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The Journal of American Science

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70	**Effect of Soil Water Content and Salinity on Daily Evaporation from Soil Column** Zhu Xue and Takeo Akae xuezhu_okayama@yahoo.com [Journal of American Science 2010; 6(8):576-580]. (ISSN: 1545-1003). **Keywords:** Non-saline and saline soils; Soil surface temperature; Soil evaporation; Soil water content	Full Text	70

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The Geology and Geochemistry of Metavolcanic Rocks from Artoli Area, Berber Province, Northern Sudan: An Implication for Petrogenetic and Tectonic Setting

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Abstract: The study investigates the geology and the rock geochemistry across an area of about 1250 km2 in the vicinity of Artoli village, Berber Province, Northern Sudan, in order to determine the petrographic characteristics of the rock assemblages, their original protoliths and tectonic environment. Field and laboratory works have revealed that the study area is entirely underlain by crystalline Proterozioc basement complex, which comprises dominantly low-grade schistosed metavolcanic rocks and minor high-grade metasediments, intruded by voluminous granitiod batholiths and covered locally by Tertiary and Recent sediments. The metavolcanics are originally rocks of variable compositions mostly of basic, intermediate to intermediate-acidic volcanic rocks parentage as confirmed by the chemical classification, which classified them as differentiated rocks of andisites, basaltic-andisites with lesser amount of dacites and basalts. The discrimination diagrams constructed enabled to identify the metavolcanic rocks of the area as sub-alkaline volcanic series carrying evolutionary trends of calc-alkaline affinity in a plate tectonic setting related to island arc environment. The overall geological and geochemical characteristics of the Artoli metavolcanic rocks provided essential evidence indicating that the area is a part of the westernmost Nubian Shield, as the features are consistent with the arc accretion models postulated in Sudan, Egypt and Saudi Arabia for the Neoproterozoic evolution of the Arabian-Nubian Shield. [Journal of American Science 2010;6(8):1-13]. (ISSN: 1545-1003).

Keywords: Artoli; Tectonics N Sudan; Artoli Metavolcanics; Geochemistry ANS

1. Introduction

In NE Sudan, there are a number of Precambrian terranes of exposed metamorphic rocks (Fig. 1B); among these is the Artoli area, which constitutes the study area for this research. It lies some 56 km northeast of the River Nile coastal city of Atbara in Berber Province, N Sudan, between latitudes 18º 11´ and 18º 20´ N and longitudes 33º 55´ and 34º 05´ E, (Fig.1A) and covers an area of about 1250 km2.

The area and the adjacent terranes are not only of important academic interest in their own right, but also are related to an evolution of auriferous gold mineralization. Only few regional studies were conducted on this suite of rock series, therefore, documented systematic field, geochemical, and geochronological studies are needed in order to provide a better understanding of the mode and nature of tectonic and geological evolution of the area and its associated ore deposits. In this study, the local geochemical and petrographic details of the metavolcanic rocks integrated with their field observations are provided in an attempt to determine the geochemical affinities and tectonic significance of the area within the Arabian-Nubian Shield and Saharan Metacraton realms.

The Artoli area is located at the southern end of the Keraf-Suture (KSZ) and between the high-grade gneissic terrane of the Bayuda Desert in the west and the spurs of the Red Sea Hills in the east, thus, it is sandwiched between the reworked older crust of Sharan Metacraton and the Neoproterozoic juvenile, accreted arc terrane of the Arabian-Nubian Shield (Kröner, et al., 1987a; and Stern, 1994, Fig.1).

The Saharan Metacraton is a heterogeneous continental crust, containing abundant pre-Neoproterozoic rocks with intense Neoproterozoic remobilization, dominated by high-grade gneisses, migmatites and supracrustal rocks of ensialic geochemical affinities (Kröner, et al., 1987a; Küster and Liégeois, 2001; Abdelsalam, et al., 2002).

The eastern boundary of the Saharan Metacraton in northeastern Sudan is defined by the N–S trending, ophiolite-decorated, 500 km-long and 30-150 km wide structural belt of Keraf- Suture zone (KSZ) (Fig. 1A). This suture has been interpreted as an arc-continental, tectono-lithological suture that resulted from NW–SE oblique collision between the Saharan Metacraton and the Arabian-Nubian Shield. The (KSZ) has rock assemblage comprising; high to medium- grade gneisses, siliciclastics, carbonate-rich low-grade metasediments, ophiolitic nappes,

molasse-type sediments and post-tectonic granitoids, (Abdelsalam et al., 2002).

The Arabian-Nubian Shield (ANS) is the northern sector of the East African Orogeny formed in the Pan-African (1100-500 Ma: Kennedy, 1964; 900-450 Ma: Kröner, 1984). It comprises a vast expanse of Neoproterozoic juvenile oceanic island arc crust extending from southern Israel through western Arabia, eastern Egypt and northeastern Sudan into Eritrea and Ethiopia and bordered on the west and east by older cratonic crust. The arc terranes are believed to be intra-oceanic island arc/back arc-basin complexes (Stern et al., 1994 and Kröner et al, 1987a). The Present widely accepted idea on the geotectonic crustal evolution of the ANS is that it has been developed by lateral crustal growth dominated by subduction-related processes and ensimatic arc construction through preponderate role of repeated plate boundary convergence, formation of intra-oceanic island arc system, subsequent ocean closure, amalgamation of the arc complexes and accretion to continental crust (Stern, 1994).

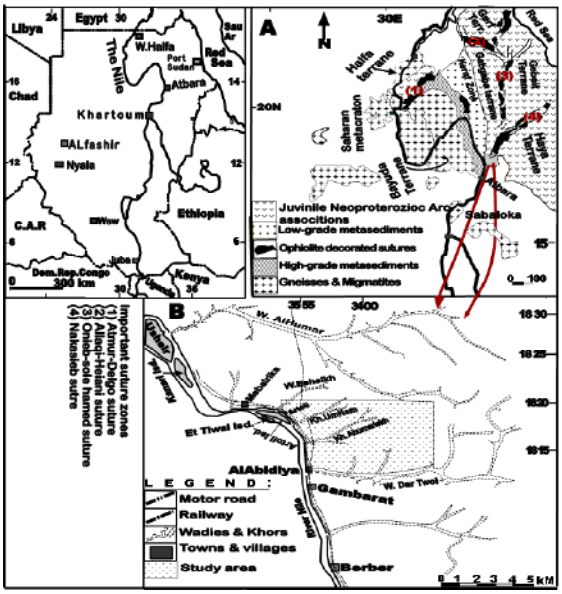

Figure 1. Locality map for; (A): A part of NE Sudan showing major terranes and important suture zones; (B): Artoli area

2. The Geology of the Study Area

The greater part of the Artoli area is underlain by crystalline basement rocks, exposed in most parts of the area except the western most (Fig. 2). The basement rocks comprise a series of spatially overlapping metamorphosed complex of schists and gneisses of volcanic and sedimentary origin cut by various generation of syn, to late-orogenic intrusive grainitiods and post-orogenic minor intrusions, all covered locally by Nubian sands and Recent superficial deposits. The contact relations among the rock types are obliterated by metamorphism and deformation, hence, integration of obvious lithological difference, metamorphic extents, field appearance and structural styles were used to classify the rock succession of the area into the following units (Fig. 2):

2.1 High-grade Metasedimentary Rocks (Paleo-Proterozoic)

Poorly defined and nearly continuous exposures of mostly high-grade metasedimentary rocks develop in the western part of the map area as a local inlier in a narrow belt extending in N-S direction (Fig. 2). The sedimentary origin of this suite, which is obvious in the field by its heterogeneous nature, frequent intercalation feature, and the short-distance facie change, is also confirmed by the geochemical and petrographic data. There is no sufficient field evidences for precise contact relations between this suite ant the other groups, as the underlying group is not exposed and the contacts with the overlying rocks are extensively modified by superimposed deformation and metamorphic events and obliterated by screes and alluvial deposits. However, the suite everywhere studied in the region ascertained to be disconformably overlying the high-grade gneisses and structurally overlain by the Low-grade volcano sedimentary series (Almond, 1982). The suite comprises variably interbedded lithologies of biotite-gneiss, quartzo-feldspathic gneiss, quartzite, marbles and amphibolites.

The biotite-gneiss, in general, is leucocratic to mesocratic, medium-grained rock exhibiting moderate gneissic layering in a NW-SE direction coinciding with the regional trend defined by parallel alignment of biotite and hornblende alternating with elongated quartz and plagioclase grains. Under the microscope the gneiss shows a simple mineral assemblage composing of angular to subangular quartz crystals within predominate orthoclase feldspars and minor plagioclase (Albite- oligoclase composition); both may be altered to turbid sericite. Of the mafic minerals, oriented yellow biotite flakes by far exceed green hornblende, muscovite and garnet. Fe-oxides are unmissed with apatite and opaques as accessories.

The quartzo-feldspathic gneiss is exposed along dry streams with the above unit at the central-west part as rather all-weathered surfaces concealed under variable thickness of alluvial sands and lag deposits. It is medium to coarse-grained, light to grayish and with detectible gneissic banding in a NW trend. Pterographically, consists of quartz, feldspars, yellowish brown biotite, few flakes of muscovite and prisms of hornblende. Secondary minerals of epidote,

sericite and chlorite are commonly developed after mafic minerals. Accessories of apatite, zircon, sphene and iron oxides are also conspicuous.

Quartzitic rocks are encountered in the eastern and the central parts of the map area as relatively outstanding ridges extending in a NE direction for considerable distance probably tracing a linear regional pattern representing fault zone (Fig. 2). The quartzites are compact, brecciated rocks or may be banded (Umtrambeish area), varying from white to ferruginous reddish brown or grey varieties. In thin section, they show abundant stout granular quartz crystals besides, few silvery muscovite flakes, plagioclase and K-feldspars.

Marbles occur in most parts of the area especially in the eastern part as irregular bands or thin layers of lenticular and tabular-shaped bodies, seldom exceed a few meters wide (0.5- 8m). Most marbles are medium to coarse-grained, massive rocks ranging from pure sugary white to impure shaded, dark gray, yellowish brown or buff coloured. However, the pure varieties show granoblastic texture, the sheared ones may show distinct cataclastic textures. 70-80 % of the rock composition is interlocking crystalline calcite with clear twin lamellae and the remaining is mostly equigranular, fine-grain quartz, sericite and plagioclase. Some mica and epidote are accessories.

Sporadic lens-shaped and patches of amphibole-rich rocks are observed in the NW part intercalating with the plutonic suite in a rather crosscutting relation and conformable with the adjacent metasediments. They are generally recognized by their dark to dark gray colour and medium-grained texture, and commonly show megascopic preferred mineral segregation banding in accord with regional trend emphasized by preferred orientation of hornblende prisms and aligned felsic minerals. The amphibolites disclose granoblastic texture and a mineral association comprising; hornblende, plagioclase and quartz as essentials, chlorite, epidote, sericite and biotite as secondary and sphene, apatite, zircon, iron oxides, garnet and pyrite as accessories ones.

2.2 The Ophiolitic Rocks (Neoproterozoic)

The occurrence of numerous dismembered fragments of obducted oceanic crust along generally NE-SW or NNE-SSW trending major belts representing suture boundaries of once existed island–arc system in the Arabian-Nubian Shield (Fig. 1A) became a general agreement among geologist nowadays (Fitches, et al, 1983; Hussein et al., 1984; Abdel Rahman, 1993). Although, Abdel Rahman, 1993, confirmed the occurrence of this suite in the study area but the present account is based merely on few insufficient filed observations, as the suite mostly lies outside the mapped area.

The term ophiolitic suite used here refers to an integrated assemblage of altered rocks found a long pinching and swelling belt (thrust bounded sheared zone) within the low-grade sequence. They represented by carbonated and serpentinized ultramafic tectonic sequence (pyroxinite and peridoditre) and retrogressed cumulous metabasic rocks mainly gabbros and banded amphibolites accompanied by altered sheeted doleritic dykes, lavas and deep sea-water sediments (Phyllitic rocks, chert and iron-rich quartzite) which may represent scattered ophiolitic fragments.

The mentioned lithologies are neither found in one stratigraphic unit nor as pure compositional varieties. The mafic-ultramafic rocks are found at scattered localities southeast and east Umtrambiesh area as strongly altered rocks of talc, talc-chlorite and chlorite-actinolite schists and serpentinites. Sheeted dykes and lavas are of great scarcity, as only scattered minute lensiod pods of lava and very small and highly altered sheets of doleritic composition found just southeast the bend of Wadi Dar Tawai (fig. 2).

2.3 Low-Grade Schistosed Metavolcanics Rocks (Neoproterozoic)

In the study area, a wide distribution of low-grade, green schist facies rocks that are predominant metavolcanics associated with relatively minor sedimentary units encountered exposed in the neighborhood of Umtrambeish ore field, in addition to small sporadic outcrops in the granitoids plain to the north (fig. 2). This group of rocks usually has a gradational boundary with the underlying units and includes metamorphosed basic to intermediate-acidic volcanic rocks that show varying degrees of deformation, ranging from massive, undeformed bodies to strongly schistosed rocks. Most of them are fine-grained with primary volcanic textures (porphyritic and sometimes amygdaloid) still recognizable. Under the microscope, most rocks contain quartz, sericitized plagioclase, chlorite and actinolite, besides K-feldspar, opaques, calcite and some rare relict pyroxene. This mineral assemblage and the shown textures are indicative features of green-schist facies metamorphism of originally volcanic rocks. Based on field and petrographic data, theses schistosed rocks are classified into; quartz-mica schist, sericite-chlorite schist and actinolite-schist.

The quartz-mica schists characterized by clear microfolds, crenulation cleavages, nearly vertical dips and well defined schistosity. They are gray to light-greenish gray coloured rocks of fine

texture and often disclose a typical low-grade mineral assemblage comprising; quartz, chlorite, muscovite, minor biotite, and subordinate sericite, plagioclase, epidote and calcite with common accessories of sphene, apatite, iron-oxides and garnet.

The sericite-chlorite schist is greenish to greenish-gray coloured rock of fine-grain texture and exhibits profound schistosity. Minerogically, it shows lepidoblastic to garnoblastic appearance in sericite, chlorite, plagioclase, quartz and minor biotite, besides epidote, iron oxides and calcite with accessory minerals of apatite, and pyrite.

The actinolite-schists are fine-gained green-coloured rocks found predominantly around Umtrambeish ore area. Under the microscope, they show almost a complete alteration testified by the high abundance of green minerals. The mineral assemblage is a combination of irregularly oriented bright green actinolite, elongated and highly altered plagioclase, dragged crystals of quartz, aggregated epidote, turbid and anhederal crystals of calcite, small, spindle-shaped granules of sphene and rounded apatite.

2.4 Syn, to Late-Orogenic Grainitiod Rocks (Neoproterozoic)

Vast masses of intermediate to acidic granitoids constitute a characteristic and dominant element of the basement rocks of the area, especially in the N and NE sectors, where they form about 50 % of the outcrops. This granitoid suite is believed to be a product of larger plutons of syn, to late-orogenic igneous activities in the late Proterozioc time that have been emplaced in both the high and low-grade sequences as evident from their xenolithic contents. Based on field and petrographic evidences, they are generally range in composition from quartz diorite, granites to micro-granites, but predominantly are hornblende granodiorites (Fig. 2).

Quartz dioritic rocks occur as excellent exposures of low to moderate relief in the NW part of the mapped area. Macroscopically, they are gray coloured rocks usually devoid of pervasive foliation, but the intense deformation caused some verities to develop a slight banding. Petrographically, they are coarse hypidiomorphic rocks in which felsic minerals constituent more than 60 %, of which quartz form about 10 %, the other minerals are slightly sericitized plagioclase, coarse subhedral hornblende, as main primaries, oriented pale-brown pleochoric biotite, mostly untwined orthoclase and perthites as minor phase and sphene, zircon, apatite, pyrite and magnetite as accessories. Secondary minerals are sericite, chlorite, epidote and carbonates.

Granodiorites constitute a wide range of rocks found in association with minor granites in the central part of area (Fig. 2). The rocks are medium to coarse-grained, gray to grayish dark in colour and mostly altered and deformed types to the extent that all gradation from the moderately massive to completely foliated types exist. Microscopic observation revealed coarse hypidiomorphic granular texture and disclosed main mineral phases in order of decreasing abundance; extremely altered (to sericite and chlorite) plagioclase, pleochoric prisms of hornblende, quartz (form about 10 %), oriented pale to dark-brown pleochoric biotite, sericite, chlorite, small columnar epidote, highly sericitized and discontinuously zoned k-feldspar, perthites, opaques, sphene, hexagonal apatite, actionlite, zircon and kaolin.

Granites and few micro-granites constitute only minor phases within the granodiorite sequence and believed to be emplacement products of the last intrusion phase to which the area was subjected. The granites are coarse to medium-grained, light to gray rocks; most of them are intensely deformed and sheared to the extent of foliation and partial destruction of the granitic features. Under the microscope, they show a porphyritic texture formed by pink K-feldspar phenocrysts in affine cloudy quartz and feldspar matrix associated with lesser amount of mafic minerals; sub-hedral flakey, brown biotite, green hornblende, chlorite, epidote and sericite and accessories of zircon, apatite and iron oxides.

2.5 Post-Orogenic Minor Intrusive rocks (Paleozoic / Early Mesozoic)

Commonly numerous quartz veins and lesser amounts of pegmatitic bodies together with scarce basic an acidic dykes are observed invading the country rocks throughout the area. Occurrence of these bodies with the associated alteration features suggests an intense hydrothermal activity. They are strongly deformed, broadly discordant, and irregular or lensiod bodies maintaining a common sinuous feature expressed mainly through swinging along N-S and NE-SW directions with steep dip westwards. In the field, the pegmatites seem to be older than most generations of quartz veins as they found terminating against some quartz veins.

The pegmatites are very coarse-grained massive rocks made up essentially of aggregates of coarse crystals of alkali-feldspar (orthoclase and microcline), quartz, some plagioclase, few mica flakes, tourmaline and apatite.

A number of scattered acidic and basic dykes are found cutting the different units of the basement complex. They are generally short and narrow bodies (0.5-1.0 m in width and rarely traceable for more than 3 m) occurring in contrast

colour with host rocks. Lithologically, typical granitic dykes (aplitic and granophyric) predominate, though dark and fine-grained basic dikes are also present.

The quartz veins represent an important episode in the history of the area since their emplacement was connected with the hydrothermal activities that brought about the gold mineralization. They are of variable sizes ranging from stringers, pods and narrow veins up to wide ones. A close examination reveals that more than a generation exists in the area; (1) the first one comprises early and widespread veins that are mainly concordant, deformed and folded with enclosing rocks (more than 200 m in length, few cm to 3 m width). The veins of this type exhibit varying colours, white, grey, milky, smoky, yellowish, brown, reddish or stained greenish depending on weather the quartz is pure, contaminated or stained by iron oxides or malachite. This type seems to be introduced along major structures and older shear zones. The veins are characterized by pinching and swell feature laterally and vertically and are associated with the main gold mineralization in Umtrambiesh area. (2) The second generation represents a younger phase of less abundant veins with more wider dimensions compared with the first generation, found striking in NW-SE or E-W direction, and occur as discontinuous, crashed and patchy bodies. This type of veins is often barren clean whitish quartz with some tourmaline crystals. Some smoky, gray and pink types are present.

2.6 The Sedimentary Cover (Mesozoic - Recent)

Sedimentary rocks assigned to the formally Nubian Sandstone Formation occur in the area as local outlier chiefly confined to a narrow land-strip stretched parallel to the eastern bank of the Nile. Most surface spreads of the formation are deeply covered with superficial deposits and loose rock screes. The sediments as else where, in he country, comprise undeformed, unmetamorphosed and bedded sequence that uncomfortably overlie the basement complex in a flat-lying attitude. Field and petrography revealed that the greater bulk of these sediments consist of medium to coarse-grained and variously cemented sandy facies of multi-colours (white, gray, dark, yellowish and brown), the coarser verities are always occupy the basal parts in fining upward sequence with rare true conglomerates.

Recent/Pleistocene superficial deposits uncomfortably overlie the above sediments and the underlying basement rocks. Gray to grayish dark heavy overburden of sandy clay and silty mud cover the Nile islands and the flood plains bordering it. The present-day beds of the dried-up streams have their floors covered by thin reddish to dark grayish loams and sandy clays of flush plain type mostly, coarse remnants of water-worn sediments comprising sub-angular to sub-rounded gravels.

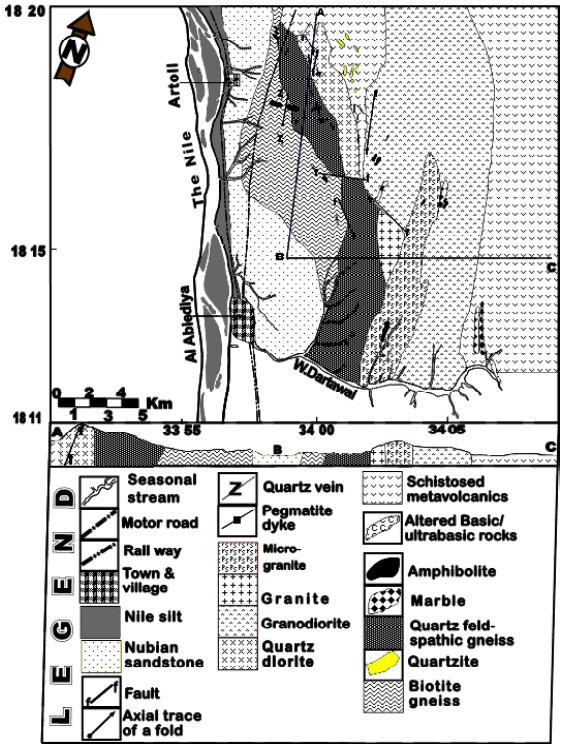

Figure 2. Geological map of the study area.

3. Material and Methods

Several representative samples encompassing the compositional and spatial range of metavolcanic rocks were collected during surface mapping from the Artoli area. Thirty-two samples were petrographically selected for whole-rock chemical analyses.

The samples were submitted through Rida Mining Company, Sudan to the ACME Analytical Laboratories, Vancouver, Canada for analytical work and calibration against international standards. The analytical results are listed in tables 1 and 2.

Whole rock element compositions were determined using inductively coupled plasma-atomic emission spectroscopy (ICP-ES) technique at ACME Analytical Laboratories, after lithium metaborate /tetraborate fusion and dilute nitric acid digestion of rock powder. Replicate analyses for some major and trace elements for some key samples were carried out by X-ray fluorescence spectrometry technique (XRF) following standard techniques and using a Phillips Venus 200 XRF instrument at the analytical laboratories of the Geological Research Authority of the Sudan (GRAS).

All major element values cited in table 1, and used in plots, were recalculated to 100 % on an anhydrous

basis. Loss on ignition (LOI) was determined from total weight loss after repeated ignition of the powdered samples at 100 °C for 1 hour and cooling. Satisfying analytical accuracy was achieved by using replicate analyses and compared with rock standards.

4. Rock Geochemistry and Tectonic Setting

The major, trace and rare earth elements abundance of the 32 whole-rock samples of the rocks believed to be metavolcanic from the area are shown in tables 1 and 2. These data are used in construction of several discrimination and variation diagrams in order to classify the rocks, decipher their geochemical nature and depict the tectonic setting of the area.

For specification of suitable discrimination and variation diagrams to be used, field and petrographic evidences were utilized in combination with geochemical data screening through TiO_2 versus SiO_2 diagram of Winchester and Max, 1984 and P_2O_5/TiO_2 vs. MgO/CaO diagram after Werner, 1987, both discriminated the rocks as of magmatic origin, (Fig. 3).

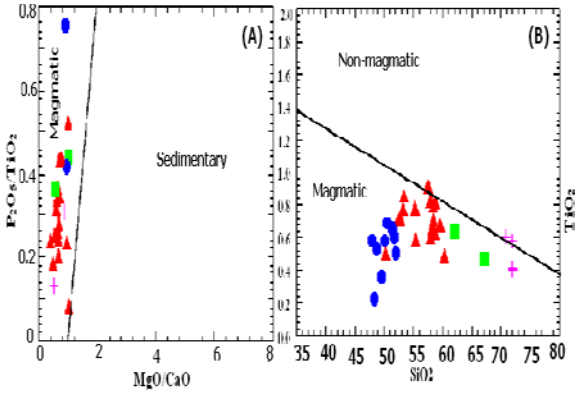

Figure 3. (A): P_2O_5 / TiO_2 vs. MgO / CaO diagram after Werner (1987); (B): SiO_2 vs. TiO_2 after Winchester and Max, 1984, diagram for discriminating between magmatic and non-magmatic rocks. *(Symbols: Closed triangle red ≈ Basalt, closed circle blue ≈ Andesite, closed cubic box green ≈ Dacite and cross pink ≈ Rhyodacite)*

4.1 Chemical Alteration and Element Mobility

In fact, all analyzed samples from the studied units are altered to some extent due to the low-grade metamorphism and/or extensive deformations, as reflected in the concentrations of the more mobile elements and the moderate loss on ignition (LOI) values (Table 1). Thus, it is likely that the present-day geochemical signature of these rocks may not be the same as the protoliths at the time of formation (Irvine and Green, 1976; Alfred and Michael, 1989**).** Therefore, and in order to elucidate the possible alteration effects of metamorphism and deformation, element mobility is noticed through plotting major and selected trace elements against Silica (SiO_2 wt %), Harker variation diagrams (Fig. 4), which show that compatible major oxides (MgO, TiO_2, Fe_2O_3t, MnO, Al_2O_3), P_2O_5 and CaO have normal, correlated and continuous differentiation trends, as they tend to decrease systematically with SiO_2 increases, (This suggests olivine, pyroxene, magnetite, and calcic plagioclase were major fractionating phases during evolution of the magma).

Sr, Ni, Zr, Y, Ba and Cr demonstrate no clear variation with SiO_2, where they remain nearly unchanged particularly in the acid varieties, thus they were probably immobile during metamorphism and other alteration processes. Some trace elements show slight gradual increase in their contents up to a maximum value of approximately 60 wt. % SiO_2 after which a decrease occurs with increasing SiO_2. It is evident from the figures 4-9 that, in spite of the low-grade metamorphism and deformation, some major and trace element signatures can be used for deciphering the original protoliths and tectonic environment.

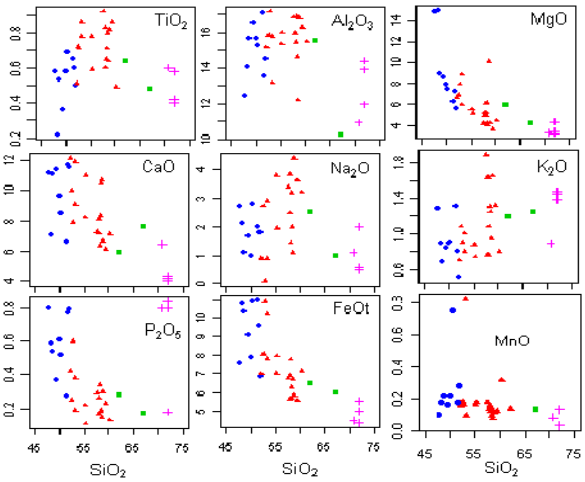

Figure 4. Harker variation diagrams; silica (SiO_2 wt %) plotted against a range of major (in wt %) for the metavolcanic rocks of the study area. *(Symbols: as in Figure 3)*

4.2 Major Element Characteristics

The major element chemistry data used with selected immobile trace and rare earth elements showed indicative characteristics, in spite of their known limited validity in classification schemes for altered and metamorphosed volcanic rocks (Alfred and Michael, 1989**)**.

Some major element concentrations span a wide compositional range (table 1), with silica SiO_2 (47.58 - 72 wt %), MgO (3.33 – 8.82 wt %) and MnO (0.03 - 0.82 wt %) suggesting diverse protolith.

Table 1. Major Elements Data of the Metavolcanic Rocks from Artoli Area

Sample	SiO_2	TiO_2	Al_2O_3	Fe_2O_3	MnO	MgO	CaO	Na_2O	K_2O	P_2O_5	L.O.I	Total
Amv1	70.78	0.60	10.93	4.96	0.08	3.33	6.44	1.11	0.89	0.80	1.5	101.42
Amv2	52.79	0.71	17.14	12.13	0.16	7.96	7.89	0.09	0.90	0.60	1.0	101.37
Amv3	72.00	0.40	14.39	4.85	0.03	3.23	4.19	0.59	1.39	0.84	1.6	103.51
Amv4	51.98	0.50	14.49	7.62	0.28	5.59	11.5	1.79	0.50	0.79	3.9	98.940
Amv5	51.70	0.60	13.50	10.6	0.17	7.29	11.71	1.77	0.80	0.77	1.5	100.41
Amv6	48.33	0.22	14.02	12.01	0.17	14.98	7.07	2.09	0.90	0.59	2.09	102.47
Amv7	49.58	0.36	16.46	10.10	0.16	8.53	11.37	1.65	0.84	0.37	1.9	101.32
Amv8	60.29	0.49	15.47	7.92	0.31	4.42	7.12	3.19	0.80	0.13	0.9	101.04
Amv9	53.28	0.86	13.15	11.36	0.09	8.82	11.88	0.90	0.80	0.38	0.8	102.32
Amv10	52.17	0.51	15.14	7.75	0.15	6.65	12.1	0.89	0.70	0.42	3.9	100.38
Amv11	50.10	0.58	15.64	8.77	0.22	7.91	9.66	0.98	0.90	0.61	3.5	98.870
Amv12	59.45	0.86	16.25	6.18	0.12	3.63	6.11	3.60	1.32	0.23	2.1	99.850
Amv13	52.50	0.72	15.83	8.98	0.18	6.88	9.98	2.72	1.08	0.25	0.6	99.720
Amv14	62.13	0.64	15.51	7.18	0.13	5.95	5.93	2.52	1.19	0.28	1.3	102.76
Amv15	55.18	0.78	15.35	7.80	0.16	5.53	8.21	3.79	0.88	0.22	1.3	99.200
Amv16	50.46	0.69	15.24	12.15	0.75	7.51	8.49	2.79	0.91	0.52	1.2	100.71
Amv17	58.58	0.64	12.18	7.43	0.07	10.12	10.7	1.10	0.95	0.15	0.8	102.72
Amv18	58.76	0.82	16.81	6.49	0.11	4.19	6.67	4.37	1.65	0.30	1.2	101.37
Amv19	47.90	0.58	12.42	8.43	0.10	14.82	11.15	2.69	1.29	0.80	0.5	100.68
Amv20	53.25	0.78	15.78	8.69	0.82	5.96	9.11	2.89	1.00	0.19	1.2	99.670
Amv21	72.00	0.42	11.96	6.14	0.03	4.33	4.31	0.50	1.45	0.80	0.5	102.44
Amv22	58.10	0.65	15.45	8.66	0.12	6.15	6.35	1.43	1.08	0.34	1.0	99.330
Amv23	58.34	0.71	16.84	6.98	0.13	4.19	8.32	3.17	1.31	0.18	0.3	100.47
Amv24	58.42	0.80	14.60	6.31	0.09	3.97	10.45	1.97	1.25	0.19	2.2	100.25
Amv25	67.13	0.48	10.27	6.64	0.13	4.23	7.60	1.00	1.25	0.17	2.6	101.50
Amv26	51.49	0.65	17.04	12.21	0.17	6.23	6.59	2.00	1.31	0.27	1.4	99.360
Amv27	72.00	0.58	13.89	5.55	0.13	3.39	4.01	1.99	1.48	0.18	0.9	104.10
Amv28	57.55	0.92	16.83	7.49	0.17	4.18	7.27	3.38	1.89	0.29	0.7	100.67
Amv29	55.33	0.59	16.00	8.86	0.17	5.12	10.97	1.93	0.74	0.11	1.0	100.82
Amv30	57.92	0.60	15.96	6.25	0.15	4.93	7.14	3.81	1.63	0.26	2.9	101.55
Amv31	48.69	0.53	15.64	11.51	0.22	8.93	11.07	1.10	0.69	0.54	1.3	100.22
Amv32	57.91	0.83	15.97	7.65	0.13	5.18	8.08	2.48	0.76	0.17	1.2	100.36

Table 2. Selected Trace and REE Abundances of the Metavolcanic Rocks from Artoli area

(The Sample of "-"symbol ≈ not analyzed)

Sample	Ni	Sc	Nb	Sr	Zr	Y	Ba	Hf	Cr	Th	La	Ce
Amv1	10	21	5	368	167	28	99	2.5	185	0.8	24.2	41
Amv3	10	15	9	223	115	16	129	1.5	435	2.1	25	35.9
Amv5	21	32	3	197	67	21	135	0.7	417	2.3	14.4	28.8
Amv7	53	45	6	134	63	14	114	1.9	418	1.23	17	36.98
Amv8	72	22	4	490	105	22	138	2.5	265	1.4	"-"	"-"
Amv9	10	22	8	443	124	29	102	2.2	287	2.2	32	49
Amv12	40.4	16	9	485	160	23	520	4.2	88	1.9	23.4	45
Amv13	65.1	18	4.8	522.7	66.5	20	282	3.7	183.4	1.3	"-"	"-"
Amv14	48	17	4	510	175	30	480	2.1	239	1.8	"-"	"-"
Amv15	93	6	6	365	85.7	22.1	231	2.17	226	3.1	10.1	20.5
Amv16	14	10	6	382	147	26	123	3.4	293	0.9	"-"	"-"
Amv17	21	22	4	398	175	24	350	2.7	279	2.3	"-"	"-"
Amv18	70.2	10	9.3	588.9	129	20	419	3.7	137	2.9	16	33
Amv19	12	12	5	222	129	32	370	1.9	298	1.3	"-"	"-"
Amv20	90.5	20	7	413	195	24	551	2.26	166	2.8	15.5	27.3
Amv21	35	12	6	225	97	29	256.4	0.98	189	0.7	"-"	"-"
Amv22	18	8	9	551	127	16	190	1.9	276	1.2	"-"	"-"
Amv23	22.3	26	6.3	268	95	24	193	2.3	53	2.4	12	25.3
Amv24	16	11	8	512	128	16	241	2.7	298	0.25	"-"	"-"
Amv25	17	13	6	395	115	21	248	1.6	389	1	"-"	"-"
Amv26	15	18	5	119	112	20	172	2.1	410	1.9	23.4	38.4
Amv27	17	7	9	501	185	41	187	1.7	418	0.9	"-"	"-"
Amv28	90	14	5.3	446	113	22.5	455.4	3.1	70.6	2.9	13.9	27.4
Amv29	30.1	13	2.9	179.8	87	15	118	0.9	78	0.82	20.3	35.6
Amv30	82.5	43	9	790	106.8	26.6	589.2	3.2	149	2.9	32	42.6
Amv31	12	10	7	248	52	18	122	1.8	319	1.6	"-"	"-"
Amv32	78.7	26	4.15	193.4	62	18.5	100.4	1.9	277	1.2	25.8	42.7

Table 2—Continued

Sample	Pr	Nd	Sm	Eu	Gd	Tb	Dy	Ho	Er	Tm	Yb	Lu
Amv1	6.1		4.3	0.95	3.45	0.5	2.9	0.61	1.8	0.3	2.15	0.4
Amv3	5.6	23.9	4.5	1.2	2.67	0.36	2.79	0.57	1.6	0.41	2.4	0.39
Amv5	3.55	14.3	3.61	1.1	2.7	0.4	2.6	0.5	1.8	0.3	2.3	0.37
Amv7	4.61	18.4	3.9	1.2	3	0.62	3.21	0.69	2.1	0.3	2.1	0.3
Amv9	7.68	28	7.1	1.6	5.1	0.71	2.3	0.4	1.3	0.29	1.2	0.22
Amv12	6.7	26.2	5.5	1.3	3.9	0.7	4	0.65	2.4	0.31	2.2	0.3
Amv15	2	12	3	1	3.1	0.4	3.8	0.6	2	0.24	2.4	0.45
Amv18	4.2	17.8	4	0.86	3.6	0.69	3.9	0.7	1.9	0.3	1.3	0.4
Amv20	2.8	15.9	3.7	1.2	3.6	0.7	3.2	0.8	2.4	0.4	2.1	0.32
Amv23	2.7	12.8	3.1	0.9	3.4	0.6	3.5	0.9	2.2	0.31	2.3	0.35
Amv26	4.8	14	5.1	1.1	4.5	0.6	4	0.7	1.7	0.4	2.3	0.41
Amv28	3.1	15.7	3.7	1.4	3.8	0.51	3.7	0.8	2.3	0.34	2.4	0.4
Amv29	4.7	14.6	2.3	0.8	1.8	0.32	2.1	0.5	1.4	0.2	1.6	0.25
Amv30	6.5	28	7.1	1.5	4.1	0.7	3.4	0.81	1.9	0.36	1.7	0.35
Amv32	5.3	16.6	2.3	0.73	2.4	0.32	2.3	0.45	1.78	0.3	2.2	0.34

Most samples show high-alumina (Al_2O_3) contents from 10.27 wt % up to 17 wt %, (mean 15.41wt %) values similar to those of calc-alkaline series rocks. Iron content (Fe_2O_3) seems to be relatively high ranging between 4.85 and 12.21 wt %, but only few samples have Fe_2O_3 contents > 12 wt % and Fe_2O_3/MgO range < 2. The high iron contents appear to reflect an abundant secondary opaque phase (magnetite?). TiO_2 values are generally < 1 %, therefore they are in the range accepted in calc-alkaline lavas (Irvine and Baragar, 1971; Pearce and Cann, 1973).

The majority of samples show relative high abundance of CaO ranging between 4.19 to less than 12 wt % which may be related to the presence of epidote and the non-intensive metamorphism (Jakes and White, 1972).

The abundances of Na_2O (mostly 0.59 – 4.37 wt %), K_2O (0.5 – 1.89 wt %), P_2O_5 (0.11–0.84 wt. %) and the Na_2O/K_2O (mostly between 1 and 3) are low compared to typical values of Jakes and White, 1971 for calc-alkaline rocks, this can surely be attributed to a loss during alteration and metamorphism. The ratios of Zr/TiO_2, Fe_2O_3/MgO and TiO_2/K_2O displayed by the rock samples are consistent with the range given by most sub-alkaline volcanic rocks of basic to intermediate composition (Pearce and Cann, 1973; Winchester and Floyd, 1977).

4.3 Trace and Rare Earth Elements Characteristics

In terms of trace elements content (Table 2), With the exception of Sr (119-790 ppm), Y (14-40 ppm) and La (12- 26.4 ppm), their abundance is generally low Nb (2 -9 ppm), Ba (99- 589 ppm), Th (0.8-2.9 ppm), Ni (10 – 90 ppm) and Zr (62-207 ppm), feature suggesting the generation of these rocks within active plate margin (Pearce and Gale, 1977). The Cr abundances is generally low (53 -300 ppm), but some samples contain comparatively high Cr content (315- 435 ppm)

The ratios of (Niobium with Yttrium) Y/Nb and Nb/Y fall in the range between 0.83 -5.75 and (0.1-0.6) respectively, Nb/Y and La/Sc ratios are mostly < 1.0 and La/Th (5- 30.3), La/Y (0.5- 1.6) implying possible approach to clac-alkaline and tholeiitic affinities (Y/Nb always < 1.0 in typical to clac-alkaline and tholeiitic basalts, Garcia, 1978).

4.4 Magma Characterization

Noting the trend displayed by the sample sets and sample falling in a specific field in the total alkali versus silica diagram of Le Bas, et al., 1986 (Fig. 5A) it is clear that the samples of the metavolcanic rocks are principally identified as low-silica andisites (53– 57 wt. % SiO_2), and only a few samples fall in the basalt field and the felsic samples are dacites. In the plot using immobile elements Nb/Y versus Zr/TiO_2 of Winchester and Floyd 1977 (Fig. 5B), the mafic samples fall in the andesite and the basalt/andesite fields, consistent with their classification in Figure 5A. However, most of the felsic samples plot in the andesite field rather than the dacite field.

For the magma type, the silica versus total alkali discrimination diagram of Irvine and Baragar, 1971(Fig 6A), defines distinct trend of sub-alkaline affinity for all samples, and the same fact is also confirmed by the (Na_2O+K_2O) vs. SiO_2 diagram after Le Bas, et al., 1986, (Fig. 5A). The K_2O vs. SiO_2 plot after Peccerillo and Taylor, 1976 (Fig, 6B), which straddle the tholeiitic and calc-alkaline boundaries indicates that the petrochemical composition of metavolcanic rocks fall within the calc-alkaline field.

In the multi-element diagram (Fig. 7) normalized to primitive mantle values of McDonough and Sun (1995), the trace element data show that the rocks possess a typical calc-alkaline island arc trace element patterns, enriched incompatible large ion lithophile elements (LILE: Sr, Ba, and K) relative to high field strength elements (HFSE: Zr, Hf, Y, Ti, and Nb) contents, that show a negative Nb and Ni anomalies.

In the chondrite-normalized REE pattern (Fig. 8), values of Sun and McDonough, 1989), all the analyzed rocks samples display enrichment in fractionated light rare earth element (LREE: La, Ce, Pr, Nd and Sm) contents relative to heavy rare earth elements (HREE: Tb, Dy, Ho, Er, Tm, Yb and Lu) contents. The Pattern is generally steep, moderately strong enrichment right-inclined type and lack Eu-anomalies similar to those from calc-alkaline series.

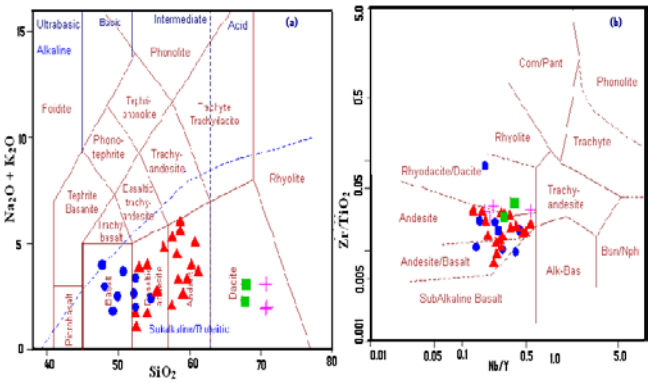

Figure 5. Chemical classification diagrams of the Artoli metavolcanics based on TAS (wt %) (Na_2O + K_2O vs. SiO_2) of Le Bas, et al., 1986 and Nb/Y-Zr/TiO_2 of Winchester and Floyd 1977. *(Symbols: as in Figure 3)*

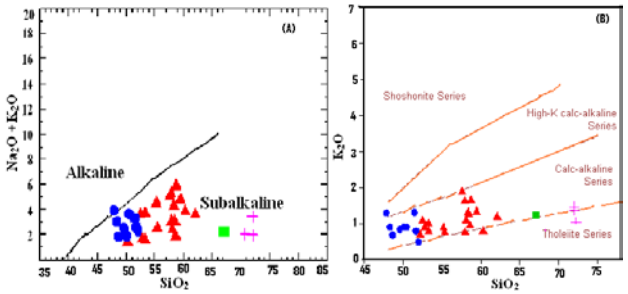

Figure 6. (A) The silica versus total alkali plot (Series boundaries after Irvine and Baragar, 1971) and (B) Weight percent K_2O vs. SiO_2 plot (Series boundaries are after Peccerillo and Taylor, 1976) illustrating clac-alkaline trends in the Artoli metavolcanic rocks. *(Symbols: as in Figure 3)*

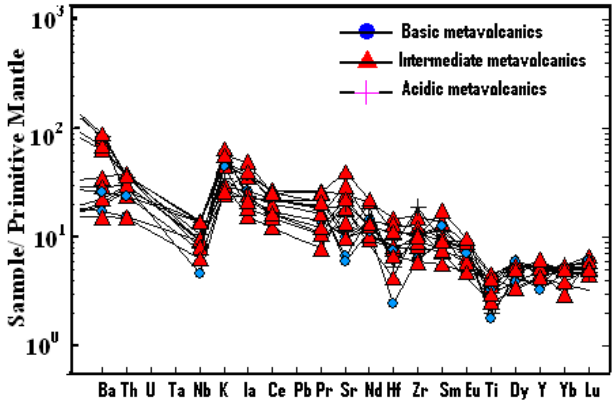

Figure 7. Mantle-normalized multi-element diagram of the bulk rock samples from the study area, (McDonough and Sun, 1995).

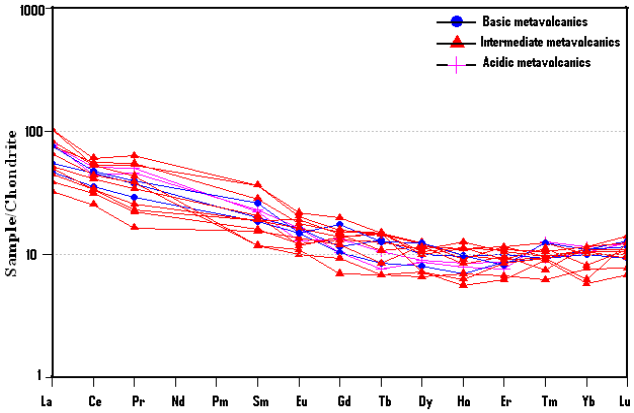

Figure 8. Chondrite-normalized REE patterns for the Artoli metavolcanics, chondrite normalization values are from Sun and McDonough (1989).

4.5 Geotectonic Setting

Samples were plotted on three immobile elements' geochemical diagrams that are proved to be effective in discriminating tectonic settings of volcanic rocks.

Plotting the immobile elements that preserve their abundance through post-formational processes following Müller, et al., 2001, in Zr vs. Y diagram, all samples discriminated as subduction-arc related rocks (Fig. 9A). In the Th-Hf-Nb tertiary discrimination diagram of Wood, 1980, most of the metavolcanic rocks (except three) plot in the calc-alkaline lavas subfield (Fig. 9B), but in the Zr vs. Ti tectonomagmatic discrimination diagram of Pearce and Cann, 1973, samples plot exclusively in the island arc calc-alkaline lavas field (Fig. 9C).

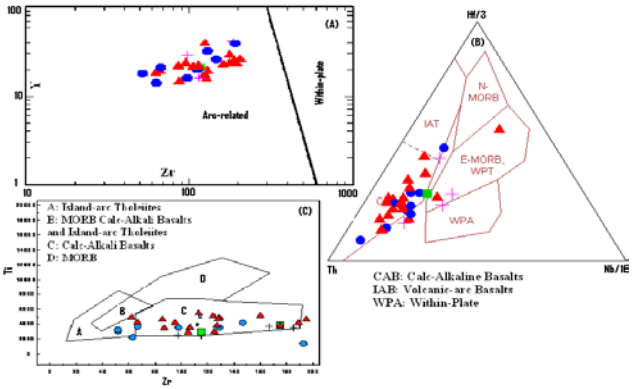

Figure 9. (A) Zr vs. Y Biaxial geochemical discrimination diagram indicating the subduction-arc features (Müller, et al., 2001), (B): Th-Hf-Nb triangular discrimination diagram (Wood 1980) and (C): Zri vs. Ti (Pearce and Cann , 1973) showing the inferred tectonic setting of the study area. *(Symbols: as in Figure 3)*

5. Discussion and Conclusions

The geological setting of the Artoli area, which characterized by predominantly low-grade metavolcanic rocks and minor high-grade metasediments is obviously different form that of the adjacent Bayuda terrane to the west (Saharan Metacraton) and the Keraf petrotectonic assemblage to the north (Suture zone), as the former is heterogeneous continental crust of high-grade gneisses, migmatites and supracrustal rocks of ensialic geochemical affinities and the latter is dominated by siliciclastic and carbonate-rich low-grade metasediments, ophiolitic nappes, and molasse-type sediments.

The dominant metamorphic rocks are from volcanic protoliths as disclosed from the field, petrographic work and geochemical petrogenesis signature (Fig. 3). It is likely that the alteration have not caused substantia1 mobility of element present in the original protoliths as reflected by the normal, correlated and continuous differentiation trends (though some show broad scatter variation) in Harker silica variation diagrams (Fig. 4) which may indicates original source. Thus, major element can be used

together with the alteration resistant trace and rare elements in classifying rocks and identifying the paleotectonic setting.

The characteristics of the major elements (a relative decrease in Al_2O_3, Fe_2O_3 MgO with silica increase, and high Al_2O_3 low TiO_2) and chemical classification diagrams (Fig. 5) confirm what was suspected from field and petrographic work, the existence of a volcanic suite consisting of basic, intermediate and intermediate-acid rock types, with fractionated rocks predominance, especially andisites, basaltic-andisites and dacites with lesser amount of basalts.

A distinctly sub-alkaline affinity is a characteristic feature of these rocks as indicted by the magma type discrimination diagrams (Fig. 6). Most element concentration features displayed by the metavolcanic rocks samples are those assigned to the characteristic features of island arc calc-alkaline rocks, among these are; the marked variation in silica content, the low values of TiO_2 (generally < 1), low Cr, Nb, Ba, Th, Ni and Nb/Y ratios (< 1.0), and the high alumina Al2O3 content (Tables 1&2), moreover, Fe2O3t, MgO, TiO2, CaO decrease systematically with increasing SiO2 (Fig. 3). The clear calc-alkaline geochemical signature is also inferred from the predominance of high proportion of basaltic andesite and andesite fractionated rocks (Fig. 5). The enrichment of large-ion lithophile elements (LILE) and light rare earth element (LREE) contents relative to heavy rare earth elements (HREE) and high field strength elements (HFSE) contents (Rollinson, 1993 and Bloomer et al., 1995), steep moderately strong right-inclined enrichment type pattern and the negative Nb-Ti anomalies (Figs. 7 & 8) all are confirming evidences.

The prominent negative Nb-Ti in spider diagrams for all samples, low Cr and Ba coupled with depletion in Y and Zr postulate subduction environment (Jakes and White, 1972; Shandelmeir et al., 1994b). The predominance of calc-alkaline andesitic rock suites suggests that, the rock series in the area are compatible with products of plate margin tectonic settings of the island arc environment, a fact confirmed by the discrimination and spider diagrams used (Figs. 5-8).

Based on the above account, a conclusion can be reached with the following outlined points derived from the field and petrographic observations combined with the interpretation of element concentrations and trends revealed by the discrimination and variation diagrams:

(1) The area under consideration is a regionally metamorphosed terrane, the rocks of which have been subjected to low-grade metamorphism and/or alterations, the dominant metamorphic rocks are from volcanic protoliths. Although, metamorphism have destroyed most original igneous features, a relationship between the geochemical groups and field characteristics is obvious, this will be helpful in separating and mapping the rock units.

(2) Fractionated rocks are the dominant among the Artoli metavolcanics, as more than two-thirds of the rocks are andesites and basaltic andesites and the remainder are dacites and basalts.

(3) Petrographic and geochemical results give clear evidences that distinctly sub-alkaline affinity is a characteristic feature of these rocks, which are dominated by calc-alkaline suites.

(4) The predominant clear calc-alkaline andesitic rocks, the LREE fractionation compared to the HREE and the enrichment of LILE relative to HFSE contents along with element contents suggest the generation of the protoliths of the metavolcanic rocks of the area in a tectonic environment of island arc setting a fact which testified by the various geochemical diagrams used.

(5) The overall geological and geochemical characteristics of the Artoli metavolcanic rocks disclosed by our study have provided essential evidence to consider the area as part of the westernmost Nubian Shield, because the features are consistent with the arc accretion models postulated in Sudan, Egypt and Saudi Arabia for the Neoproterozoic evolution of the Arabian-Nubian Shield.

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Microscopic Evaluation of the Role of Yeast Extract in Flutamide Hepatotoxicity

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Abstract: The possible protective role of yeast extract from hepatotoxicity produced by flutamide administration was evaluated using histological, quantitative histochemical and immunohistochemical methods. Twenty four adult male albino rats were divided into three groups: the control group was drenched water; the flutamide treated group was drenched 100mg/kg/ day aqueous flutamide solution; and the flutamide and yeast extract treated group was drenched 4.8mg/kg/day yeast extract for 15 days then flutamide and yeast extract for another 15 days. At the end of the experiment, the rats were sacrificed and their livers were fixed, processed for wax embedding. Six micrometer sections were prepared for study of general structural changes using Hematoxylin and eosin stain, DNA evaluation using Feulgen method, and immunohistochemical evaluation of PCNA (proliferating cell nuclear antigen). Morphometry and cytophotometric measurements were performed using Image Pro Plus image analysis software (Media Cybernetics Inc. 2002). The quantitative data were statistically analyzed using Microsoft Excel XP 2003. Liver of rats treated with flutamide presented several signs of injury which was ameliorated in livers of animals treated with yeast plus flutamide. [Journal of American Science 2010;6(8):14-18]. (ISSN: 1545-1003).

Keywords: Flutamide- yeast – Histology - Histochemistry - Immunohistochemistry –Liver - DNA

1. Introduction

Flutamide, an oral non steroidal antiandrogenic anilid compound which inhibits the uptake and binding of androgens to nuclear receptor in the prostate, is used for treatment of prostate cancer (Moguilewsky *et al.*1986). The therapeutic activity of flutamide is compromised because of its potential liver toxicity (Wysowski and Fourcroy 1996). In isolated rat hepatocytes, Flutamide toxicity can be decreased by piperonyl butoxide (an inhibitor of cytochrome p450). Liver toxicity is increased by administration of B-naphthoflavone (an inducer of cytochrome p450) indicating that cytochrome p450 plays an important role in flutamide induced liver toxicity (Wang *et al*. 2002). The inhibition of liver toxicity is bound to the action of the cellular antioxidant, glutathione. Matsuzaki *et al.* (2006) reported that signs of liver injury were detected in knockout mice fed with diet deficient in amino acid with reduced GSH. Flutamid administration was also found to result in a decrease in cellular GSH and increase in leakage of ALT, AST, and LDH (Wang et *al*.2002).

Yeast *Saccharomyces cerevisae* extract was reported to have antioxidative and antimutagenic effect due to the effect of mannan, a component of its cell wall (Krizkova *et al.*, 2001). The water soluble components of yeast extract, sulfoethyl-glucan was also reported as having antioxidant properties (Krizkova *et al.*, 2003).

The aim of the present work is to evaluate the role of yeast extract in the protection against hepatotoxicity in flutamide treated rats.

2. Materials and methods

Materials:

Yeast was produced by The Egyptian Starch, Yeast and Detergents Co.,Alexandria, Egypt. Flutamide was produced by Schering Plough co., U.S.A.

Expermintal animals and design:

Twenty four adult male albino rats weighing 170-200 g, obtained from The Animal house of National Research Center, were used in this study. The animals were fed standard laboratory pallet diet and tap water *ad libitum*. The environmental conditions were standardized with respect to temperature, humidity and light. The animals were divided into three groups, 8 rats each. The first group served as control and drenched water. The second was drenched aqueous flutamide solution (100 mg/ kg body weight (B.W.)/day) for 15 days. The animals of the third group were drenched yeast extract suspension (4.8 mg/kg/day) for 15 days, then a mixture of yeast and flutamide was administered for another 15 days.

Methods: At the end of the experiment, the rats were sacrificed and their livers were fixed, processed for wax embedding. Six µm sections were stained by Hematoxylin and eosin and Feulgen method.

For immunohistochemical evaluation of PCNA, sections were incubated with anti PCNA antibodies labeled with horse raddish peroxidase enzyme. The numbers of PCNA-positive and negative hepatocytes were counted per fixed area using computerized image analysis software. Under The percentage of PCNA positive hepatocyte nuclei was derived from nuclear count.

Phases of the cell cycle were elucidated from Feulgen stained sections subjected to measurement of optical density using Image Pro Plus image analysis software (Media Cybernetics Inc. 2002).

The quantitative data were manipulated and statistically analyzed by student (t) test and graphically presented using Microsoft Excel XP 2003.

3. Results

Treatment with flutamide resulted in the death of hepatocytes. Apoptosis was more evident in the pericentral hepatocytes while extensive oncosis was more pronounced in periportal cells (figure 1F). The central vein, portal vein and hepatic artery were highly congested with blood. Cell injury was manifested by increased eosinophilia of hepatocytes.

In liver sections of animals treated by both yeast and flutamide (Figure 1 FY), the incidence of cell injury was much less than in the flutamide treated animal liver and similar to control (Figure 1C).

In sections stained by Feulgen for DNA demonstration, hepatocytes with different features of injured and dying cells were observed in the liver of flutamide treated animals (Figure 2F). Such features were not observed in control (Figure 2C) or yeast and flutamide treated animal liver (Figure 2FY). The frequency distribution of the hepatocyte nuclear DNA of the three experimental groups is presented in figure (3). The population of cell nuclei containing sub Go/G1 complement of DNA representing dying cells was high in flutamide treated group (27.1%) compared with control (7.9%) and flutamide and yeast extract treated(4.3%) groups. On the other hand, the percentage of cells in the S-phase was very low in flutamide treated animal liver (.15%) compared with control (16.5%) and flutamide and yeast treated (24.5%) groups. Iiver sections stained by immunohistochemical method for demonstration of proliferating cell nuclear antigen (PCNA) are demonstrated in figure (4). Quantitative evaluation of the antibody bound antigen presented in figure (5) indicates a significant increase in the antigen in flutamide treated animal hepatocytes compared with control. The value for the yeast and flutamide treated group was not significantly different from control.

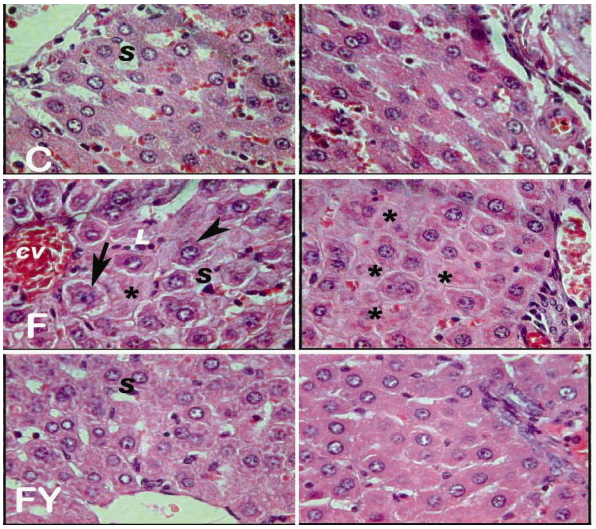


Figure 1: Histopathological changes in liver sections of different groups. The right half is pericentral and left half periportal areas. C: control, F: flutamide treated, FY flutamide and yeast treated specimens.

Notice the congested blood vessel (cv), apoptotic bodies (arrow), eosinophilic cytoplasm (arrow head) and oncotic cells (asterisk). Hx and Eosin X 400)

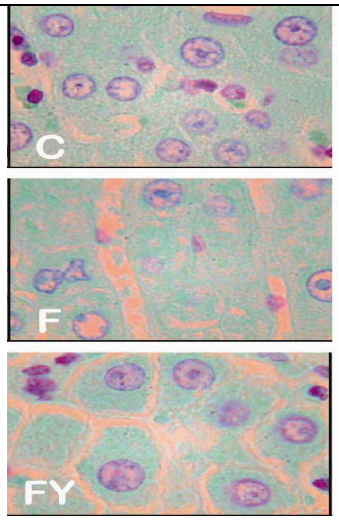
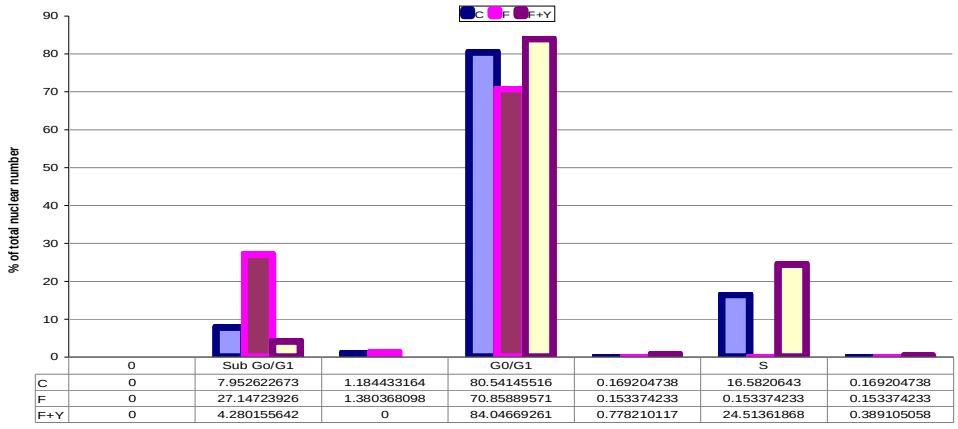


Figure 2: Feulgen stained nuclei to demonstrate DNA. C: control, F: flutamide treated and FY flutamide plus yeast



	0	Sub Go/G1		G0/G1		S	
C	0	7.952622673	1.184433164	80.54145516	0.169204738	16.5820643	0.169204738
F	0	27.14723926	1.380368098	70.85889571	0.153374233	0.153374233	0.153374233
F+Y	0	4.280155642	0	84.04669261	0.778210117	24.51361868	0.389105058

Figure 3: Frequency distribution of nuclear DNA in feulgen stained nuclei of different groups.

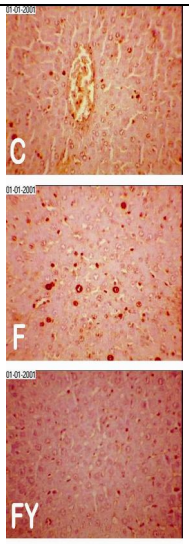


Figure 4 : immunohistochemical demonstration of proliferating cell nuclear antigen (PCNA)

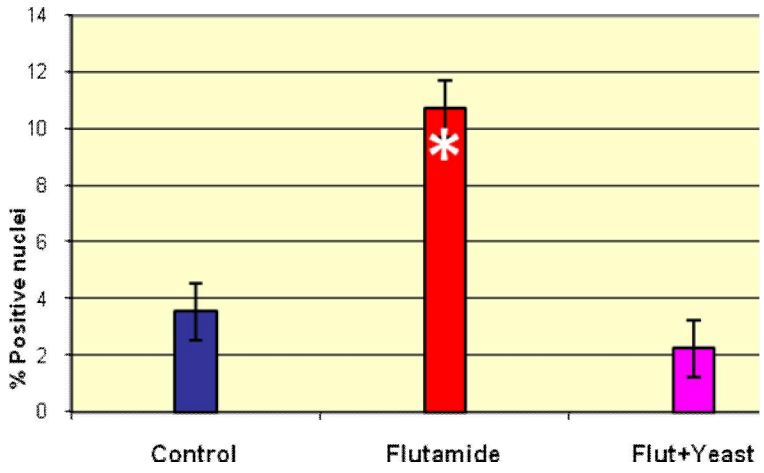


Figure 5: Mean number of PCNA positive nuclei. The error bar represents the standard deviation, the astrisk indicates statistically significant difference from the mean of control (p ≤0.001)

4. Discussion

Flutamide (2-methyl-N-[4-nitro-3-(trifluoromethyl) phenyl]-propan-amide), was the first pure nonsteroidal antiandrogen compound discovered. It is used in the treatment of prostate cancer. Patients were reported to develop serious hepatotoxicity while using flutamide. Some of these patients even died of progressive liver disease(Wysowski et al.1993). This calls for the search to avoid such effect on using the drug. The present work is aimed to study the possible protective effect of yeast extract in rats treated with flutamide.

In flutamide treated rats, signs of hepatotoxicity were demonstrated in histological sections of liver. Hepatocytes undergoing apoptotic cell death were found mainly in the pericentral area while oncosis was more abundant in hepatocytes at the portal area. Both the portal vein and central vein were congested with blood. Frequency distribution analysis of nuclear DNA content verified a high incidence of cell with sub Go/G1 values (fragmented DNA).

The observed congestion could be the result of inhibition of the vasodilator effect of testosterone by flutamide (Hutchison et al . 2005). Vascular congestion and stagnant blood flow can lead to hypoxia that affects mitochondrial respiration which was reported in flutamide hepatotoxicity (Thole et al.2004; Scatena et al. 2007). Flutamide result in the expression of genes associated with oxidative phosphorylation, fatty acid beta-oxidation, antioxidant defense, and cell death pathways(Coe et al. 2007)

Another factor that can cause cell death is oxidative stress (Hong et al.2009). The bioactivation of flumide by P450, results in the formation of hydroxylated and free radical metabolites (Tevell et al. 2006). Such metabolites have the ability to deplete reduced glutathione by oxidation and adduct formation (Kang et al.2007). Induced glutathione (GSH) depletion was reported to be the major contributor to the oncotic and apoptotic cell death induction (Tsay et al. 2009). GSH depletion can also cause the DNA fragmentation (Kanz et al. 2003) observed in the DNA frequency distribution.

The frequency of cells in the S-phase in flutamide treated population calculated from density measurement of Feulgen stained liver sections was very low. The results suggest an inhibitory effect of flutamide on hepatocyte DNA replication and cell proliferation. This effect was also reported for the proliferation of human vascular cells (Somjen et al. 1998).

The percentage of PCNA positive hepatocyte nuclei was significantly higher in flutamide treated animals compared with control. This seems controversial for the above results. However, following exposure of cells to DNA damaging agents that block the progress of the replication fork, mono-ubiquitination of PCNA mediates the switch from replicative DNA polymerases to polymerases specialized for translation synthesis (Brown et al. 2009). It is possible that flutamide generated oxidative stress and depletion of glutathione results in DNA damage that can cause such a switch replication to translation synthesis (Himmetoglu et al. 2009).

The histological, immuno- histochemical and DNA frequency distribution analysis indicate that drenching aqueous yeast suspension greatly reduced the signs of flutamide induced hepatotoxicity.

Histologically, signs of vascular congestion were absent in sections of liver treated with yeast extract and flutamide. This could be due to the antihypertensive effect (Penna et al .2008) of yeast extract (Kanauchi et al.2005) .

In sections of liver of animals treated with flutamide and yeast extract, the frequency of cells in the S-phase was high; and the mean percentage of nuclei positively stained for PCNA were statistically non significant from control. Both results support the ability of yeast extract to maintain the cell proliferating activity. The ability of yeast extract support for proliferation was recorded by Erikssona et al.(2005). They found that insect Trichoplusia ni cells were able to proliferate in serum-free culture media only in the presence of yeast extract.

The incidence of cell death was much lower in yeast extract and flutamide treated group than that of flutamide alone. This was evident in histological sections as well as the percentage of cells containing fragmented DNA. The yeast cell wall mannans was found effective in reducing DNA damage and having antioxidant properties (Krizková et al., 2001) . The same effect was also addressed to carboxymethyl-glucan (CM-G) and sulfoethyl-glucan (SE-G) both from the baker's yeast (Krizková et al., 2003).

The present study, therefore, reports that drenching yeast extract suspension to rats protected from the signs of hepatotoxicity induced by flutamide. Vascular congestion, cell death and DNA fragmentation induced in liver of flutamide treated animals were much ameliorated in the liver of rats treated by both flutamide and yeast extract.

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Hydration Characteristics of Autoclaved Cement Kiln Dust-Sludge-Silica Fume Pastes

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Abstract: Autoclaved cement kiln dust (CKD) and sludge pastes made with and without silica fume were hydrothermally hardened at a pressure of 8 atm. of saturated steam for different autoclaving ages. Hydration characteristics of the autoclaved CKD-sludge-silica fume pastes were studied by the determination of compressive strength and chemically combined water contents at different autoclaving ages. The phase composition and morphology of the formed hydrates were studied using x-ray diffraction analysis and scanning electron microscope. The replacement of silica fume in CKD-sludge mixtures results in a marked increase in strength values of the autoclaved specimens at all stages of the hydrothermal process. The results of x-ray diffraction analysis and SEM-micrographs of autoclaved specimens for various mixtures indicated that the main hydration products identified are calcium silicates hydrated and minor amounts of $CaCO_3$. [Journal of American Science 2010;6(8):19-26]. (ISSN: 1545-1003).

Key words: cement kiln dust, sludge, silica fume, hydrothermal treatment.

1. Introduction

Autoclaving building products possesses several advantages including the marked development at shorter times of autoclaving at relatively high steam pressures. In addition, the utilization of industrial solid wastes in the production of high strength building products is of prim importance for environmental protection and development.

Industrial solid wastes such as cement kiln dust and other silica and alumina bearing materials are widely used for the production of blended cement and various cementations products. In this work we studied the usage of three wastes which can act as cementations materials as new building materials namely; cement kiln dust, sludge and silica fume. Hydrothermal treatment of these wastes is almost associated with building products having improved binding characteristics. The properties of these autoclaved products are always governed by the chemical composition and physical state of the formed hydration products which act as the main binding centers [1-3]. Cement kiln dust (CKD), is produced in relatively large quantities during the production of Portland cement. Various methods for utilizing CKD in industrial applications, including existing or proposed methods for alkali removal, are reported in the literature. Bhatty [4] provides a general review of these methods. Because of the generally high lime content of CKD and subsequent ability to harden upon exposure to moisture, CKD has been used as a binder in soil stabilization suitable for a sub-base in streets and high way construction. It is commonly used as a mixture with different solid-waste materials such as waste glass, fly ash, waste water sludge with the addition of cement or other admixtures if necessary [5]. Recently, there has been a trend of utilizing it in cement products [6-10].

Sludge is a byproduct produced in electrical stations in large quantities. Many attempted of sludge disposal alternatives such as agricultural use, land filling, marine disposal and incineration, etc, have adverse environmental impacts. There is a resulting increase of interest in reuse of sludge by incorporation into construction materials [11-12].

Silica fume is a byproduct of the reduction of high-purity quartz with coal in electric furnaces in the production of silicon and ferrosilicon alloys. Silica Fume is also collected as a byproduct in the production of other silicon alloys such as ferrochromium, ferromanganese, Ferro magnesium, and calcium silicon. Silica fume consists primarily of amorphous (non-crystalline) silicon dioxide (SiO_2). The individual particles are extremely small, approximately 1/100th the size of an average cement particle. Because of its fine particles, large surface area, and the high SiO_2 content, silica fume is a very reactive pozzolana when used as a blend in cement and concrete [13-15]. The quality of silica fume is specified by ASTM C 1240 and AASHTO M 307. Toutanji et al [16] studied the effect of silica fume on the compressive and uniaxial direct tensile strength of Portland cement pastes and mortars. Many authors studied the durability of OPC blended with silica fume against sulfate ions [17-18], fire [19-20] and acid solutions [21-23].

The object of this investigation is to study the hydration characteristics of autoclaved specimens made with cement kiln dust- sludge and cement kiln dust -sludge- silica fume. The phase composition and the morphology of the formed hydrates were studied using x-ray diffraction analysis and scanning electron microscope.

2. Material and Methods

a Materials:

The starting materials used in this investigation are:
-Cement Kiln Dust (CKD) of Blain surface area about 2500 cm2/g collected during the manufacture of cement by the dry process from Suez cement company, Suez, Egypt. The chemical oxide composition was given in Table (1).
-Condensed silica fume is a byproduct of silicon or ferrosilicon alloys industries. It is obtained from ferro-silicon Co; Kom-Ombo, Egypt. It is amorphous silica with specific surface area 20 m2/g.
-Lime free sludge was obtained from West-Cairo power station , sakeel-Giza, Egypt. The chemical oxide composition was given in Table (1).

Table (1): The chemical oxide composition of CKD and sludge.

Oxide	%	
	CKD	sludge
SiO_2	15.4	60.25
Al_2O_3	3.76	2.06
Fe_2O_3	2.69	1.25
CaO	49.6	3.28
MgO	1.95	1.23
SO_3^{--}	5.23	1.28
K_2O	2.19	0.31
Na_2O	2.68	0.49
L.O.I	15.6	29.0
Free CaO	21.85	-
Free Cl^-	5.84	-

b Preparation of Mixes

Seven Mixes were prepared using different weight composition ratios of CKD, sludge and silica fume as shown in Table (2). The dry mixtures showed in Table (2) were first mixed using ethanol for one hour in order to ascertain a complete homogeneity of the mixture. After evaporation of ethanol, each dry mixture was mixed with distilled water for 3 minutes at a water/solid ratio of 0.45. From the paste produced, cylindrical specimens of 3.14 cm2 cross-section area and 2 cm height were molded at a pressure of 50 kg/cm2. The specimens were first cured at 100% humidity for 6 hours in order to attain the initial setting. The specimens of each mix were autoclaved at 8 atmosphere of saturated steam for 0.5, 2, 6, 12 and 24 hours. At the end of each autoclaving period, the specimens were dried, after being removed from the autoclave, in CO2 free atmosphere at 105°C for 24 hours to remove the free water.

At each autoclaving period, compressive strength test was carried out on the dried specimens. Then, chemically combined water contents were carried out on the crushed specimens. The phase composition of the formed hydrates is investigated using x-ray diffraction analysis. The morphology and microstructure of hydrated phases were identified using scanning electron microscopy (SEM).

Table (2): Composition percentage (w/w) of various dry mixtures and their notation.

Sample notation	Composition percentage(w/w)		
	CKD	Sludge	Silica fume
I	60	40	-
II	50	50	-
III	40	60	-
IV	50	45	5
V	50	40	10
VI	40	55	5
VII	40	50	10

3. Results and Discussion

1. Compressive Strength

The results of the compressive strength of autoclaved mixes made of weight percentage ratios 60/40, 50/50 and 40/60 of CKD / sludge (Mixes I, II and III) are shown in Fig.(1). The strength values increases with increasing age of autoclaving for all autoclaved Mixes (I –III) up to 18 hours. This mainly attributed to the hydrothermal interaction between the lime released from CKD with sludge to give calcium silicate hydrates which contribute considerably to the compressive strength of these autoclaving specimens. In addition, the presence of some alkali in CKD activates the hydrothermal reaction [1, 6]. However, the compressive strength values decrease after 24 hours of autoclaving, these results can be attributed to the stabilization of the initially formed hydrates via crystallization. Fig. (1) Shows also that the autoclaved specimens made of Mixes II and III possess the higher strength values compared to Mix I. This indicates these ratios of CKD to sludge in these Mixes possess the best hydrothermal reaction that led to the formation of hydration products having considerable hydraulic character.

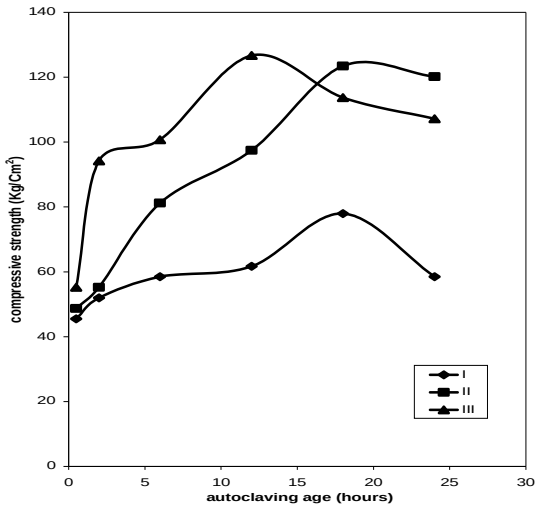


Fig.(1): Compressive strength (Kg/cm^2) of Mixes I,II and III at various autoclaving ages.

The compressive strength results of autoclaved pastes made of Mixes II, IV and V are shown in Fig.(2) indicate that the replacement of sludge in Mix II by 5 wt. % silica fume (Mix IV) or by 10 wt. % silica fume (Mix V) result in a marked increase in the strength values of the autoclaved specimens made of CKD- sludge- silica fume mixtures at all stages of the hydrothermal process. The results of Fig.(2) indicate also that the autoclaved pastes made of Mix V (containing 10 wt.% silica fume) possess relatively higher strength values compared with those of the autoclaved paste made of Mix IV (containing 5 wt. % silica fume). As the ratio of silica fume increased from 5 to 10 wt.%, the compressive strength increase, and reach a values of compressive strength in mix V (50 wt.% CKD + 40 wt.% sludge +10 wt.% silica fume) higher by 3 to 4 fold compared to mix II (50 wt.% CKD +50 wt. % sludge) at all the autoclaving times. Such results due to the high reactivity of silica fume to react with lime produced from CKD as compared to sludge in the hydrothermal reaction. At later autoclaved ages (24 hrs.) for Mix V, a slight decrease in strength values is observed, a result which is mainly associated with two factors, these are: (i) the well crystallization of the initially formed hydrates (mainly calcium silicate hydrates); the well crystallized CSH possess low hydraulic characteristics with relatively low strength and/ or (ii) the transformation of lime –rich CSH into low –lime CSH with a metastable state leads to lower mechanical properties.

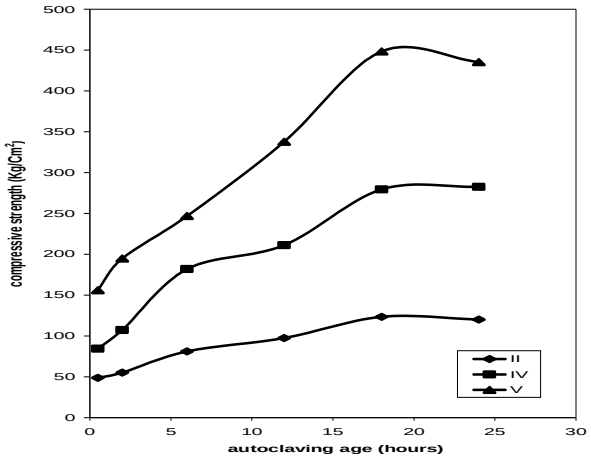


Fig.(2): Compressive strength (Kg/cm^2) of Mixes II, IV and V at various autoclaving ages.

Fig. (3) shows the compressive strength results of Mixes III, VI and VII in which a replacement of sludge in Mix III by 5 wt.% silica fume (Mix VI) or 10 wt.% silica fume (Mix VII) are made. Again, Fig. (3) shows the replacement of sludge by silica fume in the hydrothermal specimens results in a marked increase in the compressive strength at all stages of the hydrothermal process. However, the compressive strength values obtained here in these Mixes (VI and VII) are lower than those obtained in Mixes IV and V. Such results can be attributed to the weight ratios of CKD-sludge-silica fume in Mixes IV and V possess the best hydrothermal reaction compared to all autoclaving Mixes.

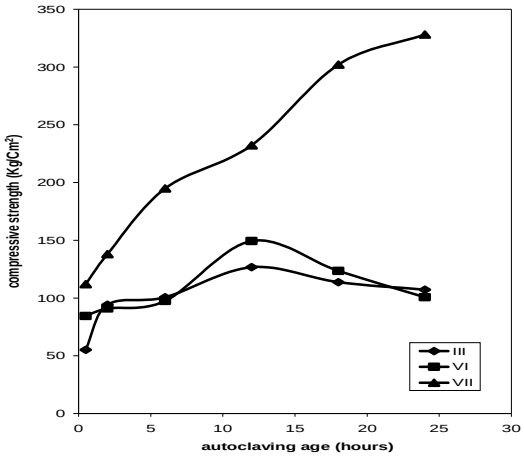


Fig.(3): Compressive strength (Kg/cm^2) of Mixes III, VI and VII at various autoclaving ages.

2. Chemically Combined Water Content:

The result of combined (non evaporable) water content (Wn, %) of mixes containing different weight ratios of CKD and sludge (mixes I-III) are shown in Fig. (4). Obviously, the combined water contents values increases with increasing the autoclaving time up to 12 hours which indicate a progress of the hydrothermal reaction and formation of more hydrates. At the period 18-24 hours, the values of the chemically combined water contents slightly decrease in all Mixes. This can be explained to the transformation of lime–rich CSH into low –lime CSH with a metastable state leads to low water content and lower mechanical properties, and/or the stabilization of the initially formed hydrates via crystallization. Crystallized CSH characterized by low water content and low compressive strength.

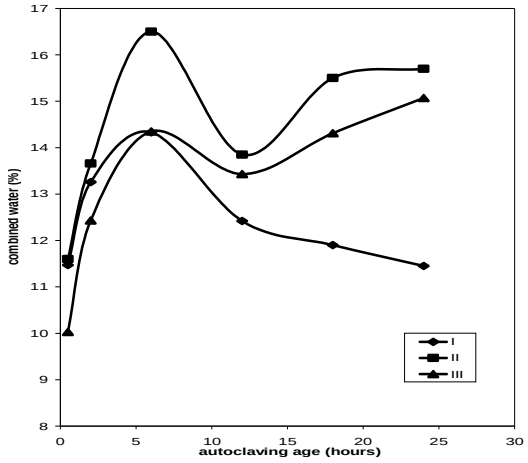


Fig.(4): Combined water contents of Mixes I, II and III at various autoclaving ages.

Combined water contents of autoclaved specimens made of Mix II, Mix IV (5 wt. % of sludge is replaced by silica fume) and Mix V (10 wt.% of sludge is replaced by silica fume) at different autoclaving times are shown in Fig.(5). Here, a similar trend of variation of chemically combined water contents of specimens containing silica fume (Mixes IV and V) to those free from silica fume (Mix II) was obtained but with relatively higher values. The increased values of chemically combined water contents indicate a formation of more hydrates and these confirm the compressive strength results. Mix (V) which composed of 50 wt. % CKD + 40 wt. % sludge and 10 wt. % silica fume shows the highest values of chemically combined water contents compared with the other investigated Mixes.

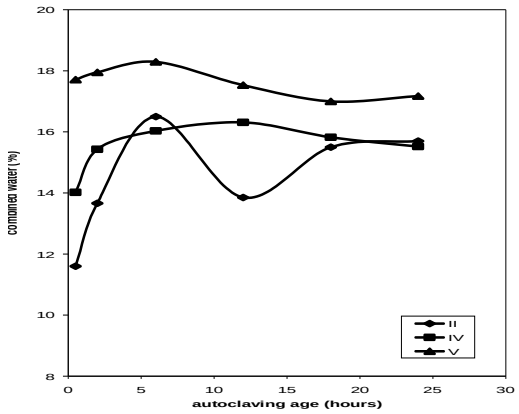


Fig.(5): Combined water contents of Mixes II, IV and V at various autoclaving ages.

Fig.(6) shows chemically combined water contents of autoclaved specimens of Mix III, Mix VI (5% of sludge is replaced by silica fume) and Mix VII (10 % of sludge is replaced by silica fume) at various autoclaving times. Again, in these specimens the replacement of sludge by silica fume led to an increase in the values of combined water contents at all the autoclaving times. These results confirm the results of compressive strength and indicate the high reactivity of silica fume compared to sludge in the hydrothermal reaction.

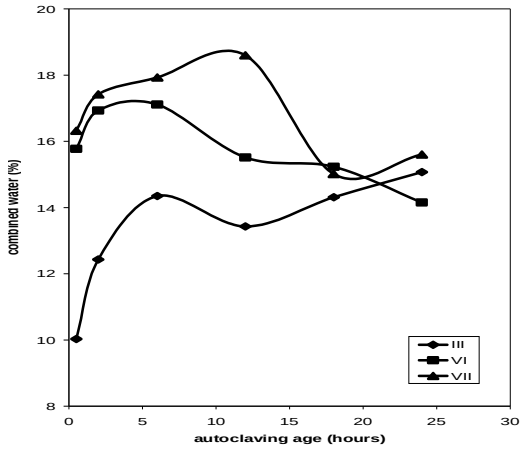


Fig.(6): Combined water contents of Mixes III, VI and VII at various autoclaving ages.

.3. X-ray diffraction analysis

The main hydration products formed as a result of hydrothermal reaction of the hardened specimens made from CKD, sludge and silica fume are identified by means of x-ray diffraction analysis.

X-ray diffraction patterns of the autoclaved CKD- sludge made of Mix II and autoclaved CKD-sludge –silica fume made of Mixes V at autoclaving ages of 0.5, 6, 18 and 24 hours are shown in Figs. (7 and 8) respectively. The distinct main hydration products identified were calcium silicate hydrates (CSH I, II) and minor amounts of $CaCO_3$. In addition, peaks characterized to unhydrated β-C2S and SiO2 were identified. At later autoclaving ages, 6, 18 and 24 hours, the intensities of the peaks characterized to CSH I,II were increased while the intensities characterized to unhydrated phases decreased. The formation of lime rich calcium silicate hydrates [mainly (CSH II] in autoclaved specimens made of Mix V (having high CKD content in dry mixture with 10 wt. % silica fume) is mainly responsible for the increased intensities characterized for hydrated phases (Fig. 8) of autoclaving of hardened CKD-sludge- silica fume pastes of Mix V compared to those of mix II. These results confirm the results of compressive strength.

4. Morphology and Microstructure

Fig.(9-a) shows the SEM micrograph of the hydration products formed after 6 hours of autoclaving of specimens made of Mix II (50 wt.% CKD + 50 wt. % sludge). The hydration products are mainly composed of semicrystalline calcium silicate hydrates beside minor amounts of CaCO3 and unhydrated phases β-C2S and SiO2. After 24 hours of the hydrothermal reaction a massive structure of calcium silicate hydrates appears as the minor hydration products (9-b).

Fig.(10-a) shows the SEM micrograph of the hydration products obtained after 6 hours of autoclaving specimens of Mix V ((50 wt.% CKD + 40 wt.% sludge +10 wt.% silica fume). The hydration products are mainly semicrystalline calcium silicate hydrates (CSH I,II). After 24 hours of autoclaving, crumpled foils with interlocking fibrous structure of calcium silicate hydrates appear in the SEM micrographs shown in Fig.(10-b).

4. Conclusion:

On the basis of the results obtained in our investigation we Concluded that:

1- Autoclaved cement kiln dust (CKD)-sludge pastes possess a considerably compressive strength at all different autoclaving ages. This mainly attributed to the hydrothermal interaction between the limes released from CKD with sludge to give calcium silicate hydrates.

2- The replacement of sludge by silica fume results in a marked increase in the compressive strength and the combined water contents of the formed hydrates at all the autoclaving ages. This mainly indicates the high reactivity of silica fume compared to sludge to react in the hydrothermal reaction.

3-X-ray diffraction analysis and SEM micrographs show the main hydration products are calcium silicate hydrates (CSH I,II) with a minor amounts of $CaCO_3$.

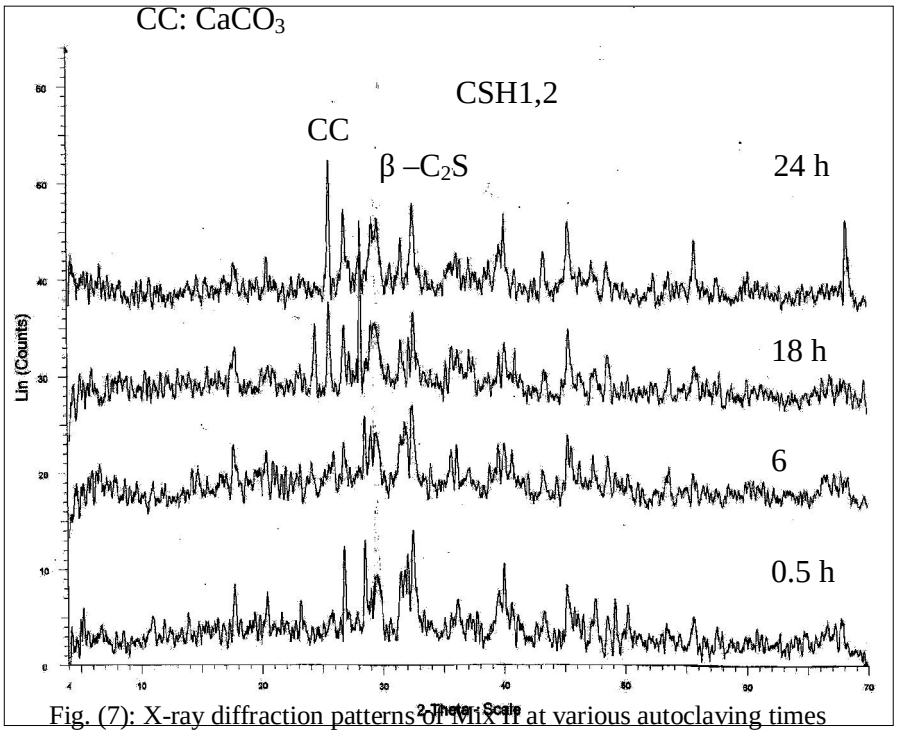


Fig. (7): X-ray diffraction patterns of Mix II at various autoclaving times

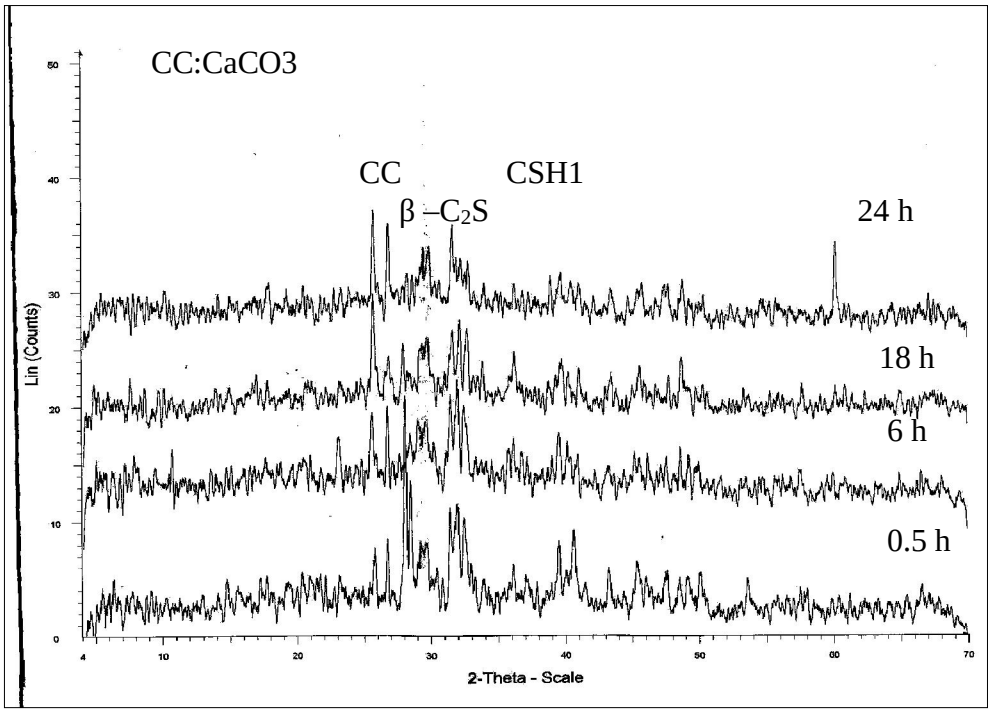
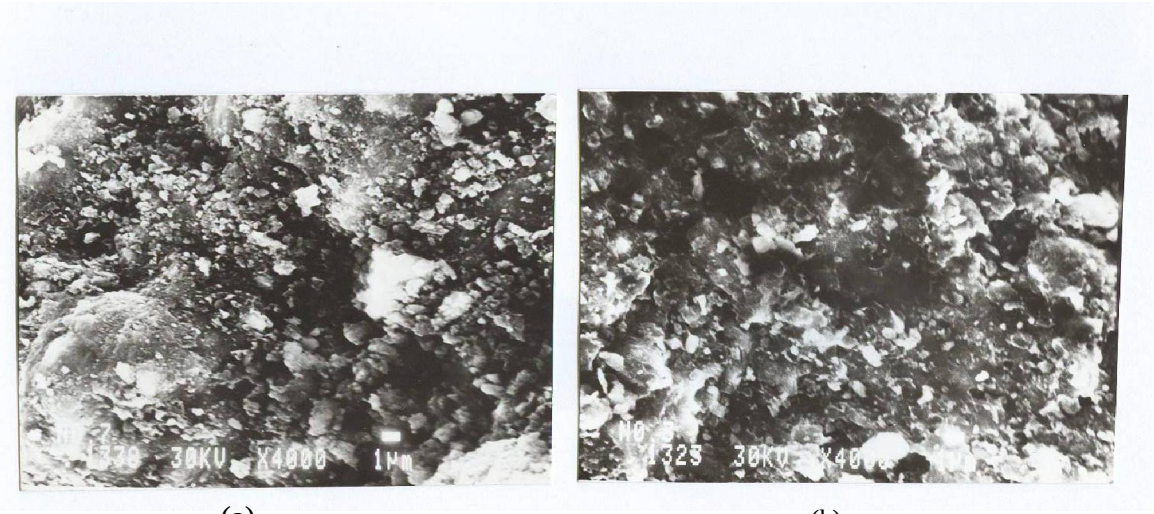
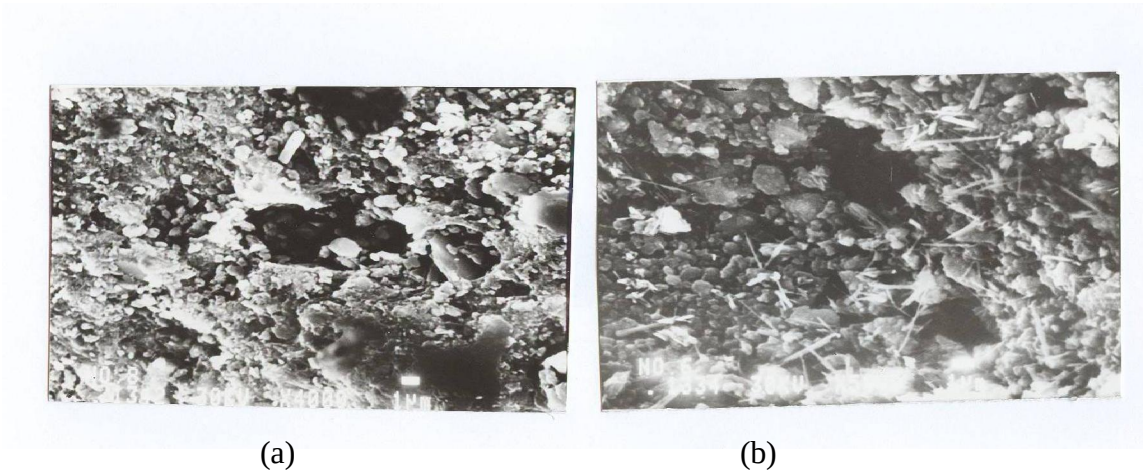


Fig.(8): X-ray diffraction patterns of Mix V at various autoclaving times



(a) (b)

Fig. (9): SEM micrographs of Mix II at at various autoclaving times
(a) after 6 hours (b)after 24 hours



(a) (b)

Fig.(10): SEM micrograph of Mix V at various autoclaving times
(a) after 6 hours (b)after 24 hours

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4/5/2010

Effect of Rose Bengal on *Hylemyia antiqa* (*Meigen*) (Diptera :Anthomyiidae)

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Abstract: Rose Bengal as photosensitizers material used as insecticide after the addition of a specific hydrocarbon to control different stages of the onion fly *Hylemyia antiqu*a (egg , larval, pupal and adult) with different doses (o.o1270 , 0.00145 , 0.00127, 0.000029 µg /L) ,with different light expose time. Most of these treatments eradicate the target pest. pupae stages of *H. antiqua* could be sensitive to photosensitizer because it can be controlled during only 15 sec. then eggs instar that could be controlled during 30 sec . but adult instar could be controlled with 60 sec. from light exposure to direct sun light and later 4th instar larvae which needed to, at lest 15 min. to be controlled with the same concentration from used photosensitizer. This is to prove that photosynthtizers can play an active role in pest control. [Journal of American Science 2010;6(8):27-30]. (ISSN: 1545-1003).

Keywords: Rose Bengal; photosensitizer; hydrocarbon; onion; fly *Hylemyia*

1. Introduction

Development of new pesticides that are efficacious, environmentally safe, and being to non target to organisms continues to be a priority for the agriculture chemistry community in order to protect and increase our food and fiber production. Over the past few decades, new and better toxic strategies have been applied to this problem. The rise and fall of the organochlorine insecticides due to long-term environmental concerns, and their replacement by the organophosphate and carbamate insecticides. The latter insecticides are under pressure at present as being too toxic to non target species. The observation of the development and the eventual difficulties of synthetic pyrethriods may, due to insect resistance . .Also, fungi in the genus *Cercospora* produce cercosporin, a potent singlet oxygen (102)-generating photosensitize that plays a critical role in the ability of these fungi to parasitize plants, mice ,bacteria and many fungi are sensitive to *cercosporin* , *Cercospora* species are resistant to its toxicity. The cellular resistance of these fungi to Cercosporin has been correlated with fungal cell surface reducing ability and ability to maintain Cercosporin in a chemically reduced state (Daub *et al*. 2000) . But for Integrated pest management (I.P.M) programmes often look for more specific ways to control pests. Biological control agents, such as the bacterium *Becillts thuringiensis* and the fungus, *Beauveria bassiana*, can control insects with minimal disturbance to the environment because of their host specify and short half – lives. Often these agents alone cannot prevent yield loss or are too expensive. Their for looking at combination of these agents and photoactive dyes like Rose Bengal, fluorescein , eosin y (Martin *et al* . 1998).

