100	39	100

Table 3
Viability of *Lactobacillus* (log_{10} CFU/g) in Talbina stored at 6±2°C

LAB	Ratio of LAB: Talbina	*Lactobacillus* count (log_{10} CFU/g)		
		Zero time	Day 7	Day 14
L. gasseri	1:1	7.740	8.113	8.901
	1:3	7.082	7.681	8.719
L. reuteri	1:1	7.698	8.079	8.880
	1:3	7.000	7.031	8.602

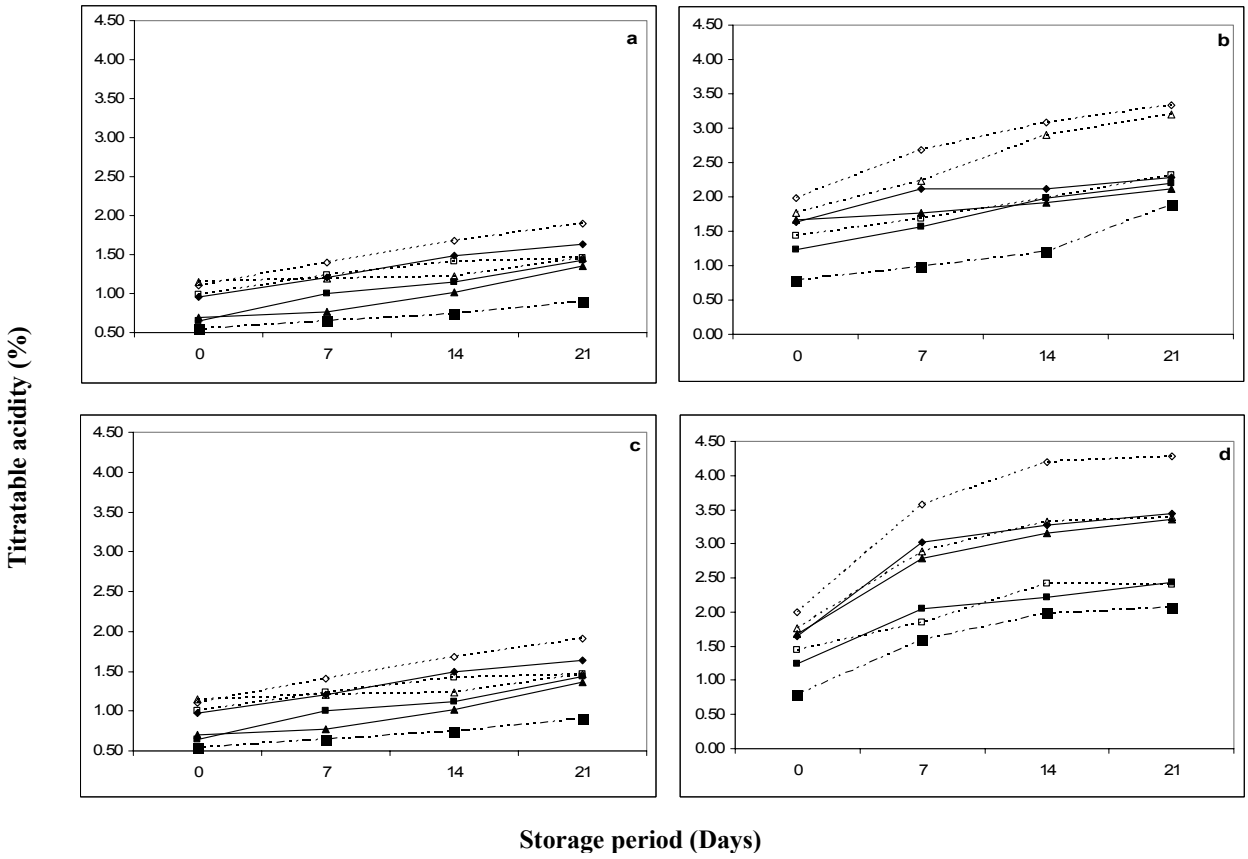


Fig.2. Changes in titratable acidity (%) in Talbina

a) UHT milk and stored at 6±2°C, b) Fresh milk and stored at 6±2°C, c) UHT milk and stored at 12±2°C, d) Fresh milk and stored at 12±2°C during storage period. ------ Ratio of LAB: Talbina 1:1 v/v, _____ Ratio of LAB: Talbina 1:3 v/v. Results revealed real significant difference $P<0.05$. ■ Control, ▲△*L. gasseri*, ■□*L. reuteri*, ◆◇Yoghurt starter

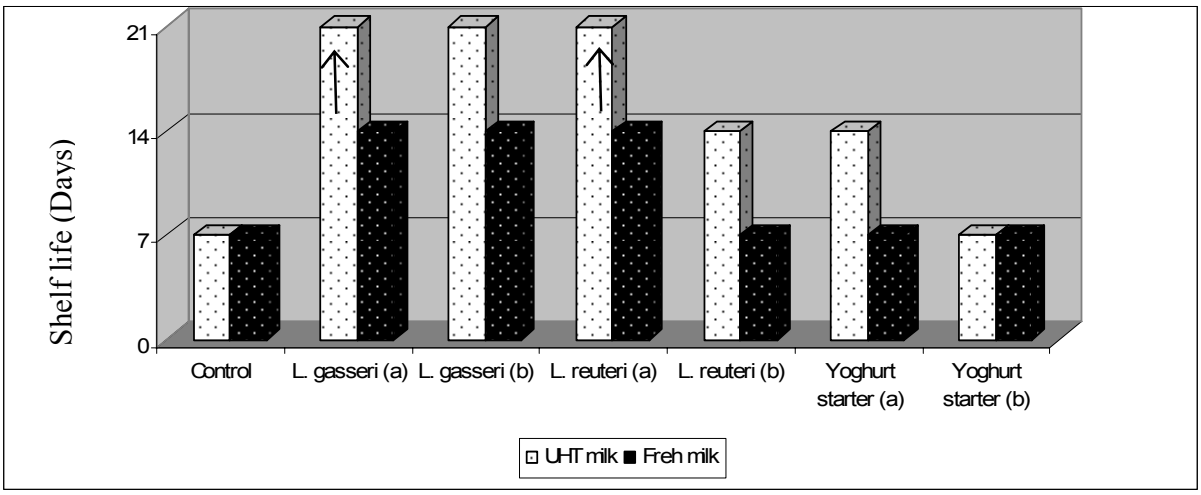


Fig.3. Shelf life of Talbina processed by UHT and fresh milk during storage at 6±2°C

Arrows means more than 21 days. a) Ratio of LAB: Talbina (1:3 v: v), b) Ratio of LAB: Talbina (1:1 v: v)

Table 4

Changes in pH value of Talbina after 21 days of storage

Treatments	Storage period (Days)	6±2°C		12±2°C	
		UHT milk	Fresh milk	UHT milk	Fresh milk
Control	Zero	6.45	6.02	6.44	5.54
	21	6.16	5.67	5.57	4.89
L. gasseri a	Zero	5.44*	5.11*	5.11*	4.79
	21	5.25*	5.00*	4.98*	4.63
L. gasseri b	Zero	5.27*	4.89*	4.95*	4.54*
	21	5.00*	4.62*	4.63*	4.40*
L. reuteri a	Zero	5.18*	5.43*	5.00*	4.93
	21	4.95*	4.95*	4.85*	4.59
L. reuteri b	Zero	4.97*	5.10*	4.79*	5.09*
	21	4.83*	4.96*	4.62*	4.10*
Yoghurt starter a	Zero	5.10*	5.11*	4.67*	4.37*
	21	4.50*	4.88*	4.19*	4.19*
Yoghurt starter b	Zero	4.70*	4.69*	4.55*	4.20*
	21	4.50*	4.20*	4.10*	4.10*

a) Ratio of LAB: Talbina (1:3 v: v)
b) Ratio of LAB: Talbina (1:1 v: v)
*Indicates real significant differences $P<0.05$

4. Conclusion

LAB provides a high preservative effect especially at low temperature <6°C causing longer shelf life to the product over 21 days. It could also be concluded that the potential of LAB to inhibit the growth of common food spoiling fungi opens up new perspectives for the bio-preservation of food products. Furthermore, Talbina as a dairy product provides the ideal food system for the delivery of these beneficial bacteria to human gut. Further research needed includes studying more LAB as biopreservative agents as well as identifying and isolation of bacteriocins produced by LAB.

Acknowledgements:

The authors wish to thank Dr. T. Saito, Faculty of Biological Resource Science, Tohoku University, Japan, for the kind donation of *L. gasseri* LA39 and *L. reuteri* LA6.

Corresponding Author:
Dr. Amal S Hathout
Toxicology & Food Contaminant Department
National Research Centre, El-Bohouth St.,
Dokki 12622, Cairo, Egypt
Tel.: +2 02 33371362;
Fax: +2 02 33370931
amal_hathout@hotmail.com

References

1. AbdAlla E, Soher A, Saleh Y, Mary S, Amal Hathout. Probiotic bacteria as a tool to produce high quality and safe Ras cheese. Egyptian Journal of Dairy Science 2008; 36 (1): 97-109.
2. AOAC, Association of Official Analytical Chemists. Official methods of analysis, 15th ed., AOAC, Washington DC, USA. 1990.
3. APHA, American Public Health Association. Compendium of methods for microbiological examinations of foods, (3rd edition), Washington DC, USA. 1992
4. Arakawa K, Kawai Y, Fujitani K, Nishimura J, Kitazawa H, Komine K, Kai K, Saito T. Bacteriocin production of probiotic *Lactobacillus gasseri* LA39 isolated from human feces in milk-based media, Rapid communication. Animal Science Journal 2008; 79: 634–640.
5. Arakawa K, Kawai Y, Lioka H, Tanioka M, Nishimura J, Kitazawa H, Tsurumi K, Saito T. Effect of gassericin A and T, bacteriocins produced by *Lactobacillus gasseri* with glycine on custard cream preservation. Journal of Dairy Science 2009; 92: 2365-2372.
6. Arihara K, Ota H, Itoh M, Kondo Y, Sameshima T, Yamanaka H. et al. *Lactobacillus acidophilus* group lactic acid bacteria applied to meat fermentation. Journal of Food Science 1998; 63: 544–547.

7. Bergamini V, Hynes E R, Quiberoni A, Suarez V B, Zalazar C A. Probiotic bacteria as adjunct starters: influence of the addition methodology on their survival in a semi-hard Argentinean cheese. Food Research International 2005; 38: 597–604
8. Bilgi B, Çelik S. Solubility and emulsifying properties of barley protein concentrate. European Food Research Technology 2004; 218: 437–441.
9. Brenier-Pinchart M P, Faure O, Garban F, Fricker-Hidalgo H, Mallaret M R, Trens A, Lebeau B, Pelloux H, Grillot R. Ten-year surveillance of fungal contamination of food within a protected haematological unit. Mycoses 2006; 49: 421–425.
10. Caplice E, Fitzgerald G F. Food fermentation: role of microorganisms in food production and preservation. International Journal of Food Microbiology 1999; 50: 131-149.
11. Carr F J, Chill D, Maida N. The lactic acid bacteria: a literature survey. Critical Review of Microbiology 2002; 28: 281-370.
12. Charteris W P, Kelly P M, Morelli L, Collins J K. Edible table (bio) spread containing potentially probiotic *Lactobacillus* and *Bifidobacterium* species. International Journal of Dairy Technology 2002; 55: 44-56.
13. Collombo M, Spahni M, Badertscher R. Optimization of storage conditions for laboratory cheese sample with acid of the chemical analysis. Scheweizeriscche Mil. Mil. Chevirstschaft Liche Forshuny 1992; 21(1):6-11.
14. Dave R I, Shah N P. Evaluation of media for selective enumeration of *S. thermophilus*, *L. delbreukii* ssp *bulgaricus* and Bifidobacteria. Journal of Dairy Science 1996; 7: 1529.
15. El Owni O A O, Hamed O I A. Effect of Storage Temperature on Weight Loss, Chemical Composition, Microbiological Properties and Sensory Characteristics of White Cheese (*Gibna Bayda*). Research Journal of Agriculture Biological Science 2009; 5 (4): 498-505.
16. Hadith. Volume 7, Book 71, Number 593: (Narrated Ursa)
17. Holzapfel W H, Geisen R, Schillinger U. Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. International Journal of Food Microbiology 1995; 24: 343-362.
18. ICMSF, International Committee on Microbiological Specifications for Foods. Microorganisms in foods, their significance and methods of enumeration. (2nd ed.). University of Toronto Press, Toronto, Canada. 1996.
19. Ishibashi N, Shimamura S. Bifidobacteria: Research and development in Japan. Food Technology 1993; 46: 126–135.
20. Ismail A M, Ismail M M, Hashem M L. Effect of some heat treatments on the quality of Edam cheese made from buffalo's milk. In the Proceeding of the 9th Egyptian Conference of Dairy Science and Technology, Cairo, Egypt. 2004.
21. Joshi V K, Sharma S. Lactic acid fermentation of Radish for shelf stability and pickling. Natural product Radiance 2009; 8 (1): 19-24.
22. Kawai Y, Ishii Y, Arakawa K, Uemura K, Saitoh B, Nishimura J, Kitazawa H, Yamazaki Y, Tateno Y, Itoh T, Saito T.. Structural and functional differences in two cyclic bacteriocins with the same sequences produced by Lactobacilli. Applied and Environmental Microbiology 2004; 70 (5): 2906-2911.
23. Kawai Y, Takahashi R, Arakawa K, Saito T. Structural and functional characteristics of gassericin A and T produced by *Lactobacillus gasseri* LA39 and LA158. Japanese Journal of Lactic Acid Bacteria 2006; 17: 32–39. (In Japanese)
24. Khalil A H, Mansour E H. Alginate encapsulated Bifidobacteria survival in mayonnaise. Journal of Food Science 1998; 63: 702–705.
25. Kuipers O P, Buist G, Kok J. Current strategies for improving food bacteria. Research Microbiology 2000; 151: 815-822.
26. Long D E. Culture and customs of Saudi Arabia. Greenwood Publishing Group. 2005: 50.
27. Majchrzak D, Mitter S, Elmadfi I. The effect of ascorbic acid on total antioxidant activity of black and green teas. Food Chemistry 2004; 488: 447.
28. Meyer A L, Elmadfa I, Herbacek I, Micksche M. Probiotic, as well as conventional yogurt, can enhance the stimulated production of pro-inflammatory cytokines. Journal of Human Nutrition Diet 2007; 20 (6): 590-598.
29. Miller H E, Rigelhof F, Marquart L, Prak-ash A, Kanter M. Whole-grain products and antioxidants. Cereal Foods World 2000; 45: 59
30. Mohsen, S. M., Amal S. Hathout, and Lamyaa E. El-Sideek. 2009. Microbiological characteristics of processed beef burger treated with lactic acid bacteria. Egyptian Journal of Biotechnology. 33: 256-266.
31. Nelson P E, Toussoun T A, Marasar W F O. An illustrated manual for identification. Published by the Pennsylvania State Union Press, London. 1983.
32. Newman C W, Newman R K. A brief history of barley foods. Cereal Foods World 2006; 51: 4–7.

33. Ong L, Henriksson A, Shah N P. Development of probiotic Cheddar cheese containing *Lactobacillus acidophilus*, *Lb. casei*, *Lb. paracasei* and *Bifidobacterium* sp. and the influence of these bacteria on proteolytic patterns and production of organic acid. International Dairy Journal 2006; 16: 446–456.
34. Reid G. Probiotic agents to protect the urogenital tract against infection. American Journal of Clinical Nutrition 2001; 73: 437S-443S.
35. Rivera-Espinoza Y, Gallardo-Navarro Y. Non-dairy probiotic products. Food Microbiology 2010; 27(1): 1-11.
36. Saneez M, Bauquis A, Carini S, Dusantois C, Serpelloni M. Inhibition of the proliferation in milk of bacteriophages of lactic acid bacteria in the presence of glucono-delta-lactone. Journal of Sciences-des-Aliments 1995; 15 (3): 291-304.
37. Shah N P. Functional cultures and health benefits. International Dairy Journal 2007; 17: 1262-1277.
38. Shehata A E, El-Nawawy M A, El-Kenany Y M, Aumara I E. Use of Bifidobacteria in Ras cheese production. I. Chemical and organoleptic properties. In the proceeding of the 9th Egyptian Conference of Dairy Science and Technology, Cairo, Egypt. 2004.
39. Stiles M E. Biopreservation by lactic acid bacteria. Antonie Van Leuwenhoek 1996; 70: 331-345.
40. Svensson U. Industrial perspectives. In Probiotic: A critical review, Horizon Scientific Press, Wymondham. 1999: 57-64.
41. Vasiljevic T, Shah N P. Probiotics—From Metchnikoff to bioactive. International Dairy Journal 2008; 18: 714-728.
42. Vignolo G, Castellano P, Belfiore C, Fadda S. A review of bacteriocinogenic lactic acid bacteria used as bio-protective cultures in fresh meat produced in Argentina. Meat Science 2008; 79(3): 483-499.
43. Vinderola C G, Bailo N, Reinheimer J A. Survival of probiotic microfora in Argentinian yoghurts during refrigerated storage. Food Research International 2000; 33: 97-102.
44. Zmysłowska I, Lewandowska D. The Effect of Storage Temperatures on the Microbiological Quality of Fish Feeds. Polish Journal of Environmental Studies 2000; 9 (3): 223-226.

10/8/2010

Immunohistochemical Study of Heat Shock Protein 70 in Psoriasis Vulgaris

Amina Gamal el Din[1]*, Hanan M Saleh[2], Nermeen Abdel Fattah[2] and Abdel Maksoud A[3]

[1].Pathology Department, Medical Research Division, National Research Centre,Dokki, Cairo-12622 (Egypt)*
[2].Dermatology & Venereology Department, Faculty of Medicine, Ain Shams University, Abbasia, Cairo-12622 (Egypt).
[3]Dermatology and Venereology Department, Medical Research Division, National Research Centre, Dokki, Cairo-12622 (Egypt).
hassaneinamina@yahoo.com

Abstract: Psoriasis, a common skin disease in Egypt, has drawn much attention to study the potential role of immunity in its pathogenesis.. Exposure of skin to microbial antigens and other stressful stimuli can induce heat shock proteins (HSPs) expression. HSPs comprise a large number of antigens against which immune responses are directed, owing to their cytokine-like effects and immunomodulatory properties. The potential role of HSP70 in pathogenesis of psoriasis is under investigation. We aimed at evaluating the differential immunohistochemical expression of HSP 70 in psoriatic skin and correlating the results with disease severity ; to elucidate its potential role in pathogenesis of psoriasis. Skin biopsies were taken from 20 patients with different severity of untreated chronic plaque-type psoriasis and from 20 healthy volunteers. Antibodies to HSP70 were analyzed immunohistochemically. Immunoreactivity intensity distribution index (IRIDI) scores including the proportion of immunoreactive cells and their staining intensity were calculated in the basal, suprabasal, superficial as well as the whole epidermal layers of patients and controls. Differential and total IRIDI scores for HSP70 expression showed highly significant higher values in psoriatic patients compared to controls. Statistical differences were found between the different groups of patients; according to their disease severity and controls. Positive correlations also existed between IRIDI scores of patients and disease severity. Based on the findings of the present study, HSP70 is suggested to play a role in the pathogenesis of psoriasis and to correlate with disease severity. Further studies on immunotherapeutic intervention are recommended, aiming at inhibiting events in an ongoing immune response which may provide new therapeutic and perhaps preventive approaches for psoriasis.
[Amina Gamal el Din, Hanan M Saleh, Nermeen Abdel Fattah and Abdel Maksoud A. **Immunohistochemical Study of Heat Shock Protein 70 in Psoriasis Vulgaris**. Journal of American Science 2010;6(12):899-908]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: psoriasis, heat shock protein 70, immunohistochemistry

1. Introduction

Psoriasis is a common, chronic inflammatory skin disease associated with both genetic and environmental risk factors (Krueger, 2002). There is growing interest in the role of innate and adaptive immunity in inflammatory diseases such as psoriasis (Curry et al,2003). The exposure of the skin to microbial pathogens and other stressful stimuli can induce heat shock proteins (HSPs) expression, as a part of the innate immunity (Boucock and Shannan,2001). Autoimmunological mechanisms triggered by microbial agents have been suggested as playing an important role in the pathogenesis of psoriasis (Jones et al, 2004; Asea et al, 2000). More recently,

HSPs have become a focus for their role in inflammatory and autoimmune diseases owing to their cytokine-like HSPs play a central role in homeostasis by their ability to bind to other peptides and to control their effects and immunomodulatory properties (Asea et al,2000). Based on molecular mass and sequence homology, HSPs are classified into families including ubiquitin, small HSPs of 20 to 28 KDa, HSP60, HSP70 and HSP90 (Curry et al, 2003). The antigenic sites of many pathogenic HSPs, particularly HSP70 are immunodominant and are thus the primary targets for B cell and T cell responses (Palleros et al, 1991). In the HSP70 family are heat shock cognate protein 70 (HSC70), which is constitutively expressed during normal cell development and differentiation (Curry el al, 2003) and the major heat or stress-inducible protein HSP70 (Palleros el al, 1991). They are nuclear and cytoplasmic proteins exhibiting approximately 95% sequence homology and forming stable complex in stressed cells. HSP27, HSP60 and HSP70 and their ligands common HSP receptor CD91 have been reported to be increased in lesional psoriatic skin (Boyman et al, 2005).

The aim of our study was to evaluate the differential immunohistochemical expression of HSP70 in psoriatic skin in comparison with normal skin to elucidate its potential involvement in the pathogenesis of psoriasis and to correlate the results with the disease severity.

2. Material and Methods

Twenty patients with untreated chronic plaque-type psoriasis and of various degree of severity were participated in the present case- control study. They were randomly selected from those attending the outpatient dermatology clinic at Ain Shams University Hospitals and fulfilling the inclusion criteria of the study. They were 13 males and 7 females with mean age 33.6 years ±12.57 (16-53) and mean disease duration 10.5 years± 6.38 (1-24). Twenty age and sex matched healthy volunteers were included as controls. Inclusion criteria included subjects with no concomitant dermatological and/or systemic diseases that could affect the outcome of the study, subjects who did not use any topical or systemic treatment for at least one month before the study. Prior to initiation of the study, every subject was informed about the aim of the study and gave an informed consent.

All subjects were subjected to the followings:

1. Full history taking.
2. General and dermatological examination.
3. Assessment of psoriasis severity using Psoriasis Area and Severity Index (PASI) score (Fredrickson and Patterson, 1978).
4. Skin biopsy: Formalin-fixed paraffin embedded 5 mm skin punch biopsy specimens were obtained from 20 clinically diagnosed and histopathologically confirmed psoriatic lesions and from 20 normal skin controls.

Immunohistochemical staining procedure was applied to all specimens. Sections, 3-4 µm thick, were cut and mounted on positively charged slides (Menzel-glazer-polysine). Monoclonal mouse antibody to HSP70Ab-2 (clone W27;Neomarkers, Fremont, CA, USA) and Ultravision universal detection kit were used. After deparaffinization of the sections in xylene, rehydrated through graded ethyl alcohol and washed with phosphate-buffered saline, endogenous peroxidase activity was prevented by using hydrogen peroxide 0.86%. Microwave treatment (x4 for 10 minutes; sections in 10 mM citrate buffer, pH 6.0) were used for antigen retrieval. Blocking of non-specific binding was done using a universal blocking reagent (ultra V block; Neomarkers). Prediluted primary monoclonal antibody was applied for 1 hour at room temperature within a humidifying chamber. DAB; Neomarkers were used as chromogen. Mayer's haematoxylin was used for counterstaining (Merck, Germany). Sections were mounted and examined under the light microscope (Olympus CX 41 Japan).

Immunohistochemical evaluation was done on light microscopic examination. Epidermis was divided into 3 layers: basal, suprabasal and superficial (stratum corneum). Each layer was evaluated for the proportion of immunoreactive cells as well as their staining intensity. The proportion of the immunoreactive cells in each layer was assessed as 0: no immunoreactive cells, 1: less than 25% of the cells are immunoreactive. 2: 25-50% of the cells are immunoreactive. 3: more than 50% of the cells are immunoreactive. As regards the staining intensity of the cells, it was graded as 0: no staining, 1: light staining, 2: moderate staining, 3: intense staining. Immunohistochemical evaluation was performed semiquantitatively according to Chaiyarit et al,1999). An immunoreactivity intensity distribution index (IRIDI) was calculated for every patient and control. IRIDI score of each layer was calculated by multiplying the score of the proportion of immunoreactive cells by the score of their staining intensity. Total IRIDI score was determined by adding the IRIDI scores of the 3 epidermal layers for every patient and control.

Statistical analysis was carried out using the Statistical Package for the Social Sciences (SPSS) version 15. Comparison of quantitative data was carried out using unpaired t-test and Mann Whitney test. ANOVA and Kruskal-Wallis tests were used to compare more than 2 groups as regards quantitative data. Post Hoc test was used to detect the least significant difference in case of significant ANOVA test. Probability (p) values ≤ 0.05 were considered statistically significant and p ≤0.001 were considered statistically highly significant. Correlation between different parameters was assessed using Pearson's correlation coefficient test. Correlation is considered significant at 0.05 level.

3. Results

The study included 20 patients with chronic plaque-type psoriasis. They were 13 males and 7 females with mean age 33.6 years ± 12.57 (16-53). Twenty age and sex-matched healthy volunteers, with mean age 32.3 years ± 12.54 (15-54) were enrolled as controls. According to PASI score, patients were divided into 3 groups:

Group 1 included patients with mild psoriasis. Their PASI score ranged from 7 to 13 (mean 10.2 ± 3.03). They were 6 patients (3 males and 3 females) with mean age 33.8 years ± 12.19 (24-53) and mean disease duration 10.17 years ± 6.62 (1-20).

Group 2 included patients with moderate psoriasis. Their PASI score ranged from 17 to 24 (mean 18.4 ± 3.09). They were 7 patients (5 males and 2 females) with mean age 39.86 years ± 10.42 (26-53) and mean disease duration 9.0 years ± 7.77 (2-24).

Group 3 included 7 patients with severe psoriasis. Their PASI score ranged from 30 to 65 (mean 50.2 ± 17.41). They were 5 males and 2 females with mean age 41 years ± 12.1 (16-53) and mean disease duration 12.43 years ± 4.99 (4-20).

Immunohistochemical evaluation of HSP70 expression: The mean IRIDI scores of HSP70 expression of psoriasis vulgaris and normal skin in the different epidermal layers (differential IRIDI scores) as well as the whole epidermis (total IRIDI scores) are summarized in Table (1). As regards normal control skin, light HSP70 immunostaining was seen within basal cell layer of the epidermis. Suprabasal cell layer showed light to moderate HSP70 immunostaining in 4 specimens only. Superficial cell layer on the otherhand, showed no HSP70 immunoreactivity. Moreover, HSP70 was not immunohistochemically detected in the dermis of normal skin. The immunohistochemical staining pattern of normal keratinocytes was diffuse; cytoplasmic and nuclear (Fig. 1). In contrast, intense HSP70 immunostaining was found in psoriatic lesions, predominantly in basal and suprabasal epidermal cell layers. In the basal cell layer, keratinocytes tended to be decorated focally and peripherally at basal side of cytoplasm with perinuclear as well as nuclear expression. In the suprabasal cell layer, it was irregularly distributed. Superficial epidermal cell layer showed light HSP70 immunoreactivity as well (Figs. 2 and 3). Only 2 cases from the severe group of psoriasis showed expression of HSP70 within the dermal inflammatory infiltrate as intense perinuclear, granular staining of lymphocytic infiltrate (Fig. 2).

Comparison between patients and controls as regards total and differential IRIDI scores of expressed HSP70: On comparing psoriatic and normal skin specimens, statistically highly significant differences were found between patients and controls as regards mean IRIDI scores of HSP70 expression in basal, suprabasal as well as superficial epidermal cell layers. Similar results were also noted on comparing patients and controls as regards total IRIDI scores (Table 1).

Comparison between different groups of patients and controls as regards total and differential IRIDI scores of HSP70 expression : Statistically highly significant differences were found between the different groups of patients and controls at all epidermal cell layers as well as regarding total IRIDI scores (Table 2). By using the post Hoc test to detect the least significant difference, statistical differences existed according to the epidermal cell layer. In the basal epidermal cell layer, significant differences were found between groups 1 and 2 (p=0.047) and between groups 1 and 3 (p=0.012). Highly significant differences were found between group 2 and controls as well as between group 3 and controls. No significant differences were however, found between groups 2 and 3 (p=0.540) and between group 1 and controls (p=0.317). In the suprabasal epidermal cell layers, significant differences existed between groups 1 and 2 (p=0.046) as well as between groups 2 and 3 (p=0.003). Statistically highly significant differences were found between groups 1 and 3, group 1 and controls, group 2 and controls and group 3 and controls.

In the superficial epidermal cell layer, significant differences between groups 1 and 2 (p=0.008), groups 1 and 3 (p=0.003) as well as between group 1 and controls (p=0.010) were found. Moreover, statistically highly significant differences existed between group 2 and controls and between group 3 and controls. No significant difference was found between groups 2 and 3 (p=0.688). As regards total IRIDI scores, there were significant differences between groups 1 and 2 (p=0.012) as well as between group 1 and controls (p=0.006). Highly significant differences existed between groups 1 and 3 as well as between group 2 and controls and between group 3 and controls. No significant difference was noted between groups 2 and 3 (p=0.072). Significant positive correlations were also found in the patients between PASI score, total and differential IRIDI scores of HSP70 expression in the basal, suprabasal and superficial epidermal cell layers (r= 0.55, 0.53, 0.61 and 0.28 respectively).

Table 1: Mean Differential and Total IRIDI Scores of HSP70 Expression in Psoriasis Patients Compared to Control

	Patients	controls	t-test	p-value
Basal cell layer	4.75± 2.40	2.35±1.04	4.099	0.001
Suprabasal cell layer	6.35±2.45	1.65±0.93	8.002	0.001
Superficial cell layer	1.6± 1.04	0.0	6.839	0.001
Whole epidermis	12.70±5.89	4.0±1.97	7.165	0.001

Table 2: Comparison between Different Groups of Patients and Controls as Regards Differential and Total IRIDI Scores of HSP70 Expression

	Group 1 Mild psoriasis	Group 2 Moderate psoriasis	Group 3 Severe psoriasis	controls	F-test	p-value
Basal cell layer	3.16±1.33	5.14±2.73	5.14±2.73	2.35±1.04	8.94	0.001
Supra Basal cell layer	4.33±0.82	6±2.24	6±2.24	1.65±0.93	43.86	0.001
Superficial cell layer	0.83±0.41	1.86±1.22	1.86±1.22	0.0	23.49	0.001
Whole epidermis	8.33±2.56	13±6.19	13±6.19	4.0±1.97	31.64	0.001

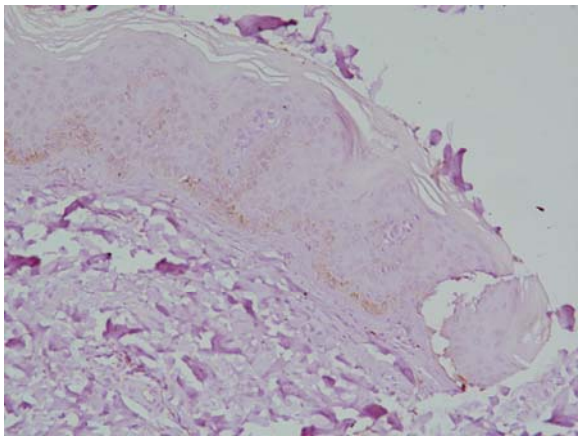


Figure 1: Normal skin: light HSP70 imunostaining in basal epidermal cell layer with negative immunoreactivity in suprabasal and superficial epidermal layers (immunoperoxidase X200)

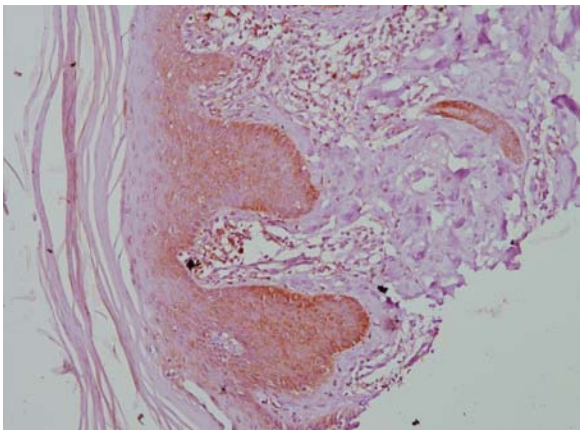


Figure 2. Psoriasis vulgaris: intense HSP70 immunostaining in basal and suprabasal epidermal cell layers with light superficial layer immunostaining and dermal inflammatory infiltrate immunoreactivity. (immunoperoxidase X 200).

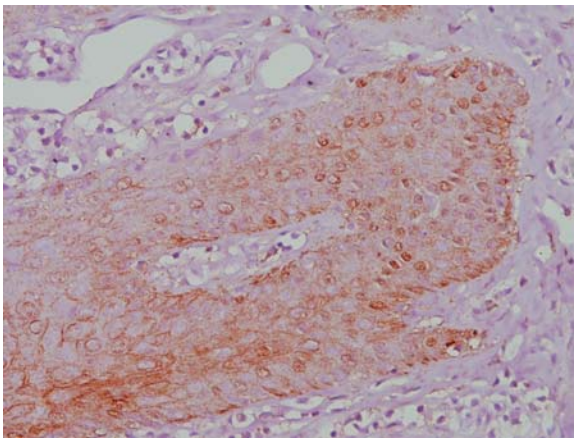


Figure 3. Psoriasis vulgaris: intense HSP70 immunostaining (nuclear and focally cytoplasmic (immunoperoxidase X 400).

4. Discussions

The differential expression of HSP70 may be related to the different expression of the stress inducible HSP70 depending on the differentiation state of keratinocytes. Expression of HSP70 may be also related to the extent of keratinocytes alteration. The following proposed scenario may explain HSP70 contribution in the development of psoriasis. Minor skin lesion(s) caused by microbial infection or physical stimuli lead(s) to stimulation of keratinocytes and secretion of cytokines, HSP70, (auto-)antigens and HSP-(auto-)antigens complexes.

Dendritic antigen presenting cells (APCs), expressing common HSP receptor CD91, bind HSP and HSP-antigen complexes. This is followed by the activation and translocation of NF-κß into the nucleus leading to the production of proinflammatory cytokines (Basu et al,2001; Boyman et al, 2004). HSP70 also facilitates the activation of dendritic APCs with IL-12 production. Presentation of processed HSP and HSP-antigen complexes, stimulation of T lymphocytes and secretion of TH-1

type cytokines then follow (Nestle et al, 1994). On the basis of this, enhanced epidermal proliferation, aberrant epidermal differentiation, recruitment of more immune cells, new blood vessel formation and finally formation of a psoriatic plaque develop. The potential role of HSP70 in the development of psoriatic lesions may therefore be attributed to its primary effect on innate system through immunomodulatory properties.

Based on the findings of the present study, we conclude that HSP70 may play an important role in the aetiopathogenesis of psoriasis, although one cannot exclude the possibility of its up-regulation secondary to inflammation. It seems that psoriasis represents a genetically determined skin disease probably initiated by hyperactivity of the triggered state of otherwise dormant cutaneous innate immunity. Recognition of the potential contribution of HSP70 in the pathophysiology of psoriasis may help clarify the mechanisms underlying the development and maintenance of psoriatic lesions with its different severity. However, further studies are recommended to determine the interplay between innate and adaptive immune responses in psoriasis and to search for immunotherapeutic intervention, aiming at inhibiting events in an ongoing immune response which may provide useful therapeutic and perhaps preventive approaches for psoriasis.

Corresponding Author:
Dr. Amina Gamal el Din
Department of Pathology
Medical Research Division, National Research Centre, Dokki, Cairo, 12622 Egypt
Phone: +202 3371382/3371 433
Mobile: +2 012 34 32 554
Fax: +202 3370931/33478742
E-mail: hassaneinamina@yahoo.com

References

1. Asea A, Kraeft SK and Kurtjones FA (2000). HSP70 stimulates cytokine production through CD14 dependent pathway, demonstrating its dual role as a chaperone and cytokine. Nat Med; 6: 435-442.
2. Basu S, Binder RJ and Ramalingam T (2001). CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70 and calreticulin. Immunity; 14: 303-313.
3. Boucock AM and Shannan W (2001). Insights into psoriasis and other inflammatory diseases from large scale gene expression studies. Hum Mol Genet; 10: 1793-1805.
4. Boyman O, Conrad C, Dudli C, Kielhorm E, Nickoloff BJ and Nestle FO (2005). Activation of dendritic antigen-presenting cells expressing common heat shock protein receptor CD91 during induction of psoriasis. Br J Dermatol; 152: 1095-1097.
5. Boyman O, Hefti HP and Conrad C, 2004. Spontaneous development of psoriasis in a new animal model shows an essential role for resident T cells and tumour necrosis factor-〈. J Exp Med; 199: 731-6.
6. Chaiyarit P, Kafrawy AH and Miles DA, (1999). Oral lichen planus: an immunohistochemical study of heat shock proteins (HSPs) and cytokeratins (CKs) and a unifying hypothesis of pathogenesis. J Oral Pathol Med; 28: 210-214.
7. Curry J, Qin JZ, Bonish B, Carrick R, Bacon P, Panella J, Robinon J and Nickoloff BJ (2003). Innate immune-related receptors in normal and psoriatic skin. Arch Pathol Lab Med; 127: 178-186.
8. Fredrickson T and Patterson U, (1978). Severe psoriasis, oral therapy with a new retinoid. Dermatologica; 157: 238-242.
9. Jones DA, Yawalkar N and Suh KY (2004). The identification of autoantigens in psoriatic plaques using expression cloning. J Inv Dermatol; 123: 93-100.
10. Krueger JG, (2002). The immunologic basis for the treatment of psoriasis with new biologic agents. J Am Acad Dermatol; 46: 1-23.
11. Nestle FO, Turka LA and Nickoloff BJ (1994). Characterization of dermal dendritic cells in psoriasis: Autostimulation of T lymphocytes and induction of Th1 type cytokines. J Clin Invest: 94: 202-209.
12. Palleros DR, Welch WJ and Fink AL (1991). Interaction of HSP70 with unfolded proteins: effect of temperature and nucleotides on the kinetics of binding. Proc Natl Acad Sci USA; 88: 5719-5723.

1/30/2010

Preparation of Glucan from *lentinula edodes* Edible Mushroom and Elucidation of its Medicinal value

George E. Rasmy[*1], William A. Botros2, SanaaS. Kabeil2, and Ayman. S. Daba2

1Biochemistry Dept., National research centre (NRC), Cairo, Egypt.
2 Mubarak City for scientific Research and Technology Applications, GEBRI, Alexandria, Egypt.
*gefahmy@gmail.com

ABSTRACT: One of the most popular mushrooms in the World is the Shiitake mushroom. In the present study *lentinula edodes (L. edodes)* mycelia were grown in submerged culture and the polysaccharides were extracted from culture broth. The structure of polysaccharides was elucidated using NMR spectra, which indicated that the polysaccharide is highly branched glucan containing mainly 1, 3 and 1, 6 linkages. The results showed that the polysaccharides posses anticancer activity against human esophageal cancer cell line. The potential of the internal transcribed spacer (ITS) region as a tool for studying molecular systematics and population genetics is significant. The results also showed that the polysaccharides enhance the immune-responses of human body thereby increasing resistance to cancer disease. Mycelia formed by growing pure cultures in submerged conditions are of constant composition, and submerged culture is the best technique for obtaining consistent and safe mushroom product.
[George E. Rasmy, William A. Botros, SanaaS. Kabeil, and Ayman. S. Daba. **Preparation of Glucan from *lentinula edodes* Edible Mushroom and Elucidation of its Medicinal value**. Journal of American Science 2010;6(12):904-909]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: Mushroom, polysaccharides, immune-responses, submerged culture, cancer.

INTRODUCTION:

Consumption of edible mushrooms has been suggested to improve health. There are thousands of different mushroom species and about 700 species have been reported to have significant pharmacological properties (Chang, 1996). Medicinal mushrooms have a long history of use in traditional Oriental therapies. Hot-water-soluble fractions of medicinal mushrooms have been used as medicine in the Far East (Wasser, 2002).

In the last three decades, numerous polysaccharides and polysaccharide-protein complexes have been isolated from mushrooms and used as a source of therapeutic agents. The most promising biopharmacological activities of these biopolymers are their immunomodulation and anti-cancer effects.

They are mainly present as glucans with different types of glycosidic linkages such as (1-->3), (1-->6)-beta-glucans and (1-->3)-alpha-glucans, and as true herteroglycans (figure 1), while others mostly bind to protein residues as polysaccharide-protein complexes (Chihara *et al.*, 1987 and Zheng *et al.*, 2005). The β-glucans have been shown to inhibit tumor growth *in vitro* and *in vivo*.

The β-glucans lentinan from *L. edodes*, schizophyllan (sonifilan) from *Schizophyllum commune*, grifolan from *Grifola frondosa*, and extracts from *Sclerotinia sclerotiorum* all have anti-tumor activity. Intratumor injection of an acid-treated fraction of *Agaricus blazei* inhibited tumor growth of that tumor as well as other tumors at remote sites [Fujimiya et al., 1998]. An extract from the *Phellinus rimosus* mushroom extended the life span of mice by 96% following injection of tumor cells in an experimental Dalton's lymphoma ascites model [Ajith and Janardhanan, 2003].

L. edodes is the first medicinal macrofungus to enter the realm of modern biotechnology. It is the second most popular edible mushroom in the global market which is attributed not only to its nutritional value but also to possible potential for therapeutic applications. *L. edodes* is used medicinally for diseases involving depressed immune function (including AIDS), cancer, environmental allergies, fungal infection, frequent flu and colds, bronchial inflammation, heart disease, hyperlipidemia (including high blood cholesterol), hypertension, infectious disease, diabetes, hepatitis and regulating urinary inconsistencies. It is the source of several well-studied preparations with proven pharmacological properties, especially the polysaccharide lentinan, eritadenine, shiitake mushroom mycelium, and culture media extracts (LEM, LAP and KS-2). Antibiotic, anti-carcinogenic and antiviral compounds have been isolated intracellularly (fruiting body and mycelia) and extracellularly (culture media). Bisen et al., 2010.

During the last 15 years the internal transcribed spacer (ITS) of nuclear DNA has been used as a target for analyzing fungal diversity in environmental samples, and has recently been selected as the standard marker for fungal DNA barcoding. Bellemain et al., 2010. The potential of

ITS region as a tool for studying molecular systematics and population genetics is significant.

Hearst et al., 2009, previously suggested that it is required to isolate and identify active compound(s) in L. edodes. They stated that once these have been identified, suitable pharmaceutical delivery systems should be explored to allow concentrated extracts to be prepared and delivered optimally, rather than crude ingestion of raw material, which could promote further bacterial resistance.

The aims of the present study were to 1- growing *L. edodes* mycelia in submerged culture and extracting the polysaccharides from culture broth. 2- to elucidated the structure of polysaccharides was using NMR spectra, and 3- to generate the phylogenetic tree of this isolate compared to other isolates. And finally 4- to posses the biological activity of the isolated polysaccharides both as antimicrobial and as anticancer (against human esophageal cancer cell line)

MATERIAL AND METHODS:

Microorganism and media

A culture of *L. edodes* was isolated from local forest in La Crosse USA. The culture was maintained on a yeast complete medium and subculture every 1 month, and the slants were incubated at 27ºC for 7 days and then stored at 4ºC. The seed cultures were grown in 250ml flasks containing 100 ml of medium containing (g/l) : glucose 10, yeast extract 1, peptone 2, KH2 PO4 0.5 , Mg SO4 0.5 at 27ºC on a rotary incubator at 150 rpm for 7 days.

Fermentation

L. edodes was initially grown on yeast complete medium in Petri dish, and then transferred to the seed culture medium by punching out 2.5mm of the agar plate culture with a sterilized cutter. After 7 days of growing in shake flasks .The fermentation medium was inoculated by 3 % of the seed culture. A 15 liters Stirred tank fermentor (BIOFLO 3000 New Brunswick, USA) was used in the experiment, the cultivation in the fermentor for 10 days. The temperature was adjusted for 27ºC and the rpm 150. Fermentation experiment was performed in duplicate.

Extraction of the polysaccharide

The fermentation broth was filtered and then centrifuged at 5000 X g for 15 minutes, and the resulting supernatant was concentrated using rotary evaporator .The concentrated supernatant was mixed with two volume of absolute ethanol, stirred vigorously and left overnight at 4ºC. The purified polysaccharide is then pooled and lyophilized.

Estimation of mycelial growth and polysaccharide production

Samples collected from the fermentor at various intervals were centrifuged at 5000 X g for 15 minutes. Polysaccharide was separated from supernatant and lyophilized until constant weight was confirmed. The dry weight of mycelia was measured after repeated washing of the mycelial pellets and drying overnight at 70ºC to a constant weight.

NMR

The ^{13}C NMR spectra were recorded on an INOVA-600 spectrometer (Varian, Palo Alto, CA, USA) at ambient temperature.

MTT assay

Human esophageal cancer cell line was kindly obtained from university of Cape Town.

The amount of yellow MTT (3- (4, 5-Dimethylthiazol- diphenyltetrazolium bromide) reduced to purple formazan is measured spectrophotometrically by a spectrometer. This reduction takes place only when mitochondrial reductase enzymes are active, and thus conversion is directly related to the number of viable cells. The production of purple formazan in cells treated with an agent is measured relative to the production in control cells, and a dose-response curve can be generated (Mosmann, 1983).

Antimicrobial Activity assay

Muller-Hinton agar medium was used as an assay medium. The agar medium at 45ºC was mixed with 0.1ml bacterial suspension. The mixture was powered into 9cm petri dish and allow to ceritified. Sterile paper diskes were placed on the dried surface of the medium. Each disk received 20ml of the culture filtrate. Perti dishes were incubated at 37ºC for 18hs. The inhibation zone was measured mm. diameter (Amade *et al.,* 1994).

DNA Extraction

DNA extraction was done using C-TAB Lysis buffer and 70% ethanol precipitation. DNA clean up via Geneclean III kit by Q-biogene Ten microlitres of each of the extracted DNA solutions were checked by an electrophoresis on a one percent agarose gel.

PCR Analysis

Total DNA was subject with primers specific to the ITS-domain (5'-CTTGGTCATTTAGAGGAAGTAA-3'), (5'-CCTCCGCTTATTGATATGC-3') (White *et al.,* 1990). DNA amplifications were carried out in a thermocycler Eppendourf PCR system with denaturing step at 95ºC for 5min and the step cycle

program set for 35 cycles (with a cycle consisting of denaturing 94ºC for 30s, annealing at 45ºC for 30s and extension step at 72ºC for 30s), followed by a final extension step at 72ºC for 10 min.

Cloning and sequencing of PCR fragments

Expected PCR-amplified fragments were excised from agarose gel and purified using Qiagen Gel Extraction kit (Qiagen, Germany) and then cloned with TOPO TA cloning kit (invetrogen, USA) in the competent E. coli strain TOPO 10. Plasmid DNA was isolated using Qia Spin mini-prep kit (Qiagen, Germany). Plasmid DNA was sequenced in both directions using BigDye Sequencing kit and Applied Biosystems 3730xl automated DNA sequencing Instrument at the University of Wisconsin, USA. Biotechnology Center.

Alignment and phylogeny

Pairwise and multiple DNA sequence alignment were carried out using CULSTALW (http://align.genome.jp). The phylogenetic tree was generating using MUSCALE 3.7 (http://www.phylogeny.fr/) according to Dereeper *et al.,* 2008). To obtain the phylogeny tree, 10 different strains were used, including ours, as illustrated in table (1).

RESULTS AND DISCUSSION

Shiitake (L. edodes (Berkeley) Pegler) is one of the most consumed mushrooms, for both therapeutic purposes and as food, therefore, the study of its biological properties is of great interest for producers and consumers. L. edodes mycelia have an excellent nutritional value. Their raw mycelia were found to include 88–92% water, protein, lipids, carbohydrates as well as vitamins and minerals. On a dry weight basis, they have a relatively high nutritional value when compared to commonly consumed vegetables. Dried shiitake mushrooms are rich in carbohydrates and protein. They contain 58–60% carbohydrates, 20–23% protein (digestibility of 80–87%), 9–10% fiber, 3–4% lipids, and 4–5% ash (Data not shown). Shiitake is one of the best-known and best-characterized mushrooms used in medicine. It is the source of several well-studied preparations with proven pharmacological properties, especially the polysaccharide. Using methods of fractionation and purification of polysaccharide reported by Chihara (1969 and 1992) exo-polysaccharides were isolated from shiitake, Chihara was one of the first to report on the anti-tumor properties of the mushroom, stating that lentinan a polysaccharide isolated from the fruit bodies ‘‘was found to almost completely regress the solid type tumors of Sarcoma 180 and several kinds of tumors including methylchloranthrene induced fibrosarcoma in synergic host–tumor system Wasser (2002).

The potential of the ITS region as a tool for studying molecular systematics and population genetics is significant (Boyer *et al.,* 2001). We compared our ITS r-RNA *L. edodes* sequence with another sequences on GenBank to detect the similarity of this region with other *L. edodes* isolates (figures 3, 4) (Hibbett *et al.,* 1995; Miyazaki *et al.,* 2007, Gao et al., 2008, Yu, 2008, Jiang et al., 2008 (a), Jiang et al., 2008 (b), Jiang et al., 2009, Cao & Bao, 2009, and Le et al., 2010).

The H NMR Spectra (Figure5) of the exopolysaccharides exhibited signals at different resonance which represent the anomeric proton and protons of the different hydroxyl groups. The C13 NMR Spectra showed the presence of 6 carbon atoms the proton magnetic resonance is the most accurate spectroscopic method used to determine the structure of new compounds. The obtained NMR spectra could be compared with those reported by Gorin (1981) for lentinan polysaccharides, a potent antitumor agent extracted from shiitake.

Mushroom polysaccharides were tested for antibacterial activity in vitro against gram positive and gram negative bacteria. The minimum inhibitory concentration reported in Table (2) shows that mushroom polysaccharide has a potent antibacterial effect against different kind of bacteria. Only a few studies have explored shiitake’s antibacterial components, and these have concentrated on their potential in terms of bacteria of oral origin. Anti-bacterial activity is an exciting result, with increasing bacterial resistance to antibiotics, improving host immunity may be the way forward in fighting bacterial infection. But, these results should be viewed with caution because the published papers in this area are confined to only one or two journals, and although these have reliable IF scores, this fact does not lend support to them being credible results. There is a lack of significant studies in this area; and none that were from researching the anti-caries aspect of anti-bacterial activity.

A postulated mechanism of lentinan’s anti-bacterial activity was by the induction of increased levels of complement C3 and C3b formation (Shouji *et al.,* 1999). Although, modulation of the non-specific immune system has also been displayed in numerous studies, and may be the potentiator of lentinan’s anti-bacterial activity.

Cytotoxic activity of isolated polysaccharides was examined using esophageal cancer cell line in vitro. Results showed that polysaccharides inhibited tumour cell growth .This result are with the conclusion of other investigators (Hibasami et al., 2003 and Zhang et al., 2007) who stated that these

polysaccharides could arrest the cell cycle and generate apoptosis. Also Miyaji et al., 2006, evaluated the aqueous extracts of the shiitake mushroom (*L. edodes* (Berkeley) Pegler) in HEp-2 cells in vitro.

Extracts of multiple varieties of mushrooms have been shown to be protective in experimental cancer models; presumably because in part they boost anti-tumor immunity. These polysaccharides and polysaccharide-protein complexes are suggested to enhance cell-mediated immune responses in vivo and in vitro and act as biological response modifiers (Borchers *et al.,* 1999). Potentiation of the host defense system may result in the activation of many kinds of immune cells that are vitally important for the maintenance of homeostasis. Polysaccharides or polysaccharide-protein complexes are considered as multi-cytokine inducers that are able to induce gene expression of various immunomodulatory cytokines and cytokine receptors (Okamoto *et al.,* 2004).

Some interesting studies focus on investigation of the relationship between their structure and antitumor activity, elucidation of their antitumor mechanism at the molecular level, and improvement of their various biological activities by chemical modifications. Israilides *et al* (2008) demonstrate cytotoxic and cell growth inhibitory (cytostatic) effect of aqueous extracts of the shiitake mushroom on MCF-7 human breast adenocarcinoma cell line. Such effect was demonstrated with fruit body and mycelial extracts, the difference being that there was no significant suppression on normal cells with the latter. Furthermore mycelial extracts did not induce any cytostatic effect in both cancer and normal cell lines based on a DNA synthesis assay. The significant suppression of the proliferation of cancer cells was reflected by the comparatively low IC (50) values and the simultaneous higher respective values on normal fibroblast cells.

There is a lot of evidence to support the anti-tumour assertions made of lentinan. A number of valuable studies have been conducted on the consequence of lentinan administration, and its acceptance into clinical medicine in Japan should perhaps highlight its efficacy. The researchers in Japan have covered much ground in the area of neutraceuticals from mushrooms and have unearthed the potential of *L. edodes*. At the moment there is not enough credible information to warrant the marketing of lentinan in the world, further investigations, look to be promising, while isolated purified shiitake constituents have received appropriate scrutiny investigations into the anti-tumour potentials of shiitake consumption are limited. There is a lack of epidemiological data as to the prophylactic effects of mushroom intake on the development of spontaneous tumours and the evidence that suggests shiitake consumption elicits anti-tumour effects are only found in murine systems. Therefore, the claim that shiitake consumption has anti-tumour effects is not fully substantiated.

CONCLUSIONS:

This study shows the potential the efficacy of the given method to isolate a highly branched glucan containing mainly 1, 3 and 1, 6 linkages. Biological examinations revealed its antimicrobial, cytotoxic activity against esophageal cancer cell line. Further research is needed to establish content and bioactivity of the many compounds present and the effect of preparation and consumption differences on their medicinal activity.

REFERENCES

Ajith T, Janardhanan KK. (2003). Cytotoxic and antitumor activities of a polypore macrofungus, Phellinus rimosus (Berk) Pilat. *J Ethnopharmacol*, 84: 157-162.

Amade P. M.; Mallea and Bouaicha, N (1994) Isolation, structural identification and biological activity of two metabolites produced by penicillium olsonii Bainier and Sartory *J. Antibiot.* , **47**, 201-207.

Bellemain E, Carlsen T, Brochmann C, Coissac E, Taberlet P, Kauserud H. 2010 . ITS as an environmental DNA barcode for fungi: an in silico approach reveals potential PCR biases. BMC Microbiol. 9;10:189.

Bisen PS, Baghel RK, Sanodiya BS, Thakur GS, Prasad GB. (2010). Lentinus edodes: a macrofungus with pharmacological activities. *Curr Med Chem*.;17 (22):2419-30.

Borchers, A. T.; Stern, J. S.; Hackman, R. M.; Keen, C. L. and Gershwin, M. E. (1999) Mushrooms, tumors, and immunity. *Proc Soc Exp Biol Med.*; **221**(**4**), 281-93.

Boyer, S. L.; Flechtner,V. R. and Johansen, J. R. (2001). Is the 16S–23S rRNA Internal Transcribed Spacer Region a Good Tool for Use in Molecular Systematics and Population Genetics? A Case Study in Cyanobacteria. *Mol. Biol. Evol.*, **18**(**6**),1057–1069.

Cao,M. and Bao,D.P. Submitted (24-SEP-2009.) Lentinula edodes strain 135 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal

RNA gene, partial sequence. (http://www.ncbi.nlm.nih.gov/nuccore/261347180). GenBank: GU001952.1

Chang R: (1996). Functional properties of edible mushrooms. *Nutr Rev*, **54**:S91-93.

Chihara, G. (1969). The antitumor polysaccharide lentinan: an overview. In T. Aoki, et al. (Eds.), Manipulation of host defense mechanism. *Nature*, **222**, 687-694).

Chihara, G. (1992). Immunopharmacology of lentinan, a polysaccharide isolated from lentinus edodes: its application as a host defense potentiator. *International Journal Oriental Medicine*, **17**, 57-77.

Chihara. G.; Hamuro, J. Maeda, Y., et al. (1987). Antitumor and metastasis inhibitory activities of lentinan as an immunomodulator. *Cancer Detect Prev*; **1**,423-43.

Dereeper, A.; Guignon. V.; Blanc. G.; Audic. S.; Buffet. S.; Chevenet. F.; Dufayard. J.-F.; Guindon. S.; Lefort. V.; Lescot. M.; Claverie. J.-M. and Gascuel. O. (2008). Phylogeny.fr: robust phylogenetic analysis for the non-specialist *Nucleic Acids Research*.; **36** (Web Server Issue):W465-9.

Fujimiya Y, Suzuki Y, Oshiman K, Kobori H, Moriguchi K, Nakashima H, Matumoto Y, Takahara S, Ebina T, Katakura R (1998). Selective tumoricidal effect of soluble proteoglucan extracted from the basidiomycete, Agaricus blazei Murill, mediated via natural killer cell activation and apoptosis. *Cancer Immunol Immunother*, **46**:147-159.

Gao,S., Zhang,J.X. and Bian,Y.B. Submitted (24-JAN-2008). Lentinula edodes strain ACCC51462 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence (http://www.ncbi.nlm.nih.gov/nuccore/257127897). GenBank: EU424320.2

Gorin, J (1981). C13 NMR spectroscopy of polysaccharides In *Advances in carbohydrate chemistry and biochemistry*, Stuart, L and Derk, H (eds), New York, Academic Press, P. 51.

Hearst R, Nelson D, McCollum G, Millar BC, Maeda Y, Goldsmith CE, Rooney PJ, Loughrey A, Rao JR, Moore JE. 2009. An examination of antibacterial and antifungal properties of constituents of Shiitake (Lentinula edodes) and oyster (Pleurotus ostreatus) mushrooms. *Complement Ther Clin Pract*.; **15**(1):5-7.

Hibasami, H.; Moteki, H., Ishikawa, K., et al. (2003) Protodioscin isolated from fenugreek (Trigonella foenum graecum L.) induces cell death and morphological change indicative of apoptosis in leukemic cell line H-60, but not in gastric cancer cell line KATO III. *Int J Mol Med;* **11**, 23-6.

Hibbett, D. S.; Donoghue, M. J.; Tsuneda, A. and Fukumasa-Nakai, Y. (1995). Lentinula edodes isolate TMI-818 , 5.8S rRNA gene, complete sequence, 18S rRNA and 25S rRNA genes, partial sequence. *Mycologia*, **87** (**5**), 618-638.

Israilides, C.; Kletsas, D.; Arapoglou, D.; Philippoussis, A.; Pratsinis, H.; Ebringerová, A.; Hríbalová, V. and Harding, S. E. (2008). In vitro cytostatic and immunomodulatory properties of the medicinal mushroom *Lentinula edodes*. *Phytomedicine*, **15**, 512-9.

Jiang, J., Cao,X., Sun,Y., Ju,X., Yin,T., Miao,Q., Li,C. and Mei,K. Submitted (22-NOV-2008a) The Key Laboratory of Biotechnology for Medicinal Plants of Jiangsu, Lentinula edodes strain xsd08112 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence. (http://www.ncbi.nlm.nih.gov/nuccore/218664616) GenBank: FJ481023.1

Jiang, J., Ju,X., Cao,X., Sun,Y., Yin,T. and Miao,Q. Submitted (19-DEC-2008b). Lentinula edodes strain xsd08092 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence. (http://www.ncbi.nlm.nih.gov/nuccore/221145757). GenBank: FJ582641.1

Jiang,J., Zhang,S., Cao,X., Li,C., Yin,T., Miao,Q., Li,C., Mei,K. and Hu,Y. ubmitted (09-MAR-2009) The Key Laboratory of Biotechnology for Medicinal Plants of Jiangsu. Lentinula edodes strain dd08110 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence (http://www.ncbi.nlm.nih.gov/nuccore/226346643). GenBank: FJ810189.1

Le, P.L.H., Nguyen,T.L.T., Le,A.T.H. and Truong,N.B Submitted Submitted (26-AUG-2010), Lentinula edodes 18S ribosomal RNA gene, partial sequence; ITS 1, 5.8S ribosomal RNA gene, and ITS 2, complete sequence; and 28S ribosomal RNA gene, partial sequence. Available on pubmed website (http://www.ncbi.nlm.nih.gov/nuccore/308055645) GenBank: HQ186261.1

Miyazaki, K.; Tsuchiya, Y. and Okuda, T. Submitted (31-OCT-2007) Forest Microbiology Group. Lentinula edodes genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 25S rRNA, partial and complete sequence, strain: G408PP-4. available on pubmed genebank website (http://www.ncbi.nlm.nih.gov/nuccore/187369517), GenBank: AB366150.1

Mosmann, T., (1983). Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays. *J. Immunol. Meth.,* **65**, 55-63.

Okamoto, T.; Kodoi, R.; Nonaka, Y.; Fukuda, I.; Hashimoto, T.; Kanazawa, K.; Mizuno, M. and Ashida, H. (2004). Lentinan from shiitake mushroom (Lentinus edodes) suppresses expression of cytochrome P450 1A subfamily in the mouse liver. *Biofactors*, **21**, 407-9.

Shouji, N., Takada, K., Fukushima, K., Hirasawa, M. and Tomotake, N. (1999). Three kinds of antibacterial substances from Lentinus edodes. *International Journal of Antimicrobial Agents,* **11**, 151-157

Wasser (2002) Medicinal mushroom as a source of antitumour and immunomodulating polysaccharides. App.Microbiol. *Biotechnology,* **60**, 258-274

White, T. J.; T. Bruns, S. Lee, and J. W. Taylor. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pp. 315-322 In: *PCR Protocols: A Guide to Methods and Applications,* eds. Innis, M. A., D. H. Gelfand, J. J. Sninsky, and T. J. White. Academic Press, Inc., New York.

Yu, Z. Submitted (22-FEB-2008) Pleurotus ostreatus isolate NW438 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence. Northwest University of Agriculture and Forestry, College of Forestry. (http://www.ncbi.nlm.nih.gov/nuccore/189162246) GenBank: EU520255.1

Zhang, M.; Cui, S. W.; Cheung, P. C. K. and Wang, Q. (2007). Wang Antitumor polysaccharides from mushrooms: a review on their isolation process, structural characteristics and antitumor activity .*Trends in Food Science & Technology*,**18**, 4-19.

Zheng, R, Jie S, Hanchuan D, Moucheng W. (2005). Characterization and immunomodulating activities of polysaccharide from Lentinus edodes. *Int Immunopharmacol.*, **5(5)**, 811-20.

10/26/2010

Assessment Removal of Heavy Metals Ions from Wastewater by Cement Kiln Dust (CKD)

Taha, A. Waly[1]; A. M. Dakroury[2]; G. O. El-Sayed[3] and S. A. El-Salam[1]

[1]Egyptian Fuel Manufacturing Pilot Plant (FMPP), [2]Hot Lab. Center and Waste Management, Atomic Energy. Authority, Egypt.
[3]Department of Chemistry, Faculty of Science, Benha University. Benha, Egypt
*aishaw95@yahoo.com

Abstract: The effective removal of HM ions from aqueous wastes is among the most important issues for many industrialized countries. The present work has been carried out to study the adsorption of Cd(II), Al(III), Co(II) and Zn(II), by adsorption technique using CKD which, are both wastes and are pollutants. The sorption process was examined in terms of its equilibria and kinetics. Batch adsorption experiments were conducted to evaluate the removal of Cd(II), Al(III), Co(II) and Zn(II), onto CKD waste over a wide range of operating conditions of sorbat concentration, pH, contact time, sorbent dose. The batch experiments showed that the most effective pH range was found from 5.5 to 8. Time-dependent experiments for the removal efficiency of HM ions showed that Al(III) required a shortest contact time, for Zn(II) and Cd(II), binding to the CKD was rapid and occurred within 20 to 40 min and completed for Co(II) within 4 hrs. High sorption capacities were observed for the four HM ions. The binding capacity experiments revealed the following amounts of HM ions bound per gram of CKD: 165.994 mg/g, 75.389 mg/g, 64.296 mg/g and 108.875 mg/g for Zn(II), Al(III), Co(II) and Cd(II), respectively. The equilibrium data for HM ions fitted both Langmuir and Freundlich models and based on Langmuir constant. The adsorption isotherm studies clearly indicated that the adsorptive behavior of HM ions on CKD satisfies not only the Langmuir assumptions but also the Freundlich assumptions, i.e. multilayer formation on the surface of the adsorbent with an exponential distribution of site energy.
[Taha, A. Waly; A. M. Dakroury; G. O. El-Sayed and S. A. El-Salam. **Assessment Removal of Heavy Metals Ions from Wastewater by Cement Kiln Dust (CKD)**. Journal of American Science 2010;6(12):910-917]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: Sorbat, Sorbent, Adsorption, Freundlich, Langmuir and Neutralization. Cement kiln dust (CKD).

1. Introduction:

Industrial and mining waste waters are the major source of pollution by heavy metals. Furthermore, in developing countries, many industries are operated at small or medium scale, or even as a family business within the residential premises of the owner. These smaller unties can generate a considerable pollution load, which, in many cases, is discharged directly into the environment without any facilities for waste-water treatment. This is because the capital investment, turnover and profit for these industries are also small. Heavy metals can pose health hazards if their concentrations exceed allowable limits. Even when the concentrations of metals do not exceed these limits, there is still a potential for long–term contamination, since heavy metals are known to be accumulated within biological systems. In recent years, increasing awareness of the environmental impact of heavy metals has prompted a demand for the purification of industrial waste water prior to discharge into natural waters. This has led to the introduction of more strict legislation to control water pollution. In this study several metal ions have been investigated such as Zinc that is present in waste water stream from steel works, rayon fiber manufacture, production and recalculating of cooling water system employing cathodic treatment also in electroplating process (Ismail, 2003). Cadmium causes intoxication of liver, kidneys, brain, lungs, heart and testicles. It is a carcinogen. Cobalt is a microelement involved in such essential processes as nitrogen fixation. The lack of cobalt can lead to abnormal angiogenesis and dangerous diseases in cattle; it is a vital component of cobalamine. On the other hand, despite of the fact that it is an essential microelement, at higher concentrations cobalt can have dangerous effects on living organisms. Cobalt compounds are classified as class II, which means they are not extremely toxic. However, one of the main potential effects of cobalt compounds is decrease of natural ability of rehabilitation of water reservoirs. In plants, cobalt becomes toxic at a concentration of 0.1 – 0.3 mg / l. People exposed to cobalt compounds were reported to suffer from defective metabolism of proteins and carbohydrates, anemia, Basedow's disease, carcinogenic and mutagenic effects (Dalia Virbalyte, 2005). Treatment

processes for metals contaminated waste streams include chemical precipitation, membrane filtration, ion exchange, adsorption, and electro deposition. Cost effective alternative technologies or sorbets for treatment of metals contaminated waste streams are needed. However, most of the methods suffer from some drawbacks as high capital and/or operational cost or the disposable of the residual metals sludge (Mohammad Aimal, 1996). Adsorption processes for heavy metals contaminated wastewater have been proved to be successful by many researchers (Bailey, 1999; Juang, 1999). In this regard the use of abundant low cost and effective adsorbent materials is of interest. Activated carbon has been recognized as a highly effective adsorbent for the treatment of heavy metals in waste water (Reed, 1994). However, it is relatively expensive to produce. Therefore, there is increasing research interest using alternative low–cost adsorbents. Many such materials have been investigated, including microbial biomass, peat, compost, leaf mould, palm press fibre, coal straw, wool fibre and rice milling by- products (Singh, 1997; Wase Daj, 1997) not all of these are effective. Therefore, it is still important to identify suitable low cost adsorbents for heavy metal removal. In this study we have investigated the interaction of CKD with Cd(II), Al(III), Co(II) and Zn(II). The cement industry in Egypt applies dry processes in cement manufacture and releases a large amount of by-pass cement kiln dust (CKD) , is both a waste and a pollutant Therefore, it was desired to make use of this waste and to save the environment.

2. Experimental

2.1. Adsorbent CKD

CKD generated in large quantities for example in Tourah Portoland Cement Factory, the production of CKD is around 5.35% of the total production of the dry kilns, which is about 9000 tons/day. So the amount of CKD about 477 tons/day. In this company 205 tons of the total CKD produced may be recycled to the wet process but the residual amount is discharged in the desert after granulation by using water. The amount of water needed to granulate this amount of CKD, was about 304m^3 /day, this calculation based only on CaO only. These big amounts of CKD produced and the amount of water needed cost the company a lot of unprofitable money and effort. As received CKD was kept all the time on a dessicator to a void the conversion of CaO to $CaCO_3$. CKD has average particle size 3µ during the adsorption process .the pH of 4g/l suspended CKD in deionized water was 10. X-ray florescence, Micromeritics GEMINI 2360 surface area analyzer, Micromeritics SEDI-GRAPH 5100 particle size analyzer and Micromeritics ACCUPYC 1330 density analyzer are devices were used for the physical and chemical analysis of CKD.

2.2. Chemicals

Analytical grade reagents were used for heavy metal solution, ACS reagent grad concentrated hydrochloric acid, NaOH and buffer solutions (E.Merck) were used to adjust pH values of samples. In all experimental work, demineralised water was used.

2.3. Adsorbate solution

Synthetic stock solution of heavy metals was prepared by dissolving required quantity of Analar grade salts in the demineralised water. The salts used are Cadmium chloride, Zinc nitrate, Cobalt chloride, Aluminum nitrate, as sources for Cd(II), Zn(II), Co(II), Al(III), respectively, for the preparation of stock solution. The stock solution was further diluted with demineralised water to desired concentration for obtaining the test solutions.

2.4. Batch mode adsorption experiments

The adsorption of heavy metals on cement kiln dust was studied by batch technique. The general method used for this study is described as below**:**

A known weight of adsorbent (e.g. 0.5–1.5 g) was equilibrated with 50 ml of the heavy metals $CdCl_2$, $CoCl_2$, $Zn(NO_3)_2$ and $Al(NO_3)_3$ with a known starting concentration in a plastic bottles at a fixed temperature (24+1) C° in a thermostatic mechanical shaker (WIDSONS Scientific) for a known period (5 min–24 h) of time. After equilibrium, the suspension of the adsorbent was separated from solution by filtration using Whatman filter paper No. 1. The concentration of heavy metal ions remaining in solution was measured by Inductive Coupled Plasma–Atomic Emission Spectroscopy (ICP-AES). The effect of several parameters, such as pH, concentrations, contact time and adsorbent dose on the adsorption was studied. The pH of the adsorptive solutions was adjusted using hydrochloric acid, sodium hydroxide and buffer solutions when required. Adsorption of metal ions on the walls of glass flasks determined by running the blank experiments was found negligible. The results of these studies were used to obtain the optimum conditions for maximum heavy metals removal from aqueous solution.

2.5. Removal and adsorption of heavy metals

The percent heavy metal removal was calculated using the following equation:

$$\%R = \frac{C_o - C}{C_o} * 100 \qquad (1)$$

Where: C_o: Initial heavy metal ion concentration of test solution, mg/l.

C : Final equilibrium concentration of test solution, mg/l.

The adsorbed phase concentration was calculated using the following equation

$$q = (C_o - C) * \frac{v}{m} \quad (2)$$

where:

q: Amount of heavy metal ions adsorbed per unite mass of adsorbent, mg/g

V: is the volume of the solution in, L

M: is the weight of dry CKD in, gm

Quality Control (QC) aspect: To insure the accuracy, reliability, and reproducibility of the collected data, all the batch experiments were carried out in duplicate and the mean values of two data sets are presented. When the relative error exceeded the relative stander deviation by more than 1.0%, the data were disagreed and a third experiment was conducted until the relative error fell within an acceptable range.

3. Results and Discussion

CKD was utilized to remove heavy metals such as: Zn (II), Co (II), Cd (II), and AL (III) from wastewater. Therefore, the parameters affecting heavy metals removal from synthetic solution such as; the effect of residence time, the effect of pH, the effect of variation sorbent amount added, the effect of variation initial sorbate concentration (Zn, Co, Cd, and Al ions) and finally studying the effect of temperature on the adsorption of these heavy metal ions. Inductive Coupled Plasma –Atomic Emission Spectroscopy (ICP-AES): It is used for analysis of trace elements in a wide range of samples and samples types.

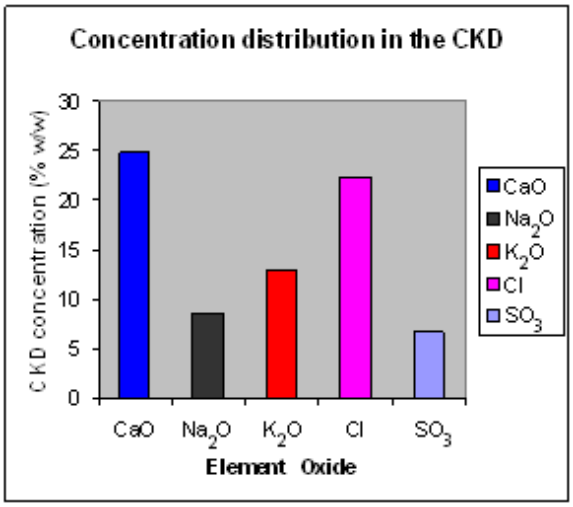
3.1. Analysis of CKD

The sample of CKD obtained from companies was analyzed by X-ray fluorescence (XRF), the percent composition of the CKD are shown in Table (1) the main ingredients or the major components of CKD are calcium oxide, besides sodium oxide, potassium oxide, sulfur-tri-oxide, silicate and chloride. The alkalinity nature of the dust makes it a good neutralizing agent for acidic wastewater stream.

Table (1): X-ray Fluorescence analysis of CKD

Ingredients	% Average Percent
SiO_2	3.14
TiO_2	0.05
Al_2O_3	0.38
Fe_2O_3	0.61
MnO	0.01
MgO	0.22
CaO	24.85
Na_2O	8.61
K_2O	12.95
P_2O_5	0.04
Cl	22.35
SO_3	6.71
L.O.I*	19.45

* loss of ignition



3.2. Effect of initial concentration of heavy metal ions

The effect of initial concentration on the percentage removal of heavy metals by CKD is shown in figure (1) It can be seen from the figure that the percentage removal decreases with the increase in initial heavy metal concentration. For Zn(II), it is seen that the percentage removal is almost complete (nearly 90-100%) throughout the initial metal ions concentration range (384.55-1825.8) mg/L for 10 g/L adsorbent dose, at pH 5.89 and equilibrium contact time. For Cd(II) and Al(III) at same adsorbent dose and equilibriums contact time, there are gradually drop in percentage removal at higher initial concentration, whereas for Co(II) the percentage removal is highly effective >80% below 600 mg/l initial concentration after which percentage removal decreases sharply to below 70%. At higher initial concentrations, Cd(II) shows greater percentage removal than Al(III). At lower initial metal ion concentrations, sufficient adsorption sites are available for adsorption of the heavy metals ions. Therefore, the fractional adsorption is independent of initial metal ion concentration. However, at higher concentrations the numbers of heavy metal ions are relatively higher compared to availability of adsorption sites. Hence, the percent removal of heavy metals depends on the initial metal ions concentration and decreases with increase in initial metal ions

concentration. The difference in percentage removal of different heavy metal ions at the same initial metal ions concentration, adsorbent dose and contact time may be attributed to the difference in their chemical affinity and ion exchange capacity with respect to the chemical functional group on the surface of the adsorbent.

The percentages of removal of Zn(II), Al(III), Co(II) and Cd(II) solutions, in which concentrations were 100–2000 mg/L, were evaluated by using CKD showed high removal efficiency for all metals. These results are shown in figure (1) in these adsorbants, high removal efficiency (95%) was obtained over the Zn(II), Cd(II) and Al(III) concentration range 100-800mg/L. However, the removal percentage gradually decreased with increasing the concentration (>800 mg/L).

3.3. Effect of adsorbent dose

The results for adsorptive removal of heavy metals with respect to adsorbent dose are shown in figure (2) over the range 10–30 g/L, at pH 5.89 and 6.17 for Al (III) and Cd (II), respectively and 8 for Zn(II) and Co(II), the percentage removal of heavy metals is seen to increase with adsorbent dose. From (Fig.2.), the percentage removal of Al (II) ions show 100% removal throughout the range of concentrations studied (within initial concentration 803.10 mg/L). It is observed that there is a sharp increase in percentage removal with adsorbent dose for Zn (II) and Cd (II) ions but in case of Co (II) ions, there is gradual increase in percentage removal with increasing dose. It is apparent that the percent removal of heavy metals increases rapidly with increase in the dose of the adsorbents due to the greater availability of the exchangeable sites or surface area. Moreover, the percentage of metal ion adsorption on adsorbent is determined by the adsorption capacity of the adsorbent for various metal ions.

3.4. Batch contact time studies

Figure (3) indicates that removal efficiency increased with an increase in contact time before equilibrium is reached. Other parameters such as dose of adsorbent, pH of solution and agitation speed was kept optimum, while temperature was kept at 30^{o}C .It is observed that for Al (III) ions, the percentage removal is nearly 100% even throughout the all contact times. Hence the Al (III) requires a shortest contact time. In case of Zn(II) and Cd(II) ions showed sharp rise in percentage removal with increasing contact time. It can be seen that Zn and Cd removal efficiency by using CKD increased from 80% to 95% when contact was increased from 20 to 40 min. On other hand, percentage removal of Co (II) increases gradually with contact time, reaching nearly 95% removals only at around 4 h. It is evident from the results that the contact time required to attain equilibrium is dependent on the initial concentration of heavy metals. For the same concentration, the percentage removal of heavy metal increases with increase of contact time till equilibrium is attained. The optimal contact time to attain equilibrium with CKD was experimentally found to be about 40 min for Zn(II), 5 min. for Al(III), 1 h for Cd (II) and 4 h for Co(II). These results are important, as equilibrium time is one of the important parameters for an economical wastewater treatment system.

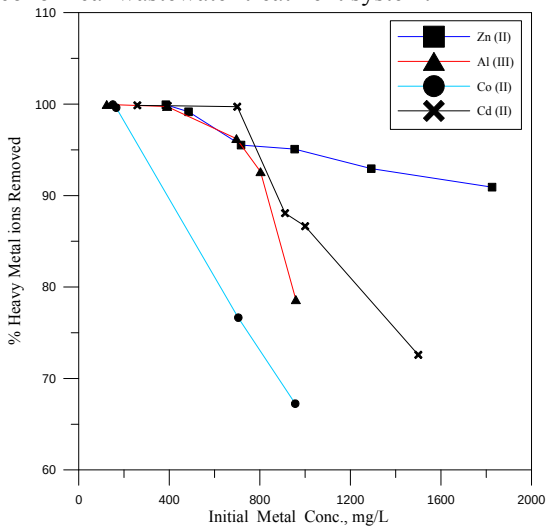


Fig. (1): Effect of initial concentration on the percent removal of heavy metal ions by using CKD.

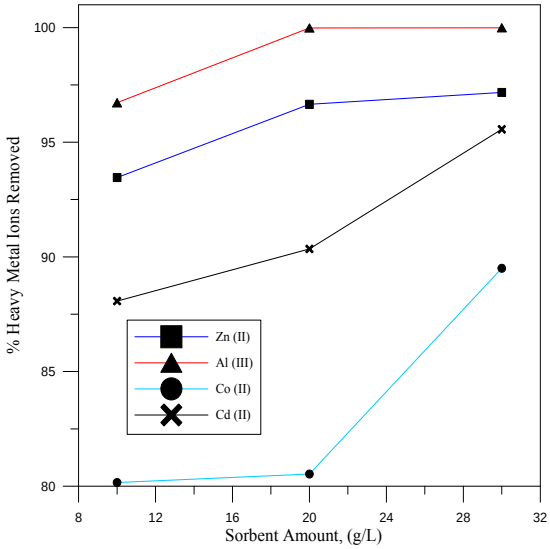


Fig. (2): Effect of variation sorbent dose on heavy metal ions removal by using CKD,at 130 rpm and temp.30^{o}C.

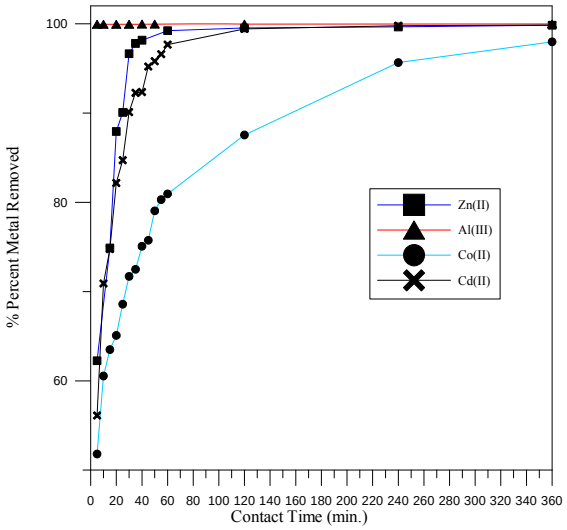


Fig. (3): Effect of contact time on the percent heavy metal ions removal by using CKD.

It has been observed that (Gardea, 1998; Panday, 1985) the mechanism of metal removal from the aqueous metal involved four steps: (i) migration of metal ions from the bulk solution to the surface of the adsorbent; (ii) diffusion through boundary layer to CKD surface; (iii) adsorption at a binding site and (iv) intra particle diffusion into the interior of the CKD. The boundary layer resistance will be affected by the rate of sorption and increasing the agitation time will reduce this resistance and increase the mobility of the ions. However, because the process is time dependent, after about 40 min, 5 min, 1 hr and 4 hr for Zn(II), Al(III), Cd(II) and Co(II), respectively of agitation, adsorption remains slightly or relatively constant. This observation is in agreement with the proposed mechanism obtained from the sorption characteristic study.

3.5. Effect of pH

pH is one of the most important parameters controlling uptake of heavy metals from wastewater and aqueous solutions. Figure (4) shows the percent removal of the four heavy metals as a function of pH at heavy metal concentrations of 384.55, 123.60, 150.40, 258.51 mg/l for Zn(II), Al(III), Co(II), Cd(II), respectively and a CKD concentration of 10 g/L. In general, the amount of heavy metal removed increased as pH increased, and sharply reached over 90% removal at a specific pH value. As shown in diagram given (Heechan , 2005), indicating that the removal of the metals was mainly accomplished by adsorption. As pH increased from 5.5 to 8, it can be expected that the CKD surface becomes more negatively charged. Thus, more favorable electrostatic attraction forces enhanced cationic metal ion adsorption as pH increased. However, the dependence of heavy metal adsorption on pH was different for each metal. The removal of zinc was about 80% at pH 6.5 and it increased to 99% at pH 8. For Aluminum, 85% was removed at pH 5 and it increased to 99% at pH 6. For cadmium, 90% was removed at pH 5.5 and it increased to 99% at pH 6.2 .For Cobalt, removal increased proportionally with increasing pH from 50% at pH 6–90% at pH 8. The percentage adsorption increases with pH to attain a maximum at pH 6-8 and thereafter it decreases with further increase in pH. The maximum removal of Zn (II) and Co(II) at pH 8 were found to be nearly 99 and 90%, respectively, whereas, for Al (III) 99% removal at pH 6 and for Cd(II) 99% removal at pH 6.2. A sharp decrease in removal percent of Al(III) was observed at very high pH values. In case of aluminum, the possible presence of species Al^{3+} and the hydroxyl forms $Al(OH)_2^+$, $Al(OH)_2^+$, $Al(OH)_3$, and $Al(OH)_4^-$. Aluminum is precipitated for pH between 4 and 10. Depending on the pH of the solution, different hydroxyl complexes: $Al(OH)_2^+$, $Al(OH)_2^+$, $Al(OH)_3$, and $Al(OH)_4^-$ can be formed by the aluminum ions Al^{3+} (Bates,1986). In all cases, over 99% of all the four metals were removed except cobalt reached 90% at pH 8. It is often suggested that the tendency of metal cations to adsorb to oxide surfaces is highly correlated with their tendency to undergo hydrolysis reactions in solution (Freundlich 1939). Metal cations in aqueous solutions hydrolyze according to the generalized expression for divalent metals.

$$M^{2+}(aq.) + nH_2O = M(OH)^{2-n} + nH^+ \qquad (3)$$

Explanation of mechanism of adsorption.

The maximum adsorption at pH 8 may be attributed to the partial hydrolysis of M^+, resulting in the formation of MOH^+ and $M(OH)_2$. $M(OH)_2$ would be adsorbed to a greater extend on the non-polar adsorbent surface compare to MOH^+.With increase of pH from 4 to 8, the metal exists as $M(OH)_2$ in the medium and surface protonation of adsorbent is minimum, leading to the enhancement of metal adsorption. At higher pH, that is, above optimum pH of 8, increase in OH^- ions cause a decrease in adsorption of metal ions at adsorbent–adsorbate interface (Langmuir, 1918). Lower solubilities of hydrolysed metal ions species may be another reason for the maximum adsorption at pH 8. Since, in lower pH range, metal is present predominantly as metal ions in the adsorptive solution, there is a competition between$^+$ and M^+ ions for adsorption at the ion-exchangeable sites, leading to a low removal of metal. The extensive repulsion of metal ions due to

protonation of the adsorbent surface at lower pH may be another reason for decrease in adsorption of metal in lower pH range.

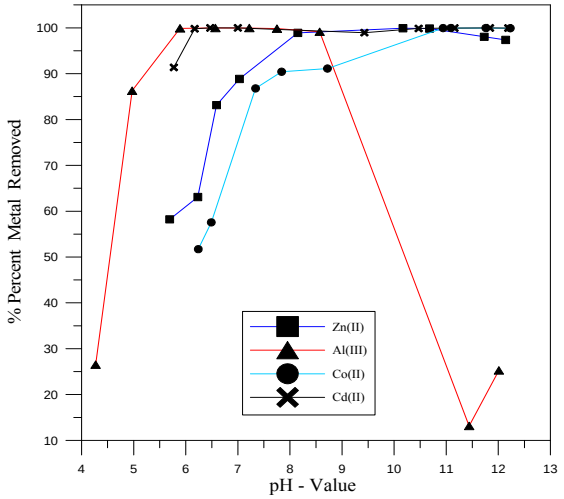


Fig. (4): Effect of different pH - values on heavy metal ions removal by using CKD.

3.6. Adsorption isotherms

The adsorption studies were conducted at fixed initial concentration of heavy metals by varying adsorbent dosage. The equilibrium data obtained were analyzed in the light of Langmuir and Freundlich isotherms.

Freundlich equation is given by (McKay, 1982).

$$q_e = K_f \; C_e^{\frac{1}{n}} \qquad (4)$$

Taking the logarithmic form of the equation

$$Log(q_e) = Log(K_f) + \tfrac{1}{n} Log(C_e) \qquad (5)$$

Langmuir equation is given by (Slejiko, 1985).

$$q_e = \frac{abC_e}{(1+bC_e)} \qquad (6)$$

The linearized expression of this equation is

$$\frac{1}{q_e} = \frac{1}{a} + \frac{1}{abC_e} \qquad (7)$$

This equation is called the **"Double-Reciprocal Langmuir Equation"** and more suitable for situations in which the distribution of equilibrium concentrations tends to be skewed towards the lower end of the range of the equilibrium concentrations. where q_e is the amount of heavy metal ions adsorbed per unit mass of adsorbent in mg/g, **Ce** the equilibrium concentration of heavy metal ions in mg/l, K_f and n are Freundlich constants, **'a'** is a Langmuir constant which is a measure of adsorption capacity expressed in mg/g, **'b'** is also Langmuir constant which is a measure of energy of adsorption expressed in l/mg. The parameters **'a'** and **'b'** have been calculated from the slope and the intercept of the plots.

Figure (5) gives the Freundlich adsorption isotherm plot of **log q_e** versus **log*C*e**. The values of K_f and **1/*n*** obtained from intercept and slope of the plot are given in Table (2). The Langmuir adsorption isotherm plot for **$1/q_e$** versus **1/Ce** is shown in figure (6) and the plots show two distinct regions, one for low $1/C_e$ values up to about 25 l/mg and another for higher $1/C_e$ values.

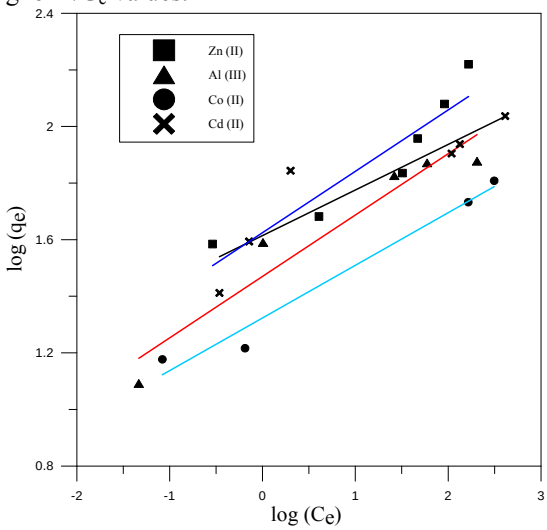


Fig. (5): Freundlich adsorption isotherm of heavy metal ions by using CKD at 30°C.

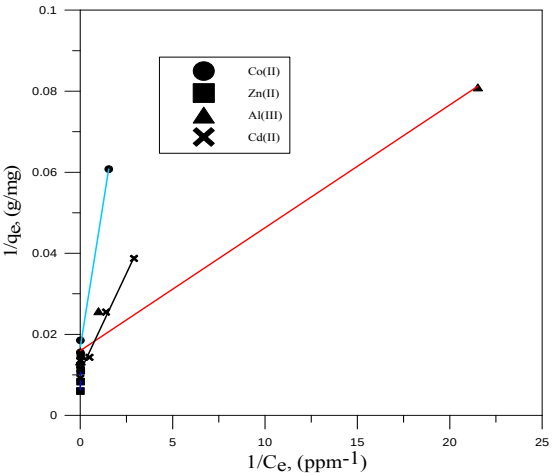


Fig. (6): Langmuir adsorption isotherm of heavy metal ions by using CKD at 30°C.

The essential characteristics of Langmuir isotherm can be described by a separation factor or equilibrium constant R_L, which is defined as,

$$R_L = \frac{1}{1+bC_i} \quad (8)$$

where C_i is the initial concentration of heavy metal ions (mg/l) and b is Langmuir constant which indicates the nature of adsorption. The separation factor R_L indicates the isotherm shape and whether the adsorption is favourable or not, as per the criteria given below.

Table 2.

R_L – values	Adsorption / Type of isotherm
$R_L > 1$	Unfavorable
$R_L = 1$	Linear
$0 < R_L < 1$	Favorable
$R_L = 0$	Irreversible

The values of Langmuir constants ***'a', 'b'*** and R_L are presented in (Tables 3, 4.) Since R_L values lie between 0 and 1 for all four adsorbants studied, it is seen that the adsorption of heavy metal ions is favourable. Adsorption capacity as indicated by value of ***'a'*** is seen to be maximum for CKD, i.e. Zn(II) (233.18 mg/g), Cd(II) (92.35 mg/g), Al(III) (62.64 mg/g), and Co(II) (58.77 mg/g) with a much lower capacities. The energies of adsorption, as indicated by ***'b'*** are seen to be highest for Al (III) (5.26 L/mg), Co(II) (4.12L/mg), Cd(II) (1.12 L/mg), and Zn(II) (0.013 L/mg) in that order. A comparison of the Freundlich adsorption isotherms for the metal ions show that ***n*** in that order Cd(II) > Co(II) > Al(II) > Zn (II). The values of **n** lie between 1 and 10 indicating favourable adsorption. K_f seen to be Zn(II) >Cd(II) >Al(II) >Co(II). This gives a similar inference as that obtained from Langmuir isotherms.

Table 3. Values of langmuir isotherm constants for adsorption of heavy metal ions by using CKD.

Metal ions	**slope**	a(mg/g)	**intercept**	b(L/mg)	R^2	R_L
Zn(II)	0.328784528	233.1785	0.004288560825	0.013044	0.992037	0.0950
Al(III)	0.003033405	62.63779	0.01596480332	5.262996	0.982052	0.0015
Co(II)	0.004133276	58.77456	0.01701416256	4.116386	0.997344	0.0016
Cd(II)	0.009701566	92.35398	0.01082790419	1.116098	0.986492	0.0034

Table 4. values of Freundlich isotherm constants for the adsorption of heavy metal ions by using CKD

Metal ions	**slope**	K_f	**intercept**	n	R^2
Zn (II)	0.2169544939	42.1260480	1.624550719	4.609262	0.862792
Al (III)	0.2168382642	29.5201782	1.470118976	4.611732	0.925075
Co (II)	0.1859835242	21.0527929	1.32330972	5.37682	0.974276
Cd (II)	0.1610804879	41.1513419	1.614384002	6.208077	0.818879

The adsorption isotherm studies clearly indicated that the adsorptive behavior of heavy metal ions on CKD satisfies not only the Langmuir assumptions but also the Freundlich assumptions, i.e. multilayer formation on the surface of the adsorbent with an exponential distribution of site energy.

On the basis of regression analysis of the experimental data on the adsorptive behavior of metal ions on CKD, it may be inferred that the adsorption behavior of metal ions on CKD is in good agreement with Langmuir model. These can be attributed to three main causes (i) the formation of monolayer coverage on the surface of CKD with minimal interaction among molecules of substrate (ii) immobile and localized adsorption and (iii) all sites having equal adsorption energies. The shapes of isotherms suggest that there are high-energy adsorption sites to favor strong adsorption at low equilibrium concentrations for the CKD.

4. Conclusions

In this study, batch adsorption experiments were performed to evaluate the use of CKD particles as an adsorbent for heavy metal ions. It was found that Aluminum required a shortest contact time 5 min, while equilibrium was reached for Zinc and cadmium within 40-60 min and completed for Cobalt at 4 hrs.

It was found that pH of the solution has a significant impact on the percentage removal of heavy metals. Between pH 5.5 and 8, where the influence of precipitation is negligible, the percent removal was 80% - 99% for zinc, 85% - 99% for Aluminum, 90% - 99% for cadmium and 50% - 90% for cobalt. The percent removal of the heavy metal

ions increased as the pH increased. Most of the metal ions had a 99% removal when the pH was 8 except cobalt had a 90% at this pH.

The adsorption isotherm studies clearly indicated that the adsorptive behavior of HM ions on CKD satisfies not only the Langmuir assumptions but also the Freundlich assumptions, i.e. multilayer formation on the surface of the adsorbent with an exponential distribution of site energy.

The results of this study have shown that CKD a cheap by- processes of cement industrial (CKD which is both a waste and a pollutant) was effective in the removal of Cd(II), Zn(II), Al(III) and Co(II) metals in wastewater.

Corresponding author
Taha, A. Waly[1]
Egyptian Fuel Manufacturing Pilot Plant (FMPP),
Atomic Energy. Authority, Egypt.

4. References:

1. Bailey S.E., Olin T.J., Bricka R.M., Adrian B.D. A review of potentially low-cost sorbents for heavy metals, Wat. Res. 1999; 33, 2469.
2. Bates Jr.F.C., R.E. Mesmer, The Hydrolysis of Cations, Robert E. Krieger Publishing Company, Malabar, FL, 1986, pp. 112–122.
3. Dalia Virbalyte et al " Interaction of heavy metal ions with cement kiln dust " EKOLOGIJA. 2005 Nr. 1 P, 31-36
4. Freundlich, H., Heller, W., Rubber die adsorption in lusungen, J. Am. Chem. Soc. 1939; 61, 2228.
5. Gardea-Torresdey JL, Gonzalez JH, Tiemann KJ, Rodrignuez O, Gamez G. Phytofiltration of hazardous cadmium, chromium, lead and zinc ions by biomass of Medicago sativa (alfalfa). J Hazard Mater 1998; 57: 29–39.
6. Heechan Cho, Dalyoung Oh, Kwanho Kim ,A study on removal characteristics of heavy metals from aqueous solution by fly ash, J. Hazard. Mater. 2005; 127: 187–195.
7. Ismail M. A. Ibrahim " Development of extraction systems for the recovery of certain metal ions from aqueous waste solutions" Thesis 2003, Helwan University
8. Juang R.S., Wu F.C. and Tseng R.L. Adsorption removal of copper(II) using chitosan from simulated rinse solutions containing chelating agents, Wat. Res. 1999; 33, 2403.
9. Langmuir, The adsorption of gases on plane surface of glass, mica and platinum, J. Am. Chem. Soc. 1918; 40, 1361.
10. McKay, G., Blair, H.S., Garden, J.R., Adsorption of dyes on chitin, equilibrium studies, J. Appl. Poly. Sci. 1982; 27: 3045–3057.
11. Mohammad Aimal, Rafaqat Ali Khan Rao and Bilquees Ara Siddiqui “Studies on the removal and recovery of Cr (VI) from electroplating waste ” Water Research, 1996; .30(3): 1478-1482.
12. Panday, K.K., Prasad, G., Singh, V.N., Copper (II) removal from aqueous solutions by fly ash, Water Res. 1985; 19: 869–873.
13. Periasamy, K., Namasivayam, C., Removal of Ni(II) from aqueous solution and nickel industry wastewater using an agricultural waste: peanut hull, Waste Manage. 1995; 15, 63.
14. Reed Be., Arunachalam S. "Removal of lead and cadmium from aqueous waste stream using granular activated carbon (GAC) columns" Environ. Prog. 1994; 13: 60-64.
15. Singh, D., Rawat, Ns. " Adsorption of heavy metals on treated and untreated low grade bituminous coal " Ind. J. Chem. Techno. 1997; 4: 39-41.
16. Slejko, F., Adsorption Technology: A Step by Step Approach to Process Evaluation and Application, Marcell Dekker, New York, 1985.
17. Wase Daj and Forster Cf "Biosorbents for metal ions" Taylor and Francis, lodon, 1997.

10/5/2010

Evaluation of Avian Influenza Vaccines used in Broiler Flocks in Egypt.

Lebdah, M.A and *Shahin, A.M.

Avian and Rabbit Medicine Department; Faculty of Vet. Med.; Zagazig University
*abeer.shahin@gmx.de

Abstract: This study was carried out to investigate the efficacy of different types of commercial Avian Influenza Vaccines (H5N1 &H5N2) used in Egypt. Three – hundred and fifty day-old broiler chicks were divided into 7 groups. Six groups of chickens were vaccinated with H5N1 and H5N2 AI vaccines at 1, 7, and 14 days-old. The chickens of group 7 were kept as negative control. All groups were fed adlibtum and kept under observation. Serum Samples were collected at day-old to evaluate the maternal immunity and after 7 weeks post vaccination with both types of vaccines from all chickens. This study revealed that, the challenge virus was highly pathogenic for control group as causing 100 % mortalities 24 hours after challenge with 10^6 EID_{50}/ 0.2 ml intranasal. Challenge of other groups showed difference in pathogenicity of the virus and immune response of the chickens according to type of vaccine and age of birds at vaccination. It could be concluded that H5N2 AI vaccine was more protective than H5N1 AI vaccine as the protection percentage and GMHI titer of experimentally broiler chicks vaccinated at day-old and fourteen days-old with H5N2 higher than chicks vaccinated with H5N1. Moreover, the vaccination of the chicks at seven days-old showed higher GMHI titer and protection percentage than vaccination at one day-old.
[Lebdah, M.A and Shahin, A.M. **Evaluation of Avian Influenza Vaccines used in Broiler Flocks in Egypt**. Journal of American Science 2010;6(12):918-926]. (ISSN: 1545-1003). http://www.americanscience.org.

1. Introduction:

Avian Influenza Virus (AIV) is a type A Orthomyxovirus and produces a variety of disease syndromes in various poultry species. On the basis of serological reactions to surface glycoprotein (hemagglutination and neuraminidase), AIV is subtyped into 16 hemagglutinin (H1-H16) and nine neuraminidase (N1-9) subtypes (Kawaoka et al., 1990; Rohm et al., 1996 and Easterday et al., 1997).

Avian Influenza become the most important disaster threat to the poultry industry all over the world after the occurrence of highly pathogenic AI (HPAI) outbreaks in many parts of the world (Alexander, 2000 and Swayne, 2003) such as H5N2 in Pennsylvania and H7N1 in Italy (Capua et al., 1999; Capua and Mutinelli, 2001; Capua and Alexander, 2004 and Manvell et al; 2000).

Beside the biosecurity and monitoring infection particularly in the densely populated poultry areas, the vaccination represents an option for control. From this point of view, the evaluation of different types of Avian Influenza Vaccines (H5N1 and H5N2) used in Egypt may provide effective vaccination strategy.

Conventional Inactivated AI vaccines are widely used all over the world. Vaccination has been shown to increase resistance to field challenge and reduce virus shedding levels in vaccinated birds and subsequently reduce transmission. Despite of wide uses of different inactivated AI vaccines program, outbreaks of AI still threat poultry flocks in Egypt. Abd El Aziz (2008) concluded that single dose of vaccination at 12 days-old have better effect on chicken immune response and protection against lethal challenge with HPAIV than one day-old vaccination which need booster vaccination for initiation of humoral immune response and maximal protection rate. The aim of this study was to obtain new insights into evaluation of Avian Influenza Vaccines used in Egypt.

2. Materials and methods

A. Materials

A.1. Experimental Chickens:

A.1. Broiler chickens: Three – hundred and fifty, day – old, Ross broiler chicks were obtained from Commercial Hatcheries-Egypt

.

A.2. Avian Vaccines:

A.2.1. ND vaccines:

a. Hitchner B1 vaccine, batch No. 0151V and titer $10^{6.5}$ EID_{50}

b. La Sota vaccine, obtained, batch No. 719 u/2 and titer $10^{6.5}$ EID_{50}

A.2.2. IB vaccines:

H120 vaccine, obtained, batch No.6m5f/3 and titer $10^{3.5}$ EID_{50}

A.2.3. AI Vaccines:

A.2.3.a. an inactivated oil emulsion H5N1 Avian Influenza vaccine, A/Goose/Guangdong/16(H5N1), batch No. 009088, and titer $10^{8.5}$ EID_{50}.

A.2.3.b. an inactivated oil emulsion H5N2 Avian Influenza vaccine, obtained from EGA Vet. Company, batch No. 0901150A, and titer $10^{8.5}$ $EID_{50.}$

A.3 Local isolated AI virus (challenge AI virus).

Locally isolated H5N1virus isolate kindly supplied by Dr. Adel Abd El-Aziz, Vet. Clinic, Faculty of Veterinary Medicine, Zagazig University with titer of 10^6 EID_{50}.

A.4. Equipments

A.4.1. Instruments and Equipments:

a. Eppendorf cups

b. Microtiter Plates

c. Automatic pipettes

A.5. Reagents: Washed RBCs 10%; sterile saline, Sterile Distilled water and Phosphate Buffered saline (PBS)

A.6. Antigen: Inactivated H5N1 antigen, obtained from Veterinary laboratories Agency, New haw, Addlestone, surrey KT153 NB, UK. Prep. Date: dec05. Lot No: 3/05. It was provided kindly by Dr. Adel El-Gamal, Animal Health Research Institute, Zagazig, Egypt.

A.7. Embryonated chicken eggs (ECE): One hundreds SPF ECE (9-11 days- old) were used for titration of the viral isolates. They were obtained from Kom-Oshim Company, El Fayoum Governorate, Egypt

B. Methods:

B.1. Experimental design:

For evaluation of both AI H5N1 and H5N2 vaccines in broilers, three hundred and fifty, day- old, Ross broiler chicks, were divided into seven groups (1-7), each group containing 50 chicks. Chicks of groups 1 and 2 were vaccinated with H5N1 and H5N2 respectively at one – day old via subcutaneous injection with dose 0.5 ml / chick. Meanwhile, chickens of group 3 and 4 were vaccinated with H5N1 and H5N2 vaccines respectively, at seven days-old, via subcutaneous injection with dose 0.5 ml /chick. In addition, chicks of group 5 and \6 were vaccinated with H5N1 and H5N2 vaccines respectively, at fourteen days-old, via subcutaneous injection with dose 0.5 ml / chick. Meanwhile, chicks of group 7 were remained non - vaccinated as non vaccinated control. All experimental chickens were challenged with H5N1 via intranasal route with dose of 106EID50/0.2ml at 28 days post vaccination with both types of AI vaccines. Blood samples were taken at were collected at day-old, 7 days, 7 weeks post vaccination and three weeks post challenge, sera were extracted for determination the level of specific antibodies against Avian Influenza by using HI test. All experimental chickens were observed for clinical signs, morbidities and mortalities. All freshly dead chickens were examined for recording PM lesions.

Table 1: Experimental design for evaluation of AI vaccines in broiler

Group No.	Type of used vaccine	No. of exp. birds	Age and route of vaccination	Serum samples collection age per day							Challenge		
				1	2	3	4	5	6	7	Age / day	route	dose
1st	H5N1	50	Day – old S/C	7	14	21	28	35	42	49	28	I/N	**0.2ml X10^6 EID_{50}**
2nd	H5N2	50	Day – old S/C	7	14	21	28	35	42	49	28	I/N	
3rd	H5N1	50	7days – old S/c	14	21	28	35	42	49	56	35	I/N	
4th	H5N2	50	7days – old S/C	14	21	28	35	42	49	56	35	I/N	
5th	H5N1	50	14 days–old S/C	21	28	35	42	49	56	63	42	I/N	
6th	H5N2	50	14days– old S/C	21	28	35	42	49	56	63	42	I/N	
7th	----	50	control	14	21	28	35	42	49	56	35	I/N	

B.2. 1. Hemagglutination inhibition (HI) test:

HI test was carried out in U bottomed microplates withantigen to contain 4 HA units according to OIE (2005).

B.3. Statistical analysis:

The statistical analysis of data of different experiments was carried out according to the statistical analysis system (SAS, 1987).

3. Results

Evaluation of avian influenza vaccines:

A- Protection %, morbidity % and mortality %:

The rate of protection, morbidity and mortality differ according to breeds and age of vaccination. The non-vaccinated, challenged control chickens of all breeds and ages were dead within 24 hours post challenge. In addition, morbidities and mortalities were 100% in non-vaccinated challenged control chickens, thus the protection % was 0%. The groups vaccinated at day-old show high mortality rate than groups vaccinated at seven days old and visa versa the groups vaccinated at day-old show low protection rate than groups vaccinated at seven days old. The protection %, mortality % and morbidity % of different groups are summarized in table (2).

Table (2) Protection, mortalities and morbidities percentage of birds.

Group No.	Type of vaccine	Age of vaccination	Protection		Protection		Mortalities %	
			%	%				
Broiler (1)	H5N1	Day - old	80%	40/50	20%	10/50	20%	10/50
Broiler (2)	H5N2	Day – old	90%	45/50	20%	10/50	10%	5/50
Broiler (3)	H5N1	7 day – old	90%	45/50	0%	0/50	10%	5/50
Broiler (4)	H5N2	7 day – old	90%	45/50	0%	0/50	10%	5/50
Broiler (5)	H5N1	14 day – old	90%	45/50	10%	5/50	10%	5/50
Broiler (6)	H5N2	14 day – old	92%	46/50	2%	1/50	8%	4/50
Broiler (7)	control	-	0%	0/50	0%	0/50	100%	50/50

The protection percentage of all experimentally vaccinated chickens with either H5N1 or H5N2 AI vaccines was arranged from 80-92 %. The broiler chicks vaccinated at day - old with H5N1 AI vaccine showed lowest protection percentage (80%). Meanwhile, broiler chickens vaccinated at 14 day-old with H5N2 AI vaccine were showed highest protection percentage (92%).

B-Serological results:

1-Mean HI titer of broiler chicks vaccinated at day-old with AI H5N2vaccine showed high titer than broiler chicks vaccinated at day-old with H5N1 vaccine. The HI titer of broiler chicks were vaccinated at day-old was summarized in table (3).

Table(3): HI titer of broiler chickens vaccinated against AI at day-old.

Group	Maternal immunity	1st week*	2nd week*	3rd week*	4th week*	5th week*	6th week*	7th week*
H5N1	1.4048± 0.1003	1.1038 ± 0.1003	1.6055 ± 0.1003	2.0069 ± 0.1003	2.3079 ± 0.1003	2.4082 ± 0.1738	2.8096 ± 0.1003	2.7093 ± 0.0000
H5N2	1.4048 ± 0.1003	1.2041 ± 0.000	1.7058 ± 0.1003	2.2076 ± 0.1003	2.4082 ± 0.1738	2.6089 ± 0.2007	2.9100 ± 0.1003	2.6089 ± 0.1003

*It mean weeks after vaccination

2- Mean HI titer of broiler chicks vaccinated at seven day-old with AI H5N1 vaccine showed high titer than broiler chicks vaccinated at seven day-old with AI H5N2 vaccine. The HI titer of broiler chicks vaccinated at seven day-old were summarized in table (4).

3- Mean HI titer of broiler chicks vaccinated at fourteen day-old with AI H5N2 vaccine similar to mean HI titer of broiler chicks vaccinated at fourteen day-old with AI H5N1 vaccine. The HI titer of broiler chicks vaccinated at fourteen day-old were summarized in table (5).

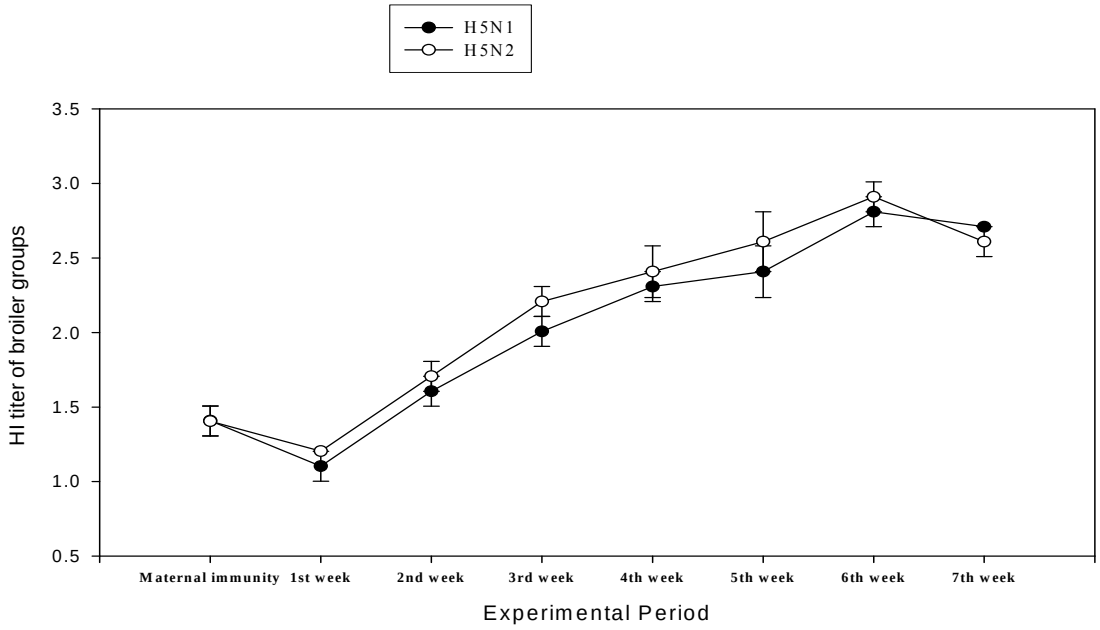


Fig (1): HI titer of broiler chickens vaccinated against AI at day-old.

Table (4): HI titer of broiler chickens vaccinated against AI at seven days-old.

Group	Maternal immunity	1st week*	2nd week*	3rd week*	4th week*	5th week*	6th week*	7th week*
H5N1	1.4048 ± 0.1003	1.20 ± 0.17	1.91 ± 0.36	2.31 ± 0.10	2.51 ± 0.20	2.81 ± 0.20	3.01 ± 0.00	2.51 ± 0.10
H5N2	1.4048 ± 0.1003	1.30 ± 0.10	1.51 ± 0.17	2.21 ± 0.20	2.41 ± 0.17	2.71 ± 0.30	2.91 ± 0.10	2.61 ± 0.27

*It mean weeks after vaccination

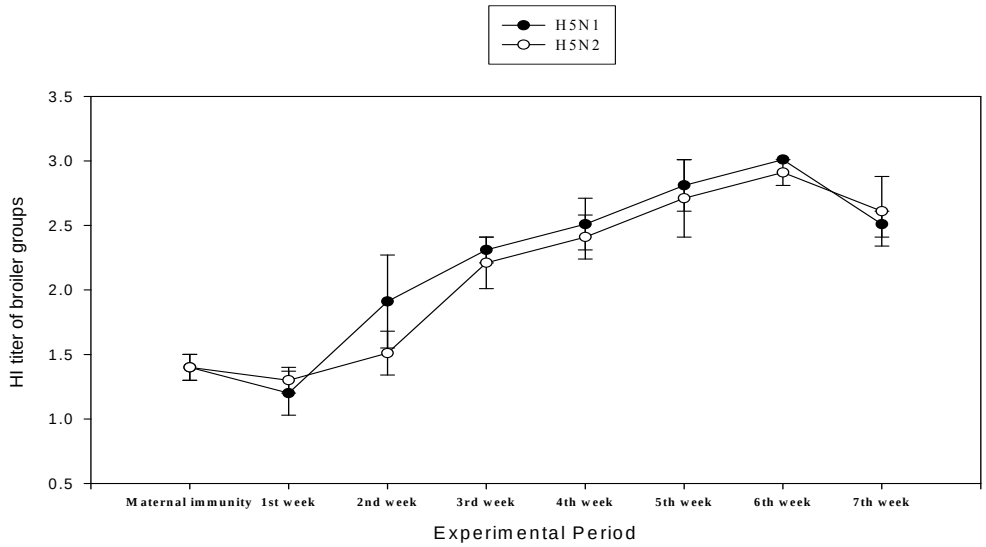


Fig (2): HI titer of broiler chickens vaccinated against AI at seven days-old

Table (5): HI titer of broiler chickens vaccinated against AI at fourteen days-old.

Group	Maternal immunity	1st week*	2nd week*	3rd week*	4th week*	5th week*	6th week*	7th week*
H5N1	1.4048 ± 0.1003	1.3045 ± 0.2007	1.8062 ± 0.1738	2.0069 ± 0.2655	2.4082 ± 0.3010	2.6089 ± 0.2655	2.8096 ± 0.1003	2.5086 ± 0.2007
H5N2	1.4048 ± 0.1003	1.2041 ± 0.1738	1.9065 ± 0.2007	2.1072 ± 0.0000	2.4082 ± 0.1738	2.6089 ± 0.1003	2.9100 ± 0.1003	2.5086 ± 0.1003

*It mean weeks after vaccination

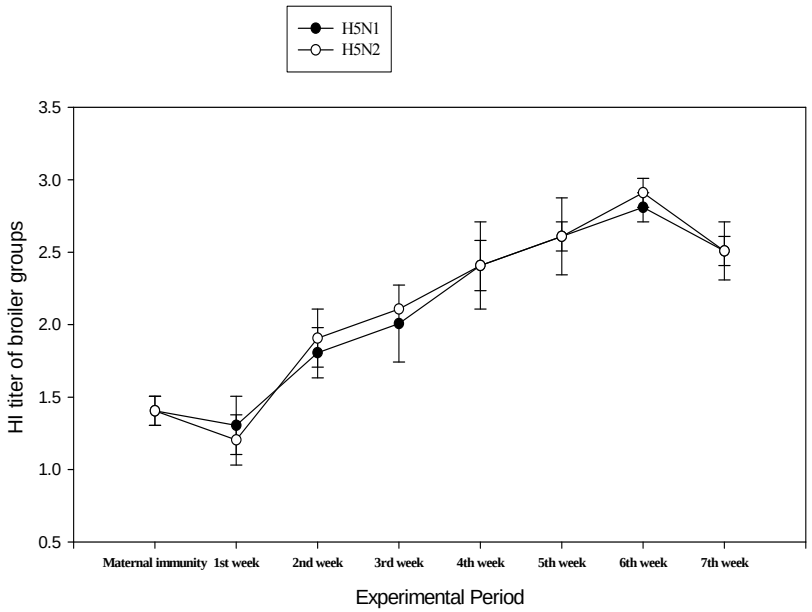


Fig (3): HI titer of broiler chickens vaccinated against AI at fourteen days-old.

C. Statistical analysis of HI titer:-

1-**Evaluation of AI (H5N1 and H5N2) vaccines of broiler chickens at day-old:**

Treatment	Mean ± Std. Error
Pre vaccination	1.4048 ± 0.1003^{b}
After vaccination	1.8187 ± 0.1017^{b}
After challenge	2.6758 ± 0.05^{a}

Means within the same column carrying different titer were significant at ($P \leq 0.05$). There was significant between after challenge and other treatment and there was no significant between pre vaccination and after vaccination

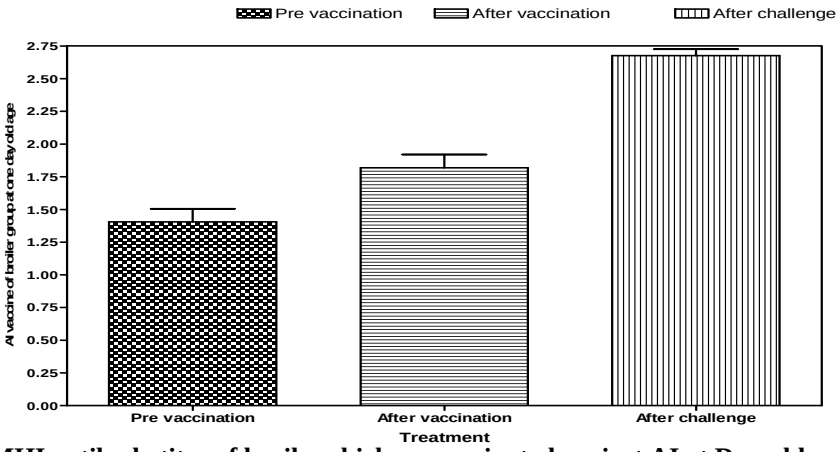


Fig (4): GMHI antibody titer of broiler chickens vaccinated against AI at Day-old.

2- Evaluation of AI (H5N1 and H5N2) vaccines of broiler chickens at seven day-old:

Treatment	Mean ± Std. Error
Pre vaccination	1.4048 ± 0.1003^{c}
After vaccination	1.9191 ± 0.1172^{b}
After challenge	2.7594 ± 0.07^{a}

Means within the same column carrying different titer were significant at (P≤0.05). There was significant between pre vaccination, after vaccination and after challenge

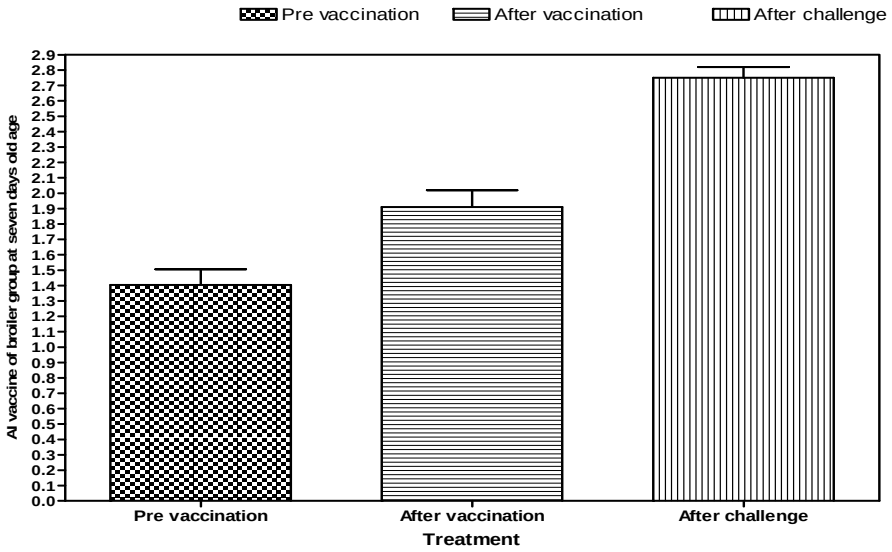


Fig (5): GMHI antibody titer of broiler chickens vaccinated against AI at seven days-old.

3- Evaluation of AI (H5N1 and H5N2) vaccines of broiler chickens at fourteen days-old:

Treatment	Mean ± Std. Error
Pre vaccination	1.4048 ± 0.1003^{c}
After vaccination	1.8940 ± 0.1064^{b}
After challenge	2.6591 ± 0.06^{a}

Means within the same column carrying different titer were significant at (P≤0.05). There was significant between pre vaccination, after vaccination and after challenge

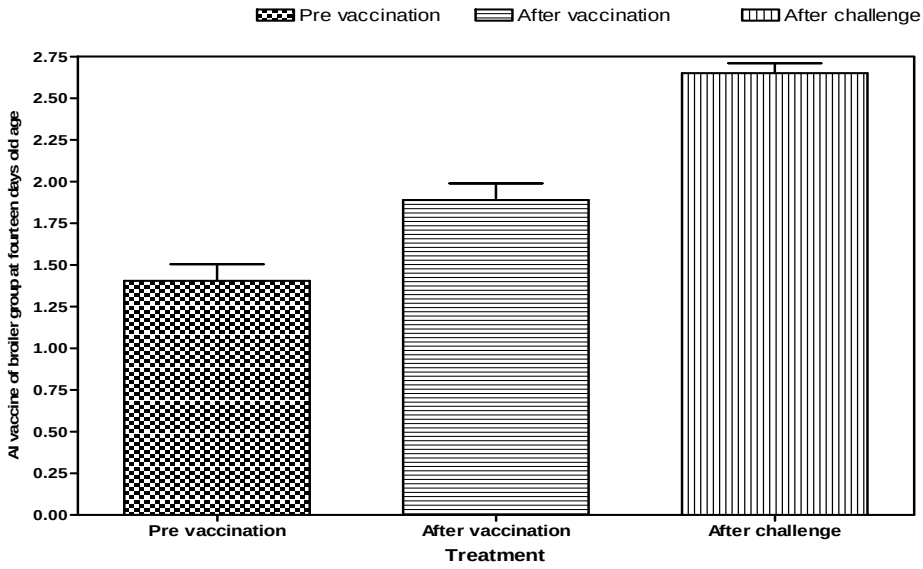


Fig (6): GMHI antibody titer of broiler chickens vaccinated against AI at fourteen days-old.

4- Evaluation of H5N1 vaccine of broiler chickens at day-old, seven day-old and fourteen days-old:

Treatment	Mean ± Std. Error
day-old	2.1861 ± 0.09[a]
seven day-old	2.2792 ± 0.09[a]
fourteen day-old	2.2219±0.08[a]

Means within the same column carrying different titer were significant at (P≤0.05). There was no significant between different treatments.

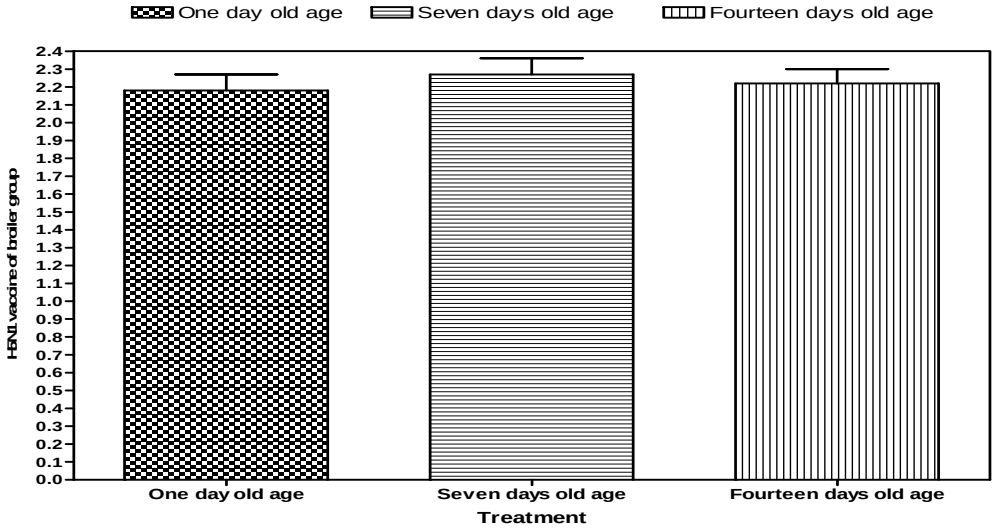


Fig (7): GMHI antibody titer of broiler chickens vaccinated against H5N1 vaccine at day-old, seven days-old and fourteen days-old.

5- Evaluation of H5N2 vaccine of broiler chickens at day-old, seven day-old and fourteen day-old:

Treatment	Mean ± Std.Error
One day old age	2.1861 ± 0.09[a]
seven days old age	2.2792 ± 0.09[a]
fourteen days old age	2.2219± 0.08[a]

Means within the same column carrying different titer were significant at (P≤0.05). There was no significant between different treatments

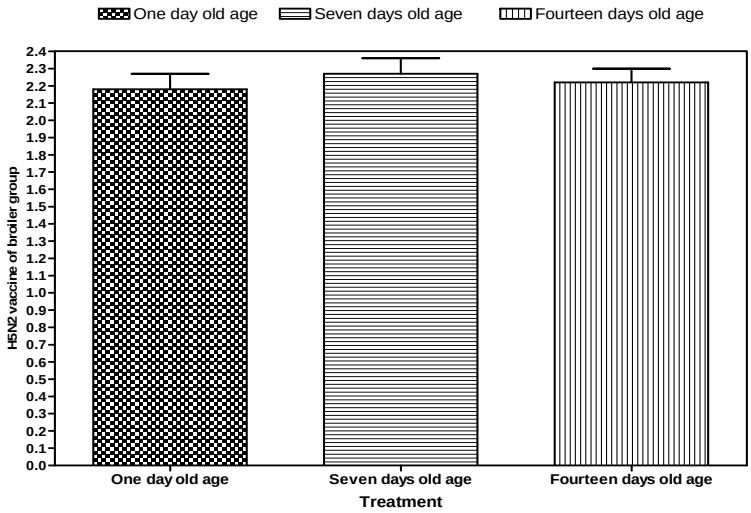


Fig (8): GMHI antibody titer of broiler chickens vaccinated against H5N2 vaccine at day-old, seven days-old and fourteen days-old.

4. Discussion:

In the present work, the effect of Avian Influenza (AI) vaccines on the chickens of different ages was recorded. In Egypt, vaccination of broilers against AI represents for now and the foreseeable future, the central strategy for prevention and control of AI.

The obtained results indicated that GMHI titer of broiler chicks vaccinated at day-old with H5N2 vaccine showed high titer than broiler chicks vaccinated at day-old with H5N1 vaccine, while mean HI titer of broiler chicks vaccinated at seven day-old with H5N1 showed high titer than broiler chicks vaccinated at seven day-old with H5N2 vaccine, but the other groups showed similarity of mean HI titer of chicks vaccinated with H5N2 or H5N1 vaccine. These results agreed with the results of Ellis et al., (2004 b) who stated that the use of killed H5N2 vaccine in the face of HPAI H5N1virus challenge was able to protect chickens from disease and can reduce virus transmission. Also, these finding were agreed with Guobin Tian et al., (2005).

Who generated a high – growth H5N21/ PR8 virus by plasmid –based reverse genetics. When chickens were immunized with 0.3 ml of the vaccine, the hemagglutinin inhibition (HI) antibody become detectable at 1 week post-vaccination and reached to the peak at 6 weeks post-vaccination then slowly declined at 43 weeks post-vaccination. When challenge test performed at 2, 3 and 43 weeks post vaccination; all the chickens were completely protected from disease signs and death. Revaccination after three weeks from the primary vaccination at saso and

Layer groups increase the GM antibody titer in both H5N1 and H5N2 vaccines leading to complete protection (100%) in some groups after lethal challenge with H5N1 virus. These results agreed with the results of Webster et al., (2006) who concluded that revaccination increase the HI antibody by about ten fold. Also these results were agreed with Abdel-Aziz (2008) who concluded that revaccination after 7 days from the primary vaccination at one day old increase the GM antibody titer about 3 folds in both H5N1and H5N2 AI vaccines, and agreed with Lee et al., (2007) who stated that one dose of 128 hemagglutinin (HA) unit of homologous H5N1 vaccine able to induce 100% protection in mortality and prevent viral shedding completely after lethal dose virus challenge, whereas one dose of 64 HA unit of heterologous H5N3 vaccine only induce50% protection in mortality, and it did not prevent viral shedding. However, two doses of 64 HA unit of heterologous H5N3 vaccine as well as one dose of 1024 HA unit of heterologous H5N3 vaccine induced 100% survival rate and could prevent viral shedding completely.

The rate of protection, morbidity and mortality after infection with the isolated H5N1Avian Influenza viruses differ according to breeds and age of vaccination. The control groups in any breeds and at any age which kept without AI vaccines, all birds of these groups died within 24 hours. The groups of chicks vaccinated at day-old showed high mortality rate (20%). On the other hand the chickens vaccinated at seven day-old showed low mortality and high protection rate (90%). These agreed with Ellis et al., (2004 a) who recorded that, when the infection spread to the recently vaccinated birds, low rate of H5N1 mortality when the chickens were between 9 and 18 days post- vaccination. However after 18 days post-vaccination, no more deaths from H5N1 AI occurred and intensive monitoring by virus isolation from these farms showed no evidence of asymptomatic shedding of the virus. This provides evidence that avian influenza vaccines can interrupt virus transmission in the field.

These results also agreed with Beato et al., (2007) who reported that the recent outbreaks of Avian Influenza were worldwide and have highlighted the difficulties in controlling this disease. Vaccination has become a recommended tool to support the eradication efforts and to limit the economic losses due to AI. The vaccination system in the poultry farms based on the use of vaccine containing a heterologous neuraminidase to the field virus. This has been shown to be very effective in reducing the viral shedding, clinical symptoms and differentiating vaccinated from infected birds. Also our results were in agreement with Bublot et al., (2007) who reported that all unvaccinated challenged birds died within 2 days, whereas 90% and 100% of chickens vaccinated with H5N9WI and H9N9It respectively were protected against morbidity and mortality. Both vaccines prevent cloacal shedding and significantly reduce oral shedding of the challenge Asian HPAI H5N1virus

It could be concluded that H5N2 vaccine gives higher protection percentage than H5N1 vaccine, and the more preferable age for vaccination is seven days-old.

Corresponding author
Shahin, A.M.
Avian and Rabbit Medicine Department; Faculty of Vet. Med.; Zagazig University
*Abeer.shahin@gmx.de

5. References:

1. Alexander, D. J. (2000): A review of avian influenza in different bird species. Vet. Microbiol 74: 3-13
2. Abdel-Aziz, A.M. (2008): syudies on avian influenza in east delta.Ph.D. Thesis (poultry and rabbit diseases). Faculty of Vet. Med. Zagazig. University.
3. Beato, M.S.; Rigoni, M.; Milani, A. and Capua, I. (2007): Generation of avian influenza reassortant viruses of the H7N5 subtype as potential vaccine candidates to be used in the framework of a "DIVA" vaccination strategy. Avian Dis.Mar; 51(1 Suppl): 479-80.
4. Bublot, M.; Le Gros, F. X.; Nieddu, D.; Pritchard, N.; Mickle, T. R. and Swayne, D. E. (2007): Efficacy of two H5N9-inactivated vaccines against challenge with a recent H5N1 highly pathogenic avian influenza isolate from a chicken in Thailand. Avian Dis.; 51 (1 suppl): 332-337.
5. Capua, I. and Alexander, D. J. (2004): Avian influenza; recent developments. Avian Pathology, 33 (4): 393-404.
6. Capua, I.; Marangon, S.; Selli, L.; Alexander, D. J.; Swayne, D. E.; Pozza, M. D.; Parenti, E. and Cancellotti, F. M. (1999): Outbreaks of highly pathogenic avian influenza)H5N2) in Italy during October 1997-January 1998. Avian Pathol. 28: 455-460.
7. Capua, I. and Mutinelli, F. (2001): Mortality in Muscovy ducks (Cairina moschata) and domestic geese (Anser anser var. domestica) associated with natural infection with a highly pathogenic avian influenza virus of H7N1 subtype. Avian Pathol. (2) 30: 197-183.
8. Easterday, B. C.; Hinshaw, V. S. and Halvorson, D. A. (1997): Avian influenza. In Diseases of Poultry Calnek, B. W.; Barnes, H. J.; Beard, C. W.; McDougald, L. R. and Saif, Y. M. (eds.). Dis. of Poultry, 10th ed. Iowa State University Press: Ames, 583-605.
9. Ellis, T. M.; Barry, B. R.; Bissett, L. A.; Dyrting, K. C.; Luk, G. S.; Tsim, S. T.; Sturm-Ramirez, K.; Webster, R. G.; Guan, Y. and Malik Peiris, J. S. (2004): Investigation of outbreaks of highly pathogenic H5N1 avian influenza in waterfowl and wild birds in Hong Kong in late 2002. Avian Pathol. 33, 492-
10. Ellis, T. M.; Sims, L. D.; Wong, H. K.; Wong, C. W.; Dyrting, K. C.; Chow, K. W.; Leung, C. and Peiris, J. S. (2006): Use of avian influenza vaccination in Hong Kong. Dev Biol (Basel). 124: 133-43.
11. Guobin Tian, Zhangb, S.; Li, Y.; Bu, Z.; Liu, P.; Zhoub, J.; Li, C.; Shi, J.; Yua, K. and Chena, H. (2005): Protective efficacy in chickens, geese and ducks of an H5N1 inactivated vaccine developed by reverse genetics. Virology: 341; 153-162.
12. Kawaoka, Y.; Yamnikova, S.; Chambers, T. M.; Lvov, D. K. and Webster, R. G. (1990): Molecular characterization of a new hemagglutinin, subtype H14, of influenza A virus. Virology 179: 759-767
13. Lee, Y. J.; Sung H. W.; Choi J. G.; Lee, E. K.; Jeong O. M.; Kwon Y. K.; Kwon, J. H.; Song C. S. and Kimd J. H. (2007): Effects of homologous and heterologous neuraminidase vaccines in chickens against H5N1 highly pathogenic avian influenza. Avian Dis.; 51: 476-480.
14. Manvell, R. J.; McKinney, P.; Wernery, U. and Frost, K. M. (2000): Isolation of a highly pathogenic influenza A virus of subtype H7N3 from a peregrine falcon. Avian Pathol. 29: 635-637.
15. OIE diagnostic and terrestial manual (2005): Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. PART 2 SECTION 2.1. Chapter 2.1.
16. Rohm, C.; Zhou, N.; Suss, J.; Mackenzie, J. and Webster, R. J. (1996): Characterization of a novel influenza hemagglutinin, H15: criteria for determination of influenza A subtype. Virology 217: 508-516.
17. Statistical Analysis System (SAS) (1987): User's guide. Statistical SAS Institute. Cary North Carolina.
18. Swayne, D. E. (2003): Introduction and Welcome. Avian Dis. 47, 783- 784.
19. Webster,R.G.;Webby R. J.; Hoffmann, E.; Rodenberg, J.; Kumar, M.; Chu, H. J.; Seiler, P.; Krauss, S. and Songserm, T. (2006): The immunogenicity and efficacy against H5N1 challenge of reverse genetics-derived H5N3 influenza vaccine in ducks and chickens. Virology. 351 (2): 303-311.

11/1/2010

Her 2/neu Gene and VEGF in Bladder Cancer in Egypt: Relationship to Schistosomiasis

Olfat A. Hammam [1], Iman Abdel Aziz [2],Ola Mahmoud [2], Manal Zahran [2], Amr Alkholy [3], Ahmed Abdel Hadi[1], Maha Akl [1], Mohamed Wishahi [3]. Bruno Voss[4] and Sabine Boehm[4]

Departments of Patholog [1], Hematology [2], Urolog[3], Theodor Bilharz Research Institute, El-Nil Street, Giza, Egypt
Research Institute for Occupational Diseases[4], Ruhr University Bochum, Germany
*totoali1@hotmail.com

Abstract: The aim of the current study was to assess Her2/neu protein on paraffin tissue sections and serum VEGF in carcinoma of the urinary bladder in a cohort of Egyptian patients. Furthermore, they were correlated to the schistosomal-associated and non-schistosomal associated bladder cancer as well as tumor types and disease stages. Immunohistochemical (IHC) procedure for Her 2/neu, FISH for detection of Her2/neu gene and serum level of VEGF by EIISA were performed in 35 patients with chronic cystitis (10 patients with nonschistosomal chronic cystitis and 25 patients with chronic schistosomal cystitis), 135 were schistosomal-associated malignant patients (75 cases of squamous cell carcinoma and 60 cases of urothelial carcinoma) and 50 cases of non schistosomal-associated urothelial carcinoma. In addition to 20 normal blood donor volunteers act as control. IHC results for Her 2/neu was overexpressed in malignant group compared to control and chronic schistosomal cystitis groups ($p<0.01$). In malignant group it was 1^+ in 33 (30%), 2^+ in 45 (40.9%) and 3^+ in 32 (29.09%). Her 2/neu incidence was significantly higher in urothelial carcinoma group 80/110 (72.2%) compared to SCC 30/75 (40%) with ($p<0.01$) and in high grade tumors than low grade tumors with ($p< 0.01$). FISH results in SCC showed that the signal ratio were 0-1.0 in 2 (6.6%), 1.1-2.0 in 18 (60%), and ≥2.0 in 10 (33.35%), which were considered positive for Her 2/neu gene amplification. In urothelial carcinoma the signal ratio was 0-1.0 in 10 patients (12.5%), 1.1-2.0 in 25 (22.3%), and ≥2.0 in 45 (58.2%). Overexpression of Her2/neu gene was significantly higher in high grades; 31 (63.6%) than in low grades; 14(56%) tumor with ($p< 0.01$), also Her2 /neu gene was significantly ($p< 0.01$) higher in invasive tumors; 45 cases (78.9%) than in non invasive tumors 10 (43.3%) with high significance ($p< 0.01$). The serum VEGF levels showed higher levels for SCC, urothelial carcinoma patients, chronic cystitis patients compared to normal controls, they were 94.7% (71/75), 89% (98/110), and 22.9% (8/35), 5% (1/20) respectively. Our results suggest that Her 2/neu overexpression might provide additional prognostic information in patients with bladder carcinomas. Because 50% of our patients harbor Her 2/neu overexpressing those patients may potentially benefit from molecular targeted therapy targeting Her 2/neu for bladder carcinoma and they should be identified by gene amplification analysis using FISH in IHC 2+ patients. In addition association between increased serum VEGF levels with high grades and invasive bladder cancer patients indicates that serum VEGF may play a role in the invasion and metastasis of cancer and may serve as an indicator of tumor progression and future recurrence and may be a candidate as a new noninvasive diagnostic tool.

[Olfat A. Hammam, Iman Abdel Aziz,Ola Mahmoud, Manal Zahran Amr Alkholy, Ahmed Abdel Hadi, Maha Akl [1], Mohamed Wishahi [3]. Bruno Voss[4] and Sabine Boehm. **Her 2/neu Gene and VEGF in Bladder Cancer in Egypt: Relationship to Schistosomiasis**. Journal of American Science 2010;6(12):927-936]. (ISSN: 1545-1003). http://www.americanscience.org.

Key Words: Her 2/neu protein; Her 2/neu Gene; IHC- FISH; Serum VEGF.

1. Introduction:

In Egypt, bladder cancer accounts for about 30% of all cancers, where it is the most common malignancy in men and the second most common malignancy in women after breast cancer (El-Mawla *et al.* 2001, Jemel *et al.* 2005). It has been associated with many pathogenetic factors most commonly bilharzial infestation, which is an endemic infection in the Nile River Valley (El Sebaie *et al.* 2005). In countries with a long history of schistosomiasis, research studies have identified the following risk factors for infection with S haematobium: male gender, an age < 20 years, living in smaller rural communities, exposure to canal waters, reagent strip detected hematuria and proteinuria, and a history of burning micturation (El-Harvey *et al.* 2000, Gabar *et al.* 2000). Most investigators have accepted the association between schistosomiasis and bladder cancer since the work of Ferguson 1911. The development of bladder cancer in a younger age group affects males more and is usually associated with schistosomal infection. Also it is accompanied with a high mortality rate and clinicopathologic features of schistosomal-associated bladder cancer (SABC) (Mostafa *et al.* 1999). The high frequency of squamous cell carcinoma (SCC) is due to schistosomiasis-infested bladders that frequently show squamous metaplasia and dysplasia of the

transitional epithelium (El Bolkainy *et al.* 1981). Recently, however, a relative increase in the frequency of transitional cell type in schistosomiasis-associated bladder cancer has been noted (Gouda *et al.* 2007, Felix *et al.* 2008, Heyns and van der Merwe 2008). The neoplastic changes in the urothelium of bladder are a multistep phenomenon (Carroll 1995). The exact genetic events leading to urothelial transformation involve the activation of oncogenes, inactivation or loss of tumor suppressor genes and alterations in the apoptotic gene products (Sandberg and Berger 1994).

The Human epidermal growth factor receptor 2 (Her 2/neu) oncoprotein, also known as (NEU, EGFR2, or ErbB2) is one of the members of the Epidermal Growth Factor Receptor (EGFR) family, which includes EGFR or (ERBB1), EGFR3 or (Her3/ErbB3) and EGFR4 or (Her4/ErbB4), is known to contribute to physiological mechanisms of cell proliferation by intrinsic tyrosine-kinase-activity (Wülfing *et al.* 2005). Binding of ligands, such as epidermal growth factor and transforming growth factor alpha (TGFα), to their extracellular ligand-binding domain initiates intracellular signaling cascades, leading to progression, proliferation, migration and survival of cancer cells (Yarden and Sliwkowski 2001).

EGFR and Her 2/neu are dysregulated in many human cancers (Slamon *et al.* 1978). Recent studies indicate the role of Her2/ neu in the development of numerous types of human cancer. Her 2/neu overexpression and/or amplification have been detected in a variety of cancer types, including non–small cell lung cancer, pancreatic carcinoma and gastric carcinoma (Gravalos and Jimeno 2008), colonic carcinoma (Schuell *et al.* 2006) ovarian carcinoma (Des Guetz *et al.* 2006, Tabernero 2007), bladder cancer (Eltze *et al.* 2005) and 10%-34% of invasive breast cancers and correlates with clinical outcome, poor prognosis, and constitute a predictor factor of poor response to chemotherapy and endocrine therapy. Her 2/neu overexpression is associated with shortened disease-free and overall survival compared with patients who have Her 2/neu-negative tumors (Kaptain *et al.* 2001).

Superficial bladder cancer (SBC) is a precursor of muscle-invasive, potentially life-threatening bladder cancer. Given the field cancerization effect and the risk of recurrence and progression, SBC appears the most suitable target for bladder cancer systemic chemoprevention. However, new risk biomarkers are demanded to select at-risk subjects and to conduct efficient clinical chemoprevention trials. Angiogenesis represents a key step for tumor progression and metastatic spread in solid tumors. Vascular Endothelial Growth Factor (VEGF) is the most known angiogenic factor and is involved in early stages of bladder carcinogenesis (Nicholson *et al.* 2001). Also expression of VEGF either as tissue or soluble form, has been reported to have prognostic significance in several cancers (Mohammed *et al.* 2007). Overexpression of Tyrosine–kinase receptors, including VEGF, and Her 2/neu, has been associated with the progression of cancer and poor prognosis in urothelial tumors (Bolenz and Lotan 2008). It is worth mentioning that all of these studies were limited to transitional cell carcinoma which was not schistosomiasis-associated. The aim of the current study was to assess Her 2/neu protein and gene and soluble VEGF in carcinoma of the urinary bladder in a cohort of Egyptian patients, and both markers were correlated to the schistosomal status as well as tumor types and disease stages.

2. Materials and methods

Materials:

This study was conducted on 220 patients (156 males and 64 females having mean age of (65.5± 11.2) patients admitted to the Urology Department at Theodor Bilharz Research Institute (TBRI) Hospital. In addition, 20 age and sex matched healthy subjects as a blood donor control group and 5 cases of cystoscopic biopsies during prostatectomies as control group for IHC & FISH; upon patient's consent were included in this investigation. Tumor specimens were taken by cystoscopy (Transurethral resection (TUR) biopsies) and cystectomies. Only cystoscopic biopsies containing muscle tissue were included, so that muscle invasion by the tumor could be assessed. The study protocol was approved by the ethical committee of TBRI according to the institutional committee for the protection of human subjects and adopted by the 18th world medical assembly, Helsinki, Finland and informed consent from all patients underwent cystoscopy and biopsy from apparent growth and lesions was taken. Patients were subjected to full clinical examination, routine laboratory investigations, complete urine analysis, abdominal and pelvic ultrasonography, general and abdominal examination, digital rectal examination (DRE), bimanual examination under anesthesia, plain x-ray of the urinary tract, intravenous urography (IVU), cystoscopy and TUR biopsies were taken from apparent growth. Accordingly they were grouped into:

- Group I: Five male patients (age range 25-50), with normal bladder urothelium, subjected to prostatectomy served as normal controls after taking their consent.
- Group II: Thirty five patients with chronic cystitis classified as:

- 10 patients with nonschistosomal chronic cystitis.
- 25 patients with chronic schistosomal cystitis, which include:

12 patients with dysplastic changes, 4 patients with squamous metaplasia and 9 patients with simple chronic schistosomal cystitis.

- Group III: One hundred and eighty five malignant patients:

- 135 were schistosomal-associated include 75 cases of SCC and 60 cases of schistosomal-associated urothelial carcinoma.
- 50 cases of non schistosomal-associated urothelial carcinoma.

Urothelail carcinoma (110 cases): Are categorized according to pathological stages into: 40 Non invasive tumors (pTa+pT1) and 70 invasive tumors (pT2+pT3) and according to pathological grades into: 46 low grade urothelial carcinoma and 64 high grade urothelial carcinoma.

Methods:

Histopathological Study

Tissues were fixed in 10% buffered formalin, paraffin embedded and processed routinely. Hematoxylin and Eosin stained slides were used to evaluate the pathological diagnosis of all bladder lesions and to assess urothelial carcinoma [transitional cell carcinoma (TCC)] cases for pathological grades according to World Health Organization (WHO) (Mostofi *et al.* 1988) and pathological tumor stage, in accordance with WHO Classification of tumors (Eble *et al.* 2004). Diagnosis of schistosomal infestation was based on detection of Schistosoma eggs in tissues and/or detection of circulating Schistosoma antibodies in sera of patients by enzyme linked immunosorbent assay (ELISA).

Immunohistochemical (IHC) procedure for Her 2/neu

For IHC, a standard 3-layer protocol was used, as previously described by

Hsu and Raine (1981). Unstained sections were processed for immunostaining with Her 2 monoclonal antibody, Endogenous peroxidase was blocked by 0.3% hydrogen peroxide for 30 minutes. The antibody-binding epitope of the antigen was retrieved by microwave treatment for 15 minutes in citrate buffer at 700°C. We used Her 2 primary antibody at a dilution of 1:50, and incubated for 24 hours in a humid chamber (Dako, Copenhagen, Denmark). The sections were then incubated with the secondary biotinylated antibody followed by avidin peroxidase complex according to the manufacturer's instructions (Universal Detection Kit, Dako, Denmark). A brown color was developed with 3,3 diaminobenzidine for 5 minutes, and counterstained with Mayer's hematoxylin. Negative controls in which the primary antibody was omitted and replaced by phosphate buffered saline were also used. Breast cancer known to express Her 2 /neu was used as a positive control.

Urothelial cells in entire sections were examined in ten consecutive fields under light microscopy at magnifications x 400 with the highest expression and the percentage was calculated from their mean. A negative staining was defined as the absence of cells expressing the marker (zero %). The intensity of reactivity was scored according to Ooi *et al.* (2004).

Fluorescence in situ hybridization (FISH) to detect Her 2/neu gene

The technique of FISH to detect Her 2/neu gene was performed according to Matsubara *et al.* (2008), after usual processing for remove of wax and hydration of the slides, we use enzymic digestion by 5 µg/ml proteinase K (Boehringer Mannheim; Mannheim, Germany) in 0.1 M Tris-HCI (pH 8.0) containing 50 mM EDTA. Disodium salt for 45 minutes at 45°C before incubation with the slides. Then incubate slides with DIG – labelled DNA probe (Zymed Lab-Sa system, USA) for 10 minutes at 95°C. Then incubate slides in humid chamber at 37°C for 45 minutes with mouse anti DIG, then add on slides goat anti mouse Cy3 at 3°C for 45 minutes. Put DAPI at 37°C for 60 seconds at 37°C for nuclear stain (blue nucleus). To preserve fluorescent labelling, sections were mounted using DABCO antifade (Sigma, Missouri, USA). Each section was mounted in approximately 5 µl of mounting medium and the slide cover is sealed with entallin. In each preparative run, positive controls (Probe Check control slides supplied by the manufacturer) were included.

An Olympus BX 61 microscope equipped with appropriate filters for DAPI, Spectrum Green and Spectrum Orange was used to score the number of signals per nucleus under magnification power x600. Images were captured using a CCD digital camera and Quips FISH imaging software (Meta Cyte scanning image cytometer, Meta Systems, Altlussheim, Germany). The number of fluorescent signals was counted in 60 urothelial cell in each case. The mean number of signals per nucleus was determined.

Serum VEGF was done using EIA technique:

From the studied subjects five milliliter of venous blood samples were collected under complete aseptic conditions, delivered into a clean tube and left to clot. Serum was separated by centrifugation at 3000 rpm for 15 min. , sera were separated and divided into small aliquots for assay of liver function tests {serum bilirubin, alanine

aminotransferase (ALT), aspartate aminotransferase (AST) and serum albumin}, kidney function tests (serum creatinin and blood urea) using autoanalyzer (Hitachi 736, Hitachi, Japan) and Determination of serum level of VEGF using quantitative sandwich enzyme immunoassay technique (ELISA). (using R&D system, USA).

Statistical Analysis

For statistical analysis, Statistical Package for the Social Sciences (SPSS, Chicago, IL, USA) version 10.0 for Windows software was used. The chi-square test was used to detect statistically significant differences between the groups, with a significance level of ($p<0.05$). Comparison between means of different groups was done using one way ANOVA. Comparison between percent of positive cases was calculated by Chi-square test.

3. Results

Clinico-pathological data and immunostaining results for the cases exhibiting overexpression of Her 2/neu are illustrated in (Table 1).

Assessement of Her 2/neu protein using IHC technique

In control cases no Her 2/neu expression was detected. Her 2/neu- positive immunostaining was limited to the cell membrane. It was overexpressed ($p<0.01$) in malignant group compared to control and chronic schistosomal cystitis groups. In malignant group it was 1^+ in 33 (30%), 2^+ in 45 (40.9%) and 3^+ in 32 (29.09%). Her 2/neu incidence was significantly ($p<0.01$) higher in urothelial carcinoma group 80/110 (72.2%) compared to SCC 30/75 (40%).

On classifying urothelial carcinoma cases on the basis of their histopathological grades into low and high grades; the numbers of cases showed overexpression of Her 2/neu were found to be significantly ($p< 0.01$) higher in high than low grade tumor.

On classifying urothelial carcinoma cases on the basis of their histopathological stages into non muscle invasive and muscle invasive urothelial carcinoma; we found Her 2/neu overexpressed in ($p< 0.01$) 23/40 (55.5%) in non muscle invasive urothelial carcinoma compared to 57/70 (81.4%) (Table 1, Figs 1A &B & C).

Assessment of Her 2/neu gene using FISH technique

In primary tumors, in SCC the signal ratio was 0-1.0 in 2 patients (6.6%), 1.1-2.0 in 18 (60%), and ≥2.0 in 10 (33.35%)., which was considered positive for Her 2/neu gene amplification. In Urothelial carcinoma the signal ratio was 0-1.0 in 10 patients (12.5%), 1.1-2.0 in 25 (22.3%), and ≥2.0 in 45 (58.2%).

According to the classification of grade, the number of cases showed over- expression of Her 2/neu gene were found to be significantly ($p< 0.01$) higher in high 31 (63.6%) than low grade 14(56%) tumor.

According to the classification of stage, we found Her 2/neu overexpressed ($p< 0.01$) in 10(43.3%) non muscle invasive urothelial carcinoma compared to those with muscle invasive 45(78.9%) (Table 1, Figs 2A&B&C).

Determination of positive VEGF rates

According to the mean level of VEGF in normal human serum (123.53 pg/ml),the normal level of VEGF was calculated as 227.20 pg/ml. Taking this as the standard, the VEGF expression positive rates for SCC, urothelial carcinoma patients, chronic cystitis patients and normal controls were 94.7% (71/75), 89 % (98/110), and 22.9% (8/35), 5% (1/20) respectively, (Table 2).

Relationship between VEGF and Schistosomiasis:

Of the 35 chronic cystitis patients, 10 were found without schistosomal infestation and 25 were chronic cystitis with schistosomiasis. There was a significant ($P< 0.01$) difference in serum VEGF level between the two groups. Of the 60 patients with schistosomal associated urothelial carcinoma, the positive rate of VEGF was 91.7% (55/60), whose VEGF level (584.77± 443.87 pg/ml), which was significantly ($P< 0.01$) higher than that of non schistosomal associated urothelial carcinoma where the positive rate of VEGF was 84% (42/50) and VEGF level was (429.41± 289.83 pg/ml) (Table 3).

Relationship between VEGF and histopathological grades

According to histopathological grades, 46 patients were classified as having low grade tumor with a VEGF level of 329.68 ± 228.45pg/ml. Sixty-four patients were classified as having high grade tumor with a VEGF level of 558.42± 370.10 pg/ml. In the two groups, the serum level of VEGF expression was significantly ($P< 0.001$) higher in histopathologically higher grade tumors, compared to lower grades, and there was a positive correlation between the serum level of VEGF and tumor grades (Table 3).

Relationship between VEGF and Histopathological stages

Superficial bladder cancer (SBC) is a precursor of muscle-invasion, potentially life-threatening bladder cancer. In this study, the levels of VEGF in the patients with non muscle invasive-

urothelial carcinoma were significantly (P< 0.001) lower than those with muscle invasive-urothelial carcinoma (418.21 ± 243. 25 pg/ml and 550.10 ± 436.60 pg/ml respectively). This finding suggested that high-level expression of VEGF predicts a higher level of muscle invasiveness and higher metastastatic tendency of the tumor (Table 3).

Table 1: Expression of Her2/neu protein using IHC and Her2/neu gene using FISH in the bladder tissue in the studied cases

Parameters	No of positive cases with Her2/neu in bladder tissue by IHC No (%)	No cases with normal expression of Her2/neu gene by FISH	No cases with over-expression of Her2/neu gene by FISH No (%)
Control (n=5)	0 (0%)	5 (100%)	--
Chr. cyst (n=35)	4 (8%)	13(86.6%)	2 (13.4%)
Ch non Schist. Cyst (n-10)	0 (0%)	5 (100%)	---
Ch Schist. Cyst (n=25)	4 (8%)	8 (80%)	2 (20%)
Malignant lesions (n=185)	**110 (59.4%)** $^{*,\wedge}$		55 (50%)$^{\wedge}$
SCC (n=75)	**30 (40%)** $^{*,\wedge}$		10(33.3%)$^{\wedge}$
Urothelial carcinoma (n=110)	**80 (72.2%)** $^{*,\wedge,\#}$		45(58.2%)$^{\wedge,\#}$
* Non Sch.Ass. urothelial carcinoma (n=50)	30 (60%)$^{*,\wedge,\$}$		13(43.3%)$^{\wedge}$
* Sch Ass.urothelial carcinoma (n=60)	50 (83.3 %)$^{*,\wedge}$		32(68%)$^{\wedge,\$}$
Histopathological grades:			
Low grade (n=46)	25 (54.3%)		14(56%)
High grade (n=64)	55 (85.9)$^{¥}$		31 (63.6%)$^{¥}$
Histopathological stages:			
Non muscle invasive urothelial carcinoma (n= 40)	23 (55.5%)		10(43.3%)
Muscle invasive urothelial carcinoma (n=70)	57 (81.4 %)$^{€}$		45(78.9%)$^{€}$

* P < 0.01 compared to control group.
$^{\wedge}$P < 0.01 compared to chronic schist. cystitis group.
$^{\#}$ P < 0.01, compared to SCC.
$^{\$}$ P < 0.05 compared to Sch TCC respectively.
$^{¥}$ P < 0.01 compared to low grade tumor .
$^{€}$ P < 0.01 compared muscle invasive urothelial carcinoma .
Chr. cyst = chronic cystitis
Ch Schist.Cyst = chronic schistosomal cystitis
Ch non Schist.Cyst = chronic non schistosomal cystitis SCC= squamous cell carcinoma
Schistosomal associated urothelial carcinoma =Sch ass. urothelial carcinoma

Table 2: Serum positive expression of VEGF in different groups

Group	n	VEGF expression	
		Negative (%)	Positive (%)
Normal Control	20	19 (95)	1(5)
Chronic Cystitis	35	27 (77.1)	8 (22.9)
SCC	75	4 (5.3)	71(94.7)
Urothelial Carcinoma	110	12 (11)	98(89.0)

Table 3: Relationship between serum expression of VEGF (pg/ml) and pathological characteristics of Urothelial Carcinoma

Pathological characteristics	N	VEGF expression	
		mean± SD	*p*
Sch.Ass. urothelial carcinoma	60	584.77± 443.87	
Non Sch.Ass. urothelial carcinoma	50	429.41± 289.83	0.016
Low grade	46	329.68 ± 228.45	
High grade	64	558.42± 370.10	0.001
Muscle invasive urothelial carcinoma	70	550.10 ± 436.60	
Non muscle invasive urothelial carcinoma	40	418.21 ± 243. 25	0.001

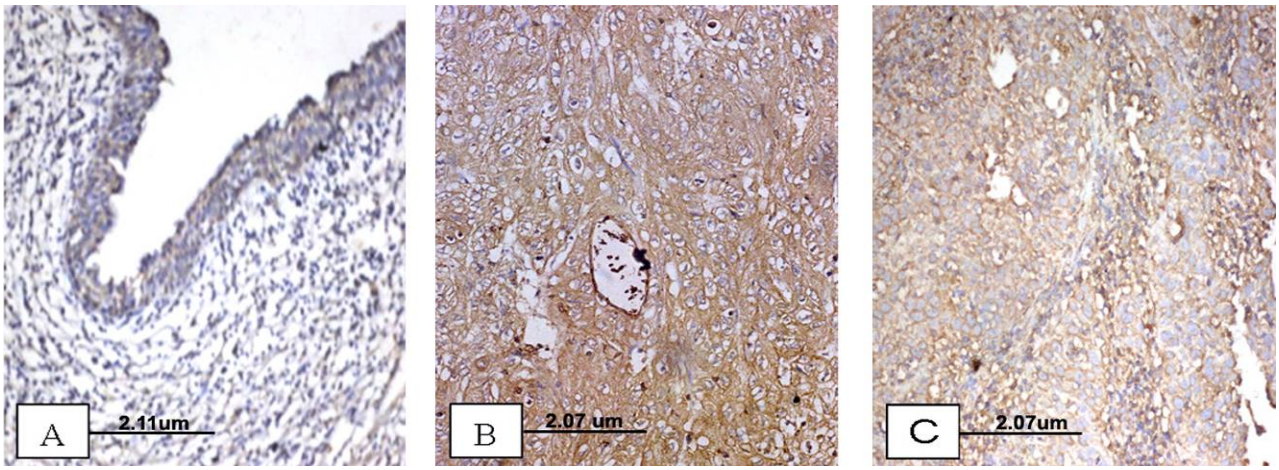


Figure 1: A) A case of chronic schistosomal cystitis, showing mild memranous expression of Her 2, involving the whole layers of the urothelium (IHC, Her2, DAB, x20). B) A case of schistosomal-associated moderately differentiated squamous cell carcinoma showing moderate membranous expression of Her 2 in about 70% of the malignant squamous cells (IHC, for Her 2 DAB, x40). C) A case of invasive urothelial cell carcinoma, high grade, showing marked membranous expression of Her2 in the majority of malignant urothelial cells (IHC for Her-2, DAB, x40).

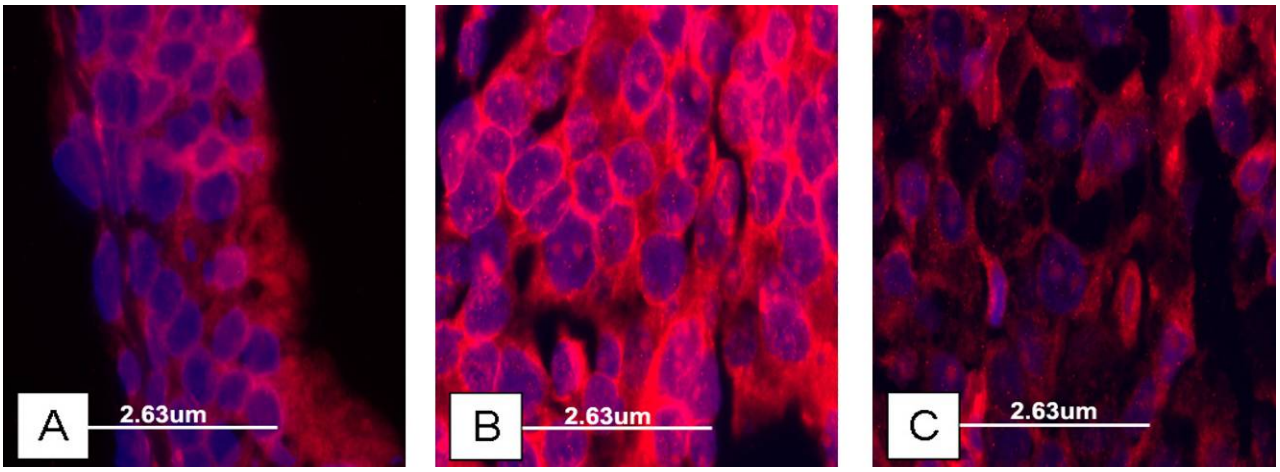


Figure 2: A) A case of chronic schistosomal cystitis, showing mild number of red signals in the nuclei of urothelial cells for Her 2 gene in the urothelium (FISH, Her2 gene, x60). B) A case of schistosomal-associated moderately differentiated squamous cell carcinoma showing moderate number of red signals in the nuclei of urothelial cells for Her 2 gene in about 50% of the malignant squamous cells (FISH, Her 2 gene x60). C) A case of invasive urothelial cell carcinoma, high grade, showing marked red signals in the nuclei of urothelial cells for Her 2 gene the majority of malignant urothelial cells (FISH, Her-2 gene, x60).

4. Discussion:

Bladder cancer is a major health problem in Egypt. The two major types of bladder cancer in Egypt are bilharzial and non-bilharzial bladder cancer. The present study included 135 bladder cancer patients infected with bilharziasis and revealed high VEGF levels compared to non-bilharzial cases. Recently, bilharzial bladder cancer was found to be positively correlated with over- expression of Bcl-2 (apoptotic marker) (Swellam *et al.* 2004a) that enhance the angiogenesis process and induced increase of VEGF protein secretion as reported by Biroccio *et al* (2000). These findings may hypothesized that molecular changes occurring in bladder cancer patients infected with bilharziasis can undergo the phenotypic (angiogenic) switch therefore able to induce phenotypic changes in endothelial cells, leading to angiogenesis (Swellam *et al.* 2004a). Our study reported significantly higher VEGF levels in SCC compared to TCC types. Previously, it was reported that SCC components are more genetically unstable and had alterations not present in TCC cases (Swellam *et al.* 2004b). Accordingly, it is possible to postulate that SCC of the bladder stimulates angiogenesis by directly secreting angiogenic substances or by activating and releasing angiogenic compounds stored within the extracellular matrix (Campbell 1997). Moreover, significantly high levels of VEGF were observed in high grade compared to low grade bladder tumors, suggesting that VEGF production increases as tumors become more anaplastic. Shinoda *et al.* (1999) and Tuttle *et al.* (2002) observed similar findings in other tumors. Our result also revealed significant association between increased VEGF levels and tumor invasion. Association between increased VEGF levels with high grade and tumor invasion in bladder cancer patients indicates that VEGF may play a role in the invasion and metastasis of cancer and may serve as an indicator of tumor progression and future recurrence.

Oncogene amplification is a common mechanism of disease progression in many solid tumors and it may be used as a prognostic marker for aggressiveness of growth and behavior in some of these malignancies (Menard *et al.* 2001).

Studies have shown that Her 2/neu overexpression induces cell transformation and that Her2/neu positive tumors are more aggressive (Eltze *et al.* 2005). With regard to the distribution of Her 2/neu in normal tissues, Her 2/neu is slightly expressed only in the liver, bile duct, gastrointestinal tract, skin, genital organs and urinary tract, with limited expression in most normal tissues (Natali *et al.* 1990, Matsubara *et al.* 2008). The methods to analyze Her 2/neu in tissues include analysis of gene amplification, mRNA overexpression, and protein overexpression; however, possible methods for use on formalin-fixed paraffin sections are IHC and FISH. It has been indicated that examination of gene amplification rather than antigen expression is a more reliable method to identify patients with Her 2/neu positive breast cancer (Mass *et al.* 2005, Matsubara *et al.* 2008).

In the present study, IHC results for Her 2/neu was overexpressed ($p<0.01$) in malignant group compared to control and chronic schistosomal cystitis groups. In malignant group it was 1+ in 33 (30%), 2+ in 45 (40.9%) and 3+ in 32 (29.09%). Her 2/neu incidence was significantly ($p<0.01$) higher in urothelial carcinoma group 80/110 (72.2%) compared to SCC 30/75 (40%). While Her 2/neu overexpression was observed in 12% (Lipponen *et al.* 1991) to 71% of urothelial cancers (Gandour-Edwards *et al.* 2002) it correlated with grade and stage in some studies (Miyamoto *et al.* 2000) but not in others (Ioachim *et al.* 2000). Several hypotheses could explain these wide variations, as well as the relatively low rate of Her 2 overexpression reported here. One of the major issues is the variability in IHC assays, related to the heterogeneity between kits, antibodies, protocols, interpretations or cut-off values, in their study performing this analysis using both IHC staining and FISH, Her 2/neu overexpression was observed in 33.3% (10), 58.2% (45) of SCC and urothelial carcinoma respectively (Lae *et al.* 2010) while Aly and Khaled, (2004) found that 9/21 (43%), and 3/16 (19%) of cases with squamous and transitional cell carcinoma had C-erb-B2 gene amplification, respectively.

In the present study, overexpression of Her2/neu gene was significantly ($p< 0.01$) higher in high 31 (63.6%) than low grade 14(56%) tumor and in high than low grade tumor with ($p< 0.01$, we found that overexpression correlate with grade, and stage, while Aly and Khaled, (2004), found that no significant association was observed between C-erb-B2 amplification and tumor grade. They suggest that relative C-erb-B2 gene amplification is associated with aggressive bladder cancer and may play an important role in tumor progression.

On comparison between IHC and FISH, Sauter *et al.* (1993) reported that Her 2/neu gene amplification was detected in only 7% (10/141) of patients with urothelial cancer (36 pTa, 42 pT1, 67 pT2-T3/20 G1, 39 G2, 46 G3 and 6 with grade and stage unknown), whereas 43% (61/141) of tumors were Her 2/neu positive by IHC. In addition, Kruger *et al.* (2002) studied 203 patients with urothelial cancer and reported that 37% (76/203) of patients were Her 2/neu positive by IHC, whereas Her 2/neu gene amplification was detected in only 5% (2/42) of

patients. Moreover, de Pinieux *et al.* (2004) reported that 23% (15/64) of patients with invasive urothelial cancer were Her 2/neu positive, while Her 2/neu gene amplification was detected in 28% (6/21) of patients.

In the current study, FISH results in SCC Her 2/neu gene amplification, was 10 (33.35%). In Urothelial carcinoma Her 2/neu gene was overexpressed in 45 (58.2%). Lae *et al.* (2010), found Her 2/neu overexpression in 9.2% of tumor samples. In comparison of Her 2/neu expression between IHC and FISH, it was suggested that the dissociation between gene amplification and overexpression could be related to a point mutation that leads to protein overexpression, translocation or transcriptional up regulation (Sauter *et al.* 1993)

Evidence from breast cancer indicates that only tumors with Her 2/neu gene amplification respond to an anti-Her 2/neu targeted therapy, such as trastuzumab. Using the same principle, 78% of muscle-invasive urothelial bladder carcinomas should be suitable for such treatment. The potential involvement of Her /neu in the proliferation of urothelial carcinoma led to the initiation of anti-Her 2/neu targeted therapy protocols in advanced disease. Single-agent data with trastuzumab in urothelial cancer are not available or limited to case reports (Peyromaure *et al.* 2005). The efficacy of molecular targeting therapy for various molecules including EGFR/VEGF/Her 2/neu has been proved clinically in a wide range of cancers (Yoon *et al.* 2004).

In the running study we found that Her 2/neu protein was expressed in 68% of cases of schistosomia associated urothelial carcinoma compared to 43.3% in nonschistosoma associated urothelial carcinoma, also in SCC in 33.3% of the cases. The study examined the prognostic value of C-erb-B2, among other markers, in bilharzial related bladder cancer (Haitel *et al.* 2001), has also suggested that C-erb-B2 expression was associated with poor prognosis.

5. Conclusion:

Her 2/neu overexpression might provide additional prognostic information in patients with muscle-invasive bladder carcinomas; Because 78.9% of our patients harbor Her 2/neu overexpressing those patients may potentially benefit from molecular targeted therapy targeting Her 2/neu for invasive bladder carcinoma and they should be identified by gene amplification analysis using FISH in IHC 3+ patients. In addition, serum VEGF levels are increased in patients with bladder carcinoma and may be a candidate as a new noninvasive diagnostic tool.

Acknowledgment

Special thanks to Prof. Dr. Bruno Voss, Head of Department of Molecular Cell Biology and Mrs. Sabina Boehm for active participation in the practical part of this work which was done in the Professional's Research Institute for Occupational Medicine BGFA, Ruhr-University, Bochum, Germany.

This work is supported by the Internal Project No 72 Sh; of Theodor Bilharz Research Institute; Principal Investigator: Prof. Maha Mahmoud Akl, Head of Clinical Laboratory Division.

Corresponding author

Olfat A. Hammam

Departments of Patholog , Theodor Bilharz Research Institute, El-Nil Street, Giza, Egypt

*totoali1@hotmail.com

5. References:

1. Aly MS and Khaled HM. (2004): Detection of C-erb B2 gene amplification in bilharzial associated bladder cancer using fluorescence in situ hybridization. Urologic Oncology: Seminars and Original Investigations 22 448 – 452.
2. Biroccio A, Candiloro A, Mottolese M, Sapora O, Albini A, Zupi G and Del Bufalo D. (2000): BCL-2 over-expression and hypoxia synergistically act to modulate VEGF expression and in vivo angiogenesis in a breast cancer cell line. FASEP J. 14: 652-660.
3. Bolenz C and Lotan Y (2008): Molecular biomarkers for urothelial carcinoma of the bladder: challenges in clinical use Nature Reviews Urology 5, 676 – 685.
4. Campbell SC (1997): Advances in angiogenesis research: relevance to urological oncology J Urol. 158: 1663-1674.
5. Carroll PR (1995): Urothelial Carcinoma: Cancers of the Bladder Ureter & Renal Pelvis. In: Tanagho EA, McAninch JW, editor. General Urology. 14. Philadelphia: Prentice-Hall International Inc, pp. 353–372.
6. Des Guetz G, Uzzan B, Nicolas P, Cucherat M, Morere JF, Benamouzig R, Breau JL and Perret GY (2006): Microvessel density and VEGF expression are prognostic factors in colorectal cancer. Meta-analysis of the literature. Br J Cancer 94: 1823–1832.
7. de Pinieux G, Colin D, Vincent-Salomon A, Couturier J, Amsellem-Ouazana D, Beuzeboc P and Vieillefond A. (2004): Confrontation of immunohistochemistry and fluorescence in situ hybridization for the assessment of HER-2/neu (c-erbb-2) status in urothelial carcinoma. Virchows Arch 444: 415-419.

8. Eble J N, Sauter G, Epstein J I and Sesterhenn I (2004): World Health Organization Classification of tumors. Pathology and Genetics of tumors of the urinary system and male genital organs. IARC Press: Lyon.
9. El-Harvey MA, Amr MM, Abdel-Rahman AB, El-Ibiary SA, Agina AM, Abdel-HafeZ AM, Waheed AA, Hussien HM, Strickland TG (2000): The epidemiology of Schistosomiasis in Egypt: Gharbia Governorate. American Journal of Tropical Medicine and Hygiene 62:42 – 48
10. Eltze E, Wülfing C, Von Struensee D, Piechota H, Buerger H and Hertle L (2005): Cox2 and Her2 co-expression in invasive bladder cancer. Int J Oncol 26: 1525-1531.
11. El Bolkainy MN, Mokhtar NM, Ghoneim MA and Hussein MH (1981): The impact of schistosomiasis on the pathology of bladder carcinoma. Cancer 48:2643-2648.
12. El-Mawla NG, El-Bolkainy MN and Khalid HM (2001): Bladder cancer in Africa: update. Semin. Oncol 28: 174-178.
13. El-Sebaie M, Zaghloul MS, Howard G and Mokhtar A (2005): Sqamous cell carcinoma of the bilharzial and non-bilharzial urinary bladder: a review of etiological features, natural history, and management. Int J Clin Oncol 10: 20-25.
14. Felix AS, Soliman AS, Khaled H, Zaghloul MS, Banerjee M, El-Baradie M, El-Kalawy M, Abd-Elsayed AA, Ismail K, Hablas A, Seifeldin IA, Ramadan M and Wilson ML (2008): The changing patterns of bladder cancer in Egypt over the past 26 years. Cancer Causes Control 19:421-429.
15. Ferguson AR (1911): Associated bilharziasis and primary malignant diseases of the urinary bladder with observation on a series of forty cases. J Pathol Bacteriol 16:76-94.
16. Gabar SN, Tarek AH, Anwar O, Eglal S, Mahmoud AK, Thomas GS (2000): Epidemiology of schistosomiasis in Egypt: Minyo Governorate. Am J Trop Med Hyg 62: 65-75
17. Gandour-Edwards R, Lara PN Jr, Folkins AK, LaSalle JM, Beckett L, Li Y, Meyers FJ and DeVere-White R. (2002): Does HER2/neu expression provide prognostic information in patients with advanced urothelial carcinoma? Cancer 95: 1009-1015.
18. Gouda I, Mokhtar N, Bilal D, El-Bolkainy T and El-Bolkainy NM (2007): Bilharziasis and bladder cancer: a time trend analysis of 9843 patients. J Egypt Natl Canc Inst 19:158-62.
19. Gravalos C and Jimeno A (2008): Her2 in gastric cancer, a new prognostic factor and a novel therapeutic target. Annals of oncology 10*:* 1093-1100.
20. Haitel A, Posch B, El-Baz M, Mokhtar AA, Susani M, Ghoneim MA and Marberger M (2001): Bilharzial related, organ confined, muscle invasive Bladder cancer: prognostic value of apoptosis markers, Proliferation markers, p53, E-cadherin, epider- mal growth factor receptor and c-erbb-2. J Urol 165:1481
21. Heyns CF and van der Merwe **A** (2008): Bladder cancer in Africa. J Urol 15:3899-3908.
22. Hsu SM and Raine L (1981): Protein A, Avidin and biotin in immunohistochemistry. J Histochem Cytochem 29 :1349-1353.
23. Jemal A, Murray T, Ward E, Samuels A, Tiwari RC, Ghafoor A, Feuer EJ, Thun MJ (2005):Cancer statistics, 2005. CA Cancer J Clin 55(1):10-30.
24. Ioachim E, Charchanti A, Stavropoulos NE, Skopelitou A, Athanassiou ED and Agnantis NJ (2000): Immunohistochemical expression of retinoblastoma gene product (Rb), p53 protein, MDM2, c-erbB-2, HLA-DR and proliferation indices in human urinary bladder carcinoma. Histol Histopathol 15: 721-727.
25. Kaptain S, Tan LK, Chen B (2001): Her2 and breast cancer. Diagon Mol Path 10*:* 139-152.
26. Krüger S, Weitsch G, Büttner H, Matthiensen A, Böhmer T, Marquardt T, Sayk F, Feller AC and Böhle A (2002): Overexpression of c-erbB-2 oncoprotein in muscle-invasive bladder carcinoma: relationship with gene amplification, clinicopathological parameters and prognostic outcome. Int J Oncol 21: 981-987.
27. Lae M, Couturier J, Oudard S, Radvanyi F, Beuzeboc P and Vieillefond V (2010): Assessing HER2 gene amplification as a potential target for therapy in invasive urothelial bladder cancer with a standardized methodology: results in 1005 patients. Annals of Oncology 21: 815–819.
28. Lipponen P, Eskelinen M, Syrjanen S, Tervahauta A and Syrjanen K(1991): Use of immunohistochemically demonstrated c-erbB-2 oncoprotein expression as a prognostic factor in transitional cell carcinoma of the urinary bladder. Eur Urol 20: 238-242.
29. Mass RD, Press MF, Anderson S, Cobleigh MA, Vogel CL, Dybdal N, Leiberman G and Slamon DJ (2005): Evaluation of clinical outcomes according to HER-2 detection by fluorescence in situ hybridization in women with metastatic breast cancer treated with trastuzumab. Clin Breast Cancer 6: 240-246.
30. Matsubara H, Yamada Y, Naruse K, Nakamura K, Aoki S, Taki T, Tobiume M , Zennami K, Katsuda R and Honda N.(2008): Potential for HER-2/neu molecular targeted therapy for invasive bladder carcinoma: Comparative study of

immunohistochemistry and fluorescent in situhybridization. Oncology Reports 19: 57-63, 2008
31. Ménard S, Casalini P, Campiglio M, Pupa S, Agresti R and Tagliabue E. (2001): HER2 overexpression in various tumor types, focusing on its relationship to the devel- opment of invasive breast cancer. Ann Oncol 12 sup 1: S15–9.
32. Miyamoto H, Kubota Y, Noguchi S, Takase K, Matsuzaki J, Moriyama M, Takebayashi S, Kitamura H and Hosaka M (2000): C-ERBB-2 gene amplification as a prognostic marker in human bladder cancer. Urology 55: 679-683.
33. Mohammed RA, Green A, El Shikh S, Paish EC, Ellis IO and Martin SG (2007): Prognostic significance of vascular endothelial cell growth factors-A, -C and -D in breast cancer and their relationship with angio- and lymphangiogenesis. Br J Cancer 96: 1092–1100.
34. Mostafa MH, Sheweita SA, O'Connor PJ (1999): Relationship between schistosomiasis and bladder cancer. Clin Microbiol Rev 12: 97-111.
35. Mostofi FK, Davis CJ, Sesterham IA (1988): Pathology of tumors of he urinary tract. In: Diagnosis and management of genitourinary cancers. Skinner DG, Leveskovesky G, 83-117. WB Sandus, Phyladelphia.
36. Natali PG, Nicotra MR, Bigotti A, Venturo I, Slamon DJ, Fendly BM and Ullrich A (1990): Expression of the p185 encoded by HER2 oncogene in normal and transformed human tissues. Int J Cancer 45: 457-461.
37. Nicholson RI, Gee JM, Harper ME (2001): EGFR and cancer prognosis. Eur J Cancer 37(Suppl 4): S9–S15.
38. Ooi A, Takehana T, Li X, Suzuki S, Kunitomo K, Iino H, Fujii H, Takeda Y and Dobashi Y(2004): Protein overexpression and gene amplification of HER-2 and EGFR in colorectal cancers: an immunohistochemical and fluorescent in situ hybridization study. Mod Pathol 17(8):895-904.
39. Peyromaure M, Scotté F, Amsellem-Ouazana D, Vieillefond A, Oudard S and Beuzeboc P. (2005): Trastuzumab (Herceptin) in metastatic transitional cell carcinoma of the urinary tract: report on six patients. Eur Urol 48: 771–775.
40. Sandberg AA and Berger CS (1994): Review of chromosome studies in urological tumors. II. Cytogenetics and molecular genetics of bladder cancer. J Urol 151:545–560.
41. Sauter G, Moch H, Moore D, Carroll P, Kerschmann R, Chew K, Mihatsch MJ, Gudat F and Waldman F. (1993): Heterogeneity of erbB-2 gene amplification in bladder cancer. Cancer Res 53: 2199-2203.
42. Schuell B, Gruenberger T, Scheithauer W, Zielinski Ch and Wrba F. (2006): Her2 protein expression in colorectal cancer. BMC Cancer 8:123.
43. Shinoda K, Ishida S, Kawashima S, Wakabayashi T, Malsuzaki T, Takayama M, Shinmura K and Yamada MM (1999): Comparision of the level of hepatocyte growth factor and VEGF in aqueous fluid and serum with grades of retinopathy in patients with diabetes mellitus. Br. J. Ophthalmol 83:834-837.
44. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A and McGuire WL (1978): Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 235:177–182.
45. Swellam M; N, Abd-Elmaksoud; M.H. Halim; H.Katab and H khiry (2004a): Incidence of BCL-2 expression in bladder cancer: relation to schistosomiasis. Clinical Biochem 37: 798-802.
46. Swellam M, Abd El-Aal AA, AbuGabel KM (2004b): Deletions of P15 and P16 in schistosomal bladder cancer correlated with transforming growth factor-α expression. Clinical Biochem 37, 1098-1104.
47. Tabernero J (2007): The role of VEGF and EGFR inhibition: implications for combining anti-VEGF and anti-EGFR agents. Mol Cancer Res 5: 203–220.
48. Tuttle R M, Fleisher M, Francis G L and Rabbins R J (2002): Serum vascular growth factor levels are elevated in metastatic differentiated thyroid cancer but not increased by short-term TSH stimulation. J. Clin. Endocrinology and Metabolism. 78:1737-1742.
49. Wülfing C, Von Struensee D, Bierer S, Bögemann M, Hertle L and Eltze E (2005): Expression of Her2/neu in locally advanced bladder cancer: implication for a molecular targeted therapy. Aktuelle Urol 36: 423-923.
50. Yarden Y, Sliwkowski MX (2001): Untangling the ErbB signalling network. Nat Rev Mol Cell Biol 2: 127–137.
51. Yoon JH, Gwak GY, Lee HS, Bronk SF, Werneburg NW, Gores GJ (2004): Enhanced epidermal growth factor receptor activation in human cholangiocarcinoma cells. J Hepatol 41: 808–814.

11/2/2010

Ultrasonic Comparative Assessment for Biodiesel Production from Rapeseed

N.N. Ibiari*, S.A. Abo El-Enin, N.K. Attia and G. El-Diwani

Chem. Eng. and Pilot Plant Dept., National Research Centre, Egypt

Abstract: The application of ultrasound during extraction and trans-esterification of oil from rapeseed was evaluated. Two methods of extraction were used, batch wise extraction and soxhlet extraction. In batch wise extraction procedure, ground rapeseeds were added to solvent and ultra-sonicated either by cleaning bath or ultrasonic generator. Conventional soxhlet extraction assisted in the soxhlet chamber by ultrasound has been developed. Ultrasonic technique reduced time required to extract oil. Using batch wise extraction procedure, percent recovery of oil increased almost 17.83% and 20.99% by using cleaning bath and ultrasonic generator respectively rather than control after 2hrs.While in using soxhlet extraction percent recovery reached 85% after 1.5 hr in case of ultrasonic and after 4 hrs without using ultrasonic. Physical and chemical properties of rapeseed oil were tested. Then the alkaline trans-esterification of rapeseed oil with methanol and potassium hydroxide for production of biodiesel was studied, using ultra-sonication and magnetic stirring. In trans-esterification the use of ultra-sonication and magnetic stirring led to similar high yields of 90% of methyl esters after approximately 10 min. of reaction time. Comparison between biodiesel obtained and standard biodiesel and diesel fuel was done.
[N.N. Ibiari, S.A. Abo El-Enin, N.K. Attia and G. El-Diwani. **Ultrasonic Comparative Assessment for Biodiesel Production from Rapeseed**. Journal of American Science 2010;6(12):937-943]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Ultrasound, extraction, biodiesel, rapeseed oil, transesterification.

1. Introduction:

Diminishing fossil fuel resources coupled with the steady increase in energy consumption have spurred research interest in alternative and renewable energy resources. Bio-diesel is a promising nontoxic and biodegradable renewable fuel comprised of mono-alkyl esters of long chain fatty acids, which are derived from vegetable oils (edible and non-edible)(Hanna 1999) . Seeds of high oil content such as sunflower, soybean and canola as edible oils, and jatropha and rapeseed as non-edible oils used as feed stock for bio-diesel production.

Extraction is one of the key processing steps in recovering oils contained in seeds. Mechanical pressing (Abu- Arabi 2000) is the simplest method of extraction, however, needs no extraction medium. It has been traditionally applied to the extraction of oils from oil seeds; the only equipment needed is a hydraulic press.

Traditional extraction processes may be classified as follows: extraction with organic solvents: percolation, maceration, and extraction using a Soxhlet apparatus; and extraction with water: infusion, decoction, and steam distillation (Silva 1998). An old method also worth mentioning is extraction with cold fat, called effleurage, used mainly for extraction of fragrances from flowers (Starmans 1996) These techniques are often time consuming and require large volume of organic solvent.

In last decade, alternative extraction techniques (non conventional extraction methods) that introduce some form of additional energy to process in order to facilitate the transfer of analysts from sample to solvent have been considered. These methods include ultrasound assisted extraction, pressurized liquid extraction, microwave extraction and supercritical fluid extraction {Huie 2002, Flores 2006, Garcia 2004, Vinatoru 2001} ,as well as vertical (turbo) extraction. The main advantage of these non-conventional methods compared to conventional methods is the increased extraction efficiency, which leads to increased yield and/or shorter extraction time.

The ideal approach would be one retaining the advantages of soxhlet extraction (namely, sample fresh solvent contact during the whole extraction step, no filtration step, simple manipulation) while circumventing its short comings by accelerating the process and minimizing environmental pollution.
With this aim, an ultrasound-assisted soxhlet extractor has been designed and constructed. The device is based on the same principles as the conventional soxhlet extractor but modified in order to allow location of the soxhlet chamber in a bath through which ultrasounds are applied by means of an ultrasonic probe. The same approach was done with the wise batch extraction.

The interest in the use of renewable fuel started with the direct use of vegetable oils as a substitute for diesel (Demirbas 2003). However, their

direct use in compression ignition engines was restricted due to high viscosity, low volatility and polyunsaturated character (Ramadhas 2005). To overcome these constrains there are at least four ways in which oils and fats can be converted into bio-diesel (Ghadge 2006), transesterification, blending, micro emulsion (Fukuda 2001)and pyrolysis. Out of these methods, trans-esterification is the most viable process. The trans-esterification process is achieved by reaction of triglyceride molecule with an excess of alcohol in the presence of a catalyst to produce glycerin and a fatty acid methyl esters "FAME" which is the bio-diesel.

Stochiometrically, three moles of alcohol are required for each mole of triglyceride, but in general, a higher molar ratio is often employed for maximum ester production depending upon the type of feed stock, amount of catalyst, temperature etc. However, the yield of bio-diesel is independent of the type of the alcohol used (methanol, ethanol, propanol and butanol) (Sharma 2008) and the selection of one of these depends on cost and performance. Methanol is preferred over others due to its low cost (Ramadhas 2005) and its physical and chemical advantages such as polar and shorter-chain alcohol (Hanna 1999). The conventional catalysts used are acid and alkali catalysts depending on the nature of the oil used (free fatty acids FFA content in the raw oil) (Fukuda 2001, Freedman 1986). FFA should not exceed a certain amount for trans-esterification to occur by an alkali catalyst (Canakci 1999, 2001).In case of high free fatty acid, acid esterification using H_2SO_4 as a catalyst was done to reduce the FFA prior to alkaline transesterification [Ramadhas 2005, Veljkovic 2006, Sahoo 2007, Shanna 2007) . The common catalyst employed during alkaline transesterification includes homogeneous catalyst such as sodium hydroxide, potassium hydroxide and sodium methoxide (CH_3ONa) (Leung 2006). Sodium methoxide proved to be more effective than NaOH, which produces a small amount of water by mixing NaOH and methanol. However, NaOH and KOH are widely used in the industrial biodiesel production process owing to its low cost (Jeong 2004). The amount of alkali catalyst was calculated on the basis of the amount needed to neutralize the unreacted acids plus 0.35% for virgin oil which came out to be 0.55% w/v KOH (Tiwari 2007).

Presence of sufficient amount of methanol during the transesterification reaction is essential to break the glycerin-fatty acid linkages (Widyan 2002). But excess of methanol should be avoided. Increasing the molar ratio of methanol/oil beyond 6:1 neither increases the product yield nor the ester content, but rather makes the ester recovery process complicated and raised its cost.

Lifka and Ondruscka (Lifka 2004) said that since basic transesterification showed no significant temperature dependence, energy required for thermo starting the reaction mixture can be saved. From the energy balance of the three mixing methods (magnetic stirrer, ultrasound and ultra turrax) employed, it was determined that energy costs are lower for ultrasonic mixing.

The main objective of the present work is to compare classical extraction techniques of oil (Soxhlet, batch wise extraction) with ultrasound-assisted extraction method, and to apply the biodiesel production technology using an alkaline catalyst to the transesterification of rapeseed oil and particularly to study the effect of ultrasonication versus mechanical stirring on methyl esters yield and reaction time.

2. Materials and methods

2.1 Materials:

- Rape Seeds used in this work were supplied by Cultivation and Production of Medicinal and Aromatic Plant Dept. at National Research Centre of Egypt.
- Solvent used for oil extraction was commercial hexane, obtained from Alexandria Petroleum Company of Egypt.
- Methanol of 99.8% analytical reagent.
- Potassium hydroxide pellets of 98% purity as catalyst.

2.2 Rape Seed Preparation:

The rape seeds were milled in an electric mill, sieved and classified into three different particle size groups ≤234, ≤600 and ≤850 µm. Moisture content of rape seeds was determined.

2. 3 Analytical Methods:

Moisture and oil content in seeds were determined gravimetrically. The fatty acid composition of rapeseed oil was determined using gas chromatographic analysis of the oil ethyl esters. Modification of the oil to its ethyl esters was made using 2% H_2SO_4 as catalyst in the presence of dry ethyl alcohol in excess. The chromatographic analysis was made using Hewlett Packard Model 6890 Chromatograph. A capillary column 30 m length and 530 µm inner diameters packed with Apiezon® was used. Detector temperature was 280°C, injection temperature was 300°C and the column temperature was programmed from 100 to 240°C at 15°C/min.

Viscosity was measured using Brookfield Viscometer Model DV-II+. The acid values are milligrams of KOH necessary to neutralize the free acidity in 1g of oil sample.

Acid Value = {(Titration (ml) *5.64) /wt of sample used} (mg KOH/g)

2.4 Extraction Procedure

Extraction was done by two different methods as shown in Fig.1 .

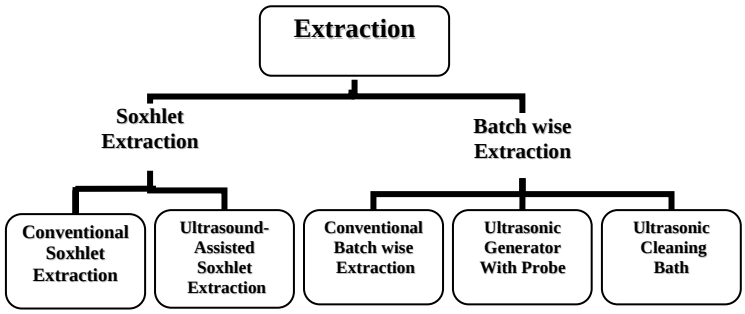


Fig (1): Extraction Methods

2.4.1 Soxhlet Extraction:

As shown in Fig. 2, the apparatus of conventional soxhlet extraction was modified by adding a water bath with an orifice at the bottom in order to enable connection with a distillation flask. A titanium alloy ultrasonic probe (2.75 mm diameter) was immersed in the water bath and used to accelerate the extraction process.

2.4.2 Batch –Wise extraction process:

2.4.2.1Conventional Extraction

Ground rape seeds are mixed in an glass reactor with hexane. Suspensions were continuously stirred at constant temperature and stirring rate (using a magnetic stirrer) for different time intervals. Suspension is filtered and extracted oil was separated from the solvent using rotary vacuum evaporator.

2.4.2.2 Ultrasonic Generator with Probe Assisted Extraction

Same steps as in conventional method except that the suspension was ultra-sonicated during extraction time using ultrasonic generator probe which was submerged in the suspension. Temperature was kept constant during sonication and extraction.

2.4.2.3 Ultrasonic Cleaning Bath Assisted Extraction

Ground rape seeds and hexane as extracting solvent were mixed in a closed flask. The sonication was performed at constant temperature for different time intervals using an ultrasonic cleaning bath.

A rotary – evaporator was used to release the solvent after each extraction.

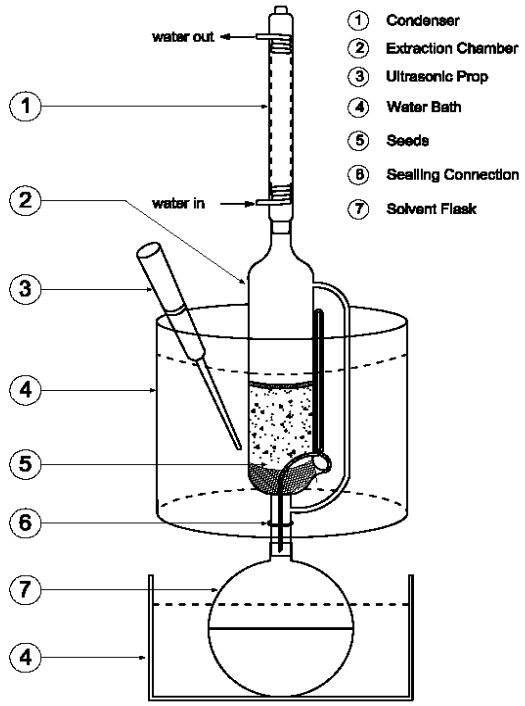


Fig (2): Soxhlet extraction apparatus

2.5 Transesterification Procedure:

Rapeseed oil produced, methanol corresponding to 6:1 molar ratio of alcohol to oil and KOH (1% w/w) were refluxed together in a glass reactor placed in water bath. Heating was achieved by means of hot plate with temperature controller. The

temperature was raised to 65°C and the mixture was stirred either using a magnetic stirrer or an ultrasonicator (ultrasonic generator with probe or ultrasonic cleaning bath).

After different reaction time intervals, the reaction was stopped and the mixture was allowed to stand for phase separation in a separating funnel. The esters mixture formed the upper layer and glycerin formed the lower layer (Stavarache 2005)

3. Results ands Discussion:

3.1. Effect of moisture content on the percentage yield of extracted oil

No significant effect in the percent oil extracted from dried seed (38.16%) and non dried seed (38.68%) using soxhlet extraction. This may be attributed to the moisture content range from 5.86 to 6%, which is less than the moisture level between 8 and 20% for optimum yield when harvesting (Loren 2007)

3.2. Effect of particle size on the percentage yield of extracted oil

The effect of seeds' particle size on the oil yield was studied by soxhlet extraction of three classes of ground rape seeds using hexane.

As can be seen from Table (1) the highest oil yield was obtained from the higher class of the ground rape seeds. Thus in the further research, the higher class of ground rape seeds were used.

Table (1) Effect of Particle Size on Extracted Oil Yield

Class	*Particle Size, mm*	*%Yield of oil*
Largest	**0.6-0.85**	**36.52**
Medium	**0.6**	**31.3**
Smallest	**0.234**	**26**

3.3. Effect of Time on the percentage yield oil extracted:

3.3.1. Using Soxhlet Extraction

The percent oil recovery by conventional soxhlet extraction and ultra-sonic assisted soxhlet extraction (UASE) with hexane using different extraction time are compared in Fig. (3). It is clear that using UASE reduces the time of extraction from about 4hr to 1.5 hr, but the percent recovery is slightly lower and this is due to the high frequency (800 KHz) ultrasound employed. When the high frequency ultrasound is employed the extraction yield did not increase significantly, however the degradation of the seed constituents was diminished. In case of low frequency sonication degradation becomes more important (Vinatoru 2001).

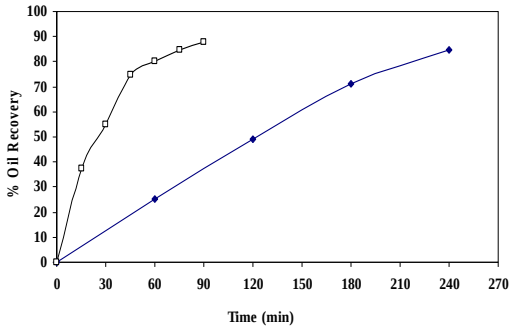
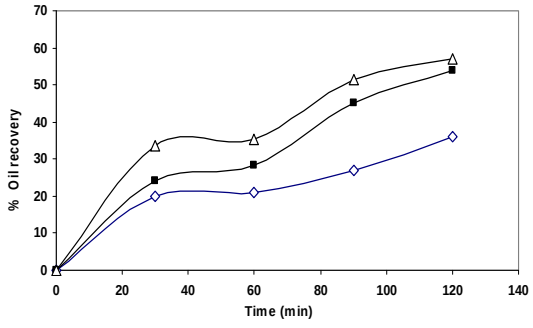


Fig (3):Effect of time on percentage recovery using soxhlet and UASE

3.3.2. Using Batch Wise Extraction Process

The oil extraction capabilities of three different methods (control, ultrasound cleaning bath and ultrasound probe) using hexane (seed: solvent (1:10 ratio)) at different extraction time are shown in Fig (4). The percentage of oil recovery is increased with application of ultra-sound. But the relative increase in the percent of oil recovery of the two methods of ultrasound differed. For sonic bath, the percent recovery increased by 4.35% at an extraction time of 30 min and 17.83% at an extraction time of 2 hr. While in case of using probe, the percent recovery increased by 13.47% at an extraction time of 30 min and 20.99 at an extraction time of 2 hr.



Fig(4):Effect of time on percent recovery using batch wise extraction process

It is interesting that the higher yield of extracted oil achieved using soxhlet extraction technique either with or without ultrasonic than using batch wise extraction process. This can be attributed not only to a higher operating temperature and much

longer extraction time, but also to a higher seed – solvent ratio applied in the Soxhlet extraction.

3.4 Effect of type of extraction on the properties of rapeseed oil

In order to evaluate if the oil composition was affected during ultrasound assisted extraction, gas liquid chromatography analyses were carried out on two different extracted samples using UASE and soxhlet. Table (2) shows that no appreciable differences in the extracts composition. This indicates that the oil composition is not affected by the use of ultrasound, and these results agree with Luque-Garcia J.L. et al (Luque 2004).

Table (2) Fatty acid composition of rapeseed oil

Constituent	% composition using UASE	% composition using soxhlet extraction
Palmitic (16:0)	4.34	4.39
Stearic (18:0)	1.55	1.67
Oleic (18:1)	60.0	60.95
Linoleic (18:2)	20.0	19.04
Linolinec (18:3)	12.5	11.78
Eicosic (20:1)	27.0	27.1
Erucic (22:1)	0.9	0.9

Average molecular weight =882.6 k.mol/kg

Characterizations of rapeseed oil are shown in Table (3).

Table (3) Characterization of rapeseed oil

Viscosity, (c.ps) at 40 °C	46.5
Free fatty acid, %	1.5
Acid value, (mg NaOH/1gm oil)	0.3

3.5 Transesterification:

A set of experiments was carried out to determine the effect of magnetic stirring and ultrasonication on the transesterification reaction. After 10 min, conversions of more than 90% were obtained. So no significant influence due to the mixing method was observed. Percent conversion to ester values is higher than those of Lifka and Ondruscka [26] who reported more than 85% conversion of rapeseed oil to methyl esters after 10 min. at 45°C, on the alkaline transesterification of rapeseed oil using NaOH at a concentration of 1 % w/w, using different mixing methods (magnetic).

Table (4): Comparison of different standard of biodiesel in worldwide with produced biodiesel from rapeseed and conventional diesel.

Property	Units	Biodiesel (Rapeseed)	US National Biodiesel	German Biodiesel	Conventional Diesel
Flash point	° C	163	100	110	54
Pour point	° C	-6			
Cloud point	° C	-3			
Kinematics viscosity at 40° C	mm^2/s	6.08	1.9-6	3.5-5	2.3
Density at 15° C	Kg/m^3	884	860	875-900	800
Carbon Residue	% (w/w)	0.258		0.3	0.3

Table (4) represents the characterization of biodiesel obtained from rapeseed and standards of biodiesel and diesel fuel in worldwide.

Flash point is the lowest temperature at which the vapor realized by a liquid, can form an ignitable mixture with air. Flash point of a fuel is a security parameter, with great importance for its transport and storage. The flash point of biodiesel is one of its main advantages (163 ° C) in our case when compared to diesel fuel (54° C).

Cloud point data consistently over predict the cold temperature limit at which start-up or performance problems may be expected to occur. The temperature at which crystal agglomeration is extensive enough to prevent free pouring of fluid is determined by measurement of its pour point

(Hochauser 1994).Feed stocks with relatively low concentrations of saturated long-chain fatty acids generally yield biodiesel with much lower cloud point and pour point. Thus, feed stocks such as linseed, olive, rapeseed, and safflower oils tend to yield biodiesel with CP ≤ 0°C; our results agree with Kalligeros (2003) and Peterson (1987).

4. Conclusion:

In this study the application of ultrasound during extraction and transesterification of oil from rapeseed was evaluated. Extraction was done by two methods, batch wise extraction (conventional and ultrasonicated) and Soxhlet extraction (conventional and ultrasound). Ultrasonic technique reduced time required to extract oil. The oil composition is not affected by the use of ultrasound. The alkaline transesterification of rapeseed oil with methanol and potassium hydroxide for production of biodiesel was studied, using ultrasonication and magnetic stirring. It is concluded that a conversion of 90% was obtained for both ultrasonicated and magnetic stirred reactions after 10 min. The characterization of biodiesel obtained is in agreement with the standard biodiesel and diesel fuel worldwide.

Corresponding author
N.N. Ibiari
Chem. Eng. and Pilot Plant Dept., National Research Centre, Egypt

5. References:

1. F. Ma, M.A. Hanna, Biodiesel production; a review. Bioresour Technol. 70 (1999) 1-15. http://www.scielo.org.ar/scielo.php?pid=S032707932006000400012&script=sci_arttext DOI: 10.1016/S0960-8524(99)00025-5
2. M.K. Abu-Arabi, M.A. Allawzi, H.S. Al-Zoubi, A. Tamimi, Extraction of jojoba oil by pressing and leaching, Chem. Eng. J. 76 (2000)61-65 http://linkinghub.elsevier.com/retrieve/pii/S1385894799001199 doi:10.1016/S1385-8947(99)00119-9
3. G.L. Silva, I.S. LEE, A.D. Kinghorn, Special problems with the extraction of plants. Pp. 343-363. In Cannell, R.J.P. (ed.): Methods in Biotechnology 4. Natural Products Isolation. Humana Press, Totowa, New Jersey, USA. 1998.
4. D.A.J. Starmans, H.H. Nijhuis, Extraction of secondary metabolites from plant material: a review, Trends Food Sci. Technol. 7 (1996) 191-197.
5. C.W. Huie, A review of modern sample preparation techniques for the extraction and analysis of medicinal plants, Anal. Bioanal. Chem.373(2002)23-30. www.springerlink.com/index/6EG8J7Y2L0HWJXQB.pdf Doi:10.1007/s00216-002-1265-3
6. P. Valéria Flores, S. Jenifer, M. S. Maria Ines, A. C. Fernanda, De A. J. Rosangela, M. M. Migdalia, C. O. Eniz, C. B. Elina, Comparison of soxhlet, ultrasound-assisted and pressurized liquid extraction of terpenes, fatty acid and vitamin E from piper gaudic haudianum kunth. Journal of Chromatography A. 1105 (2006) 115-118.http://linkinghub.elsevier.com/retrieve/pii/S0021967305016122 doi:10.1016/j.chroma.2005.07.113
7. J.L. Luque-Garcia, M.D. Luque De Castro, Ultrasound – assisted soxhlet extraction an expeditive approach for solid sample treatment. application to the extraction of total fat from oleaginous seeds. Journal of Chromatography A, 1034 (2004) 237-242. http://linkinghub.elsevier.com/retrieve/pii/S0021967304001992 doi:10.1016/j.chroma.2004.02.020
8. M. Vinatoru. An over view of the ultrasonically assisted extraction of bioactive principles from herbs, Ultrasonics Sonochemistry. 8 (2001) 303-313. http://linkinghub.elsevier.com/retrieve/pii/S1350417701000712 doi:10.1016/S1350-4177(01)00071-2
9. A. Demirbas, Biodiesel fuels from vegetable oils via catalytic and non-catalytic supercritical alcohol transesterifications and other methods: a survey, Energy Conversion and Management. 44 (2003)2093-2109. http://linkinghub.elsevier.com/retrieve/pii/S0196890402002340 doi:10.1016/S0196-8904(02)00234-0
10. A. S. Ramadhas, S. Jayaraj, C. Muraleedharan, Biodiesel Production from high FFA Rubber seed Oil, Fuel. 84 (2005) 335-40. http://linkinghub.elsevier.com/retrieve/pii/S0016236104002844 doi:10.1016/j.fuel.2004.09.016
11. SV. Ghadge, H. Raheman, Process optimization for biodiesel production from mahua (Madhuca Indica) oil using response surface methodology. Bioresour Technol. 97(2006)379-384. http://linkinghub.elsevier.com/retrieve/pii/S0960852405001744 doi:10.1016/j.biortech.2005.03.014
12. H. Fukuda, A. Kondo, H. Noda, Biodiesel fuel production by transesterification of oils. J Biosci Bioeng. 92 (2001) 405-16. http://linkinghub.elsevier.com/retrieve/pii/S1389172301802887 doi:10.1016/S1389-1723(01)80288-7
13. Y. C. Sharma, B. Singh, S.N. Upadhyay, Advancements in development and characterization of biodiesel: A review, Fuel. 87

(2008) 2355-73. http://linkinghub.elsevier.com/retrieve/pii/S00162 3610800029X doi:10.1016/j.fuel.2008.01.014
14. B. Freedman, RO. Butterfield, EH. Pryde. J. Am Oil Chem. Soc, 61 (1986) 1375-80.
15. M. Canakci, J. V. Gerpan, Biodiesel production via acid catalysis trans. Am. Soc. Agric. Eng. 42 (1999) 1203-10.
16. M. Canakci, J.V. Gerpan, Biodiesel production from oils and fats with high free fatty acid. Am. Soc. Agric. Eng. 44 (2001) 1429–36.
17. V.B. Veljkovic, S.H. Lakicevic,O.S. Stamenkovic, Z.B. Todorovic, M.L. Lazić, Biodiesel production from tobacco oil with a high content of free fatty acids, Fuel. 85 (2006) 2671-75. http://linkinghub.elsevier.com/retrieve/pii/S00162 3610600144X doi:10.1016/j.fuel.2006.04.015
18. P.K. Sahoo, L.M. Das, M.K.G. Babu, S.N. Naik, Biosiesel development from high acid value polanga seed oil and performance evaluation in a CI engine. Fuel 2007; 86, 448-54. http://linkinghub.elsevier.com/retrieve/pii/S00162 36106003024 doi:10.1016/j.fuel.2006.07.025
19. U.C. Shanna, B. Singh, Development of biodesel from karanja, a tree found in rural India, Fuel. 87 (2007) 1740-42. http://linkinghub.elsevier.com/retrieve/pii/S00162 36107003663 doi:10.1016/j.fuel.2007.08.001
20. DYC Leung, Y. Gvo, Transesterification of neat and used frying oils, Optimization for biodiesel production. Fuel Process Technol. 87 (2006) 883-90.
21. G.-T. Jeong, D.-H. Park, C.-H. Kang, W.-T. Lee, C.-S. Sunwoo, C.-H. Yoon et al, Production of biodiesel Fuel by transesterification of rape seed Oil. Applied Biochemistry and Biotechnology. 114 (2004)747-458. http://www.springerlink.com/index/DJ3J7216111 215Q7.pdf doi:10.1385/ABAB:114:1-3:747
22. Tiwari AK. Kuman A. Raheman H. Biodiesel production from Jatropha oil (Jatropha curcas) with high free fatty acids: an optimization process. Biomass Bioenergy 2007; 31:569-75.
23. Al-Widyan MI. Al-Shyoukh AO. Experimental evaluation of the transesterification of waste palm oil into biodiesel. Bioresour Technol 2002;85:253-6.
24. C. Stavarache, M. Vinatoru, R. Nishimura, Y. Maeda, Fatty acids methyl esters from vegetable oils by means of ultrasonic energy, Ultrasonic Sonochemistry 2005, 12: 367-372. http://linkinghub.elsevier.com/retrieve/pii/S13504 1770400080X doi:10.1016/j.ultsonch.2004.04.001
25. K. Loren, Manufacture of cooking oils and margarine #2, 12/27/2007 http://www.karlloren.com/Diabetes/p43.htm
26. J. Lifka, B. Ondruschka, Influence of mass transfer on the production of bio- diesel, Chemical Engineering and Technology. 27 (2004) 1156-1159.
27. Hochhauser, A.M., Gasoline and Other Motor Fuels, in Kirk-Othmer, Encyclopedia of Chemical Technology, 4th edn., edited by J.I. Kroschwitz and M. Howe-Grant, John Wiley & Sons, New York, Vol. 12, 1994, pp. 341–388.
28. Kalligeros, S., F. Zannikos, S. Stournas, E. Lois, G. Anastopoulos, Ch. Teas, and F. Sakellaropoulos, An Investigation of Using Biodiesel/Marine Diesel Blends on the Performance of a Stationary Diesel Engine, Biomass Bioenergy *24:* 141–149 (2003).
29. Peterson, C.L., R.A. Korus, P.G. Mora, and J.P. Madsen, Fumigation with Propane and Transesterification Effects on Injector Cooking with Vegetable Oils, Trans. ASAE *30:*28–35 (1987).

10/30/2010

Biochemical Changes in Glutathione Redox System and Glucose Regulation in Late Pregnant Ossimi Ewes

Ali Hafez El-Far[*1], Mohamed K. Mahfouz[2] and Hussein A. Abdel maksoud[2]

[1] Department of Biochemistry, Faculty of Veterinary Medicine, Alexandria University, Damanhour Branch (Al-Bostan), Egypt.
[2]Department of Biochemistry, Faculty of Veterinary medicine, Moshtohor, Banha University, Egypt.
[*]aboufares90@yahoo

Abstract: Pregnancy is the more prevalent stress in under feeding small ruminant with multiple bearing. Fifty Ossimi ewes of two years old and their body weight ranging between 35 and 50 kg were allotted into three groups; Group I: contains ten non pregnant non lactating ewes were used as control group. Group II: contains twenty single pregnant ewes* and Group III: contains twenty twin pregnant ewes used as experimental animals. Our study focused on the comparison between single and twin bearing ossimi ewes in the last four weeks of pregnancy and the day of parturition by measurement of reduced glutathione (GSH) level and the activities glutathione peroxidase (GSH-Px); glutathione reductase (GR-ase); glutathione-S-transferase (GST) and total superoxide dismutase (t-SOD) in erythrocytic haemolysate. In addition, glucose, non esterfied fatty acid (NEFA), Beta hydroxyl butyric acid (BHBA), cortisol, insulin and protein electrophoric patterns were measured in serum. Our results concluded that, In erythrocytic haemolysate the mean values of GSH-Px and GST in group II and III during the period of 2nd and last week before parturition and at the day of parturition were high significantly increased. While, GSH and t-SOD were high significantly decreased ($P<0.01$) and GR-ase activities were significantly decreased. While serum insulin level decreased while serum NEFA, BHBA and cortisol were increased in single and twin but in twin the values is more significant. The data showed that twin bearing ewes are more susceptible to pregnancy toxemia than single bearing that may be influence the productivity and performance of those animals.
[Ali Hafez El-Far, Mohamed K. Mahfouz and Hussein A. Abdel maksoud. **Biochemical Changes in Glutathione Redox System and Glucose Regulation in Late Pregnant Ossimi Ewes**. Journal of American Science 2010;6(12):953-959]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: pregnancy, glutathione, single bearing, twin bearing, ewes

1. Introduction:

Pregnancy, parturition, and lactation represent a physiological load to the female body. Where pregnancy toxemia (gestational ketosis) caused by negative energy balance in late gestation is commonly observed in ewes and does (Kulcsar et al, 2006), in beef cows (Rook, 2000), and also in monogastric species as rabbits, guinea pigs, dogs and in ferrets (Lewington, 2007). The background of the disease is the result of the fetal carbohydrate- or energy-demand exceeding maternal supply during the last trimester of pregnancy.

In ruminants, dietary carbohydrates provide well over one half of the energy needs for maintenance, growth and production. Glucose is a primary energy source for certain animal tissues and a precursor for lactose synthesis in the mammary gland. Consequently, understanding carbohydrate digestion and absorption, dietary glucose availability, and the involvement of gluconeogenesis in the regulation of glucose homeostasis is essential for the manipulation of the production and quality of agricultural foods (Rafael and Donald, 2007). Lipid digestion in ruminants is unique in that after ingestion feed lipids are placed into a hydrolytic and reductive environment. The result is that glycerol from triacylglycerols and phospholipids are fermented to VFA and those unsaturated fatty acids which are hydrogenated to mostly saturated fatty acids before absorption (Van Saun, 2000). In ewes, number of fetuses plays role in keeping the homeostasis. The last trimester of pregnancy is very demanding for that homeostasis, because fetuses gain over half of their weight in this period (Seidal et al., 2006). Pregnancy toxemia is a metabolic disease that commonly affects pregnant ewes with multiple fetuses and does during late gestation. It is characterized by hypoglycemia, increased concentrations of ketone bodies in the blood and elevated plasma concentrations of free fatty acids is the result of energy demand exceeding maternal supply during the last trimester of pregnancy (Kulcsar et al., 2006).

The endocrine system especially the pancreas probably is involved in the development of ruminant ketosis. Insulin inhibits ketogenesis when free fatty acids levels are high, as well as growth hormone secretions inhibited by cortisol and free fatty acids. Insulin also appears to be important in regulating the

utilization of ketone bodies as the uptake of *β*-hydroxybutyrate and acetate (Abd-Elghany et al., 2010).

Cortisol is a regulator of glucose in ruminants, which acts to increase gluconeogenesis from amino acids. In starving ruminants the gluconeogenesis is maintained by elevated levels of glucocorticoids (Azab and Abdel-Maksoud, 1999). In lactating ruminants the rate of hepatic gluconeogenesis and the relative concentrations of glycogenic precursors regulate the level of milk production (Huntington, 1990).

Ketone bodies serve as an alternative fuel for many tissues, but they probably do not or only to a minor extent contribute to the energy supply of the fetus (Battaglia and Meschia, 1988). Glucose remains the most important metabolite for fetal and placental growth. The ability of the ewe to provide a sufficient amount of glucose to the fetus from dietary sources is limited because about 70 to 75% of the dietary carbohydrate is converted in the rumen into nonglucogenic products. The remaining fraction of digestible carbohydrate provides 40 to 60% of the circulating glucose through propionate. During periods of a negative energy balance and increased demand for glucose, up to 23% of the glucose may be synthesized from liberated glycerol from the adipose tissue. Along with this glucogenic precursor, a larger amount of fatty acids is released into the circulation that may give rise to an increased rate of ketone body formation (Schlumbohm and Harmeyer, 2004). Our study aimed to investigate carbohydrate and fat metabolic changes in single and twin bearing ossimi sheep.

2. Materials and methods

A. Experimental design

The present study was carried out in field farm of Veterinary medicine, Moshtohor, Banha University. Fifty apparently healthy, multiparous Ossimi sheep, of two years old and their body weight ranging between 35 and 50 kg. All animals were kept at the same environmental and nutritional conditions. All over the experimental period, the ewes were allotted into three groups as following:

Group I: included ten ewes (non pregnant non lactating) were used as control group.

Group II: included twenty single pregnant ewes used as experimental animals.

Group III: included twenty twin pregnant ewes used as experimental animals.

Animals were fed free in feedlot. Concentrate feed mixtures were adjusted to the changing of body weight every two weeks. Concentrate mixtures were given twice daily at 10 a.m. and 2 p.m. while wheat straw was offered (*ad lib*.).

B. Blood samples

The blood samples were collected from jugular vein of all animals in the examined groups in the early morning with one week interval during the last month of pregnancy and the day of parturition. Blood samples were divided into two portions; The first portion was collected in heparinized Tube contained 20 I.U. heparin for one mL blood for preparation of haemololysate by using digitonin and washing by physiological saline according to (Kornburg and Korecker 1955).

Table (1) Chemical and cell wall constituents of feed concentrate mixture and corn stalks (on DM basis)

Items	Feed concentrate mixture ϴ	Wheat straw
Chemical composition		
DM	91.51	93.48
OM	89.64	89.58
CP	14.34	3.26
CF	8.47	40.23
EE	2.24	1.32
NFE	64.59	44.77
ASH	10.36	10.42
Cell wall constituents		
NDF	34.62	78.24
ADF	16.24	54.13
Hemi cellulose	18.38	24.11
NFC*	38.44	6.76

*NFC: Non fibrous carbohydrates= 100 - % (CP+ NDF + EE + ASH) (Calsamiglia et al., 1995).

ϴ Feed concentrate mixture consists of 18% undecorticated cotton seed meal, 4% soybean meal, 36% yellow corn, 36% wheat bran, 3% Vinass, 1.5 % limestone, 1.4% sodium chloride and 0.1% common salts.

This was used for estimation of erythrocytic GSH (Sedlak and Lindsay, 1968); t-SOD (Misra and Fridovich, 1972); GSH-Px (EC: 1.11.1.9) (Chiu et al., 1976); GR-ase (EC: 1.6.4.2) (Bergmayer, 1983); GST (EC: 2.5.1.18) (Vessey and Boyer, 1984). The second one was collected without anticoagulant for obtaining a clear non-hemolyzed serum by centrifugation of the blood sample at 3000 r.p.m for 5 minutes. The clear sera were freshly used for determining of blood glucose (Trinder, 1969), non esterified fatty acid (NEFA) and Beta hydroxyl butyric acid (BHBA) (Duncombe, 1964), Commercial radioimmunoassay kits were used to measure concentration of cortisol and insulin (Tietz, 1968 and Wilson and Miles, (1977).

C. Electrophoretic pattern of serum protein by SDS-PAGE which performed according to the method of (Laemmli, 1970).
D-Statistical analysis was done by (SAS, 1996).

3. Results

The data presented in (Table 2) revealed a high significant increase (P<0.01) in the mean values of GSH-Px and GST in group II and III during the period of 2nd and last week before parturition and at the day of parturition. In contrast, GSH and t-SOD were high significantly decreased (P<0.01) and GR-ase activities were significantly decreased (P<0.05) at the same period of experiment.

Serum glucose level (Table 3) of single pregnant ewes showed significant (P<0.05) decrease than control during the last 3 weeks of pregnancy. But of twin pregnant ewes was decreased significantly (P<0.05) during the last 4 weeks of pregnancy.

Concentration of serum non esterified fatty acid (Table 3) of single pregnant ewe showed significant (P<0.05) increase than the control during the last 3 weeks of pregnancy as well as at the day of parturition. But of twin pregnant ewes showed significant (P<0.05) increase during the last 4 weeks of pregnancy.

Serum BHBA level (Table 3) of single pregnant ewes showed significant (P<0.05) increase than the control during the last 3 weeks of pregnancy and the day of parturition. And twin pregnant ewes showed significant (P<0.05) decrease than the control during the last 4 weeks of pregnancy and the day of parturition. Serum insulin level (Table 3) of single and twin pregnant ewes showed significant (P<0.05) decrease than the control during the last 4 weeks of pregnancy and the day of parturition.

Serum cortisol level (Table 3) of single and twin pregnant ewes showed significant (P<0.05) increase than the control during the last 2 weeks of pregnancy as well as the day of parturition.

The electrophoretic pattern of serum protein revealed that albumin, alpha (α)-1-globulin, alpha (α)-2-globulin and gamma (γ) globulin of single pregnant ewes (Table, 4 and Figure, 1) were significantly decreased during the last week of pregnancy and the day of parturition. But, the concentration of serum beta (β) globulin showed significant (P<0.05) decrease during the last week of pregnancy as well as the day of parturition.

Table (2): Mean values of some biochemical parameters of group I (control), group II (single bearing ewes) and group III (twin bearing ewes)

Duration \ Parameters / Groups			GSH (umol/mg protein)	t-SOD (U/g protein)	GSH-Px (U/g protein)	GR-ase (U/g protein)	GST (U/g protein)
	Control		0.89 ±0.08	13.01 ±1.11	3.19 ±0.27	0.81 ±0.02	0.41 ±0.03
Gestation period	4th week	Group II	0.71 ±0.11	13.01 0.47±	4.01 ±0.32	0.61 ±0.10	0.59 ±0.01
		Group III	0.81 ±0.12	11.20 ±0.88	4.91 ±0.19*	0.73 ±0.09	0.67 ±0.09
	3rd week	Group II	0.79 ±0.11	10.11 ±0.49	4.19 ±0.31	0.60 ±0.11	0.91 ±0.11*
		Group III	0.77 ±0.09	9.75 ±0.17	6.11 ±0.32*	0.62 ±0.09	1.11 ±0.09*
	2nd week	Group II	0.57 ±0.11*	8.75 ±0.40	7.01 ±0.27*	0.59 ±0.11	1.10 ±.11*
		Group III	0.61 ±0.10*	8.97 ±0.51	7.33 ±0.29*	0.49 ±0.10*	1.25 ±0.21*
	1st week	Group II	0.42 ±0.11**	8.19 ±0.31*	7.41 ±0.11**	0.41 ±0.02*	1.28 ±0.12**
		Group III	0.55 ±0.10**	7.70 ±0.21*	7.51 ±0.31**	0.39 ±0.03*	1.39 ±0.20**
Day of parturition	Group II		0.39 ±0.10**	7.12 ±0.16**	8.31 ±0.70**	0.35 ±0.01*	1.75 ±0.21**
	Group III		0.40 ±0.11**	6.11 ±0.13**	9.55 ±0.29**	0.25 ±0.03*	1.90 ±0.13**

* Indicate significant difference from control at (P<0.05).
** Indicate high significant difference from control at (P<0.01).
GSH (reduced glutathione); t-SOD (total superoxide dismutase); GSH-Px (glutathione peroxidase); GR-ase (glutathione reductase) and GST (glutathione-S-transferase).

Table (3): Mean values of some biochemical parameters of group I (control), group II (single bearing ewes) and group III (twin bearing ewes)

Duration	Groups / Parameters		Glucose (mg/dl)	NEFA (g/dl)	BHBA (µmol/L)	Insulin (µU/dl)	Cortisol (µg/dl)
	Control		66..41 ±0.36	11.53 ±0.22	3.57 ±0.07	2.54 ±0.02	2.04 ±0.04
Gestation period	4th week	Group II	42.88 ±0.85*	29.20 ±0.55*	10.35 ±0.89*	0.78 ±0.03*	3.64 ±0.07*
		Group III	41.56 ±0.70*	32.64 ±1.37*	12.72 ±0.95*	0.67 ±0.06*	3.73 ±0.13*
	3rd week	Group II	44..59 ±0.80*	22.70 ±0.73*	7.85 ±0.29*	0.94 ±0.05*	2.78 ±0.04*
		Group III	43.44 ±0.36*	22.63 ±1.31*	8.92 ±0.84*	0.87 ±0.12*	2.80 ±0.11*
	2nd week	Group II	47.57 ±0.49*	22.68 ±0.43*	7.10 ±0.33*	1.10 ±0.07*	2.32 ±0.06
		Group III	46.48 ±0.46*	21.87 ±0.37*	7.62 ±0.39*	1.17 ±0.03*	2.40 ±0.05
	1st week	Group II	52..29 ±0.53	18.92 ±0.18	6.21 ±0.25*	1.31 ±0.01*	2.04 ±0.02
		Group III	50.30 ±0.47*	19.11 ±0.36*	7.82 ±0.77*	1.29 ±0.006*	2.10 ±0.01
Day of parturition	Group II		40.50 ±0.63*	25.62 ±0.81*	7.52 ±0.32*	1.15 ±0.04*	5.50 ±0.18*
	Group III		40.12 ±0.25*	25.19 ±1.07*	9.36 ±0.27*	1.23 ±0.03*	8.02 ±0.11*

* Indicate significant difference from control at (P<0.05).

Table (4): Mean vales of serum protein fractions (g/dl) in control and twin ewes

The fractions	Control	The last week of pregnancy	The day of parturition
Albumin	3.15 ± 0.06	1.82 ± 0.11*	2.25 ± 0.17*
(α)-1-globulin	0.17 ± 0.004	0.08 ± 0.004*	0.08 ± 0.008*
(α)-2-globulin	0.56 ± 0.008	0.37 ± 0.006*	0.41 ± 0.006*
(β)-globulin	0.87 ± 0.006	0.74 ± 0.01*	0.75 ± 0.01*
(γ)-globulin	2.92 ± 0.01	1.87 ± 0.013*	2.17 ± 0.05*

* Indicate significant difference from control at (P<0.05).

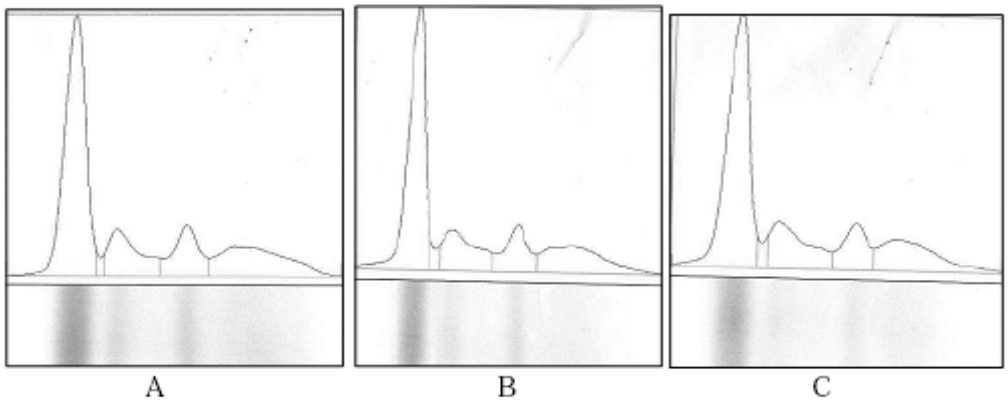


Figure (1): show the electrophoretic serum pattern of Control (A), the last week of pregnancy (B) and the day of parturition (C). In each picture, bands were arranged Albumin, Alpha (α)-1- globulin, Alpha (α)-2- globulin, Beta (β) globulin and Gamma (γ) globulin (From left to right).

4. Discussion

Our study revealed a high significant increase in the mean values of GSH-Px and GST in group II and III during the period of 2nd and last week before parturition and at the day of parturition. Also, showed high significantly decreased in GSH and t-SOD and a significant decrease in GR-ase. This result indicated that t-SOD activity decreased as it is a first line in antioxidant enzymes defense. In the second line, GSH-Px and GST were consuming GSH as a reductant cofactor. For that reason GSH-Px and GST activities were increased and GSH level was decreased. In addition, GR-ase activities were decreased because of GR-ase enzyme generates GSH (Mandour and Abou-El-Ela, 1999 and Abdel-Maksoud et al., 2000). As the glutathione assumes pivotal roles in bioreduction, protection against oxidative stress, detoxification of xenobiotics and endogenous toxic metabolites, transport, enzyme activity, and sulfur and nitrogen metabolism. Its biological significance comes from the free sulfhydryl moiety of the cysteine residue and nucleophilic properties. In cells, glutathione mainly exists in the reduced form (GSH), as the oxidized form (GSSG) (Taisuke et al., 2009). Erythrocytes are permanently in contact with potentially damaging levels of oxygen, but their metabolic activity is capable of reversing this injury under normal conditions. Erythrocytes are equipped by many defence systems representing their antioxidant capacity. This protective system includes superoxide dismutase (SOD), catalase (CAT), reduced glutathione, glutathione peroxidase (GPx), glutathione-S-transferase, and glutathione reductase (GR). However, the cellular antioxidant action is reinforced by the presence of dietary antioxidants (Nakbi et al., 2010).

The present study showed that a significant decrease in the mean values of glucose of single and twin that lower plasma glucose levels came in accordance with (Seidal et al., 2006 and Balikci et al., 2007). The observed decrease in serum glucose level may be due to the, negative energy balance increases lipid mobilization, which results in hepatic lipidosis with subsequent impairment of hepatocellular function, glucose deficiency with intermittent hypoglycemia and accumulation of ketone bodies. The hypothesis that cows suffering from stress and/or painful diseases have elevated blood glucose levels due to an increase in serum cortisol (Forslund et al., 2010).

NEFA and BHBA concentrations in single and twin pregnant ewes were significantly increased than control but in twin were more significantly increased; these results were proved by (Nazifi et al., 2002 and Moghaddam and Hassanpour, 2008).

Serum cholesterol level of single and win pregnant ewes showed significant increase than the control during the last 2 weeks of pregnancy as well as at the day of parturition. The high cortisol level inhibits the growth of the axial skeleton in the sheep fetus during the late pregnancy which enhances the parturition process (Fowden et al., 1996).

Serum insulin level of single and twin pregnant ewes showed significant decrease than the control during the last 4 weeks of pregnancy as well as at the day of parturition. The decrease of insulin level may be attributed to negative energy balance which leads to decrease in glucose level and increase the liploysis (Faulkner and Pollock, 1990). The shift of energy metabolism in a catabolic direction is characterized by a wide range of endocrine changes, such as insufficient pancreatic β-cell function with a coinciding increase in insulin resistance.

The data illustrated in table (3) showed significant increase in cortisol level than the control during the last 2 weeks of pregnancy as well as the day of parturition in Single and twin pregnant ewes. This observation may be due to a hypothesis that the known relation between stress and/or painful diseases in high yielding dairy cows and pregnant ewes may be mediated through a concurrent increased cortisol secretion leading to hyperglycaemia (Rohrbach et al., 1999). On the other hand, Forslund et al., (2010) reported significantly low levels of cortisol in Cows with ketonemia (BHBA > 1.5 mmol/l). The significant increase in cortisol and presence of significant negative correlation between plasma glucose concentration and cortisol level and the significant positive relationship with β-hydroxybutyrate may be due to increased adrenal output or to impaired ability of the fatty liver, which was a consistent finding in pregnancy toxemia, to mobilize and excrete the hormone (Ford et al., 1990 and Abd-Elghany et al., 2010).

The concentrations of serum albumin, alpha-1-globulin, alpha-2- globulin, beta globulin and gamma globulin of single pregnant ewes showed significant decrease than the control during the last week of pregnancy as well as the day of parturition. These results may attributed to consequence increase in the mother's basal metabolic rate, the maximal nutrient requirements of the placenta and the growing fetus, together with the transfer of serum albumin, immune globulins, and amino acids from the blood stream to the mammary gland for synthesis of colostrums (Batavani et al., 2006).

5. Conclusion

Late pregnancy in ewes is a very stressful period specially the late period in which in erythrocytic haemolysate the mean values of GSH-Px

and GST were high significantly increased; GSH and t-SOD were high significantly decreased (P<0.01) and GR-ase activities were significantly decreased. While, serum glucose, total protein, albumin, globulin and insulin were decreased while serum NEFA, BHBA and cortisol were increased in single and twin but in twin the values is more significant. Our research results recommended that twin bearing ewes need a special care during pregnancy and after parturition by supplementation of ewes by a demands of appositive energy balance.

Corresponding author
Ali Hafez El-Far
Department of Biochemistry, Faculty of Veterinary Medicine, Alexandria University, Damanhour Branch (Al-Bostan), Egypt.
aboufares90@yahoo

6. References:

1. Abd-Elghany, Hefnawy, S. Y. and Saad S. (2010): Some Immunohormonal Changes in Experimentally Pregnant Toxemic Goats, Veterinary Medicine International, Volume, Article ID 768438. 100- 11.
2. Abdel-Maksoud, H.A.A; Omayma A. R.; Mohamed K. M. M. and Yakout A.Y. E. (2000): Biochemical changes of glutathione redox system, some erythrocytic enzymes; serum proteins and trace elements of theileria infested buffaloes. The Egyptian Journal of Biochemistry, Vol. 18 No. 1, pp 121- 134.
3. Azab, M. E. and Abdel-Maksoud H. A. (1999): Changes in some hematological and biochemical parameters during prepartum and postpaetum in female Baladi goats. Small Ruminant Reseasch 34 : 66-85.
4. Balikci, E.; Yildiz, A. and Gurdogan, F., (2007): Blood metabolite concentrations during pregnancy and postpartum in Akkaraman ewes. Small Ruminant Research, 67, 247-251.
5. Batavani, R.A.; Ansari, M.; Asri, S., (2006): Concentrations of serum total protein and protein fractions during distrust and pregnancy in Makuii ewes. Comp Clin Pathol 15, 227-230.
6. Battaglia, F. C. and Meschia, G., (1988): Fetal nutrition. Annu. Rev. Nutr. 8:43–61.
7. Bergmayer, H.V. (1983): Methods of Enzymology. 3rd, ed. Varly chem. Weiheim. 105: 177-182.
8. Calsamiglia, S., Stern, M.D. and Frinkins, J.L. (1995): "Effects of protein sosurce on nitrogen metabolism in continuous culture and intestinal digestion in vitro" J. Anim. Sci., 73: 1819.
9. Chiu, D. I., Stults, F.H. and Tappal, A.L. (1976): purification and properties of rat lung soluble glutathione peroxidase. Biochem. Biophys. Acta, 445, 558 – 566.
10. Duncombe, W.G., (1964): The calorimetric micro determination of non-esterified fatty acids and betahydroxy butyric acid in plasma. Chem. Acta, 9: 122-125.
11. Faulkner, A. and Pollock, H.T., (1990): Metabolic responses to euglycaemic hyperinsulinaemia in lactating and non-lactating sheep in vivo. J. Endocrinol. 124, 59-66.
12. Ford E. J. H., Evans, J. and Robinson, I. (1990): Cortisol in pregnancy toxaemia of sheep, British Veterinary Journal, vol. 146, no. 6, pp. 539–542.
13. Forslund, Kristina B, Orjan A Ljungvall and Bernt V Jones (2010): Low cortisol levels in levels in blood from dairy cows with ketosis: a field study Acta Veterinaria Scandinavica, 52:31.
14. Fowden, A.L.; Szemere, J.; Hughes, B.; Gilmour, R.S. and Forhead, A.J., (1996): The effects of cortisol on the growth rate of the sheep fetus during the late gestation. J. Endocr, 151, 97-105.
15. Huntington, G.B. (1990): Energy metabolism in the digestive tract and liver of cattle: influence of physiological state and nutrition. Reprod Nutr Dev., 30:35-47.
16. Kornburg, a. and korecker, D (1955): In 'Methods in Enzymology, Acad. Press, New York, PP 201, 239.
17. Kulcsar M., Danko G., Delavaud C., Mircu C., Nikolic A.J., Gaspardy A., Cernescu H., Chilliard Y., Cseh S., Rudas P., Huszenicza Gy. (2006): Endocrine characteristics of late pregnant hyperketonaemic ewes and their reproductive performance following the induction of ovarian cyclicity out of the breeding season. Acta Veterinaria Hungarica, 54, 235–249.
18. Laemmli, A. R., (1970): Cleavage of structural protein during assembly of the head bacteriophage TA. Nature, 227: 600 – 685.
19. Lewington, J.H. (2007): Pregnancy toxaemia. In: Lewington J.H. (ed.): Ferret Husbandry, Medicine and Surgery. 2nd ed. Saunders Elsevier, Edinburgh, 94–95.
20. Mandour, M. and Abou-El-Ela, S. (1999): the protective effect of selenium on cadmium induced lipid peroxidation in rats. Egy. J. Bioch., Vol. 17; No. 1(1-21).
21. Misra, H.P. and Fridovich, S. (1972): the role of superoxide anion in the oxidation of epinephrine and a sample assay for superoxide dismutase. J. Biol. Chem., 247: 3170 – 2175.
22. Moghaddam, G. and Hassanpour, A., (2008): Comparison of blood serum glucose, beta hydroxybutyric acid, blood urea nitrogen and calcium concentration in pregnant and lambed

ewes. Journal of animal and veterinary advances, 7(3): 308-811.
23. Nakbi Amel, Wafa Tayeb, Samia Dabbou, Manel Issaoui, Abir K Grissa, Nabil Attia, and Mohamed Hammami (2010): Dietary olive oil effect on antioxidant status and fatty acid profile in the erythrocyte of 2,4-D- exposed rats. Lipids Health Dis.; 9: 89.
24. Nazifi, S.; Seb, M. and Ghavami, S.M., (2002): Serum lipid profile in Iranian fat-tailed sheep in late pregnancy, at parturition and during the post-parturition period. J. Vet. Med. Ser A. 49, 9-12.
25. Rafael A. N. and Donald C. B. (2007): Carbohydrate and Lipid Metabolism in Farm Animals, The Journal of Nutrition: 702 0022-3166.
26. Rohrbach B.W., Cannedy A.L., Freeman K, Slenning, B.D. (1999): Risk factors for abomasal displacement in dairy cows. J Am Vet Med Assoc, 214:1660-3.
27. Rook, J.S. (2000): Pregnancy toxemia of ewes, does, and beef cows: The Veterinary Clinics of North America. Food Animal Practice, 16, 293–317.
28. SAS, (1996): Statistical Analysis System. Users Guide Statistics, SAS Institute Cary, North Carolina.
29. Schlumbohm, C. and Harmeyer, J. (2004): American Dairy Science Association, Hyperketonemia Impairs Glucose Metabolism in Pregnant and Nonpregnant Ewes. J. Dairy Sci. 87:350–358.
30. Sedlak, I. and Lindsay, R.H. (1968): Estimation of total protein –bound and non-protein sulphydryl groups in tissues with Elman's reagent. Anal. Biochem. 25, 192-205.
31. Seidal, H.; Novotay, J. and Kovac, C., (2006): Selected biochemical indices in sheep during pregnancy and after parturition, Bull Vet Inst Pulway 50, 167-170.
32. Taisuke Yano, Emiko Takigami, Hiroya Yurimoto, and Yasuyoshi Sakai (2009): Yap1-Regulated Glutathione Redox System Curtails Accumulation of Formaldehyde and Reactive Oxygen Species in Methanol Metabolism of Pichia pastoris , Eukaryotic Cell, April, p. 540-549, Vol. 8, No. 4.
33. Tietz, N.W. (1968): Textbook of Clinical Chemistry, Saunders.
34. Trinder, P., (1969): Determination of Glucose in Blood Using Glucose Oxidase with an Alternative Oxygen Acceptor, Ann. Clin. Biochem 6, 24-25.
35. Van Saun R.J. (2000): Pregnancy toxaemia in a flock of sheep. Journal of the American Veterinary Medical Association, 217, 1536–1539.
36. Vessey, D.A. and Boyer, T.D. (1984): Different forms of rat liver glutathione –S-transferase by the herbicides 2,4,dichlorophenoxy acetate and 2,4,5, trichlorophenoxy acetate. Toxicol. Appl. Pharma., 73:492 – 499.
37. Wilson, M.A. and Miles, L.S.M., (1977): Radioimmunoassay of insulin. In hand book of Radioimmunoassay, G.E. Abraham (ed.), M. Dekker Inc., New York, p. 257.

11/5/2010

Economic Return of Recycling the Agricultural Wastes in Egypt and Spain

Mohamed G.M. Abou El- Azayem* and Salah S. Abd El-Ghani

Department of Agricultural Economics, National Research Centre, Dokki, Cairo, Egypt.

Abstract: Animal wastes and plant wastes are an important resource in Sustainable Agricultural Development and organic crops production for healthy food for life, when it is recycled to produce organic fertilizer (compost). It is clear that through the study of The economic returns to rotate some animal wastes and plant wastes in Egypt and Spain, And to identify. The quantity and value of losses in the content of animal wastes and plant wastes fertilizer elements (N, P, K) And also to identify Economic returns to recycling plant wastes for the production of industrial organic fertilizer (compost). Sustainable waste management means using resources efficiently to cut down on the amount of waste produced and where waste is generated, dealing with it in a way that contributes to the economic, social and environmental goals of sustainable development.

[Mohamed G.M. Abou El- Azayem and Salah S. Abd El-Ghani. **Economic Return of Recycling the Agricultural Wastes in Egypt and Spain**. Journal of American Science 2010;6(12):960-970]. (ISSN: 1545-1003). http://www.americanscience.org.

1. Introduction:

There are many complex economic and policy issues related to nutrient management. Before farmers can be convinced about applying a purchased input such as mineral or organic fertilizer, they need knowledge about such inputs and their effects on crop yield in both agronomic and economic terms. Once convinced of using fertilizers, in principle, they have to make the complex decision on how much and which fertilizer to use. Their decision on whether to use fertilizer on a particular crop is generally based on some form of economic judgment that includes experience from using such inputs, the cash or credit available, and probable produce prices. While the calculation of the economics of applying fertilizers is relatively straightforward, the economics of using nutrient sources such as animal manure, compost, crop residues, green manure crops and urban wastes is more complex. Critical elements in the calculation of the economics of using these products are their variable nutrient composition, their residual effect and the cost and availability of labour to access, process and apply them. These factors are often overlooked when advocating different nutrient management strategies.

For practical use, all agronomic data on crop responses to nutrients should always be subjected to economic analysis in order to account for differences in input and output prices and to address the basic issue of whether and to what extent fertilizer application will be profitable to the farmer. The discussion here uses mineral fertilizers as an example but the issues are also applicable to the other nutrient sources. Information on the factors that affect the returns from nutrient application is equally valuable in decision-making.

2. Material and Methods

Data sources: The study relied on sources of essential and secondary data through.

The annual statistical bulletins, periodicals, and some studies academy previous thesis of the masters and doctoral research.

Research and analytical method:

Study adopted the method of statistical analysis of descriptive and quantitative and averages, percentages, and measures of financial for the economic importance of recycling of some agricultural wastes, animal and plant through the production of organic fertilizer manufacturing in Spain, was also used some conversion factors to find out what rewards or draw the waste from the major fertilizer elements (N, P, K) in order to facilitate the economic valuation of these wastes and converted to the values of physical cash.

Reference review

Organic waste composting techniques (Julia et al., 2009) have been extensively developed in recent decades in response to the increasing concern about the amount and management of waste. Most studies focus on a specific stage or aspect in the life cycle of compost. The aim here is to determine the environmental impacts associated to the use of compost, from the collection of organic municipal solid waste to its application to tomato crops, and to compare these results with mineral fertilizer application, using life cycle assessment. Three systems were considered, depending on the fertilizing

treatment applied. The data was obtained experimentally in pilot fields and in an industrial composting facility, both located in the Mediterranean area. Treatments with compost have higher impacts than treatment with mineral fertilizer as a result of the high impact of compost production.

Growing concerns for environmentally friendly goods and services are being expressed together with those related with risks derived from intensive agriculture and broader environmental problems. This was, for example, a major issue at the World Summit on Sustainable Development held in Johannesburg in September 2002. In a recent survey (European Commission, 2005), citizen of the European Union answered that their main priorities in terms of agricultural policy were, listed in order of importance: ensuring stable and adequate incomes for farmers (36%), ensuring that agricultural products are healthy and safe (30%), promoting respect for the environment (28%), favoring and improving life in the countryside (26%) and favoring organic production (20%).

In the study for (Josep. 2006), this paper conducts an empirical study on output, costs and incomes in organic farming with a sample of Spanish firms. Financial accounting data reveals that organic and partly or transitional to organic farming do not get significantly different output than intensive farming. Farms in transition to organic farming bear significantly higher costs and obtain significantly lower income than intensive farming. Costs were recalculated incorporating opportunity costs of family work. Organic and transitional farming displayed significantly higher costs and lower relative income. However, organic farming plays a social role generating more employment than intensive farming and avoiding environmental and health damages. The article recalls for the necessity for accounting to broaden its scope and contents. It should disclose social and environmental data, as well as transactions that are not marketed, registered or valued but yield social profits and costs.

In the study for (salah, 2006) showed that maize wood was of the first rank as organic fertilizer on the total level of Egypt with L.E. 904.98 million annually and expected net return L.E. 687.78 million annually Rice straws came as second rank with total value of organic fertilizer L.E. 2317.29 million annually and total expected net returns L.E. 1761.14 million annually on Egypt's level. As for Fayoum Governorate, corn wood came in the first rank as a value of organic fertilizer L.E. 51.37 million annually and expected net return L.E. 39.04 million annually. Maize wood came in the second rank, the total of Fayoum Governorate of organic fertilizer quantity was L.E. 144.55 million annually and the total of expected net return was L.E. 109.86 million annually with 6.24% of the total Republic. As for Maize wood, it came in the first rank of quantity equivalent to Nitrogen and Rice straw came as second rank. Maize would come as first rank of quantity equivalent to phosphorous and rice straws came as second rank. Rice straws registered the first rank of equivalent quantity of potassium and corn woods were in the second rank. As for the economic retunes of organic fertilizers from agricultural wastes it was of 18% as an average according to 2004 statistics.

(Salvador et. al., 2007) Concern for the environment on a world scale is affecting all economic activities, and agriculture is not an exception. The Common Agricultural Policy (CAP) and the Policy for the Environment of the European Union (EU) have been introducing during the last years different environmental laws through their various legislating offices to facilitate the obtaining of community funding. The fundamental principles on which their measures are based are the following: "those who contaminate will have to pay", "conservationists will be paid" or "internalization of environmental costs". At present, the EU is involved in a configuration process reforming the totality of its budget policy for the programming period 2007-2013. Within this process, the community structural funds have been reviewed to conform to the new EU cohesion policy. Within this area, the new European Agricultural Fund for Rural Development (EAFRD) has been created, in which the basic lines regarding to rural development policy for the next few years are being defined. This is one of the pillars of the CAP. In this way, at least 25% of community expenditure is envisaged to be destined to environmental issues. Based on these principles, it is necessary to integrate agriculture environmentally by practicing the kind of agricultural management that respects and benefits from the opportunities offered by the environment.

3. Results and Discussion:

First: The economic returns to rotate some animal wastes in Egypt and Spain.

Can be identified on the efficiency or economic returns that accrue when to rotate some of the animal wastes and by using some conversion factors can be illustrated through the following points.

The quantity and value of losses in the content of animal waste fertilizer elements (N, P, K).

Loss occurs in the content of animal waste fertilizer elements (nitrogen, phosphorus and potassium) when these wastes are used in the production of non-conventional energy by burning them directly without the benefit with the pollution of

the environment, and can even explain it should first clarify the average content of animal waste fertilizer of these elements, which illustrated in tables (1,2) where shows the average percentage of the content of each waste of animal wastes into the study based on dry weight of the waste.

To determine the value of the content of animal waste from these elements must know the average price in euros per ton of the three fertilizer elements (nitrogen, phosphorus and potassium), and through the prices of mineral fertilizers for these items on the market (at February 2009). It can be explained prices as the price of nitrogen around (345) euros/ton, the price of some phosphorus (510) euros/ton and the price of some potassium (840) euros/ton. (www.coag.org).

Table No. (1): characteristics of the residues produced from animals

Category	*Average Weight (Kg)	*The amount of waste Dry (Kg / day)	**The number of animals in Egypt 2007	***The number of animals in Spain 2007
cattle	400	4	8974466	5740557
Horses	350	5	65714	276987
Sheep	200	0.48	5467469	18758512
Goat	50	0.24	4210714	2475710

Source:
*Samir Ahmed El-Shimi, (Dr.), "biogas", Agricultural Research Center, Department of Culture agricultural, technical publication No. 7, Egypt, 2000.
**Ministry of Agriculture, Economic Affairs Sector, Central Department of Agricultural Economics, Bulletin of Agricultural Statistics, Egypt, 2007.
***ANUARIO DE ESTADISTICA MINISTERIO DE MEDIO AMBIENTE Y MEDIO RURAL Y MARINO Madrid, 2009.

Table No. (2): average percentage of the content of animal waste dry fertilizer elements.

serial	The waste of animals	Average percentage of the content of the waste dry fertilizer elements%		
		Nitrogen (N)	Phosphorus (P)	Potassium (K)
1	**Livestock (Cows)**	1.9	0.56	1.4
2	**Sheep and goats**	1.87	0.79	0.92
3	**Horses**	1.1	0.7	0.8

Source: Parr, J.F. and colacicco, D., Organic materials as alternative nutrient sources C.F. Nutrition and pest control, Elsevier Sci. Pub. Amst. Netherlands, 1987.

It is clear from the table number (3) to the overall total of the quantity equation of nitrogen corresponding to the total animal wastes into the study at the level of both Egypt and Spain on respectively amounted to about (753.647, 631.004) thousand tons/day and reached the overall total of the corresponding value of about (260, 218) million euros/day.

Table (3): The total quantity and value of losses in the content of animal waste dry from element (nitrogen) in Egypt and Spain (2008).

Animal Type		The total amount of waste is dry tons / day	* Quantity equivalent of a nitrogen (nitrogen) per thousand tons/day	Value in million euros/day	In descending order of importance
Egypt	**Cattle**	35897864	682.059	235	**1**
	Horses	328570	3.614	1	**4**
	Sheep	2624385	49.076	17	**2**
	Goat	1010571	18.898	7	**3**
	Total	**39861390**	**753.647**	**260**	
Spain	**Cattle**	22962228	436.282	151	**1**
	Horses	1384935	15.234	5	**3**
	Sheep	9004086	168.376	58	**2**
	Goat	594170.4	11.111	4	**4**
	Total	**33945419**	**631.004**	**218**	

Source: * Calculated according to the conversion factor used.

It is clear from the table number (4) to the overall total of the amount of phosphorus equation corresponding to the total animal waste at the level of both Egypt and Spain on respectively amounted to about (232.044, 214.109) thousand tons/day and reached the overall total of the corresponding value of about (118 , 109) million euros/day.

Table (4): The total quantity and value of losses in the content of animal waste dry from element (phosphorus) in Egypt and Spain (2008).

Animal Type		The total amount of waste is dry tons / day	* Quantity equation of the element (phosphorus) in thousand tons/day	Value in million euros/day	In descending order of importance
Egypt	Cattle	35897864	201.028	103	**1**
	Horses	328570	2.300	1	**4**
	Sheep	2624385	20.733	11	**2**
	Goat	1010571	7.984	4	**3**
	Total	**39861390**	**232.044**	**118**	
Spain	Cattle	22962228	128.588	66	**1**
	Horses	1384935	9.695	5	**3**
	Sheep	9004086	71.132	36	**2**
	Goat	594170.4	4.694	2	**4**
	Total	**33945419**	**214.109**	**109**	

Source: * Calculated according to the conversion factor used.

It is clear from the table number (5) to the overall total of the quantity equation of potassium corresponding to the total animal waste at the level of both Egypt and Spain on respectively amounted to about (538.640 , 420.855) thousand tons/day and reached the overall total of the corresponding value of about (452 , 354) million euros/day.

Table (5): The total quantity and value of losses in the content of animal waste dry from element (potassium) in Egypt and Spain (2008).

Animal Type		The total amount of waste is dry tons / day	* Quantity equation of the element (potassium) in thousand tons / day	Value in million euros/day	In descending order of importance
Egypt	Cattle	35897864	502.570	422	**1**
	Horses	328570	2.629	2	**4**
	Sheep	2624385	24.144	20	**2**
	Goat	1010571	9.297	8	**3**
	Total	**39861390**	**538.640**	**452**	
Spain	Cattle	22962228	321.471	270	**1**
	Horses	1384935	11.079	9	**3**
	Sheep	9004086	82.838	70	**2**
	Goat	594170.4	5.466	5	**4**
	Total	**33945419**	**420.855**	**354**	

Source: * Calculated according to the conversion factor used.

Second: The economic returns to rotate some plant wastes in Spain

Can be identified on the efficiency or economic returns that accrue when to rotate some of the plant wastes and by using some conversion factors can be illustrated through the following points.

The quantity and value of losses in the content of plant waste fertilizer elements (N, P, K).

Loss occurs in the content of plant wastes fertilizer elements (nitrogen, phosphorus and potassium) when these wastes are used in the production of non-conventional energy by burning

them directly without the benefit with the pollution of the environment, and can even explain it should first clarify the average content of plant waste fertilizer of these elements, which illustrated in tables (6,7) where shows the average percentage of the content of each waste of plant wastes into the study based on dry weight of the waste.

To determine the value of the content of plant waste from these elements must know the average price in euros per ton of the three fertilizer elements (nitrogen, phosphorus and potassium), and through the prices of mineral fertilizers for these items on the market (at February 2009). It can be explained prices as the price of nitrogen around (345) euros/ton, the price of some phosphorus (510) euros/ton and the price of some potassium (840) euros/ton. (www.coag.org)

Table No. (6): characteristics of the waste produced from crops and various plants in Egypt and Spain (2008)..

Type of crop	*Area in thousand hectares In Egypt (2008)	**Area in thousand hectares In Spain(2008)	*** Average production per hectare in tons of waste is dry
Rice	745.1	96.1	4.3
Cotton	131.3	52.6	3.8
Maize	821.8	362.4	4.3
Sorghum	154.1	6.4	4.5
Sugar beet	108.2	52.3	8.1
tomato	240.2	57.1	7.6

Source:
*Ministry of Agriculture, Economic Affairs Sector, Central Department of Agricultural Economics, Bulletin of Agricultural Statistics, Egypt, 2008.
** ANUARIO DE ESTADISTICA MINISTERIO DE MEDIO AMBIENTE Y MEDIO RURAL Y MARINO Madrid, 2009.
***Samir Ahmed El-Shimi, (Dr.), **"biogas",** Agricultural Research Center, Department of Culture agricultural, technical publication No. 7, Egypt, 2000.

Table No. (7): average percentage of the content of dry plant waste fertilizer elements.

serial	Type of waste	Average percentage of the content of the waste dry fertilizer elements%		
		Nitrogen (N)	Phosphorus (P)	Potassium (K)
1	**Rice straw**	0.58	0.1	1.38
2	**Cotton Stalks**	0.88	0.15	1.45
3	**Maize Stalks**	0.55	0.31	1.11
4	**Sorghum Stalks**	0.55	0.31	1.11
5	**Sugar beet Thrones**	2.1	0.3	0.15
6	**tomato Thrones**	2.1	0.3	0.15

Source: Parr, J.F. and colacicco, D., Organic materials as alternative nutrient sources C.F. Nutrition and pest control, Elsevier Sci. Pub. Amst. Netherlands, 1987.

Seen from the table number (8) to Tomato Thrones came first in the equation in terms of quantity of nitrogen, which amounted to about (38.33) thousand tons per year, which are estimated at (13.22) million euros a year at the level of Egypt, and Maize Stalks comes in second place, with an overall total equation of the quantity of nitrogen at the level of Egypt about (**102.96**) thousand tons per year, with a total value of the corresponding year of around (**35.52**) million euros per year.

While Tomato Thrones came first in the equation in terms of quantity of nitrogen, which amounted to about (**9.11**) thousand tons per year, which are estimated at (**3.14**) million euros a year at the level of Spain, and Sugar beet Thrones comes in second place, with an overall total equation of the quantity of nitrogen at the level of Spain about (**30.89**) thousand tons per year, with a total value of the corresponding year of around (**10.66**) million euros per year.

Table (8): The total quantity and value of losses in the content of dry plant waste element (nitrogen) in Egypt and Spain (2008).

Type of waste		The total amount of waste is dry tones	* Quantity equivalent of the element (nitrogen) thousand tons/year	Value in million euros/year	In descending order of importance
Egypt	Rice straw	3203895	18.58	6.41	3
	Cotton Stalks	499082	4.39	1.52	5
	Maize Stalks	3533537	19.43	6.70	2
	Sorghum Stalks	693462	3.81	1.32	6
	Sugar beet Thrones	876583	18.41	6.35	4
	Tomato Thrones	1825326	38.33	13.22	1
	Total	**10631884**	**102.96**	**35.52**	
Spain	Rice straw	413230	2.40	0.83	4
	Cotton Stalks	199880	1.76	0.61	5
	Maize Stalks	1558320	8.57	2.96	3
	Sorghum Stalks	28800	0.16	0.05	6
	Sugar beet Thrones	423630	8.90	3.07	2
	Tomato Thrones	433960	9.11	3.14	1
	Total	**3057820**	**30.89**	**10.66**	

Source: * Calculated according to the conversion factor used.

Seen from the table number (9) to Maize Stalks is ranked first in terms of the quantity equation of phosphorus, which amounted to about (**10.95**) thousand tons per year, and the corresponding value of about (**5.59**) million euros a year at the level of Egypt, and Tomato Thrones comes in second place, with an overall total equation of the quantity of phosphorus on the level of about Egypt (**25.16**) thousand tons each year, with the overall total for the corresponding value of around (**12.83**) million euros per year.

While Tomato Thrones is ranked first in terms of the quantity equation of phosphorus, which amounted to about (**1.30**) thousand tons per year, and the corresponding value of about (**0.66**) million euros a year at the level of Spain, and Sugar beet Thrones comes in second place, with an overall total equation of the quantity of phosphorus on the level of about Spain (**8.21**) thousand tons each year, with the overall total for the corresponding value of around (**4.19**) million euros per year.

Table (9): quantity and value of losses in the content of dry plant waste element (phosphorus) in Egypt and Spain (2008).

Type of waste		The total amount of waste is dry tones	* Quantity equation of the element (phosphorus) thousand tons/year	Value in million euros/year	In descending order of importance
Egypt	Rice straw	3203895	3.20	1.63	3
	Cotton Stalks	499082	0.75	0.38	6
	Maize Stalks	3533537	10.95	5.59	1
	Sorghum Stalks	693462	2.15	1.10	5
	Sugar beet Thrones	876583	2.63	1.34	4
	Tomato Thrones	1825326	5.48	2.79	2
	Total	**10631884**	**25.16**	**12.83**	
Spain	Rice straw	413230	0.41	0.21	4
	Cotton Stalks	199880	0.30	0.15	5
	Maize Stalks	1558320	4.83	2.46	3
	Sorghum Stalks	28800	0.09	0.05	6
	Sugar beet Thrones	423630	1.27	0.65	2
	Tomato Thrones	433960	1.30	0.66	1
	Total	**3057820**	**8.21**	**4.19**	

Source: * Calculated according to the conversion factor used.

Seen from the table number (10) to Rice straw, which is ranked first in terms of the quantity equation of potassium, which amounted to about (**44.21**) thousand tons per year, and the value corresponding to (**37.14**) million euros a year at the level of Egypt, Maize Stalks comes in second place, with an overall total of the quantity equation of potassium at the level of about Egypt (**102.42**) thousand tons each year, with the overall total for the corresponding value of around (**86.04**) million euros per year.

While Maize Stalks, which is ranked first in terms of the quantity equation of potassium, which amounted to about (**17.30**) thousand tons per year, and the value corresponding to (**14.53**) million euros a year at the level of Spain, Rice straw comes in second place, with an overall total of the quantity equation of potassium at the level of about Spain (**27.50**) thousand tons each year, with the overall total for the corresponding value of around (**23.10**) million euros per year.

Table (10): The total quantity and value of losses in the content of dry plant waste element (potassium) in Egypt and Spain (2008).

Type of waste		The total amount of waste is dry tones	* Quantity equivalent of the element (potassium) thousand tons/year	Value in million euros/year	In descending order of importance
Egypt	Rice straw	3203895	44.21	37.14	1
	Cotton Stalks	499082	7.24	6.08	4
	Maize Stalks	3533537	39.22	32.95	2
	Sorghum Stalks	693462	7.70	6.47	3
	Sugar beet Thrones	876583	1.31	1.10	6
	Tomato Thrones	1825326	2.74	2.30	5
	Total	**10631884**	**102.42**	**86.04**	
Spain	Rice straw	413230	5.70	4.79	2
	Cotton Stalks	199880	2.90	2.43	3
	Maize Stalks	1558320	17.30	14.53	1
	Sorghum Stalks	28800	0.32	0.27	6
	Sugar beet Thrones	423630	0.64	0.53	5
	Tomato Thrones	433960	0.65	0.55	4
	Total	**3057820**	**27.50**	**23.10**	

Source: * Calculated according to the conversion factor used.

Third: Economic returns to recycling plant wastes for the production of industrial organic fertilizer (compost):

There is no doubt that reliance on mineral fertilizers under the regime of intensive agriculture leads to pollution of soil, plants and water and here endorse a need for the presence of organic matter to soils of high level of production, quality and relevance of consumer tastes and conditions of public health, so the quantities of the waste plant were considered a waste of high value-added income national agriculture as a result of the loss of organic matter and fertilizer elements, in addition to being a source of contamination of the environment in the absence of their correct use.

From this perspective, increased attention to the expansion in the production of organic fertilizers and consequently organic agriculture programs can be achieved to maintain soil fertility and improve the physical and chemical properties and biological weapons, which would lead to the production of crops with good specifications to suit the needs and requirements of foreign markets, thus providing opportunities for export of those products.

What is meant by the term organic fertilization of agricultural land is organic fertilizers made from agricultural wastes for recovery of fertilizer elements taken from the soil during various stages of plant growth, so as to maintain the fertility and vitality and restore the ecological balance of the soil, which is achieved with the reduction of environmental pollution resulting from rationalization of consumption of mineral fertilizers pesticides and other chemicals, as well as by not burning waste for disposal and the production of clean safe healthy food for both humans or animals and to obtain a high quality product and reduce the costs of agricultural production and create jobs through non-conventional stages of the production of organic fertilizers and maintain the integrity of the sources of irrigation and drainage and the economy the expenses of the clean canals, banks and reduce the chances of insect and rodent pests and harmful and increase national income through an attractive economic return.

Due attention to organic agriculture to environmental restrictions to protect humans from pollution and the difficulty of disposal of agricultural wastes, leading to increased health and environmental problems, in addition to health conditions to be met through global conventions when exporting agricultural products to world markets. All that was important to provide industrial compost (manure compost), an organic fertilizer resulting from aerobic fermentation of mixtures of plant and animal waste.

And to identify the quantity and value of industrial organic fertilizer (compost) and economic returns that can be achieved when the plant waste recycling for the production of agricultural fertilizer industrial organic (compost) for each waste of agricultural waste under study in Egypt and Spain. Can rely on the following data in the table (11,12) to determine the conversion factor which can be used is as follows:

Table No. (11): characteristics of the waste produced from crops and various plants in Egypt and Spain (2008). .

Type of waste		Area in thousand hectares	***Average production per Ha Wet tons	The total amount of waste is wet a thousand tones/year
*Egypt	Rice straw	745.1	4.8	3576.44
	Cotton Stalks	131.3	4.3	564.75
	Maize Stalks	821.8	4.8	3944.41
	Sorghum Stalks	154.1	5.0	770.51
	Sugar beet Thrones	108.2	9.5	1028.09
	tomato Thrones	240.2	10.0	2401.74
**Spain	Rice straw	96.1	4.8	461.28
	Cotton Stalks	52.6	4.3	226.18
	Maize Stalks	362.4	4.8	1739.52
	Sorghum Stalks	6.4	5.0	32
	Sugar beet Thrones	52.3	9.5	496.85
	tomato Thrones	57.1	10.0	571

Source:
*Ministry of Agriculture, Economic Affairs Sector, Central Department of Agricultural Economics, Bulletin of Agricultural Statistics, Egypt, 2008.
**ANUARIO DE ESTADISTICA MINISTERIO DE MEDIO AMBIENTE Y MEDIO RURAL Y MARINO Madrid, 2009.
***Samir Ahmed El-Shimi, (Dr.), "**biogas**", Agricultural Research Center, Department of Culture agricultural, technical publication No. 7, Egypt, 2000.

Given a ton of waste plant about 2.5 cubic meters of industrial organic fertilizer, which is equivalent to one ton (compost). As the table shows the number (12) that the estimated cost per ton of compost, equal to about (**50**) EUR/ton, on the grounds that the price per ton of it working at the equivalent of about (**150**) EUR/ton, a value representing the arithmetic average price per ton.

Table No. (12): Shows the average (cost, price) and net revenue in euros per ton of compost in Egypt and Spain (2008).

Type	Average cost euros per ton	The average price euros per ton	Net revenue euros per ton
compost	**50**	**150**	**100**

Source: www.alicanteasaja.com

Table (13) to Maize Stalks is ranked first in terms of quantity the equivalent of compost, which amounted to about (**9.86**) million cubic meters annually, an estimated cost of about (**197.2**) Euro/year and is ranked second, Rice straw, while the total amount of the equivalent of compost on Egypt's level of about (**8.94**) cubic meters annually, at a total cost amounted to about (**614.3**) euros a year on the level of Egypt.

While Maize Stalks is ranked first in terms of quantity the equivalent of compost, which amounted to about (**4.35**) million cubic meters annually, an estimated cost of about (**87.0**) Euro/year and is ranked second, Tomato Thrones, while the total

amount of the equivalent of compost on Spain's level of about (**8.82**) cubic meters annually, at a total cost amounted to about (**176.3**) euros a year on the level of Spain.

Table (13) the total quantity and cost estimates in millions of euros for the production of industrial organic fertilizer (compost) from the wet waste plant in Egypt and Spain (2008).

Type of waste		The total amount of waste is wet a thousand tones/year	* Quantity equation of compost million cubic meters/year	The estimated cost in millions of euros necessary to convert the total amount of waste to compost/year	In descending order of importance
*Egypt	Rice straw	3576.44	8.94	178.8	**2**
	Cotton Stalks	564.75	1.41	28.2	**6**
	Maize Stalks	3944.41	9.86	197.2	**1**
	Sorghum Stalks	770.51	1.93	38.5	**5**
	Sugar beet Thrones	1028.09	2.57	51.4	**4**
	Tomato Thrones	2401.74	6.00	120.1	**3**
	Total	**12285.95**	**30.71**	**614.3**	
**Spain	Rice straw	461.28	1.15	23.1	**4**
	Cotton Stalks	226.18	0.57	11.3	**5**
	Maize Stalks	1739.52	4.35	87.0	**1**
	Sorghum Stalks	32	0.08	1.6	**6**
	Sugar beet Thrones	496.85	1.24	24.8	**3**
	Tomato Thrones	571	1.43	28.6	**2**
	Total	**3526.83**	**8.82**	**176.3**	

Source: * Calculated according to the conversion factor used.

As is clear from the data table (14) to Maize Stalks comes in ranked first in Egypt in terms of manure, which amounted to about (**591.7)** million euros per year and net revenue is expected around (**394.4**) millions euros a year, comes in second place, Rice straw, while the overall total of the value of manure around (**1842.9**) millions euros annually, and the overall total of net revenue is expected around (**1228.6**) millions euros a year on the level of Egypt.

While Maize Stalks comes in ranked first in Spain in terms of manure, which amounted to about (**260.9)** million euros per year and net revenue is expected around (**174.0)** millions euros a year, comes in second place, Tomato Thrones, while the overall total of the value of manure around (**529.0**) millions euros annually, and the overall total of net revenue is expected around (**352.7**) millions euros a year on the level of Spain.

Table (14): The total value and net revenue in millions of euros expected in the production of organic fertilizer (compost) from the wet waste plant in Egypt and Spain (2008).

Type of waste		The total amount of waste is wet a thousand tones/year	* The value of industrial organic fertilizer (compost) in million euros/year	(N.R) Net revenue resulting in the production of compost in millions of euros/year	In descending order of importance
Egypt	Rice straw	3576.44	536.5	357.6	**2**
	Cotton Stalks	564.75	84.7	56.5	**6**
	Maize Stalks	3944.41	591.7	394.4	**1**
	Sorghum Stalks	770.51	115.6	77.1	**5**
	Sugar beet Thrones	1028.09	154.2	102.8	**4**
	Tomato Thrones	2401.74	360.3	240.2	**3**
	Total	**12285.95**	**1842.9**	**1228.6**	
Spain	Rice straw	461.28	69.2	46.1	**4**
	Cotton Stalks	226.18	33.9	22.6	**5**
	Maize Stalks	1739.52	260.9	174.0	**1**

	Sorghum Stalks	32	4.8	3.2	**6**
	Sugar beet Thrones	496.85	74.5	49.7	**3**
	Tomato Thrones	571	85.7	57.1	**2**
	Total	**3526.83**	**529.0**	**352.7**	

Source: * Calculated according to the conversion factor used.

4. Conclusion:

Sustai:nable waste management means using resources efficiently to cut down on the amount of waste produced and where waste is generated, dealing with it in a way that contributes to the economic, social and environmental goals of sustainable development.

The following table No. (15) shows the most important economic returns that can be obtained from the recycling of agricultural wastes in Egypt and Spain.

Country	**Egypt**			**Spain**		
Waste type	**Animal wastes**					
Type	**Q(Quantity) thousand tons/day**	**V(value) million euros/day**		**Q thousand tons/day**	**V Million euros/day**	
N	**753.647**	**260**		**631.004**	**218**	
P	**232.044**	**118**		**214.109**	**109**	
K	**538.640**	**452**		**420.855**	**354**	
Total		**830**			**681**	
Country	**Egypt**			**Spain**		
Waste type	**Plant wastes**					
Type	**Q thousand tons/year**	**V million euros/year**		**Q thousand tons/year**	**V million euros/year**	
N	**102.96**	**35.52**		**30.89**	**10.66**	
P	**25.16**	**12.83**		**8.21**	**4.19**	
K	**102.42**	**86.04**		**27.50**	**23.10**	
Total		**134.39**			**37.95**	
Waste type	**Economic returns to recycling plant wastes for the production of industrial organic fertilizer (compost)**					
Country	**Egypt**			**Spain**		
Type	**Q thousand tones/year**	**V million euros/year**	**N.R Net revenue million euros/year**	**Q thousand tones/year**	**V million euros/year**	**N.R Net revenue million euros/year**
Compost	**12285.95**	**1842.9**	**1228.6**	**3526.83**	**529.0**	**352.7**
Main opportunity	**animal wastes (Cattle, Horses, Sheep, Goat)**			**animal wastes (Cows, Sheep, Horses, Goat)**		
	plant wastes (Maize Stalks, Rice straw, Tomato Thrones, Sugar beet Thrones, Sorghum Stalks, Cotton Stalks)			**plant wastes (Maize Stalks, Tomato Thrones, Sugar beet Thrones, Rice straw, Cotton Stalks, Sorghum Stalks)**		
Main limitation	**1-lack of funding for the construction of projects in this country. 2-Lack of equipment and equipment necessary. 3-Lack of awareness of environmental issues.**			**1- lack of information centers or periodic bulletins or accurate statistics on agricultural waste. 2- Lack of manpower and high wages.**		

Source: tables from (1 to 14)

5. Recommendations:

1) Promoting cooperation between research bodies and industrial enterprises in order to facilitate the identification of problems and sources of bottlenecks and constraints that limit the optimum utilization of agricultural wastes and thus lead to the scientific and practical solutions to these problems.
2) provide the necessary support for the issuance of an annual statistical bulletin of the two countries on the types and quantities of agricultural waste.
3) Provision of machinery and equipment necessary for the establishment of small projects in the field of recycling of agricultural waste.
4) Training and employment for the construction of these projects.
5) Work to raise awareness of environmental and health among the citizens through seminars and conferences to discuss important economic, social, environmental and health resulting from the recycling of agricultural waste.

Encourage the use of organic fertilizers (compost) as alternatives to economic and safe for the production of healthy food and safe compared to chemical fertilizers are detrimental to health and environmentally.

Corresponding author:
Mohamed G.M. Abou El- Azayem
Department of Agricultural Economics, National Research Centre, Dokki, Cairo, Egypt.

6. References:

1- Anuario de Estadistica Ministerio de Medio Ambiente Y Medio Rural Y Marino Madrid, 2009.
2- Arab Organization for Agricultural Development, "Study of technical and economic feasibility of the use of agricultural waste plant", League of Arab States, Khartoum, 1997.
3- European Commission, “Europeans and the Common Agricultural Policy”, Special Euro barometer, 221/Wave 622 – TNS Opinion and Social, 2005.
4- Josep, M. A. "Empirical Study on Costs and Incomes of Organic Farming" Departament de Comptabilitat. Facultat de Ciències Econòmiques I Empresarials. Universitat de Barcelona.. Col·lecció d’Economia E07/178, p2, 2006.
5- Julia, M., Pere, M., Assumpció A., and Joan R., Life cycle assessment of the use of compost from municipal organic waste for fertilization of tomato crops. Institute of Environmental Science and Technology (ICTA). SosteniPrA. Universidad Autónoma de Barcelona (UAB). 08193 Bellaterra (Barcelona), Spain, 2009.
6- Ministry of Agriculture and Land Reclamation, "Recycling agricultural wastes to produce organic fertilizer (compost), the central administration of agricultural extension, Agricultural Research Center, Bulletin No. 693. Egypt, 2001.
7- Ministry of Agriculture and Land Reclamation, Economic Affairs Sector, Central Department of Agricultural Economics, Bulletin of Agricultural Statistics, Egypt, 2007.
8- Parr, J.F. and colacicco, D., Organic materials as alternative nutrient sources C.F. Nutrition and pest control, Elsevier Sci. Pub. Amst. Netherlands, 1987.
9- Salah, S.A.,. "Economic Return of Recycling the Agricultural Wastes; an Applied Study on Fayoum Governorate", master, thesis, Department of Agricultural Economics, Faculty of Agriculture, El- Fayoum University, Egypt, 2006.
10- Salvador P, Fernando J. A., Javier C. "Increased producer and consumer preoccupation with environment. Economic internalization of greenhouse vegetal waste impact in Almerian horticulture, Spain"2007.
11- Samir Ahmed El-Shimi, (Dr.), "biogas", Agricultural Research Center, Department of Culture agricultural, technical publication No. 7, Egypt, 2000.
12- www.alicanteasaja.com
13- www.coag.org

11/2/2010

Role of S-100B as a Serum Biochemical Marker for Brain Injury in Egyptian Patients with Phenylketonuria

Mohga S Abdallah[1], Hayat M Sharada[1], Esmat Ashour[2]*,Lamiaa Mageed [2], Nagwa Meguid [3]

[1]Biochemistry Division, Chemistry Dept., Faculty of Science , Helwan University
[2]Biochemistry Dept., Genetic Engineering & Biotechnology Division, National Research Centre, Dokki, Giza, Egypt
[3] Children with Special Needs Dept., Medical Reseach Division, National Research Centre, Dokki, Giza, Egypt
drmohgashafik@yahoo.com., hayatsharadamk@gmail.com.,esmatashour3@yahoo.com., malakalhoda@yahoo.com., Meguidna@yahoo.com

Abstract :Background: Phenylketonuria (PKU) is a metabolic disorder characterized by high phenylalanine (Phe) levels in blood. Tissue accumulation of L-phenylalanine (Phe) is the biochemical hallmark of human phenylketonuria (PKU), an inherited metabolic disorder clinically characterized by mental retardation and other neurological features. The mechanisms of brain damage observed in this disorder are poorly understood. S-100B protein is highly specific for nervous tissue where its role is not yet fully understood. **Objective:** The aim of our study was to determine the diagnostic value of measuring S-100b in the serum of PKU patients as a marker for brain lesion. Additional validity should be acquired by a comparison with plasma levels of phenylalanine. **Methods:** Nineteen PKU patients from 15 families were selected from the clinic for special needs at the National Research Centre. Their age ranged between 2 and 20 years in addition to 15 healthy controls with same age. Blood samples were drawn to investigate circulating serum levels of S-100b using ELISA technique for all the studied cases. **Results:** Statistical significant increase of serum S-100B concentrations was present in PKU patients compared to controls. Regarding sensitivity and specificity, PKU patients, serum neural protein S-100b showed high sensitivity and specificity values. In addition, there was non significant negative correlation between S100B and Phe **Conclusion:** we concluded that serum S-100B blood could be a useful peripheral marker of nervous system damage in patients with phenylketonuria.

[Mohga S Abdallah, Hayat M Sharada, Esmat Ashour, Lamiaa Mageed, Nagwa Meguid. **Role of S-100B as a Serum Biochemical Marker for Brain Injury in Egyptian Patients with Phenylketonuria**. Journal of American Science 2010;6(12):971-978]. (ISSN: 1545-1003). http://www.americanscience.org.

Key Words: Phenylketonuria, Serum neural protein S-100B

1. Introduction

Phenylketonuria is a rare metabolic disorder with an estimated prevalence of 1:10,000, i.e. an orphan disease. Inheritance is autosomal recessive caused by mutations in the phenylalanine hydroxylase gene. This enzyme converts phenylalanine to tyrosine which is also a precursor for dopamine. Partial or complete enzyme inactivity results in the accumulation of phenylalanine in tissues, blood (hyper-phenylalaninemia) and other body fluids (Albrecht *et al.,* 2009).

Untreated phenylketonuria causes severe neurological impairment, mental retardation and behavioral difficulties. Although there is no doubt that blood phenylalanine has a detrimental effect in the brain (Scriver, 2007), the ultimate causes for this effect remain hypothetical (Hoeksma *et al*., 2009). If left untreated from birth, this results in rapid accumulation of toxic concentrations of Phe in the blood, leading to severe brain damage and microcephaly (van Spronsen *et al.,* 2009). The vast majority of cases are now identified via neonatal screening programmers, allowing timely intervention to avoid severe consequences. However, insufficiently controlled Phe concentrations may still result in neuro-cognitive symptoms, such as a decrease in intelligence quotient (IQ), attention, executive function (such as planning, working memory, inhibition, flexibility and behavioral issues), and psychosocial effects in later childhood, continuing into adulthood (DeRoche et al., 2008; Waisbren *et al.,* 2007).

A low-Phe diet currently forms the principal strategy to limit Phe accumulation in the blood and, therefore, in tissues such as the brain (MacDonald and Asplin. 2006). The low-Phe diet restricts the intake of high-protein foods, and the remaining nutritional requirements must be obtained from Phe-free amino acid supplements (protein substitute) and special or natural foods that are low in Phe, the nature

and availability of which can vary geographically (Weetch and MacDonald, 2006).

The term S-100B refers to members of a multigenic family of calcium-modulated proteins (S100 proteins), mostly of low molecular mass (≅10 000 Da), that were first identified (on the basis of methods available at the time) as a protein fraction detectable in brain but not in non-neural extracts and called S100 because of their solubility in a 100%-saturated solution with ammonium sulfate (Moore, 1965). At present, at least 20 proteins have been identified as belonging to the S100 protein family, the members of which are characterized by the presence of a pair of so-called EF-hand (i.e., helix-loop-helix) calcium-binding motifs (Kawasaki *et al.,* 1998), first discovered in the crystal structure of parvalbumin (Kretsinger and Nockolds, 1973), that induce conformational changes of the protein after binding to calcium (Donato, 2001; Heizmann, 1999). This conformational change may facilitate the interaction of S100 proteins with secondary effectors: S100 proteins are generally thought to be calcium sensor proteins that modulate biological activity via calcium binding (Ikura, 1996). In addition, some S100 members (Schafer *et al.,* 2000; Nishikawa *et al.,* 1997) have been shown to bind Zn^{2+} and Cu^{2+}, suggesting the possibility that their biological activity in some cases might be regulated by Zn^{2+} and/or Cu^{2+}, rather than by Ca^{2+} (Heizmann and Cox, 1998).
Aim of this study: Since S-100B protein is a biomarker of neural tissue damage and PKU is a neurodegenerative disease, we aimed to investigate whether S-100B is related to CNS damage of PKU patients and to their blood Phe levels.

2. Material and methods

Patients screening

The present study was carried out on 19 PKU patients (12 males and 7 females) from 15 families selected from the children with Special Needs Clinic and Clinics Department, National Research Centre. Their age ranged between 2 and 20 years. In addition, 15 healthy matched subjects of age and sex were ascertained as control group.

All selected cases were selected at the time of the diagnosis of the index cases by elevation of serum levels of Phenylalanine (Phe) which was done by an enzymatic colorimetric assay (Scriver *et al.,* 1989) for all studied cases.

Participants were classified into three age groups: children with mean age below 13 years, adolescents with mean age between 13years and 18 years, and adults older more than 18 years.

All PKU patients were managed with low phe diet the time they diagnosed

Clinical Investigation: All PKU patients were on low phe diet.

- 3 patients had abnormal EEG and received Carbamazepine in addition to low phe diet
- Also 6 patients received Sodium valproate.
- On the other hand 5 patients had autistic features receiving Risperidone.

Intelligence quotient (IQ):

All the patients were tested using Stanford-Binet Intelligence scale: Fourth Edition (Thorndike et al., 1986) according to their age. The degree of mental retardation (MR) was evaluated according (Menon et al., 2003): normal ~80, borderline =70-90, mild = 51-70, moderate = 36-50, severe = 21-35, profound = 0- 20.

Clinical Investigation

Collection of Blood Samples

Blood samples were collected from (controls and PKU patients) by venous arm puncture into tubes, The tubes containing blood without adding anticoagulant were centrifuged after 30 minute at (3000 r.p.m for 10 minutes at 4^{o} C) to separate the serum. The serum samples were divided into small aliquots and kept frozen until analysis.

Laboratory Investigations

- Serum concentrations were performed by using a commercially available the RD 192090100 Human S100B ELISA Kit.
- Total serum protein was assessed by colorimetric method using commercially Stanbio Total Protein Loiqui-Color Kit. This method is based on the reports of Weischeslbaum (1946) and Gornal et al.,(1949).
- Determination of Total Calcium in serum by colorimetric method using commercially available Kit from STANBIO Company. The procedure presented is based essentially on the micro-method of Saker and Chauhan (1967).
- Ionized calcium was calculated according to Zeisler Formula (1954)

Statistical analysis of data, Package for

Social Science (SPSS for Windows Release 11; SPSS Inc., Chicago, IL, USA) was used. Data were analyzed by two-way ANOVA. As no variable was simultaneously Gaussian in the groups of study, differences were tested with the Mann–Whitney U test. Correlation coefficients were estimated with the Spearman test. For the evaluation of categorical variables, Chi square test was used. All tests were

two sided and P values < 0.05 were considered significant.

3. Results

Among the 19 patients with PKU studied, PKU was proved to have high level of Pha concentration. The diagnosis of Mental retardation was based on IQ Score (Table 1)

Table 1. Demographic and clinical data of the studied group

		#N
No. of Family groups		15
Gender	Females	7
	Males	12
	Total	19
Age (years)	0-12 yrs	12
	13-18 yrs	6
	0ver 18yrs	1
Severity of PKU	Classic(severe)	10
	Moderate	7
	MHP	2
Degree of Mental Retardation	Borderline	2
	Mild	1
	Moderate	7
	Severe	8
	Not available	1

#N.: no of cases

The results of the S100B in PKU showed that S100B level was significantly increased in PKU cases when compared to healthy control group (F=43.771, P<0.0001). In addition, Total calcium (Ca) and ionized Calcium (Ca^{+2}) were significantly decreased in PKU when compared to healthy controls (F=35.121, P<0.0001 and F=27.155, P <0.0001 respectively).In addition the results of the Total Protein (TP) in PKU Showed that there was no statistical variation between the mean values of TP in PKU (7.71±0.22gm/dL) when compared to healthy controls(7.54+ 0.19gm/dL) (F=0.333, P=0.568) (Table 2)

The best Cut off value of S100B was 1866 pg/ml (the sensitivity and PPV values were 100 % and 86.4% respectively but the specificity and NPV were 80% and 100 % respectively(Table 3) The area under the curve of S100B was 0.942 (Table 4, Fig.1). The predictive value of S100B for discriminating between individuals with and without PKU was assessed by calculating the area under the ROC curve.

Correlation between S100B and Phe, IQ, TP, Ca and ionized Ca^{+2} in PKU Cases:

There was no significant positive correlation between S100B and both of IQ, Ca and ionized Ca^{+2} (r=0.368, P=0.133, r=0.256, P=0.290 and r=0.272, P=0.260 respectively) in PKU Cases. In addition, there was non significant negative correlation between S100B and both of Phe and TP (r=0.173, P=0.479 & r=-0.157, P= 0.521 respectively) in PKU Cases (Table 5).

Table 2. Summarized for data Age, Pha Conc., IQ Score, Total Protein (TP)(g/dL), Total Calcium (Ca), Ionized Calcium (Ionized Ca^{+2}) mg/dL) and S100B (Pg/ml) in Control and PKU Groups.

Group		Age (years)	Phe (mg/dl)	IQ	TP (gm/dl)	Total Ca (mg/dl)	Ionized Ca^{+2} (mg/dl)	S100 B (pg/ml)
Controls (C)	Range	5.00 19.00	2.5 5.5	83 98	6.50 8.50	8.50 11.90	3.00 5.40	398.00 3981.00
	Mean	9.87	4.03	102.5	7.54	10.66	4.47	1541
	±SE	1.33	0.25	1.23	0.19	0.25	0.17	256.381
	N	15	15	15	15	15	15	15
PKU	Range	2.00 21.0	7.00 37.0	21. 80.	6.20 8.90	5.10 13.00	2.00 5.90	1995.00 5248.00
	Mean	9.89	20.67	41.50	7.71	7.50	3.07	3767.1
	±SE	1.19	1.71	3.83	0.22	0.43	0.20	220.07
	N	19	19	18	19	19	19	19
ANOV A test	F				0.333	35.121	27.155	43.771
	P				0.568	0.000	0.000	0.000

Table (3): Cut off, Sensitivity, Specificity, PPV, NPV, and Accuracy for S100B

Parameter	Cut off (pg/ml.)	Sensitivity	Specificity	PPV*	+NPV	Accuracy
S100B	1866	100	80	86.4	100	91.17(31/34)

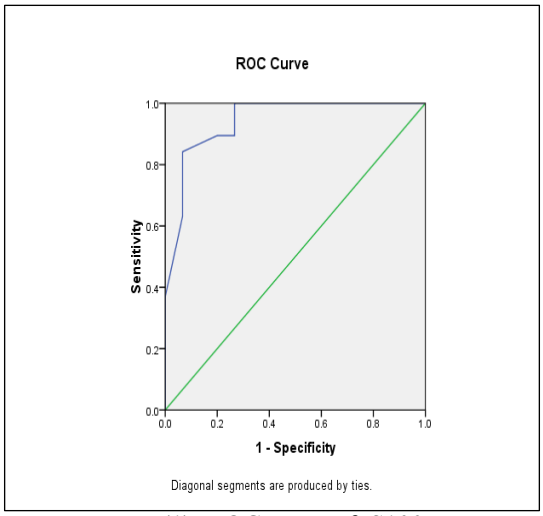


Fig (1): ROC curve of S100B

Table (4): Area under Curve of S100B

Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
0.942	0.040	0.000	0.864	1.020

Table (5): Correlation between S-100B and Other Biochemical Parameters in PKU Cases

Diagnosis			100B	Ionized Ca^{+2}	Ca	TP
KU	age	Pearson Correlation	0.285	-0.056-	-0.036-	0.217
		Sig. (2-tailed)	0.238	0.818	0.885	0.373
		N	19	19	19	19
	Phe	Pearson Correlation	-.173-	-.361-	-.320-	.405
		Sig. (2-tailed)	.479	.129	.181	.085
		N	19	19	19	19
	IQ	Pearson Correlation	.368	.262	.410	.475*
		Sig. (2-tailed)	.133	.294	.091	.046
		N	18	18	18	18
	Ca	Pearson Correlation	.256	.965**	----	-.016-
		Sig. (2-tailed)	.290	.000	-----	.947
		N	19	19	-------	19
	Ionized Ca^{+2}	Pearson Correlation	.272	----	.965**	-.242-
		Sig. (2-tailed)	.260	----	.000	.318
		N	19	----	19	19
	TP	Pearson Correlation	-.157-	-.242-	-.016-	---
		Sig. (2-tailed)	.521	.318	.947	---
		N	19	19	19	---

* P values < 0.05 were considered significant.
** P values < 0.0001 were considered very high significant.

According to the different ages group

There were no statistical variations in S-100B between different age groups [F=.050, P>0.05(P=0.826)](Fig.2). In the group of age (0-12yrs), there was no significant negative correlation between S100B and both of Phe, Ca and ionized Ca^{2+} (r=-0.0.222, P=0.0.607, r=-0.237, P=0.651 and r=-0.0.590, P=0.217 respectively) However, there was no significant positive correlation between S100B and IQ and TP (r=0.575, P=0.232 and r=-0.411, P= 0.419 respectively).

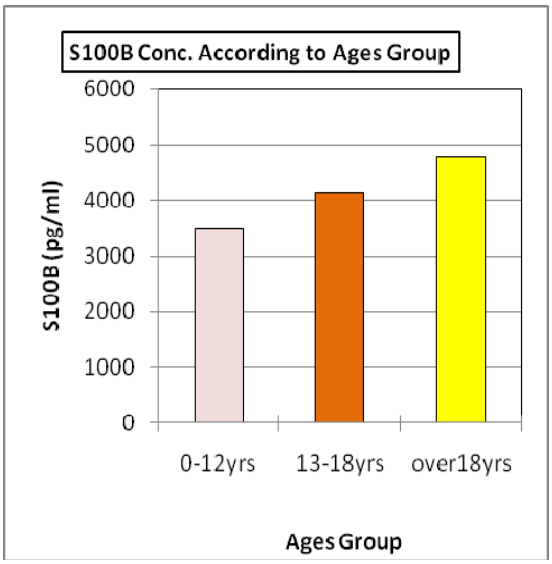


Fig. (4): Mean Values for S100B Conc. in Different Age group s of PKU

According to Severity of PKU:

The results revealed that there was no variation between different severity of PKU groups (F=2.998, P=0.104).

In Classic Phe severity. There was no significant positive correlation between S100B and both of Phe, IQ, Ca and ionized Ca^{2+} (r=0.251,P=0.0.484,r=317, P=0.373, r=0.330, P=0.352 and r=0.463, P=0.0.178 respectively). As well as in Moderate Phe severity, there was non significant positive correlation between S100B and both of Phe and IQ (r=0.490, P=0.262 and r=-0.0.233, P= 0.615 respectively), but there was non significant negative correlation between S100B and both of Ca and ionized Ca^{+2}(r=-0.083-, P=0.859 and r=-0.146-, P= 0.754 respectively)

According to Degree of Mental Retardation (Depended on IQ Score) in PKU:

The results indicated that the mean values ± SE of S100B in Borderline (B), Moderately (Mod) retarded and Severely (S) retarded of Mental retardation in PKU were 4898.5±112.5, 3941.9±252.71and 3380.1±411.26 pg/ml respectively with ranges of 4786-5011,3162-5128 and 1995-5248 pg/ml respectively, Statistically, there was no significant changes between these groups (F=0.05, P=0.826).

As well as the result showed one case have Mild Mental retardation and another one haven't IQ score.

4. Discussion

Phenylketonuria (PKU) is the most common inherited disorder of amino acid metabolism that arises from a single enzyme deficiency of phenylalanine hydroxylase, which converts the essential amino acid phenylalanine to tyrosine. Failure of this conversion results in the accumulation and elevation of phenylalanine in blood and tissues. Elevated concentrations of phenylalanine and its metabolites interfere with normal development of the central nervous system, leading to severe mental retardation. In PKU, plasma Phe concentrations may reach 400–1800 μmol/L and are harmful especially during the first year of life (Leader *et al.,* 2008).

Our study showed a significant negative correlation between concurrent Phe level (ranged from 7 to 37 mg\dl) and IQ score (ranged from 21 to 80) for the affected individuals' .Our results agreed with previous investigators (Antshel *et al*., 2003; White *et al*., 2002).

It is generally accepted that early-treated children with classic PKU suffer loss of IQ scores if the diet is discontinued, and children who are maintained on phe- restricted diet are more likely to achieve higher IQ scores and greater school achievement than those who discontinue the dietary management (Sharman *et al.,* 2009; Susan *et al.,* 2007).

On the other hand, another longitudinal study of intelligence among early-treated patients with PKU concluded that for each 300 µmol/l (5mg\dl) increase in phe level, IQ measured during pre-school years decreased by 0.5 SD (approximately 7 points) up to 10 years of age, after which time IQ remained reasonably stable (Burgard, 2007).

The measurement of biomarker of neural tissue damage such as S-100B protein may offer an alternative and direct indicator of cell damage in the nervous system when clinical and radiologic signs are not yet fully manifest and it is recommended to additional advantage of providing a quantitative

indicator of the extent of brain lesion (Steiner *et al.,* 2010).

Several reports have been published regarding the expression of S100B in patients with Phenylketonuria, Down's syndrome, in children with cerebral palsy or delayed development (Steiner *et al.,* 2010).

Previous authors reported that higher concentrations of serum S-100B protein in normal neonates and children compared to adults could reflect the ongoing central neuro-developmental processes occurring during these different stages of life (Mori *et al.,* 2009) the proliferation and maturation of glial cells, growth of neuritis and formation of synapses have been documented to be the most important functions of S-100B in morphogenesis (Tateihi *et al.,* 2006).

Our results showed a significant increase in S-100B levels in the affected cases and a significant correlation between S-100B and phe and total protein (TP) .Our data are in agreement with corresponding data reported by other investigators (Boneh *et al*., 2006; Alarcon *et al*., 2005; Foerch *et al.*, 2005; Marchi *et al*., 2004; Park *et al.,* 2004; Schulpis *et al.,* 2004).

Marchi *et al.,* (2004) reported that serum S100B is an ideal marker of blood-brain barrier integrity, because with a molecular weight of 21 kDa (S100B dimer) it may not penetrate through an intact blood-brain barrier. Furthermore, its concentration is high in central nervous system fluids and normally low in blood. Indeed, serum levels of S100B were directly correlated with an experimental damage of the blood-brain barrier.

One hypothesis is that S-100B accumulates in the extracellular space after astrocyte death or due to increased release by activated astrocytes, or after cellular disintegration of the damaged parenchyma. Under these conditions, the S-100B concentration may be in the micromolar range and the protein may become toxic due to its stimulatory effects on nitric oxide (NO) production by astrocytes and microglia. It interacts with receptor for advanced glycation end product (RAGE) on neurons and RAGE mediated neuronal apoptosis, or stimulates of interleukin-6 (IL-6) secretion by neurons (Alarcon *et al*., 2005). In this regard, it is noteworthy that RAGE is up regulated in many tissues including brain, during development and in the course of pathologic states i.e., under conditions in which many cells are destined to die by apoptosis. RAGE therefore may be an important progression factor in several disease states, and S-100B might be one crucial RAGE ligand in the brain (Alarcon *et al*., 2005).

Similarly, it was suggested that elevated S-100B levels are involved in the development of Down's syndrome; its expression was also correlated with the density of dystrophic neuritis with over-expressed β-amyloid precursor proteins (AβPP) (Medana *et al.,* 2007). Recent evidence indicates that high levels of S-100B in cerebrospinal fluid CSF increase the risk of repeated seizures in children with severe infection due to axonal injury (Medana *et al*., 2007).

Our results showed a significant negative association between S-100B and both Total Calcium (Ca) and Ionized Calcium (Ca^{2+}) in the affected cases although S-100B plays an important role in Calcium homeostasis. Our data agree with previous studies that reported similar findings (Wojda *et al.*, 2008; Mattson *et al*., 2004; Petzold *et al.,* 2003).

S-100B is involved in calcium homeostasis. For example, glial cells from mouse S100B null cerebellar cultures have been reported to display enhanced calcium transients in response to potassium chloride (KCl) or caffeine suggesting a role of S-100B in cytosolic calcium buffering. This observation is difficult to reconcile with the relatively low affinity of S-100B for calcium in vitro but one cannot exclude the possibility that normally S-100B modulates the activity of one or more effect or proteins (calcium channels/endoplasmic reticulum ATPase) that regulate the levels of cytosolic calcium with ensuing abnormality in calcium handling in the absence of S100B (Petzold *et al.,* 2003).

In conclusion, our findings support the hypothesis of a positive relationship between serum S-100B level and Phe blood level in PKU patients, and estimation of S-100B blood could be a useful informative peripheral marker monitoring the toxic effect of phe on central nervous system and direct indicator of cell damage in nervous system. Moreover, the combination of this marker with other diagnostic techniques may the way forward to improve the clinical assessment of patients with PKU.

Our study emphasized the correlation between early-dietary intervention and IQ score improvement in children with classic PKU.

Further studies with increase number of cases are highly recommended.

Corresponding author

Dr.Esmat Ashour
Biochemistry Dept.,
National Research Centre,
Dokki, Giza, Egypt.
e.mail. esmatashour3@yahoo.com.

References

- Alarcon R, Fuenzalida C, Santibanez M and von Bernhardi R 2005. Expression of scavenger receptors in glial cells. Comparing the adhesion of astrocytes and microglia from neonatal rats to urface bound beta amyloid. J Biol Chem 280:30406–30415.
- Albrecht J., Garbade S. F. , Burgard P.2009. Neuropsychological speed tests and blood phenylalanine levels in patients with phenylketonuria: A meta-analysis. Neuroscience and Biobehavioral Reviews 33 : 414–421
- Anderson P., Wood S., Francis D., Coleman L., Warwick L., Casanelia S., Anderson V., Boneh A. 2004. Neuropsychological functioning in children with early-treated phenylketonuria: impact of white matter abnormalities. Dev Med Cbild Neural 46:230-238.
- Antshel K, Waisbren S 2003. Timing is everything: executive functions in children exposed to elevated levels of phenylalanine, Neuropsychology 17: 458–468.
- Boneh A, Dorothy EM, Humphrey M, Upton J and Prters L. 2006. Three- year audit of the hyperphenylalaninemia/phenylketonuria spectrum in Victoria. Journal of pediatrics and Child Health 42: 496-498.
- Burgard P. 2007. Development of intelligence in early treated phenylketonuria. Eur. J. Pediatr. 159 :74–79.
- DeRoche K, Welsh M. 2008. Twenty-five years of research on neurocognitive outcomes in early-treated phenylketonuria: intelligence and executive function.Dev Neuropsychol; 33:474–504.
- Donato R. 2001.S100B: a multigenic family of calcium-modulated proteins of the EF-hand type with intracellular and extracellular functional roles. Int J Biochem Cell Biol; 33:637–68.
- Foerch C, Singer OC, Neumann-Haefelin T, du Mesnil de RochementR, Steinmetz H and Sitzer M. 2005. Evaluation of serum S100B as a surrogate marker for long-term outcome and infarct volume in acute middle cerebral artery infarction. Arch eurol 62:1130–1134.
- Gornal A.G, Bardawil C.J, David MM.1949. Determination of serum protein by means of the biuret reaction. J. Biol. Chem. 177: 751-766.
- Guthrie R. and Susi A..1963. A simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants. Pediatrics 32 (1963), pp. 318–34313.
- Heizmann CW, Cox JA. 1998 New perspectives on S100 proteins: a multifunctional Ca2_, Zn2_, and Cu2_-binding protein family. Biometals; 11:383–97.
- Heizmann CW. 1999. Calcium-binding proteins in the central nervous system. Neurochem Res; 24:1097–100.
- Hoeksma M, Reijngoud D.J, Pruim, H.W,. Paans A.M, van Spronsen F.J., 2009 Phenylketonuria: high plasma phenylalanine decreases cerebral protein synthesis, Mol. Genet. Metab.; 96 177–182.
- Ikura M. 1996. Calcium binding and conformational response in EF hand proteins. Trends Biochem Sci; 21:14–7.
- Kawasaki H, Nakayama S, Kretsinger RH. 1998. Classification and evolution of EF-hand proteins. Biometals; 11:277–95.
- Kretsinger RH, Nockolds CE. 1973 Carp muscle calcium-binding protein: II Structure determination and general description. J Biol.Chem; 248:3313–26.
- Leader b, Baca QJ, Golan DE. 2008. Protein therapeutics: a summary and pharmacological classification, Nature Reviews 7:21-39.
- MacDonald A, Asplin D. 2006. Phenylketonuria: practical dietary management. J Fam Health Care; 16:83–5.
- Marchi N., Cavaglia V., Fazio S. , Bhudia K. , Janigro D. 2004. Peripheral markers of blood-brain barrier damage. Clinica Chimica Acta, 342:1–12.
- Mattson MP. 2007. Calcium and neurodegeneration. Aging Cell. 6:337-350.
- Medana IM, Idro R, Newton CR. 2007. Axonal and astrocyte injury markers in the cerebrospinal fluid of Kenyan children with evere malaria. J Neurol Sci, 258:93-98.
- Menon PSN, Nair MKC, Mathur YC, Mukherjee D, Bhave S, Hathi GS, Sachdev HPS and Vijayakumar M. 2003. IAP Textbook of Pediatrics,2nd edition.
- Moore BW. 1965.A soluble protein characteristic of the nervous system. Biochem Biophys Res Commun; 19:739–44.
- Mori T, Tan J, Gary W, Koyama N, Nojima Y and Town T. 2009. Overexpression of Human S100B Exacerbates Brain Damage and Periinfarct Gliosis After Permanent Focal Ischemia. Strok (39): 2114-2121.
- Nishikawa T, Lee ISM, Shiraishi T, Ishikawa Y, Ohta M, Nishikimi I. 1997 Identification of S100b protein as copper-binding protein and its suppression of copper-induced cell damage. J Biol Chem.; 272:23037–41.

- Park IH, Yeon SI, Youn JH, Choi JE, Sasaki N, Choi IH, Shin JS. 2004.Expression of a novel secreted splice variant of the receptor foradvanced glycation end products (RAGE) in human brain astrocytesand peripheral blood mononuclear cells. Mol Immunol 40:1203–1211.
- Petzold A, Keir G, Lim D, Smith M, Thompson EJ. 2003. Cerebrospinalfluid (CSF) and serum S100B: release and wash-out pattern. Brain ResBull 61:281–285.
- Rohr F.J., Allred E.N., Turner M., Simmons J. and Levy H.L. 1996 Use of the Guthrie bacterial inhibition assay to monitor blood phenylalanine for dietary treatment of phenylketonuria. Screening 4 :. 205–211.
- Sarkar RC, Chauhan SP. A new method for determining micro quantities of calcium in biological materials. Anal Biochem 1967;20:155-156
- Schafer BW, Fritschy JM, Murmann P, Troxler H, Durussel I, Heizmann CW, and Cox JA. 2000. Brain S100A5 is a novel calcium-zinc-, and copper ion-binding protein of the EF-hand superfamily. J Biol. Chem; 275:30623–30.
- Schulpis KH, Kariyannis C, Papassotiriou I. 2004. Serum levels of neural protein S-100B in phenylketonuria.Clin Biochem. ;37(1):76-79.
- Scriver CR. 1998. Commentary on: A simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants, by Robert Guthrie and Ada Susi, 20: 120-125.
- Scriver, C.R., 2007. The PAH gene, phenylketonuria, and a paradigm shift. Hum.Mutat. 28 (9), 831–845.
- Sharman R, Sullivan K, Young R, McGill J. 2009. Biochemical markers associated with executive function in adolescents with early and continuously treated Phenylketonuria. Clin Genet, 75: 169-174.
- Sitta A ., Alethe´a G., Barschak D., Amanda T., Barden A., Giovana B., Biancini A., Paula R., Carolina F. de Souza A., Netto C ., Wajner M , Carmen R. 2009. Effect of short- and long-term exposition to high phenylalanine blood levels on oxidative damage in phenylketonuric patients. Int. J. Devl Neuroscience 27 : 243–247.
- Steiner J., Myint M., Schiltz K., Westphal S., Bernstein H., Walter M., Matthias L., Markus J., Bogerts B. 2010. S100B SerumLevels in Schizophrenia Are Presumably Related to Visceral Obesity and Insulin Resistance. Cardiovascular Psychiatry and Neurology 11:1-11.
- Tateishi N, Shimoda T, Yada N, Shinagawa R and Kagamiishi Y. 2006. S100B: astrocyte specific protein, 26(1):11-6.
- Thorndike, R. L., Hagen, E. P., & Sattler, J. M. 1986. Stanford-Binet Intelligence Scale: Fourth Edition. Itasca, IL: Riverside Publishing.
- Tsoporis N., Izhar S., Leong-Poi H., Desjardins F., Huttunen J., Parker G. 2009. S100B Interaction With the Receptor for Advanced Glycation End Products (RAGE). A Novel Receptor-Mediated Mechanism for Myocyte Apoptosis Postinfarction. American Heart Association;10: 35-42.
- van Spronsen F.J., Hoeksma M., Reijngoud D.J., 2009. Brain dysfunction in phenylketonuria: is phenylalanine toxicity the only possible cause? J. Inherit. Metab. Dis. 32 :46–51.
- Waisbren SE, Noel K, Fahrbach K, Cella C, Frame D, Dorenbaum A, Levy H.. 2007 Phenylalanine blood levels and clinical outcomes in phenylketonuria: a systematic literature review and meta-analysis. Mol Genet Metab; 92:63–70.
- Weetch E, MacDonald A. 2006 The determination of phenylalanine content of foods suitable for phenylketonuria. J Hum Nutr Diet; 19:229–36.
- Weichselbaum TE. 1946. Quantitative colorimetric determination of total protein in serum. Am J Clin Pathol 7:40-42.
- White D, Nortz M, Mandernach T, Huntington K, Steiner R. 2002. Age-related working memory impairments in children with prefrontal dysfunction associated with phenylketonuria, J. Int. Neuropsychol. Soc. 8:1–11.
- Wojda U, Salinska E, Kuznicki J. 2008. Calcium ions in neuronal degeneration. IUBMB Life, 60:575-590.
- Zeisler, E. B., Determination of diffusible serum calcium. 1954. Am. J. Clin. Pathol. 24(5), 588-593.

11/6/2010

Changes in Biochemical and Isozymes Components of Watermelon seeds during accelerated Ageing Technique

Magdy M. Rashed[1]; Magdy shallan[1]; Mahmoud Fathy[2]; and Anisa I. Ismail[2]

[1]Bio Chemistry Department, Faculty of Agriculture, Cairo University, Giza,Egypt
[2]Department of Seed Technology, Horticulture Research Institute, A R C, Ministry of Agriculture, Giza, Egypt

Abstract: The aims of this work was to study some changes in the total content of storage components of watermelon (*citrullus lanatus*) seeds during accelerated ageing technique and its relation to seeds viability. **Materials and Methods**: Before the experiment, seeds were stored for two years in store house at 25C° in the start experiment, ageing at 50°C with 17% moisture up to 24, 48, 72, and 96 hours respectively. Germination percentage was decreased, a reduction in the total content of storage components such as proteins, carbohydrates, in addition, increasing oils and decreases in the activities of various esterase enzymes under the same condition were observed. **Results:** It was clearly that 50°C with 17% moisture content could be used as a good ageing seed testing condition for watermelon seeds In the present study The treatments watermelon seeds could be identified by Biochemical analysis (Esterase isozyme and Protein) banding pattern.
[Magdy M. Rashed; Magdy shallan; Mahmoud Fathy; and Anisa I. Ismail. **Changes in Biochemical and Isozymes Components of Watermelon seeds during accelerated Ageing Technique.** Journal of American Science 2010;6(12):979-985]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: Watermelon, accelerated ageing seed, seed germination.

1. Introduction:

Watermelon (*citrullus lanatus)* is one of important horticultural crops belonging to the family cucurbitaceae mainly propagated by seeds. Ageing tests have been developed to predict emergence performance , seed vigor and comparing seed lost quality **(Yanmaz *et al.*, 1999; Matthews, 1994; McDonald, 1980).** The deterioration of the stored seed is a natural phenomenon and the seeds tend to loose viability even under ideal storage conditions **(Ayyappan *et al.*, 2006; Bhatti and Sata, 1997).**

The seeds with low viability are rejuvenated /multiplied. Frequent multiplication results in genetic drift due to which genetic integrity is impaired, it also involves high risk of out crossing and mechanical mixture during multiplication, so it is very important to prolong the seed longevity. There are number of factors that affect seed longevity in storage **(McDonald, 1999; Powell, 1998)**. Among these factors are temperature and seed moisture content/relative humidity. There are some other factors which can decrease seed longevity, e.g. varietal differences in seed viability.. Therefore, it is important to assess seed vigour and viability during storage. Seed ageing as an important parameter to assess /estimate the seed viability and vigour accelerated ageing which is a good vigour test for various crop seeds **(Anonymous,1983)** and could be used to predict storability of seed lots **(tyagi,1992)**.

Accelerated ageing treatments involve exposing seeds to severe adverse storage conditions, i.e.-raised temperature and high moisture contents for specific periods of time.

The aims of this work was studying the changes in the total content of storage components of watermelon seeds during accelerated ageing technique and its relation to seed viability, in order to reduce the time of storage experiments (which took a long time) to know the extent of changes in content of storage components of seeds.

2. Materials and Methods:

21. Materials

1) Plant material and ageing conditions:-

Watermelon (*citrullus lanatus l.cv giza1*) seeds were collected from plants grown in Moshtohr field at Horticulture Research Institute, 2007.

Seeds were stored for 2 years in store house at 25±-2°C before the experiment started. They were soaked in water for 24 hours at 4°C then air dried for 4 hours. During the experiment, seeds were aged at 50°C and 17% moisture content, stored up to 96 hours (4 days).

Samples of watermelon seeds were collected every 24 hours.

2.2. Methods:

2.2.1. Germination tests:

Germination tests were carried out on four replicates of 25 seeds. The seeds were set to germinate in between moistened paper towels. Seeds were kept at 25°C±2 for 14 days. The numbers of germinated seeds were counted daily up to 14 days in watermelon seeds. At the final count the number of normal and abnormal seedling. Then survival curves were constructed from these results, germination percentage, were recorded.

2.2.2. Determination of the total carbohydrate:

Total carbohydrates were extracted according to **A.O.A.C. (1990)** 0.1 g of air-dried sample was hydrolyzed with 1 N HCl by refluxing for 6 hrs in a boiling water bath. The obtained solution was filtered, neutralized and the total volume was made up to 100 ml with distilled water. Resulted total reducing sugars was determined calorimetrically using 1 ml of sample with alkaline potassium ferricyanide reagent. The amount of total carbohydrates was determined according to the standard curve of glucose**.**

2.2.3. Determination of total protein:

The dried parts of the plants were used. Total nitrogen in plant was determined based on micro-Kjeldahl method accorded to **Markaham (1942)**, using boric acid modification as described by **Ma and Zuazage (1942),** under steam distillation using Buchii 320 unit, and was calculated as nitrogen percent.

The protein content was calculated as follows: Protein% = Nitrogen% x 6.25.

2.2.4. Determination of total oil:

Seed Oil percentage in dry seeds was determined using Soxhlet apparatus and petroleum ether as a solvent according to **A. O. A. C. (1970).**

2.2.5. Biochemical Markers

2.2.5.1. Protein analysis

SDS-Polyacrylamide gel electrophoresis was performed in 12 % acrylamide slab gels following the system of **Laemmli (1970)** to identify their protein profiles. Young fresh leaves were collected from all studied plants and immediately ground in a mortar using liquid nitrogen. The seeds were grounded to a fine powder using a mortar and pestle, homogenized with 1 M Tris-HCl buffer; pH 6.8 in clean eppendorf tube, left in refrigerator over night, then, centrifuged at 10000 rpm for 10 min. The supernatant of each sample (contains protein extract) was kept in deep-freeze until use for electrophoretic analysis, then boiled for 5 minutes in water bath before loading in the gel.

2.2.5.2. Isozymes Electrophoresis

Native polyacrylamide gel electrophoresis was used to study isozyme variation among the ten populus accessions. The staining solution was composed of 50 ml of 1M Na-Acetate; pH 4.7, 50 ml of Methanol, 50 ml 3,3,5,5 tetra-methylbenzidine (TMBZ) and 2 ml of 30% H_2O_2 while for esterase (Est.) the staining solution composed of 50 ml of 100 mM Na – Phosphate; pH 6.0, 25 mg of α – Naphthyl Acetate and 50 ml of fast blue RR salt according to **Scandalios (1964).**

4. Results:

4.1. Effects of an Accelerated Ageing Technique on chemical Components of Watermelon seeds on total protein ,total carbohydrates, and total oil contents are illustrated in table (1) and figures (1, 2 and 3)

Table (1): Effect of Ageing on total protein (%), total carbohydrate (%) and total oil (%) in watermelon seeds.

Treatment	Total protein (%)	Total carbohydrate (%)	Total oil (%)
Untreated treatment	43.75 a	4.06 a	49.34 c
50 °C with 17% moisture for 24 hours	41.15 b	3.75 b	50.6 b
50 °C with 17% moisture for 48 hours	40.35 b	3.52 c	51.4 b
50 °C with 17% moisture for72 hours	38.9 c	3.34 c	52.4 a
50 °C with 17% moisture for 96 hours	37.75 d	3.08 d	53 a

Values in the same column followed by the same letter(s) do not significantly differ from each other according to Duncan's multiple range tests at 5% level.

Data in table 1 and Fig (1) showed high significant differences in watermelon seeds between untreated treatment seeds and accelerated ageing treatment seeds total protein was significantly decreased in ageing seeds for 50 C° with 17% moisture for (4 days) from 43.75% to 37.75%.

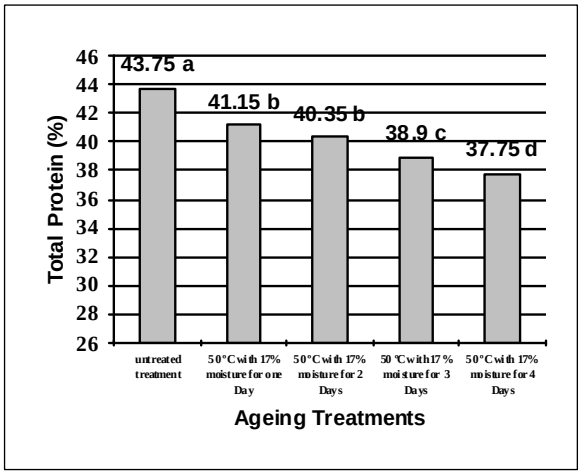


Fig 1: Changes in total protein of watermelon seeds before and during accelerated ageing technique.

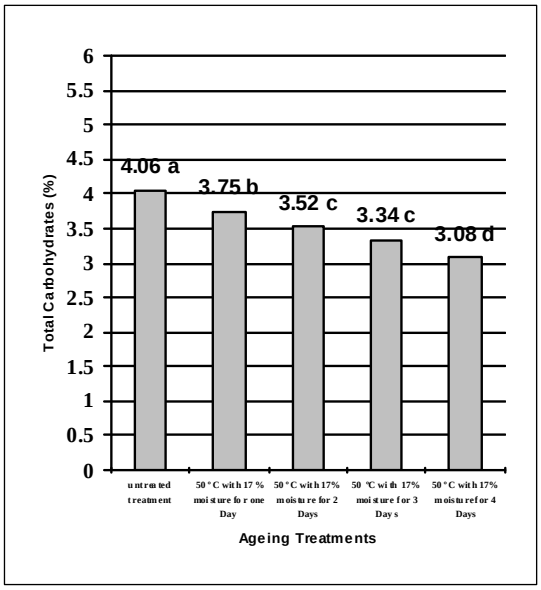


Fig 2: Changes in total Carbohydrate of watermelon seeds before and during accelerated ageing technique.

It is obvious from results shown in fig (3) that significant increase in total oil were detected due to accelerated ageing of watermelon seeds compared with untreated treatment. Total oil (53 %) were obtained from the high level of 50 ºC with 17% moisture for 4 Days while the lowest level (49.34 %) were obtained from untreated treatment.

4.2. Effects of an Accelerated Ageing Technique on Germination percentage of Watermelon Seeds.

Table (2) shows that the germination percentage of watermelon seeds decreased from 60% in untreated treatment seeds to 24% for aged seeds, after 96 hours (4 days).

Treatment	Germination (%)
Untreated treatment	60 a
50 ºC with 17% moisture for 24 hours	48 b
50 ºC with 17% moisture for 48 hours	37 c
50 ºC with 17% moisture for72 hours	30 d
50 ºC with 17% moisture for 96 hours	24 e

4.3. Effects of an Accelerated Ageing Technique on Electrophoretic banding patterns of proteins of Watermelon seeds. "Biochemical markers"

4.3.1. Watermelon seeds protein electrophoresis.

The watermelon seeds protein banding profile which was separated by using SDS-PAGE are illustrated in fig. (4). The total number of bands was 14 with molecular weights ranged from 18.4 KDa to 116 KDa. The highest number of bands was 13, detected in clone no. 1 (control) and clone no. 2 (while the lowest number of bands was 11, identified in *clone* no 4 and clone no. 5.

Fig. (4), is a dendrogram which demonstrated the distance between watermelon seeds under investigation.It showed that treatments of seeds watermelon were separated into two major groups at a distance of 2.5.

The first group included clone no. 4.

The second group involved each clone no. 1 and clone no. 2 was delimited from the first group at a distance of 10.75, while clone no. 3 was delimited, as well, from clone no. 1 and clone no. 2 at a distance of 6.75.

Demonstrative analysis of the presence and absence of bands were assessed with (1) and (0), respectively, are illustrated in Table (3). It is observed that 10 bands were mono-morphic (106.886, 85.898, 75.31, 59.478, 53.786, 44.411, 76.495, 71.709 and 70.015KDa), while 4 bands were polymorphic, giving 28.57% polymorphism. The matrix of similarity index for watermelon seeds is presented in Table (4). The

highest coefficient was 95.2 recorded between *clone no. 4 and clone no. 5* followed by 88.9 recognized between clone no.1 (control) and clone no. 2, followed by 84.2 between clone no.1 (control) and clone no.3 and clone no. 2 and clone no.3 On the other hand, the lowest coefficient value was 60 observed between clone no.1 (control) and clone no.4.

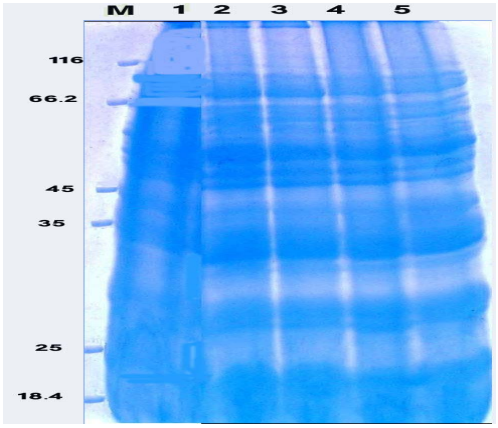


Fig. (3): SDS-protein banding patterns of seeds proteins
Clone no 1- untreated treatments
Clone no 2- ageing at 50C° with 17% moisture for one day
Clone no 3- ageing at 50C° with 17% moisture for two day
Clone no 4- ageing at 50C° with 17% moisture for three day
Clone no 5- ageing at 50C° with 17% moisture for four day

Table (3) Data matrix illustrating the presence or absence of bands in the seeds protein electrophoresis *banding patterns*.

No. bands	MW	Untreated treatment	Ageing at one day	Ageing at two day	Ageing at three day	Ageing at four day
1	106.886	1	1	1	1	1
2	85.898	1	1	1	1	1
3	75.31	1	1	1	1	1
4	68.765	0	0	0	1	1
5	60.64	1	1	0	0	0
6	59.478	1	1	1	1	1
7	53.786	1	1	1	1	1
8	44.411	1	1	1	1	1
9	33.483	1	1	1	0	0
10	76.495	1	1	1	1	1
11	76.444	1	1	1	1	1
12	71.709	1	1	1	1	1
13	70.015	1	1	1	1	1
14	18.064	1	1	1	0	0

Table(4) Proximity Matrix of protein results

	Untreated treatment	Ageing at one day	Ageing at two day	Ageing at three day	Ageing at four day
Untreated treatment		0.889	0.842	0.600	0.632
Ageing at one day	0.889		0.842	0.700	0.737
Ageing at two day	0.842	0.842		0.762	0.700
Ageing at three day	0.600	0.700	0.762		0.952
Ageing at four day	0.632	0.737	0.700	0.952	

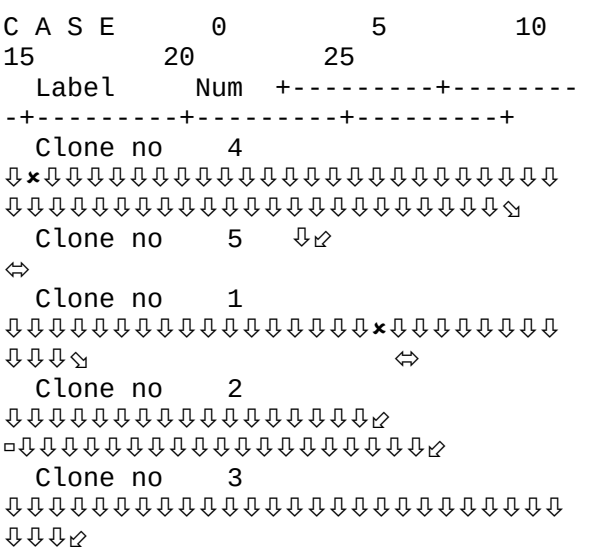


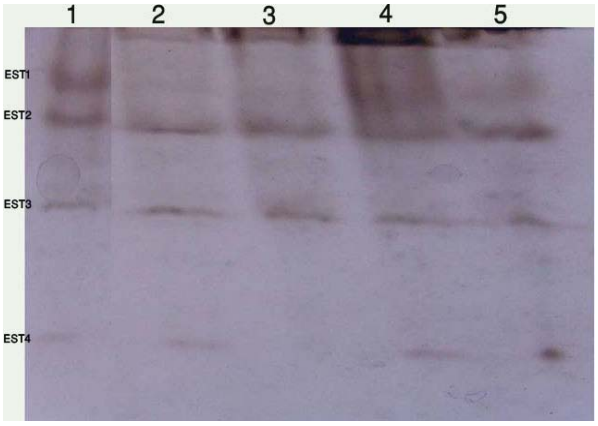
Fig (4) Dendrogram for the seeds treatment and untreated treatment of the protein data using UPGMA and Similarity matrices computed according to Dice coefficients.

Based on the similarity matrix developed by analyzing only the common bands between the different accessions representing each seeds a dendrogram (Fig. 5) was constructed. The obtained dendrogram was divided into two main clusters; one cluster included. clone no. 4 and 5, while the other one included 1, 2, and 3, the cluster no. 2 divided to two subcluster one of them included clone no. 3 while the other subcluster included clone 1 and 2 .the highest relationships was between clone no. 4 (ageing at 50C° with 17% moisture for three day) and clone no. 5 (ageing at 50 C° with 17% moisture for four day). It was found. The highest variation between clone no 1(untreated treatment) and clone no 4, 5 that indicate to the effects of accelerated ageing on banding patterns of proteins of watermelon seeds.

4.3.2. Esterase banding patterns:

Esterase banding patterns was illustrated in Fig. (6) and dendogram in fig (7).

Fig (6) represents esterase electrophoretic banding patterns among examined seeds, a total of 4 bands were identified in this study, which were presented in some seeds treatment and absent in some others. The analysis of data showed 3 bands were polymorphic with 75 % polymorphisms.



Clone no 1- untreated treatments
Clone no 2- ageing at 50C° with 17% moisture for one day
Clone no 3- ageing at 50C° with 17% moisture for two day
Clone no 4- ageing at 50C° with 17% moisture for three day
Clone no 5- ageing at 50C° with 17% moisture for four day

Fig. (6) Electrophoretic profiles of the esterase enzyme system

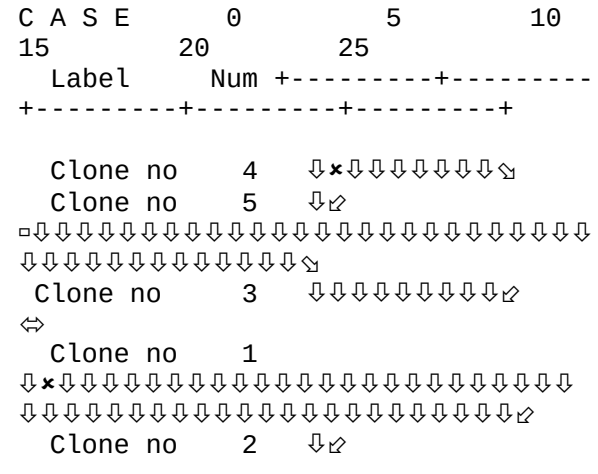


Fig. (7) Dendrogram for seeds treatment and untreated treatment of the esterase data using UPGMA and Similarity matrices computed according to Dice coefficients.

Estrase isoenzyme polymorphism detected in seeds watermelon (Fig.6), was represented by four zones of estrase activity Est.1, Est.2, Est.3 and Est.4 (in the order of increasing mobility from cathodal end). Results of seeds watermelon treatments showed that Est.1 was appeared in all treatments, results of zone Est.2 , Est.3 showed that new isoenzyme bands appeared and other disappeared, the highest close was between clone 1 (control) and clone 2, Est.3 showed decrease in its band between clone 3 and clone 4 with control.

Finally, the results obtained suggest that, the clone no. 1 (control with very close with clone 2 (Ageing at one day).

Based on the similarity matrix developed by analyzing only the common bands between the different accessions representing each seeds treatment and untreated treatment a dendrogram was constructed. The obtained dendrogram was divided into two main clusters; one cluster included. clone no. 1 and 2, while the other one included 4, 5, and 3 , the cluster no. 2 divided to two subcluster one of them included clone no. 3 while he other subcluster included clone 4 and 5. The highest relationship was between clone no. 1 (untreated treatment) and clone no. 2 (ageing at 50c with 17% moisture for one day). It was found. The highest variation between clone no 1(untreated treatment) and clone no 4, 5 that indicate to the effects of accelerated ageing on Esterase banding patterns.

4. Discussion:

The process of deterioration which occurs under these special ageing conditions is assumed to be similar to those which occur during natural ageing **(Delouche and Baskin, 1973; Perl *et al.*, 1978)**. The main difference being the speed at which these changes occur. A reduction in the total content of storage components such as proteins and carbohydrates **(Ayyappan *et al.*, 2006 and Agnieszka. *et al*., 2010)**. Increase in total oil were detected due to accelerated ageing (**Maqsood *et al.*, 2000).**

Results recorded high significant differences in watermelon seeds between untreated treatment seeds and accelerated ageing treatment seeds .Concerning total protein and total carbohydrate values, it was significantly decreased in ageing seeds for 50 C° with 17% moisture for (4 days). These results were in agreement with **Ayyappn *et al.*, 2006)** who found that total protein was decreased to half of initial content in cucumber seeds at 8th day of ageing as previously reported (**Ravikumar *et al.*, 2002)**, while the amount of free amino acids increased

gradually as previously reported (**Coolbear *et al.*, 1984**; **Ravikumar *et al.*, 2002)**. This increase in the amount of the free amino acids may be due to the hydrolysis of proteins during ageing.

Feeney and **Whitaker,(1982)**; **Ayyappn *et al.*, (2006)** who reported that the reduction of sugar content might be due to hydrolysis proved by Amadori and Maillard reactions. Starch. As well as wheat seed did not increase total carbohydrate levels as a result of accelerated ageing, on the contrary the amount of carbohydrates in these seeds slightly decreased **(Agnieszka., *et al* 2010)**.

In contrast, our results showed significant increase in total oil were detected due to accelerated ageing of watermelon seeds compared with untreated treatment. These results were harmony with (**Crowe *et al.* 1989**) who showed that addition of total fatty acids increased fusion of plant vesicles which led to an increase in membrane leakage. (**Copeland** and **McDonald 1995**) reported that continual accumulation of total fatty acids culminates in a reduction of cellular pH and is determined to normal cellular metabolism. Further more, it denatures enzymes resulting in loss of their activity. Individual cotton seeds containing 1% or more of free fatty acid usually will not germinate. The germination percentage of watermelon seeds decreased from 60% in untreated treatment seeds to 24% for aged seeds, within 4 days. The results were in agreement with **Yanmaz *et al.*, 1999** and **Alsadon *et al.* (1995)** found that cucumber seeds showed 82% of germination after storing at 24% moisture content and 45°C for 72 hours. In our experiment, at 17% moisture content and 50°C, cucumber seeds lost viability after the same period. Obviously, little variation in moisture and temperature had great effect on viability as pointed by **(Ellis and Roberts 1980)**.

Enzyme activity of esterase isozyme was parallel with the extent of germination of those seeds, since these activates owes mainly to esterase of endosperm. It was suggested that the endosperm of aged seed retains normal responsibility to the growth of seedling even if it was 2 years old (**Momotani, *et al.*, 1989**).

5. References

1. **O. A. C. (1970)**. Official methods of analysis. Of the Association Official Analysis Chemists, Th ed. Washington. D. C., USA. Pp. 94-117.
2. **O. A. C. (1990**). Official methods of analysis of the association of official analytchemists 15th Ed. Published by the association of official analytical chemists, INC suite 400, 2200 Wilson Boulevard. Arlington, Virginia. 22201 USA.
3. **Agnieszka, P. Cieslak, M. Niedzielski, D. Michalczyk, W. Luczak** and **B. Adomas. (2010**) Soluble carbohydrates in cereal (Wheat, Rye, Triticale) seed after storage under accelerated ageing condition, Acta Societatis Botanicorum Poloniae Vol. 79, No. 1: 21-25.
4. **Anonymous, A.,** 1983. Association of official seed Analysts, seed vigour testing Handbook of seed testing.
5. **Ayyappan, V.; G. Andy, S. Natesan, C. Won Choi** and **M. Markandan. (2006)** changes in L-isoaspartyl Methyltransferase, storage components and Anti-oxidant Enzymes Activities during Accelerated Ageing in Cucumber (Cucumis sativus L.) seeds. Journal of Plant Sciences (3):228-239.
6. **Bhatti, M. S.** and **D.H. Sata, 1997**. Viability testing, Annual Report, Plant genetic resources. Institute, Nat Agric. Res. Center, Islamabad.
7. **Coolbear, P., A. Francis** and **D. Grierson, 1984**. The effect of low temperature, pre-sowing treatment on the germination and membrane integrity of artificially aged tomato seed. J. EXP. Bot., 35: 1609-1617.
8. **Copeland, L. O.** and **M. B. McDonald, 1995**. Principles of Seed Science and Technology. Chapaman and Hall, p. 115. Fifth Avenue, New York, USA.
9. **Crowe, J. H., B. D. Mckerisie** and **L. M. Crowe,** 1989. Effects of free fatty acids and transition temperature on the stability of dry liposome. Biochem. Biophys. Acta, 979: 7– 10.
10. **Delouche, J. E.; and Baskin, C.C. (1973)** Acceleraled aging techniques for predicting the relative storability of seed lots. Seed Science and Technology.1: 427-452.
11. **Ellis, R. H.** and **Roberts, E.H., 1980.** Towards a Rational basis for testing seed quality. In seed production (Ed.P.D.Hebblethwaite). P605-636, London Buttterworths.
12. **Feeney, R. E.** and **W.H. Whitaker, 1982,** the Millard and its Prevention in Food Protein Deterioration. Cherry, J.P. (ED), AM. Chem. Soc. Washington, pp: 201-230.
13. **Laemmli, U. K.** (**1970)** cleavage of structural proteins during the assemble of the **head of bacteriophage T4. Natura, 227:680-685.**
14. **Ma, T.S. and Zuazage, C. (1942): Micro – Kjeldahl determination of nitrogen, a new** indicator and an improved rapid methods Industr. Eng Chem. Anal. Ed.
15. **MAQSOOD, S. A. B, Khalil. U. R** and **Sajjad. I. (2000)** Cotton Seed Deterioration: Assessment of some Physiological and Biochemical Aspects, International Journal of Agriculture & Biology. 3:195–198.

16. **Markaham, R. (1942).** A steam distillation apparatus for micro-Kjeldahl analysis. Biochemical. J. 36, 790.
17. **Mc Donald, M. B. JR., 1980.**Assesment of seed quality. Hort Science.15:784-788.
18. **Momotani, Y, Takumi. W** and **Toshihiko. Y, 1989**. Isozymes of α Amylase, Esterase and Peroxidase in aged Rice Seed. Bull. Univ. Osaka Pref., SER. B. Vol. 41.
19. **Perl, M., Luria, I.** and **Gelmond, H. (1978**) Biochemical changes in sorghum seeds affected by accelerated aging. Journal of Experimental Botany, 29: 497-509.
20. **Powell, A. A., 1998**.seed improvement by selection and invigoration.sci. Agric.
21. **Ravikumar, R., G. Ananthakrishnan, S. Girija** and **A. Ganapathia, 2002**. Seed viability and biochemical changes associated with accelerated ageing in Dendrocalamus strictus seeds. Biol. Plant, 45:153-156.
22. **Scandalios, J. C. (1964).** Tissue- specific isozyme variations in maize. J. of Heredity, 55: 281-285.
23. **Tyagi, C. S., 1992.** Evaluation of storability of soybean seed. Seeds and farms. 18:8-11.
24. **Yanmaz, R.; I. Demir** and **M. Ozcoban. (1999)** determination of appropriate ageing test conditions for cucurbit seeds. Acta Hort. 492:281-286.

10/29/2010

Effectiveness of Low Power Laser Therapy and Betamethasone in Minimizing Postoperative Edema and Trismus after Third Molar Surgery: a Clinical Trial

Dalia A. Radwan[1], Nermeen H. Mohammed[1], Ahmed A. Zaky[2]

[1] Oral and Maxillofacial Surgery, Faculty of Oral and Dental Medicine, Cairo University.
[2]Dental Laser Application, National Institute for Laser Enhancement Science, Cairo University

Corresponding author: dradwan@link.net

Abstract: Purpose: In this study the therapeutic low-power laser (LPL) and Betamethasone (as an anti-inflammatory) were compared in terms of their effects on edema and trismus associated with surgical removal of impacted mandibular third molars. **Material and methods:** 20 healthy patients divided into two equal groups were included in the study. Group (I) received LPL irradiation (energy output 6 J/cm^2 with constant power density of 100 mW, wavelength 980 nm) on the 1st and 3rd postoperative days. Group (II) received a single dose of 4 mg systemic intramuscular Betamethasone Sodium Phosphate (Diprofos) into the gluteal region immediately after suturing of the surgical wound. Both groups received the usual medical and physical postoperative recommendations. **Results:** LPL irradiation (group 1) showed remarkable reduction of postoperative edema on the 3rd postoperative day. In addition, no significance difference resulted on comparing this effect between both groups. Postoperative trismus was nearly the same in both groups. No adverse effects of the procedure or medication were observed. **Conclusion**: LPL therapy is effective than systemic Betamethasone in reducing postoperative edema after third molar surgery without statistical significant differences. However both treatment modalities have the same effect on postoperative trismus.
[Dalia A. Radwan[1], Nermeen H. Mohammed[1], Ahmed A. Zaky. Effectiveness of Low Power Laser Therapy and Betamethasone in Minimizing Postoperative Edema and Trismus after Third Molar Surgery: a Clinical Trial. Journal of American Science 2010;6(12):986-989]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: edema, trismus, low power laser therapy, Betamethasone.

1-Introduction:

Postoperative trismus and edema are consequences of tissue injury during surgery. The raising of muscular attachments results in reflexive cramp of the masticatory muscles, Jovanovic (1998), and direct trauma to the blood and lymph vessels, Petkovic and Bukurov (1987).

These conditions cause limitation of the mouth opening and fluid accumulation in the interstitial area as a result of its transudation from injured blood vessels and fibrin obstruction of lymph drainage Petkovic and Bukurov (1987). The destruction of the local tissues and severity of surgical intervention are in direct proportion to the presence of these postoperative sequelae, Gonzalez-Santana et al., (2005). These complications reach their maximal at 12–48 h after surgery, but may completely resolve in 5-7 days, Sowray (1986).

Several trials have been used to prevent the occurrence of these complications by means of preoperative and postoperative administration of corticosteroids, as they reduce the leakage of lymph and thereby the transudation of liquid. Also, non-steroidal anti-inflammatory drugs were administrated postoperatively as well as cold compresses and analgesics, Carrillo et al., (1990); Honmura et al., (1992); Tuner and Hode (1996).

The use of low power lasers (LPLs) had attracted attention. As it induces primary (photochemical, photoelectrical, and photoenergetic) and secondary biostimulation (stimulation of cell metabolism and microcirculation), Berns et al., (1990); Abt (1995); Barabash et al., (1995) and Miserendino et al., (1995). Thus it has a direct effect on lymph and blood vessels, with no adverse effects of irradiation, Lievens (1991).

So the aim of this study was to compare the effectiveness of LPL and intramuscular anti-inflammatory; Betamethasone, in minimizing postoperative edema and trismus after surgical removal of impacted lower third molars under local anesthesia (2% Mepevacaine hydrochloride/1:20,000 Levonordephrine).

2. Material and Methods

2.1. Materials:

2.1.1. Samples:

Twenty patients with the same difficult condition of mandibular third molar teeth composed the sample of this study. They were of both sex, aged 25-30y.

Clinical investigations of the LPL and Betamethasone anti-edematous and anti-trismus effects were conducted at the Oral Surgery Clinic, Faculty of Oral and Dental Medicine, Cairo University, Egypt.

The patients were divided into two equal groups.

2.2. Methods:

2.2.1. Surgery

Surgical odontectomies were performed by only one, experienced surgeon and the duration of surgery was similar in the two investigated groups, being most frequently 30 min on average.

2.2.2. Low-power laser (LPL) treatment

Patients of group (I) received GaAlAs LPL (Quanta system, Italy) sessions on the first and third postoperative days. The laser tip was directed intra-orally from a distance of 1 cm away from the surgical wound, at the buccal, distal and medial surfaces of the extraction sockets. Each surface was stimulated for 3min. The energy output was 6 J/cm^2, with constant power density of 100 mW, and wavelength of 980 nm.

2.2.3. Systemic intramuscular Betamethasone treatment

Group (II) received a single dose of 4 mg systemic intramuscular Betamethasone Sodium Phosphate (Diprofos) into the gluteal region immediately after suturing of the surgical wound. Both groups received the usual medical and physical postoperative recommendations.

2.2.4. Measurements:

The size of postoperative edema and trismus were registered in cm on the third and seventh postoperative days (the baseline level was recorded preoperatively).

2.2.4.1. Measurements of edema coefficient (Ec)

The distance between the tip of tragus and the lip commissure at the same side was measured using a graduated tape and the edema coefficient (Ec) calculated using modified formula of Carrillo et al., (1990).

Ec = postoperative distance - preoperative distance /preoperative distance x 100.

2.2.4.2. Measurements of trismus coefficient (Tc)

The trismus was determined by measuring the maximum interincisal mouth-opening ability of the patients by a sliding caliper and the trismus coefficient (Tc) calculated using Carrillo el al., (1990 formulae.

Tc= preoperative distance - postoperative distance /preoperative distance x 100.

2.2.5. Statistical analysis

Statistical analysis of edema and trismus coefficient differences between both groups was performed using the non-parametric Wilcoxon rank test.

3. Results

None of the patients showed any adverse reactions to the applied treatments.

From (Table 1) it was observed that, the average value of edema coefficient in laser stimulated patients on the 3rd postoperative day was noticeably lower than its correspondence in the Betamethasone injected ones, being 1.59 and 10.39 cm respectively. On the 7th postoperative day the average edema coefficients of both groups were almost the same.

Table 1: Comparative survey of postoperative edema coefficients in the investigated groups of patients.

Follow up intervals	Average edema coefficient in Laser group	Average edema coefficient in Betamethsone group	p-value
3rd postoperative day	1.59	10.39	0.5553
7th postoperative day	2.68	3.40	0.2538

It was noticed from table (2) that the average postoperative trismus coefficient did not show remarkable difference between both groups on the 3rd and 7th postoperative days.

Statistical analysis of edema and trismus results (Wilcoxon rank test) pointed to a non significant difference between both groups on the 3rd and 7th postoperative days.

Table 2: Comparative survey of postoperative trismus coefficients in the investigated groups of patients

Follow up intervals	Average trismus coefficient in Laser group	Average trismus coefficient in Betamethsone group	p-value
3rd postoperative day	23.47	26.33	0.7114
7th postoperative day	13.91	16.78	1.0000

4. Discussion

It is well known among oral surgeons that empirically, postoperative edema and trismus can always be expected after impacted lower third molar surgery. Operative trauma could be presumed to be fairly similar in all the study groups with regard to the need for tooth separation, drilling in bone, and duration of surgery. Both groups received their medical and physical postoperative rescue therapy, it seems reasonable to attribute the favourable results in reduction of postoperative edema or trismus primarily to the effectiveness of the treatment methodology.

Regarding the efficiency of LPL and steroids in reducing postoperative sequelae, many studies proved their anti-edematous and anti-trismus effects on experimental and clinical conditions Goldman(1980); Honmura et al., (1992). LPL is believed to induce an increase in number and diameter of lymph vessels, with a simultaneous decrease of blood vessel permeability Lievens (1988; 1991). The use of steroids, aid in inhibition of phospholipase A2 enzyme, which reduces the release of arachidonic acid in the cells of the inflamed focus. This will consequently decrease prostaglandins and thus reduces these postoperative complications, Huggman (1977). It is believed that single parenteral use of steroids, regardless of the dose, does not exert an undesired effect on the adrenal-pituitary regulation of natural steroid secretion, Montgomery (1990) ,thus implicating their safe use for this indication. The course of our investigation showed that although Wilcoxon rank test proved insignificant, yet the LPL therapy is more efficient in reducing average postoperative edema on the day of its peak occurrence. But both treatment therapies did not establish a noticeable anti-trismus effect through the follow up intervals.

The beneficial LPL irradiation effects seem to be dose-dependent. Roynesdal, 1992 verified that lasers with 3 J/cm^2 energy output or less produce no significant effects after impacted lower third molar surgery, Roynesdal (1992). In our study, we followed his recommendation and used a higher therapeutic dose (6 J/cm^2, with constant power density of 100 mW, and wavelength 980 nm). It exerted a significant anti-edematous effect on the 3rd postoperative day in comparison to the Betamethasone group.

The LPL therapy appeared to be non-invasive and without any adverse effect on the patients. This clinical finding disagreed with results of Jovanoic et al., (2004) who expected a thermal insult to tissues stimulated with 100mW power density or more Jovanovic (2004), but as we shortened the stimulation time, no adverse thermal effects was encountered. The repeated dose on the 3rd postoperative day proved unnecessary, as its' outcome on the 7th postoperative day was not notable in comparison to the results of the Betamethasone group.

It could be concluded that within the limitation of this study, LPL therapy is effective than systemic Betamethasone in reducing postoperative edema after third molar surgery. However both treatment modalities showed no statistical significant difference and have the same effect on postoperative trismus.

5. References

1. Abt E. Biostimulation and Photodynamic Therapy. In: Miserendino L, Pick R (ed.), Lasers in dentistry. Quintessence Publishing Co, Chicago-Berlin, London-Tokyo, Sao Paolo- Moscow-Warsaw, 1995: 247-257.

2. Barabash A, Tats A, Getling Z. Experience gained He-Ne laser therapy of patients with lichen planus at therapeutic stomatology clinic. Stom M 1995: 1: 20-21.

3. Berns M, Bewley W, Ho Sun Ch, Templin P. Free electron laser irradiation at 200 micrometers affects DNA synthesis in living cells. Proc Natl Acad Sci 1990; 87: 2810-2812.

4. Carrillo SJ, Calatayud J, Manso JF, Barberia E, Martinez MJ, Donado M. A randomized double-blind clinical trial on effectiveness of helium-neon laser in the prevention of pain, swelling and trismus after removal of impacted third molars. Int Dent J 1990: 40: 31–36.

5. Goldman JA. Laser therapy of rheumatoid arthritis. Lasers Surg Med 1980: 1:93–97.

6. Gonzalez-Santana H, Penarrocha-Diago M, Guarinos-Carbo J, Balaguer-Martinez J. Pain and inflammation in 41 patients following the placement of 131 dental implants. Med Oral Patol Oral Cir Bucal 2005: 10: 3: 258-263.

7. Honmura A, Yanase M, Obata J, Haruki E. Therapeutic effects of GaAlAs diode laser irradiation on experimentally induced inflammation in rats. Lasers Surg Med 1992: 12: 441–449.

8. Huggman GG: Use of methylprednisolone sodium succinate to reduce postoperative edema after removal of impacted third molars. J Oral Surg 1977; 35:198-99.

9. Jovanovic G, Buric N, Kesic L: Effect of low power laser on postoperative trismus. Facta Universitatis Medicine and Biology 2004: 11: 3: 136 –138.

10. Jovanovic G. Low power laser effect on wound healing after tooth extraction and mouth surgery. Ph.D. Thesis, Nis, 1998.

11. Lievens PC. Effects of laser treatment on the lymphatic system in wound healing. Laser 1988: 1: 12.

12. Lievens PC. The effect of the combined HeNe and IR laser treatment on the regeneration of the lymphatic system during the process of wound healing. Lasers Med Sci 1991: 6: 189–191.

13. Miserendino L, Levy G, Miserendino C. Laser interaction with biologic Tissues. In: Miserendino, L, Pick R. (Eds.), Lasers in Dentistry. Quintessence Publishing Co., Chicago-Berlin, London- Tokyo, Sao Paolo-Moscow-Warsaw 1995: 39-55.

14. Montgomery TM, Hogg PJ, Roberts LD, Redding WS. The Use of glucocorticosteroids to lessen the inflammatory sequelae following third molar surgery. J Oral Maxillofac Surg 1990: 48: 179–187.

15. Petkovic S, Bukurov S. General Surgery. Beograd-Zagreb: Medicinska knjiga 1987 (In Serb.).

16. Roynesdal A. Effect of low level laser irradiation on postoperative pain and swelling. Scand Assoc Oral Maxillofac Surg, XXIV Conf., 1992: Mariehamm, Finland.

17. Sowray JH. Oral and Maxillofacial Surgery. In: Rowe AHR, Alexander AG, Johns RB, eds: A Companion to Dental Studies. Clinical Dentistry. Oxford: Blackwell Scientific Publications 1986: 1117.

18. Tuner J, Hode L. Laser Therapy in Dentistry and Medicine. Sweden: Prima Books AD 1996: 50–52. (7).

11/1/2010

Comparism Of The Quality Parameters Of The Seed And Condiment Oil Of *Adansonia Digitata*

I.Y Chindo[1*], J.S Gushit[2], P.N Olotu[3], J. Mugana[1]and D.N. Takbal[1]

[1]Chemistry Programme, Abubakar Tafawa Balewa University, Bauchi, Nigeria.
[2]Department of Science Laboratory Technology, University of Jos, Nigeria.
[3]Department of Pharmacognosy, University of Jos, Plateau State, Nigeria.
istifanuschindo@yahoo.com

Abstracts: The oil quality parameters of the seed and condiment oil of *Adansonia digitata* were evaluated. The Iodine value, Peroxide value, Saponification value and percentage Free Fatty Acid (FFA) were 98.07g/100g, 1.4mEq/Kg, 122.60mg/g and 0.21% respectively for seed oil and 71.06g/100g, 10.20mEq/Kg, 142.80mg/g and 6.37% respectively for the condiment oil. The variation in the parameters from seed oil to condiment oil observed include increased in peroxide value, FFA and Saponification value and decreased in Iodine value. The changes have been interpreted to be due to some structural changes in the Triglyceride leading to the formation of new chemical properties and products. The Infra Red (IR) spectra have also given an identification of Rancidity of the condiment oil due to bands observed at 3400- 2700 and 1705 cm^{-1} indicating the possible formation or absence of acid and aldehyde respectively; which are products of oxidative Rancidity.

[I.Y Chindo, J.S Gushit, P.N Olotu, J. Mugana and D.N. Takbal. **Comparism Of The Quality Parameters Of The Seed And Condiment Oil Of *Adansonia Digitata***. Journal of American Science 2010;6(12):990-994]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: Rancidity, *Adansonia digitata, seed oil, condiment oil, Saponification & Infra Red.*

1. Introduction

Adansonia digitata (baobab) is a tree found in the savanna areas of Africa and Asia. In Nigeria it is found in the northern part of the country. The leaves are the major ingredient for a variety of food preparations. The pulp is also used for cooling drinks, used as an appetizer for seasoning food or curdling milk, used as a coagulant of rubber, and as a fumigant for domestic animals (Nkafamiya, 2007). The leaves, pulp and seed are used in traditional medicine for the treatment of kidney diseases, dysentery and fever, the bark of the tree is used as a substitute for quinine bark in the treatment of malaria fever. The ashes made from the seeds are rich in potash and phosphate and are used as fertilizers. The outer part of the bark is used for making packing materials and the spongy wood for making wide canoes **(**Otto, 1989**)**.

The seed oil content is about 33% of the seed bulb which consist of oleic, and linoleic acids as the major fatty acid constitute and other fatty and non fatty substances (Theodore, 1989). The high content of the linoleic and oleic acids helps in softening skin, restore and moisturize the epidermis and helps in regenerating epithelial tissues which gave the seed oil a very good carrier and useful in the cosmetic industry (Theodore, 1989).

2. Material and Methods

Sample Collection and Preparation

The seed sample of *Adansonia digitata* was obtained from Bayara village in Bauchi State, Nigeria on the 1st January, 2010. The seed coat was stone-cracked in order to collect the seeds and then crushed into powder subsequently referred to as powdered sample and was kept for condiment preparation.

Condiment Preparation

The powdered sample was made into a thick paste by mixing it in a beaker with water (100g powdered sample in 10ml of water). The resulting solution was covered with aluminium at normal room temperature and pressure for five (5) days. And was then dried at room temperature and crushed into powder subsequently referred to as condiment sample.

Oil Content Determination

The condiment sample (100g) was defatted exhaustively with petroleum ether (60- 80^{OC}) in a soxhlet apparatus and concentrated in-vacuo to produce a dark brown mass (50g). The marc was then left in the fume cardboard for about twelve hours in order to remove any spill over petroleum ether and the percentage extract recovery expressed as the weight of oil (g) divided by the weight of sample on dry mass and the resultant multiplied by one hundred percent.

***Moisture Content* Determination**

An evaporating dish was heated to a constant weight in an oven at 105^0C and its constant weight noted. 3g of the powered drug was accurately weighed into the dish. The dish with its content was then put in the oven at 105^0C and the content dried to a constant weight at 30 minutes interval after the initial drying of one hour. Two consecutive same weights confirm a constant weight. The total loss in weight (weight of the moisture) was determined by subtracting the constant weight of the dish and powered drug after heating from the weight of the dish and its content before heating. The percentage of the moisture contents with reference to the initial weight of the powered drug was then calculated.

This was achieved by dividing the weight of the moisture by the weight of the drug (condiment & oil) taken and multiplied by a hundred. Three different determinations were carried out and the average of the three gives the moisture content of the drug (Evans, 1996).

Iodine Value (IV) Determination

The method described by Marshall (2005) was adopted**.** Oil sample (0.5g) was placed in a 250ml conical flask and 10ml of anhydrous chloroform was added. This was followed by 30ml of Hanus solution and the flask was Stoppard and allowed to stand in the drawer for 30 minutes after which Potassium iodide (10ml 0f 15% v/v) was added to the content of the flask so as to wash down any iodine that might be present on the Stoppard. The resulting solution was titrated with sodium thiosulphate solution (0.14M) until the light yellow color formed disappeared. The determination for the blank was conducted in the same manner but without the oil. The iodine value was calculated as:

IV = (B - S) × M × 12.69/ sample weight (g). {Where: M = molarity of sodium thiosulphate (0.14M), 12.69 = conversion factor from Meq. Sodium thiosulphate to gram iodine, B = blank titre value, S (ml) = sample titre value}

Peroxide Value (PV) Determination

The method described by Nkafamiya et al (2007) was adopted. The oil (5g) was placed in 30ml glacial acetic acid: chloroform (3:2 v/v %) and saturated solution of potassium iodide (0.5ml) was added to liberate iodine by reacting with the peroxide. The resulting solution was titrated against sodium thiosulphate (0.01M) using starch solution (1%) as indicator, until the yellow color just disappeared. The peroxide value was calculated as follows:

PV (meq/Kg) = (S-B) × M× 1000/ sample weight (g). {Where: B = blank titre value, S (ml) = sample titre value M = molarity of sodium thiosulphate solution (0.01M)}.

Percentage Free Fatty Acid (%FFA) Determination

Nkafamiya et al (2007) procedure was used. Oil sample (2 g) was weighed into a 250 ml conical flask and 10ml of ethanol (95%) was added, the resulting mixture was titrated with sodium hydroxide (0.1 M) using phenolphthalein as indicator. The titration was done with constant shaking until a pink color persisted for 30 seconds. The %FFA was then calculated from the following equation:

%FFA = V × M × 2.82 mg/sample weight (g). {Where V (ml) = volume of sodium hydroxide solution used M = molarity of sodium hydroxide solution used, 2.82 = conversion factor for oleic acid}.

The acid value AV = % FFA × 1.99

Saponification Value (SV) Determination

The method described by Nkafamiya et al (2007) was used. The Oil sample (2 g) was added to alcoholic potassium hydroxide (4g of KOH dissolved in 100 ml of ethanol); the resulting solution was heated at 60^{o}c with constant stirring for two minutes to saponify the oil. The unreacted KOH was back titrated with HCl (1 M) using phenolphthalein as indicator. The titration took place until the solution turned pink. The Saponification value was then calculated as follows:

SV = (B-S) × M× 56.1/sample weight (g). {Where B = blank titre value, S (ml) = sample titre value M = molarity of HCl (0.1 M) and 56.1 = molecular weight of potassium hydroxide}.

PHYSICAL AND PHYSICOCHEMICAL PARAMETERS DETERMINATION

The physical parameters used include: colour, odour and specific gravity. The specific gravity is given by: weight of oil sample divided by weight of equal volume of water. Infrared (IR) analysis was performed on the oil samples and the absorption bands (cm^{-1}) showing the functional groups present in the oil was collected from the spectra.

Preparation of Reagents

All reagents used were of technical grade.
Preparation of Hanus solution

The method described by (Marshall, 1975) was adopted. Iodine crystal (3.3 g) was dissolved in small quantity of glacial acetic acid (about 10 ml) and the volume was made up to 250 ml in a volumetric flask with glacial acetic acid. The solution was then stored in brown Stoppard bottle.

Preparation of starch solution

Starch powder (1 g) was dissolved in 100 ml of distilled water, and the mixture agitated to ensure complete dissolution.

3. Results

Oil Extraction and Physical Parameters

Adansonia digitata seed oil and condiment oil were extracted by hot solvent extraction method using petroleum ether. The color, texture, odour, moisture content (%), specific gravity and oil yield and percentage recovery are shown in Table 1 below.

Table 1: Extract Recovery and Physical Quality

Sample	Oil yield of extract (ml)	Oil yield of extract (g)	Percent-age recovery (%)	Percent-age moisture content (%)	Spec-ific gravity
Seed oil	35	33	35.9	8	0.943
Condime-nt oil	33	30	31.6	5	0.909

From the Table 1, it can be seen that the seed oil has higher percent recovery and moisture content and also slightly denser than the condiment oil (which indicates possible oxidation or hydrolysis reaction of the oil during the condiment preparation). The sharp odor of the condiment oil may be due to the presence of aldehyde of medium molecular weight(heptylic or nonoic aldehydes), such compounds may be formed by oxidation and rapture of fatty acid chain which might have taken place during fermentation in the condiment preparation. The oxidation will also lead to reduction of oil content. The infrared spectroscopy analysis is shown in Fig 1, Fig 2 and Table 2.

Table 2: Samples Infrared Spectroscopy Information

Seed Oil		
Absorption (cm-1)	Absorption intensity	Inference
3000	narrow absorption	=C – H Stretching
	medium absorption	C – H Stretching
1750	medium absorption	C=O Stretching
1640	medium absorption	C = C Stretching
1250 – 1150	broad absorption	C – O – C Stretching
Condiment oil		
3400 – 2700	Broad absorption	O – H Stretching
3000	narrow absorption	=C – H Stretching
2980 – 2820	medium absorption	C – H Stretching
1750	Medium absorption	C = O Stretching
1705	Medium absorption	C = O Stretching
1640	Medium absorption	C = C Stretching
1250 – 1150	Broad absorption	C – O – C Stretching

Table 3: Sample Titre Values

Parameter	Blank titre value (ml)	Seed oil sample titre value (ml)	Condiment oil sample titre value (ml)
Iodine value	40.2	12.4	20.2
Saponification value	3.2	46.9	54.1
Peroxide value	2.0	2.7	7.1
%FFA		1.5	45.2

Table 4: Results of Chemical Parameters

Sample	PV (mEq/Kg)	IV (g/100g)	SV (mg KOH/g)	Acid value	%FFA
Seed oil	1.4	98.068	122.6	0.421	0.2115
Condiment oil	10.2	71.064	142.8	12.68	6.3732

4. Discussion

From Tables 3 and 4, the Peroxide value, the Saponification value, the %FFA are higher in the condiment oil than those of the seed oil except the iodine value which is higher for the seed oil as shown in table 4. The decrease in iodine value of the condiment oil (71.064) compared to the seed oil (98.068) is an indication of lipid oxidation, since there is a decrease in unsaturation during oxidation. The value of %FFA for the condiment oil (6.3732%) indicates a high level of free fatty acids in comparison to the seed oil (0.2115%), the increase in free fatty acids indicates that free fatty acids are formed during fermentation in the condiment preparation, and the high free fatty acid content of the condiment oil makes it unsuitable for food preparation. The higher Saponification value of the condiment oil (142.8) indicates the formation of lower molecular weight oxidation products for example aldehyde and carboxylic acids; it also explains the use of the oil as an ingredient in tooth paste. Peroxide value is also increased for the condiment oil (10.2) which indicates oil deterioration in the formation of hydro peroxide. The infrared spectra of the seed and condiment oils indicate the presence of unsaturated fatty acid and unsaturated oils. Infrared Absorbance (Table 2) at 1705 cm^{-1} in the condiment oil spectra, which is absent in seed oil spectra indicates the presence of an aldehyde in the condiment. The O – H broad absorbance (3400 – 2700cm^{-1}) for carboxylic acid in the condiment oil indicates the presence of free fatty acid in the condiment oil component.

5. Conclusion

The seed oil is very good for soap preparation due to its high Saponification value. The condiment oil is not good for use as food due to it's the high free fatty acid content, which is above the maximum acid value for edible oils; therefore it is used mainly in the preparation of soap and as an ingredient in tooth paste.

Corresponding Author:
Dr. Istifanus Y. Chindo
Department of Chemistry
Abubakar Tafawa Balewa University
Bauchi, P.M.B. 0248, Bauchi, Nigeria
E-mail: istifanuschindo@yahoo.com

REFERENCES

1. Evans W.C. Trease and Evans Pharmacognosy. Fourteenth edition. 1996.
2. Marshall, W., Schmacher, L. G. and Howell, S. Engine Exhaust Emission Evaluation of a Cummins LIONE When Fuel with Biodiesel Blend. Society of Automotive Engineers Warrandale. SAE paper No. 952363. 1975.
3. Morgan, E. D. and Robert, R. An Introduction to Organic Chemistry", The Camelot Press Ltd, Southampton. 1975:18, 84, 121 & 168.
4. Nkafamiya, I. I., Aliyu, B. A., Manji, A. J. and Modibbo, U. U. Degradation Properties of Wild *adansonia digitata* (baobab) and *prosopsis Africana* (lughu) Oils on Storage. *African journal of Biotechnology* 2007:6 (6): 751 – 755.
5. Otto W. Encyclopaedia Americana. 1989:3:207.
6. Otto W. Encyclopaedia Americana. 1989: 11:44.
7. Theodore J. Baobab. Encyclopaedia Americana. Grolier incorporated. 1989: 3:207.
8. Internet file: "what is the healthiest oil to use for cooking" www.wisegeek.com
9. Internet file: en.wikipedia.org/wiki/drying oil
10. Internet file: "chemical reaction of oil, fat, and fat based products" department of chemical engineering, in stituto. //www.inst.utl.pt/.

10/30/2010

Distribution of Gamma-Emitting Radionuclides in Soils around the Centre for Energy Research and Training (CERT) Ahmadu Bello University, Zaria, Zaria-Nigeria

Muhammad, Musa Auwal; Idris, Isa Funtua; Simon, Peter Malam and Arabi, Suleiman Abdullahi

Muhammad, Musa Auwal
Reactor Engineering Section,
Centre for Energy Research and Training (CERT), Ahmadu Bello University, P.M.B. 1014 Zaria, Nigeria
Email: mamyola@yahoo.com

Idris, Isa Funtua
Material Science and Development Section,
Centre for Energy Research and Training (CERT), Ahmadu Bello University, P.M.B. 1014 Zaria, Nigeria
Email: iifuntua@yahoo.com

Simon, Peter Malam
Health Physics and Radiation Biophysics Section,
Centre for Energy Research and Training (CERT), Ahmadu Bello University, P.M.B. 1014 Zaria, Nigeria

Arabi, Suleiman Abdullahi
Material Science and Development Section,
Centre for Energy Research and Training (CERT), Ahmadu Bello University, P.M.B. 1014 Zaria, Nigeria
Email: arabisuleiman@gmail.com

Abstract: A portable HPGe spectrometer has been employed to characterised, *in-situ* gamma activity concentration from the primordial Radionuclides ^{238}U, ^{232}Th ^{40}K in the soil at 12 monitoring points (MPs) in the environment in and around the Centre for Energy Research and Training (CERT), Ahmadu Bello University, Zaria, Nigeria. The MPs were marked-out using Global Positioning System (GPS) navigation. The measured activity concentrations due to ^{238}U range from 4.8 ± 3.0 to 11.9 ± 2.0 Bq kg^{-1} with an average of 8.3 ± 2.6 Bq kg^{-1}, ^{232}Th range from 15.5 ± 4.3 to 46.4 ± 3.5 Bq kg^{-1} with an average of 34.3 ± 3.4 Bq kg^{-1} and ^{40}K range from 317.2 ± 8.4 to 985.3 ± 7.0 Bq kg^{-1} with an average of 641.8 ± 7.3 Bq kg^{-1}.
[Muhammad, Musa Auwal; Idris, Isa Funtua; Simon, Peter Malam and Arabi, Suleiman Abdullahi. **Distribution of Gamma-Emitting Radionuclides in Soils around the Centre for Energy Research and Training (CERT) Ahmadu Bello University, Zaria, Zaria-Nigeria.** Journal of American Science 2010;6(12):995-1001]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: In-situ gamma, activity concentration, primordial Radionuclides

1. Introduction

Ionising radiation and radioactive substances are natural and permanent features of the environment, and thus the risks associated with radiation in all its forms can only be restricted, not eliminated. Moreover, the use of man-made radiation is widespread. The largest contribution to the radiation field is of natural origin: it is due to cosmic rays, the natural radioactivity generated by the decay of unstable, naturally occurring elements and the radioactive decay products of radon in the air (UNSCEAR, 1988). Artificial radioactivity may be released into the environment during the normal operations of nuclear facilities and installations such as nuclear ore processing, uranium enrichment, fuel fabrication, reactor operations, and application of radioisotopes in the fields of nuclear medicine, research, industry and agriculture (Eisenbud, 1987). These emissions are very small in normal operation, although large amounts of radioactivity could be released to the environment through accidents. The total amount of radioactivity in an environment should be accurately known and kept to a level as low as reasonably achievable (ALARA). Exposures from natural radiation are the largest component of all exposures for most people, and form the baseline

upon which exposures from manmade sources are added (UNSCEAR, 2000).

Gamma-ray spectroscopy system provides practical way to characterize dispersed Radionuclides in or on the soil to ascertain possible changes in the environmental radioactivity. Both laboratory and *in-situ* gamma spectroscopy are often used for monitoring and assessment of radioactivity and radiation dose rates in the environment due to both natural and anthropogenic sources. (Beck *et al.,* 1972; Nikl *et al*., 1988; ICRU, 1994; Othman *et al.*, 1994; Tzortzis *et al.*, 2003; Clouvas *et al*., 2004).In large-scale environmental radioactivity measurement, *in-situ* gamma spectroscopy is much favoured compared to laboratory soil analysis because of time and problems associated in cross contamination involved in the laboratory methods. It also gives the opportunity to obtain information not only of the activity concentration but also of the relative contribution of the various nuclides to activity concentration. *In-situ* techniques for measuring the activity concentration, resulting from the gamma radiation and characterizing its sources, with gamma ray spectrometer have been used successfully in the outdoor environment (Beck *et al*., 1972; Clouvas *et al*., 2001; Petalas *et al*., 2005; Auwal,M.M. 2005)

For radiation monitoring near nuclear facilities, baseline data, are indispensable for various purposes: they provide documented reference base; for the assessment of actual or potential consequence of radioactivity on health, and on the environment, due to radioactive materials or radiation fields in the environment from normal operations and accidental releases. The present work has been conducted in the Centre for Energy Research and Training (CERT), and some selected settlements within 2km radius from NIRR-1, using high-resolution portable gamma spectrometry system. The Centre is a nuclear energy (radiation) based research centre, thus, dealing with substantial quantities of artificial radioactive materials, such as neutron generator, Am-Be isotopic neutron source, a nuclear research reactor code named Nigeria Research Reactor-1 (NIRR-1); among others.

The present work puts forward a systematic method for determining environmental activity concentrations levels from *in-situ* measurements, and shows the distribution and intensities of important natural and artificial radionuclides within the studied area. It complements previous studies reported by other authors (Ibeanu *et al.,* 2002) by providing *in situ* gamma spectrometry based, baseline data, for operational and post operational monitoring, of the Centre for Energy Research and Training (CERT).

2. Materials and Methods

2.1 ***Site***

The study site is located at 007°38.523'–007°40.822'E and 11°07.830'–11°09.790' N within the Zaria sheet 102.The number of monitoring points (MPs) includes; eight locations within the Centre for Energy Research and Training (CERT), Ahmadu Bello University, Zaria, and four other locations at selected settlements around CERT within 2Km radius. Table 1 gives the description of the monitoring points (MP) with respect to the Nigeria Research Reactor 1 (NIRR1) as reference point. The chosen sites were undisturbed with little or no surface features and modest vegetation. These sites were marked-out using Global Positioning System (GPS).

2.2 ***Gamma-ray Detection system***

The experimental set-up is a stand-alone high-resolution spectroscopic system used for the in-situ measurement of the energy spectrum of the emitted gamma rays in the energy range between 50 keV and 3000 keV. The system consist of a high-purity germanium (HPGe) detector EG & G ORTEC® coaxial cylinder of crystal length of 46.8 mm and diameter 55.0 mm, with an efficiency of 23.5% 27 relative to a 7.6 x 7.6 cm^2 NaI(Tl) crystal. In addition, energy resolution (FWHM) of 2.0 keV was achieved, all for a ^{60}Co emission point source at 25 cm for 1.33 MeV. This type of detector can sustain warm up when not in use, which is a convenient feature during extended field trips. The detector was mounted on a portable (hand-held) 10 litres liquid nitrogen Dewar that features an all attitude capability. Liquid nitrogen was used for cooling the detector during operation. After filling the portable Dewar with liquid nitrogen, it requires a 6 hour cool-down time before becoming operational with 24 hours nominal holding time. The detector assembly was mounted on a 1 m tripod with the crystal end cap facing down towards the ground and the Dewar above. This orientation maximizes the flux that will be intercepted and registered by the detector (Kelvin, 1997). The detector unit was connected to a battery powered EG & G ORTEC® "Normad Plus" portable computer based spectroscopy system. High voltage and preamplifier power were supplied to the detector by the system. An advanced multi-channel analyzer (MCA) emulsion software (MAEASTRO-32) was used for data acquisition, storage display and analysis, of the acquired gamma-spectra.

Figure 1: Typical *in situ* g-ray detection system at one of the monitoring points (MP)

2.3 In-situ measurement and analysis

The current study was conducted in the months of May and June 2005. The field measurement of terrestrial gamma radiation was based on the assumption that there exist laterally uniform distribution of natural Radionuclides in the environment and that the vertical contribution from the soil is limited to the first horizon (≈ 10cm to 30cm). Measurements were performed over flat terrain; that allow source geometry to be represented as an infinite half-space; that is 2π geometry in terms of solid angle subtended by the source.

The source measured was soil sample and counting statistics for a given spectral absorption peak were obtained in a fraction of the time required for counting a small collected sample. Measurements of spectra in the field were made for a period of 5000 s. However, series of random short readings of about 600 sec were first taken, to ensure that, there is approximate uniformity, consequently, the desired counting statistics. The measuring system was routinely checked with ^{137}Cs and ^{60}Co standard sources.

A computer based multi-channel analyzer system with emulsion software (MAESTRO–32) was used for spectra acquisition. Based on two-point energy calibration as set for the operation, prominent peaks were identified in a benchmark spectrum (fig.1d) and the appropriate regions of interest were set up. These peaks, which are characteristics of typical environmental spectra, are:

- the 295, 352, 609, 1120 and 1765 keV peaks in the ^{238}U series,
- the 238, 510, 583, 911, 965 and 2615 keV peaks in the ^{232}Th series,
- the 1460 keV peak of ^{40}K.

The set energy bands define the peaks where the left and right channel markers are representative of the Compton continuum. Detector's specific calibration factors (measured efficiency) determined in an earlier experiment (Auwal, 2005), were applied to convert from net peak count rate to activity concentration. Only peaks with reasonable gamma-ray emission probabilities were considered.

3. Results and discussion

The activity concentrations for ^{232}Th, ^{238}U and ^{40}K with standard error of the measured locations are presented in table 2, while table 3, show the statistical analysis of the results and percentage contribution is shown in figure 3. The activity concentrations were calculated by the net peak areas of 511 keV (^{208}Tl) and 911 keV (^{208}Ac) for ^{232}Th, 609 keV (^{214}Bi) and 352 keV (^{214}Pb) for ^{238}U, and 1460 keV for^{40}K. The concentrations range from 15.5 ± 4.3 to 46.4 ± 3.5 Bq kg^{-1}(34.3 ± 3.4) for ^{232}Th, 4.8 ± 3 to 11.9± 2 Bq kg^{-1} (8.3 ± 2.6) for ^{238}U and 317.2 ± 8.4 to 985.3 ± 7 Bq kg^{-1} (684 ± 7.3) for ^{40}K with mean values enclosed in the brackets. The relative contribution of the different natural gamma emitters (Thorium series, Uranium series, and ^{40}K) to the total activity concentration vary among the monitoring point. This range from

3.3% to 8.3% for ^{232}Th, 0.7% to 1.7% for ^{238}U and 90.2% to 95.5% for ^{40}K .Of all the locations MP004 and MP011 appear to have the highest concentration of ^{232}Th, also MP004 exhibits the highest concentration of ^{238}U. MP012 appear to have much higher concentration of ^{40}K when compared with the concentrations at other locations, with value of 985.3 ± 7 Bq kg^{-1}. In addition, the ^{232}Th/^{238}U ratios for the locations range from 2.8 to 6.4. This shows uranium is less than thorium by a factor of three.

Table 1Site identification by Global Positioning System (GPS), and description of Monitoring Points

Site ID	Location		Description
	Latitude	Longitude	
MP001	11°08.439'N	07°39.840'E	CRMP07 about 5m from NIRR-1
MP002	11°08.426'N	07°39.844'E	CRMP08 about 105m from NIRR-1
MP003	11°08.476'N	07°39.901'E	CRMP05 about 141m from NIRR-1
MP004	11°08.497'N	07°39.851'E	CRMP06 about 100m from NIRR-1
MP011	11°08.415'N	07°39.776'E	CRMP03 about 140m from NIRR-1
MP012	11°08.358'N	07°39.912'E	CRMP04 about 220m from NIRR-1
MP013	11°08.505'N	07°39.963'E	CRMP01 about 280m from NIRR-1
MP014	11°08.549'N	07°39.823'E	CRMP02 about 220m from NIRR-1
MP111	11°07.830'N	07°39.447'E	Beside ABU Dam
MP112	11°08.205'N	07°40.186'E	LEA Unguwam Jema'a a nearby settlement
MP113	11°08.749'N	07°40.822'E	Aviation site ii
MP114	11°09.790'N	07°38.524'E	LEA Samaru a nearby settlement

Comparing the activity concentrations of the present work with the Ibeanu *et al*., (2002), in which a laboratory based NaI(Tl) detector was employed to measure the radionuclide concentrations in dry soil samples from the same measurement points, the *in situ* measurement results for ^{232}Th and ^{238}U were generally significantly lower than the laboratory data, except for some of the ^{238}U that were reported as below detection limit. On the other hand, ^{40}K has exhibited certain degree agreement between the two results, with a variation of approximately ±5% at some points.

Table 2Activity concentration of ^{232}Th, ^{238}U and ^{40}K

Concentration ±Stat. Error (Bq kg^{-1})								
Site ID	**^{232}Th**	**Contr. (%)**	**^{238}U**	**Contr. (%)**	**^{40}K**	**Contr. (%)**	**^{232}Th/^{238}U**	**Total**
MP001	21.1±2.5	3.3	7.5±2.8	1.2	609.6±5.7	95.5	2.8	638.2±9.6
MP002	45±3.5	5.5	11.9±2.0	1.5	759.9±10	93.0	3.8	816.8±15.5
MP003	37.5±2.4	4.1	10±1.8	1.1	868.4±6	94.8	3.8	915.8±10.2
MP004	46.4±3.5	5.3	11.9±2.0	1.4	809.9±10	93.2	3.9	868.1±15.5

MP011	46.2±2.9	6.8	10.5±2.5	1.5	626.2±9.1	91.7	4.4	682.9±14.5
MP012	45.9±3.6	4.4	7.2±1.8	0.7	985.3±7	94.9	6.4	1038.5±12.4
MP013	32.1±5.3	6.1	7.2±3.8	1.4	487.9±5.5	92.6	4.5	527.1±14.6
MP014	30.9±3.3	5.9	6.4±2.5	1.2	487.9±4.1	92.9	4.8	525.2±9.9
MP111	31.1±3.1	6.5	8.34±2.9	1.7	442±7.1	91.8	3.7	481.5±13.1
MP112	36.8±2.9	4.4	9.6±2.4	1.2	783.5±9.9	94.4	3.8	829.9±16.2
MP113	15.5±4.3	4.6	4.8±3.0	1.4	317±8.4	94.0	3.2	337.2±15.7
MP114	41±3.5	8.3	7.5±2.9	1.5	444±9.1	90.2	5.5	492±14.5

The reason for the difference may be mostly coming from the type of detector used in each case, the accuracy of the response at the energies used in the analyses, and the method of calibration. Moreover, soil moisture variation or actual variation in the dry concentration of radionuclides with depth is among the limiting factors for establishing comparability between in-*situ* and soil sample measurement.

Table 3Statistical data for activity concentration (Bq kg^{-1})

Nuclide	**Mean**	**Confid. ±95.000%**		**Median**	**Range**		**Std.Dev.**	**Standard error**
^{232}Th	34.3	26.8	41.9	34.5	15.5	46.4	10.5	3.3
^{238}U	8.3	6.8	9.9	7.9	4.8	11.9	2.1	0.7
^{40}K	641.8	488.8	794.8	617.9	317.0	985.3	213.9	67.6
^{232}Th/^{238}U	4.1	3.4	4.8	3.9	2.8	6.4	1.0	0.3
Total	684.4	524.9	844.0	660.6	337.2	1038.5	223.1	70.5

It may be noted that in the *in-situ* measurement, the detector samples the photon flux from a volume of soil out to a radius of approximately 10 m and down to a depth of about 30 cm, depending upon the photon energy (Kelvin, 1997). Fig. 1.c shows a pictorial representation of the relative ground area contributions to the primary (uncollided) flux at a height of 1m for a medium energy (662keV) source with typical exponential depth profile in the soil. Thus, the activity that was closer to the soil surface tends to produce a wider field of view. In effect, a field spectrum samples an area of several hundred square metres: averaging out the local inhomogenities in the distribution of the Radionuclides.

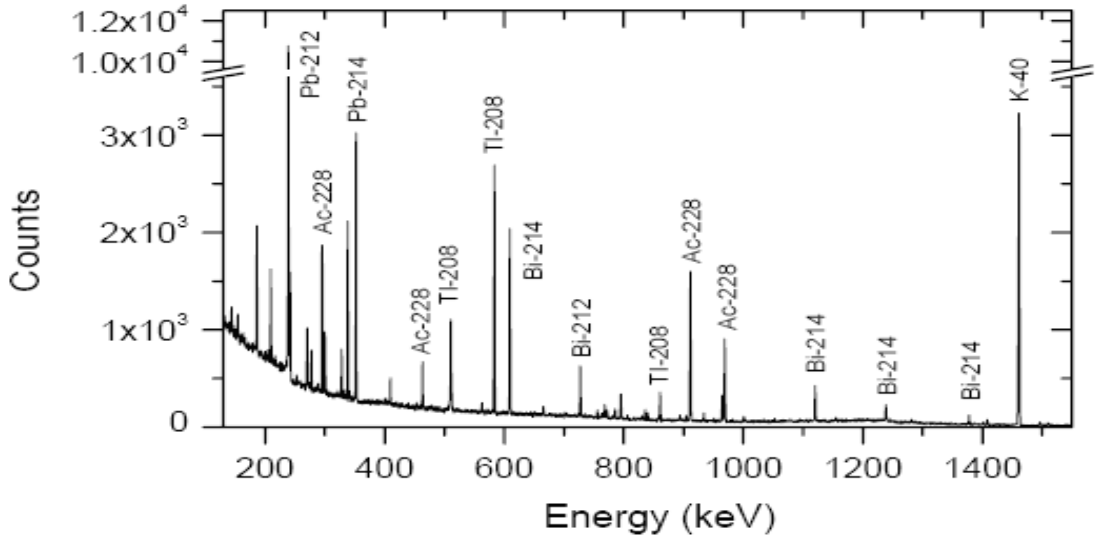


Figure 2: A typical gamma-ray spectrum showing important identified photo-peaks and their associated Radionuclides

The calculated activity mean values from the locations were 34.3, 8.3 and 641.8 Bq kg^{-1} for ^{232}Th, ^{238}U and ^{40}K respectively. Correspondingly the revised median values worldwide (UNSCEAR, 2000) are 30, 35, and 400 Bq kq^{-1} respectively. This reveals that the mean concentration level measured in the selected areas of Zaria for ^{232}Th is slightly higher than the world median value while that of ^{238}U is lower than the corresponding world value; thus ^{238}U is less than the world value by a factor of three. However, ^{40}K concentration appeared to be higher than the world median value at all the points, except MP113 with value of 317 ± 8.4 Bq kg^{-1}.

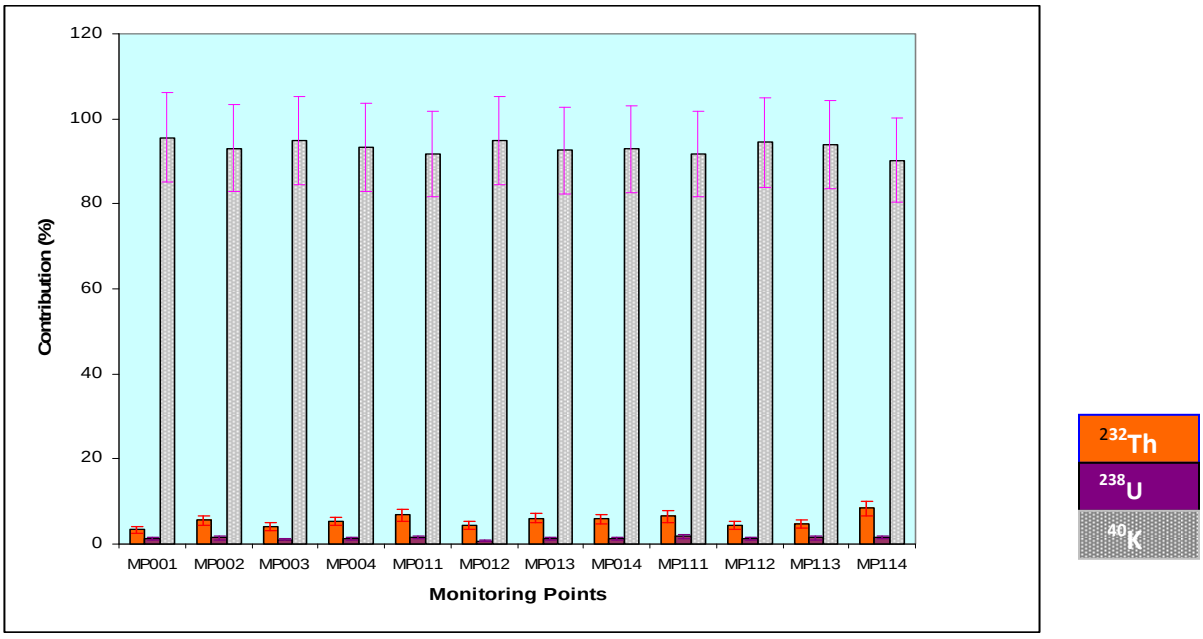


Figure 3: Percentage contribution of activity concentration

4 Conclusion

High-resolution gamma ray spectrometry was exploited to determine distribution of gamma emitting Radionuclides in soils around the Centre for Energy Research and Training, Ahmadu Bello University, Zaria. Natural radionuclide concentrations determined for ^{232}Th, and ^{238}U were significantly lower when compared with those determined by laboratory based NaI(Tl) detector system but in general agreement for ^{40}K by both techniques. The mean activity concentrations were higher than the revised world median values for ^{232}Th, and ^{40}K and lower for ^{238}U. This technique is useful for operational and post operational monitoring for the Centre, which will serve as reference for present and future assessment. The assessment of the radionuclide level of the area did not detect the presence of any artificial radionuclide and thus no significant impact of the extensive usage of radioactive materials within and around the Centre the on the radiation burden of the environment.

Acknowledgement

The authors wish to express their gratitude to the Centre for Energy Research and Training, Ahmadu Bello University, Zaria for the support given to the entire project.

Corresponding Author:
Arabi Suleiman Abdullahi
Material Science and Development Section,
Centre for Energy Research and Training,
Ahmadu Bello University, Zaria
Email: arabisuleiman@gmail.com

References

1. Auwal, M.M. Determination of Absorbed and Effective dose from Natural Background Radiation with Portable HPGe Detector. M.Sc. thesis. Ahmadu Bello University, Zaria. 200.
2. Beck, H. L., De Campo, J., Gogolak, C. In situ Ge(Li) and NaI(Tl) gamma-ray spectrometry .New York, US DOE, Environmental measurement Lab., HASL-258. 1972..
3. Clouvas, Xanthos, S., Antonopoulos-Domis, M. Extended survey of indoor and outdoor terrestrial gamma radiation in Greek Urban areas by in situ gamma spectrometry with portable Ge detector. *Radiat. Prot. Dosim.* Vol. 94, No. 3. 2001. pp. 233-245.
4. Clouvas, A., Xanthos, S., Antonopoulos-Domis, M. Radiological maps of outdoor and indoor gamma dose rates in Greek Urban areas obtained by in situ gamma spectrometry. *Radiat. Prot Dosim* Vol.112, No. 2, 2004.pp. 267-275.
5. Eisenbud, M. Environmental radioactivity from Natural, Industrial and Military Sources. (New York: Academic Press, Harcourt Brace Joanovich) 1987.
6. Ibeanu, I. G. E., Futua, I. I., Adeyemo, D. J, Bappah, A. I. and Umar, I. M. Radiation monitoring programme for the Centre for Energy Research and Training (CERT) Nuclear research reactor site and environs. Second technical report of the Centre for Energy Research and Training (CERT), Ahmadu Bello University, Zaria. CERT/MNSR/RMP/02. 2000.
7. International Commission on Radiation Units and Measurements, ICRU Report 53 Gamma-Ray Spectrometry in the Environment 1994.
8. Bethesda, MD.Kelvin, M.M. (1997. Field Gamma-Ray Spectrometry. Section 3, In EML Procedure Manual, HASL-300, 28th edHASL-300-ED.27-Vol 1DOE, Environmental Measurement Laboratory New York (DE91- 10178).
9. Nikl, I., Sztanyik, L. B. External indoor and outdoor gamma exposures in Hungary during the period of 1983-86. *Radiat. Prot. Dosim.* Vol. 24(1/4), 1988.pp. 387-389.
10. Othman, I. and Yassine, T. Natural radioactivity in the Syrian environment. *Sci.of Tot. Environ.* Vol.170, 1994.pp.119-124.
11. 11. Petalas, A. B. Vogiannis, E. Nikolopoulos, D. Halvadakis, C. P. Preliminary survey of outdoor gamma dose rates in Lesvos Island (Greece). *Radiat. Prot. Dosim*. Vol. 113, No. 3, 2000.pp. 336– 341.
12. Tzortzis M., Tsertos H., Christofides S., Christodoulides, G., Gamma-ray measurements of naturally occurring radioactive samples from Cyprus characteristic geological rocks. *Radiat. Measur*.Vol.37, 2003.pp.221-229.
13. UNSCEAR. United Nations Scientific Committee on the effects of Atomic Radiation. Source and effects of ionizing radiation. Report to General Assembly, with Scientific Annexes, United Nations, New York, 2000.

11/10/2010

An Introduction of OSCE versus Traditional Method in Nursing Education: Faculty Capacity Building & Students' Perspectives

[1*]Shadia A. Eldarir, [2]Hanan A. El Sebaae, [2]Hanaa A. El Feky, [3]Hewida A. Hussien, [1]Nagwa Abd El Fadil and [4]Inas H. El Shaeer

[1]Maternal-Newborn Health Nursing Dept., [2] Medical Surgical Nursing Dept., [3] Pediatric Nursing Dept., [4] Community Health Nursing Dept., Faculty of Nursing Cairo University, Cairo, Egypt
[*]shadiaeldarir@yahoo.com

Abstract: Background Assessment of clinical competence is of great importance when evaluating the expected learning outcomes of nursing education. Increasing number of students enrolled at Egyptian nursing faculties might increase the chances of malpractice that compromise patients' conditions. Therefore it is challenging to have such an objective assessment tool to comprehensively assess students' clinical competencies especially with increased students' number. Aims of the current project are building capacity of nursing faculties and staff members for OSCE; establishing simulated learning experiences (OSCE) in nursing practice; comparing the feasibility, utility, and effectiveness of using OSCE versus traditional clinical assessment; examining faculty and students perspectives for OSCE; and evaluating the effectiveness of OSCE versus traditional clinical assessment. Method: To achieve aims of this study needs' assessment of faculty members were carried out during conduction of raising awareness seminar about OSCE which attended by 72 faculty and staff members from both Cairo and Ain Shams Universities. A total of 7 workshops were held to build up their capacities on the scheme of OSCE and clinical scenario writings .One-hundred and forty faculty and staff members were attended and pre-post tests a were administered. Out of the 140, 31 were trained as data collectors. . Implementation of the OSCE was carried out on 400 second and third year students at the areas of critical care units. Comparison of students' achievements at traditional and OSCE methods were carried out. Faculty's and students' perspectives were investigated. Results: Needs' assessment revealed that 57% of faculty members knew nothing about OSCE and 98.6% of them had no experience in using OSCE; also a high statistical significant differences between OSCE and traditional assessment groups in the first and second trial ($t = 2.423$, $p= 0.016$), and ($t= 6.23$, $p= 0.000$) respectively. The students' achievements were better with OSCE. Faculty staff members indicated that, OSCE saves time (76.3%), prepares highly qualified competent students (62.5%) and improve students' performance (62.5%). Conclusion OSCE examination offers an attractive option for assessment of students' competency. It provided particular strengths in terms of faculty staff objectivity and reliability of the assessment process for all students, especially when compared with other methods of assessing practice.

[Shadia A. Eldarir, Hanan A. El Sebaae, Hanaa A. El Feky, Hewida A. Hussien, Nagwa Abd El Fadil and Inas H. El Shaeer. An Introduction of OSCE versus Traditional Method in Nursing Education: Faculty Capacity Building & Students' Perspectives. Journal of American Science 2010;6(12):1002-1014]. (ISSN: 1545-1003). http://www.americanscience.org.

Key Words: Assessment, OSCE, traditional method, Faculty capacity building, students' perspectives.

1. Introduction:

Assessment plays a major role in the process of nursing education, in the lives of nursing students and in society by certifying competent practitioner who can take care of the people. The objective Structured Clinical Examination (OSCE) is an approach to students' assessment in which aspects of clinical competence are evaluated in a comprehensive, consistent and structured manner, with close attention to the objectivity of the process (Byrne & Smyth, 2007). Objective Structured Clinical Examination" (OSCE) evolved from medical education in Scotland, and has been used extensively in nursing worldwide. It is now widely accepted as a fit-for-purpose instrument for measuring clinical reasoning skills with a high degree of technical fidelity (Ahmad, Ahmad & Abu Bakar, 2009).

Steady increase in number of students enrolled at Egyptian nursing faculties might increase the chances of malpractice that compromise patient's conditions, in addition to limited resources from clinical sites that might hinder the opportunity of student to practice on patient. Traditional clinical nursing examinations are not standardized to assess clinical competency, and clinical reasoning skills. Acquisition of critical thinking and problem solving skills among nursing students are difficult to manage with large groups of students. Furthermore, in traditional assessment method, teachers carrying out the assessment of student performance tend to give summative scores. Therefore it is challenging to have

such an objective assessment tool to comprehensively assess students' clinical competencies especially with increased students' number.

OSCE is now an established part of the repertoire of clinical assessment skills in many nursing schools around the world. Nursing faculties in Egypt use a range of assessment techniques that are appropriate for testing students' outcome. However, in Egypt, there is no available evidence for using OSCE in nursing education. OSCE is a new issue that needs capacity building for Egyptian nursing faculties. A baseline survey in the assessment of competency resulting from medical and nursing education in Egypt (2006) reported that skills assessed are poorly performed by four learner groups (medical & nursing undergraduates, nurse intern and house officers) in both medical and nursing faculties Furthermore, clinical training as it is currently organized and implemented for the competencies assessed is inadequate for all learner groups of all regions in Egypt (Health Workforce Development, 2006).

Assessment should measure cognitive learning, mastery of essential practice skills, and the ability to communicate effectively while using data in both critical thinking and problem solving processes (Elzubeir, & Rizk, 2003). Moreover, assessment of student's clinical competence is of paramount importance (Byrne & Smyth, 2007).

Effective and accurate clinical evaluation should be of concern to all nursing faculties and clinical instructors. There is a reasonable expectation for evaluation to be objective, fair, specific, and documented. In addition, students need to know, very clearly delineated, the specific objectives by which they are being evaluated. One type of assessment which meets these criteria is a performance based assessment. An example of a performance-based assessment is the "OSCE" (Ahmad, Ahmad & Abu Bakar, 2009).

OSCE has been widely and increasingly used since it was developed. Researches have shown that it is an effective evaluation tool to assess practical skills. In many instances the OSCE process has been adapted to test trainees from different healthcare related disciplines. In nursing education principles of OSCE can also be used in a formative way to enhance skills acquisition through simulation (Alinier, 2009).

Schoening, Sittner & Todd (2006) indicated that acquisition of critical thinking and problem solving skills among nursing students were unwieldy and difficult to manage with large groups of students; also, the nurse teachers carried out the assessment of student performance giving summative scores in traditional assessment method. While in OSCE simulation, the students find learning such skills are more beneficial because there is an immediate formative feedback following an event. Simulation-based training is superior to problem based learning for the acquisition of critical assessment and management skills. A framework for the development of clinical competence has been described by Miller (1990) who outlines four levels at which a learner can be assessed: *knows, knows how, shows how* and *does*. The OSCE conforms to the third *shows how* level of Miller's pyramid which focuses on assessment of performance of specific skills in a controlled setting (Ahmad, Ahmad & Abu Bakar, 2009). This makes it particularly relevant for the early stages of undergraduate curricula, where assessment comprises compartmentalized exercises (Miller, 2009).

Furthermore, simulated clinical learning offers significant advantages over traditional educational methods. Benefits include the provision of a safe environment for both patient and student during training in high risk procedures, unlimited exposure to rare but complicated clinical events, the ability to manipulate training opportunities rather than wait for a suitable situation to arise, the ability to provide immediate feedback, the opportunity to standardize and evaluate performance and the opportunity to repeat performance. Currently, the ability of simulation to meet the needs of practice education remains limited (Pierre, Wierenga, Barton, Branday & Christie, 2004). In addition, (Ahmad, Ahmad & Abu Bakar, 2009) added that OSCE is developed to reduce bias in the assessment of clinical competence; it is not now without the pitfalls of other assessment methods. In particular, the need for more rigorous evaluation of OSCEs in nursing education programs has been highlighted (Brosnan, Evans, Brosnan, & Brown 2006); (Miller, 2009) as these assessments are directed towards assurances that passing students can practice safely in the clinical setting with patients.

Bartfay, Rombough, Howse, & LeBlance (2004) concluded that OSCEs can be used most effectively in nurse undergraduate curricula to assess safe practice in terms of performance of psychomotor skills, as well as the declarative and schematic knowledge associated with their application. OSCEs should be integrated within a curriculum in conjunction with other relevant student evaluation methods Furthermore, as a method of clinical skills assessment; the OSCE possesses a number of intrinsic advantages. Firstly, it can include both summative and formative components, in which a judgment or evaluation of an individual's performance is made (summative) followed by the provision of feedback, from which the student can

learn (formative). Secondly, because each student is required to demonstrate specific behaviors in a simulated work environment, strict control over the clinical context is possible, while at the same time, reflecting real-life professional tasks. This control eliminates the 'luck of the draw' problem that arises when students are assessed within the 'real-world' clinical environment with actual patients as well as the risk of harm occurring to a patient. The underlying premise is that such standardized procedures ensure objectivity and maximize reliability in assessment (Bartfay et al 2004; Major 2005).

OSCE also provides an innovative learning experience for students. It offers a valid means to evaluate students' clinical performance in a holistic manner (Ahmad et al., 2009). Harden (1988) emphasized that the real power of OSCE lies in its ability to evaluate a wide rang of knowledge and skills which improves the reliability of the examination. Within OSCE reliability is based upon the interaction among students, standardized patients and assessors (Ahmad, Ahmad & Abu Bakar, 2009). These advantages made OSCE to be extensively used in nursing (Alinier, 2003; Ahmad, Ahmad & Abu Bakar, 2009).

Feedback from nursing students suggests that OSCE is an objective tool for evaluating clinical skills. Students perceived OSCE scores as a true measure for essential clinical skills being evaluated, standardized, and not affected by student's personality or social relations. The objectivity of OSCE was highlighted in the literature by many authors (Ahuja, 2009; Harden, 1988). The evaluation of OSCE by nursing students highlighted some areas that need to be enhanced in future, such a s the inadequate time of some of the stations, and the limited period of orientation about OSCE. The insufficient time at OSCE stations was one of students' complaints in some of the studies which investigated students' perspective of OSCE (Pierre et al., 2004).

OSCE generated a considerable uncertainty among students regard in g aspects of OSCE attributes, performance, scoring and objectivity. Students' uncertainty about OSCE was also reported in other studies (Pierre et al., 2004). Such uncertainty may reflect inadequate knowledge about the nature of OSCE and insufficient training on OSCE procedure. It appeared that the train in g session that students received on OSCE before the final exam was not enough for providing them with a comprehensive view of the OSCE. Brewin & Cantwell (1997) suggested that students' uncertainty about OSCE may be due to the fact that the OSCE was a new experience for all of them. From our experience, the implementation of OSCE is time consuming, and requires huge effort and extensive resources. This was also reported in other studies which implemented OSCE (Munoz, Byrne, Pugsley, & Austin 2005). It also requires a large number of qualified personnel to observe and evaluate students during OSCE (Alinier, 2003).

The measurement of clinical skills performance continues to pose a challenge for nurse educators. Experience suggests that the OSCE may be a powerful tool in the evaluation of clinical competence in nursing and that it may also be an effective facilitator for learning to perform clinical skills in nursing. Although there are a few drawbacks in using OSCEs they should not be neglected. The running cost of the OSCE is outweighed by the educational benefits (Ahmad, Ahmad & Abu Bakar, 2009) as well as the students' satisfaction to have learned something useful. The potential of OSCE as a flexible teaching method has been recognized by many lecturers from the University of Hertfordshire and might be used more regularly in several nursing curricula. This provides opportunities for students to use a number of medical pieces of equipment in a safe environment and to become more familiar with them.

Using problem-based learning scenarios, students have to employ critical thinking skills related to both the practice and theory of the task they are expected to perform. OSCE can be set up to integrate IT, communication, and critical thinking using simulation. From this it can be suggested that OSCE provide an integrated way of measuring learning outcomes in skills based learning. This has implications for work-based learning. OSCEs encourage a deep approach to learning because higher cognitive functions are tested. The OSCE sessions not only help students determining their own weaknesses, but also enable examiners or lecturers to realize what the current students' are. If required additional teaching sessions can be organized to address skills that caused problems to the students during the OSCE. The use of such sessions may well be a key element to the training of better-prepared healthcare professionals. The widespread of hybrid OSCE to other disciplines to teach and assess students on basic skills specific to the different subject of study may well occur in the near future (Alinier, 2009).

Aims of the study:

a. Capacity Building of nursing faculties and staff members for using OSCE.
b. Establish simulated learning experiences (OSCE) in nursing practice.

c. Compare the feasibility, utility, and effectiveness of using simulated learning experiences (OSCE) versus traditional clinical assessment.
d. Examine Faculty and students perspectives for OSCE.
e. Evaluate the effectiveness of OSCE Versus traditional clinical assessment.

2. Subjects and Methods

Research Design:

A time serial research design was used to accomplish aims of this study.

Sample and Setting:

A total sample of 400 second and third year students were selected randomly from Faculty of Nursing - Cairo and Ain Shams Universities. Both faculties have integrated the same curriculum of second year (Medical Surgical Nursing) and third year of (Pediatric and Maternity & New Born Health Nursing). The nursing students of both faculties' universities attended the training program of high risk and critical care nursing units.

As well, 140 faculty staff members from Faculty of Nursing - Cairo and Ain Shams Universities were participated in the current study through attending preparatory workshops. Among this group 31 faculty staff members were selected randomly for implementation of the OSCE. The involved faculty staff members at both faculties were committed and dedicated for training of nursing students at skills laboratories. Selected students were assessed by both traditional and OSCE assessment methods. Data collection was done twice: firstly through assessment of 190 students in different nursing specialties, however, the second trial was done through assessment of 210 students.

Tools of Data collection:

These tools were developed by the investigators. They were Faculty member needs assessment sheet; students' assessment and evaluation (Achievements) sheets; and student and staff perspective sheets.

a. Faculty member needs assessment sheet it was developed, used and analyzed to plan accurately TOT program.
b. Students' assessment and evaluation (Achievements) sheet covered the three domains to give an accurate judgment on student adequacy regarding the specified course knowledge, skills and attitudes.
c. Students' and staff perspective sheets that was used to assess and analyze the information about student and staff feedback, opinion as regard OSCE used as well as their recommendation to improve the newly introduced system.

Procedure:

To accomplish the aims of the study two approaches were utilized. The first approach was training of trainers for OSCE and implementing OSCE scheme on students and examined its impact on students' achievements. The second approach was assessing and analyzing the students' and faculty staff members' feedback and perspectives in regard to the newly introduced system (OSCE) as well as their recommendation to improve it. Ethical review of the study project was obtained, developing data collection tools, holding seminar for faculty staff members at both faculties to raise the awareness of OSCE. A total 72 of faculty staff members attended the seminar where needs assessments were carried out.

After reviewing related literature to fulfill the aims of the study, three different tools were designed by the research team and revised by the consultants, also content validity and expert's opinion were taken into consideration and the needed modifications were carried out., and

Face Validity of the tools was examined through a jury of three experts.

Regarding the planning phase it lasted for one month where 7 TOT - OSCE training workshops were conducted for faculty staff at Cairo and Ain Shams Nursing faculties, each for 3 days/week (approximately 20-30 trainers) at Cairo and Ain Shams Universities. Four workshops were held at faculty of nursing Cairo University and 3 workshops at Ain -Shams University. A pre-post test was administered to examine the impact of workshops in gaining knowledge about OSCE. In addition, training for putting the scheme as well as clinical scenarios was carried out; an expert for each nursing specialties attended the training workshop and was assigned to review the scenarios related to the specialties.

The implementation and data collection phase (lasted six months) where data collection was carried out using the designed tools. Training of data collectors was done where a total of 31 clinical instructors and faculty members were trained for implementing the OSCE and collecting data. through 3 workshops were carried out. OSCE were carried out at critical care units at medical-surgical, maternal-newborn health nursing and pediatric health nursing on total of 400 students at second and third year at both faculties during academic year 2008-2009 second semester as a first trial. Students were evaluated at their clinical training areas using the OSCE method Students and faculty perspectives sheets were distributed to be fulfilled at their own

pace. An oral feed back was obtained through interviewing of a focal group of students.

The second trial was conducted at the academic year 2009-2010 first semester at critical care units at maternal-newborn health nursing and pediatric health nursing at both faculties; the students were evaluated in their clinical training areas using the traditional method of evaluation in one area and the other one is evaluated using the OSCE method.

3. Results

Findings of the current study are presented in two main sections: The first one represents faculty staff members' capacity building, and the second one is concerned with students' academic achievements.

Section I: Faculty staff members' Capacity Building and Perspectives

A- Needs Assessment

This section represents findings related to description of faculty staff members according to their academic rank, computer skills, needs' assessment, pre-post test findings and their faculty perspectives about application of OSCE. Table (1) shows that more than two thirds of the study group (70.8%) was clinical instructors and assistant lecturers, while the other one third (29.5%) was faculty staff members. Their mean age was 32.31 ± SD = 8.391 years old.

Table (1): Distribution of Academic Rank and Age among Staff Members who Attended the Raising Awareness Seminar (n=72).

Academic rank	No.	%
1- Clinical instructor	33	45.8
2- Assistant lecture	18	25
3- Lectures	14	19.4
4- Assistant professor	4	5.5
5- Professor	3	4.2
Total	72	100
Mean age = 32.31±8.3 Age range: 23 : 62		

As shown in table (2) more than half (55.6%) of staff members (Cairo & Ain Shams Universities) indicated that the disadvantages of traditional clinical evaluation were being time and effort consuming, in addition to subjectivity, and shortage of resources in clinical areas AS indicated by 26.38%, and 20.83% respectively.

In relation to knowledge about OSCE; table (3), and table (4), show that 43.1% of the staff members had knowledge, most of them (73.3%) obtained their knowledge from workshops, and (n = 24 = 77.4%), and indicated that OSCE is used to evaluate knowledge, intellectual and practical objectives. The entire study group (100%) who had knowledge about OSCE reported that OSCE saves time and efforts. However, inspite of having knowledge about OSCE 98.6% of the faculty staff members who attended the raising awareness seminar did not have previous experience in utilizing OSCE. In addition, 77.45% indicated that it is a valid and reliable method of evaluation. As regard steps of developing OSCE; the great majority of those who knew OSCE (96.7%) indicated that it should start with training of the staff members, (64.5 %) indicated that it requires establishing OSCE laboratories with simulators at first, while more than half (51.6%) revealed that it should start with setting the objectives and competencies.

Table (2): Disadvantages of Traditional Clinical Evaluation as Mentioned by the Staff members who attended the raising awareness seminar (n=72).

Item	N	%
Disadvantages of traditional clinical evaluation*		
1- Time and effort consuming	40	55.6
2- Ineffective with large numbers	12	16.6
3- Subjectivity and low reliability	19	26.38
4- Shortage of resources in clinical areas	15	20.83
5- Lack of standardized cases	3	4.16

***** Responses are not mutually exclusive

Table (5) shows the distribution of the Staff Members as Regards their Knowledge about the Requirements to Establish OSCE training 58.33%,%, and skills in using OSCE as a method of evaluation in different specialties indicated by 36.15% of them.

Regarding the benefits of using OSCE as indicated by the staff members; table (6) shows that using OSCE, saves time for performing another activity in the institution (76.3%), and prepares highly qualified, competent graduates, and improves students' performance (62.5%). However, the obstacles of using OSCE were concerned with lack of maintenance, high costs, and shortage of staff (69.4%, 69.4%, and 65.2%) respectively.

B- Faculty Pre/Post test scores

Regarding to the findings of the pre/ post test about OSCE among the staff members who

attended the workshops, table (7) shows that the proportion of those who provided correct answers related to OSCE system increased significantly in the post test as compared to pretest. In addition, there was a significant improvement in the post test responses / answers regarding characteristics of OSCE, with higher mean post test scores as compared to the pre test scores, indicating high significant statistical differences.

Table (3): Frequency Distribution of the Faculty Staff Members who Attended the Raising Awareness Seminar as regards Their Knowledge about OSCE (N=72).

Items	No.	%
a-What is OSCE?		
Know	31	43.1
Does not know	41	56.9
Total	72	100
b-Types of objectives that can be evaluated by OSCE		51.4
Know:	37	
1-Knowledge and understanding	2	2.8
2-Intellectual	11	15.3
3-Practical	0	0
4-Knowledge, intellectual, and practical	24	33.3
Does not know	35	48.6
Total	72	100
c-Previous experience in using OSCE Yes	1	1.4
No	71	98.6
Total	72	100

Table (4): Sources of Knowledge, Advantages, and Steps for Developing OSCE as Indicated by the Faculty Staff (Data Collectors) who knew what is OSCE (n=31).

Item	N	%
- **Sources of Knowledge about OSCE**		
1- Workshops	22	73.3
2- The Internet	8	26.6
3- Working in another faculty "outside Egypt"	1	3.1
Total	31	100
- **Advantages of OSCE *** 1- Saves time and effort	31	100
2- Valid and reliable method of evaluation	24	77.4
- **Steps for developing OSCE***		
1- Need assessment	12	38.7
2- Establishing OSCE labs with stimulators	20	64.5
3- Preparing the examiners committee	10	32.2
4- Set the standards for student evaluation	5	16.12
5- Set the objectives and competencies	16	51.6
6- Provide training for the staff	30	96.7
7- Prepare an exam blueprint	5	16.12
8- Preparing clinical scenarios	11	35.4
9- Don't know	3	9.6

***** Responses are not mutually exclusive

Table (5):Distribution of the Staff Members by their Knowledge about the Requirements to Establish OSCE (n=72).

Items	No.	%
1-**Topics needed in the OSCE training session***		
1- How to use OSCE effectively	42	58.33
2- Designing OSCE exam	25	34.7
3- Training in OSCE lab.	14	19.4
4- Advantages and disadvantages of OSCE	25	34.7
5- Evaluation tools used in this system	16	22.2
6- Preparing the OSCE stations	6	8.33
7- How to put the exam scenarios	10	13.8
8- Don't know	19	26.38
2-Skills acquired during the OSCE training sessions*		
1- Setting clinical scenarios	5	6.9
2- Assess large numbers of students effectively without bias in short time	23	31.9
3- Developing blueprint	7	9.7
4- Using OSCE evaluation in different specialties	26	36.1
5- Be acquainted with the theoretical part of OSCE	32	44.4

***** Responses are not mutually exclusive

Table (6): Advantages of Using OSCE at the Academic Institution as Indicated by the Staff Members who attended the raising awareness seminar (n= 72).

Items	No.	%
Benefits of using OSCE in the academic institution *		
-Deal with increasing number of students	26	36.1
-Prepare highly qualified, competent graduates	45	62.5
-Improve students performance	45	62.5
-Saves time for performing another activity in the institution	55	76.3
-Help in accreditation	23	31.9
-Increase the number of newly appointed students in the institution	14	19.4
-Improving the performance of assistant staff	12	16.6
-Objectivity in clinical evaluation	22	30.5

Table (7): Comparison of OSCE Pre/Post Test Mean Scores among the Staff members who attended the preparatory workshops (n=140).

Item	Mean ± SD	t	P-value
Pre test OSCE System scores	15.5+0.93	-13.23*	0.00
Post test OSCE System scores	17.24± 1.2		
Pre test characteristics of OSCE scores	7.9 + 1.08	20.1*	0.000
Post test characteristics of OSCE scores	10.11 + 0.68		
Total Pre test	24.00±2.081	-7.635*	0.000
Total Post test	25.79±1.765		

*Significance level at $p \leq 0.05$

C- Staff Members' Perspectives about OSCE.

The following table shows staff members' opinion regarding the OSCE system. It was ranked as very satisfactory to satisfactory by more than two thirds of the staff members with mean scores of staff members' opinion 43.06 + 16.08.

Table (8): Frequency Distribution of Staff Members' Perspectives (Data collectors) Regarding the OSCE System (N=31).

Items	V. Satisfactory		Satisfactory		Poor	
	No	%	No	%	No	%
The OSCE system 1- Measures the course objectives.	18	58.06	4	12.9	9	29
2- Is credible	18	58.06	4	12.9	9	29
3- Is consistent/ reliable	19	61.3	3	9.7	9	29
4- Requires analytical questions	20	64.5	1	3.2	10	32.2
5- Relates theory to practice	17	54.8	3	9.7	11	35.5
6- Lead to increased decision making ability	13	41.93	3	9.7	15	48.4
7- Increased knowledge and understanding	17	54.8	3	9.7	11	35.5
8- Enhances teaching level	18	58.06	4	12.9	9	29
9- Enhances methods of evaluation	14	45.2	8	25.8	9	29
10- Makes exams well developed	16	51.6	5	16.1	10	32.2
11- Makes exams/ questions clear	18	58.06	3	9.7	12	38.7
12- Makes exams/ questions suitable for different students levels	15	48.4	4	12.9	12	38.7
13- Makes exams/ questions to cover most of course contents	15	48.4	4	12.9	12	38.7
Mean + SD	28.6± 10.9					

Staff members' opinion total mean scores = 43.06 ± SD =16.08

The advantages of using OSCE at the academic institution as indicated by faculty staff members, figure (1) revealed that, OSCE saves time (76.3%) prepare highly qualified competent students (62.5%) and improve students' performance (62.5%).

Figure (1): Advantages of Using OSCE at the Academic Institution as Indicated by Faculty Staff Members Who Attended the Preparatory Workshops (n=140).

Section II: Students' Achievements and Perspectives.

A- Students' Achievements

Regarding the effectiveness of OSCE, the current study indicated that, the third year students obtained higher mean scores in OSCE pediatrics exams (22.03+SD=2.56) as compared to their mean scores of the traditional method of evaluation (24.68+ SD=2.96) with a highly statistically significant differences (t= 2.015, at p≤0.046). However, the opposite picture was observed in the other two specialties (Medical-Surgical Nursing & Obstetrics) where the mean scores of the traditional method of evaluation were higher than those of OSCE. However, global comparison between the two groups of OSCE versus traditional method of evaluation revealed higher mean OSCE scores with a high significant statistical difference between the two groups in first trial (table 9).

Table (10) shows comparison of means among the students who underwent OSCE in the second trial. It is clear from the table that high statistical significance differences was found between the two groups who undergone OSCE as compared to the non OSCE groups. The same

picture was noticed regarding the total mean scores of the groups who undergone OSCE as compared to non OSCE group, indicating a high statistical significant difference in second trial (t= 6.23, at p≤ 0.000).

Table (9): Comparison of Students' OSCE versus Traditional Evaluation System Mean Scores: First Trial (N=190).

Specialties	OSCE	Traditional Evaluation	t	P-value
	Mean ± SD			
Medical-Surgical Nursing	22.03+ 2.56	24.68+ 2.96	-5.071	0.00**
Pediatrics	27.62+ 4.29	26.36 + 1.73	2.015	0.046*
Obstetrics	23.16 + 5.43	26.003+ 2.99	-3.702	0.00**
Total mean scores	24.548 + 4.65	23.544+ 4.73	2.423	0.016*

* Significance at p≤0.05 ** Significance at p≤0.001

Table (10): Comparison of OSCE versus non OSCE mean scores among Undergraduate Students after Establishment of OSCE: Second Trial (N= 210).

Specialties	OSCE	Traditional Evaluation	t	P-value
	Mean ± SD			
Obstetrics	26. 11± 2.26	24.92 ± 2.22	4.68	0.00**
Pediatrics	26.24±5.38	22.54±5.55	5.17	0.00**
Total mean scores	26.18±4.18	23.69±4.44	6.23	0.00**

* Significance at p≤0.05 ** Significance at p≤0.001

B-Students' Perspectives

The following tables (11 and 12) show students' perspectives regarding the OSCE system. It was ranked as very satisfactory to satisfactory by more than two thirds of the students; the mean score of students' opinion was 43.22 ± SD=13.59. In relation to student's perspectives regarding OSCE preparation, table (12) reveals that preparation to OSCE was ranked as very satisfactory to satisfactory by more than one third of the students regarding availability of time table, and conducting training sessions. The same rank was given to obvious preparation to OSCE by approximately half of the student's. As regards OSCE's laboratories, more than half of the students indicated that they were suitable, lighted and ventilated, clean, calm, with availability of the needed equipments and simulators. Comparison of means indicated highly statistical significant difference between preparation to OSCE mean scores and those of OSCE laboratory (t = - 16.14, p ≤ 0.000).

Table (11): Frequency Distribution of Students' Perspectives Regarding the OSCE System (N=190).

Items	Very Satisfactory		Satisfactory		Unsatisfactory	
	No	%	No	%	No	%
The OSCE system 1- Measures the course objectives.	86	45.3	48	25.3	56	29.5
2- Is credible	93	48.9	40	21.1	57	30
3- Is consistent/ reliable	81	42.6	52	27.4	57	30
4-Requires analytical questions	98	51.6	28	14.7	64	33.7
5-Relates theory to practice	102	53.7	34	17.9	54	28.5
6-Lead to increased decision making ability	98	51.6	36	18.9	56	29.5
7-Increased knowledge and understanding	90	47.4	44	23.2	56	29.5
8-Enhances teaching level	103	54.2	33	17.4	54	28.5
9-Enhances methods of evaluation	97	51.05	36	18.9	57	30
10-Makes exams well developed	98	51.6	39	20.5	53	27.9
11-Makes exams/ questions clear	81	42.6	49	25.8	60	31.6
12- Makes exams/ questions suitable for different students levels	79	41.6	45	23.7	66	34.7
13- Makes exams/ questions to cover most of course contents	80	42.1	34	17.9	76	40
Mean ± SD	28.1± 9.6					

Table (12): Frequency Distribution of Student's Perspectives Regarding Preparation to OSCE, and the OSCE laboratories. (n=190).

Items	V. Satisfactory		Satisfactory		Unsatisfactory		T	P
	No	%	No	%	No	%		
Preparation for the OSCE 1- Was obvious before establishing OSCE	53	27.9	35	18.4	102	53.7	-16.14*	0.000
2- Time tables were available and known to students	33	17.4	41	21.6	116	61.1		
3- Regular training on OSCE	31	16.3	29	15.3	130	68.4		
Mean + SD	4.8+ 2.03							
The OSCE labs. 4- 1-Suitable	46	42.2	46	24.2	88	46.5		
2-Light and ventilation	45	23.7	55	28.9	90	47.4		
3-Set up and Cleanliness	45	23.7	43	22.6	102	53.7		
5- Being calm	43	22.6	43	22.6	104	54.7		
5-Availability of needed equipments and simulators	41	21.6	44	23.2	105	55.3		
6-Suitable for student' number	45	23.7	48	25.3	97	51		
Mean + SD	10.2± 4.2							

*Significance at p≤0.05

4. Discussion:

The acquisition of clinical skills is paramount to the development of a safe and competent practitioner (Brookes, 2007). OSCE as a performance-based assessment is a well established student's assessment tool for many reasons: competency- based, valid, practical and wise effective mean of assessing clinical skills that are fundamental to the practice of nursing and other health care related professions (Ainier, 2003).

The aims of the present study were to: Build capacity of nursing faculties and staff members for using OSCE; establish simulated learning experiences (OSCE) in nursing practice; compare the feasibility, utility, and effectiveness of using simulated learning experiences (OSCE) versus traditional clinical assessment; examine faculty and students perspectives for OSCE; and evaluate the effectiveness of OSCE versus traditional clinical assessment.

A quasi-experimental research design was used to accomplish aims of this study, and a total sample of 257 students as well as 31 faculty staff members, from Cairo and Ain Shams universities were recruited for the study. The included faculty staff members represented different categories of the academic rank of them more than two thirds were instructors and assistant lecturers, and the other one third was lecturers and professors.

The faculty staff members' knowledge about evaluation was assessed and more than two thirds of them provided complete definition about evaluation and identified different methods of traditional evaluation. However they commented on the traditional clinical evaluation to have certain disadvantages such as being time and effort consuming, in addition to subjectivity, and shortage of resources in clinical practice setting. As regards staff members who attended the preparatory workshops their knowledge about OSCE was assessed using pre/ post test which indicated that the proportion of those who provided correct answers related to OSCE system increased significantly in the post test as compared to pretest. In addition, there was a significant improvement in the post test responses / answers regarding characteristics of OSCE. High significant statistical difference was found in post test mean scores as compared to pretest scores. Regarding sources of knowledge about OSCE, approximately one half of the staff members had knowledge about OSCE, three fourth of them obtained their knowledge from workshops. This could indicate that preparatory workshops had positive impact on the faculty staff members' knowledge.

The great majority of those who knew OSCE recommended that it should start with establishing OSCE laboratories with simulators at first, staff members training, in addition to setting objectives and competencies required for practical training. Moreover, faculty staff members indicated that OSCE should involve teaching certain topics in the OSCE training sessions such as how to

use OSCE effectively as a method of evaluation in different specialties.

As well, more than three fourth of the faculty staff members indicated that using OSCE has certain benefits such as saving time for performing another activity in the institution, preparing highly qualified and competent graduates, and improving students' performance. In addition, the OSCE system was ranked as very satisfactory to satisfactory by more than two thirds of the faculty staff members. However, using OSCE was described to have certain obstacles such as the need for continuous maintenance, its high costs, and shortage of staff members who can implement OSCE. These findings are in agreement with study done by Pharm and Sturpe, (2010), Turner and Dankoski, (2008) who revealed that a major obstacle in the wide implementation of OSCEs is their high cost. However, it can be set up with reasonable cost and limited resources even in smaller institutions

In relation to student's perspectives regarding OSCE preparation, it was ranked as very satisfactory to satisfactory by more than one third of the students regarding availability of time table, and conducting training sessions. The same rank was given to obvious preparation to OSCE by approximately half of the student's. Regarding OSCE's laboratories, more than half of the students indicated that they were suitable, lighted and ventilated, clean, calm, with availability of the needed equipments and simulators (Alinier, 2003).

Regarding students' perspectives toward preparation to OSCE, comparison of mean knowledge scores indicated high statistical significant difference between knowledge about preparation to OSCE mean scores and those about OSCE laboratory ($t = - 16.14$, $p \leq 0.000$) . As regards students' opinion about the OSCE system, it was ranked as very satisfactory to satisfactory by more than two thirds of the students. This feedback can suggest that OSCE is an objective tool for evaluating clinical skills. The objectivity of OSCE was highlighted in different literature such as those done by Ahuja, (2009) and AL-Omari & Shawagfa, 2010) and (Miller, 2009).

These findings are in agreement with a study conducted by El Nemer & Kandeel, (2009) who reported that most students viewed OSCE as a fair assessment tool which covered a broad area of knowledge, allowed them to compensate in some areas and minimized their chances of failing. In addition, as indicated by Pierre et al., (2004) reported favorable student's responses concerning transparency and fairness of the examination process, and the authenticity of the required tasks per station.

Moreover, as found by Pierre et al., 2004; Duffield & Spencer, (2002), most students viewed OSCE as a fair assessment tool which covered a broad area of knowledge, allowed them to compensate in some areas and minimized their chances of failing. The fairness of OSCE was also reported by other studies. As well, in a study conducted by Turner & Dankoski, (2008) to assess the validity, reliability and feasibility of OSCE team, the majority of students felt that they had been marked fairly. Most students provided positive feedback about the quality of OSCE performance in terms of the clarity of the instructions of the exam, the sequence of OSCE stations, the reflection of the tasks taught and the time at each station. These findings are consistent with Pierre et al. (2004) study results where most students viewed OSCE as comprehensive, covered a wide range of knowledge and clinical competencies and a useful practical experience. As well more than two thirds of students believed that the assessment was fair and they reported their need for more time to complete the stations. In another study done by Alinier (2003) nursing students perceived OSCE as a favorable experience that should be repeated regularly.

However, the current study findings are in agreement with a study done by Nemer, and Kandeel, (2009) where OSCE was perceived as a stressful experience and intimidating by a considerable percentage of students, particularly first year nursing students. This perception could be due to the fact that it was a new experience for nursing students which increased their anxiety. As well nursing students' stressful experience with OSCE was also reported in another studies done by Pierre et al., (2004), Byrne and Smyth (2008) who related students' stress and anxiety to the new experience with OSCE. On the same line Allen, Byrne and Smyth (2008) indicated that studies surveying student attitudes during the OSCE have documented that it can be a strong anxiety-producing experience, and that the level of anxiety changes little as student's progress through the examination. This can direct the attention toward the importance of preparing students to OSCE.

Regarding the effectiveness of OSCE, the current study pointed out that, the third year students obtained higher mean scores in OSCE pediatrics exams (22.03+SD=2.56) as compared to their mean scores of the traditional method of evaluation (24.68+ SD=2.96) with a highly statistically significant differences ($t= 2.015$, at $p\leq0.046$). However, the opposite picture was observed in the other two specialties (Medical-Surgical Nursing & Obstetrics) where the mean scores of the traditional method of evaluation were higher than those of OSCE. However, global comparison between the two groups

of OSCE versus traditional method of evaluation revealed higher mean OSCE scores with a high significant statistical difference between the two groups in first trial. These findings support the idea that OSCE is not designed to replace the traditional clinical practice assessment method, but rather it is to complement students' assessment in the clinical setting. Therefore, assessing nursing students' OSCE competency level is carried out in combination with traditional method of assessment usually done for students undergoing traditional learning mode.

As well, comparing mean scores of students who undergone OSCE in the second trial revealed high statistical significance difference between the two groups who undergone OSCE as compared to the group who undergone traditional method of evaluation. The same picture was noticed regarding the total mean scores of the groups who undergone OSCE as compared to the group who undergone traditional method of evaluation, indicating a high statistical significant difference in second trial ($t= 6.23$, at $p\leq 0.000$).

On the same line with findings of the current study, the evaluation of OSCE by nursing students highlighted some areas that need to be enhanced in future, such as the inadequate time of some of the stations, and the limited period of orientation about OSCE. The insufficient time at OSCE stations was one of students' complaints in some of the studies which investigated students' perspective of OSCE (Pharm & Sturpe, 2010).

This could be the rational of why several authors suggest that no health professional educational program should be assessed by the OSCE alone, or indeed any other single method. Rather, in the absence of a 'gold standard', successful outcome in assessments using a range of methods is repeatedly advocated as producing the most inclusive evidence of practitioner competence (Ahmad, Ahmad & Abu Bakar, 2009 and McKinley & Boulet, 2004).

5. Conclusion:

Based on the findings of the study it is concluded that:

- OSCE is a valid and reliable technique uniquely capable of assessing many fundamental clinical skills that are not being assessed in a rigorous way in most undergraduate and postgraduate programs.
- OSCE seems to offer particular strengths in terms of assessor objectivity and parity of the assessment process for all students, especially when compared with other assessment of practice processes. However, it is not without limitations, not only in terms of student stress and its considerable demands on the academic study, but also in terms of the considerable challenges of ensuring the validity and reliability of the process.
- OSCE examination offers an attractive option for assessment of practitioner competency.
- Each new OSCE should be a subject to rigorous scrutiny and piloting to ensure that the reliability and validity of that particular assessment is maximized.
- Findings of the current study highlight the need for student participation in the development of new assessment tools in nursing curricula.

Recommendations:

- Based on findings of the current study, it is recommended that OSCE must be used as an integral part of the clinical evaluation system / students' assessment at the under graduate and post graduate educational programs.
- OSCE should be used as a method of evaluating clinical practice in a combination with traditional method.
- It can be suggested that OSCE has the potential to make a very effective and meaningful contribution to 'fitness for practice' assessment.
- The level of competency in OSCE should be tested not only for nurses using the traditional methods of learning, but also for distance learning students.

Nursing implication:

OSCE as an effective and valid assessment method can be used to assess students' clinical competencies in different nursing specialties. As well, OSCE can be used in other international countries in the same situation as Egypt i.e has a large number of students and it is difficult to evaluate their skills.

Corresponding author
Shadia A. Eldarir
Maternal-Newborn Health Nursing Dept., Faculty of Nursing Cairo University, Cairo, Egypt
shadiaeldarir@yahoo.com

6. References:

1. Ahmad, C., N. Ahmad & Abu Baker R., (2009). Assessing Nursing Clinical Skills Performance Using Objective Structured Clinical Examination (OSCE) for Open Distance Learning Students in Open University Malaysia. International Conference on Information; Kuala Lumpur, 12-13 August.
2. Ahuja, J. (2009). OSCE: A Guide for Students, Part 1. Practice Nurse, 37(1), 37 – 39.

3. Alinier, G. (2009). Nursing students' and lecturers' perspectives of osce, incorporating simulation.
4. Alinier, G., (2003). Nursing students' and lecturers' perspectives of objective structured clinical examination incorporating simulation. Nurse Education Today, 23(6): 419-426.
5. Al-Omari, A. & Shawagfa, Z. M. (2010).New experience with objective structured clinical examination in Jordan. Rawal Medical Journal. Vol. 35, No.1 Jan-June.
6. Bartfay W. J., Rombough R., Howse E, & LeBlance, R. (2004). The OSCE in nursing education: objective structured clinical examinations can be vehicles for nursing education and practice by promoting the mastery of clinical skills and decision making in controlled and safe environments. The Canadian Nurse 100: 18–27.
7. Brewin, J. & Cantwell, R. (1997). Implementing the OSCE in Nottingham. Psychiatric Bulltin, 21:30-32.
8. Brosnan, M., Evans, W., Brosnan, E. & Brown, G., (2006). Implementing objective structured skills evaluation (OSCE) in nurse registration programmes in a centre in Ireland: A utilization focused evaluation. Nurse Education Today 26, 115-122.
9. Brookes, D. **(**2007) Objective Structured Clinical Examination assessment. www.nursingtimes.net
10. Byrne, E. & Smyth, S. (2007). Lecturers' experiences and perspectives of using an objective structured clinical examination, School of Nursing and Midwifery, National University of Ireland, Galway, Ireland
11. Duffield, K.E. & Spencer, J. A. (2002). A survey of medical students' views about the purposes and fairness of assessment. Medical Education, 36, 879-886.
12. Ebbert, D. W., & Connors, H. (2004). Standardized patient experiences: evaluation of clinical performance and nurse practitioner student satisfaction. Nursing Education Perspectives, 25(1), 12-15.
13. Elzubeir, M. & Rizk, D. (2003). Traditional finals and OSCEs in predicting consultant and self-reported clinical.Reliability of the MRCP(UK) Part I Examination Volume 37 Issue 7 , Pages 579 - 664 (July 2003)
14. El-Nemer, A. & Kandeel, N. (2009). Using OSCE as an Assessment Tool for Clinical Skills: Nursing Students' Feedback. AJBAS July-September, 2009 2465-2472
15. Harden, R., (1988). W hat is an OSCE? Medical Teacher, 10:19-22.
16. Health Workforce Development (HWD) Project (May 2006). Final report, Baseline survey- part I: Assessment of competency resulting from medical and nursing education in Egypt. Egypt Health Workforce Development Project.
17. Major, D., (2005) Seven years on the bandwagon: the progress of an objective structured clinical evaluation programme. Nurse Education Today 25, 442-454.
18. McKinley, D.W. & Boulet J.R. (2004). Detecting score drift in a high-stakes performance-based assessment. Advances in Health Sciences Education. 9 (1):29-38,
19. Miller, G.E. (1990). The assessment of clinical skills/competence/performance. Academic Medicine (supplement) 65, S63-S7.
20. Miller, J.K. (2009). Competency- based training: objective structured clinical experiences (OSCE) in marriage and family therapy. Journal of Marital and Family Therapy.1-13.
21. Munoz, L.Q., O, Byrne, C., Pugsley, J. & Austin, Z. (2005). Reliability, validity and generalizability of an objective structured clinical examination (OSCE) for assessment of entry-to practice in pharmacy. Pharmacy Education, 5 (1), 33-34.
22. Pierre, R., Wierenga A., Barton M., Branday J.M. & Christie, C., (2004). Student Evaluation of an OSCE in Pediatric at the University of the West Indies, Jamaica. BMC Medical Education, 4(22):1-7.
23. Pharm, D. & Sturpe, D.A. (2010). Objective structured clinical examinations in doctor of pharmacy program in the United States. American Journal of Pharmaceutical Education. 74 (8): Article 148. 1-6.
24. Schoening, A., Sittner, B., & Todd, M. (2006). Simulated clinical experience: nursing students' perceptions and the educators' role. Nurse Educator, 31(6), 253-258.
25. Turner, J.L. & Danksoki, M.E. (2008). Objective structured clinical exams: a critical review, Family Medicine. September, 574-578.

11/2/2010

Effect of Type of Aggregate on the Properties of Refractory Concrete

S.A. Ghonaim*, H.B.G. Ghazal, and M.F. Abadir*** [+]**

* Egyptian Organization for Standardization and Quality
** The High Institute of Engineering, Shorouk
*** Chemical Engineering Department, Faculty of Engineering, Cairo University
[+] Corresponding author: Magdi F. Abadir
The Chemical Engineering Department, Faculty of Engineering, Cairo University, Giza, Cairo, Egypt
Area Code: 12613; Tel: 202 35678617; Fax: 202 35678625; E–mail: magdi.abadir@yahoo.com

ABSTRACT: Low cement refractory concrete samples were prepared by mixing cement (containing 50% alumina) in percentages ranging from 10 to 20% with aggregate and the necessary amount of water. Two types of refractory aggregate were used: Bauxite containing 81% alumina and grog containing 52% alumina. Four particle sizes of each aggregate were used each time. The cast samples were left in their moulds for 24 hours in a 100% relative humidity cabin. The de–molded specimens were left in open air until their moisture content reaches 3–6%, then put in a drying oven at (110 ± 5) ºC until reaching constant weight. They were then tested for phase constitution, water absorption, bulk density, apparent porosity and cold crushing strength (after 28 days curing). It was found that bauxite based samples gave better results than those prepared with grog. It was also found using statistical analysis that the percent cement used affects all properties much more than does the particle size of aggregate.
[S.A. Ghonaim, H.B.G. Ghazal, and M.F. Abadir. **Effect of Type of Aggregate on the Properties of Refractory Concrete.** Journal of American Science 2010;6(12):1015-1027]. (ISSN: 1545-1003). http://www.americanscience.org.

Key Words: Refractory concrete – Alumina – Grog – Sodium citrate

1. INTRODUCTION

An analysis of the composition, the structure, and the properties of the conventional refractory concretes shows that their refractory properties are governed by the filler contained in them. The binder component (bonding agent) of the concretes imparts the strength required during transportation and erection; this strength is attained after setting and drying. During subsequent heating up to the temperatures preceding sintering, irreversible destructive processes occur, as a rule, in the binder. In view of the fact that the binder (along with the finely milled additives) forms a continuous matrix phase in the structure of the concrete, the thermo–mechanical characteristics of the material are adversely affected. Therefore, in order to improve the existing refractory concretes and to create new concretes, it is necessary to decrease the content of the conventional binders (e.g., high–alumina cements) in them to the maximum possible extent or to produce them without introducing the conventional (common) binders [1, 2, 3, 4].

Thermally stable aggregates combined with a bonding agent are the principal ingredients of a monolithic refractory. These raw materials are available both naturally and artificially. Raw materials available in nature unavoidably vary slightly in their compositions. But it is important to take advantage of the characteristics of these natural minerals that cannot be developed artificially rather than to avoid their use due to variations of chemical composition.

Unlike natural raw materials, artificial raw materials allow adjustment of chemical composition as well as their mineral constituents, and it is possible to get a uniform quality.

One common type of aggregate is bauxite, a raw material for alumina containing about 60% alumina. When calcined, the alumina level is usually raised above 85%. Bauxite for refractories is calcined in a rotary kiln to make a stable product. Calcined bauxite contains corundum as its principal component, mullite and a small glassy phase.

On the other hand, grog is an artificial aggregate usually obtained from crushed defective refractory bricks. Its alumina content depends on that of the original bricks. It usually ranges from 40 to 80%.

Other types of aggregate include diaspore ($Al_2O_3 . H_2O$), corundum (Al_2O_3), magnesia (MgO), zirconia (ZrO_2), etc.

In the present paper are studied the physico–mechanical properties of refractory concrete samples prepared from bauxite and grog with varying amounts of cement and varying particle size of aggregate.

2. EXPERIMENTAL

2.1 Raw Materials:

The raw materials used are:

- Refractory cement containing 50% alumina was obtained from Lafarge Cement.
- Calcined bauxite was obtained from the Alexandria Company for Refractories with an alumina content exceeding 80%.
- Grog was obtained from previously fired defective bricks that were crushed, ground and screened.

2.2 Particle Size Distribution of Aggregate:

In order to determine the grain size distribution, the procedure described by ASTM D 422/2007[(5)] was used. The standard sieves method was applied using screen apertures ranging from 6.68 mm (3 mesh) to 74 μm (200 mesh).

2.3 X–Ray Fluorescence Analysis (XRF):

X–ray fluorescence spectrometry (XRF) is a method of elemental analysis that assesses the presence and concentration of various elements by measurement of secondary X–radiation from the sample that has been excited by an X–ray source. The analysis was run on a AXIOS, panalytical 2005, Wave length Dispersive (WD–XRF) Sequential Spectrometer available at the National Research Center in Cairo.

2.4 X–Ray Diffraction (XRD):

X–Ray diffraction analysis differs from XRF in that it identifies, usually in a qualitative way, the phases present in the analyzed material rather than the elements.

For X–Ray diffraction study of bauxite and grog analysis, the aliquots for bulk mineral analysis were finely ground (–200 mesh), mounted randomly on an aluminum holder, and analyzed by a BRUKUR D8 ADVANCE COMPUTERIZED X–Ray Diffractometer apparatus (available at the Center of Metallurgical Research and Development Institute, Cairo) with mono–chromatized Cu Kα radiation, operated at 40 kV and 40 mA.

2.5 Preparation of Specimens:

Forty pastes of different size formulations for both bauxite and grog at different percentages cement (20%, 17.5%, 15%, 12.5%, 10%) by weight were kneaded with an adequate amount of water, which was determined for each batch according to the standard "good ball in hand test [(6)]". The mixed batches were then cast into cubes of 50 mm side length using a vibrating table at a frequency of 50 Hz and 4 minutes. The cast samples were left in their moulds for 24 hours in a 100% relative humidity cabinet. The hydrated samples were then demolded. The specimens were left in an open air until their moisture content reaches 3–6%, then put in the drying oven at (110 ± 5) ºC until reached constant weight. They were then tested for water absorption, bulk density and apparent porosity and cold crushing strength.

2.6 Apparent Porosity, Water Absorption, and Bulk Density:

These properties are determined according to ASTM Standards C 20/2007 [(7)]. For each test, the average measurements for five specimens at least are calculated.

The five specimens for each test are weighed to get the dry weight (D) for each. The test specimens are then placed in water and boiled for 2 h in a boiler and kept entirely covered with water with no contact with the heated bottom of the container. They are cooled to room temperature while still completely covered with water. The weight (S) of each test specimen is determined after boiling and while suspended in water. The saturated weight (W) is also determined.