Also, the encyrtid parasitoid, *c. peregrinus* has been used as a biological control agent against the mealy bags P. citri and P. ficus. With help of photoactive dyes can examine the behavior and host selection (Joyce *et al*. 2001). For medical filed, photosynthtizer plays a critical role in epidemiological characteristics of diseases (Oliveira, 2000). Later, photosynthtizer as a method to describe the effect of synthetic pyrethroid insecticides which used in protection of fruits and vegetables as well as public hygiene(Tyrkiel *et al*. 2001). As an effect of the substitution position of the suger moieties, the photosynthtizer bearing suger moieties at the meta-position of PH group showed remarkably high activity compared with para – substituted ones, and the difference could not be explained by the opticald. Confocal laser scanning microscopy revealed that meta- substituted photosynthtizers are not readily deactivated from the excited state in cellular microenvironment, this may explain their potent photocytoxicity. The phosphorescence quantum such as uranine, eosion yellowish, erythrosine B and rose Bengal or photosynthtizers was usually used as insecticides. The photodynamic action has been shown to function by one of two mechanisms Heitz and Downum (1995). In type one mechanism the dye absorbs a photon of light rises the first to singlet exited state and then drops to the exited tripled stat. The energy of the photon is then added to the target substrate molecule, making an activated form substrate. The activated molecule then adds to ground state oxygen or their oxygen radicals and becomes oxidized in the process, in type two mechanisms the

dye again absorbs a photon of light as the first step in the process. The dye rises first to the exited singlet state and then to the excited triplet state. The excited dye molecule then gives the energy to ground state oxygen thereby rising the oxygen to the exited singlet state. Finally, the excited oxygen adds to the target substrate and oxidizes it. But there are an other new ideas like, photosensitization via dye coordination a new strategy to synthesize metal nitrosyls that release NO under visible light (Harrop 2006).

Addition of hydrocarbon to Rose Bengal (Patent R. no. 1788/ 2009) is very important , that without this addition these material can not control the pest with this efficiency.

The aim of this work to define the effect of Rose Bengal as insecticide environmental friendly compound.

2. Material and Methods

Chemical preparation: Rose Bengal fluorescent stain powder was mixed with 6 mg/dl ion hydrocarbon patent R. No. 1788/2009; dissolved in water to prepare concentrations of 0.01270, 0.00145, 0,ooo127 and 0.000029 µg/L.

Insect culture : Hylemyia antiqua was reared in the laboratory of Plant Protection institute, Ministry of Agriculture, Cairo, Egypt.

Design of experiment:

Each concentration of Rose Bengal was topically applicated on each stage of insect instar by micropipette at volume of 1 µl/insect instar in groups of 10and15 replicate insects. Each group of selected treated insects exposed to direct light sun for period selected as it mentioned ,then keep it in room light. The death recorded after 24 hours after the application.

The period was selected for eggs were 30 and 60 sec.. As for larva 4th instars and pupa were 1.5, 15, 20 min and 15, 150sec. respectively. The adult form the selected time of exposure was 60 and 120 sec.

Therefore the insects were classified into 4 groups according to the concentration of Rose Bengal to reach as follow:

Group 1 at concentration was 0.01270 µg/l
Group2 at concentration was 0.00145 µ g/l
Group 3 at concentration was 0.00 0127 µg/l
Group 4 at concentration was 0.000029 µ g/l

3. Results

From table (1), LC50 for egg s stage of *H.antiqua* was 0.0 0 0 446 with slop 0.219 ± 0.006 at 3 0 seconds exposure time to direct sun light, but it was 0.0 0 0 0 84 with slop 0.516 ± 0.007 at 60 seconds that indicate the photosensitizer can control eggs stage of *H.antiqua*, but in the other hand, it was 315.022 at LC90 in 30 seconds a s time of exposure to direct sun light . But also, it was 0.0253

In 60 seconds exposure to direct sun light. However, Rose Bengal can not only control eggs stage of *H.antiqua* but also integrate this target which time of exposure to direct sun light play an active role with Rose Bengal concentration with the additional material.

But for 4th instar larvae of *H.antiqua* ,the LC50 was 0.000347 with slop 0.316 ± 0.006 at 15 min. as time of exposure to direct sun light , but it was 0. 00003 with slop 0.547 ± 0.008 at 20 min. as time of exposure to direct sun light. But 4th instarlarvae of *H.antiqua* LC90 was 3.892 at 15 min. exposure to direct sun light. In the other hand at 20 min. time exposure to direct sun light, LC90 was 0.0676. It notes that this stage take time for light exposure more than eggs stage.

For Pupae stage LC50 of *H. antiqua* was 0. 0 0 0177815 with slop 0.3843 ± 0.0654 at 15 sec. time exposure to direct sun light. But it was 0.0 0 0 0142837 with slop 0.4805 ± 0.0007 at 150 sec. time exposure to direct sun light.

For LC90, it was for the same stage o.3 8 4 with at 15 sec. time exposure to direct sun light . But, it was 0.0 0 6 6 3 741 with at 15 0 sec. time exposure to direct sun light.

For Adult stage of *H.antiqua* , the LC50 was 0.0 0 0 2 6 with slop 0.070 ± 0.0003 at 60 sec. of exposure to direct sun light. But it was 0.0 0 0 2 9 with slop1.088 ± 0.0054 at 12 0 sec. exposure to direct sun light. For LC90 to adult stage of *H.antiqua*, it was 0.0166 with slop 1.0 8 8 when it exposed 60 sec. to direct sun light, but it was 0.0 0 3 when exposed 120 second to direct sun light.

4. Discussion

From all results, time of exposure to direct sun light play an active role in controlling all stages of *H.antiqua* in order to complete photosensitizer reaction . Increasing time exposure to direct sun light decrease the used photosensitizer concentration and vice verse. However eggs stage as stable stage, it can be controlled with few seconds of light exposure to direct sun light with low concentration of photosensitize but 4th instar larvae of *H.antiqua* takes time of exposure to direct sun light, up to 20 minutes , with the same concentration of photosensitizer. Again to 15, 150 seconds of time exposure to direct sun light with the same concentration of

photosensitizer pupae stages can be controlled may be due to it is powerless stage. Also at adult stage, exposed to 60,120 sec. that can control this instar similar to the previous one . However from all treatments, pupae stages of *H. antiqua* could be sensitive to photosensitizer because it can be controlled during only 15 sec. then eggs instar that could be controlled during 30 sec . but adult instar couled be controlled with 6 0 sec. from light exposure to direct sun light and later 4th instar larvae which needed to, at lest 15 min. to be controlled with the same concentration from used photosensitizer.

Table (1): Effect of Rose Bengal on *Hylemyia antique* stages

Time	**Eggs**		
	LC_{50} and confidence limits	**Slope ± SE**	**LC_{90} and confidence limits**
30 Sec.	**0.000446 (0.000087 – 0.0020)**	**0.219 ± 0.006**	**315.022 (2.2943 – 107583)**
60 Sec.	**0.000084 (0.000036 – 0.000157)**	**0.516 ± 0.007**	**0.0253 (0.00892 – 0.1387)**
	Fourth instar larvae		
15 min.	**0.000347 (0.000119 – 0.000888)**	**0.316 ± 0.006**	**3.892 (0.2707 – 1601.33)**
20 min.	**0.00030 (0.000169 – 0.00053)**	**0.547 ± 0.008**	**0.0676 (0.0224 – 0.3862)**
	Pupae		
15 Sec	**0.000177815 0.00038094) (0.00006586 -**	**± 0.0654 0.3843**	**0.384 (0.06249954 - 12.88246017)**
150 Sec.	**0.0000142837 (0.00000301-0.00003591)**	**0.4805 ± 0.0007**	**0.00663741 (0.00253953- 0.0342026)**
	Adults		
60 Sec.	**0.00026 (0.000158 – 0.000395)**	**0.070 ± 0.0003**	**0.0166 (0.000275 –**
120 Sec.	**0.00029**	**1.088 ± 0.0054**	**0.003**

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Derivations of Tensor Product of Finite Number of Simple C*-Algebras.

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Abstract: In this paper we construct the derivations of $\overset{n}{\underset{i=1}{\otimes}}A_i$ in terms of the derivations of some simple C^*-algebras $A_i \ \forall i=1,2,.....,n.$ Also we introduce the concept of relative compatibility of finite number of A_i - derivations $\forall i=1,2,.....,n.$ We express the general form of any element c in the kernel of $\delta_{x\otimes\otimes_{k=2}^{n}I}$ where $c\in\otimes_{i=1}^{n}A_i$ and $x\in A_1$ in terms of some simple tensor product $c=I\otimes\otimes_{k=2}^{n}b_k$, $b\in\otimes_{k=2}^{n}A_k$. Finally we get a precise form of A_i-derivations $(\delta_i) \quad \forall i=1,2,3,.....,n$ in terms of a sequence of derivation $(\xi_{i_j})_{j=1}^{\infty}$ on A_i and their basis $(e_{i_j})_{j=1}^{\infty} \quad \forall i=1,2,3,.....,n$ .For resent results see [1],[3],[5] and [10]. [Journal of American Science 2010;6(8):31-38]. (ISSN: 1545-1003).

Key words: Simple C^*-algebra; Tensor product of C^*-algebra; A-derivation; Compatible derivation.

1. Introduction

If $d:\overset{n}{\underset{i=1}{\otimes}}A_i\to\overset{n}{\underset{i=1}{\otimes}}A_i$ is a derivation where $\overset{n}{\underset{i=1}{\otimes}}A_i$ be a tensor product of finite number of simple C^*- algebras.

Then for $\left(\overset{n}{\underset{i=1}{\otimes}}a_i\right),\left(\overset{n}{\underset{i=1}{\otimes}}b_i\right)in\left(\overset{n}{\underset{i=1}{\otimes}}A_i\right)$, we have

$$d\left(\left(\overset{n}{\underset{i=1}{\otimes}}a_i\right)\left(\overset{n}{\underset{i=1}{\otimes}}b_i\right)\right)=d\left(\overset{n}{\underset{i=1}{\otimes}}a_i\right)\left(\overset{n}{\underset{i=1}{\otimes}}b_i\right)+\left(\overset{n}{\underset{i=1}{\otimes}}a_i\right)d\left(\overset{n}{\underset{i=1}{\otimes}}b_i\right).$$

Let I be the identity of $A_i \quad \forall i=1,....,n.$ By $A_i \quad (\forall i=1,2,.....,n)$ we shall always mean simple C^*- algebras with countable basis. Each simple C^*-algebra A_i has the property $¢\left(A_i\right)=CI$, where $¢\left(A_i\right)$ is the centre of A_i see [9]. Recall that by a simple C^*- algebra A_i ,we shall always mean a C^*-algebras whose ideals are $\{0\}$ and A.

A linear map $D_i:A_i\to A_i, \qquad \forall i=1.2,.....,n$ is called a derivation if for each $a_i,b_i\in A_i$ $D_i\left(a_ib_i\right)=D_i\left(a_i\right)b_i+a_iD_i\left(b_i\right)$.

It is called a*-derivation if it satisfies $D_i\left(a_i\right)^*=D_i\left(a_i^*\right)\ \forall 1\le i\le n$.

For a fixed element $a_i\in A_i$, we can define $D_{a_i}:A_i\to A_i$,

where $D_{a_i}\left(b_i\right)=\left[a_i,b_i\right]=a_ib_i-b_ia_i$. It is known that D_{a_i} is a derivation,

which is called an inner derivation [9].

A derivation D_{a_i} is called approximately inner if it is the limit of a sequence of inner derivations. For more details about the definitions and results we can refer to [7]and [8].

2. Compatible derivations of finite number of simple C*- algebras.

Now we are going to define a derivation of finite number of simple C*- algebra.

Definition 2.1

Let $\overset{n}{\underset{i=1}{\otimes}}A_i$ be a tensor product of finite number of simple C^*- algebras. A linear map $\delta_i:A_i\to\overset{n}{\underset{i=1}{\otimes}}A_i$ is

called an A_i - derivation with respect to $\overset{n}{\underset{i=1}{\otimes}} A_i$, $i = 1,2,...,n$ if it satisfies,

$$\delta_1(a_1b_1) = \delta_1(a_1)\left(b_1 \otimes (\otimes_{k=2}^{n} I)\right) + \left(a_1 \otimes (\otimes_{k=2}^{n} I)\right)\delta_1(b_1),$$

$$\delta_i(a_ib_i) = \delta_i(a_i)\left((\otimes_{k=1}^{i-1} I) \otimes b_i \otimes (\otimes_{k=i+1}^{n} I)\right) + \left((\otimes_{k=1}^{i-1} I) \otimes a_i \otimes \otimes_{k=i+1}^{n} I\right)\delta_i(b_i), \quad 2 \le i \le n-1.$$

$$\delta_n(a_nb_n) = \delta_n(a_n)\left((\otimes_{k=1}^{n-1} I) \otimes b_n\right) + \left((\otimes_{k=1}^{n-1} I) \otimes a_n\right)\delta_n(b_n).$$

It is called a * - derivation with respect to $\overset{n}{\underset{i=1}{\otimes}} A_i$, if

$$\delta_i(a_i)^* = \delta_i(a_i^*) \quad \forall_i = 1,2,..,n.$$

Example 2.2

Let $C \in \overset{n}{\underset{i=1}{\otimes}} A_i$ and define

$\delta_i : A_i \to \overset{n}{\underset{i=1}{\otimes}} A_i \; i = 1,2,...,n$ by

$$\delta_1(a_1) = \delta_c\left(a_1 \otimes \overset{n}{\underset{k=2}{\otimes}} I\right)$$

$$\delta_i(a_i) = \delta_c\left(\left(\overset{i-1}{\underset{k=1}{\otimes}} I\right) \otimes a_i \otimes \left(\overset{n}{\underset{k=i+1}{\otimes}} I\right)\right)$$

$\forall i = 2,....,n-1,$

and $\delta_n(a_n) = \delta_c\left(\overset{n-1}{\underset{k=1}{\otimes}} I \otimes a_n\right)$.Then δ_i is an A_i - derivation with respect to $\overset{n}{\underset{i=1}{\otimes}} A_i \quad \forall i = 1,2,....,n$, which is called an inner A_i - derivation.

Next we are introduce the notion of compatibility of A_i - derivations $\forall i = 1,2,...,n$.

Definition 2.3

Let δ_i be A_i - derivation with respect to $\overset{n}{\underset{i=1}{\otimes}} A_i$, $i = 1,2,...,n$, then δ_i 's are compatible if the map

$d_i : \overset{n}{\underset{i=1}{\otimes}} A_i \to \overset{n}{\underset{i=1}{\otimes}} A_i$ defined by

$$d\left(\overset{n}{\underset{i=1}{\otimes}} a_i\right) = \delta_1(a_1)\left(I \otimes \overset{n}{\underset{k=2}{\otimes}} a_k\right) + \sum_{i=2}^{n-1}\left(\overset{i-1}{\underset{k=1}{\otimes}} a_k \otimes \overset{n}{\underset{k=i}{\otimes}} I\right)\delta_i(a_i)\left(\overset{i}{\underset{k=1}{\otimes}} I \otimes \overset{n}{\underset{k=i+1}{\otimes}} a_k\right) + \left(\overset{n-1}{\underset{k=1}{\otimes}} a_k \otimes I\right)\delta_n(a_n)$$

is a derivation of $\overset{n}{\underset{i=1}{\otimes}} A_i$. In this case we say that δ_i 's are the i th components of d.

Note:

We can say that δ_i , i =2,3 , …..n are compatible with δ_1 if the above condition is satisfied.

Example 2.4

Let $c \in \overset{n}{\underset{i=1}{\otimes}} A_i$ and $\forall a_i \in A_i$ let

$$\delta_i(a_i) = \begin{cases} \delta_c\left(a_1 \otimes \overset{n}{\underset{k=2}{\otimes}} I\right) & i = 1 \\ \delta_c\left(\overset{i-1}{\underset{k=1}{\otimes}} I \otimes a_i \otimes \overset{n}{\underset{k=I+1}{\otimes}} I\right) & 2 \le i < n \\ \delta_c\left(\overset{n-1}{\underset{k=1}{\otimes}} I \otimes a_n\right) & i = n. \end{cases}$$

Then δ_i 's are compatible $i = 1,2,....,n.$ Therefore we have,

$$d\left(\overset{n}{\underset{i=1}{\otimes}} a_i\right) = \delta_c\left(\overset{n}{\underset{i=1}{\otimes}} a_i\right).$$

Example 2.5

Let ξ_i be derivations on A_i , $i = 1,2,...,n,$ then

$$\delta_1(a_1) = \xi_1(a_1) \otimes \overset{n}{\underset{i=2}{\otimes}} I,$$

$$\delta_i(a_i) = \overset{i-1}{\underset{k=1}{\otimes}} I \otimes \xi_i(a_i) \otimes \overset{n}{\underset{k=i+1}{\otimes}} I \qquad , i = 2,...,n-1$$

and, $\delta_n(a_n) = \overset{n-1}{\underset{k=1}{\otimes}} I \otimes \xi_n(a_n)$, are compatible for,

$$d = \left(\xi_1 \otimes \overset{n}{\underset{k=2}{\otimes}} I\right) + \sum_{i=2}^{n-1}\left(\overset{i-1}{\underset{k=1}{\otimes}} I \otimes \xi_i \otimes \overset{n}{\underset{k=i+1}{\otimes}} I\right) + \left(\overset{n-1}{\underset{k=1}{\otimes}} I \otimes \xi_n\right)$$

see [7].

3. Main Results

We will be in need to the following Proposition which gives a necessary and sufficient condition for some A_i - derivations to be compatible.

Proposition 3.1

For $1 \le i \le n$, δ_i 's are compatible if and only if

$$\underset{I\otimes\overset{n}{\underset{k=2}{\otimes}}a_k}{\delta}(\delta_1(a_1))=\underset{a_1\otimes\overset{n}{\underset{k=2}{\otimes}}I}{\delta}(\delta_2(a_2))\left(\overset{2}{\underset{k=1}{\otimes}}I\otimes\overset{n}{\underset{k=3}{\otimes}}a_k\right)+$$

$$+\sum_{i=3}^{n-1}\left(I\otimes\overset{i-1}{\underset{k=2}{\otimes}}a_k\otimes\overset{n}{\underset{k=i}{\otimes}}I\right)\underset{a_1\otimes\overset{n}{\underset{k=2}{\otimes}}I}{\delta}(\delta_i(a_i))\left(\overset{i}{\underset{k=1}{\otimes}}I\otimes\overset{n}{\underset{k=i+1}{\otimes}}a_k\right)$$

$$+\left(I\otimes\overset{n-1}{\underset{k=2}{\otimes}}a_k\otimes I\right)\underset{a_1\otimes\overset{n}{\underset{k=2}{\otimes}}I}{\delta}(\delta_n(a_n)).$$

Proof:-

Let δ_i 's are compatible $i=1,2,...,n.$ then there exists a derivation $d:\overset{n}{\underset{i=1}{\otimes}}A_i\to\overset{n}{\underset{i=1}{\otimes}}A_i$,where

$$d\left(\overset{n}{\underset{i=1}{\otimes}}a_i\right)=\delta_1(a_1)\left(I\otimes\overset{n}{\underset{k=2}{\otimes}}a_k\right)+\sum_{i=2}^{n-1}\left(\overset{i-1}{\underset{k=1}{\otimes}}a_k\otimes\overset{n}{\underset{k=i}{\otimes}}I\right)\delta_i(a_i)\left(\overset{i}{\underset{k=1}{\otimes}}I\otimes\overset{n}{\underset{k=i+1}{\otimes}}a_k\right)$$

$$+\left(\overset{n-1}{\underset{k=1}{\otimes}}a_k\otimes I\right)\delta_n(a_n).\quad(3.1)$$

However,

$$d\left(\overset{n}{\underset{i=1}{\otimes}}a_i\right)=d\left(\left(I\otimes\overset{n}{\underset{k=2}{\otimes}}a_k\right)\left(a_1\otimes\overset{n}{\underset{k=2}{\otimes}}I\right)\right)$$

$$=d\left(I\otimes\overset{n}{\underset{k=2}{\otimes}}a_k\right)\left(a_1\otimes\overset{n}{\underset{k=2}{\otimes}}I\right)+\left(I\otimes\overset{n}{\underset{k=2}{\otimes}}a_k\right)d\left(a_1\otimes\overset{n}{\underset{k=2}{\otimes}}I\right)$$

$$=\{\ \delta_1(I)\left(I\otimes\overset{n}{\underset{k=2}{\otimes}}a_k\right)+\left(\overset{n}{\underset{k=1}{\otimes}}I\right)\delta_2(a_2)\left(\otimes^2_{k=1}I\otimes\overset{n}{\underset{k=3}{\otimes}}a_k\right)$$

$$+\sum_{i=3}^{n-1}\left(I\otimes\overset{i-1}{\underset{k=2}{\otimes}}a_k\otimes\overset{n}{\underset{k=i}{\otimes}}I\right)\delta_i(a_i)\left(\overset{i}{\underset{k=1}{\otimes}}I\otimes\overset{n}{\underset{k=i+1}{\otimes}}a_k\right)+\left(I\otimes\overset{n-1}{\underset{k=2}{\otimes}}a_k\otimes I\right)\delta_n(a_n)\}\left(a_1\otimes\overset{n}{\underset{k=2}{\otimes}}I\right)$$

$$+\left(I\otimes\overset{n}{\underset{k=2}{\otimes}}a_k\right)\delta_1(a_1)\left(\overset{n}{\underset{k=1}{\otimes}}I\right).\quad(3.2)$$

From (3.1) and (3.2) we get,

$$\left(I\otimes\overset{n}{\underset{k=2}{\otimes}}a_k\right)(\delta_1(a_1))-(\delta_1(a_1))\left(I\otimes\overset{n}{\underset{k=2}{\otimes}}a_k\right)=-(\delta_2(a_2))\left(\otimes^2_{k=1}a_k\otimes\overset{n}{\underset{k=3}{\otimes}}a_k\right)\left(a_1\otimes\overset{n}{\underset{k=2}{\otimes}}I\right)$$

$$-\sum_{i=3}^{n-1}\left(\left(I\otimes\overset{i-1}{\underset{k=2}{\otimes}}a_k\otimes\overset{n}{\underset{k=i}{\otimes}}I\right)(\delta_i(a_i))\left(\overset{i}{\underset{k=1}{\otimes}}I\otimes\overset{n}{\underset{k=i+1}{\otimes}}a_k\right)\right)\left(a_1\otimes\overset{n}{\underset{k=2}{\otimes}}I\right)$$

$$+\sum_{i=2}^{n-1}\left(\overset{i-1}{\underset{k=1}{\otimes}}a_k\otimes\overset{n}{\underset{k=i}{\otimes}}I\right)(\delta_i(a_i))\left(\overset{i}{\underset{k=1}{\otimes}}I\otimes\overset{n}{\underset{k=i+1}{\otimes}}a_k\right)+\left(I\otimes\overset{n-1}{\underset{k=2}{\otimes}}a_k\otimes I\right)(\delta_n(a_n))\left(a_1\otimes\overset{n}{\underset{k=2}{\otimes}}I\right)$$

$$+\left(\overset{n-1}{\underset{k=1}{\otimes}}a_k\otimes I\right)\delta_n(a_n).$$

Therefore,

$$\underset{I\otimes\overset{n}{\underset{k=2}{\otimes}}a_k}{\delta}(\delta_1(a_1))=-\delta_2(a_2)\left(\overset{2}{\underset{k=1}{\otimes}}I\otimes\overset{n}{\underset{k=3}{\otimes}}a_k\right)\left(a_1\otimes\overset{n}{\underset{k=2}{\otimes}}I\right)+$$

$$+\left(a_1\otimes\overset{n}{\underset{k=2}{\otimes}}I\right)(\delta_2(a_2))\left(\otimes^2_{k=1}I\otimes\overset{n}{\underset{k=3}{\otimes}}a_k\right)-\sum_{i=3}^{n-1}\left(I\otimes\overset{i-1}{\underset{k=2}{\otimes}}a_k\otimes\overset{n}{\underset{k=i}{\otimes}}I\right)(\delta_i(a_i))\left(a_1\otimes\overset{n}{\underset{k=2}{\otimes}}I\right)\left(\overset{i}{\underset{k=1}{\otimes}}I\otimes\overset{n}{\underset{k=i+1}{\otimes}}a_k\right)+$$

$$+\sum_{i=3}^{n-1}\left(I\otimes\overset{i-1}{\underset{k=2}{\otimes}}a_k\otimes\overset{n}{\underset{k=i}{\otimes}}I\right)\left(a_1\otimes\overset{n}{\underset{k=2}{\otimes}}I\right)(\delta_i(a_i))\left(\overset{i}{\underset{k=1}{\otimes}}I\otimes\overset{n}{\underset{k=i+1}{\otimes}}a_k\right)-\left(I\otimes\overset{n-1}{\underset{k=2}{\otimes}}a_k\otimes I\right)(\delta_n(a_n))\left(a_1\otimes\overset{n}{\underset{k=2}{\otimes}}I\right)$$

$$+\left(I\otimes\overset{n-1}{\underset{k=2}{\otimes}}a_k\otimes I\right)\left(a_1\otimes\overset{n}{\underset{k=2}{\otimes}}I\right)(\delta_n(a_n)).$$

Then we have,

$$\underset{I\otimes\overset{n}{\underset{k=2}{\otimes}}a_k}{\delta}(\delta_1(a_1))=\underset{a_1\otimes\overset{n}{\underset{k=2}{\otimes}}I}{\delta}(\delta_2(a_2))\left(\overset{2}{\underset{k=1}{\otimes}}I\otimes\overset{n}{\underset{k=3}{\otimes}}a_k\right)+\sum_{i=3}^{n-1}\left(I\otimes\overset{i-1}{\underset{k=2}{\otimes}}a_k\otimes\overset{n}{\underset{k=i}{\otimes}}I\right)\underset{\left(a_1\otimes\overset{n}{\underset{k=2}{\otimes}}I\right)}{\delta}(\delta_i(a_i))\left(\overset{i}{\underset{k=1}{\otimes}}I\otimes\overset{n}{\underset{k=i+1}{\otimes}}a_k\right)$$

$$+\left(I\otimes\overset{n-1}{\underset{k=2}{\otimes}}a_k\otimes I\right)(\delta_{a_1\otimes\overset{n}{\underset{k=2}{\otimes}}I}(\delta_n(a_n))).$$

On the other hand let,

$$\underset{I\otimes\overset{n}{\underset{k=2}{\otimes}}a_k}{\delta}(\delta_1(a_1))=\underset{a_1\otimes\overset{n}{\underset{k=2}{\otimes}}I}{\delta}(\delta_2(a_2))\left(\overset{2}{\underset{k=1}{\otimes}}I\otimes\overset{n}{\underset{k=3}{\otimes}}a_k\right)+\sum_{i=3}^{n-1}\left(I\otimes\overset{i-1}{\underset{k=2}{\otimes}}a_k\otimes\overset{n}{\underset{k=i}{\otimes}}I\right)\underset{a_1\otimes\overset{n}{\underset{k=2}{\otimes}}I}{\delta}(\delta_i(a_i))\left(\overset{i}{\underset{k=1}{\otimes}}I\otimes\overset{n}{\underset{k=i+1}{\otimes}}a_k\right)$$

$$+\left(I\otimes\overset{n-1}{\underset{k=2}{\otimes}}a_k\otimes I\right)\underset{a_1\otimes\overset{n}{\underset{k=2}{\otimes}}I}{\delta}(\delta_n(a_n)).$$

Now we need to show that δ_i 's are compatibles, $i=1,2,...,n$.

That is $d:\overset{n}{\underset{i=1}{\otimes}}A_i\to\overset{n}{\underset{i=1}{\otimes}}A_i$ is a derivation where for each $\left(\overset{n}{\underset{i=1}{\otimes}}a_i\right),\left(\overset{n}{\underset{i=1}{\otimes}}b_i\right)\in\overset{n}{\underset{i=1}{\otimes}}A_i$,

$$d\left(\left(\overset{n}{\underset{i=1}{\otimes}}a_i\right)\left(\overset{n}{\underset{i=1}{\otimes}}b_i\right)\right)=d\left(\overset{n}{\underset{i=1}{\otimes}}a_i\right)\left(\overset{n}{\underset{i=1}{\otimes}}b_i\right)+\left(\overset{n}{\underset{i=1}{\otimes}}a_i\right)d\left(\overset{n}{\underset{i=1}{\otimes}}b_i\right)$$

, and

$$d\left(\overset{n}{\underset{i=1}{\otimes}}a_i\right)=\delta_1(a_1)\left(I\otimes\overset{n}{\underset{k=2}{\otimes}}a_k\right)+\sum_{i=2}^{n-1}\left(\overset{i-1}{\underset{k=1}{\otimes}}a_k\otimes\overset{n}{\underset{k=i}{\otimes}}I\right)\delta_i(a_i)\left(\overset{i}{\underset{k=1}{\otimes}}I\otimes\overset{n}{\underset{k=i+1}{\otimes}}a_k\right)$$

$$+\left(\overset{n-1}{\underset{k=1}{\otimes}}a_k\otimes I\right)\delta_n(a_n).$$

Since,

$$d\left(\left(\overset{n}{\underset{i=1}{\otimes}}a_i\right)\left(\overset{n}{\underset{i=1}{\otimes}}b_i\right)\right)=d\left(\overset{n}{\underset{i=1}{\otimes}}a_ib_i\right)=\delta_1(a_1b_1)\left(I\otimes\overset{n}{\underset{k=2}{\otimes}}a_kb_k\right)+\sum_{i=2}^{n-1}\left(\overset{i-1}{\underset{k=1}{\otimes}}a_kb_k\otimes\overset{n}{\underset{k=i}{\otimes}}I\right)(\delta_i(a_ib_i))\left(\overset{i}{\underset{k=1}{\otimes}}I\otimes\overset{n}{\underset{k=i+1}{\otimes}}a_kb_k\right)+\left(\overset{n-1}{\underset{k=1}{\otimes}}a_kb_k\otimes I\right)(\delta_n(a_nb_n))$$

$$=(\delta_1(a_1))\left(I\otimes\overset{n}{\underset{k=2}{\otimes}}a_k\right)\left(\overset{n}{\underset{i=1}{\otimes}}b_i\right)+\left(a_1\otimes\overset{n}{\underset{k=2}{\otimes}}I\right)(\delta_1(b_1))\left(I\otimes\overset{n}{\underset{k=2}{\otimes}}a_kb_k\right)+\sum_{i=2}^{n-1}\left(\overset{i-1}{\underset{k=1}{\otimes}}a_kb_k\otimes\overset{n}{\underset{k=i}{\otimes}}I\right)(\delta_i(a_ib_i))\left(\overset{i}{\underset{k=1}{\otimes}}I\otimes\overset{n}{\underset{k=i+1}{\otimes}}a_kb_k\right)+\left(\overset{n-1}{\underset{k=1}{\otimes}}a_kb_k\otimes I\right)(\delta_n(a_n))\left(\overset{n-1}{\underset{k=1}{\otimes}}I\otimes b_n\right)+\left(\overset{n}{\underset{i=1}{\otimes}}a_i\right)\left(\overset{n-1}{\underset{k=1}{\otimes}}b_k\otimes I\right)(\delta_n(b_n))$$

$$=\delta_1(a_1)\left(I\otimes\overset{n}{\underset{k=2}{\otimes}}a_k\right)\left(\overset{n}{\underset{i=1}{\otimes}}b_i\right)+\left(a_1\otimes\overset{n}{\underset{k=2}{\otimes}}I\right)\left\{\delta_1(b_1)\left(I\otimes\overset{n-1}{\underset{k=2}{\otimes}}a_kb_k\otimes a_n\right)+\left(b_1\otimes\overset{n}{\underset{k=2}{\otimes}}I\right)(\delta_2(a_2b_2))\left(\otimes_{k=1}^{2}I\otimes\overset{n-1}{\underset{k=3}{\otimes}}a_kb_k\otimes a_n\right)+\sum_{i=3}^{n-2}\left(b_1\otimes\overset{i-1}{\underset{k=2}{\otimes}}a_kb_k\otimes\overset{n}{\underset{k=i}{\otimes}}I\right)(\delta_i(a_ib_i))\left(\overset{i}{\underset{k=1}{\otimes}}I\otimes\overset{n-1}{\underset{k=i+1}{\otimes}}a_kb_k\otimes a_n\right)+\left(b_1\otimes\otimes_{k=2}^{n-2}a_kb_k\otimes\otimes_{k=n-1}^{n}I\right)\delta_n(a_{n-1}b_{n-1})\left(\otimes_{k=1}^{n-1}I\otimes a_n\right)+\left(b_1\otimes\otimes_{k=2}^{n-2}a_kb_k\otimes I\right)\delta_n(a_n)\right\}\left(\overset{n-1}{\underset{k=1}{\otimes}}I\otimes b_n\right)+\left(\overset{n}{\underset{i=1}{\otimes}}a_i\right)\left(\overset{n-1}{\underset{k=1}{\otimes}}b_k\otimes I\right)(\delta_n(b_n))$$

(3.3)

Since,

$$\underset{I\otimes\overset{n-1}{\underset{k=2}{\otimes}}a_kb_k\otimes a_n}{\delta}(\delta_1(b_1))=\underset{b_1\otimes\overset{n}{\underset{k=2}{\otimes}}I}{\delta}(\delta_2(a_2b_2))\left(\overset{2}{\underset{k=1}{\otimes}}I\otimes\overset{n-1}{\underset{k=3}{\otimes}}a_kb_k\otimes a_n\right)+\sum_{i=3}^{n-2}\left(I\otimes\overset{i-1}{\underset{k=2}{\otimes}}a_kb_k\otimes\overset{n}{\underset{k=i}{\otimes}}I\right)\underset{b_1\otimes\overset{n}{\underset{k=2}{\otimes}}I}{\delta}(\delta_i(a_ib_i))\left(\overset{i}{\underset{k=1}{\otimes}}I\otimes\overset{n-1}{\underset{k=i+1}{\otimes}}a_kb_k\otimes a_n\right)$$

$$\left(I\otimes\otimes_{k=2}^{n-2}a_kb_k\otimes\otimes_{k=n-1}^{n}I\right)\underset{b_1\otimes\otimes_{k=2}^{n}I}{\delta}(\delta_n(a_{n-1}b_{n-1}))\left(\otimes_{k=1}^{n-1}I\otimes a_n\right)+\left(I\otimes\overset{n-1}{\underset{k=2}{\otimes}}a_kb_k\otimes I\right)\underset{b_1\otimes\overset{n}{\underset{k=2}{\otimes}}I}{\delta}(\delta_n(a_n))$$

Therefore,

$$\delta_1(b_1)\left(I\otimes\overset{n-1}{\underset{k=2}{\otimes}}a_kb_k\otimes a_n\right)+\left(b_1\otimes\overset{n}{\underset{k=2}{\otimes}}I\right)(\delta_2(a_2b_2))\left(\overset{2}{\underset{k=1}{\otimes}}I\otimes\overset{n-1}{\underset{k=3}{\otimes}}a_kb_k\otimes a_n\right)+\sum_{i=3}^{n-2}\left(b_1\otimes\overset{i-1}{\underset{k=2}{\otimes}}a_kb_k\otimes\overset{n}{\underset{k=i}{\otimes}}I\right)(\delta_i(a_ib_i))\left(\overset{i}{\underset{k=1}{\otimes}}I\otimes\overset{n-1}{\underset{k=i+1}{\otimes}}a_kb_k\otimes a_n\right)+\left(b_1\otimes\otimes_{k=2}^{n-2}a_kb_k\otimes\otimes_{k=n-1}^{n}I\right)(\delta_n(a_{n-1}b_{n-1}))\left(\otimes_{k=1}^{n-1}I\otimes a_n\right)+\left(b_1\otimes\otimes_{k=2}^{n-1}a_kb_k\otimes I\right)(\delta_n(a_n))\left(b_1\otimes\overset{n}{\underset{k=2}{\otimes}}I\right).$$

(3.4)

By (3.4) and (3.3) we have,

$$d\left(\left(\overset{n}{\underset{i=1}{\otimes}}a_i\right)\left(\overset{n}{\underset{i=1}{\otimes}}b_i\right)\right)=\delta_1(a_1)\left(I\otimes\overset{n}{\underset{k=2}{\otimes}}a_k\right)\left(\overset{n}{\underset{i=1}{\otimes}}b_i\right)+\left(a_1\otimes\overset{n}{\underset{k=2}{\otimes}}I\right)\left\{\left(I\otimes\overset{n-1}{\underset{k=2}{\otimes}}a_kb_k\otimes a_n\right)\delta_1(b_1)+\delta_2(a_2b_2)\left(b_1\otimes I\otimes\overset{n-1}{\underset{k=3}{\otimes}}a_kb_k\otimes a_n\right)+\sum_{i=3}^{n-2}\left(I\otimes\overset{i-1}{\underset{k=2}{\otimes}}a_kb_k\otimes\overset{n}{\underset{k=i}{\otimes}}I\right)(\delta_i(a_ib_i))\left(b_1\otimes\overset{i}{\underset{k=2}{\otimes}}I\otimes\overset{n-1}{\underset{k=i+1}{\otimes}}a_kb_k\otimes a_n\right)+\left(I\otimes\otimes_{k=2}^{n-2}a_kb_k\otimes\otimes_{k=n-1}^{n}I\right)(\delta_n(a_{n-1}b_{n-1}))\left(b_1\otimes\otimes_{k=2}^{n-1}I\otimes a_n\right)+\left(I\otimes\overset{n-1}{\underset{k=2}{\otimes}}a_kb_k\otimes I\right)(\delta_n(a_n))\left(b_1\otimes\overset{n}{\underset{k=2}{\otimes}}I\right)\right\}\left(\overset{n-1}{\underset{k=1}{\otimes}}I\otimes b_n\right)+\left(\overset{n}{\underset{i=1}{\otimes}}a_i\right)\left(\overset{n-1}{\underset{k=1}{\otimes}}b_k\otimes I\right)(\delta_n(b_n))=(\delta_1(a_1))\left(I\otimes\overset{n}{\underset{k=2}{\otimes}}a_k\right)\left(\overset{n}{\underset{i=1}{\otimes}}b_i\right)+\left(a_1\otimes\overset{n}{\underset{k=2}{\otimes}}I\right)\left\{\left(I\otimes\overset{n-1}{\underset{k=2}{\otimes}}a_kb_k\otimes a_n\right)(\delta_1(b_1))+(\delta_2(a_2))\left(\otimes_{k=1}^{2}b_k\otimes\overset{n-1}{\underset{k=3}{\otimes}}a_kb_k\otimes a_n\right)+\left(I\otimes a_2\otimes\overset{n}{\underset{k=3}{\otimes}}I\right)(\delta_2(b_2))\left(b_1\otimes I\otimes\overset{n-1}{\underset{k=3}{\otimes}}a_kb_k\otimes a_n\right)+$$

$$\sum_{i=3}^{n-1}\left(\left(I\otimes\overset{i-2}{\underset{k=2}{\otimes}}a_kb_k\otimes\overset{n}{\underset{k=i}{\otimes}}I\right)(\delta_i(a_i))\left(b_1\otimes\overset{i-1}{\underset{k=2}{\otimes}}I\otimes b_i\otimes\overset{n-1}{\underset{k=i+1}{\otimes}}a_kb_k\otimes a_n\right)+\left(I\otimes b_2\otimes\overset{n-1}{\underset{k=3}{\otimes}}a_kb_k\otimes I\right)\delta_n(a_n)\right\}\left(b_1\otimes\overset{n-1}{\underset{k=2}{\otimes}}I\otimes b_n\right)+$$

$$\left(\overset{n}{\underset{i=1}{\otimes}}a_i\right)\left(\overset{n-1}{\underset{k=1}{\otimes}}b_k\otimes I\right)(\delta_n(b_n)).$$

Repeating the above process n-1 times, we get,

$$d\left(\left(\overset{n}{\underset{i=1}{\otimes}}a_i\right)\left(\overset{n}{\underset{i=1}{\otimes}}b_i\right)\right)=$$

$$=\left\{(\delta_1(a_1))\left(I\otimes\overset{n}{\underset{k=2}{\otimes}}a_n\right)\right.$$

$$+\sum_{i=2}^{n-1}\left(\overset{i-1}{\underset{k=1}{\otimes}}a_k\otimes\overset{n}{\underset{k=i}{\otimes}}I\right)(\delta_i(a_i))\left(\overset{i}{\underset{k=1}{\otimes}}I\otimes\overset{n}{\underset{k=i+1}{\otimes}}a_k\right)$$

$$\left.+\left(\overset{n-1}{\underset{k=1}{\otimes}}a_k\otimes I\right)(\delta_n(a_n))\right\}\left(\overset{n}{\underset{i=1}{\otimes}}b_i\right)+$$

$$\left(\overset{n}{\underset{i=1}{\otimes}}a_i\right)\left\{\left(I\otimes\overset{n-1}{\underset{k=2}{\otimes}}b_k\otimes I\right)(\delta_1(b_1))\left(\overset{n-1}{\underset{k=1}{\otimes}}I\otimes b_n\right)+\left(\sum_{i=2}^{n-2}\left(\overset{i}{\underset{k=1}{\otimes}}I\otimes\overset{n-1}{\underset{k=i+1}{\otimes}}b_k\otimes I\right)(\delta_i(b_i))\left(\overset{i-1}{\underset{k=1}{\otimes}}b_k\otimes\overset{n-1}{\underset{k=i}{\otimes}}I\otimes b_n\right)\right.\right.$$

$$\left.\left.+\left(\overset{n}{\underset{k=1}{\otimes}}I\right)(\delta_{n-1}(b_{n-1}))\left(\overset{n-2}{\underset{k=1}{\otimes}}b_k\otimes I\otimes b_n\right)\right)+\left(\overset{n-1}{\underset{k=1}{\otimes}}b_k\otimes I\right)(\delta_n(b_n))\right\}$$

$$=\left(d\left(\overset{n}{\underset{i=1}{\otimes}}a_i\right)\right)\left(\overset{n}{\underset{i=1}{\otimes}}b_i\right)+\left(\overset{n}{\underset{i=1}{\otimes}}a_i\right)d\left(\overset{n}{\underset{i=1}{\otimes}}b_i\right),$$

which implies that $\delta_i,\quad i=1,2,....,n$ are compatible, that is, $d:\overset{n}{\underset{i=1}{\otimes}}A_i\to\overset{n}{\underset{i=1}{\otimes}}A_i$ is a derivation where

$$\left(I\otimes\overset{n-1}{\underset{k=2}{\otimes}}b_k\otimes I\right)\delta_1(b_1)\left(\overset{n-1}{\underset{k=1}{\otimes}}I\otimes b_n\right)+\sum_{i=2}^{n-2}\left(\overset{i}{\underset{k=1}{\otimes}}I\otimes\overset{n-1}{\underset{k=i+1}{\otimes}}b_k\otimes I\right)\delta_i(b_i)\left(\overset{i-1}{\underset{k=1}{\otimes}}b_k\otimes\overset{n-1}{\underset{k=i}{\otimes}}I\otimes b_n\right)$$

$$+\left(\overset{n}{\underset{k=1}{\otimes}}I\right)\delta_{n-1}(b_{n-1})\left(\overset{n-2}{\underset{k=1}{\otimes}}b_k\otimes I\otimes b_n\right)$$

$$+\left(\overset{n-1}{\underset{k=1}{\otimes}}b_k\otimes I\right)\delta_n(b_n)=d\left(\overset{n}{\underset{i=1}{\otimes}}b_i\right).$$

In the following we introduce the concept of relative compatibility of finite number of A_i - derivations $\forall i=1,2,.....,n.$

Definition 3.2

If δ_i *and* δ_i' $,i=2,.....,n.$ are compatible with δ_1,then we say that $(\delta_2,\delta_3,.....,\delta_n)$ *is* δ_1- compatible

to $(\delta_2', \delta_3', ..., \delta_n')$,written by

$\delta_i \equiv \delta_i' \pmod{\delta_1} \quad \forall i = 2,3,.....,n.$

We express the general form of any element c in the kernel of $\delta_{x \otimes \otimes_{k=2}^n I}$ where $c \in \otimes_{i=1}^n A_i$ and $x \in A_1$ in terms of some simple tensor product $c = I \otimes \otimes_{k=2}^n b_k$, $b \in \otimes_{k=2}^n A_k$.

Proposition 3.3

Let A_i be simple C^*- algebra $\forall i = 1,2,.....,n.$ Let $c \in \otimes_{i=1}^n A_i$, for each $x \in A_1$, $\delta_{x \otimes \otimes_{k=2}^n I}(c) = 0$. Then $c = I \otimes \otimes_{k=2}^n b_k$ for some $I \otimes \otimes_{k=2}^n b_k \in \otimes_{k=1}^n A_k$. Moreover $\delta_i \equiv \delta_i' \pmod{\delta_1} \quad \forall i = 2,3,.....,n$, if for some f_i derivations of A_i, $(\delta_i - \delta_i')(a_i) = \otimes_{k=1}^{i-1} I \otimes f_i(a_i) \otimes \otimes_{k=i+1}^n I$, for all $2 \le i \le n-1 \; and, (\delta_n - \delta_n')(a_n) = \otimes_{k=1}^{n-1} I \otimes f_n(a_n).$

Proof.

Firstly, let $c = \sum_{j=1}^{\infty} a_{1_j} \otimes \otimes_{i=2}^n b_{i_j}$,where b_{i_j} 's are linearly independent $\forall i = 2,3,.....,n.$ Since

$$\delta_{x \otimes \otimes_{k=2}^n I}(c) = \delta_c(x \otimes \otimes_{k=2}^n I) = 0.$$

$$thus, \quad c(x \otimes \otimes_{k=2}^n I) - (x \otimes \otimes_{k=2}^n I)c = 0 \quad \forall x \in A_1,$$

then ,

$$\sum_{j=1}^{\infty} (a_{1_j} \otimes \otimes_{i=2}^n b_{i_j})(x \otimes \otimes_{k=2}^n I) - (x \otimes \otimes_{k=2}^n I)(\sum_{j=1}^{\infty} (a_{1_j} \otimes \otimes_{i=2}^n b_{i_j})) = 0.$$

thus, $\sum_{j=1}^{\infty} \left((a_{1_j} x - x a_{1_j}) \otimes \otimes_{i=2}^n b_{i_j} \right) = 0$.

Secondly, since b_{i_j} 's are linearly independent $\forall i = 2,3,.....,n,$

Then $\delta_{a_{1_j}}(x) = 0 \qquad \forall x \in A_1$ see [7].

Thus , $a_{1_j} x - x a_{1_j} = 0, \; a_{1_j} \in Z(A_1) = CI$,then $a_{1_j} = \alpha_j I \quad , \quad \alpha_j \in C$ see[9].

Finally,

$$c = \sum_{j=1}^{\infty} (a_{1_j} \otimes \otimes_{i=2}^n b_{i_j}) = \sum_{j=1}^{\infty} (\alpha_j I \otimes \otimes_{i=2}^n b_{i_j}) = \sum_{j=1}^{\infty} (I \otimes (\overline{\alpha_j} \otimes_{i=2}^n b_{i_j})).$$

Now let $(\overline{\alpha_j} \otimes_{i=2}^n b_{i_j}) = \otimes_{i=2}^n b_{i_j}'$. Therefore, $c = (I \otimes \otimes_{i=2}^n b_{i_j}')$ the first part of the Proposition is proved.

Let, $(\delta_2, \delta_3, ..., \delta_n) \; be \; \delta_1 -$ compatible to $(\delta_2', \delta_3', ..., \delta_n')$,then there is a mapping

$d_i : \overset{n}{\underset{i=1}{\otimes}} A_i \to \overset{n}{\underset{i=1}{\otimes}} A_i$, defined by

$\forall \otimes_{i=1}^n a_i \in \overset{n}{\underset{i=1}{\otimes}} A_i$

$$d\left(\overset{n}{\underset{i=1}{\otimes}} a_i\right) = \delta_1(a_1)\left(I \otimes \overset{n}{\underset{k=2}{\otimes}} a_k\right) + \sum_{i=2}^{n-1}\left(\overset{i-1}{\underset{k=1}{\otimes}} a_k \otimes \overset{n}{\underset{k=i}{\otimes}} I\right)(\delta_i(a_i))\left(\overset{i}{\underset{k=1}{\otimes}} I \otimes \overset{n}{\underset{k=i+1}{\otimes}} a_k\right) + \left(\overset{n-1}{\underset{k=1}{\otimes}} a_k \otimes I\right)(\delta_n(a_n)),$$

$$= (\delta_1(a_1))\left(I \otimes \overset{n}{\underset{k=2}{\otimes}} a_k\right) + \sum_{i=2}^{n-1}\left(\overset{i-1}{\underset{k=1}{\otimes}} a_k \otimes \overset{n}{\underset{k=i}{\otimes}} I\right)(\delta_i(a_i))\left(\overset{i}{\underset{k=1}{\otimes}} I \otimes \overset{n}{\underset{k=i+1}{\otimes}} a_k\right) + \left(\overset{n-1}{\underset{k=1}{\otimes}} a_k \otimes I\right)(\delta_n(a_n)),$$

is a derivations of $\overset{n}{\underset{i=1}{\otimes}} A_i$.

Moreover,

$$\underset{I \otimes \overset{n}{\underset{k=2}{\otimes}} a_k}{\delta}(\delta_1(a_1)) = \underset{a_1 \otimes \overset{n}{\underset{k=2}{\otimes}} I}{\delta}(\delta_2(a_2))\left(\overset{2}{\underset{k=1}{\otimes}} I \otimes \overset{n}{\underset{k=3}{\otimes}} a_k\right) +$$

$$+ \sum_{i=3}^{n-1}\left(I \otimes \overset{i-1}{\underset{k=2}{\otimes}} a_k \otimes \overset{n}{\underset{k=i}{\otimes}} I\right)\underset{a_1 \otimes \overset{n}{\underset{k=2}{\otimes}} I}{\delta}(\delta_i(a_i))\left(\overset{i}{\underset{k=1}{\otimes}} I \otimes \overset{n}{\underset{k=i+1}{\otimes}} a_k\right)$$

$$+ \left(I \otimes \overset{n-1}{\underset{k=2}{\otimes}} a_k \otimes I\right)\underset{a_1 \otimes \overset{n}{\underset{k=2}{\otimes}} I}{\delta}(\delta_n(a_n)).$$

$$= \underset{a_1 \otimes \overset{n}{\underset{k=2}{\otimes}} I}{\delta}(\delta_2'(a_2))\left(\overset{2}{\underset{k=1}{\otimes}} I \otimes \overset{n}{\underset{k=3}{\otimes}} a_k\right) +$$

$$+ \sum_{i=3}^{n-1}\left(I \otimes \overset{i-1}{\underset{k=2}{\otimes}} a_k \otimes \overset{n}{\underset{k=i}{\otimes}} I\right)\underset{a_1 \otimes \overset{n}{\underset{k=2}{\otimes}} I}{\delta}(\delta_i'(a_i))\left(\overset{i}{\underset{k=1}{\otimes}} I \otimes \overset{n}{\underset{k=i+1}{\otimes}} a_k\right)$$

$$+ \left(I \otimes \overset{n-1}{\underset{k=2}{\otimes}} a_k \otimes I\right)\underset{a_1 \otimes \overset{n}{\underset{k=2}{\otimes}} I}{\delta}(\delta_n'(a_n)).$$

Then,

$$\delta_{a_1\otimes\overset{n}{\underset{k=2}{\otimes}}I}((\delta_2-\delta_2')(a_2))\left(\overset{2}{\underset{k=1}{\otimes}}I\otimes\overset{n}{\underset{k=3}{\otimes}}a_k\right)+$$

$$+\sum_{i=3}^{n-1}\left(I\otimes\overset{i-1}{\underset{k=2}{\otimes}}a_k\otimes\overset{n}{\underset{k=i}{\otimes}}I\right)\delta_{a_1\otimes\overset{n}{\underset{k=2}{\otimes}}I}((\delta_i-\delta_i')(a_i))\left(\overset{i}{\underset{k=1}{\otimes}}I\otimes\overset{n}{\underset{k=i+1}{\otimes}}a_k\right)$$

$$+\left(I\otimes\overset{n-1}{\underset{k=2}{\otimes}}a_k\otimes I\right)\delta_{a_1\otimes\overset{n}{\underset{k=2}{\otimes}}I}((\delta_n-\delta_n')(a_n))=0.$$

$$Thus,\ \delta_{a_1\otimes\overset{n}{\underset{k=2}{\otimes}}I}\{(\delta_2-\delta_2')(a_2)\left(\overset{2}{\underset{k=1}{\otimes}}I\otimes\overset{n}{\underset{k=3}{\otimes}}a_k\right)+$$

$$\sum_{i=3}^{n-1}\left(I\otimes\overset{i-1}{\underset{k=2}{\otimes}}a_k\otimes\overset{n}{\underset{k=i}{\otimes}}I\right)((\delta_i-\delta_i')(a_i))\left(\overset{i}{\underset{k=1}{\otimes}}I\otimes\overset{n}{\underset{k=i+1}{\otimes}}a_k\right)$$

$$+\left(I\otimes\overset{n-1}{\underset{k=2}{\otimes}}a_k\otimes I\right)((\delta_n-\delta_n')(a_n))\}=0.$$

Using the first part of this Proposition, we have,

$$(\delta_2-\delta_2')(a_2)\left(\overset{2}{\underset{k=1}{\otimes}}I\otimes\overset{n}{\underset{k=3}{\otimes}}a_k\right)+\sum_{i=3}^{n-1}\left(I\otimes\overset{i-1}{\underset{k=2}{\otimes}}a_k\otimes\overset{n}{\underset{k=i}{\otimes}}I\right)((\delta_i-\delta_i')(a_i))\left(\overset{i}{\underset{k=1}{\otimes}}I\otimes\overset{n}{\underset{k=i+1}{\otimes}}a_k\right)$$

$$+\left(I\otimes\overset{n-1}{\underset{k=2}{\otimes}}a_k\otimes I\right)((\delta_n-\delta_n')(a_n))=I\otimes\otimes_{k=2}^{n}b_k=d\left(I\otimes\otimes_{k=2}^{n}a_k\right)$$

where d is a derivation on $\otimes_{i=1}^{n}A_i$.

Hence,

$$(\delta_i-\delta_i')(a_i)=\otimes_{k=1}^{i-1}I\otimes f_i(a_i)\otimes\otimes_{k=i+1}^{n}I,$$

for

$$2\le i\le n-1\ \ and,\ \ (\delta_n-\delta_n')(a_n)=\otimes_{k=1}^{n-1}I\otimes f_n(a_n).$$

where, f_i are derivations of A_i $,i=2,3,.....,n.$

The other direction of the Proposition can be proved by using example (2.5), that is, let ξ_i be derivations on A_i , $i=1,2,...,n,$ then

$$\delta_1(a_1)=\xi_1(a_1)\otimes\overset{n}{\underset{k=2}{\otimes}}I,$$

$$\delta_i(a_i)=\overset{i-1}{\underset{k=1}{\otimes}}I\otimes\xi_i(a_i)\otimes\overset{n}{\underset{k=i+1}{\otimes}}I\qquad,i=2,...,n-1$$

and , $\delta_n(a_n)=\overset{n-1}{\underset{k=1}{\otimes}}I\otimes\xi_n(a_n)$,are compatibles , since

$$d=\left(\xi_1\otimes\overset{n}{\underset{k=2}{\otimes}}I\right)+\sum_{i=2}^{n-1}\left(\overset{i=1}{\underset{k=1}{\otimes}}I\otimes\xi_i\otimes\overset{n}{\underset{k=i+1}{\otimes}}I\right)+\left(\overset{n-1}{\underset{k=1}{\otimes}}I\otimes\xi_n\right)$$

.

And then the proof is completed.

Finally, we get a precise form of A_i − derivations (δ_i) $\forall i=1,2,3,.....,n$ in terms of a sequence of derivations $(\xi_{i_j})_{j=1}^{\infty}$ on A_i and their basis $(e_{i_j})_{j=1}^{\infty}$, $\forall i=1,2,3,.....,n$.

Proposition 3.4

Let $\left\{e_{i_j}\right\}_{j=1}^{\infty}$ are bases for A_i $\forall i=1,2,3,.....,n$, and δ_i be A_i − derivations. Then there is sequence $\left\{\zeta_{i_j}\right\}_{j=1}^{\infty}$ of derivations of A_i , such that,

$$\delta_i(a_i)=\begin{cases}\sum_{j=1}^{\infty}\zeta_{1_j}(a_1)\otimes\otimes_{k=2}^{n}e_{k_j} & i=1.\\ \sum_{j=1}^{\infty}(\otimes_{k=1}^{i-1}e_{k_j}\otimes\zeta_{i_j}(a_i)\otimes\otimes_{k=i+1}^{n}e_{k_j}) & 1\le i\le n.\\ \sum_{j=1}^{\infty}(\otimes_{k=1}^{n-1}e_{k_j}\otimes\zeta_{n_j}(a_n)) & i=n.\end{cases}$$

Proof.

Let $a_i,b_i\in A_i$, $\forall i=2,3,....,n-1.$

Then we have,

$$\delta_i(a_ib_i)=\sum_{j=1}^{\infty}(\otimes_{k=1}^{i-1}e_{k_j}\otimes\zeta_{i_j}(a_ib_i)\otimes\otimes_{k=i+1}^{n}e_{k_j})$$

$$=\delta_i(a_i)(\otimes_{k=1}^{i-1}I\otimes b_i\otimes\otimes_{i+1}^{n}I)+(\otimes_{k=1}^{i-1}I\otimes a_i\otimes\otimes_{i+1}^{n}I)\delta_i(b_i)$$

$$=\sum_{j=1}^{\infty}(\otimes_{k=1}^{i-1}e_{k_j}\otimes\zeta_{i_j}(a_i)b_i\otimes\otimes_{k=i+1}^{n}e_{k_j})+\sum_{j=1}^{\infty}(\otimes_{k=1}^{i-1}e_{k_j}\otimes a_i\zeta_{i_j}(b_i)\otimes\otimes_{k=i+1}^{n}e_{k_j}).$$

Thus,

$$\sum_{j=1}^{\infty}(\otimes_{k=1}^{i-1}e_{k_j}\otimes(\zeta_{i_j}(a_ib_i)-(\zeta_{i_j}(a_i)b_i+a_i\zeta_{i_j}(b_i)))\otimes\otimes_{k=i+1}^{n}e_{k_j})=0.$$

Since, $(e_{i_j})_{j=1}^{\infty}$,are linear independent $\forall i=1,2,.....,n,$ see[7].

Therefore, $\zeta_{i_j}(a_ib_i)=(\zeta_{i_j}(a_i)b_i+a_i\zeta_{i_j}(b_i))$.

Then we have $\left\{\zeta_i\right\}_j$ be sequence of derivations on A_i $\forall i=2,.....,n-1.$

Similarly, we can show that $\left\{\zeta_1\right\}_j$ and $\left\{\zeta_n\right\}_j$ are sequences of derivations on A_1 , A_n respectively.

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Study the Suitability of Cheese Whey for Bio-Butanol Production by Clostridia

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Abstract: The aim of the present paper is to investigate the feasibility of bio-butanol production by batch fermentation of cheese whey a dairy industry waste characterized by lactose concentration ranging from 4.5% to 5.0 % (w/w). The microorganisms used to carry out the fermentation processes was *Clostridium acetobutylicum* DSM 792 and *Clostridium acetobutylicum* AS 1.224. Preliminary experiments, performed in aerobic conditions on lactose medium lab experiments, have shown that *Clostridium acetobutylicum* DSM 792 was the best in the solvents production compared with AS 1.224. The bio reactor batch experiments were carried out on lactose and cheese whey media. The experimental data have demonstrated the process feasibility that cheese whey is an excellent substrate for fermentation and exhibits better performance with respect to lactose medium. [Journal of American Science 2010;6(8):39-46]. (ISSN: 1545-1003).

Key words: cheese whey, lactose, bio-butanol, bio-ethanol, *Clostridium acetobutylicum*

1. Introduction

When oil price suddenly increased in 1973, intensive research interests returned on the conversion of agricultural products into fuels and chemicals.

Biofuels used in transport are typically bioethanol which is used as a petrol substitute and biodiesel which is used as a diesel substitute. Also, butanol has sufficiently similar characteristics to gasoline to be used directly in any gasoline engine without modification and/or substitution. Butanol is superior to ethanol as a fuel additive in many regards: higher energy content, lower volatility, less hydroscopic (thus does not pick up water), and less corrosive (Dürre, 2007). Branched chain 4-carbon alcohols including isobutanol, 2-methyl-1-butanol and 3-methyl-1-butanol have higher octane numbers compared with n-butanol (Atsumi *et al*., 2008) and thus are good candidates as fuel additives (Lee *et al*., 2008).

During the early 1980s, many problems that have prevented butanol fermentation from being commercially viable were presented. These obstacles can be enumerated as: (1) use of dilute sugar solutions due to butanol inhibition; (2) low product concentration in fermentation broth which is attributed to butanol inhibition and results in cost intensive recovery; (3) low product yield, usually of the order of 0.3 due to conversion of approximately 53% of substrate into CO_2 and H_2; and (4) low reactor productivity. In spite of these problems, it has been stressed that butanol can be economically produced if cheaper substrates are used. Substrates that require minimum processing and fall in this category are by-products or waste products of agricultural industries such as molasses, and whey permeate (Dürre, 2007).

On the other hand, an estimated 90.5 billion pounds of whey was generated as a byproduct of cheese production in 2006, comprising about 85.8 billion pounds of sweet whey and 4.7 billion pounds of acid whey. Over the last 5 years from 2001 to 2006, the volume of whey increased by 15 % commensurate of whey increases in the production of cheeses. Besides the liquid carrier, the composition of whey is approximately 0.3 percent butterfat, 0.8 percent whey proteins, 4.9 percent lactose, and 0.5 percent minerals. Cumulatively, there were 4.4 billion pounds of lactose contained in the whey produced that year (Charles, 2008).

In Egypt, more than 70% of milk produced is used in cheese making to produce about 336939 tons fresh cheese annually (CAPMS, 2004), in addition to, all cheese varieties. In China, cheese production is increased rapidly from 10.000 tons (2006) to 15.000 tons (2008), which increased the amount of whey from 90.000 to 135.000 tons respectively. These are no plans for the utilization of whey in Egypt and China as in the most countries in the world. So, increasing cheese production will create pollution problems in addition to its fuel interest.

The present study is intended to investigate the possibility of using cheese whey as a source for bio-butanol production, evidencing the differences existing between lactose medium and cheese whey, which – in principle – could be used as raw materials to achieve fermentation processes aimed at bio-butanol production.

2. Material and Methods

Microorganisms

Clostridium acetobutylicum DSM 792, which corresponds to ATCC strain 824 was donated by Institute of Microbiology, Beijing, China. While, *Clostridium acetobutylicum* AS 1.224 was isolated from soil by Laboratory of Molecular Physiology and Systems Biotechnology, Department of Industrial Microbiology and Biotechnology, Institute of Microbiology Chinese Academy of Sciences, Beijing, China.

Preparation of lactose medium

The medium contained per L of deionized water: KH_2PO_4, 0.75 g, K_2HPO_4, 0.75 g, Mg SO_4 – H_2O, 0.4 g, $MnS0_4$-H_2O, 0.01g, $FeSO_4$ –7 H_2O = 0.01 g, Na CI, 1 g , Yeast extract, 5 g, Asparagine, 2 g, $(NH_4)2$ SO_4, 2 g, Cysteine-HCL , 1g, Rezurium, 50 g lactose and 1 ml. $CaCO_2$, 2 g (only for pre cultures). The pH value was adjusted to 7 using Na OH (2 N). After sterilization, the medium was sparged with nitrogen to remove the oxygen, and maintained under nitrogen pressure until its inoculation.

Preparation of cheese whey as fermentation medium

Cheese whey (containing 45– 50 g/l lactose) was obtained from Inner Mongolia Yili Industrial Group Co., Ltd., Mongolia, China. Whey was boiled for 30 min to denature and precipitate most of the whey proteins (Chen and Zall, 1982). The precipitated whey protein was separated and the supernatant was filtered through cheese cloth to remove the balance of any minute amounts of protein.

Lab batch experiment:

The 3 ml of inoculum were grown in 100-ml screw-cap bottles with rubber septa under nitrogen using 50 ml of degassed lactose medium and inoculated at 37 ^oC for 75 hrs with the agitation rate of 150 rpm, samples were taken after 50 and 75 hrs for analysis.

Bio-reactor batch experiments:

Best strain for butanol production was used to run bio-reactor batch experiments. Pre cultures were grown in 100-ml screw-cap bottles with rubber septa under nitrogen using 50 ml of degassed lactose medium and inoculated at 37 ^oC. At least two transfers were prepared before fermentor inoculated. A fermentor of 7.5-liter (New Brunswick Scientific Co., Edison, NJ) with pH and temperature control was used in batch fermentation. Two different batch experiments were done using *C. acetobutylicum* DSM 792 with lactose medium and deproteinized whey (without adding yeast extract). Lactose medium (containing 50 g of lactose /liter) reached volume of 3.0 liter was inoculated with 300 ml of the pre culture. The pH for the fermentation was regulated at 5.0 by automatic addition of ammonia solution (NH_3-H_2O), the temperature was maintained at 37°C with 150 rpm agitation rate. Samples were taken every 12 hrs for 5 days to determine different biofuels production.

HPLC analysis

The concentration of substrate and fermentation products was measured by a High-Pressure Liquid Chromatography (HPLC, Agilent 1200) with refractive index detector. The separation was obtained with an Aminex HPX-87H (Bio-Rad) column (300 by 7.8 mm). Elution was done at 15°C with 0.05 mM H_2SO_4 at a flow rate of 0.5 ml/min.

3. Results and Results

Bio-butanol formation in Lab batch experiment

Figure-1 and -2 show comparative formations of biofuels in lab batch experiment (lactose medium) using *Clostridium acetobutylicum* DSM 792, and *Clostridium acetobutylicum* AS 1.224 after 50 hrs of fermentation process.

It could be noticed that *Clostridium acetobutylicum* DSM 792 showed obvious differences from *Clostridium acetobutylicum* AS 1.224 by lactose utilization which has reflected to fermentation products. The intermediate accumulation of acetic and butyric acid was very low in *Clostridium acetobutylicum* AS 1.224 compared to *Clostridium acetobutylicum* DSM 792. These results are in agreement with those obtained by Bieb, (1999), found that butanol and acetone-producing strain DSM 2152, invalidly described as '*Clostridium saccharoperbutyl- acetonicum* ' was compared with the type strain *C. acetobutylicum, DSM 792*, with respect to solvent and acid formation at varying pH values and growth rates. Under all conditions strain DSM 2152 produced much lower amounts of butyric and acetic acids than the type strain. The pH optimum for solvent formation was higher, ie 5.5 instead of 4.5. Solvent formation occurred at higher dilution rates, but below 0.1 h^{-1} a lower solvent concentration was obtained, indicating that acid production was too low to provide a sufficient amount for acetone formation. The strain (*Clostridium acetobutylicum* AS 1.224) is a candidate for genetic improvement supplementary to the type

strain of *C. acetobutylicum* which is the subject of a rapid development in DNA recombination.

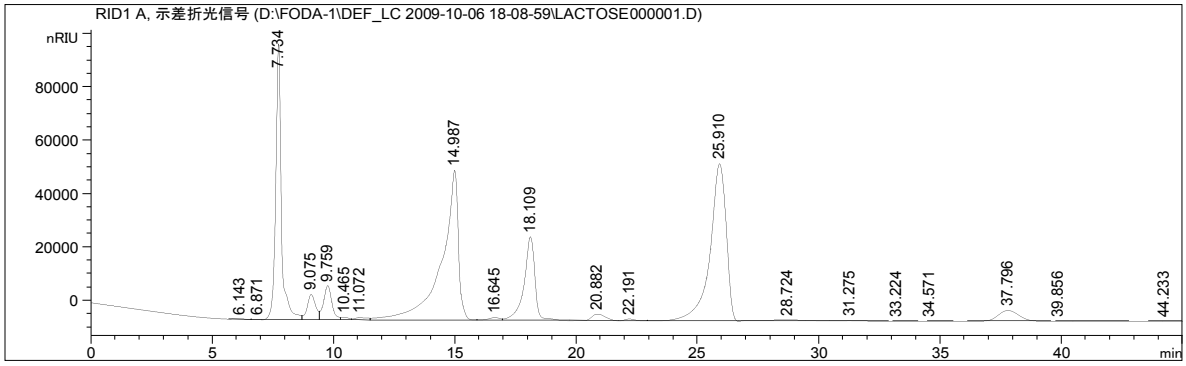


Fig. (1): HPLC-chromatogram of biofuels formation by *Clostridium acetobutylicum* DSM 792 using lactose medium (37° C, shaking velocity 150 rpm, pH 7.0) after incubation time for 50 hrs.

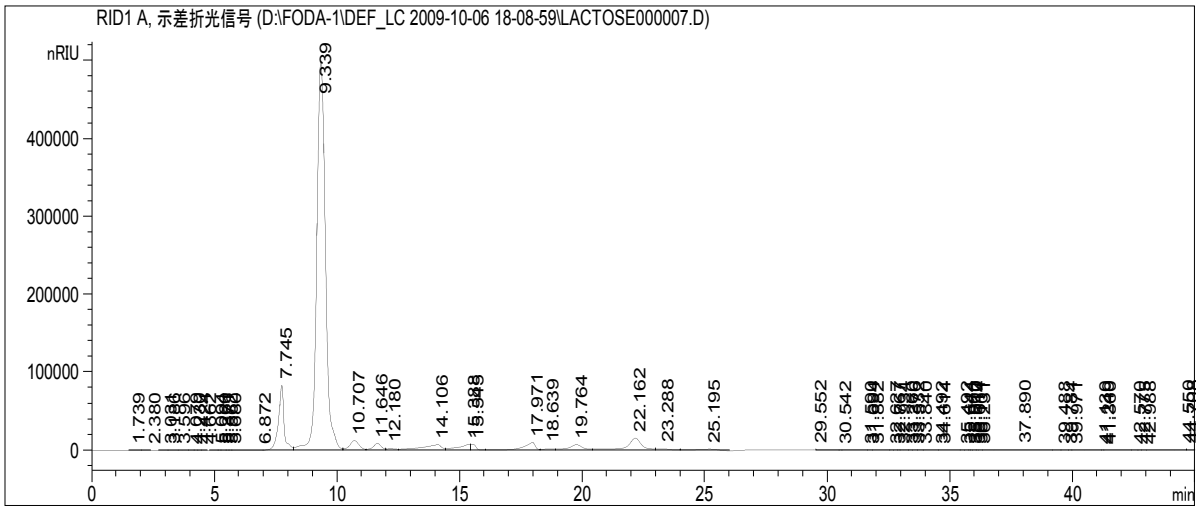


Fig. (2): HPLC-chromatogram of biofuels formation by *Clostridium acetobutylicum* AS 1.224 using lactose medium 37° C, shaking velocity 150 rpm, pH 7.0) after incubation time for 50 hrs.

Francisco and Russell (1996) reported that *Clostridium acetobutylicum* had phosphotransferase systems for glucose and lactose, and the lactose system was inducible. When *C. acetobutylicum* was provided with glucose and lactose, the cultures grew in a diauxic fashion, and glucose was used preferentially. Cells grown on lactose took up thiomethyl galactoside, and retained this non-metabolizable lactose analog for long periods of time. Because glucose inhibited thiomethyl galactoside uptake and caused the efflux of thiomethyl galactoside that had already been taken up, it appeared that *C. acetobutylicum* had inducer exclusion and inducer expulsion mechanisms similar to those found in lactic acid bacteria.

Table (1) shows lactose consumption and related biofuels production by the two Clostridium strains in lactose medium with initial pH 7 after 75 hrs. It can be seen that production of acetic and butyric acid by *Clostridium acetobutylicum* DSM 792 was higher amounts and slightly increased by prolonging the incubation time from 50 to 75 hrs. Ethanol and acetone production were decreased, while butanol amount was not changed. By *Clostridium acetobutylicum* AS 1.224, acetic acid and ethanol production was increased after 50 hrs, while other compounds did not detect. These results are in agreement with those reported by Lee *et al.,* (2008) who mentioned that the clostridia solvent production is biphasic fermentation, the first phase is the acidogenic phase, during which the acids forming pathways are activated, and acetate, butyrate, hydrogen, and carbon dioxide are produced as major products.

Table (1): Production of biofuels in lactose medium (37^0 C, shaking velocity 150 rpm, pH 7.0) by *Clostridium acetobutylicum DSM* 792 and AS 1.224 after 75 hrs.

Components (g/l)	*Clostridium acetobutylicum* DSM 792		*Clostridium acetobutylicum* AS 1.224	
	Incubation Time (Hours)			
	50	75	50	75
Lactose	5.28 ± 0.85	2.57 ± 1.84	38.28 ± 1.69	35.02 ± 1.80
Acetic acid	13.10 ± 1.00	13.41 ± 0.35	0.66 ± 0.35	2.56 ± 2.10
Ethanol	6.42 ± 0.18	4.94 ± 2.24	2.13 ± 1.52	4.38 ± 2.57
Butyric acid	10.47± 0.15	10.87 ± 0.53	0.59 ± 0.62	0.00
Acetone	0.26 ± 0.12	0.00	0.52 ± 0.40	0.00
Butanol	0.71 ± 0.06	0.71 ± 0.13	0.01± 0.01	0.00

During the first phase, the cells grow rapidly and form carboxylic acids, mostly acetate and butyrate; the excretion of these acids lowers the external pH. These acids are suggested to act as inducers for the biosynthesis of the solventogenic enzymes during a second fermentative phase (Ballongue *et al.,* 1985). The acids formed earlier re-enter the cells and act as co-substrates for the production of neutral solvents (Fond *et al.*, 1985). At this point, the production of the acids ceases as well as cell growth, and the medium pH increases slightly due to the acid uptake (Terracciano and Kashket, 1986). It has been suggested that the switch to solvent production is an adaptive response of the cells to the low medium pH resulting from acid production (Bahl *et al.,* 1982). The major end product of the fermentation is butanol, with acetone and ethanol being minor products. The bacterium, *C. acetobutylicum* is able to metabolize a great variety of carbon resources. Depending on the nature of the carbohydrate and the culture conditions, the extent of solvent conversions can vary (Compere and Griffith, 1979).

The transition from acidogenic to solventogenic phase is the result of a dramatic change in gene expression pattern (Dürre *et al.,* 1987). Bryant and Blaschek, (1988) found that the pH of the medium is very important to the biphasic acetone–butanol fermentation. In acidogenesis, rapid formation of acetic and butyric acids causes a decrease in pH. Solventogenesis starts when pH reaches a critical point, beyond which acids are reassimilated acetone and butanol, are produced. Therefore, low pH is a prerequisite for solvent production (Kim *et al.,* 1984). However, if the pH decreases below 4.5 before enough acids are formed, solventogenesis will be brief and unproductive. Increasing the buffering capacity of the medium is a simple way to increase growth and carbohydrate utilization as well as butanol production (Lee *et al.,* 2008).

Desai *et al.,* (1999) constructed a stoichiometric model for the flux analysis of acid and solvent formation pathways. As expected, higher acetate and butyrate forming fluxes were observed in acidogenesis compared with those in solventogenesis. The direction of the butyrate forming pathway was reversed in solventogenesis. During solventogenesis, the fluxes toward acetone, butanol and ethanol formation were greatly elevated. In addition, fluxes linked to the acetone forming pathway (e.g., uptake fluxes of acetate to acetyl-CoA and butyrate to butyryl-CoA) increased in solventogenesis. Transcriptome profiling strongly supported that this flux pattern was driven by phase-dependent gene expression (Alsaker *et al*., 2004).

2-Bio-butanol formation in bio-reactor batch experiments

Figure-3 shows the lactose consumption by *Clostridium acetobutylicum* DSM 792 in lactose medium and deproteinized cheese whey after 5 days of fermentation process. Lactose consumption in lactose medium goes to completion after 3 days only, while with cheese whey almost (20%) of lactose was remained. These results are similar to that obtained by Sansonetti, *et al.,* (2009) and Salman &Mohammad (2005) who found that lactose consumption goes to completion within 13 h only, i.e. much earlier than it was reported for raw cheese whey fermentation, which might also affect the process performance.

The highest fermentation product on lactose medium (50g/l) was acetic acid as shows in Fig. (4) reached the maximum formation after 3 days then decreased. The production of butyric acid and ethanol was almost constant after 2 days.

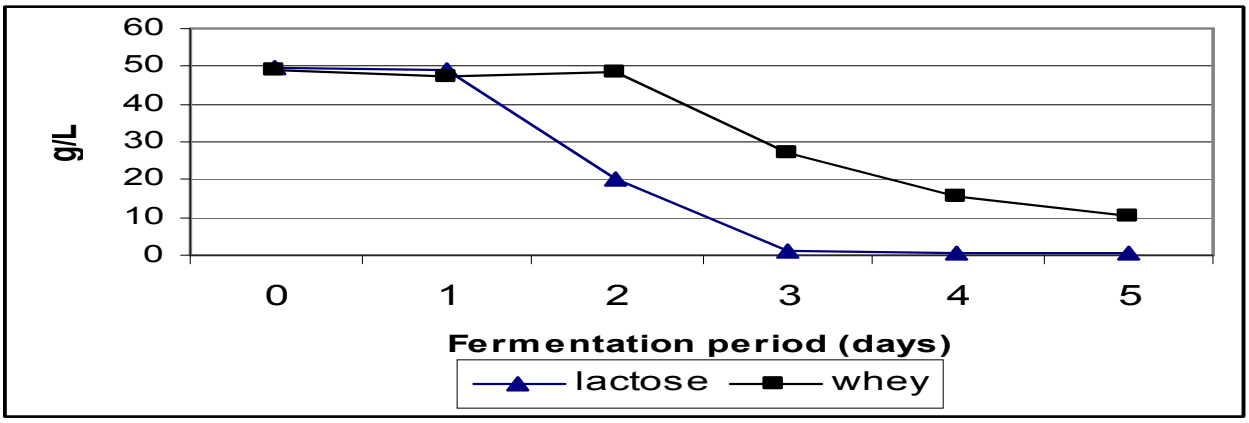
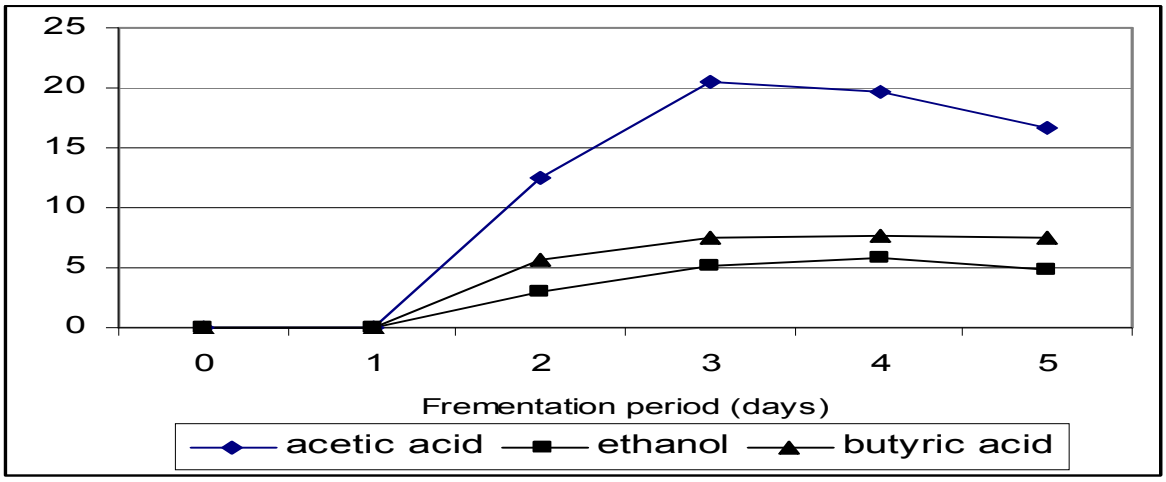


Fig. (3): Effect of fermentation media on lactose consumption by *Clostridium acetobutylicum DSM* 792 after fermentation process (37°C, shaking velocity 150 rpm, pH 5.0) for 5 days.



Lactose m

Fig. (4): Fermentation products on lactose media (37°C, shaking velocity 150 rpm, pH 5.0) by *Clostridium acetobutylicum DSM* 792 after fermentation process for 5 days.

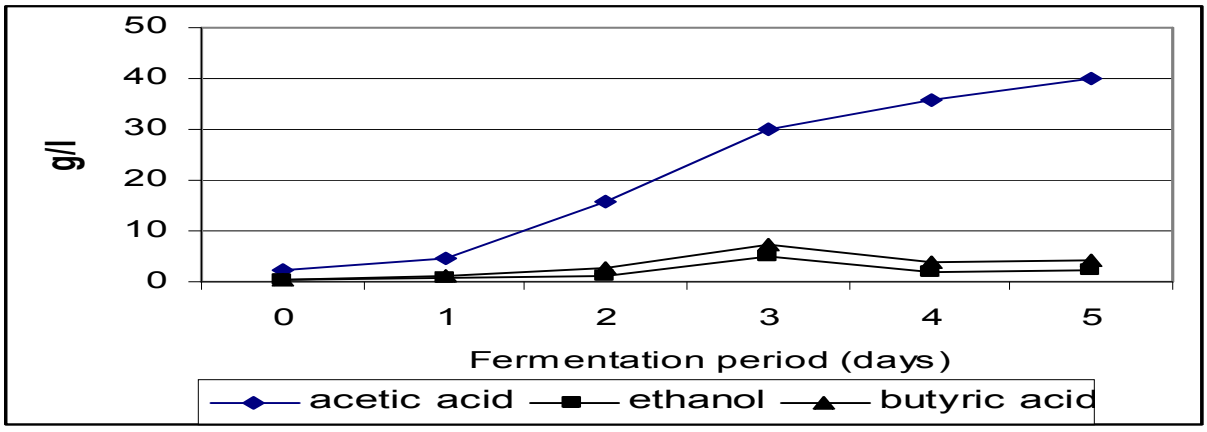


Fig. (5): Fermentation products from cheese whey medium (37°C, shaking velocity 150 rpm, pH 5.0) by *Clostridium acetobutylicum DSM* 792 after fermentation process for 5 days**.**

Figure (5) shows the production of acetic acid in cheese whey, which increased to reached the double amount compared to lactose medium. Ethanol and butyric acid were reached the maximum after 3 days then decreased to constant amount almost similar to lactose medium. These results are in agreement with those obtained by Ezeji *et al.,* (2004) who found that it takes 2–6 days to complete batch fermentation depending on the condition and the type of substrate employed. The final total concentration of solvents produced ranges from 12 to 20 g L^{-1} in batch fermentation, which can be separated from the fermentation broth by distillation. Classical fed-batch and continuous cultivation do not seem to be economically feasible, because of solvent toxicity and the biphasic nature of acetone–butanol fermentation, respectively. To overcome this problem, fed-batch culture has been coupled with an in situ recovery process.

Sansonetti *et al.,* (2009) studied the feasibility of ricotta cheese whey (scotta) fermentation process to produce bio-ethanol by *K. marxianus*. Furthermore, it was showed that scotta represents an excellent substrate since it allows attaining an ethanol yield of 97%, very close to the theoretical one. Complete lactose consumption was observed after 13 h for scotta as compared to 18 h for raw cheese whey. As far as the fermentation process is concerned, scotta is to be considered as a substrate completely different from traditional raw cheese whey and, also, from deproteinized whey, thus representing a valid alternative source to produce bio-ethanol.

Qureshi and Maddox (2005) produced acetone-butanol- ethanol (ABE) from whey permeate medium, supplemented with lactose, in a batch reactor using *Clostridium acetobutylicum* P262, coupled with ABE removal by perstraction. ABE were produced from lactose at a yield of 0.44. The ratio of acids to solvents was significantly lower in the perstraction experiment compared to the control batch process suggesting that acids were converted to solvents. Qureshi and Maddox **(**1987) immobilized cells of *Clostridium acetobutylicum* by adsorption onto bonechar and used in a packed bed reactor for the continuous production of solvents from whey permeate. A maximum solvent productivity of 4.1 g l^{-1} h^{-1}, representing a yield of 0.23 g solvent/g lactose utilized, was observed at a dilution rate of 1.0 h^{-1}. The reactor was operated under stable conditions for 61 days. High concentrations of lactose in the whey permeate favored solventogenesis, while low concentrations favored acidogenesis.

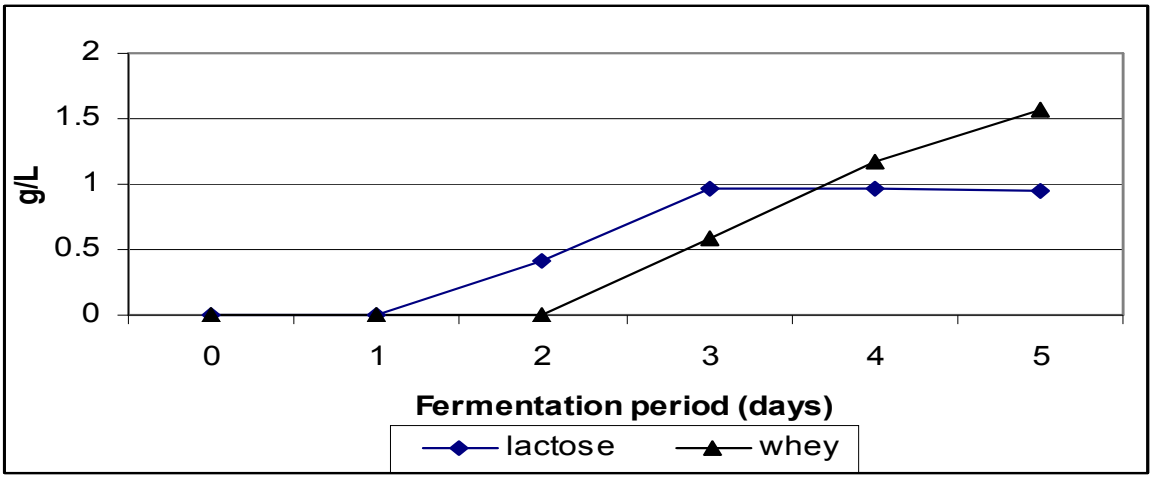


Fig (6): Butanol production from lactose and cheese whey media (37°C, shaking velocity 150 rpm, pH 5.0) by *Clostridium acetobutylicum DSM* 792 after fermentation process for 5 days.

Butanol was observed after 1 day with cheese whey medium and after 2 days when lactose medium was used as shown in Fig (6). Butanol production was increased dramatically with cheese whey during the fermentation period till the end. While, with lactose medium, the production was constant after 3 days till the end of fermentation period. This could be due to highest lactose consumption in the lactose medium compared to cheese whey (Fig-3).

These results are in contrarily with those obtained by (Qureshi *et al.,* 1988) who reported that unused lactose was toxicity due to ABE, in particular butanol. Using *C. acetobutylicum* in a batch reactor a total concentration of ABE of 20 g L^{-1} is rarely reached. At various stages of fermentation lactose utilization was fluctuated. A maximum lactose utilization rate of 0.47 gL^{-1} was recorded between 56 and 75 h of fermentation. A yield o f solvent of 0.33

was calculated from this run. During this fermentation 0.70 gL^{-1} acetic acid and 0.25 g L^{-1} butyric acid was also produced. It was reported by Zhao *et al., (*2005) that the initiation of butanol formation corresponded to the time when butyryl-P concentration reached its peak. It was suggested that the concentration of butyryl-P should be higher than 60– 70 pmol g DCW1 for butanol production. The higher butyryl-P peak concentration corresponds to higher butanol formation fluxes. Supporting this finding, the butyryl-P peak concentration never exceeded 50 pmol gDCW1 in the non-butanol producing strain lacking the megaplasmid pSOL1.

One of the most critical problems in ABE fermentation is solvent toxicity. Clostridial cellular metabolism ceases in the presence of 20 g/L or more solvents (Woods, 1995). This limits the concentration of carbon substrate that can be used
for fermentation resulting in low final solvent concentration and productivity. The lipophilic solvent butanol is more toxic than others as it disrupts the phospholipid components of the cell membrane causing an increase in membrane fluidity (Bowles and Ellefson, 1985).

Moreira *et al.*, (1981) had attempted to elucidate the mechanism of butanol toxicity in *C. acetobutylicum*. They found that 0.1–0.15 M butanol caused 50% inhibition of both cell growth and sugar uptake rate by negatively affecting the ATPase activity. Increased membrane fluidity causes destabilization of the membrane and disruption of membrane-associated functions and membrane-bound ATPase activity (Bowles and Ellefson, 1985).

In batch bio-reactor experiments it could be noticed the absent of acetone production in either lactose medium or cheese whey. This result could be due to the acetone-forming enzymes as mentioned by Mermelstein *et al.,* (1993). Who reported that the acetone producing pathway is coupled with that leading to the formation of the precursor of butanol, butyryl-CoA, from butyrate. In the fermentation of recombinant *C. acetobutylicum* with amplified *adc* (encoding acetoacetate decarboxylase) and ctfAB (encoding CoA transferase) genes, the acetone-forming enzymes became active earlier, which led to earlier induction of acetone formation.

4. Conclusion

Biotechnological production problems such as solvent toxicity have traditionally been addressed by random mutagenesis and process optimization. Development of *C. acetobutylicum* strain should be done to obtain a more solvent tolerant strain.

A more detailed investigation on the influence of fermentation parameters such as temperature, agitation velocity, pH and initial lactose concentration on butanol yield was beyond the scopes of this preliminary analysis. It would be, however, advisable to formulate a general kinetic model that could be used to improve cheese whey fermentation performance.

A deeper experimental analysis is, however, necessary to better ascertain the reasons of such a different behavior observed during fermentation experiments.

As a matter of fact, lactose solution, therefore, can be regarded as a poor fermentation substrate, as compared to cheese whey.

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Green Algae for Improving Nutritional and Environmental Status of Fish Ponds Production

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Abstract: Four large scale ponds of aquaculture (*ca*; 10.000m2x1.5m depth per each) were used in this study to investigate the effect of algal addition on water quality and fish yield without water re-newing. Prior cultivation at the first of October, each pond received 50 kg super phosphate, 50 kg urea 46.5% N and 6m^3 organic poultry manure to enhance the growth of natural flora. Two weeks later (second half of October) about 70,000 fishes were inoculated. When fish reached 20g of their fresh weight, 100kg of fresh a live algal bulk *Scenedesmus* sp. containing 75% moisture was added to each treated pond. Water analysis including E.C, water pH and nutrients as well as dissolved oxygen were periodically conducted. Comparing measurements of algal treated ponds with those of the control pond showed that the addition of algae resulted in increasing of dissolved oxygen and reducing water pH (to become around the neutral pH reaction) due to ammonium consumption, aeration and slightly to water re-newing. Decreasing electric conductivity (E.C.) of the remained water was varied with respect to the former reasons. The results also showed that night respiration of algae was slightly blocked as ponds were aerated by the circulated water pump and illuminated during night growth. Fish yield of algal treated ponds was increased by 10% increases, while such pond plus aeration was increased by about 25% as compared with the control pond. [Journal of American Science 2010;6(8):47-55]. (ISSN: 1545-1003).

Keywords: Green Algae; Nutritional; Environmental; Fish Pond

1. Introduction:

Interest in commercial of *Tilapia* sp. to meet the human needs is steadily increased. *In situ*, the common processes including re-newing of water by changing about 25% of water volume to re-new the growth medium. This process removes a part of nitrogenous fish metabolites mainly ammonical nitrogen, decreases the acid reaction (pH) and increases dissolved oxygen (DO) and zooplankton communities. In a pond of 16000m^3 capacity, about 4000m^3 of water must change every two weeks, which in turn increases the production costs including labors, power and water requirements.

In a successful aquaculture system, there must be both an organismic and chemical-balance. The first is to produce an optimal supply of natural food at all levels to ensure sufficient oxygen supply for the growth of fish and their natural food organisms, while the second is to minimize the build-up of toxic metabolic products (Colman and Edwards 1987). A wide range of fish species has been cultivated in aquaculture ponds receiving human waste, including common *Tilapia*. In some countries, a poly-culture of several fish species is used. *Tilapia* is generally cultured to a lesser extent than *Carps* in excreta-fed systems although, *Tilapia* are more technically suitable for this environment because they are able to tolerate adverse environmental conditions than *Carp* species (FAO, 1992). Several species of fish feed directly on fecal solids, use of raw sewage or fresh night-soil as influent to fish ponds should be prohibited for health reasons. Edwards (1990) represented the complex food chains in an excreta-fed fish pond involving ultimate decomposers or bacteria, phytoplankton and zooplankton. Nutrients released by bacterial degradation of organic solids in sewage, night soil or excreta are taken up by phytoplankton. Zooplankton graze phytoplankton and small detritus particles coated with bacteria, the latter also serving as food for benthic invertebrate detritivores. Phytoplankton is the major source of natural food in a fish pond. To optimize fish production in human waste fed pond, the majority of the fish should be filter feeders, to exploit the plankton growth. The advantages of using algae for that purpose include: the low cost of the operation, the possibility of recycling assimilated nitrogen and phosphorus into algae biomass as a fertilizer, avoiding sludge handling problem, and the discharge of oxygenated effluent into the water body. In addition, the process has no carbon requirement for nitrogen and phosphorus removal, which is attractive for

the treatment of secondary effluents (Aslan and Kapdan, 2006).

It is now recognized that depletion of dissolved oxygen in fertilized fish ponds is a primarily due to the high rates of respiration at night of dense concentrations of phytoplankton. There are two main processes that result in the loss or transformation of ammonia. The most important is the uptake of ammonia by algae and other plants. The other important process of ammonia transformation in fish ponds is nitrification. Bacteria oxidize ammonia in a two-step processes, first to nitrite (NO_2) and then to nitrate. Theoretically, there are several ways to reduce ammonia concentration, but most approaches are impractical for the large ponds used in commercial aquaculture. Following is a discussion of some options, their practicality and their effectiveness. Stop feeding or reduce feeding rate, increase aeration, add lime, fertilize with phosphorus, reduce pond depth, increase pond depth, flush the pond with well water, add bacterial amendments, add a source of organic carbon, add ion exchange materials and add acid (Hargreaves and Tucker, 2004).

This study was carried out in order to examine whether adding fresh microalgae to ponds would counteract the effect of leaving water without re-newing by improving environmental conditions and nutritional status.

2. Materials and Methods

The green alga *Scenedesmus* sp. (El-Sayed, 2004) was used to fulfill the current investigation. Alga was pre-cultivated in three open ponds of $15m^3$ per each one at NRC, Dokki, Cairo, Egypt. Continuous centrifugation ($5000l.h^{-1}$) was performed to obtain the algal bulk. Chemical and biochemical analyses of such bulk were done (Chapman and Pratt, 1974). Alga was transferred at 5^oC to the field and added at night to allow the adaptation potential.

A mono sexual *Tilapia* sp. was cultivated within four large scale ponds of aquaculture (*ca*; $10.000m^2$x1.5m depth). Prior cultivation, each pond received 50 kg super phosphate, 50 kg of urea 46.5% N and $6m^3$ of organic poultry manure to enhance the growth of natural flora. Two weeks later (second half of October 2006) about 70,000 fishes were inoculated ($8 fish.m^{-3}$). When fish reached about 20g of their fresh weight, 100kg of fresh a live algal bulk *Scenedesmus* sp. containing 75% moisture were added.

The ponds were treated as a) pond 1 (control) no algal addition and 25% of water was renewed every two weeks, b) pond 2, received algae without aeration and water was not renewed, d) pond 3 received algae with aeration and water was not renewed and d) pond 4, received algae with aeration, night illuminated and water was not re-newed. As growth of fish and algae was progress, water was aerated using water pump to avoid the lacking of dissolved oxygen due to algal night respiration. Night illumination was performed by white lamps.

Water analyses including E.C, pH, macro and micro-nutrients as well as dissolved oxygen were periodically conducted every week. At the end of cultivation season, fish yield was determined. The results were expressed as the mean of analyses per month.

3. Results and discussion

3.1. Algal community and growth

During the whole growth period, the dominance of the inoculated alga *Scenedesmus* sp. was observed, however other species mostly belonging to *Chlorophyta* were detected. The dominance of the local species was found with non-treated pond and the rate of their growth was periodically changed due to water re-newing which in turn led to a reduction of algal population or the growing mass. The other treated ponds represented tow growth patterns due to the effect of technical process mainly aeration, where the third and fourth ponds exhibited a massive dry weight as compared with the second pond (Fig.1a).

Night illumination support pond by additional chance to improve algal growth and also block night respiration. In addition to growth enhancement, algal growth enhancement also increases DO concentration. Fish feeding enhances bacterial degradation which in turn increases nutrients releasing to algal growth medium. Fish excretion; as well; increased by increasing the feeding rate, environmental improving and fish growth. During winter season, where low fish growth and low feeding, growth of algae could be attributed to the environmental suitability. Furthermore, the main problem in fish pond namely ammonia and ammonium increases; in many literature; that affect the growth of different algal species.

As for total chlorophyll, data was found to be proportionally to those obtained by dry weight; however the pond represented a slight decrease on total chlorophyll accumulation as compared with dry weight accumulation curves at the early months of growth (Fig. 1b). This might be attributed to the effect of water electric conductivity due to salting effect with the

absence of aeration and extra ammonical nitrogen supplementation as well as water re-newing. Comparing measurements of algal treated ponds with those of the control pond showed that the addition of algae resulted in both the effect of algal addition and aeration. Concerning algal growth, all of the treated and non-treated ponds resulted in algal blooming, but growth was surpasses with ponds received alga in the presence of aeration and night illumination. Dry weight of algal community (Fig.1a) reached the maximum in the expense of total chlorophyll. The lowest gain of total chlorophyll during cultivation season might be attributed to the effect of environmental conditions especially during the sunny season. Such effect was more obviously recorded with non-aerated ponds. During dim and cool periods, total chlorophyll was increased parallel with dry weight accumulation with aerated ponds.

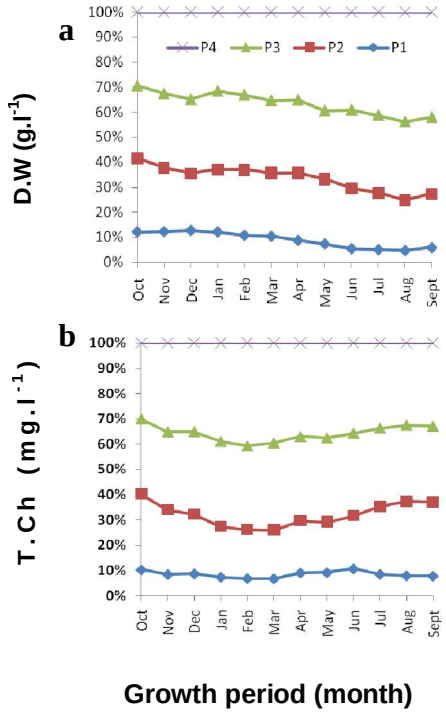

Fig.1. Percentage development of a) dry weight and b) total chlorophyll for phytoplankton community at different treated fish ponds as compared with 100% of the fourth pond.

Batterson *et al*. (1988) suggested that source water low in alkalinity (20-30 mg.l^{-1} $CaCO_3$) caused dissolved inorganic carbon (DIC), limitation of NP at low chicken-manure fertilization rates (12.5 and 25g dw.m^{-2}.w^{-1}). At higher fertilization rates, manure decomposition supplied sufficient DIC relative to the input of other nutrients, and carbon no longer limited phytoplankton growth. Otherwise, urea fertilization (0.12 g N.m^{-2}.d^{-1}) of ponds filled with untreated water resulted in NH_4 and NO_3 or N concentrations of around 0.5 mg.l^{-1}, supporting the hypothesis that nitrogen inputs were inefficiently utilized when DIC was limiting (McNabb *et al*., 1985).

The removal rates of *Chlorella pyrenoidosa* were reported as 3.4mgN.d^{-1} and 10.7mgP.d^{-1} (Tam and Wong, 1994). However, Jimenez-Perez *et al*. (2004) reported substantially higher removal rates of 20.83mgP.d^{-1} and 83mgN.d^{-1} for suspended growth culture of *Scenedesmus. intermedius* and 10.15mgP.d^{-1} and 56.06mgN.d^{-1} for *Nannochloris* sp.

The final phosphorus concentration was around 1.7mg.l^{-1} with 78% removal efficiency for (PO_4) = 7.7mg.l^{-1}. The higher concentrations resulted in mostly less than 30% removal. Although nitrogen and phosphorus uptake by algae did not provide efficient removal of these nutrients from the synthetic media at high concentrations of nutrients, final chlorophyll a content of the culture significantly increased from10.7mgl^{-1} to 27.3mg.l^{-1} with the increase in (NH_4) = 13.2mg.l^{-1} to (NH_4) = 410mg.l^{-1}. The light limitation due to excess amount of chlorophyll a could be one of the reasons for low removal efficiencies at high nutrient concentrations. These results indicate that *Chlorella vulgaris* is very effective in removing nutrient concentrations as NH_4 <22mg.l^{-1} and PO_4< 7.7mg.l^{-1}. The specific NH_4 removal rate was increased with increasing the initial NH_4 concentration. The maximum rate reached 3.0mg.mg^{-1}chl a.d^{-1}. The specific PO_4 removal rate increased from 0.2mg.mg^{-1}chl a.d^{-1} to around 0.52mg.mg^{-1}chl a.d^{-1} for the PO_4 concentrations between 7.7mg.l^{-1} and 149mg.l^{-1}. At higher concentrations it almost remained constant around 0.47mg.mg^{-1}chl a.d^{-1} (Lau *et al*., 1998).

3.2. Dissolved oxygen

Tow different patterns of the concentration of dissolved oxygen with all examined ponds were found. The first is the dissolved oxygen due to algal growth and the second is the consumed oxygen by fish during day and by algae during night respiration. The net gain of dissolved oxygen is the variation between two patterns. The original water used for all treated ponds characterized by 3.75 mg.l^{-1} dissolved oxygen after the addition of fertilizers 15 days ago. At

the early stages of *Tilapia* growth (5 months), DO concentration represented slight decreases with the first pond (control) and the variations were observed among the next treated ponds (P2, P3 and P4). The next growth period represented different manner, where the first pond exhibited a sigmoid curve on dissolved oxygen to lie between 2 and 4 mg.l^{1} (Fig.2).

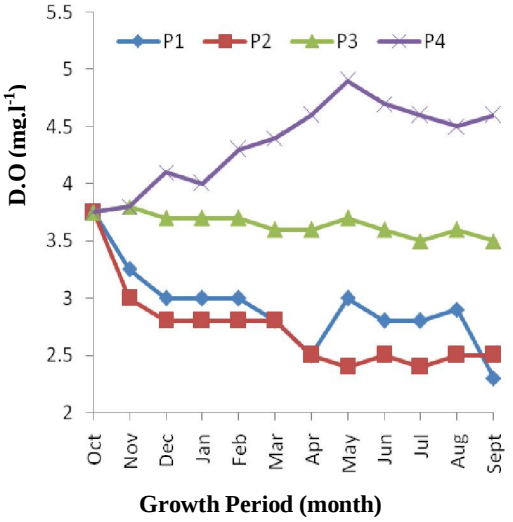

Fig.2. Dissolved oxygen (mg.l^{-1}) as affected by pond practice and algal addition at different treated ponds

The second pond results approximately constant manner (around 3.0 mg.l^{-1}). Extending increases were observed with the third and fourth pond which received algae and aerated in addition to night illumination.

The sensitivity of fish to low levels of DO varies with species, life stage (eggs, larvae, adults) and life process (feeding, growth, and reproduction). A minimum constant DO concentration of 5mg.l^{-1} is considered satisfactory, although an absolute minimum consistent with the presence of fish is probably less than 1 mg.l^{-1} (Alabaster and Lloyd, 1980).

3.3. Acid reaction (water pH)

The main source of ammonia in fish ponds is fish excretion which directly related to the feeding rate and the protein level in feed. As dietary protein is broken down in the body, some of the nitrogen is used to form protein (including muscle), some is used for energy, and some is excreted through the gills as ammonia. Another main source of ammonia in fish ponds is diffusion from the sediment. Large quantities of organic matter are produced by native algae or added to ponds as feed. Fecal solids excreted by fish and dead algae settle to the pond bottom, where they decompose. The decomposition of this organic matter produces ammonia, which diffuses from the sediment into the water column.

Algal growth affects the acid reaction of growth media and vice versa. Growth of algae led to alkaline media and the later obligate algal growth to decline and tended to shift their growth metabolites to lipid accumulation and other storage compounds other than protein. In the present case, fish growth, water type, fertilizers and addition of algae caused a complicated profile.

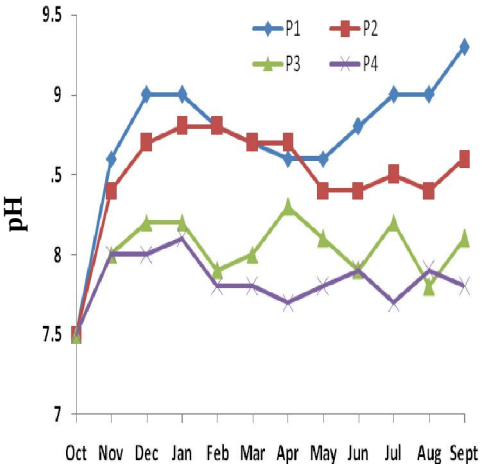

Fig.3. Acid reaction of different tested ponds as affected by algal addition, aeration and night illumination.

As shown in Fig.3, acid reaction of growth media was drastically varied, however the same curve profile was mainly observed. Values were starting to rise by the second month of cultivation due to fish growth with increasing of biological system activity and slightly warm temperature. The pH decline was observed by the fifth month due to low temperature and cultivation practice and tended to increase again concerning to the same reason. As a general, the lowest pH changes or the high stability against alkaline reaction was observed by the ponds received algae with aeration and night illumination. Here, the main effect could be attributed to algal addition that reduces pH by ammonium consumption as well as aeration in which evaporate ammonia and allow air penetration.

Many factors could affect pH values reflected on fish pond. Of these are water source and its alkalinity, fertilizers, farming, growth of fish, growth of algae and environmental factors. The net gains are nutrients releasing from fertilizers, feeding, nutrients consumption by fish and algae and finally ammonium emission. After 5 days, the level of ammonium ion removal from the wastewater by *Chlorella vulgaris* ranged from 60% to 78%, with an average of 72% from four independent repetitions (Valderrama *et al*., 2002). Ammonium is utilized as nitrogen source by planktonic algae and many species inhabit aquatic environments with very high ammonia concentrations, *e.g.* bioconversion ponds for municipal wastewater. Such ponds may become more or less alkaline due to the photosynthesis of algae and the protolytic equilibrium of ammonium–water which will be in favor of un-ionized ammonia (Alabaster and Lloyd 1980).

Fish excrete most of their excess metabolic nitrogen as unionized ammonia rather than urea. UIA is largely known to be toxic to fish at very low concentrations. Despite a rather large variation in the sensitivity of different fish species to UIA, the common range for commercial growth is narrow typically 0.05 to 0.27 mg N l^{-1}. The second excreted metabolite is toxic to the fish, but at considerably higher concentrations is CO_2 (aq). Surplus CO_2 (aq) is excreted by the fish through the gills. Accumulation of CO_2 in the aqueous phase decreases the driving gradient between CO_2 concentrations in the fish blood and the water. Consequently, CO_2 accumulates in the blood, resulting in a decrease in the oxygen carrying capacity (Lemarie *et al*., 2004). Here, the algal inoculation could solve this case by utilizing CO_2 into organic compounds. Otherwise, CO_2 dissolving to carbonate and bicarbonate might be enhances the same action.

Unionized ammonia (NH_3) is toxic to fish in the concentration range 0.2-2.0 mg.l^{-1} (Alabaster and Lloyd 1980). However, the tolerance of different species of fish varies, with *Tilapia* species being least affected by high ammonia levels. Ammonia will not easily evaporate from aqueous solutions at pH 11–11.3. Several hours of violent gas stripping are required. The toxicity of ammonium/ammonia was strongly pH dependent. The sensitivity of *Nephroselmis pyriformis* was high as compared with other species of microalgae **(**Källqvist and Svenson, 2003).

3.4. Electric conductivity and nutrients

Macro and micro-nutrients were drastically varied due to growth of algae, growth of fish, water re-newing, algae addition and fish feeding. It should be noticed that die algae and other microorganisms including phyto and zoo-plankton as well as fish excretion rough the net nutritional status. Routine grown pond represented the sigmoid curve due to water re-newing, but the removed water represented high salinity concentration. As water was re-newed, electric conductivity value (E.C) and acid reaction (pH) were decreased and alkaline reaction slightly disappeared. An opposite pattern was observed as growth took place, where alkaline reaction due to ammonical nitrogen was shown. Other ponds with modified practices showed a completely different pattern, where addition of algae increased nutrients removal and decreased the ammonical levels. Extra improving was shown due to night illumination and water movement. Thus the rate of mineral accumulation on fish flesh and bon due to algal feeding might be expected. The rough gain of nutrients uptake was expressed as the reduction on electric conductivity during the whole experimental period (Fig. 4).

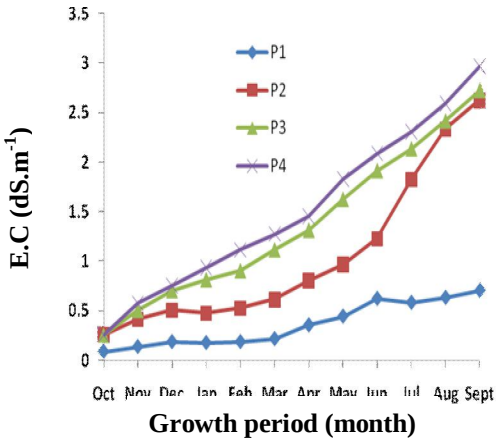

Fig.4. Nutrients status as electric conductivity changes of different tested fish ponds

Comparing water properties of different tested ponds (Original, used and drainage-water) resulted in increase of nitrogen, phosphorous and zinc, while potassium and magnesium exhibited low concentrations (Table.1). The obvious nutrient decreases were sodium and calcium; however the used pond was formed of clayly sodic soil. Increasing of nitrogen and phosphorous in drainage water might be return to fish exertion especially for phosphorous which

not utilized by algae as organic phosphate in the control pond. Otherwise microbial activity may responsible for the biodegradation of such phosphate form and makes it available for algal consumption in the blooming ponds. It may be mentioned here that nutrients or elements movements is the main reason of pond status and maintains. Nitrogen is the most required nutrient after carbon, the harmful form (ammonia) could be utilized by some algae species. Regardless the nutrition, ammonia directly affect acid reaction of pond and also affect the availability of some nutrients to planktonic grazes which indirectly increase alkalinity reaction. On the other hand, if nutrients became available, growth of algae will be accelerated and the media shift again to alkaline.

Table1. Average analysis (ppm) of the used and drainage water

Water	**D.O**		**pH**		**E.C**
Original	3.75		7.54		7.78
Treated	5.25		7.17		8.35
Drainage	2.3		8.4		1.54
	N	**P**	**K**	**Mg**	**Na**
Original	0.12	5.25	17.6	6.00	1233
Treated	0.08	5.00	19.5	6.00	122
Drainage	0.15	7.75	15.7	5.54	239
	Ca	**Fe**	**Mn**	**Zn**	**Cu**
Original	300	0.10	0.50	0.22	0.02
Treated	300	0.11	0.52	0.96	0.03
Drainage	45	Traces	Traces	0.61	0.02

In this context, the complicated relationship between these factors was considered in many ways, where treatment of the wastewater with *Chlorella vulgaris* gradually decreases the phosphorus concentration, while phosphorus in the untreated aerated water varied greatly between sampling times. The level of phosphorus ion removal from the treated wastewater ranged from 0% to 51%, with an average of 28%. *Chlorella vulgaris* treatment lowered COD after 5 days of incubation by 61% (from 3100 to 1200 $mg.l^{-1}$ in one experiment and similarly in other two repetitions (Valderrama *et al*., 2002). Furthermore, nitrogen and phosphorus removal efficiencies vary depending on the media composition and environmental conditions such as the initial nutrient concentration, the light intensity, the nitrogen/phosphorus ratio. An average of 72% nitrogen and 28% phosphorus removal by *Chlorella vulgaris* from 3–8 mg NH_4 and 1.5–3.5 mg PO_4 containing diluted ethanol and citric acid production effluent. Organic fertilizers often are added to ponds to boost fish yields by increasing primary productivity through released inorganic nutrients, or by providing organic carbon through heterotrophic pathways (Colman and Edwards, 1987).

Over 97% nitrogen and phosphorus removal was achieved by *Scenedesmus obliquus* for the nutrient concentrations of 27.4 and $11.8mgl^{-1}$, respectively (*Martinez et al., 2000*), while *Chlorella kessleri* able to uptake only 8–20% phosphorus under the light/dark cycle for PO_4 concentration of $10mg.l^{-1}$ (*Lee and Lee, 2001*). Although Dumas *et al*. (1998) reported complete phosphorus removal by *Phormidium bohneri*, Gonzales *et al*. (1997) obtained 55% phosphorus removal from agro-industrial wastewater with the total phosphorus concentration of $111mg.l^{-1}$ by 216 h batch cultivation of *Chlorella vulgaris* and *Scenedesmus dimorphus*.

In waste stabilization ponds the effects of phosphate concentration, light intensity, and temperature on luxury uptake of phosphorus by microalgae in continuous culture bioreactors was studied by Powell *et al.*, 2008. Increasing temperature had a positive effect on intracellular acid-insoluble polyphosphate concentration.

It is likely that elevated temperature increased the rate of polyphosphate accumulation, but because the biomass was not starved of phosphate, the stored acid-insoluble polyphosphate was not utilized. Increasing light intensity had no effect on acid-insoluble polyphosphate, but had a negative effect on acid-soluble polyphosphate. A possible explanation for this is that the faster growth rate at high light intensity results in this form of polyphosphate being utilized by the cells for synthesis of cellular constituents at a rate that exceeds replenishment.

The widely used microalgae cultures for nutrient removal are species of *Chlorella*, *Scenedesmus* and *Spirulina* (Lee and Lee, 2001; Gonzales *et al*., 1997, Martinez *et al*., 1999, 2000 and Olgu´ın *et al*., 2003). Nutrient removal capacities of *Nannochloris*, *Botryococcus brauinii* and *Phormidium* have also been investigated (Jimenez-Perez *et al*., 2004, An *et al*., 2003, Laliberte *et al*., 1997and Dumas *et al*., 1998).

3.5. Fish weight and yield

Fish fresh weights were improved due to nutritional and environmental modifications. Addition of algae increase dissolved oxygen (DO), consume the liberated ammonical nitrogen, increase zooplankton community and

grazing rate, regulate acid reaction (pH) and increase feeding rate. Water circulation also increases air penetration (CO_2 and O_2) into pond which in turn enhances algal photosynthesis and growth. Such practices also increased the rate of ammonical nitrogen removing out of pond and increased dissolved oxygen (DO). In addition, night illuminations break down the respiratory mode of algae and extended photosynthesis.

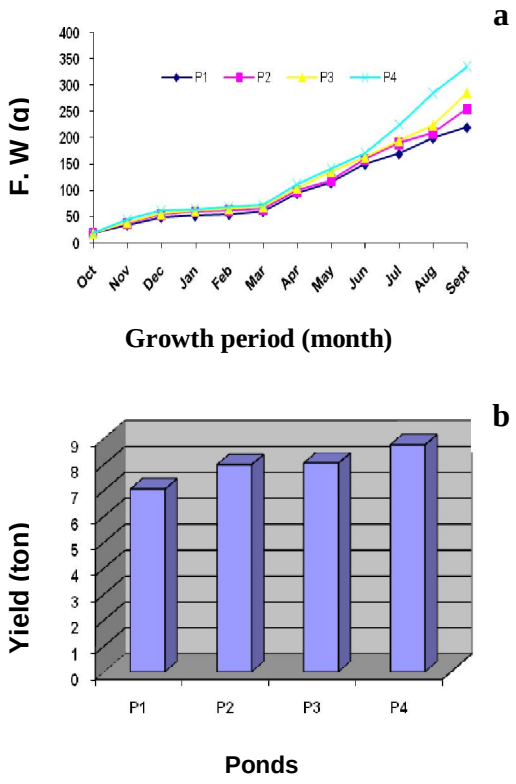

Fig. 5 a) Fish fresh weight development and b) fish yield per pond as affected by farming practice.

During the whole cultivation period, weights of randomized fish samples were periodically increased and the rate of increases was varied due to practices modification. At the end of cultivation season, ponds yield was found as 7.04, 7.98, 8.4 and 8.75 tons per fed for routine, algae supplied, algae supplied with water circulation and algae supplied with water circulation-pond plus night illumination; respectively. A wide range of yields has been reported from waste-fed aquaculture systems, for example: 2-6 tons/ha yr in Indonesia, 2.7 - 9.3 tons.ha^{-1}. yr^{-1} in China and 3.5 - 7.8 tons/ha.yr^{-1} in Taiwan. Although the majority of waste-fed fish ponds stocks *Carps*, research in Peru and Thailand has demonstrated the potential of *Ttilapia* for such systems. Management of fish ponds can have a significant effect on fish yields but the maximum attainable yield in practice is of the order of 10 - 12 tons.ha^{-1}.yr^{-1} (Edwards 1990).

4. Conclusion

Many ways could be used to improve of nutritional and environmental status of fish ponds. Nutritional status and farming practices are the most effective reasons affecting the environmental conditions as growth media. Addition of algae increases the rate of nutrients consumption rather than the production of oxygen which account as the true algal growth monitor. Accordingly, factors affecting algal growth should be improve nutritional and environmental quality offish ponds and fish production.

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Effect of Alpha Lipoic Acid and Vitamin E on Heavy Metals Intoxication in Male Albino Rats

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Abstract: The present study was carried out to investigate the effect of some nutrients on Nutritional and Health status in copper and lead intoxicated male albino rats. Fifty male albino rats weighing (125±7 g) were used. The rats were divided into 5 groups (10 rats each). The first group received the basal diet only and served as control. The second and third groups received copper (copper sulfate in water at dose of 0.1 mg /kg body weight) with alpha lipoic acid (40 mg/kg body weight) and Vitamin E (20 mg/kg body weight) respectively. Fourth and fifth groups received lead (lead acetate in water at a dose of 0.2 mg/kg body weight) with alpha lipoic acid and Vitamin E respectively.. At the end of the experimental periods, blood samples were collected from Orbital plexus, the rats sacrificed, organs were weighed, and kept for metal determination. The obtained results revealed that alpha lipoic acid (ALA) and vitamin E could improved daily food intake, body weight gain and feed efficiency ratio; reduced copper and lead levels in serum and tissues as well as diminished ALT, AST, urea and creatinine levels in lead and copper intoxicated rats. Therefore, the current study recommends that alpha lipoic acid or Vit. E (or both of them), should be administered to minimize the toxic effects of lead and copper. [Journal of American Science 2010;6(8):56-63]. (ISSN: 1545-1003).

Key words: heavy metals intoxication - copper lead - rats - alpha lipoic acid- vitamin E - liver function kidney function.

1. Introduction

Heavy metals are widely distributed in the environment. Among of them is lead and copper (Walker et al., 1995). The most common heavy metals, implicated in acute and / or chronic intoxication can effect the development and the overall health causing depression, learning difficulties and neurological disorders. These metals may cause cell damage, impairment of enzymes, functions or alter genetic material (DNA), causing cancer or birth defects, when absorbed (David, 2001). Lead and copper have been recognized as highly toxic industrial and environmental pollutants representing a continuous hazard to biological organisms due to its carcinogenic potential in humans (Kokilavanti et al., 2005). Industry produces about 2.5 million tons of lead throughout world every year. Target organs affected by lead are bones, brain, blood, kidneys and thyroid gland (Occupational Safety and Health Information Center, 1999). Most studies have shown that copper (Cu) induced hepatotoxicity in rats, and reported that free radicals may play a role in cupper induced cell toxicity because of the powerful pro-oxidant action of its salts in vitro. They indicated that the resulting oxyradicals have the potential to damage cellular lipids, nucleic acids, proteins and carbohydrates, resulting in wide ranging impairment in cellular function and integrity (Britton, 1996).

Antioxidant is a substance that prevents oxidation. In biological systems antioxidants can work in various ways, including catalytic removal of free radicals as scavengers of free radicals or in the form of proteins that minimize the availability of pro-oxidants such as metal ions. However, there are circumstances in which certain antioxidants can actually behave as pro-oxidants (halliwell et al., 1992; and halliwell, 1996).

Vitamin E is nature's major lipid soluble chain breaking antioxidant that is known to protect biological membranes and lipoproteins from oxidative stress. The main biological function of vit. E is its direct influencing of cellular responses to oxidative stress through modulation of signal transduction pathway (hsu and guo, 2002). Vit E primarily scavenges peroxyl radicals and is a major inhibitor of the free radical chain reaction of lipid peroxidation (Maxwell, 1995 and halliwell and gutteridge,1999).

Alpha lipoic acid (ALA) has the unique ability to regenerate several antioxidants such as vitamin E (Packer and Coleman, 1999). ALA has been classified as vitamin, which is a naturally occurring antioxidant (OU et al., 1995 and Femiano and Scully, 2002). It has been reported to be highly effective in improving the thiol capacity of the cell

and in reducing Lead induced oxidative stress; which suggested its possible role as a therapeutic intervention of lead poisoning in combination with a chelator (Gurer et al., 1998 and pandl, 2002).
Production of antioxidants and free radicals in the body is theoretically balanced. When conditions become favor free radical produced, a state known as oxidative stress occurs. Free radicals are directly cytotoxic. Prolonged oxidative stress can result in oxidative damage to tissues (Halliwell, 1994), specially, DNA, cellular protein and cellular lipids. It also affect cellular calcium metabolism and the cardiovascular system (Sally et al., 2003).
ALA and Vitamin E had been reported to have highly protective effect on lipid peroxidation. So, administration of ALA and Vitamin E. had significant protective effect on blood, liver and muscles against oxidative damage in diabetes (Naziroglu, 2001 and Pandl, 2002). Therefore, the aim of this work is to reduce or minimize the adverse effects of Cupper and Lead induced toxicity.

2. Material and Methods

Materials:

1) Animals:

Seventy adult male albino rates weighing (125 ± 7 g), were obtained from the laboratory Animal Colony, Ministry of Health and Population, Helwan, Cairo _ Egypt.

2) Metals and Drugs:

Lead & copper, Alpha Lipoic Acid & Vitamin E. were purchased from El Gomhoria Company for Drugs and Chemical Industries, Cairo, Egypt.

Methods:

1) Preparation of the basal diet:

Basal diet was prepared to meet (Table 1) to meet the rats nutrient requirements according to methods of Osfor (2003). It constituted of 10% protein, 3404 K. Calorie Energy, 5 % fiber, 7.5 fat, 0.5% vitamins and minerals mixture, 0.3 % salt (Sodium Chloride).

2) Experimental Design:

The present experiment was carried out on seventy male Albino rats (125 ± 7 g), that were housed in plastic cages at a room temperature maintained at 25 Cº. All rats were kept under normal healthy conditions and allowed to water and basal diet freely for one week before starting the experiment for acclimatization and then the rats were divided into main seven groups as follows:

Group - I: fed the basal diet only and served as control.

Group - II: fed the basal diet with copper in the drinking water at a dose of 0.1 mg /kg body weight.

Group - III: fed the basal diet with copper in the drinking water at a dose of 0.1 mg /kg body weight plus Alpha Lipoic acid (40 mg/kg body weight).

Group - IV: fed the basal diet with copper in the drinking water at a dose of 0.1 mg /kg body weight plus Vitamin E (at a dose of 20 mg/kg body weight).

Group - V: fed the basal diet with lead acetate in the drinking water at a dose of 0.2 mg/ kg body.

Group - VI: fed the basal diet with Lead acetate in the drinking water at a dose of 0.1 mg /kg body weight plus Alpha Lipoic acid (40 mg/kg body weight).

Group - VII: fed the basal diet with lead acetate in the drinking water at a dose of 0.1 mg /kg body weight plus vitamin E (at a dose of 20 mg/kg body weight).

3) Clinical observation:

The animals were observed daily throughout the experimental period. Complete physical examination was conducted weekly. Body weights were measured prior to offering the diet and recorded individually every week. Food consumption was calculated every other day to the nearest gram for each group of rats as the difference between the amounts of offered and residual food.

4) Clinical pathology:

Blood samples for clinical chemistry determination were obtained from the retro-orbital plexus of veins of all rats on the day before they were scheduled for euthanasia.

5) Clinical Chemistry:

Clotted blood samples were centrifuged and the serum was removed by aspiration for subsequent determination of the following:

a) Liver function tests: Glutamic Oxaloacetic Transaminase (GOT) "Unit/ dl", GlutamicPyruvic Transaminase (GPT) "Unit/ dl" were estimated according to the method of Pters et al., 1982

b) Renal function tests: Urea "mg/dl" and creatinine "mg/dl" were estimated according to the method of Sampson et al., 1980.

c) Lead and Copper Determination: Lead and Copper were determined in blood and tissues (liver and kidney), according to methods of Price, 1972, using the Atomic absorption spectrophotometer

using SPSS (2006).

6) Statistical analysis:

The obtained data were statistically analyzed

3. Results

Table (1): Composition of the basal diet:

Ingredients	Percentage
Sorghum	39
Corn yellow	31.6
Barley	8
Meat meal	8
Corn cobs	7.3
Vegetable oil	4
Lysin	0.3
Methionine	0.4
Di-calcium Phosphate	0.2
Lime stone	0.4
Sodium chloride	0.3
Vitamins and Minerals Mixture*	0.5
Calculated Nutrient Composition	
Crude protein	11.99
Energy	3404.2
Crude fiber	4.46
Ethrer extract	7.51
Lysine	0.71
Methionine	0.61
Calcium	0.45
Phosphorus	0.4

Vitamins and Minerals Mixture (g)

Copper sulphate (0.05), Ferric citrate (0.59), Zinc carbonate (0.053), Calcium carbonate (7.25), Calciumhydrogen phosphate (11.3), Di-sodium hydrogen phosphate (6.0), Potassium Iodide (0.003), Magnesium chloride (2.3), and Manganese sulphate (0.154).

Thiamine (0.3), Riboflavin (1.0), pyridoxine (0.2), Calcium carbonate (6.0), Nicotinicacid (20.0), Cyanocobalamine (0.005), Folic acid (0.2), Biotin (20.0), Inositol (60.0), Choline chloride (60.0), vitamin A (4000 IU), vitamin D (1000 IU), Vitamin E (30 IU) and Vitamin K (50 IU).

Table (2): Effect of ALA and Vit. E on food intake, body weight gain, food efficiency ratio, liver and kidney weights (g) in rats intoxicated with copper and lead:

Group	Food Intake (g)	Body weight gain (%)	Food Efficiency Ratio	Liver	Kidney
Group I	11.25 ± 0.73a	75.63 ± 4.92 a	1.44 ± 0.31 a	4.95 ± 0.50a	0.78 ± 0.10
Group II	6.18 ± 1.86b	18.77 ± 7.31 c	0.73 ± 0.03 b	2.96 ± 0.49c	0.72 ± 0.03
Group III	7.97 ± 1.63b	34.35 ±8.96 bc	0.83 ±0.04 b	3.26 ± 0.54bc	0.69 ± 0.13
Group IV	6.40 ±1.60b	21.29 ± 4.16 c	0.78 ± 0.03 b	3.94 ± 0.69bc	0.77 ± 0.13
Group V	6.72 ± 2.08b	31.79 ±16.86bc	0.85 ± 0.09 b	3.37 ± 1.04bc	0.76 ± 0.08
Group VI	10.45 ± 1.71a	53.3 ± 13.83 ab	0.99 ± 0.14 b	4.40 ± 0.42ab	0.77 ± 0.01
Group VII	10.87 ± 0.98a	58.82 ±18.86 a	1.05 ± 0.29 ab	4.75 ± 0.47a	0.83 ± 0.12

Means with different superscript are significantly different (P< 0.05)

Table (3): Effect of ALA and Vit. E on residues of copper and lead in serum and tissues of intoxicated rats:

Group	Copper			Lead		
	Serum	Liver	Kidney	Serum	Liver	Kidney
Group I	0.116±0.005c	0.67±0.19c	0.57±0.07c	0.403±0.045	0.305±0.24b	0.76±0.98b
Group II	0.435±0.052a	45.67±5.69a	2.10±0.18a	----------	----------	----------
Group III	----------	----------	----------	0.850±0.212	1.386±0.754a	3.73±0.54a
Group IV	0.247±0.13bc	39.19±4.22a	1.53±0.40b	----------	----------	----------
Group V	----------	----------	----------	0.524±0.302	0.207±0.04b	1.99±1.73ab
Group VI	0.34±0.098ab	29.71±7.15b	0.90±0.36c	----------	----------	----------
Group VII	----------	----------	----------	0.482±0.368	0.507±0.292b	2.12±0.48ab

Means with different superscript are significantly different (P< 0.05)

Table (4): Effect of ALA and Vit. E on liver and kidney functions (u / l) of intoxicated rats:

Group	Liver function tests		Kidney function tests	
	Alt	AST	Urea	Creatinine
Group I	47.00 ± 1.00c	6.33 ±0.57c	64.66 ± 1.52b	0.84 ± 0.05d
Group II	65.33 ± 3.05a	11.00 ± 1.00a	75.00 ± 5.00a	1.85 ± 0.05a
Group III	55.33 ± 3.51b	9.00 ± 1.00b	59.66 ± 1.52c	1.80 ± 0.10a
Group IV	44.66 ± 2.51c	8.00 ± 1.00bc	53.20 ± 2.30d	1.20 ± 0.10c
Group V	43.66 ± 1.52c	3.66 ± 1.52d	23.10 ± 1.85f	1.46 ± 0.15d
Group VI	41.66 ± 2.08c	3.00 ± 1.00d	46.00 ± 2.00c	1.13 ± 0.15c
Group VII	46.33 ± 1.52c	6.33 ± 0.57c	27.20 ± 2.55f	1.10 ± 0.10c

Means with different superscript are significantly different (P< 0.05)

4. Discussion

The goal of the present study was to investigate the effect of some nutrients such as alpha lipoic acid (ALA) and vitamin E (Vit. E), in" lead and copper- induced toxicity in rats. The following parameters were tested: daily food intake, body weight gain and feed efficiency ratio; levels of Cu and Pb in serum and tissues (liver and kidney), with some biochemical parameters.

Rats fed on diets containing Cupper and Lead, consumed statistically significant less food as compared to their control. Injection of Pbintoxicated rats with ALA acid or Vit. E. significantly improved food intake and body weight gain. In addition, injecting Cu- intoxicated rats with alpha lipoic acid or vit. E. and Pb- intoxicated rats with ALA had significantly improved feed efficiency ratio. Such findings might be attributed to ALA, which has two positive effects that influence weight loss. Firstly, it decreases food intake. Secondly, it increases energy expenditure. ALA exerts this effect by decreasing the levels of a key enzyme, AMP-activated protein kinase (AMPK) within the hypothalamus, whilst increasing AMPK activity within muscles.AMPK is a major regulator of cellular energy metabolism, which inhibits the synthesis of fatty acids when activated, whilst increasing both fat, and glucose metabolism, which will further enhances weight loss (Lee et al., 2005a and Lee et al., 2005b). Furthermore, ALA facilitates the production of energy in cells a nd enhances the effectiveness of other antioxidants, regenerating Vit. E. and supports healthy function of blood glucose within normal levels (Allergy Research Group, 2008).

In the present study,it was indicated that injecting Cu or Pb- intoxicated rats with ALA or Vito E significantly decreased liver enzymes levels when compared to control rat groups which received Cu or Pb only. On the protective roles of Vito E in Pb-induced damage is the preventing effects of lipid peroxidation and the inhibition of superoxide dismutase and catalase activities in liver (Chaurasia and Kar, 1997).

Sivaprasad et al (2004) tested the efficacies of lipoic acid against Pb-induced lipid peroxidation in rat liver. Rats had administered lead acetate (0.2 0/0) in drinking water for five weeks to induce toxicity and lipoic acid (25 mg /kg. body wt/day) was given during the sixth week. The authors reported that lead damage to the liver was evident through the reductions in hepatic enzymes alanine transaminase (-38%); aspartate transaminase (-42%), and lipoic acid completely ameliorated the Pb-induced oxidative damage.

The present study showed that injecting Pb or Cuintoxicated rats with ALA or Vito E significantly reduced urea and creatinine levels {as compared to the Pb- or Cuintoxicaed rats]. These findings were supported by those of (Hanafy and Soltan 2004), who reported that blood urea and creatinine levesl were significantly increased in

Norway rats when Pb and Cu were administered subcutaneously (at a dose of 0.5 mg/1 00 g body weight or as a mixture 0.25 mg/1 00 g) for 4 weeks. They also indicated that the exposure to Vito E (250 IU/1 00 g body weight) administered orally for 4 weeks and pretreatment for 7 days) minimized the higher levels of urea and creatinine in rat groups intoxicated with Cu and Pb. Pb is capable of inducing oxidative damage to brain, heart, , kidneys and reproductive organs. The mechanisms for Pb-induced oxidative stress include its effects on membranes, DNA, and antioxidant defense systems of cells (Ahamed et aI., 2005 ; Ahamed and Siddiqui, 2007). Recent epidemiological studies have reported that low-level of Pb exposure had an association with several disease outcomes such as hypertension, peripheral artery disease, kidney disease, neurodegenerative disease, and cognitive impairment (Ekong et aI., 2006; Menke et al., 2006). Acute exposure to lead acetate was shown to increase levels of urinary excretion. Progression to renal failure is significantly worsened by oxidative stress in chronic inflammatory kidney disease.

Vitamin E supplement may be beneficial in reducing and slowing progressive kidney diseases that are significantly accelerated by oxidative stress. Vitamin E therapy may also be effective in reducing cardiovascular disease associated with chronic renal failure and the uremia state. Vitamin E therapy is also considered as a mean of correcting plasma antioxidant status and attenuating the cardiovascular disease that accompanies kidney failure. The optimal dose for Vit. E that may be helpful in slowing renal failure in humans may lie between 300 to 700 IU/day (Fryer, 2000). Vit. E. protects against lipid peroxidation and copper toxicity and prevents the majority of metal-mediated damage both in vitro systems and in metalloaded animals (Gaetke and Chow, 2003 ; Valko et al., 2005).

ALA supplementation has been shown to enhance the uptake of creatinine within muscle cells (Burke et aI., 2003). This is likely to be due to ALA's ability to enhance the uptake of glucose within muscles. ALA exerts this effect via enhancing insulin sensitivity (Lee et aI., 2005a; Lee et aI., 2005b). Creatine has traditionally been combined with a high sugar drink, which increases insulin levels and helps to enhance creatine uptake by muscle fibers. Because ALA increases muscles' sensitivity to insulin, it further increases the uptake of creatine by the muscles. In vitro and animal studies suggested that lipoic acid supplementation might be a beneficial component in the treatment of heavy metal toxicity, particularly toxicity involving Pb and Cu (Packer et aI., 1995; Gurer et aI., 1999 ; Yamamoto et aI., 2001). Intraperitoneal injection of 25 mg/kg ALA given to Pb- intoxicated rats for seven days was reported to significantly alter oxidative stress induced by Pb toxicity (Gurer et aI., 1999).

The results of the present study indicated that injecting Cu- intoxicated rats with ALA significantly reduced serum copper. Moreover, whereas injecting these rats with Vito E significantly reduced liver copper. These results are supported by the findings of (Allergy Research Group, 2008), who reported that ALA is a powerful support for the detoxification processes of the liver since it reduced Pb- toxicity and excessive copper and increased cellular glutathione levels; and by the findings of (Packer et al. 1995). They indicated that ALA has been used for decades to protect the liver and to detoxify the body from heavy metal pollutants, further more concluded that ALA alone or together with Vito E is an effective treatment for lessening indices of oxidative damage and normalizing organ's functions.

ALA is a potent antioxidant (Kagan et aI., 1990; Midaoui et aI., 2003; Wollin and Jones, 2003). It was identified as an effective antidote against heavy metal induced toxicities (Anuradha and Varalakshmi., 1999; Gurer et al., 1999; Gale Group, 2006).

The present study proved that the injection of Cuintoxicated rats with either ALA or Vit. E. significantly reduced kidney copper. These findings agreed with those reported by (Hanafy and Soltan,2004), who showed that Cu-intoxication to Norway rats resulted in marked destruction and distortion of the renal tubule cells; while oral administration of Vit. E (250 lUll 00 g body weight) revealed no abnormal histological findings as compared to normal kidney (control group). In addition (Liu et al.,1992), suggested that the increased urinary excretion of copper was related to the manifestation of renal toxicity and the synthesis of metallothionen in the kidney.

The present study showed a favorable alteration in serum lead due to the injection of Pb-intoxicated rats with ALA or Vit. E, although this alteration was not statistically significant. The hematopoietic system is one of the target organs in lead toxicity. Lead has been shown to alter RBC membrane flexibility and to increase red blood cells fragility (RBC) leading to increased risk for hemolysis (Ahmed and Siddiqui, 2007). Vit. E has a known protective action in membrane stability and prevents membrane lipoproteins from oxidative damage (Packer, 1991). Alpha-tocopherol was shown to prevent RBC membrane damage in Pb- toxicity by lowering lipid peroxide levels and increasing superoxide dismutase and catalase activity (Chaurasia and Kar, 1997). Animal studies have reported that Vit. E effectively prevented lipoperoxide-related lead toxicity and was more effective than methionine r

vitamin C at decreasing lipoperoxidation in the liver and kidney of Pb-exposed rats when given at doses of 100 IU/kg body weight (Patra et aI., 2001).

Treating Pb-intoxicated rats with ALA or Vit. E significantly reduced liver lead as compared to the control. Autopsy studies ofPb- exposed humans indicated that liver tissue is the largest repository (33%) ofPb from among the soft tissues followed by kidney cortex and medulla. As environmental exposures to Pb have increased, the toxic effects of Pb on various organ systems in the body have been recognized (patrick, 2006). Rats received intragastrically 35 mg/kg of Pb2+ once a week or 70 mg /kg of Pb2+ twice a week for 7 weeks resulted in 16.8 or 32.4 mg Pb /dL blood, and caused inhibition of lipoprotein lipase in the context of arteriosclerosis (Skoczynska et aI., 1993).

Injecting Ph- intoxicated rats with ALA or Vit. E reduced kidney Pb as compared to the control rats, although this reduction was not statistically significant. Pb- intoxication had a profound effect on the structure and consequently on the function of the rat kidney (Hanafy and Soltan 2004), they also reported that four weeks of oral administration of Vit. E (250 IUIl 00 g body weight) did not show any abnormal histological findings as compared to normal kidney.

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Influence of Nano-bodies produced from Gram-negative Bacteria against infection with Pseudomonas Aeruginosa induced Bronchial Pneumonia with special references to their effect on Immune system in Male Albino Rats

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Abstract: Seventeen Gram-negative isolates were tested for Nano bodies or Membrane Vesicles production and their bacteriolytic activity against different Gram-positive and Gram-negative bacteria. The most Nano bodies producing bacterial isolates were exposed to the antibiotics Cefotaxime and Gentamycin, which induced the production of Cefotaxime membrane vesicles(c-MVs) and Gentamycin membrane Vesicles (g-MVs), respectively. The c-MVs and g-MVs are larger with higher lytic activities against the susceptible host bacteria when compared to those produced under normal growth conditions. Influence of these Nano bodies from Proteus vulgaris on the morbidity and mortality rates of albino rats was investigated and the results revealed that the animal resist the pseudomonas aeruginosa infection which induced chronic bronchial pneumonia. Also, the nano bodies from Erwinia cartovora and Proteus vulgaris were tested as vaccine in rats to protect them against Lethal (LD) and Sub-lethal (SLD) doses (acute and chronic infection) of Pseudomonas aeruginosa and surprisingly results, the animals lived till the end of the expermintal period. The vaccinated rats challenged with LD or SLD of Pseudomonas aeruginosa showed high clearance of the pathogen from Lung, Spleen, Liver and Blood when compared to the bacterial counts in control rat groups. The result of the present study proved that Nano bodies or membrane vesicles from Erwinia cartovora and Proteus vulgaris could enhance the immune response of animals and gave the protection against pseudomonas aeruginosa induced Bronchial Pneumonia. So, Nano bodies or membrane vesicles can be expressed as a new strong antigenic structure could have the ability to enhance the immune response, and also, expressed as a new hope as antibiotic,vaccine and a biological control for human and animal. Further studies on the effect of these nano bodies on other Auto-immune, cncer and different chronic diseases might be needed. [Journal of American Science 2010;6(8):64-71]. (ISSN: 1545-1003).

Keywords: Nano-bodies, Gram-negative Bacteria, Immune system.

1. Introduction

Molecular biological methods have not yet given Scientists a precise historical record of Gram-negative bacteria, but ancient stromatolites containing fossilized remained of Cyanobacteria- like Prokaryotes date back to Archaen eon-over such extraordinary periods of time (much of it when no other life existed), we can imagine that random mutation, selection and the slowly but ever-changing global environment gave rise to two fundamentally different cell wall formats in bacteria; Gram-positive and Gram-negative varieties (Beveridge, 1999).

The Gram-negative bacterium contains Lipopolysaccharids lipoprotein major components, and relatively little peptidoglycan (less than 10 % of the total cell wall) in their walls, whereas the walls of Gram-positive are mainly composed of peptidoglycan (30 – 70 % of the tota; cell wall), polysaccharids or teichoic acid (broth),or teichuronic acid (Tamm et al., 2001). So, the peptidoglycan is the only cell wall polymer common to both Gram-positive and Gram-negative, and named as basal structure (Wack, 1957), mucopeptide (Mandelstem and Rogers, 1959); glycopeptide (Strange and Kent, 1959); murein (Weidel and Pelzer, 1964) and peptidoglycan (Stromimgeret al., 1967).

Outer membrane vesicles or Nano-bodies are defined by Li et al., (1998) as bilayered membraneous particles produced by Gram-negative bacteria, into which degradative enzymes are concentrated (Autolysins, peptidoglycan hydrolase and other enzymes). It was reported that naturally produced bacterial vesicles are discrete, closed outer membrane blobs produced by growing cells (McBroom and Kuchn, 2005).

Although, outer membrane vesicles (Nano-bodies) production has been observed for more than 50 years, the machinery that allows vesicle or nano-bodies secretion while maintaining bacterial viability remains elusive. Many theories exist on the

mechanism of vesiculation based on biochemical and genetic data and are reviewed elsewhere (McBroom and Kuchn, 2005).

Nano bodies are capable of lysine a variety of Gram-positive and Gram-negative bacteria and the potency of lyses depended on the peptidoglycan chemotype of the attacked cell (Beveridge, 1999). For Gram-positive, Nano bodies adhere to the cell wall, where they break open and digest the immediate underlying peptidoglycan. It attacks the Gram-negative bacteria in much different manner. Here, it adheres to the outer membrane and rapidly fuse to it (Kadurugamuwa and Beveridge, 1996) and the luminal contents are released directly into the periplasmic space of the recipient cell. Nano bodies mediated lysis of bacteria occurs only when the recipient cells are under insufficient nutrient conditions (Beveridge, 1999).

Kadurugamuwa and Beveridge, (1995), have been reported the membrane surface-active agents such as Gentamycin could increase the production of Nano bodies. Gentamycin Nano bodies can kill Pseudomonas species, which is normally permeable to amino-glycoside antibiotics through fusing the normally impermeable outer membrane of the resistant strain and deliver gentamycin into the periplasm where it can be actively imported to the cytoplasm and inhibit protein synthesis. Another possible medical application is as vaccine agents. Nano bodies (membrane vesicles) are strongly antigenic particulate structures which also posses natural adjuvant qualities for enhancing an immune response Beveridge, (1999). This vaccine has many advantages including ease of administration and induction of a mucosal immune response at the site of infection for many pathogens.

From the above mentioned, the study aimed to produce nano bodies or membrane vesicles from different Gram-negative bacteria isolated and exposed or not exposed to antibiotics under normal growth condition. Uses of these isolate (nano bodies or membrane vesicles) against some dangerous pathogens such as Pseudomonas species, which induced Bronchial Pneumonia in Human and Animals.

2. Material and Methods

Experimental Design

Random bred male albino rats weighing approximately 110±3.71g, obtained from Animal house laboratory, National research Center, Giza, Egypt. The rats were evaluated prior to initiation of the study to ensure a sanitary Hygienic condition and acclimation to the study environment. Clinically accepted animals were randomly assigned into two major groups and each group was sub-divided into 4 groups (10rats / group).

Environmental Condition

A total of 80 rats, were housed in stainless steel wire mesh cages on a bedding of wood chips (Five animals / Cage). They were kept in an ambient temperature of 25 ± 3 oC, on a light / dark cycle of 12 / 12 hours and supplied rat chow (the diet) and fresh water ad libitum.

Diet

A basal diet (Table: 1) was formulated to meet the rat nutrient requirements as recorded by Osfor (2003).

Antibiotics:

Cefotaxime and gentamycin were purchased from the local market and they produced from T3A Pharma Group and Glaxo Wellcome Companies respectively.

Experimental design

This study was performed in two main experiments.

The first one was divided into equal 4 groups as follows:

Group I: Fed the basal diet and received the nano bodies from Erwina Caroyovora injection and served as control positive group.

Group II:Fed the basal diet and received Saline solution and challenged with Erwina Carotovora and served as control negative group.

Group III: Fed the basal diet and received the nano bodies from Erwinia Caroyovora injection and challenged with the lethal dose (2 × 105 CFU "Colony Forming Unit") of Pseudomonas Aeruginosa (after 14 days).

Group IV: Fed the basal diet and received the nano bodies from Erwina Caroyovora injection and challenged with Sub-Lethal dose (2 × 103 CFU) of Pseudomonas Aeruginosa (after 14 days).

The second experiment was also divided into equal 4 groups as follow:

Group I: Fed the basal diet and received the nano bodies from Proteus Vulgaris injection and served as control positive group.

Group II: Fed the basal diet and received Saline solution and challenged with Proteus Vulgaris injection and served as control negative group.

Group III: Fed the basal diet and received the nano bodies from Proteus Vulgaris injection and challenged with the lethal dose (2 × 105 CFU "Colony Forming Unit") of Pseudomonas Aeruginosa (after 14 days).

Group IV: Fed the basal diet and received the nano bodies from Proteus Vulgaris injection and challenged with Sub-Lethal dose (2 × 103

(CFU) of Pseudomonas Aeruginosa (after 14 days).

Bacterial Strains:

The tow main used Bacteria were chosen from 17 kinds of bacteria. They provided by Professor H.H. Martin, Institute of Microbiology, TH Darmstadt, Germany.

Culture Media:

Nutrient broth, Buffer (Phosphate & Borate) and MacConkey Agar were used for cultivation and Isolation of Bacteria from Oxford, England.

Electron Microscope Investigation:

Twenty micro-liters tested Gram-negative bacteria suspension was placed on Carbon-Coated nickel grids and stained with 2% aqueous solution of uranyl acetate for 20 Seconds. Then, examined with Zeiss EM10 transmission electron microscope operating under standard condition (60 Kilo volt).

Purification and Isolation of Nano bodies (Membrane Vesicles):

Nano bodies were purified and isolated as described by Mayrand (1986).

Clinical Observation:

The rats were observed daily throughout the experimental period to notice the morbidity and mortality rats specially after challenge with bacteria.

Clinical Pathology:

Blood samples containing EDTA as anticoagulant was used for the determination of haemoglobin content, erythrocyte and leuckocyte counts (Total and Differential).

Histopethological studies:

Immediately after sacrifice of animals, samples of Lung tissues was fixed in 10 % formal saline, dehydrated, cleared, embedded in paraffin and were sectioned at 7 µm. Paraffin sections were stained with hematozyline and eosin stain according to the method of Pearse, (1985).

Statistical Analysis:

The obtained data were statistically analyzed after Snedecor and Cochron, (1973).

3. Results and Discussion

Table (1): Composition of the basal Diet:

Ingredients	Percentage
Sorghum	39
Corn yellow	31.6
Barley	8
Meat meal	8
Corn Cobs	7.3
Vegetable Oil	4
Lysine	0.3
Methionine	0.4
Di-Calcium Phosphate	0.2
Lime Stone	0.4
Sodium Chloride	0.3
Vitamins and Mineral Mixture*	0.5
Calculated Nutrient Composition:	
Crude protein	11.99
Energy	3404.2
Crude Fibe	4.46
Ether Extract	7.51
Lysine	0.71
Methionine	0.61
Calcium	0.45
Phosphorus	0.4

*Vitamins and Minerals Mixture (g): Copper sulphate (0.05), Ferric Citrate (0.59), Zinc Carbonate (0.053), Calcium carbonate (7.25), Calcium hydrogen phosphate (11.3), Di-sodium hydrogen phosphate (6.0), Potassium Chloride (7.3), Potassium Iodide (0.003), Magnesium Chloride (2.3) and Magnesium sulphate (0.154).
Thiamine (0.3), Riboflavin (1.0), Pyridoxine (0.2), Calcium pantothinate (6.0), Nicotinic acid (20.0), Cyanocobalamine (0.005), Folic acid (0.2), Biotin (20.0), Inositol (60.0), Choline Chloride (60.0), Vitamin A (4000 IU), Vitamin E (30 IU) and Vitamin K (50 IU).

Table (2): Lytic effect of Nano bodies or membrane vesicles formed by Citrobacter Freundii and Enterobacter Cloacae on different bacteria:

Host bacteria	Clear zone (mm) caused by MV_s of Citrobacter Freundii			Clear zone (mm) caused by MV_s of Enterobacter Cloacae		
	n-MV_s	c-MV_s	g-MV_s	n-MV_s	c-MV_s	g-MV_s
Bacillus cereus	14.7±0.07	23.5±0.07	18.0±0.14	0.0	0.0	0.0
Bacillus subtilis	0.0	0.0	0.0	15.4±0.06	0.0	20.5±0.20
Enterobacter cloacae	0.0	0.0	0.0	0.0	0.0	0.0
Erwinia carotovora	0.0	0.0	0.0	17.3±0.10	0.0	26.0±0.10
Escherchia coli	17.8±0.07	24.2±0.10	22.8±0.034	17.0±0.10	0.0	25.0±0.37
Proteus vulgaris	18.7±0.23	22.0±0.12	23.1±0.12	18.1±0.14	0.0	22.6±0.06
Pseudomonas solanacearum	0.0	0.0	0.0	17.1±0.07	0.0	22.9±0.10
Pseudomonas syringae	18.6±0.01	26.0±0.10	21.5±0.35	17.6±0.06	0.0	20.6±0.28
Serratia marcescens	19.3±0.06	19.5±0.12	23.3±0.06	0.0	0.0	0.0

n-MV_s: Natural membrane vesicles

c-MV_s: Membrane vesicles produced in the presence of cefotaxime.

g-MV_s: Membrane vesicles produced in the presence of gentamycin

±: Standard error of means.

Table (3): Lytic effect of Nano bodies or membrane vesicles formed by Erwinia carotovora and Klebsiella pneumoniae on different bacteria:

Host bacteria	Clear zone (mm) caused by MV_s of Erwinia carotovora			Clear zone (mm) caused by MV_s of Klebsiella pneumoniae		
	n-MV_s	c-MV_s	g-MV_s	n-MV_s	c-MV_s	g-MV_s
Bacillus cereus	18.0±0.14	20.5±0.07	20.0±0.14	0.0	35.0±0.5	0.0
Bacillus subtilis	15.6±0.06	16.1±0.07	19.0±0.14	0.0	33.2±0.04	0.0
Enterobacter cloacae	19.5±0.07	20.2±0.11	21.8±0.04	15.5±0.07	30.0±0.12	19.5±0.07
Erwinia carotovora	0.0	23.6±0.11	21.0±0.11	0.0	31.5±0.2	0.0
Escherchia coli	19.0±0.14	19.5±0.07	21.5±0.07	19.15±0.12	31.0±0.1	23.7±0.15
Proteus vulgaris	0.0	20.5±0.07	22.2±0.24	19.97±0.20	20.3±0.15	22.5±0.07
Pseudomonas solanacearum	16.7±0.11	16.5±0.03	19.0±0.40	0.0	29.0±0.30	0.0
Pseudomonas syringae	18.5±0.07	18.8±0.11	21.6±0.03	0.0	34.0±0.14	22.0±0.10
Serratia marcescens	0.0	21.0±0.14	20.5±0.07	19.55±0.01	32.5±0.20	23.3±0.08

Table (4): Lytic effect of Nano bodies or membrane vesicles formed by Proteus vulgaris and Serratia marcescens on different bacteria:

Host bacteria	Clear zone (mm) caused by MV_s of Proteus vulgaris			Clear zone (mm) caused by MV_s of Serratia marcescens		
	n-MV_s	c-MV_s	g-MV_s	n-MV_s	c-MV_s	g-MV_s
Bacillus cereus	15.8±0.03	20.3±0.06	27.0±0.15	19.0±0.14	19.5±0.07	23.9±0.11
Bacillus subtilis	16.5±0.07	22.5±0.07	24.0±0.14	23.0±0.10	25.5±0.53	27.1±0.08
Enterobacter cloacae	18.0±0.14	27.5±0.07	26.1±0.10	0.0	0.0	0.0
Erwinia carotovora	15.6±0.06	18.4±0.10	17.6±0.06	18.3±0.07	17.3±0.06	23.1±0.12
Escherchia coli	15.8±0.03	25.0±0.14	23.4±0.07	19.0±0.28	18.5±0.07	24.25±0.04
Proteus vulgaris	0.0	0.0	0.0	16.5±0.07	20.1±0.36	21.0±0.10
Pseudomonas solanacearum	16.5±0.07	23.5±0.07	23.8±0.01	31.3±0.30	22.8±0.14	35.5±0.07
Pseudomonas syringae	0.0	0.0	0.0	30.2±0.12	24.3±0.15	23.1±0.10
Serratia marcescens	16.5±0.07	30.0±0.14	20.75±0.03	0.0	0.0	0.0

Table (5): Effect of different doses of Pseudomonas aeruginosa on mortality rate of non-immunized Animals.

Doses	First Experiment				Second Experiment			
	G-I	G-II	G-III	G-IV	G-I	G-II	G-III	G-IV
2 × 10 3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2 × 10 4	25	25	50	100	100	100	100	100
2 ×10 5	50	75	100	100	100	100	100	100
2 × 10 6	100	100	100	100	100	100	100	100

4. Discussion:

Many Gram-negative bacteria produced external nano-bodies named membrane vesicles during the normal growth. The release of these vesicles or nano-bodies from the whole cell depends on the bacterial strains as well as the nutritional conditions (Ksdurugamuwa and beveridge, 1995 and Wai et al., 1995). Nano bodies (Membrane Vesicles) entrap several periplasmic components include alkaline phosphatase, phospholipase, proelastase, protease and peptidoglycan hydrolase (Kraft et al., 1998). Nano bodies may be important during the initial phases of infection, as they concentrate such factors and convey them to host tissue (Ksdurugamuwa and beveridge, 1997). This cell wall degrading enzymes could be use to lyses surrounding dissimilar bacteria in the bacterium environment, thereby releasing organic compounds for growth. Naturally produced bacterial vesicles are discrete, closed outer membrane blebs produced by growing cells, not products of cell lyses or cell death (Yaganza et al., 2004 and McBroom & Kuehn, 2005).

In the present study, 17 bacterial strains of Gram-negative bacteria were tested for their lyses or killing effect on other 17 bacterial strains of different Gram-negative and Gram-positive bacteria under suitable condition for action and production of Nano bodies (Azab, 2004). Out of the tested isolates, ten strains had high killing effect on the tested hosts.

The electron micrograph of the bacterial strains which exhibited killing effect revealed that all of these strains produced many outer membrane nano bodies different in size and amount. They were almost spherical in shape with average diameter of 25 – 200 nm, and they had a dense materials enclosed inside them.

In this study six strains of the tested bacteria exhibited high killing effects with high nano bodies' production, were exposed to Cefotaxime and Gentamycin and the bacteriolytic activities were determined. These six strains except Enterobacter cloacae produced Nano bodies with higher lytic activities against most of the recipient bacteria after exposure to cefotaxime (β-lactam antibiotics) or gentamycin (aminogylicosid antibiotics). The higher lytic activities of the Nano-bodies might be due to the formation of large vesicles filled with enzymes and cefotaxime or gentamycin which might act

synergistically with the degradative enzymes enclosed to lyse hard-to-kill hosts.

The present study was investigated the effect of intrapritoneal injection of different doses of Proteus Vulgaris on the mortality of male albino rats and it was found that all the challenged groups lived till the end of the experimental period (ten days). No illness symptoms were noticed on the rats, and they had a significant increase in the total leuckocytic counts, and percentage of neutrophils and lymphocytes which indicated that the normal immune response of the rats could manage the pathogen and overcome it. So, no bacteria were detected in the kidney, liver and spleen tissues from the challenged animal groups. Also, the used doses of proteus vulgaris did not affect the mortality of male albino rats used, and there was noticed increase in the total leuckocytic counts, neutrophil and lymphocytes percentage. This give a conclusion that this strain of proteus vulgaris is a weak pathogen and the natural immune response of the animals can over come it. The increased total leuckocytic counts and the significant increase in lymphocytes which responsible for immune response and antibody formation may explain how rats could over come the pathogen. Also, the different in the rate of increase in the total leuckocytic counts of rats may be due to the difference in the age and species. This result was in agreement with Larsson (1980) who reported that Proteus bacteremia was dominated among the very young and the elderly patients was explained by the importance of the host-parasite relationship in Proteus bacteremia.

In he present study the effect of Proteus Aeruginosa (a human pathogen isolate) on the mortality rate of albino rats was investigated. The dose 2 × 105 Colony Forming Unit (CFU) / rat was found to be the lethal dose and causing mortality level more than 75% within 2 days, and the sub-lethal dose 2 × 103 CFU / rat caused increase in the total leuckocytic counts to 9.5 × 109 / mm3 compared the untreated control, with a high significant increase in the percentage of the neutrophils and in the lymphocytes.

These findings were agreement with Johansen et al., (1995) who reported that the rise in polymorph nucleus lymphocytes is a distinctive feature of patients with cystic fibrosis suffering from the chronic infection of lung caused by Pseudomonas aerugenosa. It was revealed also from the presented results that the eosinophil and monocyte counts were not significantly changed, and thesecan be explained according to Mbajiorgu et al., (2007) who reported that the eosinophil and monocyte counts did not contribute to the change in the total leukocytes count, since neutrophils and lymphocytes have the major role in fighting foreign organisms, the variations in leukocyte counts were essentially caused by variations in neutrophil and lymphocyte numbers. Also, it was found that the lung tissue of the rat group, who received saline and challenged with Proteus Vulgaris, was damaged and filled with polymorph nucleus lymphocytes. This result was in agreement with Hoiby, (1995) and Pedersen, (1992) whom reported that patient's lung infected with Pseudomonas aeruginosa slowly progressive damage of lung parenchyma and eventually respiratory insufficiency.

The present study was investigated the immune response for the isolated Nano bodies, as a vaccine in rats protection against Pseudomnas aeruginosa lethal (LD) and sub-lethal (SLD) doses, and the result revealed that all the vaccinated rat groups with Nano bodies (Membrane Vesicles) of Erwinia carotovora showed a high resistance against the LD and SLD of Pseudomonas aeruginosa, and all the rats lived till end of the experimental period. This means that the MVs vaccine stimulated the immune response of the rats which persist for two weeks after vaccination and help the rats to overcome the infection with Pseudomonas aeruginosa as a cross-protection immune response. This may be explained by the results of Alaniz et al., (2007) who demonstrated that MVs possess important inflammatory properties as well as B ant T cell antigens known to influence the development of salmonella specific immunity to infection in vivo. Also, these authors revealed that MVs are a functional nonviable complex vaccine for salmonella by their ability to prime protective B and T cell responses in vivo.

In the present study the bacterial count was significantly decreased in target organ (lung), parenchymal and immune organs (Spleen and Liver) and Blood of the vaccinated animal groups, when compared with the infected group. These indicate the high immune response induced by the used vaccines. On the other hand, for the group injected with LD of Pseudomonas aerugenosa specially that vaccinated with membrane vesicles of Erwinia Carotovora, the immune response induced was not solid enough to give adequate elimination of the pathogen, this could be attributed to; the shared antibodies formed were not highly specific; the intra-species variability of the pathogen or it may be the dose regimen followed in this study was not adequate.

The lung of all rat groups were examined for histopathological changes and the results revealed that the lung tissues of the rat groups vaccinated with membrane vesicles of Erwinia carotovora or Proteus Vulgaris were as normal as the control one and the groups of the tow vaccines which challenged with

SLD of Pseudomonas aerugenosa showed no change and they were almost normal. These indicated that the vaccinated animals were highly protected against damaging effect of Pseudomonas aerugenosa on the lung. On the other hand, the lungs of rat group vaccinated with membrane vesicles of Erwinia carotovora after challenged with LD of Pseudomonas aerugenosa showed some inflammation and exudates in the bronchiole space and slightly damaged parenchyma cell walls when compared with the lung tissue of the infected group. The lung of the rat group immunized with membrane vesicles of Proteus Vulgaris when challenged by LD of Pseudomonas aerugenosa had some inflammation without any damages in the cells. These results could be explained according to Bertot et al., (2007) who reported that the chronic lung infection by opportunistic pathogen, such as Pseudomonas aerugenosa and Burkholderia cepacia complex, is a major cause of morbidity and mortality in patients with cystic fibrosis. Each outer membrane preparative given to the rats intraperitoneally in a single dose injection (5 micrograms / rat) protected the animals not only in homologous but also in varying intensity in heterologous systems. Evidence was obtained that this nonspecific protection is cell mediated. Moreover, Shoemaker et al., (2005) reported that native outer membrane vesicles vaccine was administered to rabbits via different routs using different primary immunization schedules. Similar high levels of serum bactericidal activity were induced regardless of route or number on immunization.

At the end, one can conclude that the membrane vesicles of Erwinia Cartovora and Proteus Vulgaris enhanced the immune response of the rats and gave a cross-protection against Pseudomonas aerugenosa, but the result of protection from membrane vesicles of Proteus Vulgaris were more effective than that induced by Erwinia Cartovora. So, nano-bodies or membrane vesicles are strong antigenic structures and could have the ability to enhance an immune response. Nano bodies or membrane vesicles are a new hope as antibiotic, vaccine, immune enhancer and as biological control for human and animal diseases.

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Effect of Red Mite (*Dermanyssus gallinae)* Infestation on the Performance and Immune Profile in Vaccinated broiler breeder flocks

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Abstract: The article aimed to: (1) Investigate the impact of red mite infestation on performance of broiler breeders (egg production, mortality and egg- livability). (2) Assess the effect of red mite on immunological response of humeral antibodies (antibody levels) of vaccinated broiler breeders against Infectious bronchitis (IB), Infectious bursal disease (IBD), Avian Encephalomyelitis (AE), Chicken infectious anemia (CAV), Newcastle Disease (ND) and Avian Influenza (H5N1). According to the degree of infestation of the house, the obtained data were arranged into 4 groups: G1: houses of no infestation, G2: low, G3: houses of high and G4: houses of very high infestation. Results revealed that: (1) there were significant differences between the house infestation and mortality rate, egg production as well as egg livability percentage of breeders. (2) There were highly significant differences between the house infestation and the immune response (level of antibodies titre) in vaccinated breeders against:, Infectious bronchitis (IB), Infectious bursal disease (IBD), Avian Encephalomyelitis (AE) , Chicken infectious anemia (CAV) , Newcastle Disease (ND) and Avian Influenza (H5N2). [Journal of American Science 2010;6(8):72-78]. (ISSN: 1545-1003).

Keywords: Degree of infestation, Vaccination, Immune response, Egg performance

1. Introduction

The poultry red mite *Dermanyssus gallinae* is regarded as the most important external parasite of laying hens in organic as well as in conventional egg production in Europe (Maurer *et al.,* 1993; Höglund *et al.*, 1995; Kilpinen and Steenberg, 2009).It attacks the resting hens mainly at night for a short blood meal (Kirkwood, 1968; Lancaster & Meisch, 1986; Guy *et al.*, 2004; Amosova and Stanyukovich, 2008)). After feeding, the mites hide in cracks and crevices, where they also mate and lay their eggs (Hearle, 1938). Under favorable warm and moist conditions, the life cycle can be completed in less than 1 week (Kirkwood, 1968, Nordenfors and Chirico, 1999).

At high infestations, red mites can cause anemia; however low mite populations irritate the hens to an extent that they refuse to go into the henhouse or rest on the perches. The poultry red mite is currently almost impossible to control during the production cycle with traditional measure, and decrease their welfare significantly during egg production may result in poorer hen performance, associated with reduced production and cause decreased feed intake and weight loss (Williams, 2003). Scientific information on the effects of poultry red mite on hens is incomplete as information is mainly sourced from the industry and is not well documented. Researchers agree that there are indications for the following effects of poultry red mite, which include, increased water intake in infested hens and lower egg production from the flock overall(Mul *et al.*,2009).

Infested hens increase their production of new blood cells, but during periods of rapid mite population growth, blood loss exceeds blood production capacity resulting in severe anaemia (Kilpinen *et al.*, 2005). Other negative effects of poultry red mite include high mortality, stress behaviour (higher levels of preening, head scratching and gentle feather pecking), lower body weight and reduced egg quality due to blood spots (Chauve, 1998). The productivity link could be that a severe mite infestation can increase mortality, and as Arkle (2005) showed, there is a direct effect of the size of the mite population on bird mortality. This of course means lower hock productivity: however, lower egg production per hen has not been found as a result of a mite infestation (Kilpinen, 2005). Chicken mites may also act as carrier of several important disease-causing agents, e.g. Salmonella (Zeman *et al*, 1982), spirochaetosis (Hungerford & Hart, 1937), and encephalitis. Some of these survive in the mite for several months, thus forming a potential source of reinfection of new flocks, as the mite can live without feeding for up to 9 months (Nordenfors *et al*, 1999). Chicken mites are also known to cause puritic dermatosis in humans (Baselga *et al*,, 1997) and may create serious problems for workers in the poultry industry due to the nuisance of mites crawling on the skin. Furthermore, it is likely that the mite act as reservoirs for zoonotic bacteria since the mite will hide in the structure and thus be out of reach of the sanitation measures carried out between flocks (Steenberg *et al.*, 2005).

Red mite (*Dermanyssus gallinae* De Geer, 1778) is considered the most economically deleterious (Chauve, 1998). In commercial egg production, red mite is a serious problem, not only as a potential vector of several avian of pathogens, but more importantly as a direct parasite effecting both production and welfare (Nordenfors, *et al.*, 1999; Kilpinen, 2001). Exposure to red mite may induce a number of symptoms relative to the severity of infestation, including irritation, restlessness, anemia and occasionally death. This subsequently leads to reduced egg production (Axtell and Arends, 1990)., reduced egg weight and increased downgrading as a result of poor shell integrity and superficial blood staining (spotting) from engorged mites which are crushed on egg belts etc. (Chauve, 1998; Cosoroaba, 2001).

Therefore the aims of this study were: (1) to investigate the impact of red mite infestation on performance of breeders (including egg production, mortality and egg-livability). (2) to assess the probable effect of red mite-infestation on the immune response (antibody levels) of vaccinated breeders against: Infectious bronchitis (IB), Infectious bursal disease (IBD), Avian Encephalomyelitis (AE), Chicken infectious anemia (CAV), Newcastle Disease (ND) and Avian Influenza (H5N1).

2. Material and Methods

Birds and location

Seven red mite (*Dermanayssus gallinae*) infested commercial breeder farms were studied. The farms were environmentally controlled and the densities of farms were ranged between 15,000 to 50,000. Two farms of battery-cage system (five-tier wire battery cages, 5 hens\cage of Ross 308 breed and depended on artificial insemination) and 5 built up litter house system (Avian breed 48) as well as two non-infested commercial built up litter house breeder farms (Avian breed 48); as control (Environmentally controlled with 1:8 male to female ratio).The farms were in Wadi El-Natron, El- Behera and Alexanderia Governorates of Egypt. The farms were surveyed from October 2007 to October 2009.

Vaccination programme (Table1)

All flocks were received the same vaccination programme (Table 1).

Red mite population (Collection and counting of red mite)

Traps (10 traps\house) made of corrugated cardboard measuring 200 × 80 × 3 mm were placed on the floor of built up litter and battery-cage houses. The traps were collected every month to count mites in control and mite-infested houses. At low densities (below approximately 5000 mites/trap), mites were counted individually, but if they were abundant, the total number of mites were estimated by pouring them into a calibrated measuring cylinder and recording the number of mites.

Mites were counted by pouring them into a calibrated measuring cylinder and recording the number of mites (Nordenfors and Chirico, 1999). Score mite count: (1) 0 No evidence of infestation; 250 ≤ 5000 Low infestation; (2) > 5,000 ≤ 8,000 Moderate infestation; (3) >8,000 ≤ 15,000 High infestation and (4) < 15,000 Very high infestation.[Score mite counts (mite\trap)during the study period, were ranged 385- 4890;5588-7443;8227-14880 and 15983-17654 in low; moderate; high and very high infested farms, respectively.

Blood sampling

Venous blood was obtained from hens at 45, 47 and 49 wks of age. On each occasion, 1% randomly selected hens were bled directly from the wing vein to yield a volume of approximately 2 ml of blood. Blood was allowed to clot at room temperature and sera were obtained following centrifugation and stored -20°C, until required for further analysis. An enzyme linked immunosorbant assay (ELISA test-Synbiotics Corporation Company) to measure the antibody titre for Infectious bursal disease (IBD), Infectious bronchitis (IB), Chicken infectious anemia (CVA) and Avian Encephalomyelitis (AE) by using specific antigen for each one. Haemagglutination (HA) and Haemagglutination Inhibition (HI)) tests were performed to assess the immunological responses against both Newcastle Disease (ND) and Avian Influenza (AI). **Enzyme-linked immunosorbent assay** procedures were carried out according to manufactures´ protocol.

Hemagglutination and hemagglutination inhibition (HI) tests:

The recommended method use V-bottomed micro well plastic plates were applied. In which the final volume for both types of HA and HI test was 0.075 ml. The reagents required for these tests are isotonic PBS (0.1 M), pH 7.0-7.2 and RBCs. Positive and negative control antigens and antisera should be run with each test. The test was applied to quantify AIV and NDV antibodies in chicken sera according to OIE (2005)

Plate hemaggluntination inhibition (HI) test

(I) 25 µL of PBS was dispensed into each well of a plastic U-bottomed microtitre plate, except well 1 and 7 of each raw.

(ii) 25 µL of known positive antiserum (Newcastle Disease, Charles River SPAFAS ;) was placed into first and second wells of each raw of the plate.

(iii) From well two and eight of each raw two-fold dilution was made across the plate.

(iv) 25 µL of HA positive virus was added into each well and left for a minimum of 30 min at room temperature.

(v) 25 µL of 0.5% (v/v) chicken RBC was added to each well. After gentle mixing the plate was allowed to keep for about 40 min at room temperature.

(vi) In each raw two wells (six and twelve) were kept as control.

(vii) The hemagglutination inhibition activity was observed after 40 min and compared with control one. HI titres may be regarded as being positive if there is inhibition at a serum dilution of 1/16 (24 or 4 log-2 when expressed as the reciprocal) or more against 4 HAU of antigen.

Plate Hemagglutination (HA) test

This test was done to detect the hemagglutinating viruses in the collected samples. The procedure of plate hemagglutination test was as follows:

(i) 25 µL of PBS was dispensed in each well of a plastic U-bottomed microtitre plate.

(ii) 25 µL of AF was placed in the first well and mixed well.

(iii) From first well 25 µL of mixture was transferred into second well to make two fold dilutions. This process was continued up to the last well (11th) and from there 25 µL of mixture was discarded. Well 12 was used as control.

(iv) 25 µL of 0.5% (v/v) chicken RBC was dispensed into each well.

(v) The plate was tapped gently and was allowed to keep at room temperature for about 15 min.

(vi) HA was determined by tilting the plate and observing the hemagglutination of the RBC.

(vii) A uniform layer of hemagglutination covering the bottom of well of the plate was considered as positive HA and a sharp buttoning of RBC at the bottom of well of the plate was considered as negative. The titration should be read to the highest dilution giving complete HA (no streaming); this represents 1 HA unit (HAU) and can be calculated accurately from the" initial range of dilutions.

Production performance

A number of additional weekly production parameters were recorded including egg production % (actual egg production), mortality %(actual mortality) and egg- livability %).

Statistical analysis (SPSS, 2006)

Data were analyzed by analysis of variance and Pearson's correlation, which calculated the relationships between serum antibody levels and degree of mite numbers (Infestation).

3. Results and Discussion

According to the degree of infestation of the house, the obtained data were arranged into four groups: - G1: houses of no infestation, G2: low, G3: houses of high infestation and G4: houses of very high infestation. By using the analysis of variance test, we found that, **(1)** there was a difference between the degree of house infestation and the mortality rate, the egg production as well as the egg-livability percentage of breeders, Table [2-a(built up litter and cage systems) and 2-b (built up litter)] showed that, with houses of high or very high infestation having higher mortality rate, lower egg production and lower egg-livability than those that had no or low degree of infestation ($P< 0.05$). The difference between bird mortality and total mite population, suggesting that an increase in mite number (very high infestation) contributes towards an increase in total bird losses. However, the difference is relatively moderate, indicating that the relationships are likely to be effected by other variables. Arends *et al.* (1984) reported decreased egg production and feed efficiency in broiler breeder flocks caused by NFM's (North fowl mites). During two separate 1-year trials, a significant reduction in egg production was produced by mites for only 1 month in one trial, and 2 months in the other. Internal quality of eggs is not affected by the presence of NFM's on hens ((DeVaney, 1979; DeVaney 1981). DeVaney (1978) estimated an annual loss of $66 million due to external parasites causing decreases in egg production; parasite prevention might cost as much as $1.1 million. High densities of mites during egg production may result in poorer hen performance, associated with reduced production and cause decreased feed intake and weight loss (Kirkwood, 1968). The birds become susceptible to other parasites and diseases, also, mortality rate increases especially in heavy infestation (Williams, 2003).

Cencek (2003) investigated the prevalence of *Dermanyssus gallinae* in 10 systems (battery cage, perchery system and deep litter), he found that 2-10 % reduction of egg production in heavy infested farms. The severity of infestation, including irritation, restlessness, anemia and occasionally death, this subsequently leads to reduced egg production, from reduced egg weight and increased downgrading as a result of poor shell integrity and superficial blood staining (spotting) from engorged mites which are crushed on egg belts. (Axtell and Arends 1990;Chauve, 1998; Cosoroaba, 2001). In commercial egg production, red mite is a serious problem, not only as a potential vector of several avian of pathogens, but more importantly as a direct parasite effecting production and welfare (Nordenfors, *et al*,. 1999; Kilpinen, 2001), anemia (CAV), Newcastle Disease (ND) and Avian Influenza (H5N2).

In spite of there were two different systems of housing the results showed that [Tables (3-a and 3-b)], the houses of high and very high infestation had lower

immune titre, than those that had no (Control) or low infestation.

In houses of high (G3) and very high (G4) infestation which vaccinated against AE (Avian Encephalomyelitis), both had lower immune titre (2262±52 and 2440±77, respectively) than those that had no or low degree infestation (7371±73 and 7371±64 respectively) ($P < 0.001$).

Breeder houses (built-up litter system) of very high infestation (G4) which vaccinated against: IB, IBD, CAV, and AI (H5N2) had lower immune titre, than control group. Concerning the degree of infestation (ignoring the house system) ,low or high infestation, G1: houses (No evidence of infestation), G2: (low infestation) and G3: high infestation {(17956±72, 16258±67, 12258±43; 5374±44, 4873±33, 5374±67; 8632±55, 8661±44, 8632±44;8±0.4, 7.6±0.3, 7.6±0.34 and 4.2±0.7, 4±0.22, 4±0.33 ($P < 0.05$) respectively} have higher immune response than those of very high infestation,{ 9894±88, 5092±43, 6938±78, 5.4±0.44 and 3.8±0.34 ($P < 0.05$), respectively}.

Red mite shows preference for laying hens, although they have been known to engorge on a range of hosts, including humans (Bruneau *et al.*, 2001). In commercial egg production, red mite is a serious problem, not only as a potential vector of several avian of pathogens, but more importantly as a direct parasite affecting both production and welfare (Nordenfors, *et al*,. 1996).

In Summary, our data indicated that, (1) red mite infestation has deleterious impacts on the performance of broiler breeders (egg production, mortality and egg-livability). (2) it affects the immune response (Level of antibodies titre) in vaccinated breeders against:, IB(Infectious bronchitis), IBD(Infectious bursal disease),AE (Avian Encephalomyelitis),CAV (Chicken infectious anemia) , NDV(Newcastle Disease) and AV(Avian Influenza) and as a result ,it will probably have a negative effect on the transferred maternal immunity to baby chicks.

Table 1: Vaccination programmed.

Age in days	Type of vaccine	Method of vaccination
1	Infectious bronchitis (IB 120)	Spray
3	Coccidia (Coccivac B)	DW[1]
5	Chicken infectious anaemia (CVA)	S/C inj[2]. (0.2 mL/chieck)
7	Newcastle disease (HB1)	DW
8	Marek's disease	S/C inj. (0.2 mL / chieck)
10	Avian Influenza (H5N2)[a]	S/C inj. (0.2 mL / chieck)
14	Infectious bursal disease (IBD)	DW
19	Newcastle disease (LaSota)	DW
22	Infectious bursal disease (IBD)	DW
28	Newcastle disease (HB1)	DW
32	Infectious bursal disease (IBD)	DW
36	Chicken infection anaemia (CVA)	DW
44	Newcastle disease + Infectious bronchitis	I/M inj[3]. (0.5 mL / chieck)
50	Infectious bronchitis(IB)	DW
55	Fowl Pox	Wing web
58	Infectious Coryza	S/C inj. (0.2 mL / chieck)
65	Fowl Cholera	S/C inj. (0.2 mL / chieck)
77	H5N2	I/M inj (0.5 mL /poullet)
82	Infectious Coryza	S/C inj. (0.2 mL / pullet)
85	Newcastle disease (LaSota)	DW
90	Chicken infectious anaemia (CVA)	S/C inj. (0.5 mL / pullet)
95	Infectious laryngotracheitis (ILT)	Eye drop
105	Avian encephalomyelitis (AE)	DW
110	Infectious bursal disease (IBD)	S/C inj. (0.5 mL / poullet)
115	Fowl Cholera	S/C inj. (0.2 mL / poullet)
122	Infectious bronchitis(IB)	DW
128	Avian Influenza (H5N2) [a]	I/M inj. (0.5 mL/ pullet)

DW[1]: Drinking water; S/C Inj[2]. Subcutaneous injection; I/M Inj[3]: Intramuscular injection, all vaccines manufactured by Schering Plough except Avian Influenza vaccine (H5N2) [a] which produced by Ceva Sante´ company.

Asterisks indicate values that are significantly different from controls. *P <0.05, *: Significance ($P < 0.05$). [1]: 5 built up litter house system (Avian breed 48).

Asterisks indicate values that are significantly different from controls. *P <0.05. *: Significance ($P < 0.05$). [1]: (actual mortality %): the difference between bird mortality and total mite population, suggesting that an increase in mite number (high and very high infestation) contributes towards an increase in total bird losses. [2]: (actual egg production %), [3]: (actual egg livability %), [a]: built up litter house system, [b]: built up litter and cage systems.

Table 2-a. Effect of red mite infestation on Mortality %, Egg production % and Egg livability % in poultry breeders (Environmentally controlled built-up litter and battery- cage systems).

Performance (Mean SD)				
Group	Control(G1)[a]	Low(G2)[b1]	High(G3)[b2]	Very high(G4)[a]
	(n=2)	(n=2)	(n=3)	(n=2)
Parameters				
Mortality %[1]	0.38±0.04	0.45±0.08	0.55±0.1*	0.68±0.2*
Egg production %[2]	74±6.3	71±5.3*	67±6.5*	62±6.3*
Egg livability %	88±3.2	88±8.2	78±9.2*	68±7.2*

Table 2-b. Effect of red mite infestation on Mortality %, Egg production % and Egg livability %in environmentally controlled built-up litter broiler breeders[1]

Performance (Mean SD)				
Group	Control(G1)	Low(G2)	High(G3)	Very high(G4)
Parameters	(n=2)	(n=1)	(n=2)	(n=2)
Mortality %	0.38±0.04	0.42±0.09	0.54±0.2*	0.68±0.2*
Egg production	74±6.3	71.5±7.3*	65.2±8.5*	62±6.3*
Egg livability %	88±3.2	86±8.5	77± 9.4*	68±7.2

Table 3-a. Effect of red mite infestation on immune response in poultry breeders (Environmentally controlled built-up litter and cages).

	Immune responses (Mean SD)			
Group	Control(G1)[a] (n=2)	Low(G2)[b1] (n=2)	High(G3)[b2] (n=3)	Very high(G4)[a] (n=2)
Type of vaccine[1]				
IB	17956±72	16258±67	12258±43*	9894±88**
IBD	5374±44	4873±33	5374±67	5092±43
AE	7371±73	7371±64	2262±52**	2440±77**
CVA	8632±55	8661±44	8632±44	6938±7
ND	8±0.4	7.6±0.3	7.6±0.34	5.4±0.44*
AIV(H5N1)	4.2±0.7	4±0.22	4±0.33	3.8±0.34*

*: Significant ($P < 0.05$). **: Significant ($P < 0.001$). Asterisks indicate values that are significantly different from controls, [1]: three testing of antibody titre at; 45, 47 and 49 weeks of age. [a]: built up litter house system, [b1]: cage systems (1+1), [b2]: built up litter and cage systems (2+1, respectively).

Table 3-b. Effect of red mite infestation on immune response in poultry breeders (Environmentally controlled built-up litter system) [2].

Group	Immune responses (Mean SD) Control (n=2)	Low (n=1)	High (n=2)	Very high (n=2)
Type of vaccine[1]				
IB	17956±72	17254±63	12118±64*	9894±88**
IBD	5374±44	4972±27	5474±44	5092±43
AE	7371±73	7066±43	2252±44**	2440±77**
CAV	8632±55	87631±34	8552±84	6938±78*
ND	8±0.4	7.4±0.3	7.2±0.34	5.4±0.44*
AV(H5N2)	4.2±0.7	4.1±0.12	4±0.63	3.8±0.34*

Asterisks indicate values that are significantly different from controls, *: Significant ($P < 0.05$). **: Significant ($P < 0.001$). [1]: three testing of antibody titre at; 45, 47 and 49 weeks of age; [2]: 5 built up litter house system (Avian breed 48)

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Heavy Metals Bio-Remediation by Immobilized *Saccharomyces cervisiae* and *Opuntia ficus indic*a Waste

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Abstract: The working concentrations used in the biosorption medium of *Saccharomyces cervisiae* was 35 mg/l, Cu(II); 15 mg/l,Cd(II) and 25 mg/l, Fe(III) . 18 h equilibrium time needed for maximum metal removal, (100mg/l) metals adapted *S. cervisiae* hasn't prominent enhancing effect, while addition of (4 mg/l) cystine has such effect upon metal removal , with 5.5 initial pH, and 3 %(v/v) inoculums concentration while NaOH treatment resulted in 36.11, 18.11, 33.52% for Cu(II),Cd(II) and Fe(III), respectively .Alginate immobilized *S. cervisiae* cells removed 67.33 %and 45.995 % for Cu(II),Cd(II) and 60.768% for Fe(III),respectively at 30°C and 200rpm from biosorption medium advanced over polyutherene foam immobilized cells . 10%(v/v) *Opuntia ficus indica* polyelectrolyte and 150 rpm are optimum for metal removal in biosorption medium at 30°C . Wastewater treatment with alginate-, polyurethane foam - immobilized cells and natural polyelectrolyte revealed that alginate immobilized cells is the most successful. [Journal of American Science 2010;6(8):79-87]. (ISSN: 1545-1003).

Keywords: Heavy Metals; Bio-Remediation; *Saccharomyces cervisiae; Opuntia ficus indic*a

1. Introduction

The phenomenon of biosorption is defined as a metabolism independent adsorption of pollutants based on the partition process on a microbial biomass (Ringot *et al.* 2006).Or, It is a passive non-metabolically-mediated process of metal binding by biosorbent (Davis *et al.*, 2003). Bacteria, yeasts, fungi and algae have been used as biosorbents of heavy metals. Among these, yeasts are known to be selective metal biosorbents as compared to fungi, actinomycetes and bacteria (Zouboulis *et al.*, 1999, 2001). The yeast *Saccharomyces cerevisiae* as a promising biosorbent has been used to remove Cr(VI), Fe (III) (Goyal *et al*., 2003), Cd (II) (Liu *et al*., 1997), Cu (II) (Jianlong, 2002 and Machado *et al*., 2009) from aqueous solutions. Moreover,It can distinguish different metal species based on their toxicity, such as selenium, antimony and mercury. Microorganism have had to develop different mechanism of metal resistance that include cell membrane metal efflux , intracellular chelation by metallothionine protein and glutathione derived peptides called phytochelatins and metal artmentalization in vacuoles (El Aasar, 2005). Other mechanisms exist for the removal of heavy metals from aqueous solution by bacteria, fungi, ciliates, algae, mosses, macrophytes and higher plants (Holan and Volesky, 1995; Pattanapipitpaisal *et al*., 2002;Wang and Chen,2006 ,and Rehman *et al.*, 2007, 2008).As cellular response to the presence of metals includes various processes such as biosorption by cell biomass, active cell transport, binding by cytosolic molecules, entrapment into cellular capsule, precipitation and oxidation-reduction reactions (Gadd, 1990; Lovely and Coates, 1997) .

Immobilization is either artificial, having to rely on incorporation into a polymer gel, Willaert. and Baron (1996) or natural, relying on the innate properties of microbes to become entrapped in biomass support particles; Atkinson *et al.* (1980) or attached to solid supports, such as coke; Dempsey (1994), Wilkins and Yang (1996) or even polyurethane foam as it is the case of immobilization with bacteria and *Candida* sp., Quek *et al.*, (2006).

Different agriculture residues were used for heavy metal removal (Lee *et al*., 1998; Marshal *et al.*, 1999). *Opuntia,* is another source of viscous natural polyelectrolyte bearing negative surface charges (Forni *et al.*, 1994). Aim of this study is to evaluate some physiological factors of *S. cervisiae* as a biosorbent: concentration of heavy metal, equilibrium time, adaptation to higher concentration, cystine addition, , pH, inoculum concentration, and NaOH - treatment of biomass, to maximize the heavy metal removal and to find effective use of immobilized alginate and PFU as means to offset substrate toxicity. Another trail regarding the use of *Opuntia* natural electrolyte to increase metal removal ;its concentration, and agitation speed were studied, to understand the specificity of both biosorptions for the metal ions involved during the treatment .

2. Material and Methods

Biosorbent:

Culture of *Saccaromyces cervisiae* obtained from the microbial stock collection of Chemistry of Natural and Microbial products lab. NRC. Egypt.

Maintenance medium:

Stocks of strains were maintained on standard yeast extract/peptone/dextrose (YPD) rich medium comprising in (w/v) %: Glucose, 2; Yeast extract, 1 and Peptone, 2, otherwise stated agar, 2 %, Liu *et al*. (1997).

Inoculum preparation:

A loop full of (YPD) slant yeast cells was cultivated in 50 ml liquid YPD medium in 250 Erlenmeyer flask at 30 °C on a rotary shaker for 24h at 200 rpm.

Biosorption medium:

Fifty ml liquid (YPD) medium in 250 ml Erlenmeyer flask adjusted to pH 5.5 by using 0.1M HCl or 0.1M NaOH. sterilized at 121°C for 20 min., inoculated with 2%(v/v) 24h *S. cerevisiae* inoculum suspension containing 10^7 cells /ml , with the sterilized metal salts being added at different concentrations after autoclaving . The flasks were incubated at 30 °C for different time intervals shaking at 200 rpm; Pearce and Sherman(1999) .

Preparation of the heavy metal solutions:

The different metal ions were sterilized by filtration through a pore filter of 0.22 um and were added to achieve final concentrations of 15 35, 25 (mg/l) $CdSO_4.8H_2O$; $CuSO_4.5H_2O$,and $FeCl_3$ respectively in biosorption media , Liu *et al.* (1997) . At the end of incubation period, cultures were harvested, centrifuged at 10,000 x g , final metals concentration were determined and metal removal(%) was calculated .

The effect of type and initial metal concentration on *S. cerevisiae* growth :

Concentrations of heavy metals ions were adjusted between 5- 35 (mg/l) in 50 ml biosorption media in 250 ml Erlenmeyer flasks , inoculated ,incubated for 24h time and were evaluated in relation to growth.

Determination of *S. cerevisia*e growth : At different heavy metals concentrations ,optical density was quantified at 600 nm using spectrophotometer with respect to the control containing no metal at 30°C for 24h; Liu *et al.* (1997).

Effect of equilibrium time on heavy metal removal:

Final metals concentrations in cultures at different incubation time 12, 18, 24,48h at 30°C at 200 rpm were measured, (%) metal removal was calculated. Effect of adaptation to higher metals concentration;: Series of repeated 5 generations sub culturing on YPD agar plates with gradual increased concentrations from 35 mg/l of $CdSO_4.8H_2O$;$CuSO_4.5H2O_4$ and $FeCl_3$ to 100 ppm incubated for 48h at 30ºC on .The resistant. *S. cervisiae* inoculums prepared as mentioned before and used to inoculate 50 ml biosorption media in 250 ml Erlenmeyer flasks incubated for 18h. Control and higher concentrations adapted flasks were centrifuged and final heavy metals concentrations were measured in the filtrate, (%) metal removal was calculated; El Aasar (2005) .

Effect of cystine on heavy metal removal:

Different concentrations of sterilized cystine (2-6 mg/l) were added to the sets of 50 ml biosorption media flasks in 250ml Erlenmeyer flasks after autoclaving. Treated and control flasks were 2%(v/v)inoculated ,incubated for 18h at 200 rpm at 30°C , centrifuged, final metals concentrations were measured,(%) metal removal were calculated; Pearce and Sherman (1999) .

Effect of initial pH on heavy metals removal : *S.cervisiae* culture in biosorption media at 30ºC,200 rpm for 18h adjusted to different pH values (2.5 – 6.5)were harvested, centrifuged ; final metals concentrations in different flasks were measured . (%) metal removal was determined; Pearce and Sherman (1999):

Effect of inoculums concentration on heavy metal removal:

Different biosorbent inoculums concentrations (2, 3, 4, v/v %,) flasks containing biosorption media were harvested after18h , centrifuged and final metals concentrations were measured for (%) metal removal calculation.

Effect of NaOH treated biomass;

Centrifuged *S. cerevisiae* biomass was divided into 2 portions ,one part was heated in 0.75 M NaOH at 70–90°C for 10–15 min ,the cells were converted to non viable and the cell wall microstructure was altered ,washed thoroughly till the washing becomes near neutral, suspended in 5 ml sterilized water and I ml was used for inoculating 50 ml biosorption media in 250 ml Erlenmeyer flasks .Similarly, the other part was suspended in 5 ml water, 1ml suspension of untreated biomass were inoculated in biosorption media as control flasks, incubated as mentioned, harvested, and centrifuged. Final metals

concentrations in all media were evaluated and (%) metal removal were calculated; Göksungur *et al.* (2005).

S. cervisiae cells immobilization:
Calcium alginate :

S. cervisiae cells were separated by YPD centrifugation at 10,000 rpm for 10 min,. The cells were washed and re-suspended in sterile 50 ml of acetate buffer (pH , 4.0) and 10ml suspension was mixed homogeneously with 90ml volume of aliquot of Na-alginate suspension to contain 2%(w/v) Na-alginate. Alginate-biosorbent suspension was added drop wise to 500 ml of 2%(w/v) $CaCl_2$ with a pipette. Alginate drops solidified upon contact with $CaCl_2$, forming beads and thus entrapping biosorbent particles. The beads were allowed to harden for 30 min and were then washed with sterile physiological saline solution (0.85%, w/v,NaCl) to remove excess calcium ions, Equal number of control beads without cells and immobilized cell beads were used to inoculate 50 ml portions of biosorption media at 200 rpm at 30°C. After 18h, filtration to harvest the beads, centrifugation at 10,000 x g for the escaped cells and determination of final heavy metals concentrations in the filtrates by atomic absorption were carried out for control beads flasks and beads with entrapped yeast cells flasks .(%) metal removal were calculated; Ksunger *et al*.(2003)

Determination of beads dry weight according to Aloiu *et al*. (2008).
Polyurethane foam (PUF):

Preparation of Polyurethane foam (PUF): Commercial type of different densities with different pore sizes PUF was obtained from glass Industries, Egypt. PUF were washed with distilled water and then dried at 30 °C overnight and weighed; Quek *et al.* (2006).