Apparent porosity is calculated from:

$$P,\% = \frac{W - D}{V} \times 100 \qquad (1)$$

Water absorption is calculated from:

$$A,\% = \frac{W - D}{D} \times 100 \qquad (2)$$

While bulk density is calculated as follow**:**

$$\rho_B = \frac{D}{V} \times 100 \qquad (3)$$

Where:
P = apparent porosity, (%);
W = weight of the specimen as saturated with water, (g);
D = dry weight, (g);
S = weight of the specimen as suspended in water, (g);
V = exterior volume = W –S, (cm^3);
A = water absorption, (%);
ρ_B = bulk density, (g/cm^3).

2.7 Cold Crushing Strength:

This was done to determine the compression stress to failure of samples consisting of three specimens cured for 28 days. It represents an

indication of its probable performance under load. Each specimen was placed between two plates of the compression strength tester. This was followed by the application of an axial uniform load. The load at which a crack appears on the sample was noted, and it is calculated according to BS EN Standards 993–5/2000 [8]:

$$C.C.S(\sigma_c) = \frac{W}{a} \qquad (4)$$

Where:

σ_c = cold crushing strength, (MPa);

W = total maximum load at 3% deformation or at visible failure, (N);

a = average of gross areas of the two faces, (mm^2).

3. RESULTS AND DISCUSSION

3.1 Particle Size Distribution of Grog and Bauxite:

Grog and Bauxite were screened to different size fractions onto a set of standard sieves ranging form 3 mesh (Opening = 6.680 mm) down to 200 mesh (opening = 0.074 mm). The mean particle size of a fraction passing through a certain sieve and retained over the next was taken as the arithmetic average of the two openings. This way, the following mean sizes were used: 4.699 mm, 2.794 mm, 1.651 mm, 1.168mm, 0.991mm, 0.295 mm, 0.175 mm, 0.147 mm, and 0.074 mm.

Figure (1) shows the cumulative screen analyses for grog and bauxite used in the present investigation.

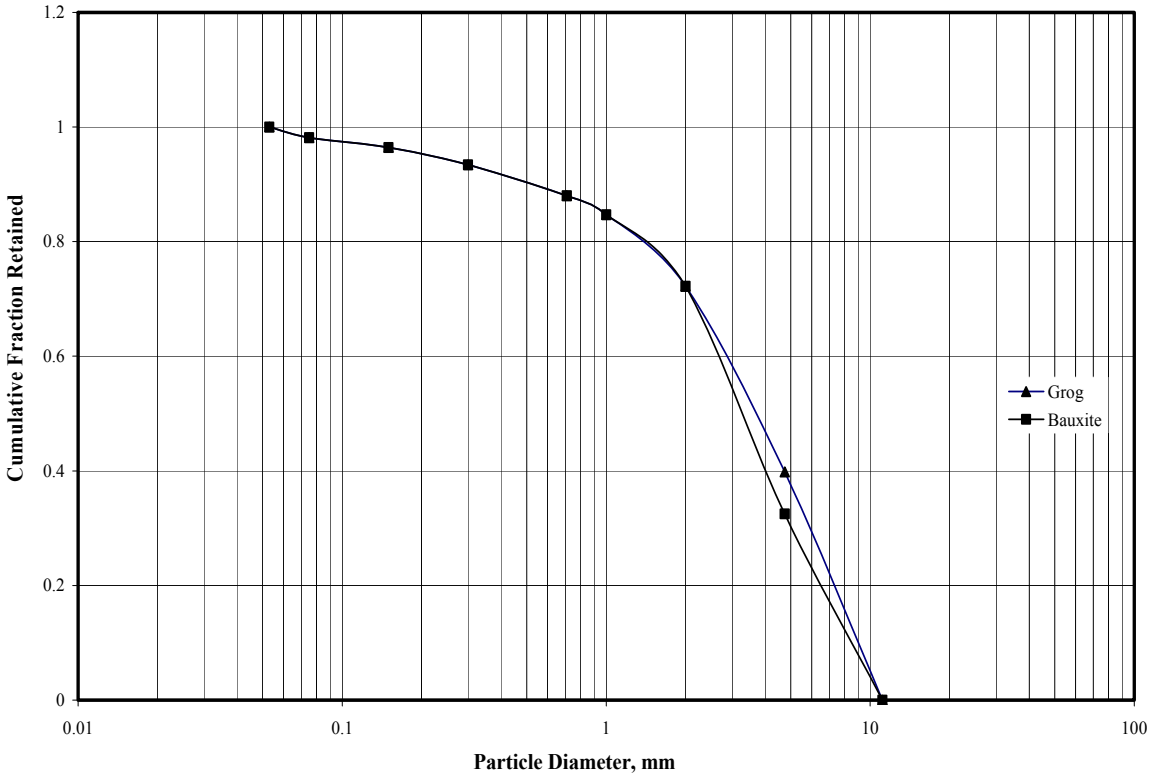


Fig. (1): Particle Size Distribution of Aggregates

3.2 Chemical Analysis of Raw Materials:

Table (1) shows the XRF results related to the chemical analysis of the refractory cement used, bauxite and grog.

Table (1): Chemical Analysis of Materials Used

Constituents (wt. %)	Cement	Bauxite Sample	Grog Sample
SiO_2	5.5	9.264	26.640
TiO_2	——	1.451	3.740
Al_2O_3	52.95	81.291	51.929
$Fe_2O_3^{tot.}$	2.5	1.816	2.994
MgO	Traces	0.372	0.510
CaO	38.05	0.435	1.215
Na_2O	< 0.1%	0.066	1.418
K_2O	< 0.1%	0.174	4.901
P_2O_5	——	0.542	1.056
SO_3	——	0.020	0.875
Cr_2O_3	——	0.120	0.069
Co_3O_4	——	0.084	0.039
Ga_2O_3	——	0.023	0.014
SrO	Traces	0.16	0.106
Y_2O_3	——	0.021	0.020
ZrO_2	——	0.272	0.190
Nb_2O_5, La_2O_3 ,CeO_2, Nd_2O_3, ThO_2	——	< 0.1%	< 0.1%
WO_3	——	0.237	——
PbO	Traces	1.6	0.011
Cl	——	0.022	4.183
L.O.I	——	1.811	——
Total	**≈100**	**≈100**	**≈100**

3.3 XRD of Raw Materials:

The following figures (2 and 3) show the XRD pattern obtained on investigating bauxite and grog. As can be seen from the figures, calcined bauxite consists exclusively of corundum (Al_2O_3) and mullite ($3Al_2O_3.2SiO_2$). This is expected from the phase equilibrium diagram $Al_2O_3 - SiO_2$ for compositions containing > 80% alumina [9]. On the other hand, the XRD pattern shows besides the expected phases of mullite and quartz some non–equilibrium phases of corundum and cristobalite, are also present lines of halite present as impurity.

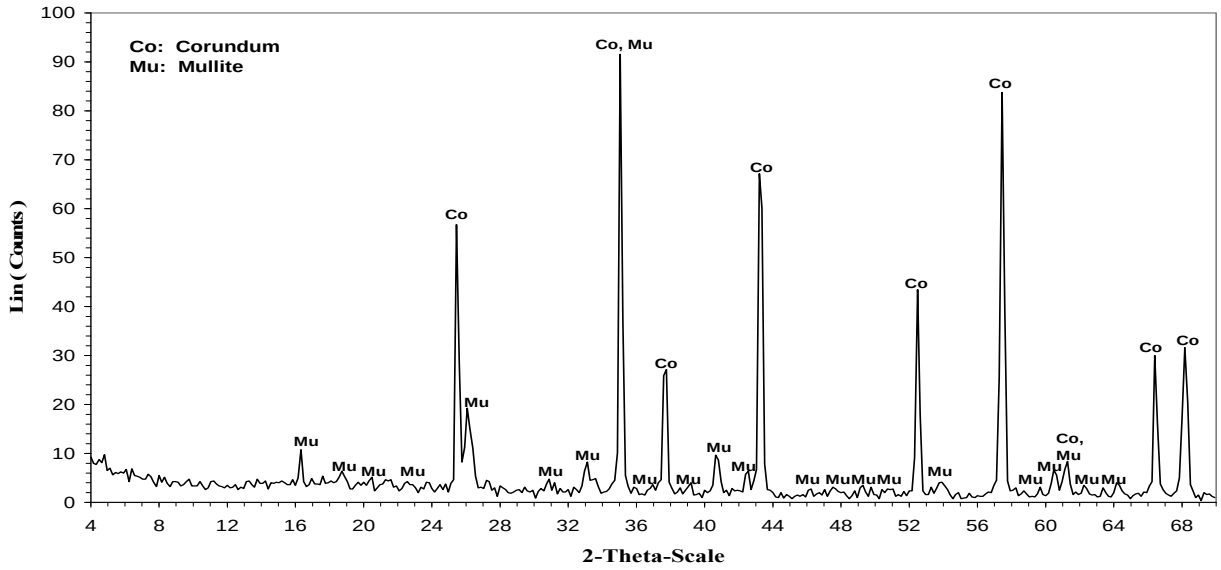


Fig. (2): XRD Pattern of Calcined Bauxite

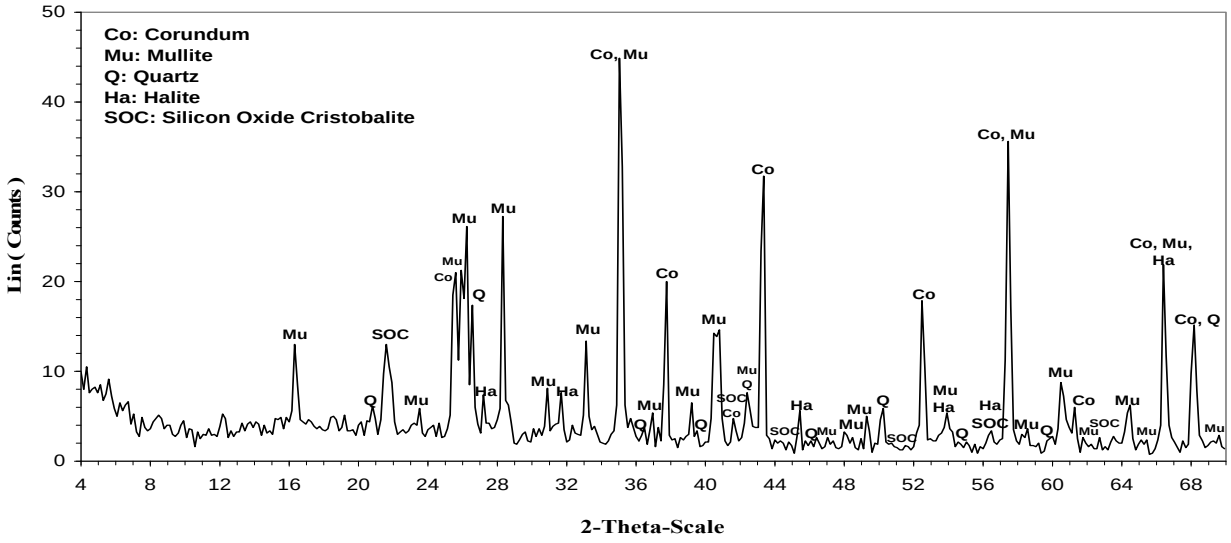


Fig. (3): XRD Pattern of Grog

3.4 Mean Particle Size of Aggregates:

In order to assess the effect of particle size of the aggregate used, bauxite and grog, on the workability, physical and mechanical properties of refractory concrete paste, four different particle size mixes were used. Each is a combination of three particle size ranges as is usually the case when wanting to maximize compactness. Table (2) shows the four mixes together with the average particle diameter of each as calculated using the method suggested by McCabe et al [(10)].

Table (2): Mean Particle Size of Aggregate Formulation Used

	% Weight			
	0–1 mm	**1–3 mm**	**3–5 mm**	**Mean Particle Size (mm)**
A	10	75	15	1.63
B	25	60	15	1.19
C	40	45	15	0.94
D	55	30	15	0.78

3.5 Effect of Mean Particle Size on Water Consumption:

During mixing, the addition ratio of water is directly affects the final product considerably [(2)]. When the amount of coarse particles in the particle size distributions used in this investigation increases, therefore the specific surface area of the particles decreases and less water is consumed because of this. The amount of consumed water as function of the mean particle size for both bauxite and grog are shown in figures (4 and 5).

It appears from these figures that the amount of water used increases with a decrease in particle size and with the amount of cement used. It is also seen that water consumption for samples containing grog is higher than for samples containing bauxite: these values range from 7.5 to 9.1% in case of bauxite based formulations against 9.5 to 10.9% in grog based formulations. This is presumably due to the presence of much more open pores in grog than in bauxite particles. To assess this point, the apparent porosity of samples of both types of particles was determined. It was found to be 6.7% for bauxite particles against 16.5% for grog particles.

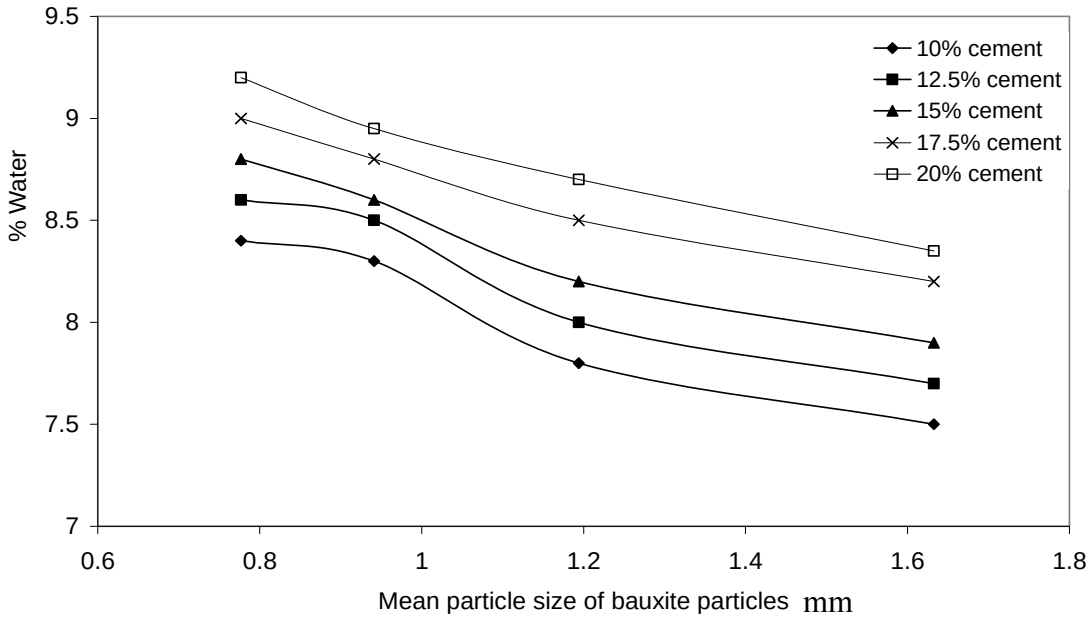


Fig. (4): Effect of Amount of Cement Used and Mean Particle Size of Aggregate on Water Consumption for Bauxite Based Mixes

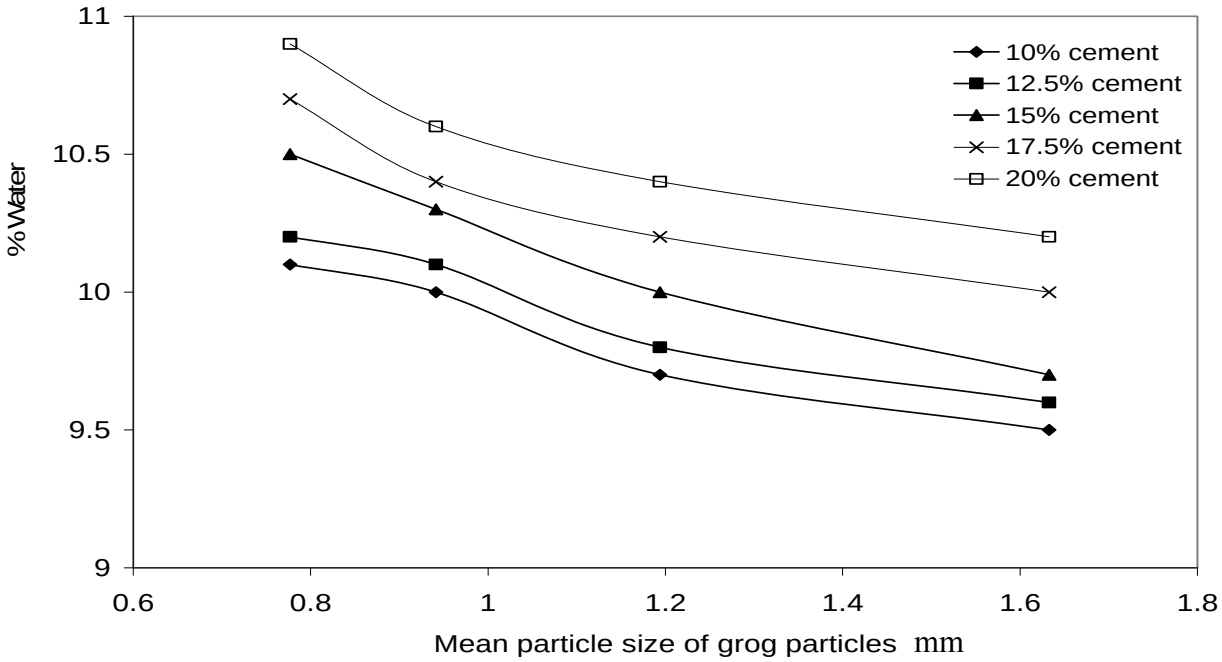


Fig. (5): Effect of Amount of Cement Used and Mean Particle Size of Aggregate on Water Consumption for Grog Based Mixes

Using the excel DATA ANALYSIS module it was possible to establish correlation tables in both cases that show the relative influence of percent cement and particle size on the percent water added. Such tables are shown below.

Table (3): Correlation Table for Water Added for Bauxite Based Mixes

	% Cement	Particle Size	% Water
% Cement	1		
Particle Size	0	1	
% Water	0.642372	− 0.7489	1

Table (4): Correlation Table for Water Added for Grog Based Mixes

	% Cement	Particle Size	% Water
% Cement	1		
Particle Size	0	1	
% Water	0.696767	− 0.68928	1

The previous tables point out to the following:

First, the relation between the percent water added and percent cement used is an increasing relation. On the other hand the negative sign associated with the effect of particle size means an inverse relation between the percent water added and particle size.

Second, it appears that in case of using bauxite the effect varying particle size on the percent water added is higher than that of varying the cement ratio. On the other hand, on using grog, the two variables have comparable effects.

3.6 Effect of Mean Particle Size on Water Absorption:

Figures (6 and 7) show the relations between the percent water absorption of cast cubes and the mean particle size of either bauxite or grog.

From these two figures, it can be seen that the percent water absorption appreciably decreases with an increase in cement content. However, as the cement content exceeds 15% its effect on water absorption diminishes. This is expected since higher cement content will have for effect to enhance the closure of available pores.

Of interest is the difference between the water absorption values observed in either case. It is clear from Fig. (6) that the values of water absorption in case of bauxite based formulations range from about 4.8% to 9.5% depending on the cement level and particle size. In case of using grog the range of water absorption is 5.3 – 10.5% showing that the use of grog has lead to higher water absorption presumably due to increased open porosity of samples.

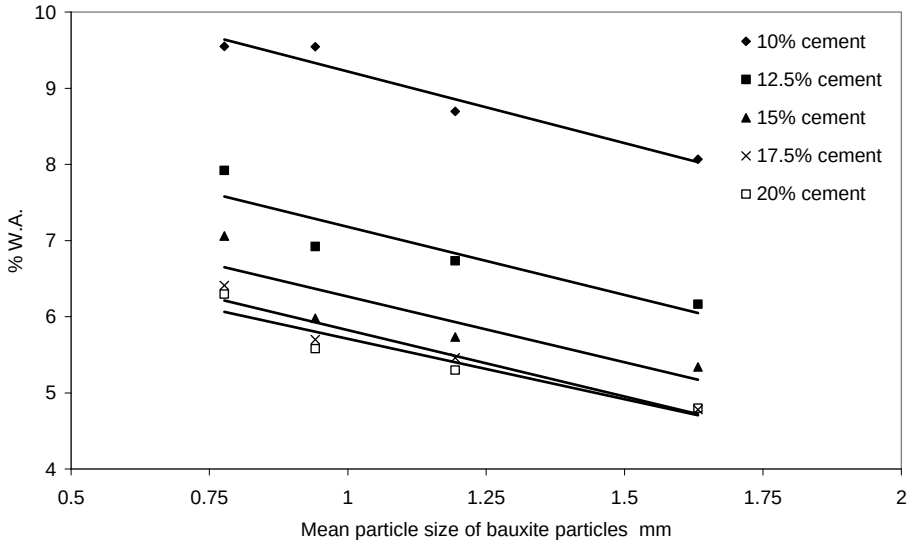


Fig. (6): Effect of Amount of Cement Used and Mean Particle Size of Aggregate on Percent Water Absorption for Bauxite Based Mixes

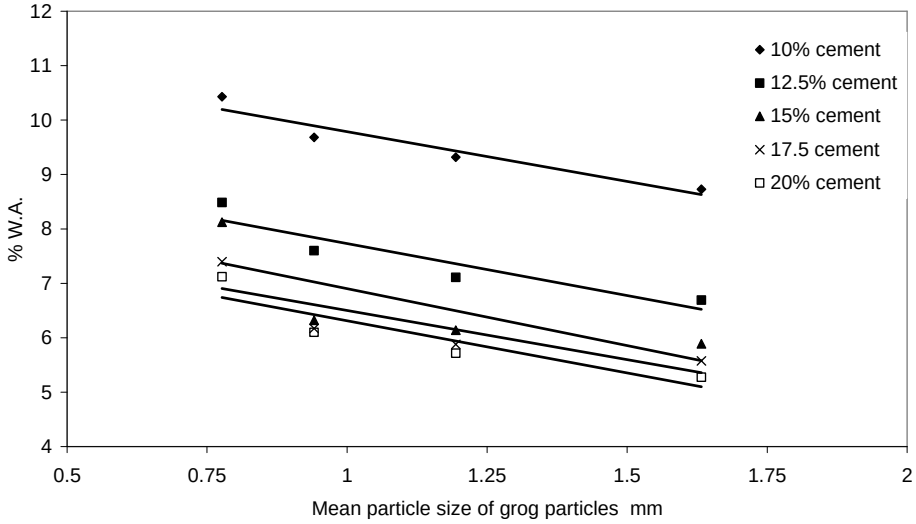


Fig. (7): Effect of Amount of Cement Used and Mean Particle Size of Aggregate on Percent Water Absorption for Grog Based Mixes

Also these figures show that the percent water absorption is only slightly affected by an increase in particle size. To assess this effect the excel DATA ANALYSIS module was used to establish correlation tables that show the relative influence of percent cement and particle size on the percent water absorption in both cases. Such tables are shown below.

Table (5): Correlation Table for Percent Water Absorption for Bauxite Based Mixes

	% Cement	Particle Size	% W.A.
% Cement	1		
Particle Size	0	1	
% W.A.	− 0.82455	− 0.39599	1

Table (6): Correlation Table for Percent Water Absorption for Grog Based Mixes

	% Cement	Particle Size	% W.A.
% Cement	1		
Particle Size	0	1	
% W.A.	− 0.79392	− 0.42302	1

These tables show that both variables affect negatively the percent water absorption where as the effect of the variation of percent cement is almost as twice as that of fineness.

3.7 Effect of mean particle Size on Apparent Porosity:

Fig. (8) and Fig. (9) show the relations between apparent porosity and the mean particle size for formulations containing bauxite and grog.

From these two figures, it can be seen that the apparent porosity decreases with an increase in cement content. This is expected since higher cement content will have for effect to enhance the closure of available pores. Also these figures show that the apparent porosity is negatively affected by an increase in particle size.

Using the excel DATA ANALYSIS module it was possible to establish correlation tables in both cases that show the relative influence of percent cement and particle size on the apparent porosity. Although such tables are not shown, their result indicates that is both variables affect negatively the apparent porosity where as the effect of the variation of percent cement on porosity is almost as twice as that of fineness.

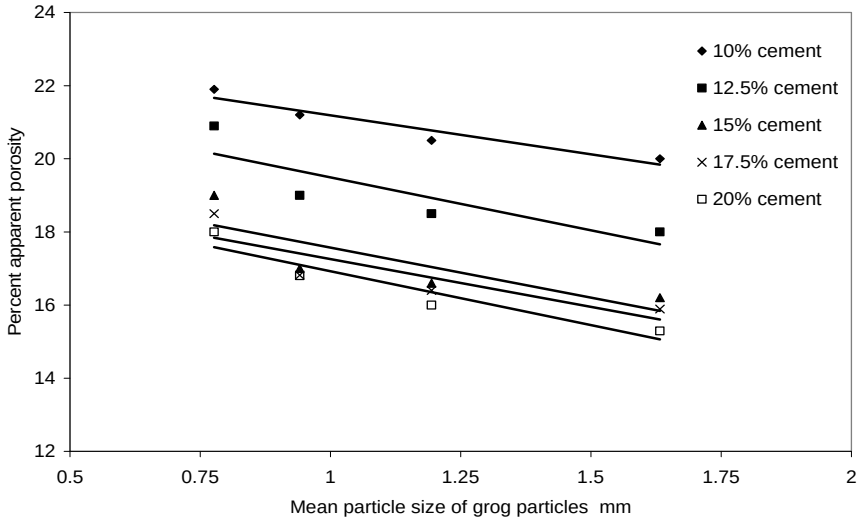


Fig. (8): Effect of Amount of Cement Used and Mean Particle Size of Aggregate on Percent Apparent Porosity for Bauxite Based Mixes

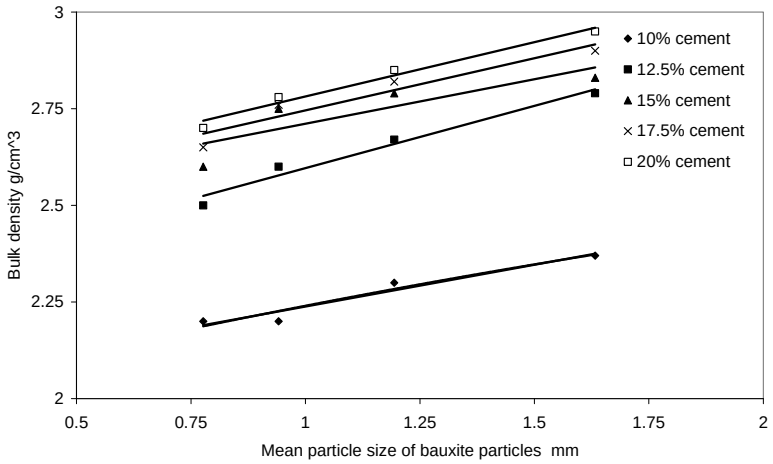


Fig. (9): Effect of Amount of Cement Used and Mean Particle Size of Aggregate on Percent Apparent Porosity for Grog Based Mixes

3.8 Effect of Particle Size Distribution on Bulk Density:

Fig. (10) and Fig. (11) show the relations between bulk density and the mean particle size for formulations containing bauxite and grog.

From these two figures, it can be seen that the bulk density increases with an increase in cement content. This is expected since higher cement content will have for effect to decrease porosity. Also these figures show that the bulk density slightly increases with an increase in particle size.

On the other hand, due to their lower porosity, the bulk density of samples containing bauxite is higher than that of samples containing grog, for the same particle size. For example, at mean particle size = 1.63 mm, the bulk density of samples containing bauxite ranged from 2.37 to 2.96 g/cm^3, depending on the amount of cement added while it ranged from 2.3 to 2.9 g/cm^3 for grog containing samples.

Also, due to the irregular shape of the grog particles with respect to bauxite, the packing efficiency of a body containing grog is less than that of bauxite. This assists the increased density in case of using bauxite.

Correlation tables were established to show the relative effect of variations in cement content and particle size on samples containing either bauxite or grog (Not shown). These tables show that that both cement content and higher particle size favor higher bulk density although the effect of cement variation on bulk density is more pronounced than that of particle size.

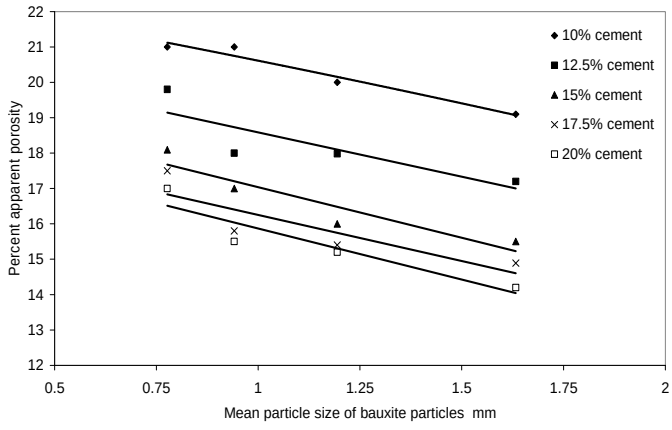


Fig. (10): Effect of Amount of Cement Used and Mean Particle Size of Aggregate on the Bulk Density of Bauxite Based Mixes

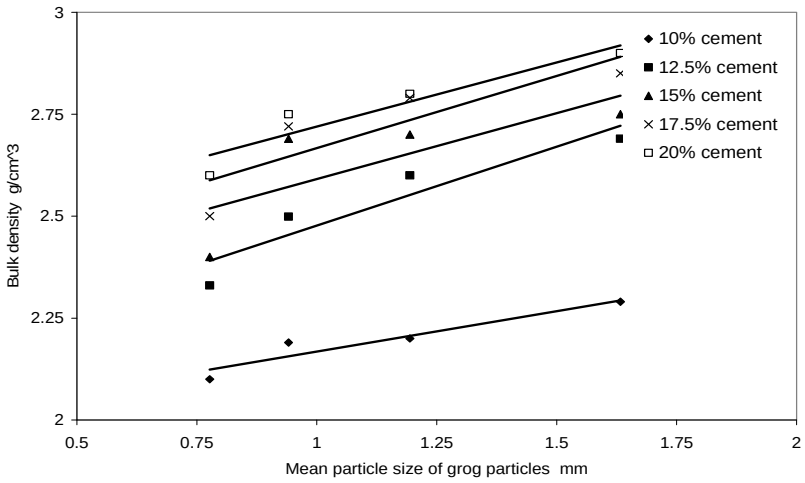


Fig. (11): Effect of Amount of Cement Used and Mean Particle Size of Aggregate on the Bulk Density of Grog Based Mixes

3.9 Effect of Particle Size Distribution on Cold Crushing Strength:

Figures (12 and 13) show the relations between cold crushing strength and the mean particle used for different cement contents (bauxite and grog). Three specimen samples were first prepared and air cured for 28 days before subjecting to the test.

From those figures, it could be seen that the compressive strength of specimens increases, as expected with an increase in cement content. The effect of mean particle size is however more complicated. All curves drawn seem to follow the same pattern: First, the cold crushing strength increases up to a mean particle size of about 0.95 mm then degreases with further increase in particle size. This mean particle size corresponds to formulation C in which the fine particles (D < 1 mm) constitute 40% of the mix where as the coarse portion represents the remaining 60% (Table 2). Such recipe approaches a state of minimum total porosity [11]. This had for effect to maximize the compactness of the mix leading to a maximum value in C.C.S.

Here also, the levels of crushing strength are higher in case of mixes containing bauxite than in those containing grog: In the former the C.C.S. ranges from 34 to 39 MPa, while ranging from 32 to 37 MPa in the latter case.

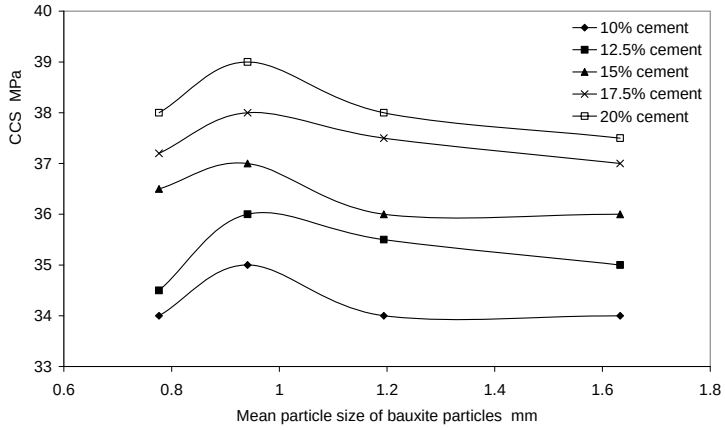


Fig. (12): Effect of Amount of Cement Used and Mean Particle Size of Aggregate on the Cold Crushing Strength of Bauxite Based Mixes

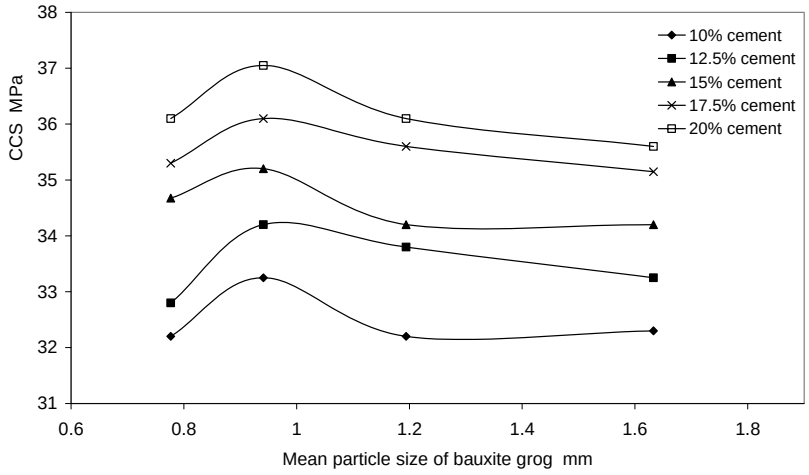


Fig. (13): Effect of Amount of Cement Used and Mean Particle Size of Aggregate on the Cold Crushing Strength of Grog Based Mixes

Tables (7) and (8) describe the relative effect of the variation in cement content and mean particle size on the variation in CCS for both types of samples. These tables show that in both cases, the variation of cement content plays a much higher role than that of particle size in assessing variations in CCS.

Table (7): Correlation Table for CCS for Bauxite Based Mixes

	% Cement	Particle Size	CCS
% Cement	1		
Particle Size	0	1	
CCS	0.945608	− 0.12905	1

Table (8): Correlation Table for CCS for Grog Based Mixes

	% Cement	Particle Size	CCS
% Cement	1		
Particle Size	0	1	
CCS	0.943331	− 0.12633	1

4. CONCLUSIONS

Samples of refractory concrete cubes were prepared using from 10 to 20% cement containing 50% alumina and two types of aggregate: calcined bauxite containing about 81% alumina and grog containing 52% alumina. These were graded to yield four portions of different mean particle size.

The following results could be deduced:

- Increasing the amount of cement added lead to higher water consumption, lower water absorption and porosity and higher bulk density.
- Using coarser aggregate resulted in a reduction in water used for mixing, lower water absorption and porosity and a higher bulk density.
- The effect of variation in cement content on the aforementioned properties is generally higher than that of variation of particle size.
- A higher cement content favored higher cold crushing strength but a maximum value was obtained at a mean aggregate particle size of about 0.95 mm

corresponding to a state of maximum compactness.

- Better results were generally obtained on using bauxite aggregate rather than grog presumably due to their lower intrinsic porosity.

REFERENCES

1. Hommer H., Wutz K., Von Seyerl J., "The Effect of Polycarboxylate Ethers as Deflocculants in Castables", 49th International Colloquium on Refractories, Aachen, Proceedings, 2006, p.p. 93−96.
2. Assis G., Parr C., Faryda and Michel Auvray J., "The Impact of Different Additive Systems Upon the Properties of a high Purity Low Cement Castable", Technical Paper, Published in Refractories World Forum, 1st of August, 2009, p.p. 58−62.
3. Sahu N.K., "Study Of Erosion Resistance Of Ulcc Based Precast With Indigenous High Alumina Cement", M.S. Thesis, Department of Ceramic Engineering National Institute of Technology Rourkela, 2009.
4. Angelescu N.G., Muthu K.U., Ionita G.G., Nicolae A.N.A, and Nicolae M.A., "Theoretical Fundaments and Applications about Special Additives", Valahia University Targoviste and Metallurgical Research Institute Bucharest, Romania, ICCBT 2008 - A - (14), 2008, p.p.165−176.
5. ASTM Standards D 422/2007, "Method for Particle–Size Analysis of Soils", Annual book of American Society for Testing of Material (ASTM), U.S.A., 2008, Vol. 4.08.
6. ASTM Standards C 1446/2007, "Standard Test Method for Measuring Consistency of Self−Flowing Castable Refractories", Annual book of American Society for Testing of Material (ASTM), U.S.A., 2008, Vol. 15.01.
7. ASTM Standards C 20/2007, "Standard Test Methods for Apparent Porosity, Water Absorption, Apparent Specific Gravity, and Bulk Density of Burned Refractory Brick and Shapes by Boiling Water", Annual book of American Society for Testing of Material (ASTM), U.S.A., 2008, Vol. 15.4.
8. BS EN Standards 993−5/2000, "Methods of test for Dense Shaped Refractory Products – Part (5): Determination of Cold Crushing Strength", British Standard, 2000.
9. Aramaki S., and Roy R., "Mullite–Corundum Boundary in the Systems $MgO–Al_2O_3–SiO_2$ and $CaO–Al_2O_3–SiO_2$", J. Am. Ceram. Soc., 42, 1959, pp. 644–645. McCabe W., Smith J.C., and Hariot P., "Unit Operations of Chemical Engineering", McGraw Hill, 7th Edition, 2004.
10. Richerson D., "Modern Ceramic Engineering", Taylor and Francis, 2nd Edition, 2006.

10/202010

Effect of Beta Radiation on Extraocular Muscles

Mohamed A. Marzouk ,MD*, Hossam E. Sayed*, Ayman A . Shoman , MD* , Hisham A .Hashim ,MD*.
* Ass. Professor – Research Institute of Ophthalmology – Giza – Egypt.

Abstract: Purpose: To evaluate the effect of different Beta radiation doses on frogs extraocular muscles. **Methods:** 50 frogs of species Rana Ridibunda were used in this study, they were divided into 5 groups, every group was treated with a different dose of radiation, and the first group was taken as control. **Results:** The estimation of soluble protein content in extraocular muscles of Beta radiated eyes showed a gradual decrease with the increase of dose. **Conclusion:** Significant changes in extraocular muscles were detected with the increase of Beta radiation dose.
[Mohamed A. Marzouk, Hossam E. Sayed, Ayman A. Shoman, Hisham A. Hashim. **Effect of Beta Radiation on Extraocular Muscles.** Journal of American Science 2010;6(12):1028-1033]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Effect; Beta Radiation; Extraocular; Muscle

Introduction:

Almost infinite variety of atomic, intra, and intermolecular changes will occur when living tissue is subjected to ionizing radiation. Some of these changes may be the goal of radiotherapy, while, of course, the others are considered to be harmful or even lethal changes [(1)].

Living tissue absorb the ionizing radiation through two mechanisms: ionization, and excitation depending on the energy of that radiation; if its energy is high enough, some molecules may be ionized causing direct damage to the other neighboring molecules, or even the far molecules through the formation of free radicals (indirect effect). If the energy of the radiation is not enough to ionize the molecules, it may cause its excitation to higher energy levels, therefore disturbing the normal balance of the organism [(2)].

Beta radiation is one of the most abundant radiation types being used in ophthalmology, it has a small penetrating power and that is to be highly considered while dealing with the eye which is one of the most radiosensitive organs of the body [(2)].

Beta radiation has been used for ocular radiotherapy since the forties of this century. Postoperative treatment of pterygium[(3)] ,and more recently primary pterygium with no prior surgical excision[(4)], may be the most pronounced application of Beta radiation in ophthalmic therapy, however, there are some other situations at which Beta rays are used successfully, like non malignant lesions of the eye lids, corneal scars, corneal vascularization, vernal conjunctivitis, superficial or deep keratitis[(5)], and in postoperative treatment of total and subtotal symblepharons[(6)] .It's also being applied in some centers now, with more advanced techniques, for the treatment of conjunctival squamous cell carcinoma , choroidal and ciliary body melanomas[(7)], also in controlling the elevated IOP by its effect on the ciliary body and wound healing alteration [(8)].

Many workers have early noticed complications due to Beta radiation such as telangiectasis of the conjunctiva, keratinization of the conjunctival epithelium that led to punctate keratitis resulted in severe lacrimation. Other late effects of beta radiation were also reported such as scleral thinning, scleral ulcers, atrophy of the sclera, superficial punctate keratitis, corneal vascularization, corneal scarring, perforation, iritis, iris atrophy, iridocyclytis, cataract, ptosis, glaucoma, and endophthalmitis[(5,9,10.11)].

These side effects became will known later and the strategies of Beta radiotherapists had to consider the dose-effect on such symptoms, so, many workers began to use smaller doses to overcome such complications, and also set dose-fraction systems to optimize therapy and minimize the side effects [(12)].

Such dose-fraction systems are being established and used in the Beta radiotherapy centers; and for the current study, the applied dose fractionation scheme for the experimental animals is that used for human application in the postoperative treatment of pterygium in the dept. of radiotherapy at the Research Inst. Of Ophthalmology, Cairo-Egypt. According to this scheme (which complies with the findings of Bahrassa et al, 1983; and El Dessoki, 1990), patients receive a total accumulated dose of 24 Gy (32 Gy in some cases) of beta radiation in three (four) divided sessions in a week[(3,13)] .

Materials And Methods:

Fifty frogs of species Rana Ridibunda, of average weight 74 gm., were used in the present study. Frogs were divided into 5 groups, each of which consist of 10 animals and labeled A, B, C, D,

and E. Group A was taken as the control (untreated) group. The other 4 groups were treated with different doses of radiation as follows: Group B received 8 Gy, group C received 16 Gy, group D received 24 Gy, and group E received 32 Gy.

The species Rana Ridibunda was chosen on the basis of its larger size and stronger musculature compared to the other available species. Eyes of the frog Rana Radibunda have close structure to human eyes and similar extraocular muscles[14].

The radiation source used in the study was a Strontium-90/Yttrium-90 source, which is contained in the eye applicator (SIA.20- Amersham, UK) found at the Research Institute of Ophthalmology, Giza, Egypt .

For all animals, the right eye was the treated eye. After irradiation, animals were left alive for up to 30 days from the last session.

For the extrusion of the extraocular muscles, the animal was slaughtered and the head was separated, the buccal cavity was opened and the lower jaw & tongue were removed and the upper membrane was peeled to uncover the eye globe and extraocular muscles. The extraocular muscles were excised from the globe and then weighted to determine the wet weight.

Due to the small size of the muscles from a single animal, a pooled sample of muscles from the individuals of each group was considered to increase the sample size. Enough impact sample of each group was preserved in formalin solution for the histological examination, and the rest of the pooled sample was then treated as described by Baldwin et al., (1955) [15].

We did use the following methods in detecting changes in extraocular Muscles:

1-Quantification of soluble protein content by Lowry method .

2-Determination of protein pattern by Gel Electrophoresis .

3-Spectrophotometric measurements.

4-Histopathological examination .

Results:

During the radiation process, the Beta applicator (type SIA 20- Amersham) was placed in contact with the cornea of the frog eye simulating the therapeutic technique used for human treatment.

This means that the cornea dose can be considered to be the same as the surface dose of the applicator[16]. Accordingly all Beta radiation doses mentioned in this study refer to the cornea dose, while that of the extraocular muscles is only 2-4% of the cornea dose which can be estimated from the dose curves of the strontium 90 Beta applicator, and this applies to all results[16].

Quantification of soluble protein content by Lowry method:

The soluble protein content of the treated extraocular muscles decreased gradually from 90% of the control and group B, (8Gy cornea dose) to 55% in group E, (32Gy cornea dose).

The decrease of soluble protein content of extraocular muscles as a result of Beta radiation indicates that some type of destructive action has occurred. Study of the protein content only is not enough to explain the possible changes. More explanations can be obtained if the different samples were subjected to electrophoresis. (Fig. 1 A).

Determination of muscle protein patterns by Gel Electrophoresis:

It can be shown from the separation pattern that the bands showed an apparent difference in the separation bands of Actin, Beta-actinin, and Tropomyosin of the last two treated groups (that received 24, and 32Gy Beta radiation corneal dose), the bands of Beta-actinin and Tropomyosin (bands 1 and 2) broadened and diffused together, while the other two bands of Actin and Myosin light chain (bands 3 and 4) became much more broadened which implies an alteration of the protein structure that may be due to the aggregation of some protein fragments in the samples. A structural deformation means malfunctioning of the affected muscle which needs further investigation in a future study. (Fig. 1 B).

Spectrophotometric measurements :

A general trend of higher absorbance values for the first two groups B&C (8 & 16 Gy corneal dose) compared to control, while the last two groups D&E (24 & 32 Gy corneal dose) which received higher radiation dosed showed lower absorbance values compared to control indicating some form of protein damage. (Fig. 1 C).

Histopathological examination:

-In group B (8 Gy):

The normal striation of muscles is unaffected, with some fatty infiltrations.

-In group C (16 Gy):

The presence of fatty infiltrations became more apparent, and the affinity to the haematotoxylin stain seems to be decreased.

-In group D (24 Gy):

The affinity to the haematotoxylin stain showed more decrease, and the muscle striation is disrupted compared to normal muscles and the myofibers showed some splitting.

-In group E (32 Gy):

The affinity to the haematotoxylin stain is much more decreased and the normal striation of myofibers is extensively disrupted. The myofibers showed more splitting and many myofibers showed the absence of nuclei (Fig. 2).

Discussion:

To our humbled knowledge this is the first study on the interaction of Beta radiation with the extraocular muscles.

Although the amount of Beta dose of the extraocular muscles of the frog was only 2-4% of the cornea dose, many changes could be found due to such doses.

The estimation of soluble protein content of extraocular muscles in Beta radiated eyes showed a gradual decrease with the increase of dose. This implies change in the nature of proteins of treated samples which increases with increase of applied dose.

Data from electrophoretic measurements also confirmed the same conclusion, where other molecular weights appeared in the separation pattern indicating the appearance of new protein fragments.

Spectrophotometric measurements focused on the effect of different Beta radiation doses on peptide bonds and nucleic acids which showed that there are different degrees of damage with different doses, which means that proteins were exposed to some type of damage which produced some new protein fragments which agrees with the previous conclusions.

The direct effect of Beta radiation can't be the only reason for such changes. The effect of Bremsstrahlung radiation can be considered to be an important factor in the appearance of many side effects of Beta radiation due to its higher penetration in tissue so that it can cause such distant changes.

Bremsstrahlung are X-rays that are emitted when high-speed charged particles suffer rapid acceleration. When a β particle passes close to a nucleus, the strong attractive Coulomb force causes the β particle to deviate sharply far from its original path. The change in direction is due to radial acceleration, and the β particle, in accordance with classical theory, loses energy by electromagnetic radiation at a rate proportional to the square of the acceleration. This means that the Bremsstrahlung photons have a continuous energy disruption that ranges downward from a theoretical maximum equal to the kinetic energy of the β particle. The likelihood of Bremsstrahlung production increases with the atomic number of the absorber [(17)].

Another factor to be considered, that is the free radicals which are formed in the aqueous medium within the tissue (which is abundant in the structure of the eye). Radiation-produced free radicals affect other molecules at a distance as they can transfer their "extra" electron to a nearby molecule, which in turn, may pass it on. The electron can be passed through a succession of molecules forming many free radicals. This chain reaction may occur in a large number of molecules and results in critical changes in organic molecules. Similarly, a free radical may "snatch" an electron from a neighbor, which then becomes a free radical. A chain of electron "snatchings" may ensue an organic molecule to be critically changed in the process. Therefore, the radiation damage that occurred in the β irradiated samples can be thought to be a result of the free-radical interactions [(17)].

Conclusion:

Therapeutic doses of Beta radiation on the eye can harmfully affect the structure and function of extraocular muscles despite its short penetrating range in tissues, and the risk to benefit ratio should be considered during its use.

We are in need for a future human studies on the extraocular muscles regarding it's balance and function after therapeutic Beta radiation.

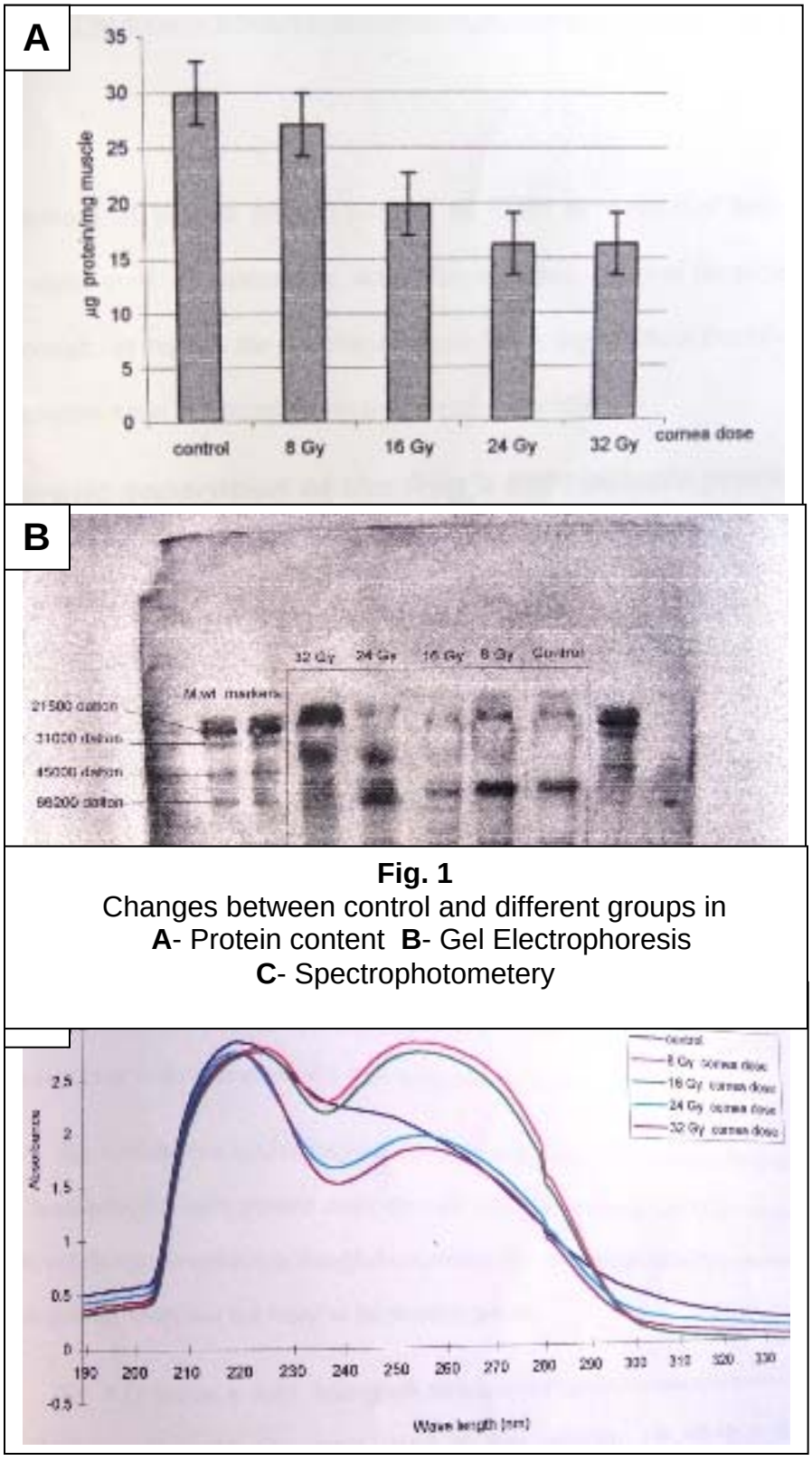


Fig. 1
Changes between control and different groups in
A- Protein content **B**- Gel Electrophoresis
C- Spectrophotometery

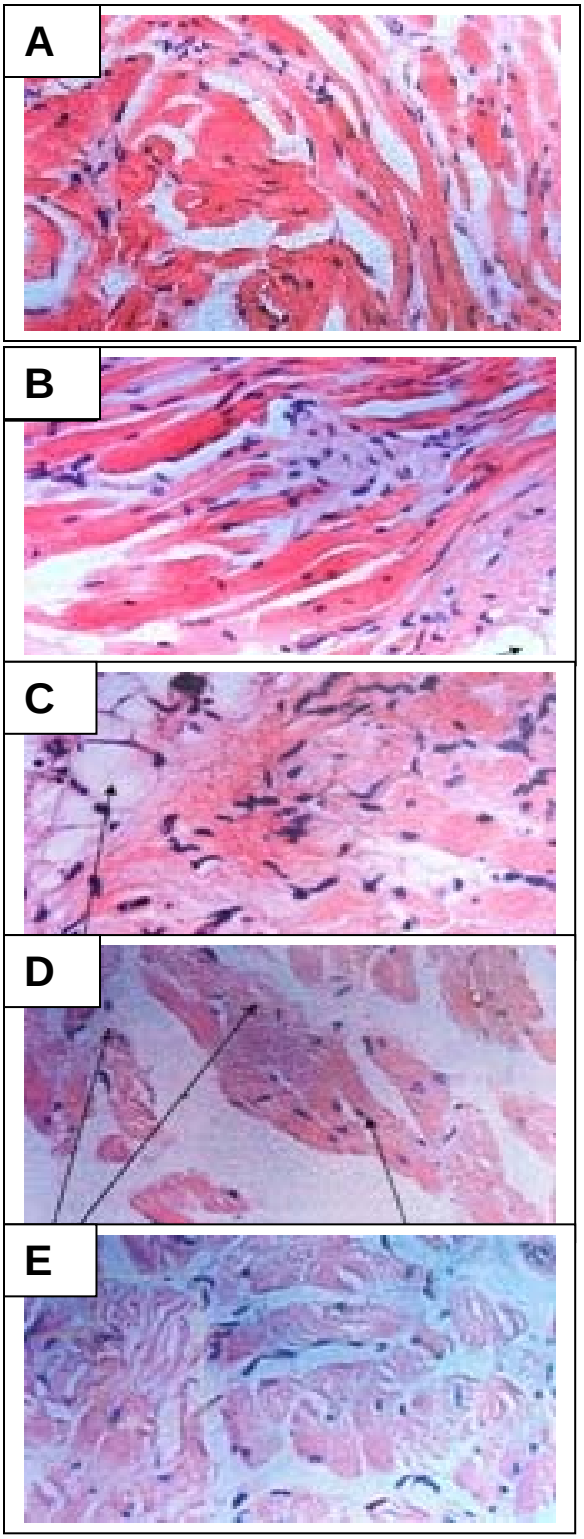


Fig. 2. Histopathological changes in different groups
A,B,C,D&E

References:

1- **Chan RC, Shukovsky LJ:** Effect of irradiation on the eye. *Radiology* 1976;120(3):673-5.

2- **Rudneva E:** The effect of ionizing radiation on the organ of vision(a review of the litreture). *Lik Sprava* 1997;(6): 34-7.

3- **Bahrassa F, Datta R:** Postoperative beta radiation treatment of pterygium. *Int J Radiant Oncol Biol Phys* 1983; 95: 679-84.

4- **Vastardis I, Pajic B, Greiner RH, Pajic-Eggspuehler B,**

Aebersold DM: Prospective study of exclusive strontium-yttrium-90 beta-irradiation of primary and recurrent pterygia with no prior surgical excision. Clinical outcome of long-term follow-up. *Strahlenther Onkol*. 2009 Dec;185(12):808-14.

5- **Iliff CE:** Injures induced to the lenses of the eyes during treatment with Sr^{90} radiations. *Arj. Ophthalmol* 1947; 38: 415-441.

6- **Fein W:** Repair of total and subtotal symblepharons *Ophthalmic Surg* 1979; 10(6): 44-47.

7- **Foerster MH, Wessing A, Meyer G, Fujishima h, Shimazaki J :** The treatment of ciliary body melanoma by beta radiation. Trans Ophthalmol Soc U K 1983; 103(1): 64-67.

8- **Kirwan JF, Rennie C, Evans JR:** Beta radiation for glaucoma surgery.*Cochrane Database Syst Rev*. 2009 Apr 15;(2):CD003433.

9- **Ruedmann AD:** Beta radiation therapy. *Br J Ophthalmol* 1949; 26: 791-794.

10- **Labib MA:** Beta rays in ophthalmic practice. *Bul Ophthalmol Soc Egypt* 1963; (56): 525.

11- **Talbot AN:** Complications of beta rays treatment of pterygia. *Trans Ophthalmol Soc N Z* 1979; 31: 62-3.

12- **Tarr KH , Constable IJ:** Late complications of pterygium treatment. *Br J Ophthalmol* 1980; 64 (7): 496-504.

13- **El Dessouky MD :** Argon laser versus beta radiation as a prophylactic treatment against the recurrence of pterygium after surgery. *Ph.D Res Inst Ophthalmol Egypt* 1990.

14- **Asmussen G :** The properties of the extraocular muscles of the frog. Acta Biol Med Ger 1978; 37(2): 313-321.

15- **Baldwin, Ernest JB, David JB:** Cole's Pract. *Physiological Chem Heffer&Sons LTD* 1955.

16- **Gleckler M, Valentine JD, Silberstein EB:** Calculating lens dose and surface dose rates from Sr^{90} ophthalmic applicators using Monte Carlo modeling. *Med Phys* 1998; 25(1): 29-36.

17- **Rubin R, Casarett GW**: Clinical radiation pathology. *Philadelphia WB Saunders Co* 1968;vol. 1.

10/24/2010

Chronic Asthmatic Chest Troubles And Their Effects On Cognitive Functions, Psychosocial Behaviour And Academic Achievment Among Children In Egypt

Samiha Samuel*, Mai Safwat*, William Morkos, Samar Salem**, Tarek El-Adly*and Abeer Mohammed.**

*Department of Paediatrics, Faculty of Medicine, Cairo University
**Department of Childhealth
National Research Center

ABSTRACT: Chronic illness is clearly an important factor affecting psychosocial state of children and adolescents. This case-control study is an effort to clarify the effect of chronic asthmatic chest troubles, as chronic illnesses, on the cognition and psychological aspects of such chronically ill children. This study was executed in the Chest Clinic of the Abou El-Reesh Children's Hospital, Cairo University. The Study was carried out on 23 children suffering from chronic asthmatic chest troubles (13 boys and 10 girls) with an age range of 6-15 years (mean age ± SD = 9.6±2.67). Twenty three age and sex matched children not suffering from any disease and living under the same socioeconomic conditions were taken as controls. WISC-R and PSCL were used to assess the cognitive and psychosocial adjustment among children while the mid-year scores for Mathematics and Arabic language were used to evaluate the academic performance. Our results indicated that chronic asthmatic disease has a negative effect on cognitive abilities, psychosocial behavior and academic achievement of such children.
[Samiha Samuel, Mai Safwat, William Morkos, Samar Salem, Tarek El-Adly and Abeer Mohammed. Chronic Asthmatic Chest Troubles And Their Effects On Cognitive Functions, Psychosocial Behaviour And Academic Achievment Among Children In Egypt. Journal of American Science 2010;6(12):1034-1043]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Chronic; Asthmatic; Chest; Cognitive Functions; Psychosocial Behaviour

INTRODUCTION

Health, happiness, independence, and productivity are basic human desires. For children, this means achieving normal growth and development, acquiring a sense of accomplishment, developing an identity, and initiating independence. Although, over time, all children face the same developmental tasks, achieving these developmental milestones depends on many conditioning factors ***(*Charron-Prochownik, 2002*)***. One conditioning factor that greatly influences developmental outcomes and quality of life is chronic illness ***(*Jackson and Vessey, 2000*)***.

Chronic diseases affect an estimated 10-20% of all children during childhood and adolescence ***(*Geist et al, 2003*)***. Chronic chest troubles are the most common cause of chronic illness in children and can affect cognition, psychosocial behavior, and school performance of children. Children with chronic illness are at higher than average risk for behavioral disorders **(Tavormina et al, 1996).** The general consensus of the literature is that chronically ill children are at risk for psychological problems. In chronic childhood conditions, as a whole, the risk of psychopathology is about 2.5 times higher than in the general population **(Noeker et al, 2005)**. One epidemiological study showed that among 4-16 years old, those with chronic health problems were 2-4 times more likely to have a diagnosable behavioral disorder than their healthy peers. **(Goldberg et al, 1997)**

Psychosocial factors in chronic illness in the pediatric population may impede optimal outcome. Overt and covert adjustment problems and psychiatric illness may present as unexplained medical symptoms, non-compliance with medical treatment, school refusal and high-risk behaviors. These signs may alert the physician to the presence of underlying issues in the child and/or the family. Before referral to a mental health professional, the doctor should try to identify the presence of underlying issues, focus on family-centered care and schedule well visits to monitor compliance and other issues.

Early detection and treatment of psychosocial problems may lead to considerable health benefits. Psychosocial problems have a high prevalence rate and lead to high costs of disease. They also cause substantial restrictions in daily functioning in later life and are the major cause of long-term work disability in young adults **(Murthy et al, 2001)** Only a minority of children with psychological or psychosocial problems are under treatment **(Reijneveld et al, 2004).** If untreated, problems are likely to persist in later life and can lead to serious limitations in daily functioning **(Verhulst and vander Ende, 1996).** Research has shown that early detection and treatment improves these

children's prognosis substantially, but a complete analysis of its cost effectiveness has yet to be carried out (**Nelson, Westhues and MacLeod, 2003)**.

The aim of this study is to assess the cognitive functions, psychosocial behavior, and school achievement in asthmatic children and compare them with healthy children in the same age.

PATIENTS & METHODS

This case-control study had been carried out on 46 Egyptian children (23 patients and 23 controls). The two groups were examined for medical and psychological evaluation, to find whether ill children have psychological problems than healthy controls. Their age ranged from 6-15 years.

The study included 23 children previously diagnosed to have chronic asthmatic chest disease and randomly selected. They regularly attended the chest clinic at Abu El- Reesh Children's Hospital, Cairo University. Inclusion criteria included children previously diagnosed to have bronchial asthma, age range between 6-15 years and both sexes. Exclusion Criteria included children less than 6 years old and those more than 15 years old, neurological diseases e.g. cerebral, mentally retarded children and asthmatic chest diseases less than 6 months duration. A control group of 23 healthy children matched for age, sex, educational level and socio-economic state as the patients group. They were selected from the brothers and sisters of the patients group. The controls were free from any chronic illness especially chronic asthmatic chest diseases.

All studied cases were subjected to the following:

1) History taking: including: age, sex, onset of disease and its duration.

2) Clinical examination: full clinical examination was done including general examination and local chest examination in order to diagnose chronic chest disease and exclude any other diseases. Diagnosis of chronic chest disease was confirmed by reviewing the laboratory and radiological findings of the patients.

3) Assessment of anthropometric measures (weight and height).

4) Assessment of cognitive abilities: They were assessed by a battery of psychological tests that covered verbal and non-verbal intelligence, memory, learning, problem solving, and attention. The children were individually assessed. All psychological evaluations were administered in one session. The tests used were:

A-The Arabic Version of the Revised Wechsler Intelligence Scale for Children (WISC-R) (Wechsler, 1977, Kamel and Ismail, 1993). This is the most widely used test for intellectual assessment and covers an age range of 6-16years. The test is scored according to a manual from which verbal and performance score and intelligent quotient are obtained from.

B-The Auditory Vigilance test: It measures the attention ability of the child. It is a measure of the efficiency of identifying signal stimuli in the context from the non-signal ones. **(Pollite, 1984).**

C-The Figural Memory Test: This is a measure of the free recall of visual objects. **(Pollite, 1984).** The free recall score is the number of items recalled correctly. The classification score is obtained by counting the number of the shifts from one category to the other, which is made by the subject during his recall. This was considered as an indicator of how he can organize aspects in his memory.

5) Assessment of psychosocial behavior: Children's behavior was evaluated by a brief version of parent-completed Pediatric Symptom Checklist (PSC-17). Although certain responses may suggest a diagnosis, the PSC is a screening tool and not a diagnostic one. If positive, the clinician should pursue a brief interview, reviewing the child's major areas of functioning (school, family, activities, friends and mood). If this brief interview supports the PSC findings, the clinician then decides whether a follow-up appointment, further evaluation or referral is indicated **(Jellinek et al, 1995).**

6) Assessment of academic Achievement: Was assessed using the mid-year test scores of Arabic language and arithmetic subjects for each child. It is considered as a good indicator of academic and learning performance **(Silver, 1989).** Each group is classified according to the mid-year scores into good achiever (the mid-year score is $\geq$ 70%) and poor achiever (the mid-year score is < 70%).

Statistical Methods: Data were statistically described in terms of range, mean ± standard deviation (± SD), frequencies (number of cases) and percentages when appropriate. Comparison of quantitative variables between the study groups was done using Student *t* test for independent samples. For comparing categorical data, Chi square (χ^2) test was performed. Exact test was used instead when the expected frequency is less than 5. A probability value (*p* value) less than 0.05 was considered statistically significant. All statistical calculations were done using the computer programs Microsoft Excel 2003 (Microsoft Corporation, NY, USA) and SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 15 for Microsoft Windows.

RESULTS

The study was conducted on 23 patients and 23 age and sex matched controls (table 1, 2).

Table (1) Age of children in the study sample

	Asthmatics	Controls
Range	6-15 y	6-15y
Mean	9.6	10.5
±SD	2.67	2.75

P value > 0.05(non significant)

Table (2) Sex distribution of children.

		Asthmatic group	Control group
F	Count	10	10
	%	43.5%	43.5%
M	Count	13	13
	%	56.5%	56.5%

P value > 0.05 (non-significant)

Anthropometric measures between the two groups were highly significant as shown in table 3.

Table (3) Results of anthropometric measures in asthmatics and controls

	Asthmatics		Controls		P value
	Mean	±SD	Mean	±SD	
Weight	25.57	10.65	35.26	7.98	.008*
Height	125.32	13.38	135.44	9.03	.010*

*P value < 0.05 (significant)

Cognitive abilities:

A- Full Scale IQ: Table (4) shows analysis of the full scale IQ for the two groups. The mean full scale IQ for asthmatics was 73.61% compared to 93.54% for the control group. It shows significant diminution in asthmatics. (P value < 0.05)

Table (4) Full scale IQ and results of Figural Memory test

	Asthmatics		Controls		P value
	Mean	±SD	Mean	±SD	
Full scale IQ	73.61%	12.79	93.54%	12.72	.000*
Free recall	8.13	3.49	11.22	2.67	.001*
Classification	2.83	2.19	4.54	1.59	.034*

B-Figural Memory test:

- **Free recall:**

The mean free recall scores for asthmatics was 8.13±3.49, while the mean free recall scores for the controls was 11.22± 2.67 which is highly significant in asthmatics (P= .001) (Fig. 1).

- **Classification :**

The mean of classification scores for asthmatics was 3.83±2.19 while the mean of classification scores for controls was 4.54±1.59. It shows significant diminution in asthmatics (P value =.034) (Fig. 1).

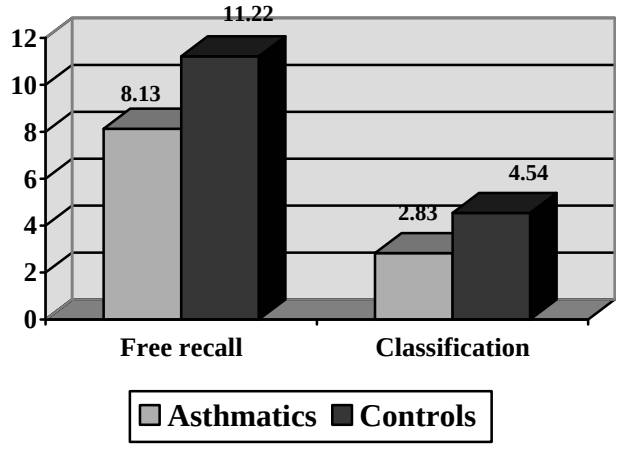


Figure (1) results of Figural Memory test in asthmatics and controls.

C-Auditory Vigilance test: Table (5) shows the results of auditory vigilance among study groups.

Table (5) Results of Auditory Vigilance test in asthmatics and controls.

		Asthmatics		Controls		P value
		Mean	±SD	Mean	±SD	
Test A	Right answers	9.83	2.25	14.00	1.28	.000*
	Wrong answers	3.17	2.25	1.02	1.27	.000*
Test B	Right answers	10.65	2.12	12.16	1.24	.001*
	Wrong answers	4.52	2.37	0.88	1.26	.000*

* P value < 0.05 (significant)

- **Test A**

The mean for right answers in asthmatics was 9.83±2.25 while the mean for right answers in controls was 14.00± 1.28 with highly significant diminution in asthmatics (P= .000) (Fig. 2). The mean for wrong answers in asthmatics was 3.17± 2.25 while the mean for wrong answers in controls was 1.02 ±.1.27 with highly significant diminution in asthmatics (P= .000) (Fig. 2).

- **Test B**

The mean for right answers in asthmatics was 10.65 ±2.12 while the mean for right answers in controls was 12.16 ±1.24 with highly significant diminution in asthmatics (P= .001) (Fig. 2). The mean for wrong answers in asthmatics was 4.52 ±2.37 while the mean for wrong answers in controls was 0.88 ±1.26 with highly significant diminution in asthmatics (P= .000) (Fig. 2).

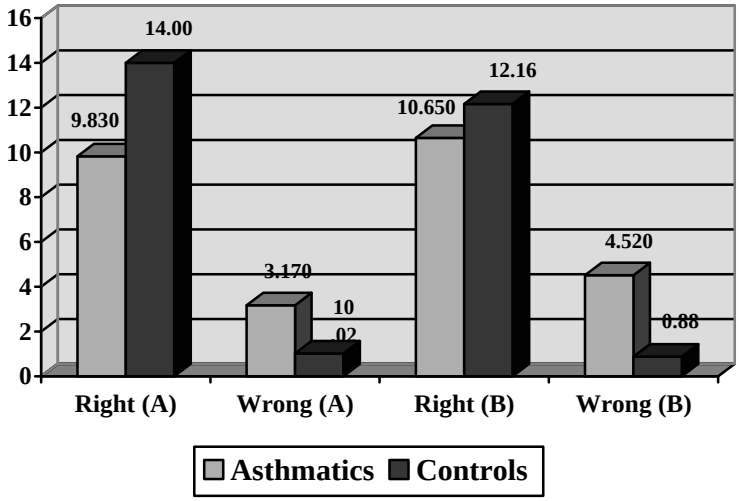


Figure (2) Results of Auditory Vigilance test in asthmatics and controls

iii.Psychosocial behavior for asthmatics and controls is shown in Table 6.
iv.

Table (6) Results of psychosocial behavior in asthmatics and controls

		Asthmatics		Controls		P value
		N	%	N	%	
Externalizing behavior	**Positive**	3	13%	0	0%	0.007*
	Negative	20	87.0%	50	100%	
Internalizing behavior	**Positive**	9	39.1%	3	6.0%	0.001*
	Negative	12	60.9%	47	94.0%	
Attention problems	**Positive**	3	13.0%	0	0%	.007*
	Negative	20	87.0%	50	100%	
Normal children		9	39.1%	47	94.0%	.000*

*P value < 0.05 (significant)

1- Externalizing abnormalities:

The behavioral questionnaire (PSCL) showed that: 3 asthmatics (13.0%) had externalizing behavior and no one had the same behavior in controls. 20 asthmatics (87.0 %) were normal compared to 50 controls (100%) with highly significant diminution in asthmatics (P= .007) (Fig. 3,4).

2- Internalizing disorders:

The internalizing behavior was found in 9 asthmatics (39.1%) as compared to 3 controls (6.0%). 12 asthmatics (60.9 %) had no internalizing behavior compared to 47 (94.0%) in controls with highly significant diminution in asthmatics (P=.001) (Fig. 3,4).

3-attention disorders:

As regard to the attention problems, there were 3 asthmatics (13.0%) compared to 0 controls (0%) had attention problems with highly significant diminution in asthmatics (P= .007) (Fig. 3,4).

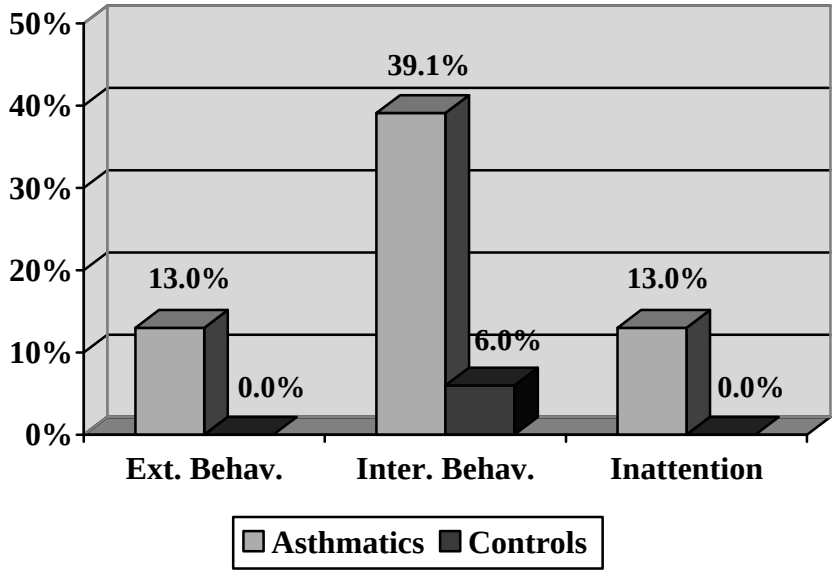


Figure (3) positive behavioral problems in asthmatics and controls.

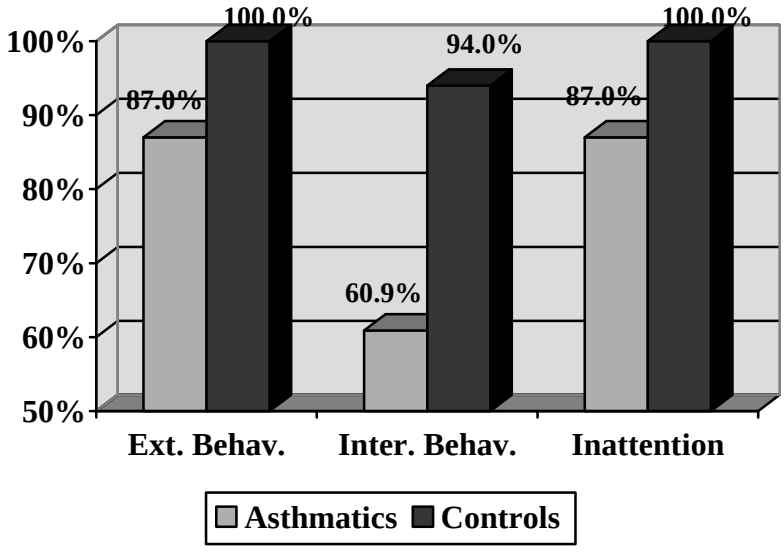


Figure (4) negative behavioral problems in asthmatics and controls.

v. **Academic Achievement:** Table (7) shows results of academic achievement among study groups.

Table (7) Results of academic achievement in asthmatics and controls

		Asthmatics		Controls		P value
		N	%	N	%	
Midyear Mathematics scores	**Good achiever**	13	56.5%	50	100%	.000*
	Poor achiever	10	43.5%	0	0%	.000*
Midyear Arabic scores	**Good achiever**	13	56.5%	50	100%	.000*
	Poor achiever	10	43.5%	0	0%	.000*

*P value < 0.05 (significant)

A- Midyear Mathematics scores:

10 (43.5 %) asthmatics compared to 0 (0%) controls were poor achievers with a highly statistically significant difference between the two groups (P= .000) (Fig. 5).

B- Midyear Arabic scores:

10 (43.5 %) asthmatics compared to 0 (0%) controls were poor achievers with highly statistically significant difference between the two groups (P= .000) (Fig. 5).

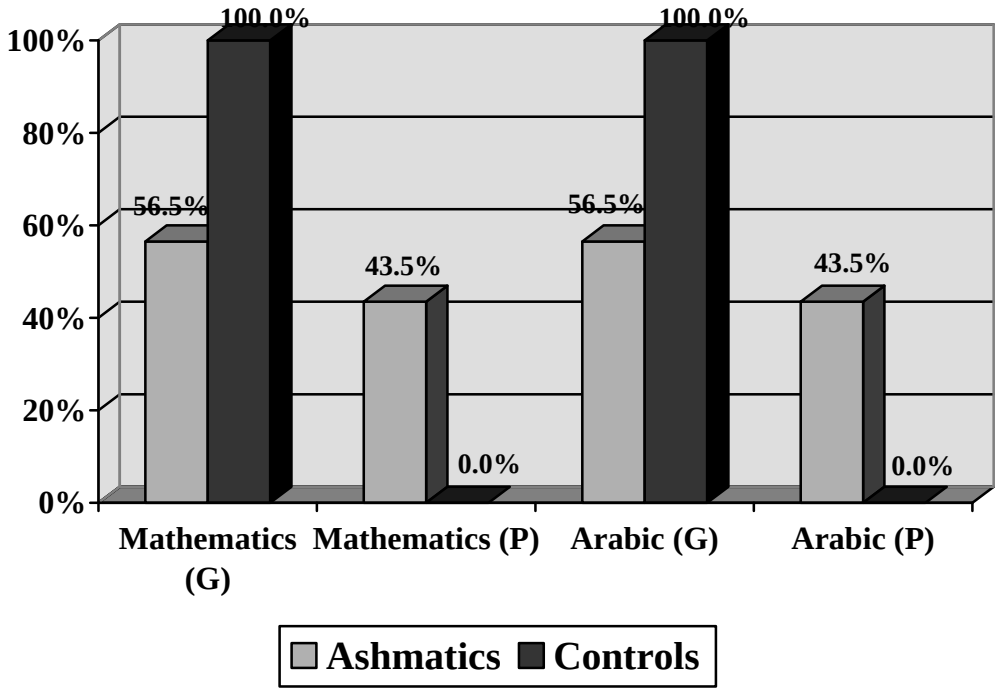


Figure (5) Results of academic achievement in asthmatics and controls.
G: good achievers P: poor achievers

iv. Nocturnal Enuresis (NE): Table 8 shows that nocturnal enuresis was found in 6 asthmatics (26.1%) compared to 0 (0%) controls with highly statistically significant difference between the two groups (P= .000).

Table (8) Results of nocturnal enuresis in asthmatics and controls

	Asthmatics		Controls	
	N	%	N	%
Positive cases	6	26.1%	0	0%
Negative cases	17	73.9%	50	100%

P= 0.000 (highly significant**)**

DISCUSSION

Epidemiologic studies indicate that up to half of pediatric visits reflect behavioral, psychosocial, and educational concerns **(Starfield et al, 1980).** Most of these problems are psychosocial problems that are not severe enough to be classified as psychiatric disorders but interfere with children's social and academic development **(Sharp et al., 1992).** Identification of parental concerns about children's behavior and evidence of problematic behavior are increasingly accepted as part of the basic responsibilities of primary care providers **(Perrin and Stancin, 2002).**

Children who have symptoms of illness for more than 3 months, or who require hospitalization or extensive home based services for more than one month in 12 months period are said to have chronic disease. **(El-Baz et al, 1995)** Contrary to El-Baz definition, **Leblan et al (2003)** suggested that the term chronic illness refers to illnesses that require at least 6 months of continuous medical care, permanent life style changes and continuous behavioral adaptation to the unpredictable course of the illness

The finding in the present study indicate that there are obvious differences between children with chronic asthmatic chest troubles and healthy children as regard to cognitive abilities, behavior and school performance. Nevertheless, pediatricians are not adequately trained and/or do not have the time to evaluate every child's psychosocial status **(Costello and Janiszewski, 1990, Jellinek et al, 1995).** One method of focusing on the limited time available to those children likely to have psychosocial problems is to use a screening procedure **(Jellinek, 1982).** As with any screening test, a psychosocial screening procedure must be economical, brief, and accurate, and easy to understand, administer and interpret **(Jellinek, 1995).**

In this study, PSCL was used as a screening test that provided a quick, valid, and reliable method for detection of psychosocial problems. It reflects parent's impressions of their child's psychosocial functioning with acceptable sensitivity and specificity (**Fielding, 1990).** Using PSCL for behavioral assessment showed that asthmatics had significantly more behavior problems across several domains compared with normal controls. **McQuaid et al. 2001**, found evidence of behavioral problems in both externalizing and internalizing domains (mainly anxiety and depression). Our findings agreed with other studies, which showed a relationship between asthma and internalizing behaviors generally **(Mrazek et al 1998).**

In a more recent review, the authors showed that in child/adolescent populations with asthma, up to one third met criteria for co-morbid anxiety disorders **(Katon et al., 2004)** particularly children with severe asthma **(Ortega et al, 2004**). Additionally, a link between higher levels of global internalizing symptoms and childhood asthma has been shown **(Gillaspy et al., 2002).** We found significant differences between asthmatics and controls as regard to attention problems. However, **Jonathan et al. (2006)** didn't find such affection.

Robert Finn (2003) studied the effect of asthma on sleep and attention of children by Questionnaires administered to the children's parents. The study revealed a significant difference between asthmatics and controls.

James et al (2007) showed that on top of physical symptoms like coughing, wheezing, and difficulty of breathing, children with asthma are also at increased risk of behavioral, emotional, and developmental problems. According to this study, parents of children with asthma were twice as likely to report that their child has severe problems with behavior, emotions, concentration, or getting along with others. The study also found that children with asthma are at increased risk for: attention deficit hyperactivity disorder (ADHD), depression behavioral and conduct problems and learning disabilities. ADHD was twice as common among children with asthma and three times more common among those with severe asthma. These results are consistent with our results in which we found significant diminution in asthmatics compared to controls as regard to psychosocial problems.

Asthma has medical, psychological and physical effects on school age children. The flare-up of asthma may lead to impaired daily function and absence from school. A study from California showed that on average, 7 children with active asthma missed 2.6% of school days per year. Sixty six percent of the studied cases mentioned that bronchial asthma affected their school attendance and they missed several school days. We have to consider this finding as a warning signal to give more attention to the role

of health education as an important preventive tool for many health problems, which in turn affect the school attendance and the scholastic achievement of the students.

Calam et al 2005 documented lower scores on a measure of attention and concentration for children with asthma which is consistent with our findings. Because behavioral problems are associated with poorer school adjustment and academic achievement, the identification and treatment of problems at this young age potentially could prevent subsequent disruptive behaviors and school difficulties (**Raver 2002**). Agreeing with our findings, a research suggested that children with asthma experience more internalizing and total behavior problems than healthy children **(Klinnert et al, 2000).**

Rosa Alati et al (2005) provided information on the association between both asthma prevalence and internalizing symptoms but didn't report any association between externalizing symptoms and prevalence of asthma. Their number of asthmatics was 5153 and they used different methods of assessment**.** We found a greater prevalence of internalizing behavior problems among children and adolescents who had asthma compared with non asthmatics. These results are consistent with the results of **Craske et al, 2001.** Consistent with the findings of **Linda et al(1989)**, in this study, academic performance and intelligence test scores indicated that, overall, the academic capabilities of children with asthma were less, compared with healthy children of the same socioeconomic status.

We noticed significant effect of bronchial asthma on cognitive and behavioral functioning of asthmatic children. These findings are consistent with **Naude and Pratorius (2003).** Children with asthma may be at risk for decreased school functioning due to acute exacerbations, increased absenteeism, iatrogenic effects of their asthma medication, and the stress associated with a chronic illness. Factors that may contribute to poor school performance among children with asthma include iatrogenic effects of oral steroids, poor medical management of the disease, and psychological problems **(Marianne et al, 1993).**

Bender and Bruce (1995) discussed the impact of asthma on children's school achievement. They found negative effect on the educational process, consistent with our results. They suggested that the cause may be due to medications that produce mild, temporary changes, affecting learning and classroom performance. For most asthmatic children, their illness does not result in permanent brain function changes that compromise their educational adaptation and performance. Increased school absence, stress of chronic illness, isolation from peers, diminished physical activities, reduced adult expectations and self esteem, and depression can compromise children's academic adaptation and progress. Co-occurrence of factors like severe illness, poverty, and family dysfunction may increase the risk for educational and psychosocial impairment. In our study, there is no opportunity to discuss the effects of such factors in the asthmatics.

Epidemiological studies showed that roughly one in ten children under the age of 15 suffers from a chronic disease. Other epidemiologic studies estimated that one third of children under 18 years of age are suffering from one or more chronic disorders or diseases**. (Costello et al 2006, Shah et al 2006 and Gallasi et al 2006)** In addition, there is an increased prevalence of learning and speech difficulties, sensory dysfunctions, mental handicaps and behavioral problems. **(Smith, 2003 and Williams et al, 2006)**. In our study chronic asthmatics and non asthmatic chest troubles (chronic diseases) were at increased risk of behavioral problems than healthy children.

CONCLUSION

1-Children with chronic asthmatic chest troubles represent a population at possible high risk for mental and psychosocial maladjustment.

2-Asthmatic children have more psychosocial problems than healthy children.

3-Asthmatic children have lower cognitive abilities and academic performance than healthy children.

4-Chronic asthmatic chest troubles have retarded weight and height compared to healthy children.

5-Medical school training needs to be more focused on the psychosocial issues and psychiatric disorders that affect adolescents with chronic illness rather than on the specific biological factor issues associated with the medical illness itself. Psychosocial issues and psychiatric disorders are clearly important factors affecting adolescents with chronic illness.

References

1-Bender and Bruce G.(1995): School Psychology Quarterly. Vol 10(4), Win 1995, 274

2- **Calam R, Gregg L, Simps**on **A, (2005):** Simps**on** B, Woodcock A, Custovic A. **Behavior** problems at study. *Am J Respir Crit Care Med.* 2005;171 :323 – 327**.**

3-Charron-Prochownik D (2002): Special needs of the chronically ill child during middle childhood: Application of a stress-coping paradigm. Journal of Pediatric Nursing 17 (6): 407-413.

4-Costello EJ and Janiszewski S. (1990): Who gets treated? factors associated with referral in children with psychiatric disorders. *Acta Psychiatr Scand*;18:523-529

5-Costello EJ., Foley DL., Angold A. (2006) Ten year research update review: the epidemiology of child and adolescent psychiatric disorders. Journal of the American Academy of Child and Adolescent Psychiatry, 45(1):8‐2.

6-Craske MG, Poulton R, Tsao JC and Plotkin D. (2001): Paths to panic disorder/agoraphobia: an exploratory analysis from age 3 to 21 in an unselected birth cohort. J Am Acad Child Adolesc Psychiatry. 2001;40(5):556-563.

7-El-Baz F., El-Shabory, AKhazbok M., Zaky E., and Negm F., (1995): Impact of Some Chronic Childhood Diseases on Physical Growth and Mental Language Development. JAC,6(4): 631-640.

8-Fielding, J.E. (1990): Psychosocial screening Questionnaires. Ambulatory Pediatrics 1st ed. By Green M. and Haggerty RS. Saunders W.B.A. Co.: 139.

9-Gallasi C., De Sario M., Biggeri A., Annibale B., Luigi C., et al (2006) Changes in prevalence of asthma and allergies among children and adolescents in Italy: Paediatrics, 117(1):34‐42.

10-Geist R, Grdisa V, and Otley A (2003): Psychosocial issues in the child with chronic condition. Best Practice & Research Clinical Gastroenterology 17 (2): 141-152.

11-Gillaspy SR, L HA, Mullins LL, Van Pelt JC, Chaney JM. (2002): Psychological distress in high-risk youth with asthma. *Journal of Pediatric Psychology.* 2002;27:363–371

12-Goldberg S; Janus M; Washington J; Simmon RJ; Maclusky I (1997): Prediction of behavioral problems in healthy and pediatric samples. Dev Behav Pediatrics 18: 304-313.

13-Jackson P and Vessey J (2000): Primary care of the child with a chronic condition (3rd ed). St. Louis: Mosby.

14-James A. Blackman, MD, MPH; Matthew J. Gurka, PhD.(2007): *Journal of Developmental and Behavioral Pediatrics*, April 12, 2007. Date reviewed: May 2007

15-Jellinek MS., (1982): The present status of child psychiatry in pediatrics. *N Engl J Med.* ;306:1227-1230.

16-Jellinek MS., (1994): Managed care: good news or bad news for children? *J Dev Behav Pediatr.* 1994;15:273-274. ISI | PUBMED

17-Jellinek MS, Little M, Murphy JM, Pagano M. (1995): The pediatric symptom checklist: Support for a role in a managed care environment. Arch Pediatr Adolesc Med. 1995; 149: 740-746

18-Jonathan M. Feldman, PH.D., Alexander L N. Ortega, PH.D. et al (2006): Psychosomatics 47:4, July-August 2006

19-Kamel M and Ismail E (1993): Wechsler Intelligence Scale for Children, Arabic Version. EL-Nahda El- Massryia, Cairo.

20-Katon WJ, Richardson L, Lozano P, and McCauley E., (2004): The relationship of asthma and anxiety disorders. *Psychosom Med.* 2004;66: 349–355

21-Linda B. Gutstadt, MEd; Jerry W. Gillette, MEd; David A. et al (1989): *Am J Dis Child.* 1989;143(4):471-475

22-Leblanc T., Goldsmith T, and Patel DR (2003): Behavioral aspects of chronic illness in children and adolescents. Pediatr Clin N Am 50: pp859-878.

23-Marianne P. Celano., Phd Robert J. Geller., MD (1993): Journal of Learning Disabilities,Vol.26,No.1,23-32,DOI10.1177/002221949302600103

24-McQuaid EL, Kopel SJ, and Nassau JH.(2001): Behavioral adjustment in children with asthma: a meta-analysis. J Dev Behav Pediatr 2001;22:430 –9.

25-Mrazek DA, Schuman WB, Klinnert M. (1998): Early asthma onset: risk of emotional and behavioral difficulties. *J Child Psychiatry*;39 :247 – 254

26-Murthy RS, Bertolote JM, Epping-Jordan J, Funk M, Prentice T et al (2001): *The World health report 2001 Mental health: new understanding, new hope.* Geneva, World Health Organization.

27-Naude H. and Pratorius E(2003): Early Child Development and Care, v173 n6 p699-709 Dec 2003

28-Nelson G, Westhues A, MacLeod J. (2003): A meta-analysis of longitudinal research on preschool prevention programs for children. *Prevention & Treatment.* 2003;**6**:1–32.

29-Noeker M, Haverkamp-Krois A, and Haverkamp F (2005): Development of mental health dysfunction in childhood epilepsy. Brain & Development 25:5-16.

30-Ortega AN, McQuaid EL, Canino G., and Goodwin RD. et al (2004): Comorbidity of asthma and anxiety and depression in Puerto Rican children. *Psychosomatics*;45:93–99.

31-Perrin EC and Stancin T. A. (2002): Continuing Dilemma: Whether and how to screen for concerns about children's behavior. Pediatr Rev.; 23 (8): 264-275

32-Pollite E, (1984): Methods for the behavioral assessment of the consequences of malnutrition. In Lockwood ER and Scrimshaw NS (eds): Methods for the evaluation of the impact of food and nutrition programs. Tokyo, UNU

33-Raver C. (2002): Emoti**on**s matter: making the case for the role of young **children**'s emoti**on**al development for early school readiness. *Soc Policy Rep.* 2002;16 :3 –18

34-Reijneveld SA, Brugman E, Verhulst FC, Verloove-Vanhorick SP. (2004): Identification and management of psychosocial problems among toddlers in Dutch preventive child health care. *Arch Pediatr Adolesc Med.* 2004;**158**:811–817. doi: 10.1001/archpedi.158.8.811. [PubMed]

35-Shah N. Harrington T., Huber M., Wellnitz C., Fridrych S., et al. (2006) Increased reported cases of tuberculosis among children younger than 5 years of age. *The Paediatric Infectious Disease Journal*, 25:151-155

36-Sharp L, Pantell RH, Murphy LO, and Lewis CL.(1992): Psychosocial problems during child health supervision visits: eliciting, then what? *Pediatrics* 1992;89:619-623.

37-Silver L.B., (1989): Learning disabilities in children; J. Amer Acad Child Adolesc Psychiat; 28: 309- 313.

38-Smith (2003) Comparative Study of Anxiety, Depression, Somatization, Functional Disability, and Illness Attribution in Adolescents With Chronic Fatigue or Migraine *Pediatrics,* 111:376-381

39-Starfield B, Gross E, Wood M, et al. (1980);Psychosocial and psychosomatic diagnoses in primary care of children. Pediatrics 66:159-167

40-Tavormina JB; Kasthner LE; Slater PM (1996): Chronically ill children: A psychologicaly and emotionally devient population. J Abn Child psychol 4: 99-110

41-Verhulst FC, van der Ende J, Koot HM(1996): Handleiding voor de CBCL/4-18 [Manual for the CBCL/4-18]. Rotterdam, the Netherlands, Erasmus University, Department of Child and Adolescent Psychiatry, Sophia Children's Hospital,

42-Wechsler D, (1977): Manual for the Wechsler Intelligence Scale for Children- Revised (WISC-R), the psychological Corporation, NY

43-Williams JG., Higgins JP., Brayne CE. (2006) Systematic review of prevalence studies of autism spectrum disorders. Archives of disease in childhood, 91(1):8-158|PageICUSNURSWEBJ,ISSUE29-30, http://www.nursing.gr.

10/25/2010

Effect of different sources of potassium fertilizers on growth yield, and chemical composition of *Calendula Officinalis*

*Yassen, A.A; **Habib, A. M.; *Sahar, M. Zaghloul, and *Khaled, S.M.
*Plant Nutrition Dept., National Research Centre, Dokki, Giza, Egypt.
**Ornamental plant Dept., Faculty of agriculture, Cairo University

azimyassen@yahoo.com

ABSTRACT: A field experiments were carried out during the two successive seasons of 2007/2008 and 2008/2009, in Qualubia Governorate, Egypt, to study the effect of different sources of potassium fertilizer (banana residue and potassium sulphate) on yield, and chemical composition of herbs and flowers of *Calendula Officinalis***.** It had been deduced that application of potassium fertilizer from different sources; potassium sulphate and banana residue were effective in increasing all tested growth yields compared with unfertilized treatment. Data also, showed that mixing potassium sulphate or/ and banana residue led to a marked increase in fresh and dry weight of herbs and flowers as compared with application of potassium sulphate or/ and banana residue solely in both seasons**.** Data also, showed that mixing potassium sulphate or/ and banana residue increased N and P and K content and uptake as compared with the control, potassium sulphate and banana residue alone.

[Yassen, A.A; Habib, A. M.; Sahar, M. Zaghloul, and Khaled, S.M. Effect of different sources of potassium fertilizers on growth yield, and chemical composition of *Calendula Officinalis*. Journal of American Science 2010;6(12):1044-1048]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: *Calendula Officinalis* - potassium fertilizer - banana residue - growth – yield - NPK.

INTRODUCTION

Calendula Officinalis (Pot Marigold) is a plant in the genus Calendula (marigolds), in the family Asteraceae. It is probably native to southern Europe through its long history of cultivation makes its precise origin unknown, and may be of garden origin. It is also widely naturalized further north in Europe (north to southern England) and elsewhere in warm temperate regions of the world.

Calendula Officinalis is used for the treatment of skin disorders and pain, and as a bactericide, antiseptic and anti-inflammatory Fuchs *et al.*, and (2005) Bolderston *et al.*, (2006). The petals and pollen contain triterpenoid esters (an anti-inflammatory) and the carotenoids flavoxanthin and auroxanthin (antioxidants, and the source of the yellow-orange coloration) Hamburger *et al.*, (2003) and Bashir *et al.*, (2006).

Potassium is a multifunctional versatile nutrient indispensable for plants. In plants, the function of K has several roles, such as enzyme activation, stimulation of assimilation and transport of assimilate anion / cation balance as well as water regulation through control of stomata Krauss and Jin Jiyun (2000) Zhou *et al.*, (2006) and TAN De-shui (2007). Dutta *et al.*, (2001) Balliu and Ibro, (2002) and Ali and Mowafy (2003) and Gent, (2004) and Liu, *et al.*, (2008) indicated that adding potassium fertilizer significantly increased number of branches and yield in crops.

Singh *et al.*, (2005 and 2007) and Munnu Singh and Ganesha Raoa (2009) found that application of N and K produced significant higher patchouli herbage and compared with controls. Similarly, N and K uptake were also higher compared with controls.

This field experiment was, therefore, conducted to investigate the influence of different sources of potassium fertilizer on yield and chemical composition of herbs and flowers of *Calendula Officinalis.*

MATERIALS AND METHODS

Two field experiments were carried out during two successive seasons of 2007/2008 and 2008/2009, in Qualubia Governorate, Egypt, to study the effect of potassium fertilizer on yield, and chemical composition of herbs and flowers of *Calendula Officinalis.*

Prior to any practices, a composite soil sample was taken from the soil surface (0-30 cm) of the experimental site, air dried, sieved by 2 mm sieve and analyzed (table 1). The physical and chemical

properties of soil were determined according to Chapman and Pratt (1961).

Table (1) some characteristics of the experimental site in two seasons

seasons	Physical properties				Chemical properties						
	Sand	Silt	Clay	Texture	pH	EC dSm^{-1}	$CaCO_3$ %	OM %	N	P	K
	%								(ppm)		
07/08	14.1	26.9	59.0	Clay	8.20	0.66	2.80	1.40	115	2.11	26.3
08/ 09	13.2	27.8	59.0	Clay	7.97	0.59	3.11	1.60	120	2.20	25.4

Potassium fertilizer at a rate of (50kg K/ fed) and two sources of potassium, one type of organic material (Banana residues) and the other inorganic potassium (potassium sulphate) were added. Application rate of organic matter used to depend on its content of potassium and the total required K (50 kg K/ fed), which added by organic material only and / or by complete the rest by K fertilizer as follows:

- Control (recommended doses)
- 50 kg K / fed (K_2SO_4)
- 10 kg K / fed (Banana residues) + 40 kg K / fed (K_2SO_4)
- 20 kg K / fed (Banana residues) + 30 kg K / fed (K_2SO_4)
- 30 kg K / fed (Banana residues) + 20 kg K / fed (K_2SO_4)
- 40 kg K / fed (Banana residues) + 10 kg K / fed (K_2SO_4)
- 50 kg K / fed (Banana residues)

From the above mentioned treatments that increasing K fertilizer associated with decreasing in the amount of organic material applications.

The seeds of *Calendula Officinalis* L. plants were kindly provided by the Department of Medicinal and Aromatic Plants, Ministry of Agriculture, Egypt.

Seeds were sown on October 1st during both seasons. The experimental design was complete randomized blocks with five replicates. The experimental area (plot) was $2m^2$ (2m x 1m) containing 4 rows; and the distance between the hills was 25cm and 50 cm apart. Thinning for one plant/hill was done 45days after sowing. The irrigation was carried out whenever plants needed.

All agricultural operations other than experimental treatments were done according to the recommendations of Ministry of Agriculture; Egypt Chemical analysis of used organic fertilizer (Banana residues) is presented in table, 2.

Table (2): Chemical analysis of Banana residues used in this study.

Contents	pH 1:20)	EC dSm^{-1} 1:20)	OM %	O.C %	N %	P %	K %
Banana residues	8.72	3.38	76.16	44.18	1.34	0.59	5.92

Plant samples and analysis

Fresh and dry weights (g/plant) of herbs and flowers of *Calendula Officinalis* which dried at 70 ^{O}C. were recorded. The following chemical analyses were determined: nitrogen, phosphorus, and potassium according to the methods described by Cottenie *et al.*, (1982). Collected data was subjected to statistical analysis of variance according to Snedecor and Cochran (1980). Physical and chemical properties of the soil were determined according to Chapman and Pratt (1961)

RESULTS AND DISCUSSION

Effect of potassium fertilizer on vegetative growth:

It is quite clear from the data presented in table (3) that application of potassium fertilizer from different sources; inorganic (potassium sulphate) and organic (banana residue), affected herbs and flowers of *Calendula Officinalis.* Fresh and dry weights were significantly increased in first and second seasons when compared with the control Singh, *et al.*, 2007 and Munnu Singh, and Ganesha Raoa, 2009) found that potassium application increased growth parameters (fresh and dry plant weight) as compared with control (no K fertilizer).

Results mentioned above indicate that superiority of fresh and dry weight of herbs and flowers under K application might be due to the role of K in the enhancement and development of plant tissues through the synthesis of simple sugars and starch and also, the translocation of carbohydrates and protein synthesis.

Comparing application of potassium fertilizer, as potassium sulphate and as compost (banana residue), data showed that mixing potassium sulphate or/ and banana residue led to marked increase in fresh and dry weight of herbs and flowers as compared with application of potassium sulphate or/ and banana residue solely in both seasons (El- Ghadban 1989), investigating *Mentha viridis*, showed that K and organic fertilizer were generally more effective in increasing fresh and dry weight of herb. The data of dry weight followed a nearly similar trend of fresh weight in herbs and flowers in both seasons.

The maximum recorded values were 14.9 and 13.2g / plant dry weight in flowers and 24.2 and 24.6g / plant in herbs in first and second seasons respectively. Similar trend noticed with fresh weight in herbs and flowers in both seasons.

From the data presented in table (3) clearly the high value of fresh and dry weight of herbs and flowers was obtained when K was added at a rate of 20kg K/ fed as potassium sulphate + 30kg K / fed as an organic source (banana compost) in both seasons.

Table (3) effect of potassium fertilizer on fresh and dry weight (g / plant) on herbs and flowers of *Calendula Officinalis.*(in both seasons)

Treatments		Flowers				Herbs			
		Fresh weight (g / plant)		Dry weight (g / plant)		Fresh weight (g / plant)		Dry weight (g / plant)	
Org.	Inorg.	1st season	2nd season	1st season	2nd season	1st season	2nd season	1st season	2nd season
Control		18.7	18.7	3.2	3.0	47.8	43.2	8.8	8.3
0 +	50	30.5	31.3	8.8	8.0	68.1	68.5	16.4	16.5
10 +	40	35.3	33.6	7.8	7.6	74.2	76.0	18.7	17.3
20 +	30	56.3	54.7	14.9	13.2	90.7	90.4	24.2	24.6
30 +	20	51.2	49.6	13.0	12.7	82.1	80.7	21.1	20.7
40 +	10	30.1	30.3	6.9	7.3	70.3	72.5	13.7	15.1
50 +	0	23.8	22.2	6.4	6.1	53.2	57.1	11.7	13.3
L.S.D	0.05								
		3.48	3.31	0.57	0.83	5.66	5.64	0.99	1.41

Org: banana residue Inorg : potassium sulphate

Chemical composition:

Data of nitrogen and phosphorus, content and uptake in the various sources of potassium fertilizers application treatments on herbs and flowers of *Calendula Officinalis* in the two successive seasons are presented in tables (4 and 5). Generally, the present results indicate that, nitrogen and phosphorus, content and uptake increased as compared with the control treatment. These results were on line with those reported by Liu *et al.,* (2008) and Munnu Singh and Ganesha Raoa (2009)

Data also, showed that application of potassium fertilizer in different sources inorganic (potassium sulphate) and organic material (banana residue) increased N and P content and uptake as compared with potassium sulphate and banana residue alone. The highest values of N and P were recorded at 30 kg K / fed as an organic source (banana residue) + 20kg K/ fed as potassium sulphate application (2.00 and 2.12% N) and(0.41 and 0.44% P) in flowers and (1.54and 1.58% N) and (0.28 and 0.26% P) in herbs in the two successive seasons, respectively.

Concerning the effect of potassium application on potassium content and uptake it is evident to data that the previous mineral in all plant organs, in the tow growing seasons, were increased by using potassium, especially by using the mixture potassium sulphate with banana residue. Similar conclusion was also reported by Singh *et al.,* 2005 and 2007.

Table (4): effect of potassium fertilizer on N, P and K content (%) on herbs and flowers of *Calendula Officinalis.* (in both seasons)

treatmens	N%				P%				K%			
	Flowers		Herbs		Flowers		Herbs		Flowers		Herbs	
Org. Inorg.	1st season	2nd season	1st season	2nd season	1st season	2nd season	1st season	2nd season	1st season	2nd season	1st season	2nd season
Control	0.97	0.95	0.67	0.70	0.18	0.20	0.11	0.09	1.10	1.07	1.45	1.53
0 + 50	1.54	1.50	1.14	1.20	0.31	0.28	0.17	0.16	1.46	1.39	2.40	2.37
10 + 40	1.78	1.80	1.35	1.30	0.33	0.35	0.21	0.20	1.55	1.50	2.53	2.46
20 + 30	1.84	1.90	1.50	1.48	0.38	0.40	0.25	0.23	1.84	1.88	2.64	2.73
30 + 20	2.00	2.10	1.54	1.58	0.41	0.44	0.28	0.26	1.93	1.88	2.89	2.94
40 + 10	1.73	1.76	1.30	1.33	0.33	0.36	0.20	0.23	1.49	1.52	2.43	2.37
50 + 0	1.49	1.50	1.20	1.27	0.35	0.31	0.18	0.20	1.35	1.40	2.38	2.31
L.S.D 0.05												
	0.14	0.15	0.11	0.11	0.04	0.03	0.02	0.02	0.13	0.12	0.20	0.22

Table (5): effect of potassium fertilizer on N,P and K uptake (%) on herbs and flowers of *Calendula Officinalis.*(in both season)

treatmens	N%				P%				K%			
	Flowers		Herbs		Flowers		Herbs		Flowers		Herbs	
Org. Inorg.	1st season	2nd season	1st season	2nd season	1st season	2nd season	1st season	2nd season	1st season	2nd season	1st season	2nd season
Control	31.04	28.50	58.96	58.10	5.76	8.20	9.68	7.47	35.20	43.87	127.60	126.99
0 + 50	135.52	120.00	186.96	198.00	27.28	22.40	27.88	26.40	128.48	111.20	393.60	391.05
10 + 40	138.84	136.80	252.45	224.90	25.74	26.60	39.27	34.60	120.90	114.00	473.11	425.58
20 + 30	274.16	250.80	363.00	364.08	56.62	52.80	60.50	56.58	274.16	248.16	638.88	671.58
30 + 20	260.00	266.70	324.94	327.06	53.30	55.88	59.08	53.82	250.90	238.76	609.79	608.58
40 + 10	119.37	128.48	178.10	200.83	22.77	26.28	27.40	34.73	102.81	110.96	332.91	357.87
50 + 0	95.36	91.50	140.40	168.91	22.40	18.91	21.06	26.60	86.40	85.40	278.46	307.23
L.S.D 0.05												
	18.98	19.26	24.98	23.64	3.88	4.03	4.47	3.95	11.85	16.81	45.82	43.46

REFERENCE

1. Ali, A.A.G. and S.A.E. Mowafy (2003) Effect of different levels of potassium and phosphorus fertilizer with the foliar application of zinc and boron on peanut in sandy soil. Zigzag J. Agric. Res., 30: 2: 335 – 358.
2. Balliu and V. Ibro, (2002), Influence of different levels of potassium fertilizers on growth, yield and ascorbic acid content of tomato fruit grown in non-heated greenhouse, Acta Horticulturae. 579 pp. 385 –388.
3. Bashir S, Janbaz KH, Jabeen Q (2006) Studies on spasmogenic and spasmolytic activities of *Calendula Officinalis* flowers. Phytother Res. 20:906-910.
4. Bolderston A, LLoyd NS, Wong RK et al. (2006). The prevention and management of acute skin reactions related to radiation therapy: a systematic review and practice guideline. Support Care Cancer. 14:802-817.
5. Chapman H.D., and Pratt P.F., (1961) Methods of Analysis for Soils, Plant and Waters. Univ. of California, Dept of Agric. Sci., USA: 5-6 and 56-58.
6. Cottenie A.M., Verloo L., Kiekens G Velghe and Camerlynck R.,(1982)Chemical Analysis of Plant and Soil. PP. 100 – 129. Laboratory of Analytical and Agrochemistry, State Univ. Ghent. Belgium.
7. Dutta, R;P.K. Gogoi, R. Lalramhluni,A.C. Thakur and N.C. Deka (2001) Effect of levels of lime and potash on production of groundnut . Crop Res. Hisar. 26:3:420 -423.

8. El- Ghadban, E.A.F. (1989)effect of some organic and in organic fertilizers on growth, oil yield and chemical composition of spearment and majoram plants. Ph.D.Thesis, Fac., Agric., Cairo Univ. Egypt

9. Fuchs SM, Schliemann-Willers S, Fischer TW, (2005) Protective effects of different marigold (*Calendula Officinalis* L.) and rosemary cream preparations against sodium-lauryl-sulfate-induced irritant contact dermatitis. Skin Pharmacol Physiol 18(4):195-200.

10. Gent, M.P.N (2004) Yield of greenhouse tomato in response to supplemental nitrogen and potassium, Acta Horticulturae. 633 pp. 341–348.

11. Hamburger M, Adler S, Baumann D, (2003) Preparative purification of the major anti-inflammatory triterpenoid esters from Marigold (*Calendula Officinalis*). Fitoterapia 2003;74(4):328-338.

12. Krauss, A. and Jin Jiyun (2000) Strategies for improving balanced fertilization. International Potash Instite, Basel Switzerland.

13. Liu, Z. H., Jiang, L. H., Li, X. L., Härdter, R., Zhang, W. J., Zhang, Y. L. and Zheng, D. F. (2008) Effect of N and K fertilizers on yield and quality of greenhouse vegetable crops. Pedosphere. 18(4): 496–502

14. Munnu Singh, a, and R.S. Ganesha Raoa(2009) influence of sources and doses of N and K on herbage, oil yield and nutrient uptake of patchouli [Pogostemon cablin (Blanco) Benth.] in semi-arid tropics. Industrial Crops and Products29, Issue 1- 229-234.

15. Singh, M. R.S. Ganesha Rao and S. Ramesh, (2005)Effects of nitrogen, phosphorous and potassium herbage, oil yield, oil quality and soil fertility status of lemongrass in a semi-arid tropical region of India, J. Hortic. Sci. Biotechnol. 80 pp. 493–497.

16. Singh, M. R.S. Ganesha Rao and S. Ramesh, (2007) Effects of N and K on growth, herbage, oil yield and nutrient uptake patterns of rosemary under semi-arid tropical conditions, J. Hortic. Sci. Biotechnol. 82 pp. 414–419

17. Snedecor,G.W. and W.G. Cochran., (1990) Statistical Methods. pp: 369-375. 11th Ed. Iowa State College Press. Ames, Iowa, U.S

18. TAN De-shui. JIN Ji-yun, a, HUANG Shao-wena, LI Shu-tiana and HE Pinga(2007) effect of long-term application of k fertilizer and wheat straw to soil on crop yield and soil k under different planting systems. Agricultural Sciences in China 6: 2:200-207

19. Zhou, TH. HP Zhang and L Liu, (2006) Studies on effect of potassium fertilizer applied on yield of Bt cotton, Chin. Agric Sci Bull 22 (8) 292–296.

10/27/2010

Effect of packing on extension of self life of retail meat

[*]Khalafalla, F.A; Nagwa, S.S. Ata; Mona. A.E. Elshabrauy, Azza, S.M. Abu Elnaga Dorgham,S.M and Khairy, A. E

[*]Faculty of vet. Medicine. Beni-Suef University, Egypt. National Research Center. Dokki, Egypt.

Abstract: The packing of meat in retail markets plays important role in controlling of microbial load. Trails for extension of shelf-life of meat was studied during chilling. The comparative between the different types of packing as well as compared with fresh and chilled meat have low available data. Therefore, this study was carried out to assessment the effect of packing (Aerobically and anaerobically) on chilled meat as compared with fresh ones in retail market.

[Khalafalla, F. A; Nagwa, S.S. Ata; Mona. A.E. Elshabrauy, Azza, S.M. Abu Elnaga Dorgham, S.M and Khairy, A. E. **Effect of packing on extension of self life of retail meat.** Journal of American Science 2010;6(12):1049-1058]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: packing; meat; retail market; microbial load

Introduction

The packing of meat in retail markets plays important role in controlling of microbial load. Trails for extension of shelf-life of meat was studied during chilling **(White et al, 1988; Nortje et al, 1990;Cliver and Riemann, 2002 and Ashton et al, 2006)** as well as the effect of packing in aerobic **(Byun et al, 2003)** and anaerobic **(Plaatjies et al, 2004)** was done for reduction the microbial load on retail meat.

The acceptable limits of microbial load in meat cuts was stated by **(ICMSF, 1986, Grau and vanderlinde, 1990 park et al, 1994 and E.O.S.Q.C, 2001-2004)** as well as the offensive odour and change in colour were appeared when the count reached 10^7CFU/g **(Jay, 1986; Shelef et al, 1997; Moje, 1999 and Byun et al, 2003).**

The comparative between the different types of packing as well as compared with fresh and chilled meat have low available data.

Therefore, this study was carried out to assessment the effect of packing (Aerobically and anaerobically) on chilled meat as compared with fresh ones in retail market.

Material & Methods

1- Experimental samples:

Seven kilograms of fresh beef were obtained from recent slaughtered animal after arrival of the meat to butcher's shop. The collected meat was taken from hindquarter after preparation (without visible fat). The collected meat was rapidly transferred as possible to laboratory in ice box with minimum delay.

2- Experimental design:

The techniques recommended by Gill et al. (2002) was applied as follows:

The collected meat was divided into two parts:

- The first part was sliced to samples; each weighed 100 g and 7 x7 x 0.5 cm in dimensions; then, kept at room temperature (about 25-30ºC) and daily examined (3 samples each time) till spoilage.
- The second part was divided into samples as previously mentioned, then kept into three groups at chilled temp (5ºC), the first group was preserved without packing (aerobic) and the secand group aerobically was packed in polyethylene bags and finally, third group, was anaerobically (vacuumed) packed. The samples were examined with 48 hours intervals (3 samples in each time).

3- Preparation of samples.

The techniques recommended by **AOAC (2000)** was applied as follows:

4- Techniques:

i) Aerobic plate count at 35ºC (mesophiles).

ii) Aerobic plate count at 25ºC (Psychrotrophs).

iii) Enumeration of coliforms (MPN).

iv) Isolation and identification of *E. coli*.

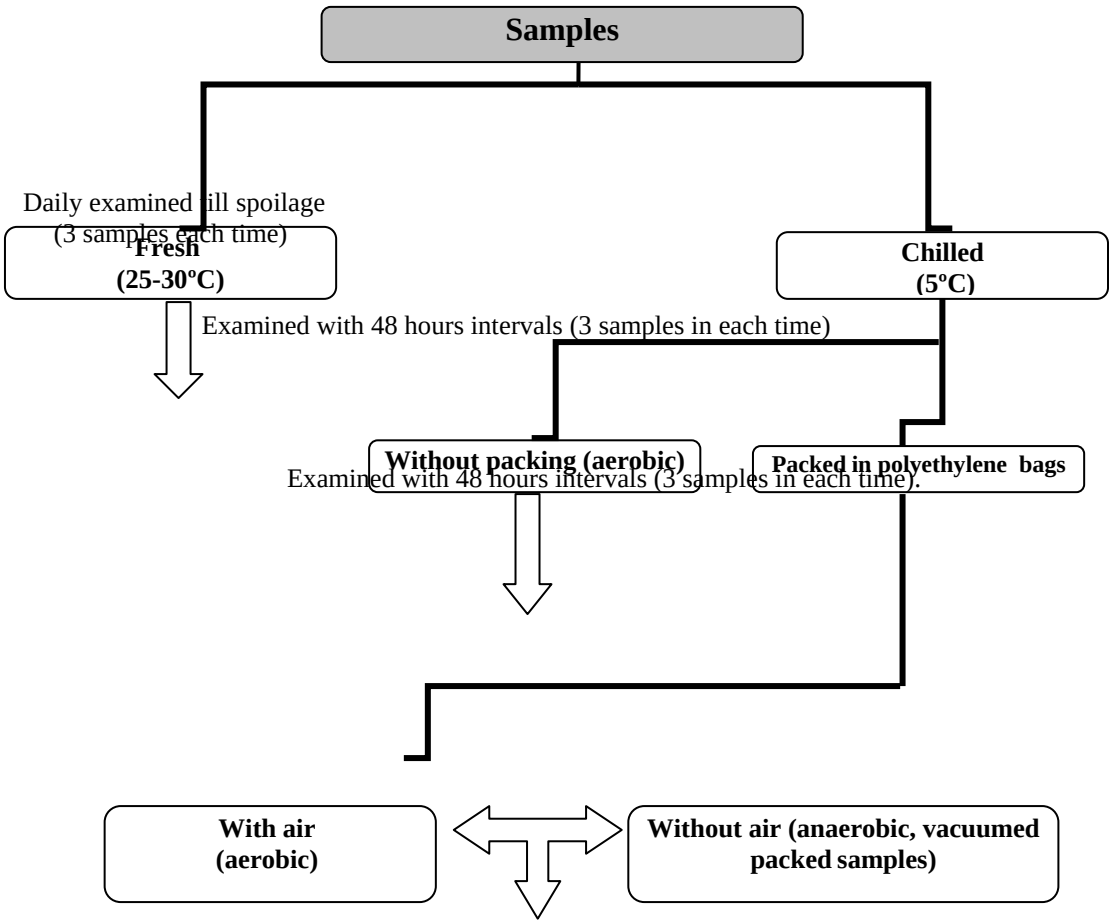
v) Isolation and Identification of Salmonellae.

vi) Determination of *Staphylococcus* count.

vii) Isolation and identification of *Staphylococcus aureus.*

viii) Isolation and identification *of Listeria monocytogenes*.

Experimental design (Gill et al., 2002):



Results

Table (1) Statistical analysis of bacteriological status of examined fresh meat samples.

Time	No. of samples	APC *	Psychrotrophic *	Coliforms bacteria (MPN) *	Fecal coliform bacteria (MPN) *	*Staphylococcus aureus* count *
1st day	3	$8\times10^{5}\pm5x10^{5}$ a	$2\times10^{4}\pm2\times10^{3}$ a	$6.7\times10\pm1.7\times10$ a	$2.8\times10\pm0.7\times10$ a	$5\times10^{2}\pm2\times10^{2}$ a
2nd day	3	$2\times10^{6}\pm9\times10^{5}$ a	$2\times10^{4}\pm1\times10^{4}$ a	$6.3\times10\pm1.5\times10$ a	$3.5\times10\pm4\times10$ a	$2\times10^{3}\pm6\times10^{2}$ b
3rd day	3	$3\times10^{7}\pm1\times10^{7}$ a	$3\times10^{5}\pm1\times10^{5}$ a	$5\times10^{2}\pm2\times10^{2}$ a b	$1\times10^{2}\pm1.9\times10$ a b	$6\times10^{3}\pm1\times10^{3}$ c e
4th day	3	$2\times10^{8}\pm1\times10^{8}$ a	$3\times10^{5}\pm1\times10^{5}$ a	$8\times10^{2}\pm2\times10^{2}$ b	$2\times10^{2}\pm1\times10^{2}$ c b	$9\times10^{3}\pm5\times10^{2}$ e
5th day	3	$9\times10^{8}\pm3\times10^{7}$ b	$9\times10^{5}\pm2\times10^{5}$ b	$1\times10^{3}\pm4\times10^{2}$ b	$1\times10^{3}\pm3\times10^{2}$ e	$2\times10^{4}\pm4\times10^{3}$ f

Mean in the same column with different alphabetical letters (a, b, c, d and f) are significant differences at ($P<0.05$).

* Mean and Standard error of three trials.

MPN = Most Probable number

Table (2) Statistical analysis of bacteriological status of examined chilled meat without packing samples during storage period.

Time	No. of samples	APC *	Psychrotrophic *	Coliforms bacteria (MPN) *	Fecal coliform bacteria (MPN) *	*Staphylococcus aureus* count *
1st day	3	$6\times10^{4}\pm2\times10^{4}$ a	$2\times10^{2}\pm5.7\times10$ a	$2.1\times10\pm0.7\times10$ a	$0.4\times10\pm0.1\times10$ a	$10^{2}\pm3\times10$ a
3rd day	3	$7\times10^{5}\pm1\times10^{5}$ a	$7\times10^{3}\pm8\times10^{2}$ a	$2\times10\pm0.9\times10$ a	$0.4\times10\pm0.09\times10$ a	$3\times10^{2}\pm8\times10$ a
5th day	3	$2\times10^{6}\pm1\times10^{6}$ a	$2\times10^{4}\pm5\times10^{3}$ a	$2.8\times10\pm0.7\times10$ a	$0.8\times10\pm0.1\times10$ a	$5\times10^{2}\pm1\times10^{2}$ a
7th day	3	$6\times10^{6}\pm1\times10^{6}$ a	$3\times10^{4}\pm1\times10^{4}$ a	$5.7\times10\pm1.8\times10$ a	$2.8\times10\pm0.8\times10$ a	$8\times10^{2}\pm1\times10^{2}$ a
9th day	3	$2\times10^{7}\pm9\times10^{6}$ a	$3\times10^{5}\pm5\times10^{4}$ a	$4\times10^{2}\pm3\times10^{2}$ a	$1\times10^{2}\pm1\times10^{2}$ a	$1\times10^{3}\pm3\times10^{2}$ c
11th day	3	$4\times10^{8}\pm1\times10^{7}$ b	$8\times10^{6}\pm1\times10^{6}$ b	$2\times10^{2}\pm1\times10^{2}$ a	$5.3\times10\pm2\times10$ a	$3\times10^{4}\pm2\times10^{4}$ b

Mean in the same column with different alphabetical letters (a, b and c) are significantly differences at ($P<0.05$).

* Mean and Standard error of three trials.

MPN = Most Probable number

period.

Time	No. of samples	APC *	Psychrotrophic *	Coliforms bacteria (MPN) *	Fecal coliform bacteria (MPN) *	*Staphylococcus aureus* count *
1st day	3	$5\times10^{3}\pm8\times10^{2\ a}$	$2\times10^{2}\pm8.8\pm\times10^{a}$	$0.4\times10\pm0.1\times10^{a}$	$0.3\times10\pm0.01\times10^{a}$	$10^{2}\pm4\times10^{a}$
3rd day	3	$3\times10^{4}\pm1\times10^{4\ a}$	$1\times10^{3}\pm8\times10^{2\ a}$	$0.8\times10\pm0.1\times10^{a}$	$0.3\times10\pm0.01\times10^{a}$	$2\times10^{2}\pm8.8\times10^{a}$
5th day	3	$1\times10^{5}\pm6\times10^{4\ a}$	$2\times10^{4}\pm1\times10^{4\ a}$	$1.7\times10\pm0.3\times10^{a\ c}$	$0.8\times10\pm0.3\times10^{a\ e}$	$2\times10^{2}\pm3.3\times10^{a}$
7th day	3	$3\times10^{6}\pm1\times10^{6\ a}$	$4\times10^{3}\pm1\times10^{3\ a}$	$3.1\times10\pm0.6\times10^{b\ c}$	$1.4\times10\pm0.4\times10^{b\ e}$	$6\times10^{2}\pm8.8\times10^{a}$
9th day	3	$7\times10^{6}\pm6\times10^{5\ a}$	$7\times10^{4}\pm5\times10^{3\ a}$	$5.7\times10\pm0.9\times10^{e}$	$2.7\times10\pm0.4\times10^{c}$	$4\times10^{3}\pm2\times10^{2\ b}$
11th day	3	$8\times10^{7}\pm5\times10^{6\ b}$	$4\times10^{5}\pm1\times10^{5\ b}$	$1\times10^{3}\pm3\times10^{2\ f}$	$1\times10^{3}\pm4\times10^{2\ f}$	$8\times10^{4}\pm3\times10^{2\ c}$

Mean in the same column with different alphabetical letters (a, b, c and e) are significantly differences at ($P<0.05$).
* Mean and Standard error of three trials.
MPN = Most Probable number

Table (4) Statistical analysis of bacteriological status of examined anaerobic packaged meat samples during storage period.

Time	No. of samples	APC *	Psychrotrophic *	Coliforms bacteria (MPN) *	Fecal coliform bacteria (MPN) *	*Staphylococcus aureus* count *
1st day	3	$1\times10^{3}\pm4\times10^{2}$ a	$10^{2}\pm2.5\times10^{a}$	$0.32\times10\pm0.2\times10^{a}$	$0.3\times10\pm0.01\times10^{a}$	$10^{2}\pm4\times10^{a}$
3rd day	3	$5\times10^{3}\pm8\times10^{2}$ a	$1\times10^{2}\pm3.3\times10^{a}$	$0.74\times10\pm0.09\times10$ a	$0.3\times10\pm0.01\times10^{a}$	$10^{2}\pm3.5\times10^{a}$
5th day	3	$3\times10^{4}\pm1\times10^{4}$ b	$3\times10^{2}\pm5.7\times10^{a}$	$1\times10\pm0.09\times10^{a}$	$0.\ 5\times10\pm0.\ 2\times10^{a}$	$10^{2}\pm4\times10^{a}$
7th day	3	$3\times10^{5}\pm2\times10^{5}$ c	$6\times10^{2}\pm5.7\times10^{a}$	1.2×10±0.2×10 a	$0.07\times10\pm0.01\times10$ a	$2\times10^{3}\pm1\times10^{3\ a}$
9th day	3	$3\times10^{6}\pm1\times10^{6}$ d	$5\times10^{3}\pm1\times10^{3\ a}$	$2.2\times10\pm0.1\times10^{a}$	$1\times10\pm0.09\times10^{a}$	$5\times10^{4}\pm3\times10^{4\ b}$
11th day	3	$7\times10^{7}\pm2\times10^{7}$ f	$1\times10^{6}\pm8\times10^{5\ b}$	$4\times10^{2}\pm3\times10^{2\ f}$	$4\times10^{2}\pm3\times10^{2\ b}$	$3\times10^{4}\pm2\times10^{4\ c}$

Mean in the same column with different alphabetical letters (a, b, c, d and f) are significantly differences at ($P<0.05$).
* Mean and Standard error of three trials.
MPN = Most Probable number

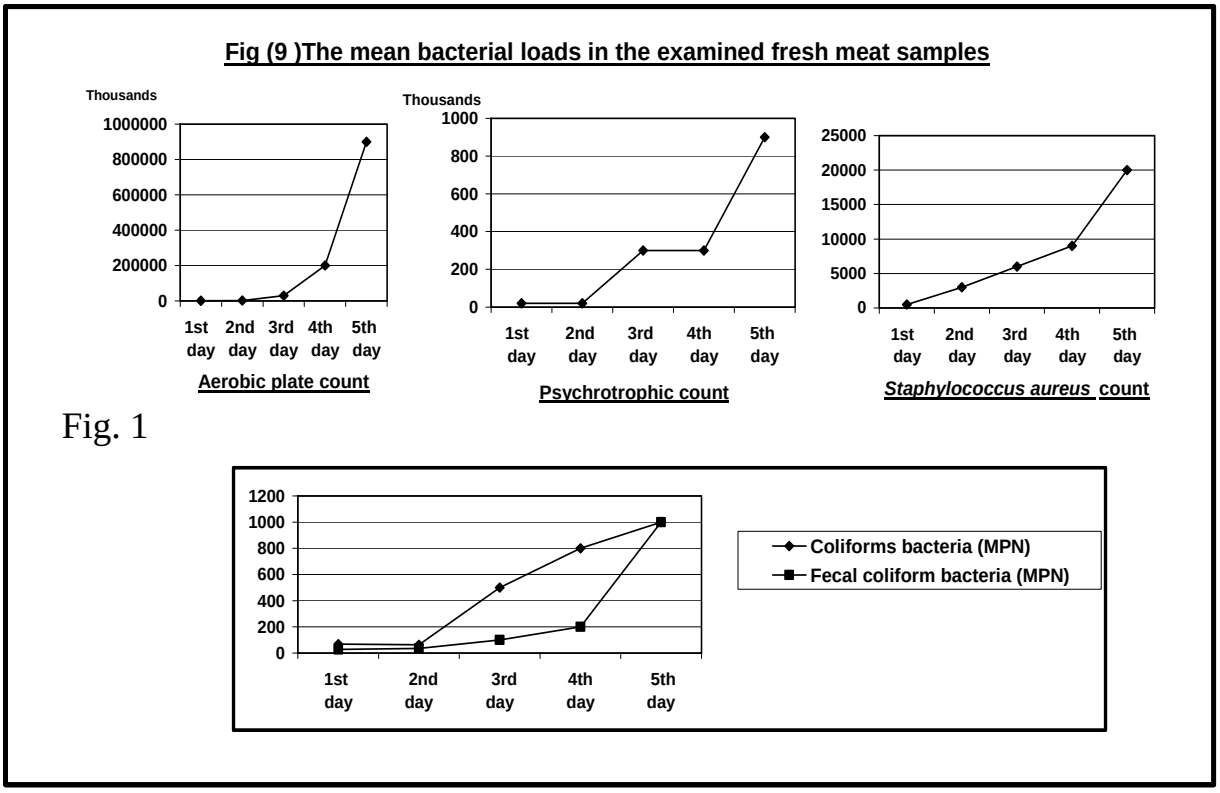


Fig (9)The mean bacterial loads in the examined fresh meat samples
Thousands
1000000
800000
600000
400000
200000
0
1st day
2nd day
3rd day
4th day
5th day
Aerobic plate count
Thousands
1000
800
600
400
200
0
Psychrotrophic count
25000
20000
15000
10000
5000
0
Staphylococcus aureus count
1200
1000
800
600
400
200
0
Coliforms bacteria (MPN)
Fecal coliform bacteria (MPN)

Fig. 1

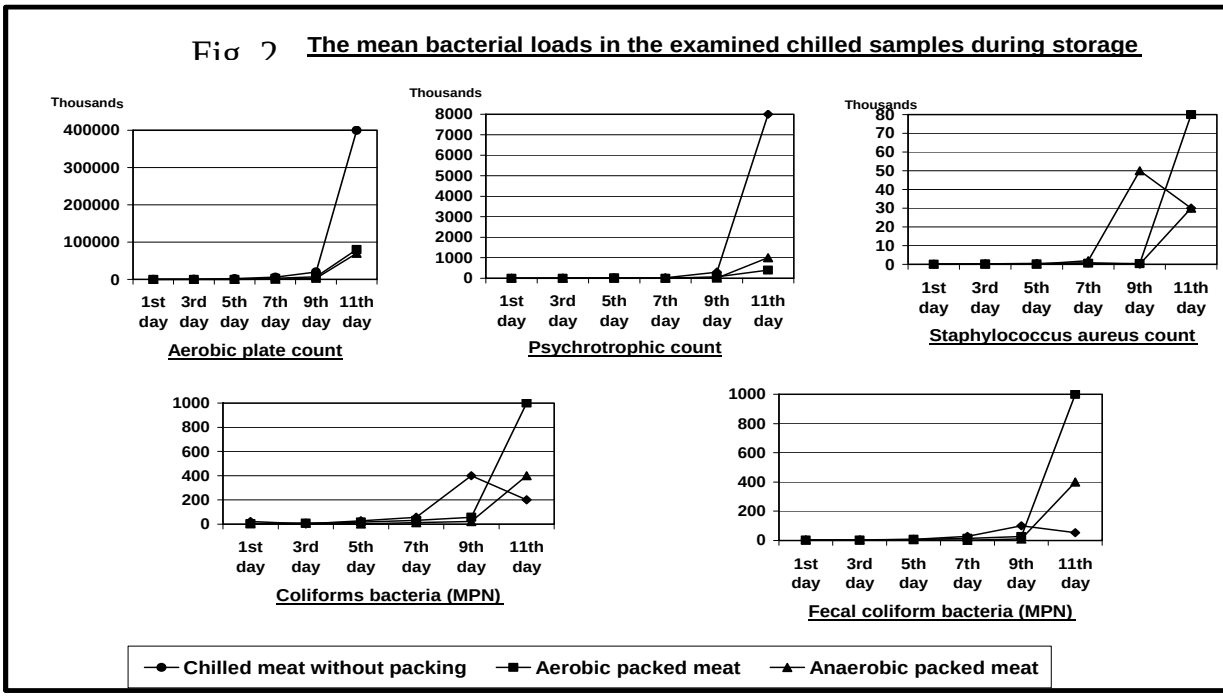


Fig. 2 The mean bacterial loads in the examined chilled samples during storage
Thousands
400000
300000
200000
100000
0
1st day
3rd day
5th day
7th day
9th day
11th day
Aerobic plate count
Thousands
8000
7000
6000
5000
4000
3000
2000
1000
0
Psychrotrophic count
Thousands
80
70
60
50
40
30
20
10
0
Staphylococcus aureus count
1000
800
600
400
200
0
Coliforms bacteria (MPN)
Fecal coliform bacteria (MPN)
Chilled meat without packing
Aerobic packed meat
Anaerobic packed meat

DISCUSSION

- From the results achieved in Table (1) fig. (1), it was evident that the mean value of aerobic plate count of fresh meat at 1st day was $8 \times 10^5 \pm 5 \times 10^5$ organisms/g while it was reached to $9 \times 10^8 \pm 10^7$ organisms/g at 5th day. Aerobic plate count was significantly increased at ($p<0.05$) at the 5th day, constituting $9 \times 10^8 \pm 10^7$ organisms/g. Concerning psychrotropic count, it was $2 \times 10^4 \pm 2 \times 10^3$ organisms/g as well as it was reached to $9 \times 10^5 \pm 2 \times 10^5$ organisms/g at 5th day. There is a significant increase in psychrotrophic count at 5th day ($9 \times 10^5 \pm 2 \times 10^5$ organisms/g.).
- Most probable number of coliforms was $6.7 \times 10 \pm 1.7 \times 10$ organisms/g at the first day while it was reached to $10^3 \pm 4 \times 10^2$ organisms/g at the 5th day. It was significant at ($P < 0.05$) at 4th and 5th days, each constituting, $8 \times 10^2 \pm 2 \times 10$ and $10^3 \pm 4 \times 10^2$ organisms/g, respectively. Dealing with most probable number of fecal coliforms, it was $2.8 \times 10 + 0.7 \times 10$ organisms/g at 1st day while it was reached to $10^3 \pm 3 \times 10^2$ organisms/g at 5th day. A significant increase in fecal coliforms (MPN) at 5th day, constituting $10^3 \pm 3 \times 10^2$ organisms/g. *Staphylococcus aureus* count was $5 \times 10^2 \pm 2 \times 10^2$ organisms/g at 1st day while it was reached to $2 \times 10^4 \pm 4 \times 10^3$ organisms/g at 5th day. There are a significant differences between *Staphylococcus aureus* counts starting from 2nd day till the 5th day, each constituting $2 \times 10^3 \pm 6 \times 10^2$ and $2 \times 10^4 \pm 4 \times 10^3$ organisms/g, respectively.
- The total bacterial counts for microbial species is freshly cut meat surfaces are likely to vary. It may be attributed to these organisms are mainly derived from exterior and the gut of animal but also from knives, other utensils; butchery tables. Therefore, variations in counts often reflect the hygienic conditions under which that meat produce. This agrees with that reported by **Nottingham (1982).** Aerobic storage of meat allowed total aerobic counts to reach high levels. The growth of initial bacterial counts in fresh meat may enhanced by the time of storage due to highly enrichment of meat with nutrient elements required for multiplication of microorganisms. The shelf-life of the meat will depend upon the rate of spoilage. Spoilage microorganisms may represent only a very small part of the initial flora they will consistently become predominant in raw meat under storage conditions **(Forsythe and Hayes, 1998 & Skandmis and Nychas, 2002).** In this respect, **Ingram (1971)** stated that some 10^8 bacterial cells per gram may be necessary to induce measurable spoilage in food over a number of days of storage. On the other hand, **Gardner (1965)** stated that sliced meats hold at 15 or 10ºC develop off-odors after to five days storage and surface slime is evident at about seven days.
- The present data in table (2) fig.(2), it is revealed that the aerobic plate count of meat at 1st day was $6 \times 10^4 \pm 2 \times 104$ organism/g. Such count was gradually increased during storage at chilling (5ºC) to reach $4 \times 10^8 \pm 10^7$ organisms/g at 11th day. Psychrotrophic count of meat/gm at 1st day of chilled storage was $2 \times 10^2 \pm 5.7 \times 10$ as well as it was highly increased to reach $8 \times 106 + 106$ organisms/g after 11th day chilled storage. There is a significant differences at ($P<0.05$) in counts of each of aerobic plate and psychrotrophic at 11th day of storage. Most probable numbers of each of coliforms and fecal coliforms were $2.1 \times 10 \pm 0.7 \times 10$ and $0.4 \times 10 \pm 0.1 \times 10$ organisms/g, respectively at the 1st day. After 11th day of chilled storage, such counts were reached to $2 \times 10^2 \pm 10^2$ and $5.3 \times 10 \pm 2 \times 10$ organisms/g; respectively. No significant variations in both most probable numbers of each of coliforms and fecal coliforms during chilled storage at $P<0.05$.
- The Staphulococcus aureus count was $10^2 \pm 3 \times 10$ organisms/g at 1st day of chilled storage while it was reached to $3 \times 10^4 \pm 2 \times 10^4$ organisms/gm after 11th day storage. There is a significant differences between the Staphylococcus counts during chilled storage at $P< 0.05$.

The obtained results were in accordance with that achieved by **Ayres (1960) and Forsythe and Hayes (1998).**

- The general viable count should be less than 10^7 organisms/g in chilled meat **(ICMSF, 1986).**
- The bacterial growth is usually inhibited at chilling room temperature, the meat continues to lose water by evaporation, and the air, becoming humid, creates a condition which is suitable for the growth of mould. This held the view reported by **Gracey and Collins (1992)** and **(Patterson and Gibbs, 1978).**
- The gradual variations in microbial counts during chill storage may be attributed to the storage in chilled temperatures at 5ºC or below a definite lag phase is apparent. The length of this phase depends on storage temperature and extends for 24 hours at 5º C before the onset of the first signs of spoilage is extended and off-odor and slime production take 8 and 12 days, respectively, to develop at 5ºC and 16ºC. This substitutes the hypothesis mentioned by **Forsythe and Hayes (1998).**
- On contrary, **Gould (1995)** stated that, in chill-stored proteinaceous foods such as meat, this generally results in the inhibition of Gram-negative e.g. Enterobacteriaceae whilst the Gram-positive

bacteria become the dominant organisms. On the other hand, **Farber (1991)** stated that the oxygen stimulate the growth of aerobic bacteria and can inhibit the growth of strictly anaerobic bacteria, although there is a very wide variation in the bacterial counts according to sensitivity to oxygen.

- From table (3) fig. (2), it was achieved that the aerobic plate count of aerobically packed meat at 1st day was $5 \times 10^3 \pm 8 \times 10^2$ organisms/g. It was reached to $3 \times 10^6 \pm 10^6$ organisms/g at7th day. Finally, it became $8 \times 10^7 \pm 5 \times 10^6$ organisms/g at the end of the experiment (11th day). Dealing with psychrotrophic count in aerobic packed meat, it was $2 \times 10^2 \pm 8.8 \times 10$.organisms/g at first day of storage. At the end of the experiment, it was reached $4 \times 10^5 \pm 10^5$ organisms/g at 11th day. There are significant variations in either of aerobic plate count and psychrotrophic count at 11th day of storage of aerobically packed meat at $P< 0.05$.
- Most Probable number of coliforms and fecal coliforms of aerobically packed meat were $4 \times 10 \pm 10$ and $0.3 \times 10 \pm 0.1 \times 10$.organisms/g, respectively at the 1st day of storage as well as they were reached to $10^3 \pm 3 \times 10^2$ organisms/g at 11th day of storage. A significant variation was observed between the Most Probable number of both coliforms and fecal coliforms during storage at ($P <0.05$). Concerning *Staphylococcus aureus*, it was $10^2 \pm 8.8 \times 10$ organisms/g, it was gradually increased; reaching $8 \times 10^4 \pm 3 \times 10^2$ organisms /g at the end of the experiment (11th day). There is a significant differences in count stating from 9th and 11th day of storage at $P <0.05$.The growth of microorganisms on vacuum-packed fresh meats may be attributed to initial bacterial contamination. Subsequent growth is slow so that by the time the final total count of 10^7 per gram will reached. The gradual changes in the spoilage flora are observed. This held with that reported by **Egan and Roberts (1987).**
- Packing of meat may be an effective method for meat shelf-life extension. The bacterial counts including the spoilage-related microbial groups had changes depending on the packing condition. When the beef was packed in air, all microbial groups showed viable counts higher than those of the other packing conditions. This in-agreement with that reported by **Skandamis and Nychas (2005); Ercolini et al. (2006) and Koutsoumanis et al. (2006).** Microbial spoilage on aerobically packed meats can be detected as off odor when surface counts reach 10^7 organisms/gm **(Jay, 1986).**
- From the present data reported here in (table 4) and fig.(2), it is evident that the aerobic plate count and psychrotrophic count of anaerobically packed meat (vacuum packed) at 1st day of storage were $10^3 \pm 4 \times 10^2$ and $10^2 \pm 25 \times 10$ organisms/g, respectively. Such counts reach $7 \times 10^7 \pm 2 \times 10^7$ and $10^6 \pm 8 \times 10^5$ organisms/g after 11th day of storage. There is a significant variations between aerobic plate counts during storage period at $P<0.05$ while this variation was significantly only on 11th day storage in psychotropic count.
- Either of Most Probable number of coliforms and fecal coliforms of anaerobic packed meat at 1st day were $0.32 \times 10 \pm 0.2 \times 10$ and $0.3 \times 10 \pm 0.1 \times 10$ orgamisms/g, respectively while it reached to $4 \times 10 \pm 3 \times 10$ and $4 \times 10^2 \pm 3 \times 10^2$ organisms/g, respectively; at the end of the experiment (at 11th day). There is only significant variation in counts during storage at 11th day in both of coliforms and fecal coliforms at $P< 0.05$.
- Concerning Staphylococcus aureus, the count was $10^2 \pm 4 \times 10$ organisms/g as well as it was not change till the 5th day. It reached to $3 \times 10^4 \pm 2 \times 10^4$ organisms/g at 11th day of storage. A significant variations ($P <0.05$) was observed in the day and continued till the end of experiment (11th day).
- The change of spoilage-related microbial flora during storage of beef under different packing condition. The large variation of gas composition during packing due to microbiological growth, which, in the contrary, is inhibited by using anaerobic condition (under vacuum). This was confirmed by suggestion reported by **Kennedy et al. (2004).**
- Vacuum packages prevent the growth of high spoilage potential aerobic microorganisms. Reaching potential spoilage numbers under anaerobic storage conditions does not necessarily coincide with the onset of spoilage. On contrary, **Sadler and Swan (1997)** stated that the storage life was shorter in vacuum-packing because a small amount of oxygen can enter the pack, allowing more rapid bacterial growth, and because there is no inhibitory carbon dioxide atmosphere.
- Vacuum packing of fresh meats provides sufficient shelf-life of primal cuts for long-term storage and intercontinental transport. Vacuum package beef held in films with oxygen permeability had a storage life of 11 weeks at 0ºC. The extension of the shelf- life of vacuum packed meat as compared with aerobic packed may be attributed to change of microflora from aerobic to anaerobic organisms in the vacuum packaged meat. This substitutes the hypothesis reported by **Pierson et al. (1970); Seideman et al. (1976) & Lee and Yoon (2001).**
- On the present data, it could be concluded that, the anaerobically packing of retail meat in markets was the preferred method for extension of shelf

life of meat as compared with aerobic packing. The suggestive measures showed that the vacuum pack of fresh meat provides sufficient shelf life of cuts at 1-5ºC for long term storage then aerobic pack of chilled beef which prefer to butchers. Finally cold storage under different packing condition for freshness of meat would benefit both consumers and meat industry**.**

- Application of HACCP (Hazard Analysis Critical Control Points) system in retail meat production and industries.

SUMMARY

This experiment was carried to assessment the effect of packing (aerobically and anaerobically) on chilled meat as compared with fresh ones. Aerobic plate count was significantly increased at $p<0.05$ at the 5th day, constituting $9 \times 10^8 \pm 10^7$ organisms/g. There is a significant increase in psychrotrophic count at 5th day $9 \times 10^5 \pm 2 \times 10^5$ organisms/g. Most probable number of coliforms was significant at $P < 0.05$ at 4th and 5th days, each constituting $8 \times 10^2 \pm 2 \times 10$ and $10^3 \pm 4 \times 10^2$ organisms/g, respectively. A significant increase in fecal coliform (MPN) at 5th day, constituting $10^3 \pm 3 \times 10^2$ organisms/g. Staphylococcus aureus count starting from 2nd day till the 5th day, each constituting $2 \times 10^3 \pm 6 \times 10^2$ and $2 \times 10^4 \pm 4 \times 10^3$ organisms/g, respectively.There is a significant differences at $P<0.05$ in count of each of aerobic plate and psychrotrophic at 11th day of storage. No significant variation in both most probable numbers of coliforms and fecal coliform during chilled storage at $P<0.05$.There is a significant differences between the Staphylococcus counts during chilled storage at $P< 0.05$. There are significant variations in either of aerobic plate count and psychrotrophic count at 11th day of storage of aerobically packed meat at $P< 0.05$. A significant variation was observed between the Most Probable number of both colifrm and fecal coliforms during storage at $P <0.05$. There is a significant difference in Staphylococcus *aureus* count stating from 9th and 11th day of storage at $P <0.05$. Significant variations between aerobic plate counts during storage period at $P <0.05$ while this variation was significantly only on 11th day storage in psychotropic count. There is only significant variation in count during storage at 11th day in both of coliforms and fecal coliforms at $P< 0.05$. A significant variation ($P <0.05$) was observed in Staphylococcus *aureus* between the days and continued till the end of experiment (11th day). Suggestive for measure extension shelf-life time of marketed retail meat in butcher's shops was discussed.

Reference

AOAC (2000):
Official Methods of Analysis.
17th ed. Gaithersburg, Maryland, USA, AOAC International.

Ashton, L.V.; Georgnaras, I.; Stopforth, J.D.; Skandamis, P.N.;Belk, K.E.; Scanga, J.A.; Smith, G.C. and Sofos, J.N. (2006):
Fate of inoculated Escherichia coli O157:H7, cultured under different conditions, on fresh and decontaminated beef transitioned from vacuum to aerobic packaging**.** J Food Prot., 69 :1273-1279.

Ayres, J.C, (1960):
The relationship of organisms of the genus Pseudomonas to the spoilage of meat, poultry and eggs. J. Appl. Bacteriol.,23 :471.

Byun, J.S; Min, J.S.; Kim, I.S; Kim, J.W.; Chung, M.S. and Lee, M. (2003):
Comparison of indicators of microbial quality of meat during aerobic cold storage.
J. Food Prot., 66: 1733: 1737.

Cliver, D.O. and Riemann, H.P (2002):
Foodborne diseases.
2nd edition. San Diego, Academic Press. London.

Egan, A.F. and Roberts T.A. (1987):
Microbiology of meat and meat products.
In: Essays in Agriculture and Food Microbiology. (Norris J.R. and Pettipher G.L.,Eds), pp &67-197, John Wiley and Sons, Chichester.

E.O.S.Q.C. (2001):
Egyptian standards for requirement of chilled beef, No., 3602.

E.O.S.Q.C. (2004):
Egyptian standards for requirement of fresh meat, No., 4334.

Ercolini, D.; Russo, F.; Torrieri, E.; Masi, P. and Villani, F. (2006):
Changes in the spoilage-related microbiota of beef during refrigerated storage under different packaging conditions.
Appl. Environ. Microbiol., 7:4663-46671.

Farber, J.M. (1991):
Microbialogical aspects of modified-atmosphere packaging technology - a review.
J. Food Prot., 54: 58-70.

Forsythe, S.J. and Hayes, P.R. (1998):
Food Hygiene, Microbiology and HACCP. Aspen Publishers, Inc. GB.

Gardner, G. A. (1965):
Changes caused by microbes in spoilage of meats.
Ph.D., Thesis, Queen's University of Belfast. Cited after Ingram, M. and Dainty, R. H. (1971). J. Appl. Bact., 34: 21.

Gill, C.O., Jones, T., Rahn, K.; Campbell, S.; LeBlanc, D.1.; HoIley, R.A. and Siark, R. (2002):
Temperatures and ages of boxed beef packed and distributed in Canada. Meat Science, 60 (in press).

Gould, G.W. (1995):
New methods of food preservation.
Blacki Academic and Professional, London, New York, Tokyo.

Gracey, J.F. and Collins D.S. (1992):
Meat Hygiene, 9th Edition. Bailliere Tindall, London.

Grau, F.H. and Vanderlinde, P.B. (1992):
Occurrence, numbers and growth of *Listeria monocytogenes* on some vacuum packaged processed meats. J. Food Prot., 55: 4.

Ingram, M. (1971):
Microbial changes in foods- general consideration. J. Appl. Bacteriol., 1-8.

International Commission on Microbiological Specifications for Foods, ICMSF (1986):
Microorganisms in Foods5.Characteristics of Microbial Pathogens. Blackie Academic & Professionals.

Jay, J.M. (1986):
Modern Food Microbiology.
Van Moster and Reinhold company. New york. 3rd ed.: 422-422.

Kennedy, C.; Buckley, D. J. and Kerry, J. P. (2004):
Display life of sheep meat retail packaged under atmospheres of various volumes and compositions. Meat Sci., 68:649-658.

Koutsoumanis, K.; Angelidis, A. S and Nychas., G. J. E. (2006):
A probabilistic modelling approach for evaluating the compliance of RTE foods with the new safety criteria for *Listeria monocytogenes*, p. 572. In Abstr. FoodMicro 2006, Food Safety Food Biotechnol. Diversity Global Impact. Bologna, Italy

Lee, K.T and Yoon C.S. (2001):
Quality changes and shelf life of imported vacuum-packaged beef chuck during storage at 0°C. Meat Sci, 59: 71-77.

Moje, M. (1999):
Chilled storage of packed fresh meat.
Fleischwirtschaft, 79: 84 – 87.

Mulder, S. J. and krol, B. (1976):
A study on the bacteriological aspect of fresh beef. I Effect of storage of slaughter cattle. A. transe Tijdschrift voor diergeneeskunde, 101:594 - 598.

Nottingham, P. M. (1982):
Microbiology of carcass meats.
In Meat Microbiology, edited by M. H. Brown. Applied Science Publishers Ltd. London & New York

Park, C. E., Akhtar, M. and Rayman, M. K. (1994):
Evaluation of commercial enzyme immunoassay kit (RIDASCREEN) for detection of staphylococcal enterototoxins A, B, C, D, and E in foods.
Appl. Environ. Microbiol., 60: 677.

Patterson, J. T. and Gibbs, P. A. (1978):
Sources and properties of some organisms isolated in two abattoirs. Meat Sci., 2:263-273.

Pierson, M. D.; Collins-Thompson, D. L and. Ordal, Z. J. (1970):
Microbiological sensory and pigment changes of aerobically and anaerobically packaged beef.
Food Techol., 24: 1 17 1.

Plaatjies, Z.; Lues1, J.F.R.; Buys, E.M. and P. Venter, P. (2004):
Staphylococcal growth in fresh vacuum-packed red meat at various storage conditions.
8th World Congress on Environmental Health; Document Transformation Technologies. Organized by SB conferences. University of Pretoria.

Sadler, D.N. and Swan, J.E. (1997):
Chilled storage life of hot boned, pre-rigor, salted minced beef. Meat Sci., 45:427-437.

Seideman , S. C.; Vanderzant, C.; Hanna, M.O.; Carpenter, Z.L. and Smith, G.C. (1976):
The effect of various type of vaccum packages and lenghth of storage on the microbial flora of wholesale and retail cuts of beef.
J. Milk Food Tecnol., 39:745- 753.

Shelef, L.A.; Mohammed, S.; Tan, W. and Webber, M.L. (1997): Rapid optical measurements of microbial contamination in raw ground beef and effect of citrate and lactate. J. Food Prot., 60: 673.

Skandamis, P.N. and Nychas, G.J. E. (2002):
Preservation of fresh meat with active and modified atmosphere packaging conditions
Int. J. Food Microbiol., 79:35-46.

Skandamis, P.N. and Nychas, G.J. E. (2005):
Fresh meat spoilage and modified atmosphere packaging (MAP), p. 461-493. In Raw material safety: meat. Woodhead Publishers, Cambridge, United Kingdom

الملخص العربي

أجريت هذه الدراسة لمعرفة مدى تأثير والتغليف (هوائى ولا هوائى) على المبردة مقارنة باللحوم الطازجة وذلك فى 4 تجارب:

1) **التجربة الأولى** : حفظ عينات اللحوم الطازجة فى درجة حرارة الحجرة 25–30 ^{5}م التى تمثل محل الجزارة حتى الفساد وكانت من يوم الذبح حتى اليوم الخامس والفحص الميكروبيولوجي لهذه العينات.
2) **التجربة الثانية** : حفظ عينات اللحوم الطازجة والغير معبأة فى درجة حرارة 1–5 ^{5}م حتى الفساد فيحدد الزمن والحالة الميكروبيولوجية لصلاحية اللحوم فى التبريد بدون تغليف حتى اليوم الحادي عشر.
3) **التجربة الثالثة** : حفظ عينات اللحوم المعبأة هوائياً فى درجة حرارة 1–5 ^{5}م حتى الفساد فى اليوم الحادي عشر.
4) **التجربة الرابعة** : حفظ عينات اللحوم المعبأة لاهوائياً فى درجة حرارة 1–5 ^{5}م حتى اليوم الحادي عشر الذى حدث به الفساد وتم الفحص الميكروبيولوجي ومنها ومنها الوصول إلى طريقة الحفظ الأمثل وتحديد درجة الحرارة المثالية لحفظ جودة اللحوم لأطول فترة ممكنة.

وأظهرت النتائج الآتية :

التجربة الأولى : متوسط العد الميكروبات الهوائية فى اليوم الأول كان العد 8×10 ^{5} ± 8× 10 ^{5} وزادت 3× 10 ^{7} ± 10 ^{7} ووصلت إلى الفساد فى اليوم الخامس وكانت 9×10 ^{8} ±10 ^{7} ميكروب/جرام ومتوسط الميكروبات المحبة للبرودة بدأت فى الزيادة من اليوم الثاني حتى اليوم الخامس ووصلت والميكروب القولوني زاد فى اليوم من اليوم الثاني إلى اليوم الخامس 10 ^{3} ± 3×10 ^{2} ميكروب/جرام.

والميكروب المكور الذهبي بدأت فى الزيادة من اليوم الثاني حتى الفساد فى اليوم الخامس من 5×10 ^{2}± 2×10 ^{2} و 2×10 ^{4} ± 4×10 ^{3} ميكروب/جرام.

وهذه الطريقة للحفظ غير مجدية حيث أنها تفسد اللحوم بسرعة ويرجع هذا إلى تلوث الأسطح الملامسة للحوم.

التجربة الثانية : متوسط العد الميكروبات الهوائية للحوم المبردة الغير مغلفة بدأت فى الزيادة والميل إلى الفساد من اليوم السابع وبلغت أقصاه فى اليوم الحادي عشر 4×10 ^{8} ± 10 ^{7}ميكروب/جرام. الميكروبات المحبة للبرودة بدأت فى الزيادة من اليوم التاسع حتى اليوم الحادي عشر 8×10 ^{6} ± 10 ^{6}. الميكروب القولوني يبدأ يتزايد من اليوم السابع حتى أقصاه فى اليوم الحادي عشر. الميكروب العنقود الذهبي بدأ فى الزيادة تدريجياً من اليوم الحادي عشر 4 × 10 ^{4} ± 2 × 10 ^{4}.

التجربة الثالثة : اللحوم المبردة المعبأة هوائياً قد يتضح أن متوسط العد الكلي للميكروبات الهوائية تبدأ فى التزايد التدريجي فى اليوم السابع حتى اليوم التاسع ويرتفع أقصاه فى اليوم الحادي عشر 5×10 ^{3} ± 8× 10 ^{2} ، 3×10 ^{6} ± 10 ^{6} ، 8× 10 ^{7} ± 5× 10 ^{6}ميكروب/جرام على التوالي.

والميكروبات المحبة للبرودة أيضا تبدأ الزيادة من اليوم السابع تدريجيا حتى اليوم التاسع يبلغ أقصاه عن النسبة المقررة فى المواصفات القياسية المصرية وكذلك الميكروب المكور العنقود الذهبي وجدت أنها تبدأ فى الزيادة من اليوم التاسع وتصل إلى الفساد فى اليوم الحادي عشر 8×10 ^{4} ± 3×10 ^{2} ميكروب/جرام. الميكروبات القولونية فقد وجدت أنها تبدأ من اليوم السابع فى الزيادة حتى اليوم التاسع ثم فى اليوم الحادي عشر.

التجربة الرابعة : اللحوم المبردة المعبأة لاهوائيا وجد أن المتوسط العد الكلي للميكروبات الهوائية والمحبة للبرودة تبدأ فى الزيادة تدريجياً حتى اليوم الحادي عشر 7×10 ^{7}± 2×10 ^{7} 10 ^{6} ± 8× 10 ^{5} ميكروب/جرام على التوالي. والميكروبات القولونية وجدت أنها تبدأ فى الزيادة فى اليوم التاسع إلى اليوم الحادي عشر حيث الفساد. وكان متوسط المكور العنقود الذهبي يبدأ فى الزيادة اليوم السابع ويصل إلى 4× 10 ^{4} اليوم الحادي عشر.

ومن هذه النتائج : اتضح ان حفظ اللحوم المبردة المعبأة لاهوائيا تحفظ اللحوم مدة 7–9أيام عند درجة حرارة 1–5م وتساعد على زيادة مدة الصلاحية لها. ثم يليها حفظ اللحوم المبردة المعبأة هوائياً فى زيادة مدة الصلاحية لحفظ اللحوم.

1. ناقشت الباحثة الأهمية الاقتصادية للميكروبات المعزولة وكذلك الأهمية الصحية لها والاقتراحات المناسبة اللازمة لحفظ اللحوم لأطول فترة ممكنة.

2010/1/11

Serum Level of Cartilage Oligomeric Matrix Protein as a Screening Modality for Osteoarthritis among Knee Joint Pain Patients

Ahmed M. Awadallah [*1], Gehan H.Sabry[1] and Tarek M.Khater[2]

[1]Departments of Clinical Pathology Faculty of Medicine, Benha University, Benha, Egypt
[2] Orthopedic Surgery Faculty of Medicine, Zagazig University, Zagazig, Egypt
[*]a_mamdouh8@hotmail.com

Abstract: Objectives: This study aimed to evaluate the diagnostic yield of estimation of serum cartilage oligomeric matrix protein (COMP) as a screening tool for osteoarthritis (OA) among patients with knee joint pain.
Patients & Methods: The study included 140 female patients with knee pain and 20 volunteers to donate blood as a control group for laboratory findings. All patients underwent full history taking, clinical examination for evaluation of pain severity using a visual analogue scale (VAS) and extent of patient mobility using mobility score (MS) and had knee anteroposterior radiographs that were scored using the Kellgren-Lawrence scoring (K-L score) system. Patients were classified according to K-L scores into: group A: pain plus no radiographic findings (K-L score=1), group B: pain plus doubtful or minimal radiographic findings (K-L score=1) and group C: pain plus radiographically determined OA (K-L score≥2). Venous blood samples were obtained from all patients and controls for erythrocyte sedimentation rate (ESR) determination and ELISA estimation of serum COMP and high-sensitivity C-reactive protein (hsCRP) levels. Results: Group C patients had significantly higher pain scores and lower MS compared to groups A and B. Mean patients' serum COMP levels was significantly higher compared to control levels and in group C compared both to controls and to groups B and A levels with significantly higher levels in group B compared to controls and group A. However, serum COMP levels were non-significantly higher levels in group A compared to control levels. There was a positive significant correlation between serum COMP levels and body mass index (BMI), pain VAS score and radiological grade and a negative significant correlation with MS. ROC curve analysis revealed that elevated serum COMP is a sensitive predictor and high BMI is a specific predictor for the presence of OA. Serum COMP at 1097.5 ng/ml was the best cutoff point with high sensitivity (87.7%), positive predictive value (PPV, 92.6%) and accuracy (84.3%) for differentiation between patient with and without OA radiological manifestations and serum COMP at 1290 ng/ml showed 100% specificity and PPV and accuracy rate of 65.7% for diagnosis of the presence of radiological findings of OA. Conclusion: Estimation of serum COMP level could be considered as screening modality for patients with knee pain and using cutoff point of 1097.5 ng/ml helps to define patients free of OA and cutoff of 1290 ng/ml could define patients with OA.
[Ahmed M. Awadallah, Gehan H. Sabry and Tarek M. Khater. **Serum Level of Cartilage Oligomeric Matrix Protein as a Screening Modality for Osteoarthritis among Knee Joint Pain Patients.** Journal of American Science 2010;6(12):1059-1066]. (ISSN: 1545-1003). http://www.americanscience.org.

1. Introduction:

Arthritis is a chronic disease with a significant impact on the population. It damages the cartilage, synovium, and bone of the joints causing pain, impairment, and disability in patients. Current methods for diagnosis and monitoring the disease are only able to detect clinical manifestations of arthritis late in the process. However, with the recent onset of successful treatments for rheumatoid arthritis and osteoarthritis, it becomes important to identify prognostic factors that can predict the evolution of arthritis. This is especially critical in the early phases of disease so that these treatments can be started as soon as possible to slow down progression of the disease, [(1)].

A valuable approach to monitor arthritis would be by measuring biological markers of cartilage degradation and repair to reflect variations in joint remodeling. One such potential biological marker of arthritis is cartilage oligomeric matrix protein (COMP). In various studies, COMP has shown promise as a diagnostic and prognostic indicator and as a marker of the disease severity and the effect of treatment. This review highlights the progress in the utilization of COMP as a biomarker of arthritis, [(2)].

Articular cartilage is a multiphasic material with at least 2 major phases: a fluid phase composed of water and electrolytes, and a solid phase composed of chondrocytes located in lacunae together with matrix molecules that include collagen and

proteoglycans. The extracellular matrix of hyaline cartilage contains an elaborated collagen fibrillar network, which imparts its tensile strength and is essential for the mechanical stability and the proper function of the tissue, [3].

As articular cartilage degenerates in OA, so chondrocytes upregulate their biosynthetic activities, including type II collagen, as if to compensate for this damage. Only after secretion, as the molecules reach the extracellular space, the non-helical domains at the end; the procollagen type II amino-terminal propeptides and procollagen type II carboxy-terminal propeptides, are cleaved from the helical domain. The c-propeptide content and release from the cartilage is directly correlated with collagen synthesis, [4].

Cartilage oligomeric matrix protein is a member of the thrombospondin family of extracellular matrix proteins, [5]. COMP consists of five 87-kDa subunits held together by interchain disulfide bonds forming a 435-kDa pentameric protein. COMP is expressed in all types of cartilage, [6]. Immunohistological staining of articular cartilage has revealed a developmentally regulated localization of COMP to the chondrocyte territorial and interterritorial matrix, [7]. COMP binds in a zinc-dependent manner to collagen type I and type II and also to collagen type IX, [8]. COMP contains type 2 (epidermal growth factor-like) and type 3 (calmodulin-like) repeats in their central domains, [9]. It is becoming known that the mechanisms of cartilage matrix destruction such as roles of degradative enzymes and cytokines, so it is important to develop the reliable biomarkers to detect the early stage of cartilage destruction. The biomarker could be useful tool not only to understand the progression of joint destruction in osteoarthritis and rheumatoid arthritis but also to develop new treatment, [10]. Thus, the present study aimed to evaluate the diagnostic yield of estimation of serum COMP as a screening tool for OA among patients with knee joint pain.

2. Patients & Methods

The present study was based as screening study of patients attending the outpatient clinic of Rheumatology with knee joint pain. Only female patients were enrolled in the study to equalize the impact of gender on the serum level of COMP. Patients with history of knee trauma, rheumatoid arthritis, previous surgery at knee joint or previous treatment of osteoarthritis were excluded of the study. All patients underwent full history including duration of symptoms, mode of onset, precipitating and relieving factors and history of medical treatment. Patients' age, weight, height and body mass index (BMI) were determined.

Pain severity was assessed using a visual analogue scale (a 10 mm-scale, with "0" indicating no pain and "10" indicating worst pain ever), [11]. Local clinical examination was conducted for evaluation of the presence of swelling, stiffness, grinding or locking. The mobility score, which provides an estimate of the patient mobility ranging between being able to walk and undertake shopping unaided (score 9), through to being bedridden (score 0) was also estimated, [12].

Radiological examination to obtain anteroposterior radiographs of the knee in a weight-bearing extended position by using a standard radiographic technique and then radiographs were scored using the Kellgren-Lawrence scoring (K-L score) system, [13]: grade 0: normal knee, grade 1: doubtful and minimal OA of the knee, grade 2: mild OA of the knee, grade 3: moderate OA of the knee and grade 4: severe OA of the knee. The anteroposterior views characterize OA of the knee in the medial and lateral femorotibial compartments, with exclusion of the patellofemoral compartment. Patients were classified according to radiological findings into 3 supposed groups: group A: pain plus no radiographically determined OA (K-L score=0), group B: pain plus doubtful or minimal radiographically determined OA (K-L score=1) and group C: pain plus radiographically determined OA (K-L score≥2). Twenty healthy volunteers with no pain plus no radiographically determined OA of the knee and mobility score of 9 and accepted to give blood samples were included as control group for comparisons of laboratory findings.

Laboratory Investigation

All patients and controls gave fasting blood samples (1.6 ml blood for each 0.4 ml sodium citrate) for determination of ESR and another a 5-ml blood sample was collected in plain tube and allowed to clot and centrifuged at 3000 rpm for 10 minutes and serum was collected for ELISA estimation of:

1. Determination of serum hsCRP level using ELISA Kit (Phoenix Pharmaceuticals, Inc.). In the assay, 100 µl of each standard and samples were added into wells, covered and incubated for 2 hours at room temperature and then washed 4 times with wash solution. Then, 100 µl/well of anti-human CRP-HRP detection antibody were added, incubated for 2 hour at room temperature with gentle shaking and wash was repeated. TMB One-Step Substrate Reagent (100 µl/well) was added and wells are incubated for 25 minutes at room temperature. Reaction was terminated with 100 µl/well of stop solution and the degree of enzymatic turnover of the substrate is determined by dual wavelength absorbance

measurement at 450 nm immediately, [14].

2. Determination of serum COMP level using ELISA Kit (AnaMar Medical, Lund, Sweden). On the 1st day afternoon, serum sample was diluted 1/50 with dilution buffer and 75 µl of each standard and diluted samples were added into the pre-incubation plate (round bottom plate). Anti-COMP reagent (75 µl/well) was added, mixed on a shaker for 5 min and incubated overnight for about 15 hours at 4°C. On the 2nd day, 100 µl were transferred from the pre-incubation plate to the antigen coated plate and incubated at room temperature for 60 minutes. After wash for 3 times with wash solution, wells are emptied by trapping the strip on an absorbent tissue. Then, 100 µl/well of conjugate are added and wells are incubated for 60 minutes. After wash and drying, substrate reagent (100 µl/well) was added and wells are incubated for 50 minutes at room temperature. The degree of enzymatic turnover of the substrate is determined by dual wavelength absorbance measurement at 450 nm immediately, [15].

Statistical analysis

The obtained results were compared using Wilcoxon Rank test (Z-test) for unrelated data. The Receiver Operating Characteristic (ROC) curve was used to evaluate the predictability of serum COMP levels for the presence of OA and results were assured using the Regression multivariate analysis (Stepwise Method) to verify the diagnostic yield of combined measurements. The validity of multiple cutoff points to identify the valid cutoff points was assessed using the test validity characters including sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy for various cutoff points. Statistical analysis was conducted using SPSS statistical program, (Version 10, 2002). P value <0.05 was considered statistically significant.

3. Results

The study included 140 female patients fulfilled the inclusion criteria. Radiological findings identified 26 patients with K-L score=0 (Group A), 52 patients had K-L score of 1 (Group B), 32 patients had K-L score of 2, 18 patients had K-L score of 3 and 12 patients had K-L score of 4; thus, group C included 62 patients. Patients included in groups B and C were significantly obese with significantly higher body weight and BMI compared both to controls and to patients included in group A with non-significantly higher body weight and BMI in group A compared to controls and in group C compared to group B, (Table 1). Patients included in group C had significantly higher pain scores and significantly lower mobility scores compared to groups A and B, with non-significant difference in favor of group A, (Table 2, Fig. 1).

All patients showed significantly higher ESR levels and serum hsCRP compared to controls with significantly higher levels in group C compared to both group A and B and a significantly higher levels in group B compared to group A, (Table 3, Fig. 2).

Mean serum COMP levels estimated in patients (1331.6±264.2; range: 890-1940 ng/ml) was significantly higher compared to levels estimated in control group. Moreover, mean COMP serum levels estimated in patients of group C were significantly higher compared both to control group and to groups B and A levels. Furthermore, mean COMP serum levels estimated in patients of group B were significantly higher compared to control group and group A levels with non-significantly higher levels in group A compared to control group, (Table 4, Fig. 3).

There was a positive significant correlation between serum COMP levels and BMI, ($r=0.246$, $p=0.040$), pain VAS score, ($r=0.521$, $p<0.001$) and radiological grade, ($r=0.778$, $p<0.001$) and a negative significant correlation ($r=-0.397$, $p=0.001$) with mobility score, (Table 5).

Evaluating the diagnostic predictability of serum COMP for the presence of OA, as judged by radiological finding, in patients with knee joint pain using ROC curve analysis versus clinical data revealed that elevated serum COMP is a sensitive and high BMI as specific predictors for the presence of OA with AUC=0.065 and 0.856, respectively, both AUC are significant ($p<0.001$) versus the null hypothesis. Knee joint mobility score was found to be specific predictor but with AUC=0.272 which despite being significant ($p=0.011$) versus the null hypothesis was less significant compared to BMI, (Fig. 4).

Verification of the obtained results for a cutoff point of serum COMP for identification of patients with OA among those who had knee joint pain, irrespective of the clinical data, defined serum COMP at 1097.5 ng/ml as the best cutoff point with high sensitivity (87.7%), positive predictive value (92.6%) and accuracy (84.3%) for differentiation between patient with and without radiological manifestations of OA. On contrary, serum COMP at 1290 ng/ml showed 100% specificity and positive predictive value and accuracy rate of 65.7% for diagnosis of the presence of radiological findings of OA, (Table 6).

Table (1): Patients' demographic data

	Control	Patients			
		Group A	Group B	Group C	Total
Number	20	26	52	62	140
Age (years)	42.5±5.4 (32-49)	44.3±4.1 (38-51)	46±4.1 (38-52)	47±5.3 (38-52)	45.4±5.3 (38-52)
Weight (Kg)	83.8±2.7 (79-89)	87±3.7* (80-93)	87.5±3.9* (80-93)	88±3.8* (79-95)	87.6±3.8* (79-95)
Height (cm)	162.6±5.4 (154-171)	165.5±2.8 (156-168)	162.5±3.8 (156-168)	160±4.3 (155-167)	162.9±4.3 (155-168)
BMI (kg/m^2)	31.8±2.3 (27.3-34.9)	31.8±1.6 (29-36.2)	33.1±1.9*† (29.8-36.2)	34.4±1.9*† (30.9-37.8)	33.1±2.9 (29-37.8)

Data are presented as mean±SD and ranges are in parenthesis
*: significant versus control group †: significant versus Group A

Table (2): Mean VAS pain and mobility scores among studied groups

	Group A	Group B	Group C
VAS Pain scores	3.9±1.4 (2-6)	5.1±1.6 (2-8)	6.2±1.3 (4-9)† ‡
Mobility score	7.1±1.7 (4-8)	6.1±0.8 (3-7)	4.9±1.1 (2-7)†‡

Data are presented as mean±SD and ranges are in parenthesis
†: significant versus Group A ‡: significant versus Group B

Table (3): Estimated ESR and serum hsCRP levels of patients categorized according to K-L scoring system compared to control levels

	Control	Group A	Group B	Group C
ESR	9.1±1.7	29.4±6.3*	34.8±8.5*†	43.1±12*†‡
Serum hsCRP (mg/l)	2.6±1.3	16.3±4.7	23.8±10*†	33.5±7.8*†‡

Data are presented as mean±SD and ranges are in parenthesis
*: significant versus control group †: significant versus Group A ‡: significant versus Group B

Table (4): Estimated serum COMP levels of patients categorized according to K-L scoring system compared to control levels

		Control	Group A	Group B	Group C
Mean±SD (ng/ml)		1015±161.6	1045.8±103.3	1250±155.8	1524±238.8
Range (ng/ml)		680-1260	890-1250	940-1520	1060-1940
Statistical analysis	Z		1.893	3.920	3.865
	p_1		>0.05	<0.001	<0.001
	Z			3.182	=3.183
	p_2			=0.001	=0.001
	Z				2.732
	p_3				=0.006

Data are presented as mean±SD and ranges are in parenthesis
p_1: significant versus control group p_2: significant versus group A p_3: significant versus group B

Table (5): Correlation coefficient between serum COMP levels and demographic and clinical data of studied patients

	"r"	p
Age	0.219	>0.05
Weight	0.164	>0.05
Height	0.169	>0.05
BMI	0.246	=0.040
VAS Pain scores	0.521	<0.001
Mobility score	-0.397	=0.001
Radiological K-L score	0.778	<0.001

Table (6): Test validity characters of estimation of serum COMP at 2 cutoff points

Cutoff point (ng/ml) Result	1065	1097.5	1290	1380	1480
True positive	54	50	33	26	18
True negative	6	9	13	13	13
False positive	7	4	0	0	0
False negative	3	7	24	31	39
Sensitivity	94.7%	87.7%	57.9%	45.6%	31.6%
Specificity	46.2%	69.2%	100%	100%	100%
PPV	88.5%	92.6%	100%	100%	100%
NPV	66.7%	56.3%	35.1%	29.5%	25%
Accuracy	85.7%	84.3%	65.7%	55.7%	44.3%

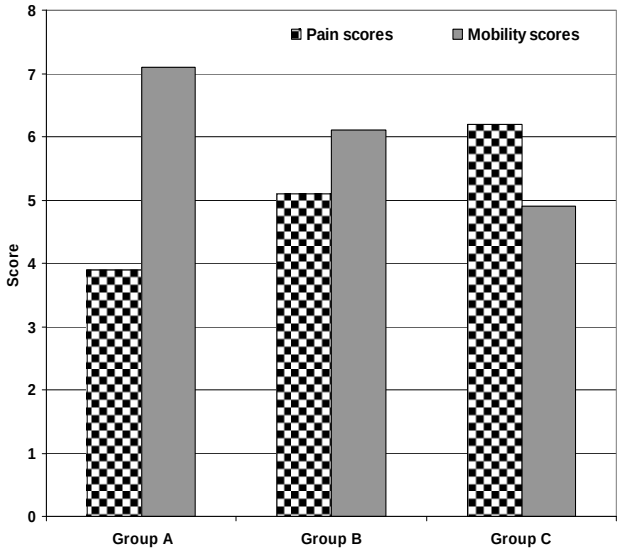


Fig. (1): Mean VAS pain and mobility scores reported in studied patients categorized according to K-L scoring system

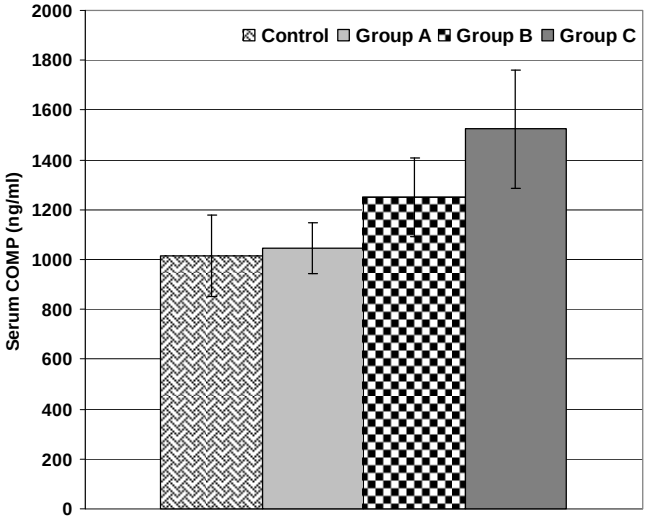


Fig. (3): Mean (±SD) of serum COMP estimated in studied patients categorized according to K-L scoring compared to control levels

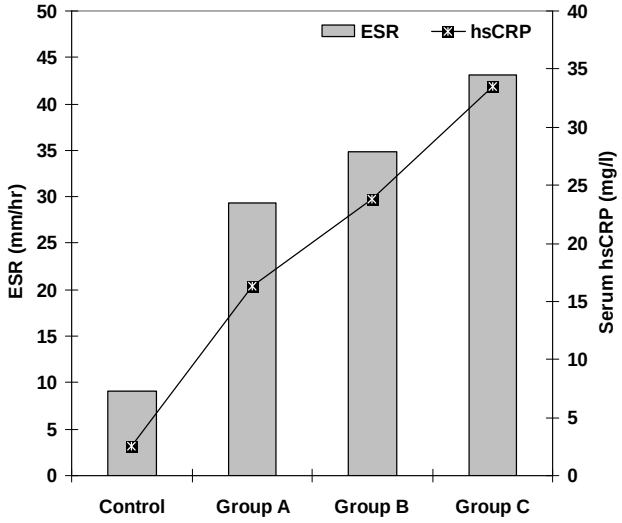


Fig. (2): Mean ESR and hsCRP estimated in studied patients categorized according to K-L scoring compared to control group

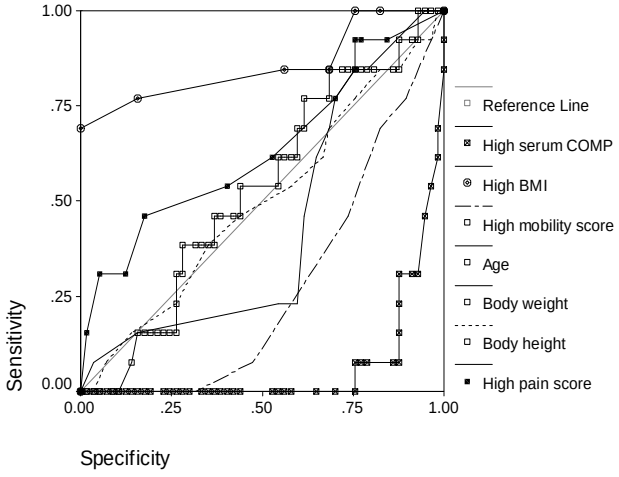


Fig. (4): ROC curve analysis for serum COMP versus demographic and clinical data for the prediction of the presence of OA in patients with knee joint pain

4. Discussion:

The detection of COMP in serum of studied population including patients and control indicated a fact that COMP, as a cartilage turnover marker is normally present in serum indicating continuous renewal of joint cartilage. This finding agreed with *Neidhart et al.,* [16] who found elevated serum COMP in seven of eight runners and during the run, the serum levels rose significantly, and gradually returned to baseline within 24 h. and attributed that elevated baseline levels of COMP might reflect increased joint matrix turnover due to prior extreme physical training, the reported significant increase during the run was possibly due to the severe physical strain on joint structures and concluded that serum COMP is a marker for distinct aspects of joint metabolism and/or damage in both disease and sport. Also, *Andersson et al.,* [17] reported that during normal daytime activities, serum COMP levels are constant and significantly decreased during the night indicating a rapid elimination of COMP once it has reached the circulation. Moreover, *Gordon et al.,* [18] tried to understand sources of variation in biomarkers for OA through evaluation of variation due to activity and food consumption and found all serum biomarkers increased after 1 h of non-exertional activity, but food consumption following activity was associated with a return of biomarker concentrations to baseline levels and demonstrated a positive association between the mean level of activity and serum COMP concentration.

Mean serum COMP levels estimated in patients was significantly ($P<0.05$) higher compared to levels estimated in control group and in patients with radiological evidence of OA compared both to control levels and to levels estimated in patients without radiological evidence of OA that were non-significantly higher compared to control levels. Moreover, there was a positive significant correlation between serum COMP levels and OA radiological severity and elevated serum COMP was found to be significant sensitive markers for differentiation between patients had or free of OA among patients had knee joint.

These results go in hand with the previously reported in literature concerning value of estimation of serum COMP in patients with OA; *Vilím et al.,* [19] found serum COMP positively correlated with knee joint space width both at baseline and after disease progression and knees that had progressed by two K-L grades were shown to have had significantly higher COMP levels at baseline and concluded that serum COMP has the potential to be a prognostic marker of disease progression. *Sharif et al.,* [20] found serum COMP concentration at baseline was significantly higher in the OA progressors compared with the non-progressors and the AUC was significantly higher in the progressors compared with the non-progressors and also that serum COMP concentrations were higher during periods of radiographic progression and identified periods of progression. *Fernandes et al.,* [21] reported that patients with symptomatic knee OA presented significantly higher serum COMP levels compared to healthy controls and to those with non-symptomatic narrowing of the articular space. Patients with clinical evidence of knee OA and without radiological abnormalities (K/L grade 0 or 1) had intermediate serum COMP levels, significantly higher than those observed in healthy controls.

Recently, *Kraus et al.,* [22] reported that serum COMP correlated negatively with total joint space narrowing burden. *Berry et al.,* [23] found COMP was significantly associated with a reduced rate of medial cartilage volume loss.

Regression analysis for estimated serum COMP levels versus clinical and demographic data for differentiation between cases with and without radiological findings defined estimation of serum COMP as significant predictor in two models and BMI in one model and test validity character determination for assigned cutoff points defined serum COMP at 1097.5 ng/ml as the best cutoff point with high sensitivity (87.7%), positive predictive value (92.6%) and accuracy (84.3%) for differentiation between patient with and without radiological manifestations of OA and serum COMP at 1290 ng/ml showed 100% specificity and positive predictive value and accuracy rate of 65.7% for diagnosis of the presence of radiological findings of OA. These finding go in hand with *Hunter et al.,* [24] who performed a logistic regression to examine the relation of levels of cartilage biomarker to the risk of cartilage loss in any knee and reported that with the exception of COMP, none of the other biomarkers was a statistically significant predictor of cartilage loss. *Kraus et al.,* [22] reported that biomarkers and demographics predicted 35-38% of variance in total burden of OA (total joint space narrowing or osteophyte).

The reported association between knee clinical and radiological features of OS, serum COMP and BMI spot line on a possible role for weight burden on the initiation and/or progression of OA and indicated the necessity for weight reduction as a prophylactic and/or therapeutic line for OA patients. Such assumption was supported by the findings of *Richette et al.,* [25] who investigated the effect of massive weight loss on knee pain and disability, low-grade inflammation and metabolic status and joint biomarkers in obese patients with knee OA and found massive weight loss improves pain and function and decreases low-grade

inflammation and concluded that change in levels of joint biomarkers with weight loss suggests a structural effect on cartilage.

In conclusion, estimation of serum COMP level could be considered as screening modality for patients with knee pain and using cutoff point of 1097.5 ng/ml helps to define patients free of OA and cutoff of 1290 ng/ml could define patients with OA. However, wider scale studies are recommended for establishment of cutoff points and defining the benefit of weight reduction as prophylactic and/or therapeutic modality.

Corresponding author
Ahmed M. Awadallah
Departments of Clinical Pathology Faculty of Medicine, Benha University, Benha, Egypt
a_mamdouh8@hotmail.com

5. Reference

1. Morita M, Yamada H, Date H, Yoshimura N: Progress of research in osteoarthritis. The evaluation of osteoarthritis with biological markers. Clin Calcium. 2009; 19(11):1586-91.
2. Tseng S, Reddi AH, Di Cesare PE: Cartilage Oligomeric Matrix Protein (COMP): A Biomarker of Arthritis. Biomark Insights. 2009; 4:33-44.
3. Aszódi A, Hunziker EB, Olsen BR & Fässler R: The role of collagen II and cartilage fibril-associated molecules in skeletal development. Osteoarthritis Cartilage. 2001;9 Suppl A:S150-9.
4. Aigner T, Zhu Y, Chansky HH, Matsen FA, III, Maloney WJ, Sandell LJ: Reexpression of type IIA procollagen by adult articular chondrocytes in osteoarthritic cartilage. Arthritis & Rheumatism 1999; 42:1443-50.
5. Adams JC, Lawler J: The thrombospondin family. Curr. Biol. 1993; 3:188-90.
6. Hedbom E, Antonsson P, Hjerpe A, Aeschlimann D, Paulsson M, Rosa-Pimentel E, Sommarin Y, Wendel M, Oldberg Å, Heinegård d: Cartilage matrix proteins. An acidic oligomeric protein (COMP) detected only in cartilage. J. Biol. Chem. 1992; 267:6132-6.
7. Chen Z, Heinegård D, Sommarin Y: Distribution and expression of cartilage oligomeric matrix protein and bone matrix protein shows marked changes during rat femoral head development. Matrix Biol. 1994; 14:773-81.
8. Rosenberg K, Olsson H, Morgelin M, Heinegard D: COMP shows high affinity Zn-dependent interaction with triple helical collagen. J. Biol. Chem. 1998; 273:20397-403.
9. Holden P, Meadows K, Chapman M, Grant K, Kadler L, Briggs M: Cartilage oligomeric matrix protein interacts with type IX collagen, and disruptions to these interactions identify a pathogenetic mechanism in a bone dysplasia family. J. Biol. Chem. 2001; 276:6046-55.
10. Kojima T: Bone and bone related biochemical examinations. Bone and collagen related metabolites. Biomarkers for cartilage matrix turnover and destruction in joint diseases. Clin Calcium. 2006; 16(6):1009-15.
11. Huskisson EC: Measurement of pain. Lancet 1974; 2: 1127–31.
12. Parker MJ, Palmer CR: A new mty score for predicting mortality after hip fracture. J Bone Joint Surg. 1993; 75B: 797–8.
13. Kellgren JH, Lawrence JS: Epidemiology of chronic rheumatism. Philadelphia, Pa: Davis, 1963.
14. Sursh MV, Singh SK, Fergussen, DA Jr., Agrrawal A. Role of the property of C-reactive protein to activate the classical pathway of complement in protecting mice from pneumococal infection. Journal of Immunology. 2006: 176(7):4.69-74.
15. Saxne T, Heinegard D: Cartilage oligomeric matrix protein: a novel marker of cartilage turnover detectable in synovial fluid and blood. Br J Rheumatol, 1992; 31:583–91
16. Neidhart M, Müller-Ladner U, Frey W, Bosserhoff AK, Colombani PC, Frey-Rindova P, Hummel KM, Gay RE, Häuselmann H, Gay S: Increased serum levels of non-collagenous matrix proteins (cartilage oligomeric matrix protein and melanoma inhibitory activity) in marathon runners. Osteoarthritis Cartilage. 2000; 8(3):222-9.
17. Andersson ML, Petersson IF, Karlsson KE, Jonsson EN, Månsson B, Heinegård D, Saxne T: Diurnal variation in serum levels of cartilage oligomeric matrix protein in patients with knee osteoarthritis or rheumatoid arthritis. Ann Rheum Dis. 2006; 65(11):1490-4.
18. Gordon CD, Stabler TV, Kraus VB: Variation in osteoarthritis biomarkers from activity not food consumption. Clin Chim Acta. 2008; 398(1-2):21-6.
19. Vilím V, Olejárová M, Machácek S, Gatterová J, Kraus VB, Pavelka K: Serum levels of cartilage oligomeric matrix protein (COMP) correlate with radiographic progression of knee osteoarthritis. Osteoarthritis Cartilage 2002; 10(9):707-13.

20. Sharif M, Kirwan JR, Elson CJ, Granell R, Clarke S: Suggestion of nonlinear or phasic progression of knee osteoarthritis based on measurements of serum cartilage oligomeric matrix protein levels over five years. Arthritis Rheum. 2004; 50(8):2479-88.
21. Fernandes FA, Pucinelli ML, da Silva NP, Feldman D: Serum cartilage oligomeric matrix protein (COMP) levels in knee osteoarthritis in a Brazilian population: clinical and radiological correlation. Scand J Rheumatol. 2007; 36:211-5.
22. Kraus VB, Kepler TB, Stabler T, Renner J, Jordan J: First qualification study of serum biomarkers as indicators of total body burden of osteoarthritis. PLoS One. 2010; 5(3):e9739.
23. Berry PA, Maciewicz RA, Wluka AE, Downey-Jones MD, Forbes A, Hellawell CJ, Cicuttini FM: Relationship of serum markers of cartilage metabolism to imaging and clinical outcome measures of knee joint structure. Ann Rheum Dis. 2010; 69(10):1816-22.
24. Hunter DJ, Li J, LaValley M, Bauer DC, Nevitt M, DeGroot J, Poole R, Eyre D, Guermazi A, Gale D, Felson DT: Cartilage markers and their association with cartilage loss on magnetic resonance imaging in knee osteoarthritis: the Boston Osteoarthritis Knee Study. Arthritis Res Ther. 2007; 9(5):R108.
25. Richette P, Poitou C, Garnero P, Vicaut E, Bouillot JL, Lacorte JM, Basdevant A, Clent K, Bardin T, Chevalier X: Benefits of massive weight loss on symptoms, systemic inflammation and cartilage turnover in obese patients with knee osteoarthritis. Ann Rheum Dis. 2010; Epub ahead of print.

11/2/2010

Biochemical Changes in Glutathione Redox System and Glucose Regulation in Late Pregnant Ossimi Ewes

Ali Hafez El-Far[*1], Mohamed K. Mahfouz[2] and Hussein A. Abdel maksoud[2]

[1] Department of Biochemistry, Faculty of Veterinary Medicine, Alexandria University, Damanhour Branch (Al-Bostan), Egypt.
[2]Department of Biochemistry, Faculty of Veterinary medicine, Moshtohor, Banha University, Egypt.
[*]aboufares90@yahoo

Abstract: Pregnancy is the more prevalent stress in under feeding small ruminant with multiple bearing. Fifty Ossimi ewes of two years old and their body weight ranging between 35 and 50 kg were allotted into three groups; Group I: contains ten non pregnant non lactating ewes were used as control group. Group II: contains twenty single pregnant ewes* and Group III: contains twenty twin pregnant ewes used as experimental animals. Our study focused on the comparison between single and twin bearing ossimi ewes in the last four weeks of pregnancy and the day of parturition by measurement of reduced glutathione (GSH) level and the activities glutathione peroxidase (GSH-Px); glutathione reductase (GR-ase); glutathione-S-transferase (GST) and total superoxide dismutase (t-SOD) in erythrocytic haemolysate. In addition, glucose, non esterfied fatty acid (NEFA), Beta hydroxyl butyric acid (BHBA), cortisol, insulin and protein electrophoric patterns were measured in serum. Our results concluded that, In erythrocytic haemolysate the mean values of GSH-Px and GST in group II and III during the period of 2nd and last week before parturition and at the day of parturition were high significantly increased. While, GSH and t-SOD were high significantly decreased ($P<0.01$) and GR-ase activities were significantly decreased. While serum insulin level decreased while serum NEFA, BHBA and cortisol were increased in single and twin but in twin the values is more significant. The data showed that twin bearing ewes are more susceptible to pregnancy toxemia than single bearing that may be influence the productivity and performance of those animals.
[Ali Hafez El-Far, Mohamed K. Mahfouz and Hussein A. Abdel maksoud. **Biochemical Changes in Glutathione Redox System and Glucose Regulation in Late Pregnant Ossimi Ewes.** Journal of American Science 2010;6(12):1067-1073]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: pregnancy, glutathione, single bearing, twin bearing, ewes

1. Introduction:

Pregnancy, parturition, and lactation represent a physiological load to the female body. Where pregnancy toxemia (gestational ketosis) caused by negative energy balance in late gestation is commonly observed in ewes and does (Kulcsar et al, 2006), in beef cows (Rook, 2000), and also in monogastric species as rabbits, guinea pigs, dogs and in ferrets (Lewington, 2007). The background of the disease is the result of the fetal carbohydrate- or energy-demand exceeding maternal supply during the last trimester of pregnancy.

In ruminants, dietary carbohydrates provide well over one half of the energy needs for maintenance, growth and production. Glucose is a primary energy source for certain animal tissues and a precursor for lactose synthesis in the mammary gland. Consequently, understanding carbohydrate digestion and absorption, dietary glucose availability, and the involvement of gluconeogenesis in the regulation of glucose homeostasis is essential for the manipulation of the production and quality of agricultural foods (Rafael and Donald, 2007). Lipid digestion in ruminants is unique in that after ingestion feed lipids are placed into a hydrolytic and reductive environment. The result is that glycerol from triacylglycerols and phospholipids are fermented to VFA and those unsaturated fatty acids which are hydrogenated to mostly saturated fatty acids before absorption (Van Saun, 2000). In ewes, number of fetuses plays role in keeping the homeostasis. The last trimester of pregnancy is very demanding for that homeostasis, because fetuses gain over half of their weight in this period (Seidal et al., 2006). Pregnancy toxemia is a metabolic disease that commonly affects pregnant ewes with multiple fetuses and does during late gestation. It is characterized by hypoglycemia, increased concentrations of ketone bodies in the blood and elevated plasma concentrations of free fatty acids is the result of energy demand exceeding maternal supply during the last trimester of pregnancy (Kulcsar et al., 2006).

The endocrine system especially the pancreas probably is involved in the development of ruminant ketosis. Insulin inhibits ketogenesis when free fatty acids levels are high, as well as growth hormone secretions inhibited by cortisol and free fatty acids. Insulin also appears to be important in regulating the utilization of ketone bodies as the uptake of β-hydroxybutyrate and acetate (Abd-Elghany et al., 2010).

Cortisol is a regulator of glucose in ruminants, which acts to increase gluconeogenesis from amino acids. In starving ruminants the gluconeogenesis is maintained by elevated levels of glucocorticoids (Azab and Abdel-Maksoud, 1999). In lactating ruminants the rate of hepatic gluconeogenesis and the relative concentrations of glycogenic precursors regulate the level of milk production (Huntington, 1990).

Ketone bodies serve as an alternative fuel for many tissues, but they probably do not or only to a minor extent contribute to energy supply of the fetus (Battaglia and Meschia, 1988). Glucose remains most important metabolite for fetal and placental growth. The ability of the ewe to provide a sufficient amount of glucose to the fetus from dietary sources is limited because about 70 to 75% of the dietary carbohydrate is converted in rumen into nonglucogenic products. The remaining fraction of digestible carbohydrate provides 40 to 60% of the circulating glucose through propionate. During periods of a negative energy balance and increased demand for glucose, up to 23% of the glucose may be synthesized from liberated glycerol from the adipose tissue. Along with this glucogenic precursor, a larger amount of fatty acids is released into circulation that may give rise to an increased rate of ketone body formation (Schlumbohm and Harmeyer, 2004). Our study aimed to investigate carbohydrate and fat metabolic changes in single and twin bearing ossimi sheep.

2. Materials and methods

A. Experimental design

The present study was carried out in field farm of Veterinary medicine, Moshtohor, Banha University. Fifty apparently healthy, multiparous Ossimi sheep, of two years old and their body weight ranging between 35 and 50 kg. All animals were kept at the same environmental and nutritional conditions. All over the experimental period, the ewes were allotted into three groups as following:

Group I: included ten ewes (non pregnant non lactating) were used as control group.

Group II: included twenty single pregnant ewes used as experimental animals.

Group III: included twenty twin pregnant ewes used as experimental animals.

Animals were fed free in feedlot. Concentrate feed mixtures were adjusted to the changing of body weight every two weeks. Concentrate mixtures were given twice daily at 10 a.m. and 2 p.m. while wheat straw was offered (*ad lib*.).

B. Blood samples

The blood samples were collected from jugular vein of all animals in the examined groups in the early morning with one week interval during the last month of pregnancy and the day of parturition. Blood samples were divided into two portions; The first portion was collected in heparinized Tube contained 20 I.U. heparin for one mL blood for preparation of haemololysate by using digitonin and washing by physiological saline according to (Kornburg and Korecker 1955).

Table (1) Chemical and cell wall constituents of feed concentrate mixture and corn stalks (on DM basis)

Items	Feed concentrate mixture ϴ	Wheat straw
Chemical composition		
DM	91.51	93.48
OM	89.64	89.58
CP	14.34	3.26
CF	8.47	40.23
EE	2.24	1.32
NFE	64.59	44.77
ASH	10.36	10.42
Cell wall constituents		
NDF	34.62	78.24
ADF	16.24	54.13
Hemi cellulose	18.38	24.11
NFC*	38.44	6.76

*NFC: Non fibrous carbohydrates= 100 - % (CP+ NDF + EE + ASH) (Calsamiglia et al., 1995).

ϴ Feed concentrate mixture consists of 18% undecorticated cotton seed meal, 4% soybean meal, 36% yellow corn, 36% wheat bran, 3% Vinass, 1.5 % limestone, 1.4% sodium chloride and 0.1% common salts.

This was used for estimation of erythrocytic GSH (Sedlak and Lindsay, 1968); t-SOD (Misra and Fridovich, 1972); GSH-Px (EC: 1.11.1.9) (Chiu et al., 1976); GR-ase (EC: 1.6.4.2) (Bergmayer, 1983); GST (EC: 2.5.1.18) (Vessey and Boyer, 1984). The second one was collected without anticoagulant for obtaining a clear non-hemolyzed serum by centrifugation of the blood sample at 3000 r.p.m for 5 minutes. The clear sera were freshly used for determining of blood glucose (Trinder, 1969), non esterified fatty acid (NEFA) and Beta hydroxyl butyric acid (BHBA) (Duncombe, 1964), Commercial radioimmunoassay kits were used to measure concentration of cortisol and insulin (Tietz, 1968 and Wilson and Miles, (1977). C. Electrophoretic pattern of serum protein by SDS-PAGE which performed according to the method of (Laemmli, 1970). D-Statistical analysis was done by (SAS, 1996).

3. Results

The data presented in (Table 2) revealed a high significant increase (P<0.01) in the mean values of GSH-Px and GST in group II and III during the period of 2nd and last week before parturition and at the day of parturition. In contrast, GSH and t-SOD were high significantly decreased (P<0.01) and GR-ase activities were significantly decreased (P<0.05) at the same period of experiment.

Serum glucose level (Table 3) of single pregnant ewes showed significant (P<0.05) decrease than control during last 3 weeks of pregnancy. But of twin pregnant ewes was decreased significantly (P<0.05) during last 4 weeks of pregnancy.

Concentration of serum non esterified fatty acid (Table 3) of single pregnant ewe showed significant (P<0.05) increase than the control during the last 3 weeks of pregnancy as well as at the day of parturition. But of twin pregnant ewes showed significant (P<0.05) increase during the last 4 weeks of pregnancy. Serum BHBA level (Table 3) of single pregnant ewes showed significant (P<0.05) increase than the control during the last 3 weeks of pregnancy and the day of parturition. And twin pregnant ewes showed significant (P<0.05) decrease than the control during the last 4 weeks of pregnancy and the day of parturition. Serum insulin level (Table 3) of single and twin pregnant ewes showed significant (P<0.05) decrease than the control during the last 4 weeks of pregnancy and the day of parturition.

Serum cortisol level (Table 3) of single and twin pregnant ewes showed significant (P<0.05) increase than the control during the last 2 weeks of pregnancy as well as the day of parturition.

The electrophoretic pattern of serum protein revealed that albumin, alpha (α)-1-globulin, alpha (α)-2-globulin and gamma (γ) globulin of single pregnant ewes (Table, 4 and Figure, 1) were significantly decreased during the last week of pregnancy and the day of parturition. But, the concentration of serum beta (β) globulin showed significant (P<0.05) decrease during the last week of pregnancy as well as the day of parturition.

Table (2): Mean values of some biochemical parameters of group I (control), group II (single bearing ewes) and group III (twin bearing ewes)

Duration / Parameters / Groups			GSH (umol/mg protein)	t-SOD (U/g protein)	GSH-Px (U/g protein)	GR-ase (U/g protein)	GST (U/g protein)
Control			0.89 ±0.08	13.01 ±1.11	3.19 ±0.27	0.81 ±0.02	0.41 ±0.03
Gestation period	4th week	Group II	0.71 ±0.11	13.01 0.47±	4.01 ±0.32	0.61 ±0.10	0.59 ±0.01
		Group III	0.81 ±0.12	11.20 ±0.88	4.91 ±0.19*	0.73 ±0.09	0.67 ±0.09
	3rd week	Group II	0.79 ±0.11	10.11 ±0.49	4.19 ±0.31	0.60 ±0.11	0.91 ±0.11*
		Group III	0.77 ±0.09	9.75 ±0.17	6.11 ±0.32*	0.62 ±0.09	1.11 ±0.09*
	2nd week	Group II	0.57 ±0.11*	8.75 ±0.40	7.01 ±0.27*	0.59 ±0.11	1.10 ±.11*
		Group III	0.61 ±0.10*	8.97 ±0.51	7.33 ±0.29*	0.49 ±0.10*	1.25 ±0.21*
	1st week	Group II	0.42 ±0.11**	8.19 ±0.31*	7.41 ±0.11**	0.41 ±0.02*	1.28 ±0.12**
		Group III	0.55 ±0.10**	7.70 ±0.21*	7.51 ±0.31**	0.39 ±0.03*	1.39 ±0.20**
Day of parturition	Group II		0.39 ±0.10**	7.12 ±0.16**	8.31 ±0.70**	0.35 ±0.01*	1.75 ±0.21**
	Group III		0.40 ±0.11**	6.11 ±0.13**	9.55 ±0.29**	0.25 ±0.03*	1.90 ±0.13**

* Indicate significant difference from control at (P<0.05).
** Indicate high significant difference from control at (P<0.01).
GSH (reduced glutathione); t-SOD (total superoxide dismutase); GSH-Px (glutathione peroxidase); GR-ase (glutathione reductase) and GST (glutathione-S-transferase).

Table (3): Mean values of some biochemical parameters of group I (control), group II (single bearing ewes) and group III (twin bearing ewes)

Duration	Parameters / Groups		Glucose (mg/dl)	NEFA (g/dl)	BHBA (µmol/L)	Insulin (µU/dl)	Cortisol (µg/dl)
	Control		66..41 ±0.36	11.53 ±0.22	3.57 ±0.07	2.54 ±0.02	2.04 ±0.04
Gestation period	4th week	Group II	42.88 ±0.85*	29.20 ±0.55*	10.35 ±0.89*	0.78 ±0.03*	3.64 ±0.07*
		Group III	41.56 ±0.70*	32.64 ±1.37*	12.72 ±0.95*	0.67 ±0.06*	3.73 ±0.13*
	3rd week	Group II	44..59 ±0.80*	22.70 ±0.73*	7.85 ±0.29*	0.94 ±0.05*	2.78 ±0.04*
		Group III	43.44 ±0.36*	22.63 ±1.31*	8.92 ±0.84*	0.87 ±0.12*	2.80 ±0.11*
	2nd week	Group II	47.57 ±0.49*	22.68 ±0.43*	7.10 ±0.33*	1.10 ±0.07*	2.32 ±0.06
		Group III	46.48 ±0.46*	21.87 ±0.37*	7.62 ±0.39*	1.17 ±0.03*	2.40 ±0.05
	1st week	Group II	52..29 ±0.53	18.92 ±0.18	6.21 ±0.25*	1.31 ±0.01*	2.04 ±0.02
		Group III	50.30 ±0.47*	19.11 ±0.36*	7.82 ±0.77*	1.29 ±0.006*	2.10 ±0.01
Day of parturition	Group II		40.50 ±0.63*	25.62 ±0.81*	7.52 ±0.32*	1.15 ±0.04*	5.50 ±0.18*
	Group III		40.12 ±0.25*	25.19 ±1.07*	9.36 ±0.27*	1.23 ±0.03*	8.02 ±0.11*

* Indicate significant difference from control at (P<0.05).

Table (4): Mean vales of serum protein fractions (g/dl) in control and twin ewes

The fractions	Control	The last week of pregnancy	The day of parturition
Albumin	3.15 ± 0.06	1.82 ± 0.11*	2.25 ± 0.17*
(α)-1-globulin	0.17 ± 0.004	0.08 ± 0.004*	0.08 ± 0.008*
(α)-2-globulin	0.56 ± 0.008	0.37 ± 0.006*	0.41 ± 0.006*
(β)-globulin	0.87 ± 0.006	0.74 ± 0.01*	0.75 ± 0.01*
(γ)-globulin	2.92 ± 0.01	1.87 ± 0.013*	2.17 ± 0.05*

* Indicate significant difference from control at (P<0.05).

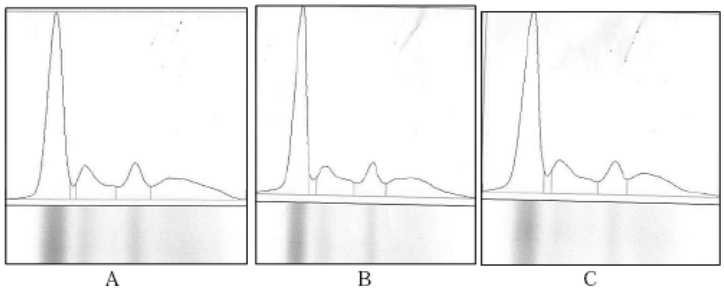


Figure (1): show the electrophoretic serum pattern of Control (A), the last week of pregnancy (B) and the day of parturition (C). In each picture, bands were arranged Albumin, Alpha (α)-1- globulin, Alpha (α)-2- globulin, Beta (β) globulin and Gamma (γ) globulin (From left to right).

4. Discussion

Our study revealed a high significant increase in the mean values of GSH-Px and GST in group II and III during the period of 2nd and last week before parturition and at the day of parturition. Also, showed high significantly decreased in GSH and t-SOD and a significant decrease in GR-ase. This result indicated that t-SOD activity decreased as it is a first line in antioxidant enzymes defense. In the second line, GSH-Px and GST were consuming GSH as a reductant cofactor. For that reason GSH-Px and GST activities were increased and GSH level was decreased. In addition, GR-ase activities were decreased because of GR-ase enzyme generates GSH (Mandour and Abou-El-Ela, 1999 and Abdel-Maksoud et al., 2000). As the glutathione assumes pivotal roles in bioreduction, protection against oxidative stress, detoxification of xenobiotics and endogenous toxic metabolites, transport, enzyme activity, and sulfur and nitrogen metabolism. Its biological significance comes from the free sulfhydryl moiety of the cysteine residue and nucleophilic properties. In cells, glutathione mainly exists in the reduced form (GSH), as the oxidized form (GSSG) (Taisuke et al., 2009). Erythrocytes are permanently in contact with potentially damaging levels of oxygen, but their metabolic activity is capable of reversing this injury under normal conditions. Erythrocytes are equipped by many defence systems representing their antioxidant capacity. This protective system includes superoxide dismutase (SOD), catalase (CAT), reduced glutathione, glutathione peroxidase (GPx), glutathione-S-transferase, and glutathione reductase (GR). However, the cellular antioxidant action is reinforced by the presence of dietary antioxidants (Nakbi et al., 2010).

The present study showed that a significant decrease in the mean values of glucose of single and twin that lower plasma glucose levels came in accordance with (Seidal et al., 2006 and Balikci et al., 2007). The observed decrease in serum glucose level may be due to the, negative energy balance increases lipid mobilization, which results in hepatic lipidosis with subsequent impairment of hepatocellular function, glucose deficiency with intermittent hypoglycemia and accumulation of ketone bodies. The hypothesis that cows suffering from stress and/or painful diseases have elevated blood glucose levels due to an increase in serum cortisol (Forslund et al., 2010).

NEFA and BHBA concentrations in single and twin pregnant ewes were significantly increased than control but in twin were more significantly increased; these results were proved by (Nazifi et al., 2002 and Moghaddam and Hassanpour, 2008).

Serum cholesterol level of single and win pregnant ewes showed significant increase than the control during the last 2 weeks of pregnancy as well as at the day of parturition. The high cortisol level inhibits the growth of the axial skeleton in the sheep fetus during the late pregnancy which enhances the parturition process (Fowden et al., 1996).

Serum insulin level of single and twin pregnant ewes showed significant decrease than the control during the last 4 weeks of pregnancy as well as at the day of parturition. The decrease of insulin level may be attributed to negative energy balance which leads to decrease in glucose level and increase the liploysis (Faulkner and Pollock, 1990). The shift of energy metabolism in a catabolic direction is characterized by a wide range of endocrine changes, such as insufficient pancreatic β-cell function with a coinciding increase in insulin resistance.

The data illustrated in table (3) showed significant increase in cortisol level than the control during the last 2 weeks of pregnancy as well as the day of parturition in Single and twin pregnant ewes. This observation may be due to a hypothesis that the known relation between stress and/or painful diseases in high yielding dairy cows and pregnant ewes may be mediated through a concurrent increased cortisol secretion leading to hyperglycaemia (Rohrbach et al., 1999). On the other hand, Forslund et al., (2010) reported significantly low levels of cortisol in Cows with ketonemia (BHBA > 1.5 mmol/l). The significant increase in cortisol and presence of significant negative correlation between plasma glucose concentration and cortisol level and the significant positive relationship with β-hydroxybutyrate may be due to increased adrenal output or to impaired ability of the fatty liver, which was a consistent finding in pregnancy toxemia, to mobilize and excrete the hormone (Ford et al., 1990 and Abd-Elghany et al., 2010).

The concentrations of serum albumin, alpha-1-globulin, alpha-2- globulin, beta globulin and gamma globulin of single pregnant ewes showed significant decrease than the control during the last week of pregnancy as well as the day of parturition. These results may attributed to consequence increase in the mother's basal metabolic rate, the maximal nutrient requirements of the placenta and the growing fetus, together with the transfer of serum albumin, immune globulins, and amino acids from the blood stream to the mammary gland for synthesis of colostrums (Batavani et al., 2006).

5. Conclusion

Late pregnancy in ewes is a very stressful period specially the late period in which in erythrocytic haemolysate the mean values of GSH-Px

and GST were high significantly increased; GSH and t-SOD were high significantly decreased (P<0.01) and GR-ase activities were significantly decreased. While, serum glucose, total protein, albumin, globulin and insulin were decreased while serum NEFA, BHBA and cortisol were increased in single and twin but in twin the values is more significant. Our research results recommended that twin bearing ewes need a special care during pregnancy and after parturition by supplementation of ewes by a demands of appositive energy balance.

Corresponding author
Ali Hafez El-Far
Department of Biochemistry, Faculty of Veterinary Medicine, Alexandria University, Damanhour Branch (Al-Bostan), Egypt.
aboufares90@yahoo

6. References:

1. Abd-Elghany, Hefnawy, S. Y. and Saad S. (2010): Some Immunohormonal Changes in Experimentally Pregnant Toxemic Goats, Veterinary Medicine International, Volume, Article ID 768438. 100- 11.
2. Abdel-Maksoud, H.A.A; Omayma A. R.; Mohamed K. M. M. and Yakout A.Y. E. (2000): Biochemical changes of glutathione redox system, some erythrocytic enzymes; serum proteins and trace elements of theileria infested buffaloes. The Egyptian Journal of Biochemistry, Vol. 18 No. 1, pp 121- 134.
3. Azab, M. E. and Abdel-Maksoud H. A. (1999): Changes in some hematological and biochemical parameters during prepartum and postpaetum in female Baladi goats. Small Ruminant Reseasch 34 : 66-85.
4. Balikci, E.; Yildiz, A. and Gurdogan, F., (2007): Blood metabolite concentrations during pregnancy and postpartum in Akkaraman ewes. Small Ruminant Research, 67, 247-251.
5. Batavani, R.A.; Ansari, M.; Asri, S., (2006): Concentrations of serum total protein and protein fractions during distrust and pregnancy in Makuii ewes. Comp Clin Pathol 15, 227-230.
6. Battaglia, F. C. and Meschia, G., (1988): Fetal nutrition. Annu. Rev. Nutr. 8:43–61.
7. Bergmayer, H.V. (1983): Methods of Enzymology. 3rd, ed. Varly chem. Weiheim. 105: 177-182.
8. Calsamiglia, S., Stern, M.D. and Frinkins, J.L. (1995): "Effects of protein sosurce on nitrogen metabolism in continuous culture and intestinal digestion in vitro" J. Anim. Sci., 73: 1819.
9. Chiu, D. I., Stults, F.H. and Tappal, A.L. (1976): purification and properties of rat lung soluble glutathione peroxidase. Biochem. Biophys. Acta, 445, 558 – 566.
10. Duncombe, W.G., (1964): The calorimetric micro determination of non-esterified fatty acids and betahydroxy butyric acid in plasma. Chem. Acta, 9: 122-125.
11. Faulkner, A. and Pollock, H.T., (1990): Metabolic responses to euglycaemic hyperinsulinaemia in lactating and non-lactating sheep in vivo. J. Endocrinol. 124, 59-66.
12. Ford E. J. H., Evans, J. and Robinson, I. (1990): Cortisol in pregnancy toxaemia of sheep, British Veterinary Journal, vol. 146, no. 6, pp. 539–542.
13. Forslund, Kristina B, Orjan A Ljungvall and Bernt V Jones (2010): Low cortisol levels in levels in blood from dairy cows with ketosis: a field study Acta Veterinaria Scandinavica, 52:31.
14. Fowden, A.L.; Szemere, J.; Hughes, B.; Gilmour, R.S. and Forhead, A.J., (1996): The effects of cortisol on the growth rate of the sheep fetus during the late gestation. J. Endocr, 151, 97-105.
15. Huntington, G.B. (1990): Energy metabolism in the digestive tract and liver of cattle: influence of physiological state and nutrition. Reprod Nutr Dev., 30:35-47.
16. Kornburg, a. and korecker, D (1955): In 'Methods in Enzymology, Acad. Press, New York, PP 201, 239.
17. Kulcsar M., Danko G., Delavaud C., Mircu C., Nikolic A.J., Gaspardy A., Cernescu H., Chilliard Y., Cseh S., Rudas P., Huszenicza Gy. (2006): Endocrine characteristics of late pregnant hyperketonaemic ewes and their reproductive performance following the induction of ovarian cyclicity out of the breeding season. Acta Veterinaria Hungarica, 54, 235–249.
18. Laemmli, A. R., (1970): Cleavage of structural protein during assembly of the head bacteriophage TA. Nature, 227: 600 – 685.
19. Lewington, J.H. (2007): Pregnancy toxaemia. In: Lewington J.H. (ed.): Ferret Husbandry, Medicine and Surgery. 2nd ed. Saunders Elsevier, Edinburgh, 94–95.
20. Mandour, M. and Abou-El-Ela, S. (1999): the protective effect of selenium on cadmium induced lipid peroxidation in rats. Egy. J. Bioch., Vol. 17; No. 1(1-21).
21. Misra, H.P. and Fridovich, S. (1972): the role of superoxide anion in the oxidation of epinephrine and a sample assay for superoxide dismutase. J. Biol. Chem., 247: 3170 – 2175.
22. Moghaddam, G. and Hassanpour, A., (2008): Comparison of blood serum glucose, beta hydroxybutyric acid, blood urea nitrogen and calcium concentration in pregnant and lambed

ewes. Journal of animal and veterinary advances, 7(3): 308-811.
23. Nakbi Amel, Wafa Tayeb, Samia Dabbou, Manel Issaoui, Abir K Grissa, Nabil Attia, and Mohamed Hammami (2010): Dietary olive oil effect on antioxidant status and fatty acid profile in the erythrocyte of 2,4-D- exposed rats. Lipids Health Dis.; 9: 89.
24. Nazifi, S.; Seb, M. and Ghavami, S.M., (2002): Serum lipid profile in Iranian fat-tailed sheep in late pregnancy, at parturition and during the post-parturition period. J. Vet. Med. Ser A. 49, 9-12.
25. Rafael A. N. and Donald C. B. (2007): Carbohydrate and Lipid Metabolism in Farm Animals, The Journal of Nutrition: 702 0022-3166.
26. Rohrbach B.W., Cannedy A.L., Freeman K, Slenning, B.D. (1999): Risk factors for abomasal displacement in dairy cows. J Am Vet Med Assoc, 214:1660-3.
27. Rook, J.S. (2000): Pregnancy toxemia of ewes, does, and beef cows: The Veterinary Clinics of North America. Food Animal Practice, 16, 293–317.
28. SAS, (1996): Statistical Analysis System. Users Guide Statistics, SAS Institute Cary, North Carolina.
29. Schlumbohm, C. and Harmeyer, J. (2004): American Dairy Science Association, Hyperketonemia Impairs Glucose Metabolism in Pregnant and Nonpregnant Ewes. J. Dairy Sci. 87:350–358.
30. Sedlak, I. and Lindsay, R.H. (1968): Estimation of total protein –bound and non-protein sulphydryl groups in tissues with Elman's reagent. Anal. Biochem. 25, 192-205.
31. Seidal, H.; Novotay, J. and Kovac, C., (2006): Selected biochemical indices in sheep during pregnancy and after parturition, Bull Vet Inst Pulway 50, 167-170.
32. Taisuke Yano, Emiko Takigami, Hiroya Yurimoto, and Yasuyoshi Sakai (2009): Yap1-Regulated Glutathione Redox System Curtails Accumulation of Formaldehyde and Reactive Oxygen Species in Methanol Metabolism of Pichia pastoris , Eukaryotic Cell, April, p. 540-549, Vol. 8, No. 4.
33. Tietz, N.W. (1968): Textbook of Clinical Chemistry, Saunders.
34. Trinder, P., (1969): Determination of Glucose in Blood Using Glucose Oxidase with an Alternative Oxygen Acceptor, Ann. Clin. Biochem 6, 24-25.
35. Van Saun R.J. (2000): Pregnancy toxaemia in a flock of sheep. Journal of the American Veterinary Medical Association, 217, 1536–1539.
36. Vessey, D.A. and Boyer, T.D. (1984): Different forms of rat liver glutathione –S-transferase by the herbicides 2,4,dichlorophenoxy acetate and 2,4,5, trichlorophenoxy acetate. Toxicol. Appl. Pharma., 73:492 – 499.
37. Wilson, M.A. and Miles, L.S.M., (1977): Radioimmunoassay of insulin. In hand book of Radioimmunoassay, G.E. Abraham (ed.), M. Dekker Inc., New York, p. 257.

11/5/2010

Atypical Bacteria in Ventilator Associated Pneumonia; an Egyptian University Hospital Experience

Nadia Abdel-Salam Mokhless[1,3] , Malaka Farouk El-Mofty[1,3] , Nesrine Fathy Hanafi[* 1,3], Akram Muhammad Fayed[2,3] and Sara Lotfy Asser[1,3].

[1]Medical Microbiology and Immunology, [2]Critical Care Medicine, [3]Faculty of Medicine University of Alexandria, Egypt

drnesra1@hotmail.com*

Abstract: Background Ventilator-associated pneumonia (VAP) is the most common hospital acquired infection seen in ICU in patients on mechanical ventilation. A diversity of microbes can cause VAP, causative agent differ according to patient populations and types of ICUs. Atypical bacteria not cultured by routinely used methods , have been implicated as causes of VAP, still no sufficient studies to assess size of their role as causative agent in VAP. In this study we aim at estimation of the potential role of atypical bacteria as Mycoplasma pneumoniae, Chlamydia pneumoniae and Legionella pneumophila in ventilator-associated pneumonia in the intensive care units of Alexandria Main University Hospital. Materials and methods: 60 endotracheal aspirates were collected from VAP ICU patients. Samples were subjected to routine culture as well as PCR amplification using specific primers for detection of the following atypical bacteria : Mycoplasma pneumoniae, Chlamydia pneumoniae and Legionella pneumophila. Results: Out of the 60 endotracheal aspirate, routine culture revealed growth of: enterobacteriaecae in 14 (23.3%) aspirate, pseudomonas in 13(21.7%), candida in14(23.3%) , and MRSA in 10 (16.7%) . In 19 (31.7%) endotracheal aspirates, no growth was encountered on routine culture. PCR reaction was positive for Atypical bacteria in 9 (15%) out of 60 samples, five were positive for mycoplasma, three for Legionella, and only one was positive for Chlamydia. Atypical bacteria positive results were encountered in 4 (21%) out of 19 aspirates with no growth culture results. Conclusion: Our results point that atypical bacteria are not an uncommon cause for VAP. This finding has to be taken into consideration while tailoring the empiric antimicrobial coverage of patients diagnosed with VAP.

[Nadia Abdel-Salam Mokhless, Malaka Farouk El-Mofty , Nesrine Fathy Hanafi, Akram Muhammad Fayed and Sara Lotfy Asser. **A Typical Bacteria in Ventilator Associated Pneumonia; an Egyptian University Hospital Experience.** Journal of American Science 2010;6(12):1074-1079]. (ISSN: 1545-1003). http://www.americanscience.org.

1. Introduction:

Ventilator-associated pneumonia (VAP) is considered as the most frequent ICU-acquired infection among patients receiving mechanical ventilation (MV). This kind of respiratory tract infection prolong the duration of Mechanical ventilation and delay the release from ICU. Most antibacterial chemotherapy prescribed in an ICU are administered for respiratory tract infections. (1)

VAP can be caused by a large variety of microorganisms . the causative agent may differ according to the population of patients in the ICU, the durations of hospitalization and stay in the ICU. "Atypical" pneumonia differ from typical one in not to be associated with shaking chills (2) and caused by atypical bacteria which cannot be grown by routinely used microbiologic culture media and techniques as *Mycoplasma pneumoniae, Chlamydia pneumoniae and Legionella pneumophila* (3-5).

Mycoplasma pneumoniae was first atypical pathogens to be identified as a cause of respiratory tract infection in 1944.(6,7) Characterized by absence of a rigid cell wall, poor staining by Gram method, and high nutritional requirements for its culture as it needs a high concentration (10-20%) of serum or supplements . This makes diagnosis of mycoplasma by conventional microbiologic examination difficult. (7) *Chlamydia pneumonia* laboratory diagnosis depended on non- specific and technically demanding techniques till 1990 but now it has been replaced by detection of chlamydial antigens or detection of DNA by PCR. (7) *Legionella pneumophila* are nutritionally fastidious, intracellular bacilli, gram negative organisms. (8) Infection with *legionella is* associated with exposure to artificial water systems ,condensers and respiratory therapy equipments .(9) Use of PCR as a rapid and specific diagnostic method for legionella infection overcame the long culture time needed for its growth (3-5 days) and the need of media supplemented with iron and cysteine as well as difficult colonial identification in mixed cultures. (7)

Accurate diagnosis of VAP remains a difficult target to achieve, that relies mainly on clinical, microbiological and radiological diagnosis.(10,11) Main clinical criteria for VAP

diagnosis have been reported to be new lung infiltrate on chest X-ray with fever , leukocytosis or leukopenia, and purulent secretions. [12-14] Inadequate antibiotic treatment have been always reported by researchers to be related to poor prognosis of VAP. [15,16] Microbiological culture and sensitivity results remains a gold standard for planning treatment for the VAP patient before empirical antibiotic administration [17] . This have been emphasized by the Guidelines from the Infectious Disease Society of America(IDSA).[18]

It is well established that Beta-lactams are not effective against such organisms because *Chlamydia pneumoniae* and Legionella species are intracellular organisms and *Mycoplasma pneumoniae* lacks a cell wall. In those cases Erythromycin and tetracycline can be useful . Other antibiotics effective against atypical bacteria, includes Macrolides, Doxycycline and Flouroquinolones.[6] Our study aims at assessment of the potential role of atypical bacteria as Mycoplasma pneumoniae, Chlamydiae pneumoniae and Legionella pneumophila in ventilator-associated pneumonia in the intensive care units of Alexandria Main University Hospital.

2. Materials and methods

Materials

The study was conducted on 60 patients on Mechanical ventilators for more than 48 hours and acquired VAP during their stay in the ICU of Critical Care Department in the Alexandria Main University Hospital in the period between April 2009 and April 2010.Cases included in this study have been informed and consented. Inclusion criteria were fever, leukocytosis, development of persistent radiographical pulmonary infiltrate during stay in the ICU and with no history of a previous pulmonary disease or pulmonary symptoms at the time of admission. Thirty ICU patients on ventilators for more than 48 hours without developing VAP, within the same period of time, were included as a control group.

Methods

Endotracheal aspirates as well as clinical data have been collected from patients and controls. Samples were subjected to routine culture as well as DNA extraction with subsequent PCR amplification for detection of specific DNA sequences of the following atypical Bacteria genus : Mycoplasma pneumoniae, Chlamydia pneumonae and Legionella pneumophila.

The endotracheal tube was previously inserted guided by a laryngoscope. A sterile suction catheter was introduced blindly into the endotracheal tube after disconnecting the ventilator. 2 ml of endottracheal aspirates were obtained by suction. The part of the catheters containing the aspirates were cut and placed in sterile test tubes and sent to the laboratory.

The endotracheal aspirates, within catheters in test tubes, were homogenized and liquefied by adding 2 ml sterile 1% N-acetyl L-cysteine (equal volume of the specimen). All tubes were centrifuged for 15 minutes at 4×10^3 rpm and then vortexed and left at room temperature for another 15 minutes.[19,20] Each homogenized specimen was then divided into 2 equal portions in 2 sterile Eppendorfs. The first part was used for conventional microbiological studies while the other was kept at -20^{o} C for PCR assay. Each aspirate was streaked on Blood agar, MacConkey's agar and Sabouraud's Dextrose agar plates. All plates were incubated aerobically at 37 ^{o}C for 24 hours. Any growth was identified according to the conventional bacteriological and mycological techniques.[19,20]

PCR assay:

Extraction of DNA was performed using QIAamp DNA blood mini kit (Qiagen). Separate PCR reactions were performed for amplification of each DNA sequence of each organism using Techne Progene thermal cycler. Reaction mixture consisted of 5µl DNA extract, 25 picomoles of each of the forward and reverse oligonucleotide primers specific for Mycoplasma pneumonia [21], Legionella pneumophila [21] and Chlamydia pneumonia ([22], 12.5 µl Taq PCR master mix (MBI Fermantas), and 4.5 µl nuclease free water. For detection of the amplified products: [21] 10 µl of the amplification products were electrophorised into 2% agarose in Tris-borate EDTA containing 0.5 µg/ml ethidium bromide at 80 volts for 45 minutes. Revealed DNA bands were visualized on an ultraviolet transilluminator.

3. Results

The mean age of the VAP study group was 43.1 ± 24.68 (18-85) year, while that of the control group was 49.7 ± 20.5 (12-70). There was no statistically significant difference between them as regards age and gender: male to female ratio was 33:27(55:45%) in patient group while in the control group, the male to female ratio was 19:11 (63:36.6%). As regards causes of hospital admission for the 60 VAP patients and the control group It has been found that cardiac problems were the most commonly encountered among both patients (38%) and controls (28%), rest of causes for admission included accidents, poisoning, Renal and hepatic problems. In the endotracheal aspirates obtained from

the 60 VAP patients included in this study, 41 specimens were positive by conventional microbiological cultures and 19 were culture negative. In the control group, 11were positive and 19 specimens were culture negative(See table 1).

The conventional microbiological culture revealed that among cultures of the patients group, *Candida* spp was the commonest organism isolated accounting for 23.3%. Only 16% were of significant count ($\geq 10^5$ CFU/ml). This was followed by *Pseudomonas aeruginosa* (21.6%) and 15% were of significant count, then the polymicrobial growth 20% and 16% with significant count, *Staphylococcus aureus* 16% and 10% with significant count, *Acinetobacter* spp 8.3% and 6.6% with significant count, *Proteus* spp 6.6% and 5% with significant count, *Klebsiella* spp 6.6% and 5% with significant count, *E-coli* 5% and 1.6% with significant count, *Coagulase-negative staphylococci* 1.6% which were of significant count and *Diphtheroids* 1.6% which were also of significant count. It is also to be noted that 31.6% of the total endotracheal aspirates of the 60 VAP cases were negative by conventional microbiological culture.

Table 1: The results of conventional microbiological culture of the endotracheal aspirates from the 60 VAP patients and the control group

	Conventional microbiologic Culture results*	
	Positive	**Negative**
Cases n=60	41 (68.3%)	19 (31.7%)
Controls n=30	11 (36.6%)	19 (63.3%)
Total n=90	52 (57.7%)	38 (42.2%)

*P value = 0.004

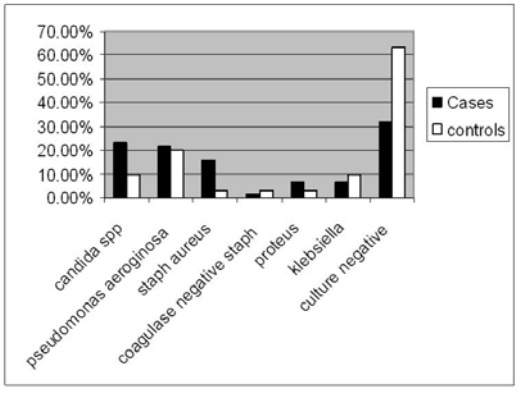


Figure 1: The conventional microbiological culture results for cases & controls group.

While in the control group, bacteria revealed from cultures were *Pseudomonas aeruginosa* accounted for 20%, *Klebsiella* spp 10%, *Candida spp* 10% also, then *Staphylococcus aureus* 3.3%, *Coagulase-negative staphylococci* 3.3% and *Proteus* spp 3.3%. In addition, 10% were culture negative and 13.3% were polymicrobial. The growth counts of the endotracheal aspirates of the control group were insignificant ($\leq 10^5$ CFU/ml). Atypical bacteria DNA detection by PCR was positive in 9 (15%) out of 60 samples, the majority of them (5) were mycoplasma, 3 were positive with Legionella, and only one sample was positive with Chlamydia. The Atypical bacteria positive results was encountered in 4 (21%) out of 19 aspirates with no growth culture results. See figure 1

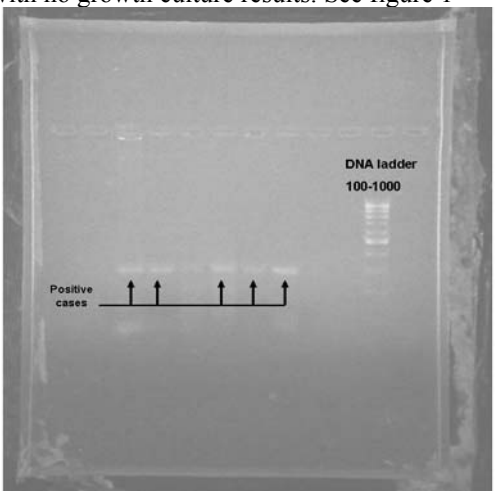


Figure 2: Gel electrophoresis showing 5 positive Mycoplasma pneumoniae cases by PCR.

Table 2: PCR and culture results of cases and control

	Culture**		PCR*	
	Culture positive	**Culture negative**	**PCR positive**	**PCR negative**
Cases	41 (68.3%)	19 (31.7%)	9 (15%)	51 (85%)
Controls	11 (36.6%)	19 (63.3%)	0 (0%)	30 (100%)

* PCR was done using primers for Mycoplasma pneumonia, Legionella pneumophila, Chlamydia pneumonia .

**All specimens were cultured on Blood agar, MacConkey's and SDA.

It was found that 5 specimens were positive for *Mycoplasma pneumoniae*, three were positive for *Legionella pneumophila* and only 1 was positive for

Chlamydia pneumoniae. Among the 5 positive specimens for *Mycoplasma pneumoniae*, 3 were positive by conventional microbiological cultures and grew other associating microorganisms, while 2 were culture negative. The 3 specimens that were positive for *Legionella pneumophila*, only 1 grew other microorganism by conventional cultures and the other 2 were culture negative. The only positive specimen for *Chlamydia pneumoniae* did not grow any microorganisms by conventional microbiological cultures. As for the control group included in this study, none of their DNA extracts were positive in the PCR assay for atypical bacteria.

Table 3: Results of PCR assay in relation to conventional microbiological culture resutls inVAP patients.

PCR assay	Conventional microbiological culture		Total
	Positive(n= 41)	Negative (n= 19)	
Mycoplasma pneumoniae	3(7%)	2 (10%)	5 (8.3%)
Legionella pneumophila	1 (2.4%)	2 (10%)	3 (5%)
Chlamydia pneumoniae	0	1 (5%)	1 (1.6%)
Total	4(9%)	5(26%)	9 (15%)

4. DISCUSSION

VAP complicates the course of 9 – 20% of mechanically ventilated patients and mortality varies greatly from 8 to 76%,. Once pneumonia is suspected, bacteriologic confirmation should be obtained and empiric therapy must be instituted as soon as possible, as a delay in therapy or inappropriate therapy greatly increases mortality.[23] Awareness of the potential microbial causes of VAP and confirmation of the specific cause in an individual patient are essential to guide optimal antibiotic therapy.[24]

Endotracheal aspirates, chosen in this study as the respiratory specimen, are used more frequently as a diagnostic method in intubated patients with suspicion of pulmonary infection, because of its simplicity and minimal training required, but the fact that the culture also contains other non-pathogenic organisms from the upper respiratory tract flora, results in a low positive predictive value of this test. However, this can be avoided by the use of the semiquantitative method of culture of the obtained specimen, with a designated threshold value above which diagnosis of VAP can be established.[25,26] Cultures revealed that *Candida* was the commonest organism isolated accounting for 23.3%, while 16% only were of significant count ($\geq 10^5$ CFU/ml). This was followed by *Pseudomonas aeruginosa* 21.6% and 15% were of significant count, then the polymicrobial growth 20% and 16% with significant count, *Staphylococcus aureus* 16% and 10% with significant count,

The results of our study agree with the results of several previous studies.[27 -31] where *Pseudomonas aeruginosa* was the predominant organism isolated by endotracheal aspiration and bronchoalveolar lavage, followed by *Staphylococcus aureas* and *Klebsiella pneumonia*.[32] The significantly high rate of Gram negative bacilli in our study and many other studies probably indicates the high incidence of prolonged hospital stay and the prolonged duration of mechanical ventilation that predisposes the patients to acquire infections from the multidrug-resistant pathogens . In contrast , Other authors reported other bacterial strains as *Acinetobacter baumanii and Streptococcus* .[33-34]

.

The results of PCR assay for atypical bacteria of the DNA extract of the endotracheal aspirates of the 60 VAP patients revealed a total of 9 positive cases (15%) for the tested microorganisms, 5 cases were positive for *Mycoplasma pneumonia* (8.3%), 3 cases were positive for *Legionella pneumophila* (5%) and only 1 case was positive for *Chlamydia pneumonia* (1.6%).

Many were studies conducted for detection of atypical bacteria by PCR. Hassan et al reported detection of legionella and Chlamydia pneumonia in VAP cases while no cases were positive for *Mycoplasma pneumonia*e.[28] Moreover, Bachinskaya et al reported that 9% of their patients were positive for *Mycoplasma pneumonia*e and 9% were also positive for *Chlamydia pneumoniae*.[35] In another study by Apfalter et al, where real time PCR was used as a fast diagnostic tool for non-conventionally cultured microorganisms, they reported that 3% of their cases were positive for *Mycoplasma pneumonia*e and 2% of cases were positive for *Chlamydia pneumoniae*. They concluded that *Mycoplasma pneumonia*e and *Chlamydia pneumoniae* should be considered as causative agents in critically ill patients who develop early-onset nosocomial ventilator-associated pneumonia. Thus, empirical antimicrobial regimens should cover *Chlamydia*, and *Mycoplasma*.[36] Furthermore, El-Ebiary et al also diagnosed six cases of *Legionella* pneumonia among patients with definite VAP. Using specific culture for *Legionella* and serology for *Legionella pneumophila, Mycoplasma pneumoniae Coxiella burnetti, and Chlamydia pneumoniae,* only

Legionella was diagnosed in 2 patients by serology and in 4 patients by culture.[37] Our results draw attention towards the possibility of these rarely diagnosed agents as being not infrequent causative agents for VAP. The prevalence of such atypical pathogens is to be taken into consideration while tailoring the empiric antimicrobial coverage of patients diagnosed with VAP.

Corresponding author
Nesrine Fathy Hanafi
Medical Microbiology and Immunology - Faculty of Medicine University of Alexandria - Egypt
drnesra1@hotmail.com

5. References:

1. Eggimann P and Pittet D. Infection Control in the ICU. Chest 2001;120;2059-2093.
2. Eggimann P, Harbarth S, Constantin MN, et al. Impact of a prevention strategy targeted at vascular-access care on incidence of infections acquired in intensive care. Lancet 2000; 355:1864–1868.
3. Rello J, Diaz E: Pneumonia in the intensive care unit. Crit Care Med 2003, 31:2544-2551.
4. Bouza E, Perez A, Munoz P, et al. Cardiovascular Infection Study Group: Ventilator-associated pneumonia after heart surgery: A prospective analysis and the value of surveillance. Crit Care Med 2003, 31:1964-1970
5. Tang W. Molecular diagnostics of atypical pneumonia. Acta Pharmacol Sin 2003; 24(12): 1308-1313
6. Blasi F. Atypical pathogens and respiratory tract infections. Eur Respir J 2004; 24: 171-181.
7. Gerald C, Barrie M et al. Mackie and McCartney Practical Medical Microbiology. 14th Ed. Tim Horne. 1996.
8. File TM ,Tan JS, Plouffe JF. The role of atypical pathogens: *Mycoplasma pneumoniae, Chlamydia pneumoniae, and Legionella pneumophila* in respiratory infection. Infect Dis Clin North Am 1998; 12: 569–592.
9. Topley W, Wilson S. Topley and Wilson's Microbiology and Microbial Infections. 10th Ed. Hodder and Arnold , UK, 2008.
10. Ilana P,David L. Diagnosis and Treatment of Ventilator-associated pneumonia. Chest 2006;130;597-604.
11. Miller PR, Johnson JC, III, Karchmer T, et al. National nosocomial infection surveillance system: from benchmark to bedside in trauma patients. J Trauma 2006; 60:98–103.
12. Luyt C-E, Chastre J, Fagon J-Y. Value of the clinical pulmonary infection score for the identification and management of ventilator-associated pneumonia. Intensive Care Med 2004; 30:844–852.
13. Schurink CAM, Van Nieuwenhoven CA, Jacobs JA, et al. Clinical pulmonary infection score for ventilator-associated pneumonia: accuracy and inter-observer variability. Intensive Care Med 2004; 30:217–224.
14. American Thoracic Society, Infectious Diseases Society of America. Guidelines for the management of adults with hospital-acquired, ventilator-associated, and healthcare-associated pneumonia. Am J Respir Crit Care Med 2005; 171: 388–416.
15. Blot F, Raynard B, Chachaty E, et al. Value of Gram stain examination of lower respiratory tract secretions for early diagnosis of nosocomial pneumonia. Am J Respir Crit Care Med 2000; 162:1731–1737.
16. Miller PR, Meredith JW, Chang MC. Optimal threshold for diagnosis of ventilator-associated pneumonia using bronchoalveolar lavage. J Trauma 2003; 55:263–268.
17. Ioanas M, Ferrer R, Angrill J, et al . Microbial investigation in ventilator-associated pneumonia . Eur Respir J 2001; 17:791-801.
18. Kristopher P, Anthony J. Atypical Pathogens and Challenges in Community-Acquired Pneumonia American Academy of Family Physicians 2004. Alan Smyth. Pneumonia due to viral and atypical organisms and their sequelae. British Medical Bulletin 2002;61: 247–262.
19. Betty F, Daniel S, et al. Bailey and Scott's Diagnostic Microbiology. 12th Ed. Mosby Elsevier. 2007.
20. Gerald C, Barrie M et al. Mackie and McCartney Practical Medical Microbiology. 14th Ed. Tim Horne. 1996.
21. Bedewy KML, Elsayed MNE, Mahmoud MI.Ventilator-Associated pneumonia: serum TNF∞ and determination of the role of some atypical bacterial pathogens by PCR. EJJM 2001; 10:27-41.
22. Bedewy KML, Ibrahim YN, Abdelfattah MA. Serum RANTES and TNF∞ levels in patients with acute coronary events: correlation with *Chlamydia pneumoniae* infection. EJMM 2003; 12: 33-46.

23. Jennifer M , Martin A. Expert Opinion on Pharmacotherapy. Pharma 2009: 10(9): 1461-1467.
24. David R. The Microbiology of Ventilator-Associated Pneumonia. Respir Care 2005; 50(6):742–763
25. Luna CM, Videla A, Mattera J, et al. Blood cultures have limited value in predicting severity of illness and as a diagnostic tool in ventilator-associated pneumonia. Chest 1999; 116:1075–1084.
26. El-Ebiary M, Torres A, González J, et al. Quantitative cultures of endotracheal aspirates for the diagnosis of ventilator-associated pneumonia. Am J Respir Crit Care Med 1993;147:1552–1557
27. Maha M, Fatma A, Tharwat E. Diagnostic value of bronchoalveolar lavage in ventilator associated pneumonia of pediatric patients. Egypt J Med Lab Sci, 2008; 17(1):11-25.
28. Hassan A, Samy A, Bedewy K, et al. VAP: The role of typical bacteria and atypical bacteria and Fungi.Comparison between endotracheal aspirates and bronchoalveolar lavage.. EJB, 2007;1(1)1: 53-61.
29. Fabrice Ml, Bruno F, Pierre B. Early Antibiotic Treatment for BAL-Confirmed Ventilator-Associated Pneumonia: A Role for Routine Endotracheal Aspirate Cultures. Chest 2005;127;589-597
30. Emine A, Andreas V. Ventilator associated pneumonia and infection control. Ann Clin Microb Antimicrob 2006, 5:7.
31. Initial Antibiotic Choice Important in Ventilator-Associated Pneumonia. AvailableURL: http://www.medpagetoday.com/MeetingCoverage/SCCM/8257
32. Combes A, Corionne F, Jean Louis T, et al. Incidence and Outcome of Polymicrobial Ventilator-Associated Pneumonia. Chest 2002; 121: 1618-1623.
33. Chien Liang W, Yang D, Wang N, Tah Kuo H, et al. Quantitative Culture of Endotracheal Ventilator-Associated Pneumonia in Patients Aspirates in the Diagnosis of With Treatment Failure. Chest 2002;122;662-668.
34. Chastre J, Fagon JY. Ventilator associated pneumonia. Am J Respir Crit Care Med 2002; 165: 867-903.
35. Bachinskaya N, Beloborodova N, Nonikov E. Efficacy of cefepime plus clarithromycin versus cefepime alone in patients with ventilator-associated pneumonia after cardiac surgery: prospective clinical trial. 16th European Congress of Clinical Microbiology and Infectious Diseases Nice, France, April 2006.
36. Apfalter, Petra , Stoiser ,et al. Community-acquired bacteria frequently detected by means of quantitative polymerase chain reaction in nosocomial early-onset ventilator-associated pneumonia . Crit Care Med 2005 ;33(7):1492-1498.
37. El-Ebiary M, Torres A, Gonzalez J, et al. Quantitative cultures of endotracheal aspirates for the diagnosis of ventilator associated pneumonia. Am Rev Respir Dis 1993; 148: 1552-7.

Synthesis of Some New Benzoxazole Acetonitrile Derivatives and Evaluation of Their Herbicidal Efficiency.

M.A.youssef[a], S.M.A.Sherif[b], A.M.A.Elkady[c] and S.E.S.Hamouda[c].

a Chemistry Department , Faculty of Science , Helwan University.

b Chemistry Department , Faculty of Science , Cairo University .

c Central Agricultural Pesticides Lab. (CAPL), Agriculture Research Center (ARC), Cairo, Egypt.

Abstract: Twenty three new 2-cyanomethyl benzoxazole derivatives were synthesized by different methods. Their structures were elucidated by many ways as elemental analysis, spectroscopic analysis and chemical methods. The herbicidal activity of the newly synthesized compounds was evaluated against wheat as pattern for monocotyledonous plants, three plant parameters were studied, seed gerimination, root and shoot growth under laboratory conditions. Compounds that showed an observable inhibition on one or more of the growth parameters under study were considered as promising compounds and needs more studies from the toxicological, soil, environmental and formulation points of view to stand on the most potent derivative that can be formulated in a suitable formulation form to be used in the field of pest control. Compounds (**16a**),(**16b**),(**16f**),(**13b**),(**10a**),(**7a**) and (**3b**) inhibited all growth parameters under study by different degrees. While compounds (**13b**) and (**13a**) were more effective on root and gerimination respectively. Most synthesized compounds inhibited markedly shoot growth.

[M.A.youssef, S.M.A.Sherif, A.M.A.Elkady and S.E.S.Hamouda. **Synthesis of Some New Benzoxazole Acetonitrile Derivatives and Evaluation of Their Herbicidal Efficiency.** Journal of American Science 2010;6(12):1080-1090]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: 2-cyanomethyl benzoxazole , 2-arylidene cyanomethyl benzoxazole, herbicidal activity, growth parameters, wheat and monocotyledonous plants.

Introduction

Our present work is a continuation of our ongoing programme in utilizing readily obtainable materials in the synthesis of different biologically active heterocylces [1].2-cyanomethyl benzoxazole and its related compounds play an important role in pharmaceutical and agricultural fields due to their broad spectrum of biological activities. Such a ring system has been reported to be used as herbicides [2-14], bactericides [15], fungicides [16-25], antiviral [26-30] and antimicrobial agents [22]. Also , 2-cyanomethyl benzoxazole connected to pyridine carbonitriles have been found to be of great interest due to their broad spectrum of biological activities [31]. So that depending on the biological activities and due to the high chemical activity of 2-cyanomethyl benzoxazole ring system , it was found that synthesis and study of the herbicidal activity of some new 2-cyanomethyl benzoxazole derivatives is a subject of great interest hoping that these new compounds could be applied as a new herbicidal agents in the field of pest control.

Results and discussion

1-Chemistry part

The key starting material 2-cyanomethyle benzoxazole (**1**) is characterized by the presence of an active methylene as well as nitrile group which makes it chemically very active so it can be used as a precursor to synthesize many biologically and chemically active ring systems. Condensation of acetonitrile (**1**) with different heterocyclic or aromatic aldehydes in ethanol and triethylamine as an organic base under reflux afforded -2- arylidene benzoxazole-2- acetonitrile (**za-e**),[31](c.f.Schem1). both elemental and spectral data of the obtained compounds are consistent with the assigned structure (c.f.Experimental). benzoxazole (**1**) as a result of the presence of the active methylene, it was allowed to react with o-hydroxy napthaldhyde (**4**) in ethanol and ammonium acetate to give coumarine (**5**)[32,33], the structure that was supported by the disappearance of the characteristic absorption band of CN with the appearance of carbonyl group band in IR spectra as well as the appearance of the signal corresponding to the ethylenic proton of coumarine ring as singlet at

9.92 ppm in 1H-NMR spectrum of compound (**5**) due to oxidation of nitrile group followed by elimination of water and cyclization on coumarine ring .

Moreover , mass spectrum of compound (**5**) showed the expected molecular ion peak at m/z=313 with relative abundance of 27.7 corresponding to the correct molecular formula . in the same way ,2-cyanomethyle benzoxaole (**1**) on treatment with different ketones (**6**) in ethanol in the presence of piperidine as a basic catalyst under reflux afforded α-(benzoxazole -2-yl)-B- alkyl (aryl) crotonitrile (**7a-c**).

Structural elucidation of derivatives (**7a-c**) was carried out by different ways as elemental analyses as well as spectroscopic data, whereas, IR spectra of compound (**7a**) revealed the appearance of the characteristic absorption band for CH aliphatic at 2225 cm^{-1} region. Also, ^{1}H-NMR (DMSO-d6) of the same derivative showed two singlets at 2.40 and 2.55 ppm corresponding to two methyl groups of compound (**7a**)

Moreover , mass spectrum of this compound showed the expected molecular ion peak at m/z= 198 with relative abundance of 50.0 corresponding to the correct molecular formula.

On the other hand, compound (**1**) was reacted with α,B – unsaturated nitriles (**8a-c**) in ethanol in the presence of piperidine as a basic catalyst under reflux to afford the corresponding dicarbonitrile derivatives (**10a-d**). structural elucidation of derivatives (**10a-d**) was carried out by different ways as elemental analyses, spectroscopic data as well as chemical ways. Whereas, IR spectra of all obtained compounds revealed two absorption bands at 1940 and 2198 cm $^{-1}$ region due to the presence of two (CN) functional groups . Also, structure (**10**) was confirmed by the presence of the characteristic signal at 5.23 ppm in ^{1}H-NMR spectrum of compound (**10b**) due to the formation of NH_2 functional group. More over the mass spectrum of compound (**10b**) showed the expected molecular ion peak at m/z=346 with relative abundance of 2.6 corresponding to the correct molecular formula .

Refluxing arylidene (**3**) with malononitrile (**a**) in ethanol in the presence of sodium ethoxide gave dicarbonitrile (**10d**) which was confirmed by m.p,mixed m.p and IR spectrum that revealed the presence of two absorption bands at 1940, 2195 cm $^{-1}$ region corresponding to two (CN) functional groups and mass spectrum of the same derivative that displayed molecular ion peak at m/z =401 with relative abundance of 2.9corresponding to the molecular formula $C_{22}H_{18}N_4O_4$.

Benzoxoazole (**1**) on treatment with the arylidene ethyl α-cyanocinnamate (**11**) in ethanol in the presence of sodium ethoxide as a basic catalyst afforded pyridine carboxylic esters (**13a-d**).

Structures (**13a-d**) was confirmed by different ways as elemental analyses and spectroscopic analyses. whereas , IR spectra of all obtained compounds revealed new absorption bands at 3375, 3237 and 1662 cm $^{-1}$ region due to the presence of NH_2 and C=O group . Also, ^{1}H-NMR (DMSO-d6) of compound (**13b**) showed signals at 1.28-1.33 (t,3H,CH_3),4.26-4.36 (q,2H,CH_2), 7.34-7.39 (m,3H,thiophene – H) , 8.07-8.23(m-5H,Ar-H)and 8.62(s,2H,NH_2). Moreover ,treatment of arylidene (3)with ethyl cyano acetate (**12**) in ethanol and sodium ethoxide as a basic catalyst under reflux gave pyridine carboxy ester(**13 a**), which was considerd as chemical tool for structural elucidation of compounds (**13b-d**) . Structure of (**13 a**) was confirmed by compatible elemental analysis and spectral data (c.f. Experimental).

In addition, 2-cyanomethyl benzoxazole (**2**)was reacted with α,β-unsaturated nitriles(**14**) in ethanol and sodium ethoxide under reflux to afford 3-cyanopyridine -2(1H)-(thi)ones(**16a-f**) was carried out by different ways as elemental analysis, Spectroscopic data as well as chemical ways. Where as IR spectra of all obtained compounds revealed new observation band at 1244 and 1657 cm^{-1} region due to the presence of c=s and c=o functional groups in pyridine thions and pyridine ones respectively .Also, ^{1}H-NMR (DMSO-d6) of compound (**16e**) showed signals at 2.72 (a,3H,cH_3) 6.62-6.86 (br.s,2H,NH_2), 7.7.49-7.96 (m,8H,Ar-H) and 8.0 (s,1H,pyridine –NH). Moreover, mass spectrum of compound (**16e**) showed the expected molecular ion peak at m/z=371 with relative abundance of 8.9 corresponding to the correct molecular formula.

As another method to elucidate structure of compound (**16**), arylidene (**3**) was treated with cyano(thio)acetamidein ethanol in the presence of catalyst amount of sodium ethoxide to afford compounds (**16d,f**).both elemental and spectral data of compounds (**16d,f**) provided satisfying evidence for the proposed structures (c.f. Experimental).

EXPERMENTAL

All melting points are uncorrected and were determined on an electric melting point (Gallenkamp) 9200 A apparatus. IR spectra were recorded (KBr) on pye Unicam SP-1000 Spectrophotometer. 1H–NMR spectra were obtained from Varian Gemini 200 MHz spectrometer and chemical shifts are expressed in δ (ppm) using TMS as internal reference. Mass spectra were recorded on a GCMS–QP 1000 mass spectrometer opening at 70 eV. Microanalytical data were obtained from the microanalytical data center at Cairo University.

2-Arylidene benzoxazole–2–acetonitrile (3a–e).

Method A

Solution of (1,3–benzoxazole–2–yl) acetonitrile (1) (1.58g, 0.01 mole) in absolute ethanol (30 ml) was stirred with triethylamine or piperidine (seven drops) for 1h and the appropriate aldhyde (2) (0.01 mole) was added gradually to the reaction mixture and stirring was maintained for about 4 hrs. The formed crystalline precipitate was filtered off, washed with ethanol, dried and crystallized from the proper solvent to afford arylidenes (3a–e).

Method B

A mixture of (1,3–benzoxazole–2–yl) acetonitrile (1) (1.58g, 0.01 mole), aromatic or heterocyclic aldhyde (2) (0.01 mole) and piperidine (five drops) in ethanol (30 ml) was heated under reflux for 2 hrs. The reaction mixture was left aside at room temperature to cool, poured into an acidified crushed ice and filtered off. The obtained solid product was crystallized from suitable solvent to give arylidenes (3a–e).

(3a) yield (2.45g, 90%); (pet.ether); mp 163–65ºC. IR (cm1): 3083 (CH–arom), 2925 (CH–aliph.), 2221 (CN), 1606 (C=N), 1583 (C=C), 1030 (C-O-C); 1H–NMR (DMSO–d6): 7.33-7.48 (m, 3H, thiophene-H), 7.81-8.18 (m,4H, benzoxazole–H), 8.73 (s, 1H, =CH). MS m/z (%): 254 (M+ +2, 19.2), 253 (M+ +1, 13.1), 252 (M+ , not detected), 251 (M+ -1, 41.5), 196 (16.9), 179 (10.8), 158 (5.4), 63 (100). Ana. calcd. for C14H8N2OS (252.27) C66.65, H3.19, N11.10, S12.71. Found: C66.29, H3.09, N11.19, S12.85%.

(3b) yield (2.73g, 80%); (n–Hexane); mp 159-61ºC. IR (cm^{-1}): 3064 (CH–arom.), 2923 (CH-aliph.) 2227 (CN), 1601 (C=N), 1542 (C=C), 1031 (C-O-C); ^{1}H–NMR (DMSO–d6): 7.49–7.90 (m, 4H, benzoxazole–H), 8.14–8.18 (m, 4H, Ar–H), 8.57 (S, 1H, =CH). MS m/z (%): 328 (M^+ + 3, 0.1), 327 (M^+ + 2, 1.0), 326 (M^+ + 1, 4.2), 325 (M^+, 4.7), 324 (M^+ -1, 4.4), 245 (100), 123 (11.3), 63 (100). Ana. calcd. for $C_{16}H_9BrN_2O$ (325.16) C 59.10, H 2.79, Br 24.57, N 8.62. Found: C 59.07, H 2.70, Br 24.53, N 8.57%.

(3c) yield (2.53g, 85%); (pet. ether); mp 163–65ºC. IR (cm^{-1}): 3055 (CH–arom.), 2926 (CH–aliph), 2229 (CN), 1608 (C=N), 1583 (C=C), 1042 (C-O-C); MS m/z (%): 282 (M^+ + 2, 2.0), 281 (M^+ + 1, 3.7), 280 (M^+ , 8.1), 279 (M^+ -1, 7.3), 245 (66.1), 216 (12.16), 63 (100). Ana. Calcd for. $C_{16}H_9ClN_2O$ (280.71) C 68.46, H 3.23, Cl 12.63, N 9.98. Found: C 68.39, H 3.17, C l12.48, N 9.75%.

(3d) yield (2.60g, 85%); (EtOH); mp 160-62ºC. IR (cm^{-1}): 3067 (CH-arom.), 2919 (CH-aliph.), 2230 (CN), 1606 (C=N), 1549 (C=C), 1060 (C-O-C); MS m/z (%): 288 (M^+, not detected), 287 (M^+ -1, 60.0), 245 (80.0), 128 (66.7), 56 (100). Ana. calcd. for C_{19} H_{16} N_2O (288.34) C 79.14, H 5.59, N 9.72. Found: C 79.06, H 5.54, N 9.57%.

(3e) yield (2.76g, 90%); (Toluene); mp 246–48ºC. IR (cm^1): 3022 (CH–arom.), 2915 (CH–aliph), 2211 (CN), 1603 (C=N), 1570 (C=C), 1063 (C–O–C); ^{1}H–NMR (DMSO–d6): 3.13(s, 6H, 2CH3), 6.88–8.08 (m, 8H, Ar-H), 8.28 (s, 1H,=CH). MS m/z (%): 290 (M^+ + 1, 13.9), 289 (M^+, 89.7), 288 (M^+ -1, 100), 144 (11.8), 127 (7.5), 63 (86.9). Ana. Calcd. for. $C_{18}H_{15}N_3O$ (289.33) C 74.42, H 5.23, N 14.52. Found: C 74.55, H 5.18, N 14.44%.

2-(-2`H-Chromn-2`-oxo-3`-yl-(5`,6`-e)-napthyl)benzoxazole (5).

To a solution of (1,3-benzoxazole-2-yl) acetonitrile **(1)** (1.58g, 0.01 mole), ammonium acetate (7.79, 0.01 mole) in ethanol (40 ml), 2-hydroxyl napthaldhyde **(4)** (1.72g, 0.01 mole) was added and the reaction mixture was heated under reflux for 4 hrs. The reaction mixture was left to cool at room temperature, poured into crushed ice, filtered off and the finally obtained solid product was crystallized from toluene to afford coumarine derivative **(5)**.

(5) yield (2.31g, 70%); (Toluene); mp 242-44ºC. IR (cm^1): 3055 (CH-arom.), 2922 (CH-**aliph**), 1746 (C=O), 1622 (C=N), 1563 (C=C), 1030 (C-O-C); ^{1}H–NMR (DMSO–d6): 7.52–7.90 (m, 6H, Ar-H), 8.10–8.73 (m, 4H, benzoxazole–H), 9.92 (s, 1H, =CH). MS m/z (%): 314 (M^+ + 1, 8.0), 313 (M^+ , 27.7), 312 (M^+ -1, 8.8), 285 (19), 228 (13.9), 164 (18.2), 138 (10.2), 63 (100). Ana. Calcd. for. $C_{20}H_{11}NO_3$ (313.31) C 76.67, H 3.54, N 4.47. Found: C 76.65, H 3.47, N 4.45%.

α-(Benzoxazole-2`-yl)-β-alkyl (aryl) crotonitrile (7).

(1,3-Benzoxazole-2-yl) acetonitrile (1.58g, 0.01 mole) was stirred in ethanol (40 ml) containing catalytic amount of piperidine (0.5 ml, 0.02 mole) for l h, ketone **(6)** (0.01 mole) was added. The reaction mixture was heated under reflux, left to cool at room temperature, poured into an acidified crushed ice, filtered off and the finally obtained solid product was crystallized from ethanol to give crotonitrile derivatives **(7a-c)**.

(7a) yield (1.94g, 90%); (EtOH); mp 128–30ºC. IR (cm^{-1}): 3060 (CH–arom.), 2922 (CH–aliph), 2225 (CN), 1603 (C=N), 1550 (C=C), 1038 (C–O–C); ^{1}H–NMR (DMSO–d6): 2.40 (S, 3H, CH_3) 2.55 (s, 3H, CH3), 7.40–7.48 (s, 2H, benzoxazole–H), 7.78–7.83 (s, 2H, benzoxazole–H). MS m/z (%): 199 (M^+ + 1, 16.7), 198 (M^+, 50.5), 159 (25.0), 158 (58.3), 63 (100). Ana. Calcd. for. $C_{12}H_{10}N_2O$ (198.22) C 72.71, H 5.08, N 14.13. Found: C 72.70, H 5.01, N 14.9%.

(7b) yield (1.619, 70%); (EtOH); mp 66–68ºC. IR (cm^{-1}): 3057 (CH–arom.), 2975 (CH-aliph), 2225 (CN), 1612 (C=N), 1552 (C=C), 1045 (C–O–C); MS m/z (%): 213 (M^+ + 1, 10.06), 212 (M^+ , 79.8), 211 (M^+ -1, 34.6) 198 (5.8), 158 (29.8), 133 (39.4), 93 (22.1), 63 (100). Ana. Calcd. for. $C_{13}H_{12}N_2O$ (212.25) C 73.56, H 5.70, N 13.20. Found: C 73.49, H 5.65, N 13.14%.

(7c) yield (2.519, 80%); (EtOH); mp 88–90ºC. IR (cm^{-1}): 3105 (CH-arom.), 2935 (CH-aliph), 2210 (CN), 1604 (C=N), 1523 (C=C), 1106 (C-O-C). Ana. Calcd. for. $C_{17}H_{11}N_3O_3$ (305.29) C 66.88, H 3.63, N 13.76. Found: C 66.64, H 6.53, N 13.72%.

1-Amino-3-aryl-3H-pyrido[2,1–b]benzoxazole-2,4-dicarbo–nitriles (10a-d).

Method A

A mixture of arylidene malonoitrile **(8a–c)** (0.01 mole), (1,3-benzoxazole-2-yl) acetonitrile **(1)** (1.58g, 0.01 mole) in ethanol (30 ml) containing catalytic amount of piperidine (0.5 ml, 0.02 mole) were heated under reflux for 5 hrs. The reaction mixture was left to cool at room temperature, poured into an acidified crushed ice, filtered off, left to dry and the obtained solid product was crystallized from a suitable solvent to afford the substituted amino pyridine dicarbonitrile derivatives **(10a–c)**.

Method B

2-arylidene benzoxazole–2–acetonitrile **(3)** (0.01 mole), sodium ethoxide [prepared from sodium metal (0.46 g, 0.02 mole) in ethanol (30 ml)] and malonoitrile **(9)** (0.66 g, 0.01 mole) was heated under reflux for 7 hrs. The reaction mixture was left aside at room temperature to cool, poured into an acidified crushed ice and the precipitated solid product was filtered off, dried and crystallized from ethanol to give dicarbonitrile derivative **(10d)**.

(10a) yield (2.54 g, 80%); (EtOH); mp 180–82ºC. IR (cm^{-1}): 3327, 3219 (NH_2), 3085 (CH–arom.), 2210, 2340 (CN), 1611 (C=C), 1033 (C–O–C); MS m/z (%): 319 (M^+ + 1, 20.7), 318 (M^+ , 31.0), 291 (34.5), 226 (17.2), 194 (20.71), 127 (17.2), 63 (100). Ana. Calcd. for. $C_{17}H_{10}N_4OS$ (318.35): C 64.14, H 3.17, N 17.60, S 10.07. Found: C 64.11, H 3.12, N 17.48, S 10.10%.

(10b) yield (2.78 g, 80%); (Acetonitrile); mp 245-47ºC. IR (cm^{-1}): 3460, 3437 (NH_2), 3061 (CH-arom.), 2198, 1940 (CN), 1642 (C=C), 1039 (C–O–C); ^{1}H-NMR (DMSO–d_6): 5.23 (s, 2H, NH_2), 6.69 (s, 1H, pyridine-H), 7.32–7.53 (m, 4H, Ar-H), 7.66–7.78 (m, 4H, benzoxazole-H). MS m/z (%): 347 (M^+ + 1, 1.1), 346 (M^+ , 2.6), 345 (M^+ -1, 1.1), 309 (2.4), 279 (12.8), 245 (100), 216 (15.6), 158 (9.6), 126 (10.4), 63 (89.8). Ana. Calcd. for. $C_{19}H_{11}ClN_4O$ (346.77): C 65.81, H 3.20, Cl 10.22, N 16.16. Found: C 65.76, H 3.11, Cl 10.19, N 16.11%.

(10c) yield (3.19 g, 90%); (EtOH); mp 248–50ºC. IR (cm^{1}): 3433, 3313 (NH_2), 3060 (CH–arom.), 2922 (CH–aliph), 2195, 1941 (CN), 1598 (C=C), 1039 (C-O-C); MS m/z (%): 358 (M^+ + 3, 29.4), 355 (M^+ , not detected), 305 (100), 262 (17.6), 181 (29.4), 174 (23.5), 77 (94.1). Ana. Calcd. for. $C_{21}H_{17}N_5O$ (355.39): C 70.97, H 4.82, N 19.71. Found: C 70.78, H 4.73, N 19.64%.

(10d) yield (3.61g, 90%); (EtOH); mp 140–42ºC.IR (cm^{-1}): 3313, 3227 (NH_2), 3081 (CH–arom.), 2920 (CH–aliph), 2195, 1940 (CN), 1627 (C=C), 1040 (C–O–C); MS m/z (%): 402 (M^+, not detected), 401(M^+ -1, 2.9), 327 (9.8), 291 (2.9), 245 (46.8), 158 (24.9), 63 (100). Ana. Calcd for. $C_{22}H_{18}N_4O_4$ (402.4): C 65.66, H 4.51, N 13.92. Found: C 65.54, H 4.41, N 13.64%.

1-Amino-3-aryl-3H-pyrido[2,1-b)benzoxazole-4-carbonitrile-2-ethyl carboxylate (13a-d).

Method A

To a solution of (1,3-benzoxazole-2-yl) acetonitrile **(1)** (1.58 g, 0.01 mole), sodium ethoxide [prepared from sodium metal (0.46 g, 0.02 mole) in absolute ethanol (40 ml)], arylidene ethyl α-cyanocinnamate **(11)** (0.01 mole) was added, and the reaction mixture was heated under reflux for 10 hrs. The reaction mixture was left to cool at room temperature, poured into an acidified crushed ice, filtered off, dried and crystallized from ethanol to afford pyridine ethyl carboxylate derivatives **(13b–d)**.

Method B

2-Arylidene benzoxazole-2-acetonitrile **(3)** (0.01 mole), catalytic amount of sodium ethoxide [prepared from sodium metal (0.46 g, 0.02 mole) in ethanol (40 ml)] and ethyl cyanoacetate **(12)** (1.13 g, 0.01 mole) was heated under reflux for 10 hrs. The reaction mixture was left to cool, then poured into ice cold water mixture and neutralized with dilute hydrochloric acid. The solid product which

precipitate was collected by filtration, washed with ethanol, dried and crystallized from ethanol to afford compound **(13a)**.

(13a) yield (2.72 g, 70%); (EtOH); mp 175–77ºC. IR (cm^{-1}): 3431, 3330 (NH_2), 3042 (CH-arom.), 2978 (CH-aliph), 2220 (CN), 1704 (C=O), 1600 (C=C) and 1023 (C–O–C); MS m/z (%): 389 (M^+ , not detected), 386 (M^+ -3, 0.17), 305 (36.38), 275 (100), 232 (15.72), 181 (13.54), 149 (13.64). Ana. Calcd. for. $C_{22}H_{19}N_3O_4$ (389.40): C 67.86, H 4.92, N 10.79. Found: C 67.84, H 4.74, N 10.65%.

(13b) yield (2.37 g, 65%); (EtOH); mp 200–02ºC. IR (cm^{-1}): 3375, 3237 (NH2), 3103 (CH–arom.), 2923 (CH–aliph), 2221 (CN), 1662 (C=O), 1584 (C=C), 1034 (C–O–C); ^{1}H–NMR (DMSO–d_6): 1.28–135 (t, 3H, CH_3), 4.26–4.36 (q, 2H. CH2), 7.34–7.39 (m, 3H, thiophene-H), 8.07–8.23 (m, 5H, Ar-H), 8.62 (s, 2H, NH_2). MS m/z (%): 366 (M^+ + 1, 9.8), 365 (M+, 4.9), 289 (100), 288 (85.2), 144 (31.1), 63 (73.8). Ana. Calcd for. $C_{19}H_{15}N_3O_3S$ (365.41): C 62.45, H 4.14, N 11.56, S 8.78. Found: C 62.34, H 4.11, N 11.44, S 8.75%.

(13c) yield (3 g, 75%); (EtOH); mp 195–97ºC. IR (cm^{-1}): 3648, 3616 (NH_2), 3022 (CH–arom.), 2912 (CH–aliph), 2211 (CN), 1793 (C=O), 1605 (C=C), 1033 (C-O-C). Ana. Calcd. for. $C_{23}H_{22}N_4O_3$ (402.45): C 68.64, H 5.51, N 13.92. Found: C 68.34, H 5.24, N 13.69%.

(13d) yield (2.74 g, 70%); (EtOH); mp 180–82ºC. IR (cm^{-1}): 3389 (NH_2), 3059 (CH-arom.), 2930 (CH-aliph), 2218 (CN), 1742 (C=O), 1613 (C=C), 1046 (C-O-C). Ana. Calcd. for. $C_{21}H_{16}N_3O_3$ (393.82): C 64.05, H 4.09, Cl 9.00, N 10.67. Found: C 63.69, H 3.97, Cl 8.93, N 10.55%.

6–Amino-5-(benzoxazole-2-yl)-4-aryl-3-cyanopyridine-2 (1H)-thio) nes) (16a-f).

Method A

A mixture of α,β-unsaturated nitrile **(14)** (0.01 mole), (1,3-benzoxazole-2-yl) acetonitrile **(1)** (1.58 g, 0.01 mole), sodium ethoxide [prepared from sodium metal (0.46 g, 0.02 mole) in ethanol (40 ml)] was heated under reflux for 12 hrs. The reaction mixture was left to cool at room temperature, poured into an acidified crushed ice, filtered off and the finally obtained solid product was crystallized from a suitable solvent to afford the substituted 3-cyano-pyridine-2(1H)-(thi) ones **(16a-c,e)**.

Method B

Equimolar amount of 2-arylidene benzoxazole-2-acetonitrile (**3**), cyano (thio) a cetamide (**15**) in ethanol (40 ml) containing sodium metal (0.46 g, 0.02 mole) was heated under reflux for 12 hrs. The reaction mixture was treated as before to produce the corresponding 3-cyanopyridine-2(1H)-(thi)one derivatives (**16d,f**).

(16a) yield (3.16 g, 90%); (ACOH); 219–20ºC. IR (cm-1): 3329, 3206 (NH2, NH), 2216 (CN), 1614 (C=N), 1555 (C=C), 1244 (C=S), 1048 (C–O–C); 1H–NMR (DMSO–d6): 6.86–7.20 (br.s, 2H, NH2), 7.5 (s, 3H, thiophene-H), 7.73 (s, 4H, benzoxazole–H), 8.98 (s, 1H, pyridine–NH). MS m/z (%): 350 (M+, 12.5), 298 (12.5), 287 (18.1), 236 (100), 146 (50), 125 (51.4). Ana. Calcd. for C17H10N4OS3 (350.42): C 58.27, H 2.88, N 15.99, S 18.30. Found: C 58.18, H 2.75, N 15.86, S 18.09%.

(16b) yield (3.19 g, 85%); (EtOH); mp 195-97ºC. IR (cm^{-1}): 3358, 3250 (NH_2, NH), 2967 (CH-aliph.), 2211 (CN), 1608 (C=N), 1564 (C=C), 1247 (C=S), 1027 (C-O-C). Ana. Calcd. for $C_{20}H_{14}N_4O_2$ (372.42): C 64.16, H 3.77, N 14.96, S 8.56. Found: C 64.03, H 3.66, N 14.89, S 8.49%.

(16c) yield (2.96, 78%); (EtOH); mp 115-17ºC. IR (cm^{-1}): 3345, 3250 (NH_2, NH), 2212 (CN), 1613 (C=N), 1552 (C=C), 1242 (C=S), 1173 (C-O-C). Ana. Calcd. for $C_{19}H_{11}ClN_4OS$ (378.83): C 60.24, H 2.93, Cl 9.36, N 14.79, S 8.46. Found: C60.18, H 2.85, Cl 9.23, N 14.64, S 8.42%.

(16d) yield (3.11 g, 80%); (ACOH); mp 183-85ºC. IR (cm^{-1}): 3353, 3206 (NH_2, NH), 2918 (CH-aliph..), 2210 (CN), 1606 (C=N), 1560 (C=C), 1242 (C=S), 1193 (C-O-C); ^{1}H–NMR (DMSO–d6): 2.94-3.02 (s, 6H, $(CH_3)_2N$), 4.5 (br.s, 2H, NH_2), 6.87-7.73 (m, 8H, Ar-H), 9.98 (s, 1H, pyridine-NH). Ana. Calcd. for $C_{21}H_{17}N_5OS$ (387.46): C 65.10, H 4.42, N 18.08, S 8.28. Found: C 64.98, H 4.38, N 17.88, S 8.19%.

(16e) yield (2.23 g, 60%); (DMF); mp > 300°C. IR (cm^{-1}): 3370, 3157 (NH_2, NH), 2924 (CH–aliph.), 2210 (CN), 1657 (C=O), 1607 (C=N), 1523 (C=C), 1168 (C-O-C); ^{1}H–NMR (DMSO–d6): 2.72 (s, 3H, CH_3), 2.89 (s, 3H, CH_3), 6.62–6.86 (br.s, 2H. NH_2), 7.49-7.96 (m, 8H, Ar-H), 8.0 (s, 1H, pyridine–NH). MS m/z (%): 372 (M^+ + 1, 2.5), 371 (H^+, 8.9), 369 (M^+ -2, 2.5), 368 (M^+ -3, 10.1), 338 (29.1), 327 (25.3), 299 (12.7), 275 (26.6), 247 (19.0), 191 (12.7), 158 (24.1), 134 (26.6), 98 (60.8), 60 (100). Ana. Calcd. for $C_{21}H_{17}N_5OS$ (371.39): C

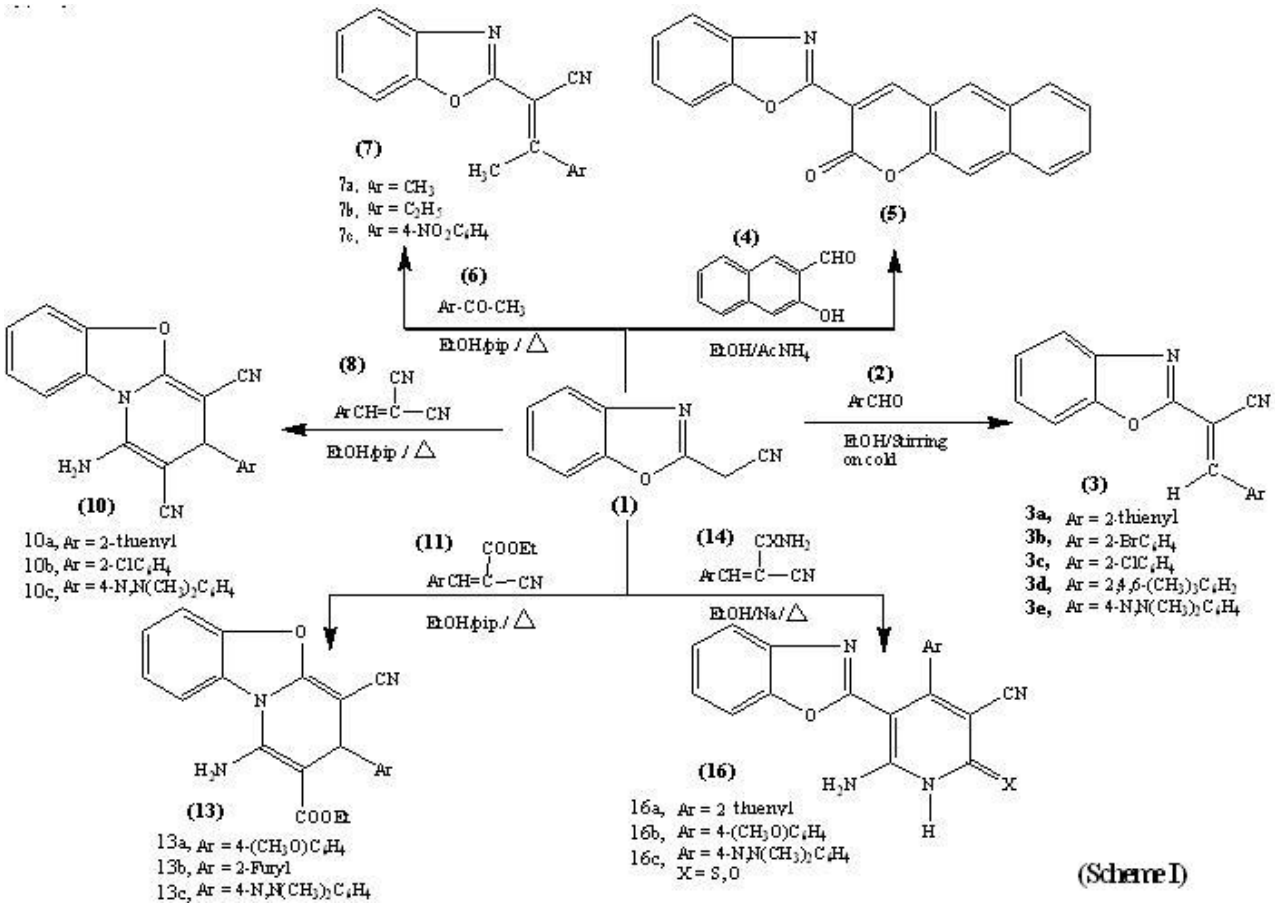
67.91, H 4.61, N 18.86. Found: C 67.88, H 4.56, N 18.79%.

(16f) yield (3.28 g, 90%); (EtOH/H_2O); mp 168–70ºC.
IR (cm^{-1}): 3743, 3357 (NH_2, NH), 2217 (CN), 1619 (C=O), 1572 (C=N), 1048 (C-O-C); ^{1}H–NMR (DMSO–d_6): 4.78 (s, 2H, NH_2), 6.95–7.68 (m, 8H, Ar–H), 11.90 (s, 1H, pyridine–NH). Ana. Calcd. for $C_{19}H_{11}ClN_4O_2$ (362.77): C 62.91, H 3.03, Cl 9.77, N 15.44. Found: C 62.75, H 3.11, Cl 9.65, N 15.37%.

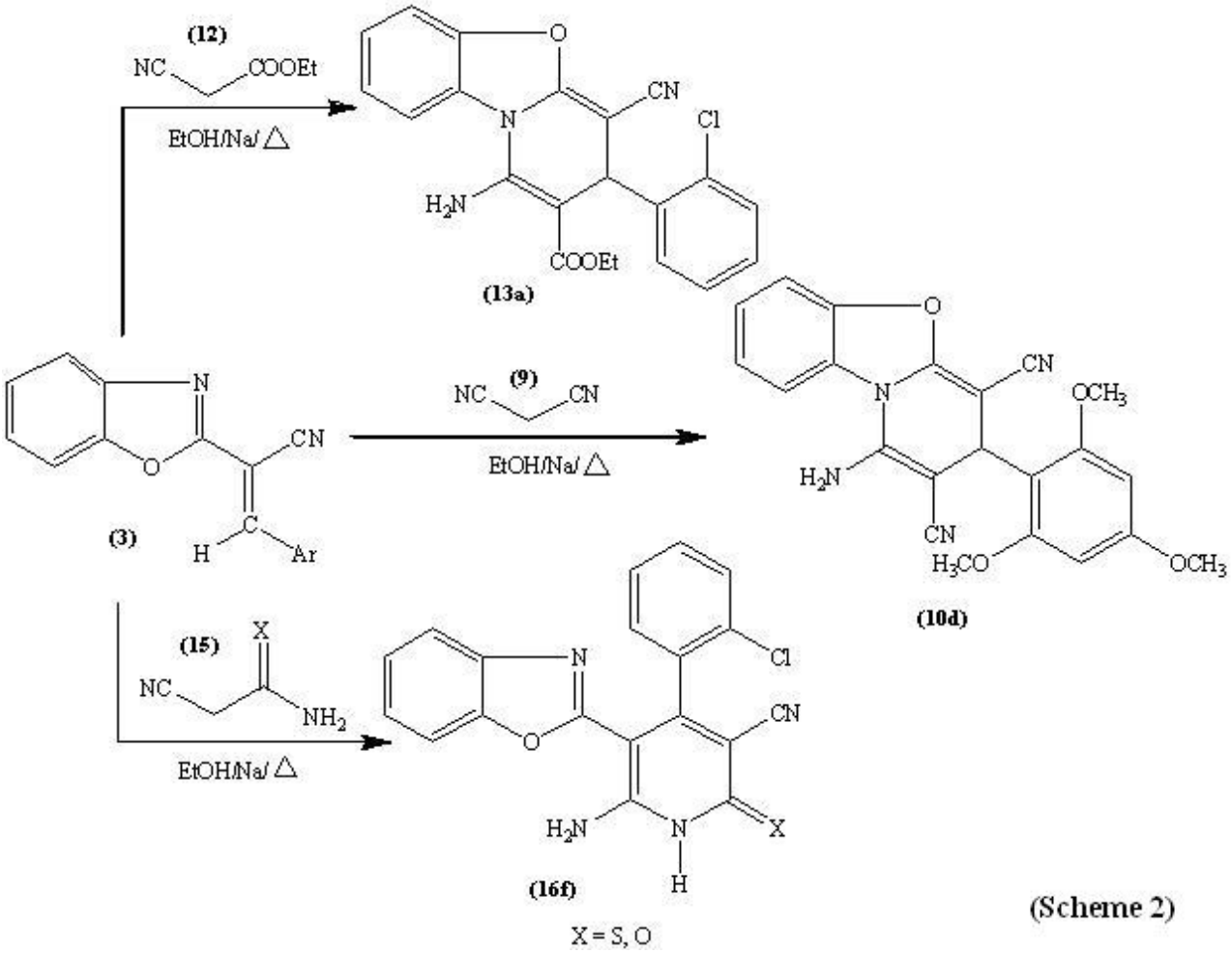
2- Biological study

The herbicidal evaluation of the newly synthesized compounds on wheat as pattern for monocotyledonous plants.

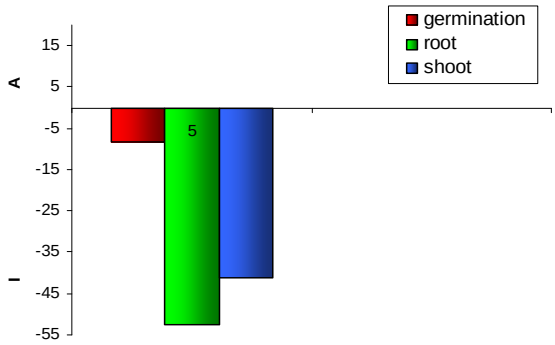
The herbicidal efficiency of the newly synthesized compounds and their derivatives was evaluated laboratory conditions against wheat as pattern for monocotyledonous plants with concentration of 2000 ppm. the percentages of inhibition of wheat growth parameters such as gerimination, root and shoot growth were taken as indicators to determine the herbicidal effect of these compounds .



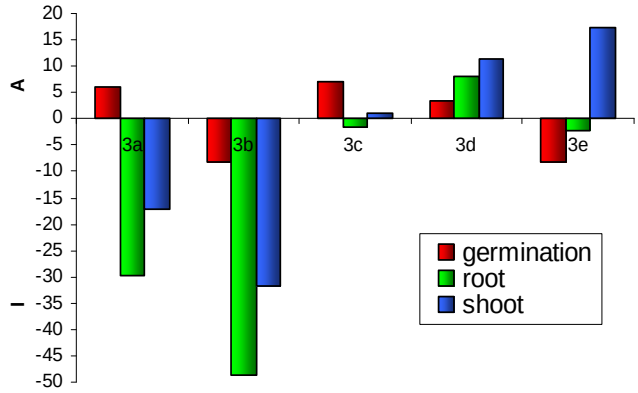
(Scheme I)



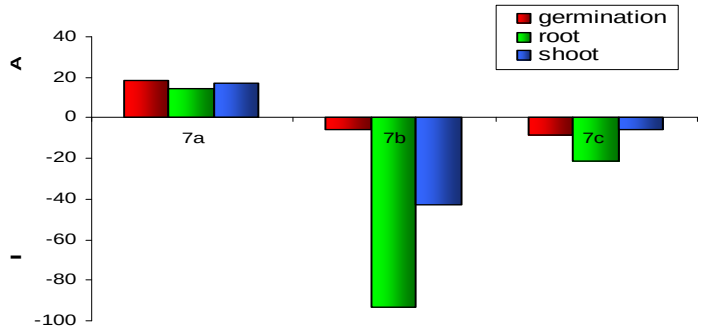
(Scheme 2)



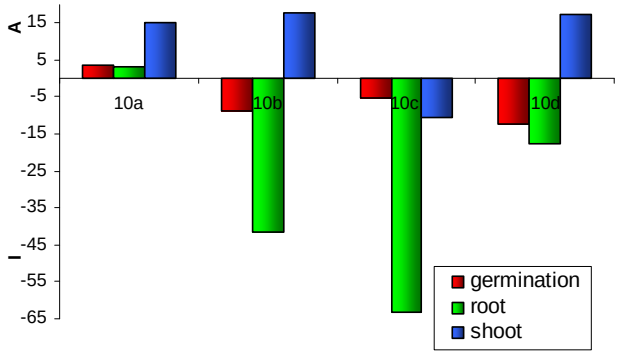
The effect of arylidenes (3a-e) on germination, root and shoot growth of wheat.



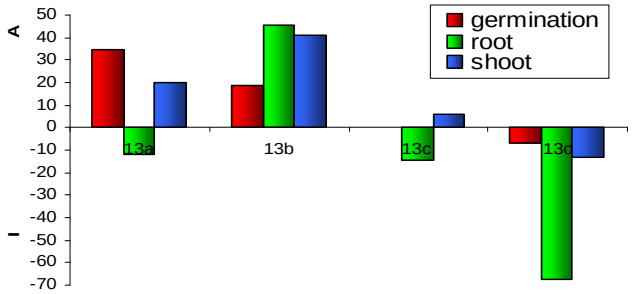
The effect of Coumarine (5) on germination, root and shoot growth of wheat.



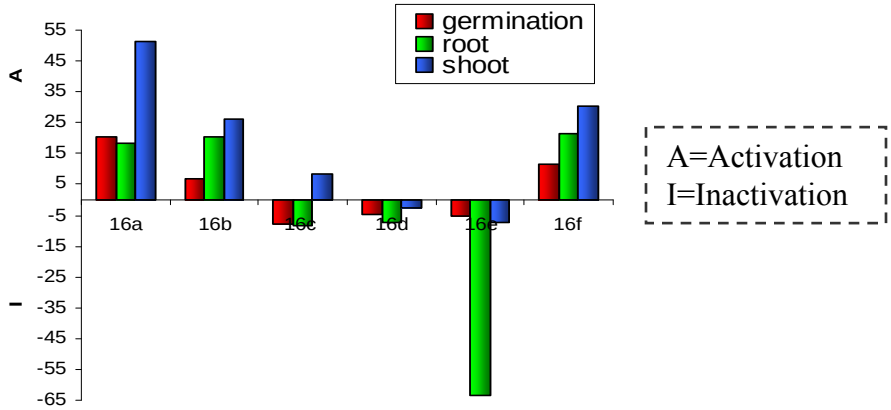
The effect of benzoxazoles (7a-c) on germination, root and shoot growth of wheat.



The effect of pyridobenzoxazoles (10a-d) on germination, root and shoot growth of wheat.



The effect of benzoxazoles (13a-d) on germination, root and shoot growth of wheat.



The effect of pyridine(thi)ones (16a-f) on germination, root and shoot growth of wheat.

According to figures (1-6) all compounds showed biological changes in growth parameters of wheat these changes were between activation to inhibition. Depending on the biological activity against growth parameters of wheat, the tested compounds could be classified as follow:

a) Compounds activated all growth parameters.
Compounds (**3b**),(**5**),(7b),(**7c**),(**10c**),(**13d**) and (**16e**) activated all growth parameters under study.

b) Compounds activated one or more of the growth parameters and inhibited the other parameter.
Compounds (**3e**),(**10d**) and(**16e**) activated gerimination and root growth, while compounds (**3c**),(1**3a**) and (**13c**) activated root growth and compound (**3a**) that activated both root and shoot growth.

c) Compounds inhibited all growth parameters.
Compounds (**16a**),(**16**b), (**16f**), (**13b**),(**10a**),(**7a**) and (**3b**) inhibited all growth parameters under study. Generally compounds that showed high inhibition percentage against all growth parameters were considered as promising compounds followed by that showed high inhibition effect against two growth parameters. So that, compounds (**16a**) ,(**16b**) ,(**16f**), (**13b**) and (**13a**) were considered as promising herbicide active ingredients . on the other hand all promising compounds except (**13b**) and (**13a**) were more effective against shoot growth than the other

growth parameters, whereas compounds (**13b**) and (**13a**) were more effective against root and gerimination respectively than the other growth parameters.

According to the obtained data , differences were found in activity between derivatives of the same compound. This differences may be explained on the basis of substitution, for example the presence of thienyl group, 2-chlorophenyl and methoxy phenyl substituents in (**16a**),(**16b**),(**16f**) respectively inhibited all growth parameters , while the presence of dimethyl amino phenyl in derivatives (**16a**) and (**16e**) resulted in an activation of all growth parameters.

From another point of view there is a relationship between some substituents and the herbicidal activity of the synthesized compounds as in case of compounds (**10a**),(**13b**) and (**16f**) due to the presence of thienyl group and as in compounds (**10a**) and (**10f**) due to the presence of 2-chorophenyl.

Conclusion

1. All synthesized compounds showed biological activity against growth parameters of wheat, the effect that was between activation to inhibition.
2. Compounds (**16a**),(**16b**),(**16f**) and (**13b**) were considered as promising herbicide active ingredients. These compounds caused high inhibition effect on all or on two from the growth parameters under study
3. Substituents play an important role in effectiveness of different derivatives of the same compound
4. There is a relationship between some Substituents and the herbicidal efficiency of the synthesized compounds including them such as thienyl and 2-chorophenyl groups .

Eperimental

Evaluation of Herbicidal Efficiency of the Newly synthesized compounds. Bioassay. Under laboratory conditions.

- Seed germination, root and shoot growth inhibition were carried out according to the procedure described by Powel and Spencer [34], some modifications were made for this work as described below.
- Serial concentrations from each compound was prepared by dissolving it in dimethyl sulfoxide and dilution with water. The calculated amount from each concentration was pipetted on thirty seeds of wheat as a test plant and agitated to coat the seed surface. Each ten seeds were transferred to petridish (90 mm diameter), lined with a dry filter paper and left at 25°C without led to grant solvent evaporation. After that, 6 ml distilled water was pipetted on the filter paper, Petridish was sealed with (PVC) electrical insulting tape. After complete germination of control (Petridishes containing untreated seeds), the number of germinated and non germinated seeds and radical length were recorded. Three replicates were done for each treatment.

References

1. youssef M.A.,Ibrahim N.A.,Attia A.E.H.and Hamouda S.E.S.;Egypt .J.chem.; (**2**)163-75 (2008).
2. Masahiko I.Seijio and Tadashi K.;JPn.Kokai Tokkyo Koho JP 06,16 ,160 (Cl.C07 C25540);C.A.,(**120**),244384r (1994).
3. Masahiko I., and Tadashi K.,JPn.Kokai Tokkyo Koho JP 06,145,007(Cl.A01 N37/34); C.A.,(**121**),1020049(1994)
4. Masahiko I., seiji N. and Tadashi K., Eur. pat. App. Ep506,373 (CL.C07C255/17) ; C.A.,(**119**),225824x(1993).
5. Masahiko I., and Seiji O.,JPn.Kokai Tokkyo Koho JP 05,339,224(Cl.C07C255/17); C.A.,(**122**),31565c(1995)
6. Masahiko I. and Seiji O., JPn.Kokai Tokkyo Koho JP 06,128,213(C1.C07D207/34); C.A., (**121**),205512n(1994).
7. Keiichiro I. and Tasuo M.;JPn Kokai Tokkyo Koho JP 06,25,160 (C1.C07D207/34); C.A., (**123**),9330y(1995).
8. Joseph D.J.;Eur.pat.Appl.7,772(C1.C07D263/58);C.A,(**93**),150241p(1980).
9. Masahiko I., Keiichiro I.,Masahiko S. and yasso M.;PCT Int.Appl. Wo 9413 ,655 (C1.C07D413/06); C.A.,(**121**),179616x(1994).
10. Alfons L., Henery M., Peter D.J., Werner F., Karl G., Haukur M.,Rene M. and Paul J.P.;Eur.Pat Appl. 12,158(C1.AON 25/32); C.A.,(**94**),78439K(1981).
11. Alfons L., Henery M., Peter D.J., Werner F., Karl G., Haukur,M.,Rene M. and Paul J.P.;Eur.Pat Appl. 12,158(C1.AON 25/32); C.A.(**97**),140287b(1982).
12. Anthony F.R. Mao C.H.L, Louis L.D. and Xavier F.W.,PCT.Int.Appl.; C.A., (**128**), 230384u (1998).

13. Huff H.P. et al; Proc.Br.crop prot.conf-Weeds;1989,(**2**),217.
14. Tomlin C.D.S.; Pesticide Manual, 2004, 13 (**3.1**), 519-20(2004).
15. Elnima I.E., Zubair U.M. and Al-bader A.A.,Antimicarb.Agents chemother., C.A.,(**94**),133032z(1981).
16. El-Shaaer, Abdel-Aziz A.S.,Allimony A.H., Ali F.V. and Abdel-Rahman M.R., Pharmazia, 52(**8**)585(1997).
17. Ersan S.Nacak S.,Berkam R. and Ozden T.; Arzenim – Forsch.;**47**(**8**): 963(1997).
18. Tiwan S., Khan M.H. and Nizamuddin; Ind.J. chem., **36B**(**8**):711(1997).
19. Oren I., Temiz O.,Yolcin I.,Sener E.Alkin A. and Ucarturk N.; Arzenim. – Forsch.;**47**(**12**):1393(1997).
20. Temiz O.,Oren I.,Sener E.,yalcin I. and Ucarturk N.; Il Farmaco; (**53**):337(1998).
21. Yamzaki K., Kenko Y.,Suwa K.,Ebara S.,Nakazwa K. and Yasuno K.; Bio.Med.chem.; (**13**):2509-22(2005).
22. Yalcin I.,Oren I.,Sener E.,Akin A. and Ucarturk N. ; Eur.J.Med.chem.,;(**27**):401-6(1991).
23. Ramalingan C., Balasubramanian S.,Kabilan S. and Vasudeuan M.;Eur.J.Med.chem.; (**39)**: 527-33(2004).
24. Sato.S and Levy G.;J.Pharm.Sci.; (**78)**:785(1989).
25. Sam J. and Valentine N.J.;J. pharm.. Sci;(**58**):1043(1969).
26. Neykova N.,Simov D.,Galunska G., Velichkava E., Galabov A.S. and Kararov A.;Arzeneim – Forsch.; **31**(**5**):747(1981).
27. Michel K.H., ; Hoehn M.M.,Jones N.D. and Chaney M.O.;J.Antibiot.; **37**(**5**):441(1984).
28. Agnihotri A.K. and Shukla S.K.; Arch. pharm. (Weinheim); (**315**):701(1982).
29. Shukla S.K., Agnihotri A.K. and Chowdhary B.L.; Ind. Drugs; **19**(**2**):59-60(1981).
30. Shiro S.,Mitsuaki H., Shinobu M. and Takuo C.;Yakugaku Zasshi; (**108**) :856-9(1988).
31. Rida M.S., Ashour A.F., El-Hawash M.A.S, Elsemary M.M.,Badr H.M. and Shalaby A.M.; Eur.J.Med.Chem.; **9**(**40**):949 – 59(2005).
32. Elgemeie H.G.; Chem.Ind.;(**19)**:653-4(1989).
33. Peter L.; Ger.Offen.;(**2**):710,285(1977).
34. Powell R.G. and Spencer G.F.; Photochemical Inhibitors of Velvet leaf gerimination as models for new biorational herbicides; Active Natural Products; ACS (Symp.Am.chem.Soc.); Washington, D-C; 1988; 211-213 (1988).

10/1/2010

Designing a Reliable Supply Chain Network Model under Disruption Risks

Nader Azad, Hamid Davoudpour

Department of Industrial Engineering, Amirkabir University of Technology, P.O. Box 15875-4413, Tehran, Iran
n.azad@aut.ac.ir

Abstract: In this paper, we consider random disruption risks in designing a reliable distribution network model. We consider the disputations in the location and the capacity of the distribution centers. In our model, the probability of disruption in distribution centers is dependent to the amount of investment for opening and operating them.
We show that this problem can be formulated as a non-linear integer programming model, and then for obtaining optimal solution, we linearize the mentioned model. In the following to solve the model in large-sized instances, a tabu search algorithm is developed. The results indicate that the tabu search method is efficient for a wide variety of problem sizes.
[Nader Azad, Hamid Davoudpour. **Designing a Reliable Supply Chain Network Model under Disruption Risks**. Journal of American Science 2010;6(12):1091-1097]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Supply chain network, Distribution network, Facility location, Random disruption risks, Tabu search.

1. Introduction

Supply chain disruptions have gained considerable attention, especially in the last ten years. Although supply chain disruptions occur with very low probability, the consequences are typically catastrophic. In the literature, disruption risks are divided in two sections: random disruption risks and premeditated disruption risks. Random disruption risks may occur at any point in the supply chain networks, for example natural hazards (earthquake…) may strike at any point in the supply chain network. Premeditated disruption risks are more likely to target a supply chain to cause the maximum disruption. Terrorists and labor union are the examples of the premeditated disruption risks.

In this study, we focus on issues related to facility random disruption risks. For a thorough review of facility location problem and supply chain network design, see Hamacher and Drezner (2002), Barmel and Simchi-Levi (2000), Daskin (1995, 2003), Qin and tang (2010) and Klibi et al. (2010).

There are some papers in the literature for designing reliable network systems. Daskin (1983), ReVelle and Hogan (1989) and Batta et al. (1989) studied on the reliable network systems that their objective function was the maximizing the expected demand coverage while Hogan and ReVelle (1986) and Batta and Mannur (1990) focused on individual demand coverage with some degree of redundancy. Ostfeld and Shamir (1993) described the reliability methods for designing water distribution networks. Drezner (1987) proposed a reliable p-median problem and used a heuristic method for solving it. Recently, Tang et al. (2008) presented a facility location model based on reliability. They considered the level of customer service in their model. Gade and Pohl (2009) developed a capacitated facility location model with unreliable facilities. They used sample average approximation algorithm to solve their model. Wagner and Neshat (2009) developed an approach based on graph theory to quantify and mitigate supply chain disruption risks. Tancel and Alpan (2010) used a timed Petri nets framework for designing supply chain networks under risk.

The papers in the literature, consider the disruptions in the location of distribution centers. They assumed that when a disruption occurs at a distribution center, it fails. In this paper, we consider the disputations in the location and the capacity of the distribution centers. In this paper is assumed that when a disruption occurs at a distribution center, it dose not fail and the distribution center misses some of the capacity to service in disruption situation. The probability of disruption in distribution centers is dependent to the amount of investment for opening and operating them, i.e., we can reduce the probability of disruption in distribution centers with additional investments. In our model, distribution centers can ship goods together under disruption situation.

The remainder of this paper is organized as follows: In Section 2, mathematical formulation of the problem is presented. Section 3 discusses the solution approach for solving the problem. Our computational results are in Section 4. Conclusions are given in Section 5.

2. Problem description

Here, we present the reliable distribution network design model considering random disruption risk. We assume that there are two types of distribution centers in the model: reliable distribution centers and unreliable distribution centers. Disruptions occur in the unreliable distribution

centers. Reliable distribution centers are safe against disruptions and also when a disruption occurs in an unreliable distribution center, it does not fail. In this case, the unreliable distribution center misses some of the capacity to service in disruption situation. In our model, the probability of disruption in unreliable distribution centers and the amount of the capacity which the unreliable distribution center misses in the disruption situation are dependent to the amount of investment for opening and operating unreliable distribution center, and finally, the reliable distribution centers can ship goods to the unreliable distribution centers in disruption situation.

Before presenting the model, let us introduce the notations that will be used throughout the paper:

Index sets

K : Set of customers.

JT : Set of potential distribution centers. $(JT = JU \bigcup JR)$

JU : Set of potential unreliable distribution centers.

JR : Set of potential reliable distribution centers.

N : Set of available investment levels for opening and operating unreliable distribution centers.

Parameters and notations

D_k : Demand of customer k , ($\forall k \in K$).

fU_{jn} : Fixed cost for opening and operating unreliable distribution center j with investment level n , $(\forall j \in JU, \forall n \in N)$.

fR_m : Fixed cost for opening and operating reliable distribution center m , $(\forall m \in JR)$.

d_{jk} : Transportation cost from unreliable distribution center j to customer k , $(\forall j \in JU, \forall k \in K)$.

l_{mk} : Transportation cost from reliable distribution center m to customer k , $(\forall m \in JR, \forall k \in K)$.

C_{mj} : Transportation cost from reliable distribution center m to unreliable distribution center j , $(\forall m \in JR, \forall j \in JU)$.

Cap_j : Capacity of unreliable distribution center j , $(\forall j \in JU)$.

a_{jn} : The percentage of total capacity of unreliable distribution center j that is affected by disruption when it is opened with investment level n , $(\forall j \in JU, \forall n \in N)$.

q_{jn} : Disruption probability in unreliable distribution center j when it is opened with investment level n , $(\forall j \in JU, \forall n \in N)$.

Decision variables

$$XU_{jn} = \begin{cases} 1 & \text{if unreliable distribution center } j \text{ is opened with investment level } n. \\ 0 & \text{otherwise} \end{cases}$$

$$XR_m = \begin{cases} 1 & \text{if reliable distribution center } m \text{ is opened.} \\ 0 & \text{otherwise} \end{cases}$$

$$YU_{jk} = \begin{cases} 1 & \text{if customer } k \text{ is assigned to unreliable distribution center } j. \\ 0 & \text{otherwise} \end{cases}$$

$$YR_{mk} = \begin{cases} 1 & \text{if customer } k \text{ is assigned to reliable distribution center } m. \\ 0 & \text{otherwise} \end{cases}$$

T_{mj} : Amount of goods that is shipped from reliable distribution center m to unreliable distribution center j , $(\forall m \in JR, \forall j \in JU)$.

In terms of the above notations, the problem formulating is as follows:

$$Min: \sum_{j\in JU}\sum_{n\in N} fU_{jn}\, XU_{jn} + \sum_{m\in JR} fR_m\, XR_m + \sum_{j\in JU}\sum_{k\in K} d_{jk}\, D_k\, YU_{jk} + \sum_{m\in JR}\sum_{k\in K} l_{mk}\, D_k\, YR_{mk} + \sum_{j\in JU}\sum_{n\in N} q_{jn}\, XU_{jn}\left(\sum_{m\in JR} T_{mj}\, C_{mj}\right) \quad (1)$$

Subject to:

$$\sum_{j\in JU} YU_{jk} + \sum_{m\in JR} YR_{mk} = 1 \quad \forall k \in K \quad (2)$$

$$\sum_{m\in JR} XR_m \geq 1 \quad (3)$$

$$XR_h + \sum_{n\in N} XU_{hn} \leq 1 \quad \forall h \in JT \quad (4)$$

$$YR_{mk} \leq XR_m \quad \forall m \in JR, k \in K \quad (5)$$

$$\sum_{k\in K} D_k\, YU_{jk} \leq \sum_{n\in N} Cap_j\, XU_{jn} \quad \forall j \in JU \quad (6)$$

$$\sum_{m\in JR} T_{mj} + \left(1 - \sum_{n\in N} a_{jn}\, XU_{jn}\right) Cap_j \geq \sum_{k\in K} D_k\, YU_{jk} \quad \forall j \in JU \tag{7}$$

$$XR_m \in \{0,1\} \quad \forall m \in JR \tag{8}$$

$$XU_{jn} \in \{0,1\} \quad \forall j \in JU, \forall n \in N \tag{9}$$

$$YR_{mk} \in \{0,1\} \quad \forall m \in JR, \forall k \in K \tag{10}$$

$$YU_{jk} \in \{0,1\} \quad \forall j \in JU, \forall k \in K \tag{11}$$

$$T_{mj} \geq 0 \quad \forall m \in JR, \forall j \in JU \tag{12}$$

The model minimizes the total expected costs of the fixed cost for opening distribution centers, the transportation cost from distribution centers to the customers, and the expected cost of disruption situation. Constraints (2) make sure that each customer is assigned exactly one distribution center. Constraints (3) ensure that we locate at least one reliable distribution center. Constraints (4) state that we can not locate both reliable and unreliable distribution center at any potential node h. Constraints (5) link the location and allocation variables. Constraints (6) are the capacity constraints associated with the unreliable distribution centers. Constraints (7) state that for each unreliable distribution center j, the sum of the goods which is shipped from reliable distribution centers and the total capacity which is not affected by disruption, must be greater than the total demands of the customers that is assigned to it. Constraints (8)-(11) enforce the integrality restrictions on the binary variables and finally constraints (12) enforce the non-negativity restrictions on the corresponding decision variables.

2.1. Linearization of the model

Formulation (1)-(12) is nonlinear. However, the only nonlinear terms are $T_{mj} \times XU_{jn}$, each being a product of a continuous variable and a binary variable. We define a new variable as follows:

$$W_{mjn} = T_{mj} \times XU_{jn} \tag{13}$$

The formulation (1)-(12) can be written as follows:

$$Min: \sum_{j\in JU}\sum_{n\in N} fU_{jn}\, XU_{jn} + \sum_{m\in JR} fR_m\, XR_m + \sum_{j\in JU}\sum_{k\in K} d_{jk}\, D_k\, YU_{jk} + \sum_{m\in JR}\sum_{k\in K} l_{mk}\, D_k\, YR_{mk} + \sum_{j\in JU}\sum_{n\in N}\sum_{m\in JR} q_{jn}\, W_{mjn}\, C_{mj} \tag{14}$$

Subject to:

(2)-(12)

$$W_{mjn} \leq T_{mj} \quad \forall m \in JR, j \in JU, n \in N \tag{15}$$

$$W_{mjn} \leq M \times XU_{jn} \quad \forall m \in JR, j \in JU, n \in N, \quad M \text{ is a large number} \tag{16}$$

$$W_{mjn} \geq T_{mj} + M\left(XU_{jn} - 1\right) \quad \forall m \in JR, j \in JU, n \in N, \quad M \text{ is a large number} \tag{17}$$

$$W_{mjn} \geq 0 \quad \forall m \in JR, j \in JU, n \in N \tag{18}$$

3. Tabu search algorithm for solving the problem

In this section, for solving the large-sized instances, a tabu search algorithm is developed. Tabu search is a well known global search heuristic method to solve the combinatorial problems such as the proposed problem. The most important feature of tabu search algorithm is to avoid search cycling by systematically preventing moves taking the solution, in the next iteration, to points in the solution space previously visited. In the next sections, we describe the tabu search algorithm which we use for solving the problem.

3.1. Initial solution construction

For obtaining the initial solution, first we assign customers to the distribution centers, randomly. The procedure for obtaining the initial solution is as follows:

Step1: Put customers into a set K'.

Step2: 1- Select a customer from K' randomly. 2- Delete the customer from K'.

Step3: Select a distribution center randomly.

Step4: If the selected distribution center is reliable then assign the customer to the reliable distribution center and go to Step 7 otherwise (the selected distribution center is unreliable) go to Step 5.

Step5: If we select this unreliable distribution center for the first time then select an investment level for this distribution center randomly.

Step6: If remaining capacity of the unreliable distribution center is greater than the demand of the customer then assign the customer to the distribution center and go to Step 7 otherwise go to Step 3 for selecting another distribution center.

Step7: Is K' empty? If yes, go to Step 8, otherwise go to Step 2.

Step8: By using the heuristic algorithm (H1), determine amount of goods must be shipped form the

reliable distribution centers to the unreliable distribution centers in disruption situation.

Before presenting the improvement phase, let us describe the heuristic algorithm (H1). The steps of (H1) are as follows:

For each of the opened distribution center (a'_j), let L'_j be the sum of the demands of the customers that are assigned to a'_j.

Step1: 1-Put all of the unreliable opened distribution centers into a set K''.

2- Put all of the reliable opened distribution centers into a set E'.

Step2: Select an opened unreliable distribution center (a'_j) from K'', randomly.

Step3: For each reliable opened distribution center m in the set E', calculate $H'_{mj} = (L'_j - (1 - a_{jn})Cap_j)C_{mj}$.

Step4: Select the supplier from E' that has the minimum value H'_{mj} (Supplier m), then $T_{mj} = H'_{mj}$.

Step5: Delete the unreliable distribution center (a'_j) from K''.

Step6: Is K'' empty? If yes stop, otherwise go to Step 2.

3.2. Improving the initial solution

In this phase, the main objective is to improve the initial solution. We apply five different types of move for generating a candidate move: mov1, mov2, mov3, mov4, mov5. We generate a candidate move (from X_0 to the candidate solution X_n) using one of the five moves randomly.

Mov1: Randomly, one of the opened distribution centers (a'_j) is closed and all of the customers are reallocated among the remaining opened distribution centers. Finally, we apply heuristic algorithm (H1) to determine amount of good that must be shipped from the reliable distribution centers to the unreliable distribution centers in disruption situation. In this move, we must check that at least one reliable distribution center is located.

Mov2: In this move we select two opened distribution centers randomly, (a'_i, a'_j), and exchange a'_i and a'_j. Finally, we use the heuristic algorithm (H1) to determine amount of good that must be shipped form the reliable distribution centers to the unreliable distribution centers in disruption situation. In this move capacities of a'_i and a'_j are checked for serving the customers. Also, we must check that at least on reliable distribution center is located.

Mov3: One of the opened distribution centers (a'_i) is closed randomly, and a closed distribution center (a'_j) is opened randomly. If the opened distribution center is unreliable, we select an investment level for this distribution center randomly. Then we assign all of the customers corresponding to the eliminated distribution center (a'_i) to the new opened distribution center (a'_j). Finally, we use the heuristic algorithm (H1) to determine amount of good that must be shipped form the reliable distribution centers to the unreliable distribution centers in disruption situation. In this move the capacity of a'_j is checked for serving the customers.

Mov4: Select two opened distribution centers, randomly, (a'_i, a'_j). Then randomly select a customer (c'_i) in a'_i and a customer (c'_j) in a'_j and exchange c'_i and c'_j. Finally, we use the heuristic algorithm (H1). In this move we must check the capacities of distribution centers.

Mov5: Select one opened unreliable distribution center, randomly. Then randomly change the investment level for this distribution center.

4. Computational results

To evaluate the performance of our overall solution procedure, extensive computational experiments are designed with respect to series of test problems. The program is coded in Visual Basic 6.

4.1. Comparison of tabu search solution with optimal solution

For evaluating the tabu search method, nineteen instances are solved by LINGO.8 software (Table 1). For each instance, the tuning of the parameters is done by carrying out random experiments. For each instance, we run the tabu search method 20 times, and the average objective value is reported in Tables 1 to 2. Also, in Tables 1, 2 the coefficient of variation for each instance is reported (Coefficient of variation for the random variable X is defined as: Standard Deviation (X)/Average (X)). In the following tables DC and CV are the abbreviations of Distribution Center and Coefficient of variation, respectively.

It can be seen that the proposed tabu search solution are optimal (or near optimal) in different problem instances (Table 1). For instances 1 to 19, the average CPU time are less than or equal to 268 seconds for the proposed tabu search method; however, the maximal average CPU time for obtaining the optimal solutions is equal to 6783 seconds, and for problem instances 15 to 19 by a reasonable amount of time limit, LINGO cannot find the optimal solution, and the tabu search solutions in these problem instances are better than the best solutions that are obtained by LINGO software.

4.2. Comparison of tabu search solution with simulated annealing (SA) solution

For evaluating the proposed tabu search algorithm, we compare the proposed tabu search with the case that we use the simulated annealing (SA) algorithm instead of tabu search algorithm. The procedure of obtaining candidate moves used in the SA algorithm is the same as that in the tabu search algorithm.

From Table 2, it can be seen that the Average costs and CV values obtained by the tabu search algorithm are better than those obtained by the SA algorithm. This shows that selection of tabu search method is a good strategy in our solution method.

5. Conclusions

In this paper, we have considered random disruption risks in designing reliable distribution network model. We considered the disputations in the location and the capacity of the distribution centers. In our model, we assumed that the distribution centers can ship goods together under disruption situation.

We showed that our model can be formulated as a linear model. Also, we presented an effective tabu search algorithm to solve the large-sized instances. We comprised the tabu search algorithm with optimal solution and simulated annealing algorithm. The computational results indicated that the tabu search method is effective for solving the problem. For future works, it is interesting to consider disruptions in the transportation costs.

Table 1. Comparison of tabu search solution and optimal solution

				Optimal Solution		Tabu Search Algorithm			
NO.	# Customers	# Potential reliable DCs	# Potential unreliableDCs	Average Cost	CPU time	Average Cost	CPU time	CV	Gap(%)
1	4	2	2	16989.7	3	16989.7	1	0	0.00
2	6	3	3	23601.6	6	23601.6	3	0	0.00
3	7	3	3	27341.7	9	27341.7	4	0	0.00
4	8	4	4	30282.0	12	30282	8	0	0.00
5	9	4	4	32877.9	18	32877.9	10	0	0.00
6	20	5	5	74805.8	96	74805.8	25	0	0.00
7	30	6	6	102040.3	135	102040.3	35	0	0.00
8	40	8	8	135337.3	241	135434.1	45	0	0.07
9	50	10	10	169934.1	368	170131.2	60	0	0.12
10	60	12	12	195877.4	731	196290.7	71	0.0001	0.21
11	70	14	14	233535.2	1324	234178	85	0.0001	0.28
12	80	16	16	278781.6	2011	279676.9	102	0.0001	0.32
13	90	18	18	320957.4	3312	321971.4	119	0.0001	0.32
14	100	20	20	350218	6783	351415.9	134	0.0001	0.34
15	120	23	23	433318.1	2 hours limit	416206.4	170	0.0001	
16	140	26	26	509494.1	3 hours limit	482168.5	201	0.0001	
17	150	27	27	539791.7	3 hours limit	507161.8	220	0.0001	
18	160	28	28	583263.6	3 hours limit	542286.4	234	0.0001	
19	180	30	30	668868.9	4 hours limit	605107.3	268	0.0001	

Gap(%)= 100*(tabu search solution value – LINGO best solution value) / LINGO best solution value.
CV= Coefficient of variation

Table 2. Comparison the results of tabu search algorithm with SA algorithm

NO.	# Customers	# Potential reliable DCs	# Potential unreliableDCs	Based on tabu algorithm			Based on SA algorithm			Percent of improved cost
				Average cost	CPU time	CV	Average cost	CPU time	CV	
1	30	8	8	102040.3	35	0	106426.9	35	0.0002	4.30
2	40	10	10	135434.1	45	0	140368.6	45	0.0002	3.64
3	50	12	12	170131.2	60	0	178109.6	59	0.0002	4.69
4	60	14	14	196290.7	71	0.0001	206081.6	71	0.0002	4.99
5	70	16	16	234178	85	0.0001	245055.6	86	0.0002	4.65
6	80	18	18	279676.9	102	0.0001	295041.5	102	0.0002	5.49
7	90	20	20	321971.4	119	0.0001	338922.3	116	0.0003	5.26
8	100	23	23	351415.9	134	0.0001	374588.4	132	0.0003	6.59
9	120	26	26	415600.8	170	0.0001	441435.8	168	0.0003	6.22
10	140	27	27	481463.5	201	0.0001	515848.3	198	0.0003	7.14
11	150	28	28	506181.8	220	0.0001	543736.9	219	0.0003	7.42
12	160	30	30	540939.4	234	0.0001	582330.2	234	0.0004	7.65
13	180	32	32	603000.8	268	0.0001	653191	265	0.0004	8.32
14	200	34	34	686791	304	0.0001	746256	302	0.0004	8.66
15	230	36	36	801394.1	360	0.0001	880129.4	357	0.0005	9.82
16	250	38	38	886215.8	396	0.0001	978251.6	392	0.0005	10.39
17	280	40	40	1010105.9	452	0.0001	1121535.8	448	0.0005	11.03
18	300	42	42	1094798.4	491	0.0001	1224386.4	488	0.0005	11.84

Corresponding Author:
Nader Azad
Department of Industrial Engineering
Amirkabir University of Technology
P.O. Box 15875-4413, Tehran, Iran
E-mail: n.azad@aut.ac.ir

References

1. Hamacher H, Drezner Z. Facility Location: Applications and Theory. Springer-Verlag, Berlin. 2002.
2. Bramel J, Simchi-Levi D. The Logic of Logistics. Springer-Verlag, New York. 2000.
3. Daskin MS. Network and Discrete Location: Models Algorithms and Applications. Wiley-Interscience, New York. 1995.
4. Daskin MS. Facility Location in Supply Chain Design Working Paper, Northwestern University, Evanston, Illinois 60208-3119, U.S.A. 2003.
5. Qin XW, Tang LX. Reliable logistics system design research under disruptions: Review. Kongzhi yu Juece/Control and Decision. 2010: 25 (2): 161-165.
6. Klibi W, Martel A, Guitouni A. The design of robust value-creating supply chain networks: A critical review. European Journal of Operational Research. 2010: 203: 283-293.
7. Daskin MS. A maximum expected covering location model: Formulation, properties and heuristic solution. Transportation Science. 1983: 17(1): 48-70.
8. ReVelle C, Hogan K. The maximum availability location problem. Transportation Science. 1989: 23(3): 192-200.
9. Batta R, Dolan JM, Krishnamurthy NN. The maximal expected covering location problem-revisited. Transportation Science. 1989: 23(4): 277-287.
10. Hogan K, ReVelle C. Concepts and applications of backup coverage. Management Science, 1986: 32(11): 1434-1444.
11. Batta R, Mannur NR. Covering-location models for emergency situations that require multiple response units. Management Science. 1990: 36(1): 16-23.
12. Ostfeld A, Shamir U. Incorporating reliability in optimal design of water distribution networks-review and new concepts. Reliability Engineering and System Safety. 1993: 42: 5-11.
13. Drezner Z. Heuristic solution methods for two location problems with unreliable facilities. Journal of Operations Research Society. 1987: 38(6): 509-514.
14. Tang XF, Mao HJ, Li XH. Logistics facility location model based on reliability within the supply chain. Proceedings of the 4th IEEE International

Conference on Management of Innovation and Technology. 2008: 1099-1103.
15. Gade D, Pohl EA. Sample average approximation applied to the capacitated-facilities location problem with unreliable facilities. Proceedings of the Institution of Mechanical Engineers, Part O: Journal of Risk and Reliability. 2009: 223(4): 259-269.
16. Wagner SM, Neshat N. Assessing the vulnerability of supply chains using graph theory. International Journal of Production Economics. 2009: doi: 10.1016/j.ijpe.2009.10.007.
17. Tuncel G, Alpan G. Risk assessment and management for supply chain networks: A case study. Computers in Industry. 2010: 61: 250-259.

2/11/2010

Serum Fetuin-A in Chronic Renal Disease Patients: Contribution to Endothelial Dysfunction and Hemostatic alteration

Nadia A. Hussein[1], Ola M. Mahmoud[1], Manal Y. Zahran[1], Manar A. Rafaat[2]

[1]Hematology and [2]Nephrology Departments, Theodor Bilharz Research Institute, Giza, Egypt

nadhussein@hotmail.com

Abstract: Background/Aim: Fetuin-A is a circulating calcium-regulatory glycoprotein that inhibits vascular calcification. In the present study, serum fetuin-A was studied as a novel risk factor for the development of endothelial dysfunction (ED) and hemostatic alteration in patients with chronic renal disease (CRD). **Patients and Methods:** 15 CRD patients on conservative treatment, 15 end stage renal disease (ESRD) patients on regular hemodialysis (HD) treatment and 15 healthy volunteers were enrolled in the study. Fetuin-A, thrombomodulin (TM), von Willebrand factor (vWF), tissue plasminogen activator (t-PA), plasminogen activator inhibitor (PAI-1), D-dimer, high sensitivity CRP (hs CRP) and IL-6 were measured by ELISA. **Results:** There was a significant reduction in Fetuin-A levels in CRD and HD patients compared to controls. A significant decrease was also detected in HD group when compared to CRD group. The inflammatory markers, hs CRP and IL-6, were significantly increased in CRD and HD patients in comparison to controls. The increase was also significant on comparing HD group to CRD group. A strong inverse correlation was found between serum fetuin-A and each of hs CRP and IL-6. In addition, regression analysis revealed that hs CRP is an independent determinant of serum fetuin-A level. The traditional markers of ED, TM and vWF, were significantly increased in CRD and HD patients compared to controls. The increase was also significant when HD patients were compared to CRD patients. The significant inverse correlation between fetuin-A and each of TM and vWF supports the hypothesis that low serum fetuin-A with subsequent vascular calcification could be one of the contributing factors for the development of ED in CKD and HD patients. The fibrinolytic parameters tPA, PAI-1 and D-dimer levels were significantly higher in CRD and HD compared to controls. HD patients had significantly higher values of the previously mentioned parameters in comparison to CRD patients. t-PA, PAI-1 and D-dimer were significantly correlated to fetuin-A in CRD and HD patients. **Conclusion:** The results of this study demonstrate that in CKD and HD patients inflammatory processes are increased and linked to low fetuin-A and vascular calcification which represents a novel risk factor for the development of ED. The interplay of these phenomena could be responsible for the development and progression of accelerated thrombogenesis that is peculiar to renal patients.

[Nadia A. Hussein, Ola M. Mahmoud, Manal Y. Zahran, Manar A. Rafaat. **Serum Fetuin-A in Chronic Renal Disease Patients: Contribution to Endothelial Dysfunction and Hemostatic alteration**. Journal of American Science 2010;6(12):1098-1105]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Serum Fetuin; Chronic Renal Disease; Patients; Endothelial Dysfunction; Hemostatic alteration

Introduction:

Hemostasis is a process of blood clot formation at site of vessel injury. When a blood vessel wall breaks, the hemostatic response must be quick, localized and carefully regulated. Bleeding or thrombosis may occur due to missing or dysfunctional moieties of the coagulation or fibrinolytic factors [1]. Disturbances in hemostasis are common complications of chronic renal disease (CRD). Their occurrence and severity correlate quite well with the progressive loss of renal function to end stage renal disease (ESRD) [2]. The association of CRD with thrombotic events is somewhat puzzling because renal disease is typically associated with increased bleeding tendency due to platelet dysfunction and disturbed plasma coagulation [3]. However, recent studies have indicated that CRD is associated with an impaired function of the vascular endothelium [4]. Profound endothelial dysfunction is a prominent pathologic feature of uraemia [5,6]. Endothelial cell injury is the probable cause due to uremic toxins retention, dyslipidemia, hypertension and secondary hyperparathyroidism as well as increased levels of interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) [1]. If such a defect also involves the endothelial fibrinolytic system, it may provide a potential mechanism of reduced thromboresistance. It is probable that disturbances in fibrinolytic activity and endothelial dysfunction may play a role in vascular complications such as stroke or ischemic heart disease [7] which represent the

leading causes of morbidity and mortality in CRD [8].

Fetuin-A, a circulating calcium-regulatory glycoprotein that inhibits vascular calcification, is predominantly synthesized in liver. It is secreted into the blood stream and deposited as a noncollagenous protein in mineralized bones and teeth. During fetal life, there is high serum concentration of fetuin-A. Its level declines following infection, acute or chronic inflammatory states and malignancy [9,10]. Low serum fetuin-A levels have been reported in CRD and dialysis patients and is associated with inflammation and outcome [11,12]. Recent data have shown a relationship between vascular calcification and endothelial dysfunction (ED) in vascular disease [13]. Therefore, low serum fetuin-A level could be one of the contributing factors for the development of ED in CRD patients.

This study was designed to unveil the possible role of fetuin-A in the development of ED and hemostatic alteration in CRD patients.

Subject and Methods

Study population

The study was conducted on 30 patients admitted to Nephrology Department and Dialysis unit (Theodor Bilharz Research Institute). The patients were divided into two groups:

Group A: This included 15 CRD patients on conservative treatment (8 males and 7 females, ages ranging between 19-70 years with a mean of 44±14.9).

Group B: This included 15 ESRD patients (9 males and 6 females with ages ranging between 33-65 years with a mean of 52±9.9) on regular hemodialysis (HD) treatment (3 sessions weekly, 4 hours each for a period of more than 3 months) using Fresenius 4008 B machine, Hemophane filters with 1.4 surface area and sodium acetate solution as a dialyzate.

The etiology of CRD was variable between the 2 studied patient groups (hypertension, diabetes mellitus, urologic and unknown causes). None of the patients received blood or blood components transfusion therapy in the past 21 days and none of them was receiving erythropoietin therapy. This study was approved by the ethical committee of Theodor Bilharz Research Institute. Informed consents were obtained from all patients in accordance with the Declaration of Helsinki.

Fifteen age and sex matched healthy subjects selected from medical and paramedical staffs were included in the study to serve as a control group.

Sampling

A sample of 6 ml blood was collected from each subject into sterile endotoxin- free vacuum blood collection tubes, of which 2 ml were collected on potassium EDTA for hemogram by automated hematology analyzer ACT Differential (Beckman Coulter, France), 2 ml on trisodium citrate to measure thrombomodulin (TM), von Willebrand factor (vWF), tissue plasminogen activator (t-PA), plasminogen activator inhibitor (PAI-1) and D-dimer levels. In addition, 2 ml were withdrawn into a plain tube and left to clot. Sera obtained were collected for kidney function tests using standard methods. The rest of the sera were aliquoted, stored and kept frozen at -70° to measure the serum levels of fetuin-A, high sensetivity CRP (hs CRP) and IL-6.

Assay methods

Quantitative analysis of fetuin-A:

Serum fetuin-A level was measured by ELISA technique using human fetuin-A kit (BioVendor, France).

Quantitative analysis of inflammatory markers:

- High sensitivity CRP (hs CRP) was assayed by ELISA technique using hs CRP kit (BioVendor, France).
- Interleukin 6 (IL-6) was measured by ELISA technique using human IL-6 kit (BioSource, USA)

Quantitative analysis of endothelial and fibrinolytic markers:

- Plasma thrombomodulin (TM) level was assayed by ELISA technique using Asserachrom TM kit (Diagnostica Stago, France).
- Plasma von Willebrand factor (vWF) antigen level was assayed by ELISA technique using an Asserachrom vWF kit (Diagnostica Stago, France).
- Plasma level of tissue plasminogen activator (t-PA) antigen was assayed by ELISA technique using ZYMUTEST t-PA antigen kit (Hyphen BioMed, France).
- Plasma level of plasminogen activator inhibitor (PAI-1) antigen was assayed by

ELISA technique using ZYMUTEST PAI-1 antigen kit (Hyphen BioMed, France).

- Plasma D-dimer level was assayed by ELISA technique using ZYMUTEST D-dimer kit (Hyphen BioMed, France).

Statistical analysis

Statistical analysis was done by a statistical software package (SPSS 16.0 for Microsoft windows, SPSS Inc.). The levels of studied parameters were analyzed by Mann-Whitney test. Data were expressed as arithmetic mean ± standard deviation. Pearson correlation coefficient 'r' was used to measure the relationship between two variables. Stepwise multiple regression analysis was employed to evaluate any association between the serum level of fetuin-A and markers of endothelial dysfunction , inflammation and fibrinolysis markers tested. P values <0.05 were considered statistically significant.

Results

The results of all parameters studied in different groups are shown in table (1). The significant correlations detected in CRF and HD groups between the studied parameters are illustrated in table (2). Stepwise multiple regression analysis revealed that hs CRP is a significant independent determinant of serum fetuin A (B= -27.946, p=0.000) table (3) and that hs CRP as a marker of inflamation together with t-PA as a marker for fibrinolysis , are significant independent determinant of serum fetuin A {(B= -31.702, p=0.000) , (B= 1366.314, p=0.000) respectively} table (4).

Table (1): Results of the parameters studied in all groups

	Control group	Group A (CRD)	Group B (HD)
Creatinine (mg/dl)	0.80±0.30	4.81±.068[a]	9.21±1.51[ab]
Fetuin-A (µg/ml)	632.71±69.53	516.00±64.45[a]	433.67±67.03[ab]
hs CRP (mg/l)	2.05±1.45	7.28±1.63[a]	11.25±1.54[ab]
IL-6 (pg/ml)	0.55±0.20	2.64±0.89[a]	5.72±1.81[ab]
TM (ng/ml)	55.40±14.41	201.20±27.93[a]	323.67±44.62[ab]
vWF (%)	53.67±6.27	92.23±11.20[a]	130.92±16.21[ab]
t-PA (ng/ml)	0.243±0.047	0.605±0.082[a]	1.59±0.33[ab]
PAI-1 (ng/ml)	6.97±1.83	13.83±2.86[a]	24.05±5.68[ab]
D-dimer (ng/ml)	262.67±49.64	427.33±52.30[a]	742.00±109.95[ab]

a: Significant difference from control group
b: Significant difference from group A (CRD group)
Significant difference: p<0.05

Table (2): Significant correlations obtained in groups A&B between parameters studied

Correlations	Group A (CRD) Correlation coefficients	Group B (HD) Correlation coefficients
Fetuin-A *vs* creatinine	- 0.924**	- 0.941**
Fetuin-A *vs* hs-CRP	-0.980**	-0.948**
Fetuin-A *vs* IL-6	- 0.953**	-0.944**
Fetuin-A *vs* TM	-0.886**	-0.976**
Fetuin-A *vs* vWF	- 0.875**	- 0.842**
Fetuin-A *vs* t-PA	- 0.835**	-0.750**
Fetuin-A *vs* PAI-1	- 0.935**	- 0.938**
Fetuin-A *vs* D-dimer	- 0.849**	-0.874**
t-PA *vs* IL-6	0.767**	0.729**
t-PA *vs* hs CRP	0.823**	0.756**
PAI-1 *vs* IL-6	0.941**	0.962**
PAI-1 *vs* hs CRP	0.934**	0.933**

** Correlation is significant at the 0.01 level

Table (3): Stepwise multiple regression analysis of serum fetuin A and hs CRP in whole patients groups

	B	T	P
Constant	733.739	35.064	0.000*
hs CRP	-27.946	-12.817	0.000*

R=0.924, R^2 = 0.854, SE= 29.90

* Statistically significant at the level of p<0.01

Table (4): Stepwise multiple regression analysis of serum fetuin A and hs CRP with T-PA in whole patients groups

	B	T	P
Constant	692.124	35.574	0.000*
hs CRP	-31.702	-16.185	0.000*
t-PA	1366.314	4.143	0.000*

R=0. 954, R^2 = 0.911, SE= 23.81

* Statistically significant at the level of p<0.01

Discussion and Conclusion

ED is common in patients with CRD and in patients on renal replacement therapy such as HD [14]. Progression of CRD is associated with both decreased endothelial function and increased prevalence of atherosclerosis, and vascular media calcification. All of which have been associated with mortality. ED precedes clinically detectable vascular disease [15-17]. It has been recognized for over 150 years that abnormalities in blood flow, vessel wall and blood components may contribute towards thrombosis (Virchow's triad) [18]. This simplified view is now modified by the recognition that the process of thrombus formation (thrombogenesis) requires complex interactions involving injury to the vascular endothelium, platelet adherence, aggregation and release, and clotting factor activation; this process eventually leads to thrombin generation and fibrin formation [19].Under physiological conditions, the vascular endothelium produces many substances which are closely associated with hemostasis, fibrinolysis, synthesis of growth factors, and the regulation of vessel tone and permeability. The etiology of ED is complex and involves dysregulation of multiple pathways [20].

In the present study, serum fetuin-A was measured in CRD and HD patients in a trial to clarify its potential contribution to ED and haemostatic alteration frequently encountered in these patients. The results obtained revealed significant reduction in Fetuin-A levels in CRD and HD patients compared to controls. These findings are in accordance with previous studies [21-24]. On the other hand, Ix et al,. [25] reported that serum fetuin-A concentration was not reduced in 970 patients with mild CRD. Caglar et al., [13] found that serum fetuin-A concentrations were decreased in all stages of CRD except stage 1. On the basis of these observations, it could be suggested that serum fetuin-A levels decline during the course of progression of CRD. This suggestion could be supported by the highly significant inverse correlation detected between serum fetuin-A and creatinine in CRD and HD patients included in the present work. The results also revealed significant decrease in serum fetuin-A levels in HD group in comparison to CRD group. Similar finding was reported by Ketteler et al [21] and Balon et al., [26]. A significant decrease of fetuin-A levels after a single HD session was also detected by Ciaccio et al., [27] and Errakonda et al., [28]. The results of the present study are in accordance with previous studies (Weinhol et al., [29] and Chang et al., [30], showing that CRD and HD patients had higher levels of inflammatory markers, as evidenced by the significant increase in hs CRP and IL-6 levels, in comparison to controls. Additionally, HD patients had significantly higher CRP levels compared to CRD patients. Chronic low-grade inflammation is a common feature of CKD. The causes of the highly prevalent state of inflammation in CKD are multiple and include factors such as volume overload, co-morbidity, intercurrent clinical events, the dialysis procedure *per se* as well as genetic factors [31, 26]. Chronic inflammation in CKD patients may contribute to down regulation of fetuin-A serum levels [32, 33, 24]. Our results revealed a strong inverse correlation between serum fetuin-A and each of hs CRP and IL-6. In addition, regression analysis revealed that hs CRP is an independent determinant of serum fetuin-A level. These findings support the hypothesis of inflammation-dependent down-regulation of fetuin-A expression and highlight the close relationship between inflammation and vascular calcification in CRD.

To evaluate the potential role of fetuin-A in endothelial cell activation and damage which could induce thrombosis, several measures of endothelial function and fibrinolytic parameters were assessed in

the current study. The traditional biomarkers of ED namely, TM and vWF were evaluated. They were significantly increased in CRD and HD patients compared to controls. The increase was also significant when HD patients were compared to CRD patients. This increase reflects the endothelial cell injury and denotes the presence of ED in CRD patients which was more pronounced in HD patients. Although ED in CRD and HD patients has been reported by many authors [7, 34, 35, 36], the precise mechanism that induces it is not clear. There are many postulations among which the accumulation of certain uremic factors [37], systemic inflammation which is closely associated with augmented oxidative stress [38], decreased oxygen supply to endothelial cells as a result of anemia [39], hypertension and shear stress [40], and the massive release of cytokines during dialysis [38]. Recent studies have demonstrated a possible role of fetuin-A in the pathogenesis of ED in CRD [41-43]. The present study demonstrated that the circulating biomarkers of ED (TM and vWF) showed a progressive and significant increase, in relation to the decrease of fetuin-A in CRD and HD patients. These findings are in accordance with those reported by previous studies [13,43] and support the hypothesized that low serum fetuin-A with subsequent vascular calcification could be one of the contributing factors for the development of ED in CKD and HD patients.

vWF is synthesized by, and stored in, endothelial cells. When released, it appears to mediate platelet adhesion and aggregation. The close association between vWF and the processes of thrombogenesis suggests that high vWF levels may be a useful indirect indicator of thrombosis [44] and also suggests that high vWF levels could contribute to thrombotic episodes in CRD and HD patients. The significant inverse correlation between fetuin-A and vWF clarifies the possible role played by fetuin-A in inducing thrombosis in CRD and HD patients.

The results of this study revealed that CRD and HD patients had significantly increased fibrinolytic parameters, t-PA, PAI-1 and D-dimer, compared to controls. HD patients had significantly higher values of the previously mentioned parameters in comparison to CRD patients. Gray et al., [45] and Bono,[46] stated that in patients with ischemic heart disease and diabetes respectively there is an approximately linear relation between t-PA and PAI-1 plasma concentrations; the equilibrium of the reaction is such that high t-PA and higher PAI-1 antigen concentrations are associated with low t-PA activity. The significantly increased t-PA, PAI-1 and D-dimer levels in our study population are consistent with a procoagulant state usually associated with the underlying ureamia, ED and proinflammatory changes. This finding was clarified by the significant inverse correlation between fetuin-A and each of t-PA, PAI-1 and D-dimer, in addition to the significant direct correlation between each of t-PA and PAI-1, and the inflammatory markers (hs CRP and IL-6) in CKD and HD patients studied. Similar findings have been reported by previous authors and they indicated that the increase in t-PA and PAI-1 reflected a mediated or facilitated endothelial cell injury as supported by certain clinical and experimental data [43, 47, 48]. An alternative explanation was that PAI-1 is an acute-phase protein that can rise in response to several stimuli, including cytokines such as IL-6. It has been speculated that the development of atherothrombotic events in CRD patients is due, at least in part, to an impaired fibrinolysis [49]. Regression analysis data revealed that hs CRP, as a marker of inflammation, together with t-PA, as a marker for fibrinolysis, are significant independent determinant of serum fetuin A which prove the impact of inflammation and fibrinolysis on serum fetuin A in patients with CRD.

In conclusion, the results of the present study seem to indicate that in CKD and HD patients inflammatory processes are increased and linked to low fetuin-A and vascular calcification which represents a novel risk factor for the development of ED. The interplay of these phenomena could be responsible for the development and progression of accelerated thrombogenesis that is peculiar to renal patients. Therefore, the use of therapeutic agents, such as sevelamer, which decrease inflammation and increase levels of the fetuin-A could be tried in CRD and HD patients to improve the endothelial function and minimize the thrombotic complications.

References

1. Malyszko J, Malysszko JS, Mysliwiec M and Buczko W. (2005): Hemostasis in chronic renal failure. Annales Academiae Medicae Bialostocensis,; 50:126-131.
2. Eberst E and Berkowitz LR(1994): Hemostasis in renal disease: Pathophysiology and management. Am J Med,; 96: 168-79.
3. Losowsky MS, Walls WD (1971): Abnormalities of hemostasis in renal failure. J R Coll Physicians Lond; 5:148–156.
4. Thambyrajah J, Landray MJ, McGlynn FJ, Jones HJ, Wheeler DC, Townend JN(2000): Abnormalities of endothelial function in patients with predialysis renal failure. Heart; 83:205–209.
5. Passauer J, Bussemaker E, Range U, et al (2000): Evidence in vivo showing increase of baseline nitric oxide generation and impair-ment of endothelium-dependent vasodilation in

normotensive patients on chronic hemodialysis. J Am Soc Nephrol; 11:1726-1734.
6. Farkas K, Nemcsik J, Kolossváry E, Járai Z, Nádory E, Farsang C and Kiss I(2005): Impairment of skin microvascular reactivity in hypertension and uraemia. Nephrol Dial Transplant;20:1821-1827.
7. Matyszko J, Matyszko JS and Mysliwiec M (2004): Endothelial cell markers in chronic renal failure on conservative treatment and continuous ambulatory peritoneal dialysis. Kidney and Blood Pressure Research; 27:71-77.
8. Qureshi AR, Alvestrand A, Divino-Filho JC, Gutierrez A, Heimburger O and Lindholm B (2002): Inflammation, Malnutrition and cardiac disease as predectors of mortality in hemodialysis patients. J. Am. Soc. Nephrol; 13:S28-36.
9. Caglar K, Yilmaz MI, Saglam M, Cakir E, Kilie S, Eyileten T, Sonmez A, Oguz Y, Oner K, Ors F, Vural A and Yenicesu M (2007): Endothelial dysfunction and fetuin-A levels befor and after kidney transplantation. Trnsplantation; 83: 392-397.
10. Brandenburg VC, Parker BD, and Ix JH (2010): Important Differences in Measurement of Fetuin-A. Annals of Internal Medicine; 153 (6): 419-420.
11. Oikawa O, Higuchi T, Yamazaki T, Yamamoto C, Fukuda N and Matsumoto K (2007): Evaluation of serum fetuin-A relationships with biochemical parameters in patients on hemodialysis. Clin Exp Nephrol; 11: 336-337.
12. Cottone S, Lorito MC, Riccobene R, Nardi E, Mule G, Buscemi S, Geraci C, Guarneri M, Arsena R and Cerasola G(2008):Oxidative stress, inflammation and cardiovascular disease in chronic renal failure. J Nephrol; 21:175-179.
13. Caglar K, Yilmaz MI, Saglam M, Cakir E, Kilic S, Sonmez A, Eyileten T, Yenicesu M, Oguz Y, Tasar M, Vural A, Ikizler TA, Stenvinkel P, Lindholm B(2008): Serum fetuin-A concentration and endothelial dysfunction in chronic kidney disease. Nephron Clin Pract;108(3):233-240.
14. Yilmaz MI, Saglam M, Qureshi AR, Carrero JJ, Caglar K, Eyileten T, Sonmez A et al (2008): Endothelial dysfunction in type-2 diabetics with early diabetic nephropathy is associated with low circulating adiponectin. Nephrol. Dial. Transplant; 23 (5): 1621-1627.
15. Dogra G, Irish A, Chan D, Watts G (2006):Insulin resistance, inflammation, and blood pressure determine vascular dysfunction in CKD. Am J Kidney Dis ; 48:926–934.
16. Fernandez-Fresnedo G, Rodrigo E, de Francisco AL, et al (2006): Role of pulse pressure on cardiovascular risk in chronic kidney disease patients. J Am Soc Nephrol; 17:S246–S249.
17. Malyszko J. (2010): Mechanism of endothelial dysfunction in chronic kidney disease. Clin Chim Acta. 2010 Oct 9;411(19-20):1412-20.
18. Virchow R (1856): Gesammelte Abhandlungen Zur Wissenschaftlichen Medizin. Frankfurt: Medinger Sohn and Co, 219–732.
19. Spiel AO, Gilbert JC, Jilma B(2008): Von Willebrand Factor in Cardiovascular Disease. Circulation ;117:1449-1459.
20. Endemann DH, Schiffrin EL. Endothelial dysfunction (2004): J Am Soc Nephrol;15:1983–1992.
21. Ketteler M, Bongartz P, Westenfeld R, Wildberger JE, Mahnken AH, Bohm R, Metzger T, Wanner C, Jahnen-Dechent W, Floege J (2003):Association of low fetuin-A (AHSG) concentrations in serum with cardiovascular mortality in patients on dialysis: A cross-sectional study. Lancet; 361: 827–833.
22. Stenvinkel P, Wang K, Qureshi AR, Axelsson J, Pecoits-Filho R, Gao P, Barany P, Lindholm B, Jogestrand T, Heimburger O, Holmes C, Schalling M, Nordfors L(2005): Low fetuin-A levels are associated with cardiovascular death: Impact of variations in the gene encoding fetuin. Kidney Int ; 67: 2383–2392.
23. Wang AY, Woo J, Lam CW, Wang M, Chan IH, Gao P, Lui SF, Li PK, Sanderson JE(2005): Associations of serum fetuin-A with malnutrition, inflammation, atherosclerosis and valvular calcification syndrome and outcome in peritoneal dialysis patients. Nephrol Dial Transplant 20: 1676–1685.
24. Hermans MM, Brandenburg V, Ketteler M, Kooman JP, van der Sande FM, Boeschoten EW, Leunissen KM, Krediet RT, Dekker FW (2007): Association of serum fetuin-A levels with mortality in dialysis patients. Kidney Int ; 72: 202–207.
25. Ix JH, Chertow GM, Shlipak MG, Brandenburg VM, Ketteler M, Whooley MA (2006): Fetuin-A and kidney function in persons with coronary artery disease: Data from the Heart and Soul Study. Nephrol Dial Transplant; 21: 2144–2151.
26. Balon BP, Knehtl M, Bevc S, Jakopin E and Gorenjak M (2010): Fetuin-A as a risk factor for mortality in hemodialysis patients. Wiener Klinische Wochenschrift; 122 (2): 63-67
27. Ciaccio M, Bivona G, Sciacca RD, Iatrino R, Natale ED, Vecchi ML and Bellia C(2008):

Changes in serum fetuin-A and inflammatory markers levels in end-stage renal disease (ESRD): effect of a single session haemodialysis. Clinical Chemical Laboratory Medicine; 46; 2: 212–214
28. Errakonda PR, Paladugu R, Bitla AR, Musturu SM, Lakshman J, Pemmaraju SR, Vishnubhotla S (2010): Effect of a single hemodialysis session on endothelial dysfunction. J Nephrol. May 12. pii: 71789013-C058-4A0B-B80F-3A6E55CEEC27.
29. Weinhold B and Ruther U (1997): Interleukin-6 dependent and independent regulation of the human C-reactive gene. Biochem J 327: 425 – 429.
30. Chang JW, Yang WS, Min WK, Lee SK, Park JS, Kim SB(2002): Effects of simvastatin on high-sensitivity C-reactive proteins and serum albumin in hemodialysis patients. Am J Kidney Dis 39 : 1213 –1217.
31. Stenvinkel P (2006): New insights on inflammation in chronic kidney disease– genetic and non-genetic factors. Néphrologie & Thérapeutique ;2: 111-119.
32. Gabay C and Kushner I (1999): Acute-phase proteins and other systemic responses to inflammation. N Engl J Med; 340: 448–454
33. Cozzolino M, Galassi A, Biondi ML, Turri O, Papagni S, Mongelli N, Civita L, Gallieni M, Brancaccio D (2006): Serum Fetuin-A Levels Link Inflammation and Cardiovascular Calcification in Hemodialysis Patients. Am J Nephrol;26:423-429
34. Nakayama M, Yamada K, Yamamoto Y, Yokoyama K, Nakano H; Kubo H; Shigematsu T; Kawaguchi Y; Sakai O (1994): Vascular endothelial dysfunction in patients on regular dialysis treatment Clin Nephrol.; 42(2):117-20:
35. Cottone S, Mule G,Amato F, Riccobene R, Vadala A, Lorito MC, Raspanti F, Cerasola G (2002): Amplified biochemical activation of endothelial function in hypertension associated with moderate to severe renal failure. J Nephrol; 15: 643-648.
36. Jacobson SH, Egberg N, Hylander B et al. (2002): Correlation between soluble markers of endothelial dysfunction in patients with renal failure. Am J Nephrol; 22: 42–47.
37. De Deyn PP, Vanholder R, D'Hooge R. Nitric oxide in uremia: Effects of several potentially toxic guanidino compounds. Kidney Int Suppl 84: S25 –S28, 2003.
38. Mezzano D, Pais EO, Aranda E, Panes O, Downey P Ortiz M, Tagle R, Gonzalez F, Quiroga T, Caceres MS, et al (2001): Inflammation, not hyperhomocysteinemia, is related to oxidative stress and hemostatic and endothelial dysfunction in uremia. Kidney Intern.; 60: 1844–1850.
39. Kuriyama S, Hopp L, Yoshida H, Hikita M, Tomonari H, Hashimoto T, Sakai O (1996): Evidence for amelioration of endothelial cell dysfunction by erythropoietin therapy in predialysis patients. Am J Hypertens, 9(5):426-31.
40 Annuk M, Zilmer M, Fellstorm B (2003): Endothelial-dependent vasodilatation and oxidative stress in chronic renal failure: Impact on cardiovascular disease. Kidny Int Suppl; 84:S50.
41. Budoff MJ, Flores F, Tsai J, Frandsen T, Yamamoto H, Takasu J.(2003): Measures of brachial artery distensibility in relation to coronary calcification. Am J Hypertens;16: 350-355.
42. Nigam A, Mitchell GF, Lambert J, Tardif JC (2003): Relation between conduit vessel stiffness (assessed by tonometry) and endothelial function (assessed by flow-mediated dilatation) in patients with and without coronary heart disease. Am J Cardiol; 92:395-399.
43. Cottone S, Palermo A, Arsena R, Riccobene R, Guarneri M, Mulè G, Tornese F, Altieri F, Vaccaro F, Previti A, Cerasola A (2010): Relationship of fetuin-A with glomerular filtration rate and endothelial dysfunction in moderate-severe chronic kidney disease. J Nephrol; 23 (01):62-69
44. Mannucci PM (1998): von Willebrand Factor A Marker of Endothelial Damage? Arteriosclerosis, Thrombosis, and Vascular Biology;18:1359-1362
45. Gray RP, Yudkin JS, Patterson DLH (1993): Plasminogen activator inhibitor: a risk factor for myocardial infarction in diabetic subjects. Br Heart J; 69:228-32.
46. Bono D (1994): Significance of raised plasma concentrations of tissue-type plasminogen activator and plasminogen activator inhibitor in patients at risk from ischaemic heart disease. Br Heart J; 71:504-507.
47. Mussoni L, Baldasarre D, Mannucci L, Sirtori CR, Tremoli E (2000): Relationship between fibrinolytic variables: A study in patients attending a lipid clinic. Ann Med 32:134 -141.
48. Segarra A, Chacón P, Martinez-Eyarre C, Argelaguer X, Vila J, Ruiz P, Fort J, Bartolomé J, Camps J, Moliner E, Pelegrí A, Marco F, Olmos A and Piera L (2001): Circulating Levels of Plasminogen Activator Inhibitor Type-1, Tissue Plasminogen Activator, and Thrombomodulin in Hemodialysis Patients:

Biochemical Correlations and Role as Independent Predictors of Coronary Artery Stenosis. J Am Soc Nephrol 12:1255-1263.
49. Opartny K Jr, Opatrna S, Vit L, Opatrny K (1998): Tissue-type plasminogen activator (tPA) and its inhibitor (PAI-1) in patients treated with continuous ambulatory peritoneal dialysis. Am J Nephrol 18: 186 –192.

11/1/2010

Electrogastrographic Findings in Cerebral Palsy Patients

Nassar M.F.[1] Aly R.H.[1], Mahmoud N.H.[2], El-Batrawy, S.R.[3] and Abdel-Kereem N.[1]

[1]Pediatrics Department and [2]Clinical Pathology Department, Faculty of Medicine, Ain Shams University,
[3]Department of Anthropology, National Institute of Research, Cairo, Egypt.
*maie_nassar@yahoo.co.uk

Abstract: Objectives: This work was designed to detect any changes in the gastric electrical activity and gastrin levels in infants with cerebral palsy (CP) and correlate them to the clinical findings.
Patients and methods: The study was conducted on 30 CP patients in comparison to 12 age and sex matched clinically healthy infants. All enrolled infants and children were initially subjected to complete history taking with special emphasis on gastrointestinal symptoms, clinical examination and routine laboratory procedures as well as total serum gastrin hormone by ELISA. Electrogastrographic (EEG) recording for gastric electrical activity was performed for all subjects upon enrollment.
Results: The initial power ratio was non-significantly higher in CP patients compared to the controls while the dominant frequency (DF) was non-significantly lower. Regarding the initial visual analysis of the EGG, ١٣ patients (٤٣.٣%) were normogastric compared to bradygastria in 16 (٥٣.٣%) of them. Initial serum gastrin was higher in CP patients compared to the controls. The regression analysis revealed that gastrin was the most determinant factor for dominant frequency values followed by the power ratio in the CP patients.
Conclusion: In conclusion, CP patients have disturbed gastric motility which explains the different proximal gastrointestinal clinical manifestations experienced by our patients and this should be considered during their nutritional rehabilitation programs.

[Nassar M.F. Aly R.H., Mahmoud N.H., El-Batrawy, S.R.and Abdel-Kereem N. **Electrogastrographic findings in cerebral palsy patients.** Journal of American Science, 2010; 6(12): 1106-1113]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: electrogastrography; gastrin; cerebral palsy.

1. Introduction:

Cerebral palsy (CP) refers to a group of childhood neurologic syndromes (pyramidal and extrapyramidal subtypes) resulting from a wide variety of genetic and acquired insults to the developing brain, with abnormalities in tone, posture, and movement on clinical examination [1, 2].

The survival of children with severe central nervous system damage has created a major challenge for medical care [1, 3]. Numerous clinical reports, in small series of patients, have indicated that brain damage may result in gastrointestinal (GI) dysfunction [4, 5].

A number of investigators have reported that neurologically handicapped children have abnormalities of lower esophageal function [6] and delayed gastric emptying is common in such patients [7]. Additionally, foregut dysmotility was previously reported in children with cerebral palsy [5, 7].

Gastrointestinal motility is defined as the action enabling the passage of food and waste products through the four main regions of the digestive system: esophagus, stomach, small intestine, and large intestine or colon. Gastric motility is regulated by a variety of mechanisms to insure that it occurs at a rate optimal for the digestion and absorption of nutrients and neutralization of gastric contents as neural and hormonal mechanisms [8]. Gastric motility can be evaluated by many methods as gastric emptying time, Electrogastrography, antroduodenal manometry, barostat studies and other newer methods for evaluation.

Gastrin is a gut hormone produced by cells called G cells in the lateral walls of the glands in the antral portion of the gastric mucosa. In addition, it is also found in the anterior and intermediate lobes of the pituitary gland, in the hypothalamus and medulla oblongata, and in the vagus and sciatic nerves. Stimulation of gastric motility is probably a physiologic action. Gastrin causes contraction of the musculature that closes the gastro esophageal junction [9]. It may have a physiological role in regulating proximal gastric mechanics by inducing gastric fundal relaxation and increasing gastric wall compliance. The effect of gastrin is dependent on acid secretion [10].

Hence, the aim of this study was to detect any changes in gastric electrical activity and serum gastrin hormone levels in CP patients and correlate them to the clinical findings.

2. Patients and methods:

This study was a cross- sectional, case-control study conducted on 30 children with CP following up in the Out-patient Pediatric Neurology Clinic, Ain-Shams University, in the period from March 2007 to September 2007.

The CP patients were 17 males and 13 females, with mean age of 6.38±3.77 years. They were compared to 12 clinically healthy children serving as a control group who were 6 males and 6 females with mean age of 7.50±2.24 years. The study was conducted after obtaining an approval from the Pediatric board at the children`s Hospital, Ain Shams University and taking an informed consent from the subjects' parents or caregivers.

Enrolled patients were free of any infection, with no history of epilepsy, or any other chronic disease or drug that might affect gastric motility and secretion.

All patients and controls were subjected to detailed history taking including perinatal, developmental and dietetic history as well as symptoms of GIT disturbance and feeding problems. Thorough clinical examination was done laying stress on anthropometric measurements and full neurological examination with special emphasis on the motor system.

Weight was measured using regularly calibrated scale with minimal clothing. Height was measured using a special board calibrated in centimeters and millimeters. Weight and height, were plotted against the percentiles to obtain the percent from the median for age. Body mass index (BMI): [Wt (kg)/ Ht2 (m)] was calculated from the previous weight and height measurements.

Routine laboratory procedures were performed including complete blood count, total protein and serum albumin as well as total serum gastrin hormone by ELISA using the Kit supplied by BIOHIT plc. (Biohitplc. Laippatie 1 Fin-0088 Helsinki, Finland). Normal fasting gastrin levels in our studied age group was considered as follows; 3-4 hours: 0.96-80.8 pmol/L, 5-6 hours: 1.44-56.3 pmol/ L, >8 hours: 0.48-60pmol/L [11].

Venous blood samples were collected under complete aseptic conditions from all enrolled subjects while fasting. The samples were divided into two halves the first was taken on EDTA for the complete blood count and the remaining part was kept in a dry sterile tube from which serum was separated by centrifugation. Samples for gastrin hormone were stored at -70^{o} C freezers till the time of test procedures.

Electrogastrographic recording for gastric electrical activity was done for all subjects upon enrollment. Recording was done from five disposable pre gelled silver/silver chloride surface electrodes placed on the upper abdomen after the skin had been carefully abraded to decrease resistance to obtain a good signal to noise ratio [12]. Infants and children under examination were kept in a reclining position to minimize motion artifact. Four electrogastrography (EGG) signals were recorded bipolarly from these five electrodes as the potential differences between each of the four electrodes. A reference electrode was placed at the left clavicle. The electrical signal was recorded with appropriate amplification and filtering. Filtering is needed to exclude cardiac and small intestinal electrical activity artifacts as well as respiratory and movement artifacts [13].

One hour recording was done while the patients were fasting then they were given a standardized test meal. The test meal was a semisolid one (milk, rice and high protein additive) which was adjusted to provide a volume of 20 cm^3/kg/feed and a caloric value of one eighth of the daily needs that are approximately 100 kcal/kg/day then post prandial recording was done for one hour [14].

EGG recordings were analyzed by computer using the Fast Fourier Transform (FFT) to detect the dominant frequencies in fasting and post prandial time periods. The FFT transforms the signal from the time domain to the frequency domain. An extension of the FFT technique is the so called Running Spectrum Analysis (RSA). In this technique power spectra of short over lapping stretches of EGG signals are computed and displayed as a function of time [15].

The following parameters were evaluated for each patient:

1. Mean dominant frequency (DF): The frequency of gastric peak was determined by the absolute peak value, and the mean frequency was computed by averaging the individual spectra.
2. Power ratio (PR): The ratio of power of the mean spectrum of post prandial state to the power of the mean spectrum of the fasting state. It is indicative of the post prandial increase in gastric motor activity and was calculated for the total post prandial period [16].

Higher harmonics were identified in the spectrum using the criteria that occur at frequencies that are exact multiples of the fundamental frequency and their power should be at least 5% of the power of the fundamental component. The early post prandial frequency dip of the normal 3 cycles per minute (cpm) gastric component was identified. A rhythmic electrical activity ranging from 2.5-3.75 cpm was defined as normal gastric electrical activity. Tachygastria was considered to be present when the power spectrum contained a sharp peak component with a frequency more than 3.75 cpm and less than

10.8 cpm. For a definite diagnosis of tachygastria it was required that at the same time the normal gastric signal was absent in all four EGG signals and that the abnormal rhythm was present for at least 2 min. When a tachygastric frequency was found in the presence of a normal gastric signal the diagnosis of tachygastria was considered probable but not definite.

Bradygastria was considered to be present when the dominant peak was less than 2.5 cpm. A dysrhythmic episode had to be present at least for 2 minutes with the absence of normal gastric signal [17].

The standard computer program SPSS for Windows, release 10.0 (SPSS Inc, USA) was used for data entry and analysis. All numeric variables were expressed as mean ± standard deviation (SD) as well as median [interquartile range (IQR)]. Kalmogrov Smirnov test was used to differentiate parametric from non parametric data. Comparison of different variables in various groups was done using student t test and Mann Whitney test for normal and nonparametric variables respectively. Chi-square (χ^2) test was used to compare frequency of qualitative variables among the different groups. Spearman's correlation test was used for correlating non-parametric variables. For all tests a probability (p) less than 0.05 was considered significant. Graphic presentation of the results was also done [18].

3. Results:

The results of the current study revealed that CP patients and the controls had matching age and sex. Repeated vomiting was present in 46.7% of our CP patients, 50% had chronic constipation and 36.7% suffered from gaseous distension. The CP patients were all non ambulatory and had significantly lower weight %, BMI as well as weight for age z scores compared to the controls (table 1). Additionally, although CP patients had lower hemoglobin levels and higher white cell counts, these comparisons were of no statistical significance.

As regard the EGG study, table (2) shows that CP patients had non-significantly higher PR and non-significantly lower DF compared to the controls. Additionally, table (3) demonstrated that by visual analysis more CP patients had bradygastria compared to the controls yet this result didn't reach statistical significance.

There was a highly significant lower DF in patients who complained of vomiting compared to those who didn't [mean values are 1.35±0.78 and 2.83±0.68 respectively and z (p) is 3.40 (<0.001)], while their mean PR was higher yet the latter comparison didn't reach statistical significance [mean values are 2.60±1.80 and 1.82±0.95 respectively and z (p) is 0.42 (>0.05)]. Additionally, 14 of the 15 children who suffered from vomiting had dysrhythmia.

Similarly, there was a highly significant lower DF in patients who complained of constipation compared to those who didn't [mean values are 1.33±0.63 and 2.95±0.66 respectively and z (p) is 4.16 (<0.001)], while their mean PR was higher yet the latter comparison didn't reach statistical significance [mean values are 2.57±1.85 and 1.84±0.97 respectively and z (p) is 0.33 (>0.05)].

Regarding serum gastrin, Fig (1) shows that the CP patients had significantly higher levels compared to the controls yet both patients and controls had their gastrin values within normal levels for age and sex.

Significant negative correlation was detected between serum gastrin levels and weight percentage for age (r = -0.495 and p<0.05) (Fig 2). Meanwhile there were no statistically significant correlations between DF and PR and weight percentage for age. There were no statistically significant correlations between serum gastrin, dominant frequency or power ratio and BMI.

Studying the effect of various studied parameters on dominant frequency of EEG in patients using multiple regression test revealed a significant effect (f = 2.50, p=0.06). Further analysis revealed that the factors causing this significance are serum gastrin (beta coefficient = -0.42 and p=0.03) and power ratio (beta coefficient = -0.42 and p=0.03) in the presence of the other studied factors.

Table (1): Comparison between the anthropometric measurements of the patients and controls.

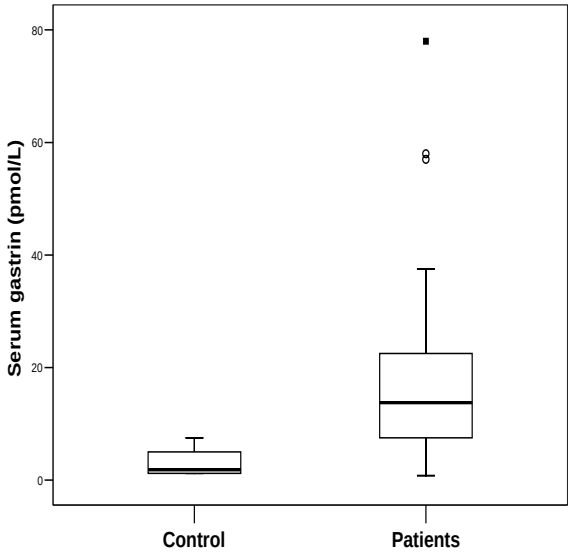
Variable	Patients		Controls		t/z*	p
	Mean±SD	Median(IQR)	Mean±SD	Median(IQR)		
Weight percentage from median for age	69.58±16.53	71.50 (22.00)	92.67±6.87	90.00 (9.00)	6.39	<0.001
Body mass index	15.13±3.82	14.05 (3.83)	20.27±5.58	18.45 (7.00)	-3.45*	<0.05
Height percentage from median for age	85.93±7.74	87.00 (12.00)	90.33±3.55	90.50 (7.00)	-1.87*	>0.05
Weight for age Z score	-2.58±1.37	-2.50 (2.10)	-0.53±0.55	-0.64 (0.81)	4.99	<0.001

Table (2): Comparison between patients and controls regarding power ratio and dominant frequency of the EGG study.

variable	Patients		Controls		z	p
	Mean±SD	Median(IQR)	Mean±SD	Median(IQR)		
Power ratio	2.21±1.50	1.49 (2.50)	1.53±0.60	1.60 (1.10)	-1.00	>0.05
Dominant frequency (cpm)	2.14±1.04	2.32 (2.20)	2.41±1.18	3.09 (2.40)	-0.84	>0.05

Table (3): Comparison between patients and controls regarding visual analysis of the EGG study

Variable		Patients number (%)	Controls number (%)	χ^2	p
Visual analysis	Normogastric	13.00 (43.30%)	8.00 (66.70)	2.05	>0.05
	Bradygastric	16.00 (53.30%)	4.00 (33.30)		
	Tachygastic	1.00 (3.30%)	0.00 (0.00)		



P=0.01

Figure (1): Serum Gastrin of patients and control groups.

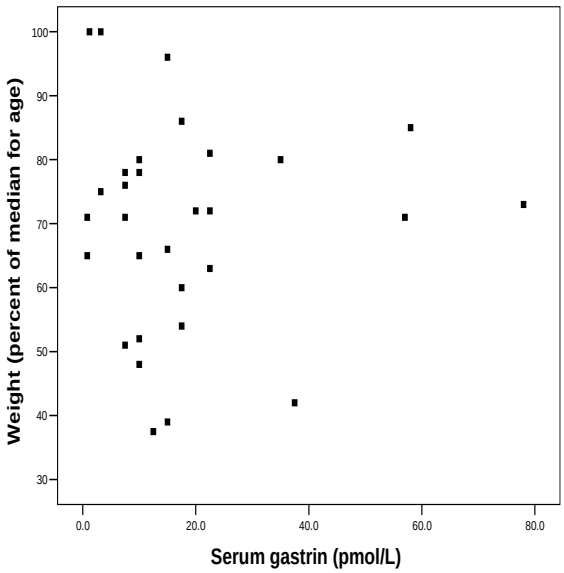


Figure (2): Serum gastrin levels with weight percentage for age of patients.

4. Discussion:

The current study revealed that the weight %, the body mass index and the weight for age Z score were statistically lower in CP patients when compared to those of the controls. Additionally, 76% of our series of CP patients had failure to thrive (mean BMI <19 [19] and Z score ≤-2 [20]).

In agreement with our results, Hurvitz et al., [21] who investigated the prevalence of overweight in a clinic-based population of children with CP and its association with gross motor function status reported that analysis of BMI in their study suggested that ambulatory children with CP had a high rate of overweight and are at risk of overweight than normal controls. On the other hand, they found that underweight is more prevelant in non ambulatory children, which is the case in our series of patients. Additionally, Feeley et al., [22] concluded that in an ambulatory CP population, patients with lower functional status or quadriplegia had significantly lower body mass index, suggesting that even highly functioning ambulatory CP patients are at risk for malnutrition.

Moreover, both Del Giudice et al., [5] who studied gastrointestinal manifestations in children with CP and Reyes et al., [23] who studied gastro esophageal reflux in children with CP, found that 52% of patients suffered from failure to thrive. Moreover, Ravelli and Milla [7] who studied vomiting and gastroesophageal motor activity in children with disorders of the central nervous system (CNS) reported that 62% of patients showed failure to thrive.

In an attempt to find the causes behind this problem, Vik et al., [24] suggested that this may in part be due to the cerebral injury and central dysfunction, in addition to the insufficient nutrition and lack of ability to self-feed.

As regards chronic GIT problems, 46.7% of our patients suffered from chronic vomiting, 50% had chronic constipation and 36.7% suffered from gaseous distension. In agreement with these findings, Ravelli and Milla, [7] found that all patients in their study suffered from vomiting and 46% of patients suffered from constipation. They concluded that in patients with CNS disorders, at least, the extrinsic innervation is abnormal, with gastric antral dysrhythmia resulting from abnormal activation of the efferent limb of the emetic reflex or from lack of inhibition of excitatory fibers as a consequence of either disturbed input to the hindbrain from higher centers or anatomic and functional disturbances of the vomiting centre and the area postrema. They demonstrated that most children who have brain damage have gastrointestinal impairments and that vomiting is a severe manifestation with significant morbidity which may persist even after surgery.

Similarly, Werlin [25] studied antroduodenal motility in neurologicaly handicapped children with feeding intolerance and found that 50% of patients suffer from vomiting. The author concluded that symptoms present in these patients might be due to an underlying forgut motor disorder.

Moreover, Del Giudice et al., [5] found that 91% of patients affected by CP had gastrointestinal symptoms. They also demonstrated that gastrointestinal motor dysfunctions are known to occur frequently in children with different degrees of brain damage and in neurologically impaired children and that the degree of GI dismotility seems to correlate with the degree of CNS damage. They confirmed that in this subset of children, chronic constipation is mainly due to prolonged transit at level of the more proximal segments of the colon. The authors further added that no child with CP presented with chronic constipation associated with soiling in contrast with neurologically normal children in whom constipation is characterized by fecal soiling.

In the current study, we found that, there was no statistical significant difference in gastric motility, as denoted by the power ratio and the dominant frequency of the EGG study, between patients and controls. However, the median values of the power ratio and the dominant frequency of the patients were lower than those of the control group. Additionally, there were no statistical significant differences between patients and controls as regard the qualitative visual analysis of the EGG study, yet the percentage of individuals with gastric motility dysfunction was more in patients (53.3% bradygastric, 3.3% tachygastric) than controls (33.3% bradygastric, 0% tachygastric).

Similarly, Ravelli and Milla [7] found that 62% (31/50) of patients with CNS disorders had antral dysrhythmia. Four had bradyarrhythmia, 12 had tachyarrhythmia, 12 had unstable electrical activity and 3 had mixed dysrhythmia in which no dominant frequency could be detected. They concluded that antral dysrhythmias could be caused by disordered intrinsic nerves of the enteric nervous system. Since their patients had various types of gastric antral dysrhythmias characterized by slower, faster, or disorganized electrical activity, they explained that this variability may be the consequence of the variable etiology, pathology, severity and site of neural lesions at the level of the CNS or the enteric nervous system.

Additionally, Werlin [25] studied antroduodenal motility in neurologically handicapped children with feeding intolerance and found that all

studied children had abnormal antroduodenal motility. The author concluded that in neurologicaly handicapped children, foregut dysmotility may be more common than is generally recognized and can explain many of the upper gastrointestinal symptoms in neurologically handicapped children.

In the current study, we found that there was a highly significant lower dominant frequency in patients who complained of vomiting compared to those who didn't; while their mean of power ratio was higher yet the latter comparison didn't reach statistical significance. Additionally, 14 of the 15 children who suffered from vomiting had dysrhythmia.

In agreement with our results, Ravelli and Milla, [7] found that 62% of children with disorders of the CNS and vomiting had antral dysrhythmia while patients without vomiting had similar dominant frequency as that of controls. They concluded that vomiting is related to gastric dysrhythmias and delayed gastric emptying possibly due to activation of the emetic reflex at least as often as gastroesophageal reflux.

Similarly, Miki et al., [26] in a study about antroduodenal motor function and gastro-oesophageal reflux in neurologically impaired children, reported that fasting antroduodenal motility was abnormal in 11 neurologically impaired children with vomiting. In 1998, Levanon and Chen [27] reported that impaired myoelectrical activity observed in the EGG is associated with disturbed motility and upper gastrointestinal symptoms (nausea, vomiting, abdominal pain) and suggested that EGG could be a useful tool in the primary evaluation of symptoms suggestive of gastroparesis.

In the current study, we found that there was highly significant lower DF in patients who had constipation compared to patients with no constipation. In agreement with our results, Ravelli and Milla [7] found that 60% of patients with disorders of the CNS and gastric dysrhythmia suffered from constipation. They concluded that chronic constipation might affect the motor activity of the stomach and the proximal small bowel.

In the current study both the patients and the control groups showed within normal fasting gastrin levels for age. Nevertheless, statistically significant higher fasting gastrin levels were found in CP patients. Meanwhile, no statistical significant difference was detected between hypertonic and hypotonic CP patients as well as those with GIT complaints versus those without.

Although many studies reported the gastrin increases gastric motility [28], yet relatively higher gastrin levels were associated with the predominance of bradygastria in our series of patients. We hypothesize that these results could be attributed to the fore-mentioned malnourished state of our CP patients which could have consequently lead to gastric mucosal atrophy [29] and/or gastric acid hyposecretion that in turn increases gastrin as a feedback mechanism [30–32]. In support of this point is the statistically significant negative correlation between serum gastrin levels and the weight percent detected in our series of patients.

Additionally, it was reported that gastrin strengthens the antral contractions against the pylorus, and constricts the pyloric sphincter, which has the effect of slowing the rate of gastric emptying [33]. Meanwhile other authors had concluded that injection of gastrin delays gastric emptying either directly [34-36] or through gastric acid action [10, 37]. The high gastrin found could thus be considered among the causes of the detected gastric motility disturbances in our series of patients. The previous point is further reinforced by the regression analysis of data which revealed that gastrin was the most determinant factor for dominant frequency values followed by the power ratio in our series of CP patients.

In conclusion, CP patients have disturbed gastric motility which explains the different proximal gastrointestinal clinical manifestations experienced by our patients and the disturbed gastrin levels could be considered among the causes. We thus recommend further larger scale studies to study the other hormonal and non hormonal factors affecting motility in CP patients; meanwhile, their feeding protocols should include small frequent meals in a semisolid rather than solid nature to overcome the gastric dysrrhythmias and delayed gastric emptying that they could subsequently suffer from.

Corresponding author
Nassar M.F
Pediatrics Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt.
maie_nassar@yahoo.co.uk

5. References:

1. Kuban KC, Leviton A. Cerebral palsy. *N Engl J Med*. 1994; 20; 330(3): 188-195.
2. Bax M, Goldstein M, Rosenbaum P, et al. Executive Committee for the Definition of Cerebral Palsy. Proposed definition and classification of cerebral palsy, April 2005. *Dev Med Child Neurol*. 2005; 47(8): 571-576.
3. Rosenbloom L. Diagnosis and management of cerebral palsy. *Arch Dis Child*. 1995;72: 350-354.
4. Staiano A, Del Giudice E. Colonic transit and anorectal manometry in children with severe brain damage. *Pediatrics*. 1994; 94: 169-173.

5. Del Giudice ED, Staiano A, Capano G, et al. Gastrointestinal manifestations in children with cerebral palsy. *Brain Dev*. 1999; 21: 307–311.
6. Gustafsson PM, Tibbling L. Gastro-oesophageal reflux and oesophageal dysfunction in children and adolescents with brain damage. *Acta Paediatr*. 1994; 83: 1081–1085.
7. Ravelli AM, Milla PJ. Vomiting and gastroesophageal motor activity in children with disorders of the central nervous system. *J Pediatr Gastroenterol Nutr*. 1998; 26: 56–63.
8. Johnson LR. Gastrointestinal physiology. In: Byrne JH, Downey JM, Goodman HM, eds. *Essential Medical Physiology*. Raven Press 1992:467.
9. Ganong WF. Regulation of gastrointestinal function In: Ganong WF, ed. *Review of Medical Physiology*, 20th edition. New York: Lange Medical Books/McGraw-Hill 2001: 464.
10. Mearadji B, Straathof JW, Lamers CB, et al. Effect of gastrin on proximal gastric motor function in humans. *Neurogastroenterol Motil* . 1999; 11: 449-455.
11. Cherner JA, Doppman JL, Norton JA, et al. Selective Venous sampling for gastrin to localize gastrinomas. A prospective assessment. *Ann Intern Med*. 1986; 105: 841-847.
12. Chen J, McCallum RW. Electrogastrography: measurement, analysis and prospective applications. *Med Biol Eng Comput*. 1991; 29:339-350.
13. Riezzo G, Porcelli P, Guerra, V, et al. Effect of different psychophysiological stressors on the cutaneous electrogastrogram in healthy subjects. *Arch Physiol Biochem*. 1996;104:282-286.
14. Parkman HP, Hasler WL, Fisher RS, American Gastroenterological Association. American Gastroenterological Association technical review on the diagnosis and treatment of gastroparesis. *Gastroenterology*. 2004;127: 1592-1622.
15. Van der Schee EJ, Grashuis JL. Running spectrum analysis as an aid in the representation and interpretation of electrogastrographic signals. *Med Biol Eng Comput*. 1987;25:57-62.
16. Riezzo G, Chiloiro M, Guerra V. Electrogastrography in healthy children: Evaluation of normal values, influence of age, gender and obesity. *Dig Dis Sci*. 1998;43:1646-1651.
17. Jebbink HJ, Van Berge-Henegouwen GP, Bruijis PP, et al. Gastric myoelectrical activity and gastrointestinal motility in patients with functional dyspepsia. *Eur J Clin Invest*. 1995; 25: 429-437.
18. Daniel WW. *Biostatistics: A foundation for analysis in the health sciences*. 6th edn. John Wiley and Sons, Inc., New York, 1995.
19. Mei Z, Grummer-Strawn LM, Pietrobelli A, et al. Validity of body mass index compared with other body-composition screening indexes for the assessment of body fatness in children and adolescents. *Am J Clin Nutr*. 2002; 75:978-985.
20. Shaan F. Nutritional indices: Z centile or percent? *Lancet*. 1993; 341: 526-527.
21. Hurvitz EA, Green LB, Hornyak JE, et al. Body mass index measures in children with cerebral palsy related to gross motor function classification: a clinic-based study. *Am J Phys Med Rehabil*. 2008; 87: 395-403.
22. Feely BT, Gollapudi K, Otsuka NY. Body mass index in ambulatory cerebral palsy patients. *J Pediatr Orthop B*. 2007; 16:165-169.
23. Reyes AL, Cash AJ, Green SH, et al. Gastroesophageal reflux in children with cerebral palsy. *Child Care Health Dev*. 1993;19:109-118.
24. Vik T, Skrove MS, Dollner H, et al. Feeding problems and growth disorders among children with cerebral palsy in south and north Trondelag. *Tidsskr Nor Laegeforen*. 2001; 121: 1570-1574. [English abstract]
25. Werlin SL. Antroduodenal motility in neurologically handicapped children with feeding intolerance. *BMC Gastroenterol*. 2004; 1; 4: 19.
26. Miki K, Harada T, Kozaiwa K, et al. Antroduodenal motor function and gastro-oesophageal reflux in neurologically impaired children, adolescents and young adults. *Eur J Pediatr*. 1998; 157: 695-696.
27. Levanon D, Chen JZ. Electrogastrography: its role in managing gastric disorders. *J Pediatr Gastroenterol Nutrition*. 1998; 27: 431-443.
28. Wolfe MM. Diagnosis of gastrinoma: much ado about nothing? *Ann Intern Med*. 1989; 111: 697-699.
29. Rösch W. Chronic gastritis. *Z Gastroenterol*. 1980; 18: 237-242. [English abstract].
30. McCloy RF, Arnold R, Bardhan KD, et al. Pathophysiological effects of long-term acid suppression in man. *Dig Dis Sci*. 1995; 40: 96S-120S.
31. Sipponen P, Valle J, Varis K, et al. Fasting levels of serum gastrin in different functional and morphological states of the antro-fundal mucosa. An analysis of 860 subjects. *Scand J Gastroenterol*. 1990; 25: 513-519.
32. Turnipseed WD, Keith LM Jr. Altered serum gastrin levels in achlorhydric states. *Am J Surg*. 1976; 131: 175-177.

33. Wikipedia. WWW. Wikipedia, the free encyclopedia: Gastrin hormone article. Available at: http:en.wikipedia.org/wiki/gastrin. Accessed November 15, 2009.
34. Gielkens HA, Nieuwenhuizen A, Biemond I, et al. Interdigestive antroduodenal motility and gastric acid secretion. *Aliment Pharmacol Ther*. 1998; 12: 27-33.
35. Lee K Y, Kim MS, Chey WY. Effects of a meal and gut hormones on plasma motilin and duodenal motility in dog. *Am J Physiol*. 1980; 238: G280-283.
36. Wingate DL, Pearce EA, Hutton M, et al. Quantitative comparison of the effects of cholecystokinin, secretin, and pentagastrin on gastrointestinal myoelectric activity in the conscious fasted dog. *Gut*. 1978; 19: 593-601.
37. Verkijk M, Gielkens HA, Lamers CB, et al. Effect of gastrin on antroduodenal motility: role of intraluminal acidity. *Am J Physiol*, 1998; 275:G1209-1216.

11/2/2010

Significance of Angiopoietin-2 as a Serum Marker for Hepatocellular Carcinoma

Shereen Shoukry Hunter*[1], Maha Sayed Hassab-Allaah[1], Mahmoud Ahmad El-Ansary[2], Faten M El Shanawani [3] and Mona M Hassan[3]

[1]Tropical Medicine Department, Faculty of Medicine, Cairo University, Cairo, Egypt
[2]Hepatology and Gastroenterology Department, Theodor Bilharz Research Institute, Cairo, Egypt
[3]Clinical Chemistry Department, Theodor Bilharz Research Institute, Cairo, Egypt
*shereenhunter@hotmail.com

Abstract: Background and study aims: Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide and one of the major causes of death. The aim of this study was to investigate the potential role of Angiopoietin-2 as a non-invasive marker for HCC. Patients and Methods: This study was conducted on 30 patients with documented HCC and 30 cirrhotic patients with no evidence of HCC; as well as 30 healthy subjects who served as control group. The levels of alfa fetoprotein (AFP) and angiopoietin-2 (Ang-2) were measured for all cases together with full clinical assessment, liver biochemical profile, viral markers, ultrasound, abdominal triphasic computerized tomography (CT) scan and guided liver biopsy for HCC cases with atypical triphasic CT pattern. Results: There was a statistically highly significant elevation ($p< 0.001$) in the mean serum AFP in HCC group (155.5 ± 271.5 ng/ml) when compared with the control group (6.3 ± 2.4 ng/ml) and also a highly significant elevation ($p<0.01$) when compared to the cirrhosis group (29.3 ± 31.2 ng/ml). There was a statistically highly significant elevation ($p< 0.001$) in the mean serum Ang-2 in HCC group (10855 ± 5321.92 pg/ml) when compared with both the control (480.67 ± 202.3 pg/ml) and cirrhosis (5578.33 ± 2928.21 pg/ml) groups. The diagnostic sensitivity of AFP at a cutoff of 200 ng/ml was 24% and the specificity was 100%. The cutoff level of Ang-2 for diagnosis of HCC in this study was 8100 pg/ml, with a sensitivity and specificity of 70% and 80% respectively. Serum Ang-2 was significantly elevated in HCC patients with portal vein thrombosis than those without. There was a significant positive correlation between the number of hepatic focal lesions and the serum level of Ang-2. The combined use of the two markers (AFP and Ang-2) led to an increase in the sensitivity of AFP from 53.3% to 83.3%. Conclusion: Serum Ang-2 is elevated in patients with cirrhosis and further elevated in patients with HCC, so its use as an independent tumor marker in the diagnosis of HCC is to be considered. Simultaneous measurement of serum AFP and Ang-2 may enhance the sensitivity of HCC detection.

[Shereen Shoukry Hunter, Maha Sayed Hassab-Allaah, Mahmoud Ahmad El-Ansary, Faten M El Shanawani and Mona M Hassan. **Significance of Angiopoietin-2 as a Serum Marker for Hepatocellular Carcinoma**. Journal of American Science 2010;6(12):1114-1123]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Hepatocellular carcinoma (HCC)**,** Alpha-fetoprotein (AFP)**,** Angiopoietin-2 (Ang-2)

1. Introduction:

Hepatocellular carcinoma (HCC) is the most common primary malignant tumor of the liver *(El Serag & Rudolph, 2007).* Over a decade (1993-2002), there was nearly a twofold increase of the proportion of HCC among chronic liver disease (CLD) patients in Egypt with a significant decline of hepatitis B virus (HBV) and slight increase of hepatitis C virus (HCV) as risk factors *(El-Zayadi et al., 2005)*. Alfa-feto protein (AFP) is an inadequate screening test for HCC *(Sherman, 2001; Bruix & Sherman, 2005),* but still has a role in the diagnosis, since in cirrhotic patients with a mass in the liver an AFP greater than 200ng/mL has a very high positive predictive value for HCC *(Trevisani et al., 2001).* Angiopoeitin (Ang)-2 is a 66 kDa protein consisting of 496 amino acid residues which was detected by homology screening as a naturally occurring antagonist for both Ang-1 and the Tie-2 receptor. In adult mice and humans, they found that Ang-2 is expressed only at the sites of vascular remodeling (*Maisonpierre et al., 1997).* As HCCs are hypervascularized tumors the generation of new arterial vessels is a prerequisite for their survival. The induction of neoangiogenesis is mainly driven by hypoxia, leading to activation of several angiogenic pathways, including the angiopoietin/Tie-2 pathway. In this context, Ang-2 [in presence of vascular endothelial growth factor (VEGF)] allows remodeling of arterial vessels not stabilized by the effects of Ang-1 *(Holash et al., 1999).* Ang-2 is overexpressed in HCC-as measured by immunohistochemistry-especially of the highly vascular type *(Sugimachi et al., 2003).* Its expression is associated with portal infiltration, microvessel density, recurrence of HCC and decreased survival *(Wada et al., 2006).*

Recent studies reported high serum Ang-2 values in patients with HCC suggesting that it might represent a useful marker for HCC and a complementary diagnostic tool *(Scholz et al., 2007).*

The aim of this work was to investigate the potential role of Ang-2 as a diagnostic serum marker for HCC in patients with liver cirrhosis and to assess its sensitivity and specificity as compared to AFP and to assess whether the combined use of both markers can improve the diagnostic power of HCC.

2. Materials and methods

Study groups:

This study was conducted on 60 patients admitted to the Hepatology and Gastroenterology Department, Theodor Bilharz Research Institute (TBRI) in the period between November 2008 and June 2009. In addition, 30 apparently healthy individuals served as a control group.

They were divided into three main groups:

Group I (HCC group):

Included 30 cirrhotic patients with HCC, 19 of them were males (63.3%) and 11 females (36.7%). Five patients with focal hepatic lesions did not show the typical HCC pattern on triphasic CT scan: these lesions were biopsied guided by ultrasound for histopathological assessment and proved to be HCC.

Group II (liver cirrhosis group):

Included 30 patients with post hepatitic liver cirrhosis; 17 of them were males (56.7%) and 13 females (43.3%).

Group III (control group):

Included 30 apparently healthy individuals, 9 of them were males (30%) and 21 females (70%). They were completely free clinically, with normal laboratory findings, negative viral hepatitis markers and normal abdominal ultrasonographic findings.

Exclusion criteria:

Inflammatory or septic conditions as spontaneous bacterial peritonitis
(SBP).

Focal hepatic lesions other than HCC (cholangiocarcinoma, hemangioma, hepatoblastoma, metastatic focal lesions…etc).

Carcinoma elsewhere.

Methodology:

Full history taking and clinical examination were done to all patients and the following routine laboratory investigations:

- Complete blood picture and erythrocyte sedimentation rate (ESR).
- Liver function tests: alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), gamma glutamyl transpeptidase (GGT), serum bilirubin (direct and indirect), serum proteins and serum albumin, prothromhin time and concentration (PT and PC) and international normalized ratio (INR).
- Renal function tests: serum urea and creatinine.
- Hepatitis markers: hepatitis B surface antigen (HBsAg), antibody to hepatitis B core antigen (HBcAb) total, antibody to hepatitis B surface antigen (HBsAb) and anti-HCV antibody.
- Fasting and 2 hours post-prandial blood sugar.

Evaluation of the severity of liver cirrhosis was assessed in each cirrhotic patient with the Modified Child score (Pugh et al., 1973).

Imaging studies:

Abdominal ultrasonography:

Abdominal ultrasonography was performed for all groups. Patients were examined using a real time machine (Hitachi, EUB-515A).

Liver was assessed for size, smoothness of the surface, texture, portal vein diameter, and thickening of portal tracts as an indicator for schistosomal hepatic fibrosis (Abdel-Wahab et al., 1992). The presence of focal lesions and their detailed description as regards number, size, site, echogenicity was reported. Doppler ultrasound was used to assess the patency of the portal vein so as to detect the presence of malignant thrombi and assess their extension. It was also used to detect any Doppler signals inside and around the lesions as the presence of intra-lesional arterial signals is highly suggestive of malignancy (Maruyama et al., 2008). A complete abdominal scanning was done to detect any other abnormality including the presence of ascites, lymph nodes or abdominal masses.

Triphasic abdominal CT scanning:

Spiral triphasic CT abdomen was done to all patients in HCC group for the diagnosis of hepatic focal lesions with specific features of HCC as previously described (Van Leeuwen et al., 1996) (Paley and Ros, 1998) (Hoon Ji et al., 2001).

Ultrasonographic-guided liver biopsy:

Biopsy was done to 5 patients with focal lesions using Trucut needles under ultrasound guidance after careful explanation of the procedure to the patient, with informed written consent to the procedure and after fulfillment of the following criteria:

1. Acceptable prothrombin time (<17 sec) and concentration (>60%), INR <2 and platelet count > 75.000/cc.
2. No ascites at the time of biopsy.
3. No other obstacles to liver biopsy (e.g. inaccessible lesion or lesion adjacent to vital structures like blood vessels).

All biopsies were histopathologically graded by Steiner-Edmondson grading system (Edmondson and Steiner, 1954).

Tumor markers:

A 15 ml blood sample was drawn from each subject immediately after diagnosis. Blood samples were centrifuged and serum aliquoted and stored at –70°C until tested for AFP and Ang-2.

1-Measurement of serum AFP (ng/dl)

AFP assay:

Serum AFP was measured by enzyme-linked immunosorbent assay (ELISA) technique using commercially available immunometric assays utilizing enhanced chemiluminescence (EQUIPAR Diagnostics, France) with cutoff 20 ng/dl as a maximum level of normal.

2- Measurement of serum Ang-2

Ang-2 assay characteristics:

Sensitivity:

The minimum detectable dose of the human Ang-2 is <6 pg/ml. This was determined by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 30 times, and calculating the corresponding concentrations.

Specificity:

Buffered solutions of a panel of substances ranging in concentrations from 10.000 to 50.000 pg/mL were assayed with the Human Ang-2 kit and found to have no cross-reactivity: Human Ang-1, Ang-4, granulocyte monocyte colony stimulating factor (GM-CSF), interferon (IFN)γ, interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10 and tumor necrosis factor (TNF)-α. Random normal serum samples from various species were also evaluated with the Human Ang-2 kit. No crossreactivity was observed with the goat, hamster, mouse and rat serum samples. There was moderate cross-reactivity with rabbit and swine, and significant cross-reactivity with fetal bovine, calf, horse and monkey serum samples.

Principle of the test:

The Human Ang-2 kit (Biosource International, USA) is a solid phase sandwich ELISA. A monoclonal antibody specific to human Ang-2 has been coated onto the wells of the microtiter strips provided. During the first incubation, standards of known human Ang-2 content, controls, and unknown samples are pipetted into the coated wells, followed by the addition of biotinylated second anti-Ang-2 antibody. After washing, streptavidin-peroxidase (enzyme) is added. This binds to the biotinylated antibody to complete the four-member sandwich. After a second incubation and washing to remove all the unbound enzyme, a substrate solution is added, which is acted upon by the bound enzyme to produce color. The intensity of this colored product is directly proportional to the concentration of human Ang-2 present in the original specimen.

Statistical Analysis

Results were expressed as mean ± standard deviation (SD). Differences between groups were analyzed either by using the Chi square test or student's t test and non-parametric (Mann Whitney test) for comparison between two groups or ANOVA test for multiple group comparison. Spearman rank correlation coefficient was used to determine significant correlations among different parameters. The analysis was performed using Statistical Analysis System, version 6.03, on an IBM at personal computer.

3. Results

The mean age ± SD within the HCC group was (50.6 ± 6.7) compared to the cirrhosis group (50.7± 6.0) and the control group (32.2± 7.2). The number of males within HCC, cirrhosis and control groups were 19/30 (63%), 17/30 (57%) and 9/30 (30%) respectively. There was no statistically significant difference in age and sex between the three studied groups. Table (1) showed the results of the laboratory liver profile of the studied groups.

Table (1): Liver profile of the studied groups

Parameters	HCC (n= 30)	Cirrhosis (n= 30)	Control (n= 30)	p value
Total bilirubin (up to 1mg/dl)	2.54 ± 2.06 a	2.67 ± 2.35 a	0.88 ± 0.22	a p< 0.01 vs. control HS
Direct bilirubin (up to 0.25mg/dl)	1.42 ± 1.45 a	1.56 ± 1.67 a	0.25 ± 0.05	a p< 0.01 vs. control HS
ALT (30-65 U/l)	56.5 ± 34.2 a	44.03 ± 27.58 a	19.37 ± 8.5	a p< 0.01 vs. control HS
AST (15-37 U/l)	60.57± 36.62 a	49.87 ± 31.04 a	24.77 ± 10.89	a p< 0.01 vs. control HS
ALP (61-171 U/l)	195.8 ± 99.07 a, b	148.5 ± 115.66 a	70.1 ± 33.1	a p< 0.01 vs. control HS b p< 0.05 vs. cirrhosis S

S= significant P< 0.05 HS= highly significant P< 0.01

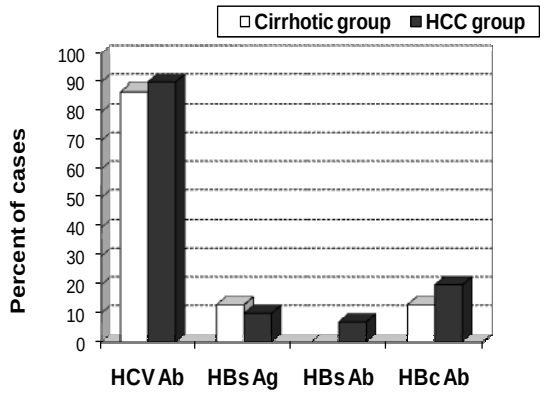


Figure (1): Viral markers of the studied groups

Among the cirrhosis group, 26 patients (86.7%) were positive for HCV antibody, while 4 patients (13.3%) had positive HBsAg and HBcAb and no patient was positive for HBsAb. Among the HCC group, 27 patients (90%) were positive for HCV antibody, 2 of those patients with HCV had previous exposure to HBV and were immune (HBsAb and HBcAb positive) and 3 patients (10%) were positive to HBsAg and HBcAb showing no statistically significant difference between both groups (Figure 1).

Five patients (17%) in the cirrhosis group were Child class A compared to 9 patients (30%) in the HCC group. Nine patients (30%) in the cirrhosis group and 10 patients (33%) of the HCC group were Child class B, while 16 patients (53%) in the cirrhosis group were Child class C as compared to 11 patients (37%) in the HCC group with no statistically significant difference between both groups.

The ultrasonographic features of the focal hepatic lesions in HCC patients showed that 19 patients (63%) had single focal lesion, 6 patients (20%) had 2 focal lesions, and 5 patients (17%) had multiple focal lesions. Four patients (13%) had focal lesions ≤ 2cms in diameter while 26 patients (87%) had focal lesions > 2cms in diameter. The focal lesions were located in the right hepatic lobe in 19 patients (63%), in the left hepatic lobe in 7 patients (23%), while 4 patients (13%) had focal lesions detected in both lobes. Twenty one lesions (70%) were hypo-echoic, 1 lesion (3.3%) was hyper-echoic and 8 lesions (26.7%) were iso-echoic. There were 5 cases (17%) of portal vein thrombosis in HCC group and none in liver cirrhosis group (0%).

As regards CT pattern of HCCs in triphasic CT scan: 25 lesions (83%) showed typical enhancement features of HCC (typical specific pattern of arterial uptake followed by venous washout in the delayed portal/venous phase), while 5 lesions (17%) showed atypical enhancement pattern on different CT scan phases and were biopsied under ultrasound guidance and examined histopathologically: 2 lesions (40%) were grade I, while the other 3 lesions were grade II (60%).

There was a statistically highly significant elevation ($p< 0.001$) in the mean serum AFP in HCC group (155.5 ± 271.5 ng/ml) when compared with the control group (6.3 ± 2.4 ng/ml) and also a highly significant elevation ($p<0.01$) when compared to the cirrhosis group (29.3 ± 31.2 ng/ml) (Table 2).

There was a statistically highly significant elevation ($p< 0.001$) in the mean serum Ang-2 in HCC group (10855 ± 5321.92 pg/ml) when compared with both the control (480.67 ± 202.3 pg/ml) and cirrhosis (5578.33 ± 2928.21 pg/ml) groups (Table 2)

Table (2): Mean levels of AFP and Ang-2 in studied groups

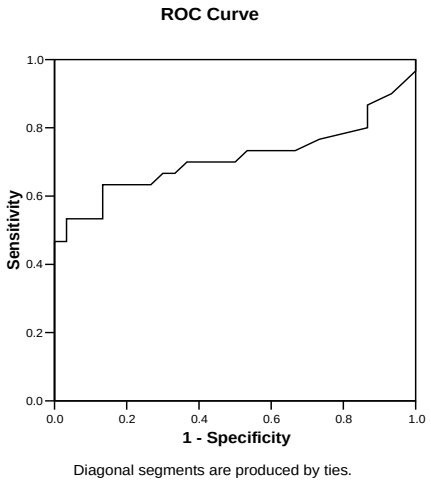
	HCC (n= 30)	Cirrhosis (n= 30)	Control (n= 30)	P value
AFP	*155.5±271.5* [a, b]	*29.3 ± 31.2*	*6.3± 2.4*	[a] *p< 0.001 vs. control* HS [b] *p< 0.01 vs. cirrhosis* HS
Ang-2	*10855.0 ± 5321.92* [ab]	*5578.33 ± 2928.21* [a]	*480.67 ± 202.30*	[a] *p< 0.001 vs. control* HS [b] *p< 0.001 vs. cirrhosis* HS

HS= highly significant P< 0.01

Specificity and sensitivity of AFP:

When using the receiver operator characterizing (ROC) curve, to improve the specificity and sensitivity of AFP in the differentiation between HCC and cirrhosis, the cutoff value of 75.5 ng/ml yielded a sensitivity and specificity of 53.3% and 86.7%, respectively (best cutoff). When the cutoff of AFP was increased to 200 ng/m1 the sensitivity dropped to 24% and specificity was 100% (Figure 2).

Figure (2): ROC curve for AFP



Specificity and sensitivity of Ang-2

1) Cirrhosis group *versus* control:

When using the ROC curve, to improve the specificity and sensitivity of Ang-2, the cutoff value of 1700 pg/ml yielded a sensitivity and specificity of 90% and 97%, respectively (best cutoff).

2) HCC group *versus* control:

When using the ROC curve, to improve the specificity and sensitivity of Ang-2, the cutoff value of 3900 pg/ml yielded a sensitivity and specificity of 93% and 100%, respectively (best cutoff).

3) HCC group *versus* cirrhosis:

When using the ROC curve, to improve the specificity and sensitivity of Ang-2, the cutoff value of 8100 pg/ml yielded a sensitivity and specificity of 70% and 80%, respectively (best cutoff) (Figure 3).

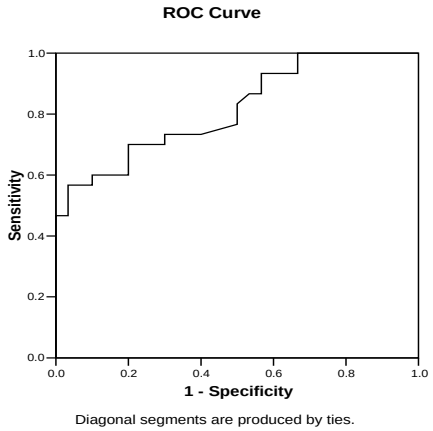


Figure (3): ROC curve for Ang-2 (cirrhosis versus HCC)

Table (3) showed the correlation between the level of Ang-2 and other parameters in the studied groups.

Figure (4) showed the inverse correlation between serum Ang-2 and serum albumin in patients with cirrhosis.

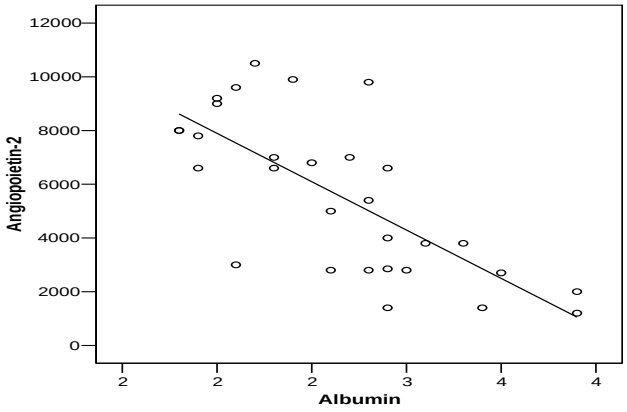


Figure (4): *Correlation between Ang-2 and albumin in cirrhosis patients.*

Table (3): ***Correlation between Ang-2 level and other parameters in the studied groups***

Variable / Correlated	Cirrhosis (n= 30)		HCC (n= 30)	
	Correlation coefficient (r)	p value	Correlation coefficient (r)	p value
Age	0.224	0.233 NS	0.117	0.537 NS
TLC	0.101	0.596 NS	0.045	0.815 NS
Haemoglobin	-0.144	0.448 NS	0.005	0.979 NS
Platelets	-0.273	0.144 NS	-0.306	0.100 NS
ESR	0.201	0.288 NS	0.176	0.352 NS
PC	-0.520	0.003 HS	-0.122	0.522 NS
T. bilirubin	0.359	0.052 NS	0.313	0.092 NS
D. bilirubin	0.415	0.023 S	0.291	0.118 NS
AST	-0.029	0.880 NS	0.030	0.877 NS
ALT	-0.204	0.279 NS	-0.163	0.389 NS
ALP	0.218	0.248 NS	0.184	0.330 NS
T. Protein	0.003	0.986 NS	-0.433	0.056 NS
Albumin	-0.737	0.001 HS	-0.327	0.077 NS
Urea	0.237	0.207 NS	-0.313	0.092 NS
Creatinine	0.214	0.256 NS	0.131	0.490 NS
FBS	0.300	0.107 NS	-0.094	0.622 NS
AFP	-0.226	0.229 NS	0.172	0.363 NS

NS= non significant P> 0.05 S= significant P< 0.05 HS= highly significant P< 0.01

Table (4) showed the correlation between serum Ang-2, serum AFP and Child score in both cirrhosis and HCC groups.

Table (4): ***Correlation between serum Ang-2, serum AFP and Child score in cirrhosis and HCC patients:***

Variable / Correlated	Cirrhosis (n=30)		HCC (n= 30)	
	Correlation coefficient (r)	**p value**	**Correlation coefficient (r)**	**p value**
Ang-2 vs. Child score	0.807	0.001 HS	0.339	0.067 NS
AFP vs. Child score	-0.222	0.238 NS	0.643	0.001 HS

NS= non significant P> 0.05 HS= highly significant P< 0.01

There was a statistically significant positive correlation between the serum Ang-2 level in HCC patients with the number of focal lesions (r= 0.95; p= 0.031) and no significant correlation between serum Ang-2 and the size of hepatic focal lesions (Table 5).

Table (5): ***Correlation between Ang-2 with the number and size of focal lesions in HCC patients***

Variable / Correlated	HCC (n= 30)	
	Correlation coefficient (r)	p value
Ang-2 vs No. of focal lesions	0.395	0.031 S
Ang-2 vs size of focal lesions	0.197	0.298 NS

NS= non significant P> 0.05 S= significant P< 0.05

There was no significant correlation between serum level of AFP and the size or number of hepatic focal lesions.

There was a significant difference ($p < 0.05$) between the mean value of Ang-2 in HCC patients with portal vein thrombosis (5 patients) (19500.00 ± 4320.88) and those without (25 patients) (*9092.5 ± 3726)*.

4. Discussion:

At present, AFP is the most commonly used tumor marker in early HCC screening in populations at high risk, however serum AFP is associated with two main problems; first, the transient high rise in the serum level of AFP during exacerbation of hepatitis on top of CLD (serum level >100 ng/ml) and slight rise in the serum AFP in chronic hepatitis and cirrhosis (serum level >20 ng/dl) causing diagnostic difficulties (low specificity) (Kuntz & Kuntz, 2006). The second is that among all patients diagnosed with HCC, AFP levels may be normal in up to 40% of patients, particularly during the early stages (low sensitivity) (Sherman., 2001).

Serum angiopoietin-2 levels were elevated in patients with cirrhosis, implicating a possible role of the angiopoietin-Tie-2 system for neoangiogenesis in cirrhosis, and were further elevated in patients with HCC, suggesting the potential use of angiopoietin-2 as a marker for the detection of cirrhosis and HCC (Scholz et al., 2007).

This study was thus done to further investigate the potential role of Ang-2 as a diagnostic serum marker for HCC in patients with liver cirrhosis and to assess its sensitivity and specificity compared to AFP and whether -in association with AFP- Ang-2 improves the diagnostic power of HCC.

Chronic HCV infection, as a cause of cirrhosis, accounted for 90% of our HCC patients reflecting the close relationship between HCV and HCC. Our results are in agreement with El-Zayadi et al. (2005) who reported that HCV accounted for 86.9% of HCC cases during a single center study for HCC in Egypt over a decade. Darwish et al. (1997) reported also that viral hepatitis is strongly associated with the development of HCC in Egyptian patients and HCV seems to play a predominant role compared with HBV.

In our study HBV carriers were 10% in HCC group which was in agreement with El-Zayadi et al. (2005) who noticed a significant decline of HBV infection in HCC patients from 38.6% to 20.5% and attributed that partially to successful control measures of blood transfusion introduced in the mid-seventies and partially to the presence of undiagnosed cases of mutant or occult HBV infection, which requires costly assays for diagnosis. Yates et al. (1999) concluded that infection with HCV and HCV-HBV double infection, but not HBV alone, is strongly correlated with HCC in Egypt.

On using the ROC curve to reach the value of the best sensitivity and specificity of AFP; it has been shown that the sensitivity and specificity of AFP varied with the different cutoff values used. At a value of 75.5 ng/ml (the best cutoff), the sensitivity was 53% and the specificity was 87%. Bruix and Sherman (2005) reported the diagnostic cutoff of HCC at 200 ng/ml. In our study, when using this cutoff, the sensitivity declined to 24% while the specificity was 100%. This finding was comparable to that of Oka et al. (1994), who reported low sensitivity (13%) and a specificity of 97% at AFP values over 200 ng/ml. When we further increased the cutoff of serum AFP> 400 ng/ml, the specificity increased and the sensitivity decreased to 10% which was close to the results obtained by Rapaccini et al. (2004), who reported a sensitivity of 7.2% at a cutoff > 400 ng/ml.

In this study, AFP was elevated (>200 ng/ml) in only 23.3% of HCC patients and this was in agreement with Huo et al. (2004), who concluded that serum AFP level was a weak diagnostic predictor in HCC patients.

In this study, no significant correlation was found between AFP levels and the number and size of focal hepatic lesions. This is in accordance with Sato et al. (1994), who concluded that the rise in the serum AFP level did not usually correlate with the tumor size. This could be explained by the fact that tumor differentiation and its ability to secrete AFP are more important than the tumor size in determining the level of AFP produced by HCC (Okaet al., 1994).

In the current study, there was a significant correlation between AFP and the Child classification in the HCC group and this may be attributed to the presence of underlying CLD with subsequent cirrhosis and the progressive deterioration of the liver condition.

Our results revealed that there was a statistically highly significant elevation ($p< 0.001$) in the mean serum Ang-2 in cirrhosis group (5578.33 ± 2928.21 pg/ml) when compared to control group (480.67 ± 202.3 pg/ml).

These results are consistent with Scholz et al. (2007) who reported a statistically highly significant elevation of Ang-2 serum levels in cirrhosis patients when compared to control subjects. Although there are few data available regarding the expression of angiopoietins in cirrhosis in humans, many theories could be speculated in explanation:

Ang-2 is released in inflammatory conditions such as chronic HCV infection (Scholz et al., 2007). Salcedo

et al. (2005) reported that chronic HCV patients showed elevated serum baseline VEGF and Ang-2 levels. After treatment by interferon alpha 2b plus ribavirin, both factors were decreased, whereas antiangiogenic sTie-2 was increased, indicating a shift toward an "anti-angiogenic" profile of serum markers in chronic HCV patients.

In situ hybridization data had shown that Ang-2 mRNA expression was absent from hepatocytes of cirrhotic livers. Apart from endothelial cells of blood vessels, Ang-2 positive cells were found also within the connective tissue strands spanning between the portal tracts. Ang-2 expressing cells may include endothelial cells as suggested by their typical cellular morphology, as well as inflammatory and mesenchymal cells (Scholz et al., 2007). These data may argue against inflammation as the sole reason for elevated Ang-2 levels in serum of cirrhotic patients and emerge the fibrosis process as an extra explanation (Scholz et al., 2007).

Angiopoietins are overexpressed in the rat liver after partial hepatectomy but their role is less fully understood (Sato et al., 2001).

Other similar findings include generation of neovessels in livers of primary biliary cirrhosis patients accompanied by the increased expression of VEGF, Ang-2, and Tie-2 (Medina et al., 2005). Taken together, these published data suggest a causative role of angiogenic factors such as Ang-2 in the remodeling of the cirrhotic liver.

Furthermore, our results revealed that there was a statistically highly significant elevation ($p< 0.001$) in the mean serum Ang-2 in HCC group (10855.0 ± 5321.92 pg/ml) when compared to cirrhosis group (5578.33 ± 2928.21 pg/ml). These results are consistent with Scholz et al. (2007) who reported a statistically highly significant elevation of Ang-2 serum levels in HCC patients when compared to cirrhotic patients and also reported that Ang-2 mRNA was expressed in most of HCC cryopreserved biopsies (using in situ hybridization) in addition to positive staining of the intratumoral vessels.

In our study, when using ROC curve to improve the specificity and sensitivity of Ang-2, the cutoff value of 8100 pg/ml yielded a sensitivity and specificity of 70% and 80%, respectively (best cutoff). These results are consistent with Scholz et al. (2007) who reported a sensitivity of 70.56% and specificity of 73.28% when the high cutoff value of Ang-2 was used (12350 pg/ml), the sensitivity and specificity were 40% and 100% respectively. These results demonstrate that the high cutoff value of serum Ang-2 may show better results of specificity than the best cutoff value of (8100 pg/ml) at the expense of much decrease of sensitivity value.

Concerning the demographic features, hematological tests, ESR and liver function tests, our study revealed that there was no significant correlation between all of the previous parameters and serum Ang-2 levels, among both cirrhosis and HCC groups, with the exception of a significant positive correlation between direct bilirubin and serum Ang-2 level in cirrhosis group, and a significant inverse correlation between serum albumin, PC and serum Ang-2 in the cirrhosis group; consequently, there was a significant positive correlation between serum Ang-2 levels and Child classification in the cirrhosis group. Although Scholz et al. (2007) had reported no significant correlation between serum Ang-2 levels and Child classification, they found that discrimination between cirrhotic and control individuals by Ang-2 serum levels improved somewhat with the progression of cirrhosis. These interesting data are in need to be further studied to assess the significance of Ang-2 as a serum marker in liver cirrhosis and illustrate its role in liver remodeling process.

On the other hand, there was no significant positive correlation between serum Ang-2 levels and Child classification in the HCC group in our study, as was the case in Scholz et al. (2007). This may be explained by the fact that Ang-2 levels increase or decrease according to degree of differentiation of HCC, tumor density and portal vein invasion and not according to the degree of deterioration in liver functions.

In addition, serum Ang-2 did not exhibit a significant correlation with the tumor pathology or the size of focal hepatic lesions which was also in agreement with Scholz et al. (2007). However, there was a significant correlation between the number of hepatic focal lesions and serum Ang-2 level, but this may be attributed to the small number of patients having more than 2 hepatic focal lesions (16.6%) as compared to those with 1 or 2 lesions (83.3%), so, further studies are needed using larger number of patients with groups of comparable sizes to verify such correlation.

In our results, there was a significant difference ($p < 0.05$) between the mean value of Ang-2 in HCC patients with portal vein thrombosis (19500.00 ± 4320.88) and those without (9092.5 ± 3726). Li et al. (2006) reported a high expression of Ang-2 mRNA (using immunohistochemistry) in HCC cases with portal vein tumor thrombosis in comparison with those without; concluding that Ang-2 can promote tumor thrombus formation by modulating angiogenesis. Also, Kuboki et al. (2008) reported a significant relationship between high Ang-2 levels in hepatic vein and portal vein invasion. However, further studies are needed to verify such

correlation using larger number of patients and imaging modalities that can differentiate between malignant and non-malignant portal vein thrombosis.
There was no correlation between serum Ang-2 and serum AFP in patients of both the cirrhosis and HCC groups in our study. This was in agreement with Scholz et al. (2007) who reported no correlation between both markers in patients with HCC and in patients with cirrhosis.

In our results, the combined use of the two markers (AFP and Ang-2) led to an increase in the sensitivity of AFP from 53.3% to 83.3%. These results were in agreement with Scholz et al. (2007) who concluded that detection rates could increase from 71.0% when using AFP alone to 89.2% when using both markers.

5. Conclusion:

• Serum Ang-2 levels are elevated in patients with liver cirrhosis secondary to HCV infection, implicating a possible role of the Ang-2 in chronic HCV infection and remodeling in cirrhotic patients.
• The use of Ang-2 as an independent tumor marker in the diagnosis of HCC is to be considered as serum Ang-2 levels were more elevated in HCC patients when compared to cirrhotic patients.
• Ang-2 may be a helpful tool in the diagnosis of vascular invasion in patients with HCC, as it was significantly elevated in HCC patients with portal vein thrombosis when compared to those without.
• AFP was found to be a weak diagnostic predictor with low sensitivity. Decreasing the cutoff value was associated with improvement of sensitivity at the expense of specificity.
• Ang-2 could be combined with AFP to increase its sensitivity in HCC detection, as combined use of both markers gave the highest index of detection of HCC.

Corresponding author
Shereen Shoukry Hunter
Tropical Medicine Department, Faculty of Medicine, Cairo University, Cairo, Egypt
shereenhunter@hotmail.com

5. References:

1. Abdel Wahab MF, Esmat G, Ramzy I et al. (1992). Schistosoma haematobium infection in Egyptian schoolchildren: demonstration of both hepatic and urinary tract morbidity by ultrasonography. Trans R Soc Trop Med Hyg; Jul-Aug;86(4):406-9.
2. Bruix J & Sherman M (2005). AASLD Practice Guidelines, Management of hepatocellular carcinoma. J Hepatol, 42(5):1208-1236.
3. Darwish MA, Amer AF, El-Moeity AA & Darwish NM (1997). Association of hepatitis C virus with liver cirrhosis and hepatocellular carcinoma compared with hepatitis B virus in Egyptian patients. J Egypt Public Health Assoc;72(5-6):569-89.
4. Edmondson HA & Steiner PE (1954). Primary carcinoma of the liver: a study of 100 cases among 48,900 necropsies. Cancer; 7: 462–503.
5. El-Serag HB & Rudolph KL (2007). Hepatocellular Carcinoma: Epidemiology and Molecular Carcinogenesis. Gastroenterology; 132:2557–2576.
6. El-Zayadi AR, Badran HM, Barakat EM et al. (2005). Hepatocellular carcinoma in Egypt: a single center study over a decade. World J Gastroenterol, Sep 7;11(33):5193-8.
7. Holash J, Wiegand SJ & Yancopoulos GD (1999). New model of tumour angiogenesis: dynamic balance between vessel regression & VEGF. Oncogene; 18 5356 -5362.
8. Hoon Ji, McTavish JD, Mortele KJ et al. (2001). Hepatic Imaging with Multidetector CT. Radiographics; Oct; 21 Spec No:S71-80.
9. Huo TI, Wu JC, Lin HC et al. (2004). Determination of the optimal model for endstage liver disease score in patients with small hepatocellular carcinoma undergoing loco-regional therapy. Liver Transpl; 10(12):1507-13.
10. Kuboki S, Shimizu H, Mitsuhashi N et al. (2008). Angiopoietin-2 levels in the hepatic vein as a useful predictor of tumor invasiveness and prognosis in human hepatocellular carcinoma. J Gastroenterol Hepatol. Jul; 23(7 Pt 2):e157-64.
11. Li Q, Xu B, Fu L et al. (2006). Correlation of four vascular specific growth factors with carcinogenesis and portal vein tumor thrombus formation in human
12. hepatocellular carcinoma. J Exp Clin Cancer Res. Sep; 25(3):403-9.
13. Maisonpierre PC, Suri C, Jones PF et al. (1997). Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science; Jul 4; 277(5322):55 60.
14. Maruyama H, Yoshikawa M, Yokosuka O (2008). Current role of ultrasound for the management of hepatocellular carcinoma. World J Gastroenterol. 2008 Mar 21;14(11):1710-9.
15. Medina J, Sanz-Cameno P, Garcia-Buey L et al. (2005). Evidence of angiogenesis in primary biliary cirrhosis: An

immunohistochemical descriptive study. J Hepatol; 42:124–31.
16. Oka H, Tamori A, Kuroki T et al. (1994). Prospective study of alpha-fetoprotein in cirrhotic patients monitored for development of hepatocellular carcinoma. Hepatology; 19:61-66.
17. Paley MR & Ros PR (1998). Hepatic metastases. Radiol Clin North Am, Mar; 36(2):349-63.
18. Pugh RN, Murray-Lyon IM, Dawson JL, Pietroni MC, Williams R. Transection of the oesophagus for bleeding oesophageal varices. Br J Surg. 1973 Aug;60(8):646-9.
19. Rapaccini GL, Pompili M, Caturelli E, et al. (2004). Hepatocellular carcinomas < 2 cm in diameter complicating cirrhosis: Ultrasound and clinical features in 153 consecutive patients. Liver Int; 24(2): 124 – 130.
20. Salcedo X, Medina J, Sanz-Cameno P et al. (2005). The potential of angiogenesis soluble markers in chronic hepatitis C. Hepatology. Sep; 42(3):696-701.
21. Sato T, El-Assal ON, Ono T et al. (2001). Sinusoidal endothelial cell proliferation and expression of angiopoietin/Tie family in regenerating rat liver. J Hepatol; 34:690–8.
22. Sato Y, Nakata K, Kato Y et al. (1994). Early recognition of hepatocellular carcinoma based on altered profiles of alpha-fetoprotein. N Engl J Med; 328: 1802-6.
23. Scholz A, Rehm VA, Rieke S et al. (2007). Angiopoietin-2 serum levels are elevated in patients with liver cirrhosis and HCC. Am J Gastroenterol, Nov; 102(11):2471-81.
24. Sherman M (2001). Alpha fetoprotein: an obituary. J Hepatol; 34: 603-605.
25. Sugimachi K, Tanaka S, Taguchi K et al. (2003). Angiopoietin switching regulates angiogenesis and progression of human hepatocellular carcinoma. J Clin Pathol., Nov; 56(11):854-60.
26. Trevisani F, D'intino PE, Morselli-Labate AM et al. (2001). Serum alphafetoprotein for diagnosis of hepatocellular carcinoma in patients with chronic liver disease: influence of HBsAg and anti-HCV status. J Hepatol, 34: 570-5.
27. Van Leeuwen MS, Noordzij J, Feldberg MA et al. (1996). Focal liver lesions; characterization with triphasic spiral CT. Radiology, 201: 327-336.
28. Wada H, Nagano H, Yamamoto H et al. (2006). Expression pattern of angiogenic factors and prognosis after hepatic resection in hepatocellular carcinoma: importance of angiopoietin-2 and hypoxia-induced factor-1 alpha. Liver Int. May 26(4):414-23.
29. Yates SC, Hafez M, Beld M et al. (1999). Hepatocellular carcinoma in Egyptians with and without a history of hepatitis B virus infection: association with hepatitis C virus (HCV) infection but not with (HCV) RNA level. Am J Trop Med Hyg, Apr; 60(4):714-20.

11/3/2010

Double -Control, Randomized Study of Antibiotic Prophylaxis during Standard Dose Chemotherapy in Cancer Patients

Fatma Zakaria[*1] and Mohamad Zakaria[2]
Departments of Clinical Oncology[1] and Microbiology[2], Faculty of Medicine, Tanta University, Tanta, Egypt

Abstract: Background: Dilemma of antibacterial prophylaxis after chemotherapy still opened. Patients and methods:Double, control trial in patients who were receiving cyclic chemotherapy for solid tumors or lymphoma and who were at risk of temporary, sever neutropenia (fewer than 500 neutrophils/ml). Patients were randomly divided into two group, the first groups assigned to receive oral 500 mg of quinolone once daily for seven days during the expected neutropenic period, while the second group received no prophlaxis (control group). The primary end point was the incidence of clinically documented febrile episodes (FE) (temperature of more than 38^{o}C) due to infection. Assessment of the risk of FE in controll group on first versus non first cycles with or without first cycle FE in the light of different pretreatment factors. Secondary end point included the incidence of all infections, severe infections, hospitalization and cost. Results: A total of 403 patients randomly divided into 201 patients received antibacterial prophylaxis quinolone (levofloxacin®) and 202 patients as control group. The tumors included breast cancer 238 (59.1 percent), lung cancer 82 (20.3%), testicular cancer 34 (8.4%) and lymphoma 49 (12.2%). During the first cycle of chemotherapy, 3.5% of patients in the quinolone group had at least one febrile episode, as compared with 8.4% in the control group (P=0.009).The per- cycle FE rate for the first cycle was 8.4% compared with 4.4% in non first cycles in control group. During the entire chemotherapy course, 9.5% of patients in the quinolone prophylactic group had at least one febrile episode; as compared with 16.3% in the control group (P ≤0.005).There was significant reduction in the rate of G3&G4 neutropenia in quinolone group (52%). The respective rates of infections were 33.8% and 42.1% (p=0.098) for quinolone versus control group. Hospitalization was required for treatment of infection in 3% of patients in the quinolone group and 7% of patients in the control group (P≤0.05). Respective rates of reduction of cost and length of stay (LOS) were 51.8% and 51.6% for infections in quinolone prophylactic group. Respective rates of sever infections were 1.0% and 2.0% (p≤0.06), for quinolone and control group, with one infection related death in each group. An organism was isolated in 194/250 cycles (77.6% of infections). Conclusions: Quinolone prophylaxis (levofloxacin is preferred) should be offered to those receiving standard dose chemotherapy for solid tumors and lymphomas to reduce incidence of fever, infection, hospitalization and cost with rational selection of patients for antibacterial prophylaxis with first cycle chemotherapy.
[Fatma Zakaria[*1] and Mohamad Zakaria. **Double -Control, Randomized Study of Antibiotic Prophylaxis during Standard Dose Chemotherapy in Cancer Patients**. Journal of American Science 2010;6(12):1124-1135]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Antibiotic Prophylaxis; standard Dose; Chemotherapy; Cancer; Patient

1. Introduction:

Chemotherapy induced neutropenia (table 1) is not only a major risk factor for infection related morbidity and mortality, but also a significant dose - limiting toxicity in cancer treatment. Patients developing sever (grade 3/4) febrile neutropenia (FN) during chemotherapy frequently underwent dose reduction and/or delay to their chemotherapy. This may impact on the success of treatment, particularly when treatment intent is either curative or to prolong survival[(1)].

Meta-analysis of nine trials (731 patients) comparing fluoroquinolone prophylaxis with no prophylaxis demonstrated significant reductions in a number of outcomes of infections[(2-3-4)].

Quinolone (Levofloxacin ®) is an agent with an acceptable side-effect profile that is administered orally once daily, thus optimizing compliance, a major issue in prophylaxis[(5)].

It is active against a wide range of gram negative pathogens, as well as some gram-positive bacteria and organisms causing atypical pneumonias[(5)].

We conducted randomized trial designed to determine the efficacy of quinolone prophylaxis offering seven days prophylaxis during the period of anticipated neutropenia in patients with solid tumors or lymphomas.

2. Patients and Methods:

Four hundred and three adult patients with solid tumors and lymphomas treated inclusively during the period from January 2006 to December 2008, at Clinical Oncology Department, Tanta University. All patients at risk of bacterial infection were randomly divided into two groups the first one 201patients assigned to receive qinolone for seven days to cover the period of anticipated neutropenia

and another control group 202 patients. Patients remained enrolled in the trial for up to six cycles of chemotherapy. A cycle of chemotherapy was defined as the standard, minimal duration of a particular regimen between the start of one treatment and the next that was sufficient to allow recovery from acute adverse effects, including myelosuppression. Exclusion criteria at the time of randomization were active infection, current antibacterial therapy, planned use of G-CSF, a history of adverse reactions to fluoroquinolones, epilepsy, a creatinine clearance below 40 ml per minute, pregnancy, and breast-feeding. All patients gave written informed consent.

End points measures:

The primary end point was the incidence of clinically documented febrile episodes (FE), (defined by a temperature exceeding 38^{o}C due to infection) with or without neutropenia with assessment of grading for neutropenia, and assessment of febrile episodes in control group on first versus non first cycles with or without first cycle febrile episode. Infections incidence were the secondary outcome measure, infections were defined by at least one of the following: a clinically documented febrile episode, other signs attributed to a systemic response to infection, such as hypothermia (temperature below 35.6^{o}C), low grade fever (temperature, 37.5 to 37.9^{o}C), tachycardia (more than 90 beats per minute),or tachypnea (more than 20 breaths per minute), signs of a focus of infection, or the use of antibacterial therapy, we were reported episodes that occurred during each chemotherapy cycle or within four weeks after the final cycle. The incidence of hospitalization for infection and the frequency of severe infection were further secondary outcome measures. Severe infections were defined by the presence of infection-related sepsis syndrome (i.e. infection causing hypotension with or without evidence of impaired organ perfusion), death from infection or both.

Microbiologic outcomes included causative organisms isolated during infection, the clinical significance of isolates was assessed by a microbiologist. For episodes of infection, study medication was withdrawn for that cycle alone, but patients could remain in the trial for subsequent cycles.

Trial medication:

Trial medication consisted of 500 mg tablets of quinolone (as prophylaxis) once daily for seven consecutive days, treatment began on day 8 for 14 day and 21-day cycles, and on the day 15 for 28-day cycles.

Cost:

Costs were derived from charges reported on patients' tickets. Total costs per patient were computed by summing individual cost, where all patients were treated at our inpatient unit.

Statistical Analysis:

Statistical presentation and analysis of the present study was conducted, using mean, median, analysis of variance [ANOVA] test and the relative differences between the treatments groups were expressed as relative risks with 95 percent confidence intervals. Data on secondary outcomes relating only to cycles with infection are presented descriptively to identify patients at greater risk of infection during chemotherapy without antibacterial prophylaxis. The analysis assesses the association of baseline patients' characteristics with FE incidence using all the patients randomly assigned to the control arm and a multivariable analysis that includes all variables in the model is also used by SPSS v.12. P-value is considered significant, if ≤ 0.05, determined by chi-square test.

Table (1):Common chemotherapy regimens associated with intermediate or high risk of febrile neutropenia[1,2,3,4,5,6,7,8,9,10]

Malignancy	FN risk category (%)	Chemotherapy regimen	FN risk
Breast cancer	>20	AC→ docetaxel	5-25
		Paclitaxel→AC	40
		Doxorubicin/docetaxel	33-48
		Doxorubicin/paclitaxel	21-32
		TAC	21-24
		DD/DDG FEC	71/59
		DDG doxorubicin→paclitaxel→cyclophosphamide	2
		DDG doxorubicin/cyclophosphamide→paclitaxel	2
		DDG epirubicin/cyclophosphamide	8

Cont. Table (1)

Malignancy	FN risk category (%)	Chemotherapy regimen	FN risk
	10-20	AC	10-20
		Doxorubicin/vinorelbine	15
		Docetaxel	16-17
		Capecitabine/docetaxel	13
		Cyclophosphamide/mitoxantrone	11
		Epidoxorubicin/cyclophosphamide	13
		CEF	14
		FEC	9-14
	<10	FEC	0-2
		CMF	0-3
		CMF oral	1
		Doxorubicin/cyclophosphamide	2
		Doxorubicin→paclitaxel→cyclophosphamide	3
		Doxorubicin/cyclophosphamide→paclitaxel	5
		FAC	5
		Epirubicin/cyclophosphamide+lonidamide	7
Small cell lung cancer	>20	ACE	24-57
		Topotecan	28
		Topotecan/paclitaxel	>20
		ICE	24
		VICE	70
		DDG ACE	34-56
		DDG ICE	18
		DDG CAV→PE	4
	10-20	CAV	14
		Etoposide/carboplatin	10-20
		Topotecan/cisplatin	19
		CODE	19
	<10	CAV	3-9
		Paclitaxel/carboplatin	9
Non-small cell lung cancer	>20	Docetaxel/carboplatin	26
		Etoposide/cisplatin	54
		VIG	25
	10-20	Paclitaxel/cisplatin	16
		Docetaxel/cisplatin	5-11
		Vinorelbine/cisplatin	1-10
	<10	Paclitaxel/carboplatin	0-9
		Gemcitabine/cisplatin	1-7
		Gemcitabine/cisplatin	4
Non-Hodgkins lymphoma	>20	DHAP	48
		ESHAP	30-64
		GHOP	17-50
		DD/DDG VAPEC-B	44/23
		DD/DDG ACVBP	78/52
	10-20	ACOD	11
		R-CHOP	19
		Fludarabine/mitoxantrone	11

Cont. Table (1)

Malignancy	FN risk category (%)	Chemotherapy regimen	FN risk
Ovarian cancer	>20	Docetaxel	33
		Paclitaxel	22
	10-20	Topotecan	10-18
	< 10	Paclitaxel/carboplatin	3-8
		Gemcitabine/cisplatin.	9
Urothelial cancer	>20	Paclitaxel/carboplatin	25
		MVAC	26
		DDG MVAC	10
Germ cell tumors	>20	BOP→VIP-B	46
		VeIP	67
	10-20	cisplatin/etoposide	10
		BEP→EP	13
Colorectal cancer	10-20	5-FU/leucovorin	1-15
		FOLFIRI1	3-14
	<10	FOLFOX	0-8
		IFL	3-7
		Irinotecan	2-7
Other malignancies	>20	TIC (head and neck cancers)	30
		MAID (sarcoma)	58
		Paclitaxel/cisplatin (cervical cancer)	28
	10-20	Gemcitabine/irinotecan (pancreatic cancer)	17
		Stanford v (Hodgkin's lymphoma)	14
	< 10	ABVD (Hodgkin's lymphoma)	4
		Doxorubicin/cisplatin (endometrial cancer)	2
		TAP (endometrial cancer)	3

Please note that these results may vary for similar regimens depending on the patient population who participated in each study. 5-FU, 5-fluorouracil; ABVD, doxorubicin / bleomycin / vinblastine / dacarbazine; AC, doxorubicin / cyclophosphamide; AC→ T, doxorubicin / cyclophosphamide followed by docetaxel; AGE, doxorubicin / cyclophosphamide / etoposide; ACOD, doxorubicin / cyclophosphamide / vincristine / prednisolone; ACVBP, doxorubicin or mitoxantrone with cyclophosphamide/ vindesine/bleomycin; BEP→ EP, bleomycin / etoposide/cisplatin followed by etoposide / cisplatin; BOP→ VIP-B, bleomycin/vincristine / cisplatin followed by cisplatin / ifosfamide / etoposide/bleomycin, CAV, cyclophosphamide / doxorubicin/vincristine; CE, cyclophosphamide/ epirubicin; CEF, cyclophosphamide / epirubicin/5-FU; CHOP-21, cyclophosphamide / doxorubicin/ vincristine / prednisone; CMF, cyclophosphamide/ methotrexate / fluorouracil; CODE, cisplatin / vincristine / doxorubicin / etoposide; DD, dose dense; DDG, dose dense with G-CSF; DHAP, cisplatin / cytarabine / dexamethasone; ESHAP, etoposide / methylprednisolone / cytarabine / cisplatin; FAC, fluorouracil / doxorubicin / cyclophosphamide; FEC, cyclophosphamide / epirubicin / fluorouracil; FMD, fludarabine / mitoxantrone; FOLFIRI, 5-FU/l-folinic acid / d,1-folinic acid / irinotecan; FOLFOX, 5-FU / folinic acid / oxaliplatin; FN, febrile neutropenia; ICE, ifosfamide / carboplatin / etoposide, IFL, irinotecan / 5-FU / calcium folinate; MAID, mesna / doxorubicin / ifosfamide / dacarbazine; MVAC, methotrexate / vinblastine / doxorubicin / cisplatin; PE, cisplatin / etoposide; Q2W, once every 2 weeks; R-CHOP-21, rituximab / GHOP; Stanford V, mustard / doxorubicin / vinblastine / vincristine / bleomycin / etoposide / predniso lone; T→AC, docetaxel followed by doxorubicin / cyclophosphamide; TAC,

docetaxel / doxorubicin / cyclophosphamide; TAP, paclitaxel; oxorubicin / cisplatin; TIC, paclitaxel / ifosfamide / carboplatin; VAPEC-B, vincristine / doxorubicin / prednisolone / etoposide / cyclophosphamide / bleomycin; VICE, vincristine/ifosfamide / carboplatin / etoposide; VIG, vinorelbine / ifosfamide / gemcitabine.

FN risk < 10% G-CSF not indicated

FN risk 10-20% { Overall FN risk ≥ 20% prophylactic G-CSF or Overall FN risk < 20%→ G-CSF not indicated

FN risk ≥ 20% prophylactic G-CSF recommended.[1]

Table (2): Grades of Neutropenia [11]

Grade	Absolute neutrophil cou 10^9/L)
0	Within normal limits
1	≥ 1.5 to < 20
2	≥ 1.0 to < 1.5
3	≥ 0.5 to < 1.0
4	< 0.5

[a] According to the National Cancer Institute, Common Toxicity Criteria, version 2.0.

3. Results

From January 2006 to December 2008, 403 patients from Tanta University Hospital, Clinical Oncology Department, underwent randomization 202 as control group and 201 to quinolone prophlaxis. A total of 94.3 percent of patients had a WHO performance status of 0 or l, and more than half were treat in the adjuvant context. More than half of the patients had breast cancer, but substantial numbers were treated for lung and testicular cancer. The treatment groups were well balanced with respect to all baseline characteristics and risk factors (table 3). A total of 2278 cycles were analyzed and the number of cycles was studied.

Infection:

Of the 403 patients, 52 patients (13.0%) had at least one febrile episode, and there were 90 cycles with febrile episodes in total 2278 cycles (4.0% of cycles). At least one infection occurred in 153 patients (38.0%), and there were total of 250 cycles with infections in total of 2278 cycles (11.0% of cycles) (table 4). A clinically documented febrile episode occurred during the first chemotherapy cycle in 7 of 201 patients in the quinolone group (3.5 %), as compared with 17 of 202 patients in the control group (8.4 %) (Table 5). The relative risk of a clinically documented febrile episode was (relative risk 1.6, 95%CI (0.40-0.71), p≤0.009), indicating a (59%) reduction in the risk of fever during the first cycle with the use of quinolone therapy, as compared with control group. There was also a significant reduction in the incidence of the more inclusive category of infections with quinolone prophylaxis, as compared with control group, resulting in 39% reduction in the risk during the first cycle of chemotherapy (relative risk 2.33,95%CI (0.79-1.22), P≤0.001).

Data obtained during the entire chemotherapy were analyzed per patient rather than per cycle, and quinolone antibacterial prophylaxis was found to confer a protective benefit similar to that identified in the analysis of the first cycle (Table 5). During the entire course of chemotherapy, 19 out of 201 patients in quinolone group had a clinically documented febrile episode (9.5 %), as compared with 33 of 202 patients in the control group (16.3%). Prophylactic quinolone with thus associated with a 42.0% relative reduction in the risk of a febrile episode (relative risk 1.31, 95%CI (0.99-1.18), p=0.051) and a 20% relative reduction in the risk of infections (relative risk 0.98, 95%CI (0.76-0.91), P=0.098). Only 14 patients (7.0%) from the quionolone had more than one febrile episode. As regared neutropenia, from G1 to G4 was present 13 cycles in quinolon group in comparison to 38 cycles in control group with reduction rate 65.8% in febrile episodes with neutropenia. Thirty four cycles with neutropenia (G1 to G4) in comparison to 73 cycles in quinolone versus control group respectively with 53.4% reduction rate of infections with neutropenia.

Hospitalization for infection:

The reduction in the incidence of febrile episodes and infection associated with quinolone prophylaxis was reflected in a significant reduction in the percentage of patients hospitalized for infection (Table 5). There was 71.6 percent reduction in the risk of hospitalization during cycle 1 with quinolone therapy, as compared with control group (relative risk 0.70, 95%CI (1.15-1.96), P=0.011) and a 45.4 percent reduction across all cycles (relative risk 0.32, 95%CI (0.13-0.82), P value= 0.04).

Severe infections:

Severe infection characterized by infection related sepsis manifestations, death, or both occurred in two patients in quinolone group as compared with four patient in the control one (relative risk 0.11,95%CI (0.24-1.12), P=0.06), one patient died in each group. Two severe infections in the quinolone group occurred outside the period in which the white cell count was expected to be lowest, in comparison to four patient severe infections in control group, causing death in one patient, which occurred during the expected nadir period (Table 5).