PUF Immobilization of *S.cervisiae* cells
Weighed PUF are placed into a 100 ml conical flask containing 18 ml of YPD broth and autoclaved at 121 °C for 15 min. Two ml of 2-day old liquid cultures were used for inoculation. Control foam flask kept un-inoculated The flasks were incubated at 30 °C with shaking at 200 rpm for 48h .YPD biosorption media was inoculated under aseptic conditions with equal number of PUF with immobilized yeast cells and control foam cubes flasks without cells , incubated at 30°C at 200rpm,for 18h;Quek *et al.* (2006).

Determination of PUF immobilized cells dry weight:. The content of the flasks was separated by filtration, using a Whatman No. 1 filter paper, dried overnight and weighed. An average control foam cube dry weight was subtracted from dry weight of foam plus cells to determine the dry weight of the attached cells .The filtrates were centrifuged at 10,000 x g for escaped cells, and the concentrations of the metals ions in the filtrates was determined for control flasks (no yeast) and immobilized cells flasks using an atomic absorption spectrophotometer and (%) metal removal was determined; Tsekoa and Petrov (2002).

The extraction of *Opuntia* natural polyelectrolytes:

The cactus waste was cut in small pieces, and approximately 132 g of cactus pieces with 750 ml of tap water were transferred to a 2 l flask and stirred for 30 min. The extraction of viscous natural polyelectrolyte (soluble sugars) was performed by decantation and keeping at 4ºC until use. Different mucilage concentrations (5-2o%,v/v) and different agitation speed (50-200rpm) were tested for maximum metal removal (%)with a control in biosorption medium and also in treating wastewater under the same incubation conditions;Olivera *et al.(*2001).

Wastewater samples were collected in screw capped Sterilized bottles from a stagnant fresh water port in Helwan , Egypt. Some physicochemical parameters of wastewater as biological and chemical oxygen demands (COD, BOD), and total suspended matter were measured according to Apha (2001). Cu (II),Cd(II), Fe(III) concentrations were determined using atomic absorption .

Wastewater treatment with immobilized yeast cells and natural polyelectrolytes:

Alginate- and PUF- immobilized cells (≈0.25, 0.12 dry weight /flask) were used to inoculate 50 ml of wastewater in 250 ml Erlenmeyer flasks that adjusted to pH 5.5, respectively, another set of 250 ml Erlenmeyer flask with 50 ml wastewater were treated with 10% (v/v) natural polyelectrolyte and a control wastewater flask kept untreated, incubated at 30ºC and 150 rpm for 18 h. and different previous parameters were evaluated.

Determination of heavy metal concentration in the filtrate:

Following metal treatment, culture filtrates were taken at certain intervals , centrifuged at 10,000 x g for 5 min and the clear supernatant liquids was used to determine heavy metals ions concentrations by using atomic absorption spectrophotometer, Varian absorption spectra AA20-NRC.,Egypt; Ksunger, *et al.*(2003).

-Metal removal (%)=Initial metal conc.(mg/l) - Residual conc.(mg/l)/Initial metal conc. (mg/l)x100; Berekaa and Hussein (2005).

3. Results and Discussion

Effect of type and concentration of heavy metal ion on growth of *S. cervisiae*:

As it could be seen from fig (1), The O.D. measurement indicated that selected working concentrations, were (35,15,25,)mg/l for Cu(II),Cd(II), and Fe(III), respectively, as it moderately inhibit the growth of *Saccaromyce*s when assayed singly compared to the biomass growth obtained under control conditions on agitation at 200 rpm for 24h at 30°C . Above 35, 15, and 25 mg/l of Cu(II),Cd (II) and Fe(III) ,respectively yeast decreased gradually in growth as indicated by O.D,fig (1). The role of the microbial cell wall in the biosorption process is to adsorb metal ions in the cell wall itself or pass through the cell membrane into the vacuoles. To balance the stimulatory or inhibitory effects of essential ions and to counteract the toxicity of nonessential metals, all organisms possess homeostatic mechanisms that properly control the cellular accumulation, distribution, and detoxification of metals, .*S. cerevisiae* provides an ideal system. Gadd (1988) stated that microorganisms can take up nickel intracellular or the presence of chelating ligands that may be present on the cell surface in trace amount even after washing the biomass thoroughly and before using in biosorption experiments. Hence, both type of the heavy metal and its concentration affect behavior of *Saccaromyces* biosorption. .

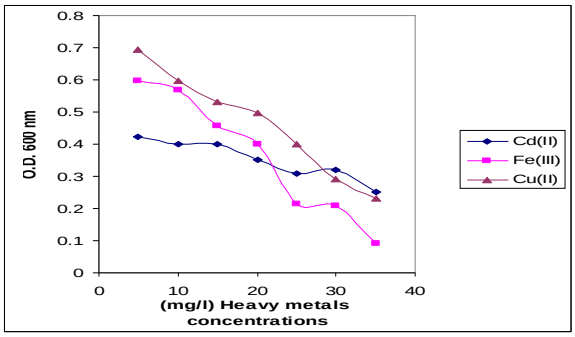

Fig.(1).The growth of *S cervisia* in the presence of cadmium, copper and iron different concentrations after 24h at 30°C and 200rpm.

Effect of equilibrium time on biosorption of heavy metal ion *by S. cervisiae*:

Table (1) indicated that with equilibrium time18 h, (%) metal removal was 14.532 in biosorption medium with Cu(II), 10.433 in Cd(II) medium and 15.594 biosorption medium with Fe(III) culture .This is may be due to the ratio of protein to hydrocarbon or as Park and Choi (2002) stated that after 24 h at 200 rpm at 30°C there is no serious metal accumulation of cadmium in the cell which was related to the cell metabolism. Equilibrium time varied according to the biosorption conditions as it was attained after 30 and 60min for dead and live cell used for removal of Cu^{2+},Machado *et al.(*2009).

Table(1). Effect of incubation equilibrium time on (%) metal removal ion by *S. cervisiae*.

Time in (h.)	(%) metal removal		
	Cu(II)	Cd(II)	Fe(III)
12	12.031	9.011	12.191
18	14.532	10.433	15.594
24	12.411	11.313	14. 356
48	11.087	12.058	11.081

The effect of adaptation of *S. cervusiae* on removal of heavy metal:

As it could be seen from fig (2), increased adaptation to 100 mg/l, for Cu (II), Cd (II) and Fe (III), has no enhanced effect on *S. cervisiae* as it showed no much advanced difference in the process of heavy metals removal compared to that of the non adapted *S. cerevisiae*. under the same incubation conditions as it exhibited (%) removal of 15.99,10.67,and 14.0 for Fe(III), Cd(II) and Cu(II) for control (untreated),respectively compared to 16.75,11.56and 14.87% for adapted biosorbent in the same order .

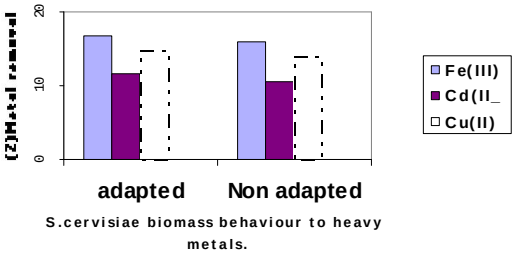

Fig.(2).Effect of (100mg/l)adapted *S.cervisiae* on (%)heavy metals removal .

Effect of cystine on the biosorption of heavy metals :

Table (2) suggested that , sensitivity to Cu(II), Cd(II), and Fe(III) can be reversed by the addition of cystine to the media to enhance the ability to synthesize this amino acid, so, a somewhat high level of cystine was required to reverse the metals toxicity .These results indicate that the intracellular cystine alleviates the toxicity of Cu (II), Cd II), and Fe (III),.As, 4 mg/l cystine resulted in (%)metal removal of 18.311in presence of copper and 15.311Cd(II) and 19. 996 % in iron biosorption medium after 18h at 200rpm and 30°C .Engle and

Kunz (1995) stated that removal carried out in three steps; rapid binding to the negatively charged groups on the cell wall and passive transport of the metal ion through the cell wall within short time 3-5 min , penetration though cell wall to the cytoplasm and accumulation of the heavy metal in the cytoplasm,. Similarly, Pearce and Sherman (1999) revealed that histidine binds divalent metals, and is routinely exploited by insertion of polyhistidine tracts into proteins, so that the protein can be bound to resins with bound divalent metals ions such as Co^{2+} and Ni^{2+}.

Table (2). Effect of cystine on(%) metal removal by *S. cervisiae*.

cystine (mg/l)	(%) metal removal		
	Cu(II)	Cd(II)	Fe(III)
2	12.332	12.413	15.454
4	18.311	15.311	19. 996
6	12.087	14.098	12.018

Initial pH effect on heavy metal removal by *S. cervisiae*:

Table(3) revealed that, pH of the solution was a critical parameter for the adsorption process (%)metal removal was 18.911 in Cu(II) medium at pH 5.5 and 15.213 in Cd(II) medium and 19.256 in Fe(III) medium after 18h at 200rpm and 30°C,in presence of 4 mg/l cystine. On increasing pH, the biosorption capacity increased as there is an increase in ligands with negative charges which results in increased binding of cations because the ligands on the cell are closely associated with the hydronium ions. With increased pH, the hydronium ions are gradually dissociated and the positively charged metal ions are associated with the free binding sites ;Ahlya *et al*.(2003). However, at pH 6.5, (%) metal removal of Cu (II),Cd(II) and Fe (III) decreased to 15.187,10.198,14.198 due to its partial precipitation. Little metal removal was observed at pH 2.5 is an indication of competition of excess of protons for the same binding sites on the cell wall. The iron, cadmium and copper uptake increased with increasing pH, to the maximum near pH 5.5 then decreased at 6.5 pH .At pH values higher than 6.5, metal ions precipitated, so it is not conducted, due to the high concentration of OH- ions in the adsorption medium to avoid possible hydroxide precipitation. and biosorption . Ahlya *et al*.(2003) indicated the importance of pH in the biosorptive process: it affects the solution chemistry of the metals, and the activity of the functional groups in the biomass. The more rapid increase in metal removal in accordance with pH may be presumed by larger degree of deprotontion of the cell wall at higher pH because the hydrolysis degree of heavy metal in the solution at a given pH was nearly constant regardless the microbial species.

Table (3). The pH effect on (%) metal removal of *S . cervisiae*.

pH	(%) metal removal		
	Cu(II)	Cd(II)	Fe(III)
2.5	3.131	1.211	2.091
4.5	12.132	11.123	12.154
5.5	18.911	15.213	19. 256
6.5	15.187	10.198	14.198

Effect of inoculum concentration on sorption of heavy metals by *S. cervisiae* :

As it could be seen from table (4), decreasing the biomass resulted in the increase of the metal removal due to exhaustion of metal ion in the biosorption medium or the interaction between the metal ion binding sites; Park and Choi(2002). The biosorption capacity profiles of Fe (II) and Cu (II) are somewhat similar. Also, it is clear that the carboxyl group, the phosphoric and amino groups in the cell wall reported to be involved on metal ion adsorption group, El Aasar (2005). For 3%,(v/v) inoculum concentration 28.44, 17.47 and 25.57% of the Cu (II),Cd (II) and Fe(III) ,respectively were biosorbed after 18 h at 200rpm and 30°C in presence of 4 mg/l cystine; the auto-aggregation of yeast biomass is rapid. High biosorbent concentrations at (4%,v/v) are known to cause cell agglomeration and consequent reduction in inter-cellular distance resulted in decrease in metal removal (%) as indicated in table (4) 20.24, 17.36. and 16.33 for Cu(II),Cd(II) and Fe(III), respectively . In other words, metal removal is higher when the inter-cellular distance is more (at low biosorbent concentration), as this condition ensures optimal electrostatic interaction between cells, a significant factor for biosorption (Itoh *et al.* 1975). The affinity for cadmium ion penetration of the cell wall during biosorption although thicker outer wall of *S.cervisiae* may inhibit the penetration through the cell wall like ex-polysaccharide pullan from *A. pullanus*. However , Park and Choi(2002) stated that many ligands on the outer cell wall mannan layer seemed to contribute to the high cadmium removal capacity by *S.cervisiae*. However, the ligand in microorganisms accumulates heavy metals on the cell wall. On the cell wall sorption, complexation, ion exchange , precipitation and accumulation of the heavy metal occurred according to the species of the microorganisms , the formation rate of the cell wall during cultivation , the ultra structure of cytoplasm and the creation rate of polysaccharide can vary ; Park and Choi(2002).

Table (4). Effect of inoculum concentration on(%) metal removal by *S. cervisiae*.

S. cervisiae cells conc. (%)v/v	(%) metal removal		
	Cu(II)	Cd(II)	Fe(III)
2	18.34	15.26	19.34
3	28.44	17.47	25.57
4	20.24	17.36	16.33

NaOH effect on heavy metal removal cells of S. *cervisiae*

Fig (3) indicated that pretreatment of biomass by NaOH enhanced metals removal 18.11% of Cd(II) and 36.11% for Cu(II) and 33.52% for Fe(III) from biosorption media after 18h at 200 rpm at 30°C,compared to untreated biosorbent which resulted 11.45,15.47,14.98 % for metals in the same order in biosorption media without additives , this is because *S.cervisiae* has the toughest cell wall structure among microorganisms hitherto with characteristic combination between glucan and mannaoproteins ,Meena and Raja (2006). So, non-living biomass appears to present specific advantages in comparison to the use of living microorganisms. Killed cells may be stored or used for extended periods at room temperature, they are not subject to metal toxicity and nutrient supply is not necessary. So, living cells can be pretreated using chemical means with the objective of increasing the metal biosorption capacity such as treating the cells with acid, or alkali chemicals showed enhancement in removal of heavy metals. As increased biosorption after pretreatment may be due to chemical modification of cell wall components or the exposure of active metal binding sites embedded in the cell wall. Ahlya *et al*. (2003) showed that alkali treatment of biomass may destroy autolytic enzymes that cause putrefaction of biomass and remove lipids and proteins that mask reactive sites. Also, Besides, the pre-treatment could release polymers such as polysaccharides that have a high affinity towards certain metal ,Ahlya *et al*.(2003).Also, Norris and Kelly (1979) revealed that the adsorption between microorganisms and heavy metals occurred initially in the cell surface by ion exchange reaction and that the plasma membrane which play a role in transmitting metal selectively must be destroyed for heavy metal to accumulate in the cell.

However, Yan and Viraraghavan, (2001) indicated in the contrary, acid pretreatment of *Mucor rouxii* significantly decreased the bioadsorption of heavy metals , this is attributed to the binding of H^+ ions to the biomass after acid treatment may be responsible for the reduction in adsorption of heavy metals, this comes in agreement with the findings of Michado *et al*. (2009).

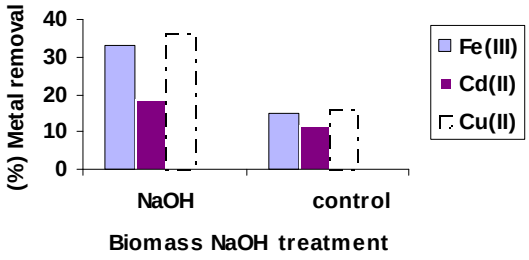

Fig (3). The effect of NaOH -treated *S. cervisiae* cells on (%)metal removal .

Effect of immobilized *S.cervisiae* cells on sorption of heavy metals:

Table (5) indicated the moderate adsorption affinity between the PEU on inert supports foam as sorbent and metals sorbets .However, with still low efficiency than alginate immobilized cells of *Saccharomyces* as (%) metal removal was 21.33 for Cu(II), 23.995 for Cd(II) and 26.768 for Fe(III) ,respectively in comparison with 67.33, 45.995, and 60.768, in the same order with alginate because of both yeast cells and the polymeric matrix of alginate beads itself in absorbing the metals, moreover, alkaline earth metal-alginate entrappers matrix is a known procedure, Meena and Raja (2006) . Stoll ,and Duncan,.(1997)stated that medium accumulation of heavy metals by microbial biomass with high surface area-to-volume ratio holds great potential for heavy metal removal in both soluble and particular forms, especially when the heavy metal concentrations are low (<50 mg/L) Immobilization offer advantages such as easier separation and reuse of cells, maintenance of higher cell concentrations and stabilization adhesion to supports ; Aloiu *et al*. (2008).

Table (5).(%) Metal removal by immobilized *S. cervisiae*.

Metal ion	(PUF) cells dry wt. (g/culture)	Alginate cells dry wt. (g/culture)	%Metal removal alginate	%Metal removal (PFU)
Cu(II)	0.211	0.576	67.33	21.33
Cd(II)	0.113	0.546	45.995	23.995
Fe(III)	0.101	0.454	60.768	26.768

Effect of agitation speed on heavy metal settlement by *Opuntia* extract:

Table (6) indicated that, 150 rpm is the optimum agitation for settlement of heavy metals in biosorption media as (%) removal of Cu(II) 32.13., 12.54 Cd(II) ,Fe(III) 22.51(mg/l) on using 20% (v/v) *Opuntia* natural polyelectrolyte under the same incubation conditions .This is may be due to mucilage hydrophilic character, several hydrogen bonds are formed between polyelectrolyte and water molecules. This association tends to occupy larger surface area causing its very high viscosity; Oliveira *et al.* (2001). This comes in agreement with La Mer and Healy (1963) and Nozaki, *et al.* (1993) who stated that natural polyelectrolyte have been used as auxiliary of flocculation and coagulation in wastewater treatment and water cleaning process .However, Olivera *et al*. (2001), stated that optimum conditions for flocculation and coagulations were: 30 sec. of strong shaking but with addition of aluminum sulfate and natural polyelectrolyte, followed by 15 min. of slow shaking, and then 30 min. for complete settlement .Iron and copper settlement increased more than 2-fold with rise in agitation speed from 50 to 150 rpm, beyond which there was no further increase.

Table (6).Effect of agitation speed on (%) metal removal by *Opuntia* polyelectrolyte.

Agitation speed(rpm)	Metat Removal(%)		
	Cu(II)	Cd(II)	Fe(III)
50	13.54	10.67	10.55
100	22.14	11.55	17.33
150	32.13	12.54	22.51
200	32. 65	12.54	22.54

Effect of natural electrolyte concentrations on heavy metal settlement:

Table (7) showed the mucilage efficiency for reducing heavy metals from biosorption media ,also, proved its flocculation with the efficiency increase with10 %(v/v) mucilage concentration resulted in 38.50%Cu (II) 16.12;Cd(II) 30.12 Fe(III).Olivera *et al.* (2001), revealed that the flocculation process induced by anionic polyelectrolyte such as the natural polyelectrolyte extracted from *Opuntia ficus indica*. The positive metals ion serves to form a bridge among the anionic polyelectrolyte and negatively charged functional groups on the colloidal particle surface. Cactus mucilage is a neutral mixture of approximately 55 high-molecular weight sugar residues composed basically of arabinose, galactose, rhamnose, xylose, and galacturonic acid. This natural product was characterized for its use as a flocculating agent. Table(7) shored that 10%, v/v of mucilage provided the optimal effectiveness for metals removal in biosorption media,. Table(7)also ,suggested that *Opuntia* mucilage proved the feasibility of applying mucilage as a method for heavy metals removal as a natural flocculating agents, as covalent bonds in vector compounds, or on cell cross-linking, that are innovative, environmentally benign, and cost-effective.

Table (7). (%)removal of heavy metals by *Opuntia* polyelectrolyte different concentrations.

Polyelectrolyte conc.% (v/v)	Metal removal(%)		
	Cu(II)	Cd(II)	Fe(III)
5	14.30	19.43	22.44
10	38.50	16.12	30.12
20	30.40	16.43	25.23

Finally, The use of this type of green chemistry shows *Opuntia* mucilage as a resource for achieving potable water, as the use of natural environmentally benign agents in the treatment of drinking water is rapidly gaining interest due to their inherently renewable character and low toxicity. As a gum-like substance, cactus mucilage, which shows excellent flocculating abilities, is an economically viable alternative for low-income communities and overcoming many problems and its use beside PUF- and alginate- immobilized cells, which have the advance of the ease of cell separation, for efficient metals removal and in decreasing the parameters indicated in table (8): total suspended solids , Cu(II),Cd(II) and Fe(III) concentrations , chemical and Biological oxygen demands(COD,BOD). Thus suggesting clarifying a local port sample regarding environmental impacts as permissible Egyptian limits Laws in 44/2000 and 48/1982 lows ,which may grant the possibility of its applications.

Table(8) .Bio-treatment of a fresh water port sample with immobilized yeast and *Opuntia* polyelectrolyte

Factor(mg/l)	Permissible limit (mg/l)		Final conc. polyelectrolyte	Final conc. immobilized Alginate	Final conc. immobilized Foam	Initial conc.
	48 /198 2	44 /200 0				
Cu(II))	1	0.2	0.106	0.065	0.101	0.512
Cd(II)	1	1.5	0.311	0.105	0.299	0.325
Fe(III)	-	1	0.321	0.177	0.349	0.633
COD	1100	60	800	900	900	900
BOD	600	80	600	700	700	700
Total suspended solids	60	800	600	500	1100	1200

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Potency of Pseudomonas fluorescens a Biotic Inducer Inhibitors Against Cucummber mosaic Cucumovirus.

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Abstract: An antiviral producing Ps. Fluorescens when designated a EG isolates from potato rhizosphere soil in Egypt were identified based on morphological; biochemical tests and protein polymorphism. The strains were grown in King's B broth medium and the culture supernatent obtained was filtered through a 0.45 µl filter. It was further boiled at 100ºC for 10 min and tested to induce LAR and SAR for its ability to control a Satellite cucumber mosaic virus (st. CMV-EG). In Local acquire resistance (LAR) boiled culture filtrate (BCF) was treated on one half of the leaves of Chenopodium amaranticolor followed by st. CMV. EG inoculated on both halves. In the systemic acquire resistance (SAR), BCF was treated on the lower leaves of Nicotiana glutinosa, and st CMV-EC mechanically inoculated onto the untreated upper leaves. In LAR. BCF treatment was able to considerably reduce the number of viral lesion and in SAR plants treated with BCF shown no visible and mild mosaic viral symptoms, compared to the King's B media and remained throughout the study period. Thus, Ps. Fluorescens was able to produce an antiviral component in the culture filtrate, which was found to be heat stable, non-phytotoxic and effective in local as well as systemic host of CMV. [Journal of American Science 2010;6(8):88-93]. (ISSN: 1545-1003).

Key words: Boiled culture filtrate (BCF), cucumber mosaic virus (CMV), local lesion, Pseudomonas fluorescens.

1. Introduction

Cucumber mosaic virus (CMV) belong to the genus cucumovirus (family bromoviridae), is one of the economically important viruses, which causes enormous losses by infecting more than 1.000 species of plants shrubs and trees world-wide. It is transmitted non-persistently into healthy plants by aphids, which acquire the virus during their brief probes on infected hosts or the symptomless carrier woods in the field (Zehnder et al, 2000). Various strategies, based on the avoidance of sources of infection, control of vectors, modification of cultural practices, and the use of resistant varieties and transgenic plants have been conventionally employed to minimize the losses caused by CMV. These strategies, however, have not been effective as control measures. Many screening studies have been conducted on antiviral agents from different sources. Most of these come from plants sources, with some showing systemic control ability against a range of viruses that infect plants (Kubo et al., 1990). Comparatively, antivirals from microbial sources have been little studied. Recently, Raupach et al. (1996); El-Badry et al. (2006); Ipper et al. (2005) and Megahed (2008) showed the systemic control of CMV in cucumbers and tomato employing rhizosphere colonization of some bacteria by a ISR mechanism. Kim, et al. (2004); Ipper et al. (2005) and Megahed (2008) used culture filtrate of Acenetobacter, species KTB3, Ps. Fluorescens Gpfol and Trichoderma sp. Respectively, to systemically control some viruses.

This study was described the antiviral activity from the heat stable culture filtrate of Ps. Fluorescens EG against st-CMV EG which produces local lesions in the hypersensitive host and systemically infected many important plants.

2. Material and Methods

Bacterial strains and growth conditions: Soil-adhered cucumber roots were obtained from a cucumber field. The roots were homogenized using demineralized water and the homogenate was serially diluted and plated onto King's B media (King et al., 1954) followed by 24 hr incubation at 28ºC. Numerous colonies with different morphologies were picked from the dilution plates. Each of these was assayed for antiviral activity using half leaf method, as described by Noordam (1973). One colony that showing maximum antiviral activity was selected. This colony was stored on nutrient broth medium containing 20% glycerol. Other bacterial strain Ps. Fluorescens ATCC, 10325 was obtained from Cairo microbiological resources center (MIRCEN) , faculty of agriculture , Ain Shams university. This strain was used for identification and comparative study of the morphological, physiological, biochemical and protein patterns of Ps. Fluorescens EG.

In order to identify the Ps. fluorescens EG strain, morphological, physiological and biochemical

tested (Table 1) were carried out as described by Sehaad et al. (1988).

Maintenance of virus: St.CMV-EG was obtained from Virology Lab., Fac. Agric., Ain Shams Univ. (Megahed, 2008). The virus was inoculated into N. glutinosa and maintained on the same host throughout the period of this study. The inoculum consisted St-CMV-EG systematically infected leaves ground in 0.01M sodium phosphate buffer (SPB) pH 7.0.

Preparation of boiled culture filtrate (BCF): Each of 5 isolates of Ps. fluorescens were inoculated onto 100 ml King's broth and grown at 28ºC for 48 hr. with shaking at 175 rpm. The cultures were centrifugated at 12.000 rpm for 10 min.The cultures supernatant were then filtered through a 0.45 µm filter. The filtrates obtained were boiled at 100ºC for 10 min and used for the antiviral assay.

Antiviral bioassay: The antiviral activity of the BCF of 5 Ps. fluorescens isolates were assayed via induced local acquired (LAR) and systemic acquired resistance (SAR), LAR was assayed on a hypersensitive host of CMV, Ch. amaranticolor using the half leaf assay. The upper right halves of the leaves were treated with the BCF, using paintbrush, and the upper left halves were left untreated. As a control treatment, the upper right halves of another leaves were treated with sterilized water, with the left halves of the leaves for both treatments. Each treatment was performed in duplicate with 3 leaves each. The local lesions were counted after 7-10 days intreated and control and calculated formula: (I-T/C) x 100 where, C is the number of LL on the control half of the leaves and T the number of LL on the treated half on the leaves.

In SAR, 5 to 6 leaved N. glutinosa plants were used under SAR. BCF was treated onto the three basal leaves, after 24 h. CMV isolate inoculated onto one upper untreated leaf. As a control treatment, the lower leaves were treated with King's B media and the CMV isolated was inoculated the upper untreated leaves. Each treatment was replicated five times. The plants were kept in a greenhouse, with 12-14 h daylight and on temperature of 28-30ºC.

Determination of protein: Protein content of Pseudomonas isolates were determined by Bradford (1976) using bovine serum albumin as a standard.

Protein analysis : Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970) under reducing conditions in discontinuous electrode buffer system. Protein samples were denatured and reduced completely by mixing the protein with in equal vol. Of 2x sample buffer (Lucine et al., 1987) and heating mixture at 92ºC in a water bath for 5 min and chilled on ice buffer. Treated protein were centrifuged at 12000 rpm for 10 min. Electrophoresis was carried out at room temperature at a constant current at 25 mA for 1 hr followed by 30 mA for 4 hr. At end of the run the gel was stained with comassie blue and distained in the stain solvent and photographed.

3. Results and Discussion

Isolation of Pseudomonas spp.: The bacteria were isolated from rhizosphere potato soil. Different morphological colonies were developed on King's B plates. Each one isolated colony was inoculated on slant medium. The bacterial isolates Pseudomonas sp. were subjected to an identification program to the genera level was performed as shown in Table (1). Pseudomonas appears fluorescence pigment on nutrient agar, short rod shape, gram negative and no spore formers. As well as, physiological and biochemical tests on Ps. fluorescens isolates showed, oxidase, proteolytic and lipolytic activities as most Ps. fluorescens isolates. As well as utilization of mannitol glucose, galactose and sobritol as most Ps. fluorescens isolates. However, no ethanol of utilization was observed. The growths at higher temperatures tests were negative. The growth of Pseudomonas isolates on butryrate, galactose as most of isolates. However no growth of isolate on Nicotiana were observed, which is usually shown by Ps. fluorescens isolate No. 1. Plotted on the basis of specific media and other test results, as shown in Table (1), indicates that these isolates are closely placed with the other Ps. fluorescens strains (Table 1). It is suggested to belong to the species Fluorscens according to criteria described by consulting Bergy's Manual of Systemic Bacteriology (Sneath, 1986). It could be given the tentative name Pseudomonas fluorescens EG isolates. The purified Pseudomonas isolates were subjected to screening against program of antiviral activities. All isolates exhibited antiviral activities against Cucumber mosaic virus (Ipper et al., 2005).

The most potent Psedomonas isolates belonging to Ps. fluorescens EG were selected for the biosynthesis of the active metabolite having antiviral activity. For this reason Ps. fluorescens EG was inoculated in nutrient broth media for antiviral production.

Antiviral, effect: The antiviral culture filtrate from Ps. fluorescens isolates (namely ATCC, 1,2,3,4 and 5) showed inhibitory inactivity against CMV with variability among isolates. The BCF treatment due to LAR whereas the half leaves of the hypersensitive host; Ch. Amaranticolor showed 85.0, 182.5, 72.9, 75.0, 68.5 and 85.4% inhibition of the production of LL respectively. Compared to the untreated the half leaves. The control plants were unable to show inhibitions of CMV-EG which is

shown 95 L.L. The average numbers of L.L. in the case of the BCF were much lower, 11.8, 18.5, 28.6, 26.4, 33.2 and 30.7 L.L than those of the sterilized water treated half leaves. The BCF due to elucidate

Table 1. Morphological, physiological and biochemical characteristics of Pseudomonas spp. isolates.

Characteristic	Ps. fluorescences ATCC 10325	Pseudomonas isolates				
		Ps1	Ps2	Ps3	Ps4	Ps5
Morphological						
Cell shape	Rod	Rod	Rod	Rod	Rod	Rod
Size (nm)	2x12	3x1.1	3x1.9	2.5x1.0	2x1.7	1x1.5
Gram reaction	SR	SR	SR	SR	SR	ST
Physiological	Diffusible	Diffusible	Diffusible	Diffusible	Diffusible	Non-diffusible
Fluorescent pigment						
Oxidase	+	+	+	+	+	+
Pectolytic activity	+	+	+	+	+	+
Lipolytic activity	+	+	+	+	+	-
Proteolytic activity	+	+	+	+	+	+
Amylolytic activity	-	+	-	-	-	+
Urease	-	V	-	-	V	-
Utilization						
Mannitol	+	+	+	+	+	+
Benzoate	-	-	-	-	-	+
Cellobiase	-	-	-	+	-	-
Starch	-	-	-	+	-	-
Serbitol	+	+	+	+	+	+
Sucrose	-	-	-	+	-	-
Glucose	+	+	+	+	+	-
Glycerol	+	+	+	+	+	+
Galactose	+	+	+	+	+	+
Xylose	-	-	-	-	+	-
Fructose	-	-	+	-	+	-
Growth on:						
Butyrate	+	+	+	+	+	+
Ethanol	-	-	-	-	-	-
Nicotinate	+	+	-	-	+	-
Growth at						
40ºC	-	-	-	-	+	-
35ºC	+	-	+	-	+	-
5ºC	+	+	+	+	+	+

V = Variable - Negative result + = Positive result

SAR, it was found that, the plants treated with BCF showed variation in viral symptom due to Ps. fluorescens isolates, ATCC and P1 revealed symptomless (0.0 L.L), P2,P3,P4 and P5 revealed mild mosaic (8.5, 10.2, 12.9 and 9.5 L.L respectively throughout the study period. The plants treated with both media (as a control) showed severe symptoms (Fig. 1(A). This results reveals the antiviral activity of BCF from Ps. fluorescens isolates was due to involvement of plant defense mechanism. In both the above LAR and SAR, no damage to the host plant was observed due to BCF treatment. BCF, thus can be characterized as a non-tioxic antiviral agent, which could give the necessary efficiency in combating CMV-EG. The activity of the inhibitory agent percent in the BCF obtained from Ps. fluorescne isolates was not destroyed by heating at 100ºC for 10 min, indicating that the antiviral agent percent in BCF is a heat stable BCF induces protection against CMV-EG in both local as well as a systemic hosts.

Virus variability and concentration were determined as local lesion morphology and mean number on Ch. amaranticolor as indicator plant for CMV ,Fig.1(B). The virus variability produced by Pseudomonas isolates considered as indication of LAR and SAR. The obtained results showed that, all isolates treatments were able to vary number and similarly (size, center, halo or without halo) produced by CMV infection. Ps. fluorescens isolates has the

lowest mean number of L.L compared to control. The same results were obtained by Zhong et al. (2002); Ipper et al. (2005); Zhao and Wu (2007) and Megahed (2008).

Protein content were determined in Pseudomonas isolates related to BSA as standard protein; the results revealed that, all isolates differed in protein content (1.305; 1.596; 1.770, 1.581, 1.602 and 1.575 mg/g fresh weight cells for ATCC, P1, P2, P3, P4 and P5 respectively.

Quantitative of antiviral protein were determined on the basis of the number, intensity, molecular weight and reproducibility of SDS-PAGE five biotic inducers bands. The total number of protein bands were 13 bands include of 10, 10, 9, 7, 7 and 7 for ATCC, P1, P2, P3, P4 and P5 respectively (Table 4 and Fig.2). Sex bands with the same mobility were treated as identical fragment for Pseudomonas isolates (monomorphic) or common fragment with 46%. Weak bands with negligible intensity and smear band were both excluded from final analysis. The numbers of scored new bands varied among isolates were 28.4, 30.5, 105.5 and 95.0 KDa-bands of ATCC, P1, P2 and P3 respectively, were treated as unique 9genetic marker) with 31%. The polymorphic (specific bands) for each isolate 80.2, 33.4 and 14.3 KDa bands.

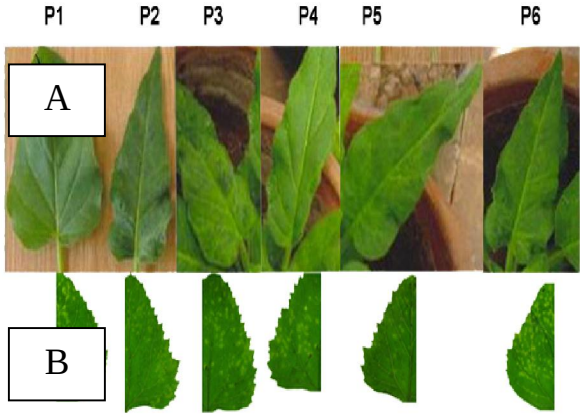

Fig.1 (A): Single local lesion diversity of CMV inoculated on Ch. Amaranticolor treated with BCF of Ps. fluorescens isolates (ATCC 13223; O1, P2, P3, P4 and P5). (B): N. glutinosa treated with BCF of Pseudomonas isolates and mechanically inoculated with CMV-EG. showing different of symptoms on leaves. Ps. ATCC, Ps.1, Ps.2, Ps. 3, Ps. 4 and Ps. 5, C = untreated with BCF.

Table (2): Effect of BCF on CMV-EG infection in Ch. amaranticolor using local lesion assay.

Ps. fluorescens	Control	LAR*			SAR*		
		BCF treated	BCF untreated	Inhibition (%)	BCF treated	BCF untreated	Inhibition (%)
ATCC	105.5**	11.8	22.5	85.0	0.0	0.0	0.0
P 1	105.5	18.5	36.1	82.5	0.0	6.5	0.0
P 2	105.5	28.6	36.7	72.9	8.5	16.3	91.0
P 3	105.5	26.4	35.8	75.0	10.2	17.5	89.2
P 4	105.5	33.2	44.2	58.5	12.9	18.5	86.4
P 5	105.5	30.7	41.5	70.9	9.5	15.1	89.9

LAR = Local acquire resistance. SAR = Systemic acquire resistance. Control = Leaf treated with sterilized water Treated -= Half or leaf treated with BCF Untreated = upper half or leaf untreated with BCF
Determinated as local lesion

The BCF of Ps. fluorescens isolates treated Ch. amaranticolor and N. glutinosa due to induce increase in the accumulation of host proteins. The induction of additional proteins may merely a disturbance in host physiology and have related to the primary mechanism responsible for the reduction of infection. It has been suggested that, the induced protein may help to limit virus spread or multiplication (Gianinazzi and Kassanis, 1974 and Chen, et al., 2006). Lochenstein (1972) considers an protein inhibitor can be an inhibite of virus replication of its effective when applied 5 to 8 hours past-virus inoculation.

Four types of antiviral protein activities have been characterized and some antiviral proteins have been capable of more than one activity. The activities were; 1- Aggregation; 2-inhibition of establishment; 3-induction of a systemic viral resistant state and 4-inhibition of replication by inactivation of protein synthesis, cited from Chessin et al. (1995).

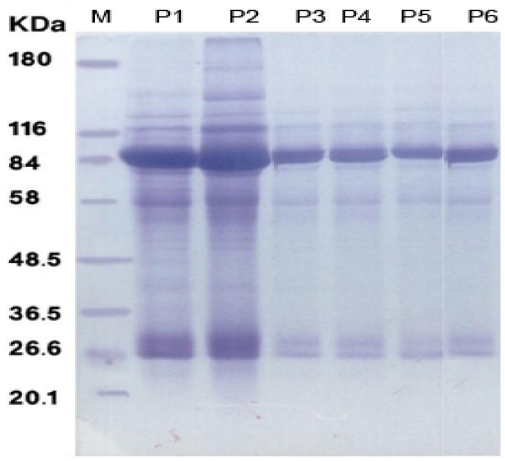

Fig. (2): SDS-PAGE 12% of protein fractions extracted from 6 Ps. fluorescens isolates (ATCC, P1, P2, P3, P4 and P5). M = marker protein (KDa).

Table (4): Protein fractions of Ps. fluorescens isolates using SDS-PAGE.

Isolates	Ps. fluorescens isolates						Polymorphism
MW (KDa)	ATCC	P4	P2	P3	P4	P5	
1058.5	-	-	+++	-	-	-	Unique
95.0	-	-	-	++	-	-	Unique
85.5	++	++	+++	+	+	+	Monomorphic
80.2	++	++	++	-	-	-	Polymorphic
75.0	++	++	+++	+	+	+	Monomorphic
54.7	+++	++++	++++	+++	+++	+++	Monomorphic
70.5	+++	+++	+++	++	++	++	Monomorphic
33.4	++	++	++	-	+	+	Polymorphic
30.5	-	++	-	-	-	-	Unique
28.4	++	-	-	-	-	-	Unique
14.3	+	+	-	-	-	-	Polymorphic
13.7	++	++	++	++	++	++	Monomorphic
12.0	++	+++	+++	++	++	++	Monomorphic
No. bands	10	10	9	7	7	7	-

Ps, fkyirescebs (ATCC 1`3223) Unique = polypeptide marker (genetic marker)
Monomorphic = or common polypeptides on all isolates Polymorphic = specific polypeptides on some isolates

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Molecular Analysis of Cucumber Mosaic Cucumovirus Symptoms Development on Squash Plants

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Abstract: A wide range of severe symptoms were appeared on inoculated squash plants (Cucmus pepo cv. El-Skandrani) with CMV under greenhouse condition. One of the first signs of systemic CMV infection, is vein clearing in the youngest leaves (about 7 days), the veins become translucent and leave produced subsequently showed a mosaic (about 10 days) severe mosaic and mottling (about 15 days). Then changed in leaves growth form i.e. little, malformation and no-Lamina giving shoestring the so-called fern leaf (about 20-25 days). The virus was transferred from each symptom to squash and chenopodium amaranticolor plants by sap mechanical inoculation then conformed by DBIA-assay. The change in chlorophyll contents to ensure that all these symptoms resulted from CMV-s EG. SDS-PAGE, peroxidase isozyme separation and RAPD-PCR to molecular analyze of S-CMV-EG symptoms development on squash plant leaves. SDS-PAGE of protein separation showed variability protein pattern and contents of healthy and S-CMV symptoms infected squash leaves (m-mosaic, S-mosaic, crinkling and malformation) with 24, 26, 28, 25, 20 and 22, 21, 20, 22, 16 soluble and unsoluble polypeptides respectively. As well as. DISC-PAGE isozyme showed 6, 7, 8 and 8 peroxidase isozymes respectively. RAPD-analysis revealed DNA polymorphic among CMV-symptom development on squash plants. RAPD analysis using two random primers revealed 8 polymorphic of total 15 amplified fragments with 53% under CMV infection. Crinkle symptoms revealed the highest number with 18 markers followed by S-mosaic 17 and malformation 16 and mild mosaic with 15 bands. [Journal of American Science 2010;6(8):94-103]. (ISSN: 1545-1003).

Key words: Squash plants, CMV symptoms development, SDS-PAGE, DISC-PAGE, PAPD PCR.

1. Introduction

Cucumber mosaic cucumorius is widely distributed in different crops in Egypt and has been reported as a main problem for squash crop production in open field and protected agriculture besides causing severe losses in cucurbit crops (Staniulus et al., 2000). The virus showed several types of symptoms including, vein clearing, mild and severe mosaic blisters, green vein banding and chlorotic local lesion followed by vein netting, yellow often necrosis and plant death, leaf distortion, crinkle and malformation, and filformshape (Gomaa, 2008). Therefore, it was important to define the etiology of this virus by symptomatology, biology, serology and protein related index tests.

Leal and Lastra (1984) revealed that the reduction in chlorophylls, soluble proteins and nitrogen contents was evident in leaves infected by tomato yellow mosaic virus that causes severe symptoms. The subcelular distraction of virus stimulates the synthesis of soluble proteins which were detected by SDS-PAGE. The most prominent as judged by the intensity of their staining, is the enhancement of 14.3, 20, 39, 58 and 97 kDa proteins (Hadidi, 1988; El-Dougdoug, 1996 and Sherif and El-Habbaa, 2000). Certain host proteins were increased dramatically as part of a general physiological response in infection by viruses and other pathoens (Comacho-Henriquez and Sanger, 1982). Viral infection with yellow leaf carl, leaf roll, mosaic, curl, crinkle and malformation leaves of tomato and squash exhibited higher activity of peroxidase polyphenol oxidase and esterase (Tag El-Din et al., 2006).

The present study aims to the study the chemical and molecular variability of CMV symptoms development in infected squash plants, using, SDS-PAGE, DISC-PAGE and RAPD-PCR.

2. Material and Methods

Virus source:

Cucumber mosaic cucumoviridae CMV-s EG isolate was obtained from the Virology Lab, (Megahed, 2008). Agric. Microbiology Dept., Fac. of Agric., Ain Shams Univ. The virus isolate maintance on Nicotiana glutinosa.

Virus detection : In order to determine the presence of CMV in inoculated and symptoms squash plants, the goat antirabbit immunoglobuline-alkaline phosphtase conjugate (Sigma A 4503) was used by Dot blot immunoassay as described by Lin et al. (1995).

The seeds of squash (Cucurbita pepo cv, Eskandarani) and indicator plants for CMV-s EG

detection were kindly provided from Virology Lab., Fac. Agic., Ain Sham Univ., Cairo, Egypt.

The squash plants were grown into steam sterilized soil in pots under greenhouse conditions until the seedling stage (cotyledon leaf). The squash leaves were mechanically inoculated with CMV-s EG isolate prepared in 0.1 M phosphate buffer pH 7.4. The symptoms were recorded at 2-6 weeks and photographed after mechanical inoculation. The plants showed symptoms were indexed by Chenopodium amaranticolor as indicator host and also by DBIA assay.

Chlorophyll content :

Chlorophyll a, b and carotenoids were extracted and estimated according to Wettstein (1957) as mg/L.

Biochemical analysis :

Protein content was determined by Bradford (1976) using bovine serum albumin as a standard.

SDS-polyacrylamide gel electrophoresis :

S odium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970). Extraction of soluble and non-soluble proteins were done according to Studier (1973).

Peroxidase (PRX) isozymes electrophoresis: Gel electrophoretic isozymes were performed according to Stegemann et al. (1985) among four symptoms leaves using one enzyme staining systems. Peroxidase revealed the most variables with good determined banding patterns.

Molecular analysis :

DNA extraction was performed by the method of Wulff et al. (2002).

DND amplification:

Random amplified polymorphic DNA (RAPD) analysis was applied according to Williams et al. (1990) using 10 mers of four random oligonucleotide primers obtained from (Metobion AG. Lena Christ Strasse, Martinsried, Deutschland) as shown in Table (1). The gels analysis was applied by programme (UVI geltec version 12.4, 1444-2005 USA).

Table 1. The four random primers used in DNA amplification

Primer name	Sequence (5`-3)
OPD-11	5`AGCGCCATTG3`
OPT-20	5`GACCAATGCC3`
2-19	5`GCACGGCGTT3`
2	5`AACGCGCAAC3`

Agarose gel electrophoresis :

PCR amplified products were analyzed using 1.2% agarose gel electrophoresis staining with ethidium bromate. The amplified DNA bands were visualized under UV light and the sizes of the fragments were estimated based on a DNA ladder of 100 to 2000 bp (manufactured by Bloron).

3. Results

Symptoms development :

Sequency of symptoms development on CMV infected squash plants (Cucurbita pepo cv. Eskandarani) showing signs of viral symptoms a wide range of severe symptoms were appeared on squash plants. One of the first signs of systemic infection is vein clearing in the youngest leaves (at 7 days), the vein become translucent and leaves produced subsequently showed a mosaic (10 days), severe mosaic yellowing, mottling (chlorosis) (20 days). Then changed in leave growth forms i.e. little, blisters, crikle, malformation and no-lamina giving shoestring (at 25-35 days). The so-called fern leaf. The symptoms were than developed into four main features on cultivated plants and thus they were classified into the following four categories:

1- The leaves were mild mosaic (Fig. 1-a); 2- The leaves were severe mosaic and blisters (Fig. 1-B). 3- The leaves were curling and narrow (Fig. 1-C) and 4- leaves showed reduction in blade and filiform or shoestring shapes (Fig. 1-D).

A number of eight plants infected squash plants showing viral different symptoms types were examined by dot blot immunoassays assay using specific polyclonal antibodies by DBIA. A purplish blue color was developed infected plants in positive reaction, whereas extracted from healthy plants remain green in the negative reactions (Fig. 2).

Another assay for viral detection and variability of symptom via local lesion diversity on Ch. amaranticolor by characteristic of morphological local lesions. It was observed variation in local lesions i.e. size, shape, colour without center and halo; chlorotic necrotic, fine and large (Fig. 1B).

Chloropyll and carotenoids :

Data presented in Table (2) showed that chlorophyll a and b were significantly decreased in infected plants showed mild and severe mosaic, where as curling and deformation plants didn't show significantly different from healthy plants. On the other hand carotenoids were not significantly changed.

Protein contents: was determined in squash leaves with individual symptoms elated to BSA (Table 2), it was revealed that, all the symptoms types due to increasing in total protein content with different values compared with healthy squash leaves.

Table 2. chlorophyll; carotenoids and protein contents in squash leaves at different periods of symptoms developments

Symptoms development / Chlorophyll* and protein contents	Symptoms development				
	Healthy	**m-Mosaic**	**S- Mosaic**	**Crinkling**	**Malformat-ion**
Chlorophyll-a	1.015	0.234	0.095	0.695	0.792
Chlorophyll-b	0.625	0.185	0.047	0.375	0.410
Carotenoids	0.412	0.425	0.445	0.495	0.485
Proteins **	7.87	4.76	5.44	6.37	6.23

* Photopigment : mg/g fresh.
** Protein content: mg/ml

SDS-PAGE proteins analysis :

SDS-PAGE profile of soluble proteins extracted from infected and uninfected squash leaves presented in Table (3) and Fig. (3). SDS-PAGE analysis revealed 32 bands with different molecular weights ranged from 275 to 10 as shown in Fig. (3).

The soluble protein bands of the four plants which revealed different symptom types were varied in number and density of bands, whereas mild mosaic, severe mosaic, crinkle and malformation were revealed the highest total number with 24, 26, 26, 25 respectively bands while healthy displayed the lower number with 20 bands.

The variability analysis of symptoms development showed some proteins bands disappeared in healthy for example 9, 7, 8 and 9 band for mild, severe mosaic, crinkling and malformed respectively (Table 2). The present of variability between symptoms development was 53% related to total bands.

The protein bands were appeared in both healthy and in under all symptoms types with 32 bands. Some other protein 17 bands were appeared under symptoms types and displayed in healthy. Some of these bands were appeared in both healthy and symptoms types such as bands number 15, 19, 18 and 16 protein bands respectively (Table 3).

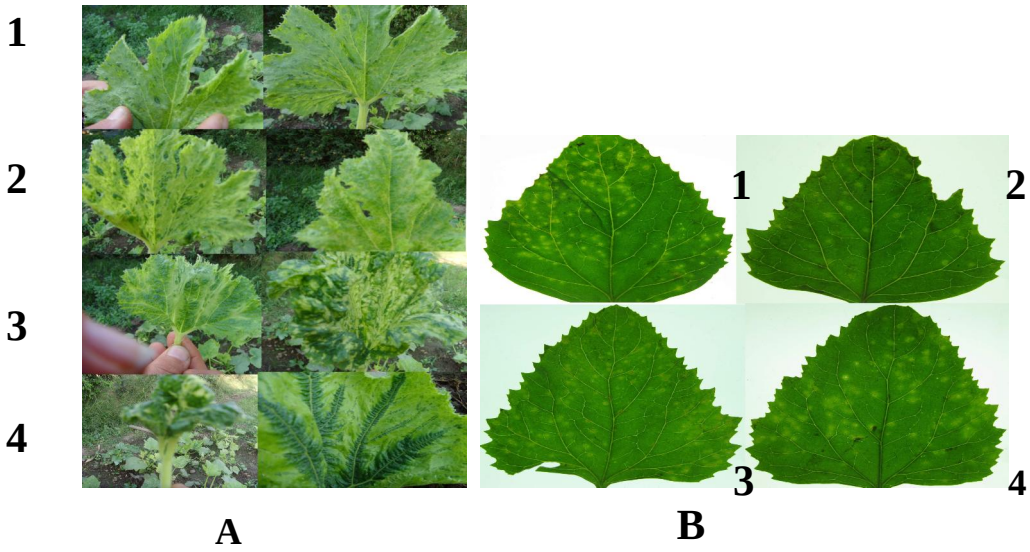

Fig. (1): Symptoms development on infected squash leaves by CMV, A. Systemic symptoms, B. Variability of local lesion on Ch. amaranticolor 1- mild mosaic; 2- severe mosaic, 3- crinkle and 4- malformation symptoms.

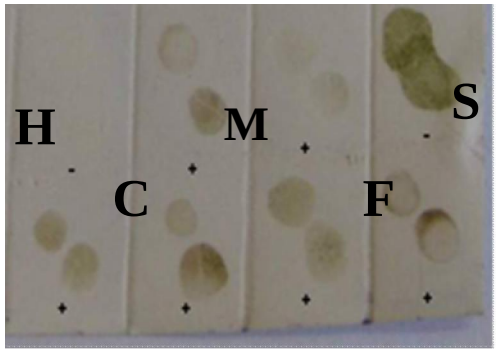

Fig. (2): Dot blot immunoassay for CMV detection in symptoms types on squash leaves against specific IgG-CMV polyclonal, H = healthy leaves, M = mild mosaic, S= severe mosaic, C = crinkle and F = malformation.

Table (3): SDS-PAGE analysis of soluble protein patterns of the variable the symptoms development related to CMV-squash inoculated.

Band no.	Healthy	Mild mosaic	Severe mosaic	crinkling	Malformation
275	-	+	+	-	+
195	-	+	+	-	+
161	-	+	-	-	-
104	+	+	+	+	+
92	+	+	+	+	+
85	+	+	+	+	-
74	-	-	-	+	+
68	-	+	+	+	+
63	+	+	+	+	-
59	+	-	+	+	+
58	+	+	-	-	-
55	+	-	+	+	+
52	+	+	+	+	+
50	-	+	+	+	+
46	+	+	+	+	+
40	-	+	-	+	-
38	+	-	+	+	+
36	+	+	+	+	+
34	+	+	+	+	+
33	-	-	+	+	+
31	+	+	+	+	+
28	+	+	+	+	+
26	-	+	+	-	-
24	+	-	+	+	-
22	+	-	+	+	+
20	+	+	+	-	+
19	+	+	+	+	+
18	+	+	+	+	+
17	-	+	+	+	+
14	-	-	-	+	+
11	+	+	+	+	+
10	-	+	-	+	+
Total bands	20	24	26	26	25

SDS-PAGE analysis revealed 26 bands with different molecular weight ranged from 238 to 20 kDa as shown in Fig. (3). The non-soluble protein of symptoms development were varied in number and density whereas 22, 21, 20, 22 bands of mild mosaic, severe mosaic, crinkling and malformation respectively, while healthy plant displayed the lower 16 bands.

SDS- profile of non-soluble proteins extracted from the infected plants with CMV and non-infected presented in Table (4). They were varied between symptoms types in numbers and density where as symptom types plants revealed increasing in protein profiles their healthy plants. The number increase was 6, 5, 4 and 6 bands for mild mosaic severe mosaic, crinkle and malformation symptom types respectively (Table 4 and Fig. 3).

On the other hand the newly induced bands under CMV infection 9 bands in symptoms types plants where disappeared in healthy plants.

The variability analysis of symptoms showed some bands disappeared in healthy and between symptoms plants. The percent of variability between symptoms plants was 48% related to total bands.

Peroxidase isozymes : Results of peroxidase isozymes are shown in Table (5) and Fig. (4). Each symptom types and healthy plants could be characterized by unique set. of isozymes. The total number of peroxidase isozymes shown in all symptoms types were 11 isozymes while those of each type 7, 8, 8 and 9 for mild mosaic severe mosaic, crinkling and malformation respectively as well as 7 bands for healthy ones. The variability between symptoms types was 60% related total peroxidase isozymes (Table 5).

Peroxidase isozyme (PRX) analysis displayed a total of 11 band whereas 5 of them were variable (polymorphic) and 5 (monomorphic) other bands among the different symptoms types of CMV-squash inoculated. As well as the control as presented in the zymogram (Table 5) and Fig. (4). The effect of CMV-EG strain infection could be observed among of 4 peroxidase bands, whereas bands No. 2, 5, 6 and 7 disappeared in the control (healthy) plants and appeared in some symptom types, on the other hand polymorphic band No. 1 appeared in healthy and symptom type 3; polymorphic band No. 2 appeared in symptoms type 1 and 4; polymorphic band No. 5 appeared in four symptoms types; polymorphic band No. 4 appeared in symptoms types 1, 2 and 4 and polymorphic band No. 6 appeared in symptoms types 3 and 4. However, 7 bands were appeared in healthy plants , 3 polymorphic bands No. 1 with symptom type 3 and No. 7 with symptoms types 3 and 4, as well as 3 monomorphic bands No. 3, 8 and 9 (Table 5 and Fig. 4).

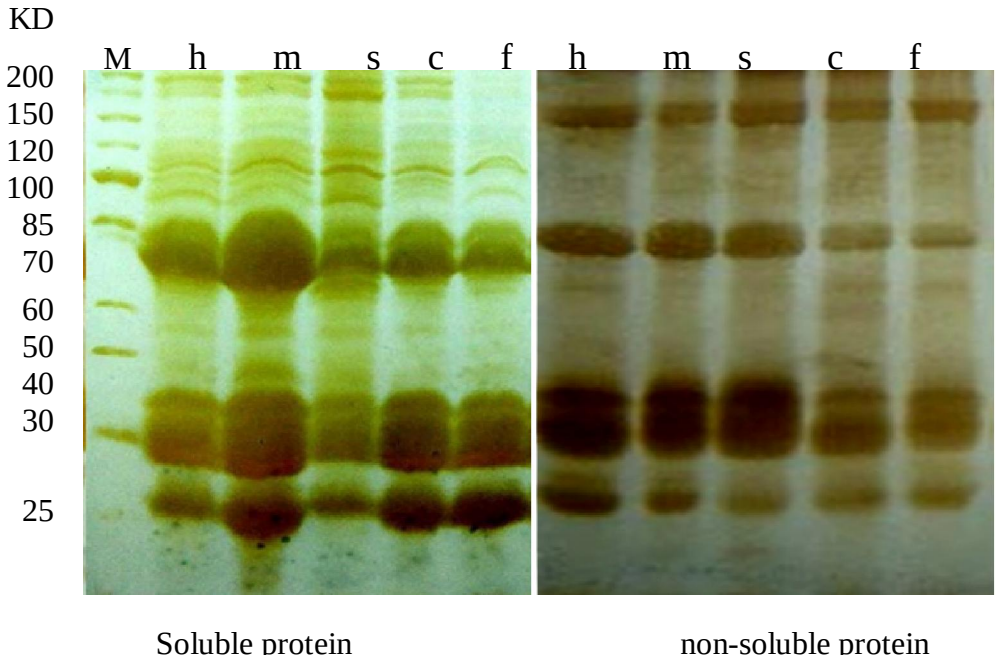

Fig. (3): SDS-PAGE (12%) of soluble protein (a) and non-soluble protein fractions extracted from the infected squash leaves with CMV. H = healthy leaves, m = mild mosaic, s = severe mosaic, c= crinkle and F = malformation.

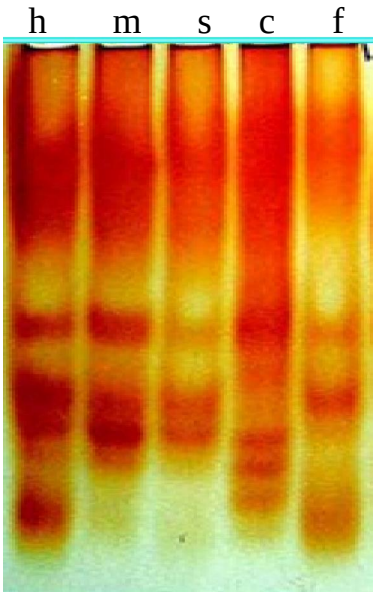

Fig. (4): Peroxidase (PRX) isozyme profiles of squash leaves infected with CMV. H = healthy leaves, m = mild mosaic, s = severe mosaic, c= crinkle and F = malformation.

Table (4):SDS-PAGE analysis of non-soluble proteins patterns of the variable the symptoms development related to CMV squash infection.

Band no.	Healthy	Mild mosaic	Severe mosaic	crinkling	Malformation
238	+	+	+	+	+
161	-	+	+	-	-
109	+	+	+	+	+
92	+	+	+	+	+
85	+	+	+	+	+
74	+	-	-	-	+
68	-	+	-	-	+
63	-	+	+	+	+
51	+	+	+	+	+
58	-	+	+	+	+
57	+	+	+	+	+
55	+	+	+	+	-
52	-	+	+	+	+
50	-	+	+	+	+
46	+	+	+	+	+
40	+	+	+	+	+
38	-	-	-	-	+
36	+	+	+	+	+
34	+	+	+	+	+
33	+	+	+	+	+
31	+	+	+	+	+
28	+	+	+	+	+
26	+	+	-	+	+
24	-	+	-	-	+
22	-	-	+	-	-
20	+	+	+	+	+
Total bands	16	22	21	20	22

Table (5): Peroxidase (PRX) isozymes analysis of the variable bands of the variable the symptoms development related to CMV squash infection

Band no.	Healthy	Symptoms development				Polymorphism
		m-mosaic	s-mosair	Crinkle	malformation	
1	+	-	-	+	-	Polymorphic
2	-	+	-	-	+	polymorphic
3	+	+	+	++	+	monomorphic
4	+	+	+	-	+	polymorphic
5	-	+	+	+	+	polymorphic
6	-	-	+	+	+	polymorphic
7	-	-	+	+	+	monomorphic
8	+	+	+	+	+	monomorphic
9	+	+	+	+	+	monomorphic
10	+	+	+	+	+	monomorphic
11	+	+	-	-	-	Polymorphic
Total bands	7	8	8	8	9	

Such new peroxidase bands may related to CMV infection in mild and severe symptoms types. In general, results in table (5) which represent the peroxidase enzyme polymorphism among the 4 symptoms types could be served as a model for analyzing the gene action and different mild and severe symptoms types.

RAPD analysis of CMV infected squash plants :

Four random primers, OPT-20, OPD-11, 2-19 and 2 were used in random amplified polymorphic DNA (RAPD) analysis in infected squash plants with CMV-s EG.

Primers OPT-20 and OPD-1 revealed 12 amplified fragments with sizes ranged from 690 to 190 bp. Whereas 8 fragments were polymorphic and 4 commonly detected among the symptom type 5 , plants with molecular size 690, 590, 340 and 380 bp. As well as the healthy squash plants revealed 16 amplified fragment (Table 6 and Fig. 5).

The healthy squash plants was varied considerably in the presence of amplified fragments where 14 fragments, thus m-mosaic, s-mosaic crinkle and malformation were 15, 16 and 18 and 16 amplified fragments respectively. On the other hand 4 amplified fragments appeared in healthy plants only and disappeared in infected plants, Table (6).

Table (6):RAPD analysis of symptom development on CMV-infected squash plants using RAPD primers

Molecular weight (bp)	**OPT-20**					OPD-11					Polymorphism
	H	**M**	**S**	**C**	**F**	**H**	**M**	**S**	**C**	**F**	
1045	+										Unique
920	+										Unique
865	+										Unique
765	+										Unique
690	+	+	+	+	+	+	+	+	+	+	Monomorphic
630	+++	+	+	++	++	-	-	-	-	-	Polymorphic
590	-	+	++	+++	+++	+	+	+	+	+	Monomorphic
415	+	-	++	+++	++++	-	-	-	-	-	Polymorphic
440	-	++	+++	+++	++++	-	-	-	+	-	Polymorphic
340	+	++	+++	++++	++++	+	+	+	+	+	Monomorphic
331	-	+++	+++	++++	++++	+	+	-	-	-	Polymorphic
320	-	+++	+++	++	++	-	-	-	-	-	Polymorphic
380	+	+	+	+	+	+	+	+	+	+	Monomorphic
240	-	+	++	+	+	-	-	+	-	-	Polymorphic
210	-	-	-	+	-	-	+	-	+	++	Polymorphic
190	-	-	-	-	-	-	+	+	+	+	Polymorphic
Total bands	9	9	10	11	10	5	6	6	7	6	

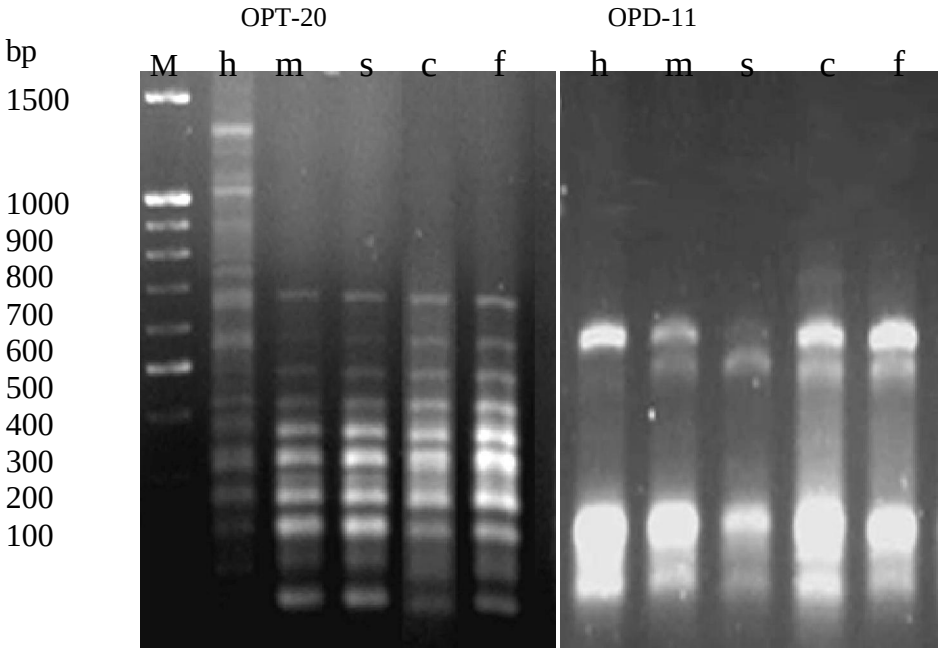

Fig. (5): RAPD amplified products of CMV-infected squash leaves using two primers. OPD-11 and OPT-20. H = healthy leaves, m = mild mosaic, s = severe mosaic, c= crinkle and F = malformation.

Moreover, the numbers of fragments of symptoms types were affected by different behaviour either by increase or by decrease compared with healthy plants. For contrast, the crinkle symptoms increased in 9 bands, S-mosaic (7) and malformation increased in 7 bands and m-mosaic increased 6 bands related healthy plants. Meanwhile, the crinkle symptoms type revealed induced amplified fragment that were nor existed either in the healthy or mild and severe mosaic and malformation symptoms (440 bp) and s-mosaic symptoms with 240 bp in OPD-11 RAPD primer. There bands resulted from RAPD-PCR amplified products could be used as RAPD markers for symptoms types against CMV infection in squash plants.

Table (7): Summarized results of biochemical and molecular variation among symptoms types of CMV-infected squash plants.

CMV symptom types	Photopigments			Proteins				RAPD primers		
	Chl a	Chl b	Carote-noid	Protein content	SP	NSP	PRX	OPD20	OPD11	Total M.M
Healthy plants	1.015	0.625	0.412	7.87	26	16	6	8	5	62
Mild-mosaic	0.234	0.185	0.425	4.76	24	22	7.	9	6	68
Severe mosaic	0.095	0.047	0.445	5.44	26	21	7	10	6	70
Crinkling	0.695	0.375	0.495	6.37	26	20	8	11	7	72
Malformation	0.792	0.410	0.485	6.23	25	22	8	10	6	71

Chl = chlorophyllNSP = non-soluble protein M.M = molecular markers SP = Soluble rotein
PRX = peroxidase isozymes

The results of biochemical and molecular variation for symptom types of CMV-infected squash plants were summarized in Table (7). Consequently the genetic analysis of CMV symptom types showed that crinkle was the highest biochemical and molecular markers (72) followed by malformation (71) severe mosaic (70) and m-mosaic (68) compared healthy with 62 molecular markers.

4. Discussion

The effects of viruses on plants are multifarious, practically and function of a plant may be disturbed. Symptoms will be named and briefly described. They may be grouped according to their effect on overall growth, colour, water content, tissue life span, shape of organs, plant anatomy and plant physiology. Most plant

virus diseases and viruses are named after their main symptoms in a particular host.

More obvious and better known are colour changes, especially of leaves. Mosaic disease has long been synonymous with virus disease. CMV-s EG mosaic of squash is one type of variegation as mentioned of tobacco and abutilon (Bos, 1983 and Mathews, 1991). Diffusely bordered variegation is often called mottling but there are various intermediates. The term vein mosaic (vein clearing) denotes an irregular mosaic along veins and vein-banding a regular one.

Actually decrease in photosynthetic rate of the infected leaves is often associated with development of the symptoms (Plat et al. 1979).

The effects are, of may be, related to changes within organs or entere plants, symptoms are variable and greatly depend on the host plant and how long it has been infected, the virus strain and the environmental conditions. For example, symptoms that are particularly noticeable in plants that have been growing in cool bright conditions since infection may become masked in host poorly-lit conditions. The time after infection before symptoms appear depends on the virus, the plant and the environmental conditions too; it is commonly a few days or weeks in herbaceous plants through it may be one or more years in woody ones.

Under greenhouse conditions the symptoms could be more clear and restricted. It should be independent of the virus and should reflect the host genetics. Symptoms of CMV disease on squash differed depending on the isolates as well as the environmental conditions (Makkouk et al., 1979 and Aref and El-Dougdoug, 1996). By comparing the data presented in this study of the viral symptoms development and mechanically transmitted with the results. CMV-s EG was detected biological and serological in infected squash plaints (Megahed, 2008), Abo El-Nasr et al. (2004), it was concluded that the three viral symptoms had ZYMV viral complex of one or more strains. Similar results were obtained for TYLCV-E (Aref and El-Dougdoug, 1996). Local lesions diversity of CMV produced n Ch. amaranticolor were used CMV identification and variability as well as isolation of CMV strains (Megahed 2008).

Protein isozymes, and DNA analysis should be independent of host genetics under virus inducer. Whereas, amino acid sequence of polypeptides are dependent on nucleotide sequence of their coding genes; therefore on electrophoretic analysis of protein and isozymes of CMV infected leaves which showing different symptoms approximates the analysis of there genetic variation. The variation of CMV symptom types was detected via determination of polypeptides by using SDS-PAGE and peroxidase isozymes using DISC-PAGE. Data presented in Table (2) indicated that the number of protein fractions increased in infected plants as recorded in severe mosaic followed crinkle and malformation than mild mosaic. Also this results indicated the CMV due to increasing protein content or certain protein fraction or both together. For example severe mosaic showed decrease in protein content but increase in protein fractions. This clearing that despite increasing the biodegradation of protein content new protein types were detected due to virus infection. The same observation was reported by (Hadidi 1988 and El-Dougdoug, 1996). The synthesis of new proteins are due to the host-virus interaction previous results also indicated that protein contents could be increased dramatically (Camacho-Henriquez and Sanger 1982) or decreased (Lead and Lastra, 1984).

The enzymatic pools and their metabolic pathways are the most important factors affecting pathogenicity especially with viruses. The results showed that the level of peroxidase in infects leaves of crinkle and severe mosaic were higher than those in healthy leaves. This high level play an important role in the defense mechanism. Increase in this enzyme activity and isozymes has been detected after infection by pathogens in different host pathogen combination (Hammeschmidt et al. 1982 and Sherif and El-Habbaa, 2000).

DNA prepared was found crucial for RAPD-PCR. The yields of DNA were determined of spectrophotometrically as, 11, 14, 15 and 13 µg/0.05 g fresh tissues. The PCR conditions for DNA analysis were optimized by investigated each factor individually, the optimized conditions were detailed in material and methods section. A total of scoroble amplified DNA fragments ranging in size 1045 to 190 bp were observed using the two primers were as polymorphic with 8 and 4 bp monomorphic fragments with detected among symptoms types. Interesting to note that, the four symptoms types were varied in there formed and developed. Where 15, 16, 18 and 16 for m-mosaic, S-mosaic, crinkle and malformed symptom, respectively compared with DNA fragments in healthy leaves. The genetic variability among symptoms types has yet 16 to be established rapid and unambiguous of genetic variability among symptoms types has greatly benefied from recent advances in DNA fragments based on the PCR and random amplified polymorphic DNA (El-Dougdoug et al. 2007 and Sharma, 2003).

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Genetic Diversity of *Shistosoma mansoni* Isolates Genome and Protein Analysis

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Abstract: Five of Shistosoma mansoni isolates which differ in disease potential show genetic variability. Esterase isozymes, protein pattern and DNA finger-print analysis successfully revealed genetic diversity. DISC PAGE esterase isozymes revealed 5 bands which differ in density and relative mobility among SM1, SM3, SM4, SM5, while 4 bands with SM2. SDS-PAGE of protein finger print varied among the five isolates in number of protein species 19.18, 17.16 and 16 bands of SM1, SM2, SM3, SM4 and SM5 isolates respectively as well as in intensity. Molecular weight and reproducibility polymorphism analysis presented 12 common fragments (monomorphic) with 54%, 8 specific bands (polymorphic) with 45% and 4 bands unique with 20%. RAPD-PCR for identification of the 5 S. monsoni isolates were conducted on the screening of primers and on the extraction of template DNA. Three out of 10 sequence 10 mer primers were successful in identification of the 5 S. monsoni isolates. PCR amplification yielded reproducible RAPD patterns which differentiated the 5 isolates examined. The difference also recognized the RAPD patterns specially of SM5. Esterase isozymes, protein and DNA fragments conformed the genetic diversity among 5 S. mansoni isolates. [Journal of American Science 2010;6(8):104-110]. (ISSN: 1545-1003).

Key *words:* *S. mansoni*, esterase isozymes DISC-PAGE, RAPD-PCR, protein and DNA fragments.

1. Introduction

Schistosoma mansoni is one of the most severe tropical diseases in the world. Egypt is one of the most highly endomic areas in some localities in the Nile Valley. Biomphalaria alexandrina snail as specific intermediate host of S. sansoni are prevalent in both upper and lower Egypt, but during the last decade, it become the most dominant species in the Nile Delta forming a main threat for Schistosomiasis transmission in the North of Egypt. This wide distribution of the disease makes the study of genomic variability extremely important. There are well known two distinct geographical strains of S. mansoni, the Puerto Rican and the Egyptian strains (Saoud, 1966). However a new geographical strain namely, Saudi Arabian strain has been reported based on differences with the Egyptian strain shown by seanning electron microscopy (Shalaby et al., 1993).

Several techniques of molecular biology for studies S1, S2, S3, S4 , S5, of genetic variability in Schistosomiasis vector have been used (Da Silva et al., 2004). An introduction of the random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) technique (Jamjoom, 2006) has amplified possibilities of polymorphism analysis and biochemical markers, as enzyme isozymes (Lockyer et al., 2000).

The present study emphasizes our interest in soil genetics to detect genetic variability among five S. mansoni using RAPD-PCR and isozymes for genetic analysis of DNA genome of S. mansoni snails and protein enzymes.

2. Material and Methods

Parasite isolates: Five adult worms isolates of repeated twice S. mansoni from (Southern region). (Delta region) and (east region) at Egypt. They were obtained from theodor Bilharz Research Institute and originally selected on the basis of healthy different size and pathogenicity according to Longand and Marand (1995).

SDS-PAGE electrophoresis: Protein content of adult worms was determined by Lowry's method (Lowry et al., 1951) using bovine serum albumin as a standard.

Protein pattern was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970) under reducing condition using 12% pore gel with 3.7% stacking gel in a discontinuous electrode buffer system. Protein samples and molecular weight markers (Sigma) were denatured and reduced completely before electrophoresis by mixing the samples with an equal volume of 2x sample buffer and heating the mixture at 95ºC in a water bath for 5 min. Treated samples were centrifuged at 12000 rpm for 10 min and chilled on ice before use. Each lane was charged with 25 µg protein of supernatant. For subsequent fractions, the lanes were field up to their full capacity (50 µl). Electrophoreis was carried out a room temperature at a constant current of 25 mA for 1 hr followed by 30 Am for 4 hr. At the end of the run, gel was stained with commassie blue and destained in the stain solvent and photographed.

Isozyme electrophoresis:

The adult worms were homogenized in Tris. Glycine buffer and centrifuged at 10000 rpm for 10 min. The suprnatent was kept at -20ºC until electrophoresis according to Jonathan an Wondell, 1990). Isozyme variability was assayed by nature polyacrylamide slob gel electrophoresis using 8% acrylamide. Each sample was mixed with bromophenol blue and applied to each well, the nels were stained with the solution contain Na-phosphate and α, β naphthyl acetate an fast blue CR salt. The get was inoculated at 4ºC in work and adding the appropriate substrate and staining solution (Jonathan and Wendell, 1990).

DNA isolation:

DNA was extracted from individual frozen adult worms and homogenized with a pestle on Eppendrof tube containing 100 µl of 10 mM Tris-HCl pH 8.0; 1 mM EDTA, 10 mM NaCl an 70 mM sucrose. Sodium dodecyl sulfate (10%) and (12 µl) proteinase 10 mg ml-1 were added to Eppendrof tube. The tubes were incubated at 57ºC for 2 h. Then extracted once with an equal volume of phenol and once with chloroform. The DNA was precipitated by 0.3 M sodium acetate; 2 volume of absolute ethanol at 20ºC overnight. The DNA was pelleted by centrifugation (15000 g/20 min at 4ºC) then rinsed with ethanol 10% dried and finally resuspended in 100 µl of TE buffer (10 mM Tris HCl pH 8.0 and 1 mM EDTA. The purified DNA concentration was determined spectrophotometrically at 260 nm and 280 nm and also by using 2% agarose gel electrophoresis.

RAPD-PCR analysis:

Three orbitrary primers, A7 (5` gaaocaaatg – 3`) A8 (5`-gtgacgtagg-3`) and A6 (5`-gtgcaagtct-3`) were used a described by Simpson et al. (1993) with modifications: 2 ng of DNA genome were amplified PCR thermal cycler). Each reaction was carried out in a final vol. Of 10 µl containing 1 unit Taq DNA polymerasem 1x PCR buffer, 0.2 mM each dNTP and 7 p mol of each random primer (Gibco). The amplification conditions were as follows: 1 cycle at 95ºC for 5 mm, 3 steps, at 95ºC for 30 sec, 30ºC for 2 min and 72ºC for min and 72ºC for 1 min and 33 cycles during which the annealing temperature was changed to 40ºC and the time of the extension step was increased to 5 min during the final cycle.

The PCR products were visualized on 1.5% agarose gels and stained with ethidium bromide to resolve amplified fragments and photographed.

3. Results

Somaclonal variations among 5 isolates of S. mansoni were detected by protein pattern, esterase activity, isozymes and DNA fingerprint.

Protein content was determined in adult warm of each isolate related to BSA (Table 1). It was revealed that the protein content was varied among 5 isolates with 480, 425, 392, 405 and 385 mg/g fresh weight of adult worms.

Esterase activity was assayed of each isolate. The specific activities of enzyme was 4.25; 4.75; 3.82; 5.12 and 3.25 µ/mg protein for SM1; SM2; SM3; SM4 and SM5 respectively (Table 1). Esterase isozymes are shown in Fig. (1.A) and calculated in Table (2). The total number of isozymes 8 bands differ in number and density among five isolate such as 4 band of SM1 isolate and 5 bands of each SM2, SM3, SM4 and SM5 out of 8 total isozymes bands. The esterase isozyme variability among isolates showed some isozymes band disappeared in SM1 (0.25, 0.35, 0.55, 0.75 Rf); SM3 (0.25 M, 0.35, 0.75 Rf); SM3 (0.25, 0.55, 0.75 SM4 (0.35, 0.55, 0.75 Rf) and SM5 (0.25, 0.35, 0.55 Rf). As well as one unique band (genetic marker) at Rf 0.25 (SM4), 0.35 (SM3); 0.55 (SM2) and 0.75 (SM5) isolates and monomorphic (common isozyme band) in five isolates at Rf 0.42, 0.45, 0.61 and 0.64.

Table (1):Protein content and esterase activity of five *Schitosoma mansoni* isolates.

	Esterase activity		
***S. monsoni* isolates**	**Protein content (mg/g)**	**Total activity (µ)**	**Specific activity (µ/mg)**
SM1	480	2040.00	4.25
SM2	425	2018.75	4.75
SM3	392	1497.44	3.82
SM4	405	2673.60	5.12
SM5	385	1251.25	3.25

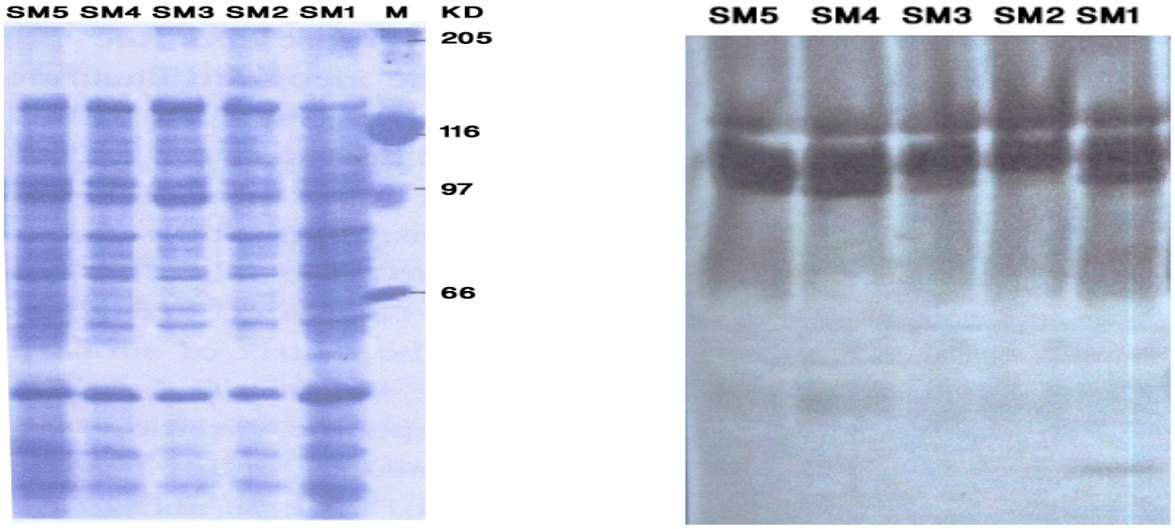
SDS-PAGE protein fractions was illustrated in Fig. (1-B) and Table (3). The results showed the variation of S. monsoni isolates in protein fractions and density bands. The variability analysis among isolates appeared 24 protein fractions. Out of them 19, 18, 17 polypeptides appeared in SM1, SM2, SM3 isolates respectively and 16 polypeptides in both SM4 and SM5. Some polypeptides bands disappeared among isolates (Table 3). The molecular weight of polypeptides were determined related to protein marker (Table 3). The most prominent alteration (polymorphic bands) among 5 isolates 122, 108, 60, 20 KDa with percentage 16%. The prominent polypeptide bands in all isolates (monomorphic or common fragments) were 156, 110, 100, 97, 85, 70, 54, 44, 36, 33, 24, 18 and 10 KDa with percentage 54%. The unique fragment (genetic markers) with 73,

39 (SM1); 15 (SM2); 65 (SM3), 175 (SM4) and 28, 26 (SM5) with percentage 30%.

Table (2): Esterases isozymes analysis of the *Schistosoma monsoni* five isolates.

Schistosoma monsoni isolates							
No.	**Rf bands**	**SM1**	**SM2**	**SM3**	**SM4**	**SM5**	**Polymorphism**
1	0.25	-	-	-	+	-	Unique
2	0.35	-	-	+	-	-	Unique
3	0.42	++	++	++	++	++	Monomorphic
4	0.45	+++	+++	+++	+++	+++	Monomorphic
5	0.55	-	++	-	-	-	Unique
6	0.61	++++	++++	++++	++++	++++	Monomorphic
7	0.64	+++	+++	+++	+++	+++	Monomorphic
8	0.75	-	-	-	-	+	Unique
Total		4	5	5	5	5	

Rf = Relative mobility Unique = Genetic marker Monomorphic or common isozyme band
Band density ++++ very strong; +++ Strong , ++ Moderate + Weak and - Absent band



A **B**

Fig. (1): Electrogram protein and isozymes profiles of five S. mansoni isolate.
1-A: SDS-PAGE (12%) of denaturated protein extracted from 5 isolates.
1-B: DISC-PAGE (12%) of native protein esterase isozymes.
M: Markers protein (KDa), S1, S2, S3, S4 and S5: S. mansoni isolates.

Table (3): Protein fractions of Schistosoma monsoni five isolates by SDS-PAGE.

Schistosoma monsoni isolates						
Molecular weight (KDa)	**S1**	**S2**	**S3**	**S4**	**S5**	**Polymorphism**
175	-	-	-	+	-	Unique
156	+++	+++	++++	+++	++	Monomorphic
122	++	++	++	+	-	Polymorphic
110	++	++	++	++	+	Monomorphic
108	+	+	+	-	-	Polymorphic
100	++	++	++	++	+	Monomorphic
97	+++	+++	+++	++	++	Monomorphic
85	+++	++++	+++	+++	+++	Monomorphic

73	+	-	-	-	-	Unique
70	+++	+++	++	++	++	Monomorphic
65	-	-	+	-	-	Unique
60	+	+	+	+	-	Polymorphic
54	++	++	++	++	++	Monomorphic
44	+++	+++	+++	+++	++	Monomorphic
39	+	-	-	-	-	Unique
36	++	++	++	+	++	Monomorphic
33	+++	+++	+++	+++	+++	Monomorphic
28	-	-	-	-	+	Unique
26	-	-	-	-	+	Unique
24	++++	+++	+++	+++	++++	Monomorphic
20	+	+	-	-	++	Polymorphic
18	++	++	+	+	+++	Monomorphic
15	-	+	-	-	-	Unique
10	++++	+++	++	++	++++	Monomorphic
Number protein	19	18	17	16	16	

DNA fingerprint: Total DNA preparation as found crucial for RAPD-PCR. The DNA yield was determined spectrophotomatically as 8 µg/ 0.15 g tissues. The DNA purity as indicated by 260/280 was 1.8. The reproducibility of RAPD analysis is known to be singly influenced by experimental conditions. It is therefore essential to optimize the PCR conditions to obtain reproducible and interpretable results before going on routine analysis. It was found that DNA quality was a good template per PCR amplification. However, treatments of DNA with RNase gave sharp and clear amplification products compared with untreated DNA.

The PCR reaction conditions, polymorphism among the five S. mansoni isolates were detected using different random primers by RAPD-PCR gave the best results of amplification expressed on average number of bands per primer. Of the three primers (Operon random primer) were screened in RAPD analysis for their ability to produce sufficient amplification products. Three random primer namely A8, A7 and A6 were more stable and reproducible and gave sufficient polymorphism among five S. manoni isolates. Therefore are focused our efforts on these primers are summarized in Table (4, 5) and Fig. (2). The RAPD analysis of DNA extracted from 5 S. mansoni isolates revealed 22 amplified bands, (8, 7 and 7 bands) with different molecular weight ranged from 1.450 ta 205 bp of primers A8, A7 and A6 respectively. The DNA amplified fragments of 5 isolates were varied in number, density and molecular weight. The variability analysis among 5 isolates showed some DNA amplified fragments absent or/and in some isolates (Table 3). The polymorphism analysis among isolates, 11 monomorphic amplified bands (common in all isolates) with 50%; 4 polymorphic amplified bands (specific bands) with 18% and 7 unique bands (genetic markers) with 32%. The genetic markers were 575 bp (S1 isolate), 275 bp (S2 isolate), 1025 bp (S3 isolate) and 1450, 1025 and 855 bp (S5 isolate).

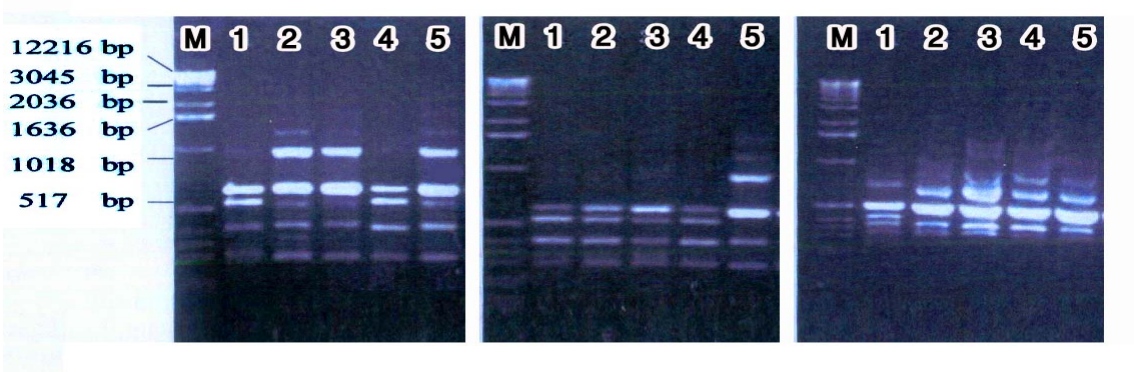


Fig. (2): Electrogram of agarose gel 15% showing DNA polymorphism based on RAPD analysis from the studied five S. mansoni against random primers (A8, A7 and A6)
M: DNA molecular, S1, S2, S3, S4 and S5 isolates, A8, A7 and A6 random primers.

Table (5): Polymorphism and genetic markers among 5 isolates of *S. mansomi* using random primers by RAPD-PCR.

Primer	Polymorphism				Genetic markers of isolates					
	TAF	MAF	PAF	Unique	M.W (bp)	S1	S2	S3	S4	S5
A8	8	4	2	2	855 275		+ 		- -	++++
A7	7	4	-	3	1450 1025 855				- - -	+ + +
A6	7	3	2	2	1025 575	- +	- -	+ -	- -	- -
Total bands	22	11	4	7	7	1	1	1	-	4
Percentage	-	50	18	32	32	4.5	4.5	4.5	-	18

4. Discussion

Schistosoma are dioecious digenetic treamatodes carrying a large (270 Mb) genome. Gaining Knowledge about the genome of these parasites is of importance for the understanding of their biology, mechanisms of drug resistance and antigenic variation that determine escape from the host's immune system (Franco et al., 2006). This aim of the present study the genetic diversity of five S. mansoni isolates selected on the basis of different in disease potential (Longand and Morand, 1998).

Several techniques of molecular biology for studies of genetic diversity in Schustosomiasis vectors have been used (Spada et al., 2002; Florence et al., 004; Hertel et al., 2004; Abdel-Hamid et al., 2006; Jamjoom, 2006 and Haggag and El-Sherbiny, 2006).

Electrophoretic protein banding pattern of an organism can be used to elucidate reliable biochemical genetic markers of this organism. It can also provide information about structural genes and their regulatory systems which control the biosynthetic pathways of that protein banding pattern (Abdel Salam et al., 1992).

In the present study, variation in protein banding patterns between five isolates were revealed using SDS-PAGE electrophoresis. The obtained protein profiles were analyzed and scanned for gel quantitations using Gel Doc 2000 instrument and quantity one. Software package (Bio-Rad). For comparisons among the five isolates of S. mansoni were analyzed in the same gel. The same procedures of scanning and quantitations were done. The observed changes in protein banding patterns in the present study could be reasonably interpreted to be the result of gene mutation. This conclusion is in accordance with Brown and Langley (1979) and Abdel Salam et al. (1992). However, other investigators El-Gamal El-Din et al. (1988) traced such changes back to the induction of chromosomal abnormalities such as bridges breakes, laggards and micronuclei which can lead to loss of some of the genetic material. Therefore, some electrophoretic bands disappeared due to the deletion of their corresponding bands.

Disappearance of some bands could also be explained on the basis of a mutational event at the regulatory genes which are suppressed at transcription level. Meanwhile, the appearance of new bands could be explained on the basis of a mutational event at the regulatory system of unexpressed gene(s) that activate them (Abd El-Salam et al., 1992).

Gel electrophoresis is a widely used tool in studies of genetic variability. The electrophoretic differences reflect the allelic variations of S. mansoni enzymes which might due to mutational events occurring in the shistosoma under stress and affecting the loci controlling the synthesis of isozymes.

Several factors may be considered as primary determinants of the number of bands observed on a gel, including. (a) The number of coding genes, (2) Their allelic states (homozygous or heterozygous) and (3) The quaternary of the protein products. The simplest case involves a single region of salinity with variant electromorphs (allozymes) observed in different individuals. Because allozymes are usually codominanly inherited, the presence and number of bands are depending on the number of polypeptide subunits contained in the active enzyme (Kahler and Allard, 1970).

An electrophoretic analysis was made on the homogenate of progeny of five S. mansoni for esterase isozymes in order to determine isozymes. Since S. mansoni were collected and from different locations, therefore separate electrophoretic runs were made for some S. mansoni from each location. Esterase isozymes are enzymes that characterized by

their common activity on many naphthyl ester substrates (Korochkin et al., 1973). The group of esterase isozymes is one of the largest and most complicated system that has been intensively studied in many organisms of animal kingdom in vertebrates, esterase isozymes are mostly monomeric. Consequently, each band of esterase activity reflects a structure of one polypeptide chain. Hence, each band on the gel represents the end product of one locus (allele). As revealed from Est zymograms, all bands were anndelly migrated and their distributions were varied, some bands were present in a certain S. mansoni and were lacking in another. A total number of 4 were present in all five S. mansoni (minomorphic) and could considered as common bans for all the five S. mansoni which seem to be necessary ones for the enzyme constitution. Bands No. 1, No. 2 and No. 3 observed only in S. mansoni isolatges S4, S3, S2 and S5 respectively which could be a result of gene expressed under stress. The result indicates that there were allelic variants in this locus in all S. mansoni. Suggesting a polymorphic type of inheritance for this enzyme.

The results obtained after RAPD-PCR are presented in Fig. (3) and molecular weights of each primer was summarized in Table (3-4). From the RAPD profiles generated by these primers, bands with MW 1450, 1025, 855, 700, 75 and 205 bp were present in some S. mansoni and absent in others (polymorphic), while the two bands MW 510 and 325 bp were present in all the five isolates (monomorphic). One unique band was detected in S1 isolate with MW of 575 bp while was not seen in the other isolates.

Fortunately, the averages of similarities among five S. mansoni were 52.5, 76.5, 70.5, 40.5 and 82.2% for S1, S, S3, S4 and S5 respectively. This we expect the similarity of genetic backgrounds of many S. mansoni isolates.

The introduction of RAPD-PCR technique has amplified the possibilities of polymorphisms analysis, as it allowed the use of small arbitrary nucleotide segments without the need of a previous knowledge of genes and/or genomic sequences (Welsh and Mc Clellend, 1990). Also, RAPD assay clearly has certain proactively advantages for detecting DNA variation. It is technically less demanding, cheaper and quicker than other molecular techniques (Stothard and Rolliknson, 1996).

Rollinson et al. (1998) reported that, snails resistant to infection occur naturally and there is a genetic basis for this resistance, in B. glabrata resistance to S. mansoni is known to be polygenic trait. So, we have initiated a preliminary search for snail genomic regions linked to, or involved in resistance by using a RAPD-PCR based approach in conjunction with progeny individual methods.

The present study, demonstrated the utility of RAPD-PCR method for the differentiation of S. mansoni isolates. The isolates studied were selected on the basis of different location and pathogenic by well characterized resistance, susceptibility phenotypes upon exposure to S. mansoni. Reproducible and inheritable stable polymorphic markes for S. mansoni were identified with one out of the ten arbitrary primers tested. Several of the primers produced monomorphic bands among the stocks, or the polymorphism identified were not reproducible. In previous studies, genetic diversity among S. mansoni snails was evaluated either by allozyme or restriction fragment length.

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Phenotypic and Genetic Variability Among Three *Bacillus Megatherium* Isolates. I. *In Viro* Evoluation of Tri-Calcium Phosphate Solubilizing Potential and Growth Pattern

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Abstract: Three *B. megatherium* isolates namely BM_1, BM_2 and BM_3 were isolated from different soil types. These isolates were evaluated phosphate solubilizing ability *in vitro*. Aleksandrov's medium (AM) supplemented with tri-calcium phosphate at concentration (0.5%). These isolates formed three morphological colony types. Circular colonies < 1 mm with entire margin and dry; flatter colonies > 1 mm irregular entire edge ith gum and convex colonies < 2 mm with entire margin and wet. The isolates have a variable degrees in release of soluble phosphate amount in culture media supplemental with insoluble phosphate. The BM_1 isolate was most powerful P-solubilizer followed by BM_2 isolates detected on AM culture medium. It was observed decreasing in pH due to an increase of total acidity and amount of soluble P in their culture media. The isolates were differed in exopolysaccharides (EPS) production. However, the capacity of P dissolution and viscosity of culture media depending on the quality of EPS produced by *B. megatherium* isolates. The BM_1 isolate grown in AM culture media produced the highest amount of EPS and viscosity compared with BM_2 and BM_3 isolates. Higher amounts of organic acid oxalic citric, tartaric and furmaric were produced by BM_2 and BM_3 in AM culture media accompanied with lowest amount of EPS. Current data showed that, inoculation AM media with 3 isolates of *B. megatherium* having a variable degrees of metabolic effectiveness led to partial degradation of P, resulting in release of higher amounts of soluble P in the culture media. [Journal of American Science 2010;6(8):111-115]. (ISSN: 1545-1003).

Key words: *B. megatherium* isolates, P rock mineral dissolution mechanism ESP, organic acid

1. Introduction

Phosphate is major essential macronutrient required for plant growth to optimize yield. Thus, the use of alternative indigenous resources and minerals such as rock phosphate are growing importance to elleviate the dependence of imported or closely commercial fertilizers (Badr et al., 2006). The use of plant growth promoting rhizobacteria (PGPR) including phosphate solubilizing bacteria as biofertilizers was suggested as a sustainable solution in improve plant nutrient and production (Vessey 2003). Increasing the bioavailability of P in soils with inoculation of PGPR or/and rock material, which may lead to increasing P uptake and plant growth (Sahin et al, 2004; Girgis, 2006 and Eweda et al., 2007).