Microbiologic outcomes:

The organism that was the probable cause of the febrile episode or episode of infection was isolated less frequently among patients in the quinolone group than among patients in the control group (46.9% vs 75.9% and 45.5% vs 60.9% respectively) (Table 6).

Adverse events:

Adverse events were reported in 38 cycles of 2278 cycles of chemotherapy (1.7 percent) there was a slight excess of adverse events in the quinolone group, owing to a higher rate of minor gastrointestinal symptoms and rash (Table 6).

Cost and length of stay (LOS):

Median duration of LOS were 6.5 days Vs 5 days for FE in quinolone group and control group per hospitalization respectively, with total length of stay 28 days in quinolone group Vs 73 days in, control group. Hospitalization for infections showed total LOS was 105 days for quinolone group in comparison to 217 days for control group with reduction of cost (51.8%) for hospitalization of infection in quinolone group (table 6).

Identifying risk factors for Infection and Hospitalization without antibacterial prophylaxis:

Tumor type: The different types carried different risks for FE across all cycles of chemotherapy and in cycle one (Table 7). Multivariable analysis identified lung and testicular cancers as the tumor types at significantly greatest risk for FE in all cycles and in cycle one (p=0.007&0.003).

Other pretreatment factors:

Poor performance status, advanced age 65 years or older and male sex have been linked to a higher risk of FE. The FE frequency was lower in patients undergoing adjuvant chemotherapy compared with those being treated for advanced disease, but this did not reach statistical significance (Table7).

First cycle versus later cycles:

Two hundred and two patients were randomly assigned to the control arm and received 1144 cycles of chemotherapy (mean, 5. 7 cycles per patient). Seventeen of them experienced a FE during the first cycle,(51.5%) (17|33).Thirty three controls, had at least one FE during the entire course of chemotherapy program,giving a per- patient FE rate of (16.3%) 33|202 .The FE rate for the first cycle was (8.4%) (17|202 cycles) compared with (4.4%)(41|942 cycles) in non first cycles.Approximately > 50% of episodes occurred in cycle one.

4. Discussion:

We studied the efficacy of antibacterial prophylaxis in patients treated for solid tumors and lymphomas with chemotherapy regimens associated with short periods of neutropenia and thus an increased risk of infection.

A simple, clinically relevant objective observation (fever, as defined by a temperature of more than 38^{o}C) attributed to infection as the primary outcome in our study,12.9 percent receiving conventional chemotherapy for solid tumors and lymphomas had at least one febrile episode with an over all incidence of 4.0% per cycle.

During the entire course of chemotherapy approximately 33.3% reduction in febrile episodes for patients in the quinolone group versus those in the control group (9.9% Vs 16.3%) and 58.9% reduction in febril episodes during the first cycle of chemotherapy for quinolone group compared tothe control group (3.5% Vs 8.4%) respectively in. There was significant reduction in grade 3&4 febril neutropenia in quinolone group (52%).

More than 50% of febrile episodes occurred in first cycle in the control group (table 5), with FE and hospitalization rates were twice more frequent in first cycle than subsequent cycle which is in agreement with other several trials[12,13,14,15,16,17] ,which recorded several explanations for this first-cycle effect, neutropenia, The explanation of this phenomena may be dose reduction of chemotherapy in subsequent cycles. The cytoreductive effects of the first chemotherapy cycle may enable resolution of a cancer related focus of infection (e.g. beyond an obstructed air way in lung cancer patients) or improvement in performance status. The first cycle of chemotherapy is noteworthy not only because of the high frequency of FE compared with later cycles, but also because FE in cycle one appears to separate patients into low and high-risk groups for subsequent episodes .[18,19]

Under pressure to limit antibacterial use, these exploratory data support offering prophylactic quinolone on cycle 1 only for myelosuppressive cancer chemotherapy and on subsequent cycles after a cycle-1 fever[19].

Pre-treatment factors and FE in first & non first cycles in control group, lung and testicular cancers in the control group were the tumor types with higher risksfor FE in first and non first cycles (p=0.007&0.003), the possible explanations for this effect may be due to, the majority of patients received etoposide which cause severe and unpredictable neutropenia[20], mucositis is also a frequent

consequence of etoposide exposure that predisposes to infection in control group. Poor PS has been linked to a higher risk of FE[21]. Age 65 years or older has been shown to confer a higher risk of FE[22], with male sex predominance for FE in first and non- first cycles without statistical significant difference (p=0.074). The reduction in the incidence of hospitalization for the treatment of infection was significant (45.9 percent), (p=0.04) for quinolone group for the entire course of chemotherapy. Seventy five percent of fever occurred outside the expected period of neutropenia (i.e. the period of prophylaxis), table (6). The two cases of sever infections and death from infection in the quinolone group occurred outside the expected period of neutropenia (i.e the period of prophylaxis), our results are supported by other studies that received prophylaxis with marked reduction in infection related outcomes, including death particularly in a cohort receiving intensified chemotherapy with G.CSF [23,24,25,26, 27].

Table (3): Patients characteristics:

Characteristics	Quinolone prophylaxis (n=201)	Control (n=202)
Sex- no%		
Male	91 (45.3%)	98(48.5%)
Female	110 (54.7%)	104 (51.5%)
WHO performance status		
0	146 (72.6%)	162 (80.2%)
1	40 (19.9%)	32 (15.8%)
2	13 (6.5%)	6 (3%)
3 or 4	2 (1.0%)	2 (1%)
Age:		
16-39 ys	40 (19.9%)	39 (19.3%)
> 40-65 ys	88 (43.8%)	83 (41.1%)
≥ 65 ys	73 (36.3%)	80 (39.6%)
Type of cancer and most commonly used chemotherapy regimens-no(%)		
Breast cancer	116 (57.7%)	122 (60.4%)
Fec	80	75
Sq T-fec	26	37
Lung cancer	42 (20.9%)	40 (19.8%)
PE	22	30
CAV	20	10
Testicular cancer	18 (9%)	16 (7.9%)
BEP	16	15
EP	2	1
Hodgkin's disease	8 (4%)	10
ABVD	8	10
Non-Hodgkin's disease	17 (8.5%)	14 (6.9%)
CHOP	17	14
Chemotherapy being given in adjuvant setting-no(%)	145 (72.1%)	151 (74.8%)
Indwelling venous catheter present no (%)	20 (10%)	26 (12.9%)
Previous myelosuppressive chemotherapy given No%	56 (27.9%)	51(25.2%)
Previous radiotherapy given no (%)	10 (5%)	14 (6.9%)

FEC denotes fluorouracil, epirubicin, and cyclophosphamide; T taxanes, PE cisplatin and etoposide, CAV cyclophosphamide, doxorubicin and vincristine, BEP bleormycin, etoposide and cisplatin; ABVD doxorubicin, bleomycin, vinblastine, and dacarbazine , CHOP cyclophosphamide, doxorubicin, vincristine, and prednisolone.

Table (4): Characteristics of 250 infections among 2278 cycles.

Variable	Focus of infection	No focus of infection	No / total (% of cycles)
Sign of probable infection	No. of probable infection (% of total)		
Fever	56(22.4%)	34 (13.6%)	(3.9) 90/2278
Other systemic signs	40(16.0%)	10(4.0%)	(2.2%) 50/2278
No systemic signs	98 (39.2%)	12* (4.8%)	(4.8%) 110/2278
Focus of infection			
Upper respiratory tract	65(26%)		
Lower respiratory tract	29(11.6%)		
Gastrointestinal tract & anal abscesses	8(3.2%)		
Urinary tract	24 (9.6%)		
Skin ad soft tissues	22(8.8%)		
Venous catheter	13(5.2%)		
Oral mucosa and teeth	24(9.6%)		
Multiple sites	9(3.6%)		
No focus of infection		56 (22.4%)	

* In these 12 episodes, the only evidence of infection was the reported use of antibacterial therapy in 8, no further data were available for the other 4 episodes.

Table (5): Incidence of febrile episodes, infections, and Hospitalization for infection

Event	Quinolone (n=201)	Control (n=202)	Relative risk &(95%) Cl	P value
Events occurring in first cycle				
-Febrile episode				
Yes	7 (3.5%)	17 (8.4%)		
No	194	185	1.60(0.40-0.71)	0.009*
-Infection				
Yes	25 (13.9%)	41 (20.2%)		
No	176	161	2.33(0.79-1.22)	0.001*
- Hospitalization for infection				
Yes	2 (0.99%)	7 (3.7%)		
No	199	195	0.70(1.15-1.96)	0.049*
Events occurring at least once in any cycle				
- Febrile episodes				
Yes for ≥ 1cycle	19 (9.5%)	33 (16.3%)		
No for all cycle	182	169	1.31(0.99-1.18)	0.051
- Infection				
Yes for ≥ l cycle	68 (33.8%)	85 (42.1%)		
No for all cycles	133	117	0.98(0.76-91)	0.098
- Hospitalization for infection				
Yes for ≥ l cycle	6(2.99%)	11(5.4%)		
No for all cycles	195	191	0.32(0.13-0.82)	0.04
-Sever infection and/or death from infection	2 (1.0%)	4 (2.0%)	0.11(0.24-1.12)	0.067

* A febrile episode was defined by temperature more than 38°C. Cl denotes confidence interval. The P values, determined by the chi-square test, are for "yes" answer as compared with "no" answer .

Table (6): Adverse events and characteristics of febrile episodes, infections, hospitalization for infections and grading of neutropenia.

Variable	Quinolone	Control
All cycles no (%)	1134	1144
Adverse events	23 (2.0%)	15 (1.3%)
Rash	6	2
Gastrointestinal effect	16	9
Central neurons system effects	--	--
Musculoskeletal effect	--	--
Multiple events including those listed above	--	3
* other	1	1
Antifungal prescribed prophylaxis	90(7.9%)-	86(7.5%)-
Incidence of mucosal candidiasis	88(7.8%)	76(6.6%)
Cycles with febrile episodes total no%	32(100%)	58(100%)
During expected nadir	8(25%)	32(55.2%)
Outside expected nadir	24 (75%)	26 (44.8%)
Hospitalization – No% duration – days	4(6.3%)	10 (8.6%)
Median	6.5 days	5days
Interquartile range	5-9 days	3-8days
Total (LOS)	28 days	73days
Grading of neutropenia at onset of infection		
G0	19	20
G1	2	13
G2	4	10
G3&4	7(21.9%)	15(25.9%)
Microbiologic analysis –No% probable causative organism.	15(46.9%)	44 (75.9%)
Cycles with infections total – no%	112(100%)	138(100%)
-During expected nadir	27(24.1%)	67(48.6%)
- Outside the expected nadir	85(75.9%)	71(51.4%)
Hospitalization for infection duration- days	15 (13.4%)	31 (22.5%)
Median	6 days	5 days
Interquartile range	4-9 days	3-9 days
Total (LOS)	105 days	217 days
Total Cost-	21195 LE	44011LE
Total (Cost) per hospitalization,		
Mean-	535.7LE	637.7LE
Median	529LE	631LE
Grading of neutropenia at onset of infection		
G0	78	65
G1	12	26
G2	10	22
G3&4	12(10.7%)	25(18.1%)
Microbiologic analysis-no% probable causative bacteria isolated	51(45.5%)	84 (60.9%)

* This category includes allergic reactions, a general feeling of malaise, breathless- ness, chest discomfort, and unspecified events, (LOS) length of stay in hospitalization.

Table (7): Number and Rate of FE by patient characteristics in controls.

Characteristic	No of patients	FE across all cycles			FE in cycle one		
		No	Rate%	P*	No.	Rate (%)	P*
Over all tumor type	202	33	16.3%	0.094	17	8.4%	0.072
Breast	122	13	10.6%		7	5.7%	
Lung	40	10	25%	0.007	5	12.5%	0.003
Testicular	16	5	31.2%		3	18.8%	
Hodjken's	10	2	20%		1	10%	
NHL	14	3	21.4%	0.052	1	7.1%	0.159
Age:							
16-39 ys	39	7	17.9%		5	12.8%	
> 40-65 ys	83	11	13.3%	0.435	7	8.4%	0.623
> 65 ys	80	15	18.8%		5	6.3%	
Sex							
Male	98	20	20.4%		10	10.2%	0.074
Female	104	13	12.5%	0.063	7	6.7%	
PS 0	162	23	14.2%		11	6.8%	
1	32	5	15.6%		5	15.6%	0.074
2	6	3	50%	0.150	1	16.5%	
3+	2	2	100%		--	--	
Adjuvant CT	151	12	7.9%	0.121	5	3.3%	0.349
Non adjuvant	51	21	41.2%		12	23.5%	
Catheter	26	5	19.2%	0.958	4	15.4%	0.099
Non catheter	176	28	15.9%		13	7.4%	
Previous RIT	14	4	28.6%	0.152	3	21.4%	0.072
No previous R|T	188	29	15.4%	0.321	14	7.4%	0.459

Abbreviations: FE febrile episode, NHL Non- Hodgkin's lymphoma ,PS performance status, CT chemotherapy, RT radiotherapy.

Microbiologic confirmation was reported in our study where organisms were isolated in 65.6% of febrile episodes and (54%) of infections, also, organisms were isolated more frequently from cultures in the control group than in the quinolone group table (6), in agreement with where was reported in Michael et al. (2005) study [12]. The lower frequency of isolation of aganiones in cases of infection may be explained by fungal or viral causes. The effect on the development of resistance with the repeated use of short periods of fluoroquinolone prophylaxis on an out patient basis on patients receiving cytotoxic chemotherapy is unknown.

In our study, there is a significant reduction of cost of hospitalization with patients in quinolone group versus the control group (45.4 percent), for infection as reported in other studies [28, 29]. Cost associated with hospitalization for FN closely correlated with length of stay in hospitalization. Despite improved medical management, Febrile neutropenia continues to be associated with substantial morbidity, mortality, and cost not only placing a significant burden on the individual patient but on the health care system as a whole as reported in Caggiano et al study[29].

In conclusion, the administration of quinolone for seven days to cover the expected period of neutropenia after cyclic, standard dose, myelosuppressive chemotherapy in patients with solid cancer or lymphoma significantly reduced incidence of febrile episodes especially with the first cycle chemotherapy, clinically documented infection, hospitalization for the treatment of neutropenic infection and cost (where use of prophylaxis antibiotic limit the share of growth factors), with minimal adverse effects.Cycle 1 infection appear to identify patients at high risk of later FE who paradoxically, may benefit from prophylactic quinolone on later cycles, but further work is required to confirm these observations.

Corresponding author
Fatma Zakaria
Department of Clinical Oncology, Faculty of Medicine, Tanta University, Tanta, Egypt

5. References:

1. Aapro MS, Cameron DA, Pettengell R, et al. EORTC guidelines for the use of granulocyte-

colony stimulating factor to reduce the incidence of chemotherapy-induced febrile neutropenia in adult patients with lymphomas and solid tumors Eur Jour of Cancer,V. 42(2006)2433-2453.

2. Tjan-Heijnen VG, Postmus PE, Ardizzoni A, et al. Reduction of chemotherapy –induced febrile leucopenia by prophylactic use of ciprofloxacin and roxithromycin in small-cell lung cancer patients: an EORTG double-blind placebo-control phase III study. Ann Oncol, 12(2001)1359-68.
3. Von Minckwitz G, Rabb G, Caputo A, et al. Doxorubicin with cyclophosphamide followed by docetaxel every 21 days compared with doxorubicin and docetaxel every 21 days compared with doxorubicin and docetaxel every 14 days as preoperative treatment in operable breast cancer: the GEPARDUO study of the German Breast Group . J Clin Oncol, 23(2005) 2676-85.
4. Martin M, Lluch A, Segui MA, et al. Prophylactic growth factor (GF) support with adjuvant docetaxel, doxorubicin, and cyclophosphamide (TAC) for node-negative breast cancer (BG): an interim safety analysis of the GEICAM 9805 study. Proc Am Soc Clin Oncol 22(2004)145. (Abstract 620).
5. Lorigan P, Woll PJ, O'Brien ME, Ashcroft, LF, Sampson MR, Thatcher N. Randomised phase III trial of dose-dense chemotherapy supported by whole-blood haematopoietic progenitors in better-prognosis small-cell lung cancer. J Natl Cancer Inst, 97(2005)666-74.
6. Edelman MJ, Chansky K, Gaspar LE, et al. Phase II trial of cisplatin/etopside and concurrent radiotherapy followed by paclitaxel/carboplatin consolidation for limited small-cell lung cancer: Southwest Oncology Group 9713. J Clin Oncol, 22(2004)127-32.
7. Papadimitriou CA, Fountzilas G, Aravantinos G, et al Hellenic Cooperative Oncology Group Study. Second-line chemotherapy with gemcitabine and carboplatin in paclitaxel-pretreated, platinum-sensitive ovarian cancer patients. A Hellenic Cooperative Oncology Group Study. Gynecol Oncol, 92(2004) 152-9.
8. Sternberg CN, de Mulder PH, Schornagel JH, et al. Randomised phase III trial of high-dose-intensity methotrexate, vinblastine, doxorubicin, and cisplatin (MVAC) chemotherapy and recombinant human granulocyte colony-stimulating factor versus classic MVAC in advanced urothelial tract tumors: European organization for Research and Treatment of Cancer (EORTC) protocol no. 30924. J Clin Oncol, 19(2001) 2638-46.
9. Douillard JY, Cunningham D, Roth AD, et al. Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: a multicentre randomized trial. Lancet, 355(2000)1041-7.
10. Fleming GF, Brunetto VL, Cella D, et al. Phase III trial of doxorubicin plus cisplatin with or without paclitaxel plus filgrastim in advanced endometrial carcinoma: a Gynecologic Oncology Group Study. J Clin Oncol, 22(2004) 2159-66.
11. National Cancer Institute. Common toxicity criteria, version 2.0. Available from URL: http://ctep. Cancer. Gov/forms/ CTCv20_4-30-992.pdf [access date January 3, (2003)].
12. Michael C, Neil S, Lucinda B et al. Antibacterial prophylaxis reduced the incidence of fever in patients receiving chemotherapy for solid tumors or lymphomas. N Engl J Med, 10(2005) 353.
13. Engels EA, lau J, Barza M. Efficacy of quinolone prophylaxis in neutropenic cancer patients: a meta-analysis. J Clin Oncol, 16(1998)1179-87.
14. Cruciani M, Rampazzo R, Malena M, et al. Prophylaxis with fluoroquinolones for bacteria infections in neutroponic patients: a meta-analysis. Clin Infect Dis, 23(1996)795-805.
15. Fu KP, Latfredo SC, Foleno B, et al. In vitro and in vivo antibacterial activities of levo-floxicin (l-ofloxacin), an optically active of loxacin. Antimicrob. Agents Chemother, 36(1992) 860-6.
16. Tjan-Heijnen VC, postmus PE, Ardizzorfi A, et al. Reduction of chemotherapy induced febrile leucopenia by prophylactic use of ciprofloxacin and roxithromycin in small-cell lung cancer patients: an EORTC double-blind placebo-control phase III study. Ann Oncol, 12(2001)1359-68.
17. Klastersky J, Paesmans M, Rubenstein EB, et al. The Multinational Association for Supportive Care in Cancer risk index: a multinational scoring system for identifying low-risk febrile neutropenic cancer patients. J Clin Oncol, 18(2000)3038-51.
18. Freifeld A, Marchigiani D, Walsh T, et al. A double-blind comparison of empirical oral and intravenous antibiotic therapy for low-risk febrile

patients with neutropenia during cancer chemotherapy. N Engl J Med, 341(1999)305-11.

19. Cullen MH, Billingham LJ, Gaunt CH, Steven NM Rational Selection of patients for antibacterial prophylaxis after chemotherapy.J CLIN ONCOL,30(2007) 4821-8.
20. Harvey VJ, Slevin ML, Johnston A, et al: The effect of dose on the bioavailability of oral etoposide. Cancer Chemother pharmacol, 16(1986) 178-181.
21. Crawford J, Dale DC, Lyman GH, Chemotherapy-induced neutropenia: Risks, consequences, and new directions for its management. Cancer, 100(2004) 228-237.
22. Lyman GH, Lyman GH, Agboola O. Risk models for predicting chemotherapy-induced neutropenia. Oncologist 10(2005) 427-37.
23. Vogel CL, Wojtukiewicz MZ, Carroll RR, et al, first and subsequent cycle use of pegfilgrastim prevents febrile neutropenia in patients with breast cancer: A multicenter, double-blind, placebo-control phase III study. J Clin Oncol, 23(2005) 1178-1184.
24. Lyman GH, Delgado DJ: Risk and timing of hospitalization for febrile neutropenia in patients receiving CHOP, CHOP-R, or CNOP chemotherapy for intermediate grade non-Hodgkin lymphoma. Cancer, 98(2003) 2402-2409.
25. Crawford J, Wolff DA, Culakova E. et al: First cycle risk of severe and febrile neutropenia in cancer patients receiving systemic chemotherapy. Results from a prospective nationwide study. Proc Am Soc Hematol, (2004) 104 (abstr 2210).
26. Timmer-Bonte JN, Tjan-Heijnen VC: Febrile neutropenia: Highlighting the role of prophylactic antibiotics and G-CSF during standard dose chemotherapy for solid tumors. Anticancer Drugs, 17(2007) 881-889.
27. Imran H,Tleyjeh IM,Arndt CA, et al,Fluoroquinolone prophylaxis in patients with neutropenia :a meta-analysis of randomized placebo-control trials.EUR J CLIN Microbiol Infect Dis,1(2008) 53-63.
28. Nicole M. Kuderer , David C. Dale , Jeffrey Crawford ,et al, Mortality, Morbidity, and cost associated with febrile neutropenia in adult cancer patients Published online 30 March (2006) in wiley Inter Science.
29. Caggiano V, Weiss RV, Rickert TS, et al. Incidence, cost and mortality of neutropenia hospitalization associated with chemotherapy. Cancer 103(2005)1916-1924., 34:257–266.

11/2/2010

Protective Effect of Broccoli and Red Cabbage Against Hepatocellular Carcinoma Induced by N- Nitrosodiethylamine in Rats

Aml F. M. Morsy*[a], Hodaa S. Ibrahim[a] and M. A. Shalaby[b]

a Nutrition and Food Science Department, Faculty of Home Economics, Helwan University, Egypt.
b Pharmacology Department Faculty of Veterinary Medicine, Cairo University, Egypt.
* dr_aml_fawzy@yahoo.com

Abstract: The hepatoprotective effect of broccoli and red cabbage extracts against hepatocellur carcinoma induced by N- Nitrosodiethyamine (NDEA) in male rats were studied. Four groups of rats were used; group (1) was used as a negative control (normal), while rats of the other groups were given NDEA as a single interperoitenial dose with subcutaneous injection of carbon tetrachloride (CCl4) once weekly for six weeks to induce hepatocellular carcinoma. Group (2) was left as a positive control, while groups (3) and (4) were pretreated with broccoli and red cabbage 10%extract, for 12 weeks, respectively. At the end of the experiment, blood samples were taken for biochemical analysis and liver tissues were histopathologically examined. The obtained results revealed that rats with hepatocellur carcinoma (HCC) had significant increase in serum levels of AST, ALT, ALP, total protein, albumin, total and direct bilirubin and malondialdehyide (MDA), as well as significant decrease in reduced glutathione (GSH), glutathione peroxidase (GPX), superoxide dismutase (SOD) and catalase (CAT) enzymes, compared to the normal control group. Liver sections of rats with HCC showed fatty infiltration of hepatocytes, cytomegaly with karyomegaly as well as vesicular active nuclei and presence of more than one nucleolus in some hepatocytes. Oral administration of broccoli and red cabbage extracts caused significant reduction in serum levels of AST, ALT, ALP, total protein, albumin, total and direct bilirubin as well as MDA and produced significant increase in GSH, GPX, SOD and CAT, compared to the positive group. Liver of these rats revealed only slight hydropic degeneration of hepatocytes, while other sections showed apparent normal hepatocytes. This study concluded that broccoli and red cabbage have a protective effect against hepatocellular carcinoma in rats, therefore this study recommends increased dietary intake of broccoli and red cabbage may be beneficial for patients with liver cancer as a preventative measures.

[Aml F. M. Morsy, Hodaa S. Ibrahim and M. A. Shalaby. **Protective Effect of Broccoli and Red Cabbage Against Hepatocellular Carcinoma Induced by N- Nitrosodiethylamine in Rats**. Journal of American Science 2010;6(12):1136-1144]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Broccoli; Red cabbage; Liver cancer; Biochemistry; Histopathology; Rat.

1. Introduction:

Hepatocellular carcinoma (HCC) is the most common form of liver cancer in adults, which accounts for about 75% of primary liver cancers. It is the 5th most common cancer in worldwide and represents 83% of all cases (Ferlay et al., 2001). In Egypt, HCC is frequent accounting for 13% of all cancer types (Inas, 2005). Liver cancers have different growth patterns; the first type begins as a single tumor that grows larger in hepatic tissue. The second type is spread through the liver almost from the beginning and is not confined to a single tumor. This is seen most often in people with liver cirrhosis. In the third type, the cancer develops as nodules in several parts of the liver (Strauss, 1995).

Risk factors for HCC include hepatitis B virus (HBV), hepatitis C virus (HCV) and aflatoxins are assumed to play an important role in the high incidence of HCC in Egypt. HBV vaccination of children and high-risk population must be the priority in reducing the incidence of HCC. Measures to reduce food spoilage by fungi and the associated dietary exposure to aflatoxins are a desirable public health goal (Wild and Hall, 2000).

Numerous compounds in the human diet have chemoprotective properties against chemical carcinogens (Wattenberg, 1990). Intake of a diet rich in fruits and vegetables is associated with a lowering risk of certain types of cancer (Steinmetz and Potter, 1991). Green leafy vegetables of all varieties and cruciferous plants such as cabbage, Brussels sprouts, cauliflower and broccoli are rich in anti-carcinogens (Block et al., 1992; Wargovich, 1999). Cruciferous vegetables contain a number of bioactive components such as folate, vitamin C, tocopherols, carotenoids, flavonoids and polyphenols (Price et al., 1998; Kurilich et al., 1999).

Broccoli is a plant of family *Brassicaceae* (formerly *Cruciferae)* which has large green flower heads (Murray and Lara, 2005). Recently, Elizabeth and Marcela, (2009) suggested that broccoli can decrease the risk for incidence of cancer. It contains many bioactive, including vitamins C and E, quercetin and kaempferol glycosides.

Red Cabbage *(Brassica oleracea* var. *capitata* f. *rubra)* is a type of cabbage, also known as Red Kraut or Blue Kraut after preparation (Michaelis et al.,

2008). The breakdown products of glucosinolates, which present in it such as isothiocyanates are considered responsible for the chemopreventive properties of red cabbage (Lynn et al., 2006).

The aim of the present study was to investigate the hepatoprotective effect of broccoli and red cabbage extracts against hepatocellur carcinoma induced by N - Nitrosodiethylamine in male rats.

2. Materials and methods:

2.1 Material:

2.1.1 Rats and Diet:

Male albino rats of Sprague Dawley strain weighing 175±5g were used. Their age between 14-16 weeks old and were purchased from the Laboratory Animal Colony, Ministry of Health and Population, Helwan, Egypt. Basal diet constituents were obtained from El-Gomhorya Company, Cairo, Egypt.

2.1. 2 Chemicals:

N-Nitrosodiethyamine (NDEA) was purchased from Sigma Chemical Company, USA. Carbon tetrachloride (CCl_4) was obtained from El-Gomhorya Company, Cairo, Egypt. Biochemical kits for serum analysis were purchased from the Gamma Trade Company for Pharmaceutical and Chemicals, Dokki, Egypt.

2. 1.3 Plants:

Broccoli and red cabbage were purchased from the local market, Cairo, Egypt. The plants were authenticated in the Botany Department, Faculty of Agriculture, Cairo University.

2. 2 Methods:

2.2.1 Preparation of plant extracts:-

Broccoli and red cabbage vegetables were cleaned, air dried and grinded into a fine powder. The powdered plants were extracted with 90% ethyl alcohol using Soxhlet apparatus and concentrated at low temperature(50Ċ) using a Rotary evaporator apparatus (manufactured in Basil, Switzerland) according to the method described by Mohamed, (2002). Each dried ethanol extract was dissolved separately in a mixture of carboxy methylcellulose and few drops of Tween 80 as a suspending agent to obtain 10% concentration liquid extract.

2. 2.2 Preparation of basal diet:

The basal diet (AIN-93M) was prepared according to Reeves et al., (1993). Diet was formulated to meet the recommended nutrients levels for rats.

2. 2.3 Experimental Design:

Forty male albino rats were fed on the basal diet and water was provided *ad libitum*. Animals were maintained under standard conditions of humidity (50-60%), temperature (20-25ºC) and light (12-h light: 12-h dark cycle) for one week before starting the experimental for acclimatization. Rats were divided into four groups of ten animals each as follows:

Group (1): Served as a control negative (normal rats) and fed on basal diet for 12 weeks.

Group (2): Kept as a control positive (with HCC) and fed on basal diet for 12 weeks.

Group (3): Fed on the basal diet and given orally 10% broccoli extract using stomach tube for 12 weeks.

Group (4): Fed on the basal diet and given red cabbage 10% extract orally by stomach tube for 12 weeks.

In first six weeks of experimental period, animals of groups (2), (3) and (4) were given a single intraperitoneal dose of NDEA (200 mg/kg b.wt.) followed by carbon tetrachloride (CCl4) tetrachloride (CCl4) given subcutaneously once weekly in a dose of 200 mg/kg b.wt. during the other 6 weeks for induction of HCC as described by Sundaresan and Subramanian (2003). At the end of the experimental period, blood samples were collected from the portal vein into dry clean centrifuge tubes for serum separation. Serum samples were frozen at -10°C until chemical analysis. Liver of sacrificed rats were kept in 10% formalin solution till processed for histopathological examination.

2.2.4 Biochemical analysis:

2. 2.4.1 Determination of liver functions:

Serum asprtate aminotransferase (AST) and alanine aminotransferase (ALT) activities were determined according to the method described by Kaplan, (1984). Serum alkaline phosphatase (ALP) was calorimetrically determined according to the method described by Roy (1970). Serum total protein concentration was calorimetrically determined according to the method described by Koller, (1984). Serum concentrations of albumin, total and direct bilirubin were determined as described by Kaplan, (1984).

2.2.4.2 Determination of malondialdehyde and reduced glutathione:

Serum malondialdehyde (MDA) was determined by the method of Draper and Hadly, (1990). Reduced glutathione concentration (GSH) in was measured by the method described by Beutler and Kelly, (1963).

2.2.4.3Determination of antioxidant enzymes:

The serum levels of glutathione peroxidase (GPX), superoxide dismutase (SOD) and catalase (CAT) were determined by autoanalyzer (Roche-Hitachi, Japan) according to the methods described by Hissin and Hilf, (1976); Kakkar et al., (1984); Sinha, (1972), respectively.

2. 2.5 Histopathological examination:

Liver of the scarified rats were taken and immersed in 10% formalin solution. The fixed specimens were then trimmed, washed and dehydrated in ascending grades of alcohol. Specimens were then cleared in xylol, embedded in paraffin, sectioned at 4-6 microns thickness, and stained with Heamtoxylin and Eosin stain for examination of the liver as described by Carleton, (1979).

2.2.6 Statistical analysis

The obtained results were expressed as Mean ± SE. Data were evaluated statistically with computerized SPSS package program (SPSS 9.00 software for Windows) using one-way analysis of variance (ANOVA). Significant differences among means were estimated at $p<0.05$ according to Snedecor and Cochran, (1986).

3. Results:

Data in Table (1) show that rats with HCC (positive control group) had significant ($p<0.05$) increase in serum level of AST (195.00±9.72 U/L), compared to normal rats (104.00±5.91 U/L). Oral administration of broccoli and red cabbage extract caused significant reduction ($p<0.05$) in serum level of AST (143.00±6.55 and 137.00±5.64 U/L, respectively) as compared to the positive control group (195.00±9.72 U/L). There were no significant changes in AST serum level between rats given broccoli or red cabbage extracts.

Table (1): Effect of oral administration of broccoli and red cabbage extracts on serum concentrations of AST, ALT and ALP of hepatocellular carcinoma rats.

(n=10 rats)

Groups	Liver enzymes (Mean ±SE)		
	AST (U/L)	ALT (U/L)	ALP (U/L)
Negative control	**e** 104.00±5.91	**d** 74.70±1.37	**d** 188.97±1.48
Positive control (HCC)	**a** 195.00±9.72	**a** 150.00±3.33	**a** 286.09±4.80
Broccoli extract (10%)	**cd** 143.00±6. 55	**cd** 84.40±3.35	**d** 193.34±2.62
Red cabbage extract (10%)	**d** 137.00±5.64	**bc** 95.00±3.87	**c** 211.79±1.31

Means with different superscripts letters are significant at $p<0.05$.

Results of hepatocellular carcinoma rats revealed that rats with HCC (positive group) had significant increase ($p<0.05$) in serum level of ALT (150.00± 3.33 U/L), compared to the negative control group (74.70±1.37 U/L). Oral administration of broccoli and red cabbage extracts to rats inflicted with HCC induced significant ($p<0.05$) reduction of levels in serum ALT (84.40±3.35 and 95.00±3.87, respectively) as compared to the control positive group. There were non-significant changes in serum levels of ALT between rats given orally either broccoli or red cabbage extract.

Tabulated results showed that serum level of ALP significantly ($p<0.05$) increased in positive control rats (286.04± 4.80 U/L) as compared to the negative control rats (188.97±1.48 U/L) as expected. Rats given orally broccoli and red cabbage extracts showed significant reduction ($p<0.05$) in serum level of ALP (193.34±2.62 and 211.79±1.31U/L, respectively), compared to the positive control group.

Results in Table (2) showed that rats with HCC had significant ($p<0.05$) increase in serum level of total protein and albumin (8.04±0.26 and 7.88±0.43 g/dL, respectively), compared to the normal rats (6.80 ± 0.26 and 4.88±0.10 g/dL, respectively). Oral administration of broccoli and red cabbage extracts caused significant ($p<0.05$) decrease in serum level of total protein and albumin as compared to the positive control group.

Table (2): Effect of oral administration of broccoli and red cabbage extracts on serum concentrations of total protein and albumin of hepatocellular carcinoma rats.

(n=1 0 rats)

Groups	Parameter (Mean ±SE)	
	Total protein (g/dL)	Total albumin (g/dL)
Negative control	**b** 6.80±0.26	**d** 4.88±0.10
Positive control (HCC)	**a** 8.04±0.26	**a** 7.88±0.43
Broccoli extract (10%)	**b** 6.49±0.33	**cd** 5.25±0.11
Red cabbage extract (10%)	**b** 6.63±0.11	**bc** 5.64±0.19

Means with different superscripts letters are significant at $p<0.05$.

Rats with HCC showed significant ($p<0.05$) increases in serum levels of total and direct bilirubin (0.71±0.01 and 0.94±0.02 mg/dL, respectively) as compared to negative control group (0.45±0.0 1 and 0.59±0.01mg/dL, respectively). Broccoli and red cabbage extracts significantly ($p<0.05$) decreased the serum levels of total and direct bilirubin, compared to the positive control group as shown in Table (3).

Results in Table (4) showed that rats with HCC had significant ($p<0.05$) increase in MDA (2.81±0.02 µmol/dL), however, GSH levels was decrease (25.42±0.25 µmol/dL), compared to the normal rats. Oral administration of broccoli and red cabbage extracts produced a significant ($p<0.05$) reductions in MDA (1.65±0.01 and 1.81±0.02 µmol/dL, respectively) and an increase in GSH levels (36.80±0.21 and 34.60±0.15 µmol/dL, respectively), compared to the positive control group.

Table (3): Effect of oral administration of broccoli and red cabbage extracts on serum concentrations of total and direct bilirubin of hepatocellular carcinoma rats.

(n=1 0 rats)

Groups	Parameter (Mean ±SE)	
	Total bilirubin (mg/dL)	Direct bilirubin (mg/dL)
Negative control	**d** 0.45±0.01	**c** 0.59±0.01
Positive control (HCC)	**a** 0.71±0.01	**a** 0.94±0.02
Broccoli extract (10%)	**cd** 0.47±0.01	**c** 0.61±0.02
Red cabbage extract (10%)	**d** 0.46±0.01	**c** 0.61±0.01

Means with different superscripts letters are significant at p<0.05.

Table (4): Effect of oral administration of broccoli and red cabbage extracts on serum concentrations of malondialdehyide and reduced glutathione of hepatocellular carcinoma rats.

(n=10 rats)

Groups	Parameter (Mean ±SE)	
	MDA (µmol/dL)	GSH (µmol/dL)
Negative control	f 1.23±0.03	a 40.21±0.12
Positive control (HCC)	a 2.81±0.02	e 25.42±0.25
Broccoli extract (10%)	d 1.65±0.01	c 36.80±0.21
Red cabbage extract (10%)	c 1.81±0.02	d 34.60±0.15

Means with different superscripts letters are significant at p<0.05

Antioxidant enzymes levels in serum of rats was significantly reduced in HCC rats compared to negative untreated group as shown in Table (5). Rats given orally broccoli and red cabbage extract showed significant (p<0.05) increases in serum levels of antioxidant enzymes as compared to the positive control group.

Histopathological examination of the liver of normal rats revealed normal histological structure of hepatic lobule as shown in Figure (1). Livers of rats with HCC showed fatty infiltration of hepatocytes, cytomegaly with karyomegaly as well as vesicular active nuclei and presence of more than one nucleolus as shown in Figure (2). Oral administration of broccoli extract at 10% to HCC rats revealed only slight hydropic degeneration of hepatocytes as shown in Figure (3). Other liver sections of the same group showed apparent normal heptocytes. Liver sections of rats with HCC given orally red cabbage extract at 10% revealed slight hydropic degeneration of some hepatocytes as shown in Figure (6), while other liver sections from the same group showed apparent normal heptocytes.

Table (5): Effect of oral administration of broccoli and red cabbage extracts on serum concentrations of antioxidant enzymes of hepatocellular carcinoma rats.

(n=10 rats)

Groups		Paramet	
	GPX (mmol/dL)	SOD (U/dL)	CAT (mmol/dL)
Negative control	a 18.70±0.13	a 95.75±0.22	a 67.00±0.56
Positive control (HCC)	f 8.25± 0.14	f 55.45±0.19	f 42.15±0.60
Broccoli extract (10%)	c 13.95±0.02	b 86.66±0.16	c 63.60±0.56
Red cabbage extract 10%)	e 9.25±0.02	e 68.61±0.10	e 51.65±0.24

Means with different superscripts letters are significant at p<0.05.

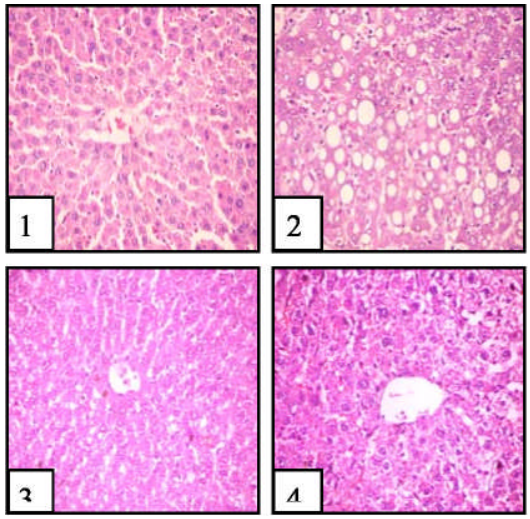


Figure (1): liver of control rat, showing the normal histological structure of hepatic lobular. (H and E x 200).

Figure (2): liver of positive group, showing fatty infiltration of hepatocytes, cytomegaly with karyomegale, viscular active nuclei and more than one nucleolus. (H and E x 200).

Figure (3): liver of rats with HCC treated with broccoli extract showing slight hydropic degeneration of heptocytes. (H and E x 200).

Figure (4): liver of rats with HCC rats treated with red cabbage extract showing slight hydropic degeneration of heptocytes. (H and E x 200).

4. Discussion:

The present study aimed to investigate the hepatoprotective effect of broccoli and red cabbage extracts at 10% against hepatocellular carcinoma induced by NDEA in rats. The biomarkers used in this study provide the measures

of carcinogen exposure in rats as an area of high risk for development of hepatocellular carcinoma. Results of this study showed that rats with HCC had significant increase in serum levels of AST, ALT, ALP, total protein, total and direct bilirubin as well as MDA. However, there were significant reductions in GSH, GPX, SOD and CAT, compared to the normal rats. The increase in these parameters in rats with HCC might be attributed to the injured structural integrity of the liver as they are released into the circulation after cellular damage induced by CCl4 and NDEA. These results agreed with those obtained by Pevicharova et al., (1997) who found that activities of AST, ALT and ALP were increased significantly following N-nitroso compounds treatment in rats. Moreover, Vozarova et al., (2002) mentioned that the elevated activities of AST, ALT and ALP enzymes were signs of impaired liver function in response to NDEA administration. Bansal et al., (2005) attributed the elevation of liver enzymes to their release from the cytoplasm into the blood circulation after rupture of the plasma membrane and cellular damage. Mittal et al., (2006) reported that NDEA administration caused a substantial liver damage as evidenced by the increases in the activities of AST and ALT enzymes in the treated rats.

Data in this study showed an increased in serum MDA concentration which may be related to the increased in free radicals. This finding was consistent with the observation that the free radicals reduced the activity of the endogenous antioxidant enzyme SOD (Conner and Grisham, 1996). It is known that free radical scavenging enzyme such as SOD protects the biological systems from oxidative stress. The current study showed a significant decrease in the activity of SOD in groups of rats administrated with NDEA (positive control). This reduction could be attributed to an enhanced production of free radicals during NDEA metabolism. Zwart et al., (1999) reported that lipid peroxidation produced several toxic byproducts such as MDA which can attack cellular targets including DNA, inducing mutagenecity and carcinogenicity. Administration of NDEA depleted the level of glutathione (GSH) in this study. Such depletion agreed with that reported by Kweon et al., (2003); Bansal et al., (2005); Sivaramakrishnan et al., (2007) and Pradeep et al., (2007) in their study. Since glutathione is required to maintain the normal reduced state of cells and to counteract all the deleterious effects of oxidative stress. Thus GSH is involved in many cellular processes including the detoxification of endogenous and exogenous compounds. NDEA is electrophilic carcinogens that interact with the large nucleophilic pool of GSH thereby reducing the macromolecule and carcinogen interaction (Ketterer and Meyer, 1989). The depletion of liver GSH in NDEA-treated rats may be responsible for the increased in lipid peroxidation. A significant decrease in the activities of GSH dependent enzymes, GPX and CAT in NDEA-treated rats may be due to decreased expression of these antioxidants during hepatocellular damage. Furthermore, the decreased levels of cellular GSH caused a reduction in their activities as GSH is a vital co-factor for these enzymes. The obtained results were in accordance with that reports by Kweon et al., (2003) who demonstrated that NDEA induced hepatocellular injury by a substantial fall in hepatic GSH, GPX and CAT activity, which then improved by administration of antioxidants. Boitier et al., (1995) reported that in hepatocellular carcinoma there is a disturbance between oxidant and antioxidant balance, which is tilted towards oxidant side.

Previous researches indicated that NEDA induced hepatocarcinogenesis in Wistar rats. It significantly elevated thiobarbituric acid reactive substances in the circulation of rats. Carcinoma indicated the higher levels of lipid peroxidation, which was accompanied by significantly decreased levels of antioxidants (reduced glutathione, glutathione peroxidase, superoxide dismutase and catalase) enzymes, as compared to the controls. Lipid peroxidation has been implicated as a major cause in cancer development (Sundaresan and Subramanian, 2003). A study by Dakshayani et al., (2005) demonstrated that the oxidative stress may be the reason for the elevated lipid peroxidation level in the liver of NDEA treated animals. These results were confirmed by Bansal et al., (2005) who reported that liver is the main site of NDEA metabolism, the production of ROS in liver may be responsible for its carcinogenic effects. In addition, Mittal et al., (2006) concluded that nitrosamines caused the generation of reactive ROS resulting in oxidative stress which alter the antioxidant defense system in the tissues.

With regard to the effect of NDEA on liver structure in rats, our results were agreed with Lijinsky, (1992) who showed that NDEA caused a wide range of tumors in all animal species and induce cancer in a variety of rodent organs, especially the liver. Devi et al., (2000) revealed that disarrangement of normal hepatic cells with centrilobular necrosis vacuolization of cytoplasm and fatty degeneration were observed in carbon tetrachloride intoxicated mice. This suggested that prolonged cell damage by chronic inflammation is critical in cancer development. Over production of nitric oxide has been implicated in the tissue damage caused by inflammation, contributing the tumor promotion (Nishikawa et al., 1998). Moreover, Singh et al., (2004) reported that oxidative stress caused by reactive oxygen species generated after administration

of NDEA has been reported in membrane lipid peroxidation, and has been associated with various stages of tumor formation process.

This study revealed oral administration of broccoli and red cabbage extracts caused significant decrease in serum levels of AST, ALT, ALP, total protein, total albumin, total and direct bilirubin and MDA. However, there were significant increases in the levels of GPX, SOD and CAT as well as improved liver structure, compared to the positive group. Consequently, administration of broccoli or red cabbage extracts could prevent or decreased the incidences of hepatocarcinogenesis in rats induced by NDEA. These results agreed with Wargovich, (1999) who reported that cruciferous vegetables namely cabbage, Brussels sprouts, cauliflower and broccoli are rich in anti-carcinogens. A possible mechanism of reduced activities of the tested enzymes and hepatoprotective effect of broccoli and red cabbage extract may be related to their antioxidant effect of the phenolic and flavonoids compounds. Previous study reported that polyphenols can inhibit nitrosation and flavonoides have hepatoprotective activities (Orhan et al., 2007). Since flavonoids are a group of potentially chemoprotective compounds and have similar structures that consist of 2 phenolic benzene rings linked to a heterocyclic pyre or pyrone (Aherne and O'Brien, 2002). It has many biological effects that play a role in cancer prevention, including free radical scavenging, antimutagenic and antiproliferative properties, regulation of cell signaling and cell cycle, and inhibition of angiogenesis (Moon et al., 2006). In vitro and vivo experimental studies suggested that flavonoids influence signal transduction pathways (Frigo et al., 2002), and inhibit proliferation in human cancer cell lines (Manthey and Guthrie, 2002).

Cruciferous vegetables contain several chemical compounds that may modulate the carcinogenic process. These compounds act as antioxidants or as inhibitors and/or inducers of phase I and phase II enzymes (Fong et al., 1990; Bradlow et al., 1991). Phytonutrients in Crucifers vegetables work at a much deeper level and actually signal genes to increase production of enzymes involved in the detoxification. Lampe and Peterson, (2002) revealed that anti-carcinogenic actions of cruciferous vegetables are attributed to their content of glucosinolates (GLS). Fowke et al., (2003) reported that cruciferous vegetables contain sulforophane, which has anticancer properties. The anticancer effects of cruciferous vegetables may attribute to organic sulfur compounds (diallyl disulfide) and isothiocyanates, which had the ability to modulate expression/activity of antioxidative and phase 2 drug-metabolizing enzymes and scavenging free radicals (Antosiewicz et al., 2008).

The cancer-preventive effect of broccoli may be due to its content of sulforaphane (Zhang et al., 1992). The chemoprotective effect of sulforaphane may to be due its ability to behave as an inducer of phase II detoxification enzymes (Prestera and Talalay, 1995). Sulforaphane was also shown to inhibit the CYP2EI isoenzyme of the cytochrome P450, thus emerging as an inhibitor of phase I enzymes (Barcelo et al., 1996). Nishikawa et al., (2009) also concluded that isothiocyanates sulforaphane presented in broccoli has inhibitory effects on tumor cell growth in vitro and in vivo. In addition to broccoli was reported to provide moderate antioxidant capacity, likely attributed to tocopherols and flavonoids (Plumb et al., 1997). However, Cindy and John, (2003) revealed that selenium-enriched broccoli activates certain pro-apoptotic genes linked to p53, NFκB and stress signal pathways in response to “danger signals” such as tumorigenesis.

The protective effect of red cabbage against HCC that reported in this study agreed with the finding of Fekadu et al., (2003) who mentioned that that the chemoprotective properties of red cabbage involve inhibition of the formation as well as development of preneoplastic lesions in liver. One of the most important mechanisms of chemoprotection is induction of phase II enzymes, which detoxify DNA-reactive metabolites and thereby inhibit the formation of initiated cells (De Flora and Ramel, 1990). Another possible mechanism of protection is the inhibition of enzymes which are involved in the activation of heterocyclic aromatic amines (Rauscher et al., 1998). The effect of red cabbage may be attributed to the prevalence of anthocyanins in its extract. In addition, Wu and Prior, (2005) reported that several highly conjugated anthocyanins were identified in red cabbage with potential antioxidant activities. However, Hagiwara et al., (2002) indicated that color extracted from red cabbage shown to inhibit adenoma and carcinoma formation in rats initiated with and subsequently fed a diet containing the heterocyclic amine. Kassie et al., (2003) also reported that administration of red cabbage extract to rats resulted in chemoprevention of liver and colon cancers induced by heterocyclic amine. On the other hand, Lynn et al., (2006) showed that breakdown products of glucosinolates such as isothiocyanates in red cabbage are responsible for the chemopreventive properties of cruciferous vegetables.

5. Conclusion:

This study concluded that broccoli and red cabbage have a protective effect against hepatocellular carcinoma in rats, therefore this study recommends increased dietary intake of broccoli and red cabbage may be beneficial for patients with liver cancer as a

preventative measures.

6. References:

[1] Aherne, S. A. and O'Brien, N. M., 2002. Dietary flavonols: chemistry, food content, and metabolism. Nutrit. 18, 75–8 1.

[2] Antosiewicz, J., Ziolkowski, W., Kar, S., Powolny, A. A. and Singh, S. V., 2008. Role of reactive oxygen intermediates in cellular responses to dietary cancer chemopreventive agents. Planta Med. 74 (13), 1570-1579.

[3] Bansal, A. K.; Bansal, M.; Soni, G and Bhatnagar, D., 2005. Protective role of Vitamin E pre-treatment on N-nitrosodiethylamine induced oxidative stress in rat liver. Chemico-Biological Interactions. 156, 101-111.

[4] Barcelo, S., Gardiner, J. M., Gescher, A. and Chipman, J. K., 1996. CYP2E1-mediated mechanism of anti-genotoxicity of the broccoli constituent sulforaphane, Carcinogenesis, 17 (2), 277–282.

[5] Block, G., Patterson, B. and Subar, A., 1992. Fruits, vegetables and cancer prevention: A review of the epidemiologic evidence. Nutri. Cancer, 18, 1-9.

[6] Beutler, E. D. and Kelly, B. M., 1963. Improved method for determination of blood glutathione. J. Lab. Clin. Med. 61, 882-888.

[7] Boitier, E., Boudia, M. M., Guillouzo, C. G., Defer, N. Picot, I. C., Leroux, J. P. and Marsac, C., 1995. Impairment of the mitochondrial respiratory chain activity in diethylnitrosamine induced rat hepatoma: possible involvement of oxygen free radicals. Cancer Res. 55 (14), 3028–3035.

[8] Bradlow, H. L., Michnovicz, J., Telang, N. T. and Osbome, M. P., 1991. Effects of dietary indole-3-carbinol on oestradiol metabolism and spontaneous mammary tumors in mice. Carcinogenesis, 12, 1571-1574.

[9] Carleton, H., 1979. Histological techniques, 4th Ed, London, Oxford University Press, New York, USA, Toronto.

[10] Cindy, D. D. and John, W. F., 2003. Effect of selenium-enriched broccoli diet on differential gene expression in liver. J. of Nutri. Biochem. 14 (4), 227-231.

[11] Conner, E. M. and Grisham, M. B., 1996. Inflammation, free radicals and antioxidants. Nutrition J. 12 (4), 274–277.

[12] Dakshayani, K., Subramanian, P., Manivasagam, T., Mohamed, M. and Manoharan, S., 2005. Melatonin Modulates The Oxidant– Antioxidant Imbalance During N-Nitrosodiethylamine Induced Hepatocarcinogenesis In Rats. J. Pharm, Pharmaceut. Sci. 8(2), 316-321.

[13] De Flora, S. and Ramel, C., 1990. Classification of mechanisms of inhibitors of mutagenesis and carcinogenesis. Basic Life Sci. 52, 461–462.

[14] Devi, G. S., Prasad, M. H., Saraswathi, I., Raghu, D., Rao, D. N. and Reddy, P. P., 2000. Free radical antioxidant enzymes and lipid peroxidation in different types of lukemias .Clin Chim Acta. 293, 53-62.

[15] Draper, H. H. and Hadley, M., 1990. Malondialdehyde determination as index of lipid peroxidation. Methods Enzymol. 186, 421-431.

[16] Elizabeth, H. J. and Marcela, A., 2009. Physiological effects of broccoli consumption. Phytochemistry Reviews. 8(1), 1568-7767.

[17] Fekadu, K., Maria, U., Sylvie, R., Bettina, G., Ruud, V., Michael, K., Monika, C., Rolf, S. and Siegfried, K., 2003. Chemoprevention of 2-amino-3-methylimidazo [4,5-f]quinoline (IQ)-induced colonic and hepatic preneoplastic lesions in the F344 rat by cruciferous vegetables administered simultaneously with the carcinogen. Carcinogenesis. 24 (2), 255-261.

[18] Ferlay, J., Bray, F., Pisani, P. and Parkin, D., 2001. Cancer incidence, mortality and prevalence worldwide, IARC Scientific Publications, IARC Press, Lyon.

[19] Fong, A. T., Swanson, H. I., Dashwood, R. H., Williams, D. E., Hendricks, J. D. and Bailey, G. S., 1990. Mechanisms of anti-carcinogenesis by indole-3-carbinol. Studies of enzyme induction, electrophile scavenging, and inhibition of aflatoxin Bl activation. Biochem. Pliannacol. 39, 19-26.

[20] Fowke, J. H., Chung, F. L., Jin, F., Qi, D., Cai, Q., Conaway, C., Cheng, J. R., Shu, X. O., Gao, Y. T. and Zheng, W., 2003. Urinary isothiocyanate levels, brassica, and human breast cancer. Cancer Res. 63, 3 980-3986.

[21] Frigo, D. E., Duong, B. N. and Melnik, L. I., 2002. Flavonoid phytochemicals regulate activator protein-1 signal transduction pathways in endometrial and kidney stable cell lines. J Nutr. 132, 1848–1853.

[22] Hagiwara, A., Yoshino, H., Ichihara, T., Kawabe, M., Taman, S., Aoki, H., Koda, T., Nakamura, M., Imaida, K., Ito, N. and Shirai, T., 2002. Prevention by natural food anthocyanins, purple sweet potato color and red cabbage color, of 2-amino-1-methyl-6-phenylimidazo [4, 5-b] pyridine (Phip)-associated colorectal carcinogenesis in rats with 1, 2-dimethylhydrazine. J of Toxicolo. Scien.. 27, 57–68.

[23] Hissin, P. L. and Hilf, R., 1976. A fluorometric method for determination of oxidized and reduced glutathione in tissues. Anal. Biochem. 74 (1), 214-226.

[24] Inas, A. E., 2005. Cancer databases in the arabe world. Ethnicity and Disease. 15 (S1), 3-5.

[25] Kaplan, A., 1984. Clin Chem the C. V. Mosby

Co. St Louis. Toronto. Princeton. Pp. 1088-1273.

[26] Kakkar, P., Das, B. and Viswanathan, P. N., 1984. A modified spectrophotometric assay of superoxide dismutase. Ind. J. Biochem. Biophyso. 21, 131-133.

[27] Kassie, F., Uhl, M., Rabot, S., Grasl-Kraupp, B., Verkerk, R. and Kundi, M., 2003. Chemoprevention of 2-amino-3-methylimidazo [4,5-f]quinoline (IQ)-induced colonic and hepatic preneoplastic lesions in the F344 rat by cruciferous vegetables administered simultaneously with the carcinogen. Carcinogenesis. 24, 255-261.

[28] Ketterer, B. and Meyer, D. J., 1989. Glutathione transferases: a possible role in the detoxification and repair of DNA and lipid hydroperoxides. Mutat. Res. 214 (1), 33-40.

[29] Koller, A., 1984. Clin Chem the C. V. Mosby Co. St Louis. Toronto. Princeton; Pp. 418, 13 16-11324.

[30] Kurilich, A. C., Tsau, G. J., Brown, A., Howard, L., Klein, B. P., Jeffery, E. H., Kushad, M., Wallig, M. A. and Juvik, J. A., 1999. Carotene, tocopherol, and ascorbate contents in subspecies of *Brassica oleracea*. J. Agric. Food Chem. 47, 1576–1581.

[31] Kweon, S., Park, K. A. and Choi, H., 2003. Chemopreventive effect of garlic powder diet in diethylnitrosamine-induced rat hepatocarcino-genesis. Life Sci. 73 (19), 2515– 2526.

[32] Lampe, J. W. and Peterson, S., 2002. Brassica, biotransformation and cancer risk: Genetic polymorphisms alter the preventive effects of cruciferous vegetables. Journal of Nutrition, 132, 2991–2994.

[33] Lijinsky, W., 1992. Chemistry and Biology of N-Nitroso Compounds. Cambridge: Cambridge University Press.

[34] Lynn, A., Collins, A., Fuller, Z., Hillman, K. and Ratcliffe, B., 2006. Cruciferous vegetables and colo-rectal cancer. Proc Nutr Soc. 65, 135-144.

[35] Manthey, J. A. and Guthrie, N., 2002. Antiproliferative activities of citrus flavonoids against six human cancer cell lines. J Agric Food Chem.50, 5837–5843.

[36] Michaelis, L., Schubert, M. P. and Smythe, C. V., 2008. Potentiometric Study of the Flavins. J. Biol. Chem. 116 (2), 587–607.

[37] Mittal, G., Brar, A.P. and Soni, G., 2006. Impact of hypercholesterolemia on toxicity of N - nitrosodiethylamine: biochemical and histopath-ological effects. Pharmacol Rep. 58(3), 413-419.

[38] Mohamed, N., 2002. Photochemical and biological studies on *convolvulus arvesis.* Ph D. Thesis submitted to Faculty of Science. Ain Shams University.

[39] Moon, Y. J., Wang, X. and Morris, M. E., 2006. Dietary flavonoids effects on xenobiotic and carcinogen metabolism. Toxicol in Vitro. 20, 187–210.

[40] Murray, M. and Lara, P., 2005. The encyclopedia of healing foods. Simon and Schuster Adult Publishing Group. 172.

[41] Nishikawa. M., Takeda, K., Sato, E. F., Kuroki, T. and Inoue, M., 1998. Nitric oxide regulates energy metabolism and Bcl-2 expression in intestinal epithelial cells. Am J Physiol. 274(5), 797-801.

[42] Nishikawa, T., Tsuno, N. H., Tsuchiya, T., Yoneyama, S., Yamada, J., Shuno, Y., Okaji, Y., Tanaka, J., Kitayama, J., Takahashi, K. and Nagawa, H., 2009. Sulforaphane stimulates activation of proapoptotic protein bax leading to apoptosis of endothelial progenitor cells. Ann Surg Oncol. 16(2), 534-543.

[43] Orhan, D. D., Orhan, N., Ergun, E. and Ergun, F., 2007. Hepatoprotective effect of Vitis vinifera L. leaves on carbon tetrachloride-induced acute liver damage in rats. J Ethnopharmacol. 112(1), 145-51.

[44] Pevicharova, G.T., Dimova, P. I. and Atanasova-Goranova, V. K., 1997. Effect of food products on endogenous generation of N nitrosamines in rats. Br. J. Nutr. 78(2), 325-345.

[45] Plumb, G. W., Price, K. R., Rhodes M. J. and Williamson, G., 1997. Antioxidant properties of the major polyphenolic compounds in broccoli. Free Radic Res. 27 (4), 429–435.

[46] Pradeep, K., Mohan, C. V., Gobianand, K. and Karthikeyan, S., 2007. Silymarin modulates the oxidant-antioxidant imbalance during diethylnitrosamine induced oxidative stress in rats. Eur J Pharmacol. 560 (2-3), 110-116.

[47] Prestera, T. and Talalay, P., 1995. Electrophile and antioxidant regulation of enzymes that detoxify carcinogens, Proc Natl Acad Sci USA, 92 (19), 8965–8969.

[48] Price, K. R., Casuscelli, F., Colquhoun, I. J. and Rhodes, M. J., 1998. Composition and content of flavonol glycosides in broccoli florets *(Brassica olearacea)* and their fate during cooking. J of the Scien of Food and Agric. 77, 468–472.

[49] Rauscher, R., Edenharder, R. and Platt, K. L., 1998. In vitro antimutagenic and *in vivo* anticlastogenic effects of carotenoids and solvent extracts from fruits and vegetables rich in carotenoids. Mutat. Res. 413, 129–142.

[50] Reeves, P. G., Nielson, F. H. and Fahmy, G. C., 1993. Reports of the American Institute of Nutrition, adhoc-wiling committee on reformulation of the AIN 93. Rodent diet. J. Nutri. 123, 1939-1951.

[51] Roy, S. E., 1970. Colorimetric determination of

serum alkaline phospohatase. Clin. Chem. 16, 43 1-432.

[52] Singh, V., Selvendiran, K., Banu, S. M., Padmavathi, R. and Sakthisekaran, D., 2004. Protective role of Apigenin on the status of lipid peroxidation and antioxidant defense against hepatocarcinogenesis in Wistar albino rats. Phytomedicine. 11, 309-314.

[53] Sinha, K. A., 1972. Colorimetric assay of catalase enzyme. Anal, Biochem. 47, 389-394.

[54] Sivaramakrishnan, V., Shilpa, P. N., Praveen, V. R. and Niranjali, D. S., 2007. Attenuation of N - nitrosodiethylamine induced hepatocellular carcinogenesis by a novel flavonol-Morin. Chemico-Biological Interactions. 171(1),79–8 8.

[55] Snedecor, G. W. and Cochran W. G., 1986. Statistical Methods. 4th Edition, Iowa State University Press. Ames, Iowa, USA, Page 91.

[56] Steinmetz, K. A. and Potter, J. D., 1991. Vegetables, fruits and cancer prevention. J. Am. Diet Assoc. 96, 1027–1039.

[57] Strauss, R. M., 1995. Hepatocellular carcinoma, clinical, diagnostic and therapeutic aspects. In: Rustgi AK, ed. Gastrointestinal Cancers. Philadelphia, Pa: Lippincott-Raven. 479-496.

[58] Sundaresan, S. and Subramanian, P., 2003. S-allylcysteine inhibits circulatory lipid peroxidation and promotes antioxidants in N - nitrosodiethylamine-induced carcinogenesis. Pol. J. Pharmacol. 55, 37–42.

[59] Vozarova, B., Stefan N., Lindsay, S. R., Saremi, A., Pratley, E. R., Bogardus, C. and Tatarnni, A. P., 2002. High alanine aminotransferase is associated with decreased hepatic insulin sensitivity and predicts the development of type 2 diabetes. Diabetes. 51, 1889-1 895.

[60] Wargovich, M. J., 1999. Nutrition and cancer: The herbal revolution.Curr Opin Clin Nutr Metab Care. 2, 421-425.

[61] Wattenberg, L.W., 1990. Chemoprevention of cancer by naturally occurring and synthetic compounds. Proc. Am. Assoc. Cancer Res. 32, 461–463.

[62] Wild, C. P. and Hall, A. J., 2000. Primary prevention of hepatocellular carcinoma in developing countries. Mutat Res. 462, 381-393.

[63] Wu, X. and Prior, R., 2005. Identification and characterization of anthocyanins by high performanceliquid chromatography electrospray ionization-tandem mass spectrometry in common foods in the United States: vegetables, nuts and grains, J Agric Food Chem. 53 (8), 3101–3113.

[64] Zhang, Y., Talalay, P., Cho, C. G. and Posner, G. H., 1992. A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure, Proc Natl Acad Sci USA. 89 (6), 2399–2403.

[65] Zwart, L. L., Meerman, J. N. and Commandeur, J. M., 1999. Biomarkers of free radical damage applications in experimental animals and humans. Free Radic. Biol. Med. 26 (1-2), 202–226.

11/2/2010

Eating Behavior and Problems in Egyptian Adolescents; Relation to Dietary Intake

Zeinab M. Monir*; Abla G Khalifa; Fawzya Hassaballa; Sawsan Tawfeek; Mohamed Abdelmonem, Moönes Abu Shady and Manal Mansour

Child Health Department, National Research Centre, Dokki, Giza, Egypt
*zeinab_monir@yahoo.com

Abstract: Objective of this study is to examine the presence of disordered eating (ED) behavior among Egyptian adolescent boys and girls and detect the influence of obesity, body image, depression, somatic symptoms, binging and weight teasing by peers and family members as well as assessing dietary intake of macronutrients and micronutrients and its correlation to obesity and eating disorder. *Subjects and Methods*: The sample consisted of 1124 adolescents (642 girls & 482 boys) aged from 14-17 years, divided according to their BMI into four groups. The questionnaires used were EAT, ACDI, body image, and teasing, 24hr- dietary recall. and sociodemographic data were collected. *Results*: we found that 25.5% & 38.6% of boys and girls reported ED that was significantly correlated to body image, bad eating habits, depression and somatic symptoms. ED is more prevalent among overweight-obese adolescents of high social class. Adolescents have deficient intake of vitamin A, calcium, thiamine and niacin; girls are more deficient in iron and boys are deficient in vitamin C. On assessing weight teasing by peers and family member by weight status and ED after adjustment for socioeconomic standard; there was statistically significant association with obesity in girls & boys. *Conclusion*: Social back ground, obesity, negative body image , depression and teasing are the main risk factors for developing ED. Early detection and intervention for ED by biological and psychological approaches, treatment of overweight and obesity using family based treatment; early detection of depression and encouraging sports practice are recommended.
[Zeinab M. Monir; Abla G Khalifa; Fawzya Hassaballa; Sawsan Tawfeek; Mohamed Abdelmonem, Moönes Abu Shady and Manal Mansour. **Eating Behavior and Problems in Egyptian Adolescents; Relation to Dietary Intake**. Journal of American Science 2010;6(12):1145-1159]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: eating disorder (ED), obesity, depressions, body image, teasing.

1. Introduction:

As the world has become a global village due to advances in communications, dietary habits have changed especially among adolescents through introduction of all types of fast food with its heavy fat content that goes hand in hand with changes in life style & physical activity. These factors lead to eating disorders, one of the most important risk factors for obesity, hypertension and cardiovascular diseases in adult life. Non Communicable Diseases (NCD) is emerging as a major health problem in Egypt, where 41 percent of all deaths are caused by chronic diseases. It is expected that NCD burden will rise to 60% by the 2020. The increasing NCD burden places a huge demand on health services in Egypt (WHO, 2005). Therefore action to reduce these major NCD should focus on preventing and controlling the risk factors in an integrated manner (WHO, 2003).

Aim of the Study:

Assessment of eating disorders among Egyptian adolescent girls and boys in relation to weight status, dietary intake, teasing, and psychosocial risk factors.

2. Methods

Study population:

The study population included 1124 adolescents (482 boys & 642 girls) attending four high schools in Giza; two public schools one for boys (264) and one for girls (458) and another two mixed private schools (218 boys & 184 girls) with age range from 14-17 years old.

Study design:

Data for the present study were drawn from project eating attitude among adolescents attending four high schools, after the approval of the scientific ethical committee at the National Research Centre and agreement of the ministry of education province office. A signed consent from parents was obtained. Survey and anthropometric data were collected from students within their classrooms under the direction of the research staff & a teacher. Height and weight were assessed by the research staff within the classrooms.

Survey development:

We preferred to use an Arabic scale for surveying eating disorders, binging and bad eating habits using Shoukeir assessment scales (Shoukeir 2002& Shoukeir 2000), derived from Marshall (1998). For Assessment of depression we used the Arabic Children's Depression Inventory (ACDI)

derived from Kovacs (1992) and Kazdin (1986) and constructed for Egyptian children by Abdel Khalek (1993). The questionnaires on body image were adopted from the original questionnaires by Moore (1998). Questionnaires for assessing teasing were quoted from Neumark (Neumark el al., 2002), that was based on the survey items developed by Thompson et al., (1995).

Measures:

Weight status was based on height and weight measurements taken by research trained staff in the classroom. Standardized equipments and procedures were employed. Body mass index (BMI) values were calculated according to WHO published standards

BMI = weight (kg)/ Height (meter)^2

Using gender, age specific cut-off points respondents were classified as underweight (BMI< 15th percentile); average weight (BMI 15th- < 85 th percentile), overweight (BMI >85 th. <95 th percentile) and obese (BMI > 95 th percentile).

We assess the frequency of teasing, source of teasing (peers or family members), and the effect of teasing, (i.e. how much it bothers those teased). The degree to which they were bothered by weight-teasing by peers and family members making them east more or less was then assessed; response categories were (1) never; (2) sometimes; (3) often.

School level and birth date were taken from the school files. Socio demographic data were based on self report. The prime determinant of socioeconomic status (SES) was parental educational level defined by 4 categories. (1) Illiterate; (2) primary-preparatory; (3) high school or similar; (4) university education; and parental occupation level defined by (1) not working; (2) workers; (3) employee; (4) professional. Family number and birth order of the participant were recorded.

Every student filled a 24hr dietary recall sheet; that was analyzed by a nutritionist to calculate the intake of macronutrients and micronutrients.

Data analysis:

Unadjusted association between eating disorder and psychosomatic variables as well as micronutrients and macronutrients intake as well other risk factors that include parental education and occupation, family number and birth order was studied. We studied also association between weight statuses, eating disorder and teasing using $\chi 2$ TEST and associations adjusted for SES and school level were conducted using logistic regression. Among the students who reported being teased by peers or family members, percentage of girls and boys bothered by teasing were examined across weight status using $\chi 2$.

Eating disorder and its association to weight teasing and weight status were examined using $\chi 2$ tests and associations adjusted for BMI, SES were examined using logistic regression. To conduct all analyses, SAS release 6.12 was used.

3. Results

Fig.1 shows that nearly 5% of boys and 3% of girls have lean body mass index (BMI) whereas, 58% of boys and 55% of girls have normal BMI; while the percentage of overweight of boys and girls were 17% & 27% and the obesity of boys and girls were 20% & 14.5 respectively, added to this obesity increases in girls with age and decreases in boys with age.

Fig.2 shows that 78% boys, 80% girls take $\geq$ 80% of RDA of their proteins; 94% boys and 93% girls receive $\geq$ 80% of RDA of their carbohydrates & 66% boys & 74% girls receive calories $\geq$ 80% RDA.

Fig.3 shows the micronutrients intake according to RDA whereas we found that the most prevalent deficient intake was in vitamin A intake and to less extent in both thiamine and niacin in boys, whereas in girls we found that the most deficient intake was in vitamin A, iron, thiamine and niacin & lesser percentage were deficient in calcium and vitamin C.

Table1 shows that BMI is significantly correlated to animal protein, animal fat in boys and girls (P = 0.00 & 0.01; P = 0.00 & 0.00 respectively). No statistically significant difference in caloric intake between normal weight and overweight-obese adolescent.

Table 2 shows that iron is negatively correlated with BMI in girls and boys (P = 0.01); vitamin A is positively correlated to BMI, and vitamin C is negatively correlated to obesity and overweight. Comparing dietary intake in girls to BMI, it was found that plant protein and vitamin C are positively correlated to obesity and overweight (P = 03.01 & 0.04 respectively); while in boys vitamin C intake only shows a significant difference between normal weight and obese (P = 0.04).

Table 3 shows psychosomatic symptoms in correlation to sex difference, it shows that: a. boys have more concern about overeating than girls (P = 0.05) and girls are concerned more about their bulimic behavior (P = 0.01). b. as regard body image there is statistically significant difference between girls and boys as girls are more concerned about their shape, size and fear of gaining weight as well, concerned for losing weight (P = 0.001). c. Somatic symptoms in boys was more significant as regard swelling of foot & hand (P = 0.000) while girls have more stomach aches (P= 0.001). d. girls show more

statistically significant difference in binging than boys (P = 0.001).

Table 4 assesses psychosomatic symptoms according to weight status in girls and boys; in girls, body image (P = 0.000), bad eating habits (P = 0.00), depression (P = 0.01) and eating disorder are positively correlated to overweight and obesity; while in boys, depression (P = 0.00), somatic symptoms (p = 0.03) and eating disorder (P = 0.01) are the factors that were correlated to overweight and obesity.

Table 5 assesses psychosomatic symptoms in relation to eating disorder in girls and boys. Body image, bad eating habits, depression and somatic symptoms have statistically significant values (P = 0.00) in both boys and girls.

Table 6 shows that girls with eating disorder shows statistical negative correlation with calcium intake (P = 0.04).

Table 7 shows that boys with eating disorder show statistical significant positive relation to animal fat (P = 0.04); calcium (P = 0.001); vitamin A (P = 0.01) and vitamin C (P = 0.01).

Table 8 shows significant statistical correlation of social status and weight status to eating disorder in boys and girls (RR = 3.48, 95%, CI = 2.37- 5.1; RR= 3.44, 95% CI= 2.6-4.55, RR= 7.83, 95% CI= 5.76 – 10.64 & RR = 2.04, 95% CI= 1.7-2.45 respectively). Eating disorder increases with highest parental education & occupation and obesity.

Association between weight status and weight teasing wer found to be statistically significant among girls and boys; high percentage of obese girls (74.3%) reported being teased sometimes (P < 0.001); 22.97% of girls reported being teased by peers (P < 0.000); and (28.88%) of girls reported being teased by family member (P < 0.000). A significant percentage of obese boys (49.0%) reported being teased sometimes (P < 0.001); 56.0% of boys reported being teased by peer (P < 0.001) and 33% of boys reported being teased by family member. There was also, a statistically significant association between underweight and weight teasing (51.9%) & (37%) of girls and boys respectively, reported being teased ; 21% & 41% of girls and boys reported frequent teasing by peers and (27% and 14%) of girls and boys reported being teased by family member. Normal weight and lean boys are more likely to be teased about their weight by peers but not by family members (table, 9).

In analysis adjusted for socio demographic characteristics overweight and obese girls but not underweight girls were at greater risk for being teased for their weight, some observation was found in boys. In analysis adjusted for sociodemographic characteristics overweight and obese girls but not under weight girls were at risk for being teased for their weight (P = 0.05 & 0.00); also over weight and obese boys were at risk for being teased for their weight (P = 0.05 & 0.00) (table, 10).

Table 11 shows number and percentage of overweight girls and boys reporting eating disorder behavior. High percentage of over weight and obese girls and boys who were teased about their weight engaged in bad eating habits and binging behavior, as compared to overweight boys & girls who were not teased about their weight. Overweight youth who experienced weight teasing were at significantly greater risk for engaging in bad eating habits and binging; all associations were statistically significant except for the association between weight teasing by family members and binging among boys, this association was of marginal statistical significance.

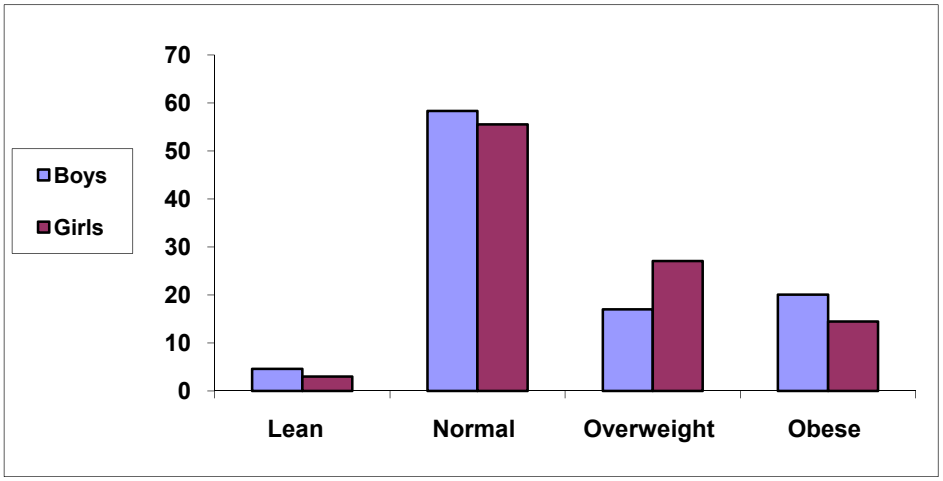


Fig. (1): Distribution of boys and girls according to weight status.

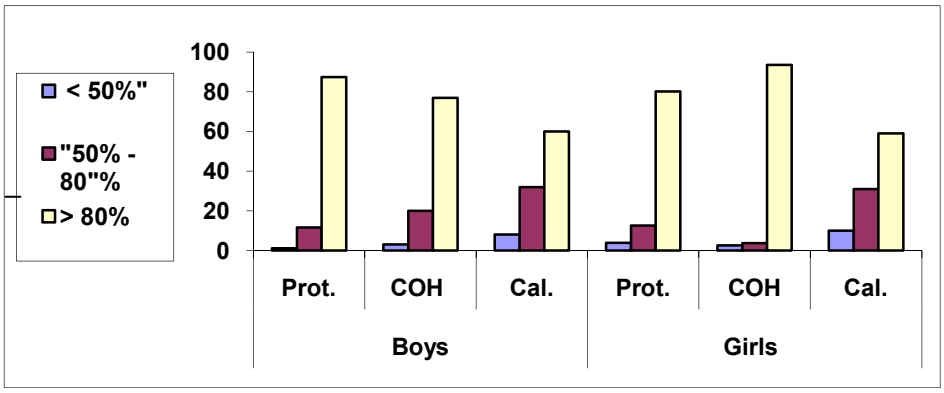
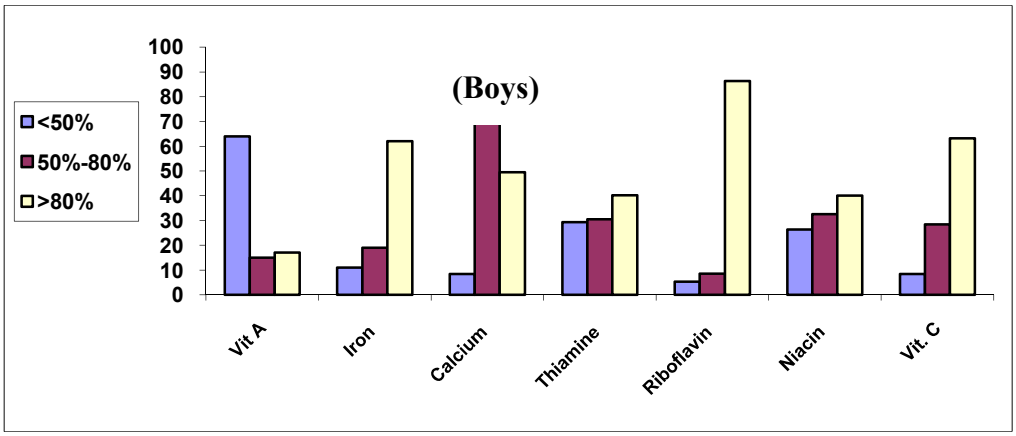


Fig. (2): Dietary intake of macronutrients of adolescent boys and girls according to RDA



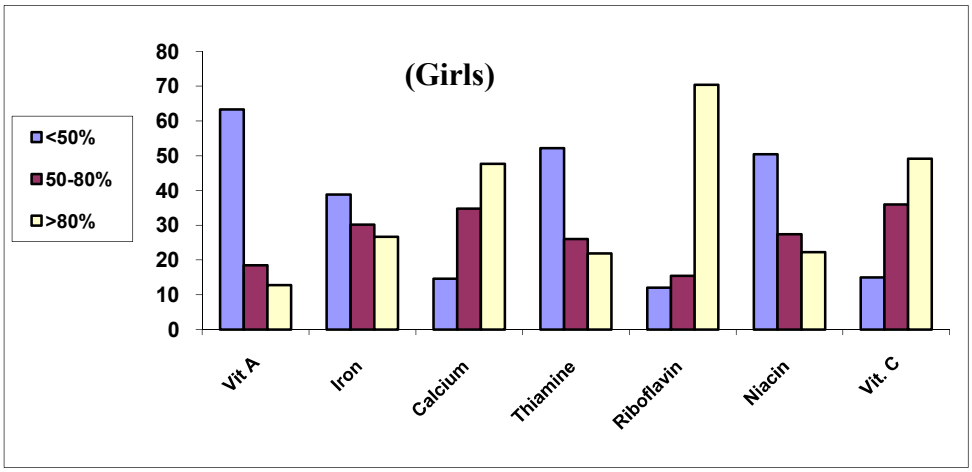


Fig. (3): Dietary intake of micro nutrient in adolescent boys and girls according to RDA

Table (1): BM1 in adolescent boys and girls in correlation to macronutrient intake.

	BMI	SEX	N	Mean	St. error of mean	t	Sig. (2- tailed)
Calories (cal./day)	Normal	M	52	1830.4	83.9	1.4	0.17
		F	155	1696.7	51.3		
	Over-obese	M	40	1776.4	97.4	0.5	0.63
		F	123	1721.2	57.6		
Protein animal (gm/day)	Normal	M	52	36.3	2.7	21.9	0.00
		F	155	27.8	1.5		
	Over-obese	M	40	34.5	3.1	2.5	0.01
		F	123	26.1	1.4		
Protein plant (gm/day)	Normal	M	52	32.8	2.1	1.2	0.24
		F	155	36.0	1.6		
	Over-obese	M	40	33.9	2.1	1.0	0.30
		F	123	31.1	1.5		
Fat animal (gm/day)	Normal	M	52	39.6	2.4	3.6	0.00
		F	155	29.1	1.6		
	Over-obese	M	40	37.8	3.1	2.7	0.00
		F	123	28.3	1.5		
Fat plant (gm/day)	Normal	M	52	31.5	2.5	-0.04	0.96
		F	155	31.6	2.0		
	Over-obese	M	40	29.6	3.4	-0.9	0.34
		F	123	33.8	3.0		
Carbohydrate (gm/day)	Normal	M	52	248.9	15.5	-0.3	0.73
		F	155	255.2	10.0		
	Over-obese	M	40	233.3	17.7	-1.3	0.20
		F	123	269.8	22.8		

Table (2): BM1 in adolescent boys and girls in correlation to micronutrient intake.

	BMI	SEX	N	Mean	St. error of mean	t	Sig. (2- tailed)
Iron (mg)	Normal	M	52	21.8	4.6	-10.1	0.01
		F	155	14.4	2.2		
	Over-obese	M	40	23.4	4.6	-11.8	0.01
		F	123	18.9	2.8		
Vit. A (*ug*)	Normal	M	52	515.1	70.9	4.8	0.000
		F	155	159.9	21.5		
	Over-obese	M	40	392.0	55.4	4.9	0.000
		F	123	107.0	17.5		
Thiamine (mg)	Normal	M	52	1.6	0.8	-0.8	0.40
		F	155	4.9	3.9		
	Over-obese	M	40	11.7	10.5	0.7	0.47
		F	123	3.8	2.7		
Riboflavin (mg)	Normal	M	52	2.1	0.2	2.6	0.01
		F	155	1.5	0.1		
	Over-obese	M	40	2.6	0.6	-0.8	0.55
		F	123	4.5	3.1		
Niacin (mg)	Normal	M	52	11.5	0.87	2.3	0.01
		F	155	8.9	0.60		
	Over-obese	M	40	11.9	1.1	4.3	0.00
		F	123	6.9	0.5		
Vit. C (mg)	Normal	M	52	61.0	12.5	-0.8	0.45
		F	155	71.8	6.8		
	Over-obese	M	40	28.8	9.1	-2.1	0.04
		F	123	52.4	6.4		

Table (3): Psychosomatic symptoms in to correlation to sex:

a. Depression

Question	Gender	No	Some times	Often	P
Nervous for polyphagia	Boy	68	19.5	12.7	0.4
	Girl	70	16.4	13.6	
No self confidence about food control	Boy	75	17.1	8	0.2
	Girl	79	14.04	6.8	
Nervous when over eat	Boy	80	14.4	5.3	0.1
	Girl	80	15.5	5	
Depressed for bulimic behavior	Boy	84	11.6	4.7	0
	Girl	79	11.9	9.1	

b. Body image

Question	Gender	No	Some times	Often	P
Concern about body shape	Boy	9.7	30.8	59.5	0
	Girl	6.3	20.2	63.4	
Worried for size	Boy	53	19.7	27.3	0
	Girl	41	16.6	42.8	
Fear of gaining weight	Boy	36	18.1	45.6	0
	Girl	23	16.3	60.6	
Unsatisfied about shape & size	Boy	42	24.2	33.9	0
	Girl	35	30.6	34.6	
Concerned for losing weight	Boy	42	28.5	29.7	0
	Girl	28	31.8	39.9	

c. Somatic symptoms

Question	Gender	No	Some times	Often	P
Suffer swelling of foot & hand	Boy	85	8.5	6.3	0
	Girl	91	3.9	4.7	
Stomach aches	Boy	52	32.7	15	0
	Girl	41	36.7	22.3	
Menstrual problems (girls)	Boy	-	-	-	
	Girl	53	23	23.8	

d. Binging

	Gender	No	Some times	Often	P
Eat huge amount every time	Boy	46	42	12	0
	Girl	63	32	5	
Swallow food without mastication	Boy	67	22	11	0
	Girl	74	18	8	
Don't enjoy binging	Boy	42	20	38	0.5
	Girl	41	18	41	
Feel nausea	Boy	69	20	11	0
	Girl	65	19	16	

Table (4): Psychosomatic symptoms by weight status in girls & boys

	Girls				Boys		
	BMI	Mean	t	P	Mean	t	P
Body image	Normal	62.7	20.3	0.000	53.2	1.5	0.22
	Over-obese	127.5			45.8		
Bad eat habits	Normal	120.8	12.7	0.005	53.8	2.3	0.13
	Over-obese	143.8			44.8		
Binging	Normal	107.3	4.6	0.20	53.2	1.5	0.22
	Over-obese	138.8			45.8		
Depression	Normal	105.4	10.2	0.01	54.9	3.4	0.00
	Over-obese	147.2			42.0		
Somatic symptoms	Normal	105.7	3.2	0.36	54.9	4.5	0.03
	Over-obese	140.4			43.0		
Eat disorder	Normal	74.3	6.7	0.03	64.1	4.1	0.01
	Over-obese	86.5			75.4		

Table (5): Psychosomatic symptoms by eating disorder score (ED) in girls and boys.

	Girls				Boys		
	ED	**Mean rank**	**M. Wh S***	**P**	**Mean rank**	**M. Wh S***	**P**
Body image	< 20	88.4	2669.0	0.000	29.70	339.0	0.002
	≥ 20	148.9			54.5		
Bad eat habits	< 20	92.7	2834.5	0.000	33.2	395.0	0.008
	≥ 20	148.2			53.8		
Binging	< 20	131.5	4350.5	0.45	37.8	438.0	0.050
	≥ 20	141.9			53.0		
Depression	< 20	97.2	3011.5	0.000	28.1	314.0	0.000
	≥ 20	147.5			54.8		
Somatic symptoms	< 20	109.2	3480.0	0.008	34.0	407.0	0.000
	≥ 20	145.6			53.7		

*=Mannwhitney score

Table (6): Dietary intake in girls by eating disorder.

	ED	**N**	**Mean**	**St. error of mean**	**t**	**Sig. (2 tailed)**
Calories	male	54	1674.57	80.29	-0.944	0.348
	female	315	1757.48	35.67		
Protein	male	54	28.38	2.57	-0.491	0.625
Animal	female	315	29.74	1.02		
Protein	male	54	32.31	1.88	-0.486	0.628
Plant	female	315	33.34	0.95		
Fat	male	54	31.51	2.56	0.097	0.923
Animal	female	315	31.79	1.08		
Fat	male	54	38.79	4.76	1.350	0.182
Plant	female	315	32.06	1.48		
Carbohydrates	male	54	245.59	16.26	-0.184	0.854
	female	315	248.82	6.50		
Calcium	male	54	420.22	34.36	-2.066	0.042
	female	315	501.98	15.38		
Iron	male	54	15.51	2.80	-0.293	0.770
	female	315	16.40	1.21		
Vitamin A	male	54	330.33	34.17	-0.767	0.445
	female	315	360.88	20.42		
Thiamine	male	54	1.82	0.60	-1.330	0.184
	female	315	5.32	0.25		
Riboflavin	male	54	1.45	0.10	-1.212	0.226
	female	315	2.93	1.20		
Niacin	male	54	8.80	0.76	-0.224	0.824
	female	315	8.99	0.39		
Vitamin C	male	54	48.00	12.83	-0.934	0.361
	female	315	60.73	4.54		

Table (7): Dietary intake in boys by eating disorder.

	ED	N	Mean	St. error of mean	Sig. (2 tailed)
Calories (cal/day)	≤ 20	15	1686.00	167.91	0.242
	> 20	81	1905.75	69.82	
Protein (gm)	≤ 20	14	35.50	6.61	0.907
Animal (gm)	> 20	81	36.32	2.03	
Protein (gm)	≤ 20	15	30.86	3.10	0.208
Plant (gm)	> 20	81	35.52	1.82	
Fat	≤ 20	14	32.14	3.53	0.041
Animal (gm)	> 20	80	41.01	2.04	
Fat	≤ 20	15	28.13	4.66	0.611
Plant (gm)	> 20	81	30.77	2.09	
Carbohydrates (gm)	≤ 20	15	217.53	23.26	0.195
	> 20	81	252.78	12.48	
Calcium (mg)	≤ 20	14	321.28	58.08	0.001
	> 20	81	275.34	35.32	
Iron (mg)	≤ 20	15	24.64	9.52	0.739
	> 20	81	21.28	2.77	
Vit. A (*ug)*	≤ 20	15	297.26	55.62	0.010
	> 20	81	504.21	52.23	
Thiamine (mg)	≤ 20	15	3.47	2.05	0.617
	> 20	81	6.28	5.20	
Riboflavin (mg)	≤ 20	15	1.80	0.24	0.091
	> 20	81	2.5	0.33	
Niacin (mg)	≤ 20	15	10.14	1.44	0.160
	> 20	80	12.53	0.79	
Vit. C (mg)	≤ 20	6	19.18	9.79	0.018
	> 20	36	56.03	10.24	

Table (8): Correlations of social status and BMI to eating attitude in boys and girls.

Eating disorder	Boys		Girls		Boys		Girls	
	High social	Low social	High social	Low social	High social	Low social	High social	Low social
> 20	95	28	198	46	83	40	98	146
< 20	143	216	159	239	18	341	61	337
	RR = 3.48 95%CI = 2.37 - 5.1		RR = 3.44 95%CI = 2.6 – 4.55		RR = 7.83 95%CI = 5.76 – 10.64		RR = 2.04 95% CI = 1.7 – 2.45	

* 25.5% of boys have ED > 20, 36.6% of girls have ED < 20.

Table (9): Number and percentage of adolescent girls and boys who report sometimes weight teasing, weight teasing by peers and weight teasing by family across weight status.

	Teasing sometimes		Weight-teasing by peers		Weight teasing by family	
	n	%	n	%	n	%
Girls	27	51.9	11	21.15	14	26.92
Lean (n = 52)	84	43.07	19	9.74	24	12.31
Normal (n = 195)	66	48.17	9	6.57	15	10.95
Overweight (n = 74)	55	74.3	17	22.97	21	28.88
Obese (n = 74)	$X^2 = 40.5$ P < 0.001		$X^2 = 23.47$ P < 0.001		$X^2 = 26.78$ P < 0.001	
Boys						
Lean (n = 22)	8	37.0	9	41.0	3	14
Normal (n = 281)	37	3.0	40	14.0	28	10

Overweight (n = 82)	18	22.0	20	26.0	14	17
Obese (n = 97)	47	9.0	57	56.0	32	33
	$X^2 = 44.5$ P < 0.001		$X^2 = 28.36$ P < 0.001		$X^2 = 25.77$ P < 0.001	

Table (10): Weight teasing among adolescent girls & boys by weight status odds ratio (OR) and 95% confidence interval (CI)[a,b]

Weight status [c]	Weight teasing			
	OR	CI	X^2	P
Girls				
Lean (n = 52)	2.977	1.47-6.01	0.647	0.42
Overweight (n = 74)	1.46	0.93-2.27	2.78	0.09
Obese (n = 74)	3.51	0.93-6.6	16.21	0.0001
Boys				
Lean (n = 22)	3.85	2.48-5.21	0.544	0.35
Overweight (n = 82)	2.06	0.93-2.84	2.85	0.05
Obese (n = 97)	4.51	0.95-8.07	15.51	0.001

a) Adjusted for SE and school level. b) Odds ratios significant (P < 0.05 when 1 is not included in the 95% CI).
c) Reference group adolescent girls and boys of average weight (BMI 15th – 85th percentile).

Table (11): Number and percentage of overweight girls and boys reporting eating disorder behavior by perceived weight leasing.

	Bad eating habits				Binge eating			
	Total No.	n[a]	%	P	Total No.	n[b]	%	P
Girls								
Frequent weight teasing								
Yes	105	89	78	0.001	73	22	30	0.0001
No	140	39	67		138	21	15	
Weight teasing by peers								
Yes	95	74	77.5	0.001	90	25	28	0.001
No	123	83	67.5		120	17	14	
Weight teasing by family								
Yes	65	54	82	0.001	83	52	27	0.001
No	132	85	64.4		130	22	16.5	
Boys								
Frequent weight teasing								
Yes	77	48	62	0.0001	76	14	18.2	0.001
No	131	56	43		130	10	7.8	
Weight teasing by peers								
Yes	94	56	60	0.0001	90	13	14	0.05
No	119	50	42		115	9	8	
Weight teasing by family								
Yes	54	35	64.5	0.01	53	33	15	0.09
No	155	71	45.5		153	14	9	

a. number reporting bad eating habits. b. number reporting binge eating.

4. Discussion:

Based an BMI cut-off points 17% & 27% of boys and girls were overweight and (20% & 14.5%) of boys and girls were obese: comparison of cross sectional data from United States and 13 European countries has shown that the prevalence of overweight varied between 5.2% - 28.9% for boys and 8.1% - 31.0% for girls; the prevalence of obesity varied between 1.9% - 13.9% for boys and 1.1-15.1% for girls among adolescents (Lissau, et al., 2004). The prevalence of overweight and obesity for Iranian has been found to be 12.1% and 7.8%

(Mohamed et al., 2004), and among adolescent Mexican 12.1% & 6.2%. In other Egyptian data the prevalence was 15.9% and 18.4%; lower than our results (Martinez et al., 2006).

Prevalence of over weight and obesity was found to be higher in boys than girls in Swedish adolescents (Berg et al., 2001) unlike our results and other Egyptian results (Martinez et al., 2006) the prevalence of over weight among girls was found to be higher than boys, in the same time the prevalence of obesity in our cases was found to be higher in boys (20%) than girls 14.5%. We found that percentage of obesity in girls increases with age while it decreases in boys with age. On the contrary Klein et al., (2008), reported that adolescents who perceived themselves to be overweight at baseline were 2-3 times more likely to be overweight at follow up compared to those with a normal weight. Boys were more likely (3 times) to transition to obesity at follow up compared with girls.

In our study according to weight status there was significantly statistical correlation of BMI status in girls to body image, bad eating habits , depression and eating disorder , whereas in boys weight status was significantly correlated to depression , somatic symptoms and eating disorder . French et al. (1995) found and inverse relationship between BMI and both self esteem and body image, also found that the most common consequences of obesity in children is poor psychological & social functioning, impaired academic success and reduced fitness & heath.

It was found that adolescents from more advantaged family backgrounds are more likely to be overweight with higher percentage of eating disorder (RR = 3.48, 95% Cl= 2.37-5.1 for boys and RR = 3.44, 95% Cl = 2.6 – 4.55 in girls & RR = 7.83, as Cl = 5.76 – 10.64 for boys & RR = 2.04, 95% Cl = 1.7 – 45 for girls) respectively; same results were found in Mexican adolescent (Martinez et al., 2006) whereas in USA adolescents from high socioeconomic level are less likely to be over weight (Needlham & Crosnoe, 2005) . Added to this, Ozmen, (2007) concluded that being male and being of higher socio economic level were predicators of obesity overweight based on BMI.

In this study, we used 24-hr dietary recall method as screening tool to throw the light on dietary pattern of Egyptian adolescents. Epidemiological studies on dietary pattern of Egyptian adolescents are scarce (Hassanyn, 2000).

Dietary assessment showed that energy consumption was inadequate among 40% of adolescent, close to 13% had low protein intake & 23% had low intake of carbohydrates. Data derived from several national surveys conducted by national nutrition institute in Egypt revealed, that Egyptian adolescents do not fulfill the recommendations of WHO for healthy diet, as only, 38% obtain their full requirement of energy (100 – 120% RDA) (Hassanyn, 2000) in our study 30% of boys & 28% of girls obtain their full requirements of energy. In our study animal protein ratio to plant protein was found to be (1.06 – 1 & 1 – 1.24 in boys & girls respectively) while in Hassanyn's study they found the total ratio to be 1-2. this reflects a problem of concern related to the low biological value of consumed protein on one hand and the low bioavailability of micronutrients mainly mineral, aggravated by the low daily intake of these mineral particularly iron & zinc.

Although it is often assumed that overweight children eat more than non-overweight children do, no data have been published to support this belief. In our study we found no significant difference in caloric intake between normal and overweight obese boys and girls. Rocandio et al., (2001) found that the percentage of energy intake was significantly lower in the overweight group compared to the non overweight children (8948 as 9590 K/day; $P < 0.01$) and carbohydrate intake was significantly greater in the non-overweight school children (250.9 + 58.8 us 221.1 + 77.4 g/day; $P < 0.01$) similar results were obtained by Bandini et al., (1999).

These findings suggested that the positive energy balance causing overweight and obesity is rather due to low energy out put due to lack of physical activity and the sedentary life adolescents are obliged to practice especially those running for high school certificate; where mostly parents stop all activities other than learning.

There was a significant difference in animal protein in boys and girls in both normal and overweight obese, where boys eat more animal protein than girls ($P < 0.001$), the same results were noticed in animal fat intake, this can be explained by the still existing cultural preference of males over females. Some studies found no differences in dietary protein and fat intakes between over weight and obese and normal weight (Ricardio et al., 2001) other studies noted that the proportion of fat in the diet was greater in obese children (Gazzaniga, and Bruns, 1993).

As regard micronutrients intake, girls have deficiency in vitamin A, iron, calcium, riboflavin, thiamine, niacin and vitamin C ; while boys have deficiency in vitamin A, thiamine, riboflavin, niacin and vitamin C .There were statistically significant difference in Iron, vitamin A & niacin intake between boys & girls normal or overweight-obese; also there was a significant difference in riboflavin intake in normal weight boys & girls, as girls have higher intake of vitamin C than overweight-obese boys.

Rocandio et al., (2001), found that vitamin A & vitamin D intakes were lower than recommended but in the contrary he found no significant difference between overweight and non-overweight. Ferandez et al.,(1996) and Failde et al.,(1997), found similar results. Szabo et al (2007), found that the average energy intake was appropriate, protein and fat intake was some what higher than the RDA; the intake of Ca and vitamin D was inadequate, Iron in girls was insufficient. In this study, 25.5% of adolescent boys have been found to have eating disorder (> 20) & 38.6% of girls were found to have scores > 20 as well, that was positively correlated to social status as it was significantly higher among high social standards as well as among those with higher BMI. Austin et al., (2008) in his study for screening eating disorder at high school's in USA observed less percentages than our results whereas 25% of girls & 11% of boys reported disordered eating (> 20); Sepulveda et al., (2008) in his study of eating disorder among Spanish university students reported the prevalence rate of students at high risk for eating disorder to be 14.9% (11.6 – 18) for males and 20.8% (181.7 – 22.8) for females (ED > 20) with statistically significant differences by gender.

Among adolescent girls, depressive symptoms for their bulimic behavior were significantly higher than boys, while boys were more concerned than girls about their overeating and binging most of the time, girls were more concerned about body image than boys; whereas boys have more physical complains than girls ; girls practice binging more than boys.

Field et al. (2008) found that among children (9-15 years old) girls show higher percentage of binging than boys (4.3% and 2.3%) as well as purging (5.3% & 0.8% respectively). On the contrary Sepulveda et al., (2008) found that males (11.6%) practice binging more than females who practice unhealthy weight control behaviors more than males (dieting, laxatives or self induced vomiting). Crosby et al., (2009) reported that depression is intimately tied to bulimic behavior and may in fact precipitate such behavior and that treatment of depressive symptoms can produce longitudinal decrease in bulimic symptoms.

According to BMI we found a significant statistical correlation of weight status in girls to body image, bad eating habits, depression, eating disorder ; whereas weight status in boys was significantly correlated to depression , somatic symptoms and eating disorder . Also, we found in our study that eating disorder (> 20) in girls was highly correlated to negative body image, bad eating habits, depression and somatic symptoms. In boys it was highly correlated to body image, bad eating habits, binging, depression and somatic symptoms. Some results were found by Costa et al., (2009) where he found that BMI and depressive symptoms for both sexes were positively associated with eating disorder symptoms. On opposition to our results; as we found that eating disorder in both sexes is higher in adolescents from higher socioeconomic level; Cost et al., (2009) found a sex effect on the association between socioeconomic status and eating disorder as girls with higher socioeconomic status and boys with lower socioeconomic status presented more eating disorder symptomatology. Suris et al., (2008) found that health risk behaviors in adolescents with chronic diseases are more than normal adolescents. Mahraj et al., (2009) stated that overweight and obesity are among the risk factors of eating disorder (11 & 7% respectively) and that many of the risk behaviors in adolescents were shown to be related to the adolescents family origin, home environment and parent child relationship as well the protective effect of the family and school connectedness and increased religiosity. Moreover Sampei et al., (2009) found that among premenarch group there is increase eating score (EAT-26) above 20 with a high correlation to body image that means that the concerns about body image develop at an earlier age.

Other studies (Mirza et al., 2005), Labera et al., (2009) and Calderon et al., (2009) stated that body weight status especially severe obesity is correlated to more concern about body image, in the same time adolescents with normal weight showed specific factor for developing eating disorders in the future (Calderon, et al., 2009). It has been suggested that body image may predict depression (Stice et al., 2000) and eating disordered symptoms that may lead to an increased prevalence of adverse psychological and health concern (Mirza, 2005).

As regard weight teasing, our results clearly showed that perceived weight teasing by both peers and family members is common among adolescents' girls who were teased more than boys; that may be due to extra sensitivity of girls towards their weight; this is evident in non-overweight girls who reported being teased for their weight more than boys. In analysis adjusted for sciodemographic characteristics, overweight and obese but not under weight girls were at greater risk for being teased for their weight by their family member, similar associations were found among boys, as they were mostly likely to be teased by family members and peers; in comparison normal weight boys and lean were more likely to be teased about their weight by peers but not by family member. Our results agree with Neumark et al., (2002) results who assessed the prevalence of weight teasing among adolescents as well, Sobal et al., (1995) who found that overweight students,

especially girls, were stigmatized regarding dating activities, added to these Jackson et al., (2000) found that obese women reported more weight and size teasing than non obese women.

We found strong associations between weight teasing and disordered eating behaviors, boys and girls who were bothered by being teased were significantly more likely to engage in these behaviors, our findings regarding association between weight teasing and unhealthy weight control/ binge eating behavior are consistent with findings from previous studies (Neumark et al., 2002, and Brown et al., 1989).

In this study, The nature of the study population (population based sample), allowed for more generalization than that of the clinical samples, added to this, collection of the anthropometric measurements was done more accurate than other studies that depend on collecting the self-reported measures. Also, its inclusion of a big number of boys and girls, and the trial to assess all the risk factors of eating disorders as well their dietary intake and the inclusion of teasing to body weight as another risk factor that may aggravate eating disorder, encourages us to assess bulimia and anorexia on those with high eating disorders scores ,that will be published later.

Early identification and treatment of disordered eating by weight control behaviors may prevent progression and reduce the risk of chronic health consequences, physician should have a high index of suspicion for eating disorders in adolescents by using the DSM-PC (Diagnostic and statistical manual for primary care); paying attention to risk factors (Musie, et al., 2003). Programs aimed at prevention and reduction of overweight and use of family-based treatment may result in a decrease in body dissatisfaction and increase in self-esteem, in turn, improving body image and self esteem may help with efforts to decrease obesity among high-risk youth population. Furthermore, school based educational interventions to learn the students about the balanced healthy food and the etiology of obesity as well as, the potential harmful effects of weight teasing and to improve the general attitude towards different body sizes and shapes. Added to this it is important for the community to change the criteria of evaluating a person only for his achievement disregarding the value of shape and size.

Acknowledgment:

I would like to thank Mrs. Wafeya Abdel Rahman for dietary intake collection and analysis.

Corresponding author
Zeinab M. Monir
Child Health Department,National Research Centre, Dokki, Giza, Egypt
zeinab_monir@yahoo.com

5. References:

1. Abdel Kalek, A. M., (1993): The construction and validation of the Arabic Children's Depressive Inventory (ACDI). Europ. J. Psychol. Asses.; 9 (1); 41-50.
2. Austin, S. B., Zigadeh, N. J., formans, S., Porkop, L. A., Keliher, A., Jacobs, D; (2008): Screening high school students for eating disorders: results of a nation wide initiative. Prev. Chronic. Dis.; 5 (4): A114. E pub. 2008 Sep. 15.
3. Berg, I. M., Simonsson, B., Branlefors B., Ringqvist, I, (2001): Prevalence of overweight and obesity in children and adolescents in a county in Sweden. Acta Pediatr., 90: 671-676.
4. Biró, L., Merei, A. R., Nagg, K., Pétez Z, Arató, G., Szabó, C., Matos, E., Antal, M., (2007): Dietary habits of school children: Representative Survey in Metropolitan Elementary Schools- Part Two. Ann Nutr. Metab., 51: 454-460.
5. Brown, T. A., Cash, T. F., Lewis K. J., (1989): Body image disturbance in adolescent female binge-purgers: a brief report of the results of a national survey in the USA. J. Child. Psychol. Psychiatr.; 30: 605-613.
6. Calderôn, C., Forns, M., Vareat V., (2009): Adolescent obesity, anxiety, congnitive and behavioral symptoms characteristic of eating disorders. An. Pediatr. (Barc); 71 (6): 489-494. Epub 2009 Oct. 7.
7. Costa, C., Ramos, E., Severo, M., Barros, H., and Lopes, C., (2008): Determinants of eating disorders symptomatology in portuguese adolescents. Arch. Pediatr. Adolesc. Med.; 162 (12): 1126-1132.
8. Crosby, R. D., Wonderlich, S.A., Engel, S. G., Simonich, H, Smyth. J. and Michell, J. E. (2009): Daily Mood Patterns and Bulinic behaviors in the natural environment. Behav. Res. Ther.; 47 (3): 181-188.
9. Failde, I., Zafra, J. A., Ruiz, E., Novalbos, J.P. (1997): Evaluation of nutrition of school children in the population of sierra de cadiz (Ubrique). Med. Clin. Barc.; 108: 254-258.
10. Field, A. E., Javaras, K. M., Aneja, P. Kitos, N., Camargo, C. A., Taylor, C. B., Laird, N. M., (2008): Family, Peer and media predictors of becoming eating disordered. Arch. Pediatr. Adolesc. Med.; 162 (6); 574-579.
11. Frenandez, T. Lôpezdel Val., T., Martinez, P., Jaunsolo, J., De Cos, A. Cilleruelo, M.L., et al., (1996): Consumo de alimentosy nutrients por edadesy sexo en escolares de la communidad

Autionoma de Madrid (CAENPE). Rev. Cin. Esp.; 196: 501-508.
12. French, S. A., Story, M. and Perry, C. L., (1995): Self-esteem and obesity in children and adolescents. A Literature review. Obes. Res., 3: 479-490.
13. Gazzanika JM., Bruns, T. L., (1993): Relationship between diet composition and body fatness, with adjustment for resting energy expenditure and physical activity in preadolescent children. Am. J. Clin. Nutr.; 58: 21-28.
14. Hassanyn, S. A., (2000): Food consumption pattern and nutrient intake among different population groups in Egypt. Final Report. (Part I). Cairo, NINI, WHO/EMRO.
15. Jackson, T. D., Grilo, C. M., and Masheb, R. M., (2000): Teasing history, Onset of obesity, Current Eating Disorder psychopathology, body dissatisfaction, and psychological functioning in binge eating disorder. Obes. Res.; 8 (6): 451-458.
16. Kaur, S., Kapil, V., Singh, P., (2005): Pattern of chronic diseases among adolescent obese children in developing countries. Current Science, 88 (7), 10.
17. Kazdin, A., Redgres, A., and Cobus, (1986): The hoplessness scale for children: psychometric characteristics and concurrent validity., J. Consult. & psychol., 54: 241-245.
18. Klein, E. G., Lytle, A., Chen, V., (2008): Scoial ecological predictors of the transition to overweight in youth. results from the Teens Eating for Energy and Nutrition at Schools (TEENS) study. J. Am. Diet. Assoc.; 108 (7): 1163-1169.
19. Kovacs, M., (1992): Children's Depressive Inventory (CDI), New York: MHS.
20. Labera, J. I., Canadu, R. J., Rios, B. P., Berriatúa, M. C., Jaramillo, D. R., Conzaloz, M. M., Millian, M. M. et al., (2009): Eating behavior and body image in a sample of adolescents from Sevilla. Nutr. Hosp.; 24 (5): 568-573.
21. Lissau, I, Overpeck, M. D., Raun, W. J., Due, P., Holstein, B. E. and Hediger, M. L., (2004): Body mass index and overweight in 13 European countries, Israel, and United Status. Arch Pediatr. Adolesc. Med., 158: 27: 33.
22. Mahraj, R. G., Nunes, P., and Renwick, S., (2009): Health risk behaviors among adolescents in the English Speeking Caribbean: a review. Child Adolesc. Psychiatry. Ment. Health, 3 (1): 10.
23. Marshall, L., (1998): Eating disorders, In Reb. Boyd, M. A. & Nihart, M. A.: Psychiatric nursing, 1st ed., Lippincott company, Ch., 21; 667-706.
24. Mirza, N. M., Davis, D., Yanovski, J. A. (2005): Body dissatisfaction, self-esteem, and overweight among inner city Hispanic children and adolescents. J. Adolesc. Health; 36 (3): 267- e16-267-e20.
25. Mohamadpour, A. B., Rashidi, A., Karandish, M., Eshrangian, M. R., Kalantari, N., (2004): Prevalance of overweight and obesity in adolescent Tahrani students, 2000-2001: an epidemic health problem. Public Health Nutr., 7: 645-648.
26. Moore, D. C., (1988): Body image and eating behavior in adolescent girls, Am J. Dis. Child.; 142: 1114- 1118.
27. Musie, A. M., Stein, D. G., and Arbes, G., (2003): Eating Disorders in adolescent Boys: A review of the adolescent and young adult literature. Adolese. Health J.; 33: 427-435.
28. National Center for health statistics (NCHS) in collaboration with National Centre For Chronic Disease Prevention and Health promotion (CDC), 2000: Body mass index for health percentiles.
29. National Research Council. Recommend dietary allowances, 10th ed. National Academy Press: Washington, DC; 1989.
30. Needlham, B. L., Crosnoe, R. C., (2005): Overweight status and depressive symptoms during adolescence. J. Adolesc. Health, 36: 48-55.
31. Neumark, S. D., Falkner, N., Story. M., Perry, C., Hannan, P.J. and Mulert S, (2002): Weight-teasing among adolescents: correlation with weight status and disordered eating behaviors. Inter. J. Obesity; 26, 123-131.
32. Ozmen, O., Ozmen E., Ergin, O., Cakmakci, T., Sen. N., Dundar, P. E. and Taskin E. O., (2007): The association of self-esteem, depression and body satisfaction with obesity among Turkish adolescents. BMC Public. Health, 7: 80.
33. Rocandio, A. M., Ansotegui, L. and Arroyo, M. (2001): Comparison of dietary intake among overweight and non-overweight school children. Inter. J. Obese., 23, 1651-1655.
34. Martinez-Salazar, E., Allen, E., Fernandez-Ortega, C., Torres-Mejia, G., Golal O, Lazcano-Ponce, E., (2006): Overweight and obesity status among adolescents from Mexico and Egypt. Arch. Med. Res., 37: 535-542.
35. Sampei, M. A., Sigulem, D. M., Novo, N. F., Julianoy., Coluganti, F. A. (2009): Eating attitude and body image in ethnic Japanese and cucasian adolescent girls in the city of Sao Paulo, Brazil. J. Pediatr. (Rioj.); 85 (2): 122-128.
36. Sepulveda, A. R., Carrobles, J. A., and Ganarillas, A. M., (2008): Gender, School and Academic Differences among Spanish University Students at high risk for developing an eating disorder: an epidemiology study. BMC Public Health; 8: 102.

37. Shaheen, F. M., Hathout, M., and I. Tawfik, A. A., (2004): National Survey of Obesity in Egypt. Final Report. Cairo, NINI.
38. Shoukier, Z. M., (2002): Eating disorders test, 2nd ed., El Nahda El Masryia bookshop, Adly. St., Cairo (In Arabic).
39. Sobal, J., Nicolopoulos, V., Lee, J., (1995): Attitudes about over weight and dating among secondary students. Int. J. Obese Relat. Metab. Disord.; 19: 376-381.
40. Suris, JC, Michaud, P. A., Akre, C., Sawyer, S. M. (2008): Health risk behaviors in adolescents with chronic conditions. Pediatr.; 1222 (5): e1113-1118.
41. WHO (2005): Preventing Chronic Diseases: A vital investment, Geneva. World Health Organization.
42. WHO, (2003): WHO Diet, Nutrition and prevention of chronic diseases. Report of a joint WHO/ FAO Expert consultation. WHO technical report series 916. Geneva, World Health Organization.

10/25/2010

Raising Awareness of Deaf Students and their School Care-Givers about First Aid Intervention in Medical Emergencies

Rabab El-Sayed Hassan[*1] and Samar Elhoseiny Abd-Elraouf[2]

[1]Pediatric Nursing, [2]Community Health Nursing, Faculty of Nursing, Mansoura University. Mansoura, Egypt
[*]biboelsayed@yahoo.com

Abstract: Objectives: To assess and raise the awareness of deaf students and their school care-givers about first aid intervention in medical emergencies. Participants: All deaf students in both the preparatory and secondary levels of education in a school for the deaf, who were under 18 years old (n = 44), in addition to school dormitories care-givers (n = 2) were participated in this study. Research Hypothesis: An implementation of first aid intervention program would have a positive outcome on raising awareness of deaf students and their school care-givers in medical emergencies. Methods: Participants' knowledge and skills were assessed using pre and post test questionnaire sheet contained thirty seven quiz multiple choices statement questions in Arabic language. Moreover, post-test intervention didactic and practical learning sessions consisted of six video films on DVD-ROM are presented to the participants accompanied by sign language translation in order to achieve the research objectives. Results: An intervention program showed a clear positive outcome on raising awareness of deaf students and their school care-givers about first aid intervention in medical emergencies. The highest percentage of deaf students (61.4%) obtained the lowest sum score lies between zero to less than 25% in the pre-intervention phase, while about half of them (45.5%) obtained sum score lies between 50 to less than 75%, and more than tenth (11.3%) obtained the highest sum score that lies between 75 to 100% in the post-intervention phase, which revealed statistical significant differences in the participants' knowledge of skills at p=0.001 and 0.000. Similarly, pre knowledge sum scores of the two school care-givers about first aid skills rose from 43.2% and 63.2% respectively reached to the mastery level of 100% in response to the study intervention programmed. Conclusion: Although not enough for all items to be statistically significant, first aid intervention program raised the awareness of deaf students and their school care-givers.

[Rabab El-Sayed Hassan and Samar Elhoseiny Abd-Elraouf. Raising Awareness of Deaf Students and their School Care-Givers about First Aid Intervention in Medical Emergencies. Journal of American Science 2010;6(12):1160-1168]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Deaf children; First aid; school-age; Care-givers.

1. Introduction:

Hearing disability has gained great interest in the last decades. This growing interest stemmed from its devastating effects on communication, person's life and the economics of countries (1). International statistics for children with hearing impairment are reported to be 2-6 per 1000 live birth (2). Hearing loss represents a significant public health problem especially when compared to other commonly known childhood diseases. In Egypt, profound hearing loss is shown to be 6-7 per 1000 child (3).

Emergency situations are challenging for everyone (4). When someone becomes ill or injured there is usually a short period of time before get a professional medical assistance. That length of time is most critical to the victim. What someone do, or don't do during that period of time can make the difference between life and death. By having some first aid training and knowing cardiopulmonary resuscitation (CPR) the trained person can have a major impact to the successful outcome of a medical emergency (5).

First aid is the initial assistance or care of a suddenly sick or injured person. It is the vital initial care we all feel an impulse to give as soon as possible after an accident or illness. So, first aid is an important part of everyday life, both at home, work or at play. Everyone should learn first aid and be willing to administer basic care until emergency assistance arrives. Not every incident requiring first aid is a life-and-death situation. First aid knowledge is commonly used to manage minor injuries at home or work (10).

Children on the whole are more capable of learning first aid and applying that knowledge sensibly. Children usually do struggle to save others lives, but at least if they know the technique they can do something better. Inspite of there are special children out there for whom the knowledge may be inappropriate, but it is a minority (6). Inclusive first aid programme helps people with disabilities play a full role in learning life-saving skills. It is a unique programme designed to work with groups or individuals who may require a more flexible approach to learning first aid skills (7). In deaf

children, a communication barrier can derail interactions between emergency responders and members of the deaf and hard of hearing community, making an already difficult situation unmanageable (4).

Personal safety skills for deaf children is a group work programme on DVD-ROM designed to help give deaf children and their caregivers the knowledge, awareness and skills they need to stay safe and make better informed life choices. Learning and practicing first aid saving skills isn't just about making someone a potential life-saver; it helps increase confidence, social skills, independence and the ability to react to a crisis (8). It also helps to strengthen the self-confidence and self-esteem of deaf children, enabling them to seek help and advice when they need it (7).

Inclusive first aid tips in the school place offers deaf students and their caregivers a chance which includes, but is not limited to, the assessment and interpretation of emergency situations and safe responses to those situations. Those students can learn the proper methods for the control of bleeding and shock, maintenance of airway, breathing and circulation, the care of burns, poisonings and other first aid techniques (8).

The loss of children's lives that results from injuries combined suggests a staggering numbers of productive lives lost to society (11). Therefore, first aid is immediate help provided to a sick or injured person and sometimes consists of procedures and techniques requiring minimal or no equipment. "Basic practical life skills will not only benefit the students but also the community at large, when such important life skills can go a long way in saving lives when needed (9).

Aims of the Study

The aims of this study were:

1-To assess deaf students and their school care-givers' awareness about providing first aid intervention in medical emergency situation.

2- To raise the awareness of deaf students and their school care-givers about providing first aid intervention in medical emergency situation.

Research Hypothesis:

It was hypothesized that:

An implementation of first aid intervention program would have a positive effect on raising awareness of deaf students and their school care-givers in medical emergencies.

Operational Definition

A care-giver is a mentally stable adult, typically over 18 years, who provides care for another(s). Child care-giver is a person who are responsible for providing direct care, protection and supervision of children in a child-care center, school for children with special needs or at home. This person is generally assigned to provide assistance to a child who is no longer able to perform the routine care or critical tasks necessary for every day survival.

2. Materials and methods

Methods

This study used a quasi experimental design.

Setting

The study was carried out at a school for the deaf students, at El-Mansoura City, in the period of the second semester of the study year 2009-2010, started from March 2nd to June 2nd, 2010.

Participants

All students inside the previously mentioned school for deaf children belonged to the preparatory phase (n=30) and those in the secondary phase (n=14) who were under 18 years old, in addition to the assigned care-givers (n=2) who stayed with at school dormitories deaf students after the end of school day in order to provide the required care and help for those children with special needs were included in the study sample.

Tools

Two tools were used to conduct the current study:

1. First Tool:

This tool was divided into 2 parts:

A) Part I:

Socio-demographic characteristics of deaf students and their families, including; age, gender, level of education, family resident, degree of deafness, place of accommodation, students' fathers and mothers educational levels, their occupation, and family history concerning the presence of deaf spouses. This part also includes the socio-demographic characteristics of the students' supervisors, such as, age, level of education, years of experience, marital status, and residency.

B) Part II:

Pre and post test questionnaire sheet in the form of 37 quiz multiple choices statement questions in Arabic language, constructed for both deaf students and their school care-givers in order to assess their awareness about providing first aid intervention in emergency situations. First aid statement questions were focused on two domains, one of them was concerned with the participants' knowledge; including emergency contact

information, basic information about first aid, vital organs information, diagnosis of hyperglycemia, intervene with poisoning, chocking, the presence of eye foreign body, epileptic fit, and an asthmatic child. The other domain was concerned with the participants' skills including; priorities of action when intervening with a casualty, and how to intervene as a first aider in different emergency situations such as; shock, burn, bleeding from cutting wound or in case of nosebleed, and musculoskeletal emergency.

2. Tool of intervention:

Post-test video films on DVD-ROM by the British Red Cross accompanied by sign language translation were used. They are specifically geared to helping the deaf and hearing impaired to learn how to follow the initial steps when intervening with a casualty**,** and the simple procedure of caring for casualties in different emergency situations, including: the steps of cardio-pulmonary resuscitation (CPR) for children, recovery position, and intervening with cutting wound, bleeding nose, burns and scalds, choking and twisting and fracture.

Intervention

1. Preparation phase

I- Administrative process

An official request to conduct the study was directed from Faculty of Nursing El-Mansoura University to the manager of Directorate of Education, Dakahlia Governorate. The approval is sent very soon to the headmaster of the school for deaf students, and the start permission is obtained.

II- Development of the study instrument

a. The quiz multiple choices questionnaire sheet was used as a pre and post test to assess both the students and their school care-givers' awareness about first aid.

Content Validity and reliability

Because there is no validation for any Arabic tool in Egypt, two bilingual lecturers belonged to the critical and pediatric nursing departments, faculty of nursing El-Mansoura University translated the tool's questions from English to Arabic language, and then this tool is back translated by another two lectures from the same departments. On conflict, the four juries met together to solve the arisen language incompatibility until the translated Arabic tool returned to its original English one. The recommended modifications that ensured tool clarity were done.

Feasibility study (pilot study) is conducted on five students chosen randomly, as student number Five from the classroom list, and those students are excluded from a full-scale study. The pilot study aimed to testing adequacy of research instruments; refine quiz multiple choices statement questions, assessing whether the research protocol is realistic and workable, and identifying logistical problems which might occur. The necessary modifications were done accordingly.

b. Training material in the form of sign language video films on DVD-ROM, laptop, data show apparatus and screen for presentation, and supplies and manikin for demonstration were prepared.

2. Implementation phase

a. The headmaster of the school for deaf students nominated an experienced teacher in sign language from the study setting itself. She accompanied the researchers as a translator throughout the study phases to facilitate and ensure complete and accurate transmission of any message to the participants. The researchers spent the first day with the translator to clarify the aim of the study, all the study phases, and to explain the content of the awareness sessions; until she understood and became well prepared for active participation in the study.
b. Ethical consideration: The two researchers introduced themselves through the translator to the participants (deaf students and their school care-givers). A simple explanation about the aims of the study was illustrated to them. A written consent notifying deaf student's agreement to share in this study signed by his/her direct care-givers is obtained. The researchers emphasized in the consent that the participation is voluntary, any participant can withdraw at any time without any need to justify his/her decision, any raised question will be answered, and the collected data will be treated confidentially and will be used only in the current study.
c. For the purpose of data collection, the researchers attended the school day and met the participants three days per week on Tuesday, Wednesday and Thursday.
d. Students of the preparatory school level are divided into six groups each group included five participants, while those of the secondary level are divided into two groups each one composed of seven participants, in addition to the two school care-givers.
e. Pre-test data collection period is started in March 3 and completed in March 17, 2010. Through this period, a group of 5-7 participants were assessed using questionnaire sheet.
f. Post-test intervention methods in the form of two presentation sessions (didactic and practical) for

each group are started from March 23 to April 15, 2010. In the first didactic session, the researchers with the translator presented some knowledge about the meaning and aim(s) of first aid, ambulance phone number, ambulance call cost, organ of breathing and that pumping blood, sites of pulse counting, the normal pulse rate among school age children, defining hypothermia, occurrence of brain damage due to asphyxia, how to diagnose hyperglycemia, and the required steps to provide first aid assistance in case of poisoning, foreign body aspiration, presence of foreign body in the eye and nose, epileptic fits and asthma. The researchers used blackboard to write the content of the session as well as a translation to sings language to ensure complete and accurate explanation. In the second practical session, each group watched six video films presented on DVD-ROM, that explained how to follow the initial steps when intervene with a casualty, and the simple procedure of caring for casualties in different emergency situations, including: the steps of cardio-pulmonary resuscitation (CPR) for children, recovery position, and intervening with cutting wounds, bleeding nose, burns and scalds, choking and twisting and fracture. After that, the participants are randomly selected to demonstrate on a manikin three procedures that were listed by order exactly as the scenarios presented in the films.

3. Evaluation phase

The post-test conducted after the completion of the presentation sessions from April 20th to June 2nd, 2010. Through this period, the participants were assessed using questionnaire sheet.

Statistical analyses

Data were analyzed using SPSS software version 14.0, and proper statistical tests were used accordingly. The obtained pre-test scores were compared to those of the post-test in order to detect the level of significance.

3. Results

Table (1) shows the total of 44 deaf students that constituted the study sample; 38.6% of them were males and (61.4%) were females. 68.2% of the sample were in the preparatory school level and (31.8%) were in the secondary level. 61.4% of students resident at urban areas, the degree of deafness among more than half (54.5%) was hard of hearing, and the majority (81.8%) were accommodate with their families.

Table (2) shows that less than half (47.7%) of deaf students' fathers and mothers were illiterate, only (11.3%) of the fathers and none of the mothers had bachelor degree. The occupation of more than half of student's fathers and mothers (59%) were as workers. Only (15.9%) of students had deaf relative(s), with (71.4%) of them had some form of hearing difficultly.

Table (3) shows that the highest percentage of deaf students (61.4%) obtained the lowest sum score that is lying between Zero to less than 25% in the pretest aimed to assess their knowledge of skills about first aid intervention in emergency situation. While, about half of the deaf students (45.5%) obtained sum score lies between 50% to less than 75% in the post-test, more than tenth of them obtained the highest sum score that lies between 75% to 100%.

Figure (1) shows that although the pre knowledge sum scores of the two school care-givers about first aid skills in emergency situations were (43.2%) and (63.2%) respectively, their post knowledge sum score were improved in to reach the mastery level of 100%.

Table (4) shows percentage distribution of deaf students' correct knowledge about first aid intervention in different emergency situations. The awareness of students' participants showed an improvement among all items of first aid, except for the average pulse of an adolescent in response to the study intervention program. Furthermore, statistically significant differences were detected in deaf students' awareness with ambulance call coast ($p=0.005$), and intervening with a poisoned casualty by drug misuse ($p=0.000$).

Table (5) shows percentage distribution of deaf students' correct practice related to providing first aid intervention in different emergency situations. Participants' skills showed an improvement among all items of first aids, with highly statistically significant differences detected among determining first aid priorities for a casualty in medical emergencies ($p=0.000$), intervening with cutting wounds ($p=0.005$), twisting and fractures ($p=0.000$), and a shocked casualty ($p=0.000$).

Table (1) Socio-Demographic Characteristics of Deaf Students (N =44)

Item	Number *(n)*	Percentage *(%)*
Gender		
Male	17	38.6
Female	27	61.4
Educational Level		
Preparatory	30	68.2
Secondary	14	31.8
Resident		
Urban	27	61.4
Rural	17	38.6
Degree of deafness		
Deafness	20	45.5
Hard of hearing	24	54.5
Accommodation		
With the family	36	81.8
Dormitories at school	8	18.2

Table (2) Socio-Demographic Characteristics of Deaf Students' Families (N = 44)

Item	Number *(n)*	Percentage *(%)*
Students' fathers educational levels		
Illiterate	21	47.7
Read & write	9	20.5
Technical secondary school	9	20.5
Bachelor Degree	5	11.3
Students' fathers occupation		
Worker	24	54.5
Technician	6	13.7
Employee	11	25
Retired	3	6.8
Students' mothers educational levels		
Illiterate	21	47.7
Read & write	15	34.1
Technical secondary school	8	18.2
Bachelor Degree	0	0.00
Student's mother occupation		
Housewife	9	20.5
Worker	26	59
Employee	9	20.5
Retired	0	0.00
Degree of relative's deafness		
Deafness	2	28.6
Hearing difficultly	5	71.4

Table (3) Knowledge of Skills of Deaf Students about First Aid Intervention, Pre vs. Post-intervention Scores

Knowledge of Skills Score	Pretest		Posttest			
	No.	(%)	No.	(%)	$\chi 2$	p-value
0% – less than 25%	27	61.4	0	0	**13.5**	**0.001***
25% – less than 50%	17	38.6	19	43.2	**18**	**0.000***
50% – less than 75%	0	0	20	45.5	**10**	**0.007**
75% – 100%	0	0	5	11.3	**2.5**	**0.3**

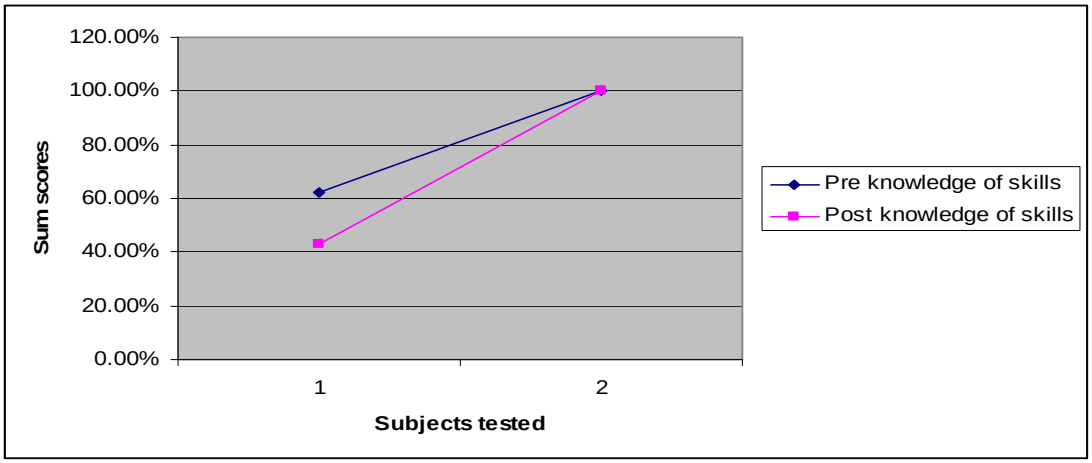


Figure (1) Knowledge of Skills of School Care-givers about First Aid Intervention, Pre vs. Post-Knowledge Sum Scores

Table (4) Number and Percent of Deaf Students' Correct Knowledge about First Aid Intervention, Pre vs. Post-intervention Phases

Statement/question	(n=44) Pre-test N (%)	(n=44) Post-test N (%)	Significance test
Participants' knowledge			
Q1: What number do you phone in case of medical emergency?	25 (56.8)	31 (70.5)	χ^2= 1.8 , **P**= 0.18
Q2: How much does it cost to call an ambulance?	20 (45.5)	33 (75.0)	χ^2= 8.0 , **P**= 0.005*
Q3: What is First Aid?	15 (34.1)	21 (47.7)	χ^2= 1.7 , **P**= 0.19
Q4: The aims of first aid are...	5 (11.4)	9 (20.5)	χ^2= 1.4 , **P**= 0.24
Q6: What organ do we breathe with?	19 (43.2)	23 (52.3)	χ^2= 0.7 , **P**=0.4
Q7: What organ pumps the blood?	7 (15.9)	15 (34.1)	χ^2= 3.9 , **P**= 0.05
Q8: For a casualty; a pulse is taken where on the body?	13 (29.5)	24 (54.5)	χ^2= 5.6 , **P**= 0.02
Q9: The average pulse of an adolescent is between:	13 (29.5)	5 (11.4)	χ^2= 4.5 , **P**= 0.03
Q10: Hypothermia develops when the body temperature drops below:	12 (27.3)	9 (20.5)	χ^2= 0.6 , **P**= 0.5
Q33: How long does it take for brain damage to occur due to lack of oxygen?	10 (22.7)	16 (36.4)	χ^2= 2.0 , **P**= 0.16
Q18: A marble is stuck up a child's nose. What do you do?	7 (15.9)	12 (27.3)	χ^2= 1.7 , **P**= 0.2
Q20: A foreign body embedded in the eye should be?	9 (20.5)	14 (31.8)	χ^2= 1.5 , **P**= 0.23
Q31: For an older child casualty that is unconscious through choking you should…	13 (29.5)	16 (36.4)	χ^2= 0.5 , **P**= 0.5
Q26: If a child is having an epileptic fit, you should...	14 (31.8)	23 (52.3)	χ^2= 3.8 , **P**= 0.05
Q27: When should you call an Ambulance when a casualty has taken an epileptic fit?	8 (18.2)	16 (36.4)	χ^2= 3.7 , **P**= 0.06
Q32: Asthma can be treated by:	11 (25.0)	20 (45.5)	χ^2= 4.0 , **P**= 0.05
Q35: Hyperglycaemia is caused when:	11 (25.0)	16 (36.4)	χ^2= 1.3 , **P**= 0.25
Q36: For a casualty that has been poisoned by drug misuse you should?	7 (15.9)	23 (52.3)	χ^2= 13.0 , **P**= 0.000*
Q37: First aid intervention in case of chemical poisoning, may include:	10 (22.7)	22 (50.0)	χ^2= 7.0 , **P**= 0.008

Table (5) Number and Percent of Correct Practice Related to First Aid Intervention, Pre vs. Post-test Phases

Statement/question	(n=44) Pre-test () N (%)	(n=44) Post-test N (%)	Significance test
Participants' skills			
Q11: What is the first thing you should do when you approach a casualty? Check…	17 (38.6)	21 (47.7)	χ^2= 0.7 , **P**= 0.39
Q5: The first aid priorities at an emergency are:	12 (27.3)	29 (65.9)	χ^2= 13.2 , **P**= 0.000*
Q12: How would you place an unconscious casualty who did not move or talk when touched or talked to?	3 (6.8)	12 (27.3)	χ^2= 6.5 , **P**= 0.01
Q34: If a person does not breathe through their mouth or nose, how else might the breath?	10 (22.7)	18 (40.9)	χ^2= 3.4 , **P**= 0.07
Q13: For a casualty with a superficial burn to the hand, you should**:**	17 (38.6)	24 (54.5)	χ^2= 2.2 , **P**= 0.14
Q14: A casualty with a full thickness burn of 2cm diameter, should:	8 (18.2)	15 (34.1)	χ^2= 2.9 , **P**= 0.09
Q15: How would you treat severe bleeding from a cutting wound?	7 (15.9)	12 (27.3)	χ^2= 1.7 , **P**= 0.2
Q16: When putting a dressing on a wound you should:	14 (31.8)	27 (61.4)	χ^2= 7.7 , **P**= 0.005*
Q17: If a piece of glass is embedded in a wound should you**:**	9 (20.5)	11 (25.0)	χ^2= 0.3 , **P**= 0.6
Q19: How would you treat a bleeding nose?	13 (29.5)	14 (31.8)	χ^2= 0.05 , **P**= 0.8
Q21: To treat a soft tissue injury you would follow which of the following sequences?	20 (45.5)	18 (40.9)	χ^2= 0.2 , **P**= 0.7
Q22: Where would you check the circulation after putting on a foot bandage?	10 (22.7)	18 (40.9)	χ^2= 3.4 , **P**= 0.07
Q23: An elevation sling should be used to treat a casualty who has**:**	8 (18.2)	26 (59.1)	χ^2= 15.5 , **P**= 0.000*
Q24: An elevation arm sling should be folded in ..…….. shape.	12 (27.3)	19 (43.2)	χ^2= 2.4 , **P**= 0.12
Q25: To treat a fractured leg you would....	1 (2.3)	17 (38.6)	χ^2= 17.9 , **P**= 0.000*
Q28: Circulatory shock is caused by...	11 (25.0)	21 (47.7)	χ^2= 4.9 , **P**= 0.03
Q29: You would treat shock by…	9 (20.5)	21 (47.7)	χ^2= 7.3 , **P**= 0.007
Q30: When a casualty is in shock you would use a blanket to**:**	2 (4.5)	15 (34.1)	χ^2= 12.3 , **P**= 0.000*

4. Discussion

Hearing loss among school-going children has been widely reported as a significant health problem in the developing world, including Egypt (Quality Inn & Suites Conference Center, 2009). The results of an earlier study revealed that the prevalence of hearing loss in school-age children was almost 10% (Riad, 1975). Moreover, an Egyptian survey has pointed to hereditary and otitis media with effusion as the commonest causes of hearing loss among preschool children (Abdel-Hamid et al. 2007). Unlike to this study and similar to the most recent ones, the findings of the current study declared that the vast majority of parents of deaf children are not deaf (ITV Signed Stories, 2008 & The National Deaf Children's Society, 2010).

A national household survey conducted to present the prevalence and patterns of hearing impairment in Egypt found that, both male and female gender are equally presented with no significant sex differences (Nationally KECO & NISO Training Current Member, 2009). The current study found that females are more enrolled than male students among the study participants. This may be interpreted in the context of school dropout due to poverty that enforces parents to introduce their deaf children to work in technical workshops to provide financial support to their families and at the same time to relieve the educational financial burden. Klare (2004), supporting this notion noted that students with disabilities who left school did so by dropping out, and concluded that students with disabilities drop out of school at twice the rate of general students. As regarding the residence of deaf students, the study findings showed rural-urban inequities, that could be interpreted in relation to parents' fears from children's disability that keep those from rural areas more prone to the risk of exposure to road accidents because of the long distance between home-place and school-place that is located at an urban city.

The findings of this study does agree with an observation in the developing world that,

childhood hearing impairment is commonest in illiterate parents and those with low socio-economic classes, because of the impact of poor hygienic conditions, inadequate medical follow-up, low immunization rate and misuse of ototoxic medications (Olusanya, Okolo & Adeosun 2004). In contrast, some other studies reported a higher prevalence of otitis media in the higher socio-economic classes, while others found no association between otitis media and socio-economic status (Quality Inn & Suites Conference Center, 2009). Given this variability, parental literacy by itself is unlikely to be a universal predictor of hearing loss in school-aged children.

Inspite of drawing attention towards children with hearing disability that has taken place during the past few years; researchers have shown that deaf children feel more neglected and less accepted by other children, so that they behave more aggressively. They also added that not only do deaf children show more anger than hearing children; most deaf children also use aggression amongst themselves. So, they are more vulnerable to school accidents that may require first aid assistance (Rieffe, & Meerum Terwogt, 2006).

The gap is still very wide between the needs of deaf children and available services due to a lot of obstacles. The more striking are those posed in communicating with children who have some sort of hearing impairment to raise their awareness about a new issue that they did not hear about it before, such as an inclusive first aid programme (AWR 186 POI).

Emergencies can occur suddenly and without any advance warning. For all individuals who have physical, sensory or cognitive disabilities, medical emergencies present a real challenge. In order to protect them, this requires planning ahead, and adequate preparedness to be self reliant during an emergency. U.S. Department of Homeland Security, (2004) and Queen's Printer for Ontario, (2007), emphasizing that disabled individuals are in the best position to know their functional abilities, and possible needs during and after exposure to an emergency situation.

The study results found deficiencies among deaf children's recall scores. This notion is supported by Liben, (2004) who suggests that deaf children's inadequate recall probably reflect insufficient knowledge of category membership. However, an educational programme to help people prepare for medical emergencies would enable vulnerable people and their caregivers to feel more in control and less anxious. An educational campaign can be conducted for deaf students and their school care-givers about emergency situations and how to help themselves (O'Brien, 2003).

First aid is the provision of initial care for an illness or injury. It is usually performed by a lay person to a sick or injured casualty until definitive medical treatment can be accessed. Certain self-limiting illnesses or minor injuries may not require further medical care past the first aid intervention. It generally consists of a series of simple and in some cases, potentially life-saving techniques that an individual can be trained to perform with minimal equipment (First Aid Medical Information, 2010).

Much of first aid is common sense. Basic principles, such as knowing to use an adhesive bandage or applying direct pressure on a bleed, are often acquired passively through life experiences. However, to provide effective, life-saving first aid interventions requires instruction and practical training (First Aid Medical Information, 2010).

Taking an immediate action is the essential principle in first aid. Often bystanders are worried about "doing the wrong thing", so don't attempt any first aid at all. If a person is sick or injured, then they need help, and they need it immediately. A casualty who is not breathing effectively, or is bleeding heavily, requires immediate aid. Prompt effective first aid gives the casualty a much better chance of a good recovery. Careful and deliberate action undertaken without too much delay is most beneficial to the casualty (Principles of First Aid - PARASOL EMT Pty Limited).

There is an increasing recognition of the need to respond to and met the health needs of vulnerable groups within society. There is a range of who may, for a variety of reasons, be vulnerable and this includes people with hearing disabilities. Those people are an integral part of society and need to be recognized and valued as equal citizens (Dolan & Holt, 2008).

5. Conclusion:

Although not enough for all items to be statistically significant, first aid intervention program raised the awareness of deaf students and their school care-givers about first aid intervention in medical emergencies.

6. Recommendations:

Future programs should focus on one section, such as emergency procedures, and emphasize the importance of it.

The educational curriculum of deaf students should include basic information about health, emphasizing on the importance of how to satisfy the

physical as well as the psychological needs of the casualty in case of exposure to medical emergencies.

Corresponding author
Rabab El-Sayed Hassan
Pediatric Nursing, Faculty of Nursing, Mansoura University. Mansoura, Egypt.
biboelsayed@yahoo.com

7. References:

1. WHO (1997): Let's Communicate: A handbook for people working with children with communication difficulties, Geneva, Section 6-Hearing Impairment.
2. Parving A. (1999): The need for neonatal hearing screening. Some aspects of epidemiology and identification. Acta pediatrics Suppl 432: 69-72
3. Mehl A. and Thompson V. (1998): Newborn hearing screening. The great omission. Pediatrics (1) 101-126
4. Deaf Notes Forum (2007). » Forums » L. LEGAL & MEDICAL ISSUES » 4. Law & Order » "Emergency Preparedness Now" Fostering Communication between Emergency Responders and the Deaf and Hard-of-Hearing Community. http://mail.google.com/mail/#inbox/11793e3c67535326.
5. http://www.sshchd.org -First Aid Tips!
6. Teaching First Aid to Children (2006). First Aid Café.co.uk.
7. Signed First Aid Tips Deaf Forum, Education (2009). Safe: Personal Safety Skills for Deaf Children, New DVD-ROM pack from NSPCC & NDCS, Google search.
8. British Red Cross (2009). What is Inclusive First id? inclusivefirstaid@redcross.org.uk.
9. MEDCARE Hospital (2007). First Aid Workshop Teaches Students to be Prepared for Emergencies, United Arab Emirates. Related Site: http://www.medcarehospital.com.
10. Nationally KECO & NISO Training Current Member (2009). Covering All Your Health & Safety Needs.
11. Allender JA & Spradely BW (2005). Community Health Nursing Promoting Protecting the Public's Health. Lipppincott Com. Philladelphia P. 643.
12. Quality Inn & Suites Conference Center. Wednesday, September 23, (2009). Iowa Protection and Advocacy Education - Workshop. Emergency Responders and the Deaf and Hard of Hearing Community. Taking the First Steps to Disaster Preparedness.
13. Riad S. (1975). A study of the hearing status of school children in Alexandria Governorate [MD thesis]. Alexandria, University of Alexandria.
14. Abdel-Hamid O., Khatib O.M.N., Aly A., Morad M. and Kamel S. (2007) Prevalence and patterns of hearing impairment in Egypt: a national household survey, Health Journal,5(13).
15. ITV Signed Stories (2008). Hearing parents with deaf children. website www.itvbabysign.com and on www.signpostbsl.com
16. The National Deaf Children's Society (NDCS) (2010). www.ndcs.org.uk/about_us/ndcs/index.html
17. Klare, M.W. (2004) prevention for students with disabilities: Recommendations for administrators. Clemson University: National Dropout Prevention for students with disabilities.
18. Olusanya BO, Okolo AA, Adeosun AA (2004). Predictors of Hearing Loss in School Entrants in a Developing Country. J Postgrad Med, 50:173-9.
19. Habpaqes Inc. and respective owners (2009). Why your child is deaf and how to manage?
20. O'Brien N. (2003). Emergency Preparedness for Older People. International Longevity Center-USA, Ltd.
21. U.S. Department of Homeland Security FEMA (2004). Preparing for Disaster for People with Disabilities and other Special Needs. American Red Cross.
22. AWR 186 POI. Emergency Responders and the Deaf and Hard of Hearing Community: Taking the First Steps to Disaster Preparedness. www.cepintdi.org/course-offerings/awr-186/program-of-instruction.
23. Queen's Printer for Ontario (2007). Emergency Preparedness Guide for People with Disabilities/ Special Needs (Disability Guide_ End. pdf).
24. Olusanya BO, Okolo AA, Adeosun AA. Predictors of Hearing Loss in School Entrants in a Developing Country. J Postgrad Med 2004;50:173-9).
25. First Aid Medical Information (2010). www.disabled-world.com/medical/first-aid/
26. Principles of First Aid - PARASOL EMT Pty Limited www.parasolemt.com.au/principles-of-first-aid/
27. Rieffe, C., & Meerum Terwogt, M. (2006). Anger communication in deaf children. Cognition and Emotion, Psychology Press, 20(8), 1261-1273.
28. Liben, L. S. (2004). Free recall by deaf and hearing children: Semantic clustering and recall in trained and untrained groups. Journal of Experimental Child Psychology, 1(27), 105-119. act., 34:257–266.

10/20/2010

Sudeck's Atrophy, Hyperhydrosis and other Hypersympathetic Syndromes, what is the Recent Proper Surgical Management?

Abulazaym A.A.[1*] and Horria H.[2]

[1]Neurosurgery Department, Faculty of medicine, Cairo University, Cairo, Egypt.
[2]General surgery Department, Faculty of medicine, Mansoura University, Mansoura, Egypt.
abaaaza51@yahoo.com*

Abstract: Twenty eight patients with upper limb Sudeck's atrophy (minor causalgia), hyperhydrosis (palmar and axillary) and causalgia were submitted to endoscopic transthoracic sympathectomy as a definitive treatment. There were 9 patients with Sudeck's atrophy, 16 patients with upper limb hyperhydrosis and 3 patients with major causalgia. The procedure was successful in curing 26 patients (92.86%) and gave mild improvement in two patients (7.14%) whom belonged to the Sudeck's atrophy (minor causalgic) group because of the advanced dystrophic changes in their limbs. The commonest side effects were compensatory sweating. The procedure is effective, very simple, and required only two nights stay, and is recommended as a method of choice for the surgical treatment of hypersympathetic syndromes of the upper limbs as Sudeck's atrophy, hyperhydrosis and major causalgia.
[Abulazaym A.A. and Horria H. **Sudeck's Atrophy, Hyperhydrosis and other Hypersympathetic Syndromes, what is the Recent Proper Surgical Management**. Journal of American Science 2010;6(12):1169-1174]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Sudeck's Atrophy, Hyperhydrosis and other Hypersympathetic Syndromes, what is the Recent Proper Surgical Management

1. Introduction:

Our modern civilization is plagued with chronic sympathetic hyperactivity secondary to chronic tension. Painful conditions such as myocardial ischemia (which is also prevailing), soft tissue trauma, bone and joints trauma or even trivial painful conditions frequently induce in those predisposed patients what is classically known as Sudeck's atrophy. Sudeck in a comprehensive series of papers beginning in 1900 described in detail the characteristics of this ambiguous chronic painful condition characterized by the predominance of muscular, bone and joint manifestations including osteoporosis. The difficult issue in this syndrome is that the chronic embarrassing pain and the gradual dystrophic changes are resistant to usual measures and medicines and thus these patients constitute a protracted problematic group. Many descriptive terms as hand-shoulder syndrome, post traumatic painful osteoporosis, acute bone atrophy, pseudo-rheumatism..etc are within a list of 26 German, 20 English and 6 French terms under which these disorders are termed; denoting their ambiguity and prevalence especially in communities of chronic tensions and frustrations as in the west communities. However, either minor causalgia or reflex sympathetic dystrophy is the term that is recently slowly dominating. These latter recent names are referring to the common denominator in the clinical picture, as also in the classic causalgia, which is the relief of the chronic heavily agonizing pain and other manifestations by effective sympathetic block. Hyperhydrosis is a more common interrelated syndrome that reflects hypersymathetic activity and affects mostly a younger age group and also responds to sympathetic block. We present in this paper our experience in a minimally invasive operation, the endoscopic trans-thoracic sympathectomy that we found effective, easy and short timed. We also stress that the endoscope is a valuable surgical indispensable armament that is very beneficial and minimally invasive and every surgeon can easily and should master it. A barrier of fear may hinder the trans-thoracic approach in surgeons and neurosurgeons, however as happened to us after few operations we found in our selves the courage and the feel at home feeling. It goes without saying that as in the second step of any operative intervention, we must (after the proper anatomical opening) do exploration and safeguard the relevant vascular, nervous and organ structures.

2. Patients and Methods

28 patients (11 men and 17 women) underwent endoscopic transthoracic sympathectomy for upper limb causalgia or hyperhidrosis. As hyperhidrosis is more common, 16 patients (12 women and 4 men) underwent 30 endoscopic transthorathic sympathectomies for upper limb and axillary hyperhidrosis. The mean age was 24.2 years (range 17-32 years). The chief complaint in all patients was excessive sweating. In 3 patients (18.75%) it was confined to the hands; in 2 patients (12.5%) it was confined to the axillae, and in 8

patients (50%) it was affecting both areas. There were 2 patients with recurrent palmar and one axillary hyperhidrosis.

The chief complaint of the major causalgic group (3 patients; all men) was the classic severe agonizing burning pain in the palm; while the chief complaint of the minor causalgic [sympathetic dystrophy] group (9 patients; 5 males and 4 females) was predominantly milder dull burning pain in the palms and distal forearms that was worse on motion and emotional excitement and associated with gradual atonic muscle atrophy and dystrophic skin changes as a series of events following the precipitating factor (myocardial infarction, angina pectoris, joints and bones trauma, soft tissue trauma, phlebitis with phlebothrombosis).

Routine laboratory and radiological screening tests were carried out preoperatively. Under general anaesthesia a 0.5 cm incision was made in the anterior axillary line over the third rib space. The Verres needle was inserted. The lung was slowly deflated by instilling one liter of CO2 into the intraplural space. The upper sympathetic chain was easily visualized lying over the necks of the ribs from the second to the six ganglia. The diathermy probe was inserted through a similar incision in the fourth space. The sympathetic chain overlying the lower part of the first, second and third ribs was treated with diathermy down to the periostium. The intercostal space left untouched. The nerve of Kuntz (a branch parallel and lateral to the main trunk) was coagulated if present. The fourth thorathic segment was coagulated in patients with axillary hyperhidrosis. In one patient with failure of a previous open surgery and recrrence of hyperhidrosis there were some pleural adhesions which were treated by the electrocautery probe. Before closure of the wound, the lung was expanded by positive endexpiratory pressure combined with suction through a small tube. A post operative chest X- ray was performed in the recovery room. The procedure took about 15-20 minutes. Patients

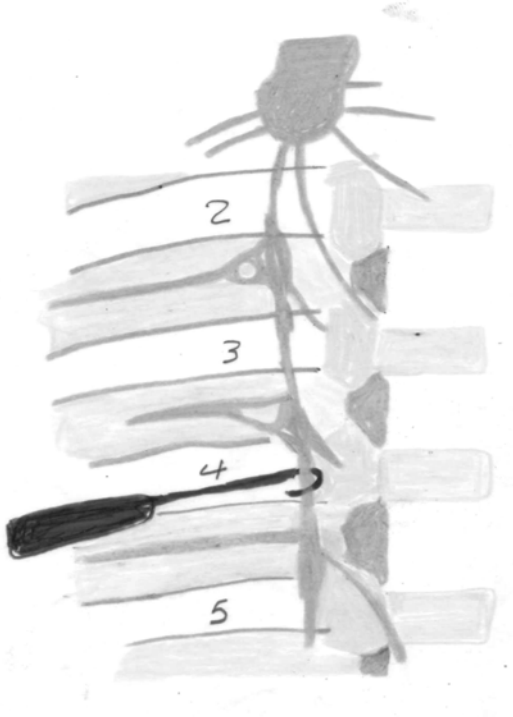


Fig. 1 The schematic drawing showing T_4 sympathectomy was performed by simple diathermic severance of the sympathetic trunk were discharged after two days (range 1-5 days) after surgery. In cases of hyperhldrosis, the contralateral sympathectomy was undertaken 6 weeks later. Patients were followed up for an average of four months (range 2-11 months) to assess the operative results and the occurrence of any complication.

3. Results:

Sixteen patients underwent endoscopic transthoracic electrocautery for hyperhidrosis of the upper limb, 14 patients underwent bilateral and 2 patients underwent unilateral surgery. There were 12 females and 4 males of mean age 24.2 years (range 17-32 years). The hands alone were affected in 3 patients (18.75%), the axillae alone in 2 patients (12.5%), and both areas in 8 patients (50%) (Table 1). There were 2 patients with recurrent palmar hyperhidrosis after the cervical approach, one with bilateral and one with unilateral recurrence. There

was another patient with recurrent unilateral axillary hyperhidrosis after open surgery. All the patients were completely relieved of sweating of the hands and axillae on returning from the operating theatre. As regard the causalgic (sympathalgic) group, the total number were 12 patients; 3 with major causalgia (all males) and 9 with minor causalgia (5 males and 4 females); all with failure of conservative treatment (including repeated sympathetic blocks done in different pain centers) to provide adequate sustained relief. The major causalgic subgroup patients presented with persistence of the typical major causalgic pain. The diagnosis could often be made from across the room based on the appearance of extreme distress shown by a patient cradling the affected limb without touching it to protect it from being touched or even to avoid a breeze inciting a paroxism of pain. The median nerve alone or with other nerves of the upper limb was involved in 2 cases and the brachial plexus in another case. The mean age was 26.7 (range 19-42). In the minor causalgic dystrophic subgroup; the extremely varied clinical presentation, often associated with features not typical of sympathetic hyperactivity with the predominence of muscular and joint symptoms as well as the usual presence of psychogenic features have led to protracted futile therapy in most patients. This eventuated in advanced dystrophic changes that were built up slowly and gradually. The mean age was 39 years (range 21-66 years).

There was no mortality and no serious morbidity. Horner's syndrome occurred in one patient with unilateral recurrent palmar hyperhidrosis, and was resolved in 6 weeks. Pneumothorax requiring a chest drain occurred in two patients. Some transient signs of pleural irritation (3 patients) and intercostal neuralgia (4 patients) were reported. The mean duration of hospital stay was two days (range 1-5 days). In the follow up period of three months, (range 3-12 months), compensatory sweating occured in 14 patients (50%) and gustatory sweating in 10 patients (35.7 1%). This compensatory and gustatory sweating were considered much less embarrassing than the original complaint. Despite the above mentioned side effects, 15 of 16 patients (93.75%) with hyperhidrosis, all patients with major causalgia and 5 patients with minor causalgia (5.55%) were very much improved in the follow up period and considered the result of endoscopic surgery highly satisfactory (Table 2). The remaining patient with hyperhidrosis (6.25%) and two patients with minor causalgia (2.22%) were moderately improved and were only satisfied. Two patients with minor causalgia (22.2%) had other residual symptoms associated with limitation of movement, stiffness of joints and other trophic changes. These were usually related to the delay between the onset and the diagnosis, which averaged 7 months. However both patients experienced gradual and moderate improvement. In a total number of 28 patients with hyperhidrosis or causalgia of the upper limb, transthoracic endoscopic sympathectomy gave excellent results in 89.28% of cases (25 cases) and mild to moderate improvement in 10.72% (3 cases).

Table (1): area affected by hyperhydrosis in 28 patients.

area	No. of patients	percent
Hands only	3	18.75
Axilla only	2	12.5
Both areas	8	50
Recurrent hands	2	12.5
Recurrent axilla	1	6.25

Table (2): subjective assessment of the results of endoscopic surgery by 28 patients.

	Improvement	Immediate postoperative	At follow up
Hyperhidrosis	Very much improved Moderately improved	15 (93.75%) 1(6.25)	15(93.75%) 1(6.25%)
Major causalgia	Very much improved Moderately improved	3(100%)	3(100%)
Minor causalgia	Very much improved Moderately improved Slightly improved	5(55.55%) 2(22.2%)	5(55.55%) 2(22.2%)

4. Discussion:

Twenty eight patients with upper limb hyperhidrosis (palmar and axillary) or causalgia (major and minor) were submitted to endoscopic transthoracic sympathectomy as a definitive treatment. There were 16 patients with upper limb hyperhidrosis (including three patients with recurrence after open surgery), and 12 patients with upper limb causalgia (3 with major causalgia and 9 with minor causalgia). The procedure was successful

in curing 25 patients (89.28%) and gave moderate improvement in 3 patients (10.72%); two of them belonged to the minor causalgic group because of the advanced dystrophic changes in their limbs. The commonest side effects were compensatory sweating. There was a high level of patient satisfaction. Horner's syndrome occurred in one patient. The procedure is effective, simple, and required only two nights stay, and is recommended as a method of choice for the surgical treatment of upper limb hyperhidrosis or causalgia.

HYPERHIDROSIS is defined as sweating above and beyond the physiological needs. It can be very distressing and the source of intense embarrassment (Symptoms usually appear at puberty with an incidence of 0.6-1.0%). Hyperhidrosis is usually localised to the palm, axillae and feet, but the face, back, groin and legs may also be affected [1]. Sympathectomy remains the cornerstone of surgical management.

Cases of causalgia of the upper limb are extremely embarrassing and renders the patient almost completely useless and miserable. Major causalgia is a clinical syndrome associated with a lesion of a peripheral nerve that contains sensory fibers and is characterized by burning pain in the affected extremity, usually in the hand or foot. It occurs as a complication in about 3% of major nerve injuries. It is most often associated with incomplete lesions of the median nerve in the upper limb and the sciatic nerve (the tibial part) in the lower limb. These two nerves account for about 60% of the cases; the rest of the cases involved primarily the nerves of the upper extemity [7]. Despite the recognition that the sympathetic nervous system is involved, the specific pathophysiology is unknown. Various theories as to the cause of causalgia have been proposed, including short - circuiting effects at the area of the injury, permitting irritation of sensory afferent fibers by the efferent sympathetic impulses, periarteritis in volving the vessels about the injured neural segments, and abnormal feedback into the internuncial centers of the spinal cord. The syndrome is one of excruciating burning pain often described as having a superimposed throbbing, aching, bursting pressure, knife like stabbing, twisting, or crushing component. In many instances (about one third); pain begins immediately after injury; it usually begins sometime during the first week. The pain is usually located in an area corresponding to the cutaneous distribution of the nerve but is not necessarily limited to this area. Another characteristic of the pain, one usually necessary for a positive diagnosis, is accentuation by stimuli to such emotions as surprise and anger and by other disturbances in the patient's environment. To touch, feel, or even hear the name of a slick object such as a paper or a sheet will cause severe increase in pain in some patients. In many instances light touch, heat, or minor movements of the trunk and extremity will increase existing pain. Some patients insist the pain seems less severe on cool, damp days or at night. After having seen a patient with severe causalgia one is not likely to forget the picture of a pain- racked patient guarding the affected extremity with extreme care. The skin of the affected part may show evidence of vasodilatation early after injury, and this may or may not be replaced later by vasoconstriction. Thus the skin of the affected part may be dark red, dry glossy, and atrophied or cold, mottled, and moist. The skin may be devoid of hair or, on the contrary, may have an abnormal growth of hair, hyperhidrosis is common. Accurate evaluation of the completeness of the nerve lesion is commonly impossible because of the extreme pain caused by handling the affected limb. When causalgia is severe, the diagnosis is usually clear and is easily confirmed by procaine block of the second and third sympathetic ganglia. Rarely will other neurologic conditions be confused with true causalgia [7].

Both major and minor causalgia seem to constitute a closely related conditions; Horowitz [17] had already concluded that (the post traumatic pain syndromes seem to be identical in type with minor causalgia), Bonica [7] has taken the logical view that effective treatment by sympathetic denervation justifies classifying the entire group under one rubric. Omar et al., [21] & Buker et al. [8] have reported successful treatment of early minor causalgic states (reflex sympathetic dystrophy) by a long series of sympathetic ganglion blocks with local anesthetics (occasionally and especially in the early stages of the disease, a series of several sympathetic blocks with procain will relieve the pain or make it tolerable); failure of this conservative measures is the indication of surgery.

Sympathectomy remains the cornerstone of surgical management to hyperhidrosis and causalgias (major and minor), various operative approaches have been described, each with its advocates; the cervical or supraclavicular approach (Tel- ford, 1935)[6], the posterior approach [2], the transaxillary approach [4], and the transthoracic endoscopic sympathectomy[3]. This study was an experience in endoscopic transthoracic electrocautery of the sympathetic chain for the treatment of upper limb hyperhidrosis or causalgia.

Endoscpic transthoracic sympathectomy is a minimally invasive procedure with several advantages over the open surgery. It provides excellent visualization of the sympathetic chain. The simplified technique described in this series of 28 patients was successful in treating around 90%

(89.28%) of the patients with upper limb hyperhidrosis or causalgia; the rest of the patients obtained moderate to mild improvement. It was not followed by any major complication, there was no wound infection and the two scars of 0.5 cm. length are almost invisible. The operative time varies, but the average for the unilateral procedure was 20 minutes. Gothberg et a! [16] reported a 25 minutes for a bilateral procedure; while Edmondson et al. who reported an average time of 45 minutes for bilateral procedure. The hospital stay average 2 days in this study which compares good with that reported by other workers.

Postoperative Homer's syndrome is largely a complication of the open approaches, but the danger of creating a Homer's syndrome (6.25%) and of pneumothorax (12.5%) was less than that reported by Edmondson et al. They reported percentages of 14% and 16% for these two complications respectively. Compansatory sweating represents a thermoregulatory response and is usually over the trunk and upper thighs in 13 patients (46.42%) in our sires, which is near the incidence reported by Adar [1]. Gustatory sweating occurred in 9 patients (32.14%) which was nearly similar to that reported by Edmondson et al. [13]. In general compensatory and gustatoy sweating were not very embarrassing to the patients.

In summary,

Endoscopic transthoracic electrocautery of the sympathetic chain for the treatment of upper limb causalgias and hyperhidrosiss is a safe, simple, fast and cosmetically acceptable procedure. It is considered the treatment of choice for these conditions.

Corresponding author
Abulazaym A.A.
Neurosurgery department, Faculty of medicine, Cairo University, Cairo, Egypt.
abaaaza51@yahoo.com

5. References:

1. ADAR R., KURCHIN A., ZWEIG A., and MOZES M.: Palmar hyperhidrosis and its surgical treatment. Ann. Surg. 186: 34-41, 1977.
2. ADSON A.W. and BROWN G.E.: Extreme hyperhidrosis of the hands and the feet treated by sympathetic ganglionectomy. Mayo Clinic. Proc. 7: 394, 1949.
3. Alric P, Branchereau P, Berthet JP, Leèger P, Mary H, Mary-Aneè C. Video-assisted thoracoscopic sympathectomy for palmar hyperhidrosis: results in 102 cases. Ann Vasc Surg 2002;16:708-713.
4. ATKINS H.H.: Preaxillary approach to the stellate and the upper thoracic sympathetic ganglia. Lancet., 11, 1949.
5. KUX E.: Thorakoskopische Engriffe am Nervebsystem. Stuttgart: Georgthieme Verlag, 1945.
6. TELFORD E.D.: The technique of sympathectomy Br. J. Surg. 23: 448-50, 1935.
7. BONICA, J.J.: Causalgia and other reflex sympathetic dystrophies. Advances in pain research and therapy, Vol. 13, New York, Raven. Press., pp. 141-166, 1979.
8. BUKER, R.H., COX, WA., SCULLY, T.J., SEITER, G. and PAULING, F.W.: Causalgia and transthorathic sympathectomy. Am. J. Surg., 124: 724727, 1972.
9. BYRNE J., WALSH T.N. and HEDERMAN W.P.: Endoscopic transthoracic electrocautery of the sympathetic chain for palmer and axillary hyperhidrosis. Br. J. Surg. 77L 1046-1049, 1990.
10. Doolabh N, Horswell S, Williams M, Huber L, Prince S, Meyer DM, Mack MJ. Thoracoscopic sympathectomy for hyperhidrosis: indications and results. Ann Thorac Surg 2004; 77:410-414.
11. DOUPE, J., CULLEN, C.H. and CHANCE, G.Q.: Post-traumatic pain and the causalgic syndrome. J. Neurol. Neurosurg. Psychiatry, 7: 33-48, 1944.
12. Dumont P, Denoyer A, Robin P. Long-term results of thoracoscopic sympathectomy for hyperhidrosis. Ann Thorac Surg 2004;78:1801-1807
13. EDMONDSON, R.A., BANERJEE A. and RENNIE, J.A.: Endoscopic transthoracic sympathectomy in the treatment of hyperhidrosis. Ann. Surg. 215-293, 1992.
14. ERDMIR, H., GELMAN, S. and GALBRAITH, J.G.: Prediction of the needed level of sympathectomy for posttraumatic reflex sympathetic dystrophy. Surg. Neurol., 17: 353-354, 1982.
15. .Georghiou GP, Berman M, Bobovnikov V, Vidne BA, Sautre M. Minimally invasive thoracoscopic sympathectomy for palmar hyperhidrosis via a transaxillary single-port approach. Interactive Cardiovasc Thorac Surg 2004;3:437-441
16. GOTHBERG G., CLAES. G. and DROTT C.: Electrocautery of the upper thoracic sympathetic chain: a simplified technique. Br. J. Surg. 8: 862, 1993.
17. HOROWITZ, S.H.: latrogenic causalgia, Classification, clinical findings, and legal ramifications. Arch. Neurol., 41: 821-824, 1984.
18. Krasna MJ, Ferguson MD. Thoracoscopic sympathectomy. General Thoracic Expert's

Techniques. Baltimore: CTSNET Experts' Techniques, 2002.
19. MALONE, P.S., DUIGNAN, J.P. and HE DERMAN, W.P.: Transthoracic electrocoagulation (T.T.E.C.) A new and simple approach to upper limb sympathectomy. Irish Med., J., 75: 20-21, 1982.
20. .Ojimba TA, Cameron EP. Drawbacks of endoscopic thoracic sympathectomy. Br J Surg 2004;91:264-269.
21. OMAR, G. and THOMAS, S.: Treatment of causalgia. Review of cases at Brook General Hospital. Tex. Med., 67: 93-96, 1971.
22. SCHWARTZMAN, R.J. and MAC LELLAN, T.L.: Reflex sympathetic dystrophy. Arch. Neurol., 44: 555-561, 1987.
23. SUNDERLAND, S.: Nerves and nerve in juries, Baltimore, The Williams and Wilkings Co., 1968.
24. WALKER, P.M. and JOHNSTON, K.W.: Predicting the success of a sympathectomy: A prospective study using discriminant function and multiple regression analysis. Surgery, 87: 216-221, 1980.

10/29/2010

Reconstructive Cervical Laminoplasty with the Preserved Fixed Spinous Processes Row as an Intervening Bone Graft; a Successful Novel Surgical Approach.

Abulazaym A.A.[*1] and Meziad M.[2]

[1]Neurosurgery Department, Faculty of Medicine, Cairo University, Cairo, Egypt
[2]Orthopedic Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt
abaaaza51@yahoo.com[*]

Abstract: Study design: A prospective study to evaluate the outcome of a novel operation for cervical myelopathy secondary to cervical canal stenosis; the Reconstructive Cervical Laminoplasty with the preserved fixed spinous processes row as an intervening bone graft. **Objective:** To explore a more effective, less invasive and more physiological operative technique for cervical myelopathy of cervical spinal canal stenosis. **Background:** The popular two cervical laminoplasties for the nowadays becoming common in elderly people cervical myelopathy of cervical spinal canal stenosis, i.e. open door laminoplasty and double doors laminoplasty are plagued with many drawbacks such as around 50% diminution in the range of cervical movements, 25% occurrence of kyphotic deformity, laminar fusions, from 10% to 50% chronic axial neck pain and nuchal musculature atrophy. A more physiological modification of this very beneficial operation is badly needed. We presented our novel reconstructive cervical laminoplasty with the preserved fixed spinous processes row as an intervening bone graft to avoid such drawbacks. **Methods:** This prospective preliminary study included 14 patients who underwent the novel reconstructive cervical laminoplasty with the preserved fixed spinous processes row as an intervening bone graft operation for their cervical myelopathy. **Results:** The novel operation is proved to be easier, more physiological and succeeded to avoid to a great extent the aforementioned drawbacks of the two popular cervical laminoplasties; only about 30% diminution of cervical movements occurred, no kyphotic deformities, post-operative axial neck pain was moderate and occurred in only 21% of the patients and the post operative nuchal musculature atrophy was avoided. **Conclusion:** Cervical myelopathy secondary to cervical spinal canal stenosis can be managed adequately with our novel cervical reconstructive myelopathy with the preserved fixed spinous processes row as an intervening bone graft. This technique obtained satisfactory outcomes and avoided the drawbacks of the popular laminoplasty operations. It can be a standard procedure for the surgical treatment of this nowadays becoming common disease.
[Abulazaym A.A. and Meziad M. **Reconstructive Cervical Laminoplasty with the Preserved Fixed Spinous Processes Row as an Intervening Bone Graft; a Successful Novel Surgical Approach**. Journal of American Science 2010;6(12):1175-1180]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Reconstructive Cervical Laminoplasty; Spinous; Processes Row; Intervening Bone Graft; Novel Surgical Approach

1. Introduction

Cervical laminoplasty is a posterior spinal operation where the position of the laminae is altered to augment the cervical spinal canal width and volume without intersegmental fusion. Laminoplasty is primarily used to treat cervical myelopathy caused by cervical spinal stenosis. Spinal multisegmental stenosis of the cervical vertebrae is common in old age secondary to moderate to severe cervical spondylosis with osteophytic bars. Laminoplasty was first described in 1968, and much of the literature regarding it comes from Asia, where cervical myelopathy from ossification of the posterior longitudinal ligament is common. In our area as well as universally, many surgeons choose and prefer laminoplasty over anterior fusion techniques when more than two levels require decompression. Laminoplasty can be used in other situations where the volume of the cervical spinal canal needs augmentation, but fusion is not desired.

2. Methodology:

Study design: A prospective (longitudinal) preliminary study on the novel cervical laminoplasty with preservation of the fixed posterior longitudinal band operation.

Inclusion criteria: Presence of cervical myelopathy with cervical spinal canal stenosis, documented tight cervical spinal canal and cervical spinal cord compression with absence of the subarchnoid space in the MRI, Pavlov ratio (canal depth / vertebral depth)= < 0.82 in the lateral view plain X ray.

Exclusion criteria: Cervical Kyposis.

Anterior osteophytic bars thickness > 7mm

Advanced myelopathy with marked spastic paraplegia or scissoring of the lower limbs and marked atrophy and weakness and of the upper limbs (grades 0, I).

Elderly debilitated patients.

Initial evaluation: 14 patients were included in this preliminary study they were submitted to complete neurological history taking and thorough examination.
Radiological assessment: cervical MRI and plain cervical antero-posterior lateral and dynamic views were done to all the patients.
Physiological studies: Electromyogram EMG of the upper limbs is done to all patients.

Operative maneuver:

Patient positioning: A general endotrachial anesthesia is used and the patient is given a preoperative dose of antibiotics and steroids. Positioning is accomplished by placing a pin-type head rest, and turning the patient prone. Chest rolls elevate the thoracic cage and decompress the abdomen, thereby lowering the venous pressure and diminishing the operative blood loss. The head and neck are positioned in a neutral position, and the head rest mechanism is locked. The shoulders are taped down to the table with 4 inch adhesive tape. We prefer and found beneficial to elevate the head by tilting the surgical table 35 degree. A standard midline approach is utilized, dissecting down to the fascia then via the cutting diathermy in the ligamentum nukae. The secret of success in this step and the following one is to keep the diathermy cutting strictly midline and restricted to the thick fibrous tissue of the ligamentum nukae to minimize blood loss and to approach the spinous processes row atraumatically and easily. A standard bilateral subperiosteal dissection is carried out including the levels immediately superior and inferior to the pathologic levels, with preservation of the midline posterior tension band consisted from the spinous processes row and the intervening interspinous ligaments via restricting the diathermy cutting dissection only bilateral to the spinous processes, i.e. neither cutting in the upper nor the lower borders of the spinous processes. Using a small drill or a thread wire saw we cut at the bases of the spinous processes leaving 6-mm projections from the laminae; the scissors are used alternatingly to cut at the bases of the interspinous ligaments. The separated spinous processes, with their ligamentous attachments, are retracted to one side. The spinous processes are making the bony components of the preserved midline posterior tension band that is left as a whole fixed upwards and downwards to the spinous processes immediately superior and inferior to the pathologic levels. The laminae (devoid from the spinous processes) are thereafter cut in the midline with the same instrument. Two bony hinges are constructed bilaterally at the junction of the laminae to the facet complexes using a high-speed drill down to thinning partially and suitably the inner cortex, taking strict care not to injure the facets. Care must be taken so that the lamina is not thinned to the point that it fractures, or that it offers no spring like resistance to deformation. The proper resistance is checked and made so that with gentle finger pressure the laminoplasty segment is opened. A gentle capacious and sufficient release and separation of the dura surrounded by its thin vascular film from the undersurface of each lamina is done, with gradual and gentle rotation of the lamina to open the segment. Then the ligamentum flavum in the mid line is opened and excised for about half a centimeter, with utmost care to avoid laceration of the epidural thin vascular film or the epidural venous plexus. Sufficient time is awaited for the dura to re-expand and regain its healthy pulsations. Now after securing hemostasis, the (to the side) retracted tension band is regained to the mid line and the spinous processes portions are used as an intervening bone grafts via drilling 1-mm holes in the medial edges of the hemilaminae and the sides of the corresponding properly opposed spinous process at each level. The spinous processes are sutured carefully into place using nonabsorbable sutures. These sutured spinous processes portions are actually the left portions after they had been cut leaving 6 mm of their bases projecting from the laminae. This induces about 4-5 mm enlargement along the anterior posterior line of the canal which was found ideal. A proper closure of the hemilaminae segments with the intervening spinous processes' portions included within the posterior tension band after expanding the spinal canal should be secured. If radiculopathy is found on the preoperative electromyogram (EMG), we always perform selective foraminotomy for the affected roots. We also always intentionally perform reconstruction of the semispinalis cervicis and re-suture it back to the C2 spinous process in both sides to strengthen the posterior tension band as well as to cover and secure water-tightness of the spinous processes-to-the medial hemilaminal edges sutures. After proper hemostasis of the back muscles, we close in layers. A rigid cervical collar is used for three weeks.

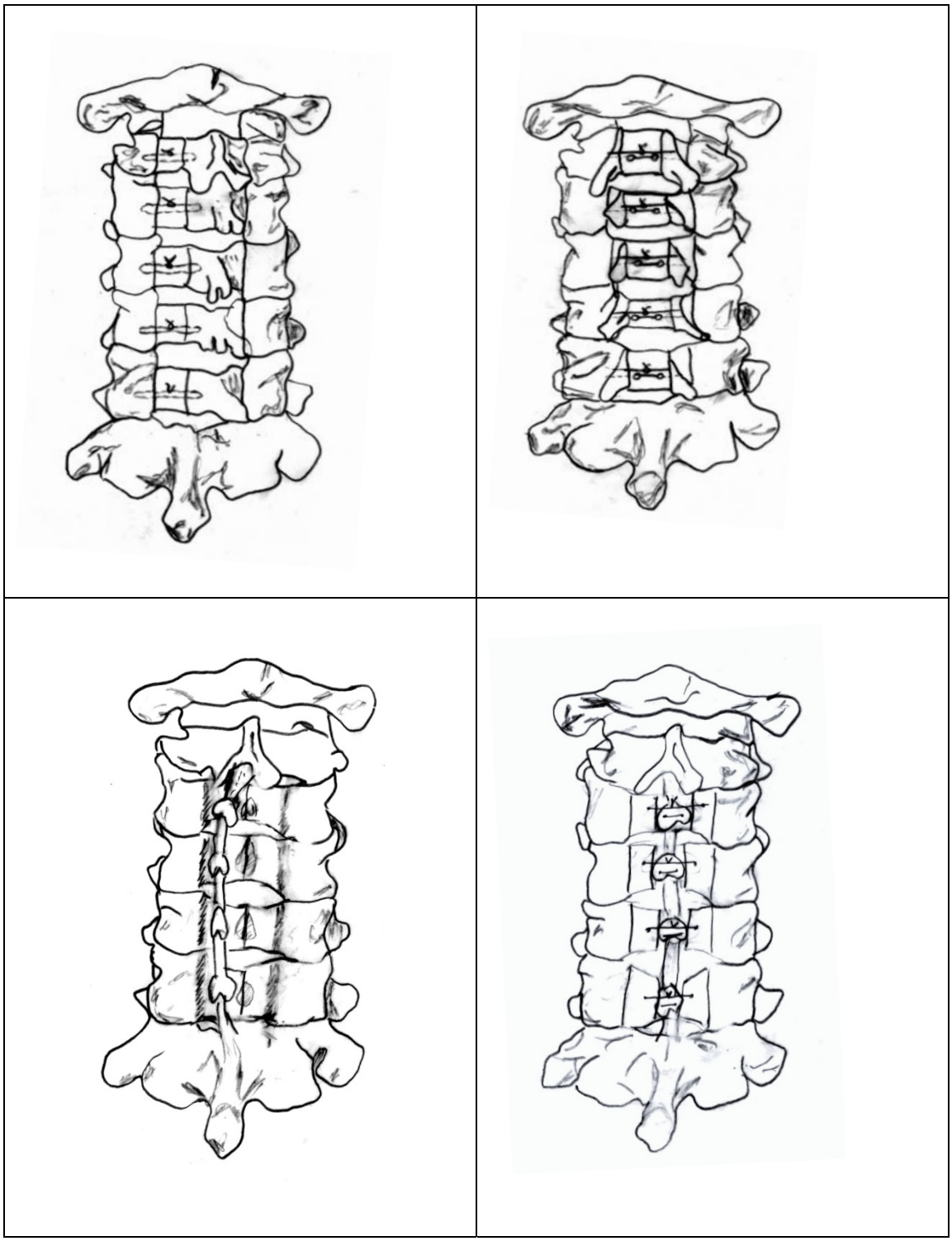


Fig. 1 Upper left: open-door laminoplasty. **Upper right:** double-door laminoplasty. **Lower left:** reconstructive laminoplasty with preservation of the fixed (to the superior and inferior spinous processes) midline longitudinal band; that is retracted to the side to permit the midline cuts in the laminae. **Lower right:** reconstructive laminoplasty with preservation of the posterior longitudinal band, after suturing of the spinous processes row in place.

3. Results:

Because most of the cervical laminoplasty operations are performed to treat myelopathy, the preoperative and post operative conditions are usually scored using the Japanese Orthopedic Association (JOA) scoring system for cervical myelopathy that gained universal acknowledgement and approval as much of the literature regarding laminoplasty comes from Asia where OPLL is prevailing. However some items in that score is strange to other communities (handling chopsticks), thus we used the same score with minimal modification that will not make any difference to suit our community. In this score:

The recovery rate percentage =100 (post operative score minus preoperative score/ 17 minus preoperative score).

Excellent recovery rate :>75%, Good:50-75%, Fair:25-50% and Poor recovery:<25%.

Ages of the patients ranged from 38 years up to 71 years. Average age was 49 years. 71% were males, the rest were females 39%.

Duration of the disease averaged 8 years (from 3 to 14 years).

To assess the success of our novel operation of laminoplasty we assessed the 14 patients as a group. Collectively, we found that there is a 72% recovery rate in JOA scoring system in our 14 patients as the following table 1.

Table 1: The collective recovery rate of our 14 patients.

	Pre-operative	**Post-operative**	**Recovery rate**
Motor dysfunction of the upper extremity 0=unable to feed oneself, 1=able to eat with spoon, 2= able to the full use of the teeth sticks with slight difficulty, 3=none	21	26	
Motor dysfunction of the lower extremity 0=unable to walk, 1=walking on flat floor with walking aid, 2= up stairs with hand rail, 3=None	24.5	29	
Sensory deficit of the upper extremity 0=severe sensory loss or pain, 1=mild sensory loss,2=none	12	16	
Sensory deficit of the lower extremity 0=severe sensory loss or pain, 1=mild sensory loss,2=none	11	16	
Trunk 0=severe sensory loss or pain, 1=mild sensory loss,2=none	10.5	17	
Sphinchteric dysfunction 0=unable to void, 1=marked difficulty in micturition, 2=difficulty in micturition, 3=none			
	28	31	
Total	**107**	**125**	**72%**

However, four factors were found related to the outcome in an ascending relevance: older age, lower JOA scores, lower Pavlov ratio (width of the canal divided by the width of the vertebral C6 body in the lateral plain X ray), and the presence of an abnormal spinal canal signal in the preoperative MRI. They were found significantly related to poorer outcome. In this preliminary study we correlated the three patients mostly affected with the previous 4 factors in relation to the rest of the patients. We found that respectively each of them correlated to 16%, 20%, 26%, and 33% diminution in the overall good result.

4. Discussion:

Two types of laminoplasty gained popularity; the open door and the double door laminoplasty. All literature on laminoplasty is class 3 data, therefore, at present; laminoplasty is listed as a treatment option for cervical spondylotic myelopathy as well as ossified posterior longitudinal ligament (OPLL) in Japan and East Asia. It is heavily documented in the literature that laminoplasty is effective in treating cervical myelopathy caused by stenosis. Theoretically, laminoplasty benefits compared to anterior fusion include preservation of cervical motion and prevention of the adjacent motion segments degeneration. Its benefits compared to the decompression laminectomy include prevention of post laminectomy kyphosis, avoiding the development

of post laminectomy membrane formation and the recurrence of stenosis as well as avoidance of the development of retrolisthesis and instability. However, unfortunately, these benefits are not always realized as there are three drawbacks that plagued this beneficial maneuver in its popular two types. It was shown by previous researchers that the range of movement diminishes approximately 50% after laminoplasty, kyphotic deformity occurs in around 25% of the patients, and intralaminar membrane formation as well as laminar fusions has been recorded frequently. Additionally there is from 10% to 60% incidence of chronic axial neck pain compared to 19% after anterior fusion.

We present our easy and straightforward novel surgical maneuver that neither necessitates any extra manipulations for bringing bone grafts from elsewhere nor is technically demanding; in addition to its benefits in avoiding to a great extent the aforementioned complications. We found that preservation of the posterior tension band that is left fixed to the spinous processes immediately superior and inferior to the pathologic motion segments prevents the tendency to kyphous deformity. This band is strengthened via the resuture of the semispinalis cervicis back to the C2 spinous process. It should be mentioned that reconstruction of this muscle alone as was done before by some researchers without the preservation of the midline structures that constitute the posterior tension band will never suffice to prevent the kyphotic tendency as the muscle tissue is a stretchable tissue.

This preservation of the normal lordotic curve was found also very beneficial to prevent the chronic axial neck pain that was repeatedly recorded by many researchers to occur in up to 60% of post-laminoplasty patients. We think that this pain arises to a great extent from the posterior uncovertebral joints either because of their injury during surgery or due to post operative abnormal stretching of their capsules secondary to the post operative kyphotic tendency. We succeeded to prevent both factors, thus the post laminoplasty axial pain was reduced to minimum as only 21% of the patients complained of it.

We also succeeded to avoid the interrelated triad of post-operative diminution of the range of movement only about 30% in our maneuver compared to 50% in the two popular manuevers, laminar fusions and perilaminar membrane formation via minimizing the surgical tissue injury and strict hemostasis. From the start we keep strictly our sharp mid line cutting within the fibrous tissues of the ligamentum nukae, we preserve the midline structures that are crucial in the biodynamichs of the cervical vertebrae as it prevents the occurrence of postoperative kyphosis which continuosly stretches and stimulates the laminar periosteum to form new bone, also we keep the posterior joints untouched.

The latter three factors are also fundamental in preventing the post laminoplasty nuchal nusculature atrophy and size reduction that was repeatedly recorded. The nuchal muscles may lose up to 25% of their preoperative size in some patients of the classical two types of laminoplasty. Avoiding this complication in our novel operation is largely due to the preservation of the midline tension band that prevents kyphosis which stretches the nuchal muscles and invite shearing tissue injury in them also may provide nutrient vasculature to the muscles in the post-operative period as well as keeping the posterior joints untouched preserve the nutrient vasculature and nerves of these muscles.

Strict hemostasis, post operative drain for 36 hours and post operative alpha chemotrypsin one amp/12 hours were also done and found very beneficial.

Limitations:

Our preliminary results are very encouraging. The novel operative maneuver is logic and straightforward. It is considered a conservative modification of the already popular and the established successful two types of cervical laminoplasty. In addition to preserving the proved very crucial posterior longitudinal tension band that is kept fixed superiorly and inferiorly; an employment of its bony element that is also kept fixed in it i.e. the spinous processes row as an intervening bone graft is easy and novel. Thus the time of the operation is greatly shortened, which is considered a welcomed element in this elderly age group. However, the limited number of our patients is considered a limitation that necessitates further studies on a greater scale.

5. Conclusion:

We presented our novel operation of reconstructive cervical laminoplasty that exploited the preserved fixed spinous processes row as an intervening bone graft. To our knowledge it is a pioneering and also a logic operation that conserves the crucially relevant posterior midline tension band fixed superiorly and inferiorly. We managed to avoid much of the drawbacks and complications of the popular two types of cervical laminoplasty, and thus our easy operative maneuver proved to be very beneficial.

Correspondence author
Abulazaym A.A.
Neurosurgery Department, Faculty of Medicine, Cairo University, Cairo, Egypt.

abaaaza51@yahoo.com

6. References:

1. Chung SS, lee CS, chung KH: factors affecting the surgical results of expansive laminoplasty for cervical spondylotic myelopathy. Int Orthop 26:334, 2002.
2. Edwards CC, Heller JG, Murakami H: Corpectomy versus laminoplasty for multilevel cervical myelopathy. Spine 27:1168-1175, 2002.
3. Fujimura Y, Nishi Y: Atrophy of the nuchal muscle and change in cervical curvature after expensive open-door laminoplasty. Arch Orthop Trauma Surg 115:203-205,1996.
4. Hirabayashi K: Expansive open-door laminoplasty for cervical spondylotic myelopathy. Shujutsu 32:1159-1163, 1978.
5. Hoshi K, Kurokawa T, Nakamura K, et al: Expansive cervical laminoplasties-Observations on comparative changes n spinous process lengths following longitudinal lamina divisions using autogenous bone or hydroxyapatite spacers. Spinal Cord 34:725-728, 1996.
6. Iseda T, Goya T, Nakano S, et al: Serial changes in signal intensities of the adjacent discs on T2-weighted sagittal images after surgical treatment of cervical spondylosis: Anterir interbody fusion versus expansive laminoplasty. Acta Neruochir (Wien) 143:710, 2001.
7. Kimura S, Homma T, Uchiyama S, et al: Posterior migration of cervical spinal cord between split lamina as a complication of laminoplasty. Spine 20:1284-1288, 1995.
8. Lee TT, Manazo GR, Green BA: Modified open door cervical expansive laminoplasty for spondylotic myelopathy: Operative technique, outcome, and predictors for gait improvement. J Neurosurg 86:64-68, 1997.
9. Nagata K, Ohashi T, Abe J, et al: Cervical myelopathy in elderly patients: Clinical results and MRI findings before and after decompression surgery. Spinal cord 34:220-226, 1996.
10. Nakano N, Nakano T: Clinical results following enlargement of the cervical spinal canal by means of laminoplasty. Jpn Orthop Assoc 62:1139-1147, 1988.
11. Sasai K, Satio T, Akagi S, et al: preventing C5 palsy after laminoplasty. Spine 28:1972-1977 2003.
12. Satomi K, Nishu Y, Kohno T,Hirabayashi K: Long-term follow-up studies of open-door expansive laminoplasty for cervical stenotic myelopathy. Spine 19(5):507-510, 1994.
13. Takayasu M, Takagi T, Nishzawa T, et al: Bilateral open-door cervical expansive laminoplasty with hydroxyapatite spacers and titanium screws. J Neurosurg (spine 1) 96:22_28, 2002.

11/5/2010

The Effectiveness of the Intervention Program on the Attitude and Self-Concept of Students with Dyslexia

Zeinab, Mihandoost[1], Prof. Habibah Elias[2], Prof. Sharifah Nor[3], Dr. Rosnaini Mahmud[4]

[1]Faculty of Educational Studies, University Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia.
[2] Faculty of Educational Studies, University Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia.
[3] Faculty of Educational Studies, University Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia.
[4] Faculty of Educational Studies, University Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia.
xozns2006@yahoo.com

Abstract: The purpose of this study is to investigate the effect of the Barton Intervention Program on the attitude and self-concept of dyslexic students. The Dyslexia Screening Instrument (DSI), and Reading Text were employed in order to identify the dyslexic students in schools in Ilam, Iran. The population of the study included 138 dyslexic students studying in elementary schools in Ilam, Iran and from this population, 64 students were selected randomly and assigned equally to an experimental group and a control group (32 students in each group). The experimental group was taught for 36 sessions using the Barton method, in two levels, and ten lessons were provided to improve their reading skills. Reading attitude and self-concept to read instruments were employed to measure their attitude and self-concept, before and after the intervention program. The reliability of the reading attitude and self-concept were confirmed. The content validity of the scales was investigated using the judgment of 10 psychology experts. The analysis of the finding through independent t-test showed a significant difference between the control group and the experimental group after the intervention, at $\rho<0.000$.

[Zeinab, Mihandoost, Prof. Habibah Elias, Prof. Sharifah Nor, Dr. Rosnaini Mahmud. **The Effectiveness of the Intervention Program on the Attitude and Self-Concept of Students with Dyslexia**. Journal of American Science 2010;6(12):1181-1191]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Intervention program; attitude; self-concept; dyslexia

1. Introduction

Most students with learning disability have problems in one or several basic skills. Dyslexia is one of the learning disability affecting these students in their basic skills (1), where dyslexia students were found to hold more negative self-concept (2), feel more helpless (3) and have negative attitudes about school learning (4). Attitude toward reading has been defined by Smith (5) "as a state of mind, accompanied by feelings and emotions, that make reading more or less probable" (p.125). A student's attitude toward reading is an essential factor affecting reading performance. Positive attitudes can compensate for relatively weak skills, while negative attitudes can prevent a student from applying existing knowledge (6). Reading attitude fulfills a pivotal role in the development and use of lifelong reading skills. Richeck, List and Lerner (7) declared that "the final success of education is strongly affected by the reader's attitude". Lipson and Wixson (8) concluded that "the student's attitude toward reading is an essential factor affecting reading performance". A number of researchers have postulated that attitudes affect one's motivation and consequently reading achievement by increasing or decreasing the amount of time that learners spend engaged in reading (9).

The result of studies by Polychroni, Koukoura and Anagnostou, (10), as well as Lazarus and Callahen (11) showed that students diagnosed with learning disability in reading have negative attitudes toward reading. Attitudes can also consist of one's affinity for a particular activity. The importance of the affective characteristics of learning- disabled students has long been noted, and these students are often attributed with negative affective

characteristics. Despite this somewhat general acceptance in the field (12), it has not been definitively ascertained whether the negative affective variables cause the learning disability, are a consequence of it, are related in origin to the actual disability, or are simply behaviors which happen to occur concurrently with the difficulty in learning. There seems to be a general agreement nevertheless, that the prolonged failure experiences of learning disabled children have a profound and lasting effect (13). Students' attitudes toward reading are positively linked to reading improvement. When students are interested in what is being taught and have access to materials that interest them, then learning, and attitudes improve (14). Reading attitude is typically viewed as a multidimensional concept related to the functions of reading. A number of attitudes to reading models of have been proposed (15-16). Across all models, the decision to read is viewed as largely determined by attitudes toward reading. Mathewson (16) supported that attitudes function as a causal agent upon the reading process. The factors that may influence children's positive attitudes toward reading are what the child believes about others' expectations; and what the child believes about his or her reading outcome and the type of prior reading experience. Thus, children's prior beliefs and cognitive-affective knowledge may affect their reading comprehension (17).

Comparisons with low-skilled, without disability students suggest that students with learning disability have negative attitudes toward reading, and some studies exist to support these inferences (10, 18). Nevertheless, there are studies which have documented that students with dyslexia who received reading instruction in special education and resource rooms expressed attitudes to academic and recreational reading that equaled or exceeded those expressed by low and average without disability students, implying that perceptions of ability are important (11). Moreover, when individuals with dyslexia get involved in voluntary reading in areas of personal interest, they improve their reading ability (19).

Many studies have examined differences between students with and without learning disability across multiple domains of functioning and these studies have revealed that their academic failure may affect their self-concept, and adjustment (20-22). Results of these studies have shown that, when compared to peers without learning disability, students with learning disability have lower level of social-emotional difficulties.

Research has been fairly consistent in demonstrating that students with learning disability have a lower self-concept than other categories of students (Bryan, Burstein, & Ergul, 2004). However, studies regarding lower self-concept among students with learning disability are somewhat equivocal (23). There is general consensus that students with learning disabilities show lower self-concept, in particular on school-specific tasks associated with their disability, such as reading (24). Research among students with learning disability particularly has shown that these students tend to attain lower school specific self-concept scores. Bender and Wall (25) suggested that there may be a developmental trend in which students with learning disability demonstrate a lower self-concept, and these students, as they grow up, may learn to think more highly of themselves in general but are predisposed toward maintaining a lower self-concept relative to academic tasks (Bender, 2008).

Several researches have shown that students with learning disability display poorer self-concepts and poorer perceived academic skills than those without disability (Bender & Wall, 1994; Elbaum & Vaughn, 2003a). Furthermore, research suggests that poorer self-concept affects social and academic achievement. Since students with learning disability experience repeated academic failure, disappointments, and frustrations, it is not surprising that many of them have low feelings of self-worth. Some students may even refuse to try a task due to fear of failure. Bryan and Pearl (13) found that the self-concepts of students with learning disability with regard to their academic performance are more negative than those without disability, while their general feelings of self-worth are equivalent to those without disability. Montgomery (26) and Kloomok, and Cosden (27) in their research found that the students with learning disability exhibited self-concepts similar to those students without disability in nonacademic areas, but displayed significantly more negative self-concepts in the areas of academics and skills. A current comprehensive meta-analytic review by Zeleke (28) showed consistent evidence that the school self-concept of students with learning disability is more negative than that of students without disability. However, empirical support on the self-concept of children with learning disability has been less then straightforward, mainly due to methodological difficulties that are reported below.

1. Given the heterogeneity of the dyslexic population and the lack of agreement on the criteria used in identifying students with learning disability, the comparisons across studies are difficult. Furthermore, the selection criteria for the low-achievement groups are also varied, including using the 25th percentile point as a cut-off score to differentiate students with learning disability from those students without

learning disability, or simply using teachers' ratings (28). 2. The majority of studies about learning disability have examined the association between general self-esteem as opposed to specific school self-esteem and achievement. Taking into consideration the multidimensional and hierarchical models of the self (29), it has been supported that this association is stronger when school self-esteem is examined in relation to children with difficulties in literacy. School self-concept is also multidimensional, having components in several academic subjects. In addition, studies on different school settings, for example mainstream schools and special schools have demonstrated that dyslexic children in special schools typically have higher school self-concept as compared to dyslexic students in mainstream schools, consistent with the social comparison theory (30-31). However, this difference is less evident in other areas of self-concept (32).

The aim of this study was to compare dyslexic students in the experimental and control group after the Barton Intervention Program. The research questions are as follows:

1. Does the Barton Intervention Program improve the reading attitude of the dyslexic students in the experimental and control group?
2. Does the Barton intervention program improve the reading self-concept of the dyslexic students in the experimental and control group?

This study is guided by the following research hypotheses:

1. There is a statistically significant difference in reading attitude between the dyslexic students in the control group and the dyslexic students in the experimental group after the Barton Intervention Program.
   - 1.1 There is a statistically significant difference in recreational reading attitude between the dyslexic students in the control group and the dyslexic students in the experimental group after the Barton Intervention Program.
   - 1.2 There is a statistically significant difference in academic reading attitude between the dyslexic students in the control group and the dyslexic students in the experimental group after the Barton Intervention Program.
2. There is a statistically significant difference in reading self-concept (RSC) between the dyslexic students in the control group and the dyslexic students in the experimental group after the Barton Intervention Program.
   - 2.1 There is a statistically significant difference in RSC-competence between the dyslexic students in the control group and the dyslexic students in the experimental group after the Barton Intervention Program.
   - 2.2 There is a statistically significant difference in RSC-difficulty between the dyslexic students in the control group and the dyslexic students in the experimental group after the Barton Intervention Program.
   - 2.3 There is a statistically significant difference in RSC-attitude of the dyslexic students in the control group and the dyslexic students in the experimental group after the Barton Intervention Program.

2. Method and Procedure

In this study, the students in the fourth and fifth grades with dyslexia were identified by first using a questionnaire called the "Dyslexia Screening Instrument". Two 100-word passages with 10 comprehension questions from the students' textbooks were selected and were assigned to the students to read. Their marks were also scrutinized in the first semester and it was found that their marks in the reading skills were lower than the marks of the students without dyslexia. To examine their IQ, Raven's test was performed, and the students with the average IQ higher than 90 made up the population of this research. Finally, 138 dyslexic students in the fourth and fifth grades in elementary schools in Ilam, Iran were selected. The population of the dyslexic students for this study consisted of 40 male and 38 female fifth grade students, and 37 male and 22 female fourth grade students. Their age ranged from 10 to 12 years. The researcher used the table of random numbers to select 64 dyslexic students from the population and assigned them equally into a control and an experimental group, with each group comprising of 32 students. "Reading Attitude" and "Reading Self-Concept" Scales were conducted on both groups. The dyslexic students were given verbal instructions on how to complete the Reading Attitude Scale (33) and Reading Self-Concept Scale (34). The items were read aloud and the students' understanding of the instrument was observed. Assistance was provided when necessary. Demographic variables such as age, gender, and IQ were obtained as well. When the students had completed answering the questionnaire (approximately 40 minutes later), they returned to their respective classrooms.

3. Intervention

The Barton Intervention Program (35) was used in this study. The Barton Reading and Spelling System includes ten levels. Each level is broken into

lessons and each lesson, in turn, is further broken into procedures. In this study, only level one and two were taught with some adjustments. Considering the fact that in the Persian language, there are 26 consonants, 6 vowels, and one digraph, 6 lessons were specified for level two. Like Barton's (2000) program, in the adjustment program, teaching procedures started with the easy level and gradually became more difficult. Since instruction tools were not available in Persian, the researcher provided the necessary tools based on the Barton program. Instruction tools included: 1) color coded letter tiles, 2) word lists, 3) cards, on which one word is written in blue consonants and red vowels respectively, 4) whiteboard, 5) blue and red markers, and 6) a notebook for dictation along with red and blue pencils, erasers and sharpeners. According to Barton's program (2000), level one is taught first. Then, 6 consonants, and one vowel were taught in each session of level two. Sometimes, due to the difficulty of some consonants or vowels, some lessons were repeated for 2 to 4 sessions. Therefore, one by one instruction was done for 36 sessions in 12 weeks, each week with three sessions and each session lasting 45 minutes. It seems necessary to note that students received the intervention in their respective schools, one to one. Instruction time was set by the tutors. If the students could not learn a lesson properly, the lesson would be repeated till she/he learned it.

4. Pilot study

The purpose of carrying out the pilot study was to evaluate the suitability and appropriateness of the use of the instruments. For the pilot study, 30 dyslexic students from Ilam, Iran with similar characteristics were selected randomly to be the participants. The students consisted of 19 males and 11 females in the fourth and fifth grades. This study was carried out from 1st March to 5th March, 2010. Then, the data was entered into SPSS version 17 Windows software to determine the reliability of the scales. The reliability test was applied by calculating the Cronbach's alpha on the variables to measure the inter-item reliability. There was consistency in the following variables: reading attitude and reading self-concept. Internal consistency is usually measured with Cronbach's alpha, a statistic calculated from the pair- wise correlation between items. Internal consistency ranges between zero and one. Cronbach's alpha coefficient of reliability and alpha of 0.70 is normally considered to indicate a reliable set of items (36). Cronbach's alpha reliabilities of the Reading Attitude and Reading Self-Concept Scales were 0.79 and 0.80 respectively. The results of the reliability coefficient showed that there is a high reliability for these instruments, so these instruments were considered appropriate to be employed in this study.

5. Validity

In this study, to ascertain the validity of the Reading Attitude, and Reading Self-Concept Scales, ten psychology experts were asked to grade the scales from 1 to 5. The acceptable degree figures are shown in Table 1 below. Although there is no statistics for content validity, a statistical figure, namely mean was introduced in Table 1. It should be stated that what has been put forward in Table 1 is the acceptability degree criteria among the judges.

Table 1. Judges Rank

Judges	Mean Self-concept	Mean Attitude
1	4.21	4.40
2	4.22	4.40
3	4.19	4.45
4	4.23	4.50
5	4.24	4.50
6	4.29	4.40
7	4.26	4.15
8	4.27	4.35
9	4.32	4.20
10	4.29	4.30

Table 1 show that Juror Rank given by experts based on Cohen (37)

6. Measures

Five instruments were utilized in this research. They are as follows: 1) the Dyslexia Screening Instrument (DSI), 2) Reading Text, 3) Reading Self-Concept Scale (RSCS), 4) Reading Attitude and 5) Raven's Progressive Matrices.

Reading Self-concept Scale: The Reading Self-Concept Scale (RSCS) (34) was used as a measure of reading self-concept. The RSCS contained 30 questions, which were read aloud individually to the dyslexic students who responded on a 5-point Likert

scale (1. Never, 2. Seldom, 3. Sometimes, 4.Often, and 5. Always). Response requirements were taught to the students by means of 4 examples and 10 practice items, which took approximately eight minutes to complete. The RSCS was developed as part of a series of experimental studies in which previous research and theory in the areas of self-concept and reading were drawn upon. The RSCS measures reading and is suitable for ages 6 and above. The Cronbach's alpha coefficient score for the scale is 0.80. The RSCS was individually administered and administration time varied between 15 and 30 minutes for each participant. Each response was scored from 1 (low reading self-concept) to 5 (high reading self-concept) with the total score calculated as the mean value of the 30 responses. Responses to the RSC-difficulty were reverse scored; this means difficulty is actually correlated to easiness in this study. Mean scores for each of the three subscales were calculated in the same manner with a total of four scores calculated; Total-RSCS, Competence, Difficulty and Attitude. In this study, scores on all RSCS sub scales show acceptable reliability (Total-RSCS α=0.88; Attitude α=0.84; Difficulty α=0.71; Competency α=0.78).

Reading Attitude: Mckenna & Kear (33) defined the Elementary Reading Attitude Survey (ERAS) as a 20-item questionnaire that asks students to rate their attitudes toward reading; each item presents a brief, simply worded statement about reading followed by four pictures of the comic strip character, Garfield the Cat in varying pictorial poses. Percentile ranks can be obtained for two component subscales: recreational reading attitude and academic reading attitude. Recreation items focus on reading for fun outside the school setting and the academic subscale examines the school environment and reading of schoolbooks. A total reading attitude percentile rank can also be computed as an additive composite of the recreational and academic scores (33) . Cronbach's alpha, a statistic developed primarily to measure the internal consistency of attitude scales (38) was calculated at each grade level for both subscales and for the composite score. These coefficients ranged from 0.74 to 0. (33). The validity of the academic subscale was tested by examining the relationship of scores to reading ability. Teachers categorized norm-group children as having low, average, or high overall reading ability. The mean of the subscale scores for the high ability readers (M=27.7) significantly exceeded the mean of low ability readers (M=27<0.001); this gave the evidence that scores were reflective of how the students truly felt about reading for academic purposes. In this research, scores on the scale have acceptable reliability (attitude=0.75).

Dyslexia Screening Instrument (DSI): The Dyslexia Screening Instrument (DSI) designed by Coon, Waguespack, and Polk in 1994 consists of checklists of basic neuropsychological skills. This instrument is a rating scale designed to describe the cluster characteristics associated with dyslexia and to discriminate between students who display the cluster characteristics and students who do not. It is designed to measure "entire populations of students or students who exhibit reading, spelling, writing, or language-processing difficulties" (39). The DSI is designed to be used with students in grades 1 through 12 (age 6 to 21). The internal consistency reliability coefficients is 0.99 for elementary students which was determined using Cronbach's coefficient alpha; and the inter rater reliability of the DSI for elementary students is 0.86 which was assessed by determining the homogeneity of the statements and consistency of ratings across examiners. Coon et al (39), stated that "content was based on an extensive review of relevant literature and on experts in the field of dyslexia". Construct validity was supported by the discriminate analysis classifications which placed the elementary and secondary students accurately (98.2% and 98.6% respectively). The DSI scale should be completed by a classroom teacher who has worked directly with the student for at least four months. This will result in a rating that will be more accurate because the teacher has observed the student over a longer period of time and can compare the student's performance to that of the students' classmates. For an elementary student, the preferred rater is the teacher who has instructed the student in a variety of subjects. The teacher should complete the DSI form based the questionnaire answers: Never exhibits, Seldom exhibits, Sometimes exhibits, often exhibits and always exhibits. In this study, the Cronbach's alpha reliability of the scale was 0.89.

Raven's Progressive Matrices test: Raven's Standard Progressive Matrices (RSPM) test was constructed to measure the educative component of g (general IQ) as defined in Spearman's theory of cognitive ability (40). Kaplan and Saccuzzo (41) stated that "research supports the RSPM as a measure of general intelligence. The advanced form of the matrices contains 48 items, presented as one set of 12 (set I), and another set of 36 (set II). Items are presented in black ink on a white background, and become increasingly difficult as progress is made

through each set. These items are appropriate for those aged 5 to 65. Lynn and Vanhanen (42) summarized a number of studies based on normative data for the test which has been collected in 61 countries. The internal consistency reliability estimate for the Raven Progressive Matrices' total raw score was 0.85 in the standardization sample of 929 individuals. This reliability estimate for the revised RSPM indicates that the total raw score on the RSPM possesses "good" internal consistency reliability as provided in the guidelines of the U.S.Department of Education (43) for interpreting a reliability coefficient. The RSPM has been widely used for decades as a measure of educative ability, which is "the ability to evolve high level constructs which make it easier to think about complex situations and events" (44) . In an extensive analysis of the cognitive processes that distinguishes between higher scoring and lower scoring examinees on the Standard Progressive Matrices and Advanced Progressive Matrices, Carpenter, Just and Shall (45) described the Raven's test "a classic test of analytic intelligence". In this research, the Cronbach's alpha reliability of the scale was 0.83.

Reading text: The reading texts were developed by the researcher based on the contents of the fourth and fifth grade textbooks. During the administration of the research, only 80 percent of the textbooks had been taught, and as such, the developed test was based on only 80 percent of the Persian text books. The tests were evaluated by the fourth and fifth grade teachers and after 3 times revisiting they evaluated it as convenient. The test included a story of one-hundred related words understandable to each education level and was followed by 10 questions which indicated the students' level of understanding. The students were required to read out the test aloud and answer the questions. , The Cronbach's alpha was employed to determine reliability. The reliability coefficients for the fourth and fifth grades' reading tests are 0.87 and 0.90 respectively.

7. Findings

SPSS (version 17) was utilized for the analysis of the data. The findings of the study are presented in two parts: descriptive findings and findings related to the hypotheses. In Table 2 and 4, the means, standard deviations, and variables of attitude and self-concept are presented. In Table 2, the means and standard deviations for attitude, recreational reading attitude and academic reading attitude are shown for both the experimental group and control group, before and after the Barton Intervention Program. The findings pertinent to the first research hypothesis are shown in Table 2.

Table 2. Mean and standard deviation for attitude

	Experimental Group		Control Group	
Test	Pretest	Posttest	Pretest	Posttest
Attitude				
M	47.25	67.51	47.66	48.06
SD	6.99	5.02	7.56	12.25
Recreational reading attitude				
M	10.71	35.45	10.73	24.63
SD	3.17	3.38	2.79	5.64
Academic reading attitude				
M	22.45	32.06	22.73	23.43
SD	4.16	2.81	4.87	6.87

The result in Table 2 shows the means and standard deviations to reading attitude, recreational reading attitude, and academic reading attitude, pre and post intervention program. This table shows that there is a significant difference in the posttest means of reading attitude, recreational reading attitude and academic reading attitude for the experimental group and the control group of students with dyslexia after the Barton Intervention Program.

Table 3. t-test results for attitude

	Test	t-value	df	sig
Pretest	Attitude	-0.219	59	0.828
	Recreation	-0.031	59	0.975
	Academic	-0.243	59	0.809
Posttest	Attitude	8.05	59	0.000
	Recreation	9.11	59	0.000
	Academic	6.45	59	0.000

The findings related to the first research hypothesis are shown in Table 3. The first hypothesis is: There is a statistically significant difference in attitude between the dyslexic students in the control group and the dyslexic students in the experimental group after the Barton Intervention Program. Independent t-test was employed to test the first

research hypothesis. As can be seen in Table 3, attitude and its subscales (Recreational and Academic Reading) after the intervention program are statistically significant (t (-0.219, -0.031, -0.243) =8.05, 9.11, 6.45 and ρ<0.000).

Table 4. Mean and standard deviation for self-concept

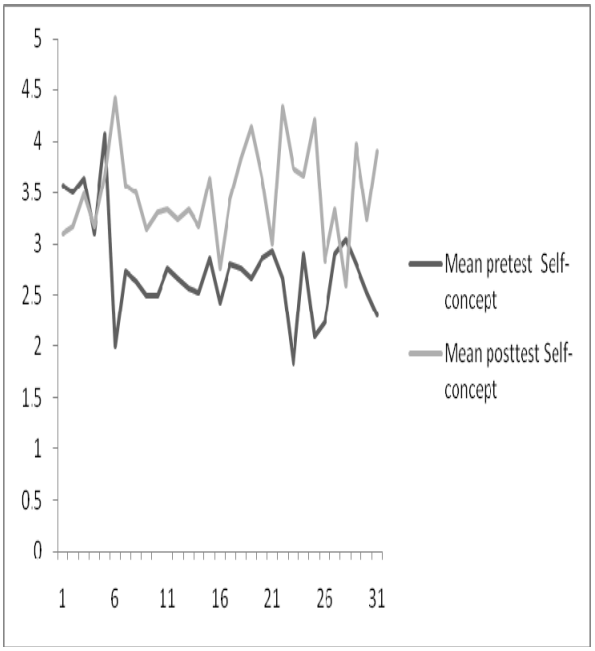
	Experimental Group		Control Group	
Test	Pretest	Posttest	Pretest	Posttest
self-concept				
M	82.64	104.06	83.63	86.63
SD	14.21	13.12	13.71	26.06
Competence				
M	28.74	35.45	28.86	29.56
SD	5.11	5.56	7.13	8.16
Difficulty				
M	26.35	29.79	26.43	27.63
SD	5.48	5.96	3.83	9.14
Attitude				
M	27.54	38.77	27.76	29.41
SD	6.77	5.70	5.39	9.71

Table 4 shows the means and standard deviations for self-concept, competence, difficulty, and attitude pre intervention program and post intervention program. This table shows that there is a significant difference in the posttest means of self-concept, competence, difficulty, and attitude for the experimental group and the control group of students with dyslexia after the Barton Intervention Program.

Table 5. t- test results for self-concept and self-concept subscale

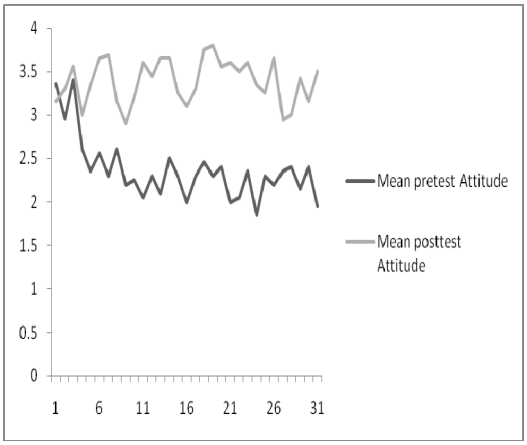
		t-value	df	sig
	Self-concept	-0.276	59	0.783
	Attitude	0.747	59	0.458
Pretest	Competence	-0.227	59	0.821
	Difficulty	-1.888	59	0.064
Posttest	Self-concept	3.316	59	0.002
	Attitude	4.776	58	0.000
	Competence	3.307	58	0.002
	Difficulty	0.396	58	0.694

The findings for the second research hypothesis are presented in Table 5. The second research hypothesis is: There is a statistically significant difference in self-concept between the dyslexic students in the control group and the dyslexic students in the experimental group after the Barton Intervention Program. As can be seen in Table 5, self-concept, competence, difficulty, and attitude are statistically significant [Self-concept (t(0.276=3.316 and ρ<0.002))], [Competence (t(-0.227=3.307 and ρ<0.002))], [Difficulty(t(-1.88=0.396 and ρ<0.694))], and [Attitude(t(-0.747=4.77 and ρ<0.000))]. Based on these results, the second research hypothesis is accepted but the subscale of difficulty is not accepted.



Reading Self-Concept

Figure 1. The pretest and posttest mean scores for self-concept. The mean score for self-concept in the pretest is lower than that of the posttest in the Standard Self-Concept Scale (Chapman & Tunmer, 1995).



Reading attitude.

Figure 2. The pretest and posttest mean scores for reading attitude. The mean score for reading attitude in the pretest is lower than that of the posttest in the Standard Reading Attitude (McKenna & Kear, 1990).

8. Discussion

The present study aimed to investigate the effect of the Barton Intervention Program on the attitude and self-concept of dyslexic students studying at fourth and fifth grades in Ilam, Iran in the academic year 2010. The first research hypothesis is: There is a statistically significant difference in attitude between the students with dyslexia in the control group and the students with dyslexia in the experimental group after the Barton Intervention Program.

The first research hypothesis is confirmed at $\rho<0.000$. The results of the study showed that the intervention program has been effective in increasing the dyslexic students' attitude towards reading. The results of this study are in line with researches such as (35, 46-47) that show intervention programs increase the academic skills of dyslexic students. Such studies show that attitude is an important factor in academic achievement.

According to Gage and Berliner (48), achievement is influenced by attitude as well as ability. "It is a well-known psychological principle that attitude influences a person's choice of activities as well as effort and persistence at tasks" (48). Alexander and Filler (49) identified several variables that seem to be associated with attitudes toward reading. These variables are achievement, the teacher and classroom, special programs and so on. As teachers attempt to improve students' attitudes toward reading, they should keep these ideas in mind; in other words, teachers need to have a positive feeling toward their students, and the students need to be commended for their efforts. The teacher's awareness of the student's attitude toward reading is essential. A student's attitude toward reading materials affects comprehension of those materials. Teachers should be well-informed that students' attitudes toward reading are formed by parents and their home environment. Studies show that reading attitude is affected by academic achievement. As such, having a positive attitude toward reading may ensure a student's success in his academic endeavor.

According to Johnson (50), attitudes toward reading are arguably formed as a result of success achievement or failure with the task of reading; therefore, students with good reading ability may have positive attitudes toward reading, while students who are poor readers often have to overcome negative reading attitudes in order to improve their reading skills. The finding of the study suggests that since the intervention program results in academic achievement of the dyslexic students, therefore, participation of the dyslexic students on a one-to-one basis in the intervention program would increase the individual capabilities of this group of students.

The second research hypothesis is: There is a statistically significant difference in self-concept between the students with dyslexia in the control group and the students with dyslexia in the experimental group after the Barton Intervention Program. The second research hypothesis is confirmed at $\rho<0.000$. In this study, it is shown that the use of the intervention program increased the self-concept of the dyslexic students; in comparison to the students with dyslexia in the control group, the dyslexic students in the experimental group performed better after the intervention program. This study is in line with researches (35, 46-47, 51-53) that show intervention programs would increase the skills and academic performance of dyslexic students. The result of the meta-analysis by Elbaum and Vaughn (54) showed that the intervention program could lead to beneficial changes in the self-perception of students with learning disabilities. The investigators noted that these findings are particularly important in light of the fact that the intervention lasted less than 12 weeks with sessions held only two or three times a week.

Studies such as Elbaum and Vaughn (55),; Davis (47); Barton (35); Torgesen (46), have shown that academic failure of students with learning disability would result in negative feelings in these students. Since the intervention program increased the academic achievement of dyslexic students (35, 46-47, 51) and since academic achievement is related to self-concept (56), it is necessary to use the intervention program in order to increase the

academic achievement and consequently, the self-concept of dyslexic students. As mentioned above, dyslexic students need a special educational program to acquire sufficient skills to complete their school assignments. Therefore, it is suggested that before running any educational program, they should be investigated regarding their ability to use the Barton educational program.

9. Recommendation for future research

Future researchers are recommended to investigate the role of such variables, for instance perception, visual memory, auditory memory, movement harmony, finding spatial direction, accuracy, creativity, and innovation on dyslexic students. Since this study was conducted on dyslexic students only and its sample was just the fourth and fifth grade students, therefore, a similar study is suggested to be carried out on other groups of students with learning disorders and other school grades. Finally, it is recommended that education officials familiarize themselves with the Barton program and carry out this method with larger groups and in several educational centers so that if positive results are observed, this method could be utilized to overcome the learning difficulties faced by dyslexic students.

10. Conclusion

The data displayed showed that the Barton intervention program does increase the students' attitude and self-concept as a result of participation and, therefore, the hypothesis is accepted. The multisensory Barton Reading and Spelling System were used on the dyslexic students in the experimental group and it improved the attitude and self-concept of these students in comparison to those students in the control group. In the final analysis, the researcher advocates the use of Strategic Barton instruction and training programs for teachers because it would be beneficial for all students.

References

1. Lerner J. Learning disabilities and related disorders, characteristics and teaching strategies. Boston: Houghton Mifflin Company; 2006.
2. Chapman JW, Tunmer WE, Prochnow JE. Early reading-related skills and performance, reading self-concept, and the development of academic self-concept: A longitudinal study. Journal of Educational Psychology. 2000;92:703-8.
3. Valas H. Students with learning disabilities and low achieving students: Peer acceptance. loneliness, self-esteem, and depression. Social Psychology of Education. 1999;3:173-92.
4. McGrady H, Lerner J, Boscardin M. The educational lives of students with learning disabilities. Boston: Allyn & Bacon.; 2001.
5. Smith MC. A longitudinal investigation of reading attitude development from childhood to adulthood. The Journal of Educational Research. 1990;83:215-9.
6. Paris, Olson, Stevenson. Learning and motivation in the classroom. Publisher: Hillsdale; 1993.
7. Richeck MA, List LK, Lerner J. Increasing the achievement of your remedial reading students. Paso Robles, CA: Bureau of Education and Research; 1989.
8. Lipson MY, Wixson KK. Assessment and instruction of reading disability an interactive approach. New York: Harper Collins; 1992.
9. Mullis IV, Jenkins LB. The reading report card, 1971–1988: Trends from the nation's report card: National Assessment of Educational Progress, Educational Testing Service, Princeton, NJ; 1990 Contract No.: Document Number|.
10. Polychroni F, Koukoura K, Anagnostou I. Academic self-concept, reading attitude and approaches to learning of children with dyslexia: do they differ from their peers? European Journal of Special Needs Education. 2006;21(4):415-30.
11. Lazarus DB, Callahan T. Attitudes toward reading expressed by elementary school students diagnosed with learning disabilities. Journal of Reading Psychology. 2000;21(4):271-82.
12. Rogers H, Saklofske DH. Self-concepts, locus of control and performance. Expectations of Learning Disabled Children. 1985;18:273-8.
13. Bryan TS, Pearl R. Self-concept and locus of control of learning disabled children. Journal of Clinical and Child Psychology. 1979;8:223-6.
14. Hidi S. Interest and its contribution as a mental resource for learning. Review of Educational Research. 1991;60(Journal Article):549-71.
15. Greaney V, Neuman SB. The functions of reading: a cross- cultural perspective. Reading Research Quartery. 1990;25(Journal Article):172-95.
16. Mathewson G. Model of attitude influence upon reading and learning to read, In: R. B. Ruddell, M. R. Ruddell & H. Singer (Eds.) Theoretical models and processes of reading (4th ed). Newark, DE: International Reading, 1131- 1161; 1994.
17. Ruddell RB, Unrau NJ. Reading as a meaning construction process: The reader, the text, and the teacher,In: R. B. Ruddell, M.R. Ruddell & H. Singer (Eds) theoretical models and processes of

reading (4th ed). Newark, DE: International reading association, 996-105; 1994.
18. van Kraayenoord CE, Schneider WE. Reading achievement, metacognition, reading self-concept and interest: a study of German students in grade 3 and 4. European Journal of Psychology of Education. 1999;14:305-24.
19. Fink R. Successful dyslexics: a constructivist study of passionate interest reading. Journal of Adolescent and Adult Literacy. 1995(39):268-80.
20. Elbum B, Vaughn S. For which students with learning disabilities are self- concept intervention effective? Journal of Learning Disabilities. 2003;36,2(Journal Article):101.
21. Heath N, Winener J. Depression and nonacademic self-perception in children with and without learning disabilities. Journal of Learning Disabilities Quarterly. 1996;9:34-44.
22. Wiener J. Do peer relationships foster behavioral adjustment in children with learning disabilities? jounal of Learning Disabilities Quarterly. 2004;27:21-30.
23. Meltzer L, Roditi B, Houser RF, Perlman M. Perceptions of academic strategies and compertence in students with learning disabilities. Journal of Learning Disabilities. 1998;31:437-51.
24. Gans AM, Kenny MC, Ghany DL. Comparing the self-concept of students with and without learning disabilities. Journal of Learning Disabilities. 2004;36(3):287-95.
25. Bender WN, Wall ME. Social-emotional development of students with learning disabilities. Learning Disability Quarterly [serial on the Internet]. 1994; 17.
26. Montgomery MS. Self-concept and children with learning disabilities: observer-child concordance across six context- dependent domains. Journal of Learning Disabilities. 1994;27:254-62.
27. Kloomok S, Cosden M. Self-concept in children with learning disabilities: The relationship between global self-concept, academic "discounting," nonacademic self-concept, and perceived social support. Learning Disability Quarterly. 1994;17:140-53.
28. Zeleke S. Self-concepts of students with learning disabilities and their normally achieving peers: a critical review. European Journal of Special Need Education. 2004;19(2):145-70.
29. Marsh HW. A multidimensional, hierarchical self-concept: theoretical and empirical justification. Educational psychology review. 1990;2:77-172.
30. Crozier WR, Ress V, Morris-Beattie A, Bellin W. Streaming self esteem and friendships within a comprehensive school. Educational Psychology in Practice. 1999;15(2):128-34.
31. Humphrey N. Teacher and pupil ratings of self-esteem in developmental dyslexia: the role of teachers and peers. British Journal of special education. 2002;29:29-39.
32. Bear GG, Minke KM, Manning MA. Self-concept of students with learning disabilities: a meta analysis, School Psychology Review. School Psychology Review. 2002;31(3):405-27.
33. McKenna MC, Kear DJ. Measuring attitude toward reading: A new tool for teachers. The Reading Teacher. 1990;43:626-39.
34. Chapman JW, Tunmer WE. Reading self-concept scale. In R. burden (Ed.), self-perceptions. Windsor, England: Nfer-Nelson; 1995.
35. Barton S. Barton Reading & Spelling System, An Orton-Gillingham Influenced Multisensory Structured, Explicit, and Systematic Phonics System. San Jose, CA: Bright Solutions for Dyslexia, LLC; 2000.
36. De vaus D. Analysing social science data: Sage Ltd; 1st edition; 2002.
37. Cohen RJ. Exercises in psychological testing and assessment, an introduction to tests and measurement. United Stat of America: McGraw-Hill; 2005.
38. Cronbach LJ. Coefficient alpha and the internal structure of tests. Psychometrika. 1951;16(3):297-334.
39. Coon KB, Waguespack MM, Polk MJ. Dyslexia screening instrument. San Antonio: TX; The Psychological Crporation: Harcourt Brace & Company; 1994.
40. Raven JRJC, Court JH. Raven manual: Section 1, general overview: Oxford, UK; 1998.
41. Kaplan RM, Saccuzzo DP. Psychological testing: Principles, applications, and issues (4th ed.) Company. Pacific Grover: Brooks Publishing; 1997.
42. Lynn R, Vanhanen. IQ and the wealth of nations. Westport CT: praeger; 2002.
43. Education USDo. Final regulation for the 1997 individuals with disabilities education act. federal register1999.
44. Raven J, Raven JC, Court JH. Raven manual: Section 4, advanced progressive matrices. Oxford, UK: Oxford Psychologists Press ltd; 1998.
45. Carpenter PA, Just MA, Shell P. What one intelligence test measures: A theoretical account of the processing in the raven progressive matrices test. Psychological review. 1990;97:404-31.
46. Torgesen JK. Recent discoveries from research on remedial interventions for children with dyslexia. In M. Snowling and C. Hulme (Eds.).

Presentations and Publications. (pp. 521-537): Oxford: Blackwell Publishers. (106 KB) 2005.
47. Davis RD. The Gift of Dyslexia. New York: Berkley Pub; 1994.
48. Gage NL, Berliner DC. Educational psychology (6th ed.). Boston: Houghton Mifflin; 1998.
49. Alexander, Filler. What Does Research Say About the Reading Interests of Children in the Intermediate Grades? The Reading Teacher. 1980;31:763-8.
50. Johnson LS. Naturally acquired learned help-lessness: the relation ship of school failure to achievement behavior, attributions, and self-concept. Journal of Educational Psychology. 1981;73:174-80.
51. Francis DJSSESKKSBAFJM. Developmental lagversus deficit models of reading disability: A longitudinal, individual growth curves analysis. Journal of Educational Psychology. 1996;88(Journal Article):3-17.
52. Foorman BR. Preventing and remediating reading difficulties. New York: York Press; 2003.
53. Blachman Bascfjmcsm. Early reading intervention: A classroom prevention study and a remediation study. In, B. R. Foorman (Ed). Preventing and Remediating Reading Difficulties: Bringing Science to Scale. New York: Timonium, MD. Press, 253-271; 2003.
54. Elbaum B, Vaughn S. Self-concept and students with learning disabilities. In H.L. Swanson, K.R. Harris, & S. Graham (Eds.) Handbook of learning disabilities. (pp. 229-241). New York: The Guilford Press; 2003.
55. Elbaum B, Vaughn S. For which students with learning disabilities are self- concept interventions effective? Journal of Learning Disabilities. 2003;36:101-8.
56. Harter S. Causes and consequences of low self-esteem in children and adolescents. In R. F. Baumeister (Ed.). Self-esteem.The puzzle of low self-regard (pp.87-116). New York: Plenum Press; 1993.

11/7/2010

Evaluation of two different implant designs for immediate placement and loading in fresh extraction sockets

Amr Zahran[1], Hisham Samy[2], Basma Mostafa [3], Ramy Rafik[4]

1. Professor, Department of Periodontology, Cairo University, Cairo, Egypt
2. Assistant Professor, Department of Periodontology, Cairo University, Cairo, Egypt
3. Researcher, Department of Surgery and Oral Medicine, National Research Centre, Cairo, Egypt
4. Assistant Researcher, Department of Surgery and Oral Medicine, National Research Centre, Cairo, Egypt

Corresponding author: Dr. Basma Mostafa Zaki, BDS, MDS, PhD
Researcher, Department of Surgery and Oral Medicine, National Research Centre,
E-mail: boshta@hotmail.com

Abstract: This study was conducted to compare between two self-tapping, self-drilling tapered one-piece implant designs used for immediate post-extraction placement with the immediate loading protocol.

Materials and Methods: Ten patients (6 males and 4 females), with a mean age of 28.5 years (range 18-39 years) were included in this study. All selected patients had two or more maxillary unrestorable hopeless anterior or premolar teeth indicated for extraction. Each patient received two implants of different designs (The OsteoCare™ Midi and Maxi-Z implants) which were placed in fresh extraction sockets and immediately loaded. Clinical criteria were survival rate, papillary bleeding index, probing depth, gingival index, Periotest M values, crestal bone level and bone density. An overall survival rate of 100% was attained. **The results** showed no significant difference in both the bleeding index and gingival index scores and also in the probing depth values, bone density measurements and crestal bone level for both implant designs after 3 and 6 months. The mean and the standard deviation of the Periotest M values (PTMV) for the Midi and the Maxi-Z implants immediately post operative were (-1.83±0.8) and (-2.57±0.9) and after 6 months were (-3.06±0.7) and (-3.11±0.7) showing a significant difference immediately postoperative and no significant difference after 6 months. Surface area analysis revealed that there is a direct relation between the initial stability and the surface area. **Conclusion**: It can be concluded that the immediate implant placement and loading using both designs is a successful treatment modality and the prognosis depends on proper case selection and treatment planning.

[Amr Zahran, Hisham Samy, Basma Mostafa, Ramy Rafik. **Evaluation of two different implant designs for immediate placement and loading in fresh extraction sockets**. Journal of American Science 2010;6(12):1192-1199. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Dental implants, immediate implant, immediate loading, two implant designs

1-Introduction:

One of the most important significant scientific breakthroughs in clinical dentistry was undoubtedly the introduction of osseointegrated implants 40 years ago (Fischer 2008). The original protocol was described by Branemark who described the two-stage surgical protocol which involves the surgical placement followed by the surgical uncovering of an implant. A healing period of 3-6 months after tooth extraction to allow for bone filling and contouring before implant placement was required, (Branemark 1977; Adell et al.; 1981).

Investigations showed that significant bone volume changes of the alveolar process take place following tooth extraction (Denissen et al.; 1994; Araujo and Lindhe 2009). It was reported that there is a 50 % reduction in bucco-lingual width of the extraction socket over a period of twelve months with two thirds of the reduction taking place during the first three months and a reduction of crestal bone level ranging from 0.7 to 1.5 mm after four to six months (Schropp et al.; 2003).

Thus, immediate post extraction implant placement into fresh extraction sockets is considered a predictable and accepted procedure of preserving the alveolar dimensions, with its consequences of better crown-implant ratio, improved soft tissue esthetics and favourable inter-arch relationship (Schulte et al.,1978; Rosenquist and Grenthe 1996; Sclar 2003; Oh et al.; 2006 and Lee et al.; 2009). Immediate implant placement has also been reported to have the advantage of reducing the treatment time required with the reduction of the number of surgeries (Gapski et al., 2003; Lorenzoni et al.; 2003; Testori et al.; 2004; Tsirlis 2005 and Wang et al.; 2006).With the improvement of implant design regarding the surface treatments and thread designs which has the purpose of achieving better primary stability and osseointegration, immediate loading became more popular and many authors have reported a high success rate with this

technique (Kahnberg 2009 and Guirado et al.; 2010).

Recent researches reported that there are three options for implant loading: conventional staged loading protocol in which the implant is loaded after insertion by 3-8 months (Esposito et al.; 2007) immediate loading protocol in which the implants are immediately loaded after insertion or within a week after placement, (Glauser et al.; 2001; Degidi et al.; 2003; De Bruyn and Collaert 2002) while early loading protocol allows the implant to be loaded after insertion by 1 week to 2 months (De Bruyn and Collaert 2002; Attard and Zarb 2005).

The combination of immediate post-extraction placement with immediate loading of dental implants has the advantage of shortening the treatment time and increasing case acceptance and reported to be safe in terms of survival rates and esthetics (Cooper et al.; 2002; Crespi et al.; 2007 and Oh et al.; 2007). An overall survival rate of 97.5 % to 98 % was reported for implant immediately loaded after placement (Calandriello et al.; 2003; Lorenzoni et al.; 2003; Drago and Lazzara 2004; Degidi et al.; 2005 and Zahran 2008).

Both the Midi and the Maxi-Z implants are machined from a piece of titanium alloy that incorporates both the implant body and an integral post or ball fixed abutment in a single component. These implants are designed with a "Buttress" thread design that has the advantage of allowing for the compression and expansion of the implant site to achieve high stability in even poor quality bone. They have grit-blasted and acid-etched (GBA) surface treatment. The conical macro-design of the Mini implants allows their placement in limited tooth-to-tooth spacing and atrophic ridges (Zahran 2008). Maxi-Z implants have a tapered body geometry which has the ability to distribute forces into the surrounding bone, thereby creating a uniform compaction in adjacent osteotomy walls when compared with parallel-walled implants. The unique design of both implants allows their placement with minimally invasive flapless procedures. Both designs the Midi and the Maxi-Z implants are tailored for immediate loading and allow for the provision of same day restorations following the concept of "a tooth in a day" (Zahran and Gauld 2007).

2.Material and Methods

2.1. Materials:

2.1.1. Subjects:

Ten patients (6 males and 4 females), with a mean age of 28.5 years (range 18-39 years) were consecutively included in this study.

All selected patients had two or more maxillary unrestorable hopeless anterior or premolar teeth indicated for extraction due to root fracture, endodontic failure or unrestorable crown fracture. The patients were required to be in good health, and had no condition that might affect the outcome of the treatment.

All patients participated in the study were thoroughly informed of the immediate loading protocol and all the risks associated with this type of procedure and signed an informed consent form.

2.1.2. Implants

Ten Midi implants (strictly, conical in shape) and ten Maxi-Z implants (tapered in shape) (OsteoCare™ Implant System, London, United Kingdom) were used in this study and placed in ten patients so that each patient received both designs.

2.2. Methods:

2.2.1. Pre-surgery evaluation:

Pre-surgical radiographic evaluation with panoramic and periapical radiographs (using standardized parallel techniques) was carried out.

All patients received oral hygiene instructions and periodontal treatment if needed.

2.2.2. Surgical Protocol and implant placement

After administration of local anaesthesia, periodontal ligament was excised using periotome, followed by careful a traumatic tooth extraction using the forceps to deliver the tooth out. After extraction, the integrity of the buccal plate of bone was checked using an osteotomy probe through the fresh extraction socket as intact buccal plate of bone was considered crucial.

The extracted roots were measured in bucco-palatal and mesio-distal dimensions at the middle third using a digital calliper and the readings were averaged, to determine the correct implant diameter. The length of the implant was obtained from the panoramic radiographs using radiographic stents.

Under copious saline irrigation to prevent heat generation and damage of bone, the 1.3 mm profile pilot drill was used to give needle point accuracy for position, angle and depth.

The osteotomy preparation extended three to five millimetres beyond the base of the extraction socket to achieve good primary stability for the implant. While for the Maxi-Z implant, when harder bone density was met, sequential drilling was performed using the 2.2 mm and the 2.75 mm drills to facilitate easier insertion of the implant without exerting undue pressure on the bone.

The type of implant was selected according to the size of the extraction socket:

-The Maxi-Z implants were always selected for the larger socket.

-In cases of equally sized sockets the implants were selected randomly.

The implant was removed from its sterile protective pouch and held using the attached plastic carrier and placed into the prepared socket and screwed

manually until a resistance was met. The plastic carrier was removed and the ratchet wrench and the hex driver were used for complete seating of the implant into its final position. Both the collar of the Midi implant and the first thread of the Maxi-Z implant were placed 3 mm below the crestal bone level confirmed by the periapical radiographs.

Establishment of primary stability of over 30N/cm was considered crucial with all the placed implants in the extraction sockets to allow for the immediate loading protocol. Primary stability of the implants was evaluated by the torque wrench.

2.2.3. Abutment Preparation and Provisional Restoration:

Immediately after implant placement, the abutment was prepared using either carbide or diamond burs with copious water irrigation to avoid overheating. Then, a temporary crown was fabricated and cemented to be completely out of functional occlusion in centric and eccentric position. The patients were instructed to avoid direct biting on the provisional restoration.

2.2.4. Post-operative care:

Oral hygiene instructions were given to the patients. Analgesics were subscribed to prevent post-surgical pain when necessary. Finally, a periapical radiograph was taken to check the final implant position and to estimate the initial bone level around the implant.

Final restorations:

The provisional acrylic resin restorations were removed after a healing period of 6 months. Final porcelain-fused-to-metal restorations were constructed and permanently cemented and checked for shade matching, marginal fitness and occlusion.

2.2.5 Post operative follow-ups and evaluation

2.2.5.1. Clinical records

Clinical records were obtained at 3 and 6 months post-operatively.

- Bleeding on probing was evaluated using papillary bleeding index (PBI) described by Muhlemann (1977) using a periodontal probe.
- Infection, swelling and gingival inflammation were assessed using the gingival index (GI) according to Loe and Silness (1963).
- Probing Depth was measured according to a standard procedure described by Glavind and Loe (1967) using periodontal probe with Williams' calibrations.
- Mobility was tested using the Periotest M (Medizintechnik Gulden, Bensheim, Germany). Loose implants show high Periotest M values, while osseointegrated implants have low Periotest M values. Periotest M values (PTMV) of (-8 to 0) were considered the ideal values that denote successful osseointegration.

2.2.5.2. Radiographic evaluations:

Standardized periapical x-rays films were taken immediately after implant insertion, three and six months post operatively to detect any change in crestal bone level and bone density around the implant using the linear measurement system of Digora software (Orion Corporation, Sordex, Finland).

2.2.5.3. Implant surface area measurements:

The surface area of the Midi and the Maxi-Z implants was measured using a 3D scanner to perform a 3D image which is then analyzed and the surface area was calculated using another program (AutoCAD 2004), to compare between the two different geometric features of the two implant designs and evaluate its effect on the primary stability.

2.2.6. Statistical Analysis

Data were presented as mean and standard deviation (SD) values. Data were explored for normality using D' Agostino and Pearson normality test. Paired t-test was used to compare between the two implant designs. The significance level was set at $P \leq 0.05$. Statistical analysis was performed with GRAPHPAD PRISM 5 for Windows.

3. Results

3.1. Complete soft tissue healing had occurred in all patients without any postoperative inconveniences during the study period.

3.2. The provisional acrylic resin restoration became loose in one patient in the fourth month after implant placement and was re-cemented in the same day.

3.3. All the 20 implants were successfully osseointegrated as revealed by clinical and radiographic examinations.

3.4. Implant survival rate of 100% was attested.

3.5. Clinically:

Results showed that the mean and the standard deviation of the papillary bleeding index after 3 months was (1.65 ±0.2) for the two implant designs and then after 6 months it was (1.4 ±0.2). There was no significant difference found, P_3months = 1.0000 and P_6months = 1.0000.

The mean and the standard deviation of the probing depth for the Midi and the Maxi-Z implants after 3 months were (3.6 ± 0.4) and (3.65 ± 0.4) respectively then at 6 months were (3.5±0.5) and (3.3 ± 0.4) with no significant difference found, P_3 months = 0.8144 and P_6 months = 0.9074).

The mean and the standard deviation of the Gingival index scores for the Midi and the Maxi-Z implants after 3 months were (1 ± 0.3) and (1.1 ± 0.3) respectively, then after 6 months were (0.82 ± 0.3) and (0.87 ± 0.3). There was no significant difference found, (P_3months = 0.6193 and P_6months = 0.6193). This means that the difference in implant design does not affect the bleeding index scores, the gingival index scores

and the pocket depth values after 3 months and also after 6 months.

The mean and the standard deviation of the Periotest M values (PTMV) for the Midi and the Maxi-Z implants immediately post-operative were (-1.83 + 0.8) and (-2.57 + 0.9) and after 6 months were (-3.06 + 0.7) and (-3.11 + 0.7). There was a significant difference found in the Periotest M values immediately post-operative which means that the difference in implant design does affect the PTMV immediately post-operative (P_immediate post-operative = 0.0122). There was no significant difference found after 6 months which means that the difference in implant design does not affect the PTMV in the second stage, P_6months = 0.8553).

Table (1): Correlation coefficients for the two implant designs.

Correlation coefficients	Initial stability	After 6 months
Surface area of Midi implant	0.8920912	0.7340346
Surface area of Maxi-Z implant	0.7824797	0.6114319

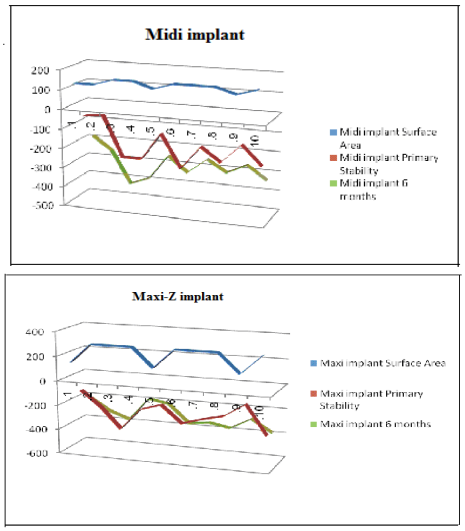


Diagram (1): showing the Midi and Maxi-Z implant surface areas, initial stability and stability after 6 months.

3.6. Radiographic evaluation:

Results revealed that the mean and the standard deviation of the crestal bone resorption for the Midi implants versus the Maxi-Z implants were (0.5 ± 0.3) and (0.6 ± 0.3) after 3 months, and was (0.67±0.3) after 6 months for the two implant designs. There was no significant difference found which means that the change in the bone level around the two implant designs was nearly the same after 3 months and after 6 months (P_3months = 0.1217andP_6months = 0.2848).

The mean and the standard deviation of the bone density values for the Midi and the Maxi-Z implants were (89.7 ± 2.1) and (88.1 ± 1.3) immediately post-operative, and after 3 months were (75.1 ± 0.84) and (76.4 ±1.6), and finally were (76.4 ± 0.7) and (76.9 ± 1.3) respectively after 6 months. There was no significant difference found which means that the difference in implant design does not affect the bone density immediately post-operative, after 3 months and after 6 months (P-immediate post-operative = 0.1075, P_3months = 0.0801, P_6months =0.3691).

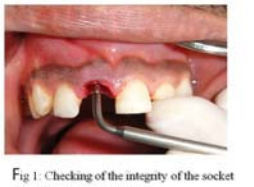


Fig 1: Checking of the integrity of the socket using the osteotomy probe


Fig 2: Clinical photograph showing implant immediately after placemen

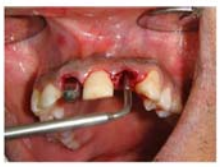


Fig 3: Checking of the integrity of the second socket using the osteotomy probe

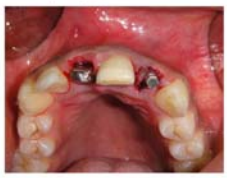


Fig 4: Clinical photograph showing both implants immediately after placement


Fig 5: Final ceramo-metal restoraton


Fig 6: Immediate postoperative panoramic radiograph

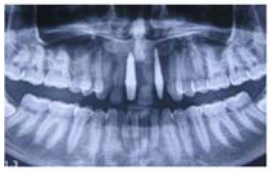


Fig 7: 3 months post operative panoramic radiograph

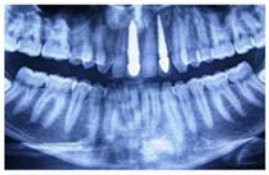


Fig 8: 6 months post operative panoramic radiograph with final crowns.

Surface area analysis showed that the calculated correlation coefficient range for the two implant designs was between 0-1 which indicated that the two variables tended to increase or decrease together. This means that there was a direct relation between the initial stability and the surface area.

4. Discussion

Success of osseointegrated implants has been validated for over 30 years as a viable alternative to fixed or removable prosthetic restorations (Albrektsson et al.; 1988; Buser et al.; 1997 and Szmukler-Moncler et al.; 2000). It has been advocated that after implant placement, surgical site should be undisturbed for at least 3-6 months depending on bone quality to allow for osseointegration. This waiting period may cause

functional and psychological problems to the patients (Chiapasco et al.; 1997 and Andersen et al.; 2002).

Several studies documented the success of the protocol of immediate implant placement in fresh extraction sockets in conjunction with immediate loading (Muhlemann 1977 and Oh et al.; 2006). Research during the last 20 years has increasingly focused on immediate loading of dental implants (Fischer 2008).

The immediate loading procedure has become a routine in the treatment of totally or partially edentulous patients and permits delivery of provisional fixed restorations the same day of implant placement (Barzilay 1993; Hahn 2000; Gapski et al.; 2003; Lorenzoni et al.; 2003 and Misch et al.; 2004a). A number of factors may influence the results of immediate implant loading. These factors could be related to the surgical procedures, patient, implant design or occlusion-related factors (Gapski et al.; 2003; Misch et al.; 2004 a,b and Zahran 2008) .

This technique is increasingly gaining popularity as an attractive advantage for both patients and clinicians alike. Today, quick delivery of implant-supported restorations immediately after extraction can be considered the standard of care in case of a missing tooth or teeth.

The present study was conducted to compare between two different implant designs for immediate placement and loading in fresh extraction sockets. All the implants were successfully osseointegrated over the six months follow-up period with a success rate of 100% with insignificant change in the crestal bone level.

The current results showed nearly similar results as that reported by (Kaj et al.; 2007) in which three implants were lost resulting in a cumulative survival rate of 97.9% after up to two years. The higher success rate which was noticed in the current study was probably attributed to the smaller sample size or the strict case selection. The results were also in agreement with those presented by Lorenzoni et al.; 2003, who evaluated the clinical outcomes of immediately loaded implants after one year of placement in the maxillary incisor region, resulting in a 100% survival rate. The results are also in agreement with Zahran et al., 2010 which evaluated the flapless immediate implant placement in fresh extraction sockets using the one piece Maxi-Z implant.

In the present study, the mobility of all implants was measured using the Periotest M immediately after placement (base line) and at 6 months post-operatively in the two implant designs. There was a significant difference between the mean Periotest values for the two implant designs at the base line but the difference was insignificant after the 6 months follow up period. This is in agreement with Orenstein et al.; 2007, who performed a study evaluating the stability of the immediately placed and immediately loaded implants using the Periotest. It was concluded that the stability of the implant through the period of the study followed the sequence of socket healing and bone remodelling.

It was observed in the present study that the initial stability attained by the Maxi-Z implant was higher than that of the Midi which was measured by the Periotest M. This could be due to the surface area of the Maxi-Z implant which is higher than that of the Midi implant. This difference in the surface area could be attributed to the modification in the body geometry of the Maxi-Z or its wider range of diameters. This coincides with the study of Langer et al.; 1993, who proposed the use of wide diameter (5.0 mm) self tapping implants to gain initial stability in the jaw bone region where low-density bone is common. The authors hypothesized that the increased contact obtained with a wider implant improved the engagement of bone and reduced the initial mobility. Increasing the diameter in a 3 mm implant by 1mm increases the surface area by 35% over the same length in overall surface. More contact area provides increased initial stability and resistance to stresses as reported by Misch 1999.

In the present study the Maxi-Z implants attained higher initial stability in wide extraction sockets than the Midi implants which in agreement with the results reported also by Jae et al.; 2005.

In the present study the bone density changes around the implants was measured. It was found that there was no significant difference in the bone densities around the two implant designs. This may be attributed to the compression of the bone trabecluae around the implants which is nearly the same for the Midi versus the Maxi-Z implants which is in similarity with a comparative study performed to evaluate implants placed in healed bony sites versus extraction sites (Diago et al.; 2008).

The first thread of the implants used in this study was placed 3 mm below the crestal bone level of the extraction sockets and this could be the reason for the minimal crestal bone resorption that occurred during the 6 months follow-up period of this study. Other studies recommended placement of the implants with their platforms below the level of the socket by 1-2mm (Lazzara 1989; Becker 2006 and Orenstein et al.; 2007).

Conclusion

Both the Midi implant and the Maxi-Z implant can be placed immediately after extraction and immediately loaded showing a 100% clinical success. The Maxi-Z implant is more suitable for bigger extraction sockets due to its wide range of diameter and its body geometry that nearly fills all the jumping gaps with better primary stability.

There is a direct correlation between the surface area and the initial stability.

5. Refrences

Adell, R., Lekholm U., Rockler B., Branemark P.I. 1981. A 15-year study of osseointegrated implants in the treatment of the edentulous jaw. Int J Oral Surgery; 10: 387–416

Araujo, M., Lindhe J. 2009. Ridge alterations following tooth extraction with and without flap elevation: an experimental study in the dog. Clin. Oral Impl. Res.; 20: 545–549.

Attard, N.J., Zarb, G.A. 2005. Immediate and early implant loading protocols: A literature review of clinical studies. J Prosthet Dent.; 94:242–248.

Andersen, E., Haanoes, H. and Knutsen, B. 2002. Immediate loading of single tooth ITI implants in the anterior maxilla: A prospective 5- year pilot study. Clin Oral Imp Res; 13:281-287.

Albrektsson, T., Dahl, E. and Enbom, L. 1988.Osseointegrated oral implants. J Perio; 59: 287-296.
Barzilay, I. 1993. Immediate implants: their current status. Int J Prosthodont; 6(2):169–175.

Becker, W. and Goldstein M. 2008. Immediate implant placement: treatment planning and surgical steps for successful outcomes. Periodontology 2000.; 47: 79–89.

Becker, W. 2006. Immediate implant placement: treatment planning and surgical steps for successful outcomes. British Dental Journal; 201: 199-205.

Branemark, P.I. 1977. Osseointegrated implants in the treatment of the edentulous jaw: experience from a 10 year period; Scand J of Plastic and Reconstructive Surgery.; 16: 1-13

Buser, D., Mericske, R. and Bernard, J. 1997. Long-term evaluation of non-submerged ITI implants. Part I: 8-year life table analysis of a prospective multi-center study with 2359 implants. Clin Oral Impl Res; 8: 161-172.

Cavicchia, F., Bravi, F. 1999. Case reports offer a challenge to treatment strategies for immediate implants. Int J Periodontics & Restorative Dent.; 19:66-81.

Calandriello, R., Tomatis, M. and Rangert, B. 2003. Immediate functional loading of Brånemark System Implants with enhanced initial stability:A prospective 1-to 2- year clinical and radiographic study. Clin Implant Dent Relat Res; 1:10-20.

Chiapasco, M., Gatti, C., Rossi, E., Haefliger, W. and Markwalder, TH. 1997. Implant-retained mandibular overdentures with immediate loading. A retrospective multicenter study on 226 consecutive cases. Clinical Oral Implants Research; 8:48–57.

Chen, S.T., Wilson, T.G. and Hammerle, C.H. 2004. Immediate or early placement of implants following tooth extraction: review of biologic basis, clinical procedures, and outcomes. International Journal of Oral & Maxillofacial Implants; 19: 12–25

Cooper, L., Rahman, A., Moriarty, J., Chaffee, N., Sacco, D. 2002. Immediate Mandibular Rehabilitation with Endosseous Implants: Simultaneous Extraction, Implant Placement, and Loading, Int J Oral Maxillofac Implants.;17:517–525.

Crespi, R., Capparè, P., Gherlone, E., Romanos, G. 2007. Immediate Occlusal Loading of Implants Placed in Fresh Sockets After Tooth Extraction, Int J Oral Maxillofac Implants.;22:955–962.

Denissen, H.W., Kalk W., Veldhuis H.A., Van Waas M.A.1993. Anatomic consideration for preventive implantation. Int J Oral Maxillofacial Implants, 82:191–196

Degidi, M., Scarano, A., Petrene, G., Piattelli, A. 2003. Histological analysis of chemically retrieved immediately loaded titanium implants: a report of 11 cases. Clin Implant Dent Relat Res.; 5(2): 89-93.

De Bruyn, H., Collaert, B. 2002. Early loading of machined-surface Branemark implants in completely edentulous mandibles: healed bone versus fresh extraction sites. Clin Implant Dent Relat Res.; 4(3): 136-142.

Degidi, M., Piattelli, A., Felice, P. and Carinci, F. 2005.Immediate Functional Loading of Edentulous Maxilla: A 5-year Retrospective Study of 388 Titanium Implant. J Periodontol; 76:1016-1024.

Diago, C.G., Araceli, B.L. and Berta, G.M. 2008. Comparative study of wide-diameter implants placed after dental extraction and implants positioned in mature bone for molar replacement. Int J Oral Maxillofac Implants; 23:497-501.

Drago, C.J. and Lazzara, R.J. 2004. Immediate

provisional restoration of Osseotite implants: A clinical report of 18-month results. Int J Oral Maxillofac Implants; 19:534-541

Esposito, M., Grusovin, M., Willings, M., Coulthard, P., Worthington H. 2007.The effectiveness of immediate, early, and conventional loading of dental implants: A cochrane systematic review of randomized controlled clinical trials. Int J Oral Maxillofac Implants.; 22:893–904.
20.

Fischer, K. 2008. On immediate/early loading of implant-supported prostheses in the maxilla. Thesis, Department of Biomaterials Institute of Clinical Sciences Sahlgrenska. Academy Göteborg University, Sweden.

Gapski, R., Wang, H.L., Mascarenhas, P., Mascarenhas, P., Lang, N.P. 2003. Critical review of immediate implant loading. Clin Oral Implants Res.; 14:515- 527.

Glauser, R., Ree, A., Lundgren, A., Gottlow,J., Hammerle, C., Scharer, P. 2001. Immediate occlusal loading of Branemark implants applied in various jawbone regions: A prospective, 1-year clinical study. Clin Implant Dent Relat Res.; 3:204-213.

Glavind, L. and Löe, H. 1967. Errors in the clinical assessment of periodontal destruction. J Periodont Res; 2: 180-188.

Guirado, J.L., Ortiz-Ruiz, A.J., Negri, B., Rodriguez, B.C. and Schlottig F. 2010. Histological and histomorphometric evaluation of immediate implant placement on a dog model with a new implant surface treatment. Clin Oral Implants Res; 21(3):308-15.

Hahn, J. 2000. Single-stage, immediate loading, and flapless surgery. Journal of Oral Implantology; 26:193-198

Jae-Hoon, L., Val, F., Keun-Woo, L. and Robert, F.W. 2005. Effect of implant size and shape on implant success rates: A Literature review. J Prosthet Dent; 94:377-81.

Kahnberg, K. 2009. Immediate implant placement in fresh extraction sockets: A clinical report. Int J Oral Maxillofac Implants; 24:282-288.

Kaj, F., Eric, R. and Joseph, T. 2007. Clinical evaluation of a prospective multicenter study on one piece implant. Part 1: Marginal bone level evaluation after 1 year of follow-up. Int J Oral Maxillofac implants; 22:226-234.

Langer, B., Langer, L., Herrmann, I., Jorneus, L. 1993. The wide fixture: a solution for special bone situations and a rescue for the compromised implant. Part 1. Int J Oral Maxillofac Implants; 8:400-408.

Lazzara, R.M. 1989. Immediate implant placement into extraction sites: Surgical and restorative advantages. Int J Periodontics Restorative Dent.; 9: 333–343.

Lee, D.H., Choi, B.H., Jeong, S.M., Xuan, F., Kim, H.R. 2009.Effects of Flapless Implant Surgery on Soft Tissue Profiles: A Prospective Clinical Study. Clinical Implant Dentistry and Related Research. (published on line).

Lorenzoni, M., Pertl, C., Zhang, K., Wimmer, G., Wegscheider, W.A. 2003. Immediate loading of single-tooth implants in the anterior maxilla. Preliminary results after one year. Clin Oral Implants Res.; 14:180–187.

Lorenzoni, M., Pertl, C., Zhang, K., Wimmer, G. and Wegscheider, W. 2003. Immediate loading of single tooth implants in the anterior maxilla.Preliminary results after one year.Clin Oral Impl Res;14:180-187.

Löe, H. and Silness, J.1963.: Periodontal disease in pregnancy (part I). Prevalence and severity. Acta Odontol Scand; 21: 533-551.

Misch, C.E., Hahn, J., Judy, K.W., Lemons, J.E., Linkow, L.I., Lozada, J.L., Mills, E., Misch, C.M., Salama, H., Sharawy, M., Testori, T. and Wang, H.L. 2004(a). Workshop guidelines on immediate loading in implant dentistry. J Oral Implantol; 30:283-288.

Misch, C.E., Misch, C.M., Sharawy, M., Lemons, J. and Judy, K.W. 2004. Rational for application of immediate load in implant dentistry: Part II. Implant Dent; 13:310-321.

Misch, C.E. 1999. Implant design consideration for the posterior region of the mouth. Implant Dent; 8:376-386.

Muhlemann, H.R. 1977. Physiology and chemical mediators of gingival health. J Prev Dent; 4: 6-20.

Oh, T.J., Shotwell, J.L., Billy, E.J., Wang, H.L. 2006. Effect of flapless implant surgery on soft tissue profile: a randomized controlled clinical trial. J Periodontol.; 77:874–882.

Oh, T.J., Shotwell, J., Billy, E., Byun, H.Y., Wang, H.L. 2007. Flapless implant surgery in the esthetic region: Advantages and precautions. Int J Periodontics Restorative Dent.; 27(1): 27 -33.

Orenstein, I., Macdonald, D., Tao, A. and Morris, H. 2007. Electronic percussive testing of the stability of an immediately provisionalized implant placed into fresh extraction sockets: A pilot evaluation. J Oral Impl; 33:69-74.

Rosenquist, B., Grenthe, B. 1996. Immediate placement of implants into extraction sockets: implant survival. Int J Oral Maxillofac Implants.; 11:205–209.

Schropp, L., Wenzel, A., Kostopoulos, L. and Karring, T. 2003. Bone healing and soft tissue contour changes following Single-tooth extraction: A clinical and radiographic 12-month prospective study. Int J Periodontics Restorative Dent; 23:313-323.

Schulte, W., Kleineikenscheidt, H., Linder, K., Schareyka, R. 1978. The Tübingen immediate implant in clinical studies; Dutch Zahnarztl Zeitschr.; 33:348–359.

Sclar, A. 2003. The importance of site preservation. In: Sclar A, ed. Soft tissue and esthetic considerations in implant therapy. Chicago, III: Quintescence publishing Co.; 76-79

Szmukler-Moncler, S., Piattelli, A. and Favero, G. 2000. Considerations preliminary to the application of early and immediate loading protocols in dental implantolgy. Clin oral Impl Res; 11: 12-25.

Testori, T., Del Fabbro, M., Galli, F., Francetti, L., Weinstein, R. 2004. Immediate occlusal loading the same day after implant placement: Comparison of 2 different time frames in totally edentulous lower jaws. J Oral Implantol.; 30:307- 313.

Tsirlis, A.T. 2005. Clinical evaluation of immediate loaded upper anterior single implants. Implant Dent.; 14:94–103.

Wang, H.L., Ormianer, Z., Palti, A., Perel, M.L., Trisi, P., Sammartino, G. 2006. Consensus conference on immediate loading: The single tooth and partial edentulous areas. Implant Dent.; 15:324–333.

Zahran, A. 2008. Clinical evaluation of OsteoCare™ Midi one-piece implants for immediate loading. Implant dentistry today; vol.2 (no. 3):26-33.

Zahran, A. and Gauld, J. 2007. Gauld's technique: Clinical innovation of flapless placement of self-tapping implants with the aid of osteotomes in the posterior maxilla. A case report. Egyptian Dental Journal; 53: 2297- 2304.

Zahran, A., Elrefai, M., Amir, T., Fouda, M., 2010.Clinical Evaluation of Flapless Free Hand Immediate Placement in Fresh Extraction Sockets. The Journal of Implant & Advanced Clinical Dentistry. Vol.2, No.8 October.

Grape seed extract alleviate reproductive toxicity caused by aluminium chloride in male rats

Hala, A.H. Khattab [a], Inas, Z.A. Abdallah[a] and Gehan, M. Kamel [b]

[a] Nutrition and Food Science Department, Faculty of Home Economics, Helwan University
[b] Pharmacology Department, Faculty of Veterinary Medicine, Cairo University

E-mail: haya_khattab@hotmail.com

Abstract: Natural dietary antioxidants are studied for their ability to protect cells from miscellaneous damage. Grape seed extract (*Vitis vinifera* L., Vitacease) is a potent antioxidant. The present study aimed to investigate the protective effect of grape seed extract (GSE) against the possible testicular dysfunction caused by aluminium chloride ($AlCl_3$) in male rats. Twenty sexually mature male albino rats were divided into four equal groups, the first served as negative control, the second received $AlCl_3$ (20 mg/kg bw, 1/ 20 LD 50), the third administered GSE (75 mg/kg bw), and the fourth received $AlCl_3$ and treated with GSE. Doses were given once daily via gavage for 70 consecutive days. The results revealed that, $AlCl_3$ induced significant decrease in final body weight, sex organs relative weight, sperm concentration, motility and viability, serum testosterone concentration and superoxide dismutase (SOD) activity, with significant increase in sperm abnormalities and thiobarbituric acid reactive substance (TBARS) concentrations. Moreover, $AlCl_3$ induced apparent alteration in the histological structure of the testis. Treatment with GSE ameliorated the harmful effects of $AlCl_3$, this was also proved histopathologically by the noticeable improvement in the testis tissues. It may be concluded that GSE may be promising as a natural therapeutic agent in $AlCl_3$-induced reproductive toxicity and oxidative stress in the male rat testes.
Hala, A.H. Khattab, Inas, Z.A. Abdallah and Gehan, M. Kamel. **Grape seed extract alleviate reproductive toxicity caused by aluminium chloride in male rats**. Journal of American Science 2010;6(12):1200-1209]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Grape seed extract- aluminium chloride- reproductive- experimental animals.

1. Introduction

Aluminium absorption and accumulation in humans can occur via the diet, drinking water, ingestion with fruit juices or citric acid causes a marked increase in both gastrointestinal absorption and urinary excretion of aluminium in healthy subjects (Venturini-Soriano and Berthon, 2001). Different forms of aluminium are environmental xenobiotics that induce free radical-mediated cytotoxicity and reproductive toxicity. Aluminium ingestion in excessive amount leads to accumulation in target organs and has been associated with damage of testicular tissues of both humans and animals (Yousef and Salama, 2009). $AlCl_3$ showed reproductive toxicity on rabbit sperm in vitro Yousef *et al*. (2007). Testicular aluminium accumulation, necrosis of spermatocytes/spermatids and significant decrease in fertility were found in male mice (Guo *et al*., 2005 a,b).

Grapevine (*Vitis Vinifera*), is cultivated today in all temperature regions of the world (Gruenwald *et al*., 2004). Its seeds contain several active components including flavonoids, polyphenols, anthocyanins, proanthicyanidins, procyanidines, and the stilbene derivative resveratrol. The grape seed extract has been reported to possess a broad spectrum of pharmacological and therapeutic effects such as antioxidative, anti-inflammatory, and antimicrobial activities, as well as having cardioprotective, hepatoprotective, and neuroprotective effects. The seeds of the grape are used in herbal medicine and as a dietary supplement (Shenoy *et al*., 2007). Chedea *et al*. (2010) reported that GSE considered as a powerful antioxidant nutritive supplement that prevent premature ageing and diseases. Maier *et al*. (2009) stated that oil produced from grape seeds is considered a rich source of polyphenolics with strong antioxidant activity.

Grape seed extract is a natural extract from the seed of grape (Asl and Hosseinzadeh, 2009). It is a rich source of one of the most beneficial groups of plant flavonoids and pro-anthocyanidins oligomers (El-Ashmawy *et al*., 2007). It has a protective effect on oxidant-induced production and deposition of extracellular matrix components (Dulundu *et al*., 2007). GSE contains mainly flavonoids, which involved in ameliorating the oxidative stress in vitro and in vivo (Martinez-Florez *et al*., 2002). Several studies have indicated that extracts obtained from grape seed inhibit enzyme systems that are responsible for the production of free radicals, and that they have antimutagenic and anticarcinogenic

effects (Li *et al*., 2001 and Maier *et al*., 2009). Aysun *et al*. (2008) reported that GSE is widely consumed as a dietary supplement and could be useful in synergizing the efficacy of chemotherapeutic agents in cancer treatment. Therefore, the present study was designed to investigate the role of GSE against $AlCl_3$- induced oxidative stress and reproductive toxicity in rat testes.

2. Material and Methods

Chemicals:

Aluminium chloride ($AlCl_3$) was obtained from Sigma Chemical Co. (St Louis, Mo, USA). Casein (> 85% protein) was obtained from Misr Scientific Company, Dokki, Giza, Egypt. Cellulose and D-L methionine were purchased from Morgan Company, Cairo, Egypt. Minerals and vitamins constituent, sucrose, glucose and ethanol absolute were obtained from El-Gomhoriya Pharm. and Chem. Ind. Co. Cairo, Egypt. Corn oil was obtained from the local market. Corn starch was obtained from Starch and Glucose Company, Helwan, Egypt.

Animals:

Twenty sexually mature male albino rats, *Sprague Dawley* strain, weighing (160 ± 10 gm) were purchased from the animal house of Ophthalmic Research Center, Giza, Egypt. The animals were housed in plastic cages, maintained on a natural light-dark cycle at room temperature of 26 ± 2º C and fed standard diet according to Reeves *et al*. (1993) and water ad libitum. Rats were kept for one week as acclimatization period before the start of the experiment. All rats were handled in accordance with the standard guide for the care and use of laboratory animals. The experiment was conducted at Faculty of Veterinary Medicine, Cairo University.

Preparation of grape seed extract:

Ripe grapes (*Vitis vinifera* L., Vitacease) were obtained from El-Behira government, Egypt. Undamaged and disease-free berries were snipped from clusters. Following manual separation of the seeds from whole berries, seeds were oven dried at 30 - 40 º C. Dried grape seeds were ground to fine powder with a grinder. The ethanolic extract was prepared by soaking 100 gm of grape seeds powdered in 300 ml ethanol (95 %) with daily shaking and kept in refrigerator covered by a piece of aluminum foil. The infusion was filtered by a piece of double gauze and the filtrate was centrifuged at 3000 rpm for 10 minutes, then the ethanol was evaporated using a rotatary evaporator apparatus (Switzerland) attached with vacuum pump. The 100 gm of dried grape seeds powder yield 26.7 gm ethanol extract.

Experimental design:

After the acclimatization period, rats were divided into four equal groups, each of five rats. **First group;** was negative control administered 3 ml distilled water orally once daily. **Second group;** was positive control group ($AlCl_3$ group) administered aluminium chloride (20 mg/kg bw, 1/ 20 LD50) dissolved in 3 ml distilled water, the LD 50 of ($AlCl_3$) when administered orally to rats was reported to be (380 - 400 mg/kg bw (Krasovskii *et al*., 1979). **Third group;** was administered grape seed extract (GSE) (75 mg/kg bw) which dissolved in 3 ml distilled water orally once daily according to El-Ashmawy *et al*. (2007). **Fourth group;** was co-administered with $AlCl_3$ and GSE in the same doses in 2nd and 3rd groups. Doses were given once daily via gavage for 70 consecutive days, for completion of the spermatogenic cycle and maturation of sperms in epididymis (Sarkar *et al*., 2003).

Blood sampling:

At the end of the experimental period, animals were fasted overnight, with free access to tap water. Rats in each group were anesthetized with diethyl ether for blood sampling by puncturing the inner canthus of the eye using heparinized microhematocrit tube. Collected blood was stored for 15 min at room temperature, then centrifuged with 3000 rpm for 20 min., and stored at - 20ºC till analysis.

Sex organs weight:

The testes and accessory sex organs (seminal vesicles and prostate glands) were dissected out, trimmed off the attached tissues and weighed. The index weight of the organ was calculated by (Index weight) = organ weight/ body weight x 100.

Determination of testosterone assay:

Serum testosterone level was estimated using method of Ismail (1986).

Semen characteristics:

Seminal content of epididymis was obtained by cutting of cuda epididymis using surgical blades and squeezed in a sterile clean watch glass. This content was diluted 10 times with 2.9 % sodium citrate dehydrate solution and thoroughly mixed to estimate the progressive motility and sperm concentration (Bearden and Fluquary, 1980). One drop of the suspension was smeared on a glass slide and stained by Eosin-nigrosin stain to determine the percentage of sperm cell viability and morphological abnormalities (Miller and Pass, 1952). Abnormal head and tails were evaluated according to (Nahas *et al*., 1989, Mori *et al*., 1991 and Okomura *et al*., 2005).

Determination of testicular antioxidant enzymes:
Preparation of testicular homogenate:

One testis of each sacrificed rat was used for estimation of oxidative enzymes and lipid peroxidation. One gram of testicular tissue was weighed after ice water washing of testes and homogenized in 9 volume buffered saline 0.9 %, centrifuged at 4000 rpm at 4 º C for 15 min., the supernatant was collected and kept at -20 º C till further investigation.

Determination of testicular thiobarbituric acid reactive substance formation (TBARS):

According to the method of Esterbauer and Cheeseman (1990), the extent of lipid peroxidation in terms of thiobarbituric acid reactive substances (TBARS) formation was measured. Tissue supernatant was mixed with 1 ml trichloroacetic acid (TCA) (20%), 2 ml thiobarbituric acid **(**TBA) (0.67 %) and heated for 1 h at 100 °C. After cooling, the precipitate was removed by centrifugation. The absorbance of the sample was measured at 535 nm using a blank containing all the reagents except the sample. As 99% of TBARS was malondialdehyde (MDA), so TBARS concentrations of the samples were calculated using the extinction co-efficient of MDA, which is 1.56 × 105 M−1 cm−1.

Determination of testicular superoxide dismutase activity (SOD):

Superoxide dismutase (SOD) activity was measured using assay kit (Cayman, MI, USA) according to (Giannopolitis and Ries, 1977). This kit utilizes a tetrazolium salt for the detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD was defined as the amount of enzyme needed to produce 50% dismutation of superoxide radical. The SOD assay measures all the three types of SOD (Cu/Zn, Mn, and Fe SOD). Enzyme activity was determined as the amount of the enzyme to reach an inhibition of 50 % nitro-blue tetrazolium (NBT) reduction rate.

Histopathological examination:

Sections were taken from testis tissues from different animals in each group immediately after sacrificed. The tissues were washed with the normal saline solution to remove blood, fixed in 10% neutral formalin for a period of at least 24 hrs, dehydrated in different grades of alcohol, and processed for paraffin embedding. Sections of 5 µm thickness were cut using a rotary microtome. The sections were processed and passed through graded alcohol series, stained with Haematoxylin and Eosin, cleared in xylene and examined microscopically according to Bancroft *et al*. (1996).

Statistical analysis:

The data were analyzed statistically by analysis of variance, for statistical significance ($p \leq 0.05$) using L.S.D. test**,** one way ANOVA, post hoc multiple comparisons according to Snedecor and Cochron (1989). An IBM computer with a software system SPSS version 16 was used for these calculations.

3. Results
Body and sex organs weight:

The effect of grape seed extract (GSE) on final body weight and weight gain in $AlCl_3$ toxicity of male rats is represented in Table (1). Oral administration of GSE had no effect on body weight gain of rats, indicating its safe use under the experimental conditions, while there were highly significant decrease in final body weight and body weight gain ($p < 0.01$) in $AlCl_3$ group (+ve) as compared with negative control group (-ve). On the other hand, there were highly significant elevation in final body weight and weight gain in $AlCl_3$ group treated with GSE as compared with untreated $AlCl_3$ group (+ve).

Table (1): Effect of grape seed extract (GSE) on body weight of $AlCl_3$ intoxicated male rats.

Experimental groups	Initial body weight (g)	Final body weight (g)	Body weight gain (g)
Control (- ve)	165.6 ± 1.21	269.2 ± 1.77	103.6 ± 2.06
$AlCl_3$ (+ ve)	166.0 ± 1.41	202.0 ± 2.43 [a**]	36.0 ± 3.61 [a**]
GSE	167.8 ± 2.65	270.8 ± 3.31	103.0 ± 3.66
$AlCl_3$ + GSE	164.4 ± 1.63	253.0 ± 1.64 [a** b**]	88.6 ± 2.23 [a** b**]

- Each value represents the mean of 5 rats ± SE.
- [a] Significant difference from control group at $p < 0.05$* and highly significant difference from control group at $p < 0.01$**.
- [b] Significant difference between $AlCl_3$ group and $AlCl_3$ group treated with GSE at $p < 0.05$* and highly significant at $p < 0.01$**.

Results indicated significant decrease in the index weights of testes and prostate ($p<0.01$ and $p<0.05$, respectively), and non-significant decrease in index weights of seminal vesicle in $AlCl_3$ group as compared with negative control group (Table 2). Treatment with GSE ameliorated the toxic effect of $AlCl_3$, the index weights of testes recorded highly significant elevation as compared with untreated $AlCl_3$ group. While, oral administration of GSE alone did not cause any significant effect on the weights of the tested tissues as compared with negative control group.

Table (2): Effect of grape seed extract (GSE) on index weights (IW) of testes and sex organs of $AlCl_3$ intoxicated male rats.

Experimental groups	Sex organs (g / 100 g body weight)		
	Testes	Seminal vesicle	Prostate
Control (-ve)	0.922 ± 0.041	0.415 ± 0.023	0.130 ± 0.006
$AlCl_3$ (+ ve)	0.488 ± 0.018 ^{a**}	0.384 ± 0.013	0.118 ± 0.004 ^{a*}
GSE	0.918 ± 0.019	0.423 ± 0.012	0.124 ± 0.0036
$AlCl_3$ + GSE	0.68 ± 0.023 $^{a**\ b**}$	0.404 ± 0.011	0.121 ± 0.002

- Each value represents the mean of 5 rats ± SE.
- ^{a} Significant difference from control group at $p < 0.05$* and highly significant difference from control group at $p < 0.01$**.
- ^{b} Significant difference between $AlCl_3$ group and $AlCl_3$ group treated with GSE at $p < 0.05$* and highly significant at $p < 0.01$** .

Sperm characteristics:

Epididymal sperm concentration, sperm motility, viability and abnormal sperm are reported in Table (3) for $AlCl_3$ and/or GSE groups. GSE group did not differ significantly from the control (-ve) in terms of sperm motility, sperm viability and abnormal sperm rates, but had highly significant elevation in sperm count ($p <0.01$) as compared with negative control group (-ve). $AlCl_3$ group (+ve) had significantly lower sperm count, motility and viability than the control group (-ve).

when total sperm abnormalities (head and tail) were analyzed, the $AlCl_3$ group (+ve) had significantly the highest level of abnormalities as compared with negative control group . On the other hand, treatment with GSE in combination with $AlCl_3$ significantly alleviated the decline in sperm count, motility, and viability, and significantly decreased the percent of dead and abnormal sperm compared to $AlCl_3$ untreated group, and this means that GSE minimized the toxicity of $AlCl_3$.

Table (3): Effect of grape seed extract (GSE) on sperm character of $AlCl_3$ intoxicated male rats.

Experimental groups	Sperm character			
	Count (10^6/ ml)	Motility (%)	Viability (%)	Sperm abnormalities (%)
Control (-ve)	62.0 ± 3.63	82.4 ± 4.53	89.6 ± 4.31	6.6 ± 0.51
$AlCl_3$ (+ ve)	28.6 ± 1.29 ^{a**}	42.0 ± 3.73 ^{a**}	52.0 ± 3.65 ^{a**}	18.4 ± 0.93 ^{a**}
GSE	75.2 ± 3.84 ^{a**}	80.6 ± 3.92	90.0 ± 4.73	5.6 ± 0.24
$AlCl_3$ + GSE	43.2 ± 3.22 $^{a**\ b**}$	58.4 ± 2.16 $^{a**\ b**}$	68.8 ± 2.85 $^{a**\ b**}$	12.0 ± 0.71 $^{a**\ b**}$

- Each value represents the mean of 5 rats ± SE.
- ^{a} Significant difference from control group at $p < 0.05$* and highly significant difference from control group at $p < 0.01$**.
- ^{b} Significant difference between $AlCl_3$ group and $AlCl_3$ group treated with GSE at $p < 0.05$* and highly significant at $p < 0.01$**.

Serum testosterone and testicular antioxidant enzymes:

Results in Table (4) showed highly significant decrease in serum testosterone concentration ($p < 0.01$) in $AlCl_3$ group compared to control group (-ve), while orally treatment with GSE induced highly significant elevation in serum testosterone concentration ($p < 0.01$) and alleviated the negative effects of $AlCl_3$ as compared with untreated $AlCl_3$ group.

On the other hand, $AlCl_3$ caused highly significant decline in SOD accompanied with highly significant elevation in TBARS compared to control group (-ve). While co-administration with GSE and $AlCl_3$, improved the toxic effect of $AlCl_3$ as manifested by highly significant increase in SOD accompanied with highly significant decrease in TBARS as compared with untreated $AlCl_3$ group.

Table (4): Effect of grape seed extract (GSE) on serum testosterone level and testicular oxidative state of $AlCl_3$ intoxicated male rats.

Experimental group	Serum testosterone (ng/ ml)	Testicular oxidative	
		SOD (u/mg protein)	TBARS (n mol/mg protein)
Control (-ve)	1.86 ± 0.049	0.068 ± 0.003	0.074 ± 0.002
$AlCl_3$ (+ ve)	0.936 ± 0.046 [a**]	0.031 ± 0.001 [a**]	0.116 ± 0.001 [a**]
GSE	1.846 ± 0.043	0.073 ± 0.003	0.066 ± 0.002
$AlCl_3$ + GSE	1.356 ± 0.05 [a** b **]	0.044 ± 0.001 [a** b **]	0.103 ± 0.004 [a** b **]

- Each value represents the mean of 5 rats ± SE.
- [a] Significant difference from control group at $p < 0.05$* and highly significant difference from control group at $p < 0.01$**.
- [b] Significant difference between $AlCl_3$ group and $AlCl_3$ group treated with GSE at $p < 0.05$* and highly significant at $p < 0.01$**.

Histopathological results:

Microscopical examination of the testis of normal negative control group (-ve) revealed the normal histopathological structure of seminiferous tubules (Fig 1). Meanwhile, testis of rat group orally administered $AlCl_3$ (+ve) showed congestion of interstitial blood vessel (Fig 2), marked degeneration and necrosis of germ cells lining seminiferous tubules (Fig 3), as well as interstitial odema and testicular degeneration with complete absence of germ cells (Fig 4). Testis of rats group received GSE showed no histopathological changes (Fig 5). In rats group received $AlCl_3$ and treated with GSE, improvement in histopathological examination was noticed in testis sections as shown in (Fig 6), where examined sections revealed no histopathological changes.

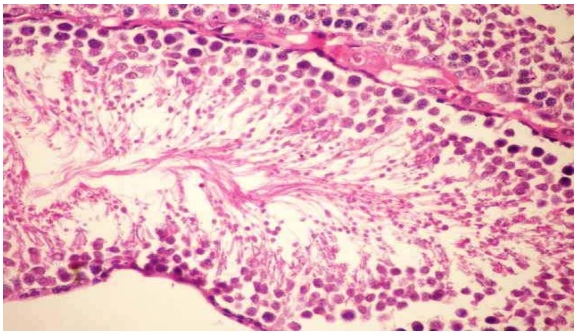


Fig (1): Testis of control group (-ve) showing normal histological structure of seminiferous tubules. (H&E x 200)

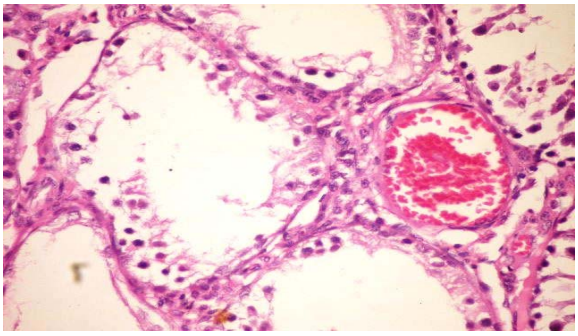


Fig (2): Testis of $AlCl_3$ group (+ve) showing congestion of interstitial blood vessel as well as marked degeneration and necrosis of germ cells lining seminiferous tubules. (H&E x 200)

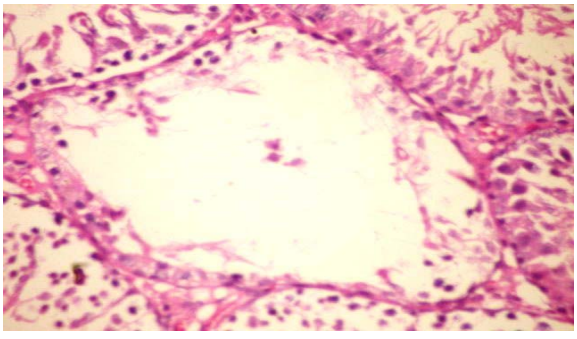


Fig (3): Testis of $AlCl_3$ group (+ve) showing marked degeneration and necrosis of germ cells lining seminiferous tubules. (H&E x 200)

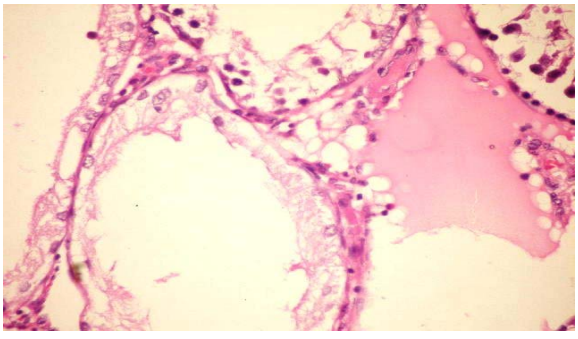


Fig (4): Testis of $AlCl_3$ group (+ve) showing interstitial odema and testicular degeneration with complete absence of germ cells. (H&E x 200)

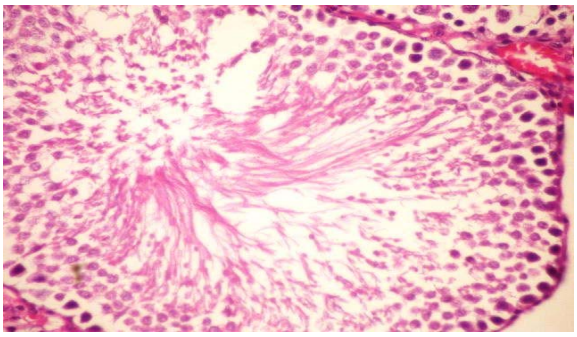


Fig (5): Testis of GSE group showing no histological changes. (H&E x 200)

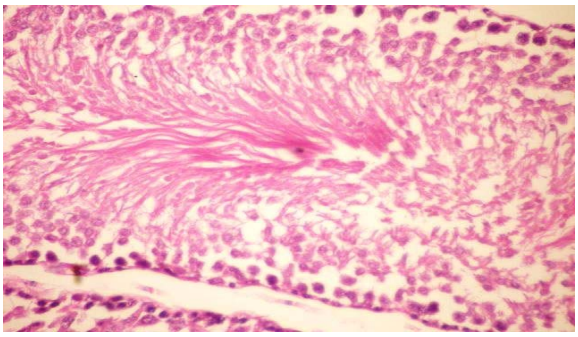


Fig (6): Testis of $AlCl_3$ + GSE group revealed no histopathological changes. (H&E x 200)

4. Discussion:

Grape seed extract is an extract by-product obtained from the seed of *Vitis vinifera*, it contains a variety of biologically active species used for protection against oxidative stress induced by free radicals and reactive oxygen spices (ROS) (Sharma *et al*., 2004). Aluminum is considered to be a non-redox active metal, it promotes biological oxidation both in vitro and in vivo because of its pro-oxidant activity (Turner and Lysiak, 2008). Testicular oxidative stress appears to be a common feature in much of what underlies male infertility, which suggests that there may be benefits to develop better antioxidant therapies for relevant cases of hypospermatogenesis (Turner and Lysiak, 2008 and Yousef and Salama, 2009). GSE has antioxidant and free radical scavenging activity (Jayaprakasha *et al*., 2003 and Caillet *et al*., 2006). Most of the beneficial health effects of GSE are attributed to their antioxidant and free radical scavenging properties (Faria *et al*., 2006).

The present results showed that oral administration of GSE had no effect on body weight gain and index weights of sex organs of rats, this confirmed its safe use and agreed with Clifton (2004) who mentioned that GSE is widely marketed as a dietary supplement and is considered safe for human consumption. Moreover, GSE- containing flavonoids are currently used as nutritional supplements, and have been shown to exert antioxidant, chemopreventive and anticancer effects (Singletary and Meline, 2001, Shi *et al*., 2003 and Mosaad *et al*., 2006). On the other hand, our results indicated significant decrease in the body weight gain and the index weights of testes and prostate ($p< 0.01$, $p <0.01$ and $p < 0.05$, respectively) in rats group received $AlCl_3$ as compared with control, and highly significant decrease in the body weight gain and the relative weights of testes ($p < 0.01$) compared with $AlCl_3$ treated with GSE.

The majority of studies that utilized chronic doses of aluminium reported significant reduction in weight gain, particularly in studies initiated in male animals, Kowalczyk *et al*. (2004) found that during three months observation of rats receiving aluminium chloride, decrease in water and food intake and transient diarrhea occurred, which resulted in lowering of final body mass of animals in comparison to the controls (differences statistically significant). The physiologic basis for this outcome is unclear, but it was reported that animals exposed to chronic doses of aluminium consumed less food. Whether general effects of aluminium on metabolic processes depress metabolism or reduce nutritional efficiency remains to be resolved. The obtained results were in agreement with Yousef *et al*. (2005) and Guo *et al*. (2005 a,b). In addition, (Bataineh *et al*., 1998) found decrease in absolute and relative testes weights and seminal vesicles weights after aluminum chloride ingestion. The decrease in the reproductive organs weights could be due to the decrease in testosterone level which was observed in the current study, that may be resulted from the oxidative damage induced in rat testes (El-Ashmawy *et al*., 2007). The amelioration effect of GSE may be due to grape seeds which are rich sources of monomeric phenolic compounds such as catechin, epicatechin, dimeric, trimeric and tetrameric proanthocynidins (Escribano-Bailon *et al*., 1992). These molecules possess a structure that confers on them an antioxidant property, which has been demonstrated to exert a novel spectrum of biological, pharmacological, therapeutic, and chemo-protective effects against oxygen free radicals and oxidative stress (Bagchi *et al*., 1997).

$AlCl_3$ induced highly significant decrease in sperm count, motility (%) and viability (%), with increase in dead and abnormal sperm count as compared to both control group (-ve) and $AlCl_3$

group treated with GSE, this means that GSE minimized the toxicity of $AlCl_3$. Moreover, rats orally administered GSE alone showed highly significant increase in sperm count, but had no effect on the other sperm character determined. Previous studies showed that, sexual behavior of male rats was suppressed after ingestion of aluminum chloride (Bataineh *et al*., 1998). Necrosis of spermatocytes/ spermatids was observed in the testes of mice exposed to aluminium (Llobrt *et al*., 1995). Yousef *et al*. (2007) showed also that $AlCl_3$ declined semen quality in vivo and vitro, and induced significant decrease in ejaculate volume, sperm concentration, total sperm output, sperm motility, total motile sperm per ejaculate, packed sperm volume, normal and live sperm, while dead and abnormal sperm were increased. The current results may be explained by aconitase, a protein that binds citrate and catalyzes its isomerization to isocitrate via the intermediate cis-aconitate in Krebs cycle, which showed decreased activity in the presence of aluminium, thus influence mitochondrial enzymes, consequently, changes in mitochondrial functions may be reflected in sperm motility and viability (Yousef *et al*., 2007). Moreover, the observed decrease in sperm motility could be attributed in part to the concomitant reduction in testosterone production following aluminium treatment (Guo *et al*.,2005a and Yousef *et al*., 2005).

Testosterone is a key hormone that regulates spermatogenesis. $AlCl_3$ induced significant ($p < 0.01$) decrease in serum testosterone concentration and SOD activity, with significant increase in TBARS levels compared to control rats. However, it was found that, GSE was capable of restoring the SOD activity and serum testosterone level in rats group administered $AlCl_3$ and treated with GSE, there were significant differences as compared with untreated $AlCl_3$ group (+ve). Also, GSE significantly reduced TBARS levels compared to $AlCl_3$ group, this means that GSE increased the process of steroidogenesis and hence testosterone production and improved sperm production and the process of fertility. These results were in line with the results reported by Yousef (2004) who showed that aluminium chloride was able to generate reactive oxygen species in rabbit's testes. Yousef *et al*. (2005) found that aluminium enhanced lipid peroxidation in plasma, testes and liver. Guo *et al*. (2005a) demonstrated also that, exposure to aluminum lowered plasma and testicular testosterone levels in mice. The authors suggested that the severe reduction in male fertility following aluminium administration might result from excessive aluminium accumulation in the testes and low testosterone concentrations. Moreover, Turner and Lysiak (2008) found over productive of ROS, which can be detrimental to sperm and associated with male infertility, and thus spermatoxic effect might be due to $AlCl_3$ induced free radicals.

There is evidence implicating androgenic hormones involved in mechanisms of aluminium toxicity on male reproduction. Guo *et al*. (2005a) found that aluminium administration significantly increased nitric oxide (NO) production and decreased both testicular adenosine 3′, 5′-cyclic monophosphate (cAMP) and testosterone levels. Excessive NO activated inducible NO synthase (NOS) which may be involved in reproductive toxicity of aluminum, hence reducing rate and motility of sperm cells, increasing their morphological abnormalities, and suppressing testosterone secretion in male rats. Moreover, these effects of $AlCl_3$ may be attributed to aluminium ability to cross the blood-testis barrier, after inducing oxidative stress and lipid peroxidation that damages the biological membranes in the testes, this in turn causes the degeneration of the spermatogenic, which disrupts spermatogenesis and reduces sperm counts (Latchoumycandane *et al*., 2002). Also, the increase in TBARS can bring negative effects on motility and sperm-oocyte fusion (Kim and Parthasarathy, 1998), which was found in the present study. Moreover, increased ROS subsequently attack almost all cell components including lipid membrane and producing lipid peroxidation (Flora *et al*., 2003). The protective effect of GSE treatment agreed with Aysun *et al*. (2008) who reported that oral intake of GSE reduced the oxidative stress. In addition, GSE treatment considerably increased the formation of antioxidant products which may be regarded to the phenolic constituents of GSE and its antioxidant activity. Flavonoids have been shown to alleviate the oxidative stress by increasing the endogenous antioxidant status, protecting cells against free-radical damage by increasing resistance to oxidative stress (Perez *et al*., 2002).

Histopathological examination of rats group orally administered $AlCl_3$ (+ve) showed apparent alteration in the testes, where it induced marked degeneration and necrosis of germ cells lining seminiferous tubules, as well as interstitial odema and complete absence of germ cells. Meanwhile, treatment of $AlCl_3$ group with GSE showed noticeable improvement in histopathological changes induced by $AlCl_3$ in testis sections. The histological changes in testes of rats administered $AlCl_3$ are in agreement with Khattab (2007) who studied the effect of $AlCl_3$ on the rat's testes. Also, Guo *et al*. (2005b) observed deleterious effects and histopathological changes in testicular tissues after 2 weeks of aluminium treatment, as well as noticeable spermatogenetic loss as necrosis in the spermatids

and spermatozoa at the 5th week of aluminium treatment. This damage effect may be explained by Yousef and Salama (2009) who reported that oxidative stress results from the production of oxygen radicals in excess of the antioxidant capacity of the stressed tissue. Many conditions or events associated with male infertility are inducers of oxidative stress, which leads to an increase in germ cell apoptosis and subsequent hypospermatogenesis, such stress condition, can cause changes in the dynamics of testicular microvascular blood flow, endocrine signaling, and germ cell apoptosis. Moreover, reactive oxygen species and oxidative damage of bimolecular may contribute to male infertility by reducing sperm function (Atessahin *et al*., 2005). Minimizing the hazard effects of $AlCl_3$ by GSE treatment may be due to the flavoniods in GSE, which exert many health-promoting effects, including the ability to increase intercellular antioxidant levels, decrease capillary permeability and fragility and scavenge oxidants and free radicals (Singth *et al*., 2004).

In conclusion, $AlCl_3$ caused fertility disturbances and testicular dysfunction, decrease in antioxidant enzymes and increase in lipid peroxidation in testes. Treatment with GSE showed protective effect against its reproductive toxicity and this may be attributed to the activity of GSE as a natural antioxidant. Oxidative stress is one of the mechanisms of $AlCl_3$ reproductive toxicity and GSE has protective effect against such oxidative damage. Additional studies are needed to demonstrate GSE efficacy in humans.

Corresponding Author:
Dr. Hala Abd El-Rahman Hassan Khattab,
Nutrition and Food Science Department, Faculty of Home Economic, Helwan University, Egypt.
haya_khattab@hotmail.com

References

1. Asl,M.N. and Hosseinzadeh,H. (2009). Review of the pharmacological effects of *vitis vinifera* (grape) and its bioactive compounds. Phytotherapy Res., 10: 1002.
2. Atessahin,A., Yilmaz,S., Karahan,I., Pirincei,I. and Tasdemir,B. (2005). The effects of vitamin E and selenium on cypermethrin-induced oxidative stress in rats. Turk. J. Vet. Anim. Sci., 29: 385-391.
3. Aysun, C., Leylagul,K., Ismail,K., Sibel,H., Recep,S., Ahmet,O., Okan,O. and Osman,S. (2008). The effect of grape seed extract on radiation- induced oxidative stress in the rat liver. Turk J. Gatroenterol., 19 (2): 92-98.
4. Bagchi,D., Garg,A. and Krohn,R.L. (1997). Oxygen free radical scavenging abilities of vitamins C and E, and a grape seed proanthocyanidin extract in vitro. Res. Commun. Mol. Pathol. Pharmacol., 95: 179-189.
5. Bancroft,D., Stevens,A. and Turmer,R. (1996). Theory and practice of histological technique, 4th ed., Churchill Living Stone, Edinburgh, London, Melbourne.p. 47-67.
6. Bataineh,H., Al-Hamood,M.H. and Elbetieha,A. M. (1998). Assessment of aggressive, sexual behavior and fertility in adult rat following long term ingestion of four industrial metals salts. Hum. Exp. Toxicol., 17: 570-579.
7. Bearden, J.H. and Fluquary, J.W. (1980). Applied animal reproduction. Reston,VA: Reston Publishers, p.157-169 .
8. Caillet, S., Salmieri,S. and Lacroix,M.(2006). Evaluation of free radical-scavenging properties of commercial grape phenol extracts by a fast colorimetric methods. Food Chem., 95: 1-8.
9. Chedea,V.S., Braicu,C. and Socaciu,C. (2010). Antioxidant/prooxidant activity of polyphenolic grape seed extract. Food Chem., 121: 132-139.
10. Clifton,P.M. (2004). Effect of grape seed extract and quercetin on cardiovascular and endothelial parameters in high risk subjects. J. Biomed. Biotechnol., 5:272-278.
11. Dulundu,E., Ozel,Y. and Topaloglu,U. (2007). Grape seed extract reduces oxidative stress and fibrosis in experimental biliary obstruction. J. Gastroenterol Hepatol., 22: 885-892.
12. El-Ashmawy,I.M, Saleh,A. and Salama, O.M. (2007). Effects of marjoram volatile oil and grape seed extract on ethanol toxicity in male rats. Basic & Clinical Pharmacology & Toxicology, 101: 320-327.
13. Escribano-Bailon,M.T., Gutierrez-Fernandez,Y. and Rivas-Gonzalo,J.C. (1992). Charaterization of procyanidins of *Vitis vinifera* variety Tinta dek Paris grape seeds. J. Agric. Food Chem., 40: 1794-1799.
14. Esterbauer,H. and Cheeseman,K.H. (1990). Determination of aldehydic lipid peroxidation products: malonaldehyde and 4– hydroxynonenal Methods enzymol., 186: 407-421.
15. Faria, A., Calhau, C., de Freitas,V. and Mateus, N. (2006). Procyanidins as antioxidants and tumor cell growth modulators. J. Agric. Food Chem., 54:2392-2397.
16. Flora,S.J.S., Mehta,A., Satsangi,K., Kannan,G. M. and Guuuupta,M. (2003). Aluminum induced oxidative stress in rat brain: response to combined administration of citric acid and HEDTA. Com. Biochem. Physiol. Part C, 134, 319-328.

17. Giannopolitis,C.N. and Ries,S.K. (1977). Superoxide dismutase. I. occurrence in higher plants. Plants Physiol., 59:309-314.
18. Gruenwald,J., Brendler,B.A. and Jaenicke,C. (2004). PDR for herbal medicines, 3rd ed., Thomson PDR: Montvale, NJ.
19. Guo,C., Lu,Y. and Hsu,G.S.W. (2005 a). The influence of aluminum exposure on male reproduction and offspring in mice. Environ. Toxicol. Pharmacol., 20:135-141.
20. Guo,C., Huang,C.J., Yeh,M.S. and Hsu,G.S.W. (2005 b). Aluminum induced suppression of testosterone through nitric oxide production in male mice. Environ. Toxicol. Pharmacol., 19: 33-40.
21. Ismail, A.A. (1986). The role of testosterone measurement in the investigation of androgen disorders. Ann Clin. Biochem., 23: 113-134.
22. Jayaprakasha,G.K., Selvi,T. and Sakaraiah,K.K. (2003). Antibacterial and antioxidant activities of grape (*Vitis vinifera*) seed extracts. Food Res. Int., 36: 117-122.
23. Khattab,I.K.F. (2007). Histological and ultrastructural studies on the testis of rat after treatment with aluminium chloride. Austr. J. Basic Appl. Sci., 1: 63-72.
24. Kim,J.G. and Parthasarathy,S. (1998). Oxidation and the spermatozoa. Semen. Reprod. Endocrinol., 16: 235-239.
25. Kowalczyk, E., Kopff1, A., Kędziora1, J., Błaszczyk, J., Kopff1, M., Niedworok, J. and Fijałkowski, P. (2004). Effect of long-term aluminium chloride intoxication on selected biochemical parameters and oxidative-antioxidative balance in experimental animals. Polish Journal of Environmental Studies, 13 (1): 41-43.
26. Krasovskii,G.N., Vasukovich,L.Y. and Chariev, O.G. (1979). Experimental study of biological effects of leads and aluminum following oral administration. Environ. Health Rerspect, 30: 47-51.
27. Latchoumycandane,C., Chitra,K.C. and Mathur, P.P. (2002). The effect of methoxychlor on the epididymal antioxidant system of adult rats. Reprod. Toxicol., 16: 161-172.
28. Li, W.G., Zhang,X.Y. and Wu.Y.J. (2001). Anti-inflammatory effect and mechanism of proanthocyanidins from grape seeds. Acta Pharmacol. Sin., 22: 1117-1120.
29. Llobrt,J.M., Colomina,M.T. and Sirvent, J.J. (1995). Reproductive toxicology of aluminum in male mice. Fund. Appl. Toxicol., 25: 45-51.
30. Maier,T., Schieber,A., Kammerer,D. and Carle,R. (2009). Residues of grape (*Vitis vinifera* L.) seed oil production as a valuable source of phenolic antioxidants. Food Chemistry, 112: 551-559.
31. Martinez-Florez,S., Gonzalez-Gallego,J. and Culebras,J.M. (2002). Flavonoids: properties and antioxidizing action. Nutr. HYosp., 17: 271-278.
32. Miller P.G. and Pass N.P. (1952). Manual of infertility and artificial insemination in cattle. Biolliere Tindall and Cox, London, U.K., p. 29 - 35.
33. Mori K., Kaido M., Fujishiro K., Inoue N., Koide O., Hori H. and Tanaka I. (1991). Dose dependent effects of inhaled ethylene oxide on spermatogenesis in rats. Brit., J. Ind. Med., 48, 270 - 274.
34. Mosaad,A.M., El-Naggar,A.M.M. and El-Far,M. (2006). Amelioration of radiation-induced oxidative stress and biochemical alteration by SOD model compounds in pre-treated γ-irradiated rats. Eur. J. Nutr., 45:251-258.
35. Nahas S., Hondt, H.A. and Abdou, H.A. (1989). Chromosome aberrations in spermatogonia and sperm abnormalities in curacron-treated mice. Mutat. Res., 222, 409 - 414.
36. Okamura, A., Kamijima, M., Shibata, E., Ohtani, K., Takagi, K., Ueyama, J., Watanabe, Y., Omura, M., Wang, H., Ichihara, G., Kondo, T. and Nakajima, T. (2005). A comprehensive evaluation of testicular toxicity of dichlorvos in wistar rats. Toxicology, 213, 129 - 137.
37. Perez,D., Strobel,P. and Foncea,R. (2002). Wine, diet, antioxidant defenses and oxidative damage. Ann NY Acad. Sci., 957: 136-145.
38. Reeves, P.G., Nielsen, F.H. and Fahey, G.C.(1993). AIN-93 Purified diets for laboratory rodents: Final report of the American Institute of Nutrition Ad Hoc Writing Committee on the Reformulation of the AIN-76. A Rodent diet. J. Nutr., 123:1939-1951.
39. Sarkar,M., Roy Chaudhury,G., Chattopadhyay, A. and Biswas,N.M. (2003). Effect of sodium arsenate on spermatogenesis, plasma gonadotrophins and testosterone in rats. Asian J. Androl., 5 :27-31.
40. Sharma,G., Tyagi,A.K. and Singh,R.P. (2004). Synergistic anti- cancer effect of grape seed extract and conventional cytotoxic agent doxorubicin against human breast carcinoma cells. Breast Cancer Res. Treat., 85:1-12.
41. Shenoy,S.F., Keen,C.L.,Kalgaonkar,S. and Polagruto,J.A. (2007). Effects of grape seed extract consumption on platelet function in postmenopausal women. Thromb. Res., 121: 431-432.
42. Shi,J., Yu,J., Pohorly,J.E. (2003). Polyphenolics in grape seeds. Biochemistry and functionality. J. Med. Food, 6:291-299.

43. Singletary,K.W. and Meline,B. (2001). Effect of grape seed proanthocyanidins on clon aberrant crypts and breast tumours in a rat dual-organ tumour model. Nutr. Cancer, 39: 252-258.
44. Singth,R.P., Tuagi,A.K., Dhanalakshmi,S., Agarwal,R. and Agarwal,C. (2004). Grape seed extract inhibits advanced human prostate tumor growth and angiogenesis and upregulates insulin-like growth factor binding protein-3. Int. J. Cancer, 108: 733-740.
45. Snedecor, G.W. and Cochron, W.G. (1989). Statistical methods. 8th ed., Lowa State Univ. Press, Ames, Lowa, USA.
46. Turner,T.T. and Lysiak,J.L. (2008). Oxidative stress: A common factor in testicular dysfunctions. J. Androl., 29: 488-498.
47. Venturini-Soriano,M. and Berthon,G. (2001). Aluminum speciation studies in biological fluids. Part 7. A quantitative investigation of aluminum (III)-malate complexequilibria and their potential implications for aluminum metabolism and toxicity. J. Inorg. Bioch., 85: 143-154.
48. Yousef,M.I. (2004). Aluminum- induced changes in hemato-biochemical parameters, lipid peroxidation and enzyme activities of male rabbits: Protective role of ascorbic acid. Toxicology, 199: 47-57.
49. Yousef,M.I., El-Morsy,A.M.A. and Hassan,M.S. (2005). Aluminium induced deterioration in reproductive performance and seminal plasma biochemistry of male rabbits: protective role of ascorbic acid. Toxicology, 215: 97-107.
50. Yousef ,M.I., Kamel, K.L., ElGuendi,M.I. and El-Demerdash, F.M. (2007). An in vitro study on reproductive toxicity of aluminium chloride on rabbit sperm: The protective role of some antioxidants. Toxicology, 239: 213-223.
51. Yousef,M.I. and Salama,A.F. (2009). Propolis protection from reproductive toxicity caused by aluminium chloride in male rats. Food and Chem. Toxicology, 47: 1168-1175.

Assessment of Body Composition, Fat Distribution and Serum lipid Profile in Obese School Children

Nayera E. Hassan[1], Sahar A. El-Masry[*1], Rokia A. El Banna[1], Mona Sallam[2], Azza M Sarry El-Din[1], Tarek S Ibrahim[2] and Mona Anwar[3]

[1]Biological Anthropology Dept., [2] Child Health Dept., [3]Medical Biochemistry Dept., National Research Centre, Dokki, Giza, Egypt
[*]masrysa@yahoo.com

Abstract: Objective: To determine the relationship between body compositions, fat distribution and blood lipid profile in obese school children aged 7 to 18 years. Methodology: In this cross-sectional study, 150 pupils between the ages of 7 to 18 years were included. Anthropometric measures of adiposity (BMI, waist circumference, waist-to-hip ratio, peripheral adiposity: as the sum of triceps and biceps skinfold thickness, central adiposity: as the sum of sub scapular, suprailiac and abdominal skinfold thickness), body composition and serum total lipids profile were assessed. Results: There are significant sex differences in ages 7 -18 years regarding BMI, abdominal skinfold thickness and TC/ HDL-C, and in peripheral adiposity at young age (7-11 years) and central one at adolescents (12-18 years). Body composition and fat distribution showed significant sex differences in adolescent period only; and in fat distribution in young age period. For young age, triglycerides and HDL-C are correlated to most of the body composition and anthropometric parameters in boys and not in girls. For adolescents, there is no correlation between any one of the lipid profile and the body composition and anthropometric parameters in either gender. Conclusion: This study has shown that in comparison to girls, the correlation of body composition, fat distribution and lipid profiles were higher in boys aged 7 – 11 years only, with a tendency to develop the higher risk level of cardio vascular disease. Particular attention should be focused on the time prevention of childhood obesity.

[Nayera E. Hassan, Sahar A. El-Masry, Rokia A. El Banna, Mona Salam, Azza M Sarry El-Din, Tarek S Ibrahim and Mona Anwar. **Assessment of Body Composition, Fat Distribution and Serum lipid Profile in Obese School Children**. Journal of American Science 2010;6(12):1210-1217]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Body composition, Anthropometry, fat distribution, lipid profile, School children, Risk of cardiovascular disease.

1. Introduction:

Obesity; which is an excessive amount of body fat; is currently a global pandemic, and represents an important health problem both in developed and developing countries [1] and Egypt is not spared.

According to the Centers for Disease Control and Prevention (CDC), the prevalence of childhood obesity in 2006 reached 27.5% in USA, 25.5% in Europe and 12.5% in Egypt and North Africa [2]. In 2008, the rate of overweight and obese children in the United States was 32% [3].

With a growing incidence of obesity in childhood, this problem is even more alarming when its progression and associations are considered because metabolic changes and the consequence of obesity, formerly observed in adults, are now observed in younger individuals [4]. The multiple associated co- morbidities increase the cardiovascular mortality risk and reduce the quality of life in adulthood. Among the different situations associated with adult obesity, hypertension, dyslipidemia and alterations in glucose metabolism have also been found in children with excess weight [4, 5]. In the school age group, early vascular complications have also been detected, and they are as feared as those in adults, particularly when their progressive character is considered [6].

The relationship of obesity to risk factors is not only defined by the level of obesity but also by distribution of fat [7]. Anthropometric measurements; including body weight , height, circumferences and skin fold thickness; can be used to assess body size and proportions as well as total body and regional body compositions especially fat distribution. The anthropometric indices of adiposity include body mass index (BMI), waist circumference, waist-to-hip ratio, peripheral adiposity; as the sum of triceps and biceps skin fold thickness; central adiposity; as the sum of sub scapular, suprailiac and abdominal skin fold thickness; and body composition [8, 9]. Waist circumference reflects central adiposity as well as general adiposity, based on waist circumference which uniquely related to disease risk [10]. The use of BMI to classify children and adolescents as overweight and obesity is well established [11].

Prospective and retrospective studies have shown that risk factors related to cardiovascular diseases (CVD) namely obesity, lipid profiles, unhealthy diets and sedentary lifestyle, have their

roots in childhood and tend to track into adulthood [12]. In childhood, obesity is associated with high levels of blood pressure, very low-density lipoprotein cholesterol (VLDL-C) and insulin, lower levels of high density lipoprotein cholesterol (HDL-C), increased heart rate and increased cardiac output [13]. Abnormal serum concentrations of lipids such as total cholesterol (TC) and low- density lipoprotein-cholesterol (LDL-C) are strongly correlated with early atherosclerotic lesions [14]. Lima et al., 2004 [15], stated that serum lipid levels may be affected by obesity or body fat distribution patterns.

Few studies have tested whether fat distribution is associated with cardiovascular risk factors irrespective of the total amount of fat in children [10, 16]. None of these studies analyzed genders separately.

The present study was planned and performed with the aim of identifying and determining the relationship between body composition, fat distribution and the serum lipid profile in obese school-aged children (aged 7 to 18 years) for each gender separately.

2. Subjects and Methods

Subjects

This research was a cross-sectional one, conducted in 6 public schools (two Primary Schools, two preparatory and two secondary schools) situated in Giza governorate, Egypt; during the period of October, 2007 to April 2009. With parental consent, 150 obese pupils (70 boys, 80 girls), were recruited for this research. These pupils were required to meet the following inclusion criteria: age, 7–18 years and BMI, greater than the 95th percentile for age and gender based on the Egyptian Growth Reference Charts [17]. Pupils were excluded if they had a prior major illness, including type 1 or 2 diabetes, recieve medications or had a condition known to influence body composition, insulin action or insulin secretion (e.g. glucocorticoid therapy, hypothyroidism and Cushing's disease). The sample was divided into 2 groups according to their age:

Group (I): including 62 pupils (32 boys and 30 girls) of the primary school, aged 7 -11 years $\pm$ 6 months.

Group (II): including 88 pupils (38 boys and 50 girls) of the preparatory and secondary schools, aged 12 -18 years $\pm$ 6 months.

Permission to perform the study was granted by the Ministry of Education, and the directors of the school included in the research. Parents were informed about the purpose of the study and their permission in the form of written consent was obtained. Approval to conduct this survey was granted by the "Ethical Committee" of the "National Research Centre".

Anthropometric measures:

Each pupil underwent a complete physical examination, including anthropometric measures. The height, weight, waist and hip circumferences and skinfold thickness were measured following the recommendations of the International Biological program [18]. The height was measured to the nearest 0.1 cm on a Holtain portable anthropometer, and the weight was determined to the nearest 0.01 kg on a Seca Scale Balance, with the subject wearing minimal clothing and no shoes. Waist circumference (central adiposity) was measured at the level of the umbilicus with the subject standing and breathing normally, hip circumference at the level of the iliac crest, using non-stretchable plastic tape to the nearest 0.1 cm. Skin folds thickness were taken at five sites: triceps, biceps, sub scapular, suprailiac and abdominal. Each skinfold was measured three times on the left side of the body; with Holtain skin fold caliper to the nearest 0.2 mm, and the mean was recorded. The following adiposity indices were calculated:

- Body mass index (BMI): as weight (in kilograms) divided by height (in meters) squared.
- Waist/ Hip ratio (cm/ cm).
- Peripheral adiposity: as the sum of triceps and biceps skinfold thickness.
- Central adiposity: as the sum of sub scapular, suprailiac and abdominal skinfold thickness.

Body Composition:

Whole body resistance and reactance (capacitance) were measured using a Bioelectrical Impedance Analyzer (HOLTAIN LIMITED). As specified by the manufacturer, the unit was calibrated before testing using 400-ohm resistor, and electrodes were placed on right wrist and ankle. By using pupil sex, age, weight and height approximated to the nearest unit, the percentage body fat (Fat %), fat mass (FM) and fat free mass (FFM) were derived.

Plasma Lipid profile:

Early morning venous blood samples were taken from each pupil for biochemical screening tests after 12-hours overnight fasting. Professional staff performed venipuncture. The blood samples were left to clot; sera were separated by centrifugation for 10 minutes at 5000 rpm then stored at –80ºC until assays. Serum concentrations of total cholesterol (TC) [19], triglycerides (TG) [20] and high-density lipoprotein-cholesterol (HDL-C) [21] were measured

using commercially available kits provided by STANBIO Laboratory Inc.(1261 North Main Street Boerne Texas 78006 USA). LDL-C was calculated according to an equation developed by Friedewald et al., 1972 [22] as follows:

LDL-C= Total cholesterol –Triglycerides/5+ HDL-C.

The ratio between total cholesterol and high-density lipoprotein-cholesterol (TC/HDL-C) was calculated.

Statistical Analysis:

All values are reported as the mean ± SD. Statistical evaluation of the results was performed with the SPSS 9.05 computer program. Student's *t test* was used to examine the sex differences. Pearson's correlation coefficients were used to assess relationships between independent variables. The level of significance was set at a probability of less than 5% ($p<0.05$).

3. Results:

The total sample included 150 students, 46.7% were boys (n: 70) and 53.3% were girls (n: 80). Descriptive data for the anthropometric measures, body composition and lipid profile for boys and girls are shown in tables I and II. Analyses of the pupils of group (I) aged 7- 11 years ± 6 months (table 1) revealed significant differences between gender for body weight, BMI, biceps, sub scapular and abdominal skinfold thickness, peripheral adiposity and TC/HDL-C ($p<0.05$). Girls had significantly higher mean values of body weight, BMI, and sub scapular skinfold thickness than boys, while skinfold thickness at the biceps and abdominal areas, peripheral adiposity and TC/HDL-C mean values were significantly higher in the boys. Insignificant sex differences were recorded regarding the body composition in this age.

Group (II) aged 12- 18 years ± 6 months (table 2) had significant differences between gender for, BMI, abdominal skinfold thickness, central adiposity, body composition parameters (fat % , fat mass , fat free mass) and TC/HDL-C ($p<0.05$). Girls had significantly higher mean values of BMI, fat %, fat mass and TC/HDL-C while abdominal skinfold thickness, central adiposity and fat free mass mean values were significantly higher in the boys. There is insignificant sex difference in TG, TC, HDL-C and LDL-C for both groups.

Correlation of lipid profiles to body composition and fat distribution for both groups by gender are presented in tables III and IV. For boys in group (I) aged 7- 11 years ± 6 months (table III) , a highly significant positive correlations were observed between triglyceride and all the anthropometric measures and indices; body composition parameters except waist/ hip ratio which show insignificant positive correlation. HDL-C showed highly significant positive correlations with weight and fat free mass, and significant positive correlations with BMI and fat%, while highly significant negative correlations were found in HDL-C and skinfold thickness at the biceps and abdominal areas, peripheral and central adiposity. Both TC and LDL-C showed significant positive correlations with suprailiac skinfold thickness, while TC only showed highly significant positive correlation with fat %, and LDL-C had significant negative correlation with fat free mass. Moreover, TC/ HDL-C showed significant negative correlations with body weight, BMI, hip circumference and fat free mass. However, none of the parameters under study showed any correlation in girls of this group, except triglyceride which had significant positive correlations with body weight, and highly significant positive correlations with hip circumference.

For group (II) aged 12- 18 years ± 6 months (table IV), none of the parameters under study showed any significant correlation in both gender except triglyceride which had significant negative correlation with biceps skinfold thickness.

4. Discussion:

Childhood obesity represents a high risk of morbidity and mortality, and it perpetuation into adulthood strongly increases the risk of cardiovascular disease [7, 23]. This constellation is caused by excessive food and decrease in physical activity which leads to accumulation of body fat [5, 24].

Obesity and changes in blood lipid profile during childhood increases cardiovascular disease risk (CVD) in adulthood. Therefore in CVD examination, one of most important parameters is the analysis of total lipid profiles [25].

BMI increase lipid mobilization leading to increase in triglycerides and LDL. Obesity appears to influence the accumulation of fat, which in turn related to the development of major risk factors. Once thought to only be inactive energy storage area in which excess calories were stored as fat, it is now known that adipose tissue also functions as an endocrine gland [26, 27]. Fat cells secrete free fatty acid, which may stimulate hepatic triglyceride and low density lipoprotein cholesterol (LDL) production in adults [28].There is evidence to suggest a similar relationship in youth [9]. So, the purpose of this research is to identify and determine the relationship between body composition, fat distribution and the serum lipid profile in obese school-aged children (aged 7 to 18 years) for each gender separately.

Table 1: Anthropometric characteristics and Lipid profile of the group (I) by sex (Age 7-11 ± 6 months)

Parameters	Boys (N = 32)		Girls (N = 30)		p
	Mean	±SD	Mean	±SD	
Weight (Kg)	53.75	±12.50	60.37	±13.19	0.047*
Height (cm)	140.40	±9.82	144.8	±10.85	0.097
BMI (Kg/cm²)	26.84	±2.49	28.41	±3.07	0.030*
Waist C (cm)	78.91	±8.34	82.62	±15.33	0.238
Hip C(cm)	91.93	±8.40	95.02	±9.31	0.174
Waist/Hip ratio	0.86	±0.04	0.88	±0.21	0.593
Skinfold (mm)					
Triceps	27.25	±4.62	25.07	±6.10	0.119
Biceps	20.63	±4.74	16.70	±5.63	0.004**
Subscaular	23.93	±3.09	26.61	±4.56	0.009**
Suprailiac	26.50	±4.13	26.57	±6.08	0.961
Abdominal	28.68	±5.44	24.36	±6.46	0.006**
Peripheral fat	47.88	±8.34	41.77	±9.50	0.010**
Central fat	79.10	±11.23	77.53	±14.54	0.638
Body Composition					
Fat %	43.39	±15.24	39.10	±4.66	0.165
Fat mass (Kg)	23.01	±8.70	25.48	±7.62	0.270
Fat free mass (Kg)	35.03	±6.85	38.86	±7.69	0.056
Lipid profile					
TG (mg/dl)	125.86	±33.99	136.19	±39.63	0.274
TC (mg/dl)	195.13	±60.94	177.70	±53.45	0.237
HDL-C(mg/dl)	37.77	±15.26	42.96	±20.55	0.265
LDL-C(mg/dl)	132.09	±61.16	108.11	±53.48	0.110
TC/HDL-C	0.048	±0.03	0.032	±0.02	0.016**

Table 2: Anthropometric characteristics and Lipid profile **of the group (II) by sex (Age 12-18 ± 6 months)**

Parameters	Boys (N=38)		Girls (N= 50)		
	Mean	±SD	Mean	±SD	p
Weight (Kg)	84.18	±13.58	85.70	±11.23	0.567
Height (cm)	162.37	±9.96	159.13	±7.12	0.093
BMI (Kg/cm²)	31.76	±2.77	33.82	±3.73	0.005**
Waist C (cm)	101.50	±13.86	96.18	±17.94	0.139
Hip C(cm)	111.77	±9.11	110.64	±11.83	0.631
Waist/Hip ratio	0.91	±0.12	0.89	±0.27	0.653
Skinfold (mm)					
Triceps	26.45	±6.09	28.23	±7.08	0.243
Biceps	20.11	±7.69	20.95	±6.82	0.602
Subscaular	31.31	±7.01	30.62	±7.49	0.674
Suprailiac	27.32	±9.42	25.28	±7.80	0.287
Abdominal	34.21	±7.69	26.24	±7.44	0.000**
Peripheral fat	46.56	±12.37	49.18	±12.62	0.355
Central fat	92.85	±20.00	82.14	±19.46	0.018**
Body Composition					
Fat %	35.13	±9.14	42.94	±3.94	0.000**
Fat mass (Kg)	30.45	±10.76	38.67	±7.97	0.000**
Fat free mass (Kg)	55.79	±11.78	49.30	± 5.04	0.001**
Lipid profile					
TG (mg/dl)	131.59	±59.98	143.36	±62.72	0.380
TC (mg/dl)	171.95	±50.71	180.84	±61.35	0.474
HDL-C(mg/dl)	41.57	±14.97	45.10	±29.64	0.477
LDL-C(mg/dl)	104.47	±53.82	108.36	±68.10	0.768
TC/HDL-C	0.03	±0.02	0.04	±0.03	0.046*

Table 3: Correlation between serum lipid profile and whole body composition and fat distribution by gender for group I (Age 7-11 ± 6 months)

	Boys					Girls				
	TG (mg/dl)	**TC** (mg/dl)	**HDL-C** (mg/dl)	**TC/HDL-C**	**LDL-C** (mg/dl)	**TG** (mg/dl)	**TC** (mg/dl)	**HDL-C** (mg/dl)	**TC/HDL-C**	**LDL-C** (mg/dl)
Weight (Kg)	0.567**	-0.004	0.457**	- 0.401*	-0.179	0.380*	-0.054	-0.175	0.190	-0.028
BMI (Kg/cm²)	0.531**	-0.164	0.353*	- 0.442*	-0.309	0.306	0.078	-0.013	0.087	0.071
Waist C (cm)	0.679**	0.190	0.150	- 0.285	0.078	-0.014	0.000	- 0.202	0.313	0.080
Hip C(cm)	0.700**	0.104	0.255	- 0.383*	-0.035	0.548**	- 0.014	0.084	- 0.090	- 0.110
Waist/Hip ratio	0.213	0.293	-0.214	0.147	0.321	-0.244	0.002	- 0.224	0.317	0.117
Skinfold (mm)										
Triceps	0.551**	0.237	-0.207	-0.006	0.228	0.045	0.225	0.119	0.024	0.202
Biceps	0.409*	-0.005	-0.698**	0.274	0.123	- 0.197	0.053	0.042	0.155	0.049
Subscaular	0.712**	0.282	-0.156	-0.111	0.242	0.121	- 0.044	- 0.032	0.257	- 0.056
Suprailiac	0.675**	0.353*	-0.296	0.144	0.352*	0.049	- 0.213	0.145	- 0.069	- 0.261
Abdominal	0.376*	-0.038	-0.650**	0.279	0.082	- 0.251	- 0.097	0.061	0.093	- 0.098
Peripheral fat	0.537**	0.129	-0.511**	0.152	0.196	- 0.088	0.176	0.098	0.098	0.154
Central fat	0.626**	0.189	-0.467**	0.158	0.236	- 0.053	- 0.146	0.077	0.092	- 0.169
Fat %	0.678**	0.530**	0.438*	-0.011	0.314	0.287	- 0.283	- 0.049	- 0.137	- 0.363
Fat mass (Kg)	0.848**	0.185	0.251	-0.172	0.019	0.253	- 0.237	- 0.233	0.079	- 0.201
Fat free mass (Kg)	0.558**	-0.242	0.536**	-0.584**	-0.447*	0.099	- 0.120	- 0.291	0.224	0.000

Table 4: Correlation between serum lipid profile and whole body composition and fat distribution by gender for group II (Age 12-18 ± 6 months)

	Boys					Girls				
	TG (mg/dl)	**TC** (mg/dl)	**HDL-C** (mg/dl)	**TC/HDL-C**	**LDL-C** (mg/dl)	**TG** (mg/dl)	**TC** (mg/dl)	**HDL-C** (mg/dl)	**TC/HDL-C**	**LDL-C** (mg/dl)
Weight (Kg)	0.010	- 0.045	0.029	- 0.046	- 0.035	0.149	0.031	0.242	- 0.238	0 0.129
BMI (Kg/cm²)	- 0.115	- 0.291	0.108	- 0.296	- 0.274	- 0.059	0.137	0.060	0.017	0.081
Waist C (cm)	- 0.154	- 0.251	0.146	- 0.145	- 0.242	0.154	0.142	- 0.230	0.271	0.199
Hip C(cm)	- 0.290	- 0.191	0.100	- 0.241	- 0.247	- 0.139	- 0.038	0.077	- 0.062	- 0.068
Waist/Hip ratio	0.003	- 0.150	0.101	- 0.009	- 0.112	0.106	0.120	- 0.211	0.246	0.178
Skinfold (mm)										
Triceps	0.033	0.201	0.096	- 0.168	0.249	0.141	- 0.180	- 0.223	- 0.059	- 0.095
Biceps	-0.428*	0.330	0.262	- 0.222	0.246	0.075	- 0.050	0.013	- 0.188	- 0.089
Subscaular	- 0.210	0.112	0.111	- 0.048	0.144	0.166	- 0.090	- 0.98	- 0.131	- 0.067
Suprailiac	- 0.030	0.150	0.142	- 0.208	0.059	0.048	- 0.110	- 0.163	- 0.033	- 0.023
Abdominal	- 0.223	0.113	0.087	- 0.020	0.098	0.269	- 0.044	- 0.205	0.056	0.016
Peripheral fat	- 0.250	0.304	0.210	- 0.223	0.276	0.120	- 0.128	- 0.119	- 0.134	- 0.101
Central fat	- 0.171	0.152	0.139	- 0.122	0.116	0.186	- 0.096	- 0.182	- 0.042	- 0.029
Fat %	- 0.160	- 0.094	0.210	- 0.207	- 0.119	- 0.182	0.023	0.030	- 0.084	- 0.075
Fat mass (Kg)	- 0.115	- 0.120	0.235	- 0.217	- 0.153	- 0.023	- 0.023	0.090	- 0.216	- 0.125
Fat free mass (Kg)	0.157	0.029	- 0.074	0.101	0.036	0.278	- 0.065	0.106	- 0.243	- 0.146

In the current research, there are significant gender differences in ages 7 -18 years regarding BMI, abdominal skin fold thickness and TC/ HDL-C, and in peripheral adiposity at young age (7-11 years) and central one at adolescents (12-18 years). Body composition showed significant sex differences in adolescent period only. In young age (7-11 years), in spite of girl's recorded significant higher values of BMI, boys had significant higher values of abdominal and peripheral adiposity. In adolescents (12-18 years), in spite of girl's recorded significant higher values of BMI, fat%, and fat mass, boys still recorded significant higher values of abdominal subcutaneous fat but with central adiposity. Regarding lipid profile, for young age, triglycerides and HDL-C are correlated nearly with all measures of adiposity (body composition and anthropometric parameters) in boys and not in girls which might have been influenced by frequency of pubertal stages in girls. Hormonal changes, found at this stage, may act as a protective factor against changes in lipid profile. Before and after menarche, changes in lipid profile are sensitive to the influence of sex hormones, especially estrogen, which has a favorable effect on lipoprotein by increasing HDL and reducing LDL [15]. This can be explained by such study found that trunk skin folds predicted cardiovascular disease risk factors to the same extent as total fat mass by DXA, and in some cases independently of total fatness [10]. In another study, Daniels et al., 1999 [29] , studied

both the percent body fat and fat distribution in a stepwise multiple linear regression analysis and found that fat distribution was a more important independent correlate of cardiovascular risk factors (high triglycerides, low HDL cholesterol, high systolic blood pressure, high left ventricular mass) than percent fat mass. The associations found only in boys between the sum of skin folds and cardiovascular risk can be due to the fact that a central fat distribution is considered as a male specific pattern and an explanation for the high prevalence of cardiovascular disease in men compared to women [30].

This study assumed that the fat distribution is more homogeneously centrally distributed in boys and its effect on cardiovascular risk factors would not be distinguished from that of subcutaneous fat mass. Conversely in girls, there is more variability in the fat distribution, from a gynoid to an android pattern, for a given level of total fat mass. The subcutaneous fat mass has a role in the relation between fat distribution and cardiovascular disease risk in these young boys.

For adolescents, there is no correlation between any one of the lipid profile and the body composition and anthropometric indices in either gender.

Our results are in partial agreement with a previous report of Hu et al 2000 [31], who found that in boys, BMI was positively correlated with triglyceride and negatively correlated with HDL-cholesterol. Triglycerides increased with waist circumference and HDL cholesterol decreased with waist circumference. Flodmark et al 1994 [32] found also that BMI was significantly correlated with serum triglycerides. Chu et al., 2001[33], proved that TG was positively associated with most anthropometric parameters. Also Okada et al, 1998 [34] found that the distribution of central-type fat accumulation was inversely correlated with the HDL-C level in both boys and girls, and showed a stronger correlation with both the triceps and the sub scapular skin fold thicknesses.

In this study both TC and LDL-C showed significant positive correlations with supra iliac skin fold thickness, while TC only showed highly significant positive correlation with fat %, TC/ HDL-C showed significant negative correlations with body weight, BMI, hip circumference and fat free mass. This coincides with the result of Takahashi et al., 1996 [35], who recorded that overweight and obese boys had significantly higher levels of total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C). Okada et al., 1998 [34] found also that the degree of obesity and the body mass index (BMI) were more strongly correlated with serum levels of lipids and apolipoproteins in boys than in girls. In boys, atherogenic lipoproteins, such as LDL-C, showed a stronger correlation with the thickness of the triceps skin fold.

5. Conclusion:

In young boys abdominal fat distribution is associated with cardiovascular risk factors, independently of overall adiposity. International definition of abdominal obesity in children is required to standardize studies and progress in the evaluation of childhood obesity and its consequences.

Corresponding author
Sahar A. El-Masry
Biological Anthropology Dept., National Research Centre, Dokki, Giza, Egypt
masrysa@yahoo.com

References:

1. Ebbeling CB, Pawlak DB, Ludwig DS: Childhood obesity: public-health crisis, common sense cure. Lancet, 2002; 360: 473-82.
2. WHO: Worldwide trends in childhood overweight and obesity. International Journal of Pediatric Obesity, 2006, 25: 1-11.
3. Ogden CL, Carroll MD, Flegal KM: "High body mass index for age among US children and adolescents, 2003-2006". *JAMA, 2008,* 299 (20): 2401–5.
4. Munakata H, Masko S, Ashraf A. Ewis, Umeno M, Yoichi S, Takro N, Sakmoto K and Yukiko Y: Prediction of children at risk for complications of childhood obesity. The Journal of Medical Investigation, 2010, 57:62- 68.
5. Korsten-Reck U, Kromeyer-Haschild K, korsten K, Baumstark M, Dickhuth h, Berg A: Frequency of secondary dyslipidemia in obese children. Vascular Health and Risk Management, 2008; 4(5):1089-1094.
6. Li S, Chen W, Srinivasan SR, Bond MG, Tang R, Urbina EM, et al: Childhood cardiovascular risk factors and carotid vascular changes in adulthood: the Bogalusa Heart Study. JAMA, 2003; 290 (17): 2271-6.
7. Kavey RE, Daniels SR, Lauer RM, et al: American Heart Association guidelines for primary prevention of atherosclerotic cardiovascular disease beginning in childhood. J Pediatr , 2003; 142:368-72.
8. Hills AP, Lyell L, Byrne NM: An evaluation of the methodology for the assessment of body composition in children and adolescents. In: Jürimae T, Hills AP (ed) Body Composition Assessment in Children and Adolescents. Med

Sport Sci Basel, Karger, Switzerland, 2001; 44:1-13.
9. Cugnetto M, Saab P, Liabre M, Goldberg R, McCalla J, Schneiderman N: Lifestyle Factors, Body Mass Index, and Lipid Profile in Adolescents.J Pediatr.Psycho., 2007, 10:1093.
10. Teixeira PJ, Sardinha LB, Going SB, et al : Total and regional fat and serum cardiovascular disease risk factors in lean and obese children and adolescents. *Obes Res,* 2001; 9(8):432–42.
11. Reilly JJ: Diagnostic accuracy of the BMI for age in paediatrics. Int J Obes (Lond), 2006; 30: 595- 7.
12. Guo SS, Huang C, Demerath E, Towne B, Chumlea WC, Siervogel RM. Body mass index during childhood, adolescenceand young adulthood in relation to adult overweight and adiposity: the Fels Longitudinal Study. Int J Obes, 2000; 24:1628-35.
13. Berenson GS, Srinivasan SR, Wattigney WA, Harsha DW: Obesity and cardiovascular risk in children. In: Williams CL and Kimm SY (ed) prevention and Treatment of childhood obesity, Annals of New York Academy of Sciences. New York 1993; 699:93-103.
14. Berenson GS, Srinivasan SR, Bao W: Association between multiple cardiovascular risk factors and athrosclerosis in children and young adult. The Bogalusa Heart study. N Engl J Med, 1998; 338:1650-6
15. Lima S, Arrais R, Almeida M, Souza Z, Pedrosa L: Plasma liped Profile and Lipid Peroxidation in overweight or obese children and adolescents. J Pediatr, 2004; 80 (1):23-8.
16. Daniels SR, Khoury PR, Morrison JA. Utility of different measures of body fat distribution in children and adolescents. *Am J Epidemiol.* 2000; 152(12):1179–84.
17. Ghalli I., Salah N., Hussien F., Erfan M., El-Ruby M., Mazen I., Sabry M., Abd El-Razik M., Saad M., Hossney L., Ismaail S.and Abd El-Dayem S.et al: Egyptian growth curves 2002 for infants, children and adolescents. Recently published in: Sartorio A, Buckler JMH and Marazzi N (2008). Crescere nel mondo. Ferring publisher.
18. Hiernaux J. and Tanner J.M: Growth and physical studies. In: Human Biology: A guide to field methods. Eds. Weiner J.S., Lourie S.A., IBP. London, Blackwell Scientific Publications. Oxford. U.K., 1969.
19. Allain CC, Poon LS, Chen CSG, Richmond W, Fu PC (1974): Enzymatic determination of total serum cholesterol. Clin. Chem., 20: 470-475.
20. Fossati P, Prencipe L (1982): Serum triglycerides determination colorimetrically with an enzyme that produces hydrogen peroxide. Clin. Chem., 28:2077-2088.
21. Burstein M, Scholnick HR, Morfin R (1970): Rapid method for the isolation of lipoproteins from human serum by precipitation with polyanions. J. Lipid Res., 11:583-595.
22. Friedewald WT, Levy RI, Fredrickson DS (1972): Estimation of the concentration of low density lipoprotein in plasma, without use of the preparative ultracentrifuge. Clin. Chem., 18:499-502.
23. Gunczler P, Lanes R, Lopez E, et al: Cardiac mass and function, carotid artery Intima-media thickness and lipoprotein (a) levels in children and adolescents with type 1 diabetes mellitus of short duration. JPediatr Endocrinol Metab, 2002; 15: 181-6.
24. Dumith SC, Ramires VV, Souza MA, Moraes DS, Petry Oliveira ES, Ramires SV, Hallal PC: Overweight/Obesity and physical fitness among children and adolescents. J Phys act Health, 2010; 7(5):641-8.
25. Gidding SS, Nehgme R, Heise C, et al: Severe obesity associated with cardiovasculardeconditioning, high prevalence of cardiovascular risk factors, diabetes mellitus /hyperinsulinemia, and respiratory compromise.J Pediatr 2004; 144: 766-9.
26. Kahn B and Filer JS. Obesity and insulin resistance. J Clinc.Investg, 2000; 106: 473-481.
27. Blüher M: The distinction of metabolically 'healthy' from 'unhealthy' obese individuals. Curr Opin Lipidol, 2010; 21(1): 38-43.
28. Weiss R, Dufour S, Taksali S, Tamborlane W, Petersen K, Bonadonna R et al: Prediabetes in obese youth: A syndrome of impaired glucose tolerance, severe insulin resistance, and altered myocellular and abdominal fat partitioning . Lancet, 2003, 362: 951-957.
29. Daniels SR, Morrison JA, Sprecher DL, et al : Association of body fat distribution and cardiovascular risk factors in children and adolescents. *Circulation.* 1999; 99(4):541–5.
30. Price JF and Fowkes FG. Risk factors and the sex differential in coronary artery disease. *Epidemiology.* 1997; 8(5): 584–91
31. Hu D, Hannah J, Gray RS, Jablonski KA, HendersonJA, Robbins DC, Lee ET, Welty TK, Howard BV: Effects of obesity and body fat distribution on lipids and lipoproteins in non diabetic American Indians: the Strong Heart Study. Obes Res, 2000; 8: 411-421.
32. Flodmark CE, Sveger T, Nilsso-Ehle P: Waist measurement correlates to a potential atherogenic lipoprotein profile in obese 12-14

year-old children. Acta Paediatr, 1994; 83: 941-5.
33. Chu NF, Spiegelman D, Hotamisligil GS, Rifai N, Stampfer M, Rimm EB: Plasma insulin, leptin, and soluble TNF receptors levels in relation to obesity-related atherogenic and thrombogenic cardiovascular disease risk factors among men. Atherosclerosis. 2001; 157: 495-503.
34. Okada T, Sato Y, Yamazaki H, Iwata F, Hara M, Misawa M, Kim H, Karasawa K, Noto N, Harada K, Ryo S: Department of Pediatrics, Nihon University School of Medicine, Tokyo, Japan Relationship between fat distribution and lipid and apolipoprotein profiles in young teenagers. Acta Paediatr Jpn, 1998; 40(1): 35-40.
35. Takahashi H, Hashimoto N, Kawasaki T, Kikuchi T, Uchiyama M: The usefulness of measuring body fat deposition for detecting obesity and atherogenesity in Japanese school children. Acta Paediatr Jpn. 1996; 38(6): 634-9.

11/21/2010

Evaluate of Head Loss, Sediment Value and Iron Removal in Rapid Sand Filter

Hossein Banejad [1], Reza Pirtaj Hamedany [1], Navab Daneshi [1]

[1.] Department of Water Engineering, Faculty of Agriculture, Bu-Ali Sina University, Hamedan, Iran
Hossein_banejad@yahoo.com

Abstract: Quality and appropriate quantity of water is necessary for human kind to survive. Along with the technology development and increasing consumption of water resources, we are experiencing low qualities in the mentioned resources. Iron is the fixed element found in the crust of the earth. This metal found variously in water resources and industrial activities. Therefore, it needs to treat the water resources from these excessive amounts. Different methods have used for this reason but the most used method during recent years has been the absorption by economic absorbers such as sand. Rapid sand filters usually used in water treatment plants for water clarification. In this research, a single layer gravity rapid sand filter has used to reduce different concentrations of iron. sediment value and head loss arising from it specially oxidized iron sediments in filter media is simulated by using combination of Carman-Kozeny, Rose and Gregory models in different discharges of rapid sand filter. Results have shown that with increasing in discharge and decreasing in input iron concentration, arriving time to given head loss, is increasing. In addition, results demonstrated that with increasing in iron concentration in influent, removal efficiency is decreasing somewhat. Results of this research can applied in (1) appropriate design of rapid sand filter to iron removal, (2) prediction of rapid sand filter ability to iron removal and (3) estimation of arising head loss during filter work thus evaluating of time interval backwash. [Hossein Banejad, Reza Pirtaj Hamedany, Navab Daneshi. Evaluate of Head Loss, Sediment Value and Iron Removal in Rapid Sand Filter
[Hossein Banejad, Reza Pirtaj Hamedany, Navab Daneshi. **Evaluate of Head Loss, Sediment Value and Iron Removal in Rapid Sand Filter**. Journal of American Science 2010;6(12):1218-1226]. (ISSN: 1545-1003).
http://www.americanscience.org.

Keywords: Sand filter, Iron concentration, Removal efficiency, Head loss.

1. Introduction

1.1. Iron content in water and its removal

In the past few decades using of heavy metals lead to increasing the concentration of this metals in water supply and environment. Discharge increasing of heavy metal from wastewater, their poisonous identity, Detroit effect on water supply (Nuhoglu et al., 2003) and indegradable in environment has caused to their special importance (saxena et al., 2006). Considering the increasing of industrial activity and problems due to the existence of heavy metals, removal or reduction of their concentration for achieving the acceptable level before discharge in environment is essential.

Iron is of the metals that found in many water supplies and they could be considerably troublesome. Soluble of iron is colorless but in exposure with air or known chemical materials convert to insoluble form and create the colors in water. Some of problems that create by high concentration of iron in water can refer to interfere in disinfection process, slime formation in piping, taste and color**.** Removal the metal ions of industrial wastewater has been achieved by ion exchange, membrane separation (Katsumata et al., 2003), evaporation (Mouflih et al., 2005) electrolysis, absorption processes and reverse osmosis (Sarioglu et al., 2005, Pehlivan, et al., 2006). Choosing the best method to water treatment depends on the concentration of heavy metals in the wastewater and the treatment expenses. Depositing has used extensively for removal of heavy metals due to low performance expenses. However, default of this method is production of high volume of sludge (Raju., 2003). On the other, hand absorption method such as ion exchange method in easy for removal of metals but ion exchanging resins are expensive (Katsumata el al., 2003, Aslam el al., 2004). Among the mentioned methods, we should look for a method that is economic and easily applicable for developing countries and can use efficiently. Adsorption method has suggested for removal of heavy metals because it is cheaper and more effective than other technologies (Pehlivan et al., 2006). A method for metal removal can be applied to industrial wastes without prior treatment using solid adsorbents such as sand and silica (Yabe et al., 2003). In recent years, liquid content iron filtration through granular media such as silica is very considerable (Aklil el al., 2004, Mouflih el al., 2005). Effluent iron concentration is an important water quality criterion used for the assessment of the performance of rapid sand filters, in addition to other criteria (Cakmakci et al., 2010).

1.2. Rapid sand filter and head loss

Filtration is the process in which the suspended particles removed from a flow by passing through a prose media (hamoda et al., 2004, vissman et al., 2004, Tebbutt, 1998, iritani, 2003). During the filtration process, water passed trough the bed under pressure or gravity. Removal of particle will vary due to size and identity of them (classen, 1998). Rapid sand filter used extensively for treatment of water and wastewater (Raju, 2003). Two factors, effective size and uniformity coefficient should consider for filter media. Usually the effective size and uniformity coefficient are considered 0.45 – 0.7 (mm) and 1.3 – 1.7 respectively in rapid sand filters (Punmia et al., 1995).

Different parameters involved in filtration affect the efficiency of these filters. Studies have shown that if the filter has smaller grains, lower rate and is deeper, the removal of manganese will be more efficient.

In drinking water treatment, granular media or rapid gravity filter is used. Filters clogged with deposits and this event lead to head loss in through of filter media. Therefore, filter backwashing have been necessary. To design an appropriate rapid sand filter utilizable effectively in removal of specific pollutant, head loss prediction before establishing is essential. Because of this, the equations that show relationship between involved hydraulic parameter must used.

1.3. Granular media hydraulic equations

During filtration, the clogging of the pores increases thus the resistance in the filter bed. When the filter reaches to the maximum available head loss, the filter needs to backwash to avoid a decrease in the filtration velocity**.** Head loss effective factors presented by below equation.

$$H_L = f(L, d, V_S, g, e, \upsilon)$$

Where H_L = head loss in L depth of filter; d= filter media diameter; V_s = flow velocity across media; g= gravity acceleration; e= filter porosity; υ = cinematic viscosity.

To calculate head loss the most common equation are (1) Carman-Kozeny, (2) Rose and (3) Gregory

1.3.1. modified Carman-Kozeny equation

The Carman-kozeny equation is a semi-empirical relationship and its extension to the particle deposition phase has to be based on experimental data because no theoretical description of the processes governing the head loss development have been developed to described the head loss as a function of time or increasing solids deposits. Summarizes of the wide variety of head loss development model during filtration by Herzig et al. (1970) and Sakthivadivel et al. (1972) also show that all head loss models have used on modifications to the Carman-Kozeny equation. The change of various parameters as probity decreases, and the internal surface and the tortusity of the flow increases during solids deposition is incorporated into the Carman-Kozeny equation (Boller et al., 1995). Must be attention that Carman-Kozeny equation can be applied to estimate head loss, but can only be applied to clean filter beds. Therefore, this promoted and modified along the time.

Most of the models lead to an equation relating the head loss gradient I at the certain floc volume deposit σ_v to the initial head loss gradient I_0 given by the general form (equation 1)

$$\frac{I}{I_o} = \left(1 + P.\frac{\sigma_V}{f_0}\right)^x . \left(1 - \frac{\sigma_V}{f_0}\right)^y \qquad (1)$$

Where *p, x, y* are empirical constant that are 35, 1.5 and -1 respectively. f_0 is the clean bed porosity (in the other word initial porosity involved in filtration).

$$I = \frac{h}{L} \qquad I_o = \frac{h_o}{L}$$

Where h, h_o and L are head loss, initial head loss and depth of purification layer respectively.

1.3.2. Rose equation

Rose equation in order to use for rapid sand filter in state that the filter bed considered homogeneous is shown as a equation 2:

$$\frac{h_0}{l} = 1.067 C_D \frac{v^2}{g \cdot d \cdot \psi} \frac{1}{f_0^4} \qquad (2)$$

Where g = gravity acceleration; h_0 = head loss between up and down of porous media; l = length of path that fluid travel through media; d = effective size of bed particles; f_o = initial porosity involved in filtration; and C_D = Newton drag coefficient.

C_D is the function of Reynolds number. Amount of C_D can achieve from equation 3:

$$C_D = (24 / R) + (3 / \sqrt{R}) + 0.34 \qquad (3)$$

R is the Reynolds number by below equation:

$$R = \frac{v\, d}{\upsilon}$$ v and υ are apparent velocity and

cinematic viscosity, respectively. ψ is the particle shape factor that achieve from below equation:

$$\psi = \frac{A_0}{A}$$

Where A_0 = area of sphere that have a same volume with filter media particle; A = real area of filter media particle. Amount of this parameter suggested between 0.79 and 1 for sand (Tebbutt., 1998).
After filter backwashing and start of filtration, due to fluid velocity in porous media, initial pressure gradient $\left(I_0 = {h_0}/{l}\right)$ produce between up and down of porous media. With gradient entrance to Rose equation, initial porosity involved in filtration (f_0) is attainable.

1.3.3. Gregory equation (Tebbutt, 1998)

Gregory equation presented by as equation 4:

$$h = h_0 + \frac{K v C_0 t}{(1 - f)} \qquad (4)$$

Where v = apparent fluid velocity; f = involved porosity in filtration with respect to head loss (h); t = time (minute); C_0 = concentration of substance in fluid that lead to lead loss; and K = Gregory equation coefficient that variable in each of condition.
In this study by combination of modified Carman-Kozeny, Rose and Gregory equation the time that head loss in granular media reach to premises level, estimated. This method is a benefit way to design the filter.

2. Materials and methods

To do this study, a single layer rapid sand filter by below characteristics is constructed. Filter surface size is 17*17 cm; length of effective layer in treatment is 70 cm that included sand with 0.42-1.8 mm diameter, actual density is 2.65 ${gr}/{cm^3}$, 0.6 mm effective size and uniformity coefficient is 1.5.

The filter media supported on base material consisting of graded gravel layers (table 1). The gravel should be free from clay, dirt, vegetable and organic matter, and should be hard , durable and round, its total depth is 120 cm and laid in the following layers (figure 1).

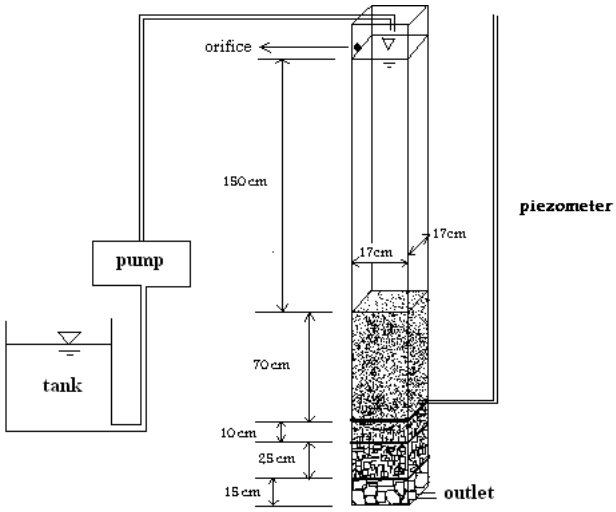


Fig 1. Schematic of filter

In order to achieve different iron concentration (25, 75, 125 and 175 ppm), nitrate salt of iron is used. Then solution separately sent to top the filter and they to passed trough the granular media in various discharge (1.5, 2, 2.5 and 2.9 ${lit}/{min}$) separately.

The characteristics of used water to making solution have shown in table 2.
Sampling carried out from established tap under filter drain. Given samples acidified immediately by nitric acid. Then iron concentration in effluent perused by atomic emission spectrometer with ICP source.

Table 1. Layer of filter

	Layer	Depth	Grasde size	Manometer below layer
1	Top most	700 mm	0.6-1.18 mm	h_1
2	Intermediate	100 mm	2.36-4.75 mm	h_2
3	Intermediate	250 mm	6.7-13.2 mm	h_3
4	Bottom most	150 mm	26-52 mm	h_4

Table 2. characteristics of used water to making solution

Unit	Amount	Characteristic
-	7.2-7.5	pH
NTU	1.5	Turbidity
mg/L	0	Chlorine
mg/L	0	Iron
mg/l	0	Manganese
mg/L as carbonate calcium	185	Hardness
$\frac{\mu mho}{cm}$	457	Electrical conductivity
^{0}**C**	23-25	Temperature

2.1. Initial porosity involved in filtration (f_0) calculating

One of most important factors in modified Carman-Kozeny equation is the f_0. Since that recognizing the amount of porosity that participate in filtration is impossible specially when deposits by complex morphology formed in granular media and f_0 will varied with each discharge to other, estimating of this factor is a hard work.

To do above aim for each discharge, initial head loss (h_0) was perused from installed piezometer at the purification layer (upper layer) below. Then C_D calculate from equation 3. In this study ψ considered equal to 0.85. f_0 calculate from Rose equation. Noticeable attention in Rose equation is on l. In the case of granular media l is length of path that fluid travel through filter. Because of this, purification layer height multiplied to tortuosity coefficient. Carrier (2003) explained that this amount is two.

2.2. Head loss in filter and porosity amount relationship with emphasis on different passed discharge

In this step, the range between initial head loss (h_0) and permissible headloss was assumed. For any discharge and assumed head loss, σ_v calculated from modified Carman-Kozeny equation. Needed f_0 in modified Carman-Kozeny equation, be achieved from step 2.1 for any discharge.

2.3. Gregory equation adaptation

Unknown parameters in Gregory equation are K and f. In each step of experiment f will be achived from below equation.

$$f = f_0 - \sigma_V$$

σ_v available from step 2.2.

To achieve K, following steps must perform.

A: Calculate iron removal efficiency by filter in various steps then figure out the concentration of trapped iron that lead to lead loss in filter (C_0).

B: h_0 peruse from installed piezometer at the beginning of filtration for each of discharges. h peruse from piezometer at certain time after filtration (in this case 50 minute) for each of discharges and inlet concentration of iron,

C: entrance C_0, f, h, h_0, v and t in Gregory equation for each of experiments step. Therefore K is available in each step of experiment.

2.4. Time estimation of certain head loss arriving

In this step, assumptive range of head loss (h) (between initial head loss and permissible head loss) is considered. Now from 2.2, decreased porosity respect to assumptive head loss (f) is available. By entrance f, h_o, C_o, v, h and K in Gregory equation for all of the situations (assumptive range of head loss, varied discharge and different concentration of inlet iron), time of reach to certain assumptive head loss (t in Gregory equation) will be accessible.

3. Results

3.1. Hydraulic parameters for different discharge

Achieved amounts for initial head loss, initial head loss gradient, Reynolds number, drag coefficient and initial porosity shown in table 3. As observed all of the Reynolds number have amount of less than one. Thus, laminar flow dominates on filter bed.

3.2. Assumptive head loss versus f diagrams for all of the discharges

Figure 2 describe relationship between head loss and decreased porosity (f) in different discharge. With attention on fig. 2 and table 2, these points figure out that with increase in discharge f_0 decreased. In addition, slop of lines in fig. 2 approximately is same. Then can be expected that porosity decreasing trend in different discharge be similar. In other word, increasing deposit rate in discharge range is similar.

Table 3. Initial head loss, initial head loss gradient, Reynolds number, drags coefficient and initial porosity amounts respect to apparent velocity .

f_0	C_D	R	I_0	h_0(cm)	v(m/sec)	Q(lit/min)
0.477130201	51.03696	0.515905	0.15714	22	0.000865	1.5
0.493656059	38.9537	0.685885	0.185	25.9	0.00115	2
0.509767521	31.5216	0.858847	0.2064	28.9	0.00144	2.5
0.519388408	27.44179	0.996024	0.224	31.4	0.00167	2.9

3.3. K (Gregory coefficient) amounts in different condition (table 4) and estimated time to arrive given head loss (minute) in different iron concentration and different discharge (fig. 4, 5, 6, 7)

To achieve C_0 in Gregory equation, removal efficiency of iron by rapid sand filter (E %), must calculate (fig. 3). Then by using below equation, C_0 be accessible.

$$C_0 = C_1 - C_2 = C_1 - (E\%) \times C_1$$

Where C_1 and C_2 is inlet and outlet concentration of iron, respectively.

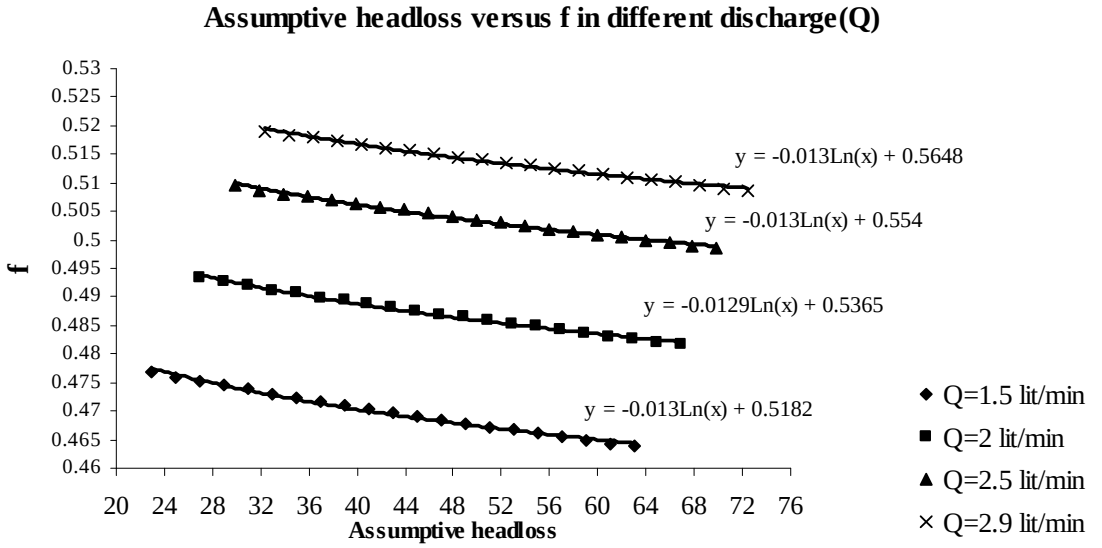


Fig.2. Assumptive head loss versus f

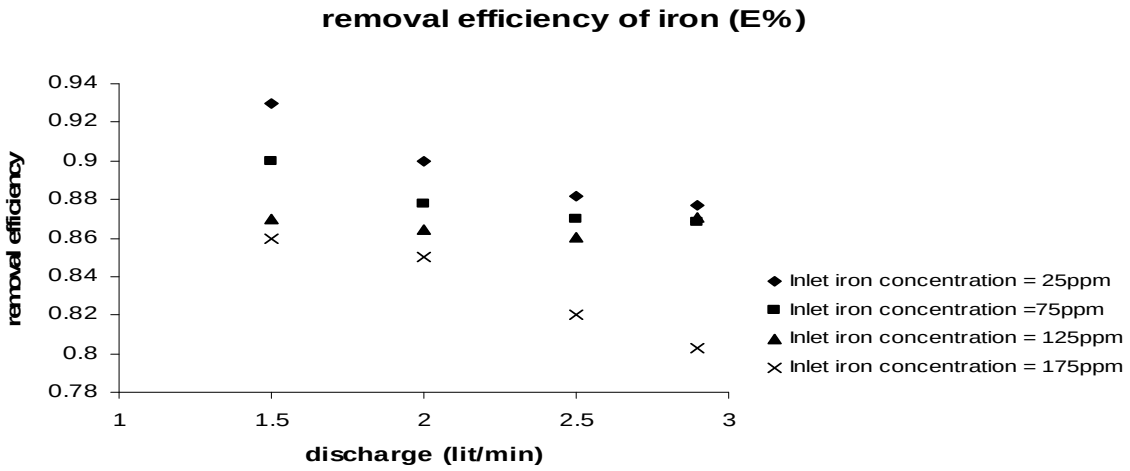


Fig.3. Removal efficiency of iron by filter

Table 4. K amounts in different condition

	Inlet iron concentration (mg/L)			
Discharge (lit/min)	**25**	**75**	**125**	**175**
1.5	0.036058668	0.01317152	0.008362015	0.00617717
2	0.010762841	0.004235695	0.002854978	0.002369632
2.5	0.006683628	0.002780231	0.002004233	0.001660144
2.9	0.003943624	0.001623595	0.001148426	0.001094495

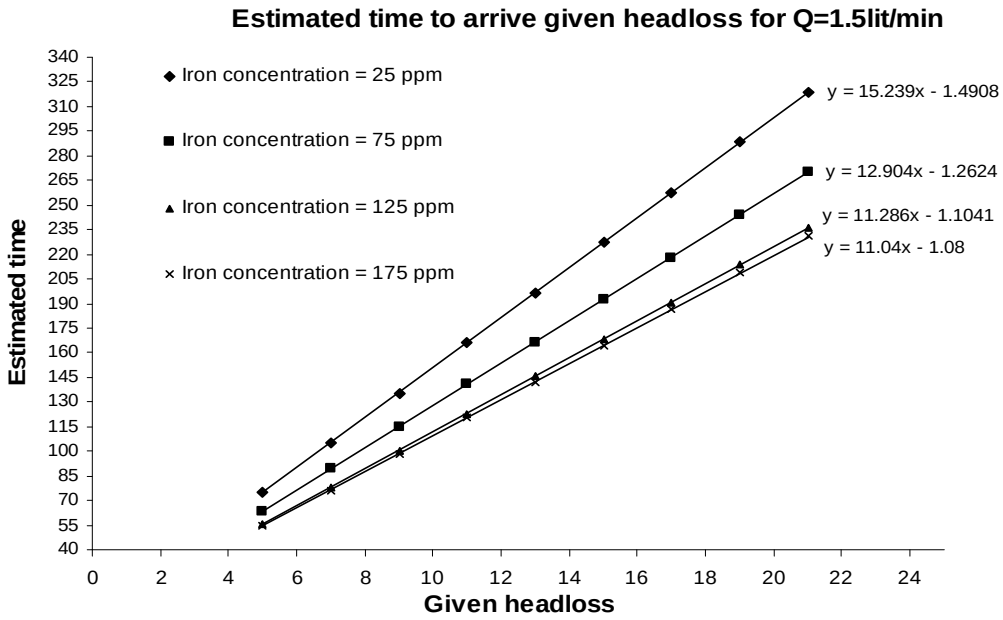


Fig.4. Time (min) versus head loss (cm) for discharge equal 1.5 (lit/min)

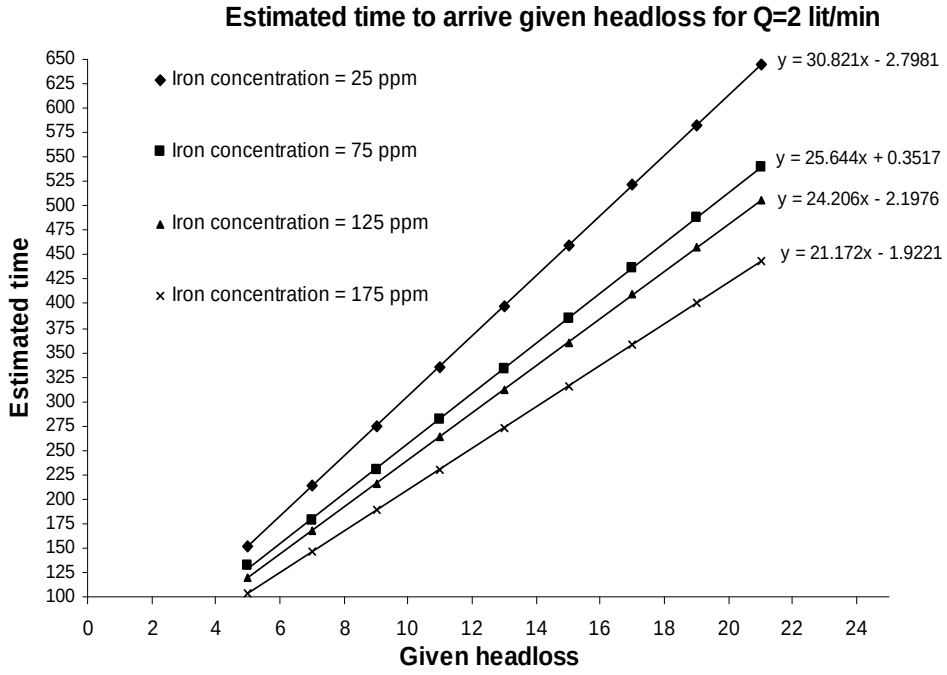


Fig.5. Time (min) versus head loss (cm) for discharge equal 2 (lit/min)

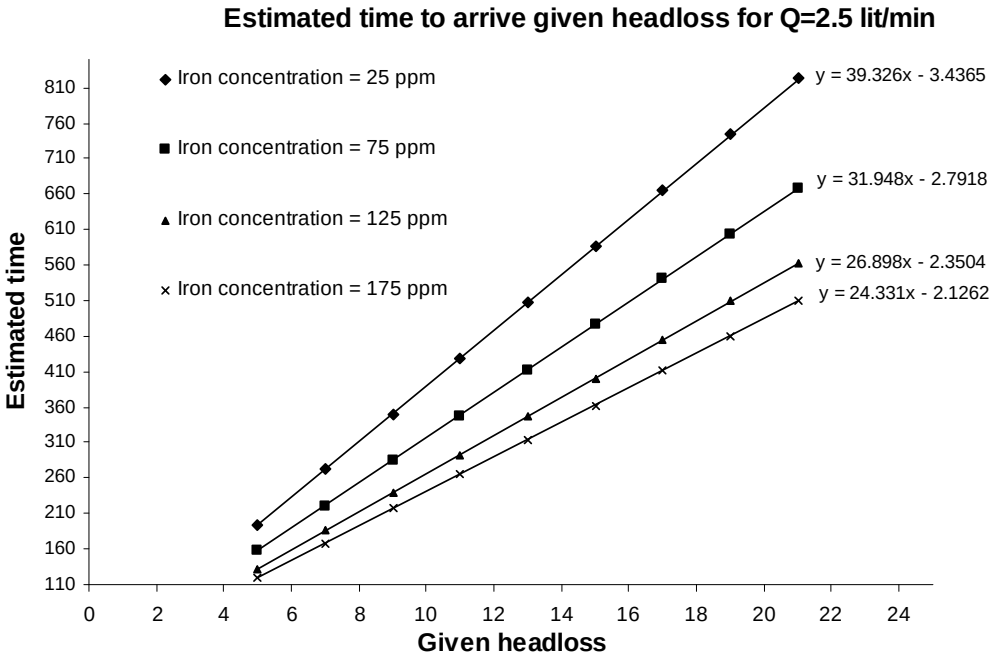


Fig. 6. Time (min) versus head loss (cm) for discharge equal 2.5 (lit/min)

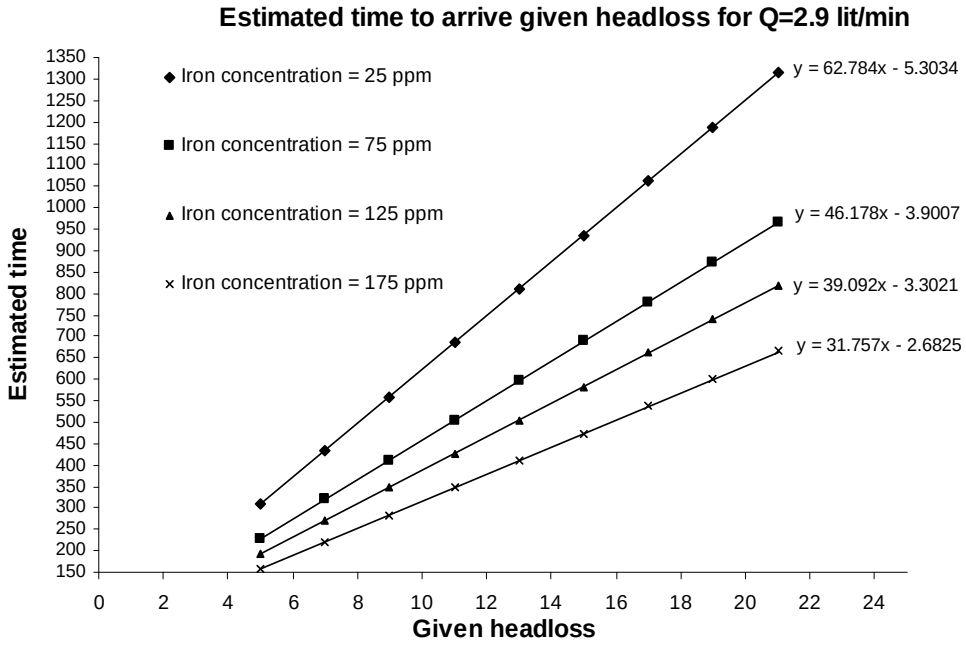


Fig 7. Time (min) versus head loss (cm) for discharge equal 2.9 (lit/min)

R^2 in figures 4, 5, 6 and 7 by linear regression is closely to 1. In addition figures 4,5,6 and 7 show that with decreasing in inlet iron concentration and increasing in discharge, arriving time to same given head loss ($h - h_0$) is increased.

Although increasing in discharge lead to entrance iron to filter is increased, the higher rate of water in bed causes that removal efficiency decreased, in addition deposit that is more compact form in granular media (because of more hydrodynamic force). Thus in same circumstance (same inlet iron concentration and given head loss), increasing in discharge lead to decreasing in σ_V . In other world, hydrodynamic force of water in iron filtration is more effective on head loss rather than inlet volume of iron.

Line slope comparison in same discharge for any of the figures 4, 5, 6 and 7 shows that in lower inlet iron concentration slope is greater. Therefore, expect that in lower inlet iron concentration, deposit distribution in depth of bed is more homogeneous. However, in higher inlet iron concentration most of deposit formed in upper layers of bed.

4. Discussions

Increasing in iron concentration lead to removal efficiency decreased. Then if high concentrations of iron exist, a series of rapid sand filters must used. Considering that rapid sand filter has relatively establishing and reclamation low cost rather than other method for iron removal, its recommend that this type of filter used for iron removal from water.

In lower inlet iron concentration, deposit distribution in depth of bed is more homogeneous. Therefore, if high concentrations of iron exist, rapid sand filters series consequence must be from filter by less depth to filter by more depth.

With increasing in discharge and decreasing in inlet iron concentration, arriving time to given head loss increased.

Following trend of this study can be useful to better rapid sand filter design (depth of filter, discharge, and grain size of filter media)

Determining of arising head loss during filtration by presented method in this research lead to more exact estimation time interval for rapid sand filter backwashing.

Using of filter media variable size in calculation and following of mentioned methodology, can aid to appropriate rapid sand filter particle size select.

Corresponding Author
Hossein Banejad
Department of Water Engineering, Faculty of Agriculture, Bu-Ali Sina University , Hamedan, Iran
Email: Hossein_banejad@yahoo.com

References

1. Nuhoglu Y, Oguz, E. Removal of copper(II) from aqueous solutions by biosorption on cone biomass of Thuja orientalis. Process Biochemistry 2003; 38:1627-1631.
2. Saxena S, Souza SFD. Heavy metal pollution abatement using rock phosphate mineral. Environment International 2006; 32:199-202.
3. Katsumata H, Satoshi K, Kentaro I, Kumiko I, Kunihiro F, Kazuaki M, Tohru S, Kiyohisa, O. Removal of heavy metals in rinsing wastewater from plating factory by adsorption with economical viable materials. Journal of Environmental Management 2003; 69(2):187-191.
4. Mouflih M, Aklil A, Sebti S. Removal of lead from aqueous solutions by activated phosphate. Jornal of Hazardous Materrials 2005; B119:183-188.
5. Sarioglu M, Atay A, Cebeci Y. Removal of copper from aqueous solutions by phosphate rock. Desalination 2005; 181:303-311.
6. Pehlivan E, Cetinand S, Yanık BH. Equilibrium studies for the sorption of zinc and copper from aqueous solutions using sugar beet pulp and fly ash. Journal of Hazardous Materials 2006 ; 135:193-199.
7. Raju B. Water supply and wastewater engineering. Tata Mc Graw-Hill 2003.
8. Aslam MM, Hasan I, Malik M. Sand as adsorbent for removal of zinc fromindustrial effluents. EJEAFche 2004; 3(6):792-798.
9. Yabe MJS, Oliveira E. Heavy metals removal in industrial effluents by sequential adsorbent treatment. Advances in environmental research 2003; 7: 263-272.
10. Aklil A, Mouflih M, Sebti S. Removal of heavy metal ions from water by using calcined phosphate as a new adsorbent. Journal of Hazardous Materials 2004; A112:183-190.
11. Cakmakci M, Kinaci K, Bayramoglu M, Yildirim Y. A modeling approach for iron concentration in sand filtration effluent using adaptive neuro-fuzzy model. Expert Systems with Applications 2010; 37:1369-1373.
12. Hamoda M, Al – Ghusain, I, Al –Mutairi, N. Sand filtration of wastewater for tertiary treatment and water reuse. Desalination 2004; 164:203-211.
13. Viessman W, Hammer J. Water supply and pollution control. Prentice Hall 2004.
14. Tebbutt T. Principle of water quality control. Butterworld Heinemann 1998.
15. Iritani E. Properties of filter cake in cake filtration and membrane filtration. Kona 2003; 21:19-40.
16. Clasen J. Efficiency control of particle removal by rapid sand filters in treatment plants fed with reservoir water: A survey of different methods. Water Science and Technology 1998; 37(2):19-26.
17. Boller M, Kavanaugh M. Particle characteristic and head loss increase in granular media filtration. Wat. Res 1995; 29(4):1139-1149.

11/10/2010

Comparative Study Of Isocratic And Gradient Elution Chromatography In Stability Indicating Assay Of An Antihypertensive Drug Combination.

M. Abdel Kawy*, A.E. El-Gendy**, E.S. Shokry**

* Faculty of pharmacy, Cairo university

** Faculty of Pharmacy, Misr International University

ABSTRACT: Inspite that chromatographers are cautioned to avoid gradient elution when isocratic elution will do. In this work, the analytical properties of gradient and isocratic elution applied to separation of a complex sample of (fosinopril, hydrochlorothiazide and their degradation products) which can be done under isocratic condition are compared. Procedures were developed for determining fosinopril and hydrochlorothiazide in presence of each other and their degradation products by HPLC in the gradient elution mode using methanol- 20 mM KH_2PO_4 (PH 2.4) containing 0.1% triethyl amine. In the isocratic mode, the same mobile phase composition was applied in a constant ratio of 60: 40 (Buffer: methanol). Separation was achieved on a cyanopropyl column (4 x 250 mm, 5 μm) known for its high selectivity for polar and hydrophilic compounds and the least retentive of hydrophobic compounds which do not normally elute on standard C18 or C8 columns. The present work shows that gradient elution gave a shorter overall analysis time with similar resolution of the critical pair without sacrificing repeatability in parameters, so many of the reasons given to avoid gradient elution deserve serious reconsideration especially for those samples that can be separated isocratically.

[M. Abdel Kawy, A.E. El-Gendy, E.S. Shokry. **Comparative Study Of Isocratic And Gradient Elution Chromatography In Stability Indicating Assay Of An Antihypertensive Drug Combination.** Journal of American Science 2010;6(12):1227-1236]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Comparative Study; Isocratic And Gradient Elution Chromatography; Stability; Antihypertensive Drug Combination

I. INTRODUCTION

Fosinopril (FOS) and hydrochlorothiazide (HCTZ) are administered as a drug combination used in treatment of hypertension marketed under the name Monozide®. Stability studies confirm that both FOS and HCTZ are liable to hydrolysis yielding fosinoprilat (FOS-at)[1-3] and 6-chloro-2,4-disulfamoyl aniline (DSA)[4-5] respectively. FOS was successfully determined in presence of its degradate in synthetic mixture and biological fluids by chromatographic[1-3,6-13] and CE[14] methods. On the other hand, spectrophotometric[15,16] and HPLC[17,18] methods were applied for determination of HCTZ in presence of its degradate or in its pharmaceutical drug combinations.

Several methods have been also presented for the analysis of pharmaceutical preparation containing mixtures of FOS and HCTZ but the analysis of a quaternary mixture of the two active ingredients simultaneously in presence of their process degradates is considered a challenge.

The complexity of this mixture arises from the great difference in hydrophobicity and physiochemical properties of the analyzed compounds, FOS (being of highest lipophilicity and excessive retention time) and HCTZ (least retentive) in addition to their degradates whose presence lead to the problem of critical pairs of almost similar chromatographic behavior which makes the separation process even more complicated.

Two approaches have been presented in this work for the analysis of this mixture, the first one is based on isocratic elution utilizing the cyanochemistry of cyanocolumns which provides a different selectivity from both phenyl and standard aliphatic (C_4, C_8, C_{18}) reversed phases, having a dual nature with both polar and hydrophobic properties. They therefore can be used as the least polar alternative of NP in normal phase applications and the least hydrophobic alternative to RP-8 in reversed

phase applications. Separations using reversed and normal phase can be carried out using this material, where combination of weak hydrophobic interactions and polar interactions enable successful separation of complex samples of compounds of different physicochemical properties[19].

In the second approach, another modulation is introduced by the use of gradient elution, which involves gradual change in the mobile phase composition by increasing the concentration of the organic modifier during the course of separation. This maintains separation of the critical pair and in the sometime decreases the retention time of the late eluting peaks by reducing the retarding strength of the aqueous component.

Combining the benefits of cyanopropyl column with gradient elution provides a complete separation of the present mixture in a much shorter runtime.

II.EXPERIMENTAL

II.1. Instrumentation

An HPLC unit equipped with an autosampler, thermostated column department, diode array detector IG1315A), quaternary pump (G13154A) and a vacuum degasser from Agilent technologies, USA. The chromatographic column from Merck KGaA, Dermstadt, Germany was lichrospher® 100 CN (4x250 mm i.d., 5µm particle size). Data acquisition was performed on Agilent LC chemstation software. All determinations were performed at 45^{o}C.

II.2. Samples and reagents

II.2.1. Samples

II.2.1.1. Authentic samples

FOS and HCTZ certified to contain 100.50% and 99.50% respectively by the manufacturer method were kindly supplied by Bristol Myers Squib. 4-amino-6-chlorobenzene-1,3 disulphonamide (DSA) certified to contain 99.25% by the manufacturer method as supplied by Sigma-Aldrich chemie, GmbH, Germany.

II.2.1.2. Market samples

A commercial pharmaceutical preparation (Monozide® tablets manufactured by (Bristol Myers Squib, Egypt, Batch no. L 93731) labeled to contain 20 mg/10 mg FOS/ HCTZ was obtained from the local market.

II.2.2. Reagent

- Methanol used was HPLC grade (Sigma – Aldrich Co., USA)
- Potassium dihydrogen phosphate (BDH, Poole, UK), triethylamine (BDH) and orthophosphoric acid used were analytical grade.
- Distilled water was used throughout the whole work.

II.2.3. Standard solutions

II.2.3.1. Standard stock solutions

II.2.3.1.1. Standard stock solutions of FOS, HCTZ and DSA

Portions equivalent to 500.00 mg of FOS and 100.00 mg of each of HCTZ and DSA were accurately weighed and transferred into separate 100 ml volumetric flasks and dissolved in the minimum amount of methanol, sonicated for 10 minutes and the volume was made up to the mark with methanol to give stock solutions of concentrations 5.0 mg.ml^{-1} and 1 mg.ml^{-1} respectively.

II.2.3.1.2. Standard stock solution of FOS -at

It was prepared by mixing a portion equivalent to 500.0 mg of FOS with 1.0 M NaOH, heated under reflux for 1 hour at 100^{o}C the solution was then cooled and neutralized with a calculated volume of 1.0 M HCl. The obtained precipitate was filtered, washed, transferred quantitatively into 100 ml volumetric flask and the volume was made up to the mark with distilled water to give a standard stock solution of concentration 5.0 mg.ml^{-1}.

II.2.3.2. Standard working solutions of HCTZ and DSA

A portion equivalent to 10.0 mg of HCTZ and DSA was accurately transferred from their standard stock solution into two 100 ml volumetric flasks. The volume was made up to the mark with methanol to give standard working solutions of concentration 100 µg.ml^{-1}.

III. Procedure

III.1. Chromatographic conditions

** For isocratic elution:*

Isocratic elution technique was utilized with the column maintained at 45°C. The mobile phase used was a mixture of 20 mM potassium dihydrogen phosphate containing 0.1% triethylamine adjusted at PH (2.4) using orhtophosphoric acid and methanol in a ratio of (60:40). The mobile phase was filtered thorough a 0.45 μm membrane filtration system (Millipore Corp., Milford, MA, USA) to remove any particulate matter then degassed by sonication for 20 minutes. The flow rate was 1.5 ml/min. samples of 20 μl, were injected onto the column and scanned at 210 nm and 220 nm and the mixtures were scanned over the range of 200-400 nm. All the chromatographic determinations were performed 3 times at 45°C.

** For gradient elution:*

Simultaneous separation and quantification of FOS, FOS-at, HCTZ and DSA was performed by the use of gradient elution with the column maintained at 45°C. The gradient was prepared from 40:60 (v/v) methanol-20 mM aqueous potassium dihydrogen phosphate containing 0.1% triethylamine adjusted at PH 2.4 (component A) and 90:10 (v/v) methanol-20 mM aqueous potassium dihydrogen phosphate containing 0.1% triethylamine adjusted at PH 2.4 (component B). The gradient elution program is presented in the following table. All the changes in gradient were linear and the re-equilibration time was 15 minutes. The mobile phase flow rate was 1.5 ml/minute. Diode array detection was performed for optimum selection of wavelengths used in quantification of the selected compounds.

Time	Component A (%)	Component B (%)
0	100	0
4	100	0
10	0	100

III.2. Method validation

III.2.1. Linearity

Aliquots equivalent to (2.0- 30.0 μg) of HCTZ and (40.0- 1000.0μg) and (6.0-600.0 μg) of FOS for isocratic and gradient elution respectively were accurately transferred from their stock and working solutions respectively into 10 ml volumetric and diluted to the volume with mobile phase. Each of those dilutions were then chromatographed by injecting an aliquot of 20 μl of each into the chromatographic system five times and processed according to the method described in this work. The mean peak areas of five determinations of each concentration were plotted against the same concentration and the regression equations were then computed.

III.2.2. Laboratory prepared mixture

A laboratory prepared mixture containing 500 $\mu g.ml^{-1}$ of FOS and FOS-at and 10 $\mu g.ml^{-1}$ of HCTZ and DSA was prepared and chromatographed by adopting the procedures mentioned under linearity. The concentrations were determined by referring to the regression equations. The percentage recoveries and the standard deviations were then calculated.

III.2.3. Accuracy

The previously mentioned procedures under linearity were applied for different concentrations of FOS, HCTZ, DSA and FOS-at. The concentrations of the studied compounds were calculated from their corresponding regression equations. The mean percentage recoveries and standard deviations were then calculated.

III.2.4. Precision

III.2.4.1. Intraday precision (Repeatability)

The previously mentioned procedures under linearity were used for the analysis of freshly prepared solutions of concentrations, (2.0, 18.0, 30.0 $\mu g.ml^{-1}$) of HCTZ, (40, 400, 1000 $\mu g.ml^{-1}$) and (6.0, 100.0, 600.0 $\mu g.ml^{-1}$) of FOS by isocratic and gradient elution respectively for the determination of intraday precision (n=5) and the relative standard deviations (R.S.D %) were calculated.

III.2.4.2. Interday precision (Intermediate precision)

The previously mentioned procedures under linearity were used for the analysis of freshly prepared solutions of concentrations, (2.0, 18.0, 30.0 $\mu g.ml^{-1}$) of HCTZ, (40, 400, 1000 $\mu g.ml^{-1}$) and (6.0, 100.0, 600.0 $\mu g.ml^{-1}$) of FOS by isocratic and gradient elution respectively for the determination of interday precision (n=5) and the relative standard deviations (R.S.D %) were calculated.

III.2.5. Application to pharmaceutical preparations

Twenty tablets of Monozide tablets were accurately weighed, and finely ground in a mortar. A portion of the powder equivalent to 100 mg of each of

FOS and HCTZ was separately extracted with the minimum amount of methanol, sonicated for 30 minutes, centrifuged for 10 minutes. The precipitate was then filtered through a micropore filter, washed into two volumetric flasks and the volume was made up to the mark with the same solvent.

An appropriate dilution was made to prepare working solutions of the studied drugs, the aliquot was then chromatographed by adopting the procedures mentioned under linearity. The concentrations of FOS and HCTZ were then calculated from their corresponding regression equations and the mean percentage recoveries were then calculated.

III.2.6. Validation by standard addition technique

This study was performed by adding known amounts of the studied compounds from their working standard solutions to a known concentration of the commercial preparations. The resulting mixtures were chromatographed by adopting the procedures mentioned under linearity.

IV. RESULT AND DISCUSSION:

When working with mixture of analytes in different compound classes, with different structures, physicochemical properties and polarities, selectivity can be a factor of many variables as type of stationary phase used, percentage of organic modifier, temperature of analysis, PH of mobile phase and flow rate.

Developments in column technology have been mainly responsible for the advances in these directions[19,20]. A chemical modification that was made to the surface functional groups by introduction of cyanobonded phases led to development of cyanocolumns which are much less hydrophobic, less sterically restricted and have lower hydrogen bond acidity and considered the least retentive of all reversed phases as discussed earlier[20-24].

Fosinopril is a phosphonate containing ACE inhibitor which is the most hydrophobic (lipophilic) and retentive among all ACE inhibitors due to the hydrophobic side chain added to fosinoprilat to modulate its ionization characteristics and promote its poor oral bioavialbility[25-29] therefore a major difficulty was faced during analysis of a mixture of not only FOS, FOS-at but also HCTZ and its degradate (DSA) due to the diverse nature in the polarity of the investigated compounds.

Method development started with the use of different columns including C8, C18 and CN columns with different lengths (15 cm and 25 cm) and CN column (25 cm) was the only one capable of eluting FOS under the chromatographic conditions which resolve the rest of the mixture isocratically but the problem faced was the very long retention time of FOS which makes the runtime very long and it being eluted as a broad peak with extensive tailing. This means that the selectivity of the stationary phase was not enough for solving this analytical problem, there arises the need for manipulating another parameters which is the column temperature.

Operation at elevated temperatures decreases the mobile phase viscosity, the column back pressure allowing the use of not only higher flow rates but also longer columns thus offers better separation for complex mixtures[19,20].

In the present work, a long cyanocolumn, 25 cm long was under being operated at 45^{o}C, where significant reduction in the column back pressure allowed the use of a flow rate of 1.5 ml/min which was considered a limitation when being used the same column at room temperature and in the same time led to faster elution of FOS as a sharp peak instead of the broad peak obtained by operating at room temperature.

Several mobile phase compositions of methanol-water were investigated but no peaks were obtained, water was then replaced by 20 mM potassium dihydrogen phosphate with subsequent adjustment of PH at different values. It was found that very minor changes in PH can have a major effect on the peak shape and the chromatographic behavior of FOS and FOS-at at where at PH 3.0, distorted or irregular shaped peaks were obtained while at PH 2.4, sharp and symmetrical peaks were obtained instead.

The composition ratios of buffer and methanol were varied to study the effect of the % of organic modifier. It was found that minor changes lead to either:

1. Complete retention of FOS, where there exists a critical concentration of methanol below which FOS is retained on the column.

2. Overlapping of the critical pair (HCTZ and DSA).

Therefore fine tuning of the mobile phases composition is considered a must in order to establish good resolution of the critical pair and elution of FOS from the column with a reasonable retention time. Triethylamine (0.1%) was added to minimize peak tailing and enhance peak symmetry. Thus the mobile phase used was a mixture of 20 mM potassium dihydrogen phosphate containing 0.1% TEA and methanol in a ratio of 40: 60 (v/v) and a representative chromatogram of the mixture is presented in figure (2).

In gradient elution:

A further refinement made to HPLC by changing the mobile phase composition during the coarse of separation termed a solvent gradient elution was generally utilized for separation of complex mixtures with a wide retention range with no loss of resolution and applied specially in the case presented here to decrease the overall runtime and in the same time the cost of analysis.

Successful separation of the investigated compounds (FOS, FOS-at, HCTZ and DSA) was carried out but with an important limitation which is the prolonged analysis time where each run lasts about 30 minutes, therefore a modification was introduced to the method which involves gradual increase in the mobile phase strength as the separation proceeds in such away that the elution of the late elute compounds (FOS and FOS-at) was continuously reduced.

The model of gradient solvent profile applied was linear, gradient shown in figure (1) where the rate of the change in methanol is linear overtime because it permits greater separation selectivity between the earlier peaks (HCTZ and DSA) as well as later peaks (FOS and FOS-at).

It starts with an isocratic mode of 60% buffer: 40% methanol for the first 4 minutes of the run to ensure complete separation of the critical pains (HCTZ and DSA) followed by linear gradient over the next 6 minutes where methanol reaches 90% by the end of the run and this produces early elution of FOS-at and FOS with no loss of their resolution.

The column was allowed to re-equilibrate for 10 minutes with less than two column volumes of the initial eluent before the next run. The detector was operated on the DAD mode, allowing scanning of the absorption of the investigated compounds for confirmation of their identity and for selection of optimum wavelength for measurement.

The main problem faced during the analysis was that the wavelength chosen for quantitative determination of HCTZ and FOS was 210 nm which is close to the cut-off λ of the solvent used (methanol) which cause slight drift in the baseline in the positive direction, therefore the linear gradient model was chosen because it involved small change in the eluent strength relative to time which helped minimize the baseline disturbance. The same flow rate (1.5 ml/min) was applied throughout the whole run for the same season. Representative chromatograms of the laboratory prepared mixture of the investigated compounds by the gradient mode are shown in figure (3).

It was found that gradient elution gave a shorter overall analysis time with similar resolution of the critical pairs compared to the isocratic elution without sacrificing repeatability in retention time, peak area, peak height or linearity of the calibration curve and this was confirmed by the values of the correlation coefficients of calibration curves constructed for Fos and HCTZ which are close to 1. The regression equations were

P.A = 18.961 C + 574.070 r =0.9999 for FOS (by isocratic elution)
P.A = 24.028 C + 11.039 r =1
for FOS (by gradient elution)
P.A = 71.534 C + 13.348 r =1
for HCTZ.

Linearity was obtained over the range of (2.0-30.0 $\mu g.ml^{-1}$) for HCTZ and (40.0 -1000.0 $\mu g.ml^{-1}$) and (6.0-600.0 $\mu g.ml^{-1}$) for FOS by isocratic and gradient elution respectively.

The results obtained from the determination of accuracy are listed in table (1). The good recoveries and low standard deviation values indicate that the method is accurate. The mean percentage recoveries ±SD were found to be 100.14±0.530 for HCTZ and 99.96±1.227 and 100.51±0.962 for FOS using isocratic and gradient elution respectively.

The methods were found selective and valid for the determination of the two intact drugs simultaneously in presence of their degradates with mean percentage recoveries 100.14±0.894 for HCTZ, 99.59±1.917 and 100.05±1.012 for FOS by isocratic and gradient elution respectively.

Satisfactory results were obtained for the recovery of the analyzed drugs in its pharmaceutical preparation (Monozide®) and were in agreement with the labeled claims. Results indicate there was no interference from the excipients used in the formulation of tablets.

The results of the assay validation parameters are discussed in table (3). The data revealed that the proposed method was found suitable for the determination of the drugs in pure powder form, pharmaceutical preparation and was proved to be precise, accurate and suitable for quality control laboratories where economy and time are essential provided that the instrument with the right specification is available. The results of the proposed method presented in table (2) were compared with the reported method, student's t- and F tests at 95% confidence level were applied and the results showed that the calculated t- and F-values didn't exceed the theoretical ones indicating no significant difference between the results obtained by the proposed method and these obtained by the reported method.

Table (1): Accuracy of the proposed HPLC method for the analysis of pure samples of fosinopril and hydrochlorothiazide.

Fos						HCTZ		
Isocratic			Gradient					
Taken ($g.ml^{-1}$)	Found ($g.ml^{-1}$)	Recovery* (%)	Taken ($g.ml^{-1}$)	Found ($g.ml^{-1}$)	Recovery (%)	Taken ($g.ml^{-1}$)	Found ($g.ml^{-1}$)	Recovery* (%)
50	49.72	99.44	230	30.45	101.5	4	3.97	99.25
100	99.56	99.56	50	50.32	100.64	8	8.06	100.75
300	297.2	99.07	150	148.2	98.8	12	12.01	100.08
500	501.67	100.33	250	253.99	101.6	16	15.95	99.69
700	717.58	102.51	350	349.62	99.89	20	20.08	100.4
900	889.89	98.88	450	453.32	100.72	24	24.15	100.63
100	999.58	99.96	550	552.24	100.41	28	28.06	100.21
Mean±SD		99.6±1.227			100.51±0.962			100.14±0.530

* Average of five determinations.

Table (2): Quantitative determination of fosinopril and hydrochlorothiazide in pharmaceutical preparation and application of standard addition technique by the proposed HPLC methods

Pharmaceutical formulation			Taken ($g.ml^{-1}$)	Found* ($g.ml^{-1}$)	Standard addition technique		
					Pure added ($g.ml^{-1}$)	Pure found ($g.ml^{-1}$)	Recovery (%)*
Monozide® plus tablets claimed to contain 20 mg/12.5 mg (Fos/HCTZ) tablets (Batch no. L 93731)	Fosinopril	Isocratic	40 $ug.ml^{-1}$	99.34±1.264	200	204	102
					400	395.38	98.85
					600	605.58	100.43
					800	793.42	99.18
							100.12±1.429
		Gradient		99.28±1.212	200	199.41	99.71
					300	299.1	99.7
					400	401.72	100.43
					500	497	99.4

	Hydrochlorothiazide	25 ug.ml^{-1}	100.56±0.602	2 3 4 5	2 2.99 4.02 5.03	100 99.67 100.5 100.6 100.19±0.436

* Average of five determinations.

Table (3): Results of assay validation parameters obtained by applying the proposed HPLC method for determination of FOS and HCTZ.

	Fosinopril		**Hydrochlorothiazide**
Parameters	**Isocratic**	**Gradient**	
Linearity	40-1000µg.ml^{-1}	6-600 µg.ml^{-1}	2-30 µg.ml^{-1}
Correlation coefficient (r)	0.999	0.9999	
Slope	18.96123495	24.02847116	13.34805027
Intercept	574.0739297	-11.03926857	71.53375862
Standard error of the slope	0.066929187	0.043138751	0.206237883
Confidence limit of the slope	18.78918799-19.1332819	23.92899303-24.1279493	71.0291127-72.03840453
Confidence limit of the intercept	37.62008927	13.03501438	3.802838654
Confidence limit of the intercept	477.3684116-670.7794478	-41.09806562-19.01952848	4.042839314-22.65326122
Standard error of estimation	59.97862445	28.45772849	5.346296957
Accuracy (mean±SD)	99.96±1.227	100.51±0.962	100.14±0.530
Selectivity			
Precision (RSD%)			
Repeatability*	0.596	1.018	1.380
Intermediate precision*	0.739	1.260	1.734

Table (4): Statistical comparison between the results obtained by the proposed HPLC method and the reference methods for determination of fosinopril and hydrochlorothiazide.

Items	HPLC method			Reported method*	
	Fosinopril		Hydrochloro-thiazide	Fosinopril	Hydrochloro-thiazide
	Isocratic	Gradient			
Mean	99.34	99.28	100.56	100.6	100.75
SD	1.264	1.212	0.602	0.528	0.404
RSD%	1.272	1.221		0.525	0.401
N	5	5	5	5	5
Variance	1.598	1.468	0.363	0.279	0.163
Student's t-test (2.306)*	2.05	2.23	0.586		
F-value (6.388)*	5.727	5.256	2.227		

* The values in parenthesis are the tabulated values.

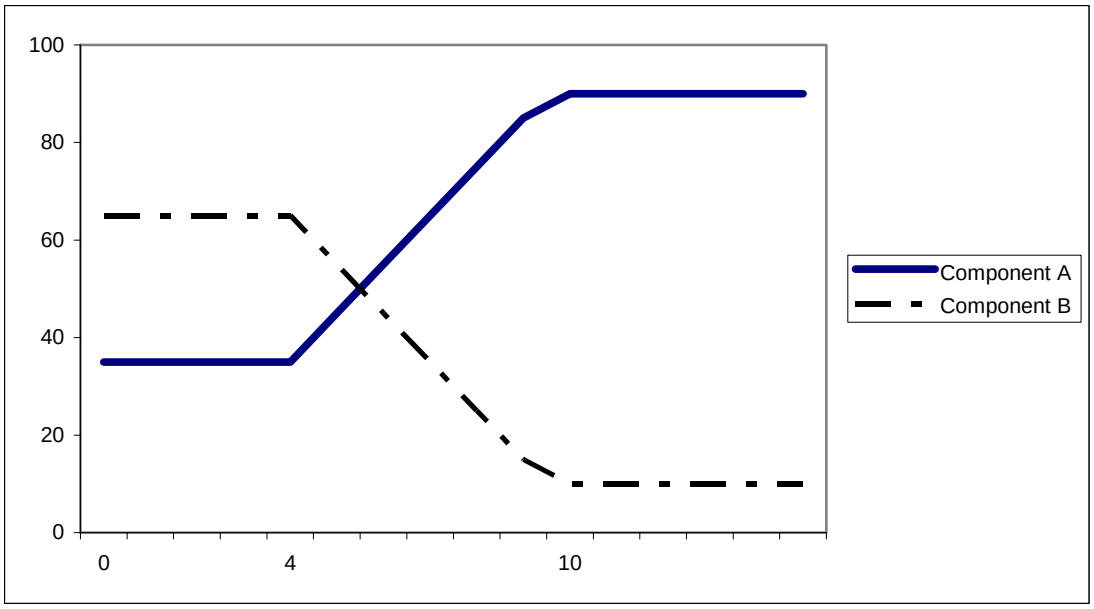


Figure (1): Linear gradient model applied in HPLC of mixture.

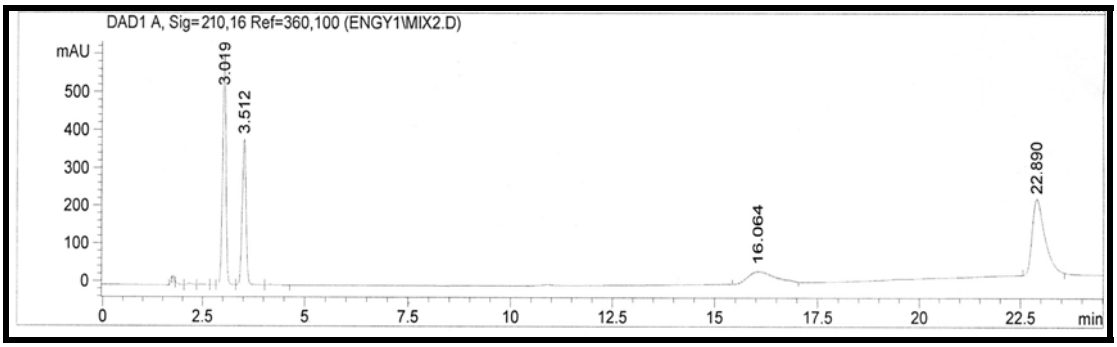


Figure (2): Scanning profile of HPLC chromatogram of fosinopril (500µg.ml^{-1}), hydrochlorothiazide (10 µg.ml^{-1}) and their process degradates fosinoprilat (500µg.ml^{-1}) and 4-amino-6-chlorobenzene-1,3 disulphonamide (10 µg.ml^{-1}).

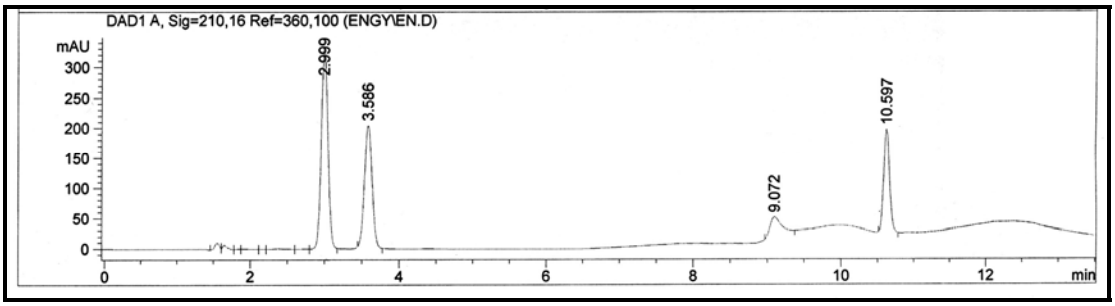


Figure (3): Scanning profile of HPLC-GE chromatogram of fosinopril (400μg.ml^{-1}), hydrochlorothiazide (10μg.ml^{-1}) and their process degradates FOS-at ((400μg.ml^{-1}) and DSA (10 μg.ml^{-1})

V. CONCLUSION

Delivering competent analytical judgment on samples in a timely manner is becoming more difficult as the sample load in quality control laboratories continues to increase. These economic pressures prompted the development of analytical technologies which can deliver high qualitative and quantitative information in a high throughout environment. Cyanopropyl columns combined with elevated temperature was presented as an approach for this purpose as well as gradient elution. Gradient elution technique was found to present tremendous chemical selectivity as well as precise retention time data under well controlled conditions. It was successful in separation of a complex mixture of fosinopril, hydrochlorothiazide and their degradates in less than 1/2 the time consumed in isocratic elution with no loss of accuracy or precision of data.

REFERENCES

1. Jancic B., Ivanovic D., Medenica M., Malenovic A.; *J. Acta Chim. Slov.*, **50**: 327-333, 2003.
2. Ivanovic D., Medenica M., Jancic B., Malenovic A. and Markovic S.; *Chromatographia*, **60**:S87-S92, 2004.
3. Biljana J., Mirjana M., Darko I., Andelija M. and Igor P.; *Chromatographia*, **67**:S123-S127, 2008.
4. Mollica J.A., Rehm C.R., Smith J.B. and Govan H.K.; *J.Pharm. Sci.*,**60**:1380-1384,1971.
5. Mollica J.A., Rehm C.R. and Smith J.B.; *J. Pharm. Sci.*,**58**:635-636,1969.
6. Odovic J., Aleksic M., STojimirovic B., Milojkovic-Opsenica D. and Tesic Z., *J. Serb. Chem. Soc*., **74** (6): 677-688, 2009.
7. Odvic J., Stojimirovic B., Aleksic M., Milojkovic-Opsenica D. and Tesic Z., *J. Serb. Chem. Soc.*, **71**(6): 621-628, 2006.
8. Hillaert S., Vander Heyden Y., VAnden Bossche W., *J. Chromatogr. A*, **978**: 231-242, 2002.
9. Hillaert S., De Grawe K. and Van den Bossche; *J.Chromatogr. A*, **924**(1-2): 439-449, 2001.
10. Jemal M., Ivashkiv E., Ribick M. and Cohen AI; *J. Chromatogr. B.*, **739**(2): 255-271, 2000.
11. Lozano R., Warren FV Jr, Perlman S. and Joseph JM; *J. Pharm. Biomed. Anal.*, **13** (2): 139-148, 1885.
12. Jancic B., Medenica M., Ivanovic D., Malenovic A. and Markovic S.; *Anal.Bioanal.Chem.*, **383**:687-694,2005.
13. Jancic B., Medenica M., Ivanovic D., Jancovic S. and Malenovic A.; *J.Chromatogr.A*, **1189**(1-2):366-373,2008.
14. Hillaert S. and Van den Bossche; *J. Pharm. And Biomed. Anal.*, **25**(5-6): 775-783, 2001.
15. Rehm C.R., Smith J.B., *Journal of the American Pharmaceutical Assosciation*, **49**(6):386-389,1960.
16. Bebawy L.I, El Koussy N.; *Analytical Letters*, **30**(7):1379-1397,1997.
17. Brigante M., Della Greca M., Previtera L., Rubino M. and Temussi F.; *Enviromental Chemistry Letters*, **2**(4):195-198,2005.

18. Franolic J., Lehr G., Barry T. and Petzinger G.; *J.Pharm. Biomed.Anal.*, **26**:651,2001.

19. Boyes B.E. and Kirkland J.J.; *Pep. Res*., **6**: 249-258,1993.

20. Yan B. et al.; *Anal. Chem.*, **72**:1253-1262, 2000.

21. Cooper W.T. and Smith P.L.; *J.Chromatogr.A.*, **355**:57-74,1986.

22. Ahuja S. and Rasmussen H.; *HPLC Method Development for Pharmaceuticals*, **8**:81,2007.

23. Zhou N.E., Mant C.T., Kirkland J.J. and Hodges R.S.; *J.Chromatogr.*, **548**:179-193,1991.

24. Waksmundzka-Hajnas M., Petruczynik A. And Hawry A., *J.Chromatogr.A*,**919**(1):39-50,2001.

25. Bratu I., Kacso I., Borodi Gh, Constantinescu D. and Dragan F.; *Spectroscopy*, **23**(1):51-58.

26. Remko M.; *Chem. Pap.*, **61**(2):133-141,2007.

27. Sparceal L., Udrescu L., Dragan L., Trandafirescu C., Szabadai Z. and Bojita M.; *Farmacia*, **58**(4):478-484,2010.

28. Lemke T.L. and Williams D.A., Roche S.W.; *Foye's Principles of Medicinal Chemistry*, 6th edition, pp. 746, 2007.

29. Sneader W.; *Drug Discovery: A History*, pp.282, 2005.

10/22/2010

Effect of *Rhazya stricta* extract on rat adiponectin gene and insulin resistance

Nabih A. Baeshen[1], Sahira A. Lari[2], Huda A. R. Al Doghaither[1] and Hassan A. I. Ramadan[1, 3]

[1]Department of Biological Sciences, Faculty of Sciences, King Abdul-Aziz University, Jeddah
[2]Department of Biochemistry, Faculty of Sciences, King Abdul-Aziz University, Jeddah
[3]Department of Cell Biology, National Research Centre, Dokki, Egypt
Corresponding Author: Nabih A. Baeshen[1] (**Nabih_Baeshen@hotmail.com**)

Abstract: *Rhazya stricta* plants have always played a major role in the treatment of human and animal diseases. The aim of this study was to study the effect of different doses of *Rhazya stricta* extract administered orally to rats, treatment period, effect on adiponectin protein, insulin resistance and finally its effect on exon 3 of adiponectin gene. Oral administration of aqueous leaves extracts of *Rhazya stricta* evoked fluctuations in adiponectin levels during eighteen weeks period of treatment. Serum adiponectin levels showed a significant increase after 2 and 4 weeks of treatments. Also a highly significant increase in adiponectin level, compared with the control group, was detected in rats treated with 0.125 gm/ml and 0.150 gm/ml after eighteen weeks of treatment. Insulin resistance is an important risk factor for type II diabetes mellitus and cardiovascular disease. Therefore, we performed HOMA-IR to check the degree of insulin resistance in rats. The results showed an inverse highly significant correlation between adiponectin levels and insulin resistance degrees after two weeks of treatment with *Rhazya stricta*. Studies published to date indicate that polymorphisms at the adiponectin gene (exon 3) are indeed predictors of circulating adiponectin levels. However, our results showed a significance increase in adiponectin levels, we did not detect any rare mutation in this locus using CSGE technique. The effects of *Rhazya stricta* extract on the increase of adiponectin levels concentrations could be promising issue (after avoiding its possible mutagenic effects) in treating diabetes, carbohydrate metabolism, hypertension, as well as inflammatory conditions.
[Nabih A. Baeshen, Sahira A. Lari, Huda A. R. Al Doghaither and Hassan A. I. Ramadan. **Effect of *Rhazya stricta* extract on rat adiponectin gene and insulin resistance**. Journal of American Science 2010;6(12):1237-1245]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: *Rhazia stricta*; rat adiponectin gene, exon 3; insulin resistance; mutagenicity

1-Introduction

Rhazya stricta plants have always played a major role in the treatment of human and animal diseases. The effect of an alkaloidal isolated from *Rhazya stricta* leaves (rhazimine) on arachidonic acid metabolism in human blood was described by Saeed *et al*. (1993). The alkaloid has been shown to be a dual and selective inhibitor of platelet activating factor (PAF)-induced platelet aggregation and arachidonic acid metabolism. It was concluded that these effects might provide additional beneficial anti-inflammatory and anti-PAF effects by comparison with classical non-steroidal anti-inflammatory drugs.

In addition, the lyophilized extract of *Rhazya stricta* was shown to relax isolated intestinal muscles of rats (Tanira *et al*., 1996a). The plant may have potential as an antispasmodic drug. This seems to corroborate the folk medicinal use of the plant in certain localities.

The effect of the lyophilized extract of *Rhazya stricta*, on some indices of the antioxidant status, was studied in rats (Ali *et al*., 2000a). It was found that the high doses were effective in significantly increasing the hepatic and renal concentrations of reduced glutathione (GSH) and ascorbic acid, and significantly reducing the degree of lipid peroxidation. The low doses were ineffective in significantly altering these indices.

A lyophilized extract of *Rhazya stricta* leaves was found to be of low toxicity in rats. In this species, the oral lethal dose (LD_{50}) has been estimated to be 16 g/kg in rats and 2.36 g/kg in mice. Subchronic treatment of rats with *Rhazya stricta* extract at doses of 0.5-2.0 g/kg/day did not significantly affect the body weights, food, water intake, urine, and faecal output during 28 days of treatment (Tanira *et al*., 1996b). Subchronic treatment also had no significant effect on haematological and plasma biochemical constituents. However, Adam, (1998) dosed Saudi Arabian sheep with powdered *Rhazya stricta* leaves in water and reported that the higher doses produced body-weight depression, bloating, diarrhea, dyspnoea and weakness of the hind limbs. Histologically, there were entero-hepatonephropaty, pulmonary congestion, haemorrhage and emphysema.

An intravenous dose of 80 mg/kg of *Rhazya stricta* extract administered to dogs, produced intense salivation, and rigor, followed by respiratory failure, convulsions and death within 15 minutes (Siddiqui and Bukhari, 1972).

Several studies were performed by Baeshin's team to assess the mutagenic potential of *Rhazya stricta* (Decne) leaf extract: It was first evaluated by using the Saccharomyces cerevisiae auxotrophic mutant test (Baeshin *et al*., 2005) then on Aspergillus terreus (Baeshin *et al*., 2009a). Also, the cytogenetic status and DNA integrity of human lymphocytes were studied after exposure to an aqueous extract of *Rhazya stricta* leaves in Vitro (Baeshin *et al*., 2009b) and its effect on plant cells were also studied on Allium cepa root tip meristem (Baeshin *et al*., 2009c). The results of this battery of tests indicated a mutagenic potential of *Rhazya stricta* Leaf extract on different organisms and tissues.

Subsequently, the biochemical parameters such as Blood Lipid Profile Concentrations, Liver Enzyme Activities and Kidney Functions in Rats were the subjects for detecting the effect of this extract in these respects and results indicated that, aqueous extract of the *R. stricta* leaves significantly decreased concentrations of TGs, LDL-c, cholesterol, uric acid and creatinin, but increased concentration of HDL-c. It triggered all these activities without affecting liver enzyme activities or kidney functions. These findings may have a positive impact on the cardiovascular patients and may provide a new therapeutic strategy to reduce hypertriglyceridemia. (Baeshin *et al*., 2010)

As far as we are aware, the effects of *Rhazya stricta* on adiponectin concentrations have not been identified yet. However, from the literature reviewed, there are similarities in the effects of *Rhazya stricta* and adiponectin on diabetes, carbohydrate metabolism, hypertension, as well as inflammatory conditions. The leaves of *Rhazya stricta* have been used, among other aliments, for the treatment of diabetes mellitus. Diabetic patients in the Arabian Gulf region commonly use water extracts of the leaves (Ali *et al*., 2000b). Tanira *et al*. (1996b) reported that acute oral treatment with the extract at a dose of 4 g/kg produced a significant and short-lived increase in plasma insulin concentration, accompanied by a significant reduction in plasma glucose concentration. On the other hand, animal studies have demonstrated that adiponectin reduces hyperglycemia in different models of obesity/diabetes mellitus (Yamauchi *et al*., 2001; Berg *et al*., 2001). In addition, multiple animal and human studies have shown a correlation between adiponectin levels and insulin sensitivity (Berg *et al*., 2001; Combs *et al*., 2002). One of the most interesting findings is that, a decline in adiponectin levels seems to identify insulin resistance before the development of diabetes (Oh *et al*., 2007).

In many studies, relatively large doses of the plant extract were used to determine the pharmacological and toxicological actions (Tanira *et al*., 1996b and Adam *et al*., 2002). Therefore, it was necessary to study the effects of this plant using doses, almost near that is used by humans in the folk medicine.

2.Material and Methods

The experimental work was conducted at the biochemistry lab of King Abdul-Aziz University Hospital (KAUH) and at King Fahd Medical Research Centre (KFMRC), Jeddah, Saudi Arabia.

2.1. Materials

2.1.1 *Rhazya stricta* plant

It was collected from the nearby areas of Jeddah, KSA. The leaves were washed, shade-dried and ground to a fine powder with a blender and the resulting powder were diluted in distilled water.

2.1.2.Experimental design (Samples)

Fifty five locally bred adult male Wistar rats initially weighing 150- 200 gm were used. They were housed in groups of five animals at a temperature of 22 °C under a 12 h dark- light cycle. They were fed standard pelleted diet (Grain Soils and Flourmills Organization Jeddah, KSA) and drinking water.

The animals were divided into four groups: Group 1 (n= 10) was the control and was dosed orally by gavage with distilled water (0.5 ml). Group 2 , 3 and 4 (n= 15), were treated orally by gavage with 0.5 ml *Rhazya stricta* extract at single doses of 0.1 gm/ml, 0.125 gm/ml and 0.150 gm/ml respectively for 18 week.

Blood samples were obtained from rats (after an overnight fast) by penetrating the retro-orbital plexus with a glass capillary tube or Pasteur pipette. Blood samples were collected after one, 2, 4, 8, 12 and 18 weeks from all treated and control groups.

2.2.Methods

2.2.1.Preparation of plasma:

Few drops of rat's whole blood were collected in an EDTA tube. The blood was centrifuged for 10 minutes at 3000 rpm. Plasma was separated carefully in a number of ependroof tubes and then stored at-80˚C.

2.2.2.Preparation of serum:

Blood was collected in a plain tube, allowed to stand to clot for one hour at room temperature and centrifuged for 10 minutes at 3000 rpm. After centrifugation, serum was separated carefully in a number of Ependrof tubes and then stored at -80˚C.

2.2.3.Determination of adiponectin levels:

Rat adiponectin kit was used in an enzyme-linked immune-sorbent assay (ELISA) for quantitative determination of adiponecin in rat serum. The intensity of color was measured at 450 nm in a plate reader.

2.2.4.Determination of insulin levels:

Rat insulin ELISA kit was purchased from Linco research and was used as recommended by the manufacturer.

2.2.5.Determination of glucose levels

Glucose was determined using enzymatic methods on automated chemical analyzer (Dimension R Clinical Chemistry System, USA). The glucose kit was used as recommended by the manufacturer.

2.2.6.Assessment of Insulin Resistance:

According to Matthews *et al*. (1985), insulin resistance can be estimated from fasting blood glucose and insulin concentrations. In the present study, degree of insulin resistance was measured by Homeostasis Model Assessment of Insulin Resistance (HOMA-IR), which was calculated with the formula: Fasting serum insulin (µ U/ml) x fasting plasma glucose (m mol/l)/ 22.5. High HOMA-IR scores denote low insulin sensitivity (insulin resistance).

2.2.7.DNA extraction:

Few drops of rat's whole blood were collected in tubes containing EDTA and genomic DNA was extracted using QIA amp DNA mini kit according to manufacturer's instructions. DNA Samples were obtained from both treated and control rats.

2.2.8.Primer design and PCR amplification:

PCR primers were designed (for exon 3 of adiponectin gene) with the computer program Primer 3.0 (Rozen and Skaletsky, 2000) and were synthesized commercially (Tib Molbiol Syntheselabor Berlin, Germany). The forward primer sequence is 5'-taa ggg tga ccc agg aga tg-3' and the reverse primer sequence is 5'-gcg gag act agg gag tgc tt-3'.

The PCR mixtures were prepared using ready to use HotStar TaqTM Master Mix as recommended by the manufacturer (Qiagen, Germany). The PCR program consisted of initial activation step for 15 minute at 95°C, followed by 35 cycles at 94°C for 30 seconds, 54°C for 1 minutes, and 72°C for 1 minute, and a final extension at 72°C for 10 minutes.

Few µl of PCR products were electrophoresed on 0.8 % agarose gel in 1X TBE buffer.. The relative size of the PCR products were determined using 100–1500 bp DNA ladder (Qiagen, Inc. USA). A visual image was obtained using Gel Documentation System (UVP Products Ltd, Cambridge, UK).

2.2.9.Conformational sensitive gel electrophoresis (CSGE):

To generate heteroduplexes for analysis by CSGE, 5µl of both control and treated PCR product (*Rhazya stricta* extract, 0.150 gm/ml) were mixed and incubated at 68°C for 30 minutes. Prior to electrophoresis, 4 µl of each PCR product was mixed with 4 µl of 20% ethylene glycol/30% formamide containing 0.025% (wt/ vol) each of Xylene Cyanol FF and Bromphenol Blue. Samples were separated by electrophoresis on a standard DNA sequencing gel apparatus [1-mm-thick gel prepared with 10% polyacrylamide made from 40% stock of acrylamide containing 99:1 acrylamide to bis-acrolylpiperazine (BAP)] using 0.5 ×TTE (Tris base, Taurine, EDTA) as the electrode buffer. The gel was pre-electrophoresed at 400V for 45 min and the samples were electrophoresed at 750 volts for 16 hr at room temperature.

After electrophoresis, one glass plate was removed and the gel on the second glass plate was stained with 1µg/ml ethidium bromide for 10 min followed by destaining in distilled water for 10 min. The bands were visualized with a UV light and the gel was then photographed under standard conditions.

3.Results

3..1 Relation between adiponection and insulin levels

Oral administration of aqueous leaves extracts of *Rhazya stricta* evoked fluctuations in adiponectin levels during eighteen weeks period of treatment with *Rhazya stricta* leaves (Table 1). Serum adiponectin levels were significantly ($p < 0.05$) lower (15.77±3.84 µg/ml) than control after one week of treatment in rats receiving 0.150 gm/ml *Rhazya stricta* aqueous extracts, On the other hand, serum adiponectin levels showed a significant increase ($p < 0.05$) after 2 and 4 weeks of treatments. This increase was clear in rats of group 4 receiving 0.150 gm/ml *Rhazya stricta* (33.40±8.38) and after 4 weeks of treatment in group 2 receiving 0.1 gm/ml *Rhazya stricta* (33.89± 5.46). Also a significant ($p < 0.01$) increase in adiponectin level, compared with the control group, was clear in rats of group 3 (20.77± 7.03 µg/ml) after eighteen weeks of treatment with *Rhazya stricta*. A highly significant ($p < 0.001$) increases in adiponectin concentration was recorded with the same dose in group 4 (24.06± 4.65 µg/ml).

Table 1: Relation between adiponection and insulin levels

Week	Variable	Group 1 Control 10 rats	Group 2 (0.1gm/ml) 15 rats	Group 3 (0.125gm/ml) 15 rats	Group 4 (0.150gm/ml) 15 rats	p-value
01	Insulin (µU/ml)	22.42±18.08	18.35±15.97	21.81±20.77	46.68±24.82	<0.01[(c)]
	HOMA-IR (µU/ml.mmol/l)	3.93±2.42	4.37±4.16	4.95±4.94	9.68±5.23	<0.01[(c)]
	Adiponectin (µg/ml)	19.01±2.76	18.60±2.00	17.46±2.94	15.77±3.84	<0.05[(c)]
02	Insulin (µU/ml)	4.62±2.50	14.81±9.66	14.92±21.01	9.53±9.42	NS
	HOMA-IR (µU/ml.mmol/l)	0.92±0.48	3.70±2.82	3.41±5.16	2.15±2.52	<0.05[(a)]
	Adiponectin (µg/ml)	27.7±7.35	24.12±4.80	24.11±5.75	33.40±8.38	<0.05[(c)]
04	Insulin (µU/ml)	8.76± 8.71	9.03± 8.13	19.24± 14.60	20.70± 12.72	<0. 05[(b,c)]
	HOMA-IR (µU/ml.mmol/l)	1.62± 1.96	1.95± 1.96	4.22± 4.31	5.50± 3.73	<0. 01[(c)]
	Adiponectin (µg/ml)	30.41± 8.96	33.89± 5.46	28.20± 3.33	27.58± 5.93	NS
08	Insulin (µU/ml)	29.70± 25	16.39± 9.96	12.83± 7.97	8.88± 3.54	<0.05[(a)] <0.01[(b)] <0.001[(c)]
	HOMA-IR (µU/ml.mmol/l)	8.30± 7.32	3.89± 29.55	2.56± 1.64	1.51± 0.65	<0.01[(a)] <0.001[(b,c)]
	Adiponectin (µg/ml)	22.85± 5.38	24.21± 4.78	23.53± 6.22	24.28± 5.26	NS
12	Insulin (µU/ml)	17.36± 18.72	12.76± 7.73	16.44± 20.83	15.86± 13.93	NS
	HOMA-IR (µU/ml.mmol/l)	4.71± 6.20	2.86± 2.08	3.99± 5.67	3.48± 3.25	NS
	Adiponectin (µg/ml)	19.62± 4.54	21.06± 4.21	18.40± 4.64	18.77± 4.58	NS
18	Insulin (µU/ml)	10.86± 8.53	7.82± 3.65	9.11± 5.95	8.52± 6.65	NS
	HOMA-IR (µU/ml.mmol/l)	2.29± 1.96	1.57± 0.83	1.84± 1.39	1.60± 1.41	NS
	Adiponectin (µg/ml)	14.61± 3.91	16.28± 2.92	20.77± 7.03	24.06± 4.65	<0.01[(b)] <0.001[(c)]

NS: Not significant
Values were represented as the mean ± SD.
(a: control vs. group 2, b: control vs. group 3,
c: control vs. group 4).
p- value ≤ 0.05 was used as a criterion of significance.
p- value ≤ 0.01and ≤ 0.001were used as a criterion of highly significance.
($p < 0.01$, r = -0.35).

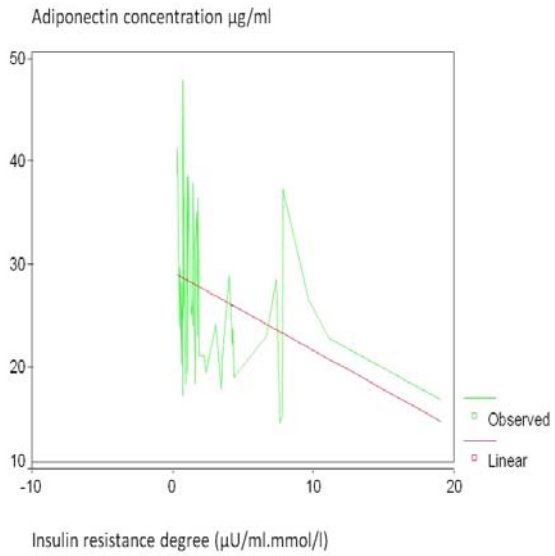


Figure 1: Correlation of adiponectin and insulin resistance degree after 2 weeks of treatment with *Rhazya stricta*

3.2.The Relationship between Adiponectin and Insulin Resistance:

Interestingly, analysis of data using Pearson correlation showed an inverse highly significant correlation between adiponectin levels and insulin resistance degrees after two weeks of treatment with *Rhazya stricta.* ($p < 0.01$, $r = -0.35$) as shown in figure 1.

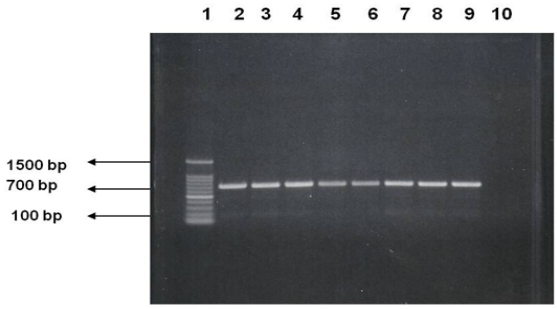


Figure 2: Gel electrophoresis image of PCR products separated on 0.8% agarose gel. Lane 1 is the DNA ladder, Lane 2-9 the 713-bp PCR products containing sequences for exon 3 (680-bp) of *Adipoq* gene. Lane 10 is the negative control (without DNA)

3.3.PCR and Gel Electrophoresis

The PCR amplification products were analyzed on 0.8% agarose gel. The electrophoresis of the PCR products is shown via an image of the ethidium bromide-stained agarose gel visualized under ultraviolet using Gel Documentation System (Figure 2). The figure shows PCR amplified bands of 713-bp containing sequences for exon 3 (680-bp) of the *Adipoq* gene.

3.4.Conformational Sensitive Gel electrophoresis (CSGE)

The PCR products were screened for mutations by conformation sensitive gel electrophoresis (CSGE). Figure 3 shows CSGE image of a 713-bp PCR product that contains the sequence for exon 3 of the *AdipoQ* gene. Lane 15 contains the control sample and Lane 1-14 contains PCR products from rats treated orally for 18 weeks with *Rhazya stricta* extracts mixed with PCR products from control rats after heteroduplexing. The CSGE analysis of the PCR products showed that there is no single-base mismatches (heteroduplexes) were detected by CSGE in a 713-bp PCR product that contains sequences for exon 3 of the *AdipoQ* gene. The results obtained showed that there is no mutation or polymorphism in exon 3 of AdipoQ genes.

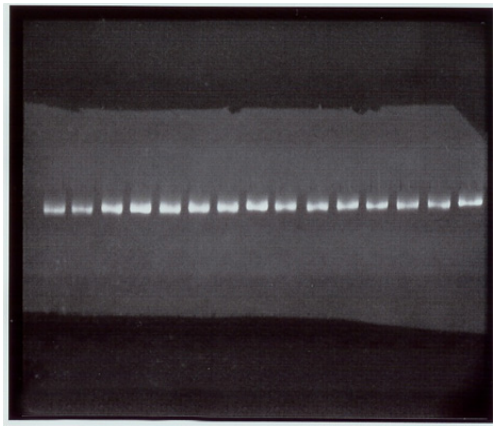


Figure 3: **CSGE image** of **a 713-bp PCR product that contains sequences for exon 3 of the *AdipoQ* gene. Fifteen lanes, lane 1-14 (from left to right) are PCR products from rats treated with the plant extract. Lane 15 is the control sample.**

4.DISCUSSION

Rhazya stricta is commonly used in folk medicine for the treatment of many diseases. In many studies, relatively large doses of the plant extract were used to determine the pharmacological and toxicological actions (Tanira *et al*., 1996b and Adam *et al*., 2002).

Recently, adiponectin has been identified as one of the adipocytokines with important metabolic

effects. It plays an important role in the development of insulin resistance and atherosclerosis. (Ekmecki and Ekmecki, 2006). It was important to realize that adipocytokine is circulating in concentrations exceeding the concentrations of any other known hormone, though still there is much uncertainty with regard to its primary physiological role especially in healthy subjects (Lihn *et al*., 2005).

The biochemical effects of *Rhazya stricta* aqueous leaves extract were examined in liver and kidney of rats by Baeshen *et al*. (2010). The same condition of the previous experiment was conducted in the present experiment to determine the effects of oral administration of aqueous leaves extract of *Rhazya stricta* on serum adiponectin concentrations and to study its molecular effects on exon 3 of adiponectin gene in rats. Also the relationship between serum adiponectin and insulin resistance after treatment with aqueous leaves extracts of *Rhazya stricta* in rats were estimated.

Oral administration of aqueous leaves extracts of *Rhazya stricta* evoked fluctuations in adiponectin levels during eighteen weeks period of treatment with *Rhazya stricta* leaves (Table 1). This increase in adiponectin concentration induced by the *Rhazya stricta* extract show the importance of using this plant as a medicinal treatment. However, the mode of modulating adiponectin concentration by *Rhazya stricta* is not clear till now.

Moreover, our results showed inverse significant correlations between adiponectin levels and serum insulin levels after week 1 and 2 of treatment with the plant extract. In agreement with our results, Wasim *et al*. (2006) reported an inverse significant correlation between adiponectin levels and insulin levels in a British South-Asian population with a high incidence of type 2 diabetes, cardiovascular disease, central obesity and metabolic syndrome.

Insulin resistance is an important risk factor for type II diabetes mellitus and cardiovascular disease (DeFronzo and Ferrannini, 1991). Therefore, we performed HOMA-IR to check the degree of insulin resistance in each rat. This method has been supported by other studies as a precise method for the assessment of insulin resistance (EL-Midaoui and de Champlain, 2002; Bonora, 2000).

The present work showed an inverse significant correlation between adiponectin and insulin resistance degrees in all treated rats after one week of treatment with *Rhazya stricta* leaves. Our finding is in consistent with previous studies that observed inverse correlation between adiponectin levels and insulin resistance (D'Anna et al., 2006; Farvid *et al*., 2006).

To our knowledge, this work is the first to study the effects of treatment with *Rhazya stricta* leaves on adipoQ gene (exon 3) and its protein (adiponectin). This study clearly shows that oral administration of *Rhazya stricta* leaves for eighteen weeks produced highly significant increases in adiponectin concentrations in treated rats especially on rats that were treated with the highest dose of the plant extract.

The adiponectin gene was shown to be associated with adiponectin levels in healthy Caucasians (Mackevics *et al*. 2006). It was reported that the presence of at least 1 non-synonymous mutation in exon3 showed evidence of association with adiponectin levels. (Cancello *et al*., 2004). The significance of the genetic variations in human adiponectin gene on its plasma concentrations and obesity were examined in Japanese subjects (Takahashi *et al*. 2000). Mutations in the adiponectin gene were screened by direct sequencing or restriction-fragment polymorphism. The levels of plasma adiponectin were determined by the enzyme-linked immunosorbent assay (ELISA). The results showed that, two nucleotide changes have been identfied in the adiponectin gene. G=T polymorphism in exon 2 was associated with neither plasma adiponectin concentrations nor the presence of obesity. A subject carrying missense mutation in exon 3 (R112C) showed markedly low plasma adiponectin concentration.

The frequency of adiponectin gene mutations in exon 3 of Polish origin patients with type 2 diabetes was 3.9%, while in the control group 0.98% and this difference was not statistically significant. It was also observed that adiponectin level is significantly lower in patients with C.331 TC mutation (Krętowski *et al*., 2005)

In summary, the studies published to date indicate that polymorphisms at the adiponectin locus (exon 3) are indeed predictors of circulating adiponectin levels, insulin sensitivity, and atherosclerosis, highlighting the pivotal role of this adipokine in the modulation of metabolism and atherogenesis (Menzaghi *et al*. 2007).

The technique of CSGE was developed for Detection of mutations in double-stranded DNA by gel electrophoresis (Ganguly and Prockop, 1995). Under the initially described conditions, no single-base mismatches (heteroduplexes) were detected by CSGE in a 713-bp PCR product that contains sequences for exon 3 of the AdipoQ gene. Since mean adiponectin levels were significantly higher ($p < 0.01$- $p < 0.001$) than the control group after 18 weeks of treatment with *Rhazya stricta* leaves extracts, and molecular analysis showed that no single base mismatches were

detected, these results indicated that no mutagenic action was practiced by *Rhazya stricta* extracts on exon- 3 of adipoQ gene . This does not exclude the possibility that it is mutagenic elsewhere in the genome of the rat since it was reported previously by Baeshin's team that it is mutagenic (Baeshin *et al*., 2005: 2009 a, b, c).

Therefore, a screening of the total genome of the rat for mutagenic action of *Rhazya stricta* liquid leaf extract will be revealing and this what it is being done in a current work in our laboratory. Furthermore a knockout mutation of the adipoQ gene will be more revealing in this respect and will be considered in future work. Adiponectin has drawn much attention because of its insulin-sensitizing and antiatherogenic actions, suggesting that genetic deficits in its production or action may contribute to insulin resistance and coronary artery disease (CAD) (Lacquemant *et al*., 2004). Diabetes is categorized into: Type 1- Insulin Dependent Diabetes Mellitus (IDDM) – which is an autoimmune destruction of Pancreatic β cells: Type 2: Non-insulin Dependent Diabetes Mellitus (NIDDM) – which is characterized by Insulin resistance in target tissues. The current focus of drugs discovery research in diabetes includes exploration of alternative medicines, discovery of new synthetic anti-diabetic agents as well as isolation of active compounds from plants which have been the source of traditional herbal medicines and have been documented and described for their anti-diabetic properties. A number of bioactive compounds have been isolated from plants which are potent α-glycosidase and /or α-amylase inhibitors and show good antidiabetic properties. The main phytochemicals with reported anti-diabetic activities were flavonoids, polyphenolic compounds, tannins, glycosides, alkaloids and terpenoids. A potent α-glycosidase inhibitor which helps in prevention of diabetes was isolated from water extract of leaves of mulberry trees (*Morus alba* L.) (Day, 2005). It is known that *Rhazya stricta* leaves extract contains the main phytochemicals with reported anti-diabetic activities such as: alkaloids, glycosides, flavonoides, tannins and triterpenes (Al-yahya *et al*., 1990: Badreldin *et al*., 2000: Szabó, 2008).

The possibility that some of the claimed therapeutic actions of *Rhazya stricta* extract may be due to immunomodulatory capacity. The alkaloidal fraction of *Rhazya stricta* significantly increased the production of IL-1 and TNF-α (Tanira *et al.,* 1998). On the other hand, it was reported that there is a highly significant correlation between IL-1 and TNF-α levels and adiponectin concentrations (Lihn *et al.,* 2003).

The effect of *Rhazya stricta* leaves extract on rat adiponectin level concentration could be a promising issue (after avoiding its possible mutagenic effects) in treating human diabetes, coronary artery disease, as well as inflammatory conditions. Future studies will be conducted for studying the mode of action of separated alkaloids of *Rhazya stricta* extract on whole adipo Q gene, adiponectin receptor loci and the identification of additional genes which might be involved in the regulation of serum adiponectin levels.

5.REFRENCES

Adam, S. E. (1998) Toxicity of *Rhazya stricta* to sheep. Vet Hum Toxicol. 10(2), 68-69.

Adam, S. E., Al-Yahya, M. A. and Al-Farhan, A. H. (2002) Toxicity of Nerium oleander and *Rhazya stricta* in Najdi sheep: hematologic and clinicopathologic alterations. Am J Chin Med. 30(2-3), 255-262.

Ali, B. H., Bashir, A. K. and Tanira, M. O. (2000a) The anti-oxidant action of *Rhazya stricta* in rats. Phytother Res. 14(6), 469-471.

Ali, B. H., Al- Qarawi, H., Bashir, A. K. and Tanira, M. O. (2000b) Phytochemistry, pharmacology and toxicity of *Rhazya stricta* Decne: a review. Phytother Res. 14, 229-234.

Al-yahya, M.A., I.A. Al-Meshal, J.S. Mossa, A.A. Al- Badr, and M. Tariq, 1990. Saudi Plants: A Phytochemical and Biochemical Approach. King Saud University Press Riyadh, Saudia Arabia.

Baeshen, N, Lari, S., Aldoghaither, H, Elkady, A (2010). Biochemical evaluation of the effect of *Rhazya stricta* aqueous leaves extract in liver and kidney functions in Rats. Nature and Science. 8(4):136-142..

Baeshin, N. A., Sabir, J. S. M., Qari, S. H. and Alhejin, A. M. (2009a): Genotoxic and molecular evaluation of genetic effects of *Rhazya stricta* (Decne) leaf extract on Aspergillus terreus, Egypt. J. Genet. Cytol., 38, 305-316.

Baeshin, N. A., Sabir, J. S. M., Abo-aba, S. E. M. and Qari, S. H. (2009b): Evaluation of the cytogenetic status and DNA integrity of human lymphocytes after exposure to an aqueous extract of *Rhazya stricta* leaves in Vitro, Journal of applied science research, 5(8): 986-994.

Baeshin, N.A., J.S. Sabir. and S.H. Qari, (2009c): Cytogenetic and molecular evaluation of genetic effects of leaf extract of *Rhazya stricta* (Decne) on Allium cepa root tip meristem. Egypt. J. Cytol., 38, 73-83.

Baeshin, N.A., N. Twaty and A. Al-Hebshi, (2005): Evaluation the genotoxicity of *Rhazya stricta* leaves extract by the Saccharomyces cerevisiae auxotrophic mutant test. Egyp. J. Nat. Toxi., 2, 87-100.

Badreldin, H., Ali, Ali A. Al-Qarawi, K. Ahmed O. Bashir Musbah Tanira, 2000. Photochemistry, pharmacology and toxicity of *Rhazya stricta* Decne: A review. Phototherapy Research, 14(4): 229 – 234.

Berg, A. H., Combs, T. P., Du, X., Brownlee, M. and Scherer, P. E. (2001) The adipocyte- secreted protein Acrp30 enhances hepatic insulin action. *Nat Med*. **7,** 947-953.

Bonora, E., Targher, G., Alberiche, M., Bonadonna, R. C., Saggiani, F., Zenere, M. B., Monauni, T. and Muggeo, M. (2000) Homeostasis model assessment closely mirrors the glucose clamp technique in the assessment of insulin sensitivity: studies in subjects with various degrees of glucose tolerance and insulin sensitivity. *Diabetes Care* **23**,57-63.

Cancello, R., Tounian, A., Poitou, C. and Clément, K. (2004) Adiposity signals, genetic and body weight regulation in humans. *Diabetes Metab*. **30**, 215-227.

Combs, T. P., Wanger, J. A., Berger, J., Doebber, T., Wang, W. J., Zhang, B. B., Tanen, M., Berg, A. H., O'Rahilly, S., Savage, D. B., Chatterjee, K., Weiss, S., Larson, P. J., Gottesdiener, K. M., Gertz, B. J., Charron, M. J., Scherer, P. E. and Moller, D. E. (2002). Induction of adipocyte complement related protein of 30 kilodaltons by PPAR-gamma agonists: a potential mechanism of insulin sensitization. *Endocrinology* **143**, 998-1007.

Day, C (2005). Are herbal remedies of use in diabetes? Diabetic Medicine, 22 (sl), 10-2.

D'Anna, R., Baviera, G., Corrado, F., Giordano, D., De Vivo, A., Nicocia, G. and Di Benedetto, A. (2006) Adiponectin and insulin resistance in early- and late-onset pre-eclampsia. *BJOG*. **113 (11),** 1264-1269.

DeFronzo, R. A. and Ferrannini, E. (1991) Insulin resistance: a multifaceted syndrome response for NIDDM, obesity, hypertension, dyslipidemia, and atherosclerotic cardiovascular disease. *Diabetes Care* **14**, 173-194.

Ekmecki, H. and Ekmecki, O. (2006) The Role of Adiponectin in Atherosclerosis and Thrombosis. *Clin Appl Thrombosis/Hemostasis.* **12(2)**, 163-168.

EL Midaoui, A. and De Champlain, J. (2002) Prevention of hypertension, insulin resistance, and oxidative stress by α-lipoic acid. *Hypertension* 39, 303-307**.**

Farvid, M. S., Ng, T. W. K., Chan, D. C., Barrett, P. H. R. and Watts, G. F.(2006). Association of adiponectin and resistin with adipose tissue compartments, insulin resistance and dyslipidaemia.*Diabet Obes Metab*. **7(4)**, 406-413.

Ganguly, A. and Prockop, D. J. (1995) Detection of mismatched bases in double stranded DNA by gel electrophoresis. Electrophoresis 16, 1830-1835.

Krętowski A, Gugała K, Okruszko A, Wawrusiewicz-Kurylonek N, Górska M (2005). Single Nucleotide Polymorphisms in exon 3 of the adiponectin gene in subjects with type 2 diabetes mellitus. *Roczniki Akademii Medycznej w Białymstoku · Vol. 50,* 148-150

Lacquemant C, Froguel P, Lobbens S, Izzo P, Dina C, Ruiz J (2004). The adiponectin gene SNP_45 is associated with coronary artery disease in type 2 (non-insulin-dependent) diabetes mellitus. Diabet Med 21, 776 –781

Lihn, A. S., Pedersen, S. B. and Richelsen, B. (2005) Adiponectin: action, regulation and association to insulin sensitivity. *Obes Rev*. **6**, 13-21

Lihn, A. S., Richelsen, B., Pedersen, S. B., Haugaard, S. B., Rathje, G. S., Madsbad, S. and Andersen, O. (2003) Increased expression of TNF-alpha, IL-6, and IL-8 in HALS: implications for reduced adiponectin expression and plasma levels. *Am J Physiol Endocrinol Metab*. **285(5),** E1072-E1080.

Mackevics, V., Heid, I., Wagner, S., Cip, P., Doppelmayr, H., Lejnieks, A., Gohlke, H., Ladurner, G., Illig, T., Iglseder, B., Kronenberg F and Paulweber B (2006). The adiponectin gene is associated with adiponectin levels but not with characteristics of the insulin resistance syndrome in healthy Caucasians. European Journal of Human Genetics 14, 349–356

Matthews, D. R., Hosker, J. P., Rudenski, A. S., Naylor, B. A., Treacher, D. F. and Turner, R. C. (1985) Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetes Care* **25(10)**, 1891-1892.

Menzaghi C., Trischitta V and Doria A. (2007). Genetic Influences of Adiponectin on Insulin Resistance, Type 2 Diabetes, and

cardiovascular Disease (2007). Diabetes 56:1198–1209

Oh, D. K., Ciaraldi, T. and Henry, R. R. (2007) Adiponectin in health and disease. *Diabet Obes Metab*. **9(3)**, 282-289

Rozen, S. and Skaletsky, H. (2000) Primer3 on the WWW for general users and for biologist programmers. *Methods Mol. Biol. 132: 365-386*.

Saeed, S. A., Simjee, R. U., Mahmood, F., Sultana, N. and Atta-ur-Rahman. (1993) Rhazimine from *Rhazya stricta*: a dual inhibitor of arachidonic acid metabolism and platelet activating factor-induced platelet aggregation. *Planta Med*. **59(6)**, 566-568.

Siddiqui, S. and Bukhari, A. (1972) I-leucopenic effect of *Rhazya stricta*. *Nature* **235,** 393.

Szabó, F.L., 2008. Rigorous Biogenetic Network for a Group of Indole Alkaloids Derived from Strictosidine. Molecules., 13: 1875-1896.

Takahashi M, AritaY, YamagataK, MatsukawayY, Okutomi K, Horie ShimomuraM, Hotta K, H KuriyamaH, KiharaS, NakamuraT, YamashitaS, FunahashiTand Matsuzawa Y(2000). Genomic structure and mutations in adiposespecific gene, adiponectin. International Journal of Obesity 24, 861-868

Tanira, M. O., Ali, B. H., Bashir, A. K., Wasfi, I. A. and Chandranath, I. (1996a) Evaluation of the relaxant activity of some United Arab Emirates plants on intestinal smooth muscles. *J Pharm Pharmacol*. **47**, 457-459.

Tanira, M. O., Ali, B. H., Bashir, A. K. and Chandranath, I. (1996b) Some pharmacologic and toxicologic studies on *Rhazya stricta* decne in rats, mice and rabbits. *Gen Pharmacol*. **27(7),** 1261-1267.

Tanira, M. O., Ali, B. H., Bashir, A. K., Stephen, S. and Lukic, M. (1998) *Rhazya stricta* enhances IL-1 and TNF-α production by macrophages in mice. *FASEB J*. **12**, A764.

Wasim, H., Al-Daghri, N., Chetty, R., McTernan, P. G., Barnett, A. H. and Kumar, S. (2006). Relationship of serum adiponectin and resistin to glucose intolerance and fat topography in south-Asians. *Cardiovasc Diabet*. **5(1),** 1-5.

Yamauchi, T., Kamon, J., Waki, H., Terauchi, Y., Kubota, N., Hara, K., Mori, Y., Ide, T., Murakami, K., Tsuboyama-Kasaoka, N., Ezaki, O., Akanuma, Y., Gavrilova, O., Vinson, C., Reitman, M. L., Kagechika, H., Shudo, K., Yoda, M., Nakano, Y., Tobe, K., Nagai, R., Kimura, S., Tomita, M., Froguel, P. and Kadowaki, T. (2001) The fat- derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nat Med*. **7**, 941- 946.

Biomonitoring Of Aquatic Ecosystem With Concept And Procedures Particular Reference To Aquatic Macro invertebrates

Shailendra Sharma, Praveen Sharma
*Department of Zoology, Shari Umiya Girls College, Mandleshwar,(M.P.) India
**Department of Chemistry, Narmada Samagra, Bhopal,(M.P.) India
shailendra.b.sharma@gmail.com

ABSTRACT: The "biological monitoring" has been widely used to assess the environmental impact of pollutant discharges. The methodology must be evaluated in terms of false positives and false negatives. A false positive is an indication that an excursion beyond previously established quality control conditions (i.e., unacceptable conditions) has occurred when, in fact, one has not. A false negative is an indication that conditions are acceptable when, in fact, they are not. Statistics must play a more important role in biological monitoring because they are capable of explicit statements of confidence in the biological monitoring results. With appropriate statistical evaluation of the data, professional judgment on whether to initiate immediate action or wait for more confirming data will be more objective and reliable. In order to optimize the usefulness of biological monitoring, the selection of biological monitoring methodology shall not be based on the investigator's favorite organism or group of organisms. Neither can be a convenient methodology adopted by regulatory agencies. The selections must be based on the compatibility of data generated with the decision making process, including the statistical establishment of confidence in the result obtained.
[Shailendra Sharma, Praveen Sharma. **Biomonitoring Of Aquatic Ecosystem With Concept And Procedures Particular Reference To Aquatic Macro invertebrates**. Journal of American Science 2010;6(12):1246-1255]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: Biomonitaring, bioindicator, diversity indices, saprobic indix, macroinvertebrates.

INTRODUCTION:

In recent years, the environment has been put to serious threat due to the discharge of harmful and toxic chemicals of various types which are primarily the byproducts of developmental activities like industrialization, urbanization, use of chemical fertilizers as well as pesticides and burning of fossil fuel emitting green house gases. Huge oil spills in the oceans and radioactive fallout are contaminating air, water and soil. In the event of large scale eco-degradation, it is necessary to monitor the nature and degree of change in environment so that the consumers may be warned and appropriate preventive and specific corrective measures may be adopted. After the UN conference on the Human Environment in Stockholm in 1972, the global Environmental Monitoring System (GEMS) has been set up. Literature is missing today. Environmental monitoring requires authentic data base and is considered as an useful tool in assesses the health of the environment. Furthermore, it is an indispensable prerequisite for Environmental Impact Assessment (EIA). Biomonitoring is an important exercise in the assessment of water quality. The present paper aims to discus the concept scope and procedure of biomonitoring. The concept of indicator species has been explained, species can be classified in tolerant facultative and sensitive groups. A holistic approach for water quality assessment has been suggested.

CONCEPT OF BIOMONITORING

Environmental monitoring is a systematic method of collecting qualitative & quantitative information about the status of environment by physico-chemical and biological methods. Monitoring by biological methods i.e. as "biomonitoring" is 'an ecological exercise where various kinds of biota are considered in ascertaining the extent of pollution in a water body'. These biota are known as bioindicators.

OBJECTIVE OF BIOLOGICAL MONITORING

Biological monitoring can be used for the following purposes:

1) To provide an early warning of a violation of quality control systems in time to avoid deleterious effects to ecosystems.
2) To detect episodic events such as accidental spills, failure of predictive models, failure of early warning systems or illegal disposal of wastes at night, etc.
3) To detect trends or cycles.
4) To determine information redundancy
5) To evaluate environment effects associated with the introduction of make it or concise part of introduction genetically engineered organisms into natural systems.

SCOPE OF BIOMONITORING

Biomonitoring has ample scope in ecology where biologists can play a meaningful role in environmental management. The idea of using appropriate organisms in the assessment of environmental quality originated at the beginning of twentieth century. It has been emphasized that nature and degree of pollution of any water body may be judged from the occurrence, abundance and composition of the inhabiting organisms. According to Forbes (1913) it is quite possible to arrange the plants and animals of a stream in order of their preference for or tolerance of organic impurities in such a way that their graded list may serve as an index to the level of contamination". Wilhm (1975) reported that environmental stresses eg. pollution induce changes in the structure and function of biological systems. Such changes may occur from the molecular to community level. In recent years biochemical, cytological and histological analysis are conducted with sophisticated instruments to assess the extent of pollution with much accuracy. Mason (1980) further stated that biological assessment of water quality involves three sequential steps: Suvey, surveillance and monitoring or research. The survey is the first step that apprises one about ecological condition of a given spot where the biomonitoring programme is to be followed. For example, In case of a lake, its geomorphology, ecogeography, nutrient status, inflow and outflows, point and non-point sources of pollution as well as biotic community may be known through survey. which should also take into account the anthropogenic influences such as socio-demographic, economic and cultural on the lake. The surveillance is vital practice of repeated measurements of the variables dependent or independent, of a particular habitat. The final step is monitoring the pollution status of the habitat concerned. The vast amount of data produced during surveillance is subjected to critical analysis for the final analysis & interpretation. The programme objective should be clearly defined and the sampling strategy outlined at the beginning of biomonitoring programme. Aquatic biota which can be are classified as follows:

1. **Plankton**: Microscopic organisms having either relatively small / one power locomotion and drift in the water due to the subject to the action of waves, currents and other forms of water motion".
2. **Periphyton:** Periphyton are assemblage of minute organisms (both plants and animals) growing on free surfaces of submerged substrata, natural (e.g. plant parts) or artificial (e.g. rocks).
3. **Nekon** includes the organisms (animals) of larger size moving freely and independently, in aquatic environment.
4. **Neuston** are the organisms resting or swimming on the surface film of water.
5. **Pleuston** are floating / submerged in higher plants water.
6. **Benthos** organisms which live in or on the bottom sediments.

SPECIES AS INDICATORS

Using of indicator organisms for the assessment of water quality a thorough knowledge of the ecological tolerance of the organisms concerned. Depending on the sensitivity to pollution Gaufin (1958) categorized species as (1) intolerant or sensitive, (2) facultative and (3) tolerant. With the onset of pollution or stress, intolerant species are either eliminated or migrate to other places, if is scopes there. Facultative species are able to withstand moderate pollution, whereas the tolerant species can endure severe pollution. It has however been postulated that through the existence of tolerant species indicate the presence of pollution but the absence of intolerant or sensitive species also indicates the occurrence of pollution. Some examples of indicator species are cited as below:

Ephemera simulans (may fly) *Acroneuria evoluta (stone fly)*	Intolerant or sensitive species
Hydropsyche bronta (caddis fly) *Agabus stagninus (beetle)*	Facultative species
Chironomus riparium (true fly) *Limnodrilus sp.* *Tubifex sp.*	Tolerant species

Further say few words how benthic involucrate are different from the above species and why they are grouped. Richardson (1925) categorized benthic Macro invertebrates into three groups on the basis of their degree of tolerance to pollution:

Pollution Level	Type of macroinvertebrates
1. Pollutional or more or less tolerant species	Tubificid worms, midge larvae etc.
2. Cleaner preference species	Current loving snails, insects, crustaceans etc.
3. Clean water species	Snails of Viviparidae, insects or insect larvae or

	nymphs of Hemiptera, Odonata, Neuroptera, Coleoptera etc.

Kolkwitz and Marsson (1908, 1909) propounded saprobien system based on the observation that a river receiving a heavy input of sewage shows zones of decreasing pollution. These zones are polysaprobic, α - mesosaprobic, β - mesosaprobic and oligosaprobic and their sequence reflects self purification. Fzerdingstand (1963) proposed nine stream zones as follows: (i) Coprozoic (ii) α - polysaprobic (iii) β - Polysaprobic (iv) χ - polysaprobic (v) α - ,mesosaprobic (vi) β - mesosaprobic (vii) χ - mesosaprobic (viii) Oligosaprobic and (ix) Katharobic. Organisms are graded into four groups on the basis of tolerance to organic enrichment as follows: Fecal coliforms, especially *Escherichia coli* is a better indicator of sewage pollution than total coliform count. The count is made by the most probable number (MPN) technique. Fecal coliform count <5000 cells / 100 ml is the minimum standard.

1.	Saprobiontic species	Occurring only in heavily polluted waters (tolerant species)
2.	Saprophilic species	Occurring generally in polluted waters but may also be found in other communities (facultative species)
3.	Saproxenous species	Generally found in unpolluted waters but are able to survive in presence of pollution (facultative species)
4.	Saprophobous species :	Unable to tolerate presence of pollution (sensitive or intolerant species)

BIOMONITORING WITH COLIFORM BACTERIA

It has been estimated that average adult excretes about 2000 000 000 coliform each day and its number is a reliable measure of fecal pollution. The presence of fecal coliforms in a water body indicates that the fecal pollution has occurred. Earlier total coliform count was used as an indicator. It is now proved that the presence in water of

BIOMONITORING BASED ON TOXICITY TEST

The presence of toxic substance in aquatic medium may be determined by toxicity test. In which suitable organisms are placed in water containing toxic chemicals and observations are made on the mortality of the test organisms. The toxicity may be acute, chronic, lethal, sublethal and cumulative. Lethal concentration is considered as the criterion of toxicity. The percentage of experience are expressed with a number, say LC_{50} which indicates the percentage (50% in this case) of animals killed at a particular concentration. The time of exposure is also important in toxicity. Eg. 48-hour $LC_{50,}$ means the concentration of a toxic substance can kill 50% of the test organisms in 48-hours. Instead of ex *situ* observation, an in *situ* continuous flow system has been devised. In such cases, caged organisms are placed in the field. In toxicity test, various organisms like algae, protozoa, rotifers, insects, microcrustaceans etc. can be used.

FISH ALARM SYSTEMS

It is well established that fishes show distinct physiological and behavioral responses to pollutants. The behavioral responses can be seen and observed by various techniques. An automatic fish monitor tank with required gadgets has been devised. If the polluted water is allowed to enter such a tank, recording of movement and other

responses will be automatically done from which the quality of water can be assessed.

BIOCHEMICAL OXYGEN DEMAND AND BIOMONITORING

The BOD values serve as good indicator of organic pollution. Although often considered as a component of chemical monitoring but in reality, it is based on a biological process and therefore, it may be regarded as an aspect of biomonitoring.

Water quality according to BOD values
(as followed in UK)

Water quality	**BOD_5 at 20^0c (mg/l)**
Very clean	<1
Clean	1-2.5
Fairly clean	2.5-4
Poor	6-10
Bad	10-15
Very bad	15-20

MISCELLANEOUS METHODS OF BIOMONITORING

Weber and McFarland (1969) used ash-free weight of periphyton and concentration of chlorophyll a in assessing the water quality. They proposed the following index (I_q) to characterize water quality I_q =

$$\frac{ash-free\ weight\ of\ perphyton\ (gm^{-2})}{Chlorophyll\ a\ (gm^{-2})}$$

According to the author the index values in unpolluted or slightly polluted waters contain mostly populations of algae and therefore, the index value should be lower than in polluted areas having large populations of filamentous bacteria and non-chlorophyll bearing organisms.

Odum (1956) found that the ratio between production and respiration (P/R) might serve the purpose of biomonitoring. According to him in the septic (polluted) zone of a stream, the respiration fax exceeds production and obviously the P/R ratio would be less than one. In the recovery zone, production increases and exceeds respiration and as a result P/R ratio would exceed one.

1.0 – 1.5	Oligosaprobic	No pollution
1.5 – 2.5	β - mesosaprobic	Weak organic pollution
2.5 – 3.5	α - mesosaprobic	Strong organic pollution
3.5 – 4.0	Polysaprobic	Very strong organic pollution

The ratio between production and biomass (P/B) may also serve as an indicator of environmental conditions. It is now known that pollution by organic phosphorous insecticides can be assessed by determining the brain acetylcholinesterase of the experimental fish. Analysis of serum and other blood components are also useful in the detection of pollution. Radioactive pollution can be determined by studying banding patterns in chromosomes.

MATHEMATICAL APPROACH IN BIOMONITORING

A large number of mathematical formulations (or indices) have been developed in biomonitoring. Surveillance programme over time produces voluminous data on various aspects of environment. Such data must be analysed to make the surveillance worthwhile. The analysis of surveillance data may be done by multivariate analysis or by using biotic or diversity indices. Multivariate analysis can be carried out on both qualitative (presence/absence) and quantitative data. Green (1979) strongly recommended that Principal Component Analysis (PCA) should from the basis of multivariate analysis. However, some biotic and diversity indices are briefly given below:

BIOTIC INDICES

A biotic index takes into account the sensitivity of tolerance of individual species or groups to pollution and assigns them a value, the sum of which gives an index of pollution for a site. The data may be qualitative (presence/absence) or quantitative (relative abundance or absolute density). These indices are designed mainly to assess the organic pollution in water bodies.

The saprobien system of Kolkwitz and Marsson (1908, 1909) is the earliest biotic index. Polysaprobic, α - mesosaprobic, β - mesosaprobic and oligosaprobic zones from the higher organic enrichment to decreasing state in a river are recognized and the presence or absence of indicator species in the said zones are recorded. This information is used to monitor pollution. Pantle and Buck (1955) also developed the saprobien system to take into account the relative abundance of organisms in a sample. They assigned a value (h) to express the abundance of each organism in the different Saprobien groups as well as a value (s) for the saprobic grouping.

The saprobic index of Pantle and Buck

The saprobic index ranges are

Saprobien groups		**Relative abundance**	
	s value		h value
Oligosaprobic	1	Occurring incidentally	1
β - mesosaprobic	2	Occurring frequently	3
α - mesosaprobic	3	Occurring abundantly	5
Polysaprobic	4		

Mean saprobic index (S) = $\frac{sh}{h}$

Trent biotic index (TBI)

Considers the presence and absence of species and species richness, but animals does not need counting. The sensitivity to organic pollution of different species or groups is used in determining the index. In grossly polluted waters, where no macro-invertebrates are present, a TBI of zero is obtained.

The maximum score in unpolluted water with a species rich invertebrate fauna is 10.

Chandler Biotic Score (CBS) –

According to Chandler Biotic Score (1970), the abundance of organisms within the community as well as the species richness, is of value assess the degree of pollution. This index has five levels of abundance, the score of each indicator species being weighted in relation to its abundance. If a species intolerant of pollution is abundant, it is given a high score of 100, whereas an abundant, pollution tolerant species is given us a lower score of 4. The allocation of scores is somewhat arbitrary because the lower limit of the score is zero while there is no upper limit when no macro-invertebrates are present,

Community Similarity Indices (Plafkin *et al*, 1989):

These indices are used in situations where a reference community exists either through sampling or through prediction for a region. Data sources or ecological data files may be available to predict a reference community to be used for comparison. These indices are designed with either species level identifications or higher taxonomic levels. Three of the many community similarity indices available are discussed as below

(Sample A = reference station [or mean of reference database]
Sample B = station of comparison)

- Community Loss Index- it measures the loss of benthic taxa between a reference station and the station of comparison. This is an index of compositional dissimilarity with values increasing as the degree of dissimilarity with the reference station

Increases. Values range from 0 to ∞. This index seems to provide greater discrimination than either of the following two community similarity indices. The formulae for the Community Loss Index is:

$$Community\ Loss = \frac{d-a}{e}$$

Where
a = number of taxa common to both samples
d = total number of taxa present in Sample A
e = total number of taxa present in Sample B

- Jaccard Coefficient of Community Similarity- Measures the degree of similarity in taxonomic composition between two stations in terms of taxon presence of absence. The Jaccard Coefficient discriminates between highly similar collections. Coefficient values, ranging from 0 to 10, increase as the degree of similarity with the reference station increases.

The formulae for the Community Loss Index and the
Jaccard Coefficient are:

$$Jaccard\ Coefficient = \frac{a}{a+b+c}$$

where
a = number of taxa common to both samples
b = number of taxa present in Sample B but not A
c = number of taxa present in Sample A but not B

- Pinkham and Pearson Community Similariy Index- Incorporates abundance and compositional information and can be calculated with either percentages or numbers. A weighting factor can be added that assigns more significance to dominant taxa. The formula is:

$$S.I._{ab} = \sum \frac{\min(x_{ia}, x_{ib})}{\max(x_{ia}, x_{ib})} \left[\frac{x_{ia}}{x_a} \cdot \frac{x_{ib}}{x_b} \div 2 \right]$$

(Weighting factor)

where

x_{ia}, x_{ib} = number of individuals in the i^{th} taxon in Sample A or B

DIVERSITY INDICES

Mason (1980) stated that biotic indices have been developed to measure responses to organic pollution and may be unsuitable for detecting other forms of pollution. Diversity indices are used to measure stress in the environment. It has been seen large number species are found in unpolluted environment, with no single species making up the majority of the community and a maximum diversity is obtained when a large number of species occur in relatively low number in a community. When an environment becomes stressed, species sensitive to that particular stress tend to disappear. As a result species richness will be reduced, and the density of the surviving species will increase. Species diversity indices usually take account of both the number of species (species richness) and their relative abundance (evenness). There are number of diversity indices but the most widely used is Shannon index of general diversity, which is based on information theory. According to Southwick (1976) "Information theory is a branch of science and mathematics which deals with measurable and quantifiable units of information". It involves the numerical analysis of

systems which transmit, process, or store information". Southwick (*op cit.*) further emphasized that "information theory provides the numerical basis for analyzing systems of all types, living as well as non-living".

Shannon index $(\bar{H})$

Where ni = importance value for each

$$\bar{H} = -\sum\left(\frac{ni}{N}\right)\log\left(\frac{ni}{N}\right) (Odum,\ 1971)$$

Where ni = importance for each species and N = total of importance values

$$H' = -\sum_{i=1}^{S} pi \log pi\ (Mason, 1980)$$

where pi is estimated from ni/N as the proportion of the total population of N individuals belonging to i^{th} species (ni)

On the basis of observation of the diversity index in a range of polluted and unpolluted streams Wilhm and Dorris (1968) reached at the following eonclusion.

H (Shannon index of diversity)	**Condition of water quality**
> 3	Clean water
1 - 3	Moderately polluted

BMWP Biotic Index (Armitage *et al,* 1983; Friedrich *et al,* 1996; Hynes, 1998; Mackie, 1998)

In order to limit the taxonomic requirement of earlier biotic indices to identify organisms to species level, some alternative indices have been developed which use only the family level of identification. An example is the Biological Monitoring Working Party-score (BMWP) which has been published as a standard method by an international panel (ISO-BMWP, 1979). This score was devised in the UK but was not specific to any single river catchment or geographical area. The new BMWP score attempted to take the advantages of earlier biotic indices. The Biological Monitoring Working Party (BMWP) score is calculated by adding the individual scores of all indicator organisms present (family level, except order Oligochaeta) (Friedrich *et al*, 1996).

The organisms are identified to the family level and then each family is allocated a score between 1 and 10. The score values (Table II-15) for individual families reflect their pollution tolerance; pollution intolerant families have high scores and pollution tolerant families have low scores. Mayfly nymphs score 10, molluscs score 3 and the least sensitive worms score 1. The number of taxa gives an indication of the diversity of the community (high diversity usually indicates a healthy environment, Friedrich *et al*, 1996).

Table II-15: Pollution sensitivity grades for families (higher levels in a few cases) of river macroinvertebrates for SIGNAL (S) and BMWP (B) scores. Families not occurring in North America have been omitted. N represents families found in N. America and are graded according to the inverse of Bode et al (1991) and Plafkin et al (1989) tolerance values to correspond to SIGNAL and BMWP scores (modified from Mackie, 1998)

Family	**Grade**			**Family**	**Grade**			**Family**	**Grade**		
	N	**B**	**S**		**N**	**B**	**S**		**N**	**B**	**S**
Acariformes	6	-	-	Gammaridae	4	6	6	Peltoperlidae	9	-	-
Aeolosomatidae	2	-	-	Gerridae	5	5	4	Perlidae	8	10	10
Aeshnidae	6	8	6	Glossiphoniidae	3	3	3	Perlodidae	8	10	-
Agrionidae	4	8	-	Glossosomatidae	10	-	8	Philopotamidae	7	8	10
Ancylidae	4	6	6	Gomphidae	6	8	7	Phryganeidae	7	-	-
Anthomyiidae	4	-	-	Gordiidae	8	10	7	Physidae	2	3	3
Anthuridae	4	-	-	Gyrinidae	5	5	5	Piscicolidae	5	4	-
Asellidae	2	3	-	Haliplidae	5	5	5	Planariidae	4	5	3
Arctiidae	5	-	-	Haplotaxidae	1	1	5	Planorbidae	3	3	3
Arrenuridae	4	-	-	Helicopsychidae	7	-	10	Platyhelminthidae	6	-	-
Astacidae	4	8	-	Helodidae	5	5	-	Pleidae	5	5	-
Athericidae	6	-	7	Heptageniidae	7	10	-	Pleuroceridae	4	-	-
Atractideidae	4	-	-	Hirudinea	0	-	-	Polycentropodidae	4	7	8
Baetidae	5	4	5	Hyalellidae	2	-	-	Polychaeta	4?	-	-
Baetiscidae	6	-	-	Hydridae	5	-	4	Polymetarcyidae	8	-	-
Belostomatidae	5	-	5	Hydrobiidae	4	3	5	Potamanthidae	6	10	-

Blephariceridae	10	-	10	Hydrometridae	5	5	5	Psephenidae	6	-	5
Branchiobdellidae	4	-	-	Hydrophilidae	5	5	5	Psychodidae	8	8	2
Brachycentridae	9	10	-	Hydropsychidae	6	5	5	Psychomyiidae	8	8	-
Caenidae	5	7	-	Hydroptilidae	5	6	6	Pteronarcidae	10	-	-
Calopterygidae	4	-	-	Hygrobiidae	5	5	5	Ptychopteridae	1	-	-
Capniidae	8	10	-	Idoteidae	5	-	-	Pyralidae	5	-	6
Ceratopogonidae	4	-	6	Isotomidae	5	-	-	Rhyacophilidae	9	-	7
Chaoboridae	2	-	-	Lebertiidae	4	-	-	Sabellidae	4	-	-
Chironomidae	1	2	1	Lepidostomatidae	10	10	-	Scirtidae	5	5	8
Chloroperlidae	10	10	-	Leptoceridae	6	10	7	Sialidae	6	4	4
Chrysomelidae	5	5	-	Leptophlebiidae	7	10	10	Simuliidae	5	-	5
Coenagrionidae	2	6	7	Lestidae	1	-	7	Siphlonuridae	8	10	-
Collembola	5?	-	-	Leuctridae	10	10	-	Sphaeriidae	4	3	6
Corbiculidae	4	-	6	Libellulidae	8	8	8	Spurchonidae	4	-	-
Corduliidae	7	8	7	Limnephilidae	7	7	8	Sisyridae	5	-	-
Cordulegasteridae	7	8	-	Limnesidae	4	-	-	Tabanidae	5	-	5
Corixidae	5	5	5	Limnocharidae	4	-	-	Taeniopterygidae	8	10	-
Corydalidae	6	-	4	Lumbriculidae	2	1	1	Talitridae	2	-	-
Culicidae	1	-	2	Lymnaeidae	4	3	-	Thiaridae	6	-	7
Dixidae	10	-	8	Mesoveliidae	5	5	4	Tipulidae	7	5	5
Dolichopodidae	6	-	-	Mideopsidae	4	-	-	Tricorythidae	6	-	-
Dreissenidae	2	-	-	Molannidae	4	10	-	Tubificidae	1	1	1
Dryopidae	5	5	-	Muscidae	4	-	3	Tyrellidae	4	-	-
Dytiscidae	5	5	5	Naididae	3	1	1	Unionidae	4	6	-
Elmidae	5	5	7	Nemouridae	8	7	-	Unionicolidae	4	-	-
Empididae	4	-	4	Nepidae	5	5	-	Valvatidae	2	3	-
Enchytreidae	1	1	-	Nepticulidae	5	-	-	Veliidae	5	-	4
Ephemerellidae	10	10	-	Notonectidae	5	5	4	Viviparidae	4	6	-
Ephemeridae	8	10	-	Odontoceridae	10	10	8				
Ephydridae	4	-	2	Oedicerotidae	4	-	-				
Erpobdellidae	3	3	3	Oligochaeta	2	-	-				

Note: The grades under (N) above should be used in the said indices (there is some question as regards the grades of the taxa which have been noted along with a `?')

BIOLOGICAL WATER QUALITY CRITERIA (BWQC)

To assess the actual health of water bodies, CPCB has derived a Biological Water Quality Criteria (BWQC) for water quality evaluation. This system is based on the range of saprobic values and diversityof the benthic macro-invertebrates families with respect to water quality. The system has been developed after making calibration study on the saprobic score and diversity score data on the presence of different taxonomic groups of benthic macro-invertebrate families in few lakes, ponds and reservoirs. To indicate changes in water quality to different grades of pollution level, the entire taxonomic groups, with their range of saprobic score from 1 to 10, in combination with the range of diversity score from 0 to 1 has been classified in to five different classes of water quality (Table 3). The abnormal combination of saprobic scorer and diversity score indicates sudden change in environmental conditions.

Table: 3 Biological Water Quality Criteria (BWQC) for Lakes/Ponds and Reservoirs

S.No. Score	Range of saprobic score	Range of diversity class	Water Quality Indicator	Water Quality	Colour
1.	7-10	0.5-1	A	Clean	Blue
2.	6-7	0.5-1	B	Slight pollution	Light blue
3.	3-6	0.3-0.9	C	Mod. Pollution	Green
4.	2-5	0.4 & less	D	Heavy pollution	Orange
5.	0-2	0-0.2	E	Severe pollution	Red

WHY BIO-MONITORING?

1. Animals and plant communities respond to intermittent pollution, which may be escaped in a chemical sampling / monitoring programme.
2. Biological communities may respond to new or unsuspected pollutants in the environment, which are difficult to analyze chemically. It would be uneconomic and impracticable to regularly determine concentration of 1500 or so known pollutants.
3. The chemical analysis is relatively expensive in terms of equipment needed and number of analysis required to achieve results with comparable reliability, to those achieved by bio-monitoring.
4. Biological monitoring can reflect the environmental pollution levels as some chemical species are accumulated in the bodies of biotic organisms.

Table 1: Comparison of Physico-chemical monitoring with Biological monitoring.

S.No.	Characteristic	Physico-chemical Monitoring	Biological Monitoring
1.	Pollutant concentration	Good	Poor
2.	Assessment of intermittent, irregular pollution discharge	Not possible unless continuously monitored	Possible without continuous monitoring
3.	Kind of pollution assessment	Good	Poor
4.	Reliability (representation of data)	Poor	Good
5.	Measure of ecological effect	Not possible	Possible
6.	Monitoring	Relatively high	Relatively low

ADVANTAGES OF BIOLOGICAL ASSESSMENT

1. The biological methods are quite quick, economical and can be integrated with other relevant studies.
2. Much less equipments are required and large area can be surveyed in less time resulting in large amount of information suitable for assessment.
3. Provide cheaper option in comparison to physico-chemical assessment, where chemical analytical equipment, manpower and operational cost are very high.
4. Biological assessment methods do not eliminate the need for chemical analysis of water samples, however, these may provide information, which may be integrated with physico-chemical information.
5. The integration of biological method with physico-chemical method may provide a system, which is not too expensive and generate necessary information with maximum efficiency.

INFORMATION GENERATED BY BIOLOGICAL BIO-ASSESSMENT

Biological assessment relies on the fact that pollution of water body will cause changes in physico-chemical environment of water and that those changes will disrupt the ecological balance of the system. The measure of extent of ecological upset will depict the severity of pollution. The extreme kind of ecological upset may be clearly visible, such as – unusual color in water, increased turbidity, or presence of dead fishes or mortality, however, many form of ecological damage cannot be assessed without detailed examination of aquatic biota.

Biological assessment of often able to indicate an effect on ecosystem arising from a particular use of the water body. It can determine and depict the general effect of anthropogenic factors on ecosystem as well as the presence and effects of common pollution problems (eutrophication, toxicity and industrial inputs etc.) Biological assessments exhibit deleterious changes in aquatic communities and provide systematic information on water quality. The pollution transformation in water and in organisms can be determined through biological surveillance. The long-term effects of polluting substances in water body may be reflected by study of bio-accumulation and bio-magnification. The biological surveillance may depict the conditions resulting from disposal of wastewater, its character and dispersion as well as assess the effectiveness of environmental protection measure. Quantify the toxicity of substances under controlled, defined laboratory conditions (e.g. toxicity studies).

1. The biological systems used as water quality indicators should have following characteristics:

2. The sampling, sorting, identification and data processing should be as simple as possible involving minimum time and manpower.

SELECTION OF BIOLOGICAL ORGANISMS AS WATER QUALITY INDICATOR

1. It is impossible to study the entire biota present at a sampling area due to constraints of time and wide variety of sampling method required for different group of organisms.
2. The biomonitoring / surveillance must therefore be based on those organisms, which are most likely to provide right information regarding pollution effects.
3. The use of single species as water quality indicator is usually avoided because individual species depict high degree of temporal and spatial variation due to habitat and biotic factors.
4. The indicator species must be able to be used to detect subtle rather than gross and obvious effect of pollution.

CONCLUSION

The term "biological monitoring" has been widely used in this discussion to include almost any type of data gathered to assess the environmental impact of discharges. In our opinion, biological monitoring is limited to a continue collection of data to establish whether explicitly stated quality control conditions are being met. If these conditions are not being met, there will be an immediate decision to take corrective action. Purpose of biological monitoring include providing early warnings of hazards, detecting spills, detecting environmental trends or cycles, determining the best and least redundant information for monitoring, and evaluating the environmental effects associated with the introduction of genetically engineered organisms into natural systems. One design will not serve each purpose, but if the researchers have clearly defined goals for the monitoring program, powerful designs are possible.

REFERENCES:

1. Chandler, J.R. 1970. A biological approach to water quality management. Wat. Pollut. Control., 69:415-422.
2. Forbes, S.A. 1913. Biological and chemical conditions on the upper Illinois River. Proc. *Fifth Meet*., III. Water supply Ass. Urbana, Illinois.
3. Fjerdingstad, E. 1963. Limnological estimation of water pollution levels. World Health Org. *WHO*/*EBL*/10.
4. Gaufin, A.R. 1958. The effect of pollution of a midwestern stream. *Ohio J. Sci.,* 58 197-208.
5. Green, R.H. 1979. Sampling design and statistical methods for environmental biologists. Weley, New York.
6. Kolkwitz, R. and Marsson, M. 1908. Okologic der pflanzlichen Saprobien. *Ber. Dt. Bot. Ges.,* 26: 505-519.
7. Kolkwitz, R. and Marsson, M. 1909. Okologic der tierische Saprobien. Beitrage zur Lehre von der biologische Gewasser-beurteilung. *Int. Revue ges. Hydrobiol. Hydrogr.* 2:126-152.
8. Mason, C.F. 1995. Biology of Freshwater pollution. Longman.
9. Pautle, R. and Buck, H. 1995. Die biologische Uberwachang der Gewasser and die Darstellung der Ergelnisse, gas-u Wasserjach, 96, 604.
10. Richardson, R.E. 1925. Illinois River bottom fauna in 1923. *Bull. Ill. Nat. Hist. Surv*., 15, 391-422.
11. Weber, C.I. and McFarland, B.H. 1969. Periphyton biomass-chlorophyll ratio as an index of water quality. Address presented at 17th Meeting of Midwest Benthological society, Gilbertsville, Kentucky.
12. Cairns, J., Jr. and W.H. van der Schalie. 1980. Biological monitoring. Part I: Early warning systems. Water Res. 14:1179-1196.
13. Eberhardt, L.L.1976b. Quantative ecology and impact assessment. J. Environ. Manag. 4:27-70.
14. Gilbert, R.O. 1987. Statistical Methods for Environmental Pollution Monitoring. Van Nostrand, NY. 384 p,
15. Hughes, R.M., S.A. Heiskary, W.J. Matthews, and C.O. Yoder. 1994. Use of Ecoregions in Biological Monitoring. In: S.L. Loeb and A. Spacie, eds., Biological Monitoring of Aquatic Systems. Lewis Publishers, Boca Raton, FL.
16. Odum, E.P. 1971. Fundamentals of Ecology. W.B. Saunders Co. Philadelphia, PA.
17. Plafkin, J.L., M. T. Barbour, K.D. Porter, S.K. Gross, and R.M. Hughes. 1989. Rapid Bioassessment Protocols for Use in Streams and Rivers: Benthic Macroinvertebrates and Fish. EPA 444/4-89-001. National Technical Information Service, Springfield, VA. 190p.

18. Best, E.P.H. and J. Haeck. 1982. Ecological Indicators for the Assessment of the Quality of Air, water, Soil and Ecosystems. D. Reidal Publishing Company, Boston. 283 p.
19. De Pauw, N. 1988. Biological assessment of surface water quality: the Belgium experience. In: "La Qualitya' delle Acque Superficiali." Atti del Convegno Internazionale, Riva del Garda, Italy. Provincia Autonoma di Trento, Italy. 285 p,
20. Ghetti, P.F. 1978. Biological Water Assessment Methods. Technical Report, Parma 8-13 October 1978. Report E.E.C., ENV/395/78 EN:1-42.
21. Woodiwiss, F.S. 1978. Biological Monitoring of Surface Water Quality. Summary Report. Commission of the European Communities. ENV/787/80-EN:45p.
22. Subba Rao, N.V. 1989. Handbook: Fresh water Molluscs of. 642 INDIA xiii. + 290 pp figs. Zoological survey of India.
23. Moog, o. and sharma, S.(1996) Biological assessment of water quality in the river Bagirathi and its tributaries, Kathmandu valley, Nepal. Proceedings of the international conference on Ecohydrology of high mountain areas 24-28 march 1996, Kathmandu, Nepal.
24. Unni K.S., 1997 Ecology of River Narmada Ashish Publishing house New Delhi. 377 PP.

7/17/2010

Effect of different phosphatic fertilizers on growth attributes of wheat (Triticum aestivum L.)

Muhammad Bilal Khan[1], Muhammad Iqbal Lone[1], Rehmat Ullah[2*], Shuaib Kaleem[3] and Muhammad Ahmed[3]

[1]Department of Soil Science & SWC, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi

[2]Soil and Water Testing Laboratory, Rajanpur-Punjab-Pakistan

[3]Agriculture Adoptive Research Complex, Dera Ghazi Khan, Punjab-Pakistan

rehmat1169@yahoo.com

Abstract: Among all the elements required by a plant, phosphorus (P) is one of the most important nutrients for crop production and emphasis is being given on the sufficient use of P fertilizer for sustainable crop production. A pot experiment was conducted in green house at the Department of Soil Science and SWC, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi during Rabi season, 2007. Phosphorus was applied at the rate of 40 and 80 kg P ha^{-1} in the form of SSP, TSP, NP and DAP. A basal doze of 100 kg N and 60 kg K ha^{-1} was applied as urea and murate of potash (MOP) respectively. All the growth parameters of wheat were significantly improved by addition of P application. It was concluded from the study that phosphorus application at the rate of 80kg P ha^{-1} as single super phosphate (SSP) showed better results as compared to triple super phosphate (TSP), nitrophos (NP) and diammonium phosphate (DAP) on phosphorus deficient soil of Balkasr area of Tehsil Chakwal.

[Muhammad Bilal Khan, Muhammad Iqbal Lone, Rehmat Ullah, Shuaib Kaleem and Muhammad Ahmed. **Effect of different phosphatic fertilizers on growth attributes of wheat (Triticum aestivum L.)**. Journal of American Science 2010;6(12):1256-1262]. (ISSN: 1545-1003). http://www.americanscience.org.

Key Words: Phosphorus, Wheat, Growth Attributes, P Fertilizer, calcareous soil

1. Introduction

Phosphorus is critical in the metabolism of plants, playing a role in cellular energy transfer, respiration, and photosynthesis. It is also a structural component of the nucleic acids of genes and chromosomes and of many coenzymes, phosphoproteins and phospholipids. Early season limitations in P availability can result in restrictions in crop growth, from which the plant will not recover, even when P supply is increased to adequate levels. An adequate supply of P is essential from the earliest stages of plant growth (Bertrand *et al.*, 2003). A growing plant may experience different stages in mineral nutrition, based on the balance among internal and external nutrient supplies and crop demand for nutrients. Initially, plants will live on their seed reserves, with external supply having little effect on plant growth. A second stage occurs when growth rate is determined by nutrient supply through a dynamic balance between internal plant factors and external (soil) supply (Grant *et al.*, 2001). In a final stage, the relative growth rate may decline for reasons other than inadequate nutrition. At this point, the growth rate of deficient and sufficient plants may converge, since the factor most limiting to growth is not nutrient supply. The length of time required for a P deficiency to show an effect on plant processes depends on the extent of P reserves in the plant. In tissues of higher plants, the majority of the P is present as inorganic P (Holloway *et al.*, 2001).

Among different factors, the role of nutrients is well recognized in crop production. The inadequate supply of the essential plant nutrients in soil is growth limiting factor towards its production. Among all the elements required by a plant, phosphorus (P) is one of the most important nutrients for crop production and emphasis is being given on the sufficient use of P fertilizer for sustainable crop production (Ryan, 2002). It is an expensive nutrient as compared to nitrogen (Nisar, 1996). Therefore it is imperative to manage it properly to achieve its maximum use efficiency. Among other factors, lack of proper balance between nitrogen (N) and phosphorus (P) is considered important as it remained mostly in favor of N and has increased to as high as 4.7:1 against the recommended ratio of 2:1 or lower (Ahmed, 2000).

Soils of Pakistan are alkaline and mostly calcareous in nature and P fixation is a serious problem in these soils (Sharif *et al.*, 2000). According to NFDC (2003), 93 percent of Pakistani soils are P deficient. When P fertilizer is added, the soil can rapidly and firmly adsorb a large amount of P from the soil solution. When P is adsorbed, it becomes unavailable to the plants and with time is difficult to release from the soil (Huang, 1998). At present DAP

(18:46:0) is the principle phosphate fertilizer used in Pakistan, with somewhat less quantities of NP (23:23:0) and much smaller amounts of SSP and TSP. The phosphorus fertilizer use can help to reduce the adverse effect of drought under rainfed conditions. The Potash and Phosphate Institute (PPI, 1999) reported that phosphorus, in balanced soil fertility program, increase water use efficiency and helps crop to achieve optimal performance under limited moisture conditions. As wheat is staple food of Pakistan, the present study was undertaken to compare the effect of different phosphatic fertilizers on wheat crop in Balkasr soil series.

2. Materials and Methods

A pot experiment was conducted to compare the effect of different phosphatic fertilizers on wheat crop in Balkasr soil series of Tehsil Chakwal. The study was conducted in green house at the Department of Soil Science and SWC, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi during Rabi season, 2007. For this, bulk soil sample from 0-30 cm depth was brought from farmer's field of Balkasr area of Tehsil Chakwal. Soil sample was air dried, ground, passed through a 2 mm sieve and mixed thoroughly. Ten kg of the prepared soil was packed in each earthen pot having height and diameter of 18 and 12, inches respectively. The pots were lined with polythene bags. Eight seeds of wheat (cv. GA 2002) were sown on 20th November, 2007. Phosphorus was applied at the rate of 40 and 80 kg P ha^{-1} in the form of SSP, TSP, NP and DAP. A basal doze of 100 kg N and 60 kg K ha^{-1} was applied as urea and murate of potash (MOP) respectively. Thinning was conducted on 22nd December, 2007 and four plants per pot were grown. Distilled water was used to bring the soil to 50 percent of maximum water holding capacity by weighing the pots as and when required. One plant per pot was harvested at 80 days growth while three plants were harvested at maturity on Saturday, May 5, 2007 at 137 days growth. Experiment was laid out in completely randomized design (CRD) with three replications.

Soil sample collected from the farmer's field of Balkasr soil series was air dried, ground, passed through a 2 mm stainless steel sieve and mixed thoroughly. This soil sample was used for physio-chemical analysis like soil texture, macronutrients (P, K), electrical conductivity (EC_e), soil pH_s, and organic matter (OM) at the beginning of experiment (Table 1). The soil used for the experiment was sandy loam in texture, alkaline in reaction (pH=7.92), poor in organic matter (0.40%), adequate in potassium (92 mg kg^{-1}) and low in available phosphorus (4.6 mg kg^{-1}). Particle Size Analysis was done by Bouyoucus Hydrometer Technique (Bouyoucus, 1962). Soil paste was prepared and saturation extract was obtained. Then the pH was determined with a pH meter (Page *et aI.*, 1982) and Electrical conductivity was measured by using electrical conductivity meter (Page *et al.*, 1982). Organic matter was determined by using Walky and Blake method (Page *et al.*, 1982). Available Phosphorus was measured by spectrophotometer (Olsen, 1982). Extractable potassium was determined by using flame photometer (Knudsen *et al.*, 1982).

2.1 STATISTICAL ANALYSIS

The data collected for various parameters were subjected to Analysis of Variance (ANOVA) and the means obtained were compared by LSD at 5 percent level of significance (Steel and Torrie, 1980).

3. RESULTS

3.1 Effect on Number of Tillers per Plant

The yield of a crop is dependent upon the combined effect of many factors. Among these factors, the number of tillers per plant has a vital position, controlling yield of wheat. The more the number of tillers, the better will be the stand of crop, which ultimately increases the yield (Jamwal and Bhagat, 2004). The data presented in Table 2 show the effect of different phosphorus sources and levels on number of tillers per plant of wheat crop (cv. GA 2002). The results revealed that the maximum numbers of tillers per plant (6.67) were found in the treatment T_6 (80 kg ha^{-1} P as SSP) and minimum numbers of tillers per plant (3.00) in treatment T_1 (control). Different P sources showed significant effect on number of tillers per plant while the number of tillers per plant increased with the increased level of phosphorus application. At both P fertilizer levels applications, Single Super Phosphate (SSP) resulted in maximum number of tillers per plant as compared to Triple Super Phosphate (TSP), Nitrophos (NP) and Diammonium Phosphate (DAP).

3.2 Effect on Plant Height (cm)

Plant height is a genetic character of a variety but its potential can be achieved by adequate crop management. The data on the effect of different P sources and levels on plant height is given in Table 2. The results showed that the maximum plant height (91.67 cm) was recorded in treatment T_6 (80 kg P ha^{-1} as SSP), while it was minimum (75.33 cm) in treatment T_1 (control). Plant height was significantly affected among all the various P sources application. It also increased linearly with the increased level of phosphorus application. Hence, among low level of various phosphorous fertilizer application,

Diammonium Phosphate had enhanced more plant height while at high level SSP resulted in maximum plant height as compared to TSP, NP and DAP.

3.4 Effect on Spike Length (cm)

The data in table-2 pertaining to spike length indicated that it was maximum (10.00 cm) in treatment T_6 (80 kg P ha^{-1} as SSP) while recorded minimum (5.00 cm) in farmer practice. Various P sources and levels application to wheat spike length varied significantly. The data concluded from the results that among low P level application, Nitrophos had enhanced more spike length. Among all high P level application, Single Super Phosphate had produced more spike length as compared to TSP, NP and DAP.

3.5 Effect on Number of Grains per Spike

Number of grains per spike is one of the easily determinable characters, which has a positive co-relation with the yield. The effect of different P sources and levels on number of grains per spike along with statistical interpretations is presented in Table 2. The results showed that maximum numbers of grains per spike (39.67) were observed in treatment T_6 (80 kg P ha^{-1} as SSP) and minimum numbers of grains per spike (33.33) were observed in treatment T_1 (control). Different P sources showed significant effect on number of grains per spike. Number of grains per spike increased with the increased level of phosphorus application. At low P level applications, DAP increased more number of grains per spike while at high P level applications, SSP had produced maximum number of grains per spike as compared to TSP, NP and DAP.

3.6 Effect on Grain Yield (g pot^{-1})

The data regarding grain yield (g pot^{-1}) shown in table-2 differed significantly in all treatment. Maximum grain yield (32.20 g pot^{-1}) was observed in T_6 (80 kg P ha^{-1} as SSP) while it was found minimum (12.00 g pot^{-1}) in treatment T_1 (control). Grain yield was significantly affected by different P sources and increased with the increased level of phosphorus application. Among low level of P application, DAP had produced maximum grain yield. Hence among all high P level applications, SSP resulted in maximum grain yield as compared to TSP, NP and DAP.

3.7 Effect on Straw Yield (g pot^{-1})

The data on the effect of different P sources and levels on straw yield (g pot^{-1}) along with statistical interpretations were shown in table-2. The straw yield also showed the similar response like the grain yield. The findings showed that the maximum straw yield (61.24 g pot^{-1}) was observed in the treatment T_6 (80 kg P ha^{-1} as SSP) while it was observed minimum (25.20 g pot^{-1}) in the treatment T_1 (control). Different P sources and levels showed significant effect on straw yield. Hence, among all P applied levels, SSP had produced maximum straw yield as compared to TSP, NP and DAP.

4. Discussion

The result of this research indicated that there existed a significant impact of P containing fertilizers on the various growth attributes of wheat crop. The crop yield is much important for enhancing the farmer and country economy. It is specifically correlated with many growth factors like number of tillers per plant, plant height, spike length, number of grains per spike and straw yield. These had lingering role to enhance or lemmatize the crop productivity. Therefore more number of tillers per plant (6.67) was found in the treatment (table-2) where 80 kg ha^{-1} P as SSP was applied while farmer practice produced very minimum numbers of tillers per plant (3.00). However, our findings showed that the number of tillers per plant increased with the increased level of phosphorus application. Among all P containing fertilizers, SSP application had produced more number of tillers per plant as compared to TSP, NP and DAP. It might be due to maximum availability of phosphorous which established more root establishments. This fact would ultimately maximum availability of mineral nutrients for optimum cell growth, reproduction, photosynthesis and transformation of sugars and starches. The results are in line with the findings of Jamwal and Bhagat (2004) who reported that basal application of the recommended dose of DAP produced significantly higher number of effective tillers m^{-1} row length which ultimately increased the grain and straw yields. Renu *et al.* (2005) also found that the number of tillers per plant was less where no fertilizer was applied as compared to phosphorus fertilizer application in case of wheat crop. Various other scientists (Baon *et al.*, 1992; Bertrand *et al.*, 2003; Glassop *et al.*, 2005) also responded similar to our findings. They also found that phosphorous had a significant impact on various cereal crops growth attributes as these are paradox for the yield enhancing factors. They also suggested for application of phosphatic fertilizers at early growth stages to support the lateral growth stages for enhancing crop productivity as per their potential.

Number of tillers per plant depicted in table-2 also elucidated the similar trend number of tillers per plant. Therefore, our findings do match to Jamwal and Bhagat (2004) who reported that basal application of the recommended dose of DAP produced significantly higher number of effective tillers m^{-1} row length which ultimately increased the grain and straw yields. Renu *et al.* (2005) also found that the number of tillers per plant was less where no fertilizer was applied as compared to phosphorus fertilizer application in case of wheat crop. Various other scientists (Baon *et al.*, 1992; Bertrand *et al.*, 2003; Glassop *et al.*, 2005) also responded similar to our findings. They also found that phosphorous had a significant impact on various cereal crops growth attributes as these are paradox for the yield enhancing factors. They also suggested for application of phosphatic fertilizers at early growth stages to support the lateral growth stages for enhancing crop productivity as per their potential.

In addition to them, plant height also contributed a significant impact on enhancing the straw yield. The table-2 showed that plant height higher plant height (91.67 cm) was also noted in treatment where80 kg P ha^{-1} as SSP, while farmer practice had attained minimum (75.33 cm) plant height. Hence DAP and SSP had enhanced higher plant height as compared to TSP, NP and DAP. Our findings are in line with the accordance of Holloway *et al.*, 2001; Khan 1975; Li *et al.*, 2005) who also reported the similar findings.

Spike length in table-2 was recorded maximum (10.00 cm) in 80 kg P ha^{-1} as SSP application and was noted minimum (5.00 cm) was found in control treatment. Different P sources and levels significantly affected spike length. However, amongst low level, NP resulted in maximum spike length while at high level SSP resulted in maximum spike length as compared to TSP, NP and DAP. Some other scientists (Lombi *et al.*, 2004; McBeath *et al.*, 2005; Mohammad *et al.*, 2004) showed the similar trend for phosphorous application to various crops. They also found that spike length of the cereal crops are the major factor to feed the livestock for the sustainability of the food web. They observed that basal application of the recommended dose of DAP produced significantly taller spike length that ultimately increased the grain and straw yields.

Number of grains per spike shown in table-2 reflected that control practice had produced minimum number of grains per spike while were noted more in treatment T_6 where 80 kg P ha^{-1} as SSP applied. However, DAP and SSP had produced more number of grains per spike as compared to other P containing fertilizers. Poulsen *et al.* (2005) recommended the phosphatic fertilizer application enhanced significantly higher number of grains per spike which ultimately increased the grain and straw yields. Various scientists (Ravnskov *et al.*, 1995; Reuter *et al.*, 1995) also showed the similar results.

In table-2, higher grain yield (32.20 g pot^{-1}) was observed in T_6 (80 kg P ha^{-1} as SSP) while it was found minimum (12.00 g pot^{-1}) in treatment T_1 (control). Grain yield was significantly affected by different P sources and increased with the increased level of phosphorus application. At low level, DAP resulted in maximum grain yield but at high level, SSP resulted in maximum grain yield as compared to TSP, NP and DAP. The results are in line with the findings of Reddy and Sigh, (2003) who observed that among the different phosphatic fertilizers, single super phosphate resulted in the highest grain yield (50.27 q ha^{-1}), followed by nitrophos (43.96 q ha^{-1}) and diammonium phosphate (43.13 q ha^{-1}). Grain yield was higher; where phosphorus fertilizer was applied as compared to that where no P fertilizer was applied in case of wheat crop (Yaseen *et al*., 1998). Highest grain and straw yields were observed where P was applied as nitrophos (Alam *et al., 2002*). Rashid *et al.* (2002) observed that substantial crop yield increases as well as plant P concentration enhancements were observed with applied P. The findings of various scientists (Schweiger and Jakobsen 1999; Smith and Smith 2005; Smith *et al.*, 2003) also responded similar to our results.

The straw yield (table-2) also showed the similar response like the grain yield. SSP had produced more straw yield as compared to TSP, NP and DAP. Straw yield was higher; where phosphorus fertilizer was applied as compared to that where no P fertilizer was applied in case of wheat crop (Yaseen *et al*., 1998). Highest grain and straw yields were observed where P was applied as NP (Alam *et al.*, 2002). Various other scientists (Rashid *et al*, 2002; Smith *et al.*, 2004; Zhu *et al.*, 2001) observed that substantial crop yield increases as well as plant P concentration enhancements were observed with the application of phosphorus.

Above discussion stated that P containing fertilizer like DAP and SSP fertilizer should be applied at the early growth stages. Hence, these would have significant affect on the lateral growth stages of the wheat crop. Hence, this study would act as a paradox for other scientist to verify this fact at large field scale for it implication.

Table 1: Basic Soil Analyses of the Selected Soil

Parameters	Units	Values
Sand	%	90.14
Silt	%	7.92
Clay	%	1.94
Textural Class		Sandy Loam
PH_s		7.90
EC_e	dS m^{-1}	1.48
Organic Matter	%	0.40
Available Phosphorus	mg kg^{-1}	4.6
Extractable Potassium	mg kg^{-1}	92

Table 2: Effect of different P sources and levels on yield and yield components of wheat crop

Treatments	Sources and Levels of phosphorus	Number of tillers per plant	Plant height (cm)	Spike length (cm)	Number of grains per spike	Grain yield (g pot^{-1})	Straw yield (g pot^{-1})
T_1	Control	3.00 c	75.33 f	5.00 c	33.33 c	12.00 e	25.20 d
T_2	40 kg P ha^{-1} as SSP	4.00 bc	83.33 bc	7.00 bc	35.67 b	20.50 d	41.21 c
T_3	40 kg P ha^{-1} as TSP	3.67 bc	79.67 e	7.67 ab	35.33 b	20.20 d	40.56 c
T_4	40 kg P ha^{-1} as NP	3.67 c	85.33 b	7.67 ab	36.33 b	20.01 d	40.27 c
T_5	40 kg P ha^{-1} as DAP	4.00 bc	82.33 cd	7.00 bc	36.67 b	21.90 d	39.90 c
T_6	80 kg P ha^{-1} as SSP	6.67 a	91.67 a	10.00 a	39.67 a	32.20 a	61.24 a
T_7	80 kg P ha^{-1} as TSP	5.33 c	80.67 d	9.33 ab	39.33 a	25.50 c	58.03 bc
T_8	80 kg P ha^{-1} as NP	5.67 b	83.00 c	8.67 ab	38.33 a	26.10 c	57.43 bc
T_9	80 kg P ha^{-1} as DAP	6.33 b	80.67 d	9.67 ab	38.67 a	29.30 b	58.84 b

- The means having different letter are significantly different from each other at 5% level of probability.

5. Conclusion

It was concluded from the study that phosphorus application at the rate of 80kg P ha^{-1} as single superphosphate (SSP) showed better results as compared to triple superphosphate (TSP), nitrophos (NP) and diammonium phosphate (DAP) on phosphorus deficient soil of Balkasr area of Tehsil Chakwal. This superiority of SSP over the other three sources could be due to presence of more Ca content and better water solubility of phosphate compound. Single super phosphate can be used on all crops and soils as a basal dressing. Its use for ailing saline/sodic soils is, however, preferred because of the ameliorative effect ascribable to its 46% gypsum content and highly acidic nature (pH 2.0). This product is also manufactured locally and easily available to farmers. However as it contains less

percentage of phosphorus therefore its storage and transportation cost is high as compared to other sources.

Acknowledgements

The author would like to acknowledge the department of Soil Science & SWC, Pir mehr Ali Shah Arid Agriculture University Rawalpindi for providing a laboratory facility for analyses purposes to complete this study.

Correspondence to:
Dr. Rehmat Ullah
Department of Soil Science & SWC,
Pir Mehr Ali Shah Arid Agriculture University,
Rawalpindi
Tel: +03336032657
Email: rehmat1169@yahoo.com

REFERENCES

1. Ahmed, N. (2000). *Integrated plant nutrition management in Pakistan Status & opportunities.* International Proceeding of Symposium on Integrated Plant Nutrition Management. NFDC, Islamabad, 18-39.

2. Alam, S. M., & Shah, S. A. (2002). *Phosphorus uptake and yield of wheat as influenced by integrated use of phosphatic fertilizers.* Paper presented at 9th International Congress of Soil Science Society of Pakistan held at NIAB, Faisalabad, 18-20.

3. Baon, J. B., Smith, S. E., Alston, A. M., & Wheeler, R. D. (1992). Phosphorus efficiency of three cereals as related to indigenous mycorrhizal infection. *Australian Journal of Agricultural Research*, 43, 479-491.

4. Bertrand, I., Holloway, R. E., Armstrong, R. D., & McLaughlin, M. J. (2003). Chemical characteristics of phosphorus in alkaline soils from southern Australia. *Australian Journal of Agricultural Research*, 41, 61-76.

5. Glassop, D., Smith, S. E., & Smith, F. W. (2005). Cereal phosphate transporters associated with the mycorrhizal pathway of phosphate uptake into roots. *Planta*, 222, 688-698.

6. Grant, C. A., Flaten, D. N., Tomasiewicz, D. J., & Sheppard, S. C. (2001). Importance of early season phosphorous nutrition. *Better Crops*, 85 (2), 18-23.

7. Holloway, R. E., Bertrand, I., Frischke, A. J., Brace, D. M., McLaughlin, M. J., & Shepperd, W. (2001). Improving fertilizer efficiency on calcareous and alkaline soils with fluid sources of P, N and Zn. *Plant and Soil*, 236, 209-219.

8. Huang, Q. N. (1998). Properties of phosphorus adsorption and desorption in red soil under a stand of Chinese fir in Fijian Nanjing. *Journal of Forestry Universal*, 22(2), 39-44.

9. Jamwal, J. S., & Bhagat, K. L. (2004). Response of wheat (*Triticum aestivum*) to top dressing of diammonium phosphate in rainfed areas of Shivalik foothills. *Indian Journal of Agronomy*, 49(4), 251-253.

10. Khan, A. G. (1975). The effect of vesicular–arbuscular mycorrhizal associations on growth of cereals. II. Effects on wheat growth. *Annals of Applied Biology*, 80, 27-36.

11. Knudsen, D., Peterson, G. A., & Pratto, P. F. (1982). *Lithium, Sodium and Potassium. In:* Page, A. L., Miller, R. H. & Keey, D. R. (eds). Methods of Soil Analysis Part 2. American Society of Agronomy. No.9. Madison, Wisconsin, USA, 228-238.

12. Li, H. Y., Zhu, Y. G., Marschner, P., Smith, F. A. & Smith, S. E. (2005). Wheat responses to arbuscular mycorrhizal fungi in a highly calcareous soil differ from those of clover, and change with plant development and P supply. *Plant and Soil*, 277, 221-232.

13. Lombi, E., McLaughlin, M. J., Johnston, C., Armstrong, R. D. & Holloway, R. E. (2004). Mobility and labiality of phosphorus from granular and fluid monoammonium phosphate differs in a calcareous soil. *Soil Science Society of America Journal*, 68, 682-689.

14. McBeath, T. M., Armstrong, R. D., Lombi, E. McLaughlin, M. J. & Holloway, R. E. (2005). Responsiveness of wheat (*Triticum aestivum*) to liquid and granular phosphorus fertilisers in southern Australian soils. *Australian Journal of Soil Research*, 43, 203-212.

15. Mohammad, A., Mitra, B., Khan, A. G. (2004). Effects of sheared-root inoculum of Glomus intraradices on wheat grown at different phosphorus levels in the field. *Agriculture Ecosystems and Environment*, 103, 245-249.

16. Nisar, A. (1996). *Annual Fertilizer Review.* NFDC Publication No. 11/96. Islamabad, 31.

17. Olsen, S. R., & Sommers, L. E. (1982). *Phosphorus.* In: Page, A. L., Miller, R. H. & Keey, D. R. eds. Methods of Soil Analysis Part 2. American Society of Agronomy No.9. Madison, Wisconsin, USA, 403-427.

18. Page, A. L., Miller, R. H. & Keey, D. R. eds. (1982). Methods of Soil Analysis Part 2. American Society of Agronomy Madison, Wisconsin, USA.

19. Phosphate and Potash institute (PPI). (1999). Phosphorus and water use efficiency. *Better crops*, 83 (1), 25-25.

20. Poulsen, K. H., Nagy, R., Gao, L. L., Smith, S. E., Bucher, M., Smith, F. A. & Jakobsen, I. (2005). Physiological and molecular evidence for Pi uptake via the symbiotic pathway in a reduced mycorrhizal colonization mutant in tomato associated with a compatible fungus. *New Phytologist*, 168, 445-453.

21. Rashid, M., Saeed, M., & Ahmed, R. (2002). Soil phosphorus levels for optimum wheat yield. *Journal of Agriculture Research*, 35(1-2), 35-39.

22. Ravnskov, S., & Jakobsen, I. (1995). Functional compatibility in arbuscular mycorrhizas measured as hyphal P transport to the plant. *New Phytologist*, 129, 611-618.

23. Reddy, M. P., & Singh, S. S. (2003). Effect of different sources of phosphatic fertilizers on growth and yield of wheat (*Triticum aestivum L.*). *Crop Research*, 26(3), 386-389.

24. Renu, P., Bhupinder, S., & Nair, T. V. R. (2005). Phosphorus use efficiency of wheat, rye and Triticale under deficient and sufficient levels of phosphorus. *Indian Journal of Plant Physiology*, 10(3), 292-296.

25. Reuter, J., Dyson, C. B., Elliott, D. E., Lewis, D. C., & Rudd, C. L. (1995). An appraisal of soil phosphorus testing data for crops and pastures in South Australia. *Australian Journal of Experimental Agriculture*, 35, 979-995.

26. Schweiger, P. F., & Jakobsen, I. (1999). Direct measurement of arbuscular mycorrhizal phosphorus uptake into field-grown winter wheat. *Agronomy Journal*, 91, 998-1002.

27. Sharif, M., Sarir, M. S., & Rabi, F. (2000). Biological and chemical transformation of phosphorus in some important soil series of NWFP. *Sarhad Journal of Agriculture*, 16 (6), 587-592.

28. Smith, F. A., & Smith. S. E. (2005). *P transport pathways in plants with low mycorrhizal responsiveness: new insights from membrane transport physiology and gene expression.* In: Plant nutrition for food security, human health and environmental protection. Beijing: Tsinghua University Press, 864-865.

29. Smith, S. E., Smith, F. A., & Jakobsen, I. (2003). Mycorrhizal fungi can dominate phosphate supply to plants irrespective of growth responses. *Plant Physiology*, 133, 16–20.

30. Smith, S. E., Smith, F. A., & Jakobsen, I. (2004). Functional diversity in arbuscular mycorrhizal (AM) symbioses: the contribution of the mycorrhizal P uptake pathway is not correlated with mycorrhizal responses in growth or total P uptake. *New Phytologist*, 162, 511-524.

31. Steel, R. G. D., & Torrie, J. H. (1980). *Principles and Procedures of Statistics*, 2nd ed. McGraw Hill Book Company Inc., New York, 507.

32. Yaseen, M., Gill, M. A., Siddique, M., Ahmed, Z., Mahmood, T., & Hamud-ur-Rehman. (1998). *Phosphorus deficiency stress tolerance and phosphorus utilization efficiency in wheat genotypes. In:* Proceeding of Symposium on plant nutrition management for sustainable agric. growth. Govt. of Pakistan, Planning and Development Division NFDC, Islamabad.

33. Zhu, Y. G., Smith, S. E., Barritt, A. R. & Smith, F. A. (2001). Phosphorus (P) efficiencies and mycorrhizal responsiveness of old and modern wheat cultivars. *Plant and Soil*, 237, 249-255.

10/3/2010

A new categorization of construction materials based on sources of waste across supply chain

Mohamad Reza Parsanejad[1], Mansor Momeni[2], Ahmad Jafarnejad[3], Ali Mohaghar[4]

[1,2,3,4] *Department of Industrial Management, Faculty of Management, University of Tehran*

parsanejad@ut.ac.ir

Abstract: Construction industry is an important part of any economy. But it does not have an appropriate performance especially in the productivity of materials. Statistics show production of billions of tons of construction waste per year in the world, and these issues threaten all beneficiaries of this industry. Thus, convenient strategies should be founded for improving waste production. This will not be achieved unless we recognize waste sources across construction supply chain. Also each material has its own source of waste, therefore exact identification of any material and after that its source will help to develop waste minimization strategies. In this research 30 questionnaires were distributed between experts. At first we prioritized waste sources, and by following the question about impact of sources on selected material, using binominal test, it observed that a category of sources had impact on some of material and another sources on another materials. Analysis of these two types of materials showed us that this result was not accidental and those materials when use in building, their dimensions is important (like brick, block, tile and etc.), those sources have impact on their waste that emphasize design parameters of building. Those material when use in building, their weight are important (like cement, gypsum, sand and etc.), those sources have impact on their waste that emphasize purchasing level of ordering and purchasing. Therefore materials categorized by their sources of waste across supply chain.

[Mohamad Reza Parsanejad, Mansor Momeni, Ahmad Jafarnejad, Ali Mohaghar. **A new categorization of construction materials Based on sources of waste across supply chain**. Journal of American Science 2010; 6(12):1263-1273]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Waste, Source of waste, Construction supply chain, Non-coordination, Dimensional, Weight based.

1) Introduction:

Statistics shows us at 2008, 16.7 million tons of wastes were disposed in Tehran landfills. Also since year 1995 to 2008 it was estimated that 150 million tons of wastes were produced in Tehran and transported to wastes centers. (Report of material section of construction and housing research center, 2008), (Report of Tehran municipal recycles organization, 2008), (Omrani et al., 2008)

Based on Tehran municipal reporters, construction wastes are transported to some places like ABALI, TELO, ARDIB and so on. The most popular of these places is ABALI that 900 thousands of construction wastes are disposed there every month. This area is more than 700 square kilometers, it means as wide as Tehran city. By growing population and flourishing demands for building, lots of problems will generate near these high amounts of wastes. (Report of environmental committee of consoling Tehran city, 2008)

These statistics shows that the amount of waste production is high in Iran but with a review it could be seen that this amount is high in another countries too. Here are some researches that show this subject:

- The amount of waste is about 1 to 10% of purchased material with average of 9% (Ekanayake and Ofori, 2004),
- The amount of waste in Brazil is between 20 to 30% of material weight entered a construction site (Pint and Agapyan, 1994),
- Construction waste in Australia is from 4% for glass to 19.6% for plaster (McDonald and Smithers, 1998),
- In USA, waste in most of buildings is 20 to 30 kilograms (Chun-Li et al., 1997),
- Average waste of block in Singapore is about 13% of purchased weight (Kang, 2000),
- The amount of waste cost is about 3 to 40% of total project cost. (Katz and Baum, 2010)
- 25% of materials in construction process waste (Hamassaki and Neto ,1994),
- 20% of material entered the site are wasted (Formoso et al., 1993),
- Construction waste is about 30% of all material weight in site. (Fishbein, 1998),
- Construction waste as a percent of solid wastes entered to the site in some countries are as below: Netherlands 26%, Australia 20-30%, USA 10-29%, Germany 19% and Finland 13-15% (Bossink and Brouwers, 1996),

These statistic excited researchers to sick and develop some solutions for management and prevention of construction wastes. Among various methodologies of waste management, a categorization is more popular. It classifies waste management

solutions to four categories : minimization, reuse, recycle and disposal. (Gavilan and Bernold, 1994; Franiran and Gaban, 1998; Begum et al., 2007; Silva and Vithana, 2008)

However material waste management leads to higher level of productivity in this business, but almost all researchers emphasize that minimization and elimination of waste is the best solution. (Gavilan and Bernold, 1994; Skoyles and Skoyles, 1987; Begum et al., 2006).

For development of waste strategies in construction, the waste and their types should be identified. Inherent properties of any material such as methods of usage, important parameters when use, how to supply and how to maintain, are effective in process of changing a material to final product. Since this process and properties are different for various materials, the trends of waste production and waste sources are different too. Many researchers have done so many studies about waste sources but there are no categorization based on inherent properties of materials until now. The purpose of this research is to identify material types which will be used for developing waste minimization strategies for any type of materials. For solving this problem we should find out that the sources across supply chain will effect on waste production in any material or not? After that, by this method the materials can be categorized.

2) Literature review

2-1) Waste

After categorizing waste to seven types by ohno (1994), Womack and jones (1996) defined waste as any activity that absorbs sources and does not have any value adding. In another word waste is the loss of any kind of sources-materials, time (labor and equipment), and capital- produced by activities that generate direct or indirect costs but do not add any value to the final product from the point of view of the client (Formoso et al., 2002).

2-2) Construction materials waste

Construction material wastes refer to materials from construction sites that are unusable for the purpose of construction and have to be discarded for whatever reason (Yahya and Boussabaine, 2006).

In another research construction waste was defined as any material apart from earth materials, which needed to be transported elsewhere from the construction site or used on the site itself other than the intended specific purpose of the project due to damage, excess or non-use or which cannot be used due to non-compliance with the speci6cations, or which is a by-product of the construction process. (Ekanayake and Ofori, 2004)

2-3) Types of Construction materials wastes

There are many studies about kinds of materials waste that have so many overlaps.

Bossink and Brouwers (1996) first intimated to waste measuring by pinto (1989) and solbeman(1994) and pinto and agopayan(1994). They compared these studies in waste of 11 materials. Next they studied material waste in five building project in Germany in three views:

1. Construction waste of a specific material as percentage of total construction waste,
2. Construction waste of a specific material as percentage of its total amount,
3. Cost of construction waste of a specific material as percentage of total waste costs. (Bossink and Brouwers, 1996)

A study in Malaysia shows, composition and percentage of material wastes: Soil 27%, wood 5%, brick and blocks 1.16%, metal product 1%, roofing material 0.20% and plastic and packaging materials 0.05% concrete and aggregate 65.80% (Begum et al., 2006).

Jones and greenwood (2003) obtained percentage of waste in ten materials as below: plaster board 36%, packaging 23%, cardboard 20%, insulation 10%, timber 4%, cateen waste 20%, chipboard 2%, plastic 1%, electric cable 1%, and rubber 1% (Yahya and Boussabaine,2006).

In another research in 2009, material wastes have been gathered in 100 building material. It contains Earthmoving transport, Concrete reinforcement ,Piles ,Reinforced concrete foundation ,Concrete ,Concrete foundation ,Catch basins ,Collectors ,Downpipe ,Concrete slabs ,Steel reinforcement ,Reinforced concrete ,Wall(chambers) ,Wall(partitions) ,Brick exterior ,Brick interior ,Roof ,Circuits ,Electric lines and derivations ,Light points ,Electric sockets ,Ground connection, Hot water pipes ,Drains ,Cold water pipes ,Tap ,Toilet, basin and bathtub ,Thermos/heaters ,Thermal insulation ,Tiling ,Plaster ,Whitewash ,Screed, Floors, Ceiling ,Finishing ,Steel frames ,Wood doors, Shades ,Glass ,Exterior paints ,Interior paints (Guzman et al., 2009).

2-4) Waste sources

It is important to know the waste sources for applying correct waste minimization methods. There are many studies about waste sources that we mention them here briefly.

Skoyles (1987) makes a distinction between direct and indirect material waste and Gavilan and Bernold(1994) grouped the causes of direct and indirect wastes into six categories, including design, procurement, material handling, operation, residual and others such as theft (Silva and Vithana, 2008).

Bossink and Brouwers (1996), based on Gavilan and Bernold (1994) and Cranen et al. (1994), classified materials wastes to seven categories and

investigated source of them. Finally they combined six sources with seven materials and reached to a table that shows any material with its sources.

Based on this categorization, among great contractors in Singapore waste sources were categorized to four categories and many subcategories. Then were scored in a likert spectrum and finally the rank of any sub category have been found. The three top subcategories in any category are as below:

- Design related:
  1) Design changes while construction is in progress,
  2) Designers' inexperience in method and sequence of construction,
  3) Lack of attention paid to dimensional coordination of products,
- Operational related:
  1) Errors by trades persons or laborers,
  2) Damage to work done due to subsequent trades,
  3) Required quantity unclear due to improper planning,
- Material handling related:
  1) Inappropriate site storage,
  2) Materials supplied loose,
  3) Use of materials which are close to work place,
- Procurement related:
  1) Ordering errors (too much or too little),
  2) Lack of possibility to order small quantities,
  3) Purchases not complying with specifications (Ekanayake and Ofori, 2004).

In another study formoso et al. (2002) extensively studied seven material wastes and their sources. They investigated waste sources for steel reinforcement, Premixed Concrete, cement, Sand and mortar, Bricks and Blocks, Ceramic Tiles, and Pipes and Wires.

In a study in china, a widespread investigation about material wastes has been implemented. A questionnaire survey was conducted to investigate the compositions of these construction waste and their sources. One hundred and ten copies were sent to governmental officers, designers, engineers, and contractors and 84 responses are received. Findings shows concrete, cement, brick, timber, tile, steel, and aluminum wastes are the main waste sources produced on construction sites and the sources of these wastes are varied (Wang et al., 2008).

In another research waste sources investigated and categorized in seven types: 1) Lack of planning 2) unclear information 3) source quality problem 4) late information 5) lack of control 6) sources misused 7) information quality problems (Serpell and Alarcon, 1998).

Design problems in many researches are known as one of waste sources. Designers think often many wastes are because of operation in sites, whereas about one third of wastes are because of design.

Keys et al (2000) explain waste production process in design period is complicated, because of many different materials have used in building and many stakeholders that impact on waste production.

Some researchers (Bossink and Brouwers, 1996; Faniran and Caban, 1998; Chandrakanthi et al., 2002) emphasis that lack of knowledge about construction technique in design process lead to waste production. Many studies (Baldwin et al., 2006; Coventry and Guthrie, 1998; Greenwood, 2003; Poon et al., 2004a) describe that designers and architectures have an important role in waste minimization (Osmani et al., 2008).

3) Methodology

In this research for categorizing of construction materials, their sources of waste across supply chain are used.

From literature review and based on interview with experts, 32 sources of waste identified. Because of research limitations all of them cannot be investigated. Thus they ranked by specialists, by First Step questionnaires, in a five options likert spectrum, and so five top sources of waste selected for more investigations.

Through interviews, 18 most important materials have been gathered and categorized considering their waste production in 12 categories. Then impact of selected sources on material waste can be surveyed. This impact can be calculated by many methods. In this research binominal test are used. Second step Questionnaire also had five options and the question was amount of impact of source on material waste .Options "very low" and "low" impact, had been located in a group, and "mediocre", "high" and "very high" impact, in another group.

Hypothesis have been designed as below:

$$\begin{cases} H0: p \leq 0.60 \\ H1: p > 0.60 \end{cases}$$

H0 shows high level of impact and H1 shows that there is no meaningful impact. The calculations were done by SPSS 15, and with amount of significant validity of questionnaires were tested.

The whole questionnaires were sent to 30 specialists in two steps. Then with analysis of impact of source on material waste categorization will be done.

4) Results and discussion

4-1) First Step Questionnaires

Analysis of data's obtained from questionnaire are presented in this section. In this research questionnaires were sent and gathered in two steps.

First Step Questionnaires Contain respondent's properties and waste sources ranking. Second Step Questionnaires Contain impact of selected sources on material waste.

Every Questionnaires will Analyzed and the results will be used in next steps.

4-1-1) Respondents' properties

The results of Questionnaires show that 73.3 % of respondents were bachelor, 23.3 % were master and 3.3 % were PHD. Also 76.7 % of them were construction engineer, 23.3 % architectural engineer and 10% mechanical engineer, and 23.3% of them had a related background between 20-30 years, 66.7% between 10-20 years and 10% between 3-10 years.

4-1-2) Ranking sources of material waste

From literature review and interviews, 32 sources of material waste were derived.

These sources of waste were ranked by questionnaire, Fig.1 shows the results.. It exhibits that the five sources have the highest rank as below:

1. Traditional construction methods,
2. Lack of design commensurate with materials exist in market,
3. Lack of coordination between supply chain,
4. Lack of proportionate material ordering of purchasing section,
5. Lack of production of material with variant dimensions,
6. Other sources were omitted because of research limitations.

4-2) Second Step Questionnaires

Whereas investigation of all construction materials and their sources of waste are higher than the capacity of this research, by using expert's opinion, some materials which had higher importance in waste, were selected for another steps. Selected materials are: Cement, gypsum, sand, tile, mosaic, ceramic, stone, gypsum board, cement board, shard, brick, block, glass, steel, reinforcement steel, water, paint, and pipe.

Then in this section result from questionnaires were entered to SPSS. It was intended to investigation of impact of sources, so the test proportion was assumed 0.6 and cut point 2.5. This is because of the options "very low" and "low" was in one side and options "average", "high" and "very high" were in another side. Using binominal test for any material a matrix was acquired that shows significants and acceptance of assumptions. Finally Table 2 shows effective sources of waste for any material. All significants are lower than 0.05 and so all of them are valid.

4-3) Analyses of Second Step Questionnaires

Here impact of five below sources on material waste are analyzed.

- Source number1 (S1): lack of design commensurate with materials exist in market,
- Source numbe2 (S2): traditional construction methods
- Source numbe3 (S3): lack of coordination between supply chain
- Source numbe4 (S4): lack of proportionate material ordering of purchasing section
- Source numbe5 (S5): lack of production of material with variant dimensions.

Some new results were achieved with observation of questionnaire as demonstrated below.

- First result: new classification of material based on waste sources, weight based material and dimensional material,
- Second result: presence of source number 3, lack of coordination between supply chain in both categories of materials effective waste sources,
- Third result: presence of source number 3, lack of production of material with variant dimensions, in both categories of materials effective waste sources,
- Forth result: presence of two incongruous sources number 1 and 4 with source number 5 in waste sources of one material, named pipe.

4-3-1) Result 1:

It was achieved that, there are separate effective sources of waste for any kind of materials. In another word in some materials the weight is the most important parameter while using.

In these materials sources number 2, 3 and 4 affect waste production. Also in some other kind of materials the dimensions is the most important parameter while using. In this case the sources number 1, 2, 3 and 5 affect waste production.

When the material is type1, source 1 and source 5 (traditional construction methods, lack of design commensurate with materials exist in market) are not effective on their waste production, and another sources like lack of proportionate material ordering of purchasing section, is effective instead of that. Vice versa if a material be type1, lack of proportionate material ordering of purchasing section is not effective.

Fig 1: Ranking of 32 sources of waste

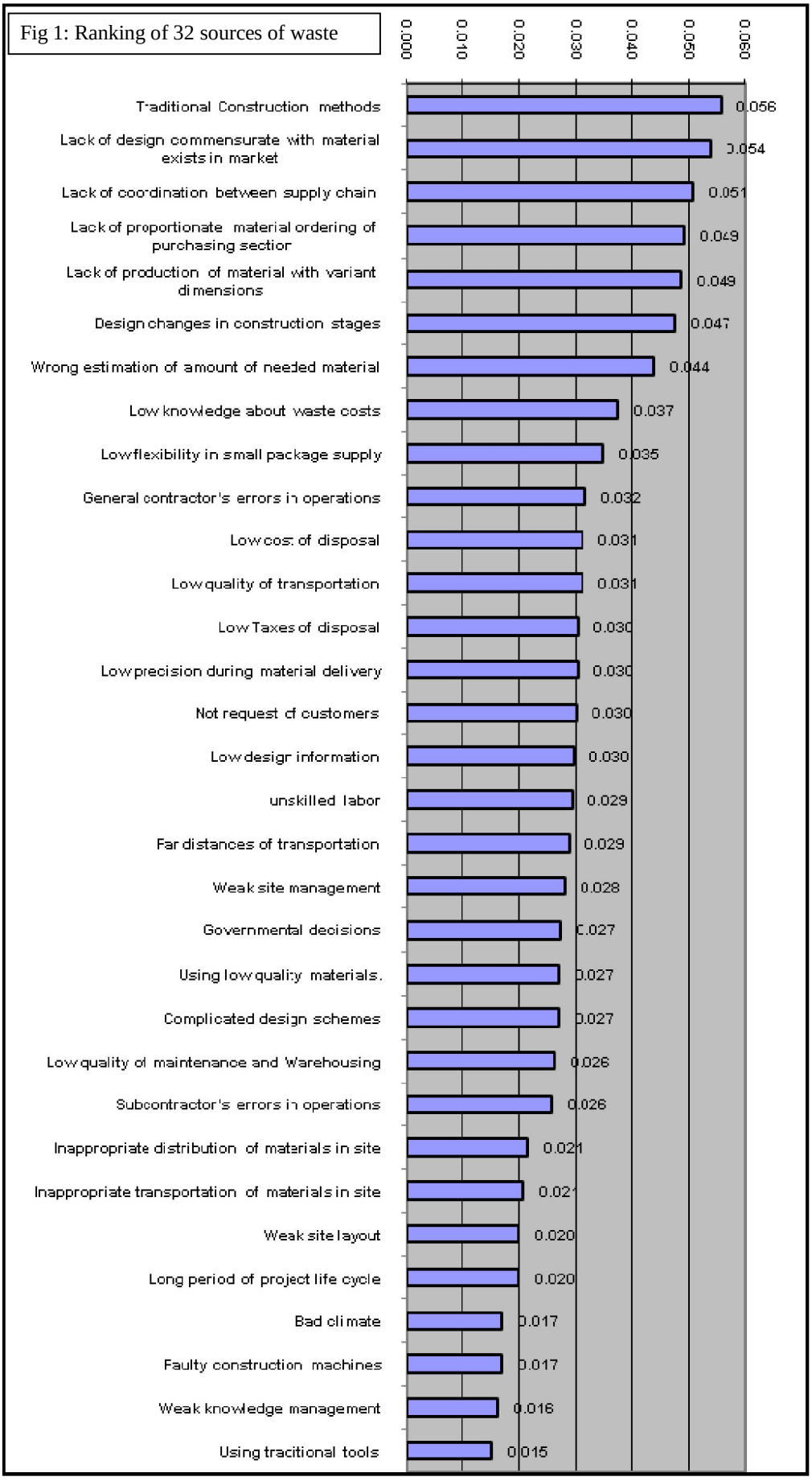


Table1: weight based and dimensional materials

Materials types	Important parameters when use	Effective sources	Non-Effective sources	Measurement units	Example of materials
Weight based materials	Material weight	2,3,4	1,5	kilogram	Cement, gypsum, sand, water and paint
Dimensional materials	Material dimensions	1,2,3,5	4	Meter	Brick, adobe , block, Tile, mosaic, ceramic, stone , steel, reinforcement steel, glass, gypsum board, cement board

Table 2: questionnaire results about Impact of selected sources on waste production of material

When sources is effective in waste production

sources	*Tile, mosaic, ceramic, stone*		*Gypsum and cement board*		*Brick, adobe , block*		*glass*		*steel*		*reinforcement steel*		*cement*		*gypsum*		*sand*		*water*		*paint*		*pipe*	
S1	**.001**	√	**.003**	√	**.000**	√	**.000**	√	**.000**	√	**.000**	√	**.000**		**.000**		**.002**		**.000**		**.002**		**.000**	√
S2	**.000**	√	**.000**	√	**.000**	√	**.000**	√	**.017**	√	**.006**	√	**.000**	√	**.000**	√	**.000**	√	**.006**	√	**.000**	√	.006	√
S3	**.003**	√	**.000**	√	**.000**	√	**.000**	√	**.000**	√	**.000**	√	**.000**	√	**.000**	√	**.000**	√	**.006**	√	**.000**	√	**.000**	√
S4	**.000**		**.002**		**.044**		**.017**		**.006**		**.017**		**.000**	√	**.000**	√	**.000**	√	**.000**	√	**.000**	√	**.000**	√
S5	**.008**	√	**.021**	√	**.000**	√	**.000**	√	**.000**	√	**.000**	√	.044		**.006**		**.006**		**.000**		**.006**		**.003**	√
Effective sources	**1,2,3,5**		**1,2,3,5**		**1,2,3,5**		**1,2,3,5**		**1,2,3,5**		**1,2,3,5**		**2,3,4**		**2,3,4**		**2,3,4**		**2,3,4**		**2,3,4**		**1,3,4,5**	
Non Effective sources	***4***		***4***		***4***		***4***		***4***		***4***		**1,5**		**1,5**		**1,5**		**1,5**		**1,5**		***2***	
Materials type	***Type2***		***Type2***		***Type2***		***Type2***		***Type2***		***Type2***		***Type1***		***Type1***		***Type1***		***Type1***		***Type1***		***Type 1 ,2***	

Table3: questionnaire results about Impact of selected sources on waste production of Pipes

pipes	Important parameter when used	Non-Effective sources Of waste	Effective sources Of waste	Materials type
Looped pipes	dimensions	3,4	1,5	Type1
Branch pipes	dimensions	1,3,5	4	Type2

It is important in explanation of locating two sources 1 and 5 close to each other, that these two sources are inherently similar. These two sources are two side of a subject and their both goal is description of non alliance between design and products of market.

If materials with variant dimensions be available in market, contractors can build any complicated design without any waste. Because they can reach to the design with selection of a module and correspond materials, and therefore there is no need to cutting and breaking materials in end of building items and elements.

In another hand if designer accepts that suppliers cannot produce materials in any variant dimensions because of economies of scale, contractors will not be enforced to do cutting works on material on site. Hence it is emphasized that sources 1 and 2 are supplement.

Thus with these analysis new categorization of material are obtained. The First category is weight base material that sources 2, 3 and 4 affect their waste, and second category is dimensional materials that sources 1, 2, 3 and 5 affect their waste. This categorization can be summarizing in Table 1.

Weight based material are materials like cement, gypsum, sand, water and paint that from contractors view, their weight are important when using them in building, and basically their dimensions are not important in their usage. For example in foundation concreting of a building, A kilogram sand with B kilogram of cement and C kilogram of water should be mixed and never be told A meter of cement or B meter of sand. Because their unit is kilogram not meter and effective parameter of their usage is their weight, not their dimensions.

In a good condition these materials are purchased in pallets or sacks and are transported to site. Often purchaseing department does not buy the required amount of material in the site and its amount is more than need. In this case some part of sacks that are semi used or none used, will be mixed with the site floor soils and will be wasted often as a powder, because of lack of packaging, poor maintenance and traditional mixing methods. Since this act repeat several times in project lifecycle, role of purchasing department is very important in coordination between orders and needs.

In a bad condition that takes place more often in projects, some of these materials are bought in mass volumes, and transported to the site with big trucks and spilled in a corner of site. Unusing materials confidently will be mixed with site floor soils and wasted. Thus source 4 is one of the effective waste sources of weight based material and sources 1 and 5 that are involved with design term are not effective in their waste.

Dimensional materials are materials like brick, shard, block, tile, mosaic, ceramic, stone, steel, reinforcement steel, and glass that in the view of contractors, when using in building, their dimensions are important and their weights don't have significant role. For example in walls it is used bricks with dimensions A*B*C meters and it isn't apply F kilogram brick, because its unit is meter not kilogram and effective parameter of its usage are its dimensions not their weight.

In usage of these materials, contractors will use products available in market and if dimensions and their modules were not accordant with building dimensions, need for cutting will browse. Here two works can be done, production of material with variant dimensions, requisition from designer to design commensurately with market's products. Thus source1 and 5 are effective sources of waste in dimensional material and sources4 are not effective in their waste.

4-3-2) Result 2:

This result is supplement of result1. It was observed that source3, lack of coordination between supply chain, is in two categories of sources of both weight based and dimensional materials. It should be noted that source3 are a general state of sources 1, 4 and 5. In another word when designers don't design commensurate with material available in market; failure happens in coordination between supply chain. This failure itself can be because of lack information, lack of willing or lack of experience and knowledge in coordination of sketch with materials available in market.

In another hand when purchasing section of a project does not coordinate with construction section of project, indeed failure in supply chain has happened. This failure also itself can be due to lack of willing or lack of experience and knowledge or lack of accurate calculation of need amount. Also when producers and suppliers cannot produce any variant dimensions of materials, failure in coordination happens between supply chain, and this failure is itself due to economic of scale. Indeed with above analysis it can be known that source3 is a general concept of all sources 1, 4 and 5.

Source3 is along with source1 and 5 in production of waste in dimensional materials, and contemporary with source4 in production of weight based materials waste. The reason is that respondents believe coordination between design section and suppliers between many coordinations in supply chain, is effective in production of waste inmaterial type2, and coordination between purchasing section and construction section is effective in production of waste in material type1. Thus source3 conceptually are effective in production of both material type1 and.

2. With summering above analysis Fig .2 and 3 will be achieved

There is some important point about Fig.2 and 3: First point is that Fig .2 and 3 shows position of any sources in supply chain, conceptual relation between sources 1 and 5, partition between sources 1 and 5 with source 4, and position of sources 1, 4 and 5 under general source3.

Another point is non-effectiveness of other non-coordination in production of waste.

Another important point is that, source4 have also its supplement like source 1 and 5. Its supplement is miss-approximation of amount of need for materials. This source had seventh rank in production of waste in material and was deleted from interring to another step. Its inducement is construction section. It means that source4 is happen in the side of purchasing section and source7 in the side of construction side.

The final point is that, source1 and 5 are kind of non- coordination between organizations and source1 and 5, are kind of non- coordination among an organization.

4-3-3) Result 3:

Presence of source2 in production of waste in both two material types should be described in other way. This presence is not similar to presence of source3. Presence of source3 was due to generality of that in comparison with sources 1, 4 and 5.

But source2 are effective in waste of all types of materials because of change in the production paradigm. In traditional construction methods amount of pre-engineering is low and often building activities are done in the construction site. But using industrialized methods, many activities are done in factory and building parts are produced in production line and transported to site to install and Montage. In this method project managers can increase portion of pre- engineering and also quality control can be done in factory.

Over above cases, since parts are produced in firm and all of them are distinct where will be used, design Sketch is completely compatible with produced parts. Thus every part are produced by contractor orders and based on design Sketch and are known where it will be used. **(**Jaillon et al., 2009; Silva and Vithana, 2008;Tam, 2008; Tam et al. 2007). In this way there are no needs to lots of adjustments in construction site.

Therefore changing production paradigm from traditional to industrialized construction will hold feasibility of mistake occurrence. Sometimes if it be happened any mistake in production line or in ordering, the part will not be used in that project never, because of non-feasibility of correction or cutting works. In this case all of building part that has produced is waste unless using that in another project and because of cost and transportation problem it is not rational. For example in prefabricated concrete elements there is no chance for correction if there is any misalliance.

Hence until the construction is in traditional paradigm, feasibility of waste production is high in any material. With these analyses presence of source2 among sources of waste in both material types is reasonable.

4-3-4) Result 4:

A new result was presence of source 1, 4 and 5 with source3 in production of waste in one material, named pipe. From result 4 it was shown that S4 is specific for waste production of material type1 and S2 and S5 are specific for waste production of material type2. Question is that how they can be among sources of waste in both material waste sources. The single reasons are that some kinds of pipe are material type1 and some others are type2. With a watchful observation it can be seen that there are two kind of pipe.

Pipes for transmission of water are in loop form in the market. Contractors can cut it in any size. In these pipes there will be no waste because of cutting works and indeed S1 and S5 are ineffective in their waste and just S4 are effective. In another word in purchase section over order, more than need, remain part will be unused and because this ordering will happen so many times in a project, feasibility of control, maintenance and management of these remained part is not easy.

In another word although important parameter in usage in these pipes are their dimensions, but because their waste sources is S4 and S1 and S5 are not effective, it will be material type1.

In another hand Pipes for transmission of sewerage and city gas are in branch form in the market. If designer does not design commensurate with existing materials in market or there are not variant materials in market, Contractors should cut pipes. In this kind of pipes remained parts of cutted pipes are waste. It cannot be used again because of technical consideration and leakage. So S1, S5 are effective waste sources thus branch pipes because of important parameter in usage of them are their dimensions, and because their waste sources is S1 and S5, and S4 are not effective, it will be material type2.

Also S3 is a generality state of S1, S4 and S5 then it will be among effective sources. By these analysis it will known that why S1 and S5 are contemporary waste sources of pipes. Table 3 summaries these analyses.

5) Conclusions

Construction wastes have a great spread, and any of them have its source . To recognize that sources, impact of selected sources on waste of selected

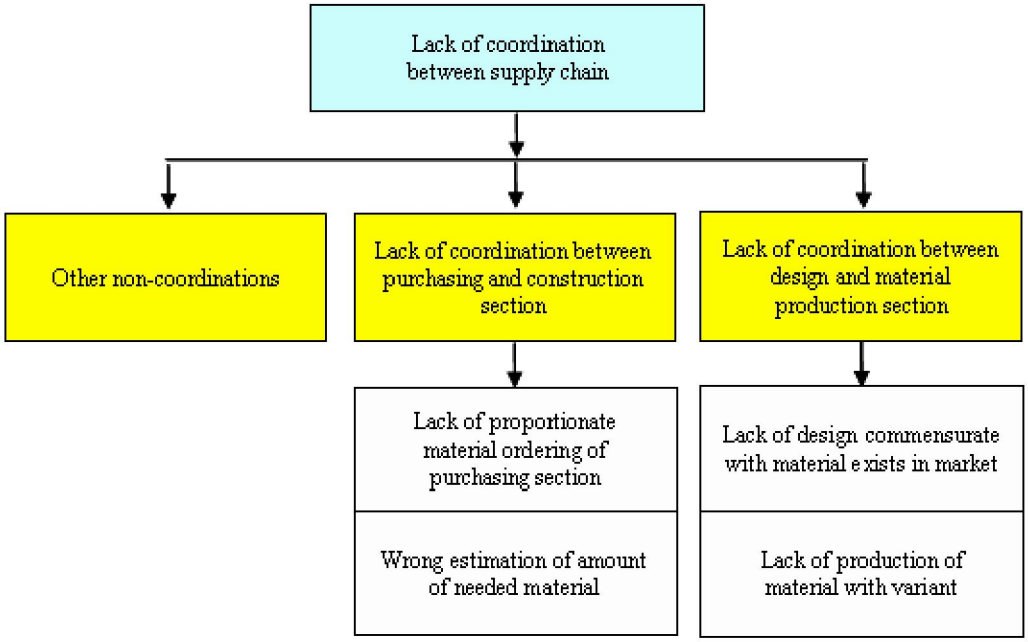


Fig. 2: non-cooardinations between supply chain

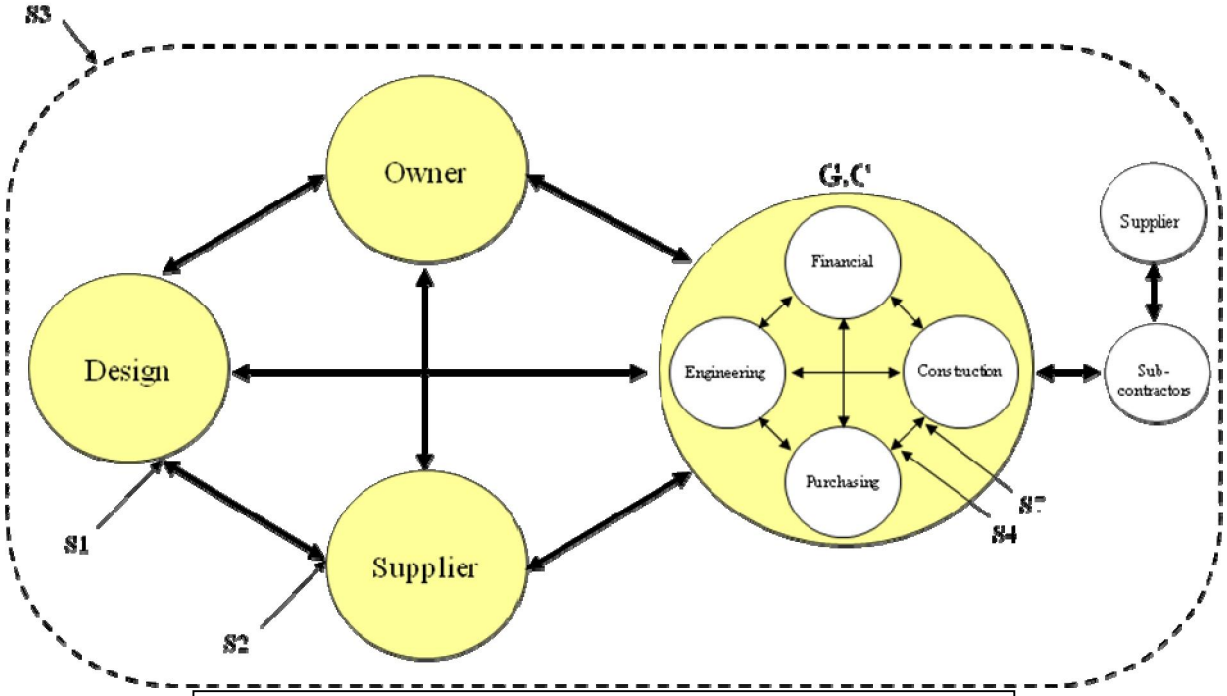


Fig.3: Position of waste sources in a typical construction supply chain

materials were investigated. Some new results were yielded. First it was clarified that materials have their specific waste sources based on their inherent properties.

Materials inherent properties are such as, methods of usage, important parameters when use, how to supply and how to maintain, measurement units, that impact on process of row material conversion to final product and therefore impact on methods that wastes are produced in any material.

With investigation of questionnaire it was identified that those material which their dimensions are important in their usage, some sources are effective in their waste that related to building design. In these materials if there were no coordination between design and materials exist in market, it will lead cutting in dimensional non-coordinations and thus waste will be produced. Whereas dimensions are the main aspect of inherent properties in these materials, their names will be dimensional materials.

Also in weight based materials, that their weight is important in their usage, some sources are effective in their waste that related to amount of purchasing. In this materials if exist any non-coordination between site need and amount of purchasing material, because of remaining material and repetition of this act in project life cycle, and lack of protection of material in site, it result in waste production. Whereas weight is the main aspect of inherent properties in these materials, their names will be weight based material. Non-coordinations between supply chain, cover both

Non- coordination between design and materials exist in market, and Non-coordination between site need and amount of purchasing material, it is effective in waste production of both two types of materials. Using traditional construction methods in contrast with industrialized methods is effective in both two types of materials too. If construction managers change their paradigm to industrialized methods, because of non-feasibility of errors in design and amount of purchasing, the feasibility of waste production will reach zero.

Acknowledgements

The authors like to thank Mapsa Company for providing relations between us and practitioners to contribute them through questionnaire and Interview. We would particularly like to acknowledge all practitioners and specialists that help us in making a good framework for data gathering and their rapid responses to questionnaires.

We also gratefully thank the comments from Dr.Jahangir Darvish and Ahmad Ghanaatian for their helpful comments in the research process.

Authors:

Mohamad Reza Parsanejad,
Mansor Momeni, Ahmad Jafarnejad, Ali Mohaghar

Department of Industrial Management,
Faculty of Management,
University of Tehran
parsanejad@ut.ac.ir

References

1. Begum, R.A., Siwar, C., Pereira, J.J., Jaafar, A.H.A., 2006.Benefit–cost analysis on the economic feasibility of construction waste minimization: The case of Malaysia. Sources, Conservation and Recycling, 48, 86–98.
2. Begum, R.A., Siwar, C., Pereira, J.J., Jaafar, A.H.A., 2007.Implementation of waste management and minimization in the construction industry of Malaysia. Sources, Conservation and Recycling, 51, 190–202.
3. Bossink, B.A.G., Brouwers, H.J.H., 1996. Construction waste quantification and source evaluation. Journal of Construction Engineering and Management, March, 55.
4. Chun, L., Scorpio, D., Kibert, C., 1997. Strategies for successful construction and demolition waste recycling operations. Construction Management and Economics.15:49–58.
5. Ekanayake, L.L, Ofori, G., 2004. Building waste assessment score: design-based tool. Building and Environment 39, 851–861.
6. Fishbein, B., 1998. Building for the future: strategies to reduce construction and demolition waste in municipal projects, Vol.5, No.1, 102 pp.
7. Formoso, C.T. Franchi, C., Soibelman, L., 1993. Developing a method for control terial waste on building sites. Economic Evaluation and the Vironment, CIB, Lisbon, Portugal.
8. Formoso, C.T., Soibelman, L., Cesare, C., Isatto, E.L., 2002. Material waste in Building Industry: Main Causes and Prevention. Journal of Construction Engineering and Management, July/August.
9. Gavilan, R.M., Bernold, L.E., 1994. Source evaluation of solid waste in building construction. Journal of Construction Engineering and Management, ASCE 120(3), 536–552.
10. Guzman, J.S., Marrero, M., Delgado, M.V.M., Arellano A.R.A., 2009. Spanish model for quantification and management of construction waste. Waste Management, 29, 2542–2548.
11. Hamassaki, L.T., Neto, C.S., 1994. Technical and econo pects of construction / demolition waste utilization. Sustainable structure (proc. 1st conf of

CIB TG 16), construction and environment, Gainesville Fla., 395-403.
12. Jaillon, L., Poon, C.S., Chiang, Y.H., 2009. Quantifying the waste reduction potential of using prefabrication in building construction in Hong Kong. Waste Management 29, 309–320.
13. Kang, Y. Wastage in bricks. Dissertation, National University of Singapore, unpublished.
14. Katz, A., Baum, H., 2010. A novel meth`odology to estimate the evolution of construction waste in construction sites. Waste Management.
15. McDonald, B., Smithers, M. Implementing a waste management plan during the construction phase of a project: a case study. Construction Management and Economics 1998; 16:71–8.
16. Omrani, G., karbasi, A., Monavari, S.M., Usefi, N., 2008. Investigation of construction wastes – case study: tehran city. Second Conferences of Waste Management and Its Position in Municipal Management.
17. Osmani, M., Glass, J., Price, A.D.F .Architects, 2008. Perspectives on construction waste reduction by design. Waste Management, 28, 1147–1158.
18. Pohjola, V.J., 2006. Formalizing waste management. Modern Formal Methods and Applications, 47–82. , Springer, Printed in the Netherlands.
19. Report of environmental committee of consoling Tehran city, 2008.
20. Report of material section of construction and housing research center, 2008.
21. Report of Tehran municipal recycles organization, 2008.
22. Serpell, A., Alarcon, L.F., 1998. Construction process improvement methodology for construction projects. International Journal of Project Management, Vol. 16, No.4, pp. 215-221.
23. Silva, N.D., Vithana, S.B.K.H., 2008. Use of PC elements for waste minimization in the Sri Lankan construction industry, Structural Survey, Emerald Group Publishing Limited., Vol. 26 No.3, pp. 188-198.
24. Skoyles, E.R, Skoyles, J.R., 1987. Waste prevention on site. London: Mitchell.
25. Tam, V.W.Y., 2008, On the effectiveness in implementing a waste-management-plan method in construction. Waste Management, 28, 1072–1080.
26. Tam, V.W.Y., Tam, C.M., Zeng, S.X., Ng, W.C.Y., 2007. Towards adoption of prefabrication in construction. Building and Environment 42, 3642–3654.
28. Yahya, K., Boussabaine, A.H., 2006. Eco-costing of construction waste. Management of Environmental Quality: An International Journal, Vol. 17 No. 1, pp. 6-19.

11/19/2010

Effect of miso (A soybean fermented food) on some human cell lines; HEPG2, MCF7 and HCT116

Abeer Abu Zaid [1, 2] and Nahla S. El-Shenawy[2,3*]

[1]Food Technology Research Institute, Agriculture Research Center, Giza, Soy product Processing Center, Egypt.
[2]Biology department, Faculty of Science, Taif University (Qurwa), Taif, Saudi Arabia.
[3]Zoology Department, Faculty of Science, Suez Canal University, Ismailia 41522, Egypt
elshenawy_nahla@hotmail.com

Abstract: The study was conducted to investigate the antitumor activity of miso, storage at different period or prepared with different starters, on human cell lines {HEPG2 (liver carcinoma), MCF7 (breast carcinoma), and HCT116 (colon carcinoma)}. The highest inhibitory effect on liver and breast carcinoma was seen when miso used after fermentation/aging zero time without storage period. Miso with different storage period (zero, 6 months and 5 years) has the same effect on colon carcinoma. Preparation of miso with different mixture of starters was also investigated on the same human tumor cell lines in culture. Miso prepared with *A. oryzae* and *Bacillus subtilis* starters inhibited the proliferation of human tumor cell lines culture with a wide variation in LC_{50} values (2.97, 3.37 and 3.37 µg/ml for MCF7, MCT116 and HEPG2, respectively). Miso prepared with *Aspergillus oryzae* and *Pleurotus ostreaus* starters inhibited human tumor cell line cultures with different LC_{50} values (10.9, 17.5 and 24.3 µg/ml for MCF7, MCT116 and HEPG2, respectively). The miso prepared with *A. oryzae* and *Rhizopus oryzae* effect only on MCF7 and HEPG2 with high LC_{50} values (25.5 and 35.8 µg/ml, respectively). We can conclude that the mixture of *A. oryzae* and *Bacillus subtilis* has the best effect among the other mixture of starters. The results indicated that all of fermented soybeans products with different mixture of starters contained higher isoflavones compounds than unfermented cooked soybeans. Moreover, soybean fermented with *B. subtilis* showed highest amount of isoflavones. Therefore, miso can be used as anticancer.

[Abeer Abu Zaid and Nahla S. El-Shenawy. **Effect of miso (A soybean fermented food) on some human cell lines; HEPG2, MCF7 and HCT116**. Journal of American Science 2010;6(12):1274-1282]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Miso; Human cell lines; Amino acid; Fatty acid; Isoflavones; *Aspergillus oryzae*; *Bacillus subtili; Rhizopus oryzae*; *Pleurotus ostreaus*.

1. Introduction

Soybeans (*Glycine max* (L.) Merr.) are highly regarded as a healthy food in several Asian countries and are widely consumed as soymilk, tofu and fermented products. Fermented soy foods, such as *paste*, are important components of the Korean diet in much of the world. *Soy paste (Doenjang)* has been traditionally manufactured from *Koji*, which is a fermented rectangular block of crushed cooked soybeans. The primary microorganisms involved in *Koji* fermentation are *Bacillus subtilis* and molds such as *Rizopus*, *Mucor*, and *Aspergillus* species (Park and KO 2005; Dajanta et al., 2009). Soy paste fermented with various filamentous fungi including *Aspergillus awamori, Aspergillus oryzae, Aspergillus sojae, Rhizopus azygosporus and Rhizopus sp.* lead to the increased antioxidant activities, total extractable phenolic compounds and anthocyanins content after fermentation (Lee et al., 2007; 2008). Fermentation of soybean and other ingredients by microbial starters is the most important step during manufacturing of soybean fermented products. During fermentation and due to excretion of hydrolytic exoenzymes by microbial starters, precursors such as phenolic compounds (genistin and diadzine) are converted by β- glucosidase into more biologically active forms of isoflavones such as genistein and daidzein (Hsiang et al., 2005). Protein and fats are also converted into amino acids and bioactive peptides, and fatty acids by proteases and lipases respectively (Dziuba et al., 2003).

In the past several years, soy and its constituents have garnered considerable attention, from both

researchers and health practitioners. Epidemiological data which indicated people from Asian cultures have lower rates of certain cancers, including cancer of the breast, prostate and colon, sparked an interest in soy as a contributing factor. Numerous epidemiological, human, animal, and *in vitro* studies have demonstrated that soy isoflavones are effective chemopreventive agents for certain types of cancer (Head, 1997). *In vitro*, high concentrations of the isoflavone genistein inhibit most types of cancer cells and some animal studies suggest that genistein inhibits metastases (Menon et al., 1998). Further controlled human trials are needed to confirm the preliminary findings reported in these studies. Jung et al. (2006) reported that miso could prevent cancer by decreasing tumor formation and increasing natural killer cell activity in spleens. Miso goes a long way towards providing people with their daily needs for the trace minerals zinc, manganese and copper. Miso has antioxidant properties and it has the ability to decrease the lipid peroxidation (El-Shenawy and Abu Zaid, 2011).

In the present study, we have tested the effect of miso that prepared with single starter (*Aspergillus oryzae*) and stored for different period (Zero, 6 months and 5 years) on three human tumor cell lines. In addition, the effects of three different mixture of starters were examined on cell proliferation of the same cell lines [HEPG2 (liver carcinoma), MCF7 (breast carcinoma) and HCT116 (colon carcinoma)].

2. Materials and Methods

2.1 Microorganisms and Media

The microorganisms included in this investigation are commonly used as starters in the fermentation of many traditional, oriental food products. The mold *Aspergillus oryzae*, is used to make traditional doenjang, a Korean fermented soy paste (Kim et al., 2009), *Rhizopus oryzea*, used in preparation of temper, Indonesian fermented food (Haron et al., 2009) and the bacterium *Bacillus subtilis* was found among the microbial community in doenjang (Yoo et al., 1999). Potato dextrose agar was used for growth and maintenance of all mold strains, and nutrient agar medium was used for the bacterium *Bacillus subtilis.*

2.2 Preparation of miso

Miso was prepared in two major steps. In the first step, microbial starters were prepared as follows: wheat bran seeded with *Aspergillus oryzea* fungi as singly at approximately 1-2 X 10^6 cfu/g. Then, it was spread at the surface of a layer of 20 Kg of boiled and steamed wheat at a ratio of 1% and incubated at 25 ºC for 3 days in order to encourage growth and multiplication of microbial starters. Secondly, the growing starter was added to 40 Kg of boiled and steamed soybean mixed thoroughly in presence of 16% salt and moisture content was controlled at approximately 12.5%. The resulting paste represents the miso at zero time. If the paste was stored in jars for fermentation and aging, the resulting miso represents 6 months or more (ISO, 2006). During all the experiment, fermentation/aging for 6 months has been used except for antitumor activity experiment; three different times of fermentation/aging [zero (1), 6 months (2) and 5 years (3)] have been investigated. Miso was also prepared with different mixture of starters; *Aspergillus oryzae* and *Pleurotus ostreaus* (4) or *A. oryzae* and *R. oryzae* (5) or *A. oryzae* and *B. subtilis* (6) (Abu Zaid *et al.* 2010). The bacteria were routinely cultured on nutrient agar and their stock cultures were maintained at -80°C in 20% glycerol. For inoculums preparation, the bacteria were grown in nutrient broth at 37°C for 24 h. The cells were then harvested, re-suspended in sterile distilled water and properly adjusted to obtain a concentration of 10^4 CFU/mL. The suspension was served as the inoculums for soybean fermentation.

2.3 Determination of miso total fatty acid and amino acids

Gas liquid chromatography was applied to identify the fatty acids present in different types of miso. Lipids were extracted by a modified method described by Xu and Beardall (1997). Amino acid determination was performed according to the method of the Official Journal of the European Communities 19-9-98 (AOAC, 1995).

2.4 Determination of miso Isoflavones

The method described by Coward et al. (1993) was used after modification. After freeze drying, samples were extracted using 80% aqueous methanol (10 ml/g) and stirred for 1.0 h at 60 °C. The mixture was centrifuged at 2500 g for 10 min and the supernatant was decanted into a round bottom flask. The pellet was re-suspended in 10.0 ml of 80% aqueous methanol and centrifuged. Then, both

supernatants were combined and taken to dryness using rotary evaporator. Then, dried extract was re-dissolved in 5.0 ml of 50% aqueous methanol, and the lipid fraction was removed by partitioning in hexane (4 X 20 ml). The aqueous methanol phase was evaporated to dryness using rotary evaporator and the dried residue dispersed in 10 ml of 80% aqueous methanol. The mixture was centrifuged using an eppendorfed by microfuge just prior to analysis by HPLC. Separation of isoflavones was achieved by high-performance liquid chromatography (HPLC) on a 30 cm x 0.45 cm, Aquapore C8 reversed – phase column with mobile phase consisting of a gradient of 0- 46.4% acetonitril in 0.1 % (v\v) aqueous trifluoracetic acid at flow rate of 1.5 ml/min. The eluting components were detected from their absorbance at 262 nm. Concentrations of the isoflavones were calculated from standard curves of area responses for authentic isoflavones standards normalized to the constant amount of fluorescein as internal standard was added to each sample.

2.5 Antitumor activities of miso on human cell lines

Antitumor activity of a colorimetric cytotoxicity assay (Skehan et al., 1990). Cell lines: HEPG2 (liver carcinoma), MCF7 (breast carcinoma) and HCT116 (colon carcinoma) were harvested from exponential phase cultures by trypsinization, counted and plated as cell monolayer in 96-multiwell plate (10^4 cells/well) for 24 h. Cells were cultured in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal calf serum (FCS) and 1 mM L-glutamine. Then different concentrations (0, 1, 2.5, 5 and 10 µg/ml in DMSO for HEPG2 and MCF7) or (0, 5, 12.5, 25, and 50 µg/ml in DMSO for HCT116) of miso after fermentation/aging for zero, 6 months and 5 years were added to the wells in triplicate. After 48 h of incubation, cells were fixed, washed and stained with Sulfo-Rhodamine-B (SRB) stain. Excess stain was washed with acetic acid and the attached stain was recovered with Tris–EDTA buffer. Color intensity was measured in an ELISA reader. The relation between survival fraction and extract concentration was plotted to give the survival curve of each tumor cell line after the specified dilution of the extract, and the LC_{50} was calculated.

2.6 Statistical analysis

Results are expressed as mean ± SE, and were obtained from at least 5 samples. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the Bonferroni's *post-hoc* test (level of significance; $P \leq 0.05$) using SPSS version 15, statistical program (Hill, 1971).

3. Results

3.1 Total fatty acid, total amino acid and total isoflavones

The highest level of fatty acid and total amino acid (Table 1) were detected in the miso that prepared with *A. oryzae* and *R. oryzae* (5) (102.55 ± 7.29 and 30.2 ± 0.27, respectively). However, the highest level of total isoflavones was determined in miso that prepared with *A. oryzae* and *B. subtilis* (6). The concentration of isoflavones in unfermented soybean was too low as compared with all other types of miso.

3.2 Effect the storage time of miso on human tumor cell lines

The survival fraction of HEPG2 and MCF7 cell line was plotted against the different concentrations (1-10 µg/ml) of miso with different storage period (0, 6 months, 5 years). The concentrations of miso that reduced survival of carcinoma cell line of liver (HEPG2) to 50% were 1.96, 2.88 and 3.5 µg/ml for zero, 6 months and 5 years of fermentation, respectively (Fig. 1). The concentrations of miso that reduced survival of breast (MCF7) carcinoma to 50% were 0.982, 1.3 and 1.14 µg/ml for zero, 6 months and 5 years, respectively (Fig. 2). The highest inhibitory effect on liver and breast carcinoma was seen when miso used immediately after fermentation (zero time). However, the survival fraction of carcinoma cell line of colon (HCT116) was plotted against the different concentrations (5-50 µg/ml) of miso (Fig. 3). Effect of different storage periods of miso on colon carcinoma was approximately the same; 14.4, 13.0 and 14.3 µg/ml for zero, 6 months and 5 years of fermentation, respectively.

3.3 Effect of different methods of miso preparation on human tumor cell lines

The survival fraction of MCF7, MCT116 and HEPG2 cell line was plotted against the different concentrations (5-50 µg/ml) of miso that prepared with different mixture of starters. The concentrations

of miso that reduced survival of breast (MCF7) carcinoma to 50% were 2.97, 10.9 and 25.6 µg/ml for 6, 4 and 5, respectively (Fig. 4). The concentrations of miso that reduced survival of carcinoma cell line of liver (HEPG2) to 50% were 3.73, 24.3 and 35.8 µg/ml for 6, 4 and 5, respectively, (Fig. 5). The highest inhibitory effect on liver and breast carcinoma was seen when miso prepared using mixture of *A. oryzae* and *B. subtilis* (6). However, effect of different preparations of miso on colon carcinoma was 3.73 and 17.5 for 6 and 4, respectively. The miso prepared by *A. oryzae* and *R. oryzae* (5) did not effect on the HCT116 (Fig. 6).

Table 1: Effect of different starters on total fatty acid, total protein and total isoflavones of miso

Miso starters	Total fatty acids (mg/ 100g)	Total amino acids (%)	Total isoflavones (mg/100g)
Raw materials (unfermented soybean)	----	----	71.46 ± 6.5
Aspergillus oryzae and *Pleurotus ostreaus* (4)	93.88 ± 6.66*,**	28.6 ± 0.23	571.02 ± 5.12**
A. oryzae and *R. oryzae (5)*	102.55 ± 7.29***	30.2 ± 0.27***	562.73 ± 4.95***
A. oryzae and *B. subtilis* (6)	77.24 ± 4.3	26.52 ± 0.24	1090.95 ± 9.69

The data presented as mean ± SE (n=5). Statistical analyses were performed with SPSS version 11.5 (SPSS Institute, Chicago, IL). One-way analysis of variance (ANOVA) was used to assess the statistical significance of the continuous variables. --- Not detected. $^{*}p < 0.01$, (4) compared with (5). $^{**}p < 0.05$, (4) compared with (6). $^{***}p < 0.05$, (5) compared with (6).

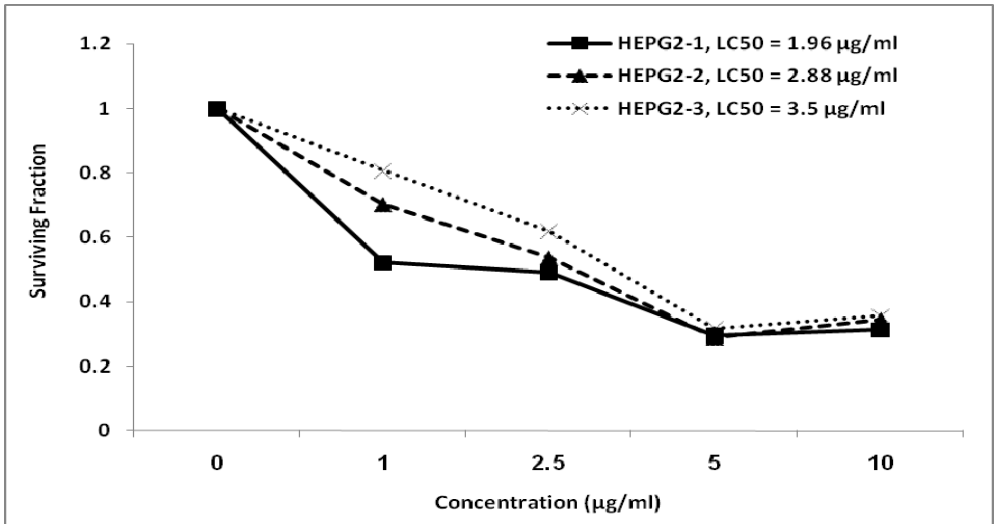


Figure 1. Potential antitumor activities of miso on human cell lines HEPG2 (liver carcinoma), with LC_{50} value denoted on curve. HEPG2-1, HEPG2-2 and HEPG2-3 represent fermentation/aging of miso for zero, 6 months and 5 years, respectively.

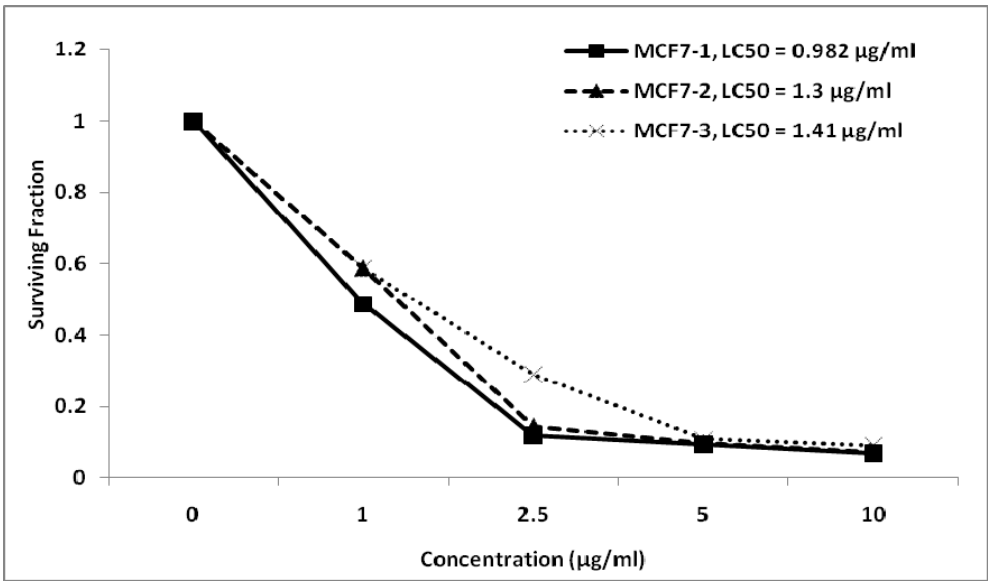


Figure 2. Potential antitumor activities of miso on human cell lines MCF7 (breast carcinoma), with LC_{50} value denoted on curve. MCF7-1, MCF7-2 and MCF7-3 represent fermentation/aging of miso for zero, 6 months and 5 years, respectively.

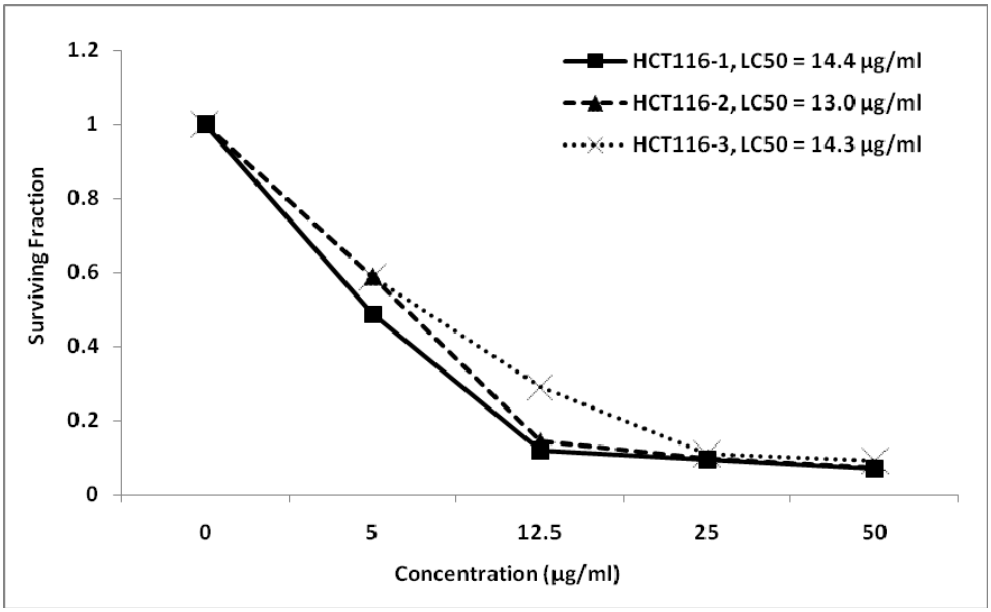


Figure 3. Potential antitumor activities of miso on human cell lines HCT116 (colon carcinoma), with LC_{50} value denoted on curve. HCT116-1, HCT116-2 and HCT116-3 represent fermentation/aging of miso for zero, 6 months and 5 years, respectively.

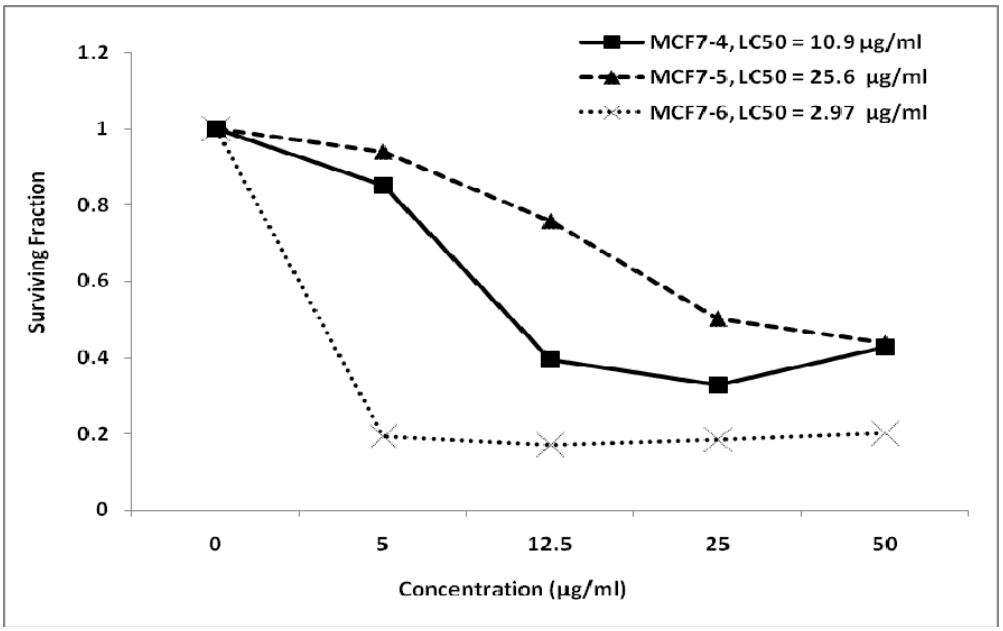


Figure 4. Potential antitumor activities of miso on human cell lines HEPG2 (liver carcinoma), with LC_{50} value denoted on curve. HEPG2-1, HEPG2-2 and HEPG2-3 represent miso for 8, 2 and 4, respectively.

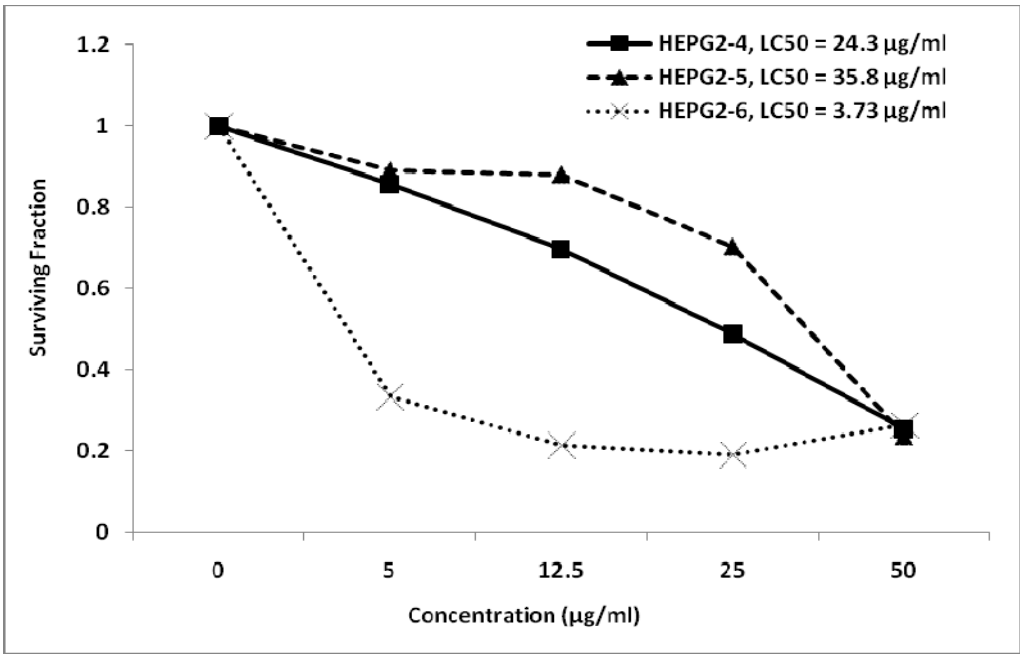


Figure 5. Potential antitumor activities of miso on human cell lines MCF7 (breast carcinoma), with LC_{50} value denoted on curve. MCF7-1, MCF7-2 and MCF7-3 represent fermentation/aging of miso for zero for 8, 2 and 4, respectively.

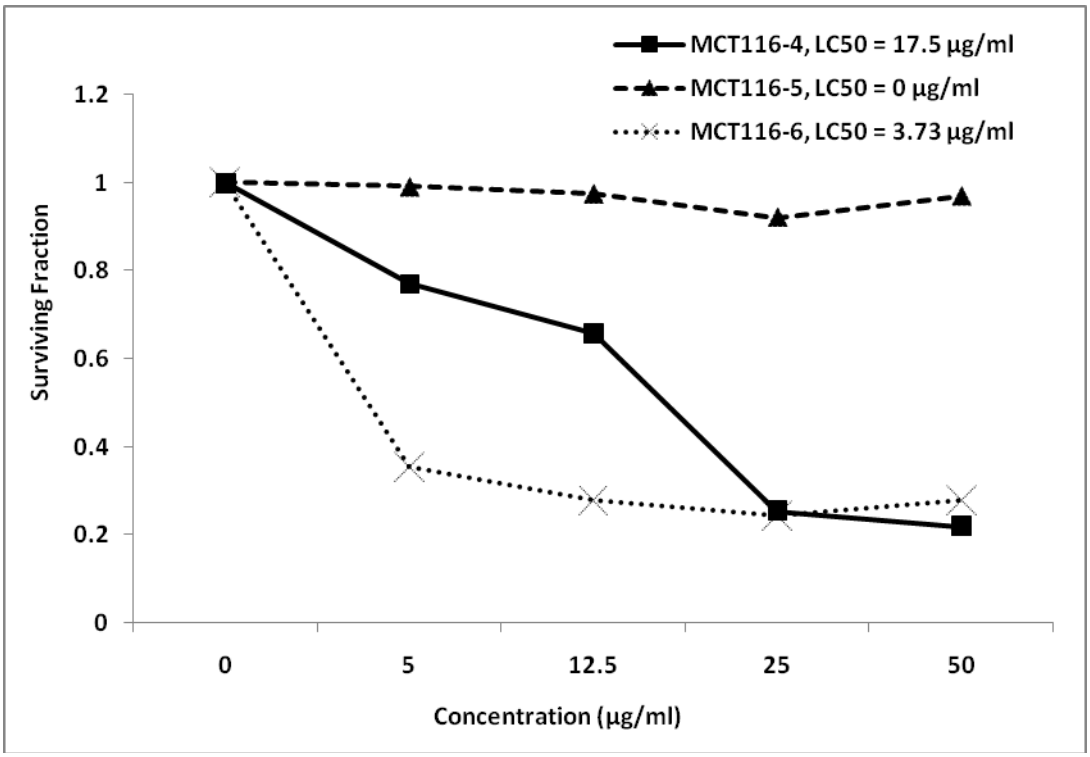


Figure 6. Potential antitumor activities of miso on human cell lines HCT116 (colon carcinoma), with LC_{50} value denoted on curve. HCT116-1, HCT116-2 and HCT116-3 represent fermentation/aging of miso for 8, 2 and 4, respectively.

4. Discussion

This study describes the *in vitro* antitumor activity of Miso, a fermented soybean food, on liver, breast and colon cancer human cell lines. Different types of miso, prepared with three starter culture mixtures and of different fermentation periods, were evaluated and their fatty acid, amino acid and isoflavone contents were determined. Finally, the effect of different miso on the inhibition of proliferation of cancer cell lines was studied.

Traditionally fermented soybeans contained higher concentration of Isoflavones than unfermented cooked soybeans. However, *B. subtilis* fermented soybeans appear to be
a better source of bioavailable soy isoflavones as it has been reported (Kano et al., 2006; Dajanta et al., 2009**).** Miso may be of particular interest for using as antitumor agents against liver, breast and colon carcinoma. The present finding indicates that miso storage for different period were able to inhibit the liver, breast and colon carcinoma. Miso has the ability to inhibit the carcinoma of liver and breast with low concentration at zero time (without storage period) after preparation. However, there is no significant different in the LD_{50} of miso that effect on colon carcinoma by using different storage fermentation period. However, Jung et al. (2006) demonstrated that prolonging the fermentation period (24 months) when making soy paste increases its antitumor effects *in vivo*. The longer storage period of miso over three years led to increase isoflavones concentrations (Yamabe et al., 2007). The time course of the isoflavone composition during the fermentation/aging process of rice-koji miso indicated that glycosides decreased from 86.4% to 44.9% after 6 months but aglycones increased from 9.6% to 53.3% (Yamabe et al., 2007). Soy

isoflavones have also been reported to reduce the risk of prostate cancer by acting as anti-cancer agents blocking the growth of hormone dependents cancers (Heald et al., 2007).

The present study proved that fresh prepared miso in better than miso after storage periods against liver and breast cancer. This result confirmed by the observation of Weed et al., 1985 and Park et al., 2003. They reported that the cooked soybeans showed less inhibition of the mutagenicity than raw soybeans, probably due to the destruction of the trypsin inhibitor by heat treatment (Weed et al., 1985). Park et al. (2003) also noted a marked antimutagenic activity in doenjang, For example, miso, tempeh and natto, fermented soybean products prepared with A. oryzae, showed an antimutagenic activity 2.5-fold more than that of unfermented milk. Soybeans inhibit mammary tumors in models of breast cancer. The lower antimutagenic activity of miso is probably due to the smaller portion of soybeans used and short fermentation period (Park et al., 2003).

In this study, the relationship between two types of starts that could be bacteria or fungi was evaluated for preparation of miso. Human cell lines have not been used as an *in vitro* model for studying the potency of miso that prepared with mixture of starters as anticancer. There is no data concerning the influence of miso on the cancer human cell lines MCF7, MCT116 and HEPG2 and for this reason it is very difficult to compare our results with those reported by other authors. Miso was prepared from *A. oryzae* and *B. subtilis* (6) has more potent effect on human tumor cell line MCF7 and reached almost plateau level at all tested concentrations (5-50 µg/ml), causing 50% cell death. There was a dose-dependent relationship of increased number of dead cells (MCF7) with increasing concentration of *A. oryzae* and *R. oryzae* (5). The concentration of miso that prepared with mixture of starters (6) has the most potent effect of the human cell lines HEPG2 and MCT116 as compared to other mixtures (4) and (5). These results could be due to the highest level of isoflavones that detected in miso that prepared with the mixture (6). This present study suggests a promising use of *Bacillus* starter cultures in improving isoflavone compounds especially the isoflavones which would benefit for novel functional food development. This result is in agreement with Dajanta et al. (2009).

We can conclude that miso decreased growth of various human cancer cells and its effect depends on the storage time of miso and the type of the starters. The results indicated that isoflavones suppressed tumor growth of the human cell lines *in vitro*. These results suggest that these different types of miso could induce inhibition of human cell lines in dose-dependent manner.

Corresponding author:

Prof. Nahla S. EL-Shenawy
Zoology Department
Faculty of Science, Suez Canal University
Ismailia 41522, Egypt.
E-mail: elshenawy_nahla@hotmail.com

References

1. Park KY, Jung KO. Fermented soybean products as functional foods: functional properties of doenjang (fermented soybean paste). In: Shi J, Ho CT, Shahidi F (eds.). Asian functional foods. Boca Raton: CRC Press (2005); pp. 555–596
2. Dajanta K, Chukeatirote E, Apichartsrangkoon A, Frazier RA. Enhanced aglycone production of fermented soybean products by *Bacillus* species. Acta Biol Szeged (2009);53(2): 93-98.
3. Lee YW, Kim JD, Zheng J, Row KH. Comparisons of isoflavones from Korean and Chinese soybean and processed products. Biochem Eng J (2007); 36:49-53.
4. Lee I-H, Hung Y-H, Chou C-C. Solid-state fermentation with fungi to enhance the antioxidative activity, total phenolic and anthocyanin contents of black bean. Intern J Food Microbiol (2008);121(2):150-156
5. Hsiang YY, Nan WS, Min HL. Chemical composition and physicochemical properties of the fiber rich materials prepared from shaya mash residue. J Agric Food Chem (2005); 53(11):4361- 436.
6. Dziuba J, Iwaniak A, Minkiewicz P. Computer-aided characteristics of proteins as potential precursors of bioactive peptides. Polimery (2003);48(1): 50-53.
7. Head KA. Isoflavones and other soy constituents in human health and disease Alt Med Rev (1997);2(6):433-450.

8. Menon LG, Kuttan R, Nair MG, Chan YC, Kuttan G. Effect of isoflavones genistein and daidzein in the inhibition of lung metastasis in mice induced by B16F-10 melanoma cells. Nutr Cancer (1998); 30: 74–77.
9. Jung KO, Park SY, Park KY. Longer aging time increases the anticancer and ant metastatic properties of doenjang. J Nutr (2006); 22: 539-545.
10. El-Shenawy NS, Abu Zaid AA. Cytotoxicity and antioxidant effect of miso on male rat. Parlar Sci. Pub. Advan.Food Sci (2011); In press.
11. Kim WT, Lee HJ, Kim ES, Park HM, Chang HC, Kim YH. Analysis of microbial communities in doenjang, a Korean fermented soybean paste, using nested PCR-denaturing gradient gel electrophoresis. Intern J Food Microbiol (2009);131(2-3): 265-271.
12. Haron H, smail A, Azlan A, Suzana S, Peng S. Daidzein and genestein contents in tempeh and selected soy products. Food Chem (2009);115:1350–1356.
13. Yoo SK, Cho WH, Kang SM, Lee SH. Isolation and dentification of microorganisms in Korean traditional soybean paste and soybean sauce. Korean. J Appl Microbiol Biotechnol (1999); 27:113–117.
14. ISO (2006) International Standard Organization. Microbiology of food and animal feeding.
15. Abu Zaid AA, Al-Salmy F, Amine G. Influence of various microbial starters on bioactive and bioavailability compounds in miso, a Japanese traditional soybean fermented food. J Agricul Food Chem,(2010); In press.
16. Xu X, Beardall J. Modification of fatty acid composition in halophilic anthracitic microaalgea. Photochemistry (1997);49:1249-1252.
17. AOAC (1995) Official Methods of Analysis of the Association of Official analytical chemists " (16 th ed). Association of Official Analytical chemists. Virginia, USA.
18. Coward L, Barnes NC, Setchell KDR, Barnes S. Genistein and daidzein, and their -glycoside conjugates: anti-tumor isoflavones in soybean foods from American and Asian diets. J Agric Food Chem (1993); 41**:**1961-1967.
19. Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenney S, Boyd MR. New colorimetric cytotoxicity assay for anti-cancer drug screening. J Natl Cancer Inst (1990); 82:1107–1112.
20. Hill AB. Principles of Medical Statistics. 9th ed., Oxford Univ. Press, Oxford, UK. (1971).
21. Kano M, Takayanagi T, Harada K, Sawada S, Ishikawa F. Bioavailability of isoflavones after ingestion of soy beverages in healthy adults. J Nutr (2006); 136:2291-2296.
22. Yamabe S, Kobayashi-Hattori K, Kaneko K, Endo H, Takita T. Effect of soybeans varieties on the content and composition of isoflavone in rice-koji miso. Food Chem (2007);100:369-374.
23. Heald CLM, Ritchie R, Bolton-Smitch C, Morton MS Alexander FE. Phytoestogens and risk of prostate Scottich men. British J Nutr (2007);98: 338-396.
24. Weed HG, McGandy RB, Kennedy AR. Protection against dimethylhydrazine-induced adenomatous tumors of the mouse colon by the dietary addition of an extract of soybeans containing the Bowman-Birk protease inhibitor. *Carcinogenesis* (1985);6:1239–1241.
25. Park KY, Jung KO, Rhee SH, Choi YH. Antimutagenic effects of doenjang (Korean fermented soypaste) and its active compounds. Mutat Res (2003);523: 43-53.

24/11/2010

Oxidative Stress in Brains of Male Rats Intoxicated With Aluminum and the Neuromodulating Effect of Some Forms of Sage (*Salvia officinalis*)

EL-Kholy, W.M.; EL-Habibi, E.M. and Mousa, A.T.
Zoology Dept., Faculty of Science, Mansoura University, Mansoura, Egypt
eelhabibi555@yahoo.com

Abstract: The present study was designed to investigate the role of oxidative stress and the status of antioxidant system in the management of aluminum chloride (AlCl3) induced brain toxicity in rats and further to elucidate the potential role of three forms of *Salvia officinalis* (sage) in alleviating such negative effects.
The results revealed that the levels of thiobarbituric acid reactive substances (TBARS) and protein carbonyl (PC) were significantly increased, however, the activities of superoxide dismutase (SOD) and catalase (CAT) as well as the reduced glutathione (GSH) content were significantly decreased in the cerebral cortex (Co) and hippocampus (Hip) of rats intoxicated with $AlCl_3$. In addition, the lipid profile, total lipids (TL), total cholesterol (TC) and triglycerides (TG) were significantly increased in serum and the mentioned brain regions, while phospholipids (PL), total protein (TP) and serum HDL-C were significantly decreased in $AlCl_3$ group. Additionally, serum and brain regions acetylcholinesterase (AChE) as well as alkaline phosphatase (ALP) and acid phosphatase (ACP) activities were significantly increased. On the other hand, the results exhibited that, sage when given in any form along with $AlCl_3$ was able to regulate the mentioned parameters and the values returned close to the normal ones. It can be concluded that Al-induced neuronal oxidative stress and inhibition of the antioxidant system, accompanied with disturbed lipid profile, total protein and enzyme activities could be the cause of $AlCl_3$ neurotoxicity. In addition three different sage forms, by their antioxidant constituents, could be able to antagonize Al neurotoxicity perhaps by reducing the oxidative stress and improving the antioxidant status and particularly by inhibiting the acetylcholinesterase activity, thus may improve memory and other brain cognitive activities.
[EL-Kholy, W.M.; EL-Habibi, E.M. and Mousa, A.T. Oxidative Stress in Brains of Rats Intoxicated With Aluminum and the Neuromodulating Effect of Different Forms of Sage. Journal of American Science 2010;6(12):1283-1297]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: Aluminum neurotoxicity- Alzheimer's disease- *Salvia officinalis* - Lipid peroxidation - Antioxidants-acetylcholinesterase

1. Introduction:

Aluminum has been implicated in many human neurodegenerative diseases; various investigations have suggested that Alzheimer's disease (AD) is more common in areas where Al content in water supplies is the highest (Lynch *et al*., 2000). Alzheimer's disease is a complex, multifactor, heterogeneous mental illness, which is characterized by an age-dependent loss of memory and an impairment of multiple cognitive functions (Mattson, 2004) and has been shown to be associated with both plaques and tangles in the brain. Indeed, the brain is a target of Al toxicity which can alter blood-brain barrier (BBB) mediating Al transport to the brain (Zatta *et al*., 2002a) and gets deposited in the cortex (Platt *et al*., 2001) and hippocampus (Struys-Ponsar *et al*., 1997). This can be occurring by altering the physiological ligands present at these barriers in states (Yokel, 2001).

Possible mechanisms of Al induced neurotoxicity have been related to cell damage via free radical production and oxidative stress (Kumar *et al*., 2009a, b). High aluminum levels exposure leads to increased central nervous system (CNS) Al concentrations that altered CNS concentrations of the essential trace elements; iron and manganese and increased the susceptibility of CNS to lipid peroxidation (LPx) (Oteiza *et al*., 1993).

Oxidative stress, caused by reactive oxygen species (ROS), is known to cause the oxidation of biomolecules leading to cellular damage. Increased lipid peroxidation (LPx) is the major consequence associated with oxidative stress. It is also speculated to be pathologically important in various neurodegenerative processes including cognitive deficits that occur during normal cerebral aging, Alzheimer's (AD), and Parkinson's diseases (Gray *et al*., 2003). Alternatively, the most remarkable biochemical change in AD patients is a reduction of acetylcholine (ACh) levels in the hippocampus and cortex of the brain (Jaen *et al*., 1996). Therefore, inhibition of acetylcholinesterase (AChE), the enzyme responsible for hydrolysis of ACh at the cholinergic synapse, is currently the most established approach to treating AD (Akhondzadeh *et al*., 2003).

On the other hand, *Salvia officinalis* (sage) specially the oil had apparent dual cholinergic activity, as it was active on both, AChE and

butyrylcholinesterase (BuChE) (Savelev *et al.*, 2004). Besides the cholinergic activity, there has already been a wider range of activities reported for the genus *Salvia*, which may be relevant for CNS disorders. These include antioxidant (Celik and Isik, 2008 and Carla *et al*., 2009), nicotinic activity (Wake *et al.*, 2000), anti-inflammatory properties (Moretti *et al*., 1997), and glutamergic activities (Kuang and Xiang, 1994). The essential oils of the plant, also, tested for its memory-enhancing effect (Akhondzadeh *et al*., 2003). Therefore, the main goal of the present study was to examine the possible mechanisms by which Al exposure could induce Alzheimer-like condition related alterations in brain of male rats, and extend to investigate the beneficial effects of sage in preventing or modulating these risks.

2. Materials and methods

Material and methods

Chemicals:

Aluminum Chloride ($AlCl_3$) was obtained from agents of Sigma Chemicals (St. Louis, MO, USA). *Salvia officinalis* (sage) oil was obtained from (NATURE'S ALCHEMY) distributed by LOTUS BRANDS, USA. Dried leaves of sage, for preparations of sage tea (water extract and ethanolic extract), were purchased from a local herb market. The taxonomic identity of the plant was confirmed by the botanist of the Department of Botany, Faculty of Science, Mansoura University, Mansoura, Egypt. All other chemicals were purchased locally and were of analytical reagent grade.

Sage extracts preparation:

1- Sage water extract (sage tea): was routinely prepared by pouring 150 ml boiling water onto 2 g of dried grounded leaves and allowing it to steep for 5 min. (Lima *et al*., 2005).

2- Sage ethanolic extract: was prepared according to the method described by Eidi *et al*. (2006). Dried grounded leaves of *Salvia officinalis* (60 g) were subjected to extraction with 300 ml of ethanol (80%) in a glass container for 72 h. The extract was decanted and filtered through Whatman No 1 filter paper into a clean flask. The same procedure was repeated a further two times. The solvent was evaporated using a rotary evaporator, and then the flask was weighed to determine dried weight of extract. The supernatant was reconstituted using 53% ethanol and assayed using serial dilutions and the dose was calculated according to Ghosh, (1971)

3- Sage oil: diluted 1:2 in sunflower oil according to Perry *et al.* (2002).

Experimental animals:

This study was carried out on 48 adult male albino rats weighing 130 ±10 g b.w., supplied by The Urology & Nephrology Center; Mansoura University. The rats were maintained under controlled humidity; temperature (25 ± 2˚C) and light (12h light/ 12h dark). They were fed standard commercial rodent pellet diet and water *ad libitum* (free access to water and food).

Experimental Protocol:

After one week of acclimatization, the rats were divided into 8 groups consisting of 6 animals each. All treatments were continued for 90 days as follows:

1. Normal control
2. Sage water extract (given instead of drinking water) according to Lima *et al*. (2005).
3. Sage ethanolic extract (given orally by stomach tube as 0.1 ml/kg b.w.) (Akhondzadeh *et al*., 2003).
4. Sage oil group (given orally by stomach tube as 100 µl/ kg b.w.) every other day (Perry *et al*., 2002).
5. Aluminum (Al) treated group (mixed with diet as 100mg $AlCl_3$/ kg b.w.) (Bilkei, 1993).
6. Al + sage water extract group (given as in groups 5&2 respectively).
7. Al + sage ethanolic extract group (given as in groups 5&3 respectively).
8. Al + sage oil group (given as in groups 5&4 respectively).

Sample preparation:

At the end of the experimental period, overnight-fasted animals were decapitated, blood samples were collected and sera were separated and stored at -20°C until biochemical assay. The brain was then gently removed; the cerebral cortex and hippocampus were separated on an ice-chilled glass plate as described elsewhere (Nayak and Chatterjee, 2001). The tissue samples were quickly frozen on dry ice, weighed, and stored at -80°C until biochemical assay. Cortex and hippocampus were chosen for the present study because; aluminum affects more severely the cortex and hippocampus regions than any other area of the central nervous system (Urano *et al*., 1997). Also, these brain regions are known to be particularly susceptible in Alzheimer's disease, and have an important role in learning and memory functions (Bihaqi *et al*., 2009).

Biochemical analysis:

Determination of lipid peroxidation product thiobarbituric acid reactive substances (TBARS) was carried out according to the method of Ohkawa *et al*., (1982). Meanwhile, protein carbonyl was

measured spectrophotometrically according to the method of Smith *et al*., (1991). On the other hand, superoxide dismutase (SOD) and catalase (CAT) activities were determined following the methods of Nishikimi *et al*., (1972) and Bock *et al*., (1980), respectively. Additionally, reduced glutathione (GSH) content was determined spectrophotometrically according to the method of Prins and Loose, (1969) as well as Acetylcholinesterase (AChE) activity was measured according to the method of Ellman *et al*., (1961). Alkaline phosphatase (ALP), acid phosphatase (ACP), total protein (TP), total lipids (TL), phospholipids (PL), triglycerides (TG), total cholesterol (TC) in (serum, cortex and hippocampus) and serum HDL cholesterol were determined by using commercial kits from (Biodiagnostic, 29 Tahreer Str., Dokki, Giza, Egypt).

Statistical analysis:

Data were presented as means ± standard error (SE). The statistical analysis was performed with one-way analysis of variance (ANOVA) using SPSS (version 17) software package for Windows followed by *Dunken test*. A p-value of less than o.o5 was considered statistically significant.

3. Results:

Cortex and Hippocampus Lipid Peroxidation (LPx), Protein Carbonyl (PC) and Antioxidants:

As observed in table (1), the statistical analysis showed that the level of cortex and hippocampus TBARS and PC were significantly ($p<0.05$) increased by Al intoxication in comparison with the control value.

Concerning sage only, there were significant ($p<0.05$) decreases and increases in hippocampus TBARS level and catalase activity respectively in the groups administered (ethanolic extract and oil) comparing to the normal control group. Regarding the antioxidants, the data indicated that, Al group exhibited significant ($p<0.05$) reduction in cortex and hippocampus SOD, CAT activities as well as GSH content compared to normal control group.

Sage administration to rats intoxicated with Al caused a significant ($p<0.05$) reduction in the elevated cortex and hippocampus TBARS and PC concentrations compared to Al intoxicated group. The reduction in hippocampus TBARS reached levels below the normal ones and was significant ($p<0.05$), only, in (Al + sage ethanolic extract) group. On the other hand, daily administration of sage preparations to Al intoxicated animals revised the decreases in the cortex and hippocampus SOD and CAT activities as well as GSH contents to marked increases compared to Al intoxicated group.

Indeed, the modulating effects of sage preparations on both the oxidative stress markers (LPx and PC) and the antioxidant system (SOD, CAT and GSH) arrived them to values within the normal ranges where non significant ($p<0.05$) alterations were seen in comparison with the normal group except a significant ($p<0.05$) reduction in Hip LPx of (Al + sage ethanolic extract) and a significant ($p<0.05$) increase in Hip CAT of (Al + sage water extract) and (Al + sage oil) groups as well as in Hip GSH of (Al + sage oil) group.

Serum, Cortex and Hippocampus Total Lipids (TL), Total Cholesterol (TC), Triglycerides (TG) and Phospholipids (PL) Concentrations:

The data presented in table (2), exhibited that, serum, cortex and hippocampus total lipids (TL), total cholesterol (TC), and triglyceride (TG) contents showed significant ($p<0.05$) increases, but phospholipids (PL) exhibited significant ($p<0.05$) reductions in Al intoxicated animals in comparison with normal control.

Concerning sage only, cortex phospholipids content was significantly ($p<0.05$) increased in the groups administered (sage water extract and oil) and in hippocampus of (sage ethanolic extract and oil) groups comparing to the normal control group. But, significant ($p<0.05$) decreases were observed in TC of cortex in (sage water extract and ethanolic extract) as well as Hip TC and TG in the groups administered (sage oil). Such results referred to the benefits of sage preparations, specially the oil for reducing TC and increasing brain PL.

On the other hand, concomitant administration of sage (different preparations) with Al reduced the elevation of serum, cortex and hippocampus TL, TC and TG contents and enhanced serum, cortex and hippocampus PL approaching most of them to the normal values except in case of cortex PL only of (Al + sage water extract) which showed a non-significant ($p<0.05$) increase compared to Al intoxicated animals indicating no protection.

However, the reduction in serum, cortex and Hip TL levels reached to values within normal levels, except Hip TL in Al + sage ethanolic extract which, still, exhibited a significant ($p<0.05$) reduction compared to normal control group. Regarding TC, the reduction was, still, significant ($p<0.05$) in both, cortex of Al + sage ethanolic extract and hippocampus of Al + sage oil groups comparing to control one. Concerning TG, the reduction arrived to values significantly ($p<0.05$) lower than normal in serum TG of Al + sage ethanolic extract and Hip TG of Al + all sage preparations groups comparing to control one.

On the other hand, the enhancement in serum and cortex PL by sage was non-significantly ($p<0.05$) changed in (Al + all sage preparations) compared to normal control. But in the Hip, PL reached to values that still significantly ($p<0.05$) lower than normal in all Al + sage different preparations groups.

Serum HDL-C, Serum, Cortex and Hippocampus Total Protein (TP) Content:

As seen in table (3) serum, cortex and hippocampus total protein content and serum HDL-C showed highly significant ($p<0.01$) decreases in Al intoxicated animals compared to the normal control group. Concerning sage only, total protein exhibited a significant ($p<0.05$) increase in hippocampus of the groups administered all sage preparations compared to the normal control group.

Administration of different preparations of sage to Al intoxicated animals reversed the decrement in serum, cortex and hippocampus total protein contents and serum HDL-C to significant ($p<0.05$) increases compared to Al group. But, there were non-significant ($p>0.05$) elevations in serum HDL-C concentration of Al + all sage preparations except Al + sage water extract group which exhibited a non-significant ($p<0.05$) reduction compared to normal control group. Interestingly, the elevation in TP arrived to the normal levels except hippocampus total protein in Al + sage water extract and Al + sage ethanolic extract which showed significant ($p<0.05$) increases compared to the normal control group. These results indicated pronounced ameliorating effects of sage oil, followed by sage ethanolic extract, the water extract showed the lowest protective effect

Serum, Cortex and Hippocampus Acetylcholinesterase (AChE), Alkaline phosphatase (ALP) and Acid Phosphatase (ACP) Activities:

As shown in table (4), in Al intoxicated rats, there were significant ($p<0.05$) elevations in serum, cortex and hippocampus AChE, ALP and ACP activities compared to normal control ones. Concerning sage only, ALP and ACP activities were significantly ($p<0.05$) decreased in hippocampus of (sage oil) group only compared to normal control one.

On the other hand, significant ($p<0.05$) reductions were seen in all Al + sage treated groups serum, cortex and hippocampus AChE, ALP and ACP activities comparing to Al intoxicated group, arriving the values within the normal levels, with the exception of AChE activity, which was still significantly ($p<0.05$) higher than normal control in cortex of Al + sage ethanolic extract group and hippocampus in (Al + sage oil) group in comparison with normal control.

Regarding ALP activity, it was still significantly ($p<0.05$) higher than the control activities in all Al + sage preparations groups except serum (Al + sage water extract) and cortex Al + sage ethanolic extract groups which showed non-significant ($p<0.05$) elevations (within normal ranges) when compared to control.

Concerning serum, cortex and Hip ACP activities, marked ameliorations were seen, where non-significant ($p<0.05$) decreases and increases were shown respectively compared to normal control animals except hippocampus ACP activity of Al + sage ethanolic extract and Al + sage oil groups which were still, significantly ($p<0.05$) declined comparing to the normal control.

3. Discussion:

Aluminium has an association with the etiology of Alzheimer's disease and some other neurodegenerative diseases. It exerts its toxic effect on nervous system especially at high concentration, causing loss of memory, speech disturbances, dysparaxia, tremors, jerking movement's impaired muscular coordination and paralysis (Drago *et al*., 2008). *Salvia officinalis* (common sage) is a medicinal plant that has strong antioxidant properties (Baricevic and Bartol, 2000). For that reason, the present study aimed to look into the antioxidant potential of various sage preparations against Al neurotoxicity.

In the present study, there were significant increases in the oxidative stress markers lipid peroxidation (LPx) and protein carbonyl (PC) contents following Al exposure for 90 days in both cerebral cortex and hippocampus regions of rats. Such results are in harmony with those obtained by Deloncle *et al*. (1999) and Johnson *et al*. (2005) who reported that the neurotoxicity of Al may be a result of LPx.. Furthermore, Nehru and Anand (2005) reported a significant increase in brain thiobarbituric acid reactive substances in rats after stimulation by Al salts which was known to be bound by the Fe^{3+} carrying protein transferrin, thus reducing the binding of Fe^{2+} and increasing free intracellular Fe^{2+} that causes the peroxidation of membrane lipids and consequently membrane damage. Aluminum, being an inert metal, has been suggested to induce oxidative damage indirectly by potentiating the peroxidative effect of Fe^{2+}. It promotes reactive oxygen species (ROS) formation. ROS subsequently attack almost all cell components including membrane lipids thus producing lipid peroxidation (Christen, 2000).

The findings of the present study, also, showed that the rise in LPx in Al treated rats was accompanied by concomitant decrease in the activity of some antioxidant enzymes involved in the detoxification of

ROS, namely SOD, CAT as well as the level of GSH in the cortex and hippocampus tissues comparing with the control declaring the prooxidant effect of Al. These findings agreed with the antecedent studies of Savory *et al*. (2003) and Johnson *et al*. (2005) whom showed that Al exposure enhanced the neuronal lipid peroxidative damage with concomitant alterations in the enzymatic antioxidant defense status, thus having serious bearing on the functional and structural development of the central nervous system (Dua and Gill, 2001). Similar data recorded a decrease in the antioxidants such as GSH (Wu and Cederbaum, 2003) and SOD activity (Yousef, 2004) in the brain of Al exposed rats (Chainy *et al*., 1996) and human (Dua and Gill, 2001).

Moreover, such results are consistent with the studies indicated that Al intake produced an oxidative stress-related change, contributed to its neurotoxicity (Flora *et al*., 2003). However, in rats, a significant relationship between Al exposure and the presence of oxidative stress was established also by Gómez *et al*. (2005). This could be caused by inflicting damage to membrane lipids, proteins and antioxidative enzyme defense system (Jyoti *et al*., 2007).

The elevation of LPx in the cortex and hippocampus in the present study and other ones (Dua and Gill, 2001) suggested participation of free-radical-induced oxidative cell injury in mediating neurotoxicity of Al. Lipid peroxidation of biological membranes results in the loss of membrane fluidity, changes in membrane potential, an increase in membrane permeability and alterations in receptor functions (Nehru and Anand, 2005 and Albendea *et al*., 2007).

However, the increased Al concentration could deleteriously affect the neurons, leading to depletion of antioxidants and metal ions (Kumar *et al*., 2008) through the induction of free radicals, that exhausting SOD and CAT which function as blockers of free radical processes. These results are in accordance with (Nehru and Anand, 2005) who recorded a significant decrease in the activities of SOD and CAT in brain of rats after Al treatment. Alternatively, the decreased enzyme activities could be related to a reduced synthesis of the enzyme proteins as a result of higher intracellular concentrations of Al (Albendea *et al*., 2007).

The data obtained by the present study illustrated, further, that administration of water, ethanolic extracts of *S. officinalis* as well as sage essential oil to Al treated rats caused a significant decrease in the level of TBARS and protein carbonyl in the cerebral cortex and hippocampus and elevated the SOD and CAT enzymes activities and GSH contents when compared with Al intoxicated rats. Moreover, the plant extracts and oil significantly, improved or restored the normal activities of the antioxidant enzymes (SOD and CAT) and GSH in both of the cortex and hippocampus regions as compared to normal control.

Generally, the antioxidant effects of sage extracts have often been attributed to phenolic and monoterpenic compounds (Ren *et al*., 2003). Flavonoids are a diverse group of polyphenols (Havsteen, 2002) rosmarinic acid being the most representative that possess several modulatory effects, either inducing or decreasing the expression of SOD and CAT enzymes depending on structure, concentration, and assay conditions. Rosmarinic acid is the predominant phenolic compound in sage (Lima *et al*., 2005) and its effects was attributed to the compound's antioxidant properties acting as scavenger of reactive oxygen species (Zheng *et al*., 2004).

In point of fact, all of the tested forms of sage have previously shown to potently suppress hydroxyl radical formation (Kosar *et al*., 2005). Additionally, the protection of cell viability conferred by sage extracts seemed to be due, mainly, to their ability to prevent GSH depletion by their main phenolic compounds, rosmarinic acid and luteolin-7-glucoside. Nevertheless, unknown compounds other than phenolics also seem to contribute to the antioxidant effects of sage on basal GSH levels (Lima *et al*., 2007). However, the later authors (Lima *et al*., 2007) besides other ones Brandstetter *et al*. (2009) showed the ability of sage (mainly the methanolic extract) to increase basal GSH levels, probably by the induction of glutathione synthesis. However, sage ethanolic extract strongly decreased the level of lipid peroxidation compared to Al intoxicated rats, such effect which may be due to its free radical scavenging potential induced by ethanol where various species of Salvia has inhibitory and quenching impact on lipid peroxidation along with enhancement of antioxidant defense system in brain tissue of rats treated with Aluminum (Zupkó *et al*., 2001).

In fact, the glutathione peroxidase system consists of several components, including GSH that effectively remove (hydrogen peroxide) and serves as a cofactor for glutathione transferase, which helps remove certain drugs and chemicals and other reactive molecules from the cells. Moreover, GSH can interact directly with certain ROS (hydroxyl radical) to detoxify them, as well as performing other critical activities in the cell. So, GSH is probably the most important antioxidant present in cells. *Salvia officinalis* had a potent increasing effect on GSH content in brain compared to Al treated rats. Also, the enzymatic antioxidant defense system including SOD

and CAT which can decompose superoxide and hydrogen peroxide in the cells are the main defense against oxidative injuries. The decreased level of these biomolecules may lead to increased severity of Al toxicities in the brain (Tripathi *et al.,* 2009). Most likely, the sage tea effects observed, herein, was a result of interactions and synergisms among the different compounds and metabolites present, which makes it difficult to attribute them to any particular compound or family of compounds (Lima *et al*., 2005).

2- Lipid profiles

The present data indicated that serum, cortex and hippocampus total lipids (TL), total cholesterol, (TC) and triglycerides, (TG) were significantly increased by aluminum ingestion, while phospholipids (PL) and serum HDL-C levels were decreased; such results are in accordance with the results reported by Yousef (2004). Similarly, Wilhelm *et al*. (1996) suggested that long-term exposure to Al specifically altered the brain lipid/phospholipid metabolism and/or their transfer to various membrane systems and resulted in significant changes in phospholipid classes and in cholesterol contents of the rat brain. Alternatively, studies in monkeys revealed the chronic effects of Al exposure on brain physiology, including alteration of the lipid composition and the activities of various membrane-bound enzymes; Al was found to decrease significantly the total lipid, glycolipid, and phospholipid concentrations in the primate brain (Sarin *et al*., 1997). In addition, cholesterol and cholesterol/phospholipid ratios were shown to be remarkably increased, indicating a relevant loss of membrane integrity, and consequently a strong effect of Al on the activity/functionality of various membrane-bound enzymes, including AChE (Atack *et al*., 1983). Similarly, the long-term exposure to $AlCl_3$ was shown to result in a 60 % decrease in the total phospholipids content while total cholesterol content increased by 55 %. It is possible that this altered lipid /phospholipid content and composition could affect the insulation properties of the myelin. The finding may thus have some bearing on loss of short-term memory in Alzheimer's disease.

The increase in serum cholesterol and total lipids due to Al administration indicated, also, a loss of membrane integrity (Sarin *et al.*, 1997). This was further confirmed when Al was found to have a significant effect on the various membrane-bound enzymes (Newairy *et al*., 2009). One possible way to explain the relatively more intense lipid peroxidation due to Al is that the susceptibility to catalyze oxidative cascades which is much easier for lipids than it is for proteins. Moreover, Al exhibited high affinity for phosphate groups and binds to the phospholipid head groups through electrostatic forces, which may disturb the order as well as the other dynamic parameters of the lipid bilayer (Martin, 1986). From the foregoing results it is clear that Al resulted in significant reduction in the phospholipid content accompanied by major compositional changes, which is consistent with membrane hypothesis of AD. According to this hypothesis, in order to make up for the choline deficiency, the neurons try to extract choline from choline containing phospholipids. These results leads to the disruption of cell membranes and ultimately to neuronal cell death (Roth *et al*., 1995). Such elevations in TL, TC together with the reduction of HDL-C following Al intoxication shown herein, represent risk factors for atherosclerosis and decreased blood flow to the brain (ischemia) which may be added to the mechanisms involved in Al-induced neurotoxicity.

On the other hand, the present study showed that treatment of rats with $AlCl_3$ plus different preparations of sage decreased serum and brain total lipids, total cholesterol and triglycerides and enhanced phospholipids and serum HDL-C levels compared to $AlCl_3$ intoxicated group. These results are in agreement with Ninomiya *et al*. (2004) and Carla *et al*. (2009) who found that oral administration of sage significantly lowered total cholesterol, triglycerides in serum of rats; and increased serum levels of HDL-C. Also, Akram and Maryam (2009) showed that oral administration of sage water extract significantly decreased serum cholesterol and triglycerides. These results suggested that *S. officinalis* tea consumption is accountable for the improvement of the lipid profile inducing an increase in the HDL-C particles, contributing, therefore, positively to the control of the dyslipidaemia observed in Type 2 diabetes but also related to other diseases (Nesto, 2005). However, sage modulating results may attributed to several sage natural components have been shown to act on cholesterol metabolism by reducing its absorption or its synthesis, such as catechins (Plana *et al*., 2008). This is in addition to the polyphenols, especially, phenolic rosmarinic acid in sage which has potent antioxidant effects protecting membrane lipids of fatty acids and phospholipids from oxidative stress (Lima *et al*., 2005).

3-Total protein content

The data of the present work showed that Al intoxication caused a significant decrease in the protein contents of serum, cortex and hippocampus protein. These results are in accordance with those

obtained by Nayak *et al*. (2006) and Newairy *et al*. (2009).

Thus, the observed alterations could be attributed to direct or indirect effects of aluminum on protein synthesis and breakdown and interaction with neurotransmitter synthesis and degradation, through a series of reactions that depends on many enzymatic pathways and regulatory mechanisms (Goncalves and Silva, 2007).

The decline in the levels of protein in Al-treated rats is in agreement with Chinoy and Memon (2001) and might be due to changes in protein synthesis and/or metabolism and could be, also, attributed on one hand to an under nutrition and on the other hand to a reduction of the protein synthesis in the liver resulted from Al intoxication as well as to reduced enzymes of protein synthesis as a result of higher intracellular concentration of Al (Tripathi *et al*., 2009).

Alternatively, since GSH has been reported to be involved in protein and DNA biosynthesis so, the reduction in its content and in the antioxidant enzymes (SOD and CAT) resulted from Al intoxication may partly explain the decline in the total protein content. Additionally, Al induced reactive oxygen species (ROS) formation and promoted oxidative stress (Exley, 2004 and Kumar *et al*., 2009 a,b) enhancing peroxidative damage to lipids and proteins of the cellular membranes (Julka and Jill, 1996) is another suggestion for protein decline. Such an explanation, which was confirmed by Jyoti *et al.* (2007), indicated that Al exposure caused oxidative stress inflicting damage to membrane lipids, proteins and antioxdative enzyme defense system. Exposure of proteins to free radicals leads to gross structural and functional modifications including protein fragmentation, formation of cross-links and aggregates, protein peroxides generation, and enzymatic oxidation and degradation or clearance (Albendea *et al*., 2007).

On the other side, the results, herein, indicated that all sage preparations enhanced the protein contents in serum and cerebral cortex and hippocampus of Al intoxicated rats reached them within or near the normal levels comparing to the control group. Regarding the protective mechanisms of sage, it has been speculated that antioxidative properties of sage components may be primarily involved, since changes related to the oxidative stress, where lipid peroxidation and oxidative DNA damage, were shown to be eliminated by sage tea consumption; possibly due, in part, to scavenging the nitrogen oxide or their radical derivatives (Lima *et al*., 2005). Since the antioxidants play an important role in the regulation and maintenance of metabolism in the body against oxidative stress. So, sage constituents with their antioxidant properties overcame the lower in the total protein content perhaps by preventing oxidative stress and protein breakdown and enhancing protein synthesis and antioxidant system. Not only phenolic (Durling and Catchpole, 2007) or other flavonoids (Wang *et al*., 2001), but all other sage components known to be participating in the series of reactions, hence the observed improvement in the present results may be due to all those components.

4- Acetylcholine system:

Cholinesterases are a large family of enzymatic proteins widely distributed throughout both neuronal and non-neuronal tissues. In Alzheimer's disease (AD), analytical as well as epidemiological studies suggested an implication of an abnormal focal accumulation of Al in the brain (Zatta *et al*., 2002b). In this devastating disease, Al may interfere with various biochemical processes including acetylcholine metabolism, and can thus act as a possible etiopathogenic cofactor. Aluminum is known to interfere with cholinergic (Amador *et al*., 2001), glutamatergic and gamma-aminobutyric acid neurotransmission. A disturbance in the enzyme activities involved in the acetylcholine metabolism has, also, been reported following Al exposure (Cordeiro *et al*., 2003).

In the present work, the data obtained showed that Al intoxication caused significant activation of AChE in the serum, cortex and hippocampus. Prior studies have reported the influence of Al on the metabolism of acetylcholine (Jankowska *et al*., 2000). They exhibited that, specifically, there was a selective loss of acetylcholine releasing neurons in the basal forebrain, hippocampus and cortex. However, impaired cholinergic function in AD has been correlated with loss of memory.

Generally, there have been some hypotheses to explain pathogenesis of the disease such as "cholinergic hypothesis" and "amyloid formation hypothesis". Nowadays, the most accepted treatment strategy in AD has been accepted as "cholinesterase inhibitors" that can inhibit acetylcholinesterase (AChE) enzyme in order to increase acetylcholine level in the brain (Akhondzadeh *et al*., 2003). In fact, the most remarkable biochemical change in AD patients is a reduction of acetylcholine (ACh) levels in the hippocampus and cortex of the brain. Therefore, the inhibition of AChE (the enzyme responsible for hydrolysis of AChE at the cholinergic synapse), is currently the most established approach to treating AD (Tariot *et al*., 2000). Al interacts with the cholinergic system, acting as a cholinotoxin According to (Kaizer *et al*., 2005) the alterations in

the lipid membrane could be a decisive factor in changing the conformational state of the AChE molecule. However, another explanation for increased AChE activity following Al exposure could be the allosteric interaction between the cation and the peripheric anionic site of the enzyme (Gulya *et al*., 1990).

On the other hand, the results of the present study showed that sage administration alone or to Al-intoxicated rats led to AChE inhibition. However, it has been reported that *Salvia officinalis* has CNS cholinergic receptor binding activities that may be relevant to enhance or restore mental functions including memory (Wake *et al*., 2000). Similar effects are also observed *in vivo* (Howes *et al.*, 2003), at least for AChE, suggesting that relevant components of *Salvia* can cross the blood– brain barrier and increase cholinergic transmission via cholinesterase inhibition (Perry *et al*., 2002).Similarly, up to date, a number of studies on AChE inhibitory activity of several *Salvia* species have been reported. Among these, the essential oil and ethanolic extract of S. officinalis have been shown to possess anti-cholinesterase activity (Perry *et al*., 1996). This finding is consistent with recent reports established sage benefits to memory following administration of the essential oil in healthy young adults (Andrew *et al*., 2008). Moreover, the essential oil as well as its major components, α-pinene, 1, 8-cineole, and camphor were determined to have uncompetitive and reversible acetylcholinesterase inhibitory activity (Perry *et al*., 2000). Additionally, the constituents contained within *Salvia* oil may combine non-linearly to produce cholinesterase inhibition. A combination of the major monoterpenoid constituents (camphor, 1,8-cineole, borneol, α-pinene and β-pinene) reconstituted in a naturally occurring ratio was significantly less potent than that of the whole oil (Perry *et al*., 2003 and Savelev *et al*., 2004). The monoterpenoids may therefore act synergistically to inhibit AChE.

The activity of the essential oils was concluded mainly to be due to its monoterpenoids. The data indicated that the terpenoids, monoterpenes in particular, may have anticholinesterase activity (Orhan *et al*., 2007). Alternatively, the ethanolic extract of *Salvia officinalis* potentiated memory retention and it has also, an interaction with muscarinic and nicotinic cholinergic systems involved in the memory retention process but to less extent than oil (Eidi *et al*., 2006).

5- Alkaline and acid phosphatases:

The present study illustrated that Al ingestion led to significant elevation in alkaline phosphatase (ALP) and acid phosphatase (ACP) activities in sera, cerebral cortex and hippocampus. Alkaline phosphatase is a membrane-associated enzyme, which predominantly concentrated in the vascular endothelium in the brain. There is a more or less continuous sheath of ALP covering all internal and external surfaces of the central nervous system including the spinal cord and thus it may functionally be part in the blood-brain barrier mechanism. On the other hand, intracellular ACP is largely confined to lysosomes, which primarily respond to cellular injury. Within the brain, the ACP is found to be concentrated in the gray matter, although it shows the activity in the white matter, also, to some extent. However, significant contribution by Al was observed to induce changes in ACP activity (Dasgupta and Ghosh, 1993).

The increased activity of ALP and ACP enzymes in serum & brain of animals treated with $AlCl_3$ are in accordance with the findings of Ochmanski and Barabasz (2000). Also, El – Demerdash (2004) found that the activities of these enzymes were increased in serum of mice fed on wheat containing Al residue of 0.2 g /kg b.w. The present results are further, in consistent with the recent findings of Esmaeili *et al*. (2009) who showed that chronic Al consumption caused significant increases in the activities of ALP and ACP enzymes which could be due to severe damage to tissue membranes.

In addition, the increase in the activity of ALP or ACP in blood might be due to the necrosis of liver, kidney and lung (Sallam *et al*., 2005). Our own interpretation for increased levels of ALP and ACP is the disruption of the blood brain barrier and oxidative damage of tissue membranes, releasing membrane bound enzymes following Al intoxication which is also confirmed previously by Exley (2004) and recently by Esmaeili *et al*. (2009).

Moreover, regarding Al enhanced serum, cortex and hippocampus ACP activities of rats, herein, it was in agreement with the earlier observations recorded altered activities of specific lysosomal hydrolytic enzymes in neuronal tissues (Suzuki *et al*., 1988) due to Al administration. From these observations it can be suggested that Al induced an increase in ACP activity of the brain may be an indication of lysosomal proliferation and increasing catabolic rate. The increased ACP activity may result in phosphate accumulation within the lysosomes, and this in turn may lead to decreased plasma inorganic phosphate concentration (Hussain *et al*., 1990).

In the present work, administration of sage tea, ethanolic extract and oil caused marked reduction in the elevated activities of ALP and ACP in Al

treated rats. Such decrease could be due to the antioxidant properties of sage constituents as polyphenols (carnosol, carnosic acid, and rosmarinic acid) and flavonoids (apigenin) that protect cellular membranes integrity from Al-induced oxidative damage and repair the antioxidant system (Carla *et al.*, 2009), consequently, improve brain structure and function against Al toxicity.

Table (1): Cortex and Hippocampus (Hip) Lipid Peroxidation (TBARS) Product Concentration and Protein Carbonyl (PC) Content (µmol /g tissue), Superoxide Dismutase (SOD), Catalase (CAT) Activities and Reduced Glutathione (GSH) Content in Different Animal Groups

		Control	Sage water extract	Sage ethanolic extract	Sage oil	Aluminum (Al)	Al + Sage water extract	Al + Sage ethanolic extract	Al + Sage oil
TBARS	Cortex (n mol/g tissue)	322.77 ± 1.51	321.25 ±1.93	323.59 ±0.98	345.65 ±8.54	426.3 ±2.01[a]	356.7 ±13.04[b]	365.18 ±16.29[b]	361.64 ±14.90[b]
	Hip (n mol/g tissue)	352.82 ±4.67	358.48 ±0.55	345.1 ±1.64[a]	337.41 ±1.94[a]	380.15 ±0.42[a]	347.32 ±0.55[b]	331.52 ±0.55[a,b]	346.45 ±0.47[b]
PC	Cortex (µ mol /g tissue)	0.16 ±0.02	0.17 ±0.01	0.14 ±0.03	0.15 ±0.02	0.45 ±0.04[a]	0.26 ±0.02[b]	0.24 ±0.04[b]	0.19 ±0.04[b]
	Hip (µ mol /g tissue)	0.٢٩ ±0.٠١	0.31 ±0.02	0.29 ±0.02	0.29 ±0.01	0.84 ±0.01[a]	0.32 ±0.02[b]	0.29 ±0.02[b]	0.30 ±0.02[b]
SOD	Cortex (U/min/g wet tissue)	189.50 ±2.51	193.52±4.17	185.47±7.70	188.41 ±5.04	163.21 ±1.91[a]	184.18±3.94[b]	185.67±4.35[b]	169.57±7.16[b]
	Hip (U/min/g wet tissue)	171.29 ±9.57	194.54 ±2.26	195.48±0.12	176.21±12.01	137.81±7.88[a]	191.97±5.53[b]	175.06±5.69[b]	169.01±1.50[b]
CAT	Cortex (µ mol/sec/g wet tissue)	1.73±0.12	2.49±0.29	2.22±0.15	1.11±0.11	0.83±0.07[a]	1.63±0.12[b]	2.06 ±0.23[b]	2.00±0.11[b]
	Hip (µ mol/sec/g wet tissue)	2.08±0.14	3.08±0.06	4.92±0.72[a]	5.59±0.26[a]	0.62±0.02[a]	3.98±0.18[a,b]	3.07±0.21[b]	5.35±0.41[a,b]
GSH	Cortex (mg /g tissue)	0.35±0.01	0.30±0.02	0.33±0.01	0.33±0.02	0.10±0.02[a]	0.29±0.26[b]	0.28±0.22[b]	0.33±0.02[b]
	Hip (mg /g tissue)	0.36±0.02	0.37±0.02	0.38±0.02	0.43±0.02	0.12±0.01[a]	0.41±0.04[b]	0.39±0.02[b]	0.49±0.04[a,b]

Values were expressed as means ±SE of six animals. P<0.05 (significant)

a = Significant difference comparing to the normal control group.

b = Significant difference comparing to the aluminum group.

Table (2): Serum, Cortex and Hippocampus (Hip) Total Lipids (TL), Total Cholesterol (TC), Triglycerides (TG) and Phospholipids (PL) Concentrations in Different Animal groups

		Control	Sage water extract	Sage ethanolic extract	Sage oil	Aluminum (Al)	Al + Sage water extract	Al + Sage ethanolic extract	Al + Sage oil
TL	Serum (mg/dl)	397.03±33.69	397.63±43.17	407.05±31.33	337.63±7.73	775.25±41.64[a]	392.07±1.85[b]	381.07±13.05[b]	393.26±10.99[b]
	Cortex (mg/g)	622.19±5.73	637.51±7.15	658.23±11.81	638.40±7.29	782.46±10.89[a]	646.20±12.26[b]	650.65±10.12[b]	657.40±6.65[b]
	Hip (mg/g)	361.39±9.26	355.60±1.81	349.20±10.69	361.38±9.90	625.77±7.65[a]	332.65±11.11[b]	212.77±0.10[a,b]	325.74±7.41[b]
TC	Serum (mg/dl)	85.20 ±1.02	83.00 ±0.95	81.60 ±0.81	82.60 ±0.81	126.11 ±2.77[a]	83.80 ±5.42[b]	86.00 ±1.00[b]	84.20 ±1.24[b]
	Cortex (m g/g)	183.60 ±9.88	122.04 ±7.03[a]	138.52 ±7.21[a]	196.97 ±0.12	363.96 ±10.28[a]	196.14 ±3.71[b]	142.27±2.01[a,b]	162.93±0.10[b]
	Hip (m g/g)	89.86 ±0.84	92.06±0.18	53.14±5.13[a]	52.45 ±0.27[a]	198.53±0.98[a]	93.70±2.35[b]	81.20 ±2.01[b]	73.20±3.71[a,b]
TG	Serum (mg/dl)	67.28 ±4.78	59.38±5.29	59.59±0.06	56.11 ±1.33	93.19±8.13[a]	52.52±3.13[b]	47.19±2.70[a,b]	49.45±2.55[b]
	Cortex (m g/g)	142.24±4.77	135.44 ±0.53	160.80±3.89	162.32±3.49	182.00±4.14[a]	127.73 ±6.31[b]	142.20±4.63[b]	143.90±4.35[b]
	Hip (m g/g)	110.34±0.01	106.82±0.89	82.36±2.75[a]	80.75±0.31[a]	143.31±6.55[a]	87.35±2.91[a,b]	57.14±4.58[a,b]	63.93±0.17[a,b]
PL	Serum (mg/dl)	142.64±8.12	143.31±20.28	109.53±1.94	131.52±6.61	102.39±0.56[a]	142.00±1.84[b]	141.21±1.02[b]	142.42±2.87[b]
	Cortex (m g/g)	149.40±1.53	232.91±13.04[a]	155.41±6.22	368.21±9.30[a]	87.29 ±2.70[a]	123.84±1.98	143.15±21.97[b]	147.71±6.96[b]
	Hip (mg/g)	150.55±1.18	149.84±0.59	172.23±0.03[a]	167.27±3.46[a]	65.52±0.05[a]	119.88±0.01[a,b]	94.49±0.41[a,b]	117.65±2.08[a,b]

Values were expressed as means ±SE of six animals. P<0.05 (significant)

a = Significant difference comparing to the normal control group.

b = Significant difference comparing to the aluminum group.

Table (3): Serum High Density Lipoproteins Cholesterol (HDL-C), Serum Cortex and Hippocampus Total Protein (TP) Content in Different Animal Groups

		Control	Sage water extract	Sage ethanolic extract	Sage oil	Aluminum (Al)	Al + Sage water extract	Al + Sage ethanolic extract	Al + Sage oil
HDL-C	**Serum (mg/dl)**	**72.75±3.73**	**75.90±5.64**	**78.27±7.12**	**80.20±2.46**	**48.67±2.22[a]**	**70.86±3.37[b]**	**84.67±4.65[b]**	**80.20±7.20[b]**
TP	**Serum (mg/dl)**	**5.99±0.12**	**4.81±0.24**	**5.68±0.13**	**5.39±0.34**	**3.91±0.32[a]**	**5.52±0.37[b]**	**5.38±0.23[b]**	**5.23±0.41[b]**
	Cortex (m g/g)	**4.65±0.12**	**5.01±0.30**	**4.93±0.56**	**5.88±0.27**	**2.89±0.24[a]**	**6.19±0.46[b]**	**4.54±0.39[b]**	**5.94±0.32[b]**
	Hip (mg/g)	**2.30±0.06**	**2.70±0.02[a]**	**2.60±0.04[a]**	**2.53±0.02[a]**	**2.07±0.03[a]**	**2.79±0.11[a,b]**	**2.62±0.01[a,b]**	**2.52±0.01[b]**

Values were expressed as means ±SE of six animals. P<0.05 (significant)

a = Significant difference comparing to the normal control group.

b = Significant difference comparing to the aluminum group.

Table (4): Serum, Cortex and Hippocampus (Hip), Acetylcholinesterase (AChE), Alkaline Phosphatase (ALP) and Acid Phosphatase (ACP) Activities in Different Animal Groups

		Control	Sage water extract	Sage ethanolic extract	Sage oil	Aluminum (Al)	Al + Sage water extract	Al + Sage ethanolic extract	Al + Sage oil
AChE	Serum (µmol SH//0.1ml/min)	2.39±0.19	2.43±0.05	1.61±0.01	2.13±0.46	5.70±0.11[a]	3.22±0.37[b]	3.27±0.24[b]	2.45±0.07[b]
	Cortex (µmol SH/g/min)	3.64±0.11	3.39±0.12	3.75±0.14	3.94±0.22	13.17±0.065[a]	4.03±0.08[b]	4.37±0.19[a,b]	4.28±0.23[b]
	Hip (µmol SH/g/min)	1.67±0.15	1.46±0.14	1.37±0.04	1.66±0.04	7.49±0.01[a]	2.82±0.19[a,b]	2.45±0.28[b]	2.69±0.28[a,b]
ALP	Serum (U/L)	78.54±2.94	81.72±1.90	84.13±0.21	74.37±4.56	189.53±1.77[a]	84.03±7.56[b]	103.02±0.56[a,b]	150.69±8.90[a,b]
	Cortex (K.A.U./g tissue)	241.18±3.68	263.55±7.70	241.31±10.99	246.99±4.93	376.95±7.46[a]	292.27±6.33[a,b]	276.73±11.48[b]	333.05±12.78[a,b]
	Hip (K.A.U./g tissue)	134.94±0.55	142.59±4.38	142.59±4.38	93.46±6.28[a]	173.20±0.79[a]	150.66±2.43[a,b]	150.86±0.29[a,b]	152.27±2.35[a,b]
ACP	Serum (U/L)	32.18±0.44	31.74±1.02	31.67±1.52	24.97±0.95	49.31±3.71[a]	30.11±2.18[b]	28.23±2.65[b]	31.66±2.06[b]
	Cortex (K.A.U./g tissue)	134.94±5.13	136.39±5.61	131.36±4.85	128.14±9.37	228.10±5.46[a]	155.85±14.23[b]	156.11±7.22[b]	154.67±7.19[b]
	Hip (K.A.U./g tissue)	68.88±0.56	71.56±0.14	47.04±0.26[a]	56.46±3.89[a]	78.62±0.41[a]	66.58±0.15[b]	32.68±1.60[a,b]	37.54±0.86[a,b]

Values were expressed as means ±SE of six animals. P<0.05 (significant)
a = Significant difference comparing to the normal control group. **b** = Significant difference comparing to the aluminum group.

4. Conclusion:

Aluminum plays a pivotal role in the neuropathology of many neurodegenerative diseases including AD and validate the fact that chronic exposure to aluminum causes oxidative damage to the membranes and neural cells affecting memory loss and other cognitive dysfunction and exhaust the antioxidant system. Moreover, it is clearly evident that sage (all forms) has a neuroprotective effect against neuronal structural dysfunction caused by aluminum which may be attributed, mainly, to their high ability to scavenge ROS and augment the repair of the antioxidant system as well as its anti-ChE activity, thus it has been suggested that sage has shown promise in the treatment of many neurodegenerative diseases including Alzheimer's disease.

No fixed pattern of protection was seen specific for the different preparations of sage, but, we can arrange them in the following order: sage oil> ethanolic extract> water extract Future study may be designed and further warrants the need for molecular studies to elucidate the mechanisms underlying the protective effects of sage and its active components.

Corresponding author
EL-Kholy, W. M.
Zoology Dept., Faculty of Science, Mansoura University, Mansoura, Egypt

5. References:

1. Akhondzadeh, S.; Noroozian, N.; Mohammadi, M.; Ohadinia, S.; Jamshidi, A.H. and Khani, M. (2003): *Salvia officinalis* extract in the treatment of patients with mild to moderate Alzheimer's disease: a double blind, randomized and placebo-controlled trial. J. Clin. Pharmacy Therap., 28: 53-59.
2. Akram, E. and Maryam, E. (2009): Antidiabetic effects of sage (*Salvia officinalis* L.) leaves in

normal and streptozotocin-induced diabetic rats Diabetes & Metabolic Syndrome: Clin. Res. Rev., 39: 40–44.

3. Albendea, C.D; Trullen, E.M.G.; Broto, L.E.; Miana-Mena, F.G.; Plano, S.M.; Gonzales, M.C.R.; Balların, E.M. and Garcia, J.J. (2007): Melatonin reduces lipid and protein oxidative damage in synaptosomes due to aluminum. J. Trace Elem. Med. Biol., 21:261–268.
4. Amador, F.C.; Santos, M.S. and Oliveira, C.R. (2001): Lipid peroxidation and aluminum effects on the cholinergic system in nerve terminals. Neurotox. Res., 3:223-33.
5. Atack, J.R.; Perry, E.K.; Bonham, J.R.; Perry, R.H.; Tomlinson, B.E.; Blessed, G. and Fairbairn, A. (1983): Molecular forms of acetylcholinesterase in senile dementia of Alzheimer type: Selective loss of intermediate (10S) forms. Neurosci. Lett., 40:199–204.
6. Baricevic, D. and Bartol, T. (2000): The biological/pharmacological activity of the Salvia genus. In Kintzios S.E. (Ed.), SAGE – The genus Salvia, Harwood Academic Publishers, Amsterdam, The Netherlands: 143-184.
7. Bihaqi, S.W.; Sharma, M.; Singh, A.P. and Tiwari, M. (2009): Neuroprotective role of *Convolvus pluricaulis* on aluminum induced neurotoxicity in rat brain. J. Ethnopharmacol., 124:409-415.
8. Bilkei, A. (1993): Neurotoxic effect of enteral aluminum. Food Chem. Toxicol., 31:357–361.
9. Bock, P.P.; Karmer, R. and Paverka, M. (1980): A simple assay for catalase determination. Cell Biol. Monoger., 7: 44-74.
10. Brandstetter, S.; Berthold, C.; Isnardy, B.; Solar, S. and Elmadfa, I. (2009): Impact of gamma-irradiation on the antioxidative propertiesof sage, thyme, and oregano. Food Chem. Toxicol., 47:2230–2235.
11. Carla, M.; Alice, S.; Ramos, A.; Azevedo, M.F.; Lima, C.F.; Fernandes-Ferreira, M. and Cristina Pereira-Wilson (2009): Sage Tea Drinking Improves Lipid Profile and Antioxidant Defences in Humans Int. J. Mol. Sci., 10:3937-3950.
12. Celik, I. and Isik, I. (2008): Determination of chemopreventive role of Foeniculum vulgare and Salvia officinalis infusion on trichloroacetic acid-induced increased serum marker enzymes lipid peroxidation and antioxidative defense systems in rats. Nat. Prod. Res., 22: 66-75.
13. Chainy, G.B.N.; Samanta, L. and Rout, N.B. (1996): Effect of aluminium on superoxide dismutase, catalase and lipid peroxidation of rat liver. Res. Commun. Mol. Pathol. Pharmacol., 94:217–20.
14. Chinoy, N.J. and Memon, M.R. (2001): Beneficial effects of some vitamins and calcium on fluoride and aluminium toxicity on gastrocnemius muscle and liver of male mice. Fluoride, 34:21–33.
15. Christen, Y. (2000): Oxidative stress and Alzheimer disease. Am. J. Clin. Nutr., 71: 6215-6295.
16. Cordeiro, J.M.; Silva, V.S.; Oliveira, C.R. and Goncalves, P.P. (2003): Aluminium-induced impairment of Ca^{2+} modulatory action on GABA transport in brain cortex nerve terminals. J. Inorg. Biochem., 97:132–142.
17. Dasgupta, S. and Ghosh, S. (1993): Nicotine induced alterations in brain acid and alkaline phosphatase activities. Ind. J. Physiol. Allied. Sci., 47: 200-206.
18. Deloncle, R.; Huguet, F.; Babin, P.; Fernandez, B.; Quellard, N. and Guillard, O. (1999): Chronic administration of aluminium L-glutamate in young mature rats: effects on iron levels and lipid peroxidation in selected brain areas. Toxicol., 104:65–73.
19. Drago, D.; Bettella, M.; Bolognin, S.; Cenddron, L.; Scancar, J.; Milacic, R.; Ricchelli, F.; Casini, A.; Messori, L.; Tognon, G. and Zatta, P. (2008): Potential pathogenic role of beta-amyloid (1–42)-aluminum complex in Alzheimer's disease. Int. J. Biochem. Cell Biol., 40:731–746.
20. Dua, R. and Gill, K.D. (2001): Aluminum phosphide exposure: implications on rat brain lipid peroxidation and antioxidant defence system. Pharmacol. Toxicol., 89:315–9.
21. Durling, N.E. and Catchpole, O.J. (2007): Extraction of phenolics and essential oil from dried sage (*Salvia officinalis*) usning ethanol-water mixtures. Food Chem., 101:1417-1424.
22. Eidi, M.; Eidi, A. and Bahar, M. (2006): Effects of *Salvia officinalis* L. (sage) leaves on memory retention and its interaction with the cholinergic system in rats. Nutr., 22:321–326.
23. El-Demerdash, F.M. (2004): Antioxidant effect of vitamin E and selenium on lipid peroxidation, enzyme activities and biochemical parameters in rats exposed to aluminum. J. Trace Elements in Med. Biol., 18:113–121.

24. Ellman, G.L.; Courtney, K.D.; Andres, V. and Featherstone, R.M. (1961): A new and rapid calorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol., 7: 88-95.

25. Esmaeili, M.A.; Sonboli, A.; Kanani, M.R. and Sadeghi, H. (2009): *Salvia sahendica* prevents tissue damages induced by alcohol in oxidative stress conditions: Effect on liver and kidney oxidative parameters. J. Med. Plants Res., 3:276-283.

26. Exley, C. (2004): The pro-oxidant activity of aluminum. Free Rad. Biol. Med., 36: 380–387.

27. Flora, S.J.S.; Mehta, A.; Satsangi, K.; Kannan, G.M. and Gupta, M. (2003): Aluminum induced oxidative stress in rat brain: response to combined administration of citric acid and HEDTA.Comp. Biochem. Physiol., Part C, 134:319–28.

28. Ghosh, M.N. (1971): Fundamentals of Experimental Pharmacology. Academic Press-New York . Pp. 85.

29. Gomez, M.; Esparza, J.L.; Nogués, M.R.; Giralt, M.; Cabré, M. and Domingo, J.L. (2005): Pro-oxidant activity of aluminum in the rat hippocampus: gene expression ofantioxidant enzymes after melatonin administration. Free Radic. Biol. Med. 38: 104–111.

30. Goncalves, P.P and Silva, S.V. (2007): Does neurotransmission impairment accompany aluminum neurotoxicity. J. Inorg. Biochem., 10: 1291–1338.

31. Gray, S.L.; Hanlon, J.T.; Landerman, L.R.; Artz, M.; Schmader, K.E. and Fillenbaum, G.G. (2003): Is antioxidant use protective of cognitive function in the community-dwelling elderly. The American Journal of Geriatric Pharmacoth., 1: 3–8.

32. Hussain, A.S.; Cantor, A.H.; Jonson, T.H. and Yokel R.A. (1990): Effect of dietary aluminum sulfate on calcium and phosphorus metabolism of broiler chicks. Poult. Sci., 69: 985–991.

33. Jaen, J.C.; Gregor, V.E.; Lee, C.; Davis, R. and Emmerling, M. (1996): Acetylcholinesterase inhibition by fused dihydroquinazoline compounds. Bioorg. Med. Chem. Lett., 6:737–742.

34. Johnson, V.J.; Kim, S.H. and Sharma, R.P. (2005): Aluminum-maltolate induces apoptosis and necrosis in neuro-2a cells: Potential role for p53 signaling. Toxicol. Sci., 83: 329-339.

35. Julka, D. and Gill, K.D. (1996): Altered calcium homeostasis: a possible mechanism of aluminium-induced neurotoxicity. Biochim. Biophys. Acta., 1315: 47–54.

36. Jyoti, A.; Sethi, P. and Sharma, D. (2007): Bacopa monniera prevents from aluminium neurotoxicity in the cerebral cortex of rat brain. J. Ethnopharmacol., 111:56–62.

37. Kosar, M.; Dorman, H.J.D. and Hiltunen, R. (2005): Effect of an acid treatment on the phytochemical and antioxidant characteristics of extracts from selected Lamiaceae species. Food Chem., 91: 525–533.

38. Kuang, P. and Xiang, J. (1994): Effect of radix Salviae miltiorrhiza on EAA a dIAA during cerebral ischemia in gerbils: a microdialysis study. J. Trad. Chin. Med., 14:45–50.

39. Kumar, P.; Taha, A.; Sharma, D.; Kale, R.K. and Baquer, N.Z. (2008): Effect of dehydroepiandrosterone (DHEA) on monoamine oxidase activity, lipid peroxidation and lipofuscin accumulation in aging rat brain regions. Biogerontol., 4: 283–284.

40. Kumar, V.; Bal, A. and Gill, K.D. (2009a): Susceptibility of mitochondrial superoxide dismutase to aluminium induced oxidative damage. Toxicol., 255: 117–123.

41. Kumar, V.; Bal, A. and Gill, K.D. (2009b): Aluminium-induced oxidative DNA damage recognition and cell-cycle disruption in different regions of rat brain. Toxicol., 264: 137–144.

42. Lima, C.F.; Andrade, P.B.; Seabra, R.M.; Fernandes- Ferreira, M. and Wilson, C.P. (2005): The drinking of a Salvia officinalis infusion improves liver antioxidant status in mice and rats" J. Ethnopharmacol., 97: 383-389.

43. Lynch, T.; Cherny, R.A. and Bush, A.I. (2000): Oxidative process in Alzheimer's disease: the role of a beta-metal interaction. Exp. Gerantol., 35: 445-451.

44. Martin, R.B. (1986): The chemistry of aluminum as related to biology and medicine. Clin. Chem., 32:1797–806.

45. Mattson, M.P. (2004): Pathways towards and away from Alzheimer's disease. Natu., 430: 631–639.

46. Moretti, M.D.L.; Satta, M. and Peana, A.T. (1997): A study on antiinflammatory and peripheral analgesic action of Salvia sclarea oil and its main components. J. Essen. Oil Res., 9: 199–204.

47. Nayak, P. and Chatterjee, A.K. (2001): Differential responses of certain brain phosphoesterases to aluminum in dietary protein adequacy or in adequacy. Food chem. Toxicol., 39: 587-592.

48. Nayak, P.; Kumar, S. and Vasudevan, D.M. (2006): Role of ethanol on Aluminum induced biochemicalchanges on rat brain. Ind. J. Clin. Biochem., 21:53-57.

49. Nehru, B. and Anand, P. (2005): Oxidative damage following chronic aluminium exposure in adult and pup rat brains. J. Tra. Elem. Med. Biol. 19:203–208.

50. Nesto, R.W. (2005): Beyond low-density lipoprotein: Addressing the atherogenic lipid triad in type 2 diabetes mellitus and the metabolic syndrome. Am. J. Cardiovasc. Drugs, 5:379-387.

51. Newairy, A.A.; Salama, A.F.; Hussien, H.M. and Yousef, M.I. (2009): Propolis alleviates aluminium-induced lipid peroxidation and biochemical parameters in male rats. Food Chem. Toxicol., 47:1093–1098.

52. Ninomiya, K.; Matsuda, H. Shimoda, H.; Nishida, N.; Kasajima, N.; Yoshino, T.; Morikawaa, T. and Yoshikawa, M. (2004): Carnosic acid, a new class of lipid absorption inhibitor from sage" Bioorg. Med. Chem. Lett. 14: 1943–1946.

53. Nishikimi, M.; Roa, N.A. and Yagi, K. (1972): Measurement of superoxide dismutase. Biophys. Res. Common., 46: 849.

54. Ochmanski, W. and Barabasz, W. (2000): Aluminum-occurrence and toxicity for organisms. Przegl. Lek., 57:665–8.

55. Ohkawa, H., Wakatsuki, A. and Kaneda, C. (1982): Assay for lipid peroxides in animals tissues by thiobarbituric acid reaction. Ana. Biochem., 95: 351 – 358.

56. Oteiza, P.L.; Keen, C.L.; Han, B. and Golub, M.S. (1993): Aluminum accumulation and neurotoxicity in swiss-webster mice after long-term dietary exposure to aluminum and citrate. Metabo., 42:1296–300.

57. Perry, N.S.L.; Houghton1, P.J.; Jenner, P.; Keith, K. and Perry, E.K. (2002): Salvia lavandulaefolia essential oil inhibits cholinesterase in vivo. Phytomed., 9: 48–51.

58. Plana, N.; Nicolle, C.; Ferre, R.; Camps, J.; Cós, R.; Villoria, J. and Masana, L. (2008): DANACOL group. Plant sterol-enriched fermented milk enhances the attainment of LDL-cholesterol goal in hypercholesterolemic subjects. Eur. J. Nutr., 47: 32-39.

59. Platt, B.; Fiddler, G.; Riedel, G. and Henderson, Z. (2001): Aluminum toxicity in the rat brain: histochemical and immunocytochemical evidences. Brain Res. Bull., 55:257–67.

60. Prins, H.K. and Loose, J.A. (1969): Glutathione in biochemical methods in red cell genetics. Edited by Yunis, J.J., Academic Press, N.Y.D. London, 126-129.

61. Ren, W.; Qiao, Z.; Wang, H.; Zhu, L. and Zhang, L. (2003): Flavonoids: promising anticancer agents. Med. Res. Rev. 23: 519–534.

62. Roth, G.S.; Joseph, J.A. and Mason, R.P. (1995): Membrane alterations as causes of impaired signal transduction in Alzheimer's disease and aging. Trends Neurosci. Sci., 18:203-206.

63. Sallam, S.M.A.; Nasser, M.E.A.; Yousef, M.S.H.; El-morsy, A.M.; Mahmoud, S.A.S. and Yousef, M.I. (2005): Influence of Aluminum Chloride and Ascorbic Acid on Performance, Digestibility, Caecal Microbial Activity and Biochemical Parameters of Rabbits. Res. J. Agric.Biol. Sci., 1:10-16.

64. Sarin, S.; Gupta, V. and Gill, K.D. (1997): Alterations in lipid composition and neuronal injury in primates following chronic aluminium exposure. Biol Trace. Elem. Res., 59:133–43.

65. Savelev, S.; Okello, E.; Perry, N.S.L.; Wilkins, R.M. and Perry, E. (2003): Synergistic and antagonistic interactions of anticholinesterase terpenoids in Salvia lavandulaefolia essential oil. Biochem. Pharmacol. Behav., 75:661–668

66. Savory, J.; Herman, M.M. and Ghribi, O. (2003): Intracellular mechanisms underlying aluminum-induced apoptosis in rabbit brain. J. Inorg. Biochem., 97: 151-154.

67. Schneider, L.S. (1996): New therapeutic approaches to Alzheimer's disease. J. Clin. Psych., 57: 30–36.

68. Smith, C. D.; Caney, J. M.; Starke-Reed, P. E.; Oliver, C. N.; Stadtman, E. R.; Floyed, R.A. and Markesbery, W.R. (1991): Excess brain protien oxidation and enzyme dysfunction in normal aging and in Alzheimer disease. Proc. Natle. Acad. Sci., 88: 10540-10543.

69. Struys-Ponsar, C.; Kerkhofs, A.; Gauthier, A.; Soffie, M. and Van den Bosch de Aguilar, P. (1997): Effects of aluminum exposure on behavioural parameters in the rat. Pharmacol. Biochem. Behav., 56:643–8.

70. Suzuki, H.; Takeda, M.; Nakamura, Y.; Tada, K.; Hariguchi, S. and Nishimura, T. (1988): Activities of lysosomal enzymes in rabbit brain with experimental neurofibrillary changes. Neurosci. Lett., 89: 234-239.

71. Tripathi, S.; Mahdia, A.A.; Nawaba, A.; Chandera, R.; Hasanb, M. Siddiquib, M.S.; Mahdic, F.; Mitrad, K. and Bajpaid, V.K. (2009): Influence of age on aluminum induced lipid peroxidation and neurolipofuscin in frontal cortex of rat brain: A behavioral, biochemical and ultrastructural study. Brain Res., 1253:107–116.

72. Urano, S.; Asai, Y.; Makabe, S. and Matsuo, M. (1997): Oxidative injury of synapse and alteration of antioxidative defence systems in rats, and its prevention by Vitamin E. Eur. J. Biochem., 245:61–70.

73. Wake, G.; Court, J.; Pickering, A.; Lewis, R.; Wilkins, R. and Perry, E. (2000): CNS acetylcholine receptor activity in European medicinal plants traditionally used to improve failing memory. J. Ethnopharmacol., 69: 105–114.

74. Wang, C.N.; Chi, C.W; Lin, Y.L.; Chen, C.F. and Shiao, Y.J. (2001): The neuroprotective effects of phytoestrogens on amyloid h protein-induced toxicity are mediated by abrogating the activation of caspase cascade in rat cortical neurones. J. Biol. Chem., 276:5287–95.

75. Wilhelm, M.; Jaeger, D.E.; Schull-Cablitz, H.; Hafner, D. and Idel, H. (1996): Hepatic clearance and retention of aluminum: studies in the isolated perfused rat liver. Toxicol. Lett., 89:257–63.

76. Wu, D. and Cederbaum, I. (2003): Alcohol, Oxidative Stress, and Free Radical Damage. Alcohol Res. Health, 27: 277-284.

77. Xu, N.; Majidi, V.; Markesbery, W.R. and Ehmann, W.D. (1992): Brain aluminum in Alzheimer's disease using an improved GFAAS method. Neurotoxicol., 13:735–44.

78. Yokel, R.A. (2001): The toxicity of aluminium in the brain: a review. Neurotoxicol., 21:813–28.

79. Yousef, M.I. (2004): Aluminum induced changes in hemato-biochemical parameters, lipid peroxidation and enzyme activities of male rabbits: protective role of ascorbic acid. Toxicol., 199:47–57.

80. Zatta, P.; Kiss, T.; Suwalsky, M. and Berthon, G. (2002a): Aluminum (III) as a promoter of cellular oxidation. Coord. Chem. Rev., 228:271-284

81. Zatta, P.; Ibn-Lkhayat-Idrissi, M.; Zambenedetti, P.; Kilyen, M. and Kiss, T. (2002b): *In vivo* and *in vitro* effects of aluminum on the activity of mouse brain acetylcholinesterase. Brain Res. Bull., 59:41–5.

82. Zheng, Q.S.; Sun, X.L.; Xubo, L.G.; Song, M. and Wang, C.H. (2004): Protective effects of luteolin-7-glucoside against liver injury caused by carbon tetrachloride in rats. Pharmazie., 59: 286–289.

83. Zhu, X.; Raina, A.K.; Lee, H.G.; Casadesus, G.; Smith, M.A. and Perry, G. (2004): Oxidative stress signaling in Alzheimer's disease. Brain Res., 1000: 32–39.

84. Zupko, I.; Hohmann, J.; Redei, D.; Falkay, G. Janicsak, G. and Mathe, I. (2001): Antioxidant activity of leaves of Salvia species in enzyme dependent and enzyme-independent systems of lipid peroxidation and their phenolic constituents, Plant. Med., 67:366–368.

11/1/2010

First Record of Microsporidium *Neonosemoides* Sp. and some Ciliates Infecting *Chrysichthus Auratus* (Bagridae) from the Damietta Branch of River Nile, Egypt

Enayat Salem Ahmed Reda

Department of Zoology, Faculty of Science, Mansoura University, Mansoura, Egypt
enayatSalem40@yahoo.com

Abstract: The present study was carried out as a general survey for the possible ectoparasites that can infect the Nile fish *Chrysichthus auratus*. A total of 52 fish specimens were collected from Damietta branch of River Nile. Examination of the investigated fish revealed that, fish were infected with four ectoparasitic species belonging to three genera. These species were: *Neonosemoides* sp., *Scyphidia* sp. 1, *Scyphidia* sp. 2 and *Ichthyophthirius multifillis*. The first three species were recorded for the first time in Egypt. The recovered parasites have pathological effects on the host fish with subsequent economic losses were discussed.
[Enayat Salem Ahmed Reda. First Record of Microsporidium *Neonosemoides* Sp. and some Ciliates Infecting *Chrysichthus Auratus* (Bagridae) from the Damietta Branch of River Nile, Egypt. Journal of American Science 2010;6(12):1298-1305]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: *Neonosemoides* sp., Ciliates, *Chrysichthus auratus*, River Nile. Egypt.

1. Introduction:

Microsporidia are obligate intracellular parasites. Infected cells usually enlarge to accommodate the proliferating parasite. In 1968 Wiessenberg coined the term "xenoma" on the host cell with completely changed structure and the parasite proliferating inside it. According to Klaus (2005) microsporidia infect most invertrbrate phyla and all classes of vertebrate. As mentioned by Klaus (2005) there are 17 genera are known to infect fishes; 13 genera infect marine fishes and 4 genera infect freshwater fishes: *Heterosporis* (Schubert, 1969), *Nosemoides* (Vinckier, 1975), *Neonosemoides* (Faye, Toguebaye and Bouix, 1996) and *Pseudoloma* (Matthew, Brown, Larison, Bishop-Stewart, Rogers and Kent, 2001).

Genus *Neonosemoides* is one of microsporidian genera parasitizing freshwater fishes and at the same time produce xenoma which is an important agent of diseases in commercial fishes.

Although there is considerable information on the species of microsporidia (Lom and Dykova, 1992; Sprague *et al*., 1992; Lom, 2002; Lom and Nilsen, 2003), little is known about those from Africa. (Sakiti and Bouix, 1987) recorded *Neonosemoides tilapia* in *Tilapia zillii* from Benin and Faye and Toguedaye, (2005) recorded 4 unidentified species in carangid fishes from Senegal.

External protozoa are cited as major problem in freshwater fishes; sessilines ciliates like genus *Scyphidia* utilize gills and skin as a substrate for attachment.

On the other hand mobilina ciliates like genus *Ichthyophthirius* which is an obligate parasite of gills, skin and fins has a worldwide distribution (Paperna, 1980). It also has been found to cause the white spot disease which is accompanied by severe morbidity and eventually end with fish mortality (Hoffman, 1970). Abu-El Wafa, (1988) and Koura *et al*., (1997), described *I. multifiliis* from some freshwater fishes.

This study aims to contribute to the ciliates fauna infecting *Chrysichthys auratus* with special emphasis on genus *Neonosemoides* as a first record in Africa and to establish a background for further studies.

2. Materials and methods

A total of 52 fish of *Chrysichthys auratus* were collected from Damietta branch of River Nile near El-Mansoura. The collected fishes were transported to the laboratory in tank with good aeration. Fishes were kept alive until required in aerated glass aquaria. Fishes were identified according to Bashai and Khalil (1997).

Fish skin, fins and gills were firstly examined by the naked eye for detection of any macroscopically visible lesions. Samples of mucus were scraped gently from the skin, fins and gills, then spread on a clean slide and freshly examined under phase-contrast microscope for the presence of ectoparasitic protozoans. Some of the positive slids were air-dried and stained according to Klein;s dry silver impregnation method. Other positive slides were also air-dried, fixed with absolute methanol and stained with 10% Giemsa stain.

Detected protozoa were examined freshly, stained and identified according to Shulman (1984) and Lom and Dykova (1992 & 2005). All measurements were taken in micrometers (µm) mean ± SD (range). Figures were drawn with aid of camera lucida.

3. Results

The detected protozoan parasites were classified into two main phyla; Microsporidia and Ciliophora as following:

Phylum: Microsporidia
Genus: *Neonosemoides*
Neonosemoides sp.

Xenomas are white spherical, inhabiting gills range in size from 50-70 µm (mean 60 µm) in diameter. Xenoma consists of a simple lamellar wall measures 2.2 µm, contains only 16 mature macrospores in direct contact with the cytoplasm of the host cells and the three lobes hypertrophic nucleus of host cell. All spores in generally are surrounded by a light zone. Fully formed xenoma appears as "a bag of spores". (Figs. 1A & 3A)

Spore description

Spores are egg-shaped with bluntly rounded poles (Fig. 1B). It measures 3.2±0.2 (3.0-3.4) µm in length X 1.6±0.3 (1.3-1.8) µm in width. The spore has a thin outer finly corrugated layer (exospore), thin inner layer (endospore) and an inner most simple cell membrane. The spore consists of three parts which determine the anterior-posterior polarity of the spore (Fig. 1C).

The anchoring disc (polar cap) is mushroom cap like-shaped and stained as a red granule by Giemsa stain (Lom & Dykova,1992) , which is highly characteristic of the group (Fig. 3A). It is eccentric (subapically) located.

The polar tube; is the first part and is inserted into the base of the polar cap. The manubrium part of the polar tube extends from the cap obliquely backwards. There is an outer sheath around the polar tube, acting as a sleeve, through which the tube slides while extruding.

The isofilar polar filament forms 4 regular and helically arranged coils around the surface of the posterior vacuole in the posterior half of the spore. The second part is the polaroplast; lamellar organelle consisting of an anterior region of closely packed membranes and posterior region of more loosely packed membranes that surrounding the basal part of the polar tube. The third part, is the posterior vacuole, that lies inside the coils of the polar tube and occupies more than one-third of the spore cavity.

The remaining space within the spore and between the polaroplast and the posterior vacuole is occupied by the infective germ itself, the sporoplasm. The nucleus is single, spherical and centrally located between the polaroplast and the posterior vacuole.

Phylum: *Ciliophora*
I-Genus: *Ichthyophthirius*
I. *multifiliis*

This parasite appears as a rounded-shaped ciliated organism (Figs. 2A, 3B & 4A). In heavily infested fish, this parasite could be easily detected by the naked eye inhabiting the gills, skin and fins. It is white in colour, tiny dots, exhibits a sluggish movement and measures 44.2-90.6 µm in diameter (mean 67.4). The body is uniformly covered by dense rows of cilia. The number of meridional kineties are ranged from 77-98 (mean 88), converging anteriorly and apically raised into a pointed elevation. The cytoplasm appears to be grossly granulated containing many small food vacuoles, the horse-shoe macronucleus measures (32.3-44.6) µm in length (mean 38.5) and lies in middle of the body. A rounded micronucleus is almost adhering to the macronucleus. There are many contractile vacuoles.

II-Genus: *Scyphidia*
Scyphidia sp. 1

This ciliate is solitary large parasite, inhabiting gills with cup-shaped body measures 57.6±3.6 (54-61.2) µm in length X 49.9±3.1 (46.8-53) µm in width. Epistomial disc is vaulted and slightly elevated above the peristomial disc. The peristomial disc is narrow and encircles the epistomial disc. The macronucleus is ribbon shaped, sinuous and measures 48.4±4.4 (44-52.8) µm in length. Micronucleus is very small. There are some scattered contractile vacuoles. Transverse striations of pellicle conspicuous and ranged from 80-110 (mean 95). There is non ciliated groove near the narrow scopula (Figs. 2B, 3C& 4B).

Scyphidia sp. 2

This peritrich is solitary parasite, inhabiting gills with cup-shaped body and measures 35.2±2(33.1-73.2) µm in length X 36.3 ±2(34.4-38.1) µm in width. Peristomial disc is narrow. Both epistomial disc and peristomial lips are at the same level. The macronucleus is ribbon-shaped, sinuous, occupies almost all the body cavity and measures 33.6±5(28.6-38.5)µm in length X 5.5±0.8(4.6-6.2). The giant micronucleus situated in close contact with the macronucleus and measures 11.3±1.4(9.9-12.6) µm in length X 2.2±0.4(1.9-2.7) µm in width. Scopula attacted to the host skin directly by a secretory layer of sticky material. Infundibulum is small and extends between the two nuclui by cytopharynx. There is a non ciliated groove situated anteriorly (Figs. 2C & 3D).

Table (1): Comparative description of *Neonosemoides tilapiae* with the present species. (Measurements are in micrometers).

Parameter	*N. tilapiae* Sakiti and Bouix, 1987	Present Species
Xenoma size	120-800	50-70
Xenoma spores number	Many micro and macrospores	16 macrospores
Nucleus	Multinuclei	One with three lobes
Spore length	2.5-3	3-3.4
Spore width	1.5-2	1.3-1.8
Polar filament coils	4-5	4
Host	Cichlid *Tilapia zillii*	Bagrid *Chrysichthys auratus*
Site	Gills	Gills
Locality	Benin (West Africa)	Egypt

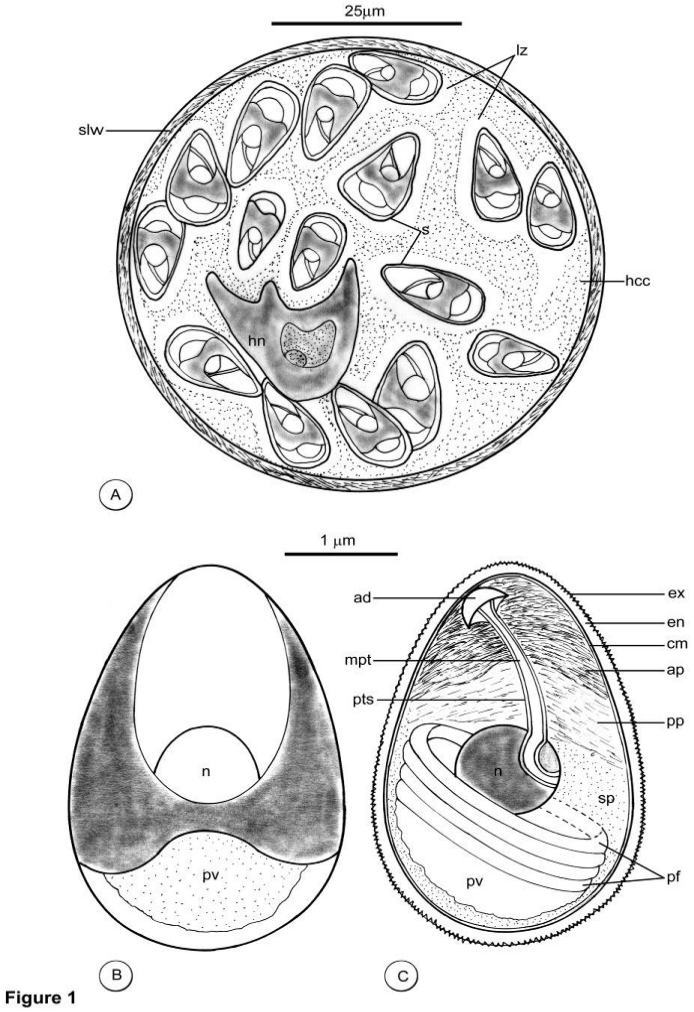


Figure 1

Fig. (1). Diagram of zenoma of *Neonosemoide* sp. (A) showing 16 macrospores, three lobes of hypertrophic nucleus and light zones. Mature spore (B) with characteristic egg-shaped and posterior vacuole. Mature spore (C) in details.

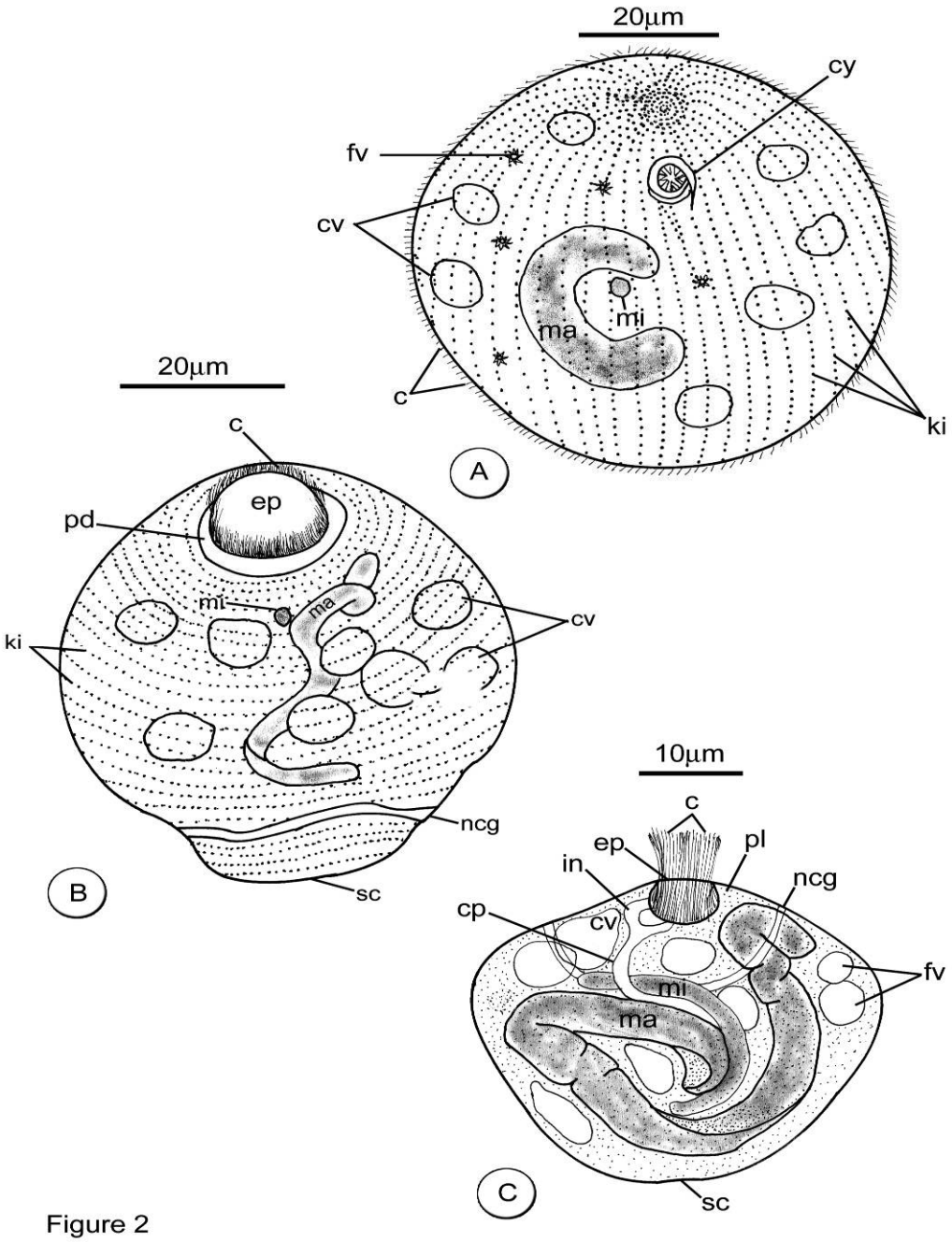


Figure 2

Fig. (2). Diagram of *Ichthyophthirius multifiils* (A). with characteristic round-shaped, horse-shoe macronucleus and meridional kineties. *Scyphidia* sp. 1(B) with transverse striation of pellicle. *Scyphidia* sp. 2(C) with cup-shaped body. Note the ribbon-shaped and sinuous macronucleus and giant micronucleus.

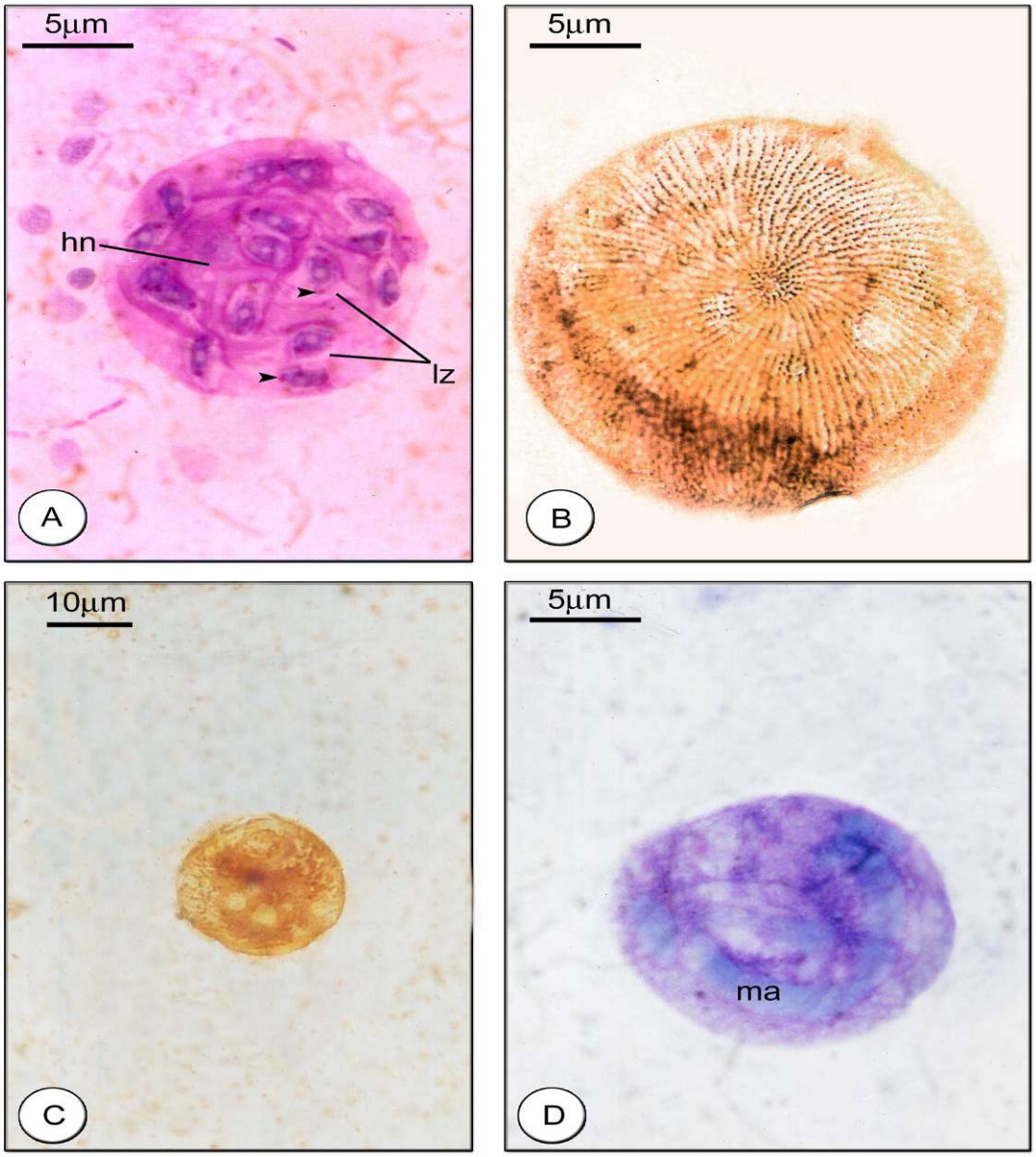


Figure 3

Fig. (3). Giemsa stain zenoma (A). Note the presence of anchoring disc as a red granule (arrowhead), silver impregnation Ichthyophthirius *multifillis* (B) and *Schyphidia* sp. 1(C) and Giemsa stain *Schyphidia* sp. 2(D). Not the sinuous ribbon-shaped macronucleus.

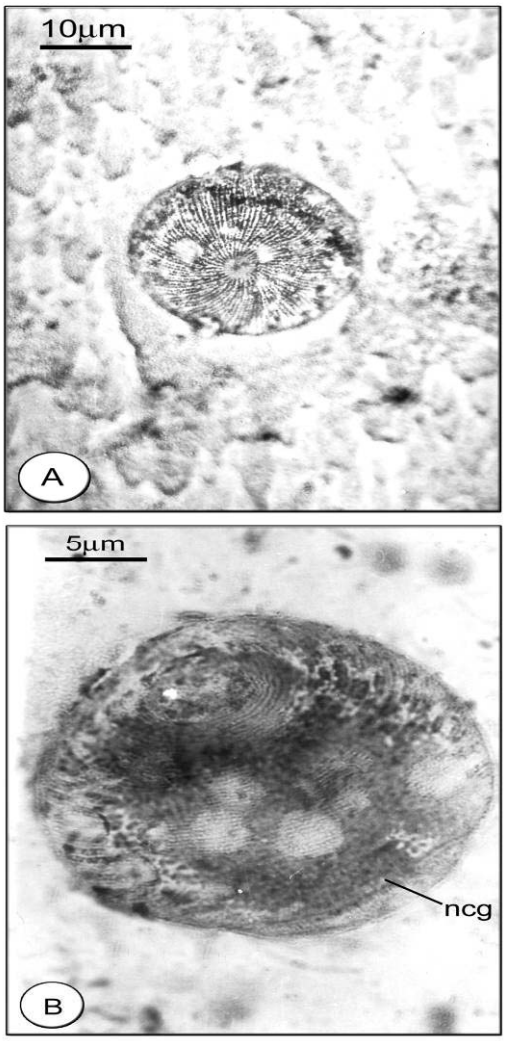


Figure 4

Fig. (4). Phase contrast microscope photograph of living specimens of *I. multifillis* (A) and *Schyphidia* sp. 1(B). Note the non ciliated groove.

Abbreviations for all figures

ad:	Anchoring disc	fv:	Food vacuole	ncg:	Non ciliated groove
ap:	Anterior part of polaroplast	hcc:	Host cell cytoplasm	pd:	Peristomial disc
c:	Cilia	hn:	Hypertrophic nucleus	pf:	Polar filament
cm:	Cell membrane	in:	Infundibulum	pl:	Peristomial lip
cp:	Cytopharynx	ki:	Kineties	pp:	Posterior part of polaroplast
cv:	Contractile vacuole	lz:	Light zones	pts:	Polar tube sleeve
cy:	Cytostome	ma:	Macronucleus	pv:	Posterior vacuole
en:	Endospore	mi:	Micronucleus	s:	Spores
ep:	Epistomial disc	mpt:	Manubrium part of polar tube	sc:	Scopula
ex:	Exospore	n:	Nucleus	slw:	Simple lamellar wall
				sp:	Sporoplasm

4. Discussion:

1-Genus *Neonosemoides*

Neonosemoides sp.

The more conspicuous characteristics of the spore; the shape, wall, polaroplast, polar filament and posterior vacuole are used to distinguish microsporidia from other taxonomic group (Sprague *et al*., 1992). According to the site of infection the present xenomas were found on the gills of freshwater fish *Chrysichthys auratus*, so it belongs to genus *Nosemoides* (Lom and Dykova, 1992). Recently (Lom and Dykova, 2005) reported that genera of microsporidia that comprise xenoma-forming species can be grouped in several categories according to xenoma wall, hypertrophic nucleus and type of spores inside xenoma. Accordingly the present investigated xenomas belong to genus *Neonosemoides*. Type and only species recorded in this genus is *Neonosemoides tilapiae* from *Tilapia zillii* (Sakiti and Bouix, 1987 and Faye *et al*., 1996) from Benin (West Africa). Comparing the present species with *N. tilapiae*, it was found many differences as listed in Table (1). So the present species assigned to the same genus but further ultrastructure and molecular study need to reveal the exact taxonomic assignment of this species.

The pathogenic effects induced by Microsporidia in host include physical disruption of cells due to occupation of intracellur space, host cell hypertrophy, change to host cell metabolism and reorganization of host cell components. The direct effects include increased mortality (Klaus, 2005). In the present work parasites are generally surrounded by a light zone the existence of which, is to be explained by the action of their proteolytic enzymes, which dissolve the host protoplasm around parasites and render it suitable for assimilation.

2- Genus: *Ichthyophthirius*

I. multifiliis

The parasite is identified by its characteristic horse-shoe shaped macronucleus in addition to the coarsely granular and vacuolated cytoplasm. Abu El-Wafa (1988) described *I. multifiliis* from different species of fishes but with smaller measurements (28 µm in diameter). He also found the same species in the grass carp *ctenopharyngodon idella* with the measurements much larger (about 710 µm in diameter). The present study (67.4 µm) is similar to Koura *et al.* (1997) described the parasite from *Oreochromis niloticus* (57.5 µm).

The first symptom of heavy infection is the presence of white spots appear over the entire body “white spots disease”. Fins begin to fray, skin starts being eroded, gills are pale (anemia). Scales may detach, eyes sunken, fish hardly move followed by death (Lom and Dykova, 1992).

3- Genus: *Scyphidia*

Scyphidia sp. 1

The present investigated parasite is resemble in shape and measerments to *Scyphidia doliaris* Chernova, 1977 (cited in Schulman 1984), but the latter has one contrctile vacuole, epistomial disc is below the peristomial disc level and there is no non ciliated groove. This species is first record in Egypt.

Scyphidia sp. 2

Scyphidia sp. investigated during this study was characterized by the cup-shaped body, ribbon-shaped irregularly twisted macronucleus, occupies almost all the cell cavity. The most characterized feature was the detection of the giant micronucleus. The present *Scyphidia* sp. 2 is similar in shape and macronucleus to *Scyphidia* sp. described by Ahmed *et al*. (2000), but the present parasite have-smaller size and has giant micronucleus. The present parasite is closely resemble *S. globularis* described by Solomatova, 1977 (cited in Shulman, 1984), but the latter has a smaller macronucleus besides the micronucleus not detected. This species is first record in Egypt.

The pathogenicity of genus *Scyphidia* is attributed to disturbance in the respiratory process of the infected fishes, leading to asphyxia. (Paperna, 1980).

Corresponding author
Enayat Salem Ahmed Reda
Department of Zoology, Faculty of Science, Mansoura University, Mansoura, Egypt
enayatSalem40@yahoo.com

5. References:

1. Abu El-Wafa, S.A.O., 1988. Protozoan parasites of some fresh-water fishes in Behera Governorate. M.V.Sc. thesis, Fac. Vet. Med. Alexandria Univ., Egypt.
2. Ahmed, A.K.; Tawfic, M.A.A. and Abbas, W.T., 2000. Some parasitic protozoa infecting fish from different localities of the River Nile, Egypt. Egypt. J. Zool., 34:59-79.
3. Bishai, H.M. and Khalil, M.T., 1997. Freshwater fishes of Egypt. Department of Natural Protectorate, Egyptian Environmental Affairs Agency (EEAA), Cabinet of Ministers, Arab Republic of Egypt. Publication of National Biodiversity Unit. No. 9.
4. Faye, N., Toguebaye, B.S. and Bouix, G., 1996. Ultrastructure and development of *Neonosemoides tilapiae* (Sakiti and Bouix, 1987)

n.g., n. comb (Protozoa: Microspora) from African cichlid fish. Eur. J. Protistol., 32: 320-326.
5. Faye, N. and Toguebaye, B.S., 2005. microsporidian parasites in four species of carangid fishes from the Senegalese Coast (West Africa). Acta Adriatica, 46(1): 21-26.
6. Hoffman, G.L., 1970. Parasites of North American freshwater fishes. Unvi. California Press (eds). Berkely and Los Angeles, London, England FDL 21.
7. Klaus, R., 2005. "Marine Parasitology" Csiro publishing, Astralia. pp565.
8. Koura, E.A.; Kamel, E.G.; Abd El-Aziz, A.M. and El-Deep, N.I., 1997. Incidence and prevalence of some protozoan parasites infecting the Nile fish *Oreochromis niloticus* in Egypt. Egypt. J. Aquat. Biol. and Fish.1: 397-430.
9. Lom, J., 2002. Acatalogue of described genera and species of microsporidians parasitic in fish. Syst. Parasitol. 53: 81-99.
10. Lom, J. and Dykova, I., 1992. Protozoan Parasites of Fishes, Elsevier, Amsterdam pp315.
11. Lom, J. and Dykova, I., 2005. Microsporidian xenomas in fish seen in wider perspective. Folia parasitological, 52: 69-81.
12. Lom, J. and Nilsen, F., 2003. Fish microsporidia: fine structural diversity and phylogeny. Int. J. parasitol., 33: 107- 127.
13. Paperna, I., 1980. parasites infections and diseases of fish in Africa CIFA Technical paper No. 72020p.
14. Sakiti, A.G. and Bouix, G., 1987. *Nosemoides tilapiae* n. sp., Microsporidie parasite de Poissons Cichlidae des eaux saumâtres du Bénin: implantation et caractères ultrastructuraus. Parasitol. Res. 73: 203-212.
15. Shulman, S.S., 1984. Key to Parasites of Freshwater Fish of the U.S.S.R. pp 430.
16. Sprague, V.; Becnel, J.J. and Hazard, E.I., 1992. Taxonomy of phylum Microspora. Crit. Rev. Microbiol. 18: 285-395.
17. Weissenberg, R., 1968. Intracellular development of the microsporidian *Glugea anomala* Moniez in hypertrophying migratory cells of the fish *Gasterosteus aculeatus* L., an example of the formation of "xenoma tumours". J., Protozool., 15: 44-57.

11/3/2010

Biochemical studies on some cotton by products Part I- Chemical constituents and cellulose extraction of Egyptian cotton stalks

*Fouad A. Ahmed; *Nadia M. Abdel-Moein ; **Amal S. Mohamed and **Shaimaa E. Ahmed

*Agric. Biochemistry Dep., Fac. Agric., Cairo University

** Cotton Research Institute, Agricultural Research Center, Giza, Egypt

shrose22@yahoo.com

ABSTRACT: The main objectives of the current investigation are to compare some chemical constituents, mainly cell wall components (cellulose and lignin), of stalks of five Egyptian cotton cultivars, as a step to convert a low valued bio-wastes of cotton plant stalks into highly value product as pure cellulose, which will, also, contributed in solving major environmental and health problem in Egypt. Lignocellulosic raw material cultivars; Giza 80, Giza 85, Giza 89, Giza 86, and Giza 90 were used in this study. They were obtained from Cotton Research Institute experimental fields. As first stage, chemical analysis comparison among aforementioned cultivars was conducted. The results showed that, there were significant differences among the five studied cultivars in moisture, Lipids, wax, crude fibers and β cellulose contents. As coincides, ash, protein, holocellulose, hemicellulose, α cellulose and lignin percentages exhibits no significant differences among cultivars. The highest percentages of moisture estimated in Giza 89 (7.74%), also in ash and lignin (3.39% and 25.75%, respectively), but it was the lowest cultivar in wax percentage (2.43%). Giza 86 showed the highest percentage in lipids and crude fibers (1.96% and 46.92, respectively), also in protein and holocellulose percentages (5.12 and 77.26 %, respectively), but it was the lowest cultivar in β cellulose (1.11%) as well as ash (2.95 %). The highest percentage in wax and β cellulose estimate (3.67% and 2.72%, respectively) was in Giza 90, but it was the lowest cultivar in Lipids (0.96%) and hemicellulose (40.04%). The highest percentages in α cellulose (49.21%) was in Giza 80 which reflected the lowest percentage in the crude fibers (38.75%). The second stage was the preparation of cellulose by removing the waxes, lignin, and hemicellulose, since cotton stalk consists of 75±2% holocellulose percentage and 44±5% α cellulose %. The third stage was conducting physical test by analyzing the sample that was prepared by X-ray, then comparison with standard cellulose sample chart to confirm its structure as cellulose.

[Fouad A. Ahmed; *Nadia M. Abdel-Moein; **Amal S. Mohamed and **Shaimaa E. Ahmed. Biochemical studies on some cotton by products Part I- Chemical constituents and cellulose extraction of Egyptian cotton stalks. Journal of American Science 2010;6(12):1306-1313]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Cotton; stalk; Delignification; Hemicellulose; Cellulose; X-ray

INTRODUCTION

In Egypt, crop residues are by-products of common crops such as cotton, wheat, maize and rice, with total amount of about 16 million tons of dry matter per year (El Saeidy, 2004). The Egyptian cotton is a unique descender of cotton that is characterized by high quality, it gained worldwide reputation for more than century and half as being of the highest lint quality among the world cottons for textile industry, beside seeds use in oil production (Bailey, 1958 and Simpson and Ogorzaly, 2001). Cotton crop area accounts for about 5% of the cultivated area in Egypt (El Saeidy, 2004). Cotton stalks produced annually as agricultural residues reached 1.9 million tons (Mona, *et al.*, 2001). These post-harvest by-product cause many severe problems; fires causing significant environmental and health disorders, in addition, eggs and larvae of harmful pink boll worm lays inside it (Amal *et al*., 2010). The cotton stalks are rich in cellulose and close to the fibrous structure of hard wood (Metcalfe and Chalk, 1950). Commercially, this material is being used as fuel in rural areas and for raising edible mushroom crops (Balasubramanya, 1981 and Pandey and Shaikh, 1987).

The largest share in cotton plant dry weight is for stem which comprises 23.15 % (Mcbryde, 1891). Cellulose comprises the major part of all plant biomass, and the source of all cellulose is the structural tissues of plants. Cellulose often occurs in close association with hemicellulose and lignin (Farone *et al*., 1998)

Brown (1927) found that cotton stalk consist of ash 3.09%, protein 4.00%, fibers 45.31% and fats

1.11% (percentage of constituents in water-free material). It was found that, as average chemical compositions of pulping woods, waxes, holocellulose, alphacellulose and lignin percentages are 1%-5%, 70-73%, 42-44% and 27%-31%, respectively (Grant, 1958). Rizk (1970) mentioned that the cotton stem fibers contained cellulose 65.43%-69.28%, lignin ranged from 21.19% to 22.93 % , wax from 1.91% to 2.06%., and ash content percentage was ranged from 1.89% to 2.53%. Kebeasy (1988) found that, the value of lipids in methanol benzene solution is (4.24%) which, mostly, belong to the waxes, whereas petroleum ether extract reflect lower values (2.93%). The estimations on cotton stalk of ash, crude protein, crude fibers, lignin, and cellulose were 2.94%, 3.48%, 47.35%, 23.6% and 35.6%, respectively. Paralikar and Bhatawdekar (1987) found that moisture content of raw cotton stalk was 9.5%. Silverstein, *et al*. (2007) reported that, the estimated percentage of acid-insoluble lignin was (27.9%), acid-soluble lignin was (2.2%) and ash was (6.0%).

Lignin is an integral part of plant cell walls and makes up one-quarter to one-third of the dry mass of wood, along with hemicellulose, is nature's cement which exploits the strength of cellulose and confers flexibility (Parajuli, 2006). Wassel (1985) found that, for sowing date, mean values of fiber cellulose and lignin were significantly increased as the time of sowing was delaying (between 67.45%-71.63% and 24.19-28%, respectively), on the contrary wax and ash percentages were (between 1.64%-1.015%, and 2.67-1.98%, respectively). The effect of retting methods was significant on the mean values of lignin and ash percentages scoring, in stagnant water retting, 23.36% and 1.79%, respectively. In 1.2% ammonium oxalate solution at 100 ºC for 1-2 hours, the highest percentage values were scored in cellulose percent, wax and ash (75.75%, 1.998% and 2.71%, respectively). On the other hand, the lowest percentages of cellulose (67.29%), lignin (21.22%) and wax (1.015%) were found in 1.2% sodium carbonate at 100 ºC for 1-2 hrs.

Acidified sodium chlorite is an effective method to remove lignin; however, during the delignification process the hydroxyl groups and reducing end groups of cellulose can also be oxidized (Fengel and Wegener, 1984).

MATERIALS AND METHODS

1. **Plant materials:** The current investigation was carried out on the dry stalks for five Egyptian cotton (*Gossypium barbadense* L.) cultivars; Giza85, Giza86, Giza89, Giza80 and Giza90.
2. **Work procedures:** The work was conducted in the Cotton Seed Technology and Natural Products Laboratory, Cotton Chemistry Department, Cotton Research Institute (CRI), Agricultural Research Center (ARC), Giza, Egypt. Cotton stalks of all studied cultivars were collected from ARC experimental fields, after complete harvesting of the economic crop, in August 2006, then cut into splinters about 2.5-4 cm in length by hand, then samples milled in a mill, using 0.4 mm screen, and finally, the milled samples were subjected to compositional analysis to compare among the five studied cotton cultivars. Mixed sample of cultivars stalks was subjected to cellulose extraction method then preparing derivatives from it. The chemical comparison among five studied cultivars was statistically analyzed as Randomized Complete Block Design (RCBD) with one factor (cultivars) in three replicates to obtain L.S.D. (at alpha 0.05) among averages for each studied character.
3. **Chemical analysis** (was determined on dry weight basis).

The chemical composition of the cotton stalks *i.e.* moisture, ash, Lipids, protein, Lignin, crude fibers, wax, (holocellulose and hemicellulose) and (α and β cellulose) were determined according to (AOAC, 1984), (AOAC, 2000), (AOAC, 1990), (AOAC, 1955), (Tanaka *et al*., 1985), (A.O.A.C., 1970), (Dorée , 1947), (whistler *et al.*, 1948) , and (Whister and Wolform, 1963), respectively.

4. **Preparation of cellulose:** The method described by Chahal *et al.*, (1979) was used for preparation of cellulose as follows:

Cotton stalks (50 g) were separately extracted with ethanol– benzene (1:2 v/v) for 6 hours in soxhlet apparatus then dried at 45 ºC. The dried samples were heated with sodium chlorite solution [Blend 23.5 g of sodium chlorite ($NaClO_2$) in 1000 ml of water containing 5 g of glacial acetic acid] at 75-80 ºC for 5 hrs. to remove the lignin. This treatment was repeated twice and the solution filtered. After filteration the residual material was washed with distilled water until free from chloride ions, then treat with ethyl alcohol to give holocellulose (hemicellulose and cellulose). To obtain the hemicellulose and cellulose fractions the

holocellulose was treated with 500 ml. NaOH 10% (w/v) for 4 hr. at 25 ºC with occasional stirring then filtered. The residue was again treated with 250 ml. NaOH 10% (w/v) for 3 hr. at 80 – 90 ºC, filtered and washed with water, ethanol, acetone and ether then air dried. This fraction contained the cellulose. The obtained cellulose was used in the preparation of hydrocellulose, cellulose acetate, cellulose nitrate

5. X-ray diffraction: The prepared cellulose sample was exposure to x-ray treatment in Central Laboratories Sector, The Egyptian Mineral Resources Authoroty (EMRA), The Ministry of Petroleum. A Philips X-Ray Diffraction equipment model PW/1710 with monochromator, Cu-radiation (λ=1.542 Å) at 40 K.V., 35 m.A. and scanning speed 0.02º/sec. were used. The reflection peaks between 2Θ = 2º and 60º, corresponding spacing (d, Å) and relative intensities (I/Iº) were obtained. The diffraction charts and relative intensities are obtained and compared with ICDD files. Obtained diffraction chart of prepared cellulose was checked against chart of standard cellulose sample (Kennedy *et al*., 1992) treated by 25% aqueous ammonia at 10 ºC for 15 and 240 min.

RESULTS AND DISCUSSION

In a trial to solve major environmental and health problem in Egypt, chemical constituents, mainly cell wall components, of five Egyptian cotton cultivars were investigated as presented in Table (a,b) and illustrated by Fig. (1a, 1b).

I- Chemical analysis:

1. Moisture %:

Data in Table (a) illustrated by Fig. (1a) indicated that there were significant differences among studied cultivars. Estimated stalk moisture ranged between 7.21 to 7.74% corresponding to studied samples. Giza 89 showed the highest value for this trait, significantly differed than Giza 90 and Giza 85 by 5.59 and 7.35%, respectively.

Table (a): Chemical composition of the cotton stalks.

Characters / Cultivars	Moisture%	Ash%	Lipids%	Wax%	Protein%
Giza 85	7.21	3.25	1.13	3.26	2.55
Giza 86	7.62	2.95	1.96	3.38	5.13
Giza 89	7.74	3.39	1.24	2.43	3.09
Giza 80	7.61	3.04	1.14	2.57	3.10
Giza 90	7.33	2.98	0.96	3.67	4.05
L.S.D at 0.05%	0.332	N.S	0.502	0.601	N.S

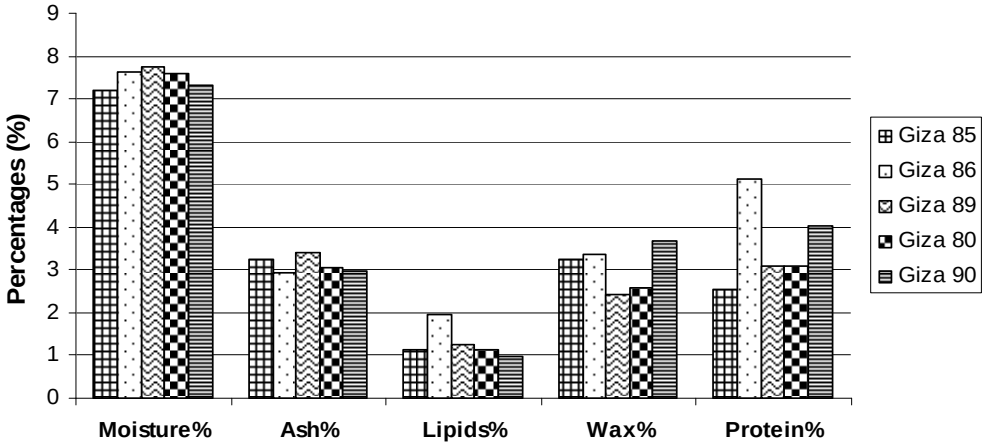


Fig. (1a): Chemical composition of cotton

2. Ash %:

No significant differences among cultivars understudied were observed, as indicated in Table (a) and Fig. (1a). Ash percentages ranged from 2.95% to 3.39%. The highest cultivar (Giza 89) increased by 14.92% than lowest one (Giza 86).

3. Lipids %:

As shown in Table (a) and Fig. (1a), there were significant differences among studied cultivars. Percentages of lipid % enclosed between 0.96% and 1.96% according to cultivar. Giza 86 showed the highest value significantly excelled Giza 89, Giza 80, Giza 85 and Giza 90 by 58.06%, 71.93%, 73.45% and 104.17%, respectively.

4. Wax %:

It was obvious of Table (a) and Fig. (1a) that, significant differences were statistically estimated among studied cultivars. Wax percentages % ranged from 2.43% to 3.67% due to cultivar. *Gossypium barbadense* cv. Giza 90 significantly exceeded Giza 80 and Giza 89 by 42.8% and 51.03%, respectively.

5. Protein %:

According to data in Table (a) and Fig. (1a), no significant differences were estimated among studied cultivars. Protein % percentages were elevates from 2.55% to 5.13% as to genotypic variations among cultivars. Giza 86 was the superior than other cultivars and exceeded Giza 85 (the lowest one) by 101.18%.

6. Crude fibers %:

Data in Table (b) and Fig. (1b.), confirmed the existence of significant differences among studied cultivars. Crude fibers % values were between 38.75% and 46.92% as to cultivar. Highest value was achieved in Giza 86 which score significant elevation by 21.08% over the lowest valued cultivar; Giza 80.

7. Lignin %:

As shown in Table (b) and Fig. (1 b), there were no significant differences among studied cultivars. Percentages of lignin % enclosed between 21.84% and 25.76% according to cultivar. Giza 89 showed the highest value exceeded Giza 85 (the lowest value) by 17.95%.

8. Holocellulose %:

It was obtained from data in Table (b) and Fig. (1b) that, no significant differences among cultivars understudied were observed. Percentages of holocellulose% ranged between 73.44% and 77.26% according to cultivar. Giza 86 showed the highest value exceeded the lowest value; Giza 85 by 5.20%.

9. Hemicellulose %:

Data in Table (b) and Fig. (1b) indicated that, there were no significant differences among studied cultivars. Estimated hemicellulose%, related to dry sample weight, ranged between 40.04% to 49.89% corresponding to studied cultivar. Giza 85 showed highest value for this trait, differed than Giza 90 which showed the least value by 24.60%.

Table (b): Chemical composition of the cotton stalks

Characters / Cultivars	Crude fibers%	Lignin%	Holo-cellulose%	Hemi-cellulose%	α Cellulose%	β Cellulose%
Giza 85	39.50	21.84	73.44	49.89	39.74	1.74
Giza 86	46.92	23.98	77.26	45.83	41.08	1.11
Giza 89	44.98	25.76	75.62	47.66	41.25	2.51
Giza 80	38.75	25.48	75.69	44.60	49.21	2.051
Giza 90	43.43	22.27	76.52	40.04	45.73	2.72
L.S.D at 0.05%	7.993	N.S	N.S	N.S	N.S	1.035

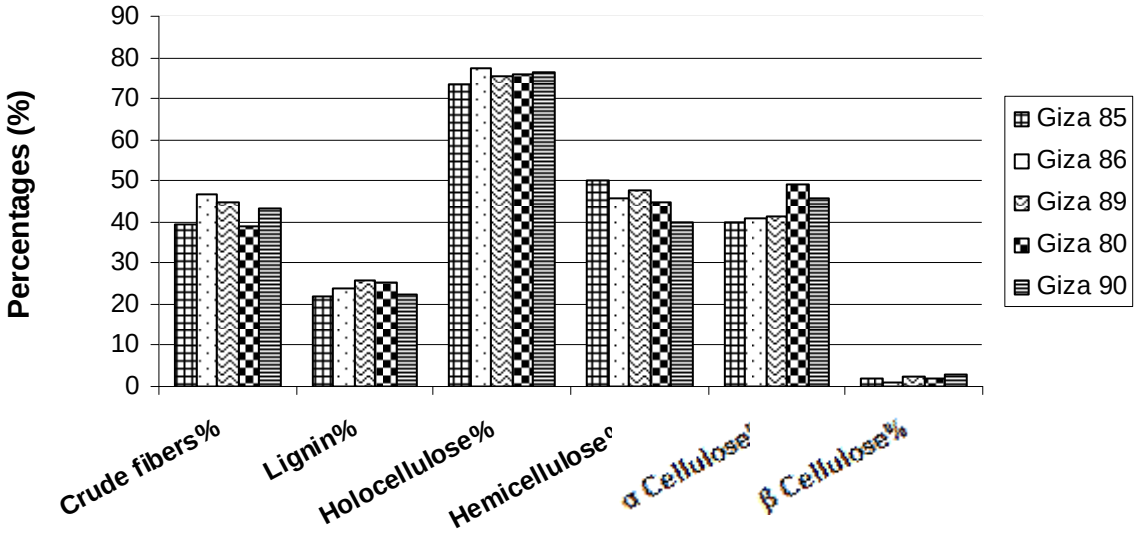


Fig. (1b): Chemical composition of cotton stalks

10. α Cellulose%

Differences among cultivars understudied were insignificant as observed in Table (b) and Fig. (1 b). Percentages of α cellulose%, related to cellulose sample weight, ranged from 39.74% to 49.21% as to studied cultivar. Highest cultivar (Giza 80) showed increment by 23.83% than lowest one (Giza 85).

11. β Cellulose%

It was obvious from Table (1 cont.) and Fig. (1 cont.) that, significant differences were statistically estimated among studied cultivars. Percentages of β Cellulose%, related to cellulose, enclosed from 1.11% to 2.72% due to cultivar. Cultivar Giza 90 significantly exceeded Giza 86 by 145.05%.

Aforementioned results are in agreement with those found by Brown, 1927, Grant, 1958 and Rizk, 1970, and partially in accordance with data estimated by Wassel, 1985 and Kebeasy, 1988. Previous results are coincides those found by Paralikar and Bhatawdekar, 1987 and Silverstein, *et al*., 2007.

II- X-ray diffraction:

Extracted cellulose sample was applied to X-Ray Diffraction equipment. Obtained diffraction chart of prepared cellulose (Fig. 3) was checked against chart of standard cellulose sample treated by 25% aqueous ammonia at 10 ºC for 15 and 240 min. (Fig 2), and this comparison showed that, the cellulose sample at D.p. (degree of polymerization) was treated with 25% aqueous ammonia for 15 and 240 min., and the tested sample, which was extracted from cotton stalks, had the same trend and enclosed in the same range. This aforementioned result confirmed that the tested extracted sample is cellulose.

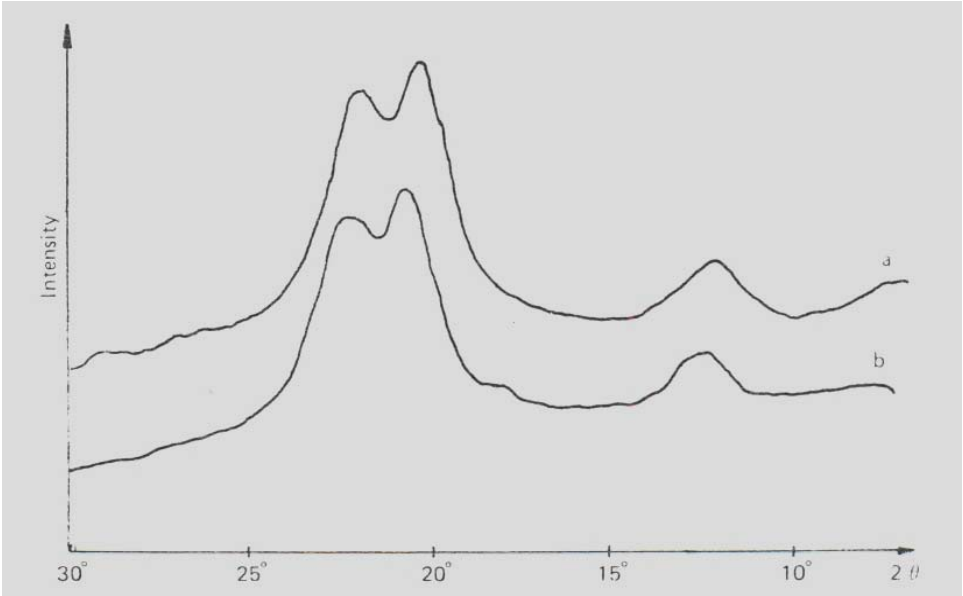


Fig. (2): X-ray diffractograms of cellulose (D.p.=425) treated by 25% aqueous ammonia at 10 ºC for :

a. 15 min.
b. 240 min.

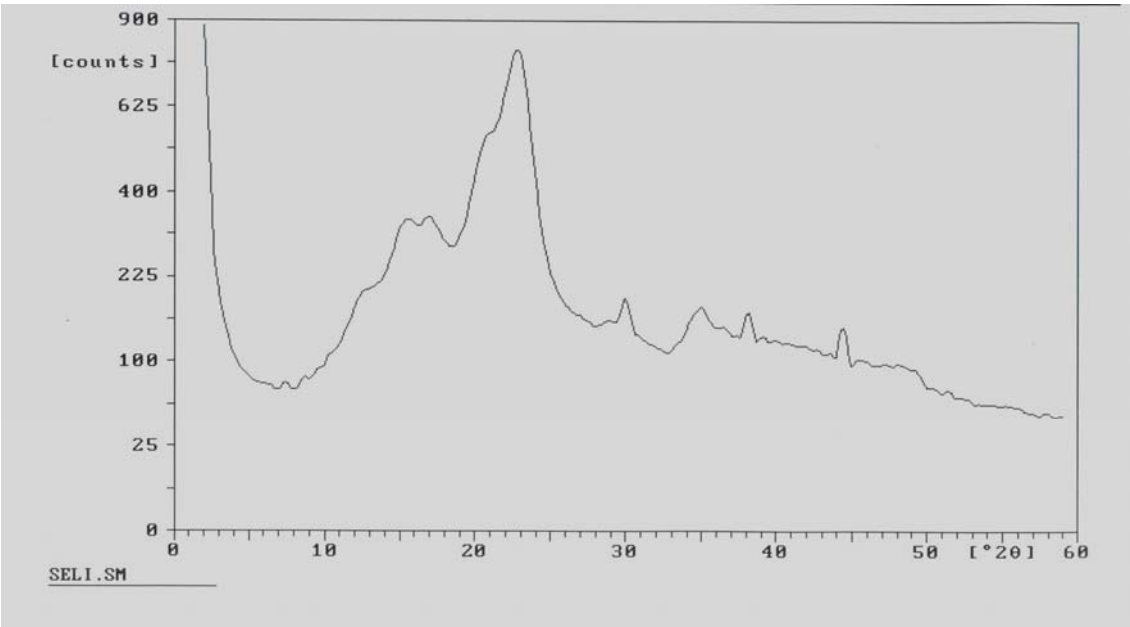


Fig (3): X-ray diffractograms of cellulose extracted from cotton stalks

CONCLUSION

Final goal of current work is achieved by extracting cellulose from cotton stalk of five cultivars understudied and use it to produce economical cellulose derivatives products. However, cultivar Giza86 has best performance for this investigation aim; it has the highest holocellulose percentage (beside some other traits) compared with other studied cultivars. Elimination of wax and lignin are prerequisite operations before cellulose extraction, thus Giza 86 has favorable (reduction in) percentage of lignin but set second (without significant difference) to Giza 90 as the highest wax percentage. Some cellulosic derivatives will be produced as future complementary investigation to current work.

REFERENCES

1. A.O.A.C. (1955). Official methods of analysis of the association of official agricultural chemists 8th ed. Association of Official Agricultural Chemists. Washington, 4 P.533.
2. A.O.A.C. (1970). Official methods of analysis of the association of official agricultural chemists 11th ed. Association of Official Agricultural Chemists. Washington, D.C., U.S.A.
3. A.O.A.C. (1984). Official methods of analysis of the association of official agricultural chemists 14th ed. Association of Official Agricultural Chemists. Washington, D.C., U.S.A.
4. A.O.A.C. (1990). Official methods of analysis of the association of official agricultural chemists 15th ed. Association of Official Agricultural Chemists. Washington, D.C., U.S.A.
5. A.O.A.C. (2000). Official methods of analysis of the association of official agricultural chemists. 17th ed. Association of Official Agricultural Chemists. Washington, DC., USA.
6. Amal, S. Mohamed, Abdel Wahab, A. F. M. ; and El –etr, Wafaa M. (2010). Recycling of Cotton Stalks with Rice Straw to Compost as Friendly Technology to The Environment. Mansoura Univ. Agricultural Chemistry and Biotechnology J., 1(1):41-52.
7. Bailey, L.H. (1958). The Standard Cyclopedia of Horticulture. The Macmillan Company, New York, USA. 3640pp.
8. Balasubramanya, R.H. (1981). An Edible Mushroom Crop on Cotton Stalks. Indian Soc. Cotton Imp. J., 6: 104-105. Cited after Shaikl (1990).
9. Brown, H.B. (1927). Cotton History, Species, Varieties, Morphology, Breeding, Culture, Diseases, Marketing, and uses. The Maple press company, New York, 509 pp.
10. Chahal, D.S.; Moo-Young, M.; and Dhillon, G.S. (1979). Bioconversion of wheat straw and wheat straw components in to single-cell protein. Canadian J. Of Microbiology, 25: 793-797.
11. Doree, Ch. (1947). The Methods of Cellulose Chemistry. Chapman and Hall, London, 2 nd ed.
12. El Saeidy, E. (2004). Technological Fundamentals of Briquetting Cotton Stalks as a Biofuel. Ph.D. Thesis, Fac. Landwirtschaftlich-Gärtnerischen Humboldt., Berlin Univ., 119 pp.
13. Farone, W. ; Cuzens, J., and Ana, C. (1998). Method of removing silica or silicates from solids resulting from the strong acid hydrolysis of cellulosic and hemicellulosic materials. U.S. Pat. No. 5,782,982.
14. Fengel D, Wegener G. (1984). Chemistry, ultrastructure, reactions. Biotechnol Bioeng 26(1984):1498–1505. Cited by Ishizawa *et al*. (2007).
15. Grant, J. (1958). Cellulose Pulp and Allied Products. Leonard Hills (books) Ltd. London. 512pp.
16. Kebeasy, M.I.M. (1988). Biochemical Studies on Farm By-products. M.Sc. Thesis, Fac. Agric., Cairo Univ., Egypt. 165pp.
17. Kennedy, J.F.; Phillips, G.O.; and Williams, P.A. (1992). Ligno-Cellulosics Science, Technology, Development and Use. 1st ed. Library of congress cataloging in- publication Data available, 867pp.
18. Mcbryde, J. B. (1891). A Chemical Study of the Cotton Plant. Bull. Tenn. Agr. Exp. Sta. 4 (5). Cited by Brown (1927).
19. Metcalfe, C.R. and Chalk L. (1950). Anatomy of the Dicotyledons; Leaves, Stem and Wood in Relation to Taxonomy with Notes on Economic Uses. Vol. 1. The Clarendon Press, Oxford, UK. 790 pp.
20. Mona A.; Medwick, V.; Byrd, J.; and Jameel, H. (2001). Soda-AQ Pulping of Cotton Stalks. TAPPI Pulping Conference and published in the conference proceedings (CD version).
21. Pandey, S. N. and Shaikh, A. J. (1987). Utilization of Cotton Plant Stalk for Production of Pulp and Paper. Biol. Wastes. 21: 63-70.

22. Parajuli,D. (2006). Development of Some Novel Lignin Derivatives For Adsorptive Removal Of Heavy Metals And Recovery Of Precious Metals .PhD. Thesis, Fac. Science and Engineering, Saga Univ., Japan, 98pp.

23. Paralikar, K.M. and Bhatawdekar, S.P. (1987). Microcrystalline cellulose from cotton stalk pulp. ISCI-Journal, Indian-Society-for-Cotton-Improvement., (September): 2 pp .

24. Rizk, M.A.M. (1970). Technological Studies on Stem Fibers of Some Egyptian Cotton Varieties. M. Sc. Thesis, Fac. Agric., Al-Azhar Univ., Egypt. Cited by Wassel. (1985).

25. Silverstein,R.; Chen, Y.; Shivappa, S.R.; Boyette, M.D.; and Osborne J. (2007). A comparison of chemical pretreatment methods for improving saccharification of cotton stalks .J. Bioresource Technology., 98: 3000–3011.

26. Simpson, B.B. and Ogorzaly, M.C. (2001). Economic Botany; Plants in Our World. 3rd ed. McGraw-Hill, New York, USA. 536 pp.

27. Tanaka, M.; Robinson, C.W.; and Moo-Young, M. (1985). Chemical and enzymatic pretreatment of corn stover to produce soluble fermentation substrates. Biotechnol. And bioeng J., 27: 362-368.

28. Wassel, O.M. (1985). Effect Of Sowing Dates On Retting And The Technological Characters of Cotton Stems. M.Sc. Thesis, Fac. Agric., Al-Azhar Univ., Egypt, 100 pp.

29. Whister, R.L. and Wolform, M.L. (1963). Methods in carbohydrate chemistry. Vol. III Academic press.

30. Whistler, R.L.; Bachrach, J.; and Bawman, D.R. (1948). Preparation and properties of corn bobs Holocellulose. Arch. Biochem.19: 25-33.

دراسات كيميائية حيوية على بعض مخلفات القطن

الجزء الاول - التركيب الكيميائى لحطب القطن المصرى

فؤاد عبد الرحيم أحمد*- نادية عبد المعين الغرباوى-*أمل صابر محمد- شيماء السيد أحمد****

***قسم الكيمياء الحيوية الزراعية - كلية الزراعة - جامعة القاهرة**

****معهد بحوث القطن- معهد البحوث الزراعية- الجيزة- مصر**

تعد الأهداف الرئيسية للبحث الحالى هى مقارنة المكونات الكيميائية وبصفة أساسية مكونات الجدار الخلوى (السليلوز و اللجنين) لحطب خمسة أصناف من الأقطان المصرية، ثم تحويل المخلفات الحيوية منخفضة القيمة إلى منتج عالى القيمة الاقتصادية كالسليلوز النقى، مما سيساهم بدوره فى حل مشكلة بيئية وصحية رئيسية فى مصر. وقد استخدم فى هذه الدراسة خمسة أصناف من أصناف القطن المصرى هى جيزة 80 وجيزة 85 وجيزة 89 وجيزة 86 وجيزة 90. ولقد تم الحصول عليهم من الحقول التجريبية لمعهد بحوث القطن. تم اجراء مقارنة للتحليل الكيميائى بين الأصناف سابقة الذكر. وقد أظهرت النتائج ما يلى

1- أن هناك اختلافات معنوية بين الأصناف المستخدمة من حيث المحتوى من الرطوبة و اللبيدات و الشمع و الألياف الخام و البيتاسليلوز

2- ليس هناك اختلافات معنوية بين الأصناف من حيث المحتوى من الرماد و البروتين والسليلوز الكلى و الهيميسليلوز و الألفا سليلوز و اللجنين

3- سجلت أعلى النسب للرطوبة فى حطب الصنف جيزة89 (7,74) أيضا فى الرماد واللجنين (3,39 و 25,75% على التوالى) ، ولكنه كان أكثر الأصناف انخفاضا فى نسبة الشمع (2,43%).

4- أظهر الصنف جيزة 86 أعلى النسب فى اللبيدات (1,96%) و أيضا فى نسب البروتين والألياف الخام والسليلوز الكلى (5,12 و46,92 و77,26 % على التوالى), بينما كان الصنف الأكثر انخفاضا فى البيتا سليلوز (1,11%) وكما فى الرماد (2,95%).

5- كانت أعلى نسبة فى الشمع (3,67%) فى جيزة 90 والذى أظهر أيضا أعلى تقدير فى البيتاسليلوز (2,72%)، ولكنه كان أكثر صنف انخفاضا فى الهيميسليلوز (40,04%) واللبيدات (0,96%).

6- كانت أعلى النسب في الألفاسليلوز (49,21%) فى جيزة 80 و الذى عكس أكثر النسب انخفاضا فى الألياف الخام (38,75%).

7- تم استخلاص سليلوز الحطب بإزالة الشموع و اللجنين والهيميسليلوز (المواد المصاحبة له) كما تم إجراء الاختبار الفيزيائى للسليلوزالمستخلص بواسطة الأشعة السينية الحيودية التى أكدت المقارنة مع الرسم البيانى لعينة سليلوزقياسية أن المستخلص هو سليلوز. يصلح لاجراء عمليات التحوير الكيميائى له لانتاج المشتقات السليلوزية وذلك فى الجزء الثانى من هذه الدراسة.

11/4/2010

Bacterial infections affecting marine fishes in Egypt

M. Moustafa [1], Laila. A. Mohamed[2], M.A. Mahmoud[3], W.S , Soliman[2], , A.E. Eissa [1] and M.Y. El-gendy[2]

[1] Department of Fish Diseases and Management, Faculty of Veterinary Medicine, Cairo University. Giza, Egypt
[2] Department of Hydrobiology, National Research Center, Dokki, Egypt
[3] Department of Pathology, Faculty of Veterinary Medicine, Cairo University. Giza, Egypt
[4] Corresponding Author

Abstract: Marine fishes are suffering from continuous depletion due to bacterial pathogens infections triggered by devastating environmental changes at their native aquatic environment. Qarun Lake and Suez Gulf are among the most vulnerable areas. 600 fish samples of Six different fish species; *Epinephelus tuvina*, *Sisganus rivulatus*, and *Dedlechilus labiosus* native to Suez-gulf at Suez governorate; *Mugil capito* , *Solea vulgaris* and *Tilapia zilli* native to Qarun Lake at El-Fayoum governorate were examined throughout the different year seasons. Gram positive and negative fish pathogenic bacteria were isolated from a total of 245 fish sample. Among those samples, the following bacteria were retrieved in the following percentages respectively, 17.55% (Vibrio*. anguillarum),* 16.73% (*Vibrio. alginolyticus*), 15.51% (*Pasteurella. piscicida*), 15.91% (*Pseudomonas. fluorescens)*, 13.46% *(Streptococus. fecalis*), 11.02% (*Aeromans . hydrophila*), 6.12% (*Aeromans. sobria)* and 3.67% were infected with *Staph. aureus*. The *Siganus rivulatus* was the highest infected fish species with a prevalence of 8.33%, while *Mugil capito* was the lowest infected species (5.67 %). The highest total prevalence of bacterial infection was recorded in summer season (40.81%) while the lowest was recorded in winter (15.91%). The aforementioned bacterial isolates were successfully re-isolated from experimentally infected fish. The retrieved isolates were confirmed by semi-automated (API 20 E) and conventional biochemical tests.
[M. Moustafa, Laila. A. Mohamed, M.A. Mahmoud, W.S, Soliman, A.E. Eissa and M.Y. El-gendy. **Bacterial infections affecting marine fishes in Egypt**. Journal of American Science 2010;6(12):1314-1324]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Marine fishes, Bacterial diseases ,Diagnosis, seasonal variation.

1-INTRODUCTION

Fish is considered as one of the main principal sources of the national income in Egypt. Like all animals, marine fish are subjected to numerous diseases, especially bacterial one, in which bacteria play the main role in producing the disease. Diseases are intensified by climatic changes that reflect negatively on the aquatic environment which is a good media for numerous pathogens **Wedemeyer, 1996)**.

The prevalence of bacterial pathogens have been well documented in several cultured and wild freshwater fish species, however; only a few bacteriological surveys on the prevalence of bacterial pathogens responsible for outbreaks in marine fishes **(Alicia., et al 2005).**

On the long run water resources will be the most limiting factor to be considered in aquaculture development in Egypt, especially freshwater aquaculture. Therefore, marine waters are the immediate alternative sources for water needed for mariculture **(Sadek, 2000).** One of the most promosing regions for mariculture in Egypt are The Gulf of Suez and Lake Qarun .

The present work was planned to isolate and identify the most predominant bacterial pathogens in some marine fishes native to both Suez Gulf, at Suez governorate and Lake Qarun at EL-Fayoum province. Further, work aimed to evaluate the seasonal variation of bacterial isolates among the examined fishes.

2. Materials and methods

2.1. Sampling and processing

Six hundred (600) marine fishes of Six different spp. were examined freshly from two localities in Egypt, (Suez Gulf and Lake Qarun). Through the different seasons of the year.

Twenty-five fish of each species were collected and examined seasonally. Fish species, numbers of fish, average body weights and localities are shown in table **(1).** Clinical and P.M examination were carried out using the methods described by **(Buller, 2004).**

Samples from gills, liver, spleen, kidney and external lesions from fishes were cultured on general and selective media; tryptic soy agar and tryptic soy broth (Difco) supplemented with 1.5% (w/v) NaCl, marine agar (Difco), and thiosulphate–citrate–bile

salt–sucrose agar (TCBS, Difco). aeromonas agar base medium supplemented with Ampicillin, pseudomonas agar base medium supplemented with CFC and 2 % NaCl , R-S agar supplemented with novobiocin and 2 % NaCl, and Azide blood agar supplemented with 2 % NaCl . All the inoculated media were incubated at 22 C for 2–5 days. clinically diseased fish, additional samples were taken from external lesions.

2.2. Identification of the isolates

Pure cultures of the isolates were identified by biochemical characterization following the criteria proposed by those described in the Bergey's Manual of Determinative Bacteriology, (**Holt et al., 1993).**Final confirmation of each strain was achieved using the analytical profile index of API20-E system **(Buller, 2004).**

2-3- Experimental infection

70 apparently healthy *O. niloticus* fish, weighting 50 ± 5 gram were selected after the 15 day of the acclimation period for determination of the pathogenicty of the most prevalent bacterial isolates. Fishes were divided into seven groups each contained 10 fish. The inocula prepared for bacterial isolates as I/P injections were prepared according to **(Austin & Austin, 1999).** Fish were observed daily for 15 days. Six groups were consistently inoculated I/P with bacterial suspension of (*Aeromonas hydrophila*, *Pseudomonas fluorescence*, *Vibrio anguillarum*, *Pasteurella piscicida*, *Streptococcus fecalis* and *Staphylococcus aureus*) at dose of 0.2 ml of (3 X 10^7 CFU) while the control group (group 7) were injected I/P with 0.2 ml of sterile tryptic soy broth according to **Hussain (2002)**.

3. Results

3-1Clinical examination

Symptoms detected in the diseased fish included Haemorrhages widely distributed into many parts of the body (Fig 1). Some fishes showed eye exophthalmia and opacity(Fig 2), scale detachment and darkness of the skin. gills were congested((Fig 3). while in others appeared to be pale and swollen with accumulation of excessive amount of mucus. Abdominal distention was observed in some fishes. Vent inflammation as well as prolapse was also seen in many cases.

Necropsy findings of the naturally infected marine fishes exhibited reddish serous fluid in the abdominal cavity. The liver was pale (Fig 4). in some fishes and congested, haemorrhagic with numerous randomly scattered whitish nodules throughout the parenchyma in others (Fig 5).. Spleen was congested and enlarged in some fishes, while in others appeared to be apparently normal. Kidneys were congested and slightly enlarged. In some fishes, white patches were found in kidneys. The intestines were haemorrhagic, inflamed with congestion of their blood vessels. In other samples, the intestines were seen filled with gases.

3.1. Isolation and identification of the bacterial isolates.

Biochemical characteristics of gram –ve bacterial spp. isolated from examined fishes are shown in Table (2).

Results indicated that 245 naturally collected fishes out of 600 were found to be infected with different types of bacteria. The culture results demonstrated that (203) fishes were found to be infected with Gram-negative bacteria and only (42) fishes were infected with Gram-positive bacteria. 17.50 % of the infected fishes were positive for *V. anguillarum,* (16.73%) for *V. alginolyticus*, (15.51%) for *P. piscicida*, (15.91%) for *Ps. fluorescens,* (13.46%) for *S. fecalis*, (11.02%) for *A. hydrophila*, (6.12%) for *A. sobria* and (3.67%) of the surveyed fishes were infected with *Staph. aureus.* The total prevalence of bacterial isolates is illustrated in tables (**3**)

Prevalence of different types of bacterial infections in the different examined fishes is illustrated in table (**3**). The results revealed that *Siganust rivulatus* was the most infected fish spp (50 %), followed by *E. tuvina* (42 %), *S. vulgaris* (41 %), *Tilapia spp.* (40 %), *M.sahlae* (38 %), while *M. capito* was the lowest infected spp (34 %).

The prevalence of bacterial infections for fishes collected from both Suez Gulf and Lake Qarun was illustrated in table (**4**). The results revealed that: The total Prevalence of bacterial infections for fishes collected from the Gulf of Suez (53.06%) was higher than that recorded for those collected from lake Qarun (46.93%).

The results indicated that, the highest total prevalence of bacterial infections among the naturally infected marine fishes was recorded in the summer season (40.81%), followed by autumn (25.71%), then spring (17.14%). On the other hand the minimal prevalence of infection was recorded in winter (15.91%). table (5).

The highest prevalence of bacterial infection among the naturally infected marine fishes in winter season, was recorded for *Ps. fluorescens* (7.75%) while the lowest one (0.40%) was recorded for *V. anguillarum.* on the other hand *P. piscicida*, *S. fecalis and Staph. aureus* were not recorded. For spring season, the highest prevalence of bacterial infection (3.67%) was recorded for *A. sobria* and *V. anguillarum*. while the lowest one (1.22%) was recorded for Ps. fluorescens and S. fecalis. on the other hand Staph. aureus were not detected. The highest prevalence of bacterial infection (9.38%) in summer season was recorded for *V. anguillarum*, while the lowest one (0.40**%**) was recorded for *A. sobria*. The highest prevalence of bacterial infection (5.30%) in autumn season was recorded for *V.alginolyticus* and *P.piscicida* while the lowest one (0.40%was recorded for *A. sobria*. The Prevalence of different types of bacterial infections in the different seasons is illustrated in table (5).

Results of experimental infection .

Mortality patterns in experimentally infected *O. niloticus* with the different bacterial isolates. Table (**6**).

experimentally infected O. niloticus with the different isolates were characterized by septicemic lesions nearly similar to those of naturally infected fish. Experimentally infected fish showed haemorrhagic patches distributed on different parts of the body surfaces. fin and tail rot (fig7). Some fish exhibited typical ulcers (fig8). Some cases showed inflammation of the vent (fig9).Pnecropsy findings showed, congestion of Liver; in some cases the liver was pale. Spleen and kidneys were congested and enlarged. Gall bladder was distended. The gut was haemorrhagic and filled with yellowish content. Serous to serosanguinous fluid in the abdominal cavity was noticed in some cases.

Re-isolation of all the injected bacterial isolates was obtained from dead and sacrificed experimentally infected fish. Moreover, the results of culture and biochemical characteristics of the re-isolated different bacterial isolates revealed the same morphochemical characteristics of the injected bacterial isolate.

Discussion

Septicemic bacterial infections such as vibrios, aeromonads, pseudomonads, photobacteria, streptococci and staphylococci have been observed in several fingerlings, juveniles, adults and brood stocks of some marine fish species **(Alicia *et al.*, 2005** and **Samuelsson *et al.*, 2006)**.

In regards to bacterial pathogens that have been isolated, results came in this study revealed that Gram-negative bacteria prevailed the Gram-positive ones with *Vibrio anguillarum*, *Vibrio alginolyticus*, *Pasteurella piscicida (photobacterium damsella subspp piscicida), Pseudomonas fluorescens, Streptococcus fecalis, Aeromonas hydrophila, Aeromonas sobria* and *Staphylococcus aureus* were the most common isolated spp. These results are supported by those reported by **Zorrilla *et al.* (2003)** who declared that the main pathogenic microorganisms isolated from diseased gilt-head seabream in the marine water at south western Spain were; *Vibrio* spp, *Pseudomonas* spp, *P. piscicida*, *Flavobacteria maritimus*, *Aeromonas* spp and Gram positive bacteria were also isolated but in low prevalence.

Table (1) Number and Weight

	Locality	Number	weight
E. tuvina	Suez Gulf	100	95±20
S. rivulatus	Suez Gulf	100	70±10
M. sahla	Suez Gulf	100	50±5
S. vulgaris	Lake Qarun	100	75±15
M capito.	Lake Qarun	100	85±10
Tilapia zilli	Lake Qarun	100	655

In concern to the total prevalence of bacterial infections in the naturally infected marine fishes at the present study (40.83 %) which may appear to be lower than those reported by some authors for the freshwater fishes as **Soliman (1999)** who noticed that the total bacterial prevalence was (65%). This difference might be due to the unfavorable effect of the salinity of marine water on the viability of bacterial pathogens.

In regard to the localities of isolation, results revealed that the prevalence of bacterial infections was higher (53.06%) in fishes collected from the Gulf of Suez than that (46.93%) recorded for those collected from Lake Qarun. the prevalence of Lake Qarun may be explaind as the lake Qarun is the largest reservoir of agricultural waste water drainage of Fayoum province as well as the drainage from fish farms established around the lake (**Mansour & Sidky, 2003**).

The high prevalence of isolation recorded from the Gulf of Suez may in part be attributed to the stress induced by high crude oil pollution at the Gulf water which is maintained by the low water

flow, low water exchange rates and daily crowded ship traffic crossing the gulf as well as industrial effluents from oil refineries. All these factors are compromising to the fish immune system ending up with marked increase in the magnitude of bacterial infections.

The study declared that marine fish can succumb MAS, as supported by **Larsen & Jensen (1977)** who isolated *A. hydrophila* from ulcer disease in Cod, *Gadus morhua* L., a strictly marine fish. Authors added that motile aeromonas group especially *A. hydrophila* is considered one of the most important pathogen responsible for haemorrhagic septicemia in a wide variety of marine water fish. Moreover, **Vethaak (1992**) isolated *A. hydrophila* from ulcers, lesions, and blood of ulcerated European flounder.

The results pointed out that the highest prevalence of *A. hydrophila* was recorded in winter season (5.71%) followed by spring (2.85%), in summer and autumn the results were the same (1.22%). These results supported by **Pathak *et al.* (1988)** who suggested that the highest isolation rates of *A. hydrophila* occurred during the late winter followed by a progressive decline in density during the summer and monsoon seasons. Moreover, **Popovic *et al.* (2000)** mentioned that there was clear seasonality in the prevalence of *A. hydrophila* as there were no isolates recovered in the summer months. On contrast, **Meyer (1970)** reported that the most epizootics of motile aeromonase were generally reported in spring and early summer.

In regards to the seasonal prevalence of *A. sobria*, our study recorded that the highest prevalence e of *A. sobria* septicemia was recorded during the spring (3.67%) followed by winter (1.63%) while the minimal prevalence e of infection (0.40%) was recorded during the summer and autumn. These results are in concordance with those obtained by **Wahli *et al.* (2005)** who noticed that mortalities due to *A. sobria* peaked during the low water temperatures of winter time and reached levels of 1% of the total fish on the farm per day. On contrary, the results are not in concordance with those obtained by **Kooj *et al.* (1988**) who demonstrated that the highest prevalence of Aeromonads in marine water have been obtained in the warmer months**.**

The pathogenicty of *A. hydrophila* for experimentally infected *Oeorchromis niloticus* with *A. hydrohila* may be attributed to the production of toxins and extracellular enzymes and lethal toxins including, proteases, amylases , lipases, enterotoxin, cytotoxins and haemolysin **(Saavedra *et al.*, 2004).**

In regards to the total prevalence of pseudomonas septicemia, the study recorded that (15.91%) of infected fish were positive for pseudomonas infection, These results are in concordance with those obtained by **Hussain (2002)** who reported that (15.27 %) of naturally infected marine fishes were positive for *Ps, flourescens* septicemia**.** on contrast, the results are lower than those reported by **Khan *et al.* (1981)** who reported that *Pseudomonas* spp accounted for (72 %) of the mortalities recorded in captive Atlantic cod, *Gadus morhus* L associated with fin rot disease.

The highest prevalence of *Ps. fluorescens* septicemia was recorded during the winter season (7.75%) followed by autumn (4.48%) summer (2.44%) and spring (1.22%), this reveals that *Ps. fluorescens* has certain affinity to low temperature for propagation and wide spreading infection **(El-Moghazy, 2004)**. The results are supported by **Golomazou *et al.* (2006)** who demonstrated that Pseudomonads were isolated mainly in cold months of winter. On contrary, the results are not in concordance with those obtained by **Hoda *et al.* (1999)** who revealed that the prevalence of pseudomonads was lower in winter than summer. This may be also attributed to amplified activity of proteinases produced by pseudomonads at the low temperature (10-25°C) that play the significant role in the pathogenesis of pseudomonas septicemia **(Hoshino *et al.*, 1997)**.

The pathogenicty of *Ps. fluorescence* for experimentally infected *Oeorchromis niloticus* may be attributed to the production of extracellular enzymes and lethal toxins **El-Attar & Moustaf (1996).**

In regards to the total prevalence of vibriosis recorded (34.28%), this result are in accordance with those reported by **Khan *et al.* (1981)** who recorded that vibrios accounted for (28%) of mortalities in captive Atlantic cod, *Gadus morhus* L associated with fin rot disease. the results are lower than those recorded by **Zorrilla *et al.* (2003)** who recorded that the prevalence of vibrios among diseased gilthead sea bream, *Sparus aurata* L in southwestern Spain was (69.90%).

V. anguillarum in this study was the *Vibrio* spp most frequently isolated as (17.55%) of the infected cases were positive for *V. anguillarum*. this is in accordance with **Zorrilla *et al.* (2003).** On the other hand *V. alginolyticus* was the cause of (16.73%) of the recorded cases. this high prevalence *of V. alginolyticus* indicates its importance in mariculture as supported by **Zhu *et al*. (2000)** who suggested that *V. alginolyticus* is one of the key diseases that have made great harm to a wide variety of marine fishes.

The highest prevalence e of *V. anguillarum* infection was recorded during the summer (9.38%), followed by autumn (4.08%), spring (3.67%), and only (0.4%) were recorded in winter. On the other hand the highest prevalence e of *V. alginolyticus* infection was recorded in summer (8.57%), autumn (5.30%), spring (2.04%) and (0.81%) in winter. The results of the seasonal prevalence of *Vibrio* spp are in concordance with those reported by **Roberts (2001)** who demonstrated that in wild, vibriosis normally occurs in fish in late summer when the temperatures are high. On the other hand, **(Golomazou *et al.* (2006**) reported that *V. alginolyticus* were not associated with a particular season.

The pathogenicty of *V. anguillarum* for experimentally infected *O. niloticus* may be attributed to the effect of the lethal and toxic effect of the extracellular toxins and enzymes produced by the bacterium **(Nottage & Birkbeck, 1987)**.

In concern to the total prevalence of *P. piscicida* in this study recorded that (15.51%) of diseased fish were positive for *P. piscicida*. The results are higher than those recorded by **Balebona *et al.* (1998)** who declared that (6.7%) of diseased gilt-head sea bream, *Sparus aurata* L. in southwestern Spain were infected with *P. piscicida*. On the other hand, our results are lower than those recorded by **Athanassopoulou *et al.* (1999)** who recorded that the prevalence of *P. piscicida* in diseased Cuvier, *Puntazzo puntazzo* L. collected from marine aquaculture systems in Greece was (80%).

Seasonally, the highest prevalence e of *P. piscicida* in our study was recorded during the summer season (7.75%) followed by the autumn (5.30%) followed by the spring (2.44%) on the other hand, it was not recorded in winter. These results are in concordance with those reported by **Magarinos *et al.* (1996) who** declared that *P. piscicida* has certain affinity to the high water temperature inducing fatal disease in fish but only when the water is warm and when water quality is low. On the other hand, **Mladineo *et al.* (2006)** suggested that temperature had not a strong influence on the course of pasteurellosis.

In regard to the pathogenicty of *P. piscicida* for experimentally injected fishes may be attributed to the effect of its toxic and harmful effect of the extracellular products produced by *P. piscicida* that possessed strong phospholipase, cytotoxic, and haemolytic activities (**Nakai *et al.*, 1992**).

In regards to the total prevalence of streptococcal septicemia. The present study recorded that (13.46 %) of infected fish were positive for streptococcal infection. These results higher than those recorded by **Zorrilla *et al.* (2003)** who recorded that 7% of bacterial infection affecting cultured gilthead sea bream, *Sparus aurata* L. was attributed to Gram-positive bacteria. **Hussain (2002)** recorded that (6.25 %) of naturally infected marine fish were positive for streptococcal septicemia.

In regards to the seasonal prevalence of streptococcal septicemia, the highest prevalence of the streptococcal infection was recorded in the summer season (6.16%) followed by autumn (4.08%) and spring (1.22 %) on the other hand it was not recorded during the winter. These results are in concordance with those obtained by **Varvarigos (1997)** who revealed that *Streptococcus* spp cause septicemia to all farmed species mainly during late spring and early summer when sea water temperatures are high.

In regards to the experimental infection *of O. niloticus* with *S. fecalis*. The pathogenicty of streptococci may be attributed to the effect of exotoxins produced by the bacterium **(Kimura & kusuda, 1979** and **Woo, 1999)**.

In regards to the total prevalence of *Staph. aureus*, the present study recorded that (3.67%) of infected fish were positive for Staphylococcal infection. These results were lower than those recorded by **Athanassopoulou *et al.* (1999)** who recoded that the total prevalence of *Staph. epidermidis* among diseased *Puntazzo puntazzo* in marine aquaculture systems in Greece was (10 %). Moreover, **Zorrilla *et al.* (2003)** recorded that (7%) of bacterial infections affecting gilthead sea bream

Sparus aurata L. were attributed to Gram-positive bacteria**.**

Seasonally the highest prevalence of Staphylococcal infection was recorded in the present study in the summer season (2.85%) followed by autumn (0.81%) while it was not recorded in spring nor in winter**.** These results are supported by **Varvarigos (2001)** who declared that *Staphylococcus* spp causing septicemia to all farmed species mainly during late spring and early summer during the high temperature of sea water. This may be explained by the high organic matters in water and the stress induced by the high temperature and hence the decrease in DO.

In regards to the experimental infection of *O. niloticus* with *Staph. aureus***.** The results are in accordance with **Huang (2000)** who indicates that staphylococci can be a possible cause of mortalities and losses among fish.

Table (2) Variable

	A. hydrophila	*A. sobria*	*Ps. fluorescens*	*V. anguillarum*	*V. alginolyticus*	*P. piscicida*
B –Galactosidase production (OPNG)	+	+	-	+	-	-
Arginine dihydrolase production (ADH)	+	+	+	+	-	+
Lysine decarboxylase production(LDC)	-	+	-	-	+	-
Ornithine decarboxylase production(ODC)	-	-	-	-	-	-
Citrate utilization (CIT)	-	Variable	-	Variable	+	-
H2S production(H2S)	-	-	-	-	-	-
Urease production(URE)	-	-	-	-	-	-
Tryptophane deaminase production (TDA)	-	-	-	-	-	-
Indole production(IND)	+	+	-	+	+	-
Acetoin production(VP)	+	+	+	+	Variable	+
Gelatinase production(CEL)	+	+	-	+	+	-
Acid from glucose(GLU)	+	+	Variable	+	+	+
Acid from manitol(MAN)	+	+	-	+	+	-
Acid from inositol(INO)	-	-	-	-	-	-
Acid from Sorbitol(SOR)	-	-	-	+	-	-
Acid from rhamnose(RHA)	+	-	-	-	-	-
Acid from sucrose(SAC)	+	+	-	+	+	-
Acid from from melibiose(MEL)	-	-	V	-	-	-
Acid from amygdalin (AMY)	Variable	-	-	-	Variable	-
Acid from arabinose (ARA)	Variable	-	Variable	Variable	-	-
Cytochrome oxidase prod(OX)	+	+	+	+	+	+

Table (**3**): Prevalence of bacterial infections in the examined marine fishes

Type of M.O / Fish spp	No. Of Exam fish	NO inf fish	*A. hydrophila*		*A. sobria*		*Ps.fluorescens*		*V. anguillarum*		*V.alginolyticus*		*P.piscicida*		*S. fecalis*		*Staph. aureus*	
			No.inf	%	No. inf	%	No. inf	%	No. inf	%	No. inf	%	No inf	%	No. Inf	%	No.inf	%
E. tuvina	100	42	2	4.76	4	9.52	7	16.66	10	23.8	8	19.04	6	14.28%	2	4.76%	3	7.14%
S. rivulatus	100	50	4	8	5	10	6	12	7	14	9	18	10	20%	8	16%	1	2%
S. vulgaris	100	41	6	14.63	0	0	5	12.19	8	19.51	7	17.07	4	9.75	9	21.95%	2	4.87%
M capito.	100	34	3	8.82	2	5.88	4	11.76	5	14.7	3	8.82	9	26.47	6	17.64%	2	5.88%
M. sahlae	100	38	7	18.42	1	2.63	8	21.05	6	15.78	10	26.31	3	7.89%	3	7.89%	0	0
Tilapia zilli	100	40	5	12.5	3	7.5	9	22.5	7	17.5	4	10	6	15	5	12.5%	1	2.5%
Total	600	245	27	11.02	15	6.12	39	15.91	43	17.55	41	16.73	38	15.51%	33	13.46%	9	3.67%

•Percentage was calculated according to the total number of infected fish.

Table (4) Prevalence of bacterial infections in Lake Qarun and Suez Gulf

Locality / M.O	Lake Qarun	Prevalence %	Suez Gulf	Prevalence %
A. hydrophila	14	5.71	13	5.30
A. sobria	5	2.04	10	4.08
Ps. fluorescens	18	7.34	21	8.57
V. anguillarum	20	8.16	23	9.38
V. alginolyticus	14	5.71	27	11.02
P. piscicida	19	7.75	19	7.75
S. fecalis	20	8.16	13	5.30
Staph. aureas	5	2.04	4	1.63
Total	115	46.93	130	53.06

Table (5): collective seasonal prevalence of bacterial infections in the examined marine fishes

Type. of M.o / season	*A.hydrophila*		*A.sobria*		*Ps.fluorescens*		*V.anguillarum*		*V.alginolyticus*		*P.piscicida*		*S.fecalis*		*Staph.aureus*		Total
	No inf	%	No. inf	%	No inf	%	No inf	%	No inf	%	No inf	%	No inf	%	No inf	%	
Winter	14	5.71	4	1.63	19	7.75	1	0.40	2	0.81	0	0	0	0	0	0	15.91
Spring	7	2.85	9	4.08	3	1.22	9	3.67	5	2.04	6	2.44	3	1.22	0	0	17.55
Summer	3	1.22%	1	0.40	6	2.44	23	9.38	21	8.57	19	7.75	20	6.16	7	2.85	40.81
Autumn	3	1.22	1	0.40	11	4.48	10	4.08	13	5.30	13	5.30	10	4.08	2	0.81	25.71
Total	27	11.02	15	6.12	39	15.91	43	17.55	41	16.73	38	15.51	33	13.46	9	3.67	

Table (6): Mortality patterns of experimentally infected *O. niloticus* with the different bacterial isolates.

Bacterial isolates	No of mortality /day												Mortality. (%)
	1	2	3	4	5	6	7	8	9	10	11	2-15	
A.*hydrophila*	-	1	-	2	-	-	2	1	-	1	1	-	80
Ps. fluorescens	-	2	-	-	1	1	2	1	1	-	1	1	100
V. anguillarum	1	1	2	1	2	-	-	1	-	1	-	1	100
P. piscicida	2	0	-	1	1	-	-	2	-	-	1	1	80
S. fecalis	1	-	1	-	-	1	1	1	1	2	-	1	90
Staph. aureas	-	1	-	2	-	-	1	1	-	-	2	-	70
Control	-	-	-	-	-	-	-	-	-	-	-	-	0

N.B. The dose of bacteria inoculated per fish were 0.2 ml of 3 X 10^7 CFU Number of I/P injected fishes per each group were 10.

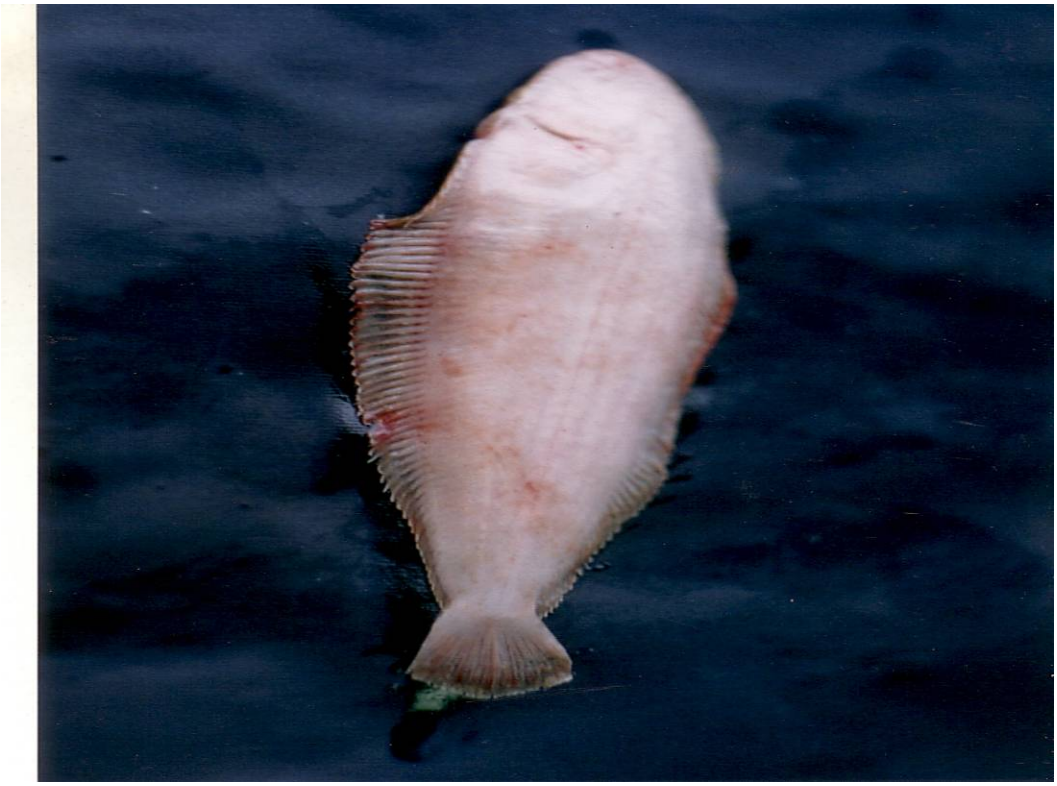


Fig (1) Fish

References

1. **Abdel-Rahman, A. M. (1996):** Studies on bacterial diseases among cultured Tilapia. Thesis, M.V.Sc., Fish Disease and Management, Fac. Vet. Med. Suez Canal Univ.
2. **Alicia, E.; Toranzo, T.; Magarinos, B. & Romalde, J. L. (2005):** A review of the main bacterial fish diseases in mariculture systems. Aquac., **246**: 37– 61**.**
3. **Athanassopoulou, F.; Prapas, T. and Rodger, H. (1999):** Diseases of Cuvier, Puntazzo puntazzo L, in marine aquaculture systems in Greece. Fish Dis., **22**: 215-218.
4. **Austin, B. & Austin, D.A. (1999): Bacterial** fish pathogens; Disaese of farmed and wild fish. 3rd ED. pulblished in association with praxis publishing Chichester.

5. **Bakopoulos, V.; Peric, Z.; Rodger, H.; Adams, A. & Richards, R., (1997):** First Report of Fish Pasteurellosis from Malta. J. Aquac. Anim. Health, **9**: 26-33.
6. **Balebona, M. C.; Andreu, M. J.; Bordas, M. A.; Zorrilla, M. I.; Morinigo, M. A. & Borrego. J. J (1998):** Pathogenicity of *Vibrio alginolyticus* for Cultured Gilt-Head Sea Bream (*Sparus aurata* L.) Appl. Environ. Microbiol., **65**: 4269–4275.
7. **Balebona, M. C.; Zorrilla, M. I; Morinigo, M. A. & Borrego. J. J. (1998):** Survey of bacterial pathologies affecting farmed gilt-head sea bream (*Sparus aurata* L.) in southwestern Spain from 1990 to 1996. Aquac., **166**:19-35.
8. **Baya, A. M.; Lupiani, B.I; Hetrick, F.M.; Robertson, B. S.; Lukacovic, R.; May, E. & Poukish, C. (1990)**: Association of *Streptococcus* spp. with fish mortality in the Chespapeak bay and its tributaries. J. Fish Dis., **13**: 251-253.
9. **Bordas, M. A.; Balebona, M. C.; Rodriguez, J. M.; Borrego, J. J. & Morinigo, M. A. (1998)**: Chemotaxis of pathogenic *Vibrio* strains towards mucus surfaces of gilt-head sea bream (*Sparus aurata* L.). Appl. Environ. Microbiol., **64**:1573–1575.
10. **Bowser, P. R.; Wooster, G. A.; Getchell, R. G. & Timmons, M. B. (1998):** *Streptococcus iniae* infection of Tilapia (*Oreochromis niloticus*) in a recirculation production facility. J.World Aquac. Soc., **29**:335–339.
11. **Bragg, R. R. & Broere, J. S. (1986)**: Streptoccocus in Rainbow trout in South afica. Bull. Eur. Ass. Fish pathol. , **6**: 179-184.
12. **Buller, N. B. (2004):** Bacteria from Fish and Other Aquatic Animals: A Practical Identification Manual. CABI Publishing, Cambridge.
13. **Carmen, P.; Octavio, R.; Juan, A. & German, N. (1990):** Pathogenicity Factors and Virulence for Rainbow Trout (*Salmo gairdneri*) of Motile *Aeromonas* spp. Isolated from a River. J. Clin. Microbiol., **35**: 350-355.
14. **Cipriano, R. (2001):** *A. hydrophila* and motile aeromonad septicemias of fish. Fish Dis.leaflet, 68.
15. **Croxatto, A.; Lauritz, J.; Chen, C. & Milton, D. L. (2007):** *Vibrio anguillarum* colonization of rainbow trout integument requires a DNA locus involved in exopolysaccharide transport and biosynthesis. Environ. Microbiol. **, 9**: 370–382.
16. **Doukas, V.; Athanassopoulou, F.; Karagouni, E. & Dotsika, E.(1998)** *Aeromonas hydrophila* infection in cultured sea bass, *Dicentrarchus labrax* L. and *Puntazzo puntazzo* from the Aegean Sea. J. Fish Dis., **21**: 317-320.
17. **Doukas, V.; Athanassopoulou, F.; Karagouni, E. & Dotsika, E.(1998)** *Aeromonas hydrophila* infection in cultured sea bass, *Dicentrarchus labrax* L. and *Puntazzo puntazzo* from the Aegean Sea. J. Fish Dis., **21**: 317-320.
18. **Ebtsam, S.H. (2002):** Studies on streptococcosis in Nile tilapia *Orechromis niloticus* .in Assut (Upper Egypt) .faculty of vet. Med .Assuit .Univ.
19. **El-Attar, A. A. & Moustaf, M. (1996):** Some studies on tail and fin rot disease among culturd Tilapia Fish .Assiut .Vet. Med. **35**:155-162.
20. **El-Moghazy, D, F. (2004):** Studies on pseudomonas septicemia in cultured *Oreochromis niloticus* fish. Thesis, M.V.Sc., Fish Disease and Management, Fac. Vet. Med. Suez Canal Univ.
21. **Fouz, B.;Toranzo, A.E. ; Milan, M. & Amaro, C. (2000):** Evidence that water transmits the disease caused by the fish pathogen *Photobacterium damselae* subsp. *Damsela.* Appl. Microbiol .,**88**: 531-535.
22. **Frerichs, G. N. & Roberts, R. J. (1989):** The bacteriology of teleosts. In: Roberts, R.J. (ed.) Fish pathology.Bailliere Tindall, London, England, pp.289-3 19.
23. **Fryer, J. L.; and Rohovec, J. S. (1984):** Principal bacterial diseases of cultured marine fish. Helgolander Meeresunters, **37**: 533-545.
24. **Gilles, L. M.; Soyez, C.; Saulnier, D.; Ansquer, D.; Christophe, J .A. & Levy. P. (1998):** Effect of hypoxic stress on the immune response and the resistance to vibriosis of the shrimp Penaeus stylirostris. Fish & Shellfish Immun., **8**: 621-629.
25. **Golomazou, E.; Athanassopoulou, F.; Vagianou, S.; Sabatakou, O. Tsantilas, H.; Rigos, G. & kokkokiris, L. (2006**): Diseases of White Sea Bream (*Diplodus sargusL.*) Reared in Experimental and Commercial Conditions in Greece Turk. J. Vet. Anim. Scin., **30**: 389-396**.**
26. **Hawke, J. P.; Plakas, S. M.; Minton, R. V.; Mcphearson, R.M.; Snider, T.G. & Guarino, A. M. (1987):** Fish Pasteurellosis of Cultured Striped Bass (*Morone saxatilis*) in Coastal Alabama. Aquac., **65**:193-204.
27. **Hawke, J. P.; Thune, R. L.; Cooper, R. K.; Judice, E. & Smith, K. M. (2003):** Molecular and phenotypic characterization of Photobacterium damselae subsp piscicida isolated from hybrid striped bass cultured in Louisiana, USA. J. Aquac. Anim. Health,**15**: 189-201.
28. **Hazen, T. C.; Fliermans, C. B.; Hirsch, R. P. & Esch, G. W. (1978):** Prevalence and distribution of aeromonas hydrophila in the united states. Appl. Environ. Microbiol. ,**36** : 731-738.
29. **Hoda, H.; Yusef, H.; Abd EL-Kader, M. & Abd EL-Latif, H. H. (1999):** Quantitave and qualitative studies on bacterial Microflora of Fish reared in fresh water fish farm in Alexandiria. J. Egyp. Microbiol., **34** : 315-330.
30. **Holt, J.G., Krieg, N.R., Sueath, P.H.A., Satley, J.T., Williams, S.T., (1993):** Bergey's Manual of Determinative Bacteriology. Williams and Wilkins, Baltimore.

31. **Hoshino, T.; Ishizaki, K.; Sakamoto, T. & Kumeta, H. (1997):** Isolation of a Pseudomonas species from fish intestine that produces aprotease active at low tempraturee. Appl. Microbiol .,**25** : 70-72.
32. **Huang, S. L.; Liao, I.C. and Chen, S.N. (2000):** Induction of apoptosis in Tilapia, *Oreochromis aureus* Steindachner, and in TO-2 cells by *Staphylococcus epidermidis*. J. Fish Dis., **23**: 363–368.
33. **Hussain, R. A. (2002):** Studies on some bacterial infections affecting certain marine fishes in the Arabian Gulf of Kingodom of Saudi Arabia. Thesis, M.V.Sc., Fac. Vet. and anim .Res. King Faisal Univ.
34. **Khan, R. A.; Campebll, J. & Lear, H. (1981):** Mortality in captive Atlantic cod, *Godus morhua* L. associasted with fin rot Disease.J. Wildlife Dis., **17**: 521-527.
35. **Kimura, H. & kusuda, R. (1979):** Studies on the pathogenesis of streptococcal infection in cultured yellowtail, *Seriola spp* L. effect of cell free culture on experimental streptococcal infection. J. Fish Dis., **2**:501-510 .
36. **Kooj, D. V. (1988):** Properties of Aeromonads and their occurrence and hygenic significance in drinking water. Zentralb. Bakt. Hyg., 187:1-17.
37. **Krovacek, K.; P, Baloda, S. B.; Soprano, V. C. M. & Dumontet. S. (1994):** Comparison of putative virulence factors in *A. hydrophila strains* isolated from the marine environment and human diarrheal cases in southern Italy Appl. Environ. Microbiol. , **60**: 1379-1382**.**
38. **Larsen, J. L. & Jensen, N. J. (1977):**. An *Aeromonas* spp implicated in ulcer-disease of the cod, *Gadus morhua* L. Nord. Veterinaermed., **29**: 199-211.
39. **Liu, P. C. Lin, J. Y. & Lee, K. K. (2003):** Virulence of *Photobacterium damselae* subsp. *Piscicida* in cultured cobia *Rachycentron canadum* L. J. Basic Microbiol., **43**: 499-507.
40. **Magarinos, B.; Santos, Y.; Romalde, J.; Rivas, C.; Barja, J. & Toranzo, A.E., (1992):** Pathogenic activities of live cells and extracellular products of the fish pathogen Pasteurella piscicida. J. Gen. Microbiol., **138**: 249-2498.
41. **Magarinos, B.; Toranzo, A. E. & Romalde, J. L. (1996):** Phenotypic and pathobiological characteristics of *Pasteurella piscicida.* Annual Rev. Fish Dis., **6**: 41-64**.**
42. **Magarinos, P.; Couso, B.; Noya, N.; Merino, P.; Toranzo, A.E. & Lamas, J. (2001):** Effect of temperature on the development of pasteurellosis in carrier gilthead seabream (*Sparus aurata*). Aquac., **195**: 17-21.
43. **Mansour, S.A. and Sidky, M. M. (2003):** Extoxological studies 6. The first comparative study between lake Qarun and wadi El-rayan wetland, with respect to contamination of their majore components. J. Food chem., **82:**181-189.
44. **Margaret, A. T. (1987):** Factors Influencing seasonal Vibriosis Mortality rates in Swedish pen reared Rainbow Aquac.,**67**: 79-85.
45. **Meyer, F. P. (1970):** Seasonal fluctuations in the incidence of disease on fish farms. Pages 21-29, in S. F. Snieszko, Ed. A symposium on diseases of fishes and shellfishes. American Fisheries Society Special Publication 5. Bethesda.
46. **Meyer, F. P. (1991):** Aquaculture disease and health management. J. Anim. Scin., **69**: 4201-4208.
47. **Mladineo, I.; Miletic, I. & Bocina, I. (2006):** *Photobacterium damselae* subsp. *piscicida* Outbreak in Cage-Reared Atlantic Bluefin Tuna, *Thunnus thynnus* L. J. Aquac. Anim. Health,**18**:51-54.
48. **Molitoris, E.; Joseph, S. W.; Krichevsky, M. I.; Sindhuhardja, W. & Colwell , R. R. (1985**): Characterization and Distribution of *Vibrio alginolyticus* and *Vibrio parahaemolyticus* isolated in Indonesia. Appl. Environ. Microbiol. , **50**: 1388-1394.
49. **Moustafa, M.; Eissa, I.A.M. & Hanafi, M. S. (1990):** Vibriosis in marine fishes of Qarun lake.J. Zag. Vet., **18**: 94-105.
50. **Muroga, K. (2001):** Viral and bacterial diseases of marine fish and shellfish in Japanese hatcheries. Aquac., **202**: 23-44.
51. **Nakai, T.; Fujiie, N.; Muroga, K.; Arimoto, M.; Mizuta, Y. & Matsuoka, S. (1992):** *Pasteurella piscicida* infection in hatchery-reared juvenile striped jack. Fish Pathol., **27**: 103-108.
52. **Nottage, A. S. & Birkbeck, T. H. (1987):** Production of protinase during experimental infection of *Osreea edulis* L. larvae with *Vibrio alginolyticus* and antigenic relation ship between protinase produced by marine Vibrios pathogenic for fish and shelfish. J. Fish Dis., **10**. 265-273.
53. **Onarheim, A. M.; Wiik, R.; Burghardt, J. & Stackebrandt, E. (1994):** Characterization and identification of two *Vibrio* species indigenous to the intestine of fish in cold sea water; description of *Vibrio iliopiscarius* sp. Nov. Syst. Appl. Microbiol .,**17**: 370–379.
54. **Oreilly, T. & Day, D. F. (1983):** Effects of Cultural Conditions on Protease Production by *A. hydrophila.* Appl. Environ. Microbiol., **45**: 1132-1135.
55. **Otoole, R.; lundberg, S.; Fredriksson, S.A.; Jansson, A.; Nilsson, & Watz, H.W. (1999):** The chemotactic response of *Vibrio anguillarum* to fish intestinal mucus is mediated by a combination of multiple mucus components. J. Bacteriol., **181**: 4308–4317.
56. **Pathak, S. P.; Bhattacherjee, J. W. ; Kalra, N. & Chandra, S. (1988):** Seasonal distribution of *A.*

hydrophila in river water and isolation from river fish. J. Appl. Bacteriol., **65**:347-52.
57. **Pedersen, K.; Inger, D. & Larsen, J. L. (1997):** *Vibrio damsela* associated with Diseased Fish in Denmark. Appl. Environ. Microbiol., **63**: 3711–3715.
58. **Popovic, T. N.; Teskeredzic, E.; Perovic, I. S. & Rakovac, R. C. (2000):** *A. hydrophila isolated* from wild fresh water fish in Croatia.Vet. Rsearch communic., **24**: 371-377.
59. **Reno. P .W. (1998):** Factores involved in the dissemination of diseases in fish population . J. Aquac. Anim. Health.**10**:160- 171.
60. **Roberts R. J. (2001):** Fish Pathology, 3rd edn. W.B. Saunders, Philadelphia, PA.
61. **Saavedra, M. J.; Novais, S. G.; Alves, A.; Marta, P. R.; Correia, T.A. & Murcia, A. M., (2004):** Resistance to β lactam antibiotics in *A. hydrophila* isolated from rainbow trout (*Oncorhynchus mykiss*) Int. Microbial., **7:**207-211**.**
62. **Sadek, S. (2000):** Sea bream culture in Egypt; status, constraints and potential. Fish Physiol. Bioch., 22: 171-178.
63. **Samuelsson, O. B.; Nerland, A. H.; Jørgensen, T.; Schrøder, M. B.; Svasand, 1. T. & Bergh, Q. (2006):** Viral and bacterial diseases of Atlantic cod, *Gadus morhua* L. their prophylaxis and treatment: a review. Dis. Aquat. Org., **71**: 239–254.
64. **Sano, T. & Fukuda, H. (1987):** Principal microbial diseases in mariculture in Japan. Aquac., **67**: 59-69.
65. **Snieszko, S. F. (1973):** Recent advances in scientific knowledge and developments pertaining to diseases of fishes. Adv.Vet. Scien. and Comp. Medic., **17**:291–314.
66. **Soliman, W. S. E. (1999):** Bacteriological studies on fresh water fish. Thesis, M.V.Sc., Microbiology, Fac.Vet. Med. Cairo. Univ.
67. **Varvarigos, P. (1997):** Marine fish diseases in Greece Fish Farmer November /December, 33-34.
68. **Varvarigos, P. (2001):** Gram positive cocci-bacteria, *Micrococcaceae, Streptococcaceae*, causing systemic disease in intensively farmed fish. December.Varvarigose .Athens, Greece.
69. **Varvarigos, P. (2001):** Gram positive cocci-bacteria, *Micrococcaceae, Streptococcaceae*, causing systemic disease in intensively farmed fish. December.Varvarigose .Athens, Greece.
70. **Vethaak, A. D. (1992):** Diseases of flounder, *Pralyatichthys flesus* L. in the Dutch Wadden Sea and their relation to stress factors. J. Sea Research, **29**: 257-272.
71. **Wahli, I. T.; Burr, S .E.; Pugovkin1, D.; Mueller, O. & Frey, J. (2005):** *Aeromonas sobria*, a causative agent of disease in farmed perch, *Perca fluviatilis* L. J. Fish Dis., **28**: 141–150.
72. **Wedemeyer, G. A. (1996):** Physiology of Fish in Intensive Culture Systems. Chapman & Hall, New York. 232 pp.
73. **Wobeser, G. A. (1981):** Diseases of wild waterfowl. Plenum.new.
74. **Woo, P. T. K. (1999)**: Fish diseases and disorders .CAB, Int. Wallingford, Oxon. UK.
75. **Yuasa, k.; kitancharoen, N.; kataoka, Y.A. & Al-Mubaty, F. A. (1999):** *Streptococcus iniae*, the causative agent of mass mortality in rabbit fish, *Siganus canaliculatus* L. in Bahrain. J. Aquac. Anim. Health, **11**:87-93.
76. **Zhu, C. H.; He, J. G. & Huang, Z. J. (2000):** Identification and pathogenicity of pathogen of *E. awoara* and *Epinephalus fario* ulceration disease. Acta Scientiarum Naturalium Universitatis Sunyatseni, **39**:278–282.
77. **Zorrilla, M.; Chabrillon, A. S.; Rosales, P. D; Manzanares, E. M; Balebona, M. C. & Morinigo, M. A. (2003**): Bacteria recovered from diseased cultured gilthead sea bream, *Sparus aurata* L. in southwestern Spain.Aquac., **218**: 11 –20.

10/10/2010

Clinical Prespective Of Repeat Breeding Syndrome In Buffaloes

Ahmed W.M., El-khadrawy H.H., Emtenan M. Hanafi , Amal H. Ali , Shalaby S.A.

Department of Animal Reproduction and Artificial Insemination, National Research Centre Dokki,Cairo, Egypt. (wahidmma@hotmail.com)

ABSTRACT: Local meat production in Egypt is in continuous decrease and can not meet the local market requirement. So this study was designed to throw light on true repeat breeding syndrome (RBS) as one of the reproductive disorders that hinders the buffalo meat and milk production. A field survey was carried out on 1358 female buffaloes which were subjected to clinical and gynecological examination , and blood samples were collected for carrying out some relevant analyses. Treatment trials were practiced using different ways to control the condition and the economic impact of this syndrome has been studied. Results revealed that the incidence of clinical repeat breeding (RB) in the examined buffalo cows was 4.34 %. Typical repeat breeders represented 7.25 % of total reproductive disorders in female buffaloes. Serum progesterone level was 1.44 ± 0.39 and 3.66±0.84 in RB and normal buffaloes (NB), respectively. Oxidant/antioxidant markers in RB buffalo-cows showed increased malondialdehyde (MDA) and nitric oxide (NO) and decreased catalase (CAT), superoxide dismutase (SOD), ascorbic acid (ASCA), reduced glutathione (R-GSH) and total antioxidant capacity (TAC). Serum zinc, copper,iron and selenium values were lower in repeat breeder cows compared to normal animals. Repeat breeder buffalo-cows responded to the treatments with mineral mixture, GnRH and Lugol's solution with recovery rates; 63.64, 61.54 and 60.00%, respectively. The study concluded that special care should be paid for food additives to control this syndrome.

[Ahmed W.M., El-khadrawy H.H., Emtenan M. Hanafi , Amal H. Ali, Shalaby S.A. **Clinical Prespective Of Repeat Breeding Syndrome In Buffaloes**. Journal of American Science 2010;6(12):1325-1331]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: Repeat breeding buffaloes - progesterone - oxidant/antioxidants and trace elements

1. INTRODUCTION

Currently, the incidence of infertility becomes relatively increased with consequent reduction of productivity of farm animals. According to global Agricultural Information Network report for 2010, the total number of Egyptian cattle and buffalo decreased from 6.256 million head in 2008 to 6.248 million in 2009 and it is expected to decline in 2010 to level lower than 2009 because of many problems that continue to limit the growth of the animal production industry [1]. The price of domestic beef increased dramatically and the imported frozen meat and liver are still important sources of protein in domestic market. The average per capita consumption of red meat including variety meats is estimated at 8.5 kilograms per year, which is quite low compared to consumption levels in other countries. The lower consumption is mainly due to limited local production combined with lower per capita income [1].

Although buffalo constitutes 49% of the above mentioned number, it is the source for high quality milk (65% of milk production), lean meat (33.9% of meat production) and preferred by most of Egyptians. However, in Egypt and most of developing countries having buffalo population, these animals are mostly raised in small holder farms under hard socioeconomic circumstances [2].

Reproductive disorders, poor nutrition, parasitic infection are the main constraints of buffalo development. Ovarian inactivity, silent heat, endometritis and repeat breeding are the main reproductive disorders in buffaloes in Egypt [3].

Typical repeat breeding (RB) is defined as the animal that did not conceive after three or more consecutive inseminations, despite; it comes normally in heat and shows clear estrous signs with no clinical detectable reproductive disorders [4].

The objective of this study was to throw light on typical repeat breeding syndrome in Egyptian buffaloes with emphasis on the oxidative status and application of some field treatment trials. Also, economic impact of this syndrome has been investigated.

2. MATERIALS AND METHODS

Animals

The current study was conducted on 1358 mature polyparous buffalo cows randomly selected from small-scale holders at Al Sherkia governorate,

lower Egypt during 2008-2010. These animals were fed on Barseem (*Trifolium alexandrinum*), wheat or rice straw and a few amount of concentrate mixture. Based on owner complains, case history, general health condition and the gynecological examination, animals were categorized into two groups; the first group (G1) bred and conceived normally after no more than 3 inseminations. The second group (G2) was those animals which did not conceive after three or more inseminations, despite no clinically detectable reproductive disorders were observed (Typical repeat breeders). Gynecological examination was carried out through rectal palpation aided by ultrasonography machine (PiaMedical Falcs e`Saote, Netherlands) with an endorectal linear array of 8.6 M hertz to register the reproductive status and/or disorders.

Sampling and Analysis:

Blood samples were drawn from the jugular vein of each animal, in tubes with and without EDTA. Serum was separated after centrifugation and stored at −20 °C until analysis. Serum progesterone level was assayed by ELISA microwell technique using kits from DIMA (Germany). The kit had a sensitivity of 2.0 pg/ml with inter- and intra- run precision coefficient of variations of 2.9 and 4.85, respectively [5]. The concentrations of malondialdehyde (MDA) [6], nitric oxide(NO) [7], catalse (CAT))[8], ascorbic acid (ASC) [9], superoxide dismutase (SOD) [10] and total antioxidants(TAC) [11] in the serum and glutathione reduced (GSH-R) [12] in the whole blood were determined by colorimetric methods using chemical kits (Biomed Egypt) and Shimdzu UV 240 spectrophotometer. Zinc, copper, iron and selenium concentrations were determined using atomic absorption spectrophotometry (Perkin Elmer, 2380) as outlined by [13]

Treatment trials:

A total number of 34 repeat breeder buffalo cows was subjected to one of the following treatments: 1- No treatment at all and kept as the control group (n=5).

2- Lugol's Iodine solution (0.5 – 2%) as a vaginal wash for 3 successive weeks (n=5).

3- Receiving 20 g from a mixture of minerals, vitamins and Lasalocids® in their ration for 10 successive days. This mixture was prepared in the laboratory by throuogh mixing of 20 g of zinc sulphate , 6.25 g of copper sulphate, 1.5 g potassium iodide, 30 mg sodium selenite, 200 g AD3E and 5 g Lasalocids®, (F-Hoffman-La Roche,Basle, Swizerland) and sodium phosphate dibasic up to 1 kg [2] (n=11)

4- Receiving an intramuscular injection of GnRH (Receptal, Hoechst Roussel Vet GmbH) (n=13)

Treatments were carried out according to the instruction of manufacturing companies. Animals were followed up during the next weeks for conception.

Economic evaluation:

Economic losses were calculated on light of decreased calf crop, prolonged calving intervals, decreased milk production and veterinary intervention services, cost of the used drugs as well as cost of repeated AI.

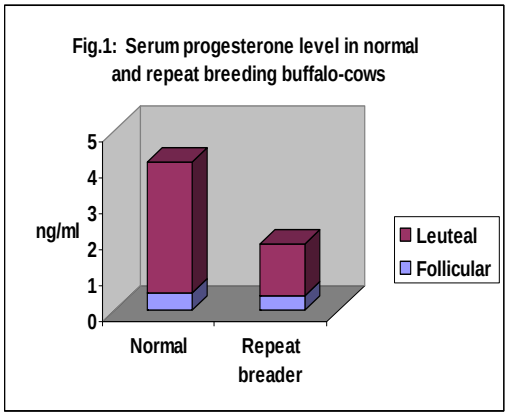
Statistical analysis:

The data were computed and statistically analyzed using PSS-10.5 software package [14].

3- RESULTS

3.1. Incidence

The present study declared that 59 (4.34%) out of 1358 examined polyparous buffalo- cows are typical repeat breeders with an average body condition and good health status. Moreover, this syndrome represented 7.25% of the total recorded reproductive disorders (813).



3.2. Reproductive aspect:

The studied buffalo cows came normally in heat inseminated in the proper time with normal proven fertile bulls and came in heat again after 20 – 22 days during the breeding season of buffaloes (September– March). Ultrasonographic examination

revealed that such animals showed no detectable clinical reproductive disorders with the corresponding normal physiological structure during the different stages of the estrous cycle. Serum progesterone level (Fig. 1) was significantly (P< 0.05) low (0.44 ± 0.39 ng/ml) during the mid -luteal phase of the estrous cycle in repeat breeder animals compared to normal animals **(**3.66±0.84 ng /ml).

3.3. Oxidative status:

Table 1 show the oxidant/ antioxidant status of the investigated animals. It was found that MDA and NO values were significantly high (P< 0.01) while, ASC, GSH-R, Zn, Cu, Fe and Se values were significantly(P< 0.01) low in repeat breeder buffalo-cows compared to normal animals.

Table (1): Oxidant / antioxidants concentrations in repeat breeder buffalo- cows (Mean ± SE).

Oxidant/antioxidant	Normal cows (N=10)	Repeat breeder buffalo-cows (N=10)
MDA (mmol/ml)	1.98 ± 0.09	3.70 ± 0.48 **
NO (mmol/L)	15.55 ± 1.58	25.17 ±0.85**
CAT (U/ml)	2.28 ± 0.4	1.99 ±0.10
ASC (µg/dl)	132.17 ± 5.12	95.16 ±2.37**
SOD (U/ml)	338.16 ± 7.11	332.12±16.14
GSH-R (mmol/L)	6.38 ± 0.11	2.66 ±0.09**
TAC (mmol/L)	1.43 ± 0.08	0.46 ±0.50
Zinc (µg/dl)	139.11 ± 2.17	120.21 ± 5.20**
Copper (µg/dl)	78.65 ± 0.13	68.33 ± 2.05**
Iron (µg/dl)	168.40 ± 4.11	152.13±2.05**
Selenium (µg/L)	144.85 ± 0.34	130.12±2.01**

** P< 0.01

3.4. Treatment trials:

Field trails to treat the typical repeat breeding syndrome using Lugol's solution, mineral mixture and GnRH indicated that 60 – 63 % of the treated animals get conceived as indicated by gynecological examination in 40 – 60 days later, while no animal from the untreated group get conceived. It was found that mineral mixture- treated group gives the highest response .

Table (2): Treatment trials for repeat breeder buffalo-cows (Mean ± SE).

Treatment	Repeat breeder-cows	Recovered animals	Recovery (%)
No treatment	5	0	00
Lugol's solution	5	3	60
Mineral mixture	11	7	63.64
GnRH	13	8	61.54

3.5. Economic evaluation

In the present study, economic losses were estimated as 1588 LE =288$ for every unsuccessful service. Moreover, such losses become greater if the animal did not get pregnant before the end of breeding season.

4- DISCUSSION

Repeat breeding is among reproductive disorders which hinder favorable productivity in buffaloes [15].

In the present study, the incidence of typical repeat breeders was 4.34% of the total examined animals and 7.25 % of all cases having reproductive disorders (813). The same result was found by [3]. Meanwhile, [16] and [17] reported a range of 8.33 - 28% for this syndrome in bovines. Variations in incidences may be attributed to the heterogeneity or multifactorial causes of the repeat-breeder syndrome as well as the effect of locality, season and year [18].

The low progesterone level that was recorded in the current study in repeat breeder buffaloes during the luteal phase was similar to the result of [19] that attributed the failure of conception in these animals to their low progesterone level. Moreover, [20] indicated that RB heifers revealed higher P4 concentrations during estrus and early metoestrus, and lower P4 concentrations during late metoestrus and onwards. In this respect, [21] suggested that the supra basal level of P4 during estrus reduced tubal contractility and delayed sperm transport to the site of fertilization and early embryonic mortality. Also, [17] mentioned that the disturbed hormone level which prolonged standing estrus and delayed ovulation causes changes in the microenvironment of the preovulatory follicle, negatively affecting the final maturation of the oocyte leading to fertilization failure in those repeat-breeder heifers. In another study, he reported a negative correlation between conception rate and skim milk progesterone level in cows artificially inseminated [22].

It is well known that in a healthy body, reactive oxygen species (ROS) and antioxidants remain in balance. When the balance is disrupted towards an overabundance of ROS, oxidative stress (OS) occurs. Also, ROS have a role in pathological processes involving the female reproductive tract, whereas, it affect multiple physiological processes from oocyte maturation to fertilization, embryo development and pregnancy [23]. This theory was confirmed in the current study where RB buffalo-cows showed increased MDA and NO and decreased CAT, SOD , ASCA, GSH-R and TAC. An endogenous NO system exists in the fallopian tubes [24].

NO has a relaxing effect on smooth muscles and it has similar effects on tubular contractility. Abnormal concentration of NO may lead to tubal motility dysfunction, resulting in retention of the ovum, delayed sperm transport and infertility. On the other hand, it was reported that increased NO levels in the fallopian tubes are cytotoxic to the invading microbes and also may be toxic to spermatozoa [24]. Moreover, [25] found that NO inhibits ovarian steroidogenesis. The presence of endothelial NO synthase in corpora lutea and its expression has been reported in the mid and early luteal phase and to a lesser extent in the late luteal phase Moreover, [26] and [27] added that NO inhibits steroidogenesis in the corpus luteum and has luteolytic action mediated through increased prostaglandins and by apoptosis.

SOD is present in the ovarian tissue and it was found that there is a correlation between SOD and Ad4BP which is a steroidogenic transcription factor that induces transcription of the steroidogenic P450 enzyme. Thus, it controls steroidogenesis in the ovaries. The correlation between Ad4BP and SOD expression suggests an association between OS and ovarian steroidogeneis [28]. The preovulatory follicle has a potent antioxidant defense, which is depleted by the intense peroxidation [29].

Glutathione peroxidase may also maintain low levels of hydroperoxides inside the follicle and thus play an important role in gametogenesis and fertilization [30]. Meanwhile, [31] reported that glutathione is present in the oocyte and tubal fluid and has a role in improving the development of the zygote beyond the 2-cell block to the morula or the blastocyst stage

Vitamin C is a chain breaking antioxidant that stops the propagation of the peroxidative process and helps to recycle oxidized vitamin E and glutathione [32].

Increase in TAC was seen in follicular fluid of oocytes that later were successfully fertilized. Therefore, lower TAC is predictive of decreased fertilization potential [33].

The low concentrations of zinc, copper, iron and selenium traced in this study coincide with [34] and [35] who recorded that serum zinc and copper

were significantly low in repeat breeders if compared to normal buffalo cows and added that when these animals were supplemented with 500 ppm zinc acetate in the drinking water and sodium phosphate 40 g/head/day in the diet for 1 month, respectively , the conception rate improved by 80%.and this explains our findings where the treatment with mineral supplementation gave the best results for conception (63.64 %) . This is in agreement with [15] who reported that 64.6 % of repeat breeder buffaloes came to estrus and 58.4 % conceived within one month after supplementation with vitamin/mineral mixture for 3 weeks. Then, he added that the hormone treatment is more effective than 3 weeks supplementation with vitamin/ mineral mixture.

Use of hormonal treatments such as GnRH or hCG, have been used by many investigators to increase the rate of pregnancy for repeat breeder cows [36, 37, 38]. It is suggested that it has a role in the expression of SOD as it is found that the Cu-Zn SOD expression in the corpora lutea paralleled with levels of progesterone and these levels rose from early to the mid luteal phase and decreased during the regression of the corpus luteum. However, in the corpus luteum from pregnant cases, the mRNA expression for Cu-Zn superoxide dismutase was significantly higher than that in midcycle corpora lutea [28]. Other investigators reported that when 36.4- 50.0 % repeat breeder buffaloes washed by 1 liter of 1% Lugol's solution conceived within one month after treatment [15].

From the economic point of view, the repeat breeding syndrome impacts the buffalo industry as it causes increased culling, reduced milk production, and reduced value of breeding stock. On the other side, the indirect costs of sound diagnosis, treatment trials, repeated artificial insemination should also be considered. RBS return the animal to service, increased time to conception and thus increased calving interval in the long-term reduced milk production or permanent infertility. The profitability of milking buffalo-cow increases with age, and culling earlier than the fourth lactation may result in net cost.Also reduced fertility is the commonest reason for culling in the UK [39], so any disease or syndrome affects fertility will have an economic impact. RBS may negatively affect milk production. Whilst an increased calving interval would reduce the number of lactations within a period of years, an RB may increase or prolong the lactation in which it happens. Thus, the impact of RB on milk production is complex and has not been fully quantified. In twenty-two Michigan dairy herds, repeat-breeder syndrome was observed in 24% of 3,309 lactations. Cost components associated with unsuccessful inseminations included costs of delayed conception, extra inseminations, extra veterinary service and losses due to culling. Lactations with repeat-breeder syndrome were associated with a loss of approximately $385. An estimated extra cost of $140 was associated with a second insemination, $279 with three inseminations, $429 with four inseminations and $612 with five inseminations [40].

It was concluded that RBS has economic impact on buffalo production and consequently, local meat and milk production in Egypt. Veterinary supervision should provide better animal health care and education to farmers regarding the risk factors that may lead to RB. Also, use of ultrasonography may help to get rapid and sound diagnosis. Great efforts should be done to catch up the breeding season not to lose the proposed new individual and lactation season. Also special care should be paid for minerals and food additive in animal's food stuff for animal welfare and breeder income.

5-REFERENCES

1- Maldonado, J. and Shreif, S. (2010). Livestock and Product Annual Global Agricultural International Network USDA Foreign Agricultural Service.

2-Ahmed, W.M., El khadrawy, H.H .and Abel Hameed, A .R. (2006): Applied investigation on ovarian inactivity in buffalo heifers. In Proc. of 3rd. Internal. Conf. Vet. Res. Div., NRC; 1-15 .

3- Ahmed, W.M.; Zaabal, M.M. and Abdel Hameed, A.R. (2010). Relationship between ovarian activity and blood lead concentration in cows and buffaloes with emphases on gene frequencies of hemoglobin. Glob. J. Biotechnol. Biochem., 5(1): 1 – 5

4- Yusuf, M.; Nakao, T.;Ranasinghe, R.B.; Gautam, G.;Long, S.T.; Yoshida, C.; Koike, K. and Hayashi,A.(2010): Reproductive performance of repeat breeders in dairy herds. Theriogenology, 73(9):1220-9.

5- Hubl, W. ; Fehert , T.; Ronde, W.; Domer, G.;Taubert, H. and Freymann, E.(1982) . Determination of progesterone. Endokrionlogie, 7 9: 165.

6- Satoh. K. (1987) : Lipid peroxide (Malondialdehyde) colorometric Methods. Clinica Chimica Acta, 90 : 37.

7- Montgomery, H.A.C and Dymock, J. (1961). Determination of nitric oxide. Analysts, 8 (6) 4- 14.

8- Aebi, H . (1984): Catalase in vivo. Methods of Enzymol., 105: 121-126 .

9- Haris, L.T. and Ray, S.N. (1945): Determination of ascorbic acid. Lancet, 71: 462

10- Misra, H. P. and A. Fridovich. (1972):The role of superoxide anion in the autooxidation of epinephrine and a simple assay for superoxide dismutase, J. Biol. Chem., 247 : 3170 – 3175.

11- Koracevic, D.; Koracevic, G.; Djordjevic, V.; Andrejevic,S. and Cosic, V. (2001) : Method for the measurement of antioxidant activity in human fluids. J. Clin. Pathol., 54 : 356-361 .

12-Beutler, E.;Duron, O. and Kelly,B.M. (1963) : Improved method for the determination of blood glutathione. J. Lab. Clin. Med., 61: 882–888.

13- Varley, H.; Gwenlock, A.H. and Bell, M. (1980): Practical Clinical Chemistry. Vol.1. General Topic's commoner test. 5th ed. William Heinemann Medical Books Ltd, London, UK.

14- Snedecor, G.W and Cochran,W.G.(1989): Statistical methods (8 th.), Iowa State University Press, Ames, Iowa

15- Sah, S.K. and Nakao, T. (2006): Characteristics of repeat breeding buffaloes in Nepal. J Reprod .Dev. , 52 (3):335-41.

16- Cebra, C. K.; Heidel, J. R.;Crisman, R. O. and Stang, B. V. (2003): The relationship between endogenous cortical, blood micronutrients and neutrophil function in postparturient Holstein cows. J. Vet. Intern. Med., 17:902-907.

17- Båge, R.;Gustafsson, H.; Larsson, B.;Forsberg, M. and Rodríguez-Martínez, H. (2002): Repeat breeding in dairy heifers: follicular dynamics and estrous cycle characteristics in relation to sexual hormone patterns. Theriogenolog, 57 (9):2257-69.

18- Ali, A.; Abdel-Razek A.Kh.; Derar, R.;Abdel-Rheem, H.A.and Shehata, S.H. (2009): Forms of reproductive disorders in cattle and buffaloes in Middle Egypt. Reprod. Domest. Anim., 44(4):580-6.

19- Rizzo, A.; Minoia, G.; Trisolini, C.; Manca, R. and Sciorsci ,R.L. (2007): Concentrations of free radicals and beta-endorphins in repeat breeder cows. Anim. Reprod. Sci., 100(3-4):257-63.

20- Singh,B.; Saravia, F.; Båge, R. and Rodríguez-Martínez ,H. (2005): Pregnancy rates in repeat-breeder heifers following multiple artificial inseminations during spontaneous oestrus . Acta Vet. Scand; 46(1): 1–12.

21-Binelli, M.; Hampton, J., Buhi, W.C. and Thatcher, W.W. (1999): Persistent dominant follicle alters pattern of oviductal secretory proteins from cows at oestrus. Biol. Reprod. , 61:127–134.

22- Båge, R. ; Petyim ,S.;Larsson, B.; Hallap, T.;Bergqvist, A.S.; Gustafsson, H.and Rodríguez-Martínez, H. (2003) : Oocyte competence in repeat-breeder heifers: Effects of an optimized ovum pick-up schedule on expression of oestrus, follicular development and fertility . Reprod. Fertil . Dev. ,15(1-2):115-23.

23- Agarwal, A.; Saleh, R.A. and Bedaiwy, M.A. (2003) : Role of reactive oxygen species in the pathophysiology of human reproduction. Fertil. Steril., 79:829–843.

24-Rosselli, M.: Dubey, R.K.;Imthurn, B.; Macas, E. and Keller, P.J. (1995) : Effects of nitric oxide on human spermatozoa : Evidence that nitric oxide decreases sperm motility and induces sperm toxicity. Hum Reprod.,10:1786–1790.

25-Seino, T.; Saito, H.; Kaneko, T.; Takahashi,T.;Kawachiya, S. and Kurachi, H .(2002): 8-hydroxy-2'-deoxyguanosine in granulosa cells is correlated with the quality of oocytes and embryos in an in vitro fertilization-embryo transfer program. Fertil.Steril., 77:1184–1190.

26- Vega, M., Urrutia, L.; Iniguez, G.;Gabler, F.;Devoto, L.and Johnson, M.C. (2000):Nitric oxide induces apoptosis in the human corpus luteum in vitro. Mol. Hum .Reprod.,6:681–687.

27- Friden, B.E., Runesson, E., Hahlin .M. and Brannstrom, M.(2000). Evidence for nitric oxide acting as a luteolytic factor in the human corpus luteum. Mol. Hum. Reprod., 6:397–403

28- Suzuki, T., Sugino, N.; Fukaya ,T.; Sugiyama, S., Uda, T.; Takaya, R.; Yajima, A . and Sasano, H. (1999) : Superoxide dismutase in normal cycling human ovaries: Immunohistochemical localization and characterization. Fertil Steril.;72:720–726.

29- Jozwik, M., Wolczynski, S. and Szamatowicz, M. (1999) : Oxidative stress markers in preovulatory follicular fluid in humans. *Mol. Hum. Reprod.*, 5:409–413.

30- Paszkowski, T.;Traub, A.I.;Robinson, S.Y.;McMaster,D.(1995): Selenium dependent glutathione peroxidase activity in human follicular fluid. Clin Chim Acta.; 236:173–180.

31- De Matos, D.G. and Furnus, C.C. (2000): The importance of having high glutathione level after bovine in vitro maturation on embryo development : Effect of b-mercaptoethanol, cysteine, and cystine. Theriogenology, 53 : 761–771.

32- Chan,A.C.(2003):Partners in defense, vitamin E and vitamin C. Can. J. Physiol . Pharmacol., 71:725–731.

33- Oyawoye, O.; Abdel Gadir , A. ; Garner, A.; Constantinovici ,N.;Perrett ,C. and Hardiman, P. (2003): Antioxidants and reactive oxygen species in follicular fluid of women undergoing IVF: Relationship to outcome. Hum. Reprod. 18:2270–2274.

34- Das, J. M.; Dutta, P; Deka, K. C. ; Biswas, R. K.;Sarmah ,B .C. and Dhali, A. (2009):Comparative study on serum macro and micro mineral profiles during oestrus in repeat breeding crossbred cattle with impaired and normal ovulation. Livestock Research for rural development, 21 (5) : http://www.lrrd.org/lrrd21/5/das21072.htm

35- Ceylan,A.;Serin,I.; Aksit,H. and Seyrek, K.(2008). Concentrations of some elements in dairy cows with reproductive disorders .Bull. Vet. Inst. Pulawy, 52:109-112 .

36- Kharche, S.D. and Srivastava, S.K. (2007). Dose dependent effect of GnRH analogue on pregnancy rate of repeat breeder crossbred cows. Anim. Reprod .Sci. ,(1-2):196-201.

37- Alnimer, M.A. and Husein, M.Q. (2007): The effect of progesterone and oestradiol benzoate on fertility of artificially inseminated repeat-breeder dairy cows during summer. Reprod. Domest .Anim. , 42(4):363-9.

38- Khoramian, B.;Farzaneh, N.;Talebkhan ; Garoussi, M. and Mohri , M. (2010): Comparison of the effects of gonadotropin-releasing hormone, human chorionic gonadotropin or progesterone on pregnancy per artificial insemination in repeat-breeder dairy cows. Res. Vet. Sci. (in press)

39- Esslemont, RJ.and Kossaibati, M.A. (1997) : Culling in 50 dairy herds in England. Vet. Rec; 140:36±9.

40-Bartletta, P.C. ; Kirka, J.H. and Mather, E.C.(1986) :Repeated insemination in Michigan Holstein-Friesian cattle: Incidence, descriptive epidemiology and estimated economic impact .Theriogenology, 26(3): 309-322.

10/12/2010

Efficiency of Natural Minerals in Presence of Different Nitrogen Forms and Potassium Dissolving Bacteria on Peanut and Sesame Yields

Gehan H. Youssef, Wafaa M. A. Seddik and Mona A. Osman
Soil, Water and Environ. Research Institute, agricultural Research Center (ARC), Giza, Egypt

ABSTRACT
A field experiment was carried out for two summer seasons at Ismailia Agric. Res. Station to study the effect of some natural minerals combined with potassium dissolving bacteria inoculation in the presence of different nitrogen forms on chemical properties of soil, nutritional status and yield of peanut-sesame. Each experiment was designed in a split-split design with three replications. Three forms of nitrogen fertilizer were included along with two natural minerals, in a presence of potassium dissolving bacteria inoculation, as well as one mineral fertilizer as source potassium fertilizer. Furthermore, data show high significant increases in available N due to the application of ammonium nitrate in combination with feldspar, and calcium nitrate in combination with potassium sulfate in a presence of inoculation for peanut and sesame, respectively. However, application of calcium nitrate combined with potassium sulfate, and ammonium nitrate in combination with feldspar, in a presence of inoculation, led to significant increases in K available in soil for peanut and sesame, respectively. Oppositely, the pH values, different to those of EC, decreased either for inoculation or non-inoculation as compared to control. In spite of that, the values of EC and pH of soil were higher with application of either bentonite or bentonite + feldspar in a presence of all nitrogen fertilizer forms. Generally, the highest EC values in soil, after the two studied seasons were encountered with calcium nitrate fertilizer as well as bentonite mineral. Moreover, applying feldspar mineral and ammonium nitrate treatments had recorded the highest values of yield components as well as nutrient (N and K) uptake by either peanut or sesame crops, particularly in the presence of inoculation as compared to those given by other treatments.
[Gehan H. Youssef, Wafaa M. A. Seddik and Mona A. Osman. **Efficiency of Natural Minerals in Presence of Different Nitrogen Forms and Potassium Dissolving Bacteria on Peanut and Sesame Yields**. Journal of American Science 2010;6(12):1332-1345]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Natural Mineral; Nitrogen; Potassium; Bacteria; Peanut; Sesame

INTRODUCTION

Sandy soils represent the most desert area in Egypt, and they are usually deficient in organic matter and plant nutrients (Abdel Wahab et al, 2003). Potassium is one of the three essential element viz., NPK, for the growth and reproduction of plants; it plays vital roles in its nutrition. The crop production in Egypt relies completely on imports to meet its annual requirement of potash fertilizers; besides the high cost of conventional, water-soluble K fertilizers constrains their use by most of the farmers in the country. In order to reduce the dependence on imported potash, feldspar potash mineral contains 11.25 % K_2O and therefore, it could be a potential K-source for crop production. New approaches are needed to unlock K from the silicate structure of this mineral in order to render K more available for plant nutrition (Badr, 2006). Many researchers showed that microbes can accelerate weathering of minerals and rocks by producing organic acids, phenolic compounds, siderophores and possibly other metabolites, which influence pH and redox conditions. In addition, direct contact between bacteria and minerals may be important in mineral alteration reaction, as microbial surfaces can be complex with metal ions. Some recent reports showed that silicate dissolving bacteria played a promotion role in the release of Si, Fe and K from feldspar (Badr, 2006).

According to Abdel Wahab et al., (2003), the highest values of growth and green yield of pea were obtained in case of organic compost application in combination with chemical or natural sources of P and K. Also, the applied natural sources of P and K gave an almost similar trend to that obtained with the chemical ones combined with the organic compost. The concentrations of phosphorus and potassium in plant tissues increased with increasing compost levels irrespective of their sources. In addition, the combined treatment of organic compost with both sources of P-K achieved the highest values of P and K concentrations in plant tissues.

Significant increases were obtained in height of main stem, branch stem length, number of branches, main stem diameter and leaf area of olive seedlings treated with compost fortified with plant guard and feldspar compared with the control treatment. The same trend was also observed concerning the application of compost and feldspar on micro and macronutrient contents in leaves of the mentioned olive seedlings (Abd El-Motty et al., 2009).

Biofertilizers have been used as sources to improve the status of plant nutrients in sustainable

agriculture. Inoculation with bacterial strain Bacillus edaphicus NBT was found to increase root and shoot growth of cotton and rape. Strain NBT could mobilize potassium efficiently in both plants when illite was added to the soil. In cotton and rape growing on soils treated with insoluble potassium and inoculated with strain NBT, the potassium content was increased by 30 and 26 %, respectively. Bacterial inoculation also resulted in higher N and P contents of aboveground plant components (Sheng, 2005).

Rock P and K applied either singly or in combination did not significantly enhance soil availability of P and K, indicating their unsuitability for direct application. PSB (phosphate solubilizing bacteria) was a more potent P-solubilizer than KSB (potassium solubilizing bacteria), and co-inoculation of PSB and KSB resulted in consistently higher P and K availability than in the control without bacterial inoculum and without rock material fertilizer. Combined together, rock material and both bacterial strains consistently increased further mineral availability, uptake and plant growth of pepper and cucumber plants, suggesting their potential use as fertilizer (Han et al., 2006).

Phosphorus and potassium nutritional status in the soil were markedly improved through inoculation with solubilizing bacteria (Bacillus mucilaginosus); groundnut plant dry matter increased by 125 % and the oil content 35.41 % were increased through bacterium inoculation (Sugumaran and Janarthanam, 2007).

Recently, the treatment of Bacillus circulans + rock phosphate + feldspar was superior in plant height, number of branches, number of nodules per plant and fresh yield (ton/fed.) of snap bean plants when compared with control and the un-inoculated plants. The NPK analysis of shoot dry matter of snap plants showed that as a result of addition of alternatives and the viability of B. circulans, there was marked increases in phosphorus and potassium solubilization (Massoud et al., 2009).

The objective of this study was to determine the efficiency of using different natural mineral as alternative potassium fertilizer in the presence of nitrogen forms and potassium dissolving bacteria inoculation adopted for peanut and sesame plants grown on sandy soil.

MATERIALS AND METHODS

Two summer successive field experiments were carried out on peanut (Arachis hypogaea) – sesame (Sesamum indicum) cropping sequence in a sandy soil under drip irrigation system at Ismailia Agric. Res. Station (A.R.C).

Some physical and chemical characteristics of the studied soil before cultivation are shown in Table (1).

Table (1): Some physical and chemical properties of soil samples representing the studied location.

Soil characteristics	Values
Particle size distribution %	
Coarse sand	50.4
Fine sand	40.4
Silt	3.20
Clay	6.0
Texture class	Sandy
Chemical properties	
CaCO3 %	1.4
pH (suspension 1:2.5)	7.92
EC dS/m (saturated paste extract)	0.37
Organic matter %	0.40
Soluble cations and anions (meq L^{-1})	
Ca^{++}	0.95
Mg^{++}	0.89
Na^{+}	1.51
K^{+}	0.45
$CO3^{--}$	-
$HCO3^{-}$	1.42
CL^{-}	1.02
$SO4^{--}$	1.36
Available nutrients (mg L^{-1})	
N	40.0
P	15.0
K	55.6

Table (2): Analysis of natural mineral constituents.

Determination	Bentonite	Feldspar
EC dS m^{-1}	2.89	0.44
pH	8.08	8.56
Available nutrients (mgL^{-1})		
N	166	216
P	2.10	5.76
K	151	400

Peanut and sesame were cultivated in a randomized split-split plot design, each treatment being replicated three times. The main plots were either inoculated or un-inoculated with potassium dissolving bacteria (Bacillus pasteurii) as (Biopotash). The sub main plots were three nitrogen sources, including ammonium sulfate, calcium nitrate and ammonium nitrate, added at the rate of 30 kg N/fed. The sources of nitrogen were added in four equal split doses after sowing. The sub- sub plots

represented the natural minerals (feldspar and bentonite), which were added individually or in combination (50% bentonite and 50% feldspar, as compared to mineral fertilizer (potassium sulfate)) at the rate of 50 kg K_2O/ fed. Phosphorus fertilizer was added at the recommended dose (200 kg/fed.) for both peanut and sesame in the form of superphosphate 15.5 % P_2O_5. Both potassium and phosphorus fertilizers were completely added to soil before cultivation.

Plant and soil were sampled at 150 days and 120 days after sowing for peanut and sesame respectively, which represent the harvesting stage.

Surface soil samples (0-15 cm depth) were taken after harvesting stage to evaluate soil pH, EC and available nutrients (N and K) were determined according to Page et al. (1982).

Peanut and sesame plant samples were taken at harvesting stage to determine the nutrients status and yield components (straw and grain yield). Plant samples were oven dried at 70 ^oC for 48 hrs up to constant dry weight, then ground and wet digested using H_2SO_4:H_2O_2 method described by Page et al. (1982). The digests were then subjected to the measurement of nutrients (N and K) according to procedures described by cottenie et al. (1982).

Obtained results were subjected to statistical analysis using STATISTICA 6.0 (statSoft, Inc, Tusla, USA) according to Hill and Lewicki (2007). Analysis of variance (ANOVA) was employed to examine the independent and interacted effects of inoculation with potassium dissolving bacteria, nitrogen and potassium sources. Also, treatments were compared by using L.S.D. at 0.05 level of probability according to Snedecor and Cochran (1980).

RESULTS AND DISCUSSION

1- Influence of nitrogen fertilizer, natural mineral and inoculation with bacteria on some soil chemical characteristics.

As for the effect of nitrogen forms and natural mineral on nitrogen and potassium availability, at the two studied seasons, results in Fig (1 A, B) reveal that calcium nitrate treatment was superior at available N while ammonium nitrate being superior at available K.

Furthermore, results showed that the highest values of available nitrogen and potassium exist in case of feldspar and potassium sulfate treatments, respectively at the first studied season. Such results are confirmed by Hagin and Shaviv (1990) who reported that the adequate supply of potassium enhances ammonium utilization and thus improves yields. An opposite trend was obtained at second season, which appeared to be highly significant with applied potassium sulfate for available nitrogen with feldspar being highly significant for available potassium. This obtained data could be due to the application of K solubilizing bacteria, which may produce bacterial acids, alkalies or chelates to enhance solubility and release of elements from potassium containing minerals in soil (Lin Qi-mei , et al., 2002 and Seddik, 2006).

To make the picture clearer, it was thought usefully to express the obtained results as interactions between the influences of both natural mineral and nitrogen fertilizer forms; such interactions are shown in Fig (2). For available nutrients (N and K) in soil, at the two studied seasons, values were significantly increased as a consequence of applied natural mineral in the presence of nitrogen fertilizer and inoculation as compared to control. A previous study (Barker, et al., 1997 and Badr, et al., 2006) confirmed that this bacterial strain produces several organic acids such as acetate, butyric, pyruvic, lactic and formic acid during their biological activities. Such acids can increase mineral dissolution rates; carboxylic acid groups, which were shown to promote dissolution of silicate, are also common in extra cellular organic materials. Moreover, some microorganisms in the soil environment contain enzymes that function in ways analogous to chitinase and celluloses. i.e. they specifically break down mineral structures and extract elements required for metabolism or structure purposes (e.g., mineralization).

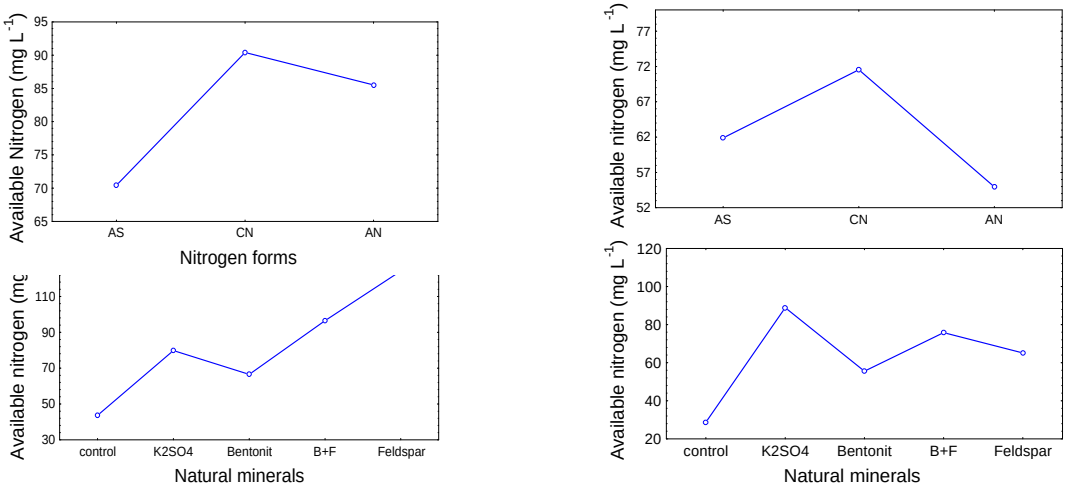
Also, data in Fig (2) show high significant increases in available N due to the application of ammonium nitrate in combination with feldspar, and calcium nitrate in combination with potassium sulfate in the presence of inoculation for peanut and sesame, respectively. However, application of calcium nitrate combined with potassium sulfate, and ammonium nitrate in combination with feldspar, in the presence of inoculation, led to significant increases in K available in soil for peanut and sesame, respectively.
Concerning EC and pH values, the highest EC values in soil following the two studied seasons were reported for calcium nitrate fertilizer as well as bentonite mineral as a source of potassium fertilizer (Fig 3.A). With respect to pH values, the highest values in soil were reported for calcium nitrate at the first season (peanut) and for ammonium nitrate at the second season (sesame). An opposite trend was generally encountered with natural mineral, particularly for applied feldspar, which led to a decrease in pH values at the two seasons as compared to control (Fig 3.B).

Again, to make the picture clearer, it was thought useful to express the obtained results as interactions between the influences of both natural mineral and nitrogen fertilizer forms; such interactions are shown in Fig (4). Data indicated that,

for both studied seasons, application of different natural mineral significantly increased the EC values but decreased the pH values of the studied soil in the presence of nitrogen fertilizer forms as compared with control, whether inoculation or non-inoculation was performed. Moreover, the indicated values of EC and pH were higher in case of applying either bentonite or bentonite + feldspar ratio in the presence of all nitrogen fertilizer forms. Soil salinity increase could be due to the relatively high content of salts in bentonite (Gouda, 1984).

Peanut **Sesame**

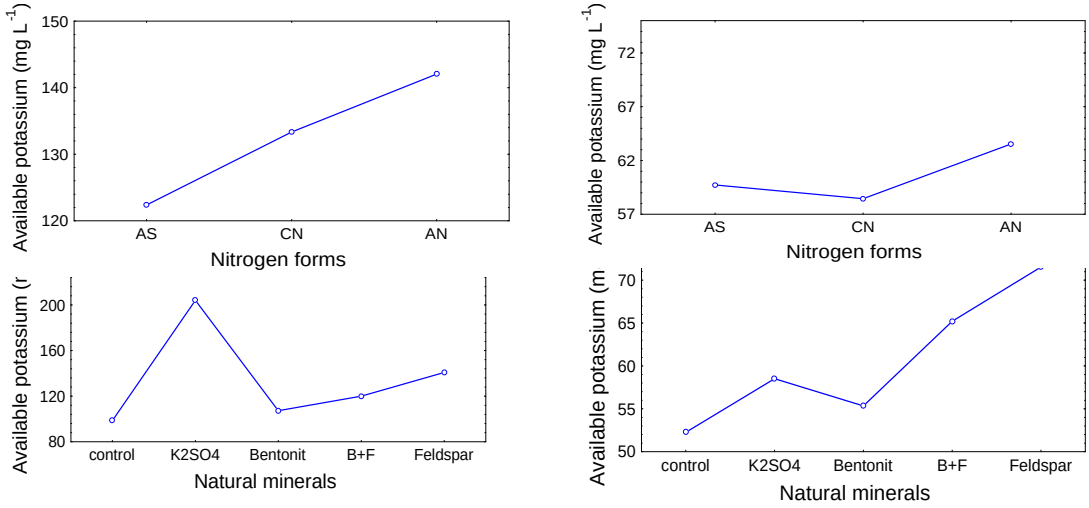
N (A)



Nitrogen forms 5.59 **6.24**

Natural mineral 8.08 **5.99**

K(B)



Natural mineral 19.5 **2.52**

Fig (1): Effect of one factor either nitrogen fertilizer or natural mineral on both nitrogen (A) and potassium (B) availability for the tested soil after peanut and sesame harvesting.

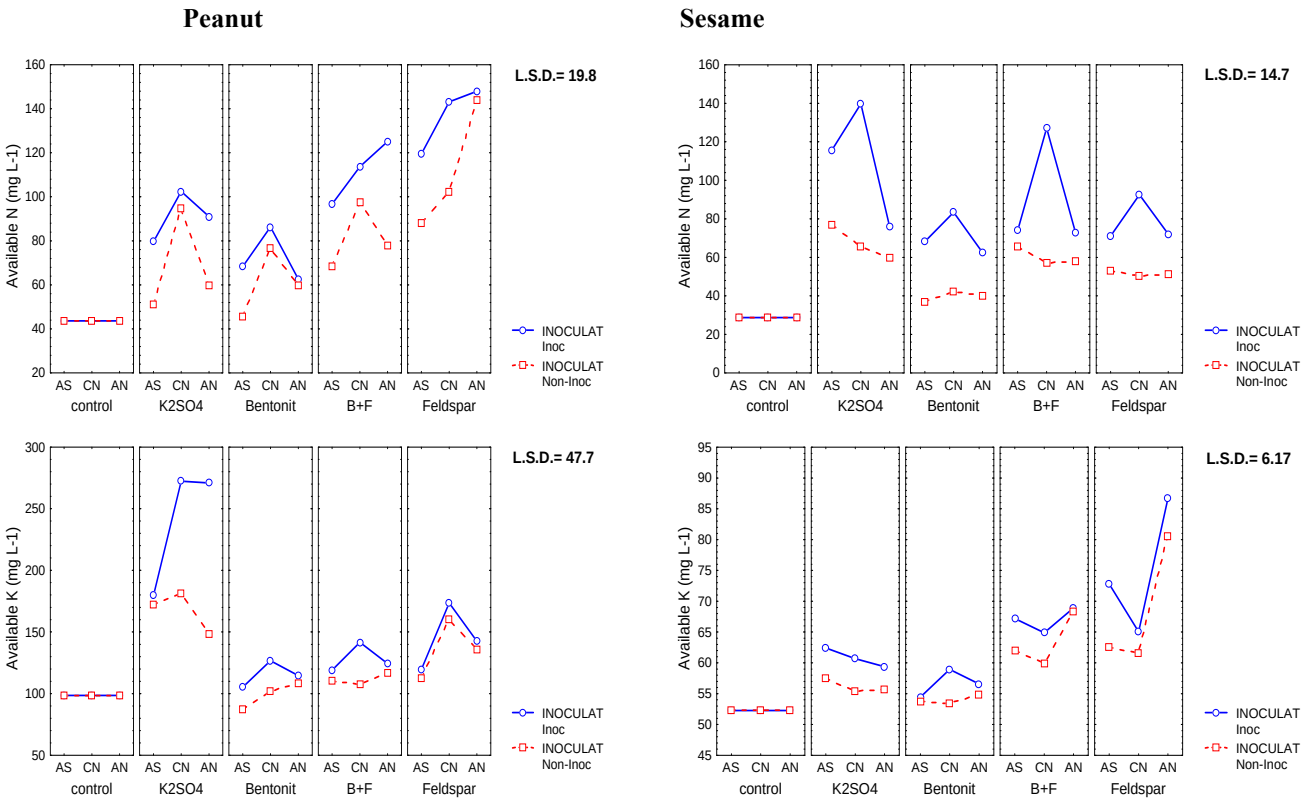
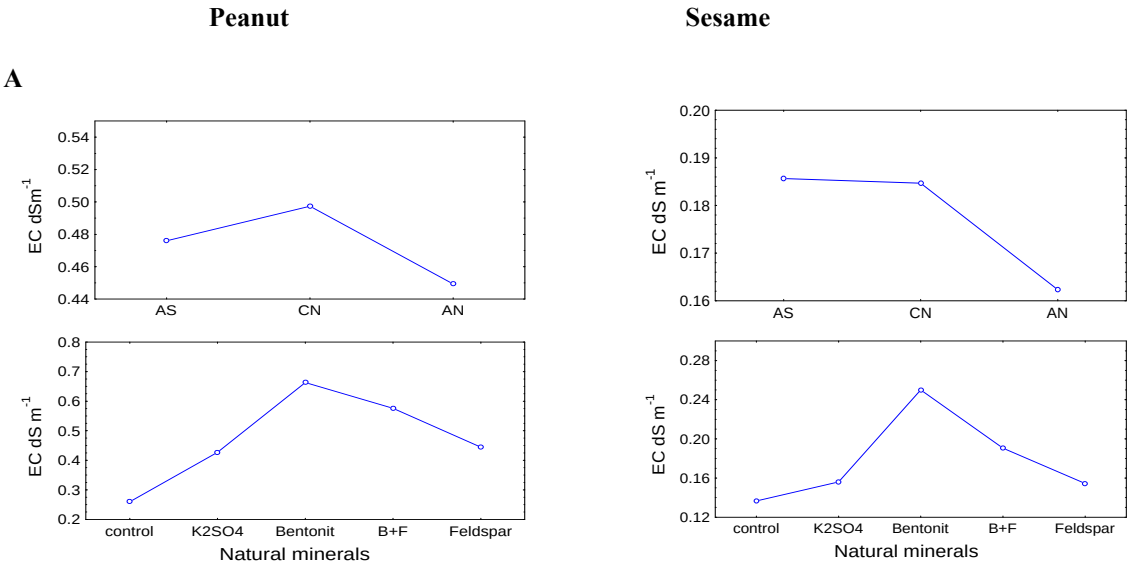


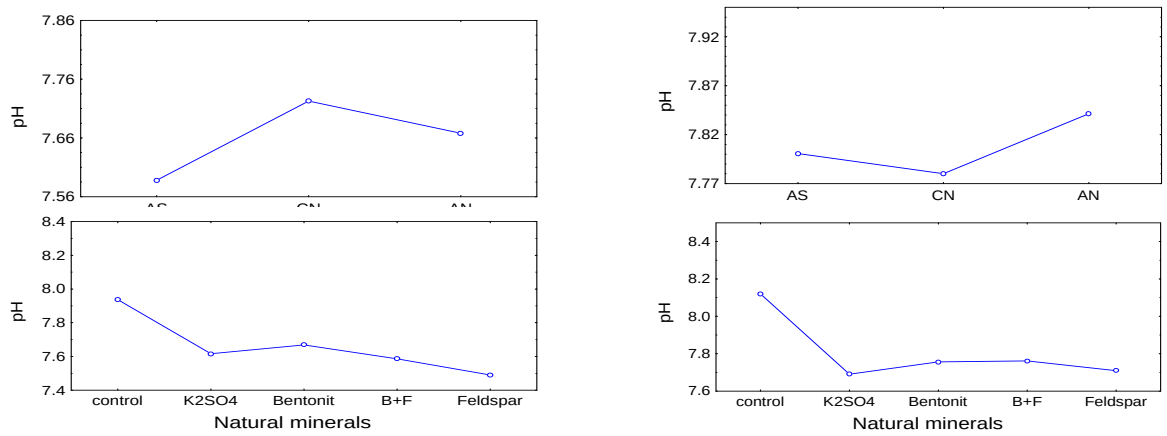
Fig (2): Effect of different sources of natural mineral, nitrogen fertilizer and/ or inoculation with potassium dissolving bacteria on available nutrients (N and K) for the tested soil after peanut and sesame harvesting.

A



L.S.D at 5%		
Nitrogen forms	**0.092**	**0.033**
Natural mineral	**0.070**	**0.042**

B



L.S.D at 5%

Nitrogen forms	**0.09**	**0.11**
Natural mineral	**0.11**	**0.15**

Fig (3): Effect of either nitrogen fertilizer or natural mineral on both electrical conductivity (EC) (A) and soil reaction (pH) (B) for the tested soil after peanut and sesame harvesting.

Peanut **Sesame**

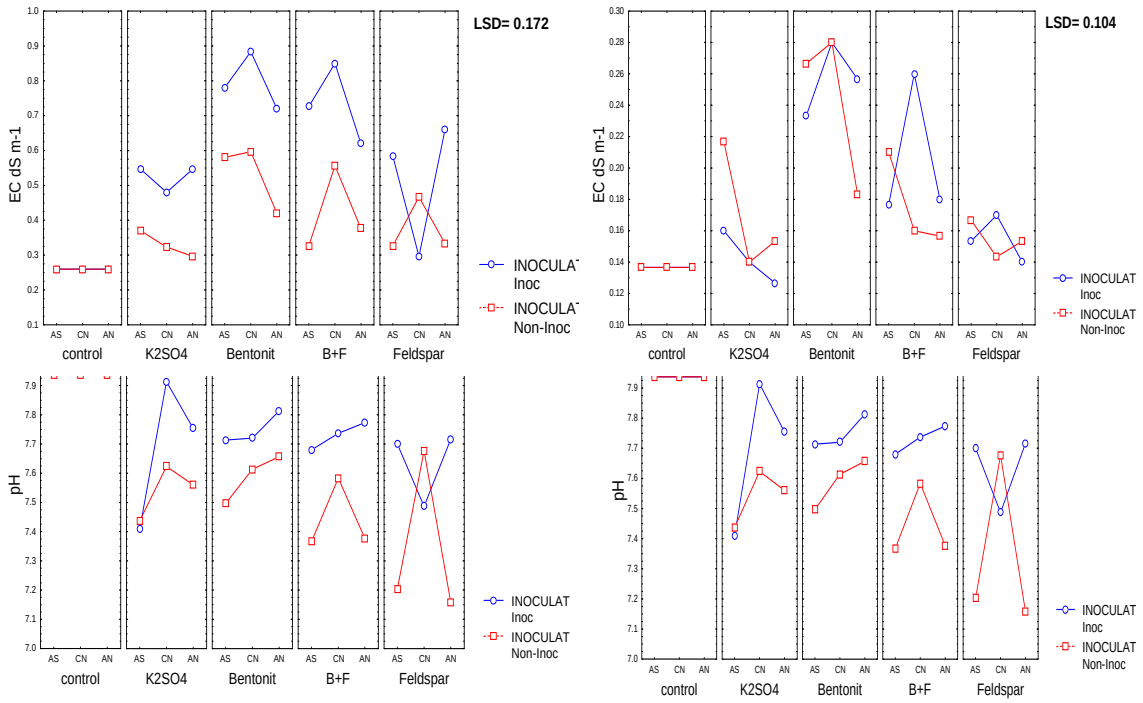
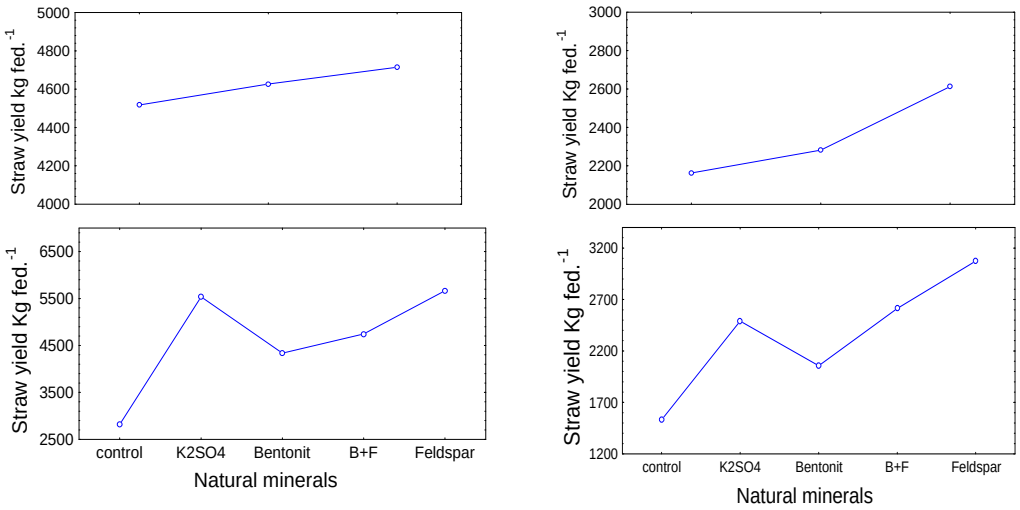


Fig (4): Effect of different sources of natural mineral, nitrogen fertilizer and/ or inoculation with potassium dissolving bacteria on both electrical conductivity (EC) and soil reaction (pH) for the tested soil after peanut and sesame harvesting.

Peanut **Sesame**

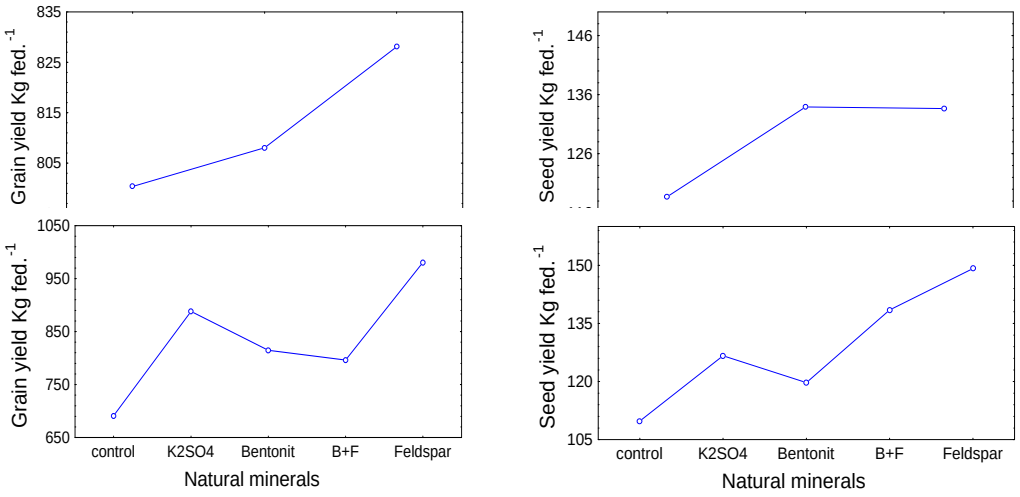
Straw (A)



L.S.D at 5%

Nitrogen forms	**315**	**205**
Natural mineral	**317**	**236**

Grain and Seeds (B)

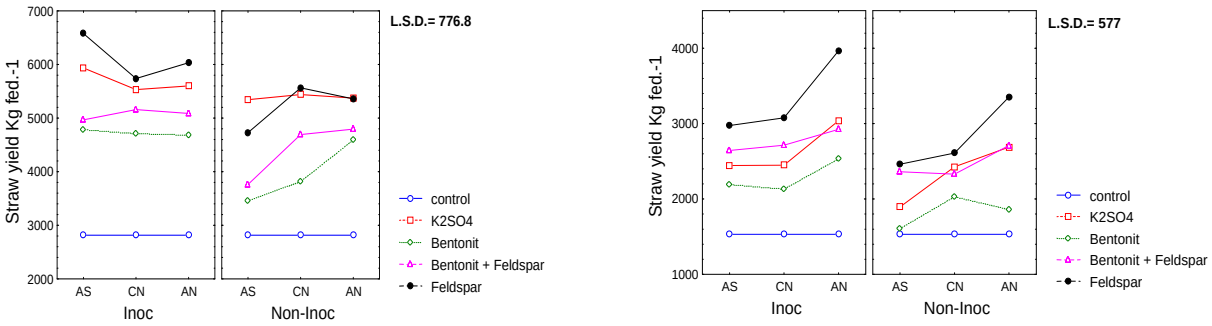


L.S.D at 5%

Nitrogen forms	**63.9**	**7.93**
Natural mineral	**77.7**	**7.49**

Fig (5): Response of yield components, straw (A) and grain or seeds (B), (Kg fed.$^{-1}$) for both peanut and sesame at two seasons to one factor either nitrogen fertilizer or natural mineral at harvest stage.

Straw of peanut **Straw of sesame**



Grain of peanut **Seeds of sesame**

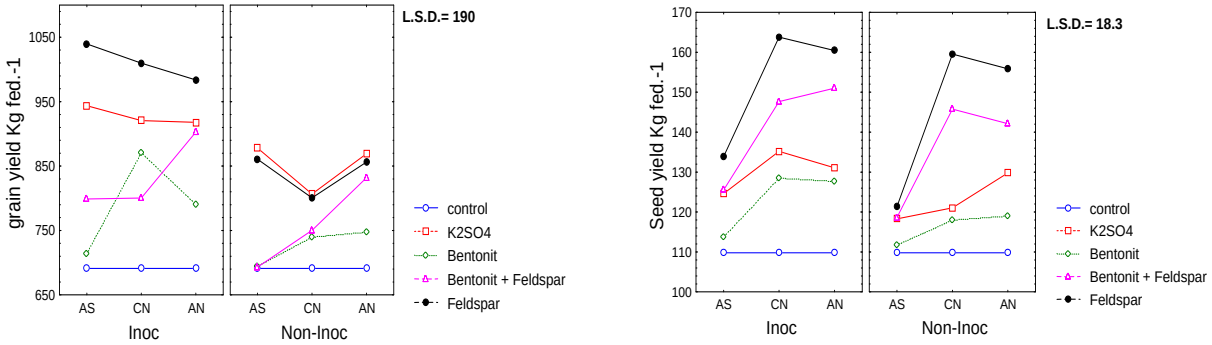
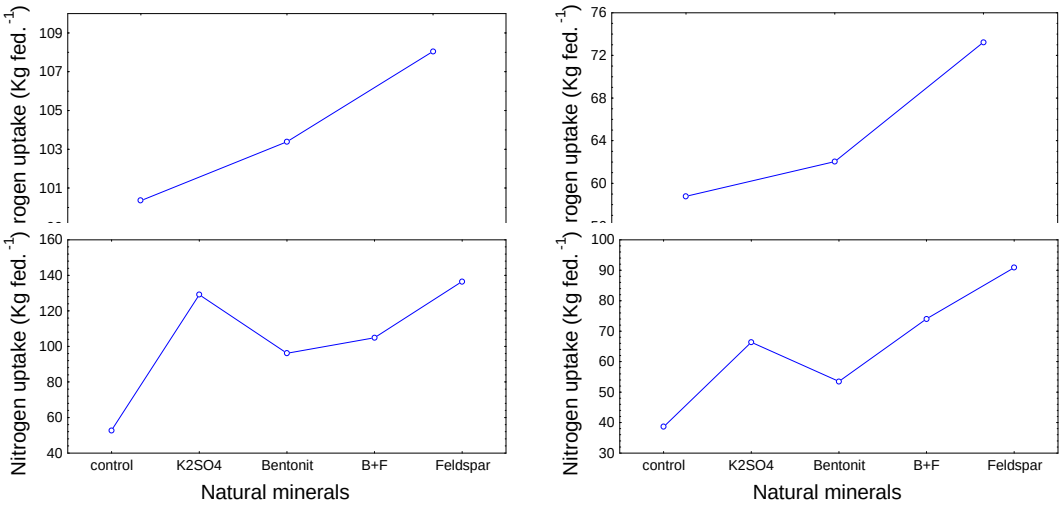


Fig (6): Response of yield components (Kg fed.$^{-1}$) for both peanut and sesame at two seasons to application of different sources of natural mineral, nitrogen fertilizer and/ or inoculation with potassium dissolving bacteria at harvest stage.

Feldspar treatment had recorded the highest values of yield components for either peanut or sesame, particularly in the presence of inoculation as compared to those given by other treatments. These increases in yield components of peanut crop, recorded 117 % and 46.2 % against 118 % and 39.4 % for sesame straw and grains or seeds as compared to control, respectively. These data agreed with the results reported by Badr (2006) who found that the better performance of feldspar-compost plus silicate dissolving bacteria could be attributed to better maintenance of soil nutrient status in the root zone, which in turn helped the plants to utilize nutrients more efficiently; release of potassium took place frequently, and thus favorably affects growth of the crop. Locascio and Hochmuch (1997) reported that potassium supply by the soil is an extremely important factor in yield production, and the high yield depends on the level of K available to the plants. Recently, Massoud et al. (2009) reported that AM-fungi inoculation combined to B.circulans is highly beneficial to the growth of plants. This combination optimizes the K solubilization from feldspar and increased microbial activity in the rhizosphere of plants. So, the weathering of feldspar by AM- mycrrhizal fungi and B. circulans bacteria enhance the release of K ions that led to encouragement for the growth and consequently, the diverse of rhizospheric microflora.

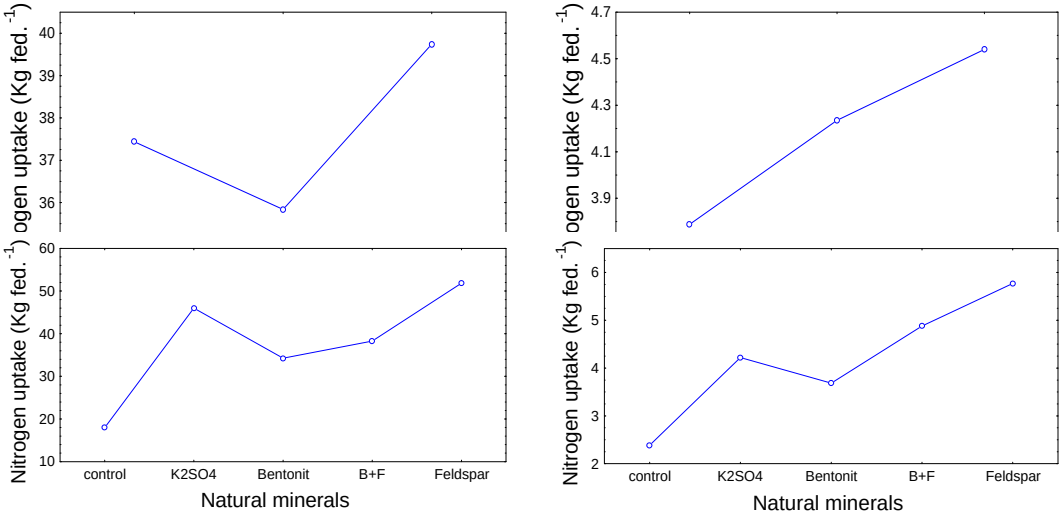
Peanut **Sesame**

Straw (A)



Natural mineral **11.5** **6.44**

Grains and Seeds (B)



L.S.D at 5%

Nitrogen forms **3.08** **0.45**

Natural mineral **4.03** **0.49**

Fig (7): Response of nitrogen uptake, straw (A) and grain or seeds (B), (Kg fed.$^{-1}$) for both peanut and sesame at two seasons to one factor either nitrogen fertilizer or natural mineral at harvest stage.

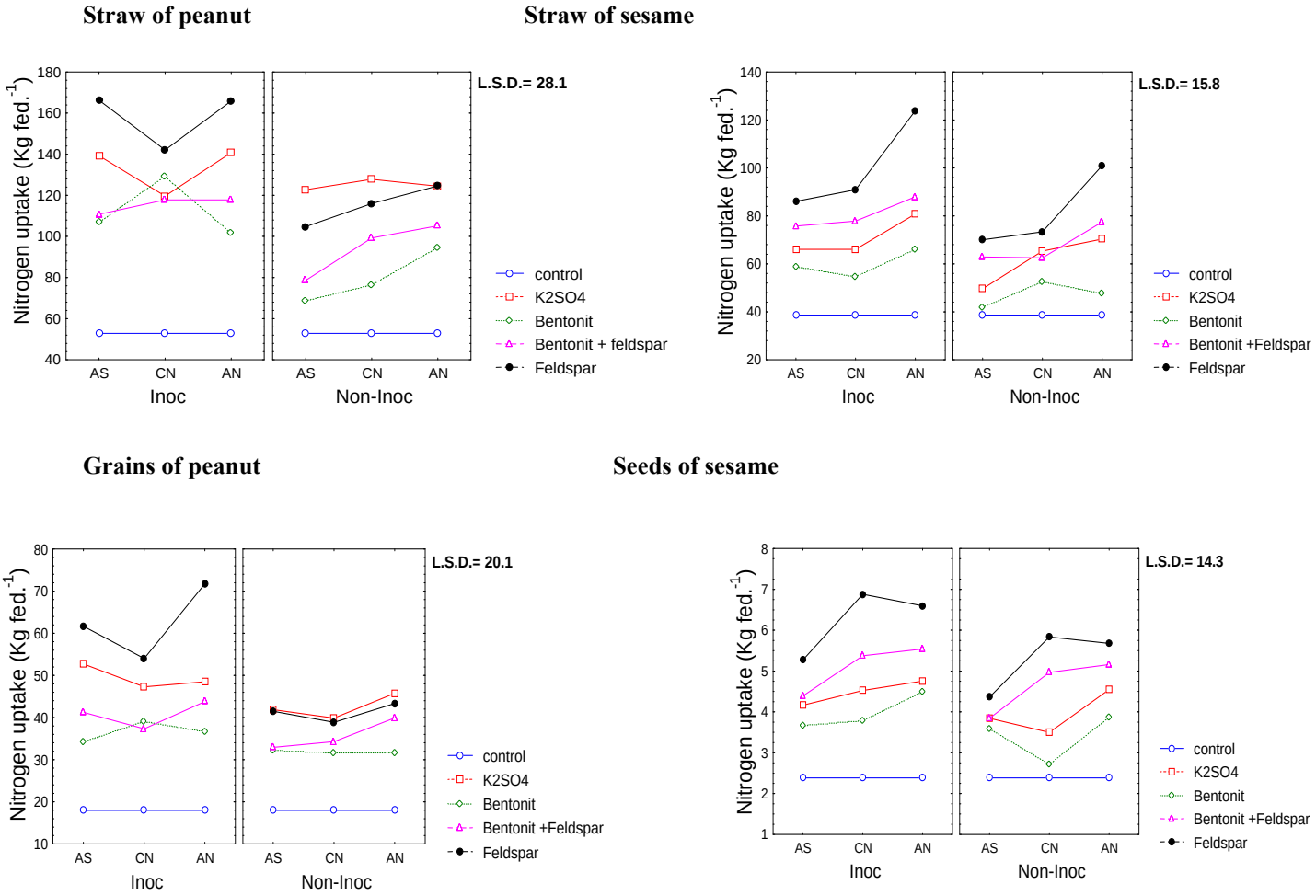


Fig (8): Response of nitrogen uptake (Kg fed.$^{-1}$) for both peanut and sesame at two seasons to application of different sources of natural mineral, nitrogen fertilizer and/ or inoculation with potassium dissolving bacteria at harvest stage.

3- Influence of nitrogen fertilizer, natural mineral and inoculation with bacteria on nutrients uptake at harvesting stage.

A- Nitrogen uptake

With respect to nitrogen uptake of peanut and sesame plants, generally, the highest significant increases for nitrogen uptake of straw and grain or seeds for either peanut or sesame yields were reported for ammonium nitrate fertilizer and feldspar mineral compared to other treatments (Fig 7., A and B). Treatments of nitrogen fertilizer may be generally arranged as follows: ammonium nitrate > calcium nitrate > ammonium sulfate for the two studied seasons. On the other hand, treatments of potassium fertilizer may be arranged as follows: feldspar > potassium sulfate > bentonite + feldspar > bentonite at the first season while arranged as follows: feldspar > bentonite + feldspar > potassium sulfate > bentonite for the second season. Also, behavior of nitrogen uptake followed the same trend of those obtained for yield components at the two studied seasons. In fact, Katai et al. (2010) indicated that the large bentonite doses reduced the nitrate- N content along with available phosphorus and potassium contents of soil, which reflected on nutrients uptake by plants.

To make the picture clearer, it was thought usefully to express the obtained results as interactions between the influences of both natural mineral and nitrogen fertilizer forms; such interactions are shown in Fig (8). Data show that values at the two studied seasons were positively affected by application of natural mineral in the presence of nitrogen forms either of inoculation or non-inoculation compared to control.

Moreover, results showed that the nitrogen uptake was significantly increased with inoculation by potassium dissolving bacteria. These results are in agreement with those of Han et al. (2006) who reported that the soil inoculation with potassium solubilizing bacteria significantly increased nutrients uptake in pepper and cucumber plants, especially when the respective rock potassium were added. Generally, pattern of nitrogen uptake followed the same trend of those obtained with yield components of both crops (peanut and sesame).

Application of feldspar with ammonium nitrate as a source of nitrogen fertilizer generally improved the uptake of nitrogen in plants, especially in the presence of inoculation compared to control. These increases in nitrogen uptake of peanut crop, recorded 214 % and 301 % against 219 % and 176 % for sesame straw and grains or seeds compared to control, respectively.

B. Potassium uptake

However, the highest response for potassium uptake of straw and grains or seeds at the two studied seasons were recorded for ammonium nitrate fertilizer as a source of nitrogen fertilizer with potassium sulfate and feldspar mineral as sources of natural mineral for peanut and sesame yield, respectively. (Fig.9, A and B).

Treatments of nitrogen fertilizer seemed to follow a trend for potassium uptake similar to those obtained for nitrogen uptake. Regarding to treatments of natural minerals, they could be arranged as follows: potassium sulfate > feldspar > bentonite + feldspar > bentonite for the peanut yield while arranged as follows: feldspar > bentonite + feldspar > potassium sulfate > bentonite for the sesame yield. The last behavior of potassium uptake, again, seemed to follow a trend similar to those obtained for nitrogen uptake for the two studied seasons. In the same concern, Badr (2006) found that the total uptake was greater when feldspar- compost plus silicate dissolving bacteria was applied followed by potassium sulfate while the lower was recorded for feldspar. This may indicate that a major portion of K present in feldspar mineral as well as in the organic materials became available for uptake and contributed considerably towards the nutritional requirements of the crop. Further, loses due to drainage, leaching and percolation of potassium from feldspar charged compost are negligible as compared to soluble potassium salts. Hence, use of feldspar in a biological form should be, further, more economical than imported potash fertilizer.

The obtained results as interactions between the influences of both natural mineral and nitrogen fertilizer forms (Fig 10) reveal positive responses for potassium uptake to application of natural mineral treatments in the presence of either nitrogen forms or inoculate as compared to control.

Application of ammonium nitrate with feldspar was generally superior, particularly in the presence of dissolving potassium bacteria. However, an exception being obtained in case of applying calcium nitrate with potassium sulfate for straw at first season compared to control. These increases in potassium uptake of peanut crop, recorded 336 % and 78.3 % against 352 % and180 % for sesame straw and grains or seeds as compared to control, respectively.

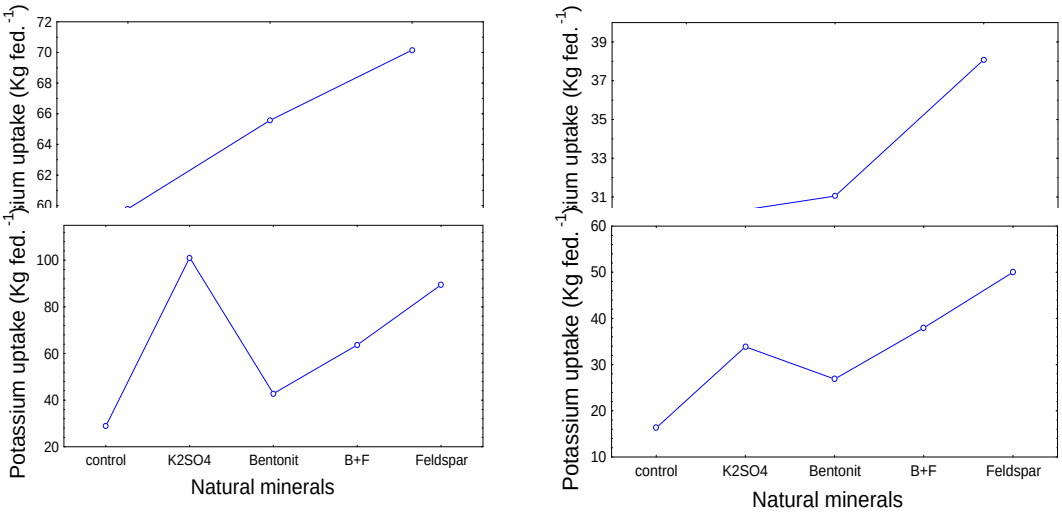
From our obtained results, it could be concluded that, the feldspar mineral and ammonium nitrate as a source of potassium and nitrogen recorded the highest values of yield components as well as nutrient (N and K) uptake for either peanut or sesame particularly in the presence of inoculation. Moreover, the effects of both feldspar and feldspar + bentonite was generally similar to that of potassium sulfate, particularly in the presence of inoculation. So, this biofertilizer is highly efficient to achieve the economy of potash fertilizer and reduce the cost of cultivation through the use of cheap and locally potash source.

Acknowledgment

The authoresses wish to express their sincere gratitude and appreciation to the Development of Soil Conditioners Project, Dept. of Chemistry and Physics of Soil, Soils, Water and Environ. Res. Inst., Agric. Res. Center (ARC), Giza, Egypt, for introducing all facilities needed to accomplish this study.

Peanut **Sesame**

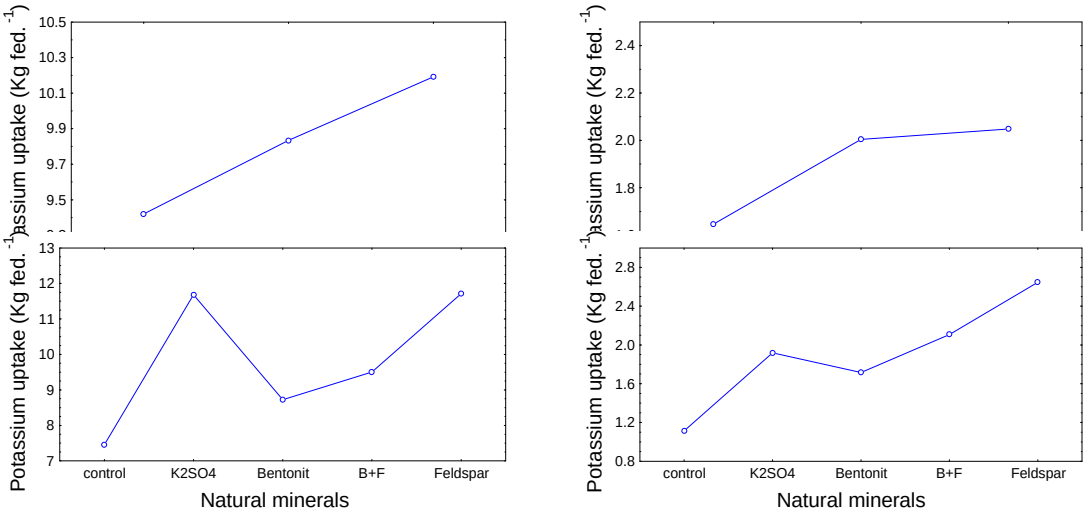
Straw (A)



L.S.D at 5%

Nitrogen forms	**7.68**	**2.81**
Natural mineral	**8.18**	**5.85**

Grains and Seeds (B)



L.S.D at 5%

Nitrogen forms	**1.01**	**0.18**
Natural mineral	**1.01**	**0.16**

Fig (9): Response of Potassium uptake, straw (A) and grain or seeds (B), (Kg fed.$^{-1}$) for both peanut and sesame at two seasons to one factor either nitrogen fertilizer or natural mineral at harvest stage.

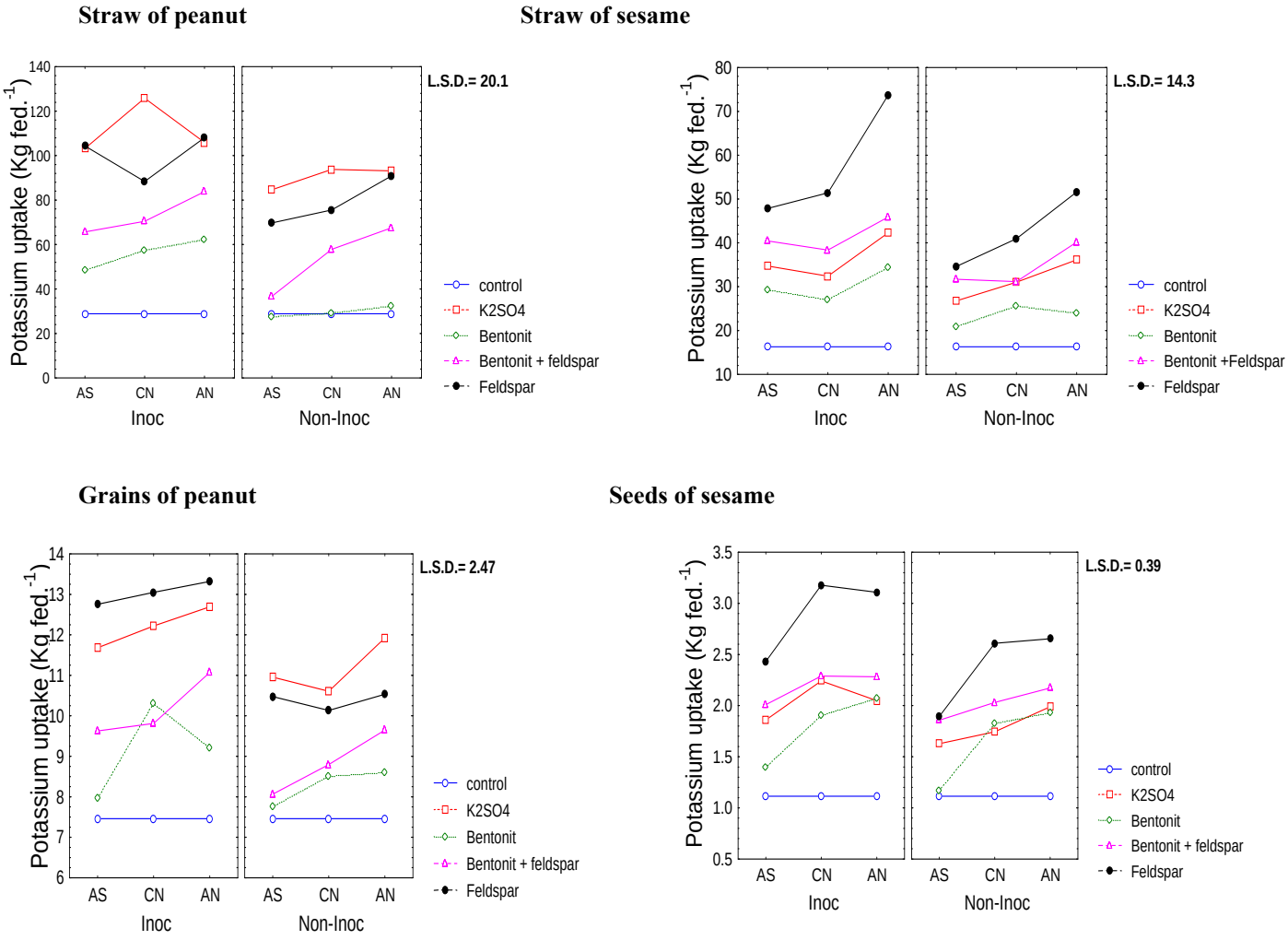


Fig (10): Response of Potassium uptake (Kg fed.$^{-1}$) for both peanut and sesame at two seasons to application of different sources of natural mineral, nitrogen fertilizer and/ or inoculation with potassium dissolving bacteria at harvest stage.

REFERENCES

1. Abd El-Motty Elham Z.; Shahin, M.F.M.; Mabd-El-Migeed, M.M. and Sahab, A.F. (2009). Comparative studies of using compost combined with plant guard and flespar on the morphological, physiological and rhizospheric microflora of olive seedlings. American-Eurasian J. Agric. and Environ. Sci., 6: 372-380.
2. Abdel Wahab, A.F.; Biomy A. H. M. and El Farghal, W.M. (2003). Effect of some natural soil amendments on biological nitrogen fixation, growth and green yield of pea plants grown on sandy soils.Foyoum J. Agric. Res. And Environ. 17: 47-54.
3. Badr, M.A. (2006). Efficiency of K-feldspar combined with organic materials and silicate dissolving bacteria on tomato yield. J. Appl. Sci. Res. 2: 1191-1198.
4. Badr, M.A.; Shafei, A.M. and Sharaf El-Deen S.H. (2006). The dissolution of K and P-bearing minerals by silicate dissolving bacteria and their effect on sorghum growth. Res. J. Agr. and Bio. Sci., 2: 5-11.
5. Barker, W.W.; Welch, S.A. and Banfield, J.F. (1997). Geomicrobiology of silicate minerals weathering. Reviews Mineralogy. 35: 391-428.
6. Cottenie, A.; Verloo, M.; Kiekns, L.; Velghe, G. and Camerlynek, R. (1982).
7. "Chemical analysis of plants and soil" Lab. Anal. And Agroch. St., state Univ., Ghent, Belgium.
8. Gouda, M. A. (1984). Soil and water management of sandy soil. Ph. D.Thesis, Fac. of Agric. Zagazig Univ., Egypt.

9. Hagin, J. and Shaviv, A. (1990). Review of interaction of ammonium- nitrate and potassium nutrition of crops. Journal of plant nutrition. 13: 1211-1226.
10. Han, H.S. and Lee, K.D. (2005). Phosphate and potassium solubilizing bacteria effect on mineral uptake, soil availability and growth of eggplant. Research J. of Agriculture and Biological Sciences. 1: 176-180.
11. Han, H.S.; Supanjani; and Lee, K.D. (2006). Effect of co-inoculation with phosphate and potassium solubilizing bacteria on mineral uptake and growth of pepper and cucumber. Plant Soil Environ. , 52: 130-136.
12. Hill, T. and Lewicki, P. (2007). "Statistics Methods and Applications" Stat Soft, Tulsa, Ok.
13. Katai, J.;Tallai, M.; Sandor, Zs and Olah Zsuposne, A. (2010). Effect of bentonite and zeolite on some characteristics of acidic sandy soil and on the biomass of a test plant. Agrokemia es Talajtan . 59: 165-174.
14. Lin Qi-mei; Rao Zheng-Hug; Sun Yan-Xing; Yao Jun and Xing Li-Jun. (2002). Identification and practical application of silicate-dissolving bacteria. Agric. Sci., China. 1: 81-85.
15. Locascio, S.J. and Hochmuch, G.J. (1997). Potassium source and rate for polyethylene-mulched tomatoes. Hort. Sci., 32: 1204-1207.
16. Massoud, O.N.; Morsy, Ebtsam M. and El-Batanony, Nadia H. (2009). Field response of snap bean (Phaseolus vulgaris L.) to N_2-fixers Bacillus circulans and Arbuscular mycorrhizal fungi inoculation through accelerating rock phosphate and feldspar weathering. Australian Journal of Basic and Applied Sciences. 3: 844-852.
17. Page, A.L.; Miller, R.H. and Keeney, D.R. (1982). " Methods of soil analysis" Amer. Soc. Of Agron. Madison, Wisconsin, U.S.A.
18. Seddik, Wafaa M.A. (2006). Effect of organic manures and feldspar application on some sandy soil physical and chemical properties and their reflection on peanut productivity. J. Agric. Sci., Mansoura Univ., 31: 6675-6687.
19. Sheng, X.F. (2005). Growth promotion and increased potassium uptake of cotton and rape by a potassium releasing strain of Bacillus edaphicus. Soil Biology and Biochemistry. 37: 1918- 1922.
20. Snedecor, G.W. and Cochran, W.G. (1980). "Statistical Methods" 7th Edition. Iowa state univ. press., Ames., IA. USA.
21. Sugumaran, P. and Janarthanam, B. (2007). Solubilization of potassium containing minerals by bacteria and their effect on plant growth. World Journal of Agricultural Sciences. 3:350-355.

كفاءة المعادن الطبيعية فى وجود صور نيتروجين مختلفة و البكتريا المذيبة للبوتاسيوم على محصولى الفول السودانى و السمسم

جيهان حسنى يوسف – وفاء محمد أحمد صديق – منى عبد العظيم عثمان
معهد بحوث الآراضى و المياه و البيئة – مركز البحوث الزراعية – الجيزة – مصر

الملخص العربى

أجريت تجربة حقلية فى موسمين صيفيين متعاقبين بمحطة البحوث الزراعية بالاسماعيلية لدراسة تاثير فاعلية بعض المعادن الطبيعية فى وجود صور مختلفة من النيتروجين مع التلقيح بالبكتريا المذيبة للبوتاسيوم وتأثير ذلك على الخواص الكيميائية و الحالة الغذائية و الانتاجية لمحصولى الفول السودانى و السمسم.
وقد صممت التجربة فى قطع منشقة فى ثلاث مكررات حيث اضيف ثلاث صور من التسميد النيتروجينى و مصدرين من المعادن الطبيعية هما الفلسبار و البنتونيت كمصدر للتسميد البوتاسى فى وجود البكتريا المذيبة للبوتاسيوم.
أظهرت النتائج ايضا زيادة معنوية فى النيتروجين الميسر نتيجة اضافة نترات الامونيوم مع الفلدسبار وكذلك نترات الكالسيوم مع كبريتات البوتاسيوم فى وجود التلقيح البكتيرى لمحصولى الفول السودانى و السمسم على التوالى. كذلك، تبين وجود زيادة معنوية فى البوتاسيوم الميسر نتيجة اضافة نترات الكالسيوم مع كبريتات البوتاسيوم وكذلك نترات الامونيوم مع الفلدسبار فى وجود التلقيح البكتيرى لمحصولى الفول السودانى و السمسم على التوالى.
أظهرت النتائج انخفاضا قيم الـ pH (مختلفة فى ذلك عن قيم الـ EC) فى وجود أو عدم وجود التلقيح البكتيرى و ذلك خلال الموسمين تحت الدراسة.
و بالرغم من ذلك، أظهرت معاملة البنتونيت و أيضا معاملة البنتونيت مخلوطا مع الفلدسبار فى وجود جميع صور النيتروجين تحت الدراسة أرتفاعا فى قيم الـ EC ، pH بحيث ظهرت أعلى قيمة للتوصيل الكهربى عند المعاملة بالبنتونيت مع نترات الكالسيوم لكلا المحصولين.
أما بالنسبة لنتائج المحصول فأظهرت المعاملة بمعدن الفلسبار مع التلقيح و نترات الامونيوم أعلى قيم للمحصول وكذلك أمتصاص العناصر (النيتروجين و البوتاسيوم) فى كل من محصولى الفول السودانى و السمسم.

2010/12/10

Mitochoderial cytochrome c oxidase subunit 1 (*cox 1*) gene sequence of the *Hymenolepis* species.

Omnia M. Kandil, Mona S. Mahmoud, Nesreen A.T. Allam, Amira H. El Namaky

Corresponding author: Kandil, O.M., Prof. of Parasitology, Parasitology and Animal Diseases Department, Veterinary Division, National Research Center, Dokki, Giza, Egypt, P.O. Box 12622, Fax +20233370931, Mobile +20105414113 kandil_om@yahoo.com

Mahmoud, M.S., Prof. of Parasitology, Parasitology and Animal Diseases Department, Veterinary Division, National Research Center, Dokki, Giza, Egypt, P.O. Box 12622, monasaid3000@yahoo.com

Allam, N. A. T., Researcher of Molecular Biology and Animal Diseases, Parasitology and Animal Diseases Department, Veterinary Division, National Research Center, Dokki, Giza, Egypt, P.O. Box 12622, nesreenallam_nrc@yahoo.com

El Namaky, A.H., Researcher of Parasitology, Parasitology and Animal Diseases Department, Veterinary Division, National Research Center, Dokki, Giza, Egypt, P.O. Box 12622, amiraelnamaky@g.mail.com

Abstract: *Hymenolepis nana* and *H. diminuta* are the most common cestodes in humans, domestic and wild rodents. Since isolates of *H. nana* species are morphologically identical, the way they can be reliably distinguished is comparing the parasite in each host using molecular techniques. In the current study, Mitochondrial Cytochrome *c* oxidase gene especially codons within subunit 1 (*cox1*) of *H. diminuta and H. nana* Egyptian isolates from different developmental stages (adult worms and eggs) and hosts origin (human and rat) were amplified, sequenced and aligned. PCR products were approximately 700 bp, 702 bp and 715 bp of *H. nana* rat isolates, *H. diminuta* rat isolates, and *H. nana* human isolates, respectively. Moreover, despite their host susceptibility differences they all gathered in one cluster with three genbank published isolates of *H. nana*; AB033412.1, AB494472.1 and AY121842.1), forming one clade with 100% similarity, which was non significantly decreased on internal nodes. In addition, clearly far away from *H. diminuta* published sequence AB033412.1 who's assumed to be genetically closely related to Egyptian *H. diminuta* than all other *H. nana* isolates. Both Egyptian murine isolates of Hymenolipidid; *H. diminuta* and *H. nana*, were closer to each other than being to *H. nana* of human origin. The annotated sequences of Egyptian isolates were deposited in GenBank under the following accession numbers; *H. diminuta* (GU433102), *H. nana* rat isolate (GU433103), and *H. nana* human isolate (GU433104). Finally, the development of effective control strategies will only be possible if complete understanding of the epidemiology of infestation is elucidated.

[Omnia M. Kandil, Mona S. Mahmoud, Nesreen A.T. Allam, Amira H. El Namaky. **Mitochoderial cytochrome c oxidase subunit 1 (*cox 1*) gene sequence of the *Hymenolepis* species**. Journal of American Science 2010;6(12):1346-1353]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Hymenolepidid, Phylogeny, Cytochrome *c* oxidase subunit 1 gene (*cox1*), Sequencing.

Introduction

Hymenolepis nana and *H. diminuta* are the most common cestodes in humans, domestic and wild rats, mice and dogs **(Macko and Hanzelova 2008)**. During hymenolepiasis various pathological, immunopathological and physiological alterations are recorded. They similarly stimulate abdominal pain accompanied by diarrhea and anorexia as well as increase in the number of mast cells and eosinophiles in the infected individuals. These uncharacteristic symptoms could not be useful in differential diagnosis clinically or on microscopical examination of fecal samples for eggs **(Raether and Hänel 2003)**. It is believed that infestations with *Hymenolipis* spp., in general, may have been under diagnosed due to sporadic egg shedding **(Thompson et al. 2001)**. Since isolates of *H. nana* infecting humans and rodents are morphologically identical, the only way they can be reliably distinguished is comparing the parasite in each host using molecular techniques **(Macnish et al. 2002a, b)**.

Mitochondrial (mt) genomes are small (usually less than 20000 bp), circular, and maternally inherited **(Boore 1999)**. In addition to high copy-number per cell which has made them attractive and more tractable targets for characterization, population genetic and phylogenetic studies **(Hu et al. 2004; McManus et al. 2004)**. Regions within the mitochondrial DNA (*mt*DNA) have been proven useful in biology, epidemiology and diagnosis of

several parasitic infestations of human and veterinary importance **(Ngarmamonpirat et al. 2005; Ando et al. 2006)**. Methods used to obtain data from flatworm *mt* genomes have included DNA sequencing, restriction fragment length polymorphism (RFLP) analysis and single-strand conformation polymorphism (PCR-SSCP) **(Boore and Brown 1998; Avise, 2000).** Intraspecific sequence variation in coding portions (genes) of the *mt* genomes seems to range from small to moderate, especially when compared with interspecific variation that have demonstrated the deep separations among strains of same species **(Littlewood et al. 2008)**.

Complete or near-complete *mt*DNA sequences are available for 12 species of parasitic flatworms; six cestodes including *Taenia crassiceps* **(Le et al. 2000)**, *Echinococcus multilocularis* **(Nakao et al. 2000)** and *Hymenolepis diminuta* **(von Nickisch-Rosenegk et al. 2001)**. Cytochrome c oxidase (COX) is a 13-subunit protein complex located on the inner mitochondrial membrane that catalyzes electron transfer, proton translocation processes, production of up to 95% of the energy of eukaryotic living cells **(Saraste 1999; Johnston 2006)**, thus directly influence metabolic performance. *mt cox* sub unit 1 is the most highly conserved among 3 genes coding for cytochrome oxidase, therefore has been employed in several phylogenetic studies **(Traversa et al. 2007)**.

DNA sequencing of informative regions within the gene encoding for the COX1 protein have emphasized specific comparative aspects without yet making a detailed genome description but revealed data for basic and applied potential differential studies on *Hymenolopis* spp. determining host specificity and transmission patterns **(Macnish et al. 2003).** Therefore, allow more appropriate approach for control of endemic infestations in Egypt, particular where rodent's population is above control limits and hygienic measures are not strictly applied. Furthermore, for diagnostic purposes since using techniques able to overcome inherent limits of the classical approaches **(Constantine 2003; Thompson et al. 2004)**. Epidemiologically, despite this infection is a hand-to-mouth rote that in general not very pathogenic, however it is extremely difficult to be controlled **(Littlewood et al. 2008)**. Till now education in hygiene is probably the only practical way to reduce the incidence in addition to rodent's eradication **(Behera et al. 2008)**. The genotyping of *Hymenolepis* isolates in different hosts will help in determine host specificity and transmission patterns and thus allow more appropriate approach to control infections in endemic communities. From a public health perspective, a better understanding of the transmission dynamics of a parasite species previously believed to be infective only to rodents will be required to answer questions about the potential for transfer of this parasite to humans and/or animals.

Since control of parasitic disease is dependent on the rapid and accurate detection of causative agents this necessitated traditional techniques being complemented by molecular tools that provide predictive data on genetic variation in and among parasites **(Thompson et al. 2004)**. Thus the present work aims is to characterize, for the first time, partial sequences of *cox1* genes of *H. diminuta* and *H. nana* Egyptian isolates to promote basic knowledge on their *mt*DNA composition, to assess the sequence variation level within local Hymenolepidid from different sources, different developmental stages (adult worms and eggs) and hosts origin (human and rat), and to discuss the potential benefits of such molecular information as record sheets for ecological, epidemiological, transmission and host-parasite interaction and as diagnostic approach of infestation in Egypt.

Materials and Methods

Parasites Samples:

H nana eggs were obtained from infected humans in Endemic Diseases institute. Approximately 2000 *H. nana* eggs were inoculated into 5-week-old male white mice **(Movsesyan et al. 2008)**. Adult worms were dissected from the small intestine approximately 14 days post-inoculation. *H. diminuta* worms were obtained from naturally infected *norvegicus* rat from Abu Rawash, Giza, Egypt. Rats were killed by cervical dislocation and entire small intestine was removed from gut. The worms and eggs washed repeatedly in phosphate buffered saline (PBS) and stored at -80 ^{o}C until used for DNA extraction.

Isolation of DNA from Adult Worms and Eggs

Templates DNA were purified from *H. nana* and *H. diminuta* using QIAmp tissue purification kit **(Qiagen, Hilden, Germany)** according to manufacturer's instructions **(Macnish et al. 2002a)**. DNA was eluted in 200 µl Tris-EDTA (TE) buffer and 1 µl of the extract was added to the polymerase chain reaction mix. Single adult worm and/or eggs for each isolate were used for DNA extraction.

Oligonucleotide Primers Design

Entire *mt* genomes of the following species were aligned *Hymenolepis diminuta* (accession number AB033412.1), *Taenia crassiceps* (accession number NC_002547), *T. solium* (accession number NC_004022), *T. asiatica* (accession number

NC_004826), *Echinococcus granulosus* (accession number NC_008075), and *E. multilocularis* (accession number NC_000928); and annotated sequence of *Hymenolepis nana* (accession number AF314223.1) **(Nakoo et al. 2000, 2002; von Nickisch-Rosenegk et al. 2001).** It was not deemed necessary to include all the available sequences from *Taenia* or *Echinococcus* as conservation of alignable positions between genera and being > 30% GC was more important for PCR primer design. PCR primers pair designed *cox1*-F 5`-ACTTCATTGCTTTTGCTTTTTAGA-3` and *cox1*-R 5`-TGCTGTCATAAATGAACCAACAGT-3` were synthesized by Metabion International AG **(Martinsried/Deutschland)**.

PCR Amplification Protocol

Fragments of the mitochondrial cytochrome *c* oxidase subunit 1 gene were amplified using designed primers and each PCR mix was prepared in 50 µl total volume with 1 µl of template (50 ng), 10 pMoles of each primer, 45 µl of Ready TaqMix Complete **(Mater Mix, AllianceBio, USA)**, and nuclease free water **(Qiagen, Germany)** to complete the total volume of the reactions. PCRs were performed in a PTC-100™ Thermal Cycler **(MJ Research Inc., USA)** using the following cycling protocol: initial denaturation at 95^0C for 3 min and then 40 cycles of 94^0C for 1 min 50 sec, 58^0C for 1 min 30 sec, and 72^0C for 1 min. Final extension was carried out at 72^0C for 7 min. A reagent blank was run as control in every PCR procedure. Positive results by PCR were retested on two further occasions several days later to examine the reproducibility of PCR. Amplified products from the PCRs were electrophorised on 1.5% agarose gels **(Bioshop Canada, Burlington, Ontario, Canada)** stained with ethidium bromide (0.5 µg/ml) **(Bioshop Canada) (Sambrook et al. 1989)**. A 100 bp ladder **(Jena Bioscince, GmbH, Germany)** was loaded in each gel then photographed under UV light with gel documentation system.

Sequencing of *cox1* Gene Products

PCR-product of each isolates were purified with QIAquick-spin PCR purification kit **(Qiagen, Germany)** then directly sequenced from both directions using ABI Prism™ Dye Terminator Cycle Sequencing Ready Reaction Kit **(Applied Biosystem, FosterCity, California)** according to manufacturer's instructions on a 3130XL Genetic Analyzer **(Applied Biosystems)**. At least two independent PCR products were used for sequencing per isolate.

Sequences Analysis

The resulting aligned output was manually adjusted **(Lee et al. 2007)**. Sequences corresponding to the PCR amplification primers were excluded prior to multiple sequence alignment and phylogenetic analysis. The confirmed sequences were then deposited in the EMBL/GenBank Data Libraries of the NCBI. In order to improve the homology statements out group included *Taenia saginata* (AB465239.1), *T. solium* (AY211880.1), *T. multiceps* (GQ228818.1), *Echinococcus granulosus* (AF314223.1), *E. multilocularis* (AF314223.1) and *Spirometra erinaceieuropaei* (AB374543.1), as well as all annotated sequences of *Hymenolepis diminuta* (AB033412.1) and *Hymenolepis nana* (AF314223.1, AY121842.1, AB494471.1, AB494472.1, AB033412.1, AF314223.1) by Basic Local Alignment Search Tool (nBLAST) (www.ncbi.nih.gov/BLAST/) in the NCBI database (National Center for Biotechnology Information, NIH, Bethesda, Maryland, USA) **(Tatusova and Madden 1999).** The alignment gaps were treated as missing data. Phylogeny of Egyptian *Hymenolipis nana* and *H. diminuta* human and rat isolates based on *coxI* gene partial sequences and multiple alignment analysis were performed with CLUSTAL W computer program **(Thompson e al. 1994).**

Phylogeny Construction

The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed **(Felsenstein 1985)**. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees and node reliability in which the associated taxa clustered together in the bootstrap test is shown next to the branches **(Felsenstein 1985)**. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method **(Zuckerkand and Pauling 1965)** and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 99 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 **(Tamura et al. 2007)**. Neighbor-Joining and UPGMA methods were used to calculate the evolutionary relationship of the Egyptian isolates with genbank references strains **(Saitou and Nei 1987).**

Results

PCR Products of *mt cox1* Gene

PCR products were amplified from *mt* genomes using synthesized primers set. Across the alignment of *mt* genomes few regions were suitably

conserved to allow primer design. The PCR products were approximately 700 bp, 702 bp and 715 bp for *mt cox1* gene of *H. nana* rat isolates, *H. diminuta* rat isolates, and *H. nana* human isolates, respectively, **(Figure 1)**.

Sequences Analysis

Variation occurred in terms of sequence length and nucleotide differences and gaps (nucleotide insertions, deletions, and substitutions)**,** but not G+C percentage where the overall numbers did not differ between amplified fragments; A (23%), C (10%), G (22%) and T (45%). Where Nucleotide alterations were found to be variable and several nucleotide insertions, deletions and substitutions were detected with gaps in the same or different positions **(Figure 2)**.

Phylogeny Construction

Similar topologies were observed in the Egyptian isolates with genbank references strains. Optimal phylogenetic tree with the sum of branch length = 1.91459848 is shown **(Figure 3)**. Egyptian species were genetically distinct from other species used in this study that are phylogenetically relating to Hymenolipidid. In addition, despite their host susceptibility differences they all gathered in one cluster with three genbank published isolates of *H. nana*; AB033412.1 (gi|6045204), AB494472.1 (gi|2262378), and AY121842.1 (gi|2221354), forming one clade with 100% similarity, which was non significantly decreased on internal nodes. Moreover, obviously far away from *H. diminuta* published sequence AB033412.1 (gi|1399136) who's assumed to be quite genetically closely related to Egyptian *H. diminuta* than all other *H. nana* isolates. Both Egyptian murine isolates of Hymenolipidid; *H. diminuta* and *H. nana*, were closer to each other than being to *H. nana* of human origin.

GenBank accession numbers of Egyptian amplicons

The annotated sequences of Egyptian isolates were then deposited in the GenBank of NCBI under the following accession numbers; *H. diminuta* (GU433102), *H. nana* rat isolate (GU433103), and *H. nana* human isolate (GU433104).

Discussion

Mitochonderia play a central role in metabolism, apoptosis, disease, and aging **(Le et al. 2002)**. They are the site of oxidative phosphorylation, essential for the production of ATP, as well as a variety of other biochemical functions. Within these subcellular organelles is a genome, separate from the nuclear chromatin, referred to as mitochondrial DNA (mtDNA), very commonly used in studies of molecular phylogenetics **(Avise 2000)**. Flatworm mitochondrial genomes have a number of distinct features including all genes are coded on the same strand **(von Nickisch-Rosenegk et al. 2001)**, utilize a unique mitochondrial genetic code **(Boore 1999)** and truncated stop codons have also been found among a number of genes **(Nakao et al. 2000, 2003)**.

In the current study the earliest genbank records of *cox1* gene of Egyptian *Hymenolepis* spp. are declared. So far, only a few is known about the relative divergence rates of mitochondrial DNA in *hymenolipis species* especially Egyptian isolates **(Vilas et al. 2005)**, hence only one species belonging to *H. diminuta* is completely sequenced and published in genbank **(Littlewood et al. 2008)**. However, PCR technology and DNA sequencing techniques permit the identification of species, strains, and populations from any stage in their life history to distinguish among morphologically similar parasites **(Boore 1999)**.

Egyptian species were genetically distinct from other species used in this study that are phylogenetically relating to Hymenolipidid. Both Egyptian murine isolates of Hymenolipidid; *H. diminuta* and *H. nana*, were closer to each other than being to *H. nana* of human origin. WHO annual reports maintained the traditional host specificity of hymenolepidid till few years ago **(Lee et al. 2007)**. Unfortunately, the unexpected discovery of a mixed infection with specie which is known to infect rodent as definitive host in surveyed individuals **(Thompson et al. 2001; Macnish et al. 2003)** as well as in dogs living in the same locality as their infected owners declared the public health impact of new infestations, and meditating urgent thorough understanding of the epidemiology of these parasites **(Jenkins and Andrew 1993; Thompson et al. 1993; Macnish et al. 2003)**. Since such deviation in patency of infestation was not previously recorded thus highlights the growing importance of using molecular techniques in both the detection and characterization of parasite species in human and animals' populations especially between morphologically similar species **(Okamoto et al. 1997; Nakao et al. 2000; von Nickisch-Rosenegk et al. 2001; Macnish et al. 2003)**.

In a comparison of genetic makeup, our result suggests that *cox1* gene is generally conserved by each isolate nucleotide sequence analysis. Despite of variation occurred in terms of sequence length and nucleotide differences and gaps (nucleotide insertions, deletions, and substitutions)**,** but not G+C percentage where the overall numbers did not differ

between amplified fragments mostly triggered by a variety of hosts' biological conditions **(Macnish et al. 2002a, b)**. Such data showed consistent patterns with other researcher groups in this regard. They reported that mitochondrial DNA sequences of the Platyhelminthes accumulate nucleotide substitutions at a much higher rate than sequences in comparisons of genetic distances **(Littlewood and Bray 2001; Vilas et al. 2005)**. Base substitutions and additions are characterized by high T content which can, at times, represent poly-T structures. In addition, this may be a consequence of frame-shift mutations or premature stop codons, however, protein-coding genes of the *mt*DNA are error-checked by translating the nucleotide sequences **(Benasson et al. 2001)**. Specific substitution rates include metabolic rates and body mass, generation time, differential fixation of slightly deleterious mutations, DNA repair mechanisms, and nucleotide composition **(Vilas et al. 2005).**

According to the inferred topology of amino acids phylogeny of Egyptian hymenolipidid, hosts effect on the evolutionary relationship between isolates was clear despite their intra species differences which agree with **Johnston (2006)**. This could explain the closer relation of *Hymenolipis* spp. (*H. nana* and *H. diminuta*) collected from rat to be arranged in one cluster despite the disparities in host species and morphology which is in continence with **Littlewood et al. (2008)**. These results agree with previous reports supported variant biological features of *H. diminuta* that are not always identical between isolates is built on genetic background **(Okamoto et al. 1997)**. However, there results are conflicting with both the characteristic cryptic species of *H. nana* **(Macnish et al. 2002a, b)**, and Schmidt classification where *H. nana* should be closer to *H. microstoma* than *H. diminuta* **(Schmidt 1986).** These observations that were revealed from the present study which should not be applied unambiguously to host-parasite associations since it does not take into consideration other factors related to the ecology of the hosts and the dynamics of the host-parasite assemblages **(Johnston 2006)**. However, it should highlight the danger of triggering changes in genetic interspecificity subsequently definitive host susceptibility. Since, *mt cox1* resultant phylogenetic tree did not support the current hypotheses on the basis of morphological evidence for the separation of species **(Littlewood et al. 2008)**.

In conclusion, molecular protocol developed in this study will provide the tools for achieving supplementary comprehensive epidemiological portrait of infestation in Egypt. Consequently, should be applied on much broader scale in screening for *Hymenolipis* spp. infestations. Sufficient clarification of evolutionary relationship of *Hymenolipis* spp. by other ribosomal DNA content, and complete *mt* genome sequencing and its genes arrangement are essentials. These data will ultimately aid investigations on dynamics of morphological and developmental evolution, as well as the biology of parasitism.

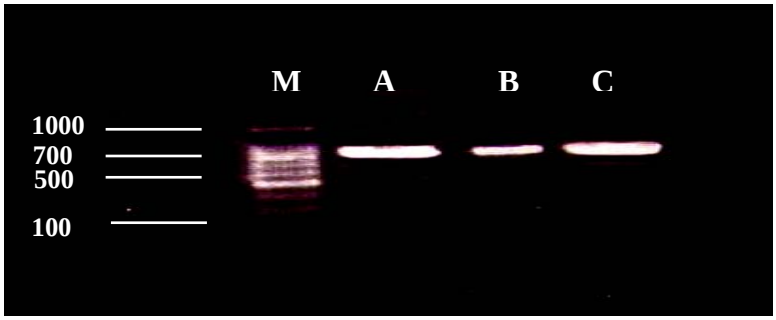


Fig. 1 PCR products of *mt cox1* gene amplified by specified primers pair from Egyptian isolates of (A) *Hymenolipis diminuta* **703 bp fragment, (B) *H. nana* rat isolate 699 bp fragment, (C) *H. nana* human isolate 715 bp fragment, and (M) 100 bp DNA Ladder.**

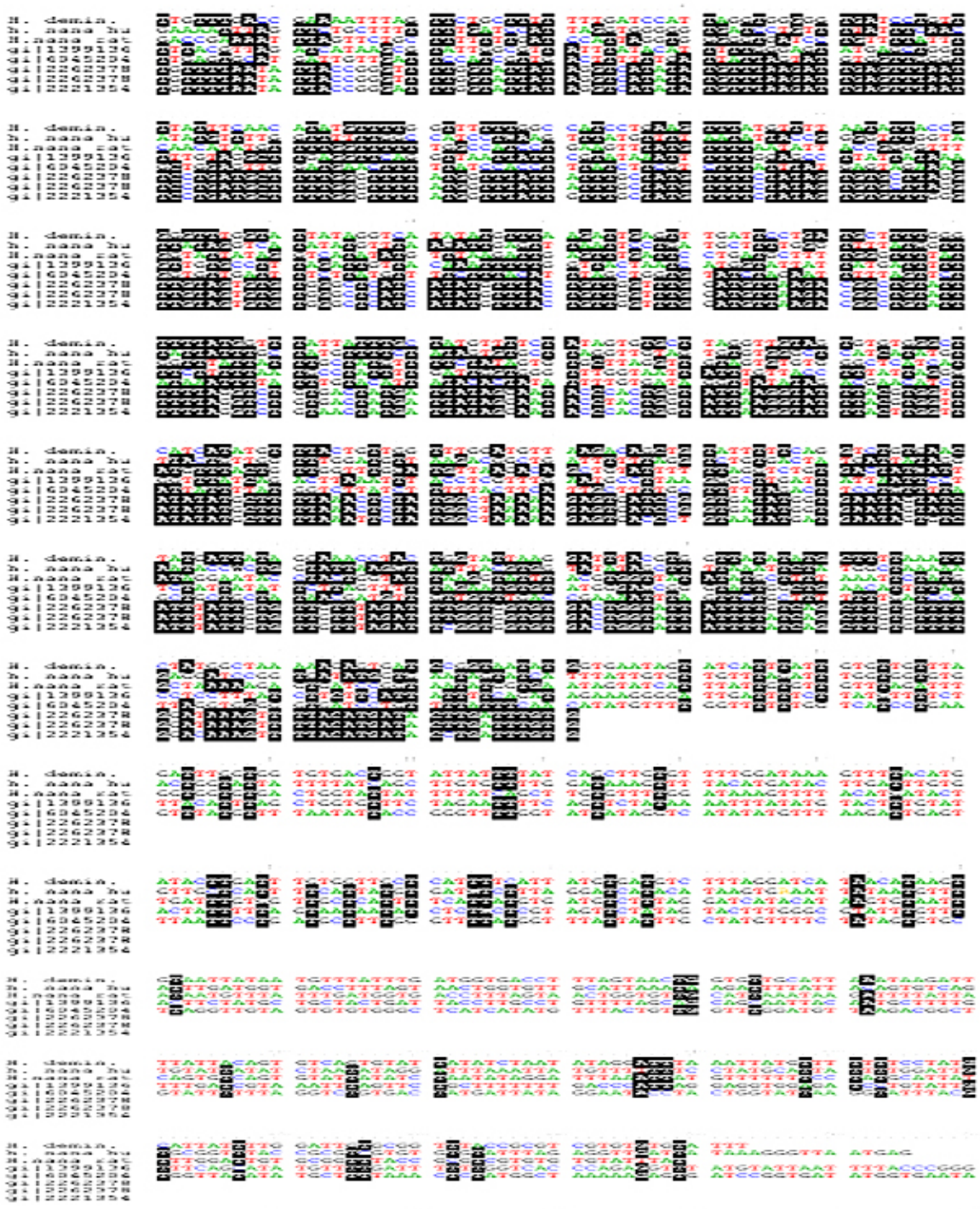


Fig. 2 Nucleotides multiple alignment of partial *mt cox1* gene sequences of Egyptian *H. diminuta*, *H. nana* human isolate, *H. nana* rat isolate, and reference gi|13991366: *H. diminuta*, gi|226237884: *H. nana* isolat: HnanaMon, gi|22213549:*H. nana*, gi|6045204: *H. nana*, gi|14009612: Echinococcus granulosus genotype 1, gi|15042575: Echinococcus equinus , gi|193884329: Spirometra erinaceieuropaei, gi|239997751: Taenia multiceps, gi|28856111: Taenia solium, and gi|260162222: Taenia saginata, isolate: TsagT017KANTH. Black columns represents homology between sequences.

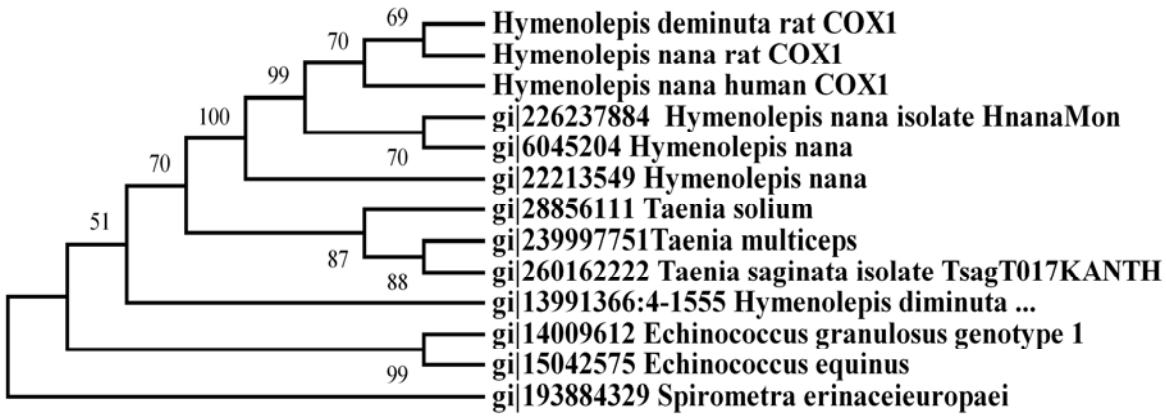


Fig. 3 Rooted phylogenetic tree based on amino acids sequences of in silico translated partial *mt cox1* gene sequences of Egyptian *H. diminuta*, *H. nana* human isolate, *H. nana* rat isolate and reference gi|13991366: *H. diminuta*, gi|226237884: *H. nana* isolate: HnanaMon, gi|22213549: *H. nana*, gi|6045204: *H. nana*, gi|14009612: *Echinococcus granulosus* genotype 1, gi|15042575: *Echinococcus equinus*, gi|193884329: *Spirometra erinaceieuropaei*, gi|239997751: *Taenia multiceps*, gi|28856111: *Taenia solium*, and gi|260162222: *Taenia saginata*, isolate: TsagT017KANTH. Similar topologies were developed when both Neighbor-Joining and UPGMA methods were applied (MEGA4 software).