Bacillus genus is ubiquitous and common soil microorganisms that play an important role in silicate biodegradation during the process of rock disintegration (Liu et al., 2006). The results of such activity involve both geochemical and structural changes in rocks and silicate and the most, powerful phosphate solubilizers. Due to its ability to produce a range of enzymes, solubilization pounded nutrients and degrade organic wastes (Kubo et al., 1994), along with the N2-fixing ability of some strains (Berge et al., 1991). Bacillus sp. seemed to be a good candidate for biofertilizers application in agriculture.

Inoculation with bacteria, which can improve P and K availability in soils by producing organic acids and other chemicals, stimulated growth and mineral uptake of plants (Lucas Garcia et al., 2004).

A considerable number of bacterial species are able to exert a beneficial effect upon plant growth. As the potential of PGPR is realized researches on their application have increased dramatically over the last few decades. A diverse array of PGPR has been shown to enhance growth and plant productivity by different mechanisms (Tilak et al., 2005).

The present study was conducted to evaluate the solubilization potential of selected Bacillus isolates to solubilize various insoluble soil phosphate mineral in vitro.

2. Material and Methods

Microbial isolates :

Bacillus megatherium were isolated from three different soils in Egypt, i.e Sandy; Sandy loam and Clay soil. The physical, mechanical chatracters of collected soils samples were determined according

to Jackson (1973) and Black et al. (1982) in the laboratory of soil Analysis; Soil, Water and Environmental Ins. Agric. Res. Center, Giza, Egypt.

Screening of B. megatherium for their phosphate solubilizing :

Soil samples were serial diluted in dH2O and inoculated on nutrient agar medium at 30ºC for 48 hr. the bacterial growth were harvested then resuspended and rinsed several times with dH2O to remove any remaining culture medium.

Bacterial isolates were screened for their phosphate solubilizing ability on Pikovskaya medium (PVK) (Pikovskayas, 1948) containing (g/l) to glucose, 0.5 (NH4)2 SO4; 0.2 NaCl, 0.1 MgSO4 7H2O; 0.2 KCL, 0.002, MnSO4 H2O, 0.002 FeSO4 8H2O; 0.5 yeast extract and pH was adjusted to 7.0. PVK medium was supplemented with tricalcium phosphate Ca2 (PO4)2, (PVK-TCP) at concentration of 0.5%. Growth experiments were carried out in 100 ml Erlenmeyer flasks containing 25 ml of PVK + TCP medium and inoculated with 1 ml of each isolate suspension (109 cells ml-1). After 48 h. of inoculation at 30ºC with shaking at 100 rpm (The biomass of Bacillus isolates was determined by direct microscopic counting. Cultures were filtered through 0.2 µm Whatman membrane filter, pH was directly measured by pH-meter. And total acidity of culture media was determined according to Helrich (1990).

The concentration of soluble P in the digested solutions were measured using spectrophotometer at 660 nm (Olsen and Sommers, 1982).

The growth curve of each isolate was calculated as follow: Each isolate was grown on 25 ml PVK broth medium in 100 Erlenmeyer flasks and incubated under continuous shaking (150 rpm) for 48 h at 30ºC. The cells of each isolate were periodically harvested by centrifugation at 4ºC from broth culture medium and washed twice with sterile water. The cells yield was determined periodically by using spectrophotometer at 600 nm optical density (Bauch and Lamb Spectronic 21). An aliquot of cellular pellet was resuspended in 0.1 N NaCl and employed for the total protein. The protein content was assayed according to the method of Bradford (1976).

The cultures fluid were treated with 6% (V/V) H2O2 and sterilized at 121ºC for 20 min to decompose the exopolysaccharides (EPS) and release the ions adsorbed by the polysaccharides. Thereafter centrifuged at 10000 rpm for 20 min (Gancel and Novel, 1994).

The total sugars content of EPS was determined by the modified phenol sulfuric acid method (Drapron and Guibot, 1962) using glucose as a standard.

Cultures viscosity was measured with a Brookfield Viscometer model (Hbovll, USA) with spindle 4 at 50 rpm/28ºC.

Determination of organic acids : The organic acids in cultures filtrate were determined by high performance liquid chromatography (HPLC) with ODS column (200 mm, 4.6 mm, 50 mm). The operating conditions consisted of 0.1% H2PO4 as the mobile phase, detector VWD (210 nm) and a constant flow rate of 1.0 ml min, the pH was adjusted to 2 by phosphoric acid and 50 µl of organic acids extract was injected. The organic acids were quantitively determined by comparing the retention times and peak areas of chromatograms standards.

3. Results and Discussion

Data in Table (1) show the soil textures of the tested soil, sandy, sandy loam and clay soil with pH levels ranged from 6.5 to 8.5. Soil salinity showed considerable variations among tested soils. (Lab. Of soil analysis, Agric. Res. Center, Giza).

Characteritsitcsof B. megatherium isolates in vitro :

Three types of colony morphology were observed after 24 hr of growth in nutrient agar, circular colonies <1 mm with entire margin and dry another flatter colonies > 1 mm, irregular edge with gum. Third type convex colonies < 2 mm with entire margin and wet. Three isolates namely BM1, BM2 and BM3.

Similar data was observed by Groudeva and Groudeva (1987), Girgis et al. (2008) and Amer (2008) whom found the varient Bradyrhizobium haponicum and Bacillus spp. Isolates produced punctiform and translucent colonies and markedly distinct from the colony morphology and the other varient showed abundant growth and acid production.

Data present in Table (2) show the differences in pH of media supplemented with insoluble tri-calcium phosphate inoculated with three isolates. For instance the level of pH 4.25, 3.91 and 5.75 in culture media supplemented with tri-calcium phosphate of BM1, BM2 and BM3 isolates respectively. Compared with medium uninoculated medium pH 7.0. The total acidity percentage were increasing due to decrease pH. This effect was observed in culture media supplemented with tri-calcium phosphate of BM2 isolate indicating that, solubilization was higher than BM1 and BM3 isolates that solubilization was higher than BM1 and BM3 isolates. The total acidity percentage recorded 4.75; 5.25 and 3.45 in culture media inoculated with BM1, BM2 and BM3 isolates respectively compared with control 0.30%, The amount of insoluble phosphate were variation among three isolates whereas BM2 isolate gave the higher amount 525.75 followed by BM1, 270.50 and BM3 145.25 mg1-1 compared with uninoculated medium 75.25.

Table (1): Physico-chemical analysis of soils testing.

Soil Typed	EC	Mechanical analysis				Chemical analysis									
		Sand %	Clay %	Silt %	CaCO5	Organic carbon	Total nitrogen	C/N	SL	SU	Ca	Mg	Na	K	PH
Sandy	0.95	42.75	22.12	14.25	3.33	1.9	0.19	5.72	7.0	1.5	2.5	1.2	8.2	0.75	8.21
Sandy loam	1.25	32.85	18.25	5.41	25.15	1.9	0.16	2.01	25.0	9.4	21.7	1.4	70.25	1.5	7.90
Clay soil	0.92	57.25	51.31	2.50	10.27	0.9	0.17	3.25	9.0	2.5	4.3	2.0	9.10	0.9	8.4

The cell densities of B. megatherium isolates were markedly increased in culture medium of BM2 (2.75 x 108); followed by BM3 (1.45 x 108) and BM1 (0.95 x 108) cell ml-1. The high density of biomass was obtained with BM2 isolate. This increase might to attributed the utilization of phosphate by the organism which is followed by effective metabolic activity on the substrate. However, it can be deduced that exopolysaccharide (EPS) may play an important role in the degradation of the tri-calcium phosphate mineral and these results were agreement with the finding by Welch and Ullman (1999), Liu et al. (2006) and Girgis et al. (2008).

Biomass, exopolysaccharide and organic acids: It was found that, the EPS production is common in all tested isolates but showed the variable degrees of metabolic effectiveness between isolates in EPS production, viscosity and biomass density. The variation in the amount of EPS production among 3 isolates were recorded in Table 2. Isolate BM2 grown in culture media supplemented with tri-calcium phosphate produced the highest amount of EPS 154.33 followed by BM1 (95.75) and BM1, 73.25 mg L-1 compared to control (uninoculated) 75.25 mg L-1. The superiority of BM2 isolate could be expected as they produced higher viscosity 24.4 while BM1 and BM3 produced only 12.5 and 14.7 mPa.s, respectively. Therfore, the viscosity of the culture media was related to the quantity of EPS for all tested isolates. The increase of total acidity with high production of EPS may explain that highest concentration of phosphate was detected in insoluble phosphate culture media. These results illustrate the effect of EPS in the degradation of insoluble soil minerals.

Table (2): Variation of three B. megatherium isolates grown on culture media supplemented with tri-calcium phosphate.

Bacterial isolates	PH	Phosphate insoluble (mg L-1)	Total acidity (%)	EPS (mg L–1)	Viscosity (mPa-s)	Cell density (X108 cell mL-1)
Control	7.0	75.25	0.30	75.25	-	-
BM1	4.25	270.50	4.75	73.25	12.5	0.95
BM2	3.91	525.75	5.25	154.32	24.2	2.75
BM3	5.75	145.25	3.45	95.75	14.7	1.45

Control = media uninoculated Eps = Exopolysaccharide

Growth pattern of B. megatherium isolates: The growth rates of isolates were measured by increases in protein content (µg/ml). It is revealed that isolates recorded the highest and lowest rates (Fig. 1) and hence the longest doubling times (Table 3). However, the isolates were shown the amount of protein produced ml-1 medium after 48 h of incubation. According to the growth in PVK medium as recorded in Table (3) the isolates could be classified into three patterns 1- good growth, the maximum amount of protein after 48 hr from incubation was 35.25 for BM3 isolate 2-intermediate growth 33.70 for BM2 isolate and 3-little growth 29.75 µg ml-1 for BM1 isolate.

Results presented in Table (3) and Fig. 1 show the variations in the organic acids quantity produced by isolates in the broth culture media containing tri-calcium phosphate. High copious amounts of organic acids i.e. citric, fumaric, oxalic and tartaric acid accompanied with solubilization of tri-calcium phosphate in culture medium were produced by isolates as well as some unknown organic acids with smaller concentrations were also detected. A high concentration of tartaric acid was in culture medium of PM2 (1875.25 µl-1) accompanied by high amount of EPS (154.32 mg L-1) and high amount of soluble phosphate (525.75 mg L-1). However, this could be explained that the EPS strongly absorb the organic acids under the effects of organic acids the minerals are partially degraded (Table 2).

Table (3): Production of organic acid by three B. megatherium isolates.

Bacteria isolates	Organic acid (ng/ml)				Growth characters			
	Citric	Formic	Oxalic	Tartatic	(1)	(12)	(3)	(4)
BM1	150.75	325.15	275.10	375.75	0.7	1.7	29.75	255.25
BM2	185.21	75.21	35.15	1875.25	0.8	1.45	33.70	282.75
BM3	420.21	150.75	45.15	265.12	0.8	1.65	35.25	305.15

1. Specific growth rate (µ). 2. Doubling time (day)
3. Growth (µg protein/ml). 4. Viability (µ mol INTF/ml)

Recently, Lin et al. (2006) and Girgis et al.(2008) proved that the polysaccharides strongly absorbed the organic acids and were attached to the surface of the mineral, resulting in an area of high concentration of organic acids near the mineral. They stated that the polysaccharides also adsorbed S1O2, which affected the equilibrium between the mineral and fluid phases. In general way, variations in organic acids quality and quantity were shown between the isolates grown in their culture media. The isolates grown in media containing insoluble phosphorus showed variations in the production of organic acids. Perhaps this is an indication that, the solubilizing ability may have a relationship with the type of organic acids produced by the isolates rather than the quantity of acid.

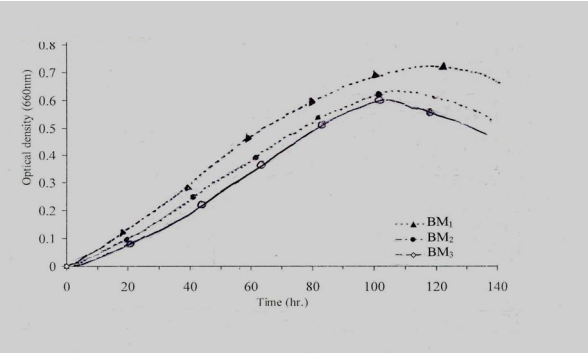

Fig.(1). Growth curves of 3 B. megatherium isolates under stirred conditions in PVK medium.

Several mechanisms have been proposed to explain the phosphate solubilization by microorganisms they are associated with the release of organic and inorganic acids (Richardson, 2001). In addition, the release of phosphatase enzyme that mineralize organic phosphate compounds has also been suggested as another mechanism involved (Marschner, 1997). Since microbial produced organic ligands include metabolic bioproducts, extracellular enzymes, chelates and both simple and complex organic acids. These substances can influence phosphate dissolution rates either by decreasing pH, forming frame work-destabilizing surface complexes or by complexing metals in solution.

The decrease in the pH values with the increase of total acidity in culture media may explain whey higher concentration of phosphate released was detected. Furthermore, linkage was observed between the final pH and the total acidity of the culture media and the amount of soluble phosphate. In this issue, Groudeva and Groudeva (1987) noted that the bacterial action on silicate and aluminosilicate is connected with the formation of mucilaginous capsules consisting of EPS as well as the production of different metabolites such as organic and amino acids. They did not exclude that the bacterial action may also be resulting of an enzymatic nature and that the bacteria are able to utilize energy released from aluminosilicate biodegradation.

4. References

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Phenotypic and Genotypic Variability among Three *Bacillus megatherium* Isolates. 2- Molecular detection of Orthocide Fungicide Biodegradation

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Abstract: Three B. megatherium isolates (BM1, BM2 and BM3) showed a great ability to degrade the fungicidal orthocide 75 (95% Captan) in vitro and in vivo. They differed in their ability to orthocide degradation in vitro with 14, 10.8 and 9.0% for BM1, BM2 and BM3 isolates respectively in the course 48 hr incubation. In addition they different in their ability to orthocide degradation in soil in the course 30 days earlier the fungicide treatment. Residual fungicide determined by GLC after 10, 20 and 30 days. It is clear that B. megatherium isolate B1 was the most active isolate degrading fungicide. The percentage of orthocide residual were 16, 20, 35 (non-sterilized soil) 43, 46 and 47% (sterilized soil) at 30 days for BM1, BM2and BM3 isolates respectively. On the other hand, the effect of orthocide on B. megatherium growth was little (non-observed). B. megatherium isolates were varied in dehalogenase activity the data showed the level of enzyme activity was found to be considerably higher in BM1 isolate followed by BM2 and BM3. Dehalogenase gene was successfully detected in total DNA genome of three isolates by polymerase chain reaction (PCR), where as showed differences among isolates related to number, density and size of isozymes (bands). In addition base pairs nucleotides (gene) were one band (478 bp) BM1, 3 bands (1059, 560, 478 bp) BM2 and 3 bands (1300, 800, 478 b[) BM3, isolates relative mobility and density. The similarity of dehalogenase activity 20% among three B. megatherium isolates. [Journal of American Science 2010;6(8):116-121]. (ISSN: 1545-1003).

Key words: Fungicide biodegradation, Dehalogenase PCR; B. megatherium, GLC.

1. Introduction

The biodegradational behaviour of pesticides on and in various edible plants was studied by many authors. However, this type of study showed be carried out under the local conditions of every country. They concluded that, the current levels of the pesticides in surface water and plants at a not constitute on acute toxicity hazard to man on a short-term basis, but as a result of its accumulation in man body it causes hazard of large term exposure. On the other hand, some researches were published about the methods of removing pesticides from soil and water by activated carbon (Hegazi et al, 1990), biodegradation by microorganisms (Yakato et al, 1987, Dahrog et al., 2006, Amer, 2008 and Azhar, 2009).

So far haloalkane dehalogenases are the only enzymes known to be capable of direct hydrolytic dehalogenation of chlorinated and brominated hydrocarbons without the requirement for enzymes or oxygen. The enzyme of Xanthomonas autorophicus GIIO's constitutively expressed 2 to 3%, of the soluble cellular protein (Keuning et al, 1985) up to 30% (Janssen et al, 1989). It has a remarkably broad substrate range which includes terminally halogenated alkanes with chain lengths up to 4 carbons for chlorinated and up to at least to carbons for brominated alkans. Other haloalkane dehalogenases of bread substrate range have been found in gram positive haloalkane, utilizing bacteria (Janssen, et al, 1989).

2. Material and Methods

Microbial cultures:

Three B. megatherium isolates (namely BM1, BM2, BM3) from different soils were grown on nutrient broth medium. These isolates were obtained from microbiology Lab. Botany Dept., Fac. Sci., Benha Univ. (Nahid, 2009).

Fungicide:

Orthocide 75 (95% captan): N-Trichloromethyl-mercapto-4-Cyclohexan- 1,2-dicarboximide, supplied by Kumia Chemical Industries Co. Ltd., Japan. Emulsifiable of the active ingredient 40% concentration of this orthocide was used in this investigation.

Detection of orthocide degradation:

In Vitro: Orthocide clearing zone: Assay medium contained (g/L): 6.0 NaCl, 1.0 (NH4)SO4; 0.5 KH2PO4; 0.1 CaCl2, 1.0 yeast extract and d H2O

up to one liter according to Vincent (1970) and supplemented with 0.5% (V/V) of emulsified orthocide (50%). This assay was carried out to determine the most potent degradation on the basis of mean diameters of clean zones (mm) by the testing B. megatherium isolates. After 24 hr petri-dishes were inspected to estimate the efficacy of orthocide degradation by millimeter ruler.

In vivo: Potted experimentally :

The soil taken from the surface layer (20 cm depth) of clay loam soil. Fourty pots (250 ml capacity) were filted with soil (about 200 pot-1). Twenty pots were sterilized and an other twenty pots were left without sterilization. The soil moisture content in all pots was raised up to 60% of WHC at 15 ibs 21 hr during 2 days. All pots were supplemented with 5000 ppm of orthocide and 100 ml broth media and mixed. Twenty ml of each broth medium contained ~ 5x108 cfu of three B. megatherium isolates used for inoculation of the soil and soil without inoculation as control. Soil samples were taken periodically after, 10, 20 and 30 days for the determination of residual orthocide using Gas liquid chromoatography (GLC) according to Vogeler (1968). As well as counted B. megatherium in sterilized and non-sterilized soil using plate count method. The soil samples were kept in freezer until analysis to determine the residual amount of orthocide.

Determination of dihalogenase activity and isozyme using polymerase chain reaction (PCR):

Enzyme activity: Cell cultures were harvested at the end of the experimental growth phase. The protein was extract from cell cultures as described by Bradford (1976). The enzyme activity was assayed according to Janssen et al (1987).

Isolation of genomic DNA:

Bacterial cells were cultured in broth medium and incubated at 28ºC with shaking at 200 rpm for 24 hr. and harvested by centrifugation. The DNA was extracted using CTAB method as described by Owen and Borman (1987).

Amplification of DNA:

The DNA was amplified by polymetase chain reaction (PCR) in 100 µl react mixture containing: 20 µl template DNA (25 mg), 0.2 µl Taq DNA polymerase (unit); 3.0 µl DNTP, (25 mM of each dATP, dCTP, dTTP, dGTP), 3.0 µl MgCl2 (25 mM); 30 µl PCR buffer (10X); 20 µl specific primer (dehalogenase encoding gen) Table (1) and 23.8 µl dH2O. The mixture was assembled on ice overlaid with 2 drop of mineral oil. The amplification was carried out in DNA thermal cycler (MWG-Biko-TECH Primuse) programmed as follows: One cycle at 94ºC for 2 min and then 30 cycles at 94ºC for 30 sec., 55ºC for 30 sec. and 72ºC for 20 sec. On cycle at 72ºC for 5 min then store in 4ºC final mix of PCR.

Table (1): Oligonucleotide sequences specific primers.

Primer sets	5` Sequence 3`
TF	tgggcggatttttggggct
TR	gtacgaaatggccagcgtcc

Gel electrophoresis analysis: Agarose gel (1.5%) was prepared in TAE buffer. Total sample, 10 µl miniprep; 2 µl 6x loading dye and 6 µl dH20) of each isolate was loaded in each well. The electrophoresis was done of 65 V for 1 hr and then stained with ethidium promide solution (10 mg/ml) for about 10-15 min. DNA amplified was visualized on UV transilluminator and photographed.

Determination of orthocide residues:

1-Extraction and clean up:

One gram of each treated soil and untreated soil for each isolates were transferred into 250 ml separating funnel and added 60 ml at 15% methylene chloride/hexane (V/V). The funnel was stoppered and shaken vigorously for 2 min and vented the pressure during shaking. The two layers were separated. The aqueous layer was drawn off into separating funnel. This procedure was repeated using 30 ml of 15% methylene chloride/hexane (V/V). The aqueous layer was discarded and the solvent extracts in both separating funnels were combined and poured on top of anhydrous sodium sulphate column. Before the solution recedes the top of sodium sulphate layer, three 10 ml rinses of 15% methylene chloride/hexane were added. The filtrates were collected for clearn up by using the US.EPA, 1988 procedures.

For clean up on a florisil column chromatography (300 mm long x 25 mm) internal diameter (i.d) with a small glass plug in the bottom was prepared by adding an activated florisil (130ºC/overnight) in small portions, while tabbing until about 10 cm high. About one and a half cm layer at anhydrous sodium sulphate was added to the top carefully without mixing with florisil.

Mature solvents of 6% eiethyl ether-petrolum ether was added then 10 ml of 15% diethyl ether/petroleum ether and 50% diethyl/petroleum ether were used for elution.

The filtered elution mixture was evaporated using rotary evaporator 40ºC for gas chromatographic determination.

2. Determination:
Separating of the residues was done on a borosilicate glass column 2 meters long, 3 mm internal diameter containing 3% oV-17 (phenyl methyl silicone) on chromosorb W, H.b. 100/120 mesh. The operating conditions were column oven temperature (150ºC) carrier gas (Nitrogen) (1.2 kg/cm), Burner gas (Hydrogen) (1.0 kg/cm), and air (1.0 kg/cm). Phillips PU4410, computerized (Gas Chromatography with FPD was used.

Soil free from Linuron was used to estimate rate of recovery by using the previous producers. The mean of the obtained recovery was 88%. All the obtained data for the residues of Linuron on treated soil were corrected by using such rate of recovery.

A series of concentration 10, 20, 30, 40, 50, 60, 70 mg of A falon analytical standard to 10 and toluene were prepared for obtaining the standard curve (Fig. 1). A suitable aliquot (5 µl) was injected from each concentration.

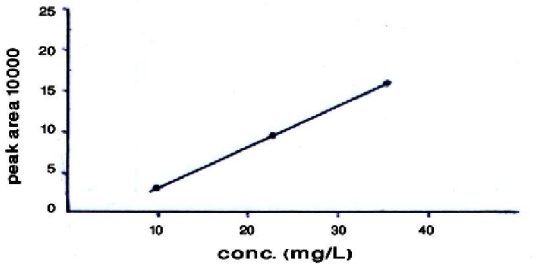

Fig. (1). Standard calibration curve orthocide.

3. Results
Bacillus megatherium isolates under study showed a great ability to degrade fungicide orthocide. They differed in their ability to orthocide degradation in vitro. Data concerning the amount of orthocide degradation with 0.70; 0.54 and 0.44 gm with 14, 10.8, 0.9% for B1, B2 and B3 isolates respectively (Table, 2).

Table (2).The potent ability of B. megatherium isolates to orthocide degradation *in vitro*.

B. megatherium isolates	Diameter clearing zone (cm)	Amount of orthocide degradation	Percent of degradation (%)
BM1	3.5	0.70	14
BM2	2.7	0.54	10.8
BM3	2.2	0.44	9.0

Orthocide amount in plate 5%.

The biodegradation of orthocide in vitro in sterilized and non-sterilized soil inoculated with B. megatherium isolates (BM1, BM2, BM3) showed that the amount of orthocide was decreased gradually through 30 days in sterilized and non sterilized soils (Table 3). The residual concentration of orthocide were 35, 40 and 47 ppm in sterilized soil for inoculated, B1, B2 and B3 respectively (Table 3). While the orthocide concentration was completely disappeared in non-sterilized inoculated soils were 16, 20 and 35 ppm after 30 days post inoculation (Table 3).

On the other hand, the total count of B. megatherium isolates were decreased in the first days then increased gradually under sterilized and non-sterilized soils (Table 3). The number of B. megatherium isolates were 5x107, 4x107 and 3x107 cfu at 30 day post inoculation with BM1, BM2 and BM3 respectively.

Table (3): Biodegradation of orthocide fungicide in soil inoculated with B. megatherium isolates.

Isolates	Non-sterilized soil			Sterilized soil		
	Orthocide (ppm)	% of orthocide residue	Total count	Orthocide (ppm)	% of orthocide residue	Total count
BM1 10th	4250	89	1 x 106	4525	91	2 x 106
20th	2750	55	2 x 106	3375	68	3 x 106
30th	775	16	5 x 107	1750	35	5 x 108
BM1 10th	4325	88	2 x 106	4500	90	2 x 106
20th	2975	60	3 x 107	3605	82	3 x 107
30th	1050	20	4 x 107	2000	40	3 x 108
BM1 10th	4350	87	1 x 106	4625	93	2 x 106
20th	3.50	61	2 x 107	3627	60	4 x 107
30th	1520	35	3 x 107	2375	47	4 x 108

* After one hour from addition.
** % of orthocide residual related to control (without inoculated).30 days post inoculation.

Dehalogenase enzyme analysis:

Dehalogenase activity was varied among three B. megatherium isolates. Theresults in Table (4) reveal the enzyme activity of three isolates. It was found higher in isolate BM1 240 followed by BM2 180 and BM2, 120 sec. (Fig. 2).

Table (4). Rate of dehalogenase activity among B. megatherium isolates.

Isolates	Dehalogenase activity		
	BM1 isolate	BM2 isolate	BM3 isolate
0 time	1.25	1.05	0.70
60 sec.	1.75	1.50	1.10
120 sec.	2.20	1.95	1.50
180 sec.	2.65	2.10	1.50
240 sec.	3.25	2.10	1.50
300 sec.	2.25	2.10	1.50
360 sec.	2.25	2.10	1.50

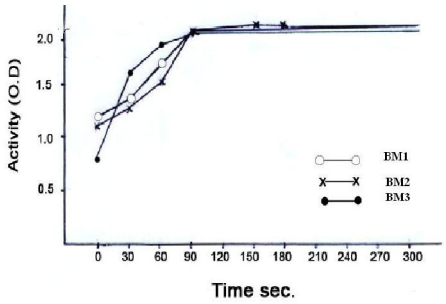

Fig. (2). Showing the rate of dehalogenase activity among three B. megatherium isolates.

Dehalogenase isozymes :

The quantity of DNA purified extracted from 3 B. megatherium isolates were conformed by UV spectrophotometer. The DNA concentration were about 30 µlg/0.5 g cells. The purity of DNA was measured on 260/280 absorbance 1:65, 1:75 and 1:50 for 3 isolates respectively, these results indicating high yield and DNA purity:

The dehalogenase gene of 3 isolates were amplified of DNA, of 3 isolates using specific primers, taq polymerase and PCR reaction mixture using one set PCR method.

PCR amplification of dehalogenase gene appeared variation among 3 B. megatherium isolates, i.e. number, density and size of PCR products. The results in Table 5 and Fig. 3 showed total 7 bands of dehalogenase isozymes in all isolates as well as one bands of dhl clone (505 bp) of dehalogenase. B. megatherium isolate B1 has one isozyme band with 478 bp and both isolates BM2, BM3 has three isozyme bands with (1059; 560; 478) and (1300, 800 and 478 bp). On the other hand, these isozyme bands were differed in density (percent of fraction). The variability among 3 isolates, one monomorphic (common) amplified band (590 bp with 14% percentage and two polymorphic (specific) amplified bands (1540 and 725 bp) with 29% percentage. The different among three B. megatherium isolate related to genetic variability.

Table (5).Genetic variability (DNA markers) of dehalogenase gene among, three B. megatherium isolates using PCR.

Rf	Dhl %	Clone MW	B. megatherium isolates						Polymor-phism
			BM1		BM2		BM3		
			%	MW	%	MW	%	MW	
0.28	-	-	-	-	-	-	54.7	1300	Unique
0.32	-	-	-	-	15.5	1059	-	-	Unique
0.34	-	-	-	-	-	-	18.3	800	Unique
0.39	100	505	-	-	78.7	560	-	-	Unique
0.42	-	-	100	478	5.8	478	27.0	478	Mono-morphic

Rf : Relative mobility. % : Percent of amplified band (DNA fraction)
MW: Size of expect band (bp). Unique : Genetic marker
Monomorphic or common amplified band.

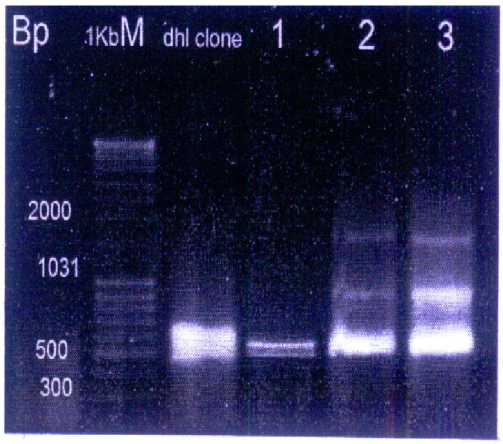

Fig. 3. Agarose gel electrophoresis stained with ethidium bromide showing the PCR products of amplified dehalogenase gene of three isolates using specific primers, Lane M: DNA molecular weight marker, Lanes gene clone, BM1, BM2 and BM3 B. megatherium isolates.

4. Discussion

In the recent years, great quantities of fungicides are consumed annually for the control of plant pathogenic fungi during our chemical warfare against a multitude of noxious organisms in the soil. It is necessary to avoid the injury of these fungicides together with their various carriers diluents and solvents on the various beneficial soil microorganisms and their biological activities contributing to soil fertility.

B. megatherium isolates showed great ability to degrade orthocide fungicide in vitro and in vivo which assimilation as a source of carbon. The analysis of orthocide residual by GLC at 10, 20 and 30 days post-inoculation showed the decrease gradually of orthocide at 20 days in sterilized and non-sterilized soils.

The results indicate that, the biodegradation of orthocide residues in inoculated sterilzized soil was high rate (16, 20, 25) followed by non-sterilized soil (43, 46, 47%) with B1, B2, B3 isolates respectively. This difference due to microbial flora in soil may be antagonistic with B. megatherium as well as it had the role in biodegradation of orthocide. In addition, this may be attributed to the concentration of soil (from drift occurrence of this fungicide.

Biodegradation of orthocide was detected in vitro with bacterial isolates but in different values (0.70, 0.54 and 0.44 ppm) of B1, B2 and B3 isolates respectively.

This obtained previous results are in agreement with those obtained by Hashish et al. (1990); Dahrog, et al. (2006), Amer, (2008) and Azhar (2009) studied the ability Pseudomonas, Bacillus, Rhizobium and Streptomyces to degrade the Linuron, Diuron, Ktozin and dichlofunanid. The actual degrade the fungicides and herbicides by microorganisms is caused by the release of enzymes that breakdown of them. The role of fungicide, herbicide and pesticide on bacterial population in treated soil was intensively studied the reported by Berger (1998), bacteria capable of significant biotransformation and reduced phenylurea concentrations in liquid culture.

Shin and Cheney (1989) and Amer, (2008), conduced a trial to determine the effect of Linuron, Simazine, alcohol and nonselective parquet on Bradyrhizobium japonicum and Rh. Leguminosarum bacteria. The alcohol and Linuron were decreased significantly by 27.4 and 57.8% respectively, while little effect was observed in simazine and marked reduction of survivals observed in 200 ppm of parquet.

The degradation of orthocide by B. megatherium isolates proceeds through the concerted enzyme actions that are specific for halogenated compound and enzymes that are involved in the metabolism of natural compounds. The former are the dehalogenase that catalyze hydrolysis of orthocide. The enzymes show a broad substrate range and one only produced by isolates that utilize halogenated substrates and thus can be considered enzymes that are acquired by this specific strain of B. metherum during genetic adaptation to degradation of chlorinated hydrocarbons. Thispaper describe present a further are lysis of the data alkane dehalogenase encoding gene dhlA. The absence of dehalogenase activities in natural of B. megatherijm allowed the identification of clones containing the dehalogenase gene. Isolation of genes involved in methanol-dehalogenase and chloroacetaldhyde dehydrogenase activity was possible by screening for complementation of mutants lacking the dehalogenase activities. In this way; harboring genes were identified and the genes were localized on different DNA segments.

The efficient expression of the halkinase dehalogenase gene in other gram-positive bacteria in not surprising in view of the fact that two regions with the consensus E. coli promoter sequence were present. Copy number probably also plays a role, since expression levels were higher in Xanthobacter autotropicus G10 (p 120) than in the wild type

isolates G110. The E. coli consensus promoter sequence is known to stimulate transcription in B. megatherium, (Dpyle et al, 1984 and Jeenes, et al. 1986) and our data suggest that it might also do so in Pseudomonas sp. In order to determine which of these sequences is the actual cause of the higher expression and whether the promoter can be used for expression of others genes in Pseudomonas spp., it well be necessary to identify the transcription short site of the gene and to study expression of different genes linked to the promoter regions.

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Production, Immobilization and Anti-tumor Activity of L-Asparaginase of Bacillus sp R36

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Abstract L-asparaginase is one of the known drugs in the treatment of cancer, especially acute lymphoblastic leukemia. In recent years several bio-conjugation protocols have been developed to improve the pharmacokinetic and immunological properties of anti-leukemic enzyme, L-asparaginase. In this study, fifty two bacilli species were newly isolated in our laboratory and screened for their ability to produce extracellular and intracellular L-asparaginase enzyme. *Bacillus* sp R36 gave the highest intracellular enzyme production. Formation physiology of the enzyme revealed that optimum culture conditions were 9:1 of air::medium ratio, with 55 x 10^5 CFU/mL inoculums size. The optimum incubation period was found to be 24 hours under shaking growth conditions. The initial pH value 5.6 was favorable for the highest enzyme production. Addition of 1% lactose or 1% raffinose resulted in a doubled enzyme productivity (yielded 204% and 209%, respectively). The enzyme was efficiently immobilized by covalent binding with activated carbon. Immobilized L-asparaginase activity was 33.0 U/g carrier; with immobilization yield of 73.6%. Characterization of the enzyme was performed on native and immobilized forms. Optimum pH value was 7.0 for free and immobilized forms. Optimum reaction temperature was 50 °C for native enzyme, while it was 60 °C for the immobilized enzyme preparation. The immobilization process greatly enhanced the thermal stability of the enzyme. Native L-asparaginase enzyme exhibited thermal stability up to 50 °C, while immobilized form retained 100% of its activity up to 80 °C. Anti-tumor and antioxidant activities were investigated. The enzyme inhibited the growth of two human cell lines including hepatocellular carcinoma (Hep-G2) and colon carcinoma (HCT-116) with IC_{50} value of 112.19 µg/mL and 218.7 µg/mL, respectively. [Journal of American Science 2010;6(8):131-140]. (ISSN: 1545-1003).

Keywords: -asparaginase, *Bacillus*, immobilization, anti-tumor effect.

1. Introduction

L-asparaginases (L-asparagine amido-hydrolase EC 3.5.1.1) hydrolyze L-asparagine to L-asparate and ammonia (Fig. 1a). Bacterial L-asparaginases are enzymes of high therapeutic value due to their use in certain kinds of cancer therapies, mainly in acute lymphoblastic leukemia (ALL) [1-3]. Many bacteria contain two L-asparaginases, a high affinity periplasmic enzyme and a low affinity cytoplasmic enzyme. These enzymes in some bacteria accumulate mainly in periplasmic space [4, 5 and 6]. In *Escherichia coli* and many other bacteria, synthesis of cytoplasmic asparaginase I is constitutive, while expression of periplasmic asparaginase II is activated during anaerobiosis. Further, only the type II enzyme has shown substantial anti-tumor activity [7]. An ideal method for the release of this enzyme would be rapid, inexpensive, gentle and compatible with downstream steps of its purification [8].

The anti-leukemic effect of L-asparaginase is a result of rapid and complete depletion of the circulating pool of L-asparagine as in a great number of patients with lymphoblastic leukemia, the malignant cells depend on exogenous source of L-asparagine to be able to survive, mean while, the normal cells are able to synthesize L-asparagine [3, 9]. The discovery of new L-asparaginase serologically different but having similar therapeutic effects is highly desired [10]. One approach to achieve improved function and properties of the enzyme is its chemical modification (immobilization) with various kinds of biocompatible polymers. Immobilization of enzymes is one of the important trends and goals of biotechnology. Enzymes have an enormous potential as biocatalysts in a wide range of industries and medicine. They offer a distinct advantage due to their specificity, high catalytic efficiency at low temperature and being biodegradable. [11] The use of immobilized enzymes lowers production costs as these can be readily separated from reaction mixture and hence can be used repeatedly and continuously. Several different methods have been employed for enzyme immobilization which includes adsorption onto insoluble materials, entrapment in polymeric gels,

encapsulation in membranes, cross linking with bifunctional or multifunctional reagents and linking to an insoluble carrier [12].

Activated carbon is a form of carbon that has been processed to make it extremely porous and thus to have a very large surface area available for chemical reactions. One gram of activated carbon has a surface area of approximately 500 m^2 with 1500 m^2 being readily achievable. Powdered activated carbon is made in particular form less than 1.0mm in size with an average diameter between 0.15 and 0.25 mm. Activated carbon is used to treat poisonings and overdoses following oral ingestion. It is thought to bind to poison and prevent its adsorption by the gastrointestinal tract. The present work introduces the production (from *Bacillus* sp R36), the physiology, the characterization, and the anti-tumor activity of a new L-asparaginase enzyme (EC 3.5.1.1) in its native and immobilized forms.

2. Material and Methods

Chemicals

Anhydrous L-asparagine, trichloroacetic acid (TCA), Nessler reagent chemicals (HgI2, KI, and sodium hydroxide), Chitin and hexane were purchased from Sigma Chemicals Co. Activated carbon, celite, carboxymethyl cellulose; silica gel and tricalcium phosphate were from Merck Chemicals. All other chemicals used were of analytical grade.

Isolation of Bacteria

One Gram of soil was transferred to a vial containing 10 mL of sterile water and kept on a rotary shaker at 100 rpm for 30 min. The bacterial suspension was pasteurized by heating at 65˚C for 15 min. The supernatant was diluted 10-folds and 0.1 mL was spread on pre-solidified nutrient agar medium composed of 5 g peptone, 3 g beef extract and 15 g agar per liter of distilled water. The plates were incubated at 30˚C for 48 h and bacterial colonies were purified on nutrient agar. Each of the purified colonies was then sub-cultured on nutrient agar slants.

Culture conditions and membrane permeablization

Bacterial isolates were screened for L-asparaginase activity on Luria – Bertani (LB) medium containing (1-1) 10 g peptone, 5 g yeast extract and 10 g NaCl at pH 7.0. A 1/100 inoculum of overnight cultures grown in LB medium was made in 40 mL LB medium in 250 mL Erlenmeyer conical flasks and incubated for 24h at 30˚C on a 200 rpm orbital rotary shaker. Cells, cultivated for L-asparaginase production, were harvested by centrifugation (6000 xg for 15 min), washed once with 0.05 mole potassium phosphate buffer pH 8.6, and re-suspended to A 600 = 5.0 in the same buffer containing n-hexane at 1% (V/V) for L-asparaginase release. The suspensions were incubated at room temperature for 1h, and briefly vortex every 10 min [8].

L-asparaginase assay

The enzyme activity was assayed according to wriston [13]. The reaction mixture contained 0.1 mL permeabilized cells free broth and 0.9 mL of 0.01 mole L-asparagine prepared in 0.05 mole tris- HCl buffer, pH 8.6 and incubated for 30 min at 37˚C. The reaction mixture was centrifuged at 6000 xg for 10 min and the ammonia released in the supernatant was determined by Nesslerization reaction. In brief, to 0.5 mL of supernatant, 1.75 mL dist. H2O, 0.25 mL of Nessler reagent was added. After 10 min. absorbance at 480 nm were read with appropriate control. One enzyme unit (U) is defined as the amount of enzyme that librates 1 µmole of ammonia per min at 37˚C. Standard curve of ammonium sulphate was used for calculating ammonia concentrations.

Immobilization of L-asparaginase

Different supports were employed for L-asparaginase immobilization according to [14]. Experimentally, 200 mg of each support was shaked in 5 mL tris –HCl buffer (0.01 mole, pH 8.6) containing 2.5 % glutaraldehyde at room temperature for 2h. The carriers were filtered off and washed with distilled water to remove the excess of glutaraldehyde then each treated carrier was incubated with 5 mL of tris HCl buffer containing 1 mL of enzyme. After being shaken for 2h at 30˚C, the unbounded enzyme was removed by washing with distilled water until no protein or activity were detected in the wash.

Cell culture

Two human cell lines were used through this work including: hepatocarcinoma (HepG2) and Colon carcinoma (HCT-116), both lines purchased from ATCC, VA, USA. Hep-G2 Cells were routinely cultured in DMEM (Dulbeco's Modified Eagle's Medium), while HCT-116 cells were cultured in Mc Coy's medium. Media were supplemented with 10 % fetal bovine serum (FBS), 2 mmole L-glutamine, containing 100 units/mL penicillin G sodium, 100 units/ml streptomycin sulphate, and 250 ng/ml amphotericin B. Cells were maintained at sub-confluence at 37ºC in humidified air containing 5 % CO2. For sub-culturing, monolayer cells were harvested after trypsin / EDTA treatment at 37°C. Tested samples were dissolved in dimethyl sulphoxide (DMSO). All cell culture material was obtained from Cambrex Bioscience (Copenhagen, Denmark). All chemicals were from Sigma/Aldrich,

USA, except mentioned. All experiments were repeated three times, unless mentioned.

Anti-tumor activity

The cytotoxic effect of the samples against Hep-G2 and HCT-116 cells was estimated by the 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay according to Hansen et al., 1989 [15]. The yellow tetrazolium salt of MTT is reduced by mitochondrial dehydrogenases in metabolically active cells to form insoluble purple formazan crystals, which are solubilized by the addition of a detergent. Cells (5 x 104 cells / well) were incubated with various concentrations of the compound at 37 ºC for 48 h in a FBS-free medium, before submitted to MTT assay. The absorbance was measured with an ELISA reader (BioRad, München, Germany) at 570 nm. The relative cell viability was determined by the amount of MTT converted to the insoluble formazan salt. The data are expressed as the mean percentage of viable cells as compared to the respective control cultures treated with the solvent. The half maximal growth inhibitory concentration IC50 values were calculated from the linear equation of the dose–dependent curve of each sample.

Antioxidant activity (scavenging of DPPH)

The antioxidant capacity of the tested samples was studied through their scavenging activity against 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radicals [16]. DPPH is a stable deep violet radical due to its unpaired electron. In the presence of an antioxidant radical scavenger, which can donate an electron to DPPH, the deep violet color decolourize to the pale yellow non-radical. The bleaching of DPPH was monitored at absorbance of 515 nm. The percentage of DPPH bleaching utilized for SC50 (half maximal scavenging concentration) was calculated as follows: 0% is the absorbance of DPPH and 100 % is the absorbance of DPPH with an efficient scavenger (10 mM ascorbic acid, AA).

3. Results and Discussion

Screening of bacterial isolates

Fifty two bacilli species were tested for enzyme production. Extracellularly, no appreciable levels of L-asparaginase were detected. After membrane permeabilization with potassium phosphate / 1% hexane system [8], *Bacillus* sp R36 gave the highest enzyme productivity in the cell suspension (20.15 U/mL). The determination of the localization of any enzyme plays a vital role in the development of bioprocess. The existence of L-asparaginase in the membrane fraction of *Tetrahymena pyriformis* and periplasmic space of *Enterobacter aerogenes* and *Pseudomonas. aeruginosa* has been reported [8, 17]. There are many reports on the production of intracellular L-asparaginase from *E. coli* [18] *Vibrio succinogenes* [19], *Erwinia aroideae* [20], *S. marcescens*[21], *E. aerogenes* [22], and *P. aeruginosa* [10, 23]. Kumar et al [24] that recently carried out a sub-cellular localization of L-asparaginase enzyme using cell fractionation techniques in various organisms. They reported that there was no trace of extracellular activity observed in the culture filtrates, which inferred that the enzyme was secreted as an intracellular product in all microorganisms tested. The maximum L-asparaginase activity was found to be 14.56 U/mL with *Pectobacterium carotovorum* MTCC 1428.

Optimization of L-asparaginase production by Bacillus sp R 36

Some factors affecting L-asparaginase productivity were studied aiming at optimization the anti-leukemic enzyme activity. With respect to the effect of aeration level, 9:1 (25 mL LB medium per 250 mL conical flask) air to medium ratio gave the highest enzyme activity reaching 22.8 U/mL (Fig 1b). Incubation period played an important role in enzyme productivity as maximum enzyme activity was obtained from 20-24 h reaching (20.0 U/mL) after 24 h only of incubation on an orbital shaker at 150 rpm. At 48 h of incubation under the same conditions, the activity decreased to 42% (8.4 U/mL) (Fig 1c). The same incubation period was reported upon production of L-asparaginase by isolated Bacillus circulans [25] and Enterobacter aerogenes and Pseudomonas aeruginosa [8]. Inoculum size in terms of colony forming unit (CFU/mL) had a positively effect on enzyme activity up to 55 x 105 CFU/mL. Higher inoculum sizes were resulting in leveling off the activity (Fig 2a). Prakasham [26] abstracted that incubation temperature, inoculums level and medium pH among all fermentation factors were major influential parameters at their individual level, and contributed to more than 60% of total L-asparaginase production. The effect of initial pH of LB medium was studied. Results revealed that 5.6 initial pH value of production medium (without adjustment) was suitable for enzyme production more than other tested pH values (Fig 2b). In all cases the harvest pH value is highly alkaline ~ 8.9 due to ammonia production in the fermentation flask. All carbon sources that were tested for L-asparaginase production enhanced enzyme formation only upon adding to Luria– Bertani (LB) medium.

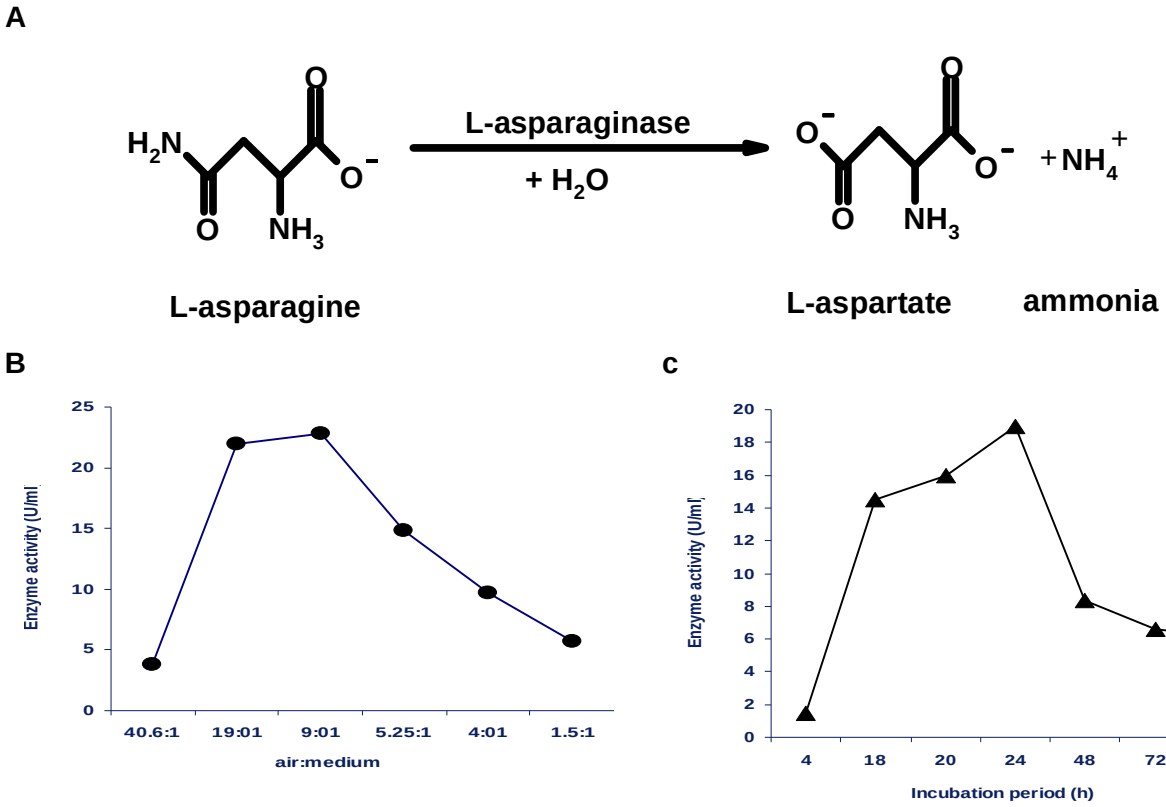


Fig (1): a. Role of L-asparaginases in hydrolysis of L-asparagine into L-asparate and ammonia. b. Effect of aeration level on L-asparaginase activity produced by Bacillus sp R36 on LB medium. All data are average value of triplicate measurements. c. Effect of incubation period on enzyme activity. The flasks were incubated on an orbital shaker at 150 rpm at the indicated times. All data are average value of triplicate measurements.

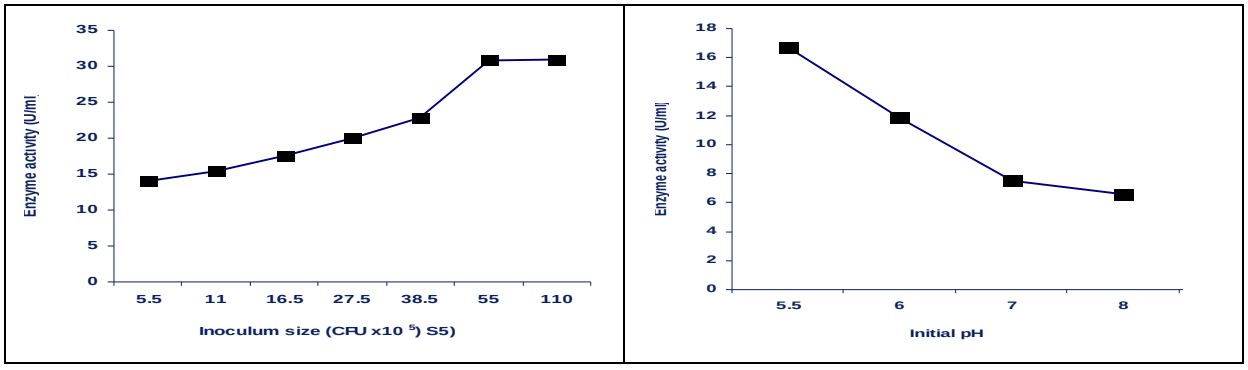


Fig (2): a. Effect of inoculum size on L-asparaginase activity. b. Effect of initial pH value of LB medium on L-asparaginase. All data are average value of triplicate measurements.

On mineral salt medium, Bacillus sp R36 neither could grow nor biosynthesize the enzyme. The obtained results revealed that addition of 1% lactose or 1% Raffinose to LB medium doubled the enzyme activity (204 % and 209 %, respectively). On the other hand, glucose was the most inhibitory carbon source compared with the control LB medium (Fig 3). The effect of carbon sources differ from organism to another, but in general, glucose was regarded as a repressor for L-asparaginase production in bacteria

[27]. Optimization of the culture conditions of L-asparaginase activity was the aim of many studies through which the enzyme productivity increased to many folds. Thus, Abdel –Fattand and olama [10] obtained more than five folds the activity in basal medium using Box-Behnken designing in solid state culture. Kumar et al [28] obtained an over all 8.3 fold in enzyme production compared to the un-optimized medium using the central composite experimental design. Hymavathi et al [29] improved L-asparaginase yield by more than 300% using fractional factorial central composite design (FFCCD).

Immobilization of L-asparaginase

Seven supports, pre-activated with glutaraldehyde, react with different degrees with the terminal amino residues of the enzyme protein. Immobilization of Bacillus sp R36 L-asparaginase by covalent binding was achieved by cross linking between the enzyme and different carriers (i.e. activated carbon, celite, chitin, carboxymethyl cellulose, egg shell, silica gel, tricalcium phosphate and chitosan) throughout glutaraldehyde. The amount of enzyme used for immobilization was 50 U/g carrier. The results (Table 1) indicated that the lowest immobilized activity and immobilization yield 7.9 U/g carrier and 17.8 % were detected with chitosan as a carrier. On the other hand, the highest immobilized activity (33.0 U/g carrier) and highest immobilization yield (73.6%) were obtained with activated carbon as a carrier. Medically activated carbon (activated charcoal) is a supplement used when accidental poisonings have occurred to absorb the poison and carry it out of the body. It is also used to treat high cholesten stomach and gas [30].

Our results are the first report for immobilization on activated carbon of L-asparaginase enzyme. In addition, immobilization of L-asparaginase R36 on activated carbon had a characteristic of simpler processing in comparison with that on silk sericin protein [31], polysaccharide levan [32] and agarose [33]. Kotzia et al [34] immobilizesd L-asparaginase of Erwinia chrysantheni 3937 on epoxy- activated Sepharose CL-6B. They reported that the immobilized enzyme retains most of its activity (60%) and shows high stability at 4°C. More recently, Tabandeh and Aminlari [35] investigated the effect of conjugation with oxidized inulin on the properties of L-Asparaginase (L- ASNase) in the form of Elspar. They found that modified L-asparaginase synthesized at ratio of 2: 1 had activity of 65% of that of native enzyme.

Characterization of L-asparaginase of Bacillus sp R36

a. Effect of pH on free and immobilized enzymes activities.

The effect of pH on L-asparaginase activity of free and immobilized preparations was studied by changing the pH value from 3.0 to 8.6 using a series of buffers namely citrate buffer (pH 3-5), phosphate buffer (pH 6-7), and Tris buffer (pH 8-8.6), (Fig. 4a). Both crude and immobilized L-asparaginase preparations exhibited maximum activity at pH 7. The immobilized form yielded more than 300 % increase in activity (333.5 %). This clearly reflects the suitability of activated carbon as a carrier for this enzyme. Our results are in agreement with that reported by Zhang et al [31] who immobilized E. coli L-asparaginase on micro particles of the natural silk sericin protein. They reported that the optimal range of pH value had no evident changes in comparison with native enzyme. Tabandeh and Aminlari [35] reported that the optimum pH of modified L- ASNase and the native enzyme is at alkaline pH (pH 8) probably due to produced L-aspartic acid acting as competitive inhibitor for enzyme in acidic condition.

b. Effect of Reaction temperature on free and immobilized L-asparaginase.

The effect of the reaction temperature on free and immobilized forms was investigated from 30 ˚C to 80 ˚C (Fig 4b). The optimum reaction temperature of free enzyme was 50 ˚C while the immobilized preparation reacted optimally at 60 ˚C reaction temperature i.e. at 10 ˚C higher than the native enzyme, with an increase of 355.8 % in enzyme activity. The same results were reported by [31], who stated that the optimum reaction temperature of immobilized enzyme was at 60 ˚C while that of free L-ASN ase was at 50 oC.

c. Thermal stability of immobilized enzyme

Residual activities after heating the enzyme at 30-90˚C for 10 min were measured (Fig 4c). An excellent thermal stability was exhibited by the immobilized enzyme preparation. The enzyme retained 100% of its L-asparaginase activity up to 80 ˚C, while 62% of it was retained upon heating for 10 min at 90˚C. The native form, maintained 100% of its activity at 50˚C while at 80˚C it retained 65% of its L-asparaginase activity. At 90 ˚C the native form maintained 57% of its activity. An improvement of thermal stability of immobilized enzyme may be acquired by multiple attachment of activated carbon to the enzyme molecule resulting in greater enzyme rigidity of enzyme conformation and increasing the activation energy for unfolding the enzyme. A marked improvement in thermal stability of modified enzyme was obtained by Tabandeh and Aminlari [35], Amiri et al [36] and Scaman et al [37]. In contrast, [31] reported that the thermo stability of the immobilized ASNase is very

similar to that of the native enzyme and there were no obvious changes in the activities.

Anti-tumor activity

Using MTT assay, the in vitro cytotoxicity effect of Bacillus sp R36 L-asparaginase enzyme on the growth of two tumor cell lines was studied. The IC50 values were calculated from the linear equation of the dose effect of the enzyme against hepatocellular carcinoma Hep G2 cells (y = -0.4069x + 95.648) and against colon carcinoma HCT-116 cells (y = -0.2233x + 98.838). The incubation of Hep G2 with gradual doses of Bacillus sp R36 L-asparaginase enzyme leads to a gradual inhibition in the cell growth as concluded from its low IC50 values 112.19 µg / mL (Fig. 5a). As shown in (Fig. 5b), the treatment of HCT- 116 with the enzyme resulted in a low anti-tumor activity with IC50 value of 218.7 µg / mL compared with the growth of untreated control cells. Cappelletti et al [38] studied in vitro cytotoxicity of a novel L-asparaginase from the pathogenic strain Helicobacter pylori CCUG 17874 against different cell lines. They reported that AGS and MKN 28 gastric epithelial cells being the most affected.

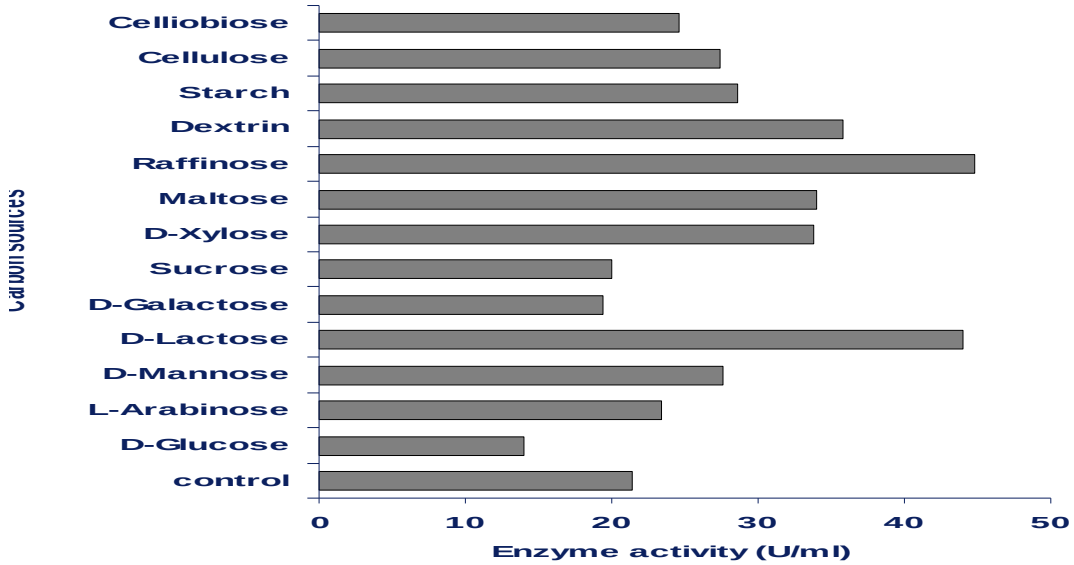


Fig (3): Effect of different carbon sources on L-asparaginase activity (final concentration 1%). All data are average value of triplicate measurements.

Table (1): Immobilization of *Bacillus* sp R 36 L-asparaginase by covalent binding with different carriers.

Carrier	Added enzyme (U/g) (A)	Unbounded enzyme (U/g) (B)	Immobilized enzyme (I)	Immobilizati-on yield I / (A-B)%
Activated carbon	**50**	**5.2**	**33.0**	**73.6**
Celite	**50**	**4.1**	**27.5**	**60.0**
Chitin	**50**	**3.5**	**14.3**	**30.7**
CMC	**50**	**11.5**	**19.8**	**51.4**
Egg Shell	**50**	**2.7**	**20.3**	**42.9**
SG	**50**	**4.8**	**28.1**	**62.1**
TCP	**50**	**7.2**	**8.6**	**20.1**
Chitosan	**50**	**5.5**	**7.9**	**17.8**

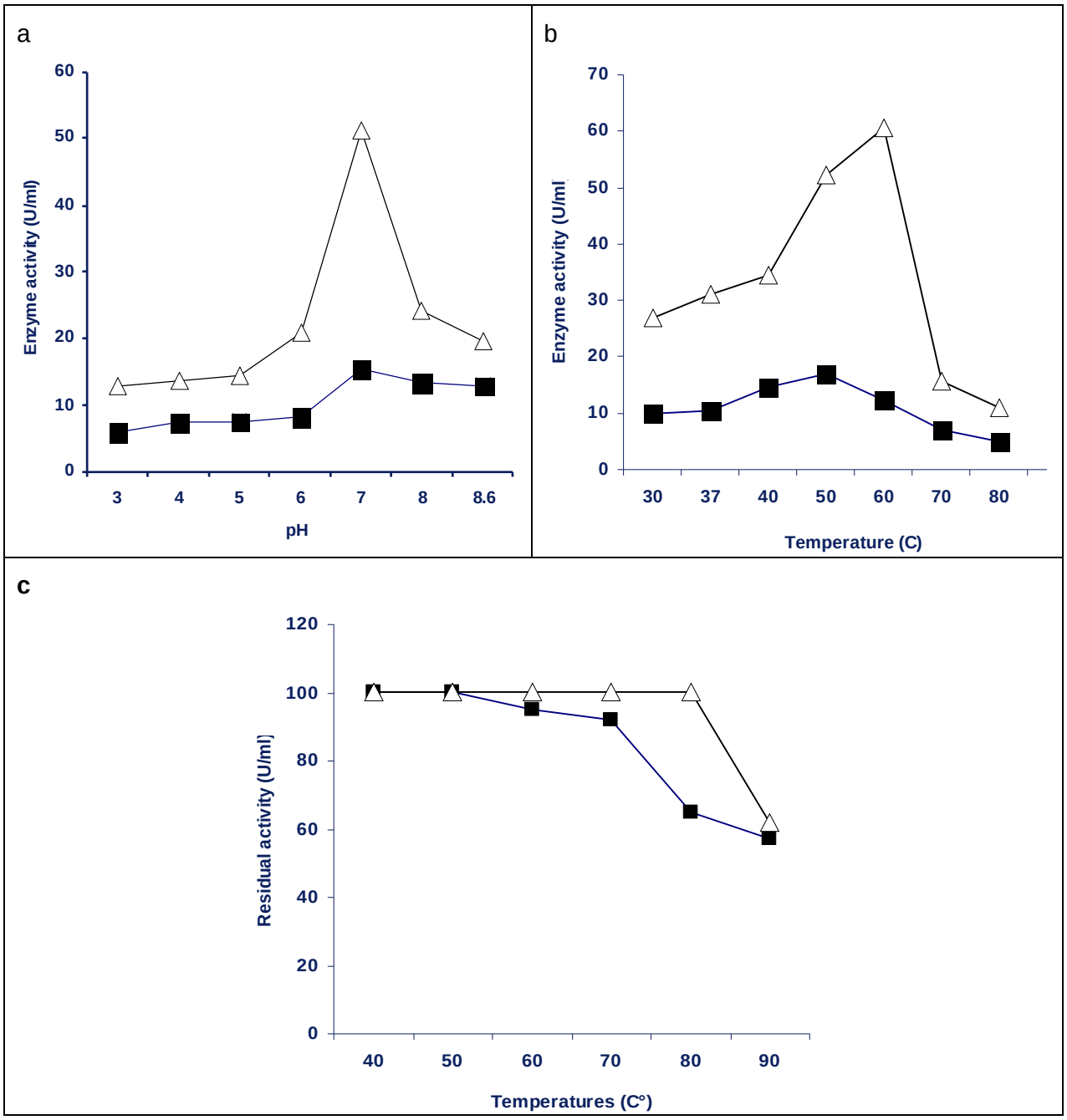
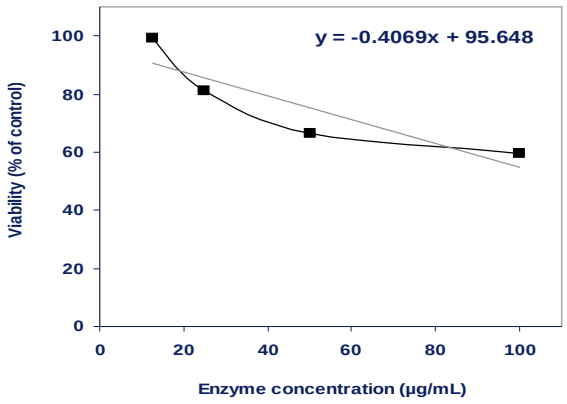


Fig (4): The effect of pH (a) and reaction temperature (b) on free (squared-line) and immobilized enzymes (triangled-line). A series of buffers was used: citrate buffer (pH 3 –

Antioxidant activity

The antioxidant capacity of the enzyme was investigated using DPPH assay. DPPH is a stable non-physiological radical, which could provide a relative figure of the radical scavenging capacity of a tested probe. The DPPH assay showed that Bacillus sp R36 possessed low scavenging activity with high SC50 values of 325.4 µg/mL compared to the scavenging activity of the well-known antioxidant (ascorbic acid, a.a., SC50 8.7 µg/mL).

b



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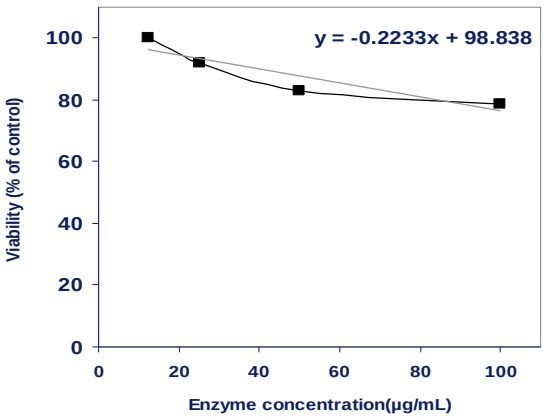

Fig (5): Anti- tumor activity against a. hepatocellular carcinoma cells (Hep-G2) and b. colon carcinoma cells (HCT-116). All data are average value of triplicate measurements.

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Growth, Yield and Seed Quality of *Lupinus termis* as Affected by Different Soil Moisture Levels and Different Ways of Yeast Application

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Abstract: Two pot experiments were carried out in the greenhouse of National Research Center. Dokki, Egypt during 2008 and 2009 seasons in order to investigate the effect of different soil moisture levels (75, 55 and 35% depletion of the available soil water) and yeast application (at rate of 8 g/L) by different ways (as a foliar, soil and foliar plus soil treatments) on growth, yield and seed quality of *Lupinus termis.* The results indicated that the highest growth parameters and RWC % observed when plants grown under the highest soil moisture level W3 and treated with yeast by foliar plus soil treatment followed by foliar treatment where the difference between the two treatments was insignificant. The number of seeds /plant, number of pods/plant and 100-seeds weight were decreased by increasing drought stress so as reached their maximum decease under the most stressed level W1, treated plants with yeast by different ways resulting in an increase in yield and yield attributes of lupinus plants. Also, both chla and chlb showed progressive increase by increasing soil moisture levels from W1 to W3, yeast application by different ways showed also progressive increase in chla and chlb. In addition, applied the highest level of soil moisture W3 combined with foliar application with yeast gave the highest significant values of nitrogen and protein percentages. Moreover, decreasing soil moisture levels caused significant increase in proline and alkaloids contents, treated plants with yeast by different ways induced significant decrease of proline and alkaloids content under different soil moisture levels. Furthermore, the highest records for carbohydrates percentage appeared in plants sprayed with yeast and grown under the highest soil moisture level W3. [Journal of American Science 2010;6(8):141-153]. (ISSN: 1545-1003).

Keywords: Growth; Yield and Seed Quality; *Lupinus termis;* Different Soil Moisture Levels; Yeast Application

1. Introduction

Lupinus termis is cultivated in a wide range of environments across Egypt. It's seed have a nutritional quality similar to soybean seed and superior to other legumes seed (Raza and Jrnsgard, 2005), and could be an important source of protein and oil. In fact, lupinus seeds have been used for human consumption and as a medicinal plant in Egypt (Kattab, 1986; ARC, 1994) and other countries for thousands of years.

The world is facing serious shortages of fresh water and growing competition for clear water, makes less water available for agriculture. The great challenge for the coming decades will be the task of increasing food production with less water, particularly in countries with limited water and land resources. Serious water shortages are developing in the arid and semi arid regions, as existing water resources are fully exploited (Jafar et al., 2007). To cope with the increasing food requirements and as drought is a major stress which adversely affects plant growth and productivity (Tawfik, 2008); it is important to develop stress tolerant crops (Mahajan and Tuteja, 2005). Improving tolerance of crop plats could be happened by different ways such using bio-stimulants which has been an important but largely unfulfilled aim of modern agricultural development (Gaballah and Gomaa, 2004). Bio-stimulants can be generally defined as some plant growth regulator or promoting substances such as vitamins, microelements, organic acids as well as preparations containing live cells (like bread yeast or Candida), which were microorganisms with the objective of increasing their number and of accelerating certain microbial process to increases the availability of nutrients elements in form which can be easily taken by plants. Thus greater attention has been directed on the use of microorganisms as bio-stimulants to provide nutrients for higher plants with out any pollution to the environment (Hayat, 2007). The objective of this work was mainly to investigate the response of growth, yield and seed quality of *lupinus termis* crop to different soil moisture levels and yeast application by different ways.

2. Material and Methods

An experiment was conducted during two winter seasons of 2008 and 2009 at the green house of the National Research Center, Dokki, Cairo, Egypt.

Treatments:
Water Treatments:

The following three water treatments were applied throughout the entire growth period of the crop:
W1= water stress maintained around 75% depletion of the available soil water and the soil water is maintained to field capacity when this depletion level is reached.
W2= water stress maintained around 55% depletion of the available soil water and the soil water is maintained to field capacity when this depletion level is reached.
W3= water level maintained around 35% depletion of the available soil water is maintained to field capacity when this depletion level is reached. The soil was sandy and it's physical and chemical analysis is shown in Table (1).

Yeast Application:

Yeast solution was prepared according to method described by Skoog and Miller (1957) at rate of 8g/L. The plants were treated with yeast in three different ways, the first was foliar application, and the second was soil application, while the third was foliar plus soil application. The plants were treated by these treatments two times during the crop life, the first application was after 45 days from planting and the second was two weeks later. The plants were sprayed until run off. The composition of yeast solution employed in the experiment was described in Table (2) as found by Nagodawithana (1991).

Planting and watering procedure:

Seeds of *Lupinus termis* (Var. Balady) provided from the Egyptian Agricultural Research Center and were thoroughly washed with distilled water and soaked for about six hours, then the seeds were directly planted in earthenware pots 40 diameter and 40 cm height with perforated bottoms, and were filed with 10 kg of sandy soil. Five seeds were planted in each pot and thinned down to two plants after emergency the number of plants per pot was determined on the basis of the area of the pot at a recommended seed rate m^2 (Soub, 1984). Each pot was maintained to water field capacity 19%, one day before starting the treatments, so that the soil moisture amount at each pot is uniform. All pots were weighted every 1 to 3 days on abeam balance. The pots were then irrigated to restore the soil to the appropriate moisture regime by adding a calculated amount of water. The general principal stated by Boutraa and Sanders (2001) was used for the water treatment application.

Design of the Experiments:

This experiment included 12 treatments which were the combination between three soil moisture levels (75, 55, 35% depletion of the available soil water) and four ways of yeast application (0, foliar, soil, foliar plus soil treatments), treatments were arranged in a split plot design with three replicates, different soil moisture levels were assigned at random in the main plots, while sub-plots were devoted to the different ways of yeast application.

Data Collection:

The following characters were either measured or computed on three lupinus plants:

Plant height (cm), number of leaves per plant, root length, fresh and dry weights of the whole plant, number of pods per plant, number of seeds per plant and 100 seeds weight were obtained. The photosynthetic pigments of fresh leaves, chlorophyll a and b were determined using for such purpose the 4th leaf from the growing point of the plant using the spectrophotometric method recommended by Metzener *et al.,* (1965). The relative water content percent was measured also on fresh leaves according to Weatherly (1962). Samples were collected and dried for 48 h at 70 °C to determine the total N in the dry tissue by the conventional micro kjeldabl method according to Bremner and Mulvaney (1982), then the protein (%) was also calculated using the equation of Alsmeyer *et al.*, (1974). Proline content was determined according to Troll (1995). Total soluble carbohydrates were determined according to Dubois et al., (1956). The total Alkaloids content of samples was assessed using a method described by Koriesh (1989).

Statistical analysis:

The collected data were subjected to statistical analysis of variance using the normal (F) test and the means separation were compared by using Least Significant Difference (LSD) at 5% level according to Snedecor and Cochran (1980).

3. Results and Discussion:

Growth Parameters:

Data presented in Tables 3 & 4 demonstrated the effect of different soil moisture levels and different ways of yeast application and their interaction on growth parameters of *Lupinus termis* plants, the data showed that increasing water stress from W3 to W1 caused significant decline in shoot length where the highest means for shoot length observed in plants grown under the highest water level W3 followed by the moderate water level W2 where the difference between to two levels was in significant, this result was true for both vegetative and flowering stages. Similar results were obtained by Mirsa and Srivastava (2000), Choi *et al.,* (2000), Ayodele (2001), Singh *et al.*, (2001) and villager and Cavagnaro (2006). Such decrease in shoot length in response to drought may either due to

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

Mechanical characteristics:	First season	Second season
Clay %	18.00	16.00
Sand %	24.75	26.25
Chemical Properties		
PH	(1:2.5) 7.25	7.9
E.C.	(1:5) 1.1dsm^{-1}	1.0
Available macro nutrients (ppm)		
Na	3.42	5.0
N	189.10	170
P	3.14	4.98
K	259.75	244.25
Ca	65.15	55.21
Mg	73.18	65.22
Available mirco nutrient (ppm)		
Fe	15.14	12.21
Mn	21.81	19.32
Zn	1.18	1.34
Cu	1.31	1.0
Cl	0.78	0.66

Table (2): The composition of active yeast.

Protein	47%		
Carbohydrates	33%		
Minerals	8%		
Nucleic acids	8%		
Lipids	4%		
The composition of minerals			
Na	0.12 mg/g	Cu	8.00 µ/g
Ca	0.75 mg/g	Se	0.10 µ/g
Fe	0.02 mg/g	Mn	0.02 µ/g
Mg	1.65 mg/g	Cr	2.20 µ/g
K	21.0 mg/g	Ni	3.00 µ/g
P	13.5 mg/g	Va	0.04 µ/g
S	13.5 mg/g	Mo	0.40 µ/g
Zn	0.17 mg/g	Sn	3.00 µ/g
Si	0.03 mg/g	Li	0.17 µ/g
The composition of Vitamins			
Thiamine	60 - 100 µ/g		
Riboflavin	35 - 50 µ/g		
Niacin	300 - 500 µ/g		
Pyridoxine HCL	28 µ/g		
Pantorhenate	70 µ/g		
Biotin	1.3 µ/g		
Cholin	40 µ/g		
Folic acid Vit. B12	5 - 13 µ/g		
Vit.B12	0.001µ/g		

decrease in cell elongation resulting from water shortage which led to decrease in each of cell turgor or cell volume and eventually cell growth (Boyer, 1988) and / or due to blocking up of xylem and phloem vessels thus hindering any translocation through (Abdalla and El-Khoshiban, 2007).

The obtained data also revealed that there was an inverse relationship between increasing drought and number of leaves/plant, where the highest records obtained under the highest soil moisture level W3 followed by the moderate soil moisture level W2 where the difference between the two levels was insignificant. This result was true for the vegetative and of flowering stages. Such decrease in number of leaves/plant with increasing stress levels detected by Sahid and Jurami (1998), Choi *et al.,* (2000), Ayodele (2001), Garg *et al.,* (2001), Singh *et al*., (2001), Koyro (2006), Martin and Stephens (2006) and Abdalla and El-Khoshiban (2007). The reduction in leaves number/plant due to water stress can be attributed to its direct effect on cell division which arose from reduction in nucleic acid synthesis and /or enhancement of its breakdown (Ashraf *et al.,* 1996). This also may be attributed to hormonal imbalance which arose from increased ABA and decreased in IAA levels in stressed plants (Xu *et al.*, 2002).

Data presented in the same table revealed also significant increase in root length with increase in stress levels, where the highest significant increase in root length obtained in plants grown under the most stressed level W1, this result was gained in both studied stages. Similar results obtained by Prior *et al.,* (1997), Chiatante *et al.,* (2000), Syman (2006) and Abdalla and El-Khoshiban (2007). Such increase in root length in response to drought was attributed to either the increase in gibberellins and cytokinin content of the root in response to drought which in turn stimulates root cell division and elongation.

It was also clear from the obtained data that both fresh and dry weights were highly significant declined under the most stressed level W1 in both stages. The decline in fresh weight may be attributed to the decrease in water content of stressed plant cells and tissue which led to loose of their turgor and thus shrink (Boyer, 1988). The results reached by Saxena and Nutiyal (2001), Fu and Huang (2001) and Monti *et al.,* (2006) confirmed our data. The decrease in both fresh and dry weights of stressed shoots revealed the influence of water on stimulating and regulating the photosynthetic enzymes which thus influence both the dry matter production and fresh weights (Abdalla and El-Khoshiban, 2007). For the effect of different ways of yeast application, the data illustrated that almost all ways of yeast application caused significant increase in growth parameters of both stages compared with control plants with few exceptions in soil treatments which revealed sometimes insignificant increase. The data also revealed that the highest significant means in growth parameters appeared in plants treated with yeast by spraying or by spraying plus soil treatments where

the difference between the two treatments was insignificant. While the lowest means observed under soil treatments. These results were gained in both vegetative and flowering stages. The positive response of yeast application is in harmony with many others as Ahmed *et al.,* (1998), Ali (2001), Gad (2001), Heikal (2005) and Hayat (2007). This increase in growth parameters as well as fresh and dry weights may be attributed to that yeast contains macro and micro nutrition, also it has growth regulators and vitamins or may be due to that yeast stimulates the plant to build up dry matters. Concerning the effect of interaction between soil moisture levels and different ways of yeast treatment the data showed that the highest significant means for growth parameters observed when plants grown under the highest soil moisture level W3 and treated with yeast by foliar plus soil treatment followed by foliar treatment where the difference between the two treatments was insignificant, except for root length where the highest significant increase obtained under the most stressed level W1 and treated with yeast by foliar plus soil treatment, these results were true for both vegetative and flowering stages.

Table (3): Growth characters of *Lupinus termis* as affected by different soil moisture levels and different ways of yeast application at the vegetative stage (combined analysis of two seasons).

Characters / Treatments		Plant height (cm)	No of leaves/ plant	Root length (cm)	Fresh weight/plant (g)	Dry weight/plant (g)
Soil moisture levels						
W1		29.50	10.83	13.96	4.01	1.34
W2		38.00	13.83	13.63	6.24	1.54
W3		38.83	14.50	9.63	6.43	1.76
LSD0.05		1.20	2.37	0.98	0.62	0.28
Ways of yeast application						
Control		32.11	10.44	11.17	4.66	1.35
Foliar		37.44	13.78	13.39	5.98	1.68
Soil		35.11	13.67	11.61	5.49	1.37
Foliar + Soil		37.11	14.33	13.44	6.11	1.77
LSD0.05		1.08	0.98	0.56	0.51	0.38
Soil moisture levels X Ways of yeast application						
W1	**Control**	29.00	8.67	13.00	2.85	1.12
	Foliar	29.67	12.00	13.33	4.62	1.52
	Soil	29.00	10.67	14.50	3.76	1.13
	Foliar + Soil	30.33	12.00	15.00	4.82	1.58
W2	**Control**	33.67	10.00	12.83	5.08	1.75
	Foliar	41.00	15.33	13.17	6.54	1.86
	Soil	33.67	14.67	14.00	6.17	1.45
	Foliar + Soil	40.00	15.33	14.50	6.34	1.81
W3	**Control**	33.67	12.67	7.67	6.05	1.19
	Foliar	41.00	15.00	8.33	6.77	1.60
	Soil	39.00	14.67	11.17	6.54	1.53
	Foliar + Soil	41.67	15.67	11.33	7.16	1.99
LSD0.05		2.01	1.26	1.01	0.98	0.66

W1 = 75% depletion of the available soil water. W2 = 55% depletion of the available soil water. W3 = 35% depletion of the available soil water.

Table (4): Growth characters of *Lupinus termis* as affected by different soil moisture levels and different ways of yeast application at the flowering stage (combined analysis of two seasons).

Characters / Treatments		Plant height (cm)	No of leaves/ plant	Root length (cm)	Fresh weight/plant (g)	Dry weight/plant (g)
Soil moisture levels						
W1		40.08	9.17	18.83	4.48	1.12
W2		59.08	23.67	16.00	8.12	1.74
W3		60.83	24.33	12.33	11.08	2.70
LSD0.05		2.63	1.02	2.23	0.93	0.21
Ways of yeast application						
Control		49.89	17.11	12.11	6.16	1.21
Foliar		56.33	21.11	16.78	9.37	2.18
Soil		50.56	17.44	16.22	9.06	1.68
Foliar + Soil		56.56	20.56	17.78	9.98	2.35
LSD0.05		1.19	0.89	1.39	0.82	0.11
Soil moisture levels X Ways of yeast application						
W1	**Control**	37.33	8.33	14.33	3.41	0.62
	Foliar	42.00	10.00	19.00	5.25	1.36
	Soil	38.33	9..00	20.00	3.93	1.00
	Foliar + Soil	42.67	9.33	22.00	5.33	1.51
W2	**Control**	56.33	19.00	12.00	6.60	1.36
	Foliar	61.00	26.67	17.67	9.30	1.90
	Soil	57.00	21.33	16.00	7.01	1.72
	Foliar + Soil	62.00	24.00	18.00	9.58	2.00
W3	**Control**	55.00	22.67	10.00	8.46	1.66
	Foliar	65.00	27.33	13.00	12.57	3.20
	Soil	57.33	23.33	12.67	10.01	2.32
	Foliar + Soil	66.00	27.67	13.67	13.27	3.63
LSD0.05		2.21	1.48	2.47	1.25	0.22

W1 = 75% depletion of the available soil water. W2 = 55% depletion of the available soil water. W3 = 35% depletion of the available soil water.

Relative water contents percent (RWC %):

Obtained data in Table 5 revealed that the RWC % of lupines leaves decreased progressively with decreasing soil moisture levels from W3 to W1 and with significant difference in both vegetative and flowering stages. Our results were confirmed with those obtained by Hayat et al., (1998), Toyagi *et al.,* (1999), Choi *et al.,* (2000), Flexas *et al.,* (2000), Phutela *et al.,* (2000), Garg *et al.,* (2001), Sanchez-Blance *et al.*, (2006) and Abdalla and Khoshiban (2007). The decline in RWC% with decrease in soil moisture level may be due to that stress causes modifications in plants metabolic pathway thus declining their osmotic and water potentials with concomitant preliminary decrease in their RWC % (Abdalla and Khashiban, 2007). Furthermore, treated plants with yeast by different ways caused significant increase in RWC% compared with control treatments in both stages, except for soil treatment of the flowering stage which revealed insignificant increase. The highest records for RWC% obtained in plants treated with foliar plus soil treatment followed by foliar treatment where the difference between the two treatments was insignificant at the flowering stage, while the lowest record obtained in soil treatment compared with control plants of the two stages. In addition, the data of interaction revealed that the highest significant means of RWC % obtained when plants grown under the highest soil moisture level W3 and treated with yeast by foliar plus soil treatment followed by foliar where the difference between the two treatments was insignificant at the flowering stage.

Yield and yield components:

Data in Table 6 revealed the effect of different soil moisture levels and different ways of yeast application on yield and yield components of *Lipinus termis*, the data indicated that the different soil moisture levels induced significant effect on number of pods/plant, number of seeds/ plant and 100 seeds weight. Also, the data indicated that there was a gradual decrease in all yield components with decrease in soil moisture level from W3 two W1. The reduction in yield and yield components of lupinus

plant as a result of water stress was in harmony with that reported by Sepaskhan (1977), Elia and Mwandemele (1986), Osman (1989) and Hayat (2007). The reduction in yield components as result of water stress may attribute to reduction of leaf area, chlorophyll content and photosynthetic rate Jafar *et al.*, (2007). Moreover, addition of adequate water decreased Absisic acid (ABA) and increased cytokinins (CYT) giberellin (GA) and indole acetic acid (IAA), which reflecting good plant growth, good carbohydrate anabolism and protein and finally attaining higher yield, it is known also that addition of water to plants has important functions in physiological processes such as minerals absorption from the soil, building different components in the plant, which in turn increase plants growth and yield (Hayat, 2007).

Table (5): Relative water content % of *Lupinus termis* leaves as affected by different soil moisture levels and different ways of yeast application (combined analysis of two seasons).

Characters / Treatments		Vegetative stage	Flowering stage
Soil moisture levels			
W1		28.52	30.38
W2		41.39	53.24
W3		53.48	60.95
LSD0.05		3.40	3.15
Ways of yeast application			
Control		36.79	44.64
Foliar		41.74	50.78
Soil		39.52	45.39
Foliar + Soil		46.48	51.95
LSD0.05		1.58	2.31
Soil moisture levels X Ways of yeast application			
W1	**Control**	25.09	27.65
	Foliar	29.60	30.95
	Soil	28.20	30.03
	Foliar + Soil	31.19	32.88
W2	**Control**	39.04	51.06
	Foliar	40.66	56.83
	Soil	40.35	50.54
	Foliar + Soil	45.52	54.51
W3	**Control**	46.23	55.22
	Foliar	54.96	64.55
	Soil	50.00	55.59
	Foliar + Soil	62.74	68.46
LSD0.05		2.65	4.00

W1 = 75% depletion of the available soil water. W2 = 55% depletion of the available soil water. W3 = 35% depletion of the available soil water.

Table (6): Yield and yield components of *Lupinus termis* as affected by different soil moisture levels and different ways of yeast application (combined analysis of two seasons).

Characters / Treatments		No of pods/plant	No of seeds/plant	100 seeds weight (g)
Soil moisture levels				
W1		3.25	7.67	1.78
W2		3.50	9.42	13.35
W3		4.83	13.17	14.82
LSD0.05		0.68	1.09	0.48
Ways of yeast application				
Control		3.00	7.67	8.40
Foliar		4.22	11.44	10.68
Soil		3.56	8.89	9.90
Foliar + Soil		4.67	12.33	10.95
LSD0.05		0.48	0.63	0.53
Soil moisture levels X Ways of yeast application				
W1	**Control**	2.67	6.00	1.60
	Foliar	3.33	8.33	1.94
	Soil	3.00	7.00	1.67
	Foliar + Soil	4.00	9.33	1.91
W2	**Control**	2.67	6.33	10.74
	Foliar	4.00	11.33	14.76
	Soil	3.00	8.00	13.37
	Foliar + Soil	4.33	12.00	14.52
W3	**Control**	3.67	10.67	12.85
	Foliar	5.33	14.67	15.60
	Soil	4.67	11.67	14.65
	Foliar + Soil	5.67	15.67	16.16
LSD0.05		0.82	1.09	0.82

W1 = 75% depletion of the available soil water. W2 = 55% depletion of the available soil water. W3 = 35% depletion of the available soil water.

Application of yeast by different ways induced significant increase in yield components, except for soil treatment of 100 seeds weight which reflect insignificant increase. The highest records of yield component obtained in foliar plus soil or foliar treatment where the difference between the two treatments was insignificant. The positive effect of yeast are in line with those obtained by Ahmed *et al.*, (1998), Eid (2001), Gad (2001), Hend (2002) and Mekki and Amal (2005) whom reported that the increase in yield components as a result of yeast treatment mainly attributed to the effect of yeast which can play a very significant role in making available nutrient elements for plants, also yeast

content macro and micro nutrients, growth regulators and vitamins or may due to that yeast stimulate the plant to build up dry matters (Heikal, 2005).

Regarding the data of interaction between different soil moisture levels and different ways of yeast application the obtained data illustrated that the maximum records obtained when plants grown under the highest soil moisture level W3 and treated with yeast by foliar plus soil or foliar treatments where the difference between two treatments was significant.

Photosynthetic pigments content:

The results presented in Table 7 showed that there was an inverse proportional relationship between increasing the severity of drought on one hand and content of leaves of chlorophyll a and b on the other hand where the highest means of chla and b obtained under the highest soil moisture level W3 and with significant difference, this result was true for both growth stages. Our result were fortified by those of Flexas *et al.*, (2000), El-Tayeb and Hassanien (2000), Misra and Srivastava (2000), Wang *et al.,* (2001), Sawhney and Singh (2002), Sanchez-Blanco *et al.,* (2006), Zhang *et al.,* (2006) and Abdalla and El-khoshiban (2007). Such reduction in the content of photosynthetic pigments in response to water stress was attributed to the ultra structural deformation of plastids including the protein membranes forming the thylakoids which in turn causes untying of photo system 2 which capture photons, so its efficiency declined, thus causing declines in electron transfer, ATP and NADPH production and eventually CO2 fixation processes (Maslenkova and Toncheva, 1997 and Zhang et al., 2006). Data in the same table revealed also that plants treated with yeast by different ways showed significant increase in chla and chlb compared with control plants in both stages. The data also showed that the highest records of chla and chlb obtained in foliar treatment of yeast and with significant difference compared with control treatment. While the lowest means obtained in soil treatment. The positive effect of yeast on chla and chlb is in harmony with that obtained by Hayat *et al.,* (2007) Stino *et al.,* (2009).

Concerning the interaction between different soil moisture levels and different ways of yeast application, the data of interaction showed significant effect on chla and chlb. Also both chla and chlb showed also progressive increase by increasing soil moisture levels from W1 to W3; it is also clear that chla and chlb showed progressive increase with yeast application by different ways under different soil moisture levels as compared with control treatments. Where the highest significant increase in chla and chlb obtained in foliar treatments of yeast and under the highest soil moisture level W3, while the lowest significant means obtained in untreated plants with yeast and under the most stressed level W1 followed by soil treatment.

Nitrogen and total protein %:

The data shown in Table 8 visualized that both nitrogen and protein % of lupinus seeds were significantly increased by increasing soil moisture level so as to reach their maximum records under the highest soil moisture level W3. These results were documented by many researches done in this field e.g Ahmed *et al.,* (1995), Hammam (1996), Yousef (1997), Neves and Lourenco (2001), Hayat (2007) and Bibi *et al.,* (2009) they all indicated that the increase in protein % may attributed to the slow hydrolysis of proteins under water stress conditions.

Application of active dry yeast by different ways resulted in significant increase in nitrogen and protein percentage as compared with control treatment. Where the maximum values obtained in plants sprayed with yeast (foliar application) compared with control treatment. These results are in harmony with those obtained by El-Kholy and Gomaa (2000), Schmid *et al.,* (2000), Ahmed (2002), Hassanein *et al.,* (2003), Gaballah and Gomaa (2004) and Hayat (2007). The increase in N and protein % could be attributed to the growth hormones produced by yeast (Gaballah and Gomaa, 2004) or may due to that yeast application stimulate the synthesis of protein (Stino, 2009). The interaction between different soil moisture levels and different ways of yeast application had significant effect on nitrogen and protein percentage. Applied the highest level of soil moisture W3 combined with foliar application with yeast gave the highest significant value of nitrogen and protein percentage. Similar results obtained by Hayat (2007) she indicated that the positive effect of yeast treatment under water stress conditions may be due to that yeast provided plants with essential nutrients elements required for protein formation.

Proline percent:

It is apparent from Table 8 that decreasing soil moisture levels from W3 to W1 showed significant increase in proline content of lupinus leaves so as to reach their maximum values in plants irrigated with the most stressed level W1. Accumulation of proline is an important indicator of drought stress tolerance in higher plants (Aspinall and Paleg, 1981). Karamanos et al., (1983) observed that increased amounts of free proline in two wheat cultivars could be associated with more effective dehydration and drought avoidance mechanisms.

Table (7): Chlorophyll content of *Lupinus termis* as affected by different soil moisture levels and different ways of yeast application (combined analysis of two seasons).

Characters / Treatments		Vegetative stage		Flowering stage	
		Chlorophyll a	Chlorophyll b	Chlorophyll a	Chlorophyll b
Soil moisture levels					
W1		1.65	0.28	0.56	0.30
W2		2.11	0.69	1.42	0.55
W3		3.60	1.51	1.97	0.82
LSD0.05		0.56	0.11	0.17	0.04
Ways of yeast application					
Control		1.70	0.63	1.09	0.45
Foliar		3.20	0.97	1.55	0.68
Soil		2.05	0.77	1.22	0.52
Foliar + Soil		2.88	0.94	1.41	0.57
LSD0.05		0.21	0.06	0.16	0.07
Soil moisture levels X Ways of yeast application					
W1	**Control**	0.40	0.17	0.28	0.11
	Foliar	2.78	0.34	0.74	0.44
	Soil	0.68	0.23	0.49	0.23
	Foliar +Soil	2.76	0.39	0.73	0.41
W2	**Control**	1.83	0.58	1.22	0.47
	Foliar	2.57	0.76	1.70	0.67
	Soil	1.94	0.64	1.43	0.49
	Foliar + Soil	2.12	0.78	1.32	0.57
W3	**Control**	2.87	1.14	1.55	0.78
	Foliar	4.26	1.82	2.21	0.94
	Soil	3.53	1.44	1.95	0.84
	Foliar + Soil	3.74	1.65	2.18	0.72
LSD0.05		0.37	0.11	0.25	0.12

W1 = 75% depletion of the available soil water. W2 = 55% depletion of the available soil water. W3 = 35% depletion of the available soil water.

A similar trend was obtained by Ashraf and Ibram (2005), Salma *et al.,* (2006), Ashraf and Foolad (2007) and Tawfik (2008) who found that osmoprotectants such as proline and glycine betaine were increased under drought stress. Production of osmolytes is general way to stabilize membranes and maintain protein conformations at low leaf water potentials, and osmolytes play major role in osmotic adjustment and also protect the cells (Pinhero *et al.,* 2001).

Application of yeast by different ways caused significant decrease in proline content of lupinus leaves as compared with control plant where the lowest means observed in foliar treatment followed by foliar plus soil treatment where the difference between the two treatments was insignificant, previous results were supported by Levitt (1980), Hasegawa *et al.,* (1986), Hathout (1996) and Gaballah and Gomaa (2004) whom reported that biofertilization of *faba bean* with Rhodotrula sp (yeast) resulted in decreasing leaves content of proline under stress conditions.

For the effect of interaction between the studied factors, the data showed that decreasing soil moisture levels caused significant increase in proline content, also treated plants with yeast by different ways induced significant decrease of proline content under different soil moisture levels Moreover, the lowest means in proline content obtained when plants sprayed (foliar application) with yeast and grown under the moderate and highest soil moisture levels (W2 and W3).

Alkaloids content:

It is clear from data in Table 8 that there was an inverse proportional relationship between increasing soil moisture levels on one hand and content of alkaloids of lupinus seeds on the other hand, where the highest significant increase in alkaloids content obtained under the most stressed level W1. Similar

results obtained by kennedy and Bush (1985) who reported that N-acetytle alkaloid increased five times under stress conditions above control treatment, while N- acetylloline increased two times under stress conditions above control treatment. Also Beverly (1987) reported an increase in alkaloids of pearl millet as influenced by drought stress, also Hőft (1996) reported similar results. In addition, treated plants with yeast by different ways caused significant decrease in alkaloids content as compared with control treatment, the highest decrease obtained in foliar plus soil treatment. For the effect of interaction between the studied factors, the data revealed significant effect on alkaloids content of lupinus seeds, increasing soil moisture levels caused significant decrease in alkaloids content, while the different ways of yeast application caused decrease in alkaloids content under different soil moisture levels, the maximum records of alkaloids content obtained in untreated plants and grown under the lowest soil moisture level W1. While the lowest means obtained in plants treated with yeast by soil plus foliar addition and grown under the highest soil moisture level W3.