References:

1. **Ando K, Tsunemori M, Akahane H, Tesana S, Hasegawa H, Chinzei Y (2006)** Comparative study on DNA sequences of ribosomal DNA and cytochrome *c* oxidase subunit 1 of mitochondrial DNA among five species of gnathostomes. J Helminthol 80:7-13
2. **Avise JC (2000)** *Phylogeography: The history and formation of species*, Harvard University Press.
3. **Behera B, Mirdha BR, Makharia GK, Bhatnagar S, Dattagupta S, Samantaray JC (2008)** Parasites in patients with malabsorption syndrome: A clinical study in children and adults. Dig Dis Sci 53:672–679
4. **Benasson D, Zhang DX, Hart DL, Hewitt GM (2001)** Mitochondrial pseudogenes: evolution's misplaced witnesses. Trends Ecol Evol 16:314-321
5. **Boore JL (1999)** Animal mitochondrial genomes. Nucleic Acids Res 27:1767–1780
6. **Boore, JL, Brown WM (1998)** Big trees from little genomes: Mitochondrial gene order as a phylogenetic tool. Curr Opin Genet Dev 8:668–674
7. **Constantine CC (2003)** Importance and pitfalls of molecular analysis to parasite epidemiology. Trends Parasitol 19:346-348.
8. **Felsenstein J (1985)** Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783-791
9. **Hu M, Chilton NB, Gasser RB (2004)** The mitochondrial genomics of parasitic nematodes of socio-economic importance: recent progress, and implications for population genetics and systematics. Adv Parasitol 56:133-212
10. **Jenkins DJ, Andrew PL (1993)** Intestinal parasites in dogs from an Aboriginal community in New South Wales. Aust Vet J 70:115–116
11. **Johnston DA (2006)** Genomes and genomics of parasitic flatworms. In: Maule AG, Marks NJ (Eds.) *Parasitic flatworms*: *molecular biology*, *biochemistry*, *immunology and physiology*. CAB International, Wallingford, pp 37-80
12. **Le TH, Blair D, Agatsuma T, Humair PF, Campbell NJ, Iwagami M, Littlewood DT, Peacock B, Johnston DA, Bartley J, Rollinson D, Herniou EA, Zarlenga DS, McManus DP (2000)** Phylogenies inferred from mitochondrial gene orders—a cautionary tale from the parasitic flatworms. Mol Biol Evol 17:1123–1125
13. **Le TH, Blair D, McManus DP (2002)** Mitochondrial genomes of parasitic flatworms. Trends Parasitol 18:206–213
14. **Lee S-U, Chung H-C, Huh S (2007)** Molecular phylogeny of parasitic Platyhelminthes based on sequences of partial 28S rDNA D1 and mitochondrial cytochrome c oxidase subunit I. Korean J Parasitol 45(3):181-189
15. **Littlewood DTJ, Bray RA (2001)** Interrelationships of the Platyhelminthes. Taylor and Francis, London, UK.
16. **Littlewood DTJ, Waeschenbach A, Nikolov PN (2008)** In search of mitochondrial markers for resolving the phylogeny of cyclophyllidean tapeworms (Platyhelminthes, Cestoda)– a test study with Davaineidae. Acta Parasitol 53(2):133–144
17. **Macko JK, Hanzelova V (2008)** New books. Helminthologia 45(4):211
18. **Macnish MG, Morgan UM, Behnke JM, Thompson RCA (2002a)** Failure to infect laboratory rodents with humans isolates of *Rodentolepis* (*Hymenolepis*) nana. J Helminthol 76:37–43

19. **Macnish MG, Morgan-Ryan UM, Monis PT, Behnke JM, Thompson RCA (2002b)** A molecular phylogeny of nuclear and mitochondrial sequences in *Hymenolepis nana* (Cestoda) supports the existence of a cryptic species. Parasitol 125:567–575
20. **Macnish MG, Ryan UM, Behnke JM, Thompson RCA (2003)** Detection of the rodent tapeworm *Rodentolepis* (*Hymenolepis*) *microstoma* in humans. A new zoonosis? Int J Parasitol 33:1079–1085
21. **McManus DP, Le TH, Blair D (2004)** Genomics of parasitic flatworms. Int J Parasitol 34:153–158
22. **Movsesyan SO, Jivanyan KA, Chubaryan FA, Malczewski A, Terenina NB, Petrossyan R, Ter-Oganyan KS (2008)** Experimental hymenolepiasis of rats: preliminary data on histopathological changes of visceral organs. Acta Parasitol 53(2):193–196
23. **Nakao M, Sako Y, Ito A (2003)** The mitochondrial genome of the tapeworm *Taenia solium*: A finding of the abbreviated stop codon U. J. Parasitol 89:633–635
24. **Nakao M, Sako Y, Yokoyama N, Fukunaga M, Ito A (2000)** Mitochonderial genetic code in cestodes. Mol Bioch Parasitol 111:415-424
25. **Nakao M, Yokoyama N, Sako Y, Fukunaga M, Ito A (2002)** The complete mitochondrial DNA sequence of the cestode *Echinococcus multilocularis* (Cyclophyllidea: Taeniidae). Mitochondrion 1:497–509
26. **Ngarmamonpirat C, Waikagul J, Petmitr S, Dekumyoy P, Rojekittikhun W, Anantapruti MT (2005)** Analysis of sequence variation in *Gnathostoma spinigerum* mitochondrial DNA by single-strand conformation polymorphism analysis and DNA sequence. Int J Parasitol 54:65-68
27. **Okamoto M, Agatsuma T, Kurosawa T, Ito A (1997)** Phylogenetic relationships of three hymenolepidid species inferred from nuclear ribosomal and mitochondrial DNA sequences. Parasitol 115:661-666
28. **Raether WH, Hänel H (2003)** Epidemiology, clinical manifestation and diagnosis of zoonotic cestode infections: An update. Parasitol Res 91:412-438
29. **Saitou N, Nei M (1987)** The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406-425
30. **Sambrook J, Fritscgh EF, Meniates A (1989)** Molecular cloning: A loboratory manual. Cold Spring Harbor Laboratory Press, New York.
31. **Saraste M (1999)** Oxidative Phosphorylation at the fin de siècle. Science 283(5407):1488-1493
32. **Schmidt GD (1986)** CRC handbook of Tapeworm identification. CRC Press, Boca Raton.
33. **Tamura K, Dudley J, Nei M, Kumar S (2007)** MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596-1599
34. **Tatusova, T.A. and Madden, T.L. (1999):** BLAST 2 Sequences, a new tool for comparing protein and nucleotide sequences. FEMS Microbiol Lett 174(2):247-250. Erratum in: FEMS Microbiol Lett 1999, 177(1):187-188
35. **Thompson RCA, Meloni BP, Hopkins RM, Deplazes P, Reynoldson JA (1993)** Observations on the endo- and ectoparasites affecting dogs and cats in Aboriginal communities in the north-west of Western Australia. Aust Vet J 70:268–269
36. **Thompson RCA, Reynoldson JA, Garrow SC, McCarthy JS, Behnke JM (2001)** Towards the eradication of hookworm in an isolated Australian community. Lancet North Am Ed 357:770–771
37. **Thompson RCA, Zarlenga DS, La Rosa G, Pozio E, Rosenthal B, Bandi C, Mortarino M, Casiraghi M, Genchi C, Gasser RB, Hu M, Chilton NB, Matthews JB, Hodgkinson JE (2004)** Molecular systematics and diagnosis. Vet Parasitol 125:69-92
38. **Traversa D, Costanzo F, Iorio R, Aroch I, Lavy E (2007)** Mitochoderial cytochrome *c* oxidase subunit 1 (*cox1*) gene sequence of *Spirocerca lupi* (Nematoda, Spirurida): Avenues for implications. Vet Parasitol 146:263-270
39. **Vilas R, Criscione CD, Blouin MS (2005)** A comparison between mitochondrial DNA and the ribosomal internal transcribed regions in prospecting for cryptic species of Platyhelminth parasites. Parasitol 131:839-846
40. **von Nickisch-Rosenegk M, Brown WM, Boore JL (2001)** Complete sequence of the mitochondrial genome of the tapeworm *Hymenolepis diminuta*: Gene arrangements indicate that Platyhelminths are Eutrochozoan. Mol Biol Evol 18(5):721–730
41. **Zuckerkandl E, Pauling L (1965)** Evolutionary divergence and convergence in proteins. In: Bryson V, Vogel HJ (Eds) Evolving Genes and Proteins, Academic Press, New York, pp 97-166

10/13/2010

Eusyllinae, Anoplosyllinae, and Exogoninae (Polychaeta: Syllidae) for the Mediterranean Coasts of Egypt, Together the Description of One New Species

F. A. Abd-Elnaby[*1] and G. San Martín[2]

[1]National Institute of Oceanography and Fisheries, Alexandria, Egypt
[2] Departamento de Biología (Zoología), Facultad de Ciencias, Universidad Autónoma de Madrid, calle Darwin, 2, 28049 Madrid, Spain.
[*]faiza_abdelnaby@yahoo.com

Abstract: In this paper, 18 species of the subfamilies Exogoninae, Anoplosyllinae, and Eusyllinae (Syllidae, Polychaeta) are reported from the Mediterranean Egyptian coasts, 8 of them are new records for the area: *Odontosyllis fulgurans* (Audouin and Milne Edwards, 1833); *Syllides japonicus* Imajima, 1966; *Salvatoria clavata* (Clapare de, 1863); *Salvatoria euritmica* (Sardá, 1984); *Sphaerosyllis glandulata* Perkins, 1981; *Parapionosyllis labornica* Cognetti, 1965; *Sphaerosyllis* sp.; and *Prosphaerosyllis* sp. Five species were reported previously in the area. Four species are new records for Mediterranean Sea: *Palposyllis prosostoma* Hartmann-Schröder, 1977; *Paraehlersia weissmaniodes* (Augener,1913); *Streptosyllis compoyi* Brito, Núñez and San Martín, 2000; and *Exogone africana* Hartmann-Schröder, 1974); *P. weissmaniodes* and *Exogone africana* are two widely distributed Indo-Pacific species, so they could be considered as Lessepsian migrants. Finally, one new species is described, *Parapionosyllis aegyptia*.
[F. A. Abd-Elnaby[*1] and G. San Martín. **Eusyllinae, Anoplosyllinae, and Exogoninae (Polychaeta: Syllidae) for the Mediterranean Coasts of Egypt, Together the Description of One New Species**. Journal of American Science 2010;6(12):1354-1363]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: *Eusyllinae, Anoplosyllinae, Exogoninae, Taxonomy, Mediterranean, Egypt, New species.*

1. Introduction:

Syllidae represent one of the most diverse and systematically challenging families of Polychaeta (Glasby, 2000; Rouse & Pleijel, 2001; San Martín, 2003, 2005; San Martín & Hutchings, 2006; Aguado & San Martín, 2009). It is a widely distributed group, found from the intertidal zone to the abyssal plains all over the world (Glasby, 2000), but less common at depth, with some species symbiotic or parasitic on other marine invertebrates (Martín and Britayev, 1998).

This family is currently divided into 5 subfamilies (Aguado& San Martín 2009): Eusyllinae Malaquin, 1893; Exogoninae Langerhans, 1879; Autolytinae Langerhans, 1879; Syllinae Grube, 1850; and the recently erected Anoplosyllinae Aguado and San Martín , 2009.

To detect newly recorded or new species we will depend on accurate taxonomic identifications and the local biodiversity. The possible existence of complexes of species, whose identity is blurred under one common specific name are present (Aguado and San Martín, 2007).

Knowledge about Polychaetes in the Egyptian waters is still far from complete; as result of less taxonomical studies and less sufficient data about this group. This paper is the second report about Egyptian Syllids, collected from the Northwestern Coast of Egypt through Salsabeel cruise, Autumn 2008 and Spring 2009, also from Gamasa (Spring 2009), under the frame work organized by National Institute of Oceanography and fisheries branch Alexandria, and from Port Said Harbour (Spring 2008), to study the benthic invertebrates. While the first report about syllid and sabellid species in the Northwestern coast of Egypt, were done by Selim (2008a & b respectively), Abd- Elnaby (2009) also studied polychaetes in Gamasa.

Generally, scarce attention has been given to the polychaetes in Egyptian waters. Fauvel (1927) recorded 8 syllid species from the Suez Canal waters of which 6 belonging to genus *Syllis*. On his work on the polychaetes collected from the fishery grounds near Alexandria, Fauvel (1937) gave a checklist of polychaetes were recorded in this area.
Only 16 species of Syllidae were recorded in that paper. More recently, Selim (1978) reported two syllids species in the Eastern Harbour of Alexandria, namely *Syllis (Typosyllis) variegata* and *Trypanosyllis zebra*. Later, the same author (Selim, 1996) added 6 syllid species from Alexandria coast (*Branchiosyllis exilis, Syllis gracilis, S. hyalinae, S. mediterranea, S. prolifera and S. variegate*). Finally, Abd-Elnaby (1999) recorded 7 syllid species, and later (2005) 21 species from Alexandria coast.

The Syllidae of the neighbouring areas were studied by several authors; from Aegean Sea by Çinar & Ergen (2002); Çinar (2003); and Çinar (2005);

from Israel and the Gulf of Elat by Ben-Eliahu (1977a & 1977b), and anteriorly Fauvel (1955, 1957), from Cyprus by Ben-Eliahu (1972), Çinar (2003a& b) and Çinar & Ergen (2003); Lebanon by Aguado & San Martín (2007), and from Turkey by Ergen (1976). A checklist, distribution, and ecological features of Syllidae and other polychaetes from Greece can be reported in Simboura & Nicolaidou (2001), also from Cyprus by many authors, the most recent one Musco et al. (2005), and the biogeographic revision on Syllidae from the Mediterranean Sea (East and West areas) was carried out by Musco & Giangrande (2005).

During the present study 18 species were recorded, 11 of which are new records for the Egyptian waters. Four species are considered as new species for Mediterranean Sea. Three species are considered as new species, although two of them are under process of description, and one species is described here as new for Science. In this paper, detailed description is given also of some interesting species

2. Materials and methods

Two cruises were carried out on the Northwestern Mediterranean coast of Egypt; on two stations (El Hammam, El Alamein), during Autumn 2008 and Spring 2009, and also one collection Spring 2008. The stations are; Port Said Harbour (station 1), in which samples were collected during Spring 2008, and Gamasa (station2, Spring 2009), depth ranging from 0 .25m to 20 m. (Fig. 1). Sediment samples were collected by a Van Veen grab; while, samples from Port Said Harbour were collected by knife and net used for collecting fauna. Sediment samples were washed up and sieved through o.3 µm sieve, then sorted under Stereomicroscope. Specimens of Syllidae were extracted and fixed in 10 % formaldehyde in sea water-solution. Examinations and identification were done by using compound microscope. Drawings were made by a camera lucida. The specimens were Preserved in the Marine Reference Collection Center of National Institute of Oceanography and Fisheries, Alexandria, under Code Number (N. Sp. 2/8/3).

3. Results

In the present study, 18 species belonging to the subfamilies Exogoninae, Anoplosyllinae and Eusyllinae (Syllidae, Polychaeta) were recorded and identified from the Mediterranean Egyptian coasts, 8 of them considered new records for the Egyptian Mediterranean waters: *Odontosyllis fulgurans* (Audouin and Milne Edwards, 1833); *Syllides japonicus* Imajima, 1966; *Salvatoria clavata* (Clapare de, 1863); *Salvatoria euritmica* (Sarda , 1984); *Sphaerosyllis glandulata* Perkins, 1981; *Parapionosyllis labronica* Cognetti, 1965; *Sphaerosyllis* sp.; and *Prosphaerosyllis* sp.; the two later are new species in process of description, although a description of both without any specific name is given by San Martín (2003). Five species were reported previously from different places of Egypt. In addition, four species are considered as a new records for the Mediterranean Sea (*Palposyllis prosostoma* Hartmann-Schröder, 1977; *Paraehlersia weissmaniodes* (Augener,1913); *Streptosyllis compoyi* Brito, Núñez and San Martín, 2000; and *Exogone africana* Hartmann-Schröder, 1974). Finally one species *Parapionosyllis aegyptia* is described as new species. The locations, dates , depth, number of specimens and geographical distribution are presented in Table (1).

Ehlersia ferrugina non Langerhans. Böggemann & Westheide, 2004: 418, fig. 6.
Paraehlersia weissmanioides San Martin & Hutchings, 2006: 312, figs. 43A-C, 47A-I, 48 A-F, 49 D-F.

Material examined. Port Said 0.25 m depth, Spring 2008, one specimen.

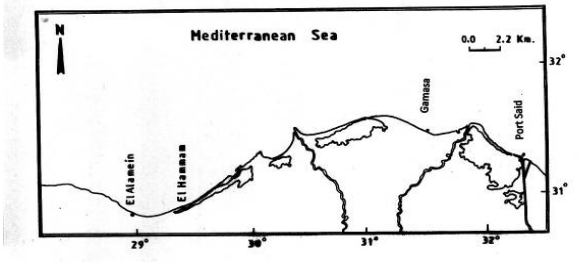


Fig. (1) Map showing the sampling sites, (North western Coast of Egypt, Gamasa and Port Said).

Table(1): Showing the Polychaete species recorded in the present study, Location, Date, Depth, Bottom, Number of specimens, and Distribution.

Name of the species	Location	Depth (m)	Date	No.	Bottom	Distribution
****Palposyllis prosostoma* Hartmann-Schröder, 1977**	**Gamasa**	**13.7**	**Spring 2009**	**1**	**S-M**	**At**
***Paraehlersia weissmaniodes* (Augener,1913)	Port Said	0.25	Spring 2008	3	F	At
*****Brevicirrosyllis weismanni* Langerhans, 1879	El Alamein	20.0	Autumn 2008	1	C-S	At, Med
****Odontosyllis fulgurans* (Audouin and Milne Edwards, 1833)	Port Said	0.25	Spring 2008	1	F	Cos
***Streptosyllis compoyi* Brito, Núñez and San Martín, 2000	Elhammam	20.0	Autumn 2008	1	C-S	At
****Syllides japonicus* Imajima, 1966	Elhammam	20.0	Spring 2009	2	C-S	At, Med, P
****Salvatoria clavata (Claparède, 1863).*	Elhammam	20.0	Spring 2009	1	C-S	Cos
****Salvatoria euritmica* (Sardá, 1984)	Elhammam	8.0	Spring 2009	1	C-S	At, Med, P
*****Salvatoria vieitezi* San Martín 1984	El Alamein	20.0	Autumn 2008	2	C-S	At, Med, P
****Sphaerosyllis glandulata* Perkins, 1981	Gamasa	13.7	Spring 2009	1	S-M	At, Med
*****Sphaerosyllis taylori* Perkins, 1981	Elhammam	20.0	Spring 2008	1	C-S	At, Med
****Sphaerosyllis* sp.	El Alamein	20.0	Autumn 2008	1	C-S	At
****Prosphaerosyllis* sp.	Elhammam	8.0	Spring 2009	1	C-S	At
***Exogone africana* Hartmann-Schröder,1974	Port Said	0.25	Spring 2008	2	F	At
***Parapionosyllis aegyptia* n. sp.**	El Alamein	20.0	Autumn 2008	2	C-S	n. sp.
*****Parapionosyllis brevicirra* Day, 1954	El Alamein	20.0	Autumn 2008	2	C-S	At, Med
*****Parapionosyllis elegans* (Pierantoni, 1903)	El Alamein	20.0	Autumn 2008	2	C-S	At, Med
****Parapionosyllis labronica* Cognetti, 1965	Gamasa	13.7	Spring 2009	1	S-M	At, Med

At= Atlantic Ocean, P= Pacific Ocean, Med= Mediterranean, Cos= Cosmopolitan, S-M= Sandy mud, C-S= Coarse Sand, F= Fouling; *= New species, **= New record for Mediterranean Sea, ***= New record for Egyptian waters, ****= Recorded before from Egyptian waters; The most important species will be described in details.

Description. Body broad anteriorly, tapered posteriorly, 11 mm long, 0.2 mm wide, with 41 chaetigers (fig. 2 A). Prostomium oval (75 µm), 4 eyes in trapezoidal arrangement, and 2 anterior eye-spots; lateral antenna 162.5 µm long, midian antenna 150 µm long. Palps broad (87.5 µm), basally fused. Dorsal tentacular cirri 147.5-162.5 µm long, ventral tentacular cirri about one third in length of dorsal tentacular cirri. Antennae, tentacular and anterior dorsal cirri; elongated, indistinctly articulated; articulation variable with short and long articles, up to 22 articles; dorsal cirri becoming progressively smoother posteriorly. Infracirral papillae not seen. Parapodia conical, slightly elongate. Ventral cirri digitiform, slightly longer than parapodial lobes. Parapodia with 12-15 falcigerous compound chaetae; blades strongly bidentate, with fine spines on margin (fig. 2 B), 2-3 distalmost ones longer than remaining (30-42.5 µm) (fig. 2 C). Most dorsal compound chaetae, spiniger-like, blades (75 µm long) on midbody, and about 93 µm on posterior parapodia with fine spines on margin (figs. 2 C, E), absent on most posterior parapodia; indistinctly bidentate. Compound falcigers becoming wider progressively along body, with stronger proximal tooth, slightly hooked (fig. 2G). Dorsal simple chaetae appear from chaetiger 19, truncate, bifid with short spines margin (fig. 2 F, H). Ventral simple chaetae on posterior parapodia, thick, with few long spines on margin, strongly bidentate, proximal tooth large, slightly hooked, and distal one shorter than proximal one (fig. 2 J).

Anterior parapodia with 2-3 slender aciculae, two of them distally rounded with small bending tip and one straight (fig. 2D); from proventricular segments onwards, acicula solitary, with oblique, short tip (fig. 2 I). Pharynx through 6 segments; pharyngeal tooth anteriorly located. Proventricle, rectangular through 4 segments with about 21 muscle cell rows.

Distribution: Australia, Seyhelles. New report for the Mediterranean Sea.

Remarks: The Egyptian specimen is similar to Australian ones; it is likely an Indo-Pacific migrant through Suez Canal.

Exogone verugera africana Hartmann-Schröder,1974a:137, figs. 164-168; 1979; 108, figs.164-168.

Exogone africana San Martín, 2005: 143, fig. 90 a-f.

Material examined. Port Said, 0.25 m depth, on fouling, Spring 2008, 2 specimens.

Description. Body small, slender, relatively broad anteriorly, 3 mm long, 0.23 mm wide, 28 chaetigers. Prostomium oval (fig.3 A); 4 eyes in trapezoidal arrangement. Antennae short, oval, close to each other, inserted between anterior to eyes; median antenna slightly longer and thicker than lateral one. Palps broad, longer than prostomium, totally fused, with a dorsal furrow (fig. 3A). Peristomium shorter than subsequent segments; one pair of small, papilliform tentacular cirri. Dorsal cirri similar to antennae and tentacular cirri, slightly longer than lateral antennae, present on all segments. Compound chaetae of two types on all parapodia: 1-2 spiniger-like, with long blades 31 µm long (fig. 3 B) on anterior parapodia, slightly short on posterior one (25-27.5 µm), distally bifid, with short marginal spines (fig. 3 C), and 4 compound chaetae with short falcigerous blades about 7.5 µm, bidentate, subdistal tooth long and distal tooth short, moderate marginal spines (fig. 3 D); posterior falcigers smaller, three in number, blades about 5µm long (fig. 3 E). Dorsal simple chaetae from anterior segments, with rounded tips (fig. 3 F), subdistally serrated, thicker posteriorly with pointed tip (fig. 3 G). Ventral simple chaetae on posterior parapodia, sigmoid, thick, with some short spines on base of teeth, bidentate, subdistal tooth longer and thicker than distal tooth (fig. 3 H). Acicula solitary, slender, distally rounded (fig. 3 I). Pharynx long, through 4 segments; pharayngeal tooth located on anterior rim. Proventricle occupying 4 segments with 18 muscle cell rows. Pygidium with 2 long anal cirri.

Distribution: Circumtropical. First report to the Mediterranean Sea.

Material examined. El Alamein 20 m depth, Autumn 2008. Holotype and Paratype, coarse sand.

Description. Holotype 3.5 mm, 0.15mm wide 29 chaetigers (fig. 4 A). Prostomium ovate, wider than long; 2 pairs of eyes, anterior pair larger than posterior ones, arranged in trapezoidal arrangement, and 2 small anterior eye-spots. Antennae spindle-shaped to bowling-pin shaped, longer than prostomium; median antenna (77.6 µm) slightly longer than lateral ones (67.5 µm), arising between anterior eyes; lateral antennae arising on anterior margin of prostomium (right one missing on holotype). Palps basally fused, shorter than prostomium. Peristomium with 2 pairs of bowling-pin shaped tentacular cirri, smaller than antennae. Parapodia somewhat elongated (32.5 µm). Dorsal cirri bowling-pin shaped, from 36- 45µm in length anteriorly to 65µm on posterior parapodia. Ventral cirri digitiform, shorter than parapodial lobes.

Anterior parapodia with 7 compound falcigers, unidentate with hooked tips and serrated margin (fig. 4 B); about 10 µm long; shafts becoming posteriorly thick with long curved acute tip, blades with serrated margin on 2 most dorsal ones, and 4-5 unidentate curved, smooth (fig. 4 D). Superior dorsal simple chaetae thin with pointed tip, present in all parapodia, except first one (fig. 4 C), become thicker posteriorly (fig. 4 E). Ventral simple chaetae unidentate, sigmoid (fig. 4 F). Acicula solitary, bent with hollow rounded tip (fig. 4 G). Pharynx extending through 3.5 setigers; mid dorsal tooth on anterior edge. Proventricle extending through 2.5 segments, with 17 rows of muscle cells. Glands small, with granular material, pair on each segment, present from first chaetiger.

Remarks. About 16 species are recognized as *Parapionosyllis*, 6 of them recorded in the Mediterranean Sea. The most similar species is *P. labronica* also found in this collection; both species have posterior compound chaetae with thick shafts, distally curved, and short, unidentate blades, smooth or almost smooth. However, the anterior compound chaetae of *Parapionosyllis aegyptia* are more elongated and provided with somewhat longer spines on margin, and the dorsal simple chaetae are different, being smooth and unidentate in *P. aegyptia* and provided with a sub-distal, thick spine and others shorter, in *P. labronica*. The remaining Mediterranean species are clearly different of these two species, because they have longer compound chaetae (see San Martín, 2003); also, other species of other seas also have more elongated compound chaetae and different dorsal simple chaeta.

Etymology. The species is named after the country in which has been found, Egypt.

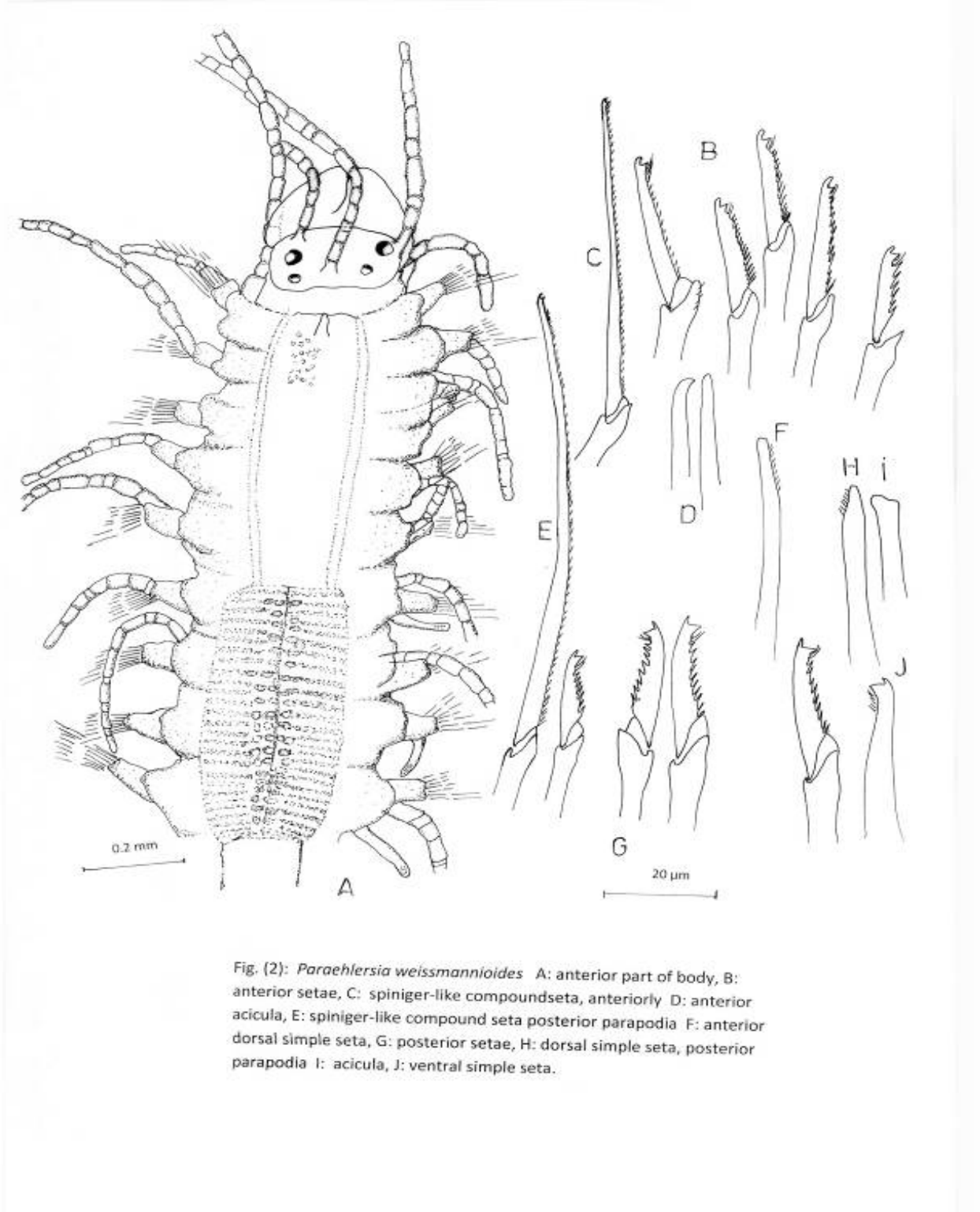


Fig. (2): *Paraehlersia weissmannioides* A: anterior part of body, B: anterior setae, C: spiniger-like compoundseta, anteriorly D: anterior acicula, E: spiniger-like compound seta posterior parapodia F: anterior dorsal simple seta, G: posterior setae, H: dorsal simple seta, posterior parapodia I: acicula, J: ventral simple seta.

Paraehlersia weissmaniodes (Augener,1913)
(Fig. 2 A-J)

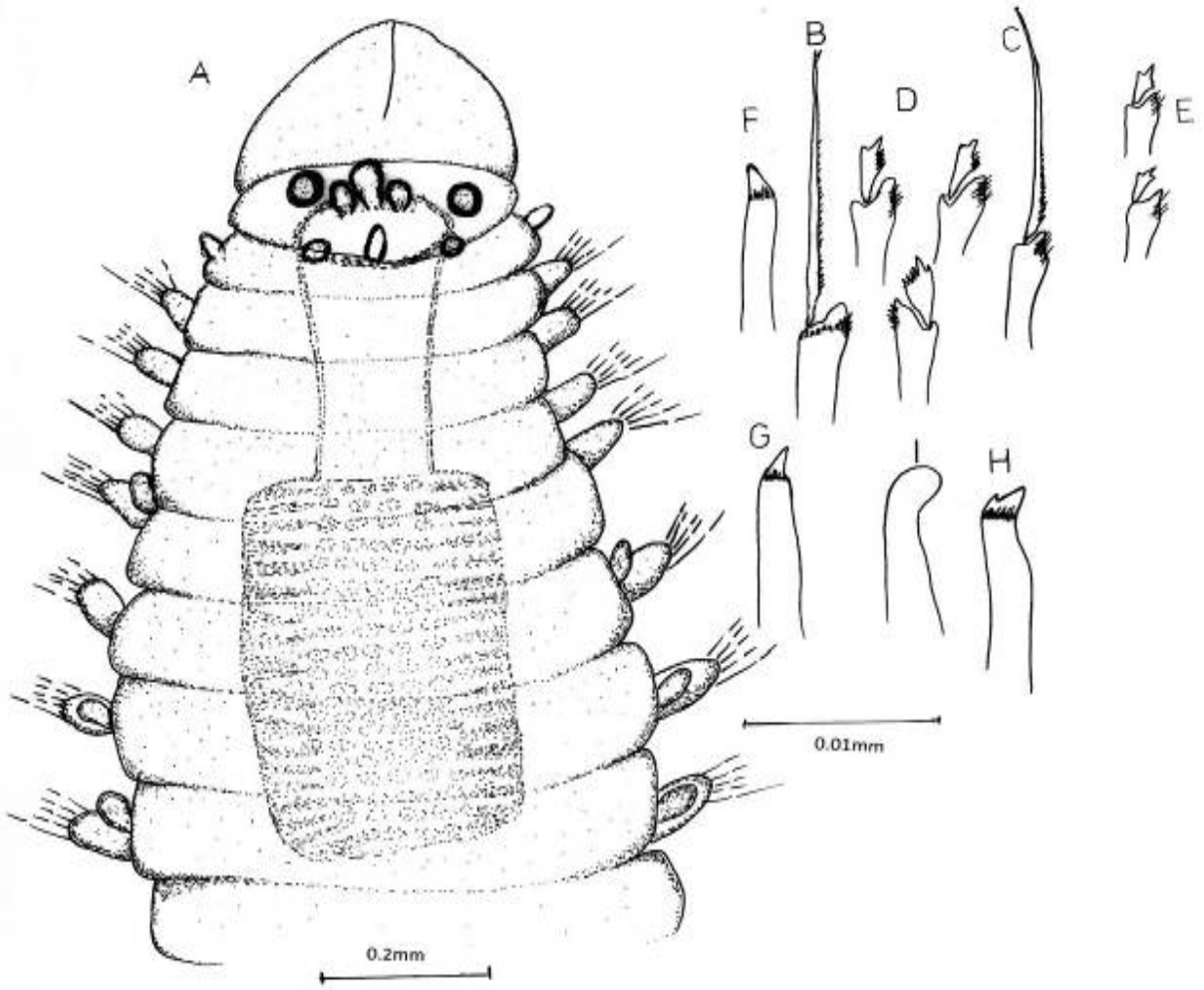


Fig. (3): *Exogone Africana* A: anterior part of body, B: anterior spiniger-like seta , C: posterior spiniger-like seta, D: anterior falcigers setae, E: posterior falcigers setae, F: anterior dorsal simple seta, G: posterior dorsal simple seta, H: ventral simple seta, I: acicula.

Exogone africana Hartmann- Schröder, 1974
(Fig. 3 A-I)

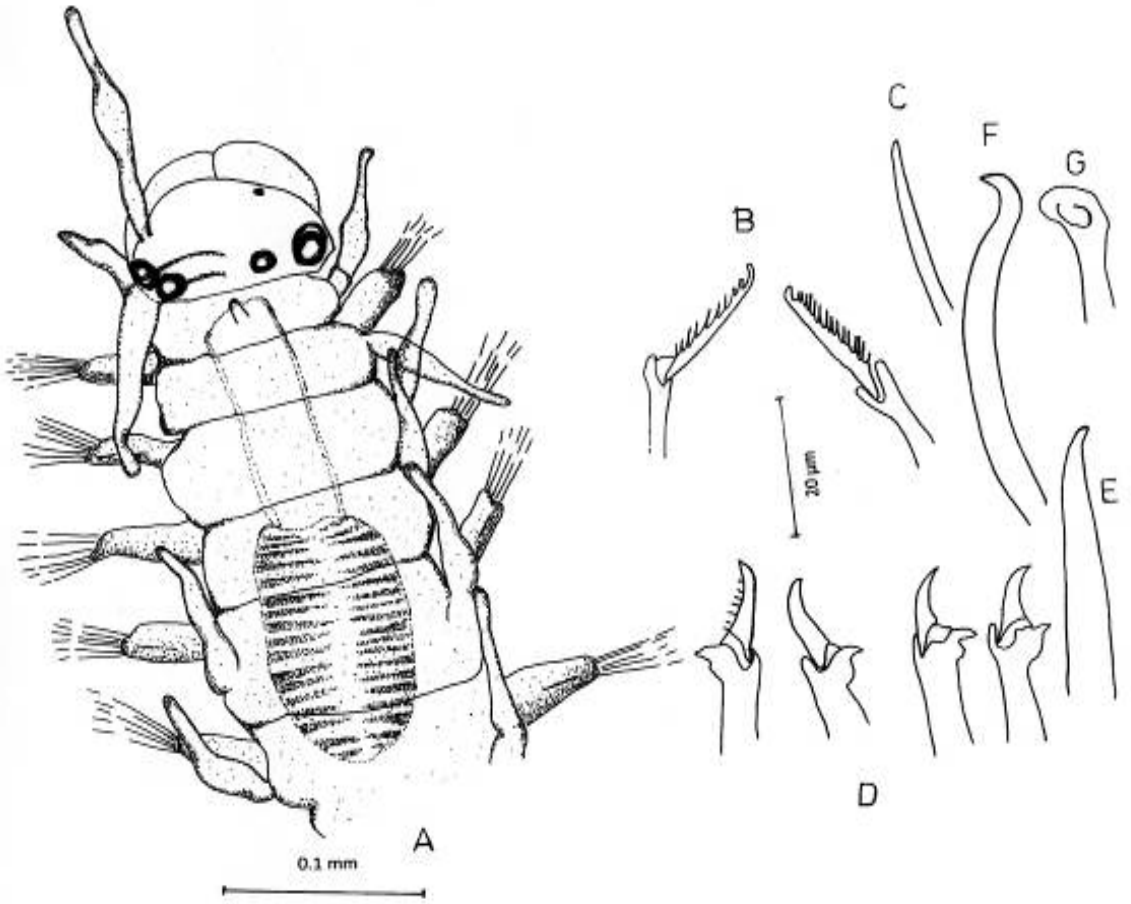


Fig. (4): *Parapionosyllis* n. sp. A: anterior part of body, B: anterior setae, C: anterior dorsal simple seta, D: posterior setae, E: posterior dorsal simple seta, F: posterior ventral simple seta, G: acicula.

Parapionosyllis aegyptia n. sp.
(Fig. 4 A-G)

4. Discussion

The number of Syllids recorded on Egyptian waters reach about 60 species, a low number when compared with the 190 Syllid species were reported by Musco and Giangrande (2005) from the whole Mediterranean waters, which represents the 31.6 % of the total Mediterranean Syllidae. Also, many other species may possibly remain unreported because of the most coastal area of Egypt are unexplored and many studies are still needed.

Tovar- Hernández *et al*. (2002) referred to the dominance and diversity of syllid members in carbonate sediments, this observation was confirmed by Selim (2008), where El Hammam and El Alamein coasts contain carbonate bottom sediments.

Most of the studied species are well known, common and widely reported for Mediterranean and Atlantic Ocean. The analysis of samples resulted into 18 species,11 of them new record for the Egyptian Mediterranean waters. Four species were reported previously, (*Brevicirrosyllis weismanni* Langerhans, 1879; *Parapionosyllis elegans* (Pierantoni, 1903); *P.brevicirra* Day, 1954 and *Sphaerosyllis taylori* Perkins, 1981, from many Mediterranean coastal areas and one more (*Salvatoria vieitezi* San Martín 1984) from the Suez Canal. Two of the studied syllids are considered apparently cosmopolitan: *Salvatoria clavata,* and *Odontosyllis fulgurans.* In addition, 11 species were known before from Spainish coasts, only 4 species were previously recorded in Greece, 9 from North West Italian, 9 from Turkish Aegean and 6 from Cyprus. Also four species are considered as a new record for Mediterranean Sea: *Palposyllis prosostoma* Hartmann-Schröder, 1977; *Paraehlersia weissmaniodes* (Augener, 1913); *Streptosyllis compoyi* Brito, Núñez and San Martín, 2000; and *Exogone africana* Hartmann-Schröder,1974.

According to geographic distribution through literature there are 9 species belong to Atlantic-Mediterranean category and one species (*Salvatoria vieitezi*) was recorded before from Suez Canal (Selim, 2009), and four species are new for Mediterranean, that means they are Lessepsian migrants; 3species are amphi-Atlantic, *Sphaerosyllis taylori*, *Shphaerosyllis glandulata* and *Salvatoria vieitezi.* Two species are considered cosmopolitan species, *Odontosyllis fulgurans* and *Salvatoria clavata* and five species are considered Atlantic-Pacific categories.

In spite of it, many new recorded species usually discovered by way in new researches, still more not recorded until now, more studies requisite to be done along the Mediterranean and Red Sea coasts of Egypt to cover this point.

The present study showed richness of Eusyllinae, Anoplosyllinae and Exogoninae species inhabiting Egyptian water benthic assemblage.

Corresponding author
F. A. Abd-Elnaby
National Institute of Oceanography and Fisheries, Alexandria, Egypt
*faiza_abdelnaby@yahoo.com

5. References:

1- Abd-Elnaby, F. A. 1999. Composition and Distribution of Some Bottom Fauna Associations Along the Alexandria Coast, Mediterranean Sea. M.Sc. Thesis, Faculty of Science, Alex. Univ. 272pp.
2- Abd- Elnaby, F. A. 2005. Systematic and Environmental Studies on Polychaetes from Alexandria Marine Water. Ph. D. Thesis, Suez Canal University. pp: 330
3- Abd-Elnaby, F. A. 2009. Polychaete Study in Northeastern Mediterranean Coast of Egypt. World Journal of Fish and Marine Sciences. 1 (2): 85-93.
4- Aguado, M. T. and San Martín, G. 2007. Syllidae (Polychaeta) from Lebanon with Two New Reports from the Mediterranean Sea. Cahiers de Biologie Marine. 48: 207-224.
5- Aguado, M. T. and San Martín, G. 2009. Phylogeny of Syllidae (Annelida, Phyllodocida) Based on Morphological Data. Zoologica Scripta. 38 (4): 379-402.
6- Ben-Eliahu, M. N. 1972. Litoral Polychaeta from Cyprus. Tethys. 4: 85-94.
7- Ben-Eliahu, M. N. 1977a. Polychaete Cryptofauna from Rims of Similar Intertidal Vermetid Reefs on the Mediterranean Coast of Israel and in the Gulf of Elat: Syllinae and Eusyllinae (Polychaeta: Errantia: Syllidae). Israel Journal of Zoology. 26: 1-58.
8- Ben-Eliahu, M. N. 1977b. Polychaete Cryptofauna from Rims of Similar Intertidal Vermetid Reefs on the Mediterranean Coast of Israel and in the Gulf of Elat: Exogoninae and Autolytinae (Polychaeta: Errantia: Syllidae). Israel Journal of Zoology. 26:59-99.
9- Brito, M. C., Núñez, J. and San Martín, G. 2000. The Genus Streptosyllis Webster and Benedict, 1884 (Polychaeta: Syllidae Eusyllinae) from Canary Islands, with the Description of a New Species Bulletin of Marine Science. 67(1): 603-615.
10- Böggemann, M. and Westheide, W. 2004. Interstitial Syllidae (Annelida: Polychaeta)

from Mahé (Seychelles). Journal of Natural History. 38: 403-446.
11- Çinar, M. E. 2003a. Ecology of Syllidae (Annelida: Polychaeta) from Northern Cyprus (Eastern Mediterranean Sea). Bulletin of Marine Science. 72: 795-811.
12- Çinar, M. E. 2003b. Ecological Features of Syllidae (Polychaeta) from Shallow-Water Benthic Environments of the Aegean Sea, Eastern Mediterranean. Journal of Marine Biological Association of United Kingdom. 83: 737-745.
13- Çinar, M. E. 2005. *Syllis ergeni*: a New Species of Syllidae (Annelida: Polychaeta) from Izmir Bay (Aegean Sea, Eastern Mediterranean Sea). Zootaxa. 1036: 43-54.
14- Çinar, M. E. & Ergen, Z. 2002. Faunistic Analysis of Syllidae (Annelida: Polychaeta) from the Aegean Sea. Cahiers de Biologie Marine. 43: 171-178.
15- Çinar, M. E., Ergen, Z. 2003. Eusyllinae and Syllinae (Annelida:Polychaeta) from Northern Cyprus (Eastern Mediterranean Sea) with a Checklist of Species Reported from the Levant Sea. Bulletin of Marine Science. 72: 769-793.
16- Çinar, M. E., Ergen, Z. and Benli, H.A. 2003. Autolytinae and Exogoninae (Polychaeta: Syllidae) from Northern Cyprus (Eastern Mediterranean Sea with a Checklist of Species Reported from the Levant Sea. Bulletin of Marine Science. 72: 741-767.
17- Ergen, Z. 1976. Taxonomy and Ecology of Polychaeta in Izmir Bay and Its Vicinity. Scientific Rapport Series of the Faculty of Science, Ege University. 209: 1-73.
18- Fauvel, P. 1927. Rapport sur les Annélides Polychètes Errantes, Cam,bridge Expedition to the Suez Canal, 1924. Transations of the Zoological Society of London. 22 : 411-440.
19- Fauvel, P. 1937. Les fonds de peche pres d'Alexandrie, XI. Anne lides polychetes. Notes Mem. Fish. Res. Dir., Cairo. 19: 1-60.
20- Fauvel, P. 1955. Contribution a la Faune des Annélides Polychètes des côtes d'Israel. Bulletin of the Sea Fisheries Research of the Station of Haifa. 10: 3-12.
21- Fauvel, P. 1957. Contribution a la Faune des Annélides Polychètes des côtes d'Israel. II. Bulletin of the Research Council of Israel. 6: 213-219.
22- Glasby, C. 2000. Family Syllidae. p. 161-167, in Polychaetes and Allies: The southern Synthesis. *Fauna of Australia*. Vol. 4a Polychaeta, Myzostomida, Pogonophora, Echiura, Spuncula, ed. P. L. Beesley. G. J. B. Ross and C. J. Glassby Melbourne. CSIRO publishing. 465pp.
23- Hartmann-Schröder, G. 1974. Zur Kenntnis des Eulitorals der Afrikanischen West-Kuste zwischen Angola und Kap der Gutten Hoffnung und der Afrikanischen Ost-Kuste von Sudafrika und Mocambique unter Bessonderer Berucksichtigung der Polychaeten und Ostracoden. Mitteilungen aus dem Hamburgischen Zoologischen Museum und Institut. 69: 95-228.
24- Hartmann-Schröder, G. 1977. Teil 2. Die Polychaeten der Tropischen Nordwestküste Australiens (Zwischen Port Samson in Norden und Port Hedland in Süden) Mitteilungen aus dem Hamburgischen Zoologischen Museum und Institut. 76: 75-218.
25- Martín, D. and Britayev, T. 1998. Symbiotic polychaetes: Review of Known Species. Oceanography and Marine Biology: An Annual Review. 36: 217-340.
26- Musco, L. and Giangrande, A. 2005. " Mediterrnean Syllidae (Annelida Polychaeta) Revisited: Biogeography, Diversity and Species Fidelity to Environmental Features", Marine Ecology Progress Series. 304: 143-153.
27- Musco, L., Çinar, M.E. & Giangrande, A. 2005b. A new Species of
28- *Sphaerosyllis* (Polychaeta, Syllidae, Exogoninae) from the Coasts of Italy and Cyprus (Eastern Mediterranean Sea). Italian Journal of Zoology. **72**: 161-166.
29- Rouse, G. W. and Pleijel, F. 2001. " Polychaetes" . Oxford University. Press, New York. 1-354.
30- San Martín, G. and Hutchings, P. 2006. Eusyllinae (Polychaeta, Syllidae) from Australia with the Description of a New Genus and Fifteen New Species. Records of the Australian Museum, 58: 257-370.
31- San Martín, G. 2003. Annelida, polychaeta II: Syllidae. In Ramos M. A., Alba J., Bellés Y.; Gosálbez J.; Guerra A.; Macpherson E.; Martin F.; Jerrano J. and Templado J. (eds) Fauna Ibérica. Museo Nacional de Ciencias Naturales, CSIC, Madrid. 21: 1-554.
32- San Martín, G. 2005. Exogoninae (polychaeta: Syllidae) from Australia with the Description of a New Genus and Twenty- Two New Species. Recods of the Australian Museum. 57: 39-152.

33- Selim, S. A. H. 1978. Systematic and Distributional Studies of Polychaetes in the Eastern Harbour, Alexandria. M. Sc. Thesis, Faculty of Science, Alexandria University, Egypt, 402pp.
34- Selim, S. A. H. 1996. On Some Syllid polychaetes from Alexandria waters, Egypt. J. Egypt Ger. Soc. Zool. 21 (D): 51-73.
35- Selim, S. A. 2008a. Eusyllinae and Exogoninae (Polychaeta: Syllidae). New Records from the Egyptian Mediterranean Coastal Waters. Egyptian Journal of Aquatic Research. 34(3) : 160-180.
36- Selim, S. A. 2008b. New records of Sabellid Species (Polychaeta: Sabellinae) from the Coastal Egyptian Waters. Egyptian Journal of Aquatic Research. 34 (1): 108-125.
37- Selim, S. A. 2009. Polychaete Fauna of the Northern Part of the Suez Canal (Port-Said-Toussoum). Egyptian Journal Aquatic Research. 35 (1): 69-88.
38- Simboura, N. & Nicolaidou, A. 2001. The Polychaetes (Annelida, Polychaeta) of Greece: Checklist, Distribution and Ecological Characteristics. Monographs on Marine Science nº4, Marine Centre for Marine Research: Athens. 115 pp.
39- Tovar- Hernández, M. A., Granados-Barbara, A. and Solis- Weiss, V. 2002. *Typosyllis papillosus*, a New Species (Annelida: Polychaeta: Syllidae) from the Southwest Gulf of Mexico", Proceedings of the Biological Society of Washington. 115 (4): 760- 768.

10/13/2010

Association of Depression and Anxiety Disorders with Weight Status among Egyptian School Children: Giza Governorate

Zeinab M Monir , Abla G Khalifa & Manal Mansour
The child health department; National Research Center, *Dokki, Giza*; Egypt,

abla_ncr@yahoo.com.

Abstract: **Background:** The prevalence of childhood obesity is rapidly increasing, and many obese children suffer from emotional and behavioral problems .**The aim** of this study was to explore the relationship between nutritional obesity and psychosocial behavior among school –children in their natural setting. ; and to examine whether social backgrounds play a role in this relationship. Target population was third; fourth and fifth grade primary school children (n=861; mean age10±0.72) attending 3 public elementary schools at Dokki District; in Giza Governorate. **Measurements:** Weight status was assessed through measurements of Body Mass Index percentiles (BAP) for age& sex using World Health Organization Growth Standards. Familial backgrounds**&** academic school achievements of the children were recorded from school files. Data on anxiety and depressive symptoms of children was assessed using standardized methods. **Results:** 23.5% of boys and18.7% of girls showed signs of depression; whereas anxiety was prevalent among 54%of boys and 52% of girls. Calculation of odds ratio (OR) showed that depression and anxiety is higher in low school achievers in girls ($p<0.05$) and boys ($p<0.01$). In a multiple regression model; depression was predicted by anxiety, age and academic achievements ($R^2=0.53$; $P\leq0.001$). Anxiety was predicted by BAP and birth order ($R^2=0.38$; $P\leq0.003$)**. Conclusion:** Obesity affects psychosocial adjustment of children raising the importance of early detection and prevention of obesity in the form of nutritional and health awareness programs and training of school health personnel.

[Zeinab M Monir[a]; Abla G Khalifa[a] & Manal Mansour. Association of Depression and Anxiety Disorders with Weight Status among Egyptian School Children: Giza Governorate. Journal of American Science 2010;6(12):1364-1373]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: Obesity - Children - Depression -Anxiety - Academic Performance.

Introduction:

The prevalence of childhood obesity has been rapidly increasing during the past two decades (1). Recent estimates suggest that 6.6% and 14% of Egyptian children are overweight and obese respectively. (2, 3) Some of the most commonly reported and striking consequences of pediatric obesity are psychosocial (4). Today's culture ascribes a negative stereotype to obese people. There is a common belief that the overweight children are unhappy with their weight and experience most psychosocial distresses, particularly depressive symptoms (5). However, existing information on this relationship comes almost exclusively form clinical samples, wherein obese children seeking treatment demonstrate increased psychopathology and social problems compared with their non obese peers. (6) Comparisons of clinical sample of obese children with non clinical samples of non obese children make it difficult to determine the independent effects of obesity regardless of clinical status on psychological factors. One notable exception to this design confound is the research conducted by Braet et al, (7) who found that a clinical group of obese children scored higher on parent-bases reports of psychopathology, including both behavioral and emotional problems, compared with a non-clinical group of obese children. Additional convergent evidence from a clinical sample includes findings that decreases in percentage of overweight during obesity treatment predict improvements in children's psychological functioning (8). Studies involving 9-years-old subject found that the heaviest children expressed the most discontent, having low body

esteem, a desire of thinness, and higher levels of dietary restraint (9).

Increased risk for depression in obese children presenting for treatment had been reported (10) whereas, Erickson et al, (5) had found a modest relation between obesity in primary school children and depression only in girls. The adult's clinical literature has also linked obesity with psychopathology in general and depression in particular (11).

In Egypt, though the school-age years of life are universally accepted to be of profound importance to the emotional and cognitive and social development; yet less psychiatric intervention occurs in this period from the school health system. The current study has attempted to assess anxiety and depression in primary school age children and in relation to family characteristics and school achievement. Relationship to weight status was as well considered.

Assessing psychological status (depression and trait anxiety) as correlates of weight-related distress in a young, , non-treatment-seeking cohort of children across a wide range of weight provides the opportunity to examine the associations between psychological characteristics and weight-related psychological distress (12). Gaining an understanding of the relationship between childhood obesity and psychological distress is important to identify what it is about obesity that may make children unhappy, early identify targets for intervention, and help clinicians identify those children in need of treatment and the types of treatments that may prove successful.

To address these issues, the present study assessed anxiety and depressive symptoms as an index of psychological distress; and their association to weight status; in a sample of elementary school boys and girls in their natural setting. Based on the existing literatures with adolescents and adults samples, the study hypothesized that obesity and psychological distress would be associated and that this would be influenced by the family backgrounds.

Subjects and Methods:

Subjects:

This study was conducted during school year 2008-2009 in three elementary schools in urban Giza Governorate, Egypt. After gaining the approval of local educational authorities; a passive consent procedure was used. Parents were informed of the study in writing. Classrooms surveys and physical measures were completed during regular school hours by trained pediatricians. A total of 436 boys and 435 girls (age ranged 9.5-10.5 years) completed the study protocol.

Methods:

Every child was subjected to a thorough clinical examination (chest, heart, abdominal and neurological examination). Children having congenital anomalies or chronic diseases were excluded from the study as we aim to assess anxiety and depression prevalence in apparently healthy normal primary school children. Age of the children was recorded from birth certificates that were already present in school files.

A-Body Mass Index:

Children's weight was measured in kilograms using a standardized portable scale. Weight was measured twice to the nearest 0.1.Kg without shoes or outer clothing and the mean was used in the analysis: Height was measured in meters by a direct reading using stadiometer. Height was measured twice to the nearest centimeter and the mean of the two measures was used in the analysis. Children Body Mass Index (BMI, calculated as weight in kilograms divided by the square of height in meters) was then calculated from measures of height and weight as a measure of obesity. BMI is the preferred measure of children adiposity for epidemiological and clinical studies (13).

BMI percentile for age (BAP) and BMI for age Z scores (BAZ) were calculated using the Anthro Plus program of the computer. (14, 15)

B- Anxiety and Depression Assessment:

Depressive Symptoms:

The Arabic Children's Depression Inventory (ACDI) derived from kovacs (16) and kazdin, (17) and constructed for Egyptian children by Abdel Khalek, (18) was used. It comprises seven factors that cover the symptoms of juvenile depression; it conations 27 items in which the children have to respond by themselves through three alternatives i.e. rarely, sometimes and often. The ACDI was found to be correlated to the Arabic version; El-Tayeb (19) of the kovac's Children's Depression Inventory (CDI)

and to the Hopelessness scale for children (16) in its Arabic form of Dowidar (20)

2- Personality Assessment Sheet for Assessment of Anxiety:

The anxiety sheet is derived from State-Trait-Anxiety Inventory (STAI) and validated by Abdel khalek (21). It contains 23 items in which the children have to respond by themselves through three alternatives, i.e. rarely; Sometimes; and often.

C-Academic achievement:

School achievement was recorded from the school files of midterm and terms examinations .Sum of Arabic Language and Mathematics Subjects were taken as an indicator of academic achievements.

D-Social Backgrounds:

Social evaluation has been made using father and mother education with scores 1, 2, 3, 4, denoting illiterate, primary school, high school and university education added to the child's birth order and the family number we gave 1st born score 3, 2nd and 3rd born score 2 more than 3rd born was given score 1, also, family number 4 was given score 3, family number-5 given score 2 and more than 5 was given score 1; social score was stratified as low social ≤ 7; middle social 8-9 and high social 10-12 to be used for the multivariate statistics.

E-Statistical Analysis:

Univariate statistics for data description was used. Bivariate statistics using $\chi 2$ test and Pearson`s correlation to assess the strength of relation of BMI and psychological distress (depression and anxiety) was applied. We used the multivariate analyses to examine independent and interactive associations of BMI and social status with each of anxiety and depressive symptoms. BMI and social status were stratified to examine the independent and interactive associations of BMI and social status with depressive symptom. SPSS program of PC (version 13) was used.

Results:

Table 1, a shows parental education of studied children: 68% of the mothers were illiterate or only read and write; 27.5% had preparatory or high school education whereas only 4.2% of the mothers were university graduated, moreover 55.9% of the fathers were illiterate or can read write and 34.8% reached to high school education and only 9% were university graduated. No significant difference between boys and girls was shown using $\chi 2$ test. ***Table 1, b*** shows that 32.1% of the children were first born child; 23.1% were second born; 18.1% were third born and the rest were fourth or more born children. Most of the children came from big families more than or equal four siblings.

Table 2 shows the weight status of boys and girls as measured by percentile BMI/age. 11% & 11.79 of boys and girls were thin; 13.7% & 12.26% of boys and girls were overweight and 7.8% and 8.01% were obese. There was no significant difference among boys and girls as regard weight status distribution.

Table 3 shows that 7.5 % of girls with low school achievement had depression scores < 44, while 2.3% of girls with high school achievement had depression scores < 44 ; and the difference was significant ($\chi 2$ =4.06, p=0.05). The same results w found among boys; where 12% of low achievers in comparison to 31% of good achievers had depression scores < 44; and the difference was significant ($\chi 2$ =6.19, p=0.017).

Table 4 shows that 19% and 11% of *high* achievers boys and girls had anxiety scores < 35 ; while 7% of boys and 8% of girls who are high achievers had anxiety scores > 35. The difference was significant ($\chi 2$ =6.89, p< 0.05 for girls &$\chi 2$ =3.9, p< 0.05 for boys).

Table 5 shows that depression scores of girls was affected by both mother`s and father`s education ($\chi 2$ =4.3, P = 0.03 & $\chi 2$ =8.1,P= 0.01 respectively),and family number ($\chi 2$ =5.8,P = 0.01); while birth order was the only factor that affected depression scores of boys ($\chi 2$ =4.9, P = 0.05).

Table 6 shows that anxiety scores of girls was affected by maternal education

($\chi 2$ =3.18, *P* = 0.05). Social backgrounds of boys have no relation with anxiety scores of boys (P > 0.05 for all parameters).

Factors affecting anxiety and depression Scores were assessed using simple correlation. ***Table 7*** shows that academic achievement was negatively statistically correlated with both anxiety and depression scores (Pearson`s correlation coefficient

r = -0.238; $p \leq 0.000$ for anxiety and r = -0.272; $p \leq 0.000$ for depression). While age; BMI/age; and height /age showed positive correlations with both anxiety and depression. Anxiety scores showed significant positive correlation with depression; (r = 0.741; $p \leq 0.000$).

Three models were applied using multiple regression analysis for prediction of factors affecting depression scores (dependent variable).

Table 8 shows that anxiety was the independent factor affecting depression in the first model with R^2=0.516; $p \leq 0.0001$. This means that 51.6% of depression states are associated with anxiety. In the second model, anxiety and age was the main predictors of depression (R^2 = 0.481; $p \leq 0.0001$). This means that 48% of anxiety states are associated with depression. The third model shows that anxiety, age and academic achievement predicted depression (R^2=0.532; $P \leq 0.0001$) .this means that 53.2% of depression cases could be predicted by anxiety, age and academic achievement; using the equation:

Depression = 0.7 anxiety +1.2 age + 2.2 academic achievement.

Table 9 shows that BMI –Z scores and Birth order was the main predictors for anxiety ($p \leq 0.03$) ;in this model 3.8% of the change in anxiety could be explained by BMI-Z scores and birth order. This means that anxiety is associated with weight status.

Discussion:

Application of ACDI and STAI on elementary school children of Giza Governorate showed that 23.5% of boys and 18.7% of girls had depressive symptoms. While 54, 1% of boys and 52% of girls reported anxiety disorders; with highly significant correlations of depression to anxiety .This finding agrees with Faith et al (22). Despite the common belief that overweight children are less happy than average weight peers, data on the relationship between obesity and depressive symptoms from a population based sample of young Egyptian children were lacking.

In a study on third grade students attending schools in Northern California, Erickson et al (5) found a modest association between depressive symptoms and body mass index for girls ;but not for boys .Girls depressive symptoms were strongly associated with overweight concerns .Other epidemiological studies have not found an association between depressive symptoms and obesity in population based samples of female adolescents and young women (23) , however some studies have found increased social, educational and psychological correlates of weight status in adolescents (24) as well.

Siegel et al (25) contributed poor health over time to overweight and depressed mood in young women. On the contrary, Neumark, (26) pointed to revaluation of psychosocial concerns among overweight adolescents. In addition to these. Ford et al (27)found that among low and increased self reported BMI, significantly impaired physical functioning rather than mental functioning were noticed.

Previous studies have suggested that gender differences in clinical depressive syndromes do not emerge until puberty ;and women are twice as likely to be depressed as men. Our data suggest that gender differences may emerge in childhood. It has been hypothesized that socioeconomic conditions of the family is one of the risk factors that affect depression in girls and boys. As we conclude from our study that parental education, birth order and family size affects depression in girls. While father education and birth order and family size are the factors that affect depression in boys (11& 22)

In this Study .there was significant negative correlations between academic achievements and each of anxiety and depression scores .Rashed et al (28) reported same results among primary school children in Alexandria Governorate. Vey et al (29); proved that school difficulties could be used in children as a substituted criterion of DSM –III criterion for major depression.

Birth order showed significant relation with depression among boys, whereas depression among girls was correlated with all social parameters (father and mother`s education; family number and birth order. This finding reflects the social behavior of the community where most girls stay at home after school; and so her mood is affected by the family circumstances. On the contrary boys are free to stay outdoors. In this study the overall prevalence of anxiety and depression among boys is high; this could be attributed to other factors other than family backgrounds. Nilzon reported significant association of anxiety and depression with social status ;but he found no sex differences (30).

In the multivariate analysis we were able to assess the independent and interactive relationships of BMI and sociodemographic status with levels of depression and anxiety. We found that BMI as well

as social status were associated with depressive symptoms in both girls and boys; after controlling for social status in a time and controlling for BMI in another time, although this study is limited in its cross-sectional design, these results suggest that anxiety and depression is affected by both obesity and social backgrounds.

In Conclusion; Obesity affects psychosocial adjustment of children raising the importance of early detection and prevention of obesity in the form of nutritional and health awareness programs and training of school health personnel for early detection of psychological disorders among children.

Table (1): Sociodemographic Backgrounds of Boys and Girls

a) Parental education.

	Mother education		Father education	
	Girls N (%)	Boys N (%)	Girls N (%)	Boys N (%)
Illiterate/read and write	294 (69)	294 (67.5)	228 (53.5)	256 (58.3)
High school	115 (27.2)	122 (27.9)	159 (37.6)	140 (32.0)
University	16 (3.8)	20 (4.6)	38 (8.9)	40 (9.7)

No significant difference using $\chi 2$ test

b) Birth order and Family number.

Birth* order (p<0.01)	Girls N (%)	Boys N (%)	**Family* Number (p<0.01)**	Girls N (%)	Boys N (%)
1st	153 (36.2)	122 (27.9)	3	10 (2.3)	2 (0.5)
2nd	88 (20.7)	112 (25.6)	4	66 (15.5)	4 (0.9)
3rd	72 (16.9)	84 (19.2)	5	152 (53.7)	6 (0.4)
$\geq$ 4th	112 (26.2)	118 (27)	> 6	197 (46.5)	426 (98.1)

*: significant differences using $\chi 2$ test

Table (2) : Weight Status in Boys & Girls According to BMI/Age Percentiles (BAP)

Weight Status* (BMI percentile/age &sex)	Boys N (%)	Girls N (%)
Lean ($\leq 3^{rd}$ Percentile)	47(11.0)	50 (11.79)
Average (15^{th} - $\leq 85^{th}$ Percentile)	295(67.6)	288 (67.92)
Overweight ($>85^{th}$ -$\leq 95^{th}$ Percentile)	60(13.6)	52 (12.26)
Obese ($> 95^{th}$ Percentile)	34(7.8)	35 (8.01)
Total N	436	425

*: No significant difference between boys & girls using $\chi 2$ test

Table (3): Depression Scores according to School Achievement among Boys & Girls

Depression scores	School Achievement			**OR**	**RR**	**$\chi 2$ (p)**
	≤50% %	**50-<70% %**	**≥70% %**			
Girls:						**4.06**
<40	**7.5**	**19.0**	**23.1**	**4.626**	**3.652**	**(0.05)**
≥40	**92.5**	**81.0**	**76.9**			
Boys:						**6.19**
<40	**12.4**	**33.5**	**30.2**	**6.631**	**5.739**	**(.017)**
≥40	**87.6**	**66.5**	**69.8**			

OR: Odds Ratio
RR: relative Risk
$\chi 2$: Chi squared test
P : level of significance

Table (4): Anxiety Scores according to School Achievement among Boys & Girls

Anxiety scores	School Achievement			OR	RR	χ^2 (p)
	≤50% %	50-<70% %	≥70% %			
Girls:						
<35	**7.9**	**17.0**	**11.7**	**2.192**	**1.139**	**6.89**
≥35	**92.1**	**83.0**	**88.3**			**(0.05)**
Boys:						
<35	**7.4**	**26.9**	**18.2**	**3.337**	**2.206**	**3.93**
≥35	**82.6**	**73.1**	**81.8**			**(0.05)**

OR: Odds Ratio
RR: relative Risk
χ2: Chi squared test
P : level of significance

Table (5) : Social Parameters according to Depression Scores among Boys &Girls

	Depression score Boys		χ^2 (P)	OR	RR	Depression score girls		χ^2 (P)	OR	RR
	≤43 %	≥44 %				≤43 %	≥44 %			
Father education										
Illiterate	17.4	36.7	22	.221	.219	15.0	43.6	8.2	8.0	4.45
Primary	13.1	24.4	(.64)			14.7	17.4	(0.01)		
High	3.3	5.6				3.2	6.1			
Mother education										
Illiterate	23.5	45.5	1.14	1.09	.06	48.2	48.2	4.26	4.2	4.31
Primary	8.5	18.8	(.79)			6.5	16.5	(.03)		
High	1.9	1.9				2.3	2.3			
Birth Order										
1st born	10.3	25.8	4.96	4.8	.099	11.0	17.0	3.44	3.4	3.03
2nd born	9.9	10.8	(.05)			9.6	16.0	(.5)		
≥3	13.6	29.6				12.4	34.0			
Family number										
≤ 4	5.2	12.7	3.98	4.04	.437	7.8	9.6	5.58	5.7	5.53
4-6	20.7	31.0	(.13)			19.7	37.7	(.01)		
> 6	18.0	22.5				5.5	19.7			
Total n	33.6	66.4				3.3	6.7			

OR: Odds Ratio
RR: relative Risk
χ2: Chi squared test
P : level of significance

Table (6) : Social Parameters according to Anxiety Scores among Boys & Girls

	Anxiety score Boys		χ2 (P)	OR	RR	Anxiety score girls		χ2 (P)	OR	RR
	≤38 (%)	≥39 (%)				≤38 (%)	≥39 (%)			
Father education										
Illiterate	11.3	42.3	2.4 (.13)	2. 3	2.2	10.1	48.6	2.8 (0.01)	8.5	2.1
Primary	9.8	27.7				8.7	23.4			
High	3.3	5.6				2.3	6.9			
Mother education										
Illiterate	16.4	52.6	.77 (.5)	.70	.31	11.9	55.5	3.1 (.05)	3.1	2.9
Primary	6.6	20.7				7.8	20.2			
High	1.4	2.3				1.4	3.2			
Birth Order										
1st born	9.4	26.6	.53 (.5)	.65	.43	6.9	21.1	2.01 .2	2.1	1.6
2nd born	5.6	15.0				6.4	19.3			
≥3	9.4	33.8				7.8	38.5			
Family number										
≤ 4	3.8	14.1	1.76 (.4)	1.7	1.3	4.1	13.3	.45 (.5)	.45	.43
4-6	14.6	37.0				12.4	45.0			
> 6	6.1	24.4				4.6	20.6			
Total n	24.4	75.6				21.1	78.9			

OR: Odds Ratio
RR: relative Risk
χ2: Chi squared test
P: level of significance

Table(7): Factors Affecting Anxiety And Depression Scores

	Anxiety		**Depression**	
	r	***p***	***r***	***p***
Academic Achievement	-0.238	0.000	-0.272	0.000
Age	0.144	0.003	0.215	0.000
HAZ	0.116	0.001	0.100	0.038
BAZ	0.147	0.002	0.148	0.002
Anxiety	-	-	0.741	0.000
Depression	0.741	0.000	-	-

r: Pearson`s correlation coefficient
p: level of significance
HAZ: height/age Z scores
BAZ: Body Mass Index/age Z scores

Table (8): Multiple Regression Analysis Using Depression as a Dependent Variable.

Model	Standardized Coefficients		
	β	t	p
(1) -Constant		**7.837**	**0.000**
-Anxiety	**0.718**	**19.614**	**0.000**
(2) **-Constant**		**0.539**	**0.590**
-Anxiety	**0.701**	**19.109**	**0.000**
- age	**0.108**	**2.934**	**0.004**
(3) **-Constant**		**1.541**	**0.124**
-Anxiety	**0.676**	**18.177**	**0.000**
-Age	**0.100**	**2.751**	**0.006**
- Academic Achievement	**0.099**	**-2.655**	**0.008**

P: level of significance

Table (9): Multiple Regression Analysis Using Anxiety as a Dependent Variable.

Model	Standardized Coefficients		
	β	***t***	***P***
(1) -Constant	**0.129**	**77.03**	**0.000**
-BAZ			**0.002**
(2) -Constant	**1.44**	**48.62**	**0.000**
-BAZ	**0.125**	**3.03**	**0.003**
- Birth order		**2.63**	**0.009**

P: level of significance

References:

1- Popkin B M; (2006): Global nutrition dynamics: the world is shifting rapidly toward a diet linked with non-communicable diseases. Am J Clin Nutr; 84: 289 –98.

2- Hafez AS; Elawady MY; Hassan NE (2000): Obesity profile among primary school children Cairo; Egyt: J Comm Med ; 18 (1) : 99-120

3- Shaheen F , Hathout M, and TawfikA (2004): National Survey of Obesity in Egypt. Final Report. Cairo,National Nutrition Institute(NINI).

4- Lamertz CM, Jacobi C, Yassouridis A,et al(2002):. Are obese adolescents and young adults at higher risk for mental disorders? A community survey. Obes Res.; 10:1152–60.

5- Erikson SJ; Robinson TN; Hayde KF; killen JD (2000): Are overweight children unhappy. Acta pediatr Adolesc Med; 154: 931- 935.

6- Anderson S; Cohen P; Naumova N;et al (2006): Association Of Depression And Anxiety Disorders With Weight Change In A Prospective Community-Based Study Of Children followed Up Into Adulthood. Arch pediatr adolesc med;160:285-291

7- Braet C; Mervieldel L; Vanderycken W (1997): Psychological aspects of childhood obesity; J. Pediatr. Psychol.; 22 : 59-71.

8- Myers MD; Rynor HA, Epslein LH1.(1998): Predictors of child psychological changes during family based treatment for obesity. Arch. Pediatr. Adolesc. Med; 152; 855-861;

9- Hill AJ. Draper E, Stack JA, (1994) : Weight on childrens minds: Body shape dissatisfactions at 9 years old. Int. J. obes. Metab. Disord., 18:383.

10- Wallace WJ; Sheslow D; Hassink S, 1993): Obesity in children: A risk for depression. Ann. N. Y. Acad. Sci., 699: 301.

11- Gibson LY, Byrne SM, Davis EA, Blair E, Jacoby P, Zubrick SR (2007): The role of family and maternal factors in childhood obesity. Med J Aust. 4; 186(11):591-5.

12- Rofey L, Kolko R, Iosif AM,. Silk G (2009): A Longitudinal Study of Childhood Depression and Anxiety in Relation to Weight Gain. Child Psychiatry Hum Dev.; 40(4): 517–526

13- Keiss W; Reich A ; Muller G; Galler A ; Kapellen T ; Raile K; Bottner A ; Seidel B and Kratzch J(2001) : Obesity in childhood and adolescence :clinical diagnosis and management. J . Pediatr. Endocrinal. Metab; 14 suppl. 6 :1431-40.

14- de Onis M, Garza C, Onyango AW, Martorell R,(2006): WHO Child Growth Standards. Acta Paediatrica Supplement;450:S5-S101.

15- World Health Organization (WHO)AnthroPlus for personal computers Manual(2009): Software for assessing growth of the world's children and adolescents. Geneva: WHO, (http://www.who.int/growthref/tools/en/).

16- Kovacs M: Children Depression inventory (CDI) New York: MHS, (1992).

17- Kazdin A; Rodgres A; Colbus (1986): The hopelessness Scale for children. Psychometric characteristics and a concurrent validity. J. consult. Psychol.; 54: 241 – 45.

18- Abdel –Khalek AM (1993): The construction and validation of the Arabic Children's Depression Inventory. Europ J Psychol. Asses ; (9) 41-50, 1993.

19- El-Tayeb MA (1992): Manual for the Children's Depression Inventory. Alexandria Dar-Al ThaKafa (in Arabic).

20- Dwidar AM (1990): Negative expectation toward the future and its relation with some personality variable among children: A psychometric study. Proceeding of The third annual congress of Egypt. Child. Ain shams Univ.; 1: 495-512 (in Arabic).

21- Abdel-Khalek,AM(1989): The development and validation of an Arabic form of STAI : Egyptian Results. Personality and Individual Differences. 10, 277-285.

22- Faith MS, Matz PE, Jorge MA(2002): Obesity-depression associations in the population. J Psychosomatic Res.; 53:935–42.

23- Sargent JD; Blanch flower DG (1994): Obesity and stature in adolescence and earnings in young adulthood: Analysis of British birth cohort. Arch Pediatr. Adolesc. Med. 148: 681.

24- Falkner NH; Neumak-Sztainer D; Story M; Jeffery RW; Beuhring T(2001): Social, educational and psychological correlates of weight status in adolescents. Obes Res 9 (1): 32 –42 .

25-Siegel JM, Yancey AK, McCarthy WJ (2000): Overweight and depressive symptoms amony African- American women. Prev. med.; 31 (3): 232-40.

26-Neumark- Sztainer D; Story M; French SA; Hannan PJ; et al(1997 : Psychosocial concerns and health – compromising behaviors among over weight and non over weight adolescents. Obes. Res.; 5 (3): 237-49.

27-Ford ES; Mariaty DG; Zack MM; Mokdad AH (2001): Self- reported body mass index and health- related quality of life. Findings from the Behavioral Risk Factor Surveillance System. Obes Res ; 9 (1): 21-31.

28-Rashed S,Hafez N; Hebeishy M (1991): childhood depression and its relation to scholastic achievement.Bull Alex Fac Med; XXXVII (4):985-7.

29-Vey P, Colberert P,Neaman B (1996): Aggressive behavior and learning difficulties as symptoms of depression in children.Child Psych hum dev ;17(10): 3-14.

30-Nilzon K A;and Palmer K (1997): The influnceof familial factors on anxiety and depression in childhood. Adolescence;32(128) : 935-43.

11/28/2010

Effect of Exercise on Plasminogen Activator Inhibitor-1(PAI-1) Level in Patients with Metabolic Syndrome

Serag Esmat[1], Randa F Abd Al Salam[*1], and Laila Rashed[2]

[1]Department of Internal Medicine, Faculty of Medicine, Cairo University.
[2]Department of Clinical Biochemistry, Faculty of Medicine, Cairo University.
randa.salam@live.com[*]

Abstract: *Back ground and aim:* Metabolic syndrome is a group of interrelated risk factors, accurate clinical diagnosis and treatment is mandatory for detection of population at risk for coronary heart disease and diabetes. Physical exercise protects against the development of cardiovascular disease, partly by lowering plasmatic total cholesterol, LDL and increased HDL .In addition it is now established that increased C - reactive protein (CRP) and plasminogen activator inhibitor-1(PAI-1) play a role in the maintenance of an inflammatory state and in the development of cardiovascular disease. This study aimed to compare plasma levels of LDL, HDL, CRP, and PAI-1 in patients with metabolic syndrome before and 6 months after moderate intensity exercise. **Methods:** Forty five obese non smoker, males with metabolic syndrome living sedentary life were included in the study .Blood samples were collected at the beginning of the study and 6 months later. However only 42 patients completed in our study The plasma lipid profile (Triglycerides, HDL, LDL, total cholesterol), fasting blood glucose, C - reactive protein and PAI-1 levels were determined. Body weight and BMI were also measured before and after the exercise. **Results:** Total cholesterol ,LDL,HDL,triglycerides,CRP,PAI-1 levels were lower after moderate intensity exercise in relation to levels before moderate intensity exercise($p<0.05$).In addition we observed a positive correlation between PAI-1 and LDL after exercise($r=0.301$,$p=0.053$),PAI and triglycerides after exercise($r=0.286$,$p=0.066$), negative correlation between HDL and PAI-1($r=-0.315$,$p=0.042$). These results indicate that moderate intensity exercise induces favourable changes in metabolic syndrome in lowering lipid profile and PAI-1 levels and may reduce risk of cardiovascular diseases.

[Serag Esmat, Randa F Abd Al Salam, Laila Rashed. **Effect Of Exercise On Plasminogen Activator Inhibitor-1(PAI-1) Level In Patients With Metabolic Syndrome**. Journal of American Science 2010;6(12):1374-1380]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Metabolic syndrome, PAI-1, exercise

1. Introduction

Metabolic syndrome is a cluster of metabolic abnormalities and related clinical syndromes most important of which are coronary artery disease and type 2 diabetes mellitus, insulin resistance, visceral adiposity, dyslipidaemia and chronic subclinical proinflammatory state are the main characteristic features of metabolic syndrome. These inflammatory process include increase in plasma proinflammatory cytokines as tumor necrosis factor alpha (TNF-$\propto$),plasminogen activator inhibitor type-1 (PAI-1), C-reactive protein (CRP) ,Interleukin 6 (IL-6) and reduction of anti-inflammatory cytokines as interleukin 10 (IL-10) and adiponectin [(1, 2)]
Sedentary lifestyle is characterized by alterations in pro-inflammatory markers in the plasma [(3,4)].

The haemostatic abnormality most likely linked to insulin resistance is the elevation of circulating plasminogen activator inhibitor-1(PAI-1).Increased PAI-1 level can be now considered a true component of the syndrome and increases risk of developing cardiovascular disease [(5)].

The aim of this study is to compare plasma levels of LDL, HDL, CRP, and PAI-1 in patients with metabolic syndrome before and 6 months after moderate intensity exercise.

2. Patients and methods:

In a cross sectional study forty five obese, non-smoker men with metabolic syndrome, participated in this study .All subjects were living sedentary life, age (30-49 years),BMI ≥30 ,all patients under went complete history taking, family history of diabetes and personal medical history, history of smoking, physical activity of each subject was defined as either sedentary (<150 min/week) or active (>150min/week) . Patients selection based on International Diabetes Federation(IDF) [(6)] ,metabolic syndrome was defined as central obesity (waist circumference ≥94 cm for men and ≥80cm for women),along with two or more of the following criteria (1) hypertriglyceridemia

(serum triglycerides ≥1.7 mmol/l(150 mg/dl) or current treatment with fibrates (2) an abnormally low HDL cholesterol concentration (<1.03mmol/l for men(<40 mg/dl) and <1.29 mmol/l for women(<50 mg/dl)(3) elevated blood pressure (systolic blood pressure ≥130mmhg and/or diastolic blood pressure≥85mmhg or current use of antihypertensive drugs or (4)elevated fasting glucose ≥5.6 mmol/l (≥100 mg/dl)or previous diagnosis of diabetes. All underwent physical examination including blood pressure recording and anthropometric measurement (BMI, waist circumference). Blood samples of all patients for fasting blood sugar, CRP, total cholesterol, triglycerides, HDL, LDL, PAI-1 were stored at -20^{0}c until analysis. Physical examination and blood samples were collected after 6 months of moderate intensity physical exercise. Only 42 patients completed in the study .The benefits and risks were explained to all patients .They all gave their oral approval to participate in the study.

Blood samples collected after an overnight fast of at least 10 h, stored at -20^{0}c until analysis.
Lipid profiles assay. total cholesterol, triglycerides and HDL were assessed by enzymatic methods using commercially available kits LDL was estimated by Friedewald formula (LDL= total cholesterol-(HDL+triglyceride/5). plasma CRP was measured by kit supplied by (Roche Diagnostics, Indianapolis, IN) and PAI-1was assessed by ELISA kit (Trinity Biotech USA, St. Louis, MO) according to manufacturer instruction .

3. Statistical Methodology:

Data were statistically described in terms of mean ± standard deviation (± SD). Comparison between pre and post data was done using paired *t* test. Correlation between various variables was done using Pearson moment correlation equation for linear relation. A probability value (*p* value) less than 0.05 was considered statistically significant. All statistical calculations were done using computer programs Microsoft Excel 2003 (Microsoft Corporation, NY, and USA) and SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 15 for Microsoft Windows

4. Results:

Results were illustrated in the form of 3 tables. Table 1 shows the mean value of patient anthropometric data(WT, BMI and Waist circumference) and the mean value of the laboratory data(PAI-1, FBS, Total cholesterol, HDL, LDL, CRP and Triglycerides), the table also shows the mean values of systolic and diastolic blood pressure of all patients before exercise.

Table 1: patient anthropometric and lab data before exercise

Parameter	Mean value of the parameters of the 42 patients
Wt(kg)	101.45
BMI(kg/m^2)	31.71
Waist(cm)	105.19
PAI-1(ng/ml)	19.279
FBS(mg/dl)	94.21
Chol(mg/dl)	233.00
HDL(mg/dl)	46.10
LDL(mg/dl)	166.40
TGs(mg/dl)	128.90
SBP(mm hg)	140.12
DBP(mm hg)	87.98
CRP(g/l)	4.12

Table 2: showing comparison in anthropometric and lab data before and after exercise (BMI, lipid profile, CRP, PAI-1) in patients before and after 6 month moderate intensity exercise. In comparison between before and after exercise all parameters show significant difference before and after exercise.

Table 2: Comparison between patient's data before and after exercise.

Parameter	Mean value before exercise	Mean value after Exercise	Correlation	p value
Wt(kg)	101.45	90.45	0.341	0.027*
BMI(kg/m^2)	31.71	28.681	0.816	0.000**
Waist(cm)	105.19	98.60	0.850	0.000**
PAI-1(ng/ml)	19.279	10.379	0.414	0.006*
FBS(mg/dl)	94.21	85.69	0.655	0.000**
Chol(mg/dl)	233.00	209.00	0.804	0.000**
HDL(mg/dl)	46.10	54.45	0.421	0.006*
LDL(mg/dl)	166.40	136.10	0.768	0.000**
TGs(mg/dl)	128.90	108.60	0.909	0.000**
SBP(mm hg)	140.12	133.93	0.665	0.000**
DBP(mg/dl)	87.98	83.81	0.434	0.004*
CRP(g/l)	4.12	2.51	0.906	0.000**

*p is significant p<0.05
**p is highly significant p<0.001

Table 3: Showing correlations between PAI-1 and different lab data 6 month after moderate intensity exercise. Positive correlation between PAI-1 and LDL(r=0.301, p=0.053), PAI and triglycerides (r=0.286, p=0.066) PAI-1 and BMI (p<0.001,r=0.589) , negative correlation between HDL and PAI-1(r=-0.315, p=0.042). Negative not statistically significant correlation between CRP and PAI-1(r=-0.016, p=0.921)

Table 3: Correlation of PAI-1 to different data before and after exercise.

		PAI-1-Pre	PAI-1-Post
Wt(kg)	Pearson Correlation	0.116	0.053
	p value	0.464	0.737
BMI(kg/m^2)	Pearson Correlation	0.507	0.589
	p value	0.001*	0.000****
Waist(cm)	Pearson Correlation	0.529	0.370
	p value	0.000**	0.016*
FBS(mg/dl)	Pearson Correlation	0.708	0.367
	p value	0.000**	0.017*
Chol(mg/dl)	Pearson Correlation	0.184	0.173
	p value	0.245	0.274
HDL(mg/dl)	Pearson Correlation	0.029	-0.315
	p value	0.855	0.042
LDL(mg/dl)	Pearson Correlation	0.336	0.301
	p value	0.030*	0.053*
TGs(mg/dl)	Pearson Correlation	0.236	0.286
	p value	0.133	0.066*
SBP(mm hg)	Pearson Correlation	0.375	0.066*
	p value	0.015	0.676
DBP(mm hg)	Pearson Correlation	0.169	0.070*
	p value	0.285	0.660
CRP(g/l)	Pearson Correlation	0.147	-0.016
	p value	0.354	0.921

*p is significant p<0.05
**p is highly significant p<0.001

5. Discussion:

The metabolic syndrome is associated with an increased risk for the development of cardiovascular disease [7,8], A number of haemostatic abnormalities have recently been associated with the metabolic syndrome ,amongst with elevated concentrations of plasminogen activator inhibitor -1(PAI-1) and tissue plasminogen activator antigen(tPA_{ag}) share the strongest associations and have been studied in the most detail [9]. Consistent associations have also been found with fibrinogen concentrations, vitamin K dependant coagulation factors (factors-vii,ix and x) ,C-reactive protein (CRP) and von willebrand factor (vWF syndrome [10, 11], increase in plasma proinflammatory cytokines as tumor necrosis factor alpha (TNF- ∝) ,Interlukin 6 (IL-6) and reduction of anti-inflammatory cytokines as interleukin 10 (IL-10) and adiponectin [1, 2]. It is now well established that impaired fibrinolysis due to elevated PAI-1 activity ($PAI\text{-}1_{act}$) is an important feature of the metabolic syndrome. [19].

PAI-1, produced by vascular endothelium is an important risk factor for CAD .Increased PAI-1levels may predispose patients to the formation of atherosclerotic plaques prone to rupture with a high lipid –to-vascular smooth muscle ratio as a result of decreased cell migration [12] Circulating levels of PAI-1 are increased in obese subjects with metabolic syndrome, as well as in patients with type2diabetes.The plasma levels of PAI-1 are directly related to the severity of metabolic syndrome [13]. The metabolic syndrome explains a major part of plasma PAI-1 level variability, PAI-1, which otherwise behaves as an acute phase reactant, was demonstrated to be independently associated with metabolic syndrome with this relationship being stronger in men than in women (45%versus 26%). Interventional studies reported that if insulin resistance is improved plasma PAI-1 levels decrease. Race seems to have a definite influence on PAI-1.Both genetic and lifestyle factors may contribute to this difference in plasma levels.[19]

Decreased plasma PAI-1 concentrations were observed after weight reduction by a hypo caloric diet and were associated with decreased body fat. In addition treatment with insulin –sensitizing drugs like metformin or troglitazone decrease plasma PAI-1 levels in subjects with type 2 diabetes and to some extent in normal obese subjects[14, 15,16]. Simvastatin in addition to reducing LDL –c it reduces inflammation in MS subjects Simvastatin also reduces circulating plasminogen activator inhibitor-1 activity in volunteers with metabolic syndrome.

The levels of PAI-1, soluble p-selectin and CD40 ligand play an important role in the

development and progression of atherosclerosis; their levels are increased in metabolic syndrome.

Metabolic syndrome has endothelial dysfunction, decreased circulating adiponectin, and high expression of angiogenic inhibitors such as PAI-1[17]

The mechanism of PAI-1overexpression during obesity is complex and it is conceivable that several inducers are involved at the same time at several sites of synthesis. Interestingly, recent in vitro and in vivo studies showed that beside its role in the atherothrombosis PAI-1 is also implicated in adipose tissue development and in the control of insulin signalling in adipocytes. These suggest that PAI-1 inhibitors serve in the control of atherothrombosis and insulin resistance [18]

The link between PAI-1 and metabolic syndrome with obesity was established many years ago. Increased PAI-1level can be now considered a true component of the syndrome [5]

Like our results in which PAI-1 had positive association between components of metabolic syndrome: BMI (p=0.001),waist circumference (p<0.001) systolic blood pressure (p=-0.015) fasting blood sugar (p<0.001),however no association with triglycerides (p=0.133) or HDL (p=0.855)

In a study done by Greyling et al[19] they observed a lack of association of plasma PAI-1 antigen concentrations with parameters of the metabolic syndrome (general and visceral adiposity, blood pressure and lipids in Africans) ,whereas these associations were prominent in Caucasian women. PAI is directly secreted by adipose tissue, especially by plasma concentrations of PAI in obesity Investigations performed on PAI deficient mice[20] and with pharmacological inhibitors of PAI reinforced the notion that the absence of PAI reduces the differentiation of adipocytes and protects against insulin resistance and diet-induced obesity, suggesting a paracrine effect of PAI on promoting of weight gain . This is also in accordance with several population based prospective studies demonstrating that PAI is the only biomarker that predicts incident metabolic syndrome and diabetes.

Metabolic syndrome subjects have raised levels of PAI-1, associated with an increased risk of MI, correlating with obesity[19,21] PAI-1 which otherwise behaves as an acute phase reactant, was demonstrated to be independently associated with metabolic syndrome, even after adjustment with HOMA-IR and other co-variables ,this finding reinforces the results of previous cross-sectional studies that considered haemostatic and inflammatory markers and observed that outstanding relationship between PAI-1 and metabolic syndrome[22]

The association between PAI_{act} and markers of the metabolic syndrome in Caucasians is well established [9, 11]

The results of the study of Bronat etal[22] indicate that HbA1C and PAI are strongly and independently related to metabolic syndrome .Moreover, the elevation of both markers seems to be a manifestation of the same pathophysiological mechanism that underlies the main characteristics of the syndrome.Therfore, PAI-1 and HbA1C should be considered as true components of metabolic syndrome and may be considered as candidates for inclusion in the list of diagnostic criteria.[22]

PAI-1 is also a valuable biomarker for predicting the metabolic syndrome(MS) in elderly residents in study done by Chou YY et al[23] mean age 79.9+/- 4 years .Elderly resident with MS had higher systolic and diastolic blood pressure (p<0.001) and higher HOMA-IR (p<0.001), CRP (p=0.008),and PAI-1 levels (p<0.001) than those without the MS .On multivariate logistic regression analysis , PAI-1was an independent risk factor for the MS. Elderly with higher waist circumferences and higher levels of plasma fasting glucose and TG and lower level HDL had higher PAI-1 levels than those without the above components[23]. Enhancing endogenous fibrinolysis by targeting PAI-1 inhibitor, the primary inhibitor of circulating plasminogen activators, has been shown to be effective in markedly attenuating the formation of arterial and venous occlusive thrombosis in animal models .In addition ,animal and human studies of PAI-1 deficiency indicate that spontaneous bleeding complications associated with even complete PAI-1 deficiency would be rare .Patients most likely to benefit from PAI-1 inhibition would be those at high risk for vascular events, with elevated PAI-1 levels , such as is in obesity ,diabetes and the metabolic syndrome .Since obesity and metabolic syndrome are now epidemic and will likely have a major adverse impact on vascular thrombotic events. It may be time to test the clinical effectiveness of PAI-1 inhibition in those populations at high risk for vascular thrombosis [24].

Sedentary lifestyle is characterized by alterations in pro-inflammatory markers in the plasma [3,4]. Several studies have consistently shown that low levels of plasma high density lipoprotein (HDL) and high levels of low and very low density lipoprotein (LDL and VLDL respectively) are linked with a sedentary life style and are strong predictor to cardiovascular disease [25, 26, 27]

It remains slightly unknown if long term moderate /high intensity exercise have lowered pro-inflammatory and increased anti-inflammatory markers than sedentary subjects.

In our study total cholesterol, LDL,HDL, triglycerides, CRP,PAI-1 levels were lower after moderate intensity exercise in relation to levels before moderate intensity exercise ($p<0.05$).In addition we observed a positive correlation between PAI-1 and LDL after exercise ($r=0.301$, $p=0.053$), PAI and triglycerides after exercise ($r=0.286$, $p=0.066$), negative correlation between HDL and PAI-1 ($r=-0.315$, $p=0.042$).

Our results were similar to Fabio etal[28], Teramoto etal[29], Coen etal[30] these studies detected the favourable effect of exercise on lipid profile and PAI-1.

In a study done by Fabio et al seven male leading sedentary life and seven highly trained athletes subjects were recruited. Blood samples were collected after an overnight fast, plasma lipid profile, glucose, adiponectin, C-reactive protein and PAI-1 levels were determined. Results of the study showed lower plasmatic lipid profile and PAI-1 levels in highly trained athletes ($p=0.01$) than sedentary subjects. In addition the total plasma cholesterol, LDL-c and TG concentration were positively correlated with PAI-1 levels. These results indicate that life style associated with high intensity and high volume exercise induces favourable changes in the lipid profile and PAI-1 levels and may reduce risk of cardiovascular diseases [28]

The effect of Short-term exercise training does not change PAI-1 levels in normal, asymptomatic men and women. In addition, modest decreases in insulin and triglyceride in individuals with elevated body fatness do not result in changes in PAI-1 after short-term training. It appears likely that decreases in PAI-1 with exercise training require decreases in adiposity and/or marked changes in metabolic variables. This was shown In study done by Bodary PF et al[31] they examined the influence of 10 days of moderate-intensity exercise training on measures of fibrinolysis. Sixteen men and 16 women between the ages of 50 and 70 yr were randomly assigned to exercise (EX) and control groups (CON) that were balanced for gender and hormone replacement therapy. Blood samples were collected on days 1, 2, 11, and 12 for measurement of plasma PAI-1, tPA, insulin, glucose, and triglyceride. Subjects in EX performed 50 min of treadmill walking at an intensity corresponding to 65% of heart rate reserve each day for 10 consecutive days.There were no significant changes in PAI-1, tPA, or associated metabolic variables between exercise and Control group during the intervention period. Within Exercise subjects, those with higher body fatness had a significant decrease in insulin and triglyceride compared with those with lower body fatness. However, no changes in fibrinolytic measures were observed within these subgroups. They concluded that Short-term exercise training does not change PAI-1 levels in normal, asymptomatic men and women. In addition, modest decreases in insulin and triglyceride in individuals with elevated body fatness do not result in changes in PAI-1 after short-term training. It appears likely that decreases in PAI-1 with exercise training [31].

6. Conclusion:

Moderate intensity exercise induces favorable changes in metabolic syndrome in lowering lipid profile and PAI-1 levels and may reduce risk of cardiovascular diseases

Correspondence Author:
Randa F Abd El Salam
Department of Internal Medicine, Cairo University.
Telephone: 002-02-2760893
Cellular phone: 02-0101407278
Email: randa.salam@live.com

7. References:

1. 1-Lira FS,Rosa JC,Zanchi NE,Yamashita AS,Lopes RD etal:Regulation of inflammation in the adipose tissue in cancer cachexia:effect of exercise.Cell Biocchem Funct .2009,27(2):71-5.
2. 2- Lira FS,Koyoma CH,Yamashita AS,Rosa JCet al:Chronic exercise decreases cytokine production in healthy rat skeletal muscle .Cell Biochem Funct. 2009, 27(7):458-61.
3. 3- Ades PA,Savage PD,Toth MJ,Harvey – Berino J,Schneider DJ,Bunn JY etal:High-calorie-expenditure exercise a new approach to cardiac rehabilitation for overweight coronary patients. Circulation .2009, 119(20):2650-2.
4. 4- King GL:The role of inflammatory cytokines in diabetes and its complications.J periodontal . 2008, 79:1527-34.
5. Singh B,Arora B,Goswami V:Metabolic syndrome: A review of emerging markers and management. Diabetes and metabolic syndrome:clinical Research and reviews.2009,(3) 240-254.
6. Alberti KG MM,Zimmet PZ, Shaw JE:The meta-bolic syndrome: A new world-wide definition from the Intrrnational Diabetes Federation Consensus.Lancet.2005;366:1059-62.
7. Bradshaw D,Scheneider M,Dorrington R,Bourne DE,Laubscher R.South African

cause of death profile in transition -1996 and future trends.S Afr Med j .2002,92:618-23.
8. Vorster HH. The emergence of cardiovascular disease during urbanization of Africans.Public Health Nutr. 2002, 5:239-43.
9. Reaven GM :Hemostatic abnormalities associated with obesity and metabolic syndrome.J Thromb Haemost.2005;3:1074-5.
10. Juhan-Vague I,Alessi MC,Mavri A, Morange PE.Plasminogen activator inhibitor-1,inflammation,obesity,insulin resistance and vascular risk.J Thromb Haemost .2003;1:1575-9.
11. Mertens I,Verrijken A,Miciels JJ,Van der planken M,Ruige JB,Van Gaal LF:Among inflammation and coagulation markers,PAI-1 is a true component of the metabolic syndrome .Int J Obes , 2006,30:1308-14.
12. Sobel BT : Increased plasminogen activator inhibitor-1 and vasculopathy .A reconcilable paradox.Circulation.1999;99:2496-8.
13. Festa A,Agostino jr R,Tracy RP,Haffner SM.Elevated levels of acute-phase proteins and plasminogen activator inhibitor-1 predict the development of type 2 diabetes.Diabetes 2002;51:1131-7.
14. Kruszynska YT,YU JG,Olefsky JM, Sobel BE: Effect of troglitazone on blood concentrations of plasminogen activator inhibitor -1 in patients with type 2 diabetes and in lean and obese normal subjects.Diabetes .2000;49:633-9.
15. Trost S,Pratkley R,Sobel B:Impaired fibrinolysis and risk for cardiovascular disease in the metabolic syndrome and type 2 diabetes. Curr. Diab Rep. 2006; 49:633-9.
16. Skurk T ,Hauner H: Obesity and impaired fibrinolysis:Role of adipose production of plasminogen activator inhibitor-1.Int J Obes Relat Metab Disord.2004;28(11):1367-64.
17. Mouquet F,Cuilleret F,Susen S ,Sautiere K et al:Metabolic syndrome and collateral vessel formation in patients with documented occluded coronary arteries :association with hyperglycaemia,insulin-resistance,adiponectin and plasminogen activator inhibitor-1.Eur Heart J.2009:3(7):840-9.
18. Alessi MC,Vague IJ:PAI-1 and the metabolic syndrome links, causes and consequences. Arterioscler Thromb Vasc Biol.2006,26:2200-7.
19. Greyling A,Pieters M,Hoekstra T,Oosthuizen W, Schutte AE:Differences in the association of PAI-1 activity with the metabolic syndrome between African and Caucasian women. Nutrrition, Metabolism and Cardiovascular Disease. 2007, 17:499-507.
20. Ma LJ,Maaosl,Taylorkl et al:Prevention of obesity and insulin resistance in mice lacking plasminogen activator inhibitor 1.Diabetes.2004;53:336-46.
21. Crandall DL, Quinet EM,El Ayachis et al. Modulation of adipose tissue development by pharmacological inhibition of PAI-1.Arterioscier Thromb Vasc Biol.2006;26:2209-15
22. Boronat M,Varillas VF,Saaverda P etal:Diadetes mellitus and impaired glucose regulation in the canary island :Prevalence and associated factors in the adult population of Telde,Gran Canaria.Diabet Med .2006,23:148-55.
23. Chou YY,Sheu WH,Tang YJ et al.Plasminogen activator type 1 (PAI-1) is a valuable biomarker for predicting the metabolic syndrome in institutionalized elderly residents in Taiwan.Arch Gerntol Geriatr.2009;49(2): s 41-5
24. Westrick RJ,Eitzman DT:Plasminogen activator inhibitor-1 in vascular thrombosis.Curr Drug Targets .2007;8(9):966-1002.
25. Hamburg NM,Mc Mackin CJ,Huang AL,Shenouda SM etal:Physical inactivity rapidly induces insulin resistance and microvascular dysfunction in healthy volunteers.Arterioscler Thromb Vasc Biol .2007 ,27(12):2650-6.
26. Elisson M,Asplund K,Evirn PE:Regular leisure time physical activity predicts high activity of tissue plasminogen activator:The Northem Sweden MONICA study.Int J Epidemiol 1996,25(6):1182-8.
27. 27-Naslund GK,Fredrikson M,Hellenius ML,De faire U:Effect of diet and physical exercise intervention programmes on coronary heart disease risk in smoking and non smoking men in Sweden.J Epidermiol Community Health 1996,50(2):131-6.
28. 28- Fabio S Lira,Jose C Rosa,Adriano E,Lima S etal:Sedentary subjects have higher PAI-1 and lipoproteins levels than highly trained athletes. Diabetology Metabolic Syndrome. 2010,2:7
29. 29- Teramato M,Golding LA:Regular exercise and plasma lipid levels associated with the risk of coronary heart disease:PA 2o-year longitudinal study.Res Q Exerc Sport. 2009,80(2):138-45.
30- Coen PM,Flynn MG,Markofski MM,Pence BD,Hannemann RE:Adding exercise training

to rosuvastatin treatment:Influence on serum lipids and biomarkers of muscle and liver damage.Metabolism .2009,58(7):1030-8.

31- Bodary PF, Yasuda N,Watson DD,Broon AS etal:Effect of short term exercise training on plasminogen activator inhibitor (PAI-1).Int J Exerc.2003;35(11):1853-8.

Reuse of Industrial Materials in Buildings To activate their application in Egypt

Nermin Mokhtar Mohamed

Researcher in Civil and Architectural Department ,Engineering division, National research centre, Egypt

ABSTRACT: Increasingly stringent rules and regulations on construction and demolition waste, diminishing landfill space and depletion of natural resources are all reasons for the push for industrial byproduct materials recovery. In Egypt, Industrial byproduct materials are generated in large volumes every day that are potentially usable materials, and must be disposed of. The main goal of this paper is to change the way Egyptians' think about waste—to see the value of a used material as a product or commodity, not as a waste, and encourage the use and recycling of these rich, largely untapped resources. Positive economic rewards and environmental results are moving our partners toward more waste reduction and materials management. This paper summarizes the proposed Egyptian industrial materials waste management guidelines to reuse in building ,which cover: (1) Identify the parties involved and the distribution of responsibilities; (2) Complementarily of roles of parties(owner, engineer, designer, and contractor) involved in the process of re-use to remove the causes that hinder the management of such material in Egypt ; and (3) Participation of the Parties to the proposed project to achieve sustainable development fields at the actual application of the project.

[Nermin Mokhtar Mohamed. **Reuse of Industrial Materials in Buildings To activate their application in Egypt**. .http://www.americanscience.org Journal of American Science 2010;6(12):1381-1393]. (ISSN: 1545-1003).

KEYWORD: reuse –industrial byproduct materials, waste management, sustainability, Egypt.

INTRODUCTION

The world is becoming increasingly conscious of the environmental implications not only of production processes but also of products discarded after use. The recycling of waste materials as a means of tackling the solid waste problem is attracting growing interest.

This is the problem of solid waste currently producing and increase with increasing Almttrd Census world population of the most serious problems and will remain polluted environment for long periods of up to thousands of years which cause environmental damage and health problems.

In Egypt, the traditional method to get rid of Industrial wastes is send to landfill as waste. Industrial wastes are more damaging to environment, and public health, In addition, Construction and demolition (C&D) materials led to urban pollution image around, as well as the economic burden and the cost of waste transportation.

In pursuit of sustainable development principles, which aims to rely on recycling waste came importance of research on alternatives, Salvaging materials for reuse can be both an economical and environmentally sound alternate to waste stream disposal, and it also saves energy and environmental impacts of producing new products from virgin materials that help communities be sustainable in infrastructure renovation, construction, and maintenance.

With the pursuit of Egypt now to the sustainable development of areas and provide jobs for young graduates, In urgent need for alternative construction materials with rising cost of wood universal waste and demolition and construction that represent a high proportion of solid waste, Find the possibility of reuse industrial materials in building that Re-cycling building materials is an essential part of ecological sustainability.

And therefore need to find research benefit from industrial materials in the possibility of exploitation as an economic environment friendly housing in Egypt organization management process participation and funding for projects on physical development, to activate application construction for environmental development, economic, social and physical in areas

Despite the government attempts to impose penalties or fines or recourse to academic studies and research centers to develop solutions for waste management but it is still a deficiency in complete control of the problem in Egypt.

Research aims to propose a comprehensive and integrated approach to establishing industrial materials and its activation in Egypt by identifying the parties involved and their respective roles for the protection of areas affected by the effects of disposal opening alternatives to creating markets for reuse industrial materials in physical development, and to conserve energy and preserve natural resources.

The main objective of research can be achieved through several sub-objectives, namely: monitor problems resulting from industrial materials pollution in Egypt, current status assessment and

study of causes that hinder the proper management of residues in Egypt, finance and operations planning part of the parties involved to upgrade the project such areas, Identify the areas of development environmental, economic, social, physical and role of parties involved in achieving these areas

1- Sustainable

Sustainable development meets the needs of the present without compromising the ability of future generations to meet their own needs. Basically, it's another term for "green" or "environmentally friendly".

Implementing sustainable projects means achieving an ecologically, socially and economically acceptable future[1].

By taking into consideration all economical aspects and the effects on people, and the environment during the planning and development phases, minimize the use of energy and resources to protect the environment, and increase the efficiency of all projects[2].

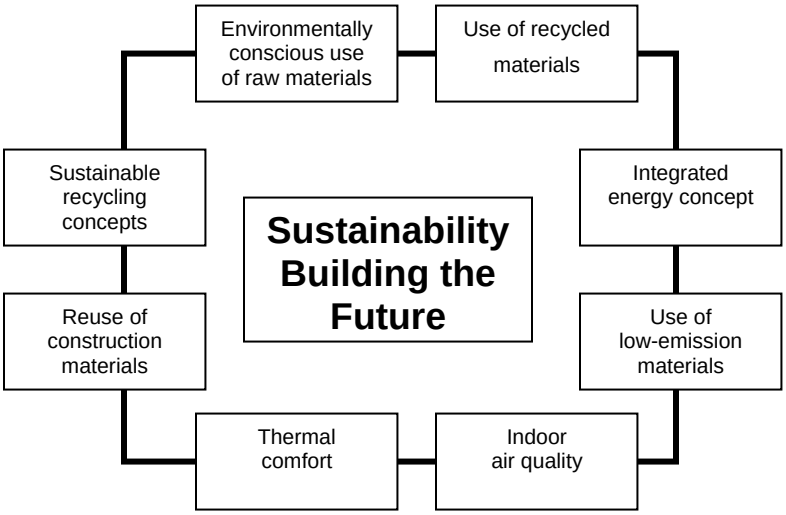


Fig. (1) Aspects of the Sustainability

Fig. (1) Aspects of the Sustainability

1-1 Choosing sustainable materials

It can be difficult to assess exactly how sustainable a product is and which materials are preferable to others[3]. There are some tools that can help you to choose the building materials, table (1).

There are many considerations that should be taken into account when choosing building materials. Since many different definitions exist concerning what constitutes an environmentally friendly or green material, this study use the following set of terms as factors in determining environmentally preferred materials and products[4].

By-product

Unused or waste material from one manufacturing or energy-producing process that can be used in another manufacturing or energy-producing process [5]

- *Agricultural by-product:* Unused or waste material from farming operations, several of which can be used in building products such as strawboard panels, etc.
- *Industrial by-product:* Unused or waste material from power plants or manufacturing operations, several of which can be used in building products, e.g. fly ash concrete, etc.

Table (1): Evaluation tools to choose the building materials

Evaluation tools	**Life-cycle assessment**	This means considering the impact during the extraction of the raw materials, manufacture, transport, handling, installation, the lifetime of its use, recycling and disposal.
	Embodied energy	The total amount of energy that is needed to produce, transport it to site and install it. For building products, it is commonly measured in Mega Joules (MJ) per unit of product.
	Renewable resources	These are resources that will be replenished with time; they include plant and animal products such as timber, paper, cork, wool and leather.
	Sustainable resources	Sustainable resources are the products of cyclic closed systems that do not require outside inputs, and do not generate waste.
	Local resources	Locally sourced products need less energy for transport and they support your local economy.
	Toxicity	Some materials are relatively harmless for humans, but their production might cause habitat destruction or release toxins into the environment. Toxic materials can also be a problem for installers or when they are disposed of at the end of their life cycle.
	Quality	The expected lifetime of the building is short; it makes little sense to use very durable materials.
	Re-use and recycling	Using second-hand or recycled materials is another option for reducing resource use.
	Uncertainty	Materials that have been tested for a long time in your local conditions are a safer choice than new materials or those which have not been proven locally.

2- Industrial materials

Industrial materials recycling, referred to as beneficial use, means reusing or recycling byproduct materials generated from industrial processes. These materials can be used as substitutions for raw materials in the manufacture of consumer products, roads, bridges, buildings, and other construction projects. Thousands of manufacturing and industrial processes and electric utility generators create hundreds of millions of tons of nonhazardous industrial materials that are often wasted[6].

2-1 Examples of practical recycling applications

Nonhazardous industrial materials, such as coal ash, foundry sand, construction and demolition materials are valuable products of industrial processes. Each material may be recycled in a variety of diverse applications, table (2). These materials have many of the same chemical and physical properties as the virgin materials they replace - they can even improve the quality of a product[7].

Table (2): The properties of industrial materials and recycling applications in building

industrial materials	The properties and the problem	recycling applications in building
Cement dust produced during the cement industry	Produced from the burning and grinding of raw materials used in the manufacture of cement, contains a high proportion of the components of the cement but in different proportions, and fails to plant one of the factories of the Egyptian cement per day at least 300 tons in the Mediterranean.	It has been used in many engineering applications, including: Partially replace cement in some industries of construction materials such as bricks, tiles, the cement industry, glass, rubber, sewage treatment, the foundation layer for roads.
Steel slag	It is a byproduct of iron and steel industry and contains a high proportion of the components of the cement, but in different proportions. The amount of (steel slag)	It is used in many engineering applications, including: as heap in concrete works of traditional and light production, types of cement

	from iron and steel sector about a million tons annually, which is a national problem as well as emissions generated from the accumulation of slag	(Ferro-cement - high iron slag - high resistance to sulfate).
Foundry Sands	It is sand that is used to make molds and cores in the metal casting process. Although generally recycled many times internally by the foundries, about 3-4 million tons of foundry sand is discarded each year. The recycling of nonhazardous, spent foundry sand can save energy, reduce the need to mine virgin materials, and may reduce costs for both producers and end users.	the spent foundry sands is used As partial replacement for fine aggregate in asphalt mixtures; in Portland cement concrete; As source material for the manufacture of Portland cement; and As a sand used in masonry mortar mixes, And in the other applications
Coal combustion products	CCPs include the following materials: Fly Ash; Ash; Boiler; Flue Gas Desulphurization Material (FGD); and Other types of material such as fluidized bed combustion ash, and scrubber residues The characteristics and physical properties of CCPs vary. In general, the size, shape, and chemical composition of these materials determines their beneficial reuse as a component of building materials or as a replacement to other virgin materials such as sand, gravel, or gypsum.	Fly ash can be used as a replacement for the Portland cement that binds traditional concrete mixes. The manufacture of Portland cement requires large inputs of energy, and it is estimated that its manufacture constitutes about 8% of all carbon dioxide emissions from human sources. Approximately 75% of the fly ash produced annually is disposed of in landfills, which makes incorporating it into concrete a resource-efficient alternative.
Pulp and paper byproducts	Two significant byproducts from the paper industry are WWTP residuals and boiler ash	There are numerous examples of other uses Building board/fixture, Brick or concrete additive, Glass or lightweight aggregate
Construction and demolition (C&D) materials[8]	It consists of the debris generated during the construction, renovation, and demolition of buildings, roads, and bridges. C&D materials often contain bulky, heavy materials, such as concrete, wood, metals, glass, and salvaged building components[9]. In Egypt, the daily quantity of construction and demolition (C&D) waste has been estimated as 10,000 tones. That is equivalent to one third of the total daily municipal solid wastes generated per day in Egypt[10].	It can make a number of products (solid cement bricks, hollow bricks, paving blocks) using the broken bricks and broken ceramics. As possible, get a light concrete using broken bricks as an alternative partial or total ruins of the great used in industry. You can also use the surplus concrete and rubble after rounding heap for the production of concrete suitable for the various structural elements.

3- Advantages and disadvantages of the process of recycling waste

The process of recycling has some of the advantages and disadvantages[11], Table (3).

4- Building Applications for Industrial Materials

The beneficial use of industrial materials that were previously considered wastes has been expanding with a number of applications gaining market and regulator acceptance. Environmental and economic benefits derived from the recycling and beneficial use of industrial materials are becoming more evident:

- Conserving energy and reducing greenhouse gas emissions by decreasing the demand for products made from energy-intensive manufacturing processes;

- Preserving natural resources by decreasing the demand for virgin materials - recycled materials have many of the same properties as the virgin material they replace, and may improve the quality of the products in which they are used;
- Decreasing the economic and environmental burdens of disposal, as well as reducing the cost of material for end users.

Table (3) Advantages and disadvantages of the process of recycling waste

disadvantages of recycling waste	**Advantages of recycling waste**
- Some materials are generally more difficult to recycle - other materials are dangerous or require more energy inputs to be recycled - The durability of some materials can be extended if they properly protected and maintained while in use - Environmentally preferable materials may be more expensive or difficult to locate - Determining a product's environmental preferability can be a complex process for which no tools exist - Prepare the materials may need more time - Need efficient labors	- Conserves energy and reduces greenhouse gas emissions by decreasing the demand for products made from energy intensive manufacturing processes - reduce the volume of materials which are sent to landfill as waste to achieve the continued development - save the embodied energy content - Preserves our natural resources by decreasing the demand for virgin materials - Saves money by decreasing disposal costs for the generator and decreasing materials costs for end users. - Local employment creation - Reuse of old buildings and use of recycled materials.

This diagram illustrates a variety of common building applications for industrial materials. Note that the availability of specific industrial materials can vary regionally, fig. (2)[12].

Fig. (2) Building Applications for Industrial Materials

Fig. (2) Building Applications for Industrial Materials

Table (4) a variety of common building applications for industrial materials

(a,b) Green Roofs & Landscaping	Green roofs are roofs covered with plants; they reduce storm runoff and provide insulation. Scrap tires can be used to make rubber tile for walkways. Bottom ash can be used as bedding material. Clean wood, recycled gypsum wallboard, and cardboard can be ground and used as soil amendments in both green roofs and landscaping applications.
(c) Landscape Furniture	Benches can be made with plastic lumber containing fly ash or with recycled C&D wood.
(d) Building Facing Material	Manufactured stone, which is concrete mixed with aggregates, is commonly used as building facing materials. fly ash can be used in the production of manufactured stone
(e) Sidewalks	Industrial materials can be used to make concrete sidewalks, and used tires can be recycled to create rubberized sidewalks. Asphalt concrete sidewalks can be made with recycled asphalt pavement and recycled asphalt shingles.
(f) Ceiling Tile	Ceiling tile can contain flue gas desulfurization (FGD) gypsum (a material resulting from burning coal to produce electricity), fly ash, recycled gypsum wallboard, or air-cooled blast furnace slag.
(g) Flooring	Industrial materials can be used in various flooring applications. (h) Carpet backing: Used tires, fly ash, or recycled carpet. (i) Wood flooring: Salvaged lumber or recycled wood. (j) Flooring tile: Fly ash, blast furnace slag. (k) Tile underpayment: Fly ash.
(l) Backfill (Foundation Support)	Backfill surrounds the building foundation, supporting it and providing drainage. Scrap tires provide superior drainage, insulation, and wall pressure relief. Blast furnace slag and recycled concrete also can be used for drainage.
(m) Foundation Structural Fill	Structural fill is an engineered fill that is constructed in layers and compacted to a desired density. Coal fly ash, bottom ash, slag, and spent foundry sand can all be used as structural fill. Concrete can be crushed and used onsite as structural fill.
(n) Poured Concrete Foundation	Concrete, which is composed of cement, aggregate, and water, is used in a wide array of building applications. Industrial materials can be recycled in cement and concrete in many ways. Here are a few examples: • Fly ash and ground granulated blast furnace slag can be used as partial cement replacements. Using these materials can produce stronger, longer-lasting concrete. • Portland cement itself can be made with fly ash, FGD gypsum, foundry sand, recycled gypsum wallboard, blast furnace, and steel slag. • Concrete aggregates can include bottom ash, foundry sand, crushed concrete, and blast furnace slag.
(o) Insulation	Air-cooled blast furnace slag can be used to produce mineral or rock wool insulation (also known as slag wool insulation).
(p)Drywall/Wallboard	FGD gypsum and recycled gypsum wall board can be used to manufacture drywall.
(q) Mortars, Grouts, Stucco	Mortars, grouts, and stucco contain aggregate (sand), binder, and water. Fly ash, foundry sand, silica fume, and slag cement can all be used as partial cement replacements.
(r) Masonry Blocks	Masonry blocks are made from cement and aggregate. Slag cement, fly ash, or silica fume can substitute partially for cement. Bottom ash, blast furnace slag, and recycled concrete aggregate can substitute for newly mined materials.
(s) Base Material	Spent foundry sand can be used in place of natural soil as base material for the building site. Recycled concrete is also commonly used as base material.

5-Case study

Home of the experiences of the World created by using industrial materials reused, table (5)

Case study	Location & Building Specs	Design	Materials Reused or Recycled	Positive Community Impacts
The Lazarus Building[13] is one of the most significant green rehabilitation projects in Columbus.  *Lazarus Before Redevelopment*  *Lazarus After Redevelopment*	Renovation of a 600,000 square foot commercial building in downtown Columbus, Ohio.  	-Reducing, reusing, and recycling materials during renovation and construction -Using recycled-content products and materials in construction -Cost savings and environmental benefits -Environmental awards and recognition -Local community revitalization	Developers retained over 75 percent of the original structure, significantly reducing the amount of materials needed for the project. The renovation employed -Coal fly ash in concrete; -Recycled glass and tile flooring containing up to 100% recycled materials; -Carpets containing recycled nylon; -Restroom partitions containing 100% post-consumer recycled plastic; -Drywall containing at least 96 percent recycled materials, including flue gas desulphurization gypsum; and -Building siding containing 60 percent recycled metal.	-cost savings and avoided local community impacts from trucks hauling debris away. - The project created local jobs, improving the local economy. -a showcase of innovative ways to reduce waste.
EPA's new building[14]  **Architectural Scale Model**  **The exterior environment**  **The interior environment**	200,000 square foot EPA Regional Headquarters office building in downtown Kansas City, Kansas.  	-Many building features contribute to exceptional energy efficiency - The building's operations and maintenance environmentally friendly. -building products and materials are more environmentally sensitive, or "greener"	-Utilizing Fly Ash in concrete design -The aluminum mullions and trim on the windows, sheer wall, sunscreens, cable trays and skylight are all constructed from recycled aluminum. -In restrooms, the floors and walls were constructed with ceramic tile made from over 70% post-industrial recycled waste glass. -The Shaw carpet is made of 25% recycled material - The wood wall base in the atrium is 100% recyclable. - The ceiling tile is made from 93%-recycled slag and the grid system from light gauge steel made from 67% recycled material.	-the reduction of smog was considered when selecting building materials. In order to help reduce the contribution of VOCs into the atmosphere. -a showcase of innovative ways to reduce waste.

6- Waste Management in Egypt

Waste Management is a component of sustainable development, which seeks to reduce the impact of human activities on public health and the environment and development.

The industrial development in Egypt is the main engine of economic growth, where is the advantage of good Waste and exhaust for different industries, one of indicators of progress in the United.

Industrial waste are serious environmental problem and that the lack of local industries based on Exploitation of the waste in other industries, making it a burden and a waste of environmental resources used In production processes, fig. (3). In fact, it can be exploited to become the waste materials with high economic value.

To sustainable improve waste and materials management in Egypt[15] will focus on the active involvement of industrial waste generators in the reduction of waste volumes at source, and will also encourage private sector participation in solid waste management services, particularly collection and recycling.

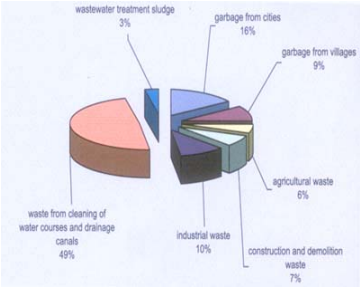


Fig. (3) The proportion of solid waste in Egypt

6-1 About a method to organize and manage the re-use of industrial materials in construction in Egypt:

This part comes to address the negative aspects which appear in the overlapping of roles and responsibilities of the parties involved in the project and overcome the causes that impede the management of re-use of industrial materials in Egypt to achieve urban development[16].

6-1-1 Identify the parties involved and the distribution of responsibilities

Parties to the proposed approach are different bodies responsible for policy development re-use of industrial materials in construction.

They are the government, institutions of technical advisory, executive agencies (investors and a group of capital and local governance), popular participation (beneficiaries), research centers, contractors and specialists (architects). In order to integrate the curriculum leading to activation of the application re-use of industrial materials in construction in Egypt should be considered interested parties and their respective roles to achieve the restructuring of the curriculum by ideal integration of limbs.

(I) the role of government

The success of the reuse industrial materials in building depends heavily on local government engagement and action. Their role is large and vital[17].

- Coordinate and facilitate partnerships to implement the reuse materials action plan.
- Lead by example in government
- Provide incentives that encourage green design, construction, and deconstruction and begin removing disincentives
- Expand capacity and markets for reusing and recycling construction and demolition materials.
- Increase awareness, knowledge, and access to reuse industrial materials
- Encourage innovative product design
- to encourage beneficiaries to participate in these projects beneficial to the environment
- amend laws and existing environmental legislation

(Ii) The role of NGOs non-governmental organizations:

- Implementation of development projects in the environment and recycling waste.
- Training of local technical staff
- Coordination between the professional societies and funding for the implementation of various development programs.

- Galvanize the efforts of various parties (government, individuals, and investors) with the coordination between them.
- Support salvaged materials collection centers.
- Subsidize warehouse space to support the collection and distribution of salvaged materials.
- Create incentives for deconstruction, recycling, and the use of salvaged or recycled materials into construction procurement contracts.

(III) The role of professional engineers[18]

Specialists are planners, architects, economists, social, the most significant roles played by professionals in the following:

- Create new buildings that save energy and water, use fewer material resources, and create less waste.
- The design appropriate to the building to suit the Egyptian environment, according to the needs of the population and location
- Created propose a method suitable for the Egyptian environment and the work of drawings.
- Technical guidance and training courses for users and individuals to create technical staff can participate in all phases of the project.
- building designers have a responsibility to specify preferred materials and methods of construction which are suited to recycling

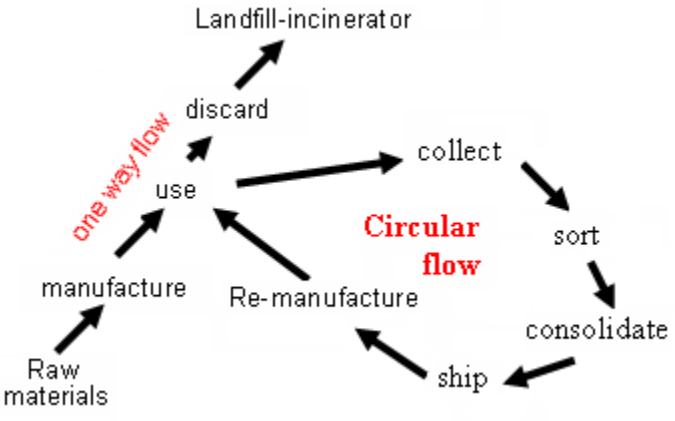


Fig.(4): Circular and linear flows of materials

(IV) Contractor` Role

- Design and plan to Prevent Waste, and develop a Construction Waste Management Plan
- Survey the Site Before Demolition or Deconstruction, Plan for Recyclable Materials
- Identify Reusable or Salvageable Items, all materials should be examined using a precautionary approach to eliminate possible toxicity or future regulatory constraints to their use and disposition.
- Select Salvage Removal Alternatives
- Estimate the Costs and Savings
- Prevent Contamination
- Separating the components will facilitate adaptation and reduce the complexity of deconstruction
- Building contractors need to exercise care during demolition, and should be prepared to re-use suitable materials on projects.


Fig. (5) Reuse –recycle can occur onsite and offsite [19]

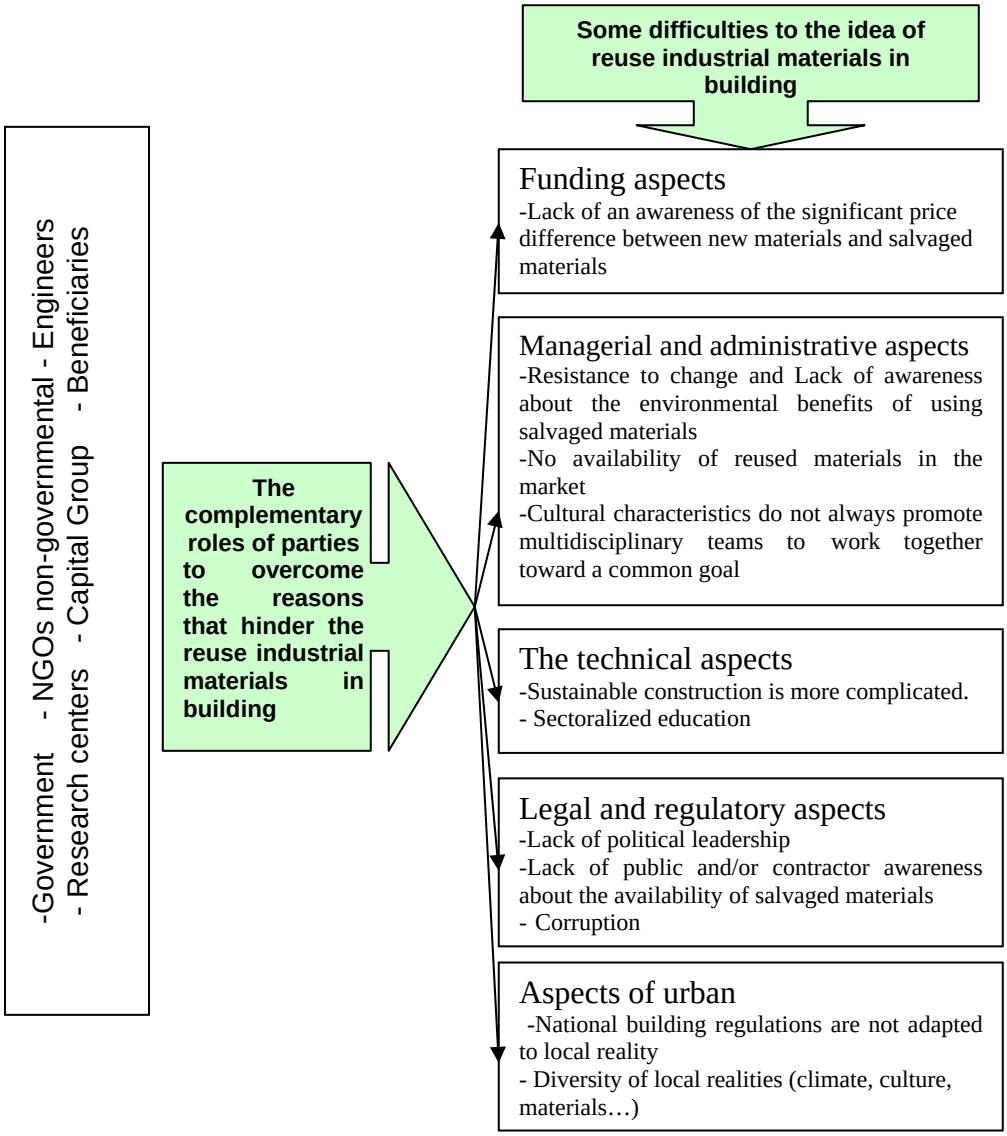


Fig (6) overcome the reasons that hinder the reuse industrial materials in building in Egypt

(V) The role of research centers

- Examine the adequacy of recycled materials used with the Egyptian environment.
- Insurance system building against fire, moisture and insects, and the work of all the tests required, and improve the implementation of this new technology.
- Study ways to improve the properties of the construction of the building using this new technology.
- Employment training to create this type of installations.
- the use of certain programs to achieve energy efficiency within enterprises, and may remove energy consumption to the minimum (Zero Energy)
- Monitoring of constraints and variables that occur in the region and draw conclusions to contribute to approve the project with the environment and the needs of the user, the study of the potential physical and technical implementation, and improve the economic viability of the project.

(Vi) The role of Group Capital (investors and businessmen, banks, real estate specialist)

- Develop and/or fund training programs. Subsidize training costs for participants.

- receipt of the site, and provide the necessary potential for re-use of building materials to be used
- coordination between manufacturers and specialists to bring in industrial materials and re-use in construction
- Follow-up after implementation of changes in operations and maintenance

(Vii) The role of beneficiaries / individuals

the user is the basis of urban development which is capable of maintaining the physical output, and can be user participation in several areas.

- participate in the implementation of self-help
- participate in the maintenance and improve the local environment
- Participating home improvement loans to upgrade facilities and infrastructure and public services.
- Owners should insist on the use of recycled materials… in the interest of ecologically sustainable development.

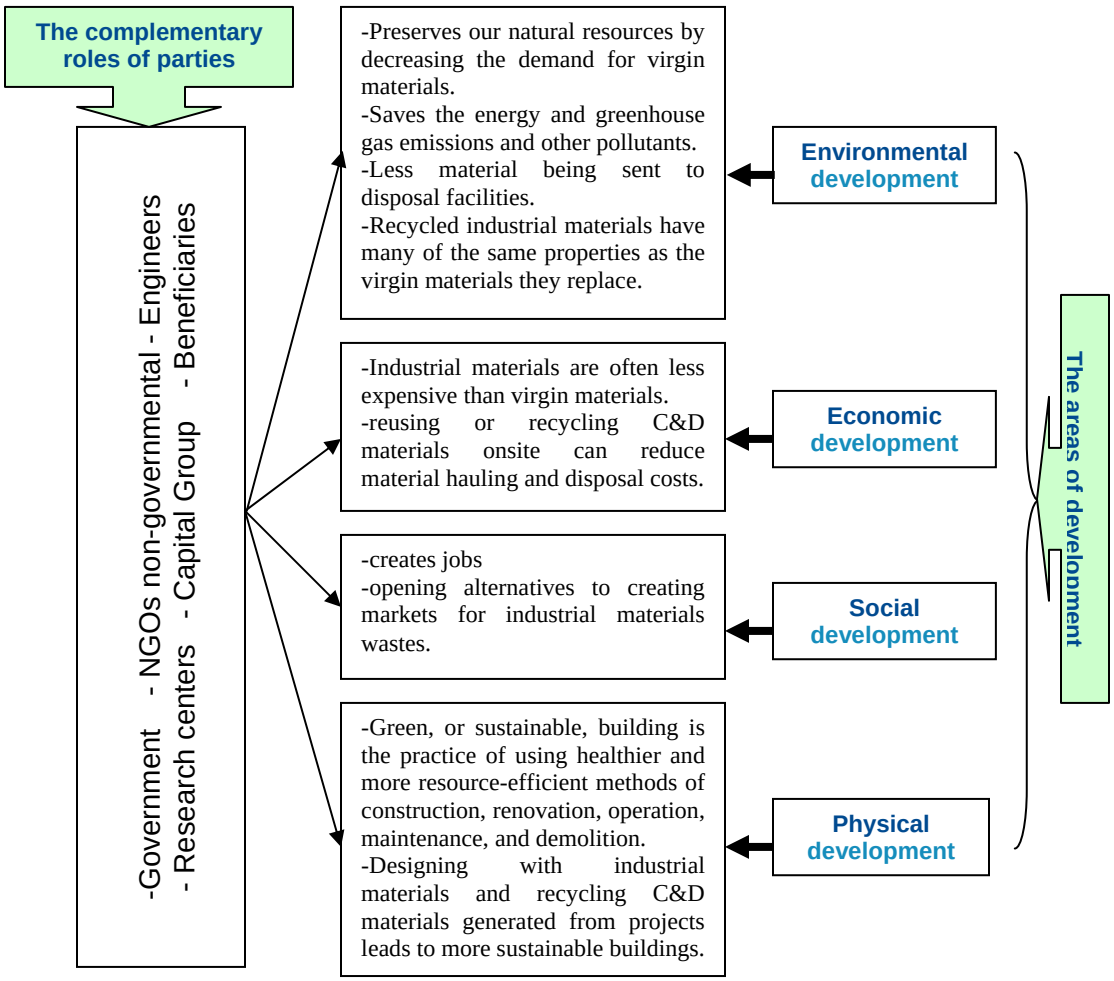


Fig (7) increase the effectiveness of the parties involved in the process of reuse industrial materials in building in various facets of development (environmental, economic, social and physical) for sustainable development

6-1-2 Complementarily of roles of parties involved in the process of re-use to remove the causes that hinder the management of such material in Egypt, figure (6).

(a) between industry and regulators to increase the understanding of the beneficial use of industrial materials and regulatory programs; (b) among state regulators to share information and experiences on beneficial use regulations

and determination processes; (c) among industries to share experiences on successful reuse and recycling, and to assess the potential to utilize each others' materials, and (d) among researchers, nonprofit representatives, industry, and regulators to share information and concerns regarding risk assessment, beneficial use determinations, environmental safety, and new research.

6-1-3 Participation of the Parties to the proposed project to achieve sustainable development fields at the actual application of the project.

Integration of the roles of the participants in the process of re-use of industrial materials in construction is conducive to the development of environmental, economic, physical and social**,** figure(7).

7- CONCLUSIONS

Based on the data collected from the literature survey, it is revealed that the production of industrial materials waste is escalating both on the international and national scales. Furthermore, environmental, safety, visual and technical related problems generated from these wastes has severely added to the long-term negative impacts of these wastes on the surrounding environment.

The essence of the recommended guidelines in this paper is to offer systematic procedures that could help in minimizing the magnitude of the industrial materials waste problem in Egypt. Therefore the disposal option can be avoided by the implementation of reuse them in building.

Waste reduction opportunities begin with the earliest choices made in the building process, including architectural design and material selection. Effectively balancing resource-efficient design concepts requires the attention of skilled and environmentally conscious building professionals. These concepts include waste prevention, durability, and recyclables.

And has been monitoring some of the negatives facing the potential to activate the application of construction materials industry and are summarized as follows:

1. Limited NGO non-governmental organizations concerned with the field of urban development in general and re-use of industrial materials in particular.
2. Does not represent low-income housing a sufficient degree of urbanization, culture and enable them to participate in such environmental projects in an effective manner.
3. Difficulty of maintaining such environmental projects for low-income.
4. Building design re-use of industrial materials zero energy consumption in Egypt still needs some time.
5. Non-participation of specialists sometimes leads to delays in implementation and increase the cost and other obstacles that may face the project when actual implementation.

8- RECOMMENDATIONS

(1) Increased awareness, acceptance and proactive government policies are critical in order to continue the upward trend of recycling and reusing materials whenever possible

(2) More political support is required to enforce the implementation of waste management scheme in the construction/building field, collect industrial material wastes under the direct supervision of authorities. Imposing a special tax levied on wastes when exceeding a certain level determined by the government.

(3) It is also recommended to extend research on the area of recycling and reusing techniques of industrial materials in building to induct feasibility studies, including cost/benefit and payback period analysis for each technique. The research should survey the Egyptian market and seek the potential possibility of using waste as raw materials in factories. This research should integrate both the construction industry and the manufacturing industry to bridge the gap between the two disciplines.

(4) Overcoming these challenges may require advocacy work to strengthen policies and incentives to reduce construction and demolition waste, intensive education and marketing to expand the demand for reused building materials, as well as smart partnerships and inventory management to keep the right mix of reused materials in stock to meet local demand.

9- REFERENCES

1) Halliday,Sandy," Sustainable Construction", Elsevier, 2008.

2) Mendler, S., Odell, W., The HOK Guidebook to Sustainable Design, New York, NY: John Wiley & Sons, Inc., 2000.

3) Ross Spiegel, Dru Meadows ," Green Building Materials", John Wiley&sons, 1999.

4) Giamberdine, R., et al, Guiding Principles of Sustainable Design, Denver, CO: The National Park Service, 1993

5) Jon Nunan," The Complete Guide to Alternative Home Building Materials & Methods", Atlantic publishing group, Inc., 2010.

6) Managing nonhazardous solid waste, http://www.epa.gov/osw/inforesources/pubs/orientat/rom2.pdf

7) 12th International Conference on Non-conventional Materials and Technologies "Materials & Technologies for Sustainable Infrastructure Systems"21-23 September, 2010 Cairo, Egypt

8) Pinto,T. P., and Agopayan, V. (1994). "Construction wastes as raw materials for low –cost construction products" Sustainable construction (Proc. 1st Conf. Of CIB TG 16), J.Kibert,ed., Ctr. For Constr. and Envir., Gainesville, Fla., 335-342.

9) Webster, M., The Use of Salvaged Structural Materials in New Construction, The Austin Papers, Brattleboro, VT: Building Green, Inc., 2002

10) Al-Ansary," Sustainable Guidelines for Managing Demolition Waste in Egypt", International RILEM Conference on the Use of Recycled Materials in Building and Structures,2004.

11) Elliott, S., 'Don't Waste Time', *International Construction* (March 2000) 15-17.

12) Using Recycled Industrial Materials in Buildings http://www.epa.gov/epawaste/conserve/rrr/imr/pdfs/recy-bldg.pdf

13) Green renovation brings a community back to life: The Lazarus Building. Available At http://www.epa.gov/epawaste/conserve/rrr/imr/pdfs/lazarus.pdf (2010)

14) Building Materials Available At http://www.epa.gov/region07/p2/offtheshelf/offshelfpage16.htm (2010)

15) Al-Ansary, M.S., 'Recommending Egyptian Guidelines for Managing Construction and Demolition (C&D) waste', MSc. Thesis, The American University in Cairo (AUC), Egypt,1999.

16) UN Documents Cooperation Circles: Our Common Future, Chapter 2: Towards Sustainable Development. http://www.un-documents.net/ocf-02.htm

17) Egyptian Environmental Law No.4 for year 1994 and its Executive Regulations, 'Promulgating a law concerning Environment', 1st Edn (Al Matba El Ameraya, Egypt, 1994)

18) Otto, K.N., Wood, K.L., Product Design: Techniques in Reverse Engineering and New Product Development, Upper Saddle, NY: Prentice Hall, 2001

19) EPA Industrial Materials Recycling—www.epa.gov/industrialmaterials

10/28/2010

Molecular Biological And Biochemical Studies On Avian Influenza Virus Receptors In Different Avian Species

Hussein I. El-Belbasi; Mohamed F. Dowidar and Safaa I. Khater

Department of Biochemistry, Faculty of Veterinary Medicine, Zagazig University,Egypt
H_elbelbasi2003@yahoo.com

Abstract: Avian influenza viruses are considered to be the key contributors to the emergence of human influenza pandemics. A major determinant of infection is the presence of virus receptors on susceptible cells to which the viral haemagglutinin is able to bind. Avian viruses preferentially bind to sialic acid α 2,3-galactose (SA α 2,3-Gal) linked receptors, whereas human strains bind to sialic acid α 2,6-galactose (SA α 2,6-Gal) linked receptors. Although ducks are the major reservoir for influenza viruses, they are typically resistant to the effects of viral infection, in contrast to the frequently severe disease observed in chickens In order to understand whether differences in receptors might contribute to this observation, we studied the expression of influenza receptors in upper and lower respiratory organs of ducks and chickens (expression of ST3Gal-III sialyltransferase and ST6Gal-I sialyltransferase genes) using semi quantitative RT-PCR. There was a marked difference in the expression of primary receptor type in the trachea of chickens and ducks. In chicken trachea, SA α 2,6-Gal was the dominant receptor type whereas in ducks SA α 2,3-Gal receptors were most abundant. This suggests that chickens could be more important as an intermediate host for the generation of influenza viruses with increased ability to bind to SA α 2,6-Gal receptors and thus greater potential for infection of humans. Chicken tracheal and intestinal epithelial cells also expressed a broader range of SA α 2,3-Gal receptors in contrast to ducks, which suggests that they may be able to support infection with a broader range of avian influenza viruses.

[Hussein I. El-Belbasi; Mohamed F. Dowidar and Safaa I. Khater. **Molecular Biological And Biochemical Studies On Avian Influenza Virus Receptors In Different Avian Species**. Journal of American Science 2010;6(12):1394-1401]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Host receptors, influenza, , chicken, duck, ST3Gal-III sialyltransferase, ST6Gal-I sialyltransferase, gene expression.

Introduction

The influenza viruses are medium-sized, comprising enveloped and negative sense RNA viruses with a segmented genome. Taxonomically, they belong to the virus family Orthomyxoviridae. There are three genetically and antigenically distinct types of influenza viruses called A, B, and C. Type A viruses are further divided into subtypes according to the combination of two main envelope glycoproteins the hemagglutinin (HA)and neuraminidase (NA). To date, 16 HA subtypes (H1–H16) and 9 NA subtypes (N1–N9) have been found (**Ghaleb, 2009**). Influenza A virus infects several hosts, including humans, birds, swine, and horses, but individual viruses are usually adapted to sustained infection in only one species. Viruses isolated from these different species bind sialic acid through their surface glycoprotein, hemagglutinin, and require this interaction for productive infection. (**Gambaryan et al., 2005).** The first step in the virus infection process is the recognition of cellular structures that act as specific receptors. This determines the virus tissue tropism and is performed by viral adhesion proteins (**Tardieu et al., 1982**) .The viral attachment to the host cell is critical for tissue and species specificity of virus infections. (**Debby et al., 2007**). Influenza virus initiates infection by binding of the viral hemagglutinin (HA) to sialic acid on the cell surface. (**Stray and Air, 2001**). The receptors for influenza viruses are sialic acids (SAs), which are usually formed 2,3 or 2,6 configuration linked to the cell-surface glycoproteins and glycolipids **(Harduin-Lepers et al.,2005).** Sialic acid is an essential component of cell surface receptors for a variety of microorganisms and microbial toxins (**Mouricout,** 1997). Sialic acid is added to the terminal sugar of glycoproteins and glycolipids by enzymes called sialyl transferases (**Harduin-lepers et al., 2005).**

Sialyl transferases (SiaTs) are required to synthesize all known sialyloligosaccharides. (**Shuichi, 1995**). The ST3Gal III, preferentially transfers sialic acid in α 2,3 linkage to the Galß1-3GlcNAc disaccharidic sequence (**Catherine et al.,1999).** While ST6Gal-I generates an α 2-6 linkage of sialic acid to underlying N-acetyllactosamine (**Weinstein et al.,1982**). The differential expression of sialic acids in the mammalian respiratory tract may help to explain the low infectivity but high pathogenicity of some avian strains. (**Gambotto et al., 2008**).

Influenza infection is initiated by virus attachment to sialic acid-containing cell-surface

molecules traditionally called viral receptors. The spectrum of sialylglycoconjugates varies substantially between viral host species as well as target tissues and cell types of the same species leading to variations in the receptor-binding specificity of viruses circulating in these hosts. It is believed that a poor fit of avian viruses to receptors in humans limits the emergence of new pandemic strains(**Matrosovich et al.,2008).** Influenza A viruses attach to host cells by binding of the hemagglutinin (HA) protein to sialosaccharides on the host cell surface. The HAs of influenza A viruses from different host species differ in their specificity of binding. For example, HAs of human influenza A viruses preferentially recognize sialic acid (SA) α 2,6-Gal-terminated saccharides(α 2,6-SA) ,whereas HAs of avian influenza viruses preferentially recognize SA α 2,3-Gal-terminated saccharides (α 2,3-SA) **(Connor et al.,1994**). These differences generally correspond with the variation in the type of SAs expressed at important sites for influenza A virus replication in the respective host species. For example, human tracheal epithelium expresses mainly α2,6-SA, whereas duck intestinal epithelium expresses mainly α 2,3-SA. Therefore, the type and distribution of SA is considered to be an important factor in the susceptibility of different host species to influenza A viruses (**Suzuki et al., 2000**). The SA recognized by influenza A virus is not only important in the host species range but also in its transmissibility(**Tumpey et al., 2007**).The HA protein mediates virus binding to sialic acid (SA)-containing host cell surface molecules and promotes the release of viral ribonucleoprotein complexes through membrane fusion.

Influenza virus infectivity is influenced by **2** entities:-
1- SA species (N-acetylneuraminic acid [NeuAc] and N- glycolylneuramic acid [NeuG].
2- the type of linkage to galactose (sialyloligosaccharides terminated by SA linked to galactose by an (alpha 2,6 linkage [Ac alpha 2,6Gal] or an alpha 2,3 linkage [Ac alpha 2,3Gal]) on the host cell surface (**Rogers et al., 1985).**

The host range selection of the receptor binding specificity of the influenza virus hemagglutinin occurs during maintenance of the virus in different host cells that express different receptor sialo-sugar chains (**Yasuo, 2005).** Ducks and chickens are important hosts of avian influenza virus (AIV) with distinctive responses to infection. Frequently, AIV infections in ducks are asymptomatic and long-lasting in contrast to the clinically apparent and transient infections observed in chickens. These differences may be due to the host response to AIV infection.(**Sean et al., 2009).**

Material and methods

Bird selection and grouping:

Four groups of healthy, four weeks aged birds are classified as fellow :

1. group 1 : 5 chicken (Baladi).
2. group 2 : 5 chicken(Hubber).
3. group 3 : 5 duck(Baladi).
4. group 4 : 5 duck(Pekeni).

Tissue preparation :

- Birds were sacrified using highly sterilized scissors (180°Cfor 6 hours) to avoid RNA degradation by RNases and latex gloves weared to minimize RNase contamination.
- After excision of trachea and lung of tested birds , they were wrapped in aluminium foil and put immediately in liquid nitrogen container to make snap-freezing of tissue and minimize action of endogenous RNase.
- Samples were taken to detect the level of gene expression of ST3Gal-III(Galβ1-3(4)GlcNAcα2,3-sialyltransferase) and ST6Gal-I (Galβ1-4GlcNAcα2,6-sialyltransferase) in that organs.

Reverse transcriptase polymerase chain reaction (RT–PCR): Using a semi-quantitative RT–PCR according to **(Mallet et al., 1995).**

A-Protocol of RNA extraction from tissue: total RNA was extracted with RNeasy Mini Kit (QIAGEN).

B-Protocol of reverse transcription polymerase chain reaction: (one step RT-PCR) by using Robus T 1 RT-PCR kit(FINNZYMES)

The protocol was as fellow:

All components, reaction mixes and samples were kept on ice. And the following reaction component were added to a nuclease free tube placed on ice.

Reaction set up:

RT-PCR mix component	**volume**
10x Robus T reaction buffer	5 µl
50 mM MgCl2	1.5 µl
dNTP mix(10mM each)	1 µl
Template RNA	5 µl
Down stream primer	10 pmol
Up stream primer	10pmol
AMV RT 5 U/ µl	1 µl
DyNAzyme EXT DNA polymerase 1U/ µl	2 µl
RNase free water	Add to 50 µl

Gently mix the components, cycling conditions have to be optimized for each amplicon. and was transferred to the thermal cycler.(2720 thermal cycler Applied Biosystems).

Cycling instructions:

1-**For ST3Gal lll gene**:- the primer for ST3Gal lll was synthesized to amplify PCR products that cross introns to avoid confusion between mRNA transcript and genomic DNA.

The primers used to amplify this gene are:

Forward: 5- CGGATGGCTTCTGGAAATCTGT- 3

Reverse: 3- AGTTTCTCAGGACCTGCGTGTT-5

The product size was 300 bp.

Cycle step	Temp	time	Number of cycle
cDNA synthesis	48 °C	30 min	1
Inactivation of AMV reverse transcriptase and denaturation of the cDNA-RNA hybrid	94 °C	2 min	1
PCR amplification Denaturation Annealing extention	 94 °C 63 °C 72 °C	 30 sec 1 min 1.5 min	 36 cycles
Final extention	72 °C	7 min	

2-**For ST6 Gal I gene:** :- the primer for ST6 Gal I were synthesized to amplify PCR products that cross introns to avoid confusion between mRNA transcript and genomic DNA.

The primers used to amplify this gene are:-

Forward: 5- TGGGCCTTGGCAGGTGTGCTGTTG-3

Reverse: 3-AGGCGAATGGTAGTTTTTGAGCCCACATC-5

The product size was 150 bp.

Cycle step	Temp	time	Number of cycle
cDNA synthesis	48 °C	30 min	1
Inactivation of AMV reverse transcriptase and denaturation of the cDNA-RNA hybrid	94 °C	2 min	1
PCR amplification Denaturation Annealing	 94 °C 50 °C 72 °C	 45sec 1 min 1 min	 35 cycles
extention			
Final extention	72 °C	7 min	

Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) were amplified parallely as internal control (481bp) and its sequence:

Forward: 5--ACTTGTGATCAATGGGCACGCCATC - 3

Reverse: 3--CTTCCCATTCAGCACAGGGATGAC-5

For the Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) amplified by 35 cycle using (2720 thermal cycler Applied Biosystems). Each cycle consist of :

Denaturation 94°C for 45 second

Annealing 62 °C for 30 second

Extention 72°C for 45 second

The PCR products were separated by agarose gel electrophoresis.

C-Agarose gel electrophoresis (Sambrook and Maniatis.,1989)

1- **Run parameters** :
   - Use 1-5 volts/ cm of the tank lenth.
   - Allow bromophenol blue to run 2/3 of the gel lenth before terminating the run.

2- stop the run and transfere the gel to a transilluminator, observe and photograph. Photographing using polarized camera and parameters are preferably 302 nm wave lenghth, 2500 uW / cm2 , or more, and using 22 A filter.

10- Using 100 bp- DNA ladder for electrophoresis of PCR product of GAPDH,ST3Gal-III and ST6Gal-I (100,200,300 ,400,500,600,700,800,900and 1000bp) from Quiagen.

11-Analysis of PCR product using GEL pro-software to detect quantitation of bands for GAPDH, ST3Gal-III and ST6Gal-I genes.

Results and discussion:

The host receptor distribution pattern in the chicken and duck upper and lower respiratory tract may be functionally significant for the evolution of viruses with a human like receptor specificity and thus for the transmission of influenza from birds and mammals. In this work, We conducted an extensive examination on the level of expression of influenza virus Receptors in trachea and lung of two different breeds of each chickens and ducks. Their was no difference in the reported results

observed due to the breed of animals, and the receptor expression was consistent between individual animals within each species. Using RT-PCR in the gene expression of ST3GAL III and ST6GALI which add sialic acids to the terminal sugar of glycoproteins and glycolipids, we found that the trachea of ducks (Baladi, Pekeni) show high expression level of ST3GAL III) while trachea of chickens(Baladi, Hubber) show law expression level. (Figure:1) but Chickens trachea (Baladi,Hubber) show very high expression level of ST6GAL I in comparson to that of ducks trachea(Baladi, Pekeni) that show lawer expression level of ST6GAL I. (Figure:3). These results were in agreement with **(Suresh et al.,2009**) who reported that The major species difference that they observed between chickens and ducks in the relative distribution of SA α 2-3 Gal and SA α 2-6 Gal receptors was along the tracheal epithelium. In chicken tracheal epithelium, SA α 2-6 Gal was the dominant receptor type, whereas in ducks the SA α 2-3Gal receptor was more abundant in the ciliated cells of the tracheal epithelium, it was found that the ratio of SA α 2-6 Gal to SA α 2-3 Gal in chickens trachea was approximately 10:1 whereas in duck the ratio was 1:20. The tracheal mucous glands of both chickens and ducks predominantly expressed SA α 2-6 Gal receptor type. The observed difference in dominant receptor type between chickens and ducks was confined to the upper airway (trachea). While the dominant SA α 2-6 Gal receptor expression pattern in chickens trachea was in contrast to a previous study **(Wan and Perez, 2006)** which, using lectin binding, found that 85% of the epithelial cells in chicken trachea were positive for SA α 2-3 Gal receptors, while only 10% were positive for SA α 2-6 Gal receptors.

Also we found that the lung of ducks (Baladi, Pekeni) showed high expression level of ST3GAL III .while lung of chickens(Baladi, Hubber) showed low expression level. but the difference between expression level of ducks trachea and lung tissues is high in case of trachea more than the lung tissue, but the expression level of chicken trachea is lower than that of lung tissue. (Figure:2). And Chickens lung (Baladi,Hubber) showed very high expression level of ST6GAL I in comparson to that of duck lung (Baladi, Pekeni) that show lower expression level of ST6GAL .

But the difference between expression level of ducks trachea and lung tissues is high in case of lung more than the trachea tissue, while the expression level of chickens lung is lower than that of trachea tissue. (Figure:4). The present results were also in agreement with the findings of **Gambaryan et al (2002),** who reported that human influenza viruses with SA α 2,6-Gal specificity bound to cell membranes isolated from chickens (but not ducks) tracheal cell membranes. Chicken alveolar cells expressed both receptor types. The difference in the predominant receptor across the tracheal epithelial lining in chickens and ducks could be an important contributing factor to influenza virus entry via the upper respiratory tract. In particular, such differences could impact on the susceptibility of each species to avian H5N1 influenza with its preferential tropism for infection of the respiratory tract rather than the intestines.

The differences in receptor expression reported in the present study suggest that they may be responsible, at least in part, for some of the differences between ducks and chickens in the pattern of disease following influenza infection. While the presence of a virus receptor is clearly not sufficient to confirm that cells or tissue support efficient virus replication or transmission, the widespread replication of influenza virus in multiple organs has been reported in both chickens **(Swayne, 1997).**
and ducks **(Londt et al., 2008)** following infection with highly pathogenic viruses.

This study suggests that some chickens and ducks tissues may facilitate entry of both human and avian viruses, with the ensuing danger of virus reassortment. However, further work is required to confirm that the tissues expressing both receptor types are able to support virus replication. The dominant presence of SA α 2-6 Gal receptor along the
chicken tracheal epithelium shows some similarities to the prevalence of the receptor in mammals such as human and pig. This suggests that chickens may be important intermediate hosts for the transmission of influenza to humans, in particular for influenza viruses such as H5N1,
which show a respiratory tropism in birds. Whilst much attention has been placed on the role of pigs as "mixing vessels", the potential importance of chickens for the evolution of humanised influenza viruses should not be overlooked and, as such, wants further studies.

Previous studies on the role of sialic acid linkage (SA α 2,3 or SA α 2,6) during influenza virus infection have shown the importance of expression of these glycans in restricting infection by viruses in different hosts.

In this work, we found the presence of both S.A α 2-3 Gal receptor and SA α 2-6 Gal receptor in chikens trachea and lung due to the expression of the two genes suggest that they may be susceptible to infection with wider range of avian influenza viruses with broader receptor specificity.

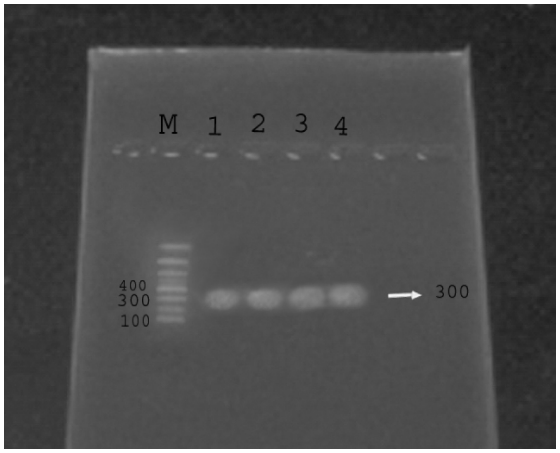
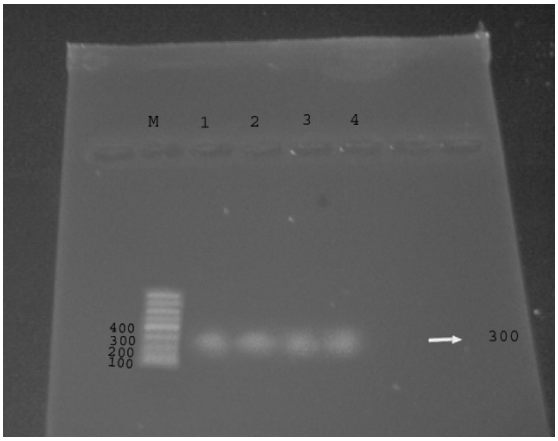
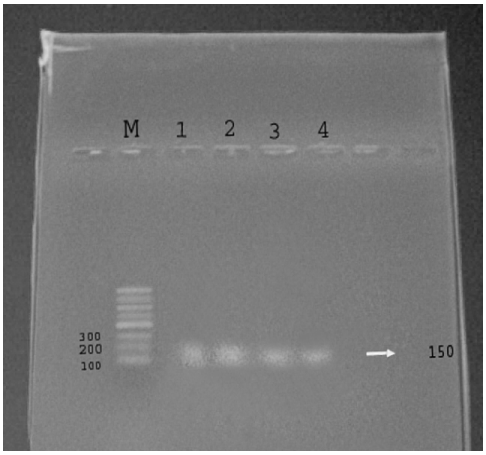


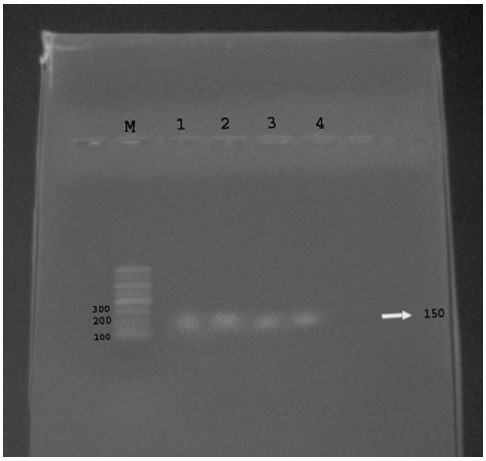
Figure (1): The elctrophoretic photograph showing the pattern of ST3GALIII mRNA expression in trachea of different birds
M: DNA ladder
Lane 1: chicken Baladi trachea
Lane 2: chicken Hubber trachea
Lane 3: Duck Baladi trachea
Lane 4: Duck Pekeni trachea



Figure(2): The elctrophoretic photograph showing ST3GAL III mRNA expression in lung the pattern o of different birds
M: DNA ladder
Lane 1: chicken Baladi trachea
Lane 2: chicken Hubber trachea
Lane 3: Duck Baladi trachea
Lane 4: Duck Pekeni trachea



Figure(3):The elctrophoretic photograph showing the pattern of ST6GALI mRNA expression in trachea of different birds
M: DNA ladder
Lane 1: chicken Baladi trachea
Lane 2: chicken Hubber trachea
Lane 3: Duck Baladi trachea
Lane 4: Duck Pekeni trachea



Figure(4): The elctrophoretic photograph showing the pattern of ST6GAL I mRNA expression in lung of different birds.
M: DNA ladder
Lane 1: chicken Baladi trachea
Lane 2: chicken Hubber trachea
Lane 3: Duck Baladi trachea
Lane 4: Duck Pekeni trachea

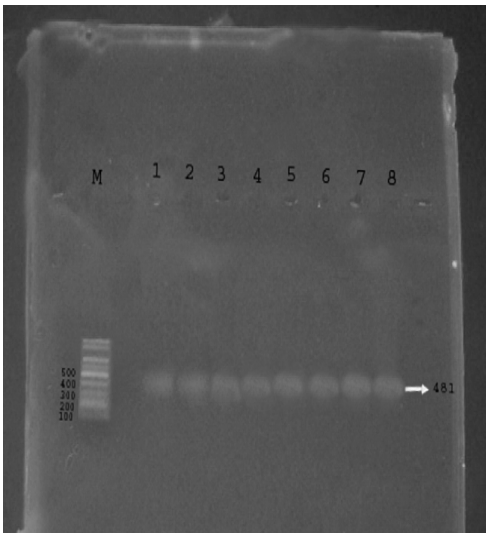


Figure (5): The elctrophoretic photograph showing the pattern of glyceraldhyde 3 phosphate dehydrogenase (GAPDH) mRNA expression in trachea and lung of different birds.
M : DNA ladder
Lane 1: chicken Baladi trachea
Lane 2: chicken Hubber trachea
Lane 3: Duck Baladi trachea
Lane 4: Duck Pekeni trachea
Lane 5: chicken Baladi lung
Lane 6: chicken Hubber lung
Lane 7: Duck Baladi lung
Lane 8: in Duck Pekeni lung

References:

1. **Catherine, C,G.; Vincent, G.; Mahfoud, A.; Alain, M. B.; Philippe, D. and Jacques, B. (1999)**: Alteration of Sialyl Lewis Epitope Expression in Pterygium. Investigative Ophthalmology andVisualScience.40:1631-1636.

2. **Connor, R.J.; Kawaoka, Y.; Webster, R.G. and Paulson, J.C. (1994):** Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates. Virology. 205 (1): 17–23.

3. **Debby, v. R.; Vincent, J.M.; Emmie, D.W.; Guus, F. R.; Ron. A.M. F; Albert, O. and Thijs K.(2007):** Human and Avian Influenza Viruses Target Different Cells in the Lower Respiratory Tract of Humans and Other Mammals (Am J Pathol. 171:1215-1223.

4. **Gambaryan, A.S.; Webster, R.** and **Matrosovich**, **M. (2002):** Differences between influenza virus receptors on target cells of duck and chicken**.** Arch Virol 147: 1197–1208**.**

5. **Gambaryan,A.S.; Yamnikova, S.; Lvov, D.; Tuzikov, A.; Chinarev, A.; Pazynina, G.; Webster, R.; Matrosovich, M. and Bovin, N. (2005):** Receptor specificity of influenza viruses from birds and mammals: new data on involvement of the inner fragments of the carbohydrate chain. Virology. 334 (2): 276-283.

6. **Gambotto, A.S.; Barratt-Boyes, S.M.; de Jong,M.D.; Neumann, G. and Kawaoka, Y.(2008):** Human infection with highly pathogenic H5N1 influenza virus. Lancet. 371, (9622):1464–75.

7. **Ghaleb, M.. A.(2009):** Characterization of H5 Hemagglutinin of H5N1 Avian Influenza Virus in the Middle East. The Islamic University Journal (Series of Natural Studies and Engineering). 17: 47- 60.

8. **Harduin-Lepers,A.; Rosella, M.; Philippe, D. and Rafael, O. (2005):** The animal sialyltransferases and sialyltransferase-related genes: a phylogenetic approach. Glycobiology. 15: 805–817.

9. **Londt, B.Z.; Nunez, A. and Banks, J. (2008)** : Pathogenesis of highly pathogenic avian influenza A/turkey/Turkey/1/2005 H5N1 in Pekin ducks (Anas platyrhynchos) infected experimentally. Avian Pathol . 37: 619-27.

10. **Mallet, F.; Oriol, G.; Mary,C.; Verrier, B. and Mandrand, B. (1995):** Continous RT-PCR using AMV-RT and taq DNA polymerase: characterization and comparison to uncoupled procedures. Biotechniques. 18: 678-687.

11. **Matrosovich, M.; Gambaryan, A. and Klenk, H. (2008):** Receptor Specificity of Influenza Viruses and Its Alteration during Interspecies Transmission. Virol. Basel, Karger. 27:134-155.

12. **Mouricout, M.(1997):** Interactions between the enteric pathogen and the host. An assortment of bacterial lectins and a set of glycoconjugate receptors. Adv. Exp. Med. Biol. 412:109-123.

13. **Rogers, G.N.; Daniels, R.S.; Skehel, J.J.; Wiley, D.C., Wang, X.F.; Higa, H.H. and**

Paulson J.C. (1985): Host-mediated selection of influenza virus receptor variants. Sialic acid-alpha 2,6Gal-specific clones of A/duck/Ukraine/1/63 revert to sialic acid-alpha 2,3Gal-specific wild type in vivo, J. Biol. Chem. 260: 7362–7367.

14. **Sambrook, F.** and Maniatis.(1989): Molecular cloning (laboratory manual). Cold Spring Harbor Laboratory Press.USA.pp: 6.9-6.13.

15. **Sean, C.A.; Zheng, X.; Jinling, L. and Carol, J.C. (2009):** Immune-related gene expression in response to H11N9 low pathogenic avian influenza virus infection in chicken and Pekin duck peripheral blood mononuclear cells. Molecular Immunology. 46: 1744–1749.

16. **Shuichi,T.(1995):** structural and functional analysis of sialyltransferases. glycobiology and glycotechnology. 8: 5-6.

17. **Stray, S.J. and Air, G.M. (2001):** Evaluation of influenza A virus receptors. In: Osterhaus, A.D., Hampson, A.W., Cox, N. (Eds.), World Congress on Options for the Control of Influenza IV. Elsevier, Amsterdam. 487– 502.

18. **Suresh, V. K.; Rahul, N.;Gavin, A. W.; Maureen, B.; Kin, C. C. and Stephen D.(2009):** Differences in influenza virus receptors in chickens and ducks: Implications for interspecies transmission. J Mol Genet Med. 3(1): 143-151.

19. **Suzuki, Y.; Ito, T.; Suzuki, T.; Holland ,J. R.E.; Chambers, T.M.; Kiso, M.; Ishida, H. and Kawaoka, Y.(2000) :** Sialic acid species as a determinant of the host range of influenza A viruses. J Virol. 74:11825-11831.

20. **Swayne, D. E. (1997):** Pathobiology of H5N2 Mexican avian influenza viruses for chickens. Veterinary Pathology. 34: 557–567.

21. **Tardieu, M.; Epstein, R.L.; Weiner, H.L. (1982):** Interaction of viruses with cell surface receptors. Int. Rev. Cytol. 80: 27–61.

22. **Tumpey, T.M.; Maines, T.R.; Van Hoeven, N.; Glaser, L.; Solorzano, A.; Pappas, C.; Cox, N.J.; Swayne, D.E.; Palese, P.; Katz, J.M. and Garcia-Sastre, A. (2007):** A two-amino acid change in the hemagglutinin of the 1918 influenza virus abolishes transmission. Science. 315: 655–659.

23. **Wan, H. and Perez,D.R.(2006):** Quail carry sialic acid receptors compatible with binding of avian and human influenza viruses. Virology. 346: 278- 286.

24. **Weinstein, J., de Souza, E. S. U. and Paulson, J. C. (1982):** Purification of a Gal beta 1 to 4GlcNAc alpha 2 to 6 sialyltransferase and a Gal beta 1 to 3(4)GlcNAc alpha 2 to 3 sialyltransferase to homogeneity from rat liver. J. Biol. Chem. 257: 13835- 13844.

25. **Yasuo, S. (2005):** Sialobiology of Influenza Molecular Mechanism of Host Range Variation of Influenza Viruses. Biol. Pharm. Bull. 28(3): 399-408.

الملخص العربى

لوحظ أن إصابة الأنواع المختلفه من الطيور بفيروس إنفلونزا الطيور يختلف إختلافا كبيرا حسب نوع الطائر حيث وجد أن إصابة الدجاج بفيروس إنفلونزا الطيور تظهر أعراضه بصوره شديده وواضحه وتصل نسبة الوفيات إلى 100% بينما فى حالة إصابة البط قد لا يظهر عليه أى عرض ولا ينتج عنه وفيات ويقوم البط كحامل للفيروس . وقد يعزى هذا إلى الإختلاف فى نوع وعدد مستقبلات فيروس إنفلونزا الطيورفى كل من الدجاج والبط .
لذا أستهدف من هذه الدراسه بحث الفرق بين مستقبلات فيروس إنفلونزا الطيور
(حامض السياليك) فى كل من الدجاج والبط من خلال دراسة التعبير الجينى لإنزيمى السياليل ترانسفيراز 6 و السياليل ترانسفيراز 3 فى الدجاج والبط.
ولقد أستخدم فى هذا البحث 5 دجاجات بلدى و 5 دجاجات أبيض و5 بطات بلدى
و 5 بطات بكينى وقد تم شراء هذه الطيور من مزارع خاصه بمحافظة الشرقيه وعمرها أربعة أسابيع وقد أخذت كافة برامج التحصينات المتبعه وكانت الطيور بحاله صحيه جيده
تم ذبح هذه الطيور وأخذت مباشرة عينات من القصبه الهوائيه والرئتين وتم وضعها فى ورق ألومنيوم ووضعت العينات فى وعاء به نيتروجين سائل.

وقد طحنت هذه العينات تحت ظروف عاليه من التعقيم لإستخلاص الحمض النووى الريبوزى الخاص بهذه العينات ثم تم حفظ هذا الحمض النووى الريبوزى فى نيتروجين سائل لإجراء تفاعل البلمره المتسلسل العكسى ثم الفصل الكهربى لهذه العينات بإستخدام الأجاروز وجهاز الفصل الكهربى. وتم تصوير النتائج بإستخدام كاميرا ديجيتال.

وقد لوحظ الأتى :
لقد أظهرت النتائج زيادة معدل الحمض النووى الريبوزى لإنزيم السياليل ترانسفيراز 6 فى القصبه الهوائيه للدجاج ولكن فى حالة البط لوحظ أن معدل الحمض النووى الريبوزى لهذا الإنزيم منخفض جدا بالمقارنه بالدجاج بينما فى حالة الرئتين لوحظ أيضا زيادة معدل الحمض النووى الريبوزى لهذا الإنزيم فى الدجاج عنه فى البط ولكن الإختلاف فى معدل الحمض النووى الريبوزى فى الرئتين ليس كبير مثلما فى حالة القصبه الهوائيه .
و لقد أظهرت النتائج أيضا زيادة معدل الحمض النووى الريبوزى لإنزيم السياليل ترانسفيراز 3 فى القصبه الهوائيه للبط وإنخفاض معدل الحمض النووى الريبوزى فى حالة القصبه الهوائيه فى الدجاج وأيضا زيادة معدل الحمض النووى الريبوزى فى رئتين البط عنها فى الدجاج.
ولوحظ أنه لايوجد إختلافا ملحوظا فى معدل الحمض النووى الريبوزى للإنزيمين نيجة إختلاف سلالة البط أوسلالة الدجاج.

2010/3/11

Synbiotic Tarhana as a functional food

*Shreef G N Gabrial, ** Ahmed H Zaghloul, ***Abd El-Rahman M Khalaf-Allah, ***Nagwa M El-Shimi, *Rasha S Mohamed and *Gamal N Gabrial

* Food Science and Nutrition Department, National Research Centre, Dokki, Cairo, Egypt.
**Dairy Science Department, National Research Centre, Dokki, Cairo, Egypt.
***Food Science Department, Faculty of Agriculture, Cairo University, Giza, Egypt.
E-mail: snoub_2000@yahoo.com

Abstract: In the present study formulated synbiotic tarhana (Turkish fermented cereal food) was produced as a functional food from the fermentation of wheat flour, some spices [salt, pepper, dill and sweet marjoram (Organum majorana)], some vegetables [tomato (Lycoprsicum esculentum), pepper (Capsicum annum) and onion (Allium cepa)], and synbiotic yoghurt which prepared with prebiotic (Inulin and lactose each 3%) and different concentrations of the probiotic culture (0.5, 1.5, 3, 4.5% DVS-ABT2 containing Streptococcus thermophilus, Lactobacillus acidophilus and Bifidobacterium bifidum). After fermentation (3 days), tarhana dough was dried in the sun. The effect of the fermentation (0, 1, 2 and 3 days) and the probiotic culture concentration on the chemical composition and the probiotic population of the wet tarhana were evaluated. The effect of the probiotic culture concentration on the chemical composition, the probiotic population and the sensory attribute of dried tarhana were evaluated. Also the effect of dried tarhana (prepared from yoghurt which was fermented by 4.5% probiotic culture) on the plasma lipid profile of human subjects was studied. The results showed that the pH value decreased while the acidity increased, acetaldehyde and diacetyl values increased during the fermentation period and by increasing the probiotic culture concentration of the wet and the dried tarhana. Neither the fermentation nor the concentration of the probiotic culture of wet and dried tarhana affected the crude protein, ether extract, crude fibre, and ash values. The numbers of probiotic bacteria increased until the second day of fermentation. However, in the following day, with an increase of the acid content their number decreased. Generally the increasing of the probiotic culture concentration increased the numbers of probiotic bacteria of the wet and dried tarhana. Also the concentration of the probiotic culture didn't affect the sensory attributes of dried tarhana. Subjects supplemented with dried tarhana showed significant reduction in total serum cholesterol, low density lipoproteins (LDL-C) and triglycerides, while high density lipoprotein (HDL-C) increased.
[Shreef G N Gabrial, Ahmed H Zaghloul, Abd El-Rahman M Khalaf-Allah, Nagwa M El-Shimi, Rasha S Mohamed and Gamal N Gabrial. **Synbiotic Tarhana as a functional food**. Journal of American Science 2010;6(12):1402-1412]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: Tarhana, functional food, fermented food, probiotic, synbiotic yoghurt, serum lipids.

1. Introduction

Fermentation as an old and economical method of producing and preserving food, it is carried out to enhance flavor, aroma, shelf-life, texture, nutritional value and other pleasant and appealing properties of foods (Steinkraus, 2002). It is possible to obtain probiotic foods from several matrices, including both fermented and non-fermented products (Rivera-Espinoza and Gallardo-Navarro 2010). Tarhana is a traditional fermented milk-cereal mixture containing lactic acid bacteria with probiotic properties. Tarhana has been considered as one of the oldest probiotic foods (Ozdemir *et al*. 2007).

Tarhana is a popular traditional Turkish fermented wheat food produced both commercially and in homes. It is mainly used in the form of a thick and creamy soup consumed at lunch or dinner and is easily digested (Bilgicli and Elgun 2005). Tarhana is prepared by mixing wheat flour, yoghurt, yeast and a variety of cooked vegetables and spices (tomatoes, onions, salt, mint, paprika) followed by fermentation for one to seven days (Daglioglu 2000). Lactic acid bacteria and the yeast are responsible for the acid formation during fermentation and the leavening effect. The dough at fermentation is called as wet tarhana. Afterwards, the dough is dried in the sun or by dryer as a lamp, nugget or thin layers to obtain dry tarhana. Also the tarhana is locally consumed as snack after being dried as thin layer or nugget, not to be ground. Since there is no standard production method, nutritional properties of tarhana strictly depend on ingredients and their ratios in the recipe (Erbas *et al*. 2006).

Tarhana is a good source of minerals, organic acids and free amino acids which make it healthy for children, the elderly and medical patients. In addition, it is a good source of vitamins such as thiamine, riboflavin and vitamin B12 (Ibanoglu *et al*. 1995). Ascorbic acid, niacin, pantothenic and folic

acid are also present (Ekinci, 2005, Ekinci and Kadakal 2005). Lacticacid bacteria (LAB) from yoghurt also aid in absorption of nutrients, which would otherwise, be indigestible or poorly digestible. (Farnworth, 2003).

Fermentation of tarhana dough is generally carried out using yoghurt bacteria, such as Lactobacillus bulgaricus, Streptococcus thermophilus and Baker's yeast (Saccharomyces cerevisiae) (Bilgicli and Ibanoglu 2007). This is similar to other natural systems (e.g. Kefir grains) in which associations of LAB and yeasts are used in food fermentation (Gobbetti 1998). The fermentations occur simultaneously during this aspect of production (Bilgicli et al. 2006). Yeast fermentation proceeds through the Embden-Meyerhof pathway (EMP), in which glucose is transformed into ethanol (via piruvate and acetaldehyde), carbon dioxide, and traces of other acids and carbonyl compounds (Gobbetti 1998, Gelinas and McKinnon 2000). According to Mugula et al. (2003) a combined culture of yeasts and lactobacilli cause a more significant decrease in pH (increase in acidity), than with the use of single cultures in the fermented millet.

The present study was initiated to produce synbiotic tarhana and evaluate it as a functional food. The effect of fermentation time (0, 1, 2 and 3 days) and starter concentrations (0.5, 1.5, 3 and 4.5%) on the chemical composition and the probiotic bacterial counts of wet tarhana were evaluated. Also the effect of starter concentrations (0.5, 1.5, 3 and 4.5%) on the sensory attributes, chemical composition and the probiotic bacterial counts of dried tarhana were evaluated. The hypocholesterolemic effect of dried tarhana on human subjects was studied.

2. Material and Methods

Tarhana ingredients: Vegetables [Tomato (Lycopersicum Esculentum), Green Pepper (Capsicum Annum), Chicory (Cicohorium Intybus) and Onion (Allium Cepa)], Cereals [Wheat (Triticum oestivum)], Spices [salt, pepper, dill and sweet marjoram (Origanum majorana)], Yeast (Saccharomyces cervisiae, press form) were purchased from the local market, Cairo, Egypt. Lactulose syrup (52.40% lactulose, 4.3% lactulose and 2.5 galactose) was obtained from the Egyptian International Industries Company (EIPICO), Cairo, Egypt and Spray dried skim milk (low heat) was obtained from Dina for Agriculture Investments, Egypt.

Probiotic Culture: DVS-ABT2 (containing Streptococcus thermophilus, Lactobacillus acidophilus and Bifidobacterium bifidum) were obtained from Chr. Hansen's Lab., Copenhagen Denmark. M17 agar, MRS-Salccin agar, violet red bile agar (VRBA), potato dextrose agar, and MRS agar. All media were purchased from Oxoid LTD, London.

Preparation of Chicory Water-Soluble Extract

Chicory Water-Soluble Extract was prepared according to the methods described by Kim and Shin (1998) as follows: 10g dried chicory plant was dissolved into 200 ml distillated water, soaked for 24 hours under refrigeration, heated at 70 ^{o}C for 15 min. then filtered. Chicory extract was then added to the synbiotic yoghurt.

Preparation of synbiotic yoghurt

Milk samples were standardized by adding skim milk powder to achieve 16% total solids content, pasteurized (15 min. at 85 ^{o}C) and cooled to 40 ^{o}C. Chicory extract and lactulose syrup (3% each) were individually added to milk samples, then inoculated with different concentrations (0.5, 1.5, 3, 4.5%) of the DVS-ABT2 culture, then milk was dispensed into pasteurized plastic cups (100 ml), capped, incubated (5 hours at 44 ^{o}C) cooled and stored in the refrigerator at 5 ^{o}C to prepare synbiotic tarhana.

Preparation of synbiotic tarhana

The ingredients of tarhana are presented in Table (1). Production method of tarhana is presented in fig. (1). All ingredients were prepared (cleaned, peeled and cut), then mixed, blended, pasteurized (30 min. at 65^{o}C) and cooled at 25^{o}C, whole flour, salt, synbiotic yoghurt (with different concentrations of the probiotic culture) and Baker's yeast were added to the mixture, then kneaded to form tarhana dough. The dough was fermented (3 days at 25^{o}C) in an incubator. The samples were withdrawn at time intervals (0, 1, 2 and 3 days) for chemical analysis and microbial analysis. Also tarhana samples (fermented for 3 days) were dried in the sun, filled in small packages and stored. The dried tarhana were subject to chemical, microbial and sensory evaluation and it was also used in human studies to evaluate its hypolipidemic effect.

Chemical analysis

pH value, total acidity, crude proteins, ether extract, crude fiber and ash were determined according to AOAC (2000). Acetaldehyde was estimated as described by Lees and Jaco (1969). Diacetyl was determined according to Lees and Jaco (1970).

Microbial analysis

Bifidobacterium bifidum was enumerated according to Dave and Shah (1969) using the modified MRS agar supplemented with 0.05% L. cysteine-HCl. The plates were anaerobically incubated at 37°C for 48 hours using anaerogen sheets.

Lactobacillus acidophilus count was estimated according to Dave and Shah (1969) on MRS-salccin agar. Incubation was carried out at 37°C for 48 hours.

Streptococcus thermophilus count was estimated according to Terzaghi and Sandine, (1975) using M17 agar. Incubation was carried out at 25 °C for 48 hours.

Moulds were enumerated according to standard methods for examination of dairy products (APHA, 1994). Incubation was carried out at 25 °C for 4 – 5 days.

Coliform group bacteria were enumerated according to standard methods for examination of dairy products (APHA, 1994) using violet red bile agar (VRBA). Incubation was carried out at 37 °C for 48 hours.

Sensory evaluations

Synbiotic tarhana samples were organoleptically evaluated by 10 panelists from the staff members of food science and nutrition department of the National Research Center, Dokki, Cairo, Egypt. The panelists evaluated the samples using a five point Hedonic scale (5 = Liked Extremely to 1 = Unacceptable) adopted from (Iwe, 2000). All samples were evaluated for appearance, taste and general acceptability. The samples were filled in small white porcelain bowl (150 ml) and they were coded with numbers and served to the panelists at random.

Human experiment

Fifteen hyperlipidemic volunteers aged between 40 and 55 years old were studied, all were in good general health, with no history of cardiovascular or gallbladder disease, non of the volunteers were taking any medications. They were given their regular diet which was daily supplemented with 200g of synbiotic tarhana (prepared from yoghurt which was inoculated by 4.5% probiotic culture) for 45 days.

Blood sampling

Blood samples were collected from each volunteer, before supplementation and at the end of the experimental period, after overnight fasting and withdrawn through heparinized tubes for serum. The blood was allowed to clot at room temperature for one hour, and then the serum was separated by centrifugation at 3000 rpm for 15 minutes, clear serum was divided into aliquots and stored at 20°C until analyzed.

Biochemical analysis

Blood lipids were estimated according to the following methods, total cholesterol (Allain et al., 1974), total triglycerides (Fossati and Prencipe 1982), high density lipoproteins (Lopes-Virella et al., 1977), and low density lipoproteins (Friedewald et al., 1977).

3. Results

Evaluation of tarhana dough

Changes in some of the chemical components of tarhana dough samples [prepared with yoghurt and inoculated by different concentrations (0.5, 1.5, 3 and 4.5%) of the probiotic culture (DVS-ABT2)] were studied in relation to different fermentation time. Table 2 shows that for all tested tarhana dough samples, fermentation time had no effect on crude fiber, ether extract and ash. However, fermentation time had an effect on pH value and acidity of tarhana dough. The acidity content of all studied tarhana dough samples increased by increasing the fermentation time and consequently the pH values decreased until the end of fermentation course. Also increasing of probiotic culture concentrations from 0.5 to 4.5% decreased the pH values and increased the acidity content of the resultant tarhana dough samples. The pH value decreased from 5.47, 4.89, 4.62 and 4.57 to 4.89, 4.09, 4.08 and 3.92 while the acidity increased from 3.9, 5.0, 6.8 and 7.7 to 7.4, 9.7, 10.2 and 13.6 respectively during the fermentation time when a probiotic culture inoculation of 0.5, 1.5, 3 and 4.5% respectively was added.

Acetaldehyde and diacetyl contents of tarhana dough samples increased during fermentation. Also they increased with increasing of the probiotic culture concentrations in tarhana dough as shown in Figure 2 and 3.The effect on fermentation time on microbial counts of tarhana dough samples are presented in Table 3. Results indicate that the microbial counts numbers increased by increasing the fermentation time reaching the highest level at the second day of fermentation, then these decreases by increasing that period to record the lowest level at the end of fermentation (3 days). It was found that increasing the probiotic culture concentrations 0.5, 1.5, 3 and 4.5% (samples A, B, C and D) increased the numbers of probiotic bacteria as shown in Table 3. The highest population of probiotic bacteria from tarhana dough samples that contained 0.5, 1.5, 3 and 4.5% probiotic culture was recorded at the second day of fermentation being 8.5 x 10^7, 5.9 x

10^9. 9.7 x 10^{10} and 9.9 x 10^{10} (cfu/g) for *L. acidophilus*, 7.2 x 10^7, 6.4 x 10^9, 6.1 x 10^{10} and 7.6 x 10^9 (cfu/g) for *S. thermophilus* and 6.4 x 10^7, 8.6 x 10^8, 8.2 x 10^9 and 9.0 x 10^9 (cfu/g) for *B. bifidum*. Also, the results indicate that all tarhana dough samples were free from coliform and mold during the fermentation period, indicating no contamination occurred from the environment or the row materials.

Sensory characteristics (flavor, body and texture and appearance) of dried tarhana samples (A, B, C and D) prepared with different concentrations of probiotic culture (0.5, 1.5, 3 and 4.5%) were evaluated as shown in Table 4. The obtained results show that the sensory evaluation properties of dried tarhana had good scores and were acceptable for all the samples which contained different concentrations of probiotic culture.

Chemical composition of dried tarhana samples prepared by yoghurt inoculated with different concentrations of probiotic culture (0.5, 1.5, 3 and 4.5%) is presented in Table 5. Results indicate that all dried samples (A, B, C and D) had a protein content that ranged between (19.87-19.88%). All dried samples (A, B, C and D) had a fiber and ash content that ranged between (3.80-3.82%) and (9.93-9.95%) respectively. All dried samples (A, B, C and D) had an ether extract that ranged between (2.90-2.92%). Concerning pH value and acidity content of dried tarhana sample results Table 5 disclose that the acidity increased and pH value decreased by increasing the concentration of the probiotic culture. The highest acidity (13.4) and the lowest pH value (3.9) were recorded for dried tarhana sample D (containing 4.5% probiotic culture). Acetaldehyde and diacetyl contents of the dried tarhana are shown in figure 4. Acetaldehyde and diacetyl contents of the dried tarhana samples increased by increasing probiotic culture concentration. The highest content of Acetaldehyde (0.65 µmol/ml) and diacetyl (0.55 µmol/ml) were obtained for tarhana samples contained 4.5% probiotic culture, while the lowest contents were obtained for sample having 0.5% probiotic culture being 0.46 and 0.31 µmol/ml consecutively.

Effect of sun drying on microbial population of tarhana samples (A, B, C, and D) is shown in Table 6, data presented disclose that all dried tarhana samples recorded a sharp decrease in probiotic bacterial counts after drying compared to the corresponding values at the end of fermentation (day three) as shown in Table 3.

Hypolipidemic effect

Table 7 shows the changes in total cholesterol, total triglycerides, low and high density lipoprotein of the subjects that consumed dried synbiotic tarhana for 45 days (prepared from yoghurt which was inoculated by 4.5% probiotic culture). Results show a significant hypocholesterolemic effect where the mean of the serum cholesterol concentration was (222.0 ± 5.2) at the start of experiment then decreased to (202.6 ± 8.5) at the end of the experiment, triglyceride level showed a highly significant reduction from (179.8 ± 5.4) at the start of experiment to (169.0 ± 5.5) at the end of the experiment, high-density lipoprotein cholesterol was significantly raised from (50.1 ± 1.0) to (57.8 ± 0.9) at the end of the experiment. As for the low-density lipoprotein cholesterol there was no significant change with a value of (92.7 ± 0.7) at the start of experiment to (81.9 ± 0.5) at the end of the experiment.

Table 1: Synbiotic Tarhana Ingredients (% w/w)

Ingredients	% w/w
Whole wheat flour	35
Synbiotic Yoghurt	25
Fresh onions	12
Fresh tomato	10
Fresh red pepper	6
Green pepper	4
Baker's yeast	4
Salt	2
Dill powder	1
Sweet marjoram	1

Table 2: Changing in pH value, acidity, crude protein, crude fibre, ether extract and ash of tarhana dough samples prepared with yoghurt fermented by different concentrations of DVS-ABT2 culture during fermentation.

Tarhana samples	Fermentation time (days)	pH	Acidity	Crude protein	Ether Extract	Crude Fibre	Ash
				(g/100g)			
A	0	5.47	3.9	19.88	2.90	3.80	9.94
	1	5.24	4.2	19.88	2.91	3.81	9.93
	2	4.90	7.4	19.87	2.91	3.81	9.94
	3	4.89	7.4	19.88	2.92	3.80	9.91

B	0	4.89	5.0	19.89	2.91	3.81	9.95
	1	4.67	6.9	19.90	2.92	3.80	9.97
	2	4.11	9.5	19.87	2.92	3.81	9.93
	3	4.09	9.7	19.88	2.91	3.81	9.94
C	0	4.62	6.8	19.89	2.91	3.82	9.96
	1	4.44	6.5	19.90	2.90	3.83	9.94
	2	4.10	10.0	19.88	2.93	3.82	9.95
	3	4.08	10.2	19.87	2.91	3.81	9.95
D	0	4.57	7.7	19.89	2.91	3.80	9.91
	1	4.36	9.2	19.89	2.90	3.82	9.94
	2	4.94	13.5	19.87	2.91	3.81	9.95
	3	3.92	13.6	19.88	2.93	3.81	9.93

(A): prepared using yoghurt incubation with 0.5% probiotic culture. (B): prepared using yoghurt incubation with 1.5% probiotic culture. (C): prepared using yoghurt incubation with 3% probiotic culture. (D): prepared using yoghurt incubation with 4.5% probiotic culture.

Table 3: Effect of fermentation time on microbial counts (cfu/g) of tarhana dough prepared with yoghurt fermented by different concentrations of DVS-ABT2 culture.

Tarhana samples	Fermentation time (days)	Microbial counts (cfu/g)				
		L. acidophilus	S. thermophilus	B. bifidum	Molds	Coliform
A	0	9.3×10^4	8.7×10^4	2.2×10^4	ND	ND
	1	7.9×10^5	6.7×10^5	5.3×10^5	ND	ND
	2	8.5×10^7	7.2×10^7	6.4×10^7	ND	ND
	3	4.0×10^6	6.9×10^5	5.0×10^6	ND	ND
B	0	5.9×10^7	7.2×10^7	8.3×10^6	ND	ND
	1	6.1×10^8	3.9×10^8	5.9×10^7	ND	ND
	2	5.9×10^9	6.4×10^9	8.6×10^8	ND	ND
	3	7.5×10^8	6.6×10^8	9.2×10^7	ND	ND
C	0	8.0×10^8	5.5×10^8	3.8×10^7	ND	ND
	1	2.9×10^9	2.5×10^9	1.1×10^8	ND	ND
	2	9.7×10^{10}	6.1×10^{10}	8.2×10^9	ND	ND
	3	8.4×10^9	6.9×10^9	8.0×10^8	ND	ND
D	0	8.9×10^8	7.3×10^9	8.8×10^7	ND	ND
	1	5.3×10^9	6.1×10^9	3.6×10^8	ND	ND
	2	9.9×10^{10}	7.6×10^{10}	9.0×10^9	ND	ND
	3	7.0×10^9	6.5×10^9	9.1×10^8	ND	ND

(A): Prepared using yoghurt incubation with 0.5% probiotic culture. (B): Prepared using yoghurt incubation with 1.5% probiotic culture. (C): Prepared using yoghurt incubation with 3% probiotic culture. (D): Prepared using yoghurt incubation with 4.5% probiotic culture.
ND = Not Detected

Table 4: sensory attributes of dried tarhana prepared with yoghurt fermented by different concentrations of DVS-ABT2 culture.

Tarhana samples	flavor	body and texture	appearance	Total (15)
A	4.5	4.6	3.5	12.6
B	4.6	4.6	4.0	13.2
C	4.5	4.5	4.0	13
D	4.5	4.6	3.5	12.6

Each value represents the mean of ten panel's degree
(A): Prepared using yoghurt incubation with 0.5% probiotic culture. (B): Prepared using yoghurt incubation with 1.5% probiotic culture. (C): Prepared using yoghurt incubation with 3% probiotic culture. (D): Prepared using yoghurt incubation with 4.5% probiotic culture.

Table 5: chemical composition of dried tarhana prepared with yoghurt fermented by different concentrations of DVS-ABT2 culture.

Components	Tarhana samples			
	A	B	C	D
pH	4.90	4.08	4.10	3.94
Acidity	7.5	9.5	10.4	13.4
Crude protein(g/100g)	19.87	19.88	19.87	19.89
Ether extract (g/100g)	2.92	2.92	2.91	2.90
Crude fiber (g/100g)	3.81	3.82	3.82	3.80
Ash (g/100g)	9.93	9.94	9.98	9.93

(A): Prepared using yoghurt incubation with 0.5% probiotic culture. (B): Prepared using yoghurt incubation with 1.5% probiotic culture. (C): Prepared using yoghurt incubation with 3% probiotic culture. (D): Prepared using yoghurt incubation with 4.5% probiotic culture.

Table 6: Microbial counts of dried tarhana samples prepared with yoghurt fermented by different concentrations of DVS-ABT2 culture.

Microorganisms (CFU/g)	Tarhana samples			
	A	B	C	D
L. acidophilus	5.0×10^2	6.2×10^3	4.0×10^4	7.4×10^4
S. thermophilus	9.2×10^2	4.0×10^3	3.6×10^4	7.9×10^4
B. bifidum	3.4×10^2	5.1×10^3	4.1×10^4	8.8×10^4
Molds	ND	ND	ND	ND
Coliform	ND	ND	ND	ND

(A): Prepared using yoghurt incubation with 0.5% probiotic culture. (B): Prepared using yoghurt incubation with 1.5% probiotic culture. (C): Prepared using yoghurt incubation with 3% probiotic culture. (D): Prepared using yoghurt incubation with 4.5% probiotic culture.
ND = Not Detected

Table 7: Plasma lipid profile of experimental group before and after 45 days of dietary supplement. [1]

Parameters	Before (Mean±SE)	After (Mean±SE)
Cholesterol (mg/dl)	222.0 ± 5.2	202.6 ± 8.5*
TGs (mg/dl)	179.8 ± 5.4	169.0 ± 5.5**
HDL-Ch (mg/dl)	50.1 ± 1.0	57.8 ± 0.9**
LDL-Ch (mg/dl)	92.7 ± 0.7	81.9 ± 0.5

[1] Supplement by dried synbiotic tarhana (prepared from yoghurt which was inoculated by 4.5% probiotic culture), n=15, *= significant $p<0.05$ **= high significant $p<0.01$
TGs = Triglycerides, HDL-Ch = high-density lipoprotein cholesterol , LDL-Ch = low-density lipoprotein cholesterol.

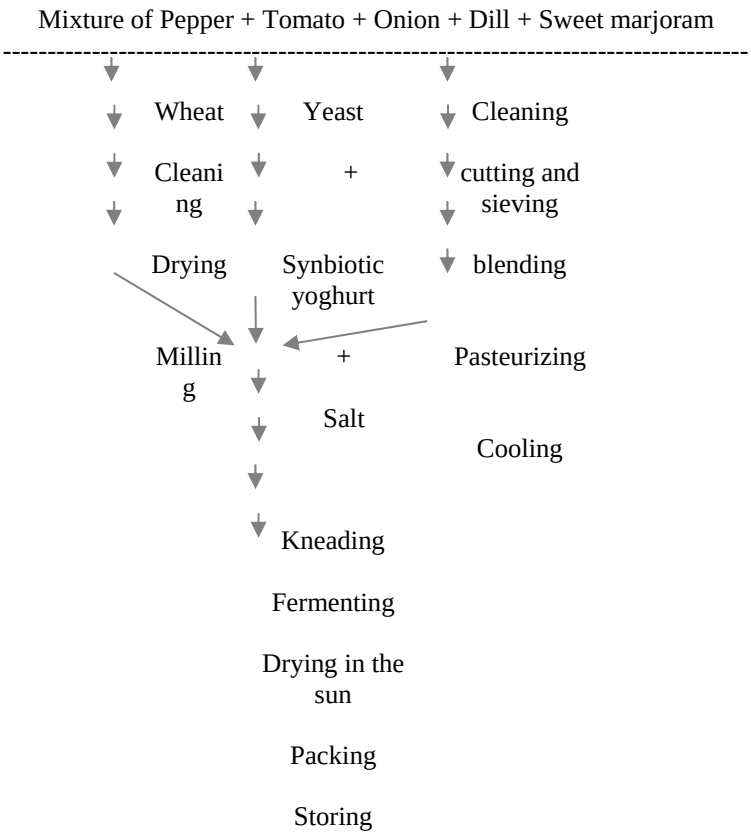


Figure 1: Flow chart for the preparation of tarhana.

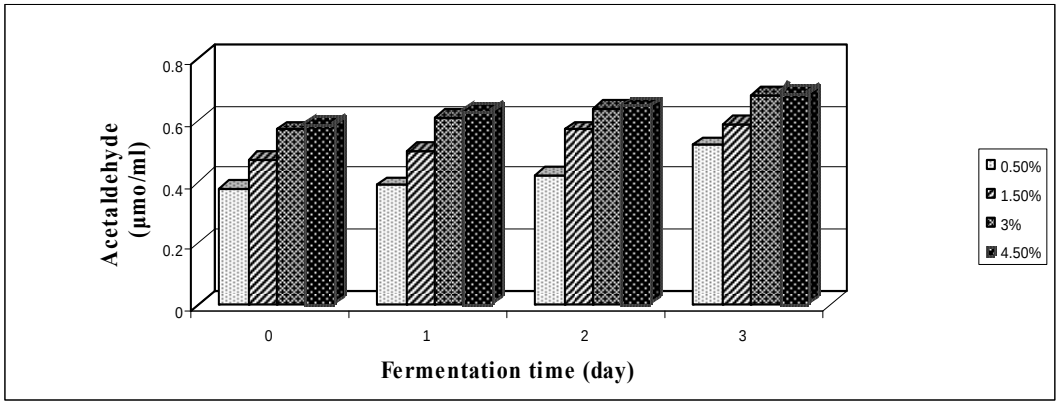


Figure 2: Effect of fermentation time on acetaldehyde contents (µmo/ml) of tarhana dough prepared with yoghurt fermented by different concentration (0.5, 1.5, 3 and 4.5%) of DVS-ABT2 culture.

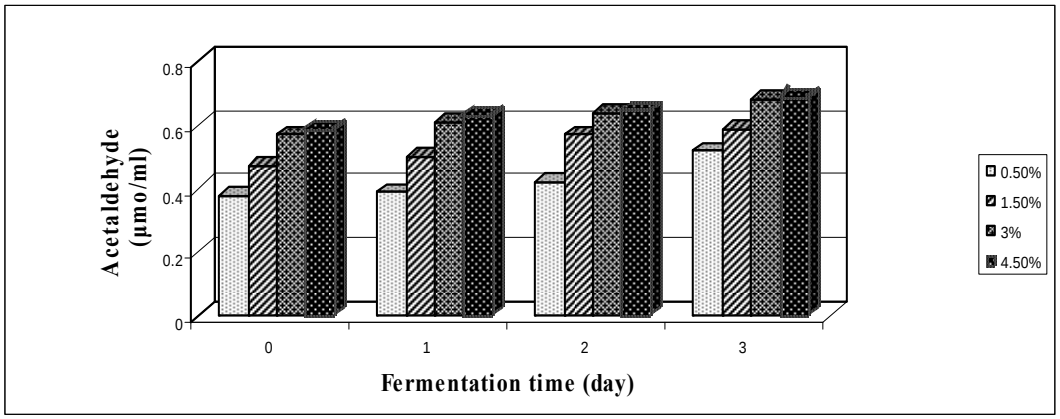


Figure 3: Effect of fermentation time on diacetyl contents (µmo/ml) of tarhana dough prepared with yohgurt fermented by different concentration (0.5, 1.5, 3 and 4.5%) of DVS-ABT2 culture.

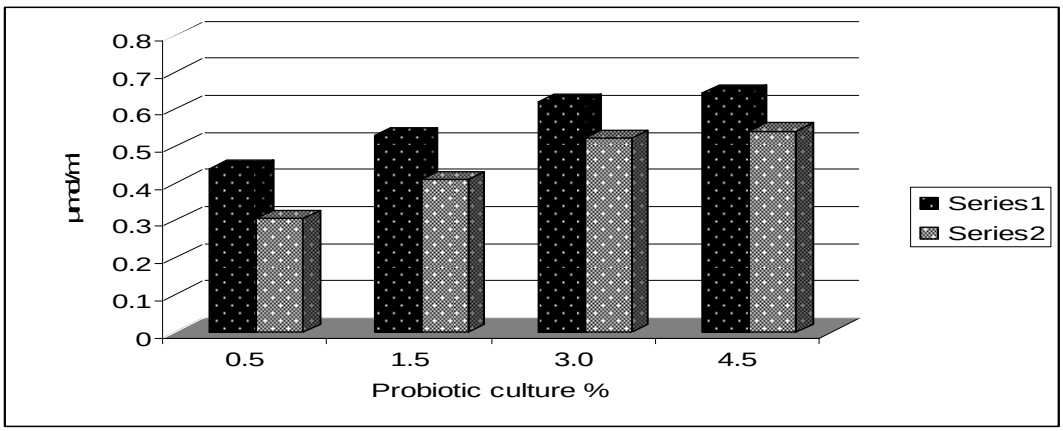


Figure 4: Acetaldehyde and diacetyl contents (µmol/ml) of the dried tarhana prepared with yoghurt fermented by different concentrations of DVS-ABT2 culture.

4. Discussion:

In the present study, changes in some of the chemical components of tarhana dough samples were studied in relation to different fermentation time. Results show that for all tested tarhana dough samples, fermentation time had no effect on crude fiber, ether extract and ash. This is in the agreement with the results obtained by Erbas *et al*. (2005), who found out that fermentation had no significant effect on dry matter, crude protein, ether extract and ash.

Data obtained revealed that the acidity content of all studied tarhana dough samples increased by increasing the fermentation time and consequently the pH values decreased until the end of fermentation course. The increase in the acidity followed by a decrease in pH value may be due to the formation of organic acids from fermentation of sugars mostly by probiotic bacteria. Such findings coincide with those reported by Ibanoglu *et al*. (1995) and Erbas *et al*. (2005), who demonstrated that the acidity of tarhana dough increased and the pH decreased during fermentation.

Acetaldehyde and diacetyl are two important aromatic compound. Acetaldehyde and diacetyl contents of the tarhana dough samples increased with increasing of the probiotic culture concentrations in tarhana dough. Acetaldehyde content is attributed to probiotic bacteria (Erbas *et al*. 2005).

Results indicate that the microbial counts numbers increased by increasing the fermentation time reaching the highest level at the second day of fermentation, then it decreases by increasing that period to record the lowest level at the end of fermentation (3 days). These results are in accordance with those obtained by Daglioglu *et al*. (2002) using Turkish tarhana. The results of bacterial counts are close to those reported by Capela *et al*. (2006), who, reported that the viability of probiotic organisms in yoghurt that had been inoculated with 4% was more than in yoghurt that had been inoculated with 2% before and after fermentation. Ibanoglu *et al*. (1999) found that the increasing of yoghurt amount from 500g to 1000g in tarhana

during the fermentation increased the population of probiotic bacteria of tarhana.

Sun drying is a slower but a more common and economical approach for traditional tarhana production. As for the drying process the critical moisture value is 13 – 15% for the inhibition of undesirable microbial growth in dry recipes produced from wheat flour (Bozkurt and gurbuz, 2008). The moisture content of tarhana is low, that it can be stored for 2 or 3 years without deterioration (Ibanoglu et al. 1999, Tarakc et al. 2004).

The results of the sensory analysis show that the use of yeast in the tarhana formula had a positive effect on the sensory properties. This shows that yoghurt bacteria and yeast together produce lactic acid, ethyl alcohol, carbon dioxide, and other fermentation products, which give tarhana its characteristic taste and flavour (Koca et al. 2002).

The protein content of the dried samples was higher than that obtained by Daglioglu (2000), Kose and Cagndi (2002) who reported that the crude protein content of dried tarhana were between 14.5 – 16 %. The latter author added that dried tarhana is a good source of protein. This can be explained by the differences in the tarhana formulas produced in different regions of Turkey.

The fiber and ash content in the present study were higher than that obtained by Daglioglu (2000), Erbas *et al.* (2006) who reported that the crude fiber and ash content of dried tarhana were 1% and 6.2%. The ether extract content was less than that obtained by Daglioglu (2000), Erbas *et al*. (2006) who reported that the ether extract content of dried tarhana was 5.4%. This can be explained by the differences in the tarhana formulas produced in different regions of Turkey.

Drying treatments can slightly affect the increase in the pH and the decrease in acidity contents of the tarhana samples. The acetaldehyde and diacetyl contents of the dried tarhana may be due to the formation of aromatic components which is attributed to the presence of probiotic bacteria as reported by Erbas et al. (2005).

The sharp decrease in probiotic bacterial counts after drying coincide with the results reported by Daglioglu et al. (2002) who observed a sharp drop in L.A.B. population of tarhana samples after conventional drying. Also Erbas *et al.* (2005) attributed the decrease of L.A.B. population to the low water content of dried tarhana samples.

In the present study it was found that increasing the probiotic culture concentrations increased the number of probiotic bacteria of dried tarhana. These results are in agreement with those reported by Capela *et al.* (2006) who, disclosed that the viability of probiotic organisms in freeze-dried yoghurt was increased by increasing the inoculums volume from 2 to 4 %.

Hypolipidemic effect

The relationship between atherosclerotic cardiovascular disease and nutrition is very important. Many functional foods have been found to be potentially beneficial in the prevention and treatment of cardiovascular disease. (Anderson 2003).

The hypocholesterolemic effect of dried tarhana may be due to its content of probiotic bacteria and prebiotic inulin because it is soluble in water and not hydrolyzed by human digestive enzymes, it is expected to behave like a soluble fiber and to have a hypolipidemic effect (Kim and Shin 1998), wheat flour which as explained by Illman et al. 1993 lowers plasma cholesterol and increases cecal steroids relative to whole wheat flour, wheat bran and wheat pollard in rats. Also wheat is among cereals containing high concentrations of ß-glucan which is known to have a cholesterol lowering effect (Newman et al. 1989, Mc Intoch et al. 1991). Vegetables such as onion have hypocholesterolemic effect by inhibiting hepatic cholesterol biosynthesis (Gupta and Porter 2001, Singh and Porter, 2006) , Lycopene from tomatoe led to reduction of serum total cholesterol (Agarwal and Rao 1998) and green pepper which prevents arteriosclerosis and lower cholesterol (Mezzetti et al. 1995).

5. Conclusion

Fermentation process is an important stage for the development of sensory profile of tarhana. Fermentation and increasing probiotic culture concentrations decreased the pH values and increased acidity, acetaldehyde and diacetyl values while neither the fermentation nor the concentrations of the probiotic culture affected crude protein, ether extract, crude fibre and ash values of wet and dried tarhana.

The increasing of the probiotic culture concentration from 0.5 to 3% ensured probiotic bacteria population of wet and dried tarhana at satisfactory level while increasing the probiotic culture concentration from 3 to 4.5% slightly increased probiotic bacteria population. Generally drying process decreased the viability of probiotic bacteria as drying decreased the water activity. So it has a poor medium for pathogens and spoilage organisms.

Since tarhana is a good source of B vitamins, minerals, organic acids, and free amino acids, and since it is a product of L.A.B. and yeast fermentation, it may be considered a functional and probiotic food with hypolipidemic effect.

Corresponding Author:
Shreef G.N. Gabrial
Food Science and Nutrition Department,
National Research Centre (NRC), Dokki, Cairo 12311, Egypt
E-mail:snoub_2000@yahoo.com

6. References

1. AOAC, Official Methods of Analysis. The Association of Official Agricultural Chem., 17th ed., U.S.A. (2000).
2. Agarwal, S and Rao, AV, Tomato lycopene and low density lipoprotein oxidation: a human dietary intervention study. *Lipids* 33:981- 4 (1998).
3. Allain CC, Poon LS, Chon CSG, Richmond U, Fu PC, Enzymatic determination of total serum cholesterol. Clin Chem 20:470–475 (1974).
4. American Public Health Association (APHA), Standard methods of the examination of dairy products. 16th ed., Washington, U.S.A. (1994).
5. Anderson, JW, Whole grains protect against atherosclerotic cardiovascular disease. *Proc.Nutr.Soc.* 62(1):135-142 (2003).
6. Bilgicli, N and Elgun, A, Changes in some physical and nutritional properties of tarhana, a Turkish fermented cereal food, added various phytase sources. *Food Science and Technology International*, 11(5):383-389 (2005).
7. Bilgicli, N and Ibanoglu, S, Effect of wheat germ and wheat bran on the fermentation activity, phytic acid content and colour or tarhana, a wheat flour- yoghurt mixture. *Journal of Food Engineering*, 78(2):681 – 686 (2007).
8. Bilgicli N, Elgun A, Herken EN, Turker S, Ertas N. and Ibanoglu, S, Effect of wheat and sensory quality of tarhana, a fermented wheat flour-yoghurt germ/bran addition on the chemical, nutritional product. *Journal of Food Engineering*, 77(3):680 – 686 (2006).
9. Bozkurt O and Gurbuz O, Comparison of lactic acid contents between dried and frozen tarhana. *Food Chemistry* 108:198 – 204 (2008).
10. Capela P, Hay TKC and Shah NP, Effect of cryoprotectants, preboitic and microencapsulatin on survival of probiotic organisms in yoghurt and freez-dried yoghurt. *Food Research International* 39:203 – 211 (2006).
11. Cavallero A, Empilli S, Brighenti F and Stanca AM, High (1-3, 1-4)-b-glucan barley fractions in bread making and their effect on human glycemic response. *Journal of Cereal Science* 36:59-66 (2002).
12. Daglioglu O, Tarhana as a traditional Turkish fermented cereal food. Its recipe, production and composition. *Die Nahrung* 44(2):85- 88 (2000).
13. Daglioglu O, Arici M, Konyali M and Gumus T, Effects of tarhana fermentation and drying methods on the fate of Escherichia coli O157:H7 and Staphylococcus aureus. *European Food Research and Technology* 215(6):515-519 (2002).
14. Dave RI, and Shah NP, Evaluation of media for selective enumeration of Streptococcus thermophilus, Lactobaccilus deblrueckii ssp. Bulgaricus, Lactobaccilus acidophilus and Bifidobacterium. *J. Dairy. Sci.* 79:1592 (1996).
15. Davidson MH, Maki KC and Synecki C, Evaluation of the influence of dietary inulin on serum lipids in adults with hypercholesterolemia. *Nutrition* 18: 503-17 (1998).
16. Ekinci R, The effect of fermentation and drying on the water-soluble vitamin content of tarhana, a traditional Turkish cereal food. *Food Chemistry* 90(2):127-132(2005).
17. Ekinci R and Kadakal C, Determination of seven water-soluble vitamins in tarhana, a traditional Turkish cereal food, by high-performance liquid chromatography. *Acta Chromatographica* 15:289-297 (2005).
18. Erbas M, Certel M and Uslu MK, Microbiological and chemical properties of tarhana during fermentation and storage as wet-sensorial properties of tarhana soup. *Swiss Society of Food Science and Technology* 38:409-416 (2005).
19. Erbas M, Uslu MK, Erbas MO and Certel M, Effects of fermentation and storage on the organic and fatty acid contents of tarhana, a Turkish fermented cereal food. *Journal of Food Composition and Analysis* 19:294-301(2006).
20. Farnworth ER, Handbook of fermented functional foods. Boca Raton, FL: CRC Press (2003).
21. Fossati P and Prencipe I, Serum triglyceride determintation colorimetrically with an enzyme that produce hydrogen peroxide. *Clin. Chem*. 28:2077 (1982).
22. Friedewald WT, Levy RI andFredrickson DS, Estimation of the concentration of low density lipoprotein cholesterol in plasma without the use of the preparative ultracentrifuge. Clin Chem 18:499–502, (1972).
23. Gelinas P and McKinnon C, Fermentation and microbiological processes in cereal foods. In Kulp K & Ponte JJ (Eds.). Handbook of cereal science and technology. New York: Marcel Dekker, Inc. vol. 99:741-754(2000).
24. Gobbetti M, The sour dough microflora: interactions of lactic acid bacteria and yeasts. *Trends in food science and technology* 9(7),267-274 (1998).

25. Gupta N and Porter TD, Garlic and garlic-derived compounds inhibit human squalene mono-oxygenase. *The Journal of Nutrition* 131:1662-1667 (2001).
26. Hayta M, Alpaslan M and Baysar A, Effect of drying methods on functional properties of Tarhana: a wheat flour-yoghurt mixture. *Journal of Food Science* 67(2):740-744 (2002).
27. Ibanoglu S, Ainsworth P, Wilson G and Hayes GD, The effect of fermentation conditions on the nutrients and acceptability of tarhana. *Food Chemistry* 53(2):143-147 (1995).
28. Ibanoglu S, Ibanoglu E and Ainsworth P, Effect of different ingredients on the fermentation activity in tarhana. *Food Chemistry* 64(1):103-106 (1999).
29. Illman R J, Storer G B and Topping D L, White wheat flour lowers plasma cholesterol and increases cecal steroids relative to whole wheat flour, wheat bran and wheat pollard in rats. *J. Nutr.* 123: 1094–1100 (1993).
30. Iwe MO, Sensory methods and analysis. *Regional Com., Aba, Nig.* 86-89 (2000).
31. Jacobs DR Jr, Marquart L, Slavin J and Kushi LH, Whole-grain intake and cancer: an expanded review and meta-analysis. *Nutrition and Cancer* 30(2):85-96 (1998).
32. Kim M and Shin HK The water-soluble extract of chicory influences serum and liver lipid concentrations, cecal short-chain fatty acid concentrations and fecal lipid excretion in rats. *The Journal of Nutrition* 128:1731-1736 (1998).
33. Koca AF, Yazici F, Anil M, Utilization of soy yoghurt in tarhana production. *European Food Research and Technology* 215: 293–297(2002).
34. Kose E and Cagindi OS An investigation into the use of different flours in tarhana. *Int. J. Food Sci. Tech.* 37(2):219-222 (2002).
35. Lees GJ and Jaco GR, Method for the estimation of acetaldehyde in cultured dairy products. Aust. J. Dairy Tech. 24:181 (1969).
36. Lees GJ and Jaco GR, The estimation of diacetyl in cultured in the presence of other carbonyl compounds. *J. Dairy Res.* 37:129 (1970).
37. Lopes-Virella MF, Stone P, Ellis S and Coldwell JA, Cholesterol determinations in high density liproproteins separated by three methods. Clin Chem 23:882–884, (1977).
38. McIntoch GH, Whyte J, McArther R and Nestel PJ, Barley and wheat foods: influence on plasma cholesterol concentrations in hypercholesterolemic men. *Am J Clin Nutr* 53(5):1205-1209 (1991).
39. Mensah P, Fermentation the key to food safety assurance in Africa. *Food Control* 8:271-278(1997).
40. Mezzetti A, Lapenna D, Piedromenico SD, Cala.ore AM, Constantini F, Riario-Sforza G, Imbastaro T, Neri M and Cuccurullo F, Vitamin E, C and lipid peroxidation in plasma and arterial tissue of smokers and non smokers, *Atherosclerosis* 112:91-99 (1995).
41. Mugula JK, Narvhus JA and Sorhaug T, Use of starter cultures of lactic acid bacteria and yeasts in the preparation of togwa, a Tanzanian fermented food. *International Journal of Food Microbiology*, 83(3):307-318 (2003).
42. Newman RK, Lewis SE, Newman CW, Boik RJ and Ramage RT, Hypocholesterolemic effect of barley foods on healthy men. *Nutritional Reports International* 39:749-760 (1989).
43. Nout MJR and Motarjemi Y, Assessment of fermentation as a household technology for improving food safety: a joint FAO/WHO workshop. *Food Control* 8:221-226 (1997).
44. Ozdemir S, Gocemen D and Kumral Y, A traditional Turkish fermented cereal food: Tarhana. *Food Rev. Int.* 23: 107-121 (2007).
45. Rivera-Espinoza Y and Gallardo-Navarro Y, Non dairy probiotic products. *Food Microbiology* 27:1-11 (2010).
46. Singh DK and Porter TD, Inhibition of sterol 4 alpha-methyl oxidase is the principal mechanism by which garlic decreases cholesterol synthesis. *Journal of Nutrition* 136:759-764 (2006).
47. Steinkraus KH, Fermentations in world food processing. Comprehensive Reviews in Food Science and Food Safety 1:23-30 (2002).
48. Tarakc Z, Dogan IS and Koca AF, A traditional fermented Turkish soup, tarhana, formulated with corn flour and whey. International Journal of Food Science and Technology 39:455-458 (2004).
49. Terzaghi BE and Sandine WE, Improved medium for lactic Streptococci and their bacterophages. Appl. Microbial 29:807 (1975).

11/7/2010

Adiponectin level in Protein Energy Malnutrition and its Role in Predicting the Disease Severity

Nassar M.F.[*1], Badawy N.B.[1], Soha Ezz El-Arab[2], El-Batrawy, S.R.[3] and Kamal M.A.[1]

Departments of [1]Pediatrics and [2]Clinical Pathology, Faculty of Medicine, Ain Shams University, Cairo, Egypt
[3]Department of Anthropology, National Institute of Research, Cairo, Egypt.
[*]maie_nassar@yahoo.co.uk

Abstract: Background and aims: Since adiponectin plays an important role in the pathogenesis of both obesity and anorexia nervosa, its level might be altered in protein energy malnutrition (PEM) patients which would highlight the pathogenesis of the morbidities that those patients suffer from. This work was thus designed to assess the adiponectin level in PEM and its relation to the severity of the condition.
Methods: The present study was conducted on 20 PEM infants in comparison to ten clinically healthy infants. Dietetic history and anthropometric measurements were taken. Routine laboratory investigations were done as well as serum adiponectin level by ELISA. Patients were re-evaluated after nutritional rehabilitation.
Results: Initial serum adiponectin levels were significantly higher in PEM patients in comparison to the controls and decreased after nutritional rehabilitation. Distinct negative correlation was found between initial adiponectin levels and both the z score and serum albumin in all studied patients.
Conclusions: Adiponectin level is increased in PEM patients and decreases upon nutritional rehabilitation which further proves the inverse relationship between adiponectin level and both weight and body fat. This up regulated adiponectin level in PEM may have an anti-inflammatory role which awaits further studies.

[Nassar M.F., Badawy N.B., Soha Ezz El-Arab, El-Batrawy, S.R. and Kamal M.A. **Adiponectin level in Protein Energy Malnutrition and its Role in Predicting the Disease Severity**. Journal of American Science 2010;6(12):1413-1422]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: Adiponectin; Protein energy malnutrition; Nutritional rehabilitation..

1. Introduction:

Adiponectin, the most abundant adipose-specific protein, is suggested to possess anti-hyperglycemic, anti-atherogenic and anti-inflammatory properties [1]. Low levels have been associated with obesity, insulin resistance, Type II diabetes and cardiovascular disease [2]. In anorexia nervosa an inverse linear relationship was reported between adiponectin level and fat mass [3].

Protein energy malnutrition (PEM) is by far the most lethal form of malnutrition. Children are its most visible victims. Malnutrition "the silent emergency" is an accomplice in at least half of the 10.9 million child deaths each year [4]. PEM is manifested primarily by inadequate dietary intake of protein and energy. However, PEM is almost always accompanied by deficiencies of other nutrients. For this reason, the term severe childhood undernutrition (SCU), which more accurately describes the condition, is preferred [5]. The pathogenesis of the affection of various body systems in PEM is still the center of attention of many researchers. There is definite change in body weight and fat content as well as many inflammatory changes.

We hypothesize that since adiponectin plays an important role in the pathogenesis of both obesity and anorexia nervosa, its level might be altered in PEM patients which would highlight the pathogenesis of the morbidities that those patients suffer from. This work was thus designed to assess the adiponectin level in PEM and its relation to the severity of the condition. The effect of nutritional rehabilitation on these levels was studied as well.

2. Patients and methods:

The present study was conducted on 20 infants having protein energy malnutrition (PEM) based on the criteria of [6]. Their ages ranged between 6-24 months, with mean age of 11.15 ± 4.85 months. They were 8 males and 12 females.

The PEM patients were recruited from the Outpatient Clinic, Nutrition Clinic and Inpatient Department, Children's Hospital, Faculty of Medicine, Ain-Shams University in the period from June 2005 till April 2006. They were free from any chronic renal or liver diseases, any neurological deficits, chromosomal or congenital malformations and were born full term.

They were classified on the basis of Z score of weight for length and presence or absence edema according to *Gernaat and Voorhoeve classification* [7] into two groups. The non-edematous PEM group which included 12 infants, their Z score of weight for length was < -2 SD with no edema. The second group is the edematous PEM group: Which included 8 infants, their Z score of weight for length was < -2 SD with edema.

Ten clinically healthy, age and sex matched infants having their anthropometric measurements within the normal range for age and sex according to *Ogden et al.,* [8] were included as the control group. They were 5 males and 5 females with mean age of 13.65 ± 5.57 months.

After the approval of the ethical committee of the Children Hospital, Faculty of Medicine, Ain Shams University, an informed verbal consent was obtained from parents or legal guardians. Enrolled cases were then subjected to complete history taking with special emphasis on dietetic history including 24 hours recall of feeding. Thorough clinical examination was done laying stress on the anthropometric measurements, signs of PEM and associated vitamin deficiencies as well as systemic examination.

Weight was measured using regularly calibrated scale with minimal clothing. Length was measured using a special calibrated board. Skull circumference (SC) was measured by passing a tape over the most prominent part of the occiput and just above the supraorbital ridges. Readings was approximately to the nearest millimeter. Mid arm circumference (MAC) was measured midway between the tip of acromial process and the epicondyle of the humerus. The measuring was the mean of two reading taken the nearest millimeter. Skinfold thickness (SFT) was measured using Harpenden skinfold callipers over the triceps, supra-iliac and under the scapula, applying a constant pressure of 10 g/mm2 at a contact surface of 20–40 mm. The measurements were read 2 s after full pressure was exerted by the jaws of the caliber. Weight, height and skull circumference were plotted against the percentiles to obtain the percent from the median for age according to *Ogden et al.,* [8].

Venous blood was collected under complete aseptic condition. The sample was divided into two tubes one containing EDTA and designed for the complete blood count using coulter GEN-S (Coulter Corporation USA) and the other was plain to allow blood to clot for 30 minutes before being centrifuged at 3000g for 15 minutes at room temperature. Plasma were rapidly separated and part was used to estimate the liver and kidney functions by Hitachi 917 automated analyzer and the rest of the sample was stored at -40 degree centigrade until used later to determine serum adiponectin level by ELISA using the kit purchased from A&D Company.

Patients entered a nutritional rehabilitation program according to the *WHO,* [9] for a period of 2 – 4 months then they were re-evaluated using the previously mentioned clinical and laboratory parameters.

Regarding the statistical methods, the standard computer program SPSS for Windows, release 10.0 (SPSS Inc, USA) was used for data entry and analysis. All numeric variables were expressed as mean ± standard deviation (SD). Comparison of different variables in various groups was done using student t test and Mann Whitney test for normal and nonparametric variables respectively. Paired t or Wilcoxon signed ranks tests were used to compare variables before and after therapy. Multiple regression analysis was also performed to determine effect of various factors on a dependent variable. Pearson's and Spearman's correlation test were used for correlating normal and non-parametric variables respectively. For all tests a probability (p) less than 0.05 was considered significant. Graphic presentation of the results was also done [10].

3. Results

The results of the present study reveal lower anthropometric measurements in non-edematous as well as edematous PEM patients compared to those of the controls (table 1). These measurements show significant improvement after nutritional rehabilitation (yet not reaching the control values in most of them) (table 2).

Tables (1) and (2) also show that both serum albumin and hemoglobin (Hb) levels are significantly lower in both groups of PEM compared to the controls and their values show significant improvement after nutritional rehabilitation yet not reaching the control values.

Figures (1) and (2) demonstrate that initial serum adiponectin levels are significantly higher in non-edematous and edematous PEM patients in comparison to the controls. After nutritional rehabilitation these levels decrease yet they are still significantly higher than the controls. Additionally, no significant difference is detected between the adiponectin level of non-edematous and edematous PEM patients whether before or after nutritional rehabilitation [t = 0.32 (p>0.05) and t = -0.61 (p>0.05) respectively].

The correlation studies reveal distinct negative correlation between initial adiponectin levels and both the z score and serum albumin in all

studied patients yet these correlations don't reach statistical significance (Figures 3 and 4).

4. Discussion:

The results of the current study revealed statistically initial lower values of the studied anthropometric measurements in both studied groups of PEM in comparison to the controls with significant improvement after nutritional rehabilitation. The decreased initial values of weight and length agree with Wellcome's classification of PEM [6] and are fulfilling one of the constant features of malnutrition, namely growth retardation in weight and height [11]. The lower skull circumference measurement agrees with *Needlman* [12] who stated that severe under nutrition depresses head growth and this is considered as ominous predictor of later cognitive disability. As regards the low measures of mid arm circumference, it is reinforced by *Castiglia,* [13] who stated that mid arm circumference reflects the state of muscle and subcutaneous fat so it is affected in PEM especially the non-edematous. Additionally, similar to our results *Curran and Barness,* [14] reported decreased skin fold thickness in PEM patients.

As regards the hematological parameters we found that the initial hemoglobin and albumin levels were significantly lower in both groups of patients in comparison to the control group with significant

Table (1): Comparison between anthropometric measurements, and laboratory parameters of protein energy malnutrition patients and that of the controls before nutritional rehabilitation.

Studied parameter	*Non-edematous Group I n=12* Mean ± SD [Median (interquartile range)]	*Edematous Group II n=8* Mean ± SD [Median (interquartile range)]	*Control Group III n=10* Mean ± SD [Median (interquartile range)]	Group I Vs III t/z* (p)	Group II Vs III t/z* (p)	Group I Vs II t/z* (p)
Weight % from median for age	**50.92 ± 5.90 [51.32(8.56)]**	**69.01 ± 4.64 [68.60(5.87)]**	**98.5 ± 6.17 [100.13(10.52)]**	**-11.19 (p<0.001)**	**-11.19 (p<0.001)**	**-7.27 (p<0.001)**
Length % from median for age	**84.92 ± 5.55 [85.99(10.31)]**	**92.31 ± 3.33 [91.16(5.44)]**	**97.55 ± 2.93 [98.31(2.67)]**	**-6.46 (p<0.001)**	**-3.55 (p<0.01)**	**-3.36 (p<0.05)**
Z score	**-2.65 ± 0.65 [-2.54 (0.98)]**	**-2.07 ± 0.93 [-2.13 (1.50)]**	**0.71 ± 0.68 [0.97(1.19)]**	**-3.96* (p<0.001)**	**-3.56* (p<0.001)**	**-1.54* (p>0.05)**
SC% from median for age	**91.83 ± 1.83 [92.55(3.71)]**	**94.98 ± 2.45 [95.01(3.79)]**	**98.67 ± 2.25 [98.51(4.11)]**	**-5.64 (p<0.001)***	**-3.33 (p<0.01)**	**-2.34 (p<0.05)**
MAC (cm)	**8.92 ± 1.46 [9(1.88)]**	**11.81 ± 0.65 [12.00(0.88)]**	**13.45 ± 1.40 [13.75(1.50)]**	**-7.38 (p<0.001)**	**-3.04 (p<0.01)**	**-5.24 (p<0.001)**
SFT (mm)	**7.13 ± 1.48 [7.50(2.00)]**	**9.38 ± 1.19 [9.00(2.50)]**	**11.20 ± 1.69 [11.00(2.25)]**	**-6.04 (p<0.001)**	**-2.58 (p<0.05)**	**-3.51 (p<0.01)**
Albumin (gm/dL)	**3.42 ± 0.48 [3.35(0.65)]**	**2.25 ± 0.42 [2.15(0.80)]**	**4.01 ± 0.40 [4.00(0.50)]**	**-3.06 (p>0.01)**	**-9.02 (p<0.001)**	**5.60 (p<0.001)**
Hb (gm/dL)	**8.24 ± 1.75 [8.55(1.78)]**	**8.25 ± 1.14 [7.90(2.08)]**	**12.63 ± 0.83 [12.85(1.50)]**	**-7.27 (p<0.001)**	**-9.46 (p<0.001)**	**-0.01 (p>0.05)**

*Non-parametric data detected by Shapiro-Wilk test.
The test of significance used here is Mann-Whitney test.
P<0.05 is significant, p<0.01 is highly significant, p<0.001 is very highly significant and p>0.05 is non-significant. Vs means versus.

Table (2): Comparison between anthropometric measurements and laboratory parameters of protein energy malnutrition patients before and after nutritional rehabilitation.

Studied Parameter	*Non-edematous (n=12)* Mean ± SD [Median (interquartile range)]			*Edematous (n=8)* Mean ± SD [Median (interquartile range)]		
	Before	After	t/z*(p)	Before	After	t/z*(p)
Weight % from median for age	50.92 ± 5.9 [51.32(8.56)]	96.56 ± 4.35 [96.48(4.35)]	-18.71 (p<0.00)	69.01 ± 4.64 [68.60(5.87)]	98.09 ± 9.05 [97.56(18.89)]	-7.47 (p<0.001)
Length % from median for age	84.92 ± 5.55 [85.99(10.31]	96.59 ± 1.19 [96.51(1.49)]	-3.06* (p<0.01)	92.31 ± 3.33 [91.16(5.44)]	96.59 ± 3.91 [95.98(7.87)]	-8.55 (p>0.05)
Z score	-2.65 ± 0.65 [-2.54 (0.98)]	0.74 ± 0.21 [0.71(0.31)]	-3.06* (p<0.01)	-2.07 ± 0.93 [-2.13 (1.50)]	1.11 ± 0.55 [1.04(1.11)]	-2.52* (p<0.05)
SC % from median for age	91.83 ± 1.83 [92.55(3.71)]	97.90 ± 2.86 [97.47(4.74)]	-4.55 (p<0.01)	94.98 ± 2.45 [95.01(3.79)]	97.90 ± 2.86 [97.70(5.50)]	-1.61 (p>0.05)
MAC (cm)	8.92 ± 1.46 [9(1.88)]	14.00 ± 0.8 [14.00(0.5)]	-3.06* (p<0.01)	11.81 ± 0.65 [12.00(0.88)]	13.75 ± 1.75 [14.50(3.25)]	-2.21* (p<0.05)
SFT (mm)	7.13 ± 1.48 [7.50(2.00)]	11.83 ± 0.72 [12.00(1.00)]	-3.09* (p<0.01)	9.38 ± 1.19 [9.00(2.50)]	11.50 ± 1.60 [12.00(3.00)]	-2.20* (p<0.05)
Albumin (gm/dL)	3.42 ± 0.48 [3.35(0.65)]	4.00 ± 0.13 [4.00(0.20)]	-4.06 (p<0.01)	2.25 ± 0.42 [2.15(0.80)]	4.02 ± 0.21 [4.00(0.43)]	-10.26 (p<0.001)
Hemoglobin (gm/dL)	8.24 ± 1.75 [8.55(1.78)]	12.99 ± 0.82 [12.85(1.55)]	-9.85 (p<0.00)	8.25 ± 1.14 [7.90(2.08)]	12.15 ± 1.38 [12.40(2.85)]	-8.03 (p<0.001)

*Non-parametric data detected by Shapiro-Wilk test.
The test of significance used here is Wilcoxon matched pairs test.
p<0.01 is highly significant and p<0.001 is very highly significant.

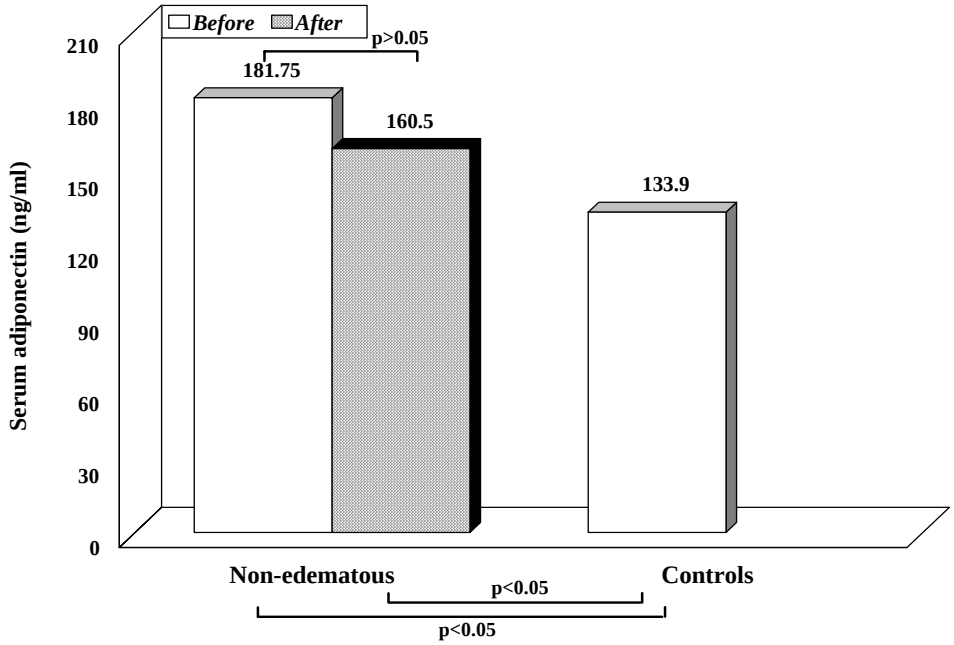


Figure (1): Serum adiponectin levels of non-edematous PEM patients before and after nutritional rehabilitation and that of the control group.

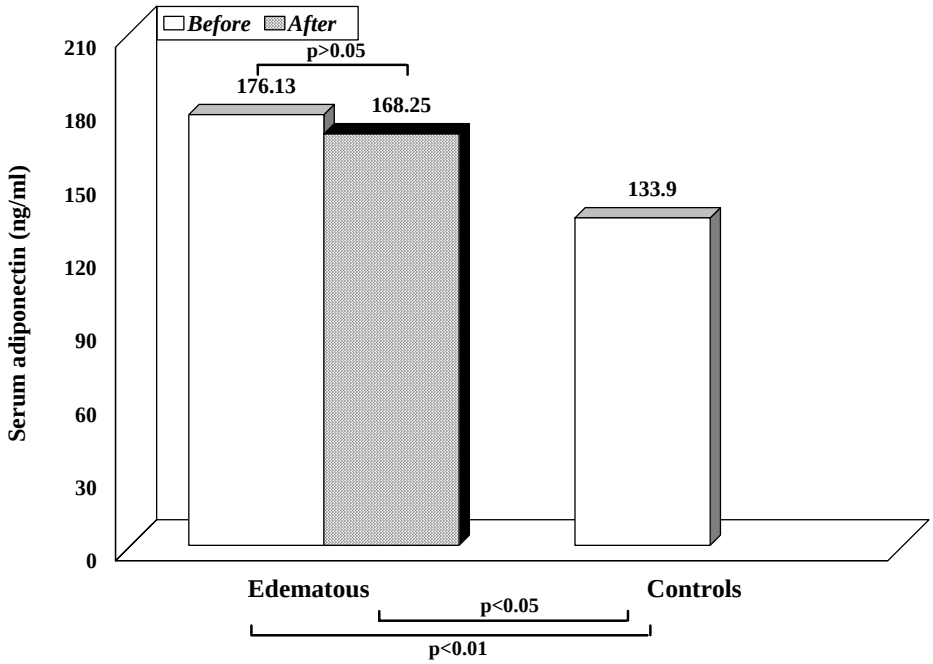


Figure (2): Serum adiponectin levels of edematous PEM patients before and after nutritional rehabilitation and that of the control group.

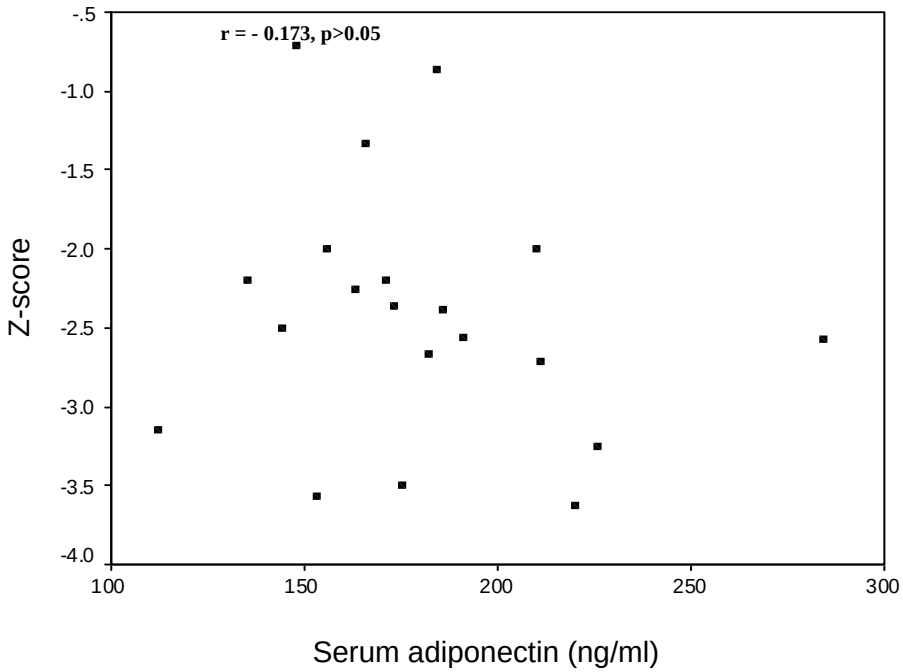


Figure (3): Correlation between serum adiponectin levels and Z score in PEM patients.

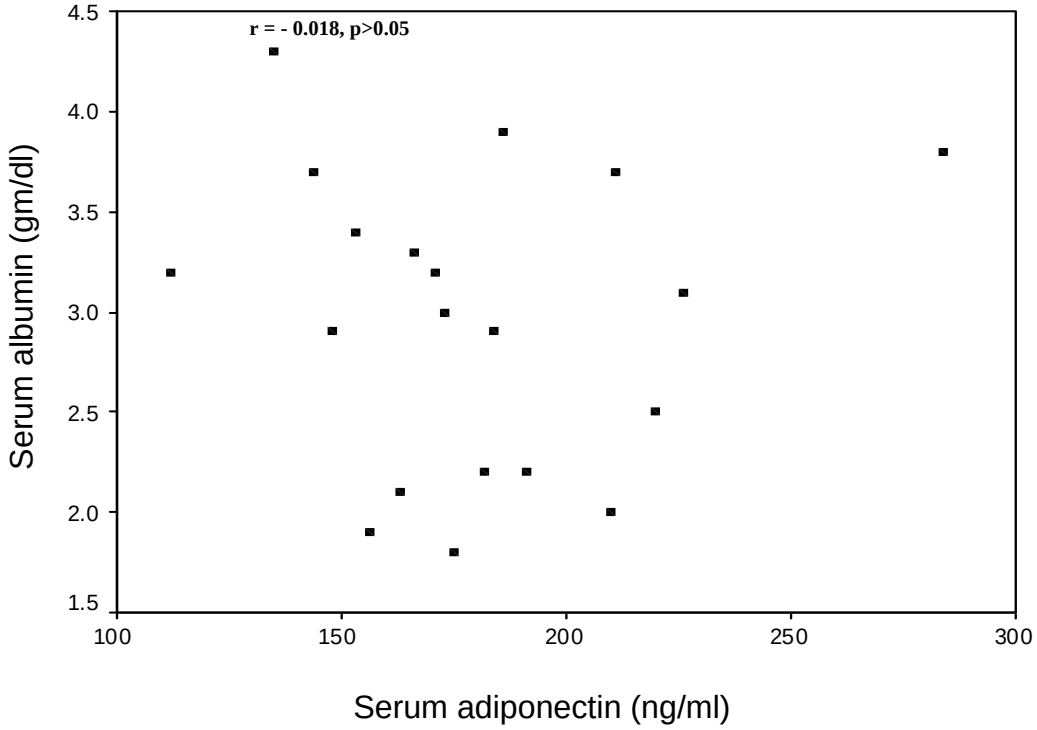


Figure (4): Correlation between serum adiponectin levels and serum albumin in PEM patients.

improvement after nutritional rehabilitation which further proves the success of its implementation. The anemia initially detected in our patients comes in agreement with *Sive et al.,* [15] *and Ashour et al.,* [16]. Regarding the hypoalbuminemia it agrees with the results of *Morlese et al.,* [17], who detected hypoalbuminemia in both edematous and non-edematous groups of PEM cases. They explained its deficiency by increased rate of catabolism rather than impairment of synthesis and attributed this mainly to infection. Additionally, *Karp,* [18], suggested that hypoalbuminemia here comes from the decrease in its synthesis.

Serum adiponectin level was measured in both groups of PEM patients and both showed significantly higher values before nutritional rehabilitation as compared to the control group. After nutritional rehabilitation the serum adiponectin level showed non-significant decrease in both groups, but it was still higher than control group values.

The current study shows increase in adiponectin level in PEM patients and decrease in this level upon nutritional rehabilitation which entails an increase in body weight. Our results denote an inverse relation between circulating adiponectin level and weight. Comparatively, circulating adiponectin level was reported to be down regulated in obesity [19], and increased upon weight reduction [20].

Considering the relation between body fat and adiponectin level *Hotta et al.,* [21] reported significant decrease in adiponectin levels in cases of obesity who suffer from excess fat accumulation. Conversely, PEM is characterized by loss of body fat [22] and this explains the increased adiponectin level in our series of patients. These findings, regarding PEM patients, further prove the inverse relation between body fat and adiponectin serum level that is found in obese subjects.

Additionally, *Pannacciulli et al.,* [23] found that anorexia nervosa is associated with increased adiponectin serum levels. This agrees with our research, when taking into consideration that anorexia nervosa is characterized by decreased caloric intake, low weight and reduced body fat [24]. Thus the findings of the former authors further support our conclusion about the relation between body fat and adiponectin levels in PEM patients.

On the other hand *Tagami et al.,* [25], found that adiponectin levels were significantly low in the patients with AN and bulimia nervosa, compared with normal weight control subjects, and hypoadiponectinemia was reversed by weight recovery in the patients with AN. The authors suggested that this might be normal physiological response to starvation or abnormal feeding behavior.

The distinct negative yet non significant correlation between serum adiponectin level in PEM patients and serum albumin and Z score revealed in the current study proves that the increase in adiponectin level in PEM patients is judged by the severity of the condition.

From another perspective *Engeli et al.,* [26], found an inverse relation between inflammatory mediators and adiponectin levels in obese women which was explained by *Ajuwon and Spurlock* [27] by the fact that adiponectin directly targets the adipocytes to suppress IL-6 and TNF mRNA expression. It has been reported earlier that chronic undernutrition and micronutrient deficiency compromise cytokines response and affect immune cell trafficking [28]. We thus hypothesis that the increased adiponectin level in our series of patients may be in part responsible for their altered inflammatory mediators response. In a way this increased level can be considered as a protective mechanism that the body utilizes to overcome hard times.

In conclusion, adiponectin level is increased in PEM patients disregards its type

and decreases upon nutritional rehabilitation which further proves the inverse relationship between adiponectin level and both weight and body fat. This up regulated adiponectin level in PEM may have an anti-inflammatory role. Large multi-centric study are thus recommended to highlight the level of adiponectin as well as other growth factors in PEM patients in a trail to detect the interrelation between them and the feedback mechanism controlling their levels in such disease. Further studies should also concentrate on exploring the anti-inflammatory and the possible anti-atherogenic role of the upgraded adiponectin level in PEM patients.

Acknowledgements

We acknowledge the help provided by Professor Dr Ehab El-Hakeem to analyze the data and perform the required statistical tests.

Funding

We, the authors, declare that we did not receive any form of funding throughout the whole steps of the study. Ain Shams University gave us the privilege of using the laboratory instruments and the kits used for the hormonal assay was purchased by the authors themselves.

Corresponding author

Nassar M.F

Pediatrics Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt.

maie_nassar@yahoo.co.uk

5. References:

1. Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, Yamashita S, Noda M, Kita S, Ueki K, Eto K, Akanuma Y, Froguel P, Foufelle F, Ferre P, Caling D, Kimura S, Nagai R, Kahn BB, Kadowaki T. Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. Nat Med 2002, 8: 1288-1295.
2. Fu Y, Luo N, Klein RL, Garvey WT. Adiponectin promotes adipocyte differentiation, insulin sensitivity, and lipid accumulation. J Lipid Res 2005, 46:1369-1379.
3. Bosy-Westphal A., Brabant G, Haas V, Onur S, Paul T, Nutzinger D, Klein H, Hauer M, Muller MJ. Determinants of plasma adiponectin levels in patients with anorexia nervosa examined before and after weight gain. Euro J Nutr 2005, 44: 355-359.
4. WHO. Turning the tide of malnutrition: responding to the challenge of the 21st century. Geneva: World Health Organization, 2000: 4 (WHO/NHD/00.7).
5. Heird WC. Food insecurity, hunger and undernutrition. In: *Nelson Textbook of Pediatrics*, 18th edition. Edited by Behrman RE, Kliegman RM, Jenson HB, Stanton BF. Saunders Elsevier, 2008: 225-232.
6. Wellcome T. Wellcome trust working study: Committee on nutrition. Lancet 1970, 2: 302.
7. Gernaat HB, Voorhoeve HW. A new classification of acute protein-energy malnutrition. J Trop Pediatr 2000, 46: 97-106.
8. Ogden CL, Kuczmarski RJ, Flegal KM, Mei Z, Guo S, WeiR, Grummer-Strawn LM, Curtin LR, Roche AF, Johnson CL. Centers for disease and prevention 2000 growth charts for the Unites States: Improvements to the 1997 National Center for Health Statistics version. Pediatrics 2002, 109: 45-60.
9. WHO. Management of severe malnutrition: a manual for physicians and other senior health workers. Geneva: World health organization, 1999.
10. Daniel WW. *Biostatistics: A foundation for analysis in the health sciences*, 6th edn. John Wiley and Sons, Inc., New York, 1995.
11. Jelliffe DB, Jelliffe EF. Causation of kwashiorkor: toward a multifactorial consensus. Pediatrics 1992, 90: 110-113.

12. Needlman R. Failure to thrive: parental neglect or well-meaning ignorance? Am Fam Physician 2001, 63: 1867-1869.
13. Castiglia PT. Protein-energy malnutrition (kwashiorkor and marasmus). J Pediatr Health Care 1996, 10: 28-30.
14. Curran SJ, Barness LA. Nutritional requirements and malnutrition. In: *Nelson textbook of pediatrics*, 17th edition. Edited by Behrman RE, Kliegman RM, Jenson HB. WB Saunders, Philadelphia, London, 2004: 169-172.
15. Sive AA, Subotzky EF, Malan H, Dampster WS, Heese HD. Red blood cell antioxidant enzyme concentration in KWO and Marasmus. Ann Trop Pediatr 1993, 13:33-38.
16. Ashour MN, Salam SI, El-Gadban HM, Elwan NM, Basu TK. Antioxidant status in children with protein energy malnutrition living in Cairo: Egypt. Eur J Clin Nutr 1999, 53: 669-673.
17. Morlese JF, Terrence F, Asha B, Melanie DR, Margaret F, Farook J. Albumin kinetics in edematous and non-edematous protein energy malnourished children. Am J Clin Nutr 1996, 64: 952-959.
18. Karp R. Malnutrition among children in the United States "The impact of poverty". In: *Modern nutrition in health and disease*, 9th edition. Edited by Shils ME, Olson JA, Shike M. Baltimore, Williams and Wilkins, 1999: 989-1001.
19. Arita Y, Kihara S, Ouchi N, Takahashi M, Maeda K, Miyagawa J, Hotta, K, Shimomura I, NakamuraT, Miyaoka K, Kuriyama H, Nishida M, Yamashita S, Okubo K, Matsubara K, Muraguchi M, Ohmoto Y, Funahashi T, Matsuzawa Y. Paradoxical decrease of an adipocyte-specific protein, adiponectin, in obesity. Biochem Biophys Res Commun 1999, 257: 79-83.
20. Milan G, Granzotto M, Scarda A, Calcagno A, Pagano C, Federspil G, Vettor R. Resistin and adiponectin expression in visceral fat of obese rats: effect of weight loss. Obes Res 2002, 10: 1095-1103.
21. Hotta K, FunahashiT, Arita Y, Takahashi M, Matsuda M, Okamoto Y, Iwahashi H, Kuriyama H, Ouchi N, Maeda K, Nishida M, Kihara S, Sakai N, Nakajima T, Hasegawa K, Muraguchi M, Ohmoto Y, Nakamura T, Yamashita S, Hanafusa T, Matsuzawa Y. Plasma concentrations of a novel adipose-specific protein, adiponectin in type 2 diabetic patients. Arterioscler Thromb Vasc Biol 2000; 20:1595-1599.
22. Torun B, Viteri FE. Protein energy malnutrition. In: *Modern nutrition in health and disease*, 7th edition. Edited by Shils ME, Young VR. Philadelphia, Lea and Febringer, 1988: 746.
23. Pannacciulli N, Vettor R, Milan G, Granzotto M, Catucci A, Federspil G, De Giacomo P, Giorgino R, De Pergola G. Anorexia nervosa is characterized by increased adiponectin plasma levels and reduced non-oxidative glucose metabolism. J Clin Endocrinol Metab 2003, 88: 1748-1752.
24. Rubin RT, Kaye WH. Anorexia nervosa and other eating disorders. In: *Rubenstein Endocrinology*, 4th edition. Edited by DeGroot LJ, Jameson JL, Burger HG, Loriaux DL, Marshall JC, Melmed S, Odell WD, Potts Jr JT, AH. New York: WB Saunders, 2001: 631–641.
25. Tagami T, Satoh N, Usui T, Yamada K, Shimatsu, A, Kuzuya H. Adiponectin in Anorexia Nervosa and Bulimia Nervosa. J Clin Endocrinol Metab 2004; 89:1833-1837.
26. Engeli S, Feldpausch M, Gorzelniak K, Hartwig F, Heintze U, Janke J, Mohlig M, Pfeiffer AF, Luft FC, Sharma AM. Association between adiponectin and mediators of inflammation in obese women. Diabetes 2003, 52: 942-947.
27. Ajuwon KM, Spurlock ME. Adiponectin inhibits LPS-induced NF-kappaB activation and IL-6 production and increases

PPARgamma2 expression in adipocytes. Am J Physiol Regul Integr Comp Physiol 2005; 288(5): R1220–1225.

28. Cunningham-Rundles S, McNeeley DF, Moon A. Mechanisms of nutrient modulation of the immune response. J Allergy Clin Immunol 2005; 115(6):1119-1128.

11/8/2010

Atypical Bacteria in Ventilator Associated Pneumonia; an Egyptian University Hospital Experience

Egypt

dr_mona_zaki@yahoo.co.uk

Background Ventilator-associated pneumonia (VAP) is the most common hospital acquired infection seen in ICU in patients on mechanical ventilation. A diversity of microbes can cause VAP, causative agent differ according to patient populations and types of ICUs. Atypical bacteria not cultured by routinely used methods , have been implicated as causes of VAP, still no sufficient studies to assess size of their role as causative agent in VAP. In this study we aim at estimation of the potential role of atypical bacteria as Mycoplasma pneumoniae, Chlamydia pneumoniae and Legionella pneumophila in ventilator-associated pneumonia in the intensive care units of Alexandria Main University Hospital. **Materials and methods:** 60 endotracheal aspirates were collected from VAP ICU patients. Samples were subjected to routine culture as well as PCR amplification using specific primers for detection of the following atypical bacteria : Mycoplasma pneumoniae, Chlamydia pneumoniae and Legionella pneumophila. **Results:** Out of the 60 endotracheal aspirate, routine culture revealed growth of: enterobacteriaecae in 14 (23.3%) aspirate, pseudomonas in 13(21.7%), candida in14(23.3%) , and MRSA in 10 (16.7%) . In 19 (31.7%) endotracheal aspirates, no growth was encountered on routine culture. PCR reaction was positive for Atypical bacteria in 9 (15%) out of 60 samples, five were positive for mycoplasma, three for Legionella, and only one was positive for Chlamydia. Atypical bacteria positive results were encountered in 4 (21%) out of 19 aspirates with no growth culture results. **Conclusion:** Our results point that atypical bacteria are not an uncommon cause for VAP. This finding has to be taken into consideration while tailoring the empiric antimicrobial coverage of patients diagnosed with VAP.

[Egypt. **Atypical Bacteria in Ventilator Associated Pneumonia; an Egyptian University Hospital Experience**. Journal of American Science 2010;6(12):1423-1428]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Atypical Bacteria; Ventilator; Pneumonia

Introduction

Ventilator-associated pneumonia (VAP) is considered as the most frequent ICU-acquired infection among patients receiving mechanical ventilation (MV). This kind of respiratory tract infection prolong the duration of Mechanical ventilation and delay the release from ICU. Most antibacterial chemotherapy prescribed in an ICU are administered for respiratory tract infections. [1]

VAP can be caused by a large variety of microorganisms . the causative agent may differ according to the population of patients in the ICU, the durations of hospitalization and stay in the ICU. "Atypical" pneumonia differ from typical one in not to be associated with shaking chills [2] and caused by atypical bacteria which cannot be grown by routinely used microbiologic culture media and techniques as *Mycoplasma pneumoniae, Chlamydia pneumoniae and Legionella pneumophila* [3-5].

Mycoplasma pneumoniae was first atypical pathogens to be identified as a cause of respiratory tract infection in 1944.[6,7] Characterized by absence of a rigid cell wall, poor staining by Gram method, and high nutritional requirements for its culture as it needs a high concentration (10-20%) of serum or supplements . This makes diagnosis of mycoplasma by conventional microbiologic examination difficult. [7] *Chlamydia pneumonia* laboratory diagnosis depended on non- specific and technically demanding techniques till 1990 but now it has been replaced by detection of chlamydial antigens or detection of DNA by PCR. [7] *Legionella pneumophila* are nutritionally fastidious, intracellular bacilli, gram negative organisms. [8] Infection with *legionella is* associated with exposure to artificial water systems ,condensers and respiratory therapy equipments .[9] Use of PCR as a rapid and specific diagnostic method for legionella infection overcame the long culture time needed for its growth (3-5 days) and the need of media supplemented with iron and cysteine as well as difficult colonial identification in mixed cultures. [7]

Accurate diagnosis of VAP remains a difficult target to achieve, that relies mainly on clinical, microbiological and radiological diagnosis.[10,11] Main clinical criteria for VAP diagnosis have been reported to be new lung infiltrate on chest X-ray with fever , leukocytosis or leukopenia, and purulent secretions. [12-14] Inadequate antibiotic treatment have been always reported by researchers to be related to poor prognosis of VAP. [15,16] Microbiological culture and sensitivity results remains a gold standard for planning treatment for the VAP patient before empirical antibiotic administration [17] . This have been emphasized by

the Guidelines from the Infectious Disease Society of America (IDSA).[18]

It is well established that Beta-lactams are not effective against such organisms because *Chlamydia pneumoniae* and Legionella species are intracellular organisms and *Mycoplasma pneumoniae* lacks a cell wall. In those cases Erythromycin and tetracycline can be useful . Other antibiotics effective against atypical bacteria, includes Macrolides, Doxycycline and Flouroquinolones.[6] Our study aims at assessment of the potential role of atypical bacteria as Mycoplasma pneumoniae, Chlamydiae pneumoniae and Legionella pneumophila in ventilator-associated pneumonia in the intensive care units of Alexandria Main University Hospital.

Materials

The study was conducted on 60 patients on Mechanical ventilators for more than 48 hours and acquired VAP during their stay in the ICU of Critical Care Department in the Alexandria Main University Hospital in the period between April 2009 and April 2010.Cases included in this study have been informed and consented. Inclusion criteria were fever, leukocytosis, development of persistent radiographical pulmonary infiltrate during stay in the ICU and with no history of a previous pulmonary disease or pulmonary symptoms at the time of admission. Thirty ICU patients on ventilators for more than 48 hours without developing VAP, within the same period of time, were included as a control group.

Methods

Endotracheal aspirates as well as clinical data have been collected from patients and controls. Samples were subjected to routine culture as well as DNA extraction with subsequent PCR amplification for detection of specific DNA sequences of the following atypical Bacteria genus : Mycoplasma pneumoniae, Chlamydia pneumonae and Legionella pneumophila.

The endotracheal tube was previously inserted guided by a laryngoscope. A sterile suction catheter was introduced blindly into the endotracheal tube after disconnecting ventilator. 2 ml of endottracheal aspirates were obtained by suction. The part of the catheters containing the aspirates were cut and placed in sterile test tubes and sent to the laboratory.

The endotracheal aspirates, within catheters in test tubes, were homogenized and liquefied by adding 2 ml sterile 1% N-acetyl L-cysteine (equal volume of the specimen). All tubes were centrifuged for 15 minutes at 4×10^3 rpm and then vortexed and left at room temperature for another 15 minutes.[19,20] Each homogenized specimen was then divided into 2 equal portions in 2 sterile Eppendorfs. The first part was used for conventional microbiological studies while the other was kept at -20^{o}C for PCR assay. Each aspirate was streaked on Blood agar, MacConkey's agar and Sabouraud's Dextrose agar plates. All plates were incubated aerobically at 37 ^{o} C for 24 hours. Any growth was identified according to the conventional bacteriological and mycological techniques.[19,20]

PCR assay: Extraction of DNA was performed using QIAamp DNA blood mini kit (Qiagen). Separate PCR reactions were performed for amplification of each DNA sequence of each organism using Techne Progene thermal cycler. Reaction mixture consisted of 5µl DNA extract, 25 picomoles of each of the forward and reverse oligonucleotide primers specific for Mycoplasma pneumonia [21] , Legionella pneumophila [21] and Chlamydia pneumonia [22] , 12.5 µl Taq PCR master mix (MBI Fermantas), and 4.5 µl nuclease free water. For detection of the amplified products: [21] 10 µl of the amplification products were electrophorised into 2% agarose in Tris-borate EDTA containing 0.5 µg/ml ethidium bromide at 80 volts for 45 minutes. Revealed DNA bands were visualized on an ultraviolet transilluminator.

Results

The mean age of the VAP study group was 43.1 ± 24.68 (18-85) year, while that of the control group was 49.7 ± 20.5 (12-70). There was no statistically significant difference between them as regards age and gender: male to female ratio was 33:27(55:45%) in patient group while in the control group, the male to female ratio was 19:11 (63:36.6%). As regards causes of hospital admission for the 60 VAP patients and the control group It has been found that cardiac problems were the most commonly encountered among both patients (38%) and controls (28%), rest of causes for admission included accidents, poisoning, Renal and hepatic problems. In the endotracheal aspirates obtained from the 60 VAP patients included in this study, 41 specimens were positive by conventional microbiological cultures and 19 were culture negative. In the control group, 11were positive and 19 specimens were culture negative. See table 1.

The conventional microbiological culture revealed that among cultures of the patients group, *Candida* spp was the commonest organism isolated accounting for 23.3%. Only 16% were of significant count ($\geq 10^5$ CFU/ml). This was followed by *Pseudomonas aeruginosa* (21.6%) and 15% were of significant count, then the polymicrobial growth 20% and 16% with significant count, *Staphylococcus*

aureus 16% and 10% with significant count, *Acinetobacter* spp 8.3% and 6.6% with significant count, *Proteus* spp 6.6% and 5% with significant count, *Klebsiella* spp 6.6% and 5% with significant count, *E-coli* 5% and 1.6% with significant count, *Coagulase-negative staphylococci* 1.6% which were of significant count and *Diphtheroids* 1.6% which were also of significant count. It is also to be noted that 31.6% of the total endotracheal aspirates of the 60 VAP cases were negative by conventional microbiological culture.

Table 1: The results of conventional microbiological culture of the endotracheal aspirates from the 60 VAP patients and the control group

	Conventional microbiologic Culture results*	
	Positive	**Negative**
Cases n=60	41 (68.3%)	19 (31.7%)
Controls n=30	11 (36.6%)	19 (63.3%)
Total n=90	52 (57.7%)	38 (42.2%)

*P value = 0.004

While in the control group, bacteria revealed from cultures were *Pseudomonas aeruginosa* accounted for 20%, *Klebsiella* spp 10%, *Candida spp* 10% also, then *Staphylococcus aureus* 3.3%, *Coagulase-negative staphylococci* 3.3% and *Proteus* spp 3.3%. In addition, 10% were culture negative and 13.3% were polymicrobial. The growth counts of the endotracheal aspirates of the control group were insignificant ($\leq 10^5$ CFU/ml). Atypical bacteria DNA detection by PCR was positive in 9 (15%) out of 60 samples, the majority of them (5) were mycoplasma, 3 were positive with Legionella, and only one sample was positive with Chlamydia. The Atypical bacteria positive results was encountered in 4 (21%) out of 19 aspirates with no growth culture results. See figure 1.

It was found that 5 specimens were positive for *Mycoplasma pneumoniae*, three were positive for *Legionella pneumophila* and only 1 was positive for *Chlamydia pneumoniae.* Among the 5 positive specimens for *Mycoplasma pneumoniae*, 3 were positive by conventional microbiological cultures and grew other associating microorganisms, while 2 were culture negative. The 3 specimens that were positive for *Legionella pneumophila*, only 1 grew other microorganism by conventional cultures and the other 2 were culture negative. The only positive specimen for *Chlamydia pneumoniae* did not grow any microorganisms by conventional microbiological cultures. As for the control group included in this study, none of their DNA extracts were positive in the PCR assay for atypical bacteria.

Table 2: PCR and culture results of cases and control

	Culture**		PCR*	
	Culture positive	**Culture negative**	**PCR positive**	**PCR negative**
Cases	41 (68.3%)	19 (31.7%)	9 (15%)	51 (85%)
Controls	11 (36.6%)	19 (63.3%)	0 (0%)	30 (100%)

* PCR was done using primers for Mycoplasma pneumonia, Legionella pneumophila, Chlamydia pneumonia.
**All specimens were cultured on Blood agar, MacConkey's and SDA.

Table 3: Results of PCR assay in relation to conventional microbiological culture resutls inVAP patients.

PCR assay	Conventional microbiological culture		Total
	Positive(n= 41)	**Negative (n= 19)**	
Mycoplasma pneumoniae	3(7%)	2 (10%)	5 (8.3%)
Legionella pneumophila	1 (2.4%)	2 (10%)	3 (5%)
Chlamydia pneumoniae	0	1 (5%)	1 (1.6%)
Total	4(9%)	5(26%)	9 (15%)

DISCUSSION

VAP complicates the course of 9 – 20% of mechanically ventilated patients and mortality varies greatly from 8 to 76%,. Once pneumonia is suspected, bacteriologic confirmation should be obtained and empiric therapy must be instituted as soon as possible, as a delay in therapy or inappropriate therapy greatly increases mortality.[23] Awareness of the potential microbial causes of VAP and confirmation of the specific cause in an individual patient are essential to guide optimal antibiotic therapy.[24]

Endotracheal aspirates, chosen in this study as the respiratory specimen, are used more frequently as a diagnostic method in intubated patients with suspicion of pulmonary infection, because of its

simplicity and minimal training required, but the fact that the culture also contains other non-pathogenic organisms from the upper respiratory tract flora, results in a low positive predictive value of this test. However, this can be avoided by the use of the semiquantitative method of culture of the obtained specimen, with a designated threshold value above which diagnosis of VAP can be established.[25,26] Cultures revealed that *Candida* was the commonest organism isolated accounting for 23.3%, while 16% only were of significant count ($\geq 10^5$ CFU/ml). This was followed by *Pseudomonas aeruginosa* 21.6% and 15% were of significant count, then the polymicrobial growth 20% and 16% with significant count, *Staphylococcus aureus* 16% and 10% with significant count.

The results of our study agree with the results of several previous studies.[27 -31] where *Pseudomonas aeruginosa* was the predominant organism isolated by endotracheal aspiration and bronchoalveolar lavage, followed by *Staphylococcus aureas* and *Klebsiella pneumonia*.[32] The significantly high rate of Gram negative bacilli in our study and many other studies probably indicates the high incidence of prolonged hospital stay and the prolonged duration of mechanical ventilation that predisposes the patients to acquire infections from the multidrug-resistant pathogens . In contrast , Other authors reported other bacterial strains as *Acinetobacter baumanii and Streptococcus* .[33-34]

.

The results of PCR assay for atypical bacteria of the DNA extract of the endotracheal aspirates of the 60 VAP patients revealed a total of 9 positive cases (15%) for the tested microorganisms, 5 cases were positive for *Mycoplasma pneumonia* (8.3%), 3 cases were positive for *Legionella pneumophila* (5%) and only 1 case was positive for *Chlamydia pneumonia* (1.6%).

Many were studies conducted for detection of atypical bacteria by PCR. Hassan et al reported detection of legionella and Chlamydia pneumonia in VAP cases while no cases were positive for *Mycoplasma pneumonia*e.[28] Moreover, Bachinskaya et al reported that 9% of their patients were positive for *Mycoplasma pneumonia*e and 9% were also positive for *Chlamydia pneumoniae.*[35] In another study by Apfalter et al, where real time PCR was used as a fast diagnostic tool for non-conventionally cultured microorganisms, they reported that 3% of their cases were positive for *Mycoplasma pneumonia*e and 2% of cases were positive for *Chlamydia pneumoniae.* They concluded that *Mycoplasma pneumonia*e and *Chlamydia pneumoniae* should be considered as causative agents in critically ill patients who develop early-onset nosocomial ventilator-associated pneumonia. Thus, empirical antimicrobial regimens should cover *Chlamydia,* and *Mycoplasma*.[36] Furthermore, El-Ebiary et al also diagnosed six cases of *Legionella* pneumonia among patients with definite VAP. Using specific culture for *Legionella* and serology for *Legionella pneumophila, Mycoplasma pneumoniae Coxiella burnetti, and Chlamydia pneumoniae,* only *Legionella* was diagnosed in 2 patients by serology and in 4 patients by culture.[37] Our results draw attention towards the possibility of these rarely diagnosed agents as being not infrequent causative agents for VAP. The prevalence of such atypical pathogens is to be taken into consideration while tailoring the empiric antimicrobial coverage of patients diagnosed with VAP.

References

1- Eggimann P and Pittet D. Infection Control in the ICU. Chest 2001;120;2059-2093.

2- Eggimann P, Harbarth S, Constantin MN, et al. Impact of a prevention strategy targeted at vascular-access care on incidence of infections acquired in intensive care. Lancet 2000; 355:1864–1868.

3- Rello J, Diaz E: Pneumonia in the intensive care unit. Crit Care Med 2003, 31:2544-2551.

4- Bouza E, Perez A, Munoz P, et al. Cardiovascular Infection Study Group: Ventilator-associated pneumonia after heart surgery: A prospective analysis and the value of surveillance. Crit Care Med 2003, 31:1964-1970

5- Tang W. Molecular diagnostics of atypical pneumonia. Acta Pharmacol Sin 2003; 24(12): 1308-1313

6- Blasi F. Atypical pathogens and respiratory tract infections. Eur Respir J 2004; 24: 171-181.

7- Gerald C, Barrie M et al. Mackie and McCartney Practical Medical Microbiology. 14th Ed. Tim Horne. 1996.

8- File TM ,Tan JS, Plouffe JF. The role of atypical pathogens: *Mycoplasma pneumoniae, Chlamydia pneumoniae, and Legionella pneumophila* in respiratory infection. Infect Dis Clin North Am 1998; 12: 569–592.

9- Topley W, Wilson S. Topley and Wilson's Microbiology and Microbial Infections. 10th Ed. Hodder and Arnold , UK, 2008.

10- Ilana P,David L. Diagnosis and Treatment of Ventilator-associated pneumonia. Chest 2006;130;597-604.

11- Miller PR, Johnson JC, III, Karchmer T, et al. National nosocomial infection surveillance

system: from benchmark to bedside in trauma patients. J Trauma 2006; 60:98–103.
12- Luyt C-E, Chastre J, Fagon J-Y. Value of the clinical pulmonary infection score for the identification and management of ventilator-associated pneumonia. Intensive Care Med 2004; 30:844–852.
13- Schurink CAM, Van Nieuwenhoven CA, Jacobs JA, et al. Clinical pulmonary infection score for ventilator-associated pneumonia: accuracy and inter-observer variability. Intensive Care Med 2004; 30:217–224.
14- American Thoracic Society, Infectious Diseases Society of America. Guidelines for the management of adults with hospital-acquired, ventilator-associated, and healthcare-associated pneumonia. Am J Respir Crit Care Med 2005; 171: 388–416.
15- Blot F, Raynard B, Chachaty E, et al. Value of Gram stain examination of lower respiratory tract secretions for early diagnosis of nosocomial pneumonia. Am J Respir Crit Care Med 2000; 162:1731–1737.
16- Miller PR, Meredith JW, Chang MC. Optimal threshold for diagnosis of ventilator-associated pneumonia using bronchoalveolar lavage. J Trauma 2003; 55:263–268.
17- Ioanas M, Ferrer R, Angrill J, et al . Microbial investigation in ventilator-associated pneumonia . Eur Respir J 2001; 17:791-801.
18- Kristopher P, Anthony J. Atypical Pathogens and Challenges in Community-Acquired Pneumonia American Academy of Family Physicians 2004. Alan Smyth. Pneumonia due to viral and atypical organisms and their sequelae. British Medical Bulletin 2002;61: 247–262.
19- Betty F, Daniel S, et al. Bailey and Scott's Diagnostic Microbiology. 12th Ed. Mosby Elsevier. 2007.
20- Gerald C, Barrie M et al. Mackie and McCartney Practical Medical Microbiology. 14th Ed. Tim Horne. 1996.
21- Bedewy KML, Elsayed MNE, Mahmoud MI.Ventilator-Associated pneumonia: serum TNF∞ and determination of the role of some atypical bacterial pathogens by PCR. EJJM 2001; 10:27-41.
22- Bedewy KML, Ibrahim YN, Abdelfattah MA. Serum RANTES and TNF∞ levels in patients with acute coronary events: correlation with *Chlamydia pneumoniae* infection. EJMM 2003; 12: 33-46.
23- Jennifer M , Martin A. Expert Opinion on Pharmacotherapy. Pharma 2009: 10(9): 1461-1467.
24- David R. The Microbiology of Ventilator-Associated Pneumonia. Respir Care 2005; 50(6):742–763
25- Luna CM, Videla A, Mattera J, et al. Blood cultures have limited value in predicting severity of illness and as a diagnostic tool in ventilator-associated pneumonia. Chest 1999; 116:1075–1084.
26- El-Ebiary M, Torres A, González J, et al. Quantitative cultures of endotracheal aspirates for the diagnosis of ventilator-associated pneumonia. Am J Respir Crit Care Med 1993;147:1552–1557
27- Maha M, Fatma A, Tharwat E. Diagnostic value of bronchoalveolar lavage in ventilator associated pneumonia of pediatric patients. Egypt J Med Lab Sci, 2008; 17(1):11-25.
28- Hassan A, Samy A, Bedewy K, et al. VAP: The role of typical bacteria and atypical bacteria and Fungi.Comparison between endotracheal aspirates and bronchoalveolar lavage.. EJB, 2007;1(1)1: 53-61.
29- Fabrice Ml, Bruno F, Pierre B. Early Antibiotic Treatment for BAL-Confirmed Ventilator-Associated Pneumonia: A Role for Routine Endotracheal Aspirate Cultures. Chest 2005;127;589-597
30- Emine A, Andreas V. Ventilator associated pneumonia and infection control. Ann Clin Microb Antimicrob 2006, 5:7.
31- Initial Antibiotic Choice Important in Ventilator-Associated Pneumonia. AvailableURL: http://www.medpagetoday.com/MeetingCoverage/SCCM/8257
32- Combes A, Corionne F, Jean Louis T, et al. Incidence and Outcome of Polymicrobial Ventilator-Associated Pneumonia. Chest 2002; 121: 1618-1623.
33- Chien Liang W, Yang D, Wang N, Tah Kuo H, et al. Quantitative Culture of Endotracheal Ventilator-Associated Pneumonia in Patients Aspirates in the Diagnosis of With Treatment Failure. Chest 2002;122;662-668.
34- Chastre J, Fagon JY. Ventilator associated pneumonia. Am J Respir Crit Care Med 2002; 165: 867-903.
35- Bachinskaya N, Beloborodova N, Nonikov E. Efficacy of cefepime plus clarithromycin versus cefepime alone in patients with ventilator-associated pneumonia after cardiac surgery: prospective clinical trial. 16th European Congress of Clinical Microbiology and Infectious Diseases Nice, France, April 2006.

36- Apfalter, Petra , Stoiser ,et al. Community-acquired bacteria frequently detected by means of quantitative polymerase chain reaction in nosocomial early-onset ventilator-associated pneumonia. Crit Care Med 2005 ;33(7):1492-1498.

37- El-Ebiary M, Torres A, Gonzalez J, et al. Quantitative cultures of endotracheal aspirates for the diagnosis of ventilator associated pneumonia. Am Rev Respir Dis 1993; 148: 1552-7.

11/11/2010

The neuroprotection role of heat shock protein 70 (HSP70) against microwave radiation induced DNA damage in male Wistar rat brain

Magda Mohamed El-Ezabi

Department of Zoology – Faculty of Science – Benha University- Egypt
E-mail address:dr.rosa@hotmail.com

Abstract : This investigation aims to study the effect of radiofrequency (RF) radiation on DNA damage in brain cells of male Wistar rats using the comet assay and to investigate the role of HSP70 as a protective molecular chaperone that increases stress tolerance of brain cells. Male Wistar rats (118±20g) were divided into three groups. The 1st (exposed) group was subdivided into three subgroups and exposed for 15 min to activated cell phone emitting a frequency radiation of 900 MHz, at non-thermal specific absorption rate (SAR) of 2.9×10^{-3} W/Kg. The 2nd (exposed) group was also subdivided into three subgroups but was exposed for 30 min to the cell phone. The third group was the sham-exposed (control). Animals in each group were sacrificed after 1, 3 and 7 days recovery period. The comet assay parameters showed significantly increased DNA damage in brain cells after 1 and 7 days in the first group and after 7 days in the second group. The HSP70 showed significantly increased levels after 7 days in both exposure groups. Meanwhile, HSP70 showed significantly decreased levels after 1 day in the second group. The results of the present study demonstrate a damaging effect of RF radiation on DNA of the brain cells. This damaging effect initially inhibits the synthesis of HSP70; But after a 7 day recovery period, the levels of HSP70 increase significantly possibly due to powerful capacity of the cells for recovery.
[Magda Mohamed El-Ezabi. **The neuroprotection role of heat shock protein 70 (HSP70) against microwave radiation induced DNA damage in male Wistar rat brain**. Journal of American Science 2010;6(12):1429-1435]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: comet assay, DNA damage, brain cells, heat shock protein 70, radiofrequency radiation

Introduction

The cellular response to stress is represented at the molecular level by the induced synthesis of a set of highly conserved proteins, heat shock proteins (HSPs) (Park et al., 2000) which protect cells and organisms against oxidative stress and often prevent cell death. While prolonged exposure to conditions of extreme stress is harmful, and can lead to cell and tissue death, induction of HSP synthesis can result in stress tolerance and cytoprotection. The cytoprotective effect of HSPs has been attributed to one of the major HSPs, HSP70 (Lee et al., 2001) that is particularly relevant as it is the major protein induced transcriptionally by various forms of stress including lethal heat shock, anoxia, heavy metals, ionizing and non-ionizing electromagnetic radiation (Santoro, 2000, Calini et al., 2003).

In recent years, there has been growing concerns about the potential effects of radiofrequency electromagnetic fields (RF-EMFs; 10MHz—300GHz) on human health, especially the influence on DNA damage (Panagopoulos et al., 2007, 2010; Yan et al., 2007; DeIuliis et al., 2009), because of the wide use of mobile phones. The proximal distance of mobile phone to the head has raised anxieties about the biologic effects of microwave radiation (300MHz – 300 GHz) on the brain (Lai and Singh, 2005; Paulraj and Behari, 2006; Luukkonen et al., 2009). It has been demonstrated that mobile phones affect neural function in human. These effects range from changes in the permeability of the blood brain barrier (Grigor'ev, 2005; Nittby et al., 2009) to changes in electroencephalogram (EEG) pattern (Croft et al., 2002) suggesting that exposure to an active cell phone affects the resting EEG in humans. Functions such as sleep (Hamblin and Wood, 2002), attention (Lee et al., 2001) or learning and memory (Koivisto et al., 2000) have been also shown to be ″mobile phone sensitive″. Cell phone exposure may also lead to brain cancer and that link is via the heat shock response (French et al., 2000).

Although many studies have reported that RF-EMF does not induce genotoxic effects (Maes et al., 2001; McNamee et al., 2003; Scarfi et al., 2006; Zeni et al., 2008), several independent studies (Sykes et al., 2001; Baohong et al., 2005; Lixia et al., 2006; Yao et al., 2008; Luukkonen et al., 2009), have provided evidence for DNA damaging alterations. Since RF-microwave EMF is non-ionizing, it is not considered to induce direct alterations in DNA, the mechanism is unclear. Previous studies have shown that RF-EMF increased the formation of reactive oxygen species (ROS) which have been shown to induce DNA damage (Moustafa et al., 2001; Stopczyk et al., 2005; Yao et al., 2008). Oxygen free radicals may play a role in mechanism of biological effects induced by RF-EMF. In aerobic cells, ROS

are generated as a by-product of normal mitochondrial activity. If not properly controlled, ROS can cause severe damage to cellular macromolecules, especially DNA (Barzilai and Yamamoto, 2004). There may be some association between the overproduction of ROS and DNA damage induced by RF-EMF.

The comet assay is considered a sensitive assay for detecting DNA single strand breaks, double strand breaks, alkali labile sites, incomplete excision and repair sites (Fairbairn et al., 1995). So, the present study aims to investigate whether RF-EMF induces DNA damage in male Wistar rat brain. The comet assay was used to measure DNA damage after two different exposures to GSM radiation (15 and 30 min) and the animals were sacrificed after a recovery period of 1, 3 and 7 days. The stress response to RF-EMF was also determined by measuring the levels of HSP70 in male Wistar rat brain.

Materials and Methods

Animals

Male Wistar rats weighing about 118±20g were obtained from Helwan Farm for Vaccine and Biological Preparations. The animals were housed in cages 5 animals in each cage in the laboratory for one week before the beginning of the experiment. The animals were maintained on 12h dark/light cycle and were given food and water *ad libitum*.

Experimental design

After the acclimation period, animals were randomly divided into two exposed groups and one sham-exposed (control) group:

Group 1: exposed 15 min (n=15).
Group 2: exposed 30 min. (n=15).
Group 3: sham-exposed (control) (n=15).

Animals in groups 1 and 2 were kept for a recovery period of 1, 3 and 7 days (with 5 animals in each) after which they were sacrificed.

Method of exposure

During irradiation, each animal was placed in its own restrainer rocket Plexiglas (15 cm length, 6 cm diameter) and a cone (3 cm length) in which the rat inserted its head. A cell phone in the ″on″ mode (spiking mode) was placed against the cone directly above the rat́s head. The end of the cone was opened and holes were made in the rocket to facilitate breathing and minimize body temperature elevation. A Plexiglas disk was placed at the back to prevent the rat from backing out of the rocket. The cell phone was manufactured by Nokia (model 6300 type RM-217, GSM 900MHz, SAR 1.6 W/Kg) in the “on” mode (spiking mode) was placed with its antenna above the head of the rat . Control animals were treated identically as the exposed ones; but the cell phone is ´switched off˝ during of the sham-exposed.

Preparation of brain samples

Rats from each group were anesthetized by anesthetic ether and then decapitated and their brains were dissected out. Whole brain was washed three times with phosphate buffer solution (PBS: NaCl, 8.0g, KCl, 0.2g, $Na_2HPO_4.12\ H_2O$ 2.8g, $KH_2PO_4$0.2g, pH 7.4), cut into pieces with stainless steel scissors, homogenized with the appropriate amount of PBS at 4ºC and pH 7.4 in a glass homogenizer at 0-4ºC, and then sifted through a 300-µm sieve. The slides were previously stained by trypan blue (3,3'-{[3,3'-Dimethyl(1,1'-biphenyl)-4,4'-diyl]bis(azo)}bis(5-amino-4-hydroxy)-2,7-naphthalenedisulfonic acid, tetra-sodium salt), and under a microscope up to 90% of the brain cells were found to be alive. The cells were resuspended at approximately 10^6 cells per ml in PBS and then used immediately for comet assay.

The comet assay

To measure the potential DNA damaging effect of microwave radiation in Wistar rat brain, the comet assay was carried out as described by Ge *et al.* (2005) with some modifications. At least 100 cells per slide subjected were analyzed (original magnification ^{x}200) under a fluorescent microscope (BX51, Olympus) equipped with a green light excitation and at 590-nm barrier filter. Comets form as the broken ends of a negatively charged DNA molecule becomes free to migrate in the electric field toward the anode. For each cell, the length of DNA migration (comet tail length) was measured in micrometers from the center of nucleus to the end of the tail. The percentage of damaged DNA concentration in the comet tail was determined by measuring the total intensity of ethidium bromide fluroscence in the cells, which was taken as 100% and determining what percentage of this total intensity correspond to the intensity measured only in the tail.

Determination of HSP70 levels

The levels of HSP70 (pg/ml) in the brain samples were determined according to the method described by Oc et al. (2008) using ELISA Kit (DUOSEt®IC, US).

Statistical Analysis

Data were expressed as a mean ± standard error (SE). Differences between the control and treated groups were tested using Student's t-test with the help of statistical software origin 7.5. Differences between control and exposed animals were considered statistically significant when $P< 0.05$.

Results

Table (1) shows the mean tail length and the mean % of damaged DNA (comet assay parameters) performed on rat brain cells after exposure to 900 MHz of RF radiation for 15 min and sacrificed after 1, 3 and 7 days recovery period. Mean tail length and mean % of damaged DNA showed significantly (P<0.05) increased levels after 1 and 7 days of the recovery period as compared to the control animals. No significant differences in the comet assay parameters were observed after 3 days of the recovery period.

After 7 days of the recovery period, the mean levels of HSP70 showed significantly (P<0.05) increased levels (Table.1). Meanwhile, HSP70 levels showed non significant decreased levels after 1 and 3 days recovery period as compared to the control group.

The data of the comet assay parameters (mean tail length and mean % of DNA damage) performed on rat brain cells after exposure to 900 MHz microwaves for 30 min and sacrificed after 1, 3 and 7 days recovery period are summarized in table (2). As shown in the table, there were non significant increased levels of the comet assay parameters after 1 and 3 days of exposure, while after 7 days recovery period, the comet assay parameters showed significantly (P<0.05) increased values as compared to the control ones.

Mean levels of HSP70 of rat brain exposed to 900 MHz microwaves for 30 min showed significantly (P<0.05) decreased levels after 1 day recovery period, non significantly decreased levels after 3 days and significantly increased levels after 7 days recovery period as compared to the control group (Table 2).

Table 1. Comet assay parameters (mean tail length and mean % of damaged DNA) and mean HSP70 levels performed on rat brain after exposure for 15 min to 900MHz RF radiation and sacrificed after 1, 3 and 7 days recovery period as compared to the control group.

Parameter	control	1 day	3 days	7 days
TL (μm)	1.585±0.22	4.632±0.90	3.585±0.78	10.233±1.05*
% of damaged DNA	1.270±0.20	3.628±0.42	3.135±0.69	6.063±0.51*
HSP 70 (pg/ml)	204.776±3.62	186.87±16.97	202.399±17.66	302.458±5.28*

Results are means ± SE of 5 animals.
* statistically significant difference between control and exposed group at (P<0.05).
Number of cells (100 cells per each animal).

Table 2. Comet assay parameters (mean tail length and mean % of damaged DNA) and mean HSP70 levels performed on rat brain after exposure for 30 min to 900MHz RF radiation and sacrificed after 1,3 and 7 days recovery period as compared to the control group.

Parameter	control	1 day	3 days	7 days
TL (μm)	1.642±0.17	3.322±0.73	3.269±1.16	3.126±0.24*
% of damaged DNA	1.333±0.15	2.699±0.64	2.184±0.51	2.495±0.13*
HSP 70 (pg/ml)	1.99.524±1.66	153.856±0.82*	160.098±18.45	207.852±1.72*

Results are means ± SE of 5 animals.
* statistically significant difference between control and exposed group at (P<0.05).
Number of cells (100 cells per each animal).

Discussion

The need for research regarding biological effects of electromagnetic fields is justified by the dramatic increase of RF-EMF sources and therefore, the population exposed during recent years (Zotti-Martelli et al., 2005). Mobile phones emit RF radiation into the heads of their users and the brain is one of the energy absorbing structures in the body. This resulted into a variety of neurological effects such as headaches, change in sleep patterns, modification in the electroencephalogram (EEG) and increase in blood pressure (Ilhan et al., 2004). Because DNA damage is closely related to every aspect of physiological and pathological activity of cells, one of the most active areas of RF radiation investigation is the assessment of direct and indirect effects on DNA (Brusick et al., 1998).

The comet assay is a sensitive method for detecting DNA damage in eukaryotic cells. It has become one of the standard methods for assessing genome damage in genotoxicity tests as well as in fundamental research of DNA damage and repair (Garaj-Vrhovac et al., 2009).

The present study, provides evidence that RF radiation from GSM cell phone exposure results in damaging effect on DNA of the brain cells, with remaining observable effects 7 days after the exposure. The study also shows that 15 min exposure to GSM has no effect on comet assay parameters after 3 days recovery period and after 1 and 3 days recovery period of the exposure time 30 min. These observations suggest that exposure to RF radiation can induce damaging effect on DNA in rat brain. DNA damage is closely related to human risk particularly DNA damage in brain cells could affect neurological functions and possibly lead to neurodegenerative diseases (Lai and Singh, 1996). The exact mechanism by which RF radiation induced DNA damage is still unclear. As is well known reactive oxygen species (ROS) are reactive and readily damage biological molecules, including DNA (Barzilai and Yamamato, 2004). Stopczyk et al. (2005) found that oxidative stress after exposure to microwave may be the reason for many adverse changes in cells. The study of Moustafa et al. (2001), indicated that acute exposure to the RF-EMF of commercially available cellular phones may modulate the oxidative stress of free radicals by enhancing lipid per oxidation and reducing the activation of superoxide dismutase and glutathione peroxidase. A number of studies have indicated that exposure to RF-EMF could lead to DNA damage through free radicals and interaction with transitional metals (e.g: iron) (Zmyslony et al., 2000; Jajte et al., 2001; Lai and Singh 2004; Phillips et al., 2009). Iron have also implicated to play a role in the genotoxic effects of RF-EMF exposure.

Several reports have indicted that EMF enhances free radical activity in cells (Lai and Singh, 2005; Oral et al.; 2006, Simkó, 2007; Phillipis et al., 2009) particularly via the Fenton reaction (Lai and Singh, 2004). The Fenton reaction is a process catalyzed by iron in which hydrogen peroxide, a product of oxidative respiration in the mitochondria, is converted into hydroxyl free radicals which are very potent and cytotoxic molecules. This supports the view that RF-EMF affects DNA via indirect secondary process, since the energy level associated with EMF exposure is not sufficient to cause direct breakage of chemical bonds within molecules, the effects are probably indirect and secondary to other induced biochemical changes in cells.

Since the brain is exposed to rather high levels of EMF during cell phone use, the consequences of EMF induced genetic damage in brain cells are of particular importance. Brain cells have high levels of iron. Special molecular pumps are present on nerve cell nuclear membranes to pump iron into the nucleus. Iron atoms have been found to intercalate within DNA molecules. In addition, nerve cells have a low capacity for DNA repair and DNA breaks could easily accumulate. Another concern is the presence of iron particles in body tissues, particularly in the brain. These particles could enhance free radical activity in cells and thus increase the cellular-damaging effects of EMF. These factors make nerve cells more vulnerable to EMF. Thus, the effect of EMF on DNA could conceivably be more significant on nerve cells than other cell types of the body (Phillips et al., 2009). Since nerve cells do not divide and are not likely to become cancerous, the more likely consequences of DNA damage in nerve cells include changes in cellular functions and in cell death, which could either lead to or accelerate the development of neurodegenerative diseases. Double-strand breaks, If not properly repaired are known to lead to cell death. Cumulative DNA damage in nerve cells of the brain has been associated with neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases. However, another type of brain cell, the glial cell, can become cancerous as a result of DNA damage. Mausset-Bonnefont et al. (2004) showed an increase in the glial reactivity after 3 days recovery period of acute exposure to 900 MHz EMF. The authors concluded this increase in glial reactivity was due to an astrocyte hypertrophy as a result of the effect of microwaves. These observations are in agreement with the results of the present study, since the increased DNA damage was observed after 1 and 7 days of exposure to EMF.

It has been shown that exposure to mobile phone signals can influence cellular processes such as proliferation (French et al., 1997, Velizarov et al.,1999), cell morphology (Donnellan et al.,1997) and the level of heat shock protein expression (Kwee et al.,2001; Leszczynski et al.,2002). The results demonstrated here showed that HSP70 levels decreased 1 and 3 days after 15 and 30 min exposure to mobile phone radiation and then increased significantly after 7 days. This could be due to the possibility that after 1 and 3 days of recovery period, the basal content of HSP70 which protects brain cells from microwave exposure is consumed. Another thing is that some neurons do not appear to express HSP70 during stress.

There are two characteristics that determine a cell's response to a stress factor: (i) its original pre-stress level of HSP70 and (ii) its ability to rapidly accumulate the protein. Nollen et al. (1999) reported that there are basal levels of HSP70 which can protect a cell against harmful conditions without the need for additional synthesis of the protein. Although the protective system based on HSP70 exists in all tissues and organs, some cell types do not appear to express the protein. Among these are certain types of neurons (Sprang and Brown, 1987).

The results of the present study demonstrates three interesting findings: one is the finding of detectable DNA damage after 1 and 7 days from the 15 min exposure and after 7 days from the 30 min exposure. The second is significant decreased levels of HSP70 after 1 day from the 30 min exposure. The third is the significant increased levels of HSP70 after 7 days from both exposure durations. This means that exposure to GSM 900 MHz RF radiation at SAR 2.9×10^{-3} w/Kg induces a transient DNA damage in rat brain. The significant decrease in HSP70 levels after 1 day recovery from the 30 min exposure means that the disrupting action of RF radiation on DNA delayed the induction of this protein. The significant increase in the levels of HSP70 after both exposure durations means that brain cells induced the synthesis of the protein for protecting themselves from DNA damage and thus have a powerful capacity for recovery from damage induced by RF radiation.

In conclusion, this study confirms the existence of genotoxic effect of RF radiation on brain cells, this effect is not statistically significant but it becomes significant 7 days after the exposure. The data presented here show that HSP70 can be induced with exposure of brain cells to a GSM signal of 900MHz at SAR 2.9×10^{-3} W/Kg. But the cells have a powerful capacity for recovery from damage induced by RF radiation. The study also prove the potential for HSP70 to be used directly as cytoprotective agents in wide variety of clinical situations involving neurodegenerative diseases.

Coressponding Author
Magda Mohamed El-Ezabi
Department of Zoology – Faculty of Science – Benha University- Egypt
dr.rosa@hotmail.com

References

1. Baohong, W., Jiliang, H., Lifen, J., Deqiang, L., Wei, Z., Jianlin, L., Hongping, D., 2005. Studying the synergistic damage effects induced by 1.8 GHz radiofrequency field radiation (RFR) with four chemical mutagens on human lymphocyte DNA using comet assay in vitro. Mutat. Res. 578, 149-157.
2. Barzilai, A., Yamamoto, K. 2004. DNA damage responses to oxidative stress. DNA Repair, 3, 1109-1115.
3. Brusick, D., Albertini, R., McRee, D., Peterson, D., Williams, G.,Hanawalt, P., Preston, J. 1998. Genotoxicity of radio-frequency radiation. DNA/Genetox Expert Panel, Environ. Mol. Mutagen 32 (1), 1-16.
4. Calini, V., Urani, C., Camatini, M. 2003. Overexpression of HSP70 is induced by ionizing radiation in C3H10T1/2 cells and protects from DNA damage.Toxicol. in Vitro 17, 561-566.
5. Croft, R. J., Chandler, J. S., Burgess, A. P., Barry, R. J., Williams, J. D., Clarke, A. R. 2002. Acute mobile phone operation affects neural function in humans. Clin. Neurophysiol. 113, 1623-1632.
6. De Iuliis, G.N., Newey, R.J., King, B.V.; Aitken, R.J. 2009. Mobile phone Radiation induces reactive oxygen species production and DNA damage in human spermatozoa in Vitro. Plos one, 4 (7).
7. Donnellan, M., McKenzie, D. R., French, P. W. 1997. Effects of exposure to electromagnetic radiation at 835 MHz on growth, morphology and secretory characteristics of a mast cell analogue, RBL-2H3. Cell Biol. Int. 21, 427-439.
8. Fairbairn, D. W., Olive P.L., O'Neill, K. L. 1995. The comet assay: a comprehensive review. Mut. Res. 339, 37-59.
9. French, P. W., Donnellan, M., McKenzie, D. R. 1997. Electromagnetic radiation at 835 MHz changes the morphology and inhibits proliferation of a human astrocytoma cell line. Bioelectrochem. Bioenerg. 43, 13-18.
10. French, P. W., Penny, R., Laurence, J. 2000. Mobile phones, heat shock proteins and cancer. Differentiation 67, 93-97.

11. Garaj-Vrhovac, V., Gajski, G., Trosic, I., Pavicic, I. 2009. Evaluation of basal DNA damage and oxidative stress in Wistar rat leukocytes after exposure to microwave radiation. Toxicol. 259, 107-112.
12. Ge, Y., Ning, H., Wang, S., Wang, J. 2005. Comet assay of DNA damage in brain cells of adult rats exposed to high fluoride and low iodine. Fluoride, 38 (3), 209-214
13. Grigor'ev, I. U. G. 2005. The electromagnetic fields of cellular phones and the health of children and teenagers (the situation requiring to take an urgent measure). Radiats. Biol. Radioecol. 45, 442-450.
14. Hamblin, D.L., Wood, A.W. 2002. Effects of mobile phone emissions on human brain activity and sleep variables. Int. J. Radiat. Biol. 78, 659-669.
15. Ilhan, A., Gurel, A., Armutcu, F., Kamisli, S., Iraz, M., Akyol, O., Ozen, S. 2004. Ginko biloba prevents mobile phone-induced oxidative stress in rat brain. Clin. Chim. Acta 340, 153-162.
16. Jajte, J., Zmyslony, M., Palus, J., Dziubaltowska, E., Rajkowska, E. 2001.
17. Protective effect of melatonin against In vitro iron ions and 7mT 50 Hz magnetic field-induced DNA damage in rat lymphocytes. Mutat. Res. 483, 57-64.
18. Koivisto, M., Krause, C. M., Revonsuo, A., Laine, M., Hamalainen, H. 2000. The effects of electromagnetic field emitted by GSM phones on working memory. NeuroReport 11, 1641-1643.
19. Kwee, S., Raskmark, P., Velizarov, S. 2001. Changes in cellular proteins due to environmental non-ionizing radiation. 1. Heat shock proteins. Electro Magnetobiol. 20, 165-176.
20. Lai, H., Singh, N. P. 2005. Effects of microwaves and a temporally incoherent magnetic field on single and double DNA strand breaks in rat brain cells. Electromag. Biol. Med. 24, 23-29.
21. Lai, H.; Singh, N. P. 2004. Magnetic-field-induced DNA Strand breaks in brain cells of the rat. Environ. Health Perspect. 112, 687-694.
22. Lai, H., Singh, N. P. 1996. Single and double-strand DNA breaks in rat brain cells after acute exposure to radiofrequency electromagnetic radiation. Int. J. Radiat. Biol. 69, 513-521.
23. Lee, S. J., Choi, S.A., Lee, K. H., Chung, H. Y., Kim, T. H., Cho, C. K., Lee, Y. S. (2001). Role of inducible heat shock protein 70 in radiation-induced cell death. Cell Stress and Chaperones, 6, 273-281.
24. Lee, T. M. C., Ho, S. M. Y., Tsang, L. Y. H., Yang, S.Y. C.; Li, L. S. W., Chan, C. C. H. 2001. Effect on human attention of exposure to the electromagnetic field emitted by mobile phones. NeuroReport 12, 729-731.
25. Leszczynski, D., Joenvaara, S., Reivinen, J., Kuokka, R. 2002. Non-thermal activation of the hsp27/p38MAPK stress pathway by mobile phone radiation in human endothelial cells: Molecular mechanism for cancer-and blood-brain barrier-related effects. Differentiation, 70, 120-129.
26. Lixia, S., Ke, Y., Kaijun, W., Deqiang, L., Huajun, H., Xiangwei, G., Baohong, W., Wei, Z., Jianling, L., Wei, W. 2006. Effects of 1.8 GHz radiofrequency field on DNA damage and expression of heat shock protein 70 in human lens epithelial cells. Mutat. Res. 602, 135-142.
27. Luukkonen, J., Hakulinen, P., Maki-Paakkanen, J., Juutilainen, J., Naarala, J. 2009. Enhancement of chemically induced reactive oxygen species production and DNA damage in human SH-SY5Y neuroblastoma cells by 872 MHz radiofrequency radiation. Mutat. Res. 662, 54-58.
28. Maes, A., Collier, M., Verschaeve, L. 2001. Cytogenetic effects of 900 MHz (GSM) microwaves on human lymphocytes. Bioelectromagnetics 22, 91-96.
29. Mausset-Bonnefont, A-L., Hirbec, H., Bonnefont, X., Privat, A., Vignon, J., de-Seze, R. 2004. Acute exposure to GSM 900-MHz electromagnetic fields induces glial reactivity and biochemical modifications in the rat brain. Neurobiol. Dis. 17, 445-454.
30. McNamee, J.P., Bellier, P. V., Gajda, G. B.; Lavallee, B.F.; Marro, L.; Lemay, E.; Thansandote, A. 2003. No evidence for genotoxic effects from 24 h exposure of human leukocytes to 1.9 GHz radiofrequency fields. Radiat. Res. 159, 693-697.
31. Moustafa, Y. M., Moustafa, R. M., Belacy, A., Abou-El-Ela, S. H., Ali, F. M. 2001. Effects of acute exposure to the radiofrequency fields of cellular phones on plasma lipid peroxide and antioxidase activities in human erythrocytes. J. Pharmaceu. Biomed. Anal. 26, 605-608.
32. Nittby, H., Brun, A., Eberhardt, J., Malmgren, L., Persson, B. R.R., Salford, L. G. 2009. Increased blood-brain barrier permeability in mammalian brain 7 days after exposure to the radiation from a GSM-900 mobile phone. Pathophysiol., 16:103-112.
33. Nollen, E. A., Brunsting, J. F., Roelofsen, H., Weber, L. A., Kampinga, H. H. 1999. In vivo

chaperone activity of heat shock protein 70 and thermotolerance. Mol. Cell Biol. 19, 2069-2079.
34. Oc, M., Ucar, H.I., Pinar, A., Akbulut, B., Oc, B., Akyon, Y., Kanbak, M., Dogan, R. 2008. Heat shock protein 70: a new marker for subsequent atrial fibrillation development? Artif Organs. 32(11), 846-850.
35. Oral, B., Guney, M., Ozguner, F.;, Karahan, N., Mungan, T., Comlekci, S., Cesur, G. 2006. Endometrial apoptosis induced by a 900-MHz mobile phone: preventive effects of vitamins E and C. Adv. Ther. 23, 957-973.
36. Panagopoulos, D.J.; Chavdoula, E.D.; Nezis, I.P.; Margaritis, L.H. 2007.
37. Cell death induced by GSM 900MHz and DCS 1800MHz mobile telephony radiation. Mutat. Res. 626, 69-78.
38. Panagopoulos, D.J.; Chavdoula, E.D.; Margaritis, L.H. 2010. Bio effects of Mobile telephony radiation in relation to its intensity or distance from the antenna. Internation. J. Radia. Biol. 86(5),345-357.
39. Park, S. H.; Lee, S. J.; Chung, H. Y.; Kim, T. H.; Cho, C. K.; Yoo, S. Y.; Lee, Y. S. (2000). Inducible heat-shock protein 70 is involved in the radioadaptive response. Radiat. Res., 153:318-326.
40. Paulraj, R., Behari, J. 2006. Single strand DNA breaks in rat brain cells exposed to microwave radiation. Mutat. Res. 596, 76-80.
41. Phillips, J. L., Singh, N. P., Lai, H. 2009. Electromagnetic fields and DNA damage. Pathophysiol. 16, 79-88.
42. Santoro, M. G. 2000. Heat shock factors and the control of stress response. Biochem. Pharmacol. 59, 55-63.
43. Scarfi, M. R. Fresegna, A. M., Villani, P., Pinto, R., Marino, C., Sarti, M., Altavista, P., Sannino, A., Lovisolo, G. A. 2006. Exposure to radiofrequency radiation (900 MHz, GSM signal) does not affect micronucleus frequency and cell proliferation in human peripheral blood lymphocytes: an interlaboratory study. Radiat. Res. 165, 655-663.
44. Simko, M. 2007. Cell type specific redox status is responsible for diverse electromagnetic field effects. Curr. Med. Chem. 14, 1141-1152.
45. Sprang, G. K., Brown, I. R. 1987. Selective induction of a heat shock gene in fibre tracts and cerebellar neurons of the rabbit brain detected by in situ hybridization. Mol. Brain Res. 3, 89-93.
46. Stopczyk, D., Gnitecki, W., Buczynski, A., Kowalski, W., Buczynska, M., Kroc, A. 2005. Effect of electromagnetic field produced by mobile phones on the activity of superoxide dismutase (SOD-1)-in vitro researches. Ann. Acad. Med. Stetin. 51, 125-128.
47. Sykes, P. J., McCallum, B. D., Bangay, M. J., Hooker, A. M., Morley, A. 2001. Effect of exposure to 900 MHz radiofrequency radiation on intrachromosomal recombination in pkz1 mice. Radiat. Res. 156, 495-502.
48. Velizarov, S. Kwee, S. Raskmark, P. 1999. The effects of radiofrequency fields on cell proliferation are non-thermal. Bioelectrochem. Bioenerg. 48, 177-180.
49. Yan, J.G.; Agresti, M.; Bruce, T.; Yan,Y.H.; Granlund, A.; Matloub, H.S. 2007. Effects of cellular phone emissions on sperm motility in rats. Fertil Steril. 88(4): 957-964.
50. Yao, K., Wu, W., Wang, K., Ni, S., Ye, P., Yu, Y., Ye, J., Sun, L. 2008. Electromagnetic noise inhibits radiofrequency radiation-induced DNA damage and reactive oxygen species increase in human lens epithelial cells. Mol. Vis. 14, 964-969.
51. Zeni, O., Schiavoni, A., Perrotta, A., Forigo, D., Deplano, M., Scarfi, M.R. 2008. Evaluation of genotoxic effects in human leukocytes after in vitro exposure to 1950 MHz UMTS radiofrequency field. Bioelectromagnetics 29, 177-184.
52. Zmyslony, M., Palus, J., Jajte, J., Dziubaltowska, E., Rajkowska, E. 2000. DNA damage in rat lymphocytes treated in vitro with iron cations and exposed to 7 mT magnetic fields (static or 50 Hz). Mutat. Res. 453, 89-96.
53. Zotti-Martelli, L., Peccatori, M., Maggini, V., Ballardin, M., Barale R. 2005. Individual responsiveness to induction of micronuclei in human lymphocytes after exposure in vitro to 1800-MHz microwave radiation. Mutat. Res. 582, 42-52.

11/11/2010

Perception Of Student Nurses Towards The Use Of Portfolio In A Faculty Of Nursing

*Salwa S. Kaml and **Nagwa R. Attia

***Assistant Professor Medical Surgical Nursing, Faculty of Nursing, Ain Shams University**
****Lecturer Medical Surgical Nursing, Faculty of Nursing Tanta University**

Sal232@hotmail.com

Abstract: The use of portfolio for learning and assessment within nursing education has recently increased. The main purposes of developing a portfolio is to link understanding about clinical experiences and theoretical knowledge, promotion of student-centered learning and reflective learning. It is important that students developing a portfolio understand the process. Unless portfolio is perceived by students to be relevant and useful, they will not be committed to using portfolios to their full potential. This paper aimed to identify perception of student nurses towards the use of portfolio and to compare the perception of first and second year student nurses towards the use of portfolio. The sample of the study composed of 376 first and second year students studying medical surgical nursing, in a faculty of nursing. Students were asked to respond voluntary to portfolio perception questionnaire, which was developed by researchers. The results of the present showed that students stated that portfolio encourage their independent learning, understanding and utilization of basic concepts. The results showed also some discrepancies between first and second year students in their perceptions toward portfolio. Students expressed how the portfolio process could be improved and they recommended the continued use of portfolio in subsequent study years.

[Salwa S. Kamal and Nagwa R. Attia. **Perception Of Student Nurses Towards The Use Of Portfolio In A Faculty Of Nursing**. Journal of American Science 2010;6(12):1436-1446]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Portfolio, Perception, Nursing education.

1. Introduction:

Professionals, such as architects, engineers, and graphic artists, have long used portfolios as authentic evidence of their professional accomplishments (Kear and Bear, 2007)[1]. In recent years, the use of portfolios as learning and assessment tools has become more widespread across the range of health professions (Buckley et al, 2009)[2].

There is evidence within the literature of an increasing use of portfolios in nursing education in North America (Tracy et al, 2000, and Robertson et al, 2004)[3,4], Australia (Emden et al, 2003)[5] and United Kingdom (Buckley et al, 2009)[2], in pharmacy education (Plaza et al., 2007)[6], and in engineering education (Saker and Hu, 2006)[7].

McMullan et al (2003)[8] stated that, a portfolio is a collection of evidence of both the products and process of learning that attests to achievement and personal and professional development. Portfolio-based learning is an interactive process. Each student identifies learning experiences, integrates new knowledge into practice, identifies areas for improvement or personal growth, and envisions a plan for meeting further learning needs. Thus the portfolio is a qualitative and interpretive record of learning that is amenable to continuous assessment (Bell, 2001)[9].

A comprehensive literature review into the use of portfolios as a tool for assessing competence in nursing has been carried out by McMullan et al (2003)[8]. They identified that portfolios are being used for a variety of theoretical and clinical reasons. These include using them to assess clinical competence; to reduce the theory practice gap; monitor student learning over a period of time; and for personal and professional development.

Morgan (1999)[10] identified different types of portfolio based on the National Education Association definition that "a portfolio is a record of learning that focuses on student's work and his/her reflection on that work". These included 'assessment portfolios' documenting student learning on specific curriculum outcomes and 'skills area portfolios' demonstrating acquired skills in specific areas such as problem solving Gannon et al (2001)[11] point out that in preregistration nursing the portfolio of evidence must enable the student to demonstrate that they are fit to practice as registered nurses.

Items typically included in a comprehensive portfolio are reflective writings, samples of work and written evaluations. Both cognitive and affective

learning are demonstrated as the student briefly describes the experience, applies the pertinent concepts, and then offers personal thoughts on the transaction (Kear and Bear, 2007)[1].

For the portfolio process to be successful, it is vital that students own their work (Challis, 1999)[12]. For this to happen, students need to invest time and effort into the portfolio process and to assume responsibility for the content and direction of their work (Harris et al, 2001)[13]. In this way, they learn to decide for themselves, which aspects to include in their portfolio. This is consistent with Knowles (1975)[14] concept of adult learning and with life long and self directed learning (Brown, 2002)[15].

The educational value of portfolios is accepted with regard to the promotion of student-centered learning deep learning and reflective learning (Davis et al, 2009)[16]. The potential of portfolios to drive student learning in an educationally desirable direction and the importance of identifying individual strengths and weaknesses with regard to limits of competence as part of professional clinical practice may be some of the reasons for the current wave of enthusiasm for portfolios in the health care professions (Challis, 2001)[17].

According to Saker and Hu (2006)[7] portfolios emphasize the process of learning and the learners' comments on their success in meeting the learning outcomes. Thus students' confidence and motivation are enhanced as they reflect upon their improvements. By enabling students to assess themselves, they begin to take responsibility for their own learning. Portfolio learning has the potential advantage of encouraging students to draw on a wide range of sources of evidence to demonstrate their competence; that is demonstrating their academic and clinical learning outcomes.

There are several studies in both higher education (Zeichmer and Wray, 2001)[18] and nursing education (Dolan et al, 2004)[19] that have identified that, unless portfolio is perceived by students to be relevant and useful, they will not be committed to using portfolios to their full potential.

Significance of the study:

In today's challenging health care environment, nurses must be able to clearly articulate what they do. The development of clearly defined outcomes and identification to measure nurse's competencies is critical to the nursing education. So, it is an important challenge to prepare nursing students to meet and document competencies desirable for nurses practicing in the 21st century.

The use of portfolios in nursing education could help students to promote lifelong learning and professional goal achievement (Bell, 2001)[9].

Internationally, portfolios are used in nurse education, there has been researches into how portfolios are perceived, understood and used by student nurses (McMullan, 2008; Williams et al, 2009)[20,21]. Students should perceive portfolio to be relevant and useful to be able to use in their professional development, therefore the aims of this study is to identify and compare the perception of first and second year nursing students. In Egypt, the researchers did not find any published researches concerning portfolios in nursing education during the period of 2000-2007. This study is an attempt to start researching in this important issue.

Aim of the study:

The aims of this study were to identify and compare the perception of first and second year student nurses towards the use of portfolio.

Research questions:

1. What is the perception of student nurses toward the use of portfolio?
2. Are there any differences in the perception of first and second year student nurses towards the use of portfolios?

2. Subjects and Methods:

Study design:

A descriptive comparative research design was utilized in this study.

Setting:

This study was conducted in the Faculty of Nursing Tanta University in Egypt.

Sampling:

The eligible sample for this study was 709 first and second year student nurses. The time table when students were in classroom settings provided the researchers with a potential sample of 400 students in two classroom settings in the period of data collection. In total 376 students completed the questionnaire, which gave us a response rate of 94% and representation of 53% of the total pre-registration student population. We compared the demographic statistics with the portfolios of the 709 eligible students and found that the present research sample of students is representative across gender and age. Slightly more than half (52%) of the students (195) were in their first year and (48%) in the second year (181).

Tools:

A students portfolio perception questionnaire was specifically developed for this study constructed by the researchers dependent on the review of the literature. The questionnaire composed of three parts:

Part I: Used to gather students perception toward building of portfolio. Composed of six statements related to the students' perception of receiving clear complete portfolio guidelines, its importance, frequent teachers feedback, searching necessary information, participation by students to build portfolio and monitoring of student progress.

Part II: Included data about students perception toward portfolio as a learning activity. Composed of three statements, that portfolio helped them to improve their understanding and utilization of basic concepts as problem solving and communication skills, statement two concerned with portfolio encouraged students to be responsible for their independent learning, theory-practice link, decision making, using information technology and self-assessment. Statement three related to portfolio reflects the students' knowledge activities and skills.

Part III: Concerned with data about students' perception toward usefulness of portfolio compared with other teaching/learning methods as clinical placement, academic assignments, classroom based teaching/learning and usefulness of portfolio for personal life and professional career development.

Students were asked to rate the statements in part I, II and III using a 3-point Likert scale where 1=disagree, 2= neither and 3=agree.

In addition, students were asked to add hand written open comments toward portfolio.

Tool development:

The students portfolio perception questionnaire was tested for content and face validity by seven assistant professors and lecturers in the fields of Medical Surgical Nursing, education and psychology at the Faculty of Nursing and Faculty of Arts. All experts were affiliated to Ain Shams University, Cairo, Egypt. Modifications were done accordingly to ascertain relevance and completeness.

Ethical consideration:

Ethical approval was given by the Dean of Nursing Faculty, Tanta University. Students were verbally informed about the purpose and procedure of the study. The voluntary nature of their participation and that anonymity and confidentiality would be maintained when the questionnaires were distributed. This was reinforced in the front page of the questionnaire. No individuals were identified in the study.

Pilot study:

The pilot study was conducted on 10% of the sample to evaluate the developed tool for clarity; applicability and then necessary modifications were carried out. The data obtained from the pilot study were not included in the actual study.

Procedure:

The questionnaires were handed to the students studying Medical Surgical Nursing during classroom session identified previously at the end of the semester. The questionnaire took nearly fifteen to twenty minutes to be completed. Students were asked to complete the questionnaire at the beginning of the session data collected during April-May 2008.

Statistical analysis:

Results were collected and tabulated. Statistical analysis was done using statistical package for social science (SPSS version 13). Chi-square test (X^2) was used for comparison between two groups regarding qualitative data. Results were considered significant at $p \leq 0.05$.

The context of the study:

The study reported here was developed specifically to identify student's perception towards the use of portfolio within a Faculty of Nursing, Tanta University. Portfolio was introduced into the pre-registration-nursing curriculum in this faculty of nursing two years ago when the nursing curriculum was reviewed.

It was anticipated that as portfolio was introduced into the curriculum that over time students demonstrate an increasing ability to effectively link between theory and practice during the course of the study, to be self independent learner, and to achieve clinical learning outcomes. The evidence for these achievements is recorded in their practice record which is assessed at the end of each clinical placement by the registered nurse acting as their

clinical instructor during their placement. The clinical instructor is responsible for assessing the student's level of clinical competence and enabling the students to achieve their clinical learning outcomes. An academic lecturer in a Faculty of nursing also has responsibilities in terms of portfolio building and development. The academic lecturer assures that a clinical instructor has evaluated the student's achievement of clinical learning outcomes and reviews the content and structure of the student's portfolio to ensure that it reflects ongoing professional development.

The components of the portfolio include patient assessment sheet, planning and practice recording, student evaluation sheet, assignments, case study, activities and projects. Portfolio component is summative assessed by the clinical instructor and verified by the lecturer to ensure student achievement of expected learning outcomes.

Guidelines for building of portfolio:

The guidelines were discussed and handed to students at the beginning of the semester to help students build their portfolios. Two main features of the guidelines were selection of content and reflection on learning, as the following:

- Selection of contents, the following points was suggested for consideration of items to be included in portfolio.
  - Re-reading the course syllabus focusing on the learning outcomes.
  - Select the written works that demonstrate best work.
- Reflection.
  - The purpose of portfolio.
  - What are the activities performed to achieve learning outcomes?
  - What skills were developed to meet requirements of the course?
  - How the knowledge and skills acquired during the course could be applied in their personal life and professional career development?

3. Results:

Table (1) showed nursing students' perception regarding the building of portfolio. The majority of the students in group I agreed that they receive clear and complete portfolio guidelines (purpose, learning outcomes) and important to them to receive it 98%, 95%, 90, 87% respectively, while for the second year nursing students (group II) the percentage of agreement were decreased for the same items as 81%, 70%, 71%, 35% respectively (X^2 = 30. 83 , X^2 =42.40* , X^2 =24.80* , X^2 =117.45* at P level 0.0001) respectively.

The same table illustrated that nearly all first year students (92%) and less than half of the second year students (48%) stated that frequent teacher's feedback is important during the semester. The majority of students in group I (80.5%) and less than half of the students in group two (43%) stated that portfolio encourages them to search necessary information by themselves. Students in first and second group verbated that they participated in the building of portfolio (85%, 57%) respectively. Also, students in first and second group stated that portfolio helped them to monitor their progress (88%, 58%) respectively (X^2 = 88.88*, X^2 =68.16*, X^2 =38.56*, X^2 =45.99* at P level 0.0001) respectively.

Table (2) regarding to the role of portfolios as a learning activity. The students in group I and II stated that portfolio helps them to improve understanding and utilization of problem solving and communication skills. 93%, 94%, 56%, 59% respectively at significance (X^2 = 67.89*, X^2=69.60*, at P level 0.0001) respectively. The same table showed that students in group I and II stated that portfolio encourages them to be responsible for independent learning, theory practice link, decision making, using information technology and self assessment. 89%, 91%, 92%, 70%, 91%, 62%, 58%, 48%, 48% and 59% respectively at significance (X^2 =41.50*, X^2 = 55.45*, X^2 =88.51*, X^2 =22.78*, X^2 =55.50* at P level 0.0001) respectively. The majority of students in group I and less than half of students in group II reported that portfolio help them in their personal life and professional career 79%, 44% respectively (X^2 =48.42 at P level 0.0001).

Table (3) illustrated that the nursing students in group I agreed that building a portfolio is the most useful learning activity followed by classroom based teaching/learning, academic assignments and clinical placements representing 89%, 87%, 72% and 69% respectively X^2= 39.88*, X^2 =54.20*, X^2 =18.67* and X^2 = 8.04*. While the students in group II agreed that the portfolio building is the most useful learning activity followed by clinical placements, classroom based teaching/learning: 62%, 55%, 53%, and 51% respectively. The same table showed that portfolios are useful for career development as stated by nursing students in group I and group II: 94%, 79% respectively (X^2 =19.44* at p level 0.0001).

Table (4) showed the mean scores of nursing students' perceptions toward portfolio. It was apparent that students in both groups perceive portfolio as a learning activity followed by building portfolio and then its usefulness (t=15.43; 12.01; 7.89, respectively at p level 0.0001).

Table (5) showed the students comments toward portfolio. Students in group I and II stated some negative comments as too much paperwork and time consuming, little emphasis and interfere with clinical practice, little guidance and difficult in writing and not certain what to include: 69%, 15%, 3%; 30%, 56%, 27% respectively. Some students (18%) in group I required teachers to provide weekly portfolio ungraded feedback during the semester. Some positive comments were given only by students in group I as portfolios help them to be confident to learn independently, reflects their abilities, knowledge, skills and improves students' communication and with the faculty staff: 21%, 18%, 8% respectively. The same table indicated that nearly more than half (51%) of the students in group I recommended to continue use of portfolio in subsequent years.

4. Discussion:

Portfolio-based learning is an active learning strategy that is individualized, learner centered, outcome oriented, and promotes contextual learning and valuing learning. Portfolios provide a mechanism for documenting the application of knowledge in practice (Kear and Bear, 2007)[1].

It was essential to consider the guidelines provided to build a portfolio as important, particularly if the portfolio is being assessed. It can be seen that nearly all first year students and majority of second year nursing students receive complete and clear portfolio guidelines. However, there, was some confusion among the second year students, although this was not their first experience building a portfolio. This was reinforced by responses to the open-ended questions, "little guidelines on how to make the portfolio; not certain what to include".

This is in accordance with the study done by Saker and Hu (2006)[7] in that students stated in one of the responses to the open-ended questions, "give more on how to make the portfolio". Students need to be given clear guidelines about the purpose, content and structure of portfolio to balance the open-ended nature of its content and structure (Gannon et al, 2001; McMullan et al, 2003)[11, 8].

In contrast the findings of the present study Dolan et al (2004)[19] found that the majority of preregistration nursing students surveyed at their university rarely discussed the contents of their portfolio with their academic tutors. They speculate that this may have occurred because the portfolio was not summatively assessed.

As stated by Nairn et al (2006)[22] in their study to examine the knowledge, skills and attitudes of student nurses about the value and purpose of portfolio, they concluded that nursing students believed that academic tutors did not make the purpose of a portfolio clear and more than half of students did not consider that the content of portfolio was sufficiently explained. McMullan (2006)[23] concluded that portfolios can be very effective as an assessment and learning tool, but it is essential that both students and mentors receive clear guidelines on and comprehensive support with their use.

The present study showed that feedback was considered as important factor in building and using a portfolio, it was provided by clinical instructor once, in the middle of the semester. It was suggested by students in open-ended questions that portfolio be assessed weekly to encourage students to use it more seriously and efficiently. However, weekly assessment might not be practical in many cases because it would significantly increase the workload of the instructors, especially in a large class. The frequency of the feedback on portfolio needs to be based on factors such as number of students, weight of portfolio in the overall assessment and instructor's workload.

The present study results showed that the majority of first year students and more than half of second year students agreed that portfolio building helped them better understand and apply concepts as communication and problem solving. In contrast to this result, Nairn et al (2006)[22] found that students did not generally consider portfolios useful for developing their communication skills. Despite the portfolio being one way to communicate learning from academic and clinical activities.

The present study results showed that portfolio encouraged students to be responsible for their own learning, theory practice link, using information technology, self assessment and reflecting on their learning activities. In general, the students' responses support the use of a portfolio for better understanding of the concepts keeping track of learning progress through reflection. This supports the findings of Tillema and Smith (2000)[24], that portfolio assist reflection of the learning process by focusing students' attention on the unit outcomes.

Portfolios are commonly used in nursing education, both as a tool for reflective learning and as an innovative way of documenting student learning and evaluating clinical competence (McMullan et al, 2003)[8].

The present study results were supported by McMullan (2008)[20] who conducted a study to obtain nursing students' perception on using portfolio for their clinical practice learning and assessments. Students stated that portfolios helped them in their development of self-awareness and independent learning.

On the same line, Bukley et al (2009)[2] concluded that, studies reported direct measurement of changes in student skills or attitudes, the main benefits of portfolio use identified as improvement in student knowledge and understanding, greater self-awareness encouragement to reflection and the ability to learn independently. While, on the contrary to the results of the present study, the same author indicated that students found portfolios do not sufficiently address the assessment of their clinical skills and the integration of theory and practice.

The present study comprehensively documented that the process of building a portfolio increases students' reflective abilities. This is in agreement with Syndre et al (1998)[25] and Klenowski (2002)[26] as they indicated that reflection is made during the portfolio process because there is a requirement that students document knowledge and clinical practice as they co-evolve. To do this students require specific teaching and support to develop the cognitive processes of critical reflection, self-learning and assessment, which will help them to develop this work of portfolio. The teacher's role as a facilitator is vital here to provide support and regular feedback. This develops the student's confidence, independence and ownership of the process (McMullan et al, 2003)[8] and has positive effects on students' perceptions toward portfolio learning (Klenowski, 2002)[26].

In a study done by Davis et al (2009)[16] to identify students perception to the portfolio assessment, they concluded that students perceived that portfolio construction increased their understanding of the learning outcomes and enabled reflection on their work. Student reactions to the portfolio process were initially negative, although they become familiar with their work over time.

It was possible that student perceptions towards portfolios could influence the degree with which they embrace or reject the portfolio as a method for effective learning. To see the level of positive or negative perceptions towards portfolio some items evaluated the perceptions that students held towards portfolios as an important approach to teaching and learning compared to other methods and as being useful for career development.

The present study results indicated that portfolios were seen as more useful activity than other teaching and learning activities for both first and second year nursing students. Also the majority of first year nursing students and nearly half of second year nursing students found that classroom based teaching and learning were useful to them. Clinical placements were seen as a least useful activity by the first year nursing students, whereas, academic assignments were seen as a least useful activity by the second year nursing students.

On the contrary, Nairn et al (2006)[22] reported that nursing students did not see portfolios as more useful than other teaching and learning activities. Moreover (91%) nursing students found teaching and learning within clinical placements useful compared to (81%) of students having a positive attitude towards classroom based teaching and learning.

As related to the perception of nursing students towards portfolios as being useful for career development. Nearly all first year students and the majority of second year students agreed that portfolios were useful for personal life and professional career development. It could be concluded that the perceptions about portfolios as facilitating career development are maintained throughout the first and second years. McMullan (2006)[23] stated that portfolios encourages both personal and professional career development through the process of reflective practice and critical analysis.

The students in the present study found that portfolio building interfered with clinical learning. The students' mentioned that there was too much paperwork in the portfolio process and it was time consuming. In the same line McMullan (2006)[23] the students reported that portfolios were very time consuming, causing them a great deal of anxiety, and were not very effective in developing and assessing their learning.

On the contrary, Dolan et al (2004)[19] in their study to investigate the students and staff usage and perceptions of portfolio, concluded that the majority of students felt that too little time was spent on the portfolio than any other aspect of the course, although many students and staff appreciate the potential value of using the portfolio, it is not a requirement of the course and so tends not to be treated as a high priority. Davis et al (2009)[16] stated

that reduction in portfolio content may be responsible for improvements in student perceptions to portfolio process.

In responses to the open-ended questions, some students stated that they need more guidance on the portfolio, have difficulties in writing and not certain what to include. These responses were reported mainly by second year nursing students. They did not give any positive response in open ended questions and they have the lower proportion of agreement on the perception statements compared to first year nursing students.

Second year nursing students who participated in this study were the first group-within the faculty of nursing where the study was undertake who have required to maintain a portfolio building to demonstrate academic progression and achievement of clinical learning outcomes and competencies. This could be due to that lecturers and clinical instructors, might be failing to fulfill the need for support and guidance on portfolio building and use which they where the students were in their first year.

Nairn et al (2006)[22] reported that students in their third year were less optimistic about the use of portfolio than students in the first year.

Contrary to the present study findings Robinson (2000)[27] found positive attitudinal change towards portfolio assessment and the initial feeling of student uncertainty and resentment is a finding common to several portfolio assessment studies. Robinson (2000)[27] added that although some frustration was exhibited by several students at the beginning, this tapered off quickly as students become familiar with portfolio development process.

In a study done by Williams et al (2009)[21] to measure lecturers and students perceptions toward portfolio, they found that third and fourth-years students were the least positive compared to lecturers, added that the value of portfolios becomes less salient to student nurse towards the end of their training course.

The study results indicated that portfolio improved communication and relationship between first year students and faculty staff. In the same line Harris et al (2001)[13]; McMullan et al (2003)[23] stated that student-faculty link is an important component in the reflective portfolio process. Challis (1999)[12] suggested that the student-faculty link could take many forms ranging from regularly scheduled formal meetings with a mentor to telephone or e-mail communication as needed. This student-faculty link could potentially reduce negativity and confusion associated with the use of portfolio, as well as provides valuable formative assessment information to both students and faculty a like.

Findings of this study revealed that first year nursing students in their open ended responses stated that portfolio helped them to be confident and efficient in their independent learning and that portfolio could be continued through the years of the study.

Saker and Hu (2006)[7] concluded that in engineering education students reported that creating a learning portfolio generally helped their learning since they took responsibility for their own learning.

5. Conclusion and Recommendations:

Student nurses in the present study stated that portfolio process helped their learning since they took more responsibility for their own learning. Also, portfolio helped them to understand the basic concepts, improve the students-teacher relationship and portfolios are useful for their professional career development.

There were same discrepancies between first and second year nursing students in their perceptions towards portfolio. The value of portfolio becomes less salient to second year nursing students.

Some aspects considered important by students in building a portfolio including: provision of clear guidelines, regular frequent feedback, decrease the paperwork required and increase the marks allocated for portfolio appropriate to the workload involved.

Portfolios were seen by student nurses as more useful learning activity than other teaching and learning methods. The positive feedback from students also supported the continued use of a portfolio in subsequent study years.

Based on the findings of the present study, it could be recommended that:

- Portfolio can be very effective learning method but it is essential that students receive clear guidelines on and comprehensive support with their use.
- Portfolios should be designed in such a way that they are clear, relevant, objective and not time-consuming with refined content and should be linked with learning outcomes.
- Lecturers and instructors need to look at students' perceptions and why some students' perceptions deteriorate. There could be regular discussion with students to see how and why the students begin to see portfolios less useful for

their education and continual professional development.

- Provision of portfolio template could help students to make decisions about the type, quality and content of documents to include in their portfolios.
- The small class size in this study limits the generalization of the findings. Further studies based on a larger sample size are recommended to strengthen the evidence for portfolios use, particularly studies which observe changes in students' perceptions, knowledge and abilities.

Table (1): Student nurses' perception toward building of portfolio.

Statement	Group I (1st year) (n=195)						Group II (2nd year) (n=181)						X^2	P
	Agree		Neither		Disagree		Agree		Neither		Disagree			
	No	%	No	%	No	%	No	%	No	%	No	%		
1. Receive clear and complete portfolio guidelines:														
a. Purpose of portfolio	191	98	3	2	1	1	146	81	14	8	21	12	30.83*	0.0001*
b. Content	185	95	9	5	1	1	127	70	32	18	22	12	42.40*	0.0001*
c. Expected clinical learning outcomes	176	90	15	8	4	2	128	71	32	18	21	12	24.80*	0.0001*
2. Important to me to receive portfolio guidelines	170	87	24	12	1	1	63	35	66	37	52	29	117.45*	0.0001*
3. Frequent teacher's feedback is important during semester	179	92	8	4	8	4	86	48	36	20	59	33	88.88*	0.0001*
4. Portfolio encourage me to search information myself	157	81	33	17	5	3	78	43	52	29	51	28	68.16*	0.0001*
5. Participated actively in the building of portfolio	166	85	20	10	9	5	103	57	41	23	37	20	38.56*	0.0001*
6. Portfolio helps me to monitor my progress	171	88	17	9	7	4	105	58	34	19	42	23	45.99*	0.0001*

**Significant p<0.05*

Table (2): Student nurses' perception towards portfolios as a learning activity.

Statement	Group I (1st year) (n=195)						Group II (2nd year) (n=181)						X^2	P
	Agree		Neither		Disagree		Agree		Neither		Disagree			
	No	%	No	%	No	%	No	%	No	%	No	%		
I. Portfolio helps me to improve understanding and utilizing of basic concepts as …..														
- Problem solving skills	181	93	11	6	3	2	102	56	48	27	31	17	67.89*	0.0001*
- Communication skills	184	94	9	5	2	1.0	106	59	40	22	35	19	69.60*	0.0001*
II. Portfolio encourages me to be responsible for……														
- Independent learning	174	89	17	8	4	2	112	62	39	22	30	17	41.50*	0.0001*
- Theory-practice link	177	91	13	7	5	3	104	58	49	27	28	16	55.45*	0.0001*
- Decision making	179	92	10	5	6	3	86	48	63	35	32	18	88.51*	0.0001*
- Using information technology	137	70	31	16	27	14	87	48	65	36	29	16.0	22.78*	0.0001*
- Self-assessment	178	91	14	7	3	2	106	59	49	27	26	14	55.50*	0.0001*
III. Portfolio reflects my knowledge, activities and skills	154	79	30	15	11	6	80	44	77	43	24	13	48.42*	0.0001*

**Significant p<0.05*

Table (3): Usefulness of portfolio as perceived by student nurses compared with other teaching and learning methods.

Statements	Group I (1st year) (n=195)						Group II (2nd year) (n=181)						X^2	P
	Agree		Neither		Disagree		Agree		Neither		Disagree			
	No	%	No	%	No	%	No	%	No	%	No	%		
- The following are useful to me……														
- Building portfolio	174	89	18	9	3	2	112	62	49	27	20	11.0	39.88*	0.0001*
- Clinical placements	135	69	50	26	10	5	100	55	65	36	16	9	8.04*	0.018*
- Academic	140	72	37	19.0	18	9	93	51	69	38	19	11	18.67*	0.0001*

assignments - Classroom based teaching/learning	170	87	13	7	12	6	96	53	57	32	28	16	54.20*	0.0001*
- Portfolios are useful for my personal life and professional career development	183	94	10	5	2	1.0	142	79	28	16	11	6	19.44*	0.0001*

**Significant p<0.05*

Table (4): Mean scores of student nurses' perceptions toward portfolio (building – learning activity – usefulness).

Variables	Group I (n=195)	Group II (n=181)	t-test	P
	Range Mean±SD	Range Mean±SD		
Building of portfolio	17-24 23.11±1.41	8-24 19.03±3.43	15.43*	0.0001*
Portfolio as a learning activity	22-50 28.62±2.41	4-30 23.57±5.37	12.01*	0.0001*
Usefulness of portfolio	7-24 14.04±1.53	6-15 12.55±2.12	7.89*	0.0001*

**Significant p<0.05*

Table (5): Student nurses' comments toward portfolio.

Nursing students' comments toward portfolio	Group I (n=195)		Group II (n=181)	
	No	%	No	%
1. Too much paper work, time consuming	135	69	54	30
2. Little emphasis, and interfere with clinical learning	29	15	102	56
3. Little guidance, difficulties in writing, not certain what to include	5	3	48	27
4. Provide weekly feedback during the semester	35	18	-	-
5. Portfolio help me to be confident, to develop myself, able to independent-learning efficiently	40	21	-	-
6. Reflects my ability, learning activities	35	18	-	-
7. portfolio improve communication relationship between students and faculty staff	14	8	-	-
8. Continue use of portfolio next years	99	51	-	-

References:

(1) Kear, M. and Bear, M. (2007). Using portfolio evaluation for program outcome assessment. Journal of Nursing Education, 46, 3, 109-114.

(2) Buckley, S., Coleman, J., Davison, J, Khan, R., Zamora, J, Malick, S. (2009). The educational effects of portfolios on undergraduate student learning: a best evidence medical Education systematic review. BEME Guide No. 11, Medical teacher, 31, 4, 282-298.

(3) Tracy, S., Gail, L., Richo, K. and Daly, E. (2000). The clinical achievement portfolios: an outcomes-based assessment project. Nurse Educator, 25, 241-246.

(4) Robertson, J., Elster, S. and Kruse, G. (2004). Portfolio outcome assessment: Lessons learned. Nurse Educator, 29, 52-53.

(5) Emden, C., Hutt, D., Bruce, M. (2003). Portfolio learning/assessment in nursing and midwifery: an innovation in progress. Contemporary Nurse 16, 124-132.

(6) Plaza, C.M., Draugalis, J.R., Slack, M., Skrepnek, G.H. & Sauer, K. (2007). Use of reflective portfolios in health sciences education. American Journal of pharmaceutical Education, 71, 2, 1-6.

(7) Saker, P. and Hu, Y. (2006). Portfolio assessment to enhance student learning: 2006 Evaluations and assessment conference refereed papers [Electronic Version].

(8) McMullan, M., Endacott, R., Gray, M., Jasper, M, Miller, C., Scholes, J. and Webb, C. (2003). Portfolios and assessment of competence: A Review of the Literature. Journal of Advanced Nursing, 41, 283-294.

(9) Bell, S.K. (2001). Professional nurse's portfolio. Nursing Administration Quarterly, 25, 6-73.

(10) Morgan, B. (1999). Portfolios in a pre-service teacher field based programme: evaluation of a rubric for performance assessment. Education, 119, 416.

(11) Gannon, F., Draper, P., Watson, R., Proctor, S. and Norman, I. (2001). Putting portfolios in their place. Nurse Education Today, 21, 534-540.

(12) Challis, M. (1999). Portfolio-based learning and assessment in Medical Education Teacher, 21, 370-386.

(13) Harris, S., Dolan, G. and Fairburn, G. (2001). Reflecting on the use of student portfolios. Nurse Education Today, 21, 278-286.

(14) Knowles, M. (1975). Self directed learning: A guide for learners and teachers. Follett, Chicago.

(15) Brown, J. (2002). Theyself: The impact of portfolio development on adult learning: Adult Education Quarterly, 52, 228-245.

(16) Davis, M., Ponnamperuma, G. and Ker, J. (2009). Student perceptions of a portfolio assessment process. Medical Education, 43, 89-98.

(17) Challis, M. (2001). Portfolios and assessment: meeting the challenge Medical Teacher, 43, 437-440.

(18) Zeichmer, K and Wray, S. (2001). The teaching portfolios in US teacher education programs: what we know and what we need to know. Teaching and Teacher Education, 17, 613-621.

(19) Dolan, G., Fairbairn, G & Harris, S. (2004).Is our student portfolio valued? Nurse Education Today, 24, 1, 4-13.

(20) McMullan, M. (2008). Using portfolios for clinical practice learning and assessment: The pre-registration nursing student's perception. Nurse Education today, 28, 7, 873-879.

(21) Williams, G.A., Park, J.R., Traynor, V., Nairn, S., O'Brien, E., Chapple, M. and Johnson, S. (2009). Lecturers' and Students' perceptions of portfolio in an English School of Nursing. Journal of Clinical Nursing, 18, 1113-1122.

(22) Nairn, S., O'Brien, E., Traynor, V., Williams, G., Chapple, M. and Johnson, S. (2006). Student nurses' knowledge, skills and attitudes towards the sue of portfolios in a school of nursing. Journal of clinical nursing, 15, 1509-1520.

(23) McMullan, M. (2006). Students' perceptions on the use of portfolios in pre-registration nursing education: a questionnaire survey. International Journal of Nursing studies, 43, 3, 333-343.

(24) Tillema, H.H. and Smith, K. (2000). Learning from portfolios: Different use of feedback in portfolio construction. Studies in Educational Evaluation, 26, 193-210.

(25) Snyder, J., Lippincott, A. and Bower, D. (1998). Portfolios in teacher education: Technical or transformational? In with portfolio in hand; validating the new teacher professionalism (lyons Ned.). Teachers College press, New York, pp. 123-124.

(26) Klenouski, V. (2002). Developing portfolios for learning and assessment: process and principles. Routledge Falmer, London.

(27) Robinson, M. (2000). College students' attitudes toward portfolio assessment. ITFORUM. http://it.coe.uga.edu/itforum/paper47/paper 47. htm.[accessed22october2007.].

11/25/2010

Utilization of Alfalfa and Atriplex for Feeding Sheep under Saline Conditions of South Sinai, Egypt

Afaf M. Fayed*, Abeer, M. El- Essawy, E.Y. Eid, H. G. Helal, Ahlam, R. Abdou & H. M. El Shaer

Department of Animal & Poultry Nutrition, Desert Research Center, Mataria, Cairo, Egypt
a_fayed2007@yahoo.com*

Abstract: The objective of this study was to assess the influence of replacing percent of alfalfa by percent of Atriplex as roughage fed to animals. Twenty four adult Barki lambs weighed an average 49± 77 kg and age 3 years were used in six digestibility trials (4 animals each). Experimental diets were made of alfalfa and *Atriplex nummularia* in different ratios to each other as follow:R1: 75 % alf + 25% At, R2: 50 % alf + 50 % At, R3: 25 % alf + 75 % At, R4: 100 % At, T5: 100 % alf furthermore R6: berseem hay (BH) . All animals were fed barley at 25 % of energy requirements in R1, R2, R3, R4 and R5 while R6 fed concentrate feed mixture (CFM) .

Results obtained indicated that: all experimental diets had comparable values of dry matter (DM). Mixing of plants affecting chemical composition of feed ingredients where highest crud protein (CP) was recorded in R5 and least one was in R4. Crude fiber (CF) values were decreased as follow in R6, R5, R1, R2, R3 and R4, respectively. R6 and R4 had comparable values of condensed tannins while highest saponin levels were recorded in R4. Animals fed on R5 showed highest DM and total digestible nutrients (TDN) intake g/kg BW followed by those fed R1 and R2. Nitrogen intake showed higher values in R5 followed by R1, R2, R3, R6 then R4. Nitrogen retention also was maximum in R5 and minimum in R6. Animals fed At alone showed highest water intake with significant ($P< 0.05$) differences when compared with other treatments. There is a sampling time effect (zero and 6 hrs post feeding) on serum metabolites, liver enzymes and some minerals. Indeed the prefeeding rumen parameters ($NH_3 - N$) and TVFA,s were increased significantly to reach the peak value at 8 hr post feeding. Some minerals Na, K, Ca and P were analyzed.Na and P intake increased with increasing Atriplex level while Ca and K intake increased with increasing alfalfa level. Finally R2 is nutritious despite the generally low nutritive value and energy content.

[Afaf M. Fayed, Abeer, M. El- Essawy, E.Y. Eid, H. G. Helal, Ahlam, R. Abdou & H. M. El Shaer. **Utilization of Alfalfa and Atriplex for Feeding Sheep under Saline Conditions of South Sinai, Egypt**. Journal of American Science 2010;6(12):1447-1461]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: Salt tolerant fodders, digestibility, antinutritional factors, voulantry intake, rumen and blood metabolites, sheep

1. Introduction:

South Sinai is hyper arid region with salt affected natural resources (water, soil, plants, etc). Therefore, feed resources in the region are that represent one of the main obstacles for animal production development in the region. Salt tolerant forage species could play an important role in the region. *Atriplex nummularia* has great potentialities since it is known to be tolerant to salinity and drought (El Shaer., 2010). In addition to high content of crude protein, fiber and mineral contents.. However, it is deficient in energy and around 65% of nitrogen is non- protein nitrogen (Ben Salem *et al*, 2005 b). The high salt level in Atriplex limits its intake and digestion by ruminants (Hassan, 2009). Also *Atriplex* species contain some secondary metabolites as condensed tannins which may restrict feed intake and lead to a negative impact on animal performance (Ben salem., 2005). Alfalfa is the main cultivated legume crops in saline lands due to its high productivity,its high nutritional value and palatability (Anon , 2009). Mixing alfalfa with Atriplex as green fodders to sheep may increase the palatability and consequently intake and utilization of Atriplex which lead to improvement of the performance of animal . The objective of the study was to compare the nutritional evaluation of mixtures of alfalfa and Atriplex with different proportions fed to sheep in South Sinai.

2. Materials and methods

The study was carried out at South Sinai Research Station, Ras Sudr, South Sinai Governorate where the experimental forages diets *Atriplex nummularia* (At) and Alfalfa (*Medicago sativa*) (alf) were cultivated in saline soils and irrigated with saline water (total salinity 8500 ppm in ground water)

Animals and feeding management

Twenty four adult Barki lambs, weighing 49 ±1.62 kg and 3 years old, were used in the study where the sheep were equally divided into six

treatments (4 animals / treatment) and allocated to one of six dietary treatments. Animals received the experimental diets for 30 days as a preliminary feeding period followed by a 15- day digestibility trial where animals were kept in individual metabolic cages. The first ten days of the digestibility trial were devoted as an adjustment period. The weighed tested forages were offered twice daily, at 8.00 am and 3.00 pm. Measured amounts of drinking fresh water were left free choice for all animals. Barley grains were offered once daily at 12.00 pm as an energy source to all animals in R1, R2, R3, R4 and R5 and CFM to aimals in R6. Feed and refusals were collected, weighed and recorded during the preliminary period. In the following five days (collection period) measurements of 24 hours urine and fecal samples were collected for chemical analysis. At the end of the collection period, blood samples were taken before the morning feeding, then 6 hours post feeding. Rumen liquor were collected by stomach tube before feeding, 4 and 6 hrs post feeding. Live body weight and forage intake were recorded. Alf and (At) grown at the experimental farm were daily harvested, collected separately and chopped into small pieces (2-3 inches) then mixed in different ratios in the following basal rations (R) as follows:
R1: 75% alf + 25% At; R2: 50% alf + 50% AT; R3: 25% alf + 75% At; R4: 100% At; R5: 100% alf and R6: Berseem (*Trifolium alexandrinum*, 4th cut) hay (BH) as a control group. All animals were given feed supplements to cover 25% of TDN maintenance requirements according to Kearl (1982). Barley grains were given to animals in R1, R2, R3, R4, and R5 where animals in the control treatment (R6) were fed concentrate feed mixture (CFM). The CFM consisted of 25% cotton seed cake, 30% yellow corn, 35% wheat bran, 3% rice bran, 3% molasses, 1% urea, 2% limestone and 1% common salt.

Analytical methods

Proximate chemical analysis for all feed ingredients, refusals, fecal samples and urine were determine according to(A.O.A.C., 1997). Neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) (Goering and Van Soest, 1970). In addition, Sodium (Na) and potassium (K) were measured using flame model (Jenway PFP7) photometer and calcium (Ca) and phosphorus (P) were determined using Atomic absorption Spectrophotometer model (Unicam 929) in all dietary, fecal and urinary samples.

Anti-nutritional factors (ANF) analysis

Qualitative and quantitative estimation of condensed tannins (CT) and saponins (Sap) as the main ANF in all feed ingredients was carried out by Porter *et al.* (1986) and Balbaa *et al.* (1981), respectively.

Rumen and blood analysis:

Rumen liquor was withdrawn by stomach tube just after the end of the collection period before feeding, 4 hr and 8 hrs post feeding. Blood samples were collected before morning feeding and 6 hrs post feeding. The pH of rumen liquor was immediately recorded using Gallen Kamp pH Stick pH K-120 – B. Rumen liquor samples were analysed to determine total volatile fatty acids (TVFA 's) according to Warner, (1964) and ammonia – nitrogen (NH_3) (A. O. A. C. 1997). All serum samples were analyzed for Sodium (Na) and potassium (K) using flame photometer model (Jenway PFP7), calcium (Gitelman, 1967), inorganic phosphorus (Goodwin, 1970), triglycerides (Trinder, 1969), cholesterol (Roeschlau *et al.*, 1974), total protein (Reinhold, 1953), albumin (Rodkey, 1965), urea –N (Berthelot, 1959), creatinine (Seelig and Wust, 1969), alanine amino transferase (ALT) and aspartate amino transferase (AST) (Wilkison *et al.*, 1972). All kits used from Human Co. (Germany) using Jenway spectrophotometers (UK).

Statistical analysis:

Analysis of variance (ANOVA) was used to test the obtained data using the general linear modeling procedure (SAS, 2000). The used design was one way analysis. Duncan 's multiple tests (1955) were applied for comparison of means .

3. Results and Discussion:

Chemical composition:

Considerable variations were observed among the chemical composition of the studied forage crops (Table 1). Berseem hay (BH) fed to animals in R6 and *Atriplex nummularia* (At) had similar CP concentrations (averaged 12.25%) while alfalfa (alf) contained higher CP concentration (19.25%). Similar figures were recorded by El-Shaer *et al.* (2001) and Ben Salem *et al.*,(2005). However, the CP of *At* may lower than those reported by other workers Aganga *et al.* (2003) that could be attributed to different climatic, environmental, stage of growth factors. At, as a halophyticshrurb, contained lower concentrations of energy (324.087 GE, Mcal / kgDM) and organic matter (81.93%) compared to the other feed materials. Therefore, CP, OM and energy values in the mixture forage treatments were decreased as alfalfa ratio decreased and the increased portions of Atriplex in the forages diets. Such results are in agreement with those found by El –Shaer *et al.* (1991) and Ben Salem *et al.* (2005)

The OM was highest in BH followed by alf diet, while it was the lowest in At. On the other hand, NDF, ADF and ADL concentrations were higher in At compared to alf and BH. At attained the highest lignin content followed by R3, R2, R6, R1 then R5 (alf). Fiber fractions in the forage mixtures diets (R1, R2, R3) were increased by increasing the inclusion of At (R4) portion in the diets.

Data in Table (1) showed that Na, K, Ca and P levels were higher than probable dietary requirements (0.09 – 0.18, 0.50 – 0.80, 0.36 – 0.42 and 0.29 – 0.31%, for Na, K, Ca and P, respectively, as described by (NRC, 1985) but still below maximum tolerable levels (4.0, 2.0, 1.5 and 0.6 %, respectively as reported by (NRC, 1980) . The higher levels of minerals Na, K, Ca and P in saltbush offered to sheep could be due to the high content of these minerals in saltbush leaves. Saltbush leaves are more palatable than other plant parts (Gihad and El – Shaer, 1992). P value in alf was similar to those reported by Karabulut *et al*. (2006) while values of Na, K and Ca were different. These differences may possibly be due to maturity differences in alfalfa used in this experiment.

Phytochemical screening of the experimental feed materials revealed that they contained saponins (sap) and condensed tannins (CT) except the BH. which was free from sap.. Also, barley grains as energy source and concentrate feed mixture (CFM) were free from both (sap) and (CT). Therefore, quantitative determination for such materials was necessary before using such feed materials for feeding sheep.

The levels of ANF's are varied from plant to plant and from season to season (El-Shaer *et al*., 2005). At. (R4) and BH. (R6) showed the highest levels of CT followed by R3, R2, R1 then R5, respectively. Thus, the concentrations of CT declined as the proportions of At. decreased in the dietary mixture. The present levels of CT ranged from 1.48 to 2.30 mg/ 100g DM. So, it is safe for animals according to the previous studies.

At. (R4) showed the greatest level of sap. followed by R3, R5. R2 then R1

Table (1): Chemical composition and secondary metabolites levels of the feed ingredients(roughages,barley and CFM) on DM% bases.

Item	Rations							
	R1	R2	R3	R4	R5	R6	Barley grains	CFM
DM%	91.91	92.44	92.95	92.35	91.39	92.90	93.69	92.98
OM	80.76	77.83	74.89	71.97	83.69	86.32	96.9	93.95
CP	17.66	15.75	14.00	12.25	19.25	12.25	9.38	15.28
CF	24.86	24.15	23.42	22.71	25.57	29.22	3.53	9.57
EE	2.22	1.93	1.64	1.34	2.51	1.95	2.43	1.91
NFE	36.o2	36.00	35.84	35.67	36.36	42.90	81.56	67.19
Ash	19.24	22.17	25.10	28.03	16.31	13.68	3.10	6.05
GE Mean/kgDM	372.4	344.58	340.4	324.08	389.36	386.8	428.9	422.8
NDF	48.83	52.65	56.47	60.30	45.0	58.0	18.0	36.0
ADF	34.70	37.41	40.11	42.81	32.0	43.0	7.0	29.0
ADL	11.97	13.95	15.92	17.89	10.0	12.0	2.0	11.0
Cellulose	22.73	23.46	24.19	24.92	22.0	31.0	5.0	7.0
Hemicellulose	14.13	15.24	16.36	17.49	13.0	15.0	11.0	18.0
Sodium (Na)%	1.03	1.32	1.64	1.93	0.709	1.42	4.94	0.631
Potassium (K)%	1.26	1.29	1.31	1.34	1.24	1.72	5.72	0.759
Calcium (Ca)%	0.717	0.745	0.786	0.815	0.682	0.926	3.5	0.359
Phosphorus (P)%	0.351	0.465	0.583	0.702	0.233	0.666	1.35	0.511
CT	1.58	1.80	1.98	2.29	1.48	2.30	Nil	Nil
Saponins	2.08	2.84	3.60	4.42	3.04	Nil	Nil	Nil

R1: 75% alfalfa + 25% Atriplex, R2: 50% alfalfa + 50% Atriplex
R3: 25% alfalfa + 75% Atriplex, R4: 100% Atriplex
R5: 100% alfalfa, R6: berseem hay
CFM: Concentrate feed mixture, CT: Condensed tannins
GE (Mcal / kgDM) = CP× 5.65+ CF×4.15+EE×9.40+NFE×4.15 (Blaxter, 1968).

Feed intake, digestion coefficients and nutritive values:

Results in Table (2) indicated that the highest DMI (P≤ 0.05) was observed for animals fed alf (R5) followed by those in R1, R2, R3, R4 while the lowest value was recorded for animals fed R6 (the control group). These findings may be due to the fact that fresh alf. and At. were more palatable compared to berseem hay (BH) in R6. These results were in-agreement with those reported by Shawket and Ahmed (2009).

The present data clearly indicated a significant (P≤ 0.05) effect of the roughage type on DMI. Daily DMI was negatively affected by saltbush. This is attributed to higher salt content and higher levels of fiber especially ADL and NDF in At. Similar results were reported by Ben Salem *et al*. (2005), Masters *et al*. (2006), and Hassan (2009). Moreover, due to high ash content, at forage is relatively low in energy. As shown in table (2); the apparent digestibility of OM, CF, NFE, ADF and ADL were higher with fresh alf than other groups containing At with no significant difference. However the control group R6 has the lowest (p≤0.05) CP digestibility. Similar trend was observed by Abdel – Malik *et al*. (2001) who found that CP digestibility of berseem was lower than that of alf. Introducing of At. instead of alf in other treatments slightly decreased the digestibility coefficients of CP, CF, NFE, cellulose with no significant differences. Similarly, Al Owaimer *et al*. (2008) concluded the same result. These results may be due to higher salt content of Atriplex which is the major negative component in Atriplex species (Wilson, 1992) thus leading to increment of animal water intake and shortening the rumen turnover time with consequential influences on rumen physiology and metabolism (Warner and Casson, 1992 and Konig, 1993).

Table (2): Average daily feed intake, digestion coefficients and nutritive values of animals fed the experimental diets

Item	Rations						
	R1	R2	R3	R4	R5	R6	±SE
DM intake g/h/d							
Concentrate	6.71 ^{b}	6.68 ^{b}	6.62 ^{b}	6.71 ^{b}	6.87 ^{b}	10.98 ^{a}	0.719
Roughage	29.09 ^{b}	25.59 ^{c}	23.65 ^{cd}	22.64 ^{d}	31.29 ^{a}	17.76 ^{e}	0.228
Total	35.80 ^{b}	32.27 ^{c}	30.27 ^{cd}	29.35 ^{d}	38.16 ^{a}	28.74 ^{d}	3.40
Digestion coefficient:							
DM	60.32	64.55	63.93	67.0	66.86	63.77	5.97
OM	65.78	65.72	64.88	59.49	66.57	64.59	4.75
CP	72.78 ^{a}	70.99 ^{a}	70.69 ^{a}	70.08 ^{a}	77.34 ^{a}	59.28 ^{b}	2.27
CF	50.04	50.87	46.16	45.21	49.70	47.19	3.39
EE	65.39	66.44	67.82	77.09	65.49	58.76	5.38
NFE	67.69	72.72	69.93	67.49	74.20	68.45	3.73
NDF	52.53	51.24	49.93	50.22	51.50	51.81	1.69
ADF	48.82	48.08	47.45	48.36	51.25	47.10	3.39
ADL	43.01	41.46	42.90	41.69	45.91	43.69	1.55
Hemicellulose	55.37	57.90	54.41	60.83	62.24	50.14	5.23
Cellulose	53.03	52.81	51.38	44.17	54.93	49.65	5.04
CT	56.59 ^{b}	64.83 ^{b}	63.68 ^{b}	86.00 ^{a}	33.05 ^{c}	40.0 ^{c}	9.69
Saponin	89.20 ^{c}	92.30 ^{cb}	92.23 ^{cb}	96.41 ^{a}	93.06 ^{b}	n. ev.	1.83
Nutritive value:							
TDN g/kg B.W	22.06 ^{a}	18.17 ^{b}	16.07 ^{bc}	15.07 ^{bc}	22.49 ^{a}	15.67 ^{c}	0.058
TDN %	63.09 ^{a}	56.28 ^{ab}	53.24 ^{ab}	51.32 ^{b}	58.91 ^{ab}	58.63 ^{ab}	0.483
DCP g/kg B.W	4.520 ^{b}	3.44 ^{c}	2.86 ^{cd}	2.55 ^{de}	5.44 ^{a}	2.17 ^{e}	0.781
DCP %	12.48 ^{b}	10.65 ^{c}	9.45 ^{cd}	8.69 ^{ed}	14.24 ^{a}	7.51 ^{e}	4.750
ME_Mcal /kg DM	20.18 ^{ab}	20.82 ^{a}	18.25 ^{ab}	17.32 ^{b}	21.35 ^{a}	21.24 ^{a}	0.934

ME,M cal/ kg DM = (TDN × 3.6) /100 (Church and Pond, 1982).
R1: 75% Alfalfa + 25% Atriplex + barley , R2: 50 % Alfalfa + 50 % Atriplex+ barley
R3: 25 % Alfalfa + 75 % Atriplex+ barley , R4: 100 % Atriplex+ barley
R5: 100 % Alfalfa+ barley , R6: Berseem hay + CFM
a, b, c and d : values with different letters in the same row means statistically significant at P<0.05
n. ev.: means not evaluated

Such results might be also attributed to the secondary metabolites in At which include oxalates, tannins and saponins which might decrease the production of volatile fatty acids in the rumen. The same results were reported by Shawket and Ahmed (2009) and Abu – Zanat and Tabbaa (2005). Inclusion of Atriplex in diet up to 50% insignificantly increased OM, CP, CF, NFE, NDF and cellulose than At alone. Getachew *et al*. (2008) found that digestibility of NDF and nitrogen was reduced by all levels of tannic acid compared with control.

Furthermore, Positive digestibilities of the CT were reported in the present study and the variations among groups were significant (P≤ 0.05). The highest digestibility of CT was in R4 followed by those fed R2, R3, R1, R6 then R5 (alf), respectively.

It is clear that R4 contained the highest concentration of CT as mentioned before in table (1). This finding could be explained by DM intake. In general, as DM intake increased, apparent digestibility decreased in sheep which could be due to higher rumen turnover rates observed in both sheep and cattle (Mulligan et al., 2001). Also, the same finding was reported in case of saponins.

R4 fed animals showed the highest saponin digestibility followed by R5, R3 and R2 showed a comparable value of digestibility then R1 was the lowest one. Saponin was absent from R6 (BH) so, it is not evaluated in BH fed animals.

When the nutritive values expressed as TDN and DCP a significant difference (P≤ 0.05) was observed for TDN g/kg BW values with alfalfa diets and the lowest in R4. Moreover, TDN% of intake was significantly (P≤ 0.05) increased in R1 followed by R5, R6, R2, R3 and R4, respectively. The elevation of TDN in alf. may be due to the highest digestibility of OM, CP, CF and NFE than those of At. Al- Owaimer *et al*. (2008) consistent with our results where they noticed that lambs fed alf hay had higher TDN than those fed At Ahmed *et al*. (2001) showed that the value of TDN for ration containing At was lower by 7.15% than that containing BH. Shawket, (1999) reported that the nutritive value expressed as TDN was 55.7 and 67.8%, respectively for diets containing At alone and with energy supplement.

Digestible crude protein (DCP) g/kg BW value increased (P≤ 0.05) significantly on (R5) followed by R1, R2, R3, R4 then R6. Similar observations were reported by Abdel – Malik *et al*. (2001). Results obtained may be attributed to higher CP content of alf and its rapid fermentation followed by At and BH. The percentage of DCP of intake had the same trend.

ME, Mcal / Kg DM varied significantly (P≤ 0.05) among treatments. Results indicated that the values of ME Mcal / Kg DM were decreased (P≤ 0.05) with 100% and 75% At. than other treatments. However 50% At + 50% alf expressed comparable values with those of 75% alf +25% At.

This finding may be due to lower energy content of At than alf and BH. Shawket, (1999) concluded similar results, she found that the utilization of At was enhanced with the energy supplementation. Ben Salem *et al*. (2005) found that animal performance was improved by feeding At supplemented with barley than those fed At alone.

Nitrogen balance:

As shown in Table (3); Total nitrogen intake (NI) expressed as gm / head / day for animals fed R5 was the highest (p≤0.05) followed by R1 and the lowest one was R4. These differences may be due to the type of forage and its content of nitrogen and also these findings were matched with the CP intake in the experiment. These results are in agreement with those of Shawket *et al.,* (2005) and Al – Owaimer *et al*., (2008). Results showed that the main pathway of N excretion was through urine for all tested roughages except BH group.

Table (3): Nitrogen balance (gm / head / day) of animals fed the experimental diets

Item	Rations						
	R1	R2	R3	R4	R5	R6	±SE
Nitrogen intake gm/head/day	43.63 ^{b}	38.18^{cb}	32.24^{c}	28.74^{c}	64.68^{a}	29.53^{c}	5.06
Excreted nitrogen: (g/h/d) :Fecal nitrogen	11.85^{ab}	10.69^{b}	9.17^{dc}	7.78^{d}	14.92^{a}	14.32^{ab}	2.026
Urinary nitrogen	19.23 ^{b}	19.32 ^{b}	17.49^{cb}	14.81 ^{cb}	33.84^{a}	12.97^{c}	2.52
Total excretion(g/h/d)	31.08 ^{b}	30.01^{b}	26.66^{cb}	22.59 ^{c}	48.76^{a}	27.29^{c}	3.73
Nitrogen retention	12.54 ^{b}	8.17^{b}	5.58^{cb}	6.15^{cb}	15.92^{a}	5.14^{c}	1.57
N R % of intake	28.75 ^{a}	21.40^{ab}	17.31^{b}	21.40 ^{ab}	24.61^{a}	17.69^{b}	3.28
F N % of intake	27.17 ^{b}	27.99^{b}	28.51^{b}	26.84 ^{b}	22.84^{b}	49.50^{a}	5.51
U N % of intake	44.08	50.61	54.34	51.86	52.82	44.98	5.63

R1: 75% Alfalfa + 25% Atriplex+ barley , R2: 50 % Alfalfa + 50 % Atriplex+ barley.
R3: 25 % Alfalfa + 75 % Atriplex+ barley , R4: 100 % Atriplex+ barley. R5: 100 % Alfalfa+ barley ,
R6: Berseem hay + CFM.
F N: fecal nitrogen , U N : urinary nitrogen , N R : nitrogen retention
a, b, c and d : values with different letters in the same row means statistically significant at P<0.05

These findings may be attributed to the rapid hydrolysis of alf and At CP in the rumen which led to accumulation of ammonia (Weston *et al*., 1970) which is inefficiently increase urinary nitrogen excretion. Values of total nitrogen excretion clearly indicated that animals fed on 100% alf lost higher ($p \leq 0.05$) values of nitrogen followed by R1 and R2 and the lowest one was R4.

Nitrogen retention (NR) was higher in animals fed R5 and the lowest value was in R6. However R3 and R4 had comparable values of NR which have higher percent of At. This may be due to higher CP content and its higher digestibility in alf than At and BH. Data of NR% of intake showed that R1 indicated the highest ($p \leq 0.05$) nitrogen utilization while R6 was the lowest one.

Water balance:

As shown in Table (4); the highest water intake was observed in animals of R4 and R3 and this attributed to At. with high salt content. The lowest amount of water intake was recorded in sheep fed control diet with significant ($p \leq 0.05$) differences among the experimental groups.These results are inharmony with those reported by Bhatti *et al*. (2009), Hassan (2009), Shawket &Ahmed (2009) and Shawket *et al*. (2005) who reported that the salt content of At. can influence the animal water requirements because additional water is required to excrete their high salt content.

As expected urinary water was higher in R4 followed by R3 ($P \leq 0.05$), respectively and R6 was the lowest one. These findings are in agreement with El Aich (1987), Eid (2003) and Allam *et al*. (2006) who indicated that the high content of ash in halophytes lead to push animals to increase excretion of urine as natural channel to excrete minerals.

Water balance ml/h/day values for sheep fed R2 and R3 are significantly higher ($p \leq 0.05$) than those of animals fed R1 and R5. While the lowest was R6.

Table (4): Water balance (ml / head / day) of animals fed the experimental diets.

Item	Rations						
	R1	R2	R3	R4	R5	R6	±SE
Drinking water (ml / head / day)	1500^{c}	2446^{c}	3176^{b}	4142^{a}	1284^{c}	1825^{cb}	216
Combined water (ml / head / day)	1368^{b}	1133^{cb}	1039^{c}	760^{d}	1800^{a}	118^{d}	207
Metabolic water (ml / head / day)	563^{b}	531^{b}	482^{b}	475^{b}	813^{a}	451^{b}	50
Total water intake (ml / head / day)	3431^{b}	4110^{b}	4697^{ab}	5377^{a}	3897^{b}	2394^{d}	298
Urinary water (ml / head / day)	1710^{c}	1986^{c}	2743^{b}	4232^{a}	2036^{c}	1477^{d}	141
Fecal water (ml / head / day)	655	551	524	515	805	572	107
Total water excreted (ml / head / day)	2365	2537^{cb}	3267^{ab}	4747^{a}	2841^{bc}	2049^{d}	184
Water balance (ml / head / day)	1066^{b}	1573^{a}	1430^{a}	630^{c}	1056^{b}	345^{d}	210

R1: 75% Alfalfa + 25% Atriplex+ barley , R2: 50 % Alfalfa + 50 % Atriplex+ barley. R3: 25 % Alfalfa + 75 % Atriplex+ barley , R4: 100 % Atriplex+ barley.
R5: 100 % Alfalfa+ barley , R6: Berseem hay + CFM.
a, b, c and d : values with different letters in the same row means statistically significant at $P<0.05$.

Rumen parameters:

Data revealed that the ammonia–N (NH_3–N) concentration mg/100ml rumen liquor (RL) was significantly ($p \leq 0.05$) higher in R5 which is rich diet with CP than those of R1, R6, R2,R3 and R4, respectively (Figure 1).

The prefeeding ammonia–N values increased significantly to reach to the peak value at 8 hr post feeding (Fig 1). Mehrez *et al*. (2001) found that 20.61mg/100 ml RL NH3–N would satisfy microbial needs for N and hence maximize the rate of fermentation of the experimental diet in the rumen. Hassan (2009) also reported that ruminal microbial protein synthesis requires an adequate supply of nitrogen to achieve maximal efficiency. Also, because of lower nitrogen content uncoupled fermentation could occur (McMeniman, 1976).

The average values of ruminal total volatile fatty acids (TVFA's) (Fig. 2) showed that the highest ($p \leq 0.05$) value was recorded in R5 and the lowest in R4 . This result may be due to higher salt and lower energy contents of Atriplex which shortening the rumen turnover time with consequential influences on rumen physiology and metabolism (Warner and Casson; 1992 and Konig, 1993) and decrease the production of VFA 's in the rumen (Shawket and Ahmed, 2009).

The prefeeding TVFA's values (Fig.2) significantly ($p \leq 0.05$) increased to reach the peak value at 8 hr post feeding.

Blood metabolites:

Blood metabolites (Table 5) were used to monitor nutrient status. In the current study, there

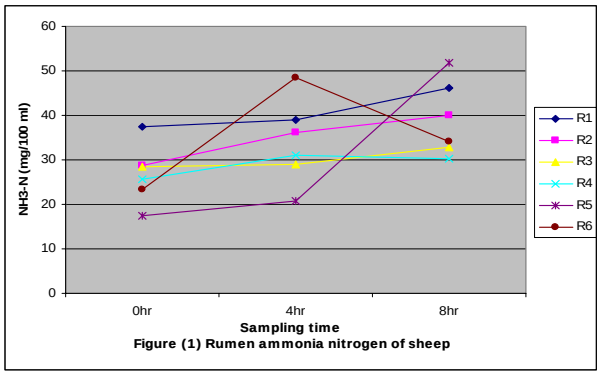


Figure (1) Rumen ammonia nitrogen of sheep

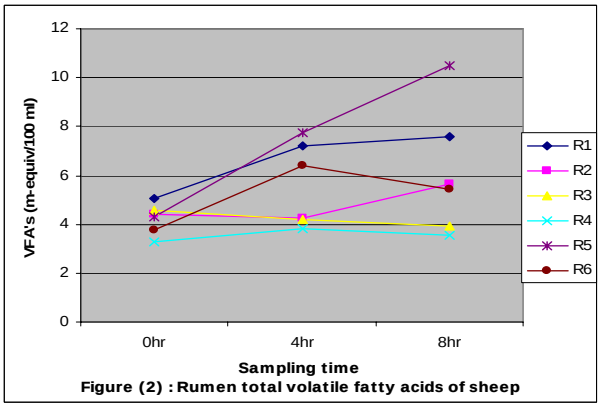


Figure (2) : Rumen total volatile fatty acids of sheep

was a sampling time effect after feeding for all the studied parameters. Serum biochemistry was within the range that was reported by other authors (Rankins *et al*. 1991 and Getachew *et al*. 2008) and there were different variations among the studied groups before and after feeding.

Serum total protein (TP) showed a significant ($p \leq 0.05$) elevation post feeding (Table 5). The lowest value was recorded in control group and the highest one in R5. These findings agree with the fact of dietary CP which was highest in R5 (alfalfa) and lowest in BH and R4 (At). Moreover, dietary CT was highest in BH and R4 and lowest in R5. Although Getachew *et al*. (2008) found that alfalfa protein is poorly utilized by ruminants due to its rapid degradation in rumen. Indeed, the alfalfa digestibility was reduced in the diet containing barley grains (Jahani-Azizabadi et al.,2009). CT may share in these variations as CP forms a complex with tannins under aerobic conditions causing a lowering in the available protein intake (Wina et al, 1999). Also, the ability of tannins to precipitate different proteins varies considerably (Bennick, 2002).

BH was free from saponins as previously reported and At (R4) was the highest ration in saponins. Saponins reduce protein digestibility by the formation of less digestible saponin – protein complexes affecting the nutritive value of the diet (Potter et al,1993). *In addition* the complementarity's of tannin – rich shrubs and a saponin-rich shrub positively influenced biomass intake (Rogosic et al,2006) . Alao, CT and saponin alleviate the adverse effect of each other (Makkar, 2003).Neglecting the sampling time, there was no significant variations among experimental rations. Albumin showed the same pattern among groups where the highest levels were recorded in R5 and the lowest ones in R6 . Significant ($p \leq 0.05$) differences were reported when neglecting sampling time. Globulin showed significant ($p \leq 0.05$) diet effect among the studied groups.

Urea –N was decreased ($P \leq 0.01$) in post feeding and when neglecting time (Table 5) reflecting low protein intake by sheep fed the experimental diets. Present results were in agreement with those reported by Rankins et al,(1991) who mentioned that low protein intake although serum total protein, albumin and globulin were elevated but protein intake was not low enough to compromise protein synthesis by liver. Samanta *et al*. (2003) showed that plasma urea – N reflects the dietary CP intake. Getachew *et al*. (2008) found that only blood urea – N was affected by both the level of tannin and sampling time after feeding although other metabolites were affected by sampling time. They proved the blood urea –N was elevated at 2h after feeding and started to decline to the pre – feeding level. Romero *et al.* (2000) also showed that sheep fed with tanniferous diet had reduced blood urea-N level than sheep fed lower dietary tannin.

Creatinine levels were elevated significantly ($p \leq 0.05$) before and after feeding and indicating impairment of renal functions (Brenner *et al*., 1987).

As can be observed, considerable decrease in cholesterol levels in all studied groups except R5 and R6 was recorded before and after feeding even when neglecting time. This result is consistent with those reported by Potter *et al*. (1993) and Matsuura (2001) who found that saponins from different sources causing lower serum cholesterol levels in a variety of animals as several dietary saponins have a hypocholesterolaemic action (Francis et al, 2002). Also, if causes a delaying of the intestinal absorption of dietary fat by inhibiting pancreatic lipase activity (Han *et al*., 2000). On the other hand, tannins play a considerable role in lipids digestibility by complexing with fatty acids(Romero *et al*., 2000) causing a decrease in cholesterol absorption and increase in fat excretion (Bravo *et al*., 1993).

Cocerning serum enzymes, AST was significantly ($p \leq 0.05$) increased post feeding and on neglecting the time factor. This elevation could be attributed to tannin content of the diet. Reed (1995) and Silanikove *et al*. (1996) reported hepatotoxicity and elevated AST in goats and cattle fed on

Table (5): Some blood parameters in animals fed the experimental diets .

Item	Ratios						
	R1	R2	R3	R4	R5	R6	±SE
T.P.g/dl							
0 hr	6.466	6.163	6.296	6.023	6.200	6.500	1.380
6 hr	8.143 ^{ab}	6.446 ^{b}	6.913 ^{ab}	7.163 ^{ab}	9.100 ^{a}	6.333 ^{b}	1.282
mean	7.305^{a}	6.305^{a}	6.605^{a}	6.593^{a}	7.650^{a}	6.416^{a}	0.588
Albumin g/dl							
0 hr	2.82	2.95	2.85	3.06	2.90	2.77	0.31
6 hr	3.13 ^{ab}	3.07 ^{ab}	3.07 ^{ab}	3.26 ^{ab}	3.77 ^{a}	2.83 ^{b}	0.36
mean	2.98^{ab}	3.01^{ab}	2.96^{ab}	3.16^{ab}	3.33^{a}	2.79^{b}	0.15
Globulin g/dl							
0 hr	3.640	3.220	2.310	2.960	3.300	3.730	1.150
6 hr	5.020 ^{ab}	2.810^{c}	3.850^{abc}	3.200 ^{bc}	5.330^{a}	3.510 ^{abc}	1.090
mean	4.330	3.020	3.080	3.080	4.320	3.620	0.490
Urea mg/dl							
0 hr	24.90	27.17	27.60	30.60	33.00	34.05	9.54
6 hr	30.63 ^{b}	22.23^{b}	25.87 ^{b}	20.10 ^{b}	61.33 ^{a}	40.27 ^{b}	11.29
mean	36.07^{cb}	24.65^{d}	26.73^{cd}	25.35^{cd}	50.35^{a}	46.26^{ab}	3.58
Creatinine mg/dl							
0 hr	1.23 ^{b}	1.17 ^{b}	1.40 ^{b}	1.18 ^{b}	1.40 ^{b}	2.42 ^{a}	0.18
6 hr	0.89 ^{b}	1.06 ^{b}	1.24 ^{ab}	1.08 ^{b}	1.20 ^{ab}	1.82 ^{a}	0.19
mean	1.06^{b}	1.11 ^{b}	1.32 ^{b}	1.13 ^{b}	1.30 ^{b}	1.95 ^{a}	0.14
Cholesterol mg/dl							
0 hr	32.20 ^{b}	34.53 ^{b}	37.67 ^{b}	44.33 ^{b}	64.83 ^{a}	63.50 ^{a}	7.66
6 hr	48.33 ^{dc}	64.00 ^{bc}	37.67 ^{d}	47.0^{dc}	85.20 ^{a}	80.67 ^{ab}	11.29
mean	40.27 ^{b}	51.60 ^{b}	37.67 ^{b}	45.67 ^{b}	75.01 ^{a}	72.08^{a}	5.07
Triglycerides mg/dl							
0 hr	49.5 ^{b}	78.24 ^{a}	80.27 ^{a}	51.83 ^{b}	43.53 ^{b}	48.19 ^{b}	12.70
6 hr	42.88 ^{b}	48.83 ^{b}	38.89 ^{b}	49.22 ^{b}	86.02 ^{a}	94.45 ^{a}	5.55
mean	46.11 ^{b}	63.54 ^{ab}	52.52 ^{ab}	50.53 ^{ab}	64.78 ^{ab}	71.32 ^{a}	7.28
GOT U /L							
0 hr	55.0	50.0	60.33	57.0	40.67	34.67	15.24
6 hr	43.67^{ab}	45.33 ^{ab}	53.33 ^{a}	47.00 ^{ab}	32.0 ^{bc}	24.33 ^{c}	9.47
mean	49.33 ^{ab}	47.67 ^{ab}	56.83 ^{a}	52.00 ^{ab}	36.33 ^{bc}	29.50 ^{c}	5.08
GPT U / L							
O hr	16.00	17.00	19.00	21.50	19.0	18.33	3.84
6 hr	13.33 ^{b}	19.50 ^{a}	13.50 ^{b}	8.67 ^{c}	17.33 ^{a}	16.33 ^{ab}	2.00
mean	14.67	18.58	16.17	15.00	18.17	17.33	1.74
Sodium mmol/l							
0 hr	144.0	142.0	143.8	143.0	147.5	140.5	3.22
6 hr	146.0	142.8	145.0	147.0	148.0	142.0	2.98
mean	145.0	142.4	144.4	145.0	147.8	141.3	3.0
Potassium mmol/l							
0 hr	4.400	4.700	4.600	4.800	4.500	4.900	0.670
6 hr	4.600	5.000	4.800	5.100	4.700	5.000	0.770
mean	4.500	4.900	4.700	5.000	4.600	5.000	0.710
Calcium mg/dl							
0 hr	10.53 ^{b}	13.90 ^{a}	11.90 ^{ab}	10.13 ^{b}	10.20 ^{b}	6.73 ^{c}	1.58
6 hr	13.97 ^{a}	10.67^{ab}	10.40 ^{b}	9.30 ^{b}	11.13 ^{ab}	11.77 ^{ab}	1.73
mean	12.25 ^{a}	12.28 ^{a}	11.15 ^{ab}	9.72 ^{ab}	10.67 ^{ab}	9.25^{b}	0.89
Phosphorus mg/dl							
0 hr	5.03 ^{ab}	4.70 ^{b}	5.47 ^{ab}	4.53 ^{b}	6.23 ^{ab}	6.73 ^{a}	0.98
6 hr	5.27 ^{ab}	5.57 ^{ab}	5.80 ^{ab}	5.17 ^{b}	6.57 ^{a}	6.07 ^{ab}	0.69
mean	5.15^{b}	51.33^{b}	5.63 ^{ab}	4.85 ^{b}	6.40 ^{a}	6.40 ^{a}	0.33

0 hr: means before feeding. 6 hr: means six hours after feeding

R1: 75% Alfalfa + 25% Atriplex+ barley , R2: 50 % Alfalfa + 50 % Atriplex+ barley.

R3: 25 % Alfalfa + 75 % Atriplex+ barley , R4: 100 % Atriplex+ barley.

R5: 100 % Alfalfa , R6: Berseem hay + CFM.

a, b, c and d : values with different letters in the same row means statistically significant at P<0.05.

tanniferous forages, while Romero *et al*. (2000) did not find such elevation. Fitcher (1993) explained the elevated AST to be due to muscle destrophy. ALT showed nonsignificant variations except post feeding values. Makkar *et al*. (1988) assumed that the inhibitory or stimulatory effect of tannins on enzyme activity may result from a change in the conformation of the enzyme in the presence of tannins leading to a variable variability of substrate at the catalytic site of the enzyme.

As show in Table (5), indicated that Sodium and potassium levels in blood serum of the studied animals were not differed significantly among the studied groups neither at 0 hr nor at 6 hr post feeding. Na and K were within the normal concentrations which are in agreement with the findings of El – Shaer *et al*. (2001). On the other hand sheep fed R2 had higher (P≤ 0.05) Ca level than sheep fed saltbush alone, alfalfa or the traditional ration (BH) at 0 hr. There were also considerable variations in P level in the blood serum of the studied sheep where the highest P levels were recorded in those fed R6. This elevated P level in this group could be due to that P content in At and BH was slightly higher than maximum tolerable levels (NRC, 1980) (0.702 and 0.666 % vs. 0.6%. Generally, these hypocalcemia in R6 could be attributed to the presence of high levels of CT in BH because tannins can disturb the absorption of minerals by chelation of them within the gastrointestinal tract of the animal (Cowieson *et al*., 2004) and / or increase the endogenous losses of the minerals such as Ca (Mansoori and Acamovic, 1997). Also, BH may contain oxalic acid which binds with Ca to form a non soluble, non digestible Ca oxalate (Cheeke, 1995). Moreover, the imbalanced Ca: P ratio in this group might be responsible for decreasing Ca availability of sheep in the same group (McDowell, 1992) while the rest of animal groups were within normal levels of both minerals (9 -12, and 4.5-6mg/dl) respectively for Ca and P as described by Kincaid (1993).

Minerals retention:

As shown in Table (6), Na intake was increased as the ratio of Atriplex increased. This results was matched with the fact of that Atriplex contain the highest concentration of Na (table 1). Sodium intake increased in the following order from R1, R5, R2, R6, R3 and R4, respectively. Wilson (1996) consistent with our results in that high amount of Na and K salts found in Atriplex species. Differences in Na intake among studied groups were significant (P≤0.05) while fecal Na excreted without significant differences . Urine is generally accepted as main pathway for the excretion of Na (McDowell and Arthington, 2005). Significant (p≤0.05) urinary sodium loss was noticed among tested animals . The highest urinary sodium was reported in R3 and R4 (the highest Na intake groups and highest percent of Atriplex). Sodium balance also follows the same pattern where Na balance increased as a result of increasing Atriplex (from R1, R2, R3 and R4). R5 (100% alf) revealed the least sodium balance with significant (p≤0.05) differences.

It is clear that animals fed alfalfa alone had the highest K intake followed by control animals R6 then R1, R4, R2 then R3. Differences among groups were significant (P≤0.05). Potassium content of alf and Atriplex were comparable but K intake in R5 could be attributed to highest DM intake in R5 (Table 2) however, the lowest K intake in R3 was attributed to lowest DM intake in the same group. Fecal and urinary K output varied significantly (p≤0.05) among treatments but most of the excreted K was via urine and this consistent with the fact that urine is the major pathway for the excretion of potassium (McDowell and Arthington, 2005). Potassium balance was slightly different among groups.

The highest (p≤0.05) Ca intake was recorded in R5 while R1, R2 and R3 revealed comparable values of Ca intake. R4 and R6 were also comparable. Fecal Ca revealed significant (p≤0.05) differences among groups . It is well known that feces is the main route of Ca excretion (McDowell and Arthington, 2005). The present study revealed that fecal Ca was affected by saltbush percentage in the diet which contains high levels of secondary metabolites mainly oxalic acid which binds with Ca to form calcium oxalate, non – soluble, non – digestible compound (Alazzeh and Abu Zanat, 2004 :Cheeke, 1995). Generally, urinary Ca loss tended to remain low and constant. There are no differences among groups in urinary Ca . Calcium balance was positive for all different groups with significant (p≤0.05) differences.

The higher levels of P intake in diets as a result of increasing saltbush to reach its peak in R4 could be due to the high content of P in saltbush as reported in Table (1). Al azzeh and Abu – Zanat (2004) reported similar conclusion where saltbush leaves are rich in minerals and more palatable than other parts of the plant. Feces is the main pathway for P excretion in ruminants (McDowell and Arthington, 2005). There were no differences among studied animals in fecal phosphorus. The highest fecal P was recorded in R4 and this finding consistent with the experimental findings which recorded that saltbush had the highest level of P and animals of this group showed highest P intake. However, the least level was in R6 where animals fed traditional ration.

Urinary P was different significantly (P≤0.05) among groups. The highest urinary P was recorded in

R3 and R4. R1 and R2 showed comparable values. The lowest levels were noticed in R5 and R6, respectively. Phosphorus balance was positive in the present study with significant ($p \leq 0.05$) variations .

Feeding saltbush to sheep affecting minerals status in animal body leading to a decrease in Ca and P levels in blood as noticed in Table (5) whether saltbush was introduced as a complementary feed or alone. Also, all studied minerals were in positive balance because alf can alleviate constrains of saltbush in diets. Therefore, special attention should be taken when feeding saltbush to sheep for long periods.

Table (6): Mineral balance (gm/ dl) of sheep fed the experimental roughage

Mineral	Item	R1	R2	R3	R4	R5	R6	± SE
	Intake (g/d)	13.45 ^{d}	15.10 ^{cd}	18.47 ^{b}	23.91 ^{a}	14.45 ^{cd}	16.93 ^{cb}	0.950
Na	Feces (g/d)	1.43	1.58	1.55	2.34	0.98	1.34	0.507
	Urine (g/d)	1.86 ^{cd}	2.87 ^{cb}	5.15 ^{a}	5.11 ^{a}	3.49 ^{b}	0.81 ^{d}	0.425
	Balance	10.16 ^{b}	10.65 ^{b}	11.77 ^{b}	16.46 ^{a}	9.98 ^{b}	14.78 ^{a}	0.916
	Intake (g/d)	18.76 ^{b}	17.67 ^{b}	17.19 ^{b}	18.03 ^{b}	25.74 ^{a}	20.48 ^{b}	1.153
K	Feces (g/d)	2.34 ^{bc}	2.87 ^{ab}	1.11 ^{c}	2.31 ^{bc}	3.83 ^{a}	3.74 ^{a}	0.423
	Urine (g/d)	7.51 ^{ab}	7.29 ^{ab}	6.39 ^{ab}	8.65 ^{a}	2.28 ^{b}	5.26 ^{ab}	1.707
	Balance	8.91 ^{b}	7.51 ^{b}	9.69 ^{b}	7. 07 ^{b}	19.63 ^{a}	11.48 ^{b}	1.497
	Intake (g/d)	9.79 ^{b}	9.36 ^{b}	9.31 ^{b}	10.06 ^{b}	13.37 ^{a}	10.72 ^{b}	0.623
Ca	Feces (g/d)	5.76 ^{a}	3.03 ^{ab}	3.08 ^{ab}	2.23 ^{ab}	1.62 ^{b}	3.59 ^{ab}	1.074
	Urine (g/d)	1.82	1.90	2.38	1.90	2.13	2.01	0.377
	Balance	2.21 ^{c}	4.43 ^{bc}	3.85 ^{bc}	5.93 ^{b}	9.62 ^{a}	5.12 ^{cb}	1.057
	Intake (g/d)	6.45^{c}	7.06^{b}	8.41 ^{b}	10.59 ^{a}	6.83 ^{c}	9.34 ^{ab}	0.415
P	Feces (g/d)	0.41	0.37	0.20	0.41	0.13	0.08	0.109
	Urine (g/d)	0.19^{b}	0.19^{b}	0.36 ^{a}	0.35 ^{a}	0.15 ^{b}	0.11 ^{b}	0.027
	Balance	5.85^{c}	6.50^{c}	7.85 ^{b}	9.83 ^{a}	6.55 ^{c}	9.15 ^{a}	0.364

R1: 75% Alfalfa + 25% Atriplex+ barley , R2: 50 % Alfalfa + 50 % Atriplex+ barley.
R3: 25 % Alfalfa + 75 % Atriplex+ barley , R4: 100 % Atriplex+ barley.
R5: 100 % Alfalfa+ barley , R6: Berseem hay + CFM.
a, b, c and d : values with different letters in the same row means statistically significant at $P<0.05$.

4. Conclusion and recommendation:

From the aforementioned results we could conclude that*:* *Atriplex nummularia* had a pronounced nutritive value as a fodder component when mixed with other plant species for livestock as previously detected in (R1, R2 and R3) rather than its intake alone (R4). The main disadvantages of using *A. nummularia* alone as animal feed are: high in ash and minerals contents and has insufficient energy density for sheep. This fact affecting negatively on its DMI, DCP and TDN as appeared in R4. Finally R2 is nutritious despite the generally low the nutritive value and energy content.

Corresponding author
Afaf M. Fayed
Department of Animal & Poultry Nutrition, Desert Research Center, Mataria, Cairo, Egypt
a_fayed2007@yahoo.com

5. References:

1. Abdel–Malik, W. H.; El–Sayes, M. F.; Khinizy, A. E. M.; and Soliman,E. (2001): Yield and quality of Berseem and Alfalfa cultivated in newly reclaimed sandy soil in response to foliar trace mineral fertilization and its effect on lamb performance. Egyptian J. Nutr. And Feeds (4), Special Issue: 167 – 177.

2. Abu – Zanat, M. M. W. and Tabbaa, M. J. (2005): Effect of feeding Atriplex brows to lactating ewes on milk yield and growth rate of their lambs. Small Ruminant Research, 1-10.
3. Aganga, A. A. ; J. K. Mthetho and S. Tshwenyane. (2003): *Atriplex Nummularia* (Old Man Saltbush) : A Potential Forage Crop for Arid Regions of Botswana. Pakistan Journal of Nutrition 2 (2): 72-75.
4. Ahmed, M.H., Khatab, I.M., Borhami, E.B. and W.G. Fahmy. (2001): Effect of energy source supplementation of the utilization of some desert forage by growing Lambs. J. Adv. Agric Res., 6: 255-277.
5. Alazzah, A. Y. and Abu – Zanat, M. M. (2004): Impact of feeding saltbush (Atriplex sp.) on some mineral concentrations in the blood serum of lactating Awassi ewes. Small Ruminant Research, 54: 81 – 88.
6. Allam, Sabbah, M.; Youssef, K. M.; Ali, M. A. and AboBakr, S. Y. (2006): Using some fodder shrubs and industrial by products in different forms for feeding goats in Sinai. J. Agric. Sci. Mansoura Univ., 31(3): 1371 – 1385.
7. Al-Owaimer A. N.; Zahra, S. M. and Al-Bassam, B. A. (2008): Effect of feeding some types of Atriplex spp. in complete diet on growth performance and digestibility of growing lambs. Res. Butt., No. (161), Food Sci. and Agric. Res. Center, King Saud Univ. pp (5 – 19).
8. Anon, (2009): Introduction of salt tolerant forage production systems to salt – affected lands in Sinai Peninsula in Egypt: a pilot demomstration project. Final Report, DRC, Egypt – ICBA, UAE.
9. A.O. A. C. (1997): Official Methods of Analysis. 16th Ed. Assoc. Offic. Anal. Chem., Arlington, VA.
10. Balbaa, S.I.; S.H. Hilal and A.Y. Zaki (1981): Medicinal plants constituents. 3rd ed. General Organization for University Books, Cairo, Egypt. 644 pp.
11. Bennick, A. (2002) : Interaction of plant polyphenols with salivary proteins. Critical reviews in Oral Biology and Medicine 13, 184 – 196.
12. Ben Salem, H.; Nefzaoui, A.; Makkar, H. P. S.; Hochlef, H.; Ben Salem, I. and Ben Salem, L. (2005b) : Effect of early experience and adaptation period on voluntary intake, digestion and growth in Barbarine lambs given tannin – containing (Acacia cyanophylla Lindl. foliage) or tannin – free (oaten hay) diets. Anim. Feed Sci. and Tech. 122, 59 – 77.
13. Ben Salem,H.; Makkar,H.P.S.; Nefzaoui,A.; Hassayoum,L. and Abidi, S. (2005): Benefit from association of small amounts of tannin – rich shrub foliage (Acacia cyanophylla LindI.) with soya bean meal given as supplements to Barbarine sheep fed on oaten hay. Anim. Feed Sci. Technol., 122: 173 – 186.
14. Berthelot, M. (1959): Estimation of serum urea. Report Chem. Applique 1: 248.
15. Bhatti, J. A., M. Younas, M. Abdullah, M. E. Babar and H. Nawaz. (2009): Feed intake, weight gain and haematology in nili-ravi buffalo heifers fed on mott grass and berseem fodder substituted with saltbush (*atriplex amnicola*). Pakistan Vet. j., 2009, 29(3): 133-137.
16. Blaxter, K. L. (1968): The energy metabolism of ruminants. 2nd Ed. Hutchinson and Co. Lted. London.
17. Bravo, L.; Manas, E. and Caixto, F. S. (1993): Dietary non extractable condensed tannins as indigestible compound. Effect on fecal weight and protein and fat excretion . J. Sci. Food Agric., 63: 68.
18. Brenner, B.; Coe, F. L. and Rector, F. C. jr. (1987): Renal physiology in health and disease. W. B. Saunders, Philadelphia
19. Cheek, P. R. (1995): Endogenous toxins and mycotoxins in forage grasses and their effect on livestock. J. Anim. Sci. 73, 909 – 918.
20. Church, D . C . and W . G . Pond (1982): Basic Animal Nutrition and Feeding , 2 nd ed .Johnwiley and Sons, New York, U. S .A .

21. Cowieson, A. J.; Acamovic, T. and Bedford, M. R. (2004): The effects of phytase and phytic acid on loss of endogenous amino acids and minerals from broiler chickens. British Poultry Sci., 45: 101 – 10
22. Duncan, D. B., (1955): Multiple range and multiple Ftests. Biometrics, 11: 1-42.
23. Eid, E. Y. A. (2003): Feed utilization and performance of animal fed the natural and cultivated fodder shrubs in Sinai. Ph D. Thesis, Fac. Of Agric. Cairo univ. Egypt.
24. El Aich, A.,(1987): Fodder trees and Shrubs in range and farming systems in North Africa. Food and Agricultural (FAO) report. Rome.
25. El – Shaer, H. M. (2010); Halophytes and salt tolerant plants as potential forage for ruminants in the Near East region. Small Ruminant Res. 91: 3- 12.
26. El-Shaer, H. M.; Ali F.T. ; Morcos N.Y.S. ; Emam S.S. and Essawy A.M. (2005): Seasonal changes of some anti-nutritional factors contents of some halophytic shrubs and the effect of processing treatments on their utilization by sheep under desert conditions of Egypt. EgyptianJ.Nutr.Feeds,8 (1) SpecialIssue:417-431.
27. El – Shaer H. M.; Fahmy, A. A.; Abdul Aziz, G. M.; Shalaby, A. S. and Abd El Gawad, A. M. (2001): Nutritional evaluation of non – conventional fattening diets fed to sheep under arid conditions of Egypt. Proceeding of International Conference, Tunisia, 8 -10 Nov.
28. El-Shaer, H.M., Kandil, N.M. and Khamis, H. S. (1991): Salt marsh plants ensiled with dried broiler litter as feedstuff for sheep and goats. J. Agric. Sci. Mansoura Univ., 16(3): 1524 – 1534.
29. Fitcher, L.R. (1993): Grazing ryegrass endophyte associations and their effect on animal health and performance. In: Proc. 2nd . Int. Symp. On Acremonium Grass Interactions: Plenary papers. P. 115. Ag Research. Grasslands Research center, palmerston.
30. Francis, G.; Kerem, Z.; Makkar H. P. S. and Klaus Becker. (2002): The biological action of saponins in animal systems: a review. British Journal of Nutrition, 88, 587–605
31. Getachew, G.; Pittroff, W.; DePeters, E. J.; Putnam, D. H.; Dandekar, A. and Goyal, S. (2008): Influence of tannic acid application on alfalfa hay: in vitro rumen fermentation, serum metabolites and nitrogen balance in sheep. Animal, 2:3, pp381 – 390.
32. Gihad, E. A. and El – Shaer, H. M. (1992): Utilization of halophytes by livestock on rangelands. In: Squires, V. R.; Ayoub, A. T. (Eds.), Halophytes as a source for livestock and for rehabilitation of degraded lands. Kluwer Academic publishers, Dordrecht, pp. 77 – 96.
33. Gitelman, H. (1967): Determination of serum calcium using O-cresalphathalein complexone. Annal. Biochem. 18: 521.
34. Goering, H.K. and P.J. Van Soest (1970): Forage fiber analysis. Agricultural Handbook, No. 379, USDA, Washington. DC, U. S. A.
35. Goodwin, J.F. (1970): Quantitative estimation of inorganic phosphorus. Clin. Chem., 16 (9): 776 – 80.
36. Han, L.K.; Xu B.J, Kimura Y, Zheng ,Y.N. and Okuda, H. (2000): Platycodi radix affects lipid metabolism in mice with high fat dietinduced obesity. Journal of Nutrition 130, 2760–2764.
37. Hassan, A. A. (2009): Effect of some Enrichment and Nawaz biologicaltreatments on a melioration utilization of *Atriplex nummularia* fed by sheep. Egyptian J. Nut. And Feed. (12) : (3) Special Issue : 553-566.
38. Jahani–Azizabada, H.; Mesgaran, D. M.; Valizadeh, R. and Moghaddam, N. H. (2009): Comparison of in vivo with in situ mobile bag and three step enzymatic procedures to evaluate protein disappearance of alfalfa hay and barley grain. Iranian J. of Veterinary Research, Shiraz University, Vol. 10, No. 3, Ser. No. 28, 2009.
39. Karabulut, A.; OzgurOzkan, C.; Kamalak, A. and Canbolat, O. (2006): Comparison of the nutritive value of a native Turkish forages, tumbleweed hay (*Gundelia tournefortii L.*), wheat straw and alfalfa hay using in situ and in vitro

measurements with sheep. Arch Latinoam. Prod. Anim. Vol. 14 (3): 78 – 83.

40. Kearl, L.C. (1982): Nutrient requirements of ruminants in developing countries. Utah Agric. Exp. St., Utah State Univ. Logan, UT, U. S. A.
41. Kincaid, R. (1993):Macroelements for ruminants. In: Church, D. C. (Ed.), Ruminant Animal Digestive Physiology and Nutrition. Prentice – Hall, Englewood Clifs, NJ, pp: 326 – 341.
42. Konig, K. W. R. (1993): Influence of saltbush (Atriplex spp.) as diet component on performance of sheep and goats under semi arid range condition. Ph D. dissertation, Reihe Agrarwissenschaft, Institute for animal production in the tropic and subtropics, Aachen, Germany (ISBN: 3 – 86 111-706-1).
43. Makkar, H.P.S. (2003): Effects and fate of tannins in rumunant animals, adaptation to tannins, and strategies to overcome detrimental effects of feeding tannin-rich feeds. Small Rumin. Res. 49: 241-256.
44. Makkar, H. P. S.; Singh, B. and Dawra, R. K. (1988): Effect of tannin- rich leaves of oak (Quercus incana) on various microbial enzyme activities of the bovine rumen. Br. J. Nutr., 60: 287 – 296.
45. Mansoori, B. and Acamovic, T. (1997): The excretion of minerals from broilers fed tannic acid with and without gelatin. Proc. Spring Meeting of the world poultry Science Association. Pp. 25 – 26 .
46. Masters D. G., N. Edwards, M. Sillence, A. Avery, D.Revell, M. Friend, P. Sanford, G. Saul, C. Beverly and J. Young, (2006): The role of livestock in the management of dryland salinity. Aust. J. Exp. Agri., 46(7): 733- 741.
47. Matsuura, M. (2001): Saponins in garlic as modifiers of the risk of cardiovascular disease. Journal of Nutrition 131, s1000S–1005S.
48. McDowell, L. R. and Arthington, J.D. (2005): Minerals for grazing ruminants in tropical regions. 5th Ed. 86pp. University of Florida, Gainesville, F1.
49. McDowell, L. R. (1992): Minerals in animal and human nutrition. Academic Press, New York, pp. 265 – 293 .
50. McMeniman, N. P. (1976) : Studies on the supplementary feeding of sheep fed mulga under pen conditions. Australian J. of Experimental Agricultural and Animal Husbandry. 16: 818 – 822.
51. Mehrez, A. Z.; Soliman, E. M.; El – Ayek, M. Y.; El Ayouty, E. A. and El Kholany, M. E. (2001): Influence of roughage to concentrate ratio and type of roughage on digestibility, some rumen parameters and fiber fractions degradability of treated rations with ruminants. Egyptian J. Nutrition and feeds (4). Special Issue: 193
52. Mulligan, F. J.; Caffrey, P. J.; rath,, M.; Callan, J. J. and O ' Mara, F. P. (2001): The relationship between feeding level, rumen particulate and fluid turnover rate and the digestibility of soya hulls in cattle and sheep (including a comparison of Cr-mordanted soya hul;ls and Cr2o3 as particulate markers in cattle. Livestock Production Science 70,191 – 202.
53. NRC (National Research Council), (1980): Mineral Tolerance Of Domestic Animals. Washington, D. C. National Academy Press.
54. NRC (National Research Council), (1985); Nutrient Requirements of sheep. National Academy Press, Washington, DC, pp. 45 – 48.
55. Porter, L.J.; L.N. Hrstich and B.G. Chan (1986): The conversion of procyanidins and prodelphinidins to cyaniding and delphinidin. Phytochemistry 1: 223- 230.
56. Potter, S.M.; R. Jimenez-Flores; J. Pollack; T.A. Lone and M.D. Berber-Jemenez (1993): Protein saponin interaction and its influence on blood lipids. J. Agric. Food Chem. 41: 1287 – 1291.
57. Rankins, Jr., D.L.; G.S. Smith and D.M. Hallford (1991): Altered metabolic hormones, impaired nitrogen retention and hepatotoxicosis in lambs fed Kochia scoparia hay. J. Anim. Sci. 69: 2932.
58. Rasool, E.; Rafique, S.; Haq, I. U.; Khan, A. G.; Thomson, E. F. (1996): Impact of fourwing saltbush on feed and water

intake and on blood serum profile in sheep. Asian – Australian. J. Anim. Sci, 9: 123 – 126.

59. Reed, J. D. (1995): Nutritional toxicology of tannins and related polyphenols in forage legumes. J. Anim. Sci. 73: 1516 – 1528 .
60. Reinhold, J.G. (1953): Total protein, albumin and globulin. In: M. Sleigson ed.: Standard Methods of Clinical Chemistry. Academic Press, New York, vol. 1: p. 88.
61. Rodkey, F.L. (1965): Separation and determination of the total globulins of human serum. Clin. Chem. 11: 488 – 494.
62. Roeschlau, P.; E. Bernt and W.J. Gruber (1974): Estimation of cholesterol. Clin. Chem. Clin. Biochem. 12: 403.
63. Rogosic, J.; R.E. Estell and D. Skobic (2006): Role of species diversity and secondary compound complementarity on diet selection of Mediterranean shrubs by goats. J. Chem. Ecol. 32: 1279 – 1287 .
64. Romero, M.J.; J. Madrid; F. Hernandez and J.J. Ceron (2000) : Digestibility and Voluntary Intake of Vine Leaves (Vitis vinifera L.) by sheep, Small Rumin. Res. 38: (2) 191-195.
65. Samanta, A. K.; Singh, K. K; Das, M. M.; Maity, S. B and Kundu, S. S. (2003) : Effect of complete feed block on nutrient utilization and rumen fermentation in Barbari goats. J. small Rum. Reas., 48: 95 – 102.
66. SAS (2000) : Statistical analysis systems, release 8.01 Statistical analysis systems institute Inc., Cary, NC
67. Seelig, H.P. and H. Wust (1969): Determination of serum creatinine. Arztl Labor, 15: 34.
68. Shawket, S.M. (1999): Effect of energy level supplementation on the utilization of some pasture plants by goats. J. Agric. Sci. Mansoura Univ., 24: 4565-4573.
69. Shawket, M. Safinaz ; Ahmed, M. H. Borhami, B. E. and Allam, A. M. (2002): A comparative study on the performance of lambs and goats fed on halophytic forage and supplemented with different sources of energy under arid and semi – arid conditions. Int. Symp. On Optimum Resources Utilization In Salt – Affected Ecosystems In Arid and Semi – Arid Regions, Cairo, 8 – 11 Apr. 2002.
70. Shawket, M. Safinaz and Ahmed, M. H. (2009): Effect of prolonged feeding Atriplex (saltbush) to camels on digestibility, nutritive value and nitrogen utilization. Egyptian J. Nutr. And Feeds 12 (3) Special Issue: 205- 214.
71. Shawket, M. Safinaz ; Kewan, Z. K.; Nour, M. A. and Mamdouh, A. A.S. (2005): Atriplex and Acacia shrubs as feed stuffs for young male camels and Egyptian semi – arid condition. Egyptian J. Nutr. And Feeds (8): (1) Special Issue: 225 – 241.
72. Silanikove, N.; N. Gilboa; A. Perevolotsky and Z. Nitsan (1996): Goats fed tannin–containing leaves do not exhibit toxic syndromes. Small Rumin. Res., 21(3): 195–201.
73. Trinder, P. (1969): Quantitative enzymatic–colorimetric determination of total and HDL cholesterol in serum or plasma. Ann. Clin. Biochem. 6:24.
74. Warner, A. C. J. (1964): Production of volatial fatty acids ln the rumens methods of measurments.Nut. Abst. Rev., 34 : 39.
75. Warner, B. E. and Casson, T. (1992).:Performance of sheep grazing salt tolerant forages on revegetated salt – land. Aust. Soc. Anim. Prod., 19: 237 – 241.
76. Weston, R. H.; Hogan, J. P. and Hemsly, J. H. (1970): Some aspects of the digestion of *Atriplex nummularia* by sheep. Aust. Soc. Anim. Prod., 8: 517.
77. Wilson, A. D. (1996): The intake and excretion of sodium by sheep fed on species of Atriplex (saltbush) and Kochia (Bluebush). Aust. J. Agric. Res., 17: 155 – 163. Wilson, R. T. (1992): Factors affecting weight and growth in one–humped camels. Proc. 1st Camel Conf. Dubai, UAE. Pub. R & W.
78. Wilkison, J.H.; D.N. Baron; D.W. Moss and P.G. Walker (1972): Standardization of clinical enzyme assays: A reference method for aspartate and alanine transaminases. J. Clin. Pathol., 25: 940.

79. Wina, E.; Tangedjaja, B.; Palmer, B. and Brooker, J. D. (1999):The effect of drying conditions on condensed tannin estimates in *callindra calothyrsus*. In: Proc. Internat. Workshop Tannins in livestock and human nutrition. Adelaide, Australia, pp. 106 – 110 .

11/25/2010

Oxidative stress in brains of rats intoxicated with aluminum and the neuromodulating effect of different forms of sage

EL-Kholy, W.M.; EL-Habibi, E.M. and Mousa, A.T.
Zoology Dept., Faculty of Science, Mansoura University, Mansoura, Egypt

ABSTRACT: The present study was designed to investigate the role of oxidative stress and the status of antioxidant system in the management of aluminum chloride (AlCl3) induced brain toxicity in rats and further to elucidate the potential role of three forms of *Salvia officinalis* (sage) in alleviating such negative effects. The results revealed that the levels of thiobarbituric acid reactive substances (TBARS) and protein carbonyl (PC) were significantly increased, while the activities of superoxide dismutase (SOD) and catalase (CAT) as well as the reduced glutathione (GSH) content were significantly decreased in the cerebral cortex (Co) and hippocampus (Hip) of rats intoxicated with $AlCl_3$. Regarding the lipid profile, total lipids (TL), total cholesterol (TC) and triglycerides (TG) were significantly increased in serum and the mentioned brain regions, while phospholipids (PL), total protein (TP) and serum HDL-C were significantly decreased in $AlCl_3$ group. Additionally, serum and brain regions acetylcholinesterase (AChE) as well as alkaline phosphatase (ALP) and acid phosphatase (ACP) activities were significantly increased. On the other hand, the results exhibited that, sage when given in any form along with $AlCl_3$ was able to regulate the mentioned parameters and the values returned close to the normal ones. It can be concluded that Al-induced neuronal oxidative stress and inhibition of the antioxidant system, and the consequent disturbed lipid profile, total protein and enzyme activities could be the mechanisms of $AlCl_3$ neurotoxicity. Moreover, the results suggested that the different sage forms, by their antioxidant constituents, could be able to antagonize Al neurotoxicity perhaps by reducing the oxidative stress and improving the antioxidant status and particularly by inhibiting the acetylcholinesterase activity, thus may improve memory and other brain cognitive activities.

[EL-Kholy, W.M.; EL-Habibi, E.M. and Mousa, A.T. Oxidative stress in brains of rats intoxicated with aluminum and the neuromodulating effect of different forms of sage. Journal of American Science 2010;6(12):1462-1474]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: Aluminum neurotoxicity- Alzheimer's disease- *Salvia officinalis* - Lipid peroxidation - ntioxidants-acetylcholinesterase

INTRODUCTION

Aluminum has been implicated in many human neurodegenerative diseases; various investigations have suggested that Alzheimer's disease (AD) is more common in areas where Al content in water supplies is the highest **(Lynch *et al*., 2000)**. Alzheimer's disease is a complex, multifactor, heterogeneous mental illness, which is characterized by an age-dependent loss of memory and an impairment of multiple cognitive functions **(Mattson, 2004)** and has been shown to be associated with both plaques and tangles in the brain. Indeed, the brain is a target of Al toxicity which can alter blood-brain barrier (BBB) mediating Al transport to the brain **(Zatta *et al*., 2002a)** and gets deposited in the cortex **(Platt *et al*., 2001)** and hippocampus **(Struys-Ponsar *et al*., 1997)**. This can be occurring by altering the physiological ligands present at these barriers in states **(Yokel, 2001)**.

Possible mechanisms of Al induced neurotoxicity have been related to cell damage via free radical production and oxidative stress **(Kumar *et al*., 2009a, b)**. High aluminum levels exposure leads to increased central nervous system (CNS) Al concentrations that altered CNS concentrations of the essential trace elements; iron and manganese and increased the susceptibility of CNS to lipid peroxidation (LPx) **(Oteiza *et al*., 1993)**.

Oxidative stress, caused by reactive oxygen species (ROS), is known to cause the oxidation of biomolecules leading to cellular damage. Increased lipid peroxidation (LPx) is the major consequence associated with oxidative stress. It is also speculated to be pathologically important in various neurodegenerative processes including cognitive deficits that occur during normal cerebral aging, Alzheimer's (AD), and Parkinson's diseases **(Gray *et al*., 2003)**. Alternatively, the most remarkable biochemical change in AD patients is a reduction of acetylcholine (ACh) levels in the hippocampus and

cortex of the brain **(Jaen *et al*., 1996)**. Therefore, inhibition of acetylcholinesterase (AChE), the enzyme responsible for hydrolysis of ACh at the cholinergic synapse, is currently the most established approach to treating AD **(Akhondzadeh *et al*., 2003)**.

On the other hand, *Salvia officinalis* (sage) specially the oil had apparent dual cholinergic activity, as it was active on both, AChE and butyrylcholinesterase (BuChE) **(Savelev *et al.*, 2004).** Besides the cholinergic activity, there has already been a wider range of activities reported for the genus *Salvia*, which may be relevant for CNS disorders. These include antioxidant **(Celik and Isik, 2008 and Carla *et al*., 2009),** nicotinic activity **(Wake *et al*., 2000),** anti-inflammatory properties **(Moretti *et al*., 1997), and** glutamergic activities **(Kuang and Xiang, 1994)**. The essential oils of the plant, also, tested for its memory-enhancing effect **(Akhondzadeh *et al*., 2003)**. Therefore, the main goal of the present study was to examine the possible mechanisms by which Al exposure could induce Alzheimer-like condition related alterations in brain of male rats, and extend to investigate the beneficial effects of sage in preventing or modulating these risks.

Material and methods

Chemicals:

Aluminum Chloride ($AlCl_3$) was obtained from agents of Sigma Chemicals (St. Louis, MO, USA). *Salvia officinalis* (sage) oil was obtained from (NATURE'S ALCHEMY) distributed by LOTUS BRANDS, USA. Dried leaves of sage, for preparations of sage tea (water extract and ethanolic extract), were purchased from a local herb market. The taxonomic identity of the plant was confirmed by the botanist of the Department of Botany, Faculty of Science, Mansoura University, Mansoura, Egypt. All other chemicals were purchased locally and were of analytical reagent grade.

Sage extracts preparation:

1- Sage water extract (sage tea): was routinely prepared by pouring 150 ml boiling water onto 2 g of dried grounded leaves and allowing it to steep for 5 min. **(Lima *et al*., 2005)**.

2- Sage ethanolic extract: was prepared according to the method described by **Eidi *et al*. (2006)**. Dried grounded leaves of *Salvia officinalis* (60 g) were subjected to extraction with 300 ml of ethanol (80%) in a glass container for 72 h. The extract was decanted and filtered through Whatman No 1 filter paper into a clean flask. This same procedure was repeated a further two times. The solvent was evaporated using a rotary evaporator, and then the flask was weighed to determine dried weight of extract. The supernatant was reconstituted using 53% ethanol and assayed using serial dilutions and the dose was calculated according to **(Ghosh, 1971)**

3- Sage oil: diluted 1:2 in sunflower oil according to **Perry *et al.* (2002)**.

Experimental animals:

This study was carried out on 48 adult male albino rats weighing 130 ±10 g b.w., supplied by The Urology & Nephrology Center; Mansoura University. The rats were maintained under controlled humidity; temperature (25 ± 2˚C) and light (12h light/ 12h dark). They were fed standard commercial rodent pellet diet and water *ad libitum* (free access to water and food).

Experimental Protocol:

After one week of acclimatization, the rats were divided into 8 groups consisting of 6 animals each. All treatments were continued for 90 days as follows:

1- Normal control
2- Sage water extract (given instead of drinking water) according to **Lima *et al*. (2005)**.
3- Sage ethanolic extract (given orally by stomach tube as 0.1 ml/kg b.w.) **(Akhondzadeh *et al*., 2003)**.
4- Sage oil group (given orally by stomach tube as 100 µl/ kg b.w.) every other day **(Perry *et al*., 2002)**.
5- Aluminum (Al) treated group (mixed with diet as 100mg $AlCl_3$/ kg b.w.) **(Bilkei, 1993)**.
6- Al + sage water extract group (given as in groups 5&2 respectively).
7- Al + sage ethanolic extract group (given as in groups 5&3 respectively).
8- Al + sage oil group (given as in groups 5&4 respectively).

Sample preparation:

At the end of the experimental period, overnight-fasted animals were decapitated, blood samples were collected and sera were separated and stored at -20°C until biochemical assay. The brain was then gently removed; the cerebral cortex and hippocampus were separated on an ice-chilled glass

plate as described elsewhere **(Nayak and Chatterjee, 2001)**. The tissue samples were quickly frozen on dry ice, weighed, and stored at -80°C until biochemical assay. Cortex and hippocampus were chosen for the present study because; aluminum affects more severely the cortex and hippocampus regions than any other area of the central nervous system **(Urano *et al*., 1997)**. Also, these brain regions are known to be particularly susceptible in Alzheimer's disease, and have an important role in learning and memory functions **(Bihaqi *et al*., 2009)**.

Biochemical analysis:

Determination of lipid peroxidation product thiobarbituric acid reactive substances (TBARS) was carried out according to the method of **(Ohkawa *et al*., 1982)**. Meanwhile, protein carbonyl was measured spectrophotometrically according to the method of **(Smith *et al*., 1991)**. On the other hand, superoxide dismutase (SOD) and catalase (CAT) activities were determined following the methods of **(Nishikimi *et al*., 1972)** and **(Bock *et al*., 1980)**, respectively. Additionally, reduced glutathione (GSH) content was determined spectrophotometrically according to the method of **(Prins and Loose, 1969)** as well as Acetylcholinesterase (AChE) activity was measured according to the method of **(Ellman *et al*., 1961)**. On the other hand, alkaline phosphatase (ALP), acid phosphatase (ACP), total protein (TP), total lipids (TL), phospholipids (PL), triglycerides (TG), total cholesterol (TC) in (serum, cortex and hippocampus) and serum HDL cholesterol were determined by using commercial kits from (Biodiagnostic, 29 Tahreer St., Dokki, Giza, Egypt).

Statistical analysis:

Data were presented as means ± standard error (SE). The statistical analysis was performed with one-way analysis of variance (ANOVA) using SPSS (version 17) software package for Windows followed by *Dunken test*. A p-value of less than o.o5 was considered statistically significant.

RESULTS

Cortex and Hippocampus Lipid Peroxidation (LPx), Protein Carbonyl (PC) and Antioxidants:

As observed in table (1), the statistical analysis showed that the level of cortex and hippocampus TBARS and PC were significantly increased by Al intoxication in comparison with the control value.

Concerning sage only, there were significant decreases and increases in hippocampus TBARS level and catalase activity respectively in the groups administered (ethanolic extract and oil) comparing to the normal control group. Regarding the antioxidants, the data indicated that, Al group exhibited significant reduction in cortex and hippocampus SOD, CAT activities as well as GSH content compared to normal control group.

Sage administration to rats intoxicated with Al caused a significant reduction in the elevated cortex and hippocampus TBARS and PC concentrations compared to Al intoxicated group. The reduction in hippocampus TBARS reached levels below the normal ones and was significant, only, in (Al + sage ethanolic extract) group. On the other hand, daily administration of sage preparations to Al intoxicated animals revised the decreases in the cortex and hippocampus SOD and CAT activities as well as GSH contents to marked increases compared to Al intoxicated group.

Indeed, the modulating effects of sage preparations on both the oxidative stress markers (LPx and PC) and the antioxidant system (SOD, CAT and GSH) arrived them to values within the normal ranges where non significant alterations were seen in comparison with the normal group except a significant reduction in Hip LPx of (Al + sage ethanolic extract) and a significant increase in Hip CAT of (Al + sage water extract) and (Al + sage oil) groups as well as in Hip GSH of (Al + sage oil) group.

Serum, Cortex and Hippocampus Total Lipids (TL), Total Cholesterol (TC), Triglycerides (TG) and Phospholipids (PL) Concentrations:

The data presented in table (2), exhibited that, serum, cortex and hippocampus total lipids (TL), total cholesterol (TC), and triglyceride (TG) contents showed significant increases, but phospholipids (PL) exhibited significant reductions in Al intoxicated animals in comparison with normal control.

Concerning sage only, cortex phospholipids content was significantly increased in the groups administered (sage water extract and oil) and in hippocampus of (sage ethanolic extract and oil) groups comparing to the normal control group. But, significant decreases were observed in TC of cortex in (sage water extract and ethanolic extract) as well as Hip TC and TG in the groups administered (sage oil). Such results referred to the benefits of sage preparations, specially the oil for reducing TC and increasing brain PL.

On the other hand, concomitant administration of sage (different preparations) with Al reduced the elevation of serum, cortex and hippocampus TL, TC and TG contents and enhanced serum, cortex and hippocampus PL approaching most of them to the normal values except in case of cortex PL only of (Al + sage water extract) which showed a non-significant increase compared to Al intoxicated animals indicating no protection.

However, the reduction in serum, cortex and Hip TL levels reached to values within normal levels, except Hip TL in (Al + sage ethanolic extract) which, still, exhibited a significant reduction compared to normal control group. Regarding TC, the reduction was, still, significant in both, cortex of (Al + sage ethanolic extract) and hippocampus of (Al + sage oil) groups comparing to control one. Concerning TG, the reduction arrived to values significantly lower than normal in serum TG of (Al + sage ethanolic extract) and Hip TG of (Al + all sage preparations) groups comparing to control one.

On the other hand, the enhancement in serum and cortex PL by sage was non-significantly changed in (Al + all sage preparations) compared to normal control. But in the Hip, PL reached to values that still significantly lower than normal in all (Al + sage different preparations) groups.

Serum HDL-C, Serum, Cortex and Hippocampus Total Protein (TP) Content:

As seen in table (3) serum, cortex and hippocampus total protein content and serum HDL-C showed highly significant decreases in Al intoxicated animals compared to the normal control group. Concerning sage only, total protein exhibited a significant increase in hippocampus of the groups administered all sage preparations compared to the normal control group.

Administration of different preparations of sage to Al intoxicated animals reversed the decrement in serum, cortex and hippocampus total protein contents and serum HDL-C to significant increases compared to Al group. But, there were non-significant elevations in serum HDL-C conc. of (Al + all sage preparations) except (Al + sage water extract) group, exhibited a non-significant reduction compared to normal control group. Interestingly, the elevation in TP arrived to the normal levels except hippocampus total protein in (Al + sage water extract) and (Al + sage ethanolic extract) which showed significant increases compared to the normal control group. These results indicated pronounced ameliorating effects of sage oil, followed by sage ethanolic extract, the water extract showed the lowest protective effect

Serum, Cortex and Hippocampus Acetylcholinesterase (AChE), Alkaline phosphatase (ALP) and Acid Phosphatase (ACP) Activities:

As shown in table (4), in Al intoxicated rats, there were significant elevations in serum, cortex and hippocampus AChE, ALP and ACP activities compared to normal control ones. Concerning sage only, ALP and ACP activities were significantly decreased in hippocampus of (sage oil) group only compared to normal control one.

On the other hand, significant reductions were seen in all (Al + sage treated) groups serum, cortex and hippocampus AChE, ALP and ACP activities comparing to Al intoxicated group, arriving the values within the normal levels, with the exception of AChE activity, which was still significantly higher than normal control in cortex of (Al + sage ethanolic extract) group and hippocampus in (Al + sage oil) group in comparison with normal control.

Regarding ALP activity, it was still significantly higher than the control activities in all (Al + sage preparations) groups except serum (Al + sage water extract) and cortex (Al + sage ethanolic extract) groups which showed non-significant elevations (within normal ranges) when compared to control.

Concerning serum, cortex and Hip ACP activities, marked ameliorations were seen, where non-significant decreases and increases were shown respectively compared to normal control animals except hippocampus ACP activity of (Al + sage ethanolic extract) and (Al + sage oil) groups which were still, significantly declined comparing to the normal control.

DISCUSSION

Aluminium has an association with the etiology of Alzheimer's disease and some other neurodegenerative diseases. It exerts its toxic effect on nervous system especially at high concentration, causing loss of memory, speech disturbances, dysparaxia, tremors, jerking movement's impaired muscular coordination and paralysis **(Drago *et al.*, 2008)**. *Salvia officinalis* (common sage) is a medicinal plant that has strong antioxidant properties **(Baricevic and Bartol, 2000)**. For that reason, the present study aimed to look into

the antioxidant potential of various sage preparations against Al neurotoxicity.

In the present study, there were significant increases in the oxidative stress markers lipid peroxidation (LPx) and protein carbonyl (PC) contents following Al exposure for 90 days in both cerebral cortex and hippocampus regions of rats. Such results are in harmony with those obtained by **Deloncle *et al.* (1999)** and **Johnson *et al.* (2005**) who reported that the neurotoxicity of Al may be a result of LPx.. Furthermore, **Nehru and Anand (2005)** reported a significant increase in brain thiobarbituric acid reactive substances in rats after stimulation by Al salts which was known to be bound by the Fe^{3+} carrying protein transferrin, thus reducing the binding of Fe^{2+} and increasing free intracellular Fe^{2+} that causes the peroxidation of membrane lipids and consequently membrane damage. Aluminum, being an inert metal, has been suggested to induce oxidative damage indirectly by potentiating the peroxidative effect of Fe^{2+}. It promotes reactive oxygen species (ROS) formation**.** ROS subsequently attack almost all cell components including membrane lipids thus producing lipid peroxidation **(Christen, 2000)**.

The findings of the present study, also, showed that the rise in LPx in Al treated rats was accompanied by concomitant decrease in the activity of some antioxidant enzymes involved in the detoxification of ROS, namely SOD, CAT as well as the level of GSH in the cortex and hippocampus tissues comparing with the control declaring the prooxidant effect of Al. These findings agreed with the antecedent studies of **Savory *et al.* (2003) and Johnson *et al.* (2005)** whom showed that Al exposure enhanced the neuronal lipid peroxidative damage with concomitant alterations in the enzymatic antioxidant defense status, thus having serious bearing on the functional and structural development of the central nervous system **(Dua and Gill, 2001)**. Similar data recorded a decrease in the antioxidants such as GSH **(Wu and Cederbaum, 2003)** and SOD activity **(Yousef, 2004)** in the brain of Al exposed rats **(Chainy *et al.*, 1996)** and human **(Dua and Gill, 2001)**.

Moreover, such results are consistent with the studies indicated that Al intake produced an oxidative stress-related change, contributed to its neurotoxicity **(Flora *et al.*, 2003)**. However, in rats, a significant relationship between Al exposure and the presence of oxidative stress was established also by **Gómez *et al.* (2005)**. This could be caused by inflicting damage to membrane lipids, proteins and antioxidative enzyme defense system **(Jyoti *et al.*, 2007)**.

The elevation of LPx in the cortex and hippocampus in the present study and other ones **(Dua and Gill, 2001)** suggested participation of free-radical-induced oxidative cell injury in mediating neurotoxicity of Al. Lipid peroxidation of biological membranes results in the loss of membrane fluidity, changes in membrane potential, an increase in membrane permeability and alterations in receptor functions **(Nehru and Anand, 2005 and Albendea *et al.*, 2007)**.

However, the increased Al concentration could deleteriously affect the neurons, leading to depletion of antioxidants and metal ions **(Kumar *et al.*, 2008)** through the induction of free radicals, that exhausting SOD and CAT which function as blockers of free radical processes. These results are in accordance with (**Nehru and Anand, 2005)** who recorded a significant decrease in the activities of SOD and CAT in brain of rats after Al treatment. Alternatively, the decreased enzyme activities could be related to a reduced synthesis of the enzyme proteins as a result of higher intracellular concentrations of Al **(Albendea *et al.*, 2007)**.

The data obtained by the present study illustrated, further, that administration of water, ethanolic extracts of *S. officinalis* as well as sage essential oil to Al treated rats caused a significant decrease in the level of TBARS and protein carbonyl in the cerebral cortex and hippocampus and elevated the SOD and CAT enzymes activities and GSH contents when compared with Al intoxicated rats. Moreover, the plant extracts and oil significantly, improved or restored the normal activities of the antioxidant enzymes (SOD and CAT) and GSH in both of the cortex and hippocampus regions as compared to normal control.

Generally, the antioxidant effects of sage extracts have often been attributed to phenolic and monoterpenic compounds **(Ren *et al.*, 2003)**. Flavonoids are a diverse group of polyphenols **(Havsteen, 2002)** rosmarinic acid being the most representative that possess several modulatory effects, either inducing or decreasing the expression of SOD and CAT enzymes depending on structure, concentration, and assay conditions. Rosmarinic acid is the predominant phenolic compound in sage **(Lima *et al.*, 2005)** and its effects was attributed to the compound's antioxidant properties acting as scavenger of reactive oxygen species **(Zheng *et al.*, 2004)**.

In point of fact, all of the tested forms of sage have previously shown to potently suppress hydroxyl

radical formation **(Kosar *et al*., 2005)**. Additionally, the protection of cell viability conferred by sage extracts seemed to be due, mainly, to their ability to prevent GSH depletion by their main phenolic compounds, rosmarinic acid and luteolin-7-glucoside. Nevertheless, unknown compounds other than phenolics also seem to contribute to the antioxidant effects of sage on basal GSH levels **(Lima *et al*., 2007)**. However, the later authors **(Lima *et al*., 2007)** besides other ones **Brandstetter *et al*. (2009)** showed the ability of sage (mainly the methanolic extract) to increase basal GSH levels, probably by the induction of glutathione synthesis. However, sage ethanolic extract strongly decreased the level of lipid peroxidation compared to Al intoxicated rats, such effect which may be due to its free radical scavenging potential induced by ethanol where various species of Salvia has inhibitory and quenching impact on lipid peroxidation along with enhancement of antioxidant defense system in brain tissue of rats treated with Aluminum **(Zupkó *et al*., 2001)**.

In fact, the glutathione peroxidase system consists of several components, including GSH that effectively remove (hydrogen peroxide) and serves as a cofactor for glutathione transferase, which helps remove certain drugs and chemicals and other reactive molecules from the cells. Moreover, GSH can interact directly with certain ROS (hydroxyl radical) to detoxify them, as well as performing other critical activities in the cell. So, GSH is probably the most important antioxidant present in cells. *Salvia officinalis* had a potent increasing effect on GSH content in brain compared to Al treated rats. Also, the enzymatic antioxidant defense system including SOD and CAT which can decompose superoxide and hydrogen peroxide in the cells are the main defense against oxidative injuries. The decreased level of these biomolecules may lead to increased severity of Al toxicities in the brain **(Tripathi *et al.*, 2009)**. Most likely, the sage tea effects observed, herein, was a result of interactions and synergisms among the different compounds and metabolites present, which makes it difficult to attribute them to any particular compound or family of compounds **(Lima *et al*., 2005).**

2- Lipid profiles

The present data indicated that serum, cortex and hippocampus total lipids (TL), total cholesterol, (TC) and triglycerides, (TG) were significantly increased by aluminum ingestion, while phospholipids (PL) and serum HDL-C levels were decreased; such results are in accordance with the results reported by **Yousef (2004)**. Similarly, **Wilhelm *et al*. (1996)** suggested that long-term exposure to Al specifically altered the brain lipid/phospholipid metabolism and/or their transfer to various membrane systems and resulted in significant changes in phospholipid classes and in cholesterol contents of the rat brain. Alternatively, studies in monkeys revealed the chronic effects of Al exposure on brain physiology, including alteration of the lipid composition and the activities of various membrane-bound enzymes; Al was found to decrease significantly the total lipid, glycolipid, and phospholipid concentrations in the primate brain **(Sarin *et al*., 1997)**. In addition, cholesterol and cholesterol/phospholipid ratios were shown to be remarkably increased, indicating a relevant loss of membrane integrity, and consequently a strong effect of Al on the activity/functionality of various membrane-bound enzymes, including AChE **(Atack *et al*., 1983)**. Similarly, the long-term exposure to $AlCl_3$ was shown to result in a 60 % decrease in the total phospholipids content while total cholesterol content increased by 55 %. It is possible that this altered lipid /phospholipid content and composition could affect the insulation properties of the myelin. The finding may thus have some bearing on loss of short-term memory in Alzheimer's disease .

The increase in serum cholesterol and total lipids due to Al administration indicated, also, a loss of membrane integrity **(Sarin *et al*., 1997)**. This was further confirmed when Al was found to have a significant effect on the various membrane-bound enzymes **(Newairy *et al*., 2009).** One possible way to explain the relatively more intense lipid peroxidation due to Al is that the susceptibility to catalyze oxidative cascades which is much easier for lipids than it is for proteins. Moreover, Al exhibited high affinity for phosphate groups and binds to the phospholipid head groups through electrostatic forces, which may disturb the order as well as the other dynamic parameters of the lipid bilayer **(Martin, 1986)**. From the foregoing results it is clear that Al resulted in significant reduction in the phospholipid content accompanied by major compositional changes, which is consistent with membrane hypothesis of AD. According to this hypothesis, in order to make up for the choline deficiency, the neurons try to extract choline from choline containing phospholipids. These results leads to the disruption of cell membranes and ultimately to neuronal cell death **(Roth *et al*., 1995)**. Such elevations in TL, TC together with the reduction of HDL-C following Al intoxication shown herein, represent risk factors for atherosclerosis and decreased blood flow to the brain (ischemia) which may be added to the mechanisms involved in Al-induced neurotoxicity.

On the other hand, the present study showed that treatment of rats with $AlCl_3$ plus different preparations of sage decreased serum and brain total lipids, total cholesterol and triglycerides and enhanced phospholipids and serum HDL-C levels compared to $AlCl_3$ intoxicated group. These results are in agreement with **Ninomiya *et al.* (2004)** and **Carla *et al.* (2009)** who found that oral administration of sage significantly lowered total cholesterol, triglycerides in serum of rats; and increased serum levels of HDL-C. Also, **Akram and Maryam (2009**) showed that oral administration of sage water extract significantly decreased serum cholesterol and triglycerides. These results suggested that *S. officinalis* tea consumption is accountable for the improvement of the lipid profile inducing an increase in the HDL-C particles, contributing, therefore, positively to the control of the dyslipidaemia observed in Type 2 diabetes but also related to other diseases **(Nesto, 2005)**. However, sage modulating results may attributed to several sage natural components have been shown to act on cholesterol metabolism by reducing its absorption or its synthesis, such as catechins **(Plana *et al*., 2008)**. This is in addition to the polyphenols, especially, phenolic rosmarinic acid in sage which has potent antioxidant effects protecting membrane lipids of fatty acids and phospholipids from oxidative stress (**Lima *et al*., 2005)**.

3-Total protein content

The data of the present work showed that Al intoxication caused a significant decrease in the protein contents of serum, cortex and hippocampus protein. These results are in good accordance with those obtained by **Nayak *et al.* (2006) and Newairy *et al.* (2009)**.

Thus, the observed alterations could be attributed to direct or indirect effects of aluminum on protein synthesis and breakdown and interaction with neurotransmitter synthesis and degradation, through a series of reactions that depends on many enzymatic pathways and regulatory mechanisms **(Goncalves and Silva, 2007)**.

The decline in the levels of protein in Al-treated rats is in close agreement with **Chinoy and Memon (2001)** and might be due to changes in protein synthesis and/or metabolism and could be, also, attributed on one hand to an under nutrition and on the other hand to a reduction of the protein synthesis in the liver resulted from Al intoxication as well as to reduced enzymes of protein synthesis as a result of higher intracellular concentration of Al **(Tripathi *et al*., 2009)**.

Alternatively, since GSH has been reported to be involved in protein and DNA biosynthesis so, the reduction in its content and in the antioxidant enzymes (SOD and CAT) resulted from Al intoxication may partly explain the decline in the total protein content. Additionally, Al induced reactive oxygen species (ROS) formation and promoted oxidative stress **(Exley, 2004 and Kumar *et al*., 2009 a,b)** enhancing peroxidative damage to lipids and proteins of the cellular membranes **(Julka and Jill, 1996)** is another suggestion for protein decline. Such an explanation, which was confirmed by **Jyoti *et al.* (2007),** indicated that Al exposure caused oxidative stress inflicting damage to membrane lipids, proteins and antioxdative enzyme defense system. Exposure of proteins to free radicals leads to gross structural and functional modifications including protein fragmentation, formation of cross-links and aggregates, protein peroxides generation, and enzymatic oxidation and degradation or clearance **(Albendea *et al*., 2007)**.

On the other side, the results, herein, indicated that all sage preparations enhanced the protein contents in serum and cerebral cortex and hippocampus of Al intoxicated rats reached them within or near the normal levels comparing to the control group. Regarding the protective mechanisms of sage, it has been speculated that antioxidative properties of sage components may be primarily involved, since changes related to the oxidative stress, where lipid peroxidation and oxidative DNA damage, were shown to be eliminated by sage tea consumption; possibly due, in part, to scavenging the nitrogen oxide or their radical derivatives **(Lima *et al*., 2005)**. Since the antioxidants play an important role in the regulation and maintenance of metabolism in the body against oxidative stress. So, sage constituents with their antioxidant properties overcame the lower in the total protein content perhaps by preventing oxidative stress and protein breakdown and enhancing protein synthesis and antioxidant system. Not only phenolic **(Durling and Catchpole, 2007)** or other flavonoids **(Wang *et al*., 2001)**, but all other sage components known to be participating in the series of reactions, hence the observed improvement in the present results may be due to all those components.

4- Acetylcholine system:

Cholinesterases are a large family of enzymatic proteins widely distributed throughout both neuronal and non-neuronal tissues. In Alzheimer's disease (AD), analytical as well as epidemiological studies suggested an implication of an abnormal focal accumulation of Al in the brain **(Zatta *et al*., 2002b)**.

In this devastating disease, Al may interfere with various biochemical processes including acetylcholine metabolism, and can thus act as a possible etiopathogenic cofactor. Aluminum is known to interfere with cholinergic **(Amador *et al*., 2001)**, glutamatergic and gamma-aminobutyric acid neurotransmission. A disturbance in the enzyme activities involved in the acetylcholine metabolism has, also, been reported following Al exposure **(Cordeiro *et al*., 2003)**.

In the present work, the data obtained showed that Al intoxication caused significant activation of AChE in the serum, cortex and hippocampus. Prior studies have reported the influence of Al on the metabolism of acetylcholine **(Jankowska *et al*., 2000)**. They exhibited that, specifically, there was a selective loss of acetylcholine releasing neurons in the basal forebrain, hippocampus and cortex. However, impaired cholinergic function in AD has been correlated with loss of memory.

Generally, there have been some hypotheses to explain pathogenesis of the disease such as "cholinergic hypothesis" and "amyloid formation hypothesis". Nowadays, the most accepted treatment strategy in AD has been accepted as "cholinesterase inhibitors" that can inhibit acetylcholinesterase (AChE) enzyme in order to increase acetylcholine level in the brain **(Akhondzadeh *et al*., 2003)**. In fact, the most remarkable biochemical change in AD patients is a reduction of acetylcholine (ACh) levels in the hippocampus and cortex of the brain. Therefore, the inhibition of AChE (the enzyme responsible for hydrolysis of AChE at the cholinergic synapse), is currently the most established approach to treating AD **(Tariot *et al*., 2000)**. Al interacts with the cholinergic system, acting as a cholinotoxin According to **(Kaizer *et al*., 2005)** the alterations in the lipid membrane could be a decisive factor in changing the conformational state of the AChE molecule. However, another explanation for increased AChE activity following Al exposure could be the allosteric interaction between the cation and the peripheric anionic site of the enzyme **(Gulya *et al*., 1990)**.

On the other hand, the results of the present study showed that sage administration alone or to Al-intoxicated rats led to AChE inhibition. However, it has been reported that *Salvia officinalis* has CNS cholinergic receptor binding activities that may be relevant to enhance or restore mental functions including memory **(Wake *et al*., 2000)**. Similar effects are also observed *in vivo* **(Howes *et al*., 2003),** at least for AChE, suggesting that relevant components of *Salvia* can cross the blood– brain barrier and increase cholinergic transmission via cholinesterase inhibition **(Perry *et al*., 2002)**.Similarly, up to date, a number of studies on AChE inhibitory activity of several *Salvia* species have been reported. Among these, the essential oil and ethanolic extract of S. officinalis have been shown to possess anti-cholinesterase activity **(Perry *et al*., 1996)**. This finding is consistent with recent reports established sage benefits to memory following administration of the essential oil in healthy young adults **(Andrew *et al*., 2008)**. Moreover, the essential oil as well as its major components, α-pinene, 1, 8-cineole, and camphor were determined to have uncompetitive and reversible acetylcholinesterase inhibitory activity **(Perry *et al*., 2000)**. Additionally, the constituents contained within *Salvia* oil may combine non-linearly to produce cholinesterase inhibition. A combination of the major monoterpenoid constituents (camphor, 1,8-cineole, borneol, α-pinene and β-pinene) reconstituted in a naturally occurring ratio was significantly less potent than that of the whole oil **(Perry *et al*., 2003 and Savelev *et al*., 2004)**. The monoterpenoids may therefore act synergistically to inhibit AChE.

The activity of the essential oils was concluded mainly to be due to its monoterpenoids. The data indicated that the terpenoids, monoterpenes in particular, may have anticholinesterase activity **(Orhan *et al*., 2007)**. Alternatively, the ethanolic extract of *Salvia officinalis* potentiated memory retention and it has also, an interaction with muscarinic and nicotinic cholinergic systems involved in the memory retention process **(Eidi *et al*., 2006)** but to less extent than oil.

5- Alkaline and acid phosphatases

The present study illustrated that Al ingestion led to significant elevation in alkaline phosphatase (ALP) and acid phosphatase (ACP) activities in sera, cerebral cortex and hippocampus. Alkaline phosphatase is a membrane-associated enzyme, which predominantly concentrated in the vascular endothelium in the brain. There is a more or less continuous sheath of ALP covering all internal and external surfaces of the central nervous system including the spinal cord and thus it may functionally be part in the blood-brain barrier mechanism. On the other hand, intracellular ACP is largely confined to lysosomes, which primarily respond to cellular injury. Within the brain, the ACP is found to be concentrated in the gray matter, although it shows the activity in the white matter, also, to some extent**.** However, significant contribution by Al was observed to induce changes in ACP activity **(Dasgupta and Ghosh, 1993)**.

The increased activity of ALP and ACP enzymes in serum & brain of animals treated with $AlCl_3$ are in accordance with the findings of **Ochmanski and Barabasz (2000)**. Also, **El – Demerdash (2004)** found that the activities of these enzymes were increased in serum of mice fed on wheat containing Al residue of 0.2 g /kg b.w. The present results are further, in consistent with the recent findings of **Esmaeili *et al.* (2009)** who showed that chronic Al consumption caused significant increases in the activities of ALP and ACP enzymes which could be due to severe damage to tissue membranes.

In addition, the increase in the activity of ALP or ACP in blood might be due to the necrosis of liver, kidney and lung **(Sallam *et al.*, 2005)**. Our own interpretation for increased levels of ALP and ACP is the disruption of the blood brain barrier and oxidative damage of tissue membranes, releasing membrane bound enzymes following Al intoxication which is also confirmed previously by **Exley (2004)** and recently by **Esmaeili *et al.* (2009)**.

Moreover, regarding Al enhanced serum, cortex and hippocampus ACP activities of rats, herein, it was in agreement with the earlier observations recorded altered activities of specific lysosomal hydrolytic enzymes in neuronal tissues **(Suzuki *et al.*, 1988)** due to Al administration. From these observations it can be suggested that Al induced an increase in ACP activity of the brain may be an indication of lysosomal proliferation and increasing catabolic rate. The increased ACP activity may result in phosphate accumulation within the lysosomes, and this in turn may lead to decreased plasma inorganic phosphate concentration **(Hussain *et al.*, 1990)**.

In the present work, administration of sage tea, ethanolic extract and oil caused marked reduction in the elevated activities of ALP and ACP in Al treated rats. Such decrease could be due to the antioxidant properties of sage constituents as polyphenols (carnosol, carnosic acid, and rosmarinic acid) and flavonoids (apigenin) that protect cellular membranes integrity from Al-induced oxidative damage and repair the antioxidant system (**Carla *et al.*, 2009**) ,consequently, improve brain structure and function against Al toxicity.

CONCLOSION

From the data presented here, there is ample evidence supports the fact that aluminum plays a pivotal role in the neuropathology of many neurodegenerative diseases including AD and validate the fact that chronic exposure to aluminum causes oxidative damage to the membranes and neural cells leading to memory loss and other cognitive dysfunction and exhaust the antioxidant system. Further, it clearly demonstrated that sage (all forms) has a neuroprotective effect against aluminum induced neuronal structural dysfunction.

The overall beneficial effects of sage different preparations against aluminum disturbances may be attributed, mainly, to their high ability to scavenge ROS and augment the repair of the antioxidant system as well as its anti-ChE activity, thus it has been suggested that sage has shown promise in the treatment of many neurodegenerative diseases including Alzheimer's disease. No fixed pattern of protection was seen specific for the different preparations of sage, but, we can arrange them in the following order: sage oil> ethanolic extract> water extract Future study may be designed and further warrants the need for molecular studies to elucidate the mechanisms underlying the protective effects of sage and its active components.

REFERENCES

Akhondzadeh, S.; Noroozian, N.; Mohammadi, M.; Ohadinia, S.; Jamshidi, A.H. and Khani, M. (2003): *Salvia officinalis* extract in the treatment of patients with mild to moderate Alzheimer's disease: a double blind, randomized and placebo-controlled trial. J. Clin. Pharmacy Therap., 28: 53-59.

Akram, E. and Maryam, E. (2009): Antidiabetic effects of sage (*Salvia officinalis* L.) leaves in normal and streptozotocin-induced diabetic rats Diabetes & Metabolic Syndrome: Clin. Res. Rev., 39: 40–44.

Albendea, C.D; Trullen, E.M.G.; Broto, L.E.; Miana-Mena, F.G.; Plano, S.M.; Gonzales, M.C.R.; Balların, E.M. and Garcia, J.J. (2007): Melatonin reduces lipid and protein oxidative damage in synaptosomes due to aluminum. J. Trace Elem. Med. Biol., 21:261–268.

Amador, F.C.; Santos, M.S. and Oliveira, C.R. (2001): Lipid peroxidation and aluminum effects on the cholinergic system in nerve terminals. Neurotox. Res., 3:223-33.

Atack, J.R.; Perry, E.K.; Bonham, J.R.; Perry, R.H.; Tomlinson, B.E.; Blessed, G. and Fairbairn, A. (1983): Molecular forms of acetylcholinesterase in senile dementia of Alzheimer type: Selective loss of intermediate (10S) forms. Neurosci. Lett., 40:199–204.

Baricevic, D. and Bartol, T. (2000): The biological/pharmacological activity of the Salvia genus. In Kintzios S.E. (Ed.), SAGE – The genus Salvia, Harwood Academic Publishers, Amsterdam, The Netherlands: 143-184.

Bihaqi, S.W.; Sharma, M.; Singh, A.P. and Tiwari, M. (2009): Neuroprotective role of *Convolvus pluricaulis* on aluminum induced neurotoxicity in rat brain. J. Ethnopharmacol., 124:409-415.

Bilkei, A. (1993): Neurotoxic effect of enteral aluminum. Food Chem. Toxicol., 31:357–361.

Bock, P.P.; Karmer, R. and Paverka, M. (1980): A simple assay for catalase determination. Cell Biol. Monoger., 7: 44-74.

Brandstetter, S.; Berthold, C.; Isnardy, B.; Solar, S. and Elmadfa, I. (2009): Impact of gamma-irradiation on the antioxidative propertiesof sage, thyme, and oregano. Food Chem. Toxicol., 47:2230–2235.

Carla, M.; Alice, S.; Ramos, A.; Azevedo, M.F.; Lima, C.F.; Fernandes-Ferreira, M. and Cristina Pereira-Wilson (2009): Sage Tea Drinking Improves Lipid Profile and Antioxidant Defences in Humans Int. J. Mol. Sci., 10:3937-3950.

Celik, I. and Isik, I. (2008): Determination of chemopreventive role of Foeniculum vulgare and Salvia officinalis infusion on trichloroacetic acid-induced increased serum marker enzymes lipid peroxidation and antioxidative defense systems in rats. Nat. Prod. Res., 22: 66-75.

Chainy, G.B.N.; Samanta, L. and Rout, N.B. (1996): Effect of aluminium on superoxide dismutase, catalase and lipid peroxidation of rat liver. Res. Commun. Mol. Pathol. Pharmacol., 94:217–20.

Chinoy, N.J. and Memon, M.R. (2001): Beneficial effects of some vitamins and calcium on fluoride and aluminium toxicity on gastrocnemius muscle and liver of male mice. Fluoride, 34:21–33.

Christen, Y. (2000): Oxidative stress and Alzheimer disease. Am. J. Clin. Nutr., 71: 6215-6295.

Cordeiro, J.M.; Silva, V.S.; Oliveira, C.R. and Goncalves, P.P. (2003): Aluminium-induced impairment of Ca^{2+} modulatory action on GABA transport in brain cortex nerve terminals. J. Inorg. Biochem., 97:132–142.

Dasgupta, S. and Ghosh, S. (1993): Nicotine induced alterations in brain acid and alkaline phosphatase activities. Ind. J. Physiol. Allied. Sci., 47: 200-206.

Deloncle, R.; Huguet, F.; Babin, P.; Fernandez, B.; Quellard, N. and Guillard, O. (1999): Chronic administration of aluminium L-glutamate in young mature rats: effects on iron levels and lipid peroxidation in selected brain areas. Toxicol., 104:65–73.

Drago, D.; Bettella, M.; Bolognin, S.; Cenddron, L.; Scancar, J.; Milacic, R.; Ricchelli, F.; Casini, A.; Messori, L.; Tognon, G. and Zatta, P. (2008): Potential pathogenic role of beta-amyloid (1–42)-aluminum complex in Alzheimer's disease. Int. J. Biochem. Cell Biol., 40:731–746.

Dua, R. and Gill, K.D. (2001): Aluminum phosphide exposure: implications on rat brain lipid peroxidation and antioxidant defence system. Pharmacol. Toxicol., 89:315–9.

Durling, N.E. and Catchpole, O.J. (2007): Extraction of phenolics and essential oil from dried sage (*Salvia officinalis*) usning ethanol-water mixtures. Food Chem., 101:1417-1424.

Eidi, M.; Eidi, A. and Bahar, M. (2006): Effects of *Salvia officinalis* L. (sage) leaves on memory retention and its interaction with the cholinergic system in rats. Nutr., 22:321–326.

El-Demerdash, F.M. (2004): Antioxidant effect of vitamin E and selenium on lipid peroxidation, enzyme activities and biochemical parameters in rats exposed to aluminum. J. Trace Elements in Med. Biol., 18:113–121.

Ellman, G.L.; Courtney, K.D.; Andres, V. and Featherstone, R.M. (1961): A new and rapid calorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol., 7: 88-95.

Esmaeili, M.A.; Sonboli, A.; Kanani, M.R. and Sadeghi, H. (2009): *Salvia sahendica* prevents tissue damages induced by alcohol in oxidative stress conditions: Effect on liver and kidney oxidative parameters. J. Med. Plants Res., 3:276-283.

Exley, C. (2004): The pro-oxidant activity of aluminum. Free Rad. Biol. Med., 36: 380–387.

Flora, S.J.S.; Mehta, A.; Satsangi, K.; Kannan, G.M. and Gupta, M. (2003): Aluminum induced oxidative stress in rat brain: response to combined administration of citric acid and

HEDTA.Comp. Biochem. Physiol., Part C, 134:319–28.

Ghosh, M.N. (1971): Fundamentals of Experimental Pharmacology. Academic Press- New York . Pp. 85.

Goḿez, M.; Esparza, J.L.; Nogués, M.R.; Giralt, M.; Cabré, M. and Domingo, J.L. (2005): Pro-oxidant activity of aluminum in the rat hippocampus: gene expression ofantioxidant enzymes after melatonin administration. Free Radic. Biol. Med. 38: 104–111.

Goncalves, P.P and Silva, S.V. (2007): Does neurotransmission impairment accompany aluminum neurotoxicity? J. Inorg. Biochem., 10: 1291–1338.

Gray, S.L.; Hanlon, J.T.; Landerman, L.R.; Artz, M.; Schmader, K.E. and Fillenbaum, G.G. (2003): Is antioxidant use protective of cognitive function in the community-dwelling elderly? The American Journal of Geriatric Pharmacoth., 1: 3–8.

Hussain, A.S.; Cantor, A.H.; Jonson, T.H. and Yokel R.A. (1990): Effect of dietary aluminum sulfate on calcium and phosphorus metabolism of broiler chicks. Poult. Sci., 69: 985–991.

Jaen, J.C.; Gregor, V.E.; Lee, C.; Davis, R. and Emmerling, M. (1996): Acetylcholinesterase inhibition by fused dihydroquinazoline compounds. Bioorg. Med. Chem. Lett., 6:737–742.

Johnson, V.J.; Kim, S.H. and Sharma, R.P. (2005): Aluminum-maltolate induces apoptosis and necrosis in neuro-2a cells: Potential role for p53 signaling. Toxicol. Sci., 83: 329-339.

Julka, D. and Gill, K.D. (1996): Altered calcium homeostasis: a possible mechanism of aluminium-induced neurotoxicity. Biochim. Biophys. Acta., 1315: 47–54.

Jyoti, A.; Sethi, P. and Sharma, D. (2007): Bacopa monniera prevents from aluminium neurotoxicity in the cerebral cortex of rat brain. J. Ethnopharmacol., 111:56–62.

Kosar, M.; Dorman, H.J.D. and Hiltunen, R. (2005): Effect of an acid treatment on the phytochemical and antioxidant characteristics of extracts from selected Lamiaceae species. Food Chem., 91: 525–533.

Kuang, P. and Xiang, J. (1994): Effect of radix Salviae miltiorrhiza on EAA a dIAA during cerebral ischemia in gerbils: a microdialysis study. J. Trad. Chin. Med., 14:45–50.

Kumar, P.; Taha, A.; Sharma, D.; Kale, R.K. and Baquer, N.Z. (2008): Effect of dehydroepiandrosterone (DHEA) on monoamine oxidase activity, lipid peroxidation and lipofuscin accumulation in aging rat brain regions. Biogerontol., 4: 283–284.

Kumar, V.; Bal, A. and Gill, K.D. (2009a): Susceptibility of mitochondrial superoxide dismutase to aluminium induced oxidative damage. Toxicol., 255: 117–123.

Kumar, V.; Bal, A. and Gill, K.D. (2009b): Aluminium-induced oxidative DNA damage recognition and cell-cycle disruption in different regions of rat brain. Toxicol., 264: 137–144.

Lima, C.F.; Andrade, P.B.; Seabra, R.M.; Fernandes- Ferreira, M. and Wilson, C.P. (2005): The drinking of a Salvia officinalis infusion improves liver antioxidant status in mice and rats" J. Ethnopharmacol., 97: 383-389.

Lynch, T.; Cherny, R.A. and Bush, A.I. (2000): Oxidative process in Alzheimer's disease: the role of a beta-metal interaction. Exp. Gerantol., 35: 445-451.

Martin, R.B. (1986): The chemistry of aluminum as related to biology and medicine. Clin. Chem., 32:1797–806.

Mattson, M.P. (2004): Pathways towards and away from Alzheimer's disease. Natu., 430: 631–639.

Moretti, M.D.L.; Satta, M. and Peana, A.T. (1997): A study on antiinflammatory and peripheral analgesic action of Salvia sclarea oil and its main components. J. Essen. Oil Res., 9: 199–204.

Nayak, P. and Chatterjee, A.K. (2001): Differential responses of certain brain phosphoesterases to aluminum in dietary protein adequacy or in adequacy. Food chem. Toxicol., 39: 587-592.

Nayak, P.; Kumar, S. and Vasudevan, D.M. (2006): Role of ethanol on Aluminum induced biochemicalchanges on rat brain. Ind. J. Clin. Biochem., 21:53-57.

Nehru, B. and Anand, P. (2005): Oxidative damage following chronic aluminium exposure in adult and pup rat brains. J. Tra. Elem. Med. Biol. 19:203–208.

Nesto, R.W. (2005): Beyond low-density lipoprotein: Addressing the atherogenic lipid triad in type 2 diabetes mellitus and the metabolic syndrome. Am. J. Cardiovasc. Drugs, 5:379-387.

Newairy, A.A.; Salama, A.F.; Hussien, H.M. and Yousef, M.I. (2009): Propolis alleviates aluminium-induced lipid peroxidation and biochemical parameters in male rats. Food Chem. Toxicol., 47:1093–1098.

Ninomiya, K.; Matsuda, H. Shimoda, H.; Nishida, N.; Kasajima, N.; Yoshino, T.; Morikawaa, T. and Yoshikawa, M. (2004): Carnosic acid, a new class of lipid absorption inhibitor from sage" Bioorg. Med. Chem. Lett. 14: 1943–1946.

Nishikimi, M.; Roa, N.A. and Yagi, K. (1972): Measurement of superoxide dismutase. Biophys. Res. Common., 46: 849.

Ochmanski, W. and Barabasz, W. (2000): Aluminum-occurrence and toxicity for organisms. Przegl. Lek., 57:665–8.

Ohkawa, H., Wakatsuki, A. and Kaneda, C. (1982): Assay for lipid peroxides in animals tissues by thiobarbituric acid reaction. Ana. Biochem., 95: 351 – 358.

Oteiza, P.L.; Keen, C.L.; Han, B. and Golub, M.S. (1993): Aluminum accumulation and neurotoxicity in swiss-webster mice after long-term dietary exposure to aluminum and citrate. Metabo., 42:1296–300.

Perry, N.S.L.; Houghton1, P.J.; Jenner, P.; Keith, K. and Perry, E.K. (2002): Salvia lavandulaefolia essential oil inhibits cholinesterase in vivo. Phytomed., 9: 48–51.

Plana, N.; Nicolle, C.; Ferre, R.; Camps, J.; Cós, R.; Villoria, J. and Masana, L. (2008): DANACOL group. Plant sterol-enriched fermented milk enhances the attainment of LDL-cholesterol goal in hypercholesterolemic subjects. Eur. J. Nutr., 47: 32-39.

Platt, B.; Fiddler, G.; Riedel, G. and Henderson, Z. (2001): Aluminum toxicity in the rat brain: histochemical and immunocytochemical evidences. Brain Res. Bull., 55:257–67.

Prins, H.K. and Loose, J.A. (1969): Glutathione in biochemical methods in red cell genetics. Edited by Yunis, J.J., Academic Press, N.Y.D. London, 126-129.

Ren, W.; Qiao, Z.; Wang, H.; Zhu, L. and Zhang, L. (2003): Flavonoids: promising anticancer agents. Med. Res. Rev. 23: 519–534.

Roth, G.S.; Joseph, J.A. and Mason, R.P. (1995): Membrane alterations as causes of impaired signal transduction in Alzheimer's disease and aging. Trends Neurosci. Sci., 18:203-206.

Sallam, S.M.A.; Nasser, M.E.A.; Yousef, M.S.H.; El-morsy, A.M.; Mahmoud, S.A.S. and Yousef, M.I. (2005): Influence of Aluminum Chloride and Ascorbic Acid on Performance, Digestibility, Caecal Microbial Activity and Biochemical Parameters of Rabbits. Res. J. Agric.Biol. Sci., 1:10-16.

Sarin, S.; Gupta, V. and Gill, K.D. (1997): Alterations in lipid composition and neuronal injury in primates following chronic aluminium exposure. Biol Trace. Elem. Res., 59:133–43.

Savelev, S.; Okello, E.; Perry, N.S.L.; Wilkins, R.M. and Perry, E. (2003): Synergistic and antagonistic interactions of anticholinesterase terpenoids in Salvia lavandulaefolia essential oil. Biochem. Pharmacol. Behav., 75:661–668

Savory, J.; Herman, M.M. and Ghribi, O. (2003): Intracellular mechanisms underlying aluminum-induced apoptosis in rabbit brain. J. Inorg. Biochem., 97: 151-154.

Schneider, L.S. (1996): New therapeutic approaches to Alzheimer's disease. J. Clin. Psych., 57: 30–36.

Smith, C. D.; Caney, J. M.; Starke-Reed, P. E.; Oliver, C. N.; Stadtman, E. R.; Floyed, R.A. and Markesbery, W.R. (1991): Excess brain protien oxidation and enzyme dysfunction in normal aging and in Alzheimer disease. Proc. Natle. Acad. Sci., 88: 10540-10543.

Struys-Ponsar, C.; Kerkhofs, A.; Gauthier, A.; Soffie, M. and Van den Bosch de Aguilar, P. (1997): Effects of aluminum exposure on behavioural parameters in the rat. Pharmacol. Biochem. Behav., 56:643–8.

Suzuki, H.; Takeda, M.; Nakamura, Y.; Tada, K.; Hariguchi, S. and Nishimura, T. (1988): Activities of lysosomal enzymes in rabbit brain with experimental neurofibrillary changes. Neurosci. Lett., 89: 234-239.

Tripathi, S.; Mahdia, A.A.; Nawaba, A.; Chandera, R.; Hasanb, M. Siddiquib, M.S.; Mahdic, F.; Mitrad, K. and Bajpaid, V.K. (2009): Influence of age on aluminum induced lipid

peroxidation and neurolipofuscin in frontal cortex of rat brain: A behavioral, biochemical and ultrastructural study. Brain Res., 1253:107–116.

Urano, S.; Asai, Y.; Makabe, S. and Matsuo, M. (1997): Oxidative injury of synapse and alteration of antioxidative defence systems in rats, and its prevention by Vitamin E. Eur. J. Biochem., 245:61–70.

Wake, G.; Court, J.; Pickering, A.; Lewis, R.; Wilkins, R. and Perry, E. (2000): CNS acetylcholine receptor activity in European medicinal plants traditionally used to improve failing memory. J. Ethnopharmacol., 69: 105–114.

Wang, C.N.; Chi, C.W; Lin, Y.L.; Chen, C.F. and Shiao, Y.J. (2001): The neuroprotective effects of phytoestrogens on amyloid h protein-induced toxicity are mediated by abrogating the activation of caspase cascade in rat cortical neurones. J. Biol. Chem., 276:5287–95.

Wilhelm, M.; Jaeger, D.E.; Schull-Cablitz, H.; Hafner, D. and Idel, H. (1996): Hepatic clearance and retention of aluminum: studies in the isolated perfused rat liver. Toxicol. Lett., 89:257–63.

Wu, D. and Cederbaum, I. (2003): Alcohol, Oxidative Stress, and Free Radical Damage. Alcohol Res. Health, 27: 277-284.

Xu, N.; Majidi, V.; Markesbery, W.R. and Ehmann, W.D. (1992): Brain aluminum in Alzheimer's disease using an improved GFAAS method. Neurotoxicol., 13:735–44.

Yokel, R.A. (2001): The toxicity of aluminium in the brain: a review. Neurotoxicol., 21:813–28.

Yousef, M.I. (2004): Aluminum induced changes in hemato-biochemical parameters, lipid peroxidation and enzyme activities of male rabbits: protective role of ascorbic acid. Toxicol., 199:47–57.

Zatta, P.; Kiss, T.; Suwalsky, M. and Berthon, G. (2002a): Aluminum (III) as a promoter of cellular oxidation. Coord. Chem. Rev., 228:271-284

Zatta, P.; Ibn-Lkhayat-Idrissi, M.; Zambenedetti, P.; Kilyen, M. and Kiss, T. (2002b): *In vivo* and *in vitro* effects of aluminum on the activity of mouse brain acetylcholinesterase. Brain Res. Bull., 59:41–5.

Zheng, Q.S.; Sun, X.L.; Xubo, L.G.; Song, M. and Wang, C.H. (2004): Protective effects of luteolin-7-glucoside against liver injury caused by carbon tetrachloride in rats. Pharmazie., 59: 286–289.

Zhu, X.; Raina, A.K.; Lee, H.G.; Casadesus, G.; Smith, M.A. and Perry, G. (2004): Oxidative stress signaling in Alzheimer's disease. Brain Res., 1000: 32–39.

Zupko, I.; Hohmann, J.; Redei, D.; Falkay, G. Janicsak, G. and Mathe, I. (2001): Antioxidant activity of leaves of Salvia species in enzyme dependent and enzyme-independent systems of lipid peroxidation and their phenolic constituents, Plant. Med., 67:366–368.

Study on immune response of quail for avian influenza vaccines

M.A.saad ,A.I.Abd-Elhady, A.EL-nagar

Central laboratory for evaluation of veterinary biologics Quail influenza

Abstract: This study was a trial to evaluate: The immune responses of quails vaccinated with common AI commercial vaccines in Egypt The results revealed that: There were high to moderate levels of maternal immunity against AIV (H5N1and H5N2) on the 1st, 5th day of age and low levels on the 7th day of age. There was no significant difference concerning the immune response of H5N1 and H5N2 AI vaccines ($P < 0.05$) in vaccinated quails. Vaccination at 8-days of age with 0.5ml of vaccine, gave satisfactory titers, on the 3rd week post vaccination. By the 4th week post vaccination quails exhibited highest titers and continued to the 5th week post vaccination(age of slaughter or marketing of quail) against AIV.

[M.A.saad, A.I.Abd-Elhady, A.EL-nagar. **Study on immune response of quail for avian influenza vaccines**. Journal of American Science 2010;6(12):1475-1478]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: immune response; quail; avian influenza; vaccines

Introduction

Avian Influenza (AI) is a disease of poultry that has occurred worldwide over the past 100 years (Easterday et al., 1997). Two clinical forms are seen in the field: a mild disease affecting the respiratory, reproductive and or urinary tracts, and a severe systemic disease, causing high morbidity and mortality. AI viruses are classified as highly pathogenic (HP), mildly pathogenic (MP) and a non-pathogenic (NP) based on the mortality rates (Senne et al., 1986; US Animal Health Association (USAHA, 1994). Over the past decade, the emergent HPAI viruses have shifted to increased virulence for chickens. HPAI viruses typically produce a similar severe, systemic disease with high mortality in chickens and other gallinaceous birds (Swayne, 2007). 26 epizootics of HPAI have occurred in the world since 1995. The largest of these outbreaks has been the H5N1 HPAI which has caused problems in poultry and some wild birds in over 60 countries of Asia, Europe and Africa since beginning in 1996 (Maines *et al.*, 2005). In Africa, H5N1 HPAI cases approved in February 2006 in several countries. It began in Nigeria then other African countries including Egypt. (Swayne, 2008). On 17 February 2006, the Egyptian government confirmed that bird flu had broken out in the nation's poultry.

Quails are migratory game birds belonging to the same family as the domestic fowl (Weatherbee and Jacobs, 1961).

Highly pathogenic avian influenza (HPAI) virus subtype H5N1 has caused significant losses in Thailand's poultry industry since its initial detection in January 2004 (Tiensin *et al.*, 2005). Chickens and quail are highly susceptible to HPAI H5N1 infection; however ducks, considered more resistant, are probable "Trojan horses" or carriers (Hulse-Post et al., 2005; Tiensin et al., 2005).

AI virus was detected in quail and chickens muscles and organs by indirect immunofluorescent assay (Antarasena, C. et al. (2006).

The HI test against AI showing positive results in quail sera collected random samples from Egypt. (Elmahdy et al. 2009).

A formalin-inactivated oil-emulsion vaccine was prepared from a high-growth H5N1/PR8 virus (Chen *et al.* 2005). Vaccine candidates of influenza A viruses of H5N1 subtype have been generated in several laboratories (Lu *et al.*, 2007). In the face of disease outbreaks in quail industry and the potential pandemic threat to humans caused by the highly pathogenic avian influenza viruses (HPAIVs) of H5N1 subtype, improvement in biosecurity and the use of inactivated vaccines are two main options for the control of this disease, for that we designed our present study to measure the immune response of quail to AI vaccines H5N1 and H5N2.

Materials and Methods

1-Quails: two hundred and fifty, one day old quail were used in this experiment.

2-AI Vaccine: commercial AI H5N1 and H5N2 vaccines, used for vaccination of quail.

3-Serum samples: quail blood samples were collected and sera were separated to apply HI test.

4- AI antigen: local inactivated HPAI virus was obtained from CLEVB and used as AI antigen with a titer of 2^6 HA units/ml. and was used at a final concentration of 4 HA in HI test for the tested serum samples.

5- HA haemagglutination test: HA test were carried out according to (Anon 1971) to estimate the HA titer of used antigens.

6- HI haemagglutination inhibition test: Was carried out according to (Takatsy 1956) the test was applied

to quantify AIV antibodies in sera according to OIE (2008)

Experimental design

Experiment 1

Maternal immunity: fifty quails were selected for determination of maternal immunity that acquired from vaccinated parents by HI test.

Experiment 2

Immune response of the vaccinated quail: 200

Quails were used for:

Determination of the immune response of quails by vaccinated S/C with either inactivated oil-emulsion H5N1 or H5N2 vaccines. Commercially available oil emulsion vaccines were used: H5N1 (subtype, Re-1 strain - A/chicken / China, Puerto - Rico) and H5N2 (subtype chicken / England, Mexico) of = 10^4 EID50 haemagglutination antigen content. The dosage was 0.3ml at age 4-days and 0.5ml at age 8-days Blood samples were collected 1, 2, 3, 4 and 5 weeks post vaccination. The flocks were arranged as follows:

A- Vaccination at 4 days old with 0.3ml of vaccine.

B- Vaccination at 8 days old with 0.5ml of vaccine.

Results and Discussion

Table 1: Maternal immune wading in quails § acquired from vaccinated parents by AIV inactivated oil-emulsion vaccines H5N1 and H5N2.

Age of Quail	H5N1	H5N2
	HI	HI
1 day	5.3	4.8
5 days	4.4	4.3
7 days	4.0	3.6
10 days	2.9	2.8
14 days	2.0	2.0

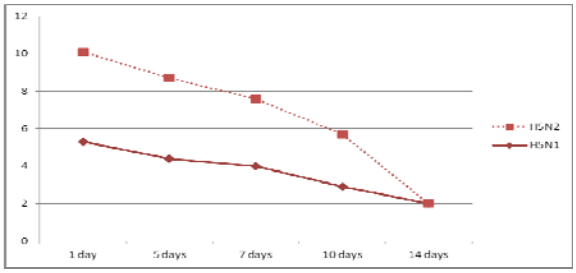


Figure 1: Maternal immune wading in quails § acquired from vaccinated parents by AIV inactivated oil-emulsion vaccines H5N1 and H5N2 .

Table 2: The immune response of quails vaccinated by AIV inactivated oil-emulsion vaccines H5N1 and H5N2 .

Group No.	Type of vaccine	Age of vaccine	Dose	HI titer post vaccination				
				1^{ST} W	2^{nd}W	3^{rd} W	4^{th} W	5^{th} W
1	H5N1	4 days	0.3 ml	2.1	3.0	5.7	5.9	5.0
2	H5N1	8 days	0.3 ml	1.8	2.6	5.5	6.2	5.3
3	H5N1	4 days	0.5 ml	2.3	3.2	6.1	6.4	5.4
4	H5N1	8 days	0.5 ml	2.0	2.8	6.5	6.8	6.0
5	H5N2	4 days	0.3 ml	2.0	2.8	5.2	5.7	4.7
6	H5N2	8 days	0.3 ml	1.7	2.4	5.4	5.9	5.0
7	H5N2	4 days	0.5 ml	2.2	3.1	5.7	6.1	5.2
8	H5N2	8 days	0.5 ml	1.9	2.6	5.9	6.3	5.7

Now quails are raised commercially for meat and egg production and kept as pet birds and experimental birds in most parts of the world. (Lima et al., 2004). Quail are resistant to many diseases but they are susceptible to most naturally occurring viral diseases of chickens, especially when reared under poor management conditions. However the reports of the naturally occurring diseases are few when compared to those of chickens and this may be due to the fact that there are few quail farms (Ratnanohan, 1993). we have recently shown that quail are highly susceptible to infection with highly pathogenic H5N1 viruses isolated from geese. These viruses cause disease in quail; however, infected quail have a longer disease period than do chickens and thus are more likely to transmit the virus (Webster et al., 2003).

Table 1 and Fig.1 illustrated The results of maternal immunity,they show that:

1: There were high to moderate levels of maternal antibodies against AI (H5N1) and (H5N2) on the 1st and 5th day of age and low levels on the 7th day of age [HI mean values were 5.3, 4.4, 4.0, 2.9 and 2.0 (log-2) respectively]for H5N1 . On the other hand, quails vaccinated by H5N2 at ages of one-day, 5-days and 7-days, were 4.8, 4.3, 3.6, 2.8 and 2.0 respectively (HI titer values).

2: After the age of 7 days the level of maternal immunity was greatly reduced and it was fade at the age of 14 days.

3: There was no significant difference concerning the immune response of H5N1 and H5N2 AI vaccines (P <0.05).

Determination of immune response in quails vaccinated with inactivated oil-emulsion H5N1 and

H5N2 vaccines Vaccination at 4-days old: In one hand, Table 2 Showed that: H5N1 and H5N2 vaccination at 4-days of age (0.3ml of vaccine) resulted in positive antibody response on the 1st week post vaccination (HI titers were, 2.1 and 2.0 (log-2) respectively). The antibody response was gradually increased up to the 4th

week post vaccination (HI titers were, 5.9 and 5.7, log-2

respectively).while at 4-days of age (0.5ml of vaccine) resulted in positive antibody response on the 1st week post vaccination(HI titers were, 2.0 and 1.9 (log-2) respectively) The antibody response was gradually increased up to the 4^{th} week post vaccination (HI titers were, 6.4 and 6.1, log-2

respectively)

Vaccination at 8-days old: On the other hand,

Vaccination at 8 - days of age with 0.3ml of vaccine,

Gave satisfactory titers, 3 weeks post vaccination (HI

Titers were, 5.5 and 5.4 (log2) respectively), but highest

Titers were exhibited on the 4th week post vaccination (HI

Titers were, 5.9 and 5.7 (log2) respectively) and then

Continued to the 5th week post vaccination. While 0.5ml of vaccine gave satisfactory titers, 3 weeks post vaccination (HI

Titers were, 6.5 and 5.9 (log2) respectively), but highest

Titers were exhibited on the 4th week post vaccination (HI

Titers were, 6.8 and 6.3 (log2) respectively) and then

Continued to the 5th week post vaccination.

Our results pointed out that, vaccines do not sufficiently

Reduce the probability of infection up to 3 weeks post vaccination and this is indicated by the low HI titers. Although H5N1 or H5N2 vaccination at the age of 8-

Days, gave protection 3 weeks post vaccination where,

The titer ranged from 4.2 to 5.7 (log2), but maximum

Levels of HI titers occurred 4 weeks post vaccination (4.6 to

6.1, log2) and continue with protective titer to five weeks of quail age (age of slaughter or marketing of quail) .Our results agreed with Swayne, D.E. 1999. Study The influence of vaccine strain and antigen mass on the ability of inactivated avian influenza (AI) viruses to protect chicks from a lethal, highly pathogenic (HP) AI virus challenge were they affect the immune response to AV vaccine.

Further studied needed by application of challenge test to estimate the vaccine efficacy in quails but these test need critical high registrations to use the virulent AV virus to apply these test.

Our results suggested in regard to The immune response of vaccinated quails Against AIV.that The ideal age for quails Vaccination by AI vaccine is between 4 and 8-days of age, otherwise quails maternal immunity should be considered if they vaccinated at one-day of age. Quails one-day old of age which have low or no maternal immunity should be vaccinated at one-day old (with a dose of 0.3ml, H5N2), followed by a second dose (0.5ml) at 15-21-days of age.

The effectiveness of the available commercial vaccines in protection against the disease required . Two main categories for the control of this disease:

1: The use of efficient inactivated vaccines (targeted Control strategies).

2: Improved, strict and satisfactory biosecurity measures.

References

1. Anon, (1971). Methods for examining poultry biologics and quantifying avian pathogens. National Acd. Of Sci. Washington.
2. Antarasena, C.; Sirimujalin, R.; Prommuang, P.; Blackshell, S.D. ; Promkundtod, N. and Prommuang, P. Tissue tropism of a Thailand strain of high-pathogenic avian influenza (H5N!) in tissues of naturally infected native chickens (Gallus gallus), Japanese quail (Coturnix coturnix japonica) and ducks (Anas spp.) Avian Pathology 35(3), 250-253.(June 2006)

3. Chen, H., G. Tian, S. Zhang, Y. Li, Z. Bu, P. Liu, J. Zhou, C. Li, J. Shi and K. Yu, 2005. Oct. 10, Virol., 341: 153-62.
4. Easterday, B.C.; Hinshaw, V.S. and Halvorson, D.A. (1997). Influenza. In: B.W. Calnek, H. J. Barnes, C. W. Beard, L. R. Elmahdy, S.; Saber, S.; Taha M. and Elebiary, E. (2009). Prevalence of antibodies against poultry viral diseases in sera of quails. J. of Egy. Vet. Assoc.69(4) 141-149.
5. Hulse-Post, D.J.; Sturm-Ramirez, K.M>; Humberd, J.; Seiler, P.; Govorkova, E.A.; Krauss, S.; Scholtissek, C.; Puthavathana, P.; Buranathai, C.; Nguyen, T.D.; Long, H.T.; Naipospos, T.S.; Chen, H.; Ellis, T.M.; Guan, Y.; Peiris, J.S. and Webster, R.G. (2005). Role of domestic ducks in the propagation and biological evolution of highly pathogenic H5N1 influenza viruses in Asia. Proceedings of the National Academy of Sciences USA, 102, 10682-10687.
6. Lima, F.S.; Santin, E.; Antonio, C.P. and Luciano, D.K. (2004). Evaluation of different programs of NDV in Japanese quail. Inter. J. Poul. Sci., 3 (5).
7. Lu, J., H. Shi, X.F. Liu, X. Zhang, S. Chen and L. Sun,2007. Generation of an attenuated H5N1 avian influenza virus vaccine with all eight genes from avian viruses, 25: 7379-84.
8. Chen, H., G. Tian, S. Zhang, Y. Li, Z. Bu, P. Liu, J. Zhou, C. Li, J. Shi and K. Yu, 2005. Oct. 10, Virol., 341:153-62.
9. Maines, T.R., X.H. Lu, S.M. Erb, L. Edwards, J. Guarner, P.W. Greer, D.C. Nguyen, K.J. Szretter, L. Chen, P. Thawatsupha, M. Chittaganpitch, S. Waicharoen, D.T. Nguyen, T. Nguyen, H.H.T. Nguyen, J. Kim, L.T. Hoang and C. Kang. L.S. Phuong, W.L., S. Zaki, R.O. Donis, N.J. Cox, J.M. Katz and T.M. Tumpey, 2005. Avian Influenza (H5N1) Viruses Isolated from Humans in Asia in 2004. Exhibit Increased Virulence in Mammals. J. Virol., pp: 11788-11800.
10. Ratnanohan, N. (1993). Virus disease of Japanese quail. Virus infection of birds, Elsevier Science Publishers, B.V. Chp44.
11. Senne, D.A.; Pearson, J.E.; Kawaoka, Y.; Carbrey, E.A. and Webster, R.G. (1986). Alternative methods for evaluation of pathogenicity of chicken Pennsylvania H5N2 viruses. In: B.C. Easterday (Ed.), Proceedings of the 2nd International Symposium on Avian Influenza (pp. 246-257). Richmond, VA: US Animal Health Association.
12. Swayne, D.E.; Beck, J.R.; Garcia, M. and Stone, H.D. (1999). Influence of virus strain and antigen mass on efficacy of H5 avian influenza inactivated vaccines. Avian Pathology, 28, 245-255.
13. Swayne, D.E., 2007. Understanding the complex pathobiology of high pathogenicity avian influenza viruses in birds. Avian dis. Mar, PMID: 17494560, 51: 242-9.
14. Swayne, D.E., 2008. Current status of avian influenza with emphasis on pathobiology, ecology, disease diagnosis and control. 8th Sci. Conf. EVPA, Giza (Egypt), 10-13 March.
15. Takatsy, G.Y. (1956). The use of spiral loops in serological and virological methods. Acta Microbiol. Hung. 3: 191.
16. Tiensin, T.; Chaitaweesub, P.; Songserm, T.; Chaisingh, A. ; Hoonsuwan, W. ; Buranathai, C.; Parakamawongsa, T.; Premashthira, S.; Amonsin, A.; Gilbert, M.; Nielen, M. and Stegeman, A. (2005). Highly pathogenic avian influenza H5N1. Thailand, 2004. Emerging Infectious Diseases, 11, 1664-1672.
17. USAHA (1994). Report of the Committee on Transmissible Diseases of Poultry and other Avian Species. Criteria for determining that an AI virus isolation causing an outbreak must be considered for eradication In: Proceedings of the 98th Annual Meeting US Animal Health Association (p.522). Grand Rapids: US Animal Health Association.
18. Weatherbee, D.K. and Jacobs, K.F. (1961). Migration of the common coturnix in North America Oklahoma Dept. Wildlife Conservation, 32. 85-91.
19. Webster, R.G. (2003). Role of quail in the interspecies transmission of H9 Influenza A viruses: Molecular changes on HA that correspond to adaptation from ducks to chickens. Journal of Virology, 77(5) 3148-3156.

9/2/2010

First Record Of Microsporidium *Neonosemoides* Sp. And Some Ciliates Infecting *Chrysichthus Auratus* (Bagridae) From The Damietta Branch Of River Nile, Egypt

Enayat Salem Ahmed Reda
Department of Zoology, Faculty of Science, Mansoura University, Mansoura, Egypt

Abstract: The present study was carried out as a general survey for the possible ectoparasites that can infect the Nile fish *Chrysichthus auratus*. A total of 52 fish specimens were collected from Damietta branch of River Nile. Examination of the investigated fish revealed that, fish were infected with four ectoparasitic species belonging to three genera. These species were: *Neonosemoides* sp., *Scyphidia* sp. 1, *Scyphidia* sp. 2 and *Ichthyophthirius multifillis*. The first three species were recorded for the first time in Egypt. The recovered parasites have pathological effects on the host fish with subsequent economic losses were discussed.

[Enayat Salem Ahmed Reda. **First Record Of Microsporidium *Neonosemoides* Sp. And Some Ciliates Infecting *Chrysichthus Auratus* (Bagridae) From The Damietta Branch Of River Nile, Egypt.** Journal of American Science 2010;6(12):1479-1482]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: *Neonosemoides* sp., Ciliates, *Chrysichthus auratus*, River Nile. Egypt.

Introduction

Microsporidia are obligate intracellular parasites. Infected cells usually enlarge to accommodate the proliferating parasite. In 1968 Wiessenberg coined the term "xenoma" on the host cell with completely changed structure and the parasite proliferating inside it. According to Klaus Rohde (2005) microsporidia infect most invertrbrate phyla and all classes of vertebrate. There are 17 genera are known to infect fishes; 13 genera infect marine fishes and 4 genera infect freshwater fishes: *Heterosporis* (Schubert, 1969), *Nosemoides* (Vinckier, 1975), *Neonosemoides* (Faye, Toguebaye and Bouix, 1996) and *Pseudoloma* Matthew, Brown, Larison, Bishop-Stewart, Rogers and Kent, 2001 (Klaus Rohde 2005).

Genus *Neonosemoides* is one of microsporidian genera parasitizing freshwater fishes and at the same time produce xenoma which play an important agent of diseases in commercial fishes.

Although there is considerable information on the species of microsporidia (Lom and Dykova, 1992; Sprague *et al*., 1992; Lom, 2002; Lom and Nilsen, 2003), little is known about those from Africa. Sakiti and Bouix, 1987 recorded *Neonosemoides tilapia* from *Tilapia zillii* from Benin and Faye and Toguedaye, 2005 recorded 4 unidentified species from carangid fishes from Senegalse.

External protozoa are cited as major problem in freshwater fishes; sessilines ciliates like genus *Scyphidia* utilize gills and skin as a substrate for attachment.

On the other hand mobilina ciliates like genus *Ichthyophthirius* which is an obligate parasite of gills, skin and fins has a worldwide distribution (Paperna, 1980) also has been found to cause the white spot disease which is accompanied by severe morbidity and eventually end with fish mortality (Hoffman, 1970). Abu-El Wafa, (1988) and Koura *et al*., (1997), described *I. multifiliis* from some freshwater fishes. This study aims to contribute to the ciliates fauna infecting *Chrysichthys auratus* with special emphasis on genus *Neonosemoides* as a first record in Africa and to establish a background for further studies.

\

Materials and Methods

A total of 52 fish of *Chrysichthys auratus* were collected from Damietta branch of River Nile near El-Mansoura. The collected fishes were transported to the laboratory in tank with good aeration. Fishes were kept a live until required in aerated glass aquaria. Fishes were identified according to Bashai and Khalil (1997).

Fish skin, fins and gills were firstly examined by the naked eye for detection of any macroscopically visible lesions. Samples of mucus were scraped gently from the skin, fins and gills, then spread on a clean slide and freshly examined under phase-contrast microscope for the presence of ectoparasitic protozoans. Some of the positive slids were air-dried and stained according to Klein;s dry silver impregnation method. Other positive slides were also air-dried, fixed with absolute methanol and stained with 10% Giemsa stain.

Detected protozoa were examined freshly, stained and identified according to Shulman (1984) and Lom and Dykova (1992 & 2005). All measurements were taken in micrometers (µm) mean

± SD (range). Figures were drawn with aid of camera lucida.

Results

The detected protozoan parasites were classified into two main phyla; Microsporidia and Ciliophora as following:

Phylum: Microsporidia
Genus: *Neonosemoides*
***Neonosemoides* sp.**

Xenomas are white spherical, inhabiting gills range in size from 50-70 µm (mean 60 µm) in diameter. Xenoma consists of a simple lamellar wall measures 2.2 µm, contains only 16 mature macrospores in direct contact with the cytoplasm of the host cells and the three lobes hypertrophic nucleus of host cell. All spores in generally are surrounded by a light zone. Fully formed xenoma appears as "a bag of spores". (Figs. 1A & 3A).

Spore description

Spores are egg-shaped with bluntly rounded poles (Fig. 1B). It measures 3.2±0.2 (3.0-3.4) µm in length X 1.6±0.3 (1.3-1.8) µm in width. The spore has a thin outer finly corrugated layer (exospore), thin inner layer (endospore) and an inner most simple cell membrane. The spore consists of three parts which determine the anterior-posterior polarity of the spore (Fig. 1C).

The anchoring disc (polar cap) is mushroom cap like-shaped and stained as a red granule by Giemsa stain (Lom & Dykova,1992) , which is highly characteristic of the group (Fig. 3A). It is eccentric (subapically) located.

The polar tube; is the first part and is inserted into the base of the polar cap. The manubrium part of the polar tube extends from the cap opliquely backwards. There is an outer sheath around the polar tube, acting as a sleeve, through which the tube slides while extruding.

The isofilar polar filament forms 4 regular and helically arranged coils around the surface of the posterior vacuole in the posterior half of the spore. The second part is the polaroplast; lamellar organelle consisting of an anterior region of closely packed membranes and posterior region of more loosely packed membranes that surrounding the basal part of the polar tube. The third part, is the posterior vacuole, that lies inside the coils of the polar tube and occupies more than one-third of the spore cavity.

The remaining space within the spore and between the polaroplast and the posterior vacuole is occupied by the infective germ itself, the sporoplasm. The nucleus is single, spherical and centrally located between the polaroplast and the posterior vacuole.

Phylum: Ciliophora
I-Genus: *Ichthyophthirius*
I. multifiliis

This parasite appears as a rounded-shaped ciliated organism (Figs. 2A, 3B & 4A). In heavily infested fish, this parasite could be easily detected by the naked eye inhabiting the gills, skin and fins. It is white in colour, tiny dots, exhibits a sluggish movement and measures 44.2-90.6 µm in diameter (mean 67.4). The body is uniformly covered by dense rows of cilia. The number of meridional kineties are ranged from 77-98 (mean 88), converging anteriorly and apically raised into a pointed elevation. The cytoplasm appears to be grossly granulated containing many small food vacuoles, the horse-shoe macronucleus measures (32.3-44.6) µm in length (mean 38.5) and lies in middle of the body. A rounded micronucleus is almost adhering to the macronucleus. There are many contractile vacuoles.

II-Genus: *Scyphidia*
***Scyphidia* sp. 1**

This ciliate is solitary large parasite, inhabiting gills with cup-shaped body measures 57.6±3.6 (54-61.2) µm in length X 49.9±3.1 (46.8-53) µm in width. Epistomial disc is vaulted and slightly elevated above the peristomial disc. The peristomial disc is narrow and encircles the epistomial disc. The macronucleus is ribbon shaped, sinuous and measures 48.4±4.4 (44-52.8) µm in length. Micronucleus is very small. There are some scattered contractile vacuoles. Transverse striations of pellicle conspicuous and ranged from 80-110 (mean 95). There is non ciliated groove near the narrow scopula (Figs. 2B, 3C& 4B).

***Scyphidia* sp. 2**

This peritrich is solitary parasite, inhabiting gills with cup-shaped body and measures 35.2±2(33.1-73.2) µm in length X 36.3 ±2(34.4-38.1) µm in width. Peristomial disc is narrow. Both epistomial disc and peristomial lips are at the same level. The macronucleus is ribbon-shaped, sinuous, occupies almost all the body cavity and measures 33.6±5(28.6-38.5)µm in length X 5.5±0.8(4.6-6.2). The giant micronucleus situated in close contact with the macronucleus and measures 11.3±1.4(9.9-12.6) µm in length X 2.2±0.4(1.9-2.7) µm in width. Scopula attacted to the host skin directly by a secretory layer of sticky material. Infundibulum small and extends between the two nuclui by cytopharynx. There is a non ciliated groove situated anteriorly (Figs. 2C & 3D).

Discussion

1-Genus *Neonosemoides*

Neonosemoides sp.

The more conspicuous characteristics of the spore, the shape, wall, polaroplast, polar filament and posterior vacuole are used to distinguish microsporidia from other taxonomic group (Sprague *et al*., 1992). According to the site of infection the present xenomas were found on the gills of freshwater fish *Chrysichthys auratus*, so it belong to genus *Nosemoides* (Lom and Dykova, 1992). Recently (Lom and Dykova, 2005) reported that genera of microsporidia that comprise xenoma-forming species can be grouped in several categories according to xenoma wall, hypertrophic nucleus and type of spores inside xenoma. Accordingly the present investigated xenomas belong to genus *Neonosemoides*. Type and only species recorded in this genus is *Neonosemoides tilapiae* from *Tilapia zillii* (Sakiti and Bouix, 1987 and Faye *et al*., 1996) from Benin (West Africa). Comparing the present species with *N. tilapiae*, it was found many differences as listed in Table (1). So the present species assigned to the same genus but further ultrastructure and molecular study need to reveal the exact taxonomic assignment of this species.

The pathogenic effects induced by Microsporidia in host include physical disruption of cells due to occupation of intracellur space, host cell hypertrophy, change to host cell metabolism and reorganization of host cell components. The direct effects include increased mortality (Klaus Rohde, 2005). In the present work parasites are generally surrounded by a light zone the existence of which, is to be explained by the action of their proteolytic enzymes, which dissolve the host protoplasm around parasites and render it suitable for assimilation.

2- Genus: *Ichthyophthirius*

I. multifiliis

The parasite is identified by its characteristic horse-shoe shaped macronucleus in addition to the coarsely granular and vacuolated cytoplasm. Abu El-Wafa (1988) described *I. multifiliis* from different species of fishes but with smaller measurements (28 µm in diameter). He also found the same species in the grass carp *ctenopharyngodon idella* with the measurements much larger (about 710 µm in diameter). The present study (67.4 µm) is similar to Koura *et al.* (1997) described the parasite from *Oreochromis niloticus* (57.5 µm).

The first symptom of heavy infection, white spots appear over the entire body "white spots disease". Fins begin to fray, skin starts being eroded, gills are pale (anemia). Scales may detach, eyes sunken, fish hardly move followed by death (Lom and Dykova, 1992).

3- Genus: ***Scyphidia***

***Scyphidia* sp. 1**

The present investigated parasite is resemble in shape and measerments to *Scyphidia doliaris* Chernova, 1977 (cited in Schulman 1984), but the latter has one contrctile vacuole, epistomial disc is below the peristomial disc level and there is no non ciliated groove. This species is first record in Egypt.

***Scyphidia* sp. 2**

Scyphidia sp. investigated during this study was characterized by the cup-shaped body, ribbon-shaped irregularly twisted macronucleus, occupies almost all the cell cavity. The most characterized feature was the detection of the giant micronucleus. The present *Scyphidia* sp. 2 is similar in shape and macronucleus to *Scyphidia* sp. described by Ahmed *et al*. (2000), but the present parasite have-smaller size and has giant micronucleus. The present parasite is closely resemble *S. globularis* described by Solomatova, 1977 (cited in Shulman, 1984), but the latter has a smaller macronucleus besides the micronucleus not detected. This species is first record in Egypt.

The pathogenicity of genus *Scyphidia* is attributed to disturbance in the respiratory process of the infected fishes, leading to asphyxia. (Paperna, 1980).

Explanation of figures

Fig. (1). Diagram of zenoma of *Neonosemoide* sp. (A) showing 16 macrospores, three lobes of hypertrophic nucleus and light zones. Mature spore (B) with characteristic egg-shaped and posterior vacuole. Mature spore (C) in details.

Fig. (2). Diagram of *Ichthyophthirius multifiils* (A). with characteristic round-shaped, horse-shoe macronucleus and meridional kineties. *Scyphidia* sp. 1(B) with transverse striation of pellicle. *Scyphidia* sp. 2(C) with cup-shaped body. Note the ribbon-shaped and sinuous macronucleus and giant micronucleus.

Fig. (3). Giemsa stain zenoma (A). Note the presence of anchoring disc as a red granule (arrowhead), silver impregnation Ichthyophthirius *multifillis* (B) and *Schyphidia* sp. 1(C) and Giemsa stain *Schyphidia* sp. 2(D). Not the sinuous ribbon-shaped macronucleus.

Fig. (4). Phase contrast microscope photograph of living specimens of *I. multifillis* (A) and *Schyphidia* sp. 1(B). Note the non ciliated groove.

Abbreviations for all figures

ad:	Anchoring disc
ap:	Anterior part of polaroplast
c:	Cilia
cm:	Cell membrane
cp:	Cytopharynx
cv:	Contractile vacuole
cy:	Cytostome
en:	Endospore
ep:	Epistomial disc
ex:	Exospore
fv:	Food vacuole
hcc:	Host cell cytoplasm
hn:	Hypertrophic nucleus
in:	Infundibulum
ki:	Kineties
lz:	Light zones
ma:	Macronucleus
mi:	Micronucleus
mpt:	Manubrium part of polar tube
n:	Nucleus
ncg:	Non ciliated groove
pd:	Peristomial disc
pf:	Polar filament
pl:	Peristomial lip
pp:	Posterior part of polaroplast
pts:	Polar tube sleeve
pv:	Posterior vacuole
s:	Spores
sc:	Scopula
slw:	Simple lamellar wall
sp:	Sporoplasm

Table (1): Comparative description of *Neonosemoides tilapiae* with the present species. (Measurements are in micrometers).

Parameter	*N. tilapiae* Sakiti and Bouix, 1987	Present Species
Xenoma size	120-800	50-70
Xenoma spores number	Many micro & macrospores	16 macrospores
Nucleus	Multinuclei	One with three lobes
Spore length	2.5-3	3-3.4
Spore width	1.5-2	1.3-1.8
Polar filament coils	4-5	4
Host	Cichlid *Tilapia zillii*	Bagrid *Chrysichthys auratus*
Site	Gills	Gills
Locality	Benin (West Africa)	Egypt

References

1. Abu El-Wafa, S.A.O., 1988. Protozoan parasites of some fresh-water fishes in Behera Governorate. M.V.Sc. thesis, Fac. Vet. Med. Alexandria Univ., Egypt.
2. Ahmed, A.K.; Tawfic, M.A.A. and Abbas, W.T., 2000. Some parasitic protozoa infecting fish from different localities of the River Nile, Egypt. Egypt. J. Zool., 34:59-79.
3. Bishai, H.M. and Khalil, M.T., 1997. Freshwater fishes of Egypt. Department of Natural Protectorate, Egyptian Environmental Affairs Agency (EEAA), Cabinet of Ministers, Arab Republic of Egypt. Publication of National Biodiversity Unit. No. 9.
4. Faye, N., Toguebaye, B.S. and Bouix, G., 1996. Ultrastructure and development of *Neonosemoides tilapiae* (Sakiti and Bouix, 1987) n.g., n. comb (Protozoa: Microspora) from African cichlid fish. Eur. J. Protistol., 32: 320-326.
5. Faye, N. and Toguebaye, B.S., 2005. microsporidian parasites in four species of carangid fishes from the Senegalese Coast (West Africa). Acta Adriatica, 46(1): 21-26.
6. Hoffman, G.L., 1970. Parasites of North American freshwater fishes. Unvi. California Press (eds). Berkely and Los Angeles, London, England FDL 21.
7. Klaus, R., 2005. "Marine Parasitology" Csiro publishing, Astralia. pp565.
8. Koura, E.A.; Kamel, E.G.; Abd El-Aziz, A.M. and El-Deep, N.I., 1997. Incidence and prevalence of some protozoan parasites infecting the Nile fish *Oreochromis niloticus* in Egypt. Egypt. J. Aquat. Biol. and Fish.1: 397-430.
9. Lom, J., 2002. Acatalogue of described genera and species of microsporidians parasitic in fish. Syst. Parasitol. 53: 81-99.
10. Lom, J. and Dykova, I., 1992. Protozoan Parasites of Fishes, Elsevier, Amsterdam pp315.
11. Lom, J. and Dykova, I., 2005. Microsporidian xenomas in fish seen in wider perspective. Folia parasitological, 52: 69-81.
12. Lom, J. and Nilsen, F., 2003. Fish microsporidia: fine structural diversity and phylogeny. Int. J. parasitol., 33: 107- 127.
13. Paperna, I., 1980. parasites infections and diseases of fish in Africa CIFA Technical paper No. 72020p.
14. Sakiti, A.G. and Bouix, G., 1987. *Nosemoides tilapiae* n. sp., Microsporidie parasite de Poissons Cichlidae des eaux saumâtres du Bénin: implantation et caractères ultrastructuraus. Parasitol. Res. 73: 203-212.
15. Shulman, S.S., 1984. Key to Parasites of Freshwater Fish of the U.S.S.R. pp 430.
16. Sprague, V.; Becnel, J.J. and Hazard, E.I., 1992. Taxonomy of phylum Microspora. Crit. Rev. Microbiol. 18: 285-395.
17. Weissenberg, R., 1968. Intracellular development of the microsporidian *Glugea anomala* Moniez in hypertrophying migratory cells of the fish *Gasterosteus aculeatus* L., an example of the formation of "xenoma tumours". J., Protozool., 15: 44-57.

11/25/2010

A Novel Self-adaptive Behavior Quantum Evolutionary Algorithm

Hassan K. Khalafi

School of Mathematics and Computer Science, Damghan University,
Damghan, Semnan, P.O. Box 36715-364 Iran
h_kadkhodaei@du.ac.ir

Abstract: In accordance with tradition quantum evolutionary algorithms can obtain excellent results in the optimization of Multi-peak functions. In any case, they are easy to be trapped to hurriedness. In this article, A Novel Self-adaptive Behavior Quantum Evolutionary Algorithm is recommending on the basis of the concepts and tenet of quantum evolutionary algorithms in order to enhance the efficiency. Firstly, Self-adaptive Behavior triploid chromosome is constructed to keep the population variety; Secondly, double mutation is used to make sure the variety of the swarm, then individual chromosome cross will be imported into this new algorithm in order to achieve the information communication between the chromosomes and enlarge the search scope in the available space. Experiments on test functions of varied intricacies are implemented and compared with other EAs. The result indicates that the new algorithm in this article can search and get the global most efficient solution in a shorter time.
[Hassan K. Khalafi. **A Novel Self-adaptive Behavior Quantum Evolutionary Algorithm**. Journal of American Science 2010;6(12):1483-1486]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Self-adaptive Behavior; Evolutionary Algorithm; QEA; Double Mutation; Discrete Cross

1. Introduction

Evolutionary algorithms (EAs) such as Genetic Algorithm [1] , Evolutionary Programming [2] , Evolutionary Strategies [3] , which can find out the best solution when they are used to deal with the complexity problems Cso they have widely applied into parameters estimate, mode recognition, machine learning, neural network, industry control and so on. However, when they are used to deal with the optimization of non-linear system, both of them often exist in the problem of prematurity and slow convergence speed.

The quantum evolutionary algorithm [4] is proposed in order to solve above problems. QEA is a new probability optimization method based on quantum computing theory. In recent years, much attention is paid to QEA because it has the characteristics of strong parallelism, rapid convergence, good search capability, short computing time, and small population size. Some QEAs and its improvement have been proposed for some combinatorial optimization problems. Although the QEA show better performance in solving combinatorial optimization problems than the traditional evolutionary algorithm, it is not suitable for solving complex optimization problems. So this paper presents a real-coded quantum evolutionary algorithm (RCQEA). RCQEA uses the variable component of the solving complex functions and qubit to construct a real-coded triploid chromosome in order to increase the diversity of the swarm, then all of the individuals (chromosomes) will evolve based on the double mutation and random discrete cross in order to achieve a balance between the local search and the global search. Standard simulation on the testified function shows that RCQEA which is used for solving complex optimization function has a very good performance.

2. Overview of QEA

As a new research field, QEA combines the quantum computing with genetic evolutionary algorithm. QC deals with investigations on quantum mechanical computers and quantum mechanics like qubits representation and superposition of states. QC can process huge numbers of quantum states simultaneously in parallel.

In QEA, the smallest unit of information stored in two-state quantum computer is called qubit, which maybe in the “1” state, or in the “0” state, or in any superposition of the two. The state of a qubit can be described as

$$|\psi\rangle = \alpha|0\rangle + \beta|1\rangle$$

Where α, β are complex numbers that specify the probability amplitudes of the corresponding states. $|\alpha|^2$ gives the probability that the qubit will be found in the 0 state and $|\beta|^2$ gives the probability that the qubit will be found in the 1 state. Normalization of the state to unity guarantees

$$|\alpha|^2 + |\beta|^2 = 1$$

The state of a qubit can be changed by the operation with a quantum gate. Inspired by the concept of quantum computing, QEA is designed with a novel Q-bit representation, a Q-gate as a variation operator, and an observation process.

A Q-bit individual as a string of n Q-bits is defined as

$$q_j^t = \begin{bmatrix} \alpha_{j1}^t & \alpha_{j2}^t & \cdots & \alpha_{jm}^t \\ \beta_{j1}^t & \beta_{j2}^t & \cdots & \beta_{jm}^t \end{bmatrix} \quad (3)$$

Where m is the number of qubit, i.e., the string length of the qubit individual, and $j = 1, 2, \cdots, n$ The following rotation gate is used as a Q-gate in QEA, such

$$U(\theta) = \begin{bmatrix} \cos(\theta) & -\sin(\theta) \\ \sin(\theta) & \cos(\theta) \end{bmatrix} \quad (4)$$

Where θ is a rotation angle of each Q-bit toward either 0 or 1 state depending on its sign.

The structure of QEA implies that most operations of QEA are based on the probabilistic, and QEA can not use any prior knowledge in the whole computing process.

The basic structure of QEA is described in the following [5] :

Procedure QEA
Begin
$t \leftarrow 0$
Initialize $Q(t)$
Make $P(t)$ by observing the states of $Q(t)$
Evaluate $P(t)$
Store the best solutions among $P(t)$ into $B(t)$
While (not termination condition) do
Begin
$t \leftarrow t+1$
Make $P(t)$ by observing the states of $Q(t-1)$
Evaluate $P(t)$
Update $Q(t)$ using Q-gates
Store the best solutions among $B(t-1)$ and $P(t)$ into $B(t)$
Store the best solution b among $B(t)$
If (global migration condition)
Then migrate b to $B(t)$ globally
Else if (local migration condition)
Then migrate b to $B(t)$ locally
End
End

Where $Q(t)$ is a population of qubit chromosomes at generation t , and $P(t)$ is a set of binary solutions ate generation f .

3. A New Real-Coded Quantum Evolutionary Algorithm

In this paper, the basic idea of RCQEA is as follows: Firstly, to reject the traditional encoding method in order to form a structure of real-coded triploid chromosome; then the chromosomes will be evolved based on the specific design of the real-coded triploid chromosome; Thirdly, double mutation is used to make sure the diversity of the swarm, then discrete chromosome cross will be imported into this new algorithm in order to achieve the information communication between the chromosomes and enlarge the search scope in the available space. At last, the new algorithm create the new chromosome through improved quantum gate.

3.1. Real-coded structure

As the multi-peak function optimization problem, it generally consists of two parts: The objective function:

$$Min(f(X)) = f(x_1, x_2, \cdots, x_n)$$

Subject to: $L \le X \le U$

$$X = \{(x_1, x_2, \ldots, x_i, \ldots, x_n) \mid l_i \le x_i \le u_i\} \} \in R^n$$

$$i = 1, 2, \ldots, n$$

Where $L = (l_1, l_2, \ldots, l_n)$, $U = (u_1, u_2, \ldots, u_n)$, L and U are the solution space, as for the since the variables x_i , its lower bound is l_i , and its upper bound is u_i . Real-coded triploid chromosome is composed of a variable component of the function and qubit. That is to say, the new chromosome is described as follows:

$$\begin{bmatrix} \alpha_1 & \cdots & \alpha_i & \cdots & \alpha_n \\ \beta_1 & \cdots & \beta_i & \cdots & \beta_n \\ x_1 & \cdots & x_i & \cdots & x_n \end{bmatrix}$$

Where $|\alpha|^2 + |\beta|^2 = 1$

3.2. Double mutation and random discretecross

In this paper, every new chromosome in the swarm will be mutated in one gene bit, that is, only one gene bit in the chromosome will be mutated every time, and other gene bits in this chromosome will keep invariability in order to build a new chromosome. The experiment has show that single gene mutation has higher search efficiency than the entire gene mutation [6].

Suppose the swarm in the t generation is:

$$P(t) = \{p_1^t, p_2^t, \ldots, p_j^t, \ldots, p_N^t\}$$

As a chromosome p_j^t , the new algorithm will select one gene bit random:

$$[\alpha_{ji}^t, \beta_{ji}^t, x_{ji}^t]^T \ , i = 1,2,\ldots,n$$

variable x_{ji}^t can be mutated as follows:

$$x_{ji}^{t+1} = x_{ji}^t + \sigma_{ji}^t N(0,1)$$

$$\sigma_{ji}^t = (u_j - l_j)\exp(\frac{-\mu k + \sqrt{\mu k}}{2})$$

Where $0 \le \mu \le 0.3$, μ is a step, which will be changed in a smaller range. The value of μ is related to the expectations precision of the objective function, k is the number of generation. In order to control the variation does not exceed the value of the domain, the parameters is adjusted as follows:

If $x_{ji}^{t+1} + 1 > u_j$

Then $x_{ji}^{t+1} = 2 \times u_j - x_{ji}^{t+1}$

If $x_{ji}^{t+1} < l$

Then $x_{ji}^{t+1} = 2 \times l_j - x_{ji}^{t+1}$

Repeat this process until x_{ji}^{t+1} can meet the conditions of a viable solution. If the new chromosome after mutation is better than the original chromosome, then this process is considered as a beneficial mutation for the evolution; otherwise as a invalid mutation for the evolution. As to the α_{ji}^t , β_{ji}^t in the original chromosome, the new algorithm will make mutation in its low bit (multi-point mutation), which can represent a larger search space, increase the diversity of the swarm. In this paper, when the solving function is very complex and the variables have strong correlation, so the algorithm will carry out random separated-cross in order to avoid the algorithm trapped into the local optimization, the process is as follows: Select an appointed chromosome p_u^t , and select another chromosome p_v^t random from the swarm, $(u \ne v)$. Then both of them will carry out random separated-cross as follows:

$$i = fin(random \times N)$$

Where i is the cross location in the chromosome, $fin(x)$ is a function, its result will be the maximum integer which is less small than x . $random$ will create a new random number between $[0,1]$; N is the length of chromosome.

The new two chromosomes after the random separated-cross are through the gene bit exchange in the location of i .

3.3 The procedure of RCQEA

Begin

Step 1. $t = 0$ initialize $Q(t) = \{q_1^t, q_2^t, \ldots, q_n^t\}$ according to the formula 6, define a empty memory storeroom;

Step 2. Make $R(t)$ by observing the states of $Q(t)$;

Step 3. Evaluate the whole swarm, and select the best chromosome;

Step 4. While the termination is true, the output of RCQEA is p_{best}^t , and the algorithm ends, otherwise, go to the next step;

Step 5. The chromosomes are evolved according to the 3.2 ;

Step 6. $t = t + 1$ go to step 4. End

4. Experimental results

$0/1$ knapsack problem [7] is referred to n articles with various value and weight, as well as partial articles are selected. To each article, there are two ways: select or not. The total weight of selected articles can't overrun that of knapsack appointed boundary and should reach the maximum of total value. If the total weight of all articles is less than that of the knapsack, then the problem will be extremely simple. And the benefit is equal to the total value of the whole articles. But actually, the knapsack's weight is always less than the total weight of the articles.

$0/1$ knapsack problem is an effective criterion to verify the performance of all kinds of algorithms. $0/1$ knapsack problem is a typical combinatorial optimization problem, it belongs to a NP-complete, we can describe it as follows:

Suppose something related to travel, its number is n , the quality and value of each one is $w_i(w_i > 0), c_i(c_i > 0), (i = 1, 2, \ldots, n)$. The capacity of this knapsack is $V(V > 0)$, then to solve an answer $x = (x_1, x_2, \ldots, x_n)$ to make sure the total value of this knapsack which is loaded with a lot of things is the most largest. We can describe this question as follows:

$$\max \quad f(x_1, x_2, \ldots, x_n) = \sum_{i=1}^{n} c_i x_i$$

Subject to

$\sum_{i=1}^{n} w_i x_i \le V$, $x \in \{0,1\}$, $(i = 1,2,...,n)$, x_i is a decision variable, if $x_i = 1$, which means res i has loaded in this knapsack; if $x_i = 0$, which means res i has not loaded in this knapsack.

In order to get a result, all the test data in this experiment have the strong correlation between weight and value.

$$w_i = random([1,10]) \ (Equably)$$

$$c_i = w_i + 5$$

And the average capacity of the knapsack:

$$V = 0.5\sum_{i=1}^{m} w_i$$

Before we solve knapsack problem, we have some prior knowledge, which are:

• When people begin to install a knapsack, they will choose bigger value of "profit/weight" firstly;

• When people begin to eliminate the capacity of knapsack, they will choose smaller value of "profit/weigh" to discard firstly.

So this is character information, and we will use them in our new RCQEA. In order to compare the new RCQEA with others in this paper, we adopt traditional GA, QEA, and RCQEA to validate the knapsack problem. The matlab 7.0 software was adopted for the implementation of the approach described previously. The number in knapsack problem is 250, and 500 respectively, and the size of population is 100 in GA and QEA. The size of population is 50 in RCQEA. The three evolutionary algorithms run 50 times and get their statistical results, respectively. The statistical results are in Table 1. We can find that the RCQEA's performance improve obviously compared with GA and QEA.

Table 1. statistical results

Number	Value	GA	QAE	RCQEA
300	Best	1653.4	1681.3	1732.1
	Average	1507.6	1595	1671.2
	Worst	1302.7	1421.4	1620.4
50	Best	2722.3	2907.2	3301.5
	Average	2599.4	2767.1	3113.4
	Worst	2278.2	2635.9	2997.1

5. Conclusions

This paper proposed a new algorithm RCQEA, inspired by the concept of Real-Coded. Compared with the traditional quantum evolutionary algorithm, its encoding method is convenient, running time is short, the speed of convergence is fast, and its global search ability is strong. So it is suitable for optimizing the complex functions such as 0/1 knapsack problems.

Corresponding Author:

Hassan K. Khalafi
School of Mathematics and Computer Sciences
Damghan University
Damghan, Semnan, P.O. Box 36715-364, Iran
E-mail: h_kadkhodaei@du.ac.ir

References:

1. Holland J H. Genetic algorithms and classifier systems: foundations and their applications[C], Proceedings of the Second International Conference on Genetic Algorithms DHillsdale, NJ : F Lawrence Erlbaum Associates, 1987, pp82-89.
2. Fogel L J, Owens A J, Walsh M J, Artificial Intelligence Through Simulated Evolution[M], Chichester: John Wiley,1966
3. Hans Paul Schwefel, Evolution and Optimum Seeking: A Wiley Interscience Publication, John Wiley & Sons, Inc., New York, NY, 1994.
4. Ren Ailian, Zhou Liang, Qu Hongjian, An Improved Quantum Evolutionary Algorithm and Its Application [C], Proceedings of the 3rd International Conference on Product Innovation Management, 2008, 10, pp1428-1431
5. Kuk-Hyun Han, Jong-Hwan Kim, On the analysis of the quantum-inspired evolutionary algorithm with a single individual, in: Proceedings of 2006 IEEE Congress on Evolutionary Computation, Vancouver. 2006, pp2622-2629
6. Wang Xiangzhong, Yu Shouyi, Improved evolution strategies for high-dimensional optimization[J], Control Theory & Applications, Vol.23, No.1, 2006, pp148-151
7. Hanxiao SHI, Solution on 0/1 Knapsack Problem Based on Improved Ant Colony Algorithm, Proceedings of the 2006 IEEE International Conference on Information Acquisition, 2006, 8, pp1062-1066

The Factors For Free Flow Speed On Urban Arterials – Empirical Evidences From Nigeria

Ibrahim Tunde Yusuf
Department of Civil Engineering,
University of Ilorin, P.M.B. 1515,
Ilorin, Kwara State of Nigeria (zip code: 234).
Email: ityusuf@yahoo.com; tunde_aroes@hotmail.com

ABSTRACT: Many generic factors of the weather, environment, vehicles (machines), fixed facilities (roadway) characteristics, humane (driver) and traffic streams either singly or in combination influence the free flow speed. The quantitative measures of these factors are desirable for reliable system design, analysis and evaluation for effectiveness, especially as reflect the typical humane-machine-environment system prevailing in Nigeria. This paper therefore presents the outcome of the quantitative evaluation of the influence of some factors on the free flow speed on an arterial in a medium sized urban settlement in Nigeria with a view to determining the probable analytical values for towns of similar hierarchy in Nigeria. Instantaneous speeds of forty test vehicles were observed in-vehicle at lull periods on the 7.1km Offa Garage-Emir's Market urban Road, Ilorin with simultaneous collection of data on age of driver, age of vehicle, passenger occupancy, roadside packed vehicles and businesses. The geometric properties of the arterial ware earlier established and segmented to four uniform sections. The data were computed using the category and statistical analysis approach. The results of the study indicated that the three factors of the environment (weather), humane and roadway geometry have negative influences on the free flow speed on an urban arterial. Estimates of the reduction of the various factors were detailed in the paper which was recommended for adoption for design and analysis of traffic stream in Nigeria and other medium sized towns in Nigeria.

[Ibrahim Tunde Yusuf. **Factors for Free Flow Speed on Urban Arterials – Empirical Evidences from Nigeria**. Journal of American Science 2010;6(12):1487-1497]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Free flow, instantaneous speed, lull period, roadway geometry, environment, in-vehicle data**,** time speed and traffic stream.

INTRODUCTION

Free flow speed can be defined as the drivers' desired average space mean speed in a low volume traffic stream when the density and flow theoretically tend to zero as mathematically represented by the fundamental traffic equation; $q = U_sD$, where q is flow (veh/hr), D is density (veh/km) and U_s is space mean speed (km/hr) (Agent et al (1998) and Dixson et al (199)). It occurs when no obstructions to flow either in terms of operational delays (congestion) or other adverse conditions prevail. Three principal factors of (a) roadway geometry and condition (b) drivers attributes and (c) environment have been reported to influence the values in the American urban traffic streams and highways (Kyte et. al. (2000) and Lamm et. al. (1990)). These authors showed from different studies that the percentage reduction due to the environment in the free flow speed are 8, 14, 15 and 17 – 18 respectively for wet pavement, high wind (greater than 24 km/ hr), low visibility (less than 0.28km) and rains; with a combined effect of all in the range of 30-38%. Lamm et al (1990) additionally observed that drivers also adjust speeds by as much as 10km/hr less during heavy rains when visibility becomes substantially obstructed, while Dowling et al (1997) equally identified drivers attributes and vehicle characteristics as the major factors in the free flow speed in the urban traffic. Younger drivers (in age), level of passenger occupancy, age of vehicles and trip purpose have impacted higher values on the speed while horizontal / vertical alignments and parked vehicles along the road impacted negatively on free flow speeds; (Blake, 1989).

The free flow speed is very important in traffic stream analysis for incidents and bottlenecks and hence the factors influencing its value should attract equal, if not higher attention because it is the source and not the effect. Besides it shall be helpful in sensitivity analysis. No wonder the Highway Capacity Manual (HCM 2000) underscores the importance of the various factors as pertaining to lane width/lateral clearance, the number of lanes and the driver's lateral wander etc. Indeed, Liang et al (1998) presented a chapter of detailed analysis of the effect of human factors (drivers') on the free flow speeds in the premise of person-machine control system. The phenomenon of perception-reaction time, control movement time responses to movement of other vehicles, handling of hazards in the roadway and the peculiarities of the different segments of driving population were examined, while the evolving formulation were tested with the American traffic situations and various models. Two of such models

are the (a) Hick-Heyman's law for perception-response time and (b) Fit's law of braking inputs.

Of particular interest for Nigerian situation are the age of drivers, age and condition of vehicles, the roadway geometry and pavement condition. It is an open secret that majority of vehicles on Nigerian roads are old and second hand that have already operated on the streets of country of manufacture before being imported to this country. Obviously, the enumerated models can not completely address the Nigerian situation because Nigerian drivers must have, at least characteristic vehicle handling practices, which must reflect on the traffic stream operation.

Traffic stream analysis and design in Nigeria presently apply either the American or British values which are empirical evidences of the operation in the developed countries. Hence the assessment of the influence of some of these factors to reflect the traffic operation in Nigeria shall be a worthwhile exercise, in that the closeness of the prevailing values to those usually borrowed from other countries' traffic situation, environment and humane (driver) attributes, shall offer the desired confidence and reliability when used for Nigeria traffics. It will appropriately reflect on Nigeria's peculiarities, her drivers' characteristic behaviors behind the wheels, the vehicle conditions/age, passengers' perceptions of trip purpose and other latent issues or combined effects.

The aim of this study is therefore to establish both quantitative and qualitative measures of the influence of the roadway factors (geometry), pavement condition, human (drivers) and the environment; on the free flow speeds on a typical Nigeria urban arterial. The specific objectives include (1) determination of traffic volume trend on different sections of a typical urban arterial in Nigeria, the Offa Garage – Emirs road, (2) determination of the inventory and geometric characteristics of each section of the arterial, (3) determination of the respective free flow speed at different weather conditions, (4) comparison of the free flow speed at each section, at different weather conditions and level of various factors, (5) to catalogue the various factors responsible for the prevailing free flow speed at each section and hence (6) postulate how the different factors influence the free flow speed on the Murtala Muhammad road in Ilorin, a medium sized urban centre in Nigeria or other Nigerian towns with the same political, historical and transportation hierarchy. Actually one would need to examine more than fifty geometric parameters for an attempt at comprehensive summation evaluation of the effects of factors on any parameter for traffic such as the free flow speed. This study is however limited to a few roadway environment (wet/dry condition), roadway geometry and vehicles (carriageway, roadway shoulder and width, lane width, number and obstruction, vehicle age and passenger occupancy); and driver' age.

MATERIALS AND METHODS

Studied Arterial

The studied arterial is 7.1km in length with the last 5km dualised. It originates from the Offa Garage and terminates at the Emir's palace, Ilorin, the Kwara State capital in the central western part of Nigeria. The road traverses four distinct sections; in terms of different levels of the road way characteristic, land use (road side development and business) and traffic volume. The respective partitions are designated as sections A, B, C and D.

Section A: Offa garage to Gaa Akanbi Junction, which is the section without median, Section B: Gaa Akanbi Junction to "A" Division which is a section with isolated and dedicated educational or public buildings which is not densely populated and thereby having a low level of activities, Section C: "A" Division to Post Office, which is a central business district of the town and Section D: Post Office to Oja-Oba, the predominantly market and indigenous area of the town which is densely populated and thereby highly characterized by a high level of activities. Figure 1 presents the diagrammatic sketch of the studied arterial.

Study Methods and Test Data

1. Road and Traffic Characteristics.

The influence of the three factors of roadway characteristics, drivers (human) and environment were studied using the manual observatory approach for the road inventory, traffic volume and drivers characteristics at different weather conditions. The lull period was established in order to determine the timings for the speed measurement. Table 1 presents the geometric properties of the studied arterial while Table 2 presents the probable measurement timings of free flow speed for the various days of the week.

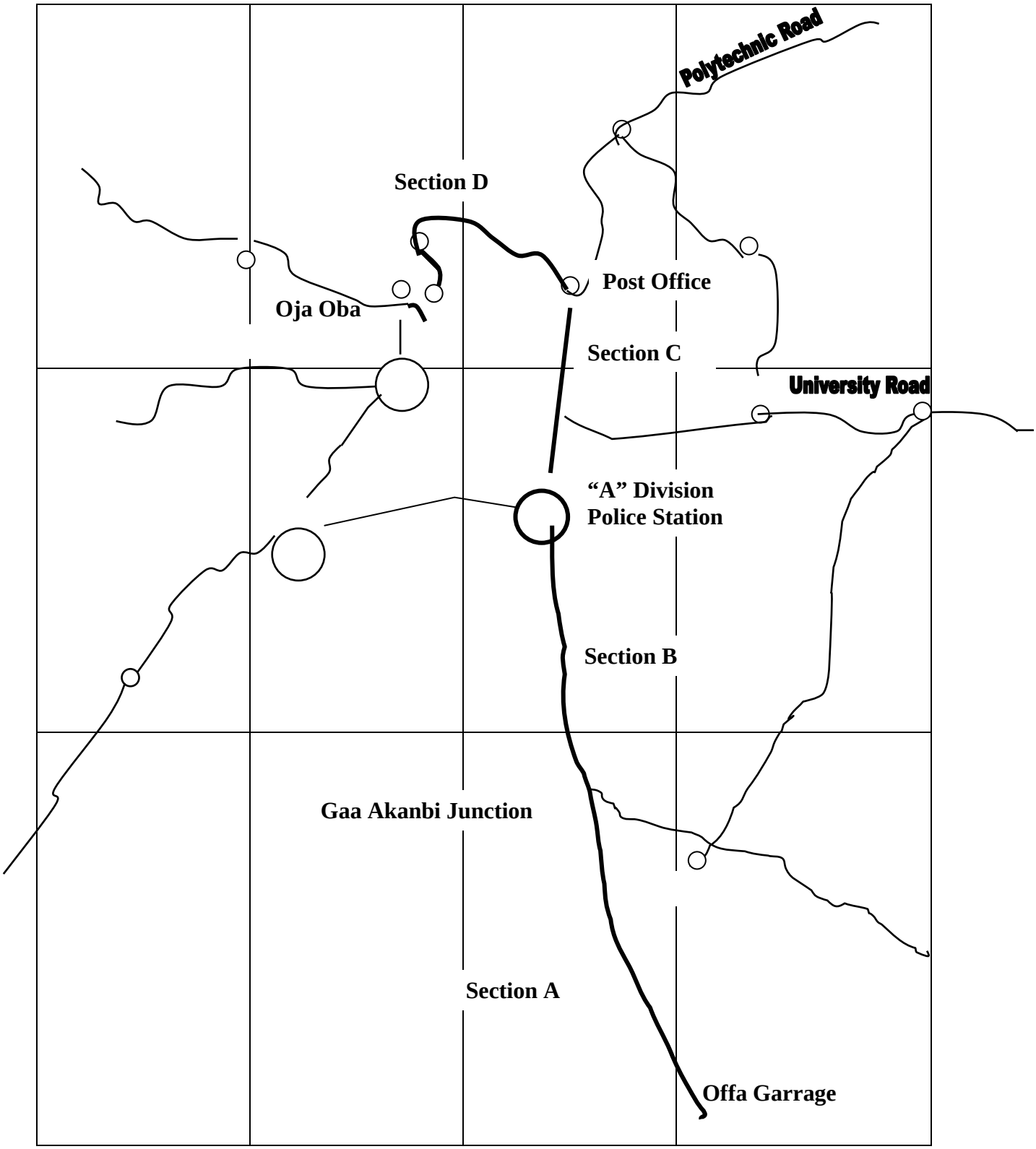


Figure 1: Geographical Presentation of Study Area

Table 1: Geometric Characteristics of the studied Arterial

Section	A	B	C	D
Length (km)	2.3	1.5	0.9	2.4
Overall width (m)	20.8	29.2	28.6	19.5
Roadway (m)	8.10	10.7	10.7	7.10
Shoulder (m)	2.3	3.0	2.70	2.20
Median (m)	1.8	1.8	1.8	1.5
Number of lanes	2	3/2	3/2	2/2
Number of intersections	11	9	5	15
Number of traffic controls	1	-	1	1

Table 2: Traffic Lull Periods on the Arterial

	Section A		**Section B**		**Section C**		**Section D**	
	Morning	Afternoon	Morning	Afternoon	Morning	Afternoon	Morning	Afternoon
Monday	7-8am	6-7pm	7-8am	6-7pm	7-8am	6-7pm	10-11am	6-7pm
Tuesday	7-8am	6-7pm	7-8am	6-7pm	7-8am	6-7pm	7-8am	6-7pm
Thursday	11-12noon	1-2pm	7-8am	1-2pm	7-8am	3-4pm	7-8am	6-7pm
Friday	7-8am	5-6pm	7-8am	12-1pm	7-8am	6-7pm	10-11am	6-7pm
Saturday	7-8am	6-7pm	7-8am	12-1pm	7-8am	6-7pm	7-8am	6-7pm

2. Free Flow Speed Data and Factors

The free flow speed measurement data was carried out simultaneously with drivers' attributes data collection using the in–vehicle method at pre-determined lull periods with two observers. Forty (40) test vehicles were selected ensuring that the two predominant passenger commuting vehicles (taxis) and small buses ("turo–turo") on the township roads were sampled. Observer (1) noted the instantaneous speeds (the speedometer reading) at three spots, approximately third portion along the length of each section of the arterial, while simultaneously observer (2) recorded the numbers of parked vehicles along the roadsides and passenger occupancy of the test car. Thereafter both observers interviewed the drivers to obtain the relevant information about respective attributes of age, sex, vehicle age and condition and pavement condition. A category analysis of the mean speed with the various parameters or factors was carried out. A summary of the free flow speed with respect to various factors for section A is presented in the Appendix.

RESULTS

1 Free Flow Factors Relationship

Table 3 presents the matrix of the relationship between the average free flow speed and the driver's age for the four sections and subjected to further analysis. For instance, the average free flow speed for cars driving along section A and driven by drivers within the age range of 60 – 69 years is (61.7 + 66.7 + 60.0)/3 which equals 62.8km/h. The average free flow speeds for the other three sections under consideration were similarly found for each age range and summarized in Table 4. These average values were then plotted against their corresponding ages as shown in Figure 2 whose slope was computed to be 0.46 km/hr/yr.

Table 3: Matrix of Free Flow Speed against Driver's Age

Driver's Age	Section A	Section B	Section C	Section D
20-29	1, 8, 10, 11, 19 21, 30, 33, 35, 44, 46, 48	1, 2, 7, 8, 20, 32, 33, 36, 42, 43, 44	7, 8, 11, 14, 19, 22, 25, 26, 27, 34, 40, 42, 48, 49	9, 17, 21, 27, 32, 38, 39, 41, 49
30-39	2, 3, 5, 7, 9, 12, 18, 20, 24, 31, 32, 34, 36, 37, 41, 42, 45, 37	3, 4, 5, 6, 9, 10, 11, 16, 17, 19, 23, 24, 25, 29, 31, 37, 38, 45, 48, 49, 50	1, 2, 3, 9, 15, 17, 18, 23, 32, 35, 36, 41, 43, 44, 50	4, 8, 11, 12, 13, 14, 16, 19, 20, 22, 26, 33, 40, 44, 48
40-49	4, 6, 13, 14, 16, 17, 22, 23, 27, 28, 38, 40, 49, 50	12, 14, 18, 21, 22, 26, 27, 28, 30, 39, 40, 41, 46, 47	6, 10, 12, 13, 16, 21, 24, 28, 29, 31, 37, 45, 47	1, 5, 6, 7, 10, 18, 23, 25, 29, 30, 31, 34, 36, 42, 43, 45, 46, 47, 50
50-59	15, 25, 39	13, 15, 35	4, 5, 20, 33, 38, 39, 46	2, 15, 24, 28, 36, 37
60-69	26, 29, 43	34	30	3

Table 4: Average Free Flow Speed – Driver's Age

Driver's Age	Section A	Section B	Section C	Section D
20-29	77.9	78.9	74.3	64.4
30-39	73.1	75.9	67.9	59.8
40-49	73.3	77.9	77.0	55.9
50-59	52.8	66.1	68.6	52.5
60-69	62.8	46.7	46.7	48.3

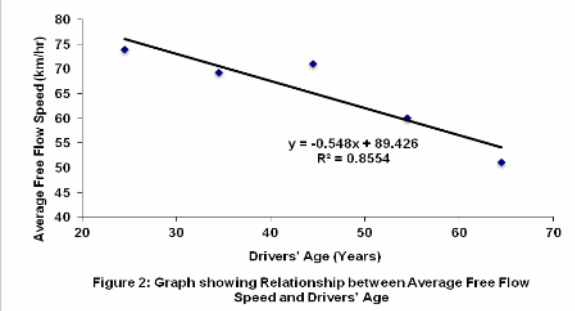


Figure 2: Graph showing Relationship between Average Free Flow Speed and Drivers' Age

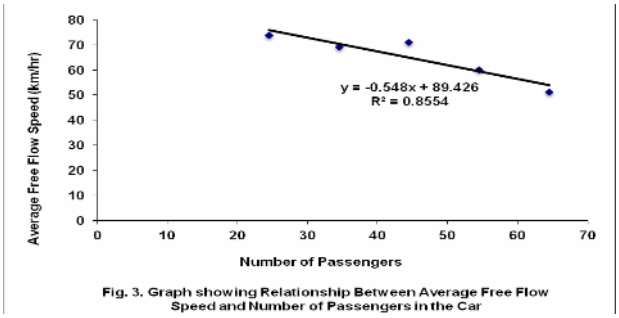


Fig. 3. Graph showing Relationship Between Average Free Flow Speed and Number of Passengers in the Car

Table 5: Average Free Flow Speed – Number of Passengers in Cars Relationship

No of Passengers	Section A	Section B	Section C	Section D	Average free flow speed
2	76.5	71.8	66.8	52.6	66.9
3	74.1	71.3	71.7	56.5	68.4
4	67.9	75.5	71.5	60.2	68.8
5	62.2	82.5	71.7	61.0	69.4
6	71.3	80.8	81.6	66.0	75.0

This procedure was repeated for the other factors of j number of passengers in the car, car age and number of parked cars along roadsides with corresponding results as represented in Tables 5, 6 and 7. The results were correspondingly displayed in Figures 3, 4 and 5. From the graph of average flow speed against number of passengers in car as shown in Fig. 3, the slope value in km/hr/passenger is -0.548. From the graph of average free flow speed against car age shown in Figure 4, the slope in km/hr/yr of age of car is -1.59. The graph of average free flow speed against number of parked cars is shown in Figure 5. The relationship is of quadratic form with peak at about 25 parked cars.

Table 6: Average Free Flow Speed – Car Age Relationship

Car Age	Section A	Section B	Section C	Section D	Average urban arterial free flow speed (AUAS)
1-2	77.1	80.5	77.0	57.4	73.0
3-4	73.0	77.2	70.5	56.5	69.3
5-6	62.0	77.1	67.2	62.3	67.2
7-8	53.3	69.3	72.7	48.3	60.9
9-10	54.2	58.4	66.7	65.9	61.3

Table 7: Average Free Flow Speed – Number of parked Cars Relationship

Number of parked Cars	Section A	Section B	Section C	Section D	Average urban arterial free flow speed (AUAS)
10-19	73.6	74.2	76.4	-	56.1
20-29	69.8	83.0	73.0	61.7	71.9
30-39	76.3	72.5	65.2	59.0	68.3
40-49	67.5	-	66.7	56.5	47.7
50-59	-	-	-	52.7	13.2

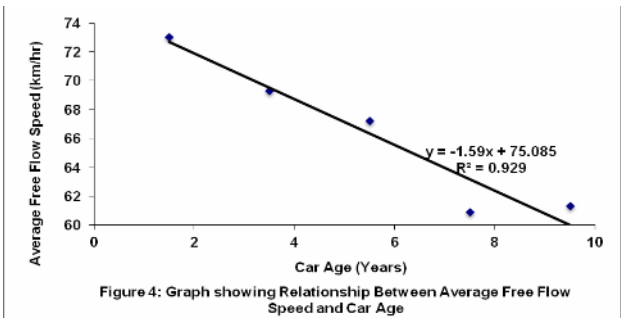


Figure 4: Graph showing Relationship Between Average Free Flow Speed and Car Age

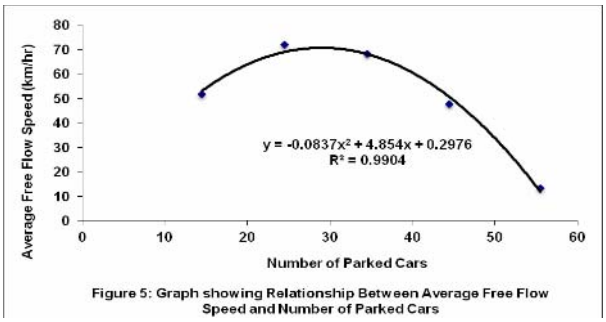


Figure 5: Graph showing Relationship Between Average Free Flow Speed and Number of Parked Cars

2. Free Flow Speed – Environmental Factor Relationship

The mean free flow speed was calculated respectively for dry and wet pavement conditions for the four different sections. Table 8 presents the summary of statistic data (mean and standard deviation) for section A while Table 9 is the corresponding summarized true speed at 95% upper confidence level (mean + 1.96 x standard error). There was a reduction of 0.5km/hr to 14.0km/hr on the road due to the wet condition which approximates to percentage reduction of 0.9% to 21.0%. The corresponding values based on time means at 95% confidence levels were 1.7km/hr to 9.98km/hr.

3 Free Flow Speed and Road Geometry/Condition

Table 10 shows the summary of average free flow speed and geometric condition at each section of the road. The graph of average free flow speed against each of the road geometry i.e. road width, number of lanes, shoulder width, number of intersections, travel way width and number of traffic control units are shown in Figures 6-11. These outcomes can form the basis of adjustment factors for the free flow speed of traffic stream on an urban arterial. For instance, a road width of 8metres or more will not attract any reduction in operating value but lower widths will attract at a unit rate of 17km/hr or less. The corresponding values for the different factors are summarized in Table 11.

Table 8: Mean Free Flow Speed Statistics (mean + standard deviation) (km/hr)

Section	A	B	C	D
Wet condition	73.8±11.10	66.8±14.99	71.1±10.72	57.5 ±6.10
Dry condition	72.0±12.00	80.8±7.18	74.2±5.00	58.0 ±7.76
Reduction (km/hr)	1.8	14.0	3.1	0.5
% Reduction	2.2	21.0	4.2	0.87

Table 9: True Free Flow Speed/pavement Condition Relationship

Section / Pavement Condition	Section A	Section B	Section C	Section D	Average Free Flow Speed (km/h)
Wet	78.44	73.32	74.75	60.05	71.64
Dry	76.37	83.30	76.44	60.85	74.24

Percentage reduction in free flow speed on wet pavement
=100(74.24 – 71.64)/74.24 = 3.5%

Table10: Summary of Free Flow Speed – Road Geometry Relationship

	Road width (m)	Shoulder with (m)	Number of lanes	Travel road width	Number of intersection	Number of traffic control unit	Mean free flow speed (km/h)
Section A	8.10	2.3	2	20.8	11	1	75.31
Section B	10.7	3.0	3	29.2	9	-	75.5
Section C	10.7	2.7	3	28.6	5	1	73.7
Section D	7.1	2.2	1	19.5	15	1	59.0

Table 11: Quantitative Measure of Geometric Effect on Free Flow Speed

S/No.	Factor	Unit	Critical value	Rate of reduction (km/hr/unit)
1	Road width	m	8	17.0
2	No. of Lanes	No.	2	15.0
3	Shoulder width	m	1.2	0.5
4	No. of intersections	No.	4	0.5
5	Overall roadway	M	20	17.0
6	No. of control Units	No.	1	-

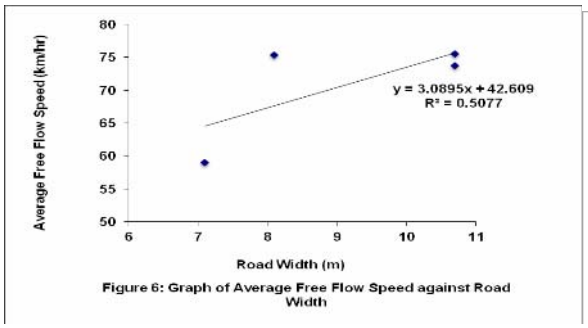


Figure 6: Graph of Average Free Flow Speed against Road Width

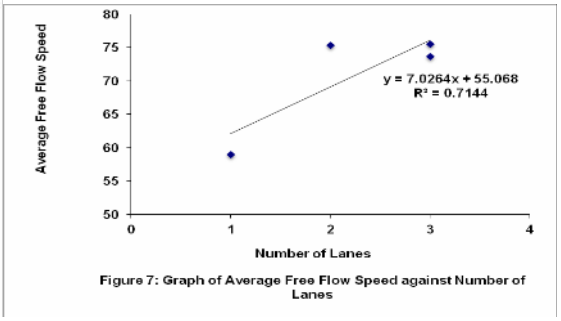


Figure 7: Graph of Average Free Flow Speed against Number of Lanes

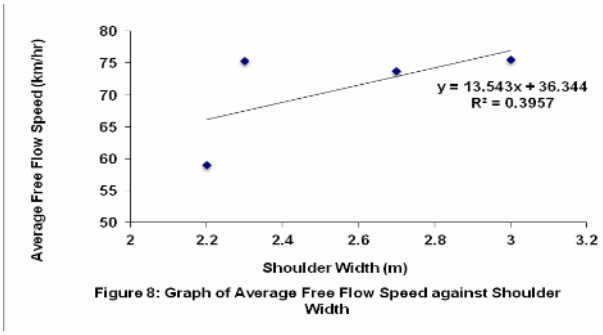


Figure 8: Graph of Average Free Flow Speed against Shoulder Width

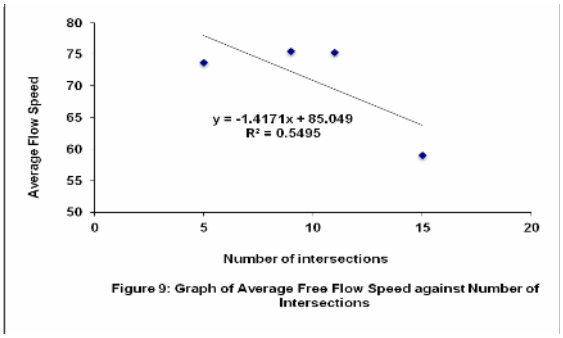


Figure 9: Graph of Average Free Flow Speed against Number of Intersections

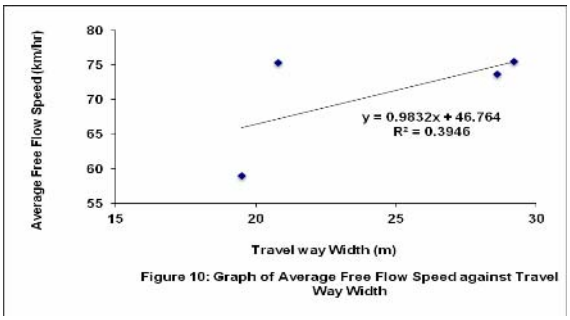


Figure 10: Graph of Average Free Flow Speed against Travel Way Width

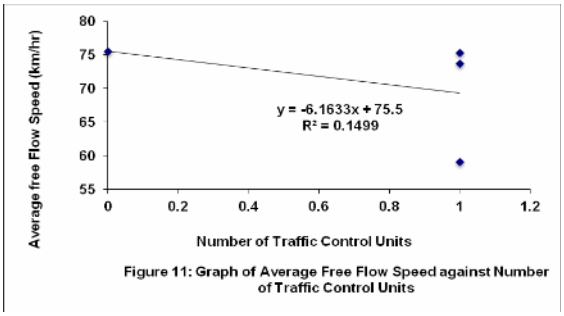


Figure 11: Graph of Average Free Flow Speed against Number of Traffic Control Units

DISCUSSIONS

As can be observed from Tables 4-11 and the corresponding Figures 2-11, the results of this study established that the drivers' desired speed on an urban arterial are influenced quantitatively by the various factors. For instance, the pavement condition when wet could impact as much as 10-14km/hr or 12-21% reduction in free flow speed; which are relatively higher than the reported 10km/hr for rains and 8% for wet pavement for other countries. This observation could be explained by many reasons, among which include the general state of pavement condition (various degrees of failures - potholes, edge breaks etc.), driver's age and state of mind, roadside

land-use and density, businesses and parking. The variation in free flow speed values (66.8, 71.1 and 57.5km/hr (wet) and 80.8, 74.2 and 58.0km/hr (dry)) for the different sections, especially B (with exception of section A) even attested to this deduction. Their corresponding coefficient of variation (CV = 100xSD/mean) of 22.4%, 15.1% and 10.6% for wet and 8.9%, 6.7% and 13.9% for dry show a reasonable consistency of the vehicles movement and by implication the behaviour of the drivers on the arterial. The better performances of the drivers during dry weather clearly show the influence. However, the results for section A showed otherwise which is absolutely a negation of the common logic.

However, it could still be explained by the fact that this section, being the only undivided portion allows the drivers more latitude to demonstrate their personal attribute and desire at overtaking and perform other movement manouvres. The simple principle for car following theory with which traffic stream flow operations are analyzed can easily breakdown. An organized movement is more probable on the divided arterial because the interactions with on-coming vehicles have been completely eliminated. It is at the intersections that it may manifest and will involve only for the left turning vehicles. Even, the highest values obtained for this section during the wet period is still more suspect. Another reason is that it is a sub-urban and hence the operating free flow speed should be close to that of the freeway. This is even more probable because the coefficient of variation for the two pavement conditions is 15% and 16.7% implying a good consistency of the data and person-machine control-road response system. The instantaneous speed is the most discerning indication of the overall duration for the performance of all the necessary activities for safe driving on a road.

Table 4 clearly shows the influence of drivers' age on free flow speeds. Their quantitative measures are averagely in the range of 0.36 and 0.68km/hr. As expected, the younger drivers operate on higher speeds than the older ones and it is consistent for all the sections. However, an interesting observation is noted for the drivers of age 40-49years. The drivers in the group operate on a speed same with or atimes higher than those in the 20-29 age brackets. This can be explained by their probable better understanding of the roads due to long time of driving coupled with their energy. As drivers grow older, such energy decreases in obedience to the law of nature – older bones get weaker and hence fewer propensities for higher work rate. Driving age in Nigeria commences from 18years while there is no official terminal age. It depends on how the body can cope. An equally relevant and related factor is the age of the vehicles. It is definite that age of the vehicle impact negatively on the performance of the road. The quantitative measurements are 1.30, 2.86 and 1.3625km/hr/year age of vehicle; with an average of 2.07km/hr/yr unit reduction factor. The reasons for this are obvious. The performance of vehicles decreases as they age and it is at old age they are converted to transportation operations. Both vehicles and drivers have their respective lifespans.

Another factor is the level of passenger occupancy of the vehicles. The speeds are averagely higher as the passenger capacity for each vehicle is being met. The measure of this effect was estimated for sections B, C and D to be in the range of 2.46-2.73km/hr/passenger. Table 5 and Figure 3 show that for section A (undivided), there is a reduction in speed because the occupants of a vehicle fast arriving from a non-urban journey shall be alighting at their destinations. This implies that the only activity predominating in the mind of the driver is how to reach the destination and return for more passengers. The usual stops to either drop or pick a passenger no longer exist. The various effect of the road geometry is succinctly summarized in Table 11. The critical values and the unit values reasonably agree with applicable modifications to various operational parameters for traffic stream analysis. The carriageway width of 8.0m, number of lanes of 2 and shoulder width of 1.4m are reasonable as they compare with standards. The same applies to other factors.

CONCLUSIONS

The following conclusions were reached from the study.

1. The traffic volume distribution on an urban arterial in Ilorin is trimodal with morning, noon and evening peaks and lulls in between.
2. The changes in the road geometry on each section of the arterial in terms of shoulder width, number of lanes, road/carriageway width and distance of nearest obstruction to the edge of the road do affect the instantaneous speeds. The corresponding numerical values of these factors on the Offa Garage-Emir's Road are about 0.5, 15, 27 and17km/hr for sections A, B and C respectively.
3. Different free flow speeds operate on distinct sections of the arterial. Respectively, the values are 72.0, 80.8, 74.2 and 58.0km/hr for section A, B, C and D when the pavement is dry, but correspondingly for wet conditions they are 73.9, 66.8, 71.1 and 57.5km/hr. Section B (Gaa Akanbi junction to "A" Division) has the highest average free flow speed while Section D (Post Office to Oja-Oba) has the lowest average free flow speed. The density

or level of usage reflects the deteriorating values along the routes.

4. A linear relationship exists between free flow speed of commuter vehicles and drivers' age, number of passengers in the car and car age with following respective rates of (a) decrease of 0.46km/hr/yr of driver's age, (b) an increase of 0.53km/hr per passenger in the car, (c) a decrease of 1.48 km/hr/yr of age of car.

5. A linear relationship does not exist between free flow speed and road side parking situation due to other prevailing factors.

6. The average free flow speed of commercial saloon cars is lower on wet pavement than on dry pavement, the percentage reduction being about 12%.

7. There are variations in free flow speed with changes in road geometry and characteristics.

RECOMMENDATIONS

The estimates of the various factor modification parameters obtained from this study can be used in urban road highway planning and analysis on Offa Garrage-Emir's road and other Nigerian roads with similar characteristics with the Ilorin transportation system evolution. The free flow speed modifications can be applied in the analysis and design of Ilorin urban arterial. This study should be conducted on arterials in other towns in Nigeria so that a national operational data base value could be established.

AKNOWLEDGEMENTS

This paper cannot be put together without the tremendous background information made available by various research workers, authors of excellent books and articles which have been referred to and listed in my references. I thank them.

REFERENCES

1. Adeniyi, A. A. (2006), "The Study of Factors Influencing Free Flow Speed on
2. Urban Arterials in Ilorin – A case Study of Offa Garage to Emir's Road", B.Eng
3. (Civil) Project, University of Ilorin, Unpublished, pp 18-40.
4. Agent, K. R., Pigman, J. G. and Weber, J. M. (1998), "Evaluation of Speed Limits in Kentucky", Transportation Research Record No. 1640, Transportation Research Board, Washington, D. C., pp.57-64.
5. Akcelik, R. (1982), "Traffic Signals: Capacity and Timing Analysis", Research Report No. 123, Australian Road Research Board, Victoria pp 3-5.
6. Blake, L.S. (1989), "Civil Engineering Reference Book", 4th edition, Butter Work and Co. Publishers Ltd. New York, pp 25-26.
7. Dixson, K. K., Wu, C.H, Sarasua, W. and Daniel, J. (1999), "Posted and Free-Flow Speeds for Rural Multilane Highways in Georgia", ASCE Journal of Transportation Engineering (1999), Vol.125, No.6, pp 54-61.
8. Dowling, R., Kittelson, W., Zegeer, J. and Skabardonis, A. (1997), "Planning Techniques to Estimate Speeds and Service Volumes for Planning Applications", NCHRP Report 387, Transportation Research Board, pp 12-15.
9. Institute of Transportation (2001), Taiwan Area Highway Capacity Manual, 90-16-1183, Ministry of Transportation and Communication, Taipei, pp 11-12.
10. Kyte. M., Khatib, Z., Shannon, P. and Kitchener, F. (2000), "Effect of Environmental Factors on Free-Flow Speed", Transportation Research Circular E-C018: 4th International Symposium on Highway Capacity, pp 34-42.
11. Lamm, R., Choueiri, E.M. and Mailaender, T. (1990), "Comparison of Operating Speeds on Dry and Wet Pavements of Two-Lane Rural Highways", Transportation Research record 1280, Transportation Research Board, Washington, D.C.
12. Liang, W.L., Kyte, F., Kitchener, F. and Shannon, P. (1998), "Effect of Environmental Factors on Driver speed", Transportation Research record 1635, Transportation Research Board. Washington, D.C.
13. O' Flaherty, C.A. (1974), "Highways and Traffic (Vol. 1)", 2nd edition, Edward Arnold Ltd., pp 21-26.
14. Teply, S., Allingham, D. I., Richardson, D. B. and Stephenson, B. W. (1995), "Canadian Capacity Guide for Signalized Intersections", Institute of Transportation Engineers, District 7.
15. Transportation Research Board (2000), "Highway Capacity Manual", National Research Council, Washington, D. C.

APPENDIX

Free Flow Speed Data in Respect of Various Factors for Section A

S/NO	Speed 1 (Km/h)	Speed 2 (Km/h)	Speed 3 (Km/h)	Average Speed (km/h)	DA	DS	NPIC	PC	CA	NPC
1	75	80	90	81.6	29	M	4	DRY	1	15
2	65	70	55	63.3	35	M	2	DRY	3	18
3	85	65	70	73.3	43	M	4	DRY	1	10
4	80	80	85	82.6	47	M	6	DRY	2	14
5	70	85	65	73.3	35	M	3	DRY	1	22
6	75	70	80	75.0	40	M	2	DRY	2	16
7	60	60	80	66.7	32	M	2	DRY	2	19
8	70	65	80	71.6	28	M	3	DRY	1	21
9	80	70	85	78.3	30	M	2	DRY	1	17
10	70	85	65	73.3	29	M	2	DRY	3	24
11	85	70	75	76.7	24	M	4	DRY	4	12
12	90	60	90	80.0	39	M	2	DRY	2	30
13	70	60	50	60.0	43	M	6	DRY	1	18
14	65	65	70	66.7	45	M	4	DRY	4	15
15	40	50	50	46.7	58	M	6	DRY	9	26
16	55	70	85	70.0	42	M	2	DRY	1	14
17	80	75	50	68.3	48	M	5	DRY	2	16
18	65	50	40	51.7	32	M	5	DRY	5	20
19	75	80	70	75.0	28	M	4	DRY	1	29
20	90	90	95	91.7	31	M	3	DRY	1	18
21	100	80	80	86.7	26	M	3	DRY	2	15
22	80	55	40	58.3	43	M	4	DRY	4	25
23	95	100	90	95.0	47	M	2	DRY	3	32
24	75	75	80	76.7	38	M	3	DRY	6	16
25	55	45	50	50.0	54	M	4	DRY	5	37
26	70	55	60	61.7	62	M	3	DRY	9	29
27	80	80	85	81.7	41	M	3	DRY	6	16
28	90	70	65	75.0	40	M	2	DRY	4	19
29	60	70	70	66.7	60	M	5	WET	2	17
30	70	75	90	78.3	28	M	3	WET	3	20
31	65	70	75	70.0	33	M	3	WET	3	15
32	75	65	65	68.3	37	M	4	WET	4	17
33	80	85	80	81.6	29	M	6	WET	3	13
34	85	85	100	90.0	36	M	2	WET	2	12
35	90	70	60	73.3	24	M	3	WET	3	17
36	65	85	90	80.0	28	M	2	WET	4	25
37	60	50	70	60.0	34	M	3	WET	3	17
38	45	60	55	53.3	48	M	3	WET	7	19
39	55	70	60	61.7	53	M	4	WET	4	21
40	80	90	90	86.7	42	M	6	WET	2	20

Author's information

Age: 47
Sex: Male
Height: 1.6m
Hobby: Reading

Submission date: 24th October, 2010.

Biochemical and Molecular genetic Evaluation of some conifers genetic resources

Soliman.M.H., ⋆ Gad,Mervat,M.A.⋆⋆ Hussein, H.Mona, ⋆ Mohamed,A.S.⋆⋆

⋆ Department of Genetics, Faculty of Agriculture, Cairo University, Giza, Egypt.

⋆⋆ Horticulture Research Institute, Agricultural Research Center , Giza.

rfr2000@live.com

Abstract: Genetic polymorphism was investigated in six conifers representing four Pinus species, i.e (*P.halepensis, P.canariensis, P.pinea, and P.roxburghii)* which belong to family *Pinaceae* and two members of family *Taxodiaceae, i.e. (Sequoia sempervirens and Taxodium distichum).* In this respect, genetic biochemical (proteins and isozymes), as well as molecular (RAPDs and ISSRs) analysis were investigated. Proteins and peroxidase banding patterns resulted in extensive polymorphism among conifers under investigation, however, Adh isozyme banding patterns were not satisfactory in this concern. RAPD analysis exhibited a total of 66 bands, out of them 25 bands were polymorphic (37.88%). Five ISSR primers generated reproducible and informative amplified products. those were used to distinguish between the six conifers, since 38 bands were polymorphic out of total 81 bands with 47.95% of polymorphism which can be considered as useful markers for identifying conifers. Based on combined data obtained by proteins, peroxidase, RAPD and ISSR analysis, it was possible to discriminate between the six conifer trees under investigation. The present study indicates that the application of biochemical and molecular fingerprinting of the six conifers provided a solid ground that will allow an easier and faster genetic identification of other woody trees species.

[Soliman.M.H., Gad,Mervat,M.A. Hussein, H.Mona, Mohamed, A.S. **Biochemical and Molecular genetic Evaluation of some conifers genetic resources.** Journal of American Science 2010;6(12):1498-1509]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Conifers, *Pinus*, *Sequoia*, *Taxodium*, fingerprint, RAPD, ISSR, SDS-PAGE, Peroxidase, alcoholdehydrogenase.

1. Introduction:

In forestry, genomic discovery will support genetic improvement of tree varieties for solid wood, pulp and paper, biofuels, and biomaterials through integration into traditional breeding approaches in domesticated tree population (Neale, 2007).

Characterization of the genetic diversity and examination of the genetic relationship among conifers are important for the sustainable conservation and increase use of plant genetic resources. Traditionally, comparative vegetative anatomy and plant systematic were two common strategies to assess the relationships among conifers (Wang, *et al.*, 2009).

Tree breeding generally involves recurrent selection and population improvement in each cycle of breeding and each cycle can take many years (White, *et al.*, 2007). In trees, breeding populations are often large and genetically diverse in their composition, as opposed to line breeding in many agricultural crops where the number of genotype is often limited. Tree breeders could then use genomic selection directly for population improvement or, more likely, combine genomic selection approach to maximize genetic gain per unit time (Neale, *et al.*, 2007).

The genus *Pinus* is among the most widely distributed and prominent genera of trees in the world, including many of the most economically valuable species of forest trees. The taxonomy of *Pinus* is based mainly on morphological and partially on molecular data that are incomplete for many taxa (Strauss and Doerksen, 1990).

Taxodium disticum is a deciduous conifer in the family *Taxodiaceae*, which has numerous attributes that qualify it as a supreme urban landscape tree and as a species to mediate harsh coastal wetland and flood plains of major rivers in the south (Zhou, 2007). *Sequoia sempervirens* has a very decay and fire resistant wood, as well, resistant to weather, insects and fungus attacks. The bark is used as hog fuel, insulation, or garden mulch. This species lives up to 1500 years, and reaches 116 m height (as the tallest stands record). The economic value of the tree is so high, however, little attention has yet been paid to it (Clark and Scheffer, 1983). There is only one *Sequoia sempervirens* tree in Egypt which had been threatened, never produces seeds, neither vegetatively propagated, however, it was possible to propagate through tissue culture techniques (Gad, *et al.*, 2006).In Egypt, no attention has been paid to evaluate the genetic relationships between such conifers which were introduced and grew well under the local environmental conditions. This study was designed to assess the pattern of genetic variation between different genetic resources of conifers genera and species. This was achieved through the use of electrophoretic (protein and isozymes) and molecular (RAPD and ISSR) techniques which have been increasingly applied to the study of tree species in

recent years, to provide information in support of conservation planning and management. Therefore, the objective of this study is to identify and characterize species-specific biochemical and molecular markers among the conifers of the two families (*Pinaceae* and *Taxodiacea*e) in order to establish genetic relationships among them. Moreover, the resulted genetic relationships can be directed towards tree genetic resources sustainable conservation as well as to exploit these investigations in breeding programs to maximize genetic gain per unit time in order to support genetic improvement of tees under investigation.

2.Materials and Methods:

2.1.Materials:

2.1.1.Plant materials

This investigation was achieved during the period from 2006 to 2010 at Biotechnology Research Laboratory, Horticulture Research Institute (HRI), Agricultural Research Center (ARC), Ministry of Agriculture, Egypt.

Genetic resources of six mother trees, morphologically identified conifers located at Orman Botanic Garden, Giza, were used as germplasm sources for this research work, namely *Pinus halepensis*, *Pinus canariensis*, *Pinus pinea*, *Pinus roxburghii*,(belong to family *Pinaceae*), *Sequoia sempervirens* and *Taxodium distichum*,(belong to family *Taxodiacea*)

2..2.2.Primers:

Table (1): List of the RAPDs primers codes and their nucleotide sequences

No	Code	Sequence	No	Code	Sequence
1	OP - F01	5` ACGGATCCTG 3`	4	OP-C11	5` AAAGCTGCGG 3`
2	OP-F05	5` CCGAATTCCC 3`	5	OP-Z01	5` TCTGTGCCAC 3`
3	OP-F08	5` GGGATATCGG - 3`			`

Table (2): List of used ISSR primer codes and their nucleotide sequences.

No.	Code	Sequences	No.	Code	Sequences
1	A98	5` ACACACACACACA 3	4	HB 12	5`CACCAC CAC GC 3`
2	HB 8	5` GTGTGTGTG TGTGG 3`	5	HB 13	5`GAGGAGGAGGC 3`
3	HB 10	5`GAGAGAGAGAGACC 3`			

2.2.Methods:

2.2.1.Biochemical genetic identification.

2.2.1.1. Protein electrophoresis:

Young fresh leaves were collected from conifers under investigation and were ground into a fine powder by using liquid nitrogen (-196 °C) and a mortar and pestle. A sample of 0.5 g was homogenized with 0.9 ml extraction buffer (10 ml 0.5 M Tris pH 6.8, 16 ml 10% SDS, 30 ml D.W.). The extracts were transferred into Eppendorf tubes and centrifuged for 10 min. at 1000 rpm under cooling (4 ºC). Supernatants (containing protein extract) were transferred into clean tubes and used for SDS-PAGE analysis. Isozymes were extracted, as described by Jonathan and Weeden (1990). A volume of 120 µl of protein extract was loaded into sodium dodecyle sulphate polyacrylamide gel (SDS-PAGE), 12% W/V vertical slab using BIORAD Techware 1.5 mm according to the method of Laemmli (1970) and modified by Studier (1973). The molecular weights of proteins were estimated relative to a standard protein marker with a wide range of molecular weight (Fermentas .com).

2.2.1.2. Isozymes electrophoresis

Native – polyacrylamide gel electrophoresis (Native - PAGE) was conducted to identify isozyme variations among the six studied conifers using two isozyme systems using 12% (W/V) slab gel according to Stegmann *et al.* (1985). Isozymes were extracted from 0.2 g of fresh and young leaves samples in a 1 ml of 0.125 M Tris – borate buffer, pH 8.9. A volume of 50 µl extract of each sample was mixed with 12.5 µl of glycerol, and 60 µl from this mixture was applied to each gel well.

2.2.1.2.1. Peroxidase Detection:

Peroxidase was detected by incubating the gel in a darkness for one hour at 37°C in a mixture of 15 ml of 10% benzidine (in 95% ethanol); 50ml of 1mM potassium acetate and 1 ml of 1% H_2O_2 (pH 4.7). After the incubation period the gel was rinsed in a distilled water and fixed in a 50% glycerol for one hour. The gel was placed into this solution and 5 drops of hydrogen peroxide solution were added. The gel was incubated at room temperature until bands appeared, (Brown, 1978).

2.2.1.2.2 Alcohol dehydrogenase (*Adh*):

Gel was placed in a solution composed of 100 ml of 0.1M Tris-pH (7.5), NAD 30 mg, MTT 20 mg, phenazine methosulfate (PMS) 5 mg and ethanol 6 ml, and incubated at 30 ºC for 30 min. until bands appeared.

2.2.1.2.3 Gel documentation

Gels were digitally photographed and analyzed using Gel Doc Viller Lourmat system to capture the image and to calculate band intensities.

2.2.2.Molecular genetic identification

DNA extraction:

Total genomic DNA was extracted and purified from 0.1 g of freeze – dried powered samples as described by Dellaporta *et al.* (1983). DNA present in the supernatant was precipitated according to the

described protocol, re-dissolved in a sterile, distilled water (D.W.) and quantified.

2.2.2.1. Randomly amplified polymorphic DNA (RAPD).

Amplification of genomic DNA using polymerase Chain Reaction (PCR)

Different preliminary experiments were carried out in order to optimize the factors leading to clear reproducible amplification products. A total of ten random DNA oligonucleotide primers were independently used according to Williams *et al.* (1990) in each PCR reaction.

Only five primers (Operon biotechnologies, Inc. Germany) were succeeded to generate reproducible polymorphic DNA products. Table (1) displays the base sequence of these DNA primers those produced informative polymorphic bands.

PCR was performed in a 30µl reaction volume containing the following : 3.0µl of dNTPs (2.5 mM), 3.0µl Mg-Cl_2 (25mM), 3.0 µl of 10 x buffer, 2.0 µl of primer (10 ρmol), 0.20 µl of Taq DNA polymerase (5u/ µl), 2.0 µl of template DNA (50.0 ng/ µl), 16.80 µl H_2o (sterile D.W.). The DNA amplifications were performed in an automated thermal cycle (Techn.TC-512 PCR system). The reaction was subjected to one cycle at 94ºC for 4 min. followed by 45 cycles of 1 min. at 94 ºC, 1 min. at 36 ºC, and 2 min. at 72 ºC. The reaction was finally stored at 72 ºC for 10 min.

The amplification products were separated in 1% (w/v) agarose gel in 1 x TBE buffer and visualized by staining with ethidum bromide. Reproducibility of DNA profiles was determined by replicating all RAPD reactions at least three times.

After electrophoresis, the RAPD patterns were visualized with UV transilluminator and photographed by gel documentation system (Gel Doc Bio Rad 2000). RAPD markers were scored from the gel as DNA fragments present or absent in all lanes. Each experiment was repeated twice and only stable products were scored.

2.2.2.2. Intersimple sequence repeats (ISSR)

ISSR- PCR reactions were conducted using five primers. PCR was performed according to Wang, *et al.* (2002) in 30 µl reaction volume containing 2.5 µl dNTPs (2.5 mM), 2.5 µl Mg Cl_2 (25 mM), 2.5 µl buffer (10 X), 3.0 µl primer, 2.5 µl Taq DNA polymerase (1 U/1 µl), 2.0 µl Template DNA (25 ng) and 11 µl H_2O (sterile D.W.).

The PCRs were programmed for one cycle at 94 ºC, for 4 min. followed by 45 cycles for 1 min. at 94 ºC, 1 min. at 57 ºC and 2 min. at 72 ºC then 12 min. at 72 ºC for one cycle and the reaction was finally hold at 4 ºC. The PCR products were separated on 1.5 % agarose gels and fragments sizes were estimated with the 100 bp ladder marker. Table (2) illustrates the base sequence of the DNA primers, those produced informative polymorphic bands.

2.2.3.Data analysis:

The similarity matrices were done using Gel works ID advanced software UVP- England Program.

The relationships among genotypes and species as revealed by dendrograms were done by using SPSS windows (Version 10) program. Dice computer package was used to calculate the pairwise difference matrix and plot the phenogram among conifers genotype under investigation. The resultant similarity matrix was employed to construct a dendrogram using Sequential Agglomerative Hierarchical Nesting (SAHN) based Unweighted Pair-Group Method with Arithmetic Means (UPGMA) to infer genetic relationships and phylogeny(Sensi, et al., 2003)

3.Results:

3.1.Biochemical genetic identifications

3.1.1. SDS- PAGE protein banding patterns of conifers leaves.

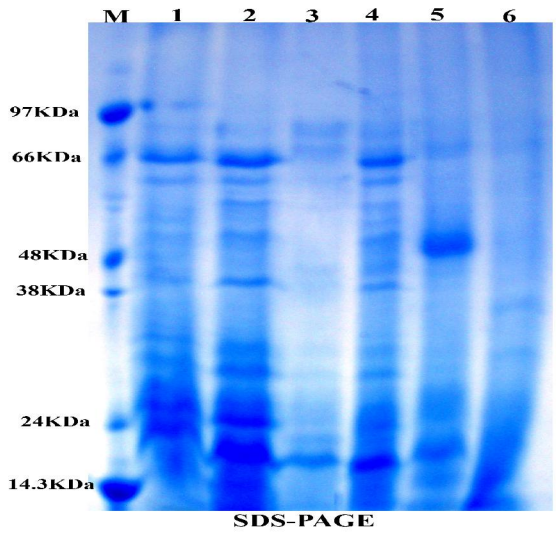
The needle leaves protein banding profile which was separated using SDS-PAGE of the six genera and species of conifers are illustrated in Fig. (1). The total number of bands was 23 with molecular weights ranged from 15.798 KDa to 100.751 KDa (Table 3). The highest number of bands was 16, detected in *Pinus roxburghii* while the lowest number of bands was 8, identified in *Taxodium distichum.*

Demonstrative analysis of the presence and absence of bands were assessed with (1) and (0), respectively, are illustrated in Table (3). It is observed that two bands were monomorphic (29.677 and 21.287 KDa), while 18 bands were polymorphic, giving 91.304% polymorphism and 3 unique bands (100.751, 92.825 and 24.963 KDa) among the examined conifers.

The matrix of similarity index for the six conifers germplasm is presented in Table (4). The highest coefficient was 86.7 recorded between *Pinus canariens*is and *P.roxburghii*, followed by 80.0 recognized between *Sequoia sempervirens* and *Taxodium distichum*, followed by 75.9 between *P.roxburghii* and *P.halepensis* and finally 74.1 between each of (*P.halepensis* and *P.canariensis*) and (*P.roxburghii* and *P.pinea*). On the other hand, the lowest coefficient value was 28.6 observed between *P.halepensis* and *Taxodium distichum.*

3.1.2. Isozymes banding patterns.

The isozymes banding pattern of peroxidase and alcoholdehydrogenase (*Adh*) isozymes of the six conifers genotypes is presented in Fig. 3 (a and b), while data are scored in Table (5).



Fig(1): SDS-PAGE protein banding patterns of leaves of the six conifers

[M :standard protein (KDa) ,
1-Pinus halepensis,
2- P.canariensis,
3- P.pinea,
4-P.roxburghii,
5-Sequoia sempervirens,
6-Taxodium distichum].

The peroxidase patterns exhibited a total number of 20 bands, 13 of them are polymorphic, with (100%) polymorphism while 7 bands are unique, though, no monomorphic bands were scored. Polymorphism exhibited by this pattern completely discriminated between the studied conifers.

Table (4) A Proximity matrix of protein banding patterns

Case	Matrix file input					
	1	2	3	4	5	6
1		0.741	0.583	0.759	0.400	0.286
2	0.741		0.720	0.867	0.538	0.455
3	0.583	0.720		0.741	0.522	0.316
4	0.759	0.867	0.471		0.500	0.333
5	0.400	0.538	0.522	0.500		0.800
6	0.286	0.455	0.316	0.333	0.800	

Pinus roxburghii was discriminated by the presence of the unique bands with Rf values (0.105 and 0.361). Besides, *Sequoia sempervirens* was distinguished by the absence of the unique negative bands with Rf values (0.279, 0.336 and 0.387) and the presence of unique bands with Rf values (0.731, 0.790, 0.836 and 0.900). As well, *Taxodium distichum* was identified by the presence of (0.055) and the absence of (0.597) unique bands, in addition, to other polymorphic bands recognized in each conifer.

Table (3): Data matrix illustrating the presence or absence of bands in the leaves protein electrophoresis banding patterns for the six conifers.

Band number	MW (KDa)	P.halepensis	P canariensis	P.pinea	P.roxburghii	S.sempervirens	T.distichum
1	100.751	0	0	0	1	0	0
2	92.825	1	0	0	0	0	0
3	84.94	1	1	1	1	0	0
4	77.725	0	0	0	0	1	1
5	77.196	0	0	1	0	1	0
6	73.593	1	1	0	1	0	0
7	65.826	1	1	1	1	0	0
8	59.283	0	1	0	1	0	0
9	54.993	1	1	1	1	0	0
10	49.639	1	1	0	1	1	0
11	49.414	0	0	0	0	1	1
12	43.600	0	0	1	1	1	0
13	40.445	1	1	1	1	1	0
14	36.341	0	0	0	0	1	1
15	31.847	1	0	0	1	0	0
16	29.677	1	1	1	1	1	1
17	26.727	0	1	1	1	0	0
18	24.963	1	0	0	0	0	0
19	23.690	1	1	0	1	1	1
20	21.287	1	1	1	1	1	1
21	19.171	0	1	1	1	1	1
22	17.662	1	1	1	1	0	0
23	15.798	0	1	0	0	1	1
Total		13	14	10	16	12	8
Polymorphism %	91.304						

In Fig (3-b) and Table (5) there is a quite evidentence that only four polymorphic bands were identified out of five total scorable bands with 80% polymorphism, while one monomorphic band (Rf 0.863) was recognized as well as 1 unique band was scored in the illustrated *Adh* profile. Only *Taxodium distichum* was discriminated with the presence of the unique band with (0.205) and the absence of (0.268) Rf values, while the other taxa shared the presence and absence of several bands. Each of *P. halepensis* and *P. canariensis* shared the presence of the bands with RF values (0.268, 0.363 and 0.863) and the absence of (0.205 and 0.739) bands. Besides, each of *P. pinea*, *P.roxburghii* and *S. sempervirens* shared the presence of bands with Rf values (0.268, 0.739 and 0.863) and the absence of the bands (0.205 and 0.363) Rf values. Therefore, *Adh* isozyme patterns were not satisfactory to detect

phylogenetic relationships among conifers genotypes used in this study.

Consequently, isozyme profile permitted the identification of three conifers under investigation, *i.e. Pinus roxburghii*, *Sequoia sempervirens* and *Taxodium distichum* by the presence of two unique markers found in peroxidase isozyme with *P. roxburghii*, four with *Sequoia sempervirens* and absence of three bands and the presence of one with *Taxodium distichum* with absence of one unique band.

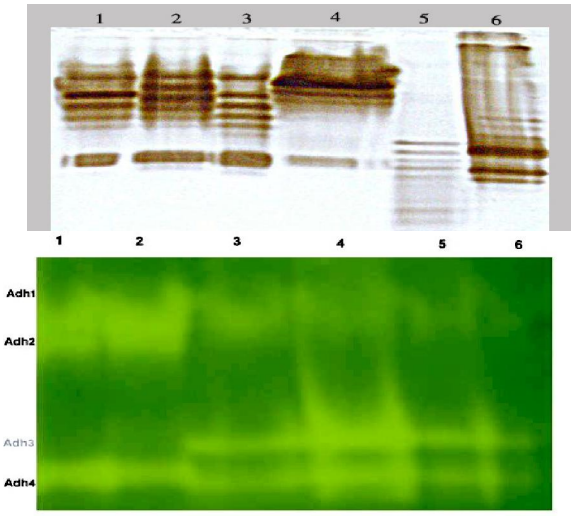


Fig. (3): Zymogram of: (a) peroxidase and (b) Adh isozymes of the six conifers :
1-*Pinus halepensis*
2-*P.canariensis*
3-*P.pinea*
4-P.roxburghii
5-*Sequoia sempervirens*
6-*Taxodium distichum*

3.2.Molecular markers:

3.2.1. Randomly Amplified Polymorphic DNA (RAPDs) analysis

Out of ten decamer (RAPD) primers tested, five revealed distinct polymorphism among the six conifers under investigation. A total of 66 DNA bands were detected; 25 of them showed polymorphism. Out of these polymorphic bands, 10 unique bands were scored (Table 6). However, polymorphism ranged between 18.182% (primers OP-F01, OP-F05 and OP-C11) and 58.824% (primer OP-Z01). The range of DNA bands size was between 97.071 and 882.55 bp (Fig 4).

From Table (6) and Fig (4) it could be noticed that a maximum of two polymorphic (18.182% polymorphism) and nine monomorphic DNA bands were recorded in the RAPD profiles generated by the primer OP-C11, while no polymorphic-unique bands were scored. The first polymorphic band (about 350 bp) was observed in all conifers genera and species, except in *P.halepensis*, while the second polymorphic band (313.4 bp) was identified in all conifers, except in *S.sempervirens*. The absence of the forementioned polymorphic bands could be considered as negative unique bands for both genera.

It could be observed that the primer OP-F01 generated a total of two polymorphic bands, inducing 18.182% polymorphism and nine monomorphic DNA bands in the studied species and genotypes, while one unique band (positive) was identified out of the total polymorphic bands. This unique band (molecular size 587.065 bp) was detected in *Sequoia sempervirens*, while the absence of the (negative) band (molecular size 473.151 bp) was observed in *Pinus canariensis*. These unique bands (positive and negative) clearly discriminate both of *Sequoia sempervirens* and *P.canariensis* from the pool of conifers investigated.

Table (6) and Fig (4) illustrate the RAPD profile generated by the primer OP-F05, which produced nine monomorphic and two polymorphic DNA bands with 18.182% polymorphism. One polymorphic band was identified as a unique band (with fragment size 337.427 bp) in *Taxodium distichum*. The other polymorphic, non-unique band, was detected at about 592.501 bp distinguishing the four *Pinus* species by its presence, while it was absent in each of *S.sempervirens* and *T.distichum*. This primer could discriminate each of *S.sempervirens* and *T.distichum* from the group of *Pinus* species, since *S.sempervirens* exhibited no polymorphic bands while *T.distichum* was distinguished by the presence of 337.427 bp unique band.

A total of nine polymorphic bands out of 16 total observed bands with 56.25% polymorphism were generated by the primer OP-F08, three bands were scored as unique, out of the polymorphic bands. All the three unique bands (with 562.785, 366.313 and 341.174 bp) discriminated *S.sempervirens* from the other genera. On the other hand, seven monomorphic bands were scored at about 386.7, 328.8, 290.1, 271.8, 212.2, 180.4 and 158.8 bp. Each of the six conifers is characterized by polymorphic non unique bands, *i.e. P.halepensis* recorded 521.2, 441.9 and 97.07 bp polymorphic bands, *.canariensis* scored only 97.07 bp as polymorphic band while *P.pinea* and *P.roxburghii* involved 235.06 and 97.07 bp. On the other hand, each of 443.2, 343.1 and 235 bp bands were observed in both of *S.sempervirens* and *T.distichum*, in addition to 521, 441.9, 212.2, 180.4 and 158.8 bp bands in *S.sempervirens*.

The primer OP-Z01 generated a total of ten polymorphic (58.824% polymorphism) and seven monomorphic DNA bands in the studied conifers genotypes and species. Five unique bands were scored out of the total identified polymorphic bands in the conifers under investigation. *S.sempervirens* was characterized by the presence of the unique bands with fragment size 838.8, 483.3 and 98.9 bp while *T.distichum* was distinguished by the unique bands (235.5 and 219.5 bp). Moreover, *P.pinea* scored the polymorphic band (364.7 bp) as well as *P.roxburghii*,

S.sempervirens and *T.distichum*. In addition, the polymorphic band (453 bp) was recorded in each of *P.roxburghii*, *S.sempervirens* and *T.distichum*. Besides, *S.sempervirens* and *T.distichum* were both discriminated by the polymorphic bands (546.6 and 269.66 bp), while each of *P.roxburghii* and *T.distichum* were characterized by the presence of (270.4 bp) polymorphic band. However, non of *P.halepensis* and *P.canariensis* scored polymorphic bands. The forementioned polymorphic and unique bands generated by the primer OP-Z01 represent the most distinct ones, therefore, these bands provide additional potentiality for discrimination among the studied genotypes and conifers species.

Table (5): Isomers of peroxidase and Adh enzymes (0/1) and their Rf value.

Band number	Rf	*P.halepensis*	*P.canariensis*	*P.pinea*	*P.roxburghii*	*S.sempervirens*	*T.distichum*
Peroxidase							
1	0.055	0.00	0.00	0.00	0.00	0.00	1.00
2	0.105	0.00	0.00	0.00	1.00	0.00	0.00
3	0.138	1.00	1.00	0.00	1.00	0.00	1.00
4	0.195	1.00	1.00	1.00	0.00	0.00	1.00
5	0.235	0.00	1.00	0.00	1.00	0.00	1.00
6	0.279	1.00	1.00	1.00	1.00	0.00	1.00
7	0.336	1.00	1.00	1.00	1.00	0.00	1.00
8	0.361	0.00	0.00	0.00	1.00	0.00	0.00
9	0.387	1.00	1.00	1.00	1.00	0.00	1.00
10	0.436	1.00	1.00	1.00	0.00	0.00	1.00
11	0.477	0.00	0.00	1.00	0.00	0.00	1.00
12	0.521	0.00	0.00	0.00	0.00	1.00	1.00
13	0.559	0.00	0.00	0.00	0.00	1.00	1.00
14	0.597	1.00	1.00	1.00	1.00	1.00	0.00
15	0.654	0.00	0.00	0.00	0.00	1.00	1.00
16	0.696	0.00	0.00	0.00	0.00	1.00	1.00
17	0.731	0.00	0.00	0.00	0.00	1.00	0.00
18	0.790	0.00	0.00	0.00	0.00	1.00	0.00
19	0.836	0.00	0.00	0.00	0.00	1.00	0.00
20	0.900	0.00	0.00	0.00	0.00	1.00	0.00
Polymorphism%					100%		
Adh							
1	0.205	0.00	0.00	0.00	0.00	0.00	1.00
2	0.268	1.00	1.00	1.00	1.00	1.00	0.00
3	0.363	1.00	1.00	0.00	0.00	0.00	0.00
4	0.739	0.00	0.00	1.00	1.00	1.00	1.00
5	0.863	1.00	1.00	1.00	1.00	1.00	1.00
Polymorphism%					80%		

Table (6): Type and number of the amplified DNA bands generated by five DNA random primers (RAPD) used for the identification of the six conifers.

Primer code	Total amplicones	Monomorphic amplicones	Polymorphic amplicones	Unique amplicones	Polymorphism %
OP-C11	11	9	2	0	18.182
OP-F01	11	9	1	1	18.182
OP-F05	11	9	1	1	18.182
OP-F08	16	7	6	3	56.250
OP-Z01	16	7	5	5	58.824
Total	66	41	15	10	
Average Polymorphism %					37.88

Data illustrated in Table (7) reveal that the highest similarity coefficient was 98% between *P.roxburghii* and *P.pinea*, followed by 96.8% between *P.pinea* and *P.canariensis* then 95.7% between *P.canariensis* and *P.halepensis*. On the other hand, the lowest similarity coefficient was 80.8% between *S.sempervirens* and *P.canariensis* followed by 83% between *S.sempervirens* and *P.halepensis* then 84.1% between *S.sempervirens and P.pinea.*

Table (7): Similarity coefficient among the six conifers as estimated by RAPD analysis.

Species	*P.halepensis*	*P.canariensis*	*P.pinea*	*P.roxburghii*	*S.sempervirens*	*T.distichum*
P.halepensis	1.00					
P.canariensis	0.957	.				
P.pinea	0.947	0.968				
P.roxburghii	0.928	0.947	0.980			
S.sempervirens	0.830	0.808	0.841	0.844		
T.distichum	0.843	0.860	0.893	0.914	0.877	1.00

3.2.2. **Genotype identification by RAPD markers**

The RAPD assay permitted the identification of the six conifers under investigation by unique positive and / or negative markers, as well as the polymorphic markers, as recorded in Table (6) and Fig. (4). *Pinus halepensis* was characterized by the presence of the polymorphic markers with molecular size (521.2, 441.9 and 97.07 bp) obtained from the primer OP-F08 and the polymorphic marker (313.4 bp) obtained from the primer OP-C11. *Pinus canariensis* was distinguished by the absence of the negative unique marker (473.1 bp) revealed with primer OP-F01 and the presence of the polymorphic marker (97.07 bp) obtained with the primer OP-F08. However, *P.pinea* was poorly recognized by the presence of the polymorphic marker (364.7 bp) only as revealed by the primer OP-Z01. *Pinus roxburghii* was characterized by the presence of the polymorphic markers (453, 364.7 and 270.4 bp) all together revealed by the primer OP-Z01. On the other hand, each of *S.sempervirens* and *T.distichum* were highly discriminated from other conifers, as *S.sempervirens* was distinguished by the presence of the unique marker (587.1 bp) by the primer OP-F01, the unique markers (562.8, 366.3 and 341.2 bp) by the primer OP-F08 and the unique markers (838.8, 483.3 and 98.9 bp) out of the primer OP-Z01. Besides, eight polymorphic markers obtained by the primer OP-F08 (521.2, 443.2, 441.9, 343.1, 235, 212.2, 180.4 and 158.8 bp) provided additional potentiality for the discrimination of *S.sempervirens* from the other conifers. *Taxodium distichum*, as well, was highly differentiated by the presence of the unique marker (337.4 bp) out of the primer OP-F05, and the unique markers (235.5 and 219.5 bp) by the primer OP-C11. Moreover, the presence of the polymorphic markers (443.2, 343.1 and 235 bp) from the primer OP-F08 and the polymorphic markers (546.6, 453, 364.7, 270.4 and 269.6 bp) obtained from the primer OP-Z01 and the polymorphic markers (350 and 313.4 bp) recorded by the primer OP-C11 played a considerable role in discriminating *T.distichum*.

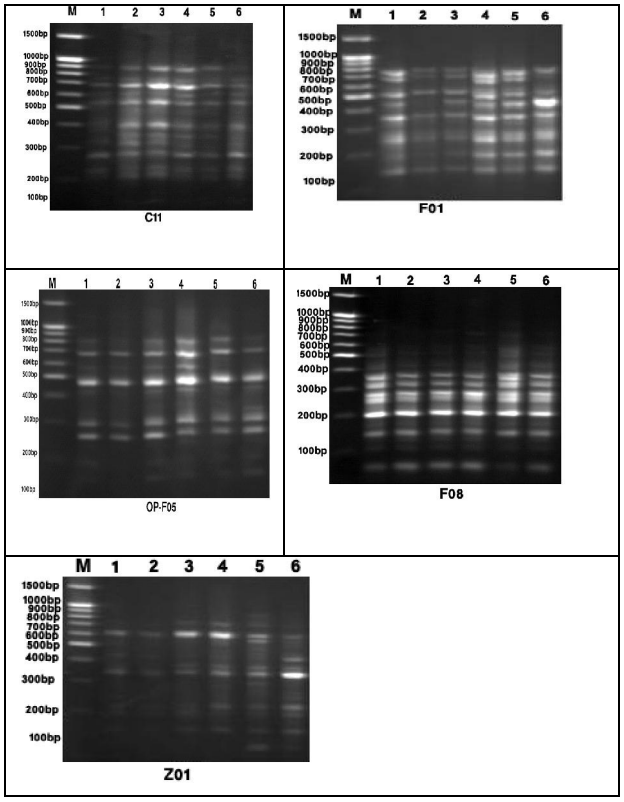


Fig. (4): RAPD profiles for the six conifers as detected with primers: OP-C11, OP-FO1, OP-F05, OP-F08 and OP-Z01 [Lanes 1to6 repr*esent: P. halepensis, P.canariensis, P.pinea, P.roxburghii, S.sempervirens* and *T.distichum.* M: bp ladder DNA marker]

3.2.3 Inter Simple Sequence Repeats (ISSRs)

Five ISSR primers successfully amplified DNA fragments of the six conifers under investigation with total number of 81 fragments producing 38 polymorphic bands (7 unique + 31 non-unique) with 47.95% of mean polymorphism, as demonstrated in Table (8). Besides polymorphism range was recorded between 42.11% (primer A98) and 55.56% (primer HB12) and the range of DNA band size was between (120 – 1460) bp.

Table (8) and Fig. (5) illustrate that primer A98 produced 8 polymorphic bands (42.11% polymorphism) and 11 monomorphic DNA bands in the studied conifers, while 1 unique band was observed out of the polymorphic bands. Four unique bands (491, 436, 305 and 276 bp) were recorded in *P.halepensis*, other four unique bands (389, 303, 278 and 215 bp) were observed in *P.canariensis*, two unique bands (214 and 135 bp) were seen in *P.pinea* and one unique band (478 bp) in *P.roxburghii*. However, *S.sempervirens* and *T.distichum* did not record any unique band concerning for the primer A98. Moreover, *P.halepensis* did not show any non-unique polymorphic band with the primer A98.

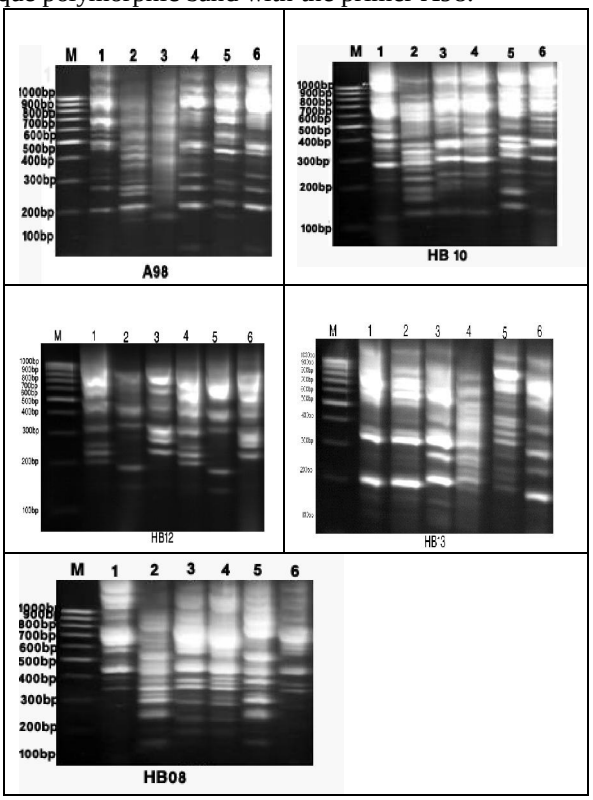


Fig. (5): ISSR profiles for the six conifers as detected with primers:OP-A98,OP-HB10,OP-HB12,OP-HB13, OP-HB08

Primer HB08 resulted in the amplification of twenty DNA fragments with molecular size range from 139 to 1460 bp, with nine polymorphic bands (45% polymorphism), two of them were unique (384 and 598 bp) in *P.roxburghii* and *T.distichum*, respectively. The primer HB 10 produced total of seventeen DNA fragments with molecular size range from 131 to 1288 bp, eight of them were polymorphic (47.06% polymorphism) with two unique DNA bands (256 and 1288 bp) considered as plant specific markers to *P.canariensis* and *T.distichum*, respectively.

Table (8): Type and number of amplified DNA bands generated by five DNA-ISSR primers used for the identification of the six conifers.

Primer code / Band type	A98	HB08	HB10	HB12	HB13	Total
Monomorphic	11	11	9	4	8	43
Unique	1	2	2	0	2	7
Polymorphic (non unique)	7	7	6	5	6	31
Total bands	19	20	17	9	16	81
Polymorphism (%)	42.11	45.00	47.06	55.56	50.00	Mean: 47.95
Fragment size range (bp)	134-1446	139-1460	131-1288	126-763	120-1018	

On the other hand, the primer HB12 resulted in nine DNA fragments with molecular size range from 126 to 763 bp, but did not produce any unique bands, only recorded five polymorphic bands (55.56% polymorphism). Besides, the ISSR profile generated by the primer HB13 (Fig. 5 and Table 8) produced eight polymorphic bands (50% polymorphism) out of them two bands were identified as unique (187 and 147 bp) both were noticed in *P.roxburghii*. The molecular size range generated by the primer HB13 was between 120 and 1018 bp

Each conifer subjected to this study could be discriminated from the others by ISSR-PCR specific markers, except *P.pinea* and *S.sempervirens*. *Pinus halepensis* was characterized by the presence of the unique band 491 bp (primer A98), *P.canariensis* was distinguished by the presence of the unique band 256 bp by the primer HB10. While *P.roxburghii* was discriminated by the unique band 384 bp (primer HB08) and (187 and 147 bp) generated by HB13 primer. Besides, *T.distichum* was distinguished by 598 bp unique band from HB08 primer and 1288 bp generated by the primer HB10

It is evident from Table (9) that the highest similarity coefficient values are (93.4%) between *P.canariensis* and *P.halepensis*, (91.2%) between *P.roxburghii* and*P.halepensis* and 91% between *P.roxburghii* and *P.pinea*. On the other hand, the lowest values are observed between *T.distichum* and *P.pinea* (78.7%) followed by *T.distichum* and *P.halepensis* (79%) and between *T.distichum* and *P.roxburghii* (79.7%).

Table (9): Similarity coefficient among the six conifers as estimated by ISSR analysis

species	*P.halepenses*	*P.canariensis*	*P.pinea*	*P.roxburghii*	*S.sempervirens*	*T.distichum*
P.halepenses	1.00					
P.canariensis	0.934	1.00				
P.pinea	.0.88	0.904	1.00			
P.roxburghii	0.912	0.885	0.910	1.0		
S.sempervirens	0.868	0.855	0.85	0.829	1.0	
T.distichum	0.790	0.840	00.878	0.797	0.865	1.0

3.3. The overall polymorphism detected by the biochemical and molecular markers:

The relationship among the six conifers based on the overall polymorphism detected by the biochemical assays (leaves protein and isozyme polymorphism) and the molecular markers (RAPDs and ISSRs) are presented in Table (10). The total produced bands were 190 out of all markers, 104 (77 non-unique polymorphic and 27 unique bands) were polymorphic bands with 54.74% polymorphism and 86 monomprphic bands. Peroxidase marker gave the highest proportion of polymorphism (100%) followed by protein marker (91.3%), while ISSR and RAPD marker resulted in lower propotion of polymorphism (46.91% and 37.9%), respectively and overall mean (54.74%) of polymorphism % out of the whole assays

Table (10): Polymorphism detected by each marker system in the six conifers

Marker number	Marker system		Monomorphic bands	Polymorphic bands	Unique bands	Total	Polymorphism %
1	Protein		2	18	3	23	91.30
2	Peroxidase isozyme		0	13	7	20	100.0
3	RAPD	OP-F01	9	1	1	11	
4		OP-F05	9	1	1	11	
5		OP-F08	7	6	3	15	
6		OP-Z01	7	5	5	17	
7		OP-C11	9	2	0	11	
	Total RAPD bands		41	15	10	66	37.9
8	ISSR	A98	11	7	1	19	
9		HB08	11	7	2	20	
10		HB10	9	6	2	17	
11		HB12	4	5	0	9	
12		HB13	8	6	2	16	
	Total ISSR bands		43	31	7	81	46.91
	Total number of bands		86	77	27	190	
	Mean polymorphism %						54.74

3.4.Genetic relationships based on protein, peroxidase, RAPD-PCR and ISSR-PCR analysis.

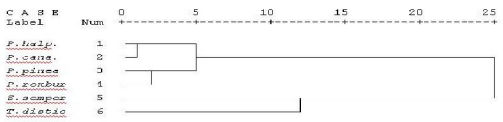
The matrix of similarity index for the six conifers under investigation is presented in Table (11). The maximum similarity coefficient value (91.8%) recorded between *P.halepensis* and *P.canariensis* followed by (91.1%) observed between *P.pinea* and *P.roxburghii*, then (90.5%) between *P. canariensis* and *P.pinea* followed by (90.0%) between *P.canariensis* and *P.roxburghii*. However, the lowest similarity coefficient value (75.3%) was noticed between *P.halepensis* and *T.distichum*. Similarity coefficient range value between *Pinus* species was observed between 91.8% (between *P.halepensis* and *P.canariensis*) and 87.7% (between *P.halepensis* and *P.pinea*), while similarity coefficient between *S.sempervirens* and *T.distichum* was 85.4%. The range of similarity coefficient between *S.sempervirens* and *Pinus* species was between 77.4% (with *P.roxburghii*) and 79.1% with *P.pinea*. On the other hand, the highest similarity coefficient value between *T.distichum* and *Pinus* species was 79.4 (with *P.canariensis*) and the lowest value (75.3%) with *P.halepensis*

Table (11): Similarity coefficient among the six conifers as estimated by protein, peroxidase isozyme, RAPD and ISSR analysis.

Species	P.halepensis	P.canariensis	P.pinea	P.roxburghii	S.sempervirens	T.distichum
P.halepensis	1.00					
P.canariensis	0.918	1.00				
P.pinea	0.877	0.905	1.00			
P.roxburghii	0.895	0.900	0.911	1.00		
S.sempervirens	0.784	0.779	0.791	0.774	1.00	
T.distichum	0.753	0.794	0.775	0.780	0.854	1.00

Data illustrated in Fig. (6) exhibit the dendrogram of the genetic distance between the six investigated conifers, they are grouped into two main clusters, at a distance of 25. The first cluster included *T.distichum* and *S.sempervirens* and the second cluster involved the four *Pinus* species which subdivided into two groups, at a distance of 0.5, the first group included *P.roxburghii* and *P.pinea* and the second group contained *P.canariensis* and *P.halepensis*.

Fig. (6): Clustering dendrogram of the genetic distance between the six conifers, based on proteins, peroxidase, RAPD and ISSR analysis data.



4. Discussion:

Characterization of the genetic diversity and examination of the genetic relationship among conifers are important for the sustainable conservation and increase use of plant genetic resources. In our study, Genetic polymorphism was investigated in six conifers representing four *Pinus*

species, i.e(P.halepensis, P.canariensis, P.pinea, and *P.roxburghii)which* belong to *family Pinaceae* and two members of family *Taxodiaceae, (Sequoia sempervirens and Taxodium distichum).* Genetic biochemical (proteins and isozymes), as well as molecular (RAPDs and ISSRs) analysis were investigated. Proteins and peroxidase banding patterns resulted in extensive polymorphism among conifers under investigation.

The needle leaves protein banding profile was separated using SDS-PAGE of the six genera and species of conifers. The highest number of bands was 16, detected in *Pinus roxburghii* while the lowest number of bands was 8, identified in *Taxodium distichum.*

The protein assay permitted the identification of only three conifers under investigation by unique positive and negative markers. In this regard, Piovesan, *et al.* (1993) stated that the genus *Pinus* has maintained a considerable homogeneity, may be attributed to speciation processes mainly due to gene mutations in which hybridization plays a major role.

In addition, isozyme profile permitted the identification of three conifers under investigation, *i.e. Pinus roxburghii*, *Sequoia sempervirens* and *Taxodium distichum* by the presence of two unique markers found in peroxidase isozyme with *P. roxburghii*, four with *Sequoia sempervirens* and absence of three bands and the presence of one with *Taxodium distichum* with absence of one unique band.

In this respect, González-Andrés, *et al.* (1999) could distinguish between *P.canariensis, P.halepensis, P.pinaster* and *P.pinea* by using *ACP*, *GOT* and *SOD* isozymes banding patterns. They found that *P.canariensis* and *P.pinea* had similarity level of 0.6, while *P.halepensis* presented the lowest similarity level with the other species.

RAPD assay permitted the identification of the six conifers under investigation by unique positive and / or negative markers, as well as the polymorphic markers. Our results revealed that the RAPD marker produced 66 bands with mean polymorphism 37.88%, out of them 10 unique markers were recorded across the six conifers under investigation, besides of 15 polymorphic markers. Each of *S. sempervirns* and *T. distichum* produced the highest unique markers (seven positive markers with *S. sempervirens* and three positive markers with *T. distichum*) followed by *P. canariensis* (one negative marker), while the other *Pinus* species showed no unique markers. In this concern, Cuesta, *et al.* (2010) postulated that stone pine (*Pinus pinea*), the exceptionally low genetic polymorphism of the species has been confirmed in studies applying different markers, such as isozymes, chloroplast and nuclear microsatellites and RAPD. Moreover, Klaus (1989) noted that *P.pinea, P.canariensis* share many cone and vegetative characters, while Frankis (1993) combined *P.canariensis, P.halepensis* in one subsection, *Pinaster*, but he still placed *P.pinea* in a separate subsection. However, Wang, *et al.* (1999) grouped *P.pinea* together with *P.canariensis* and *P.halepensis* in the same subsection.

ISSR results confirm the role of fragments polymorphism on conifers identification. The results were in harmony with those recorded by Wang, *et al.* (2009), and Wang, *et al.* (1999), since they concluded that the ISSR markers are believed to be distributed throughout the whole genome. Most of them may have no direct effect on morphological phenotypes because these DNA sequences are not amplified from functional genes. Also, results suggested the ISSR genetic diversity did not necessarily match the morphological trait difference among the used species (*Dendrobium*). Application of multiple DNA marker systems would help reveal more accurately the phylogenetic relationships among the species based on differentiation of their whole genome organization.

In this investigation, the observed polymorphism in different biochemical (proteins and isozymes patterns) as well as in molecular patterns (RAPDs and ISSRs) recorded with the six conifers, exhibited different similarities between some *Pinus* species which does not match those of taxonomy. This could be explained on the bases of adaptations to the local environmental conditions associated with selection processes through many generations or may be attributed to the occurrence of mutational events that alter the performance of genes encoding some isozymes as well as changes in the annealing site of a random primer. This suggestion is in agreement with Rottenberg, *et al*. (2000).

Newton, *et al*. (2002) postulated that pines, genetically were among the most variable of organisms and characterized by high variation both within and between populations, as indicated by assessments of both quantitative and isozyme variation. The high diversity generally recorded within populations of pines was attributed to reproductive characteristics such as wind pollination, high reproductive capacity, effective mechanisms of seed dispersal, and flexible mating systems which permit inbreeding and selfing in isolated trees. Pines were monoecious, and in most species of the genus, mechanisms of self-incompatibility appeared to be lacking. However, rates of out crossing were generally very high, which appeared to be mentioned by partial self-sterility.

Kusumi, *et al.* (2000) concluded that *Sequoia* were formed in a clade with *Metasequoia* and *Sequoiadendron*, while *Taxodium* formed another clade together while *Glyptostrobus*, both in the family *Taxodiaceae*.

On the other hand, Gadek, *et al*. in the same year (2000) related *Taxodium* and *Seqouia* to two subfamilies (*Taxodioideae* and *Sequoioideae*), respectively under the family *Cupressaceae*.

On the other hand, Liston, *et al.* (1999) found that Himalayan *P.roxburghii* was paraphyletic to the Asian and Mediterranean hard pines, and that the strong

morphological resemblance of *P. roxburghii* to *P.canariensis* has promoted the classification of the two taxa into the same subsection, *Canarienses*. Klaus (1989) suggested that *P.roxburghii* originated from Mediterranean ancestors of P. *canariensis* which reached the Himalayan region and led to the rise of *P. roxburghii.* However, Mirove (1967) suggested an eastern Asian origin of *P.roxburghii* from where it purportedly migrated to the Himalayas and extended to the west, hence he proposed the closely related *P. canariensis* reached to the canary Islands. Moreover, Liston, *et al*. (1999) concluded that *P.roxburghii* might represent an ancestral stock to the Eurasian hard pines.

It is necessary to search extensively at genome scale for more ISSR markers or molecular markers of other types for molecular diagnosis tool specific for additional conifers genus and species.

The ISSR markers reported in the present study will facilitate the understanding of inter- species gene flow, genetic structure of species, genetic diversity and evolutionary relationships in the conifers under investigation. Remaining challenges, as incorporating additional nuclear loci into the molecular analysis, comparing and combining the molecular results with morphology-based phylogenetic analyses.

In conclusion, the present data distinguished the genetic relationship between the six conifers under investigation and established the genetic similarities. In fact, both biochemical and molecular identification were useful in the discrimination between conifers, generally characterized by a high level of polymorphism.

5.References:

Brown, A.H.D. (1978). Isozymes, plant population, genetic structure and genetic conservation. Theoretical and Applied Genetic, 52:145-157.

Clark, J. and Scheffer, Th. C. (1983). Natural decay resistance of heartwood of coast redwood *Sequoia sempervirens* (D. Don) Endl. Forest Products Journal, 33 (5): 15-20.

Cuesta, C.; Ordás, R. J.; Rodriguez, A. and Fernández, B. (2010). PCR-based molecular markers for assessment of somaclonal variation in *Pinus pinea* micropropagated *in vitro*. Biol. Plant., 54 (3): 435-442.

Dellaporta, S. L.; Wood, J. and Hicks, J. B. (1983). A plant mini-preparation: Version II. Plant Mol. Biol. Report, 1: 19-21.

Frankis, M. P. (1993). Morphology and affinities of *Pinus brutia*. In: International Symposium on *Pinus brutia* Ten., 11-18. Ministry of Foresty, Ankara.

Gad, Mervat M. A; Salem, M. A. and El-Sayed, Hwida, M. F. (2006). Clonal Propagation and Mass Production of *Sequoia sempervirens*, D. Don, Endl. Through tissue culture techniques Proceedings of First International Conference on Strategy of Botanic Garden, Bulletin of CAIM- Herbarium, Vol. 7, May 10-12, 2006, Giza, Egypt. 53-69.

Gadek, P. A.; Alpers, D. L.; Heslewood, M. M. and Quinn, Ch. J. (2002). Relationships within *Cupressaceae* Sensu Lato: A combined morphological and molecular approach. Amer. J. Bot., 87 (7): 1044-1057.

González-Andrés, F.; Pita, J.-M. and Ortiz, J.-M. (1999). Identification of Iberian and Canarian species of the genus *Pinus* with four isoenzyme systems. Biochem. Syst. Ecol., 27: 235-242.

Jonathan, F. W. and Weeden, N. F. (1990). Visualization and interpretation of plant isozymes. Isozymes in Plant Biology. D. E. Soltis and P. S. Soltis (eds.). London Chapman and Hall, pp. 5-45.

Klaus, W. (1989). Mediterranean pines and their history. Plant Systematics and Evolution, 162: 133-163.

Kusumi, J.; Tsumura, Y.; Yostimaru, H. and Tachida, H. (2000). Phylogenetic relationships in *Taxodiaceae* and *Cupressaceae* Sensu Strico based on matK gene, ch/L gene, trnL-trnF IG S region, and trnL intron sequences. American J. Bot., 87 (10): 1480-1488. (Bot. Soc. Of America, Inc.)**.**

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680-685.

Liston, A.; Robinson, W. A.; Pinero, D.; and Alvarez –Buylla, E. R. (1999). Phylogenetics of *Pinus* (*Pinaceae*)based on nuclear ribosomal DNA internal transcribed spacer region sequences. Molecular phylogenetics and Evolution, 11: 95-109.

Mirove, N. T. (1967). The genus *Pinus.* Ronald Press Company, New York, NY.

Neale, D. B. (2007). Genomics o tree breeding and forest health. Current Opinion in Genetics and development, 17: 539-544.

Newton, A. C.; Allnutt, T. R.; Dvorak, W. S.; Del Castillo, R. F. and Ennos, R. A. (2002). Patterns of genetic variation in *Pinus chiapensis***,** a threatened Mexican pine, detected by RAPD and mitochondrial DNA-RFLP markers. Heredity, 89: 191-198.

Piovesan, G.; Pelosi, C.; Schirone, A. and Schirone, B. (1993). Taxonomic evaluations of the genus *Pinus* (*Pinaceae*) based on electrophoretic data of salt soluble and insoluble seed storage proteins. Pl. Syst. Evol., 186: 57-68.

Rottenberg, A.; Nevo, E. and Zohary, D. (2000). Genetic variability in dimorphic and monomorphic populations of *Populus euphratica* (*Salicaceae*). Candian J. Forest Research, 30 (3): 482-486.

Sensi, E.; Vignani, R.; Scali, M.; Masi, E. and Cresti, M. (2003). DNA fingerprinting and genetic relatedness among cultivated varieties of Olea europaea L. Estimated by AFLP analysis. Scientia Horticulturae, 97: 379-388.

Stegmann, H.; Affify, A.M.R. and Hussein, K.R.F. (1985). Cultivar identification of dates (*Phoenix daectylifrea*) by protein patterns. 2nd International Symposium of Biochemical Approaches to Identification of Cultivars. Braunschweig. West Germany. 44 pp.

Strauss, S.H. and Doerksen, A.H.(1990). Restriction fragment analysis of pine phylogeny. Evolution, 44:1081-1096.

Studier, F. W. (1973). Analysis of Bacteriophage T4 early RNAs and proteins of slab gel. J. Mol. Bio., 79: 237-248.

Wang, H.-Z.; Feng, Sh.-G.; Lu, J.-J.; Shi, N.-N. and Liu, J.-J. (2009). Phylogenetic study and molecular identification of 31 *Dendrobium species* using inter- simple sequence repeat (ISSR) markers. Scientia Horticulture, 122: 440-447.

Wang, X.-R.; Tsumura, Y.; Yoshimaro, H.; Nagasaka, K.; and Szmidt, A. E. (1999). Phylogenetic relationships of Eurasian Pines (*Pinus*, *Pinaceae*) based on chloroplast RBCL, MATK, RPL20-RPS18 spacer, and TRNV intron sequences. Am. J. Bot., 86 (12): 1742-1753.

Wang, Y; Georgi, L. L.; Reighard, G.; Scorza, R. and Abbott, A.G. (2002). Genetic mapping of the evergrowing gene in peach [***Pruns persica*** (L.) Batsch] J. of Heredity, 93:352-358.

White, T. L.; Admas, W. T. and Neale., D. B. (2007). Forest Genetics. CABI publishing.

Williams, J.G.K.; Kubelick, A.R.; Livak, K.J.; Rafalski, J.A. and Tingey, S.V. (1990). DNA polymorphism amplified by arbitrarly primers are useful as genetic markers. Nucleic Acids Res., 18: 6531- 6535.

Zhou, L. (2007). Salt Tolerance, Propagation and Provenance Evaluation of *Taxodium* as a Landscape and Coastal Wetland Tree. M.Sc. Thesis, Fac. of the Graduate School of Stephen F. Austin State University, Texas, USA.

11/5/2010

Study on immune response of quail for avian influenza vaccines

M.A.saad, A.I.Abd-Elhady, A.EL-nagar

Central laboratory for evaluation of veterinary biologics Quail influenza
Saad940@yahoo.com

Abstract: This study was a trial to evaluate: The immune responses of quails vaccinated with common avian influenza (AI) commercial vaccines in Egypt The results revealed that: There were high to moderate levels of maternal immunity against AIV (H5N1and H5N2) on the 1st, 5th day of age and low levels on the 7th day of age. There was no significant difference concerning the immune response of H5N1 and H5N2 AI vaccines ($P < 0.05$) in vaccinated quails. Vaccination at 8-days of age with 0.5ml of vaccine, gave satisfactory titers, on the 3rd week post vaccination. By the 4th week post vaccination quails exhibited highest titers and continued to the 5th week post vaccination(age of slaughter or marketing of quail) against AIV.
[M.A.saad ,A.I.Abd-Elhady, A.EL-nagar. **Study on immune response of quail for avian influenza vaccines**. Journal of American Science 2010;6(12):1510-1514]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: immune response avian influenza - vaccines.

1. Introduction:

Avian Influenza (AI) is a disease of poultry that has occurred worldwide over the past 100 years (Easterday et al., 1997). Two clinical forms are seen in the field: a mild disease affecting the respiratory, reproductive and or urinary tracts, and a severe systemic disease, causing high morbidity and mortality. AI viruses are classified as highly pathogenic (HP), mildly pathogenic (MP) and a non-pathogenic (NP) based on the mortality rates (Senne et al., 1986; US Animal Health Association (USAHA, 1994). Over the past decade, the emergent HPAI viruses have shifted to increased virulence for chickens. HPAI viruses typically produce a similar severe, systemic disease with high mortality in chickens and other gallinaceous birds (Swayne, 2007). 26 epizootics of HPAI have occurred in the world since 1995. The largest of these outbreaks has been the H5N1 HPAI which has caused problems in poultry and some wild birds in over 60 countries of Asia, Europe and Africa since beginning in 1996 (Maines *et al.*, 2005). In Africa, H5N1 HPAI cases approved in February 2006 in several countries. It began in Nigeria then other African countries including Egypt. (Swayne, 2008). On 17 February 2006, the Egyptian government confirmed that bird flu had broken out in the nation's poultry.

Quails are migratory game birds belonging to the same family as the domestic fowl (Weatherbee and Jacobs, 1961). *Sccharomyces cerevisiae* yeast has the ability to reduce the toxic effect of AFB1 in quail. It was also apparent that the higher the inclusion rates of SC in the diet of quail (2.5 mg/kg) the more the effective it is.(Mariam et al.,2010)

Highly pathogenic avian influenza (HPAI) virus subtype H5N1 has caused significant losses in Thailand's poultry industry since its initial detection in January 2004 (Tiensin *et al.*, 2005). Chickens and quail are highly susceptible to HPAI H5N1 infection; however ducks, considered more resistant, are probable "Trojan horses" or carriers (Hulse-Post et al., 2005; Tiensin et al., 2005).

AI virus was detected in quail and chickens muscles and organs by indirect immunofluorescent assay (Antarasena,. et al. 2006).
The HI test against AI showing positive results in quail sera collected random samples from Egypt. (Elmahdy et al. 2009).

A formalin-inactivated oil-emulsion vaccine was prepared from a high-growth H5N1/PR8 virus (Chen *et al.* 2005). Vaccine candidates of influenza A viruses of H5N1 subtype have been generated in several laboratories (Lu *et al.*, 2007). In the face of disease outbreaks in quail industry and the potential pandemic threat to humans caused by the highly pathogenic avian influenza viruses (HPAIVs) of H5N1 subtype, improvement in biosecurity and the use of inactivated vaccines are two main options for the control of this disease, for that we designed our present study to measure the immune response of quail to AI vaccines H5N1 and H5N2.

2. Materials and methods

1-Quails: two hundred and fifty, one day old quail were used in this experiment.
2-AI Vaccine: commercial AI H5N1 and H5N2 vaccines, used for vaccination of quail.
3-Serum samples: quail blood samples were collected and sera were separated to apply HI test.

4- AI antigen: local inactivated HPAI virus was obtained from CLEVB and used as AI antigen with a concentration of 4 HA in HI test for the tested serum samples.
5- HA haemagglutination test: HA test were carried out according to (Anon 1971) to estimate the HA titer of used antigens.
6- HI haemagglutination inhibition test: Was carried out according to (Takatsy 1956) the test was applied to quantify AIV antibodies in sera according to OIE (2008)

Experimental design

Experiment 1
Maternal immunity: fifty quails were selected for determination of maternal immunity that acquired from vaccinated parents by HI test.

Experiment 2
Immune response of the vaccinated quail:
200 Quails were used for Determination of the immune response of quails by vaccinated S/C with either inactivated oil-emulsion H5N1 or H5N2 vaccines. Commercially available oil emulsion vaccines were used: H5N1 (subtype, Re-1 strain - A/chicken / China, Puerto - Rico) and H5N2 (Subtype chicken / England, Mexico) of = 10^4 EID50 haemagglutination antigen content. The dosage was 0.3ml at age 4-days and 0.5mlat age 8-days (inoculate at two different sites) Blood samples were collected 1, 2, 3, 4 and 5 weeks post vaccination. The flocks were arranged as follows:
A- Vaccination at 4 days old with 0.3ml of vaccine.
B- Vaccination at 8 days old with 0.5ml of vaccine.

titer of 2^6 HA units/ml. and was used at a final

3. Results and Discussion

Table 1: Maternal immune wading in quails § acquired from vaccinated parents by AIV inactivated oil-emulsion vaccines H5N1 and H5N2.

Age of Quail	H5N1	H5N2
	HI	HI
1 day	5.3	4.8
5 days	4.4	4.3
7 days	4.0	3.6
10 days	2.9	2.8
14 days	2.0	2.0

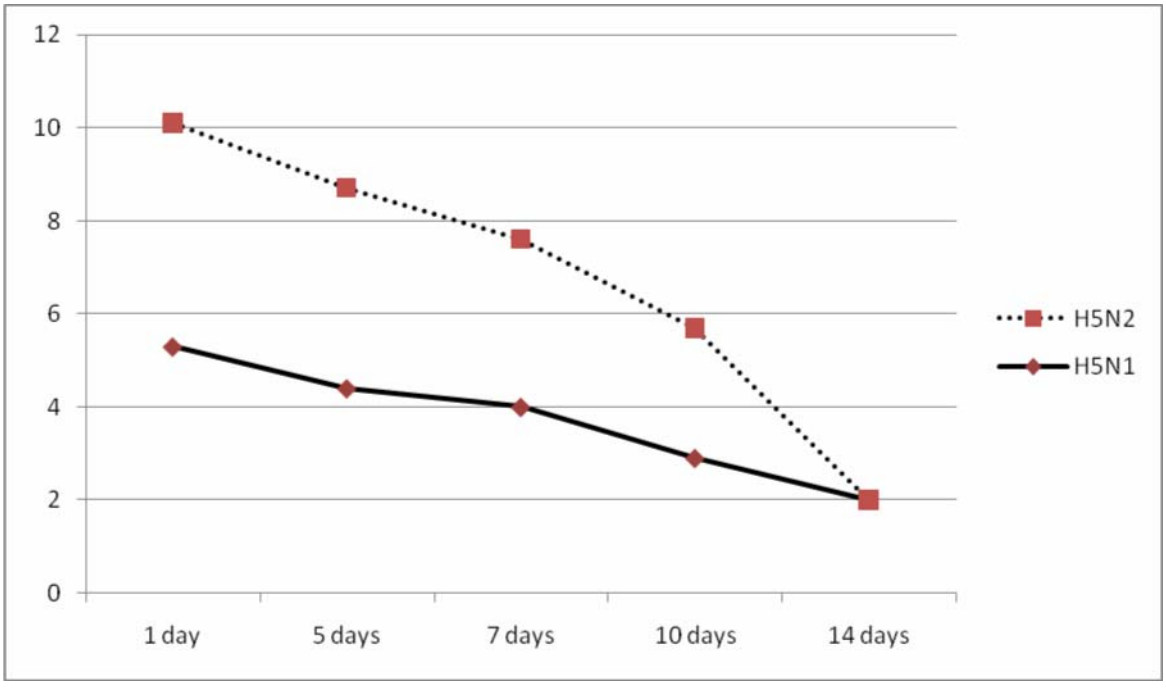


Figure 1: Maternal immune wading in quails § acquired from vaccinated parents by AIV inactivated oil-emulsion vaccines H5N1 and H5N2 .

Table 2: The immune response of quails vaccinated by AIV inactivated oil-emulsion vaccines H5N1 and H5N2 .

Group No.	Type of vaccine	Age of vaccine	Dose	HI titer post vaccination				
				1ST W	2ndW	3rd W	4th W	5th W
1	H5N1	4 days	0.3 ml	2.1	3.0	5.7	5.9	5.0
2	H5N1	8 days	0.3 ml	1.8	2.6	5.5	6.2	5.3
3	H5N1	4 days	0.5 ml	2.3	3.2	6.1	6.4	5.4
4	H5N1	8 days	0.5 ml	2.0	2.8	6.5	6.8	6.0
5	H5N2	4 days	0.3 ml	2.0	2.8	5.2	5.7	4.7
6	H5N2	8 days	0.3 ml	1.7	2.4	5.4	5.9	5.0
7	H5N2	4 days	0.5 ml	2.2	3.1	5.7	6.1	5.2
8	H5N2	8 days	0.5 ml	1.9	2.6	5.9	6.3	5.7

Now quails are raised commercially for meat and egg production and kept as pet birds and experimental birds in most parts of the world. (Lima et al., 2004). Quail are resistant to many diseases but they are susceptible to most naturally occurring viral diseases of chickens, especially when reared under poor management conditions. However the reports of the naturally occurring diseases are few when compared to those of chickens and this may be due to the fact that there are few quail farms (Ratnanohan, 1993). We have recently shown that quail are highly susceptible to infection with highly pathogenic H5N1 viruses isolated from geese. These viruses cause disease in quail; however, infected quail have a longer disease period than do chickens and thus are more likely to transmit the virus (Webster et al., 2003).

Table 1 and Fig.1 illustrated The results of maternal immunity,they show that:

1: There were high to moderate levels of maternal antibodies against AI (H5N1) and (H5N2) on the 1st and 5th day of age and low levels on the 7th day of age [HI mean values were 5.3, 4.4, 4.0, 2.9 and 2.0 (log-2) respectively] for H5N1. On the other hand, quails vaccinated by H5N2 at ages of one-day, 5-days and 7-days, were 4.8, 4.3, 3.6, 2.8 and 2.0 respectively (HI titer values).
2: After the age of 7 days the level of maternal immunity was greatly reduced and it was fade at the age of 14 days.
3: There was no significant difference concerning the immune response of H5N1 and H5N2 AI vaccines ($P < 0.05$).

Determination of immune response in quails vaccinated with inactivated oil-emulsion H5N1 and H5N2 vaccines Vaccination at 4-days old:

In one hand, Table 2 Showed that: H5N1 and H5N2 vaccination at 4-days of age (0.3ml of vaccine) resulted in positive antibody response on the 1st week post vaccination (HI titers were, 2.1 and 2.0 (log-2) respectively). The antibody response was gradually increased up to the 4th week post vaccination (HI titers were, 5.9 and 5.7, log-2 respectively).while at 4-days of age (0.5ml of vaccine) resulted in positive antibody response on the 1st week post vaccination (HI titers were, 2.0 and 1.9 (log-2) respectively) The antibody response was gradually increased up to the 4th week post vaccination (HI titers were, 6.4 and 6.1, log-2 respectively)

Vaccination at 8-days old:

On the other hand, Vaccination at 8 - days of age with 0.3ml of vaccine,Gave satisfactory titers, 3 weeks post vaccination (HITiters were, 5.5 and 5.4 (log2) respectively), but highest Titers were exhibited on the 4th week post vaccination (HI Titers were, 5.9 and 5.7 (log2) respectively) and then Continued to the 5th week post vaccination. While 0.5ml of vaccine gave satisfactory titers, 3 weeks post vaccination (HI Titers were, 6.5 and 5.9 (log2) respectively), but highest Titers were exhibited on the 4th week post vaccination (HI Titers were, 6.8 and 6.3 (log2) respectively) and then Continued to the 5th week post vaccination.

Our results pointed out that, vaccines do not sufficiently reduce the probability of infection up to 3

weeks post vaccination and this is indicated by the low HI titers. Although H5N1 or H5N2 vaccination at the age of 8- Days, gave protection 3 weeks post vaccination where, The titer ranged from 4.2 to 5.7 (log2), but maximum Levels of HI titers occurred 4 weeks post vaccination (4.6 to 6.1, log2) and continue with protective titer to five weeks of quail age (age of slaughter or marketing of quail) .Our results agreed with (Swayne, etal. 1999). Study The influence of vaccine strain and antigen mass on the ability of inactivated avian influenza (AI) viruses to protect chicks from a lethal, highly pathogenic (HP) AI virus challenge were they affect the immune response to AV vaccine, and also our results supported by (OIE 2008) which referred that the positive HI titer must be more than 4 log2 for AI.

Further studied needed by application of challenge test to estimate the vaccine efficacy in quails but these test need critical high registrations to use the virulent AV virus to apply these test. Our results suggested in regard to The immune response of vaccinated quails Against AIV.that The ideal age for quails Vaccination by AI vaccine is between 4 and 8-days of age, otherwise quails maternal immunity should be considered if they vaccinated at one-day of age. Quails one-day old of age which have low or no maternal immunity should be vaccinated at one-day old (with a dose of 0.3ml, H5N2), followed by a second dose (0.5ml) at 15-21-days of age.

The effectiveness of the available commercial vaccines in protection against the disease required. Two main categories for the control of this disease:

1: The use of efficient inactivated vaccines (targeted Control strategies).

2: Improved, strict and satisfactory biosecurity measures.

References:

1. Anon, (1971). Methods for examining poultry biologics and quantifying avian pathogens. National Acd. Of Sci. Washington.
2. Antarasena, C.; Sirimujalin, R.; Prommuang, P.; Blackshell, S.D. ; Promkundtod, N. and Prommuang, P(2006). Tissue tropism of a Thailand strain of high-pathogenic avian influenza (H5N!) in tissues of naturally infected native chickens (Gallus gallus), Japanese quail (Coturnix coturnix japonica) and ducks (Anas spp.) Avian Pathology 35(3), 250-253.
3. Chen, H., G. Tian, S. Zhang, Y. Li, Z. Bu, P. Liu, J. Zhou, C. Li, J. Shi and K. Yu, (2005). Oct. 10, Virol., 341: 153-62.
4. Easterday, B.C.; Hinshaw, V.S. and Halvorson, D.A. (1997). Influenza. In: B.W. Calnek, H. J. Barnes, C. W. Beard, L. R.
5. Elmahdy, S.; Saber, S.; Taha M. and Elebiary, E. (2009). Prevalence of antibodies against poultry viral diseases in sera of quails. J. of Egy. Vet. Assoc.69(4) 141-149.
6. Hulse-Post, D.J.; Sturm-Ramirez, K.M>; Humberd, J.; Seiler, P.; Govorkova, E.A.; Krauss, S.; Scholtissek, C.; Puthavathana, P.; Buranathai, C.; Nguyen, T.D.; Long, H.T.; Naipospos, T.S.; Chen, H.; Ellis, T.M.; Guan, Y.; Peiris, J.S. and Webster, R.G. (2005). Role of domestic ducks in the propagation and biological evolution of highly pathogenic H5N1 influenza viruses in Asia. Proceedings of the National Academy of Sciences USA, 102, 10682-10687.
7. Lima, F.S.; Santin, E.; Antonio, C.P. and Luciano, D.K. (2004). Evaluation of different programs of NDV in Japanese quail. Inter. J. Poul. Sci., 3 (5).
8. Lu, J., H. Shi, X.F. Liu, X. Zhang, S. Chen and L. Sun, 2007. Generation of an attenuated H5N1 avian influenza virus vaccine with all eight genes from avian viruses, 25: 7379-84.
9. Maines, T.R., X.H. Lu, S.M. Erb, L. Edwards, J. Guarner, P.W. Greer, D.C. Nguyen, K.J. Szretter, L. Chen, P. Thawatsupha, M. Chittaganpitch, S. Waicharoen, D.T. Nguyen, T. Nguyen, H.H.T.
10. Nguyen, J. Kim, L.T. Hoang and C. Kang. L.S. Phuong, W.L., S. Zaki, R.O. Donis, N.J. Cox, J.M. Katz and T.M. Tumpey, 2005. Avian Influenza (H5N1) Viruses Isolated from Humans in Asia in 2004. Exhibit Increased Virulence in Mammals. J. Virol., pp: 11788-11800.
11. Mariam G. E, Wagdy K.B. K, Eman M. H, Ibrahim M. F, Fadel.M and Farid K.R. S. : Effect of yeast (Saccharomyces cerevisiae) on reduction of aflatoxicosis, enhancement of growth performance and expression of neural and gonadal genes in Japanese quail Journal of American Science, 2010;6(12).
12. OIE, (2008): Manual of diagnostic tests and vaccines for terrestrial animals: avian influenza. Chap. 2.3.4.
13. Ratnanohan, N. (1993). Virus disease of Japanese quail. Virus infection of birds, Elsevier Science Publishers, B.V. Chp44.
14. Swayne, D.E.; Beck, J.R.; Garcia, M. and Stone, H.D. (1999). Influence of virus strain and antigen mass on efficacy of H5 avian influenza inactivated vaccines. Avian Pathology, 28, 245-255.
15. Swayne, D.E., (2007). Understanding the complex pathobiology of high pathogenicity

avian influenza viruses in birds. Avian dis. Mar, PMID: 17494560, 51: 242-9.
16. Swayne, D.E., (2008). Current status of avian influenza with emphasis on pathobiology, ecology, disease diagnosis and control. 8th Sci. Conf. EVPA, Giza (Egypt), 10-13 March.
17. Takatsy, G.Y. (1956). The use of spiral loops in serological and virological methods. Acta Microbiol. Hung. 3: 191.
18. Tiensin, T.; Chaitaweesub, P.; Songserm, T.; Chaisingh, A. ; Hoonsuwan, W. ; Buranathai, C.; Parakamawongsa, T.; Premashthira, S.; Amonsin, A.; Gilbert, M.; Nielen, M. and Stegeman, A. (2005). Highly pathogenic avian influenza H5N1. Thailand, 2004. Emerging Infectious Diseases, 11, 1664-1672.
19. USAHA (1994). Report of the Committee on Transmissible Diseases of Poultry and other Avian Species. Criteria for determining that an AI virus isolation causing an outbreak must be considered for eradication In: Proceedings of the 98th Annual Meeting US Animal Health Association (p.522). Grand Rapids: US Animal Health Association.
20. Weatherbee, D.K. and Jacobs, K.F. (1961). Migration of the common coturnix in North America Oklahoma Dept. Wildlife Conservation, 32. 85-91.
21. Webster, R.G. (2003). Role of quail in the interspecies transmission of H9 Influenza A viruses: Molecular changes on HA that correspond to adaptation from ducks to chickens. Journal of Virology, 77(5) 3148-3156.

11/4/2010

Modification of Silk for Improvement of Weighting and Properties

S.M.Gawish*, A.M.Ramadan, S.M.Abo El-Ola

Textile Research Division, National Research Centre, Tahrir Street, Dokki, Cairo, Egypt
*Samihagawish@yahoo.com

Abstract: Silk was modified by 2-hydroxy ethyl methacrylate (HEMA) and glycidyl methacrylate (GMA) and GMA/ derivatives to increase the weight, improve silk properties, antibacterial and fungicidal activities. Thus silk was grafted using a chemical method to different percentage add-on HEMA and GMA. Modified silk / GMA were further reacted with Diethylene triamine (DETA) at 85ºC for one hour to yield bactericidal and fungicidal silk fabrics. The weight of silk was increased and the properties were improved including moisture regain, crease recovery angles, abrasion resistance, whiteness and decrease of yellowness index. Characterization of modified fabric was done by FTIR, thermal gravimetric analysis (TGA) and SEM.
[S. M. Gawish, A. M. Ramadan, S. M. Abo El-Ola. **Modification of Silk for Improvement of Weighting and Properties.** Journal of American Science 2010;6(12):1515-1520]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: modified silk, chemical redox method, HEMA, GMA/derivatives, weighting moisture regain, crease recovery angle, abrasion resistance, whiteness, yellowness index, bactericidal and fungicidal activities.

1. Introduction

Silk is one of the most expensive fabrics. It has excellent properties such as soft, smooth handle, pleasant luster and gives brilliant color shades on dyeing. Silk is used for the apparel women dressing and it is a traditional dress in some countries like India, Japan, and Sudan etc.

Nowadays, grafting modification of silk[1-7] is considered an alternative procedure for the old weighting technique using inorganic salts [8] which was used for a long time ago with the purpose to increase silk weight resulting from the demumming process. Graft copolymerization of active vinyl monomers onto silk by different techniques acquired a great interest on an industrial scale by researchers all over the world to increase silk weight and improve its properties.

In addition to the well known chemical redox method [9-13] and silk irradiation using electron beam or γ–rays[14] followed by grafting, other methods were investigated using micro-wave irradiation [15] or plasma grafting techniques [16].

Thus, silk grafting is not only an effective method to increase the silk weight but it is an excellent method for improving silk properties such crease recovery angle, abrasion resistance, photo yellowing, dimensional stability and inducing antimicrobial activity for biomedical applications.

In the present article, silk fabric was grafted by the chemical redox method with the aim of weighting, improving silk properties and inducing antimicrobial activity to the fabric. For this purpose ammonium persulfate (APS) was used as initiator followed by grafting GMA or HEMA. GMA was reacted with DETA to produce silk/GMA/DETA or silk/HEMA fabrics. Grafted silk fabrics were characterized by FTIR, TGA and SEM. The properties of silk /HEMA were evaluated including moisture regain, crease recovery angle (dry and wet). Abrasion resistance, whiteness and yellowness index, which were improved. In addition silk/ GMA/ DETA had imparted bactericidal and fungicidal activities to the fabric.

Experimental

2. Materials

Egyptian plain construction silk fabric (63 gm/m^2) was scoured by 2g/1 of monionic surfactant solution at 50ºC for one hour. The silk fabric was thoroughly washed with warm water, then with cold water and finally squeezed and air dried.

Reagents

2-hydroxy ethyl methacrylate (HEMA) and glycidyl methacrylate (GMA), were purchased from Aldrich Co. and were used without further purification. Potassium persulfate (PPS), ammonium persulfate (APS), copper sulfate (CuSo4, 5 H2O), Diethylene triarnine (DETA) and acetone were all analytical grade reagents.

Polymerization procedure

A graft coplymerization reaction was carried out by treating silk fabric with a solution (2% w/w) of PPS or APS for 20 minutes at room temperature. The fabric was removed, thoroughly washed with distilled water, squeezed and dried at room temperature.

Silk fabric pretreated with (PPS or APS) were introduced into a stoppered Erlenmeyer flask containing 100 ml of the solution. The solution consisted of water, monomer and copper sulfate. The stoppered flask was kept in a thermostated shaking water bath at the desired temperature during the polymerization reaction. After a desired time, the reaction mixture was then washed several times with warm water and then with acetone , dried in oven at 105ºC for one hr and cooled to room temperature in a desiccator until constant weight. The graft yield was calculated as follows.

$$\% \text{ Graft yield } (G^o) = \frac{W - W^o}{W^o} \times 100$$

Where W is the weight of grafted silk sample and Wº is the weight of original silk fabric.

Grafting silk with GMA derivatives

Silk was grafted with GMA using the previous conditions and gave a fabric of the following composition: silk/109.7 GMA. Then it is allowed to react with DETA solution at a liquor ratio 1:50 (the solution mixture is 30 ml distilled water and 20 ml DETA) in an alkaline medium (addition of 1gm pyrophosphate). The reaction temperature was adjusted at 85ºC for one hr. The dry weight increase of the fabric was determined and the fabric was of the following composition:
Silk/109.7% GMA/7.7% DETA

Measurements

FTIR Spectroscopy

The FTIR spectrums of the silk fabrics were recorded on a Pirken Elmer 781 Infrared Spectro-photometer, as KBr disks.

Thermogravimetric analysis

Thermogravimetric analysis TGA was carried out using Pirken Elemer 7 Series instrument. The samples were placed on an aluminium holder and covered with gold. The rate of heating was adjusted at 10ºC/min. The thermograms were recorded from 50ºC to 600ºC under normal atmosphere.

Crease recovery angle measurement

The crease recovery angle of silk was measured by an iron recovery apparatus (Type FF-07 Metrepex) for 10 min crease period. The sum of crease angles of the warp (W) and weft (F) directions were measured.

Abrasion resistance measurement

One hundred fifty cycles were used for each fabric sample using the Universal Textile Abrasion Tester (Custom type, ASTMD). The percentage weight loss was determined.

Yellowness and Whiteness Indexes

The yellowness and whiteness indexes of silk fabric were measured using Erichsen Model 299/300, Erichsen GMBH and Co, Germany.

Antimicrobial activity

Antimicrobial activity was done by the diffusion disc method. A sample of the fabric is placed in a Petri dish containing solid bacterial medium (nutrient agar broth) or fungal medium (Doxs medium) which has been heavily seeded with the spores suspension of the tested organism. After inoculation, the sample is incubated at 37ºC for 24 or 48 hrs. The diameter of the clear zone of inhibition surrounding the sample is taken as a measure of the fabric inhibition action against the particular test organism[18-21].

Scanning electron microscopy

SEM micrographs were done on JEOL Model JSM-T20 instrument operating at 19 KV Photos which were prepared at a magnification range 2500X. The samples were placed on aluminium holder and covered with gold.

3. Results and Discussions

Effect of cupric ions concentration

The effect of copper sulfate concentration on the percentage graft yield of HEMA onto silk fabric is shown in Figure1. It was observed that the presence of cupric ions played a significant role in increasing the grafting reaction. An increasing in the graft yield was achieved when Cu^{2+} ion concentration increased gradually and reached 100% at 0.1 m.mol/1. The presence of Cu^{2+} ion caused an accelerating effect on the decomposition of PPS and by further increasing of Cu^{2+} concentration, the graft yield became constant.

Effect of HEMA concentration on the graft yield

The effect of HEMA concentration on percentage HEMA graft yield onto silk fabric is shown in Figure 2. It was observed that the percentage graft yield increases by increasing the HEMA concentration to 1.2×10^{-4} mmol/1 and attains a corresponding graft yield of 78%.

FTIR Study

FTIR were done for the degummed silk Figure 3 (a) and for the modified silk ie silk/GMA (b), silk/GMA/DETA (c) silk/ HEMA (d).

In the spectrum (a) the ungrafted silk[17] shows the absorption bands at 1516 and 1647 cm^{-1} assigned for=C=O stretching of amide I and amide II respectively. The peaks at 2925 and 3301 cm^{-1} are attributed to the C-H stretching and N-H stretching deformation.

In case of spectrum (b) for grafted silk, it revealed new absorption bands of GMA-grafted silk at 1151 cm^{-1} which is attributed to C-O stretching and at 1734 cm-1 which is due to C=O stretching of ester groups and increase absorption bands of epoxide group at 1261 cm^{-1} and appearance of extra bands at 906 and 847 cm^{-1}. In case of spectrum (c) silk/GMA/TETA the appearance of the bands at 906 and 847 cm^{-1} which correspond to the unreacted epoxide group. For spectrum (d) silk/HEMA, it reveals the same previous ester bands and a sharp peak at 3296 cm^{-1} corresponding to the hydroxyl group of HEMA.

Effect of grafted HEMA on crease recovery angle

The result of this study revealed that both dry wet crease recovery angles were improved due to grafting of silk fabric with HEMA, even at low graft yield which agree with our previous work [1]. Thus as shown in Table 1, dry crease recovery angles increased from 159º to 198º (29.3% HEMA) and then slightly decreased to 191º as the graft yield increases to 35.5% of HEMA, which may be attributed to steric hindrance.

The dry crease recovery angles of silk were improved due to grafting of silk by HEMA even at low HEMA graft yield, which is shown in Table 1.

The wet crease recovery angles (wetted in 1% nonionic detergent and then well squeezed) increased from 106ºC for the ungrafted silk to approximately mean constant value of 119ºC for all the HEMA graft yield (9.48 to 35.5% HEMA) i.e. increase grafting has the same effect on the wet crease recovery angles.

Table 1. Effect of grafted HEMA on the dry and wet crease recovery angles of silk.

HEMA Grafted Silk %	Dry Crease Recovery Angle (W+F)º	Wet Crease Recovery Angle (W+F)º
Zero	159	106
9.48	183	115
15	189	121
29.3	198	122
35.5	191	116

Effect of grafted HEMA on the abrasion resistance of silk fabric

Grating of silk by HEMA increases the abrasion resistances as shown in Table 2. It was observed that the percentage weight loss decreases from 11.59 for the control silk fabric (ungrafted) to 1.25% for 35.5% HEMA grafted silk fabric. As a result of grafting, the percentage weight gains by grafting compensate the percentage abrasion weight loss. As a conclusion grafting of silk fabric by HEMA leads to improvement of its abrasion resistance.

Table 2. Effect of grafted HEMA on the abrasion resistance of silk fabric.

HEMA Grafted Silk%	Weight loss%
Control sample	11.59
9.48	7.26
15	3.5
29.3	2.4
355	1.25

Yellowness and Whiteness Indexes

It is shown in Figure 4 that the photo yellowing index of modified silk fibers is decreased from 14 (ungrafted silk) to 9 for (20% grafted HEMA). The reverse is clear with the whiteness since the whiteness index increases from 12.5 (ungrafted silk) to 19 for (35.5% grafted HEMA). Therefore it can be deduced that grafting silk with HEMA reduced the native silk yellowing and thus protected silk against sunlight damage.

Moisture Regain

Table 3 shows that the percentage moisture regain increased by increasing HEMA yield induced by grafting silk, i.e. it increased from the 7.3% (blank silk) to 8.31% (for 15% grafted HEMA). This can be attributed to HEMA itself as it is contains hydroxyl groups grafted in its chemical structure, which are hydrophilic in nature and consequently increase moisture absorption of silk.

Table 3. Effect of percentage grafted HEMA on the moisture regain of silk.

Grafted HEMA %	Moisture regain%
Zero	7.3
9.48	7.52
15	8.31

Thermo gravimetric analysis TGA

TGA Study Table 4 and Fig.4 revealed that grafted silk fabric with (silk/108.6% GMA/7.7% DETA is slightly increased at 374ºC to 600º. On the other hand the grafted silk fabric of composition 29.3% HEMA, its thermal stability slightly decreased than that of silk at temperature beyond 250ºC to 600ºC.

SEM

SEM micrographs of ungrafted and grafted HEMA 35.5% silk fabrics are shown in Figure 6 (a,b). The ungrafted silk fabrics (a) has a smooth surface compared to that of silk/HEMA grafted fabrics (b) which shows a large amount of side chains, confirming the grafting of silk.

Antimicrobial activity

The modified silk fabric of composition silk/109.6% GMA/7.7 DETA showed antimicrobial activity against *Escherichia* Coli (G^-) and Staphylococcus *Aureus* (G^+). Table 4 shows that the inhibition zones were 25 mm and 28 mm/cm fabric respectively for 24 hrs incubation. Also the same fabric gave fungicidal activity against *Candida Albicans and Saccharomyces Cereviseaes* and the inhibition zones were 24 and 26 mm/cm fabric respectively for 24 hrs incubation. Ungrafted Silk fabric was inactive against the above microorganism. These findings support the mechanism of the antimicrobial action whereby a hydrophobic polycationic chain penetrate bacterial cell membranes/ walls and fatally damage them [22-23]. The bactericidal modified silk prepared are active not only to pathogenic bacteria but to fungi as well.

Table 4. Effect of percentage grafted silk on the inhibition growth of bacteria

Samples	Inhibition Zones Diameter (mm/cm sample)							
	E. coli (G-)		S. aureus(G+)		C. albicans (Fungus)		S. cereviseae (Fungus)	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
Grafted silk	25	25	28	28	24	24	26	26
Blank	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Grafted silk: 109.6% GMA/7.7% DETA; G: Gram reaction.

Table 5. Thermal Gravimetric Analysis (TGA) for grafted & native silk.

Temperature °C	Silk control	Silk/29.3% HEMA (2)	Silk/109.6% GMA/7.7% TETA (3)
	Residual weight %		
200	94.917	95.325	93.386
250	93.568	93.904	91.227
300	85.432	83.414	79.225
350	61.923	52.680	58.543
400	45.168	37.535	46.922
450	33.544	2.816	37.922
500	21.839	18.414	26.922
550	4.962	6.285	13.22
600	-	-	3.269

References

1. Ramadan, A.M.; Mosleh, S.; Gawish, S.M.; J Appl Polym Sci; **93**, 1743 (2004) 2004.
2. Watt, I.C.; J Macromol Sin Rev, Macromol Chem; C5, 176, (1970).
3. Arai K. In Block and Graft Copolymerization; Wiley: London, **1**, 193, (1973)
4. Nayak, P.L.; J Macromol Sin Rev, Macromol Chem. **C14**, 192, (1976).
5. Lenka, S.; J Macromol Sin Rev, Macromol Chem Phys. **C22**, 303, (1982).
6. Freddi, G.; Tsukada, M.; Polym Mater Encycl. **10,** 7734, (1996)..
7. Czerny, A.R.; Vbler, A.M.; Schindler, W.; Melliand Textil Brichte **71**, 211, (1990).
8. Peng, W.; Chen, D.; Zhou, Z.; Zhong, A.; Du, Z.; J Appl Polym Sci. **69**, 247 (1998).
9. Nayak, P.L.; Lenka, S.; Pati, N.C. J Appl Polym Sci. **23**, 1345 (1979).
10. Nayak, P.L.; Lenka, S.; Mishra, M.K. J Appl Polym Chem Ed. **18**, 2247 (1980).
11. Pati, N.C.; Lenka, S.; Nayak, P.L.; J Macromol Sci Chem. **A16**, 487 (1981).
12. Maji, T.K.; Banerjee, A.N.; J Appl Polym Sci. **62**, 595 (1996).
13. Schindler, W.; Dietel, A,Melliand Textilberchte, **74**, 81, (1996).
14. [Tsukada, M; Islam, S; Arai, T; Boschi, A.; Freddi G; Autex Research Journal, **5 (1)**, 40 (2005).
15. Zhu, Y.Q.; Kang, E.T.; Neoh, K.G.; Osipowicz, T.; Chan,, I.; J of the Electrochemical Soxiety, **152 (9)**, F 107-F114 (2005).
16. Prochayawarakorn, J.; Klairatsamee, W.; Songklanakarin, T.; J Sci Technol; **27(6)**, 1233 (2005)
17. Grayer, R.J.; Harbone J.B.; Phytochemistry; **37**, 19 (1994)
18. [18] Irob, O.N.; Moo-Young, M.; Anderson, W.A.; Int J Pharmacog, **34**, 87 (1996)/
19. Jawetz, E.; Melnick, J.L.; Adelberg, E.A. Review of Medical <icrobiology 13th Edition, Lange Medical Publisher: Los AltoCA, 1974.
20. Muanza, D. N.; Kim, B.W.; Euler, K.L.; Williams, L.; Int. J. Pharmacog. 32, 337 (1994).

21. Trommsdorff, H.; GmbH & Co.; K.G. Arzneimittel Fabrik Fr. Pat. 2209582 (1974).
22. Tiller, J.C.; Liano C.J.; Lewis K.; Klibanov A.M.; Proc Natl Acad. Sci USA, 98, 5981 (2001).
23. Lin J.; Qiu S.; Lewis K.; Klibanov A.M.; Biotechnology and bioengineering, 83(2), July 20 (2003).

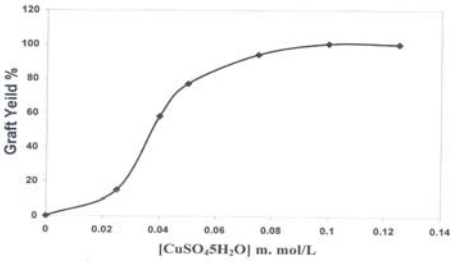


Figure 1: Effect of cupper sulfate concentration on percentage of HEMA graft yield

Grafting Condition: HEMA concentration 165 mol/l, $K_2S_2O_8$ 0 .74 x 10^{-4} mol/L, grafting temperature 80 °C, Liquor ratio 1:100 , reaction time 60 min.

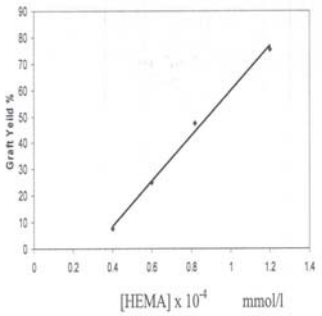


Figure 2: Effect of HEMA concentration on its graft yield %
Grafting Conditions: [$K_2S_2O_8$] 0.74x 10^{-4} mol/l, Cu SO_4 0.1 mole/l, grafting temperature 80 ^{0}C, reaction time 60 min, liquor ratio 1:100

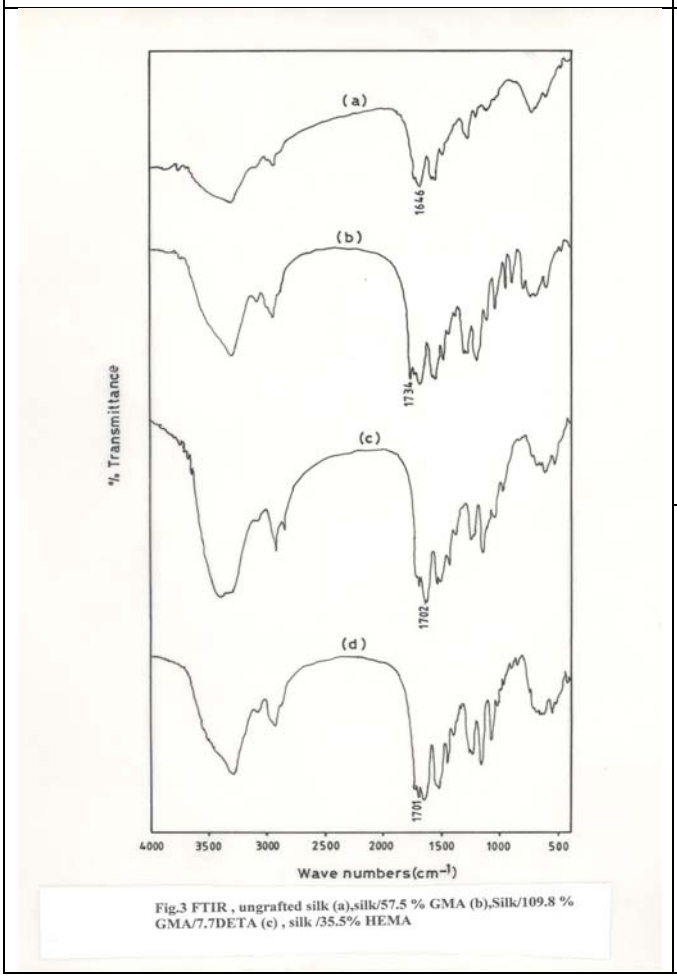
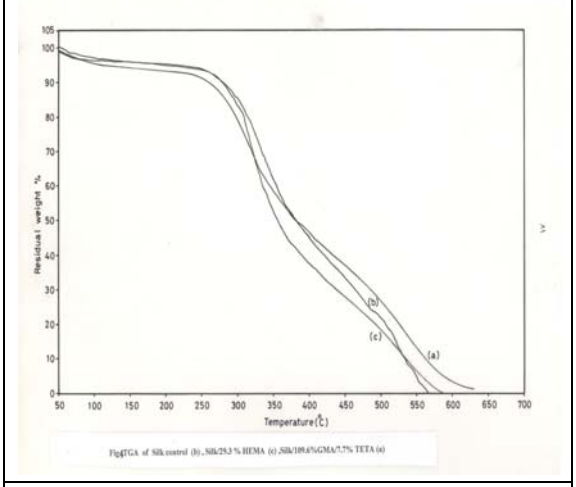


Fig.3 FTIR , ungrafted silk (a),silk/57.5 % GMA (b),Silk/109.8 % GMA/7.7DETA (c) , silk /35.5% HEMA



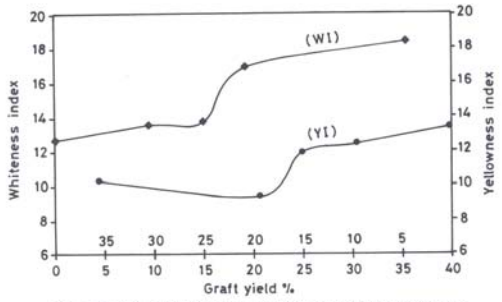


Fig. 5 Effect of SilK/HEMA modification on the whiteness &yellowness indexes

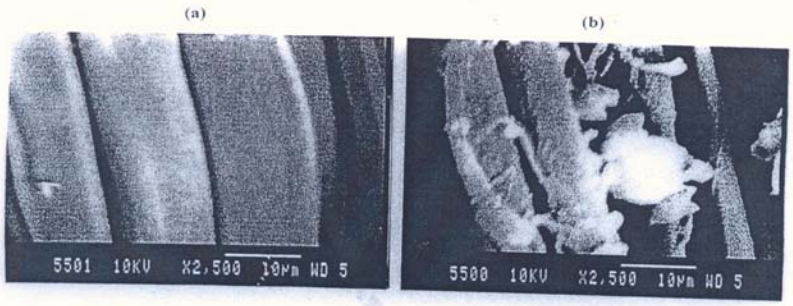


Fig. 5 SEM micrographs of ungrafted silk (a) and HEMA 3505% grafted silk (b)

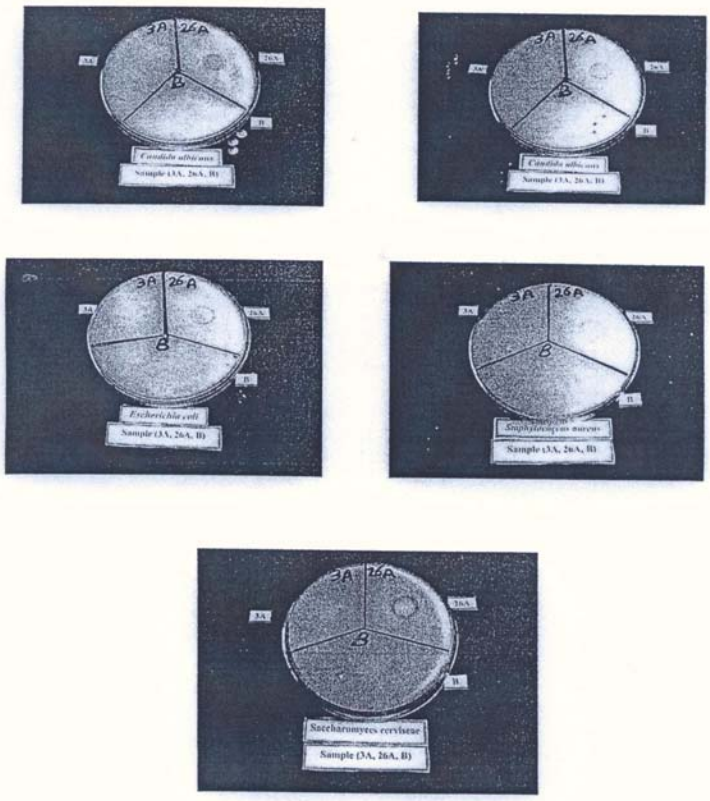


Fig.. 6 Effect of grafted silk on the inhibition bacteria growth zones.
Blank silk (B), silk / 109.6% GMA / 7.7% TATA (26A)

11/6/2010

Different Bone Resorption Levels Effect on Stresses Distribution for Different Implant Design

Mohamed M. EL Zawahry[1], Mohamed I. El-Anwar[2], and Ahmed F. El-ragi[3]

[1] Researcher, Prosthodontic Dept., National Research Centre, Egypt.
[2] Researcher, Mechanical Engineering Dept., National Research Centre, Egypt.
[1,2] Member Scientific Excellence Center (Applied Programming Network),
[3] Ass. Professor, Civil Engineering Dept., Faculty of Engineering, Fayoum University, Egypt.

Abstract: Aim :Different bone resorption levels effect, and better understanding of the effect of implant design parameters such as implant length on the stability of implants, stress values and distribution in surrounding bone are targeted in this study. **Materials and Methods**: Nine cases (implant-bone conditions) were numerically analyzed in 3D by Finite Element Method (FEM). Three bone levels were tested versus three implant lengths, while one type of loading was applied. **Results** showed that implant stability decreases as bone level decreases. The level of instability depends on implant design parameters. Bone stresses increase as bone level decreases with varying values depending on implant parameters. Approximate design curves were obtained.
[Mohamed M. EL Zawahry[1], Mohamed I. El-Anwar[2], and Ahmed F. El-ragi. Different Bone Resorption Levels Effect on Stresses Distribution for Different Implant Design. Journal of American Science 2010;6(12):1521-1525]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: Bone resorption ,implants,

Introduction:

The resorption of the dental bones after the dental implant surgery is one of the main factors that lead to biological and/or mechanical failure of implant-supported dental restorations.

From the biomechanics point of view, bone resorption decreases the bone density, therefore leading to the reduction of the overall stiffness of the bone. This could worsen the stress/strain distribution and the bone's role as the support to the implant, and in some cases, cause bacteria to be trapped in the resorbed notch area, therefore further worsening the resorption phenomenon. Clinically, two factors are primarily identified and associated with the incidence of bone resorption: bacterial infection due to the plaque in the bone/implant interface; and occlusal overload in the newly formed bones in the gap between the implant surface and the hosting bone, thereby damaging mineralized cellular tissues and their responses, thus preventing proper osseointegration, Daniel et al., (2009).

The exact reason for bone resorption varies between patients and has not been pinpointed yet. In general, the crestal bone level remains more or less the same and does not change much. Nevertheless, several literature studies have reported the marginal bone loss following the dental implantation surgeries, Nowzari et al., (2006). It was reported the alveolar bone loss occurs at an average rate of 0.17mm per year after implantation, Corn et al .,(1989). While Kol et al (2003);Nowzari et al .,(2006) reported the alveolar bone loss between 0.2 and 0.4mm over the first twelve months of implantation, followed by an additional 0.1mm bone loss between months 12 and 18.Overall, it can be seen that the average dental bone loss due to resorption is approximately around 0.2-0.4mm over the first twelve months, and can be said to reach a more stable status after months 12 to 18. Clinically, the bone resorption usually occurs in the cortical region surrounding the neck of the implant. Micro-damage and macro-damage may be differentiated in such construct of artificial (implant) and natural (bone) biomaterials. A certain level of micro-damage in bone tissues could promote positive bone remodeling, while excessive micro-damage may lead to certain local micro-cracking of mineralized tissues and the loss of bone strength, consequently weakening the implantation as a whole. Thereafter, the damage could reduce the magnitude of stress and strain induction in such bone tissues. Bone remodeling on the other side is a damage repair process, Li et al.,(2007). Bone resorption can therefore be viewed as the result of excessive damage in the bone tissues, where the damage takes place at a rate too rapid for the bone to repair and remodel, McNamara et al.,(1997).

Currently, placement of dental implants is a gold standard for replacement of missing teeth. Although successful clinical treatments by dental implants have

been reported Andersen et al.,(2002). failure of osseointegration still sometimes occurs Chee and Jivraj (2007). In contrast to natural teeth, there is no periodontal ligament between dental implants and their surrounding bone. The periodontal ligament acts as a shock absorber between a root and surrounding bone and it also contains mechanoreceptors.
Without the periodontal ligament,
mechanoreceptors will also be absent. The absence of mechanoreceptors results in poor detection of bite forces with small magnitudes.
This subsequently increases the tendency of occlusal overloading, which can cause peri-implant bone loss and implant failure, Kim et al.,(2005). When dental implants are used, it is therefore advisable to remove the sources of occlusal overloading as much as possible.
The Finite Element Method (FEM) is frequently used to determine stresses in bone around dental implants, Dejak and Mlotkowski(2008). Complex FE analysis methods such as the FE contact analysis can be very beneficial for modeling different clinical situations,Lin et al.,(2008).
Modeling of a bone resorption level problem, can actually be done by employing FE contact analysis. With having the maxilla, mandible, periodontal ligament and all teeth placed in the actual positions. The main reason may be that the FE contact analysis is expensive since it needs both large human and computational resources. Nevertheless, studies of bone resorption levels by experiments, both in humans and animals, are even more difficult and more expensive. In fact, some information, such as stresses in human bone, can be very difficult to obtain from experiments. To obtain stresses in bone from experiments, strains in bone must be first measured and, after that, stresses can be computed from the measured strains. The measurement of strains in bone is complex and can be quite expensive. The difficulties in performing experiments discourage studies of premature contacts with varied parameters, Takayama et al.,(2001)

2.Material and Methods:

2.1.Materials:

2.1.1.Implant designs:

Fixed implant diameter of 3.9 mm, and three different implant lengths: 9, 11 and 13 mm were modelled. Vertical load of 100 N perpendiculars in the middle for implant head top surface and coincident with bone (cortical and spongy) centreline were applied. Cortical and spongy bones were modelled as two co-axial cylinders of 24 and 22 mm diameter, and 16 and 14 mm height respectively. Where cortical bone cylinder is hollow, and has a constant thickness of 1 mm.

2. 2. Methods:

2.2.1.Experimental design

-Three different bone resorption levels at: 0, 2, and 4 mm were studied.
-Four types of stresses: Tensile stress, Von Mises stress, Compressive stress and Shear stress were obtained and focused by this study.
-The modulus of elasticity and possion ratio for the cortical and spongy bone besides the titanium implant was provided to the F.E.package,Table (1).
-Boundary condition was just supporting the bottom level of the cortical bone cylinder.
-The solid modeling (Figure 1) and finite element analysis were performed on a personal computer Intel Pentium IV, processor 2.8 GHz, 1.0GB RAM. The meshing software was ANSYS version 9.0 and the used element in meshing all three-dimensional models is 8 nodes Brick element (SOLID45), which has three degrees of freedom (translations in the global directions) Peter Kohnke (1994)
Figure (2).

Table 1. Material properties used in the analysis

Material	Modulus of Elasticity (MPa)	Poisson's Ratio
Cortical bone [Ishigaki etal.,2003]	14,500	0.323
Spongy bone [Caglar etal.,2006]	1,370	0.3
Titanium [Mellal etal.,2004]	110,000	0.3

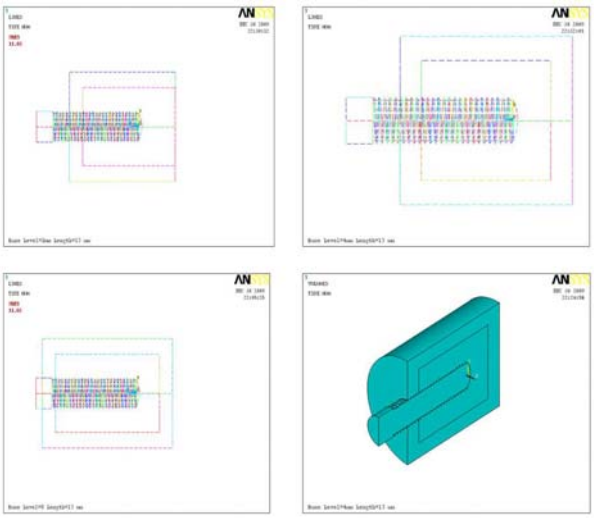


Figure 1: Different levels of bone resorption (screen shots from FE package)

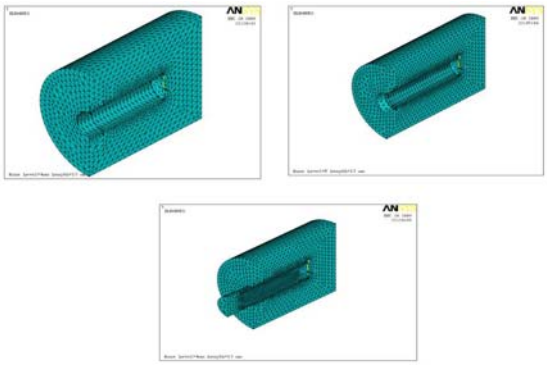


Figure 2: Meshed models for different bone resorption level

3.Results:

3.1. Running the F.E. package for the nine planned cases

This resulted in tons of data, and screen shots for the detailed analysis.

Figure(3)showed the maximum tensile stress generated on the 13mm implant at resorption level of 4mm. The maximum stresses were localized at implant-cortical bone interface. Generally all cases showed safe implant stresses levels, therefore, no further demonstrations for implant results presented in this study.

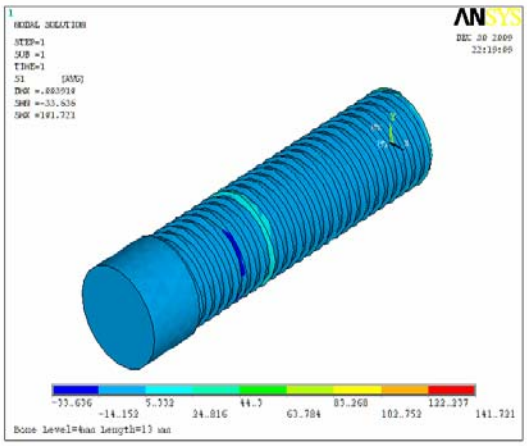
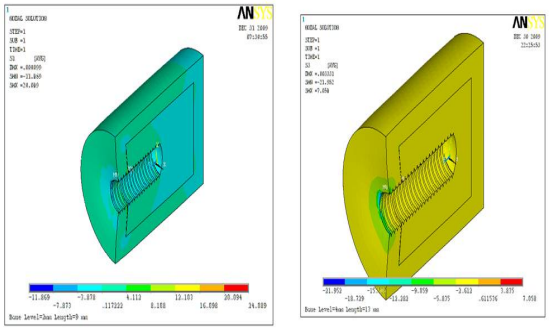


Figure 3: Maximum tensile stress in 13mm implant
length, bone level 4mm

3.2. Bone stresses distributions and levels:

Bone stresses distributions and levels resulted on fixed stresses distribution where the maximum stresses were found at cortical bone interface with the implant (Figure 4).



(a) **(b)**

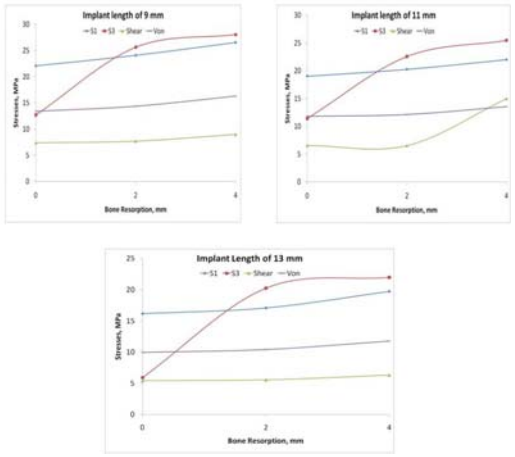
(a) 9mm implant length / 2mm bone level Maximum tensile Stress

b) **13mm implant length / 4mm bone level Maximum compressive Stress**

Figure 4: Spongy and Cortical bones stresses distribution

3.3.**Comparing stresses values with different resorption levels** :

This comparison could lead to approximate design curves, and solid conclusions. Figure (5) showed these comparisons between bone maximum tensile, maximum compressive, shear, and Von Mises.



Bone maximum stresses are generally increased with bone resorption level increase

Figure 5: Comparison between the nine cases

4.Discussion:

Consistently, the most prominent stress peaks occured in the bone (either cancellous or compact) near the protruding part of the stem. The values were probably under-estimated, since slip might occur between implant and tissue - contrary to the models' assumptions-thus at the least reducing amount of load transmitted by shear and tension. The resulting compressive stressed in the mandible were still locally approaching the upper limit of temporarily-acting physiological stresses, Borchers and Reichart(1983).

Crestal bone resorption primarily occurred during the first 4 weeks after uncovering, and although the cellular mechanism has not yet been identified, the micro-gap elicited an inflammatory response and subsequent bone loss, Xavier et al.,(2006). Pattern of stresses showed the distributions of stresses within the implant and the bone highlighting the location of the maximum and minimum values. An increase of all types of stresses occurred with higher bone resorption proportionate to degree of resorption. The compression stress showed an increased stress more than the others. For all bone levels the locations of maximum stresses were the same. On the other hand increasing implant length (in other words implant side area) ensured more stable fixation, and less stresses values which was preferred for weak bone patients. All stresses types could be said that it increased linearly with resorption level except maximum compressive stress (S_3). Which dramatically increased, by 100 to 400%, when resorption level increasing from zero to four millimetres.

Conclusions:

In the present study, the influence of a premature contact on stress and strain distributions in bone around an STDI was investigated. Bone resorption increased stresses for both implant & bone with all types of stresses namely, tension, Von Mises, shear & compression; with the later showing maximal increase. The increase of compressive stress in the bone showed more than one hundred percent increase. The location of the maximum stresses in the bone was the same for all levels of resorption in all implant designs. The increase of the implant lengths reduced the rate of increase in bone stresses with increasing bone resorption.

5.Refrences:

Andersen E, Haanaes HR, and Knutsen BM., "Immediate loading of single-tooth ITI implants in the anterior maxilla: a prospective 5-year pilot study", Clin Oral Implants Res 2002; 13: 281-7.

Borchers.L and Reichart.P. "Three-dimensional Stress Distribution Around a Dental Implant at Different Stages of Interface Development", J Dent Res 62(2):155-159, February 1983

Caglar A, Aydin C, Ozen J, Yilmaz C, Korkmaz T.:"Effects of mesiodistal inclination of implants on stress distribution in implant-supported fixed prostheses", Int J Oral Maxillofac Implants 2006; 21: 36-44.

Chee W, and Jivraj S.:"Failures in implant dentistry", Br Dent J 2007; 202: 123-9.

Corn, H. and Marks M.H.: "Basic Biological concepts associated with adult orthodontics. Atlas of adult orthodontics", 1989; Philadelphia: Lea & Febiger. 7-56.

Daniel L, Qing L, Wei L, Pim R and Michael S., "Bone Resorption Induced By Dental Implants with Ceramics Crowns", Journal of the Australian Ceramic Society Volume 45[2], 2009, 1-7

Dejak B, and Mlotkowski A., "Three-dimensional finite element analysis of strength and adhesion of composite resin versus ceramic inlays in molars", J Prosthet Dent 2008; 99: 131-40.3.

Ishigaki S, Nakano T, Yamada S, Nakamura T, Takashima F. "Biomechanical stress in bone

surrounding an implant under simulated chewing", Clin Oral Implants Res 2003; 14: 97-102.

Kol C.C., Douglas W.H., DeLong R., Rohrer M.D., Swift J.Q., Hodges J.S., An K.-N., and Ritman E.L.: "Effects of Implant Healing Time on Crestal Bone Loss of a Controlled-load Dental Implant", *J Dent Res* 82(8):585-591, 2003

Kim Y, Oh TJ, Misch CE, and Wang HL., "Occlusal considerations in implant therapy: clinical guidelines with biomechanical rationale", Clin Oral Implants Res 2005; 16: 26-35.

Li, I., Li H., Shi L., Fok A.S.L., Ucer C., Deulin H., Horner K., and Silikas N., "A mathematical model for simulating the bon remodeling process under mechanical stimulus", Dental Materials, 2007; 23(9): p. 1073-1078.

Lin CL, Wang JC, Chang WJ.: "Biomechanical interactions in tooth-implant-supported fixed partial dentures with variations in the number of splinted teeth and connector type: a finite element analysis", Clin Oral Implants Res 2008; 19: 107-17.

Mellal A, Wiskott HW, Botsis J, Scherrer SS, Belser UC.: "Stimulating effect of implant loading on surrounding bone; Comparison of three numerical models and validation by *in vivo* data", Clin Oral Implants Res 2004; 15: 239-48.

McNamara, B.P., Taylo D., and Prendergast P.J., "Computer prediction of adaptive bone remodeling around non cemented femoral prostheses: the relationship between damage based and strain-based algorithms", Medical Engineering & Physics, 1997. 19(5): p. 454- 463.

Nowzari, H., Chee W., Yi K., Pak M., Chung W.H, and Rich S.: Scalloped dental implants: A retrospective analysis of radiographic and clinical outcomes of 17 Nobel Perfect (TM) implants in 6 patients", Clinical Implant Dentistry and Related Research, 2006. 8(1): p.1-10.

Peter Kohnke, "ANSYS Theory Reference Manual", Ansys Inc., 1994

Takayama Y, Yamada T, Araki O, Seki T, Kawasaki T., "The dynamic behaviour of a lower complete denture during unilateral loads: analysis using the finite element method", J Oral Rehabil 2001; 28:1064-74.

Xavier VN, Xavier RC, Carlos RA, and Maribel ST., "Benefits of an Implant Platform Modification Technique to Reduce Crestal Bone Resorption", Implant Dent.2006; 15:313-20.

11/9/2010

Diagnosis of Egyptian Bovine Meat Borne Zoonosis

Nawal, A. Hassanain; Mohey, A. Hassanain; Raafat, M. Shaapan; Hassan, A. Fadaly and Ashraf, M. Barakat

Department of Zoonotic Diseases, National Research Center,
nnawalah@yahoo.com

Abstract: Food borne zoonoses have a major health impact in both industrialized and developing countries. Meat might be infected with some bacterial and parasitic agents; that could be threatening on human health. One hundred and eight meat samples (20 buffaloes and 88 cows) were collected from different Cairo abattoirs and examined parasitologically and bacteriologically. Results showed that 16.67% and 34.26% of the examined meat samples were infected with bacteria and parasites, respectively. The bacterial isolates were non typhoid *Salmonella* (50%), *E. coli* (38.89%) and *Mycobacterium bovis* (1.11%). Three out of the *E. coli* isolates (16.67%) were identified as *E. coli* O157:H7. The liberated parasites were *Cysticercus bovis* (51.35%) and *Toxoplasma gondii* (48.65%). ELISA results showed that seroprevalence of toxoplasmosis was 47, 22.7 and 38.42% in human, cows and buffaloes, respectively. The immunoreactive profiles of *C. bovis* (167.82, 137.32, 88.839, 66.859, 59.851, 54.660 and 48.480 KDa) and *T. gondii* local tachyzoite (158, 111, 102, 86, 55 and 33 KDa) antigens probed with rabbit hyper immune serum showed one immunoreactive band at 55 KDa. While those of *E. coli* (182.01, 144.90, 72.558, 60.324, 28.312 and 18.392 KDa) and non typhoid *Salmonella* (91.967, 60.955 and 20.031 KDa) antigens displayed one common immunoreactive band at 60 KDa. It can be concluded that although immunoblotting help in identification of strains and detection of common cross reactive epitopes between different pathogens, there still exist many challenges and opportunities to improve the current technology of food pathogen detection.
[Nawal, A. Hassanain; Mohey, A. Hassanain; Raafat, M. Shaapan; Hassan, A. Fadaly and Ashraf, M. Barakat. **Diagnosis of Egyptian Bovine Meat Borne Zoonosis**. Journal of American Science 2010;6(12):1526-1533]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Meat borne, human health, parasites, bacteria, ELISA, SDS-PAGE, immunoblotting.

1. Introduction:

Food borne diseases continue to be a major public health problem and constitute an important cause of morbidity and mortality in both developed and developing countries. The annual incidence of some 1.5 billion episodes of diarrhea in children less than 5 years of age, and the more than 3 million resultant deaths are an indication of the magnitude of the problem (NIH, 2006). Most of these diseases are infections caused by a variety of bacteria, viruses and parasites (Smith, 2003).

Meat is an important source of protein and a valuable commodity in resource poor communities. In many developing countries, there is lack of appropriate slaughtering facilities, unsatisfactory slaughtering techniques and slaughtering places are frequently contaminated and may not be protected against dogs, rodents and insects. Meat products coming from such conditions are often deteriorated due to bacterial infection or contaminated, which may cause food poisoning or diseases in consumers. Moreover, in many developing countries, regulations concerning meat inspection and/or control are inadequate or non-existent allowing consumers to be exposed to pathogens including zoonotic parasites (Joshi et al, 2003).

In many countries of the world, bacterial food borne zoonotic infections are the most common cause of human intestinal disease **(**Thorns, 2000). Salmonellosis is the most common food borne bacterial disease in the world. *Salmonella* is a significant pathogen for food-producing animals and these animals are the primary source of salmonellosis. It is estimated that herd prevalence varies between 0% and 90%, depending on the animal species and region (Plym and Wierup, 2006). Shiga toxin-producing *E. coli* (O157: H7) usually do not cause disease in animals but may cause watery diarrhea, hemorrhagic colitis, and/or hemolytic uremic syndrome in humans. Cattle and other ruminants are the most important reservoir of zoonotic *E. coli* O157, which are transmitted to humans through the ingestion of foods or water contaminated with animal feces (Fairbrother and Nadeau, 2006). Mycobacterium bovis infections are of major importance in many developed and developing countries. Transmission of *M. bovis* can occur between animals, from animals to humans and vice versa and rarely, between humans. As with *M. tuberculosis*, transmission is most common by the aerosol route and also through the ingestion of milk and meat from infected animals. Infection acquired

through ingesting *M. bovis* is more likely to result in non-pulmonary forms of disease (Oloya et al., 2007).

Parasitic food borne diseases remain a major public health problem affecting hundred millions of people and animals, particularly in tropical developing countries (Tagboto and Townson, 2001). Bovine cysticercosis is a zoonosis caused by the larval stage (cysticercus) of the human tapeworm *Taenia saginata*. Humans get infected by eating raw or undercooked meat containing viable cysticerci. They penetrate the intestinal wall and invade subcutaneous tissue, brain, eye, muscle, heart, liver, lung, and peritoneum. Bovine cysticercosis occurs world-wide, but is at particularly high prevalence in Africa (Eddi et al., 2003). Meat borne toxoplasmosis in humans may result from ingestion of tissue cysts contained in meat of many different animals (Tenter, 2009). Up to 50% of *T. gondii* infections are transmitted by ingesting undercooked meat, making toxoplasmosis one of the most clinically significant food borne diseases in pregnant women (Ogunmodede et al., 2005).

The types of food borne diseases are constantly changing. A century ago; typhoid and Cholera were common food borne diseases. Improvement in food safety as pasteurization of milk, safe canning and disinfection of water has conquered those diseases. Today new food borne diseases took their place as *Salmonella Enteritidis*, *Campylobacter and E. coli* 0157: H7. In future, other diseases; whose origin is currently unknown may turn out to be related to food born infections. So, in this investigation we shall try to diagnose bovine bacterial and parasitic meat borne infections common in Egypt.

2. Material and Methods

1. Sample collection:

a. Meat samples: One hundred and eight cattle meat samples (20 buffaloes and 88 cows) were collected from different Cairo abattoirs and subjected for bacteriological and parasitological examination.

b. Blood samples: Two hundreds, 20 and 88 blood samples were collected from the contact persons, the investigated buffaloes and cows, respectively and used for the determination of the prevalence of *T. gondii* infection.

2. Bacteriological meat examination:

Samples were taken from the infected lymph nodes, lungs, liver, spleen and kidneys of the infected animals (buffaloes and cows) and inoculated on different bacteriological media {nutrient agar, MacConkey agar, blood agar, Salmonella Shigella (SS) agar, Lowenstein-Jensen}. Samples were also enriched with tryptone soya broth containing novobiocin and sub cultured on sorbitol MacConkey agar for isolation of *E. coli* O157. The inoculated plates were incubated at 37°C for 48hr but cultures on Lowenstein-Jensen were incubated up to 8 weeks. Suspected colonies appearing on the different media were identified according to Holt et al. (1994) and Roberts et al. (1991) for *Mycobacteria*. The recovered *Salmonella* isolates were identified serologically using the diagnostic polyvalent and monovalent antisera according to Kauffmann (1972). *E. coli* O 157 suspected colonies were tested by the *E. coli* O157:H7agglutination latex test (Oxoid).

3. Parasitological meat examination:

a. Cysticercus bovis cysts were collected from liver, heart and masseater muscles of infected cattle.

b. *T. gondii* local isolate was isolated according to procedures described by Dubey and Beattie (1988) after many trials of feeding 20 kittens with 250g meat samples from cattle carcasses and their feces examined daily for identification of *T. gondii* oocysts using concentration floatation technique with saturated NaCl solution (sp gr. 1.15). Sporulation of the recovered *T. gondii* oocysts were done and mice were experimentally infected with the recovered sporulated oocysts by oral inoculation (1000 oocysts / mouse) and After 6 - 8 days, the peritoneal exudates of these injected mice were examined to obtain the tachyzoites of *T. gondii.*

4. Preparation of parasitic and bacterial antigens:

a. *C. bovis* antigen: Crude whole cyst antigens were prepared by homogenization of cysts in phosphate buffer saline (PBS) and centrifuged at 10,000 g for 30 min. following the method of Cheng and Ko (1991). The supernatant was collected and its protein content was determined by the method of Lowry et al. (1951). **b. *Toxoplasma gondii* antigen**: Local isolate antigen (LA) of *T. gondii* tachyzoites was prepared using the method described by Waltman et al. (1984). Briefly, tachyzoites were repeatedly freezed and thawed to rupture the parasite wall, sonicated and centrifuged at 12,000 rpm for 45 min at 4°C. The supernatants were separately collected and its protein content was determined by the method of Lowry et al. (1951).

c. *E. coli* and *Salmonella* antigens: Whole cell protein antigens of the isolated and identified *E. coli* and non typhoid *Salmonella* from meat samples were prepared according to Khan et al. (1996). Overnight bacterial culture of each strain in brain heart infusion broth was harvested by centrifugation; washed with phosphate buffer saline (PBS), pH 7.2 and resuspended in sample buffer (6.25 mM Tris {pH 6.8}; 2% SDS; 5% B-Mercaptoethanol; 10% glycerol

and 0.002% bromophenol blue). The sample was boiled in a boiling water bath for 10 min. Protein concentration was determined according to Lowry et al. (1951).

5. Elisa:

The optimum antigen, serum and conjugate concentrations were determined by checkerboard titration and test procedures were carried out according to Lind et al. (1997) with little modifications. The cut off values of optical density (OD) were calculated according to Hillyer et al. (1992).

6. Rabbit immunization:

Two white New-Zealand rabbits were immunized with 400 µg protein per rabbit of each of the prepared parasitic and bacterial antigens emulsified in equal volumes of Freund's complete adjuvant, and two rabbits were kept as control. Booster injections were administered at 14, 21 and 28 days post-immunization in Freund's incomplete adjuvant (Alkarmi and Faubert, 1985). Serum samples were collected 7 days after the last booster injection.

7. SDS-PAGE and immunoblot:

SDS-PAGE of the prepared crude parasitic and whole cell protein bacterial antigens was carried out as described by Laemmli (1970) using pre-stained high molecular weight marker. The electrophoresed proteins were transferred to a nitrocellulose membrane and the immunoblotting was done according to Towbin et al. (1979).

3. Results

Microbiological examination of meat samples revealed that 18 out of 108 (16.67%) gave cultures of microorganisms which were as follows; 11 cows (10.19%) and 7 buffaloes (6.48%). The identified bacterial isolates were non typhoid *Salmonella* (*S. enteritidis* and *S. typhimurium*) (50%) (cows 38.89 and buffaloes 11.11%), *E. coli* (38.89%)(cows 33.33 and buffaloes 5.56%) and *Mycobacterium bovis* (1.11%)(cows 1.11 and buffaloes 0%) (Table 1). Three out of the *E. coli* isolates (16.67%) were identified as *E. coli* O157: H7.

Parasitic examination showed that 34.26% of the collected meat samples were infected with parasites which were as follows; 31 cows (28.70%) and 6 buffaloes (5.56%). The liberated parasites were *C. bovis* (51.35%) (cows 37.84 and buffaloes 13.51%) and *T. gondii* (48.65%) (cows 18.92 and buffaloes 29.73%)(Table 2).

ELISA results showed that seroprevalence of *T. gondii* in human, cows and buffaloes were 47%, 22.7 and 38.42%, respectively.

The electrophoretic profile of the local antigen (LA) of *T. gondii* tachyzoites and *C. bovis* antigens consists of 12 (137-25.1 KDa) and 12 (185.32-24.977 KDa) protein bands, respectively (Fig. 1& 3). SDS-PAGE of the whole cell protein antigens of *E. coli* and Non typhoid *Salmonella* consists of 10 (182.01, 169.49, 144.90, 108.05, 89.032, 72.558, 60.324, 47.803, 28.312 and 18.392 KDa) and 11 (182.01, 168.20, 129.22, 91.967, 75.325, 60.955, 47.720, 43.905, 27.068, 20.91 and 14.691 KDa) polypeptide bands, respectively (Fig. 5).

The immunoblot profile of LA of *T. gondii* tachyzoites and *C. bovis* antigens probed with the homologous rabbit hyper immune serum showed 6 (158, 111, 102, 86, 55 and 33 KDa) and 7 (167.82, 137.32, 88.839, 66.859, 59.851, 54.660 and 48.480 KDa) reactive bands, respectively (Fig. 2 & 4). The Immunoblotting profiles of *E. coli* and non typhoid *Salmonella* probed with the homologous rabbit hyper immune serum displayed 6 (182.01, 144.90, 72.558, 60.324, 28.312 and 18.392 KDa) and 3 (91.967, 60.955 and 20.031 KDa) immunoreactive bands, respectively (Fig. 6).

Table 1: Results of bacteriological examination of meat samples

Meat samples	**Total samples**	**positive samples**	**(%) of positive samples**	**Non typhoidal *Salmonella***	**(%) of positive samples**	***E. coli***	**(%)of positive samples**	***M. bovis***	**(%)of positive samples**
Cow	**88**	**11**	**10.19**	**7**	**38.89**	**6**	**33.33**	**2**	**1.11**
Buffalo	**20**	**7**	**6.48**	**2**	**11.11**	**1**	**5.56**	**-**	**-**
Total	**108**	**18**	**16.67**	**9**	**50**	**7**	**38.89**	**2**	**1.11**

Table 2: Results of parasitological examination of meat samples

Meat samples	Total samples	positive samples	(%) of positive samples	*C. bovis*	(%) of positive samples	*T. gondii*	(%)of positive samples
Cow	88	31	28.70	14	37.84	7	18.92
Buffalo	20	6	5.56	5	13.51	11	29.73
Total	108	37	34.26	19	51.35	18	48.65

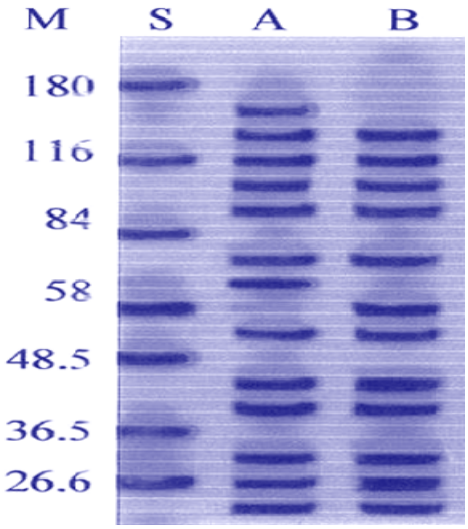


Fig. (1): Comparative electrophoretic profiles of *T. gondii* antigens: RH (lane A) and local strain (lane B). Standard molecular weight marker (lane S).

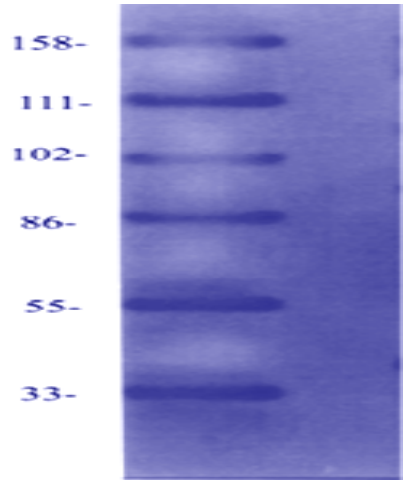


Fig. (2): Immunoreactive bands of *T. gondii* local strain antigen identified by rabbit hyperimmune serum using immunoblot.

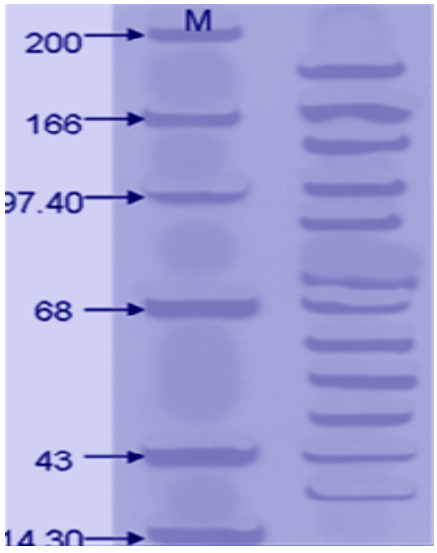


Fig. (3): SDS-PAGE of *C. bovis* antigen. Lane(M): Standard molecular weight marker.

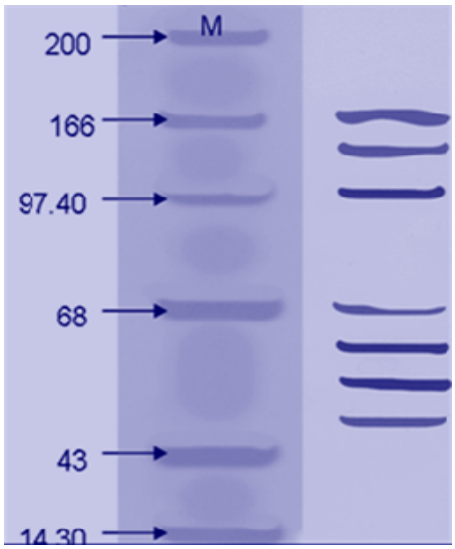


Fig. (4): Western blot showing reactivity of *C. bovis* with rabbit hyperimmune serum. Lane (M): Standard molecular weight marker.

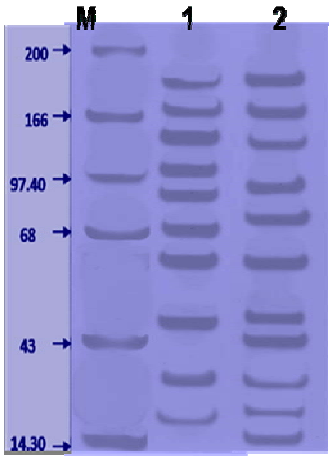


Fig. (5): SDS-PAGE of whole cell protein antigens of *E .coli* (1) and non typhoid *Salmonella* (2), lane (M): Standard molecular weight marker.

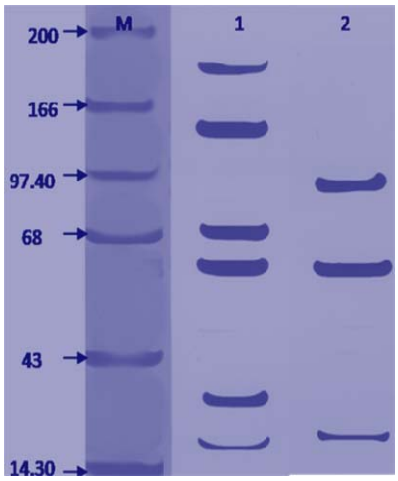


Fig. (6): Western blot showing reactivity of whole cell protein antigens of *E .coli* (1) and non typhoid *Salmonella* (2) with rabbit hyper immune serum, lane (M): Standard molecular weight marker.

4 .Discussion

Diagnosis of food borne bacterial diseases is carried out through the isolation of organisms from implicated food or feces and detection of toxins. Serologic testing is an important method for detecting parasitic infections, and includes immunofluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA), competitive-inhibition ELISA, Western blotting, and direct agglutination test (DAT) (Jenkins et al., 2002; Huang et al., 2004).

In the current study, *E. coli* (38.89% as 33.33 cows & 5.56% buffaloes) and *E. coli* O157:H7 (16.67%) were identified. Stampi et al. (2004) detected *E. coli* and *E. coli* O157 in 30.2 and 2% of the 149 bovine meat samples examined, respectively. Fairbrother and Nadeau (2006) isolated *E. coli* O157 from 14.3% of bovine carcasses. Also, non typhoid *Salmonella* (50% as 38.89 cows & 11.11% buffaloes) was isolated. Beach et al. (2002) recorded isolation rate of salmonellae of 19% from carcass samples of cattle. While, Al-Lahham et al. (1990), Branham et al. (2005) and Maharjan et al. (2006) reported isolation rate of *Salmonella* (6%), *E. coli* and *Salmonella* spp. in cattle (1.25%) and *Salmonella* from raw buffalo meat (13.5%), respectively. Phillips et al. (2008) isolated *E. coli* and *Salmonella* from 17.8% and 1.1% of ground beef samples, respectively. *E. coli* O157 was recovered from 0.3% of samples.

Microbiological examination of meat samples revealed that 1.11% was infected with *M. bovis* (cows 1.11 and buffaloes 0%). Teklul et al. (2004) found that of the 751 carcasses examined, 4.5% were found to have tuberculous lesions. Routine abattoir inspection detected only 29.4% of the carcasses with visible lesions. In addition, *M. bovis* was isolated from a carcass that presented no gross tuberculosis lesions. The low sensitivity of routine abattoir inspection demonstrates that existing necropsy procedures should be improved. Tadayon et al. (2006) recorded that 1.43 and 72.22% of the examined specimens from cows and buffaloes, respectively were positive for *M. bovis*.

In this study, parasitic examination showed that 34.26% (37/108) of the collected meat samples were infected with parasites (cows 28.70 and buffaloes 5.56%). The liberated parasites were *C. bovis* (51.35%) (cows 37.84 and buffaloes 13.51%) and *T. gondii* (48.65%) (cows 18.92 and buffaloes 29.73%). Wanzala et al. (2003) found cysticerci in 12/25 (48%) and 24/25 (96%) of infested cow by inspection and total dissection, respectively and indicated that except for the dead, degenerate or calcified cysticerci a careless meat inspector will most likely miss out quite a number of viable cysticerci, and be passed on for human consumption, becoming the source of bovine cysticercosis. Megersa et al. (2010) reported that of the total of 500 inspected cattle, 22 animals had varying number of *C. bovis* giving an overall prevalence 4.4% (22/500). On the other hand, Pearse et al. (2010) reported that of the 23 samples submitted, none was positive for C. bovis by either diagnostic method.

Although meat examination revealed that

18.92 and 29.73% of the examined cow and buffalo meat were infected with *T. gondii* cysts, ELISA results showed that the seroprevalence of *T. gondii* in cows and buffaloes were 22.7% and 38.42%, respectively. The higher incidence of *T. gondii* in the tested animals by ELISA is probably attributed to cross-reactivity, particularly with the use of crude extracts not purified antigens. Lower seroprevalence (8.8%) was recorded in buffaloes examined by Navidpour and Hoghooghi (1998). On the other hand, Selvaraj et al. (2007) found that sera of 99 she-buffaloes slaughtered showed 100% seropositivity for antibodies to *Toxoplasma gondii* by modified direct agglutination test at a dilution of 1:200. Also, our results recorded 47% prevalence of toxoplasmosis in human. Ibrahim et al. (2009) detected anti-*T. gondii* antibodies in pregnant women (51.49%) and cattle (10.75%) examined at Northen Egypt and stated that the high prevalence of toxoplasmosis in cattle affects the development of the livestock industry and is also an important infective source for human infection in Egypt.

In the present investigation, the electrophoretic profile of *C. bovis* antigen displays 12 polypeptide bands located at 185.32- 24.97 KDa. Similar results were recorded by Kandil et al. (2003).

The whole cell protein electrophoretic patterns of *E. coli* and non typhoid *Salmonella* displayed 3 common bands at 182, 60 and 47 KDa. The non typhoid *Salmonella* whole cell protein SDS-PAGE profile showed a 20.91 KDa protein band specific for *Salmonella*. Gupta et al. (2005) compared the protein profiles of selected *Salmonella* serovars with *E. coli* to identify genus specific protein(s) for *Salmonella* and stated that a protein of molecular weight 20.89 KDa was found in all *Salmonella* serovars, but not in *E. coli* suggesting its genus specific attribute.

Our results showed that the electrophoretic profile of the whole cell protein antigen of *E. coli* consisted of 10 proteins with molecular weight of 182-18.392 KDa. While, Uçan et al. (2005) reported that SDS-PAGE of the whole cell protein extracts of *E. coli* produces patterns containing 26 to 35 discrete bands with molecular weights of 6500-200 KDa. Also, Nawal (2008) found that the electrophoretic profile of the whole cell protein antigen of *E. coli* contains 10 protein bands with molecular weight range 775- 12 KDa.

In the present study, the immunoblotting profiles of *E. coli* and non typhoid *Salmonella* probed with its homologous rabbit hyper immune serum displayed 6 (182.01, 144.90, 72.558, 60.324, 28.312 and 18.392 KDa) and 3 (91.967, 60.955 and 20.031 KDa) protein bands, respectively. While, Nawal (2008) reported that the immunoblot fingerprinting of *E. coli* and non typhoid *Salmonella* whole cell protein antigens probed with human serum showed 6 (75.00, 70.36, 45.15, 43.00, 25.83 and 14.965 KDa) and 6 (138.48, 56.04, 46.36, 25.83, 20.91 and 14.00 KDa) protein bands, respectively.

In the current study, the immunoreactive profiles of *C. bovis* (167.82, 137.32, 88.839, 66.859, 59.851, 54.660 and 48.480 KDa) & *T. gondii* local tachyzoite (158, 111, 102, 86, 55 and 33 KDa) and that of *E. coli* (182.01, 144.90, 72.558, 60.324, 28.312 and 18.392 KDa) & non typhoid *Salmonella* (91.967, 60.955 and 20.031 KD) antigens showed common bands at 55 and 60 KDa, respectively. So, it can be concluded that beside immunoblotting helps in the identification of the different bacterial and parasitic strains, it can also detect common (cross reactive) immunoreactive epitopes between the antigens of the respective strains which may be beneficial in making shared vaccines.

It can be concluded that despite the recent advances in food pathogen detection, there still exist many challenges and opportunities to improve the current technology. Immunoblotting can help in the identification of pathogens and finding common immunoreactive epitopes between them with the possibility of manufacturing common cross protective vaccines.

References:

1. Alkarmi, T.O. and Faubert, G. M. (1985): Studies on the antigenic difference between the larvae of *Trichinella pseudospiralis* and *Trichinella spiralis* by gel diffusion and immuno-electrophoretic techniques. In Kim, C. V.ed Trichinellosis. New York, University Press, New York.
2. Al-Lahham, A.B., M. Abu-Saud and A.A. Shehabi, 1990. Prevalence of *Salmonella*, *Shigella* and intestinal parasites in food handlers in Irbid, Jordan. J. Diarrhea Disease Res., 8: 160-162.
3. Beach, J.C., E.A. Murano and G.R. Acuff, 2002. Serotyping and antibiotic resistance profiling of *Salmonella* in feedlot and nonfeedlot beef cattle. J. Food Protect., 65: 1694-1699.
4. Branham, L.A., M.A. Carr and C.B. Scott, 2005. *E. coli* O157 and *Salmonella* spp. in white-tailed and livestock. Current Issue Intestinal Microbiol., 6: 25-29.
5. Cheng, R. W. K. and Ko, R. C., (1991): Cross-reaction between crude antigens of larval *Taenia solium (Cysticercus cellulosae)* and other helminthes of pigs. Vet. Parasitol., 39: 161-170.
6. Dubey, J. P. and Beattie, C.P., (1988): Toxoplasmosis of Animals and Man. CRC Press, Boca Raton, FL, pp. 1–220.
7. Eddi, C.; Nari, A. and Amanfu, W. (2003): *Taenia solium* cysticercosis/taeniosis: potential

linkage with FAO activities; FAO support possibilities. Acta Trop., 87: 145-148.
8. Fairbrother J.M. and Nadeau E. 2006. *Escherichia coli*: on-farm contamination of animals. Rev. Sci. Tech. 25:555-69.
9. Gupta, M., V.D. Sharma and R.S. Gupta, 2005. Comparative protein profiles of *Salmonella* and *E. coli*. Indian J. Exp. Biol., 43: 475-478.
10. Hillyer, G.M.; Soler De Galanes, M., Rodriguez-Perez, J., Bjorland, J., De Lagrava, M.S., Guzman, S.R., Bryan, R.T. (1992): Use of the falcon assay screening test-enzyme-linked immunosorbent assay (FAST-ELISA) and the enzyme linked immunoelectrotransfer blot (EITB) to determine the prevalence of human fascioliasis in the Bolivian Altiplano. Am. J. Trop. Med. Hyg., 46: 603-609.
11. Holt, J.G.; Krieg, N.R.; Sneath, P.H.A.; Staley, J.T.; and Williams, S.T. (1994): Bergey'sManual of Determinative Bacteriology, 9th ed., Williams & Wilkins, Baltimore.
12. Huang X, Xuan X, Hirata H, Yokoyama N, Xu L, Suzuki N, Igarashi I, 2004. Rapid immunochromatographic test using recombinant SAG2 for detection of antibodies against *Toxoplasma gondii* in cats. J Clin Microbiol *42:* 351–353.
13. Ibrahim H. M., Huang, P, Salem T. A., Talaat R. M., Nasr M. I., Xuenan X. and Nishikawa Y**.** (2009). Prevalence of *Neospora caninum* and *Toxoplasma gondii* Antibodies in Northern Egypt Am. J. Trop. Med. Hyg., 80: 263-267.
14. Jenkins M, Baszler T, Bjorkman C, Schares G, Williams D, 2002. Diagnosis and seroepidemiology of *Neospora caninum*-associated bovine abortion. Int. J. Parasitol., *32:* 631–636.
15. Joshi, D.D., M. Maharjan and M.V. Johansen, 2003. Improving meat inspection and control in resource poor communities: the Nepal example. Acta Tropica, 87: 119-127.
16. Kandil, O. M.; Mahmoud, M. S. and Khalil, F. A.M.(2003): Characterization and demonstration of species specific and cross reactive components of cysticercosis and hydaitosis. J. Egypt. Vet. Med. Assoc., 63: 277-289.
17. Khan, F. G.; Rattan, A.; Khan, I. A. And Kalia, A. (1996): A preliminary study of fingerprinting of *Pseudomonas aeruginosa* by whole cell protein analysis by SDS-PAGE. Indian J. Med. Res., 104: 342-348.
18. Kauffmann, F., 1972. Serological Diagnosis of *Salmonella* Species. Kauffmann White Scheme Minkagaard, Copenhagen, Denmark. 16. Krikler, S.J., T.H. Pennington and Petri,
19. Laemmli, U.K. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature, London, 227: 680-685.
20. Lind, P; Haugegaard, J; Wingstrand, A. and Henrisken, S. A. (1997): The time course of the specific antibody response by various ELISAs in pigs experimentally infected with *Toxoplasma gondii.* Vet. Parasitol., 71: 1 – 15.
21. Lowry, O.H.; Rosenbrough, N.J.; Farr, A.I. and Randall, R.J. (1951): Protein measurement with Folin-phenol reagent. J. Biol. Chem. 193: 265-275.
22. Maharjan, M., V. Joshi and D. Joshi, 2006. Prevalence of *Salmonella* species in various raw meat samples of a local market in Kathmandu. Ann. Rep. New York Acad. Sci., 1081: 249-256.
23. Megersa,B.; Tesfaye, E.; Regassa, A.; Abebe, R.; Abunna, F. (2010). Bovine cysticercosis in cattle slaughtered at Jimma Municipal Abattoir, South western Ethiopia:Prevalence, Cyst viability and Its Socio-economic importance. Vet. World, 3: 257-262
24. Nawal A. Hassanain (2008): Detection of antibodies against zoonotic food borne pathogens in sera of food handlers. Global Veterinaria, 2: 385-289.
25. Navidpour Sh. and N. Hoghooghi-rad. (1998). Seroprevalence of anti-*Toxoplasma gondii* antibodies in buffaloes in Khoozestan province, Iran**.** Vet. Para**.,** 77: 191-194.
26. NIH (2006): Food poisoning. National Institute of Health. Medline Plus Medical Encyclopedia F.
27. Ogunmodede F, Scheftel J, Jones JL, Lynfield R. 2005. Toxoplasmosis prevention knowledge among pregnant women in Minnesota. Minn. Med. 88:32-34.
28. Oloya, J; Opuda-Asibo, J; Kazwala, R; Demelash, AB; Skjerve, E; Lund, A; Johansen, TB; Djonne, B. (2007). Mycobacteria causing human cervical lymphadenitis in pastoral communities in the Karamoja region of Uganda. Epidemiol Infect, 34: 1–8.
29. Pearse BHG, RJ Traub, A Davis, R Cobbold, PB Vanderlinde (2010). Prevalence of Cysticercus bovis in Australian cattle. Australian Vet. J. Article first published online: 21 JUN 2010
30. Phillips D, Jordan D, Morris S, Jenson I, Sumner J. 2008. A national survey of the microbiological quality of retail raw meats in Australia. J. Food Prot. 71:1232-1236.
31. Plym F and Wierup M. 2006. *Salmonella* contamination: a significant challenge to the global marketing of animal food products. Rev. Sci. Tech. 25: 541-554.

32. Roberts, G.D., E.W. Koneman and Y.K. Kim, 1991. Mycobacterium. In: Manual of Clinical Microbiology, 5th Edn. Balows, A. American Society for Microbiology. Washington, D.C., USA. , pp: 304-339.
33. Selvaraj,J., Manohar,B., Murali, S. and Balachandran, C. (2007). Seroprevalence of *Toxoplasma gondii* in buffaloes. J. Vet. Para., 21: 23-27.
34. Smith, J. (2003): Food born illness. Report of Center of Diseases Control and Prevention. National Center of Infectious Diseases, USA, Sept. 2003.
35. Stampi S, Caprioli A, De Luca G, Quaglio P, Sacchetti R, Zanetti F. 2004. Detection of *Escherichia coli* O157 in bovine meat products in northern Italy. Int. J. Food Microbiol., 90:257-62.
36. Tadayon K., N. Mosavari, A. H. Shahmoradi, F. Sadeghi, A. Azarvand, K. Forbes. (2006).
37. The Epidemiology of Mycobacterium bovis in Buffalo in Iran. Journal of Vet. Med., Series B, 53: 41–42.
38. Tagboto, S. and Townson, S. (2001): Antiparasitic properties of medicinal plants and other naturally occurring products. Adv. Parasitol., 50: 199-295.
39. Teklul A, Asseged B, Yimer E, Gebeyehu M, Woldesenbet Z. 2004. Tuberculous lesions not detected by routine abattoir inspection: the experience of the Hossana municipal abattoir, southern Ethiopia. Rev Sci Tech. 3:957-964.
40. Tenter A.M. 2009. *Toxoplasma gondii* in animals used for human consumption. Mem. Inst. Oswaldo Cruz.104:364-369.
41. Thorns C.J. 2000. Bacterial food-borne zoonoses. Rev. Sci. Tech. 19: 226-239.
42. Towbin, H.; Staehelin, T. and Gordon, J. (1979): Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Nat. Acad. Sci. USA., 176: 4350-4354.
43. Uçan, U.S., L. Açik and A. Çelebu, 2005. Plasmids and protein patterns of *E. coli* isolated from bovine mastitis in Konya, Turkey. Turkish. J. Vet. Anim. Sci., 29: 475-480
44. Waltman, W. D; Dreesen, D. W; Prickett, D. M; Blue, J. L. and Oliver, D. G. (1984): Enzyme-linked immunosorbant assay for the detection of toxoplasmosis in swine: interpreting assay results and comparing with other serologic tests. Am. J. Vet. Res., 45: 1719 -1725.
45. Wanzala W, Onyango-Abuje JA, Kang'ethe EK, Zessin KH, Kyule NM, Baumann MP, Ochanda H, Harrison LJ (2003). Control of *Taenia saginata* by post-mortem examination of carcasses. Afr Health Sci., 3(2):68-76.

11/19/2010

Effect of Titanium oxide toxicity on Biochemical, Haematological and clinicopathological Changes in *Clarias lazera* Present in the River Nile

Mona, S. Zaki[*1], Refat A. Yossef [2] and Nadia M. Taha[3]

Department of uHydribuilogy[1], National Research Centre, Cairo, Egypt
Soil and Water Uses Department[2] National Research Centre, Cairo, Egypt
Department of Physiology[3], Faculty of Verti. Medicine Cairo University
[*]dr_mona_zaki@yahoo.co.uk

Abstract: The effect of dietary carbohydrates and titanium oxide on haematalogical profile, blood chemistry and hormonal level was studied in cat fish *Clarias lazera*. Fish were divided into 3 groups (n=10) ant exposedto different doses of titanium oxide and carbohydrate. Groupi was served as control. Group 2 was fed with carbohydrate and titanium oxide (10 mg Kg^{-1} diet ration), group 3 was fed with carbohydrate and titanium oxide (1 5 rng Kg^{-1} diet ration). There is a significant decrease in hemoglobin and P .C.V in group (3). There is a significant increase in serum corlisol, cholesterol, AST, ALT, urea, creatinine and alkaline phosphorous in r group (3), also there is a significant decrease in serum phosphorous, sodium and potassium in treated fish. There is a significant high level of titanium content in kidney muscles, heart and spleen in group (3) suggesting toxic effects of titanium on cat fish *Clarias lazera*. The total viable count of bacteria identified higher in fish fed on carbohydrate titanium. Predominate bacteria were identified as Aeromona, E. coli, Streptococcous, Pseudomonas, Fluorscences and Lacto bacilus species. We emphasize the finding that increase carbohydrate concentration causes harmful pathological effects which reduces humoral immure responses and enhances dietary titanium toxicity.
[Mona, S. Zaki, Refat A. Yossef and Nadia M. Taha. **Effect of Titanium oxide toxicity on Biochemical, Haematological and clinicopathological Changes in *Clarias lazera* Present in the River Nile**. Journal of American Science 2010;6(12):1534-1539]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: *Clarias lazera*, titanium pollution, haematalogical, biochemical, clinicopathological, Bacteria account

1. Introduction:

Fish plays an important role, not only in human food diets but also in animal and poultry rations. It is a palatabie and easily digested food which is rich in vitamins, calcium, phosphon.rs and iodine. In Egypt, fish is considered as a cheap food article if compared with other foods of animal origin. The flesh of healthy fish is considered as a marker for the natural aquatic environment.

Titanium is a rare, element found combined in certain minerals and used mainly to produce certain alloys. Most of the vanadium (about 80%) produced is used as ferrovanadium or as a steel additive. Mixed with aluminiurn in titanium alloys is used in jet engines and high speed air-frames and steel alloys are used in axles, crankshafts, gears and other critical components. Titanium oxide (Ti_2O_5) is used as a catalyst in manufacturing sulfuric acid and in making ceramics. It is added to glass to produce green or blue tint [1]

Titanium oxide is never found unbound in nature. Titanium oxide occurs in carbon containing deposits such as crude oil, coal, oil shale and tar sands. Titanium is abundant in most soils, in variable amounts, especially in areas where chemicals or petrochemicals complex were located, where these areas showed a significant increase in its concentration [2].

Humans may be exposed to excessive vanadium in several situations for example, overconscription of vanadium-rich foods (e.g. seafood) [3], ingestion of certain dietary regimens specially that of body building, or inhalation of vanadium-rich environmental pollutants in certain occupations including boilermakers and power plant workers, who are often exposed to high levels of vanadium-rich compounds at work.

Titanium was first discovered in 1971 as a trace element that is essential for normal growth. Since then, Titanium has been found to regulate the activity of various enzymes that induce pronounced changes in metabolic function.

Because titanium is vasoactive, individuals expose to excessive titanium may develop adverse vascular effects [4] especially pulmonary vascular diseases [5] as well as nonoparticulate of titanium oxide potential titanium toxicity in human cells [6] and Niclel and titanium rich pollutant dust could be responsible for therespiratory problems reported [7].

Titanium is one of the eight most abundant in the earth's crust and consequently enters the food chain to some degree. Human are estimated to consume approximately 300g/titanium/day in (Dundord et al., 1997). Moreover, TiO2 accounts forabout 70% of the total volume of pigment production world wide (Bann et al., 2006).

The Federal Regulations of US Government limit usage of TiO2 In food products to 1% by weight (Ghoropade et al., 1995 and Wang et al., 2007). Oral routs is a potential exposure route for general population due to TiO2 used as white pigment on tooth paste, drug capsule, in tableted drug products (Ghoropade et al., 1995), in dairy based products as a whitener in manufacture of different types of cheese (Leone, 1973), dairy base drinks, chocolate, milk, coca, soybean products, milk powder, margarine, processed meat, table and soda water, sausage casing in bread flour and in the confectionary. Also, TiO2 therapeutically used in sunscreens and cosmetic creams. There have been a relatively few systematic studies that have employed pigmentary TiO2. Wang et el. (2007) reported that, until now, most studies on TiO2 toxicity in mammals were focused on the pulmonary impact of inhaled. Mahrousa (2004) reported that 4mg/kg body weight of TiO2 for 90 days in rates resulted in non significant change in DNA, and RNA content in liver and testis

Chronic exposure to titanium oxide dust and fumes may cause severe irritation of the eye, skin, upper respiratory tract, persistent inflammations of the trachea and bronchi, pulmonary edema and systemic poisoning. Signs and symptoms of overexposure include; conjunctivitis, nasopharyngitis, cough, labored breathing, rapid heart beat, lung changes, chronic bronchitis skin pallor, greenish-black tongue and an allergic skin rash[1,7].

In animals, titanium oxides cause inhibition of certain enzymes, which has several neurological effects. Next to the neurological effects vanadium can cause breathing disorders, paralyses and negative effects on the liver and kidneys. Laboratory tests with test animals have shown that vanadium can cause harm to the reproductive system of male animals and rat it accumulates in the female placenta. Vanadium can be found in fishes and many other species. In mussels and crabs vanadium strongly bioaccumulates, which can lead to concentrations of about 10^5 to 10^6 times greater than the concentrations that are found in seawater [8]

In recent years, much attention had been paid to the possible danger of metals poisoning in human as a result of consumption of contaminated fishes. So, the present study was carried out to elucidate the impact of titanium on catfish *Clarias lazera*. It's haematological, biochemical and hormonal parameters were studied as well as the bacteriological and clinopathological investigation.

2. Materials and methods

Experimental design:

Thirty catfish *Clarias lazera* were used to assess the effects of titanium oxide. Fish weighting from 180-250 were obtained from Nile revier and were kept in glass aquaria supplied with dechlorinate tap water at rate of one litter for each cm of fish's body. Fish were acclimated to the laboratory conditions for two weeks before the beginning of the experiment, they were fed with a commercial fish diet[9], the experiment was determined after 4 weeks. Fish were divided into three groups (n=10) and exposed to different doses of titanium oxide and carbohydrate. Group 1 was served as control, group2 was fed with carbohydrate and titanium oxide (10 mg kg-1 diet rations), group 3 was fed with carbohydrate and titanium oxide (15 mg/kg^{-1} diet ration)

Mean of the initial body, weight of the each examined fish at the beginning of the experiment then after 2-4weeks of exposure.

Blood samples:

Blood samples were collected from the caudal vein after 4 weeks of exposure. Each sample was divided into two parts the first one was heparinized for haematological investigations, while the second was centrifuged at 3000 rpm for 5 minutes to obtain serum for biochemical studies.

Hematological Analysis:

Haematological studies were performed according to Sandnes et al. [10], where blood haemologlobin (Hb) and haematocrit (Ht) values were evaluated.

Biochemical Analysis:

The activities of alkaline phosphatase, aspartic aminotransferase (AST) and alanine aminotrarsferase (ALT) as well as cholesterol urea and creatinine level were determined according to the method of Varley et al. [11] by using commercial kits (Bio Merieus, France)

Total scrum protein was estimated according to Drupt [12]. Serum cortisol was analyzed by a Gamma counter using 125 I cortisol radioimmunassay Kit (Baxter Health Care Corporation USA) according to the method described by Pickering and Pottinger [13]. Potassium, Sodium and Phosphorous concentrations were determined by atomic absorption spectrophotometer [11].

Tissue analysis:

Liver, kidney and spleen samples were washed with distilled water then dried in hot air oven. sulphuric acid and hydrogen peroxide were added on samples then heated until the mixture became transparent after performing a wet ash digestion according to the method of Issac and Kerber [14].

Identification of bacteria:

The liver, kidney, spleen, muscle, stomach and gill from each examined fish were diluted immediately after sampling in sterile 0.9% saline and 0.1 ml volumes of appropriate dilutions and were spread over the surface of the typtic soy agar (oxide).The plates were incubate at22^{O}C and inspected daily for up to 4 weeks.

The isolates were classified and identified according to Steverson [15] and Quirm et al. [16]. The data were evaluated statistically according to Gad-Weil [17].

Water samples:

Two water f samples were collected from River Nile (Hehvan) as well as two water samples from any heavy metal pollution El-Kasr El-Eini (control) were analyzed for titanium concentration byatomic absorption spectrophotometer.

3. Results:

Data in Table I showed that, the titanium level in Hehvan region was clearly higher than the maximum allowable concentration for human consumption as recommended internationally according to WHO (World Health Organization). Nadal et al. [2] concluded that the occurrence of titanium in nature and its use in various industrial processes has increased its inputs in the environmental. From the present study it is clear that the low titanium levels were reported in water samples collected from areas far from industrial discharges, while high titanium levels in the present study may be due to the collection of samples from areas subjected to industrial pollution.

In Table 3 there is a significant decrease in body weight in group 3 (fish fed 1.5 mg titanium for 4 weeks) than in group 1 (control) and group 2 (fish fed l0 mg titanium), this results agree with that reported by Khalaf-Allah [18].

The results present in Table 6 showed the comparison of cholesterol levels between groups. The level was significantly increased in group 3 (fish fed on l5 mg vanadium) than in group 1(control). Hypercirolestremia might be due to necrotic changes occurring in liver with liberation of cholesterol as a byproduct of cell destruction. The present data suggest that impaired liver function lead to increased serum levels of alkaline phosphat, AST and ALT among group 3 (fish fed on l5 mg titanium) and among group 2 (fish fed 10 mg titanium) compared to group 1(control). In this concern Khalaf-Allah [8] concluded that ALT and AST enzymes are good indices for the health status of liver parenchymatous, tissue necrosis is considered as the main source of AST and its increase in the serum of catfish *Clarias Lazera* declared these necrotic changes [18]. In addition, exposure of fish to environmental pollutants might result h stimulation or depression of the enzyme activity depending on the concentration of pollutant and the duration of exposure [19, 20].

Regarding the effect of titanium on serum cortisol level in cat fish *Claias lazera* highest level was obtained in group 3 (fish fed on 15 mg titanium) then in group 2 (fish fed on 10 mg vanadium) as compared to that obtained in group 1 (control). The significant increase of cortisol level is probably due to the activation of hypothalamus pituitary internal axis [21].

From the data present in Table 6, it is clear that elevation of titanium level in the diets fed to *Clarias lazera* was positively correlated to hemoglobin (Hb) levels and haematocrit (Ht). A marked decrease in the IIb and Ht was recorded after feeding diet containing 15 mg and 10 mg titanium, respectively. Reduced Hb reflect metabolic adjustment according to reduced need for oxygen by change in blood PH.

Moyle and Ceeh, Hall and Cliffs recorded actived acetchlinesterase of erthrocytes [22,23] Further more Pickeringand Dusten concluded that a consistent effect of cortisol was the reduction in the hemoglobin and iron levels as a result of decrease in appetite in rainbow trout fish or more likely to be the direct-result of catabolic effect of cortisol in the fish tissues [24].

The mean phosphorus, sodium and potassium values in the serum of fish of group 3 (fish fed 15 mg titanium) were significantly increased respectively than those recorded in the group 1 (control). This retention maybe attributing to kidney dysfunction, whereas, the kidney is the normal pass for sodium and potassium.

This kidney dysfunction may also explain the increase in serurn urea and creatinine especially in group3, but little known about the mechanisms involved in this association.

The results displayed also in Table 6 showed that there was general decrease in the mean total protein value in serum samples collected from the fish of group 3 and 2, respectively. The mean value of these parameters was lower than in group 1. Jagadeesh et al. estimated marked decrease in glycogen in tissues of fresh water fish after exposure to vanadium [25]

This experiment showed that the body weight of the examined fish was significantly decreased than the initial body weight after 4 weeks of exposure to 15mg titanium. Also, Hilton and Better recorded a significantly reduced growth and increased mortality among feeding diets of titanium (0, 10, 100, 1000, 10000mg Kg-') [26]. The increase in muscles and

tissue lactic acid (2 fold) in association with decrease in pyruvic acid (72 in muscles +26% in liver) reflect a shift towards an anaerobic metabolism of fish following long term exposure to titanium [26]

Table 4 showed that, the bacterial isolates and counts were increased by feeding the fish with CHO and titanium. The carbohydrates affect immunity and resistance to infection as recorded by Waagbo et al. [19] Utility of vanadate, mimetic protein phosphate inhibitors to protect fish from microorganism [27]. The increase of bacterial count among the fish fed on titanium may be related to the increased level of corlisol which decreases the host immunity.

In the course of experiment, a high concentration or titanium levels has been found in kidney, liver, spleen, heart and muscles of catfish *Clarias lazera* fed 15 mg titanium (Table 5). This suggests that these organs could be useful as a marker for vanadium in the aquatic environment. In this concern Ray et al. recorded a high concentration of titanium in kidney liver and other organs of catfish as the concentration of titanium in the tissues increased with its concentration in the aquatic environment and exposure time[28].After exposure of fish to increased doses for 4 days, the titanium content in the muscle then increased in all tissues [20, 25, 26] The capability of titanium to be present in fish muscle is of particular interest in assessing the exposure of man to environmental titanium as ingested by food.

Clinicopathological observations:

Abnormal swimming darking of the skin, scale loss and haemorrhasges, water seen on the external body surface. In addition to congestion of gills, eyes mouth, liver, kidney, spleen, and intestine. This was notice in fish exposed to titanium oxide 15mg (group 3) but not in fish exposed to titanium oxide 10 mg (group 2).

In conclusion: we emphasize that, the reported finding increase of carbohydrate concentrations causes harmful physiological effects, reduces hormonal immune response and enhances dietary toxicity.

Table 1: titanium concentration in water samples collected from two areas in Egypt.

Areas	No	Concentration of titanium p.p.m
Helwan	1	1.05
	2	1.28
Ak-Kasr El-Aini	3	0.156
	4	0.164

Table 2: Ingredients and Proximate composition of diets used in the experiments with titanium supplementation

Ingredients	Diet Control	Diet 2	Diet 3
Fish meal	25	25	30
Meat and bone meal	5	5	10
Wheat bran	20	20	20
Skimmed milk	12	12	7
Yeast	10	10	15
Starch	-	10	15
Cod liver oil	2	2	2
Vitamin premix	1	1	1
Titanium Mg	-	10	15
Crude protein %	40.35	35.95	38.89
Metabolizable energy k cal/kg]	2205.4	2551.78	2315.4
Ether extract%	4.29	4.21	2.86
Crude fiber %	4.46	3.73	4.27
Ash%	5.56	6.26	10.25
Lysine%	2.13	1.88	2.29
Methionine %	0.62	0.55	0.613

Mineral and vitamin premix per/kg of pellet food

Vit A, 8000 g/u, vit D 900 g/u vit E/u, vit k 4mg, vit B2 3.6 niacin 20mg, pyridoxine 0.2mg Vit B1 25, Mn 70mg, Sn 60mg

Table 3: Changes in body weight in cat fish (*Clarias lazera*) fed on different levels of dietery carbohydrate s in addition to titanium oxide.

Group	Group 1	Group 2	Group 3
Initial body weight g	74±0.15	86±0.16	93±0.23
After 2 weeks g	109±0.45	101±0.23	96±0.67
After 4 weeks g	150±0.27	123±0.63	94±0.63*

Table 4: Bacterial isolates recorded from the examined fish

No of examined fish 10/group	Bacterial isolates	Site of isolation	Bacterial count
Group 3	-Aeromonas	Kideny, spleen, muscle	4X104
	-E. Coli	-Muscles	-----
	-Streptococcus	-External surface, Stomach	3X103
	-E. Coli	Gills	----
	-Aeromonas	Gills, Stomach	5.6X104
	-Lactobacillus	Gills	
Group 2	-Enterbacter	Liver, Kidney	4X103
	-Pseudomonas	-Spleen, Muscles	5X103
	-Fluroscences	-Stomach	3X106
	-Lactobacillus	-Gills	----

Table 5: The mean titanium concentration in the organs of fish mg/g net weight

Groups	Muscles	Spleen	Heart	Kidney	Liver
Group1	0.28±0.14	0.51±0.83	0.68±0.49	3.25±0.72	2.21±0.69
Group2	0.47±0.25	0.61±0.71	0.73±0.41	4.20±0.83	3.21±0.70
Group3	0.58±26	0.92±0.41	0.87±0.24	7.74±0.74	6.23±0.05

Table 6: Some haematological, biochemical parameters in catfish *Clarias lazera* on different levels of dietery carbohydrates in addition to titanium oxide

Group	Group 1	Group 2	Group 3
Parameters	6.5±0.23	6.5±0.23	6.52±0.12*
Hemoglobin g/dl	37.2±0.27	37.2±0.27	32.5±0.24*
HCT %	0.83±0.21	0.94±0.10	1.50±0.67*
Cartisol ng/dl	9.7±0.64	9.3±0.27	82±0.67*
Phosphorous mg/dl	123±1.24	112±0.75	102±0.14*
Sodium M.EQ	7.23±0.82	7.02±0.44	62±0.74*
Potassium M. EQ	21.42 ±3.2	22±0.73	27±0.72*
Alkphosphatase U/L	134±0.41	132±0.88	143±0.23*
AST U/L	22±0.17	24±0.74	37±0.28*
Cholestrol mg	143±0.25	148±0.13	171±0.54*
Total protein g/dl	9.2±0.76	9.02±0.81	8.02±0.72*
Urea mg/dl	3.1±0.78	34±0.76	4.7±0.23*
Creatinine mg/dl	0.77±0.23	0.73±0.76	0.93±0.52*

Corresponding author
Mona, S. Zaki
Department of uHydribuilogy
National Research Centre
Cairo, Egypt
dr_mona_zaki@yahoo.co.uk.

6. References:

1. Copyright @ 1998-2007. Lenntech Water treatment & air purification Holding B.V. Rotterdamseweg 402 M 2629 HH Delft, The Netherlands e-mail: info@lenntech.com
2. Nadal, M., M and Schulmacherand J.L. Dommgo, 2007. Levels of metals, PCB's PCN's and PAH's in soils of highly industrialized chemical/petrochemical area chemosphere, 66: 267 -76.
3. Bu-Olayan, A.H. and S. Al-Yakoob, 1998. Lead Nickel and Vanadium in sea food an exposure assessment for Kuwait consumers. Sci. Environ.. 2-3: 8l-86.
4. Huang, Y.C. and A.J. Ghio, 2006. Vascular effects of ambient pollutant particles and metals, curr. Vasc. Pharmacol , 4:199-203.

5. Li, Z., J.D. Carter, L.A. Dailey and Y.C.T. Huang, 2004 4-vanadyl sulfate inhibits NO production via threonine phosphorylation of eNOS, Environ Health Prospect, 112:20l-206.
6. Worle, J.M., K. Kem C. Schelh A.C. Helmy, C. Feldman and H.F. Krug, 2007. Nanoparticulate vanadium oxide potentiated vanadium toxicity in human lung cells, Environ. Sci. Technol., 41 331-6.
7. Figuero, D.A., C.I. Rodriquez-Sierra and B.D. Jimenez-velez 2006. Toxicol & health, 22:87-99.
8. Bu-Olayan, A.H. and M.N.V. Subranmanyam, 1996. Trace metal in fish from Kuwait coast using microrvave acid digestion technique. Environ-lnter., 22: 753-758.
9. Waagbo, R., J. Glette, K. Sandnes and G.F. Hemre, 1994 .J. Fish Dis.,1 7:l 45.
10. Sarndnes, K., Q. Lee and R. Waagatn, 1998. J. of Fish Biol., 3l: I19.
11. Varley, H., A. HI. Gwenbek and M. Bell, 1980. Practical clinical chemistry, vol. I General I top's comneuer test 5th ed. London, William medical books Ltd.
12. Dmpt, F., 1974. Pharm. Biol, 9: 77.
13. Pickering, A.D. and I. Pottinger, 1983. Gen Comp Ender. 49: 232
14. Issac, R.A. and kerber, I 971. Amer. Madison. 17.
15. Steverson P., 1987. "Field Guide Systematic Bacteriology". University of Cuelphontaria, Canada, pp: 280.
16. Quinn, P.J., M.E. Carter, B.K. Makey and G.R Carter, 1994. "Clinical Veterinary Microbiology", wolf publishing Mosby, Year Book Europe limited.
17. Gad, S.C. and C.S. Weil, 1986. Statistics for toxicologists. In, Hages A.W. (2nd ed.), "Principles and Methods of toxicology", Raven Press, New York, pp: 273-32
18. Khalaf-Allah, 1998. Screening the effect of water pollution with some pesticides on the immune response in *Oreochromis nilolicus* fish. Vet. Mid. J. Giza., 46: 883-393.
19. Venberg, F.G. andW.B. Venbcrg, 1974. Pollution and Physiology of marine organisms. Academic Press New York, pp: 59.
20. Edel, J. and E. Sabioni, 1993. Accumulation and distribution of mussel myails edulis and the gold fish carassits auratus. Sci. Total-Envilon. 133: 139-151.
21. Carballo, M.J., M.J. Torroba, C. Munoz, D.V. Sanchez, I.D. Txazora and J. Dominguez, 1992. J. Fish and Shell Fish Immunology, Z. 121.
22. Moyle, P.B. and J.J. Cech 1982. Blood And Its Circulation In Fish-An Introduction to Ichthyology" (Ed. by, P.B. Moyle, J.H. Cech) pp: 52-73 Prentice.
23. Hall, I. and E. Cliffs, 1982. Aspects and energy response in an Indian catfish Babachus, Biometais, 11: 95 – 100.
24. Pickering, A.P. and J. Duston1983. J. Fish Biol., 23: 163.
25. Jagadeesh K.B., S.A. Shaffi and S. Jeelani, 1989 Acta Physiologia Hungarica 74: 43.
26. Hitton" J.W. and W.G. Bettgeo, 1988. Aquatic Toxicology, l2: 63.
27. Evans-Donald L. and L. Jaso. Friedmarur, 2001. Protection of *Teleost fish*. Biotclr, Nav., 15: 777.
28. Ray, D., S.K. Panerjeo and M.I. Chattejee, 1999. Bioaccumulation of Nickel and Vanadium in tissues of Catfish Batracchus. J.Inorg-Biochem., 38: I 69-173.
29. Dunford, D. K.; Salinaro, A.; Car, L.; Serpone M. N.; Harikoshi, S.; Hidaka, H. and Knowland, J. (1997): Chemical oxidation and DNA damage catalyzed by inorganic sunscreen ingredients. FEBS letters 418: 97-90.
30. Bann, R.; Straif, K.; Grosse, Y.; Secreton, B.; Ghissassi, F.F. and Cogliano, V. (2006): Carcinogenicity of carbon black, titanium dioxide and talc. J. of the Lancet Oncol.7, 295-296.
31. Ghoropade,V.M.; Desphande,S.S.and Salunkhe, D.K. (1995): Food colors in food additive toxicology by Joseph ,A.M. and Authony,T.Tu, , New York . Basal, Hony Kong. Chapter 4 Page 214.
32. Wang, J.; Zhou, G.; Chen,C.; Yu, H. ; Wang, T.; Ma, Y.; Jia, G.; Gao, Y.; Li, B.; Sun,J. ; Li, Y. ; Jiao, F.; Zhao, Y. and Chai, Z. (2007): Acute toxicity and biodistribution of different sized titanium dioxide particles in mice after oral administration . Toxicology Letters, 168: 176-185.
33. Leone, J. (1973): Collaborative study of the quantitative determination of titanium dioxide in cheese. J. Assoc. Offic. Anal Chem., 56: 535-558.
34. Mahrousa, M. H. Kandiel (2004): Cytogenetic and biochemical effects of some food colors in rats. Ph. Dr. Thesis Submitted to animal Production department, Faculty of Agriculture, Cairo University.

11/25/2010

The Influence of Technological Changes on Labour Availability: A Case of Cocoa Farming Households in Ogun State, Nigeria.

[1]Oluyole, Kayode A and [2] Egbetokun, Olugbenga A

[1]Economics and Statistics Division, Cocoa Research Institute of Nigeria, PMB 5244, Ibadan, Nigeria
[2]Institute of Agricultural Research and Training, PMB 5029, Apata, Ibadan
greatjoge@yahoo.com

Abstract: This study examined the effects of technological changes on labour availability. Primary data was collected using structured questionnaires administered to a purposive sample of eighty cocoa farmers in Ogun state of Nigeria. The data collected was analyzed using descriptive statistics, Analysis of Variance (ANOVA) and Multi-variate regression analysis. Descriptive analysis revealed that some technologies such as improved spacing and fertilizer application require the employment of more labour while some technologies like mechanization and herbicide application displace labour. The result of the ANOVA shows that there is significant difference in the magnitude of labour used in different technological groups. Multi-variate regression analysis revealed that availability of labour is influenced by the extent of cultivation as well as the expenditure on improved technologies ($P<0.01$). The study recommended that small scale processing industries should be established in the rural areas to take the advantage of the available excess rural labour resulting from the displacement by some technologies thereby eliminating the problem of unemployment that is likely to be generated as a result of the adoption of the technologies.

[Oluyole, Kayode A and Egbetokun, Olugbenga A. **Toxic Effects of *Grewia mollis* Stem Bark in Experimental Rats**. Journal of American Science 2010;6(12):1540-1543]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Effects, technological changes, labour availability, cocoa farming households, Nigeria, Analysis of Variance, multivariate regression analysis.

1. Introduction

Nigerian agricultural sector is dominated by small scale farmers whose farms vary between 0.10 and 5.99 hectares and constitute about 80.35% of all the 29.800 million holdings in Nigeria (Olayide *et al*, 1980; Ogunwale, 2005). Their farmers used traditional technologies called hoe-cutlass culture. Their capital structure is in form of small tools and predominant usage of family labour (Adegeye, 1995). Among the other problems that are associated with small scale farming are the problems of low productivity due to the problems of pest and diseases infestation and the problems of aged crop trees (cocoa trees) (Adegeye, 1995). Giving the increasing population pressures and consequent increase in food demand, government found it imperative to search for ways by which agricultural sectors could be improved. In line with this, a lot of programmes have been embarked upon and some institutions have been established. Such institutions include the National Seed Service (NSS), National Accelerated Food Production Programme (NAFPP), Agricultural Development Project (ADP) and others. It shall be noted that the main objective among others of these programmes is revolving around the development and dissemination of improved technologies in farming practice.

Improved technologies are the various new "technical know-how" for the promotion and development of agriculture. It alters the structure of agricultural production process through acting as a sure value for changing physical and value productivity of farm resources (Olayide, 1982). Some of these improved technologies are the use of tractors, application of fertilizers and insecticides, adoption of improved spacing, treatment of seed before planting, improved storage techniques and a host of others (Oluyole, 2005). These have taken over from the use of traditional technology which is characterized with the problems of deterioration in the vigour and stability of human labour in a stand environment of high and humidity (Olayide, 1980).

In Nigerian agriculture, hired labour is predominantly used. In fact it carries 88% of total labour used on farms (Okuneye, 2000). But apart from hired labour, the other type of labour that could be employed are family labour and cooperative labour. The availability of labour has been found to have impact on planting precision, better weed control, timely complete harvesting and crop processing (Oluyole, *et al* 2007). Therefore, labour is a major constraint in peasant production especially during the early planting, weeding and harvesting (Gocowski and Oduwole, 2003).

However, there is a strange relationship between the technological changes and labour. The classical economists such as Richado, Mathus, Stuart Mill and Marx were particularly concerned with the problem of employment implications of technological change. The introduction of improved technologies (such as machines) by making production more efficient can lead to the reduction in the employment of labour.

The need for this study is the persistent high demand for labour for most farm operations and to determine the extent to which agricultural innovations has relieved the shortages of labour force in farm operations and subsequent improvement of farm operations as well as standard of living of small scale farmers. This study will be undertaken through the following objectives: To investigate the pattern of adoption of improved technologies in the study area; to determine the magnitude of labour requirements by different improved technologies; to determine whether there is significant change in labour use among the different technological combinations and to determine the factors that affect the availability of labour in the study area.

Hypothesis testing

There is no significant difference in the means of labour used among the different technological groupings.

METHODOLOGY

The study was carried out in Ogun state of Nigeria. The state is one of the fourteen cocoa producing states in Nigeria (NCDC, 2006). Four cocoa producing Local Government Areas (LGAs) were chosen for the study. The LGAs are Abeokuta North, Abeokuta South, Odeda and Owode. Twenty respondents were purposively randomly selected from each LGA making a total of eighty respondents in all for the study.

Respondents were classified into three technological groups depending on the number of technologies adopted by the respondent. The technological groups are Low Technology (LT), Medum Technology (MT) and High Technology (HT). Low Technology is the adoption of a maximum of two technologies; Medium Technology is the adoption of between two and five technologies while High Technology is the adoption of more than five technologies. Information was collected from the respondents with the aid of structured questionnaires and the data collected was analysed using Descriptive Statistics, Analysis of Variance (ANOVA) and Multi-variate Regression analysis.

Descriptive Statistics was used to analyse the pattern of adoption of technologies as well as the magnitude of labour requirements by different technologies. ANOVA was used to assess whether there is significant difference in the amount of labour used among the three technological groups. Multi-variate Regression analysis was used to evaluate the effects of the income of farmer, extent of cultivation, wage rate as well as expenditure on improved technologies on the availability in the study area.

$$\ln LAB = \ln\alpha_o + \alpha_1 \ln INC + \alpha_2 \ln EXT + \alpha_3 \ln WAG + \alpha_4 \ln EXP + e_i$$

Where:
LAB = Avaiability of labour (Mandays);
INC = Income of farmers (₦);
EXT = Extent of cultivation (Ha);
WAG = Wage rate (₦);
EXP = Expenditure on improved technologies;
e_i = Stochastic random error.

RESULTS

Table 1: Distribution of farmers by the technologies used

Technologies adopted	Number of farmers	Percentage
Rehabilitation techniques	36	45
Mechanization (mechanical clearing)	3	3.8
Improved seedlings	64	80
Fertilizer	40	50
Improved spacing	63	79
Herbicides	13	16
Insecticides	15	19
Fungicides	65	81

Source: Field survey, 2006

Table 2: Labour requirements per hectare in different farm operations

Farm operations	Labour used (mandays)
Manual clearing	12
Mechanization (mechanical clearing)	2
Herbicides application	3
Planting with unimproved spacing	5
Planting with improved spacing	8

Fertilizer application	6
Insecticides application	3

Source: Field survey, 2006

Table 3: Estimated regression coefficients for the determinants of the availability of labour

Variables	Linear	Semi-log	Double-log
Constant	11.71	157.18	2.21
	(0.93)ns	(2.49)**	(5.48)***
Income of farmer	-0.000083	14.51	0.0575
	(-1.44)ns	(1.40)ns	(0.87)ns
Extent of cultivation	44.6	143.08	1.16
	(9.45)***	(5.92)***	(7.52)***
Wage rate	0.0453	7.25	0.082
	(-0.57)ns	(0.36)ns	(0.65)ns
Expenditure on improved Technologies	0.00480	-57.83	-0.082
	(-7.08)***	(-4.58)***	(-4.21)***
R^2 value	0.640	0.569	0.734
F- value	33.37	24.71	51.74
Std. Error	25.28	27.68	0.1765

Source: Computed from field survey data, 2006.
Note:
Figures in parentheses are t- values
*** Significant at 1% level
** Significant at 5% level
ns = Not significant

DISCUSSION AND CONCLUSIONS

Patterns of technological adoption by cocoa farmers

The improved technologies that have been introduced into the study area are rehabilitation techniques, mechanization, improved seedlings, fertilizer application, improved spacing, herbicides, insecticides and fungicides.

From table 1, it could be observed that improved seedlings, fungicides as well as spacing are widely adopted in the study area. They have the proportion 80 percent, 81 percent and 79 percent of the total sampled farmers respectively. This shows that the impact of extension personnel as regards the dissemination of information particularly on the improved technologies is greatly felt in the study area. As for mechanization, herbicides and insecticides, these are marginally adopted in the study area. They carry 3.8 percent, 16 percent and 19 percent of the total sampled farmers in the study areas respectively.

Magnitude of labour requirements per hectare in different farm operations

Table 2 shows that some operations require more labour. Such operations include manual clearing, planting with improved spacing and fertilizer application which requires 12, 8 and 6 mandays respectively. However, some operations such as mechanization, herbicide application and insecticide application require less labour (2, 3 and 3mandays respectively). Hence, some improved technologies such as spacing and fertilizer application add labour, while other improved technologies such as mechanization, herbicides and insecticides application reduce labour requirements. It should be noted that spacing increases labour due to the fact that most improved spacings are aimed at maximizing the use of land, thus bringing in more crop stands and more crop stands would definitely require more labour.

Variations in the quantity of labour used in different technological groupings

In order to determine whether there is significant difference in the number of mandays used in different technological groupings, the computer result of the analysis of variance in the labour used among the three technological groupings was used. The result showed that F calculated is 44.42. Meanwhile, F tabulated at 1% is 4.88. Since F calculated is greater than F tabulated, the null hypothesis [Ho] which says that there's no significant difference in the amount of labour used in the three technological groupings is rejected while the alternative hypothesis is rejected. Therefore, there is significant difference in the amount of labour used among the three technological grouping. The differences might be due to the fact that some of the adopted improved technologies such as herbicides displaced labour. However, some technologies such as fertilizer and improved spacing added labour but their impact might not be as high as those of labour displacing improved technologies.

Determinants of the availability of labour

Multivariate regression analysis was used to determine the factors affecting the availability of

labour and the result of the analysis is presented in Table 3. The Table shows the result of the three functional forms of Ordinary Least Square Regression analysis. However, out of the three functional results, double log regression result was chosen based on the value of the standard error, value of the coefficient of determination and the number of variables that are significant. The result of the lead equation shows the coefficient of determination [R^2] of 73.4%, that is, the independent variables are able to explain 73.4% of the total variations in dependent variable. Table 3 also revealed that out of the four factors regressed against the dependent variable, two were found to be significantly affected the availability of labour. These factors are extent of cultivation as well as expenditure on improved technologies [$p<0.01$]. The significance of the extent of cultivation could be attributed to the fact that size of farm determines the number of labour, that is, the larger the farm the more the number of labour that would work on such a farm and vice versa. As for the expenditure on improved technologies, as more money is been spent on improved technologies, less labour will be employed since the improved technologies (such as herbicides) would displace labour thus requiring few labour to be employed.

Based on the findings, the study concludes that the introduction of improved technologies (like mechanization and herbicides) unequivocally reduce labour use thereby reduces the cost of employing farm labour thus reduces the overall cost of farm operations.

However, the study recommends as follows: Government should assist to make improved technologies available to farmers anytime they are needed and at subsidized prices. This will enable the farmers to adopt more improved technologies. Farmers should organize themselves into groups to enable them have access to credit facilities for them to be able to procure improved technologies.

Small scale processing industries should be established in the rural areas to take the advantage of the available excess rural labour resulting from the displacement by some improved technologies thereby eliminating the problem of unemployment that is likely to be generated as a result of the adoption of the improved technologies.

Corresponding Author:
Egbetokun, Olugbenga Aderemi
Institute of Agricultural Research and Training
P M B 5029 Apata Ibadan
Oyo State Nigeria
E mail address: greatjoge@yahoo.com

References

1. Adegeye, A.J. Production and Marketing of Cocoa in Nigeria. In Adegeye, A.J. and W.O. Ajayi (eds.). Cocoa Revolution in Nigeria. *Proceedings of a National Seminar on Revolutionizing Nigeria's Cocoa Industry.* University of Ibadan. 1995, 28-30th November.
2. Ezeh, C.O. and D.F. Omokare Rural Labour Dynamics and Seasonal Farm Production in Ikara and Makarfi LGAs of Kaduna State. Economic Spatial Considerations. Paper presented at the 3rd Annual Conference of the Farm Management Association of Nigeria. 1986 Pp.1-2.
3. Gocowski, J and S. Oduwole. Labour Practices in the Cocoa Sector of Southwest Nigeria with a special focus on the role of children. STCP/IITA monograph IITA, Ibadan, Nigeria. 2003.
4. National Cocoa Development Committee.The National Cocoa Development Committee Intervention. *Cocoa Rebirth,* 2006 Pp.21
5. Olayide, S.O., Ewewa, J.A. and Bello-Osagie, V.E. *Nigeria Small Farmers: Problems and Prospects in Integrated Rural Development.* Centre for Rural Development. University of Ibadan. 1980
6. Olayide, S.O. *Agricultural Technology and Small Scale Farmers.* University of Ibadan. 1982
7. Ogunwale, A.B.. A case study of Small Scale Farmers participation in Nigeria Agricultural Development Programmes: Oyo and Osun State Agricultural Development Programmes. *Journal of Rural Development.* 2005 28 (1): 85-96.
8. Okuneye, P.A. "Employment Generating Potentials of Agricultural Processing and Storage Technology: Additional Gain in Increased Food Availability Pursuit" Paper presented at the workshop for local government officials in Lagos state. 2000
9. Oluyole, K.A. Evaluation of the Economics of Post Harvest Processing of Cocoa in Cross River State, Nigeria. *Journal of Agriculture, Forestry and the Social Sciences*. 2005 3(2): 58-64.
10. Oluyole, K.A., Adebiyi, S. and Adejumo, M.O. An Assessment of the Adoption of Cocoa Rehabilitation Techniques among Cocoa Farmers in Ijebu East Local Government Area of Ogun state. *Journal of Agricultural Research and Policies*, 2007*l.2(1):56-60*

02/04/2010

Toxic Effects of *Grewia mollis* Stem Bark in Experimental Rats

Wilson Obidah[†], Julius L. Godwin[†], Joseph Z. Fate[#] , Mike A. Madusolumuo[†]

[†]Department of Biochemistry, Federal University of Technology Yola, P.M.B.2076 Yola, Nigeria.
[#]Department of Science Laboratory Technology, Federal Polytechnic Mubi, P.M.B. 35 Mubi, Nigeria.
domwam@yahoo.com

Abstract: *Grewia mollis* stem bark used locally in Nigeria as food additive was mixed with the normal diet at 0, 1, 5 and 10% and fed male Wister rats over a four week period. No deaths or remarkable changes in general appearance or behaviour were observed in treated animals. Significant ($p < 0.05$) increases in serum transaminases activities, accompanied by decreased food intake were observed in rats fed the stem bark at 10% dietary level. Treatments had no effect on serum alkaline phosphatase activity, urea, creatinine, triglycerides, cholesterol, glucose concentrations and body and organ weights determined. These findings suggest that dietary exposure of rats to *Grewia mollis* stem bark at high concentrations may cause some adverse effects, especially liver injury.

[**Toxic Effects of *Grewia mollis* Stem Bark in Experimental Rats. Toxic Effects of *Grewia mollis* Stem Bark in Experimental Rats**. Journal of American Science 2010;6(12):1544-1548]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: *Grewia mollis;* stem bark ; additive; toxicity; rat

1.0 Introduction

Plant materials have been used as alternatives to chemical food additives to enhance the quality of food products. Recently, there has been a general increased interest in the use of natural additives presumably due to their relatively higher availability, affordability, and the perceived lower risk compared to synthetic additives. Consequently, various plant preparations have now been introduced as additives in food. Although generally regarded as safe, plant materials differ in their chemical composition and toxicity resulting from consumption of some plant products have been reported (Burkhard *et al.*,1999;Haller and Benowitz,2000; Nortier *et al.*,2000;Ernst 2002), indicating the need for their continual safety assessment.

Grewia mollis (Tiliaceae) is a shrub or tree widely distributed in northern Nigeria and some African countries. Various parts of the plant are used in food and medicine. In Nigeria, the stem bark powder or mucilage is used as a thickener in local cakes made from beans or corn flour commonly called "Kosai" and "Punkasau" in Hausa (Nigeria), respectively . The dried stem bark is ground and the powder mixed with beans or corn flour thereby enhancing the texture of the food product. Some findings demonstrated that the mucilage obtained from the stem bark can serve as a good binder in paracetamol formulations (Martins *et al.,* 2008 and Muazu *et al.,* 2009). In addition, the mucilaginous property of the bark is used traditionally by the Yoruba people of Nigeria at child birth. Phytochemical studies on the leaves and stem bark of *Grewia mollis* indicated the presence of tannins, saponins, flavonoids, glycosides, phenols, steroids and the absence of alkaloids (Onwuliri *et al.,* 2006). Although *Grewia mollis* stem bark powder or mucilage is widely used in Nigeria, few studies have been reported on the safety of the stem bark to consumers as a food additive. The purpose of this study was to determine the potential toxic effects of *Grewia mollis* stem bark powder in male Wister rats after dietary exposure for four weeks.

2.0 Materials and Methods

2.1 Plant material and preparation of the stem bark powder.

Fresh samples of the stem bark of *Grewia mollis* were collected along Ganye-Sugu road, Adamawa state, Nigeria. The specimens were identified at Forestry Department, Federal University of Technology Yola, Adamawa state, Nigeria. The hard outer part of the stem bark was removed by peeling manually and the remaining inner soft tissue cut into small pieces and dried to constant weight in an oven at about 50^0C. The dried samples were pulverized using pestle and mortar, and sieved to obtain a fine powder that was stored in brown glass containers at room temperature until use in the experimental diet.

2.2 Experimental diet

A pelleted standard diet purchased from Grand cereals Ltd., Jos, Nigeria, was used in the entire study. The diet was made to powder using pestle and mortar to obtain a good homogeneity when admixed with *Grewia mollis* stem bark powder. Diets

containing the stem bark were prepared on a daily basis.

2.3 Animals

Male Wister rats weighing 170±10g were purchased from the animal unit of the National Veterinary Research Institute (NVRI) Vom, Plateau state, Nigeria. The animals were housed in plastic cages, kept at room temperature, allowed free access to water and fed standard diet with or without the stem bark powder *ad libitum*. The animals were allowed to acclimatize for two weeks prior to the commencement of the treatment.

2.4 Experimental design

Rats were randomized and divided into four groups of six animals each and fed diet containing 0, 1, 5 or 10% (w/w) *Grewia mollis* stem bark daily for four weeks. Rats were fasted overnight at the completion of the treatment period. Blood samples were collected from rats by cardiac puncture under diethylether anaesthesia and used for the determination of serum biochemical parameters. Clinical signs and general appearance of animals were observed daily for signs of toxicity. Body weights and food intake were also monitored. The animal weights were measured immediately prior to the commencement of the study at weekly intervals and at the end of the study. Final mean body weights and body weight gain were calculated.

The animal diet was weighed and given to animals daily. Food intake was calculated by subtracting the mass of the feed remaining from the known mass provided to the rats and the food consumption per g/rat/day determined. Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), alkaline phosphatase (ALP) activities and serum urea, creatinine, triglycerides, cholesterol and glucose concentrations were assayed using commercial kits (Randox laboratories Co.Antrium, UK) following the manufacturer's instructions. The kidney and liver of all animals were removed immediately after sacrifice and weighed. The relative organ weights for each group were calculated.

2.5 Statistical analysis

All results are expressed as mean ± SEM. The data were analyzed by ANOVA and Dunnett's test (Dunnett, 1955). The level of significance was set at $p<0.05$.

3.0 Results

Clinical signs observed among the rats appeared normal and no deaths were recorded during the four weeks of the experiment. *Table 1,* shows the effects of *Grewia mollis* stem bark administration on some liver marker enzymes in rats. Incorporation of *Grewia mollis* stem bark powder at 10% in the normal diet of rat induced significant ($p< 0.05$) increases in serum transaminases (ALT and AST) activities. There were no significant ($p> 0.05$) differences in serum ALP activity between treated and control animals. The effects of dietary exposure of rats to *Grewia mollis* stem bark on some serum constituents in rats are shown in *Table 2.* No significant ($p> 0.05$) difference in serum urea and creatinine concentrations were observed between control and treated groups. Serum glucose, triglycerides and cholesterol concentrations were also not affected by administration of the stem bark in rats. Mean body weight gain, food intake, absolute and relative organ weight of rats treated with *Grewia mollis* stem bark powder are shown in *Table 3.* Food intake decrease significantly ($p< 0.05$) in animals fed with 10% stem bark powder as compared to control animals. No remarkable changes were observed in mean body weight gain, absolute and relative liver and kidney weights.

Table 1: Effects of *Grewia mollis* stem bark administration on some liver marker enzymes activities in rats.

Dose group (%)	ALT (U/L)	AST (U/L)	ALP (U/L)
0 (control)	74.80±3.20	93.60±2.01	18.58±2.44
1	75.60±2.33	87.80±3.06	21.93±1.84
5	78.76±3.11	96.23±2.67	20.28±2.31
10	94.80±3.21*	107.80±2.75*	19.42±5.61

Tabulated Values are Mean±SEM, n =6

ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; ALP, Alkaline phosphatase

*Significantly different from control group at $p<0.05$

Table 2: Effects of *Grewia mollis* stem bark administration on serum urea, creatinine, triglycerides, cholesterol and glucose concentrations in rats.

Dose group (%)	UREA (mmol/l)	CREATININE (mmol/l)	TRIGLYCERIDES (mmol/l)	CHOLESTEROL (mmol/l)	GLUCOSE (mmol/l)
0 (control)	63.60±3.01	168.56±19.80	5.26±1.98	5.04±1.32	9.56±1.53
1	63.40±4.09	140.75±15.25	4.20±1.91	5.10±1.43	8.71±1.40
5	59.76±5.27	155.08±12.75	3.10±1.34	4.96±1.34	8.98±2.57
10	65.18±3.52	154.23±16.19	4.83±1.77	4.78±1.18	8.72±1.87

Tabulated Values are Mean±SEM, n =6

Table 3: Effects of *Grewia mollis* stem bark administration on body weight gain, food intake, absolute and relative organ weights (g/100g body weight) of rats.

Dose group (%)	Body weight gain (g)	Food intake (g/rat/day)	Absolute organ weight		Relative organ weight	
			Liver	*Kidney*	*Liver*	*Kidney*
0 (control)	44.60±5.20	17.60±2.19	6.58±0.13	1.06±0.02	4.12±0.12	0.67±0.03
1	47.63±4.17	17.73±2.14	6.54±0.16	1.12±0.03	4.20±0.17	0.71±0.02
5	46.41±3.12	15.61±2.56	5.54±0.05	1.06±0.02	3.08±0.05	0.69±0.01
10	39.56±4.23	14.24±2.16*	6.12±0.16	1.12±0.01	4.05±0.18	0.74±0.03

Tabulated Values are Mean±SEM, n =6
*Significantly different from control group at $p<0.05$

4.0 Discussion

The results suggest adverse effects that may involve the liver. Consumption of high concentrations of the *Grewia mollis* stem bark powder resulted in increased serum transaminases (ALT and AST) activities and decreased food intake in rats. Liver cell injury is usually characterised by a rise in plasma transaminases with serum ALT activity more pronounced than AST (Stroev and Makarova, 1989). The elevated levels of ALT and AST activities were considered to reflect the cytosolic release of liver associated enzymes into serum, resulting from the necrotic and degenerative responses of hepatocytes (Lavrijsen, 1992;Kew 2000; Dobbs 2003; Matsumoto, *et al.*, 2006). The absence of significant increases in serum alkaline phosphatase activity may indicate that the stem bark administered to rats had little or no effect on the induction of hepatobilliary distruption (Varley *et al.*, 1980; Somchit *et al.*, 2004).

Since no significant change in serum urea and creatinine concentrations were observed during the course of the study, it was suggested that the administration of *Grewia mollis* stem bark did not interfere with the renal capacity to excrete metabolites. Blood urea and creatinine concentrations are commonly used as indicators of renal injury and are elevated during kidney disease (Imai *et al.*,1981; Narama *et al.*, 1993;Chawla, 1999). Similarly, the non significant changes in serum triglycerides, cholesterol and glucose concentrations suggest normal absorption and metabolism of these substances.

Mean food intake decreased significantly in animals fed with 10% of the stem bark powder. Whether this decrease in mean food intake was due to the toxic effects of the plant or other factors remain to be ascertained. However, incorporation of the *Grewia mollis* stem bark in the fed diet at high concentrations may reduce food palatability and consequently, appetite and food intake in experimental animals. For instance, the presence of some constituents in the stem bark such as tannins (Onwuliri *et al.*, 2006) may cause the observed reduced food intake. Tannins have been shown to reduce feed intake in animals fed tannin diets, which was attributed to the astringent property of tannins and induction of internal malaise in mammals (Hotellier and Delaveau, 1975; Toma *et al.*, 2009). Therefore, the decrease in food intake may not necessarily reflect the severity of the stem bark toxicity. This view is further strengthened by the fact that the body weight gain, absolute and relative liver and kidney weights of the rats were not affected by administration of the stem bark powder.These parameters usually indicate the pathological and

physiological status in man and animals (Ramesh, 2007 and Sellers *et al.*, 2007).

Conclusion

Dietary exposure to *Grewia mollis* stem bark powder at high concentrations (10%) resulted in significant reduction in food intake and elevated serum transaminases activities in rats. It is therefore, reasonable to presume that consumption of the plant material at high concentrations may elicit hepatotoxic effects in rats and possibly in humans.

Correspondence to:
Wilson Obidah
Department of Biochemistry,
Federal University of Technology Yola,
P.M.B 2076 Yola,
Adamawa State,
Nigeria.
Email: domwam@yahoo.com

Acknowledgement

The authors wish to thank Mr. M.Raji of Biochemistry Department, Federal University of Technology Yola for his assistance in animal sacrifice.

References

1. Burkhad PR Burkhad K, Haenggeli C.-A , Landis T.Plant-inducedseizures:reappearance of an old problem. J. Neurol. 1999; 246:667-670.
2. Haller CA, Benowitz NL. Adverse Cardio-Vascular and central nervous system events associated with diet a supplements containing ephedra alkaloids. New Engl. J. Med.2000;343 (25):1833-1838.
3. Nortier JL, Muniz Martinez M.-C, Schmeiser HH, Arlt VM, Bieler CA, Petein M, Depierreux MF, De Paw L, Abramowicz D, Vereerstraeten P, Vanherwegheim J.-L. Urothelial carcinoma associated with the use of a Chinese herb (Aristolochia fanghi). New Engl. J.Med. 2000;342(23):1686-1692.
4. Ernst E. The risk-benefit profile of commonly used herbal therapies:Ginkgo, St. John's Wort, Ginseng, Echinacea, Saw Palmetto, and Kava. Ann. Intern. Med. 2002;136: 42-53.
5. Martins E, Christiana I, Olobayo K. Effect of Grewia gum on the mechanical properties of paracetamol tablet formulations. Afr. J.Pharm. Pharmacol. 2008;2:1-6.
6. Muazu J, Musa H, Musa KY. Compression, mechanical and release Properties of paracetamol tablets containing acid treated Grewia gum. J. Pharmaceutical Sci. Technol. 2009;1(2):74-79.
7. Onwuliri F.C,Mawak JD, Wonang DL,Onwuliri EA. Phytochemical, toxicological and Histopathological studies of some medicinal plants in Nigeria. Int. J. Nat. Appl. Sci. 2006; 2(3):225-229.
8. Dunnett CWA. Multiple comparison procedure For comparing several treatments with a control. J. Am. Stat. Assoc. 1955;12:1096-1121.
9. Stroev AE,Makarova GV. Laboratory Manual in Biochemistry. Mir publishers, Moscow, 1989; 162-164.
10. Lavrijsen APM, Balmus KJ, Nugteren-Huying WM, Roldaan AC, Vant Wout JW, Stricker BHC. Hepatic injury associated with itraconazole.Lancet, 1992;340:251-252
11. Kew ML. Serum aminotransferase concentration as evidence of hepatocellular damage. Lancet, 2000;335:951-952.
12. Dobbs NA, Twelve CJ, Cruickshanka C, Richard MA, Ruben RD. Epirubicin in patients with Liver dysfunction development and evaluation of a novel dose modification scheme. Eur. J. Cancer,2003; 39:580-586.
13. Matsumoto M, Also S, Umeda Y, Arito H, Nagano K, Yamamoto S, Matsushima T. Thirteen week oral toxicity of para- and ortho-chloronitrobenzene in rats and mice. J.Toxicol. Sci. 2006;31(1):9 -12.
14. Toma I, Karumi Y, Geidam MA. Phytochemical Screening and toxicity studies of the aqueous extract of the pods pulp of cassia sieberiana DC (Cassia Kotchiyana Oliv.). Afr. J. Pure. Appl. Chem. 2009;3(2):26 - 30.
15. Somchit N, Norshahida RA, Hasiah HA, Zuraini A, Sulaiman RM, Noordin MM. Hepatotoxicity induced by antifungal drugs. Intraconazole and fluconazole in rats: a comparative in vivo study. Hum.Exp. Toxicol. 2004;23:519-525.
16. Imai K, Yashimura S, Ohtaki T, Hashimoto K .Experimental toxicity study of an angiotensin I (ACE I) converting enzyme inhibitor captopril in the rats. J. Toxicol. Sci. 1981;6:189 – 214
17. Narama I, Nakajima H, Fujishima H, Kurio W, Maeda H, Ozaki K, Kumagai Y, Hiramatsu Y, Takabatake E. Twelve-month oral toxicity study of tranpolapril (RU44570) in rats. J.Toxicol. Sci. 1993;18:37- 92.
18. Chawla R. Practical Clinical Biochemistry (Methods and Interpretations). Jaypee Brothers Medical Publishers, New Delhi, India, 1999;127-134.
19. Hotellier FP, Delaveau P. Nauclefine and

Naucletine constituents of Nauclea latifolia. Phytochem. 1975;14:1407 - 1411.
20. Toma I, Karumi Y, Geidam MA. Phytochemical screening and toxicity studies of the aqueous extract of the pods pulp of cassia sieberiana DC (Cassia Kotchiyana Oliv.). Afr. J. Pure. Appl. Chem. 2009;3(2):26 - 30.
21. Ramesh T,Lee K, Lee WH, Kim JS. Acute oral toxicity study of Asiasari radix extract in mice. Int. J. Toxicol. 2007;26: 247-251.
22. Sellers S.R, Mortan D, Michael B, Roome N, Johnson K.J, Yano L.B, Perry R, Schafer K. Organ weight recommendations for toxicology studies. Toxic. Pathol. 2007; 35: 751-755.

2/12/2010

Rapid leaf Area Estimation of *Crytorchid monteiroae*

Olosunde, M.A[1]. Dauda, T.O[2] and. Aiyelaagbe, I.O.O[1].,
1. Department of Horticulture
Unversity of Agriculturre, Abeokuta, Nigeria.
2. Institute of Agricultural Research and Training, Obafemi Awolowo University, PMB 5029, Moor Plantation, Ibadan, Nigeria.
taofikdaud@yahoo.com, taofik.iart@gmail.com

Abstract: Leaf area measurement of *Crytorchid monteiroae* was carried out using non –destructive methods at the University of Agriculture, Abeokuta, Nigeria in 2008. The objective of this study was to assess rapid leaf area estimation from both destructive and nondestructive sampling method for *Crytorchid monteiroae*. Leaf samples were randomly selected from lower, middle and upper parts of the plant. Leaf length, leaf width leaf dry weight and leaf area from the graphical method were determined. The results showed that leaf width has the minimum variance (2.083) while leaf length ***x*** leaf with had the maximum variance (428.497). Also, all the considered growth indices were directly and significantly correlated. Of the entire investigated model, cubic model of the relationships between leaf area and the leaf length ***x*** leaf width gave the best result in term of minimum residual variance and highest coefficient of determination.
[Olosunde, M.A. Dauda, T.O and. Aiyelaagbe, I.O.O. **Rapid leaf Area Estimation of *Crytorchid monteiroae***. Journal of American Science 2010;6(12):1549-1553]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words- Exponential model, cubic model, residual variance, monopodial

Introduction

Cytorchis monterioae is a member of the family Orchidaceae. It is one of the two members of the genus of fifteen species that are indigenous to Africa, including Nigeria and, found occasionally in cultivation for decorative, food and medicinal purpose (Arditti, 1992; Cullen, 1992). It is a monopodial epiphyte with long stem and leaves are in two ranks, thick, fleshy and leathery, oblong with unequal two lobes at their apices and usually folded when young.

Leaves normally represent a plant's assimilating area and determine its photosynthetic and dry matter production. Several methods used to determine leaf area of plants include using a planimeter (Nautiyal, *et al*,, 1990), tracing out on graph sheet (graph method), measuring of weight of leaves, length of midrib or width, and multiplying by width (Wahua, 1985; Aiyelaagbe and Fawusi, 1988; Aiyelagbe, 1990; Monterro *et al* 2000). Based on the relationship between the actual leaf area and the corresponding length of midrib, width of leaf dry weight or product of length and width, formulae for rapid determination of leaf had been suggested for Okra (Asif, 1977); watermelon (Oseni and Fawusi, 1984); pawpaw (Aiyelaagbe and Fawusi, 1988); Guava (Aiyelaagbe, 1990); pumkin (Salau and Olasantan,, 2004), and Queen of the Philippines (Olosunde, 2007). Leaf area is important in the study of physiological process of plants as well as in assessment of plant growth and development. Orchids are grown primarily as ornamentals and as cut flowers; they are multibillion dollar worth (Ugese, *et al*, 2008) industries in many countries of the world unlike in Nigeria exporters of potted orchids include Taiwan, Thailand, the United States (Law, 2002). Majority of cultivable orchids exist in the world in Nigeria (Vang and Cribb, 1983) and little work were known to popularize its cultivation and production for local and foreign markets which could enhance tourism in particular and Nigeria's economy in general. The development of an appropriate rapid nondestructive and accurate measurement of leaf area for *Cytorchis monteiroae* is a useful tool in agronomical and physiological studies for implementation of the domestication of the program and its establishment for commercial production for export and domestic uses. It is essential for sustainable production of the orchid through reduction of destruction of leave through sampling. Similarly, this work would provide an alternative method of leaf area determination where the graph method is unavailable its establishment for commercial production export and domestic uses. This study was undertaken to assess rapid leaf area estimation from both destructive and nondestructive sampling method for C.M

Materials and Methods.

The study was conducted in the year 2008 at the University of Agriculture, Abeokuta (7 15' N, 3 25'E) Ogun state, Nigeria Two methods, (nondestructive and destructive methods) of leaf area estimation. For leaf area measurements 200 matured- fully expanded, healthy leaves were randomly selected from the lower, middle and upper parts of the plant. In the non – destructive method, the length of midrib, the width

and the product of length and width of each of the 200 leaves were determined while for the destructive method, the leaf areas of the whole 200 leaves were estimated by graph paper tracing. The corresponding leaf dry weights were obtained after oven drying at 70 ^{0}C for 36hrs and weighed.

The data were subjected to descriptive statistics (like mean, variance and sums) and correlation analysis. Relationship between leaf area (by graphical method) and leaf length, leaf width, dry weight and leaf length ***x*** breadth was rapidly determined using the following regression models;

$$Y = \alpha + \beta x_1 + \gamma x_2 + \lambda x_3 ,$$

$$Y = \alpha + \beta \ln(x_1) + \gamma \ln(x_2) + \lambda \ln(x_3)$$

$$Y = \alpha + \beta x_1 + \gamma x_2 + \lambda x_3 + \delta x^2_1 + \tau x^2_2 + \eta x^2_3$$

	Dry weight	***Leaf Length***	***Leaf width***	***Length x width***	***Leaf area***
Dry weight	-	0.541**	0.160*	0.292**	0.778**
Leaf Length		-	0.137*	0.396**	0.867**
Leaf width			-	0.963**	0.193**
Lenght x width				-	0.414**
Leaf area					-

$$Y = f(\lambda^{x_i})$$

where Y = leaf area and x_i = leaf length, leaf width and leaf length x width.

The models were further subjected to analysis of variance to test for existence of significance difference in the means of the dependent and independent variables. Also, the co efficient of determination (R^2) as well as the variance of the residuals was computed.

Results and Discussion.

The descriptive statistics (Table 1) showed that mean leaf length, leaf width and length ***x*** width were respectively; 12.744cm, 2.124cm, and 37.651cm respectively. The variance ranges between 2.083 for width and 428.497 for leaf length *x* leaf width. It was observed that leaf width have minimum variance. The implication of this is that the variance is a function of the magnitude of the data. The correlation analysis returned correlation coefficient ranging between 0.14 (correlation between length and width) and 0.963 (correlation between width and interaction of length width). All the correlation coefficient were significant at 0.05 level of significance. Similarly, leaf length returned the highest correlation coefficient (0.867) with the leaf area while the least was the correlation between leaf area and leaf width (0.193). Neither negative nor zero coefficient was obtained (Table 2). The implication of this result is that all the growth indices are directly related though the relationship might be low.

Table 1. Descriptive statistics of the growth Indicies.

Parameter	**Mean**	**Variance**	**Minimum**	**Maximum**	**Sum**
Leaf Length	12.744	4.034	6.8	16.1	2548.8
Leaf width	2.924	2.083	1.9	22.5	584.7
Length x width	37.651	428.497	13.6	299.25	7530.24

The different models produced different sets of coefficient of determination (R^2) as well as of varying pattern.

Table 2. Correlation table for the growth indices.

For the models and for all plant indices, the cubic model ($Y = \alpha + \beta x + \lambda x^2 + \partial x^3$) gave the highest coefficient of determination (R^2). The implication is that the cubic model in each gives the highest predictive capacity. Each of the investigated models has different components and which are uniform across the different growth indices used in the models. The only exception is the cubic model which is not uniform across the growth indices (Table 3).

The linear model consists of 3 components;

- *Y*, the dependent variable,
- α , the constant/intercept and
- *x*, the independent variable

The quadratic model consists of 4 components which are;

- *Y*, the dependent variable,
- α , the constant/intercept
- *x*, the independent variable and
- x^2 the second order of the independent variable

The logarithmic models have 3 components;

- *Y*, the dependent variable,
- α , the constant/intercept and
- *ln x*, the natural log of the independent variable

The exponential models compose of,

- *Y*, the dependent variable,
- α , the constant/intercept and

- λ^x , the exponential of the independent variable
- For the cubic models,
- *Y*, the dependent variable,
- α , the constant/intercept and
- *x*, the independent variable
- x^2 the second order of the independent variable
- x^3 the third order of the independent variables.

In this cubic model, the coefficient of any of the components apart from the dependent and the constant were found to be zero for some indices (Table 3).

The F statistics for the different models of the growth indices ranged from 7.650 (for the linear relationship between leaf area and leaf width) to 855.624 (for the quadratic models of the relationships between leaf area and leaf length by leaf width).

All these F statistics were significant at 0.05 level of significant because they were all greater than their values at the different thresholds (Table 3). Similarly, the variance of the residuals ranged between 4.913 for the cubic model of the relationship between leaf area and leaf length by width and the 60.784 for the exponential models of the same relationship. The implication of this is that the cubic model of the relationship between leaf area and leaf length by width have the residuals with minimum variance. This model (cubic models of the relationship between leaf area and leaf length by width) in addition gave the highest coefficient of determination as well as a relatively high and significant F statistics. Based on these facts therefore, it is wise to choose the cubic model, $Y = -1.773 + 0.984x - 0.004x^2 + 0.00000239x^3$ to predict the leaf area of *Crytorchid monteiroae.*

The plot of the predicted values against the observed showed that the trend is random and non stationary. This showed that the error term would be random and thus plausibility of the model is implied. Model simulation for the chosen model (cubic model) showed that the validity range of this model is $1 < x = \infty$ (Figure 1B). At x = 1, the leaf area becomes negative and it is absurd. This model falls in line with Tsialtas and Maslaris (2008)'s quadratic model for estimating the leaf area of sugar beet. The model explored the relationship between the leaf length and leaf width to estimate the leaf area using quadratic model while this study uses cubic model for the same relationship. The difference in the model type could be related to: difference in the plant species used and No report of attempt to investigate the cubic model in Tsialtas and Maslaris (2008).

Similarly, Kathirvelan and Kaliselvan (2007) have explored the interaction between leaf length and width in the allometric relation between leaf area and linear measurement (length by width) of groundnut leaf.

From these facts, the importance of linear measurement (leaf length x leaf width) in leaf area estimation have been stressed. Also, the choice of the cubic model for the estimation of the leaf area for *Crytorchid monteiroae* is justified.

Conclusion.

1. This model (cubic regression model) developed in this study is adoptable for rapid and sufficient (that is with minimal residual's variance) estimation of leaf area otherwise known as adaxial leaf area.
2. This model provides a better estimation over the quadratic form that are widely in use for other plants because of its minimum variance as well as higher coefficient of variations (R^2).
3. Non destructive determination of the leaf area through this model assures sustainable experimental monitoring and research.
4. The model provides a good substitute where graphing method's materials as well as expertise are unavailable.

It is thus recommended that spatial and temporal dependence of the model be investigate in future research to determine its reliability across time and space.

Table 3. Summary of the different models, their coefficient of determination and their residuals' variances.

Leaf area against	Model	Constant	*x*	x^2	x^3	In *x*	λ^x	R^2	F	σ^2
Dry weight	Linear	5.846	65.323	-	-	-	-	0.606	304.457	18.770
	Logarithmic	53.453	-	-	-	22.973	-	0.616	317.269	18.303
	Quadratic	- 7.449	141.882	- 104.557	-	-	-	0.620	160.524	18.113
	Cubic	34.468	- 217.040	877.504	- 861.902	-	-	0.631	111.501	17.598
	Exponential	12.448	-	-	-	-	2.304	0.596	291.635	20.281
Leaf length	Linear	- 8.989	2.978	-	-	-	-	0.751	597.847	11.850
	Log	- 57.753	-	-	-	34.257	-	0.712	488.380	13.7403
	Quard	24.241	- 2.624	0.229	-	-	-	0.778	345.095	10.577
	Cubic	15.185	0	- 0.013	0.007	-	-	0.779	347.789	10.513
	Exp	7.126	-	-	-	-	0.108	0.777	690.0559	10.702
Leaf width	Linear	26.270	0.922	-	-	-	-	0.037	7.650	45.860
	Log	11.459	-	-	-	16.839	-	0.240	62.520	36.201
	Quad	- 13.677	17.031	- 0.673	-	-	-	0.534	112.795	22.205
	Cubic	- 9.384	13.776	-	-0.024	-	-	0.535	113.483	22.1327
	Exp	25.517	-	-	-	-	0.033	0.038	7.927	47.556
Length x width	Linear	23.767	0.138	-	-	-	-	0.172	41.011	39.459
	Log	- 37.557	-	-	-	18.628	-	0.652	371.603	16.557
	Quad	- 0.833	0.927	- 0.003	-	-	-	0.897	855.624	4.917
	Cubic	-1.773	0.984	-0.004	0.00000239	-	-	0.897	568.094	4.913
	Exp	23.347	-	-	-	-	0.005	0.174	41.822	60.784

NB: - implies not applicable for such model while "0" implied zero coefficient for such component.

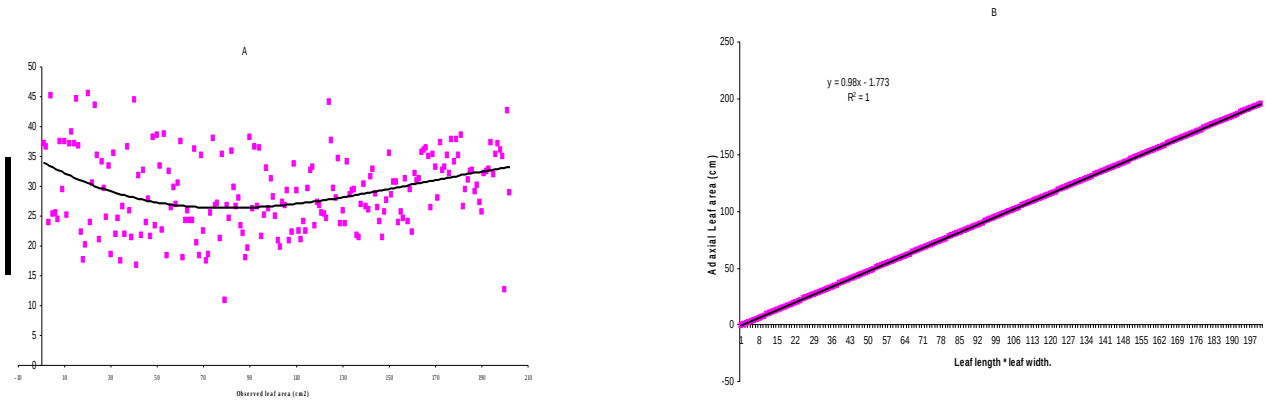


Figure 1. Predicted leave area against the observed leaf area (A) and Model Simulation for the cubic Model (B).

Reference:

1. **Aiyelaagbe I.O.O**. (1990). Rapid estimation of leaf area of Guava (Psidium guajava). *India journal of Agricultural Sciences* 60(8): 574.
2. **Aiyelaagbe I.O.O., Fawusi M.O.A.** Estimation of the area of detached or intact leaves of Papaya (Carica papaya), *India Journal of Agriculture Sciences* 1988:58(4): 192.
3. **Asif M.I.**. Estimation of leaf area of Okra (*Abelmoschus esculentus* (L) Moench). *Tropical Agriculture* 1977: 54 (2):192.
4. **Kathervelan, P. and Kalaiselvan, P**. Groundnut (*Arachis hypogae* L.) Leaf Area Estimation using allometric model in *Research Journal of Agriculture and Biological Sciences*, 2007; 3:1, 59 - 61.
5. **Montero, F. S., Dejuan, J. A., Guesta. A. Brasa A.**. Non destructive methods of Estimate leaf area of Vitis vinifera L. *HortScience* 2000: 35 (4) 696-698
6. **Nautiyal M.C., Singh P.K., Shukla R.N., Prakash. S. Kumar. A.** 1990. Correcting Leaf Area Measurement by Conventional Methods; A New Approach for Apple (Malus domestica Borkh). J. of *Horticultural Science* 1985:65(1): 15-18.
7. **Olosunde, O.M.** Rapid leaf area measurement method for Queen of the Philippines (*Mussaenda philippica* A. Rich) ASSET Series A (2007) 7(1): 152-158
8. **Oseni, T.O., Fawusi M.O.A.**. Rapid Determination of Leaf Area of Intact Leaves of Watermelon. *Indian Journal of Agricultural Science* 1984: 54 (11):1009.
9. **Salau, A. W., Olasantan, F. O.** 2004. Rapid leaf Area Estimation of Pumpkin (Cucurbita maxima). *ASSET Series A.* 2006: 6(1):255-258.
10. **Tsialtas, J.T. and Maslaris, N**. Leaf area prediction model for sugar beet (Beta vulgaris L.) cultivars. In *Biomedical and Life Sciences Journal*, 2008:.46 (2); 291 - 293.
11. **Ugese, F. D., Bayeri, K.P. and Mbah, B.N**. Leaf area determination of shea butter tree (*Vitellaria paradoxa* C.F. Gaertn.) in *International Agrophysics*, 2008: 22: 167 - 170.
12. **Wahua, T.A.T**. Effect of Melon (Colocynthis Vulgaris) population density on intercropped maize (Zea mays) and melon. *Experimental Agriculture*, vol. 21, pp 281 – 289.
13. **Wahua T.A.T.** *Applied Statistics for Scientific Studies*, African Link Books. 1999: 282 –283.

Molecular analysis of monoclonal antibodies specific to *Cucumber mosaic virus* coat protein: restricted light chains and alternative heavy chain partners

Haggag S. Zein[1,3] , Jaime A. Teixeira da Silva[2] and Kazutaka Miyatake[3]

[1.] Department of Genetics, Faculty of Agriculture, Cairo University, 12613, Egypt
[2.] Faculty of Agriculture and Graduate School of Agriculture, Kagawa University, Miki-cho, Ikenobe 2393, Kagawa-ken, 761-0795, Japan
[3.] Department of Applied Biological Chemistry, Graduate School of Agriculture and Biological Sciences, Osaka Prefecture University, 1-1, Gakuen-Cho, Sakai, Osaka, 599-8531, Japan
haggagsalah@gmail.com

Abstract: A total of 10 hybridomas were generated from five fusions of BALB/c mice immunized with *Cucumber mosaic virus* coat protein (CMV-CP) subgroup I. A modified reverse-transcriptase PCR protocol was used to amplify and sequence the V-genes' light and heavy chains. Database analysis of the sequences that encoded the V-genes showed that the light chains of the 10 hybridomas expressed the family Vκ2 gene, bd2, while four of the heavy chains genes expressed four genes of the V_H1/J558 family, three of V_H5/V_H7183, and three of V_H8/V_H3609. There was frequent addition of the N region and expected variation in the lengths of CDR3 regions which form the center of the antigen binding site. Somatic mutation, junctional diversity and alternative light chains collectively impart specificity to these serologically distinct epitopes. Apparent dissociation constants (K_d) of mAbs were determined by direct ELISA yielding K_d = 3.0-38.5 nM. We conclude that high-affinity CMV-binding antibodies can arise without extensive somatic hypermutation in the variable-region genes because of the expression of appropriate HCDR3s. This information permits analysis, not previously possible, of the relationship between antibody H and L chain genes and the antigenic domains on antigen. Knowledge of the specific immunoglobulin genes for common epitope may lead to insights on pathogen-host co-evolution and helps blocking the virus infections in plants

[Haggag S. Zein, Jaime A. Teixeira da Silva and Kazutaka Miyatake. **Molecular analysis of monoclonal antibodies specific to *Cucumber mosaic virus* coat protein: restricted light chains and alternative heavy chain partners**. Journal of American Science 2010;6(12):1554-1564]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Monoclonal Antibodies; *Cucumber mosaic virus*; antibody genes; affinity; ELISA

1. Introduction

Cucumber mosaic virus (CMV) is the type species in the genus *Cucumovirus*, family *Bromoviridae*. CMV has the broadest host range of any known virus, infecting more than 1,000 species of plants, including monocots and dicots, herbaceous plants, shrubs, and trees (Palukaitis et al. 1992). The antigenic structures of many plant viruses have been investigated through the identification of epitopes recognized by monoclonal and polyclonal antisera, which has been accomplished largely through the use of synthetic peptides, although early work was done with coat protein (CP)-derived peptide fragments and some information has emerged from sequence and mutational analysis. Serologically, the two subgroups are closely related, as shown by the cross reactions of polyclonal antibodies. Some monoclonal antibodies produced against the CPs of subgroups I and II can differentiate the two, indicating the presence of unique epitopes for each (Zein et al. 2007). Antibody genes from hybridomas, as well as diverse repertoires of antibody genes from immunised and non-immunised donors have been displayed in this way (Marks and Marks, 1996). Accurate diagnosis depends on the affinity and specificity of the antibody preparation used, and high affinity antibodies are essential for the detection of very small amounts of pathogen. Monoclonal and recombinant antibodies can be produced in potentially unlimited quantities, and the epitopes with which they react can be identified, thus making well characterised preparations of these reagents the preferred choice for inclusion in standard assays. Also, assay specificity can be designed to detect one or more antigens by incorporating several different antibodies (Zein and Miyatake, 2007). Previously, the molecular structure of CMV was determined at 23 Å resolution by cryoelectron microscopy and image reconstruction. In examining which capsid protein domains are clearly exposed on the virus, Liu et al. (2002) noted that the amino acid sequence of the βH-βI loop forms a conspicuous, negatively charged

electrostatic field on the surfaces of virions (Liu et al. 2002) and has fundamental aspects that are conserved among cucumoviruses: its structure plays a role in aphid vector transmission (Perry et al. 1998), A prominent feature on the surface of the *Cucumber mosaic virus* (CMV) capsid is a negatively charged loop structure, the βH-βI loop. Six of eight amino acids in this protein loop are highly conserved among strains of CMV and other members of the cucumovirus family. Liu et al. (2002) individually changed five of these amino acids to alanine or lysine (positively charged), creating nine mutants. The mutants formed virons and accumulated to wild-type levels, but eight of the nine mutants were defective in aphid vector transmission. Since the disruption of charged amino acid residues in the βH-βI loop reduces or eliminates vector transmissibility without grossly affecting infectivity or viron formation, they proposed that this sequence or structure has been conserved to facilitate aphid vector transmission. The long-term goal of generating CMV-resistant transgenic plants using antibody genes depends on the molecular basis of antibody structure and diversity. Knowledge of the molecular structure of epitopes provides the possibility to predict *in silico* the cross-reactivity of antibodies.

The epitopes can directly be used to screen the databases for identical or similar sequences, either by performing a general search or a specific search among family members. Antibody-based resistance is a novel strategy for generating transgenic plants resistant to pathogens (Schillberg et al. 2000). Ectopic expression of recombinant antibodies (rAbs) has great potential to prevent viral infection. The strategy of using antibodies to increase pathogen resistance has been successfully demonstrated for human viruses (Marasco 1995) but the application in plant virology has been limited. Recent advances in gene isolation and an understanding of 'antibody-based resistance', an approach in which expressed antibodies bind to essential proteins, were used to interfere with pathogenesis. The introduction of antibodies is an attractive alternative and effective resistance against a number of plant viruses has been demonstrated (Zimmermann et al., 1998) as well resistance against other phytopathogens including fungi (Peschen et al., 2004).

The aim of this work was to examine the molecular structure, in particular, the nucleotide sequences of the heavy and light chain genes which are specific to CMV-coat protein (CP) specific for subgroup I, and their respective affinity. We also attempt to understand the antibody structure and the immunoglobulin genes that encode the biding sites of antibodies against CMV-CP, the high affinity heavy V_H and light V_L chains variable regions (Fab) of these antibodies for long-term CMV resistance of important transgenic crop plants.

2. Material and Methods

2-1 Plant material and virus purification

Tobacco plants (*Nicotiana tabacum* cv. 'Xanthi-nc') and *Nicotiana benthamiana* plants at the five-leaf stage were used for inoculation. The pepo strain of CMV (subgroup IA) was originally obtained from *Cucurbita pepo* in Japan; CMV propagated in tobacco was purified as described by Nitta *et al.* (1988). Plants were inoculated mechanically with purified CMV strain pepo, diluted to a final concentration of 50 μg ml^{-1} in 100 mM phosphate buffer, pH 7.0. Inoculated plants were grown in a growth chamber (NK systems) at 24°C with a 14 h light/10 h dark cycle.

2-2 Immunization

Immunized eight-weeks old BALB/c mice (Nippon SLC Co., Japan) were injected subcutaneously with 100 μg of purified CMV strain pepo in 0.1 ml phosphate-buffered saline (PBS; 0.01 M phosphate and 0.015 M sodium chloride, pH 7.5), which was mixed with an equal volume of adjuvant containing TDM plus MPL (Sigma). Three injections were administered at two-week intervals. Three days after the fourth injection, the mice were given a peritoneal injection of 200 μg of virus in 0.2 ml PBS. The mice were sacrificed three days later and their spleens were harvested. Fusion experiments were carried out in which lymphocytes from the spleens of the immunized mice were mixed at a 5:1 ratio with non-secreting P3X63-Ag8-U1 myeloma cells in polyethylene glycol 6000 (50%, w/v). The cells were distributed to 96 well plates at a concentration of 10^5 cells/well with HAT medium (100 μM hypoxanthine, 0.4 μM amino protein, 16 μM thymidine, 6 mM Hepes, and 200 μM 2-mercaptoethanol). Clones that successfully secreted antibodies specific to CMV were examined by both ELISA and western blotting. Furthermore, these positive hybridoma cells were subcloned by a limiting dilution method in the presence of thymocytes of BALB/c mice as feeder cells according to standard protocols (Harlow and Lane, 1988).

2-3 Production of monoclonal antibodies (mAbs)

mAbs were produced following the intraperitoneal injection of 10^7 hybridoma cells into intraperitoneal cavities of BALB/c mice primed two-weeks previously with 0.5 ml pristine (2, 6, 10, 14-tetramethylpentadecane), and the antibodies were

purified from the isolated ascetic fluid by affinity chromatography protein.

2-4 Preparation of IgG

The immunoglobulin (IgG) fraction was separated from ascitic fluid by using protein A affinity purification kit (Bio-Rad, Hercules, CA). Ascitic fluid was diluted with binding buffer 1:2 (v/v), centrifuged, and filtered through a PF syringe filter, 0.2 μm Acrodisc. Nine milliliters of the diluted ascitic fluid was applied to a column filled with 3 ml of sorbent and then allowed to flow through the chromatographic column with immobilized protein A. The column was washed with 10 bed volumes of washing buffer, eluted with 3-4 bed volumes of eluting buffer, and neutralized with Tris-HCl buffer. After dialysis against PBS buffer, the IgGs were incorporated into indirect ELISA to establish assay parameters of the respective antibodies.

2-5 Determination of the real binding Affinity mAbs to CMV-CP

The binding affinity of each mAb was determined by virus inhibition, with each mAb used at an appropriate concentration that gave 50% maximal binding (Fujii et al., 1998). Diluted optimum concentrations were prepared: mAbs-(4, 5, 8, and 52) used 100 ng/ml; mAb-(6 and 7) used 200 ng/ml; mAb-8 used 400 ng/ml. These dilutions were preincubated for 2 h at room temperature (RT) with an equal volume of buffer containing a range of CMV-CP concentrations (1, 3, 30, 90 μg/ml). Competitive ELISA was performed on 96-well microtiter plates coated with CMV-CP at a constant concentration (1 μg/ml in carbonate buffer (100 μl/well) at 4°C overnight. Wells were aspirated and remaining free sites on the microtiter plates were then saturated using 1% Block Ace (Nippon SLC Co., Japan) in TBS (200 μl/well), and incubated for 2 h at RT. Using polypropylene tubes, the CMV-CP was concentrated to(1, 3, 30, 90 μg/ml) into optimum concentrations of antibody solution, and the amount of free mAb in the antibody inhibitor mixture was placed for 30 min at RT using CMV-precoated plates. The average mAb affinity was calculated according to the method of Bobrovnik (2003).

1-6 RNA isolation and cDNA synthesis

Total RNAs prepared from about 10^7 hybridoma cells were cultured in minimum essential medium containing 15% (v/v) fetal bovine serum, 2% (v/v) glutamine (200 mM), and 1% (v/v) gentamicin (10 mg/ml) in a 5% CO_2 humidified incubator (BIO-RAD) using ISOGEN RNA extraction buffer (Nippon Gene Co., Tokyo, Japan). Chloroform was added, followed by vigorous agitation, and incubated at RT for 2-5 min, centrifuged, and the upper aqueous phase was retrieved then incubated with isopropanol at RT for 10 min to precipitate the RNA. The RNA pellet washed with 75% ethanol, air-dried, and dissolved in 0.1% diethylpyrocarbonate water (Sigma). RNA concentration and purity were gauged using $OD_{260/280}$. The mRNAs were isolated on Oligotex-dT30 (Super) columns (Takara, Kyoto, Japan), as specified by the manufacturer's instructions. The primers used in PCR amplification were based on data by Huse et al. (1989): for V_H, 5′-AGGTCCAACTG**CTCGAG**TCAGG-3′ and 5′-AGGCTT**ACTAGT**ACAATCCCTGGGCACAAT-3′, where the underlined portion of the 5′ primers incorporates an *Xho*I site and that of the 3′ primer an *Spe*I restriction site. Primers for the Vκ genes were 5′-CCAGATGT**GAGCTC**GTGATGACCCAGACT-CCA-3′ and 5′-GCGCCG**TCTAGA**ATTAACACT-CTTCCTGTTGAA-3′ where the underlined portion of the 5′ primer incorporates a *Sac*I restriction site and that of the 3′ primer an *Xba*I restriction site for amplification of the Fd and κLc regions, respectively. First-strand cDNA was synthesized from mRNA template with the Moloney murine leukemia virus M-MLV Reverse Transcriptase kit (Takara, Kyoto, Japan) using oligo-dT20 primers (Pharmacia Biotech). Variable heavy and light chains were amplified from first-strand cDNA using Ex-*Taq* DNA polymerase with 30 cycles of PCR (1 cycle = 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C) in 50 μl of the following reaction mixture: 78 mM Tris-HCl (pH 8.8), 17 mM $(NH_4)_2SO_4$, 10 mM β-mercaptoethanol, 2 mM $MgCl_2$, 0.05% W-1 detergent (Takara, Kyoto, Japan), 0.2 mg of BSA/ml, 200 mM each of dATP, dCTP, dGTP, and dTTP, 1 mM of each primer, 10 ng of cDNA, and 2.5 U of EX*Taq* DNA polymerase (Takara, Kyoto, Japan). The PCR products were analyzed on a 2% low-melting-point agarose-Tris acetate-EDTA (TAE) gel and visualized with ethidium bromide. PCR products of expected size about 650 bp were excised from the gel and purified with a QIAGEN gel extraction kit as specified by the manufacturer. The amplified fragments were cloned into pGEM-T Easy Vector (1:1, 3:1, 10:1) respectively, according to manufacture protocol (Promega, Biotech) and ligated with Ligation High Kit (Takara, Kyoto, Japan), for the purpose of transforming into competent *E. coli* DH5α cells.

2-7 DNA sequence of variable region of the heavy and light chain genes

Direct sequencing of the treated DNA fragments was made using M13 primer and ABI PRISM BigDye Primer Cycle Sequencing Kit reagent following the manufacturer's instructions (Applied Biosystems) and run on an ABI Prism 310 Genetic

Analyzer (Applied Biosystems) using ABI Prism Sequencing Analysis 3.7 software for data analysis. The PCR product was analyzed and sequenced using M13 primer sequencing of the V regions. Cyclic sequencing of these DNAs was performed in both directions using a commercial kit (Thermo Sequence kit, Amersham Pharmacia Biotech), M13 forward (5′-CACGACG-TTGTAAAAACGAC-3′) and reverse (5′-GGATAACAATTTCAC-ACAGG-3′) primers set (Pharmacia, Biotech). Fd or Lc sequences were "blasted" against the publicly accessible "Ig-Blast" database of mouse Ig sequences at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/igblast) to determine the closest germline gene of origin, and to identify potential mutations. CDR position and numbering adopted Kabat numbering (Martin, 1996) and CDR definition was adopted from Andrew's web site (www.bioinf.org.uk/abs/).

3. Results

3-1 Production and characterisation of CMV-CP specific mAbs

Immunization of nonautoimmune BALB/c mice with native CMV-CP–stimulated antibodies was intriguing. Hybridoma technology allows the production of hybrid cell lines from B cells that secret a single monoclonal antibody with specific binding, and can potentially produce unlimited quantities. The serological differentiation of CMV isolates is important in plant breeding for disease resistance and to study disease epidemiology. Specific mAbs have been developed, and one of the goals in their development being to provide tools for serotyping viral isolates. mAbs with serotype specificity have been previously described (Hsu et al. 2000). The antibodies bind a broad variety of antigens with high affinity and specificity and the structural information about the molecular interactions between them helps us to understand the effect of mutations on the affinity and specificity of the antibodies.

In this study, 10 mouse hybridoma cell lines secreting monoclonal antibodies specific to CMV-CP subgroup I were established, and the immunoglobulin classes and subclasses for each were determined (Table 1). The antibodies were produced in ascites fluids in mice, and the optimum dilution of the antibody solutions for use in ELISA ranged from 0.05 to 0.4 µg/ml (Table 1). In testing the recognition specificity of the mAbs, the binding affinity and specificity of each mAb were examined with ELISA and western blotting (data not shown).

3-2 Measurement of the Affinity constant of mAbs to CMV-CP by ELISA

The affinity constant of MAbs to CMV-CP was measured by ELISA binding specificity by demonstrating that soluble CMV-CP inhibited mAbs interactions with immobilized CMV. The mAb was incubated in solution with the antigen until equilibrium was reached. Then, the antibody, which remains free at equilibrium, was captured by binding to antigen on the microtiter plate and measured by classical indirect ELISA. The binding constant was calculated from the experimental curve with equation [1/Ai vs. (A0-Ai)* Li] plot linear regression analysis according to Bobrovnik (2003) (Figure 1). Two mAbs, -5 and -8, showed the highest binding activity, 3.0 and 5.0 $\times 10^{-9}$, respectively while the binding activity of mAbs-(4, 52, 521, and 6) against CMV-CP was 1.36, 1.43, 2.7, and 3.85 $\times 10^{-8}$, respectively (Table 1). This method was valuable for measuring true dissociation and was found to be simple, reproducible, and accurate. It is therefore possible to assess the contribution of different germlines in the assay by affinity maturation even when they have

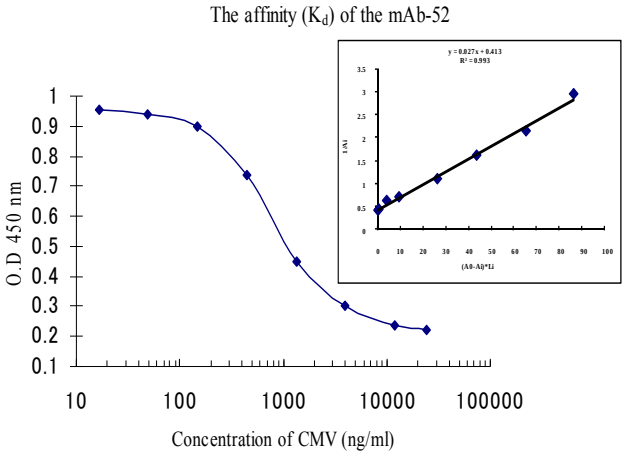


Figure 1. Competitive-ELISA for measuring the binding affinity of CMV-specific mAb-52. Different concentrations of CMV were incubated with a constant concentration of MAb-52 (0.45 µg/ml). The binding constant was calculated from the experimental curve with equation [1/Ai vs. (A0-Ai)* Li] plot linear regression analysis according to Bobrovnik (2003).

A. V_H-8/V_H 3609.CB17

```
          ←--------------HFW1-------------><-HCDR1->←----HFW2-----><------HCDR2-----><---------HFW3--
                                      31! 35!ab             50!             !65
      CB17  QVTLKESGPGILQPSQTLSLTCSFSGFSLS TSNMGIG WIRQPSGKGLEWLA HIWWNDDKYYNPSLKS RLTISKDTSNNQV
       VH6  ............................. ..G..V. ............. ......S.....A... ........Y....
       VH7  ............................. ..G..V. ............. ..L...S.....A... ........Y....
     VH122  ............................. ..D..V. .....P.......L ..L...S.....A... ........Y....
             --------------------><---------HCDR3--------><---HFW4--->
            82!  abc      90!        100!abcdefghijk    !102
      CB17  FLKITTVDTADTATYXXXX N         D      J
       VH6  ....AS.........YCAR MGVI YYYGSSYVGY- FDV WGQGTTLTVSS
       VH7  ....AN..............  ....  ...........  ...  ...........
     VH122  ....AN..............  ....  ...........  ...  ...........

B. VH-5/VH7183.68-5N
          ←--------------HFW1------><-HCDR1->←----HFW2-----><------HCDR2-------><---------HFW3-------
                                      31!   !35             50! a!             !65
      68-5N      GLVQPGGSLKLSCAASGFTFS SYGMS WVRQTPDKRLELVA TINSNGGSTYYPDSVKG RFTISRDNAKNTLYLQMSS
       VH-8  ESGG..................... ..... ............... ................. ...................
              -------------><---HCDR--->←---HFW4--->
            82! abc 90!    95!               !102
      68-5N  LKSEDTAMYYCAR N   D      J
       VH-8  .............  EE YY GKAWFVY WGQGTLVTVSA

C.VH-5/VH7183.14
            ←--------------HFW1-------------><-HCDR1->←----HFW2-----><------HCDR2-----><---------HFW3----
                                      31!    !35            50! a!              !65
   VH7183.14  EVKLVESGGGLVKPGGSLKLSCAASGFAFS SYDMS WVRQTPEKRLEWVA TISSGGSYTYYPDSVKG RFTISRDNARNTLY
       VH5  ............................. ..... ............... ......GS......... .........K....
       VH52 ............................. ..... ............... Y.....GS.....T... .........K....

            ------------------->←-----HCDR----><---HFW4---->
            82! abc       90!     95!              !102
   VH7183.14  LQMSSLRSEDTALYYCAR N   D      J
       VH5  ......K.....M..... EE YY GKAWFVY WGQGTLVTVSA
       VH52 ......K.....M..... .G .. NGYEGD. .....S....S

D. VH-1/VHJ558.104B
            ←--------------HFW1-------------><-HCDR1->←----HFW2-----><------HCDR2--->←---------HFW3----
                                      31!   !35              50! a!              !65
     VH104B  QVQLQQPGSVLVRPGTSVKLSCKASGYTFT SYWMH WAKQRPGQGLEWIG EIHPNCGNINYNEKFKG KATLTVDTSSSTAY
       VH-11  ............................. ..... .V............ .....S........... ..............
            ------------------><-HCDR--><---HFW4--->
            82! abc        90!    95!         !102
     VH104B  VDLSSLTSEDSAVYYCAR N   D  J
       VH-11  ................I GG YY FDY WGQGTTLTVAS

E.VH-1/VHJ558.45
            ←--------------HFW1-------------><-HCDR1->←----HFW2-----><------HCDR2--->←---------HFW3----
                                      31!   !35              50! a!              !65
     J558.45  QVQLQQSGAELAKPGASVKMSCKASGYTFT SYWMH WVKQRPGQGLEWIG YINPSTGYTEYNQKFKD KATLTADKSSSTAY
       VH-521 .....E..P..................... K.... .............. .....S...D......G .....V........
              ------------------->←-HCDR-->←---HFW4--->
             82! abc        90!    95!         !102
     J558.45  MQLSSLTSEDSAVYYCAR N   D   J
       VH-521 .E.H...........TN   PYY RYDY WGQGTTLTVSS

F. VH-1/VHJ558.51
            ←--------------HFW1-------------><-HCDR1->←----HFW2-----><------HCDR2--->←---------HFW3--
                                      31!   !35              50! a!            !65
     J558.51  QVQLQQSGAELVRPGASVTLSCKASGYTFT DYEMH WVKQTPVHGLEWIG AIDPETGGTAYNQK FKGKATLTADKSSSTAY
       VH4  ..................K...R.LI.... ...V. .............. ..L.GN.N.....................
       VH9  ..................K...R.LI.... ...V. .............. ..L.GN.N.....I .................
              ------------------><--HCDR--->←---HFW4--->
            82! abc        90!    95!       !102
     J558.51  MELRSLTSEDSAVYYCTL N   D   J
       VH4  ...S............N   PYY RFDY WGQGTTLTVSS
       VH9  ...S............N   ... .Y.. ...........
```

Figure 2. Multalignments of the amino acid sequences of V_H genes with germline gene in GenBank database. (A) Alignment of amino acid of V_H genes of the mAbs-(6, 7, and 122) with the most closely related germline family V_H-8/V_H 3609, gene CB17. (B) Alignment of amino acid of V_H genes of mAb-8 with the most closely related germline family V_H-5/V_H7183, gene 68-5N. (C) Alignment of amino acid of V_H genes of the mAbs-(5 and 52) with the most closely related germline family V_H-5/V_H7183, gene 14. (D) Alignment of amino acid of V_H genes of mAb-11 with the most closely related germline family V_H-1/V_HJ558, gene 104B. (E) Alignment of amino acid of V_H genes of mAb-521 with the most closely related germline family V_H-1/V_HJ558, gene 45. (F) Alignment of amino acid of V_H genes of mAbs-(4 and 9) with the most closely related germline family V_H-1/V_HJ558, gene 51. A dot in the individual sequences denotes amino acids that are the same as the consensus. A dash in the individual sequences denotes a deletion. The framework and complementarity determining regions (CDR) are indicated above the appropriate sequence segments. The numbering of amino acid residues is according to Kabat (1991).

Fig. 3

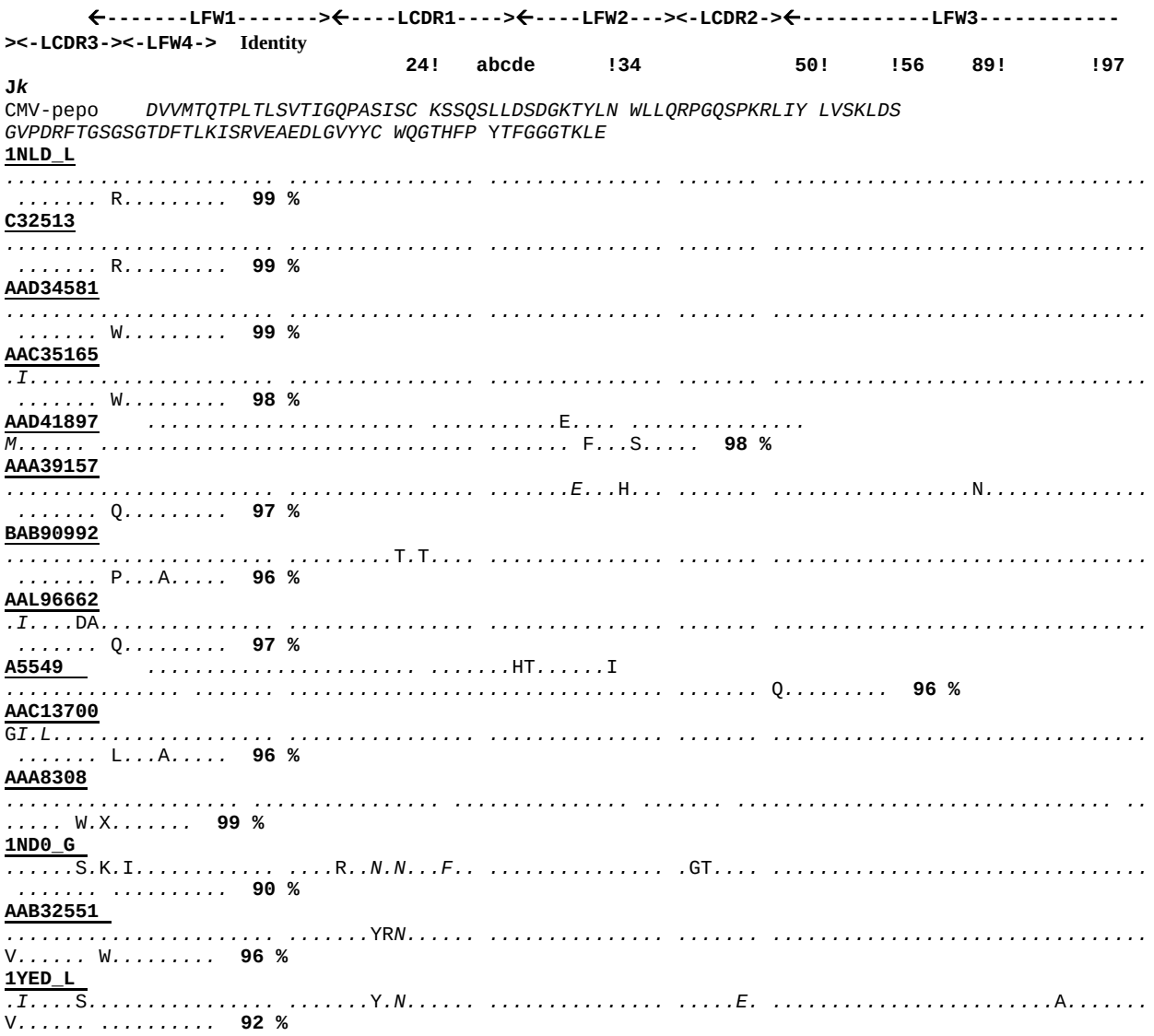


Figure 3. Multialignments of the amino acid sequence of light chain gene of CMV-specific mAbs with GenBank database VL accession numbers. Dots represent residues identical to the corresponding germline. The numbering of amino acid residues is according to Kabat (1991).

Table 1. The real affinity of CMV-CP specific mAbs with indirect competitive ELISA

Fusion	Clones	Immunogen	Optimum μg/ml	Affinity [a] (Kd) M
1	4	Pepo	0.20	1.36×10^{-8}
	9		nd	nd
	11		nd	nd
	521		0.10	1.43×10^{-8}
2	5	Pepo	0.05	3.0×10^{-9}

	8		0.10	5.0×10^{-9}
	52		0.45	2.7×10^{-8}
3	6	Pepo	0.2	3.85×10^{-8}
	7		nd	nd
	122		nd	nd

[a] The specific reactivity of the mAbs to CMV-CP. The affinity of the MAbs was determined by indirect enzyme linked immunosorbent assay as described in the materials and methods.

3-3 Utilization of the V gene segments of the H and L chain genes

The V_H and Vκ regions of 10 CMV-specific mAbs generated from three different fusions of BALB/c mice were sequenced. These sequences were almost homologous with corresponding germline genes published in the GenBank database, outlined in Table 2, which summarizes the V_H, D, and J_H fragments of variable heavy chain genes and Vκ, and Jκ of variable light chain genes. The nucleotide and deduced amino acid sequences of the expressed light chain germline gene assignments confidently to very restricted germline family Vκ2, gene bd2 (ten mAbs), GenBank accession nos. (**EF672211**, **EF672212**, **EF672213**, **EF672214**, **EF672215**, **EF672216, EF672217, EF672218**, **EF672219**, and **EF672220)** (Table 2). The identity of the V genes used was determined by searching the GenBank database for homologies to known V genes using the BLAST protocol (Altschul et al., 1997). On the other hand, the nucleotide and deduced amino acid sequences of the expressed V_H genes of the 10 anti-CMV antibodies are shown in Figs. 2, 3, and 4. The V_H genes belong to the following GenBank accession nos.: V_H1/V_HJ558 (four antibodies) (**EF672206**, **EF672197**, **EF672202**, **EF672203**); V_H5/V_H7183 (three antibodies) (**EF672198**, **EF672205**, **EF672201**); V_H8/V_H3609 (three antibodies) **(EF672199**, **EF672200**, **EF672204**) (Table 2). In addition, the V_H genes of the IgG antibodies were more somatically mutated. D segment usage also appears to be restricted with 7 mAbs of V_H using the DSP2 segment, while three mAbs were used for another segment, DFL16 (Figs 2, 3, and 4). On the other hand, it does not appear to be an obvious restriction in J_H segment usage. Interestingly, most antibodies could group into three sets based on their use of the same or highly similar V_H and V_L genes. Gene rearrangement entails the joining of heavy-chain V, D and J germline genes followed by the joining of light-chain V and J genes. The heavy chains belonged to three different families classified into three subgroups. The first included four mAbs (4, 9, 11, and 521) and belongs to the V_HJ558 germline family with different genes; the homology of the amino acid sequences were: $V_HJ558.51$ (89%), $V_HJ558.51$ (93%), $V_HJ558.45$ (94%), and V_H104B (99%) (Table 2; Cohen and Givol, 1983; Haines et al., 2001). The second subgroup included three mAbs-(5, 8, and 52) whose V_H gene segments were from the V_H7183 germline family (Gubbins et al., 2004). The mAbs-(5 and 52) V_H genes were derived from the same germline gene $V_H7183.14$ with 97 and 95% amino acid homology, respectively (Table 2; Chukwuocha et al., 1994). The third subgroup included mAbs-(6, 7, and 122) V_H genes which were derived from the same V_H3609 germline family, CB17H.10 gene (Gubbins et al., 2004) with 96, 96, and 95% homology, respectively (Table 2).

3-4 Somatic mutation and affinity maturation

Based on the sequence analyses of V genes in specific acquired immune responses to foreign antigens, somatic hypermutations were found to occur mainly in complementarity-determining regions (CDR) of V genes during the process of affinity maturation. The combined processes of immunoglobulin gene rearrangement and somatic hypermutation allowed for the creation of an extremely diverse antibody repertoire. V_H-521 showed 16 mutations, five of which were silent, while 11 others led to the mutation of amino acid no. 6 glutamine in germline to glutamic acid ($Gln6^HGlu$); $Ala9^HPro$; $Ser31^HLys$; $Thr54^HSer$; $Glu58^HAsp$; $Asp65^HGly$; $Ala71^HVal$; $Gln80^HGlu$; $Ser82^HHis$; $Ala94^HThr$; and $Arg95^HAsn$ (Fig. 2F). V_H-(4 and 9) showed 18 mutants, 7 silent and 11 amino acid replacements: $Thr19^HLys$; $Lys23^HArg$; $Ser25^HLue$; $Gly26^HIle$; $Met34^HVal$; $Asp52^HLue$; $Glu53^HGly$; $The54^HAsn$; and $Gly56^HAsn$; $Arg82A^HSer$; and $Lue94^HAsn$. The only difference between two antibodies is a one-point mutation in the V_H gene in CDRH2 $Lys65^HIle$ and another in the DSP2 segment of $Phe99^HTyr$ (Fig. 2H). In contrast, V_H-11 reveled only two substitutions, the first in CDRH2 with $Cys54^HSer$ and the second in FW3 with $Arg94^HIle$ (Fig. 2D). V_H-5 revealed 7 mutants: 2 were silent and 5 were substitutions: $Ser55^HGly$; $Tyr56^HSer$; $Arg75^HLys$; $Arg83^HLys$; $Lue89^HMet$. V_H-52 revealed 10 mutations: 3 were silent and 7 were substitutions, 5 being typical as Fd-5 with two more substitutions; $Thr50^HTyr$ and $Ser62^HThr$ (Fig. 2C). V_H-6 has 10

mutants, 3 silent and 7 substitutions: Asn33^{H}Gly; Ile35A^{H}Val; Asp56^{H}Ser; Ser62^{H}Ala; Ser74^{H}Tyr; Thr82A^{H}Ala; and Thr82B^{H}Ser. V_H-72 showed 11 mutants, 3 silent and 8 substitutions, similar to Fd-6 substitutions, except for Trp52^{H}Lue and Thr82B^{H}Asn. V_H-122 showed 13 mutants, 3 silent and 10 substitutions, similar to Fd-72, except for Asn33^{H}Asp and Ser41^{H}Pro; Ala49^{H}Lue. As the frequency of the PCR error used in this study was one in 5000-10000 nucleotides, the intraclonal sequence heterogeneity observed here might not be derived from PCR errors.

3-5 CDR3 length, D regions, and number of N insertions

The length of the H-CDR3 varied from 27 nucleotides in mAb-4 to 51 nucleotides in mAb-6 (Table 3). It has been suggested that the presence of Tyr and Trp residues in H-CDR3 confer flexibility upon the Ab molecule. Consequently, V_H-(6, 7, and 122) has five Tyr residues in this region, while the other V_H has three (Table 3). There are different D and J_H regions used in the CMV-specific V_H and the number of N insertions between these regions (Table 3). On the basis of N insertions at both the V-D and the D-J_H junctions, a third subgroup, V_H showed 10 nucleotides on the V_H-D side and three nucleotides on the other side, D-J_H. V_H-(5 and 8) showed 6 and 4 nucleotides on the V_H-D side, respectively, while only one nucleotide on the D-J_H side. V_H-52 showed 7 nucleotides on the V_H-D side and 5 nucleotides in the D-J_H side. V_H of the first subgroup showed only one sided V_H-D, with 7 or 5 nucleotide insertions.

Table 2 Summary of variable region gene V, (D), and J genes of CMV-CP specific mAbs.

Heavy Chain								Light Chain				
Accession Number	**Clone**	**Isotype**	**V_H**	**Germline gene**	**Homology germline (%)**	**D gene**	**J_H**	**Accession Number**	**V_K**	**Germline gene**	**Homology Germline (%)**	**J_K**
EF672206	521	IgG1	J558	J558.45	94	DSP2.11	2	EF672220	V_K2	bd2	99	2
EF672197	4	IgG1	J558	J558.51	94	DSP2.11	2	EF672211	V_K2	bd2	100	1
EF672202	9	IgG1	J558	J558.51	93	DSP2.11	2	EF672216	V_K2	bd2	99	2
EF672203	11	IgG1	J558	VH104B	99	DSP2.9	2	EF672217	V_K2	bd2	100	2
EF672198	5	IgG1	7183	7183.14	97	DSP2.7	3	EF672212	V_K2	bd2	100	2
EF672205	52	IgG1	7183	7183.14	95	DFL16.2	4	EF672219	V_K2	bd2	98	1
EF672201	8	IgG1	7183	68-5N	100	DSP2.7	3	EF672215	V_K2	bd2	100	2
EF672199	6	IgG1	3609	CB17H.10	96	DFL16.1	1	EF672213	V_K2	bd2	98	1
EF672200	7	IgG2b	3609	CB17H.10	96	DFL16.1	1	EF672214	V_K2	bd2	99	1
EF672204	122	IgG1	3609	CB17H.10	95	DFL16.1	1	EF672218	V_K2	bd2	100	2

[a] Closest matches from either the GenBank Database, the germline assignments were based on the published DNA sequences.

Table 3. Nucleotide sequence of the CDR3 region of the V_H CMV-specific mAbs.

mAbs	N	D segment	N	J_H	Length
V_H 6,7, 122	ATGGGGGTGA	TTTATTACTACGGTAGTAGCTAC	GTA		
GGGTACTTCGATGTCTGGGGCGCAGGGACCACGGTCACCGTCTCCTCA				J_H1	84
V_H 5	GAAGAA	TACTATGGTAA	A		
GCCTGGTTTGCTTACTGGGGCCAAGGGACTCTGGTCACTGTCTCTGCA				J_H3	66
V_H 8	AGAA	TACTATGGTAA	A		
GCCTGGTTTGTTTACTGGGGCCAAGGGACTCTGGTCACTGTCTCTGCA				J_H3	64
V_H 52	AGGGTTA	TTATAACGGCTACG	AGGGG		
GACTACTGGGGTCAAGGAACCTCAGTCACCGTCTCCTC				J_H4	64
V_H 521	ACAAACC	CCTACTATAGGTAC			
GACTACTGGGGCCAAGGCACCACTCTCACAGTCTCCTCA				J_H2	60
V_H 4	AAACC	CCTACTATAGG			
TTCGACTACTGGGGCCAAGGCACCACTCTCACAGTCTCCTCA				J_H2	58
V_H 9	AAACC	CCTACTATAGG			
TACGACTACTGGGGCCAAGGCACCACTCTCACAGTCTCCTCA				J_H2	58

V_H 11		ATCGG	CGGTTA	
CTACTTTGACTACTGGGGCCAAGGCACCACTCTCACAGTCGCCTCA			J_H2	57

Contribution of N- "random nucleotides insertion between V_H and D, D and J_H"

4. Discussions

Antibody fragments may have advantages for treatment of infections due to their small size and lack of Fc portion. It has been known for some time that small Ab molecules such as Fv's could have the potential for therapeutic application. However non-covalently assembled V_H and V_L tend to dissociate in the range of 10^{-4}-10^{-7} M depending on the sequence of interacting complementarity-determining regions (King et al., 1995). Therefore techniques have been developed to covalently link both V regions and stabilize them for *in vivo* applications. Using data from known crystal structures of Antibody and computer modeling, a series of linkers were designed and evaluated as potential candidates to genetically connect the V_H and V_L regions. The resulting scFv molecules were evaluated for their functional activities and relative affinity (Wörn and Plückthun, 2001). Very little molecular characterization of CMV with monoclonal antibodies (mAbs) has been achieved so far. Three different clones of a human synthetic antibody library specific to CMV-CP have been isolated; their V_H belongs to the human V_H1 family (Ziegler et al., 1995). A ScFv phage display library was constructed from mice immunized with CMV specific to the subgroup of both isolates I and II. V_H belongs to germline family V_HJ558 and subfamily V130.3, while the V_L gene belongs to germline Vκ4/5, gene ap4. Synthesis of an scFv antibody targeting CMV-CP, V_H belongs to germline family V_HI/J558, gene V_HF102, while V_L belongs to germline Vκ4/5, gene at4 (Chae et al., 2001). Several clones with high reactivity against CMV-CP were isolated from a large semi-synthetic scFv phage display library based on chicken immunoglobulin genes (van Wyngaardt et al., 2004). Due to the activation of the immune system as a response to a foreign antigen, maturation of the antibody response takes place, resulting in the production of specific, high-affinity antibodies. Therefore, specific antibodies can be selected using a relatively small, random combinatorial V-gene library derived from an immunized donor. Briefly, the procedure followed included the isolation of the variable heavy and light chain domains of the murine monoclonal antibody from mRNA of hybridoma cells, followed by cloning, sequencing and characterization of the Fab. V_H-gene. Usage was determined and compared to V_H-genes used by antibody fragments of a germline database. The V_H and Vκ regions of 10 anti-CMV mAbs generated from three different fusions of BALB/c mice were immunized with native CMV-CP, and the V_H, D, J_H, Vκ, and Jκ were determined (Table 2). Based on nucleotide sequence homology of the mAbs, V_H and V_L genes were classified into three subgroups. All the antibodies were found to derive from distinct B cells because they had utilized diverse V_H, D_H, and J_H gene combinations, and because the length of the CDR3 region ranged from 7 to 17 amino acid residues (Table 2). An abundance of V_H genes from the J558 family was observed (4/10) but each represented a separate member of the family (Table 2). CMV-CP is capable of inducing a variety of B cells that have distinct phenotypic and genotypic paratopes. Antibody-binding kinetics measured by surface plasmon resonance showed that antibodies from 'naïve' repertoires have comparable on-rates for antibodies from immune repertoires but faster off-rates (Winter et al., 1994). Frequently it is necessary to improve the affinity of antibodies isolated from a 'naïve' library by affinity maturation techniques that include chain shuffling, error-prone PCR (Fujii et. al., 1998), or parsimonious mutagenesis (mutagenesis in a few sites in which most residues remain parental) (Glaser et. al., 1992). Interestingly, the high affinity antibody specificity was encoded by germline genes such as mAb-8 (Figure 1). Furthermore, analysis of the affinity measurements and nucleotide sequences shows a strong correlation with the germline heavy chains, in which mAbs-(5, 52 and 521) were derived from V_H7183, showing high affinity to CMV-CP (Table 1). Although it is unlikely that all of these mutated residues are involved in CMV binding, increasing mutation in the heavy chain CDRs with increasing affinity of the antibodies is quite striking. It is difficult to determine the contribution of the CDR3 of the heavy chain, or of individual amino acids to affinity. Higher affinity scFv were produced by evolving the center of the antigen binding pocket by sequentially randomizing amino acid residues located within the heavy and light chain CDR3. Amino acid residues conferring higher affinity were located in loops within the CDRs and had solvent accessible side chains. In contrast, residues known to have a structural role were conserved. Isolation of higher affinity antibodies required a stringent selection process using limiting concentrations of soluble biotiylated antigen, use of a biosensor-based screening process

to identify higher affinity antibodies, and optimization of elution conditions (Schier et al., 1996).

One of the important aspects of V_L and V_H amino acid sequences is the study of the structural analysis of the antigen binding loops by molecular modeling and simulation of molecular dynamics (research currently in progress). Through these findings, it will be suggested that amino acids residues may play a crucial role in the antigen-antibody interaction.

Knowledge of specific immunoglobulin genes for a common epitope may lead to insight on pathogen-host co-evolution and help block a virus from infecting plants over the long-term, which is useful for antibody-based resistance. CMV-CP domains are clearly exposed on the virus; in addition, the amino acid sequence of the βH-βI loop forms a conspicuous, negatively charged electrostatic field on the surfaces of virions, which is a fundamental aspect that is conserved among *Cucumoviruses*, and this structure plays a role in aphid vector transmission (Liu et al., 2002). The βH-βI loop of the CP is a critical determinant of viral pathogenicity and has been shown to contain major immunodominant neutralization domains (He et al., 1998). The decapeptide sequence DDKLEKDE (aa198–205) probably contains essential contact residues, in which K (lysine) and E (glutamic acid) are both hydrophilic and negatively charged and might be important to constitute the epitope (Liu et al., 2002). Tyrosine side chains that exist in the antigen combining site might be capable of mediating most of the contacts necessary for high-affinity antigen recognition, and, thus, it seems likely that the overabundance of tyrosine in natural antigen-binding sites is a consequence of the side chain being particularly well suited for making productive contacts with antigen (Fellouse et al., 2004). Interestingly, the genes encoding the heavy chain variable region of these antibodies displayed evidence of only minimal somatic hypermutation. The crucial role of heavy-chain CDR3 in high-affinity CMV recognition is suggested. We propose that high-affinity CMV-binding antibodies can arise without extensive somatic hypermutation in the variable-region genes because of the expression of appropriate HCDR3s. We consider that the negative charge on the acetate group in the CMV-CP was partially neutralized by a hydrogen bond with the phenolic hydroxyl group of tyrosine, which exists in HCDR3. We consider that the negative charge on the acetate group in the CMV-CP was partially neutralized by a hydrogen bond with the phenolic hydroxyl group of tyrosine that exists in HCDR3. Therefore, we speculate and expect that the HCDR3-peptide be used as tool for plant virus resistance depending on the peptide-neutralizing epitope.

Acknowledgements:

Authors are grateful to to Professor Dr. Ikuo Fujii for his advice and helpful discussion.

Corresponding Author:
Dr. Haggag Salah Zein
Department of Genetics, Faculty of Agriculture, Cairo University, 12613, Egypt
E-mail: haggagsalah@gmail.com

References

1. Palukaitis P, Roossinck MJ, Dietzgen RG, Francki RIB. Cucumber mosaic virus. Adv Virus Res 1992;41:281-348.
2. Zein HS, Mona HH, Miyatake K. Characterization of *Cucumber mosaic virus* monoclonal antibodies (mAbs): Specificity, neutralization of infectivity and gene sequence. Plant Viruses 2007;1(1):193-9.
3. Marks C, Marks JD. Phage libraries a new route to clinically useful antibodies. New Engl J Med 1996;335(10)**:**730-3.
4. Zein HS, Miyatake K. Cloning, sequencing and expression of immunoglobulin variable regions of murine monoclonal antibodies specific to cucumber mosaic virus coat protein. Plant Viruses 2007;1(1):201-7.
5. Liu S, He X, Park G, Josefsson C, Perry KL. A conserved capsid protein surface domain of Cucumber mosaic virus is essential for efficient aphid vector transmission. J Virol 2002;76(19): 9756-62.
6. Perry KL, Zhang L, Palukaitis P.. Amino acid changes in the coat protein of cucumber mosac virus differentially affect transmission by the aphids Myzus persicae and Aphis gossypii. Virology 1998;242(1):204-10.
7. Schillberg S, Zimmermann S, Findlay K, Fischer R. Plasma membrane display of anti-viral single chain Fv fragments confers resistance to tobacco mosaic virus. Mol Breed 2000;6(10):317-26.
8. Marasco WA. Intracellular antibodies (intrabodies) as research reagents and therapeutic molecules for gene therapy. Immunotechnology 1995;1(1):1-19.
9. Zimmermann S, Schillberg S, Liao YC, Fischer R. Intracellular expression of TMV-specific single-chain Fv fragments leads to improved virus resistance in Nicotiana tabacum. Mol Breed 1998;4(4):369-79.

10. Peschen D, Li HP, Fischer R, Kreuzaler F, Liao YC. Fusion proteins comprising a fusarium-specific antibody linked to antifungal peptides protect plants against a fungal pathogen. Nat Biotechnol 2004;22(6):732-8.
11. Nitta N, Masuta C, Kuwata S, Takanami Y, Comparative studies on the nucleotide sequence of Cucumber mosaic virus RNA3 between Y strain and Q strain. Ann Phytopathol Soc Japan 1988;54(1):516-22.
12. Harlow E, Lane D. Antibodies. A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1988.NY.
13. Fujii I, Fukuyama S, Iwabuchi Y, Tanimura R. Evolving catalytic antibodies in a phage-displayed combinatorial library. Nat Biotechnol 1998;16(5):463-7.
14. Bobrovnik SA. Determination of antibody affinity by ELISA. Theory J Biochem Biophys Methods 2003;57(3):213-36.
15. Huse WD, Sastry L, Iverson SA, Kang AS, Alting-Mees M, Burton DR, Benkovic SJ, Lerner RA. Generation of a large combinatorial library of the immunoglobulin repertoire in phage lambda. Science 1989;246(4935):1275-81.
16. Martin ACR. Accessing the Kabat antibody sequence database by computer proteins: structure, function and genetics 1996;25(1): 130-3.
17. Hsu HT, Barzuna L, Hsu YH, Bliss W, Perry KL. Identification and subgrouping of Cucumber mosaic cucumoviruses with mouse monoclonal antibodies. Phytopathology 2000;90(6):615-20.
18. Kabat EA, Wu TT, Perry HM, Gottesman KS, Foeller C. Sequences of proteins of immunological interest, 5th ed., Public Health Service, National Institutes of Health, 1991; publication no. 91-3242, Washington, DC.
19. Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucl. Acids Res. 25, 3389-402.
20. Gubbins MJ, Plummer FA, Yuan XY, Johnstone D, Drebot MA, Andonova M, Andonov A, Berry JD. Molecular characterization of a panel of murine monoclonal antibodies specific for the SARS-coronavirus. Mol Immunol 2004;42(1), 125-36.
21. King DJ, Antoniw P, Owens RJ, Adair JR, Haines AMR, Farnsworth APH, Finney H, Lawson ADG, Lyons A, Baker TS, Baldock D, Mackintosh J, Gofton C, Yarranton GT, McWilliams W, Shochat D, Leichner PH, Welt S, Old L J, Mountain A. Preparation and preclinical evaluation of humanised A33 immunoconjugates for radioimmunotherapy. Br J Cancer 1995;72(6):1364-72.
22. Wörn A, Plückthun A. Stability engineering of antibody single-chain Fv fragments. J Mol Biol 2001;305(5):989-1010.
23. Chae JS, Choi JK, Lim HT, Cha SH. Generation of a murine single chain Fv (scFv) antibody specific for cucumber mosaic virus (CMV) using a phage display library. Mol Cells 2001:11(1), 7-12.
24. Van Wyngaardt W, Malatji T, Mashau C, Fehrsen J, Jordaan F, Miltiadou D, du Plessis DH. A large semi-synthetic single-chain Fv phage display library based on chicken immunoglobulin genes. BMC Biotechnol. 2004;1(6):4-6.
25. Winter G, Griffiths AD, Hawkins RE, Hoogenboom HR. Making antibodies by phage display technology. Ann Rev Immunol 1994;12:433-55.
26. Glaser SM, Yelton DE, Huse WD. Antibody engineering by codon-based mutagenesis in filamentous phage vector system. J Immunol 1992;149(12):3903-13.
27. Schier R, Bye J, Apell G, McCall A, Adams GP, Malmqvist M, Weiner LM, Marks JD. Isolation of high-affinity monomeric human anti-c-erbB-2 single chain Fv using affinity-driven selection. J Mol Biol 1996;**255**(1):28-43
28. Fellouse FA, Wiesmann C, Sidhu SS. Synthetic antibodies from a four-amino-acid code: A dominant role for tyrosine in antigen recognition. Proc Natl Acad Sci USA 2004;101(34):12467-72.

1 5th February 2010

Including Effectiveness of the blue crab (*portunus pelagicus*) antioxidants inhibit oxidative stress

Ashraf Jazayeri[1*], Ahmad savari[1], Mehran Hossein-Zadeh[2], Forough papan[3], Manijeh kadkhodaei[2]

1. Marine Science and Technology of Khorramshahr University
2.Professor of medical Sciences, Jondi Shapur University of Ahwaz
3. Professor of marine biology, Shahid Chamran university of Ahwaz
[*]Corresponding Arthur: *jazayeriashraf@yahoo.com*

Abstract: today, the role and importance of the human body antioxidant defense system in prevention of many diseases has been proved completely on the other hand the need to strengthen the immune system is inevitable that the consumption of synthetic antioxidants in numerous adverse effects Experts are always following the use of natural antioxidants in this regard must recommend extensive research to find natural resources and the needs of modern human antioxidant is ongoing results of many studies have shown that marine resources are rich in antioxidants are the blue swimmer crab research in this respect were fractions antioxidant extracted from muscle tissue extracts such, antioxidant capacity showed significant antioxidant addition fractions such significant effects in inhibiting oxidative stress, including inhibition hemolysis of red blood cells and protection of Thiol groups of blood showed in a laboratory.

[Ashraf Jazayeri, Ahmad savari, Mehran Hossein-Zadeh, Forough papan, Manijeh kadkhodaei. **Including Effectiveness of the blue crab (*portunus pelagicus*) antioxidants inhibit oxidative stress.** Journal of American .http://www.americanscience.orgScience 2010;6(12):1565-11569]. (ISSN: 1545-1003).

Keywords: antioxidant capacity, blue crab, oxidative stress

1. Introduction

The human body in a state health system, a balance between free radicals and anti oxidant rate each factor that causes interference can be above the equilibrium incidence of some diseases cause. Including atherosclerotic cancer and premature aging recent research necessary to strengthen the body's antioxidant system through diet or supplements nutritional shows, although the late twentieth century as the only plant sources of natural antioxidant sources are identified in the recent decade extensive research on fish showed that some species of marine plants and animals significant amounts of anti-tumor compounds, anti-viral and contain antioxidants, blue crab research in the Gulf of antioxidant capacity consideration Therefore, the species was muscle tissue above the isolation and the environment was then Buffering homogenize blue crab muscle tissue for extraction process Sequestration antioxidants ammonium sulfate and dialysis, and then it was sad, extract the above route by the end of gel from 150, then the maximum for the fraction of total antioxidants was selected in 5 different concentrations Measurement for efficacy in inhibiting oxidative damage was used in this section of the human red blood cell model used and the effectiveness of the blue crab fractions antioxidant in human erythrocyte hemolysis inhibition and the amount of blood Thiol protecting groups was Calculated reviews and tests show that the blue crab at the level of antioxidants can significantly inhibit human erythrocyte hemolysis and blood Thiol protecting groups are the results of this study blue crab as a rich source of natural antioxidants that can be defined as the a source of marine biotechnology in food production complexes and compounds for drug prevention and treatment of disease pathogenesis that free radicals are used.

2. Material and Methods

Crab fishing by trawler were incubated with samples of ice and immediately transferred to the laboratory, the samples described tissue, muscle separation and buffer Tracy (5%, 5.7PH =) transferred along with muscle tissue was then centrifuged buffer homogenize iceman temperature for 10 minutes c 40 and g × 6000, was centrifuged, sedimentary layer was discarded, for the separation layer supernatant Sequestration method was used proteins thus under the terms of the first magnetic mixer adding the necessary amounts of solid ammonium sulfate saturation level was 50 percent, after centrifugation (g × 6000, c40) for 30 min, sedimentation at this stage was maintained and sup layer according to the previous stage saturation of 70 percent required rate of solid ammonium sulfate was added after this stage centrifugal sedimentation with sediment before step combined amount of the buffer size after mixing Tracy added, for 12-hour dialysis, the dialysis bag was transferred (input buffer during the dialysis, buffer was Tracy was replaced every 2 hours) after dialysis Inundation by sucrose for 10 hours was the end result of solution outside the dialysis bags to human transmission found and the

potential for antioxidants extraction chromatography was used in the first chromatography column containing seeds Pharmacia diameter 5.2 cm and 60 cm in height, Tracy salt buffer mobile phase, flow rate 40 ml per hour was elected , fractions obtained by the end of the device fraction collector column, were collected (170 fraction) then all the above fractions light absorption wavelength 280 nm and 540 nm was read and help protein and Bioret kit by Randox rate Each antioxidant was calculated fractions (122-117 and 97-93 and 58-52 and 26-20), which had the maximum amount of antioxidants to second column purification transferred to the second phase chromatography column containing DEAE cellulose column diameter 5.1 cm and 60 cm height Tracy salt buffer mobile phase, flow rate was 40 ml per hour by the end of the column device 150 fraction collector raised before the rate matching step, the amount of total protein and antioxidants, all calculated fractions was then fractions (89-84 and 71-68 and 48-42 and 27-25 and 16-12), which had the maximum amount of antioxidants microbial filter method, is sterile in order to review the effectiveness of the inhibition of oxidative damage were used.

2.1. The impact on blue crab antioxidants inhibits oxidative damage in a laboratory

2.1.1. Liz inhibition of red blood cells
A - **Suspension the blood**:

The blood in healthy individuals heparin pipe and then collected by centrifugation, plasma was separated from red blood cells, Rbc above three times by saline washing, and ultimately pure red blood cells were prepared from pure erythrocyte above dilution method in phosphate buffer salt, 2 percent suspension (equivalent hematocrit 12) was prepared.

B - Hemolysis of red blood cells

Of antioxidant fractions sterile, the pipe with concentrations (100%, 75%, 50%, 25%) was produced (with dilution buffer were PBC), then pipe that includes Prepare the mixture for 2 hours the temperature was then incubated with 370 centrifuges (g × 1000) of 10 minutes and the upper layer of light absorption wavelength 415 nm was read (represents the amount of hemolysis) and the control tube (No extract fractions) the rate of hemolysis were compared with values based on percent inhibition of hemolysis in the vicinity with fractions, compared to the control rate (percentage of hemolysis showed percent) was calculated.

2.2. Protection of Thiol groups

After the suspension of red blood cells, 50 micro litter of the suspension Rbc 1000 Tracy and 50th micro litter buffer fraction antioxidant (concentrations of four) the composition and light absorption wavelength of 4 micro litter 12 nm was read then pipe above 20 micro litter reagent DTNB added and 30 minutes and then incubated in the laboratory temperature optical absorption was read at 412 nm, pipe controls included buffer suspension cells and introduced Tracy DTNB prepared and its optical absorption 413 nm was read , then read the values in light absorption were the following formula according to the amount of computation protected Thiol groups were obtained Thiol protecting groups for the amount of each of the five branches fractions antioxidant concentrations separately tested and was measured at the end of percentage protection for each concentration fractions Thiol groups compared with control was calculated.

3. Results

Determine the antioxidant capacity of a blue crab: After chromatography, fractions from each stage (gel filtration, ion exchange) levels of total protein and total antioxidant were analyzed (Tables 1 and 2).

3.1. effect of antioxidant fractions blue crab in the inhibition of hemolysis of red blood cells

Fractions group of the A_1 the most total antioxidants were also dilution, fractions with total antioxidant levels (12, 9, 6 and 3 and 2 / 1) mmol liter in the equivalent concentrations (100, 75, 50, 25 and 10) respectively were prepared, then each fraction cells were in the vicinity, after adding a certain amount of AAPH for 2 hours incubation period temperature was 37 degrees then created hemolysis rate for all pipes and pipe control (No fraction antioxidant) spectrophotometric method at 415 nm wavelength was read and the percentage hemolysis inhibition was calculated (compared to control samples), all the numbers obtained in 0.05> p was considered significant (Table 3).

As is clear in Table 3 fractions antioxidant concentration of 100 percent blue crab (the amount of antioxidants in 12 mmol L) no effect on hemolysis of red blood cells did not fraction concentration of 75 percent (total antioxidant levels in 9 mmol L) maximum effectiveness in inhibiting the rate of hemolysis showed 95 percent plus extract dilution effects of the above reduction in hemolysis fraction the lowest effect concentration and the rate of 10 percent antioxidant 2 / 1 mmol l showed that (a rate 7 / 19 percent).

Table 1: Results of total protein and total measured antioxidant derived from gel filtration chromatography blue crab muscle tissue

step purification	fraction	Total protein(mg/l)	Total antioxidant (m mol / Lit)
(sephadex G100)	crude tissue extract	1360	42.5
	group A (26-20)	580.5	21.2
	group B (58-52)	204.9	6.8
	Group C (97-93)	405	14.7
	Group D (122-117)	167	5.3

Table 2: Results of measurement of protein and total antioxidant fractions A group from ion exchange chromatography

stage purification	fraction	Total protein (mg/l)	Total antioxidant
DEAE cellulose	group A (26-20)	580/5	21/2
	A_1	199	12
	A_2	105.4	7.3
	A_3	174	10.2
	A_4	59.5	5.1
	A_5	29	4.6

3.2. The effectiveness of the blue crab fractions antioxidant protection Thiol groups (-SH) blood

In this section the concentrations of five triple antioxidant fractions blue crab was prepared the same step, then any effects in protecting blood Thiol groups were determined according to procedures based on mg protein vs control calculation, all numbers obtained in 05/0p <are considered significant. (Table4).

Table 3: The rate the effectiveness of the blue crab fractions antioxidant in inhibiting hemolysis of red blood cells

Fraction	Total antioxidant (mmol/l)	Inhibition percent
control	0	0
concentration 100 percent (Group A)	12	0
concentration of 75 percent	9	98.5
concentration of 50 percent	6	89.2
concentration of 25 percent	3	75.9
concentration of 10 percent	1/2	46

Table 4: Effect of blue crab fractions antioxidant protection Thiol groups fraction type and amount of antioxidant

fraction type and amount of antioxidant	Protein (mol/mg)	Percent to control
control	930	100
12	920.7	99
9	890	95.7
6	821.1	88.3
3	605.4	65.1
1.2	404.5	43.5

4. Discussion

The antioxidant capacity measurement showed that blue crab fractions muscle tissue from this species contain considerable quantities of antioxidants average 5 / 10 mmol were lit. Toresin and Kerstin 1998 (8) also reviews some fish, including salmon successful extraction of synthetic antioxidants were Astaxantin name. Clark and colleagues (1998), while reviews of gel were able to jack in the antioxidant properties of these prove. Smith and Bell 1996 (9), while crab carcinus menas review declared that Super oxide enzyme extracted from a laboratory in such a strong antioxidant properties shows. According to World Health Organization (WHO 2007) standard rate of serum antioxidant capacity in normal individuals, 1.03 - 1.77 mmol l that this value depends on the age, sex,

nutrition and the geographical conditions of the Hang the tea and colleagues Watch 2007 (16) antioxidant capacity in such a way the average shrimp Penaeus monodon have announced 4 compared with values above antioxidant capacity blue crab is significantly more.

For measuring the effectiveness of antioxidants blue crab model of red blood cells were used. Cruz Silva and colleagues (2000) (17) red blood cell model ideal model to evaluate stress Oxidative laboratory environments experience and introduced them announced that free radicals attack cells, causing lipid per oxidation and membrane proteins and eventually to hemolysis are, in this study results showed that fractions A1 (100 percent concentration and antioxidant levels in 12 mmol L) not only to prevent blood cell hemolysis, but also has had the effect per oxidant.

1999 Partasaraty and colleagues announced that the antioxidants in different concentrations can be different effects of antioxidant vitamin E Per oxidant even have the same feature to update itself, State hawthorn 1998 (18) showed that concentrations of vitamin C in 60-100 property antioxidant concentrations, but not more than this amount will Per oxidant properties, Frag and Partners 1999 (19) announced that its antioxidant properties and chemical components of each combination of concentration is related. Other fractions blue crab concentrations 75, 50 and 25 percent respectively, the values 9, 6, 3 and 1.2 mmol l antioxidants were all showed inhibition of addition, as Table 3 shows the reduced dose antioxidant effect Atpase considerably reduced. Burton and colleagues 2009 declared that what proteins Thiol groups who are building cell membranes and how those buildings hemoglobin (especially glutathione) exist, a very important factor in membrane stability and are soluble oxidative stress during this groups to the oxide and formation of disulfide bond to protect the cells against free radicals are combined, so if Thiol groups against oxidation in the cell's ability to protect against oxidative stress is taken up.

This study showed that blue crab fractions antioxidant to protect the good Thiol groups have a protective effect above fraction dilution and reduced antioxidant capacity, it declined (Table 4).

Considering the importance of antioxidants and their role in prevention of the pathogenesis and treatment of disease free radicals (13) The results of this study showed that blue crab having significant amounts of antioxidants in this area is of great import can be a natural source of antioxidant should be exploited.

Corresponding Author:
Dr. Ashraf Jazayeri
Department of biology
Shahid Chamran University of Ahwaz
00989161414883
jazayeriashraf@yahoo.com

References

1 - Tabrstany M, hematology medical. Tehran publisher. 2000:55-98
2 - Hussein-pour M, Cancer Medicine 2004
3 – Mohammad R, Medical Biochemistry 2009
4 – Mazry, Laboratory methods in diagnosis of blood diseases(2007)
5 – Ahmadzadeh. Myrjlaly, attitude and chemotherapy and future perspectives (2007)
6 - Shariat-Zadeh, M. AS free radicals and antioxidants (2000)
7 - cooper EL, Hrzenja k, Grdisa Alternative sources of Antimicrobial and anti cancer molecules (2004)
8 - Haefner. B, Drugs from deep marine products as drug candidates (2006)
9 - Kim. J, park EJ. Cytotoxic anticancer candidates from natural resources (2004)
10 – zilelekaT, Natural products with anti-HIV activity from marine organisms (2003)
11 – omuras, Prospect of chemotherapy in the 21 century (2009)
12 - Galler S. R Biomedical application of horseshoe crab (1999)
13 - Kicking. P, The secret of the blue blood1998)
14 – mduda M.K., Antimicrobial isolated from horseshoe crab hemocytes Antimicrobial Agents and chemotherapy (1993)
15 - Kuashing. Li Traditional and modern Biomedical prospecting (1994)
16 - Haefner. B, Drugs from The deep, marine natural products as drug candidates (2003)
17 - Dnia. B, marine products and potential application as anti infective agents (2002)
18 – Enhan. MT, cing marine natural products structure diversity and bioactivity and biocatalysts (1998)
19 - Kim. Japers. EJ, Cytotoxic anticancer candidates from marine resources (2000)
20 - Journal of medicinal food volume 10 (3). Page 401-407(2007)
21 - Journal of food science volume 56 (1) page 143-148(2005)
22 – Halliwell. B, Guttering Free radical in biological system (1999)
23 – Fariver. M. Alain, Analysis of free radical in biological system (2008)
34- Finger man. M, Rachakonda Biomaterial from aquatic organism (2007)

35 – Burton G.W, Antioxidant effects in human blood and RBC model (2006)
36 – Lund. L., A method to measure total Antioxidant capacity in aquatic organisms (2005)
37 – Cruz. M., Silva, A biological model for oxidative damage in vitro (2000)
38 – State H., Lake L., Free Radical & antioxidants in Biochemical Methods (1998)
39 – Miller. Davis, Free Radical & biomedical sciences (2003)
40 - Genj Tao. L & Ettal protective action of seven natural compounds against per oxidative damage to biomembranes (1999)
41 – Frage. G. & Ettal Antioxidant compound in biological system & Biochemical Assay (1999)

11/28/2010

Production of *Potato Spindle Tuber Viroid*-Free Potato Plant Materials *in Vitro*

Sherin A. Mahfouze [*1]; Kh. A. El-Dougdoug[2] and E. K. Allam[2]

[1]Genetic Engineering and Biotechnology Division, Genetic and Cytology Department, National Research Center, El-Beehoth street, 12622, Dokki, Giza, Egypt .
[2]Microbiology Department (Virology lab.), Faculty of Agricultue, Ain Shams University, Shoubra, El-Kheima, Cairo, Egypt.
Sherinmahfouze@yahoo.com

Abstract: PSTVd-$_{EG}$ strain was isolated from infected potato plants cv. Diamond during autumn season. The PSTVd-$_{EG}$ was eliminated from these plants by different methods. The meristem-tip culture with size (0.25 mm) gave the high percentage of plantlets PSTVd-$_{EG}$-free was 83.33%. The chemotherapy with ASA, 2-TU and Virazole was applied in culture media with concentrations 10, 20, 30, 40 and 50 ppm. It was found that, the percentage of PSTVd-$_{EG}$-free plantlets was increased by increasing chemical concentrations. The thermotherapy of plantlets in jars (21, 3-4, 5, 8 and 21 $^{\circ}$C /4 mon. due to PSTVd-$_{EG}$ elimination). In addition to, the combination cold treatment of tubers plus meristem-tip culture is more effective for PSTVd-elimination *in Vitro*. As well as, the exposure of the tubers for electricity 5/5, 5/10, 10/5, 10/10, 15/5 and 15/10 mA/min. due to PSTVd-$_{EG}$ elimination particularly the exposure at 10/10; 15/5 and 15/10 mA/min. The results were confirmed by dot-blot hybridization assay.
[Sherin A. Mahfouze; Kh. A. El-Dougdoug and E. K. Allam. **Production of *Potato Spindle Tuber Viroid*-Free Potato Plant Materials *in Vitro*.** Journal of American Science 2010;6(12):1570-1577]. (ISSN: 1545-1003).
http://www.americanscience.org.

Key words: Potato, PSTVd, Meristem-tip, Chemotherapy, Thermotherapy, Electrotherapy

Abbreviation
2-TU= 2-Thiouracil, ASA =Acetyl salicylic acid, GA_3= Gibberillic acid, IAA= Indol acetic acid.
MS= Murashiage and Skoog, NAA= Nephthaline acetic acid, NASH= Nucleic Acid Spot Hybridization.
PSTVd-$_{EG}$ = Potato Spindle Tuber Viroid (Egyptian Strain).

1. Introduction

Potato Spindle Tuber Viroid (PSTVd) is the type member of the genus *Pospiviroid* (Family *Pospiviroidae*) (ICTV, 2008). It is a circular single-stranded, RNA molecule, measuring between 356-361 nt. in length and un-encapsidated (Schnöelzer *et al*. 1985). Higher percentage of PSTVd-free was obtained from apical domes, followed by meristem and lower percentage from shoot tips (Lizárraga *et al*. 1982 and Salazer *et al*. 1985).Lower temperature (cold therapy) due to reduce or stopping viroid replication and translocation through phloem tissue (Helms & Wardlaw 1976). Whenever, PSTVd-$_{EG}$ was not detected in plantlets grown at 5ºC for 3 and 6 mon. and low light intensity but viroid was present in tissue when plantlets was transferred to 25ºC for 1 mon. (Lizárraga *et al*. 1980).

There are many antiviroid chemicals such as piperonyl butoxide (Singh, 1977); Silver nitrate and ethylene (Conejero, 1982)**;** Ribavirin (Belles *et al*., 1986); ethephon against Citrus exocortis viroid (CEVd) infection (Belles *et al*., 1990). In addition to, Chitosan (1-4 glucosamine polymers) has been shown to stimulate different responses in plants (Ryan, 1988). Amantadine, Ribavirin and Thiouracil used to eliminate viroids from infected plants (Kryczyński 1992). *Potato Virus X* was eliminated by exposure potato stems to 5, 10 or 15 mA for 5 or 10 min. followed by immediate planting the axillary buds tips *in vitro*. The highest TE values were obtained at 15 mA for 5 min. under these conditions, 40% to 80% of the buds regenerated and 60% to 100% of the regenerated plantlets tested virus negative Lozoya *et al*. (1996).

The first concern of the present study was production of PSTVd-$_{EG}$-free potato plantlets from infected tuber potato through applied meristem-tip, chemotherapy, thermotherapy electrotherapy techniques and combination with them.

2. Materias and Methods

Two-month-old PSTVd-$_{EG}$ infected potato plants (*Solanum tuberosum* L.) cv. Diamond growing under greenhouse conditions were used for PSTVd elimination by meristem culture, chemical anti-viroid, thermotherapy and electrotherapy.

Detection of Potato viruses and viroid

DAS-ELISA technique was applied for detection potato viruses (PVX, PVY, PLRV, PVS, PVA, CMV and TRV) as described by Clark and Adams (1977) using an ELISA kits (completely ready for use supplied by Sanofi, Sante Animals, Paris, France).

Dot-blot hybridization for detection PSTVd was done according to (Owens *et al*., 1986).

Sterilization of stem nodes

The stem nodes were cut from PSTVd-$_{EG}$ infected potato plants cv. Diamond. Nodal cutting and sprouts were washed in running tap water for 1 h. and then transferred to 5% solution of commercial bleach (sodium hypochlorite 5.25% active ingredient) containing 0.1% tween-20, Then were rinsed 3 times with sterile dsH_2O at 2, 5 and 15 min. sequently.

Meristem-tip culture

Twenty-five shoots (2-3 cm length) under the steromicroscope (CETI WF 10X) the outer leaves and leaf primordia until the youngest leaves (2 primordia) were removed. The dome with two leaf primordia was excised with 0.25 mm length by scalpel. Excised the dome meristems were cultivated on MS-medium containing (0.1 mg/L NAA + 0.5 mg/L kinetin + 2.25 gm/L phytagel) (Edriss *et al*., 1996) then incubated at 25ºC and light intensity 2.000 Lux for 16 h. lightday. After 21 days, the plantlets were transferred to MS propagation medium until rooting.

Chemotherapy

Stem cuttings with one leaf node were cut from potato plants cv. Diamond (6 wks-old) infected with PSTVd-$_{EG}$ and healthy. The stem cuttings were cultured on MS medium solidified with agar (8 gm/L) in jar (250 ml vol.). Jars were incubated at 18ºC/16 h. daylight. The plantlets were multiplied *in vitro* as nodal cuttings in jars vol. 500 ml (5 subcultures). The nodal cuttings were transferred on MS media containing three antiviroid separated [1-β-D Ribofuranosyl- 1, 2, 4-triazole-3-carboxamide (Ribavirin or virazole), (ASA) and 2-TU]. Thirty nodal cuttings were cultivated on Paper Bridge in jars (500 ml) for each concentration. The jars were incubated on the same mentioned conditions. Percentage of survival cuttings were counted for each conc. after 30 days. The survival nodal cuttings were transferred in anti-viroid free media. After 4 wks. plantlets were detected by dot-blot hybridization against PSTVd-$_{EG}$.

Thermotherapy

Fivety-six PSTVd-$_{EG}$ infected tubers of cultivar Diamond and PVX, PVY and PLRV virus tested as well as four healthy ones was used in this study. Tubers were storaged at different temperatures and periods (3-4, 5, 7-8 ºC/4 mons. in refrigerators and 21±0.2ºC/4 mons. in incubator. Fourteen tubers/treatment from each temperature. The tuber sprouts were separated and meristems with two leaves primordia were excised. The sprout and meristem tips were cultured on MS media and were incubated as above mentioned. The percentages of survival and PSTVd-$_{EG}$-free plantlets were counted post storage for 4 mons.

Electrotherapy

Seventy-two PSTVd-$_{EG}$ infected tubers cultivar Diamond were exposed to electrictherapy treatment (Lozoya-Saldaña, 1996) The tubers were treated as following: current intensity-time combinations: 5, 10 or 15 (mA) for 5 or 10 min. Electricity was applied by an electrophoresis power supply (consort 600V-500 mA E865). Immediately after treatment 12 sprouts per treatment were removed from tubers. Sprouts were excised and were planted *in vitro* in a semi-solid MS medium. Then growing tips 1.9 mm long were excised and were planted in medium consisting of basic MS salts and supplemented with 0.25 ppm GA_3, 2.0 ppm calcium pantothenate (B_5), 3% sucrose and 2.25 mg/L phytagel (Espinoza *et al*., 1985). After 30 days the plantlets were transferred to shoot differentiation medium containing the basic MS salts supplemented with 0.3 ppm IAA, 0.3 ppm kinetin, 4% sucrose and 2.25 gm/L phytagel (Lozoya and Dawson 1982). The plantlets were incubated at 16 h. daylight/18ºC, for 30 days in the shoot differentiation medium. The percentages of survival and PSTVd-$_{EG}$-free plantlets were recorded.

- Viroid detection by Dot-blot hybridization

Dot-blot hybridization was used for PSTVd-$_{EG}$ indexing of plantlets arised from tissue culture experiment after micropropagation reindexing was performed after meristem-tip, chemotherapy, thermotherapy and electrotherapy treaments.

3. Results

Dot-blot hybridization assay was used to detect PSTVd-$_{EG}$ in potato plants and tubers cv. Diamond virus tested which used to produce PSTVd-$_{EG}$ -free plants as well as micropropagated plantlets. These plants were divided into three groups. Group one: was used for excised meristem-tip, Group two: for treated chemotherapy of plantlets

micropropagated *in vitro* and Group three: PSTVd-$_{EG}$- infected tubers were treated with cold and electrotherapy treatments.

Meristem-tips size 0.25 mm was excised from PSTVd-$_{EG}$ potato plants cv. Diamond under stereomicroscope. They were cultivated on MS medium and incubated under convenient conditions. After four subcultures the meristems were developed to shoot (Fig. 1), the survival of potato plantlets was 75% and percentage of PSTVd-$_{EG}$-free plantlets was 83.33%. These results were confirmed by dot-blot hybridization (Table 1).

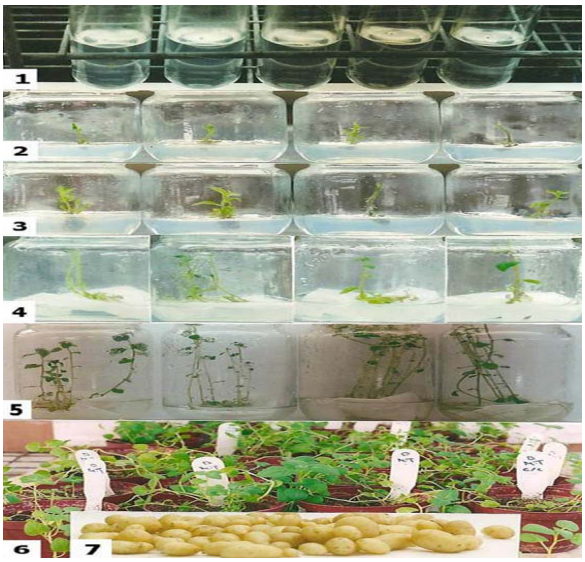


Figure 1. Micropropagation of PSTVd -free potato plantlets by meristem tip on MS-media.

1- Meristem-tip (0.25 mm size), 2, 3- Establishment stage. 4- Multiplication stage, 5- Rooting stage, 6- Adaptation, 7- Minitubers.

The plantlets were cultured on MS media treated with three different anti-viroid compounds. These compounds [Ribavirin (Virazole), 2-TU and ASA were incorporated individually into MS medium with concentrations 10, 20, 30, 40 and 50 ppm. The plantlets were incubated for 30 days. Incorporation of anti-viroid virazole, 2-TU and ASA in culture medium at conc. of 10, 20, 30, 40 and 50 ppm progressively increased the percentages of viroid-free plantlets to 57; 71.4; 77.7; 87.5 and 87.5 for virazole, 50, 63.63, 66.6, 77.7 and 85.7 for 2-TU and 42.8, 50, 71.42, 75 and 83.3 for ASA respectively. In addition, the percentage of survival decreased with increment conc. of the chemical antiviroid (Table 1).

Table 1. Effect of chemotherapy and meristem-tip on production of PSTVd-$_{EG}$ -free plantlets *in vitro*.

Chemotherapy treatments	% survival*		% viroid elimination
	H	I	
Meristem-Tip••(0.25 mm)	75.0	75.0	83.3
Chemotherapy •			
10 mg/L virazole	100	100	57.0
20 mg/L virazole	87.7	73.3	71.4
30 mg/L virazole	80.0	73.3	77.7
40 mg/L virazole	76.6	54.0	87.5
50 mg/L virazole	76.6	42.0	87.5
10 mg/L Thiouracil	93.3	66.6	50.0
20 mg/L Thiouracil	70.0	63.3	63.6
30 mg/L Thiouracil	36.6	30.0	66.6
40 mg/L Thiouracil	35.0	28.3	77.7
50 mg/L Thiouracil	33.3	25.3	85.7
10 mg/L Salicylic	95.0	100	42.8
20 mg/L Salicylic	90.0	84.4	50.0
30 mg/L Salicylic	88.3	72.2	71.4
40 mg/L Salicylic	78.3	61.6	75.0
50 mg/L Salicylic	65.5	33.3	83.3

* Jar contaiing 30 plantlets. •Total number of tested plantlets 240 (8 jars x 30 plantlets), ••Total number of tested plantlets 25 (one meristem per tube).

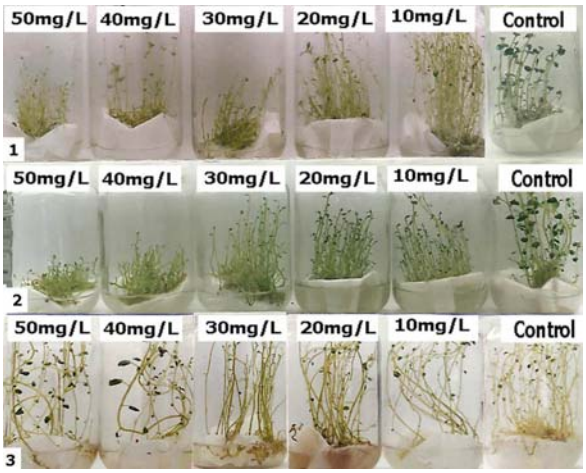


Figure 2. Micropropagation of plantlets on MS media treated with different concentrations of virazole. 1- Healthy plantlets treated with different concentrations of virazole, 2- PSTVd infected plantlets treated with different concentrations of virazole., 3- Re-subculture of plantlets on fresh media without virazole.

On the contrary, the effect of chemical antiviroid on the development plantlets was different such as virazole proved to be somewhat phytotoxic in

highest concs. 40 and 50 ppm causes severe stunting in plantlets, thin stem and stunted leaflets. Whereas, concentrations 30, 40, 50 ppm from 2-TU cause deleterious effects on growth of potato shoots, and concentrations 20, 30 and 40 ppm from ASA cause stunting of plantlets and concs 30, 40 and 50 ppm encourage formation of callus in some plantlets, while conc. 50 ppm induced formation of microtuber *in vitro*. But this phytotoxic effect was removed when subcultured on fresh medium without antiviroid-compunds. These results were confirmed by NASH (Table 1) and (Figures. 2, 3 and 4).

Potato tubers cv. Diamond infected with PSTVd-$_{EG}$ isolate were exposed to low-temperatures and cold therapy in refrigerators at 3-4; 5, 8ºC/4 mon. and incubator 21ºC/4 mon. (Table 2). Sprouts were excised with 0.5 mm buds. As well as, meristem-tip (0.25 mm) separated from sprout then micropropagated on MS media. It was found that percentage of sprout survival was 100%. Whenever, PSTVd-$_{EG}$ elemination percentages from sprouts were 61.50, 71.4, 71.4 and 64.2 at temperatures 21, 3-4, 5 and 8ºC respectively. On the other hand, meristem-tip excised from sprouts exposured for low-temperatures 3-4, 5, 8ºC for 4 mon. were the best treatments for viroid elimination which gave 100%, whereas 21ºC/4 mon. gave 77.7 %. However, survival rate of meristem-tip was 85.7; 71.4, 57.1 and 57.1% for temperatures 21, 3-4, 5, 8ºC for 4 mon. respectively (Table 2). These plantlets were indexed for PSTVd isolate by dot-blot hybridization assay.

Each 12 potato tubers cv. Diamond infected with PSTVd-$_{EG}$ isolate were exposed to 5, 10 and 15 mA for 5, 10 min. The sprouts of these tubers were excised and cuttings with 0.5 mm (explant). The explants were planting on MS media. The shoot-tips of plantlets were excised from plantlets and planting on MS media. It was found that treatments 10/10; 15/5 and 15/10 mA/min were the more effective for PSTVd-$_{EG}$ elimination (100 %) (Table 2). While, 5/5; 5/10 and 10/5 mA/min treatments gave 66.7; 0 and 0 % respectively. On the other hand, it was observed that regeneration Increased in treated sprouts with electrically compared with untreated electrotherapy ones. On the contrary, the survival rate decreases with increase electricity and treatment time (Figure 5 and Table 2). These results were confirmed by NASH.

Figure 3. Micropropagation of plantlets on MS-media treated with different concentrations of 2-TU.

1-Healthy plantlets treated with different concentrations of 2-TU, 2- PSTVd infected plantlets treated with Different concentrations of 2-TU, 3- Re-subculture of plantlets on fresh media without 2-TU.

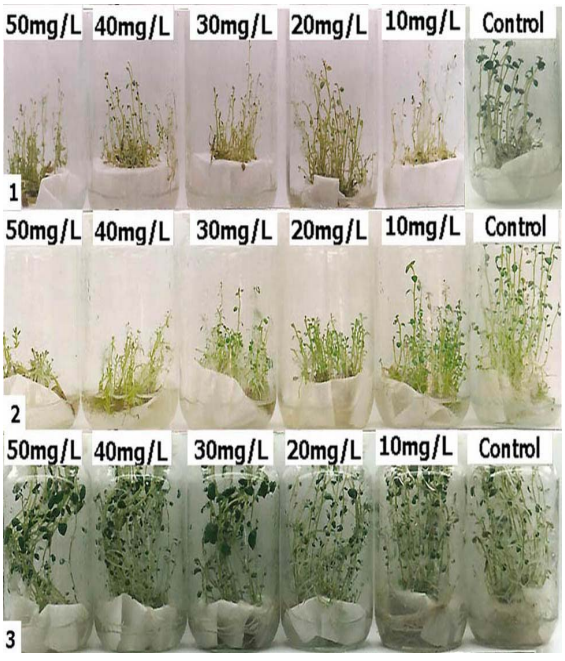


Figure 4. Micropropagation of plantlets on MS media treated with different concentrations of ASA.

1- Healthy plantlets treated with different concentrations of ASA., 2- PSTVd infected plantlets treated with different concentrations of ASA., 3- Re-subculture of plantlets on fresh media without ASA.

4. Discussion

The size 0.25 mm of meristem-tips was excised from PSTVd-$_{EG}$ infected potato plants cv. Diamond under stereomicroscope and was cultured on meristem-tip media**.** The meristem- tip was developed of complete plantlets with 75% the survival of potato plantlets.

Table 2. Elimination of PSTVd from infected tubers by thermotherapy and electrotherapy.

Treatment	Explant treated	% of survival plantlets	% of PSTVd elimination**
Thermotherapy.			
21°C / 4mon.	Sprout	14/14 (100%)	
	Meristem-tip	12/14	
	Sprout	(85.74%)	61.50
3-4°C / 4mon.	Meristem-tip	14/14 (100%)	77.7
	Sprout	10/14	71.4
	Meristem-tip	(71.4%)	100
5°C / 4mon.		14/14 (100%)	71.4
	Sprout	8/14 (57.1)	100
	Meristem-tip	14/14 (100%)	64.2
8°C / 4mon.		8/14 (57.1)	100
Electrotherapy.	Shoot-tip	(6/12) 50.0	(4/6) 66.7
(mA/min)		(8/12) 66.6	(8/8) 0
5/5		(8/12) 66.6	(8/8) 0
5/10		(6/12) 50.0	(0/6) 100
10/5		(5/12) 42.0	(0/5) 100
10/10		(6/12) 50.0	(0/6) 100
15/5			
15/10			

*****Plantlets survived/Total of treated samples.
** Plantlets PSTVd–free/Total Plantlets survived.

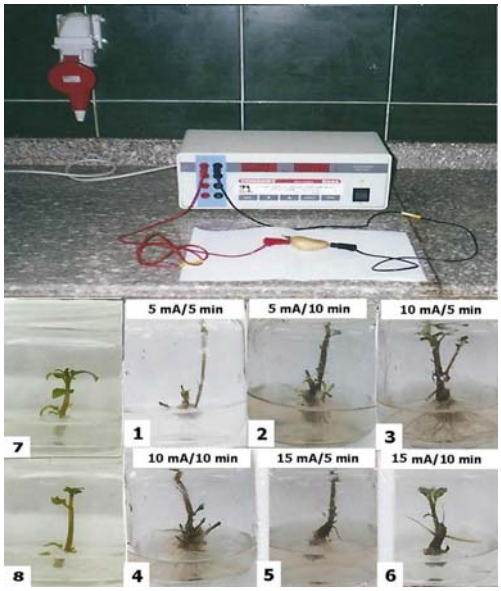


Figure 5. The electrotherapy procedure of PSTVd infected tubers and micropropagation of sprouts on MS media, 1-6) Sprouts of treated tubers cultured on MS media, 7) Sprout of untreated healthy tuber, 8) Sprout of untreated PSTVd- infected tuber.

As well as, the plantlets survived PSTVd-$_{EG}$-free were 83.33%, where as gave negative result against PSTVd-$_{EG}$ by dot-blot hybridization. The same results were obtained by (Stace-Smith, 1989 and Edriss *et al*., 1996) who mentioned that viroids were eliminated from shoot apical meristems with ranging size 0.2 to 0.5 mm long, but not obtained from longer ones. Lizáraga *et al*. (1982) and Salazar *et al*. (1985) showed that eradication of PSTVd was higher in the smaller tissue reactions and the higher percentage of eradication PSTVd was obtained from apical domes, followed by meristem-tip and in lower percentage from shoot-tips. They discussed this result where PSTVd no infection meristem cells since no phloem elements are found in apical domes and phloem elements disconnected from the rest of the plant vascular system are present in meristem-tips larger portions of apical tissue (like shoot-tips) already contain sieve tubes which are connected to the vascular system of the plant.

Three different anti-viroid compounds namely virazole (Ribavirin), 2-TU and ASA were incorporated individually into MS-medium with 10, 20, 30, 40 and 50 ppm. Potato explants (Nodal cuttings) were taken from PSTVd-$_{EG}$ infected potato plants cv. Diamond and were cultured on media. It was found that, using concentrations of 10, 20, 30, 40, and 50 ppm progressively increased the percentage of PSTVd-$_{EG}$-free plantlets for three compounds. But on the contrary, the percentage of survival decreased with increment concentration compared to the control. Thus, was found that Virazole proved to be somewhat phytotoxic in highest concentrations (40 and 50 ppm) and it causes stunting in plantlets, thin stem and stunted leaflets. The same results were obtained by many authors Kryczyński (1992) explained that the virazole inhibited viroid multiplication by RNA breaking. Also, they found that Ribavirin has been successful in reducing PSTVd concentration in *Scopolia sinensis* plants, but in shoot-tip culture or cuttings grown from Ribavirin-treated plants, viroid conc. rapidly reappears. Belles *et al*. (1986) showed that Ribavirin has been tested against viroids; it was active in eliminating *Citrus exocortis viroid* from *Gynura aurantiaca* as foliar applications. They found that concentrations. The lower than 30 mg L^{-1} was not effective, while phytotoxicity occurred at concentrations of 1600-2000 mgL^{-1}.

It was found that the highest concentrations 30, 40 and 50 ppm of 2-TU cause deleterious effects on growth of potato shoots. These results were similar with Commoner and Mercer (1951) who

reported that 2-TU (Pyrimidine analogue) is a powerful inhibitor of virus synthesis and replication. Also, Clerence & Agrawal (1972) mentioned that 2-TU affect on the metabolism of the plant itself; the young leaves become pale and apical growth is arrested**.** Kryczyński (1992) mentioned that amantadine, ribavirine and thiouracil used to eliminate viroids from infected plants.

In related to, ASA it was found that concentrations 20, 30, 40 and 50 ppm cause stunting of plantlets and concentrations 30, 40 and 50 ppm encourage formation of calli in some plantlets, while concentration 50 ppm induce formation of microtubers *in vitro***.** These results agreed with those reported by Malamy and Klessig (1992) who observed that ASA improve callus growth and/or regeneration in some culture media. Yu *et al*. (1997) found that ASA induce systemic acquired resistance (SAR). It has been proposed that SAR is mediated by an endogenous signal that is produced in the infected leaf and translocated in the phloem to other plant parts where it activates resistance mechaisms SAR is often induced by avirulent pathogens carrying an avirulence (*avr*.) gene. The production of an *avr* gene is recognized by the production of a resistance *R*-gene in plants (gene-for-gene recognition). In the case of viroid infection, however, involvement of *R*-genes is unlikely since viroids do not encode proteins. Therefore it will be of great interest to elucidate the PSTVd-activated pathway that is cross-linked with a SAR pathway to activate common down-strain genes encoding PR-1 and β 1, 3 glucanse (Itaya *et al*., 2002). Our results indicate that PSTVd-$_{EG}$ can be more successfully eliminated by treated PSTVd-infected plants cv. Diamond under conditions of low temperature and more efficiency when used a combination of low temperature treatment and subsequent meristem-tip culture. Thus showed that PSTVd-$_{EG}$ elimination percentage from sprouts were 61.50; 71.4; 71.4 and 64.2 at temperatures 21; 3-4; 5 and 8ºC respectively. On the other hand, meristem-tip excised from sprouts exposured for low temperatures 3-4; 5; 8ºC for 4 mons. were more efficiency for viroid elimination (100%) than in those at 21ºC/4 mon. (77.7%). Whenever, the percentage of sprout survival was 100% for meristem-tip was 85.7; 71.4; 57.1 and 57.1% for 21, 3-4; 5 and 8ºC/4 mons. The same result was obtained by (Lizàrraga *et al*., 1980). Hadidi *et al*. (2003) who mentioned that cold treatment of tubers, cuttings and plants must be pronged, even for mons. to increase the percentage of viroid-free plantlets, propably, the length of the treatment could be reduced when applied to *in vitro* cultured germplasm. The reduced size and tenderness of plant tissue could increase the effect of temperature allowing it to reduce the time of exposure. Also, the percentage of viroid elimination differed according to the isolate. Paduch-Cichel and Kryczński (1987) reported that prolonged a low temperature therapy (longer than 3 mon.) in eradication of PSTVd. The PSTVd-free plants were obtained from meristem-tips of sprouts from infected tubers after 6 months of therapy at 6 to 7ºC in the dark. The efficiency of 6 mon. therapy varied from 18.5 to 80% depending on viroid and plant material. A 3 mon therapy period at the same temperature proved to be too short.

Potato tubers cv. Diamond infected with PSTVd-$_{EG}$ were exposed to 5, 10 and 15 mA for 5; 10 min. followed by immediate planting the sprout *in vitro* then shoot-tips were excised and cultured on MS media. Temperature was increased from 4 to 10ºC in the tissue during the exposure to the electricity. After a 40 days growing period, electricity was influenced by the severity of treatment since organogenesis and viroid elimination were both stimulated by the electricity. The highest PSTVd-$_{EG}$-free values were obtained at 10/10; 15/15 and 15/10 mA/min. On the contrary, the survival rate decreases with increase electricity and treatment time. These results were compatible with that found by. Hadidi *et al*. (2003) mentioned that the viroids also may be eliminated if plants are treated simultaneously at low (2-5ºC) or high (37-38ºC) temperatures, especially if plants are exposed simultaneously to low irradiance lighting followed by shoot-tip culture. Also, Lozoya-Saldaña *et al*., (1996) who observed that *Potato Virus X* was eliminated by exposure potato stems to 5, 10 or 15 mA for 5 or 10 min. followed by immediate planting the axillary buds tips *in vitro*. Temperature increased from 4 to 10ºC in the tissues during the exposure to the electricity. After 60 days growing period, therapy efficiency (TE = % plant regeneration X % virus-free resulting plants) was influenced by the severity of treatment, since organogenesis and virus elimination were both stimulated by the electricity. The highest TE values were obtained at 15 mA for 5 min. under these conditions, 40% to 80% of the buds regenerated and 60% to 100% of the regenerated plantlets tested virus negative.

Corresponding Author:
Dr. Sherin Amin Mahfouze
Genetic Engineering and Biotechnology Division, Genetic and Cytology Department, National Research Center, El-Beehoth street, 12622, Dokki, Giza, Egypt
E-mail: Sherinmahfouze@yahoo.com

References

1. Belles JM, Hansen AJ, Granell A, Conejero V. Antiviroid effects of ribavirin on citrus exocortis viroid infection in *Gynura aurantiaca*. DC. Physiology Molecular Plant Pathology 1986:28: 61-65.
2. Belles JM, Granell A, Conejero V.. Impairment of viroid infection in *Gynura aurantiaca* plants by treatment with 2-chloroethylphonic acid (ethephon). Canadian Journal of Plant Pathology 1990:12: 175-179.
3. Clarence I Kado, Hari O. Agrawal. Principles and Techniques in Plant Virology, Van Nostrand Reinhold Company, New York, 1972 pp. 32-41.
4. Clark MF, Adams SAN. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of Plant Viruses. Journal of General Virology 1977:34: 475-483.
5. Commoner B, Mercer F. Inhibition of the biosynthesis of *tobacco mosaic virus* by thiouracil. Nature 1951:168:113-114.
6. Conejero V. Do viroid elicit host pre-existing mechanisms of response? IV International Conference on Comparative Virology. Control of Viral Diseases. Banff. Alberta, Canad, Abstract W 1982:12-1.
7. Edriss MH, Badawy MA, Fathi S, El-Barkouki TM. Propagation of potato using tissue culture technique. Acta Horticulture 1996:434: 413-418.
8. Epinoza N, Estrada R, Tovar P, Bryan JH. Cultivo de Tejidos: Micropropagación, Conservación y Exportación de germoplasma de papa. Documento de Tecnologia Especializada 1. CIP, Lima, Peru. 1985:18 pp.
9. Hadidi A, Flores R, John Randles W, Joseph S Semancik. Viroids-Casiro, Australia 2003:370 pp.
10. Helms K and Wardlaw IF. Movement of viruses in plants: long distance movement of *tobacco mosaic virus* in *Nicotiana glutinosa*. In: Transport and transfer processes in plants ,eds. Wardlaw, I.F. and J.B. Passiour, 1976:PP. 283-294. Academic Press, New York.
11. ICTV, (2008) (http://tak.ictvonline.org).
12. Itaya A, Matsuda Y, Gonzales RA, Nelson RS, Ding B. *Potato spindle tuber viroid* strains of different pathogenicity induces and suppresses expression of common and unique genes in infected tomato. Molecular plant microbe Interactions 2002:15 (10): 990-999.
13. Kryczyński S. Plant viroids in Plant. In: Plant disease of viral, viroid, mycoplasma and uncertain etiology. Published in India by Mohan Primlani for Oxford & IBH, 1992:pp. 45-65.
14. Lizárraga RE, Salazar LF, Roca WM, Schilde-Rentschler L. Elimination of *potato spindle tuber viroid* by low temperature and meristem culture. Phytopathology 1980:70: 754-755.
15. Lizárraga RE, Salazar LF, Shilde-Rentschler L. Effect of meristem size on eradication of *potato spindle tuber viroid*. In: Research for the potato in the year 2000. Proc. Int. Congress 10th Anniv, ed. Hooker, W.J. 1982:PP. 118-119. Int. Potato Center, February 1982, Lima, Peru.
16. Lozoya Saldăna H, Dawson WO. Effect of alternating temperature regimes on reduction or elimination of viruses in plant tissue. Phytopathology 1982:72: 1059-1064.
17. Lozoya Saldăna J, Albelló FH, Garciá de la RG. Electrotherapy and shoot tip culture eliminate *potato virus X* in potatoes. American Potato Journal 1996:73:149-154.
18. Malamy C, Klessig DF. Salicylic acid and plant disease resistance. The Plant Journal 1992:2(5): 643-654.
19. Owens RA, Hammond RW, Gardner RC, Kiefer CM, Thompson SM, Cross DE. Site-specific mutagenesis of *potato spindle tuber viroid* cDNA. Plant Molecular Biology 1986:6: 179-192.
20. Paduch C, Kryczyński S. A low temperature therapy and meristem tip culture for eliminating four viroids from infected plants. Journal of Phytopathology 1987:118(4): 341-346.
21. Ryan CA. Oligosaccharides as recognition signals for the expression of defensive genes in plants. Biochemistry 1988:27: 8879-8883.
22. Salazar LF, Schilde-Rentschler L and Lizarraga R. Elimination of *potato spindle tuber viroid* from potato by cold treatment and meristem culture. In: Subviral pathogens of plants and animals: Viroids and prions. The international potato center lima, peru. 1985: pp. 137-150.
23. Schnöelzer M, Hass B, Ramm K, Hofmann H and Saenger L. Correlation between structure and pathogenicity of *potato spindle tuber*

viroid (PSTVd). EMBO Journal. 1985:4(9): 2181-2190.

24. Singh KP. Piperonyl butoxide as a protectant against potato spindle tuber viroid infection. Phytopathology 1977:67: 933-935.

25. Stace-Smith R. Tissue culture. In: Plant viruses, Volume II Pathology CRC Press. Inc. United State 1989.

26. Yu D, Liu Y, Fan B, Klessing DF and Chen Z . Is the high basal level salicylic acid important for disease resistance in potato?. *Plant Physiol*ogy 1997:115, 343-349.