Table (8): Nitrogen, protein and Carbohydrates percentages, as well as Alkaloids and Proline content of *Lupinus termis* as affected by different soil moisture levels and different ways of yeast application(combined analysis of two seasons).

Characters / Treatments		N%	Total Protein %	Alkaloids (mg/g)	Proline (mg/100g)	Carbohydrates%
Soil moisture levels						
W1		3.63	22.63	16.67	1.06	42.52
W2		4.81	30.04	14.81	0.75	52.79
W3		5.71	34.92	10.78	0.63	60.79
LSD0.05		0.42	2.64	0.73	0.09	2.28
Ways of yeast application						
Control		4.01	25.09	16.30	0.99	46.65
Foliar		5.47	33.20	15.91	0.69	59.69
Soil		4.58	28.61	13.71	0.84	48.89
Foliar +Soil		4.79	29.90	10.42	0.73	52.89
LSD0.05		0.26	1.60	0.31	0.05	1.00
Soil moisture levels X Ways of yeast application						
W1	**Control**	2.70	16.88	19.07	1.34	29.76
	Foliar	4.27	26.67	18.90	0.98	52.22
	Soil	3.91	24.44	17.67	1.04	43.62
	Foliar + Soil	3.62	22.54	11.03	0.87	44.47
W2	**Control**	4.27	26.67	16.87	0.89	50.64
	Foliar	5.48	34.25	16.43	0.54	59.32
	Soil	4.43	27.71	15.20	0.84	48.08
	Foliar + Soil	5.05	31.54	10.73	0.74	53.12
W3	**Control**	5.08	31.73	12.97	0.73	59.55
	Foliar	6.67	38.67	12.40	0.54	67.54
	Soil	5.39	33.67	9.50	0.65	54.98
	Foliar + Soil	5.70	35.63	8.26	0.59	61.07
LSD0.05		1.23	2.88	0.54	0.09	1.56

W1 = 75% depletion of the available soil water. W2 = 55% depletion of the available soil water. W3 = 35% depletion of the available soil water.

Total carbohydrates %:

It is evident from Table 8 that the values of total carbohydrates of lupinus seeds were significantly increased with increasing soil moisture levels from W1 to W3 and reached their maximum values under the highest soil moisture level W3. Our results were shown to be similar to those of Wang and Quebedeaux (1997), Osman (2000). Martinez *et al.*, (2004), Wu and Xia (2006), Abdalla and El-Khoshiban (2007) and Hayat (2007). We attributed the above decline in total carbohydrates percentages with decline in soil moisture to its bad effect on certain chemical stimulus mostly

ABA through xylem vessels to leaves and seeds of stressed plants which led to stomata closure, reduction of each of stomata conductance, CO2 concentration in leaf tissues, electron transport system, CO2 fixation, rate of photosynthesis and eventually quantity of photosynthates, thus causing decline in growth rates. Or it may be attributed to the decrease in chlorophyll content under stress conditions.

Furthermore, treated plants with yeast by different ways caused significant increase in total carbohydrates percentage compared with control treatment, where the highest significant increase in carbohydrates percentage obtained in plants sprayed (foliar spray) with yeast. While the lowest means obtained in plants treated with yeast in soil. The positive effect of yeast obtained also by Bahr and Gomaa, (2002), Gaballah and Gomaa (2004), Heikal (2005) and Hayat (2007). The increase in total carbohydrates % as a result of yeast application in our search may be due to the increases in chlorophyll a and b or may be due to that yeast application had enhancing role in cell division, cell elongation producing more leaf area (Hayat, 2007).

The data of bi-interaction indicates that the highest records of carbohydrates percentage appeared in plants sprayed with yeast and grown under the highest soil moisture level W3 as compared with other treatments, while the lowest values obtained under the most stressed levels W1 in control plants.

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5/1/2010

Pericentric inversion of chromosome 1 and 9 in a case with recurrent miscarriage in Egypt

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Abstract: We report phenotypically normal female carrying structural variants on both chromosomes 1 and 9. She was referred to the Recurrent Pregnancy Loss Clinic, National Research Centre, with a complaint of repeated miscarriage (5 consecutive first trimester miscarriages). Conventional cytogenetic study of the peripheral blood of the wife revealed 46, XX inv (1) (p32 q44) in all cells and inversion (9) (p12 q12) in 30% of the studied cells, while the husband was normal 46, XY. FISH study was done to confirm and clarify the findings. To the best of our knowledge, this is the first report of a phenotypically normal female carrying structural variant on both chromosomes 1 and 9 leading to recurrent miscarriage. Our study highlights the deleterious effect of pericentric inversion of chromosomes 1 and 9 on recurrent pregnancy loss. It also underlines the importance of performing cytogenetic studies for couples with such complaint. In such cases, a well informed genetic counseling should be given to the couple and prenatal diagnosis should be offered in future pregnancies. [Journal of American Science 2010;6(8):154-156]. (ISSN: 1545-1003).

Keywords: Pericentric inversion; chromosome; pregnancy

1. Introduction

Recurrent pregnancy loss is a serious health problem that affects many couples. Miscarriage occurs in up to 15% of pregnant women and is considered the most common complication of pregnancy [1]. Although 15% of clinically recognized pregnancies miscarry, total reproductive losses are closer to 50% [2]. It has been estimated that 2 to 5% of women have 3 or more miscarriages [1].

Recurrent pregnancy loss requires a multidisciplinary approach since genetic, endocrinologic, anatomic, immunologic, thrombophilic, infections and iatrogenic factors may require evaluation and management [3]. Structural chromosomal abnormalities are the single most common cause for pregnancy loss. Among first trimester abortions, 50 to 60% show chromosomal abnormalities [4, 5]. Published studies have shown a prevalence of chromosomal abnormalities that varies from 2 to 8% of couples with recurrent miscarriage [6, 7, 8].

In about 4% of couples with recurrent pregnancy loss, one partner carries a balanced reciprocal or Roberstonian translocation [9, 10]. Carriers of balanced reciprocal translocation are phenotypically normal, but 50 to 70% of their gametes are unbalanced, because of abnormal segregation at meiosis. The reproductive risk conferred by chromosome rearrangements is dependent on the type of rearrangement and whether it is carried by the female or the male partner [11].

Pericentric inversions have been described for all chromosomes. They are considered the less common structural aberrations in human and their incidence differs strongly from chromosome to chromosome [12]. The frequency of pericentric inversions in the general population vary from 0.089 to 0.34 [13, 14].

Pericentric inversion of chromosome 9 is usually a normal polymorphism and its incidence has been reported to be approximately 1 to 2% in the general population [15]. Although its clinical consequences remain unclear, studies have implicated it with recurrent miscarriages [16]. Demirhan et al., [17] indicated a correlation between pericentric inversions of 9 and recurrent miscarriages. Inversions in chromosomes 1, 8 and 16 are rare. A possible reason for such findings may be unequal recombination causing lethality and thus, limited detection [12]. Also, the additional genetic material from chromosome 1 leads to early termination of pregnancy and poor fetal development [18].

As inversions do not seem to influence female fertility to any noticeable degree apart from an increasing miscarriage rate [12], reproductive guidance for the management of recurrent miscarriage recommends chromosome analysis in both partners.

2. Material and Methods

Pedigree analysis of 3 generations of the couple having reproductive failure was performed in order to determine the presence of consanguinity or any other similar cases in the family.

Detailed medical and gynecologic evaluation was performed to exclude other causes for the pregnancy loss. This included: hysterosalpingogram (HSG) for anatomical evaluation, in addition to hormonal, immunologic and haematologic investigations.

Chromosomal analysis for the couple was done using G-banding technique according to the method described by Seabright [19] and Verma and Babu [20], a total of 25 metaphases were analyzed for each case. Structural or numerical anomalies were recorded and karyotyped according to the ISCN [21]. Fluorescence in situ Hybridization (FISH) analysis was used for further identification of the rearrangement type and the respective breakpoints.

3. Results

The female partner was of average height and weight (1.68 m and 72 kg respectively). Her basal hormone profile showed: FSH 6 mU/ml; LH 3.6 mU/ml; prolactin 19 ng/ml; progesterone 16 ng/ml (day 22 of the menstrual cycle) and her serum TSH was 2.8 mIU/ml. The results of lupus anticoagulant and anticardiolipin antibodies (Ig G and M) were negative. In addition, anatomical evaluation by ultrasonography and HSG revealed normal uterine cavity.

Semen analysis of the male partner revealed normal parameters with less than 20% abnormal forms.

The karyotype of the husband revealed normal male 46, XY, while that of the wife revealed 46, XX, pericentric inversion (1) (p32q44) in all cells and inversion (9) (p12q12) in 30% of the cells as shown in Fig. [1, 2].

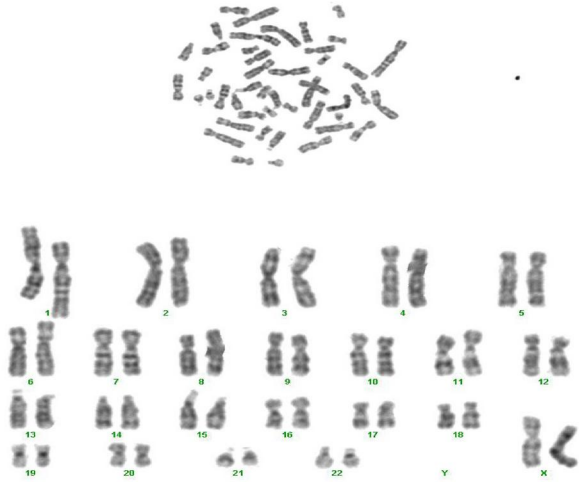

Fig. [1]: Karyotype of the wife revealed 46, XX, pericentric inversion (1) (p32q44).

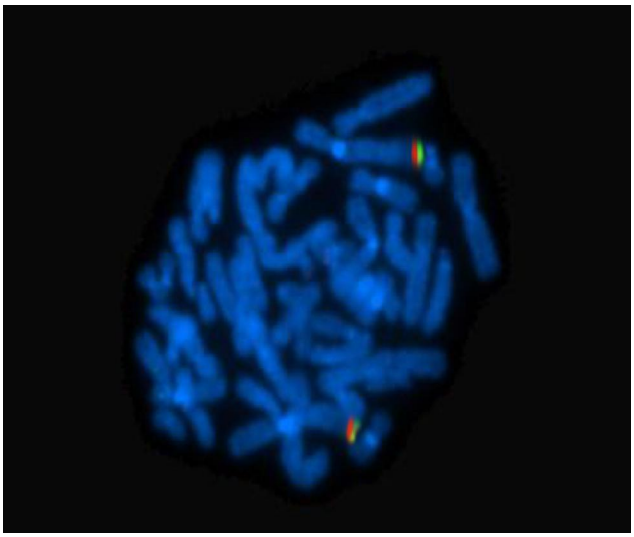

Fig. [2]: FISH using LSI p58 probe hybridized to band region 1p36 (spectrum orange) and band region 1p telomere (spectrum green) showing the involved break points.

4. Discussion

Chromosomal inversions are associated with a higher risk of pregnancy wastage; pericentric inversion 9 is considered a population variant [2]. There are a number of studies showing a correlation with reproductive failure. As previously hypothesized, the large amount of additional genetic material from chromosome 1 leads to a very early termination of pregnancy and poor fetal development [3, 4, 5].

To the best of our knowledge, this is the first report of a phenotypically normal mother carrying structural variants on both chromosomes 1 and 9 leading to repeated early miscarriage.

5. Conclusion

Genetic counseling is mandatory after the diagnosis of pericentric inversion of chromosome 1 and 9 and prenatal diagnosis should be offered in a subsequent pregnancy.

Competing interests

The authors declare that they have no competing interest

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5/6/2010

Production, Immobilization and Anti-tumor Activity of L-Asparaginase of Bacillus sp R36

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Abstract L-asparaginase is one of the known drugs in the treatment of cancer, especially acute lymphoblastic leukemia. In recent years several bio-conjugation protocols have been developed to improve the pharmacokinetic and immunological properties of anti-leukemic enzyme, L-asparaginase. In this study, fifty two bacilli species were newly isolated in our laboratory and screened for their ability to produce extracellular and intracellular L-asparaginase enzyme. *Bacillus* sp R36 gave the highest intracellular enzyme production. Formation physiology of the enzyme revealed that optimum culture conditions were 9:1 of air::medium ratio, with 55 x 10^5 CFU/mL inoculums size. The optimum incubation period was found to be 24 hours under shaking growth conditions. The initial pH value 5.6 was favorable for the highest enzyme production. Addition of 1% lactose or 1% raffinose resulted in a doubled enzyme productivity (yielded 204% and 209%, respectively). The enzyme was efficiently immobilized by covalent binding with activated carbon. Immobilized L-asparaginase activity was 33.0 U/g carrier; with immobilization yield of 73.6%. Characterization of the enzyme was performed on native and immobilized forms. Optimum pH value was 7.0 for free and immobilized forms. Optimum reaction temperature was 50 °C for native enzyme, while it was 60 °C for the immobilized enzyme preparation. The immobilization process greatly enhanced the thermal stability of the enzyme. Native L-asparaginase enzyme exhibited thermal stability up to 50 °C, while immobilized form retained 100% of its activity up to 80 °C. Anti-tumor and antioxidant activities were investigated. The enzyme inhibited the growth of two human cell lines including hepatocellular carcinoma (Hep-G2) and colon carcinoma (HCT-116) with IC_{50} value of 112.19 µg/mL and 218.7 µg/mL, respectively. [Journal of American Science 2010;6(8):157-165]. (ISSN: 1545-1003).

Keywords: -asparaginase, *Bacillus*, immobilization, anti-tumor effect.

1. Introduction

L-asparaginases (L-asparagine amido-hydrolase EC 3.5.1.1) hydrolyze L-asparagine to L-asparate and ammonia (Fig. 1a). Bacterial L-asparaginases are enzymes of high therapeutic value due to their use in certain kinds of cancer therapies, mainly in acute lymphoblastic leukemia (ALL) [1-3]. Many bacteria contain two L-asparaginases, a high affinity periplasmic enzyme and a low affinity cytoplasmic enzyme. These enzymes in some bacteria accumulate mainly in periplasmic space [4, 5 and 6]. In *Escherichia coli* and many other bacteria, synthesis of cytoplasmic asparaginase I is constitutive, while expression of periplasmic asparaginase II is activated during anaerobiosis. Further, only the type II enzyme has shown substantial anti-tumor activity [7]. An ideal method for the release of this enzyme would be rapid, inexpensive, gentle and compatible with downstream steps of its purification [8].

The anti-leukemic effect of L-asparaginase is a result of rapid and complete depletion of the circulating pool of L-asparagine as in a great number of patients with lymphoblastic leukemia, the malignant cells depend on exogenous source of L-asparagine to be able to survive, mean while, the normal cells are able to synthesize L-asparagine [3, 9]. The discovery of new L-asparaginase serologically different but having similar therapeutic effects is highly desired [10]. One approach to achieve improved function and properties of the enzyme is its chemical modification (immobilization) with various kinds of biocompatible polymers. Immobilization of enzymes is one of the important trends and goals of biotechnology. Enzymes have an enormous potential as biocatalysts in a wide range of industries and medicine. They offer a distinct advantage due to their specificity, high catalytic efficiency at low temperature and being biodegradable. [11] The use of immobilized enzymes lowers production costs as these can be readily separated from reaction mixture and hence can be used repeatedly and continuously. Several different methods have been employed for enzyme immobilization which includes adsorption onto insoluble materials, entrapment in polymeric gels, encapsulation in

membranes, cross linking with bifunctional or multifunctional reagents and linking to an insoluble carrier [12].

Activated carbon is a form of carbon that has been processed to make it extremely porous and thus to have a very large surface area available for chemical reactions. One gram of activated carbon has a surface area of approximately 500 m^2 with 1500 m^2 being readily achievable. Powdered activated carbon is made in particular form less than 1.0mm in size with an average diameter between 0.15 and 0.25 mm. Activated carbon is used to treat poisonings and overdoses following oral ingestion. It is thought to bind to poison and prevent its adsorption by the gastrointestinal tract. The present work introduces the production (from *Bacillus* sp R36), the physiology, the characterization, and the anti-tumor activity of a new L-asparaginase enzyme (EC 3.5.1.1) in its native and immobilized forms.

2. Material and Methods

Chemicals

Anhydrous L-asparagine, trichloroacetic acid (TCA), Nessler reagent chemicals (HgI2, KI, and sodium hydroxide), Chitin and hexane were purchased from Sigma Chemicals Co. Activated carbon, celite, carboxymethyl cellulose; silica gel and tricalcium phosphate were from Merck Chemicals. All other chemicals used were of analytical grade.

Isolation of Bacteria

One Gram of soil was transferred to a vial containing 10 mL of sterile water and kept on a rotary shaker at 100 rpm for 30 min. The bacterial suspension was pasteurized by heating at 65˚C for 15 min. The supernatant was diluted 10-folds and 0.1 mL was spread on pre-solidified nutrient agar medium composed of 5 g peptone, 3 g beef extract and 15 g agar per liter of distilled water. The plates were incubated at 30˚C for 48 h and bacterial colonies were purified on nutrient agar. Each of the purified colonies was then sub-cultured on nutrient agar slants.

Culture conditions and membrane permeablization

Bacterial isolates were screened for L-asparaginase activity on Luria – Bertani (LB) medium containing (1-1) 10 g peptone, 5 g yeast extract and 10 g NaCl at pH 7.0. A 1/100 inoculum of overnight cultures grown in LB medium was made in 40 mL LB medium in 250 mL Erlenmeyer conical flasks and incubated for 24h at 30˚C on a 200 rpm orbital rotary shaker. Cells, cultivated for L-asparaginase production, were harvested by centrifugation (6000 xg for 15 min), washed once with 0.05 mole potassium phosphate buffer pH 8.6, and re-suspended to A 600 = 5.0 in the same buffer containing n-hexane at 1% (V/V) for L-asparaginase release. The suspensions were incubated at room temperature for 1h, and briefly vortex every 10 min [8].

L-asparaginase assay

The enzyme activity was assayed according to wriston [13]. The reaction mixture contained 0.1 mL permeabilized cells free broth and 0.9 mL of 0.01 mole L-asparagine prepared in 0.05 mole tris- HCl buffer, pH 8.6 and incubated for 30 min at 37˚C. The reaction mixture was centrifuged at 6000 xg for 10 min and the ammonia released in the supernatant was determined by Nesslerization reaction. In brief, to 0.5 mL of supernatant, 1.75 mL dist. H2O, 0.25 mL of Nessler reagent was added. After 10 min. absorbance at 480 nm were read with appropriate control. One enzyme unit (U) is defined as the amount of enzyme that librates 1 µmole of ammonia per min at 37˚C. Standard curve of ammonium sulphate was used for calculating ammonia concentrations.

Immobilization of L-asparaginase

Different supports were employed for L-asparaginase immobilization according to [14]. Experimentally, 200 mg of each support was shaked in 5 mL tris –HCl buffer (0.01 mole, pH 8.6) containing 2.5 % glutaraldehyde at room temperature for 2h. The carriers were filtered off and washed with distilled water to remove the excess of glutaraldehyde then each treated carrier was incubated with 5 mL of tris HCl buffer containing 1 mL of enzyme. After being shaken for 2h at 30˚C, the unbounded enzyme was removed by washing with distilled water until no protein or activity were detected in the wash.

Cell culture

Two human cell lines were used through this work including: hepatocarcinoma (HepG2) and Colon carcinoma (HCT-116), both lines purchased from ATCC, VA, USA. Hep-G2 Cells were routinely cultured in DMEM (Dulbeco's Modified Eagle's Medium), while HCT-116 cells were cultured in Mc Coy's medium. Media were supplemented with 10 % fetal bovine serum (FBS), 2 mmole L-glutamine, containing 100 units/mL penicillin G sodium, 100 units/ml streptomycin sulphate, and 250 ng/ml amphotericin B. Cells were maintained at sub-confluence at 37ºC in humidified air containing 5 % CO2. For sub-culturing, monolayer cells were harvested after trypsin / EDTA treatment at 37°C. Tested samples were dissolved in dimethyl sulphoxide (DMSO). All cell culture material was obtained from Cambrex Bioscience (Copenhagen, Denmark). All chemicals were from Sigma/Aldrich, USA, except mentioned. All experiments were repeated three times, unless mentioned.

Anti-tumor activity

The cytotoxic effect of the samples against Hep-G2 and HCT-116 cells was estimated by the 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay according to Hansen et al., 1989 [15]. The yellow tetrazolium salt of MTT is reduced by mitochondrial dehydrogenases in metabolically active cells to form insoluble purple formazan crystals, which are solubilized by the addition of a detergent. Cells (5 x 104 cells / well) were incubated with various concentrations of the compound at 37 ºC for 48 h in a FBS-free medium, before submitted to MTT assay. The absorbance was measured with an ELISA reader (BioRad, München, Germany) at 570 nm. The relative cell viability was determined by the amount of MTT converted to the insoluble formazan salt. The data are expressed as the mean percentage of viable cells as compared to the respective control cultures treated with the solvent. The half maximal growth inhibitory concentration IC50 values were calculated from the linear equation of the dose–dependent curve of each sample.

Antioxidant activity (scavenging of DPPH)

The antioxidant capacity of the tested samples was studied through their scavenging activity against 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radicals [16]. DPPH is a stable deep violet radical due to its unpaired electron. In the presence of an antioxidant radical scavenger, which can donate an electron to DPPH, the deep violet color decolourize to the pale yellow non-radical. The bleaching of DPPH was monitored at absorbance of 515 nm. The percentage of DPPH bleaching utilized for SC50 (half maximal scavenging concentration) was calculated as follows: 0% is the absorbance of DPPH and 100 % is the absorbance of DPPH with an efficient scavenger (10 mM ascorbic acid, AA).

3. Results and Discussion

Screening of bacterial isolates

Fifty two bacilli species were tested for enzyme production. Extracellularly, no appreciable levels of L-asparaginase were detected. After membrane permeabilization with potassium phosphate / 1% hexane system [8], *Bacillus* sp R36 gave the highest enzyme productivity in the cell suspension (20.15 U/mL). The determination of the localization of any enzyme plays a vital role in the development of bioprocess. The existence of L-asparaginase in the membrane fraction of *Tetrahymena pyriformis* and periplasmic space of *Enterobacter aerogenes* and *Pseudomonas. aeruginosa* has been reported [8, 17]. There are many reports on the production of intracellular L-asparaginase from *E. coli* [18] *Vibrio succinogenes* [19], *Erwinia aroideae* [20], *S. marcescens*[21], *E. aerogenes* [22], and *P. aeruginosa* [10, 23]. Kumar et al [24] that recently carried out a sub-cellular localization of L-asparaginase enzyme using cell fractionation techniques in various organisms. They reported that there was no trace of extracellular activity observed in the culture filtrates, which inferred that the enzyme was secreted as an intracellular product in all microorganisms tested. The maximum L-asparaginase activity was found to be 14.56 U/mL with *Pectobacterium carotovorum* MTCC 1428.

Optimization of L-asparaginase production by Bacillus sp R 36

Some factors affecting L-asparaginase productivity were studied aiming at optimization the anti-leukemic enzyme activity. With respect to the effect of aeration level, 9:1 (25 mL LB medium per 250 mL conical flask) air to medium ratio gave the highest enzyme activity reaching 22.8 U/mL (Fig 1b). Incubation period played an important role in enzyme productivity as maximum enzyme activity was obtained from 20-24 h reaching (20.0 U/mL) after 24 h only of incubation on an orbital shaker at 150 rpm. At 48 h of incubation under the same conditions, the activity decreased to 42% (8.4 U/mL) (Fig 1c). The same incubation period was reported upon production of L-asparaginase by isolated Bacillus circulans [25] and Enterobacter aerogenes and Pseudomonas aeruginosa [8]. Inoculum size in terms of colony forming unit (CFU/mL) had a positively effect on enzyme activity up to 55 x 105 CFU/mL. Higher inoculum sizes were resulting in leveling off the activity (Fig 2a). Prakasham [26] abstracted that incubation temperature, inoculums level and medium pH among all fermentation factors were major influential parameters at their individual level, and contributed to more than 60% of total L-asparaginase production. The effect of initial pH of LB medium was studied. Results revealed that 5.6 initial pH value of production medium (without adjustment) was suitable for enzyme production more than other tested pH values (Fig 2b). In all cases the harvest pH value is highly alkaline ~ 8.9 due to ammonia production in the fermentation flask. All carbon sources that were tested for L-asparaginase production enhanced enzyme formation only upon adding to Luria– Bertani (LB) medium.

On mineral salt medium, Bacillus sp R36 neither could grow nor biosynthesize the enzyme. The obtained results revealed that addition of 1% lactose or 1% Raffinose to LB medium doubled the enzyme activity (204 % and 209 %, respectively). On the other hand, glucose was the most inhibitory carbon source compared with the control LB medium (Fig 3). The effect of carbon sources differ from organism to

another, but in general, glucose was regarded as a repressor for L-asparaginase production in bacteria [27]. Optimization of the culture conditions of L-asparaginase activity was the aim of many studies through which the enzyme productivity increased to many folds. Thus, Abdel –Fattand and olama [10] obtained more than five folds the activity in basal medium using Box-Behnken designing in solid state culture. Kumar et al [28] obtained an over all 8.3 fold in enzyme production compared to the un-optimized medium using the central composite experimental design. Hymavathi et al [29] improved L-asparaginase yield by more than 300% using fractional factorial central composite design (FFCCD).

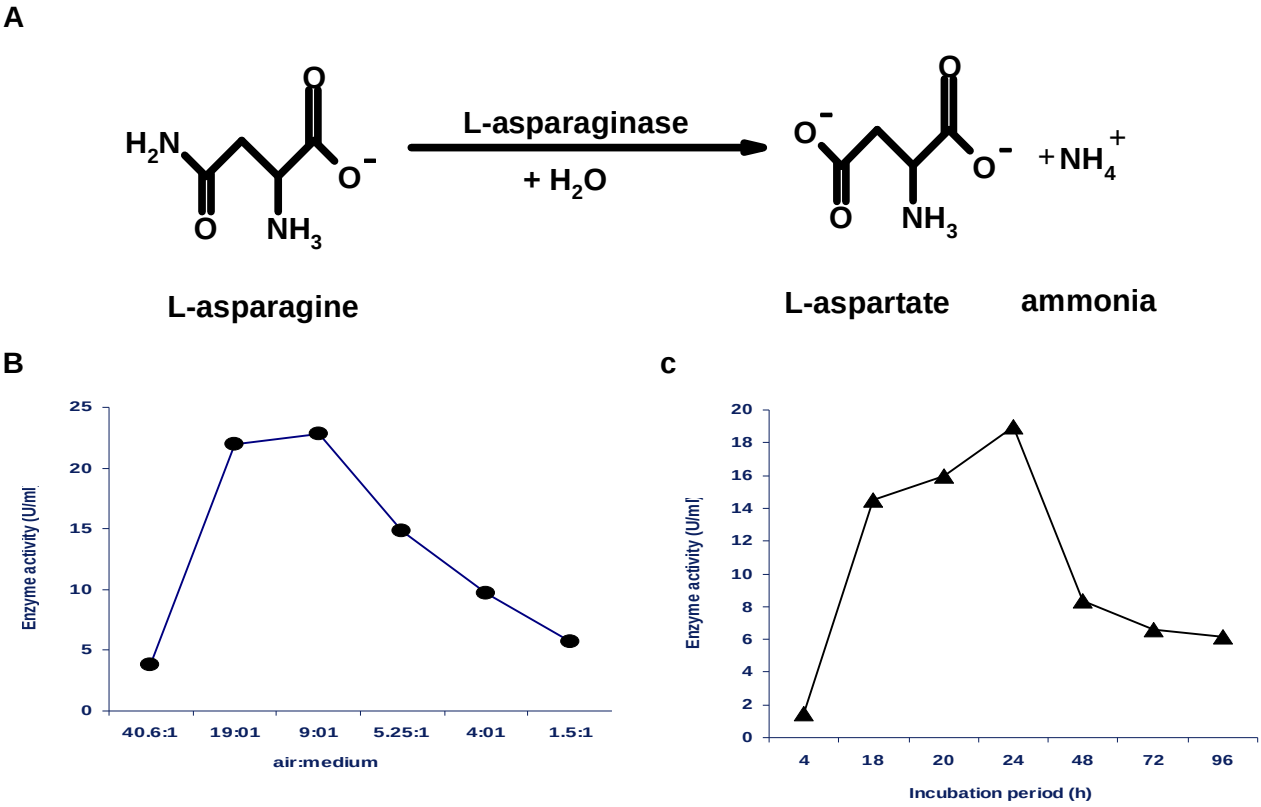

Fig (1): a. Role of L-asparaginases in hydrolysis of L-asparagine into L-asparate and ammonia. b. Effect of aeration level on L-asparaginase activity produced by Bacillus sp R36 on LB medium. All data are average value of triplicate measurements. c. Effect of incubation period on enzyme activity. The flasks were incubated on an orbital shaker at 150 rpm at the indicated times. All data are average value of triplicate measurements.

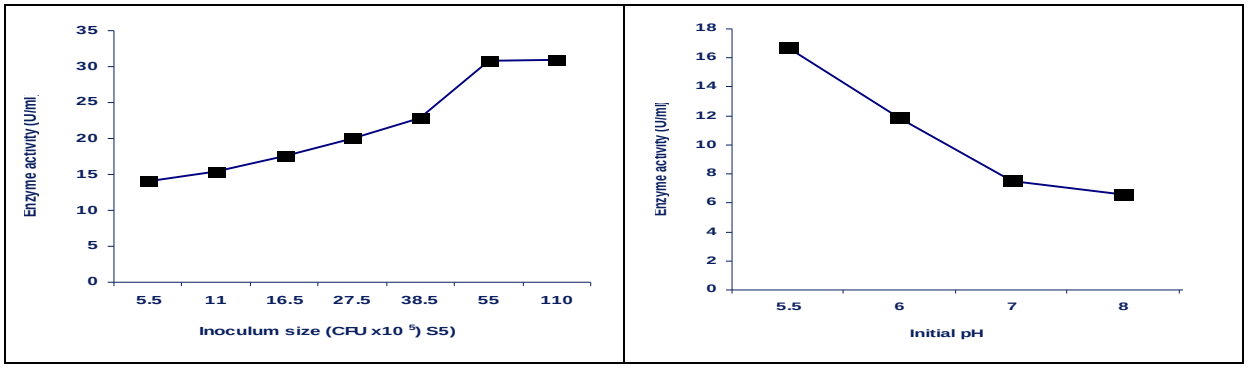

Fig (2): a. Effect of inoculum size on L-asparaginase activity. b. Effect of initial pH value of LB medium on L-asparaginase. All data are average value of triplicate measurements.

Immobilization of L-asparaginase

Seven supports, pre-activated with glutaraldehyde, react with different degrees with the terminal amino residues of the enzyme protein. Immobilization of Bacillus sp R36 L-asparaginase by covalent binding was achieved by cross linking between the enzyme and different carriers (i.e. activated carbon, celite, chitin, carboxymethyl cellulose, egg shell, silica gel, tricalcium phosphate and chitosan) throughout glutaraldehyde. The amount of enzyme used for immobilization was 50 U/g carrier. The results (Table 1) indicated that the lowest immobilized activity and immobilization yield 7.9 U/g carrier and 17.8 % were detected with chitosan as a carrier. On the other hand, the highest immobilized activity (33.0 U/g carrier) and highest immobilization yield (73.6%) were obtained with activated carbon as a carrier. Medically activated carbon (activated charcoal) is a supplement used when accidental poisonings have occurred to absorb the poison and carry it out of the body. It is also used to treat high cholesten stomach and gas [30].

Our results are the first report for immobilization on activated carbon of L-asparaginase enzyme. In addition, immobilization of L-asparaginase R36 on activated carbon had a characteristic of simpler processing in comparison with that on silk sericin protein [31], polysaccharide levan [32] and agarose [33]. Kotzia et al [34] immobilizesd L-asparaginase of Erwinia chrysantheni 3937 on epoxy- activated Sepharose CL-6B. They reported that the immobilized enzyme retains most of its activity (60%) and shows high stability at 4°C. More recently, Tabandeh and Aminlari [35] investigated the effect of conjugation with oxidized inulin on the properties of L-Asparaginase (L- ASNase) in the form of Elspar. They found that modified L-asparaginase synthesized at ratio of 2: 1 had activity of 65% of that of native enzyme.

Characterization of L-asparaginase of Bacillus sp R36

a. Effect of pH on free and immobilized enzymes activities.

The effect of pH on L-asparaginase activity of free and immobilized preparations was studied by changing the pH value from 3.0 to 8.6 using a series of buffers namely citrate buffer (pH 3-5), phosphate buffer (pH 6-7), and Tris buffer (pH 8-8.6), (Fig. 4a). Both crude and immobilized L-asparaginase preparations exhibited maximum activity at pH 7. The immobilized form yielded more than 300 % increase in activity (333.5 %). This clearly reflects the suitability of activated carbon as a carrier for this enzyme. Our results are in agreement with that reported by Zhang et al [31] who immobilized E. coli L-asparaginase on micro particles of the natural silk sericin protein. They reported that the optimal range of pH value had no evident changes in comparison with native enzyme. Tabandeh and Aminlari [35] reported that the optimum pH of modified L- ASNase and the native enzyme is at alkaline pH (pH 8) probably due to produced L-aspartic acid acting as competitive inhibitor for enzyme in acidic condition.

b. Effect of Reaction temperature on free and immobilized L-asparaginase.

The effect of the reaction temperature on free and immobilized forms was investigated from 30˚C to 80˚C (Fig 4b). The optimum reaction temperature of free enzyme was 50˚C while the immobilized preparation reacted optimally at 60 ˚C reaction temperature i.e. at 10 ˚C higher than the native enzyme, with an increase of 355.8 % in enzyme activity. The same results were reported by [31], who stated that the optimum reaction temperature of immobilized enzyme was at 60˚C while that of free L-ASN ase was at 50˚C.

c. Thermal stability of immobilized enzyme

Residual activities after heating the enzyme at 30 - 90 ˚C for 10 min were measured (Fig 4c). An excellent thermal stability was exhibited by the immobilized enzyme preparation. The enzyme retained 100 % of its L-asparaginase activity up to 80 ˚C, while 62 % of it was retained upon heating for 10 min at 90 ˚C. The native form, maintained 100 % of its activity at 50 ˚C while at 80˚C it retained 65 % of its L-asparaginase activity. At 90 ˚C the native form maintained 57% of its activity. An improvement of thermal stability of immobilized enzyme may be acquired by multiple attachment of activated carbon to the enzyme molecule resulting in greater enzyme rigidity of enzyme conformation and increasing the activation energy for unfolding the enzyme. A marked improvement in thermal stability of modified enzyme was obtained by Tabandeh and Aminlari [35], Amiri et al [36] and Scaman et al [37]. In contrast, [31] reported that the thermo stability of the immobilized ASNase is very similar to that of the native enzyme and there were no obvious changes in the activities.

Anti-tumor activity

Using MTT assay, the in vitro cytotoxicity effect of Bacillus sp R36 L-asparaginase enzyme on the growth of two tumor cell lines was studied. The IC50 values were calculated from the linear equation of the dose effect of the enzyme against hepatocellular carcinoma Hep G2 cells ($y = -0.4069x + 95.648$) and against colon carcinoma HCT-116 cells ($y = -0.2233x + 98.838$). The incubation of Hep G2 with gradual doses of Bacillus sp R36 L-asparaginase enzyme leads to a gradual inhibition in

the cell growth as concluded from its low IC50 values 112.19 µg / mL (Fig. 5a). As shown in (Fig. 5b), the treatment of HCT- 116 with the enzyme resulted in a low anti-tumor activity with IC50 value of 218.7 µg / mL compared with the growth of untreated control cells. Cappelletti et al [38] studied in vitro cytotoxicity of a novel L-asparaginase from the pathogenic strain Helicobacter pylori CCUG 17874 against different cell lines. They reported that AGS and MKN 28 gastric epithelial cells being the most affected.

Antioxidant activity

The antioxidant capacity of the enzyme was investigated using DPPH assay. DPPH is a stable non-physiological radical, which could provide a relative figure of the radical scavenging capacity of a tested probe. The DPPH assay showed that Bacillus sp R36 possessed low scavenging activity with high SC50 values of 325.4 µg/mL compared to the scavenging activity of the well-known antioxidant (ascorbic acid, a.a., SC50 8.7 µg/mL).

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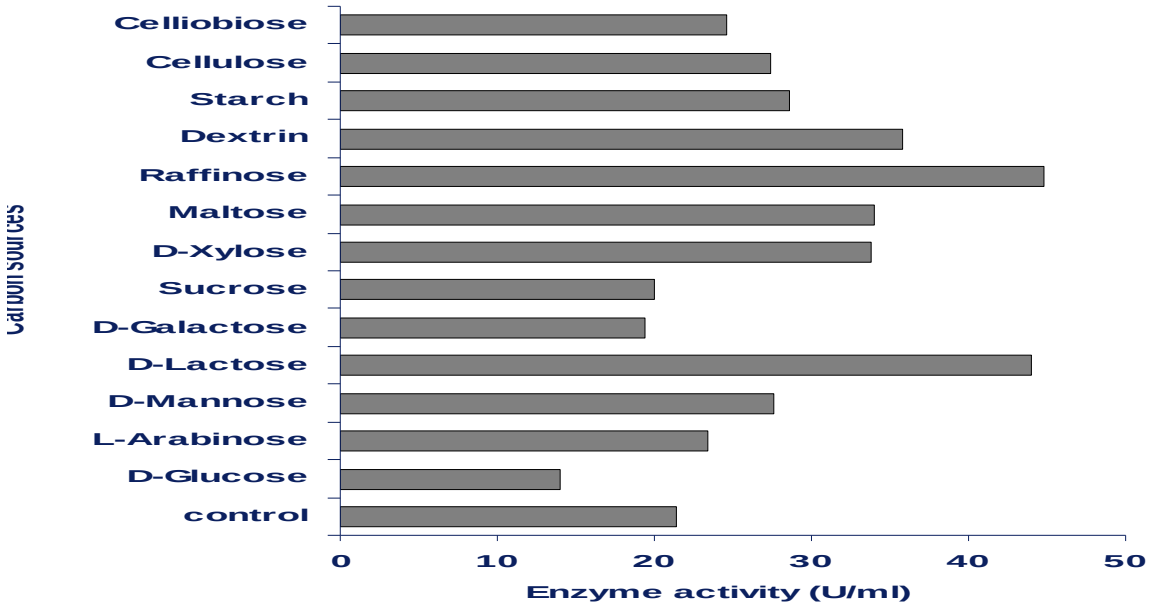


Fig (3): Effect of different carbon sources on L-asparaginase activity (final concentration 1%). All data are average value of triplicate measurements.

Table (1): Immobilization of *Bacillus* sp R 36 L-asparaginase by covalent binding with different carriers.

Carrier	Added enzyme (U/g) (A)	Unbounded enzyme (U/g) (B)	Immobilized enzyme (I)	Immobilizati-on yield I / (A-B)%
Activated carbon	50	5.2	33.0	73.6
Celite	50	4.1	27.5	60.0
Chitin	50	3.5	14.3	30.7
CMC	50	11.5	19.8	51.4
Egg Shell	50	2.7	20.3	42.9
SG	50	4.8	28.1	62.1
TCP	50	7.2	8.6	20.1
Chitosan	50	5.5	7.9	17.8

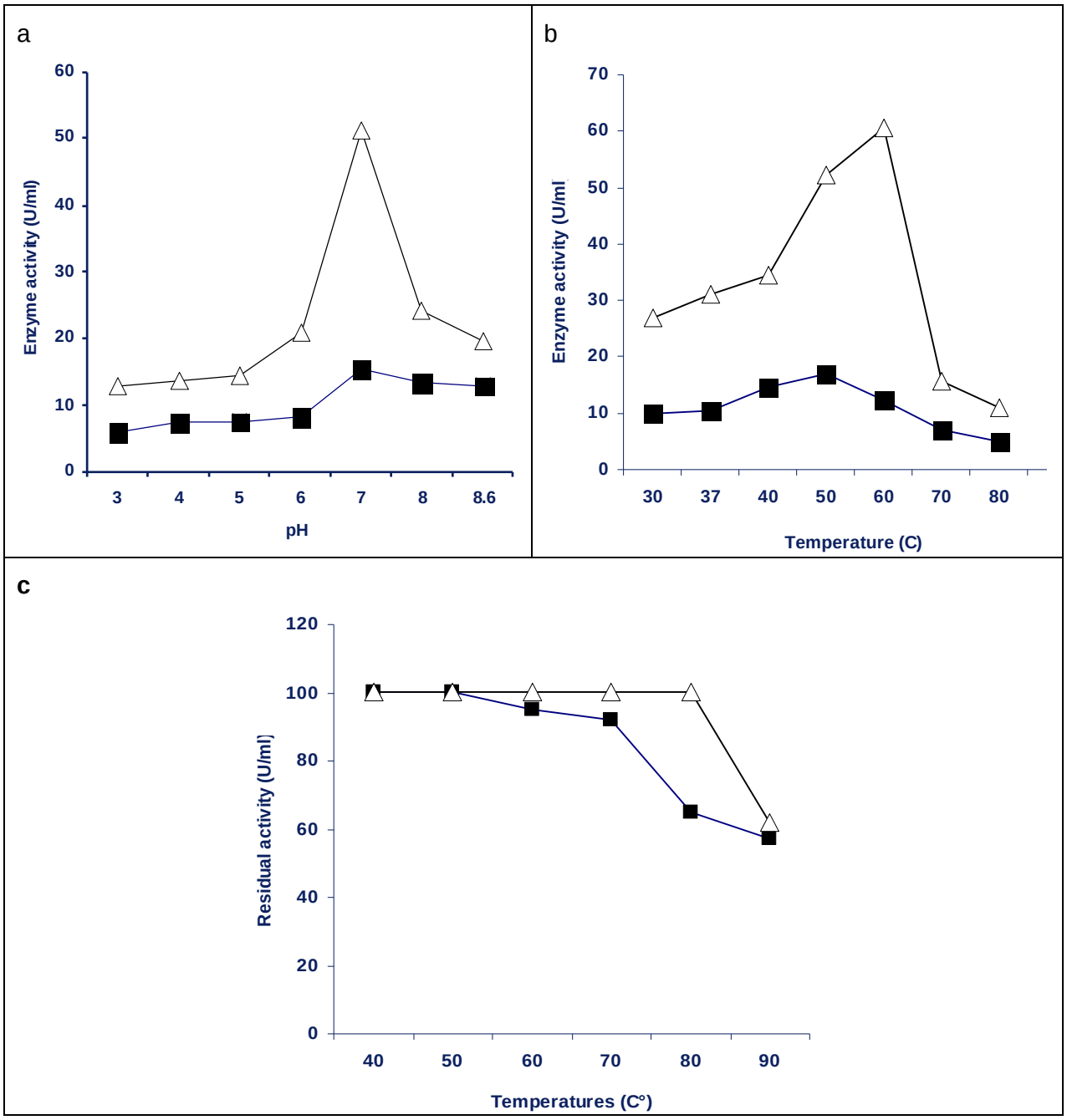
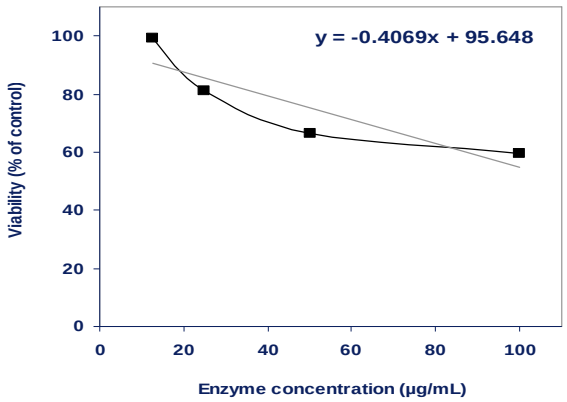


Fig (4): The effect of pH (a) and reaction temperature (b) on free (squared-line) and immobilized enzymes (triangled-line). A series of buffers was used: citrate buffer (pH 3 –

b



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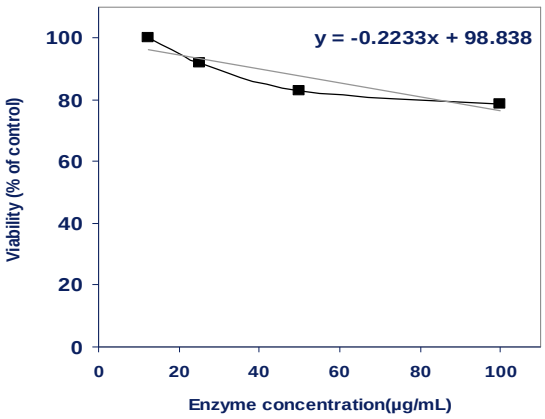

Fig (5): Anti- tumor activity against a. hepatocellular carcinoma cells (Hep-G2) and b. colon carcinoma cells (HCT-116). All data are average value of triplicate measurements.

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Context-aware Ubiquitous Data Mining Framework to Predict Malicious Activities

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Abstract: Advances in wireless sensor networks (WSN), handheld computational and communicational devices, communication systems, sensor stream processing, and data mining techniques, etc. lead us to develop such systems that perform in real-time fashion. These state of the art technologies can be very helpful to predict hazardous and criminal activities in time so that necessary actions can be taken to minimize social, economic and humanitarian loss. Context-aware computing can further increase the overall performance of the systems. A lot of research has been carried out individually in each of WSN, sensor stream processing, data mining, context-aware computing, etc. but very little attention is given to develop systems that utilize all these technologies collectively. In this paper we propose a framework to predict criminal activities and suggest suitable necessary actions on the basis its knowledge-base. The system proposed here gathers information from heterogeneous sensors, fuse that information, and generate real-time alerts to minimize the likelihood of disaster. This model utilizes real-time data from sensors; apply novel context-aware sensor stream association rule mining technique for prediction and decision tree (a machine learning technique) to take necessary action. This model makes ubiquitous data mining process more dependable and improves the reliability of the entire system. [Journal of American Science 2010;6(8):166-171]. (ISSN: 1545-1003).

Keywords: Ubiquitous Data Mining; Sensor Streams Mining; Association Rule Mining; Security System; Terror detection

1. Introduction

A large number of human fatalities and economic losses around the world daily are due to criminal activities. These activities are alarming in the modern world and thus need due attention. Daily news both from electronic and print media depict considerable numbers of lives lost everyday all over the world due to criminal activities, which disturbs the sustainability of society and makes people afraid to move around in society due to danger. Typically, busier place with increased human activity, such as shopping malls, stadiums,

busy streets, and bus stands, are more prone to criminal activities.

Information Technology can help make society more secure and sustainable. Specifically, Sensor Network Technology monitors environments by collecting and analyzing data from sensors monitoring particular environments.

The aim of this paper is to present the architecture of a Sensor Stream Mining framework that will analyze the real-time contextual information of a particular environment although such information will of course impede the computational and communicative powers of ubiquitous devices. In this paper, the contextual information analyzed in real-time includes persons' movement, actions, physical expressions, as well as current location and the importance of that location. In addition, we also considered personal demographics such as area of residence, criminal record, and security particulars to understand individual behavior and in turn design necessary precautionary measures. In the event of anomalous behavior, alerts and alarms caution security personnel to take necessary actions in a timely manner. These alerts help minimize the chances of disrupting the smooth flow of daily life and make society more sustainable as well as more dependable.

In the next sub-section of this paper we provide an overview of Ubiquitous Data Mining (UDM) and its potential for implementation in real-world scenarios. This emergent technology can play a vital role in predicting individual behavior in a desired location. Next sub-section describes the use of Sensor Stream and Ubiquitous Data Mining. After that next sub-section discusses integrating ubiquitous data mining and contextual information to predict intentions of a person. In Section 2, we present the architectural design of the system, including all sub-modules and interactions among modules. Finally, Section 3 concludes and describes implications for future research.

1.1 Ubiquitous Data Mining

Data mining is the process of extracting useful, hidden, and interesting patterns. Data mining technology classify data, identify clusters, and extract associations in the data. When performing data mining tasks, it is important to pay attention to the computational and communicative powers of systems. In conventional data mining systems, data is normally gathered at some central location in the form of a data warehouse to perform data analysis by incorporating statistical techniques and machine learning algorithms. The emergence of wireless and mobile devices has introduced a new dimension and enabled access to a large amount of data located at distributed and remote locations in the form of continuous streams. Ubiquitous Data Mining (UDM) is the process of analyzing data and information retrieved from remote systems on ubiquitous or mobile devices for time-critical applications [2]. Ubiquitous computing and data mining enables users to monitor, retrieve and analyze data from distributed and heterogeneous devices like sensors and mobiles [3, 4, 5, 6]. Continuous increases in the computational power of wireless and portable devices provides an opportunity to intelligibly analyze and monitor data in the form of streams by considering spatial and temporal constraints [7, 8]. The basic techniques for analyzing data and extracting hidden patterns are usually derived from traditional data mining, statistical techniques and machine learning methodologies. However, there is a need to alter those techniques in such a way that they are able to adapt on the bases of available resources. In UDM, resources are constrained in terms of memory, computational power, communication bandwidth, and availability for time-critical applications.

It is important to note that, as compared to traditional data mining, ubiquitous data mining is unable to analyze data in real-time while working in resource constrained environments with the same accuracy and quality of results. It is evident that the quality and accuracy of traditional data mining will be better than that of ubiquitous data mining, since applying the sliding window in a time-series-analysis fashion in combination with one-pass data analyzing algorithms will overcome memory and computational constraints [9, 10].

Handheld and portable devices need ubiquitous data mining software to receive continuous streams of data either from sensors that continuously read/gather data from their environment, or from external sources such as stock exchanges, web click streams, among other things. For time-critical applications, UDM modules analyze this continuous data in close to real-time and transfer the relevant retrieved information to central modules for aggregation or personalization.

Data-intensive applications are starting to appear on PDAs and cell phones such as cell-phone-based patient monitoring systems [21, 17], vehicles and driving monitoring systems [19], and wireless security systems. In the near future, some of the applications to be exercised include monitoring and analyzing data in embedded devices for smart applications, and the use of nano-scale devices for on-board monitoring. Thus, it is necessary to provide support for such applications in terms of advanced data analysis and prediction. Such applications pose various challenges and problems in order to analyze data and apply data mining techniques, which, in this domain, include:

- Developing efficient and effective techniques to analyze data in the form of continuous streams;
- Visualizing results and incorporating results on the screens of hand-held devices;
- Overcoming low bandwidth in wireless networks by introducing algorithms that minimize communication as well as number of hops. One solution is to increase dynamism in data traversing in order to minimize overhead for those devices that are under high computational activities or lacking memory [11, 5].

Further, the problem of limited batteries or battery power is another barrier to fully exploiting the potential of ubiquitous data mining. However, research has explored different methods to optimal utilization of this limited and vital resource to increase the overall lifespan of the network [14, 15].

1.2 Related Work

Data mining equips us with a number of techniques that can be used to model crime detection. In traditional data mining, data is gathered at some central location, after which a suitable data mining method is employed to detect useful as well as interesting hidden patterns from that data. Grouping or classifying similar data into clusters and then separating it from dissimilar patterns can help to solve crime problems that otherwise seem unsolvable by normal or manual techniques [16]. Similarly, sensor networks are used to monitor human activities at home or in offices [17].

Context-aware ubiquitous data mining is applied to detect car accidents as well as to warn drivers before the crash occurs. These precautionary alarms are generated on the basis of contextual information gathered from both on-board sensing devices and the environmental sensor network infrastructure [18, 19]. To minimize car accidents, an architectural model is developed by incorporating road

conditions and the psychological factors of drivers [1]. This early breakthrough utilizes contextual information to warn individuals about their driving and provide the necessary information to help them perform better under their current circumstances. It is expected that context awareness will decrease the number of car accidents and in turn lives lost. Detecting divergent patterns and critically analyzing those patterns in detail reveals that anomalous patterns normally cause alarming situations.

Similarly, researchers have also experimented with monitoring patients who have been in emergencies and putting their information onto mobile devices on a contextual basis [17, 21]. The relevant patient information facilitates in the treatment process as it is displayed on the handheld devices of the attending practitioner or nurses.

To detect human behavior in real time, unsupervised feature extraction is incorporated and human activities are made discrete. To preserve privacy, the analysis of human activities is performed locally on small-scale devices, considering factors such as location visited, goods purchased, tasks performed and routes adopted for traveling, etc., so that any unusual behavior can be detected by applying link analysis techniques [20].

To date, no research has attempted to combine contextual information, personal profiles, and location-based data in order to detect society's vulnerability, nor has ubiquitous data mining had the power to enable prediction processes in (close to) real-time. Therefore, combining heterogeneous sensor streams and predicting human intentions in real-time enables us to make society more secure and reliable

1.3 Context-aware UDM to predict uneven situations

A situation is the state of affairs of an entity, and discussing both situation and awareness collectively requires knowing about the state of affairs of an entity. Context-awareness includes information about the location, person and the environment in which the person is currently located, among other things. Compared with others in the same environment, information about a person can detect his or her behavior in the current environment, as well as any sort of discrepancy in movement. Information about the environment enables us to determine the importance of the location as well as the likelihood of criminal activities regarding the importance of location based on busyness, for example, in crowded locations such as bus stops, airports, and railway stations. Security personnel have PDAs capable of applying UDM techniques that analyze readings on contextual bases from sensors distributed around the environment. Based on such contextual information, the unusual behavior of each individual is determined.

An automatic alert informs security personals about the expected intentions of a specific person and suggests a set of actions appropriate for necessary countermeasures. Information about the person including age, race and colour, place of residence, current residence, history, purchasing habits and recent purchases, and traveling history may help determine the likelihood that a person will engage in malicious activities. Similarly, information about place or location include importance of location, activities performed there, number of people present, number of key persons or prominent folk present, and criticality of place. Combined, these things make up the scenario, and can help detect vulnerability to risks. Recognizing such situations in a manner that is timely, reliable, and cost-effective is challenging. Similarly, it is also very difficult to select and perform appropriate countermeasures in a timely and reliable manner.

2. Proposed Methodology

There is a great diversity of engineering and safety in life and society security areas. However, research and development have not yet considered the advantages of combining situation and environment awareness, personal bio-data and history, and criticality of location to protect society from criminal activities. To consider this shortcoming, we propose a conceptual model that employs ubiquitous data mining for situation detection in real-time. This model uses context-awareness and proposes countermeasures for the event detected. Information about these events and countermeasures will be propagated to security personnel through hand-held devices. The conceptual architecture is presented in Fig. 1.

Our approach is novel in that it integrates personal behavior, contextual information and environmental factors, historic data about criminal activities, and personal profile or bio-data. Similarly, it is also innovative in that it uses personal behavior and history with ubiquitous data mining to detect and predict the possibility of anomalous events. These factors have yet to be used collectively to assess risk factors and predict a person's intentions in a given location.

Information about the person's location and environment is acquired to measure the risk of criminal activities from each individual. To achieve this objective, we incorporate a wide range of technological devices such as sensors to gather contextual information about movement in terms of directions as

well as frequency. We define movement pattern at some specific location by observing the movement of the majority of the people in that vicinity at a given time.

In real-time, we analyze our data on PDA'S or hand-held devices available to security personnel in order to assess the risk of criminal activities. Using past patterns of criminal activity, historic data, personal profiles, and current contextual information, the model detects odd events. For example, if a person had been involved in some criminal activity, has been identified as suspicious, or his/her current contextual information clusters him in a criminal category, the model alerts security personnel to take necessary countermeasures.

Initially, we may train the model on past/historic data of criminal activities, or we may use simulated data. Subsequently, our database will continue to grow, as the number of criminal activities around the world are substantial. Patterns incorporated from past incidents provide further insight into the causes of particular incidents. One of our research goals is to extract the most important and influential factors in detecting criminal activities. In the future, if any combination of these factors is found, security personnel are alerted about the criticality of the situation before a criminal event occurs.

As uneven event detection at critical locations require quick and robust process following appropriate actions therefore in our ubiquitous framework three levels are employed. Firstly experts at monitoring centers continuously check the streaming data from the locations under observation and alerts the security personals at the location but all this process is automated. Secondly uneven events can also be detected by analyzing the streaming data collected and stored in the data centers for futuristic use. This helps to improve performance of the emergency detection system. Thirdly motion detectors and video cameras installed at location transmit abnormal motions to the devices of security personals in the same vicinity as well as transmitting those to the monitoring centers. An early action can be taken by the security staff before receiving an alert from the monitoring centers hence improving performance.

Before taking any action, the probability of an unusual event must be calculated. If the results are above a specified threshold, security personnel are alerted. On the other hand, if the results are lower than the specified threshold, but within some predetermined range, then that person will be placed under close observation to avoid the possibility of vulnerability. The success and failure rates of the alerts generated, as well as the resulting predictions will be analyzed and the main repository database will be updated. This process of continuous learning improves system performance and future decisions become more accurate and reliable.

To save energy and communication cost, data sampling is performed. Only that data is recorded which is predicted as malicious and propagated towards main repository database for futuristic use. It will decrease network traffic and utilization of communication channel is expected to optimize. This mechanism increase overall lifetime as well as performance of network.

To convert this proposed model into practical shape, several issues need consideration and catering those factors is necessary to obtain expected results as well as accuracy. Few factors are:

- **Data:** multidimensional data from heterogeneous sensors arrive in the form on continuous streams at a high rate. To deal with such a substantial amount of data in real-time is a complex challenge. Moreover, classifying this continuous stream of data by incorporating a predictive model requires access to historic data about criminal events. Normally, data about a location where criminal action has occurred is available, as is information about the person who committed the crime. However, personal information about history of the criminal is often unavailable. Similarly, the movements and actions performed before committing a crime are not available as there is no existing system to record such information. To obtain such data, a simulator is ideal at initial stages; later, continuous learning processes enable models to become more realistic.
- **Analysis:** As lightweight algorithms have already been developed which perform well in resource constraint environment but there is a need to optimize those algorithms and cater those so that data obtained from integration of heterogeneous streams can be analyzed and process in effective manner [12, 13].

Human Rights and Legal Issues: Unfortunately, public organizations, constitutional rights, and basic rules of independence are core hindrances in the implementation of this model. To deploy this model, constitutional shelter as well as acceptance by citizens is required, given that such a system will be perceived an invasion of their privacy due to modern ethics, which do not allow viewing an individual’s personal details without sufficient and appealing proof.

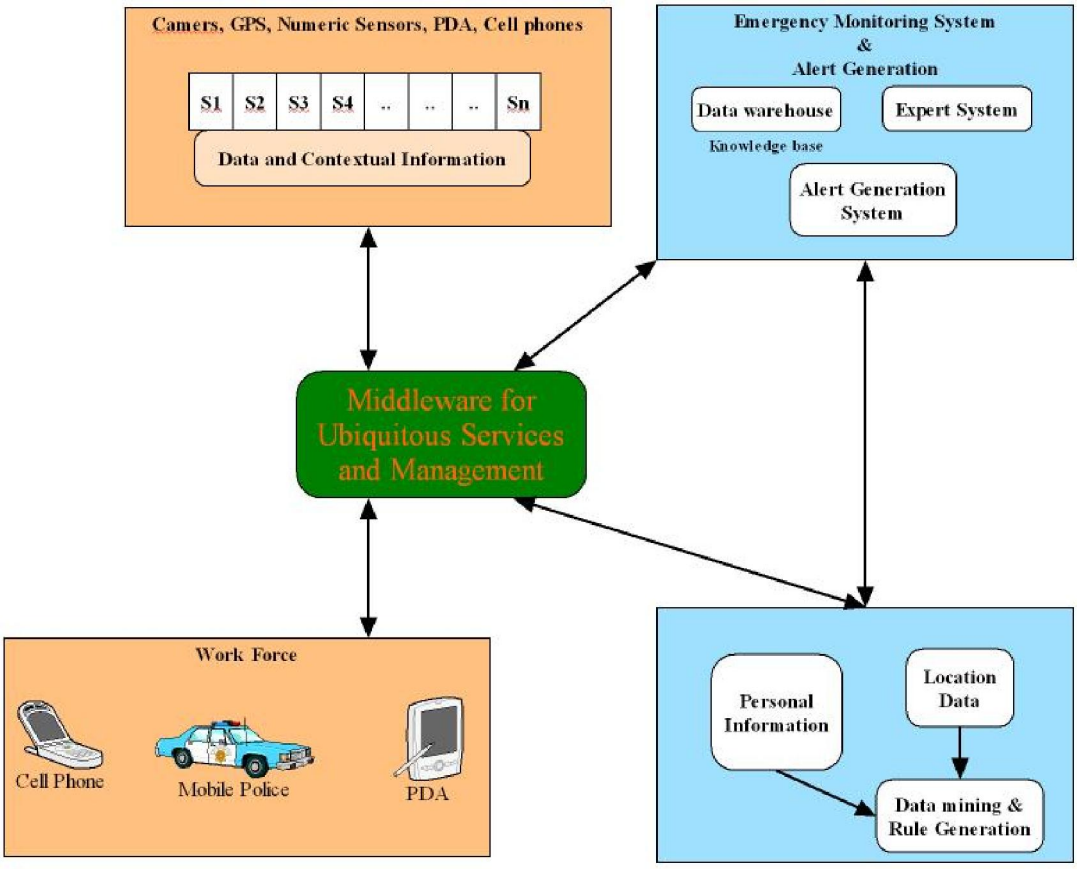

Fig. 1 Ubiquitous Data Mining and Context-awareness to Predict Uneven Events

3. Conclusion and Future Work

There is large toll of human fatalities and economic losses on daily bases due to criminal activities all over the world. Securities and law enforcing agencies have taken several measures to reduce causalities and financial losses to make society sustainable, dependable and more reliable. The methods applied so far have not caused some significant improvement in this regard. Therefore some advanced and alternate method to develop a sustainable society by incorporating state of the art technology is proposed. This paper presented a novel approach to detect risk by analyzing factors including movements of persons, their track record, historic data and location. Other potential factors can also be found and mined to improve overall performance and accuracy of the system. Using this model enable security personals to predict intensions of people in some specific vicinity and take necessary actions in timely manner. It is expected that this technique will bring considerable improvement in the reliability of society.

As a next step, we are planning for laboratory implementation of this system and will propose necessary actions in case of detected risks. After successful completion of laboratory implementation, real life deployment will be considered with necessary changes.

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The Influence of Temperature, Light and Pre-treatment on the Seed Germination of Critically Endangered Sikkim Himalayan Rhododendron (*R. niveum* Hook f.)

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Abstract: *R. niveum* Hook f. is a beautiful and endangered rhododendron that has limited distribution in the Sikkim Himalaya. In an effort to improve and promote the propagation of this over-exploited plant, the effect of temperature and light on the germination of seeds was investigated with various presoaking treatments of plant growth substances (GA_3, Kinetin and BAP) and nitrogenous compound (KNO_3). The combined effect of GA_3 with Kinetin or BAP (25 µM) was also examined. Seeds were given a presoaking treatment with GA_3, BAP or a combination of both to influence germination. A temperature of 21^{o}C was found optimum and showed 34.33% germination, with 21 days for onset and 50 days for final germination under 16 hr light condition. The seeds of *R. niveum* need light to trigger the germination and no germination was observed in darkness. Though the seed viability was 86% as determined by tetrazolium staining, maximum germination of 63.67 % was obtained only when the seed was soaked in GA_3 + BAP (25 µM each) solution for 24 h and incubated for germination at 21^{o}C, constant temperatures in 16 hr photoperiod. The other treatments were far less effective in promoting the germination of this endangered species. The present study indicates that constant 21^{o}C, temperature incubation and 16 hr photoperiod have a positive relationship with seed germination of *R. niveum* even under no pre-treatments. Seeds stored at low temperature (4 ^{o}C) could maintain viability for less than six month. Here, it is the first time we have described the seed germination requirements of *R. niveum*, which are under threat due to anthropogenic pressure [Journal of American Science 2010;6(8):172-177]. (ISSN: 1545-1003).

Keywords: *Rhododendron niveum*; seed germination; temperature; light; Sikkim Himalaya.

1. Introduction

The genus *Rhododendron* L. (family Ericaceae) includes a number of decorative species and cultivars that are often utilized in accent, garden and park architecture designs. In India, it is represented by about 80 species, of which 36 species are found in Sikkim (Pradhan and Lachungpa, 1990; Singh et al., 2003; Bhattacharyya and Sanjappa, 2008). Among these, *R. niveum* is considered to be the most beautiful with lilac-purple flowers and dull green foliage having creamish white undersurface and was declared as the State Tree of Sikkim. Over exploitation of this species has caused a serious threat. The regeneration status in the form of available seedlings/saplings is very poor due to the above situation for many of the rhododendrons (Semwal and Purohit 1980; Singh et al., 2008a; Singh et al., 2008b; Singh 2009). So far no attempt has been made to develop suitable germination protocols and large scale production of rhododendron species which are under the threat of extinction. Germination is a complex process that is controlled by several biological (species, seed viability, seed dormancy, seed size) and environmental (moisture availability, temperature, relative humidity, light intensity and duration) factors. Since plant species vary in their response to these factors, it is important to determine the optimum conditions and seed treatments for germination and seedling establishment under the prevailing climatic conditions. Though studies have been conducted on different aspects of *R. niveum* (Singh et al., 2008a; Singh et al., 2009), no information is available on seed germination and seed storage aspects of this species.

The aim of the present study was therefore to investigate the effects of growth regulators and nitrogenous compound on the germination of *R. niveum* seeds under different temperature and conditions both in light and darkness.

2. Material and methods

2.1 Brief description of plant material: *R. niveum* Hook. f. [Snow-leaved Rhododendron, Nepali: Hiun-pate Gurans)]; First report by Hooker (1849). More or less localized within 3500-4500 m elevations of the Sikkim Hills, *R. niveum* is scarce in the Darjeeling hills, almost a rare element. Within the rhododendron community of the region, *R. niveum* is a comparatively large tree, attaining heights of over 3 m. It is quite a distinctive species as far as its general habit and flowers are concerned. Flowers of deep magenta or lilac with darker nectar pouches appear in

April and May. The leaves are hairy underneath. Under our recent field exploration it has been found out that the *R. niveum,* endemic to Sikkim *(*also the state tree of Sikkim) is limited to a microniche at a place called Yakchey in northern Sikkim, and individual count shows less than 45 plants. Flowering-April; Fruiting-July. As the species is much less in number and at the verge of extinction it needs high-priority conservation measures.

2.2 Seed Collection: Seed lots of *R. niveum* Hook. f. were collected in October 2008 in Yakchey in North Sikkim (Longitude 27^o 43' North, and Latitude 88^o 45' East with an elevation 3500 m amsl). Seeds were collected in small cotton bags. Immediately after collection, capsule were dried at room temperature for 1 week, then stored in small plastic bags at 4^{o}C.

2.3 Seed viability assessment: To ensure that the seeds used for the experiment were viable and of high quality, the sample lots were subjected to viability test using the tetrazolium technique (Peters, 2000). Seed viability was observed immediately at the time of collection and after 2, 4, 6, 10 and 12 months of storage at 4^{o}C.

2.4 Effects of temperature and light: To determine the effect of different temperatures, the seeds were incubated at 7 ^{o}C, 10 ^{o}C, 13 ^{o}C, 17 ^{o}C, 21 ^{o}C, 25 ^{o}C and 30 ^{o}C constant temperatures under either a 16 h photoperiod or total darkness. To determine the effect of different photoperiod, the seeds were incubated at 21, and 25^{o}C constant temperatures. These treatments were conducted in 0 (total darkness), 2, 4, 8, 12, 16, 20 or 24 hr photoperiod conditions for 30 consecutive days in the seed germinator. Daily photoperiod treatments were regulated by removal and placement of the petri dishes into black carbon paper. Seed germinators were equipped with cool-white fluorescent lamps that provided a photosynthetic photon flux (400-700 nm) of approximately 40 $\mu mol.m^{-2}\cdot s^{-1}$.

2.5 Effects of pre-treatment: Various seed treatments were attempted for improving germination percentage. To test germination, seeds were disinfected with 0.05% aqueous solution of $HgCl_2$ for 3 min, washed thoroughly with double distilled water, and dipped in various pre-treatment solutions, viz. gibberellic acid (GA_3); (25, 100, 250 µM), 6-benzylaminopurine (BAP); (25, 100, 250 µM), Kinetin (Kin); (25, 100, 250 µM), GA_3 + BAP (25 µM each), GA_3 + BAP (250 µM each), GA_3 + Kin (25 µM each), GA_3 + Kin (250 µM each), potassium nitrate (KNO_3); (50, 100, 150 mM) for 24 h. Control was maintained using double distilled water. After treatment, seeds were washed with distilled three times and placed in glass petri dishes (90 mm) on a single layer of Whatman No. 1 filter paper with about 5 ml of distilled water. A set of seeds without any treatment was used as control. The moisture levels of filter paper were maintained by adding distilled water as required. For prevent of infection and evaporation of solution, all of plates were closed with parafilm. All operations were done under laminar flow. Germination was monitored daily from the date of seed sowing. Seeds showing signs of decay were removed immediately from the Petri plates. Seeds were considered germinated when the radicals reached ≥1 mm in length. Germination percentage was recorded every day until no further germination was found. Observations on germination of seeds kept in dark were taken in dull green light. Percent germination was calculated as a mean of four replications per treatment.

2.6 Statistical analysis: Every treatment has three replicates of 25 seeds each because of the limited availability of seeds. Standard error of the mean was calculated. Least significance difference (LSD) at $P<0.05$ level was calculated following the method of Snedecor and Cochran (1967).

3. Results and discussion

3.1 Seed viability: In view of the low germination rates, a tetrazolium test was carried out, which showed that 86% of the seeds were viable at the time of collection. It was noted that after two months of storage at 4^{o}C, seed viability decreased significantly (P = 0.05) and the end of twelve months, it was only 8% (Figure 1). Loss of moisture content and very low reserves of nutrients are the chief cause of deterioration of seeds under storage condition. Seeds from species of the genus *Rhododendron*, tend to be relatively short-lived compared with species from other families and/or genera (Troup, 1981; Hay, 2006).

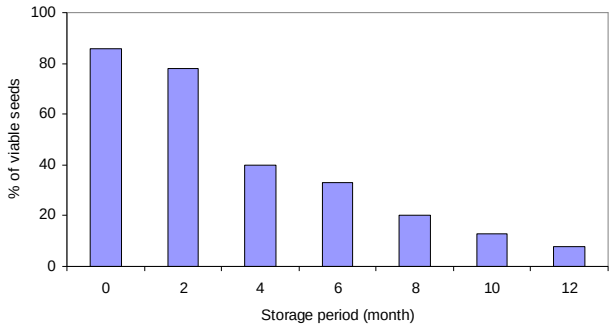

Figure 1. Changes in seed viability of *R. niveum* during storage at 4ºC.

3.2 Effects of temperature and light: In this study, effects of temperatures and light treatments on germination rate of *R. niveum* are presented in Table 1. Since the threshold temperature for germination was 13°C, the presentation of the 7 and 10°C variants were omitted in the Table 1. The rate of germination increased with increasing temperature, and then decreased at temperatures above 25 °C. At 13 °C, the percentage germination was significantly lower than those at other temperature (Table 1). Furthermore, variation germinability at different temperatures was significant (F = 4.36; P = 0.05). The optimum temperatures for germination of the seeds of these species are 17-21 °C and no difference in germination was observed between seeds incubated at 21-25 °C, but at 7 °C the seed was not germinated. Seedlings germinated at 25 °C and 30 °C under light condition were not seems to be healthy and most of them will die after producing from radicles. High temperature is well known to prevent radicle and shoot

Table 1. Seed germination at different temperatures under 16 h light and dark conditions

Temperature/ light or dark	Days required for onset of germination	Days required for completion of germination	Germination percentage
13°C, Light	30	55	14.33 ± 1.86
17°C, Light	26	54	25.00 ± 1.73
21°C, Light	21	50	34.33 ± 0.88
25°C, Light	25	48	35.00 ± 0.58
30°C, Light	36	49	33.33 ±1.76
Alternate temperature (21°C in light and 10°C in dark for 12 h)	21	50	32.66 ± 3.38

F = 4.36 (significant at P = 0.05).
Values are the mean ± SE of four replicates and the experiment was repeated twice.
At 13°C, 17°C, 21°C, 25°C, 30°C temperature under dark condition no germination took place.

elongation by inhibiting synthesis of protein and nucleic acid (Hegarty and Ross, 1979; Sivaramakrishnan et al., 1990). Germination at alternate temperature (21°C in light for 12 h and 10°C in dark for another 12 h) was also not very successful (Table 1). Seeds of *R. niveum* required light for germination regardless of temperature. This effect is illustrated by comparing germination percentages between 21 °C and 25 °C at different photoperiods (Figure 2). Among different photoperiods, 16 hr duration was found optimum for germination. At 21 °C, increasing photoperiods increased germination with 22.67 % and 34.33 % germination occurring by day 30 for the 12- and 16 hr photoperiods, respectively.

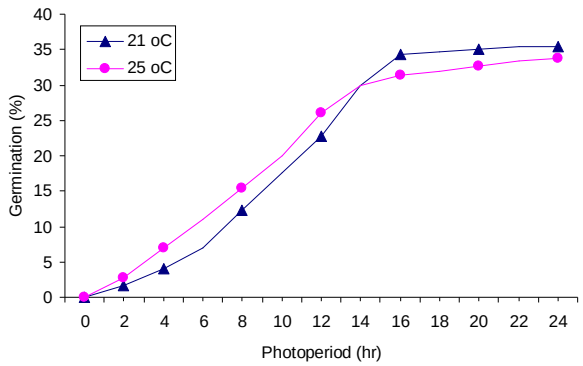

Figure 2. Cumulative (30-day) seed germination of *R. niveum* as influenced by photoperiod and temperature. The effect of photoperiod on germination percentage was significant (F = 4.68; P = 0.05) for both temperatures.

Seeds of *R. niveum* in dark did not germinate at stipulated experimental time. It has been conclude that most of the Himalayan species do not show intrinsic dormancy and that germination in most of

these is better in light than in darkness (Semwal and Purohit, 1980). Our results indicate that light is a necessary factor for germination of *R. niveum* seeds. The analysis of variance proved that increasing photoperiods have had significant effects ($F = 4.68$; $P = 0.05$) on percent germination at both temperatures of 21°C and 25°C. Generally, 8-12 hr day light is necessary for maximum germination and growth in forest trees (Kozlowski, 1971), whereas in the present studies a 16 hr photoperiod was found optimum for seed germination and seedling growth. A similar effect has been reported for other species of rhododendron (Blazich et al., 1991; Blazich et al., 1993; Tort 1996; Arocha et al., 1999; Kumar et al., 2004; Faravani and Bakar 2007). In general, absence of light has a negative effect on germination in several Rhododendron species (Juntilla, 1972; Singh, 2008).

3.3 Effects of pre-treatment: The effects of different pre-treatments on *R. niveum* seed germination are shown in Table 2. In the control treatment, *R. niveum* showed the lowest germination percentage and longest time to germination. Of the growth regulator pretreatments alone, GA_3 and BAP shortened the time required for germination by alleviating its germination-delaying effect. On the other hand, double combinations of these regulators much more successfully shortened the period of germination. Seeds treated with GA_3 began germinating sooner and germination was completed earlier than that of untreated seeds at low temperature (21ºC). Germination of untreated seeds also began after 21 days. They germinated slowly, but after 30 days, the rate began to accelerate. All concentrations of GA_3 had higher germination and increased seedling vigour over control, where its highest concentration (250 µM) was most effective. More than two fold improvement in germination was recorded in seeds treated with GA_3 + BAP (25 µM each). The other treatments were far less effective in promoting the germination of this endangered species. The present study indicates that constant 21°C, temperature incubation and 16 hr/8 hr (light/dark) photoperiod have a positive relationship with seed germination of *R. niveum* even under no pre-treatments. However, on the basis of ANOVA, significant variation ($F = 4.48$; $P = 0.05$) was found in germination due to these hormonal treatments. However, this study demonstrates that exposure of seeds of *R. niveum* to the GA_3 + BAP (25 µM each) for 24 h and incubation at 21°C in 16 h light photoperiod conditions can result in almost 63.67% germination and should provide a method to assist in the *ex situ* management of *R. niveum.* Several studies from recent years have shown that gibberellin is an effective germination stimulator (Thompson, 1969; Juntilla, 1972). According to the magnification of stimulatory effects of GA_3 and BAP in this study, conclusion can be drawn that GA_3 are permitted to reach their active sites through the modifying influence

Table 2. Effects of various pretreatments on seed germination of *R. niveum* at 21ºC and 16 h photoperiod.

Treatments	Days required for onset of germination	Days required for completion of germination	Germination percentage
Control	21	50	32.00 ±1.15
GA_3 (25 µM)	18	27	34.67 ± 0.88
GA_3 (100 µM)	17	24	46.33 ±0.88
GA_3 (250 µM)	18	23	51.00 ±1.52
Kinetin (25 µM)	22	47	35.00 ± 0.58
Kinetin (100 µM)	23	47	36.33 ± 0.33
Kinetin (250 µM)	20	48	33.67 ± 0.77
BAP (25 µM)	18	31	34.67 ± 0.67
BAP (100 µM)	17	32	35.67 ± 1.45
BAP (250 µM)	18	30	39.00 ± 0.49

GA_3 + Kinetin (25 µM each)	25	44	36.00 ± 0.58
GA_3 + Kinetin (250 µM each)	21	44	29.67 ± 0.88
GA_3 + BAP (25 µM each)	15	22	63.67 ± 1.45
GA_3 + BAP (250 µM each)	14	22	56.00 ± 0.58
KNO_3 (50 mM)	22	46	34.33 ±1.20
KNO_3 (100 mM)	21	45	33.67 ± 0.67
KNO_3 (150 mM)	21	45	32.67 ± 1.45

F = 4.48 (significant at *P* = 0.05).
Values are the mean ± SE of four replicates and the experiment was repeated twice.

of cytokinins on transport across membranes and are thus able to initiate the biochemical processes necessary for germination to occur (Thomas et al., 1975). The cytokinin probably penetrates the testa and neutralizes the inhibitors present in the embryo, thus enabling the embryo to rupture the seed coats (Khan, 1971).

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17/2/2010

Numeric modeling of carbon dioxide sequestration in deep saline aquifers in Wangchang Oilfield-Jianghan Basin, China

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Abstract
Climate change has become one of the most pressing issues globally with the increased emissions of greenhouse gases (GHG) into the atmosphere and the resultant impacts on the environment threatening development and life on Earth. Anthropogenic emissions of carbon dioxide (CO_2) into the atmosphere are largely blamed for this effect and carbon capture and storage (CCS) in geologic media is an enabling technology that can be utilized to mitigate global warming whilst still continuing to use fossil fuel until the end of the fossil era. Deep saline aquifers in sedimentary basins have a large potential for CO_2 storage. China has a rapidly growing economy with large remaining coals reserves hence her future CO_2 emissions are bound to increase from the present high levels. We have developed a simple 2-dimensional homogenous model to study the spatial and temporal distribution and storage of CO_2 injection into Wangchang Oilfield, Jianghan Basin, one of China's largest inland sedimentary basins. A 10 year injection phase followed by 90 years shut-in is modeled. During the injection period, most of the gas exists in a supercritical state trapped beneath the caprock. When injection ceases, the gas gradually dissolves into the formation brine. Increase in vertical to horizontal permeability ratio, residual gas saturation and salinity all have different effects on the CO_2 spatial migration, dissolution in brine and overall storage. [Journal of American Science 2010; 6(8):178-187]. (ISSN: 1545-1003).

Key words: *Geological storage; deep saline aquifers; carbon dioxide; numerical modeling; Wangchang Oilfield-Jianghan Basin; China.*

1. Introduction

Climate change has become one of the most pressing issues globally with both the developed and developing countries decrying the increasing emissions of GHG into the atmosphere (IPCC, 2005, Bekker et al, 2009). Combustion of fossil fuel mainly from power production and transportation is largely blamed for this effect with CO_2 being the leading GHG largely responsible warming in global temperatures with a warming forecast of 1.8-4.0^oC if current trends persist (IPCC, 2007). CO_2 emissions in the atmosphere have increased over the past 200 years from about 280 parts per million (ppm) to the current 380 ppm levels, a 30% increase (IPCC, 2007). If effective and urgent mitigation measures are not put in place, under the current "business as usual" scenario, these levels could double over the next 50 years (IPCC, 2007). CCS in geologic media is regarded as one of the possible mitigation options for reducing anthropogenic GHG emissions in the atmosphere (IPCC, 2005). Potential geologic formations include porous and permeable depleting or depleted oil and gas fields, unmineable coal seams and deep saline aquifers in sedimentary basins (IPCC, 2005; Holloway, 2008).

CCS is a process consisting of the separation of CO_2 from industrial and energy–related sources

(capture), transporting it to a storage location and subsequently disposing it in geologic media either permanently (sequestration) or for significant periods of time (storage) (IPCC, 2005, Bachu, 2008). Therefore, CCS can be applied to large sources of CO_2 including large fossil-fuel and biomass energy facilities; major CO_2-emitting industries; natural gas production plants; synthetic fuel plants and fossil-fuel-based hydrogen production plants, among others. Globally, large-scale field injections are already being done at Alberta, Canada; Sleipner Vest Field, Norway; Gippsland Basin, Australia (Holloway, 2005; Bachu, 2008) and Nagaoka, Japan (Sasaki et al, 2008). Other GHG emission mitigation options include switching to more energy-efficient machinery, use of less carbon-intensive fuels, nuclear power, renewable energy sources, enhancement of biological sinks and the reduction of non-CO_2 GHG emissions in addition to socio-economic and policy changes (IPCC, 2005; IPCC, 2007; Celia and Nordbotten, 2009).

China is the second largest emitter of CO_2 globally after the USA and it is projected to be the world's largest emitter of GHG by 2025, thus China's

action to reduce its emissions is requisite (Meng, et al, 2007, Li et al., 2009). In addition, it has large remaining coal reserves with a rapidly growing economy contributing high amounts of CO_2 emissions into the atmosphere making CCS a viable mitigation option the country. China has 629 power plants emitting 72% of 3,890 million tons CO_2 per annum with the remaining percentage split among 994 sources from other industrial sectors (Li, et al., 2009). These large emitters are concentrated along the heavily industrialized North China plain and the coastal zones (Li, et al., 2009). Fortunately, China has a large CO_2 storage capacity in both onshore and offshore sedimentary basins with continental deep saline aquifers offering over 90 percent of total geological storage (Li, et al., 2009).

CO_2 can be stored in deep saline aquifers by three principle mechanisms. (1) *structural trapping/hydrodynamic trapping* where CO_2 is trapped as a supercritical fluid (hereinafter referred to as 'gas') below a low permeability confining caprock according to the structural lithology of the storage zone or as an immobile phase trapped in the pore spaces of the reservoir rocks (*residual trapping)* (IPCC, 2005; Audigane et al., 2007); (2) *solubility trapping* where CO_2 dissolves *in situ* in the formation brine, a process which increases the brine density and lowers the pH (increases the acidity) (Xu et al., 2004; Ghanbari et al, 2006); and (3) the decreased brine pH induces *mineral trapping* where CO_2 reacts with carbonate minerals such as calcite, magnesite, siderite, dolomite and/or dawsonite through a series of reactions (dissolution and complexing) with the dissolved ions found in the brine aquifer as well as with minerals composing the host rock matrix leading to chemical precipitation of secondary carbonates (Allen et al., 2005; IPCC, 2005; Xu et al., 2005). The interaction of CO_2 with alkali aluminosilicate minerals will result in the formation of dissolved alkali carbonate and bicarbonates thereby enhancing solubility trapping (Xu, et al, 2006, Xu, 2009). These four trapping mechanisms occur at different time scales (see figure 1). Initially, structural trapping is the dominant mechanism but with time, residual and solubility trapping become significant. The advantage of residual, solubility and mineral trapping and is that they do not rely directly on the integrity of the cap-rock as physical trapping does hence they enhance storage security (Suekane, 2008). The objective of this paper was to numerically simulate the temporal and spatial distribution and storage of CO_2 injection in Wangchang Oilfield, Jianghan Basin, China. A short-term period of 100 with 10 years injection followed by 90 years shut-in is modeled. Three sensitivity tests are done for permeability, residual gas saturation and salinity.

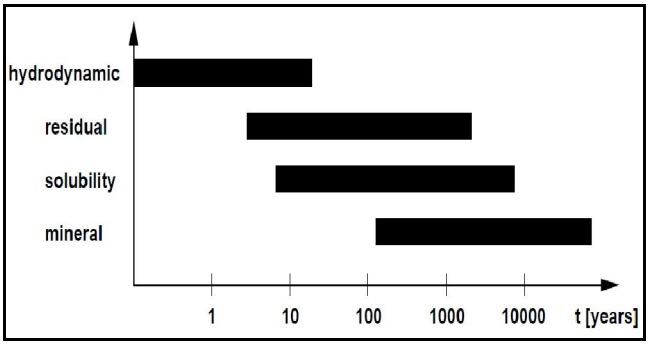

Figure 1. Predominant CO_2 trapping mechanisms and their estimated times within saline aquifers (Bieliski, 2007, p7)

2. 0. Numerical method

2.1. Simulation tool

Modeling geochemical systems to predict future outcomes is an indispensable tool in geochemical engineering. This is because the geo-environment is highly complex and investigation by laboratory experiments can only apply to subsystems and processes (van Gaans, 1998). For CO_2 sequestration research, numeric simulation is essential prior to experimental and field demonstrations on account of feasibility and cost and also because mineral alteration rates to CO_2 injection in saline aquifers are very slow especially with regard to aluminosilicates which are not amenable to experimental study (Xu, et al, 2007). In our research, simulations were done using TOUGH2 version 2 (Transport of Unsaturated Groundwater and Heat), a general purpose simulator program for multi-dimensional fluid and heat flow of multiphase, multi-component fluid mixtures in 1-, 2- and 3-dimensional porous and fractured media (Pruess, et al, 1999). It is a program that has wide application including geothermal reservoir engineering; nuclear waste isolation studies; environmental assessment and remediation; and flow and transport in variably saturated media and aquifers (Pruess, 1999; Wu and Pruess, 2000).

In our simulation, ECO2N, a fluid and property module of TOUGH2 designed for applications of geologic sequestration of CO_2 in saline aquifers is used (Pruess, 2005). ECO2N gives an ample description of the thermodynamics and thermophysical properties of mixtures of water, brine and CO_2 under conditions typically encountered in saline aquifers targeted for CO_2 disposal and gives results largely within experimental error for

temperature, pressure and salinity conditions within the range of $10^oC \leq T \leq 110^oC$; $P \leq 600$ bar and salinity of up to full halite saturation (Pruess, 2005). Flow processes may be modeled isothermally or non-isothermally and phase conditions represented may include a single aqueous or CO_2-rich phase as well as two-phase mixtures (Pruess and Sypcher, 2007). Solid salt may precipitate or dissolve adding a third active phase in the system (Pruess and Spycher, 2007). Mutual solubilities for CO_2 and H_2O in gas and brine phases are calculated from the correlations of Spycher and Pruess (2005). Spatial discretization is achieved through the Integral Finite Difference method (IFD). Time is discretized fully implicitly as a first-order backward difference (Pruess et al., 1999).

2.2. Model setup

A simple two-dimensional radial model with a cylindrical geometric configuration is used to study the temporal and spatial distribution of CO_2 as injected into the first segment of Qian reservoir of Wangchang Oilfield in Jianghan Basin, Hubei Province, China (hereinafter referred to as Wangchang for simplicity) (PRC, 1987). Jianghan Basin (see figure 2) in Hubei Province is one of China's largest inland petroleum reservoirs with an area of 28,000km^2 (PRC, 1987) and a potential for CO_2 storage by solubility trapping in deep saline aquifers of 52,800 million tons CO_2 (Li et al., 2009). Wangchang lies within Qiangjiang depression and is mainly composed of alternating sequence of sandstone and mudstone or sandstone and salt (PRC, 1987). The mudstone and salt offer good sealing conditions while the sandstone are a good storage formation making the Wangchang a suitable reservoir for CO_2 injection. The salinity at Wangchang ranges between 120 to 300mg/l. The air permeability of Qian 1 lies within 1880 x 10^{-9} to 2537 x 10^{-9} mm^2 and the porosity ranges between 23 to 28.5 percent (PRC, 1987).

In our model, CO_2 was injected into a homogenous sandstone aquifer of 100 meter thickness. The depth at the top of the reservoir is 1000 meters below the earth surface. The aquifer is discretized into 10 grids of constant 10 meters spacing in the vertical direction while in the horizontal direction, a distance of 10,000 meters is modeled with a radial spacing that increases gradually away from the 0.3 meter injection well positioned at the center of the domain. A total of 65 radial grid elements are used including the injection well. The outer boundary was assumed to be infinite acting by making the last grid inactive while the top and bottom cap-rock layers are assumed to be impermeable. A hydrostatic pressure distribution over the depth was initially specified and local phase equilibrium is assumed with a constant temperature. CO_2 is injected at a supercritical state of 31.04^{o}C temperature and 73.82 bar pressure into the lower segment at a constant rate of 50kg/s for 10 years, equivalent to 1.6 metric tons per year followed by 90 years shut-in period. Other hydrological and thermodynamical parameters used in the simulations are in table 1 below.

3. Results and discussion

3.1. Base-case

As CO_2 is injected at the lower segment of the sandstone reservoir, it migrates rapidly upwards by buoyancy forces towards the impermeable cap-rock because the density of supercritical CO_2 is lower than that of aqueous brine in the formation (Figure 3). Upon reaching the cap-rock, the gas spreads horizontally along the impermeable cap-rock and this upward and horizontal spread continues even after gas injection stops. During the injection phase, most of the free CO_2 gas accumulates below the cap-rock (hydrodynamic trapping) but in this same upper region, some of gas will dissolve in the formation brine (solubility trapping) thereby increasing the density of the CO_2-brine mixture which starts to sink downwards due to gravity (figure 4). The sinking heavier CO_2-brine mixture vertically displaces the lower fresh brine which is pushed upwards giving rise to a fluid circulation mixing process that enhances dissolution of free CO_2 gas in the fresh brine (Ennis-King et al., 2005, Audigane et al., 2007).

This fluid circulation process between fresh formation brine and the CO_2-brine mixture is mainly controlled by vertical permeability (Audigane, et al, 2007) and is mainly evident after hundreds of years hence it is not covered in the present work but is nonetheless important in CO_2 solubility trapping (Audigane et al., 2007 and references therein). Even after injection stops, CO_2 dissolution in the brine continues trapping the gas in solution thus enhancing solubility trapping. As CO_2 migration in the media perpetuates, some of it is trapped in the formation interstices between pores of the grains in the rocks leaving a trace of residual saturation (occurring at a residual gas saturation value of less than 0.05) where a certain part of the pore volume is filled with CO_2 that is immobilized due to the effect of capillary pressure (Bachu, 2008) and interfacial tension (Chalbaud, et al, 2009; Suekane, et al, 2008) - residual trapping. Thus with increased time, solubility trapping increases while gas trapping decreases. Mineral trapping is not covered here because it occurs after hundreds of years.