21/2/2010

The Environmental Impact of Industrial Agriculture: The Case of Mulindi Tea Plantations in Rwanda

JIWEN GE[1,2] , MUPENZI JEAN DE LA PAIX[1]*, GABRIEL HABIYAREMYE，JEAN DE DIEU BAZIMENYERA

[1]China university of Geosciences, Institute of Ecology and Environmental Sciences
School of Environmental Studies, 388 Lumo Road, Hongshan Administrative District,
Wuhan, Hubei 430074, China
[2]Hubei Wetland Evolution & Ecological Restoration Key Laboratory, 388 Lumo Road, Hongshan Administrative District,Wuhan, Hubei 430074, China
E-mail: jeandelapaixmup@yahoo.fr, gejiwen2002@yahoo.com.cn

Abstract: The aim of this study is to assess the impact of industrial agriculture on the environment in Rwanda taking at Mulindi tea plantations as a case study. Soil samples collected in three zones of Mulindi Valley were analyzed in the laboratory through PH Meter and the results showed that pH of all soil samples is less than 5 (pH<5), which implies that the soil in that valley is acidic. During this study, soil erosion caused by deforestation has been noticed and the sediments carried down and deposited in valley were became a peat after process of acidification. On the another hand, the analysis of water samples from the tank in polyethylene of three streams of Mulindi using spectroscopic techniques revealed a high concentration of elements like: Na, Ca NO^{3-}, H^{+}, H_2NO_3, Cu and S. and elements with low concentration : Fe, NO_3, K, and $al^{3.}$ This pollution may be due to agrochemicals used. Finally we proposed the methods which can be applied in the country in order to ensure a sustainable tea agriculture and better environmental conservation**.**

[JIWEN GE , MUPENZI JEAN DE LA PAIX, GABRIEL HABIYAREMYE，JEAN DE DIEU BAZIMENYERA. **The Environmental Impact of Industrial Agriculture: The Case of Mulindi Tea Plantations in Rwanda**. Journal of American Science 2010;6(12):1578-1590]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Environment,;Soil degradation,; Deforestation; Tea and Water pollution; Rwanda

1.Introduction

The environment which is defined as an area in which something exists or lives (WordNet3.0@ Princeton University, 2006) must be imperatively protected and every effort is important. In Rwanda, one of the smallest countries in Africa with a land area of 26 338 km^2 (with water: 1,390 km^2 and land: 24,948 km^2), many factors make it difficult to conserve the environment. The country's hilly topography is the reason why it is called a land of thousand hills. Most of the Rwandan area lies at high altitudes. The average elevation throughout the country is about 5,200 feet; 1,585 meters above sea level .The Rwandan topography (Figure1) includes steep hills, densely forested mountains, plateaus and savannas. The country has five regions which are: (i) The Vilunga mountains region in the north-west with five volcanoes: KALISIMBI the highest point of the country with 4, 507 m; BISOKE with 3,710 m; SABYINYO with 3,636 m, MUHABURA with 3500 m and GAHINGA with 3300 m. (ii) The Albertine Rift Valley region which is a branch of Great Rift Valley, it's located at the western border of country with an altitude between 3000 m and

3300m. (iii) The Congo-Nile Ridge with an altitude between 2500m and 3000m. (iv) The Central plateau which consists of gently hills with an altitude between 1500m and 2000m

(v) The Eastern savanna and Lowland of the south-west with an altitude between 900 m and 1500 m. (Thomas Streissguth , 2000)

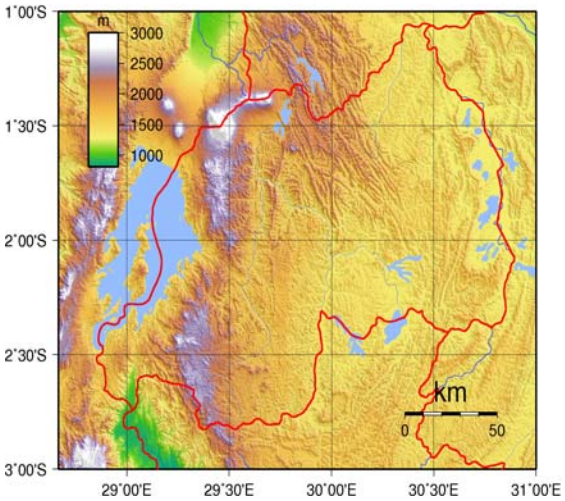


Figure 1 Topographic map of Rwanda (Wikipedia, 2009)

Another factor is the population density. Rwanda is one of the most densely populated countries in Africa with a population density of about 310 inhabitants per km^2 and an annual population growth rate of around 3.1% (MINISANTE/ONAPO,2003). In 1999, about 91% of her population earned their living, directly or indirectly, from agriculture; this is only applicable especially in some mountainside, in the alluvial valleys and in the volcanic soils of the northwest. About 1.1 million hectares (2.8 million acres) are under cultivation. Subsistence agriculture predominates, and the basic agricultural unit is the small family farm about one hectare (2.5 acres) (*nationsencyclopedia.com/Africa, 2009*). Rwanda's industrial agriculture is dominated by tea, coffee and Pyrethrum.

Tea agriculture requires a large area, in 1999 the total area for tea cultivation was 12,541 ha and 14,394 ha in 2008 with 1,407ha not exploited (Rwanda Privatization 2009, Lawrence w. Reed, 2002). Although Rwanda had made modest attempts to grow tea since the Second World War, it was only in the 1960's that the industrial cultivation of tea was really established.

Under the impulse of the FED, which considered tea as "a major agricultural opportunity" for Rwanda; the first tea unit was created in 1960 at Mulindi (Ex Byumba province actual Northern Province). Ten years later, already six tea units were functioning.

In 1964, the Office for Industrial Cultivation in Rwanda (Office des Cultures Industrialles du Rwanda - OCIR) was established, with the mission to manage the tea and coffee branches. Mulindi Tea plantation covers the largest area with 1,909 ha and its factory is the biggest in the country. Tea agriculture has positive and negative impacts on the environment. This is why this paper intended to provide a preliminary investigation of the environmental impact of industrial agriculture in Rwanda, particularly the case of Mulindi tea Plantations. This study shows the impact of tea plantation in Rwanda through the following factors: soil degradation, deforestation and soil erosion, air pollution and water pollution in Mulindi river, agrochemical use, tea agriculture methods, and finally it proposes the methods and techniques or strategies which can be used for conserving the environment and ensure sustainable development.

2. Materials and Methods

The study area: General state of Rwanda

The Rwanda is a small country located in Great lakes region of east central Africa, bordered by Uganda to the north, Tanzania to the East, Burundi to the South and Democratic Republic of Congo to the West where it is separated by Lake

Kivu and Ruzizi river (Siuberski, Philippe, 2008).This small country is also located near the centre of Africa, a few degrees south of the Equator. By this localization, the climate in Rwanda is cooled by the high altitude. It is warm throughout most of the country but cooler in the mountains. There are two rainy seasons: mid January to April and mid October to mid December (RICP, 2008). The capital, Kigali, is located in the centre of the country. In 2006, it was announced that they had located the longest headstream of the River Nile in Nyungwe forest (Team researches Nile's true source, 2006); the relief is mountainous with altitude declining from west to east. The country has few natural resources and its economy is based mostly on agriculture by local farmers using simple tools. It is estimated that 90% of the active population are in the agriculture and this sector occupied about 39.4% of GDP in 2006 (Special report on Rwanda, 97). Crops grown in the country include coffee, tea, pyrethrum, bananas, beans, sorghum and potatoes; but tea and coffee are the major cash crops for export (WTO Doha Round Bulletin , 2004)

Brief story of tea agriculture in Rwanda

Tea growing was introduced in Rwanda as an industrial crop and purely for export purposes to generate foreign income as early as 1960. Black tea manufacturing followed in 1965 at Mulindi tea factory in the actual Northern Province. Since then the tea sector has become the most important source of export earnings after the coffee market plunged in recent years. Tea is now number one export earner; contributing up to 34% of the total national exports. Today, the tea sector in Rwanda consists of six state owned production units, Gisovu, Kitabi, Mata, Mulindi, Shagasha, Gisakura and four private owned production units, Cyohoha (SORWATHE), Pfunda (Pfunda Tea Company), Nyabihu and Rubaya (Rwanda Mountain tea). Nshili-Kivu is another private owned production unity with a factory which is still under construction. The current tea sector consists of 10 tea factories, 8 governments owned plantations known as Blocs Industrials (BI), 3 tea cooperatives known as Coopthés and 11 tea small holders associations known as Thé Villageois, spread in the country as indicated in Table 1.The total planted area is 12,989 ha but only 12,862 hectares are exploited. The factories installed capacity is 15,500 tones of made tea per annum. The tea sector provides employment to 52,838 people, tea farmers and workers together (OCIR –THE , 2006)

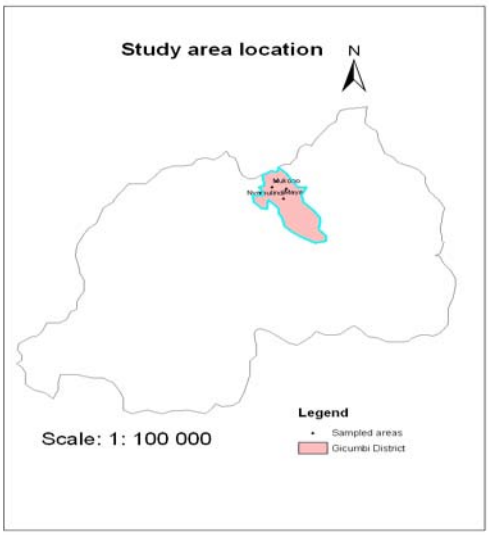


Figure 2 Study Area locations

Mulindi tea plantation is located in Gicumbi district in Northern Province and is home to about 47,000 people. Its capital is Byumba, which is also the ex provincial capital. The district lays due-north of Kigali, straddling the major road from Kigali to Kampala. It is a hilly district and is divided into 21 sectors called Imirenge in local language (Francis, 2008). Tea plantations are located in eleven Administrative sectors among 21 sectors of Gicumbi district which are: Kaniga in which Mulindi tea factory is located, Mukarange, Cyumba, Rushaki, Bwisige, Nyankenke, Manyagiro,

Byumba, Rubaya, and Shangasha. Mulindi tea factory is located at 14km to Gatuna board and at a 1 hour journey from Kigali and at 5 km from Kigali-Gatuna road in Kaniga Sector. The factory is the biggest in terms of production but as it does not have its own plantations; it depends on COOPTHÉ and Village's Tea which is called COOTHEVM for its green leaf supply. The COOPTHÉ constitute 35% with 585 ha exploited, and Village's Tea, 55% with 1150 ha.

The industrial block has only 174.4045ha; it means a total of 1909.4045 ha. All these tea plantations are distributed around ten agriculture sectors (Nyamulindi, Rushaki I and II, Bushara, Kaniga, Maya, Ngondore, Muturirwa, Rubaya and Mukono). About 90% of this area is located in valley or swamp and 10% only is located on versants of mountains. The factory was built in 1962 with capacity of 3200T. During 1994-1996 the factory and tea plantations were rehabilitated following the war. The factory only has 224 ha of woodlands that are not sufficient, the reason why the factory is obliged to buy the wood fuel from the population around.

Methods

The first phase of this study used a survey among the governmental and private tea factories through different plantation management companies (Coopthe, village's tea and estates tea plantations which called B.I).

During this phase, a questionnaire was used to collect information about different activities carried out in tea plantations such as plucking, drainage, pruning, fertilization etc. This questionnaire was sent to 11 tea plantation managers, representing the total number of the tea factories in the country. This questionnaire was also helpful in getting information concerning pesticides and types of fertilizers used in Rwanda's tea plantations. It was also designed in order to identify different techniques used to fight against erosion and to protect the environment. Lastly the questionnaire was used to get information about the evolution of tea production. All tea plantation managers submitted their completed responses.

The second phase of this project concerned particularly Mulindi tea plantation, where we took soil samples by dryer (or Terri ere in French) on three locations which are Nyamulindi, Maya and Mukono for getting information relating to soil degradation through laboratory analysis using pHMeter. We also took water samples by tank in polyethylene and we put on bottles very clean for a good transportation from three locations of Mulindi streams (Nyamulindi, Maya and Mukono) for analyzing and quantifying water pollution resulting from tea preparation activities in laboratory.

These water samples were acidified with purified nitric acid and were analyzed through atomic mass spectroscopy. The measure of pH in the solution used the followings elements: ammoniumacetae ammonia (pH 6-8), sodium acetate- acetic acid (pH 3-6), and hydrochloric acid-glycine (p h 1-3).

The analysis of the relationship between production and utilization of wood fuel in factory has been carried out using SPSS, Origin pro 7.5 and excel. The deforestation in that region is remarkable, and sediment deposition and different floods was a good indicator of the presence soil erosion in region.

The water samples taken in three streams of Mulindi River using the tank in polyethylene before rinsed with ultra pure water and it was taken 1L by location. This study started from 1st April 2008 and it was planned to get all responses of the questionnaire by 30th April 2009; by chance, all the respondents submitted their responses on time. The final report was completed by the 20th June 2009

3. Results

State of tea plantations and Evolution of production.

Tea plantations in Mulindi region are divided in two categories. A big part of all tea plantations around 90% are for private sector which are called Cooperatives with COOPTHE and COOTHEVM, and the small part of tea plantations (10%) which are called BI are controlled by Tea factory headed by Rwanda Tea Authority (Ocir the). Coopthe Mulindi is a cooperative with one thousand members and produces about 35% of green leaves. Tea plantations in this Cooperative get a special treatment like regular sarclage, good table of plucking and regular drainage. It is the same for the small plantations under the control of the Tea factory.

The Coothe.VM is a villages' cooperative with around three thousands members and every member has his field and he is responsible for all activities except plucking and transport.

The coothevm produces about 55% of green leaves. In general, all tea plantations in these villages' cooperatives do not receive good services and support such as drainage, plucking and pruning. Most of Mulindi tea plantations are composed by the stamps and few clones from Kenya. About 5000 workers are employed in tea plantations including those who work in the tea factory in different activities; accounting for about 10.64% of all people of Gicumbi District and 25% the active population of that District. Tea plucking is the first activity which employs many works, about 3500. Others activities (pruning, drainage) are occupied by about 1500 people. In Mulindi valley composed by Maya, Mukono, Nyamulindi, Ngondore and Muturirwa, they are many empty spaces caused by erosion and regular floods between April and May every year.

Table 1. Tea growing areas and their location in Rwanda

				state factories				
Factories	localization	started	state	coopthe	Vill.tea	BI	PRIVATE	TOTAL
MULINDI	N-Gicumbi	1962	Swamp	867	1428	3	0	2298
SHAGASHA	W-Nyamasheke	1969	Hill/swamp	515	1033	7	30	1585
GISAKURA	W-Nyamasheke	1975	Hill/swamp	608	330	357	0	1295
MATA	S-Nyaruguru	1981	Hill/swamp	0	475	610	0	1085
KITABI	S-Nyamagabe	1977	Hill	0	650	350	0	1000
GISOVU	W-Karongi	1983	Hill	0	732	340	0	1072
Sub-total				1990	4648	1667	30	8335
			private factories					
SORWATHE	N-Rulindo	1978	Swamp	0	880	0	261	1141
PFUNDA T.C	W-Ngororero	1972	Hill/swamp	0	786	0	124	910
RUBAYA	W-Ngororero	1979	Hill	0	342	0	647	989
NYABIHU	W-Nyabihu	1950	Hill/swamp	0	31	0	627	658
SHILI-KIVU	S-Nyamagabe	1983	Hill	0	225	0	731	956
Sub-total				0	2264	0	2390	4654
National Total				1990	6912	1667	2420	12989

Table 2 Production, wood fuel, fertilizers, area in Mulindi Tea Plantations from 1996 to 2008

Years	Green leaves/kg	wood fuel used/T	fertilizers/kg	Area/ha
1996	3282152	3090	0	921.4
1997	3241815	9115	0	921.4
1998	4757300	11300	0	1026.9
1999	8853154	6847	165662	1614.7
2000	9238657	7732	153413	1642.2
2001	13750123	14406	450000	1772.5
2002	10072611	10629	370800	1762.5
2003	9286082	10896	166850	1770
2004	8731340	10581	470700	1909.4
2005	11271253	12492	470800	1909.4
2006	11148716	11945	235574	1909.4
2007	13904538	15836	514616	2298
2008	13678308	12774	370385	2298
Total	121216049	137643	3368800	

Table 3 Concentration of H^+, Cu, H_2NO_3, Al^{3+}, NO_3, NO_2, Mg, Ca, Fe, K, P, and S in Mulindi streams (mg/L) in 2008

Samples no	Na	H+	cu	H_2NO_3	Al^{3+}	NO_3	NO_2	Mg	Ca	Fe	K	P	S
1.Nyamulindi	31.85	26.4	13.2	13.1	2.06	1.14	20.3	12.2	46.1	0.18	3.8	0.26	20.2
2.Maya	28.3	38.6	6.3	11.6	3.15	1.64	19.6	13.1	51.9	0.14	9.3	0.65	18.9
3.Mukono	33.3	21.9	8.4	10.8	4.04	0.96	26.4	14.5	41	0.09	8.1	0.48	19.3

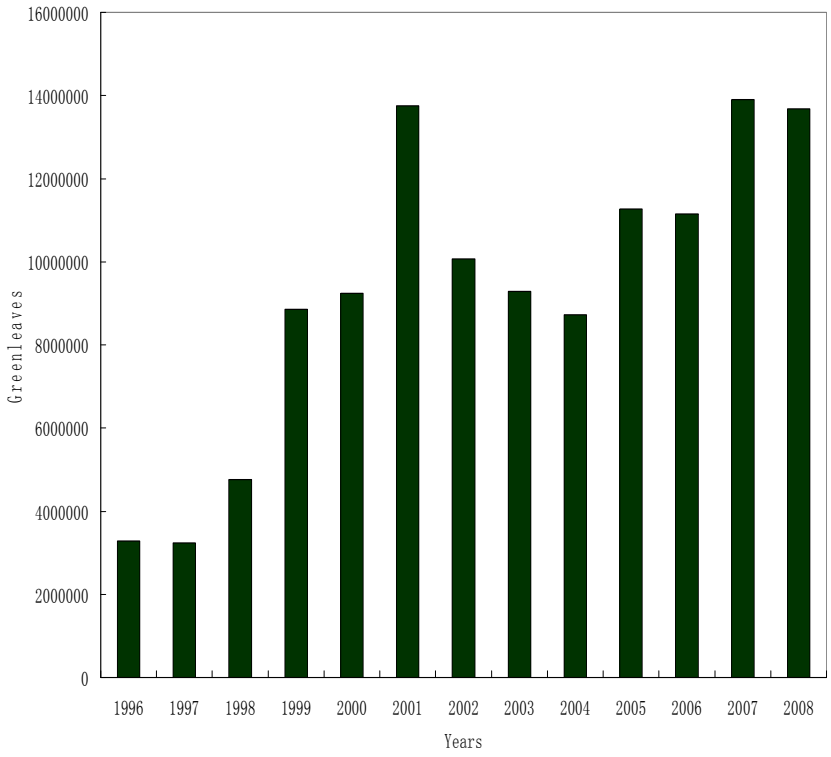


Figure 3 Evolution of tea Production / kg

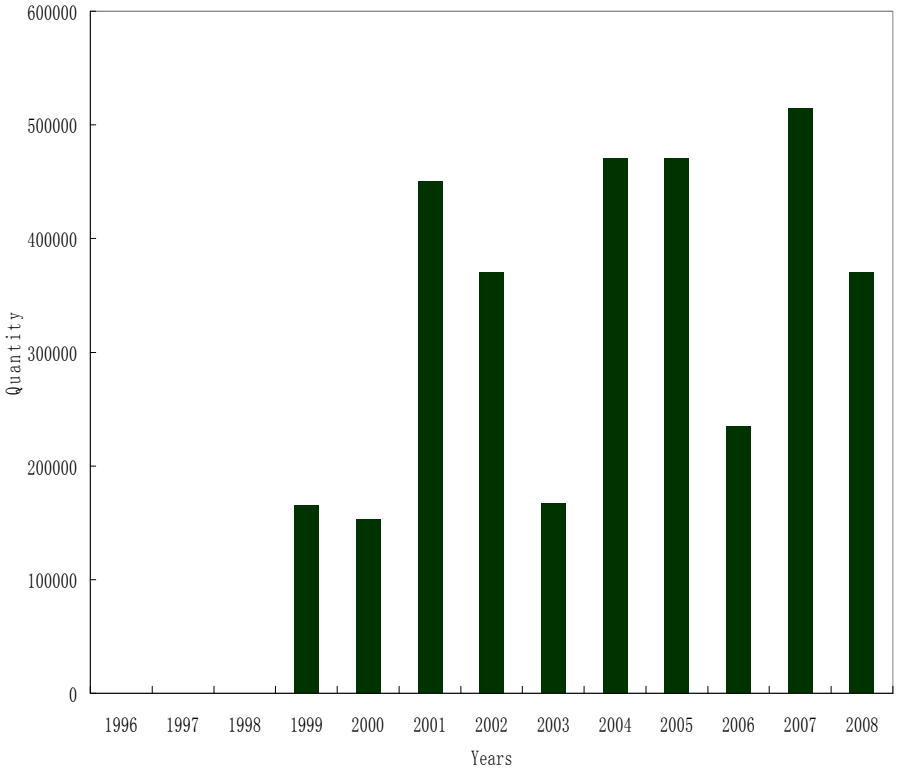


Figure 4 Fertilizers (NPK) used at Mulindi Tea Plantations from 1996 to 2008

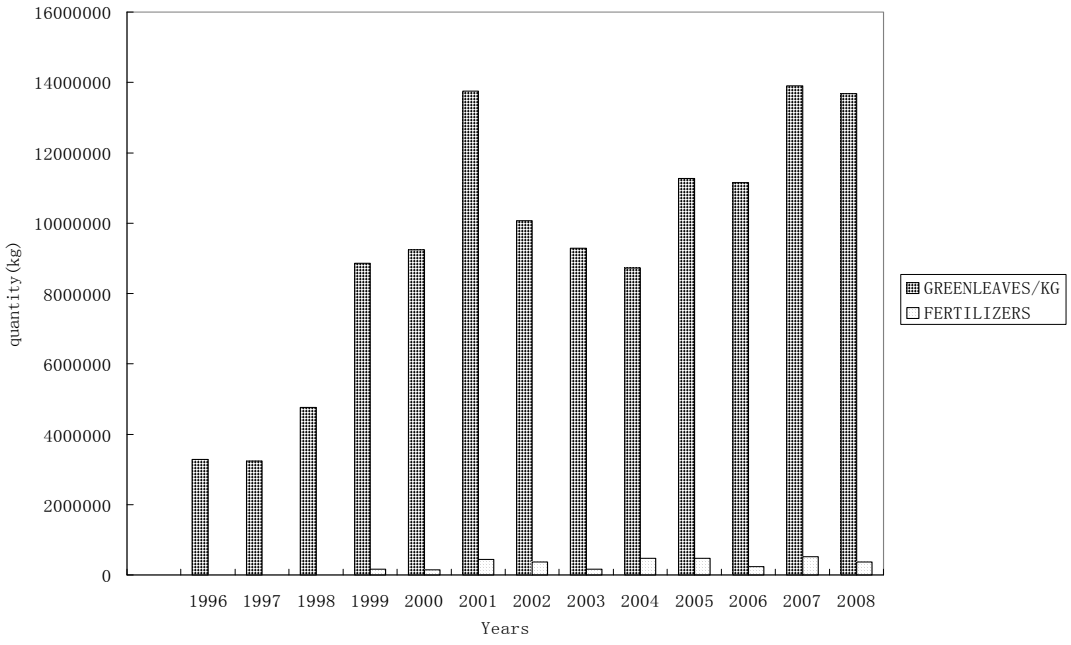


Figure 5 Relationship between product and fertilizers

Analysis of Pollution

Figure 6 A floods in Rebero land in September 2008

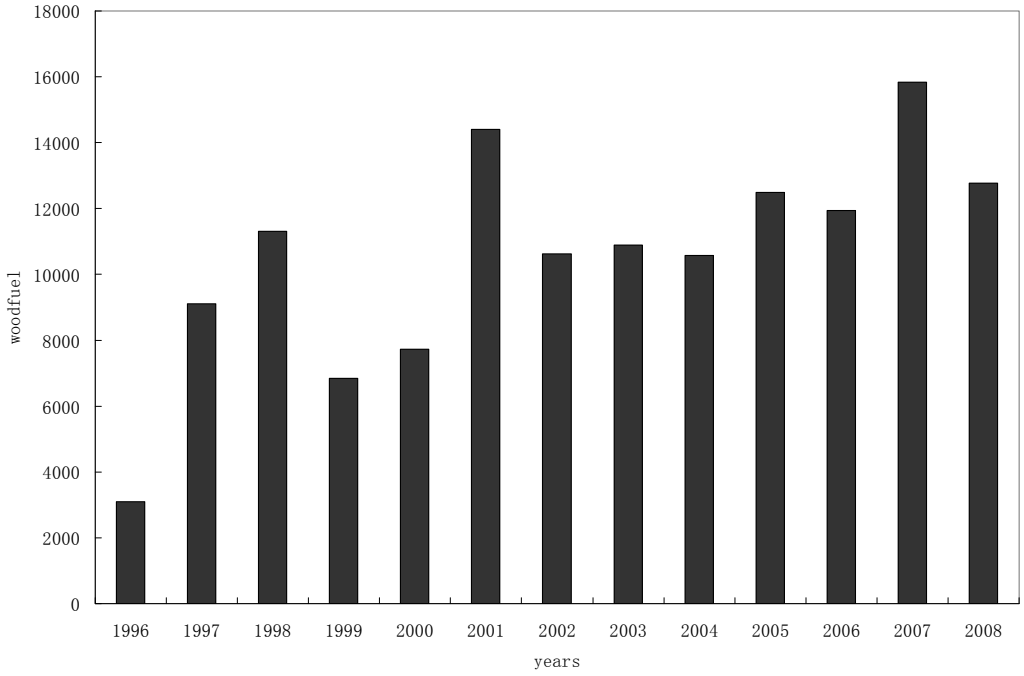


Figure 7. Evolution of Wood fuel (on tones) used at Mulindi Tea Factory

4. Discussions

During 13 years from 1996 to 2008, the production of green leaves at Mulindi tea plantations (table 2) increased by 10396156kg (316.74816%). During that period the using of wood fuel had an increase of 9684tones or 313.39806% while the use of fertilizers increased from 0 kg in 1996 to 370385kg in 2008. The total area was around 1900ha in 1996 but only 921.4ha were rehabilitated, and it was 2298ha in 2008 including the young tea and empty area inside of tea plantations but only 1909.4045ha give a production. By the Figure3 it was noticed that the years 2007, 2001 and 2008 were very productive with the

following production respectively 13904538 kg in 2007, 13750123 kg in 2001 and 13678308 kg in 2008.The good climate with enough rainfall, high quantity of fertilizers and good plucking are the favorable factors which can explain that increase of production. Also we found that the first three years from 1996 to 1998, had low production of green leaves because of it was a short period after genocide; many tea plantations were not taken care, few workers and it was difficult for the country to get fertilizers

Agrochemical: The use of chemical fertilizers led to the decline of soil fertility (Fared, 1996). To determine the impact of fertilizers in addition to laboratory experiment, in this study, we tried to use previous studies carried out in other study areas. For example, studies in India have shown that as much as 70% of soil biota has been lost on tea plantations The Figure 4 show that the use of fertilizers at Mulindi Tea Plantation was not regular; this can be explained by the following factors: Some farmers cannot afford the cost of fertilizers while others lack knowledge about the importance of fertilizers to nearby natural habitat, especially in areas that workers and machinery pass over (Senapati et al. 2002). In general, Rwanda tea plantations and at Mulindi in particular, they use NPK 25-5-5 +1Mg and NPK 26-6-8 +2Mg very frequently as fertilizers. Other pesticides which are used in different tea plantations are Round up for eliminating of weed; urea, methane and dime thane for young tea or in nurseries. .It was found through the Fig. 5 that the high quantity of fertilizers implies high productivity of green leaves. During the three most productive years 2001, 2007 and 2008, the use of fertilizers was also at very high rate, carried out in other study areas. For example, studies in India have showed that as much as 70% of soil biota has been lost on tea plantations as compared to nearby natural habitat, especially in areas that workers and machinery pass over (Senapati et al. , 2002). In general, in Rwanda tea plantations and at Mulindi in particular, the farmers use NPK 25-5-5 +1Mg and NPK 26-6-8 +2Mg frequently as fertilizers. Other pesticides which are used in different tea plantations are Round up for eliminating of weed; urea, dithane and dime thane for young tea or in nurseries

Soil Degradation at Mulindi was among the Main objectives of this study; for itsmeasurements, we used pH as indicator. For that case pHMeter was used to analyze the soil acidity. It was found that the pH of soil samples collected on three locations of Mulindi valley was 5.2 at Nyamulindi, 4.8 at Maya and 4.6 at Mukono. This means that the pH of soil in all Mulindi valleys is less than 7 and all soils are classified among the acidic soils. This acidic of soil can be explained by the following factors. First, the rock in which the soil came from, Mulindi valley belongs to the sedimentary rock. And then the using of fertilizers with NO_3^+ and an augmentation of aluminums ions and H^+ in soil. (www.document: calibrate a pHMeter, 2009). .

The concentration of major elements in water at Mulindi valley varied by site. The result in table 3 showed that the concentration of Na, H^+, H_2NO_3, Al^{3+}, No_3, NO_2, Mg, Ca, Fe, K, P, and S were important in all sites with 31.85, 26.4, 13.2, 13.1, 2.06, 1.14, 20.3, 12.17, 46.12, 0.18, 3.8, 0.26, 20.18 respectively at Nyamulindi, with 28.3, 38.6, 6.3, 11.6, 3.15, 1.64, 19.6, 13.05, 51.87, 0.14,9.3, 0.65, 18.9 respectively at Maya, and with 33.3, 21.9, 8.4, 10.8, 4.04, 0.96,26.4, 14.46,41.04, 0.09, 8.1, 0.48, 19.3 respectively at Mukono. The study also showed that the concentration of Fe, NO_3, K, Al^{3+} were no important than other elements in all Mulindi valley, but the concentration of Ca, Na, H^+, NO_2, S, H2NO_3 and cu ere highest in all valley and this concentration indicate that the water in Mulindi Valley are acidic and the pollution has its origin from oxide of nitrate (NO_2) and the use of many fertilizers with high level of MPK+ Mg . Note that this instrument (experimental) technique has been

used by many researchers in environmental studies such as (Filgueiras et al; 2002) and several schemes are existing (Smichowski et al; 2005).

Deforestation impact on Soil Degradation

Soil degradation is also associated with off-site problems of sedimentation, carbon emissions affecting climate change (Smyth and Dumanski, 1993). Soil degradation accelerated by erosion is a big problem in Rwanda and the erosion is caused by not only the topography of the country but also by deforestation. At Gicumbi district, particularly on the mountains around Mulindi where the climate is classified as tropic, we observed serious erosion caused by deforestation. From 1996 to 2008, the use of wood fuel in Mulindi tea factory was increasing as the production increased. Because of deforestation for example, over the years, the lands of Rebero (Figure 6) one among regions surrounding Mulindi tea plantation in Gicumbi District north of the Rwanda were washed away by rainfall. The study in India showed that the production of eucalyptus can vary from 11.9T/hm^2 in three year old plantation to 146T/hm^2 in 9 year old plantation in moist region and from 5.65t/ha in 5 year plantation to 135.5T/ha in 9 year old plantation in dry tropic region (Vijan Rawat, et. al, 2004) From 1996 to 2008, Mulindi tea factory used around 137 643 T of wood fires of eucalyptus, and it was noticed that the production of eucalyptus was 170t/ha for 7 year old plantation; it means that 809.66471ha of forests were cut in 13 years. If we consider that a forest is able to give a good production after 7 years, it is clear that an area equals to 404.83235ha of forest were cutting after 7 years; Its means that 57.833193 ha of forest were cutting every year for only Mulindi tea factory. Because of the deforestation we found a high quantity of peat in all valleys which surrounding Mulindi tea plantations caused by soil erosion through many types of sediment from the mountains. Mulindi tea factory has 227ha of forest and the government of Rwanda tried to mobilize its population about forest protection and environmental protection. In order to prevent soil erosion, this government tried also to mobilize its people to plant trees as a necessary call to restore the lost forest cover in Rwanda. Selected seedlings are planted in all provinces of the country by environmentalists in collaboration with all stakeholders and local community (Ministry of Lands, Resettlement and Environment, 2003).

This study through Figure 7 shows that the increase of quantity of wood fuel used is highly influenced by the increase of tea production and the deforestation is among the crucial problems that affect conservation in developing countries in which Rwanda is included. The human pressure on natural resources, poverty, low education level and lack of integration of local population in environmental protection activities are the major barriers for environmental conservation in those countries. Note that the use of wood fuel is not only for the tea factories but also in general the rural population use wood fires in preparation of their food. Also wood fires are used by different institutions like schools, hospitals and prisons. In Gicumbi district the use of high quantity of wood fires was also identified in GIHEMBE Refugees Camp.

5. Conclusion & Recommendations

Industrial tea agriculture introduced in Rwanda from 1960 has a negative impact on environment .The soil and water samples taken from Mulindi tea plantation indicate that this part of the country has been polluted by different contaminants.The fertilizers used in the tea plantations were the cause of soil degradation and through those fertilizers the water was polluted. Also the deforestation resulted in soil erosion which led to floods and the deposit of sediments on the valley. For that case, some recommendations have been proposed. First, the government should apply

lime on Mulindi tea plantations and on other tea plantations which have big quantities of peat. Second, anti-erosion canals should be dug around all tea plantations which are located on mountainside. Third, find alternative sources of energy for treating tea other than wood fuel in order to protect the environment. And finally to proceed by analysis of all rivers streams in order to ensure the quality of drinking water.

ACKNOWLEDGEMENTS

Our thanks go directly to the Government of Rwanda for their financial support, to National University of Rwanda and China university of Geosciences for laboratories, Prof Bukome Itangwa and Dr. Habumugisha Jean de Dieu (NUR) for their helpful comments and suggestions.

Abbreviations

1. B.I: Blocs Industrials

2. COOPTHE: Cooperative theicole or Cooperative of tea cultivation

3. COOTHEVM: Cooperative du the villagois de Mulindi or Villagois tea cooperative of Mulindi

4. Dr: Doctor

5. FED: Federalism

6. Fig: Figure

7. GDP: Gross Domestic Product

8. Ha: hectares

9. Mg: Magnesium

10. **MINISANTE**: Ministere de la santé/Ministry of health

11. N.P.K: Sodium phosphate potassium

12. NUR: National University of Rwanda

13. OCIR: Office for Industrial Cultivation in Rwanda

14. ONAPO: Rwanda's national office of Population

15. P h: potential of hydrogen

16. RICP: Rwanda Investment Climate Project.

17. SORWATHE:Rwanda society of tea

18. T: Tones

References

1. Almeida, R.R et. Al. *Veneer production from Eucalyptus grand is & Eucalyptus Urophylla hybrid clones wood. Forest Production Specia*l, Dehradun / Indian 2004:5(2) 53-76.
2. Climate zone.com. Climate information of Rwanda: Energy Foe Development; The World Bank Perspectives 2008 and beyond. *ESMAP -ASTAE, Washington* 2008: 15-30
3. FAO and WFP (Special Report on Rwanda, July 1997, *Crop and Food supply Assessment mission to Rwanda*. Retrieved on 2009.02.04. 1997: 16-32.
4. 4. Filgueiras, A.V, Filgueiras, I. Lavilla and C.Bendicho et al; Chemical sequential extraction for metal partitioning in environmental solid samples. DOI: 10.1039/b20.1514c 2002: 720-823
5. Francis. ICT Dream to the rural communities thanks to telecentres. Kigali. 2008: 1-2
6. Lawrence w. Reed. A Privatization Revolution-in a Most Unlikely Place. *General Article*. Mackinac Center 2002: 2-3
7. MINISANTE/ONAPO Politique Nationale de la population pour le developpement durable au Rwanda. Ministry of health/National office for Family planning. *InterScience,* Kigali /Rwanda 2003 : 8-16
8. Ministry of Lands, Resettlement and Environment. National environment policy in consultation with other state institutions, *United Nations Agencies, NGOs and civil society*. Kigali, 2003: 2-3.
9. Nationsencyclopedia.com/Africa. Rwanda agriculture. 2009:2
10. OCIR-THE. A New tea sector strategy for Rwanda year 2005-2010. *Tea strategy***.** Kigali 2006:2-12.
11. RICP. Rwanda climate, weather, Temperature,

Rainfall. *World Travel Guide*. Kigali 2008:1-2.

12. Rwanda Privatization. Tea special in our magazine. Rwanda privatization No11.Kigali 2009:6.
13. Samuel Kanyamibwa. Impact of war on conservation: Rwanda environment and wildlife in agony. *Biodiversity and Conservation* 1998:7(11): 1399-1406.
14. Senapati, S., Keiper, J. S., DeSimone, J. M., Wagnall, G. D., Melnichenko, Y. B., Frielinghaus, H. and Berkowitz, M. L. Structure of Phosphate Fluor surfactant Based Reverse Micelles in Supercritical Carbon Dioxide. *Langmuir* 2002: 18(2): 7371.
15. Siuberski, Philippe. Women run the show in a recovering Rwanda. *Washingtonpost.com Woman's World* 2008: 01.
16. Smichowski, P., G. Polla and D. Gomez et al;. Metal fractionation of atmospheric aerosols via Sequential Chemical extraction: *A review, Anal. Bioana*l. Chem. 2005: 381:302-316.
17. Smyth and Dumanski. Cited Ivan Good body, Elizabeth M. Thomas-Hope (1994) Natural Resource management for Sustainable Development in the Caribbean. *Barbadon. Jamaica. Trinidad* 1993: 323-372.
18. Team researches Nile's true source BBC. News (2006) Retrieved on 2006.12.04.
19. Thomas streissgnth Juvenile Nonfiction. *Rwanda in picture*. 2007: 70-8
20. Vijay Rawat, Negi, J.D.S. Biomass production of *Eucalyptus tereticornis* in different agro ecological regions of India. *Cahiers Agricultures* 2004: *130 (7*): 6-12.
21. Wikipedia,The free encyclopedia. Geography of Rwanda: *Topographic map* of *Rwanda*. Satellite image of *Rwanda*, generated from raster graphics data supplied by The *Map* Library. 2009: 2-3.
22. WTO Doha Round Bulletin. Continued reforms and technical assistance should help Rwanda in its efforts to achieve a dynamic economy: Trade policy Review: *Rwanda. World trade organization 2004:* 2-3.

1/11/2010

Antimicrobial Activity Of *Waltheria Indica*

[*]Zailani, A. Hauwa , [#]Jada, S, Mahmud and [+]Wurochekke, U Abdullahi.

Department of Biochemistry, Federal University of Technology, P.M.B,2076 Yola, Adamawa State, Nigeria.
+wchekke@yahoo.co.uk; #amjnennelushi@yahoo.com; *howwrkoulou@yahoo.com

Abstract: *Waltheria indica* is used in Nigeria traditional medicine for the treatment of diarrhoea, infertility, skin diseases, gonorrhea and for relieving pains. Phytochemical analysis revealed the presence of steroids, tannins, saponins, and cardiac glycosides in all parts of the plant; flavonoids were detected only in the leaves and stem, while terpenoids and alkaloids were detected in the leaves only. No part of the plant showed the presence of anthraquinones. Antimicrobial activity of different parts of the plant on *E.coli, Pseudomonas aeroginosa and Salmonella typhi* showed the leaves having highest activity against *E.coli and pseudomonas aeroginosa* with the stem having the lowest activity against the three organisms. Column chromatography of crude extracts of the leaves gave fractions I, II and III that were eluted with ethylacetate/methanol benzene/methanol and acetic acid/methanol respectively. Of these extracts, fraction III showed highest activity against *E.coli and Salmonella typhi.* These findings support the traditional use of the plant as an anti diarrhoeal agent.
[Zailani, A. Hauwa , Jada, S, Mahmud and Wurochekke, U Abdullahi. **Antimicrobial Activity of *Waltheria Indica***. Journal of American Science 2010; 6 (12):1591-1594]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: *Waltheria indica,* phytochemical analysis, antimicrobial activity, *E.coli, Pseudomonas aeroginosa,* and *Salmonella typhi.*

1. Introduction:

The plant *Waltheria indica* belongs to the family *Sterculiacae*. It is widespread in West Africa (Akobunda and Agyakwa, 1998). Locally, the plant is called 'hankufah' or 'hankubah' in Hausa, 'kafafi' in Fulfulde, 'korikodi' in Yoruba and 'efu-abe in Nupe (Hutchinson and Dalziel 1958; Irvine, 1961). The uses of the plant are diverse; it is used in Northern Nigeria by the Hausas for the treatment of skin diseases, impotence, and infertility, as an aphrodisiac, and as children's medicine at birth and during teething (Mohammed *et al.*, 2007). In the Fulani community, the aqueous extract of the root is used in relieving aches and pains during the 'Sharo' festival. Among the Yoruba, the aqueous extract of the root and stem is used in treating syphilis, internal haemorrhage, and as a restorative during the labours of harvesting (Mohammed *et al.*, 2007). Yerra *et al.*, (2005) investigated the inhibitory effects of the flavonoids isolated from *Waltheria indica* on the production of nitric oxide (NO), tumor necrosis factor alpha (TNF)-a and interleukin 12 (IL)-12, the study supports the use of the plant for the treatment of inflammatory diseases in traditional medicine. The extract of *Waltheria indica* was among the six plants from Northern Cote D'voire that showed promising *in vitro* antibacterial activity against *pneumococcus* including strains resistant to penicillin (Kone *et al.*, 2007). The analgesic activity and/or anti-inflammatory effects have been reported with flavonoids as well as tannins content of the plant (Ahmadiani *et al.*, 1998). Here we present the antimicrobial activity of the crude and partially separated extract of the plant.

2. MATERIALS AND METHODS

Plant material

The whole plant of *Waltheria indica* was collected from uncultivated farmlands located at Yola South Local Government, Adamawa State, Nigeria. The plant was identified and authenticated at the department of Biological sciences, Federal University of Technology, Yola, Nigeria.

Micro-organisms

The micro-organisms used were obtained from the Department of Microbiology, Federal University of Technology, Yola, Nigeria. The organisms include: *E.coli, Pseudomonas aerogenosa,* and *Salmonella typhi.*

Extract preparation

The different parts of the plant namely: leaves, stem and root were removed from the whole plant and air dried separately at room temperature for five days. The dried parts were ground to powder and 50g of each was macerated in distilled water and left overnight. The mixtures obtained were filtered and evaporated using a rotary evaporator.

Phytochemical analysis

Chemical tests were carried out on the aqueous extract and on the powdered samples using standard procedures to identify the constituents as described by Sofowora (1993); Trease and Evans (1989).

Antimicrobial activity testing

The crude extract and individual components were tested using the method of Emeruwa (1982). Wells were made on the surface of 19ml nutrient agar plates previously seeded with 0.1ml of 10^{-6} test organisms. 0.5ml of each extract was aseptically introduced into the wells made. 0.5ml of distilled water was used as control in a separate well. The plates were allowed to stand on the work bench for 30 minutes and were then incubated for 24 hours at 37^{o} C in an incubator. The presence of zone of inhibition was regarded as the presence of antimicrobial activity. From the inhibition zones, the antimicrobial activity was expressed in terms of average diameter of the zone of inhibition that was measured.

Separation of crude extract

The method of Nok *et al*., (1993) was used. Slurry was prepared by dissolving 30g silica gel in 100ml methanol: water (1:1) and packed in a column (1.5x30cm). The column was loaded with 15m of the crude extract and sequentially eluted with ethyl acetate/methanol (19:1) benzene/methanol (9:1) and acetic acid/methanol (1:1). The fractions were collected separately and concentrated under pressure using a rotary evaporator.

Result

Phytochemical analysis:

The screening of leaves, stems, and roots of *Waltheria indica* using aqueous extract and powdered samples was carried out to determine the presence of tannins. Saponins, flavonoids, steroids, alkaloids, terpenoids, cardiac glycosides and anthraquinones. The results are presented in table 1.

Antimicrobial activity of crude extracts

The antimicrobial activity of crude extracts of leaves, stem and root were tested on *E.coli, Pseudomonas aerogenosa, and Salmonella typhi.* The diameter of zone of inhibition is summarized in table 2.

Table2: Diameter of zone of inhibition of crude extracts (mm).

Chemical constituents	leaves	stem	root
E.coli	14	10	11
Pseudomonas aerogenosa	10	10	12
Salmonella typhi	12	8	10

Antimicrobial activity of the separated eluents of the leaves.

The recovered extracts from the column weighted 0.5, 0.6 and 0.5g for fractions I, II and III respectively. The antimicrobial activity of the separated eluents of the leaves is summarized in table 3 below:

Table 3: Antimicrobial activity of the separated eluents of the leaves.

	Diameter of zone of inhibition (mm)		
	Fraction I	**Fraction II**	**Fraction III**
E.coli	9	8	10
Pseudomonas aerogenosa	8	12	9
Salmonella typhi	8	7	20

Control (distilled Water) = 4mm
Fraction I=and ethyl acetate/methanol
Fraction II=benzene/methanol
Fraction III= acetic acid/methanol

Table 1: Result of phytochemical analysis of leaves, stem and root of *Waltheria indica*

Chemical constituents	leaves	stem	root
Tannins	++	++	+
Saponins	++	+	+
Flavonoids	++	+	-
Steroids	+	+	+
Alkaloids	+	-	-
Terpenoids	+	-	-
Cardiac glycosides	++	+	+

Key: ++= Highly present +=Present -=absent

Discussion

Phytochemical analysis of the extract of the plant revealed the presence of steroids, tannins, saponins, and cardiac glycosides in all parts of the plant. The presence of flavonoids in leaves and stem in this study is in contrast with the opinion of Mohammed *et al.*, (2007) who noted that flavonoids are only present in the leaves with no part of the plant showing the presence of anthraquinones (Table 1). The observed difference could be due to environmental changes where the plants were collected or seasonal changes that could have altered the plant components. It could also have been as a result of changes during extraction and/or storage. Antimicrobial activities of different parts of the plant were tested on *E.coli, Pseudomonas aerogenosa, and Salmonella typhi.*

The leaves showed highest activity against two organisms- *E.coli* and *Pseudomonas aerogenosa* (Table 1). This could be due to its high composition of cardiac glycosides. Genzolel and Mathel (1982) demonstrated that high composition of cardiac glycosides have been found to inhibit microbial growth and is capable of protection against microbial infection. The phytochemical screening results also

showed the presence of alkaloids and saponins, these classes of compounds have earlier been reported to have antimicrobial activity (Hostettman and Nakanishi, 1979). Therefore, these compounds may be responsible for the observed antibacterial activity of the leaves of *Waltheria indica*.

The root had the highest activity against *Pseudomonas aerogenosa* while the stem had the lowest activity against the three organisms (Table 2). The different components of the leaves i.e. fractions I, II, and III were tested using the same three organisms; fraction III had the highest activity on the organisms, more specifically on *Salmonella typhi.* The result suggests that water is not the most effective solvent for extracting the pharmacologically active compounds and it is a good indication that the most active ingredient of the *Waltheria indica* crude extract is contained in fraction III. It could also mean that the active ingredient maybe different for the different organisms. The fractions reduced the activity of the leaves against the organisms except fraction II, which increased the activity against *Pseudomonas aerogenosa* as compared to the initial crude extract of the leaves (Tables 2 and 3). The observed reduction in activity could have occurred during fractionation.

Gunners (1991) reported that different solvent extracts of some plants may exhibit different pharmacological properties against the same species of microorganisms. Crude extracts may be very active because of synergistic relationship between the components of the plant although sometimes the relationship maybe antagonistic. The observed pattern of antimicrobial activity of crude and various fractions of the plant parts underscores the need to study all parts of the plant before generalization is made on the plant's pharmacological and therapeutic potentials. This is more so because *in vivo*, biotransformation reactions would occur and may likely give rise to new results.

These findings confirm the basis of traditional use of *Waltheria indica* for treating diseases such diarrhoea. The mechanism of action of the constituents of *Waltheria indica* may be difficult to speculate; however, many antibacterial agents may exhibit their action through inhibition of nucleic acid, protein and membrane phospholipids biosynthesis (Franklin *et al.*, 1987). It is probable that the antibacterial agent(s) in the extract of *Waltheria indica* act via some of the above mechanisms. Further studies on the *in vivo* activity, isolation and structural elucidation of the component(s) and toxicological studies of the plant extract are recommended.

Corresponding Author:
Zailani, Ahmed Hauwa
Department of Biochemistry,
Federal University of Technology, P.M.B 2076, Yola, Adamawa State, Nigeria.
Email: howwrkoulou@yahoo.com

REFERENCES

Ahmadiani, A, Fereidoni M., Semnanian, S., Kamalinejad, M., and Saremi, S.. Anti-nociceptive and anti- inflammatory effects of *Sambucus ebulus* rhizhome extract in rats. J. Ethnopharmacol. 1998(61)229-235.

Akobundu, I.O., and Agyaklwa, C.W. A handbook of West African weeds. 2nd edition, INTEC printers, Ibadan. . 1998: 406

Emeruwa, A.C. Antibacterial substances from *Carica papaya* fruit, Liodydia. 1982: 45(2)123-127.

Franklin T.J., Snow, G.A Barrett-Bee, K.J., and Nolan R.D. Biochemistry of antimicrobial action. Fourth edition, Chapman and Hall London New York. 1987: 71-73; 112.

Gonzalel, L., and Mather, S.B. Identification of terpenoids from the leaves of *Piptocarpa peritora* and their biological activities. Journal of Natural Products. 1982 (45):495-496.

Gunner, B. McGraw Hill encyclopedia of science and technology vol 1;8th edition. 1991: 205

Hostettman, K., and Nakanishi, K. Moronic acid, a simple triterpenoid keto acid with antimicrobial activity isolated from *Ozoroa Mucroanta* J. Med. Plant Res. 1979 (31) 358-366.

Hutchinson, M.J., and Dalziel, J.M. Flora of West Tropical Africa. Crow Agent for oversea Government and Administration, Mill Bank, London. 1958:319.

Irvine, F.R.. Woody plants of Ghana, Oxford University Press, London. 1961:185.

Kone, M.W., Antindehou, K.K., Kacaou-N'Douba, A., and Dosso, M. Evalution of 17 medical plants from Northern Cote D'voire for their *in vivo* activity against *Streptococcus pneumonias.* African Journal of Traditional, Complementary and Alternative Medicines. 2007:4(1) 17-22.

Mohammed, Z., Shok, M., IIyas, N., Musa, K,Y., and Yaro, A.H. Analgesic activity of *Waltheria indica*, European Journal of Scientific Research. 2007:16 (1) 1-6.

Nok, A.J., Esievo, K.A.N., Longdet , I., Arowosafe, S., Onyenekwe, P.C., Gimba, C.E., and Kagbu, J.A.

Trypanocidal potentials of *Asadirachta indica*: *in vitro* activity of leaf extract against Trypanosama brucei. J. Clin. Biochem. Nutr; 1993(15) 113-118.

Sofowora, E.A. Medicinal plants and traditional medicine in Africa, University of Ife Press, Nigeria. 1993:1-23

Yerra, K. R., Shih-hua, F., and Yew-min, T. Inhibitory effects of flavonoids isolated from *Waltheria indica* on the production of NO, TNF-α and IL-2 in activated macrophages. Biological and Pharmacetical Bulletin. 2005(28),5912.

22/3/2010

Allelopathic effect of leaf extract of *Azardirachta indica* and *Chromolaena odorata* against post harvest and transit rot of tomato (*Lycopersicum esculentum* L)

[1*] Ijato James Yeni, [1]Oyeyemi Sunday Dele, [2]Ijadunola John Ademola, [3]Ademuyiwa Justus Adeniran

[1]Department of Plant Science, Faculty of Science, University of Ado–Ekiti, P.M.B 5363. Nigeria.

E-mail: jamesyeni@yahoo.com; GSM: 08067335124

[2]Federal College of Agriculture, Institute of Agricultural Research and Training, Moor Plantation, Ibadan, Nigeria.

[3]Department of Statistics, Federal Polytechnic, Ado-Ekiti, Nigeria.

Abstract: The aim of the present research was focused on the allelopathic effects of *Azardirachta indica* and *Chromolaena odorata* via *in vitro* approach. The aqueous and organic solvents (water and ethanol) extracts from leaves of *Azardirachta indica* Adr.Juss (*Meliaceae*) and *Chromoolaena odorata* (*Asteraceae*) where tested against fungal pathogens of rotten tomato (*Aspergillus niger*, *Fusarium oxysporum*, *Rhizopus stolonifer* and *Geotrichum candidium*) by poisoned food method. The results showed promising antifungal activity against the fungi tested. Among the various solvents with varying concentrations, aqueous extracts of 80% *Azardirachta indica* was found to have more inhibitory effect (65.20%) against *Rhizopus stolonifer* compared with other concentrations of 80% *Chromolaena odorata* (52.60%). Ethanol extracts of 30% *Azardirachta indica* had the best inhibitory effect (83.30%) against *Aspergillus niger* followed by 30% ethanol extract of *Chromolaena odorata* (80.00%) against *Geotrichum candidium* comparatively, 20% ethanol extract of *Azardirachta indica* (75.20%) against G. candidium inhibited than 20% ethanol extract of *Chromolaena odorata* (69.80%) against *Geotrichum candidium*. This finding proved the potentiality of the plant extracts for the control of post harvest and transit fungal rot of tomato fruit.

Ijato James Yeni, Oyeyemi Sunday Dele, Ijadunola John Ademola, Ademuyiwa Justus Adeniran. **Allelopathic effect of leaf extract of *Azardirachta indica* and *Chromolaena odorata* against post harvest and transit rot of tomato (*Lycopersicum esculentum* L).** Journal of American Science 2010;6(12):1595-1599]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: *Azadirachta indica*, *Chromolaena odorata*, allelopathy, tomato fungal rot, Pathogens.

Introduction.

Tomato (*Lycopersicum esculentum*) originated from the Central America where it was then cultivated as an ornamental plant. Tomato belongs to the family *Solanaceae*. As a common vegetable crop, it rated second in significance to potato in many countries. Tomato is an important commercial crop in the world. Nutritional value of tomato makes it a widely accepted vegetable by consumers. Nevertheless, tomato is a very perishable crop with a short shelf life as well as high vulnerability to mycotic diseases. During extended storage, tomato fruits are prone to post harvest disease caused by various fungal pathogens. Control of tomato fruit rot has been by application of synthetic chemicals. However, these days' consumers request less use of chemicals and still want food devoid of corruptions, microbial growth, toxins as well as other qualities-deteriorating factors (Lingk, 1991).

Ripe tomato fruit has high vitamin C and potassium. More importantly, previous researches indicated that consumption of cooked tomatoes reduced cholesterol related cardiac diseases (Simon and Schuster, 1996). Tomato is essential in human diet; it can be prepared into stew, puree, salad etc. Despite the human need of tomato, its low yield as a result of disease infestation has being source of serious concern. Natural plant products and their analogues have been found as important sources of agricultural bio-pesticide which serve as antimicrobial properties of plant extracts (Cardelina, 1995, Okigbo 2009).

Antifungal properties of *Azardirachta indica* and *Chromolaena odorata* on fungal rot pathogens of post harvest tomato fruits are therefore aimed at in this finding. This is to serve as a relative alternative to the use of synthetic chemicals to extend the shelf life of tomato so as to reduce or eliminate loss due to post

harvest rot caused by phyto pathogens mainly fungi and the resultant economic loss to the farmers, traders and consumers.

Materials and Methods.

Collection of tomato fruits

Tomato fruits with symptoms of rot were randomly collected from the market stalls at Ado-Ekiti, Ekiti state, Nigeria. Softness of tissues of tomatoes was identified as being biologically deteriorated. Fresh and healthy tomatoes were also collected and packed into a sterile polythene bag already lined with soft paper and taken to the laboratory for further studies.

Collection of plant materials.

Azardirachta indica and *Chromolaena odorata* were collected in the premises of the University of Ado- Ekiti, Ekiti state Nigeria. These plants were taken to the herbarium unit of the University for Identification

Isolation of spoilage fungi from rotten tomato fruits.

Pieces of tomato were washed in a running tap these were cut from the periphery of a rotten tomato, these were surface sterilized in the plate with 70% ethanol for just 1minute, dropped on sterile soft paper and culture out on (Potato dextrose agar) already mixed with streptomycin. A minimum of four replicates pieces from each of the rotten tomato were cultured out. The Petri-dishes were incubated at 28 $\pm$ 2^0c for five days and observed for fungal growth. Fungi associated were re-cultured to obtain pure culture and the pure isolates stored in slant for further use. The frequency of occurrence was determined using the method of Okigbo and Ikediugwu (2000).

Pathogenicity test.

Cylindrical cores of 1cm deep were taken away from different spots of a fresh and healthy tomato fruits with the aid of sterile 5mm cork borer and then disc of 4mm was taken from the periphery/ core of a colony of five days old test fungus was placed downward into each hole in the tomato fruit. The core of the tomato fruit was replaced after 2mm pieces had been cut off to compensate for the thickness of the agar inoculum and then replaced core sealed with Vaseline (jelly). Sterilized potato dextrose agar (PDA) was used in place of the culture discs in the control set up**.**

Preparation of leaf extracts.

Azardirachta indica (leaf) and *Chromolaena odorota* (leaf) were collected and washed thoroughly under running water and allowed to air-dry for 7 days. These were grounded separately. Thirty grams of each sample was added to 15ml of distilled water in separate flasks. This was vigorously shaken and left to stand for 24hrs. The sample was filtered with 3-layer cheese cloth and filtrate extract preparation of 80% and 60% concentrations were used as the extracts. The same procedure was used for 30% and 20% ethanol extract.

Effect of plant extracts on mycelia growth of fungi

The approach of Amadioha and Obi (1999) was used to evaluate the allelopathic effect of the extracts on fungal growth by creating four equal sections on each plate by drawing two perpendicular lines at the bottom of the plate. The point of intersection indicated the centre of the plates. This was done before dispensing PDA into each of the plates. The extracts were poured into the flask, plugged with cotton and heated for about 10minutes to avoid contamination (Madari and Singh, 2005). About 2ml of the extract of various plant materials was separated introduced into the Petri-dish containing the media, (poisoned food method) (Nene and Thapilyal, 2002). A disc of 4mm diameter of the pure isolate each was placed on the extract in plate with PDA at the point of intersection of the two perpendicular lines drawn at the bottom of the plate. Control experiments were without addition of any plant extract but sterile distilled water. Fungitoxicity was determined in term of percentage colony inhibition % = $\frac{DC - DT}{DC} \times \frac{100}{1}$

Where DC - Average diameter of control

DT - Average diameter of fungal colony with treatment

Results

"Table 1 Percentage inhibition of radial growth of rot fungi cultured in potato dextrose agar poisoned with aqueous plant extracts of 60% and 80% concentrations."

Table 1. Plant extracts (% inhibition of mycelia growth)

Rot Fungi	*Azardirahta indica*		*Chromolaena odorata*		Control (mm)
	60%	80%	60%	80%	
Aspergillus niger	59.90a	60.00a	52.20a	53.30a	16.00
Fusarium oxysporum	48.20bc	62.50a	42.80c	45.20b	15.00
Rhizopus stolonifer	56.40ab	65.20a	54.80a	52.60a	16.00
Geotrichum candidium	46.70c	59.20a	45.60bc	52.30a	17.00

Each of the data is a mean of three replications. Each data followed by the same alphabet along the columns not significantly different at P= 0.05, using (DMRT) Duncan Multiple Range Test to separate the means.

Table 2: Percentage inhibition of mycelia growth of rot fungi grown in potato dextrose agar poisoned with ethanol plant extract of 20% and 30% concentration

Rot Fungi	Plant extract (% inhibition of mycelia growth)				
	Azardirachta indica		*Chromolalena odorata*		Control (mm)
	20%	30%	20%	30%	
Aspergillus niger	63.30b	83.30a	50.00bc	72.60ab	15.00
Fusarium *oxysporum*	69.60ab	79.80a	62.00a	74.70ab	17.00
Rhizopus stolonifer	63.30b	83.60a	47.80c	70.20b	16.00
Geotrichum candidium	75.20a	80.20a	69.80a	80.00a	16.00

Each of the data is a mean of three replacements. Each data followed by the same alphabet along the columns not significantly different at P= 0.05, using DMRT Duncan Multiple Test to separate the means.

The isolated spoilage fungi from infected tissues included: *Aspergillus niger*, *Fusarium* o*xysporum*, *Rhizopus stolonifer* and *Geotrichum candidium*. The results of the pathogenicity test showed that these rot fungi were able to cause deterioration after 7 days of inoculation. Both aqueous and ethanol extracts of the test plants of *Azardirachta indica* and *Chromolaena odorata* were able to reduce the radial growth of the fungal mycelia. (Tables 1 & 2), 80% aqueous extract of both *Azardirachta indica* and *Chromolaena odorata* reduced the mycelia growth more than the compared 60% aqueous extract of the respective plants (Table 1). The same thing was recorded in 30% ethanol extract inhibited in both test plants than 20% ethanol (Table 2).

The highest of 80% aqueous extract of *Azardirachta indica* inhibition of 65.20% was recorded against *Rhizopus stolonifer* (Table 1). *Azardirachta indica* was able to cause inhibition up to 83.60% and 83.30% using 30% ethanol against *Rhizopus stolonifer* and *Aspergillus niger* respectively. Inhibition 75.20% was attained with 20% ethanol extract of *Azardirachta indica* against *Geotrichum candidium*. *Chromolaena odorata* using 30% ethanol extract had the highest inhibition of 80.00% against *Geotrichum candidium*. Higher concentration percentage of ethanol extract favoured better inhibition also aqueous extract. *Azardirachta. indica* seemed to possess more efficacy cumulatively than *Chromolaena odorata* irrespective of the extractive mode (solvents).

Discussion

The microbes linked with the post harvest deterioration in this finding were: *Geotrichum candidium*, *Rhizopus stolonifer*, *Fusarium oxysporum* and *Aspergillus niger*. Both the test plants: *Azardirachta indica* and *Chromolaena odorata* had inhibitory effect on post harvest rot pathogens of

tomatoes in aqueous and ethanol solvent of varied concentrations. Extracts of *Azardirachta indica* and *Chromolaena odorata* have been reported to have antimicrobial properties. *Azardirachta indica* of the family *Meliaceae* contains secondary metabolite of alkaloid and terpenes which can be used to cure dermal diseases.

The derivatives of *Azardirachta indica* are of great use in agriculture, public health, medicine, cosmetics and many more. Its leaves, bark, seed and flower are bitter, astringent, acrid, depurative, refrigerant, demulcent, insecticidal, expectorant, liver tonic etc. *Chromolaena odorata* of the family Asteraceae is used to cure diabetic mellitus and malarial. Hycenth (2008) reported the antifungal effect of *Azardirachta indica* against yam rot pathogens (*Rhizopus stolonifer*). Siva *et al.* (2008) used *Azardirachta indica* to inhibit *Fusarium oxysporum* (wilt pathogen) of *Solanum melogena* (egg plant). Nahed (2007) improved biological control of fusarium root rot in cucumber (*Cucumis sativum* L) by *Azardirachta indica*. Vigorous inhibition of soil borne pathogenic fungal growth using *Azardirachta indica* was reported by Paul and Sharma (2002). Okigbo and Ajalie (2005) inhibited some human pathogens with *Chromolaena odorata*. Amadioha (2000) was able to control rice blast *in vitro* and *in vivo* with extract of *Azardirachta indica.* In Nigeria, plant extracts have been used to inhibit fungal diseases of plants such as cowpea (Alabi, *et al* 2005),banana (Okigbo and Emoghene, 2004), yam (Okigbo and Nmeka 2005), cocoyam (Eunice, et al 2008), and sweet potato (Amienyo and Ataga, 2007) . The extracts of *Azardirachta indica* and *Chromolaena odorata* could be used as bio-pesticides against tomato fruit rot caused by fungal pathogens, these plants are economical and save to handle.

Correspondent Author:

Ijato James Yeni.

Department of Plant Science, Faculty of Science, University of Ado–Ekiti, P.M.B 5363. Nigeria.

jamesyeni@yahoo.com

GSM: 08067335124

References.

Okigbo, R.N. Variation in phytochemical properties of selected fungicidal aqueous extract of some plant leaves in Kogi State, Nigeria. A*merican-Eurasian J. of Sust. Agric.* 2009; 3 (3): 407-409.

Hycenth, N. Effect of different plant extracts in the control of yam (*Dioscorea* sp) in Yola, Adamawa state, Nigeria. *Nigeria. Agric. J.* 2008; 3 (5): 382-387.

Siva, N, Ganesan, S, Banumatty, N and Mutthuchelian. Antifungal effect of leaf extract of some medicinal plant against *Fusarium oxysporum* causing wilt disease of *Solanum melongena* L. *Ethnobot. Leaflets* 2008; 12:156-163.

Paul, P.K and P.D Sharma. *Azardirachta indica* leaf extract induced resistance in barley against leaf stripe disease. *Physiol .Molec. Pl. Pathol.* 2002; 16:3-13

Okigbo, R.N and Ajalie, A.N. Inhibition of some human pathogens with tropical plants extracts *Chromolaena odorata* and *Citrus aurantifolia* and some antibiotics. *Int. J. Mol. Adv. Sci. (Pakistan).* 2005 1 (1): 34-40.

Madari,S and R.P Singh. Management of mushroom pathogens through botanicals. *Ind. Phyto Patho*l. 2005; 58:189-193.

Okigbo, R.N and Ikediugwu, F.E.O. Studies on biological control of post-harvest rot of yam *Dioscorea* spp with *Trichoderma viride*. *J. Phytophathol*. 2000; 148:351-355.

Amadioha, A.C and Obi,V.I. Control of anthracnose disease of cowpea *Cymbopogon citratus* and *Ocimum gratissimum*. *Acta phytopathol. Entomol. Hungeric*a.1999; 34(92): 85-89.

Nahed, Z.H. Improving Biological Control of *Fusarium* root rot in Cucumber (*Cucumis sativus L.*) by allelopathic plant extracts. *Int. J. of Agric. and Biol*. 2007; 1560-8530/2007/09-3-459-461.

Lingk, W. Health risk evaluation of Pesticides contamination in drinking water. *Gesunde Pflaunge*, 1991; 43:21-25.

Cardelina, J.H. Natural products in the search for new agrochemicals. In H. G. Gulter, (Ed). Biologically active natural products. Potential use in agriculture. 1995; Pp 305-311

Nene, Z.H and Thapilyal. Management of mushroom pathogens through botanicals. *Ind. Phytopathol*. 2002; 58:189-193.

Amienyo, C.A and Ataga, A.E. Use of indigenous plant extracts for the protection of mechanically injured sweet potato *(Ipomea batatas* (L) Lam) tubers. *Scientific Research and Essay*. 2007; Vol 2 (5) pp. 169 – 170

Alabi; D.A, Oyero, I. A. Jimoh, Amusa, N.A. Fungitoxic and phytotoxic effect of *Venonia amygdalina* (L), *Bryophllum pinnatum* (known) *Ocimum gratissimum* (Closium) L. and *Eucalytus globule* (Caliptos) Labil water extract on cowpea and cowpea seedling pathogens in Ago-Iwoye, South West Nigeria. *World of Agric Sci*. 2005; (1) 70-75

Eunice O, Nwachukwu, Osuji J.O. Evaluation of plant extract for antifungal activity of *Cassia alata* and *Dennetia tripetala* against *Sclerotina rolfsi* causing cocoyam cornel rot in storage. *J. of Agric and Biol. Sci.* 2008; 4(6): 784-788

Okigbo R. N. and Nmeka A. I. Control of yam tuber rot with leaf extracts of *Xylopia aethiopica* and *Zingiber officinale. Afri. J. Biotechnol.* 2005; 4(8): 804 – 807

Okigbo, R. N and Emoghene A. O. Antifungal activity of leaf extract of some plant species on *Mycospharella fijiensis* merelet, the causal organism of black sigatoka disease of banana (*Musa acuminata*). *KMITL. Sci. J.* 2004; 4:20-31

Amadioha,A. C. Controlling rice *in vitro* and *in vivo* with extracts of *Azardirachta Indica*. *Crop Protection.* 2000; 19:287-290

11/20/2010

Purification and properties of alanine aminopeptidase from water buffalo kidney

Mahmoud A. Ibrahim[1], Abdel-Hady M.Ghazy[1], Mohamed N. Mosaad[2] and Doaa A. Darwish[1]

[1.] Molecular Biology Department, National Research Centre, Dokki, Cairo, Egypt
[2.] Zoology Department, Faculty of Science, Banha University
ibrahimm70@hotmail.com

Abstract: Aminopeptidases participate in the development of flavour in food products. The present study aims at production of aminopeptidase(s) from the safe mammalian locally available rich sources. Three forms of alanine aminopeptidase AAP1, AAP2 and AAP3 isoenzymes were purified to homogeneity from the kidney cortex of water buffalo. The purification procedures involved anion exchange chromatography on DEAE-cellulose column and gel filtration through Sephacryl S-300 column. All of the purified isoenzymes turned out to be homogeneous as judged by native polyacrylamide gel electrophoresis. The molecular weights of the native isoenzymes AAP1, AAP2 and AAP3 were determined by gel filtration to be 120, 400 and 310 kDa. AAP1 was a homodimer of 60 kDa subunits. AAP2 was a homohexamer of 67 kDa subunits. AAP3 was a homohexamer of 53 kDa. AAP1, AAP2 and AAP3 displayed their maximum activity at pH 8, 7.8 and 7.8 and their isoelectric point (pI) values at pH 6.4, 6.2 and 6.6 respectively. The type of inhibition of AAP1 by dithiothreitol and AAP2 and AAP3 by 1,10 phenanthroline was found to be competitive. One binding site was deduced on each isoenzyme for its corresponding inhibitor.
[Mahmoud A. Ibrahim, Abdel-Hady M.Ghazy, Mohamed N. Mosaad and Doaa A. Darwish. Purification and properties of alanine aminopeptidase from water buffalo kidney. Journal of American Science 2010;6(12):1600-1613]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Alanine aminopeptidase; water buffalo; kidney

1. Introduction

Aminopeptidases are ubiquitously distributed in animals, plants, bacteria as well as in fungi and catalyze the sequential removal of amino acid residues from the amino termini of peptides, polypeptides and proteins (Matsui et al., 2006; Bogra et al., 2009; Mane et al., 2010; Renwrantz and Lam, 2010).

Aminopeptidases are also vital for metabolic pathway regulation, cell maturation and turnover of proteins, including utilization of exogenous proteins as nutrient substances and elimination of non-functional proteins (Liu et al., 2008).

Aminopeptidase N (APN; EC 3.4.11.2), also called alanyl aminopeptidase (AAP) that cleaves neutral amino acids from the N-terminus of oligopeptides (Gabrilovac et al., 2005).

Also AAP are widely distributed in mammalian tissues and body fluids such as human seminal plasma (Huang et al., 1997), human liver (Yamamoto et al., 2000), rat liver (Yamamoto et al., 1998), human placenta (Mizutani et al., 1993), human pancreas (Sidorowicz et al., 1980), human kidney (Mantle et al., 1990), porcine kidney (Itoh and Nagamatsu, 1995), human gallbladder bile (Offner et al., 1994) and human skeletal muscle (Mantle et al., 1983). These enzymes from mammals are considered to participate in the metabolism of hormones and neurotransmitters (Hiroi et al., 1992).

AAP is an enzyme that is used as a biomarker to detect damage to the kidneys, and that may be used to help diagnose certain kidney disorders. It is found at high levels in the urine when there are kidney problems (Flynn, 1990).

Aminopeptidases participate in the development of flavour in food products, either directly, by hydrolyzing bitter peptides which are generally rich in hydrophobic amino acids and therefore good substrates for its action, or indirectly, where aminopeptidases could be involved in the liberation of aromatic amino acids which are important precursors of aroma compounds identified in cheese (Martinez-Cuesta et al., 2001).

The main goal of our research project is the production of industrial enzymes from the locally available rich sources. In the case of such enzyme (AAP), there is a need to develop economical and large-scale production methods for use in food industries. This work is the first report describes a simple purification procedure and some properties of aminopeptidase from the water buffalo kidney as a safe mammalian locally available rich source.

2. Material and Methods

Kidney materials

Fresh kidneys from water buffalo Bubalus bubalis, were obtained fresh from a local slaughter-house. The cortex and medulla were separated.

Chemicals:

Ampholyte solution, pH 3.5-10, ampholyte solution, pH 3-7, DL-alanine *β*-naphthylamide,1,4 Dithiothreitol (DTT), *p*-Chloromercuribenzoate, *p*-Hydroxy-mercuribenzoate, 1,10 phenanthroline, Phenymethylsulfonyl-fluoride (PMSF), diethyl-aminoethyl-cellulose (DEAE-Cellulose), bestatin, puromycin, glutathione reduced form, N-Tosylamide- L-phenyl-alanine chloromethyl ketone (TPCK), *Na-p*-Tosyl-L-Iysine chloromethyl ketone (TLCK), Phenylmethylsulfonyl fluoride (PMSF), Pepstatin A, soyabean trypsin inhibitor and Sephacryl S-300 were purchased from Sigma Chemical Co. The other chemicals were of analytical grade.

Purification of the kidney AAP

1- Preparation of crude extract

All of the procedures were performed at 4˚C unless stated otherwise. Thirty gm of frozen kidney cortex or medulla were sliced and homogenized in omni-mixer (Sorvall DuPont Instruments), with two volumes (w/v) of 0.02 M Tris-HCl buffer pH 7.8 containing 1 mM $MgCl_2$ and 0.2 mM PMSF. After centrifugation at 12,000 xg for 30 min at 4˚C, the supernatant was saved and designated crude extract.

2- DEAE-cellulose column Chromatography

The crude extract was chromatographed on DEAE-cellulose column previously equilibrated with 0.02 M Tris-HCl buffer pH 7.8 containing 1 mM $MgCl_2$ and 0.2 mM PMSF. The proteins were eluted with stepwise NaCl gradient ranging from 0 to 1 M followed by 0.5 % Triton X-100 prepared in the equilibration buffer. Fractions of 5 ml were collected at a flow rate of 60 ml / h and the fractions of the peaks containing the alanine aminopeptidase activity were pooled.

3- Sephacryl S-300 column Chromatography

The concentrated material containing the AAP activity was applied onto a Sephacryl S-300 column (142 cm x 1.75 cm i.d.). The column was equilibrated and developed with 0.02 M Tris-HCl buffer pH 7.8 containing 1 mM $MgCl_2$ and 0.2 mM PMSF at a flow rate of 30 ml / h. 2 ml fractions were collected. The Sephacryl S-300 column was used for molecular weight determination of the buffalo kidney cortex native AAP according to the method of Andrews, (1964 and 1965). The above described Sephacryl S-300 column was calibrated with ferritin (440 kDa), catalase (240 kDa), *β*-amylase (200 kDa) alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), carbonic anhydrase (29 kDa), myoglobin (17.2 kDa), and cytochrome C (12.4 kDa).

Assay of aminopeptidase activity

(A) Using *β*-naphthylamide derivatives

The aminopeptidase activity was assayed according to Kawata et al., (1980) by measuring the liberated *β*-naphthylamine. The reaction mixture 1.5 ml contained; 100 mM Tris-HCl buffer pH 7.8, suitable dilution of the enzyme extract and 0.4 mM DL-alanine-*β*-naphthylamide HCl. The reaction was initiated by adding 0.1 ml of the substrate (6 mM stock solution). The reaction was terminated by the addition of 0.5 ml of Fast Garnet GBC solution (1 mg / ml) in 1 M Na-acetate buffer pH 4.2 containing 10% Tween 20. The absorbance of the liberated *β*-naphthylamine was determined spectrophoto-metrically at 525 nm. One unit of AAP activity was defined as the amount of the enzyme which catalyzes the liberation of 1 nmol of *β*-naphthylamine per hour at 37˚C. The specific activity is expressed in units / mg protein. The *β*-naphthylamine concentration was determined from a previously constructed curve for *β*-naphthylamine treated similarly.

(B) By using *p*-nitroanilide derivatives

The aminopeptidase activity was determined according to Niven, (1995) by measuring the liberated *p*-nitroaniline in 1 ml reaction mixture containing 100 mM Tris-HCl buffer pH 7.8 and suitable dilution of the enzyme extract. The reaction was initiated by addition of substrate; 5 mM amino acyl *p*-nitroanilide derivatives dissolved in dimethylsulfoxide (DMSO). The reaction was terminated by the addition of 0.5 ml 30 % (v / v) acetic acid and the mixtures were centrifuged for 10 min at 10 000 xg. The absorbance at 405 nm was recorded against control lacking the enzyme and the *p*-nitroaniline concentration was determined from a previously constructed standard curve for *p*-nitroaniline treated similarly.

Electrophoretic analysis

Native gel electrophoresis was carried out with 7% polyacrylamide gel according to Smith, (1969). SDS-PAGE was performed with 12% polyacrylamide according to Laemmli, (1970). The molecular weights of the purified AAP subunits were determined by SDS-PAGE as described by Weber and Osborn, (1969). The isoelectric point (pI) of the purified AAP was analysed on native 5% polyacrylamide vertical slabs (Robertson et al., 1987). The proteins were stained with 0.25% coomassie brilliant blue R-250.

Isoelectrofocusing marker proteins and their pI values; trypsinogen (9.3), lectin 1 (8.8), lectin 2 (8.6) lectin 3 (8.2), myoglobin 1 (7.2), myoglobin 2 (6.8), carbonic anhydrase 1 (6.6), carbonic anhydrase 2 (5.9), *β*-lactoglobulin (5.1), trypsin inhibitor (4.6)

and amyloglucosidase (3.6) were used to construct a calibration curve by plotting the distance from anode of each marker protein versus its isoelectric point pI value (Ubuka et al., 1987).

Staining of the AAP activity

Detection of AAP activity on gel was performed as described by Chien et al. (2002). After the native PAGE, the gel was incubated in the staining solution; 0.1 M Na-phosphate buffer pH 5.8 containing 1 mM $CoCl_2$, 0.06% DL-Alanine β-naphthylamide HCl and 0.06% Fast Garnet GBC until the development of the red bands. The stained gel was washed with water and then fixed in 7% acetic acid.

Protein determination

Protein was determined by the dye binding assay method (Bradford, 1976). Bovine serum albumin was used as a standard protein.

3. Results

Comparison of the AAP activity in the kidney cortex and medulla:

The AAP activity was assayed and compared in the kidney cortex and medulla. The AAP showed a higher specific activity in the cortex (1616.13 ± 91.77 units / mg protein) than the medulla (194.4 ± 10.7 units / mg protein) representing more than 8 folds Table (1). Therefore, the kidney cortex is selected for the AAP purification.

Table (1), AAP specific activity in the water buffalo kidney cortex and medulla.

Specific activity* of the water buffalo kidney AAP by using DL-alanine β-naphthylamide	
Cortex	Medulla
1616.1 ± 91.8	194.4 ± 10.7

Purification of AAP from the water buffalo kidney cortex

A typical purification scheme of the AAP from water buffalo kidney cortex is presented in Table (2). The procedure involved anion exchange chromatography on DEAE-cellulose column (Fig. 1) followed by gel filtration chromatography on Sephacryl S-300 column (Fig. 2 A, B and C). The starting specific activity in the crude extract was 1704.25 unit / mg protein. The chromatography on the DEAE-cellulose column revealed the presence of three peaks of the AAP activity eluted with 0.1 M NaCl (AAP1), 0.2 M NaCl (AAP2) and 0.5% Triton X-l00 (AAP3) (Fig. 1).

For further purification, the concentrated pooled fractions of AAP1, AAP2 and AAP3 were applied separately on a Sephacryl S-300 column. The column was equilibrated and developed with 0.02 M Tris-HCl buffer pH 7.8 containing 1 mM $MgCl_2$ and 0.2 mM PMSF. The elution profiles of AAP1, AAP2 and AAP3 (Fig. 2 A, B and C) revealed the presence of one peak of AAP activity in each one. After the gel filtration on the Sephacryl S-300 column the specific activity of AAP1, AAP2 and AAP3 were increased to 2972.1, 4038.06 and 11655.32 units / mg protein which represents 1.74, 2.36 and 6.83 fold purification over the crude extract with 5.18 %, 8.6 % and 51.31 % recovery respectively.

The molecular weights of the native AAPs were calculated from the calibration curve to be 120 kDa, 400 kDa and 310 kDa for AAP1, AAP2 and AAP3 respectively. The elution volumes (Ve) of AAP1, AAP2 and AAP3 are 190 ml, 140 ml and 146 ml respectively from the Sephacryl S-300 column (Fig. 2A, 2B and 2C) and the column void volume (Vo) was 132 ml as determined by the dextran blue (2000 kDa).

Characterization of the water buffalo kidney cortex AAPs

Electrophoretic analyses

` Samples from the different purification steps (crude extract, DEAE-cellulose and Sephacryl S-300 fractions) of AAP1, AAP2 and AAP3 were analyzed electrophoretically on native 7% polyacrylamide gel (Fig. 3). The isoenzyme pattern of the crude extract and the three isoenzymes confirmed that the water buffalo kidney cortex contain three distinct AAP isoenzymes. The AAP activity was visualized on 7% native PAGE. AAP1 and AAP2 isoenzymes migrated faster than AAP3 due to their higher negative charge (Fig. 4).

The native and denatured 12% SDS-PAGE confirmed the purity of water buffalo kidney cortex AAP1, AAP2 and AAP3 (Fig. 6). The subunit molecular weights of AAP1, AAP2 and AAP3 were estimated to be 60 ± 1 kDa, 67 ± 1 kDa and 53 ± 1 kDa respectively (Fig. 6).

Samples of the purified AAP1, AAP2 and AAP3 isoenzymes were electrofocused (Fig. 5). AAP1, AAP2 and AAP3 isoenzymes showed isoelectric points (p*I*) value at pH 5.6, 4.9 and 4.6 respectively.

Substrate specificity

The substrate specificity of the purified water buffalo kidney AAP1, AAP2 and AAP3 were screened toward various substrates and presented in (Table 3). The three isoenzymes AAP1, AAP2 and AAP3 cleaved preferentially alanyl residue (100 % relative activity).

The rate of hydrolysis of DL-alanine -*β*-naphthylamide HCl (0.311), (0.327) and (0.827) were more than the rate of hydrolysis of L- alanine *p*-nitroanilide (0.231), (0.244) and (0.763) for AAP1, AAP2 and AAP3 respectively.

Effect of pH on AAP1, AAP2 and AAP3

The enzyme activity of AAP1, AAP2 and AAP3 isoenzymes toward DL-alanine-*β*-naphthylamide HCl were measured in 100 mM Tris-HCl buffer of various pH values from 7.2 to 9. The optimum pH was found to be at pH 8, 7.8 and 7.8 for AAP1, AAP2 and AAP3 respectively (Fig. 7).

Table (2). A typical purification scheme of the water buffalo kidney cortex AAP.

Purification step	Total protein (mg)	Total units	Recovery (%)	Specific activity	Fold purification
Crude extract	400	681700.6	100.0	1704.3	1.0
DEAE-cellulose fractions					
0.1 M NaCl (AAP1)	61.6	62714.5	9.1	1017.6	0.6
0.2 M NaCl (AAP2)	70.4	70444.5	10.3	1000.2	0.6
Triton X-l00 (AAP3)	107.3	466587.4	68.4	4346.1	2.6
Sephacryl S-300					
0.1 M NaCl (AAP1)	11.9	35349.0	5.2	2972.1	1.7
0.2 M NaCl (AAP2)	14.5	58632.1	8.6	4038.1	2.4
Triton X-l00 (AAP3)	30.0	349793.1	51.3	11655.3	6.8

1- All data based on 15 gm water buffalo kidney cortex.
2- One unit of alanine aminopeptidase activity was defined as the amount of the enzyme which catalyzes the liberation of 1 nmol of *β*-naphthylamine per hour at 37°c.
3- The specific activity is expressed as units / mg protein.

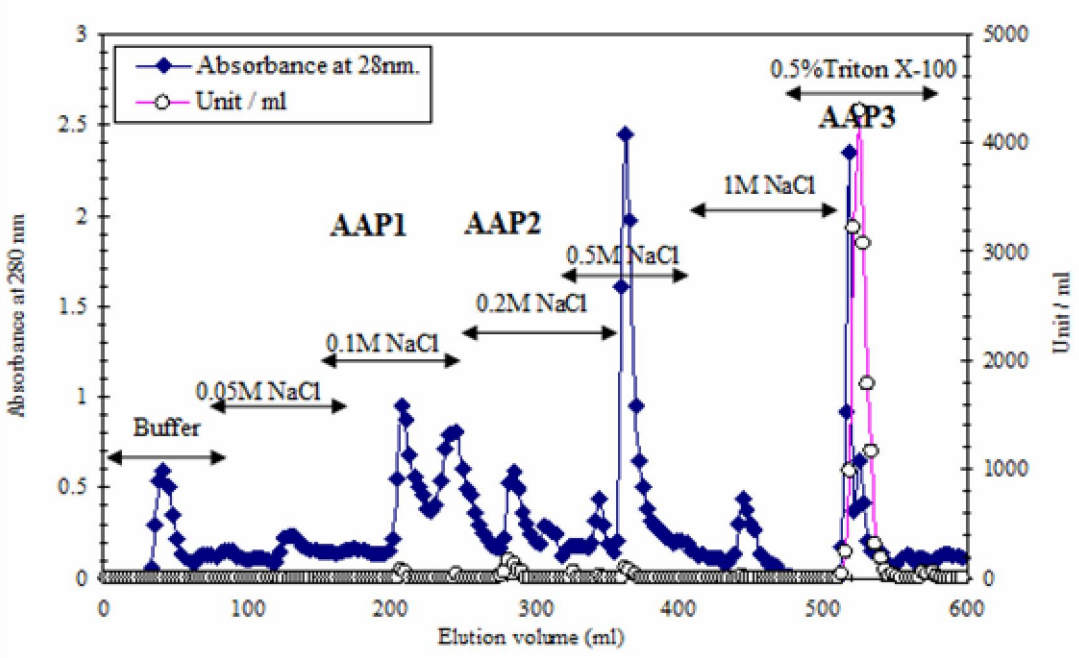


Figure 1. A typical elution profile for the chromatography of the water buffalo kidney cortex crude extract on DEAE-cellulose column (43 cm x 2.6 cm i.d.).

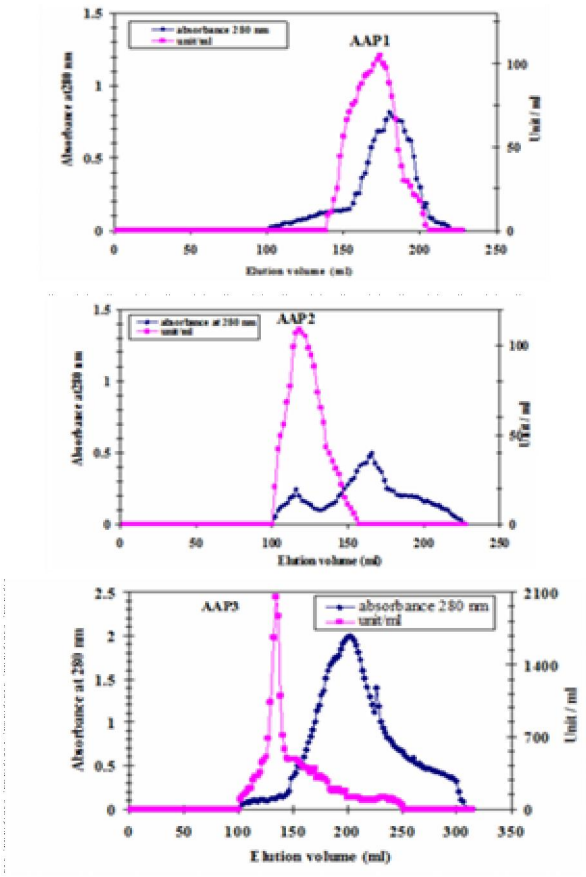


Figure 2. A typical elution profiles for the chromatography of concentrated pooled DEAE-cellulose fractions AAP1, AAP2 and AAP3 on Sephacryl S-300 column (142 cm x 1.75 cm i.d.).

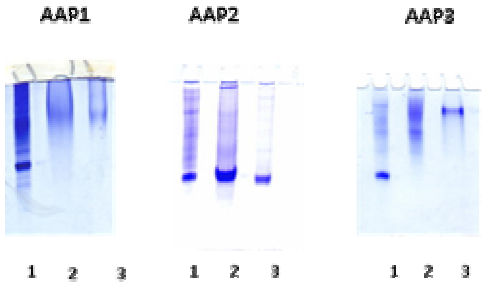


Figure 3. Electrophoretic analysis of protein pattern of the different purification steps of AAP on 7% native polyacrylamide gel: (1) water buffalo kidney cortex crude extract, (2) concentrated DEAE-cellulose fraction and (3) Sephacryl S-300 purified fraction.

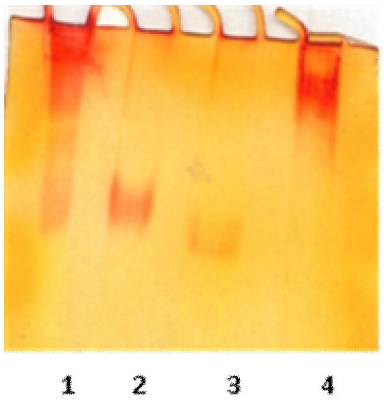


Figure 4. Isoenzyme pattern on 7% native polyacrylamide gel: (1) crude extract, (2) AAP1, (3) AAP2 and (4) AAP3.

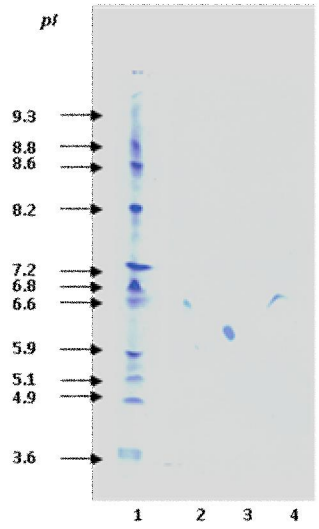


Figure 5. Isoelectrofocusing; [1] Isoelectric point (*pI*) marker proteins, [2] AAP1, [3] AAP2 and [4] AAP3.

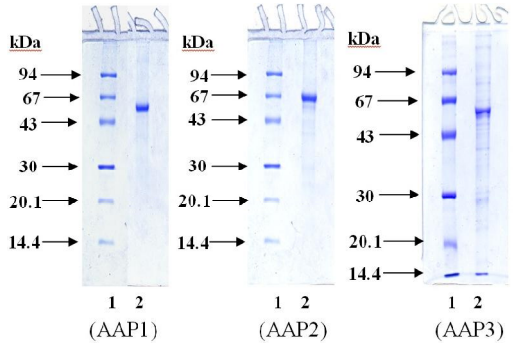


Figure 6. 12% SDS-polyacrylamide gel electrophoresis of (1) low molecular weight marker proteins (2) denatured purified water buffalo kidney cortex AAP.

Table (3), Substrate specificity of the water buffalo kidney cortex AAP1, AAP2 and AAP3.

Substrate	Concentration	AAP1		AAP2		AAP3	
		Rate of hydrolysis	Relative activity	Rate of hydrolysis	Relative activity	Rate of hydrolysis	Relative activity
L-Alanine *p*-nitroanilide HCI	1mM	0.231	100.0 %	0.244	100.0 %	0.763	100.0 %
L-Leucine *p*-nitroanilide	1mM	0.129	55.8 %	0.208	85.2 %	0.689	90.3 %
Glycine *p*-nitroanilide	1mM	0.046	19.9 %	0.06	24.6 %	0.192	25.2 %
S-Benzyl-L-Cysteine *p*-nitroanilide	1mM	0.134	13.4 %	0.052	21.3 %	0.171	22.4 %
L-Lysine *p*-nitroanilide 2HBr	1mM	0.079	34.2 %	0.111	45.4 %	0.252	33.0 %
L-Arginine *p*-nitroanilide 2HCI	1mM	0.068	29.4 %	0.097	39.7 %	0.325	42.6 %
L-Valine-*p*-nitroanilide HCl	1mM	0.011	4.7 %	0.024	9.8 %	0.15	19.7 %
L-Phenylalanine *p*-nitroanilide HCI	1mM	0.037	16.0 %	0.083	34.0 %	0.316	41.4 %
L-Glutamic acid y-(*p*-nitroanilide)	1mM	0.164	70.9 %	0.183	75.0 %	0.616	80.7 %
L-Proline *p*-nitroanilide	1mM	0.031	13.4 %	0.04	16.4 %	0.163	21.4 %
DL-Alanine *β*-naphthylamide HCI	0.4mM	0.311	100.0 %	0.327	100.0 %	0.827	100.0 %
Glycine *β*-naphthylamide HCI	0.4mM	0.078	25.0 %	0.052	15.9 %	0.099	12.0 %
L-Leucine *β*-naphthylamide HCI	0.4mM	0.206	66.0 %	0.229	70.0 %	0.569	68.8 %

Michaelis-Menten constant (Km) value

The purified AAP1, AAP2 and AAP3 isoenzymes were incubated with increasing concentrations of DL- alanine -*β*-naphthylamide HCl. The plots of substrate concentration [s] versus reaction velocity (v) (Fig. 8) were used to calculate the Michaelis-Menten constants (Km). The Km values were found to be 0.15, 0.17 and 0.125 mM and the corresponding maximum velocities (Vmax) were calculated to be 1694, 1143 and 66129 units / mg protein for AAP1, AAP2 and AAP3 respectively.

Effect of divalent cations on AAP1, AAP2 and AAP3

The purified AAP1, AAP2 and AAP3 isoenzymes were pre-incubated with 0.5 and 1.0 mM of each divalent cation at 37ºC and the activity was assayed. Table (4) shows the activity of AAP1, AAP2 and AAP3 in the presence of each cation. A control test based on the rate of hydrolysis of DL-alanine-*β*-naphthylamide HCl without any cation was taken as 100 % relative activity.

The activity of AAP3 was increased 112.5 % and 108.2 % in the presence of 0.5 and 1.0 mM $MgCl_2$ and 107.9 % and 100.8 % in the presence of 0.5 and 1.0 mM $CaCl_2$ respectively. The three isoenzymes were inhibited by the metal ions of Cu^{2+}, Mn^{2+} and Ni^{2+}, while Co^{2+} and Fe^{2+} have non-significant effect on AAP3 but have inhibitory effects on AAP1 and AAP2. The metal ion Zn^{2+} has a great inhibitory effect on the three isoenzymes AAP1, AAP2 and AAP3.

Effect of amino acids on AAP1, AAP2 and AAP3

Prior to the reaction initiation with the substrate, 1 mM of each tested amino acid was incubated with the enzyme in the assay reaction mixture. Table (5) presents the effect of the different amino acids on the purified water buffalo kidney cortex AAP1, AAP2 and AAP3 isoenzymes.

Almost all amino acids increase the activity of AAP2 except L- tyrosine, phenylalnine and L-leucine caused either slight or moderate inhibition of the enzyme. The isoenzymes AAP1 and AAP3 were inhibited 47.4 % and 32.9 % in the presence of 1 mM of tyrosine, 30 and 17.8 % by 1 mM phenylalnine and 25 % and 7.7 % by 1 mM serine respectively. The other tested amino acids caused slight inhibition of the isoenzymes AAP1 and AAP3.

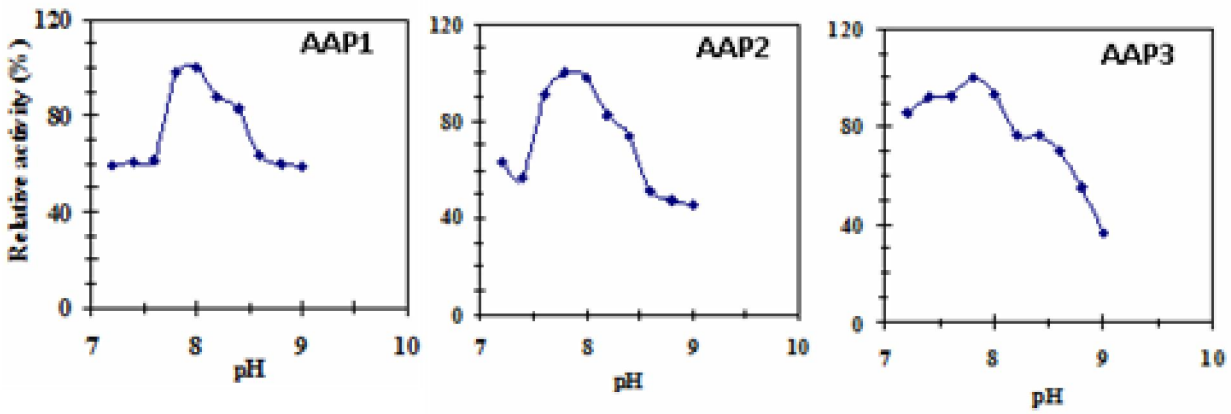


Figure 7. Effect of pH on the purified water buffalo kidney cortex AAP1, AAP2 and AAP3 using DL-alanine-*β*-naphthylamide HCl as substrate in 100 mM Tris-HCl buffer of various pH values from 7 to 9.

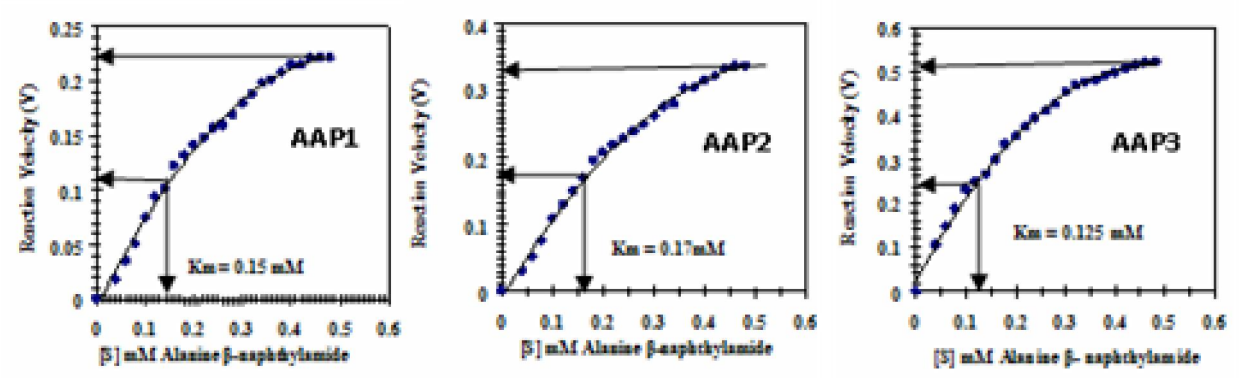


Figure 8. Effect of the substrate DL-alanine-β-naphthylamide HCl concentration in mM on the reaction velocity of the purified water buffalo kidney cortex AAP1, AAP2 and AAP3. The reaction velocity is the change in absorbance at 525nm per 30 min.

Effect of inhibitors on AAP1, AAP2 and AAP3

The purified AAP1, AAP2 and AAP3 isoenzymes were pre-incubated with a suitable concentration of each inhibitor at 37ºC for 5 min and the residual activity was assayed (Table 6).

The DL-dithiothreitol (DTT) was an obvious inhibitor of AAP1 since it caused 97.9 % inhibition at a concentration of 0.4 mM, while 1,10 phenanthroline was an inhibitor of both AAP2 and AAP3 since it caused 94.4 % and 96.5 % inhibition at a concentration of 10 mM.

The bestatin was a potent inhibitor of AAP1, AAP2 and AAP3 since it caused 72 %, 82.1 % and 79.56 % inhibition respectively at a concentration of 1 µM.

The isoenzymes AAP2 and AAP3 were sensitive to the thiol compound *β*-mercaptoethanol since it inhibited 60.13 % and 58.6 % of the enzyme activity at a concentration of 0.4 mM and also they were sensitive to reduced glutathione (GSH), which inhibited 57.69 % and 36.18 % of the enzyme activity respectively.

Kinetics of AAP1, AAP2 and AAP3 inhibition by DTT and 1,10 Phenanthroline

The effect of varying concentrations of DTT on the AAP1 activity is shown in Fig. (9). A maximum inhibition of AAP1 by DTT 92 % was reached at a concentration of 0.4 mM. A linear relationship was observed by constructing the Hill

plot for the inhibition of the purified water buffalo kidney cortex AAP1 by DTT, a straight line was obtained with slope of about 1.1 for AAP1 Fig. (9). Also, the effect of varying concentrations of 1,10-phenanthroline on the AAP2 and AAP3 activity are shown in (Fig. 10 and 11). A maximum inhibition of AAP2 and AAP3 by 1,10 phenanthroline 94.1 % and 91.2 % was reached at 10 mM 1,10-phenanthroline. The slopes of the Hill plot were found to be 0.95 and 0.78 for AAP2 and AAP3 (Fig. 10 and 11)**.**

Table (4**),** Effect of divalent cations on water buffalo kidney cortex AAP1, AAP2 and AAP3.

Reagent	**Final concentration (mM)**	**Residual activity (%)**		
		AAP1	**AAP2**	**AAP3**
Control	-	100.0	100.0	100.0
$CuCl_2$	0.5	64.7	87.6	74.1
	1.0	60.6	80.3	67.2
$MnCl_2$	0.5	45.5	84.3	88.9
	1.0	34.7	71.3	85.2
$ZnCl_2$	0.5	17.6	19.7	1.7
	1.0	15.0	11.8	0.0
$NiCl_2$	0.5	43.5	48.9	55.6
	1.0	37.3	34.3	40.0
$CoCl_2$	0.5	70.9	73.0	100.0
	1.0	65.2	75.2	99.1
$CaCl_2$	0.5	64.2	69.1	107.9
	1.0	61.6	67.4	100.8
$FeCl_2$	0.5	89.6	83.7	99.1
	1.0	86.5	86.5	95.6
$MgCl_2$	0.5	92.2	84.8	112.5
	1.0	86.5	87.6	108.2

The inhibition of AAP1, AAP2 and AAP3 by DTT and 1,10 phenanthroline were competitive type since the presence of the inhibitors did not alter the Vmax value but increases the Km value (Fig. 9, 10 and11).

The Ki value of AAP1 inhibition by DTT is determined to be 47 µM directly from the intercept of the X axis of the plot, while the Ki values of AAP2 and AAP3 inhibition by 1,10 phenanthroline are determined to be 1.3 mM and 1.9 mM respectively (Fig. 9, 10 and 11).

Table (5), Effect of amino acids (1 mM final concentration) on water buffalo kidney cortex AAP1, AAP2 and AAP3.

Amino acid	**Residual activity (%)**		
	AAP1	**AAP2**	**AAP3**
Control	100.0	100.0	100.0
L-Alanine	93.5	127.3	99.2
L-Tyrosine	67.1	83.1	52.6
DL-Phenylalnine	82.1	80.4	69.8
DL-Tryptophane	73.1	114.5	86.4
L-Lysine	90.4	118.0	98.7
L-Methionine	95.9	125.0	99.3
L-Histidine	90.4	120.0	93.2
L-Leucine	84.5	85.7	83.4
L-Arginine	86.8	121.9	98.3
L-Glutamine	90.4	123.8	97.7
L-Serine	92.2	130.0	74.9

4. Discussion

Among various aminopeptidases, the alanyl aminopeptidases (AAPs) which preferentially liberate amino-terminal neutral amino acids, such as Ala, Met, Leu and Tyr of peptides are widely distributed in mammalian tissues and body fluids (Mane et al., 2010).

In this study, a simple, convenient and reproducible purification procedure of the water buffalo kidney cortex AAP is carried out by a combination of anion exchange chromatography on DEAE-cellulose column followed by gel filtration chromatography on Sephacryl S-300 column. The native enzyme was obtained since both the extraction buffer and the equilibration buffer of the DEAE-cellulose column contained 0.2 mM phenylmethylsulfonylfluoride (PMSF), a serine protease inhibitor to avoid the action of the endogenous protease.

On the basis of the protein charge, the chromatography on DEAE-cellulose column revealed the presence of three forms of water buffalo kidney

cortex alanine aminopeptidases AAP1, AAP2 and AAP3 (Fig. 1). A considerable yield and purity of the AAP isoenzymes from water buffalo kidney cortex was obtained (Table 2). The recovery % of the enzyme units was 9.1, 10.3 and 68.44 for AAP1, AAP2 and AAP3 respectively. The total recovery is more than 87%. The alanine aminopeptidase from chicken intestine has a yield of 3.43% (Mane et al., 2010).

Table (6), Effect of inhibitors on the purified water buffalo kidney AAP1, AAP2 and AAP3.

Inhibitor	**Final concentration**	**Inhibition %**		
		AAP1	**AAP2**	**AAP3**
Control		0.0	0.0	0.0
Bestatin HCI	1.0 µM	72.0	82.1	79.6
Puromycin	1.0 mM	30.7	6.6	27.4
p-Chloromercuribenzoic acid (*p*CMB)	0.2 mM	0.0	5.9	29.3
p-Hydroxymercuribenzoate (*p*HMB)	0.2 mM	33.6	34.6	14.7
β - Mercaptoethanol	0.4 mM	46.0	60.1	58.6
DL-Dithiothreitol (DTT)	0.4 mM	97.9	88.5	73.9
L-Cysteine	1.0 mM	0.0	19.2	13.3
Glutathione reduced form (GSH)	1.0 mM	22.0	57.7	36.2
N-Ethylmaleimide	1.0 mM	0.0	15.0	2.7
N-Tosylamide-L-phenylalanine chloromethyl ketone (TPCK)	1.0 mM	0.0	30.4	15.0
EDTA	1.0 mM	0.0	3.8	0.0
Na-p-Tosyl-L-Iysine chloromethyl ketone (TLCK)	1.0 mM	0.0	39.5	2.0
Phenylmethylsulfonylfluoride (PMSF)	1.0 mM	7.6	15.0	12.5
1,10 Phenanthroline	10 mM	91.0	94.4	96.5
Pepstatin A	10 mM	0.0	0.0	0.0
Iodoacetic acid	10 mM	0.0	0.0	0.0
Soya bean trypsin inhibitor	15 µg	0.0	0.0	0.0

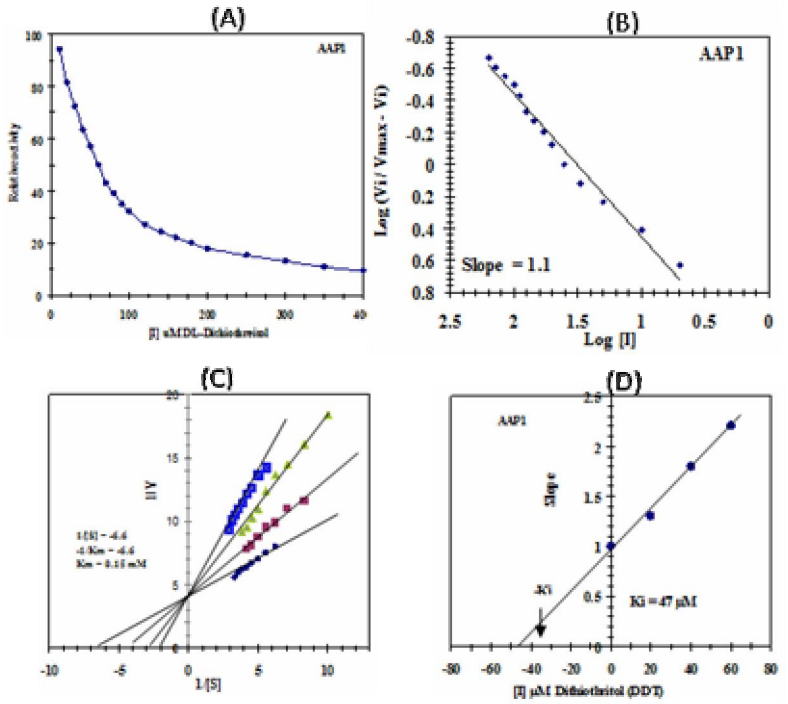


Figure 9. (A) Inhibition AAP1 by varying concentrations of DTT. (B): Hill plot for inhibition of AAP1 by varying concentrations of DTT. (C): Lineweaver-Burk plots showing the type of inhibition of AAP1 by DTT. (D): Determination of the inhibition constant (Ki) value for the inhibition of t AAP1 by DTT.

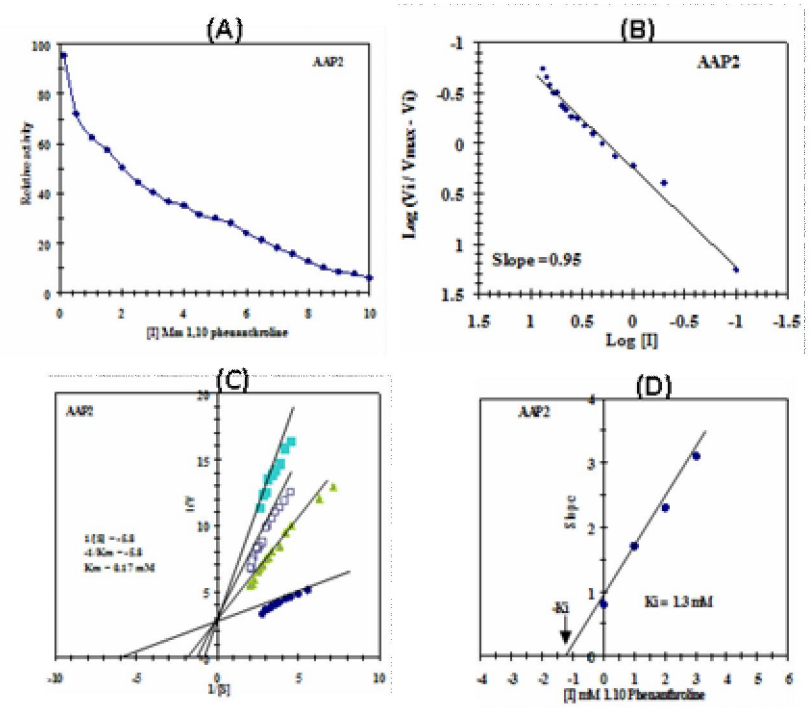


Figure 10. (A) Inhibition of the purified AAP2 by varying concentrations of 1,10-phenanthroline. (B): Hill plot for inhibition of AAP2 by varying concentrations of 1,10-phenanthroline. (C): Lineweaver-Burk plots showing the type of inhibition of AAP2 by 1,10-phenanthroline. (D): Determination of the inhibition constant (Ki) value for the inhibition of AAP2 by 1,10-phenanthroline.

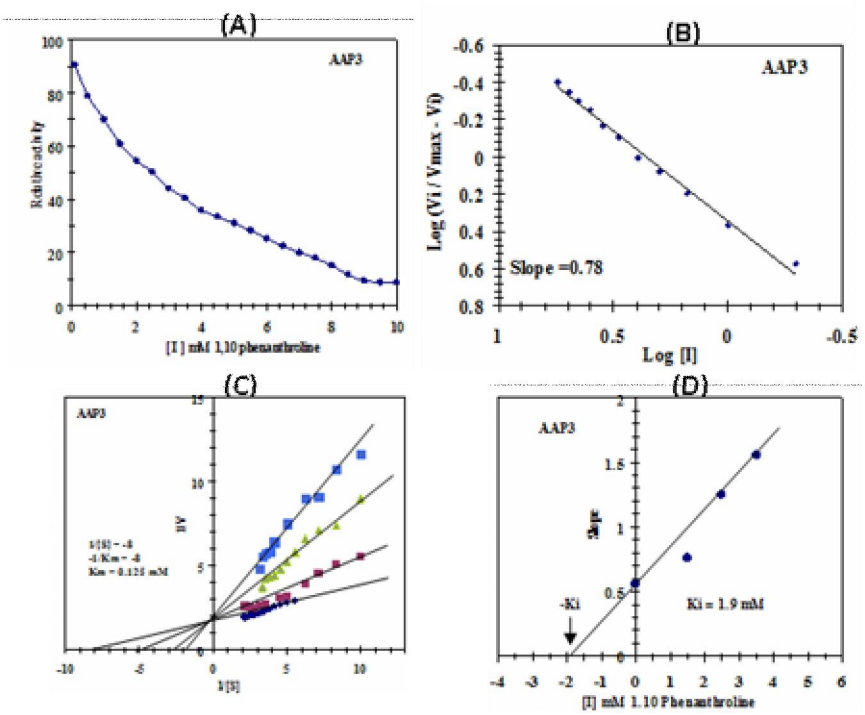


Figure 11. (A) Inhibition of the purified AAP3 by varying concentrations of 1,10-phenanthroline. (B): Hill plot for inhibition of AAP3 by varying concentrations of 1,10-phenanthroline. (C): Lineweaver-Burk plots showing the type of inhibition of AAP3 by 1,10-phenanthroline. (D): Determination of the inhibition constant (Ki) value for the inhibition of AAP3 by 1,10-phenanthroline.

The molecular weights of the native isoenzymes were determined by gel filtration to be 120 kDa, 400 kDa and 310 kDa for AAP1, AAP2 and AAP3 respectively (Fig. 2). The molecular mass of AAP1 was different from those of 180 kDa from chicken intestine alanine aminopeptidase (Mane et al., 2010), 153 kDa from human seminal plasma (Huang et al., 1997), 140 kDa from rat kidney (Watt et al., 1989), 106 kDa from porcine skeletal muscle (Flores *et al.*, 1996), 102 kDa from human skeletal muscle (Mantle et al., 1983) and 98 kDa from human liver cytosol (Yamamoto et al., 2000). The molecular masses of AAP2 and AAP3 were more or less similar to those of 420 kDa from gypsy moth *Lymantria dispar* (Masler and Kovaleva, 1997), 375 kDa from eosinophilic blood cells of *Mytilus edulis* (Renwrantz and Lam, 2010), 320 kDa from human placenta (Lampelo et al., 1982), 290 kDa from ostrich duodenal mucosa (Roos et al., 1993), 280 kDa from pig kidney (Wacker, 1976) and 236 kDa from human kidney (Kao et al., 1978).

All of the AAP1, AAP2 and AAP3 turned out to be homogenous as indicated by both native (Fig. 3) and SDS-PAGE (Fig. 6). Also, the single band of the enzyme subunit (Fig. 5) confirms the purity of the enzyme.

The subunits molecular weight of the purified buffalo kidney cortex AAP1 is determined by SDS-PAGE to be 60 ± 1 kDa indicating a homodimeric structure composed of two identical subunits, while AAP2 was 67 ± 1 kDa indicating a homohexameric structure composed of six identical subunits and AAP3 was 53 ± 1 kDa suggesting also a homohexameric structure composed of six identical subunits. (Fig. 6). However, aminopeptidases exhibit molecular weights ranging from 53–140 kDa per subunits and exist as monomers, hexamers and octamers (Božić et al., 2008).

The estimated p*I* values at pH 6.4, 6.2 and 6.6 for AAP1, AAP2 and AAP3 respectively (Fig. 5) are slightly higher than that described previously. The isoelectric point (pI) value is found to be 5.0 for membrane alanyl aminopeptidase (Riemann et al., 1999), between pH 4.5 and 5.8 for the soluble and surface-bound aminopeptidase in *Mytilus edulis* blood cells (Renwrantz and Lam, 2010) and 4.9 for that from rat liver (Hiroi et al., 1992).

The isoenzymes AAP1, AAP2 and AAP3 cleaved preferentially alanyl residue (Table 3)

The Km values using alanine *β*-naphthylamide as substrate were found to be 0.15, 0.17 and 0.125 mM for AAP1, AAP2 and AAP3 respectively (Fig. 8). These values were considerably

close to alanyl aminopeptidase from chicken intestine, since Km value of preferentially hydrolyzed Leu-β-NA was found to be 0.1 mM (Mane et al., 2010) indicating the high affinity of the buffalo kidney cortex AAPs toward the alanine β-naphthylamide.

The water buffalo kidney cortex AAP1, AAP2 and AAP3 displayed their optimum activity at pH 8, 7.8 and 7.8 respectively (Fig. 7). These pH values are similar to that of monomeric alanine aminopeptidase from bovine skeletal muscle which was optimal at pH 8.0 (Ye and Ng, 2011) and pH 7.9 for human pancreas alanine aminopeptidase (Sidorowicz et al., 1981).

The activity of AAP3 was increased in the presence of $MgCl_2$ and $CaCl_2$. The metal ion Zn^{2+} has a great inhibitory effect on the three isoenzymes AAP1, AAP2 and AAP3 (Table 4). High concentrations of Zn^{2+} (in the mM range) often inhibit metallopeptidases due to the formation of zinc monohydroxide that bridges the catalytic Zn^{2+} ion to a side chain in the active site of the enzyme (Božić et al., 2008). The chicken intestinal alanine aminopeptidase show that presence of cations (Zn^{2+} and Mn^{2+}) slightly activated the enzyme activity (Mane et al., 2010). The bovine skeletal muscle monomeric alanine aminopeptidase activity was totally abolished by Co^{2+} and Zn^{2+} ions, and almost completely inhibited by Mn^{2+}, the activity was strongly inactivated by Mg^{2+}, and Fe^{3+} ions. However the activity was not affected by Ca^{2+} (Ye and Ng, 2011).

The susceptibility of aminopeptidases to inhibition by free amino acids, suggesting in turn that the degradation of oligopeptides to amino acids may play a key role in control of the overall protein turnover process **(**Toldra et al., 1996). It has been suggested that the levels of the free amino acids within the cell may regulate the protein synthesis/degradation cycle, via a feedback inhibition type **(**Mader, 1988**)**; because aminopeptidase enzymes are in turn subject to inhibition by free amino acids **(**McDonald and Barrett, 1986**)**, it follows that the ultimate function of soluble aminopeptidases may be involved in the control of cellular protein turnover **(**Mantle, 1992**)**. Therefore, the effect of amino acids on the three isoenzymes AAP1, AAP2 and AAP3 was studied and presented in Table (5). The tyrosin inhibited the three isoenzymes obviously.

In the present study, the effect of different specific and characteristic inhibitors on the water buffalo kidney cortex AAPs is presented in Table (6). The purified isoenzymes are resistant to the serine protease inhibitors PMSF, N-Tosylamide-L-phenylalanine chloromethyl ketone (TPCK), Nα-*p*-Tosyl-L-lysine chloromethyl ketone (TLCK) and soya bean trypsin inhibitor indicating that the enzyme active site does not contain a serine residue and this was the reason why PMSF was added to the tissue homogenization buffer to inhibit the endogenous serine proteases. Also, they do not belong to the acid or thiol proteases groups since both the acid protease inhibitor, pepstatin A and thiol protease inhibitor, iodoacetic acid did not affect the purified AAP isoenzymes. The three isoenzymes AAP1, AAP2 and AAP3 are not cysteine proteases due to their resistance to the cysteinyl protease inhibitors, *p*-chloromercuribenzoic acid (*p*CMB), *p*-hydroxymercuribenzoate (*p*HMB) and N-ethylmaleimide (Table 6). Lack of enzyme inhibition with cysteine and serine protease inhibitors suggests that purified aminopeptidase does not have any endopeptidase activity (Pokharel and Rathaur, 2008).

The inhibition of the AAP isoenzymes by the thiol compound DTT indicates the role of sulfhydryl group in enzyme catalysis and their inhibition by 1,10 phenanthroline indicates that the isoenzymes are metalloenzymes.

Similarly, aminopeptidases from porcine liver (Imamura et al., 1983) and rabbit kidney (Oliveira et al., 1999) are found to be metalloenzymes. Inhibition by chelating agents such as 1,10-phenanthroline indicates the presence of at least one divalent zinc cation associated with the enzyme active site and should be considered as a zinc-aminopeptidase (Pokharel and Rathaur, 2008). Bestatin is not only LAP inhibitor but also a well-recognized inhibitor of membrane alanyl aminopeptidase although considerably less potent than amastatin or probestin **(**Tieku and Hooper, 1992). In this study, bestatin was found to be a potent inhibitor of the three AAP isoenzymes (Table 6) confirming that all of them are alanine aminopeptidase.

From the titration curves data a linear relationship was observed by constructing the Hill plot for the inhibition of the purified AAP1 by DTT and AAP2 and AAP3 by 1, 10 phenanthroline. The slope of the Hill plot was found to be 1.1 for AAP1 whereas 0.95 and 0.78 for AAP2 and AAP3 indicating the existence of one binding site for DTT and 1, 10 phenanthroline (Fig. 9, 10 and 11).

The type of inhibition of AAP1 by DTT, and AAP2 and AAP3 by 1, 10 phenanthroline were found to be competitive where the presence of DTT and 1, 10 phenanthroline did not alter the Vmax value but increased the Km value. For the determination of the Ki value, the slopes of the reciprocal plots lines were plotted against the DDT and 1, 10 phenanthroline concentrations. The Ki value of the AAP1 inhibition by DTT was determined to be 47 µM, whereas the Ki values of AAP2 and AAP3 inhibition by 1, 10 phenanthroline were determined to be 1.3 mM and

1.9 mM respectively directly from the intercept of the X axis of the plot (Fig. 9, 10 and 11).

The hexameric structure of AAP2 (400 kDa) and AAP3 (310 kDa) and their similar catalytic properties suggest that AAP2 may be a precursor of AAP3 and proteolytic modification is involved in the conversion of AAP2 to AAP3. On the other hand, AAP1 could be considered a unique isoform.

In conclusion, this study presents a simple, convenient and reproducible method for the purification of a well characterized alanine aminopeptidases from the water buffalo kidney cortex as a safe locally available rich source. Production of these enzymes on large scale will allow their use in various applications such as food industries and also investigation of the protein primary structure.

Corresponding Author:
Dr. Mahmoud A. Ibrahim.
Molecular Biology Department.
National Research Centre, Dokki, Cairo, Egypt.
E-mail: ibrahimm70@hotmail.com

References

1. Andrews, P. (1965): The gel filtration behaviour of proteins related to their molecular weights over a wide range. Biochem. J. 96: 595-606.
2. Andrews, P. (1964): Estimation of the molecular weights of proteins by sephadex gel filteration. Biochem. J. 91: 222 – 233.
3. Bogra, P.; Singh, J. and Singh. H. (2009): Purification and characterization of aminopeptidase B from goat brain. Process Biochemistry 44: 776 – 780.
4. Božić, N.; Vujčić, Z.; Nenadović, V. and Ivanović, J. (2008): Purification and properties of major midgut leucyl aminopeptidase of Morimus funereus (Coleoptera, Cerambycidae) larvae. Comp. Biochem. Physiol. B (149): 454 – 462.
5. Bradford, M. M. (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248 - 254.
6. Chien, H.-C. R.; Lin, L.-L.; Chao, S.-H.; Chen, C.-C.; Wang, W.-C.; Shaw, C.-Y.; Tsai, Y.-C.; Hu, H.-Y. and Hsu, W.-H. (2002): Purification, characterization, and genetic analysis of a leucine aminopeptidase from *Aspergillus sojae*. Biochim. Biophys. Acta 1576: 119 – 126.
7. Flores, M.; Aristoy, M. C. and Toldra, F. (1996): HPLC purification and characterization of soluble alanyl aminopeptidase from porcine skeletal muscle. J. Agric. Food Chem. 44: 2578 – 2583.
8. Flynn, F. V. (1990): Assessment of renal function: selected developments. Clin. Biochem. 23: 49 - 54.
9. Gabrilovac, J.; Breljak, D.; Cupic, B. and Ambriovic´-Ristov, A. (2005): Regulation of aminopeptidase N (EC 3.4.11.2; APN; CD13) by interferon-γ on the HL-60 cell line. Life Sciences 76: 2681 – 2697.
10. Hiroi, Y.; Endo, Y. and Natori, Y. (1992): Purification and properties of an aminopeptidase from rat-liver cytosol. Arch. Biochem. Biophys. 294: 440 – 445.
11. Huang, K.; Takahara, S.; Kinouchi, T.; Takeyama, M.; Ishida, T.; Ueyama, H.; Nishi, K. and Ohkubo, I. (1997): Alanyl amino- peptidase from human seminal plasma: purification, characterization and immunohistochemical localization in the male genital tract. J. Biochem. 122: 779 – 787.
12. Imamura, T.; Kawata, S.; Ninomiya, K. and Makisumi, S. (1983): Porcine liver aminopeptidase. Further characterization of its sulfhydryl groups. J. Biochem. 94: 267 - 273.
13. Itoh, C. and Nagamatsu, A. (1995): An aminopeptidase activity from porcine kidney that hydrolyzes oxytocin and vasopressin: Purification and partial characterization. Biochim. Biophys. Acta 1243: 203 – 208.
14. Kao, Y. J.; Starnes, W. L. and Behal, F. J. (1978): Human kidney alanine aminopeptidase: physical and kinetic properties of a sialic acid containing glycoprotein. Biochemistry 17: 2990 – 2994.
15. Kawata, S.; Takayama, S.; Ninomiya, K. and Makisumi, S. (1980): Purification and some properties of porcine liver aminopeptidase B. J. Biochem. 88: 1025 - 1032.
16. Laemmli, U. K. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680 – 68.
17. Lampelo, S.; Lalu, K. and Vanha-Perttula, T (1982): Purification and partial characterization of human placental particle-bound aminopeptidase. Placenta 3: 379-393.
18. Liu, B-X.; Du, X-L.; Zhou, L-G.; Hara, K.; Su, W-J. and Cao, M-J. (2008): Purification and characterization of a leucine aminopeptidase from the skeletal muscle of common carp (Cyprinus carpio). Food Chemistry 108 : 140 – 147.
19. Mader, A. A (1988) transcription translation activation feedback circuit as a function of protein degradation, with the quality of protein mass adaptation related to average functional load. J. Theor. Biol. 134: 135 – 137.
20. Mane, S.; Damle, M.; Harikumar, P.; Jamdar, S. and Gade, W. (2010): Purification and characterization of aminopeptidase N from chicken intestine with potential application in debittering Process. Biochemistry 45: 1011–1016.
21. Mantle, D. (1992) Comparison of soluble aminopeptidases in human cerebral cortex, skeletal muscle and kidney tissues. Clin. Chim. Acta 207: 107 – 118.
22. Mantle, D.; Hardy, M. F.; Lauffart, B.; McDermott, J. R.; Smith, A. I. and Pennington, R. J. T. (1983): Purification and characterization of the major aminopeptidase from human skeletal muscle. Biochem. J. 211: 567 – 573.
23. Mantle, D.; Lauffart, B.; McDermott, J. and Gibson, A. (1990): Characterization of aminopeptidases in

human kidney soluble fraction. Clin. Chim. Acta 187: 105 - 113.
24. Martinez-Cuesta, M. C.; Fernandez de Palencia, P.; Requena, T. and Pelaez, C. (2001): Enzymatic ability of Lactobacillus casei subsp. casei IFPL731 for flavour development in cheese. Internl. Dairy J. 11: 577 - 585.
25. Masler, E. P. and Kovaleva, E. S. (1997): Aminopeptidase-Like Activity in Hemolymph Plasma from Larvae of the Gypsy Moth, *Lymantria dispar* (Lepidoptera: Lymantriidae). Comp. Biochem. Physiol. B (116): 11-18.
26. Matsui, M.; Fowler, J. H. and Walling, L. L. (2006): Leucine aminopeptidases: diversity in structure and function. Biol. Chem. 387: 1535 – 1544.
27. McDonald, J. K. and Barrett, A. J. (1986) Mammalian Proteases: A Glossary and Bibliography, vol. 2: Exopeptidases. London: Academic Press, pp. 59 - 71.
28. Mizutani, S.; Goto, K. and Nomura, S. (1993): Possible action of human placental aminopeptidase N in feto-placental unit. Res. Commun. Chem. Pathol. Pharmacol. 82: 65 – 80.
29. Niven, G. W. (1995): The characterization of two aminopeptidase activities from the cyanobacterium *Anabaena flos-aquae*. Biochim. Biophys. Acta. 1253: 193 - 198.
30. Offner, G. D.; Gong, D. and Afdhal, N. H. (1994): Identification of a 130-kilodalton human biliary concanavalin A binding protein as aminopeptidase N. Gastroenterology. 106: 755 – 762.
31. Oliveira, S. M.; Freitas, J. O. and Alves, K. B. (1999): Rabbit kidney aminopeptidases: purification and some properties. Immunopharmacol. 45: 215 – 221.
32. Pokharel, D. R. and Rathaur, S (2008): Purification and characterization of a leucine aminopeptidase from the bovine filarial parasite *Setaria cervi*. Acta Tropica. 106: 1 – 8.
33. Renwrantz, L. and Lam, A. (2010): Soluble and surface-bound aminopeptidase in eosinophilic blood cells from Mytilus edulis. Journal of Invertebrate Pathology. 103: 68 – 70.
34. Riemann, D.; Kehlen, A. and Langner, J. (1999): CD13 – not just a marker in leukemia typing. Immunol. Today 20: 83 – 88.
35. Robertson, E. F.; Dannelly, H. K.; Malloy, P. J. and Reeves, H. C. (1987): Rapid isoelectric focusing in a vertical polyacrylamide minigel system. Anal. Biochem., 167: 290 – 294.
36. Roos, A-A.; Naudé, R. J. and Oelofsen, W. (1993): The isolation and partial characterization of alanine aminopeptidase from ostrich (*Struthio camelus*) duodenal mucosa. Comp. Biochem. Physiol. B (104): 817-823.
37. Sidorowicz, W.; Zownir, O. and Behal, F. J. (1981): Action of human pancreas alanine aminopeptidase on biologically active pep tides: kinin converting activity. Clin. Chim. Acta. 111: 69-79.
38. Sidorowicz, W.; Jackson, G. C. and Behal, F. J. (1980): Multiple molecular forms of human pancreas alanine aminopeptidase. Clin. Chim. Acta. 104: 169 – 179.
39. Smith, I. (1969): Acrylamide gel disc electrophoresis. Electrophoretic techniques (Edited by Smith, I.) Academic press, New York, pp. 365 – 515.
40. Tieku, S. and Hooper, N. M. (1992) Inhibition of aminopeptidases N, A and W. A re-evaluation of the actions of bestatin and inhibitors of angiotensin converting enzyme. Biochem. Pharmacol. 44: 1725 - 1730.
41. Toldra, F.; Falkous, G.; Flores, M. and Mantle, D. (1996) Comparison of Aminopeptidase Inhibition by Amino Acids in Human and Porcine Skeletal Muscle Tissues *In Vitro*. Comp. Biochem. Physiol. Vol. 115B: 445 – 450.
42. Ubuka, T.; Masuoka, N.; Yoshida, S. and Ishino, K. (1987): Determination of isoelectric point value of 3-Mercaptopyruvate sulfurtransferase by isoelectric focusing using ribonuclease A-glutathione mixed disulfides as standards. Anal. Biochem. 167: 284 – 289.
43. Wacker, H. (1976): The role of carbohydrate moieties in the activity and properties of aminopeptidase from pig kidney. Biochim. Biophys. Acta 334: 417-422.
44. Watt, V. M. and Yip, C. C. (1989): Amino acid sequence deduced from a rat kidney cDNA suggests it encodes the Zn-peptidase aminopeptidase N. J. Biol. Chem. 264: 5480 – 5487.
45. Weber, K. and Osborn, M. (1969): The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244: 4406 – 4412.
46. Yamamoto, Y.; Li, Y. H.; Huang, K.; Ohkubo, I. and Nishi, K. (1998): Isolation and characterization of an alanyl aminopeptidase from rat liver cytosol as a puromycin-sensitive enkephalin-degrading aminoipeptidase. Biol. Chem. 379: 711 - 719.
47. Yamamoto, Y.; Li, Y. H.; Ushiyama, I.; Nishimura, A.; Ohkubo, I. and Nishi, K. (2000): Puromycin-sensitive alanyl aminopeptidase from human liver cytosol: purification and characterization. Forensic Sci. Int. 113: 143 – 146.
48. Ye, X. J.; Ng, T. B. (2011): Purification and characterization of an alanine aminopeptidase from bovine skeletal muscle. Food Chemistry 124: 634-639.

11/22/2010

Engineering aspects and associated problems of flood plain deposits in Sohag Governorate, Upper Egypt

EL-SAYED SEDEK ABU SEIF* AND EL-SHATER, A.A.

Geology Department, Faculty of Science, Sohag University, 82524 Sohag, Egypt.
*e-mail: elsayed_71@Yahoo.com

Abstract: The flood plain sediments occurred on both sides of the River Nile course which are dominated by alluvial sediments. Signs of deterioration have been seen which characterized by cracking of the superstructures. This due to low bearing capacity, ground settlement and shrinkage and swelling of these soils. The sediments of the floodplain were accumulated during the annual inundation of the Nile causing deposition of fine materials before the construction of the High Dam. Five types of clay minerals were identified throughout the studied sequence, namely smectite and kaolinite were the predominant clay minerals present in all samples, mixed layer (smectite-illite), chlorite and illite. In general, for each unit the SPT "N" values increase downwards with depth. The high C_C values of the studied clayey soil (A-Unit) is ranged between 0.24 and 0.32, that indicated to the loose and very high compressible nature of this type of soil. The geotechnical associated problems of the River Nile flood plain sediments area: the low bearing capacity of the sediments, ground settlement and Shrinkage and swelling.
[EL-SAYED SEDEK ABU SEIF AND EL-SHATER, A.A. **Engineering aspects and associated problems of flood plain deposits in Sohag Governorate, Upper Egypt**. Journal of American Science 2010;6(12):1614-1623]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Geotechnical problems; Standard penetration test (SPT); Undrained shear strength (S_U); Coefficient of compressibility (Co); Nile flood plain sediments; Clay minerals.

1. Introduction

Urban areas in Egypt occupy about 5 % of the territory and they are concentrated along the River Nile between Aswan and the Delta. The flood plain sediments occurred on both sides of the River Nile course which are dominated by muddy sediments in the upper most part. The average thickness of these mud deposits is 10 m between Aswan and Cairo.

Sohag is criss-crossed by a River Nile and most of the Nile Valley of the country is covered by of alluvial sediments. Due to insufficient accommodation for the increasing population. During the last three decades, a large numbers of structures include homes, schools, and offices in Sohag Governorate were built on soft alluvial soil of Nile flood plain sediments. Signs of deterioration have been seen which characterized by cracking of the superstructures. This due to low bearing capacity, ground settlement and shrinkage and swelling of these soils.

The suitability of soils for engineering purposes depends largely on their ability to remain in place and to support whatever loads may be placed upon them either by a permanent structure or transient loads. The civil engineer should be informed among other data of the quantity and type of clay minerals that are present and of their properties in order to evaluate their potential influence on the engineering project. Therefore, it is very important to determine the main geotechnical properties of these soil samples. When civil engineering projects are based on limited site geological conditions, geotechnical problems frequently arise and the work suffers cost increases and delays. To reduce such problems, investigation is recommended to correctly characterize the site and define land units with similar behaviour (Anonymous, 1972, 1976). This information allows the engineering geologist or the geotechnical engineer to develop a conceptual geological model (Fookes, 1997), useful to the site-specific project or to others in the same geological terrain. This model helps to understand site geological conditions, to identify the main geological problems, and to make realistic estimates of material properties.

This paper represents a comprehensive assessment of ground conditions and soil characteristics as well as causes of particular problems

2. Geological setting

From a geologic point of view, Sohag Governorate situated in the Upper Egypt occupying a major section (about 125 km long) from the Nile Valley with an average width varying from 16 to 20 km. Egyptian Nile Valley, where the Nile soil extended along the two banks of the River Nile for about 10 km. Sohag is located 467 km south of Cairo. To the east it is bounded by the Red-Sea Governorate and the Eastern desert and to the west by the New Valley Governorate and the Western desert (Fig. 1). It occupies a region including both the floodplain and the desert fringes between longitudes 31o 15' and 32o 15'E and latitudes 26o 00' and 27o 00'N. It is occupying a major section (about 125 km long) from the Nile Valley with an average width varying from 16 to 20 km, where the Nile flood plain soil extended

along the two banks of the River Nile for about 10 km. Contrarily, it constitutes only a very narrow strip along the eastern side and, moreover, it frequently disappears. Geologically the area has been studied by a variety of authors (e.g. Said, 1975, 1981, 1983, 1990; Issawi et al., 1978; Paulissen and Vermeersch, 1987; Issawi and MC Cauley, 1992; Mahran 1992 and 1993; Omer, 1996; Omer and Issawi, 1998 and Hassan, 2005). The sediments of the floodplain were accumulated during the annual inundation of the Nile causing deposition of fine materials before the construction of the High Dam These sediments are mainly muddy in nature and accumulated during the successive stages of the annual Nile floods for thousands of years till the construction of the High Dam.

3. Geotechnical investigation

3.1. Site soil conditions

The first step of site investigation was to collect undisturbed and disturbed samples of recent alluvial deposits from 0- to 30-m depth by drilling three boreholes extending along Sohag Governorate. The investigation sites from north to south are: Tema City (site I), Sohag City (site II) and Gerga City (site III), Theses samples were tested in the field and in laboratory to determine the index engineering properties. A subsurface profile is shown in Fig. (2). It is noted that the proportion of gravels and sands are increase downward at the expense of the proportion of silts and clays.

The alluvial deposits in the studied boreholes can be subdivide lithologically and geotechnically into three distinctive subunits (A, B and C). The soil sequences together with their Unified Soil Classification are given below:

Unit A (floodplain deposits): Inorganic clays of low to medium plasticity, sandy/silty/lean clays (CL): The top soil sequence is predominantly composed of sandy silt varying from one meter to 12 m in thickness especially in site III. This soil sequence is characterized by a low standard penetration test (SPT) resistance indicating a loose condition (Table 1). A floodplain is the low-lying, generally flat area adjacent to a River Nile channel which has been deposited during many thousands of years when river discharge exceeds the capacity of the channel, water rises over the channel banks and floods the surrounding low-lying lands. The average rate of sediment accumulation was about 9cm per century (Ball, 1939). Also, This type of soil are highly porous and permeable (El-Haddad and El-Shater, 1988).

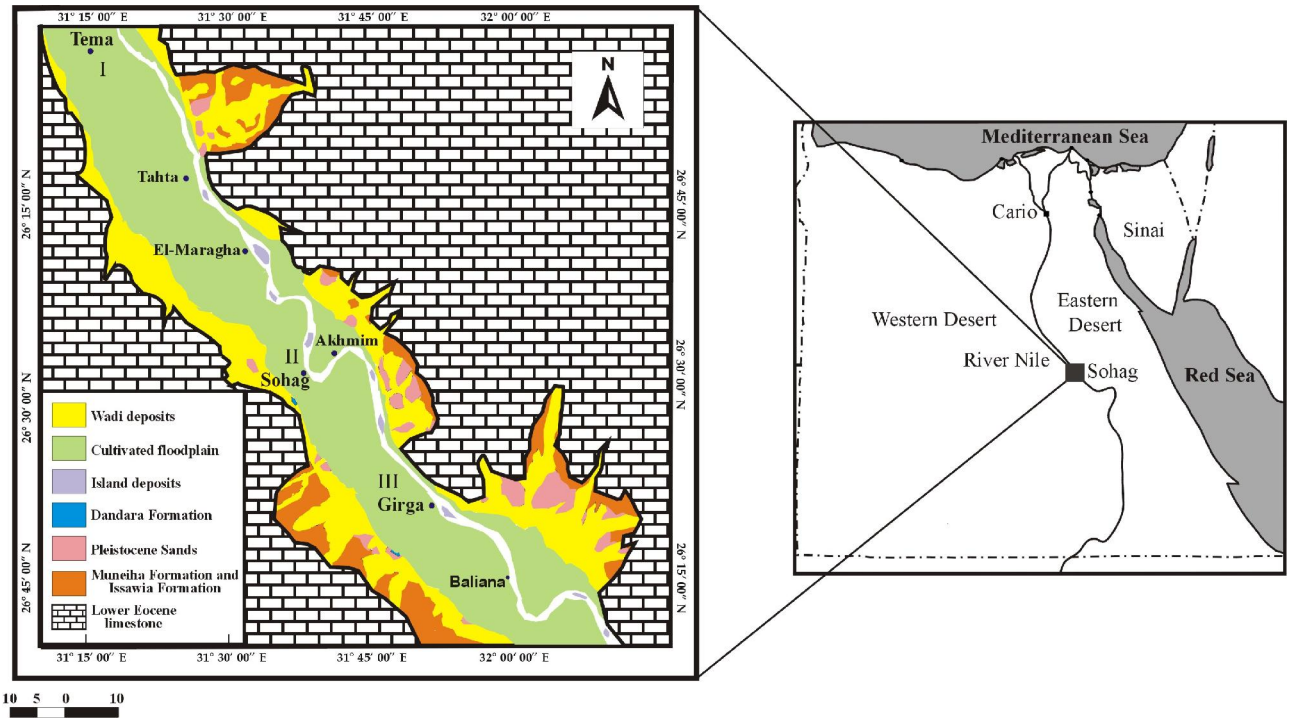


Fig. 1: Geological map of Sohag Governorate, Upper Egypt

editor@americanscience.org

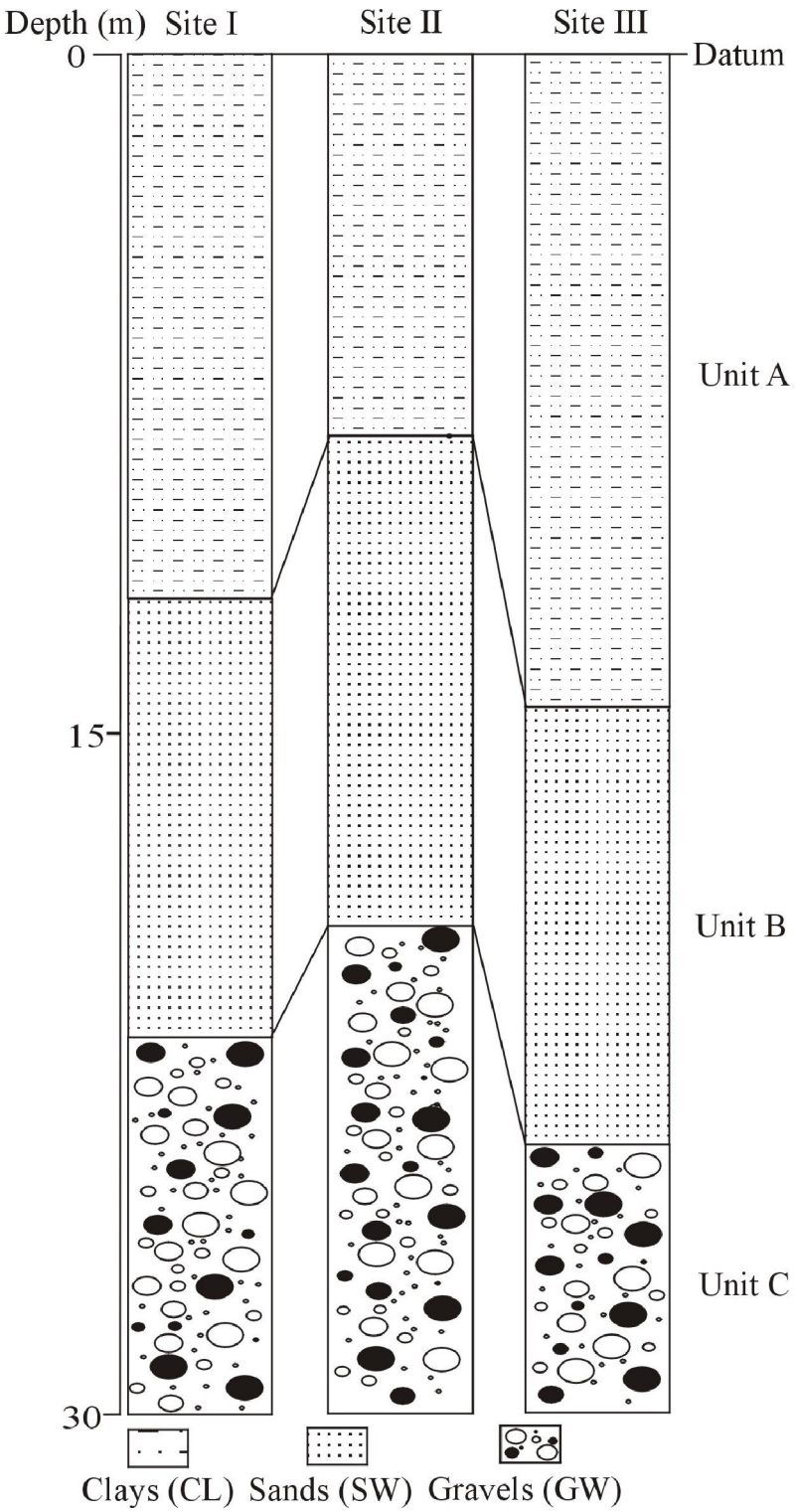


Fig.2: Typical Subsurface profile of the studied sites

Table 1a. Basic geotechnical properties of the Nile Flood Plain sediments.

Age	Depth (m)	Gravel %	Sand %	Silt %	Clay %	LL	PL	PI	Soil Type	SPT (N)	Consistency	Su, (kPa)	CC	Swelling Free	Percent% Swell	Pressure Swell	Swell	Smectite	Kaolinite	Mixed laver	Chlorite	Illite
Recent	1	0	25	4	26	4	2	1	CL	3	Soft	15-	0.									
	2	0	26	5	21	4	2	1	""	4	""		0.	6	9.	1.	4	3	1	5.	1.	
	3	0	27	5	19	4	2	1	""	5	Mediu	30-	0.									
	4	0.	29	4	22	4	2	1	""	7	""	""	0.									
	5	0.	33	4	21	4	2	1	""	8	Stiff	""	0.	7	10	1.	5	2	1	5.	2.	
	6	1.	40	4	17	4	2	1	""	10	""	60-	0.									
	7	1.	41	4	17	4	2	2	""	11	""	""	0.									
	8	1.	42	4	14	4	2	2	""	11	""	""	0.	7	12	2.	5	2	1	5.	2.	
	9	2.	42	4	15	4	2	2	""	12	""	""	0.									
	10	3.	45	3	17	4	2	2	""	12	""	""	0.	7	11	1.	5	2	1	5	2	
Late Pleistocene	11	1	58	2	6				S	14	""											
	12	1	56	2	9				""	14	""											
	13	1	55	2	6				""	15	""											
	14	1	56	2	6				""	15	""											
	15	1	57	1	7				""	15	""											
	16	1	55	2	8				""	16	Very											
	17	1	58	1	6				""	17	""											
	18	2	61	1	5				""	18	""											
	19	5	42	7	0				G	19	""											
	20	5	39	6	0				""	20	""											
	21	5	39	5	0				""	21	""											
	22	5	40	3	0				""	22	""											
	23	5	39	3	0				""	23	""											
	24	5	40	2	0				""	23	""											
	25	5	39	3	0				""	26	""											
	26	5	40	3	0				""	27	""											
	27	5	38	4	0				""	30	""											
	28	5	40	3	0				""	30	""											
	29	5	38	3	0				""	32	Hard											
	30	6	37	3	0				""	33	""											

Table 1b. Basic geotechnical properties of the Nile Flood Plain sediments.

Age	Depth (m)	Gravel %	Sand %	Silt %	Clay %	LL	PL	PI	Soil Type	SPT (N)	Consistency	Su, (kPa)	CC	Swelling Free	Percent% Swell	Pressure Swell	Swell	Smectite	Kaolinite	Mixed laver	Chlorite	Illite
Recent	1	0	1	6	20	3	2	1	CL	3	Soft	15-	0.									
	2	0	1	6	18	3	`	1	""	3	""	""	0.	7	10	1.	5	2	1	7.	1.	
	3	0	2	5	17	3	2	1	""	4	""	""	0.									
	4	0	2	5	15	3	2	1	""	5	Mediu	30-	0.									
	5	0.	3	5	15	3	2	1	""	6	""	""	0.	7	13	2.	5	2	1	6.	1.	
	6	0.	3	3	23	3	1	1	""	7	""	""	0.									
	7	1.	4	3	21	3	2	1	""	9	Stiff	60-	0.	7	10	1.	5	3	1	7	3	
Lat	8	4.	5	2	14				S	10	""											
	9	6	5	2	17				""	11	""											

editor@americanscience.org http://www.americanscience.org

	10	5.	5	2	16				""	12	""										
	11	7	5	2	16				""	13	""										
	12	8	5	1	15				""	14	""										
	13	9	6	1	13				""	14	""										
	14	10	6	1	11				""	14	""										
	15	11	6	1	10				""	15	""										
	16	12	6	1	5.				""	15	""										
	17	51	4	5	0				G	18	Very										
	18	54	4	5	0				""	18	""										
	19	55	4	4	0				""	22	""										
	20	61	3	5	0				""	22	""										
	21	62	3	6	0				""	22	""										
	22	59	3	4	0				""	25	""										
	23	60	3	4	0				""	26	""										
	24	61	3	3	0				""	26	""										
	25	62	3	3	0				""	27	""										
	26	60	3	3	0				""	27	""										
	27	61	3	4	0				""	29	""										
	28	59	3	4	0				""	30	""										
	29	57	3	5	0				""	32	Hard										
	30	56	4	3	0				""	33	""										

Table 1c. Basic geotechnical properties of the Nile Flood Plain sediments.

Age	Depth (m)	Gravel %	Sand %	Silt %	Clay %	LL	PL	PI	Soil Type	SPT (N)	Consistency	Su, (kPa)	CC	Free Swelling	Swell Percent%	Swell Pressure	Smectite	Kaolinite	Mixed laver	Chlorite	Illite
Recent	1	0	2	5	23	4	2	1	CL	3	Soft	15-	0.								
	2	0	2	5	20	3	2	1	""	4	""	""	0.								
	3	0	2	5	15	3	2	1	""	4	""	""	0.	6	10	1.	4	2	1	9	2
	4	0	2	5	14	4	2	2	""	5	Mediu	30-	0.								
	5	0	2	5	15	3	2	1	""	6	""	""	0.								
	6	0	2	5	16	3	2	1	""	6	""	""	0.								
	7	0.	2	5	19	4	2	2	""	6	Stiff	60-	0.	7	11	1.	5	2	1	7.	2.
	8	0.	3	5	14	4	2	2	""	7	""	""	0.								
	9	0.	3	5	14	3	2	1	""	7	""	""	0.								
	10	1.	3	5	13	4	2	1	""	8	""	""	0.								
	11	1.	3	5	11	4	2	2	""	8	""	""	0.	7	13	2.	5	2	1	6.	2
	12	1.	3	4	16	3	2	1	""	9	""	""	0.								
Late Pleistocene	13	1	5	2	5				S	12	""										
	14	1	5	2	8				""	14	""										
	15	1	5	1	9				""	15	""										
	16	1	5	1	8				""	16	""										
	17	1	5	1	8				""	15	""										
	18	1	5	2	7				""	17	Very										
	19	1	5	2	6				""	17	""										
	20	2	4	2	7				""	17	""										
	21	5	3	1	0				G	19	""										
	22	5	3	1	0				""	21	""										

	23	6	2	9	0		""	22	""
	24	6	2	7	0		""	26	""
	25	6	3	6	0		""	26	""
	26	6	3	5	0		""	28	""
	27	5	3	6	0		""	29	""
	28	5	3	5	0		""	30	""
	29	5	4	3	0		""	32	Hard
	30	5	4	4	0		""	33	""

Unit B (channel sands); Well-graded sand (SW): This zone is predominantly well-graded sands of channel sediments. The SPT 'N' values range from 10 to over 18 showing a general increasing downwards (Table 1). The shape of the grain size distribution curve of this type is considered "smooth.", C_U value > 6, C_C value from 1 to 3 is required (Fig. 3).

Similarly, unit C (channel gravels): Well-graded gravel (GW): This zone is predominantly well-graded gravels. The SPT 'N' values range from 18 to over 33 showing a general increasing downward (Table 1). The shape of the grain size distribution curve of this type is considered "smooth.", C_U value > 6, C_C value from 1 to 3 is required (Fig. 3).

The channel sands and gravels of Pleistocene age generally friable and highly porous and permeable. They represent the main aquifer which yields large quantities of groundwater in the Nile Valley. It has an average thickness of 150 m in Sohag area (Abdel-Moneim, 1992).

3.2. X-ray diffraction (XRD)

The XRD test was done on ten representative samples were chosen for mineralogical investigation from the three selected sites. Each sample was examined in three forms: as an oriented clay sample (untreated); as an oriented clay sample treated with ethylene glycol; and as an oriented clay sample heated to 550°C for 2h. The clay particle sizes in the samples were <2µm. The identification of the clay minerals was based on the basal reflections (001), according to the X-ray powder diffraction results of Weaver (1958 and 1967), Carrol (1970), Millot (1970), Chen (1977) and the ASTM cards. Five types of clay minerals were identified throughout the studied sequence, namely smectite and kaolinite were the predominant clay minerals present in all samples, mixed layer (smectite-illite), chlorite and illite (Table 1, Fig. 4). These results were agreed with a number of studies have previously been made on clay fractions in the alluvial soils of Egypt (Hamdi et. al., 1968; Abdel-Kader and Abdel-Hamid, 1974 and Hanna and Beckmann, 1975). According to these investigations, the dominant minerals in the clay fraction as a whole are smectite, kaolinite, and illite. They (op. cit.) found the mineralogical composition of the clay fractions is nearly the same.

3.3. Standard penetration test (SPT-N) and undrained shear strength (SU)

Engineering properties can be determined by means of tests carried out in the field and laboratory. In order to avoid certain difficulties during sampling processes in coarse-grained soils and the disturbance of the sampling in fine-grained soils, in situ tests are frequently used. The standard penetration test (SPT) is used for soil exploration in geotechnical applications and foundation design (Sivrikaya and Toğrol, 2006). The SPT has the advantages with the easiness of the test procedure and the simplicity of the equipment employed. This test is the most commonly used penetration test in Egypt. In geotechnical engineering, the engineering properties of soil layers must be known down to the required depths. The SPT is widely used around the world. The SPT test is carried out in various types of soils ranging from soft clay and loose sand to very stiff clay and dense sand. It is possible to estimate the undrained shear strength of fine-grained soils from SPT data.

SPT "N" values, are used to calculate important engineering properties of soils such as the internal friction angle (ϕ′), relative density (Dr), and bearing capacity and settlement of coarse grained soils (Schmertmann and Palacios, 1979; Kovacs *et al.*, 1981; Farrar *et al.*, 1998). The undrained compressive strength (qu) is an important characteristic for fine-grained soils and it gives an idea about their consistency. In addition, it is used to estimate both the undrained shear strength (Su) and the sensitivity of clays (Sivrikaya and Toğrol, 2006).

The SU-values based on the SPT "N" values and the consistency of the studied samples were calculated according to Tschebotarioff (1973) as shown in Table (1). The SPT "N" values range between 3 and 12 in the case of clayey soil (Unit A) that indicating a loose condition. SPT "N" values vary from 10 to 18 in the case of well graded sand soil (Unit B) and ranged from 18 to 33 in case of well graded gravels (Unit C).

In general, for each unit the SPT "N" values increase downwards with depth.

3.4. Compressibility characteristics of clayey soil

The compressibility of a soil is conditioned by the capacity of the soil voids to decrease in volume under a pressure. It is also assumed that the pore volume decrease due to a large degree of packing of soil grains. Settlement is the most common reason for failure of foundation and it is therefore of great importance to understand the mechanics of settlement. Compressibility of clayey soils is due to mechanical (deformation, reorientation and sliding of particles or domains with respect to one another, expulsion of pore fluid etc.) and physicochemical factors. Physicochemical factors may play dominant role in the compressibility of clayey soils depending on the clay mineral composition and the type of exchangeable cations present in the exchange complex (Blot 1956; Olson and Mesri 1970; Mitchell 1993).

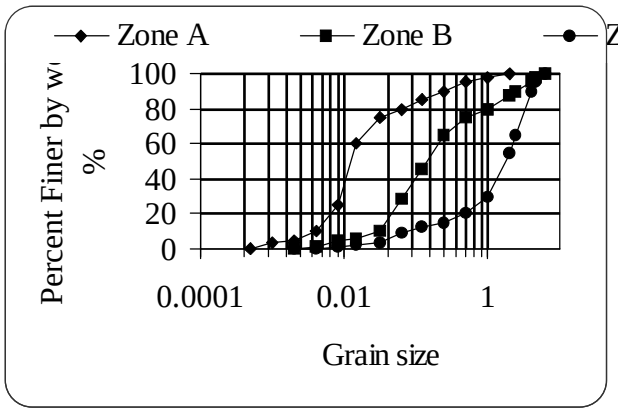


Fig. 3: Grain-size distribution curves for studied sediments.

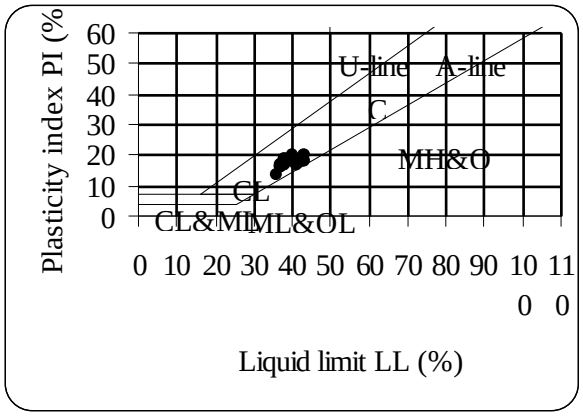


Fig. 4: XRD chart of clay minerals of unit A sediments.

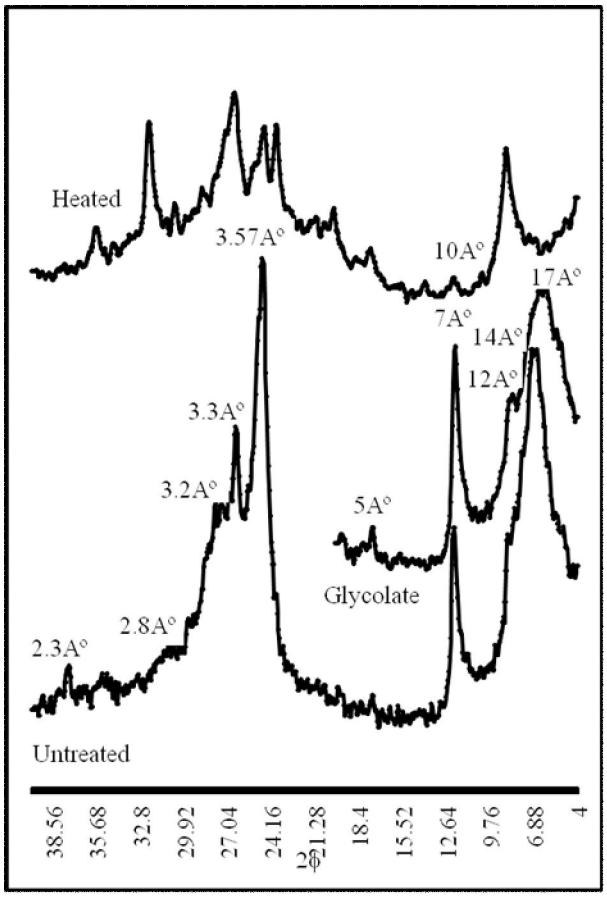


Fig. 5: Swelling potential classification of unit A sediments.

Plasticity and compressibility are typical properties of clays. Atterberg's limits of a clayey soil reflect the clay content and clay type of a soil. Compression index is also a clay dependent parameter. Among different correlations between the engineering and index properties of soils, which are often used to lessen the work load of a soil investigation program, Skempton's relationship (1944) between compression index (CC) and liquid limit (wL) given as Cc=0.009 (wL-10). The CC values were calculated using the Skempton (1944) formula: Co=0.009 (LL-10). The high CC values of the studied clayey soil (A-Unit) is ranged between 0.24 and 0.32 (Table 1, Fig. 5), that indicated to the loose and very high compressible nature of this type of soil (Maslov, 1987)

4. Geotechnical associated problems

Many parts of the world suffer from constructing problems that associated with flood plain clay soils. These problems include, ground settlement, low bearing capacity and swelling. These problems which greatly affect the safe and economic land utilization of the plains.

Alluvial deposits are generally granular and present favorable conditions, however, development activities in Egypt on these deposits are encountered with numerous geotechnical problems which greatly affect the safe and economic land utilization of the plains. These geotechnical problems: the low bearing capacity of the sediments, ground settlement and Shrinkage and swelling

.

4.1. Bearing capacity of soils

The low bearing capacity of the studied soils is the principal foundation problems in Sohag Governorate which related to the low resistance of the underlying soil to shearing resistance. Low bearing capacity is a problem that mainly affects the clay soil (CL) A-Unit of the flood plain sediments. Building foundations on these soils in this area must take into account the low shear strength of sediments and accordingly there are two frequently-used solutions. For low rise buildings, the most commonly used is a raft foundation, 1 or 1.5m deep and 60 to 80 cm thick. This type of foundation has the advantage of better support for differential ground settlement. Because the flood plain sediments are heterogeneous, geotechnical properties vary greatly over a short distance. In practice, various types of shallow foundations such as pad, strip and compensating types are used for light structures with pressures ranging from 25 to 40 kPa at depths between 1.5 and 3.0 m (Delgado, *et. al*., 2003).

The soil top layer (A) is unsuitable soil for carrying spread foundations safely. Soil improvement techniques are suggested. If these methods prove to be more economic than using deep foundations, they will be adopted:

1-It is economic to remove this layer and replace it with compacted sand or gravel.

2-Soil improvement techniques include compaction, deep compaction, preloading accompanied by sand drains, use of stone columns, injections, and concrete piles. Selection of the suitable methods depends on economy and nature of project.

4.2. Ground settlement

Ground settlement constitutes the principal geotechnical difficulty of the fine grained flood plain sediments. This problem is associated with fine sediments because the compressibility of sands and gravels is generally low (Smith and Smith, 1998). Settlement is particularly important in the flood plain sediments. Settlement is another major foundation problem in Sohag Governorate related to the loose and compressible nature of the surficial soil. The compression index, extracted from the record, ranged between 0.24 and 0.32 (Table 1). That means the surficial clay soil is of loose and very high compressible type (Maslov, 1987).

Structural settlement in the Sohag Governorate varies from a few centimeters to more than 50cm. Where, loading is high, deep foundations are usually used to reduce settlement. Another solution is ground improvement by vibro-replacement (gravel columns) of the ground to a depth of up to 10–15 m (Delgado, *et. al.*, 2003).

4.3. Shrinkage and swelling

Shrinkage and swelling are well-known phenomena causing damage to building foundations, roads, aircraft runways, underground service lines, etc. It is caused by a deficiency or excess of water (Youssef *et al.*, 1957; Popesco, 1980). Shrinkage and swelling soils are often characterized by high LL and PI caused by a variable content of more active clay minerals. Plasticity values of the studied soils are given in Table 1. The data are not strictly comparable because they are collected at different sites.

Undisturbed representative samples were chosen (Table 1), to determine swelling pressure as well as swelling percent using oedometer test. The Swelling pressure of the studied fine-grained soil of flood plain sediments varies from 1.8 to 2.4 kg/cm^2 (medium expansion, Chen, 1975). The free swell test was carried out typically as described by Holtz and Gibbs (1956), the free swell values of the studied samples ranged between 65 and 70% (Table 1). Ranganatham and Satyanarayana (1965) (quoted by Derriche *et. al.,* 1998) used plasticity index (PI) as classification indicator, PI-values of the studied clayey soil ranged from 14 to 20 (low swelling potential). Swelling measurements values are indicative of the low-medium swelling potential. For light structures, this can cause excessive uplift pressure inflicting damage. The problem of swelling can be handled, among others, by designing structures sufficiently rigid or flexible to accommodate the anticipated movement. Therefore, footings, piers, foundations, etc. should be placed at sufficient depths. It is common to place compacted sand (or sand and gravel or pit run gravel) cushion as buffer flexible layer between the foundation and the swelling clay.

9. Summary and Recommendations

This work can be considered as a simple geotechnical model of the Nile flood plain sediments in Upper Egypt. Thus, the following four points are of special interest and should be connected with the future projects.

(1) The geological features of the Nile flood plain soil in Sohag Governorate, Upper Egypt, had a great influence on the evolution of ground conditions and soil properties.

(2) Soil test data from the development projects cover almost the entire Nile flood plain soil in Sohag

Governorate indicate that the subsoil is dominated by sandy clay and sandy materials. A typical subsurface profile shows that the cover has a variable thickness comprising soft fine-grained cohesive soils graded down ward into well-graded sandy soil. There are broad similarities with respect to the textural composition over large distances..

(3) Sohag Governorate especially in flood plain region has encountered numerous specific construction problems because of its unique sedimentation history. The top most soils are too weak to support heavy foundations.

(4) A sufficient safety factor must be done in the design of any construction founded on this soil type.

Corresponding author
El-Sayed Sedek Abu Seif*
Geology Department, Faculty of Science, Sohag University, 82524 Sohag, Egypt.
*e-mail: elsayed_71@Yahoo.com

References

1. Abdel Moneim, A.A., (1992): Numerical simulation and groundwater management of the Sohag Aquifer. Ph.D. Thesis, Civil Eng. Dept., University of Strathclyde, U.K.
2. Abdel-Kader, F.H. and Abdel-Hamid N.E (1974): Comparative micromorphology of some soil types of Lower Egypt. Geoderma, Vol. 12, No. 3, pp. 245-262.
3. Anonymous, C. (1972): The preparation of maps and plans in terms of engineering geology. Q. J. Eng. Geol. Vol. 5, pp. 293– 381.
4. Anonymous, C. (1976): Engineering Geological Maps: A Guide to their Preparation. The UNESCO Press, Paris.
5. Ball, J. (1939): Contributions to the geography of Egypt. Survey & Mines Dept., Cairo, 308p.
6. Blot, G.H. (1956): Physico-chemical analysis of the compressibility of pure clays. Geotchnique, vol. VI, No. 2, London, S. W. I. P. 86.
7. Carrol, D. (1970): Clay minerals: a guide to their X-ray identification. Geol. Soc. Am. Spec. paper No. 126, 75p.
8. Chen, F.H. (1975): Foundation on Expansive Soil. Elsevier, Amsterdam, 464 pp.
9. Chen, P.Y. (1977): Table of key lines in x-ray powder identification patterns of minerals in clays and associated rocks. Occas. Ind. Geol. Surv., paper No. 21, pp. 67.
10. Delgado, J., Alfaro , P.J., Andreua, A.M., Cuencab, C. Dome´nechc, C., Este´vez, A. Soriaa, J.M., Toma´sd, R. and Ye´benes, A. (2003): Engineering-geological model of the Segura River flood plain (SE Spain): a case study for engineering planning. Engineering Geology, Vol. 68, pp. 171–187.
11. Derriche, Z. Kebailim. (1998): Prevision du gonlement des argiles d In-Amenas. Bulletin des laboratories des Ponts et Chaussees, 218, November- decembre, pp. 15-23.
12. El-Haddad, A. and El-Shater, A. (1988): Sediments characteristics as a controlling factor of pollution of the groundwater from disposed of wastes, Sohag, Egypt. Sohag Pure & Appl. Sci. Bull. Fac. Sci., V.4, pp.145-162.
13. Farrar, J.A., Nickell, J., Alien, M.G., Goble, G., and Berger, J. (1998): Energy loss in long rod penetration testing — Terminus Dam liquefaction investigation. Proceedings of the ASCE Specialty Conference on Geotechnical Earthquake Engineering and Soil Dynamics III, Seattle, vol. 75, pp. 554–567.
14. Fookes, P.G. (1997): Geology for engineers: the geological model, prediction, and performance. Q. J. Eng. Geol. 30, 293– 424.
15. Hamdi, H., Salah E.M. and Bishay Guindi, B. (1968): A comparative study of hydraulic conductivity in the alluvial soils of Egypt. Geoderma, Vol. 2, No. 1, pp. 27-32.
16. Hanna, F. S. and Beckmann, H. (1975): Clay minerals of some soils of the Nile Valley in Egypt. Geoderma, Vo. 14, No. 2, pp. 159-170.
17. Hassan, A.M. (2005): Geochemical characteristics of the superficial Nile Basin sediments and their environmental Relevance, Sohag Area, Egypt. M. Sc. Thesis, Fac. of Sci. South Valley University (Sogah Branch).
18. Holtz, W.G., and Gibbs, H.J. (1956): Engineering properties of expansive clays. Transactions of ASCE, vol.121, pp. 641-663
19. Issawi, B and McCauley, J.F. (1992): The Cenozoic rivers of Egypt: the Nile problem. In: Freidman, R. and Adams, B. (Eds)., The Followers of Hours: Studies asoc. Public., No.2, Oxbow Mong. 20, Park End Place, Oxford, p.121-138.
20. Issawi, B.; Hassan, M.W. and Osman, R. (1978): Geological studies in the area of Kom Ombo, Eastern Desert, Egypt. Ann. Geol. Survey, Egypt, V.VIII, pp.187-235.
21. Kovacs, W.D., Salomone, L.A., and Yokel, F.Y. (1981): Energy Measurement in the Standard Penetration Test. U.S. Department of Commerce and National Bureau of Standards, Washington D.C.
22. Mahran, T.M. (1992): Sedimentological development of the Upper Pliocene-Pleistocene sediments in the areas of El-Salamony and El-Sawamha Sharq, NE Sohag, Nile Valley, Egypt. Sohag Pure & Appl. Sc. Bull. Fac. Sc., Vol. 8, pp. 251-275.

23. Mahran, T.M. (1993): Sedimentology of Upper Pliocene-Pleistocene sediments of the Nile Valley area: A model around Awlad Toq Sharq Environs., SE of Sohag, Egypt. Bull. Fac. Sci Assuit University Assuit, Egypt. Vol. 22, No. 2 pp. 1-25.
24. Maslov, N.N. (1987): Basic engineering geology and soil mechanics. Mir Publishers Moscow, 551p.
25. Millot, G. (1970): Geology of Clays. Springer Verlag, New York, 429P.
26. Mitchell, J.K. (1993): Fundamentals of Soil Behavior, 2nd Edition, John Wiley & Sons, Inc., New York.
27. Olson, R.E., and Mesri, G. (1970): Mechanism controlling compressibility of clay. Journal of the Soil Mechanics and Foundations Division. ASCE, vol. 96, pp. 1863-1878.
28. Omer, A.A.M. (1996): Geological, mineralogical and geochemical studies on the Neogene and Quaternary Nile basin deposits, Qena-Assiut stretch, Egypt. Ph.D. Thesis, Geology Dept., Fac. Sci. Sohag, South Valley University. 320p.
29. Omer, A.A.M., and Issawi, B. (1998): Lithostratigraphical, mineralogical and geochemical studies on the Neogene and Quaternary Nile basin deposits, Qena-Assiut stretch, Egypt. The 4Th International Conference on Geology of the Arab World, Cairo. (Abstract).
30. Paulissen, M. and Vermeersch, P.M. (1987): Earth, man and climate in the Egyptian Nile Valley during the Pleistocene. In: Close, A. (Ed.), Prehistory of North Africa. Southern Methodist University Press, Dallas, p.29-67.
31. Popesco, M.E. (1980): Engineering problems associated with expansive clays from Romania. Eng. Geol., 14: 43-53.
32. Said, R. (1975): The geological evolution of the river Nile. In: Problems in Prehistory of Northern Africa and the Levant. Wendorf, F. and Marks, A. F. (Eds.). Southern Methodist University Press. Dallas, Texas, pp.1-44.
33. Said, R. (1981): The geological evolution of the River Nile. Springer-Verlag, New York. 151p.
34. Said, R. (1983): Proposed classification of the Quaternary of Egypt. J. African Earth Sciences., V.1, p.41-45.
35. Said, R. (1990): The geology of Egypt. Egyptian General Corporation, Conoco Hurghada Inc. and Repsol Exploration, S. A., Balkema, Rotterdam, Brookfield, 731p.
36. Schmertmann, J.H., Palacios, A. (1979): Energy dynamics of SPT. ASCE Journal of Geotechnical Engineering Division 105 (GT8),909–926.
37. Sivrikaya, O., and Togˇrol, E. (2006): Determination of undrained strength of fine-grained soils by means of SPT and its application in Turkey. Engineering Geology, Vol. 86, pp. 52-69.
38. Skempton, A. W. (1944): Notes on the compressibility of clays. Q.J. Geol. Soc. London, C:119-135.
39. Smith, G.N., Smith, I.G.N. (1998): Elements of Soil Mechanics, 7th ed. Blackwell, Oxford.
40. Tschebotarioff, G.P., (1973): Foundations, Retaining, and Earth Structures, 2nd edition. McGraw-Hill, New York.
41. Weaver, C.E. (1958): Geological interpretation of clay minerals in sedimentary rock, Clay Minerl., 5th Nat. Conf., Nat. Res. Councel Publ., Vol. 566, pp. 159-175.
42. Weaver, C.E. (1967): The signficance of clay minerals in sediments, in fundamental aspects of petrol. Geochemistry, Nagy and Colombo, Ed. El-Sevier
43. Youssef, M.S., Sabry, A.A. and Tawfiq, M.M. (1957): Substantial consolidation and swelling of clay caused by two intersecting cases of serious damage to hospital building in Egypt. Int. Conf. Soil Mech. Found. Eng., 1: 462-46.

11/3/2010

A Multi-Objective Approach for Multi Capacity warehouse Location within Distribution Supply Chain Problem

Mehrdad Rezazadeh [1], Sajjad Farahani [2]

[1.] School of Industrial Engineering, Islamic Azad University - South Tehran Branch, Tehran, Iran.
[2.] Industrial Engineering department at Amirkabir University of Technology, Tehran, Iran.
Sajad.farahani@gmail.com

Abstract: In this paper, we propose a mixed integer programming formulation for a location distribution problem. We have a two layer supply chain, central warehouses/stocks, regional warehouses and customers. Stocks should satisfy the multi-commodity customers demand. Our objectives are to minimize transportation cost of goods, from stocks to regional warehouses and from regional warehouses to customers, and installing cost of warehouses and to maximize average service level of customers. Our model determines a set of Pareto optimal solution for considering these two conflicting objectives. We have a three type alternatives for both stocks and regional warehouses with varying installing costs and capacities. Regarding the long term decision making for a location problem, we consider time value of money to have more assumptions of real worlds. As a result, a case study is indicated to show efficiency of model to solve the industrial problems; a sensitivity analysis is also implemented upon the rate of return and the life of cycle of the supply chain system.
[Mehrdad Rezazadeh, Sajjad Farahani. **A Multi-Objective Approach for Multi Capacity warehouse Location within Distribution Supply Chain Problem**. Journal of American Science 2010;6(12):1624-1628]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Supply chain, facility location, mixed integer programming, time value of money

1. Introduction

Supply chain management (SCM) is the process of planning, implementing and controlling the operations of the supply chain in an efficient way. SCM spans all movements and storage of raw materials, work-in-process inventory, and finished goods from the point-of-origin to the point-of-consumption (Council of Supply Chain Management Professionals 2007, Simchi-Levi et al. 2004).

There are more works in literature considering concepts of SCM in variant areas that we state some of them as follows. Altiparmak et al. (2006) developed a multi-objective genetic algorithm (MOGA) to find a set of optimal pareto solution for Supply chain network (SCN) design. Thanh et al. (2008) proposed a mixed integer programming (MIP) formulation to design and plan a production – distribution system along the supply chain. Pujari et al. (2008) presented an integrated approach for incorporation of location, production, inventory and transportation issues within a supply chain. Shu and Karimi (2009) developed two heuristic algorithms for considering concept of safety stock in supply chain networks. Kaminsky and Kaya (2008) proposed effective heuristics for inventory positioning in supply chain networks involving several centrally managed production facilities and external suppliers. Monthatipkul and Yenradee (2008) introduced an MIP model to find an optimal inventory/distribution plan (IDP) control system for a one-warehouse/multi-retailer supply chain system. Chauhan et al. (2009) designed a heuristic for Multi-commodity supply network planning and a branch and price for large-sized problems. Khouja formulated a three-stage supply chain model and investigate effect of change from two-stage from three-stage in cost reduction. Seliaman and Ahmad consider three-stage supply chain with stochastic demand to optimize inventory decision. Santoso et al. (2005) proposes a stochastic programming formulation for supply chain under uncertain environment. Newly, a single vendor and multiple retailers supply chain retailers is modeled (Darwish and Odah, to be published). For more detailed study, Gunasekaran and Ngai (2009) and Minner (2003) can be useful.

Facility location is and has been a well established research area within Operations Research (OR). Numerous papers and books are witnesses of this fact. The development of SCM started independently of OR and only step by step did OR enter into SCM. As a consequence, facility location models have been gradually proposed within the supply chain context (including reverse logistics), thus opening an extremely interesting and fruitful application domain (Melo et al. 2009). Here, some previous researches worked on location within supply chain, are described. Gebennini presented a model for location-allocation problem to optimize safety stocks and customer service level. Snyder et al. (2007) proposed a stochastic version of the location model with risk pooling (LMRP) that include location, inventory, and allocation decisions under uncertainty.

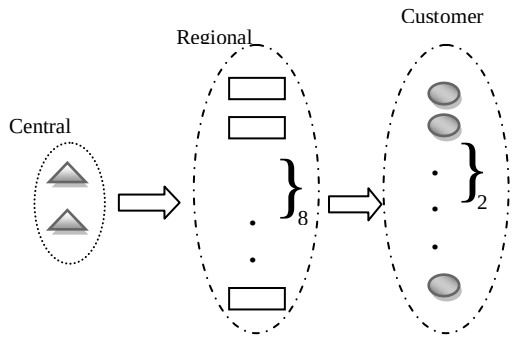
Syam (2002) extend facility location problem considering several concepts of logistic as holding, ordering, and transportation costs. He used two lagrangian relaxation and a simulated annealing (SA) based heuristics algorithm for comparing experimental results. Thanh et al. (2008) consider facility location problem in supply chain within planning horizon. Melo et al. (2009) reviewed facility location problem in a well-organized way that it can be useful for being depth in this area. There are other problems derived from facility location problem as transfer point location, hub location and etc.

In this paper, we present a multi-objective mixed integer programming formulation for location within network distribution problem considering time value of money, TVM. Objectives are to minimize total cost including establishment and transportation cost and to maximize customer satisfaction. The problem describes two location layers in single period. We determine the volume of the inventory in both stocks and middle warehouses. There is a few resea considering TVM as Rastpour and Esfehani (2010). They proposed a mathematical formulation for input/output point of department in facility layout considering time value of money. The remainders of paper are as follows. Model description is stated in section II. In, Section III, mathematical model is formulated, computational results are indicated in section IV and conclusions are discussed in section V.

2. Model formulation

The Components of supply chain such as are illustrated in Figure 1 are introduced. Central warehouses: the main stocks of supply chain that demands are supplied here. There are two potential location for central warehouses, capital of country and south port. Regional warehouses: stocks between central warehouses and customers that demands are distributed here. There are 8 potential locations for regional warehouses that they are in the capital of provinces. Customers: there are 28 customers that are located in the cities of the provinces. Goods: Five types of commodities can be supplied for the customers demanding five families of cars.

Figure 1. Components of supply chain



Assumptions of problem are as follows:

1. There are two potential central warehouses that at least one of them should be located,
2. There are limited capacities for both central and regional warehouses.
3. Transportation cost per unit is as a coefficient of distance between central and regional warehouses and between regional warehouses and customers.
4. There is a minimum level of customer satisfaction.

There are two objectives for supply chain, minimizing total cost including establishment and transportation cost and maximizing customer satisfaction.

Sets and indices

P Sets of central warehouses $(|P|=p, k \in P)$,
M Sets of regional warehouses $(|M|=m, j \in M)$,
N Sets of customers $(|N|=n, i \in N)$,
O Sets of good types $(|O|=o, t \in O)$, .
L Sets of type of warehouses $(|L|=L, l \in L)$,

Variables

v_{kl} is 1 if the potential point k is selected as a stock with capacity type l, otherwise 0.
u_{jl} is 1 if the potential point of j is selected for a regional warehouse with capacity type l.
x_{ijt} Percentage of demand customer i for commodity t that is supplied by regional warehouse j,
y_{jkt} Percentage of demand regional warehouse j for commodity t that is supplied by central warehouse k

Parameters

M Number of month in a year,
a_{it} Demand of customer i for commodity t,
b_{ijt} Capacity of regional warehouse j with type l for commodity t,
c Cost of transportation per unit,
d_{ij} Distance between regional warehouse j and customer i,
d'_{jk} Distance between regional warehouse j and central warehouse k,
e_{ktl} Capacity of central warehouse k with type l for commodity t,
P Coefficient of total cost in objective function,
q_{kl} Cost of installation central warehouse k with type l,
S_{it} Minimum level of customer satisfaction i for commodity t
W_{jl} Cost of installation regional warehouse j with

type l,

$$\min z_1 = P.\sum_{t=1}^{o}\sum_{j=1}^{m}\sum_{i=1}^{n} cd_{ij}a_{it}x_{ijt} + P\sum_{t=1}^{o}\sum_{k=1}^{p}\sum_{j=1}^{m} cd'_{jk}a_{it}y_{jkt} + P\left(\sum_{l=1}^{L}\sum_{j=1}^{m} w_{jl}u_{jl} + \sum_{k=1}^{p} q_{kl}v_{kl}\right)(A/P, i/M\%, nM)$$

$$\max Z_2 = (1-P)\sum_{t=1}^{o}\sum_{i=1}^{n}\sum_{j=1}^{m} x_{ijt}/no$$

$$\sum_{t=1}^{o}\sum_{i=1}^{n} x_{ijt} \le no\sum_{l=1}^{L} u_{lj} \qquad \forall j \qquad (1)$$

$$\sum_{t=1}^{o}\sum_{j=1}^{m} y_{jkt} \le mo\sum_{l=1}^{L} v_{lk} \qquad \forall k \qquad (2)$$

$$\sum_{t=1}^{o}\sum_{i=1}^{n} a_{it}x_{ijt} \le \sum_{t=1}^{o}\sum_{l=1}^{L} b_{jtl}u_{lj} \qquad \forall j \qquad (3)$$

$$\sum_{i=1}^{n}\sum_{j=1}^{m}\sum_{t=1}^{o} y_{jkt}a_{it} \le \sum_{l=1}^{L}\sum_{t=1}^{o} e_{ktl}v_{kl} \qquad \forall k \qquad (4)$$

$$\sum_{j=1}^{m} x_{ijt}a_{it} \ge s_{it}a_{it} \qquad \forall i,t \qquad (5)$$

$$\sum_{k=1}^{p} b_{jtl}y_{jkt} + \sum_{i=1}^{n}\sum_{t=1}^{o} a_{it}(1-u_{jl}) \ge \sum_{i=1}^{n} a_{it}x_{ijt} \qquad \forall j,t,l \qquad (6)$$

$$\sum_{i=1}^{n} x_{ijt} \le 1 \qquad \forall j,t \qquad (7)$$

$$u_{jl}, v_{kl} \in \{0,1\}$$

First objective Z_1, is summation of present cost, installation cost, and annually cost, transportation cost: Transportation cost between central and regional warehouses, $\sum_{t=1}^{o}\sum_{j=1}^{m}\sum_{i=1}^{n} c.d_{ij}a_{it}x_{ijt}$ Transportation cost between regional warehouses $\sum_{t=1}^{o}\sum_{k=1}^{p}\sum_{j=1}^{m} c.d'_{jk}a_{it}x_{ijt}$ and customer, Installation cost for central warehouses, $\sum_{l=1}^{L}\sum_{j=1}^{m} w_{jl}u_{jl}$ and Installation cost for regional warehouses, $\sum_{l=1}^{L}\sum_{k=1}^{p} q_{kl}v_{kl}$ that is multiplied by weighted coefficient P. Second objective, Z_2 is the summation of the level of the customer satisfaction that is multiplied by $(1-P)$. Constraints (1) and (2) states if regional warehouse j or central warehouse k satisfy the demand, it has been installed. Constraints (3) and (4) show capacity restriction for each regional warehouse. Constraint (5) implies that there is a minimum level of customer satisfaction i for commodity t. Constraint (6) considers that amount of supply should be greater than amount of demand. Finally, constraint (7) state that for service level of each goods for each customer is less than 100%.

3. Computational Results

We run the model without considering TVM and with considering TVM. Table 1 and 2 show the results of the model. We show Pareto set of solution the model both before and after TVM (Figure 2) and we show effect of rate and life cycle in the service level (Fig. 3).

Table 1. Computational result before considering TVM in the model

n=20						n=30					
i=10%			i=20%			i=10%			i=20%		
α	Z_1×10^3	Z_2%	α	Z_1×10^3	Z_2%	α	Z_1×10^3	Z_2%	α	Z_1×10^3	Z_2%
1	10.26	29.5	1	5.8	20.3	1	11.25	30.3	1	5.91	30
0.9	10.27	29.7	0.9	5.81	20.4	0.9	11.26	30.4	0.9	5.93	30.1
0.8	10.27	29.8	0.8	5.82	20.4	0.8	11.28	31.8	0.8	5.94	30.3
0.7	10.28	30	0.7	5.83	22.1	0.7	11.29	31.9	0.7	5.94	30.4
0.6	10.29	31.9	0.6	5.84	22.2	0.6	11.3	32	0.6	5.95	31.9
0.5	10.31	32.1	0.5	5.86	22.4	0.5	11.3	32.2	0.5	5.95	32
0.4	10.32	32.2	0.4	5.87	23.2	0.4	11.31	32.3	0.4	5.95	32.5
0.3	10.32	32.3	0.3	5.87	35.5	0.3	11.32	32	0.3	6	35.5
0.2	10.34	35.5	0.2	5.89	36.5	0.2	11.33	35.5	0.2	6.1	36.5
0.1	10.34	37	0.1	5.92	39	0.1	11.34	36.5	0.1	6.1	39
0	10.6	43	0	6.31	46.5	0	11.6	42.5	0	6.3	46

Table 2. Computational result after considering TVM in the model

n=20						*n*=30					
i=10%			*i*=20%			*i*=10%			*i*=20%		
α	$Z_1 \times 10^3$	Z_2%	α	$Z_1 \times 10^3$	Z_2%	α	$Z_1 \times 10^3$	Z_2%	α	$Z_1 \times 10^3$	Z_2%
1	9.51	32	1	5.4	32	1	10.5	32	1	5.5	32
0.9	9.56	40.5	0.9	5.44	41	0.9	10.5	40.5	0.9	5.5	41
0.8	9.59	41.4	0.8	5.49	42.7	0.8	10.6	41.4	0.8	5.5	42.7
0.7	9.67	42.7	0.7	5.49	42.7	0.7	10.6	42.7	0.7	5.5	42.7
0.6	9.67	42.7	0.6	5.63	44	0.6	10.6	42.7	0.6	5.7	44
0.5	9.7	43	0.5	7.36	55	0.5	10.6	42.7	0.5	7.4	54.5
0.4	10.4	46	0.4	7.8	57	0.4	11.1	45	0.4	8	57
0.3	13.4	56	0.3	7.85	57.2	0.3	14.6	56	0.3	8	57
0.2	13.8	57.1	0.2	7.85	57.2	0.2	15.2	57.2	0.2	8	57
0.1	13.8	57.2	0.1	7.85	57.2	0.1	15.2	57.2	0.1	8	57
0	13.8	57.2	0	7.85	57.2	0	17.4	57.2	0	8	57
0.95	9.53	39	0.55	5.75	45	0.35	14.1	54.5	0.55	5.7	44
0.98	9.52	36.5	0.525	5.9	46	0.38	11.3	45.5	0.525	6	46
0.35	12.9	55	0.51	7.27	54.5	0.36	11.4	46	0.5125	7.4	54.5
0.38	12.8	54.5	0.517	7.27	54.5	0.355	14.1	54.5	0.51875	6	46
0.39	10.4	46	0.52	7.27	54.5	0.358	14.1	54.5	0.5156	7.4	54.5
0.385	10.4	46	0.5225	5.9	46	0.359	14.1	54.5	0.5172	6	46
0.382	12.8	54.5	0.5212	5.9	46	0.3595	14.1	54.5	0.5164	7.4	54.5
0.383	10.4	46	0.5206	7.27	54.5	0.3598	14.1	54.5	0.5168	7.4	54.5
0.3825	10.4	46	0.5209	7.27	54.5	0.3599	11.4	46	0.517	6	46
0.3827	10.4	46	0.5211	7.27	54.5	0.35985	14.1	54.5	0.5169	7.4	54.5

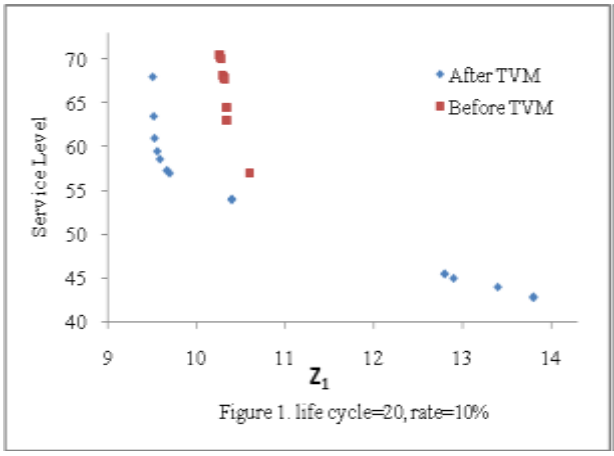


Figure 1. life cycle=20, rate=10%

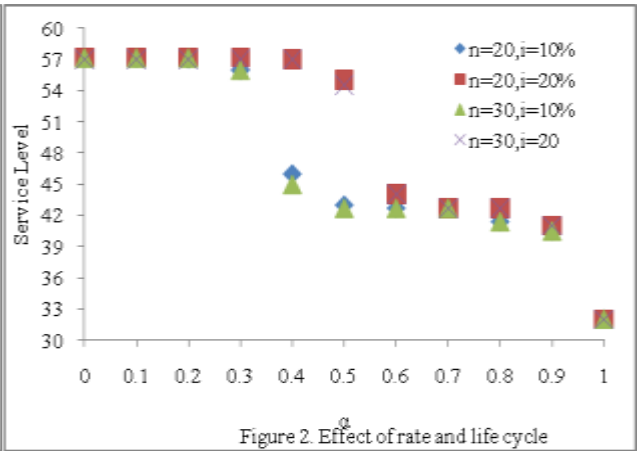


Figure 2. Effect of rate and life cycle

4. Conclusions

In this paper, we present a new mixed integer programming formulation for multi-capacity multi level location distribution problem. Regarding, location problem is a long term, so we consider time value of money in period of using SCM system. We consider different cost of installment for each size of warehouses and stocks according to real world assumptions. Computational results show effect of TVM in objective function. As future research, we suggest to present a multi capacity multi period location distribution problem in which we have a different demand in each period. We show Pareto set of solution the model both before and after TVM (See Figure 2) and we show effect of rate and life cycle in the service level (Fig. 3).

References

[1] Altiparmak F, Gen M, Lin L, Paksoy T, A genetic algorithm approach for multi-objective optimization of supply chain networks, Computers & Industrial Engineering 51 (2006) 196–215.

[2] Chauhan S. S, Frayret J-M, LeBel L, Multi-commodity supply network planning in the forest supply chain, European Journal of Operational Research 196 (2009) 688–696.

[3] Council of Supply Chain Management Professionals, <http://cscmp.org>, 2007.

[4] D. Simchi-Levi, P. Kaminsky, E. Simchi-Levi, Managing the Supply Chain: The Definitive Guide for the Business Professional, McGraw-Hill, New York, 2004.

[5] Darwish M. A, Odah O. M, Vendor Managed Inventory Model for Single-Vendor Multi-Retailer Supply Chains, To be published in European Journal of Operational Research.

[6] Gebennini E, Gamberini R, Manzini R, An integrated production–distribution model for the dynamic location and allocation problem with safety stock optimization, International Journal of Production Economics 122(2009)286–304.

[7] Gunasekaran A, Ngai E. W.T. Modeling and analysis of build-to-order supply chains, European Journal of Operational Research 195 (2009) 319–334.

[8] Kaminsky P, Kaya O, Inventory positioning, scheduling and lead-time quotation in supply chains, International Journal of Production Economics 114 (2008) 276–293.

[9] Khouja M, Optimizing inventory decisions in a multi-stage multi-customer supply chain, Transportation Research Part E 39 (2003) 193–208.

[10] Melo M.T, Nickel S, Saldanha-da-Gama F, Facility location and supply chain management – A review, European Journal of Operational Research 196 (2009) 401–412.

[11] Minner S, Multiple-supplier inventory models in supply chain management: A review, International Journal of Production Economics 81–82 (2003) 265–279.

[12] Monthatipkul C, Yenradee P, Inventory/distribution control system in a one-warehouse/multi-retailer supply chain, International Journal of Production Economics, 114 (2008) 119–133.

[13] Pujari N. A, Hale T. S, Huq F, A continuous approximation procedure for determining inventory distribution schemas within supply chains, European Journal of Operational Research 186 (2008) 405–422.

[14] Rastpour A, Esfehani M. S, Mathematical models for selection of optimal place and size of connections considering the time-value of money, European Journal of Operational Research, 200(3), 764-773.

[15] Santoso T, Ahmed S, Goetschalckx M, Shapiro A, A stochastic programming approach for supply chain network design under uncertainty, European Journal of Operational Research 167 (2005) 96–115.

[16] Seliaman M.E, Ahmad A A, Optimizing inventory decisions in a multi-stage supply chain under stochastic demands, Applied Mathematics and Computation 206 (2008) 538–542.

[17] Shu j, Karimi I. A, Efficient heuristics for inventory placement in acyclic networks, Computers & Operations Research 36 (2009) 2899-2904.

[18] Snyder L. V, Daskin M. S, Teo C-T, The stochastic location model with risk pooling, European Journal of Operational Research 179 (2007) 1221–1238.

[19] Syam S. S, A model and methodologies for the location problem with logistical components, Computers & Operations Research 29 (2002) 1173-1193.

[20] Thanh P. N, Bostel N, Pe´ton O, A dynamic model for facility location in the design of complex supply chains, International Journal of Production Economics 113 (2008) 678–693.

12/25/2010

Patient Satisfaction And Its Related Factors Within Emergency Care Departments: A Study Of Iranian Military Hospitals

Ahmad Ameriyon[1],Mohammadkarim Bahadori[1*], Mohammad Meskarpour Amiri[2], Hosein Amini Anabad[2]

[1.] Health Management Research Centre, Baqyattallah University of Medical Sciences, Tehran, Iran
[2.] Department of Health Economics, School of Medicine, Shahed University, Tehran, Iran
bahadorihealth@gmail.com

Abstract: Background: Today, researchers pay special attention to patient satisfaction with emergency care services, the first line of hospital healthcare services. However, the nature of emergency medicine (EM) has changed significantly in recent years, and related factors in patient satisfaction have changed over time. The aim of this study was assessment of patient satisfaction and its related factors with emergency care services in six Iranian military hospitals. **Materials and Methods:** In this cross-sectional study, the satisfaction levels of 360 patients of emergency care services in six military hospitals of Iran in 2007 were assessed. After discharge from the emergency ward, a checklist of basic information and a 12-item questionnaire about satisfaction levels was completed for each patient. A 5-level Likert scale was used for the responses. Scores from 20-100 were allocated to each response (completely dissatisfied to completely satisfied), respectively. **Results:** 3,559/4,220 responses (82.4 percent) were completely satisfied or satisfied. In respect to priority, "Observation of ethical issues," "giving information “and” behavior of reception personnel" had the highest scores. "Variety of medical specialists," "emergency ward facilities," and "speed in calling doctor" scored the lowest. The total satisfaction score reported by patients older than 35 year ($p=0.022$), insurance coverage ($p=0.002$) and with history of previous referring to that emergency ward ($p=0.017$) was significantly higher than others. Gender, marital status, and educational level had no statistical correlation with the total satisfaction score ($p>0.05$). **Conclusion:** The findings of this study revealed favorable satisfaction levels for patients receiving emergency care services at military hospitals. However, using a variety of expert physicians and more facilities and also improving the process of calling doctors into the emergency ward are aspects that need more attention from healthcare managers in emergency centers.

[Ahmad Ameriyon, Mohammadkarim Bahadori, Mohammad Meskarpour Amiri, Hosein. **Patient Satisfaction And Its Related Factors Within Emergency Care Departments: A Study Of Iranian Military Hospitals**. Journal of American Science 2010;6(12):1629-1635]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: satisfaction; emergency departments; military hospital

1. Introduction

Respect for patients’ needs and wishes is critical to any healthcare system (Gani et al, 2007; Soufi et al, 2010; Nguyen et al,2002). The quality of health services has traditionally been based on professional practice standards. However, over the last decade, patient perceptions about healthcare have been predominantly accepted as an important indicator of healthcare quality and a critical component of performance improvement and clinical effectiveness (Woodring et al, 2004; Kikwilu et al, 2009). Since the 1990s, measuring patient satisfaction has come to be regarded as the method of choice for obtaining patient views about care and has been widely adopted as an indicator of quality of care (Soufi et al, 2010; Hjortdahl et al, 1992). Measuring healthcare quality and improving patient satisfaction have become increasingly prevalent, especially among healthcare providers and purchasers of healthcare as consumers become more knowledgeable about healthcare (Howard et al, 2007). Indeed, patient satisfaction is widely considered an integral part of quality of care (Bernard et al, 2007; Benson et al, 1987; Ball, 1996; Tamaki et al, 2005). Pascoe has defined it as a recipient’s reaction to salient aspects of his experience of a service. In his formulation, satisfaction consists of a cognitive evaluation and an emotional reaction to the structure, process, and outcome of healthcare services (Pascoe, 1983). So patient satisfaction is a quality indicator that can potentially provide valuable information about the care delivered by providers. This indicator is considered an important marker of quality by paramedics (Greenberg et al, 1997; Holt, 2006; Institution of Medicine of National Academy, 2008). Patient satisfaction is important outcome of healthcare services and can affect compliance with medical advice, service utilization, and the clinician-patient relationship (Hjortdahl et al, 1992; Howard et al, 2007; Pascoe, 1983). Most researchers agree that patient satisfaction is a multidimensional concept, but, no consensus exists regarding which dimensions of care should be evaluated to measure patient satisfaction (Acorn, 1999; Schulmeister et al, 2005).

Several approaches have been used to identify the factors contributing to healthcare satisfaction. A distinction is made between those based on expectations, those focusing on health service attributes, those emanating from economic theory, and those that are holistic in nature (Soufi et al, 2010; Moore, 1999; Ware, 1981).

Approaches based on health service attributes attempt to clarify the concept of satisfaction. They also focus on consumers' evaluations of health service attributes. These methods use reviews of the available literature or primary research to produce lists of critical features that affect healthcare satisfaction. These features are often incorporated into factor or principal-components analysis to validate definable dimensions of the care process. The resulting classifications may subsequently form the basis of the development of instruments to measure satisfaction (Eriksen, 1995; Greeneich et al, 1992; Bennan, 1995). In line with previous studies and the literature, demographics, socioeconomics, and patient health characteristics were explored(Laschinger et al, 2005; Sitzia et al, 1997).

Also emergency departments (EDs) provide emergency healthcare to all those who present with acute emergencies (Salazar et al, 2006; Ochoa et al, 2000). EDs are overcrowded with patients who often seem dissatisfied with emergency health services(Sinclair, 2007; Coughlan et al, 2007; McCarthy et al,2008). Objective information about patient satisfaction to ensure the quality of care delivered by emergency medical service systems is in demand by governmental agencies, insurance companies, and customers (Bernard et al, 2007; Moore, 1999). But the nature of emergency medicine has changed significantly in recent years with the advent of new treatment options and the availability of more medical technology [30, 31]. So related factors in patient satisfaction have changed over the time and standard quality indicators such as response time and outcome data may not reflect everything that patients consider important (Bernard et al, 2007; Greenberg et al, 1997). In this study, patient satisfaction and its related factors were examined through large data sets that were collected from emergency departments of Iranian military hospitals.

2. Material and Methods

In this cross-sectional study, subjects were randomly chosen from patients referred to emergency military hospitals located in six cities: Shiraz, Isfahan, Mashhad, Kerman, Kermanshah, and Tabriz in July and August 2008 . In total, 360 patients (60 patients from each emergency center) participated in this study. Inclusion criteria were: minimum age of 15 and admission to the ED for more than five hours. Exit conditions from the study were inability to answer questions (such as patients with decreased level of consciousness or patients with severe psychiatric disorders). After the ED stay was complete, subjects were interviewed by our colleagues. The interviewers completed the checklists (without name) that included information such as age, gender, marital status, education level, insurance type, history of previous admissions, and name of the city. This checklist was completed for all patients by interviewers.

The questionnaire determined satisfaction levels of emergency services for all patients. It let patients express their level of satisfaction with emergency care services through a series of 12 questions covering topics such as giving information, the reception process, speed in calling doctors, continuous presence of doctors and nurses at the patient's bedside, quick action by caring medical staff, diversity of medical specialties, reception personnel's performance and behavior, laboratory personnel's performance and behavior, financial personnel's performance and behavior, compliance with ethical issues by clinical staff, facilities of emergency ward, and emergency cleanliness.

The five-part Likert scale (completely satisfied, satisfied, not satisfied and not dissatisfied, dissatisfied, and completely dissatisfied) was the response vehicle for each item. Scores from 20-100 were assigned to each response (from completely dissatisfied to completely satisfied), respectively. Statistical analysis using SPSS 13 software was performed. A description of the qualitative variables and quantitative variables has been done by frequency tables and calculating of average (standard deviation), respectively. The independent samples test related to the level of patient satisfaction and two-way variables such as sex, age, marital status, insurance coverage, and history of referring. The ANOVA test was used for more than two variables such as educational level and the city. The significant level (p-value) was considered less than 0.05.

3. Results

3.1. Basic information

Of all patients, 228 (63 percent) were male and 256 (71 percent) were married. Regarding patient age, 202 people (56 percent) 35 years old or younger and 158 people (44 percent) were more 35 years old. For education, 147 people (41 percent) held lower educational diplomas, 122 people (34 percent) helped diplomas, and 91 people (25 percent) had higher than diplomas. In this study, 232 people (65 percent) have a history of previously referring people to the facility

and 39 people (11 percent) didn't have any insurance coverage as they entered the emergency centers.

3.2. Satisfaction with emergency different services

Of the 4,220 total responses, 1,320 responses (30.6 percent) were completely satisfied, 2,239 responses (51.8 percent) were satisfied, 585 responses (13.5 percent) were not satisfied and not dissatisfied, 137 responses (3.2 percent) were dissatisfied, and 39 cases (0.9 percent) were completely dissatisfied. Thus, from total of 4,220 patient responses, 3,559 responses (82.4 percent) were satisfied or completely satisfied with emergency care services received.

Table 1: Results of patient satisfaction quality and quantity with services provided in emergency centers for each service

Items	**Average (Standard Deviation)**	**Satisfaction levels**				
		Completely Satisfied	Satisfied	Not Satisfied and Not Dissatisfied	Dissatisfied	Completely Dissatisfied
Giving information	85/8± 13/9	145(40%)	184(51%)	22(6%)	9(3%)	0
Patients' reception process	82± 7/14	93(26%)	228(63%)	24(7%)	11(3%)	4(1%)
Speed in calling doctor	79/6 ± 16/2	93(26%)	186(52%)	167(18%)	10(3%)	4(1%)
Continuous presence of doctors and nurses at patient's bedside	80/5 ± 16/4	103(29%)	183(51%)	57(16%)	15(4%)	2(1%)
Acting quickly and caring medical staff	79/8± 16/4	98(27%)	178(49%)	71(20%)	9(3%)	4(1%)
Diversity of medical specialties	78/7± 18/4	95(26%)	187(52%)	46(13%)	25(7%)	7(2%)
Reception personnel's performance and behavior	82/4± 14/9	110(31%)	193(54%)	48(13%)	7(2%)	2(1%)
Laboratory personnel's performance and behavior	79/8± 0/17	100(28%)	180(50%)	62(17%)	14(4%)	4(1%)
Finance personnel's performance and behavior	82/1± 15/2	108(30%)	188(52%)	47(13%)	13(4%)	4(1%)
Compliance with Ethical issues	87/3± 15/2	176(49%)	153(43%)	22(6%)	4(1%)	4(1%)
Emergency facilities	79/5±15/7	93(26%)	178(49%)	78(22%)	10(3%)	0
Cleanliness	82/1± 15/2	106(29%)	201(56%)	41(11%)	9(3%)	3(1%)
Total satisfaction	81/6± 10/6	1320(30/6%)	2239(51/8%)	585(13/5%)	137(3/2%)	39(0/9%)

Thus, from total of 4,220 patient responses, 3,559 responses (82.4 percent) were satisfied or completely satisfied with emergency care services received. This number increases to 95.9 percent when blank answers are included. Based on mean (standard deviation) of satisfaction scores related to various sectors of service, "Observation of ethical issues," "Style of giving information," and reception personnel's performance and behavior in emergency centers had the highest scores with 87.3±15.2, 85.8±13.9, and 82.4±14.9, respectively. "Enjoy the diversity of medical specialties," "Emergency facilities," and acting quickly to inform the physician scored the lowest scores with 78.7±18.4, 79.5±15.7, and 79.6±16.2 respectively. Frequency and average (standard deviation) of the total satisfaction and satisfaction to each of the separate parts of the emergency services is shown separately in Table 1.

3.3. Factors associated with overall satisfaction score of patients with emergency services

The total satisfaction score reported by patients older than 35 years was significantly higher than those 35 years or less (p=0.022). The scores

reported from patients without insurance coverage were significantly higher than those from patients covered by insurance (p=0/002). Also total satisfaction scores reported by patients with a history of previous referrals to the emergency centers under study was significantly higher than others (p=0.017). The total satisfaction reported by men vs. women and single people vs. married showed no significant difference. Likewise, the total satisfaction reported from patients with less, equal, and higher education wasn't statistically significant (p>0.05) (Table 2).

Table 2: Factors associated with patients' satisfaction scores

	Sig. level	Subgroups	Total satisfaction
gender	9590/*	Men	81/7 ± 10/9
		Women	81/8 ± 9/2
Age (year)	0220/*	≥35	80/6 ± 10/5
		<35	83/2 ± 10/4
Marital status	3120/*	Married	82 ± 10/5
		Single	80/7± 10/6
Education	6190/**	Less than diplomas	82/3 ± 10/2
		diplomas	81/2 ± 10/1
		Higher than diplomas	81/1± 11/3
Insurance coverage	0020*	Yes	82/2 ± 10/2
		No	76/5 ± 10/6
History of previous admission	017/*0	Yes	82/3 ± 10/4
		No	79/4 ± 10/9
Location	001/0< **	Isfahan	82/9 ± 13/2
		kerman	80/8 ± 4/3
		keremanshah	79/8 ± 13/9
		Mashhad	79/1 ± 7/5
		Shiraz	87 ± 8/6
		Tabriz	79/9 ± 0/12

3.4. Comparing patient satisfaction with emergency services in various cities

The total satisfaction score reported by patients in different cities varied by a statistically significant amount (p<0.001). Closer examination of the results through post hoc tests reveals that patient satisfaction scores in Shiraz City was significantly higher than cities of Kerman (p=0.016), Mashhad (p=0.001), Kermanshah (0.003), and Tabriz (0.003). The patient satisfaction scores with emergency services weren't statistically significant in other cities (p>0.05) (Table2).

4. Discussions

This study showed that patient satisfaction with emergency services in Iranian military hospitals was desirable in more than 82 percent of all instances. Results of previous studies of this topic were completely different. Satisfaction with emergency services in previous studies found extensive variability - from 44-98 percent. Patient satisfaction with emergency services in some training hospitals that depend on Tehran University of Medical Sciences (44 percent) (Omidvari et al, 2008)., Tehran Imam Khomeini Hospital (62 percent)(Jalili et al, 2007)., Lorestan (64 percent)(Rezaei et al, 2002)., Ardebil (78 percent)(Entezariasl et al, 2003)., and Army (81 percent)(Khoshjan et al, 2005). were, in some cases, far lower than what this study found. On the other hand, patient satisfaction with emergency services in Tabriz training hospitals (88 percent) (Behshid et al, 2005)., and Gazvin (98 percent) was better what the present study found (Sarchami, 2001). Nevertheless, the dramatic differences in the findings from other studies call for closer examination. It's clear that these differences may be related to the emergency services provided in each ED under study. We must also consider factors such as differences related to the studied population, the frequency of emergency centers under study, and most important of all, different ways of measuring satisfaction (Trout, 2000).

As stated before, one application of patient satisfaction is to enable health managers to identify strengths and weaknesses and improve service quality(Sun et al, 200). In this study, the lack of diversity of expertise, facilities, and delay in calling doctors into emergency centers led to the highest dissatisfaction levels among emergency different services. A review of findings from previous studies revealed that, despite a variety of assessments, in the internal studies similar to this study, the largest cause of dissatisfaction stemmed from a lack of emergency facilities [Omidvari et al, 2008; Jalili et al, 2007; Rezaei et al, 2002; Sarchami, 2001).Therefore, healthcare managers and executives should pay added attention to this issue to noticeably increase patient satisfaction and ultimately enhance the ED. Also, in line with these results, patient dissatisfaction with personnel Acting quickly and wait times to receive emergency services were another matter that in all studies were among the main reasons for dissatisfaction (Omidvari et al, 2008; Jalili et al, 2007; Sun et al , 2001,2002). Patient wait times may occur in different stages - such as triage, encounter with a doctor, Para clinical services, the interpretation of

results, and finally a medical consultation and admission/discharge (Booth et al 1992). This is an important matter because lengthy waiting times lead as many as 30-60 percent of patients to leave an emergency before a medical examination is completed. On the other hand, faster wait times were associated with greater satisfaction - up to 75 percent in other studies (Krishel, 1993). Nevertheless, some researchers believe that patients' perceptions of wait times play a critical role in patient satisfaction than just the waiting time itself. (Sun et al , 2001; Hall, 1996). For instance a lengthy wait time can be mitigated by appropriate personnel behavior, communication/explanation, and estimation of wait times for patients (Hall, 1996). Therefore, emergency care managers should devise and implement solutions to reduce patient dissatisfaction with wait times.

This study also examined patient background factors and found that there were no meaningful statistical differences between men and women, singles and married, and different levels of education. However, older patients expressed more satisfaction with care services. Also, patients covered by insurance or who have a history of previous visits were more satisfied with services compared to others. In several earlier studies, the impact of patient demographics on satisfaction levels has varied(Taylor et al, 2004).Some studies reported that gender [Omidvari et al, 2008; Quintana et al, 2006). age (Omidvari et al, 2008; Hargraves, 2001), and level of education (Hedges et al, 2002) impacted patient satisfaction. Other studies reported that these factors were irrelevant to satisfaction (Jalili et al, 2007; Quintana et al, 2006). On closer examination, findings of Omidvarim et al.(Omidvari et al, 2008;)., the study within our country found that that men and older patients with lower education levels have more satisfaction. Marital status was irrelevant to satisfaction. However, Sarchami and Sheikh's study (Trout et al,2000) ("Patients' satisfaction level with quality of emergency services in training hospitals which depended on Qazvin University of Medical Sciences") found that women, younger patients, patients with history of previous referrals, and patients lacking insurance expressed greater satisfaction than other groups. These results showed noticeable differences - even the impact of background factors on patient satisfaction level was contradictory.

As mentioned, several causes could explain the differences among the various studies. The first factor is the differences among populations under study. Generally, the entry and exit conditions from the study had noticeable differences and common selection and measurement criteria were not used (Quintana et al, 2006). There are different ways to assess satisfaction in different studies, which may have led to the different findings. For example, the study of qualitative and quantitative satisfaction can lead to different findings regarding the impact of background factors. Hence, different methodologies may be necessary when studying patient satisfaction with healthcare.

The use of six different locations for this study of patient satisfaction with emergency care was a strength of this study. However, the use of more vague questions and the lack of variables such as disease type, severity, and clinical outcome were deficiencies of this study. We suggest that a confirming study must seek this additional information from a more comprehensive questionnaire.

The findings of this study revealed that patients' satisfaction level with emergency care services in military hospitals is desirable. Nonetheless, it seems to require skilled manpower and facilities and also reformed processes for referring physicians to emergencies. Managers, policy makers, and planners should pay special attention to these aspects of emergency care services

Acknowledgements:

This work supported by the Trauma Research Center and Health Management Research Center. The authors would wish to thank the administrators of these centers for their support in preparation of this paper..

Corresponding Author:
Mohammadkarim Bahadori
PhD in Health Service Management
Health Management Research Centre, Baqyattallah University of Medical Sciences, Mollasadra Street, Tehran, Iran
Tell: +98.2182482416
Fax: +98.2188057022
E-mail: bahadorihealth@gmail.com

References

1. Ball R. Practical marketing for dentistry; relationship marketing and patient/consumer satisfaction. Br Dent J, 1996, 180(1);467-472.
2. Behshid M., Soami M.H., Akbarzadeh F. & Zamanzadeh V. Evaluation of patients' satisfaction with emergency services in hospitals of Tabriz University of Medical Sciences, 2000. http://dbase.irandoc.ac.ir/00689/00689512.htm
3. Bennan P. Patient satisfaction and normative decision theory. J Am Inf Assoc, 1995, 2:250-9.
4. Benson D.S., Gratner C., Anderson J. & Schweer H. The ambulatory care parameter: A structured approach to quality assurance in the ambulatory

care setting. QRB Qual Rrv Bull, 1987,13(2);51-55.
5. Bernard A., Lindsell Ch., Handel D. & Collett L. Postal survey methodology to assess patient satisfaction in a suburban emergency medical services system: An observational study. BMC Emergency Medicine, 2007, 7:5.
6. Booth A.J., Harrison C.J., Gardener G.J. & Gray A.J. Waiting times and patient satisfaction in the accident and emergency department. Arch Emerg Med, 1992, 9:162–8.
7. Coughlan M. & Corry M. The experiences of patients and relatives/significant others of crowding in accident and emergency in Ireland: A qualitative descriptive study. Accid Emerg Nurs., 2007, 15:201-9.
8. Entezariasl M. & Motamedi F. Degree of satisfaction among patients referring to the emergency rooms of hospital of Ardabil University of Medical Sciences, 2000. Journal Of Ardabil University Of Medical Sciences (JAUMS), 2003, 2(8):20-27.
9. Eriksen L. Patient satisfaction with nursing care: Concept clarification. J Nurs Meas, 1995, 3:59-76.
10. George G., Jell C. & Todd B.S. Effect of population aging on emergency department speed and efficiency: A historical perspective from a district general hospital in the UK. Emerg Med J., 2006, 23:379-83.
11. Greenberg M.D., Garrison H.G., Delbridge T.R., Miller W.R., Mosesso V.N., Roth R.N. Quality indicators for out-of-hospital emergency medical services: The paramedics' perspective. Prehosp Emerg Care, 1997, 1:23-27.
12. Greeneich D., Long C. & Miller B. Patient satisfaction update: Research applied to practice. Appl Nurs Res, 1992, 5:43-8.
13. Gany F., Leng J., Shapiro E. & Abramson D. Patient Satisfaction with Different Interpreting Methods: A Randomized Controlled Trial. Society of General Internal Medicine, 2007, 22(2):312–8.
14. Holt V.P. Patient satisfaction questionnaire: How to do them successfully. Dent Update, 2006:38(1); 338-346.
15. Acorn S. & Barnett J. Patient satisfaction: Issues in measurement. Can Nurse, 1999, 95:33-36.
16. Hall M.F. & Press, I. Keys to patient satisfaction in the emergency department: Results of a multiple facility study. Hosp Health Serv Adm, 1996, 41:515-32.
17. Hargraves J.L., Wilson I.B., Zaslavsky A. & James C. Adjusting for patient characteristics when analyzing report patients about hospital care. Medical Care, 2001, 39: 635-41.
18. Hedges J.R., Trout A. & Magnusson A.R. Satisfied Patients Exiting the Emergency Department (SPEED) Study. Acad Emerg Med, 2002, 9:15–21.
19. Hjortdahl P. & Laerum E. Continuity of care in general practice: Effect on patient satisfaction. BMJ, 1992, 304(6837):1287-1290.
20. Howard M., Goertzen J., Hutchison B. & Kaczorowski J. Patient Satisfaction With Care for Urgent Health Problems: A Survey of Family Practice Patients. Ann Fam Med, 2007;5:419-424.
21. Institution of Medicine of National Academy. Crossing the Quality Chasm: The IOM health care quality initiative. Available online 2008 at: www.iom.edu/CMS/8089.aspx>
22. Jalili M., Mostashar Nezami M. & Siahtir M. Evaluation of patient satisfaction with emergency medical services in Emam Khomeini Hospital. 2nd Annual Congress on Emergency Medicine. Ardabil University of Medical Sciences Faculty of Medicine. 2007.
23. Jayaprakash N., Sullivan R., Bey T. & Suleman S. Crowding and Delivery of Healthcare in Emergency Departments: The European Perspective. Western Journal of Emergency Medicine, 2009:40(3);233-239.
24. Khoshjan A., Mohammadi R., Tamaddonfar M. & Hosseini F. Emergency wards and satisfaction level of their patients and families. Iran Journal of Nursing, 2005, 18(41-42):49-60.
25. Kikwilu E., Kahabuka F., Masalu J. & Senkoro A. Satisfaction with urgent oral care among adult Tanzanians. Journal of Oral Science, 2009, 51(1):47-54.
26. Krishel S. & Baraff L.J. Effect of emergency department information on patient satisfaction. Ann Emerg Med, 1993, 22:568–72.
27. Laschinger H.S., Hall L.M., Pedersen C. & Almost J. A psychometric analysis of the patient satisfaction with nursing care quality questionnaire. J Nurs Care Qual, 2005, 20:220-30.
28. McCarthy M.L., Aronsky D., Jones I., et al. The emergency department occupancy rate: A simple measure of emergency department crowding. Ann Emerg Med., 2008, 51:15-24.
29. Moore L. Measuring quality and effectiveness of prehospital EMS. Prehosp Emerg Care, 1999, 3:325-331.
30. Nguyen Thi PL., Briançon S., Empereur F. & Guillemin F. Factors determining inpatient satisfaction with care. Soc Sci Med, 2002, 54:493-504.
31. Ochoa J., Ramalle-Gonar E., Villar A., et al. Visitas inapropiadas al Servicio de Urgencias de

un hospital general. Med Clin (Barc), 2000;10:377–8.
32. Omidvari S., Shahidzadeh Mahani A., Montazeri A., Azin S.A., Harirchi A.M., Souri H., Jafari H. & Goudarzi F. Patient satisfaction with emergency departments. Payesh J, 2008, 7(2):141-152.
33. Pascoe G. C. Patient satisfaction in primary health care: A literature review analysis. Eval Program Plann, 1983, 6:185–210.
34. Quintana J.M., Gonzalez N., Bilbao A. & Aizpuru F. Predictors of patient satisfaction with hospital health care. BMC Health Services Research, 2006, 6: 102.
35. Rezaei K., Asad Beygi M., Toulabi T. & Tarahi M.J. Patients' satisfaction of receiving services in the emergency wards. Yaft-E, 2002, 4(3 (14)):33-38.
36. Salazar A., Juan A., Ballbe R., et al. ED short-stay unit: An effective alternative to in-hospital admission for acute COPD exacerbation. Am J Emerg Med, 2006 in press.
37. Sarchami R. & Sheikhi M.R. Patients' satisfaction of the quality services in emergency departments. The Journal Of Qazvin University Of Medical Sciences, 2001, 5(18):64-68.
38. Schulmeister L., Quiett K. & Mayer K. Quality of life, quality of care, and patient satisfaction: Perceptions of patients undergoing outpatient autologous stem cell transplantation. Oncol Nurs Forum, 2005, 32:57-67.
39. Sinclair D. Emergency department crowding – Implications for pediatric emergency medicine. Paediatr Child Health, 2007, 12:491-4.
40. Sitzia J. & Wood N. Patient satisfaction: A review of issues and concepts. Soc Sci Med, 1997, 45:1829-43.
41. Soufi Gh., Belayachi J., Himmich S. & Ahid S. Patient satisfaction in an acute medicine department in Morocco. BMC Health Services Research, 2010, 10:149.
42. Sun B.C., Adams J., Orav E.J., Rucker D.W., Brennan T.A. & Burstin H.R. Determinants of patient satisfaction and willingness to return with emergency care. Ann Emerg Med, 2000, 35:426-34.
43. Sun B.C., Adams J.G. & Burstin H.R. Validating a model of patient satisfaction with emergency care. Ann Emerg Med., 2001, 38:527–32.
44. Tamaki Y., Nomura Y,. Nishikawara F.& Motegi M. Correlation between patient satisfaction and dental clinic credibility in regular dental check-ups in Japan. J Oral Sci, 2005:47(1);97-101.
45. Taylor C. & Benger J. Patient satisfaction in emergency medicine. Emerg Med J., September 2004, 21(5): 528–532.
46. Trout A., Magnusson A.R. & Hedges J.R. Patient satisfaction investigations and the emergency department: What does the literature say? Acad Emerg Med, 2000, 7:695–709.
47. Ware J.J. How to survey patient satisfaction. Drug Intell Clin Pharm, 1981, 15:892-9.
48. Woodring S., Polomano R.C., Haack M.M. & Nunn R.R. Development and testing patient satisfaction measure for inpatient psychiatry care. J Nurs Care Qual, 2004, 19:137-47.

26/11/2010

Growth Pattern in Anemic Children and Adolescents, aged 12-14 years

Sanaa Kamal *; Moushira Erfan *; Shams Mohamad Kholoussi; and karima Abd Elfattah Bahgat*****

*Biological anthropology Department, Medical Research Division, National Research Centre (NRC) **Immuno-genetics Department, Human Genetics and Genome Research Division, NRC
***Pediatric Department faculty of Medicine (Girls), Al-Azhar University.
moushiraz@yahoo.com

Abstract: Iron deficiency anemia among children and adolescents is a large health problem worldwide. Adolescence is characterized by a large growth spurt and the acquisition of adult phenotypes and biologic rhythms. During this period, iron requirements increase dramatically in both boys and girls. Anemia due to iron deficiency often coexists with zinc deficiency. Deficits in macronutrients or micronutrients can impair growth. Menstruation increases the risk for iron deficiency anemia among girls throughout their adolescence. The aim of the present work is to assess growth pattern in anemic boys and girls and to study relations between anthropometric parameters and hemoglobin, iron and zinc levels. The sample consisted of 60 anemic children and adolescents aged from 12- 14 years (30 boys and 30 girls) and 30 normal healthy children (15 boys and 15girls). Weight, height, mid upper arm circumference (MUAC), waist and hip circumferences were measured and body mass index (BMI) was calculated. Sex- and age-independent SD scores (SDS) were calculated for all anthropometric measurements with the use of the Egyptian reference data. Hemoglobin concentration, serum ferritin, iron and zinc were measured for patients and control. Anemic girls showed significant association between height SDS, weight SDS, BMI SDS and hemoglobin concentration level and also between MUAC SDS and zinc level. Anemic boys showed less marked growth delay. The study showed that growth delay was pronounced among anemic girls during adolescent growth spurt. Thus, age and sex are the factors most predicative of growth delay among Egyptian adolescents. The study emphasized that iron and zinc are essential micronutrients for normal growth and anemia has a negative impact on growth. The study suggests regular nutrition assessment of adolescents and recommends behavior modification to get dietary change among adolescents. The inhibited growth rate, induced by the iron-deficient diet could be reversed by giving a diet supplemented with iron.
[Sanaa Kamal; Moushira Erfan; Shams Mohamad Kholoussi; and karima Abd Elfatta Bahgat. **Growth Pattern in Anemic Children and Adolescents, aged 12-14 years**. Journal of American Science 2010;6(12):1636-1646]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: Iron deficiency, phenotypes, Growth Pattern.

1. Introduction:

Adolescence is a time of intense physical, psychosocial, and cognitive development. During this period they gain up to 50% of their adult weight, more than 20% of their adult height, and 50% of their adult skeletal mass. Thus the nutritional needs for energy, protein, and many vitamins and minerals are increased. Inadequate stores or intake of nutrients during this period can have adverse effects on the physical growth and cognitive development. So adolescence is a vulnerable period for the development of nutritional anemia (DiMeglio, 2000; Chaudhary and Dhage, 2008).

Deficits in macronutrients or micronutrients can impair growth (Sen and Kanani, 2006). Iron and zinc deficiencies are the main micronutrient deficiencies of public health significance. The deficiency of these nutrients arise from inadequate intakes, impaired absorption and or utilization, excessive losses, or a combination of these factors and are exacerbated during times of greater physiological need such as adolescent (Ijarotimi,2004).

Anemia is a major public health problem at all ages worldwide (Maninder and Kochar, 2010). It may be due to iron deficiency; insufficient hematopoiesis due to deficiencies of folic acid or vitamin B 12; hemorrhagic anemia secondary to blood loss; hemolytic anemia with premature red blood cell plasma membrane rupture; hemoglobinopathies such as sickle cell anemia and thalassemia; or aplastic anemia with destruction of bone marrow. Iron-deficiency anemia is the most common type of several causes of anemia. Deficiencies of folic acid or vitamin B 12 can also cause anemia but are rare, and usually occur because of impaired vitamin absorption. Diseases of the bone marrow will also cause anemia. Fortunately, these conditions are rarer (Hoffman et al., 2008).

Iron deficiency anemia (IDA) is the main micronutrient deficiency that may affect adolescent. It is one of the most universally prevalent diseases in the world today particularly developing countries and

it is a major public health .Iron deficiency has been considered an important risk factor for ill health and is estimated to affect 2 billion people worldwide (WHO, 2002). Iron is essential for all tissues in a young child's developing body. It is present in the brain from very early in life, when it participates in the neural myelination processes. Beard (2001) and other authors (Haas and Brownlie, 2001) have been postulated other roles of iron that would affect growth and immune function. It is essential for the expansion of blood volume and muscle mass. Functions of iron include involvement in energy metabolism, gene regulation, cell growth and differentiation, oxygen binding and transport, muscle oxygen use and storage, enzyme reactions, neurotransmitter synthesis, and protein synthesis. Attention deficits, poor performance in intelligence tests, behavioral and mood changes, tiredness, and below-average school performance are associated with iron deficiency in adolescents (Amy et al., 2007).

Iron needs are highest in males during peak pubertal development because of a greater increase in blood volume, muscle mass and myoglobin (Hyder et al., 2007). However, after menarche, iron needs continue to remain high in females to replace menstrual blood loss (Paknahad et al; 2003). So an adolescent girl is 10 times more likely to develop anemia than a boy because of their irregular eating habits (caused by concerns about body image) compounded by normal menstrual blood loss. James et al., (2006) reported that Iron deficiency is the most prevalent micronutrient deficiency disease in the world and occurs in young women in the United States.

The detrimental effects of IDA on physical growth have been attributed to poor appetite, altered endocrinological profile and neurotransmitter metabolism consequent to iron deficiency. The appetite is seen to decrease in IDA independently of plasma leptin levels (Topaloglu et al., 2001). In IDA plasma norepinephrine as well as urinary excretion of epinephrine and norepinephrine is increased .An elevated cortisol and parathormone level along with altered metabolism of calcium, phosphorus and magnesium has also been observed (Campos et al., 1998). The effects of IDA on physical growth have been shown to be resistant even to the administration of growth hormone (Ceppi and Blum, 1994). The thyroid gland metabolism is also affected (Zimmermann et al., 2000) with impaired thermoregulation and a hyperadrenergic state is seen in hypothyroid individuals suffering from iron deficiency (Shakir et al., 2000).

Zinc, which is an essential cofactor for nearly 200 enzymes, participates in cellular growth as a cofactor for enzymes necessary for the synthesis of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA), and controls, growth and development. Adolescents need more zinc/kg than adults, due to the role of this metal in growth and maturation. It is important to assess the levels of iron, and zinc in adolescents during the pubertal spurt (Urbano et al., 2002). Zinc deficiency has profound and far-reaching effect on health and well being of humans. There is convincing evidence linking zinc deficiency to childhood growth stunting (villalpando et al., 2003). Growth retardation has been associated with anemia and zinc deficiency in adolescent populations (Wajeunnesa et al., 2009).

Iron and zinc are essential micronutrients for human health. Deficiencies in these 2 nutrients remain a global problem, especially in developing countries. Anemia due to iron deficiency often coexists with zinc deficiency. It was apparently first recorded by Prasad et al., in that year (Nishiyama et al., 1998). Clinical findings included growth stunting, anemia, low serum iron and plasma zinc levels, low urine, sweat and hair zinc levels. Iron and zinc nutrition are often associated. Red meat is the most important common dietary source of bioavailable iron and zinc. Phytate inhibits iron and zinc retention. Consequences of these associations were first described by Prasad, Halsted and their associates in Egyptian and Iranian adolescents whose diets were nearly devoid of meat and were based on bread prepared from whole grain wheat flour rich in phytate (Paknahad et al., 2007). Instead, vegetables, whole grain cereals, legumes and nuts are their major food sources of these trace elements. However, these same foods also contain high levels of phytic acid and dietary fiber; components that may interfere with the absorption of iron and zinc, thus leading to decreased bioavailability The aim of the present study is to assess growth pattern in anemic boys and girls and to study correlations between anthropometric parameters and hemoglobin, iron and zinc levels.

2. Patients and methods:

The subjects of this study comprised a total number of 90 children and adolescents: 60 were anemic (30 boys and 30 girls), and 30 were normal healthy one and randomly chosen of both sexes (15 boys and 15girls). Their age ranged from 12- 14 years. A formal consent letter from the parents of each child included in the study was obtained after explaining to them the whole procedure. This study was approved by the Ethics Committee of the Hospital. They were attending the Pediatric Clinics of Al-Zahraa Hospital, Al-Azhar University complaining of easy fatigability, pale colour and decreased school performance. According to WHO criteria for diagnosis of anemia (Beard and Stoltzfus,

2001) the children with hemoglobin concentration< 12 g/dL were assigned to the anemic group and those with hemoglobin concentration ≥ 12 g/dL were assigned to the control group.

Children suffering from any chronic illness e.g. asthma, rheumatic heart disease etc, or receiving any long term drug treatment were excluded from the study. All Cases were subjected to: appropriate and thorough medical history, physical examination, routine laboratory investigations were also done including: Complete blood picture, erythrocyte sedimentation rate, and C-reactive protein. Hemoglobin concentration values and RBC indices were measured using automated hematology cell counter (Coulter T 890), serum iron values along with peripheral blood smear to study RBC morphology were analyzed. Serum iron was measured by method described by International Committee for standardization in Haematology and TIBC was measured by Ressler and Zak (1958) method. Transferrin saturation was determined by dividing serum iron by total iron binding capacity (looker et al .1995). According to the cut-offs values used by WHO the three indicators of abnormal iron status were applied: serum ferritin < 12 ng/mL, transferrin saturation < 16%, and red blood cell distribution width (RDW) >15%. Calorimetric test for the determination of Zinc in sera of all groups was performed by atomic absorption spectro-photometer (Gupta et al., 1992) using Zinc fluid Monoreagent (Centronic Gmbh-Germany).

The anthropometric measurements and instruments used followed the International Biological Programme (IBP) (Tanner et al., 1969). Measurements were taken on the left side of the body and included: weight, height, mid upper arm circumference (MUAC), waist and hip circumference. Body mass index (BMI; in kg/m2); and waist to hip ratio (WHR) were calculated.

Physical growth was assessed for each child by determining the standard deviation scores of weight, height, BMI and mid-upper arm circumference, using the Egyptian growth reference data (Ghalli et al., 2002), we calculated standard deviation score (SDS) independent of sex and age that is, child measurement minus population mean/population SD.

Waist to hip ratio was also calculated and compared to the control group in the present study. Statistical presentation and analysis of the results were carried out using SPSS software version 11. Statistical tests used included chi-square test, student's't' test , analysis of variance, and tukey tests. Correlations were tested between growth parameters and hemoglobin and serum zinc by linear regression analysis.

3. Results

Table 1 shows means of growth parameters SDS in anemic boys and girls. The values of SDS for the weight, height , BMI, and MUAC lied at the lower limits of reference Egyptian growth data with no statistical significant difference between boys and girls (P<0.188). However, girls had more delyed growth parameters than boys. Also, the waist to hip ratio had lower values, with no statistical significant difference between both sexes as well as when compared to the non anemic control group.

Table 2 shows means of growth parameters SDS in anemic girls. As the age of the girls increase the growth delay is more pronounced in all anthropometric parameters with no statistical significant differences between the age groups.

Table 3 shows means of growth parameters SDS in anemic boys. The values of SDS for height among the studied adolescent boys lied at the lower limits of reference Egyptian growth data nearly with the same lower value in all age groups, with no statistical significant difference between the age groups. However the SDS for weight, BMI, and MUAC showed lower values than Egyptian reference growth data, but at age 14 years the growth delay is more pronounced than that at age 12 years, with no statistical significant difference.

Fig. 1 shows the regression analysis between Hb (gm/dl) and serum zinc levels (µg/dl) among the studied anemic adolescents. The Hb values were negatively correlated with serum zinc in the studied anemic adolescents with significance (P<0.05).

Fig. 2 shows the regression analysis between Hb (gm/dl) and SDS for the weight in anemic girls. It shows significant correlation between Hb (gm/dl) and SDS for the weight in anemic females (P<0.05).

Fig .3 represents the regression analysis between Hb (gm/dl) and SDS for the height in anemic females. The Hb values were negatively correlated with SDS for the height in the studied anemic girls with significance.

Fig. 4 shows the regression analysis between Zinc and MUAC SDS among the anemic girls. Regression analysis shows significant correlation between Zinc and Z MUAC in anemic females (P<0.05).

The linear regression analysis in boys shows no significant correlations between the values of SDS for the weight, height, BMI, and MUAC, hemoglobin and serum zinc (P>0.05).

Table 1: The mean SDS of growth parameters in anemic boys and girls

Gender	Growth Parameters				
	Weight SDS **Mean** ± SD	**Height SDS** **Mean** ± SD	**BMI SDS** **Mean** ± SD	**MUAC SDS** **Mean** ± SD	**WHR** **Mean** ± SD
Boys	-1.5 ± 1.45	-1.8 ± 0.98	-1.76 ± 0.89	-1.23 ± 1.89	. 72 ± .99
Girls	-1.9 ± 1. 38	- 2.6 ± 1.57	-1.68 ± 1.370	-1. 98 ± 0.87	. 69 ± .87

Table 2: The mean SDS of growth parameters in anemic girls by age

Age	**Weight SDS** **Mean** ± SD	**Height SDS** **Mean** ± SD	**BMI SDS** **Mean** ± SD	**MUAC SDS** **Mean** ± SD	**WHR** **Mean** ± SD
12years	-0.15 ± 0.556	0.40 ± 0.67	-0.16 ± 0.74	-0.78 ± 0.49	.63 ± .77
13 years	-0.94 ± 1.07	-0.56 ± 0.267	-0.86 ± 1.49	-1.57± 0.763	.65± .99
14 years	-1.07±1.03	-0.72 ± 1.13	-1.02 ±1.042	-0.55 ± 0.702	.62± .98

Table 3: The mean SDS of growth parameters in anemic boys by age

Age	**WTSDS** **Mean**±SD	**HTSDS** **Mean**±SD	**BMI SDS** **Mean**±SD	**MUAC SDS** **Mean**±SD	**WHR** **Mean** ± SD
12years	-0.07 ± 1.02	-1.14 ± 1.09	-0.37 ± 0.66	-0.17 ±1.57	**.**67 ± .67
13 years	-0.92 ± 0.65	-1.32 ± 0.45	-0.43±0.85	-1.44 ± 0.88	.68 ± .85
14 years	-1.51 ± 0.66	-1.09 ±1.60	-1.19± 0.64	-1.81 ± 0.41	.77 ± .55

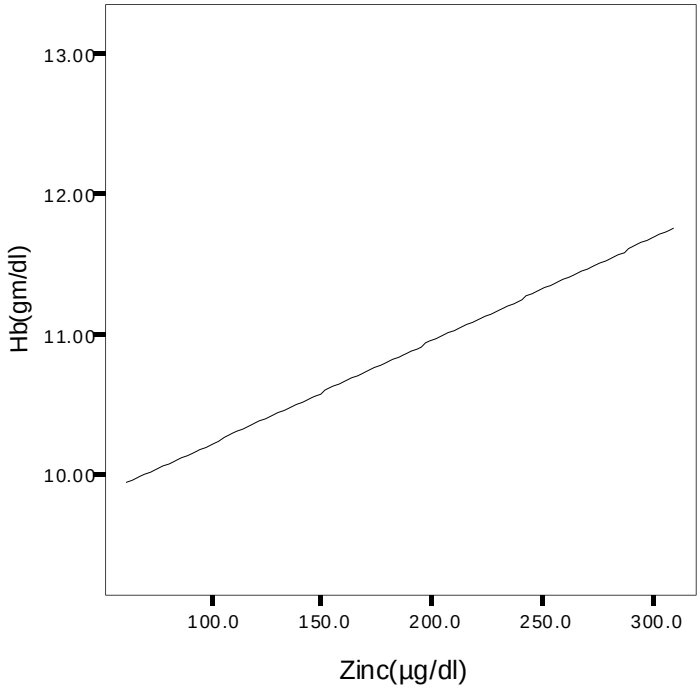


Figure 1 Regression analysis between Hb (g/dl) and serum Zinc levels (µg/dl) among the studied anemic adolescents.

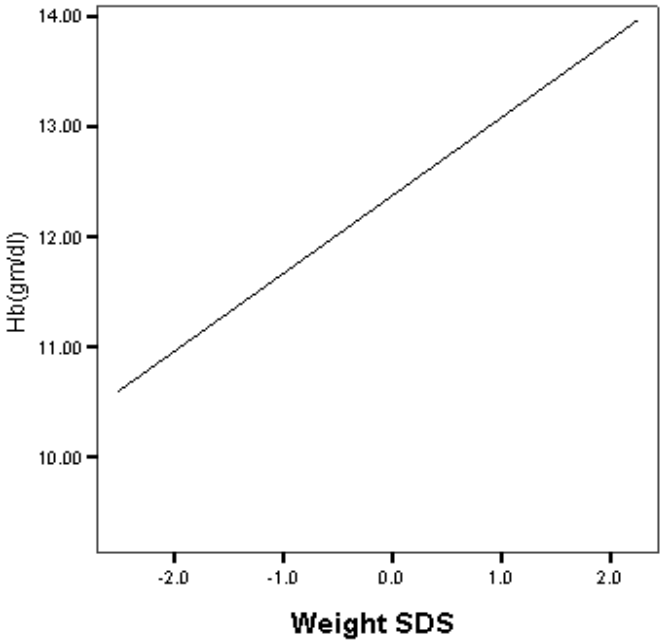


Figure 2 Regression analysis between Hb (gm/dl) and weight SDS in anemic girls

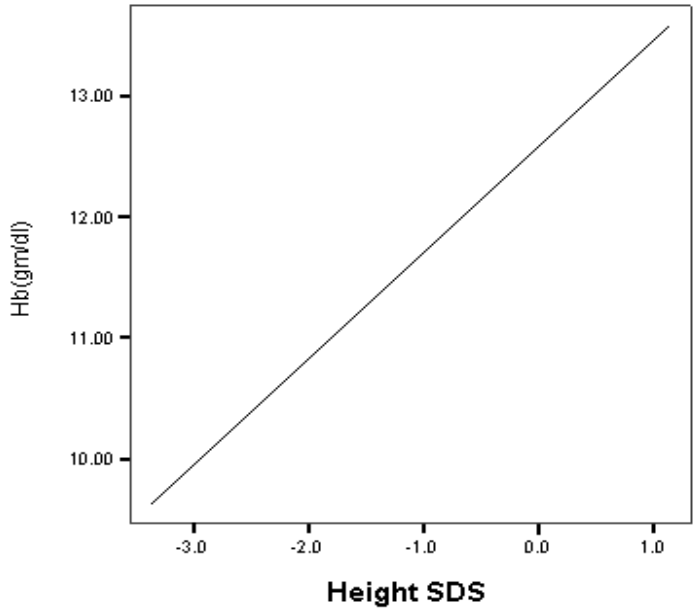


Figure 3 Regression analysis between Hb (gm/dl) and Height SDS in anemic girls

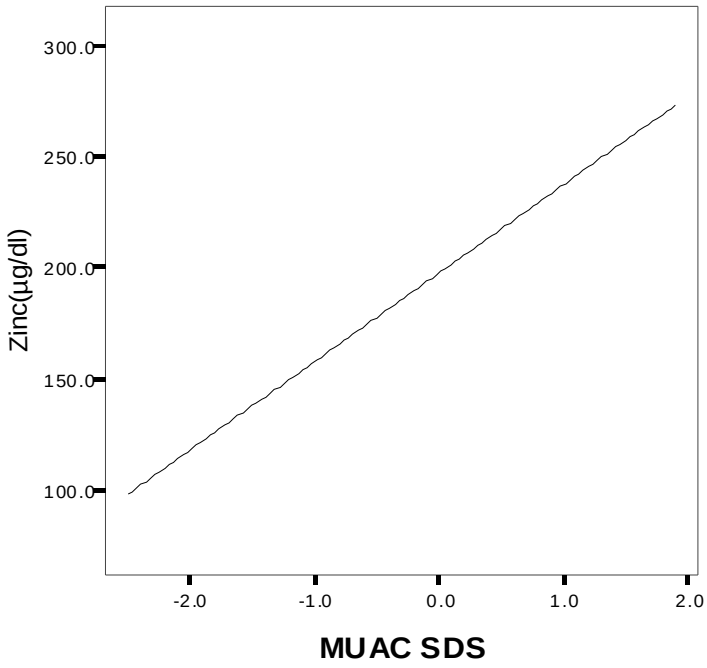


Figure 4 Regression analysis between Zinc and MUAC SDS in anemic girls

4. Discussion:

There exist general feelings in the society that adolescent years are normally free from major health problems. On the contrary it is a crucial period, because adolescent is still a developing child. Significant proportion of young people in developing countries suffers from nutritional anemia, in spite of impressive gains in the field of health and nutrition. Iron deficiency is the most prevalent micronutrient deficiency disease in the world (Stoltzfus, 2001). It affects more than 3.5 billion people in the developing countries (WHO, 2001). Twenty two percent of the Egyptian population is currently adolescents. A survey conducted in 1997 Ibrahim et al., (1999) found that 47% of adolescent girls and boys are anemic. El-Sahn et al., (2000) reported that the prevalence of anemia among adolescents in Egypt was 46.6 %. To address the high anemia rates, the Egyptian government and the Student Health Insurance Program (SHIP) began a targeted program to lower those rates through a dynamic school-based program. The task of improving the health and nutrition of adolescents by strengthening the preventive health program of SHIP was part of this work and was called the Adolescent Anemia Prevention Program. The program is implemented in all governorates all over Egypt.

High rates of anemia have been found in other developing countries, such as India (55%), Nepal (42%) and Cameroon (32%) (Kurz and Johnson-Welch, 1994). Among school children, the prevalence of anemia ranged from 32% in Bahrain to 78% in Oman (Verster and van der Pols; 1995). Irrespective of the severity, the prevalence of anemia ranges between 12-100% in the Region South-East Asian Countries (WHO, 2006). The prevalence of iron deficiency anemia was 6.2 ± 0.8% in Mexican American females and 2.3 ± 0.4% in non-Hispanic white females (Amy et al ,2000).

In the present study, the values of SDS for the weight, height, BMI, and MUAC among the studied anemic adolescents, showed values that represented the extreme lower end of the distribution of reference growth data of the normal age-matched Egyptian reference growth data (Ghalli et al., 2002). This is in agreement with the study of Kanani and Poojara (2000) on Indian adolescents, they recorded that anemia has a negative impact on growth and it may compromise pubertal growth spurt. Beard (2000) has been reported also, that iron deficiency anemia causes poor growth in humans especially among adolescents due to the increased iron requirements and growth spurt. Growth retardation has been associated with anemia in adolescent populations (Alton, 2005). The iron needs are high in adolescents because of the increased requirements for expansion of blood volume associated with the adolescent growth spurt. The low iron status among adolescents may limit their growth spurt. Poor eating habits are the main reason for the high rates of anemia among adolescents in Egypt. The typical Egyptian diet has few iron-rich foods, or foods that enhance iron absorption, and often considerable amounts of foods that inhibit iron absorption such as tea and whole wheat bread. WHO (2006) reported that anemia has a serious negative impact on growth and development. Studies conducted in different countries, reveal that nutritional deprivation affects almost all growth parameters and final adult body size resulting in thinness and stunting (Kanani and Poojara, 2000; Beard 2000; Alton, 2005; Hyder et al., 2007). However, the effect of malnutrition is more pronounced at the time of peak growth.

In the present study anemic adolescents displayed less development of circumferential measurements (waist and hip circumference) as compared to the non anemic control. Likewise, Colin-Ramirez et al., (2003) also observed that anemic subjects had lower waist and hip circumference than the non anemic subjects. Similar finding were noticed by Maninder and Kochar (2010) in anemic rural Haryanvi Jat women in India. It was observed that decrease in hemoglobin concentration reduces the availability of oxygen to the tissues, which in turn affects development of circumferential measurements among anemic subjects.

Muñoz et al., (2009) reported that adolescent iron requirements are higher in developing countries because of infectious diseases and parasitic infestations that cause iron loss, and because of low bioavailability of iron from diets limited in hem iron. Increased iron requirements, limited external supply, and increased blood loss may lead to iron deficiency anemia. Our findings suggest that physical growth spurt may be playing a larger role in causation of growth delay in anemic adolescents.

In our study the growth delay was evident within each gender across age groups in both boys and girls. However, regression analysis shows significant correlation between Hb (gm/dl) and SDS for the weight and height in anemic girls and no significant correlation in boys. This denotes that adverse impact of anemia on growth is more evident in adolescent girls than boys. This is in agreement with the study of Sen and Kanani (2006) on Indian adolescent girls and demonstrated adverse impact of anemia on growth. Bergstrom et al., (1995) evaluated iron status in healthy Swedish adolescents and found that low heme iron intakes increased the risk of low iron stores in girls but not in boys. They suggested that the differences in iron status between boys and girls in adolescence results primarily from biological

differences other than menstrual bleeding or insufficient iron intake. Moreover, adolescent girls have high nutrient needs and are susceptible to micronutrient deficiencies (Hyder et al., 2007). So boys are less at risk of growth delay secondary to anemia than girls who lose blood and as a result, iron through menstruation. The World Health Organization (2001) estimated that about 30-55% of adolescent girls suffer from anemia. Egypt Demographic and Health Survey conducted in 2000-2005 recorded the prevalence of anemia in adolescent girls and boys are 35.6% and 26% respectively (El Ashry, 2009). After menarche, iron needs continue to remain high in females to replace menstrual blood loss (Meier et al.، 2003). An adolescent girl is 10 times more likely to develop anemia than a boy because their irregular eating habits, caused by concerns about body image compounded by normal menstrual blood loss. The girl begins her adolescent growth spurt at an average of about 10 years and grows at peak velocity at about 12 years. The boy starts his adolescent growth spurt around 12 years of age and in a year or two overtakes the girl (WHO, 2006).This can explain another superimposed factor for growth delay in adolescent girls in the present study.

Iron and zinc are essential micronutrients for human health and normal growth. Their deficiencies usually coexist in the same individual and make anemia with worst effect on growth. By regression analysis, our study revealed significant positive correlation between Hb values and serum zinc levels in anemic adolescents, which was consistent with the observation of Paknahad et al., (2007) in Iran. However Kogirima et al., (2007) found a positive correlation between zinc intake and serum zinc levels. Serum zinc was significantly correlated with hematocrit and hemoglobin (P was 0.027 and 0.02 respectively). This is in agreement with the study of Cole et al., (2010) who reported that anemic African American children had an increased risk of zinc deficiency, and serum zinc is correlated with hemoglobin ($r = 0.24$, $P < 0.001$). Several studies have highlighted that supplementation of zinc along with iron improves hemoglobin level and is beneficial in iron deficiency anemia (Kolesteren et al., 1999). At the other hand zinc is clearly involved in several aspects of normal hematopoiesis by virtue of its role in many enzyme systems involved with DNA synthesis including thymidine kinase and DNA polymerase (Nishiyama et al., 1998). Zn deficiency should be considered as one of etiologic factors in some children with unexplained short stature. Oral Zn supplementation may be considered as the growth-promoting therapy for children with short stature once marginal zinc deficiency is established. Zinc is well known to be essential for somatic growth of children. Zinc has a close relationship with the endocrine system; it sustains normal growth (Kaji and Nishi, 2006). However, the interrelationships among Zn, growth, and GH-IGF-I axis appear to be complex and deserve further investigation.

In our study we didn't find any relationship between serum zinc and age. Villalpando et al., (2003) findings showed results similar to our study. Regression analysis showed significant correlation of zinc and MUAC SDS in anemic girls only. Consequently, zinc intake influence anthropometric measurements and has effects on the mid-upper arm circumference. Keith et al., (2005) findings showed results similar to our study. MUAC is a useful as an indirect measure of muscle size to determine whether a person has depleted lean body mass and is an excellent indicator of the severity of malnutrition (Rodriguez et al, 2000). An MUAC measurement was easier to perform on severely malnourished adults than BMI assessment. During famine; MUAC may be better suited to screening admissions to adult feeding centers than BMI. In rural Kenya Berkleyet al., (2005) found that MUAC is a practical screening tool that performs at least as well as WHZ in predicting subsequent inpatient mortality among severely malnourished children. Dasgupta et al., (2010) stated that the mid arm circumference measurement is a reliable and a feasible method of assessment of nutritional status of adolescents. The evidence indicates that adolescence boys have greater MUAC than girls in our study and anemic girls showed more growth delay during the adolescent growth spurt.

In conclusion, the anthropometric parameters are low in the anemic children as compared to Egyptian national reference data. Differences were evident within each gender across age groups. Several studies have suggested that lower growth rates and impaired physical performance are the adverse effects of iron deficiency anemia in children (Bandhu et al., 2003; Kanani and Poojara, 2000; Beard, 2000). Age and sex were the factors most predicative of anemia and growth delay among Egyptian adolescents. Boys had higher values of both hemoglobin and zinc concentrations than girls which may be due to the biological difference. Anemic girls were especially at risk of growth delay. A significant correlation between growth parameters and anemia in children, particularly girls during adolescent growth spurt was observed. Iron and zinc are playing a larger role in causation of growth delay in anemic adolescents. The inhibited growth rate, induced by the iron-deficient diet could be reversed by giving a diet supplemented with iron. Aggressive interventions are imperative to correct iron and zinc

deficiency and by so doing avoid their deleterious effect on growth. The main strategies suggested for improving adolescent nutrition can be food-based strategies like food fortification, for ensuring adequate nutrition at household level; addressing behavior modification to bring about dietary change in adolescents. This may be achieved through school-based nutrition interventions; regular nutrition assessment and counseling of adolescents; intersectional linkages at community level and building linkages with adolescent friendly health services.

Corresponding author
Prof. Dr. Moushira Erfan
Biological anthropology department
E-mail:moushiraz@yahoo.com
Tel.: 0102331348

References:

1. Alton I. Iron Deficiency Anemia. In: Stang M, Story M, eds. Guidelines for Adolescent Nutrition Services. Minneapolis, MN: Center for Leadership, Education and Training in Maternal and Child Nutrition, Division of Epidemiology and Community Health, School of Public Health, University of Minnesota; 2005;101-08.
2. Frith-Terhune AL, Cogswell ME, Khan LK, Will JC, and Ramakrishnan U.. Iron deficiency anemia: higher prevalence in Mexican American than in non-Hispanic white females in the third National Health and Nutrition Examination Survey, 1988-1994. Am J Clin Nutr 2007; 72(4):963-968.
3. Bandhu R, Shankar N. and Tandon OP. .Effect of Iron on Growth in Iron Deficient Anemic School Going Children. Indian J Physiol Pharmacol 2003; Volume 47 - No.1
4. Beard JL. Iron requirements in adolescent females. J Nutr 2000; 130: 440S-442S.
5. Beard JL. Iron biology in immune function, muscle metabolism and neuronal functioning. J Nutr 2001;131:568S–80S.
6. Beard J, Stoltzfus R, eds. Iron-deficiency anaemia: reexamining the nature and magnitude of the public health problem. Proceedings of the WHO-INACG Conference, Belmont, MD, USA, 2000. Journal of Nutrition, 2001; Supplement 131.
7. Bergström E, Hernell O, Lönnerdal B, Persson LA. Sex differences in iron stores of adolescents: what is normal? J Pediatr Gastroenterol Nut.1995; Feb; 20(2):215-24.
8. Berkley J, Mwangi I, Paeds M, Griffiths K, Ahmed I, Mithwani S, Newton C, Maitland K. , Assessment of Severe Malnutrition Among Hospitalized Children in Rural Kenya Comparison of Weight for Height and Mid Upper Arm Circumference *JAMA.* 2005; No. 5294:591-597.
9. Campos MS, Barrionuevo M, Alferez MJ, Gomez-Ayala AE, Rodriguez-Matas MC, Lopez Aliaga I, Lisbona F. Interactions among iron, calcium, phosphorus and magnesium in the nutritionally iron deficient rat. Exp Physiol 1998; 83(6): 771-781.
10. Ceppi A, Blum JW. Effect of growth hormone on growth performance, hematology, metabolites and hormones in iron deficient veal calves. Zentralbl Veterinarmed A 1994; 41(6): 443-458.
11. Chaudhary SM, Dhage VR. A study of anemia among adolescent females in the urban area of Nagpur. Indian J Community Med 2008; 33:243-5
Cole CR, Grant FK, Swab-Ellis ED, Smith JL, Jacques A, Northrop-Clewes CA, Caldwell KL, Pfeiffer CM, Ziegler TR. Zinc and iron deficiency and their interrelations in low-income African American and Hispanic children in Atlanta. Am J Clin Nutr 2010; 91: 1027-1034.
12. Colin-Ramirez E, Castillo-Martinez L, Orea-Tejeda A. Body Composition and Echocardio-Graphic Abnormalities Associated to Anemia and Volume Overload in Heart Failure Patients. Clinical Nutrition 2003; 25(5): 746-757.
13. Dasgupta A., Butt A, Kanti Saha T. , Basu G., Chattopadhyay A., Mukherjee A. Assessment of malnutrition among adolescents: Can BMI be replaced by MUAC. Indian Journal of community medicine Year : 2010 ; 35 (2) Page : 276-279
14. DiMeglio, G. Nutrition in Adolescence.Pediatrics in Review. 2000; 21:32-33. doi:10.1542/10.1542/pir.21-1-32.
15. El Ashry N. May Adolescents Anemia Prevention Program. The Egyptian Experience, SAHCD/SHIP. Director, School Age Health Care Department Ministry of Health & Population. 2009.
16. El-Sahn F, Sallam S, Mandil A, Galal O. Anaemia among Egyptian adolescents: prevalence and determinants. East Mediterr Health J. 2000; 6(5-6):1017-25.
17. Ghalli, I., Salah, N., Hussien, F., Erfan, M., El-Ruby, M., Mazen, I., Sabry, M., Abd El-Razik, M., Saad, M., Hossney, L., Ismaail, S. and Abd El-Dayem, S., et al. In: Proceedings of the 1st

National Congress for Egyptian Growth Curves, Cairo University, 11 December 2003. Cairo, Published in Cresceve Nelmondo, 2008, Ferring Company. Eds: Sartorio, A., Buckler, J.M.H. and Marazzi, N. Egyptian Growth Curves 2002 for Infants, Children and Adolescents.
18. Gupta RK, Bhattagcharya SK, Sundar S, Kumar K, Kachhawaha JS and Sen PC: A correlative study of serum zinc and in vivo cell mediated immune status in rheumatic heart disease. Acta Cardiol. 1992; 47(4): 297-304.
19. Haas JD, Brownlie T: Iron deficiency and reduced work capacity: A critical review of the research to determine a causal relationship. J Nutr 2001; 131:676S –690S.
20. Hoffman R, Benz EJ, Shattil SS, et al. Hematology: Basic Principles and Practice; 2008. 5th ed. Philadelphia, Pa: Elsevier Churchill Livingstone.
21. Hyder SM, Haseen F, Khan M, Schaetzel T, Jalal CS, Rahman M, Lönnerdal B, Mannar V, Mehansho H. A multiple-micronutrient-fortified beverage affects hemoglobin, iron, and vitamin A status and growth in adolescent girls in rural Bangladesh. J Nutr. 2007; Sep; 137(9):2147-53.
22. James P. McClung, PhD, Louis J. Marchitelli, MS, Karl E. Friedl, PhD and Andrew J. Young, PhD. Prevalence of Iron Deficiency and Iron Deficiency Anemia among Three Populations of Female Military Personnel in the US Army Journal of the American College of Nutrition, 2006;. 25 (1): 64-69.
23. Kaji M , and Nishi Y. Growth and mineral: Zinc.GGH Journal March 2006; Volume 22, Issue 1.
24. Kanani S, Poojara R. Supplementation with iron folic acid enhances growth in adolescent Indian Girls. J Nutr 2000; 130: 452S-455S.
25. Keith P, West J, Caballero B, and Black RE. "Nutrition". In Michael H. Merson, Robert E. Black, Anne Mills. International public health: diseases, programs, systems, and policies (2005),(2nd Ed.). Jones & Bartlett Publishers. pp. 227.
26. Kogirima M, Kurasawa R, Kubori S, Sarukura N; Nakamori M; Okada S; Kamioka H and Yamamoto S. Ratio of low serum zinc levels in elderly Japanese people living in the central part of JapanRatio of low serum zinc levels in elderly Japanese European Journal of Clinical Nutrition March 2007; 61, 375-381.
27. Kolsteren P; Rahman S.R. ; Hilderband K. ;and Diniz A. ,. Treatment for Iron deficiency anemia with a combined supplementation of Iron, vitamin A, and Zinc, in women of Dinajpur, Bangladesh. Eur. J. Clin. Nutr.1999; 53: 102-106.
28. Kurz KM, Johnson-Welch S. The nutrition and lives of adolescents in developing countries,1994: Findings from the Nutrition of Adolescent Girls Research Program. Washington DC, International Center for Research on Women.
29. Ijarotimi, OS. Evaluation of Energy and Micronutrients Intake of Nigerian Adolescent Females:A Case Study of Institutionalized Secondary Schools in Akure South Local Government Area, Ondo State, Nigeria. Pakistan Journal of Nutrition 2004; 3 (4): 250-253.
30. Looker AC, Gunter EW, Johnson CL: Methods to assess iron status in various NHANES surveys. Nutr Rev1995; 53:246 –254.
31. Maninder K and Kochar GK. :Impact of Anaemia on Morpho-physiological Variables of Rural Jat Women J Life Sci.,2010; 2(1): 17-20
32. Meier PR, James Nickerson H, Olson KA, Berg RL, Meyer JA. Prevention of Iron Deficiency Anemia in Adolescent and Adult Pregnancies Clinical Medicine & Research, 2003 ;Volume 1, Number 1: 29 – 36.
33. Muñoz M, Villar I., José Antonio García-Erce. An update on iron physiology World J Gastroenterol October 7, 2009 Volume 15 Number 37.
34. Nishiyama, S, Irisa K, Matsubasa TA. Higashi and I Matsuda,. Zinc status relates to Hematological Deficits in Middle aged Women: J. Am. College Nutr. 1998; 17: 291-295.
35. Paknahad Z, Mahdavi R, Mahboob S, Ghaemmaghami SJ, Omidvar N, Ebrahimi M, Ostadrahimi A, Afiat Milani Sh. Iron and Zinc Nutritional and Biochemical Status and Their Relationship among Child Bearing Women in Marand Province. Pakistan Journal of Nutrition 2007; 6 (6): 672-675.
36. Ressler N, Zak B. Serum unsaturated iron binding capacity. Am J Clin Pathol 1958; 3087-3090.
37. Rodriguez, G. L.A. Mo reno, A. Sarria, et al, . Assessment of nutiritional status and body composition in children. Pediatr. Nutr. 2000; 30 (a): 305-8.
38. Sen A. and Kanani S.J. Deleterious Functional Impact of Anemia on Young Adolescent School Girls. Indian Pediatrics 2006; 43:219-226.
39. Shakir KM, Turton D, Aprill BS, Drake AJ, Eisold JF. Anemia : a cause of intolerance to thyroxine sodium. Mayo Clin Proc 2000; 75(2): 189-192.

40. Stoltzfus RJ .Defining iron-deficiency anemia in public health terms: time for reflection. J Nutr.2001; 131:565S –567S.
41. Tanner, JM, J Hiernaux, S Jerman. Human Biology 1969. A guide to Field Methods. IBP Hand book. (ed) by Weiner J, lourie J. Blackwell Scientific Publications, Oxford and Edinburgh. U. K.
42. The World Health Report 2006.Adolescent Nutrition: A Review of the Situation in Selected South- East Asian countries.
43. The World Health Report 2002: Reducing risks, promoting healthy life. Geneva, World Health Organization, Geneva, Switzerland.
44. Topaloglu AK, Hallioglu O, Canim A, Duzovali O, Yilgor E. Lack of association between plasma leptin levels and appetite in children with iron deficiency. Nutrition 2001; 17(7-8): 657-659.
45. Urbano MRD, Vitalle MSS, Juliano Y, Amancio OMS. Iron, copper and zinc in adolescents during pubertal growth spurt. J Pediatr 2002; 78 (4): 327-34.
46. Verster A, van der Pols JC. Anaemia in the Eastern Mediterranean Region. Eastern Mediterranean health journal 1995; 1:64-79.
47. Villalpando S, García-Guerra A, Ramírez-Silva, CI; Mejía-Rodríguez F, Matute G, Shamah-Levy T, Rivera JA.. Iron, zinc and iodide status in Mexican children under 12 years and women 12-49 years of age. A probabilistic national survey.Salud pública Méx 2003;vol.45 suppl.4 Cuernavaca .
48. Wajeunnesa M, Begum N, Ferdousi S, Akhter S, Begum S. Serum Zinc and Copper in Iron Deficient Adolescents Journal of Bangladesh Society of Physiologist 2009; Vol 4, No 2 .
49. World Health Organization. Iron Deficiency Anaemia: assessment, prevention, and control. A guide for programme managers. Geneva, World Health Organization 2001 (WHO/NHD/01.3).
50. Zimmermann M, Adou P, Torresani T, Zeder C, Hurrell R. Iron supplementation in goitrous iron deficient children improves their response to oral iodized oil. Eur J Endocrinol 2000; 142(3): 217-223.

11/21/2010

Effect of Spraying with some Nutrient Elements on Tolerance Beachilyfolia Pear Rootstock to Salinity

Faten, H. M. Ismaeil[*1] and Wahdan, M. T. [2]

[1]Agric. Botany. Dept. Fac. of Agric. Benha Univ. Benha, Egypt
[2] Hort. Dept. Fac. of Agric. Suez Chanel Univ. Egypt
fatenesmaeil@yahoo.com[*]

Abstract: The present investigation was carried out during 2008 and 2009 seasons in the experimental farm belonging to El-Kanater Horticultural Research Station, Kalyubeia Governorate Egypt to study effect of some nutrient elements on tolerance beachilyfolia pear rootstock to salinity. The following measurements were recorded: vegetative growth, nutritional status and some physiological properties of Beachilyfolia pear rootstock, irrigated with saline solution at 6000 ppm with 6 SAR and high chloride level (Cl : So4). Zinc at 50 ppm, Potassium at 250 ppm and Phosphorus at 250 ppm were used in this study to give more explanation about the protect against salt injury. The results revealed that, foliar spray treatments caused a significant increase of some growth measurements (stem height, root length, number of branches & leaves, leaf area, stem diameter and fresh & dry weights of plant organs), leaf photosynthetic pigments content (chlorophyll A, B and carotenoids), leaf mineral content (N, P, K, Na, Fe Mn and Zn), physiological properties (leaf succulence grade, leaf water potential and leaf relative turgidity) of beachilyfolia pear rootstock transplants during 2008 and 2009 consecutive seasons. On the contrary, leaf sodium and proline contents and leaf osmotic pressure took the other way around during the study.
[Faten, H. M. Ismaeil and Wahdan, M. T. **Effect of Spraying with some Nutrient Elements on Tolerance Beachilyfolia Pear Rootstock to Salinity**. Journal of American Science 2010;6(12):1647-1654]. (ISSN: 1545-1003). http://www.americanscience.org.

Key wards: Beachilyfolia, pear, rootstock, nutrient elements, salinity.

1. Introduction:

Pear is one of the most important deciduous fruits in Egypt. For that, in recent years there has been a steady increase in the area cultivated with pear to meet the continuous rise in demand for pear fruits for local consumption in Egyptian markets.

Undoubtedly, the expansion of agricultural land needs a great amounts of suitable irrigation water which already is not sufficient to meet all the expected demands in this respect. In addition to that, the limited amounts of water is an ever growing crisis that may face us in Egypt in future due to the natural aridity in the region, the increasing population and land reclamation projects which represented a very important sector in the agricultural development programs for increasing the cultivated area. Salinity is one of the most serious and oldest environmental problems affecting approximately one third of earth's irrigation land. There are many factors affecting the salinity-yield relationship such as the physical and chemical conditions of the soil, climate and farming practices (Schreiner and Ludders, 1992).

The possibility of using saline water for irrigation, especially underground water is considered as a limiting factor and great value for the success of the projects of new land reclamation, which it is still very limited source until now, however many problems are expected to arise. These problems would be related to the excessive accumulation of saline salts in the soil because this water contains a considerable amount of harmful salts as an actual limiting factor for growth and productivity of transplants and fruit trees (Sharaf *et al.*, 2006).

There is a little of available information for fruit growers about the possibility of some pear and other deciduous rootstocks to grow under conditions of new reclaimed lands and probability of these rootstocks to tolerance for irrigation with saline water (Kabeel, 1985, Bondok *et al.*, 1995, Osman, 2005 and khamis *et al.*, 2008) on some deciduous rootstocks transplants.

The present study carried out to investigate the effect of spraying with some nutrient elements (K, P and Zn) on tolerance beachilyfolia pear rootstock to salinity.

2. Materials and methods

The present investigation was carried out throughout the two consecutive seasons of 2008 and 2009 in the experimental farm belonging to El-Kanater Horticultural Research Station, Kalyubeia Governorate, Egypt.

Uniform and healthy one-year-old transplants of *Pyrus beachilyfolia* rootstock was the plant materials used in this study. In both seasons of study and during the first week of February, pear rootstock transplants were transplanted individually each in clay pot of 35 cm. in diameter that previously

had been field with specific weight of media consisting of clay and sand at equal proportion (by volume). Mechanical and chemical analysis of the experimental soil from 0 to 30 cm. depth just before pear investigated treatments had been started are shown in Table (1). These standard methods used in this respect described by A.O.A.C, (1990).

Table (1): Physical and chemical analyses of the experimental soil.

Depth (cm.)	Sand %	Silt %	Clay %	Soil texture	Soil extract pH 1:25	E.C. (1:5) ds/m	Soluble cations				Soluble anions			
							Ca^{++}	Mg^{++}	Na^{+}	K^{+}	CO_3^{--}	HCO_3^{-}	Cl^{-}	$SO_4^{=}$
0-30	21.10	25.90	52.60	Clay loam	7.86	0.195	0.74	0.22	0.68	0.17	0.55	0.80	-	0.47

Irrigation with the saline solution was carried out twice weekly by adding (¾) liter per each pot starting from the first week of March until the last week of September throughout the two seasons of study. To prevent salts accumulation pots irrigated with tap water every 12 days, then rewatering with salt solutions applied the next day. Control treatment was supplied periodically two times every week with tap water only at (¾) liter/pot. The following treatments are used:

1- Tap water irrigation (control).
2- Irrigation with 6000 ppm saline solution of SAR6 and high Cl: SO_4 level plus tap water spray.
3- Irrigation with 6000 ppm saline solution of SAR6 and high Cl: SO_4 level + Zn at 50 ppm spray.
4- Irrigation with 6000 ppm saline solution of SAR6 and high Cl: SO_4 level + K at 250 ppm spray.
5- Irrigation with 6000 ppm saline solution of SAR6 and high Cl: SO_4 level + P at 250 ppm spray.

The different treatments arranged in a complete randomized block design where each treatment was replicated three times with two transplants for each replicate. Regarding the foliar spray solutions with some nutrition elements were periodically sprayed at one month interval beginning from mid- March until mid September. The abovementioned saline solution was prepared as shown in Table (2).

Salts added in grams were estimated as anhydrous form.

*SAR = Meq

$$\frac{Na}{\sqrt{\frac{Ca + Mg}{2}}}$$

Methodology as has been followed in this investigation is being determined as follows:

Table (2): Preparation of saline solution used.

	Saline solution (6000 ppm SAR6 high Cl)		
Salt added per liter	$CaCl_2$	g	1,67
		meq.	30,00
	$MgSO_4$	g	0,60
		meq.	10,00
	KCl	g	0,44
		meq.	5,84
	K_2SO_4	g	1,65
		meq.	8,96
	Na_2SO_4	g	0,45
		meq.	6,34
	NaCl	g	1,20
		meq.	20,51
	SAR*		6,00
	Cl meq./L		56,35
	SO_4 meq./L		35,29
	Cl / SO_4 ratio		1,60

Morphological characteristics (vegetative growth measurements):

On mid week of October during both seasons as the experiment was ended, the effect of the different studied treatments on some vegetative growth measurements were evaluated by the following growth parameters: Stem height (cm), Stem diameter (mm.), Root length (cm), Number of branches per plant, Number of leaves per plant and Dry weights of plant organs (leaves, stems and roots in gm).

Transplants of each replicate were carefully taken out from pots then washed with tap water and followed by distilled water to free them any residues. Thereafter, each transplant was divided individually into its three organs (leaves, stem and root) to be air dried in an electrical oven at 70 °C. until a constant weight then weighed then as average dry weight for each plant organ for every replicate was estimated and recorded.

Leaf physiological characteristics:

Determination of leaf osmotic pressure (in bar).

Adequate leaf samples were immediately frozen, the cell sap was extracted in the laboratory with a piston pressure. When the frozen tissue has been thawed. The sap total soluble solid (TSS) was determined by refractometer and the equivalent values of the osmotic pressure (in bars) were estimated according to Gusov (1960).

Leaf succulence grade (L.S.G.).

Leaf succulence grade (L.S.G.) was calculated as gms. H_2O/cm^2 of leaf according to the following equation according to Nomir (1994).

$$L.S.G. = \frac{\text{Leaf water content (gm.)}}{\text{Leaf area } (DC^2)}$$

Whereas, leaf water content (gm) =

$$\frac{\text{Fresh weight – dry weight of the leaves}}{\text{Number of leaves}} \times 100$$

Leaf relative turgidity (L.R.T.).

Discs of about one cm. in diameter were removed from each leaf sample to determine their fresh weight immediately, then placed in a closed containers (Petri dishes) until they became constant in weight (after 24 hours) at room temperature 22 ± 2° in shade. The discs were surface dried with paper and weighed for their turgid weight. Dry weight of each ten discs was determined after 24 hours. Leaf relative turgidity was estimated according to the following equation described by Nomir (1994):

$$L.R.T. = \frac{\text{Fresh weight – dry weight}}{\text{Turgid weight – dry weight}} \times 100$$

Leaf water potential (L.W.P.):

The method and the equation for the calculations have been suggested by Nomir (1994).

$$(L.W.P.) = \frac{\text{Fresh weight – dry weight}}{\text{Fresh weight}} \times 100$$

Chemical analysis:

In this regard, leaf photosynthetic pigments (chlorophylls A, B and carotenoids), leaf proline content and leaf mineral composition as well as shoot content of total carbohydrates in response to different studied treatments included were concerned.

Leaf photosynthetic pigments determination:

The quantitative analysis of photosynthetic pigments in response to treatments under study were determined and calculated according to the methods described by A.O.A.C. (1990).

Leaf proline content:

The proline content was estimated in fresh leaves according to the method described by Batels *et al*., (1973) and confirmed by Draz (1986).

Estimation of total carbohydrates:

Total carbohydrates in dry shoots (0.1 gm) were determined photometrically at 490 µm., according to the method described by the method of A.O.A.C. (1990).

Determination of leaf minerals content:

At last week of October in both seasons samples were collected and cleaned from adherent dust and dried at 70° C for 72 hours, ground to fine powder and digested according to Chapman and Pratt, (1961). The ground dried materials of leaf samples were analyzed for total nitrogen, phosphorus, potassium, sodium, iron, manganese and zinc by the method of A.O.A.C, (1990).

- Statistical analysis:

All data obtained during each season of this study were subjected to statistical analysis according to the method described by Snedecor and Cochran (1980). However, means values for every studied parameter were compared according to the Duncan's multiple range test (Duncan, 1955).

3. Results

Growth measurements:

Stem diameter, length of (stem and root); number of (branches & leaves per plant); average leaf area; fresh and dry weights of different plant organs

(leaves, stem and root) were investigated regarding their response to the treatments.

Regarding the effect of sprayed nutrient elements, results in Tables (3, 4, 5 and 6) declared that, all investigated growth measurements of the salinity stressed transplants were significantly increased by any of three nutrient elements however, Zn at 50 ppm foliar spray proved to be the most effective in this regard followed in a descending order by K and P each at 250 ppm foliar spray during two seasons of study.

Leaf physiological properties:

Four physiological characteristics (leaf water potential; leaf osmotic pressure; leaf relative turgidity and leaf succulence grade) were investigating regarding their response to effects of sprayed three nutrient elements (Zinc, Potassium and Phosphorus).

With regard to effect of sprayed three nutrient elements (Zinc, Potassium and Phosphorus), data in Table (7) revealed that, two conflicted trends were detected. Herein, leaf succulence grade, leaf water potential and leaf relative turgidity were significantly increased by any of three nutrient elements sprayed, but Zn foliar spray was more effective for (leaf water potential and leaf relative turgidity) and K spray showed the greatest increase in leaf succulence grade. On the contrary, the trend of response for leaf osmotic pressure as influenced by three nutrient elements (Zn, K and P) spray took the other way around; where characteristic was significantly decreased by any foliar application.

Chemical composition:

Photosynthetic pigments:

Leaf chlorophyll (A & B) and carotenes contents of salt stressed pear rootstock in response to effects of sprayed with nutrient elements were investigated.

The obtained results and tabulated in Table (8) revealed that, both (K and P) foliar spray each at 250 ppm and Zn at 50 ppm increased three photosynthetic pigments, while Zn foliar spray was more effective descendingly followed in this respect by (K and P) foliar spray during the study.

Stem total carbohydrates:

As for the effect of sprayed nutrient elements (Zn, K and P), it is quite clear that, total carbohydrates was increased. Zn foliar spray at 50 ppm was more effective followed in a descending order by K and/or P each at 250 ppm as increase in total carbohydrates (Table, 9).

Leaf proline contents:

With regard to effect of Zn, K and P sprays reduced significantly proline; however, Zn foliar spray was statistically the most depressive in this concern during the study (Table, 9).

Leaf mineral composition:

In this regard, effects of sprayed nutrient elements on leaf (N, P, K, Na, Fe, Mn and Zn) contents of salt stressed pear rootstock transplants were investigated. As for the effect of sprayed nutrient elements on leaf mineral composition of salt stressed pear transplants, data obtained in Tables (10 and 11) revealed obviously that, the response varied from one element to another. Foliar spray with Zn and K as well as P solely increased leaf N, P, K, Fe; Mn and Zn contents, but decreased leaf Na contents.

Table (3): Effect of some nutrient elements on some growth measurements of beachifolia pear rootstock transplants irrigated with saline water during 2008 and 2009 seasons.

Treatments	Stem height (cm)		Root length (cm)		Stem diameter (cm)	
	2008	2009	2008	2009	2008	2009
Tap water (control)	155.90 A	151.20 A	69.33 A	71.66 A	0.74 A	0.71 A
Saline water(6000/6/H)	81.22 D	78.36 C	33.66 D	33.17 D	0.45 C	0.43 E
Saline water(6000/6/H) + Zn at 50 ppm	90.19 B	86.47 B	42.57 B	41.67 B	0.70 A	0.63 B
Saline water(6000/6/H) + K at 250 ppm	88.33 BC	85.49 B	41.19 BC	40.52 BC	0.57 B	0.59 C
Saline water(6000/6/H) + P at 250 ppm	86.61 C	84.48 B	38.56 C	38.47 C	0.48 C	0.48 D

Means followed by the same letter are not significantly different at 5% level

Table (4): Effect of some nutrient elements on some growth measurements of beachifolia pear rootstock transplants irrigated with saline water during 2008 and 2009 seasons.

Treatments	No. of branches/plant		No. of leaves/plant		Leaf area (cm^2)	
	2008	2009	2008	2009	2008	2009
Tap water (control)	10.00 A	9.33 A	157.80 A	155.30 A	42.18 A	42.68 A
Saline water (6000/6/H)	6.33 D	5.33 D	66.80 E	65.33 D	22.67 E	22.88 D
Saline water (6000/6/H) + Zn at 50 ppm	9.33 AB	8.67 A	78.67 B	77.67 B	26.33 B	24.64 B

Saline water (6000/6/H) + K at 250 ppm	**8.33 BC**	**7.33 B**	**75.33 C**	**75.33 C**	**25.76 C**	**23.88 C**
Saline water (6000/6/H) + P at 250 ppm	**7.33 CD**	**6.33 C**	**74.33 D**	**74.00 C**	**24.39 D**	**22.58 D**

Means followed by the same letter are not significantly different at 5% level

Table (5): Effect of some nutrient elements on fresh weight (F.W.) of beachifolia pear rootstock transplants irrigated with saline water during 2008 and 2009 seasons.

Treatments	Leaves F.W. (gm)		Stem F.W. (gm)		Roots F.W. (gm)		Total plant F.W. (gm)	
	2008	2009	2008	2009	2008	2009	2008	2009
Tap water (control)	**42.42 A**	**41.31 A**	**97.75 A**	**82. 85 A**	**83.95 A**	**81.90 A**	**224.10 A**	**205.80 A**
Saline water (6000/6/H)	**17.14 D**	**17.88 D**	**38.68 D**	**37.41 D**	**32.60 C**	**34.40 D**	**88.41 D**	**89.69 E**
Saline water (6000/6/H) + Zn at 50 ppm	**25.54 B**	**24.52 B**	**48.57 B**	**47.61 B**	**44.23 B**	**47.41 B**	**118.30 B**	**119.50 B**
Saline water (6000/6/H) + K at 250 ppm	**24.26 B**	**23.13 B**	**45.78 BC**	**44.88 BC**	**43.46 B**	**44.71 C**	**120.20 B**	**112.70 C**
Saline water (6000/6/H) + P at 250 ppm	**22 .68 C**	**21 .42 C**	**43.76 C**	**42.67 C**	**42.58 B**	**43.66 C**	**109.00 C**	**107.80 D**

Means followed by the same letter are not significantly different at 5% level

Table (6): Effect of some nutrient elements on dry weight (D.W.) of beachifolia pear rootstock transplants irrigated with saline water during 2008 and 2009 seasons.

Treatments	Leaves D.W. (gm)		Stem D.W. (gm)		Roots D.W. (gm)		Total plant D.W. (gm)	
	2008	2009	2008	2009	2008	2009	2008	2009
Tap water (control)	**11.53 A**	**11.25 A**	**35.16 A**	**31.77 A**	**36.19A**	**35.30A**	**82.88 A**	**78.32 A**
Saline water (6000/6/H)	**7.87 C**	**7.11 D**	**18.16 C**	**17.55 D**	**15.14C**	**16.03C**	**41.17 C**	**40.69 C**
Saline water (6000/6/H) + Zn at 50 ppm	**9.50 B**	**9.72 B**	**22.81 B**	**22.46 B**	**19.15 B**	**20.44 B**	**51.46 B**	**52.62 B**
Saline water (6000/6/H) + K at 250 ppm	**9.02 B**	**8.19 C**	**21.50 BC**	**21.07 BC**	**18.82 B**	**19.28 C**	**49.34 B**	**48.54 B**
Saline water (6000/6/H) + P at 250 ppm	**8.44 BC**	**7.97 CD**	**20.55 BC**	**20.23 C**	**18.28 B**	**18.82 C**	**47.27 B**	**47.02 B**

Means followed by the same letter are not significantly different at 5% level

Table (7): Effect of some nutrient elements on some leaf physiological properties of beachifolia pear rootstock transplants irrigated with saline water during 2008 and 2009 seasons.

Treatments	L. O. P.		L. R. T.		L. W. P.		L. S.G.	
	2008	2009	2008	2009	2008	2009	2008	2009
Tap water (control)	**11.73 E**	**11.73 E**	**57.29 A**	**57.46 A**	**73.39A**	**72.43A**	**1.577A**	**1.569A**
Saline water (6000/6/H)	**19.83 A**	**19.83 A**	**23.16 E**	**23.42 E**	**54.31 D**	**52.83 D**	**1.558 D**	**1.456 C**
Saline water 6000/6/H) + Zn at 50 ppm	**12.62 D**	**12.62 D**	**29.43 D**	**28.51 D**	**61.01C**	**59.88C**	**1.565C**	**1.552B**
Saline water (6000/6/H) + K at 250 ppm	**14.88 B**	**14.88 B**	**39.57 B**	**39.33 B**	**68.52B**	**67.78B**	**1.570B**	**1.564B**
Saline water (6000/6/H) + P at 250 ppm	**14.26 C**	**14.26 C**	**32.65 C**	**33.17 C**	**61.00C**	**59.87C**	**1.563C**	**1.554B**

Means followed by the same letter are not significantly different at 5% level

Table (8): Effect of some nutrient elements on leaf photosynthetic pigments content of beachifolia pear rootstock transplants irrigated with saline water during 2008 and 2009 seasons.

Treatments	Chlorophyll (A)		Chlorophyll (B)		Carotene	
	2008	2009	2008	2009	2008	2009
Tap water (control)	1.33 A	1.16 A	1.41 A	1.39 A	1.01 A	0.98 A
Saline water (6000/6/H)	0.58 D	0.51 D	0.77 D	0.75 D	0.78 C	0.76 E
Saline water (6000/6/H) + Zn at 50 ppm	0.86 B	0.77 B	0.93 B	0.96 B	0.91 B	0.92 B
Saline water (6000/6/H) + K at 250 ppm	0.74 C	0.72 BC	0.93 B	0.86 C	0.87 B	0.87 C
Saline water (6000/6/H) + P at 250 ppm	0.62 CD	0.65 C	0.79 C	0.78 D	0.79 C	0.78 D

Means followed by the same letter are not significantly different at 5% level

Table (9): Effect of some nutrient elements on leaf carbohydrates and praline content of beachifolia pear rootstock transplants irrigated with saline water during 2008 and 2009 seasons.

Treatments	Carbohydrate		praline	
	2008	2009	2008	2009
Tap water (control)	39.42 A	38.16 A	0.13 D	0.13 D
Saline water(6000/6/H)	19.97 E	18.52 E	0.39 A	0.40 A
Saline water(6000/6/H) + Zn at 50 ppm	31.58 B	28.56 B	0.20 C	0.21 C
Saline water(6000/6/H) + K at 250 ppm	25.33 C	25.11 C	0.29 B	0.30 B
Saline water(6000/6/H) + P at 250 ppm	23.13 D	21.73 D	0.29 B	0.29 B

Means followed by the same letter are not significantly different at 5% level

Table (10): Effect of some nutrient elements on leaf N,P,K and Na content of communis pear rootstock transplants irrigated with saline water during 2008 and 2009 seasons.

Treatments	Nitrogen (%)		Phosphorus (%)		Potassium (%)		Sodium (%)	
	2008	2009	2008	2009	2008	2009	2008	2009
Tap water (control)	2.67 A	2.61 A	0.24 A	0.23 A	2.16 A	2.25 A	0.24 E	0.24 E
Saline water (6000/6/H)	1.20 D	1.18 D	0.11 B	0.12 D	0.91 C	0.74 C	0.55 A	0.62 A
Saline water (6000/6/H) + Zn at 50 ppm	1.68 B	1.52 B	0.12 B	0.12 D	0.85 E	0.71 C	0.33 C	0.35 D
Saline water (6000/6/H) + K at 250 ppm	1.43 C	1.34 C	0.12 B	0.14 C	1.57 B	1.43 B	0.38 C	0.39 C
Saline water (6000/6/H) + P at 250 ppm	1.10 D	0.86 E	0.23 A	0.21 B	0.87 D	0.74 D	0.41 B	0.42 B

Means followed by the same letter are not significantly different at 5% level

Table (11): Effect of some nutrient elements on leaf Fe, Mn and Zn content of beachifolia pear rootstock transplants irrigated with saline water during 2008 and 2009 seasons.

Treatments	Iron (ppm)		Manganese (ppm)		Zinc (ppm)	
	2008	2009	2008	2009	2008	2009
Tap water (control)	126.60 A	127.10 A	52.43 A	55.69 A	29.37 A	28.59 A
Saline water (6000/6/H)	57.69 D	55.53 C	29.23 CD	31.07 C	15.65 C	14.39 D
Saline water(6000/6/H) + Zn at 50 ppm	69.51 B	67.38 B	34.54 B	35.18 B	20.31 B	19.43 B
Saline water (6000/6/H) + K at 250 ppm	50.14 E	50.18 D	19.76 C	31.31 C	16.11 C	15.13 C
Saline water (6000/6/H) + P at 250 ppm	61.48 C	57.44 C	28.56 D	31.11 D	15.17 C	14.61 D

Means followed by the same letter are not significantly different at 5% level

4. Discussions

Zn stimulated cell elongation by encouraging cell walls to stretch (Nason, 1950), as a result of its function for process of tryptophan biosynthesis, the precursor of the IAA plant auxin. Bouat *et al*., (1954) showed that, there was a continuous demand for phosphorus for best vegetative growth of Arbequine olive. Also, Miller and Deidda, (1975) demonstrated that some parameters of young olive trees were positively affected by phosphorus application.

On the other words, potassium not only ameliorated the harmful effect of salinity, but also encouraged the vegetative growth of the pear rootstock. Huffaker and Wallace (1966) found that, the high rate of K fertilizer prevent the absorption of Na by plants to which Na is not a nutritive element by plants that need a high ratio of (Ca + Mg) : (K +Na) in the nutritional requirements.

Moreover, Rajput *et al*., (1976) on mango trees who found that spraying $ZnSO_4$ at 0.2- 0.8% in January increased length of the terminal shoot. In addition, Khamis *et al*., (1985) on grape rooted cuttings and Osman (2005) on some apple rootstocks reported that, spraying saline stressed with P or K reduced the salinity damage and improved growth.

The present results pertaining the response to foliar sprays with some nutrient elements findings Omar (1996) on mango and apricot plants, Abd- El-Mageid (1998) on almond seedlings, Hasan (2005) on olive transplants and Osman (2005) on some apple rootstocks transplants gave a real support in this regard.

These results regarding the influence of nutrient elements and growth retardants application on leaf photosynthetic pigments of salt stressed transplants are in accordance with those found by Kucherova *et al*., (1979); Nomir and El-Deeb (2000) and Gowda (2002), all reported that, nutrient elements and growth retardants increased foliar pigments contents i.e., chlorophyll (A & B) and carotenoids compounds.

The present result regarding the response of total carbohydrates was in harmony with that found by Gowda (2002).

The present result regarding the effect of nutrient elements on leaf proline contents goes in line with those found by Anju-Thakur and Singh (1998).

Such results are in general agreement with finding of Khamis *et al.*, (1985) on Thompson and American grape rooted cuttings who found that, P foliar spray caused significant decrease in leaf N and K content and increased significantly leaf P, Fe and Mn content over that of salinity stressed rooted cuttings.. However, K foliar spray increased leaf – N content. In addition, Behairy *et al.*, (1985) reported that, foliar spray with Zn increased leaf N, Fe and Mn content and decreased leaf P and K content of salt stressed transplants. Also, Omar, (1996) on mango and apricot plants and Abd- El- Mageid, (1998) on almond seedlings gave a real support in this regard.

5. Conclusion:

Foliar spray Beachilyfolia pear transplants with Zn at 50 ppm caused a significant increase of some growth measurements, leaf photosynthetic pigments content (chlorophyll A, B and carotenoids), leaf mineral content (N, P, K, Na, Fe Mn and Zn), Physiological properties (leaf succulence grade, leaf water potential and leaf relative turgidity) of beachilyfolia pear rootstock transplants. On the contrary, leaf sodium and proline contents and leaf osmotic pressure took the other way around during the study.

Corresponding Author
Faten, H. M. Ismaeil
Agric. Botany. Dept. Fac. of Agric. Moshtohor, Kalyubeia Governorate, Egypt.
Box. P. 13736
fatenesmaeil@yahoo.com
Bakry672003@ yahoo.com

6. References:

1. A.O.A.C. 1990 Association of Official Analytical Chemists. Official an tentative methods of analysis, 15th Ed., 1008pp, Washington. D.C., USA.
2. Abd El- Mageid, I. S. 1998 Physiological studies on salinity tolerance of almond seedlings. M.Sc. Thesis, Fac. of Agric. Moshtohor, Zagazige Univ., Egypt.
3. Anju-Thakur, P. S. and Singh, R. P. 1998 Influence of paclobutrazol and triacotanol on growth and water relations in olive varieties under water stress. Indian Jour. of Plant Physiology, 3 (2): 116-120.
4. Batels, L. S.; Waldren, R. P. and Teare, I. D. 1973 Rapid determination of free proline for water stress studies. Plant and Soil; 939: 205-207.
5. Behairy, Z. H.; M. M. Sharaf and Khamis, M. A. 1985 Micronutrients influence on salt tolerance of guava and olive seedlings. Ann. of Agric., Sci., Moshtohor, Vol. 23, No. (8)
6. Bondok, A.; Tawfik, H.; Shaltout, A. and Abd El-Hamid, N. 1995 Effect of salt stress on growth and chemical constituents of three peach rootstocks. Assiut J. of Agric. Sci., 26(1):173-194.
7. Bouat, A.; Renava, P. and Dulac, J. 1954 Study on the physiology of olive nutrition. Ann. Agron., 5: 459-489.
8. Chapman, H. D. and P. F. Pratt 1961 Methods of analysis for soils, plants and waters. Div. Agric. Sci. Univ. California, Berkely pp. 309.
9. Draz, M. Y. 1986 Response of bitter almond seedling to different water regime. Ph. D. Thesis Fac. of Agric., Cairo Univ. Egypt.
10. Duncan, B. D. 1955 Multiple test range and multiple F tests. Biometries, 11-142.
11. Gowda, M. A. 2002 Physiological studies on drought tolerance some fruit transplants. Ph.D. Thesis, Fac. Agric., Minia Univ., Egypt.

12. Gusov, N. A. 1960 Some methods in studying plant water relations. Leningrad Acad. of Sci., USSR.
13. Hasan, A. E. 2005 Physiological studies on the effect of salt stress on some olive cultivars. Ph. D. Thesis, Fac. of Agric. Moshtohor, Benha University.
14. Huffaker, R. C. and Wallace, A. 1966 Effect of potassium and sodium levels on Sodium distribution in some plant species. Soil Sci., 88: 80-82.
15. Kabeel, H. 1985 Physiological studies on salt tolerance of some deciduous fruit species. M. Sc. Thesis, Fac. Agric. Moshtohor, Zagazig, Univ. Egypt.
16. Khamis, M. A.; Sharaf, M. M. and Behairy, Z. H. 1985 Salt tolerance in Thompson seedlings and American grape plants as affected by some macro-element. Ann. of Agric. Sci. Vol. 23, No. (9).
17. Khamis, M. A., Bakry, KH. A. and Ismaeil, E. A. 2008 Physiological studies on growth and salt tolerance of some fruit plants. Annals oF Agric. Sci., Moshtohor, Vol. 46 (2) 87- 100.
18. Kucherova, T. P.; Lishchuk, A. I. and Stadnik, S. A. 1979 Effect of chlorocholine chloride and latex on the water regime, pigment content and bioelectric reaction of apricot cultivars with different degree of drought resistance. Botaicheskuu sad, Yalta, Crimea, USSR. (C. F. Hort. Abst. 49. (9): 6603).
19. Mieller, A. and Deidda, P. 1975 Mineral nutrition of olives in artificial cultivation. Italia. Agricola, 112 (11).
20. Nason, A. 1950 Effect of Zn- deficiency on the synthesis of tryptophan by Newrospora extracts. Hortscience, 20 (4) : 111-12.
21. Nomir, S. A. 1994 Physiological studies on kaki. Ph. D. Thesis., Fac. of Agric. Zagazig Univ. Egypt.
22. Nomir, S. A. and El-Deeb, M. A. 2000 Response of Washington Navel orange transplants to some CCC and PP_{333} treatments. Zagazig J. Agric. Res. Vol.27, No. 4:931-945.
23. Omar, T. A. 1996 Physiological studies on tolerance of apricot and mango seedlings to salinity. M.Sc. Thesis, Fac. Agric. Moshtohor., Zagazig Univ. Egypt.
24. Osman, A. H. 2005 Physiological studies on growth and salt tolerance of some apple rootstocks. Ph. D. Thesis, Fac. of Agric. Benha Univ., Egypt.
25. Rajput, C. B.; Singh, B. P. and Singh, S. B. 1976 Effect of foliar application of zinc sulphat on vegetative growth of mango (*Mangifera indica*, L.). Hortscience, 46(2):23-24 (En, 4 ref.).
26. Schreiner, M. and Ludders, P. 1992 Effect of sodium salt on the vegetative growth of Golden Delicious apple tress at different levels of potassium nutrition. Mitteilungen-Klosterneuburg, Rebe und wein, obsttbau und fruchtcvcrwcrtung. 42 (1): 34-38 (C.F. Hort. Abst., 64: 5).
27. Sharaf, M. M.; Bakry, KH. A. and Darwesh, D. R. 2006 Studies on salinity tolerance in some pomes rootstocks. 1-Vegetative growth, photosynthetic pigments and total carbohydrates. J. Biol. Chem. Environ. Sci., 1(4): 913-981.
28. Snedecor, G. W. and Cochran, W. G. 1980 Statistical Methods. 6th ed. The Iowa state. Univ. Press, Amer, Iowa, U.S.A. PP. 593.

11/2/2010

Black Tea Forestalls Sodium Fluoride-Induced Neurobehavioral Toxicity in Laboratory Rats

Mervat M. Kamel[1*], Heba S. El-lethey[1], Iman B. Shaheed[2], and Gehan M. Kamel[3]

[1]Department of Animal Hygiene and Management, [2]Department of Pathology, [3]Department of Pharmacology, Faculty of veterinary Medicine, Cairo University, Cairo, Egypt, mevy58@yahoo.com*

Abstract: The present study aimed to investigate the main effects as well as the interaction effect of supplemental Na-F and black tea on emotional reactivity and learning and memory capacities in rats using a variety of behavioural tasks. Eighty weanling 32-days old Wistar male rats randomly distributed into four groups of 20 animals each, were supplemented with Na-F at 100 ppm and 2% black tea in a factorial pattern to constitute 4 experimental treatments. Brain tissue specimens, representing all treatments, were taken for biochemical and histopathological investigations. In the open field test, Na-F-treated rats displayed higher levels of anxiety that were significantly reduced when black tea was concomitantly administered. Marked impairment in habituation was a significant remark for Na-F group. A superior learning and memory ability was recorded for black tea-supplemented rats during classic maze test, where black tea significantly recovered the intervention observed in Na-F-exposed rats. Moreover, black tea significantly enhanced spatial cognition learning ability and successfully alleviated Na-F-induced spatial memory impairment. Rats administered Na-F displayed distinct neurodegenerative changes of nerve cells especially in hippocampus, accompanied by inhibition of brain acetylcholinesterase (AChE) activity with increased oxidative stress. Administration of black tea along with Na-F was able to afford protection against these Na-F-induced alterations. Our findings suggest a profound ameliorative effect of black tea on Na-F-induced adverse alterations in the brain of rats as indicated by hindrance of learning and memory performance, and argue for concurrent administration of black tea to Na-F-exposed individuals in order to help alleviate fluoride intoxication.

[Mervat M. Kamel, Heba S. El-lethey, Iman B. Shaheed, and Gehan M. Kamel. **Black Tea Forestalls Sodium Fluoride-Induced Neurobehavioral Toxicity in Laboratory Rats**. Journal of American Science 2010;6(12):1655-1673]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: Sodium fluoride, black tea, SOD, TBARs, learning, memory, anxiety-like behaviour

1. Introduction:

Toxic effects of excessive intake of fluoride are matters of serious international concern (Verma et al., 2006). Fluorides are naturally occurring contaminants in the environment and commonly involved in toothpastes, mouth rinses, processed beverages and food as well as public water in order to prevent dental caries (Buzalaf et al., 2004). In humans, exposure to elevated fluoride drinking water in endemic areas has been found to cause headache, followed by lethargy and insomnia with reduced Intelligence Quotient (IQ) of children and lowered levels of mental work capacity of adults (Lu et al., 2000, Xiang et al., 2003, Sharma et al., 2009).

Since fluoride was evidenced to cross blood brain barrier, a link between excessive exposure to fluoride and dysfunction of the central nervous system has established (Blaylock, 2004, Ge et al., 2005). High levels of fluoride in drinking water (3-11 ppm) are known to cause definite harm to the central nervous system as manifested in diminished mental acuity with alterations in learning and memory processes directly without first causing the physical deformities of skeletal fluorosis (Wu et al., 2006, Chioca et al., 2008, Zhang et al., 2008). Rats exposed to fluorosis showed neuroinflamation in the brain, including demylinization, a reduction in the number of Purkinje cells, thickening and disappearance of dentrites, swelling of mitochondria and dilation of endoplasmic reticulum in neurons (Guan et al., 1998). Moreover, disturbances in brain development in offspring rats with chronic fluorosis have been reported (Liu et al., 1989).

Prolonged exposure to sodium flouride has been reported to induce deleterious effects on soft tissues (Patel and Chinoy, 1997, Purohit et al., 1999) and changes in behaviour through locomotor activity impairment (Ekambaram and Paul, 2001, 2003).

The central nervous system is principally more susceptible to oxidative damage due to its high oxygen consumption along with high tissue concentrations of iron and comparatively low levels of some antioxidants system (DrÖge and Schipper, 2007; Korkmaz et al., 2007). It has been emphasized that higher levels of fluoride provoke oxidative stress in the brain through excessive production of reactive oxygen species (ROS) free radicals taxing the compensatory task of antioxidant system with increased levels of lipid peroxidation, accounting for neuronal dysfunction with cognitive decline (Guan et al., 1998, Chirumari and reddy, 2007). Moreover, fluoride intoxication has been shown to induce changes in central neurotransmission presenting inhibited acetylcholinesterase (AChE) activity (Gao

et al., 2009). Thus, prevention of fluoride-induced oxidative damage to the brain is likely to be beneficial for the maintenance of cognitive function.

There is a growing awareness of the role of certain nutritional components including flavonoid polyphenols, a group of dietary-derived phytochemicals, found in fruits, vegetables and beverages like tea in maintenance of health and prevention of chronic diseases (Youdim et al., 2002). Flavonoids have been reported to induce improvement in memory, learning and cognition (Spencer, 2009). Several studies suggested that flavonoids might improve cognitive function by protecting vulnerable neurons against injury induced by neurotoxins, suppressing neuroinflamation, and enhancing existing neuronal function or by stimulating neuronal regeneration (Mandel and Youdim, 2004, Spencer, 2008, Rendeiro et al., 2009). Most of these studies have been conducted in rodents as models for human in order to predict effects of flavonoids on human cognitive performance (Casadesus et al., 2004, Lee et al., 2005, Williams et al., 2008).

Although six types of teas are distinguished, the three main types are black tea (fully fermented), oolong tea (semi-fermented) and green tea (non-fermented). Black tea accounts for 80% of the world's total tea production (Krisnamoorthy, 1991). While black tea is the most common type of tea consumed in Egypt and widely consumed beverages, second to water, few studies have investigated the effects of black tea on cognitive performance.

The type of flavonoid polyphenols founds in different types of teas, and constitutes 93% of total tea phenolic compounds (Lakenbrink et al., 2000), depends on the degree of fermentation during manufacture. Black tea contains more complex flavonoids than green tea. Although catechin content of black tea is lower (3-10%), but it has a higher content of theaflavins and thearubigins as a result of catechins enzymatic polymerization under substantial oxidation during processing (Unno and Hoshino, 2007). In addition, tea leaf also contains theanine, caffeine and other chemical components (Xu and Chen, 2002).

Tea flavonoids are brain permeable (Nakagawa and Miyazawa, 1997) and have been reported to possess potent cognitive protective effect through antioxidative and radical scavenging properties that can help to ameliorate neurodegenerative disorders such as Alzheimers and Parkinson diseases (Weinreb et al., 2004, Mandel and Youdim, 2004, Sutherland and Rahman, 2006).

In a previous study, clear deleterious effects of Na-F on the brain of rats were revealed as indicated by impaired cognitive abilities (El-lethey et al., 2010). Therefore, the present study was undertaken to evaluate the possible ameliorative effect of black tea, an important source of dietary antioxidants, against Na-F-induced alterations in the brain of rats with negative manifestation on learning and memory performance. For further investigation for the mechanisms of Na-F-induced cognitive disorders, and the potential protective effect of black tea on brain oxidative stress, the later parameter was also quantified in the current study.

2. Materials and methods

2.1. Animals and housing:

Since estrogen has been found to enhance memory in females, only male rats were employed in our study in order to eliminate estrogen related beneficial influence on memory (Wolf and Kirschbaum, 2002). Eighty weanling 32-days old Wistar male rats, approximately 45g weight were obtained from the Unit for Laboratory Animals at Faculty of Veterinary Medicine, Cairo University and used in our study. They were housed in standard polypropylene cages with stainless steel wire lids, bedded with wood shavings. Animals were maintained on a 12-h light/dark cycle at a room temperature of 20-22°c and 60% humidity with free access to feed (standard laboratory pellets) and water throughout the study. The procedures concerning animal care and experiment protocols were carried out in accordance with guidelines from Cairo University Policy on Animal Care and Use.

2.2. Experimental design:

All males were randomly distributed into four groups of 20 animals each, divided on 2 replicates and orally administered our treatments throughout the study till its completion at 106 days of age, in a 2 x 2 factorial design as follows:

Group (1) control (C), n=20: Weanling pups were administered plain water.

Group (2) Na-F group (F), n=20: Weanling pups were exposed to *ad libitum* supply of Na-F alone (Sigma Chemical Company) in drinking distilled water at 100 ppm on a mg/kg/day basis of 10.77 Na-F (Chioca et al., 2008).

Group (3) black tea group (T), n=20: Weanling pups were exposed to *ad libitum* supply of 2% black tea alone in drinking water (Trivedi et al., 2006). Twenty grams of black tea solids (Lipton Yellow label, Unilever Limited, India) and 1000 ml boiled drinking water were used to produce a 2% tea solution.

Group (4) ameliorated group (Na-F+T), n=20: Weanling pups were exposed to *ad libitum* supply of 100 ppm Na-F in combination with 2% black tea solution.

2.3. Behavioural assessment:

All behavioural testing were conducted by the same personnel throughout, started at 90 days and ended at 106 days of animals' age.

2.3.1. Open field test

The open field has been long established as an appropriate test for measuring situational anxiety in rodents (Millan, 2003). The process of habituation, a form of non-associative learning, was also measured in the open field test (Mello e Souza et al., 2000; Chioca et al., 2008). The open field used was a square wooden arena measured (90 x 90 x 25cm). The wood of the apparatus is covered with a plastic laminate (formica), which prevents absorption of fluids (urine of rats). The floor was divided by black lines into 36 small squares (15 x 15cm). All testing was conducted between 09:00 and 15:00 h. All treatment groups were tested at the same day in a random array. Rats were gently placed into a corner of the arena and allowed to confront this novel aversive situation (Belzung, 1999) for 3 minutes.

During the three minutes test duration, assessment of anxiety included measuring time spent freezing (immobility), exploratory behaviours in the form of ambulation (horizontal locomotion) and rearing (vertical activity) as well as non-exploratory measures comprising only vegetative behaviours in the form of number of faecal boluses (defecation) and number of times of urination (Kalueff et al., 2006). These parameters have all been labeled measures for anxiety (Ivinskis, 1970, Archer, 1973).

Ambulation (horizontal locomotion) is assessed in relation to lines drawn on the floor (the number of squares crossed). A crossed square was defined as the rat placing its two forepaws in the next square and moving forward (Chioca et al., 2008), whereas rearing was defined as the number of times an animal stood erect on its hind legs with its fore legs in the air or leaning against the wall of the open field (Brown et al., 1999). Hand operated counters and stop watches were used to score the behaviour of animals.

After the 3 minutes test session, the rat was returned to its home cage and the open field was cleaned using 70% ethyl alcohol (to avoid odour cues) and permitted to dry between tests. To assess the process of habituation to the novelty of arena, rats were exposed to the apparatus for a 3 minutes test session, on three consecutive days.

2.3.2. Classic maze test

Associative learning was assessed using classic maze test. The base of the maze measured (100 x 100cm) with walls height of 25cm. The entire maze was made of plywood with a glass cover in order to prevent escape of animals and allow observation. Testing was carried out between 09:00 and 15:00 h, where all groups were randomly allowed for testing at the same day in a randomized order. Rats were deprived from feed for a 23 hours period before start of testing. Rats were given their daily feed amount as a reward at the end of the maze. Animals were given one trial per day for five consecutive days. Time elapsed to locate the feed at the end of the maze and numbers of entries of blind alleys (errors) were recorded according to Staddon (1983).

2.3.3. Spatial Y-maze memory

The test was conducted only once for all treatment groups randomly divided on two consecutive days, between 09:00 and 15:00 h. The Y-maze apparatus consisted of three arms (labeled A, B and C). Each arm was 40cm long, 30cm high, 15cm wide and positioned at an equal angle converged in an equilateral triangular central area with 15cm at its longest axis. The maze has no floor, but placed on a clean sheet of paper on a table-top, where the sheet has to be changed for each animal in order to prevent the use of odour cues in maze navigation. Each rat, was randomly placed at the end of one arm and allowed to move freely through the maze for eight minutes session. Entry was considered complete when the hind paws of the rat had completely entered the arm. The sequence of arm entries was recorded (i.e. ABCABABCACABACAB, etc.). A spontaneous alternation behaviour, a measure of spatial memory, was defined as consecutive entries into all three different arms without repetitions in overlapping triplet sets (Rasoolijazi et al., 2007). Percentage of alternation was calculated as the ratio of actual to all possible alternations (total number of arm entries minus two) multiplied by 100.

2.4. Biochemical and histopathological examination:

On completion of all behavioural assessments, five rats per treatment were sacrificed by cervical decapitation under ether anaesthesia for whole brain tissue extraction. Brain tissue specimens were dissected out carefully and cut into two sagital sections for execution of biochemical and histopathological examination.

2.4.1. Biochemical estimation

Brain tissue specimens were homogenized (10% w/v) in ice-cold 1.15% KCl-0.01M, sodium potassium phosphate buffer (pH 7.4) using a Teflon mechanical homogenizer. The homogenate was then centrifuged at 4000 rpm for 15 min at 4^{o}C and the supernatant was used for the studied enzymatic assay.

2.4.1.1. Determination of acetylcholinestrase (AChE) activity

AChE activity (µmoles/min/mg protein) was estimated in the brain by an improved Ellman's colrimetric method, employing acetylcholine iodide as a substrate for the reaction (Darreh-Shori et al., 2002).

2.4.1.2. Determination of super oxide dismutase (SOD) activity

SOD activity (µmoles/min/mg protein) was measured according to Giannopolitis and Ries (1977), by means of SOD assay kit (Cayman, MI, USA). The kit utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD was defined as the amount of enzyme needed to produce 50% dismutation of superoxide radical. The SOD assay measures all three types of SOD; Cu/Zn, Mn, and Fe SOD. Enzyme activity was determined as the amount of the enzyme required to induce 50% inhibition of nitro-blue tetrazolium (NBT) reduction rate.

2.4.1.3. Determination of thiobarbituric acid reactive substance (TBARS) formation

The level of lipid peroxidation, in terms of TBARS formation (nmoles/min/mg protein) was determined (Esterbauer and Cheeseman, 1990). Tissue supernatant was mixed with 1ml of 20% trichloroacetic acid (TCA), 2ml of 0.67% thiobarbituric acid (TBA) and then heated at 100°C for 1h. After cooling, the precipitate was removed by centrifugation. The absorbance of the sample was measured at 535nm using a blank containing all the reagents except the sample. Since, 99% of TBARS was malondialdehyde (MDA), so TBARS concentrations of the samples were calculated using the extinction co-efficient of MDA, which is $1.56\times105\ M^{-1}cm^{-1.}$

2.4.1.4. Estimation of total protein

Total protein content (mg/100 mg fresh tissue weight) was identified in brain tissue homogenate as described by Lowry et al. (1951).

2.4.1.5. Estimation of lipids

Lipids were extracted from brain tissues using a mixture of (2:1 v/v) chloroform-methanol (Folch et al., 1957) and the contents were expressed as mg/100mg tissue.

Total cholesterol was estimated by the method of Zlatkis et al. (1953), where 9.9ml of ferric chloride-acetic acid reagent was added to 0.1ml of lipid extract, allowed to stand for 15 min, centrifuged and the supernatant fluid was collected. 3ml of conc. H_2SO_4 were then added to 5ml of the collected supernatant fluid. The colour developed was red after 20 min at 560nm against a reagent blank and values were expressed as mg/100mg tissue.

Triglycerides were estimated by the method of Foster and Dunn (1973). An aliquot of lipid extract was evaporated till dryness in glass tubes, 0.1ml methanol was then added followed by 4ml isopropranol and 0.4g alumina and centrifuged at 3000rpm for 15 min. 2ml of the supernatant fluid was transferred to labeled tubes to which 0.6ml of a saponification reagent was added, and then placed in a water bath at 65°c for 15 min for saponificatioin. 0.5ml of acetyl acetone reagent was added, mixed and the tubes were kept in a water bath at 65°c for 1h. The contents were then cooled and the absorbance was red at 420nm. The triglyceride content was expressed as mg/100mg tissue.

Low-Density Lipoprotein-Cholesterol (LDL-c) was calculated using Friedewald formula (Friedewald et al., 1972), based on the assumption that LDL-c is present in a concentration equal to one-fifth of the triglycerides. Values are expressed as mg/100mg tissue.

2.4.2. Neurohistopathology

The dissected brain tissue specimens were fixed in 10% neutral buffer formalin, processed by paraffin embedding method, sectioned at 4-5µm and stained with Hematoxylin and Eosin stain (Bancroft et al., 1996). Stained sections were fixed on slides, and lesions were then confirmed by microscopic examination.

2.5. Statistical analysis

Data analysis for all variables were carried out by means of analyses of variance (ANOVA) to judge the effect of administration of Na-F, black tea to rats as well as session factor for behavioural tests using the general linear models procedure in SPSS® statistical software (SPSS, 2006). After confirmation of significant effects in the overall ANOVA, data for different groups were compared using post hoc Tukey HSD test. For all tests, the criterion for statistical significance was $p<0.05$. Results are reported as mean ± SEM.

3. Results

3.1. Open field test:

Anxiety state of the animals was assessed on first occurrence in the open field test as shown in Table 1. Administration of Na-F to rats produced an anxiogenic profile of behavioural changes as indicated by increased time spent freezing ($F_{(1, 36)} = 19.35$; $p = 0.00$), higher vertical activity (numbers of rearing; ($F_{(1, 36)} = 36.16$; $p = 0.00$) as well as elevated defecation scores ($F_{(1, 36)} = 21.92$; $p = 0.00$). Also,

rats administered black tea solution alone displayed higher levels of anxiety-related behaviours than others in the control group. However, the level of enhancement noted for anxiety measurements was significantly different in individuals of Na-F group compared to counterparts in T group. On the other hand, our treatments had no significant influence on horizontal activity (numbers of crossed squares) as well as urination scores. Furthermore, administration of black tea along with Na-F significantly reduced levels of Na-F-induced anxiety. Similar levels of anxiety were recorded for rats in the ameliorated group and those in T group.

A significant degree of formation of memory of habituation over three sessions in the open field test was noted regarding time spent freezing ($F_{(2, 117)} = 11.61$; $p = 0.00$), horizontal activities (numbers of squares, ($F_{(2, 117)} = 27.16$; $p = 0.00$), vertical activity (numbers of rearing, ($F_{(2, 117)} = 11.34$; $p = 0.00$) and defecation scores ($F_{(2, 135)} = 9.57$; $p = 0.00$) for all treatments but not for Na-F group. In the later group, an equivalent assessment were significantly noted in the first day of induction compared to second and third test sessions ($p = 0.91$ and 0.08), ($p = 0.78$ and 0.09), ($p = 0.66$ and 0.16), ($p = 0.62$ and 0.27) for all previous parameters, respectively.

3.2. Maze test:

The ability of learning and memory of animals over the five days of maze test was presented in Table 2. Administration of Na-F to rats significantly increased both latency to locate the feed at the end of the maze ($F_{(1, 180)} = 122.87$; $p = 0.00$) and frequencies to enter the blind alleys ($F_{(1, 180)} = 127.82$; $p = 0.00$) compared to control group. In contrast, learning and memory were superior in group of rats exposed to black tea solution compared to the control group, as shown by reduced latency to end the maze ($F_{(1, 180)} = 62.83$; $p = 0.00$) accompanied with less numbers of errors (numbers of entries for blind alleys, ($F_{(1, 180)} = 90.19$; $p = 0.00$). Moreover, Administration of black tea to Na-F-treated rats significantly improved the parameters studied to the level of control group.

On testing the retention of the task over five days of maze test, rats in all treatments required steadily less time to terminate the maze ($F_{(4, 180)} = 16.46$; $p = 0.00$) with reduced numbers of errors ($F_{(4, 180)} = 21.01$; $p = 0.00$), except for Na-F-treated rats. Concerning Na-F group, values for latency as well as numbers of errors weren't significantly different during acquisition on first day when compared to their retention values on the four consecutive days of testing ($p = 1.00$, 1.00, 1.00 and 0.97), ($p = 1.00$, 0.61, 0.33, and 0.33), respectively, indicating poorer memory retention in Na-F treated rats compared to all other treatments.

3.3. Spatial Y-maze memory:

Spatial Y-maze performance was illustrated in Table 3. Spatial memory on the Y-maze was seriously impaired in rats administered Na-F as indicated by reduced percentages of spontaneous alternation behaviour in comparison with rats in all other treatments ($F_{(1, 36)} = 49.31$; $p = 0.00$). On the other hand, rats supplemented with black tea solution significantly improved spatial cognition learning ability than controls ($F_{(1, 36)} = 23.08$; $p = 0.00$). Furthermore, administration of black tea significantly alleviated Na-F-induced spatial memory impairment, where rats in the ameliorated group exhibited as better performance in the Y-maze test as their counterparts in the control group.

3.4. Biochemical analysis:

3.4.1. Brain acetylcholinesterase (AChE) activity:

The activity of AChE in the brain of rats was demonstrated in Table 4. Na-F-treated rats have shown a significant reduction in AChE activity compared to their counterparts in the control group. Administration of black tea has no significant influence on AChE level in the fluoride group. No significant differences were detected between rats in tea group and those in the control one.

3.4.2. Oxidative stress parameters:

SOD activity, TBARs formation as well as total protein contents in the brain tissues of rats were presented in table 5. SOD activity was significantly lowered in Na-F-exposed rats compared to Na-F-free rats. Significant alleviation of this reduction was recorded when black tea was concomitantly administered. Black tea alone significantly enhanced SOD activity in rats compared to the control group.

The Level of TBARs formation was significantly higher in case of Na-F-treated rats. This elevation was significantly diminished by administration of black tea. Compared to control rats, treatment with black tea alone had no significant influence on brain tissue TBARs level .

Total protein content was significantly decreased in brain tissues of rats administered Na-F when compared to those in the control group. The later reduction was significantly improved when black tea was concomitantly administered. The observed increase in total protein contents in black tea-treated rats, compared to control one, was not statistically significant.

3.4.3. Lipid profile:

Total cholesterol (TC), triglycerides (TG) and low density lipoproteins cholesterol (LDL-c) were significantly increased when Na-F was administered to rats (Table 6). Administration of black tea significantly lightened this noted increase in Na-F-exposed rats. Rats administered black tea alone have revealed a significant decrease in lipid profile parameters when compared to their counterparts in the control group.

3.5. Histopathological examination:

No histopathological changes could be detected in the brain of rats in both of the control and tea-treated groups.

Examination of fluoride-treated rats revealed severe pathological alterations as evidenced by congestion of the meningeal, cerebral and cerebellum blood capillaries, in addition to congestion of choroid plexus in the ventricle (Fig. 1). Large hemorrhagic areas were also detected in the cerebral cortex, cerebellum white matter as well as in the ventricles around choroid plexuses (Fig. 2). Neurodegenerative changes were noticed in nerve cells especially at hippocampus and large nerve cells of cerebral cortex, as represented by the accumulation of neurofilaments in the cytoplasm of nerve cells and axons (Fig. 3).

In addition, nerve cells of cerebral cortex revealed sever edema, central chermatolysis, atrophy, necrosis and neuronophagia (Fig. 4), whereas the pyramidal cells of Ammon's horn of hippocampus showed atrophy and necrosis (Fig. 5). Either focal or diffused Gliosis were noticed in the cerebral cortex with severe demylination of the nerve fibers in the neuropil shown in the cerebrum, and accompanied with axonal swelling (Fig. 6). Glial fibers was detected under the ependymal cells lined the ventricles. The cerebellum showed necrosis of Purkinje cells and edema with necrosis in the granular cell layer.

Concomitant administration of tea to fluoride-intoxicated rats resulted in mild to moderate pathological changes compared to their counterparts exposed to fluoride alone. Small hemorrhagic areas were noticed in the ameliorated group, especially in the ventricle around the choroid plexuses (Fig. 7). Only edema was detected in the large nerve cells of the cerebral cortex (Fig. 8). Few numbers of large pyramidal cells of hippocampus appeared slightly atrophied (Fig. 9). Focal glial cells and fibers were detected in few numbers of the ameliorated examined cases (Fig. 10).

Table 1. Effect of Na-F and its amelioration by black tea on anxiety measurements on first occurrence in open field test in rats.

	Experimental Groups			
	(C) Group	**(Na-F) Group**	**(T) Group**	**(Na-F+T) Group**
Freezing time(s)	**2.00±0.37[a]**	**7.30±3.82[b]**	**4.50±3.09[c]**	**4.70±3.09[c]**
Horizontal activity	**50.3±3.93[a]**	**55.8±1.26[a]**	**46.9±4.44[a]**	**51.8±3.14[a]**
Vertical activity	**6.80±7.36[a]**	**15.80±3.82[b]**	**10.20±3.09[c]**	**11.60±3.09[c]**
Defecation scores	**0.50±7.36[a]**	**5.40±3.82[b]**	**2.70±3.09[c]**	**3.00±3.09[c]**
Urination scores	**0.2±0.13[a]**	**0.6±0.22[a]**	**0.4±0.16[a]**	**0.5±0.22[a]**

(C) Group: Animals received plain water without any treatment and served as a control.
(Na-F) Group: Animals received 100 ppm Na-F.
(T) Group: Animals received 1% black tea solution alone.
(Na-F+T) Group: Animals received 100 ppm Na-F + 1% black tea solution.
[a-c]Values within row with unlike superscripts differ significantly **($p<0.05$)**, according to ANOVA.
Values represent mean±SEM of 10 animals per treatment.

Table 2. Effect of Na-F and its amelioration by black tea on measurements of maze test over the course of five days in rats.

	Experimental Groups			
	(C) Group	**(Na-F) Group**	**(T) Group**	**(Na-F+T) Group**
Latency (s)	**87.48±21.25[a]**	**215.38±7.91[b]**	**49.1±18.79[c]**	**115.1±24.05[a]**
No. of entries of blind alleys	**2.32±0.56[a]**	**6.38±0.44[b]**	**1.32±0.52[c]**	**2.76±0.74[a]**

(C) Group: Animals received plain water without any treatment and served as a control.
(Na-F) Group: Animals received 100 ppm Na-F.
(T) Group: Animals received 1% black tea solution alone.
(Na-F+T) Group: Animals received 100 ppm Na-F + 1% black tea solution.
[a-c]Values within row with unlike superscripts differ significantly **($p<0.05$)**, according to ANOVA.
Values represent mean±SEM of 50 animals per treatment.

Table 3. Effect of Na-F and its amelioration by black tea on spatial Y-maze memory in rats.

	Experimental Groups			
	(C) Group	**(Na-F) Group**	**(T) Group**	**(Na-F+T) Group**
Spontaneous alternation behaviour (%)	**71.08±4.07[a]**	**23.10±9.22[b]**	**95.00±4.48[c]**	**57.59±5.13[a]**

(C) Group: Animals received plain water without any treatment and served as a control.
(Na-F) Group: Animals received 100 ppm Na-F.
(T) Group: Animals received 1% black tea solution alone.
(Na-F+T) Group: Animals received 100 ppm Na-F + 1% black tea solution.
[a-c]Values within row with unlike superscripts differ significantly **($p<0.05$)**, according to ANOVA.
Values represent mean±SEM of 10 animals per treatment.

Table 4. Effect of Na-F and its amelioration by black tea on the level of AChE activity in the brain of rats.

	Experimental Groups			
	(C) Group	**(Na-F) Group**	**(T) Group**	**(Na-F+T) Group**
AchE (µmoles/min/mg protein)	**0.18±0.01[a]**	**0.10±0.01[b]**	**0.18±0.01[a]**	**0.11±0.01[b]**

(C) Group: Animals received plain water without any treatment and served as a control.
(Na-F) Group: Animals received 100 ppm Na-F. (T) Group: Animals received 1% black tea solution alone.
(Na-F+T) Group: Animals received 100 ppm Na-F + 1% black tea solution.
[a-c]Values within row with unlike superscripts differ significantly **($p<0.05$)**, according to ANOVA.
Values represent mean±SEM of 5 animals per treatment.

Table 5. Effect of Na-F and its amelioration by black tea on various parameters of oxidative stress in the brain of rats.

	Experimental Groups			
	(C) Group	**(Na-F) Group**	**(T) Group**	**(Na-F+T) Group**
SOD (µmoles/min/mg protein)	**0.07±0.01[a]**	**0.02±0.00[b]**	**0.09±0.01[c]**	**0.04±0.01[d]**
TBARs (nmoles/min/mg protein)	**0.10±0.01[a]**	**0.18±0.01[b]**	**0.10±0.01[a]**	**0.15±0.01[c]**
Total protein (mg%)	**71.20±6.31[a]**	**43.15±4.68[b]**	**73.28±7.00[a]**	**56.28±4.27[c]**

(C) Group: Animals received plain water without any treatment and served as a control.
(Na-F) Group: Animals received 100 ppm Na-F.
(T) Group: Animals received 1% black tea solution alone.
(Na-F+T) Group: Animals received 100 ppm Na-F + 1% black tea solution.
[a-c]Values within row with unlike superscripts differ significantly **($p<0.05$)**, according to ANOVA.
Values represent mean±SEM of 5 animals per treatment.

Table 6. Effect of Na-F and its amelioration by black tea on lipid profile in the brain of rats.

	Experimental Groups			
	(C) Group	(Na-F) Group	(T) Group	(Na-F+T) Group
TC (mg/100mg tissue)	1038.59±59.25[a]	1582.47±70.14[b]	823.18±55.47[c]	1283.55±67.22[d]
TG (mg/100mg tissue)	293.18±13.94[a]	474.37±15.22[b]	242.08±12.83[c]	315.92±15.47[a]
LDL-c (mg/100mg tissue)	58.64 ±2.31[a]	74.88±3.64[b]	48.42±2.56[c]	63.18±2.32[a]

(C) Group: Animals received plain water without any treatment and served as a control.
(Na-F) Group: Animals received 100 ppm Na-F.
(T) Group: Animals received 1% black tea solution alone.
(Na-F+T) Group: Animals received 100 ppm Na-F + 1% black tea solution.
[a-c]Values within row with unlike superscripts differ significantly **($p<0.05$)**, according to ANOVA.
Values represent mean±SEM of 5 animals per treatment.

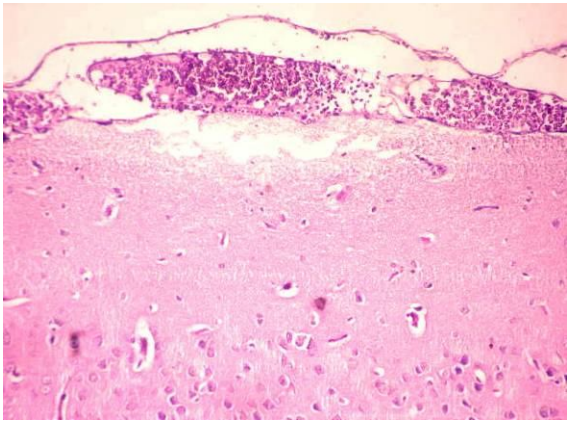


Figure 1: Brain of fluoride-treated rats showing congestion of meningeal blood vessels. H&E X 200.

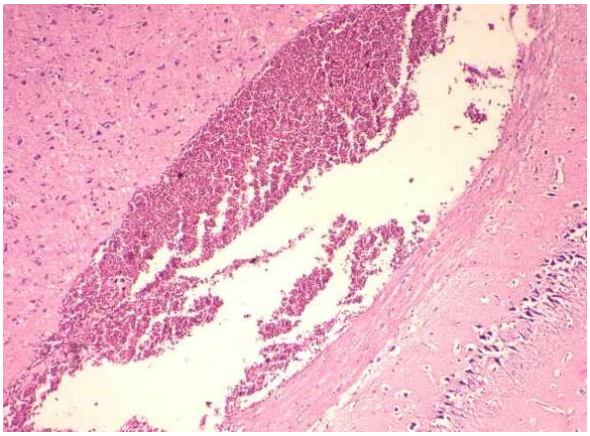


Figure 2: Brain of fluoride-treated rats showing large area of hemorrhage. H&E X 200.

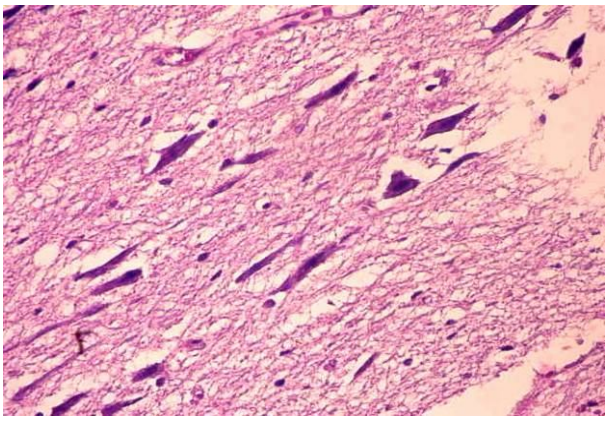


Figure 3: Brain of fluoride-treated rats showing neurofilaments accumulation in the cytoplasm of nerve cells and axons. H&E X 200

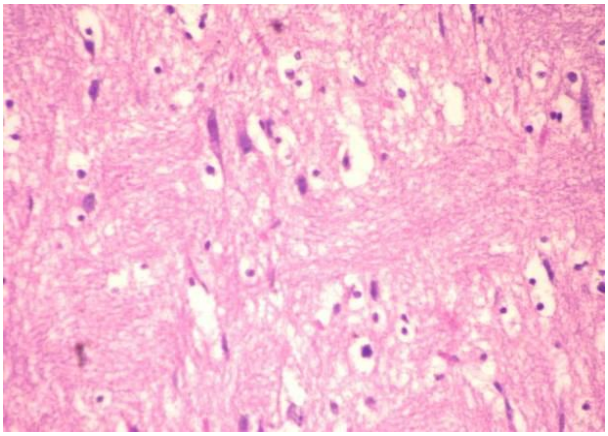


Figure 4: Brain of fluoride-treated rats showing cellular edema, atrophy and necrosis. H&E X 400.

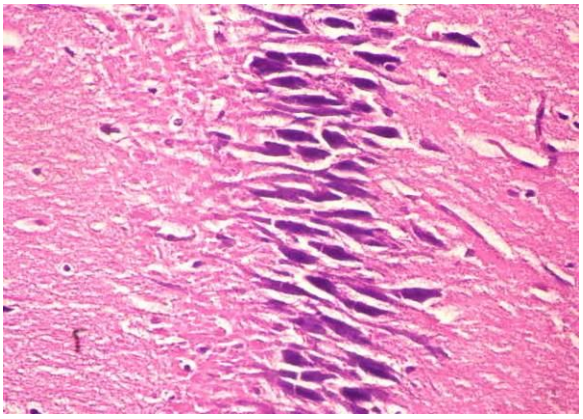


Figure 5: Brain of fluoride-treated rats showing atrophy and necrosis of pyramidal cells of Ammon's horn of hippocampus. H&E X 200.

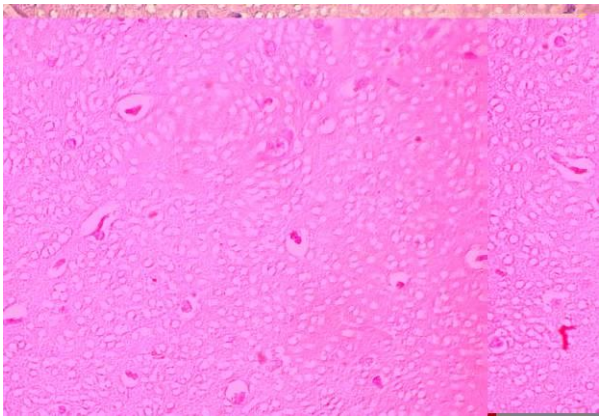


Figure 6: Brain of fluoride-treated rats showing demyelination of nerve fibers. H&E X 100.

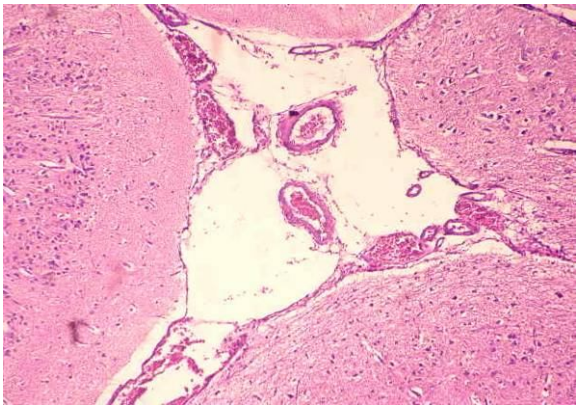


Figure 7: Brain of fluoride-treated rats concomitantly administered black tea showing few numbers of free RBC's around chroid plexuses. H&E X 200.

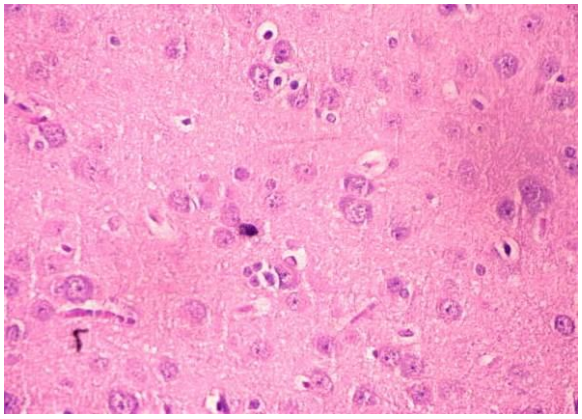


Figure 8: Brain of fluoride-treated rats concomitantly administered black tea showing edema in few numbers of large nerve cells of cerebrum. H&E X 400.

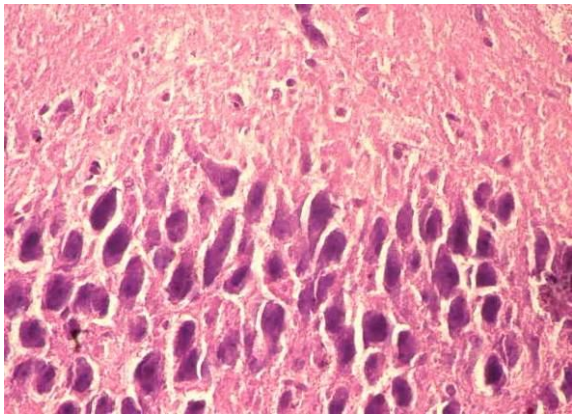


Figure 9: Brain of fluoride-treated rats concomitantly administered black tea showing atrophy of few numbers of pyramidal cells of hippocampus. H&E X 200.

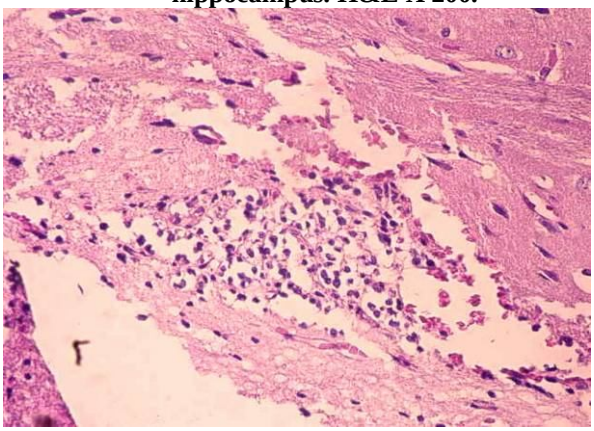


Figure 10: Brain of fluoride-treated rats concomitantly administered black tea showing glial cells and glial fibers in the cerebral cortex. H&E X 200.

4. Discussion:

For assessment of emotionality, the open field is one of the simplest and most popular tests currently in use (Weiss and Greenberg, 1996; Brain & Marrow; 1999; Crawley, 1999). The test is employed to assess anxiety-related conflict arising between the drive to explore by venturing into the center of the arena and safety by remaining in a corner or along a wall (Weisstaub et al., 2006).

To the best of our knowledge, few researches have implemented open field test to investigate Na-F influence on anxiety levels in rats. In compliance with our earlier study, rats exposed to Na-F spent more time freezing on the first exposure to open field test (El-lethey et al., 2010). Increased immobility in the open field is characteristic for increased levels of anxiety (Homberg et al., 2002; Fromm et al., 2004; Kalueff & Tuohimaa, 2004). Unlike horizontal locomotion, higher levels of vertical activity were detected in Na-F-treated rats. This finding supports the fact that vertical exploration is more sensitive to anxiety than horizontal locomtion (Lapin et al., 1995). Although the locality of the activities were not recorded here, the enhancement in vertical activity at first exposure might be on account of novelty-provoked more fear-related behaviour in rats such as activity in the corners and walls of the open field (Choleris et al., 2001). Moreover, rearing at the perimeter of the enclosure has been evidenced to indicate anxiety and an animal's attempts to escape; whereas rearing in the inner arena may be more indicative for curiosity, namely exploration of the upper parts of the apparatus (Anderson & Hughes, 2008). Although urination scores were not influenced by Na-F, our results revealed a profound increase in faecal boluses deposited in the field. These data were in agreement with Ivinskis (1968) who suggested that defecation is a more reliable measure than urination to assess emotionality in the open field. Also, Brain & Marrow (1999) have confirmed defection to be a satisfactory measure for anxiety, with increased defecation indicating higher anxiety. Taken together, these reports and the results presented here suggest a Na-F-induced increase of anxiety-like behaviour.

Where the decrement in exploratory response to successive exposure to a novel environment is taken as an index for memory of habituation, a non-associative learning task (Bolivar et al. 2000**;** Izquierdo et al., 2001; Winogard & Viola, 2004), an impairment in habituation was shown in Na-F-treated rats. Consistent with our finding, a decrement in habituation signifying learning and memory impairment was previously shown in rats exposed to 100 ppm Na-F, the identical concentration employed in the current study (Chioca et al., 2008; Pereira et al., 2009; El-lethey et al., 2010).

Tea consumption in many cases is the main source of caffeine (Haider et al., 1998). Caffeine content in black tea is greater than that in a green one, 3.86% versus 2.04%, respectively (Komes et al., 2009). The anxiogenic effect of caffeine has been documented in a substantial literature (Brice & Smith, 2002; Botella & Parra, 2003). Caffeine has been reported to elicit a dose-dependent, subjective feeling of anxiety, even at low doses (Kaplan et al., 1997). The stimulation of anxiety in response to caffeine may be as a result of increased levels of lactate in the brain (Tancer et al., 1991, 1994). Here, the enhancing effect of tea on rearing activity might be explainable in terms of caffeine-induced anxiety in a novel environment. In agreement with our findings, increased activity of animals was experienced after administration of low or moderate doses of caffeine (Buckholtz and Middaugh, 1987). Moreover, Haleem et al. (1994) have observed an increase in both home cage and open field activity in rats following low doses of caffeine. Enhancement of activity was not entirely due to caffeine per se, where presence of theophylline, another alkaloid present in tea, might be also attributable for promoting locomotor activity (Haider et al., 1998). In agreement with our data, no change on numbers of squares crossed in the open field was reported after four weeks of tea administration in rats (Haider et al., 1998). The stress effect of novelty on exposure to open field could suppress locomotor enhancing effects of stimulants present in tea, resulting in lack of effect on horizontal locomotion in our study. In addition, partial tolerance to the locomotor-enhancing effects of caffeine has been evidenced to develop following administration of high doses of caffeine for about a week resulting in lack of effect on locomotion (Haleem et al. (1994). Administration of tea to Na-F-treated rats brought about recovery in anxiety parameters almost to tea administration level alone, suggesting the potential ameliorative effect of tea on Na-F-induced symptoms of anxiety.

Although associative processes have been evidenced to play a real role in spatial learning, almost no studies have employed classic maze to address spatial memory (Leising, 2009). The animal must build a cohesive spatial representation of the maze to end with the food. On repeating the maze experiment several times, the changes in latency, and errors made to reach the food are indicators for learning and memory abilities of the rat. In the present study, the defective ability of Na-F-treated rats to navigate the maze for food reinforcement with higher errors on successive days of testing signifying inferior memory retention compared with other

treatments. Consistent with these findings, impairment in the learning capacity during classic maze was formerly experienced in Na-F-exposed rats (El-lethey et al., 2010). Bhatnagar et al. (2002) and Gao et al. (2009) have also reported poor performance of fluoride-intoxicated animals during maze test with increased inability to perform well with higher fluoride concentration in drinking water.

The alternation behavior observed during spatial Y-maze memory testing has been motivated by the detection of novelty, where animals are attracted to a stimulus that is novel relative to previous stimuli experienced, being a sign for the degree of learning ability in rats (Gaffan and Davies, 1982). Confirming the results of classic maze, more evidences for impaired cognitive capacity were derived from the data for Y-maze test, where Na-F provoked a remarkable hindrance in spatial memory of rats as indicated by a notable decrease in spontaneous alteration behaviour. Our findings are in accordance with previous reports from Niu et al. (2008) that have demonstrated a spatial memory deficit in rats during Y-maze after Na-F exposure.

Consumption of flavonoid-rich foods, such as tea that makes a significant contribution to dietary intake of flavonoids by 82% (Hertog et al., 1997), throughout life holds a potential to limit the neurodegeneration associated with a variety of neurological disorders and to prevent or reverse normal or abnormal deteriorations in cognitive performance. Interestingly, there is evidence to suggest that tea polyphenols can localize within the brain following dietary consumption and thus be available to promote actions resulting in cognitive improvements (Matsuoka et al., 1995; Suganuma et al., 1998). Our results are in line with these earlier reports, where administration of black tea has greatly improved cognitive performance during the two tasks applied here for evaluating spatial memory. Also, the observed attenuation in cognitive capacity in Na-F-exposed rats has been blunted to normal level when black tea was concomitantly administered. Accordingly, the potential cognitive protective effect of tea has been reported in various experimental animal models (Kim et al., 2004; Unno et al., 2004). Long-term administration of green tea catechins has been reported to improve the performance in radial maze tasks (Haque et al., 2006). Likewise, the effectiveness of tea extract in enhancing learning and memory, and hence, reversing age-related deficits in aged rats has been proved, as signified in performance in elevated maze and passive avoidance tests (Kaur et al., 2008).

The neuroprotective effect of dietary flavonoids involves a number of actions within the brain, including a potential to protect neurons against neurotoxins-induced injury, an ability to suppress neuroinflamation, and to promote memory, learning and cognitive function (Spencer, 2009). This multiplicity of effects appears to be attained by two processes. Firstly, flavonoids interact with important neuronal signaling cascades leading to an inhibition of neurotoxins-triggered apoptosis, as well as a promotion of neuronal survival and differentiation. Secondly, they induce peripheral and cerebral vascular blood flow in a manner leading to induction of angiogenesis, and new nerve cell growth in the hippocampus.

Complex mechanisms are underlying the neuroprotective effect of tea.

As regards tea catechins, possible mechanisms might involve their antioxidant and iron-chelating properties, as well as modulation of cell-signaling and cell survival pathways (Mandel and Youdim, 2004; Weinreb et al., 2004). As for black tea, since catechins undergo enzymatic transformation during fermentation, their amount is generally lower than in green tea (Luczaj and Skrzydlewska, 2005; Unno and Hoshino, 2007). Nonetheless, the conversion of catechins to theaflavins and thearubigins during fermentation does not significantly alter their free radical-scavenging activity, and both black tea and its components possess strong antioxidative properties (Leung et al., 2001; Henning et al., 2004)**.** In addition to tea polyphenols, theanine, an amino acid uniquely found in tea leaf, might also possess neuroprotective effect and enhance the positive cognitive effects (Nathan et al., 2006; Haskell et al., 2008)**.** Because tea leaf contains various other phytochemicals, including vitamin C, it is likely that the cognitive protective effect of tea is not due to a single compound but rather to the synergistic effect of many of its chemical components (Tze-Pin et al., 2008).

Acetylcholine is an important neurotransmitter present at cholinergic nerve terminal and plays a key role in cholinergic neurotransmission (Garcia-Ayllon et al., 2006). A tight correlation has been established between acetylcholinestrase (AChE) activity and cognition. Estimation of AChE, a substrate specific enzyme degrading neurotransmitter acetylcholine in nerve synapses, provides an easy and valuable assessment of cholinergic function. 90% of AChE in the brain is membrane bound and only 10% is in a soluble form (Atack et al., 1986; Mortensen et al., 1998). Since, estimation of the soluble form of AChE is a relatively simple and sufficiently reliable indicator of relative changes of AChE activity in the brain (Muller et al., 1985; Zakut et al., 1985), quantifying the soluble form of this enzyme was only considered in our study.

Here, the marked reduction in AChE activity experienced in brain tissue of Na-F-treated rats is in

agreement with previous findings in rats (Wang et al., 2004; Wu et al., 2006). Further studies have reported a significant decline in brain AChE activity after excessive intake of Na-F in mice (Vani and Reddy, 2000; Bhatnagar et al., 2006).

The observed inhibition of AChE might be attributable to the loss of neuron cell bodies in the hippocampus as well as loss of synaptic structures (Bhatnagar et al., 2003; Ge et al., 2005). Anion hydrolysis products of methyl phosphoric difluoride were also implicated to cause an inhibition of AChE in both rats and guinea pigs (Dahl et al., 1987). Toxic inhibition of the AChE will cause high concentration of acetylcholine to accumulate in the body, whereas excessive induction of the acetylcholine will lead to down-regulatory effects through more hydrolysis of the acetylcholine into acetate and choline in order to reduce the concentration of acetylcholine in the body and counteract excess acetylcholinergic activity (Teh et al., 2010). Therefore, the diminution in AChE level may lead to altered utilization of acetylcholine, thus interfering with the synaptic transmission in brain, being accountable for cognitive dysfunctions observed in our study (Wang et al., 2004).

Contrary to our findings, an elevated level of AChE activity was detected with severe brain tissue damage accompanied by high fluoride intake in rats and mice (Chen and Bai 1995; Sun et al., 2000). This contradiction in Na-F effect on AChE activity might be due to the exposed level of Na-F, where the inhibited activity of AChE activity is mostly induced with moderate fluorosis, whereas severe fluorosis may exert a stimulatory effect on the activity of AChE (Gao et al., 2009).

There are numerous reports about inhibitory effect of antioxidants, namely polyphenols, on AChE acivity. Kim et al. (2004) showed that tea polyphenols exhibit a dramatic inhibitory effect on AChE activity and might be useful in the treatment of Alzheimer's disease (AD). Additionally, Kulisic-Bilusic et al. (2008) reported about high inhibitory activity of aqueous tea infusions on AChE activity. In contrast to these earlier findings, there was a lack of influence of black tea on brain tissue AChE activity in our study. This discrepancy might be on account of different polyphenols production as a result of different degrees of fermentation during tea manufacturing as well as variation in place of origin.

The brain and nervous system are prone to oxidative stress, and also inadequately equipped with an antioxidant defense system to prevent ongoing oxidative damage (Halliwell, 2006). In addition, the brain consumes large quantities of oxygen that contributes to the formation of reactive oxygen species (ROS). In the current study, rats exposed to Na-F showed a significant reduction in the activity of antioxidant enzyme (SOD) and a concomitant enhancement in lipid peroxidation (TBARS), in accordance with earlier reports in brain tissues of fluoridated rats and mice (Mullenix et al., 1995; Vani and Reddy, 2000; Shivarajashankara et al., 2001; Bhatnagar et al., 2006; Chirumari and Reddy, 2007; Bharti and Srivastava, 2009). These detected alterations suggest Na-F-induced-oxidative stress in the brain thereby disturbing the antioxidant defense, in compliance with earlier findings (Chlubek, 2003; Inkielewicz and Krechniak, 2004). Increased oxidative stress could therefore be one of the mediating factors in the pathogenesis of Na-F-induced neurotoxicity experienced in the present work.

Tea polyphenols can pass through the brain-blood barrier to exert antioxidant activity and potent neuroprotective effects (Nie et al., 2002). One of the possible mechanisms may be their directly scavenging ROS produced either outside or inside the cell or both. Earlier data have shown that tea polyphenols can scavenge different kinds of ROS and organic free radicals, for example, superoxide anion, hydroxyl radical, singlet oxygen, and lipid free radicals (Zhao et al., 1989; Guo et al., 1996; Guo et al., 1999). Here, the elevated level of free radical enzyme (SOD) with lowered level of lipid peroxidation when black tea was administered to Na-F treated rats suggests the role of black tea in amelioration of Na-F-induced oxidative stress. This ameliorative effect of black tea may be due to the presence of monomeric catechins that affect plasma antioxidant biomarkers and energy metabolism (Williamson and Manach, 2005; Trivedi et al., 2006; Verma et al., 2006). Moreover, it has been reported that quercetin, a unique flavanol present in black tea extract, can reduce free radicals (Pietri et al., 1997). Polyphenols are also well known for their ability to reduce membrane lipid peroxidation that can prevent Na-F-induced-oxidative damage. Further support derived from former studies, where oral administration of a flavonoid-rich tea extract prevented iron-salt-induced lipid peroxide accumulation and suppressed age-related accumulation of neurotoxic lipid peroxides in rat brain (Inanami et al., 1995; Yoneda et al., 1995).

Brain protein is important to maintain normal brain physiological function and learning-memory ability (Liu et al., 1999). In the present study, protein contents have been shown to be reduced in the brains of rats subsequent to fluoride exposure. Decreased protein contents have been formerly reported in the brain of rats treated with Na-F (Wang et al., 2004; Wu et al., 2006). The Na-F-induced reduction in brain protein contents might be as a result of either increased proteolysis or decreased

rate of cellular protein synthesis through impairment of peptide chain initiation (Trivedi et al., 2007). Moreover, fluoride has been evidenced to inhibit oxidative decarboxylation of branched chain amino acids and simultaneously promote protein breakdown. Reduced activities of glutamine synthetase that catalyzing certain stages of amino acid biosynthesis as well as methionine activating enzymes of the liver have been also implicated in fluorosis-induced disturbance in protein synthesizing system (Zahvaronkov and Strochkova, 1981).

Brain triglycerides levels as well as cholesterol content were highly elevated in rats exposed to Na-F in the present study. In a study on rabbits, fluoride induced alterations in brain lipid metabolism similar to lipidosis, a disorder of lipid metabolism leading to abnormal fat accumulation in the body tissues, particularly in the liver and brain (Shashi, 1992). Fluoride intoxication-induced hyperlipidemia might occur due to enzymatic defect and inability of brain to degrade the lipid in the body. Furthermore, as lipids are transported in association with a carrier protein, a defect in lipoprotein metabolism as evidenced by reduced LDL-c in the current study might be on account for hyperlipidemia. Deficiency of a lipotropic agent has been evidenced to cause triglycerides to accumulate, leading to a decrease in free fatty acids synthesis during fluoride intoxication (Shashi, 1988; Shashi et al., 1989). Further evidence derived from a study for Trivedi et al. (2009) where accumulation of cholesterol and total lipids was reported in Na-F administered mice. Here, the observed hypercholesterolemia may be due to Na-F-induced deficiency of liposomal lipase which hydrolyzes cholesterol esters taken up by the cell, reducing the release of free fatty acids and glycerol as well as enhancing lipogenesis. (Shashi, 1992). The hypercholesterolemia and hypertriglyceridemia have been reported in previous studies to indicate excessive fat immobilization (Vatassery et al., 1980; Shashi et al., 1989). In our study, the alteration in lipid metabolism might also be due to increase in lipid peroxidation activity (Shivarajashankara et al., 2001).

In line with findings of Trivedi et al. (2009), amelioration of fluoride-induced rise in brain triglycerides and cholesterol contents by administration of black tea was recorded in our study. Black tea has been evidenced to inhibit the synthesis of cholesterol and decrease its concentration in the brain (Maron et al., 2003). Since the antilipogenic effect of black tea might be due to its radical scavenging activity, the alleviation of Na-F-induced alteration in brain by black tea might be due to its potent antioxidative properties (Katiyar and Mukhtar, 1997; Du Toit et al., 2001).

Given the crucial involvement of hippocampal activity in many aspects of learning and memory (Strack et al., 2000; Leussis & Bolivar, 2006), and since fluoride is known to accumulate in various parts of rat brain, especially in the hippocampus (Burgstahler and Colquhoun, 1996), detection of neurotoxic alterations in the hippocampus was monitored in the present study. Here, the demonstrated histopathological changes triggered by fluoride intoxication in the brain, especially hippocampus and cerebrum regions, are attributed to neurotoxic effect of fluoride. Previous studies have evidenced that accumulation of fluoride in the hippocampus generates neuronal dysfunction by mechanisms involving decreased aerobic metabolism with elevated levels of free radicals (Spittle, 1998; Chirumari and Reddy, 2007). Fluoride binds to antioxidants in the body such as N-acetyl cysteine and glutathione and other free-radical destroying enzymes, thereby disturbing the antioxidant defense, triggering oxidative stress that initiates nerve cell damage especially cell membrane and even cell apoptosis (Anuradha et al., 2001; Gao et al., 2009).

In the current study, presence of neurofilament in the nerve cells might be attributable to the interference of fluoride with various steps of protein synthesis inside nerve cells (Miu et al., 2003). Fluoride may also accumulate in both neurons and astrocytes, resulting in strong morphological changes such as clustering, degeneration and finally death (Mullenix et al., 1995).

In our study, the ameliorative effect of black tea on Na-F-induced histopathological alterations in the brain of rats could be explained on the basis of flavanoids-exhibited antioxidant activity via radical scavenging capacity (Trivedi et al., 2007).

In conclusion, our study demonstrated a potential effect of black tea drinking in protection against Na-F intoxication-induced cognitive decline in rats. Since, black tea is cheap, non toxic, and widely consumed, thus its regular consumption is greatly recommended in order to promote cognitive health and lessen the risk of cognitive impairment elicited by neurotoxins, particularly fluorides.

Corresponding author
Mervat M. Kamel
Department of Animal Hygiene and Management, Faculty of veterinary Medicine, Cairo University, Cairo, Egypt
mevy58@yahoo.com

5. References:

1. Anderson, N., Hughes, R. (2008): Increased emotional reactivity in rats following exposure to caffeine during adolescence. Neurotoxicol Teratol, 30: 195-201.
2. Anuradha, C., Kanno, S., Hirano, S. (2001): Oxidative damage to mitochondria is a preliminary step to caspase-3 activation in fluoride-induced apoptosis in HL-60 cells. Free Radic Biol Med, 31: 367-373.
3. Archer, J. (1973): Tests for emotionality in rats and mice: A review. Animal Behav, 21: 205-235.
4. Atack J., Perry E., Bonham J., Candy J., Perry R. (1986): Molecular forms of acetylcholinesterase and butyrylcholinesterase in the aged human central nervous system. J Neurochem 47: 263-277.
5. Bancroft, J., Stevens, A., Turner, D. (1996): Theory and practice of histological techniques. 4th Ed. New York, Churchill, Livingstone.
6. Belzung, C. (1999): Measuring rodent exploratory behavior. In: Crusio, W. & Gerlai, R. (eds.), Handbook of molecular-genetic techniques for brain and behavior research, Vol. 13, pp. 738-749. Amsterdam: Elsevier Science B.V.
7. Bharti, V., Srivastava, R. (2009): Fluoride-induced oxidative stress in rat's brain and its amelioration by buffalo (*Bubalus bubalis*) pineal proteins and melatonin. Biol Trace Element Res, 130(2): 131-140.
8. Bhatnagar, M., Rao, P., Bhatnagar, C., Bhatnagar, R. (2003): Trace element concentration in various tissues following fluoride administration to female mice. Ind J Exp Biol, 41: 652-654.
9. Bhatnagar, M., Rao, P., Jain, S., Bhatnagar, R. (2002): Neurotoxicity of fluoride: neurodegeneration in hippocampus of female mice. Indian J Exp Biol, 40(5): 546-554.
10. Bhatnagar, M., Rao, P., Saxena, A, Bhatnagar, R., Meena, P., Barbar, S., Chouhan, A., Vimal, S. (2006): Biochemical changes in brain and other tissues of female mice from F in their drinking water. Fluoride 39(4): 280-284.
11. Blaylock, R. (2004): Excitotoxicity: a possible central mechanism in fluoride neurotoxicity. Fluoride, 37: 301-314.
12. Bolivar, V., Caldarone, B., Reilly, A., Lorraine, F. (2000): Habituation of activity in an open field: a survey of inbred strains and F1 hybrids. Behav Genet, 30: 285-293.
13. Botella, P., Parra, A. (2003): Coffee increases state anxiety in males but not in females. Hum Psychopharmacol, 18(2): 141-143.
14. Brain P., Marrow, L. (1999): Rodent models of human neuroses and psychoses. In: Haug, M., Whale, R. (eds.), Animal models of human emotion and cognition. Washington: American Psychological Association, pp. 59-75.
15. Brice, C., Smith, A. (2002): Effects of caffeine on mood and performance: a study of realistic consumption. Psychopharmacol, 164: 188-192.
16. Brown, R., Corey, S., Moore, A. (1999): Differences in measures of exploration and fear in MHC-congenic C57BL/6J and B6-H-2K mice. Behav Gen 29(4): 263-271.
17. Buckholtz, N., Middaugh, L. (1987): Effects of caffeine and L-phenylisopropyladenosine on locomotor activity of mice. Pharmacol Biochem Behav, 28: 179-185.
18. Burgstahler, A., Colquhoun, J. (1996): Neurotoxicity of fluoride. Fluoride 29: 57-58.
19. Buzalaf, M., de Almeida, B., Cardoso, V., Ollympio, K., Furlani, T., de, A. (2004): Total and acid soluble fluoride content of infant cereals, beverages and biscuits from Brazil. Food Addit Contam, 21: 210-215.
20. Casadesus, G., Shukitt-Hale, B., Stellwagen, H., Zhu, X., Lee, H., Smith, M., Joseph, J. (2004): Modulation of hippocampal plasticity and cognitive behaviour by short-term blueberry supplementation in aged rats. Nutr Neurosci, 7(5-6): 309-316.
21. Chen, Y., Bai, X. (1995): Approach to the mechanism of increased whole blood ChE activity due to fluoride and the antagonism of Zn Mn Mo in rats. Chin J Contr Endem Dis, 10: 1-4.
22. Chioca, L., Raupp, I., Da Cunha, C., Losso, E., Andreatini, R. (2008): Subchronic fluoride intake induces impairment in habituation and active avoidance tasks in rats. Eur J Pharm, 579(1-3): 196-201.
23. Chirumari, K., Reddy, P. (2007): Dose-dependent effects of fluoride on neurochemical milieu in the hippocampus and neocortex of rat brain. Fluoride, 40(2): 101-110.
24. Chlubek, D. (2003): Fluoride and oxidative stress. Fluoride, 36: 217-228.
25. Choleris, E., Thomas, A., Kavaliers, M., Prato, F. (2001): A detailed ethological analysis of the mouse open field test: effects of diazepam, chlordiazepoxide and an extremely low frequency pulsed maynetiz field. Neurosci Biobehav Rev, 25: 235-260.
26. Crawley, J. (1999): Evaluating anxiety in rodents. In: Crusio, W. and Gerlai, R. (eds.), Handbook of Molecular-Genetic Techniques for Brain and Behavior Research, Elsevier, Amsterdam, pp. 667-673.

27. Dahl, A., Hobbs, C., Marshall, T. (1987): The inhibition of rat and guinea pig cholinesterases by anionic hydrolysis products of methylphosphonic difluoride (Difluoro). Fluoride, 20(1): 47(Abstract).
28. Darreh-Shori, T., Almkvist, O., Guan, Z., Garlind, A., Strandberg, B., Svensson, A., (2002): Sustained cholinesterase inhibition in AD patients receiving rivastigmine for 12 months. Neurol, 59: 563-572.
29. DrÖge, W., Schipper, H. (2007): Oxidative stress and aberrant signaling in aging and cognitive decline. Aging Cell, 6: 361-370.
30. Du Toit, R., Vosteedt, Y., Apostolides, Z. (2001): Comparison of the antioxidant content of fruits, vegetables and teas measured as vitamins C equivalents. Toxicol, 166: 63-69.
31. Ekambaram, P., Paul, V. (2001): Calcium preventing locomotor behavioural and dental toxicities of fluoride by decreasing serum fluoride level in rats. Environ Toxicol Pharmacol, 9: 141-146.
32. Ekambaram, P., Paul, V. (2003): Effect of vitamin D on chronic behavioural and dental toxicities of sodium fluoride in rats. Fluoride, 36: 189-197.
33. El-lethey, H., Kamel, M., Shaheed, I. (2010): Neurobehavioural toxicity produced by sodium fluoride in drinking water of laboratory rats. J Am Sci, 6(3): 54-63.
34. Esterbauer, H., Cheeseman, K. (1990): Determination of aldehydic lipid peroxidation products: Malonaldehyde and 4-hydroxynonenal. In: Packer, L. & Glazer, A. (eds.), Methods in enzymology, 186: 407-421.
35. Folch, A., Lees, M., Stanley, G. (1957): A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem, 226: 497-509.
36. Foster, L., Dunn, R. (1973): Stable reagents for determination of serum triglycerides by a colorimetric Hantzsch condensation method. Clin Chem, 19: 338-340.
37. Friedewald, W., Levy, R., Frederickson, D. (1972): Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem**,** 18: 499-502.
38. Fromm, L., Heath, D., Vink, R., Nimmo, A. (2004): Magnesium attenuates posttraumatic depression/anxiety following diffuse traumatic brain injury in rats. J Am Coll Nutr, 23: 529S-533.
39. Gaffan, E., Davies, J. (1982): Reward, novelty and spontaneous alternation. Q J Exp Psych, 34B: 31-47.
40. Gao, Q., Liu, Y., Guan, Z. (2009): Decreased learning and memory ability in rats with fluorosis: increased oxidative stress and reduced cholinesterase activity in the brain. Fluoride, 42(4): 277-285.
41. Garcia-Ayllon, M., Silveyra, M., Candela, A., Compan, A., Claria, J., Jover, R. (2006): Changes in liver and plasma acetylcholinesterase in rats with cirrhosis induced by bile duct ligation. Hepatol, 43(3): 444-453.
42. Ge, Y., Ning, H., Wang, S., Wang, J. (2005): Effect of high fluoride and low iodine on brain effects of high fluoride and low iodine on brain histopathology in offspring rats. Fluoride, 38: 127-132.
43. Giannopolitis, C., Ries, S. (1977): Superoxide dismutase. I. Occurrence in higher plants. Plant Physiol, 59: 309-314.
44. Guan, Z., Wang, Y., Xiao, K., Dai, D., Chen, Y., Liu, J., Sindelar, P., Dallner, G. (1998): Influence of chronic fluorosis on membrane lipids in rat brain. Neurotoxicol Teratol, 20: 537-542.
45. Guo, Q., Zhao, B., Li, M., Shen, S., Xin, W. (1996): Studies on protective mechanisms of four components of green tea polyphenols against lipid peroxidation in synaptosomes. Biochim Biophys Acta, 1304: 210-222.
46. Guo, Q., Zhao, B., Shen, S., Hou, J., Hu, J., Xin, W. (1999): ESR study on the structureantioxidant activity relationship of tea catechins and their epimers. Biochim. Biophys. Acta, 1427: 13–23.
47. Haider, S., Yasmeen, A., Parveen, T., Haleem, D. (1998): Neurochemical and behavioural effects of long term intake of tea. Pak J Pharm Sci, 11(2): 55-60.
48. Haleem, D., Yasmeen, A., Parveen T., Zafar A. (1994): Enhancement of hepatic tryptophan pyrrolase activity and decrease of open field locomotion following single and repeated administration of high doses of caffeine in rats. Life Sci. 54: 297-304.
49. Halliwell, B. (2006): Oxidative stress and neurodegeneration: where are we now? J Neurochem, 97:1634-1658.
50. Haskell, C., Kennedy, D., Milne, A., Wesnes, K., Scholey, A. (2008): The effects of L-theanine, caffeine and their combination on cognition and mood. Biol Psychol, 77(2): 113-122.
51. Haque A., Hashimoto, M., Katakura, M., Tanabe, Y., Hara, Y., Shido, O. (2006): Longterm administration of green tea catechins

improves spatial cognition learning ability in rats. J Nutr, 136(4):1043-1047.
52. Henning, S., Niu, Y., Lee, N., Thomas, G., Minutti, R., Wang, H., Go, V., Heber, D. (2004): Bioavailability and antioxidant activity of tea flavanols after consumption of green tea, black tea, or a green tea extract supplement. Am J Clin Nutr, 80(6): 1558-1564.
53. Hertog, M., Sweetnam, P., Fehily, A., Elwood, P., Kromhout, D. (1997): Antioxidant flavonols and ischemic heart disease in a Welsh population of men: the Caerphilly Study. Am J Clin Nutr, 65: 1489-1494.
54. Homberg, J., van den Akker, M., Raaso, H., Wardeh, G., Binnekade, R., Schoffelmeer, A., de Vries T. (2002): Enhanced motivation to self-administer cocaine is predicted by self-grooming behaviour and relates to dopamine release in the rat medial prefrontal cortex and amygdala. Eur J Neurosci, 15: 1542-1550.
55. Inanami, O., Asanuma, T., Inukai, N., Jin, T., Shimokawa, S., Kasai, N., Nakano, M., Sato, F., Kuwabara, M. (1995): Neurosci Lett, 196: 85-88.
56. Inkielewicz I, Krechniak J. (2004): Fluoride effects on glutathione peroxidase and lipid peroxidation in rats. Fluoride, 37: 7-12.
57. Ivinskis, A. (1968): The reliability of behavioural measures obtained in the open field. Aust J Psychol, 20(3): 173-177.
58. Ivinskis, A. (1970): A study of validity of open-field measures. Aust J Psychol, 22(2): 175-183.
59. Izquierdo, L., Viola, H., Barros, D., Alonso, M., Vianna, M., Furman, M., Levi de Stein, M., Szapiro, G., Rodrigues, C., Choi, H., Medina, J., Izquierdo, I. (2001): Novelty enhances retrieval: molecular mechanisms involved in rat hippocampus. Eur J Neurosci, 33: 1-5.
60. Kalueff, A., Keisala, T., Minasyan, A., Kuuslahti, M., Tuohimaa, P. (2006): Temporal stability of novelty exploration in mice exposed to different open field tests. Behav Proc, 72: 104-112.
61. Kalueff, A., Tuohimaa, P. (2004): Experimental modeling of anxiety and depression. Acta Neurobiol Exp, 64: 439-448.
62. Kaplan, G., Greenblatt, D., Ehrenberg, B., Goddard, J., Contreau, M., Harmatz, J., Shader, R. (1997): Dose-dependent pharmacokinetics and psychomotor effects of caffeine in humans. J Clin Pharmacol, 37: 693-703.
63. Katiyar, S., Mukhtar, H. (1997): Tea antioxidants in cancer chemoprevention. J Cell Biochem Suppl, 27: 59-67.
64. Kaur, T., Pathak, C., Pandhi, P., Khanduja, K. (2008): Effects of green tea extract on learning, memory, behavior and acetylcholinesterase activity in young and old male rats. Brain cogn, 67(1): 25-30.
65. Kim, H., Kim, M., Kim, S., Kim, M., Chung, J. (2004): Effects of green tea polyphenol on cognitive and acetylcholinesterase activities. Biosci Biotechnol Biochem, 68(9): 1977- 1979.
66. Komes, D., Horžić, D., Belščak, A., Ganič, K., Baljakak, A. (2009): Determination of caffeine content in tea and maté tea by using different methods. Czech J Food Sci, 27, Special Issue.
67. Korkmaz, A., Oter, S., Sadir, S., Topal, T., Uysal, B., Özler, M., Ay Hakan, Akin, A. (2007): Exposure time related oxidative action of hyperbaric oxygen in rat brain. Neurochem Res, 33(1): 160-166.
68. Krisnamoorthy, K. (1991): The nutritional and therapeutic value of tea. In: Proceedings of International Symposium on Tea Science. Organizing Comitte of ISTS, Shizuoka, Japan.
69. Kulisic-Bilusic, T., Katalinic, V., Dragovic-Uzelac, V., Ljubenkov, I., Krisko, A., Dejanovic, B., Jukic, M., Politeo, O., Pifat, G., Milos, M. (2008). Antioxidant and acetylcholinesterase inhibiting activity of several aqueous tea infusions *in vitro*. Food Technol Biotechnol, 46(4): 368-375.
70. Lakenbrink, C., Lapczynski, S., Maiwald, B., Engelhardt, U. (2000): Flavonoids and other polyphenols in consumer brews of tea and other caffeinated beverages. J Agric Food Chem, 48: 2848-2852.
71. Lapin, I., Khaunina, R., Mirzaev, S. (1995): Vertical motor activity of mice is slowed by lower doses of psychotropic drugs than horizontal. Bull Eksp Biol Med, 120: 385-387.
72. Lee, Y., Lee, H., Sohn, H. (2005): Soy isoflavones and cognitive function. J Nutr Biochem, 16(11): 641-649.
73. Leising, K. (2009). Associative basis of landmark learning and integration in vertebrates. Comp Cogn Behav Rev, 4: 80-102.
74. Leung, L., Su, Y., Chen, R., Zhang, Z., Huang, Y., Chen, Z. (2001): Theaflavins in black tea and catechins in green tea are equally effective antioxidants. J Nutr, 131(9): 2248- 2251.
75. Leussis, M., Bolivar, V. (2006): Habituation in rodents: a review of behavior, neurobiology, and genetics. Neurosci Biobehav Rev, 30: 1045-1064.
76. Liu, J., Xie, L., Hong, A., Chen, S. (1999): Animal model for Alzheimer's disease based on the blockade of neuronal nicotinic acetylcholine receptors. J Hyg Res, 28: 218-20.
77. Liu, W., Dong, Z., Liu, J. (1989): Experimental study of behaviour and cerebral morphology of

rat pups generated by fluorotic female rat. Chin J Pathol 18: 290-292.
78. Lowry, O., Rosebrough, N., Farr, A., Randall, R. (1951): Protein measurement with the Folin phenol reagent. J Biol Chem, 193**:** 265-275.
79. Lu, Y., Sun, Z., Wu, L., Wang, X., Lu, W., Liu, S. (2000): Effect of high-fluoride water on intelligence in children. Fluoride, 33: 74-78.
80. Luczaj, W., Skrzydlewska, E. (2005): Antioxidative properties of black tea. Prev Med, 40(6): 910-918.
81. Mandel, S., Youdim, M. (2004): Catechin polyphenols: neurodegeneration and neuroprotection in neurodegenerative diseases. Free Radic Biol Med, 37(3): 304-317.
82. Maron D., Lu, G., Cai, N., Wu, Z., Li, Y., Chen, H., Zhu, J., Jin, X., Wouters, B., Zhao, J. (2003): Cholesterol-lowering effect of a theaflavin enriched green tea extract: a randomized controlled trial. Arch Intern Med, 163: 1448-1453.
83. Matsuoka, Y., Hasegawa, H., Okuda, S., Muraki, T., Uruno, T., Kubota, K. (1995): Ameliorative effects of tea catechins on active oxygen-related nerve cell injuries. J Pharmacol Exp Ther, 274: 602-608.
84. Mello e Souza, T., Rohden, A., Meinhardt, M., Goncalves, C., Quillfeldt, J. (2000): S100B infusion into the rat hippocampus facilitates memory for the inhibitory avoidance task but not for the open-field habituation. Physiol Behav, 71: 29-33.
85. Millan, M. (2003): The neurobiology and control of anxious states. Prog Neurobiol, 70: 83-244.
86. Miu, A., Andreescu, C., Va-siu, R., Olteanu, A. (2003): A behavioral and histological study of the effects of long- term exposure of adults rats to aluminum. Int J Neurosci, 113(9): 1197-1211.
87. Mortensen, S., Hooper, M., Padilla, S. (1998): Rat brain acetylcholinesterase activity: developmental profile and maturational sensitivity to carbamate and organophosphorus inhibitors. Toxicol, 125: 13-19.
88. Mullenix P., Denbesten P., Schunior A., Kernan W. (1995): Neurotoxicity of sodium fluoride in rats. Neurotoxicol Teratol, 17(2): 169-177.
89. Muller, F., Dumez, Y. Massoulie´, J. (1985): Molecular forms and solubility of acetylcholinesterase during the embryonic development of rat and human brain. Brain Res, 331: 295-302.
90. Nakagawa, K., Miyazawa, T. (1997): Absorption and distribution of tea catechin, (_) epigallocatechin-3-gallate, in the rat. J Nutr Sci Vitaminol, 43: 679-684.
91. Nathan P., Lu, K., Gray, M., Oliver, C. (2006): The neuropharmacology of L-theanine (N-ethyl-L-glutamine): a possible neuroprotective and cognitive enhancing agent. J Herb Pharmacother, 6(2):21-30.
92. Nie, G., Jin, C., Cao, Y., Shen,S., Zhao, B. (2002): Distinct effects of tea catechins on 6-Hydroxydopamine-induced apoptosis in PC12 cells. Arch Biochem Biophys, 397(1): 84- 90.
93. Niu, R., Sun, Z., Wang, J., Cheng, Z., Wang, J. (2008): Effects of fluoride and lead on locomotor behaviour and expression of nissl body in brain of adult rats. Fluoride 41(4): 276-282.
94. Patel, D., Chinoy, N. (1997): Synergistic action of ascorbic acid and calcium in mitigation of fluoride induced toxicity in uterus of mice. Indian J Environ Toxicol, 7: 16-19.
95. Pereira, M., Dombrowski, P., Losso, E., Chioca, L., Da Cunha, C., Andreatini, R. (2009): Memory Impairment Induced by Sodium Fluoride is Associated with Changes in Brain Monoamine Levels. Neurotox Res, (Epub).
96. Pietri, S., Seguin, J., Arbigny D., Drieuk, A., Culcasi, M. (1997): Ginkgo Bilober extract (EGb 761)pretreatment coronary by pass surgery. Cardiovasc Drug Ther, 11: 121-31.
97. Purohit, S., Gupta, R., Mathur, A., Gupta, N., Jeswani, I., Purohit, S. (1999): Experimental purmonary fluorosis. Indian J Chest Dis Allied Sci, 41: 27-34.
98. Rasoolijazi, H., Joghataie, M., Roghani, M., Nobakht, M. (2007): The beneficial effect of (-) Epigallocatechin-3-Gallate in an experimental model of Alzheimer's disease in rat: a behavioral analysis. Iran Biomed J., 11(4): 237-243.
99. Renderio, C., Spencer, J., Vauzour, D., Butler, L., Ellis, J., Williams, C. (2009): The impact of flavonoids on spatial memory in rodents: from behaviour to underlying hippocampal mechanisms. Genes Nutr, 4: 251-270.
100. Sharma, J., Sohu, D., Jain, P. (2009): Prevalance of neurological manifestation in a human population exposed to fluoride in drinking water. Fluoride, 42: 127-132.
101. Shashi, A. (1988): Biochemical effects of fluoride on thyroid gland during experimental fluorosis. Fluoride, 21: 127-130.
102. Shashi, A. (1992): Studies on alterations in brain lipid metabolism following experimental fluorosis. Fluoride, 25(2): 77-84.
103. Shashi, A., Singh, J., Thapar, S. (1989): Effect of fluoride in excess on lipid constituents of respiratory organs in albino rabbits. Fluoride, 22: 33-39.

104. Shivarajashankara, Y., Shivashankara, A., Bhat, P., Rao, S. (2001): Effect of fluoride intoxication on lipid peroxidation and antioxidant systems in rats. Fluoride, 34: 108-113.
105. Spencer, J. (2008): Flavonoids: modulators of brain function? Br J Nutr, 99(1): 60-77.
106. Spencer, J. (2009): Flavonoids and brain health: multiple effects underpinned by common mechanisms. Genes Nutr, 4(4): 243-250.
107. Spittle, B. (1998): Comments on the finding of toxin-induced blood vessel inclusions and alterations in neuronal and cerebrovascular integrity following the chronic administration of aluminum fluoride and sodium fluoride. Fluoride, 31(2):89-90.
108. SPSS (2006): SPSS for Windows 14.0.
109. Staddon, J. (1983): Memory and temporal control. In: J. E. R. Staddon (Ed.), Adaptive behaviour and learning (pp. 354-394). Cambridge University Press, Cambridge.
110. Strack, S., McNeill, R., Colbran, R. (2000): Mechanism and regulation of calcium/calmodulin-dependent protein kinase II targeting to the NR2B subunit of the Nmethyl-D-aspartate receptor. J Biol Chem, 275**:** 23798-23806
111. Suganuma, M., Okabe, S., Oniyama, M., Tada, Y., Ito, H., Fujiki, H. (1998): Wide distribution of [3H](-)-epigallocatechin gallate, a cancer preventive tea polyphenol, in mouse tissue. Carcinogenesis, 19(10): 1771-1776.
112. Sun, Z., Liu, F., Wu, L., Lü, Y., Yu, D. (2000): Effects of high fluoride drinking water on the cerebral functions of mice. Chin J Endemiol, 19: 262-263.
113. Sutherland, B., Rahman, R. (2006): Appleton I. Mechanisms of action of green tea catechins, with a focus on ischemia-induced neurodegeneration. J Nutr Biochem, 17(5):291-306.
114. Tancer, M., Stein, M., Uhde, T. (1991): Lactate response to caffeine in panic disorder: a replication using an “anxious’ control group. Biol Psychatry, 29: 57.
115. Tancer, M., Stein, M., Uhde, T. (1994): Lactic acid response to caffeine in panic disorder: comparison with social phobics and normal controls. Anxiety, 1: 138-140.
116. Teh, C., Khoo, Z., Khursiah, F., Rao, N., Chin, J. (2010): Acetylcholinesterase inhibitory activity of star fruit and its effect on serum lipid profiles in rats. Int Food Res J, 17: 987-994.
117. Trivedi, M., Verma, R., Chinoy, N. (2006): Amelioration by black tea of changes induced by sodium fluoride in protein content of liver and kidney in mice. Fluoride 39(4): 269- 273.
118. Trivedi M., Verma R., Chinoy, N. (2007): Amelioration by black tea of sodium fluorideinduced changes in protein content of cerebral hemisphere, cerebellum, and medulla oblongata in brain region of mice. Acta Pol Pharma-Drug Res, 64(3): 221-225.
119. Trivedi, M., Verma, R., Chinoy, N. (2009): Mitigation of NaF induced toxicity in mice brain by black tea infusion. Fluoride, 42(1): 29-33.
120. Tze-Pin, N., Feng, L., Niti, M., Kua, E., Yap, K. (2008): Tea consumption and cognitive impairment and decline in older Chinese adults. Am J Clin Nutr, 88: 224-231
121. Unno, K., Hoshino, M. (2007): Brain science and neuroprotective dietary components. Central Nervous System Agents in Medicinal Chemistry, 7: 109-114.
122. Unno, K., Takabayashi, F., Kishido, T., Oku, N. (2004): Suppressive effect of green tea catechins on morphologic and functional regression of the brain in aged mice with accelerated senescence (SAMP10). Exp Gerontol, 39(7): 1027-1034.
123. Vani, L., Reddy, K. (2000): Effects of fluoride accumulation on some enzymes of brain and gastrocnemius muscle of mice. Fluoride, 33(1): 17-26.
124. Vatassery, G., Ophaug R., Singer L. (1980): The effect of fluoride intake on the total lipid, cholesterol, carbohydrate and tricarboxylic acid metabolism in Guinea pigs. Fluoride, 20: 137-141.
125. Verma, R., Trivedi, M., Chinoy, N. (2006): Amelioration by black tea extract of sodium fluoride induced hemolysis of human red blood cell corpuscles. Fluoride, 39(4): 261-265.
126. Wang, J., Ge, Y., Ning, H., Wang, S. (2004): Effect of high fluoride and low iodine on oxidative stress and antioxidant defense of the brain in offspring rats. Fluoride, 37: 264-270.
127. Weinreb, O., Mandel, S., Amit, T., Youdim, M. (2004): Neurological mechanisms of green tea polyphenols in Alzheimer’s and Parkinson’s diseases. J Nutr Biochem, 15(9): 506-516.
128. Weiss, E., Greenberg, G. (1996): Open field procedures. In: Greenberg, G., and Haraway, M. (eds.), Comparative Psychology: A Handbook, Garland, New York, pp. 603-662.
129. Weisstaub, N., Zhou, M., Lira, A., Lambe, E., Gonzalez-Maeso, J., Hornung, J., Sibille, E., Underwood, M., Itohara, S., Dauer, W., Ansorge, M., Morelli, E., Mann, J. Toth, M., Aghajanian, G., Sealfon, S., Hen, R., Gingrich, J. (2006): Cortical 5-HT2A receptor signaling

modulates anxiety- like behaviors in mice. Sci, 313: 536-540.
130. Williams, C., El Mohsen, M., Vauzour, D., Renderio, C., Butler, L., Ellis, J., Whiteman, M., Spencer, J. (2008): Blueberry-induced changes in spatial working memory correlate with changes in hippocampal CREB phosphorylation and brain-derived neuro-tropic factor (BDNF) levels. Free Radic Biol Med, 45(3): 295-305.
131. Williamson, G., Manach, C. (2005): Bioavailability and bioefficacy of polyphenols in humans. II Review of 93 intervention studies. Am J Clin Nutr, 81(I Suppl): 243S-255S.
132. Winograd, M., Viola, H. (2004): Detection of novelty, but not memory of spatial habituation, is associated with an increase in phosphorylated cAMP response element binding protein levels in the hippocampus. Hippocampus, 14: 117-123.
133. Wolf, O., Kirschbaum, C. (2002): Endogenous estradiol and testosterone levels are associated with cognitive performance in older women and men. Horm Behv, 41: 259-266.
134. Wu, C., Gu, X., Ge, Y., Zhang, J., Wanga, J. (2006). Effects of high fluoride and arsenic on brain biochemical indexes and learning-memory in rats. Fluoride, 39(4): 274-279.
135. Xiang, Q., Liang, Y., Chen, L., Wang, C., Chen, B., Chen, X., Zhou, M. (2003): Effect of fluoride in drinking water on children's intelligence. Fluoride, 36: 84-94.
136. Xu, N., Chen, Z. (2002): Green tea, black tea, and semi-fermented tea. In: Zhen Y., ed. TZea: bioactivity and therapeutic potential. London: CRC Press UK, pp. 35-54.
137. Yoneda, T., Hiramatsu, M., Skamoto, N., Togasaki, K., Komastu, M., Yamaguchi, K. (1995): Antioxidant effects of "beta catechins". Biochem Mol Biol Int, 35: 995-1008.
138. Youdim, K., Spencer, J., Schroeter, H., Rice-Evans, C. (2002): Dietary flavonoids as potential neuroprotectants. Biol Chem, 383: 503-519.
139. Zahvaronkov, A., Strochkova, L. (1981): Fluorosis: Geographical pathology and some experimental findings. Fluoride, 14: 182-191.
140. Zakut, H., Matzkel, A., Schejter, E., Avni, A., Soreq, H. (1985): Polymorphism of acetylcholinesterase in discrete regions of the developing human fetal brain. J Neurochem, 45: 382-389.
141. Zhang, Z., Xu, X., Shen, X., Xu, X. (2008): Effect of fluoride exposure on synaptic structure of brain areas related to learning-memory in mice. Fluoride, 41(2): 139-143.
142. Zhao, B., Li, X., He, R., Cheng, S., Xin, W. (1989): Scavenging effect of extracts of green tea and natural antioxidants on active oxygen radicals. Cell Biophys, 14: 175-185.
143. Zlatkis, A., Zak, B., Boyle, A., Mich, D. (1953): A new method for the direct determination of serum cholesterol. J Lab Clin Med, 41:486-492.

11/6/2010

Epidemiological Studies of Urinary Tract Infection (UTI) among Post-menopausal Women in Uyo Metropolis, South-South, Nigeria.

[1]Akinjogunla, O. J., [2]Odeyemi, A. T. and [3]Olasehinde, G. I.

[1]Department of Microbiology, Faculty of Science, University of Uyo, P.M.B. 1017, Uyo, Akwa Ibom State, Nigeria.
[2]Department of Microbiology, Faculty of Science, University of Ado-Ekiti, P.M.B.5363, Ado-Ekiti, Ekiti State, Nigeria.
[3]Department of Biological Sciences, College of Science and Technology, Covenant University, Ota, Ogun State, Nigeria.
papajyde2000@yahoo.com

ABSTRACT: Cross-sectional studies of UTI among post menopausal women were carried out between January and June, 2009 using standard microbiological techniques. The result obtained showed that 42 (39.6%) out of 106 postmenopausal women had urinary tract infections with highest prevalence among women aged 56-60 and lowest among those aged ≥ 61 . Microscopic examinations of forty-two (42) mid-stream urine samples revealed the presence of 13(30.9%) epithelial cells, 5 (11.9%) phosphate crystals, 16 (38.1%) pus cell , 9 (21.4%) yeast cells, 7(16.7%) red blood cells and eggs of *Schistoma haematobium* 2(4.8%). Bacteria isolated were: *Escherichia coli* 20 (25.3%), followed by *Staphylococcus aureus* 16 (20.3%), *Pseudomonas aureginosa* 10 (12.7%), Coagulase negative *Staphylococcus* spp 9 (11.4%), *Streptococcus pyogenes* 6 (7.6%), *Serratia marcescens* 6 (7.6%), *Enterobacter* spp 5 (6.3%). *Klebsiella* spp. 4 (5.1%) and *Enterococcus faecalis* 3(3.8%). *E. coli* showed low percentage resistance to ciprofloxacin, ceftazidime and ceftriaxone. *Enterobacter* spp. were susceptible to ciprofloxacin and cotrimoxazole in 80%, respectively. Between 60-80% of *Pseudomonas aeruginosa* and *Enterobacter* spp were susceptible to all the tested antibiotics, while 4(66.7%) *Streptococcus pyogenes,* 6 (66.7%) CON-*Staphylococcus* spp and 4(66.7%) *Serratia marcescens* were sensitive to ceftazidime. All the *Enterococcus faecalis* and *Klebsiella* spp isolated were sensitive to ciprofloxacin. The phenotypic determination identified a low ESβL rate of 28.8 % (13 of 45 isolates). ESBLs were detected among the following species: 5 *Escherichia coli* (25.0%), 3 *Pseudomonas* spp (30.0%), 1 *Klebsiella* spp (25.0%), *Serratia marcescens*2 (33.3%) and *Enterobacter* spp. 2 (40.0%). The result also showed that 18.9 % of the bacteria were resistant to at least 3 antibiotics with (MAR) index ranging from 0.2 to 0.8. The results obtained in this study are statistically significant ($p \leq 0.05$). However, continuous surveillance to monitor the prevalence of UTI and antimicrobial resistance among post menopausal women is overwhelmingly necessary.
[Akinjogunla, O. J., Odeyemi, A. T. and Olasehinde, G. I. **Epidemiological Studies of Urinary Tract Infection (UTI) among Post-menopausal Women in Uyo Metropolis, South-South, Nigeria.** Journal of American Science 2010;6(12):1674-1681]. (ISSN: 1545-1003). http://www.americanscience.org.

Key Words: Post-menopausal, Prevalence, Infection, Susceptibility, ESβL, Uyo

INTRODUCTION

The urinary tract consists of various organs of the body involved in the production, storage and excretion of urine. (Sotelo and Westney, 2003; Beer *et al*., 2006). Urinary tract infection (UTI) is caused by pathogenic invasion of the urinary tract, which leads to an inflammation of the urothelium. The invading microbes may affect the entire tract or be restricted to either the upper region or lower region (Stamm and Norrby, 2001). Urinary tract infection (UTI) is one of the most common causes of hospitalization and referral to outpatient, having an estimated figure of 150 million per annum worldwide. (Stamm and Norrby, 2001; Fakhrossadat and Narges, 2009). The urinary tract infections may be asymptomatic, acute, chronic and complicated or uncomplicated and the clinical manifestations of UTI depend on the portion of the urinary tract involved, the etiologic organisms, the severity of the infection, and the patient's ability to mount an immune response to it. Acute urinary tract infection is an extremely common entity that affects almost half of women in The United states of America (Foxman, 2002). The prevalence and incidence of urinary tract infection is higher in women than in men, which is likely the result of several clinical factors including hormonal effects, behaviour patterns or their having a short urethra and vaginal vestibule which can easily be contaminated (Harding and Ronald, 1994). Prevalence of UTIs increases with advancing age and in females the prevalence ranged from 3% in young girls under the age of 10 to a peak of 10% in post-menopausal women between the ages of 55 and 64 (Young and Koda-Kimble, 1995; Sotelo and Westney, 2003).The leading causes of acute and uncomplicated UTIs in patients have been reported to be due to *Escherichia coli, Staphylococcus aureus, Proteus spp,*

Klebsiella spp and *Pseudomonas aeruginosa* (Manges *et al*., 2006; Akram *et al*., 2007; Akortha and Ibadin, 2008). In Nigeria, *E. coli*, *Proteus spp* and *Klebsiella spp* have been isolated in 90% of UTI reposted cases (Obaseiki-Ebor, 1988; Foxman, 1990). The symptoms of UTIs which include fever, burning sensations while urinating, lower abdominal pain, itching, formation of blisters and ulcers in the genital area, genital and suprapubic pain, and pyuria generally depend on the age of the person infected and the location of the urinary tract infected (Amali *et al*., 2009). UTIs are associated with a high risk of morbidity and mortality and account for significant health care costs in spite of the availability and use of the antimicrobial drugs.

Hormonal changes that characterize menopause are likely to influence the habits of women. A number of observational epidemiological studies have dealt with risk factors of chronic diseases, namely cardiovascular disease (CVD), osteoporosis, and urinary tract infection. In post-menopausal women, susceptibility to UTI increases due to a deficiency of oestrogen (Foxman,1999). Sensitivity of bacterial to antibiotics shows a great geographical and historical variability due to different antibiotic treatments (Akinjogunla *et al*., 2009). So knowledge of the sensitivity pattern of common uropathogens according to local epidemiological studies is necessary for selection of appropriate antibiotics for empirical treatment. Researches have not been extensively carried out to determine the prevalence of symptomatic or asymptomatic bacteriuria among the postmenopausal women in this environment , this therefore necessitated this study that determined the prevalence and sensitivity pattern of uropathogens isolated from post-menopausal women in Uyo Metropolis.

MATERIALS AND METHODS

STUDY SETTING

This cross-sectional study was designed to cover post-menopausal women in Uyo metropolis. Uyo is the capital city of Akwa –Ibom State located in the South-South (S/S) part of Nigeria. Uyo metropolis was selected for the study due to its dense population. Demographic information such as age, and time of stoppage of their menstrual cycle of each post-menopausal woman obtained were kept confidential.

COLLECTION OF MID STREAM URINE SAMPLES

In a prospective study from January to June, 2009, a total of 106 mid-stream urine (MSU) samples from apparently healthy postmenopausal women who gave verbal informed consent were aseptically collected into sterile McCartney bottles and stored at 4^{o}C.

MICROSCOPIC EXAMINATION OF MID STREAM URINE SAMPLES

Two loopful of uniformly mixed uncentrifuged urine samples were aseptically placed on a clean grease-free slide and covered with a cover slip**.** The preparation was examined microscopically to detect the presence of pus cell, epithelial cell, red blood cell, yeast cell, phosphate crystal using 10x and 40 x condenser iris closed sufficiently to give good contrast.

ISOLATION AND IDENTIFICATION OF UROPATHOGENS

The uncentrifuged, uniformly mixed mid stream urine (MSU) samples were inoculated on Cysteine lactose electrolyte deficient (CLED), MacConkey agar (MCA) and Blood agar (BA) media and incubated at 37°C aerobically for 24 hrs. After incubation the cultures developed on media were observed and the colonies were counted by colony counter. Significant urinary tract infection (UTI) was defined as the presence of $\geq 10^5$ colony-forming units per millimeter in the culture of an appropriately collected urine sample. Standard identification procedures of colony morphology, Gram staining reaction, motility, catalase test, oxidase test, urease test, coagulase test, sugar fermentation, indole production and IMViC (Indole, Methyl red, Voges-Proskauer, and Citrate) tests were used to determine the uropathogens present in the urine samples.

THE ANTIBIOTIC SENSITIVITY TESTING

The antibiotic sensitivity of the bacterial species isolated from the mid stream urine samples was performed by disk diffusion method on Muller-Hinton agar plates as described by the National Committee for Clinical Laboratory Standards (presently called as Clinical Laboratory Standard Institute). 0.1 ml of each bacterial isolates was seeded into each of the Petri dishes containing Mueller-Hinton agar and were allowed to stand for 30 mins to enable the inoculated organisms to pre-diffuse. The commercially available discs containing the following antibiotics: Ceftazidime, (Caz,30ug), Streptomycin (Stp,30ug) Ciprofloxacin (Cpf,5ug) Ceftriaxone (Cef,30ug) and Cotrimoxazole (Cot, 30ug) (Oxoid, UK) were aseptically placed on the surfaces of the sensitivity agar plates with a sterile forceps and were incubated for 18 - 24hrs at 37°C. Zones of inhibition after incubation were observed and

the diameters of inhibitory zones were measured in millimeters. The interpretation of the measurement as Sensitive (S) and Resistant (R) was made according to the manufacturer's standard zone size interpretive manual. The percentage resistance was calculated using the formula *PR=a/b×100, where 'PR' was* percentage resistance, '*a*' was the number of resistant isolates and '*b*' was the number of isolates tested with the antibiotic. The percentage sensitivity was calculated using the formula *PS=c/d×100, where 'PS' was* percentage sensitivity, '*c*' was the number of sensitive isolates and '*d*' was the number of isolates tested with the antibiotic.

DETERMINATION OF MULTIPLE ANTIBIOTIC RESISTANCE INDEX (MAR)

Multiple antibiotic resistance index (MAR) was determined using the formula MAR=*x/y*, where *x* was the number of antibiotics to which test isolate displayed resistance and *y* is the total number of antibiotics to which the test organism has been evaluated for sensitivity (Akinjogunla and Enabulele, 2010)

ESBL SCREENING AND DETECTION

All the Gram negative bacteria that are resistant to either ceftazidime or ceftriaxone (≤15) were screened using double-disc synergy tests (DDST) to detect ESBL-producing isolates (Akinjogunla *et al*., 2010). 0.1 ml of each bacterial isolates was seeded into each of the Petri dishes containing Mueller-Hinton agar and were allowed to stand for 30 mins to enable the inoculated organisms to pre-diffuse. An augmentin was placed at the center of the Petri-dish and ceftriaxone (30 µg), ceftazidime (30) at 15mm from the augmentin. An inhibitory zone of 37 mm for ceftriaxone and 22 mm for ceftazidime indicated that the isolated strains are probably ESBL producers.

STATISTICAL ANALYSIS OF RESULTS

Frequencies and percentages were calculated for study variables. Chi-square ($\chi 2$) test was used to calculate probabilities and determine significance. A p-value of less than or equal to 0.05 was considered to be statistically significant ($p \leq 0.05$), while p-value more than 0.05 was considered to be statistically not significant (NS).

RESULTS:

The result obtained showed 42 (39.6%) out of 106 postmenopausal women included in the study had urinary tract infections and the prevalence was highest among post-menopausal women aged 56-60 and lowest among those aged ≥ 61 (Table 1) . Microscopic examinations of forty-two (42) mid-stream urine samples of post menopausal women with urinary tract infections revealed the presence of epithelial cell 13 (30.9%), phosphate crystals 5 (11.9%), pus cell 16 (38.1%), yeast cells 9 (21.4%), eggs of *Schistoma haematobium* 2(4.8%) and red blood cells 7(16.7%) (Table2).

Out of the 42 midstream urine samples of the post menopausal women with UTI, 79 bacterial isolates consisting both Gram positive and Gram negative bacteria were recovered using their morphological and biochemical characteristics such Gram's reaction, motility, catalase, oxidase, coagulase, indole, urease, indole, sugar and IMViC (Indole, Methyl red, Voges-Proskauer, and Citrate) tests (Tables 3 and 4). Of these, *Escherichia coli*. had the highest prevalence of 20 (25.3%), followed by *Staphylococcus aureus* 16 (20.3%), *Pseudomonas aureginosa* 10 (12.7%), Coagulase negative *Staphylococcus* spp 9 (11.4%), *Streptococcus pyogenes* 6 (7.6%), *Serratia marcescens* 6 (7.6%), *Enterobacter* spp 5 (6.3%). *Klebsiella* spp. 4 (5.1%), *Enterococcus faecalis* 3 (3.8%), (Table 4). The incidence of sensitivity of uro-pathogens isolated to five antibiotics routinely used to treat UTI infections are shown in (Table 5). *E. coli* as the predominant cause of UTI, showed low percentage resistance to ciprofloxacin in 25.0% , ceftazidime in 25.0% and the lowest resistance to ceftriaxone in 20.0%. *Enterobacter* spp. displayed a similar resistance pattern and were susceptible to ciprofloxacin and cotrimoxazole in 80%, respectively. Between 60-80% of *Pseudomonas aeruginosa* and *Enterobacter* spp. were susceptible to ceftazidime, streptomycin, ciprofloxacin, ceftriaxone and cotrimoxazole, while 4(66.7%) *Streptococcus pyogenes*, 6 (66.7%) CON-*Staphylococcus* spp and 4(66.7%) *Serratia marcescens* were sensitive to ceftazidime. All the *Enterococcus faecalis* and *Klebsiella* spp. isolated from post-menopausal women with UTI were sensitive to ciprofloxacin (Table 5). 13 (28.9%) out of 45 Gram negative bacterial species produced extended spectrum betalactamase with *Escherichia coli* producing the highest, followed by *Pseudomonas aeruginosa* 3 (30.0%), while *Klebsiella* spp, *Serratia marcescens and Enterobacter* spp had 1 (25.0%)., 2 (33.3%), 2 (40.0%), respectively.(Table 6). The result also showed that 15 (18.9 %) of the bacteria were resistant to 3 or more antibiotics with MAR index ranging from 0.2 to 0.8 in *Escherichia coli, Staphylococcus aureus* and CON-*Staphylococcus* spp., respectively. *Pseudomonas aureginosa and Enterococcus faecalis* had MAR index ranging from 0.2 to 0.6, while lowest MAR index of 0.2-0.4 was obtained in *Klebsiella* spp. and *Enterobacter* spp (Table 7).

Table 1: Age Groups and Occurrence of UTI among Post –menopausal women

Age / Yrs	No of Samples Collected	No / Percentages (%) Positive for UTI
≤ 50	30	11 (36.7)
51-55	20	8 (40.0)
56-60	35	17(48.6)
≥ 61	21	6 (28.6)
Total	106	42 (39.6)

Table 2: Microscopic Examination of Mid-stream 42 Urine Samples of Post-menopausal Women with Urinary Tract Infection

Microscopic Examination	Number of Occurrence	Percentage of Occurrence (%)
Epithelial cell	13	30.9
Phosphate crystals	5	11.9
Pus cell	16	38.1
Yeast cells	9	21.4
Eggs of *Schistoma haematobium*	2	4.8
Red blood cells	7	16.7

Table 3: Morphological and Biochemical Characteristics of Bacteria Isolated from 42 Mid-stream Urine Samples of Post- menopausal women with Urinary Tract Infection

	Isolates								
PARAMETERS	a	b	c	d	e	f	g	h	i
Grams reaction	+/cocci	+/cocci	+/cocci	-/rod	-/rod	+/cocci	-/ rod	-/rod	-/ rod
Catalase test	-	+	+	-	-	-	-	-	-
Citrate test	-	-	-	-	-	-	+	+	-
Oxidase test	-	-	-	-	-	-	-	-	+
Coagulase test	-	+	-	-	-	-	-	-	-
Indole test	-	-	-	+	-	-	-	-	-
Urease activity	-	-	-	-	-	-	-	+	-
Glucose	+	+	+	+	+	+	+	+	+
Lactose	-	-	-	+	+	-	-	+	-
Sucrose	-	-	-	-	-	-	+	+	-
Mannitol	+	+	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+	-	+
Voges Proskauer	-	+	+	-	+	-	+	+	-

Keys a: *Streptococcus* spp.; b: *Staphylococcus aureus*; c: *CON-Staphylococcus* spp.; d: *Escherichia coli*; e: *Enterobacter* spp.; f: *Enterococcus faecalis*; g*: Serratia marcescens*; h: *Klebsiella* spp i*: Pseudomonas aeruginosa*;

Table 4: Occurrence of Bacteria Associated with 42 Mid Stream Urine Samples of Post-menopausal Women with Urinary Tract Infection

Bacterial spp	Age Groups ≤ 50	51-55	56-60	≥ 61	Total Number / Percentage of Occurrence
Escherichia coli.	8	3	7	2	20 (25.3)
Klebsiella spp.	2	0	2	0	4 (5.1)
Staphylococcus aureus	6	3	6	1	16 (20.3)
Streptococcus pyogenes	2	2	2	0	6 (7.6)
Pseudomonas aeruginosa	2	2	4	2	10 (12.7)
CON-*Staphylococcus* spp	1	3	3	2	9 (11.4)
Serratia marcescens	0	1	2	3	6 (7.6)
Enterobacter spp	1	1	2	1	5(6.3)
Enterococcus faecalis	0	1	1	1	3(3.8)
Total	22	16	29	12	79 (100)

Table 5: Antibiotic Susceptibility Profile of Bacteria Isolated from 42 Mid-stream Urine Samples of Post-menopausal Women with Urinary Tract Infection

Bacterial Isolated	Caz^s n (%)	Caz^r n (%)	Stp^s n (%)	Stp^r n (%)	Cpf^s n (%)	Cpf^r n (%)	Cef^s n(%)	Cef^r n(%)	Cot^s n(%)	Cot^r n(%)
Escherichia coli. (20)	15(75.0)	5(25.0)	13(65.0)	7(35.0)	15(75.0)	5(25.0)	16 (80.0)	4(20.0)	11(55.0)	9(45.0)
Klebsiella spp. (4)	3(75.0)	1(25.0)	1(25.0)	3(75.0)	4(100.0)	0(0.0)	4(100.0)	0(0.0)	2(50.0)	2(50.0)
Staphylococcus aureus (16)	7(43.8)	9(56.3)	9(56.3)	7(43.8)	12(75.0)	4(25.0)	10(62.5)	6(37.5)	9(56.3)	7(43.8)
Streptococcus pyogenes (6)	4(66.7)	2(33.3)	2(33.3)	4(66.7)	2(33.3)	4(66.7)	3(50.0)	3(50.0)	2(33.3)	4(66.7)
Pseudomonas aeruginosa (10)	7(70.0)	3(30.0)	6(60.0)	4(40.0)	7(70.0)	3(30.0)	8(80.0)	2(20.0)	6(60.0)	4(40.0)
CON-*Staphylococcus* spp (9)	6 (66.7)	3(33.3)	5(55.6)	4(44.4)	7 (77.8)	2(22.2)	5(55.6)	4(44.4)	6(66.7)	3(33.3)
Serratia marcescens (6)	4(66.7)	2(33.3)	3 (50.0)	3(50.0)	3(50.0)	3(50.0)	4(66.7)	2(33.3)	2(33.3)	4(66.7)
Enterobacter spp (5)	3(60.0)	2(40.0)	4(80.0)	1(20.0)	4(80.0)	1(20.0)	3(60.0)	2(40.0)	4(80.0)	1(20.0)
Enterococcus faecalis (3)	2(66.7)	1(33.3)	1(33.3)	2(66.7)	3(100.0)	0(0.0)	2(66.7)	1(33.3)	1(33.3)	2(66.7)

Keys : Caz : Ceftazidime ; Stp : Streptomycin ; Cpf : Ciprofloxacin ; Cef : Ceftriaxone ; Cot : Cotrimoxazole
s : sensitive ; r : resistant

Table 6: Prevalence of Extended Spectrum Betalactamase among Gram Negative Bacteria Isolated from 42 Mid-stream Urine of Post-menopausal Women with Urinary Tract Infections

Bacterial spp.	Number Isolated	Occurrence (%) of ESβL	Occurrence (%) of non-ESβL
Escherichia coli	20	5(25.0)	15(75.0)
Pseudomonas aeruginosa	10	3(30.0)	7(70.0)
Klebsiella spp.	4	1(25.0)	3(75.0)
Serratia marcescens	6	2 (33.3)	4(66.7)
Enterobacter spp.	5	2 (40.0)	3(60.0)
Total	45	13 (28.9)	32(71.1)

Table 7: Multiple Antibiotic Resistance Index of Bacteria Isolated from 42 Mid-stream Urine Samples of Post-menopausal Women with Urinary Tract Infection

	Multiple Antibiotic Resistance (MAR) Index				
Bacterial Isolated	0.2	0.4	0.6	0.8	Total
Escherichia coli.	14	4	1	1	20
Klebsiella spp.	2	2	-	-	4
Staphylococcus aureus	6	7	2	1	16
Streptococcus pyogenes	-	2	3	1	6
Pseudomonas aureginosa	5	4	1	-	10
CON-*Staphylococcus* spp.	4	4	-	1	9
Serratia marcescens	1	2	3	-	6
Enterobacter spp.	3	2	-	-	5
Enterococcus faecalis	1	1	1	-	3
Total	36	28	11	4	79

DISCUSSION

Mid stream urine samples are among the most numerous of specimens sent for microbiological analysis in order to reduce the morbidity and mortality caused by UTI. Very few data exist concerning UTIs in developing countries, especially among the post-menopausal women in Africa. Microscopic examinations of mid-stream urine samples of post menopausal women with urinary tract infections revealed the presence of epithelial cell, phosphate crystals, pus cell, yeast cells, eggs of *Schistoma haematobium* and red blood cells. The presence of pus cell in mid-stream urine has been recorded by Merila et al. (1987). Occurrence of *Escherichia coli, Serratia marcescens, Enterobacter* spp, *Enterococcus faecalis, Klebsiella* spp. *Pseudomonas aeruginosa,* CON-*Staphylococcus* spp, *Streptococcus pyogenes* and *Staphylococcus aureus* in mid-stream urine samples in this study is similar to the previous reports by Ahmed et al (2000) . *E. coli* was isolated from highest number of cases, followed by *Staphylococcus aureus,* this results is similar with the previous studies by Brosnema *et al.*, (1993); Weber *et al.*, (1997) ; De-Mouy et al (1999); Ahmed et al (2000); Gupta et al (2001); Hryniewicz et al (2001); Hima-Lerible et al (2003). The frequency of occurrence of *Klebsiella* spp obtained from the mid-stream urine samples in this study is lower than the values obtained by Randrianirina et al. (2007).

There was occurrence of UTIs among post meno-pausal women with age less than or equal to 50 to age greater than or equal to 60. The occurrence of UTI among women above 60 years have also been recorded by Randrianirina et al (2007). The age distribution of UTIs among post meno-pausal women showed that the highest carrier rate was in the age group of 56-60 years (48.6%), and lowest among aged ≥ 61years .This result suggest that UTI is not an age moderated disease or infection.

The prevalent pathogens of UTIs have been found to be resistant to most chemotherapeutic agents, though the antimicrobial susceptibilities of these pathogens are highly predictable. Development of resistance to these antimicrobial agents in UTI cases will therefore affect future treatment and management of the infection with these drugs. Majority of the treatments begins or is done completely empirically, the knowledge of the organisms their epidemiological characteristics and their antibacterial susceptibility is therefore mandatory.

The isolated bacteria were resistant to streptomycin. This observed resistance to these drugs is a probable indication of earlier exposure of the isolates to these drugs. Shittu and Mandare (1999) in slight contrast to this study, reported *S. aureus* as 100% sensitive to cephalosporins**.** The high sensitivity of *Staphylococcus aureus, E. coli* and *Pseudomonas aeruginosa* to ciprofloxacin in this study is similar to the valued obtained by Ehinmidu (2003). The resistant of *Staphylococcus aureus* and *E. coli* to streptomycin in this study is in agreement with the result obtained by Ehinmidu (2003). Randrianirina et al (2007) reported the resistance to of uro-pathogens to third-generation cephalosporins and ciprofloxacin and this is in conformity with this study.

Multiple-antibiotic resistant bacteria are important pathogens and commonly express ESBL enzymes belonging to the SHV family; encoded by blaSHV genes (Jones et al., 2005). Many reports from different countries and regions have showed different prevalence rates of ESBLs producing *Enterobacteriaceae* causing urinary tract infections**.** The occurrence of ESBL-producing *Klebsiella* spp and *E. coli* in this study is similar to the results of Jones et al. (2005) and Ktari et al. (2006). Genes encoding

ESBL such as blaTEM, blaSHV, and blaCTX-M are usually located on conjugative plasmid. The increasingly prevalent of Extended-spectrum B-lactamases producing *E. coli* bacteria have been also reported (Naas et al., 2007; Akinjogunla et al., 2010). The prevalence of ESβL-producing isolates among the post menopausal women may be presumably due to overuse of some antibiotics resulting in selective pressure on new types of betalactamase antibiotics most especially cephalosporins. The production of extended spectrum β- lactamase (ESBL) among *E. coli* and *K. pneumoniae* also contributed significantly to the resistance of these isolates (Jenks *et al*., 1995). Multiple antibiotics resistance (MAR) index is a tool that reveals the spread of bacteria resistance in a given population (Krumpermann, 1983). An MAR index greater than 0.2 implies that the strains of such bacteria originate from an environment where several antibiotics are used. The MAR indices obtained in this study is similar to the valued obtained by Ehinmidu (2003), and this shows that a very large proportion of the bacteria isolates have been exposed to several antibiotics. Moreover these differences in sensitivity profile of the bacteria isolated may be attributed to practices of self medication, the drug abuse and indiscriminate misuse of antibiotics among the post-menopausal women, which has favoured the emergence of resistance strains.

In conclusion, therapy against UTIs should be guided by antimicrobial susceptibilities as increasing numbers of urinary isolates are developing resistance to commonly use antibiotics. Increasing antimicrobial resistance of uropathogens has led to reconsideration of traditional treatment of recommendations in many areas. This retrospective study should be followed by a multicentre study on antimicrobial resistance among post-menopausal women in Uyo and other regions as there is no data concerning the antibiotic susceptibility spectrum of bacteria isolated from Post-menopausal women with UTIs in South-South part of Nigeria have been published to date.

Corresponding Author
E–mail: papajyde2000@yahoo.com
Tel No: +2348064069404

REFERENCES

1. Ahmed, A.A., Osman, H. and Mansour, A.M. 2000. Antimicrobial agent resistance in bacterial isolates from patients with diarrhea and urinary tract infection in Sudan. American Journal of Tropical Medicines and Hygiene. 63: 259–263.
2. Akinjogunla, O J., Eghafona, N. O. and Ekoi, O. H. 2009. Diarrheagenic *Escherichia coli* (DEC): Prevalence among in and ambulatory patients and susceptibility to antimicrobial chemotherapeutic agents. Journal of Bacteriology Research, 1 (3).34–38
3. Akinjogunla, O. J., Eghafona, N. O., Enabulele, I. O., Mboto, C. I. and Ogbemudia, F. O. 2010. Antibacterial activity of ethanolic extracts *of Phyllanthus amarus* against extended spectrum β-lactamase producing *Escherichia coli* isolated from stool samples of HIV sero- positive patients with or without diarrhoea African Journal of Pharmacy and Pharmacology 4(6):402-407.
4. Akinjogunla, O. J. and Enabulele, I. O. 2010. Virulence Factors, Plasmid Profiling and Curing analysis of Multi-drug Resistant *Staphylococcus aureus* and Coagulase negative *Staphylococcus* spp. isolated from Patients with Acute Otitis Media : Journal of American Science 6(11):1022-1033
5. Akortha, E.E. and Ibadin, O.K. 2008. Incidence and antibiotic susceptibility pattern of *Staphylococcus aureus* amongst patients with urinary tract infection (UTI) in UBTH Benin City, Nigeria. African Journal Biotechnolo gy. 7(11):1637-1640.
6. Akram, M., Shahid, M. and khan, A. 2007. Etiology and antibiotic resistance pattern of community acquired urinary tract infection in JNMC Hospital India. Annals of Clinical Microbiology and Antimicrobial. 6(4):1-7.
7. Amali, O., Indinyero, M.D., Umeh, E.U. and Awodi, N.O. 2009. Urinary Tract Infections among Female Students of The University Of Agriculture, Makurdi, Benue State, Nigeria. The Internet Journal of Microbiology. 7 (1):1-5.
8. Beers, M.H., Porter, R.S., Jones, T.V., Kaplan, J.L. and Berkwits, M. 2006. The Merck Manual of Diagnosis and Therapy. 18th edition. New Jersey: Merck Research Laboratories; pp1-85
9. Brosnema, D.A., Adams, J.R., Pallares, R. and Wenzel, R.P. 1993. Secular trends in rates and etiology of nosocomial urinary tract infections at a university hospital. Journal of Urology. 150:414-416.
10. De-Mouy. D., Cavallo, J.D, Armengaud, M. 1999. Infections urinaires en pratique de ville: e´tiologies et sensibilite´ aux antibiotiques en function des ante´ce´dents. Presse Med; 28: 1624–28.
11. Ehinmidu, J. O. 2003. Antibiotics susceptibility patterns of urine bacterial isolates in

Zaria, Nigeria. Tropical Journal of Pharmaceutical Research, 2 (2): 223-228.
12. Fakhrossadat, M. and Narges, S. 2009. Changing patterns in sensitivity of bacterial uropathogens to antibiotics in children. Pakistan Journal of Medical Sciences 25(5):801-805.
13. Foxman, B. 1990. Recurring urinary tract infection: Incidence and risk factors. American Journal of Public Health, 80, 331-333.
14. Foxman, B. 1999. Urinary tract infection in postmenopausal women. Current Infectious diseases Reports. 1(40):367-370
15. Foxman, B. 2002. Epidemiology of urinary tract infections: incidence, morbidity, and economic costs. Diseases. 49(2):53-70.
16. Gupta, K., Sahm, D.F. and Mayfield, D. 2001. Antimicrobial resistance among uropathogens that cause community-acquired urinary tract infections in women: a nationwide analysis. Clinical Infectious Diseases. 33: 89–94.
17. Harding, G. and Ronald, R 1994. The management of urinary infections: what have we learned in the past decade? International Journal of Antimicrobial Agents 4: 83–88.
18. Hima-Lerible, H., Me´nard, D. and Talarmin, A. 2003. Antimicrobial resistance among uropathogens that cause community-acquired urinary tract infections in Bangui, Central African Republic. Journal of Antimicrobial Chemotherapy. 51: 192–194.
19. Hryniewicz, K., Szczypa, K. and Sulikowska, A. 2001. Antibiotic susceptibility of bacterial strains isolated from urinary tract infections in Poland. Journal of Antimicrobial Chemotherapy. 47:773–80.
20. Jenks, P.J., Hu, Y.M., Danel, F., Mehtar, S. and Livermore, D.M. 1995. Plasmid mediated
21. production of class I (AmpC) β-lactamase by two *Klebsiella pneumoniae*. Journal of Antimicrobial Chemotherapy. 35: 235-236.
22. Jones, L.A., McIver, C.J., Kim, M.J., Rawlinson, W.D. and White, P. 2005. The *aad*B gene cassette is associated with blaSHV genes in *Klebsiella* species producing extended-spectrum ß- lactamases. Antimicrobial Agents and Chemotherapy. 49(2): 794-797.
23. Krumpermann, P.H. 1983. Multiple Antibiotics Resistance Indexing of *E. coli t*o identify high risks sources of faecal contamination of foods. Applied Environmental Microbiology 46:165-170.
24. Ktari, S., Arlet, G., Mnif, B., Gautier, V., Mahjoubi, F., Jmeaa, M.B., Bouaziz, M. and Hammami, A.2006. Emergence of multi-drug resistant *Klebsiella pneumoniae* isolates producing VIM- 4 metallo-ß-lactamase, CTX-M-15 extended-spectrum ß-lactamase, and CMY-4 AmpC ß- lactamase in a Tunisian university hospital. Antimicrobial Agents and Chemotherapy. 50(12): 4198-4201.
25. Manges, A.R., Natarajan, P., Solberg, O.D., Dietrich, P.S. and Riley, L.W. 2006. The changing prevalence of drug-resistant Enterobacteriaceae groups in a community: evidence for community outbreaks of urinary tract infections. Epidemiology and Infections. 134: 425– 431.
26. Merila, S., Raisanen, M., Ylitalo, P. and Eskelinen, K. 1987.Microscopic examination of bacteria and cells in urine: International Urology and Nephrology 19(2): 109-113.
27. Naas, T., Oxacelay, C. and Nordmann, P. 2007. Identification of CTX-M-type extended-spectrum- ß-lactamase genes using real-time PCR and pyrosequencing. Antimicrobial Agents and Chemotherapy. 51(1): 223-230.
28. Obaseike-Ebor, E. E. 1988. Trimethoprim / Sulphamethoxazole resistance in *Escherichia coli* and *Klebsiella* spp. urinary isolates. African Journal of Medical Science. 17: 133-140.
29. Randrianirina, F., Jean-Louis, S., Jean-Francois, C., Elisoa, R., Pierre G and Antoine T. 2007. Antimicrobial resistance among uropathogens that cause community-acquired urinary tract infections in Antananarivo, Madagascar. Journal of Antimicrobial Chemotherapy.59, 309–312
30. Shittu, S.O. and Mandere, M.U. 1999. Asymptomatic bacteriuria in antenatal patients in A.U.TH Zaria. Tropical Journal of Obstetrics and Gynecology. 16(1): 41.
31. Sotelo, T. and Westney, O.L. 2003. Recurrent urinary tract infections in women. Curr Womens Health Rep.3; 313-318.
32. Stamm, W. E. and Norrby, S. R .2001. Urinary tract infections: disease panorama and challenges Journal of Infectious Diseases. 183(1): S1-4.
33. Weber, G., Riesenberg, K., Schlaeffer, F., Peled, N., Borer, A. and Yagupsky, P. 1997. Changing trends in frequency and antimicrobial resistance of urinary pathogens in outpatient clinics and a hospital in Southern Israel, 1991-1995. European Journal of Clinical Microbiology and Infectious Diseases. 16: 834- 838.
34. Young, L.Y. and Koda-Kimble, M. 1995. Applied therapeutics: The clinical use of drugs. 6th edition. pp 1-74

Morphology And Wall Structure Of Some Turonian Rudists (Bivalvia, Hippuritoida) Of Gabal Yelleg, Northern Sinai, Egypt

Hosni Hamama

Geology Department, Faculty of Science, Mansura University, Mansura 35516, Egypt
hamamahem@hotmail.com

Abstract: The Turonian succession exposed at the northern extremity of Gabal Yelleg at Northern Sinai yields many rudists. Most of these rudists exhibit polymorphism. Identification, systematic, wall structure and the biostratigraphy of the rudists are made. Rudists encountered are found to belong to: family RADIOLITIDAE Gray, 1848 which includes species related to subfamily RADIOLITINAE Gray, 1948: *Radiolites* cf *polyconilites* Orbigny, *Radiolites peroni* (Choffat), *Radiolites sauvagesi* (d'Holmis-Firmas), *Gorjanovicia costata* Polsăk and *Praeradiolites biskraensis* (Coquand); subfamily BIRADIOLITINAE Douvillé: *Milovanovicia heraki* Polsăk 1968; Subfamily SAUVAGESIINAE Douville´: *Suvagesia sharpie* (Bayle), *Durania* gaensis (Dacque), *Suvagesia nicaisei* (Coquand), *Durania barakatensis* nov. sp, *Durania cornupastoris* (Des Moulins) and *Durania arnaudi* (Choffat) and subfamily LAPEIROUSIINAE Kühn: *Lapeirousella aumalensis* (Douville´). From the family HIPPURITIDAE Gray, 1948 only species *Hippurites (Hppuritella)* cf. *castroi* Vidal was identified. One species among the rudists of Gabal Yelleg is suggested as new species: *Durania barakatensis* nov. sp. Fourteen thin sections representing the described Turonian rudists were prepared to study the wall structure of rudists, and the evaluation of such structure in classification of the studied rudists is discussed.
[Hosni Hamama. **Morphology And Wall Structure Of Some Turonian Rudists (Bivalvia, Hippuritoida) Of Gabal Yelleg, Northern Sinai, Egypt**. Journal of American Science 2010; 6(12):1682-1701]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: Turonian rudists, Bivalvia, Hippuritoida, Gabal yelleg.

1. Introduction:

General view:

Ghorab (1961) divided the Upper Cretaceous in Ras Gharib oil field into five formations, namely: Raha Fm., Abu Qada Fm, Wata Fm., Matulla Fm. And Sudr Chalk. Moon and Sadek (1921) studied the Cretaceous succession exposed at Gabal Yelleg starting from the Lower Cretaceous to the Campanian and Maastrichtian forming the ground of Gabal Yelleg.

Omran, A. M. (1997) divided the Upper Cretaceous succession of Wadi Um Said in the southeastern flank of Gabal Yelleg into Halal Fm. (Cenomanian), Wata Fm. (102.8m, Turonian), Matulla Fm. (63m, Coniacian- Santonian) and Sudr Chalk (77m, Campanian).The lower part of the latter formation, the Markha Member (40m) was assigned to Campanian and the upper part, the Abu Zenima Member (30.8m) was related by Omran to the Masstrichtian.

El-Sabbagh and El-Hedeny (2003) recorded seven radiolitids from the Upper Turonian of the Acteonella Series of Abu Roach. Theses are *Durania cornupastoris* (Des Moulins), *D. gaensis* (Dacque), *D. humei* (Douville´) *Lapeirousella aumalensis* (Douville') *Sauvagesia sharpei* (Bayle) and *S. toucasi* Pamouktchiev and *S. nicaisei* (Coquand).

Abdel-Gawad et al. (2004) recorded four species of rudists from Gebel Yelleg: *Praeradiolites irregularis* Douville´, *Durania arnaudi* (Choffat) and *Praeradiolites ponsianus* (d´,Archiac) from the Middle Turonian, Wata Formation, and *Eoradiolites liratus* (Conrad) from the Lower Cenomanian, Galala Formation).

Aly et al. (2005) identified 17 rudist species from the Cenomanian –Turonian rocks (Halal Fm. and Wata Fm.) of northern Sinai in sections of Gabal El-Minsherah, Gabal Yelleg and Gabal Maaza. These species belongs to genera: *Eoradiolites, Radiolites, Praeradiolites, Distefanella, Bournonia, Durania* and *Ichthyosarcolites.* The geological map of the Gabal Yelleg is given in (Fig. 1) after Omran, A. M. (1997).

In the present work a section was measured representing the topmost part of the Upper Cretaceous succession exposed at the northern flank of Gabal Yellig. A brief description of the measured section is given in (Fig.2):

Lithostratigraphy:

A section of about 130 meters of the Wata Formation was measured and divided into 18 beds. The lithology is mainly represented by marls and limestone and their intercalations. Marl and little shale, dominate the lower part of the section and limestone is the essential component all over the section. The limestone becomes gradually chalky toward the top of the section until it becomes entirely

chalk in the topmost part. Many rudist lisothsomes are encountered from which rudists are collected for this study. A bed of variegated sandstone attaining 3 meters in thickness found in the middle of the section is used as a marker bed. Accordingly, the section is tentatively divided into Lower Turonian and Upper Turonian. The top of the section consists of the chalk which probably related either to the Wata Formation or to the above formation.

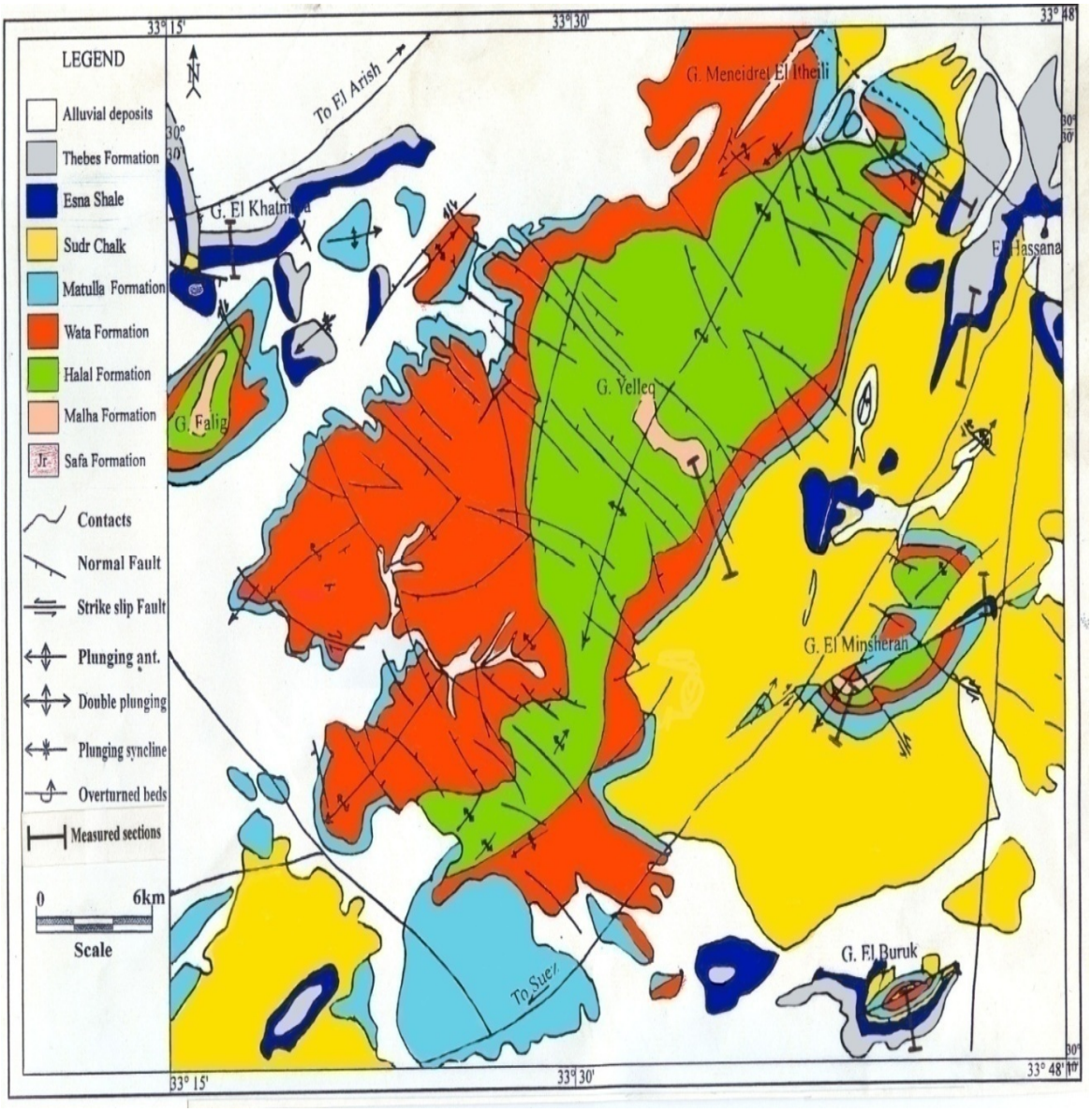


Fig (1): Geological map of Gabal Yelleg (After Omran, A. M. 1997)

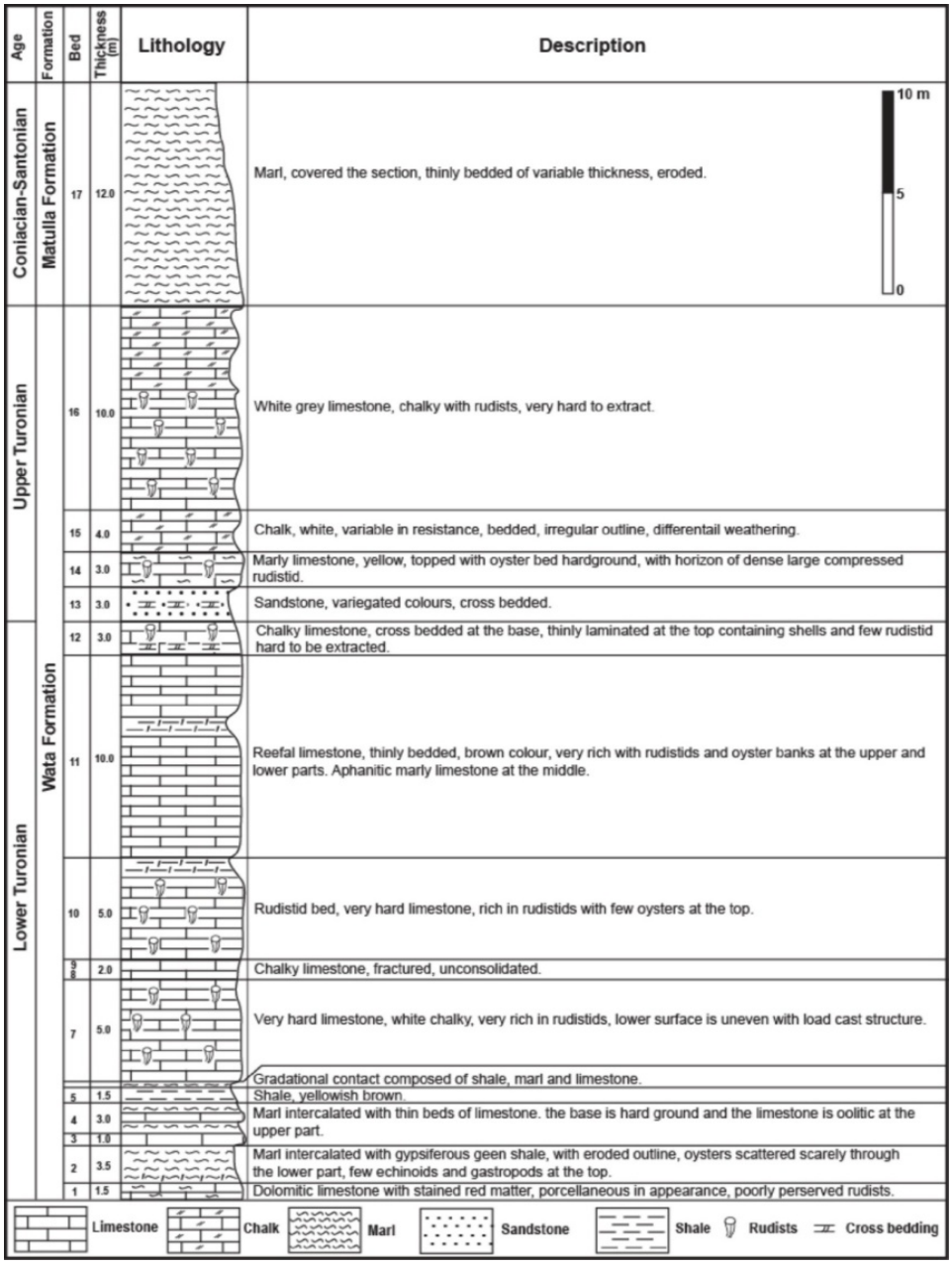


Fig (2): Stratigraphic column of Turonian succession at Gabal Yelleg, north Sinai, Egypt.

2. Materials and methods:

The shell of radiolitids consists of a hypostracum of aragonite and an ostracum of calcite (Zapfe 1937; Kennedy & Taylor 1968; Amico 1978; Cestari & Sartorio, 1995). In the attatched valve (AV) of most radiolitids, three outer layers of ostracum and an inner layer of hypostracum can be distinguished (Sanders, D. &Pons, J.M. 1995 & Sanders, 1999). Sanders recognized three layers in the wall of radiolitids: **(1)** an outermost ostracal layer of delicate calcite lamellae**, (2)** a thick layer of 'boxwork ostracum' built of radial funnel plates and cell walls, **(3)** a thin, inner 'ostracal layer **3'** of thick-walled boxwork, and **(4)** the hypostracum that formed the innermost shell layer (Fig. 3 after Sanders, 1999).

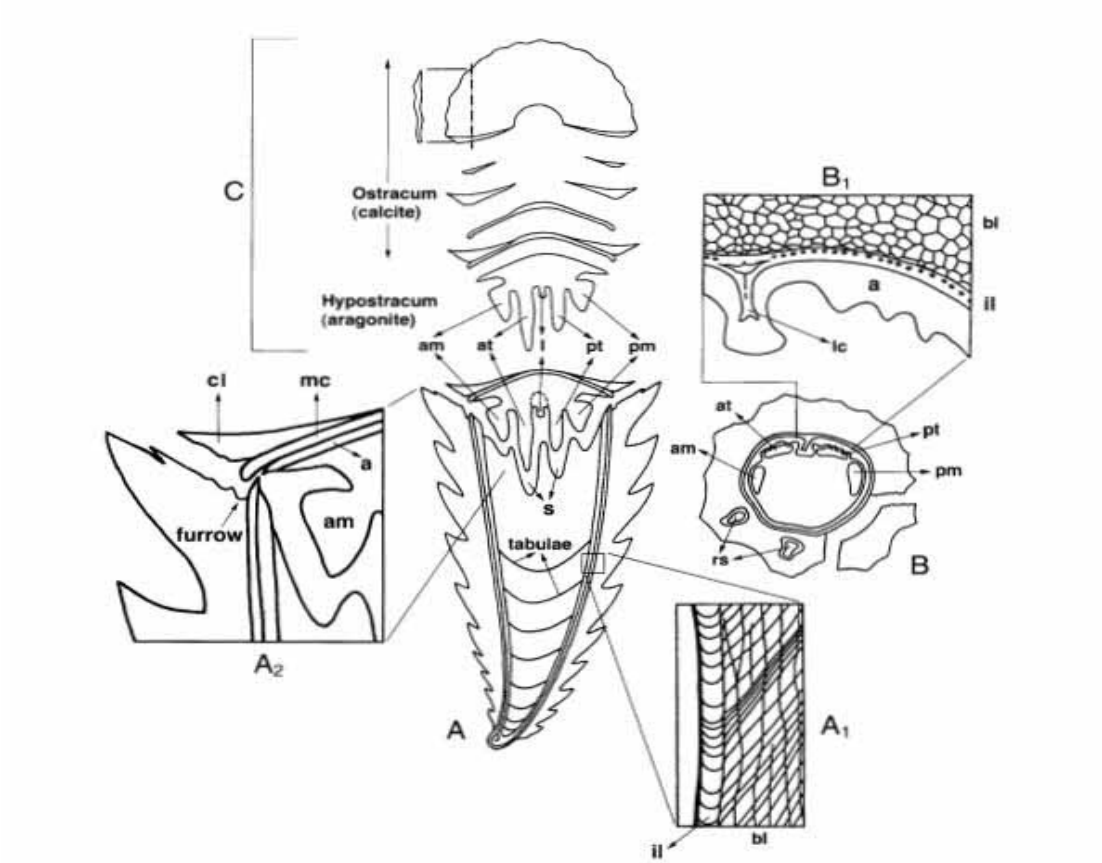


Fig (3): Main features of radiolitid shell (after Sanders, 1999) with some abbreviations: (s) socket; (am) anterior myophore; (pm) posterior myophore (at) anterior tooth; (pt) posterior tooth (L) ligament; (il) inner layer; (bl) thick outer layer of thin-walled boxwork; (a) the aragonitic hypostracum; (mc) layer of massive calcite; (rs) radial structures; ligamentary crest (lc).

The terminology used in description of rudistids in this paper is used after Moore (editor) in the Treatise on Invertebrate Paleontology- Mollusca, vol. 2, 1969, after Yanin, B.T. (1989).

Size: Concerning the size of the mature individuals, the scale proposed by Yanin was accepted, where:
Height (length) Small: up to 5cm; Medium: 6-10 cm, Large: 11-20 cm and Very large: 21-30cm. Bar in all text-figures equals 1mm.

Orientation**: AV:** attached valve**; FV:** free valve.

Ligamental Structures:

Ligamental groove**: (**Ligamental zone**,** LB**)** or furrow on the exterior of the shell on the lateral cardinal side of the lower valve.

Ligamental cavity**:** within the shell wall**.**

Ligamental ridge or pit on the interior of the shell.

L: Ligamental crest (truncated, small truncation and rounded).

Siphonal Structures (pillars, bands, pseudopillars, fossettes, and oscules):

Sp: first pillar

Ep: second pillar

Sb, **Eb**: siphonal bands (smooth, shallow depresses areas) in posterior-lateral side corresponding **S** and **E**.

Es and **Ss**: pseudopillars.

Myophore**:** am and pm, anterior and posterior myophore.

The material is deposited in the Geological Museum of Faculty of Science- Mansura University, Egypt.

SYSTEMATIC PALAEONTOLOGY

Class: BIVALVIA

Order: HIPPURITIDA Newell, 1965

Suborder: HIPPURITINA Newell, 1965

Superfamily: HIPPURITOIDEA Gray, 1848

Family: HIPPURITIDAE Gray, 1848

Genus *Hippurites* Lamarck, 1801

Type species: *Hippurites biloculata;* M

***Hippurites* (*Hppuritella*) aff. *castroi* Vidal (pl. 3, fig. 3)**

1960 aff. *Orbingnya vlasovi:* Bobkova, p. 117, pl. 25, fig.3

1977 *hippurites* (*Hppuritella*) *castroi* Vidal: Pons, pl. X

1989 aff. *Hippurites vlasovi* (Bobkova): Yanin, Pl. XIV, Fig S. 3-6.

Dimensions (mm)**:**				
Specimen no	Length	Width	Diameter opening	wall thickness
……29	61.50	25.40	30.30	?
…….. .30	72.00	22.10	22.10	7.50

Description:
Shell medium sized, LV curved cone to sub cylindrical form, wall thick; transverse section circular; surface covered with numerous longitudinal rounded smooth thin ribs, ribs regularly spaced forming network; anterodorsal aspect, growth laminae crowded in the lower part of the attached valve and widened near the commissure, at posterodorsal aspect four narrow concave siphonal bands and raised interbands having the same thickness as the bands.
Remarks: The described specimens has some affinity to *Hippurites vlasovi* (Bobkova, 1960), but the latter species is vey large and found in a higher stratigraphic level (Maastrichtian).
Locality: beds 15 & 16, Wata Formation, Upper Turonian, Gabal Yelleg.