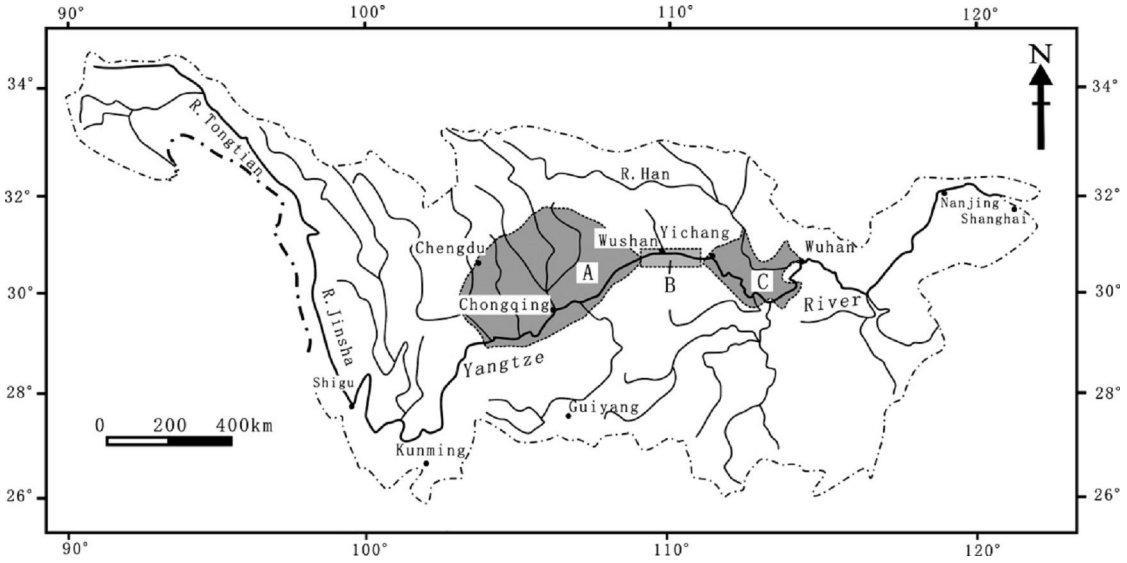

Figure 2. Location map, shaded areas are: A. – Sichuan Basin, B. – Three Gorges, C. – Jianghan Basin. (Fang, et al., 2007, p250)

Table 1: Hydrogeological and thermodynamical parameters used in the simulations

Parameter	Aquifer
Rock grain specific heat (J/kg °C)	920
Permeability	
Horizontal	2×10^{-12} m^2
Vertical	2×10^{-13} m^2
Porosity (%)	26
Pressure (bar)	110
Temperature (°C)	48
Salinity (% by weight)	12
CO_2 injection rate (kg/s)	50
Relative permeability model:	
Liquid (van Genutchen, 1980)	
$k_{rl} = \sqrt{S^*}\left\{1-(1-[S^*]^{1/m})^m\right\}^2$	$S^* = (S_l - S_{lr})/(1-S_{lr})$
S_{lr} : residual water saturation	S_{lr} = 0.30
m : exponent	m = 0.457
Gas (Corey, 1954)	
$k_{rg} = (1-\hat{S})^2(1-\hat{S}^2)$	$\hat{S} = (S_l - S_{lr})/(S_l - S_{lr} - S_{gr})$
S_{gr} : residual gas saturation	S_{gr} = 0.05
Capillary pressure model (van Genutchen)	
$P_{cap} = -P_0([S^*]^{-1/m} - 1)^{1-m}$	$S^* = (S_l - S_{lr})/(1-S_{lr})$
S_{lr} : residual water saturation	S_{lr} = 0.00
m : exponent	m = 0.457
P_0 : strength coefficient	P_0 = 19.61 kPa
Rock grain density (kg/m^3)	2600
Formation heat conductivity (W/m °C)	2.51

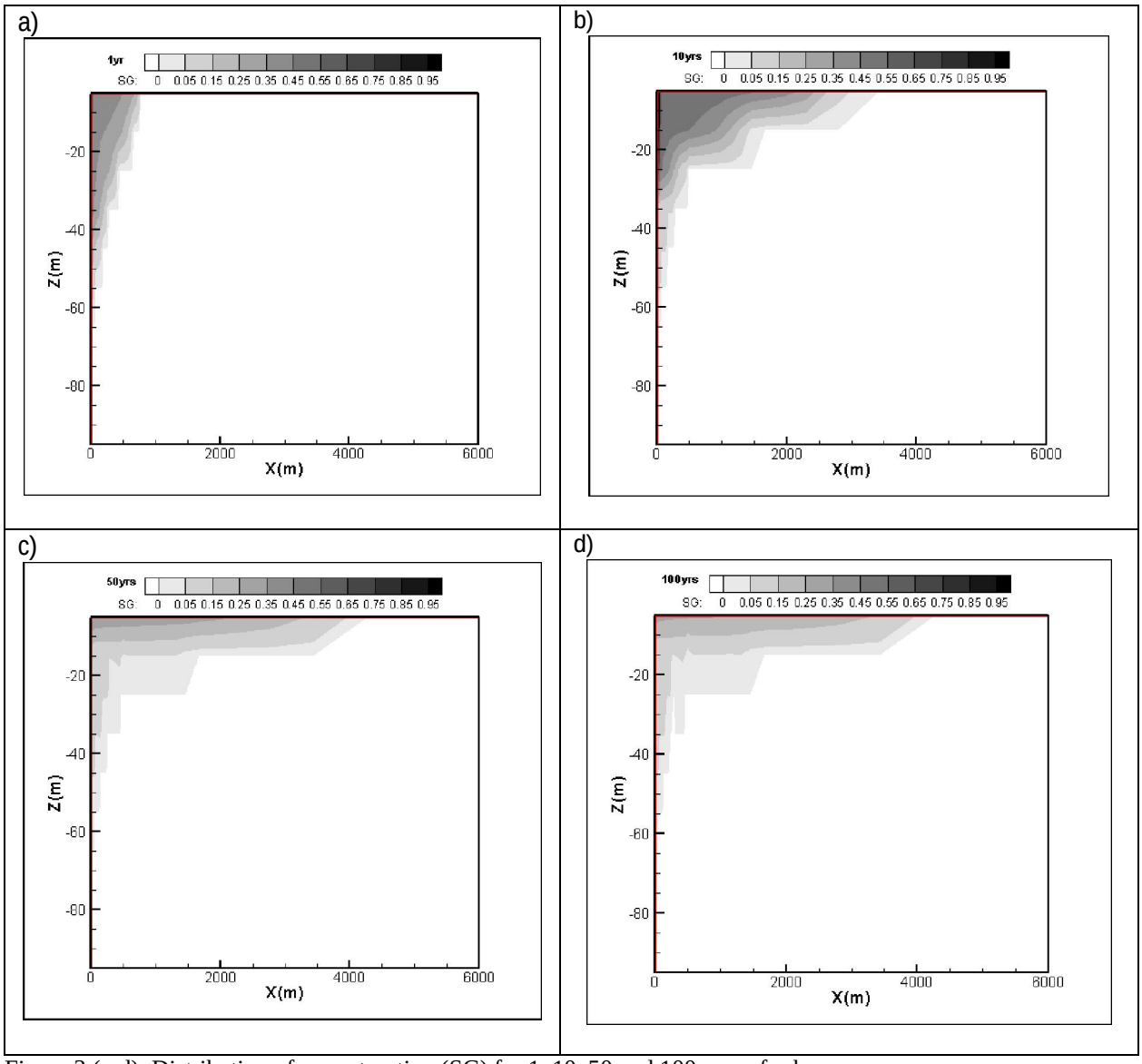

Figure 3 (a-d). Distribution of gas saturation (SG) for 1, 10, 50 and 100 years for base-case

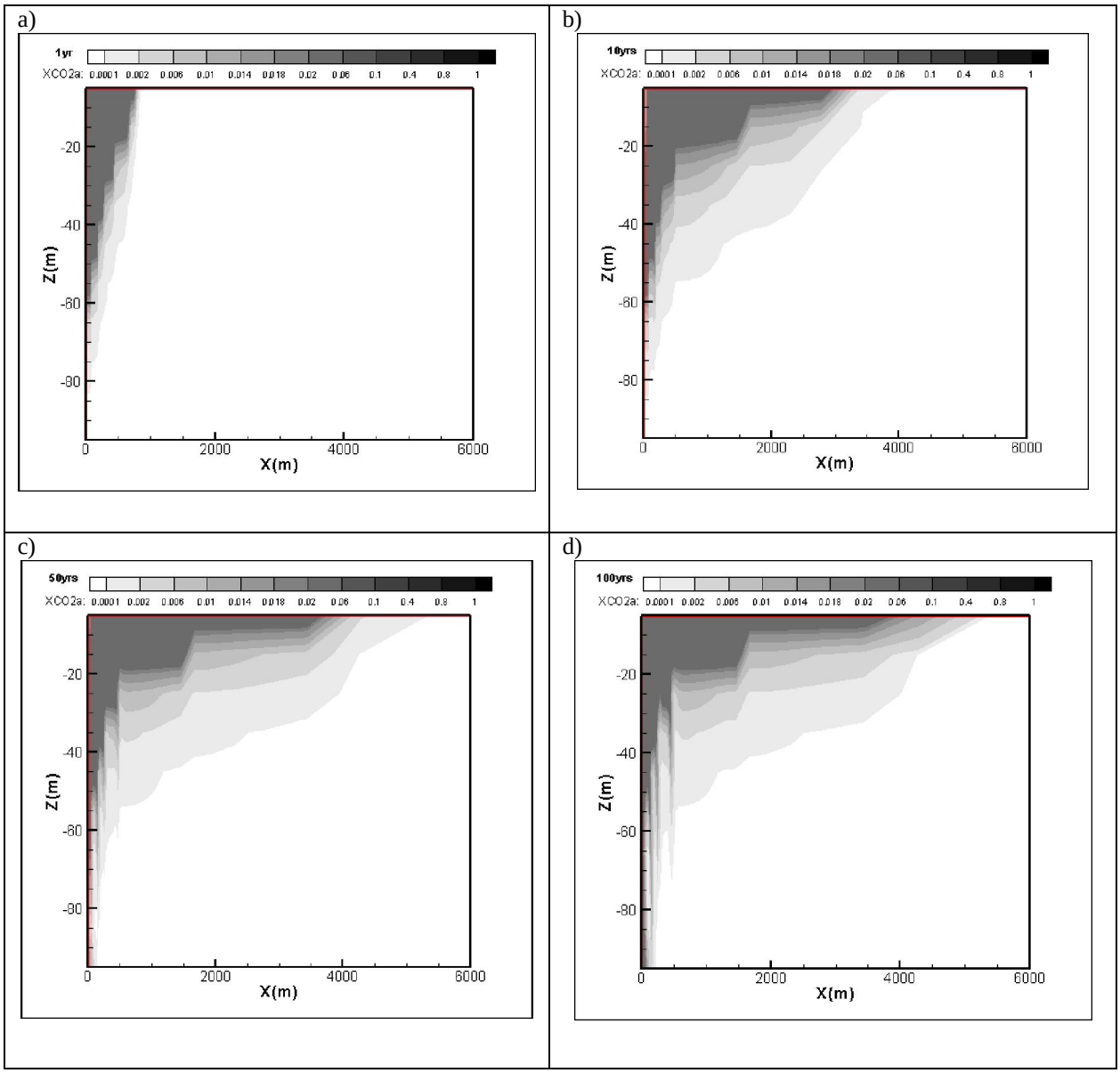

Figure 4(a-d). Distribution of concentration of dissolved CO_2 mass fraction (XCO2a) for 1, 10, 50 and 100 years for base-case

Table 2. Values used in sensitivity analysis and impact on dissolved CO_2 after 100 years

Sensitivity test	**Parameter**	**CO_2 dissolved in formation water after 100 years (%)**
Vertical permeability(Kv)	2X10-13 (K_v/K_h=0.1) base case 1x10-12 (k_v/k_h=0.5) case 2	33.7 34.5
Residual gas saturation (S_{gr})	0.05 (base-case) 0.25 (case 3)	33.7 21.5
Salinity	12% by weight (base case) 24% by weight (case 4)	33.7 23.2

3.2. Sensitivity studies

Accuracy of site-specific parameters is critical if the numeric simulations are to give a true representation of the reality. In this regard, sensitivity tests have been conducted for three parameters which may affect CO_2 spatial distribution and overall storage in the formation. These are vertical to horizontal permeability ratio (K_v/K_h) that has been increased from 0.1 (base-case) to 0.5 (case 2); residual gas saturation (S_{gr}) that has been raised from 0.05 (base-case) to 0.25 (case 3), and salinity (X_{NaCl}) whose value has been increased from 0.12 (base-case) to 0.24 (case 4) - see table 2.

3.2.1. Change in vertical to horizontal permeability ratio – case 2

Increase in the K_v/K_h ratio (case 2) increases the amount of CO_2 that is trapped by solubility trapping from 33.7% to 34.5% (table 2). In our case, vertical permeability was increased while the horizontal permeability remained unchanged. This in turn leads to greater vertical gas migration during the injection phase (figure 5a). At the end of 100 simulation time, there is greater slumping of the CO_2-brine mixture due to higher vertical permeability which promotes dissolution of CO_2 in the fresh brine that is displaced by the sinking CO_2-brine mixture - figure 5b (Ghanbari et al., 2006). However, although higher vertical permeability promotes solubility trapping through enhanced fluid circulation (Audigane et al., 2007), it may increase chances of CO_2 vertical leakage if the caprock has fractures, faults or there exists pre-existing poorly-sealed abandoned oil wells in Wangchang in turn threatening CO_2 storage safety.

3.2.2. Change in residual gas saturation – case 3

Increase in S_{gr} from 0.05 to 0.25 leads to reduced solubility trapping and increase in gas trapping. At the end of 100 years simulation time, only 21.5% of total CO_2 injected is stored in solution form compared to base-case's 33.7% (table 2). This in turn means that more gas is stored as free gas hydrodynamically or by residual trapping when compared to base-case (figure 5c). Consequently, higher S_{gr} limits movement in the gas within rock interstices and pores which leads to decreased contact between CO_2 gas and formation brine thereby decreasing dissolution of CO_2 (Zhang, et al., 2009).

3.2.3. Effect of salinity - Case 4

Wangchang has highly halide waters thus a sensitivity test for salinity was done by increasing the base-case value of 12%-by-weight two-fold (case 4). An increase in salinity reduced the amount of CO_2 stored by solubility trapping at the end of the 100 years simulation time with case 4 having 23.2% compared to 33.7% for base-case (table 2 and figure 5d). Increase in salinity increases the partial molar volume of water hence the difference between the partial molar volume of water and CO_2 decreases in turn reducing solubility through convective mixing (Ghanbari et al., 2006). This therefore means that saline aquifers in continental sedimentary basins may be more ideal for CO_2 storage compared to marine sedimentary ones (Zhao et al., 2009).

Conclusion

A short-term 100 year simulation of CO_2 injection into a deep saline sandstone aquifer has been done using a simple homogenous 2-dimensional radial fluid flow model to study CO_2 spatial and temporal distribution and storage. Parameters from Wangchang have been used in the model and three sensitivity tests have been carried out. Based on our studies, the following conclusions can be drawn out. When CO_2 is injected at the lower segment of the model, it will tend to migrate upwards towards the cap-rock and then horizontally upon reaching the cap-rock barrier driven by buoyancy forces because of density differences between the supercritical CO_2 and aquifer formation brine. Thereafter, the gradually gas dissolves in the formation water even after injection ceases. Increase in ratio of K_v/K_h, S_{gr} and salinity have different effects on overall storage. Increase in K_v/K_h increases CO_2 dissolution in brine but may be a danger in cases of caprock imperfections leading to vertical leakage of the gas to overlying shallow aquifers. Higher S_{gr} and increased brine salinity both reduce the amount of CO_2 dissolved in formation brine thus more gas is trapped structurally but at a different degree of effect. Overall, 33.7% of gas is stored in solution form in base-case compared to 33.4% in case 2, 21.5% case 3 and 23.2% in case 4 (table 2).

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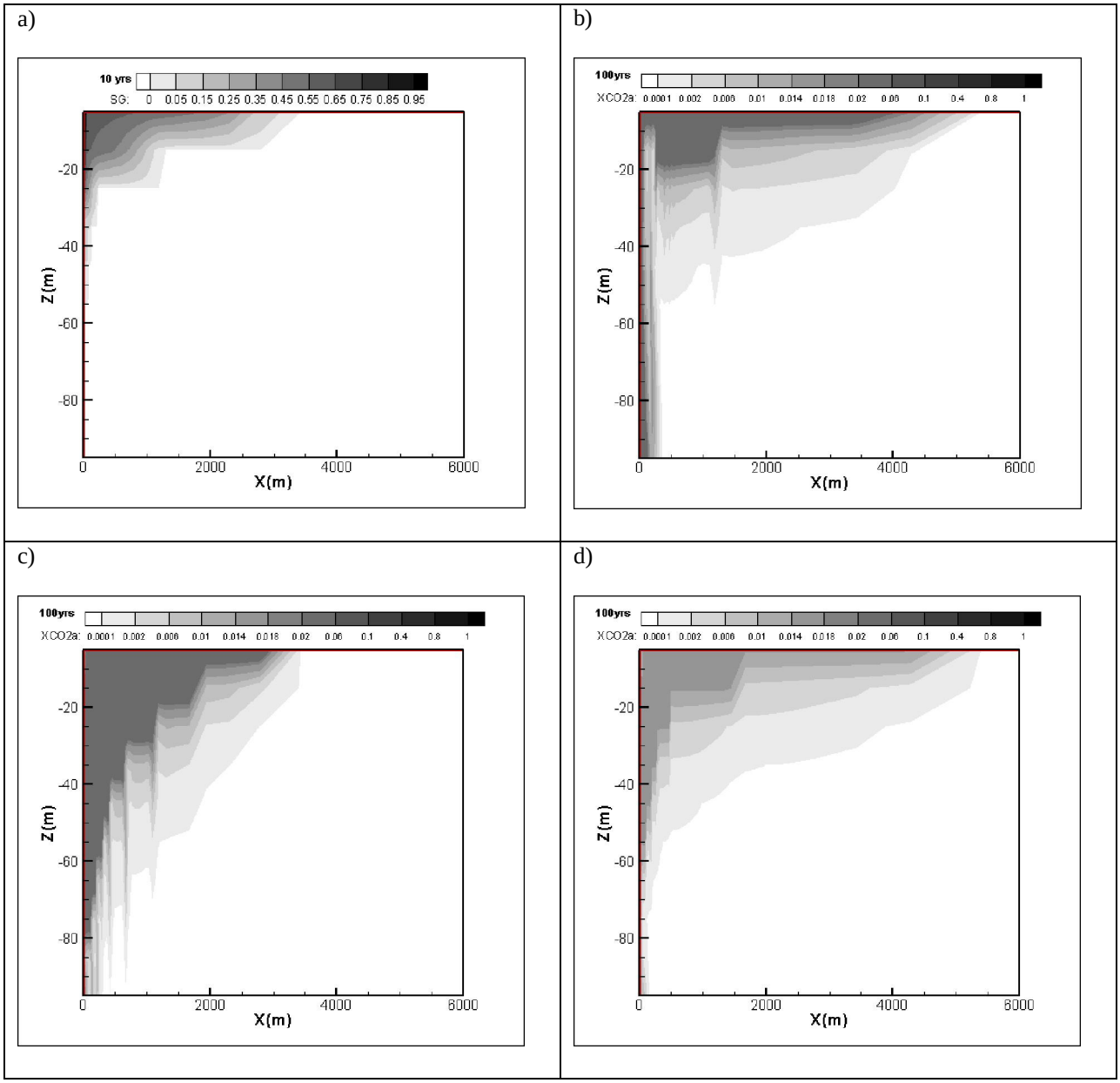

Figure 5a) Gas saturation profile at 10 years for case 2; b to d - spatial distribution of concentration of dissolved CO_2 in brine at 100 years for case 2, 3 and 4 respectively.

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26th February 2010

Time develoment of local scour at a bridge pier using square collar in a 180 degree flume bend

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Abstract: Local scouring around the bridges pier occurs because of flow separation and developing several vortexes around the bridge pier. In this study, the use of square collars for reducing the effects of local scour at a bridge pier is presented together with the time aspect of the scour development. The study was conducted using in a 180 degree laboratory flume bend with a relative radius of Rc/b=4.67 operated under clear-water conditions. Tests were conducted using one pier with 60 mm diameter in positions of 60 degree under one flow conditions. Investigated was the effect of size and elevation collar on the time development of scour and its efficacy at preventing scour at a bridge pier. The time development of the scour hole around the model pier with and without a square collar installed was compared with similar studies on bridge piers. Several equations for the temporal development of scour depth and those for the prediction of the equilibrium scour depth were tested as part of this study. The depth of the scour hole increases as the duration of the increased flow that initiates the scour increases. It was observed that, as the minimum depth of scour occurs for the square collar at width of 3D placed at elevation of 0.1D below the bed and the size of a collar plate increases, the scour decreases. [Journal of American Science 2010;6(8):188-195]. (ISSN: 1545-1003).

Keywords: Square Collar, Time development, Circular pier, 180 degree channel bend, Scour depth

1. Introduction

While flood damages typically involve widespread inundation of agricultural land, destruction of homes and businesses, and disruption of economic activity, a less obvious threat is the existence of bridges over waterways that cause flow obstruction and scour around the bridge foundations with possible failure of the bridges. In recent years, flood waters have closed many highways and local roads as well as interstate highways, and caused scour that damaged many bridges and even resulted in loss of life.

For example, one thousand bridges have collapsed over the last 30 years in the United States and the leading cause is hydraulic failure, resulting in large financial losses. In Georgia, the total financial loss from tropical storm Alberto in 1994 was approximately $130 million because more than 100 bridges had to be replaced and repaired due to flooding (Richardson and Davis, 2001).

During the 1993 upper Mississippi River basin flooding, more than 258 million dollars in federal assistance was requested for repair and/or replacement of bridges, embankments, and roadways (Parola et al. 1997). Bridge failures can be also lead to loss of life such as in the 1987 failure of the I-90 bridge over Schoharie Creek near Albany, New York, the US 51 bridge over the Hatchie River in Tennessee in 1989, and the I-5 bridges over Arroyo Pasajero in California in 1995 (Morris and Pagan-Ortiz, 1999).

Breusers et al. (1977) defined scour as a natural phenomenon caused by the flow of water in rivers and streams. It is the consequence of the erosive action of flowing water, which removes and erodes material from the bed and banks of streams and also from the vicinity of bridge piers and abutments.

The mechanism has the potential to threaten the structural integrity of bridges and hydraulic structures, ultimately causing failure when the foundation of the structures is undermined.

The mechanism of bridge foundation failure is due to processes of local scour at the piers caused by flow obstruction, downflow, and formation of a horseshoe vortex that wraps around the obstructions (Fig.1).

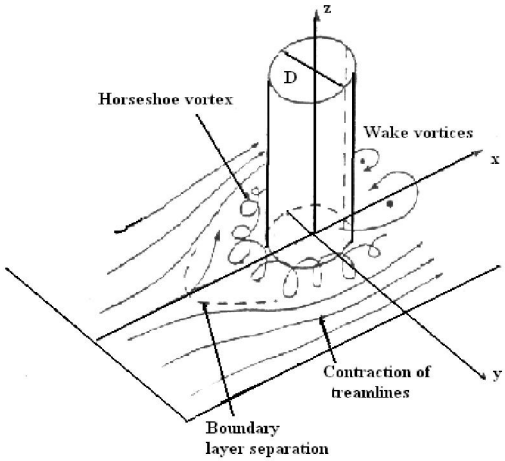

Figure 1. Flow and scour pattern at a circular pier

One way of reducing pier scour is to combat the erosive action of the horseshoe vortex by armoring the riverbed using larger size materials such as stone riprap. Another approach is to weaken the down-flow and thus the formation of the horseshoe vortex using collar.

Chabert and Engeldinger (1956) found that a single circular plate placed 0.4D below the original bed elevation and having a diameter of 3D, where D is the pier diameter, could reduce the depth of scour by 60%.

Ettema (1980) conducted a series of experiments to ascertain the possibility of using a thin collar to mitigate against local scour at a circular bridge pier. Collars were installed on a circular pier at various elevations on, above and below the channel bed. A 0.4 mm thick, circular, brass collar of width two times the pier diameter was installed on the circular pier at four different locations, viz. yc/D = 0.5, 0, -0.5 and -0.1. It was observed that, when a collar of width twice the size of the pier diameter was installed at an elevation of half the diameter (yc/D = 0.5) above the channel bed, the collar was not effective at reducing the scour depth. However, the effectiveness of a collar at reducing scour became noticeable when the collar was installed at the channel bed. No scour developed below the collar when the collar was installed at yc/D = -0.1.

From the study of Singh et al. (2001) it was concluded that the efficacy of a collar in preventing scour is a function of its width and its elevation relative to the bed surface. They found that collar of width of 1.5D, 2D and 2.5D placed on the bed reduced the scour depth by 50%, 68% and 100%, respectively; collar of 2D wide placed at -0.1D resulted a maximum reduction in scour depth.

Mashahir and Zarrati (2002) and Zarrati et al. (2004) worked on the application of a collar to control the scouring around rectangular shape piers having a rounded nose. Their funding also confirmed the previous results. Comparison of the results for rectangular piers aligned with the flow and the previous experiments on circular piers showed that a collar of 3D wide is more effective at reducing the depth of the scour hole for rectangular piers than for circular piers.

The use of collar as a countermeasure at bridge abutment was studied by Kayaturk et al. (2004). The effects of various sizes of collars placed at different elevations on the scour depth at the abutment. Their experimental results showed that not only did the presence of a collar reduce the scour depth; the rate of temporal development of the scour hole was also reduced. According to Kayaturk et al. (2004), a 67% reduction in the scour depth was achieved when the collar was positioned at an elevation of 50 mm below the bed.

Zarrati et al. (2006) studied the use of independent and continuous pier collars in combination with riprap for reducing local scour around bridge pier groups. Their results showed that with two piers in line, a combination of continuous collars and riprap led to a scour reduction of about 50% and 60% for the front and rear piers, respectively. In another experiment with two piers in line, independent collars showed better efficiency than a continuous collar around both the pier. It was also observed that the efficiency of collars is more on a rectangular pier aligned to the flow than two piers in line.

Mashahir et al. (2009) studied the effectiveness of different countermeasures to control scour around bridge piers including application of riprap and installing a collar around piers. Piers aligned with the flow and skewed at 5°, 10° and 20° to the flow were tested. A 3D wide collar installed around the piers at the streambed level has been tested. The size and extent of stable riprap stones for prevention of scouring around the piers was found to decrease when using collar.

The scour geometry around a circular pier in a bend depends on channel geometry (channel width, channel radius and bed slope), pier characteristics (pier diameter and location in bend), collar characteristics (collar size, collar shape and location in bed), flow conditions (approach depth and discharge or velocity), sediment properties (specific gravity, grain size and friction angle), fluid parameters (density and viscosity) and time. Therefore for depth of scour ds can write:

$$ds = f(D, W, H, \theta, Y, b, S_0, V, g, d_{50}, R, \rho_s, \phi, \rho, \mu, t) \quad (1)$$

in which D is diameter of pier, W is size of collar, H is location of collar in bed, θ is location of pier in bend, Y is approach flow depth, b is channel width, S0 is bed slope, V is approached flow velocity, g is gravitational acceleration, d50 is median grain size, R is radius of bend, ρs is density of sediment,φ is friction angle of sediment,ρ is density of fluid,μ is viscosity of fluid, and t is time of scour. Using dimensional analysis, Eq. (2) can be written as:

$$\frac{ds}{D} = f\left(\frac{W}{D}, \frac{Y}{D}, \frac{b}{D}, Fr, \frac{d_{50}}{D}, \frac{R}{\lambda}, \mathrm{Re}, \frac{H}{D}, \frac{\theta}{\lambda}, \frac{\phi}{\lambda}, \frac{\rho_s}{\rho}, S_0, \frac{t}{t_e}\right) \quad (2)$$

in which Fr is approach Froude number, λ is angle of bend, te is maximum of time development of scour and Re is Reynolds number. After simplification of above equation and eliminating the parameters with constant values, one can have:

$$\frac{ds}{D} = f\left(\frac{W}{D}, \frac{H}{D}, \frac{t}{te}\right) \quad (3)$$

Majority of researches on scour at bridge pier are conducted at a straight flume. In this study many examples where the bridges cross the river bends. In this work, results on the effect of square collar on time development of scour at pier in positions of 60 degree in a 180 degree flume bend under a clear water regime are reported.

2. Experimentals

Experiments were carried out at the Hydraulic Laboratory of Islamic Azad University, Ahwaz. The main channel consisted of a 9 m long upstream and a 6 m long downstream straight reaches (Fig. 2). A 180 degree channel bend was located between the two straight reaches with a relative radius of Rc/b=4.67. The study conducted at hydraulic laboratory of Islamic Azad university of Ahwaz during March 2009 to October 2009.

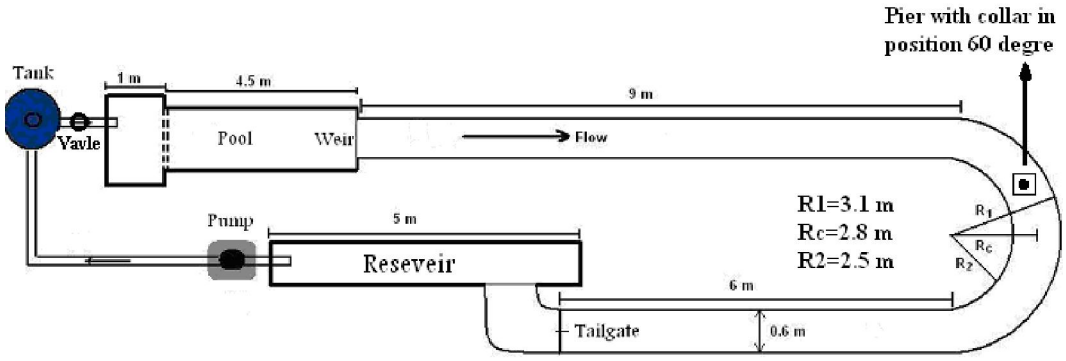

Figure 2. Illustration of the experimental setup (Plan)

The bed of channel was made of Aluminum and sides was made of Plexiglass supported by metal frame. Measurement of discharge was done by a 60 degree triangular weir was used at the upstream section of the flume. Depth of flow and bed profile was measured by a digital point gauge having an accuracy of ± 0.01mm. A sluice gate was located at the end of the main channel to control the flow depth. Uniform sediment with a median size d50 = 2 mm and standard deviation was 1.7 used with a thickness of 20cm and covered the total length of flume. Chiew and Melville (1987) was found that to avoid wall effect on scouring, pier diameter should not be more than 10% of flume width. Therefore in this study a circular pier of diameter 60 mm fabricated from Polyvinyl Chloride (PVC) pipe was used. Collars were made of Plexiglas having a thickness of 5 mm. Figure 3 shows a schematic illustration of a pier fitted with a square. Four different collar widths of 1.5D, 2D, 2.5D and 3D (in which D is the pier diameter) were used. For all of the tests with a collar, the collar was positioned at the bed level,0.1D,0.5D and 1D below the bed in accordance with the recommendations of earlier researchers (Ettema 1980; Kumar et al. 1999 and Singh et al.2001).

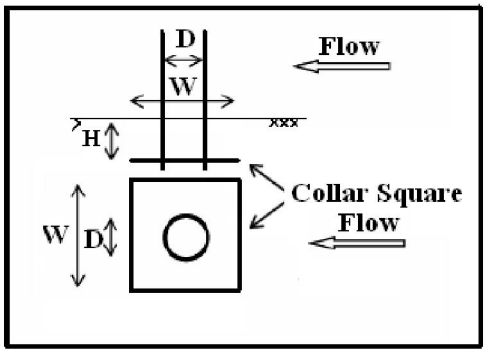

Figure 3. Circular pier fitted with a square collar

The duration of experiments was kept equal to 24 hrs at which equilibrium time condition occurs. Experiment was conducted at Froude number of 0.41 at the position of 60 degree for a pier without collar. The results are shown in Fig.4. As it can be seen approximately 92% of scouring occurs during the first 4 hours. Therefore in all remaining of our experimental tests, duration of 4 hours was selected for each test.

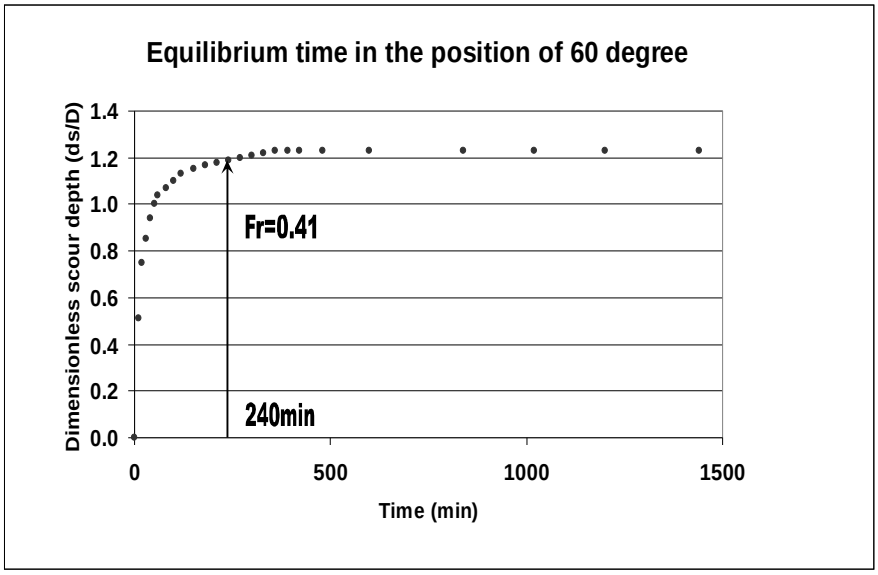

Figure 4. Equilibrium time in the position of 60 degree for a pier without collar

Experiments on scour were performed near threshold condition in straight channel i.e. 0.9<U/Uc<1 (U is approach velocity and Uc is critical velocity for sediment movement). Initially the bed surface was leveled by a plate attached to the carriage mounted on the channel. Then inlet valve was opened slowly, the discharge increased to a predetermined value so that no scour occurs at the straight reaches of flume. Scour depth in upstream of collar in test time was measured by a digital point gauge having an accuracy of ± 0.01mm (Fig. 5).

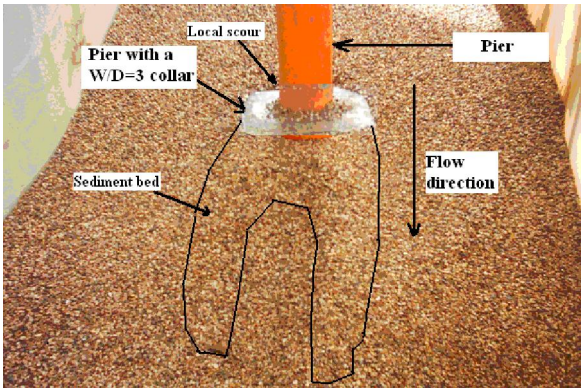

Figure 5. Scour pattern at the end of a test

3-1. Various sizes of collar fitted at time development of scour

Figure 6 shows the results obtained time development for four different collar widths, W=1.5D,2D,2.5D and 3D were tested at the bed level. Results shown, the size of a collar plate

increases, the scour decreases. Collar plates of size W/D=3 gave a maximum reduction in scour depth.

Figure 7 shows the maximum scour depth with and without collar plates for four different elevations were used at the bed level, 0.1D, 0.5D and 1D below bed with collar sizes of 1.5D, 2D, 2.5D and 3D. As shown by the results in Figure 7, the depth of the scour in square collars reduced increases because of the square collar is sharp-pointed and reduced horseshoe vortex.

As concluded in the study by Mashahir et al. (2004), placing the collar below the channel bed level did not lead to an appreciable increase in the efficacy of the collar. This was so because the depth of the sand sediments above the collar will itself become part of the scour hole as this is swept away very fast by the erosive action of the flow. Comparison of the results for rectangular piers aligned with the flow and the previous experiments on circular piers by Zarrati et al. (2004) and Mashahir et al. (2009) showed that a collar of W/D = 3 is more effective at reducing the depth of the scour hole for rectangular piers than for circular piers.

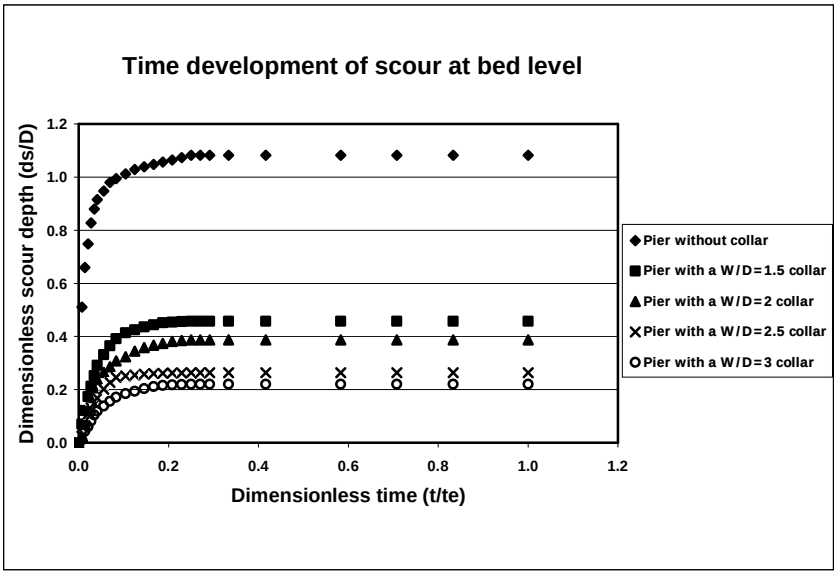

Figure 6. Time development of scour for different collar sizes

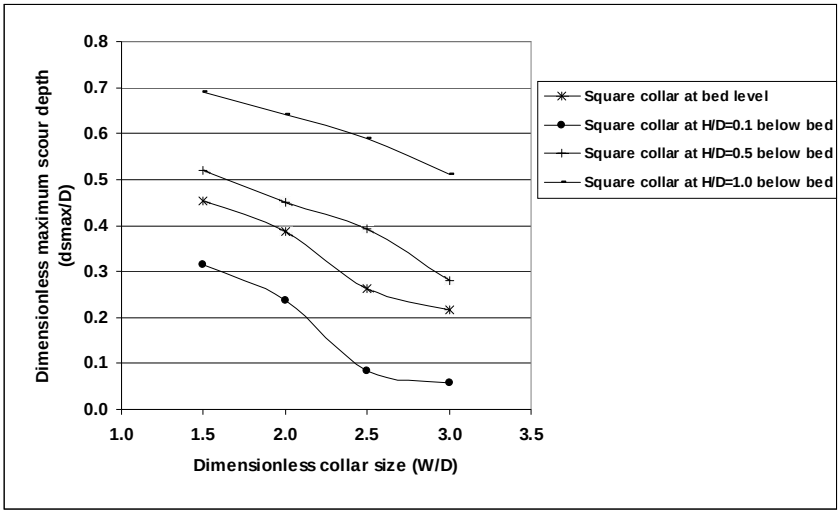

Figure 7. Maximum scour depth for different collar sizes

3-2. Various elevations of collar fitted at time development of scour

Figure 8 shows the time development of the local scour around the pier fitted with and without collar plates for four different elevations were used at the bed level, 0.1D, 0.5D and 1D below the bed with collar widths 3D. As shown by the results in Figure 8, the depth of the scour hole reduced because of the collar irrespective of the collar size and vertical position. The experimental results signified that not only did the presence of a collar reduce the scour depth, the rate of temporal development of the scour hole was also reduced.

From Fig.9 it is obvious that, all collar of any width, installed at 0.1D below the bed results maximum reduction in scour depth. The main reason of such finding is that the strength of the downward vortex decreases when the collar placed at 01D below the bed.

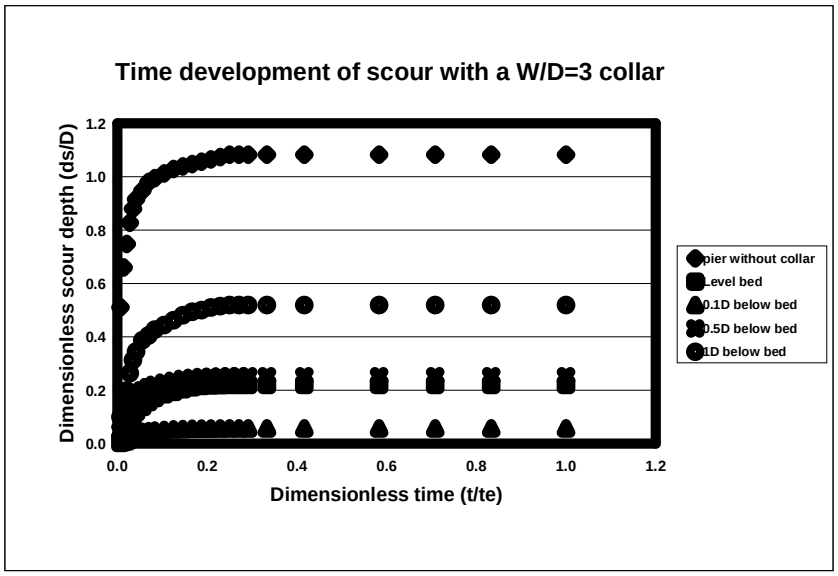

Figure 8. Time development of scour for different elevations

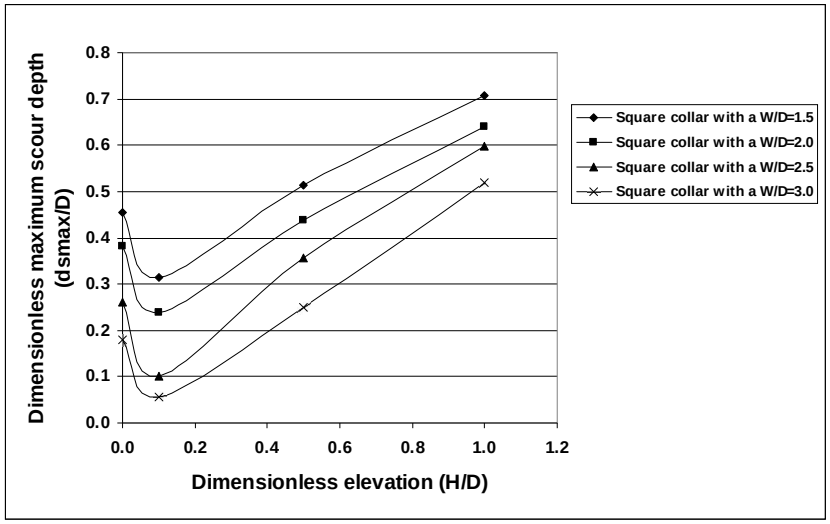

Figure 9. Maximum scour depth for different elevations

Table 1 shows the reduction in the scour depth around the pier fitted with collar plates for four different elevations were used at the bed level,0.1D,0.5D and 1D below the bed with collar widths of 1.5D, 2D, 2.5D and 3D. Table 2 shows collar plates of size W = 3D when placed at 0.1D below the bed surface gives a maximum reduction in scour depth equal to 96% of the scour depth without collar.

Table1. Percent reduction in the scour depth

Elevations Sizes of collars	Bed level	H/D=0.1	H/D=0.5	H/D=1.0
Square collar with a W/D=1.5	53	71	51	36
Square collar with a W/D=2.0	62	78	59	43
Square collar with a W/D=2.5	73	90	61	44
Square collar with a W/D=3.0	80	96	73	52

4. Equation for scour depth

The equation (3) can be written as:

(4)

$$\frac{ds}{B} = a\left(\frac{W}{B}\right)^{b}\left(\frac{H+c}{B}\right)^{d} Ln\left(\frac{t+e}{t_e}\right)^{f}$$

in which a, b, c, d, e and f are empirical constants and can be found using experimental data. By using least squares method for all the data it was found. Therefore, equation (4) can be written as:

(5)

$$\frac{ds}{B} = 0.85\left(\frac{W}{B}\right)^{0.8}\left(\frac{H+1}{B}\right)^{1.35} Ln\left(\frac{t+360}{t_e}\right)^{0.185}$$

with regression coefficient of 0.96. Figure 10 shows the comparison of calculated values with use to Eq. (5) and tested values of relative maximum scour depth. It is evident that Eq. (5) predicts the maximum scour depth with acceptable accuracy.

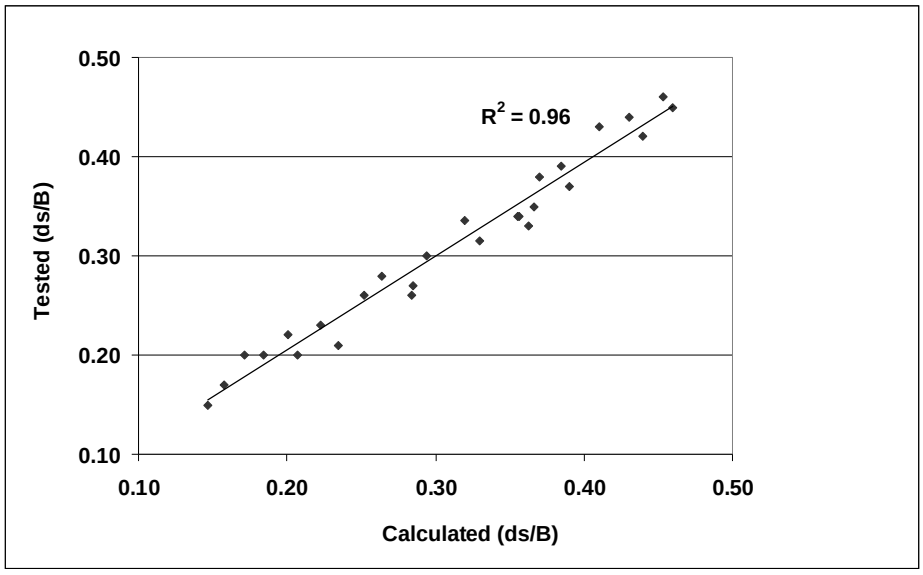

Figure 10. Comparison of calculated and tested scour depth

4. conclusions

In this work, presented the results of several long duration scour laboratory experiments around a circular pier located in a 180 degree flume bend. Detailed measurements of the erosion holes allowed the evaluation of scoured volumes. The analysis of the results shows the following.

For a test duration of about 4 hours, the maximum scour depth was observed at the upstream face of the pier for an unprotected pier while the position of the maximum scour depth for a pier protected with a collar of W= 3D.

The pathway to an equilibrium scour depth is different depending on whether the pier is fitted with a collar or not. With a collar in place, the development of the scour hole is considerably delayed.

There was a reduction in the scour depth when a collar of W=3D was used to protect the pier when compared with a W=1.5D, 2D and 2.5D.

All collars of any diameter, at 0.1D below the bed resulted in maximum reduction in scour depth.

Minimum depth of scour occurs for a collar of W=3D placed at 0.1D below the bed. The percent of reduction by 96% compare to without collar.

The equilibrium scour depth in clear water is less than in 25% of total time.

Acknowledge

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5/1/2010

Biomimetic Synthesis Of Guided-Tissue Regeneration Hydroxyapatite/Polyvinl Alcohol Nanocomposite Scaffolds: Influence Of Alignate On Mechanical And Biological Properties.

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Abstract: This paper presents a part of a major research, in which HA/PVA/alginate scaffolds -with different alginate compositions -up to 20wt% were fabricated by a modified freeze-extraction method. This method includes the physical cross-linking of PVA and chemical cross-linking of the alginate. Characterization of the prepared scaffolds was performed by morphology observations using scanning electron microscopy (SEM). Different physical properties – as porosity and density-were measured. It was noticed that by increasing alginate composition scaffolds exhibited highly porous, open-cellular pore structures with almost porosity about 90%, regardless of alginate composition and the pore sizes from about 150 to about 300μm.The In Vitro bioactivity and biodegradability of nano-composite scaffolds were investigated by incubation in simulated body fluid (SBF) and water under osteoclastic resorption conditions, respectively. The in-vitro bioactivity test indicating the higher bone-bonding ability of the biomimetically synthesized a scaffold that is awarded by the fast formation of bonelike apatite on their surfaces within one day. Also The addition of alginate to HA/PVA scaffolds increased the biodegradability compared with that one without alginate. Mechanical behavior of scaffolds was investigated under axial loading. Scaffolds stress strain behavior, maximum true stress, and elastic moduli, were calculated. It was found that increasing alginate content from 0 to 20% by weight, decreased the compressive modulus from 85.3 to 44.7 MPa, whereas the maximum compressive strength decreased from 6 to 5 MPa. Finally, it was concluded that the proposed scaffolds expressed promising performance, despite of the resulting degradation in their mechanical behavior. The obtained compressive strength and modulus of elasticity were still within satisfactory limits. [Journal of American Science 2010;6(8):196-207]. (ISSN: 1545-1003).

Keywords: Tissue re-generation, Poly(vinyl), composites, scaffolds

1. Introduction

One of the main goals in bone tissue engineering is to develop biodegradable materials as bone graft substitutes, especially for filling large defects [1-3]. Besides bone forming cells and growth factors, synthetic biomaterials served as scaffolds play a critical role in bone tissue engineering and osteogenesis. They provide a three dimensional (3-D) space for cell growth and extracellular matrix (ECM) formation, and structural support for the newly formed bone tissue. The ideal synthetic scaffolds for osteogenesis should meet certain criteria to serve this function, including good biocompatibility, sufficient mechanical properties, and adequate biodegradability at a controlled rate commensurate with remodeling [4, 5]. Being the major mineral component of natural bone and structurally similar to the bone, hydroxyapatite (HA) is the prospective one of inorganic biomaterials for bone regeneration[6,7]. HA ceramics were high biocompatible in bone tissue and had a high osteogenic potential (osteo-conduction and osteo-integration), but the brittleness and fatigue failure in the body limit their clinical applications only for unloading bearing repair and substitute [8, 9]. This fact restricts the use of these materials in a wide range of applications. One alternative that is being considered and studied is the production of composites and hybrid systems. Hence, the development of composite materials is regarded as a promising approach for preparing bioactive scaffolds [10]. Composites, which include synthetic and biological polymers with HA, have the potential capability of combining bioactive behavior with adequate mechanical properties.

Among several choices of polymers, poly(vinyl alcohol) (PVA), a water-soluble polyhydroxy polymer, has been frequently explored as an implant material in biomedical applications such as drug delivery systems, dialysis membranes, wound dressing, artificial skin, cardiovascular devices and surgical repairs because of its excellent mechanical strength, biocompatibility and non-toxicity [11,12]. PVA is considered as a biologically friendly material but with some reduced integration to living tissues due to its relative limited biodegradability and bioactivity, compared to poly(ethylene oxide) (PEO), poly(lactic-co-glycolic acid) (PLGA) and others. Therefore, it is promising to blend PVA with biopolymers such as alginate to produce a new polymeric system applicable for a variety of purposes [13]. In order to overcome the limited biological performance of synthetic polymers

and to enhance the mechanical characteristics of biopolymers, a new class of specially designed materials, 'bioartificial polymeric materials', has been introduced [14]. These materials based on blends of both synthetic and natural polymers such as alginate could be usefully employed as biomaterials. Alginate, a polysaccharide derived from brown seaweed, has been widely utilized as cell carriers since the 1970s [15] due to the simplicity for fabricating cell-immobilized beads or 3D porous scaffolds, low price compared to proteins or other natural polymers and non-toxicity to cells. It is also approved by the Food and Drug Administration (FDA) for human use as wound dressing material and food additive. The alginate, the monovalent salt form of alginic acid is a linear block copolymer composed of *β*-D-mannuronate (M-block) and *α*-L-guluronate (G-block) linked by 1,4-glycoside linkage [16]. The G-block of alginate has correspondingly high affinities for divalent ions such as calcium (Ca2+), strontium (Sr2+) and barium (Ba2+) at room temperature and thus in an aqueous solution of divalent ions, the alginate chains are rapidly cross-linked *via* the stacking of G-blocks to form an egg-box structure and subsequently become gel [17].

In our previous work, HA/PVA nanocomposite was synthesized by using biomimetic method and subject to freeze extraction technique in conjugation with liquid descant drying regime to obtain the three dimensional HA/PVA scaffolds[18]. The resultant scaffolds exhibit a high interconnectivity and maximum pore size of 150 µm, indicating preferred morphology for tissue engineering application.

In the presented study, a series of HA/PVA/Alginate scaffolds with 0, 5, 10 and 20% alginate were fabricated by freeze extraction method followed by chemical cross-linking of alginate. The porosity, pore size, mechanical property of the scaffolds was investigated as functions of alginate content. In addition, the in vitro bioactivity and biodegradability of nano-composite scaffolds were investigated by incubation in simulated body fluid (SBF) and water under osteoclastic resorption conditions, respectively. The emphasis of this study was placed on the development of a novel scaffold suitable for hepatic tissue-engineering application.

Mechanical properties of a scaffold are important both for integrity during handling and implantation, and for support of tissue subject to load during healing. Measures to improve mechanical properties without sacrificing other properties (biocompatibility, osteoconductivity) are thus highly desirable. There are numerous reports in literature describing the improvement of the mechanical properties of polymers by adding bioactive inorganic materials, such as hydroxyapatite (HA). As with the modulus, it is natural for the strength of the scaffold to increase with increasing volume fraction of the filler content, especially if there is strong interaction between the matrix and the filler. A careful literature survey has shown that polymer/HA composites are complex systems [27-29].

2. Experimental procedure for scaffolds manufacturing.

2.1. Materials

Through out the entire preparation part of the work, double distilled water as well as the following reagent-grade chemicals were used calcium hydroxide [$Ca(OH)_2$],BDH-Laboratory,England; ammonium dihydrogen phosphate($NH_4H_2PO_4$), MERCK, Germany; PVA with an average molecular weight of 124,000 and degree of hydrolysis 98 - 99%, Loba Chemie, India. Sodium alginate (also called algin or alginic acid sodium salt) from Sigma (USA). Ammonia solution (NH_4OH) (33%), absolute Ethanol and pure Acetone, El Nasr Pharmaceuticals and Chemicals Co. Egypt.

2.2. Samples preparation

2.2.1. Synthesis of HA/PVA/ alginate gels

HA/PVA/alginate gels with different alginate compositions (0, 5, 10, and 20 wt %) were fabricated by using the freeze extraction method. Making a deviation from our early employed post precipitation technique, including freeze extraction method (18), the present study involves chemical cross-linking of alginate extracted gels. In brief, Sodium alginate was easily soluble in cold water. Then, the solution was added to the synthesized HA/PVA mix solution. The mixture was then left under stirring for approximately 4 h at room temperature until a completely homogenous solution was obtained. After each the mixture solutions were poured in cylindrical Teflon vials. The freeze extraction step was used necessary to obtain the physically coherent gels having the ability to keep their original shape (mold shape) before alginate cross-linking. The obtained gels were then immersed in 5 wt% $CaCl_2$ solution for 12 h to chemical cross-linking of alginate with calcium ions. The resultant gels with 0, 5, 10 and 20% alginate were designated by the abbreviations AHP, 5AHPG, 10AHPG and 20AHPG, respectively.

3. Samples characterization

3.1. Bulk density and apparent porosity measurement

The bulk density and apparent porosity of the porous scaffolds were measured by using the Archimedes' principle [19]. This method is based on soaking the samples under kerosene for 2hrs in a vacuum desiccator to saturate their open-pore structure

with the latter. The weight of saturated sample suspended in kerosene (Wi) and its saturated weight in air after removal of kerosene film from outer surface (Ws) were recorded. Triplicate measurements were carried out to obtain their mean values. Bulk density (D) and apparent porosity (P) were calculated according to the following equations (1):

$$D=\frac{W}{(Ws-Wi)}x\rho$$

$$P=\frac{(Ws-W)}{(Ws-Wi)}x100\% \qquad (1)$$

ρ= specific gravity of kerosene (0.78).

3.2. Morphological observations

Morphological observation of the resultant scaffolds was preformed by Philips XL 30 Scanning Electron Microscope (SEM) with an acceleration voltage of 30 kV. Whereby specimens were cut from liquid nitrogen-treated (frozen) scaffold to avoid disturbing of the pore structure. Specimens were placed on a stub using a carbon sticker and were examined under the microscope after the samples were sputtered with a thin coat of gold.

3.3- Axial Compression Mechanical test

3.3.1-Instrumentation and test setup

A universal (SHUMADZU) testing machine was utilized to conduct the compression mechanical test. A computer-controlled module monitored and controlled the entire testing. Input and output data were recorded related to compression test using the machine acquisition system. Three specimens were cast for each of the investigated scaffolds: $AHP_{10\text{-}6}$, and $20AHPG_{10\text{-}6}$. Dimensions of each sample were measured prior to testing. Averages of measured dimensions are 16 and 14 mm (diameter and height respectively.) Tests were conducted in room temperature. The crosshead speed was set at 0.5 mm/min.

3.4 (In-Vitro Test):

3.4.1- Water-binding capacity testing

The dry scaffolds ($AHP_{10\text{-}6}$, $5AHPG_{10\text{-}6}$, $10AHPG_{10\text{-}6}$ and $20AHPG_{10\text{-}6}$) were immersed were placed in a small bottle containing 20 ml water (pH 7.4) and incubated at 37°C for 24h. The water binding capacity (expressed as a percentage) was calculated by comparing the initial weight (W_0) with the wet weight after swelling (W_1), as shown in Equation (2). Three individual experiments were performed, and then the average value was gained.

$$\text{Water - binding capacity} = \frac{W1-Wo}{Wo}x100 \qquad (2)$$

3.4.2- Biodegradation testing

The in-vitro biodegradation study of the resultant scaffolds ($AHP_{10\text{-}6}$, $5AHPG_{10\text{-}6}$, $10AHPG_{10\text{-}6}$ and $20AHPG_{10\text{-}6}$) was carried out under the conditions of osteoclastic bone resorption, i.e. at a pH of about 4.5 in order to simulate the general remodelling of the skeletal system (20). Samples were immersed into water (100 ml each) at pH 4.5 and temperature 37 °C. The pH was checked and adjusted at regular intervals (2 h). If the pH was increased due to neutralization of the basic calcium phosphate, 0.001 N HNO_3 was added in order to maintain an average pH of 4.5. The samples were taken out after 72 h and weighed after being dried. The biodegradation was monitored as the change in sample weight over time. Weight loss was calculated by comparing the initial weight (W_0) with the mass measured at a given time point (W_2), as shown in Equation 2. Three individual experiments were performed, and then the average value was gained:

$$\text{Weight loss} = \frac{W2-Wo}{Wo}x\ 100 \qquad (3)$$

3.4.3- Bioactivity testing

Standard in vitro bioactivity test was carried out to evaluate the formation of an apatite layer onto the surface of the samples. In order to study the bioactivity, the samples were soaked in simulated body fluid (SBF) which was proposed by Kokubo et al. [21] at 37 ºC and pH 7.4 for several periods of times (1, 2, 4 and 8 days)**.** The specimens were removed from the SBF solution and were abundantly rinsed with water in order to remove the soluble inorganic salts and to stop the reaction.The formation and elemental composition of the bioactive layer were characterized using Philips XL 30 Scanning Electron Microscope (SEM) with an acceleration voltage of 30 kV coupled with energy dispersive X-ray analysis (EDXA) detector with an accelerating voltage of 20 kV.

4. Results

4.1.Morphological observations

The extracted gels were dried via liquid desiccant method. The result indicating that there was a great improvement in the shrinkage values with the chemical cross-linking of alginate with Ca^{2+} ions (Figure 1). As experimentally found the alginate gels attained a level of 62% and 51% for 5 and $10AHPG_{10\text{-}6}$ gels, respectively, much more than $20AHPG_{10\text{-}6}$ one, which shrunk by an amount of 38% as shown in Table (1). The density and porosity of the obtained scaffolds are presented in the same table. Results revealed that the porosity of the alginate scaffolds varied from 69.9% to 89.7% depending on the percentage addition of alginate. This indicates that the rigid structure of alginate formed by chemical cross-linking tends to

regulate the overall pore size as well as the interconnectivity of the HA/PVA scaffolds. On the other hand, the density of the scaffold exhibited a downward trend with alginate addition. Regarding the physical property values quoted in Table (1), it goes without saying that there is a complete agreement between them. Saying in practical terms, as the shrinkage decreases the porosity and pore size substantially increase, meanwhile the density decreases greatly.

The alginate-containing scaffolds were prepared by freeze extraction method followed by chemical cross-linking in $CaCl_2$ solution, displayed different pore morphology, as judged by SEM examination. Figure (2) depicts the SEM images of alginate scaffolds taken at two different magnifications (50× and 800×). This may be due to The G-block of alginate has correspondingly high affinities for divalent calcium ions. The resultant porous structure was found to be comprised of directionally organized pores and the pore size increases with alginate addition. The pores appear to have an elongated shape. The alginate chains appear to be orderly mineralized with HA nano-particles that cover the flat area of the polymeric ribbons which are organized in a network. A further increase of alginate causes the formation of a typical egg-box structure which is the result of anisotropic growth of isomorphological molecular units [13]. It can be seen that, the alginate-containing scaffolds have tubule-like oriented pore structure, whereas their diameter, wall thickness, and structure regularity are different. With increase in alginate content, the regularity of the oriented pore structure of the scaffold was improved. For the $20AHPG_{10\text{-}6}$, the diameters of the tubule-like macropores reached 300 μm, formed well shape conjugations by regular arrayed organization, as shown in Figure (2). There was a systematic increase in the number and size of these egg-box structures with increasing the alginate contents.

4.2. Mechanical characteristics

Figure (3) presents one of the samples during the compression test, and samples shape after compression test was conducted. Some differences in failure patterns were noticed. The barrel shape resulting from friction is clear in sample 3. As for sample 1 and 2 homogenous compression is noticed, and slight compressive instability at the edges due to work-softening of material

Using engineering stress- strain relationship did not seem convenient for such highly deformable material. So load versus displacement were captured from the output acquisition system, to be interpreted into true stress- strain curve for each tested sample. The following equations were applied to obtain true stresses and strains, taking in consideration the actual dimensions of the sample at each captured result:

Compressive true stress=load × (h_0-h) / vol of sample,

& Compressive true strain=lnh_0 /h

Where: h_0=initial thickness, and h=instantaneous thickness during compression.

Figure (4) presents the average true stress =strain curves for investigated scaffolds.

Straightening out (re-arrangement) in material is noticed at the first non-linear zone of all the curves. The next part is the elastic portion of the curve to be used in calculating the Young's modulus of the material (Table 2). This Linear zone located between 0.02 to 0.09 strains. Non-linear plastic zone extended from 0.07 strains till end of test, indicating matrix degradation towards failure. Maximum average true stress for sample $AHP_{10\text{-}6}$ was 6 MPa, while that for sample $20AHPG_{10\text{-}6}$ was 5Mpa.

4.3- (In-Vitro Test):

4.3.1 Water-binding capacity

Figure 5 shows the water binding capacity of the AHPG scaffolds with different alginate content. It can be observed that after 1 day of immersion, compared with HA/PVA scaffold, the water content of all the alginate scaffolds increased with the introduction of alginate, which contains negatively charged carboxylic and hydroxyl groups that can form hydrogen bonds with water. A mass increase of 320% is observed for $AHP_{10\text{-}6}$ scaffolds, while for $AHPG_{10\text{-}6}$ scaffolds, this increase ranges from 356% to 443%. In additions, the water binding ability of the scaffolds gradually increases with further increase of alginate contents due to the increase in porosity. This result agrees with the results of the porosity analysis (Table 1).

4.3.2 Biodegradation test

Figure (6) shows the relative weights of the obtained scaffolds after 72 h in osteoclastic resorption conditions (pH=4.5) [20]. It can be observed that the rate of weight loss of the scaffolds increased with alginate additions. Among the four scaffolds, it can be seen that the scaffold with a 0 wt% alginate had a lowest weight loss rate, while the scaffolds with 20% alginate had a highest rate owing to the highest porosity and also the water binding capacity.

4.3.3 Bioactivity behavior

The $AHP_{10\text{-}6}$ and $20AHPG_{10\text{-}6}$ scaffolds were subjected to the in vitro bioactivity test. The two types of scaffolds showed same biomineralization behaviors in SBF. Figure (7) shows the SEM micrographs of the porous HA/PVA composite scaffolds after 1, 2, 4 and 8 days of immersion in SBF. After 1day immersion, a lot of tiny apatite crystals deposited on the surface of the

specimens, as shown in Figure 7-b. The particles formed in vitro have a near-spherical shape. Except some large particles with a diameter of about 1–2µm, reflecting the nucleation of new mineral particles. After a longer immersion (e.g. 8 days), a more compact mineral layer was found on the samples surfaces (Figure 7-d). As reported in other study [22], the Ca–P nucleus formed on the surface of biomaterials increased both in number and coalesced with an increase in immersion time and as a consequence of particle growth from the nuclei formed at different times, there was a wide size distribution. The average particle diameter, density (number of particles per unit surface area), and total apatite mass increased with incubation time. They attributed the nucleation and growth of mineral layer on the surface to the enhanced water-uptake capability of the HA induced by the presence of polymer in the nanocomposite[23].

The presence of Ca and P was confirmed by the EDX analysis of the newly formed crystals on the AHP (Figure 8). The Ca/P peak intensity ratio was 1.5, which might be indicative of the presence of a Ca-deficient apatite. This Ca–P deposition is of greater biological interest than stoichiometric HA since the Ca/P ratio in natural bone is lower than 1.67 [24].

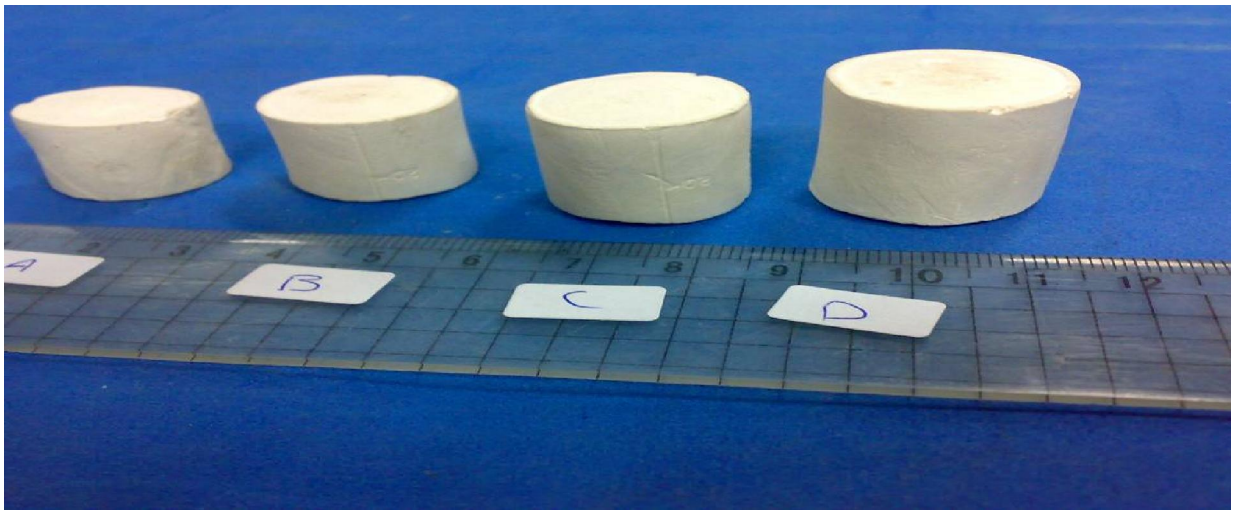


Figure (1). Photographs of scaffolds (a) AHP (b) 5AHPG (c) 10AHPG (d) 20AHPG.

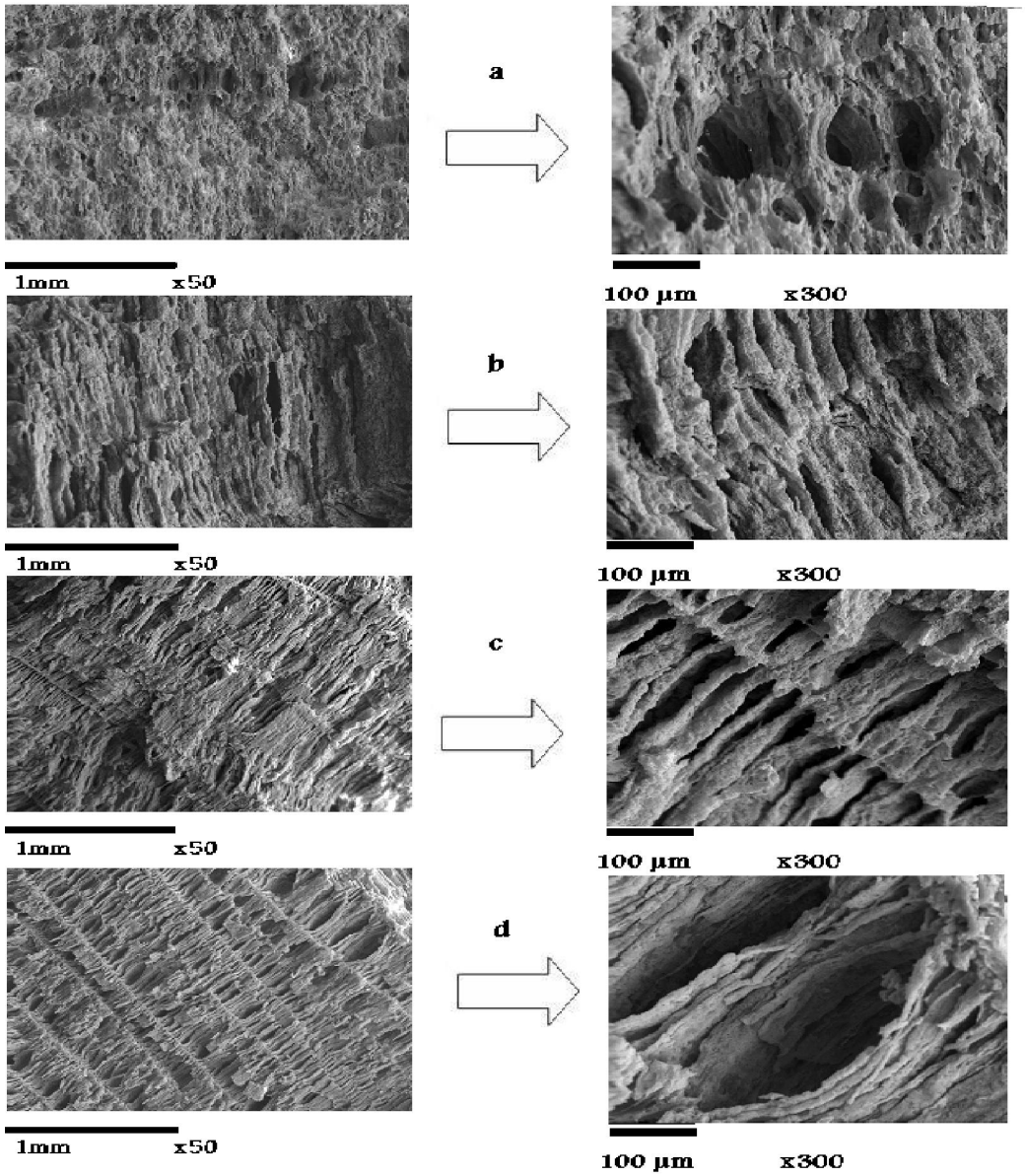


Figure (2). SEM micrographs of resultant scaffolds (a) AHP (b) 5AHPG (c) 10AHPG (d) 20AHPG.

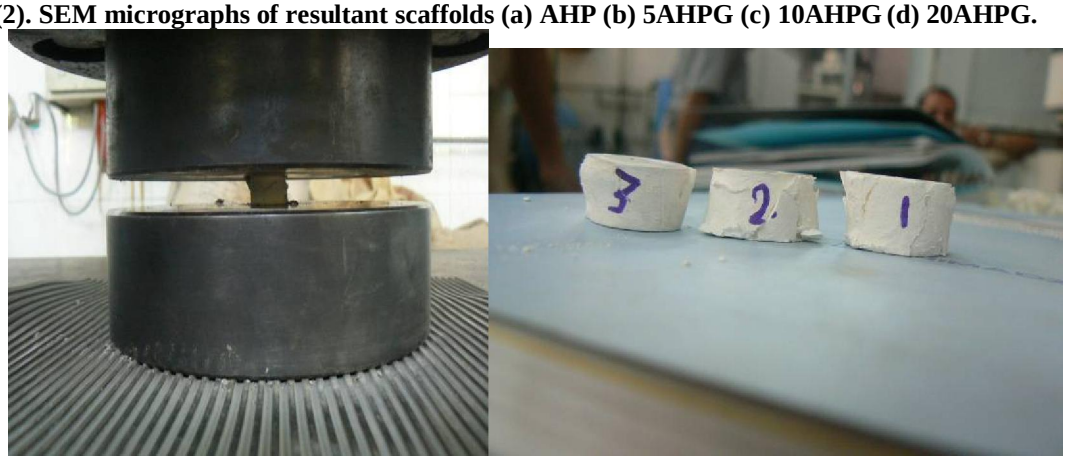
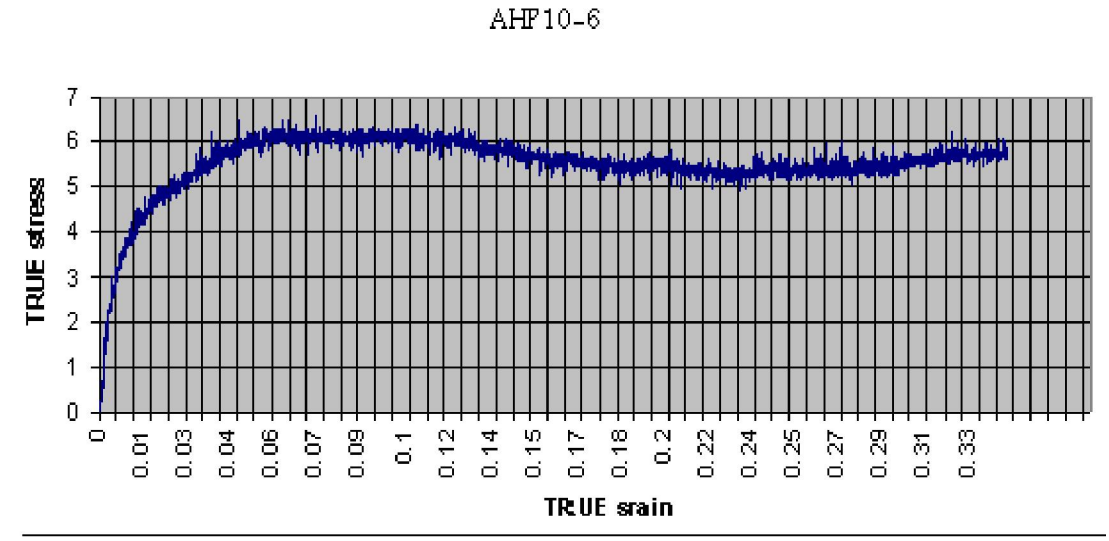


Figure (3): Failure patterns of some of the tested specimens.



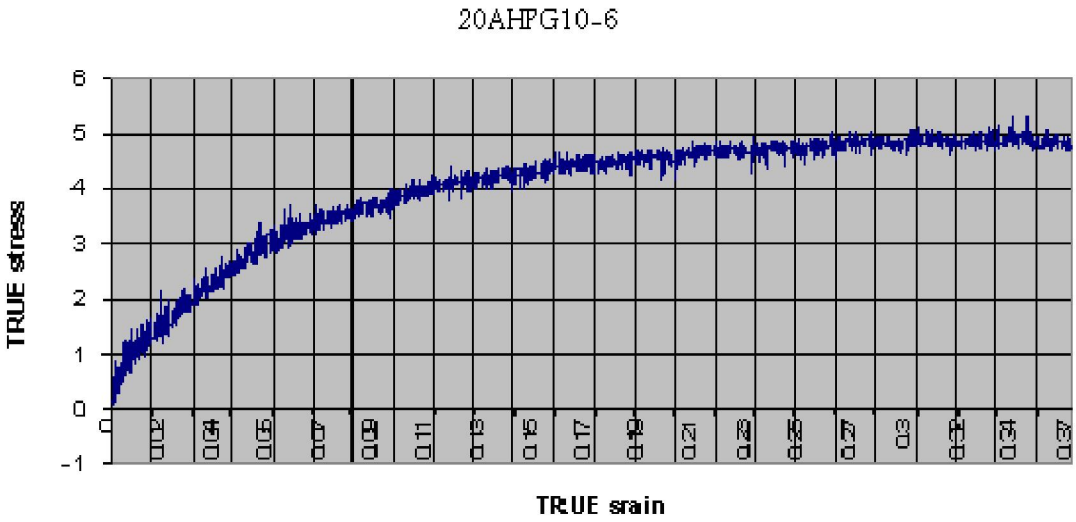


Figure (4): True stress- strain curves of tested scaffolds.

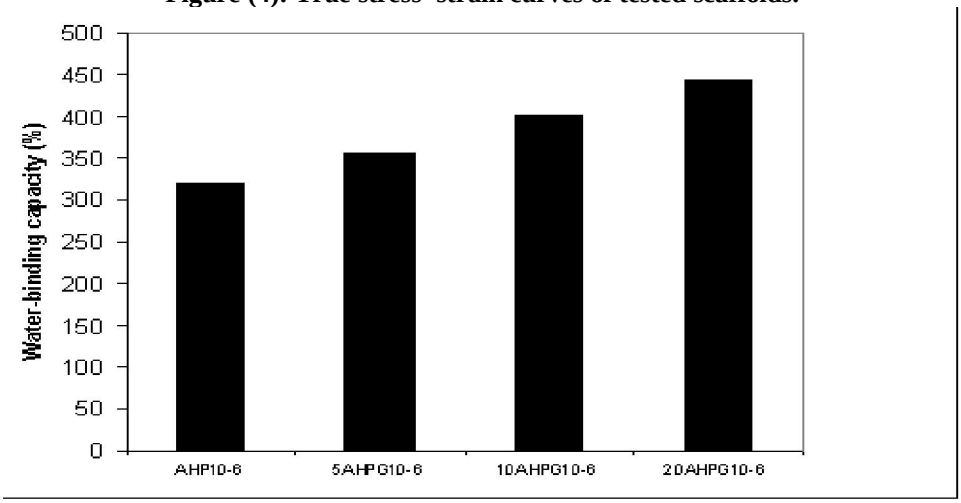


Figure (5). Water binding capacity of resultant scaffolds after one day of immersion in water.

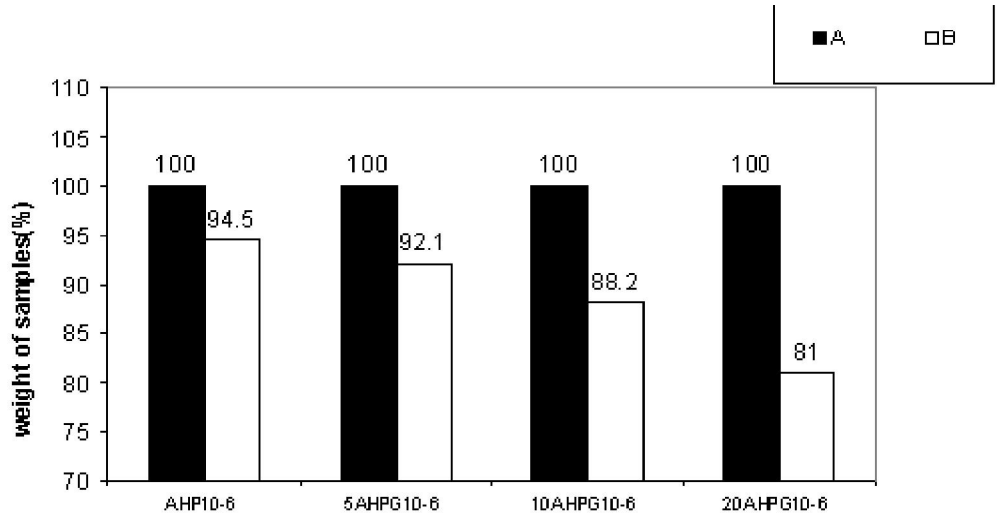


Figure (6). Biodegradation of scaffolds in water at pH = 4.5 (simulation of osteoclastic resorption). A, 0 h; and B, 72 h.

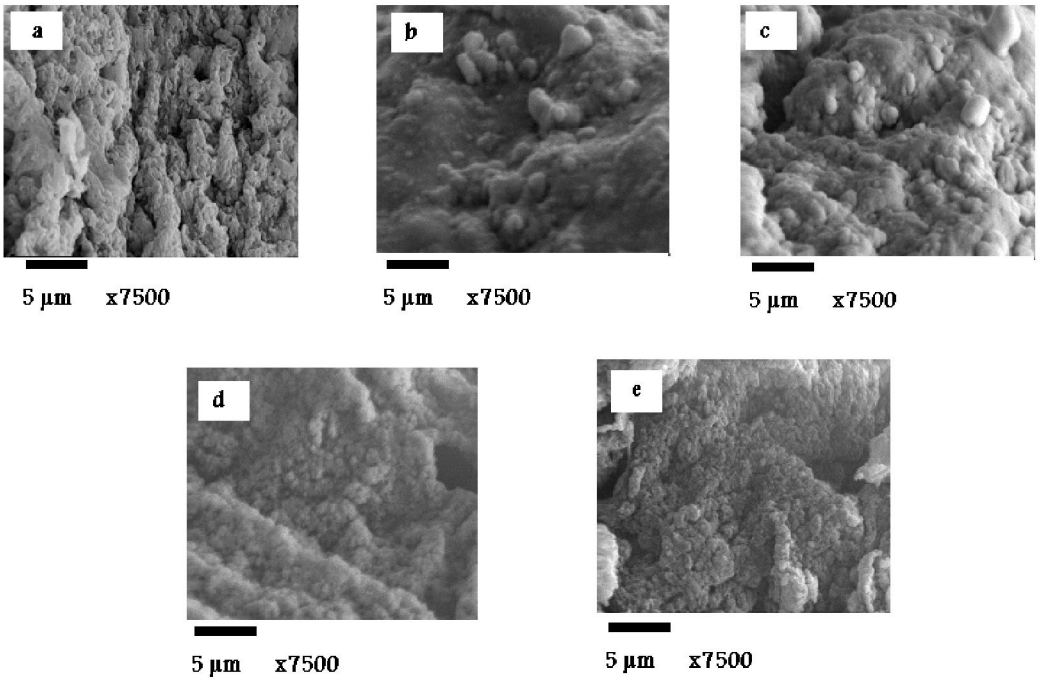


Figure (7). Morphology of the porous AHP_{10-6} scaffolds after immersion in SBF for different periods, observed by SEM: (a) 0, (b) 1, (c) 2 (d) 4 and (e) 8 days.

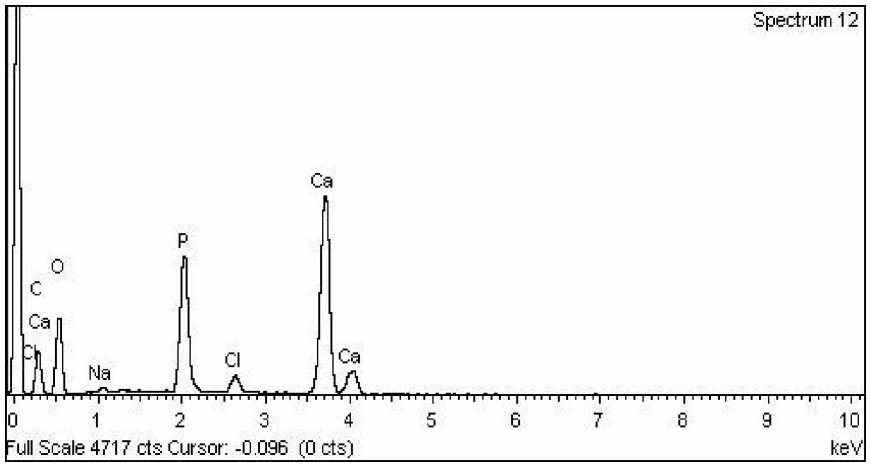


Figure (8). EDX analysis of the AHP_{10-6} scaffold after immersion in SBF for 4 days.

Table (1). Density, Porosity and Related properties of the fabricated HA/PVA and alginate scaffolds.

Samples designation	Density (g/cm^3)	Porosity (%)	Maximum pore size (µm)	Degree of pores interconnectivity	Shrinkage (%)
AHP_{10-6}	0.89	62.3	150	medium	70
$5AHPG_{10-6}$	0.75	69.6	150	High	62
$10AHPG_{10-6}$	0.61	75.1	200	High	51
$20AHPG_{10-6}$	0.48	89.7	300	High	38

Table (2). Maximum compressive strength and elastic modulus of the resultant scaffolds along with cancellous bone.

Samples designation	Maximum compression strength (MPa)	Elasticity modules (MPa)
AHP_{10-6}	6	85.3
$20AHPG_{10-6}$	5	44.7
Cancellous bone	2 - 12	50 - 500

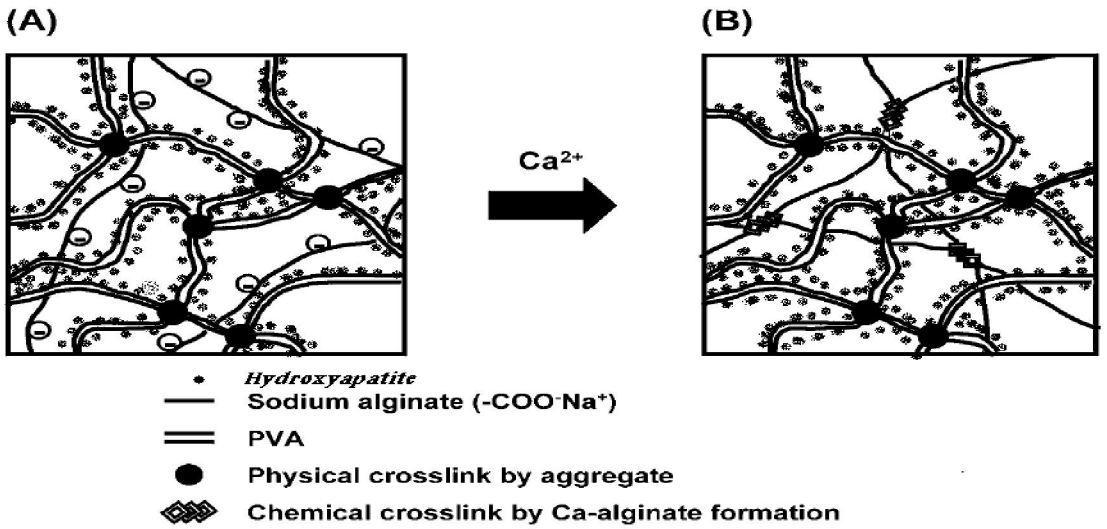


Figure (9). Schematic diagrams showing (a) physical cross-links of PVA chains by freeze extraction and (b) the following chemical cross-links of sodium alginate chains by $CaCl_2$.

5. Discussion

In this study, 3D porous HA/PVA/alginate scaffolds with different alginate compositions (up to 20 wt %) were prepared from the HA/PVA/alginate mixture solutions by freeze extraction and the following chemical cross-linking of alginate. The ultimate porous morphology of a scaffold, including pore size, pore shape, porosity, mechanical and biological properties was affected by alginate addition. The mixture solutions did not show any phase separation for the mixing ratios used in this study owing to their good compatibility. The freeze-extraction step before alginate cross-linking was necessary to keep the original shape of the mixture gel disk, where the freeze-extraction process induces the physical cross-linking of polymer chains by the hydrogen bonding [13]. Hydrogen bonding is thought to be the directional interaction that causes a physical cross-link to form in the gel. The latter was then chemically cross-linked in $CaCl_2$ solution to form interpenetrating networks, as demonstrated in Figure (9).

From SEM observations, it can be seen that the scaffolds dried via liquid desiccant method exhibit the interconnected macroporous network with interconnected pore diameter of 150–300 μm. Generally, for the ideal scaffolds for bone tissue regeneration, the macropore diameter and the macropore interconnections should be larger than 100 μm [25]. If the diameters of these interconnected macropores are not large enough, tissue ingrowth and vascularization will not occur efficiently In Vivo and the damaged tissue cannot be fully regenerated [26]. Most of the researchers have found that the pores between 100 μm determine osteoid growth and the pores larger than 100 lm facilitate proliferation of cells, vascular ingrowth, and internal mineralized bone formation. Therefore, here these $AHP_{10\text{-}6}$ and $AHPG_{10\text{-}6}$ scaffolds have suitable macroporous structure to allow cell migration, bone ingrowth and vascularization.

The difference in the processing technique, the degree of porosity and interconnectivity of the pores in the scaffolds are some of the factors which may have contributed to the variations in the mechanical properties of the scaffolds. In the present study, the decreased compressive strength values can be attributed to presence of macropores within the scaffold; it is well known that it is difficult to achieve high compressive strength for porous materials because of the negative effects of the porous structure. So it is reasonable in this case that the compressive strength and modulus decreased with the growth of scaffold's porosity.

The degradation of the prepared scaffolds at osteoclastic resorption conditions, increased with decrease in alginate content due to the increase in obtained porosity. These in-vitro results suggested the possibility of preparing scaffolds with different degrees of solubility for the needs for different applications. The scaffold with higher alginate composition have higher water uptake. This event may be attributed to the hydrophilicity of alginate. The scaffold with more alginate composition suffered from greater weight loss because the higher water uptake causes faster degradation of the scaffold. In this context, When the HA/PVA and alginate containing composites implanted in body using as tissue scaffold, the degradation of the composite makes room for the growth of new bone and

then is substituted by new bone completely. Moreover, the surface of composite is hydrophilic; this was also confirmed by the rate of water binding capacity, which can facilitate cell adhesion, proliferation and differentiation [25]. So the obtained scaffolds, used as bone substitutes, are hopeful to activate the regeneration and remodeling of bone tissue.

The formation of HA is regarded as the essential requirement for implanted materials to bond to bone in the living body. It has been widely accepted that SBF can well reproduce the in vivo surface changes of certain biomaterials. Many factors affect the nucleation and growth of Ca–P in the SBF solution. The results imply HA particles were rapidly formed on the surface of the scaffolds during SBF soaking. This may be due to the enhanced water-uptake capability (increasing of hydroxyls groups) of the composite induced by the addition of PVA and alginate. This kinetics difference of surface structural change indicating the higher bone-bonding ability of the biomimetically synthesized HA/PVA and alginate containing scaffolds that is awarded by the fast formation of bone-like apatite on their surfaces within one day.

The obtained compressive strength of $AHP_{10\text{-}6}$ (contain 70wt% HA) was 6 MPa. Whereas the alginate-containing ($20AHPG_{10\text{-}6}$) possessed a compressive strength of 5MPa Comparing the obtained results with Sinha et al. [30] who conducted a study on the fabrication of HA/PVA scaffolds for tissue engineering applications using the freeze thawing technique, though their attempts to improve the mechanical properties of the porous scaffolds by incorporating HA. The obtained results show an enhancement in the compressive strength from 2.5MPa to 21.0MPa, for samples comprising 25wt, 40wt and 50wt% HA.

6. Conclusions

- A series of characteristic interconnected open pore microstructures with pore sizes ranging from 150 to 300 microns were fabricated by a modified freeze-extraction method including the physical cross-linking of PVA and the following chemical cross-linking of alginate.
- The porosity of the scaffolds was found to vary from 62.9% to 89.7% depending on the percentage addition of alginate. This indicates that the rigid structure of alginate formed by chemical cross-linking tends to regulate the overall pore size as well as the interconnectivity of the scaffolds. On the other hand, the density of the scaffold exhibited a downward trend with alginate addition.
- The compressive strength of spongy bone is in the range of *2–12MPa*. Hence, the measured compressive strength *(5-6 MPa)* of the present scaffolds falls in this range.
- In-vitro water binding capacity and biodegradation studies, clearly demonstrates that it is possible to fine-tune the biodegradation and correspondingly the biological lifetime of the scaffolds by varying the alginate contents ratios. The characteristics of the resultant nanocomposite scaffolds indicating that the addition of alginate provided a more promising scaffold for tissue engineering applications.
- A biologically active apatite layer forms within a short period on composite scaffolds after its immersion in SBF, demonstrating high *in vitro* bioactivity of the composite.
- The goal of an ideal scaffold that provides good mechanical support temporarily while maintaining bioactivity and that can biodegrade at later stages at a tailorable rate is achievable with the developed alginate containing scaffolds.

7- Acknowledgement

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Utilization *of Opuntia ficus indica* waste for production of *Phanerochaete chrysosporium* bioprotein

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Abstract: The highest Opuntia *ficus indica* waste saccarification was 75.6 % obtained with 1% (w/v) NaOH treatment. *Phanerochaete chrysosporium* was the most potential fungus among the tested microorganisms : *A. terreus* and *R. oryzae* for bioprotein recovery of 0.073/100 g waste.*Opuntia ficus indica* peels proved to be a suitable substrate among the other agricultural wastes, corn cob shred , and sugar cane bagasse which were used as carbon sources for *Phanerochaete chrysosporium* bioprotein production. Also ,The most optimum fermentation conditions were : 10(g /L)*Opuntia* waste as carbon source ; phosphate buffer for bioprotein extraction ; 3% (v/v) inoculum size; supplementation of Modified Czapek Dox medium (MCD) with 0.3 %(w/v) CSL; the initial PH , 4 at 150 rpm., and 75 ml (MCD)medium was suitable volume resulted in 0.123 /100 g bioprotein after 7 days of fermentation at 30°C. [Journal of American Science 2010;6(8):208-216]. (ISSN: 1545-1003).

Keywords: Utilization *of Opuntia ficus india;* waste; *Phanerochaete chrysosporium* bioprotein

Introduction

Opuntia ficus indica is one of the most promissory by presenting the largest part of alimentation furnished to the animals during the drought. This increases the availability of fodder and alleviates the problems of the water supplement to these animals. It is also rich in sugars,minerals, calcium, iron and vitamin A. It presents an elevated texture of soluble carbohydrates besides presenting a high coefficient of digestibility of the dry matter and high productivity Lúcia de Fátima *et al., (*2005). Besides, improper handling of solid waste is a health hazard and causes damage to the environment. The main risks to human health arise from the breeding of disease vectors, like flies and rats. Healthy life and cleaner environment is the end result of solving these problems in such a way by utilizing the waste into valuable by-products ; Nigam *et al*., (2009). Mycelium biomass from *Rhizopus oryzae*, can partly substitute high-quality fishmeal in diets to rainbow trout without causing any major short-term adverse effects on growth, nitrogen and amino acids digestibility. Nutrient digestibility of diets containing mycelium biomass of *R. oryzae* are in carnivorous fish and larvae ; Uysal *et al*. ,(2002) and Olsen *et al*.,(2006)). For humans, it is also considered as food additive to improve flavor, fat binding and more recently as a replacement for animal protein in the diet(Jamal *et al.,* 2007). Bioprotein can also be used as additives in certain chemical and pharmaceutical industries. Bioprotein is the protein extracted from cultivated microbial biomass that can be produced using a number of different microorganisms and such as low carbon cost, high energy sources agricultural wastes .Agro- wastes can be regarded as new sources for bioprotein production, which have a high nutritional value, do not compete with food for human consumption, economically feasible and locally available ;(Anupama and Ravindra, 2000, Uysal *et al.,* 2002).

Lignocellulosic waste is a complex mixture of cellulose, hemicellulose, lignin along with extractives,wastes pretreatment is therefore a necessary process in order to achieve high yield. Grinding and milling are the primary physical accomplished by using acids or bases . The objectives of this study were beneficial in production of nutritional bioprotein from a cheaper carbon source.

Materials and method

Microotganisms:

Three different fungi were used ; culture of *Phanerochaete chrysosporium* ATCC 28236 obtained from Cairo MERCIN , Ein Shams University; culture of *Rhizopus oryzae* obtained from Dr.M. U. Noaman, Biochemistry Dept. and *Aspergillus terreus* obtained from Dr.A.F. Salah, Plant Pathology Dept. NRC.,Egypt.

Maintenance medium*:*

All strains were maintained by subculturing on PDA slants for 7 days then stored at 4ºC .

Inoculum preparation:

Spores were harvested from a week old slants in five ml of sterile distilled water. 0.5 ml of spore suspension was added to fifty ml broth with pretreated substrate based medium in 250 ml conical flasks. The inoculated flasks were incubated at rotary shaker 100 rpm for 30°C for 7 days. (Anupama and Ravindra, 2000).

Preparation of lignocellulosic substrates

-Fresh *Opuntia* peels were collected from local markets during summer season stored in a refrigerator until use. The fresh *Opuntia* skins were washed and cut into smaller pieces (1-2 cm) dried ,grind to a very small pieces , before use;Olivera *et al.,(*2001).

-Corn cob shred: Obtained from Sugar and Starch plant, Mustrud , Egypt,washed, dried in an oven at 60-100°C to a constant weight. The dried substrate was ground, sieved ,before use.

-The sugar cane bagasse: The sugarcane dust was obtained from a market . The sugarcane dust was washed, dried at a temperature of 60 °C for 96 h, was grind and sieved before use.

lignocellulosic waste acid treatment:

Five grams of fine carbon sources; corn cob , *Opuntia* and bagasse were suspended in 50ml of 1%(v/v) HCL for I hr in a boiling water bath ,reducing sugars were estimated .The treated materials was washed until the wash was neutral; (Gupta *et al.*,(1972).

lignocellulosic waste a lkali treatmrnt:

25 g of different substrates were suspended in 500 of 1 % NaOH solution in 1 liter conical flask boiling for 1 h in a water bath ; Choudhury *et al.*(1980).reducing sugars were estimated . The treated material was washed until the supernatant was alkali free as checked by pH meter

Fermentation medium:

0. 25 g of an accurately weighed treated lignocellulosic waste(corn cob , sugar cane bagasse and *Opunti*a) were placed in a 250 ml conical flask containing fifty mls of modified Czapek Dox medium (MCD medium) in order to obtain the biomass. MCD medium contained the following ingredients (g/100 ml): K_2HPO_4, 0.12; $MgSO_4$, 0.06; $FeSO_4$, 0.05; KCl, 0.02; $NaNO_3$, 0.3 and sucrose,3; Anupama and Ravindra (2001), adjusted to pH 4, autoclaved at 121°C for 15 min. Inoculated with 1 %(v/v)from 7 days old slant ,then incubated on a rotary shaker at 100 rpm and 30°C for seven days fermentation.

Culture Harvesting and biomass concentration :

The cultures were harvested by filtering the biomass through a weighed Whatman no. 1 filter paper, the fungal biomass washed with distilled water to remove medium adhered to the mycelium .The filter papers along with the biomass were dried at 60°C for 48 h to constant weight,APHA (1989), left in desiccators(Cardoso,1981), ground in a mortar to a very fine powder and kept desiccated.

The biomass means the mycelium together with unfermented substrate residue and then analyzed for their protein content .

Chemical analysis:

The amount of total sugar in the homogenized *Opuntia* samples was determined. For estimation **of total sugars**, one g of the dried substrate was suspended in 60 ml of distilled water. This was kept at an ambient temperature for12 h for the extraction of sugars that are measured by the phenol-sulfuric acid method with glucose as a standard (Dupois *et al.*,1965). The moisture content of the sample was determined according to methods A.O.A.C.(1980) and total protein by Lowry *et al.* (1951).

Extraction buffer of microbial protein:

0.25 g of fermented moldy biomass was soaked with 50 mL

of **phosphate buffer**, pH(6.9) and stirred for 30 min. The extract was collected by filtration. The temperature during the course of extraction was maintained at 4°C. The supernatant obtained was used for estimating protein content. The extraction process was repeated five times, all the extractants were transferred to a flask, and the final volume was made up to 100 mL with distilled water; Oliver *et al*.,(2001).

Total protein : Protein determination was measured according to Lowry *et al.* (1951) method using folin-phenol reagent and bovine serum albumin standard .All spectrophotometer readings were recorded at 660 after 20 minutes.

Determination of total reducing sugar:

Reducing sugars were determined according to Nelson (1944).

Culture conditions

Effect of carbon source

Different carbon sources were used; *Opuntia* waste ,bagasses and corn cob in concentration of 0.5% (w/v).

Substrate concentration

Varying concentration of alkali treated *Opuntia ficus indica* substrate 0. 5-1.5 % (w/v)were investigated.

Effect of initial pH: (MCD) medium was adjusted to different pH values before autoclaving.

Effect of agitation speed:
The fermentation medium were shaked at different speed ranging from 50 – 200 rpm at 30°C.

Effect of nitrogen supplementation:
0.3 % (w/v) ammonium sulphate ,peptone, yeast extract and CSL were supplemented instead of sodium nitate in the(MCD) broth.
CSL(corn steep liquor) obtained from Sugar and Starch company, Mustrud, Egypt .

Effect of aeration:
To study the effect of air /medium ratio on bioprotein production ,different volumes of MCD cultivation medium in 250 ml Erlenmyer flask were used.

Calculations:
-(%)_saccharification =Amount of reducing sugars(mg/ml)/Amount of substrate(mg/ml) x 0.9 x100; Dey *et al*., (1992).
Protein recovery(g/l) = protein content(%) x biomass (g/l);Ibrahim(1998)-
Protein recovery (g/100g) = protein recovery (g/l) / substrate used (g) / L(.Ebrahim,1998)

Results and Discussion

Ligbocellulosic waste Treatment with 1% NaOH :
On using NaOH pretreated,corn cob, sugar cane bagasse and *Opuntia* wastes saccarification reached 75.6
% from *Opuntia* skin waste as a potential, low cost and high content of carbohydrate make it more suitable compared to other wastes ; 63.9 and 56.16 % for sugar cane bagasse and corn cob waste ,respectively . Corn cob is a very hard lignocellulosic waste of corn industry that must be deliginfy as it cannot be utilized efficiently by microbes without pretreatment for single cell protein *with P. chrysosporiu*m;Asad *et al*.,(2000) .