Family: RADIOLITIDAE Gray, 1848
Subfamily RADIOLITINAE, Gray, 1848
Genus : *Radiolites* Lamarck. 1801
Type species: *Ostracites angeiodes* Picot De Lapeirouse, 1781

***Radiolites* cf. *polyconilites* Orbigny.**
(pl.1, fig.7)
1851: *Radiolites* cf. *polyconilites* Orbigny, pl.547, fig. 3 &4.
Material: one specimen of AV.

Dimensions:	Length (mm)	Width (mm)	commissural diameter	wall thickness
Specimen1.	87.82	22.22	36.50	?

Diagnosis: Horn-shaped AV, smooth with a triangular tooth and two coma shape sockets to receive the teeth of FV; concave radial bands and elevated ligamental ridge in between.
Locality: Gabal Yelleg, bed 1, Lower Turonian.
Geographic distribution: Cretaceous France; Cenomanian, Mexico.

***Radiolites peroni* (Choffat, 1886)**
(pl.1, figs.1a-b & 2)
1886 *Spheriolites peroni*: Choffat, p.33, pl. V, fig. 1-8.
1974 *Radiolites peroni* (Choffat): Atabican & Babkova (in Atlas fauna Azerbaijan), p.220, pl. 116, fig.3.
1981 *Radiolites peroni* (Choffat): Tzankov, p. 183, pl. XCII.
Material: 8 well preserved specimens of AV.

Dimensions:	Length	Width	commissural diameter	wall thickness
Specimen no.2	70.00	37.00	35.00	5.30
……..…..3	50.60	23.00	26.60	9.00
……4	52.80	21.18	24.68	5.70

Diagnosis: conical and horn-shaped AV, deep growth layers, irregular polygonal and vermiform cells, siphonal fossetes structures.
Description: Av medium size, elongate, curved cone, wall very thick, transverse section globular; external surface covered with thick raised folds, zigzag wavy growth laminae; external surface ornamented with longitudinal interrupted sharp ribs; siphonal zones large wide bands. Siphonal furrows longitudinal shallow. Wall structure with compressed elongate vermiform and smaller granular cells and fasciculate aspect (fig.4a-c)
Remarks: the described specimens differ from those identified by Aly *et al.* (2005) as *Eoradiolites sinaiticus* Douvillé: (pl.1, figs. 1-3) by their small size, coarse ribbing and development of fossetes siphonal structures of the outer layer; however a similarity in wall structure is sometimes obvious**.**
Locality: Gabal Yelleg, Wata Formation, bed 7, Lower Turonian.
Geographic distribution: U. Cenomanian: Middle Asia, Portugal, Libya, Egypt, Syria, Azerbaijan, Tajikistan, Little Caucasus; U. Cenomanian - L. Turonian: South France, Albania, Tunisia, Egypt, Iran, Karakorum; U. Cenomanian and L. Turonian: Albania, Tunisia, Egypt, Iran, Little Caucasus; Lower Turonian: Portugal, Armenia, Azerbaijan; Turonian: Albania; U. Turonian: Bulgaria, Romania, Greece, Tunisia, Iran, West China. Upper Cenomanian and Lower Turonian Azerbaijan, Upper Cenomanian Tadzhikistan, Lower Turonian Portugal, Upper Cenomanian – Lower Turonian south France, Albania, Tunisia, Egypt, Iran, Karakorum.(Ali-Zade,1988).

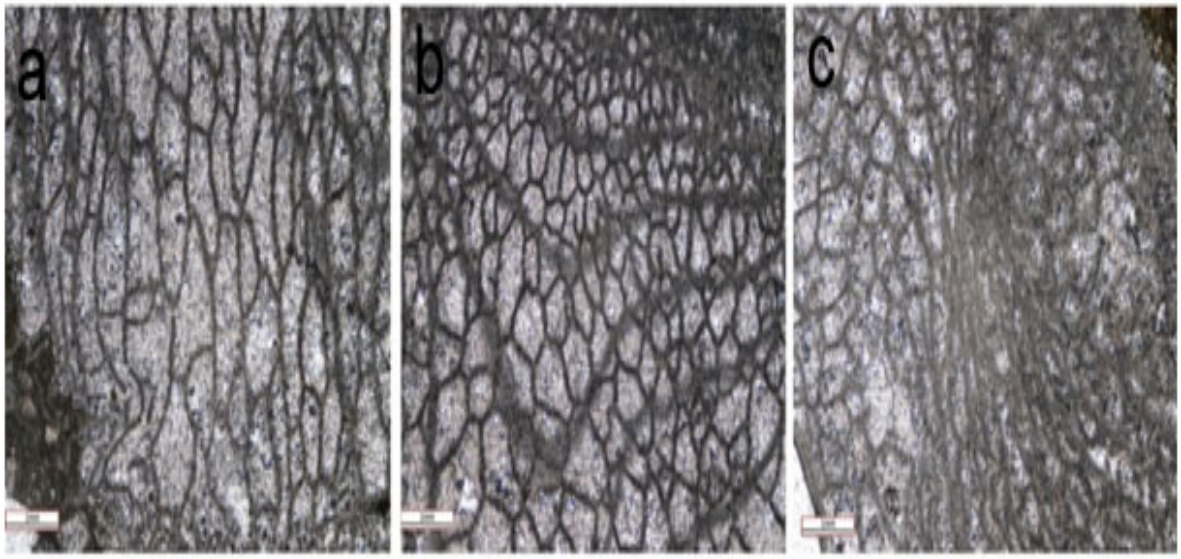


Fig (4) Transverse section in *Radiolites peroni* (Choffat, 1886): a- vermiform elongated cells; b- wavy plates and cellular polygons; c- siphonal fossetes structures. Bar equals 1mm.

Radiolites sauvagesi (d'Holmis – **Firmas, 1838)**

(Pl.1, figs. 3-5)

1851 *Radiolites sauvagesi* Orbigny: Orbigny, pl.553, fig.1-8.
1908 *Radiolites sauvagesi* (d ´Holmis - Firmas): Toucas, p.65, pl.12, fig 10.
1981 *Praeradiolites subtocasi* Toucas: Tzanov et al., p.189, pl. LXXV, fig.3.
2004 *Praeradiolites ponsianus* (d Archiac): Abdel-Gawad, p. 292, pl. 9, figs. 7 & 10.
2005 *Radiolites sauvagesi* (d´Holmis-Firmas): Aly, p. 263, pl.6, figs 4-5 & pl. 7, fig. 1a-b.
2009: *Radiolites sauvagesi* (d´Holmis - Firmas); Gil, *et al*., p.533, fig.6
Material: 10 specimens.

Dimensions:	Length	Width	commissural diameter	wall thickness
Specimen no 5	99.70	39.22	39.22	11.20
…….6	5 0.40	39.60	39.60	12.30
……7	52.00	40.10	39.80	15.20

Diagnosis: dense growth laminae, horn and cylindrical shape, high amplitude wavy plates and closed funnels.
Description: Av medium size, conical and subcylindirical, thick recrystallized wall; transverse section circular; external surface covered with dense wavy zigzagged growth laminae; siphonal bands smooth, concave toward the commissure, Es and Eb well developed, Eb wider than Es., pentagonal cells and closed elliptical funnels of the outer shell layer (fig. 5).
Locality: Gabal Yelleg, Wata Formation, bed 7, lower Turonian.
Geographic distribution. The species was recorded from higher stratigraphic levels than the Turonian.

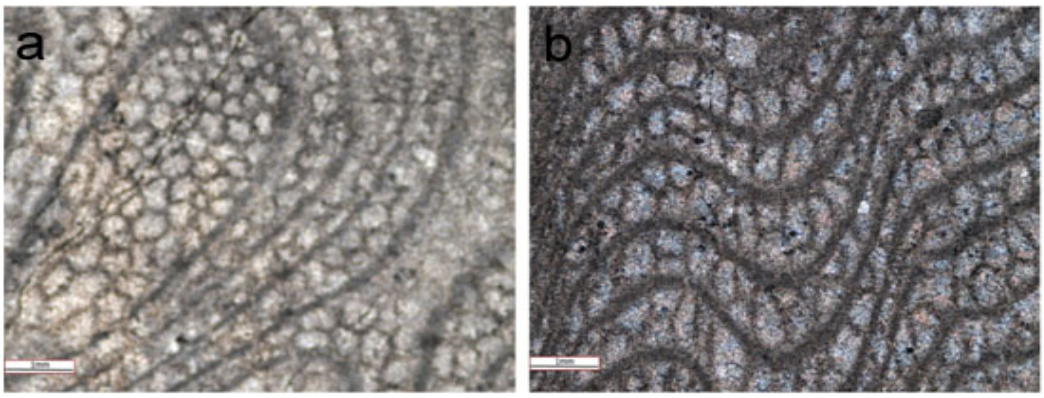


Fig (5) Transverse section of *Radiolites sauvagesi* (d´Holmis-Firmas): a- elliptical funnel plates; b- . ,nm, hz

Genus: *Gorjanovicia* Polsăk, 1968
Type species: *Gorjanovicia costata;* OD.
Gorjanovicia costata Polsăk, 1968
(pl.1, fig. 6)
1960: *Gorjanovicia costata* Polsak: Moore R.C. (ed.); Part N, vol. 2/3, Mollusca 6, N 808, fig E268-
Material: Tow well preserved specimens.
Dimensions: Length (89.66) Width (28.20), commissural diameter (20.50), wall thickness (8.30), specimen no 8.
Diagnostic feature: compressed subcylindirical Av, network of the outer shell layer composing of uniform rhombohedral cells, slightly undulation of plates.
Description: Av medium sized, thick-walled, elongate, cylindrical-conical, slender, slightly impressed with long dimension normal to the commissure and extending from the apex of the valve to commissure; longitudinal ribs salient, ribs thin and inter- ribs regularly spaced and relatively broad and deep, with fine zigzag growth layers, pointed upward; siphonal zones well developed Sb and Eb and Sb concave broader, both with dense flat laminae; ligamental groove in the exterior of the shell, very narrow pit on the interior of the shell; network of the outer shell layer composing of uniform rhombohedral cells; laminae with a definite pattern of arrangement of repetitions of two or three narrow spaced layers followed by fine distant layers crossed by muri; inner layer thin with fragments of disaggregate rod-like spines (fig. 6).
Remarks: The rhombs of the network differentiate the described specimen from the lamellar structure shown in *Sauvagesia nicaisei* (Coquand) figured by El-Sabbagh & El-Hedeny (2003, pl. 7, fig 2. Also the internal structure of *Praeradiolites* cf *irregularis* Douvillé described by Aly et al (2005) is similar to the present species; both are quite different morphology.
Locality: Gabal Yelleg, bed 11, Wata Formation, Lower Turonian
Italy, Yugoslavia and Turkey.

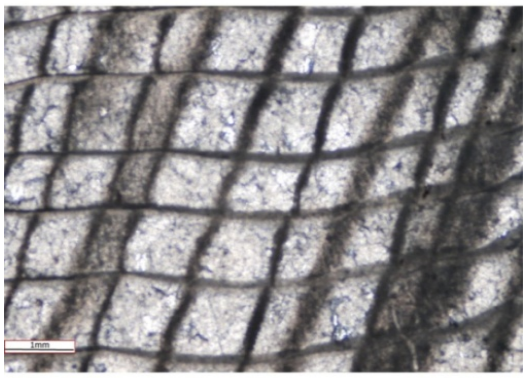


Fig (6) Transverse section of *Gorjanovicia costata*: compact rhombohedral network.

Praeradiolites Douvillé, 1902
Type species: *Radiolites fleuriaui* d´Orbigny, 1842
***Praeradiolites biskraensis* (Coquand, 1880)**
(pl. 3, fig. 1)
2004 *Praeradiolites biskraensis* (Coquand): Abdel-Gawad et al., p.292, pl.9, fig.1.
Material: one well preserved AV.

Dimensions:	Length	Width	commissural diameter	wall thickness
Specimen no 9	88.82	47.66	44.52	9.52

Diagnosis: Strong longitudinal regular folds, concave bands and prominent ridge.

Description: Attached valve (AV) medium size, conical shape, thick recrystallized wall, broad-rounded posterior margin and very narrow anterior margin; surface coarsely reticulate, ornamented with strong longitudinal regular folds, nearly 16 ribs pre diameter 20.5mm assuming bundle- shape of longitudinal digits, unequally spaced, condensed and sharp in early stage and large and divergent near commissure; growth laminae regularly spaced and crowded as growth proceeds ; growth laminae covered the surface, concave upwards at the ribs and convex downward in inter-ribs; siphonal furrow shallow longitudinal slit bounded by rounded raised prominent siphonal ridge; internal shell cavity narrow ellipse, anterior myophore (ma) large kidney- shaped tangent the inner wall, posterior myophore (mp) inclined at the inner wall.

Locality: Wata Formation, bed13, Upper Turonian, Gabal Yelleg.

Locality: Gabal Yelleg, Wata Formation, bed 7, Lower Turonian.

Subfamily SAUVAGESIINAE, Douvillé, 1908
Subfamily BIRADIOLITINAE Douvillé, 1902
Genus: ***Milovanovicia*** Polsăk, 1968

Type species: *Milovanovicia heraki;* OD.

***Milovanovicia heraki* Polsak 1968**
(PL. 2, figs. 1-5)

1969 *Milovanovicia heraki* Polsăk: Moore (editor), Mollusca 6. Bivalvia, p.N810, fig271, 1
2007 *Milovanovicia heraki* Polsăk: Bilal Sari & Sacit Ozer.

Material: 10 specimens.

Dimensions:	Length	Width	commissural diameter	wall thickness
Specimen no 10	150.00	21, 60	59.62	8.13

Description: AV cylindo-conical to conical, very elongate, slightly curved. External ornamentation consists of few large massive, elongate folds separated by deep furrows; both with fine longitudinal striae; ligamental structure on the exterior of the shell very deep; siphonal bands smooth or finely costulate (fig. 7).

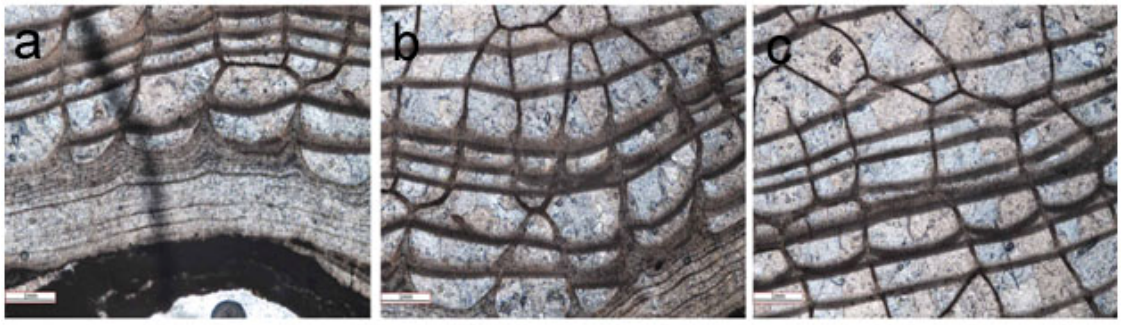


Fig (7) Transverse section of Radiolites *Milovanovicia heraki* Polsăk: a. of the exterior of the outer layer; b. tow radial bands (Eb, Es) and interband (ib); c. thin inner layer and irregular polygons of the outer layer.

Locality: Gabal Yelleg, Wata Formation, bed 16, Upper Turonian.

Geographic distribution: Middle Upper Turonian, Turkey; Turonian Yugoslavia; Upper Turonian.

Subfamily SAUVAGESIINAE Douvillé, 1908
***Suvagesia* Choffat, 1886**
***Suvagesia sharpei* (Bayle, 1857)**
(Pl.2, fig.6)

1886 *Suvagesia sharpei* Bayle: Choffat, p.29, pl.29, pl. 4, fig. 1.
1902 *Suvagesia sharpei* (Bayle): Choffat, p.171, pl.8, fig. 14.
1969: *Vautrinia Syriaca* (Vautrin): Moore R.C. (ed.); Part N, vol. 2/3, Mollusca 6, N777, pl. 243 , fig. 5-6 and N815, pl. 274, fig. 2.
1974 *Vautrinia Syriaca* (Vautrin, 1933): Atabican , A.A and Babkova, H. H., P.221, Pl. 117, fig. 1-2, pl. 118, fig.1-2.
1977 *Hippurites* (*Orbigny*) *radiosus* Des Moulins: Jose Maria Pons, pl. XXX, fig. 1-2.
2003 *Suvagesia sharpei* (Bayle): El-Sabbagh & El-Hedeny, p.252, pl.3, figs. 2-4.

Materials: Two fragments of AV.

Dimensions: length of AV = 70.20mm, commissure diameter = 59.60.mm, specimen no 15.

Diagnosis: cylindrical Av, regular ribbing and growth laminae and network of uniform polygons**.**
Description: AV very large, cylindrical, wall very thick; surface covered by thin rectilinear ribs with nearly equal spaces between them, thin regular growth layers concave toward the commissure; siphonal bands large and flat finely costulate, Eb broader than Es. Cellular structure consists of rectangular polygons forming with the parallel muri a net work of uniform polygons. Siphonal bands consist of well developed funnels (fig. 8).
Locality: Locality: Occurrence: Bed 13, Wata Formation, Upper Turonian, Gabal Yelleg.

Geographic distribution: Upper Cretaceous, Syria, Turkey, Iran, Azerbaijan and Egypt.

***Suvagesia nicaisei* (Coquand, 1826)**
Pl. 2, figs 7-8)
1862 *Suvagesia nicaisei:* Coquand, p. 223, pl, 17, fig. 12.
2003 *Suvagesia nicaisei* (Coquand): El-Sabbagh & El-Hedeny, p.251, pl. 2, figs. 5-6.
Material: 6 specimens of AV.

Dimensions:	Length	Width	commissural diameter	wall thickness
Specimen no 16	111.2O	?	63. 00	52.00
……17	130.00	?	56.0	12.00

Diagnosis: Long and large AV, conical shape, deep narrow smooth radial costulate bands, well developed ligamental cavity, sloping wide growth laminae
Description: Av conical with broad commissure, ornament with radial thin ribs, inter-ribs regularly spaced; growth layers broad and regularly spaced; siphonal bands slightly concave finely costulate folds, interbands narrow.
Locality: Gabal Yelleg, Wata Formation, bed 16, Upper Turonian.

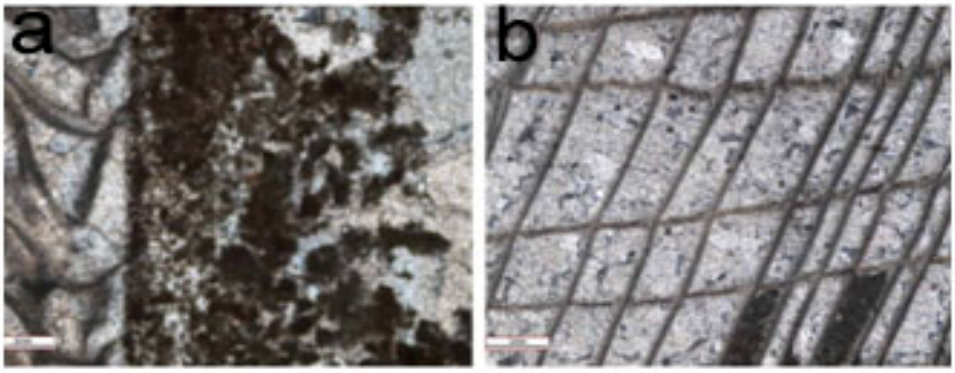


Fig (8) Transverse section of *Suvagesia sharpei* (Bayle): a. radial band with funnel shaped laminae; b. radial rectangular cells and parallel muri, sample no 15.

Genus: ***Durania*** Douvillé, 1908
Type species: *Hippurites cornupastoris* Des Moulins, 1827, p. 288; OD

***Durania barakatensis* nov. sp.**
(pl. 3, fig. 2a-c)
Derivation of name: on the honor of Gabir Barakat, professor of Paleontology, Cairo University.

Dimensions:	Length	Width	commissural diameter	wall thickness
Specimen no 18	83.10	41.10	50.60	9.70
…… 19	87.00	49.30	48.10	6.48

Description: AV vase-shaped, short cone, medium size, expanding upward rapidly; surface ornamented with folded laminae and sharp radial ribs, inter-ribs wide and slightly concave; radial bands narrow deeply concave, with one sharp rib carrying chevrons pointing downward; interbands wider than bands, ridge with growth layers concave upward; wall relatively thick; transverse section subcircular to quadrate; inner layer thin composing of 7 wavy laminae divided by pillars; cells of the outer layer increase in size toward the periphery, polygons rounded near the inner layer and becoming rectangular toward the commissure; rod-like bearing bifurcated feather-like structure (fig. 9).
Locality: Beds 11 & 13, Gabal Yelleg, Wata Formation, Upper Turonian.

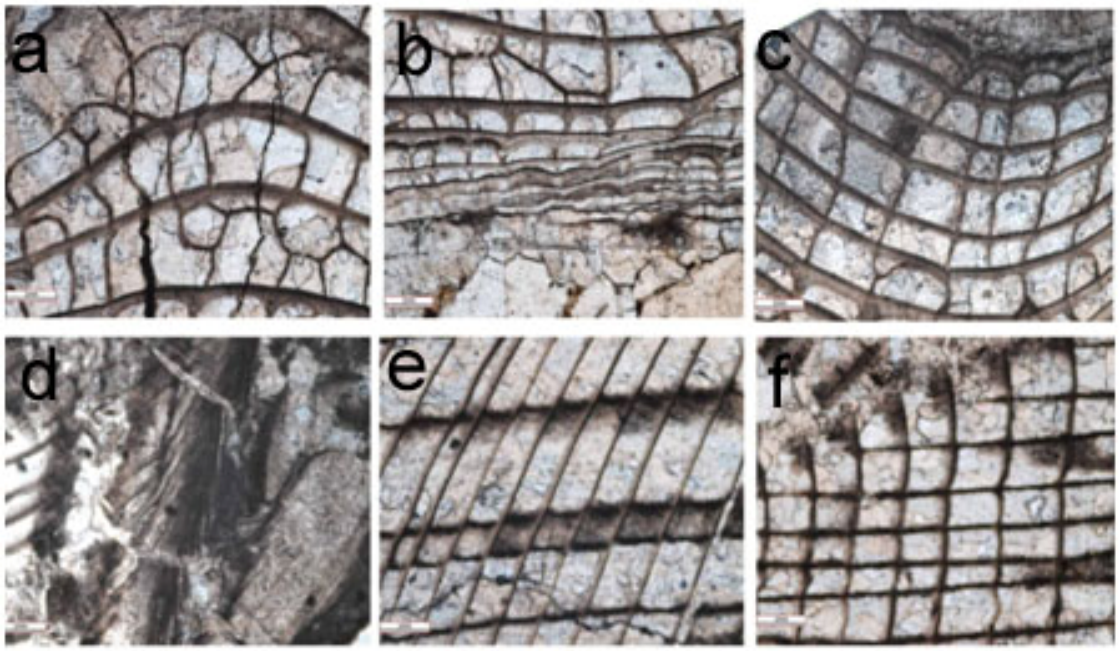


Fig (9): Transverse section of *Durania barakatensis* nov. sp. showing irregular polygonal muri, laminated inner layer, regular semi-quadrate polygons, branching funnels, network of elongated and quadrate cells from a to f respectively.

***Durania gaensis* (Dacque, 1903)**
(Pl. 4. Fig.1a-c)

1903 Radiolites *gaensis* Dacque: Dacqué, p. 374, pl. 35, figs. 7-9.
1903 *Praeradiolites boucheroni*: Toucas, p. 32, pl. 3, fig. 10-12
1977 *Hippurites* (*Orbignya*) *toucasianus* d' Orbigny: Jose Maria Pons, pl. XL, fig. 1.
2003 *Durania gaensis* (Dacqué): El-Sabbagh & El-Hedeny, p.284, pl.1, figs. 5-6)
2004 *Durania gaensis* (Dacqué): Abdel-Gawad *et al.*, p. 292, pl.9, fig.6.

Dimensions:	Length	Width	commissural diameter	wall thickness
Specimen no 20	88.82	47.66	44.52	9.52
……21	75.80	38.40	45.20	10.90
…….. …..22	77.10	31.4	48.90	14.20

Description: AV vase shape, conical to subcylindirical, shell beginning very narrow, after that increases by continuous accretion gradually during growth; radial bands deeply concave, very narrow, with fine chevrons, 5 in number, interband bud-shaped, broader than the bands; external surface ornamented with folded branching laminae; thick of regular polygons,, radial bands mostly with hexagonal polygons, ligamental pit in the interior of the shell (fig. 10)
Locality: Gabal Yelleg, bed 11, Wata Formation.

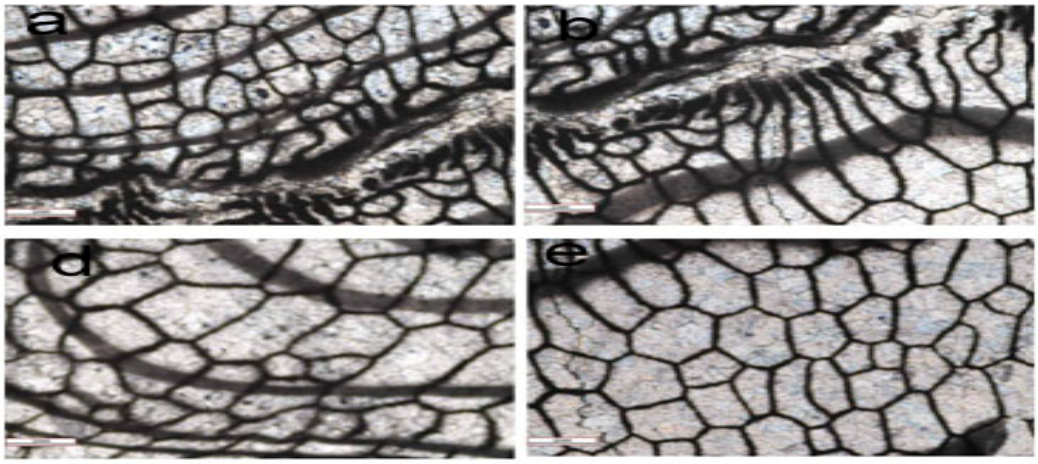


Fig (10): transverse section of *Durania gaensis* (Dacqu`) showing free muri (a &b) ending in subcircular bores, pentagonal, hexagonal or more faces and curved laminae (c & d).

Durania cornupastoris (Des Moulins)
(pl.3, fig. 3a-c)

Material: One good preserved specimen of AV.

Dimensions:	Length	Width	commissural diameter	wall thickness
Specimen no 23	82.20	16.20	34.80	9.4019

1969: ***Durania cornupastoris (***Des Moulins): Moore (editor), Mollusca 6, N.813, fig. E 272, 4.
2003: ***Durania cornupastoris (***Des Moulins): El-Sabbagh & El-Hedeny, pl.1, fig. 1-4.
Description: AV conical, curved, medium sized; transverse section oval; external wall ornamented with fine widely spaced ribs, inter ribs flat to slightly concaved; growth layers zigzagged with chevrons in the inter ribs and inverted chevrons across costae; ribs coarse in the ventral aspect; Siphonal bands Sb and Eb finely costulate, cells hexagonal in siphonal bands, normal the commissure, laminae folded (8 crenulations) in the outer part of the outer layer (fig. 11)
. **Locality**: Gabal Yelleg, Wata Formation bed 14, Upper Turonian.
Geographic distribution: Upper Turonian in the Tethyan Province.

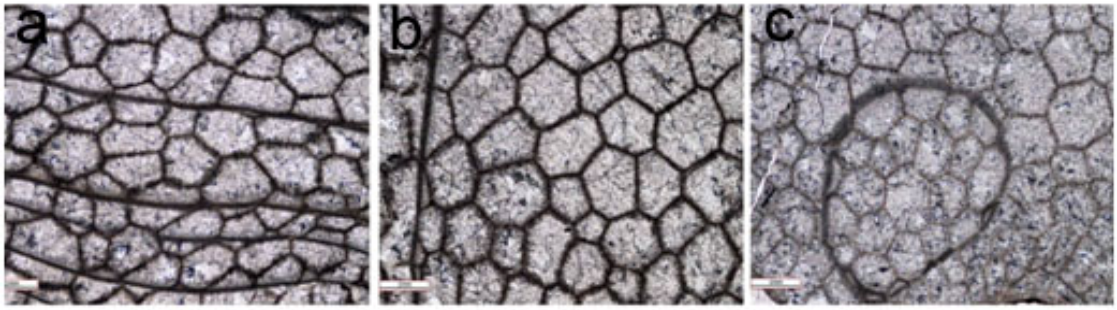


Fig (11): Transverse section of *Durania cornupastoris (*Des Moulins) showing: a-shell outer layer and superposed lamellae, b- one siphonal band with compact polygons (6-7 faces), c- ligamental pit.

Durania arnaudi (Choffat, 1891)
(pl.4, figs. 3-8 & 10-11)

1891 *Durania arnaudi:* Choffat, pp.203, 210 and 211.
1901 *Biradiolites arnaudi*: (Choffat); p. 138, pl. VI & pl. VII.
2004 *Durania arnaudi* (Choffat), Abdel-Gawad et al. p.289, pl 9, figs 4-5.
2005 *Durania arnaudi* (Choffat), Aly et al., p. 273, p. 10, Fig. 9.

Material: Ten complete and fragments of AV.

Dimensions:	Length	Width	commissural diameter	wall thickness
Specimen no 24	30.00	24.00	24.00	0.80
……25	26.00	25.oo	25.00	0.80
…… 26	55. 70	20.20	22.00	0.50
…… 27	71.90	30.00	38.00	0.70

Description: Shell medium sized, according shape three forms are presented: typical cylindrical, conical with curved anterior part and rounded commissure, conical with elliptical commissure; surface covered with sharp fine rounded radial uniform ribs with flat to slightly concave smooth inter-ribs; wall relatively thick, crystallized with finger print – like; ligamental cavity within the shell wall and ligamental ridge in the interior of the third mentioned form (fig. 12) and (pl. 4, fig.7b); siphonal structures with well developed Eb and Sb bands, the bands deeply concave separated by raised interband (ib).

Remarks: The conical form with broad commissure is similar to *Durania cornupastoris* (Des Moulins) described by El-Sabbagh & El-Hedeny (2003) in plate1, fig 2&4 from the Upper Turonian of el-Hassana Dome in Abu Roach area, but our mature shells are smaller in size. On the other hand, the typical cylindrical form is a typical the same species figured by Abdel-Gawad *et al.*, (2004) from the Middle Turonian of Gebel Yelleg and those of Aly *et al.* (2005) from the Turonian of the same locality.

Locality: Gable Yelleg, bed 11, Wata Formation, Lower Turonian.

Geographic distribution: Tethyan Province.

***Durania humei* Douvillé 1913**

(Fig. 13)

1913 *Durania humei*: Douville, p.254, pl.16, figs. 3-5

2003 *Durania humei* Douville: El-Sabbagh & El-Hedeny, p.248, pl. 2, figs. 1-2.

2004 *Durania arnaudi* (Choffat, 1891): Abdel-Gawad et al., p292, p. 9, fig.4-5.

Material: Two specimen of AV.

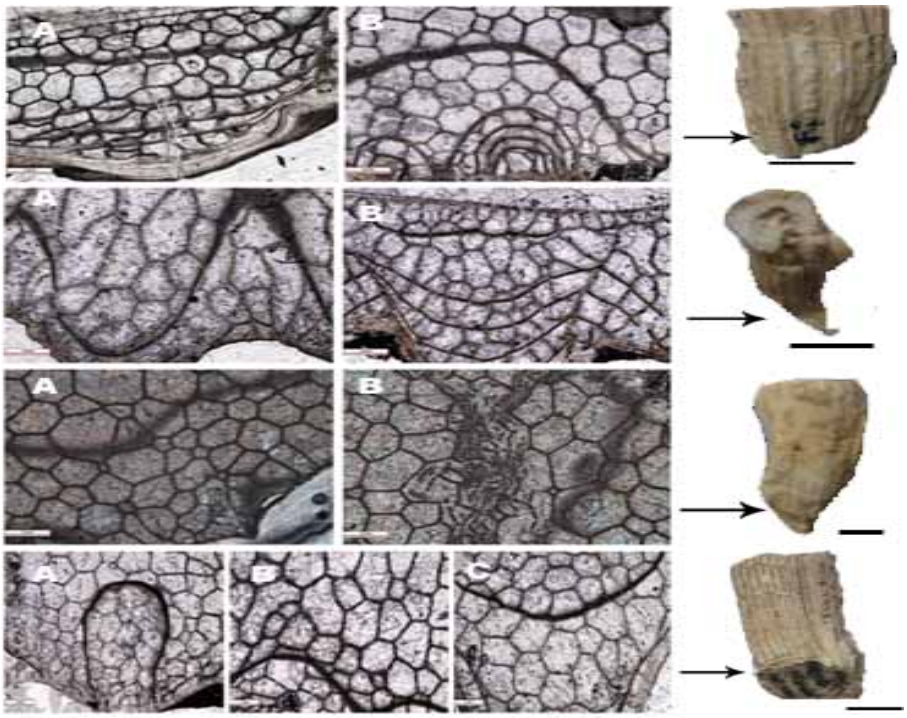


Fig (12) Transverse sections of *Durania arnaudi* (Choffat, 1891): first row- typical cylindrical form with radial structure (rs) in the outermost ostracal layer; second row- conical form showing the sockets for the teeth of the FV and ligament in between; third row-conical form with elliptical commissure showing relic shell by sapling of the ostracal layer; fourth row- typical cylindrical form showing the ligamentary crest (lc), with the hypostracal, aragonitic shell layer (a) indicated.

Dimensions LV (mm):				
Specimen no 29	Length	Width	commissural diameter	wall thickness
	62.00	40.5o	42.50	36.00

Diagnosis: Ligamental groove, deep narrow siphonal bands and different number in faces of polygons.
Description: Av conical shape, wall thick; surface covered with dense thin radial ribs; siphonal bands deep and narrow, Eb slightly deeper , interband broad, raised and broader than bands; presence of ligamental groove, pentagonal-hexagonal and pentagonal polygons, some cells oblique and normal to axial radial lamminae and the others parallel to lamminae (fig. 13).
Locality: bed 7, Wata Formation, Lower Turonian, Gabal Yelleg.

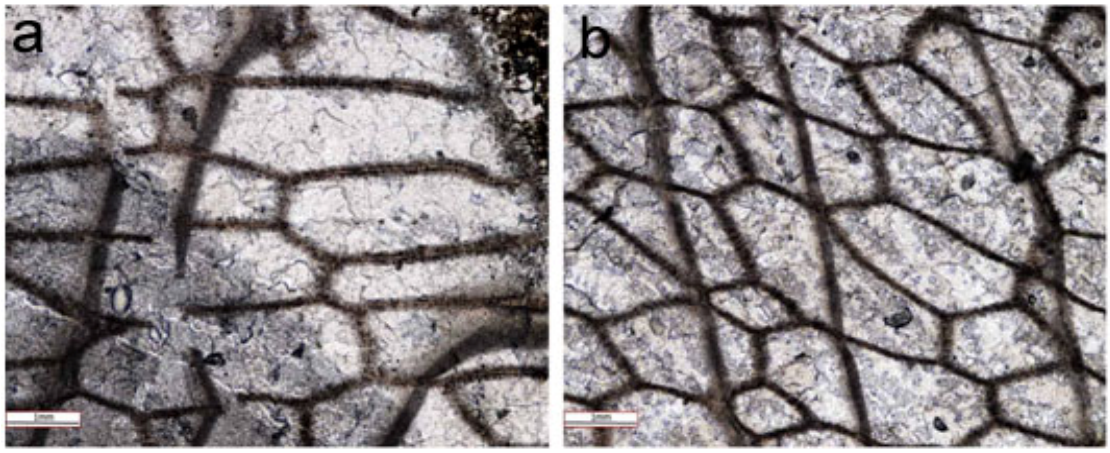


Fig (13): Transverse section of *Durania humei* Douvillé showing: a- elongated polygonal cells; b- polygons oblique to the siphonal bands.

Subfamily LAPEIROUSIINAE Kühn, 1932
Lapeirousella Milanovanović, 1938
***Lapeirousella aumalensis* (Douvillé, 1915)**
(pl. 4, fig. 9)
1915 *Lapeirousia aumalensis*: Douvillé, p. 26, text-fig. 1.
1988? *Durania bertholoni* Yanin**:** Yanin, (in Cretaceous fauna Azerbaijan: editor, Ali-Zad et al.), p.288, pl. XVII, fig. 6; pl. XVIII, fig.1-3, pl. XIX, FIG.1.
1989? *Durania bertholoni* Yanin**:** Yanin, P.288, pl. XIV, fig. 1-2
2003 *Lapeirousella aumalensis* (Douvillé):El-Sabbagh & El-Hedeny, p. 250, pl. 2, figs. 3-4.
Material: 10 specimens deformed perpendicular to commissure.

Dimensions:	Length	Width	commissural diameter	wall thickness
Specimen no 30	90.00	50.00	50.00	10.00

Diagnosis: conical to subcylindrica with more or less regular longitudinal ribs, outer layer with braided and structure surrounding cavities, pseudopillar giving rise to ropy structure.
Description: AV medium to large size, elongate, subcylindrical, slender, deformed; transverse section elliptical shape, narrow squeezed; surface of the attached valve covered on the dorsal side with radial straight thin dense similar, longitudinal ribs strong and raised between bands on the ventral aspect; Sb and Eb shallow depressed separated by terraces which covered by radial costae, Eb wider than Sb (fig. 14).
Remarks: the described specimens have some affinity to *Durania bertholoni* Yanin which identified from the Upper Cretaceous of Azerbaijan.(1988, p. 288, pl. XVII, fig. 6, pl. XVIII, fig. 1-3 and pl. XIV, figs. 1-2)
Locality: Gabal Yelleg, Wata Formation, bed 14, Upper Turonian.
Geographic distribution: ? Upper Coniacian of Caucasus, Coniacian of Tunisia, and Turonian of Egypt.

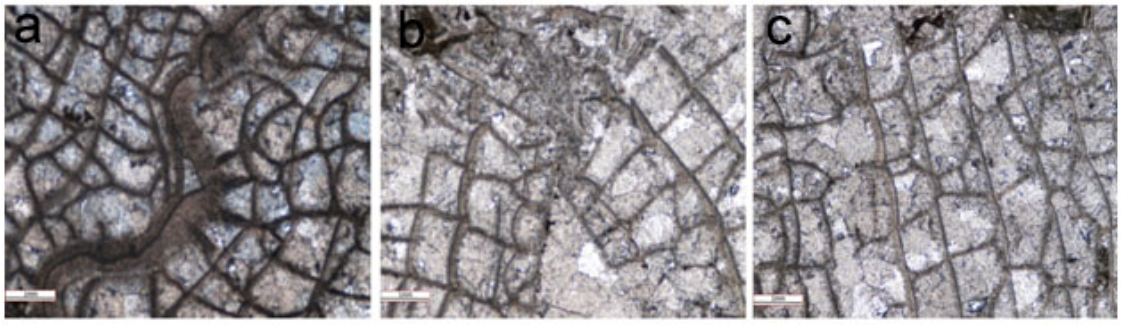


Fig **(14):** Transverse section of wall of ***Lapeirousella aumalensis*** **(Douvillé) showing: a- pseudopillar and longitudinal layers; b & c braided structure surrounding ligamental cavity parallel to commissure.**

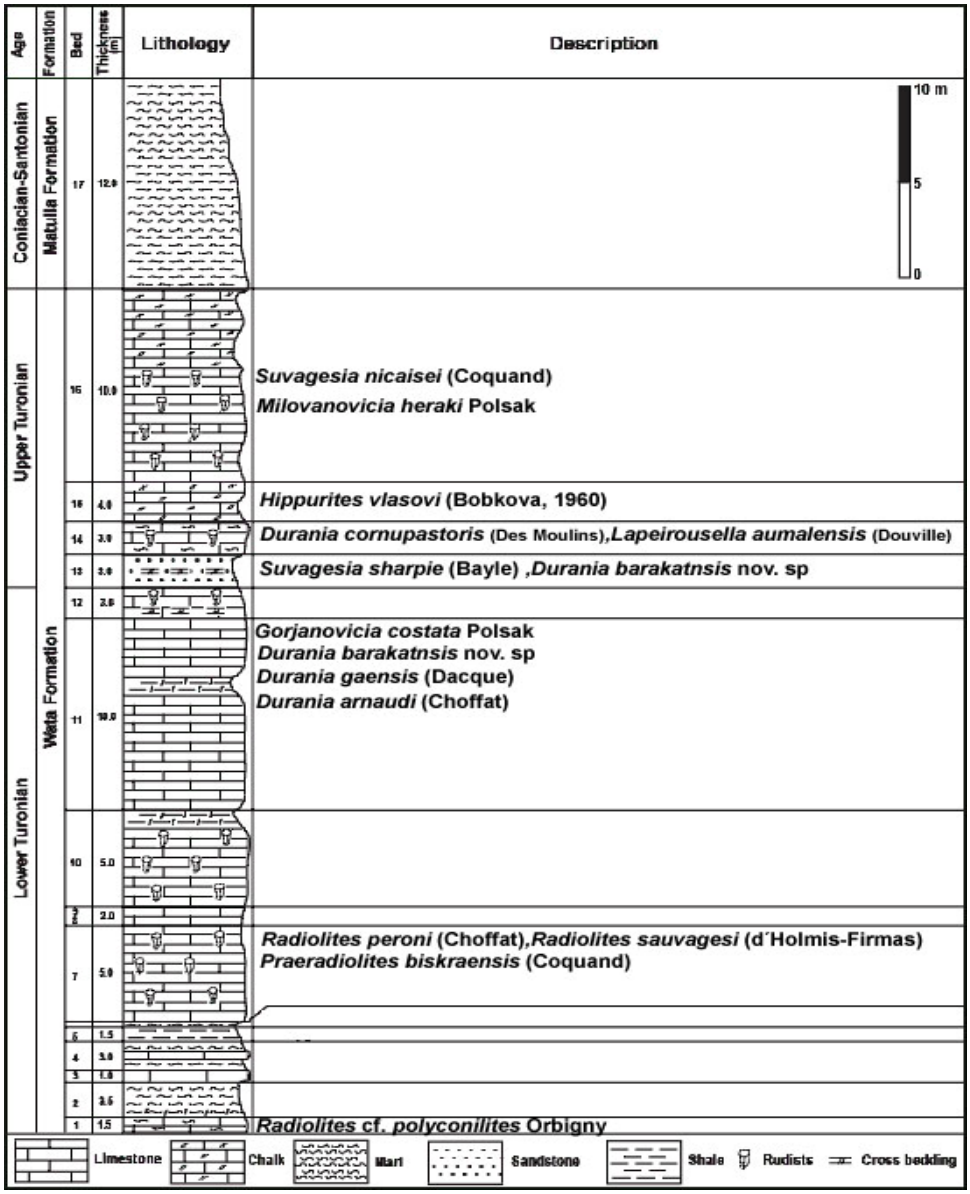


Fig (15): Distribution of the identified rudists though The Turonian rocks of Gabal Yelleg, North Sinai.

MORPHOLOGIAL VARIABILITY AND SIGNIFICANCE OF WALL STRUCTURE

Cretaceous rudist formations have long been studied with respect to paleontology, sedimentology and diagenesis (e.g. Toucas 1903; Zapfe 1937; Kühn 1967; Skelton 1976, Skelton *et al.* 1995; Bebout &Loucks 1977; Pons 1977, 1982; Enos 1988; Minero 1988; Köch et *al.* 1989; Ross & Skelton 1993; Sanders 1998a; Sanders & Baron-Szabo 1997; Sanders ***et*** *al.* 1997; Sanders & Pons 1999.

In general radiolitids demonstrate wide intraspecific morphological variability (Gill, *et al.*, 2009; Senders, 1999; El-Sabbagh and El-Hedeny, 2003). Such variability markedly appears in rich assemblage, as the number of species and individuals increase the variability is well demonstrated. Concerning the studied specimens the some remarks of the variability in form and ornament are observed in the following species:

1- *Radiolites peroni* (Choffat, 1886): Two forms are recorded: horn and conical form with well developed concave tabulae and branched muri. The outermost ostracal layer is not preserved, it may be disintegrated. A thick layer of 'boxwork ostracum' is built of radial funnel plates and cell walls. The cells take two forms: cellular structure of pentagonal and hexagonal polygons and vermiform structure of elongated polygons with 2mm diameter. Very characteristic is the presence of siphonal fossetes structures (fig. 4).

2- *Radiolites sauvagesi* d'Holmis – Firmas, 1838): There are two forms, horn and cylindrical. Radial funnel plates are well developed, closed funnel is a characteristic feature for this species (fig. 5).

3- *Gorjanovicia costata* Pols˘ak, 1968: A profound feature of this radiolitids is network of the radial bands composing of rhombs of calcite and the narrow interbands (fig. 6).

4- *Milovanovicia heraki* Polsăk 1968: It is very large among all the studied specimens. Its wall is characterized by (a) an outermost thick ostracal layer of delicate calcite lamellae and the ligamental crests (lc). The latter penetrate the inner hypostracum that form the thin inner layer (il). The thick outer layer (bl) has a well developed radial band (rb) and interband (ib). Muri form what look-like ropy structure and the polygons are irregular hexagonal (fig. 7)

5- *Suvagesia sharpei* (Bayle): The cylindrical form is the only recorded form in this species. The Lamellar network and a well defined funnels are well developed (fig. 8).

6- *Durania barakatensis* nov. sp.: It is one of the large species in the rudist assemblage. The main features of the wall are highly laminated hypostracum with ligamental crests, rhombs of calcite, radial lamellae, branching muri, thick outer layer and branching funnels (fig. 9).

7- *Durania gaensis* (Dacqué,1903): The characteristic features of such species include a typical honey bee cellular structure of the inner layer, free muri ending in a vesicular pores and curved radial laminae(fig. 10)

8- *Durania cornupastoris* (Des Moulins): It has broad radial bands of hexagonal polygon and vesicular pore (fig. 11).

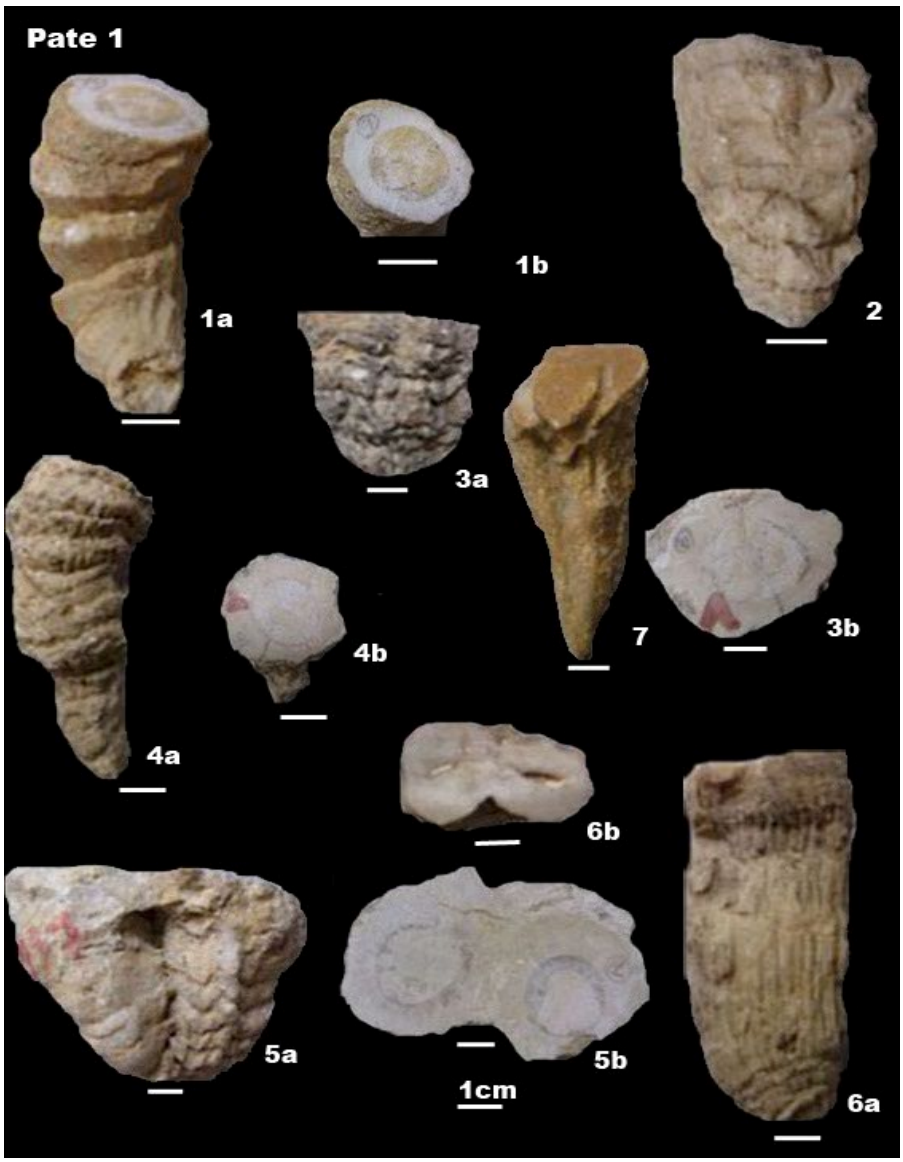
9- *Durania arnaudi* (Choffat, 1891): This species exhibits highest variability in form among the identified rudists. Three forms are recognized including conical form with rounded commissure, conical form with elliptical commissure and typical cylindrical form. The aspects include disintegrated laminae in the inner layer (fig, 12)

10-*Durania humei Douvillé* 1913: The large, oblique polygons and the developed radial bands are very characteristic features of the species (fig.13)

References:

1. Abdel-Gawad, G.I, El-Sheikh, H.A., Abdel Hamid, M.A., El-Beshtawy, M.K., Abed, M.M. and Qot, G.M. (2004a): Stratigraphic studies on some Upper Cretaceous successions in Sinai, Egypt. Egypt Jour. Paleontol., Vol., 4, p.263-303.
2. Abdel-Gawad, G.I, Orabi, O.H. and Ayoub, W.S. (2004b): Macrofauna and biostratigraphy of the Cretaceous section of Gebel El-Fallig area, northwest Sinai, Egypt. Egypt Jour. Paleontol., Vol. 4, p.305-333.
3. Akobiana,V. T. ed. (1974): Atlas fauna Armenia. AH Armenia CCCP, EREBAN, 836pp.
4. Ali Zad (1988): Cretaceous Fauna Azerbaijan. Publication Academic Science Azerbaijan, ELM puplication , Baku 455 p.
5. Aly, M.F., Saber, S.G., Abdel-Gawad, G.I and Ferieg, Y.F. (2005): Cenomanian-Turonian rudists of Northern Sinai, Egypt. Egypt. Jour. Paleontol., vol. 5, p. 253-286.
6. Amico, S. (1978): Recherché sur la structure du test des Radiolitidae. *Travaux du Laboratoire de Gdologie historiqrre* et *de Palkontologie* 8, 1- 131. Universite de Provence, Marseille.
7. Atabkian, A.A. and Bobkova (1974): Cretaceous molluscan bivalvs., Atlas fossil Fauna Armenia USSR..Erivan Science Publisher, pp 211-221.
8. Babkova, H.H. (1960): New Upper Cretaceous rudists SW Middle Asia. Govergeoltec, vol. 2, p.p 115-120.
9. Bebout, D.G. & Loucks, R.G. (eds.) 1977: Cretaceous carbonates of Texas and Mexico. Applications to Subsurface Exploration. *Bureau of Economic Geoiogy and University of Texas at Austin, Report oflnvestigations* 89. 332 pp.
10. Cestari, R. & Sartorio, D. (1995): Rudists and Facies of the Periadriatic Domain .p 207 Agip, San Donato Milanese.
11. Choffat, P. (1886-1901): Faune Crétacique du Portugal.lisbonnequand, H. (1862): Geologie.
12. Coquand. H. (1862): Geologie et paleontology de la region sud de la province de Constantine, mem. De la Soc. d`Emulation de la Provence, 2:5-342, 31 figs, 35 pls.

13. Dacqué, E. (1903): Mittheilungen Uber den Kreidecomplex von Abu Roash bei Kairo. Palaeontographica VOL. 30, p. 337-391
14. Douvillé, H. (1913): Description des rudists de lEgypt. Mém. Inst. Egypt, 6, pp. 237-256.
15. El-Hedeny, M.M. and El-Sabbagh, A.M...(2005): *Eoradiolites liratus* (Bivalvia, Radiolitidae) from the Upper Cenomanian Galala Formation at Saint Paul, Eastern Desert, Egypt. Cretaceous Research 26, p.551-566.
16. El-Sabbagh, A.M. and El-Hedeny, M.M.(2003) Upper Turonian Radiolitidae rudist bivalves) from the Acteonella Series, El-Hassana Dome, Abu Roash, Egypt. Egypt Jour. Paleontol., 3, p.243-269.
17. Enos, P. (1988): Evolution of pore space in the Poza Rica trend (Mid-Cretaceous, Mexico). *Sedimentology* 35,287-32
18. Ghorab, M. A. (1961(: Abnormal stratigraphic features in Ras Gharib oilfield. 3rd Arab. Petrol. Congr., 10pp.; Alexandria.
19. Gil, J., Pons, J. M. and Segura, M. (2009): Succession of rudistid lithosomes along the western coastal margin of the Iberian Basin (Coniacian, Castrojimeno Section, centralSpain). Facies, pp. 55:523–538
20. Kennedy, W.J. & Taylor, J.D. 1968: Aragonite in rudists. *Proceedings of the Geological Society of London* 1645, 325-331.
21. Köch, R., Ogorelec, B. & Orehek, S. (1989): Microfacies and diagenesis of Lower and Middle Cretaceous carbonate rocks of N\V Yugoslavia (Slovenia, Trnovo Area). *Facies* 21, 135-170.
22. Kuhn, O. (1967): Rudistenhorizonte als okologische und stratigraphische Indikatoren. *Geologische Rundschau 56,* 186189.
23. Moore, R. C., eds, (1960): Treatise on invertebrate paleontology, part I, Mollusca 1, Geological Society of America, Inc and the University of Kansas, 351pp.
24. Minero, C.J. 1988. Sedimentation and diagenesis along an island-shelteredplatform margin, El Abra Formation, Cretaceous of Mexko. *In* James, N.P. & Choquette, P.W. (eds.): *Paleokarst,* 385-406. Springer, New York, N.Y.Sarı, B., Steuber, T., Özer, S. (2004): First record of Upper Turonian rudists (Mollusca, Hippuritoidea) in the Bey Dag˘ları carbonate platform, Western Taurides (Turkey): taxonomy and strontium isotope stratigraphy of Vaccinites Praegiganteus (Toucas, 1904). Cretaceous Research 25, 235–248.
25. Moon, F.W. and Sadek, H. (1921): Topography and geology of North Sinai, Egypt. Petrol. Res, Bull., 10: pp154, Cairo.
26. Moore, R. C., eds, (1969): Treatise on invertebrate paleontology, part N, Mollusca 6, Bivalvia. Geological Society of America, Inc and the University of Kansas, P. 491- 952.
27. Omran, A. M. (1997): Stratigraphical studies of the Upper Cretaceous – Lower Tertiary successions in some localities, Northern Sinai. PhD thesis in Geology, Suez Canal University, 330 p, 29 plates.
28. Orbigny, A. (1851): Les animaux mollusque et rayonnés, Atlas. Paris
29. Pons, J.M. (1977): Estudio estratigrafico y paleontologico de 10s yacimientos de rudistidos del Cretacico Superior del Prepireneo del la Provincia de Lerida. *Universitat Autdnorna* ***de*** *Barcelona, Publicaciones de Geologia* 3.pp105.
30. Pons, J.M. (1982): Distribucion de 10s rudistas (Bivalvia) del Cretacico Superior sudpirenaico. *Cuadernos de Geologia Iberica* 8, 1027-1033.
31. Ross, D.J. & Skelton, P.W. (1993): Rudist formations of the Cretaceous: apaleoecological, sedimentological and stratigraphical review. *In* Wright, P. (ed.): *Sedimentology Review* 1, 73-91. Blackwell, Oxford.
32. Sari, B. Steuber, T. and Ozer, S. (2004):First record of Upper Turonianrudists (Mollusca, Hippuritoidea) in the Bey Daglari carbonate platform, western Turides,(Turkey): taxomomy and strontium isotope stratigraphy of *Vaccinites praegiganteus (Toucas).Cretaceous Research 25, pp 235-248.*
33. Sarı, B., Özer, S. & Taslı, K., (2005): Upper Cretaceous (Middle Cenomanian-Upper Turonian) benthonic foramnifera and rudist assemblages of the Bey dağları carbonate platform between Elmalı and Ağlasun, Western Taurides, Turkey: International Earth Sciences Colloquium on the Aegean Region, IESCA-2005, İzmir, Abstracts, 103.
34. Sanders, D. (1999): Shell disintegration and taphnomic loss in rudist biostromes. Lethaia, vol. 32, pp. 101-112.
35. Sanders, D. 1998a: Upper Cretaceous rudist formations. Proceedings from the Fourth Meeting of the Austrian Paleontological Society.
36. Sanders, D. & Baron-Szabo, R.C. (1997): Coral-rudist bioconstructions in the Upper Cretaceous Haidach section (Northern Calcareous Alps, Austria). *Facies 36,* 69-90.
37. Sanders, D., Kollmann, H. & Wagreich, M. (1997): Sequence development and biotic assemblages on an active continental margin: The Turonian- Campanian of the Northern Calcareous Alps. *Bulletin de la*
38. Sanders, D. &Pons, J.M. 1995: Rudist formations in mixed siliciclasticcarbonate depositional environments, Upper Cretaceous, Austria: Stratigraphy, sedimentology, and models of development. Palaeo*geography,* Palaeoclimafofogy, Palaeoecology 148,249-284.
39. Skelton, P.W. 1976: Functional morphology of the Hippuritidae. *Lethaia 9,* 83-100.
40. Skelton, P.W., Gili, E., Vicens, E. Obrador, A. (1995): The growth fabric of gregarious rudist elevators (hippuritids) in a Santonian carbonate
41. Steuber, T. (1999): Cretaceous rudists of Boeotia, central Greece. Palentolology, Spec paper, pp. 61:1–229.
42. Steuber T, Rauch M, Masse J-P, Graaf J, Malkoc M (2005) Low-latitude seasonality of Cretaceous temperatures in warm and cold episodes. Nature 437:1341–1344.
43. Toucas, A. 1903: Classification et l'evolution des hippuritides. *Memoires de la sociéte gkologique de France* 30. 127 pp
44. Tzankov, V. Pamouktchiev, A., Tchechmedjieva, V. and Motekova, N. (1981): LLes fossils de Bul Bulgarie, v Cretaceous Superiere. Edition de l Academie Bulgare des ssciences, Sofia, 214 pp, pls.I-XCVIII.
45. Yanin, B.T.(1989) : Jurassic and Cretaceous Rudists . Science Publication, Moscow, 214p.
46. Zapfe, H. (1937): Palaobiologische Untersuchungen an Hippuritenvorkommen der nordalpinen Gosauschichten. Verhandlungen der Zoologisch Botanischen Gesellschafr in Wien 86/87, p. 73-124.



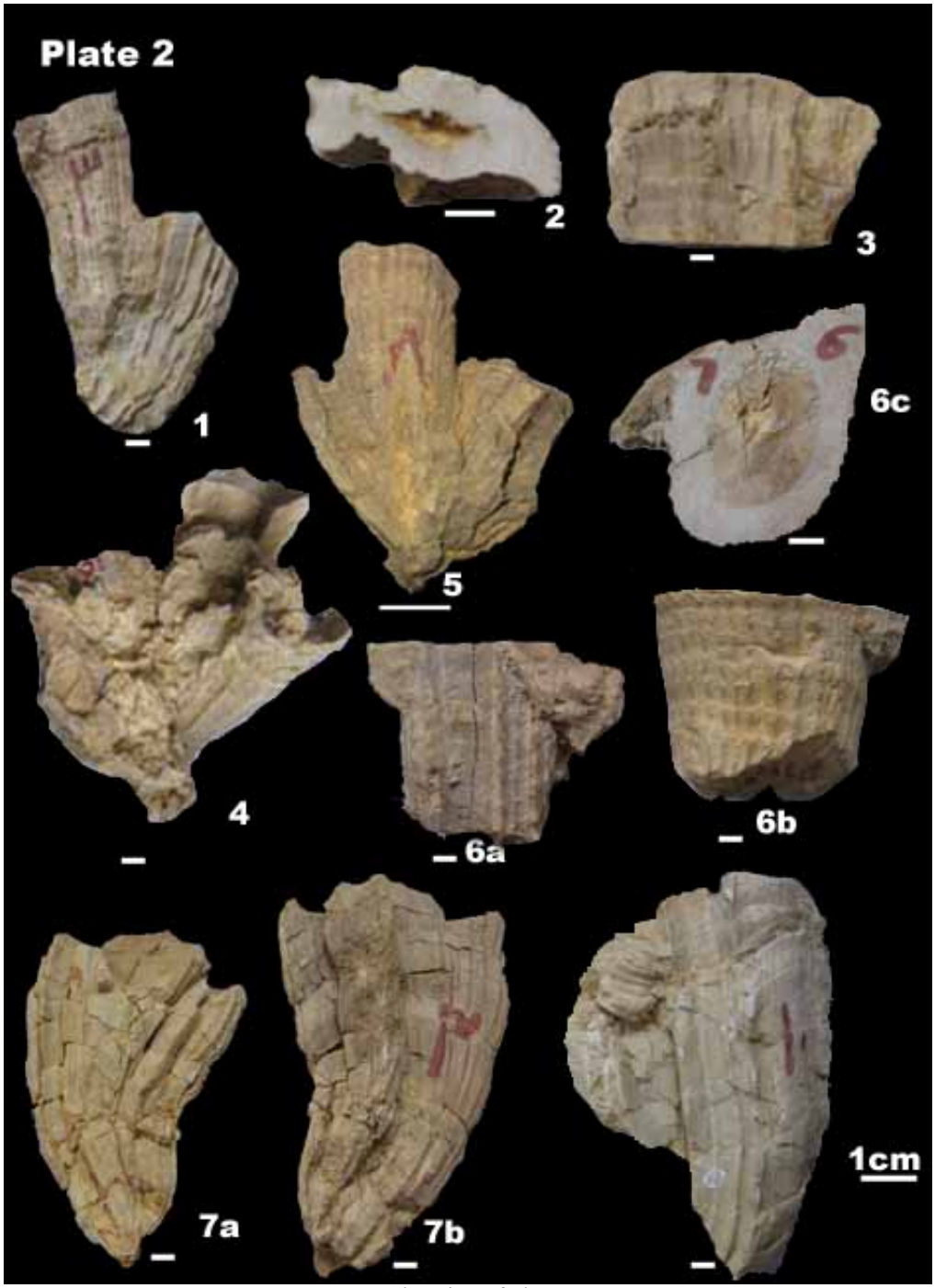
Explanation of plate 1

Figs. 1-2- *Radiolites peroni* (Choffat): 1a. Posterodorsal aspect; 1b. Adapical view, specimen no 2; 2. anterodorsal aspect, specimen no 3. Wata Formation, bed no 7, Lower Turonian, Gabal Yelleg.

Figs. 3-5- *Radiolites sauvagesi* (d´Holmis-Firmas): 3a. Dorsal aspect shows dense wavy growth lamellae, specimen no; 3b. Transverse section showing thick wall, specimen no 6; 4a. dorsal aspect, 4b. transverse section. specimen no 5, 4b. Transverse section of AV, 5a. Anterodorsal and posterodorsal aspects of two coagulate individuals, 5b. Transverse section of AV, specimen no 7 (length = 52mm). bed no 7, Wata Formation, Lower Turonian, Gabal Yelleg.

Fig. 6 - *Gorjanovicia costata* Pols˘ak,: 6a. Dorsal aspect, 6b, Transverse section of AV of a pair of *G. Costata,* specimen no 8. Wata Formation, bed no11, Lower Turonian, Gabal Yelleg.

Fig.7- Radiolites cf. *polyconilites* Orbigny, specimen no 1. Bed no 1, Wata Formation, L. Turonian, Gabal Yelleg.

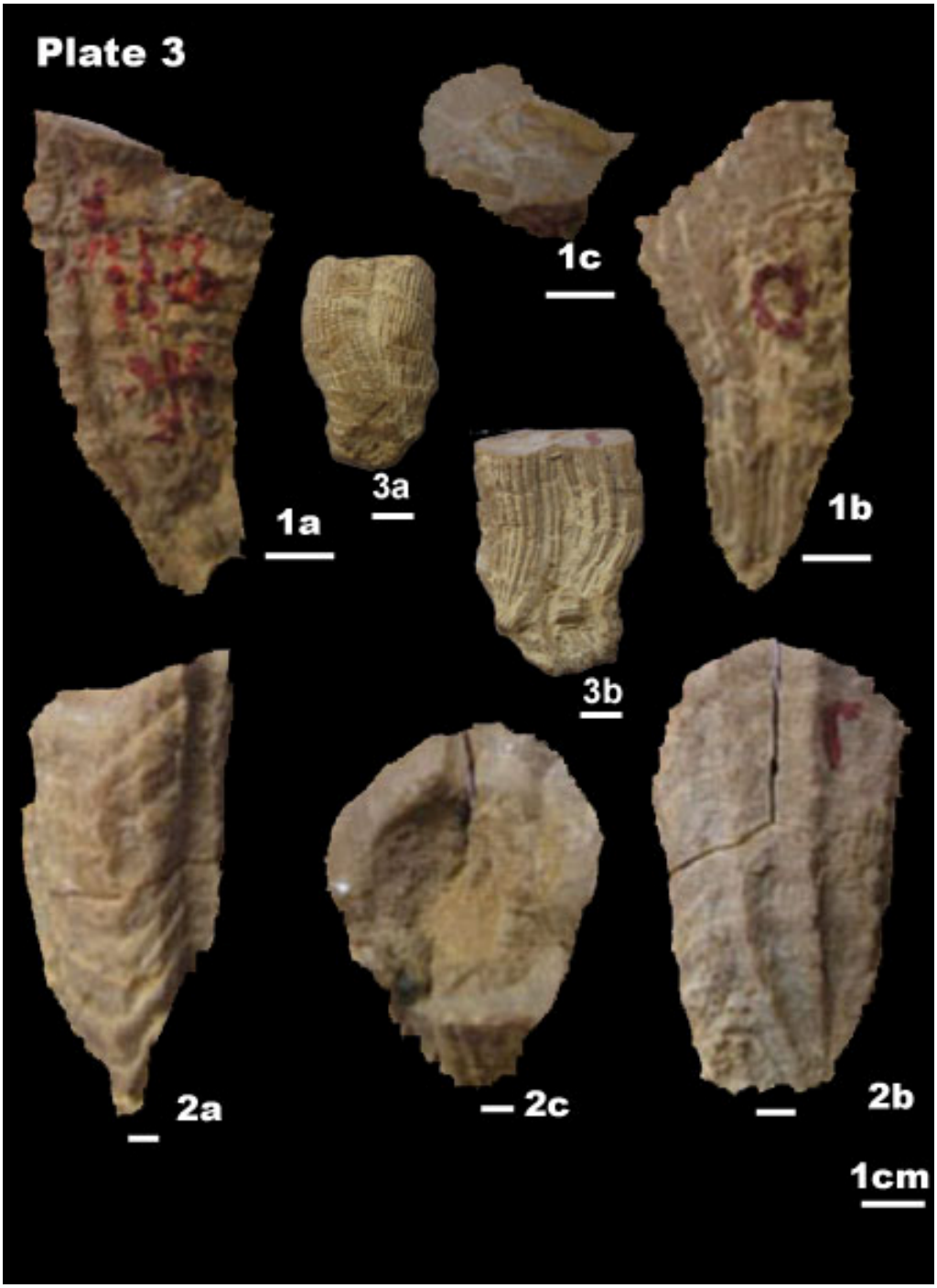


Explanation of plate 2

Figs. 1-5: *Milovanovicia heraki* Pols˘ak, side view, specimen no 10. Bed 16, Wata Formation, Upper Turonian, Gabal Yelleg.

Fig. 6a-c: *Suvagesia sharpei* (Bayle): 6a.dorsal aspect showing normal thin ribs interrupted by regularly spaced growth layer; 6b. Radial bands (Eb wider) with ligamental furrow; 6c.transverse section showing thick wall and ligamental pillar, specimen no 15, bed no 13, Wata Formation, Upper Turonian, Gabal Yelleg. .

Figs. 7-8: *Suvagesia nicaisei* (Coquand): 7 a & b. posteroventral & dorsal aspects, with radial costulate and flat bands, specimen no 16; 8 dorsal aspect, specimen17. Bed no 16, Wata Formation, Upper Turonian, Gabal Yelleg.

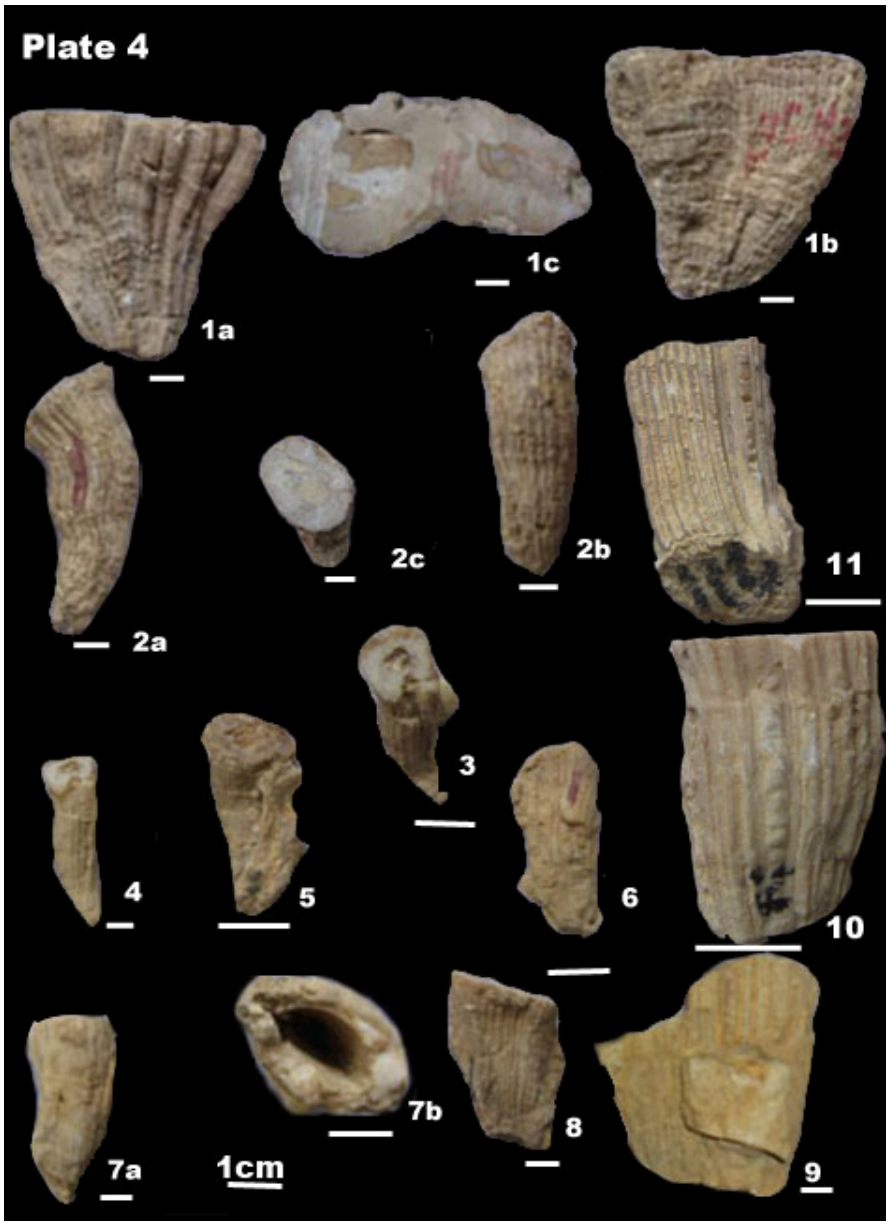


Explanation of plate 3

Fig.1a-c: *Praeradiolites biskraensis* (Coquand): 1a. Posteroventral aspect with concave siphonal band ligamental furrow; 1b. ornament with divergent radial folds, specimen no 9. Bed 7, Wata Formation, Lower Turonian, Gabal Yelleg.

Fig. 2a-c: *Durania* barakatensis nov. sp.: 2a. Concave siphonal bands and raised interband with concave growth laminae; 2b. Adapical view of AV; 2c. sharp ribs and wide inter-ribs, specimens no 18&19, Beds 11&13, Wata Formation, Upper Turonian, Gabal Yelleg.

Fig. 3a-b: *Hippurites (Hppuritella)* aff. *castroi* Vidal, specimens 29&30, beds 15 & 16, Wata Formation, Upper Turonian, Gabal Yelleg.



Explanation of plate 4

Fig. 1a-c: *Durania gaensis* (Dacque): 1a. Posteroventral aspect showing radial bands deeply, and plicate interbands; 1b. longitudinal and growth laminae; 1c. Transverse section, specimen no 20, bed 11, Wata Formation, Upper Turonian Gabal Yelleg., .

Fig. 2a-c: *Durania cornupastoris (*Des Moulins): 2a. Ventral with siphonal bands; 2b. dorsal aspect, 2c. transverse section, specimen no 23. Bed 14, Upper Turonian, Gabal Yelleg.

Fig. 3-8 & 10-11: *Durania arnaudi* (Choffat). 3-6: conical form (fig. 4 = specimen 26);7-8: conical form with broad commissure (fig. 7 = specimen 27), 10-11: cylindrical form (specimen no 24 & 25 , sample no 24-27. Bed 11, Wata Formation, Lower Turonian, Gabal Yelleg..

Fig. 9: *Lapeirousella aumalensis* (Douville), ventral view showing band and interbands, specimen no 30, bed 14, Upper Turonian, Gabal Yelleg.

11/8/2010

Barremian and Aptian Mollusca of Gabal Mistan and Gabal Um Mitmani, Al-Maghara Area, Northern Sinai, Egypt

HOSNI HAMAMA
Geology Department, Faculty of Science, Mansura University, Mansura 35516, Egypt.
hamamahem@hotmail.com

Abstract: A very rich assemblage of 40 Molluscan species was identified from the Lower Cretaceous succession of Gabal Mistan and Um Mitmani lying at the extremity of the northern flank of Gabal Al-Maghara, northern Sinai. These are used to date the investigated material as Barremian and Aptian. Comparison of the Sinai material with coeval deposits in the northern Caucasus and Western Europe signifies a possible direct marine connection between these areas.

[Hosni Hamama. **Barremian And Aptian Mollusca Of Gabal Mistan And Gabal Um Mitmani Al-Maghara Area, Northern Sinai, Egypt**. Journal of American Science 2010; 6(12):1702-1714]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Barremian, Aptian, Albian**,** Mollusca, North Sinai.

1. Introduction

Aim and Material: The stratigraphic boundaries of the Lower Cretaceous chronostratigraphic units are a matter of great deal. Ammonites are used successfully for this achievement. The author focuses attention to delineate the stages of Barremian, Aptian and Albian (Hegab *et al*.; Hamama, 1992, 1993 and 2000). Rich Molluscan specimens were collected by the author during field excursions from the area east of Gabal Lagama at Gabal Mistan and Gabal Um Mitmani. The identified molluscan fauna, especially ammonites were used to subdivide the Barremian and Aptian into substages and to determine some biozones of the Lower Cretaceous succession at the area of study.

Recent detailed important works on the Lower Cretaceous of north Sinai were made by Aboul – Ela *et al.* (1991), Aly, M. and Abdel-Gawad (2001 &2006), Hewaidy *et al.* (1998), Aly, M.(2006), Abu Zied R. H. (2006 & 2008), Mekawy, M. S. and Abu-Zeid, R. H (2008) at Gabal Manzour, Gabal Lagama and Gabal Abu Ruqum. The identity of the identified ammonite species with those of Northern Caucasus and Western Europe is taken as an indication to the presence of a marine seaway which connected North Sinai with Tethyan Province.

Stratigraphy: The measured Lower Cretaceous section of Gabal Mistan and Gabal Um Mitmani (Fig. 1) is represented by the Risan Aneiza Formation. The lower part of this formation consists mainly of sandstones, marls and intercalation of thin limestone beds, whereas the Upper part is composed mainly of marls and limestone with few sandstone intercalation. The Lower part of the succession was named Um Mitmam Member and the Upper part is Manzour Member (Hegab *et al.* (1989), Hamama (1992 & 2009), Hamama and Gabir (2001). The studied Mollusca were collected from Um Mitmam Member.

2. Systematic Paleontology

More than forty five species of ammonites, gastropods, Pelecyopods were identified. All the collected specimens were collected by the author and they were deposited at the Geological Museum of the Geology Department, Mansura University. The systematic of ammonites are adopted after Moore (1996), and for the Gastropods and Pelecyopods we use the systematic of Pcelincev and Korobkov (1960).

AMMONOIDEA
1-Order AMMONOIDEA Zittel, 1848
Suborder ANCYLCERATINA Wiedmann, 1966
Superfamily DOUVILLEICERATACEAE Parona & Bonarelli, 1897
Family DOUVILLEICERATIDAE Parona & Bonarelli, 1897
Subfamily CHELONICERATINAE Spath, 1923
CHeloniceras Hyatt, 1903
C. (*Epicheloniceras*) Casey, 1954a

***C.* (*Epicheloniceras*) *subnodosocostatum* Sinzow, 1954a**
(pl.2, fig. 1a-c)

1907 Kilian: *Douvilleiceras martini* Orbigny, pl. 2, fig. 5.
1915 Nikchitch: *Douvilleiceras seminodosum,* pl.1, fig. 9.
1960 Drushchitz, &. Kudriavteseva (Eds): *Epicheloniceras subnodosocostatum* Sinzow**,** p. 341, pl.XXI, Fig.3; pl. XXII, Fig 4&5.-b.
1989 Follmi: *Cheloniceras subnodosocostatum* (Sinzow), p. 134, Pl .6, Figs.17 – 20.

Remarks: The identified specimen is very similar to many *C.* (*Epicheloniceras*) species described by some authors. It differs by faint ribbing and distinct nodes from *Cheloniceras* (*Epicheloniceras*) *martini caucasica* described by Drushchitz &.Kudriavteseva (p.339, pl.

XVII, Fig.4a, pl. XX, fig3). The species is strongly similar to *Epicheloniceras tschernyschewi* described by the same authors (p.339, pl.XIX, Fig.2a-b), but it differs by distinct ventrolateral nodes. Moreover the described specimen may represent an embryonic stage of *C.* (*Epicheloniceras*) *tschernyschewi* described by Moore, (P. 269, Fig. 208, 5. C).

Age: Middle Aptian, *subnodosocostatum* Zone, Gabal Mistan.

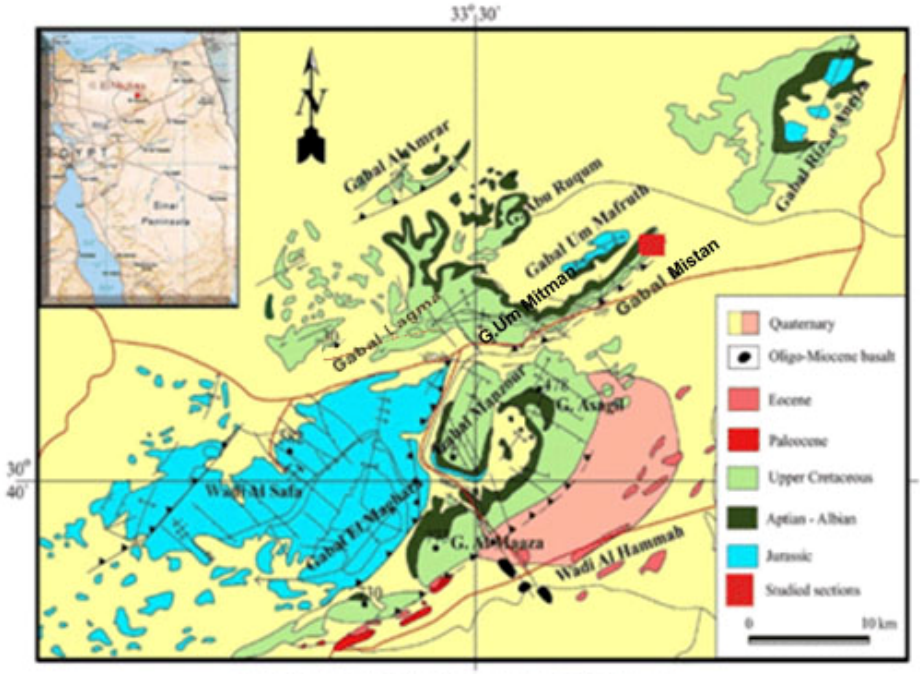


Fig.(1): Geological map of Maghara area, showing the study area
(After Geological Atlas of Sinai, 2004)

Text- Fig.1: Geological map of Al- Maghara area with Lower Cretaceous rocks surrounded the Al-Maghara Massif (after Geological Atlas of Sinai, 2004)

Superfamily DESHAYESITACEAE Stoyanow, 1949
Family DESHAYESITIDAE Stoyanow, 1949
Subfamily DESHAYESITINAE Stoyanow, 1949
Deshayesites Kazansky, 1914

***Deshayesites deshayesi* (Leymerie MS.) Orbigny**
(pl. 1, figs.1-2)

1842 Orbigny: *Ammonites deshayesi,* p. 288, pl. 85, fig. 1-4.
1936 Rengarten: *Deshayesites dechyi* Papp, pl. II, fig. 2
1960 Drushchitz, &. Kudriavteseva (Eds): *Deshayesites deshayesi* Leym, p.309, Pl .I, fig. 2 & 5.
1960 Casey: *Deshayesites d*eshayesi (ORBIGNY), p. 300, Text-Fig 106, *e, f. g.*
1996 Moore, R. ed.: *Deshayesites* deshayesi (Orbigny) p. 271, fig.211, 1a-c

Remarks*:* The described specimen is very similar to *D. Deshayesi* (Orbigny) var. *stringosus* Casey (p. 300, Text-Fig 106, h), but the latter has dens ribbing.

Age: Early Aptian, ***deshayesi*** Zone, Gabal Mistan.

***Deshayesites lavaschensis* Kazansky, 1914**
(pl. 1, fig.3)

1914 Kasansky: *Hoplites (Deshayesites) lavaschensis*, p. 105, pl. VI, fig. 87.
1960 Drushchitz, & M. P. Kudriavteseva: *Deshayesites lavaschensis* Kazansky, p. 311, p. lI, fig. 4a – b.
1964 Casey: *Deshayesites forbesi*, p.314, pl. XLVII, fig. 6a, Text-Fig 109, b & c.

Remarks: According Casey (1964, part v, p.314), the *D. forbesi* differs from *D. deshayesi* by an oblique umbilical wall and a more feebly ribbed nucleus, and from the earliest times it has been misidentified as *D. deshayesi.* The described specimen is similar to *Deshayesites multicostatus* Swinnerton described and figured by Casey (1964, p.304, pl. XLIII, fig. 5), however the latter has normal s-shaped ribs. The species is similar to *Deshayesites weissiformis* Bogdanova.

Age: Early Aptian, Gabal Mistan.

Superfamily ANCYLOCERATACEAE Meek, 1876
Family HAMITIDAE Gill, 1871
Hamites Parkinson, 1811

***Hamites intermedius* Sowerby 1814**
(pl. 2, fig. 10)

1889 Follmi: *Hamites intermedius* Sowerby, p. 124, pl.4, figs. 17-23.

Remarks: The ribs of the described species are thin relative to the Lower Aptian *Leptoceras biplex* Koenen (Drushchitz and Kudriavteseva, 1960, p.295, pl. XXXIX, fig., and 3). The described species is very similar to *Tonohamites aequicingulatus* (von Koenen) figured by

Casey (1960, part I, pl. IX, figs 2-4), but our specimen is slender with relatively thin and straight ribs.
Age: Early Aptian, Gabal Um Mitmani.

Family HAMULINIDAE Gill, 1871
Anahamulina Hyatt, 1900
Anahamulina lorioli Uhlig, 1883
(pl. 1, fig. 4)
1960: Drushchitz & M. P. Kudriavteseva.: *Anahamulina lorioli* Uhlig, p.265, pl. X, fig.
Remarks: From *Anahamulina subcylindrica* Orbigny, the described species differs by the presence of a pair of ventrolateral tubercles (Moore, 1996, p. 231, fig.181, 1a-c; Drushchitz & Kudriavteseva, 1960, p.364, pl. X, fig.2a-b).
Age: Barremian, Gabal Um Mitmani.

Suborder AMMONITINA Hyatt, 1953
Family DESMOCERATIDAE Zittel, 1895
Subfamily BARREMITNAE Breskovski, 1977
Barremites Kilian, 1913
***Barremites subdifficilis* (Karakasch, 1907)**
(PL. 1, figs. 5 – 7)
1907 Karakasch: *Desmoceras subdifficile,* p. 58, pl.6, fig.1a-b.
1960 Drushchitz & Kudriavteseva: *Barremites subdifficilis* Karakasch, 299, pl. XLII, fig. 2.
1974 Akobiana: *Barremites subdifficilis* Karakasch, p.269, pl. 92, fig. 8.
1996 Moore: *Barremites difficilis* (Orbigny), p.69, Fig.50, 1a-b.
Remarks: *Barremites difficilis* (Orbigny) and *Barremites subdifficilis* Karakasch is thought to be a dimorphic pair deferring in the height of the whorl section, one with very high whorl section and the other with relatively low section.
Age: Barremian, Gabal Mistan.

***Barremites charrierianus* Orbigny, 1840**
(Pl. 1, fig. 8)
1883 Uhlig: *Haploceras psilotatus*, p.226, pl. 16, figs. 2-3.
1960 Drushchitz & Kudriavteseva: *Barremites charrierianus* Orbigny, p. 300, PL XLII, fig. 4-5.
1974 Akobiana: *Barremites charrierianus* (Orbigny), p.270, pl. 92, fig. 5.
AGE: Barremian, Gabal, Um Mitmani.

***Barremites psilotatus* (Uhlig, 1883)**
(pl. 1, fig. 11)
1838 Uhlig: *Haploceras psilotatus*, p.226, pl. 16, figs. 2 and 3.
1960 Drushchitz & Kudriavteseva: *Barremites psilotatus* Uhlig, p. 73, 299, PL XLII, fig. 3a, b.
1972: Vasicek, Z: *Barremites psilotatus* Uhlig, p. pl. XII, fig.2, 3.
Remarks: The wide sinuous constrictions, the large size of the adult specimen, the presence of feeble lirae and the relatively low oval whorl section characterize the described specimen from the *Barremites difficilis* Orbigny described by Moore (1996, Fig 50, 1a-b) and Drushchitz & Kudriavteseva(1960, pl. XLII, fig. 1a-b).
Age: Barremian, Gabal Um Mitmani and Gabal Mistan

Subfamily PUZOSIINAE Spath, 1922
***Puzusia (Puzusia) matheroni* Orbigny**
(Pl. 1, fig. 12)
1916 Douville: *Puzusia matheroni* Orbigny, p.103, pl.XIII, figs. 1 – 7.
1960 Drushchitz & Kudriavteseva: *Spitidiscus seunesi* Kil., p. 306, pl. XLVII, fig.4.
1996 Moore: *Spitidiscus Rotula* (Sowerby), p.69, fig. 49, 1a-c.
Remarks: The described specimen is identical to that figured by Douville'. From *S. seunesi* it is relatively compressed. The three species of *Spitidiscus* Killian figured by Drushchitz & Kudriavteseva (1960, p. 305-306, pl. XLVII) seam to me as polymorphic forms of Lower Barremian ammonites. It is similar to *Puzosia quenstedti media* Seitz from the Albian of Poland by less inflated sides (1990, Marcinwski, pl. 6, fig.1).
Age: Upper Barremian-Early Aptian, Gabal Um Mitmani and Gabal Mistan.

Valdedorsella Breistroffer, 1947b
***Valdedorsella akuschense* (Anthula)**
(Pl. 1, figs. 15 & 16)
1960 Drushchitz & Kudriavteseva: *Valdedorsella akuschense* Anthula, p. 301, fig.3.
1996 Moore: *Valdedorsella akuschansis* (Anthula), p. 71, fig. 61, 2a – b.
Age: Late Aptian, Gabal Um Mitmani and Gabal Mistan.

Subfamily BEUDANTICERATINAE Breistroffer, 1953
Zuercherella Casey, 1954a
***Zuercherella* aff. *Zuercheri* (Jacob)**
(pl.1, fig. 13)
1996 Moore: *Zuercherella zuercheri* (Jacob), p.80, fig. 61, 2a – b.
Age: Late Aptian, Gabal Um Mitmani and Gabal Mistan.

Uhligella Jacob 1907
***Uhligella clansayensis* (Jacob)**
(Pl. 1, fig. 14)
1996 Moore: *Uhligella clansayensis* (Jacob), p.80, fig. 61, 3b-c.
Remarks: Although the described specimen is similar to *Uhligella walleranti* Jacob (Marcinwski, 1990, pl. 6, fig.5a-b), the latter species is highly inflated.
Age: Late Aptian, Gabal Um Mitmani and Gabal Mistan.
Suborder PHYLLOCERATINA Arkell, 1950
Family PHYLLOCERATIDAE Zittel, 1884
Subfamily PHYLLOCERATINAE Zittel, 1884

Macrophylloceras Spath
***Macrophylloceras ptychostoma* Benecke**
(pl.1, figs. 9&10)

1960 Drushchitz & Kudriavteseva: *Macrophylloceras ptychostoma* Benecke, p. 252, pl. II, fig. 6.
Age: Barremian, Gabal Mistan.

Phylloceras (*Hypophylloceras*) Salfeld, 1924
***Phylloceras* (*Hypophylloceras*) *velledae* (Michelin, 1842)**
(pl. 2, fig. 6)

1834 Michelin: *Ammonites velledae*, p.280, pl.35
1960 Drushchitz & Kudriavteseva: *Euphylloceras velledae* MICHELIN, p. 252, PL II, fig. 5a-b.
1990 Marcinowski & Wiedman: *Phylloceras* (*Hypophylloceras*) *velledae velledae* (Michelin), pl. 1, fig.1.
Remarks: It seems to me that the *Phylloceras semistriatum* ORBIGNY described by Douvillé from the Barremian of Sinai (1916, Pl. XII, fig 1.) is identical to *Euphylloceras Ponticuli* Rousseau described by Drushchitzs & Kudriavteseva (p. 251, PL I, fig. 9a-b.) from the Lower Barremian of Crimea. Also the described species is similar to *Euphylloceras velledae* Michelin, and it also collected from higher stratigraphic position comparable with that of *Euphylloceras velledae*.
Age: Middle Aptian, Gabal Mistan and Gabal Um Mitmani.
Age: Middle Aptian, Gabal Mistan

***Phylloceras* (*Hypophylloceras*) *moreti* (Mahmoud)**
pl.2, fig 4

1956 *Salfeldiella (Goretphylloceras)* Moreti Mahmoud; p. 67, fig. 44, pl. 5: 2-4
1964 *Phylloceras* (*Hypophylloceras*) *moreti* (Mahmoud): Weidmann, p. 200, fig. 46, pl. 19:2.
1990 Marcinowski and Wiedman:*Ph.(Hypophylloceras) moreti* (Mahmoud); p. 21, pl. 1, fig. 6.
Remarks: The species is known from the Lower and Middle Albian of the Sinai Peninsula (see discussion), and was recently described from the Aptian-Albian boundary of Solovakian Carpathians (Marcinowski and Wiedman), 1990. Therefore it used herein to determine This Contact in the studied section
Age: Late Aptian, Gabal Mistan and Gabal Um Mitmani.

Order LYTOCERATIDAE Hyatt, 1889
Suborder LYTOCERATINA Hyatt, 1889
Superfamily LYTOCERATACEAE Neumayr, 1875
Family LYTOCERATIDAE Neumayr, 1875
Subfamily LYTOCERATINAE Neumayr, 1875
Protetragonites Hyatt, 1900
***Protetragonites crebrisulcatus* (Uhlig, 1883)**
(pl. 1, figs. 19 – 21)

1960 Drushchitz & Kudriavteseva, *Protetragonites crebrisulcatus* Uhlig, p. 260, PL VIII, fig. 1a, b.
Remarks: It is probable that the *Protetragonites crebrisulcatus* Uhlig and *Protetragonites karakaschi* Druczczic are one and the same species (Drushchits & Kudriavteseva, 1960, pl.VIII, fig 1a-b and pl.VIII, fig. 2a-b). Both species were collected from the Upper and the Lower Barremian of Crimea respectively.
Age: Late Barremian, Gabal Mistan.

Superfamily TETRAGONITACEAE Hyatt, 1900
Family TETRAGONITIDAE Hyatt, 1900
Subfamily TETRAGONITINAE Hyatt, 1900
Tet1ragonites Kossmat, 1895
***Tetragonites (Tetragonites)* aff. *heterosulcatus* Anthula 1899**
(Pl. 2, fig. 3, 5 and 7)

1960 Drushchitz & Kudriavteseva: *Tetragonites heterosulcatus* Anthula, p. 260, pl. VIII, fig. 3a, b.
Remarks: The described specimens are differentiated from *Tetragonaites*(*Tetragonites*) *nautilodes* (Pictet) figured by Marcinowski & Wiedman (1990, pl. 1, figs. 12-13) by less inflated shell and much constrictions. The present species has affinity to the same species described by Drushchitz & Kudriavteseva , but it characterized by rectangular whorl section, little inflation and narrower venter.
Age: Middle - Late Aptian, Gabal Mistan and Gabal Um Mitmani.

Tetragonites Kossmat, 1895
***Tetragonaites(Tetragonites) nautilodes* (Pictet, 1847)**
(Pl.2, figs.8 &9)

1847 Pictet: *Ammonites Timotheanus* var. *nautiloides*, p. 296, pl. 3, fig. 2.
1967 Murphy: *T. (Tetragonites) nautilodes* (Pictet), p.27, pl. 2, figs. 5-10.
1989 Follmi: *Tetragonites nautilodes* (Pictet), p. 119, pl. 3, figs. 13, - 15.
1990 Marcinowski & Wiedman: *T. (Tetragonites) nautilodes* (Pictet), pl. 1, and figs.12 - 13).
Age: Late Aptian – Early Albian, Gabal Um Mitmani.

***Tetragonites* (*Tetragonites*) sp.**
(Pl.2, fig. 2)

Description: Small size, inflated shell with rounded sides and broad venter, whorl section rectangular.
AGE: Middle - Late Aptian, Gabal Mistan ..

Class GASTROPODA Cuvier, 1797
Order PROSOBRANCHIA Milne Edwards, 1848
Superfamily TROCHACEA Rafinesque, 1815
Family TROCIDAE Rafinesque, 1815
Subfamily TROCHINAE Stoliczka, 1868
Discotectus Faver, 1913
***Discotectus* (*Discotectus*) sp.**
(Pl. 3, figs. 6 &12)

Description: Very small size; low cone with relatively broad base; apex obtuse; surface ornamented with axial raised, intercostae flush and /or relatively depressed of width two times as costae.
Age: **AGE**: Middle - Late Aptian, Gabal Mistan.

Superfamily NERINEACEA
Family NERINEIDAE Zittel, 1873
Subfamily NERINEINAE Pcelincev
Nerinea Defrance, 1825
***Nerinea monocarinata* Pcelincev**
(pl. 3, fig. 18)

1960 Pcelincev and Korobkov (Eds): *Nerinea monocarinata* Pcelincev, p. 120, pl. XII, fig. 9.

1974 Collignon: *Nerinea* (*Ptygmatis) hottingera* Collignon, p.17, pl. 4, fig.7.

Remarks: The described species is very similar to *Nerinea archimedi* Orbigny identified by Pcelincev and Korobkov (P.123, fig 206) from the Lower Cretaceous , western Europe, but the latter has a wavy outline. Also *Nerinea* (*Ptygmatis) hottingera* Collignon described from Maroc meridional (Collignon, 1972. P.17, pl.4, fig. 7**)** has some affinity to the present identified species.

AGE: Late Barremian, Gabal Mistan.

Superfamily PSEUDOMELANIACEA Pcelincev, 1960.

Family TRAJANELLIDAE Pcelincev, 1953

Pseudomesalia Douville, 1916

***Pseudomesalia deserti Douville*, 1916**

(pl. 3, fig. 4)

1916: Douvillé: *Pseudomesalia deserti Douville*, pl. XVIII, figs. 18-25.

1949 Collignon: *Tympanotonus hourcqi* Collignon, P. 110, pl. XVII (V).

1991 Aboul Ela et al: *Pseudomesalia deserti Douvillé*, p. 208, pl. 2, figs. 10-11.

Remarks: Many species of *Pseudomesalia* recorded from the Cenomanian and Turonian of Armenia such as *P. brevis* Douvillé, *P. imbricate* Pcelincev, *P. angustata* Pcelincev differ from the present described *P. deserti* Douvillé by the presence of sharp strong spiral costae and depressed sutures (Okobiana, 1974, pl.119).

Age: Late Aptian, Gabal Mistan and Gabal Um Mitmani.

***Pseudomesalia* sp.**

(pl. 3, figs. 2 & 3)

1916 Douville': *Pseudomesalia bilineata* Douville', 1916, pl. XVII, fig.27)

Description: Very small, shell conical with acute apical angle, 6 to 7 rounded whorls with depressed sutures.

Remarks: The unknown *Pseudomesalia* sp. Is similar to *Nerinea mistanensis* Awad, 1952 descibed from the Middle Albian by Mekawy and Abu-Zied (2008, p. 316, pl. 4, fig. 20). But the latter species has slightly broader body whorl. If they may be encountered in the same horizon, they may probably represent a dimorphic pair.

Age: Late Aptian, Gabal Mistan.

Suborder MESOGASTROPODA

Family POTAMIDIDAE

Pyrazus Montofort, 1810

***Pyrazus* (*Echinobethra) magharensis* var. *rekebensis* Abbass.**

(pl. 3, fig. 8)

1991 Aboul Ela et al: *Pyrazus* (*Echinobethra*) maghareensis var. rekebensis Abbass, p. 208, pl. 2, fig. 21.

AGE: Late Aptian, Gabal, Mistan.

***Pyrazus* (*Echinobethra*) *sexangulatus* Ze'k**

(pl. 4, figs. 17-20)

2008 Mekawy and Abu- Zied: ***Pyrazus* (*Echinobethra*) *sexangulatus* Ze'k**, p. 208, pl. 4, fig. 13.

Remarks: This species is more or less typical to *Pyrazus valeriae* Vern. et Lor, however the axial nodes of the latter species is coarse forming alternating axial rows.

AGE: Late Aptian, Gabal, Mistan.

***Pyrazus valeriae* aff. *valeriae* (Vern.& Lor., 1868)**

(Pl. 3, fig. 9)

1916 Douvillé: *Pyrazus valeriae* Vern. et Lor., p. 136, pl. XVIII.

1972 Collignon: Confusiscala dupiniana (Orbigny), p.14, pl.2, figs.6 and 8.

Remarks: The described specimen has slightly fine axial ornamentation and smaller size relative to the original species.

AGE: Late Aptian, Gabal, Mistan.

Superfamily NATICACEA Forbes, 1883

Family AMPULLINIDAE (EUSPIRIDAE Cossman, 1907)

Tylostoma Sharpe, 1849

***Tylostoma (T.) canalliculata* Abdel Gawad**

(pl. 3, fig.1)

1991 Aboul Ela, et al: *Tylostoma (T.) canalliculata* Abdel Gawad, p. 210, pl. 3, figs.1 and 2,, Early Albian.

Remarks: This species shows a great affinity to species identified by Collignon as *Ampullospira (Euspirocrommium) exaltata* Goldf (Collignon, 1949, p. 104, Pl. XVI (IV), figs 9, 9a-b.

AGE: Late Aptian, Gabal, Um Mitmani

***Tylostoma (T.) maghareensis* Abbass**

(pl.3, figs. 13, 14, 16, 17; pl. 4, figs. 13 - 16)

1991 Aboul Ela, et al: *Tylostoma (T.) maghareensis* Abbass, p. 210, pl. 3, fig. 3.

Remarks: The described specimens are similar *to Natica laevigata* Deshayes from the Hauterivian of Crimea which described by Drushchitz & Kudriavteseva (1960, p. 159, Pl, VII, fig. 2)

AGE: Late Aptian, Gabal Um Mitmani.

***Tylostoma (T.) gloposum* Sharpe, 1849**

(pl. 3, fig. 21)

1991 Abdel – Gawad: *Tylostoma (T.) Gloposum* Sharpe, p. 211, pl.4, fig. 1.

Remarks: The described specimen is similar to? *Tylostoma* sp. Identified by Aboul-Ela *et al*. (1991, p. 211, pl. 4 figs. 2-3) of the Late Albian of Gabal Mannzur, but while the former is more globosely, the later species posses higher body whorl.

Age: Late Aptian - Early Albian, Gabal Mistan.

***Tylostoma (T.) zaghloulum* nov. sp.**

(pl.3, figs. 19 & 20)

Description: shell with short spire, consisting of four whorls; the body whorl incomplete, about one half of the spire; distribution of varices follows regular pattern when viewed in plan from above the apex as in Cassididae; aperture incomplete.

Derivation of name: The name of the species is derived in the memory of Professor Zaki Zaghloul.

Remarks: The nominated species has a short spire relative to Tylostoma sp. assigned to Late Albian by Aboul Ela *et al.* (1991, p.211, pl.3, fig. 5)

AGE: Late Aptian, Gabal Um Mitman.

Amauropsell Bayle, 1885

***Amauropsell holzapfeli* Cossmat.**

(pl.4, fig. 22)
1949 Collignon: *Amauropsell holzapfeli* Cossmat, p. 104, Pl. XVI (IV), figs. 8-8a.
Age: Late Aptian, Gabal Mistan.

Superfamily VOLUTACEA
Family VASIDAE
Tudicla Bolten, 1798
***Tudicla (Tudicla) spindllus* nov. sp.**
(pl. 3, fig.5)
Derivation of name: From the spindle - shaped y form of the shell.

Remarks: The species is identical to species identified by Aboul Ela, et al (1991, p. 211, pl.3, fig. 6).
AGE: Late Aptian, Gabal Mistan.

Superfamily SCALACEA
Family SCALIDAE
Confusiscala Boury, 1910
***Confusiscala dupiniana* Orbigny, 1842**
(pl. 3, fig. 7)
1960 Pcelincev and Korobkov (Eds): *Confusiscala dupiniana* Orbigny, p.173, fig 414, Albian France.
Remarks: The described species is very similar to *Scala* (*Criposcala*) primitive Collignon (Collignon, 1949, p.102, pl. XVI (IV), fig.4, but the costae of the second species is distant. Moreover, the costae of the present species are coarser than those of Confusiscala *dupiniana* Orbigny.
Age: Late Aptian, Gabal Mistan and Gabal Um Mitmani.

Superfamily PROCERITHIACEA
Family PROCERITHIIDAE Cossmann, 1905
Subfamily PARACERITHIINAE Cossmann, 1906
Cirsocerithium Cossmann, 1906
***Cirocerithium subspinosum* Deshayes**
(pl. 3, figs.10 &11)
1960 Drushchitz & Kudriavteseva: *Cirocerithium subspinosum*, p. 156, pl. VI, fig. 3a- b.
Age: Late Aptian, Gabal Mistan and Gabal Um Mitmani.

Order OPITHOBRANCHIA
Suborder TECTIBRANCHIA
Superfamily ACTEONACEAE
Family SCAPHANDRIDAE
Cylichna Loven, 1846
***Cylichna* sp.**
(pl. 3, fig. 15)
Description: obical cone, absent or very reduced spire, flat topped, smooth, aperture siphostomatus with canal.
Age: late Aptian, Gabal Um Mitmani.

Class BIVALVIA Linnaeus, 1758
Order HETERODONTA
Superfamily ASTARTACEA
Family Crassatellidae Ferussac, 1821
Crassatella Lamarck, 1799
***Crassatella (Rochella) seguenzai* (Thomas & Peron, *1890-1891*)**
(pl. 4, fig. 8)
1972 Collignon: *Crassatella (Rochella) seguenzai* (Thomas & Peron). 1916 Douville: *Trigonia analoga* Douvilllé, p.162, pl.XXI, fig.6.
AGE: Late Aptian, Gabal Mistan.

Superfamily CARDITACEA
Family CARDITIDAE Ferussac, 1821
Cardita Bruguiere, 1792
Cardita dupini Orbigny var. *deserti* Douvillé
(pl.4, fig.1)
1916 Douvillé: *Cardita dupini* Orbigny var.*deserti,* p.162, pl.XXI, fig.1, 2.
AGE: Late Aptian, Gabal Mistan.

Superfamily CYPRINACEA
Family CYPRINIDAE Adams, 1858
Cyprin Lamarck, 1812
***Cyprina (Anisocardia) hermitei* Choffat**
(pl. 4, figs. 2 & 5)
1916 Douvillé: *Cyprina (Anisocardia) hermitei* Choffat, p.156, pl.XIX, figs. 14-16.
AGE: Late Aptian, Gabal Mistan.

Superfamily VENERACEA Rafinesque, 1815
Family VENERIDAE Rafinesque, 1815
Subfamily VENERINAE Rafinesque, 1815
Meretrix Lamarck, 1799
***Meretrix* (*Flaventia*) *deserti* Douvillé, 1916**
(pl.4, fig.6)
1916 Douville: *Meretrix* (*Flaventia*) *deserti* Douvillé, p.151, pl.XIX, fig.10.
AGE: Late Aptian, Gabal Mistan and Gabal Um Mitmani.

Subfamily TAPETINAE Adams & Adams, 1857F
Flaventia Jukes - Browne
***Flaventia brongniartina* (Lymerie)**
(pl.4, figs. 3 & 4)
1991 Aboul Ela, et al: *Flventina brongniartina* (Lymerie), p. 213, pl. 5, figs 14-15.
AGE: Late Aptian, Gabal Um Mitmani.

Superfamily GLOSSACEA Gray, 1847
Family DICEROCARDIIDAE, Kutassy, 1934
Megalocardia Beringer, 1914
***Megalocardia* (?) *simplex* (Mahmoud)**
(pl.4, fig. 10)
1991 Aboul Ela, et al: *Megalocardia* (?) *simplex* (Mahmoud), p. 215, pl. 6, figs 1-2.
AGE: Late Aptian, Gabal Um Mitmani.

Order SCHIZODONTA
Superfamily TRIGONIACEA
Family TRIGONIIDAE Lamarck, 1819
Scabrotrigonia Deecke, 1925
***Scabrotrigonia scabra* (Lamarck)**
(pl. 4, figs.7, 9 & 11)
1958 Saveleev: *Scabrotrigonia scabra* (Lamarck), p.119, pl. LVIII, fig. 4a-b., Turonian, France.
1991 Aboul- Ela, *et al*.: *Pterotrigonia* (*Scabrotrigonia*) *scabra* (Lamarck) p. 213, pl.5, figs 6-7.
1916 Douville: *Trigonia orientais* Douvillé, p.162, pl.XXI, fig.1, 2.

AGE: Late Aptian, Gabal Mistan.

3- Discussion and Conclusion

1: Vertical faunal distribution as an approach for Barremian and Aptian biozonation.

The identified ammonites (19 species), gastropods (15 species) and pelecypods (7 species) were used to subdivide the Barremian from the Aptian. These species help to subdivide these two stages and to establish tentatively some biozones. The measured Lower Cretaceous of Gabal Mistan and Gabal Um Mitmani are divided into the following stages:

Barremian: The Barremian is represented by about 100 meters of marl and sandstone comprising the lower part of Risan Aneiza Formation. Many species of ammonites characterized the stage including: *Barremites charrierianus* Orbigny, *Barremites psilotatus* (Uhlig), *Macrophylloceras ptychostoma*, *Puzusia (P.) matheroni*, *Barremites subdifficilis*, *Anahamulina lorioli* Uhlig and *Protetragonites crebrisulcatus* (Uhlig). The first four species record the Lower Barremian.

Aptian: The Aptian rocks attain a thickness of about 200 meters of clastic rocks intercalated with thin beds of limestone. The Lower Aptian is defined by the presence of *Deshayesites deshayesi* (Leymerie) Orbigny and *Deshayesites lavaschensis* Kazansky. The zonal index species *C.* (*Epicheloniceras*) *subnodosocostatum* Sinzow in addition to *P.* (*Hypophylloceras*) *velledae* (Michelin,) *T. (Tetragonites) heterosulcatus* Anthula define the Middle Aptian. The Upper Aptian is defined by the presence of ammonite species *Uhligella clansayensis* (Jacob), *Valdedorsella akuschense* (Anthula) and *Zuercherella* aff. *Zuercheri* (Jacob). The Aptian is characterized by rich assemblage of gastropods and pelecypods in addition to abundant sceleractinids.

Albian: worthwhile to mention that many authors in the old literatures assigned the Clansayesian to the Albian, therefore what was considered as a basal Albian is actually Upper Aptian. All the gastropods and pelecypods are collected from low stratigraphic position below the Middle and Upper Albian. The Albian fauna is outside the scope of the present study. In the studied section, it is difficult to define the Aptian/ Albian boundary in the absence of the Lower ammonite *Leymeriella tardefurcata* and *Douvilleiceras mammillatum*. The contact is based on lithological variation between Um Mitmam Member and Manzour Member. Herein the facies changes from dominant siliciclastic to become calcareous facies.

2: Paleobiogeography

The Aptian-Albian interval (124.5-97.0 Ma) was a critical time both globally and for the Tethyan domain. In the Tethyan domain it was the time when a united Neo-Tethyan subduction zone became established between the future site of the Alps and Southeast Asia and greatly accelerated the rate of north-south convergence throughout the Tethyan region **(**Naci Görür, 1991**).** This is had been confirmed in the area of study by the identity of the identified ammonites between north Sinai, the Caucasus and the Atlantic region. The ammonite distribution shows that the faunal composition is ecologically controlled **(**Fabrizio Cecca**,** 1998). The diversity of molluscs in the studied section reflects an increase of the number of niches.

Sinai Peninsula lies within the South Tethyan region (North Africa, Middle East, Iran) **(**Damotte, R., 1990). Generally, the faunal assemblages recovered from the studied sections have Tethyan affinities. They indicate rather warm waters of normal to slightly hypersaline conditions that represent a shallow, near shore environment in which the water depth did not exceed 100 m (Abu-Elaa, *et al*., 1991).

In many regions it is impossible to distinguish pre-Barremian stages (Louise Beauvais, 1992). In Sinai as a whole and in Egypt in general, the pre-Barremian interval is assessted on microfossil specially palynomorphs in some rare exposures and in many boreholes (**(**Mahmud, M.S. and. Moawad**,** A**.**M., 2000). Rare and signals on macrofauna were mentioned in literatures. The missing of some Barremian and Aptian ammonite index species in the area of study, in addition to the small sizes of the molluscan shells including ammonites refer to crises. Crises in species richness and abundance during the Early and mid-Cretaceous were coeval with oceanic anoxia associated with platform drowning. These crises can be attributed to regional environmental, induced by either oceanic anoxia or tectonic movements (Steuber, T. and and LöserH., 2000).

A step-wise demise of the carbonate platform biota transpired in the latest Aptian to Middle Albian interval was recorded by many authors (Iba, Y. and Sano, S.**,** 2007**;** Coccioni*, et al.***,** 2006). In the Pacific Province nerineacean gastropods disappeared at the Late Aptian to Early Albian transition (Iba, Y. and Sano, S.**,** 2007), hoever in the area of study, they are well represented. The missing of Barremian zonal index ammonites of the Tethyan Province such as *Colchidites securiformis* Sim, *Imerites densecostatus* Renng., *Matheronites ridzewski* Kar., *Acrioceras furcatum* Orb., …etc. may refer to such step-wise demise. Also the same phenomenon is confirmed in the area of study by missing the Aptian indes zonal ammonites of *Turkmenicera turkmenicum* Tovb., *Deshayesites weissi* Neum.,*Procheloniceas albrechtiaustriae* Hoh., *Dufrenoya furcata* Sow, *Colombiceras crassicostatun* Orb., *Parahoplites melchioris* Anth., *Acanthohoplites nolani* Seun*., and Hypacanthoplites Jacobi* Coll; in addition to absence of the most basal Albian *Leymeriella tardefurcata* Leym., and *Douvilleiceras mammillatum*.

In Tunisia, the so called "Aptian Crisis" of the south Tethyan margin is suggested by Adel Rigane *et al.* (2004) due to deficiency in organic deposits except where the medusa coral are encountered. The presence of red beds of the studied section, in addition to hard grounds may refer to the change from generally humid to arid climates during the Barremian. In Western Europe it is

though to have been linked to the lowering of the sea level (Ruffell and Batten, 1990). Many hard grounds forming ledges are encountered in the area of Gabal Mistan and Gabal Um Mitmani.

On a global scale, major transgressions were stepwise enlarged in space and time from the Neocomian, via Aptian-Albian, to the Late Cretaceous, and the post-Cretaceous regression was very remarkable. Tectono-eustasy may have been the main cause of the phenomena of transgression-regression in the Cretaceous (T. Matsumoto, T., 1980). According to El-Azaby and El-Araby (2005) and Abd-Elshafy**,** E. & Abd El-Azeam, S. (2010), the Lower Cretaceous sequence is dominated by sandy braided-river deposits with minor overbank fines and basal debris flow conglomerate.

Three second order depositional sequences were recorded in the carbonate platform of the eastern Levant. These three second-order depositional sequences mid-Cretaceous succession are: (MCEL-1: Upper Barremian–Lower Aptian, MCEL-2: uppermost Lower Aptian–middle Upper Aptian and MCEL-3: middle Upper Aptian–Middle Albian. Moreover eight third-order depositional sequences were observed in the Upper Barremian–Albian interval. They comprise successions of the inner ramp facies from open marine to restricted lagoons or tidal flats **(**Bachmann, M. and Hirsch, F., 2006)**.** In the northern Sinai Upper Aptian to Middle Cenomanian succession represents an example of a carbonate platform, 18 sequences superimpose the second-order sea-level change: 3 sequences in the Upper Aptian and 11 sequences in the Albian (Bachmann, *et al*., 2003). Without hesitation, there was a direct link between Caucasus and North Sinai.

References

1. Abdel-Shafy, E. and Abd El Azeam, S. (2010): Paleogeographic relation of the Egyptian Northern Galala with the Tethys during the Cretaceous Period. Cretaceous Research, Volume 31, Issue 3, Pages 291-303.
2. Aboul Ela, N. M., Abdel-Gawad, G. I. and Aly, M. F. (1991): Albian fauna of Gebel Manzour, north Sinai, Egypt. Jour. Afr., Earth Sci., 13 (2):201-220.
3. Abu Zied, R. F. (2006): Biostratigraphy framework of some Lower Cretaceous outcrops from Sinai, Egypt (based on Cephalopoda).Egyptian Journal Paleontology, 6, p. 125-158.
4. Abu Zied, R. F. (2008): Lithostratigraphy and biostratigraphy of some Lower Cretaceous outcrops from Northern Sinai, Egypt, Jour. Paleontology., Cretaceous Research 29 (2008), 603-624.
5. Akobiana,V. T. ed. (1974): Atlas fauna Armenia. AH Armenia CCCP, EREBAN, 836pp.
6. Aly, M. F. (2006): Aptian cephalopods from Gabal Abu Ruqum, North Sinai, Egypt. Journal of Paleontology, 6, 2006, 89-123.
7. Aly, M. F, and Abdel-Gawad, G.I. (2001): Early Cretaceous ammonites of Gebel Lagama, north Sinai, Egypt. Palaeontographica, Stuttgart, A. Bd. 262: 25-52.
8. Aly, M. F, and Abdel-Gawad, G.I. (2006): Uppermost Albian-basal Cenomanian ammonites from North Sinai, Egypt. Journal of Paleontology, 5: 347-385.
9. Bachmann, M. and Hirsch, F. (2006): Lower carbonate platform of the eastern Levant (Galilee and the Golan Heights): Stratigraphy and second-order sea-level change. Cretaceous Research. Volume 27, Issue 4, Pages 487-512.
10. Bachmann, M., Bassiouni, M. and Kuss, J. (2003) Timing of mid- Cretaceous carbonate platform depositional cycles, northern Sinai Egypt. Palaeogeography, Palaeoclimatology, Palaeoecology, Volume 200, Issues 1-4, Pages 131-162.
11. Beauvais, L.(1992):Palaeobiogeography of the Early Cretaceous corals. Palaeogeography, Palaeoclimatology, Palaeoecology, Volume 92, Issues 3-4, Pages 233-247.
12. Casey, R, (1960): A monograph of the ammonoidea of the Lower Greensand, Parts I-9, Paleontographical Society. London, xxxv + 225 fig., 112 pl., 660pp; 1- 44: Pl. I - X.
13. Coccioni, R., Luciani, V. and Marsili, A. (2006): Cretaceous oceanic anoxic events and radially elongated chambered planktonic foraminifera: Paleoecological and paleoceanographic implications.Palaeogeography, Palaeoclimatology, Palaeoecology, Volume 235, Issues 1-3, Pages 66-92.
14. Collignon, M. (1949b): Recherches sur les faunes Albiennes de Madagascar. Annales Geologiques du Service des Mines, Madagascr, fascicle 16: 1-128, fig. p. 1-22. 143 pp.
15. Collignon, M, ed. (1972): Le basin cotier de Tarfya (Maroc meridional), Tom III, paleontology. Geologique du Maroc, Rabat, 18 plates, 248 pp.
16. Damotte, R. (1990): Early Cretaceous from Tethyan regions from Tethyan regions. Cretaceous Research, Volume 11, Issue 3 , Pages 307-311
17. Douvillé, M. H. (1916): Les terrains secondaires dans le massif du Moghara, a L' Est de L' istheme de Suez. – Memoires de.L' Academie des Sciences (series 2) 54: 184 p., 21 pls.
18. Drushchitz, V. V. and Kudriavteseva, M. P., Eds (1950): Nizhnemelovoi funny severnova Kaukaaza ei Kryma [Atlas of the Lower Cretaceous faunas of the Northern Caucasus and Crimea] Vsesoiuznyi Nauchno-Issleddovatelskii Institute Prirodnykh Gasov, Moscow, p 770.
19. El-Azaby, M. H. and El-Araby, A. (2005): Depositional facies, environments and sequence stratigraphic interpretation of the Middle Triassic–

Lower Cretaceous (pre-Late Albian) succession in Arif El-Naga anticline, northeast Sinai, Egypt. Journal of African Earth Sciences, Volume 41, Issues 1-2Pages 119-143.
20. Fabrizio Cecca (1998): Early Cretaceous (Pre – Aptian) ammonites of the Mediterranean Tethys: palaeoecology and palaeobiogeography. Palaeogeography, Palaeoclimatology, Palaeoecology Volume 138, Issues 1-4, P. 305-323
21. Follmi, K., B. (1989): Beschreibung neugefundener Ammonoidea aus der Vorarlberger Garschella-Formation (Aptian-Albian), Jb. Geol. - A., ISSN 0016-7800, Band 132, Heft 1, S. 105-189.
22. Hamama, H. H. (2009): Sexual dimorphism and polymorphism of the Middle Albian ammonites from Risan Aneiza and Gabal Manzour, north Sinai. Egypt Jour. Paleontol., vol. 9, p. 239 – 265.
23. Hamama, H. and Gabir, M. (2001): Lower Cretaceous (Barremian – Albian) ammonites of Gabal Risan Aneiza, Egypt. In: Proceedings of the 2nd International Conference of the Geology of Africa, ASSUIT. PP. 421 – 441.
24. Hegab, O. A., Hamama, H. H. and Attia, N. A. (1998): Stratigraphy, facies and environment of the Lower Cretaceous of Gebel Um Mitmam, Maghara area, north Sinai. Proc 2nd Conf. Geol. Sinai Develop., Ismailia, p. 110 – 120.
25. Hewaidy, A. G. A, Azab, M. M. and Kamel, D. E. (1988): Macrobiostratigraphy of the Lower/Middle Cretaceous sequence in some parts of northern Egypt. Proceeding 11th Symposium, Phanerozoic, Development, Egypt, P.39-77.
26. Iba, Y. and Sano, S. (2007): Mid-Cretaceous step-wise demise of the carbonate platform biota in the Northwest Pacific and establishment of the North Pacific biotic province. Palaeogeography, Palaeoclimatology, Palaeoecology.Volume 245, Issues 3-4, Pages 462-482.
27. Karakasch, N. E. (1907): Lower Cretaceous deposits of the Cremia and their fauna.work CPB, ob-va, t. XXXII, issue 5.
28. Kasansky,P., A. ((1913-1914): Description of collection of Cephalopoda of Cretaceous sediments of Daghistan, t. XXXII, kh. 4.
29. Kilian, W. (1907-19013): Unterkreide (Palaaeocretacicum). Lethaea geognosticaII Theil. Das Mesozoicum, bed. 3, Kreide.
30. Lehmann, J., Heldt, M.,Bachmann, M. and Hedi Negra, M.b(2009): Aptian (Lower Cretaceous) biostratigraphy and cephalopods from north central Tunisia. Cretaceous Research, Volume 30, Issue 4, Pages 895-910.
31. Luppov, N. P., & Drushchits, eds. (1958): Molliuski—Golovonogie II. Ammonoidea (tseratity I ammonity) Vnucrennerakovinny prilozhenie: Konikonkhii (Mollusca—Cephalopoda II, Ammonoidea (ceratites and ammonites) and Endococochlia). IU. A. orlov, ed. Osnovy paleontologii [Fundamentals of Paleontology], vol. 6. Gosudarstvennoe Nauchno-tekhnicheskoe Izdatelstvo Literatury po Geologii I Okhrane Neder. Moscow, 168 fig., 360 pp
32. Mahmoud, M.S. and Moawad, A.M. (2000): Jurassic-Cretaceous (Bathonian to Cenomanian) palynology and stratigraphy of the West Tiba-1 borehole, northern Western Desert, Egypt. Journal of African Earth Sciences, Volume 30, Issue 2, February 2000, Pages 401-41.
33. Marcinowski, R, and Wiedmann, J., (1990): The Albian ammonites of Poland. Warszawa Krakova, , 25 Pl, pp 94.
34. Matsumoto, T. (1980): Inter-regional correlation of transgressions and regressions in the Cretaceous period. Cretaceous Research, Volume 1, Issue 4, Pages 359-373.
35. Mekawy, M. S.) and Abu-Zied (2008) : Lower Cretaceous Molluscan fauna from north Sinai, Maghara area, Egypt. Egypt. Jour. Paleontol., vol. 8, p. 291-334.
36. Michelin, H. (1838): Note sur une argile du Gault observe' au Gaty depart. De l'Aube. Me'm. Soc. Geol. France, t. III
37. Moore, R. C., eds, (1960): Treatise on invertebrate paleontology, part I, Mollusca 1, Geological Society of America, Inc and the University of Kansas, 351pp.
38. Moore, R. C., eds, (1960): Treatise on invertebrate paleontology, part N, Mollusca 6, Bivalvia. Geological Society of America, Inc and the University of Kansas, P. 491- 952.
39. Moore, R. C., eds. (1996) Treatise on Invertebrate Paleontology. Part. L. Mollusca 4, revised, vol. 4, Cretaceous Ammonoidea. The Geological Society of America, INCA and Kansas Univ. Press., New York and Lawrence. XX + 362p. 216 fig., 2 tab.
40. Murphy, M. A. (1967): The Aptian-Cenomanian members of the ammonite genus *Tetragonites*. 93 pp
41. Pcelincev, V. F. and Korobkov, E.A. (1960): Ocnova Paleontologii Spravoschnek dlia Paleotologov ei geologov, Molluski-Brokhionogea.Gasoderstevnoe Naoschno-Tekhneschkoe Ezdatelstva Letraturi po geologii ei Okhrany NEDR, Moscova, 28 pl, 360pp.
42. Naci Görür (1991): Aptian – Albian palaeogeography of the Neo-Tethyan domain. Palaeogeography, Palaeoclimatology, Palaeoecology, vol. 87, issues 1-4, p 267-288.
43. Orbigny, A. (1840-1842): A. Paleontolologie Francaise. Terrains cretaaces, t. I, Cephalopodes.
44. Pcelincev, V. F. and Korobkov, E.A. (1960): Ocnova Paleontologii Spravoschnek dlia Paleotologov ei geologov, Molluski-Brokhionogea.Gasoderstevnoe Naoschno-Tekhneschkoe Ezdatelstva Letraturi po geologii ei Okhrany NEDR, Moscova, 28 pl, 360pp

45. Pictet, F. J. (1847): De'scription des Mollusques fossils. Qui se trouvent dans les Gre's Verts des environs de Gene've. I, Ce'phalopodes. Me'm. Soc. Phys. Hist. nat. Gene've, 11, pp 257-412.
46. Rengarten, V. (1926): Fauna of the Cretaceous rocks in Caucasus.Tr. Geol. Kom., new seies, T. 147.
47. Rigane, A., Moncef, F., Claude, G., Mabrouk, M. et al.(2004): The "Aptian Crisis" of the South-Tethyan margin: New tectonic data in Tunisia. Earth and Planetary Science Letters, Volume 223, Issues 3-4, P. 303-318.
48. Ruffell, A. H. and Batten, D. J. (1979): The Barremian-Aptian arid phase in Western Europe. Palaeogeography, Palaeoclimatology, Palaeoecology, Vol. 80, Issues -4, P. 197-212
49. Saveleev, A.A. (1958): Lower Cretaceous Trigoniids Mangishlakvol and west Turkmenia, Leningrad, 516pp.
50. Steuber, T. and Löser, H. (2000): Species richness and abundance pattern of Tethyan Cretaceous rudist bivalves (Mollusca: Hippuritacea) in the central-eastern Mediterranean and Middle East, analysed from a palaeontological database. Palaeogeography, Palaeoclimatology, Palaeoecology, Volume 162, Issues 1-2, Pages 75-104.
51. Vasicek, Z., (1972): Ammonoidea of the Tesin – Hradiste Formation (Lower Cretaceous) in the Moravskoslezske Beskydy Mts. Vydal Ustredni ustav geologicky, Praha, Pl. I - XVI, P.10
52. Uhlig, V. (1883): Die cephalopodenfauna der Wernsdorfer Schichten. Denkschr d. k. k. Akad. D. Wissensh. Math. Naturwissensch. Kl., Bd. XLVI.

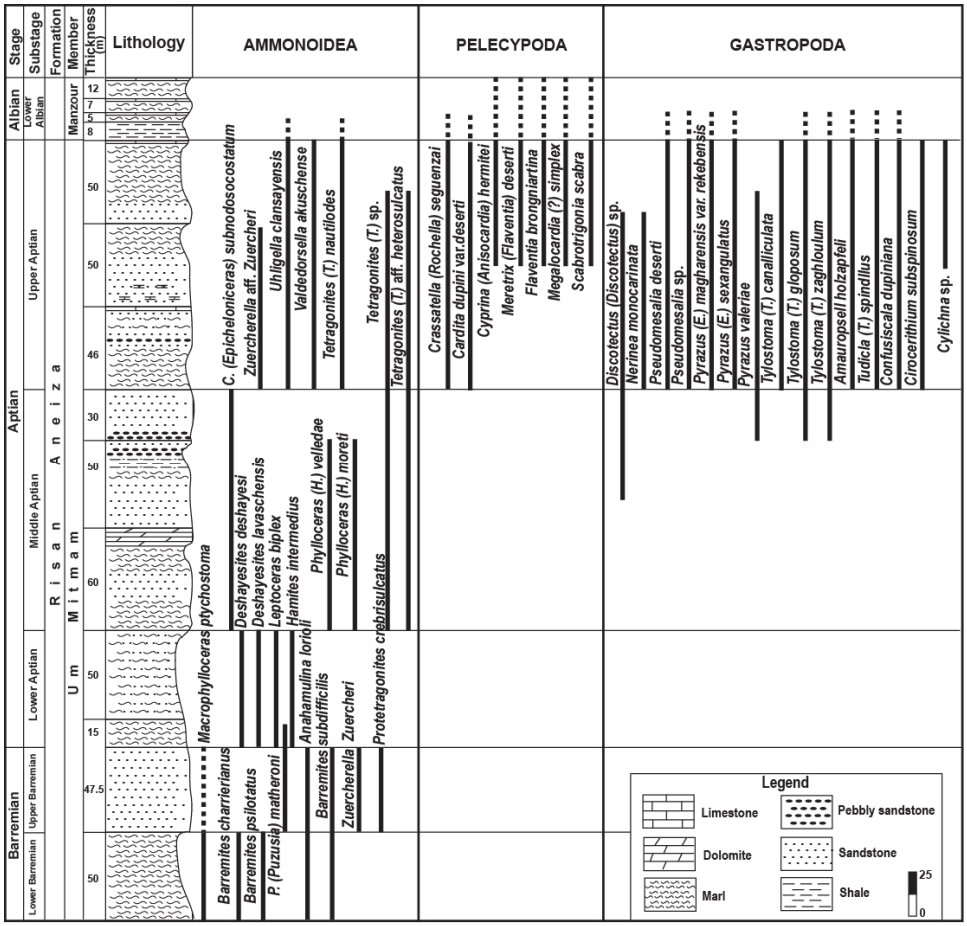
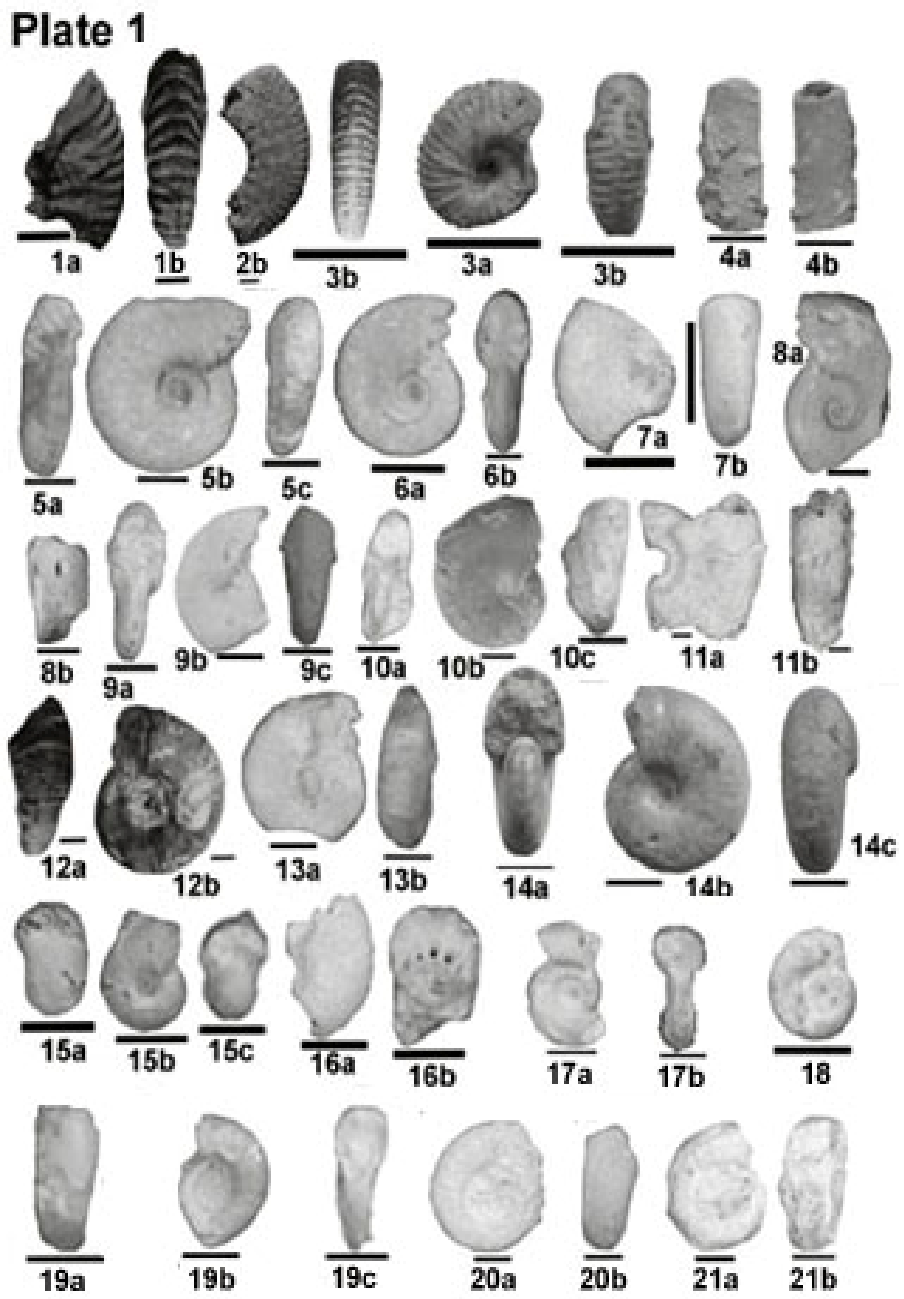
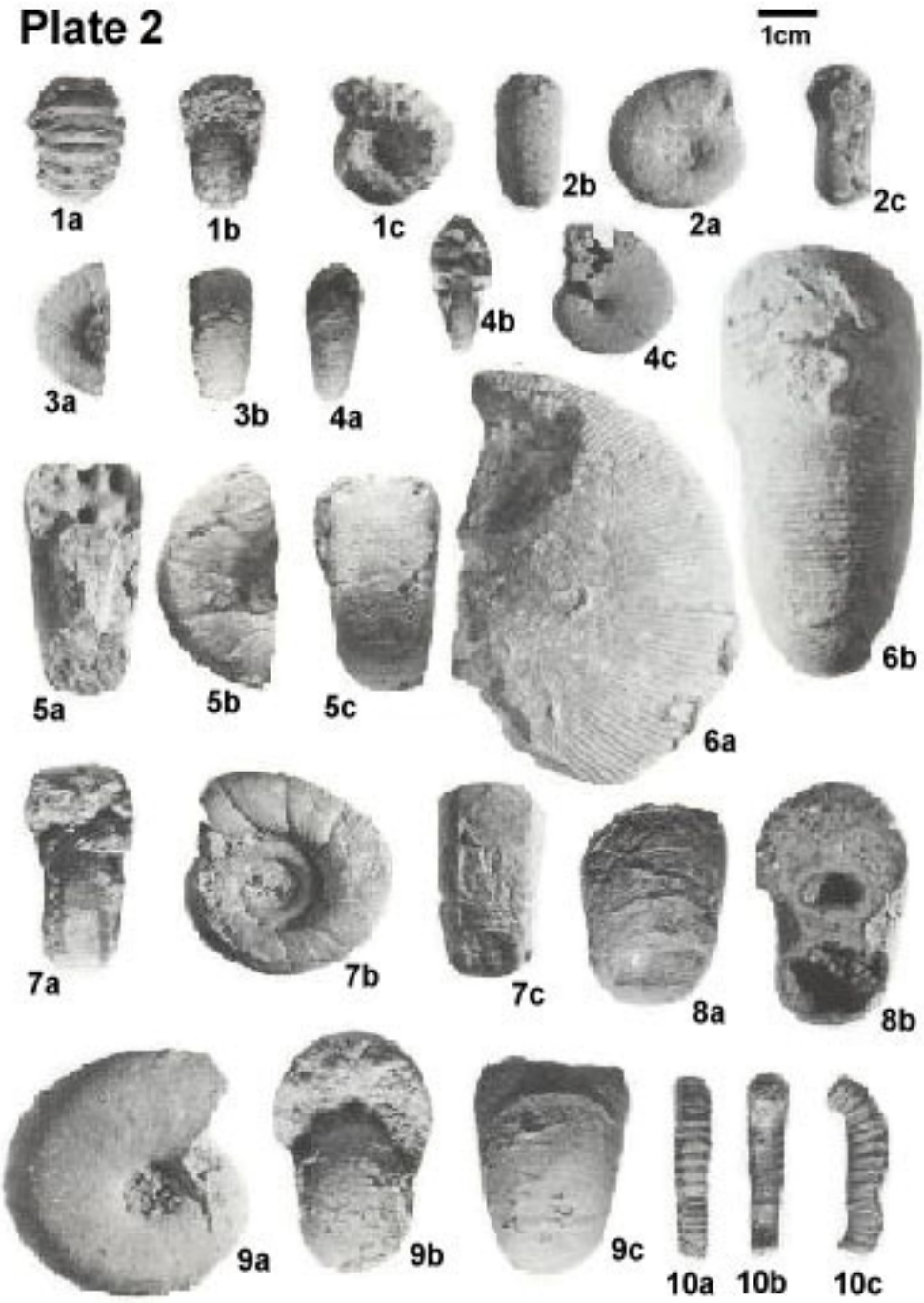


Fig. 2: Text- Fig (2): Distribution chart of the Aptian and Albian Mollusca of Um Mitmani and Gabal Mistan.



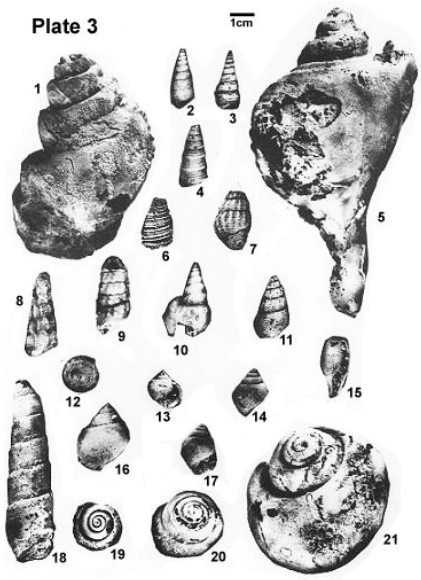
Explanation of Plate 1(bar=1cm)

Figs (1&2)*: Deshayesites deshayesi* (Leymerie MS.) Orbigny. Fig (3): *Deshayesites lavaschensis* Kazansky. Fig (4): *Anahamulina lorioli* Uhlig. Figs (5 – 7): *Barremites subdifficilis* (Karakasch, 1907). Fig (8): *Barremites charrierianus* Orbigny. Figs (9&10): *Macrophylloceras ptychostoma* Benecke, Fig (11): *Barremites psilotatus* (Uhlig). Fig (12): *Puzusia (Puzusia) matheroni* OrbignyFig (13): *Zuercherella* aff. *Zuercheri* (Jacob). Fig (14): *Uhligella clansayensis* (Jacob) Figs (15&16): *Valdedorsella akuschense* (Anthula). Figs (17 – 21): *Protetragonites crebrisulcatus* (Uhlig, 1883)



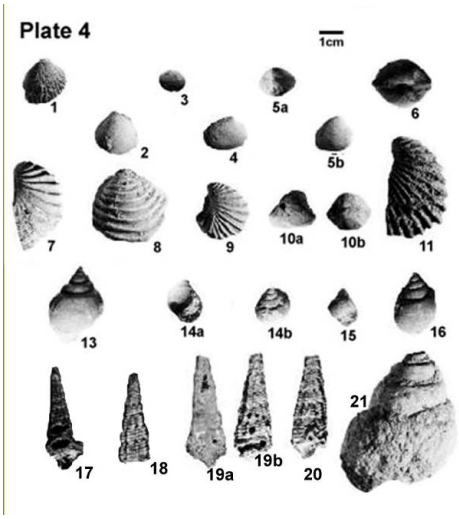
Explanation of Plate: 2

Figs (1)**:** *C.* (*Epicheloniceras*) *subnodosocostatum* Sinzow. Fig (2)**:** *T.* (*Tetragonites*) sp. Figs (3, 5&7): *Tetragonites* (*Tetragonites*) aff. *Heterosulcatus* Anthula. Fig (4)**:** *Phylloceras* (*Hypophylloceras*) *moreti* (Mahmoud). Fig (6)**:** *Phylloceras* (*Hypophylloceras*) *velledae* (Michelin). Figs (8 &9): *T. (Tetragonites) nautilodes* (Pictet). 10**.** *Hamites intermedius* Sowerby.



Explanation of Plate 3

Fig (1): *Tylostoma (T.) canalliculata* Abdel Gawad. Fig (2-3): *Pseudomesalia* sp. Fig (4): *Pseudomesalia deserti Douville´*. Fig (5): *Tudicla (Tudicla) spindllus* nov. sp. Fig (6&12)*: Discotectus* (*Discotectus*) sp. Fig (7): *Confusiscala dupiniana* Orbigny. Fig (8): *Pyrazus* (*Echinobethra) magharensis* var. *rekebensis* Abbass; Fig (9): *Pyrazus* aff. *valeriae* (Vern. & Lor). Figs (10- 11): *Cirocerithium subspinosum* Deshayes. Figs (13-14, 16-17): *Tylostoma (T.) maghareensis* Abbass. Fig (15): *Cylichna* sp. Fig (18): *Nerinea monocarinata* Pcelincev. Figs (19-20): *Tylostoma (T.) zaghloulum* nov. sp. Fig (21): *Tylostoma (T.) gloposum* Sharpe.



Explanation of Plate 4

Fig (1): *Cardita dupini* Orbigny var.*deserti* Douvillé. Fig (2 & 5): *Cyprina (Anisocardia) hermitei* Choffat. Figs (3 & 4): *Flaventia brongniartina* (Lymerie). Fig (6): *Meretrix* (*Flaventia*) *deserti* Douville. Figs (7, 9 &11): *Scabrotrigonia scabra (*Lamarck). Fig (8): *Crassatella (Rochella) seguenzai* (Thomas & Peron). Fig (10): *Megalocardia* (?) *simplex* (Mahmoud). Fig (13-16): *Tylostoma (T.) maghareensis* Abbass. Figs (17-20): *Pyrazus* (*Echinobethra*) *sexangulatus* Zek. Fig (21): *Amauropsell holzapfeli* Cossmat.

Phenotypic and Gene-technological Methods for the Identification of Clinically Isolated *Streptococcus pneumoniae* from Egyptian Children

Fouz Mohamed El-Antably[*1], Salah Abdalla[2], Alaa El-Dien MS Hosney[3] and Gehan Saddik El-Hadidy[4]

[1]Ministry of Heath, Egypt, [2]Department of Microbiology & Immunology, Faculty of Pharmacy, Suez Canal University, Egypt, [3]Department of Microbiology & Immunology, Faculty of Pharmacy, Cairo University, Egypt, [4] Department of Microbiology & Immunology, Faculty of Medicine, Suez Canal University, Egypt
fouzelantably@yahoo.com[*]

Abstract: *Streptococcus pneumoniae* is an important human pathogen that causes both serious invasive infections, such as septicemia, meningitis and pneumonia, as well as mild upper respiratory infections. The purpose of the study was to identify the *Streptococcus pneumoniae* using the conventional phenotypic methods and the PCR assay; especially, to evaluate their usefulness in the identification of the suspected pneumococcal isolates lacking one or more of their typical phenotypic characteristics. A total of 123 nasopharyngeal specimens obtained from children under five years of age, with acute upper respiratory tract infection were subcultured and identified by conventional and gene-technological methods. Forty-one isolates were identified as *Streptococcus pneumoniae*. Approximately (7.31%) were found to be atypical optochin-resistant, while, (4.87%) were bile insoluble. A 209-bp fragment indicative the pneumolysin (ply) gene was obtained from all typical and atypical isolates. The bile solubility test is more specific than the optochin test for identification of *Streptococcus pneumoniae*. Genetic test (PCR) for *ply* could be used to evaluate any isolates giving questionable results by any of the other phenotypic methods.

[Fouz Mohamed El-Antably, Salah Abdalla, Alaa El-Dien MS Hosney and Gehan Saddik El-Hadidy. **Phenotypic and Gene-technological Methods for the Identification of Clinically Isolated *Streptococcus pneumoniae* from Egyptian Children**. Journal of American Science 2010;6(12):1715-1719]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: *Streptococcus pneumonia*; optochin susceptibility test; bile solubility; *pneumolysin* PCR.

1. Introduction:

Streptococcus pneumoniae (pneumococcus) is a major bacterial infection worldwide, ranging from common infections such as otitis media to life threatening invasive infections such as sepsis, meningitis and pneumonia. Pneumococcus is the sixth most frequently isolated organism from human patients (Harakeh *et al.*, 2006). It has one of the largest public health and economic impacts of any bacterial infectious disease agent in both developing and industrialized countries (O'Brien *et al*., 2003). Pneumococcal disease kills over 1.6 million people each year. The vast majority of its victims come from the world's poorest countries (All-Party Parliamentary Group (APPG) on Pneumococcal Disease Prevention in the Developing World, 2008). It affects people of all ages, but its incidence is especially high in children less than 2 years and in adults more than 65 years (Domínguez *et al*., 2002). World Health Organization estimates that between 700 000 and 1 million children under five die from pneumococcal diseases each year (World Health Organization, 2005), and at least one child dies of pneumococcal disease every minute (All-Party Parliamentary Group (APPG) on Pneumococcal Disease Prevention in the Developing World, 2008).

Streptococcus pneumoniae is a member of the *Streptococcus mitis-Streptococcus oralis* group (the Smit group) of viridans group streptococci, which includes *Streptococcus mitis*, *Streptococcus oralis*, *Streptococcus cristatus*, *Streptococcus infantis*, *and Streptococcus peroris* (Arbique *et al*., 2004). There are now 90 recognised serotypes of *Streptococcus pneumoniae*, and the involvement of different serotypes in invasive disease varies between countries and between different age groups within the same country (Mckenzie *et al*., 2000). Most of the ninety pneumococcal serotypes immunologically distinguishable by their polysaccharide capsules are potentially pathogenic (García-Suárez *et al*., 2006).

Rapid and accurate diagnosis of pneumococcus infections plays an important role in treatment, effective management and control of outbreaks. The laboratory identification of

Streptococcus pneumoniae is based on the hemolysis pattern when it is cultured on blood agar plates and by confirmatory tests that include optochin (ethylhydrocupreine hydrochloride) sensitivity, bile solubility, miniaturized manual systems such as the API 20 Strep system, reaction with specific antisera, and PCR assays (Rudolph *et al*., 1993; Gardman & Miller,1998; Kellogg *et al*., 2001; Scott *et al*., 2003; Verhelst *et al*., 2003; Arbique *et al*., 2004; Messmer *et al*., 2004; Saukkoriipi et al., 2004; Slotved *et al*., 2004).

The purpose of the study was to identify the *Streptococcus pneumoniae* using the conventional phenotypic methods and the PCR assay; especially, to evaluate their usefulness in the identification of suspected pneumococcal isolates lacking one or more of their typical phenotypic characteristics.

2. Materials and methods

Bacterial strains:

A total of 123 nasopharyngeal specimens were obtained from children under five years of age suffering from acute upper respiratory tract infections defined as an illness having a sudden onset with rhinorrhea, pharyngitis, or cough, indicating mucosal involvement of the nose, throat, or bronchus. The cases were visiting the ENT Department of Ismailia General Hospital and the outpatient Department of Ismailia Fever Hospital. Samples were collected between September and November 2007. All specimens were immediately submerged into test tubes containing 2 ml of Skim-milk tryptone glucose glycerol (STGG) transport medium and cultured within 3-4 hours of collection.

Optochin sensitivity test: The suspected α-hemolytic colonies were touched with a sterile loop and streaked onto a tryptic soy agar plate with 5% defibrinated sheep blood in a straight line. Then, aseptically place an optochin disk (Oxoid) with a diameter of 6 mm containing 5 µg of ethylhydrocupreine HCl on the streak of inoculum. The plates were incubated in 5% CO_2 in a candle-jar at 35°C for 18–24 hours. Zone of inhibition of growth ≥14 mm in diameter indicated positive result.

Tube bile solubility test: Cells from fresh growth on agar plate were suspended in 2ml of sterile saline similar to that of a 2.0 McFarland or greater turbidity standard. The suspension was divided into two equal amounts (1ml per tube), 1ml of 0.9% saline was added to one tube (control), and 1ml of 10% sodium deoxycholate was added to the other. The tubes were shaked gently and incubated up to 30 minutes at 35°C. The tubes were visually compared; if clearing of turbidity occurred in the tube containing bile reagent, the tube was considered positive, indicating *Streptococcus pneumonia*e. Partial clearing was not accepted as a positive result (Messmer *et al*., 2004).

API 20 Strep system: Biochemical test was carried out according to the instructions of the manufacturer.

DNA extraction: Five to ten colonies were suspended in 100µl sterile distill H_2O and incubate at 100°C for 10 min. Then, a centrifugation at 12000g for 1 min was carried out. The supernatant was collected and stored at -20°C until used (Morrison *et al*., 2000; Mayoral *et al*., 2005).

Ply gene PCR assay: The oligonucleotide primers used for the amplification are IIa, (5'-CCC ACT CTT CTT GCG GTT GA-3') and IIb, (5'-TGA GCC GTT ATT TTT TCA TAC TG-3') amplify a 209-bp region of the *ply* gene. This target DNA sequence was used in developing the PCR assay according to Verhelst et al., 2003. The PCR mixture (20µl) contained 10µl of 2x TaqMix complete (Alliance Bio), 50 pmoles for each primer, 4µl Sterile distill H_2O, and finally 5µl of the extracted DNA was added. Amplification was performed using an automated thermal cycler (BIO-RAD) with the following parameters: Predenaturation 94°C for 10 min, followed by 30 cycles of (30 s at 94°C, 30 s at 55°C, and 30 s at 72°C for denaturing, annealing, and extension, respectively) with a final post-extension at 72°C for 6 min. Approximately 10 µl of each PCR amplicon was electrophoresed using a 2.0% agarose/1XTAE buffer gels and subsequently stained with ethidium bromide and visualized with a UV transilluminator. Amplified product size was determined by comparison with a 100bp ladder DNA marker (Axygen biosciences).

Control strain: A positive control (*Streptococcus pneumoniae* ATCC 49619) was included in all assays.

3. Results

Out of 123 isolates**,** 41(33.3%) isolates surrounded by a greenish zone of α- hemolysis after incubation on tryptic soy blood agar medium plates in a 5% CO_2 atmosphere for 18-24hours. Thirty-eight of them had typical pneumococcal colonial morphology and showed optochin inhibition zones: range, 18 to 22 mm with a clear zone, and they were bile soluble. Three isolates (7.31%) were found to be atypical optochin-resistant with no inhibition zone: Two isolates of them (4.87%) were small, dry colonies and bile insoluble, while, one isolate was typical colonial morphology and bile soluble. The API 20 Strep system (bioMérieux, Marcy L'Etoile, France) failed to definitively identify any of the isolates. Applying conventional PCR technique for *ply* gene, a 209-bp fragment was obtained from all isolates (Fig; 1).

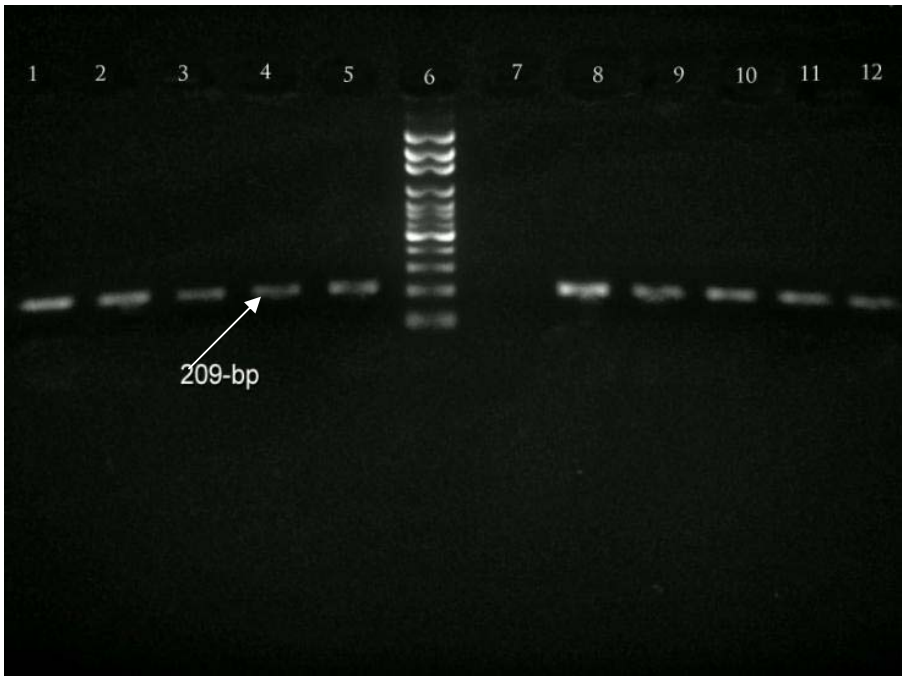


Fig. (1): Agarose gel electrophoresis of PCR-amplified products
Lane 6: Molecular weight marker (Twelve discrete fragments ranging from 100bp to 3000bp);
Lane 7: Negative control; Lanes 1-5, 8- 12: PCR positive

4. Discussion:

The accurate identification of pneumococci isolates has traditionally relied on observations of colony morphology, α-hemolysis on sheep blood agar, optochin susceptibility, and bile solubility tests. Atypical (nontypeable) pneumococci have been previously reported (Kearns *et al*., 2000; Whatmore *et al*., 2000; Obregón *et al*., 2002; Messmer *et al*., 2004). They may produce atypical reactions in one or more of the standard tests, leading to misidentification and thus may influence diagnosis and treatment. PCR for the *ply* gene has been observed to be sensitive and reliable in detecting of pneumococcus. Our result showed that all typical and atypical isolates showed positive band at 209-bp and thus were confirmed to be pneumococci. However, PCR requires special skills and equipment; therefore, at this stage, we suggested that the *ply*-PCR is a basic tool for the identification of "difficult" isolates suspected of being pneumococci.

Bile solubility and optochin sensitivity have shown to have almost complete correlation, but in 10% of cases the interpretation was considered uncertain. In our study, the optochin resistant was observed in 7.31% of isolates that had no inhibition zone. The resistance results from point mutation in the atpC gene, which prevents optochin from disrupting the H^+ transport path, by this way the strains lose their susceptibility to this compound (Pikis *et al*., 2001). However, some studies have presented that the number of colonies and the optochin discs that are used may influence the optochin sensitivity (Wasilauskas & Hampton, 1984; Gardman & Miller, 1998). Kaijalainen *et al.* 2002, reported that the density of colonies has only a small effect on the result of the optochin sensitivity test. However, when heavy inoculums is used, the diameter of the optochin sensitivity test is smaller than when a light inoculums is used, and in borderline cases the result of the optochin sensitivity test should be interpreted as sensitive or the test should be repeated.

On the other hand, the result of the present study suggests that bile solubility test is more sensitive than the optochin sensitivity test as only 4.87% was found to be bile insoluble. This result is consistent with other reported series (Burdash & West, 1982; Wasilauskas & Hampton, 1984; Davis *et al*., 1992; Kellogg *et al*., 2001), on the contrary, Kaijalainen *et al.* 2002 reported that the optochin sensitivity test is still a reliable and practical test for identifying pneumococcus from invasive as well as respiratory infections, and even from nasopharyngeal specimens. A false-positive bile solubility result will occur more often when the test is performed directly

on colonies on the agar surface rather than on those in broth medium (Denys & Carey, 1992).

Biochemical identification of pneumococcus has been proved to be quite difficult. The API 20 STREP system could not identify any of the isolates tested, and in all cases, additional testing was required before identification could be made. Some studies are in agreement with these finding (Fordymacki *et al*., 1998; Verhelst *et al*., 2003; Arbique *et al*., 2004). Other serological tests provide simpler and more rapid serological identification of *Streptococcus pneumonia* from culture (Smith & Washington, 1984; Wasilauskas & Hampton, 1984). These rely on visible detection of an antigen-antibody complex resulting from the reaction between pneumococcal surface antigens and type-specific antibodies. However, pneumococcal strains lacking a polysaccharide capsule cannot be identified by serological tests (Arbique *et al*., 2004).

On the basis of our observations, it is recommended that the genetic test for *ply* could be used to evaluate any isolates giving questionable results by any of the other phenotypic methods. On the other hand, the tube deoxycholate bile solubility test is preferred over the optochin susceptibility assay as a primary means of identification of most routine isolates of *Streptococcus pneumoniae*. Because the latter assay requires overnight incubation and the number of false negative tests were higher.

Acknowledgment

I would like to thank Dr. Ahmed Samir Mohamed, Lecturer of Microbiology, Faculty f Veterinary Medicine, Cairo University, for offering the reference strain (*Streptococcus pneumoniae* ATCC 49619) used in this study.

Corresponding author
Fouz Mohamed El-Antably
Ministry of Heath, Egypt
fouzelantably@yahoo.com

5. References:

1- All-Party Parliamentary Group (APPG) on Pneumococcal Disease Prevention in the eveloping World (2008). Improving global health by preventing pneumococcal disease.
2- Arbique, J., C., Poyart, C., Trieu-Cuot, P., Quesne, G., Carvalho Mda, G., Steigerwalt, A., G., Morey, R., E., Jackson, D., Davidson, R., J., Facklam, R., R. (2004). Accuracy of phenotypic and genotypic testing for identification of *Streptococcus pneumoniae* and description of *Streptococcus pseudopneumoniae* sp. nov. *J Clin Microbiol* 42: 4686–4696.
3- Burdash, N., M., West, M., E. (1982). Identification of *Streptococcus pneumoniae* by the phadebact coagglutination test. *J Clin Microbiol* ., 15:391-394.
4- Davis, T., E., Fuller, D., D., Aeschleman, E., C. (1992). Rapid, direct identification of *Staphylococcus aureus* and *Streptococcus pneumoniae* from blood cultures using commercial immunologic kits and modified conventional tests. *Diagn Microbiol Infec*t *Dis* 15:295–300.
5- Denys, G., A., Carey, R., B. (1992). Identification of *Streptococcus pneumoniae* with a DNA probe. *J Clin Microbiol* 30:2725-2727.
6- Domínguez, A., Salleras, L., Cardeñosa, N., Ciruela, P., Carmona, G., Martínez, G., Torner, N., Fuentes, M. (2002). The epidemiology of invasive *Streptococcus pneumoniae* disease in Catalonia (Spain) A hospital-based study. *Vaccine*, 20: 2989–2994.
7- Fordymacki, P., Kochman, M., Lawrynowicz-Paciorek, M., Kałuzewski, S. (1998) Evaluation of the usefulness of selected commercial systems for identification of Streptococcus species (Abstract). *Med Dosw Mikrobiol* ., 50:171-177.
8- García-Suárez Mdel, M., Villaverde, R., Caldevilla, A., F., Méndez, F., J., Vázquez, F. (2006). Serotype distribution and antimicrobial resistance of invasive and non-invasive neumococccal isolates in Asturias, Spain. *Jpn J Infect Dis*., 59:299-305.
9- Gardman, M., A., Miller, M., A. (1998). Optochin revisited: defining the optimal type of blood agar for presumptive identification of *Streptococcus pneumoniae*. *J Clin Microbiol* , 36:833-834.
10- Harakeh, S., Yassine, H., El-Fadel, M. (2006). Antimicrobial-resistance of *Streptococcus Pneumoniae* isolated from the Lebanese environment. *Mar Environ Res*., 62:181–193.
11- Kaijalainen, T., Rintamäki, S., Herva, E., Leinonen, M. (2002). Evaluation of gene-technological and conventional methods in the identification of *Streptococcus pneumonia*. *J Microbiol Methods*, 51:111-118.
12- Kearns, A., M., Wheeler, J., Freeman, R., Seiders, P., R., Perry, J., Whatmore, A., M., Dowson, C., G. (2000). Pneumolysin detection identifies atypical isolates of *Streptococcus pneumoniae*. *J Clin Microbiol* ., 38:1309–1310.
13- Kellogg, J., A., Bankert, D., A., Elder, C., J., Gibbs, J., L., Smith, M., C. (2001). Identification of *Streptococcus pneumoniae* revisited. *J Clin Microbiol*., 39:3373-3375.
14- Mayoral, C., Noroña, M., Baroni, M., R., Giani, R., Zalazar, F. (2005). Evaluation of a nested-PCR assay for *Streptococcus pneumoniae*

detection in pediatric patients with community acquired pneumonia. *Rev Argent de Microbiol*., 37: 184-188.

15- Mckenzie, H., Reid, N., Dijkhuizen, R., S. (2000). Clinical and microbiological epidemiology of *Streptococcus pneumoniae* bacteremia. *J Med Microbiol*., 49:361-366.

16- Messmer, T., O., Sampson, J., S., Stinson, A., Wong, B., Carlone, G., M., Facklam, R., R. (2004). Comparison of four polymerase chain reaction assays for specificity in the dentification of *Streptococcus pneumoniae*. *Diagn Microbiol Infect Dis.,* 49:249–254.

17- Morrison, K., E., Lake, D., Crook, J., Carlone, G., M., Ades, E., Facklam, R., Sampson, J., S. (2004). nasopharyngeal secretions of healthy children: comparison of real-time PCR and culture from STGG-transport medium. *Mol Cell Probes*, 18:147–153.

18- Obregón, V., Garcia, P., Garcia, E., Fenoll, A., López, R., Garcia, J., L. (2002). Molecular peculiarities of the *lytA* gene isolated from clinical pneumococcal strains that are bile insoluble. *J Clin Microbiol*., 40: 2545-2554.

19- O'Brien, K., L., Nohynek, H., and the Pneumococcal Vaccine Trials Carriage Working group (2003). Report from a WHO Working Group: standard method for detecting upper espiratory carriage of *Streptococcus pneumoniae*. *Pediatr Infect Dis.,* 22: e1-11.

20- Pikis, A., Campos, J., M., Rodriguez, W., J., Keith, J., M. (2001). Optochin resistance in *Streptococcus pneumoniae*: Mechanism, significance, and clinical implications. *J Infect Dis*., 184:582– 590.

21- Rudolph, K. M., Parkinson, A., J., Black, C., M., Mayer, L., W. (1993). Evaluation of polymerase chain reaction for diagnosis of pneumococcal pneumonia. *J Clin Microbiol.,* 31: 2661-2666.

22- Saukkoriipi, A., Leskelä, K., Herva, E., Leinonen, M. (2004). *Streptococcus pneumoniae* in nasopharyngeal secretions of healthy children: comparison of real-time PCR and culture from STGG-transport medium. *Mol Cell Probes.* 18:147–153.

23- Scott, J., G., Marston, E., L., Hall, A., J., Marsh, K. (2003). Diagnosis of pneumococcal pneumonia by PsaA PCR analysis of lung aspirates from adult patients in Kenya. *J Clin Microbiol*., 41: 2554–2559.

24- Slotved, H., C., Kaltoft, M., Skovsted, I., C., Kerrn, M., B., Espersen, F. (2004). Simple, rapid latex agglutination test for serotyping of Pneumococci (Pneumotest-Latex). *J Clin icrobiol* 42:2518-2522.

25- Smith, S., K., Washington, JA2nd. (1984). Evaluation of the Pneumoslide latex agglutination test for identification of *Streptococcus pneunoniae*. *J Clin Microbiol* ., 20:592-593.

26- Verhelst, R., Kaijalainen, T., De Baere, T., Verschraegen, G., Claeys, G., Van Simaey, L., De Ganck, C., Vaneechoutte, M. (2003). Comparison of five genotypic techniques for identification of optochin-resistant pneumococcus-like isolates. *J Clin Microbiol*., 41: 3521–3525.

27- Wasilauskas, B., L., Hampton, K., D. (1984).An analysis of *Streptococcus pneumoniae* identification using biochemical and serological procedures. *Diagn Microbiol Infect Dis*., 2:301–307.

28- Whatmore, A., M., Efstratiou, A., Pickerill, A., P., Broughton, K., Woodard, G., Sturgeon, D., George, R., Dowson, C., G. (2000). Genetic relationships between clinical isolates of *Streptococcus pneumoniae, Streptococcus oralis, and Streptococcus mitis:* characterization of "atypical" pneumococci and organisms allied to *S. mitis* harboring *S. pneumoniae* virulence factor encoding genes. *Infect Immun*., 68:1374–1382.

29- World Health Organization (2005). Vaccinating African children against pneumococcal disease saves lives. World Health Organization, Geneva.

11/22/2010

Relationship between Nursing Procedures and Oxygen Saturation Level of Preterm Infants with Respiratory Distress Syndrome

Randa El-Sayed Mohammed El-Sayed*; Gamalat El-Sayed Mansy; Bahaa Salah El-Din Hamad and Nehad Sabry Basiouny

Pediatric Nursing, Faculty of Nursing, Alexandria University, Alexandria, Egypt
*randa_7373@yahoo.com

Abstract: The present study was conducted to determine the relationship between nursing procedures (Suctioning, change of position, Heel stick) and blood oxygen saturation level, using pulse oximter monitoring. Fifty preterm infants with respiratory distress syndrome were monitored during performing the nursing procedures at the Neonatal Intensive Care Unit, in Maternity University Hospital at EI-Shatby in Alexandria. An assessment sheet was developed for monitoring the oxygen level before, during, and after each of the three nursing procedures. The main results were the preterm neonates with respiratory distress syndrome reacted to nursing care procedures with decrease in oxygen saturation (SPO_2) during different positioning and repositioning, suctioning and heelstick. After the procedures, all preterm neonates returned to pre-procedure average of oxygen saturation except after repositioning from side- lying to supine, from supine to prone position, and after suctioning. The supine position contributed to a slight decrease in oxygenation. Both prone position and suctioning contributed to an increase in oxygenation after the procedures. The main recommendation is that continuous monitoring of oxygen saturation before, during and after performing the nursing procedures is mandatory.

[Randa El-Sayed Mohammed El-Sayed*; Gamalat El-Sayed Mansy; Bahaa Salah El-Din Hamad and Nehad Sabry Basiouny. **Relationship between Nursing Procedures and Oxygen Saturation Level of Preterm Infants with Respiratory Distress Syndrome**. Journal of American Science 2010;6(12):1720-1732]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Relationship; Nursing; Oxygen Saturation; Preterm Infants; Respiratory Distress Syndrome

1. Introduction:

During pregnancy, the fetus grows in the mother's uterus usually for 37 to 42 weeks. Infants born less than 37 weeks are called preterm or premature infants.(1) Those infants are at increased risk of growth problems, developmental delays and complex medical problems. (2)

According to World Health Organization (WHO)2009 data showed that, worldwide more than one million infants die each year because they are born early (or 28% of total newborn death) .The highest preterm delivery rate in the world are found in Africa (11.9%) , followed by North America (United States and Canada). (3) In the U.S.A, the preterm birth rate has risen steadily from 9.4% in 1981, to 10.4% in 2000, to 10.6% in 2005.(3,4) In Canada, there has been a slight increase in preterm birth for 7.5% in 2000, to 7.7 in 2003, to 7.9% in 2004 % , to 10.6 in 2005.(5-7)

Worldwide, in 2005 the preterm birth rate was estimated as 9.6 %-representing about 12.9 million infants.(3) In Alexandria University Hospital, the rate of preterm admission in El-Shatby Neonatal Intensive Care Unit was 74.7% in 2009. Records show that mortality rate of preterm infants has dramatically improved in the neonatal intensive care unit (NICU) from 75.1% in 2002, to 44.8% in 2004, to 35.7 % in 2009. These, records showed that respiratory distress syndrome was the second main reason for admission to the unit (11.4%) during the year 2009.(8)

The tenuous respiratory status of many preterm infants in the neonatal intensive care units places them at high risk from the potential stress of repetitive diagnostic, nursing and therapeutic procedures. These procedures are associated with alteration in physiological responses, which may result in undetected subclinical episodes of hypoxemia or hyperoxemia. (8) Handling during routine nursing care (i.e., vital signs, changing diaper or position, vein puncture for blood draws or feeding, heelsticks, and suction) can result in increased intracranial pressure, episodes of apnea/bradycardia, agitation, and increased or decreased heart rate and blood pressure. (9 - 11)

The development of oxygen saturation measurement techniques provides a tool with which oxygenation may be continuously and non-invasively monitored during nursing care procedures.(11,12) Care should be taken to include oxygen saturation target in daily practice to avoid prolonged or alternating episodes of hypoxia /hyperoxia in infant breathing supplemental oxygen.(13)

The minimum duration of hypoxia that cause brain damage is not known. Until this critical

period is determined, hypoxia for any length of time should be avoided. If the infant is unable to recover spontaneously, significant hypoxia can end in assisted ventilation and prolonged intubation. To avoid this risk, the arterial oxygen tension of critically ill preterm infants needs to be maintained at 50-70 mmHg. [14]

In the neonatal intensive care unit (NICU), preterm infants often require support of ventilation and oxygenation through endotracheal intubation and invasive positive-pressure ventilation (IPPV). [15] The presence of an endotracheal tube causes soft-tissue irritation and increased secretions. Intubation can lead to thickening of secretions. Increased or thick secretions add to the risks of endotracheal-tube blockage, lobar collapse, and compromised gas exchange. [16]Suctioning the endotracheal tube in infants with RDS is one of the stressful nursing procedures that should be done if there is secretion, and significantly affects gas exchange and should be kept to a minimum. There are many complications associated with endotracheal suctioning which include hypoxemia, bradycardia, tachycardia, pneumonia, fluctuation in blood and intracranial pressure, localized trauma to the airway, infection and tube dislodgement. [17, 18]

Repositioning of preterm infants is a basic nursing care. [19]It includes supine, prone, side lying, and head up tilted position. Several studies demonstrated variable outcomes affected by different positioning of preterm infants .The American Academy of Pediatrics recommended that the prone position is more appropriate for sick preterm infants because oxygenation is better. [20] Therefore, proper positioning may improve oxygenation and reduce the need for supplemental oxygen and mechanical ventilation. Suitable positioning to maintain normal oxygen saturation is a priority in the nursing care.[21] Preterm infants regularly are subjected to a multitude of diagnostic and therapeutic procedures that are painful but medically necessary. The most frequently performed procedure is heelstick.[22-23] Infants undergoing this procedure experience significant pain manifested by crying, facial expressions, body movements and physiologic changes .[24] Physiological indicators of pain are changes in heart rate, respiratory rate, blood pressure, oxygen saturation (SaO_2), vagal tone, palmar sweating, facial expression and intensity of cry.[25] One reason that heelstick blood collection is painful and distressing is the prolonged squeezing of the heel required to complete the sample.[26]

Many events consistently lead to hypoxemia in preterm infants: as drops in transcutaneous oxygenation $TcPo_2$ during the insertion of an intravenous needle, taking vital signs and pharyngeal suctioning.[27] Daily procedures such as feeding, examination, and diaper changes were sometimes associated with hypoxemia.[12] The physiological and behavioral effects of a supposedly beneficial procedure, a sponge bath, on preterm infant may increase heart rate, cardiac oxygen demand, and decreases in oxygen saturation . [28]

From these facts and clinical observation, changes in nursing care of the preterm infant has taken place faster than their evaluation . Meticulous attention must be given to subtle changes in the infant oxygenation status. So, nursing care should be altered and adapted based on specific observations of each infant's response to care giving. When care is structured to support the individual infant's physiologic stability and neurobehavioral organization, stress is reduced for that infant.[29]Certain routine procedures may be more harmful than beneficial to the infant. There are three routine nursing procedures that may be associated with hypoxemia as suctioning, repositioning and performing heelstick. In the present study procedures are selected because of the frequency with which they are performed in the usual management of preterm infants. The effect of nursing care procedures on the fluctuation of the oxygenation level need to be evaluated. [14]

Aim of the study

The aim of the present study was to determine the relationship between nursing procedures (Suctioning, change of position, Heel stick) and blood oxygen saturation level in preterm infants with respiratory distress syndrome.

2. Materials and methods:

Materials:

Research design: It is a descriptive study.

Setting: The study was conducted at the Neonatal Intensive Care Unit, in Maternity University Hospital at EI-Shatby in Alexandria.

Subjects:

A convenient sample of fifty preterm infants with respiratory distress syndrome who had the following criteria:

-Born at 26 -34 weeks of gestation.
- On mechanical ventilator or continuous positive air way pressure (CPAP).
-Free from any congenital malformation.
-Having normal body temperature and hemoglobin level.
-Having normal oxygen tension before nursing procedure (to exclude hyperoxia)

Tool: It comprised three parts:

Part (I):-

Neonate's biodemographic data such as: name, age, sex, birth weight, gestational age and the type of delivery.

Part (II):-

- Physiological indicators as temperature, heart rate and respiratory rate.
- Temperature of incubator.
- Hemoglobin level.
- If the infants had received surfactant or not.
- Method of oxygen administration such as: tracheal CPAP (Continuous Positive Airway Pressure) or mechanical ventilator.
- Continuous Positive Airway Pressure data included:
  * Fraction of Inspired Oxygen (FiO_2) and Positive End Expiratory Pressure (PEEP).
- Ventilator data included:
  * Mode of ventilator, ventilator category: high or low pressure setting,
  * Duration of stay on ventilator, Peak Inspiratory Pressure (PIP), (PEEP),(FIO_2),Inspiratory Time (Ti), Expiratory Time(Te), Mean Airway Pressure (MAP).
- Arterial blood gas less than 2hrs before procedure which included:
  * PH, Partial pressure of oxygen (PaO_2), Partial pressure of carbon dioxide ($PaCO_2$), Oxygen saturation (SpO_2), Bicarbonate (HCO_3).

Part (III):-

- An assessment sheet for monitoring oxygen saturation level before, during, and after each of the three nursing procedures:
- Change of positioning
  From supine to side, from side to supine, and from supine to prone.
- Endotracheal suctioning.
- Heelstick.

Method:

1. An official approval for conducting the study was obtained from the responsible administrative personnel.
2. The tool was developed by the researcher after thorough review of literatures.
3. The developed tool was validated by five experts in the field in nursing and medical field, modification proposed was implemented and the validity was 0.88.
4. Tool reliability was ascertained where the researcher observed five infants using the assessment sheet for two times with an interval period of 48 hours .The reliability of this tool was 0.82 tested by Cronobach alpha test.
5. A pilot study was done on 5 neonates to test the applicability of the tool; these five neonates were excluded from the sample
6. Each infant was observed by the researcher during the morning and afternoon shifts while the nurse was performing endotracheal suctioning, repositioning, and heelstick.
7. Arterial blood gases were obtained from the neonates by the nurse less than 2 hrs before the procedures.
8. Ventilator data were recorded immediately before each of the three procedures.
9. Temperature, heart rate and respiratory rate were measured 10 minutes before the nursing procedures.
10. Heart rate and oxygen saturation were recorded immediately before the procedure.
11. During baseline monitoring, the infants were not disturbed.
12. Oxygen saturation and heart rate were collected while the infant was receiving the nursing care (Change of positioning - suctioning - heelstick)
13. Oxygen saturation level of every procedure was recorded at 30 seconds before starting, during, and 30 seconds after each procedure until reach to average pre-procedure level.
14. The duration of procedures was recorded.
15. The length of time to return to normal O_2 saturation was measured.
16. The observer didn't interfere with the routine care of the infants. The only request made to the nurse performing the procedure was to allow the infants to return to the pre-procedure oxygen saturation average (88%- 95%) before disturbing again .
17. Several parameters were used to determine the relation of nursing procedures and oxygen saturation. These were (a) desaturation: the incidence of oxygen saturation was less than 88%; (b) high oxygen saturation: the incidence of oxygen saturation was 95% or more;(c) bradycardia : heart rate less than 100 b/m ; (d) tachycardia : heart rate more than 160 b/m.
18. Oxygen saturation was measured by pulse oximeter Nelcor-560 made in Korea or Massimo Rad–9 made in U.S.A with Max –N disposable neonatal sensors .A tiny, lighted probe placed on the infant's hand or foot projects a beam of light through the capillary beds in the tissue .The light beam is converted into an electric signal by a photo detector in the probe that is processed within the module and displayed as both a waveform and a digital value for both the oxygen saturation SpO_2 and the heart rate.

19. The three procedures were performed in the following manner consistent with routine practice in NICU:

A-Suctioning:

a. Tracheal suctioning was performed only when there was a clinical need.

b. The following considerations were followed:

- Suction Catheter was selected according to the endotracheal tube size.
- Negative suction pressure was 40-60 mmHg, it was applied intermittently and only during catheter withdrawal while simultaneously rotating the catheter.
- Hyperoxygenation of the neonates was performed before, and after suction through the ventilator by increasing fraction of inspired oxygen (FiO_2) 10-20% above the baseline.
- The endotracheal tube was disconnected at Y piece from the ventilator.
- The suction catheter was inserted into the endotracheal tube for 10-15 seconds and repeated if needed.
- The attending resident did lung recruitment procedure by the ambu bag 3-5 puffs manually.
- The endotracheal tube was reconnected at Y piece to the ventilator, and gradual decrease of FiO_2 to the pre suction level.
- The oxygen saturation level and heart rate was continuously monitored before, during and after the procedure using a pulse oximeter.

B- Changing positioning:

The paediatric nurse picked up the preterm infant during a calm period one continuous motion from back to one side or from one side to back or from back to prone.

First, the supine position was performed by supporting head, feet and body in the midline by using soft rolls around the infant. A roll under the shoulders was placed to support the newborn's airway and allowed slightly forward flexion of the head.

Second, the prone position was performed by putting the newborn's body prone. The arms should be close to the body with the hands symmetrically close up to the mouth. Flexion of the legs can be encouraged with the knees brought up to the chest, raising the hips slightly. This position was maintained by using a rolled blanket to make a boundary. Position device for prone include a small hip roll to assist in maintaining flexion. Use of a rolled cloth placed under the infant (from top of the head to umbilicus) to provide elevation of the body.

Third, the side lying position was performed by ensuring that the head and trunk should be maintained in neutral alignment. The nurse should use a roll along the infant's back (not touching the back of the head as the infant may be stimulated to push back into it). The legs should be flexed and the upper leg supported in neutral position by the use of roll between the legs.

The oxygen saturation level and heart rate were recorded during and after the procedure using a pulse oximeter.

C- Heel stick:

The nurse warmed the heel for one a minute with a warm washcloth, handled the infant's foot, and appliedmild pressure between thumb and fingers to hold ankle in dorsiflexion , cleaned the heel with alcohol swab. The puncture was made at the outer aspect of the heel with micro lancet. One drop of blood was obtained and a dressing was applied to the heel. The oxygen saturation level and heart rate were recoded before, during, and after the procedure using a pulse oximeter.

Data analysis:

Data collected were coded and transferred into specially designed formats to be suitable for computer Feeding: the SPSS version 15.0 statistical program was utilized for results .Descriptive measures included : Percentage, mean, standard deviation , “t” test, F-test ,and Chi-square were used for test of significant. Level of significant was 5% level.

3. Results

Table (1) shows the general characteristics of the study subjects. Male constituted 62.0%. Age ranged from 1-17 days with a mean 4.96 ±2.28 days. Moreover, the gestational age of the neonates ranged between 27-34 weeks with a mean 30.04±2.09 weeks. The highest percentage of preterm neonates (84%) had a gestational age less than 33 weeks.

The weight of the neonates ranged from 600-2020 gms with a mean of 1128.78±261.92 gms. More than half of the sample (54%) had a Very low birth weight (VLBW) i.e weighing 1000 to less than 1500 gms, while 20% of the sample had Extremely low birth weight (ELBW) i.e weighing less than 1000 gms .Concerning the type of delivery, 68.0% were delivered by caesarean section, while 32.0% of the preterm neonates had delivered normaly .

Figure I describes the percent of preterm neonates regarding oxygen saturation when repositioning from supine to side. The present result reveals that 96.0% of neonates had normal oxygen saturation (88-94%) before repositioning. During the procedure, it declined to 68.0%. After the procedure, it increased to 80.0% at half a minute, it was kept the

same at 2.0 minutes, and then it increased to 84.0% at 5.0 minutes. In the rest of neonates, the percent of those who had oxygen saturation ≥ 95% increased. There were statistically significant differences between the percent of neonates before repositioning and their percent during the procedure, and at half a minute, 2 minutes, and 5 minutes after.(p=0.0021, 0.0159, 0.033, 0.048 respectively)

Figure II illustrates the percent of the preterm neonates regarding oxygen saturation when repositioning from side to supine. It demonstrates that before the procedure, the percent of neonates who had normal oxygen saturation (88-94%) were 96.0%. During the procedure, their percent decreased to 54.0%. After repositioning, the percent of neonates declined to 52.0% at half a minute. This decline was apparent in the percent of neonates who had O_2 saturation< 88%. At 2.0 minutes and 5.0 minutes after the procedure, the percent of neonates increased to 74.0%, 84.0% respectively. This difference in the percent of neonates before repositioning and at 5.0 minutes after the procedure shows either increase in the percent of neonates who had O_2 saturation< 88% , or in the percent of neonates whose O_2 saturation ≥ 95% . There was a statistically significant difference between the percent of neonates before repositioning and their percent during, at half a minute, 2 minutes, and 5 minutes later.(p=0.001, 0.001, 0.036, 0.011 respectively)

Figure III illustrates the percent of the preterm neonates regarding oxygen saturation when repositioned from supine to prone. The figure reveals that 98% of the neonates who had normal oxygen saturation between 88-94% before repositioning declined to 44.0% during repositioning. This decline appears in the percent of neonates who had O_2 saturation< 88%. After the procedure, the percent of neonates increased to 88.0% at half a minute, and then kept nearly the same 86.0% at 2.0 minutes, and then it decreased to 52.0% after 5.0 minutes which is parallel by an increase in the percent of neonates who had oxygen saturation ≥ 95%. There was a statistically significant difference between the percent of neonates before repositioning and their percent during, after half, 2.0, and 5.0 minutes.(p=0.001, 0.039, 0.045, 0.001 respectively)

Figure IV shows the percent of preterm neonates regarding oxygen saturation before, during, and after suctioning. It is clear from the figure that before suctioning, all the neonates (100%) had normal oxygen saturation (88%-94%).During the procedure, their percent decreased to 20.0 %. This decline shows an increase in the percent of neonates who had O_2 saturation< 88% .After the procedure, it was observed that, the oxygen saturation of neonates improved when the percent of neonates increased to 74.0%, and 82.0% at half a minute and at 2.0 minutes respectively. At 5.0 minutes, the percent of neonates decreased to 62.0%. The decrease in percent of neonates is apparent in those who had O_2 saturation ≥95%. There were statistically significant differences between the percent of neonates before and their percent at different periods of suctioning except at 2.0 minutes after. (p=0.0001, 0.0013, 0.002 respectively)

Figure V portrays the percent of preterm neonates regarding oxygen saturation before, during, and after heelstick. It is clear that 100% of neonates had normal oxygen saturation (88-94%) before heelstick. During the procedure, the percent of neonates decreased to 72.0% .This decline is apparent in the percent of neonates who had O_2 saturation< 88%. After the procedure, the percent of neonates increased to 80.0% and 88.0% at half a minute and at 2.0 minutes respectively, and then it decreased to 82.0% at 5.0 minutes which is paralleled by an increase in the percent of neonates who had oxygen saturation ≥ 95%. Statistically significant differences between the percent of neonates before heelstick and their percent during the procedure, at half a minute , at 2.0, and at 5.0 minutes after were observed.(p=0.0032, 0.039, 0.0043, 0.045 respectively)

Table II shows the mean values of oxygen saturation of preterm neonates before, during and after the nursing procedures. It is observed that the mean oxygen saturation value was 91.49± 9.25% before repositioning from supine to side, it decreased to 88.32± 8.65% during the procedure, and to 87.86± 9.65% at half a minute after, then it gradually increased to 91.04 ± 9.25% , to 92.68± 10.6 % at 2 .0 minutes and at 5.0 minutes respectively after repositioning. There was statistically significant difference between mean oxygen saturation before the procedure and each of during, and at half a minute after the procedure (p=0.045, and 0.033 respectively). It is clear from the table that the mean value of oxygen saturation was 92.16± 7.89% before repositioning from side to supine, it decreased to 87.44± 7.25% during the procedure, then it gradually increased to 88.9 ±7.65% , to 89.86± 7.65%,and to 90.48±7.98% after the procedure at half a minute , 2.0 minutes , and 5.0 minutes respectively. There was statistically significant difference between mean oxygen saturation before the procedure and each of during, and half a minute after the procedure (p=0.048, and 0.05 respectively).

It is observed also that the mean value of oxygen saturation when repositioning of neonates from supine to prone was 90.34± 6.98% before repositioning, it decreased to 86.84±6.99% during the

procedure, then it increased to 89.38± 8.02%, 92.54 ± 8.06%,and 95.8± 11.3% at half , 2.0, and 5.0 minutes respectively after repositioning. Statistically significant difference was observed between mean value of oxygen saturation before the procedure and each of during the procedure and 5 minutes after repositioning (p=0.047, and 0.021 respectively).

In relation to Endotracheal Tube(ETT) suctioning, the same table shows that the mean value of oxygen saturation was 89.96±7.54% before the procedure, it decreased to 84.86 ± 6.52% during the procedure, then gradually increased to 90.3 ±7.36%, 92.88±7.65%, and 93.62 ±10.6% at half, at 2, and at 5 minutes after suctioning respectively. There was a statistically significant difference between mean value of oxygen saturation before the procedure and each of during the procedure and 5 minutes after repositioning (p=0.041, and 0.048 respectively).

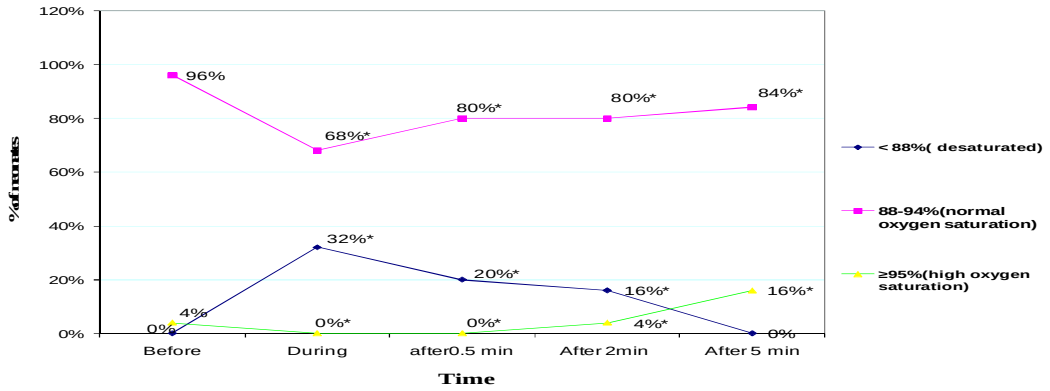
Concerning the heelstick, the mean value of oxygen saturation before the procedure was 92.26±6.98%, it declined to 85.65± 7.41% during the procedure, then it increased to 88.1±5.11%, 92.86±8.04%, and 92.96± 9.65 % at half a minute, 2 minutes and 5 minutes after the procedure. There was a statistically significant difference between mean value of oxygen saturation before the procedure and each of during the procedure and at half a minute after repositioning (p=0.036, and 0.046 respectively).While no statistically significant differences were found between mean oxygen saturation before and during the different nursing procedures.

Table (1): Percent distribution of the preterm neonates according to biodemoographic data.

Biodemographic Characteristics	N	%
Sex		
Male	31	62.0
Female	19	38.0
Age (days)		
• 1-	28	56.0
• 5-	15	30.0
• 9-	5	10.0
• 13-17	2	4.0
Range	1-17 days	
Mean±S.D.	4.96 ±2.28	
Gestational age(weeks)		
• 27-	12	24.0
• 30-	30	60.0
• 33-34	8	16.0
Range	27 – 34 weeks	
Mean±S.D.	30.04±2.09	
Weight (gm)		
• ELBW <1000 -	10	20
• VLBW 1000 -	27	54
• LBW 1500-2020	13	26
Range	600 – 2020 gm	
Mean ± S.D.	1128.78 ±261.92	
Type of delivery		
• Caesarean section	34	68.0
• Normal vaginal delivery	16	32.0
Total	50	100.0

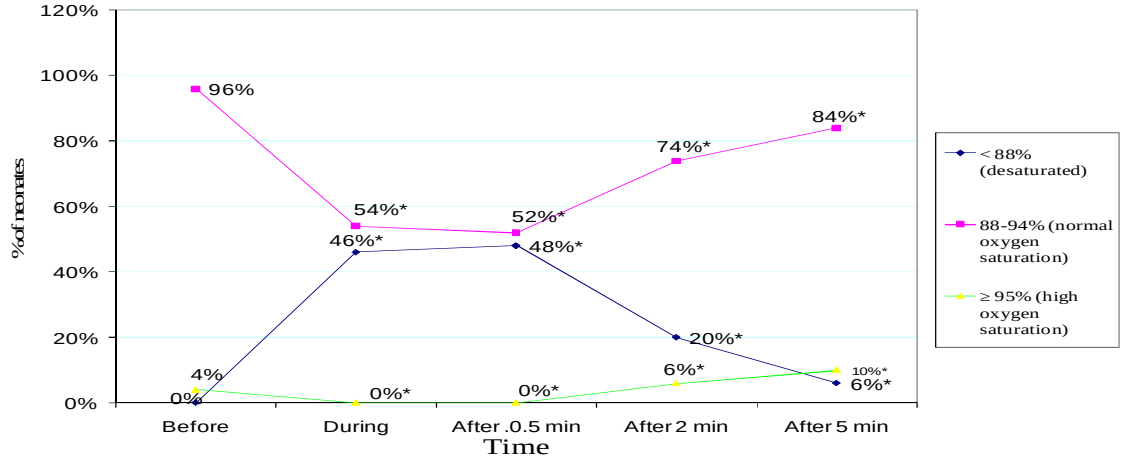
- Extremely low birth weight (ELBW): weighing less than 1000 gms.
- Very low birth weight (VLBW): weighing 1000 to less than 1500 gms.
- Low birth weight LBW: weighing less than 2500 gms

Figure (I): Percent Distribution of preterm neonates regarding oxygen saturation before , during and after repositioning from supine to side



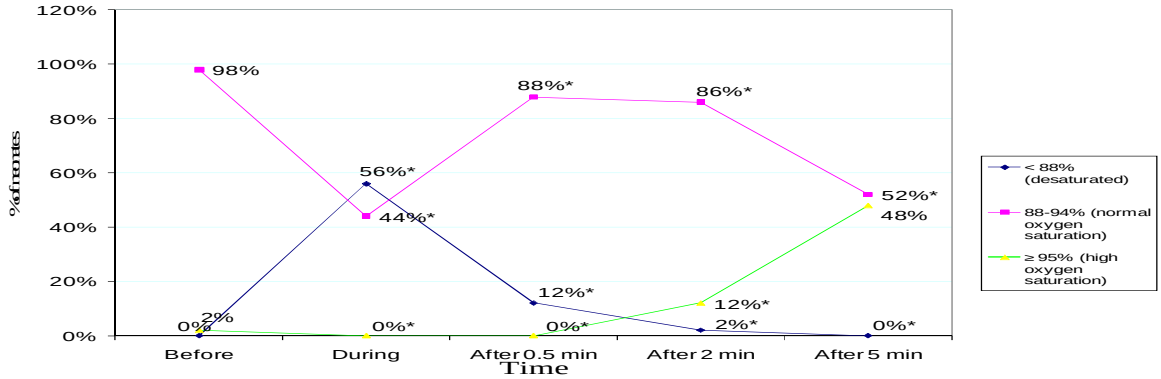
*Statistically significant at P ≤ 0.05

Figure (II): Percent Distribution of preterm neonates regarding oxygen saturation level before, during and after repositioning from side to supine



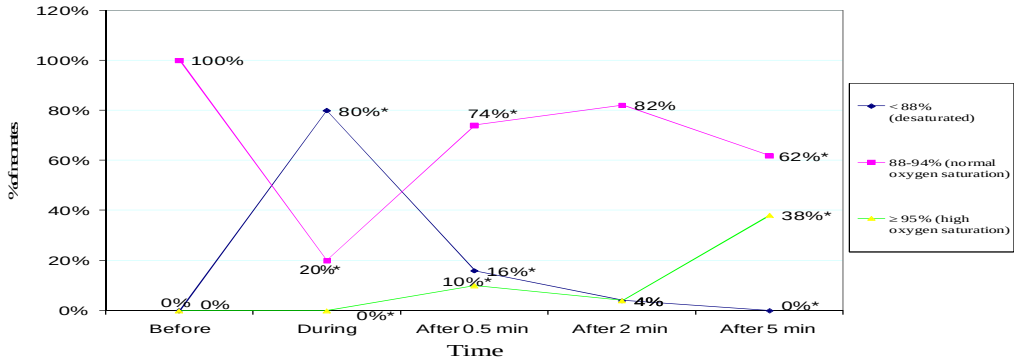
*Statistically significant at P ≤ 0.05

Figure (III): Percent distribution of preterm neonates regarding oxygen saturation before , during and after repositioning from supine to prone



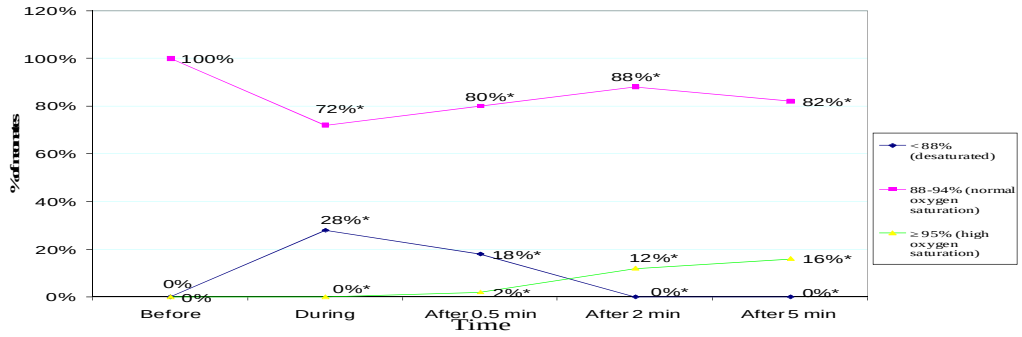
*Statistically significant at P ≤ 0.05

Figure (IV): Percent Distribution of preterm neonates regarding oxygen saturation before, during and after suctioning



*Statistically significant at P ≤ 0.05

Figure (V): Percent distribution of preterm neonates regarding oxygen saturation before, during and after heelstick



*Statistically significant at P ≤ 0.05

Table (II): Comparison of preterm neonates' mean oxygen saturation before, during and after nursing procedures

Nursing procedures	Oxygen saturation of preterm neonates				
	Before	During	After the procedures		
			0.5 min. after the procedure	2.0 min. after the procedure	5.0 min. after the procedure
(I)Reposition (1)From Supine to Side	91.49±9.25	88.32±8.65	87.86±9.65	91.04±9.25	92.68±10.6
t-test		1.89	2.11	0.48	0.62
P		0.045*	0.033*	0.62	0.45
(2)From Side to Supine	92.16±7.89	87.44±7.25	88.9±7.65	89.86±7.65	90.48±7.98
t-test		1.82	1.79	1.62	1.089
P		0.048*	0.05*	0.069	0.244
(3)From Supine to prone	90.34±6.98	86.84±6.99	89.38±8.02	92.54±8.06	95.8±11.3
t-test		1.89	1.54	0.98	2.32
P		0.047*	0.086	0.21	0.021*
(II)Endotracheal Suctioning	89.96±7.54	84.86±6.52	90.3±7.36	92.88±7.65	93.62±10.6
t-test		1.89	0.84	1.25	1.98
P		0.041*	0.46	0.098	0.048-*
(III)Heelstick	92.26±6.98	85.65±7.41	88.1±5.11	92.86±8.04	92.96±9.65
t-test		2.01	1.85	0.35	0.38
P		0.036*	0.046*	0.65	0.71
ANOVA	2.65	3.04	1.65	0.85	1.25
p	0.108	0.099	0.28	0.39	0.27

*Statistically significant at P ≤ 0.05

4. Discussion:

The fetus is brought into a world for which he is not physiologically prepared which constitutes an obstacle to normal development. Preterm infants are at high risk for chronic lung disease, intraventricular hemorrhage, retinopathy of prematurity and necrotizing entrocolitis. Moreover, possible insults to the preterm neonates during the time she/he is in the intensive care unit are exposure to stress and pain caused by medical and nursing procedures. [30,31]

In caring for the preterm neonates with respiratory distress syndrome, one of the aims of therapeutic intervention is to maintain optimal oxygenation. Therefore; the fragile preterm infants might need mechanical ventilator support .The appropriate use of oxygen in the preterm infant has been the source of concern and study for more than half a century. Oxygen therapy has been causally linked to adverse neonatal outcomes including retinopathy of prematurity and chronic lung disease due to prolonged exposure to high oxygen tensions.

Lowering oxygen saturation targets in preterm infants in the first few weeks of life has been shown to reduce the incidence of certain complications; however prolonged periods of hypoxemia may result in poor growth, cardiopulmonary complications of chronic lung disease, neurodevelopmental disabilities, or increased mortality. [32]

Preterm infants who require extended mechanical ventilator support are at high risk of the potential stress of repetitive diagnostic, nursing and therapeutic procedures. Routine nursing care (i.e., vital signs, changing diaper or position, vein puncture for blood draws, feeding, heelstick, suction, and physical or neurological examination) may result in unnoticed subclinical episodes of hypoxemia or hyperoxemia. These episodes could be detected by arterial oxygen saturation (SpO_2) monitoring by pulse oximetry. Newborn intensive care unit personnel respond to high / low Spo_2 alarms but any delay of response can prolong exposure to unnecessary high concentration of supplemental oxygen or periods of hypoxemia that may increase the risk for its complications. [10, 33, 34]

As regards positioning of preterm neonates from supine to side-lying, the finding of the present study showed that a statistically significant fall in oxygen saturation was observed during the procedure, then after the procedure there was a gradual slight increase in oxygen saturation that did not reach significance . In accordance to the current finding, Yottiem et al (2004) stated that infants with side lying position had higher mean oxygen saturation than infants with regular position.[32] Furthermore, the neonatology clinical guidelines (2006) recommended that side-lying position can be used to treat unilateral lung disease, with better oxygenation being achieved by positioning the 'good ' lung uppermost. This also supports a better oxygenation. [34]

The present results revealed a significant decrease of oxygen saturation during repositioning from side to supine and short period after the procedure. Within the five minutes observation period the supine position was associated with a slight reduction in oxygen saturation and 6% of infants did not reach the pre-procedure average of oxygen saturation. Such findings raise the doubts about the benefit of supine position .It could be justified that in the supine position the abdominal organs tend to be forced by gravity against the diaphragm. This makes it difficult for the diaphragm to contract and for the newborn to breathe. [35]The present results are supported by Dimitriou et al (2002) and Woragidpoonpol (2001) who reported that the supine position was associated with lower oxygen saturation than the prone**.** [36, 37]

In the current study, although repositioning from supine to prone resulted in a transient fall in oxygen saturation during the procedure, yet the prone position was associated with improvement in oxygen saturation after the procedure.It could be justified that when the infant is placed in the prone, stability of the chest wall is enhanced, particularly during inspiration. This fixation of chest wall allows for an increased thoracic volume when needed without additional work of breathing. This occurs because additional muscles of inspiration are not needed to overcome diaphragmatic contraction. [38,39]

In the same context, Bhat et al (2003) found that oxygenation was better in the prone posture than in the supine posture. The researchers explained that prone placement might contribute to better SpO_2 and lower respiratory resistance, which is attributed to a more stable rib cage. [40]Similarly, Ammari et al (2009) observed an increase in oxygenation when the infants were turned from supine to prone. [41]This is in agreement with Poets et al (2007), Picheansthian and Woragidpoolpol (2003) who reported that infants who are born prematurely exhibit less apnoea and intermittent hypoxia have better thoracoabdominal synchrony, higher lung volumes and better oxygenation when nursed in the prone position. [42, 43]According to Chang et al (2002) the prone position produced fewer episodes of desaturation and lesser levels of activity. [44]

The findings of the present study revealed that the incidence of desaturation increased during repositioning from supine to side, from side to supine, and from supine to prone .It could be

explained that motion results in disruption of the calmed infant when picked up and turned from one position to another. This explanation is in accordance with Claure and Bancalari (2009), Esquer et al (2007), and Bolivar et al (1995) who found that the episodes of hypoxemia have been associated with increased activity of preterm infants.[45-47]The current result is also in agreement with Browne(2000), and Evans(1991) who stated that preterm infants who required assisted ventilation are susceptible to oxygen desaturation because of physiological instability and vulnerability to handling.[48,49] Mourdoch and Darlow (1984) studied handling during neonatal intensive care and mentioned that nursing care like alteration of position was associated with reduction of trascutaneous oxygenation.[50]

The present result revealed no significant difference in mean oxygen saturation between side-lying, supine, and prone position. The findings are congruent with the results of Bozynski et al (1988) who found no significant difference in the median of $TcPaO_2$ value between the lateral positioning and supine position, when examining the effect of side lying versus supine on transcutaneous oxygen saturation $TcPaO_2$ of 18 mechanically ventilated neonates .[51] Crane et al (1990) mentioned that there was no significant difference in oxygen saturation between supine and side-lying position. [52] Elder et al (2005) who also reported that there was no significant difference in oxygen saturation between supine and prone position. [53]The results of the present research are in line with a systemic review done by Balaguer et al (2006) who reported that no evidence concerning whether particular body positions during mechanical ventilation of the neonate are effective in producing sustained and clinically relevant improvements , prone position was found to slightly improve oxygenation.[54]

The practice of endotracheal suctioning (ETT) of ventilated preterm neonates is necessary for removing secretions to prevent obstruction of endotracheal tube.[55,56] The present results show that a statistically significant decrease in oxygen saturation was present in 80% of infants during open endotracheal suctioning .One explanation for the decline in oxygenation during suctioning may be the interruption of the infant's airway by suction catheter as this procedure necessitated the disconnection of the infants from the ventilator and disturbance in oxygen delivery. Another explanation is that, suctioning involved some degree of repositioning of the head and torso, so the effect of the two procedures may have been compounded. [15] Seckel (2008) and Jondgreden et al (2007) added that during open endotracheal suctioning, there was a drop in airway pressure, loss of lung volume and decreased in oxygen saturation. [57,58] In addition, Lindgren (2007) also reported that disconnection of breathing apparatus and negative pressure application during suctioning result in atelectasis, pulmonary shunting and venous return producing hypoxemia and lung compliance change. [59]In addition , Hooser (2002) mentioned that during open suctioning the gas drawn from the lung was replaced by air drawn from the atmosphere through the space left around the catheter which decreases the oxygen saturation .[60]

The finding of the current study are congruent with Hoellering et al (2008), Kalyn et al (2003) and Slevin et al (1998) who mentioned that open ETT suctioning could contribute to disturbance in ventilation, leading to greater degree of hypoxemia. [61-63]After performing of endotracheal suctioning, the present result shows an increase in oxygen saturation up to 5 minutes. This may be due to removal of secretions that partially obstructed the endotracheal tube. It can also be interpreted by the fact that hyperoxygenation before and after ETT suctioning may have a positive effect in increasing oxygen saturation.

Heelstick is one of the most common painful invasive procedures in NICU .It is the conventional method of blood sampling in neonates for screening tests or measurements of serum bilirubin or glucose or blood gas. Sick preterm infants admitted to neonatal intensive care units (NICUs) are exposed to this procedure repeatedly as part of their routine care. Painful procedures are harmful to the infant's physiological stability and ability to self regulate. [64, 65]

The result of current study shows a significant transient decrease of oxygen saturation during squeezing the heel and a short period after the procedure, and then it returned quickly to the baseline oxygen saturation . The result can also be attributed to the fact that decreased oxygen saturation is one of the physiological cues that might indicate pain and distress. The finding is congruent with Newnham et al (2009) who reported that heel prick was associated with hypoxemic episodes. [66]The current study is in agreement with Cong et al (2009) who examined the effect of kangaroo care on heelstick pain. They found that oxygen saturation was lower during heelstick than another phases in kangaroo group and in incubator heel group, supporting evidence that heelstick is a stressful event. [67] Hummel and Van (2006) and Walden & Gibbins (2008) explored that brief, acute noxious stimuli result in decreasing in oxygen saturation during the pain. [68, 69] On the contrary, Herrington et al (2007) found that no significant differences were noted in oxygen saturation in any phases of heelstick procedure. [70] Norris et al (1981) also concluded that transcutaneous

oxygen saturation did not differ significantly during heelstick procedure. (15)

5. Conclusion

Based on the findings of the present study, it is concluded that preterm neonates with respiratory distress syndrome reacted to nursing care procedures with decrease in oxygen saturation (SPO_2) during repositioning from supine to side- lying, from side-lying to supine, and from supine to prone position as well as during suctioning, and during heelstick. After the procedures, all preterm neonates returned to pre-procedure average of oxygen saturation except after repositioning from side- lying to supine, from supine to prone position, and after suctioning. The supine position contributed to a slight decrease in oxygenation. Both prone position and suctioning contributed to an increase in oxygenation after the procedures.

Recommendations

Individual neonates show varied response to nursing intervention especially preterm infants, therefore continuous monitoring of oxygen saturation is mandatory before, during and after performing the nursing procedures because an infant's status can change rapidly.

The need for caution among nurses and other professionals in delivery of care to this vulnerable infant is warranted. In planning care for preterm infant with RDS, the infant's response to procedures should be predicted consideration should be given to the benefits of the procedure in relation to its risk.

Routine intensive care should be altered and adapted based on specific observations of each infant's response to the care given and avoid prolonged exposure to unnecessary high concentration of supplemental oxygen or periods of hypoxemia that may increase the risk for their complications.

Neonatal intensive care units should include updated policies related to oxygen therapy, repositioning, suctioning, heelstick and other nursing care procedures for preterm neonates.

Care protocols for preterm neonates should incorporate a principle of minimizing the number of disruptions in care as much as possible and avoid sudden interruption.

Corresponding author
Randa El-Sayed Mohammed El-Sayed
Pediatric Nursing, Faculty of Nursing, Alexandria University, Alexandria, Egypt
randa_7373@yahoo.com

6. References:

1. Robert M. Fetal and Neonatal Medicine. In: Behrman RD, Kliegman RM. Nelson Essential of Pediatric. 4th ed. Philadelphia: W.B. Saunders Co. 2002; 214-5.
2. Ricci SS. Essential of Maternity, Newborn, and Women's health Nursing. Philadelphia: Lippincott Co. 2007; 644.
3. Beck S, Wojdyla D, Say L, Betran AP, Merialdi M. WHO Systemic Review on Maternal mortality and morbidity: The global burden of preterm birth 2009. The Bulletin of the World Health Organization. available at http://www.eurekalert.org/pub.../2009.../modfgdt100209.phd
4. Hamilton BE, Martin JA, Ventura SJ. Births: Preliminary data for 2007. National vital statistics reports 2009; 57(12):1-5. Available at http://*www.cdc.gov/nchs/data/nvsr/nvsr57/nvsr5712.pdf*
5. Henrik, Hagberg. Preterm birth and Perinatal morbidity and mortality: Incidence of Preterm birth in different countries. Subgroups of preterm birth. Available at http://*www.kbh.uu.se/pdf/Quiip/Preterm-Birth.pdf*
6. MacDorman MF, Mathews TJ. Recent Trends in Infant Mortality in the United States. NCHS data brief, no 9. Hyattsville, MD: National Center for Health Statistics. 2008. Available at http://*www.cdc.gov/nchs/data/databriefs/db09.htm*.
7. Rate of preterm birth (percent of live birth) ,Canada,2002-2004 source : public health Agency of Canada using statistics Canada, Canadian vital statistics, birth database available at http://www.phcspc.gc.ca/publicat/2007/Ibrd.
8. Un-published Statistical Records of Neonatal Department, Children University Hospital at El-Shatby in Alexandria, 2009.
9. Danford D, Miske S, Headly J, Nelson R. Effect of routine Care procedures on transcutaneous oxygen in neonates: a quantitative approach. Journal of Archives of disease in childhood 1983; 85:20-30.
10. Peters K. Does routine nursing care complicate the physiologic status of the premature neonate with respiratory distress syndrome? .Journal of Perinatal Neonatal Nurse 1992; 9:67.
11. Gagnon R, Leung A, Macnab A. Variations in regional cerebral blood volume in neonates associated with nursery events. Journal of American Perinatology1999; 16:7.
12. Gonsalves S, Mercer J .Physiological correlates of painful stimulation in preterm infants. Clinical Journal of Pain 1993; 9(2):88-93.
13. Long J, Philip A, Lucey J .Excessive handling as a cause of hypoxemia .Journal of Pediatrics 1980; 65(2):203-7.
14. Claure N. Automated Regulation of inspired oxygen in preterm infants: Oxygenation stability and clinical

workload. Journal of Anesthesia and Analgesia 2007; 105(6):537-41.
15. Norris S, Campebll, Brenerl S. Nursing procedures and alteration in transcutaneous oxygen tension in premature infants. Journal of Nursing Research 1981; (4): 330-6.
16. Demaray W. Suction for Intubated Patients. RT. For Decision Makers in Respiratory Care June/July 2002.Available at http://www.rtmagazine.com/issues/articles/2002-0606.asp.
17. Woodgate PG, Flenady V. Tracheal suctioning without disconnection in intubated ventilated neonates. Cochrane Database Systematic Reviews 2001; 4:1-14.
18. Rennie J, Morley CJ, Sinha S, King M, Danielian p. Guidelines for good practice in the management of neonatal respiratory distress syndrome .1998. Available at http://www.bapm.org/documents/publications/rds
19. Morrow B, Futter M,and Argent A. Effect of endotracheal suctioning on lung dynamic in mechanically –ventilated pediatric patients . Australian Journal of Physiotherapy 2006; 52:121.
20. Heimler R, Langlois J, Hodel D. Effect of positioning on breathing pattern of preterm infants. Journal of Archives Disease in Childhood 1992; 67(3):312-4.
21. American Academy of pediatrics Task Force on Infant and SIDS. Positioning and sudden infant death syndrome (SIDS): update. Journal of Pediatrics 1996; 98 (6 Pt 1):1216-8.
22. American Academy of pediatrics Task Force on Infant and SIDS. Changing concepts of sudden infant death syndrome: implication for infant sleeping environment and sleep position. Journal of Pediatrics 2000; 105: 650-6.
23. James A. Lemons, William P, and Hugh M. Prevention and Management of Pain and Stress in the Neonate. Journal of Pediatrics 2000; 105(2):454-61.
24. Abu-Saad H, Bours G, Stevens B, Hamers J. Assessment of pain in neonates. Journal of Semin Perinatol 1998; 22:402-16.
25. Gibbins F, Maddallena P, Moulsdale W. Pain assessment and pharmacological management for infant with NEC: a retrospective chart audit. Neonatal Net Work 2006;25(5):339-45.Available at http://www.ncbi.nlm.nih.gov/pubmed/16989133
26. Gibbins F, Stevens B. The influence of gestational age on the efficacy and short term safety of sucrose for procedural pain relief .Advances in Neonatal Care 2003; 4(3):126-40.
27. Folk L. Capillary Heelstick Blood Sampling .In: MacDonald M, Ramasethu J, Seigafuse S. Atlas of Procedures in Neonatology, 4th ed. Philadelphia: Lippincott Williams & Wilkins Co. 2007; pp. 93-6 .
28. Peters K. Bathing Premature Infants: Physiological and Behavioral Consequences. American Journal of Critical Care 1998; 7:90-100.
29. Goubet N. Effects of repeated heelsticks on premature newborn. Doctorate Dissertation, Massachusetts Amherst University, 1998; 12-3
30. Datta P.Pediatric Nursing.New Delhi: Jaypee Brothers Co. 2007; 105-6 .
31. Greenspan J, Goldsmith J .Oxygen therapy in preterm infants: Hitting the target .Journal of Pediatrics 2006; 118:1740-1.
32. Yottiem P, Tilokskulchai F, Vitchisukon K, Kolatat T. The effect of position in premature infants with assisted ventilation. Asian Journal of Nursing Studies 2004; 7(3):36-42.
33. Claure N. Automated Regulation of Inspired Oxygen in Preterm Infants: Oxygenation Stability and Clinician Workload. International Anesthesia Research Society 2007; 105(6): S37.
34. Neonatology Clinical Guidelines. Metabolic management. 2006. Available at http://www.kemh.health.wa.gov.au/services/nccu/guidelines/documents/7467.pdf
35. James J, Baker C, Swain H, Principles of Science for Nurses. Australia: Blackwell Co., 2002; pp.148.
36. Dimitriou G, Greenough A, Pink L, Mc Ghce A. Effect of posture on oxygenation and respiratory muscles strength in convalescent infant. Journal of Arch Dis Child 2002 ; 86: 147-50.
37. Woragidpoonpol P . The effect of positioning on the oxygenation and sleep state in preterm infants. Journal of Chiang Mai Med Bull 2001; 40(2):53-60.
38. Bowling S, McEvory C, Cozum E. Prone position improves functional residual capacity: respiratory compliance (CRSl) and oxygenation in intubated preterm infants less than 1259 grams. American Journal of Respiratory Critical Care Medicine 1998; 157:A373.
39. Mendoza IC, RobertsIL, Cook LN. Postural effects on pulmonary function and heart rate of preterm infants with lung disease. Journal of Pediatrics 1991; 118:445-8.
40. Bhat RY, Leipälä JA, Singh NR, Rafferty GF, Hannam S, Greenough A. Effect of posture on oxygenation, lung volume, and respiratory mechanics in premature infants studied before discharge. Journal of Pediatrics 2003; 112 (1 Pt 1):29-32.
41. Ammari A, Schulze K, Kist K, Kashyap S, Fifer W. Effects of body position on thermal, cardiorespiratory and metabolic activity in low birth weight infants. Early Human Development 2009 .Available at http://www.ScienceDirect.
42. Poets C, Bodman A . Placing preterm infants for sleep: first prone, then supine. Journal of Arch Dis Child Fetal Neonatal Ed 2007; 92: F331- 2.
43. Picheansathian W, Woragidpoolpol P. A systemic review of positioning of preterm infants for optimal

physiological development .2003. Available at http://www.joannabriggs.edu.eu/protocols/position.
44. Chang YJ, Anderson GC, Dowling D, Lin CH .Decreased activity and oxygen desaturation in prone ventilated preterm infants during the first postnatal week. Journal of Heart Lung 2002; 31(1):34-2.
45. Claure N, Bancalari E. Automated respiratory support in newborn infants. Journal of Seminars in Fetal and Neonatal Medicine 2009; 14(1): 35-41.
46. Esquer, N. Claure C. D'Ugard , Bancalari E . The role of abdominal muscle activity on the duration and severity of hypoxemia episodes in mechanically ventilated preterm infants. Journal of Neonatology 2007; 92: 182– 6.
47. Bolivar J, Gerhardt T, Gonzalez A .Mechanisms for episodes of hypoxemia in preterm infants undergoing mechanical ventilation, Journal of Pediatrics 1995; 127: 767–73.
48. Browne J. Developmental Care -Considerations for Touch and Massage in the Neonatal Intensive Care Unit. Neonatal Network 2000; 19 (1). Available at http://www.preemie-l.org/massage.html
49. Evans JC. Incidence of hypoxemia associated with care giving in premature infants. Neonatal Network 1991;10(2): 17 -24
50. Murdoch D, Darlow B. Handling during neonatal intensive care. Journal of Archives of Disease in Childhood 1984; 59: 957- 61.
51. Bozynski ME, Naglie RA, Nicks JJ, Burpee B, Johnson RV. Lateral positioning of the stable ventilated very-low-birth-weight infant. Effect on transcutaneous oxygen and carbon dioxide. American Journal of Diseases of Children 1988; 142(2):200-2.
52. Crane LD, Snyder JE, Knight P, Philips JB, Cassady G. Effects of position changes on transcutaneous carbon dioxide tension in neonates with respiratory distress. Journal of Perinatology 1990; 10:35-7.
53. Elder DE, Campbell AJ, Doherty DA. Prone or supine for infants with chronic lung disease at neonatal discharge?. Journal of Paediatrics Child Health .2005; 41: 189-5
54. Balaguer A, Escribano J, Roqué M. Infant position in neonates receiving mechanical ventilation. Cochrane Database Syst Rev 2006 ;(4):CD003668.
55. Siempos K, Vardakas M, Falagas E. Closed Tracheal Suction Systems for Prevention of Ventilator-associated Pneumonia. British Journal of Anaesthesia 2008;100(3):299-306
56. Wong S, Guillermo S,Kacmareck R ,Hess D . A bench evaluation of alveolar pressure changes during endotracheal suction. Journal of Respir Care 2002; 45(10):1070.
57. Seckel M. Does the use of a closed suction system help to prevent ventilator-associated pneumonia? Journal of Critical Care Nurse 2008; 28: 65-6.
58. Jongerden IP, Rovers MM, Grypdonck MH. Open and closed endotracheal suction systems mechanically ventilated intensive care patients: a meta-analysis. Journal of Critical Care Medicine 2007; 35:260–70.
59. Lindgren S. Open and closed suctioning experimental and human studies. Göteborg University 2007 *Available at* http://www. gupea.ub .gu.se/ dspace/ bitstream/ .../Ram% 20Sophie%20Lindgren.pdf
60. Hooser TV. Airway clearance with closed system suctioning. American Association of Critical Care Nurses. Columbia: 2002; 1-12.
61. Hoellering AB , Copnell B , Dargaville PA. Lung volume and cardiorespiratory changes during open and closed endotracheal suction in ventilated newborn infants. Journal of Arch Dis Child Fetal Neonatal Ed 2008; 93(6):F436-41.
62. Kalyn A, Blatz S, Feuerstake SR, Paes B. Closed Suctioning of Intubated Neonates Maintains Better Physiologic Stability: A Randomized Trial. Journal of Perinatology 2003; 23:218–22.
63. Slevin M, Daly L, Murphy J. Preterm infants stress responses to an invasive NICU event: Endotracheal suctioning. Journal of Reproductive and Infant Psychology 1998; 16(4): 285 – 92.
64. Shah V, Ohlsson A. Vein puncture versus heel lance for blood sampling in term neonates. A Cochrane review in The Cochrane Library, Issue 4, 2007.Available at http://www.thecochranelibrary.com
65. Johnston C , Filion F, Campbell M, Goulet C, Bell L. Kangaroo mother care diminishes pain from heel lance in very preterm neonates. BMC Pediatrics 2008; 8(13)Available at www.pubmedcentral.nih.gov/articlerender.fcgi?artid =2383886
66. Newnham C , Inder T, Milgrom J . Measuring preterm cumulative stressors within the NICU: The neonatal infant stressor scale. Journal of Early Human Development 2009. Available at http://www.ScienceDirect
67. Cong X, Ludington-Hoe S, McCain G. Kangaroo Care modifies preterm infant heart rate variability in response to heel stick pain: Pilot study. Journal of Early Human Development 2009. Available at http://www.ScienceDirect
68. Hummel P, Van D. Pain assessment: Current status and challenges.Journal of Seminars in Fetal and Neonatal Medicine 2006; 11:237-45.
69. Walden M, Gibbins S .Pain assessment and Pain management Guidelines for practice 2nd ed .2008 .Available at http://www.NANN.org
70. Herrington C. Reducing pain of heelstick in premature infants with gentale humen touch .Doctorate Dissertation, MSU College of Nursing, University of Wayne State, 2007; 39-40.

11/21/2010

Overcoming Early Shoot Senescence of *Colutea istria* Miller Propagated *In Vitro*

Ghada Abd El-moneim Hegazi and Mahdia Farid Gabr

Tissue Culture Unit, Plant Genetic Resources Department, Ecology and Dry Land Agriculture Division, Desert Research Center, 11753 El-matarya, 1 Mathaf El-matarya St., El-matarya, Cairo, Egypt.

Corresponding author: hegazighada@yahoo.com or hegazi_ghada@yahoo.com

Abstract: Seedlings of the leguminous shrub; *Colutea istria* Mill. were used as explants for the micropropagation of this vulnerable species. Cotyledonary nodes, stem node sections and shoot tips from the *in vitro* germinated seedlings were examined for micropropagation. The *in vitro* establishment of explants was attempted by using various concentrations of 6-benzylaminopurine (BA), Thidiazuron (TDZ) and N6-(2-isopentenyl) adenine (2iP) (0.5, 1, 2 mg/L each) in combination with NAA at 0.1 and 0.2 mg/L incorporated into Murashige and Skoog (MS) and Gamborg's (B5) media, in addition to the MS and B5 media without plant growth regulators (PGRs). The best results were obtained on MS medium supplemented with NAA and BA, in addition to PGRs free MS medium. And the best average number and length of shoots were obtained by using cotyledonary nodes as explants. For multiplication, the explants were cultured on MS medium containing BA at concentrations of 0.25, 0.5 and 1 mg/L either individually or in combination with 2iP at a concentration of 0.5 mg/L. The combination of BA and 2iP is recommended for multiplying the established shoots produced from colyledonary nodes and stem node sections due to the significantly higher average number of shoots/explant comparing to the media containing BA singly. However, BA is better for the multiplication of shoot tip explants. When axillary shoots were subcultured on the same medium, the shoots failed to multiply and began to senesce. The senescence progressed to the entire shoot, and growth ceased. Reducing the duration of the subculture to 3 weeks is necessary to prevent this problem. Explants rooted on MS medium containing 0.5 mg/L of both Indole-3-butyric acid (IBA) and NAA and plantlets with well developed shoots and roots transferred to soil and grew normally without loss of green colour and wilting.

[Ghada Abd El-moneim Hegazi and Mahdia Farid Gabr. **Overcoming Early Shoot Senescence of *Colutea istria* Miller Propagated *In Vitro***. Journal of American Science 2010; 6 (12): 1733-1738]. (ISSN: 1545-1003). http://www.americanscience.org.

Key Words: *Colutea Istria,* Leguminosae, micropropagation, *in vitro* culture, seedlings, yellowing, senescence.

Abbreviations: AgNO3, Silver Nitrate; BA, 6-Benzyladenine; CaCl2, Calcium Chloride; IBA, Indole-3-Butyric Acid; 2iP, N6-(2-Isopentenyl) Adenine; MS, Murashige and Skoog; NAA, δ-Naphthalene Acetic Acid; NaOCl Sodium Hypochlorite; PGRs, Plant Growth Regulators; TDZ, Thidiazuron.

Introduction

The economical and ecological importance of leguminous forest trees necessitated the application of micropropagation technique for their clonal multiplication **(Pradhan *et al,* 1998)**, furthermore, the regeneration rate of leguminous trees in natural habitats is low **(Dewan *et al,* 1992)**. Also, as stated by **Nanda and Rout (2003)**, due to poor germination and death of young seedlings under natural conditions, propagation through seeds, as with most leguminous trees is unreliable. Tissue culture techniques have been used for rapid clonal multiplication of selected genotypes of a number of forest trees including some woody legumes **(Dhawan, 1989 and Trigiano *et al,* 1993)**. During the past few years, a number of woody legumes have been successfully propagated *in vitro* using juvenile as well as mature plant parts **(Pradhan *et al,* 1998)**.

Colutea istria Mill. is a very rare species in Egypt and confined to Sinai which seems to be the westernmost limit of the species distribution. Its Arabic name is Yasser. The genus *Colutea* is belonging to the family Fabaceae (Leguminosae). It is a nano-phanerophyte which grows on crevices of smooth-faced rocky outcrops. Flowering and fruiting of this species is from February to April. *Colutea istria* is a vulnerable shrub. Its vulnerability can be related to its small population size combined with cutting by the natives for the preparation of ropes from the bark fibers. Older branches are used as firewood grazing by domestic livestock leading to the gradual decline of the species. Two populations of *Colutea istria* in Gebel El Rabba (Sant Catherine, South Sinai) and Gebel El Maghara (North Sinai) are being wildlife sanctuaries. Cultivation in botanical gardens as an ornamental is another useful method to protect the species from extinction. The plant is of horticultural value, because of its habit and decorative flowers **(El-Hadidi *et al,* 1991)**. The bark and fibers are used for the preparation of strong ropes **(Danin, 1983)**. It is a highly palatable, has a high nutritive value and is used as a fodder plant.

The seed oils of *Colutea* contained linoleic, oleic and linolenic acids as their major components. Palmitic and stearic acids were the major saturated fatty acids in the seed oils **(Bagci, 2004)**. Sixteen flavonoidal compounds from *Colutea istria* were isolated and identified as: quercetin-3-glucoside; quercetin-3-gentiobioside; quercetin-3,7-diglucoside; isorhamnetin-3-gentiobioside; isorhamnetin-3-rutinoside; kaempferide-3,7-diglucoside; 2',4,4'trihydroxychalcone; 2',4,4',6'tetrahydroxychalcone; formononetin; daidzein; calycosin; cladrin; rhamnocitrin-3-neohesperidoside; rhamnocitrin-3-glucoside; rhamnocitrin-3-galactoside and rhamnocitrin aglycone **(Shabana *et al,* 2005)**. Two new isoflavonoids have been isolated from the aerial parts of *Colutea istria* of Egyptian origin and identified as (3)-7-hydroxy-3',4'-dimethoxyisoflavan-2',5'-quinone and 6,3'-dihydroxy-7,4'-dimethoxyisoflavone **(Radwan, 2008)**.

Hegazi (2005) studied the response of *Colutea istria* Mill. to *in vitro* propagation from nodal segments and shoot tip explants from the mature plants. Explants were cultured on Murashige and Skoog (MS) nutrient medium **(Murashige and Skoog, 1962)** supplemented with different concentrations of 6-benzyladenine (BA) alone or in combination with 0.1 mg/L δ-Naphthalene acetic acid (NAA). In the establishment stage of the nodal segments; MS nutrient medium supplemented with 3 mg/L BA attained the highest average number of axillary shoots/explants. However, MS nutrient medium with 3 mg/L BA and 0.1 mg/L gave the highest value of the average length

of axillary shoots. MS medium supplemented with 4 mg/L BA with or without 0.1 mg/L NAA were the best media for the establishment of shoot tip explants. Nodal segments showed better response to the *in vitro* establishment than shoot tips. The regenerated shoots from both types of explants were multiplied on the best establishment medium. Shoots showed a reduced ability to elongate and became senescent, then abscised. To overcome this problem, different treatments were applied as the elimination of BA from the medium, doubling the amount of Calcium Cloride (CaCl2) in the medium, using the double phase medium, addition of Silver nitrate (AgNO3) to the medium, at different concentrations (1, 2 and 5 mg/L) and reducing the sucrose concentration to 20 g/L. All these treatments did not prevent the yellowing and necrosis of the shoots.

The aim of this study is to develop a micropropagation protocol for *Colutea istria* Miller using seedling explant materials because juvenile explants generally perform better in tissue culture than do mature explants, and to overcome early shoot senescence and obtain complete plantlets of this important endangered plant, to conserve and protect it from extinction. Since, there is no information on the micropropagation of Genus *Colutea* except the previous review.

Materials and Methods

Plant material, media and culture conditions

Seeds of *Colutea istria* Mill. were collected from their natural habitat; Gebel El-Maghara in Wadi El-Arousia. They were washed under running tap water and surface sterilized by immersion into 50% concentration of commercial bleach (active ingredient, 5.25% NaOCl) for 20 min, and then rinsed five times with sterile distilled water.

Seeds were individually planted on MS medium free from plant growth regulators (PGRs). The percentage of survival and seed germination were recorded after three weeks of culture. Cotyledonary nodes, stem node sections and shoot tips from the seedlings were examined for *in vitro* propagation. The *in vitro* establishment of explants was attempted by using various concentrations of BA, TDZ and 2iP (0.5, 1, 2 mg/L each) in combination with NAA at 0.1 or 0.2 mg/L incorporated into MS and Gamborg's (B5) media, in addition to the control media without PGRs, to select the optimum hormonal combination and concentration for shoot development.

All media were supplemented with 100 mg/L myo-inositol, 30 g/L sucrose and gelled with 2.5 g/L phytagel. The pH was set at 5.7-5.8, adjusted with 0.1N NaOH and HCl. Fifteen ml volumes of the media were dispensed into 25×150 mm glass culture tubes for explant establishment and rooting, or 45-50 ml volumes into large jars for seed germination and proliferation of shoots. All containers were closed with autoclavable polypropylene caps and autoclaved for 15 minutes at 121˚C under a pressure of 1.1 Kg/cm². The cultures were incubated at a temperature of 26±2˚C and were exposed to a 16-h photoperiod supplied by a bank of cool-white florescent tubes of 2 K lux light intensity.

For multiplication, the explants were cultured on MS medium containing BA at concentrations of 0.25, 0.5 and 1 mg/L and 2iP at a concentration of 0.5 mg/L – either individually or in combination, to obtain stock materials to be used for the following stages. MS medium without PGRs was also tested.

Thirty replicates were used for each treatment, and each experiment was repeated thrice during 2008-2010. The percentage of explants regenerating axillary growth, the average number of axillary shoots/explant and the average length of axillary shoots (cm) were recorded after 3 weeks of culture.

For root induction, the *in vitro* developed shoots were planted on MS medium supplemented with various concentrations of IBA and NAA (0.1, 0.2, 0.5, 1, 2 mg/L) individually and in combination, as following; 0.1 mg/L IBA+0.1 mg/L NAA, 1 mg/L IBA+0.2 mg/L NAA, 1 mg/L IBA+1 mg/L NAA and IBA at concentrations of 0.5, 1 and 2 in combination with 0.5 mg/L NAA. Explants with well developed roots were placed in pots containing garden soil and peat moss in a ratio of 1:1 and covered with plastic bags to maintain high humidity. The pots were irrigated regularly.

Treatments for overcoming shoot senescence

Treatments tested to overcome shoot senescence and necrosis of the plantlets were 1. Addition of L-glutamine at 100 mg/L – either individually or in combination with thiamine-HCl at 40 mg/L. 2. Reduction of the duration of the subculture from 4-6 weeks as recorded by **Hegazi (2005)** to 3 weeks. 3. Reducing the concentration of BA and addition of 2iP to the medium. 4. Transferring the shoots into growth regulators free medium after each subculture

Analysis of data

Analysis of variance (ANOVA) and Duncan's multiple range test were performed to analyze the obtained data. The differences among means for all treatments were tested for significance at 5% level. Means followed by the same letter are not significantly different at $P \leq 0.05$.

Results and discussion

Seeds germination and establishment stage

The seeds were germinated on MS medium free from PGRs and germination percentage reached 44.2% after 3 weeks of culture (Figure 1A). An experiment was conducted testing the establishment of the three types of explants developed from the germinated seedlings; cotyledonary nodes, stem node sections and shoot tips, cultured on MS and B5 media supplemented with NAA at 0.1 or 0.2 mg/L in addition to three different cytokinins; BA, TDZ and 2iP, at concentrations of 0.5, 1 and 2 mg/L as shown in table 1 (Plate 1B).

Concerning the establishment stage (Figure 1B), table 1 indicates that the use of MS nutrient medium was more effective than B5 medium with respect to the average number and length of axillay shoots for all types of explants. This finding is agreed with **Vengadesan *et al* (2002)** who reported that MS medium was preferred in the micropropagation of many leguminous trees. For shoot tips there was insignificant difference in the average shoot length between MS medium supplemented with 0.1 mg/L NAA+2 mg/L BA and B5 medium supplemented with 0.2 mg/L NAA+0.5 or 1 mg/L BA. And the highest value of the average length of axillary shoots was obtained on MS medium containing 0.1 mg/L NAA+0.5 or 1 mg/L BA. Shoot tips gave the highest percentage of explants regenerated axillary growth (100%) on most of the tested media if compared with the other types of explants. The suitability of shoot tip explants for regeneration and its sensitivity to various hormones is due to the activity of meristematic cells, which are actively dividing and are known to have dense cytoplasm with much more uniform and homogenous composition **(Mathur *et al*, 2002a)**. Also, cotyledonary nodes gave 100% of explants regenerated axillary growth on B5 medium without PGRs and B5 medium+0.2 mg/L NAA+0.5 and 2 mg/L 2iP. However, stem node sections gave the highest percentage of explants regenerated growth (83%) on MS medium supplemented with 0.1 mg/L NAA+1 mg/L BA.

It is noticed that the best medium with respect to the average number of axillary shoots derived from cotyledonary nodes, was MS medium containing 0.1 mg/L NAA+0.5 mg/L BA. However, MS medium without PGRs gave the best average length of axillary shoots (3.6). **Abd Alhady *et al* (2010)** have also recorded similar results on

MS control medium in the establishment of *Periploca angustifolia*. Stem node sections gave the highest number of axillary shoots/explant on MS medium supplemented with 0.1 mg/L NAA+2 mg/L BA, and it significantly decreased with the decrease of BA concentration. The highest average length of axillary shoots was obtained on MS medium supplemented with 0.1 mg/L NAA+0.5 mg/L BA. Shoot tips developed the maximum number of axillary shoots/explant on MS medium containing 0.1 mg/L NAA+0.5 and 1 mg/L BA which decreased significantly with the increase of BA concentration.

Comparing the three tested cytokinins added to B5 medium; differences were observed in the data obtained. It could be noticed that TDZ at 0.5 mg/L followed by 2iP at 2 mg/L gave the highest average number of axillary shoots/explants, and 2iP at concentrations of 1 and 2 mg/L proved to be the best with respect to the average length of axillary shoots developed from cotyledonary nodes. Also, **Scholten (1998)** found that Lilac tree (*Syringa vulgaris*) needed 2iP for elongation and it is used to induce bud growth in many *Acacia* species **(Vengadesan *et al*, 2002)**. Concerning stem node sections; BA and TDZ at concentrations of 1 and 2 mg/L in addition to all the tested concentrations of 2iP (0.5, 1 and 2 mg/L) were insignificantly different in the average number of axillary shoots/explant. Also, the average length of axillary shoots was insignificantly different on all B5 tested media. With respect to shoot tip explants, there were insignificant differences between the three tested cytokinins in the average number of axillary shoots/explants. Although, average length of axillary shoots was significantly the highest on B5 medium containing 0.5, 1 mg/L BA and 2 mg/L TDZ. The B5 control medium without PGRs and B5 medium containing 0.2 mg/L NAA are not recommended for the *in vitro* establishment of *C. istria* plant due to the least or no response of the explants.

In conclusion, the best medium for establishment of all tested explants was MS medium supplemented with NAA and BA, in addition to PGRs free MS medium. This result confirmed by **Al-Wasel (2000)** who obtained multiple shoots from shoot tips of seedlings of *Acacia seyal* when BA and NAA were used together. He found that no clear trend was observed by TDZ. BA in combination with an auxin was also found to be essential for multiple shoot induction in some other leguminous trees as many *Acacia* species **(Shekhawat *et al*, 1993; Bhaskar and Subhash, 1996** and **Vengadesan *et al*, 2002)**. In addition, **Abd Alhady *et al* (2010)** found that the combination of the two cytokinins (BA and 2iP) was more ideal for shoot multiplication than 2iP singly. The best average number and length of axillary shoots were obtained by using cotyledonary nodes as explants. It may contribute to the role of cotyledonary nodes in shoot production from seedling explants as they supply endogenous growth regulators to the cultures as reviewed by **Audiehya (1999)**. Also, cotyledonary nodes were used successfully for the micropropagation of *Acacia mangium* **(Vengadesan *et al*, 2002)**.

Multiplication stage

With respect to the multiplication stage (Figure 1C), data shown in table 2 indicates that cotyledonary nodes gave the maximum average number of axillary shoots/explant on MS medium supplemented with 0.5 mg/L of both BA and 2iP followed by 0.25 mg/L BA which is the best medium in the average length of axillary shoots. The average length of axillary shoots decreased with the increase in BA concentration. Similar observation was reported by **Abd Alhady *et al* (2010)**. There were insignificant differences in the average length of axillary shoots between the MS medium containing 0.5 and 1 mg/L BA and that containing 0.25 and 0.5 mg/L BA+0.5 mg/L 2iP. Concerning stem node sections, MS medium containing 0.25 mg/L BA+0.5 mg/L 2iP induced the highest average number of axillary shoots/explants, followed by MS medium without PGRs and MS+0.5 mg/L of both BA and 2iP which is insignificantly different from the same concentration of BA (0.5 mg/L) without 2iP. Both 0.25 and 0.5 mg/L BA were optimum for the production of the highest average length of axillary shoots, followed by 0.5 mg/L 2iP. Shoot tip explants gave the highest number of axillary shoots/explant on MS medium with 1 mg/L BA and decreased with the decrease of BA concentration or addition of 2iP to the medium. With respect to the average length of axillary shoots, it reached the highest value on MS medium containing 0.25 mg/L BA comparing to the other tested media. BA as a cytokinin proved to be effective *in vitro* with many woody species **(Murashige, 1974; Sharma *et al*, 1981 and Bennett and Davies, 1986)**. Also, it is the most frequently used compound in enhancing the production of proliferated shoots **(Thomas and Blackesly, 1987)**.

In conclusion, the combination of BA and 2iP is recommended for multiplying the established shoots produced from colyledonary nodes and stem node sections due to the significantly higher average number of axillary shoots/explant comparing to the media containing BA singly. However, BA is better for the multiplication of shoot tip explants. It was reported that an excess of synthetic cytokinin like BA is the most effective for the *in vitro* microporopagation of *Acacia mangium* **(Darus, 1993)**.

When axillary shoots were subcultured on the same medium, the shoots failed to multiply and leaves began to senesce. At the same time the leaf senescence progressed to the entire shoot and growth ceased. A similar observation was reported by **Hegazi (2005)** during the multiplication of of *Colutea istria* shoot tips and nodal segments from mature plants, and in other leguminous plants as *Leucaena leucocephala* **(Dhawan and Bhojwani, 1985)**, *Dalbergia latifolia* **(Swamy *et al*, 1992)** and *Dalbergia sissoo* **(Gulati and Jaiwal, 1996)**. For this reason it was important to apply a series of treatments to overcome such a problem.

Treatments for overcoming shoot senescence

Addition of L-glutamine at 100 mg/L either singly or in combination with Thiamine-HCl at 40 mg/L failed to inhibit yellowing and caused complete death of the explants. This result is in contrary with that of **Ranga Rao and Prasad (1991)** who mentioned that the amino acid L-glutamine increases shoot bud regeneration, and **Vengadesan *et al* (2002)** who found that it is ideal for shoot bud induction in *Acacia catechu* and *A. nilotica*. Also, **Mathur *et al* (2002b)** reported that incorporation of additives as glutamine and thiamine HCl was found to be most effective in shoot elongation as well as accelerating multiple shoot proliferation. Glutamine proved to be most effective to stop leaf-fall in multiple shoots, and thiamine is known to be one of the most important vitamin additives. Although green plant parts normally synthesize thiamine, additional amounts to the culture medium appeared to stimulate explants growth and may enhance root growth in the rooting stage.

The reduction of the duration of the subculture from 4-6 weeks as recorded by **Hegazi (2005)** to 3 weeks was very effective in the deterioration of the yellowing of the explants. This observation is supported by **Vengadesan *et al* (2002)** who found that in genus *Acacia*, frequent subculture of explants in constant intervals (25-30 days) make significant improvement in enhancing the number of multiple shoots. Repeated sub-culturing caused activation and conditioning of meristems. Two continuous subcultures excluding the initial culture favoured maximum shoot multiplication in *A. mangium*, *A. nilotica*, *A. sinulata*. Transferring the cultures to fresh

medium after three weeks found to be essential to prevent culture deterioration and sustained shoot growth.

Also, reducing the concentration of BA and addition of 2iP to the medium, gave promising results in the reduction of leaf senescence, since **Hegazi (2005)** used BA at high concentrations reached to 4 mg/L which caused shoot senescence in the multiplication stage. In this experiment the concentrations of BA reduced (0.25, 0.5 and 1 mg/L) and the percentage of explants regenerated growth and survived from yellowing ranged between 40% and 100%. Growth regeneration decreased with the increase of cytokinins concentration or the elimination of BA. It is concluded that BA is very important for the multiplication of shoots but if added with high concentrations it causes yellowing and necrosis of the explants. Also, the regular transferring of the shoots into fresh PGRs free medium after each subculture gave promising results in protecting the shoots. In this respect, **Al-Wasel (2000)** found that shoots of *Acacia seyal* needed to be subcultured into PGRs-free medium to allow and maintain shoot survival and elongation. These results are in harmony with that recorded by **Galiana *et al* (1991)** and **Darus (1993)** who mentioned that high concentrations of BA were observed to stimulate greatly the potential for axillary shoot formation during the first subcultures, but resulted quickly in a noticeable and often irreversible organogenic culture decline. It may rapidly become phytotoxic after a small number of subcultures, as noticed in other species. Also, **Rosu *et al* (1995)** reported that BA appears to have a positive effect only in the initial stage of culture establishment. This effect did not persist because of the leaf senescence which was presumably induced by ethylene. BA has been shown to stimulate ethylene biosynthesis *in vitro* **(Escalettes** and **Dosba, 1993)**.

Rooting and acclimatization

Only two media gave response in root induction; MS medium supplemented with 0.1 mg/L NAA which induced the rooting of 10% of axillary shoots and MS medium containing 0.5 mg/L of both IBA and NAA, on which 40% of explants developed roots (Figure 1D). These results is attributed to the presence of a number of explants that became senescent and caused the yellowing which didn't permit the auxins tested for promoting root formation. Fifty percent of the plantlets with well developed shoots and roots transferred to soil and grew normally without loss of green colour and wilting over 4 weeks observation period (Figure 1E).

Correspondence to:
Ghada Abd El-moneim Hegazi
Researcher
Tissue Culture Unit
Plant Genetic Resources Department
Ecology and Dry Land Agriculture Division
Desert Research Center
11753 El-matarya, 1 Mathaf El-matarya St., El-matarya, Cairo, Egypt.
Telephone No.: Home: (+202) 24729019; Mobile: (+2012)7550486
E-mails: hegazighada@yahoo.com or hegazi_ghada@yahoo.com

References

1. Abd Alhady M R A, Abd Alla M M, Hegazi A Ghada, Gabr F Mahdia. Rapid propagation of *Periploca angustifolia* Labill. by tissue culture. International Journal of Plant Developmental Biology 2010; 4(1): 15-18. Global Science Books.
2. Al-Wasel A S. Micropropagation of *Acacia seyal* Del. *in vitro*. Journal of Arid Environments 2000; 46: 425-431.
3. Andres E F, Sanchez F J, Catalan G, Tenorio J L, Ayerbe L. Vegetative propagation of *Colutea istria* Mill. from leafy stem cuttings. Agroforestry Systems 2005; 63: 1, 7-14. 21 ref.
4. Audiehya M. Regeneration studies on oil containing seed through tissue culture. Ph.D. Thesis, Univ. of Rajasthan, Jaipur 1999.
5. Bagci E, Bruehl L, Ozcelik H, Aitzetmuller K, Vural M, Sahim A. A study of the fatty acid and tocochromanol patterns of some Fabaceae (Leguminosae) plants from Turkey I. Grasas y Aceites (Sevilla) 2004; 55: 4, 378-384. 45 ref.
6. Bennett L K, Davies F T. *In vitro* propagation of *Quercus shumardii* seedlings. Hortscience 1986; 21: 1045-1047.
7. Bhaskar P, Subhash K. Micropropagation of *Acacia mangium* Willd through nodal bud culture. Indian Journal of Experimental Biology 1996; 34: 590-591.
8. Danin A. Desert vegetation of Israel and Sinai. Jerusalem 1983; pp.148.
9. Darus H A. Vegetative propagation. In: *Acacia mangium*: growing and utilization. Eds. Kamis Awang and
10. Dewan A, Nanda K, Gupta S C. *In vitro* micropropagation of *Acacia nilotica* subsp. *indica* Brenan *via* cotyledonary nodes. Plant Cell Reports 1992; 12 (1): 18-21.
11. Dhawan V, Bhojwani S S. *In vitro* vegetative propagation of *Leucaena leucocephala* (Lam) de Wit. Plant Cell Rep. 1985; 4: 315-318.
12. Dhawan V. Micropropagation and nodulation of tree legumes. In: Application of biotechnology in forestry and horticulture 1989; Ed. Dhawan, V., pp. 285-296. Plenum Publishing Corp., New York.
13. El-Hadidi M N, Batanouny K H, Fahmy A G. The Egyptian plant red data book. Vol. I Trees and Shrubs. Faculty of Science, Cairo Univ. 1991; 226 pp.
14. Escalettes V, Dosba F. *In vitro* adventitious shoot regeneration from leaves of *Prunus* sp. Plant Science 1993; 90: 201-209.
15. Galiana A, Tibok A, Duhoux E. *In vitro* propagation of nitrogen fixing tree legume *Acacia mangium* Willd. Plant and Soil 1991; 135: 151-159.
16. Gulati A, Jaiwal P K. Micropropagation of *Dalbergia sissoo* from nodal explants of mature trees. Biol. Plant. 1996; 38: 169-175.
17. Hegazi A Ghada. Ecophysiological and biotechnological studies of endangered plants from Sinai Peninsula. Ph.D. Thesis, Botany Dep. Faculty of Science, Ain Shams Univ. Cairo, Egypt 2005.
18. Mathur S, Shekhawat G S, Batra A. An efficient *in vitro* method for mass propagation of *Salvadora persica via* apical meristem. Journal of Plant Biochemistry and Biotechnology 2002a; 11: 125-127.
19. Mathur S, Shekhawat G S, Batra A. Micropropagation of *Salvadora persica* Linn. *via* cotyledonary nodes. Indian Journal of Biotechnology 2002b; 1: 197-200.
20. Murashige T and Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 1962; 15: 473-497.
21. Murashige T. Plant propagation through tissue culture. Annu. Rev. Plant Physiol. 1974, 25: 135-166.
22. Nanda R M, Rout G R. *In vitro* somatic embryogenesis and plant regeneration in *Acacia arabica*. Plant Cell, Tissue and Organ Culture 2003, 73:131-135.
23. Pradhan C, Kar S, Pattnaik S, Chand P K. Propagation of *Dalbergia sissoo* Roxb. through *in vitro* shoot proliferation

from cotyledonary nodes. Plant Cell Reports 1998; 18: 122-126.
24. Radwan M M. Isoflavonoids from an Egyptian collection of *Colutea istria*. Natural Product Communications 2008; 3 (9): 1491-1494.
25. Ranga Rao G V, Prasad M N V. Plant regeneration from the hypocotyls callus of *Acacia auriculiformis* multipurpose tree legume. J. Plant Physiol. 1991; 137: 625-627.
26. Rosu A, Skirvin R M, Bein A, Norton M A, Kushad M, Otterbacher A G. The development of putative adventitious shoots from a chimeral thornless rose (*Rosa multiflora* Thunb. Ex J. Murr.) *in vitro*. Journal of Horticultural Science 1995; 70 (6): 901-907.
27. Scholten H J. Effect of polyamines on the growth and development of some horticultural crops in micropropagation. Scientia Horticultuae 1998; 77: 83-88.
28. Shabana M H, Saleh N A M, Mansour R M, Shabana M M. Chemical constituents and biological activity of some Egyptian members of the Leguminosae. Bulletin of the National Research Centre (Cairo) 2005; 30: 1, 45-55. 27 ref.
29. Sharma A K, Prasad R N, Chaturvedi H C. Clonal propagation of *Bougainvillea glabra* "Magnifica" through shoot apex culture. Plant Cell, Tissue and Organ Culture 1981; 1: 33-38.
30. Shekhawat N S, Rathore T S, Singh R P, Deora N S, Rao S R. Factors affecting *in vitro* clonal propagation of *Prosopis cineraria*. Plant Growth Regulation 1993; 12: 273-280.
31. Swamy R B V, Himabindu K, Lakshmi Gita G. *In vitro* micropropagation of elite rosewood (*Dalbergia latifolia* Roxb.). Plant Cell Rep. 1992; 11: 126-131.
32. Taylor, D. MPTS monograph series no. 3 Bangkok: Winrock International and FAO 1993; 59-74.
33. Thomas T H, Blackesley D. Practical and potential uses of cytokinin in agriculture and horticulture. British Plant Growth Regulator Group. Monograph 1987; 14: 69.
34. Trigiano R N, Geneve R L, Merkle S A, Preece J E. Tissue and Cell cultures of woody legumes. Hot. Rev. 1993; 14: 265-332.
35. Vengadesan G, Ganapathi A, Amutha S, Selvaraj N. *In vitro* propagation of *Acacia* species-a review. Plant Science 2002; 163: 663-671.

11/12/2010

Table 1. Establishment of different seedling's explants of *Colutea istria* cultured on MS and B5 nutrient media supplemented with different growth regulators.

Nutrient medium	PGR concentration (mg/L)				Cotyledonary nodes			Nodal segments			Shoot tips		
	NAA	BA	TDZ	2iP	% of explants regenerated growth	Average number of axillary shoots/ explant	Average length of axillary shoots (cm)	% of explants regenerated growth	Average number of axillary shoots/ explant	Average length of axillary shoots (cm)	% of explants regenerated growth	Average number of axillary shoots/ explant	Average length of axillary shoots (cm)
MS	-	-	-	-	66.66	2.4 c	3.60 a	33.33	1.6 b	2.70 b	100.0	2.2 c	1.04 bc
	0.1	0.5	-	-	66.66	6.0 a	2.00 b	66.66	1.6 b	3.46 a	66.66	5.4 a	2.10 a
	0.1	1	-	-	66.66	3.4 b	2.00 b	83.33	2.0 b	1.82 c	66.66	5.2 a	2.52 a
	0.1	2	-	-	66.66	3.0 b	1.90 b	66.66	3.0 a	1.34 c	66.66	3.0 b	1.20 b
B5	-	-	-	-	100.0	1.0 de	1.52 bc	00.00	0.0	0.00	100.0	1.0 d	0.50 cd
	0.2	-	-	-	00.00	0.0	0.00	00.00	0.0	0.00	100.0	1.0 d	0.58 cd
	0.2	0.5	-	-	66.66	1.0 de	0.82 def	00.00	0.0	0.00	100.0	1.4 d	1.48 b
	0.2	1	-	-	66.66	1.0 de	0.50 efg	20.00	1.0 c	0.24 d	50.00	1.0 d	1.20 b
	0.2	2	-	-	66.66	0.6 e	0.22 g	80.00	1.0 c	0.56 d	100.0	1.0 d	0.38 d
	0.2	-	0.5	-	33.33	2.0 c	1.00 cde	00.00	0.0	0.00	100.0	1.0 d	1.00 bc
	0.2	-	1	-	66.66	1.0 de	0.50 efg	40.00	1.0 c	0.24 d	100.0	1.0 d	1.00 bc
	0.2	-	2	-	33.33	1.0 de	0.48 efg	20.00	1.0 c	0.22 d	100.0	1.0 d	1.16 b
	0.2	-	-	0.5	100.0	1.0 de	0.38 fg	40.00	1.0 c	0.20 d	66.66	1.0 d	0.50 cd
	0.2	-	-	1	66.66	1.0 de	1.06 cd	20.00	1.0 c	0.22 d	66.66	1.0 d	0.92 bcd
	0.2	-	-	2	100.0	1.4 d	1.28 cd	80.00	1.0 c	0.22 d	100.0	1.0 d	0.90 bcd

Table 2. Multiplication of different seedling's explants of *Colutea istria* cultured on MS nutrient medium supplemented with BA and 2iP.

Cytokinin concentration (mg/L)		Cotyledonary nodes			Nodal segments			Shoot tips		
BA	2iP	% of explants regenerated growth	Average number of axillary shoots/ explant	Average length of axillary shoots (cm)	% of explants regenerated growth	Average number of axillary shoots/ explant	Average length of axillary shoots (cm)	% of explants regenerated growth	Average number of axillary shoots/ explant	Average length of axillary shoots (cm)
-	-	66.66	1.8 abc	2.20 b	50.00	2.0 b	1.1 bc	62.50	1.6 ab	1.8 b
0.25	-	100.0	2.2 ab	3.60 a	50.00	1.0 d	1.0 bc	100.0	1.6 ab	2.62 a
0.50	-	100.0	1.0 cd	2.40 ab	100.0	2.0 b	2.6 a	100.0	1.8 ab	1.5 b
1.00	-	100.0	1.0 cd	2.70 ab	100.0	1.0 d	0.5 cd	60.00	2.3 a	1.7 b
-	**0.5**	95.00	1.6 abcd	1.90 b	41.33	1.4 c	1.7 b	96.66	1.4 ab	1.5 b
0.25	**0.5**	100.0	1.4 bcd	2.80 ab	100.0	3.0 a	2.6 a	100.0	1.0 b	0.5 c
0.50	**0.5**	100.0	2.6 a	2.40 ab	100.0	2.0 b	1.0 bc	100.0	1.4 ab	1 bc
1.00	**0.5**	40.00	0.6 d	0.16 c	00.00	0.0	0.0	100.0	1.4 ab	1.8 b

A

B

C

D

E

Figure 1. Different stages of micropropagation of *Colutea istria*.
A. *In vitro* germinated seedlings on MS medium free from PGRs. B. Establishment of cotyledonary nodes and shoot tip explants. C. Multiplication of axillary shoots on MS medium supplemented with BA and 2iP. D. Rooted plantlets on MS medium+0.5 mg/L NAA+0.5 mg/L IBA. E. Complete plantlets transfered to soil.

The influence of amaryl on genetic alterations and sperm abnormalities of rats with alloxan-induced hyperglycemia

Abeer H. Abd El-Rahim [1], Hasnaa A. Radwan [1], Omaima M. Abd El-Moneim [1], Ibrahim M. Farag [1], Somaia A. Nada [2]
1-Cell Biology Department, National Research Centre, Dokki, Cairo, Egypt.
2-Pharmacology Department, National Research Centre, Dokki, Cairo, Egypt.
Corresponding author: faragimdiab@yahoo. Com

Abstract: Amaryl (Glimepiride) is the third generation antidiabetic sulphonylurea known to possess the antioxidant effect in streptozotocin (STZ) induced diabetes. In this study, the antimutagenic activity of amaryl (0.03mg/kg.po daily for 30 days) was evaluated against the cytotoxic effect of alloxan (150mg/kg) on somatic and germinal cells of male and female albino rats . Somatic cells included bone marrow (for chromosome abnormality and micronucleus tests) and liver (for DNA fragmentation test). In germinal cells , sperm shape and count were studied . The present results showed that the glucose levels significantly increased in alloxan diabetic rats compared to those found in the controls. The alloxans diabetes of rats (males or females) had higher frequencies of structural and numerical chromosome aberrations compared to normal control. The diabetic condition in both male and female rats also increased the populations of each of micronucleated erythrocytes and DNA fragmentation. Moreover, the diabetic condition of male rats significantly increased the sperm shape abnormalities besides significant reducing of caudal sperm count. In contrast , the administrations of amaryl (glimepiride) to the alloxan diabetic rats had reduced the blood glucose level, abnormalities of genetic materials (chromosomal aberrations, the population of micronucleated erythrocytes, DNA fragmentation) and sperm shape abnormalities besides enhancing the sperm count. In conclusion, the present findings add that the antioxidant property of amaryl could have contributed for its ability to decrease the alloxan mediated defects in somatic and germinal cells.

[Abeer H. Abd El-Rahim, Hasnaa A. Radwan, Omaima M. Abd El-Moneim, Ibrahim M. Farag and Somaia A. Nada. **The influence of amaryl on genetic alterations and sperm abnormalities of rats with alloxan-induced hyperglycemia**. Journal of American Science 2010; 6(12):1739-1748]. (ISSN: 1545-1003).
http://www.americanscience.org.

Key words: Hyperglycemia, amaryl, alloxan, genetic alterations, sperm abnormalities, rats.

1. Introduction

Several studies showed that hyperglycemia and oxidative stress play a central role in diabetic tissue damage (Mastrocola et al., 2005; Somefai. et al., 2006; Kuhad et al., 2008; Rabbani et al., 2009). High level of blood sugar determines overproduction of reactive oxygen species (ROS) by the mitochondria electron transport chain. Also, in diabetic condition, both nitric oxide levels and mitochondrial nitric oxide synthase expressions were found to be increased in brain mitochondria, whereas antioxidant defences, such as glutathione (GSH) peroxidase activity and manganese superoxide dismutase protein content were reduced (Mastrocola et al., 2005; Rabbani et al., 2009). ROS virtually damages all cellular components, leading to DNA and protein modification (Rehman et al., 1999; Rabbani et al., 2009). In consequence, the diabetic patients suffer from an increased risk of oxidative stress-related diseases not only in the present generation but can also transmit the nuclear defects to their progeny (Blasiak et al., 2004).

Induction of diabetes in laboratory animals is a convenient and useful strategy in the understanding and treatment of the disease. An appropriate dose of alloxan was used to induce experimental diabetes. Alloxan (AL) was the first cytotoxic compound reported to cause inhibition of glucose – induced insulin secretion and selective beta-cell damage (Lenzen and Panten, 1988). The cytotoxic activity of this compound seems to be achieved through its penetration into the pancreatic beta-cells, a phenomenon which by itself depends on the expression of the glucose transporter proteins-2 (Schnedl et al., 1994; Bloch et al., 2000). An addition contributor to beta cells sensitivity to alloxan or to various toxins related to their poor antioxidant enzyme defence system (Tiege et al; 1997).

Glimepiride is a sulfonylurea was known to possess antioxidant effect against the oxidative stress induced by streptozotocin –diabetes (Krauss et al., 2003; Rabbani et al., 2009). The mutagenic studies conducted by battery of in vitro and in vivo methods concluded that glimepiride do not have mutagenic potential (Donaubauer and Mayer, 1993; Rabbani et

al., 2009). Since quenching the free radicals generated in the oxidative stress is one of the possible mechanisms to prevent the mutagenic defects in diabetes and there is a need for antidiabetic regimen that also reduce the ROS induced health complications (Johansen et al., 2005; Rabbani et al., 2009), the present study has been planned to evaluate the anti-mutagenic effect of amaryl (glimepiride) in alloxan – induced diabetic rats.

2.Materials and Methods

2.1. Materials

2.1.1. Animals:

Males and females of adult albino rats weighing 150-160 g, bred in the Animal House Lab, National Research Centre, Cairo, Egypt, were used. These animals were maintained under standard laboratory conditions and provided a standard diet and water *ad libitum*.

2.1.2.Drugs:

Alloxan and glucose oxidase peroxidase diagnostic enzyme kit was purchased from Sigma (St. Louis, MO., USA).

Amaryl (Glimepiride tablet) was obtained from local pharmacies, Cairo, Egypt and ground using a mortar. The powder was dissolved in distilled water and orally administrated at dose 0.03 mg / kg b.wt / d 1 for 30 days. This dose equals the dose of acceptable daily intake of amaryl for human (4 mg/ Kg), after modification to suit the small weight of rats. The dose of amaryl was based on previous studies (Sato et al., 1993; Nieszner et al., 2002).

2.2. Methods:

2.2.1. Induction and assessment of diabetes:

A single dose of alloxan monohydrate (150 mg/ kg) was prepared in 10 % saline solution and injected intraperitoneally to induce diabetes (El-Shabrawy and Nada. 1996). Diabetes was confirmed after 72 h of alloxan injection, the blood samples were collected via retro-orbital venous plexus and serum glucose levels were estimated by enzymatic GOD-PAP (glucose oxidase peroxidase) diagnosis kit method (Kuhad et al., 2008 and Rabbani et al., 2009). The rats with serum glucose level above 200 mg / d1 were selected and used for the present study (Kuhad et al., 2008).

Also, blood glucose values were determined just prior to killing the animals at the end of experiment. The animals were fasted for three hours then blood was collected from orbital sinus.

2.2.2. Experimental design:

Male or female rats were randomly selected and divided in three groups of six animals each. First group consisted of non-diabetic control animals, second group was the diabetic control (D), and third group consisted of diabetic animals treated with amaryl (DT).

2.2.3. Chromosome preparations:

For chromosome analysis both treated (D or DT) and control animals were sacrificed by cervical dislocation at the end of experiment. One hour and half or two hours before sacrifice, rats were injected with 4 mg colchicine / kg. b.w. Femurs were removed and the bone marrow cells were aspirated using saline solution. Metaphase spreads were prepared using the method of Preston et al. (1987). Fifty metaphase spreads per animals were analyzed, for scoring the different types of chromosome aberrations.

2.2.4. Micronucleus test:

Bone marrow slides were prepared according to the method described by Krishna and Hayashi (2000). The bone marrow was washed with 1 ml of fetal calf serum and then smeared on clean slides. The slides were left to air dry and then fixed in methanol for 5 minutes, followed by staining in May-Grunwald-Giemsa for 5 minutes then washed in distilled water and mounted. For each animal, 2000 polychromatic erythrocytes (PCEs) were examined for the presence of micronuclei.

2.2.5. DNA fragmentation:

Liver samples were collected immediately after sacrificing the animals. The tissues were lysed in 0.5 ml lysis buffer containing 10mM tris-HCL (PH.8), 1 mM EDTA, 0.2 % triton X-100, centrifuged at 10000 r.p.m. (Eppendorf) for 20 minutes at 4ºC. The pellets were resuspended in 0.5 ml of lysis buffer. To the pellets (P) and supernatants (S), 1.5 ml of 10 % trichloroacetic acid (TCA) was added and incubated at 4ºC for 10 minutes. The samples were centrifuged for 20 minutes at 10000 r.p.m. (Eppendorf) at 4ºC and the pellets were suspended in 750 µl of 5 % TCA, followed by incubation at 100 ºC for 20 minutes. Subsequently, to each sample 2 ml of DPA solution [200 mg DPA in 10 ml glacial acetic acid, 150 µl of sulfuric acid and 60 µl acetaldehyde] was added and incubated at room temperature for 24 hour (Gibb et al., 1997). The proportion of fragmented DNA was calculated from absorbance reading at 600 nm using the formula:

$$\text{DNA fragmentation} = \frac{\text{OD of fragmented DNA (S)}}{\text{OD of fragmented DNA (S)} + \text{OD of intact DNA (P)}} \times 100$$

2.2.6. Sperm analysis:

For sperm-shape analysis, the epididimus excised and minced in about 8 ml of physiological saline, dispersed and filtered to exclude large tissue fragments. Smears were prepared after staining the sperms with Eosin Y (aqueous), according to the

methods of Wyrobek and Bruce (1978) and Farag et al. (2002). At least 3000 sperms per group were assessed for morphological abnormalities. The sperm abnormalities were evaluated according to standard method of Narayana (2008). Epididymal sperm count was also determined by hemocytometer as described by Pant and Srivastava (2003).

2.2.7. Statistical analysis:

Statistical analysis was performed with SPSS software. Data were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's post hoc test for comparison between different treatment in the same sex. However, two-way analysis of variance (ANOVA) followed by Duncan's post hoc test were used in the DNA fragmentation test. Moreover Chi-Square test was used for comparison between male and female rats for inducing micronuclei and chromosome aberrations in diabetic condition and amaryl-treated diabetes. Results were reported as mean ± S.E. and differences were considered as significant when $P<0.05$.

3. Results

3.1. Blood glucose levels:

Blood glucose levels significantly increased in alloxan diabetic rats (Fig. 1, Table 1) compared to those in the normal controls (normal males or normal females). Whereas the blood glucose levels were significantly reduced in alloxan diabetic rats after 30 days of amaryl treated compared to those found in the D groups.

Fig.1:Alloxan - diabetic rats treated with amaryl (4mg/Kg)

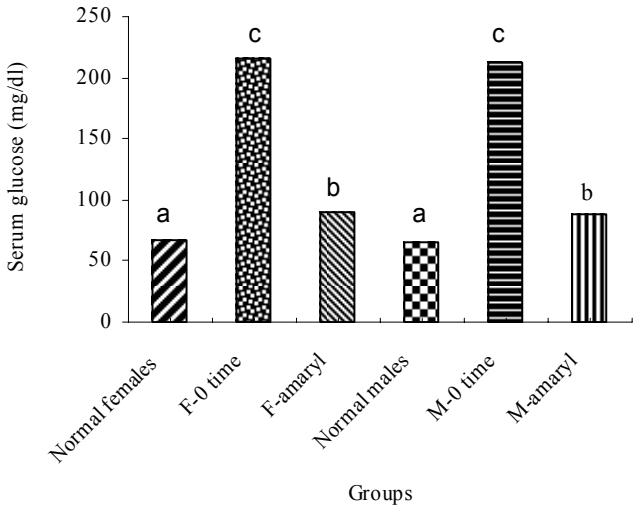


Table (1): Effect of amaryl (4 mg/Kg) on alloxan-induced diabetic female and male rats

Female			Male		
Normal females	F-0 time	F-amaryl	Normal males	M-0 time	M-amaryl
66.33± 1.35 [a]	215.58± 2.46 [c]	89.92 ± 1.36 [b]	66.00 ± 2.24 [a]	212.50 ± 1.86 [c]	88.75 ± 1.86 [b]

Data were expressed as mean ± S.E.
Means with different superscript letters (a, b, c) are significantly different ($P<0.05$)
F= Females M=Males

3.2. Chromosome examinations in diabetic male rats:

Chromosome examinations (Table, 2) showed that there were structural and numerical chromosome aberrations. Structural aberrations consisted of chromatic gaps and breaks, deletions, fragments, centromeric attenuation (C.A) and endomitosis. Numerical aberrations were aneuploidy and polyploidy. From the present results it was found that, the diabetic male rats (D group) had higher frequencies of structural and numerical aberrations than control group. Deletions, C.A., endomitosis and aneuploidy were more frequent than other aberrations. Statistical analysis showed that there were significant differences between diabetic and normal rats for the frequencies of total structural aberrations (except fragments) and total numerical aberrations.

On the other hand, the diabetic males treated with amaryl drug (DT group) had decreases in the frequencies of structural and numerical aberrations compared to D group. These decreases were significant ($P< 0.05$) for the frequencies of gaps, C.A, total structural aberrations, aneuploidy and total numerical aberrations.

3.3. Chromosome examinations in diabetic female rats:

Chromosome examinations (Table, 3) showed that the diabetic female rats had higher frequencies of structural and numerical chromosome aberrations compared to normal females. Statistical analysis showed that there were significant differences between diabetic and normal rats for the frequencies of chromatic gaps and breaks, deletions, fragments, C.A, endomitosis, total structural aberrations and polyploidy.

In contrast, the diabetic females treated with amaryl drug (DT) had decreases in the frequencies of structural and numerical chromosome aberrations compared to D group. These decreases were significant for the frequencies of deletions, fragments, C.A and total structural aberrations.

From the present study, it was observed that structural chromosomal aberrations were increased in diabetic females than those found in diabetic males

(Table, 4). These increases were significant ($P < 0.05$). On the other hand, numerical aberrations (especially aneuploidy) were more frequent in diabetic males than those found in diabetic females. However, the statistical analysis showed that the differences for the frequencies of numerical aberrations between the two sexes were not significant.

Moreover, the treatment with amaryl drug led to decrease of the structural and numerical chromosome aberrations in both diabetic sexes. However, the DT females were more response for decreasing of structural aberrations than DT males. Whereas, the frequencies of numerical aberrations were lowered in DT males than those of DT females.

Table (2): Effect of amaryl on the frequency of chromosome aberrations in alloxan-induced diabetic male rats

Treatment	Structural chromosomal aberrations							Numerical chromosomal aberrations		
	Gap	Break	Deletion	Fragment	C.A	End.	Total structural	Aneuploidy	Polyploidy	Total numerical
Control	1.0± 0.26 ^{b}	0.0± 0.0 ^{b}	0.67± 0.33 ^{b}	0.17± 0.17 ^{a}	2.33± 0.21 ^{b}	0.17± 0.17 ^{b}	4.33± 0.33 ^{c}	2.50± 0.50 ^{b}	0.0± 0.0 ^{b}	2.50± 0.50 ^{b}
D	2.67± 0.42 ^{a}	1.33± 0.42 ^{a}	3.0± 0.37 ^{a}	1.0± 0.52 ^{a}	5.0± 0.52 ^{a}	2.5± 0.34 ^{a}	15.50± 1.23 ^{a}	6.50± 1.20 ^{a}	2.0± 0.68 ^{a}	8.50± 1.18 ^{a}
DT	1.17± 0.31 ^{b}	0.83± 0.31 ^{ab}	2.67± 0.56 ^{a}	0.67± 0.33 ^{a}	3.17± 0.48 ^{b}	2.0± 0.58 ^{a}	10.50± 0.76 ^{b}	4.17± 0.31 ^{b}	0.83± 0.40 ^{ab}	5.0± 0.68 ^{b}

Data were expressed as mean ± S.E.
Means with different superscript letters (a, b, c) are significantly different ($P<0.05$)
D: diabetic condition
DT: diabetic treated with amaryl
C.A: Centromeric attenuations, End.: Endomitosis

Table (3): Effect of amaryl on the frequency of chromosome aberrations in alloxan- induced diabetic female rats

Treatment	Structural chromosomal aberrations							Numerical chromosomal aberrations		
	Gap	Break	Deletion	Fragment	C.A	End.	Total structural	Aneuploidy	Polyploidy	Total numerical
Control	0.50± 0.22 ^{b}	0.17± 0.17 ^{b}	1.50± 0.22 ^{b}	0.0± 0.0 ^{b}	3.0± 0.37 ^{b}	0.33± 0.21 ^{b}	5.50± 0.72 ^{c}	3.17± 0.70 ^{a}	0.33± 0.21 ^{b}	3.50± 0.76 ^{a}
D	3.50± 0.50 ^{a}	2.0± 0.68 ^{a}	3.83± 0.75 ^{a}	1.83± 0.31 ^{a}	6.50± 0.76 ^{a}	2.0± 0.26 ^{a}	19.67± 1.14 ^{a}	4.67± 1.17 ^{a}	2.17± 0.79 ^{a}	6.83± 1.92 ^{a}
DT	2.50± 0.43 ^{a}	1.33± 0.42 ^{ab}	1.17± 0.48 ^{b}	0.83± 0.40 ^{b}	4.17± 0.70 ^{b}	1.17± 0.40 ^{ab}	11.17± 0.91 ^{b}	4.50± 0.43 ^{a}	0.83± 0.40 ^{ab}	5.33± 0.72 ^{a}

Data were expressed as mean ± S.E.
Means with different superscript letters (a, b, c) are significantly different ($P<0.05$)
D: diabetic condition
DT: diabetic treated with amaryl
C.A: Centromeric attenuations, End.: Endomitosis

Table (4): Comparison between male and female rats for inducing chromosomal aberrations in diabetic condition and amaryl-treated diabetes

Treatment	Structural chromosomal aberrations							Numerical chromosomal aberrations		
	Gap	Break	Deletion	Fragment	C.A	End.	Total structural	Aneuploidy	Polyploidy	Total numerical
Control ♂	1.0±0.26	0.0±0.0	0.67±0.33	0.17±0.17	2.33±0.21	0.17±0.17	4.33±0.33	2.50±0.50	0.0±0.0	2.50±0.50
Control ♀	0.50±0.22	0.17±0.17	1.5±0.22	0.0±0.0	3.0±0.37	0.33±0.21	5.50±0.72	3.17±0.70	0.33±0.21	3.50±0.76
X^2 values	1.02	1.0	1.96	1.0	0.53	0.33	0.92	2.22	2.0	1.06
D ♂	2.67±0.42	1.33±0.42	3.0±0.37	1.0±0.52	5.0±0.52	2.5±0.34	15.50±1.23	6.50±1.20	2.0±0.68	8.50±1.18
D ♀	3.50±0.50	2.0±0.68	3.83±0.75	1.83±0.31	6.50±0.76	2.0±0.26	19.67±1.14	4.67±1.17	2.17±0.79	6.83±1.92
X^2 values	0.71	0.83	0.65	1.51	1.32	0.35	4.57*	2.04	0.04	1.29
DT ♂	1.17±0.31	0.83±0.31	2.67±0.56	0.67±0.33	3.17±0.48	2.0±0.58	10.50±0.76	4.17±0.31	0.83±0.40	5.0±0.68
DT ♀	2.50±0.43	1.33±0.42	1.17±0.48	0.83±0.40	4.17±0.70	1.17±0.40	11.17±0.91	4.50±0.43	0.83±0.40	5.33±0.72
X^2 values	3.02	0.71	3.66	0.11	0.89	1.36	0.15	0.09	0.0	0.07

*Significant at $P<0.05$.
C.A: Centromeric attenuations, End.: Endomitosis
D: diabetic condition
DT: diabetic treated with amaryl

3.4. Micronucleus assay:

In male groups: As shown in table (5), the frequencies of micronuclei were significantly higher ($P < 0.01$) in diabetic males (D group) than those found in the control group. On the other hand, the frequencies of micronuclei significantly decreased ($P < 0.05$) in diabetic males which treated with amaryl drug (DT group) compared with those found in D group.

Table (5): Effect of amaryl on the frequency of micronucleated polychromatic erythrocytes in alloxan-induced diabetic male rats

Treatment	No. of animals	No. of examined cells	Mean values of MNPE
Control	6	12,000	6.50±0.99 [c]
D	6	12,000	16.50±1.78 [a]
DT	6	12,000	11.67±1.05 [b]

Data were expressed as mean ± S.E.
Means with different superscript letters (a, b, c) are significantly different (P<0.05)
D: diabetic condition
DT: diabetic treated with amaryl

In female groups:

Micronuclei results in female groups (Table, 6) were similar with those recorded in male groups.

Moreover, the diabetic females had more frequencies of MNPE than diabetic males. However, statistical analysis showed no differences between the two sexes (Table, 7). On the other hand, the treatment of amaryl drug led to decrease of MNPE in both sexes. However, the DT females were more response for amaryl treatment and had the lowest frequencies of MNPE than DT males.

Table (6): Effect of amaryl on the frequency of micronucleated polychromatic erythrocytes in alloxan-induced diabetic female rats

Treatment	No. of animals	No. of examined cells	Mean values of MNPE
Control	6	12,000	6.33±0.88 [c]
D	6	12,000	20.50±1.48 [a]
DT	6	12,000	12.83±0.83 [b]

Data were expressed as mean ± S.E.
Means with different superscript letters (a, b, c) are significantly different (P<0.05)
D: diabetic condition
DT: diabetic treated with amaryl

Table (7): Comparison between male and female rats for inducing micronucleated polychromatic erythrocytes in diabetic condition and amaryl-treated diabetes.

Treatment	MNPE
Control ♂	6.50±0.99
Control ♀	6.33±0.88
X^2 values	0.01
D ♂	16.50±1.78
D ♀	20.50±1.48
X^2 values	2.61
DT ♂	11.67±1.05
DT ♀	12.83±0.83
X^2 values	0.33

D: diabetic condition
DT: diabetic treated with amaryl

3.5. DNA fragmentation:

In male groups:

The present results (Table, 8) showed that, the rates of DNA fragmentation were significantly increased ($P < 0.05$) in diabetic males than those in control group. On the other hand, the rates of DNA fragmentation significantly decreased ($P < 0.05$) in diabetic males treated with amaryl drug (DT) compared to D group of males.

In female groups:

The diabetic females had more frequent ($P < 0.01$) of rates of DNA fragmentation. In contrast, the rates of DNA fragmentation significantly decreased ($P<0.05$) in diabetic females treated with amaryl drug (DT) compared to D group of female.

Moreover, diabetic females had higher frequent ($P<0.05$) of DNA fragmentation than diabetic males. On the other hand, the treatment with amaryl drug led to decrease the rates of DNA fragmentation in both diabetic sexes. The DT females were more response for decreasing of DNA fragmentation than DT males. However, statistical analysis showed no differences for the frequencies of DNA fragmentation between two sexes (DT males and DT females).

Table (8): Effect of amaryl treated upon DNA fragmentation of rat livers. The livers were obtained from control, diabetic and amaryl-treated diabetic rats

Sex	Treatment	Rate of DNA fragmentation
Males	**Control**	19.78 ± 0.32 [de]
	D	34.17 ± 1.19 [b]
	DT	23.16 ± 1.38 [cd]
Females	**Control**	19.46 ± 0.22 [e]
	D	41.02 ± 2.08 [a]
	DT	24.68 ± 0.19 [c]

Data were expressed as mean ± S.E. Means with different superscript letters are significantly different (P<0.05)
D: diabetic condition DT: diabetic treated with amaryl

3.6. Sperm-shape analysis:

Sperm examination (Table, 9) showed that the sperm abnormalities (head and tail) were more frequent in diabetic male rats than those of the control. Statistical analysis showed the differences of the frequencies of head abnormalities (such as amorphous, total head abnormalities) and total sperm abnormalities (head and tail) were significant ($P < 0.05$ or $P < 0.01$) between diabetic and normal rats. On the other hand, the sperm abnormalities especially in head decreased in males diabetic treated with amaryl drug (DT) compared to those of the diabetic animals (D group). These decreases were significant ($P < 0.05$) in the frequencies of amorphous, without hock, total head and total sperm abnormalities. Exception to this,

the frequencies of tail abnormalities were few in each of D (0.17) and DT (0.33) groups; these abnormalities non-significant increased in DT group than those found in D group.

Sperm count:

Sperm counts significantly decreased ($P < 0.01$) in diabetic rats than those found in control group. In contrast, sperm counts significantly increased ($P < 0.05$) in diabetic rats treated with amaryl drug (DT) compared to those of the D group.

Table (9): Sperm abnormalities in diabetic condition of male rats and amaryl-treated diabetes

Treatment	Head abnormalities						Tail abnormalities	Total abnormalities	Sperm count
	Amorphous	Without hock	Small shape	Big shape	Banana	Total	Coiled		
Control	0.67± 0.33 [b]	2.33± 0.42 [c]	0.50± 0.34 [a]	0.0± 0.0 [a]	0.17± 0.17 [a]	3.67± 0.84 [c]	0.0± 0.0 [a]	3.67± 0.84 [c]	76.60± 4.06 [a]
D	4.50± 1.06 [a]	5.83± 0.60 [a]	1.17± 0.40 [a]	0.33± 0.21 [a]	0.33± 0.21 [a]	12.17± 1.35 [a]	0.17± 0.17 [a]	12.33± 1.26 [a]	46.96± 3.64 [c]
DT	2.17± 0.54 [b]	4.0± 0.52 [b]	0.33± 0.21 [a]	0.0± 0.0 [a]	0.33± 0.21 [a]	6.83± 0.60 [b]	0.33± 0.21 [a]	7.17± 0.54 [b]	62.33± 2.73 [b]

Data were expressed as mean ± S.E.
Means with different superscript letters (a, b, c) are significantly different ($P<0.05$)
D: diabetic condition
DT: diabetic treated with amaryl

4. Discussion

In the present study, the blood glucose levels significantly increased in alloxan diabetic rats compared to those in the normal controls. Our results are in agreement with that reported on observed hyperglycemia in alloxan diabetic mice (Diamond et al., 1989) and rats (El-Shabrawy and Nada, 1996). The observed hyperglycemia may be due to cytotoxic effect of alloxan compound on level of glucose transporter protein-2 (GLUT-2) expression in the pancreatic beta-cells causing inhibition of glucose-induced insulin secretion (Lenzen and Panten, 1988; Schnedl et al., 1994, Bloch et al., 2000). The administration of amaryl for 30 days at dose of 0.03mg/g b.wt. caused a significant reduction of the serum glucose level in alloxan diabetic rats. Similarly, Rabbani et al. (2009) found that the administration of glimepiride to nicotinamide (NA)-streptozotocin (STZ) diabetic rats had reduced the serum glucose level. As known amaryl (glimepiride) belongs to third generation sulphonylureas and chemically it is a carboxamido phenyl pyrroline sulphonylurea. The primary mechanism of action of glimepiride in lowering the blood glucose appears to be dependent on stimulating the release of insulin from functioning pancreatic beta-cells (Kramer et al., 1996; Rabbani et al., 2009). The extra pancreatic glucose reducing effects include inhibition of gluconeogenesis, ketogenesis, stimulation of peripheral glucose transport, glucogen synthase activity and glycerol-3-P-acyltransferase activity (Muller et al., 1995; Rabbani et al., 2009). Also, Kecskemeti et al. (2002) and Yassin and Mwafy (2007) indicated that the main effect of the sulfonylureas is enhancement of insulin secretion and improvement of metabolism both by pancreatic and extra-pancreatic mechanisms.

The present results showed that the alloxan diabetes of rats (males and females) had higher frequencies of structural and numerical chromosome aberrations compared to normal control. The diabetic condition in male and female rats also increased the populations of each of micronucleated erythrocytes and DNA fragmentation. Moreover, the diabetic

condition of male rats significantly increased the sperm shape abnormality besides significant reducing of caudal sperm count. These effects appeared to be mediated through the oxidative stress and inducing of ROS generated due to hyperglycemia (Vikram et al., 2007; Shrilatha and Muralidhara, 2007). The generation of ROS in diabetes was considered to be the major cause for mutagenecity including chromosome aberrations, DNA fragmentation, micronuclei and sperm abnormalities (Chauhan et al., 2000; Otton et al., 2004; Rabbani et al., 2009). Cytogenetic analysis by Tollinger et al.(1974) and Bloch et al. (2000) indicated that the majority of alloxan- induced diabetic rats was composed of hypodiploid cell with a chromosome number of 38 to 41 (2n=42). Also, chromosome abnormalities (aneuploidies and polyploidies) have been reported to be increased frequency in embryos of diabetic mice (Yamamoto et al., 1971). The influence of alloxan diabetes on first meiotic segregation behaviour in female and male mice was studied by Wauben-Penris and Prins (1983) who found in primary spermatocytes higher chiasma frequencies in the translocation multivalent in diabetic males than in controls. Also, the analysis of metaphase -II cells in the females revealed less 3:1 segregation and more adjacent-II segregation in the diabetics. So, they concluded that diabetes influences the meiotic segregation behaviour of chromosomes and that chromosomes showing higher incidence of unbalanced segregation behaviour. Also, alloxan diabetes caused fragmentation of nuclear beta-cell DNA (Okamoto, 1996). The occurrence of DNA fragmentation in lymphocytes obtained from alloxan – induced diabetic rats was found to be 81% compared to 45% of untreated cells from the control (Otton et al., 2004).So, an association between diabetes and both chromosomal abnormalities and DNA fragmentation has been found, and alloxan has been used as a means of studying this association. Despite, the fact that alloxan has been used in chromosomal and DNA fragmentation studies, no information is available concerning alloxan as a cause of micronuclei and sperm abnormalities. However, the increase micronuclei frequency and sperm abnormality has been reported in streptozotocin (STZ) diabetes of rats and mice(Vikram et al., 2007; Shrilatha and Muralidhara, 2007). Also, the results of Rabbani et al. (2009) indicated that STZ diabetes of rats increased the population of micronucleated erythrocytes and sperm shape abnormality besides reducing of caudal sperm count. Several studies reported that suggested mechanism for inducing damage of nuclear component and sperm abnormalities in diabetic condition include the activation of several damaging pathways by the ROS such as accelerated formation of advanced glyccation end production (AGE), polyol pathway, hexosamine pathway, protein kinase (PKC) or increase of lipid peroxidation (LPO) (Piconi et al., 2003; Valko et al., 2007 and Rabbani et al., 2009).

LPO occurs when ROS attacked the poly unsaturated fatty acid residues of phospholipids of cell membrane which is extremely sensitive to the oxidation. Host cell like spermatozoa are highly susceptible to the damage by excess concentrations of ROS due to high content of polyunsaturated fatty acid within their plasma membrane. Increased LPO and altered membrane can affect the sperm function through impaired metabolism, motility, acrosome reaction as well as oxidative damage to sperm DNA leading to increase of morphological changes in sperm and decrease of caudal sperm count (Tramer et al., 1998; Kumar et al., 2002; Sanchez et al., 2006; Rabbani et al., 2009). Also, Sikka (2001) reported that the decrease in sperm count can be attributed to the influence of hyperglycemia on late stages of spermatogenesis, possibly through an increase of reactive oxygen species (ROS). The consequences of such oxidative damage could include loss of motility due to lipid peroxidation. Moreover, Hemachand and Shaba (2003) indicated that the increased hydroperoxide level can affect the spermatogenesis process, since germ cells are more susceptible to peroxidative damage.

In contrast, the administration of amaryl (glimepiride) to the alloxan (AL) diabetic rats in the present study had reduced the abnormalities of genetic materials (chromosomal aberrations, DNA fragmentation, the population of micronucleated erythrocytes) and sperm shape abnormalities besides enhancing the sperm count. These findings indicated that amaryl (glimepiried) inhibited the AL mediated changes in the genetic materials and sperm abnormality and enhanced the antioxidant defence. These observations suggest that the antioxidant property of amaryl could have contributed for its ability to decrease the AL mediated defects in somatic and germinal cells. Similar results were observed by Rabbani et al. (2009) who reported that the administration of glimepiride to the STZ diabetic rats had reduced the population of micronucleated erythrocytes and sperm shape abnormalities besides enhancing the sperm count compared to diabetes control. They also found that this drug has enhanced the serum levels of antioxidant enzymes (CAT, SOD and GPx) and reduced the LPO and hyperglycemia. So they suggested that the increasing levels of CAT, SOD and GPx and reducing the LPO could minimize the cytogenetic damage in somatic and germinal

cells. The same authors indicated that the compound possessing glucose lowering property along with an antioxidant effect play a beneficial role in preventing the ROS mediated DNA damages. Krishnamoorthy et al. (2007) reported that antioxidants limit the nuclear damage by preventing the free radical action.

The antioxidant activity of glimepiride on rats with streptozotocin -induced hyperglycemia was also previously reported by Krauss et al. (2003) who found that the administration of glimepiride had increased the plasma levels of SOD, GPx besides reducing the levels of H2O2 and malondialdehyde. So, they suggested that glimepiride by increasing the level of antioxidant enzymes lead to decrease the ROS mediated damage in the host cells. Kono (1978) and Valko et al. (2007). Rabbani et al. (2009) reported that SOD is an enzyme that catalyses the dismutation of superoxide ion in to oxygen and hydrogen peroxide, thus protecting the cell from the superoxide toxicity. Moreover, Valko et al. (2007) and Rabbani et al. (2009) indicated that the function of GPx is to remove the H2O2 generated by metabolic action or oxidative stress. Another study by Yassin and Mwafy (2007) found in diabetic rats, that serum triglycerides, cholesterol and urea concentration were markedly elevated. However, glimepiride therapy returned such changes towards normal.

In conclusion, the present findings add that the antioxidant property of amaryl could have contributed for its ability to decrease the alloxan mediated defects in somatic and germinal cells.

5. Refrences:

1. Blasiak, J., Arabski, M., Krupa, R., Wozniak, K., Zadrozny, M., Kasznicki, J., Zurawska, M. and Drezewoski, J. (2004): DNA damage and repair in type 2 diabetes mellitus. Mutat. Res., 554 (1-2): 297-304.
2. Bloch, K. O., Zemel, R., Bloch, O. V., Grief, H. and Vardi, P. (2000): Streptozotocin and alloxan – based selection improves toxin resistance of insulin-producing RINm cells. Int. Experimental Diab. Res., 1: 211-219.
3. Chauhan, L. K. S., Pant, N., Gupta, S. K. and Srivastava, S. P. (2000): Induction of chromosome aberrations, micronucleus formation and sperm abnormalities in mouse following carbafuran exposure. Mutat. Res., 465 (1): 123-129.
4. Diamond, M.P., Moley, K.L., Pellicer, A., Vaughn, W. K. and De Cherney, A. H. (1989): Effect of streptozotocin – and alloxan- induced diabetes mellitus on mouse follicular and early embryo development. J. Reprod. Fert., 86 (1): 1-10.
5. Donaubauer, H. H. and Mayer, D. (1993): Acute, subchronic and chronic toxicity of the new sulfonylurea glimepiride in rats. Drug Res., 43 (5): 547-549.
6. El-Shabrawy, O. A. and Nada, S. A. (1996): biological evaluation of multicomponent of tea used as hypoglycemic in rats. Fitoterapia, Volume LXVII (2): 99-102.
7. Farag, I.M., Abdou, H.S.A., Ayesh, A.M. and Osfr, M.M.H. (2002): Chromosomal and sperm studies on the mutagenic effect of over heated meat and the protective role of green tea and gingeng on rats. Al-Azhar Bull. Sci., 13: 105 - 120.
8. Gibb, R., K., Taylar D.D., Wan, T., Oconnor, D. M., Doering, D.L., and Gercel- Taylor, C., (1997) Apoptosis as a measure of chemo sensitivity to cisplatin and taxol therapy in ovarian cancer cell lines. Gynecologic Oncology, 65: 13-22.
9. Hemachand, T. and Shaba, C. (2003): Functional role of sperm surface glutathione s-transferases and extracellular glutathione in the haploid spermatozoa under oxidative stress. FEBS lett., 538:14 -18.
10. Johansen, J. S., Harris, A. K., Rychly, D. J. and Ergul, A. (2005): Oxidative stress and the use of antioxidants in Diabetes: Linking basic science to clinical practice. Cardovasc. Diabetol. , 4 (1): 5-16.
11. Kecskemeti, V. Bagi, Z. and Pacher, P. (2002): New trends in development of oral antidiabetic drugs . Curr. Med. Chem., 9 (1): 53 – 71.
12. Kono, Y. (1978): Generation of superoxide radical during antoxidation of hydroxylamine and an assay for superoxide. Arch. Biochem. Biophys., 186: 189-195.
13. Kramer, W., Mullet, G. and Geisen, K. (1996): Characterization of the molecular mode of action of sulphonylurea, glimepiride at beta cells. Horm . Metab. Res., 28 (9): 464- 468.
14. Krauss, H., Kozlik, J., Grzymislawski, M., Sosnowski, P. Mikrut, K., Piatek, J. and Paluszak, J. (2003): The influence of glimepiride on the oxidative state of rats with streptozotocin-induced hyperglycaemic. Med. Sci. Monit., 9 (11): 389-393.
15. Krishna, G. and Hayashi, M. (2000): In vivo rodent micronucleus assay: protocol, conduct and data interpretation. Mutation Research, 455 (1-2): 155 – 166.

16. Krishnamoorthy, G. Venkataraman, P., Arunkumar, A., Vignesh, R., C., Aruldas, M. M.and Arunakaran, J. (2007): Ameliorative effect of vitamins (α-tocopherol and ascorbic acid) on PCB (Aroclor 1254) induced oxidative stress in rat epididymal sperm . Reprod. Toxicol., 23 (2): 239 -245.
17. Kuhad, A., Sethi, R .and Chopra, K. (2008): Lycopene attenuates diabetes –associated cognitive decline in rats. Life sciences, 83 (3-4) : 128 – 134 .
18. Kumar, T. R., Doreswamy, K. Shrilatha, B. and Muralidhara, (2002): oxidative stress associated DNA damage in testis of mice: Induction of abnormal sperms and effects on fertility. Mutat. Res., 513(1-2): 103-111.
19. Lenzen, S. and Panten, U. (1988): Alloxan: history and mechanism of action. Diabetologia, 31 (6): 337 – 342.
20. Mastrocola, R., Restivo, F., Vercellinatto, L., Danni, O., Brignardello, E., Aragno, M., and Boccuzzi, G., (2005): Oxidative and nitrosative stress in brain mitocondria of diabetic rats. Journal of Endocrinology, 187: 37 -44
21. Muller, G., Satoh, Y. and Geisen, K. (1995): Extrapancreatic effects of sulphonylurea- a comparison between glimpiride and conventional sulphonylureas. Diabetes Res, Clin. Pract., 28: 115-137.
22. Narayana, K. (2008): An aminoglycoside antibiotic gentamycin induces oxidative stress, reduces antioxidant reserve and impairs spermatogenesis in rats. J. Toxicol. Sci., 33: 85 – 96.
23. Nieszner, E, Posa, I., Pogatsa, G et al (2002): Influence of diabetic state and that of different sulfonylureas on the size of myocardial infraction with and without ischemic preconditioning in rabbits. Exp. Clin. Endocrinol Diabetes, 110 (5): 212- 218 .
24. Okamoto, H. (1996) : Okamoto model for B-cell damage : recent advances , In : Lessons from animal diabetes , edited by Shafrir ,E. pp, 97-112 , Boston , Birkhauser.
25. Otton, R., Soriano, F. G., Verlengia, R. and Curi, R. (2004): Diabetes induces apoptosis in lymphocytes. Journal of Endocrinology,182: 145-156.
26. Pant, N. and Srivastava, S. P. (2003): Testicular and spermatotoxic effect of quinaphos in rats . J. Appl. Toxicol., 23 (4):271-274.
27. Piconi, L., Quagliaro, L. and Ceriello, A. (2003): Oxidative stress in diabetes. Clin. Chem. Lab. Med. 41: 1144 - 1149.
28. Preston, R. J., Dean, B. J., Galloway, S., Holden, H., McFee, A. F., and Shelby, M. (1987): Mammalian in vivo cytogenetic assays: Analysis of chromosome aberrations in bone marrow cells. Mutat. Res., 189 (2):157-165.
29. Rabbani, S. I., Devi, K. and Khanam, S. (2009): Inhibitory effect of glimepiride on nicotinamide – streptozotocin induced nuclear damage and sperm abnormality in diabetic Wister rats. Indian Journal of experimental Biology, 47: 804-810.
30. Rehman, A., Nourooz-Zadeh, J., Moller, W., Tritschler, H., Pereira, P. and Halliwell, B., (1999): Increased oxidative damage to all DNA bases in patients with type - II diabetes mellitus. FEBS Letters, 448(1):120-122.
31. Sanchez E. E. T., Marquette M. L., Brown D. B. and Ansari N.H. (2006): The effect of oxidative stress on human sperm morphology . Fertil. Steril. , 86 (suppl 1). S444. In: Am. Soc. Reprod. Med. 62 nd Annual Meeting.
32. Sato, J., Ohsawa, I., Oshida, Y., et al. (1993): Effects of glimepiride on vivo insulin action in normal and diabetic rats. Diabetes Res. Clin. Pract., 22: 3-9.
33. Schmid, W. (1975): The micronucleus test. Mutat. Res., 31: 9 – 15.
34. Schnedl, W. J., Ferber, S., Johnson, J. H. and Newgard, C.B. (1994): STZ transport and cytotoxicity. Specific enhancement in GLUT-2 expressing cells. Diabetes, 43(11): 1326 – 1333.
35. Shrilatha, B. and Muralidhara, (2007): Early oxidative stress in testis and epididymal sperm in streptozotocin –induced diabetic mice: in progression and genotoxic consequences. Report. Toxicol, 23(4):578-587.
36. Sikka, S.C. (2001): Relative impact of oxidative stress on male reproductive function. Curr. Med. Chem., 8(7): 851 – 862.
37. Somfai, G. M., Knippel, B., Ruzicska, E., Stadler, K., Toth, M., Salacz, G., Magyar, K., Somogyi, A. (2006): soluble semicarbazide – sensitive amine oxidase (SSAO) activity is related to oxidative stress and subchronic inflammation in streptozotocin – induced diabetic rats. Neurochemical International, 48(8):746 -752.
38. Tiege M., Lortz S Drinkgern J. and Lenzen S. (1997): Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin-producing cells. Diabetes, 46: 1733- 1742.
39. Tollinger, C. D., Chrisman, C. L. and Doolittle, D. P. (1974): Alloxan – induced aneuploidy in mice. The Journal of Heredity, 63 : 345-348

40. Tramer, F., Rocco, F., Micali, F., Sandri, G. and Panfili, E. (1998): Antioxidant systems in rat epididymal spermatozoa . Biol. Reprod, 59(4): 753-758.
41. Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T.D., Mazur M. and Telser, J (2007): Free radicals and antioxidant in normal physiological function and human disease. Int J biochem. Cell Biol., 39: 44-84.
42. Vikram, A. Tripathi, D. N., Ramarao, P. and Jene, G. B. (2007): Evaluation of streptozotocin genotoxicity in rats from different ages using the micronucleus assay. Regulatory Toxicology and Pharmacology , 49, (3): 238-244
43. Wauben-Prins, P. J. J. and Prins, J.-B. (1983): Meiotic behavior of alloxan –treated diabetic and non diabetic T (1; 13) 70H/+mice . Human Genetics, 63 (3): 268-273.
44. Wyrobek, A. J. and Bruce, W. R. (1978): The induction of sperm shape abnormalities in mice and humans. Chem. Mutagens, 5: 257 – 285.
45. Yamamoto, M., Endo, A., Watanabe, G. and Ingalls T.H. (1971): Chromosomal aneuploidies and polyploidies in embryos of diabetic mice. Archs Enviro. Health, 22(4):468-475
46. Yassin, M.M. and Mwafy, S.N. (2007): Protective potential of glimepiride and Nerium oleander extract on lipid profile, body growth rate, and renal function in streptozotocin – induced diabetic rats. Turk J Biol., 31: 95 – 102.

.

7/12/2010

Comparative study of three calcium hydroxide based root canal sealers using different cultivating techniques.

Omar N.*, Negem M. **, Kataia M. **, and Zaazou M. *

* Restorative and dental material department, National Research center, Cairo, Egypt.
** Department of endodontics, Faculty of Oral and Dental Medicine Cairo University.

Corresponding author: mohamedzaazou@yahoo.com

Abstract: The aim of this study was to evaluate the antimicrobial properties of three calcium hydroxide based endodontic sealers: Apexit sealer (Ivocalr/Vivdent, Schaan and Liechtenstein), CRCS sealer (Coltene-Whaledent, U.S.A) and Sealapex sealer (Kerr Co, Italy) against *Streptococcus α haemolyticus* (facultative anaerobic bacteria). **Materials and Methods**: Three methods were used to evaluate the antibacterial activities of the three root canal sealers against *Streptococcus α haemolyticus* using "Agar diffusion test". The strains were prepared and inoculated into 5ml broth and incubated at 37^{o}C for 24 h. The freshly mixed sealers were placed into the prepared wells of agar plates (inoculated with the test microorganisms). The antimicrobial effect of each sealer was determined by measuring the diameter of zones of inhibition in millimeters at one and three days period. Five plates were prepared for every sealer in each method. **Results** showed that Sealapex gave the highest mean of inhibition zone diameter. This was followed by CRCS and Apexit showed the lowest mean of inhibition zone after one day. While at the three days period, the Sealapex gave the largest inhibition zone diameter and there was no statistically significant difference between CRCS and Apexit groups. The three methods used confirm these results.
[Omar N., Negem M., Kataia M., and Zaazou M. Comparative study of three calcium hydroxide based root canal sealers using different cultivating techniques. Journal of American Science 2010;6(12):1749-1753]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: Root canal sealers, *Streptococcus α haemolyticus,* Agar diffusion test

1.Introduction

Among the ideal criteria set up by Grossman in the 1980's, for the selection of root canal sealer; *"It must possess bactericidal properties or at least has bacteriostatic effect"* Fraunhofer and Branstetter (1982).Flora of root canal infections are polymicrobial. While some are asymptomatic, others are associated with serious infections. The seriousness of an infection beyond the apex of a tooth is related to number, virulence, host resistance, and associated anatomic structures. If the infection spread beyond the tooth socket, it may localize or continue to spread through the bone and soft tissues as diffuse abscess or cellulitis, Khemaleelakul et al.,(2002).

Many researchers investigated the composition of the microbiata from acute and chronic endodontic infections. Fox and Isenberg(1967) studied the bacterial populations of 381 positive cultures from root canals and their antibiotic resistance pattern. They found that incidence of *Enterococci* which was second to *Viridans streptococci* in the study, Fox and Isenberg(1967). Ibrahim and Aisa(1973) found that 58.8% (204 out of 381) pure positive cultures taken from infected root canals before treatment were *Streptococci.*Ibrahim and Aisa (1973) . Infective endocarditis caused by the *Viridans* group of organisms is generally a result of their invading into the blood stream during intra oral procedures; tooth extraction, endodontic treatment, or even during tooth brushing,Samaranayake(1996).The *Viridans* group or other species of *Streptococci* typically shows alpha hemolysis on blood agar. Various biological properties of calcium hydroxide are due to its strong alkalinity, (pH 12.5). Thus, several bacterial species commonly found in infected root canals are eliminated after short period when in direct contact with this substance. Most of the endo-pathogens are unable to survive in such highly alkaline environment, Siqueira and Lopes (1999). Mirijana and Branka(2006) evaluated the antimicrobial activity of five root canal sealers: AH26 (a resin based paste);Apexit (calcium hydroxide based paste); Endo-methasone and Tubliseal (zinc oxide eugenol-based materials) and Ketac Endo Aplicap, (glass ionomer based sealer). Antimicrobial activity was tested against *S. mutans* 70C and *L.casei* ATCC 27773 using ADT (agar diffusion inhibitory test) on TYC SB, blood and MRS agars. The results confirmed that epoxy resin and zinc oxide-eugenol based sealers had the greatest antimicrobial effect. Calcium hydroxide and glass ionomer based sealers showed significantly lower antimicrobial activity compared to AH26, Endo-methasone and Tubliseal. The greatest antimicrobial activity was found for epoxy resin based sealer (AH26) for both tested microorganisms. In several studies, the antibacterial activity of calcium hydroxide[$Ca(OH)_2$]containing sealers was tested by 'Agar diffusion test' (Al-Khatib et al.(1990);. Therefore this study aimed to evaluate the antibacterial effect of three $Ca(OH)_2$ based sealers using different cultivating techniques.

2.Materials and methods

2.1.Materials

2.1.1. The root canal

The root canal sealers studied were:

1. Apexit sealer (Ivocalr/Vivdent, Schaan and Liechtenstein).
2. CRCS sealer (Coltene-Whaledent, U.S.A).
3. Sealapex (Kerr, Italy)

All of them were mixed according to the manufacturers' instructions.

2.1.2. Microorganism:

Microorganism used :in this study was *Streptococcus α haemolyticus*.

It is facultative anaerobic bacteria.

2.2.Methods:

Three methods were used to evaluate the antibacterial activities of the three root canal sealers against the *streptococcus α haemolyticus.*

The strains were prepared and inoculated in 5ml broth and incubated at 37^{o}C for 24 h.

2.2.1.Method (1)

1ml of the prepared bacterial suspension was impregnated and mixed by hand shacking with the blood agar while it is still liquid, then poured into sterile Petri dishes. Plates were left for 5 minutes to allow the media to harden. Wells were then punched using sterile disposable micropipette tips of 5mm diameter and 5mm depth in each agar plate and filled with the freshly mixed sealers.

2.2.2.Method (2)

Mueller-Hinton Agar was used instead of blood agar plates. Typical formula of Mueller-Hinton Agar by g/l contains: beef, dehydrated infusion from 300.0, casein hydrolysate 17.5, starch 1.5, agar 17.0, supplemented with yeast extract 0.1% and glucose extract 0.1%. Seeding was done using sterile cotton swabs, by spreading/brushing the bacteria across the surface of the blood agar, vertically and horizontally from the bacterial suspension. Similar to that tube # 3 of the Mc Farland scale. Plates were left for 5 min to allow the absorption of the inoculum.

2.2.3.Method (3)

Petri dishes containing blood agar were prepared .Seeding was done using sterile cotton swabs, by spreading/brushing the bacteria across the surface of the blood agar, vertically and horizontally from the bacterial suspension. Similar to that tube # 3 of the Mc Farland scale. Plates were left for 5 min to allow the absorption of the inoculums. Wells of 5mm diameter and 5mm depth were punched in each agar plate and filled with the freshly mixed sealers.

The agar plates were incubated into anaerobic jars at 37^{0}C for 24 and 72 hrs. The antimicrobial effect of each sealer was determined by measuring the diameter of zones of inhibition in millimeters. Five plates were prepared for every sealer in each method.

3.Results

3.1. Results of Blood agar impregnated with 1 ml of bacterial suspension (Method 1)

(Blood agar impregnated with 1 ml of bacterial suspension-method 1) at one day period showed that there was a statistically significant difference between the three groups (P < 0.001) by ANOVA test.

Duncan's test showed that Sealapex gave the statistically significantly highest mean inhibition zone diameter. This was followed by CRCS. Apexit showed the statistically significantly lowest mean as shown in figures (26, 27, and 28)

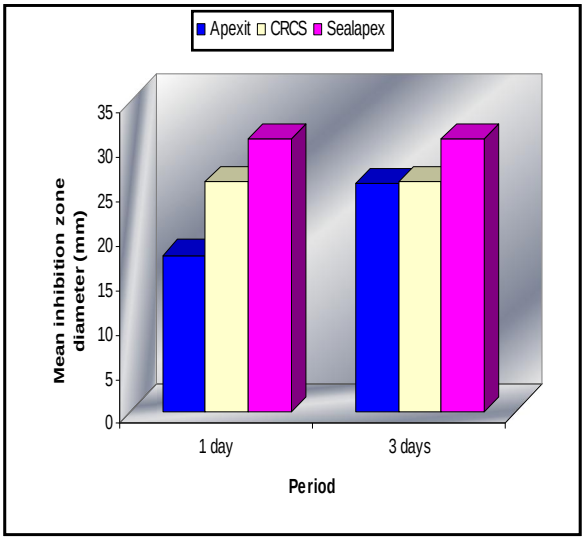
While After 3 days period ANOVA test showed that there was a statistically significant difference between the three groups (P = 0.037).

Duncan's test showed that Sealapex gave the statistically significantly highest mean inhibition zone diameter. There was no statistically significant difference between CRCS and Apexit which showed the statistical significant lowest means of inhibition zones.

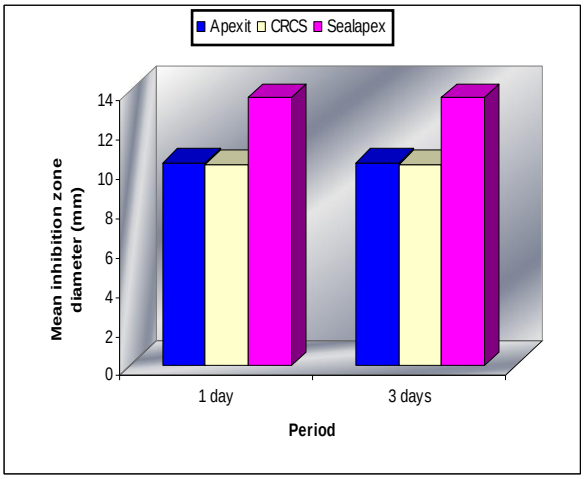
3.2. Results of Meuller Hinton agar (method 2)

After 1 day and 3 days of incubation ,ANOVA test showed that there was a statistically significant difference between the three groups (P < 0.001).

Duncan's test showed that Sealapex gave the statistically significantly highest mean inhibition zone diameter. There was no statistically significant difference between CRCS and Apexit which showed the statistical significant lowest means as shown in figures (1,2,3).



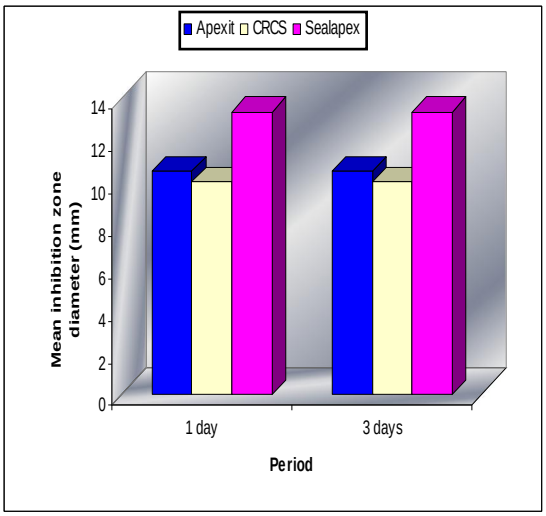
Mean inhibition zone diameter in the three groups (method 1) Fig (1)



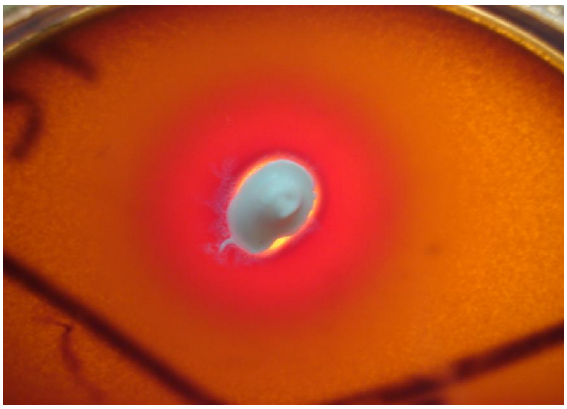
Mean inhibition zone diameter in the three groups (method 2) Fig (2)

3.3.Results of Blood agar with bacterial swab (method 3) :

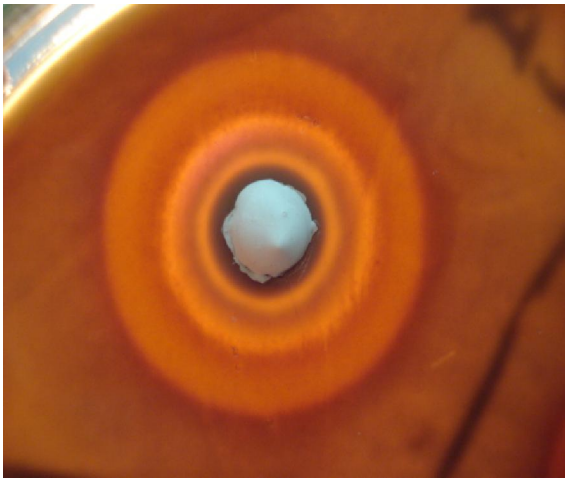
After 1 day and 3 days incubation, ANOVA test showed that there was a statistically significant difference between the three groups ($P = 0.009$). Duncan's test showed that Sealapex gave the statistically significantly highest mean inhibition zone diameter. There was no statistically significant difference between CRCS and Apexit which showed the statistical significant lowest means as shown in fig (3).



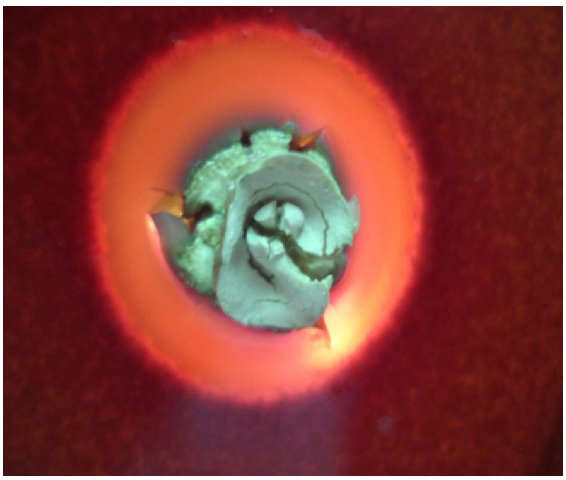
Mean inhibition zone diameter in the three groups (method3) Fig (3)



Inhibition zone produced by Apex



Inhibition zone produced by CRCS.



Inhibition zone produced by Sealapex

4. Discussion

The endodontic microbiota of untreated teeth was dominated by strict anaerobic bacteria, Siqueira (2000) .However, aerobic and facultative anaerobe microorganisms are usually minor constituents of primary infections, but they have been found in cases in which the treatment had been protracted, in flare-

ups, and associated with endodontic failures. These microorganisms can invade the root canal system during the treatment, survive the treatment procedures, and persist after obturation. Therefore, they cause secondary infections, Waltimo et al.,(1997); Sundqvist et al.,(1998); Siqueira(2000).

So, it is important to evaluate the antimicrobial activity of endodontic materials against aerobic and facultative anaerobic microorganisms.

The microorganism used in this study was *Streptococcus α haemolyticus*. It is facultative anaerobic bacteria. It includes two main groups, which were: *Viridans* group and *Pneumococci* group. The *Viridans* group of *Streptococci* principally lives in oropharynx and the oral cavity. It comprises roughly one-quarter of the total cultivable flora from supra-gingival , gingival plaque, and one-half of the isolates from the tongue and saliva. Thus, they can easily release to the blood stream during dental procedures, and sometimes during tooth brushing. Usually the bacteria released during dental procedures settle on damaged heart valves causing infective endocarditis(60% of the cases are due to this organism),Samaranayake (1996).

Three methods of the agar diffusion test (ADT) were used for antimicrobial evaluation of these tested sealers for the confirmation of the results.

The three methods revealed that after 24 h duration, Sealapex gave the highest mean inhibition zone followed by Apexit and CRCS. Where there was no statistical difference between their means. However, the means of inhibition zones by Apexit were slightly bigger than those of CRCS. This may be due to the high molecular size of CRCS and thus the low diffusion of the antimicrobial component of the sealer. CRCS contains zinc oxide, eugenol, eucalyptol, and small components of calcium hydroxide, Fuss et al.,(1997) .

Its antibacterial effect is probably due to the eugenol content rather than to the calcium hydroxide, Al-Khatib et al.,(1990).

Blood agar impregnated with 1ml of bacterial suspension gave the same results but with higher values in the mean inhibition zone diameters of the three groups in the 24 h period. This is probably due to more inoculum's number. As the microorganisms was totally mixed with the blood agar. Thus the bacteria were available in the whole thickness of the agar, increasing the sealer/ agar contact. Again in the 24h period Sealapex gave the highest statistically significant mean diameter of inhibition zone (30.6 mm), followed by CRCS (25.8mm) and then Apexit (917.6 mm) which showed the lowest mean inhibition zone diameter. At the three days period, the mean inhibition zone diameter of Sealapex and CRCS did not change. However, there was a significant increase in the inhibition zone diameter of Apexit group. This may be due to more diffusion of the material through the agar.

The anti microbial substance must diffuse through the aqueous agar medium and thus only the water soluble agents can be tested, Samaranayake(1996). On the other hand, the results of the media Mueller-Hinton agar (method B) and blood agar (method C) with bacterial swabbing were almost the same in both 24h and 3 days periods.

This confirmed the results that Sealapex had the highest antimicrobial effect against *Streptococcus α haemolyticus*, followed by Apexit and CRCS. These results came in agreement with Estrela et al.(2000) ; Lai et al.,(2001) ; Saleh et al.(2004) and Sipert et al.,(2005).

On the other hand, these results were in consistent with Fuss et al.,(1997) ; Siqueira et al., ,(1997);Mirjana and Branka (2006).

Fuss et al.,(1997) concluded that CRCS had stronger antibacterial effect than Sealapex with fresh samples. They referred their findings to the antibacterial effect of the eugenol content of CRCS rather than the calcium hydroxide content. While, Sealapex is based on polymeric resin and calcium formula. Thus, needs more time for polymerization and the release of hydroxyl ions. Fuss and his colleagues used direct contact test, and recorded their results every 30 min for 15h while in the present study, ADT was used, and the inhibition zones were measured after 1 and 3 days ,Fuss et al.,(1997)

Mirjana and Branka (2006) reported no antimicrobial effect of Apexit against *S. mutans* (*Streptococcus α haemolyticus*) on both types of agar. This is possibly because they measure the inhibition zone exactly after the application of freshly mixed specimens. Also, Apexit is based on resin and calcium hydroxide. Thus time was needed for polymerization of the materials, and accordingly there will be no hydroxyl ions which elevate the pH and having its antimicrobial effect, Mirjana and Branka (2006).

Siqueira et al.(1997) concluded that Sealapex presented low antibacterial activity when exposed to human saliva. This decreased activity might be due to the saliva buffering system, which was provided by proteins and by phosphate and bicarbonate buffering system. Therefore, even if Sealapex released hydroxyl ions which elevated the pH, that might reach to the toxic levels of the bacteria present in saliva. So when these hydroxyl ions were exposed to large volume of saliva, it is likely that the chemical effect of calcium hydroxide be rapidly neutralized by the buffer system, Siqueira et al.(1997)

Conclusions

This study was conducted to compare the antibacterial activity of three calcium hydroxide

based sealers namely: Apexit, CRCS, and Sealapex were three methods were used to evaluate the antibacterial activity of the three sealers. Sealapex had the highest antimicrobial effect. While there was no change in the means of the inhibition zones after three days for the other sealers.

All the calcium hydroxide tested sealers release calcium ions and are able to provide an alkaline medium and thus having antimicrobial property.

The prolonged setting time of Sealapex may render it beneficial for the long-term antimicrobial effect, and thus provide high pH even after complete observation.

5. References

1. Al- Khatib ZZ, Baum RH, Morse DR, Yesiloy C, Bhambhani S, Furst ML. the antibacterial effect of various endodontic sealers. Oral Surg 1990; 70:784-90.
2. Esterla C , Bammann L L , Esterla C R , Silva R , Pecora J. Antimicrobial and Chemical Study of MTA, Portland Cement, Calcium Hydroxide Paste, Sealapex and Dycal. Braz Dent J 2000 11: 3-9.
3. Fox J, Isenberg H D. Antibiotic resistance of microorganisms isolated from root canals. Oral Surg 1967; 23:230- 5
4. Fraunhofer V., Branstetter J. The physical properties of four endodontic sealer cements. J Endodon 1982; 8:126- 30
5. Fuss Z, Weiss E I, Shalhav M. Antibacterial activity of calcium hydroxide containing endodontic sealers on *Enterococcus Faecalis* in vitro. Int Endod J 1997; 30: 397-402.
6. Ibrahim S M, Aisa H A. Incidence and identity of microorganisms present in the root canal. Egypt Dent J 1973; 19:65.
7. Khemaleelakul S, Baumgartner C, Pruksakorn S, Mai C. Identification of bacteria in acute endodontic infections and their antimicrobial susceptibility. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2002; 94:746-55.
8. Lai C C · Huang F M · Chan H· Huang M· Chang M. Antimicrobial activity of four root canal sealers against endodontic pathogens. Clin Oral Invest 2001 5:236–239
9. Mirjana V, Branka R. Antimicrobial activity of root canal sealers. Serbian Dental J 2006; 53:104-112.
10. Saleh I M, Ruyter I E, Haapasalo M, Orstavik D. Survival of Enterococcus Faecalis in infected dentinal tubules after root canal filling with different root canal sealers invitro. Int Endod J 2004; 37: 193-198.
11. Samaranayake L.P. Essential Microbiology for Dentistry1996, 1st ed, 73-6
12. Sipert CR, Hussne RP, Nishiyama CK, Torres SA. In vitro antimicrobial activity of Fill Canal, Sealapex, Mineral Trioxide Aggregate, Portland cement and EndoRez. Int Endod J 2005; 38: 539–543.
13. Siqueira J F , Favieri A G, Sergio M. M., Moraes S R. Lima K C, Lopes H P. Antimicrobial Activity and Flow Rate of Newer and Established Root Canal Sealers. J Endodon 2000; 26:274-277.
14. Siqueira JF Jr, Rocas IN, Lopes HP, de Uzeda M. Coronal leakage of two root canal sealers containing calcium hydroxide after exposure to human saliva. J Endodon 1999; 25:14-6.
15. Sundqvist G, Figdor D, Persson S, Siogren U. Microbiologic analysis of teeth with failed endodontic treatment and the outcome of conservative re-treatment. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1998; 85:86-93.
16. Waltimo TMT, Siren EK, Torkko HLK, Olsen I, Haapasalo MPP. Fungi in therapy-resistant apical periodontitis. Int Endod J 1997; 30:96-101.

11/24/2010

Influence of the Etiological Factors for Gingival Enlargement on some Angiogenic and Inflammatory Mediators: An immunohistochemical study

Abeer Gawish [a], Amira M. Gamal-Eldeen [b*], Salah H.Sherif [c] Amany Neamat [d]

[a] Assoc. prof.of Oral Medicine, Periodontology, Oral Diagnosis and Radiology, Faculty of Dental Medicine (Girls'), Al-Azhar University, Cairo, Egypt
[b] Assoc. prof. in Cancer Biology Laboratory, Centre of Excellence for Advanced Sciences, National Research Centre, Cairo, Egypt
[c] prof. of Oral pathology. Dean of Faculty of dentistry Misr international University
d Prof of Oral Pathology in Department of Surgery and Oral Medicine Researches, National Research Centre, Cairo, Egypt
Corresponding Author: dr_amany@hotmail.com

Abstract: Inflammatory gingival enlargement is the most common inflammatory gingival disease and is associated with multiple factors including inflammation due to bacterial plaque colonization, as aside-effect of systemic medications, prolonged orthodontic treatment, and other etiological factors. This study investigated the effect of different etiological causes of gingival enlargement; including the treatment with cyclosporine A, the plaque, and the orthodontic treatment; on the angiogenic inflammatory mediators such as vascular endothelial growth factor (VEGF), cycloxygenase-2 (COX-2), 5-lipoxygenase (5-LO), and tumor necrosis factor-α (TNF- α), using histopathological and immune-histochemical analysis. The results of the immune-histochemical analysis of different angiogenic inflammatory mediators in the gingival enlargement samples indicated that in cyclosporine A-induced enlargement neither of VEGF, COX-2, 5-LO, nor TNF-α were affected, while there is a remarkable general over-expression of VEGF, COX-2, and 5-LO in the parakeratinized epithelial surface, the epithelial layer, connective tissue and in the fiber bundles regions of plaque-induced enlargement gingival. Additionally, orthodontic treatment samples indicated that there is a very high expression of VEGF in the epithelial layer of gingival but not in the connective tissue nor in the fiber bundles regions with no change in COX-2, 5-LO, nor TNF-α expression. In conclusion, this report indicated that the expression of different angiogenic and inflammatory mediators in gingival enlargement is influenced by the etiological factor that initially induced this enlargement.
[Abeer Gawish, Amira M. Gamal-Eldeen, Salah H.Sherif, Amany Neamat. **Influence of the Etiological Factors for Gingival Enlargement on some Angiogenic and Inflammatory Mediators: An immunohistochemical study.** Journal of American Science 2010;6(12):1754-1760]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: gingival enlargement, plaque, cyclosporine A, orthodontic treatment; nickel, angiogenic, inflammatory, VEGF, COX-2, 5-LO, TNF-α, immunohistochemical.

1.Introduction

The inflammatory gingival enlargement is caused by bacterial plaque colonization on tooth surfaces and subsequent invasion of the micro-organisms. Clinically detectable fibrotic overgrowth of gingiva is caused by a variety of etiological factors and is aggravated by local bacterial plaque accumulation. Affected gingival tissues are red, oedematous, soft in consistency, and may bleed when gently probed Clocheret et al.,(2003). In some pathological conditions, gingivitis caused by plaque accumulation can be more severe. Gingival enlargement can be inherited, or is of idiopathic origin, or is sometimes associated with other systemic diseases. The majority of cases, however, occur as a side-effect of systemic medications. There is now general agreement that gingival overgrowth lesions all contain fibrotic or expanded connective tissues with various levels of inflammation and an enlarged gingival epithelium Clocheret et al.,(2003). The degrees of inflammation, fibrosis, and cellularity depend on the duration, dose, and identity of the drug, on the quality of oral hygiene, and on individual susceptibility that stems from genetic factors and environmental influences Trackman and Kantarci (2004).

The clinical presentation of gingival overgrowth varies from a non-inflamed, firm and fibrous condition to an edematous, hemorrhagic appearance with a tendency to spontaneous bleeding. It is usually begins at the interdental papilla in a lobular form, while at later stages it affects the entire gingival and extends coronally, leading to functional, esthetic and phonetic complication, Costa et al.,(2007). A patient history helps establish the inflammatory enlargement as acute or chronic, localized or generalized. Chronic enlargements are generally painless and slow to progress, whereas acute enlargements are characterized by a painful, rapid onset, Lundergan (2003).Chronic inflammatory gingivalenlargements are generally associated with identifiable systemic or

local factors. The primary local factor associated with these enlargements is plaque. Secondary local factors can include calculus, poor dental restorations, prolonged orthodontic treatment, orthodontic braces, caries, tooth crowding or misalignment, open contacts with food impaction, mouth breathing, and removable appliances. A gingival enlargement that is inflammatory and fibrotic can represent an enlargement that was initially fibrotic with secondary inflammation or an enlargement that was initially inflammatory but has become secondarily fibrotic, Lundergan (2003)..

During the past few years, the list of the medications causing a similar gingival overgrowth condition has increased. These medications include the anti-seizure drug phenytoin, the immune suppressor cyclosporin A, and certain anti-hypertensive dihydropyridine calcium-channel-blockers, most notably nifedipine, Trackman and Kantarci (2004).

Cyclosporine A is a lipophilic, hydrophobic, c yclicendecapeptide used to prevent rejection of transplanted organs and to treat various autoimmune diseases, Seymour RA, Jacobs(1992) .A common side effect of cyclosporine is gingival overgrowth which is characterized by epithelial hyperplasia, interstitial fibrosis, changes in blood vessel profile, and focal inflammatory cell infiltrate, Chiu et al.,(2008). A diagnosis of drug induced gingival enlargement can be made if the development of the fibrotic enlargement coincided with the administration of one of these medications. The gingival tissues are enlarged, oedematous, soft, and tender to touch, with a tendency to bleed easily. The gingiva is bluish-red with some pseudo-membrane plaques covering ulcerated surfaces. In some conditions, gingival enlargement can progress rapidly into destructive periodontal diseases, as a result of the altered immune response of the gingiva to the bacterial plaque,Trackman and Kantarci (2004).

Orthodontic treatment may initiate oral clinical manifestations, such as labial desquamation, Lindsten and Kurol (1997) gingival enlargement, Bishara et al.,(1993); Genelhu et al.,(2005); Kouraki et al.,(2005)and multiform erythema, Starkjaer and Menne(1990)gingivitis Shelley (1981).Such manifestations are usually associated with the inflammatory response induced by the corrosion of orthodontic appliances, and major emphasis has been placed on nickel, Eliades et al.,(2003). Inflammatory response to nickel is considered as type IV hypersensitivity and is manifested as nickel allergic contact stomatitis, Holmstrup(1999); Vanarsdall(2000).

Gingival enlargement is a more common sequela of orthodontic treatment than other manifestations, Genelhu et al.,(2005); Genelhu et al.,(2005); Gursoy et al.,(2007). Fibrous gingival enlargements associated with fixed orthodontic appliances seem to be transitory, and it is generally thought that enlargement resolves after orthodontic therapy, Carranza (2006)However, there are also studies reporting that this resolution is not complete Ramadan (2004).Orthodontic treatment-induced gingival overgrowth shows a specific fibrous and thickened gingival appearance, different from fragile gingiva with marginal gingival redness, which is seen in allergic or inflammatory gingival lesions, Ramadan (2004).; Gursoy et al.,(2007) .

This study investigated the effect of different etiological causes of gingival enlargement; including the treatment with cyclosporine A, the plaque, and the orthodontic treatment; on the angiogenic inflammatory mediators such as vascular endothelial growth factor (VEGF), cycloxygenase-2 (COX-2), 5-lipoxygenase (5-LO), and tumor necrosis factor-α (TNF- α), using histopathological and immunohistochemical analysis.

2.Material and methods

2.1.Materials:

2.1.1.Study population

A total of 30 subjects (24 males and 6 females aged 22- 45 years) were submitted to this study. They were attending the Oral Medicine, Periodontology, Oral Diagnosis and Radiology Department, Faculty of Dental Medicine Girls' Al-Azhar University. They included 10 renal transplant patients receiving cyclosporine A therapy for at least 6 months without any other drugs reported to cause drug- induced gingival overgrowth. Ten healthy subjects with teeth plaque and had no systemic disease and none of them had taken medications that could affect their periodontal status for at least 3 months prior to the study. The last 10 subjects were orthodontically treated with fixed orthodontic appliance for a period of 2 years suffering from hyperplastic enlargements covering one third to two thirds of the clinical crown. Smokers, pregnant and post moneposal women were excluded. The patients signed approval consents and samples were collected under the approval of the ethical Committee of the National Research Centre, Cairo, Egypt.

2.2.Methods:

2.2.1.Clinical procedures

Clinical recordings including plaque index O'leary et al.,(1993) gingival index Löe and Silness (1963)and probing depth were performed as shown in table 1. All selected subjects did not have any evidence of clinical attachment loss or bone resorption as diagnosed by radiographs and had not undergone any periodontal therapy. Gingival biopsies were obtained from diseased gingival during gingivictomy for

cyclosporine A treated group as well as in orthodontically treated group as a part of their routine clinical management, which also included intensive plaque control, while the samples were taken during pre-prosthetic crown lengthining procedure for plaque induced gingival enlargement subjects.

2.2.2.Histopathology

The biopsies were immersed in 10% formalin and decalcified in multiple baths of 10% trichloroacetic acid. After decalcification, the blocks were immersed in paraffin, and semi-serial 4 µm histologic sections were stained with haematoxylin and eosin (HE).

2.2.3.Immunohistochemistry

Paraffin sections of the 4 µm of biopsies were collected on Superfrost Plus slides (Menzel-Gläser), the paraffin was removed and the sections were rehydrated again. Before staining, the slides were treated with 0.1 % trypsin 250 (DIFCO Laboratories, Detroit, USA) for 10 minutes and rinsed with PBS. Thereafter, the slides were treated with 3 % H_2O_2 in PBS for 30 minutes to block endogenous peroxidase and rinsed in PBS. All sections were pre-incubated with 5 % bovine serum albumin (PBSA; Sigma Chemical Co., St Louis, Missouri, USA) in PBS buffer. VEGF, COX-2, 5-LO, and TNF-α were detected using their corresponinga rabbit antibodies (Abcam, Cambridge, UK), After being washed, bound antibody was detected using goat anti-rabbit antibody linked to horseradish peroxidase (Cambridge, UK) and bound complexes were detected using O-phenylenediaminedihydrochloride (OPD) (Sigma Aldrich, VA, USA). Representative sections were photographed on a Leitz DMRD Microscope (Leica, Wetzlar, Germany).

3.Results:

3.1. Histopathological examination :

3.1.1.The gingival specimens

Histopathological examination of the gingival specimens in cyclosperin of the treated group revealed a parakeratinized surface epithelium of the attached gingival of variable thickness in different parts of the attached gingival of variable thickness in different parts, connective tissue stroma formed of longitudinal bundles of hyalinized delicate collagen fibrils which showed various distribution, either dense or finely arranged. They were interspersed with a heavy chronic inflammatory infiltrate (mainly lymphocytes and plasma cells) and quit a noticeable number of dilated blood vessels (Fig. 1).

3.1.2.In plaque induced gingival enlargement group:

Histopathological examination in plaque induced gingival enlargement group showed a dense thick band of collagenous fibrous tissue arranged subepithelially demarcating a heavy zone of chronic inflammatory cell infiltrate packed with plasma cells and lymphocytes with fewer dilated blood vessels were found (Fig. 1).

3.2.3. In orthododontic group specimens :

Histopathological examination In orthododontic group specimens showed thinner parakeratinized surface epithelium covering a connective tissue stroma with contentious dense organized collagen bundles with chronic inflammatory infiltrate and a fewer dilated blood vessels were observed (Fig. 1).

3.2.The effect of the immune suppressor drug; cyclosporine A

This effect on the gingival expression of different angigenic inflammatory mediators using immunohistochemical analysis revealed that neither of VEGF (Fig. 2), COX-2 (Fig. 3), 5-LO (Fig.4), nor TNF-α (Fig. 5) were affected (Table 2), as indicated by negatively stained gingival enlargement samples. Despite extensive research, Results from table (1) indicated that there was a remarkable general overexpression of VEGF (Fig. 2), COX-2 (Fig. 3), and 5-LO (Fig. 4) in the parakeratinized epithelial surface, the epithelial layer, connective tissue and in the fiber bundles regions of gingival. Additionally, plaque was found to no influence of the gingival content of TNF-α (Fig. 5).

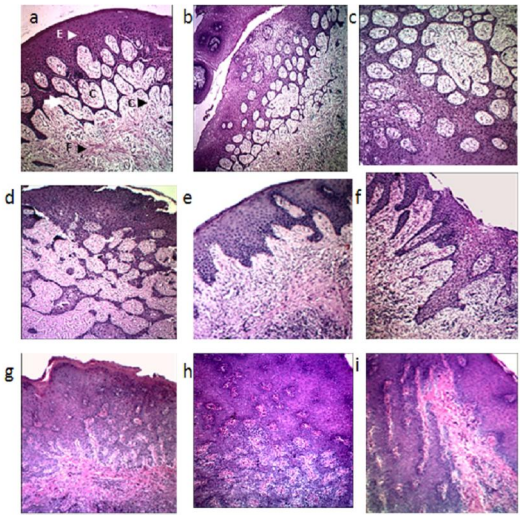


Fig. 1: Histochemical analysis using HE stain of sections represent different gingival enlargement causes including cyclosporine A-induced enlargement (A-C), plaque-induced enlargement (D-F), and the orthodontic treatment-induced enlargement (G-I). The photos labels are E= epithelium, C= connective tissue, F= fibre bundles. Microscopic magnification was X100.

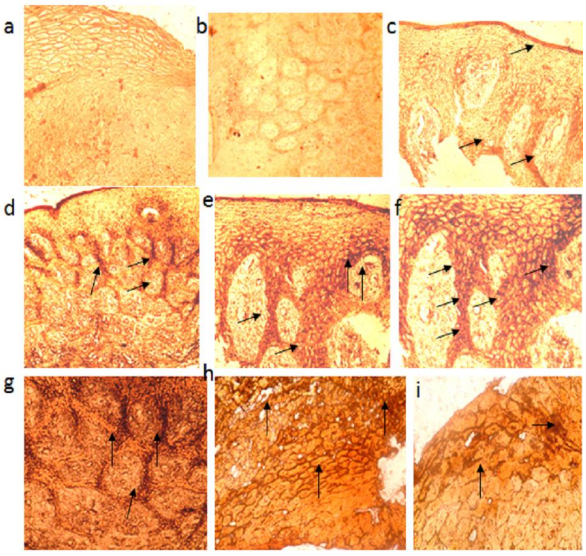


Fig. 2: Immunohistochemical analysis of VEGF expression in sections represent different gingival enlargement causes including cyclosporine A-induced enlargement (A, B), plaque-induced enlargement (C-G), and the orthodontic treatment-induced enlargement (H-I). Microscopic magnification was X100 (A-E) and X400 (F-1)Representative regions of high VEGF expression were marked by black arrows.

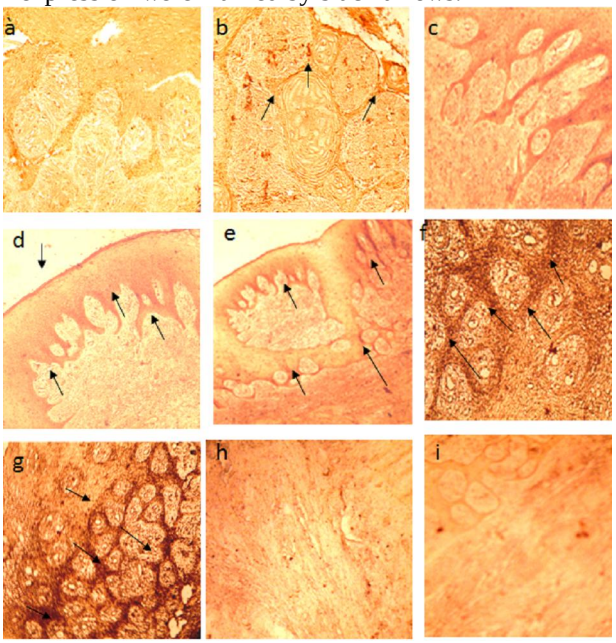


Fig.3:Immunohistochemical analysis of COX-2 expression in sections represent different gingival enlargement causes including cyclosporine A-induced enlargement (A, B), plaque-induced enlargement (C-G), and the orthodontic treatment-induced enlargement (H, I). All microscopic magnification was X100, except in (F, G) it was X400. Representative regions of high COX-2 expression were marked by black arrows.

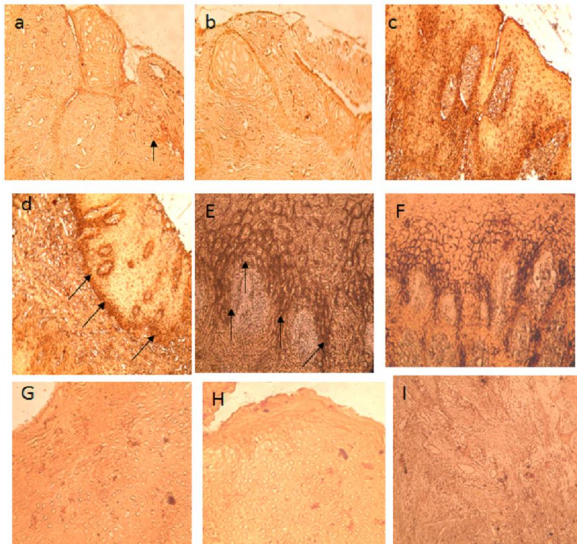
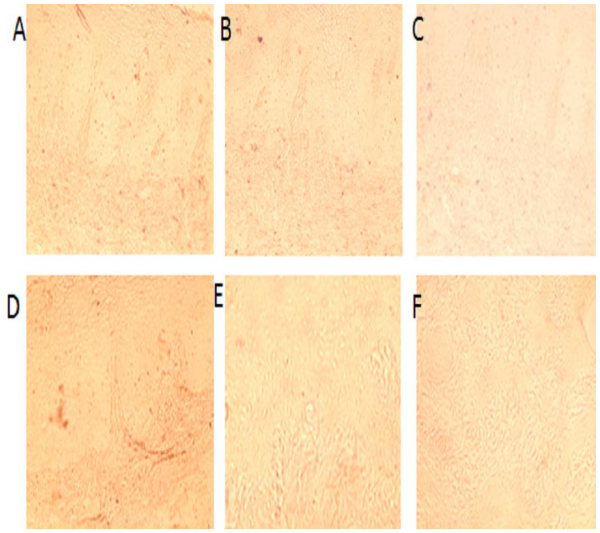


Fig. 4: Immunohistochemical analysis of 5-LO expression in sections represent different gingival enlargement causes including cyclosporine A-induced enlargement (A, B), plaque-induced enlargement (C-F), and the orthodontic treatment-induced enlargement (G- I). All microscopic magnification was X100, except in (E, F) it was X400. Representative regions of high 5-LO expression were marked by black arrows.



Fig(5):Immunohistochemical analysis of TNF-α expression in sections represent different gingival enlargement causes including cyclosporine A-induced enlargement (A, B), plaque-induced enlargement (C, D), and the orthodontic treatment-induced enlargement (E, F). All microscopic magnification was X100, except in (F) it was X400.

3.3. Immunohistochemical analysis of plaque-induced gingival enlargement

Table 1: Clinical characteristics of the gingival enlargement groups that was induced by different etiological factors including Cyclosporine A treatment (n = 10), Plaque (n = 10), and Orthodontic treatment (n = 10); (mean ± SD).

Marker	Cyclosporine A group	Plaque group	Orthodontic group
VEGF	0/10	10/10	8/10
COX-2	0/10	10/10	2/10
5-LO	0/10	8/10	2/10
TNF- α	0/10	0/10	0/10

Table 2: Number of patients with positively high expression of different angiogenic inflammatory markers, as assayed by immunohistochemical analysis, of the gingival enlargement groups that was induced by different etiological factors including Cyclosporine A treatment (n = 10), Plaque (n = 10), and Orthodontic treatment (n = 10).

	Cyclosporine A group	Plaque group	Orthodontic group
Plaque index	1.45 ± 0.67	1.58 ± 0.64	1.08 ± 0.47
Gingival index	1.24 ± 0.36	1.38 ± 0.29	1.02 ± 0.21
Probing depth	4.17 ± 0.85	3.38 ± 0.47	3.29 ±0.67

Data was represented as: (number of patients with positive high expression) / (total number of patients in the group).

3.**4. Immunohistochemical analysis of gingival enlargement samples from orthodontic treatment** : Patients indicated that there was a very high expression of VEGF in the epithelial layer of gingival but not in the connective tissue nor in the fibre bundles regions. On the other hand, as shown in table 2, orthodontic treatment was found to have no influence of the gingival content of COX-2 (fig 3), 5-LO (fig. 4), nor TNF-α (Fig. 5).

4. Discussion

Gingival tissues are generally in a state of injury and repair that involves repetitive cycles of production of chemotactic factors, angiogenic factors, inflammatory cell recruitment, and tissue resorption, replacement, and remodelling, Clark (1998) . Collagen turnover is unusually high in periodontal tissues, Trackman and Kantarci (2004).

Wound healing and connective tissue turnover are largely controlled by chemokines and cytokines secreted by inflammatory cells such as macrophages and lymphocytes and, to a lesser degree, by fibroblasts. Proliferation and differentiation of connective tissue cells and production of extracellular matrix are controlled by cytokines that initiate signalling cascades mediated by specific receptors, Trackman and Kantarci (2004).

In this regard, the histopathological examination in the present work of the gingival specimens in cyclosperin A treated group revealed a parakeratinized surface epithelium of the attached gingiva of variable thickness in different parts of the attached gingival connective tissue stroma formed of longitudinal bundles of hyalinized delicate collagen fibrils which showed various distribution, either dense or finely arranged. They were interspersed with a heavy chronic inflammatory infiltrate (mainly lymphocytes and plasma cells) and quit a noticeable number of dilated blood vessels. In plaque induced gingival enlargement group a dense thick band of collagenous fibrous tissue arranged subepithelially demarcating a heavy zone of chronic inflammatory cell infiltrate packed with plasma cells and lymphocytes with fewer dilated blood vessels. In orthododontic treated group specimens thinner parakeratinized surface epithelium covering a connective tissue stroma with contentious dense organized collagen bundles with chronic inflammatory infiltrate and a fewer dilated blood vessels were present.

Gingival hyperplasia is a common side-effect of immunosuppression with cyclosporine A. Exploring the effect of the immune suppressor drug; cyclosporine A on the gingival expression of different angigenic inflammatory mediators using immunohistochemical analysis revealed that neither of VEGF, COX-2 ,5-LO, nor TNF-α were affected .Despite extensive research, the mechanism leading to the accumulation of abnormal amounts of gingival tissue in cyclosporine A-induced gingival overgrowth is unclear. Fibroblasts are the main cell type residing in the gingival connective tissue, and are responsible for the formation and turnover of the extracellular matrix. Studies on the effect of cyclosporine A on gingival fibroblast activity have reported conflicting

findings, and it is uncertain if cyclosporine A can induce gingival overgrowth by directly altering the function of fibroblasts Voulgari and Drosos(2002). Recently cyclosporine A was reported to increase both IL-6 and TGF-beta1 levels Chae et al.,(2006)and to inhibit COX-2 Chiang et al.,(2007).

Plaque induced gingival enlargement can progress rapidly into destructive periodontal diseases, as a result of the altered immune response of the gingiva to the bacterial plaque. In present study, the immunohistochemical analysis of plaque-induced gingival enlargement patients indicated that there was a remarkable general over-expression of VEGF ,COX-2, and 5-LO in the parakeratinized epithelial surface, the epithelial layer, connective tissue and in the fiber bundles regions of gingival. Additionally, plaque was found to no influence of the gingival content of TNF-α . Gursoy et al.,(2007).

Nickel, the most common metal used in orthodontic appliances, may activate monocytes and epithelial cells, suppressing or promoting the expression of intracellular adhesion molecule 1 by endothelial cells, mostly depending on its concentration. Nickel ions can also intracellularly accumulate in human oral mucosal cells and human HaCaT keratinocytes, Ermolli et al.,(2001); Faccioni et al.,(2003).

Nickel concentrations, which do not significantly modify oral epithelial cell viability and inflammatory cytokines release (<1.3 mM) can stimulate apoptosis in vitro Trombetta et al.,(2005.)On the contrary, human primary cultured keratinocytes and HaCaT cells also proliferate in response to nickel ions Jia et al.,(1999).Nickel-containing orthodontic wires can reduce cell viability and stimulate apoptosis in three-dimensional cell culture models Vannet et al.,(2005). Even though nickel is related to allergic response seen in orthodontic therapy, Holmstrup (1999); Vanarsdall (2000), its influence of angiogenic inflammatory mediators in gingival overgrowth has not been studied yet.

In the present study, the immunohistochemical analysis of gingival enlargement samples from orthodontic treatment patients indicated that there was a very high expression of VEGF in the epithelial layer of gingival but not in the connective tissue nor in the fibre bundles regions. On the other hand, orthodontic treatment was found to have no influence of the gingival content of COX-2 , 5-LO nor TNF-α . TNF-α was expected to be secreted from the immune cell in the intracellular infiltrate within the inflammatory gingiva, but this was not noticed in all of different gingival enlargement samples. Although there was highly noticed inflammatory cell infiltrate in orthodontic treatment samples, no positively stained TNF-α was noticed. As a positive control, samples of bacterial lipopolysaccharide-treated human macrophages were stained positively for TNF-α.

Nickel has been stated to be corrosive in the oral cavity, Jia et al.,(1999).An average release of 40 µg nickel per day from a stimulated full-mouth fixed appliance has been reported, Park and Shearer (1983) and also nickel accumulation was found to be higher in dental plaque samples of patients receiving orthodontic therapy in comparison with untreated subjects, Fors and Persson(2006). However, it is also suggested that the release of nickel is not necessarily proportional to the nickel content of the alloy, Grimsdottir et al.,(1992).The in vivo and in vitro results of a previous study suggested that low-dose continuing nickel release from orthodontic appliances might be the initiating factor for gingival overgrowth, as it has the capability of increasing epithelial cell proliferation, Gursoy et al.,(2007). Depending on the induced VEGF results of the present study, nickel might be responsible for such induction that may subsequently lead to neo-vascularization, which in turn may support nourishment of overgrown tissue.

In conclusion, the immunohistochemical analysis of different angigenic inflammatory mediators in gingival enlargement samples indicated that in cyclosporine A-induced enlargement neither of VEGF, COX-2, 5-LO, nor TNF-α were affected, while there was a remarkable general over-expression of VEGF, COX-2, and 5-LO in the parakeratinized epithelial surface, the epithelial layer, connective tissue and in the fiber bundles regions of plaque-induced enlargement gingival. Additionally, orthodontic treatment samples indicated that there was a very high expression of VEGF in the epithelial layer of gingival but not in the connective tissue nor in the fiber bundles regions with no change in COX-2, 5-LO, nor TNF-α expression. Taken together, this report indicated that the expression of different angiogenic and inflammatory mediators in gingival enlargement is influenced by the etiological factor that initially induced this enlargement.

5. Refrences:

1. Bishara S E, Barrett R D, Selim M. Biodegradation of orthodontic appliances. Part II. Changes in the blood level of nickel. *Am J Orthod Dentofacial Orthop* 1993; **103**: 115 – 119
2. Carranza Jr FA 1996 Gingival enlargement. In: Carranza Jr F A, Newman M G (eds) Clinical periodontology, 8th edn. W.B. Saunders Company, Philadelphia , pp. 233 – 234
3. Chae H-J, Ha M-S, Yun D-H, Pae H-O, Chung H-T, Chae S-W, Jung Y-K, Kim H-R, Mechanism of Cyclosporine-induced Overgrowth in Gingiva, *J Dent Res* 2006; **85**: 515-519.
4. Chiang CY, Chen YT, Hung FM, Tu HP, Fu MM, Fu E. Cyclosporin-A inhibits the expression of

cyclooxygenase-2 in gingival. *J Periodontal Res* 2007; **42**:443-449.
5. Chiu HC, Lan GL, Chiang CY. Upregulation of heme oxygenase-1 expression in gingival after cyclosporine A treatment. *J Periodontol* 2008; **79**: 2200-2206.
6. Clark RAF (editor). The molecular and cellular biology of wound repair. New York: Plenum Press, 1998, 98-105.
7. Clocheret K, Dekeyser C, Carels C, Willems G. Idiopathic gingival hyperplasia and orthodontic treatment: a case report. *J Orthodont* 2003; **30**:13–19.
8. Costa FO, Ferreira SD, Lages EJ, Oliveria AM, Cota LO. Demographic, pharmacologic, and periodontal variables for gingival overgrowth in subjects medicated with cyclosporine in the absence of calcium channel blockers. *J Periodontol* 2007; **78**: 254-261.
9. Eliades T, Trapalis C, Eliades G, Katsavrias E. Salivary metal levels of orthodontic patients: a novel methodological and analytical approach. *Eur Orthod* 2003; **25**:103–106
10. Ermolli M, Menne C, Pozzi G, Serra M A, Clerici L A. Nickel, cobalt and chromium-induced cytotoxicity and intracellular accumulation in human hacat keratinocytes . *Toxicology* 2001; **159**: 23 – 31.
11. Faccioni F , Franceschetti P , Cerpelloni M , Fracasso ME. In vitro study on metal release from fi xed orthodontic appliances and DNA damage in oral mucosal cells. American *Am J Orthod Dentofacial Orthop* 2003; **124**: 687–693.
12. Fors R, Persson M. Nickel in dental plaque and saliva in patients with and without orthodontic appliances . *Eur J Orthod* 2006; **28**:292-297.
13. Genelhu MCLS, Marigo M, Alves-Oliveira LF, Malaquias LCC, Gomez RS. Characterization of nickel-induced allergic contact stomatitis associated with fixed orthodontic appliances. *Am J Orthod Dentofacial Orthop* 2005; **128**: 378 – 381.
14. Grimsdottir MR, Gjerdet NR, Hensten-Pettersen A. Composition and in vitro corrosion of orthodontic appliances. *Am J Orthod Dentofacial Orthop* 1992; **101**: 525 – 532
15. Gursoy UK, Sokucu O, Uitto V-J, Aydin A, Demirer S, Toker H, Erdem O, Sayal A. The role of nickel accumulation and epithelial cell proliferation in orthodontic treatment-induced gingival overgrowth. *Eur J Orthod 2007;* **29**:555–558.
16. Holmstrup P. Non-plaque induced gingival lesions . *Ann Periodontol* 1999 ; **4** : 20 – 29Lindsten R , Kurol J. Orthodontic appliances in relation to nickel hypersensitivity: a review. *J Orofacial Orthop* 1997; **58**:100 –108.
17. Jia W, Beatty MW, Reinhardt RA, Petro TM, Cohen DM, Maze CR, Strom EA, Hoffman M. Nickel release from orthodontic arch wires and cellular immune response to various nickel concentrations . *J Biomed Mat Res* 1999; **48**:488 – 495
18. Löe H, Silness J. Periodontal disease in pregnancy. I. Prevalence and severity. *Acta Odontolica Scadinavia* 1963; **21**:533-538.
19. Lundergan WP, Diagnosis of Gingival Enlargement. In: WB Hall (eds) Critical Decisions in Periodontology, 4th Ed., BC Decker Inc, Hamilton, London, 2003, 58-59.
20. Lundergan WP, Treatment of Gingival Enlargements, In: WB Hall (eds) Critical Decisions in Periodontology, 4th Ed.,, BC Decker Inc, Hamilton, London, 2003, 138-139.
21. Park HY, Shearer TR. In vitro release of nickel and chromium from simulated orthodontic appliances . *Am J Orthod* 1983; **84**:156-159 O'leary TJ, Drake RP, Naylor JE. The plaque control record. *J Periodontol* 1972; 34-8
22. Ramadan AAF. Effect of nickel and chromium on gingival tissues during orthodontic treatment: a longitudinal study. *World J Orthod* 2004; **5**:230 – 235.
23. Seymour RA, Jacobs DJ. Cyclosporin and the gingival tissues, *J Clinc Periodontol* 1992; **19**:1-11.
24. Shelley BW. Gingival hyperplasia from dental braces. *Cutis* 1981; **28**:149–15011. Kouraki E , Bissada N F , Palomo J M , Ficara A J Gingival enlargement and resolution during and after orthodontic therapy . *NY Dent J* 2005; **71**:34 – 37.
25. Starkjaer L, Menne T. Nickel allergy and orthodontic treatment. *Eur J Orthod* 1990; **12**:284–289.
26. Trackman PC, Kantarci A. Connective tissue metabolism and gingival overgrowth. *Crit Rev Oral Biol Med* 2004; **15**:165-175.
27. Trombetta D, Mondello MR, Cimino F, Cristiani M, Pergolizzi S, Saija A. Toxic effect of nickel in an in vitro model of human oral epithelium
28. Vanarsdall Jr RL, Periodontal/orthodontic interrelationships. In:Graber T M , Vanarsdall Jr R L (eds) Orthodontics. Current principles and techniques, 3rd ed. Mosby Inc, St Louis, 2000, 820-828
29. Vannet V.B, Mohebbian N, Wehrbein H. Toxicity of used orthodontic archwires assessed by three-dimensional cell culture. *Eur J Orthod* 2006; **28**: 426 – 432.
30. Voulgari PV, Drosos AA. Gingival hyperplasia associated with cyclosporin A. *J Rheumatol.* 2002; **29**:2466-2471

11/25/2010

Technical knowledge of biological plants America and localizes it for energy production from agricultural residues in IRAN (Khuzestan province)

Ashraf jazayeri[1*], Tayeb Saki Nejad[2], Sorosh zarrin abadi[3]

1- Department of biology, Shahid Chamran University
2-Assistant Professor Department of Agronomy Physiology, Islamic Azad University, Ahvaz branch
3- Assistant Professor Department of science, Islamic Azad University, Ahvaz branch
jazayeriashraf@yahoo.com* Corresponding Arthur:

Abstract: Power generation business in the United States with approximately 9,733 MW of installed capacity from agricultural waste and non-agricultural sector, the largest source of non-renewable water power in the world has created. The capacity of 9733 MW including 5,886 MW of forest plants and agricultural residues, 3,308 MW power generation capacities of 539 MW waste urban and other materials, such as the gas is obtained from buried waste. Maximum electricity production from biomass to electricity load based on the existing electricity distribution system is used. More than 200 companies from non-wood products and food industries in the United States produce electricity biomass. Four power generation systems using biomass there. Direct fuel, the fuel mixture (with coal), and plants gasify module. Most biomass power plants are direct systems such as traditional fossil fuel power plants often act. Biomass production in North America is 180 million tons of which 43 percent of the amount of agricultural residues in plants using advanced biological anaerobic bacteria and gas production and energy production are a combination of fuel between the available biomass Potential country of Iran 22 million is a system of energy production from residue agriculture often is that this residue in a tank Amplifier as burnt is fuel ash and gas artificial is that gas result can be thermal energy used or by the generator to electrical energy to become today the ability to produce 15 billion cubic meters of gas household artificial residue agriculture there is fuel derived from technologies convert biomass or state gas (Environmental gas) or liquid (methanol, ethanol and biodiesel), which for produce electricity and heat are used. It is estimated that if only 10 percent of farms and forests to provide and providing allocated biomass, annual production of energy from biomass, equivalent to four-fifths of world energy consumption will be present. Developing communities that almost three-quarters of the world's population are included, 35 percent of energy consumption comes from biomass. If the process can be used to power advanced production techniques such as biological America, collecting, etc. in areas such as agriculture in Khuzestan, which remains almost "Between 25-18 percent of products is very high figure is in addition to performing and indigenous on energy production, burning farms and destruction of ecosystems, soil, water, air and will prevent .... Use of biomass resources, one of the best and most economical solutions to provide basic energy needs of people in remote areas, and environmental benefits this type of environment, renewable energy and its development, its application, is reasonable and affordable.
[Ashraf jazayeri, Tayeb Saki Nejad, Sorosh zarrin abadi. **Technical knowledge of biological plants America and localizes it for energy production from agricultural residues in IRAN (Khuzestan province).** Journal of American Science 2010;6(12):1761-1765]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: biological plants, infrastructure, agriculture remains

1. Introduction

Where power producers to provide low cost biomass have access to selected conditions using biomass fuels in combination caused their development of market competition is. This especially in the near future for power generation companies will find the truth if their method of electricity generation from biomass with coal combination to select. This method will save on fuel costs. Many active people engaged in the electricity market to offer to purchase electricity generated by the Complies with environmental laws, including electricity from biomass are. This move in response to consumer demand and emergencies is legal As we know, electricity from the most basic needs of life in the new century is considered, such as lighting applications ranging from homes and streets in the use of telephone and television as well as industrial and medical uses, such as metals and machinery and medical instruments ... Shows that this energy unmatched role in the development of human and community needs are. World statistics on the growing world demand shows that about 70 percent of global investment in energy sector development - equivalent to 11 trillion dollars - over the next three decades, is dedicated to providing electricity as compared to three times the equivalent figure last three decades is. International Energy Agency estimates that electricity than any other energy in final energy demand growth rate of 2.8 percent will face. On the other hand the resources scattered in different parts of the world energy supply has caused the country, different methods are used to provide energy. For example, in Norway probe, ninety-nine percent of the country electricity power

plant - water is produced. While in America in less than 10 percent of its electricity this way comes. In a prediction that the next 50 years, 30 percent of plants in the world of fuel coal, 10 percent of gas used, 15 percent of renewable energy sources (water, wind, solar and thermal energy the earth ...), and 15 percent of atomic energy for power plant use, biological plants in this way as fossil fuel power plants with fuel for thermal energy needed to be used. The difference is that the fuel and products of biological organisms (plants, animal waste, crop residues and ...) will be provided. Because this method requires extensive agricultural land is facing many constraints, but with shrinking fossil resources and rising prices, a serious rival for these fuels will be considered.

2. Biological metabolism of plants Fuel biology (biological) Biomass

Means any organic material, known source of renewable energy is considered. Waste sawdust, residues of agricultural products, and organic material from urban and industrial waste can be burned when electricity and liquid fuel directly used in transportation becomes. State Florida, agricultural fuel inventory of about 20% of biomass fuel in this state provide. Agriculture remains a wide variety of compounds are, of course, some for use as fuel are not appropriate. Agricultural residues suitable for fuel in power plants, solid fuel biomass materials are included in the following types:

- Food processing residues such as nuclei, conveyors, and skin
- Destruction of gardens and vineyards
- Pruning of gardens and vineyards
- Straw farm

Cereals and oilseeds major industrial inputs used in production of more ethanol, biodiesel and bio products are used today. Food and feed processing residues and a third residue after the consumer in order to produce a low amount of electricity are used. Of course, this amount of biomass than small agricultural biomass resources currently available, and little of the full potential of agriculture is. With appropriate economic stimulus measures and advanced technologies such as crop plants with increased yield and more efficient harvesting equipment, sufficient amounts of residual agricultural products and food and feed processing residues for the stability can be produced. In addition, the amount of sustainable biomass obtained from agricultural land can be devoted more land to produce wood products and increased herbaceous perennial.

3. Biological metabolism of plants in America

North America produced 180 million tons of biomass is that 43 percent of the amount of agricultural residues that plants using advanced biological anaerobic bacteria and gas production and energy production are a combination of fuel between the available biomass Potential country of Iran 22 million is a system of energy production from residue agriculture often Gasification is that this residue in a tank called Gassy Amplifier as Term chemical burned is Vials fuel ash and gas artificial is that gas result can be thermal energy used or by the generator to electrical energy to become today the ability to produce 15 billion cubic meters of gas household artificial residue agriculture there is fuel derived from technologies convert biomass or state gas (Environmental gas) or liquid (methanol, ethanol and biodiesel), which for produce electricity and heat are used. Large amounts of agricultural crop residue to form straw production that even if outdoors and plowing fields, so that usage can be 30 to 40 percent of these are leftover waste (between and Amos, 2003). This residue can be collected and processed to fuel power plants would. Fuel-based straw are expensive and their low mass density and hardness of some problems in handling their burning creates. Consequently very little material to help fuel sources. Even with the current level of use of biomass fuels leading agricultural areas, enormous amounts of agricultural residues for use as fuel in power plants still open are burned. Fate for most other agricultural residues used for fuel is burned in open space; even a small percentage of these applications in the absence of fuel or plowing are buried. Almost one-third of biomass energy plant in California (as a leading regional) areas Farm state have been constructed to use the residue as fuel in many capital absorbed to compensate for pollution that if some residue biomass in the case of absorption by the plant was not open as it was burned and in these conditions For energy to be used. For example, U.S. agriculture from the beginning to the twentieth century has changed considerably.

Key stimulus for this change in technology and mechanization increased performance fiber in cereal products were the main. Mechanization notably a need for horses, and consequently reduced production of oats (as livestock feed) much reduced. Mechanization, while the same performance per unit area in the period 1915 to 1978 wheat yields per unit area to more than double corn also fulfilled the function of the pan more than tripled its former reach the same performance side shows more product (straw) is equal to two or three. Of course, biotechnology and genetically modified products and high product varieties produced in this way has a role to reward. The point that should be noted here is that many of

the input potential of biomass, like straw, ash problem lies is that software behavior which sedimentation problems are getting.

Researcher's conversion and marine algae befouls and the use of methyl (root univalent hydrocarbon) Gas to convert methane from natural gas components to the remarkable success received. Although ethanol full biological energy known today, but just barely Energy is considered distressed situation. Researchers working to develop the more organic materials such as sugar cane, seaweed, grass, sewage and hospital waste, even for purposes of transportation and power generation to energy with less pollution have become. The current production of biological energy around 643 thousand barrels per day in 2050 that requires about 34 million barrels per day is estimated.

Canals in Venice, one of the important sources of energy known as the green features that can generate electricity at the commercial level will find. A Power 272.6 million dollars in expected electricity production license from biological fuel Canal hast algae. Fuel power plants, algae collected in a canal 26 feet planted biological reactor (with Gas fertilize the plant) and dried and then extracted the juice and add alkaline materials to be converted into befoul. Predicted plant in 2011 could power 40 MW (with the lowest carbon pollution) that production costs in the supply, it provides other types of ships and vessels in the port side should be taken.

After adjustment for maximum boiler power generation with biomass by adding a very small loss in efficiency occurs or no does not make any losses. In these conditions, the energy in biomass with high efficiency (about 33 to 37 percent) coal power plant will become.

Biomass converter system with gas heating biomass in the environment act and there is analysis of solid biomass and flammable gas that is rising. In this way energy than directly burning biomass is superior. Biogas biomass analysis can be cleaned and the spent filters and chemical composition of the device in which it separated. This gas can be more efficient power generation systems that use its combined cycle is called. In this system, gas turbine to generate electricity and steam turbines are combined together. The system efficiency can be increased to 60 percent.

Systems can be converted to gas fuel cell systems for future applications can be combined. Fuel cell using the process (and heat) to hydrogen gas turns it into electricity. Then much of the substance that is water vapor in the air will be rising Iranian researcher with Ohio State University discovered bacteria capable of complex molecules break down cellulose in the cow digestive system for the first time successfully developed microbial fuel cell system capable of generating electricity from waste cellulose was . Innovative Fuel Cell system that also can be a source of renewable energy, clean and efficient electricity supply required for remote control systems and also areas away from electricity transmission network is used, an attractive and promising perspective in making bio-refineries maps in which they discovered by using bacteria, organic waste and waste, electricity, methane and hydrogen is produced. The young Iranian researcher, although already successful researchers and other research groups to design microbial fuel cells systems are available, but mainly from simple ingredients and still qualify for food value, such as simple sugars and starches used for electricity production This is the first time possible to produce electricity from decomposition of complex cellulose molecules that abound in agricultural and industrial waste exist, is provided. Rope Hamid Yazdi, PhD student in Biotechnology Engineering America Ohio State University (2009) who led the investigation is responsible for technology reporter in an interview with Iranian Students News Agency (ISNA) said: The new fuel cell using bacteria that have been discovered in the cow rumen, cellulose from waste paper, wood products, and vegetable waste in the fields after harvesting, parsing, and electricity is produced. He noted: Cellulose is a complex chemical compound that only a few bacteria have the ability to break down the bacteria and the ability to transfer electrons produced from the metabolism of cellulose to have the electrode was not discovered until now. Since the collection of rumen bacteria in ruminants as the best-developed and cellulose. Users can work in anaerobic conditions are therefore used in this way were considered as the result was very good.

Yazdi (2009) rope emphasized: microbial fuel cells a new technology for the production of alternative and sustainable energies in which the use of specific bacteria as catalysts for the production of free electrons from agricultural waste and even sewage electric current is produced. The significance of this lack of technology gases and uses renewable organic sources and thus is infinite. He noted: One of the most important sources of renewable cellulosic waste from agricultural and industrial units that many researchers in the University of Ohio and other universities in America and the world to convert the waste compounds can be used to try, but technically and economically difficulties in the enjoyment of them exists. Our innovative approach lies in the composition of chemical energy directly into electrical energy without combustion and thus produces pollutants and high efficiency electricity is produced. This method, especially in areas that were due to natural disasters or in areas having not

connected to the power grid as well as remote control systems that frequent battery replacement difficult or impossible, they can be used.

In the future we as a bio-refinery oil refinery to create that organic waste (agricultural - industrial) and excretion into the water and electricity, hydrogen and methane, which is outside. According to this scholar, innovative fuel cell system can currently up 3.5 watt per cubic meter volume of the anode, producing electricity with the hope that more progress is research about the result, it can increase efficiency.

Cell Two anode and cathode compartment is composed. The anode compartment anaerobic decomposition of cellulose electrons through electron transport chain to transfer outside the cell is rendered. These electrons are then both directly or by a carrier made of graphite electrode in the anode being transferred and from there through an external circuit to the cathode used. While the hydrogen ions from the bacterial metabolism through a selective membrane to the cathode part is transmitted in this section is that aerobic electrons with input from the anode the hydrogen ions and oxygen in the cathode electrode surface it is made from graphite compound and water are formed. Fuel Cell cycle thus formed. He admitted to the microbial fuel cell that already has been designed, said: This is a very important point in this project have been able for the first time electricity we cellulosic compounds, while other groups of very simple compounds such as sugar and starch that can be used both by humans is their use. Yazdi rope at the end about why use of fuel cells and bacteria in microbial fuel cells to the advantage of fuel cells commonly said: Conventional fuel cells use hydrogen as fuel and purpose platinum catalyst to apply directly, but if compounds organics like cellulose in the cell to produce electricity to use, the catalysts have the ability to break them, so bacteria as biological catalysts will be the best option. Such bacteria also have the ability to break down the complex molecules have such, should be able to directly or indirectly generated electrons are transferred to the electrode.

Technology led to the use of bio fuels for the future will be cleaner but the industry should worry about the supply of food available to solve Washington - The biomass of at least 4000 years ago man first wood burns, simple and reliable source for energy supply have been. Today, the urgent need to reduce dependence on fossil fuels, technological advances had paved the way for the use of biomass in the future. Many believe that bio fuels as an important subset of biomass, clean and renewable alternative to fossil fuels in the transport process considered. Many others say that bio fuels such as ethanol derived from grain, agricultural land and provide food in the world would be dangerous to cast and still being applied materials technology much time remains. Any biomass organic waste material, including sawmills, fruit trees and destroyed the forests, agricultural byproducts, and animal feces and human elements of urban and industrial organic waste and many other refers.

From biomass package organic materials in their manufacturing various products such as plastics, polymers, carpets, textiles, detergent, softener materials and oil and fuel needed for transportation is used. Biomass can be contrary to other sources of renewable energy like solar and wind energy, directly to liquid bio fuels such as ethanol and biodiesel into. This fuels some cases the use of biomass that have the fastest growth. According to the Renewable Fuels Association in Washington, the rate of ethanol production in the world in 2006 was about 51.1 billion liters and the countries to reduce oil imports boost rural economies and help air cleanliness, the amount of ethanol is rising. Also expected to increase concerns about emissions and low world oil resources will also increase the production of ethanol.

Bill, a member of the board of American Council of Renewable Energy, told America.gov: ethanol production is increasing rapidly. United States has the largest production in Brazil is ranked second. Other countries developing their own ethanol industry, China, India and Latin America as well as developing and advancing its bio fuels programs are. Food versus fuel ethanol is an alcohol fuel from sugar in cereals such as sorghum and wheat and skin potatoes, rice, sugar cane and sugar beet is obtained. Tom Fawcett, director of technological applications of biomass renewable energy National Laboratory (NREL) United States Department of Energy, told America.gov: Brazilian ethanol from sugar cane to exclusively produce and the United States almost exclusively from corn ethanol to come. University of Arkansas (UA) in research methods to reduce production costs for bio fuel, alternative fuel from vegetable oil, animal fats or algae, renewable fuel that can replace oil with foundation, they are. Students using three methods of combining sound waves for rapid mixing, factor interplay of solid and chemical conditions close to the explosion at elevated temperatures and pressures to improve production of bio fuels to review.

3. Work in Iran

Study the feasibility for plants with fuel waste city with a capacity of 10 MW in cooperation with consulting and Iranian colleague Germans started that is currently available is an action, he existence of more than 400 million tons of residue

agriculture, forestry and waste of livestock, more than 20 million Tons of waste and more than 5 billion cubic meters of sewage in the human potential of this is based on the feasibility study for construction of urban waste-fueled power plant with a capacity of 10 MW in cooperation with a German colleague of Consulting and began and now Action is in hand. In 2004 about 170 thousand hours of electricity from renewal sources production was ranked second after power plants - water is the primary Reviews shows that in the amount of potential energy potential of biomass resources can be obtained from the equivalent of 6 / 15 million crude oil in 2000 is that the rate of 59 percent share of agriculture and forestry, 11 percent share of urban waste, 28 per cent share of livestock waste, urban sewage and 2 percent share of 5 percent share of food. I attempting to compile the atlas for biomass and municipal waste biomass source 4 includes agricultural residue - forests, livestock waste, and waste human and industrial waste has been. The project to identify suitable sites for energy utilization can be obtained from biomass resources, promotion of culture resources in the production of biomass energy and fuel through the recognition of laws and regulations and provide necessary suggestions, technical and economic studies appropriate extraction technologies biomass energy sources and ways to expand their knowledge and capacities and resources of biomass energy extraction runs.

Reference:

1- www.nasimjonoub.com
2- www.cityweb.blogfa.com
3- www.yousefi.us
4- www.azfarshid.blogsky.us
5- http://www.hamshahrionline.ir/News/?id=98001
6- http://news.tavanir.org.ir/press/press_detail.php?id=1961
7- http://www.ksna.ir/engineering/elecrtic/2040--------10---.html

10/12/2010

Optical and mechanical effects of different bleaching regimens on enamel surface

Nagy S *, Zoghbi A M El **, Zaazou MH.*, Abd alsabour K K ***, Taher HA **

*Department of restorative dentistry and dental materials ,national research center; ** Department of operative dentistry, faculty of oral and dental medicine, Cairo University; ***Laser Institute, Cairo university. Corresponding author :mohamedzaazou@yahoo.com

Abstract: Purpose: This study was designed to assess the effect of 3 different bleaching regimens on color and microhardness of enamel. **Materials and Methods**: Ninety bovine upper central incisors were divided into three main groups according to the bleaching regimen used; chemically activated (Viva Style Paint on Plus), photo activated (Zoom 2), and laser activate (Opalescence X-Boost) bleaching agents. Each group was stained with (tea, carbonated beverage or a combination of both tea and carbonated beverage for 1 day or 6 days. Color was assessed using computerized image analysis in terms of grey scale, while Vickers microhardness tester was used to assess change in enamel microhardness. **Results:** Computerized image analysis revealed statistically significant decrease in the mean grey scale value of all teeth immersed in the three staining solutions used. The results also revealed that color change become intense as the immersion time increased. After bleaching with the three bleaching regimens the results revealed increase in the mean grey scale value of all the three bleaching regimens used with statistically significant increase in the mean grey scale value of both photo and laser activated bleaching agents than did chemical activated bleaching agent. Microhardness results revealed that there was statistically significant decrease in enamel microhardness after immersion in the three solutions, where the carbonated beverage group showed the lowest mean microhardness value than did the tea and the combination solutions. After bleaching with the three bleaching regimens enamel revealed a significant decrease in its microhardness. For all groups, no correlation was found between color change of enamel surface and its microhardness. **Conclusion**: Tea and Carbonated beverages have the ability to discolor teeth and alter their microhardness. Different bleaching regimens are lightening the color of discolored teeth but adversely affect enamel microhardnes.

[Nagy S, Zoghbi A M El, Zaazou MH, Abd alsabour KK, Taher HA. **Optical and mechanical effects of different bleaching regimens on enamel surface.** Journal of American Science 2010;6(12):1766-1773]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Optical and mechanical effects of different bleaching regimens on enamel surface

Introduction

Nowadays, esthetic of dentition is of great concern for many individuals seeking dental treatment due to the increase of the patient's awareness of the ability to improve their smile by lightening discolored teeth through few visits to dentists.

Bleaching is considered the most conservative approach to obtain esthetic than aggressive methods such as veneering, crowning or bonding ***(Matis at el, 2000).*** Therefore aesthetic dentistry has turned its attention to develop a series of bleaching techniques and materials to offer the patient a successful pain-free bleaching of both intrinsic and extrinsic stains. These techniques include peroxide based, light-activated, laser-activated or combination therapies. A broad range of peroxide based treatments are currently available including those that are professionally administered (in-office), professionally dispensed (custom-tray based system) and self-directed (over the counter) ***(Zanin et al, 2004).***

Power bleaching is an in-office whitening technique developed to bleach teeth in single office visit with a combination of a whitening agent such as peroxide and an auxiliary light. It has the advantage that all the smile-line teeth are whitened simultaneously ***(Tavares et al, 2003).*** There are several different available types of light sources to accelerate the in office bleaching procedure. The techniques that use coherent or incoherent light sources, have the advantage of being quick and convenient ***(Wetter et al, 2004).***

The mechanism by which teeth are bleached by H2O2 is not completely understood; it is thought that, as the peroxide diffuses into the tooth structure, it may react with organic colored materials found within the tooth structure leading to reduction in color ***(Luk et al, 2004).*** Unfortunately improvement in tooth color may, however, be at the expense of tooth strength.

Thus it was thought that a study aiming at evaluating the effect of different bleaching

regimens on the color change and micro-hardness of enamel surface might be of value.

2. Materials and methods:

2.1. MATERIALS:

2.1.1. TEETH SELECTION AND GROUPING:

Ninety recently extracted sound bovine upper central incisors were selected and stored in isotonic saline (0.2% sodium azide NaN_3) at room temperature until use to avoid bacterial growth and dehydration. The ninety teeth specimens were divided into three main groups of 30 teeth each, according to the bleaching regimen used (B). Where B1 referred to Viva Style paint on plus bleaching agent **(**IvoclarVivadentAG, FL-949schaan/ Liechenstein) B2 referred to photo activated bleaching agent Zoom2 bleaching system (Discus Dental, Inc. Culver City, CA 90232 USA) and B3 referred to Opalescence X-tra Boost + laser activation bleaching gel (Ultradent products, South Jordan, UT, USA).. Each main group was further subdivided into two subgroups, 15 teeth each, according to time of immersion in the staining solutions (T). T1 referred to 1 day and T2 referred to 6 days immersion time in the staining solutions. One day staining was equivalent to one month of three times drinking tea cups or carbonated beverage daily, while six days was equivalent to six month ***(Guler et al, 2005).*** Each subgroup was further subdivided into three classes according to the staining solution (S), where S1 represented tea, S2 represented carbonated beverage and S3 represented a combination of both tea and carbonated beverage one by one respectively.

2.2. Methods:

2.2.1. BLEACHING TREATMENT:

2.2.1.1. Paint on plus bleaching agent 6% hydrogen peroxide (HP):

A thin layer of Viva Style paint on plus bleaching agent was applied to the labial tooth surface using a brush. According to the manufacturer's instructions, this layer was washed out after 10 minutes with water and toothbrush. That procedure was repeated 14 times according to the manufacturer's instructions. Then the specimens were stored in saline till testing.

2.2.1.2. ZOOM 2 photo-activated bleaching agent 25% HP:

The gel was spread on the labial teeth surfaces with the supplied brush for three 15-minutes sessions with the zoom light (short-arc metal halide bulb -wavelength of 365-500 nm). At the end of the treatment the teeth were rinsed with water and each subgroup was stored in a separate bottle filled with normal saline till testing.

2.2.1.3. palescence X-tra Boost 38%HP+laser activation:

The Opalescence X-tra Boost laser activated bleaching agent was applied on the labial surfaces of the teeth for 15 minutes according to the manufactures instructions then the gel was rinsed with water and the teeth was dried with gauze. The bleaching agent was again applied on the labial surface of the teeth and activated with diode laser (wave length 904nm, power 6Wfrequency 500MZ) for 1 minute time exposure. (The diode laser exposure was done in laser technology services center, Cairo University). At the end of laser bleaching the teeth were rinsed with water and each sub-group was stored in a labeled bottle filled with normal saline till testing.

2.2.2. COLOR CHANGE ASSESSMENT:

Each bovine tooth was mounted vertically in a mould of self-cured acrylic resins using Teflon ring with dimension of 2x2cm. Color assessment was made at baseline (control before staining), post-staining and post-bleaching using the grey scale image analyzer software (soft ware image ware, J-13, microsoft, USA). Each tooth was photomicrographed using zoom stereomicroscope (Olympus-SZ-PT,Japan) with charged couple device digital camera(Olympus-DP10,Japan). Four points were randomly selected and measured for each specimen where the mean values of the four points were calculated.

2.2.3. MICRO-HARDNESS MEASUREMENT:

Each bovine tooth was mounted horizontally in a mould of self-cured acrylic resins using Teflon ring with dimension of 3x3cm.The micro-hardness test of enamel surface was made at baseline (control before staining,) after staining and after bleaching. The micro-hardness values of the specimens were determined using a Vickers micro-hardness tester. Three indentations were made with a static load of 200 grams for 20 seconds dwell time. The indentation dimensions were registered. The mean VHN values of the three indentations were calculated.

2.2.4. TATISTICAL ANALYSIS

The recorded data for color change and micro-hardness assessment were collected, tabulated and statistically analyzed using ANOVA and Tukey's tests. Pearson's correlation coefficient was used to determine significant correlation between mean percentage changes in color and microhardness after bleaching with the three different bleaching agents.

3. RESULTS:

3.1. COLOR MEASUREMENTS

3.1.1. Color measurements before (control) and after staining with the staining solutions and after bleaching with the different bleaching gels (Table 1):

Table 1. Mean, standard deviation values, results of ANOVA and Tukey's tests for the effect of the three staining solutions on color and the color change after bleaching with the three bleaching gels:

Bleaching agent	Control		Storage time	Solution / Measurement	Tea		Carbonated beverages		combination		p-value
	Mean	SD			Mean	SD	Mean	SD	Mean	SD	
Paint - on Plus	141.8 [a]	4.5	1 day	After staining	129.5 [b]	1.6	134 [b]	1.9	130.9 [b]	2.3	**<0.001***
	141.8 [a]	4.5		After bleaching	133.4 [c]	2	138.2 [b]	2.6	136.5 [b]	3.4	**0.007***
	141.8 [a]	4.5	6 days	After staining	119.4 [c]	3.2	121.9 [b]	3.3	124.8 [b]	2.6	**<0.001***
	141.8 [a]	4.5		After bleaching	128.4 [b]	4.5	132.4 [b]	3.6	133 [b]	2.3	**<0.001***
Zoom 2+ Photo activation	141.8 [a]	4.5	1 day	After staining	129.1 [c]	1.7	133 [b]	2.9	132.4 [b]	1.9	**<0.001***
	141.8	4.5		After bleaching	138.5	1.7	141.3	0.8	142.6	2.7	**0.148**
	141.8 [a]	4.5	6 days	After staining	116.3 [c]	3.5	121.2 [b]	2	124.1 [b]	3.7	**<0.001***
	141.8	4.5		After bleaching	139.3	4.7	139.1	1.6	139.6	1.9	**0.590**
Opalecence Xtra- boost + laser activation	141.8 [a]	4.5	1day	After staining	128.7 [c]	2	133.4 [b]	3.5	131.1 [b]	1.4	**<0.001***
	141.8	4.5		After bleaching	139.8	0.6	141.7	2.2	141.3	2.2	**0.626**
	141.8 [a]	4.5	6 days	After staining	118.9 [c]	3	122.5 [b]	1.8	124.1 [b]	3.3	**<0.001***
	141.8	4.5		After bleaching	137.9	2.2	137	2.8	139.4	1.9	**0.104**

SD: Standard Deviation, *: Significant at $P \leq 0.05$, Means with different letters are statistically significantly different according to Tukey's test.

3.1.1.1. For Paint on plus:

-1 day storage:

After staining,

Control group showed the statistically significantly highest mean color measurement.

There was no statistically significant difference between staining with tea, carbonated beverage and combination which showed the statistically significantly lowest means.

After bleaching,

Control group showed the statistically significantly highest mean color measurement.

There was no statistically significant difference between staining with tea, carbonated beverage and combination which showed the statistically significantly lowest means.

3.1.1.2. For Zoom2 bleaching:

-1 day storage:

After staining, Control group showed the statistically significantly highest mean color measurement. There was no statistically significant difference between staining with carbonated beverage and combination which showed lower mean values.

Staining with tea showed the statistically significantly lowest mean.

After bleaching, there was no statistically significant difference between the four groups.

-6 days storage:

After staining,

Control group showed the statistically significantly highest mean color measurement.

There was no statistically significant difference between staining with carbonated beverage and combination which showed lower mean values.

Staining with tea showed the statistically significantly lowest mean. After bleaching, there was no statistically significant difference between the four groups.

3.1.1.3. For opalesence extra-boost+ laser-activation bleaching:

-1 day storage:

After staining,

Control group showed the statistically significantly highest mean color measurement.

There was no statistically significant difference between staining with carbonated beverage and combination which showed lower mean values.

Staining with tea showed the statistically significantly lowest mean. After bleaching, there was no statistically significant difference between the four groups.

-6 days storage:

After staining,

Control groupshowed the statistically significantly highest mean color measurement. There was no statistically significant difference between staining with carbonated beverage and combination which showed lower mean values.

Staining with tea showed the statistically significantly lowest mean. After bleaching, there was no statistically significant difference between the four groups.

3.2. MICROHARDNESS RESULTS:

3.2.1. Microhardness measurements before (control) and after staining with the three staining solutions and after bleaching with the three different bleaching gels (Table 2):

Table 2. Mean, standard deviation values, results of ANOVA and Tukey's tests for the microhardness measurments.

bleaching agent	Control		Storage ti	Solution / Measurement	Tea		Carbonated beverages		combination		p-value
	Mean	SD			Mean	SD	Mean	SD	Mean	SD	
Paint on Plus.	135 [a]	7.6	1 day	After staining	132.2 [b]	9.6	124 [c]	7.6	130.6 [b]	5.8	**0.023***
	135 [a]	7.6		After bleaching	115.8 [b]	9.6	111.2 [b]	5.3	108.6 [b]	7	**<0.001***
	135 [a]	7.6	6 days	After staining	131.6 [b]	7.2	121.4 [c]	7.5	128.7 [b]	6.1	**0.034***
	135 [a]	7.6		After bleaching	118.4 [b]	5.9	111.6 [c]	8.2	109.7 [c]	6.3	**<0.001***
Zoom2+ Photo activation	135 [a]	7.6	1 day	After staining	133.1 [a]	4.9	126 [b]	6.6	132.3 [a]	7.5	**<0.001***
	135 [a]	7.6		After bleaching	116.6 [b]	7	89.6 [c]	6	110.6 [b]	5	**0.148**
	135 [a]	7.6	6 days	After staining	131.2 [a]	7.9	125.1 [b]	4.7	131 [a]	8.2	**<0.001***
	135 [a]	7.6		After bleaching	117.4 [b]	5.8	104.2 [c]	5.1	111.4 [b]	7.1	**0.590**
Opalescence Xtra-boost.	135 [a]	7.6	1day	After staining	133.9 [a]	7.2	122.6 [b]	7	131 [a]	6.4	**0.001***
	135 [a]	7.6		After bleaching	120 [b]	5.7	112.8 [c]	6.6	105.1 [c]	7.2	**<0.001***
	135 [a]	7.6	6 days	After staining	133.5 [a]	2.9	120.4 [b]	6.6	130.4 [a]	9.3	**<0.001***
	135 [a]	7.6		After bleaching	119 [b]	7.2	111.7 [c]	7.3	108.2 [c]	9.4	**<0.001***

SD: Standard Deviation, *: Significant at P ≤ 0.05, Means with different letters are statistically significantly different according to Tukey's test.

3.2.1.1.Paint on plus:

1 day storage:

After staining, the highest mean microhardness both tea group (132.2) and combination group (130.6) which showed no statistically significantly difference between them which showed lower mean. The lowest mean microhardness was for carbonated beverage group (124). **After bleaching**, the highest mean microhardness was the control group (135), followed by the other 3 groups, tea group (115.8), carbonated beverage group (111.2) and combination group (108.6) which showed no statistically significantly difference between them which showed lower mean.

6 days storage:

After staining, the highest mean microhardness was the control group (135), followed by both tea group (131.6) and combination group (128.7) which showed no statistically significantly difference between them which showed lower mean. The lowest mean microhardness was for carbonated beverage group (121.4). **After bleaching,** the highest mean microhardness was the control group (135), followed by both tea group (118.4) and combination group (109.7) which showed no statistically significantly difference between them. The lowest mean was for carbonated beverage group (111.6).

3.2.1.2.Zoom2 gel+ photo activation.

1 day storage:

After staining, the highest mean microhardness was the control (135), tea group (133.1) and combination group (132.3) which showed no statistically significantly difference between them. The lowest mean value was for carbonated beverage group (126).

After bleaching,the highest mean microhardness was the control group (135), followed by both tea group (116.6) and combination group (110.6) which showed no statistically significantly difference between them which showed lower mean. The lowest mean microhardness was for carbonated beverage group (89.6).

6 days storage:

After staining, the highest mean microhardness was the control group (135), followed by both tea group (131.2) and

combination group (131) which showed no statistically significantly difference between them The lowest mean was for carbonated beverage group (125.1). **After bleaching**, the highest mean microhardness was the control group (135), followed by both tea group (117.4) and combination group (111.4) which showed no statistically significantly difference between them which showed lower mean. The lowest mean microhardness was for carbonated beverage group (104.2).

3.2.1.3.Alesencextra-boost+Laser activation.

1 day storage:

After staining, the highest mean microhardness was the control group (135), followed by both tea group (133.9) and combination group (131) which showed no statistically significantly difference between them The lowest mean was for carbonated beverage group (122.6).

After bleaching, the highest mean microhardness was the control group (135), followed by tea group (120). Both carbonated beverage (112.8) and combination groups (105.1) showed the lowest mean with no statistically significant difference between them.

6 days storage:

After staining, the highest mean microhardness was the control group (135), tea group (133.5) and combination group (130.4) which showed no statistically significantly difference between them. The lowest mean was for carbonated beverage group (120.4).

After bleaching, the highest mean microhardness was the control group (135), followed by tea group (119). Both carbonated beverage (111.7) and combination groups (108.2) showed the lowest mean with no statistically significant difference between them.

4. DISCUSION

Since the last decade, methods to improve the esthetics of the dentition by tooth whitening were of interest to dentists, their patients and the public. Thus, dental bleaching has become an alternative to change the color of discolored teeth.

In the current study bovine teeth were used as it was practical and suitable because of its large labial surface area and its validity in a sound, non carious form. Bovine teeth were also used in previous studies according to **Adeyemi et al, 2006; Al Salehi et al, 2007**. The results and observations in studies by **Titley et al, 1991 & 1993** utilized human teeth generally support the results and observations recorded for bovine teeth.

In this study, the effects of tea and carbonated beverages on enamel surface were investigated, as they are the most commonly used by the population. Moreover carbonated beverage was chosen as it continues to replace milk and other nutrient-dense foods and beverages in which patients consider it harmless, **Jain et al, 2007; Owens and Kitchens, 2007.**

Three peroxide based bleaching regimens were used in this study. As peroxide based products are effective in achieving a wide range of shade enhancement and are available by professional application in-office or through professional dispensing for daily use (Custem-tray based system) and self-directed (over the counter)**; Bernie et al, 2003; Zanin et al, 2004.**

In this study, chemical activated bleaching agent was tested as it is the most commonly used and available bleaching regimen, **Sulieman et al, 2006; Luo et al, 2007.** In addition photo and laser activated bleaching agents were also tested to accelerate lightening of discolored teeth during chair side treatments, where immediate whitening occurs. This was in accordance with previous studies by **Wetter et al in 2004 and Buchalla and Attin in 2007;** who used Light and laser for the activation of the bleaching agents. In the current study color change and microhardness were measured to assess the effect of the three bleaching regimens used on enamel surface.

Color changes could be assessed visually using shade guide systems and digital photographic means or digitally using colorimeter, spectrophotometer and digital analysis; **Sulieman et al, 2006; Braun et al, 2007; Luo et al, 2007.** However each system has its own limitation. In this study Color change was assessed using computerized image analysis in term of grey scale (ranging from 0= black and 255= white) as it is one of the objective measuring procedure that eliminates the potential for human variability. Microhardness was assessed using Vickers hardness tester as it was used in previous studies by **Unlu et al in 2004 and Al-Salehi et al in 2007**.

Results of computerized image analysis revealed statistically significant decrease in the mean grey scale value of all the teeth immersed in the three solutions used. As the normal color of the teeth is determined by the blue, green and pink tints of the enamel and is reinforced by the yellow through to brown shades of dentine beneath. Direct staining has multifactorial etiology with chromogens derived from dietary sources or habitually placed in the mouth. These organic chromogens are taken up by the enamel pellicle and color imparted is determined by the natural color of the chromogen as stated by

Watts and Addy in 2001. Moreover the chromogens diffuse rapidly into the dentine to saturate binding sites. The diffusion occurs mainly through the dentinal tubule system, although diffusion through inter-tubular dentine is possible and must occur to produce staining in the body of the dentine which is the main cause of showing the translucent enamel discolored. Teeth immersed in tea showed the lowest mean grey scale value this result was in agreement with **Sulieman et al in 2003 and 2005** where they revealed considerable colour changes in all L*a*b* values with the most marked value change in L* indicating tooth darkening. The change in a* and b* were of interest since these moved in the direction of red and yellow, this could be attributed to the polyphenolic chromogens found in tea namely, the theorubigins and theoflavins, which are red and yellow, respectively. As well as results revealed that as the immersion time increased the color change become intense.

After bleaching with the three bleaching regimens results revealed increase in the mean grey scale value of all the three bleaching regimens used. This result is in agreement with **Bartlett, 2001; Reinhard et al, 1993; Matis et al, 2002 & 2003; Zekonis et al, 2003; Joiner et al, 2004; Duschner et al, 2006 and Sulieman et al 2006.** This could be attributed to the fact that when hydrogen peroxide interacts with a tooth, it decomposes into hydroxyl radicals or into water and oxygen molecules, depending on the mechanism of hydrogen peroxide decomposition. The free radicals released are unstable and immediately seek an available target with which they may react. The reaction may decompose organic materials, including stains on enamel, from larger-chained, darker-colored molecules into smaller, shorter-chained, light- colored molecules. In the course of decomposition, a color change occurs on the enamel surface and the decomposed organic materials are dissolved in the hydrogen peroxide solution.

Moreover results of computerized image analysis revealed statistically significant increase in the mean grey scale value of both photo and laser activated bleaching agents than did chemical activated bleaching agent.

This could be attributed to the difference in hydrogen peroxide concentrations between the bleaching agents used where the chemical activated bleaching agent has 6% H2O2 while the photo and laser activated bleaching agents have 25% and 38% H2O2 respectively. These results indicate that bleaching efficiency might be concentration dependant. This result is in agreement with the results of **Matis et al in 2000 and Braun et al in 2007.**

Moreover this may be also attributed to under photo-chemically initiated reactions using light or laser, the formation of hydroxyl radicals from hydrogen peroxide has been shown to increase, by a rise in temperature according to the following equation: H2O2+211 KJ/mol----2HO. This is in accordance with increase in speed of decomposition of a factor of 2.2 for each temperature rise of 10ºc. Due to the increased release of hydroxyl-radicals (thermocatalysis), an increase in efficacy is conceivable. If light is projected onto a bleaching gel a small fraction is absorbed and its energy is converted into heat. Most likely,

this is the main mechanism of action of all light activated bleaching procedures. Moreover the bleaching process is a chemical reaction composed of different factors that determine the rate of the chemical reaction and the increase of the temperature. Concentration of the reactants and intensity of light in a photo-chemical reaction are all proportional to the rate of chemical reaction of the tooth whitening. Similar results have been suggested by **Sun et al in 2000; Luk et al in 2004; Perdigo et al in 2004 and Buchalla et al, 2007.**

However the results suggested by **Zekonis et al in 2003 and Sulieman et al in 2006** were contradicting. This might be to significant variations in the bleaching agents, time of application and color assessment methods used.

Microhardness results revealed that there was statistically significant decrease in enamel microhardness after immersion in the three solutions, where the carbonated beverage group showed the lowest mean microhardness value than did the tea and the combination solutions. This is in agreement with the results of previous studies by **Kim et al in 2001; Willershausen and Dobrick in 2004; Jain et al in 2007; Owens and Kitchens in 2007.** This may be due to the acidic nature of the carbonated beverages that may result in enamel erosion. In addition, there are many other factors affecting the rate of enamel erosion and dissolution, which are the total exposure time that would depend on the actual amount of beverage consumed, the frequency of consumption (that is, if small sips are taken at frequent intervals or the entire can/bottle is consumed quickly), if the consumer uses a straw to drink these beverages (reducing the enamel's exposure as a result), and so forth. This explanation matches that of **Willershausen and Dobrick in 2004; Joiner et al in 2004 and Jain et al in 2007.**

Moreover, microhardness results after bleaching with the three bleaching regimens revealed a significant decrease in enamel microhardness. This is with agreement with

Park et al, 2004; Pinto et al, 2004; Unlu et al, 2004; Junior et al, 1996; Rotstein et al, 1996. This may be attributed to surface degradation, resulting from the complicated oxidation process of free radicals. Since the organic materials (proteins, lipids or dental staining substance) are distributed mainly in the inter-zone of inorganic structures, the removal of such organic materials makes the surface uneven. Hydrogen peroxide can also interact with inorganic materials and dissolves the enamel surface gradually by removing the mineral elements. As the main building block of enamel is hydroxy-apitite crystal that is composed of calcium and phosphorus. Therefore changes in ca/p ratio indicate alteration in the microhardness, since microhardness directly related to the mineral content of enamel.

However, the results suggested by **Joiner et al in 2004; Duschner et al in 2006 and Ferreira et al in 2006;** were contradicting. This can be due to variations in the methodology applied, such as time of exposure, pH of the immersion solution, type of teeth and mainly the storage environment. When the specimens are stored in artificial saliva or exposed to oral environment in situ, no change in the superficial hardness of enamel is observed, considering that saliva presents a large remineralization potential. The enamel contact with the bleaching solution slightly below the critical pH for a short period followed by the contact for a longer period with a hypermineralized solution of artificial saliva seems to be unable to result in demineralization.

For all groups, no correlation was found between color change of enamel surface and its microhardness. This is in agreement with **Basting et al, 2003 and Joiner et al, 2004**. However, other study by **Rodrigues et al in 2005** have shown that the increase in the concentration, duration and frequency of exposure of tooth structure to hydrogen peroxide is directly proportional to the increase in the bleaching action and the associated sequelae such as effect on enamel microhardness.

Nevertheless, in vitro procedures are not necessarily representative of the in vivo situation. By the lack of the positive outward pressure along the dentinal tubules, which in vivo might retard the penetration of any bleaching agent clinically with vital teeth. The use of extracted teeth that were devoid of dentinal fluid also probably allowed the agent to penetrate the tooth more quickly than would be the case clinically as stated by **Sulieman et al, 2003.**

Conclusion:

Tea and carbonated beveraves have the ability to discolor teeth and alter their microhardness. Different bleaching regimens are lightening the color of discolored teeth but adversely affect enamel microhardness.

Enamel microhardness is directly proportional with hydrogen peroxide concentratin in the bleaching agent**.**

5. References:

1. Adeyemi AA, Jarad FD, Pender N, Higham SM: comparison of quantitative light-induced fluorescence (QLF) and digital imaging applied for the detection and quantification of staining and removal on teeth. Journal of Dentistry, 34: 460-466, 2006.
2. Al-Salehi SK, Hatton PV, Woods DJ: The effect of 24h non-stop hydrogen peroxide concentration on bovine enamel and dentine mineral content and microhardness. Journal of Dentistry, vol 35, issue 11:845-850, Nov, 2007.
3. Bartlett D: Bleaching discolored teeth. Dental update, 28: 14-18, 2001..
4. Basting RT, Rodrigues AI, Serra MC: Peroxide bleaching agents on enamel microhardness over time. Journal of American Dental Association , vol 134: 1335-1342, 2003.
5. Bernie KM: Maintaining tooth-whitening results. Journal of practical hygiene, sep-oct, 2003
6. Braun A, Jepsen S, Krause F: Spectrophotometric and visual evaluation of vital tooth bleaching employing different carbamide peroxide concentrations. Dental Materials Journal, 23:165-169, 2007.
7. Buchalla W, Attin T: External bleaching therapy with activation by heat, light or laser-a systemic review. Dental Materials Journal, vol 23,(5): 586-596, 2007.
8. Duschner H, Götz H, White D J, Kozak KM, Zoladz J R: The effects of hydrogen peroxide bleaching strips on tooth surface color, surface microhardness, surface and subsurface ultrastructure, and microchemical (Raman spectroscopic) composition. Journal of Clinical Dentistry, 17 (3):72-8, 2006.
9. Ferreira IA, Lopes GC, Vieira LCC, Araujo E: Effect of hydrogen-peroxide-based home bleaching agents on enamel hardness. Brazilian Journal of Oral Sciences, Vol. 5, No. 18, pp. 1090-1093, July-September 2006.
10. Jain p, Nihill P, Sobkowski J, Agustin MZ: Commercial soft drinks: pH and in vitro dissolution of enamel. General Dentistry, vol 55, issue 2, 2007.
11. Joiner A, thakker C, Cooper Y: Evaluation of a 6% Hydrogen peroxide tooth whitening gel on enamel and dentine micro-hardness in vitro. Journal of Dentistry, 32:27-34, 2004.
12. Jonior A: The bleaching of teeth. Journal of Dentistry, vol.34: 412-419, 2006.

13. Kim JW, Jang KT, Lee SH, Kim CC, Hahn SH, Godoy GF: In vivo rehardening of enamel eroded by a cola drink. ASDC Journal of Dentistry for Children. Mar-Apr; 68 (2):122-142, 2001.
14. Luk K, Tam L, Hubert M: Effect of light energy on peroxide tooth bleaching. Journal of American Dental Association, vol 134:194-201, 2004.
15. Luo W, Westland S, Brunton P, Ellwood R, Pretty IA, Mohan N: Comparison of the ability of different colour indices to assess changes in tooth whiteness. Journal of Dentistry, (35): 109-116, 2007.
16. Matis BA, Mousa HN, Cochran MA, Eckert GJ: Clinical evaluation of bleaching agents of different concentration. Quintessence International, vol 31: 303-310, 2000.
17. Matis BA, Yousef M, Cochran MA, Eckert GJ: Degradation of bleaching gels in vivo as a function of tray design and carbamide peroxide concentration. Operative Dentistry, 27: 12-18, 2002.
18. Owens BN, Kitchens M: The erosive potential of soft drinks on enamel surface substrate: an in vitro scanning electron microscopy investigation. Journal Contemporary Dental Practice, vol 8 (7): 11-2, 2007.
19. Park HJ, Kwon TY, Nam SH, Kim HJ, Kim KH, Kim YJ: Change in bovine enamel after treatment with a 30% hydrogen peroxide bleaching agent. Dental Materials Journal, 23 (4) 517-521, 2004.
20. Perdigo J, Baralieri LM, Arcaci GM: Contemporary trends and techniques in tooth whitening: a review. Practical procedures & Aesthetic Dentistry, 16 (3), 185-192, 2004.
21. Pinto CF, Oliveria RD, Cavalli V, Giannini M: Peroxide bleaching agents effect on enamel surface microhardness, roughness and morphology. Brazilian Oral Research, vol. 18 (4), 2004.
22. Reinhardt JW, Eivins SE, Swift EJ, Denehy GE: A clinical study of night guard vital bleaching. Quintessence International, vol 24, no 6, 1993.
23. Rodrigues JA, Marchi GM, Ambrosano GMB, Heymann HO, Pimenta LAF: Microhardness evaluation of in situ bleaching on human dental enamel using a novel study design. Dental Materials Journal, 21: 1059-1067, 2005.
24. Rotstein I, Dankner E, Goldman A, Meling I, Stabholz A, Zalkind M: Histochemical analysis of dental hard tissues following bleaching. American Association of Endodontics, vol 22, no. 1, January, 1996.
25. Sulieman M, Addy M, Macdonald E, Ress J S: The bleaching depth of a 35% hydrogen peroxide based in-office product. Journal of Dentistry, vol 33: 33-40, 2005.
26. Sulieman M, Addy M, Rees JS: development and evaluation of a method in vitro to study the effectiveness of tooth bleaching. Journal of Dentistry, 31: 415-422, 2003.
27. Sulieman M, Macdonald E, Rees J S, Newcombe RG, Addy M: Tooth bleaching by different concentrations of carbamide peroxide and hydrogen peroxide whitening strips: An in vitro study. Journal of Esthetic and Restorative Dentistry, 18: 93-100, 2006.
28. Sun G: The role of lasers in cosmetic dentistry. Dental clinics of North America, vol 44, no 4, October 2000.
29. Tavares M, Stultz, Newman M, Smith V, Kent R, Carpino E, Goodson JM: Light augments tooth whitening with peroxide. Journal of American Dental Association, vol. 134, February, 2003.
30. Titley KC, Torneck, Ruse,Krmec D: Adhesion of a resin composite to bleached and unbleached human enamel. Journal of Endodontics, vol 19 (3), 1993.
31. Titley KC, Torneck, Smith, Chernecky R, Adibfar A: Scanning electron microscopy observations on the penetration and structure of resin tags in bleached and unbleached bovine enamel. Journal of Endodontics, vol 17, no 2,1991.
32. Unlu N, Combankara F K, Agttinoz C, Ozer F: Effect of home bleaching agents on the microhardness of human enamel and dentin. Journal of oral Rehablitation 31: 57-61, 2004.
33. Watts A, Addy M: Tooth discoloration and staining: a view of literature: British Dental Journal, vol 190 no 6 March, 2001.
34. Wetter NU, Barroso MCS, Pelino JEP: Dental bleaching efficacy with diode laser and LED irradiation: An in vitro study. Laser in Surgery and Medicine, 35: 254 - 258, 2004.
35. Willershausen B, Dobrick BS: in vitro study on dental erosion provoked by various beverages using electron probe microanalysis. European Journal of Medical Research, 9: 432-438, 2004.
36. Zanin F, Junior AB, Marchesan M, Pecora JD: Laser and led external teeth bleaching. Proceeding SPIE (international society for optics and photons) vol 5610, 2004.
37. Zantner C, Schwarzbacha NB, Neumannb K, Kielbassaa AM: Surface microhardness of enamel after different home bleaching procedures. Dental Materials Journal, 23; 243–250, 2007.
38. Zekonis R, Matis BA, Cochran MA, Al Shetri SE, Eckert GJ, Carlson TJ: Clinical evaluation of in-office and at- home bleaching treatments. Operative Dentistry, 28:114-121, 2003.

11/21/2010

Evaluation of Conical Self-tapping One-piece Implants for Immediate Loading of Maxillary Overdentures

Amr Zahran[1], Mona Darhous[2], Mohamed Sherien[3], Tarek El-Nimr[4],
Basma Mostafa[5], Tamer Amir[6]
1. Professor of Periodontology, Cairo University, Cairo, Egypt.
2. Professor and Chairman of the Department of Periodontology, Cairo University, Cairo, Egypt.
3. Professor of Periodontology, Cairo University, Cairo, Egypt.
4. Lecturer of Maxillofacial Surgery, MSA University, Cairo, Egypt.
5. Researcher, Department of Surgery and Oral Medicine, National Research Center, Cairo, Egypt.
6. Researcher, Department of Periodontology, Cairo University, Cairo, Egypt.
Corresponding author: Dr. Basma Mostafa Zaki, BDS, MDS, PhD
Researcher, Department of Surgery and Oral Medicine, National Research Centre,
E-mail: boshta@hotmail.com

Abstract: Studies of maxillary overdentures supported by conventional implants often show a high implant failure rate. It was believed that maxillary implants should be splinted to retain a removable maxillary overdenture in order to maintain osseointegration. **Materials and Methods:** The present study evaluated the clinical performance of new generation of OsteoCare's Midi self-tapping self-drilling one-piece (ball type) implants for the support of maxillary overdentures. Seventy five implants were placed in the anterior maxillary region of 14 patients. A transmucosal flapless procedure was used to place four to six implants for each patient and followed by immediate delivery of an overdenture. The patients were evaluated at 6-month intervals for a follow-up period of 18 months. The clinical criteria to be checked were survival rate, Periotest values, radiographic crestal bone level and patient satisfaction. The **results** showed that 73 implants had successfully osseointegrated as indicated by the clinical and radiographic examinations. Implant survival rate of 97.3% was attested. The accumulated mean marginal bone loss was 0.88mm at the end of the follow-up period. Patients showed a very high degree of satisfaction of the treatment outcome due to the highly improved retention with partial palatal coverage using horse shoe designed maxillary over-dentures. This procedure has many advantages which include implant placement with minimally invasive transmucosal flapless surgery, decreased postoperative pain and a decreased cost of treatment. Single-stage one-piece implant placement, immediate loading, and transmucosal flapless surgery can result in high success rates when proper techniques are utilized with appropriate patient selection. In **conclusion**, the use of the Osteocare's Midi one-piece (ball type) implants is a valid unique simple treatment modality to support maxillary overdentures.
[Amr Zahran, Mona Darhous, Mohamed Sherien, Tarek El-Nimr, Basma Mostafa, Tamer Amir **Evaluation of Conical Self-tapping One-piece Implants for Immediate Loading of Maxillary Overdentures.** Journal of American Science 2010;6(12):1774-1781]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Midi one-piece implants, immediate loading, maxillary overdentures.

1- Introduction

Implants supported overdentures appear to be highly successful in the edentulous mandible, Burns et al (1995); Feine et al (2002); Chiapasco and Gatti (2003); Zahran (2008). In contrast, treatment outcome with maxillary overdentures seems to be less predictable in comparison to other prosthetic indications with conventional implants. To this date, maxillary overdentures have not been adequately addressed in the literature. Over viewing of the few available maxillary over denture case presentations and studies, reveals a low implant survival rate , Jemt and Lekholm (1992); Jemt (1993); Mericske et al (2002).

With the conventional implant designs and the traditional surgical techniques, maxillary implants appear to cause more problems than mandibular implants supporting overdentures in patients with poor bone quality and severely resorbed maxillae , Misch et al (2004).

In some cases, the retention of the upper dentures is difficult while some patients suffer from the big size of the maxillary denture with full palatal coverage that restricts their tongue movements. The placement of implants to support a maxillary overdenture allows for optimal results which include retention, function, phonetics and patient satisfaction ,Cleave (2000).

The traditional original Brånemark's 2-stage protocol initially calls for the submerging of the implants, which remain load-free for a healing period of 3-6 months to ensure successful osseointegration , Branemark et al (1977); Adell et al (1981). The actual need for healing periods of such duration has been greatly questioned because they were determined on empirical basis, De Vasconsellos et al (2006). Many clinicians, however, are unaware that the concept of immediate loading of implants actually began more than 30 years ago , Hahn (2005). For a long period

of time, the success documented for Brånemark's protocol convinced clinicians that this was the only acceptable protocol. On the other hand, earlier results with immediately loaded implants were often unpredictable , Gapski et al (2003).

Recently, the evolution of the science of Dental Implantology yielded technological breakthroughs of the macro- and the micro-design of the dental implants, including improved implant shape, thread patterns and surface treatments that have demonstrably fostered greater primary stability and faster osseointegration , Stanford (2002); Jones and Cochran (2006); Sakoh et al (2006). These modern implants were designed for the immediate loading procedures and were applied to rehabilitate the edentulous mandible with high predictability. In parallel with the recent technical advances of the implant designs, the better understanding of biology had led to shifting towards the minimally invasive or the a traumatic flapless surgical procedures , Al-Ansari and Morris (1998); Hahn (2000); Kan et al (2000); Becker et al (2005); Zahran and Gauld (2007); Zahran (2008). Appropriate patient selection, single-stage surgery, immediate loading, and flapless site preparation are dependable treatment approaches that offer favorable long-term prognosis, Fortin et al (2006).

Nowadays, many clinical studies validate the immediate loading protocols as a viable therapeutic alternative to the original Brånemark's protocol in its appropriate conditions , Misch et al (2004). The ultimate goal of an immediate loading protocol is to reduce the number of surgical interventions and shorten the time frame between surgery and prosthesis delivery, all without compromising the success rate of the procedure , Fortin et al.; (2006).

The use of the Midi one-piece (ball type) implants is a unique simple treatment modality which have been specially designed to support over dentures. They are considered an alternative to the conventional implantation regimen and are ideal for immediate loading in varying bone qualities as well as thin atrophic ridges. They allow minimally invasive trans-mucosal flapless placement and limit the requirement for hard tissue grafting procedures. The conical macro-design of the Midi implants, the special buttress thread pattern and the undersized drilling using one drill result in compression or condensation of the bone with the increase of the initial stability of the implants , Zahran and Gauld (2007); Zahran (2008); Zahran (2008 a).

The aim of the present study was to evaluate the clinical performance of the new generation of self-tapping Midi one-piece (ball type) implants for supporting of maxillary overdentures.

2-Materials and Methods

2.1. Materials:

2.1.1. Subjects:

A total of 14 patients, including 8 males and 6 females, were consecutively included in this study. The average age at the time of implant placement was 60.42 years (range 52-72 years). Six patients were completely edentulous. The other patients had partially edentulous mandibles. All patients were completely edentulous in the maxilla except one patient who has 2 second molars. All the implants were placed well spaced, in the anterior part of the maxilla between the left and right second premolars area to avoid the maxillary sinus.

Table (1): Overview of clinical data of patients and number of implants included in the study.

Patient	Age	Sex	Number of implants	Opposing arch	Comments
1	63	♂	6	Implant supported overdenture	
2	58	♂	6	Partially edentulous	
3	72	♀	6	Implant supported overdenture	
4	52	♂	4	Partially edentulous	
5	54	♀	4	Implant supported overdenture	
6	60	♂	4	Implant supported overdenture	
7	57	♀	6	Implant supported overdenture	1 failed implant
8	62	♂	6	Implant supported overdenture	
9	58	♂	5	Partially edentulous	
10	64	♂	6	Partially edentulous	
11	62	♂	6	Implant supported overdenture	
12	66	♀	6	Partially edentulous	
13	58	♀	4	Implant supported overdenture	1 failed implant
14	60	♀	6	Implant supported overdenture	

All patients had at least 5mm of ridge width for the placement of implants. The ridge width of each patient is evaluated by ridge mapping or by using bone callipers. The patients received Midi implants with diameters of 3.3mm, 3.8mm and 4.3mm and length of 13mm. The patients were thoroughly informed of the immediate loading protocol and of all the risks associated with this type of procedure. They all gave their full informed consent. Clinical evaluation included the ridge

width and shape, the opposite jaw (being partially or completely edentulous with an overdenture) and the occlusal forces. The selected patients were systemically healthy and not heavy smokers.

2.1.2. Implants

The treatment plan for the patients in this study included placement of 4-6 Midi implants in the anterior maxillary area and the premolar region of the alveolus bilaterally. Eight patients received six implants, one patient received five implants and five patients received four implants. The implants were placed in healed bony sites with bone types (D1 to D3). The 75 implants used in the study were OsteoCare's Midi one-piece (ball type) implants (OsteoCare™ Implant System, London, United Kingdom). Midi implants have range of diameters (3.30, 3.80 and 4.30mm) and lengths (10, 13, and 16 mm). The implants have blasted and acid etched surface, and a high load "buttress" thread that has the advantage of allowing maximum bone-to-implant contact. This results in achieving high initial stability in even poor quality bone. The conical macro-design of the Midi implants has the advantage of allowing for the compression and expansion of the site.

Table (2): Implant number, diameter (mm) and length (mm).

Size of implants	Number	Failed Implants
3.3x 13 mm	4	1
3.8x 13 mm	65	1
4.3x13mm	6	0
Total	75	2

2.2. Methods:

2.2.1. Pre-surgery evaluation:

Pre-surgical radiographic evaluation was carried out with panoramic radiographs, periapical radiographs and cone beam volumetric tomography (CBVT) whenever indicated.

The ridge width was evaluated through the diagnostic casts, ridge mapping or directly in the patients' mouth using callipers.

Before surgery, final impressions of the arches were made, and working models were casted. The models were mounted in an articulator after bite registration on occlusal rims for establishing the centric relation. Tooth settings try-in were made and confirmed by the patients.

2.2.2. Surgical Protocol and implant placement (using Flapless trans-mucosal technique):

2.2.2.1.Marking of the drilling sites:

Using a skin marker, marks were made directly onto the patient's dried mucosa covering the alveolar ridge, to determine the drilling positions of the implants as planned from the diagnostic casts and the panoramic radiograph.

2.2.2.2. Site preparation:

The implant surgical procedures were performed under local anesthesia and without sedation. Only one perforation profile drill (1.3mm diameter) was used for site preparation to give needlepoint accuracy for position, angle and depth. The use of saline was paramount when making the perforation. When the drill passed through the mucosa (trans-mucosal), it reached firstly the cortical bone then the cancellous bone. Confirmation of reaching the cancellous bone was achieved via the physical feel; the drilling was harder through the tough cortical plate and became far easier when engaging the softer cancellous bone. Preparation of the osteotomy was shorter than the implant length as Midi implants have a strong self-tapping self-drilling property.

2.2.2.3. Implant Placement:

The implant was removed from its protective pouch and offered to the site. The implant was manually placed after the trans-mucosal site preparation and was rotated clockwise for approximately three revolutions or until the plastic carrier could no longer rotate the implant manually. Then the over-hex driver with the ratchet wrench was used to complete the seating of the implants.

2.2.2.4.Immediate Loading (Same day of implant placement):

The Initial stability (primary fixation) of the Midi implants was carefully checked by the torque wrench to confirm that the initial primary fixation was exceeding 30N/cm which was crucial to start loading.

2.2.2.5.Relief of Denture to Accommodate the Housings:

Holes were made in the denture at the pre-marked locations by using a laboratory bur. The polycarbonate housings were placed on the implants, and were checked to make sure that they were securely seated with full passivity. Try in of the denture was made to check full seating without binding on the housings.

2.2.2.6.Pick-up of the Housing (chair-side pick-up procedures)

Once the spaces for the housings had been relieved, they were filled with self-cured acrylic resin and the denture was placed over the housings. The patient was allowed to bite in centric occlusion.

After setting of the self-cured acrylic resin, all the excess was removed and the denture was trimmed and polished.

2.2.3. Post-operative care:

After the implants placement and delivery of the overdenture, the patients were instructed to consume easily chewable food for 2 months. No preoperative or postoperative antibiotics were prescribed. Analgesics were used when needed.

2.2.4 Post operative follow-ups and evaluation

The patients were evaluated at 6-month intervals for 18 months. The clinical criteria to be checked were survival rate, Periotest values and radiographic crestal bone level.

The following criteria were applied to evaluate implant success:

(1) Absence of clinically detectable mobility when tested with opposing instrument pressure.

(2) No evidence of peri-implant radiolucency on periapical radiographs.

(3) Absence of recurrent or persistent peri-implant infection.

(4) No complaint of pain at the site of treatment.

(5) No complaint of neuropathies or paraesthesia,

(6) Crestal bone loss not exceeding 1.5 mm by the end of first year of functional loading and less than 0.2 mm/year in the ensuing years (according to the criteria proposed by Albrektsson et al.; (1986) up to the 18 months of the follow-up period.

Panoramic and periapical radiographs were obtained at implant insertion and subsequently at 6-month intervals up to 18 months postoperatively to evaluate crestal bone loss. The linear measurement obtained by means of conventional radiographs and indirect digital images evaluated by the Digora software for Windows, version 1.5 (Soredex, Helsinki - Finland), Kawauchi et al (2004).

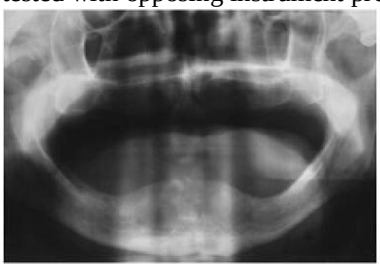


Figure 1a: The preoperative panoramic radiograph of patient no. 1

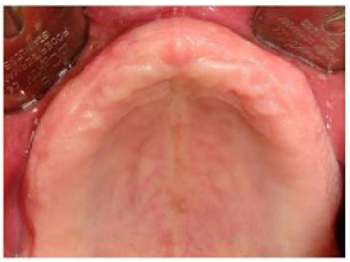


Figure 1b: The clinical picture of the fully edentulous atrophic maxilla of patient no.1

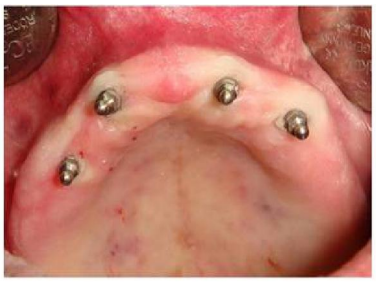


Figure 1c: Immediate postoperative photograph of the placed four Midi implants

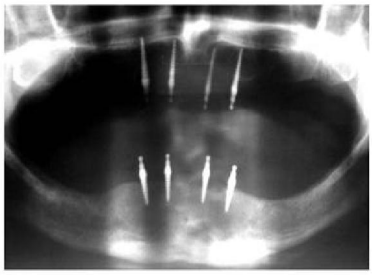


Figure 1d: The immediate postoperative panoramic radiograph

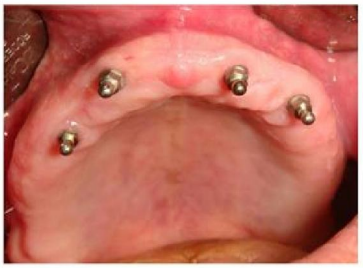


Figure 1e: Clinical aspect at 6 months

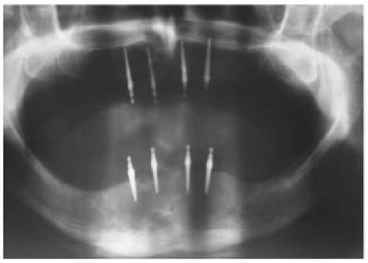


Figure 1f: Panoramic radiograph at 18 months

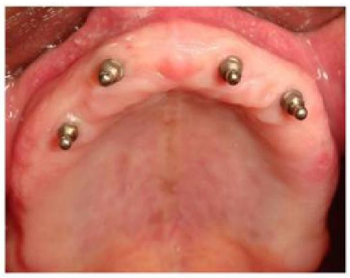


Figure 1g: Clinical aspect at 18 months

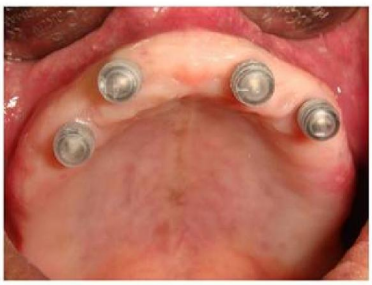


Figure 1h: Placement of the polycarbonate housings on the implants

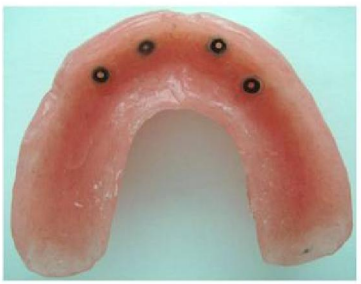


Figure 1i: The finished overdenture with the housings

The Periotest M (Medizintechnik Gulden, Bensheim, Germany) was used to evaluate the clinical stability. Periotest values (PT) of (-8 to 0) were considered the ideal values that denote successful osseointegration.

For the evaluation of the patient satisfaction, questionnaires were filled by the patients at the 6 months follow-up visit. The questions were based on the questionnaire proposed by Brånemark et al (1999).

3. Results:

3.1. Complete soft tissue healing was generally uneventful in all patients within the first 2 weeks after implants placement. The patients reported minimal postoperative swelling or pain experiences with no occurrence of hematoma and minimal need for analgesics. Most patients returned to their normal lives the day following surgery.

3.2. The number of implants placed, status of the opposing arch and the implant diameter and length are summarized in table (1) and (2). All patients demonstrated bone type D2 or D3 as determined by tactile perception during the time of osteotomy preparation and implant placement.

Figure 2a: Immediate postoperative photograph of the placed six Midi implants of patient no. 2

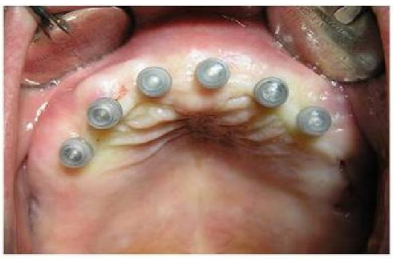


Figure 2b: Placement of the polycarbonate housings on he implants

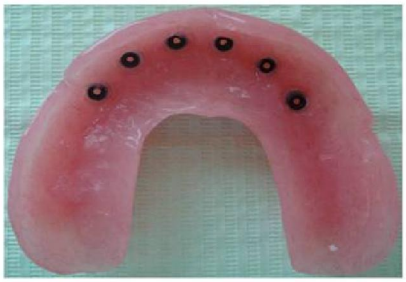


Figure 2c: The finished overdenture with the housings

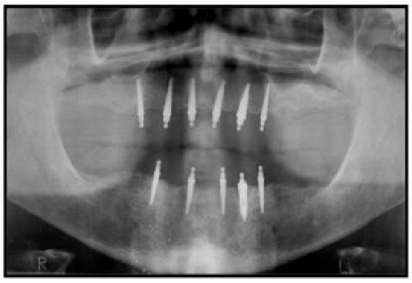


Figure 2d: Panoramic radiograph at 18 months

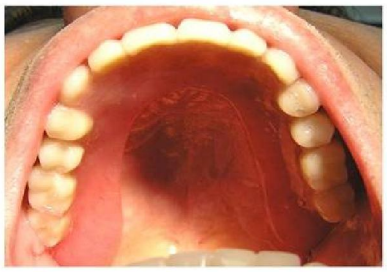


Figure 2e: Immediate delivery of the overdenture

3.3. During the 18 months postoperative follow-up period, all patients showed no postoperative inconveniences. Seventy three Midi implants were successfully osseointegrated as revealed by clinical and radiographic examinations. Implant survival rate of 97.3% was attested.

3.4. The mean marginal bone loss was 0.72mm at 12 months, while it was 0.88mm at 18 months. The mean values of linear radiographic measurements were recorded using digital programs.

3.5. The Periotest values (PT) during the 18 months follow-up period never exceeded a maximum of (PT= 0) and the minimum value was (PT= -5) for all the successfully osseointegrated implants.

3.6. Reviewing of the patient satisfaction questionnaires showed subjective answers that demonstrated a very high degree of satisfaction of the treatment outcome. All patients have verbally indicated their comfort with the horse shoe denture design due to their partial palatal coverage. It

provides them with more room for their tongue and exposes more palatal tissues and improves the feeling of the texture of their food.

4. Discussion

Immediate loading of dental implants is becoming a widespread therapeutic procedure for the rehabilitation of patients with edentulous jaws. In general, patients with completely edentulous maxillary jaws are restored with an implant supported overdenture. They are at the highest risk of occlusal overload for immediate loading protocols when conventional implants are used to support a maxillary overdenture , Jemt and Lekholm (1992); Jemt (1993); Mericske et al (2002).

The high failure rate of the maxillary over dentures supported by conventional implants is related to the inadequate bone volume and the low bone density of the completely edentulous maxilla. Generally, the bone is less dense in the anterior maxilla than the anterior mandible. The maxilla presents very thin porous cortical labial bone and the trabecular bone is usually very fine, Cleave (2000); Misch et al (2004).

Several factors may influence the results of immediate implant loading. These could be divided into the following categories: surgery, host, implant, and occlusion-related factors. Surgical factors consist of primary implant stability and surgical technique. Host factors comprise the quality and quantity of bone, and wound healing. Implant factors include the macro and the micro designs, surface textures, and dimensions of the implant. Occlusal factors involve the quality and quantity of force and prosthetic design ,Gapski (2003).

The 97.3% successful results of the present study illustrated that the new generation of OsteoCare's Midi dental implants present the opportunity to provide patients with a minimally invasive, less costly, less complicated, and less surgically intensive treatment in a high percentage of cases that would be difficult to treat with the current inventory of conventional root-form implants for supporting of maxillary overdentures.

The OsteoCare's Midi one-piece dental implants have a number of unique points that set them apart from their conventional counterparts. There is no similarity between the OsteoCare Midi implants that were placed in this study and the other commercially available conventional implants.

All the 75 Midi implants reached high initial stability over 30 N/cm due to their conical design, buttress threads and the roughened surface (grit blasted and acid etched). Also, the under dimensioned drilling and the bone condensing property of the Midi implants have been used to increase initial stability as well as to improve the bone quality , Zahran and Gauld (2007); Zahran (2008); Zahran (a) 2008).

It was reported that conical implant design in combination with the use of an undersized form drill could lead to higher initial stability than conventional implants, O'Sullivan et al (2000); Sakon et al (2006). Also experimental and clinical studies proved that the implant surface roughness and the thread design are major factors in achieving success with immediate loading Stanford (2002).

The trans-mucosal flapless procedure for placement of the Midi implants resulted in minimal swelling and pain with no occurence of hematoma.

The patients required minimal postoperative medication. The flapless procedure resulted in a very high increase of the patient acceptance and satisfaction of this treatment modality. It was reported that flapless surgery also admits a maintained better blood supply to the marginal bone, thus reducing the likelihood of bone resorption ,Al-Ansari and Morris (1998); Hahn (2000); Kan et al (2000).

Although flapless implant placement is considered a blind surgical procedure, there is a learning curve with every surgical procedure, after which it becomes routine. There are many advantages for the patient as well as for the surgeon, since the procedure is less time consuming, bleeding is minimal, implant placement is expedited, and there is no need to place and remove sutures ,Becker et al (2005).

The one-piece implant design eliminates the need for placing healing collars and makes it possible to avoid manipulation of the soft tissue portion after initial healing. The implant-abutment junction in a two-piece implant design constitutes a structural weakness that may complicate the procedures, Hahn (2005).

The polycarbonate housings with rubber O-rings were successfully used for retention of the overdentures. O-rings possess a number of advantages, including ease of use and maintenance and low cost. The patients were pleased with the function and esthetics of the overdenture O-ring prosthesis. Clinical comparisons of ball and bar designs for mandibular over-dentures revealed a significantly higher number of complications and/or repairs for the bar group, Trakas et al (2006).

Implant retained over-dentures could be considered the treatment of choice for most patients of advanced age who are already denture wearers, Romanos (2004). They have an increased probability of having medical problems such as diabetes mellitus or using anticoagulant therapy, so they need a simple a traumatic surgical protocol as offered by the use of the Mini and Midi implants. Advantages of this procedure include implant placement without any bone augmentation surgery, minimally invasive surgery resulting in virtually no

bleeding, decreased pain and a decreased cost of treatment. Another important advantage is the possibility of removal of the palatal part of the maxillary overdenture that results in having smaller horse shoe designed denture that gives bigger space for the patient's tongue. This will result in improvement of phonetics, taste sensation as well as patient's self confidence.

Conclusion

The use of four to six Midi one-piece (ball type) implants in the maxilla is a feasible treatment option to support maxillary overdentures. These implants have a number of distinct features that set them apart from their conventional counterparts. They allow minimally invasive flapless trans-mucosal placement. Immediate loading is also possible and they are ideal for most types of bone qualities, quantities and for atrophic ridges. They are reliable and cost effective implants that bring secure dentures within the reach of many patients, who are medically or financially compromised. This technique can contribute to a higher degree of implant treatment acceptance due to less discomfort and generally shorter treatment times.

5. References:

1. Adell R., Lekholm U., Rockler B., Branemark P-I. 1981. A 15-year study of osseointegrated implants in the treatment of the edentulous jaw. Int J Oral Surgery; 10: 387–416.
2. Al-Ansari B.H., Morris R.R. 1998. Placement of dental implants without flap surgery: A clinical report. Int J Oral Maxillofac Implants; 13:861-865.
3. Albrektsson T., Zarb G., Worthington P., Eriksson A.R. 1986. The long-term efficacy of currently used dental implants: a review and proposed criteria for success. Int J Oral Maxillofac Implants.; 1:11–25.
4. Becker W., Goldstein M., Becker B.E., Sennerby L. 2005. Minimally invasive flapless implant surgery: A prospective multicenter study. Clin Implant Dent Rel Res; 7(suppl 1): 1-7.
5. Brånemark P-I., Engstrand P., Ohrnell L.O. 1999. Brånemark Novum: A new treatment concept for rehabilitation of the edentulous mandible. Preliminary results from a prospective clinical follow up study. Clin Implant Dent Res; 12: 2-16.
6. Brånemark P-I., Hansson B.O., Adell R., Breine U., Lindström J., Hallen O. , Öhman A. 1977. Osseointegrated implants in the treatment of the edentulous jaw. Experience from a 10-year period. Scandinavian J Plastic and Reconstructive Surgery; 16: 1-132.
7. Burns D.R., Unger J.W., Elswick R.K., Beck D.A. 1995. Prospective clinical evaluation of mandibular implant overdentures. J Prosthet Dent; 73:354-363.
8. Chiapasco M., Gatti C. 2003. Implant-retained mandibular overdentures with immediate loading: a 3- to 8-year prospective study on 328 implants. Clin Implant Dent Relat Res; 5:29-38.
9. Cleave R.J. 2000. Maxillary insufficiency implant and tooth-retained maxillary overdenture: A clinical case report. J Oral Implantol; 26: 29-33.
10. De Vasconsellos D.K., Bottino M.A., Saad P.A. 2006. A new device in immediately loaded implant treatment in the edentulous mandible. Int J Oral Maxillofac Implants; 21:615-622.
11. Feine J.S., Carlsson G.E., Awad M.A. 2002. The McGill consensus statement on overdentures. Mandibular two-implant overdentures as the first choice standard of care for edentulous patients. Gerodontology; 19:3-4.
12. Fortin T., Bosson J.L., Isidori M., Blanchet E. 2006. Effect of flapless surgery on pain experienced in implant placement using an image-guided system. Int J Oral Maxillofac Implants; 21:298-304.
13. Gapski R., Wang H.L., Mascarenhas P., Mascarenhas P., Lang N.P. 2003. Critical review of immediate implant loading. Clin Oral Implants Res;14:515-527.
14. Hahn J. 2000. Single-stage, immediate loading, and flapless surgery. J Oral Implantol; 26: 193-198.
15. Hahn J. 2005. One-piece root-form implants: A return to simplicity. J Oral Implantol; 31:77-84.
16. Jemt T. 1993. Implant treatment in resorbed edentulous upper jaws: a three-year follow up on 70 patients. Clin Oral Implants Res 1993; 4: 187-194.
17. Jemt T. and Lekholm U. 1992.Implant treatment in edentulous maxillae: a 5 year follow- up on patients with different degrees of jaw resorption. Clin Oral Implants Int J Oral Maxillofac Implants; 7: 513-522.
18. Jones A.A., Cochran D.L. 2006. Consequences of implant design. Dent Clin N Am; 50:339-360.
19. Kan J.Y.K., Rungcharassaeng K., Ojano M., Goodacre C.J. 2000.Flapless anterior implant surgery: A surgical and prosthodontic rationale. Pract Periodont Aesthet Dent; 12: 467-474.
20. Kawauchi N., Bullen I., Chinellato L. 2004. Evaluation of the linear measurements by

conventional radiographs and indirect digital images in the endodontic treatment. J Appl Oral Sci; 12(4): 330-336.
21. Mericske-Stern R., Oetterli M., Kiener P. and Mericske E. 2002. A follow-up study of maxillary implants supporting an overdenture: Clinical and radiographic results. Int J Oral Maxillofac Implants; 17: 678-686.
22. Misch C.E., Hahn J., Judy K.W., Lemons J.E., Linkow L.I., Lozada J.L., Mills E., Misch C.M., Salama H., Sharawy M., Testori T., Wang H.L. 2004. Workshop guidelines on immediate loading in implant dentistry. J Oral Implantol; 30: 283-288.
23. O'Sullivan D., Sennerby L., Meredith N. 2000.Measurements comparing the initial stability of five designs of dental implants: A human cadaver study. Clin Implant Dent Relat Res; 2:85-92.
24. Romanos G.E. 2004. Present status of immediate loading of oral implants. J Oral Implantol; 30:189-197.
25. Sakoh J., Wahlmann U., Stender E., Al-Nawas B., Wagner W. 2006. Primary stability of a conical implant and a hybrid, cylindric screw-type implant in vitro. Int J Oral Maxillofac Implants; 21:560-566.
26. Stanford C.M. 2002. Surface modifications of implants. Oral Maxillofacial Surg Clin N Am; 14:39-51.
27. Trakas T., Michalakis K., Kang K., Hiramaya H. 2006. Attachment systems for implant retained overdentures: A literature review. Implant Dent; 15:24-34.
28. Zahran A. (a). 2008. Clinical evaluation of the OsteoCare™'s Midi one-piece implants for immediate loading. Implant Dentistry Today, 2(3): 26-33.
29. Zahran A. 2008. Clinical evaluation of the OsteoCare™'s Mini and Midi implants for immediate loading of mandibular overdentures. Implant Dentistry Today, 2(1): 54-59.
30. Zahran A., Gauld J. 2007.Gauld's technique: Clinical innovation of flapless placement of self-tapping implants with the aid of osteotomes in the posterior maxilla. A case report. Egyptian Dental Journal, 53: 2297: 2304.

11/18/2010

The Journal of American Science

ISSN: 1545-1003

The international academic journal, "***The Journal of American Science*"** (ISSN: 1545-1003)**,** is registered in the United States, and invites you to publish your papers.

Any valuable papers that describe natural phenomena and existence or any reports that convey scientific research and pursuit are welcome, including both natural and social sciences. Papers submitted could be reviews, objective descriptions, research reports, opinions/debates, news, letters, and other types of writings that are nature and science related. All the manuscripts will be processed in a professional peer review. After the peer review, the journal will make the best efforts to publish all the valuable works as soon as possible.

Here is a new avenue to publish your outstanding reports and ideas. Please also help spread this to your colleagues and friends and invite them to contribute papers to the journal. Let's work together to disseminate our research results and our opinions.

Papers in all fields are welcome, including articles of natural science and social science.

Please send your manuscript to editor@americanscience.org;
sciencepub@gmail.com;
americansciencej@gmail.com

For more information, please visit http://www.americanscience.org;
http://www.sciencepub.net; http://www.sciencepub.org

Marsland Press
PO Box 180432, Richmond Hill, New York 11418, USA
The United States
Telephone: 347-321-7172; 718-404-5362; 517-349-2362
Email: editor@americanscience.org;
sciencepub@gmail.com;
americansciencej@gmail.com
Website: http://www.americanscience.org
http://www.sciencepub.net; http://www.sciencepub.org

The Journal of American Science

ISSN 1545-1003

Marsland Press

102-34 92nd Avenue

Queens, New York 11418, USA

Telephone: 347-321-7172

2158 Butternut Drive
Okemos, Michigan 48864
The United States
Telephone: 517-349-2362

Emails: editor@americanscience.org;
americansciencej@gmail.com

Websites: http://www.americanscience.org;
http://www.sciencepub.net

ISSN 1545-1003