Table(1). % Saccarification of carbon sources after treatment with 1% Na OH .

Agro-waste	Total reducing sugar concn(mg/ml)	(%))Saccarification
Opuntia ficus indica	4.2	75.6
Sugar cane bagasse	3.55	63.9
Corn cob	3.12	56.16

Ligbocellulosic waste treatment with 1% HCL:

As it could be seen from Table 2. 64.8 % saccharification could be obtained with *Opuntia* skin with HCL treatment compared to 45.9 % and 44.1 % for sugar cane bagasse and corn cob wastes .Such low cost agro-cultural lignocellulosic wastes must be treated by physical/chemical methods to liberate cellulose from lignins. Since cellulose in lignin-hemicellulose-cellulose complex is not accessible to enzymatic hydrolysis ;Asad *et al.* (2000. Table (1,2) showed that alkali treatment is more effective in preparing *Opuntia* skin for fermentation, so, this pretreatment will be applied in the next experiments. Many pre-treatment methods have been reported which vary from alkali or acid treatment, steam explosion or even x-ray radiation; Rodriguez-Vazquez *et al*.(1992).
Table(2). % Saccarification of carbon sources after treatment with 1% HCl.

Agro-waste	Total reducing sugar concn(mg/ml)	(%)Saccarification
Opuntia ficus indica	3.60	64.8
Sugar cane bagasse	2.55	45.90
Corn cob	2.45	44.1

Evaluation of potential microorganism:
As could be indicated from table 3, the total protein % production by each strain differ from the other. As many fungal species are used as a protein-rich food. They provide the B-complex group of vitamins and thy also show a low level of nucleic acid content. Biomass obtained from *P. chrysosporium* has been found to contain most of the essential amino acids (Balagopalan and Padmaja, 2000). However, as stated by Jamal (2007),the fermentation time for maximum production of bioprotein was different for every microorganism, e.g. *A .niger* and *P. chrysosporium* showed highest concentration on sixth day.Also, As it could be seen from table 3, *P. chrysosporium* is of importance due to the highest bioprotein production 6.90 /100g substrate together with the highest biomass 5.95 g/l compared to the other microorganisms. . Other strains gives 3.374, 1.016 /l00g *Opuntia* for *A.terreus* and *R.oryzae,* respectively. The protein from microorganisms is cheap and competitive with other protein sources. It may have good nutritive value depending, however upon their amino acid composition. It is necessary to use microorganisms which is non toxic to the animal. Most organisms used in direct single cell protein are fungi as *Aspergillus , Fusarium and Trichoderma*

Table (3). Screening on microbial bioprotein production using *Opuntia ficus indica peels waste.*

Microog anism	Bi om ass (g/ l)	Total prote in(%)	Total protei n recove ry(g/l)	recove ry protein (g/100 gsubst rate)
P. chrysosporiu m	5.9 5	5.8	0.345	.069
A. Terreus	4.8 2	3.5	0.169	0.034
R.oryzae	4.6 2	2.2	0.101	0.20

Initia1 substrate concentration is 5 g/ l broth.

Production of *P. chrysosporium* bioprotein using different carbon sources :

The proximate composition of *Opuntia w*aste used was as follows : 0.05% total protein, moisture 85%, and 55.25%. total carbohydrates of its weight.

The term single cell protein (SCP) refers to dead, dry cells of micro-organisms such as yeast, bacteria, fungi and algae which grow on different carbon sources. As it could be seen from table 4, *P.chrysosporium* protein recovery reached 0.073 /l00g .Hence, *Opuntia* waste could be used as one of less expensive means of increasing the protein quality such as cassava and wheat flour ;Ghaly *et al.,* (2004) than sugar cane bagasse and corb cob which produce 0.051and 0.046/l00 g substrate ,respectively . Other potential substrates for SCP include citrus wastes, sulphite waste liquor, sewage ,molasses, animal manure, whey, starch and wheat flour, Jamal *et al.,* (2008).

Table (4). *P.chrysosporium* bioprotein on using *different carbon sources* .

Waste	Biomass (g/l)	Total protein (%)	Protein recovery (g/l)	Protein recovery (g/100g substrate)
Opuntia	5.6	6.5	0.364	0.073
Sugar cane bagasse	4.59	5. 6	0.257	0.051
Corn cob shred	4.61	5. 1	0.234	0.046

Initia1 substrate concentration is 5 g/ l broth.

Effect of carbon source concentration on *P.chrysosporium* bioprotein production*:*

The biomass values on using different concentrations of *Opuntia* skin is shown in table (5) . It could be stated that reduced substrate, result in greater protein yield as more oxygen required for the oxidation of the substrate. 1%(w/v) substrate concentration result in 0.086 /100 g *Opuntia* waste so, it will be used in the next experiments . 2%(w/v) substrate result in 0.049/100g waste, this may be due to exhaustion of nutrients required in fermentation medium other than the *Opuntia* waste.

One of the main advantages of SCP compared to other types of protein is the small doubling time of cells as in yeast. Due to this property, the productivity of protein form micro-organisms is greater than that of traditional proteins. For the assessment of the nutritional value of SCP, factors such as nutrient composition, amino acid profile, vitamin and nucleic acid content as well as palatability must be evaluated.

Table (5). Effect of varying concentrations of *Opuntia ficus indica* skin on *P. chrysosporium* protein production.

* (substrate concentration %(w/v)	Biomass (g/l)	Total protein(%)	Protein recovery (g/l).	Protein recovery(g/100g substrate).
0.5	6.6	5,5	0.363	0.073
1.0	8.15	10.5	0.856	0.086
1.5	14.4	5.6	0.806	0.054
2.0	19.1	5.1	0.974	0.049

Initia1 substrate concentration is 5,10,15,20 g/ l broth.

Effect of *P. chrysosporium* inoculum size on bioprotein production :.

As it could be seen from fig 1,the total protein content of the biomass was only 8.02 %with 1*%(v/v) P. chrysosporium* inoculum when *Opuntia* based medium incubated for 7 days at 30°C on 100 rpm .The total protein was found to be increased to 9. 139 %l with elevation of the inoculum size to 3 (v/v)%.However, no increase in total protein was noticed with 4(v/v) %, as it gave 4.5% total protein . So, 3% (v/v) inoculum will be used in the next experiment s .

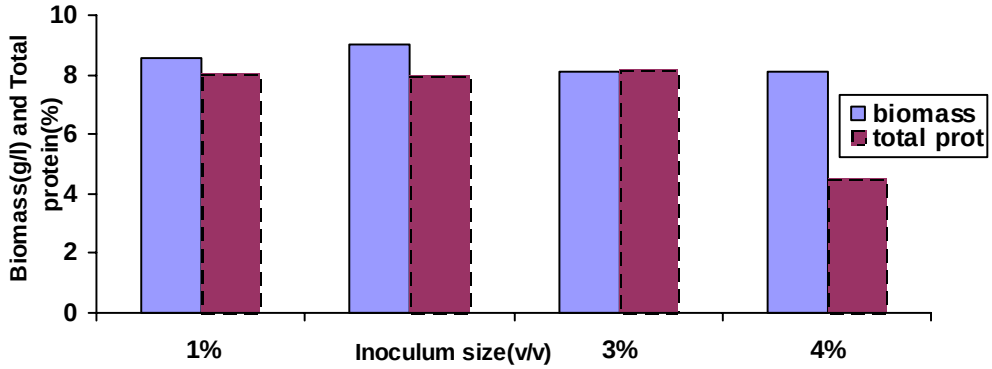

Fig1. Effect of inoculum size on P. chrysosporium bioprotein production.

Effect of (MCD) nitrogen source on *P. chrysosporium* bio protein production:

As it could be seen from Table (6), CSL resulted in 0.096 /100g *Opuntia cactus*. This was essential in supporting and enhancing the growth of *P. chrysosporium*, as it gave 8.10 g /l biomass as well as to promote the production of bioprotein, this comes in agreement with Chahal *et al.* (1987) who produced maximum biomass protei
n (40%) from CSL as additional nitrogen source is required to support both microbial and biomass production. These results are also in accordance with those by (Nacib *et al*., 2001); who stated that the production of lactic acid by using date juice as fermentation medium could be increased after supplementing it with nitrogen sources, and also Rosma and Cheong (2007) who stated that addition of inorganic nitrogen source as the main components in the growth of biomass and building block of proteins is due to the lack of nitrogen sources in pineapple juice. The effect of inorganic nitrogen source was studied by Chung and Muhammad (2000) to reach the highest yield of cellulose..The importance of using carbon and nitrogen source were reported to be essential in providing a suitable growth media for *P. chrysosporium* which was used in the molasses wastewater (MWW) (Ahmadi *et al*., 2006), while there are also many reports on use of C and/or N sources to support of *P. chrysosporium* growth (Kirk *et al.,* 1978; Jansheker and Fiechter, 1983).Urea, and peptone when supplemented to the medium along with *Opuntia* peels waste gave higher protein yield compared to medium supplemented by $(NH_4)_2SO_4$ as they gave, 0.082 , 0.066 and 0.061/100 *Opuntia* substrate, respectively .So ,CSL will be used in the next experiments. CSL is an unexpensive source of nutrient in the fermentation medium, also used as the sole source of nitrogen ,vitamin ,growth stimulant and other nutritional requirement;Amarty and Jeffries(1994).

Table (6). Effect of nitrogen source for the production of *P. chrysosporium* bio protein *using Opuntia ficus indica* based medium.

Nitrogen source in MCD)(	Biomass(g/l)	Total protein(%)	Total protein recovery (g/l)	Recovery protein (g/100g substrate)
CSL	8.10	11.9	0.964	0.096
Urea	7.12	11.6	0.825	0.082
Peptone	7.76	8.5	0.659	0.066
$(NH_4)_2SO_4$	7.82	7.8	0.611	0.061

Initia1 substrate concentration is 10 g/ l broth.

Initial pH effect on *P. chrysosporium* bioprotein production.

On optimizing medium conditions for attaining higher production of bioprotein ,as it could be seen from Table(8) pH 4 result in 0.092 g /100 g waste recovery protein , while higher pH values reduce the bioprotein,this findings are in agreement with Jamal *et al*., (2009) who stated that any increase in pH medium becomes inhibitory for the organism and induction of enzyme synthesis.

Table(7) . Effect of initial pH on bio protein production by *P. chrysosporium* grown on *Opuntia ficus indica* based medium .

Initial PH	Biomass (g/l);BM	Total protein(%)	Protein recovery g/l)(	Recovery protein(g/100g substrate)
2	7.4	3.3	0.244	0.024
4	8.12	11..4	0.925	0.092
6	7.12	1. 6	0.113	0.011
8	7.1	1. 1	0.078	0.008

Initia1 substrate concentration is 10 g/ l broth.

Effect of agitation on *P. chrysosporium* bioprotein production using *Opuntia ficus* indica based medium.

As could be seen from fig (2), with 200 rpm , lower protein content 6.7 % protein on 7 th day of fermentation at 30°, this is in agreement with Daugulis, (2004) who stated that , higher agitation rates results in reduced levels of microbial protein. While,with 150 rpm speed resulted in 10.85 % *protein* whereas at 200 rpm the biomass still growing 9.11 g/l.So,150 rpm will be used in the next experiment.

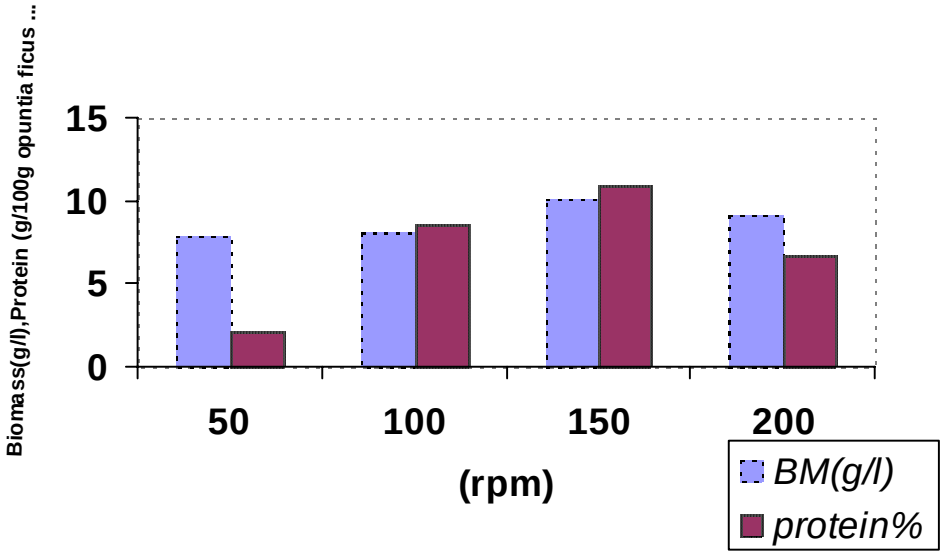

Fig.2.The effect of agitation on Phanerochaete chrysosporium bioprotein production.

Effect of aeration on *P. chrysosporium* bioprotein production using *Opuntia ficus indica* based medium :

As it could be seen from Table(8),with 50 ml (MCD)medium the biomass was 8.9 g/l while protein was.0.105 g/100 g *Opuntia* substrate. With 75 ml broth volume /250 ml flask capacity at pH 4, when inoculated with 3% spore suspension and incubated for 7 days at 30°C on 150 rpm , resulted in 0.123*g P. chrysosporium* bioprotein /100 *g Opuntia*

waste, with 9.5 g biomass /l concentration This represents the highest biomass concentration because it reached maximum growth yet and were still in growth phase.The biomass should increase exponentially as the cell is growing and when only the cells enter the decline phase or death phase, biomass will decrease. With 125 ml culture volume , the reduction in protein content 0.032 g/100g *Opuntia* waste because of the decreased aeration which may reduce the growth of the organism.It should be mentioned although there is an increase in the bioprotein on applying the optimal cultivation conditions, yet the amount is still small compared to the amount of protein initially present , this may be due to the protein extraction method, the buffer used or even the cultivation season of *Opuntia.*

Table(8). Effect of aeration on *P. chrysosporium* bio protein production by using *Opuntia ficus indica* based medium .

Culture volume(ml)	Biomass (g/)l	Total protein(%)	Total protein recovery (g/l)	recovery protein(g/100g substrate)
50	8.9	11.85	1.054	0.105
75	9.5	12.98	1.233	0.123
100	8.5	4.12	0.350	0.035
125	7.8	4.12	0.321	0.032

Initia1 substrate concentration is 10 g/ l broth.

Hatem *et al.,* (2001) stated that the extract obtained from nopal peel at neutral pH of carbohydrate polymers from *Opuntia ficus-indica* and their physicochemical characterization indicated that they are polysaccharides .Their sugar composition indicates that all the polysaccharides obtained contain anionic moieties, galacturonic acid residues and are typical of pectin. It could be deduced the valuable reuse of this carbohydrate rich solid waste in minimization of costs associated with nutritional supplements in a fermentation medium ,it is also essential for economic large-scale production, which somehow contribute to the pollution problem in the environment, either for the production of useful bioprotein or other beneficial metabolites .

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Comparative Evaluation of Different Organic Fertilizers on Soil Fertility Improvement, Leaf Mineral Composition and Growth Performance of African Cherry Nut (*Chrysophyllum Albidium* L) Seedlings

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Abstract

The effect of wood ash, poultry manure and pig manure as source of fertilizers on soil fertility improvement, leaf mineral composition and growth performance of African Cherry nut (*Chrysophyllum albidium* L) seedlings was investigated at Akure in the rainforest zone of Nigeria. Three organic fertilizer treatments namely poultry manure, wood ash and pig manure were applied at 40g per 10kg soil filled poly bag (8t/ha) along with a reference treatment 400kg/ha NPK 15-15-15 fertilizer (2g per 10kg), replicated four times and arranged in a completely randomized design (CRD). The results showed that these organic fertilizers increased significantly ($P<0.05$) the growth parameters (plant height, stem girth, leaf area, leaf number, root length and fresh shoot weight), soil and leaf N, P, K, Ca, Mg, Soil pH and organic matter (O.M) compared to the control treatment. Poultry manure treatment had the highest values of plant height, leaf area, stem girth, leaf number, root length and fresh shoot weight (kg) of African cherry nut seedlings compared to wood ash, pig manure, NPK 15-15-15 and control treatments respectively. For-instance, poultry manure treatment increased the plant height, lea area, stem girth, leaf number, root length and fresh shoot weight of African cherry nut seedlings by 13%, 8.4%, 4.3%, 7.8%, 15.7% and 30% respectively compared to wood ash. When compared with NPK 15-15-15 fertilizer, poultry manure treatment also increased the plant height, leaf area, stem girth, leaf number, root length and fresh shoot weight of African cherry nut by 9%, 13%, 7%, 18%, 16.9% and 37% respectively. For leaf chemical composition of African cherry nut seedlings, poultry manure had the highest values of leaf N and P while wood ash also had the highest values of leaf K, Ca and Mg compared to others. When compared with NPK 15-15-15 fertilizer treatment, wood ash treatment increased the leaf K, Ca and Mg by 51.7%, 99.3% and 99.5% respectively. However, NPK fertilizer increased leaf N and P by 50.5% and 26.3% compared to wood ash. For soil chemical composition, poultry manure treatment had the highest values of soil O.M, N, P while wood ash also had the highest values of soil pH, K and Mg compared to others. For-example, poultry manure increased the soil O.M, N and P by 6.6%, 44% and 29% compared to wood ash. When compared to NPK 15-15-15 fertilizer treatment, poultry manure increased soil pH, O.M, N, Ca and Mg by 29%, 85%, 34%, 98% and 97% except soil P and K where NPK fertilizer increased these nutrients. The highest soil K.Ca, K/Mg, P/Mg and P/Ca ratios in the NPK 15-15-15 fertilizer caused imbalance in the supply of P, K, Ca and Mg nutrients to African cherry nut seedlings. From this study, poultry manure applied at 8t/ha (40g/10 kg soil) was the most effective treatment in improving African cherry nut growth parameters, soil and leaf mineral composition. [Journal of American Science 2010;6(8):217-223]. (ISSN: 1545-1003).

Key words: Organic fertilizers, soil fertility improvement, growth performance, leaf mineral composition and African cherry nut seedlings.

1. Introduction

African cherry nut (*Chrysophyllum albidium* L) belongs to the family sapotaceae which is up to 25-30m in height with a mature girth varying between 1.5-2 m and simple elliptic to oblong leaves. It produces fleshy fruits which are popularly taken by the people as refreshment, the juice are gum like and can be fermented for the production of wine and alcohol through distillation. Opeke (2005).

Despite the economic and nutritional importance of African cherry nut to the nations, the trees are going into extinction because of the scarcity of young seedlings to replace the few ageing ones in the field. This replacement is becoming difficult due to the problems of raising the plant in the nursery and continued decline in soil fertility status.

Efforts to increase the soil nutrient status is limited by high cost of purchase and scarcity at the farmers

level. Hossian (2000). Therefore, there is justification for the adoption of low cost and sustainable organic fertilizers to grow African cherry nut.

Except the previous works of Moyin-Jesu and Atoyosoye (2002); Moyin-Jesu (2008); Obatolu (1995) who worked on cocoa, dika nut and coffee seedlings using different organic fertilizers, there is paucity of research information on the use of poultry manure, wood ash and pig manure to grow African cherry nut both in the nursery and field.

The objectives of this research work are:

(i) To determine the effect of different organic fertilizers on the growth of African cherry nut seedlings in the nursery.
(ii) To determine the influence of the organic fertilizers on the leaf mineral composition of African cherry nut and soil chemical composition after the experiment.

2. Materials and Methods

The experiment was carried out at Akure in the rainforest zone of Nigeria in 2008 and was repeated in 2009 to validate the results. The soil is sandy, clay loam, skeletal, kalinitic, isohyperthermic oxic paleustalf (Alfisol). Soil Survey Staff (1999). The annual rainfall is between 1100 and 1500mm while the average temperature is 24^{o}C.

Soil sampling and analysis before planting

Thirty core samples were collected randomly from 0-15cm depth on the site using soil auger, mixed thoroughly and the bulk sample was taken to the laboratory, air-dried, and sieved to pass through a 2mm screen for chemical analysis.

The soil pH (1:1 soil/water) and (1:2 soil/O.O1M $CaCl_2$) solution was determined by using a glass calomel electrode system Crockford and Nowell (1956) while organic matter was determined by the wet oxidation chromic acid digestion method. Walkley and Black (1934). The total nitrogen was determined by the microkjedahl method (AOAC, 1970) while available soil phosphorus (P) was extracted by the Bray P_1 extractant and measured by the Murphy blue colouration and determined on a spectronic 20 at 882Um (Murphy and Riley, 1962).

Soil K, Ca, Mg and Na were extracted with 1M NH_4OAC pH_7 solutions. The K, Ca and Na contents were determined with flame photometer while Mg was determined with an atomic absorption spectrophotometer (Jackson, 1958).

The soil exchangeable acidity (H^+ and Al^{3+}) were determined using O.O1M HCl extracts and titrated with O.1M NaOH (McLean, 1965) while the micronurients (Mn, Cu, Fe and Zn) were extracted with 0.1M HCl (Ogunwale and Udo, 1978) and read on Perkin Elmer atomic absorption spectrophotometer. The mechanical analysis of the soil for soil texture determination was done by the hydrometer method (Bouycous, 1951).

Source and Preparation of Organic Fertilizers

Wood ash, poultry and pig manure were obtained from the cassava processing unit and livestock unit of Federal College of Agriculture, Akure respectively. The organic materials were processed to allow decomposition for easy release of nutrients.

Wood ash was sieved to remove the pebbles, stones and unburnt shafts while pig and poultry manures were air-dried to allow quick mineralization process.

Chemical Analysis of the Organic Materials

Two grams each of the processed forms of the organic fertilizers were analyzed. The percent nitrogen content was determined by Kjedahl method. Jackson (1964) while the determination of other nutrients such as P, K, Ca, Mg, Fe, Zn, Cu and Mn was done using the wet digestion method based on 25-5-5ml of HNO_3-H_2SO_4-$HClO_4$ acids (AOAC, 1970). The organic carbon was determined by wet oxidation method through chromic acid digestion. Walkley and Black (1934).

Collection of African cherry nut seeds for planting

Ripe fruits of African cherry nut were obtained from the few trees at Federal College of Agriculture, Akure. The fruits were carefully opened to remove the seeds embedded with whitish mucilage, carefully washed in water, air-dried for 10 days, parked inside poly bags, labeled and stored for the planting in the nursery experiment.

Nursery establishment of African cherry nut seedlings

The site was cleared to remove weeds and other debris and a shed was erected for the nursery. The bulk soil taken from the site (0-15cm depth) was sieved to remove stones and plant debris and 10kg of the sieved soil was weighed into a poly bag (30x17cm).

There were three organic fertilizer treatments namely wood ash, poultry manure and pig manure applied at 8 tonnes/hectare each (40g of each treatment per 10kg soil) with four replications and arranged in a completely randomized block design (CRD). A reference treatment with 400kg/ha NPK 15-15-15 fertilizer (2g per 10 kg soil) was used along with a control treatment (no fertilizer, no manure).

The treatments were incorporated into the soil using hand trowel and allowed to decompose for one week before planting African cherry nut seeds to the poly bags. Watering was done immediately and

continued every morning and evening until the rain was steady.

The seeds African cherry nut germinated 28 days after planting. Manual weeding started two weeks after planting and continued at every two weeks interval until 22 weeks after planting. Spraying of karate (Lamba cyhalotrin) at 2ml a.i per 6 litres of water against grasshoppers and army worms was done at 3 weeks interval.

The measurement of growth parameters such as plant height, leaf area, number of leaves and stem, girth started at two weeks after planting and continued weekly until 22 weeks after planting.

Representative leaf samples from the top, middle and lower parts of the germinated seedlings were randomly taken at 23 weeks after planting per each treatment using a secateur, packed into labeled envelopes and oven dried for 24 hours at 70^{o}C.

The dried leaf samples were dry-ashed using a muffle furnace at 450^{o}C for 6 hours and the ash was made into solution, filtered and analyzed for N, P, K, Ca and Mg as described earlier.

At 26 weeks after planting in the nursery, the seedlings were ready for final transplanting in the field and at the end of the experiment, soil samples were also collected from each treatment, air-dried, sieved using 2mm sieve and analyzed for N, P, K, Ca, Mg, soil pH and organic matter.

Statistical Analysis

The average data obtained for the growth parameters such as plant height, leaf area, number of leaves and stem girth, soil chemical composition and leaf mineral composition of African cherry nut seedlings in 2008 and 2009 were analyzed using ANOVA F-test while the treatment means were separated using Duncan Multiple Range Test at 5% level. Gomez and Gomez (1984).

3. Results

Soil chemical composition before planting

Table 1: Soil fertility status before planting African Cherry nut

Soil parameters	Values	
Soil pH (H_2O)		5.45
Soil pH O.O1M $CaCl_2$	5.32	
Organic matter (%)		0.56
Nitrogen (%)		0.05
Available P (mg/kg)		6.10
Exchangeable bases		
K^+(mmol/kg)		0.10
Ca^{2+}(mmol/kg)	0.11	
Mg^{2+}(mmol/kg)	0.09	
Al^{3+}(mmol/kg)		1.48
Fe(mg/kg)		8.50
Zn (mg/kg)		3.75
Mn (mg/kg)		1.80
Cu (mg/kg)		2.0
Sand (%)		79.10
Silt (%)	15.20	
Clay (%)		5.70
Soil bulk density (mgm^{-3})	1.60	
% Porosity		41.81

The soil chemical properties before planting are presented in Table1. Based on the established critical levels for soils in South West Nigeria, the soil was acidic with pH 5.45 and low in organic matter compared to the critical level of 3% organic matter (Agboola and Corey 1973).

The total nitrogen (0.05%) is far less than 0.15% which is considered optimal for most crops (Sobulo and Osiname, 1981) while the available P is less than 10mg/kg P critical level (Agboola and Corey 1973).

The exchangeable K (0.10), Ca (0.11) and Mg (0.09mmol/kg) were lower than 0.20cmol/kg critical levels considered as adequate for crops (Folorunso *et al*, 2000). The soil textural class is sandy loam and it is classified as Akure soil series which is equivalent to Alfisol (*Isohyperthermic oxic paleustalf*). Soil Survey Staff (1999).

Chemical composition of the organic fertilizers used

Table 2: Chemical analysis of the organic fertilizers used for the experiment.

Treatments (organic fertilizers)	C	% N	% ratio	C/N mg/kg	P	K %	Ca	Mg	Fe mg/kg	Cu	Zn
Poultry manure		32.10	4.53	7.08	385	0.97	0.32	0.41	37.85	0.15	1.26
Pig manure	30.00	3.72	8.06	312	1.44	0.31	0.40	34.0	0.17	1.3	
Wood ash		18.00	1.53	11.76	86	2.30	0.94	0.85	65.55	0.66	1.83

The chemical analysis of the organic fertilizers materials used for growing African cherry nut seedlings is presented in Table 2. Poultry manure had the highest values of N, P and lower C/N ratio compared to pig manure and wood ash respectively. Wood ash had the highest values of K, Ca and Mg contents compared to the poultry and pit manures.

Effect of organic fertilizers on the growth parameters of African cherry nut seedlings

Table 3: Effect of different organic fertilizers on the growth parameters of African Cherry nut seedlings.

Treatment	Plant height (cm)	Leaf area (cm^2)	Stem girth (cm)	Leaf number	Root length (cm)	Fresh weight (kg)
Poultry manure	35.24e	87.62d	3.69e	14.1d	8.3d	3.0e
Wood ash	30.75b	80.25c	3.53cd	13.0c	7.0b	2.1bc
Pig manure	32.55d	80.65c	3.50c	13.2c	7.6c	2.4d
NPK 15-15-15	32.02c	76.29b	3.42b	11.6b	6.9b	1.9b
Control	27.19a	61.68a	1.82a	7.5a	5.7a	0.9a

Treatment means within each column followed by the same letters are not significantly different from each other using Duncan Multiple Range Test at 5% level.

There were significant increases ($P<0.05$) in the plant height, stem girth, leaf area, leaf number, root length and fresh weight (kg) under different organic fertilizers compared to the control treatment. (Table 3).

Poultry manure treatment had the highest values of plant height, leaf area, stem girth, leaf number, root length and fresh shoot weight of African cherry nut seedlings compared to wood ash, pig manure, NPK 15-15-15 and control treatments respectively.

For instance, poultry manure treatment increased the plant height, leaf area, stem girth, leaf number, length and fresh shoot weight of African cherry nut seedlings by 13%, 8.4%, 4.3%, 7.8%, 15.7% and 30% respectively compared to wood ash.

When compared with NPK 15-15-15 fertilizer, poultry manure treatment increased the plant height, leaf area, stem girth, leaf number, root length and fresh shoot weight of African cherry nut seedlings by 9%, 13%, 7%, 18%, 16.9% and 37% respectively.

All the treatments had better values of growth parameters of African cherry nut seedlings than that of control treatment.

Effect of organic fertilizers on the leaf chemical composition (%) of African cherry nut seedlings

Table 4: Leaf analysis of African Cherry nut seedlings under different organic fertilizers treatments.

Treatment	N %	P %	K %	Ca (%)	Mg (%)
Poultry manure	1.27d	0.99d	0.65c	0.53d	0.32d
Wood ash	0.89b	0.67c	1.45e	0.73e	0.66e
Pig manure	0.92bc	0.56b	0.59b	0.45c	0.24c
NPK 15-15-15	1.80e	0.91d	0.70d	0.005a	0.003a
Control	0.04a	0.024a	0.03a	0.04b	0.05b

Treatment means within each column followed by the same letters are not significant different from each other using Duncan Multiple Range Test at 5% level.

There were significant increases ($P<0.05$) in leaf N, P, K, Ca and Mg of African cherry nut seedlings compared to the control treatment. (Table 4).

Among the organic fertilizer treatments, poultry manure had the highest values of leaf N and P while wood ash also had the highest values of leaf K, Ca and Mg contents compared to others.

For example, poultry manure treatment increased leaf N and P of African cherry nut seedlings by 27.5% and 43% compared to pig manure while wood ash treatment increased the leaf K, Ca and Mg by 55%, 27% and 52% respectively compared to the poultry manure treatment.

When compared to NPK 15-15-15 treatment, wood ash treatment increased leaf K, Ca and Mg of African cherry nut seedlings by 51.7%, 99.3% and 99.5% respectively. However, NPK 15-15-15 fertilizer increased leaf N and P by 50.5% and 26.3% compared to wood ash.

Generally, NPK 15-15-15 fertilizer had the highest values of leaf N content compared to poultry manure, wood ash, pig manure and control treatments.

The leaf K/Ca and K/Mg ratios were 140:1 and 223:1 under NPK 15-15-15 fertilizer treatments compared to K/Ca (1:1) and K/Mg (2:1) under the poultry manure treatment.

Effect of organic fertilizers on soil chemical properties after removing African cherry nut seedlings

Table 5: Soil Chemical Analysis after Experiment under different organic fertilizers.

Treatment	Soil pH (H_2O)	O.M %	N %	Available P(mg/kg)	K mmol/kg	Ca mmol/kg	Mg mmol/kg
Poultry manure	6.13c	1.97c	0.32d	24.0d	2.6c	2.0d	1.23c
Wood ash	6.80d	1.84b	0.18b	17.0b	2.72cd	1.97c	1.32d
Pig manure	6.50c	1.90b	0.20c	21.2c	2.38b	1.48b	1.08b
NPK 15-15-15	4.36a	0.30a	0.21c	25.9e	3.55e	0.03a	0.04a
Control	5.35b	0.35a	0.03a	3.66a	0.04a	0.06a	0.07a

Treatment means within each column followed by the same letters are not significant different from each other using Duncan Multiple Range Test at 5% level.

There were significant increases ($P<0.05$) in soil pH N, P, K, Ca, Mg and O.M compared to the control treatment under different organic fertilizer treatments (Table 5).

Poultry manure treatment had the highest values of soil O.M, %N and P while wood ash also had the highest values of soil pH, K and Mg. For instance, poultry manure treatment increased the soil O.M, N and P by 6.6%, 44% and 29% compared to wood ash.

In-addition, wood ash increased the soil pH, K, Ca and Mg contents by 4.5%, 12.5%, 25% and 18% respectively compared to pig manure treatment.

When compared to NPK 15-15-15 fertilizer treatment, poultry manure increased soil pH, O.M, N, Ca and Mg by 29%, 85%, 34%, 98.5% and 97%. However, NPK 15-15-15 fertilizer increased soil P and K by 7.3% and 26.7% compared to poultry manure.

NPK fertilizer treatment decreased significantly ($P<0.05$) soil pH, O.M, Ca and Mg contents after removing the African cherry nut seedlings compared to the initial soil fertility status.

NPK fertilizer treatment had higher ratios of K/Ca, K/Mg, P/Mg and P/Ca interactions. For-instance, the soil K/Ca, K/Mg, P/Mg and P/Ca interactions were 118:1, 89:1, 647:1 and 863:1 respectively in NPK 15-15-15 fertilizer treatment compared to K/Ca (1:1), K/Mg (2:1), P/Mg (20:1) and P/Ca (12:1) under poultry manure treatment.

4. Discussion

The increase in growth parameters such as plant height, leaf area, stem girth, leaf number, root length and fresh shoot weight of African cherry nut seedlings could be attributed to the nutrient contents of the organic fertilizers used which encouraged better seedlings growth. This observation agreed with Adebayo and Akoun (2000) and Moyin-Jesu (2007) who reported that organic manures supported crop growth performance and increased crop yield.

Poultry manure gave the best performances on the growth, leaf and moderate soil fertility and this could be as a result of its high values of N, P, K and moderate Ca and Mg. This finding also agreed with Babalola *et al* (2000) who reported that poultry manure when used as fertilizer usually stimulated microbial activities and thereby enhanced the release of organic nitrogen and phosphorus in the soil. Soil nitrogen had been reported by Ojeniyi (1984) to aid vegetable growth and yields of crops.

The values reported for soil pH before the experiment was acidic but the application of wood ash increased the soil pH to a near neutral level. This could be due to the fact that wood ash was rich in K, Ca and Mg which created a liming effect in soil and stability of the soil buffering capacity. The observation agreed with the work of Gordon, 1998 and Moyin-Jesu 2009. Soil pH had been reported to influence nutrient availability and uptake by crops (Aduayi, 1980).

The application of NPK 15-15-15 fertilizer at 400kg/ha has led to high soil K/Ca, K/Mg, and P/Ca ratios which made difficult the availability of K, Ca and Mg nutrients to crops. This could be responsible for the lower values of soil Ca, Mg, O.M and pH compared to poultry manure, wood ash and pig manure respectively. Hence, the above observation was further supported by the fact that NPK 15-15-15 fertilizer decreased soil pH from 5.45 to 4.36.

Therefore, Obi and Ofonduru (1997) reported that the continuous use of mineral fertilizers such as NPK, Urea and Ammonium sulphate had led to degradation of soil physical qualities and low soil organic matter level.

The least values recorded for the soil, growth and leaf parameters of African cherry nut seedlings under the control treatment could be attributed to the initial poor soil fertility status and continuous cultivation of the land without replenishing with appropriate fertilizer. This observation agreed with Woomer and Muchena (1993) who reported that continuous productivity of tropical soils is associated with maintenance and improvement of soil physical characteristics which can be further improved by applying organic fertilizers.

5. Conclusion and Recommendation

The application of organic fertilizers such as poultry manure, wood ash and pig manure at 8 t/ha (40g/10kg soil) increased significantly the soil, leaf N,P, K, Ca, Mg, Soil pH and O.M, plant height, stem girth, leaf number, leaf area, root length and fresh shoot weight of African cherry nut seedlings. It is recommended that poultry manure applied at 8t/ha (40g/10kg soil) was the most effective fertilizer material for improving the nutrient availability and ensuring sustainable cultivation of African cherry nut seedlings on a commercial basis.

This recommendation corroborates with the fact that inorganic fertilizers are becoming too expensive to purchase by small scale farmers of African cherry nut. Besides, these organic fertilizers appear to have a strong beneficial secondary effects on the soil properties and could be environmental friendly.

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Biochemical And Molecular Profiles Of Gibberellic Acid Exposed Albino Rats

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ABSTRACT: The present study casts the light on the influence of the plant growth regulator ,Gibberellic acid (GA3), on antioxidant defense systems [glutathione peroxidase, superoxide dismutase (SOD), and catalase (CAT)] , lipid peroxidation level (malondialdehyde = MDA), AST, ALT , alkaline phosphatase , creatinine, total protein , albumin globulin , total lipids , total cholesterol, calcium and glucose . Moreover, histopathological examination of kidney and liver was done . On the molecular level the DNA damage was determined . The rats were received 75 ppm of GA3 in drinking water ad libitum for 50 days. Gibberellic acid (GA3) treatments caused different effects on the estimated parameters compared to control . Gibberellic acid exposure induced significant elevations of plasma AST, ALT , alkaline phosphatase , creatinine and malondialdehyde . However , Gibberellic acid produced non significant alterations in plasma total protein , albumin globulin , total lipids , total cholesterol, calcium and glucose . On the other hand, exposure elucidate significant reductions of catalase , superoxide dismutase and glutathione peroxidase in comparison to control group. The histopathological findings revealed that Kidney sections of Gibberellic acid treated rats suffered from areas of interstitial fibrosis which appear as segmental and global glomerular sclerosis tubulointerstitial injury . On the similar ground, liver section of Gibberellic acid treated rats , revealed that Gibberellic acid induced liver fibrosis; fatty metamorphosis and necrosis. The total genomic DNA electrophoretic pattern of lymphocytes deprived from Gibberellic acid treated rats revealed strong and obvious DNA damage as represented by a lot of fragments migrated from the wells. As a conclusion, Gibberellic acid(75 ppm) produce hepatonephrotoxicity , subsequently has oxidative stress role and DNA damage in albino rats 50 days post treatment. [Journal of American Science 2010;6(8):224_229]. (ISSN: 1545-1003).

Keywords: Biochemical; Molecular Profile; Gibberellic Acid; Rats

INTRODUCTION

Gibberellic acids are a group of plant growth regulators that have been identified in
different plants(MacMillian et al. ,1961)and they are used in agriculture as plant regulators
to stimulate both cell division and cell elongation that affect leaves as well as stems(Taiz and Zeige, 1991). Gibberellic acid (actually a group of related substances called Gibberellic acids) was discovered as a metabolic by product of the fungus *Gibberella fujikuroi* (Riley, 1987). If gibberellic acid or one of its metabolites is applied to dwarf varieties of peas, broad beans or maize, growth is greatly accelerated(Jones , 1973).

In *Alstroemeria hybrida*, leaf senescence is retarded effectively by application of Gibberellic acids(Kappers et al., 1997).Feeding toads, *Bufo regularis*, with Gibberellic acid A3 induced hepatocellular carcinomas in 16% of the animals.Moreover, it was showed that Gibberellic acid A3 induced breast and lung adenocarcinomas in mice(El-Mofty and Sakr ,1988). Gibberellic acid was found to induce chromosomal aberrations in human lymphocytes(Zalinian et al., 1990)and mice(Bakr et al., 1999). The World Health Organization(1990) classified Gibberellic acid-A3 as a plant growth regulators related to pesticides.

Gibberellic acid (Gibberellic acid A3) is used extensively in Egypt to increase the growth of some fruits (such as strawberries and grapes) and some vegetables (such as tomatoes, cabbages and cauliflower)(Weaver et al., 1961). Recently ,Kamel et al.(2009) recorded that Male rabbits treated with Gibberellic acid at all studied doses caused a significant increase in semen ejaculate volume, sperm concentration, total sperm out-put and sperm motility (%) and has direct androgenic-like action on testes compared to the control group.

The present investigation aimed to cast the light on the possible effects of Gibberellic acid on biochemical profile, histopathological image and DNA integrity of exposed rats.

MATERIALS AND METHODS

Twenty sexually mature male albino rats weighing 170±10 g were used. Animals were kept in the laboratory under constant temperature (24±2 ^{o}C) for at least one week before and throughout the experimental work. They were maintained on a standard diet and water were available *ad libitum.*

Animals were divided into two groups. Ten rats in the first group were orally given Gibberellic acid-A3 (Berelex®, BDH chemical, Pool, UK) at 75 ppm in drinking water were continuously administered orally to rats ad libitum for 50 days (Celik et al.,2007). Animals in the second group (10 rats) were served as controls. The treated animals and their controls were sacrificed by decapitation after the end of treatment.

For enzyme determination, Plasma were obtained by centrifugation of the blood samples and stored at –20^{o}C until assayed for the biochemical parameters. Transaminases (ALT, AST) and alkaline phosphatase activities were determined on the basis of King(1965).

Plasma creatinine levels were measured using the photometric determination according to the Jaffe method (Ecoline Mega, DiaSys Diagnostic Systems GmbH, Holzheim, Germany) described earlier(van Dokkum et al ., 2004) .

Plasma total protein concentration as (g/dl) was measured by the Biuret method as described by Armstrong and Carr (1964). Albumin (A) concentration as (g/dl) was determined by the method of Doumas *et.al*. (1971). Globulin (G) concentration as (g/dl) was calculated as the difference between total protein and albumin. Plasma total lipids (PTL) concentration as (g/dl) were estimated according to Frings *et.al*. (1972). Total cholesterol (TCh) concentration as (mg/dl) was determined according to Richmond (1973). Plasma glucose (PG) concentration as (mg/dl) was estimated according to the method of Trinder (1969). Serum calcium (SCa) concentration as (mg/dl) was measured according to the method of Sarkar and Chauhan (1967) using commercial kits (Stanbio kits).

Catalase activity was estimated by the method of Cakmak and Horst (1991). The reaction mixture contained 100 crude enzyme extract, 500 µL 10 mM H_2O_2 and 1400 µL 25 mM sodium phosphate buffer. CAT activity of the extract was expressed as CAT units. Superoxide dismutase activity was determined with the reaction mixture contained 100 µL 1 µM riboflavin, 100 µL 12 mM L-methionine, 100 µL 0.1 mM EDTA (pH 7.8), 100 µL 50 mM Na_2CO_3 (pH 10.2) and 100 µL 75 µM Nitroblue Tetrazolium (NBT) in 2300 µL 25 mM sodium phosphate buffer (pH 6.8), 200 µL crude enzyme extract in a final volume of 3 mL. SOD activity was assayed by measuring the ability of the enzyme extract to inhibit the photochemical reduction of NBT glass test tubes containing the mixture were illuminated with a fluorescent lamp (120 W); identical tubes that were not illuminated served as blanks. After illumination for 15 min, the absorbance was measured at 560 nm(Hernandez *et al*., 2000). Glutathione peroxidase (GSH-Px) was measured by spectrophotometric method developed by Paglia and Valentine (1967). One unit of GSH-Px activity was defined as the amount of the enzyme that converted 1 µM NADPH, substrate to NADP+ per minute. All the assays were carried out in triplicate using a spectrophotometer (Hitachi U-2000, Hitachi Ltd., Tokyo, Japan).

Malondialdehyde (MDA) was measured by colorimetric method (Stewart and Bewley, 1980).

Histological Examination

Rat kidneys were removed and portions fixed in 4% paraformaldehyde (PFA). Other kidney portions were frozen in optimum cutting temperature (OCT) compound (Miles, Elkhart, IN, U.S.A.) and stored at -80 °C. Fixed renal tissues were embedded in paraffin and cut into 4-µm-thick sections. The sections were stained with periodic acid-Schiff (PAS) stain and Masson trichrome stain to reveal histological changes and areas of interstitial fibrosis.

Liver tissue sections were fixed in 100 ml/L formalin saline in phosphate buffer and processed in paraffin wax. Sections from blocks were stained with hematoxylin-eosin , histological analyses were performed blindly using light microscope **.**

Total genomic damage of DNA analysis

DNA was extracted from lymphocytes of blood samples using DNA extraction Kit (Fermentas life Siences, Lithuania -Cat. #K0513).

Gel was prepared with 1.5% electrophoretic grade agarose (BRL) and 0.2% polyvinyl pyrolidine (PVP, Sigma). The agarose and PVP were boiled with Tris Borate EDTA buffer (TBE buffer; 89 mM Tris, 89 mM boric acid, 2mM EDTA, pH 8.3). 0.5 mg ethidium bromide /ml distilled water (Sigma) was added to the gel at 40^{o}C. Gel was poured and allowed to solidify at room temperature for 1h before samples were loaded (15 µl of extracted DNA/well). Electrophoresis was performed for 2 hrs at 50 volt using lX TBE buffer as a running buffer. Gel was photographed using a polaroid camera while DNA was visualized using a 312 nm UV light under a transilluminator (Herolab, Germany) . The photographes were analyzed using Phortex software version 3.0, UK to determine the degree of DNA damage (Cressman et al. ,1999) .

Statistical analysis

All values were expressed as mean ± standard error (SE). All statistical analyses were performed using SAS (version 8.02). Statistical differences among the experimental groups were assessed by one-way ANOVA. Tukey's test was used as a follow-up test and significance was defined at $p<0.05$.

RESULTS

Table (1) revealed that Gibberellic acid-A3 exposure induced significant elevations of plasma AST, ALT , alkaline phosphatase , creatinine and malondialdehyde . However , Gibberellic acid produced non significant alterations in plasma total protein , albumin globulin , total lipids , total cholesterol, calcium and glucose . On the other hand, Gibberellic acid exposure elucidate significant reductions of catalase , superoxide dismutase and glutathione peroxidase in comparison with control group.

Table(1): Effect of Gibberellic acid-A3 exposure on some biochemical parameters of rats.

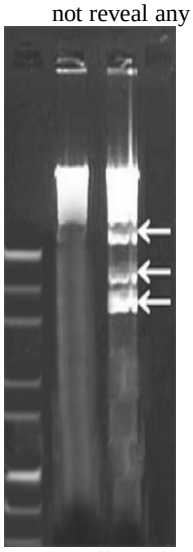
Items	**Control group**	**Exposed group**
AST (iu/l)	52.28 ± 4.28	66.85 ±4.51*
ALT(iu/l)	33.27 ± 4.33	44.31±3.07*
Alkaline phosphatase (iu/l)	103.85 ± 12.36	137.08±5.88*
Creatinine (mg/dl)	0.346 ± 0.16	0.636±0.09*

Total protein (gm/dl)	8.82 ± 1.61	8.91±1.55
Albumin (gm/dl)	4.42 ± 0.54	4.35±0.52
Globulin (gm/dl)	4.37 ± 0.33	3.61±0.21
Total lipids (gm/l)	4.208 ± 0.59	4.34± 0.18
Total cholesterol (mg/dl)	72.58 ± 5.78	75.89±5.48
Glucose (mg/dl)	138.24 ± 10.95	145.35±7.51
calcium (mg/dl)	1.66 ± 0.33	2.15±0.05
Catalase (nmol/min/ml)	18.77 ± 1.91	15.04±0.33*
Superoxide dismutase (u/ml)	105.28 ± 10.58	85.28±5.71*
Glutathione peroxidase (nmol/min/ml)	90.48 ± 10.84	74.33±5.49*
Malondialdehyde (nmol/dl)	4.33 ± 1.63	6.85±0.35*

*significant at $p < 0.05$

Total genomic damage of DNA :

Figure (1) shows the total genomic DNA electrophoretic pattern of lymphocytes deprived from control and Gibberellic acid treated groups. Gibberellic acid revealed strong and obvious DNA damage at the tested concentrations as represented by a lot of fragments migrated from the wells. On the other hand, control group did not reveal any damage of DNA .



Fig(1): Effect of Gibberellic acid-A3 treatment on DNA damage of DNA of rats, **arrows** indicate that fragmented DNA bands in Gibberellic acid treated group.

Histological Examination

Kidney sections of Gibberellic acid treated rats which stained with periodic acid-Schiff (PAS) stain and Masson trichrome stain to reveal histological changes and areas of interstitial fibrosis appear as segmental and global glomerular sclerosis tubulointerstitial injury (tubular dilatation, atrophy of tubular epithelial cells) (Photo , 1) .

Histopathological examination of Hematoxylin and eosin-stained liver section of normal and Gibberellic acid treated rats with magnification -400 X , revealed that Gibberellic acid induced liver fibrosis; fatty metamorphosis and necrosis (Photo, 2).

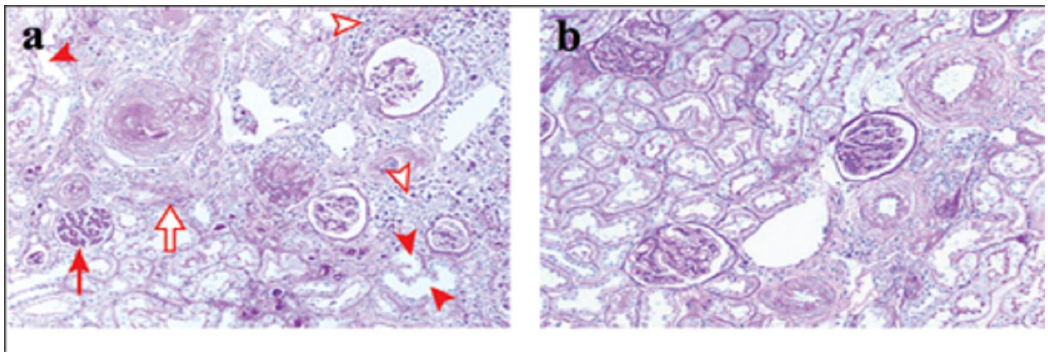


Photo (1): Rat renal tissue sections with PAS staining arrows indicating global glomerular sclerosis and tubulointerstitial injury: arrow heads; fibrosis: open arrow; and infiltration of inflammatory cells: open arrow heads) in Gibberellic acid treated (a) and control (b).

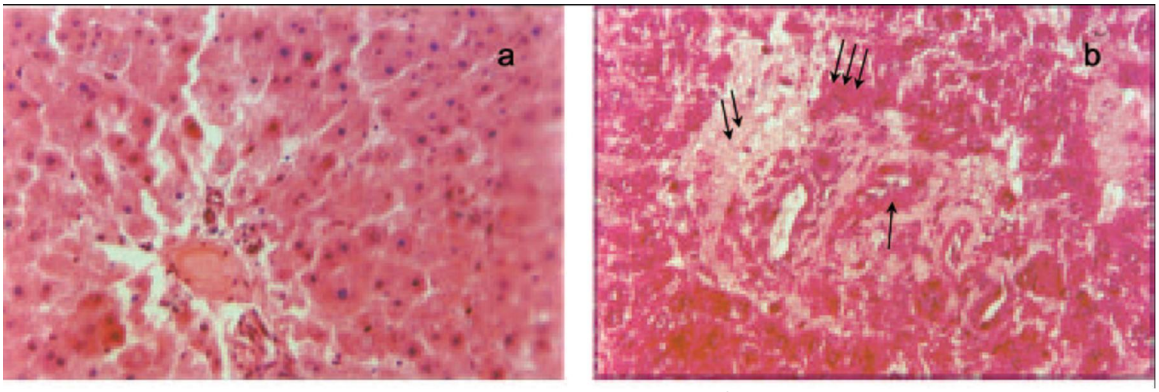


Photo (2):Liver tissue sections were stained with haematoxylin and eosine for light microscope observation . **(a**) control . (**b**) Gibberellic acid treated. ↓↓, fibrosis; ↓↓↓, fatty metamorphosis; ↑, necrosis

DISCUSSION

Gibberellic acid is a type of plant hormone which regulates growth. There are 126 known Gibberellic acids, divided into two classes, and many more may be discovered in the future. Plants produce these hormones naturally through biosynthesis as they grow, ensuring that they have the hormones they need to develop normally, and these hormones can also be applied to plants by gardeners and farmers to achieve specific desired outcomes (**Fernandez** and **Rodriguez**,1979).

In the present study, Gibberellic acid induced significant elevations of plasma AST, ALT , alkaline phosphatase, creatinine and malondialdehyde. However, Gibberellic acid produced non significant alterations in plasma total protein, albumin globulin, total lipids, total cholesterol, calcium and glucose .on the other hand, Gibberellic acid exposure elucidate significant reductions of catalase, superoxide dismutase and glutathione peroxidase in comparison with control group.

The histopathological findings revealed that Kidney sections of Gibberellic acid treated rats suffered from areas of interstitial fibrosis appear as segmental and global glomerular sclerosis tubulointerstitial injury .

On the similar ground , liver section of Gibberellic acid treated rats with magnification -400 X , revealed that Gibberellic acid induced liver fibrosis; fatty metamorphosis and necrosis.

The previous findings were in accordance with the recorded findings of Sakr et al.(2003), who recorded that Gibberellic acid induced histopathological changes in the liver such as cytoplasmic vacuolization of the hepatocytes with pyknotic nuclei, blood vessel congestion and inflammatory leucocytic infiltrations. Histochemical observations revealed marked reduction in total carbohydrates and total protein contents in the hepatocytes. These changes proved to be time dependent.

On the same hand, Celik et al. (2007) recorded that gibberellic acid (GA3) has deleterious effect on the antioxidant defense systems [reduced glutathione (GSH), glutathione reductase (GR), superoxide dismutase (SOD), glutathione-S-transferase (GST) and catalase (CAT)] and lipid peroxidation level (malondialdehyde = MDA) in various tissues of the rat were investigated during treatment as a drinking water 75 ppm of ABA and GA3 in drinking water were continuously administered orally to rats for 50 days. The

lipid peroxidation end product MDA significantly increased in the lungs, heart and kidney of rats treated with GA3. The GSH levels were significantly depleted in the spleen, lungs and stomach of rats treated with GA3. SOD significantly decreased in the spleen, heart and kidney. While CAT activity significantly decreased in the lungs of rats treated with GA3. The drug metabolizing enzyme GST activity significantly decreased in the lungs of rats treated with ABA but increased in the stomach of rats treated with GA3. the authors concluded that GA3 produced substantial systemic organ toxicity in the spleen, lungs, stomach, heart and kidney during a 50-day period of subchronic exposure.

The present study disclose that the total genomic DNA electrophoretic pattern of lymphocytes deprived from control and Gibberellic acid treated groups. Gibberellic acid revealed strong and obvious DNA damage at the tested concentrations as represented by a lot of fragments migrated from the wells. On the other hand, control group did not reveal any damage of DNA .

These data on the same way of the findings of Hassab-Elnabi and Sallam (2002).
They recorded that higher concentration of Gibberellic acid induced total genomic damage of DNA. By the application of modified comet assay (single cell gel electrophoresis) technique, DNA damage was found at all applied doses. Also, Bakr et al. (1999) reported a significant increase in the incidence of total chromosomal aberrations induced by gibberellic acid in bone marrow cells of albino mice.

The illustrated results is fully agreed with that recorded by Zalinian *et al.* (1990). They reported that gibberellic acid induced chromosomal aberrations in human lymphocyte cultures. Gibberellic acid in this demonstration induced a significant damage of DNA. This result agreed with the data that declared by Abou-Eisha (2001). He showed that Gibberlic acid induces a dose-dependent increase in the level of DNA breakage in human blood cells , this increase attaining statistical significance at the highest concentrations tested (25, 100, 150µg/ml), which would confirm its genotoxicity.

So, the DNA damage may attributed to the direct attack of DNA by gibberellic acid causing alkali labile and single strand breaks and total genomic damage as revealed by our demonstration, or may be due to accumulation of nucleases as reported by Fath *et al.* (1999). The mechanism of gebrilic acid to induce DNA damage may be attribute to elevation of oxidative stress markers such as (ROS and GSH), Bcl-2 protein expression, mitochondrial membrane potential and caspase-3 activity. The sequel of these events lead to mitochondrial membrane depolarization and caspase-3 activation followed by apoptosis(Abou-Eisha , 2001).

From the recoded data , it could be concluded that Gibberellic acid(75 ppm) produce hepatonephrotoxicity , subsequently has oxidative stress role and DNA damage in albino rats 50 days post treatment.

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Study on Models of Commuter Mode Choice beyond Fuel Prices Based on Ordered Logit Models

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Abstract: To ease the traffic congestion, domestic and foreign scholars have paid more attention to the measures of an increased ridership of public traffic and some traffic management measures to reduce vehicle usage. The travel cost and availability of parking are important determinants of private automobile use and of the balance between automobile and transit use. The paper deals with the question of how travelers with private cars would react to fuel prices rising above the high fuel levels that were attained in mid-2008 for commuting purpose. Revealed and Stated Preference surveys were constructed and carried out including vehicle travelers' socio-demographic characteristics, commuter trip characteristics and potential trip mode choices in different hypothetical choice situations which includes travelers' choice under different fuel price rising. The influence factors of commuter mode choice behavior are analyzed and commuter mode choice model corresponding to fuel price was established based on ordered logit model. And the result show that Annual income, work organizations parking condition, household kids' number, and monthly fuel expenditure have marked influences on the results of commuter mode choice. Finally the margin effects of the variables are given. The forecast veracity of the model is satisfied and the precision of this model is high which can provide the datum sustain for the traffic management measures to reduce vehicle usage. [Journal of American Science 2010; 6(8):230-235]. (ISSN: 1545-1003).

Key words: Fuel prices; ordered logit model; Stated Preference survey; commuter characteristics

1 Introduction

With the development of the economy and the rapid increase of vehicle in China, more and more commuters' arrivals are made by private cars, hence creating traffic congestion. To solve this problem, domestic and foreign scholars have paid more attention to the measures which can establish an effective accessibility public traffic system, and especially an encouraging trend is an increased ridership in rail transit and BRT in recent time (Han, 2009). On the other hand, some traffic management measures to reduce vehicle usage such as license plate rule restriction measure, cost increase, parking policy (Qing, 2009) and etc. have been taken to encourage the travelers to transfer from car to public transport. Fuel prices rising and fax is one of the methods to reduce vehicle usage which is applied in China recently. The motivation for the present study were the rising fuel prices in recent time and an interest in how private car travelers would react to further fuel cost increases during the commuter course. The price for a liter of regular non-leaded fuel was 5.6RMB at July 2009, and now is about around 6.92 RMB in April 2010 which gets to the highest in Beijing. This circumstance provided the opportunity to conduct Stated Preference (SP) experiments implementing a much greater bandwidth in pricing schemes than was the case in the previous studies during the world economic recovery period.

Foreign scholars paid attention to those researches on the fuel elasticities and the relationship between fuel and households' vehicle utilization and usage choices. Acutt and Dodgson (1996) derives that the car cross-elasticities of demand with respect to the public transport fares range from 0.0005 for London Buses to 0.0118 for intercity rail. The public transport cross-elasticities with respect to the price of petrol range from 0.013 for local buses to 0.094 for intercity rail, within the range reported for studies from the 1970s and 1980s. Golob and Brownstone (2005) reveal that annual fuel consumption per vehicle declines a bit more sharply with increasing housing density than does annual miles driven, generating a positive relationship between fuel efficiency and density. Vrtic et al. (2007) investigate the effects that potential mobility pricing schemes would have on travelers' tactical (mode choice) and strategic (long-term) decisions. Hao Audrey Fang（2008）develops a Bayesian Multivariate Ordered Probit & Tobit (BMOPT) model to estimate a joint system of vehicle fuel efficiency choice and vehicle utilization in response to varying residential density. Claude WEIS（2009）estimated multinomial logit models for mode and fleet choice based on the SP data While demand elasticity are expected to increase non-linearly with rising prices. From the introduction above, it can be seen foreign scholars have done some studies on fuel prices which are in low fuel price level and do not focus on the price elasticity of high fuel price level. And whether the results are suitable for

China has not been confirmed yet. Domestic scholars pay more attention to the parking policy and fuel fax policy. Whether or not fuel fee rising will induce the reduction of vehicle usage and a compositional shift from car to public transport has not been widely studied in China. And also at what fuel price will the commuters reduce vehicle usage for one day in one week or even give up driving has not been studied. This paper tries to solve this question and emphasizes the impact of both individual and household attributes and relevant variables such as parking and public transport accessibility on the vehicle commuter behavior.

The research comprises of a number of sections. Section 2 presents the vehicle commuter behavior survey and analysis consisting of the structure and design of the stated preference experiments. Section 3 provides explorative statistics of the sample. Section 4 covers the mode choice model of vehicle commuters based on Ordered Logit model. Section 5 presents the results of the model, followed by summary and conclusions.

2 Mode choice behavior survey and analysis

2.1 Recruitment of Respondents

The customers' reactions to further fuel cost increases can be divided into short- and long-term (C.Weis, 2009). In the short term, the interest focuses on modeling individuals' mode choice under the modified pricing schemes. In long-term (strategic) choices, the interest focuses on customers' sets of mobility tools, such as new vehicle purchase and a re-distribution of yearly mileages. Also as we all know mode choice behaviors of the travelers are different according to the trip objects such as commuter, shopping and etc. The survey discussed in this paper only considered the traveler's short term reaction. And trips for commuter purpose over the past week are recorded along with their relevant characteristics such ad origin and destination, travel and waiting times, distances, and so on.

During the survey, respondents owning a car driving license were recruited for the SP survey. Limiting the recruitment process to driving license owners made sure that the car alternative presented in the mode choice experiments was realistic to the respondents.

2.2 Design of Stated Preference Survey

According to the above analysis, the subject of this survey was the stated preference of vehicle commuters in short-term under different hypothetical fuel fee prices assumed the customers' reactions vary with the costs. The survey was conducted in Beijing over the time period from September 8th through April 9th, 2010. The survey consisted of three parts, and the first part concerned respondents' socio-demographic characteristics such as age, gender, occupation, number of adults, number of family driving license adults, number of children, car possession, annual income, zone type of the residence area and work organization. The second part encompasses questions on respondents' commuter trip characteristics such as origin and destination, travel times, distances, weekly workday number, month mileages, parking characteristics of the residence and work organization, parking fee, pay condition of the trip cost, month fuel fee and etc. The third part yielded information about the respondents' potential trip mode choices in different hypothetical choice situations which includes travelers' choice under different fuel price increases by 10%, 20% , 50%, 70%, 100%, 150% and no thinking about the fuel price respectively, and their substitute mode when they do not go to work with private car. In the Stated Preference Survey(Guan,2004), the respondent was faced with 7 mode choice situations, and for each choice situation, explanations on how the car trip costs were computed were displayed in order to clarify the underlying assumptions to the respondents.

The survey takes the mode of quiz face to face mainly (Yao, 2007). And there are about 230 participants to which the questionnaires were sent by internet investigation. A total of 1200 respondents were selected.

3 Sample statistics

Descriptive statistics for the data used in the application are all analyzed.

3.1 Statistics of Revealed Preference survey

Monthly fuel expenditures of respondents and the commuting distance distribution are demonstrated in figure 1 and figure 2.

As shown in Figure 1, the proportions of the respondents of whose monthly fuel expenditure are below 300 RMB and higher than 1500 RMB are 10% and 5% respectively. Most of monthly fuel expenditures of the respondents are between 500 and 1500 RMB. And 46% of monthly fuel expenditures of the respondents are between 500 and 1000 RMB for commuting purpose.

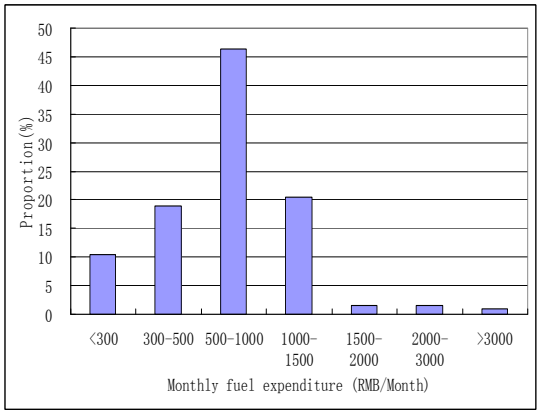

Figure 1. Proportion of the commuter monthly fuel expenditure

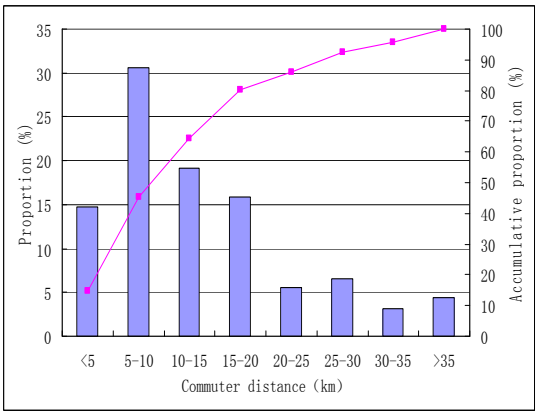

Figure 2. Accumulative proportion of the commuter distances

Figure 2 indicates that, the proportion of the respondents whose everyday commuting distance is below 25 km is about 85%. And the proportion of commuting distance about 5~10, 10~15, 15~20 km are 30.6%, 19.1% and 15.9% respectively.

The parking condition of the residence and work organization such as parking price, supply condition of the parking spaces and average parking time are all investigated to discuss how parking condition will affect commuters' choices. The survey result shows that 45% of commuters choose to drive because they are supplied with abundant employee parking and it will take about 25% of the commuters more than 5 minutes to park. And also 58% of the commuters choose to drive alone because employee parking is free which will stimulate and induce the usage of the vehicles for commuting purpose.

3.2 Statistics of Stated Preference survey

The fuel fee is one of the important factors which influence the trip mode choice of the commuters whose reactions are assumed to vary with the costs. In the survey the stated preference of the commuters by vehicle mode were asked that at what fuel price they will reduce car usage for one day every week considering the increase of fuel price. Using the investigate datum, the proportion of the commuters who will reduce car usage one day per week under different fuel prices were given in Figure 3 and Figure 4.

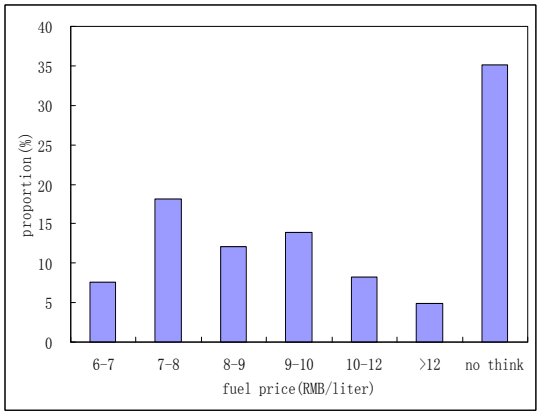

Figure 3. Proportion of the commuters who will reduce car usage for one day per week under different fuel prices

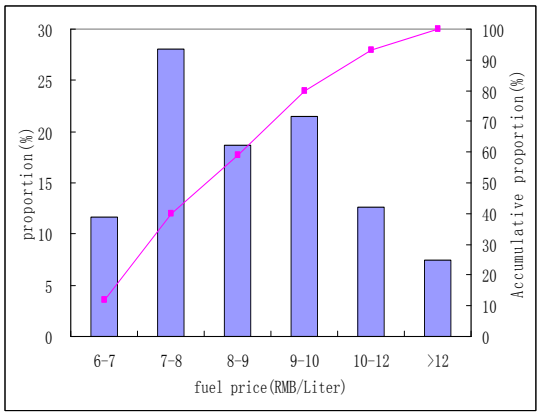

Figure 4. Accumulative proportion of the commuters who will reduce car usage for one day per week under different fuel prices

As shown in Figure 3, about 35% of the respondents will not reduce the car usage for one day every week no matter what the fuel price is. These travelers are defined as rigid travelers who do not have any elasticity with the fuel price and their commuter behaviors will not change with the fuel price. And 65% of the respondents have elasticity with the fuel price. And it can be seen from Figure 4 that when the fuel price gets to 9-10 RMB/liter, 80 % of them will reduce the car usage for one day every week.

4 Formulation and estimation of the discrete choice models

To illustrate the relationship between the travel behavior character of commuters to reduce car usage for one day every week and fuel prices, fuel price choice decisions are modeled as ordered and the ordered choice for fuel price would be zero, one, two, three, four, five and six, which represents fuel price increases by 10%, 20%, 50%, 70%, 100%, more than150% and no thinking about the fuel price respectively for each commuter in the sample.

4.1 Model Specifications

The Ordered model platform is an underlying random utility model or latent regression model. (William H.Greene, 2009)

$$y_i^* = V_i + \varepsilon_i = \beta' x_i + \varepsilon_i,\ i = 1,..., n, \quad (1)$$

Where: $\mathbf{x}_i$ is a set of K covariates that are assumed to be strictly independent of ε_i; β' is a vector of K parameters that is the object of estimation and inference. V_i is the deterministic component of utility for user i (fixed item).

In which the continuous latent utility or measure, y_i^* is observed in discrete form through a censoring mechanisms;

$$\begin{aligned} y_i &= 0 \text{ if } \mu_{-1} < y_i^* < \mu_0, \\ &= 1 \text{ if } \mu_0 < y_i^* < \mu_1, \\ &= 2 \text{ if } \mu_1 < y_i^* < \mu_2 \\ &= ... \\ &= 6 \text{ if } \mu_5 < y_i^* \end{aligned} \quad (2)$$

The thresholds, μ_j are specific to the person and number (J-1) where J is the number of possible ratings (here, six) – J-1 values are needed to divide the range of utility into J cells. The thresholds are an important element of the model; they divide the range of utility into cells that are then identified with the observed ratings.

With the full set of normalizations in place, the likelihood function for estimation of the model parameters is based on the implied probabilities,

$$\text{Prob}[y_i = j \mid \mathbf{x}_i] = [F(\mu_j - \beta'\mathbf{x}_i) - F(\mu_{j-1} - \beta'\mathbf{x}_i)] > 0, \quad j = 0, 1, \ldots, 6. \quad (3)$$

$$\text{Prob}([y_i = 0 \mid \mathbf{x}_i] = F(\mu_0 - \beta'\mathbf{x}_i)$$
$$\text{Prob}[y_i = 1 \mid \mathbf{x}_i] = [F(\mu_1 - \beta'\mathbf{x}_i) - F(\mu_0 - \beta'\mathbf{x}_i)]$$
$$\text{Prob}[y_i = 2 \mid \mathbf{x}_i] = [F(\mu_2 - \beta'\mathbf{x}_i) - F(\mu_1 - \beta'\mathbf{x}_i)]$$
$$\text{Prob}[y_i = 3 \mid \mathbf{x}_i] = [F(\mu_3 - \beta'\mathbf{x}_i) - F(\mu_2 - \beta'\mathbf{x}_i)]$$
$$\text{Prob}[y_i = 4 \mid \mathbf{x}_i] = [F(\mu_4 - \beta'\mathbf{x}_i) - F(\mu_3 - \beta'\mathbf{x}_i)]$$
$$\text{Prob}[y_i = 5 \mid \mathbf{x}_i] = [F(\mu_5 - \beta'\mathbf{x}_i) - F(\mu_4 - \beta'\mathbf{x}_i)]$$
$$\text{Prob}[yi = 6 \mid xi] = [1 - F(\mu 5 - \beta' xi)]$$

The standard treatment in the received literature completes the ordered choice model by assuming either a standard normal distribution for εi, producing the "ordered probit" model or a standardized logistic distribution (mean zero, variance π2/3), which produces the "ordered logit" model. The ordered logit model is given in this paper. For the determination of the attribute combinations to be used in the SP experiments, the software LIMDEP was used.

4.2 Variable Filtration and Grouping

Using the survey datum, the influence of the travelers' household characteristics and trip characteristics are analyzed through crosstables chi-square test. It can be concluded that discrete parameters such as age, occupation, income, parking characteristics of the residence and work organization, and continuous parameters such as number of household children, car possession number, distances between residence and work organization, weekly driving day number, monthly fuel expenditure have marked influences on the results of fuel price choice modes, and other parameters collected in the survey are not significant to choice modes.

Variables filtration and grouping were decided as following by correlation analysis: 1)Age is divided into three groups: Age1 is between 18 and 30 years old,Age2 is between 30 and 50 years old and age3 is others; 2)Occupation is divided into three groups: Occupation1 is private owner, senior manager; Occupation2 is engineering and technical personnel, office clerk and Occupation3 is others; 3)Income is divided into three groups:Income1 is below 60000 RMB/year, Income2 is between 60000 and 150000 RMB/year and Income3 is over 150000 RMB/year.

4.3 Calibration of Model

Using the Ordered logit model and survey datum, vehicle commuter behavior model based on different fuel prices is proposed as shown in Table 1.

According to the statistic analysis, annual income, parking condition of work organization, household kids' number, and distance between residence and work organization and monthly fuel expenditure are chosen as the independent variables of the model. The parameter estimates for the final model are displayed in Table 1. Parameter values are displayed along with the corresponding t-statistics (where absolute values above 1.96 indicate significance of the parameters at the 5% level), as well as general model fit information. The absolute values of t-statistics of HHKIDS, income1, income2, WRKPARK, CARDIST,MFUEL are all higher than 1.96 at a 5 percent confidence level, which indicates that these seven influential factors are significant for the choice probability. The adjusted ρ^2 value of 0.369 indicates a good model fit, and all the included variables are of the expected sign and statistically significant.

For example , let's forecast the probability of junior manager whose household kids number is two ,annual income is between 60000 and 150000 RMB, monthly fuel expenditure is about 1000 RMB ,parking spaces in his work organization is not abundant and distance between residence and work organization is about 25km. The result is listed in table 2.

Table 1. Estimated Ordered Choice Model

Variable		Coef.	t statistic	P
Discrete	Income1	-1.076	-2.886	0.010
	Income2	-1.098	-2.920	0.008

	WRKPARK	-0.778	2.51	0.012
	CARDIST	-0.028	8.278	0.004
Continuous	HHKIDS	0.686	3.164	0.001
	MFUEL	0.001	3.128	0.002
Thresholds		Coef.	Thresholds	Coef.
u_0		-3.315	u_3	-0.589
u_1		-1.805	u_4	-0.250
u_2		-1.225	u_5	-0.015
$L(0)$= -1931.325 ρ^2 = 0.371		$L(\hat{\theta})$ = -1214.344	$-2(L(0)-L(\hat{\theta}))$ =1433.963 *Adjusted* $\bar{\rho}^2$ = 0.369	

HHKIDS =household kids number; Income1 = annual income is below 60000 RMB. Income2= annual income is between 60000 and 150000 RMB; MFUEL = Monthly Fuel expenditure, WRKPARK= Parking condition of work organization; CARDIST=Distance between residence and work organization.

Table 2. Estimated probabilities

yi	0	1	2	3	4	5	6
Prob.	0.020	0.063	0.099	0.132	0.163	0.180	0.343

As shown in table 2, the probability of choose y_i =6 choice is equal to 0.343 and it is the biggest in all of the choices which represents the junior manager will not reduce the car usage for one day every week no matter what the fuel price is. The elasticity of fuel price is very small and his commuter behaviors will not change with the fuel price. Suppose If the probability of choose y_i =4 choice is the biggest of all the choices, it can be concluded that the junior manager will reduce the car usage for one day every week when the fuel price increases and is about 10~12RMB/liter.

4.4 Interpretation of the Model – Partial Effects

Interpretation of the coefficients in the ordered logit model is more complicated than in the ordinary regression setting. The implication of the preceding result is that the effect of a change in one of the variables in the model depends on all the model parameters, the data, and which probability (cell) is of interest. It can be negative or positive. The partial effects are shown in Table 3.

Table 3 Summary of Marginal Effects for Ordered Probability Model

Variable	y_i =0	y_i =1	y_i =2	y_i =3	y_i =4	y_i =5	y_i =6
CARDIST	0.0000	0.0001	0.0006	0.0015	-0.0015	-0.0001	.0000
WKPARK	-.0001	-.0002	-.0031	.0032	.0001	.0001	.0000
INCOME1	.0001	.0002	.0050	-.0050	-.0002	-.0001	.0000
INCOME2	.0000	.0001	.0021	-.0021	-.0001	.0000	.0000
MFUEL	-.0001	-.0006	-.0016	.0015	.0001	.0000	.0000
HHKIDS	.0000	-.0002	-.0020	-.0510	.0499	.0030	.0002

HHKIDS =household kids number; Income1 = annual income is below 60000 RMB. Income2= annual income is between 60000 and 150000 RMB; MFUEL = Monthly Fuel expenditure, WRKPARK= Parking condition of work organization; CARDIST=Distance between residence and work organization.

The partial effects give the impacts on the specific probabilities per unit change in the stimulus or repressors. For example, for continuous variable HHKIDS, we find partial effects for the ordered logit model for the seven cells of 0.000，-0.0002, -0.0020, -0.0510, 0.0499, 0.0030, 0.0002 respectively, which give the expected change on the probabilities per additional number of household kid. For the WKPARK variable, for the (y_i =5) cell, the estimated partial effect is 0.0001; however, some care is needed in interpreting this in terms of a unit change. The WKPARK variable has a mean of 0.8875 and a standard deviation of 0.2601. A full unit change in WKPARK would put the average individual nearly six standard deviations above the mean. Thus, for the marginal impact of WKPARK, one might want to measure a change in standard deviation units. Thus, an assessment of the impact of a change in WKPARK on the probability of the (y_i =5) cell probability might be 0.0001×0.2601 = 0.00026. Precisely how this computation should be done will vary from one application to another.

5 Conclusions

Based on survey data and ordered logit model, commuter mode choice model corresponding to fuel price was established. Conclusions following can be obtained by analyzing the mode. About 35% of the respondents who are defined as rigid travelers will not reduce the car usage for one day every week no matter what the fuel price is. 65% of the respondents have elasticity with the fuel price. And when the fuel price get to 9-10RMB/liter, 80 % of them will reduce the car usage for one day every week. Annual income, work organizations parking condition, household kids' number, and monthly fuel expenditure have marked influences on the results of commuter choice. The forecast veracity of the model is satisfied and the

precision of this model is high. For advanced research we could take advantage of SP (Stated Preference) survey data to establish choice behavior models which include more convenient factors.

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Zinc and Boron Fertilization on Concentration and Uptake of Iron and Manganese in the Corn Grain

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Abstract: A farm experiment with maize plant grown on Zn and B deficient soil was conducted to study the effect of Zn and B interaction on the concentration and total uptake of Fe and Mn in corn grain during 2009 at Fars Province, Iran. Treatments including five levels of Zn (0, 8, 16 and 24 kg ha^{-1} and Zn foliar spray) and four levels of B (0, 3, and 6 kg ha^{-1} and B foliar spray) in a completely randomized block design were set up. The findings showed that the effects of Zn and B and the interaction of Zn and B on the Fe concentration in the grain were insignificant. The effect of Zn on Fe uptake in the grain was insignificant and among different levels of B, only application of 3 kg/ha B increased Fe uptake in the grain. Boron use had no effect on Fe uptake in the grain in any level. Only at high level of B (6 kg/ha B), application of 16 kg/ha Zn increased Fe uptake in the grain. Boron use and Zn and B interaction was not significant on Mn concentration in the grain. Application of 24 kg/ha Zn increased Mn concentration in the grain from 3.67 to 4.75 mg/kg but other levels of Zn had no effect. Application of Zn to the soil and spraying it increased Mn uptake in the grain. Application of B to the soil increased Mn uptake in the grain but B spraying had no effect on it. Application of B at low levels and Zn spraying had no effect on Mn uptake in the grain but at higher Zn levels (16 and 24 kg/ha Zn) increased Mn uptake in the grain. Application of Zn at zero and 6 kg/ha B levels increased Mn uptake in the grain but at other B levels, it had no significant effect on Mn uptake. [Journal of American Science 2010;6(8):236-242]. (ISSN: 1545-1003).

Keywords: Interaction, zinc, boron, iron, manganese, concentration, corn grain

1. Introduction

Zinc (Zn) deficiency is a very important nutrient problem in the world's soils. Total Zn concentration is in sufficient level in many agricultural areas, but available Zn concentration is in deficient level because of different soil and climatic conditions. Soil pH, lime content, organic matter amount, clay type and amount and the amount of applied phosphorus fertilizer affect the available Zn concentration in soil (Adiloglu et al., 2006). Zinc deficiency rate was determined as a 30 % in the world (Sillanpaa, 1982). The interaction among nutrient elements is very important for plant nutrition. Boron x Zn interaction among these interactions has been curicial in the Zn deficient soils, in recent years (Alkan et al., 1998). Plant root cell membrane permeability increases in Zn deficient soils[5], which may lead to accumulate B and other nutrient elements in plant roots. Therefore, excess B uptakes by plants may cause B toxicity for the plants in this soil conditions (Singh et al., 1990).

Zinc and Fe are antagonistic; of course this is not always the case and even in some cases they are combined and used, they can play a useful and effective role on plant growth and increase in the harvest (Baybordi et al., 2000; Haghnia et al., 1989). when the corn plant is under Zn stress, it absorbs a large amount of Fe and Mn, worsening the adverse effects of Zn deficiency (Tisdale et al., 1993; Warnocke et al., 1973). Moreover, an interaction exists between B and Mn; a high B content leads into appearance of signs of Mn deficiency in the plant. For B uptake, N and K must be present in desired amounts. An Fe-B antagonism as well has been reported. Of course, in B deficiency conditions, B uptake increased by adding Fe. Also, an antagonism between B and Fe in the plant has been observed (Salardini, 1994). Kumar et al. (1981) reported that adding Zn to the soil reduced the plant Fe, Mn and Cu and increased plant Zn content. Concurrent Zn and Fe application delayed plant aging and increased the grain carbohydrate content (Hemantaranjan and Gray, 1988). Zinc use, by increasing harvest, reduces the concentration of other elements, including Fe and Mn, due to the dilution effect (Rengel and Graham, 1995). Ming and Yin (1992) reported that the use of each of fertilizers containing Zn and Mn, reduced the concentrations of other elements. Graham et al. (1987) reported that whenever the soil Zn content reduced, the barley plant P, K and Cu content increased while its Fe content was not affected. Parker et al. (1992) showed that Zn application increased plant Mn content but had no effect on its Fe and Cu contents. Mozafar (1987), by studying the effects of 12 nutrients on corn graining, observed that the corn ear Zn, B, Cu, Mn, ca, Mg, N, P, K and sodium contents and unripe ear stem Zn, B, Fe, Cu, Mn, molybdenum, ca and P contents had the same status and showed no significant difference. Studies made on wheat showed that by increase in wheat grain Zn content, the grain K, ca, Mn and Fe decreased, significantly

while the grain N, P, Cu, and Zn content increased and Mg content did not change (Ming and Yin, 1992). According to Rengel and Graham (1995) report, by increase in wheat grain Zn content, Fe, Mn, Cu, B, ca, Mg, sodium, K and P contents decreased. Touchton and Boswell (1975), in a study on the effect of B on the corn observed that by B application, the plant B content increased but the Fe, Zn, Mn, Cu, P and K contents did not change.

Considering the numerous applications of corn in human, animal and poultry nutrition and extraction of about 500 different products from it, richness of its grain in Fe and Mn elements plays an effective role in the human health. Also, the grain's being rich in these elements reflects an increase in the grain harvest, qualitatively and quantitatively. Therefore, by studying the effect of Zn-B interaction on the grain, while enriching the grain, we can use its indirect effects to know about its indirect effects, which are the increase in grain harvest, qualitatively and quantitatively. Moreover, it has been established that if grains rich in these elements are used ad seeds, the harvest will increase, qualitatively and quantitatively.

2. Materials and Methods

This research was conducted in the field experiment of Firouz Abad University, Fars province of Iran, during April 2009. Before implementing the project sampling from the soil (0-30 cm depths) was made in order to select a zone in which the available amount of Zn and B was low (less than 1mg/kg extracted by methods DTPA and hot water, respectively). This soil had a loam texture, pH of 8.4, 0.78 % organic matter, 210 mg kg^{-1} exchangeable potassium (K), 9.9 mg kg^{-1} available phosphorus, DTPA extractable Fe, Mn, Zn and Cu concentration were 1.4, 6, 0.38 and 1 mg kg^{-1} and available B with hot water extractable was 0.9 mg kg^{-1}.

For performing the experiment, 20 treatments in 3 replications in the form of randomized complete block design and factorial were considered. The treatments used consisted of 5 levels Zn (0, 8, 16 and 24 kg/ha Zn and Zn spray with a 0.5 percent concentration) and 4 B levels (0, 3 and 6 kg/ha B and spray with a 0.5 percent concentration). Nitrogen, P and K, from sources of urea, triple super phosphate and potassium sulfate at 350, 200 and 200 kg/ha, respectively, were added to all treatments (plots). Moreover, 50% of the urea was used when planting and the remainder two times: At veg**e**tative growth and when the corn ears were formed. Zinc and B, from Zinc sulfate and boric acid sources, respectively, were used by two methods, adding to the soil and spraying. Addition to the soil was made at the time of plantation and the sprayings were made at 5 per thousand (0.5%) Zinc sulfate and 3 per thousand (0.3%) boric acid two times: one at vegetative growth stage and the other after corn ears formation. Each experimental plot was 8m length and 3m width, had 5 beds and 4 rows, equally spaced, and seeds 20cm apart on the rows.

Analysis of the grain was carried out using common lab procedures. Phosphorous was measured by Olsen method, available K by acetate ammonium extraction method and K assessment in the extract by flame photometer, organic carbon by the Walkley and Black method. Available Fe, Zn, Mn and Cu in the soil were first extracted by DTPA and then were read by atomic absorption setup. The soil's available B was extracted by hot water and then was measured by spectrophotometer by Curcamin method, considering the intensity of the color produced. Digestion method by dry burning was used to measure Fe and Mn, and then they were measured by atomic absorption setup. Statistical analysis of data was made using MSTATC and SAS software with Duncan test and regression equations via the SPSS program.

3. Result and Discussion

Soil test results from soil samples taken in the spring of 2008 are presented in Table 1.

Table 1.The result of soil analysis

Depth of soil (cm)	Soil texture	pH	EC (ds m^{-1})	Organic matter (%)	P	K	Fe	Mn	Zn	Cu	B
					mg kg^{-1}						
0-30	Loam	8.4	2	0.78	9.9	210	1.5	6	0.38	1	0.9

3.1. The grain Fe content

Different Zn levels showed no significant on the grain Fe content (mg/kg) (table 2). Application of B to the soil and its spraying had no effect on the grain Fe content relative to no B level but B spraying significantly decreased the grain Fe content relative to B application to the soil (3 and 6 kg/ha B). The minimum and the maximum grain Fe content, 40.07 and 57.93 mg/kg, were observed at B spraying and 3 kg/ha B levels, respectively, but showed no significant difference as compared with the no B level. The Zn-B interaction effect on the grain Fe content was insignificant at 5% level.

Table 2. The effect of Zn and B on Fe concentration in the grain (mg kg^{-1})

B (kg ha^{-1})	Zn (kg ha^{-1})					
	0	8	16	24	Foliar Spray	Mean
0	64.47 ab	39.67 bc	46 abc	50 abc	43.67 abc	48.8 ab
3	49.67 abc	66.33 ab	62.33 ab	70.33 a	41 bc	57.93 a
6	46.67 abc	40.67 bc	63.33 ab	52 abc	56.33 abc	51.8 a
Foliar Spray	47.33 abc	41.33 bc	35 c	34.67 a	42 bc	40.07 b
Mean	52.08 a	47 a	51.67 a	51.75 a	45.75 a	

***Means with same letters lack a significant difference at 5% level by Duncan's test**

3.2. Iron uptake by the grain

Different Zn levels showed no significant on the Fe uptake by the grain (g/ha) but the main B effect on Fe uptake was significant at 5% level (table 3). The minimum mean Fe uptake by the grain, 354.77 g/ha, was at the B spraying level but showed no significant difference relative to the no B level. Application of 3 kg/ha B significantly increased Fe uptake by the grain, from 378.77 to 517.95 g/ha (36.74 % increase relative to no B use); but more B use (6 kg/ha B), showed no significant difference from no B and 3 kg/ha B levels. Boron spraying significantly reduced Fe uptake by the grain relative to 3 kg/ha B; but showed no significant difference from the no B level. The maximum Fe uptake by the grain, 517.95 g/ha, was seen at 3 kg/ha B level.

The Zn-B interaction effect on Fe uptake by the grain showed that B application left no significant difference from the no B use in any of Zn levels. At high B levels (6 kg/ha B), only the use of 16 kg/ha Zn increased Fe uptake by the grain from 335.1 to 617.1 g/ha (84.15 percent increase relative to no Zn use at that B level). But at other B levels, Zn application had no significant effect on Fe uptake by the grain.

The maximum and the minimum Fe uptake by the grain, 662.1 and 335.1 g/ha, were seen by application of 24 kg/ha Zn + 3 kg/ha B and 6 kg/ha B, respectively; showing 52.24 % increase and 22.94 % decrease relative to the control, with an uptake of 434.9 g/ha, respectively. No treatment, including those with the maximum and the minimum Fe uptake by the grain, showed a significant difference from the control.

Table 3. The effect of Zn and B on Fe uptake by the grain (gr ha^{-1})

B (kg ha^{-1})	Zn (kg ha^{-1})					
	0	8	16	24	Foliar Spray	Mean
0	439.6 abc	346.6 bc	346.1 bc	394.9 abc	370 bc	378.77 b
3	411.1 abc	589.7 Abc	563.3 abc	662.1 a	363.6 bc	517.95 a
6	335.1 c	352.6 bc	617.1 ab	420.4 abc	513 abc	447.64 ab
Foliar Spray	356.9 bc	352.4 bc	344.3 bc	354.9 bc	365.4 bc	354.77 b
Mean	384.49 a	410.33 a	467.7 a	458.06 a	403.01 a	

***Means with same letters lack a significant difference at 5% level by Duncan's test**

3.3. The grain Mn content

Application of Zn at a high level (24 kg/ha Zn), increased grain Mn content from 3.67 to 4.75 mg/kg (29.42% increase relative to no B level), but the use of other Zn levels showed no significant effect on the grain Mn content relative to no Zn level (table 4). The maximum and the minimum mean grain Mn content, 3.67 and 4.75 mg/kg, were seen at no Zn and 24 kg/ha Zn levels, respectively.

The use of different B levels had no significant effect on the grain Mn content relative to no B level but there was a significant difference between B application to the soil and B spraying; B spraying significantly decreased grain Mn content relative to 3 and 6 kg/ha B levels. The minimum mean grain Mn content, 3.73 mg/kg, was seen at B

spraying level but showed no significant difference relative to no B level.

The effect of Zn-B interaction on grain Mn content was not significant at 5% level. Joint use of 6 kg/ha B and 16 kg/ha Zn made the maximum increase in grain Mn content (6mg/kg) showing a 100% increase relative to the control (3 mg/kg). The minimum grain Mn content, 3 mg/kg, was seen at no Zn and B use (control). Except for the treatment with the highest grain Mn content, other treatments showed no significant difference from the control.

Table 4. The effect of Zn and B on Mn concentration in the grain **(mg kg^{-1})**

B (kg ha^{-1})	Zn (kg ha^{-1})					
	0	8	16	24	Foliar Spray	Mean
0	3 c	5 abc	4.67 abc	4 abc	4.33 abc	4.2 ab
3	4 abc	5.33 abc	3.33 bc	5.57 ab	5.67 ab	4.8 a
6	4 abc	4.33 abc	6 a	4.67 abc	5.33 abc	4.87 a
Foliar Spray	3.67 abc	3.33 bc	3.67 abc	4.67 abc	3.33 bc	3.73 b
Mean	3.67 b	4.5 ab	4.42 ab	4.75 a	4.67 ab	

***Means with same letters lack a significant difference at 5% level by Duncan's test**

3.4. Manganese uptake by the grain

Zinc application at all levels significantly increased Mn uptake by the grain (g/ha) relative to the no Zn level (table 5). The minimum and the maximum Mn uptake by grain, 27.54 and 42.06 g/ha, were seen at no Zn and 24 kg/ha Zn levels, respectively. The use of 8, 16, and 24 kg/ha Zn, significantly increased Mn uptake by the grain from 27.54 at no Zn level to 39.53, 39.64 and 42.06 g/ha, respectively (43.53, 43.93 and 52.72 % increase in that order); but there was no significant difference at application to the soil levels. Zinc spraying increased Mn uptake by the grain from 27.54 to 40.72 g/ha, a 47.86 % increase relative to no Zn level, but showed no significant difference from the Zn applied to the soil level. Thus a synergism was seen between Zn application and Mn uptake by the grain.

The main effect of B on Mn uptake by the grain was significant at 5% level. Boron application to the soil increased Mn uptake by the grain relative to no B level but B spraying had no significant effect on Mn uptake. The minimum mean Mn uptake by the grain, 33.48 g/ha, was seen at no B level. The use of 3 and 6 kg/ha B, significantly increased Mn uptake by the grain from 33.48 to 42.44 and 41.97 g/ha, respectively (26.76 and 25.36 % increase relative to no B use, in that order), but there was no significant difference between the two. Boron spraying showed a significant reduction relative to application of B to the soil.

The Zn-B interaction effect on Mn uptake by the grain showed that application of Zn at low levels (0 and 8 kg/ha Zn) and Zn spraying had no effect on the Mn uptake by the grain but at higher Zn levels (16 and 24 kg/ha Zn), it increased Mn uptake by the grain. At 16 kg/ha Zn, only the use of 6 kg/ha B increased Mn uptake by the grain from 35.1 to 57.8 g/ha (64.67% increase relative to no B use). The use of 3 kg/ha B at 24 kg/ha Zn level, increased Mn uptake by the grain from 29.67 to 52.13 g /ha (75.7 % increase); but other levels of B had no significant effect.

Zinc application at zero and 6 kg/ha B levels increased Mn uptake by the grain but at other B levels (3 kg/ha and B spraying) it had no significant effect on Mn uptake. At no B level, only the use of 8 kg/ha Zn increased Mn uptake by the grain from 20.2 to 44.07 g/ha (118 % increase). At 6 kg/ha B as well, only application of 16 kg/ha Zn increased Mn uptake by the grain from 29.33 to 57.8 g/ha (97 % increase).

The least Mn uptake by the grain, 20.2 g/ha, was by no Zn and B use (the control). The highest Mn uptake by the grain, 57.8 g/ha, was by joint use of 6 kg/ha B and 16 kg/ha Zn, a 186 % increase relative to the control.

Table 5. The effect of Zn and B on Mn uptake by the grain **(gr ha^{-1})**

B (kg ha^{-1})	Zn (kg ha^{-1})					
	0	8	16	24	Foliar Spray	Mean
0	202 d	44.07 abc	35.1 bcd	29.67 cd	38.37 abcd	33.48 b
3	32.93 bcd	48.5 abc	30.23 bcd	52.13 ab	48.4 abc	42.44 a
6	29.33 cd	37.23 abcd	57.8 a	38.6 abcd	46.9 abc	41.97 a
Foliar Spray	27.7 cd	28.33 cd	35.43 bcd	47.83 abc	29.2 cd	33.7 b
Mean	27.54 b	39.53 a	39.64 a	42.06 a	40.72 a	

***Means with same letters lack a significant difference at 5% level by Duncan's test**

3.5. Correlation between the concentration and total uptake of Fe and Mn in grain with other variables

The correlation coefficients (R) between different variables by the Pearson method and the relevant equations were obtained by the step by step method using the SPSS software. One can use each of the following equations depending on what are the variables measured and R and R^2, but the last equation derived, is the most complete equation containing dependent and independent variables and we must measure more variables to derive that equation. The symbols * and ** in equations and correlation coefficients (R or R^2), are significance at 5% $(\alpha = 0.05)$ and 1% $(\alpha = 0.01)$ levels.

3.5.1. The grain Fe content

The grain Fe content showed a positive correlation with the grain P content (R=0.35), and the Fe uptake by the grain (R=0.89**) and a negative correlation with the leaf K content (R=-0.49*). The equations of which were:

1) FeG = 10.322 + 0.0926 FeUG R = 0.892**
2) FeG = 52.315 + 0.108 FeUG - 0.00568 TGY $R^2 = 0.984^{**}$
3) FeG = 63.22 + 0.109 FeUG - 0.00598 TGY - 0.0753 FeL $R^2 = 0.989^{**}$
4) FeG = 69.025 + 0.109 FeUG - 0.00603 TGY - 0.0749 FeL - 0.219 ZnG $R^2 = 0.993^{**}$
5) FeG = 76.78 + 0.11 FeUG - 0.00597 TGY - 0.0726 FeL - 0.181 ZnG - 0.0284 GTW $R^2 = 0.995^{**}$
6) FeG = 83.4 + 0.11 FeUG - 0.00572 TGY - 0.0503 FeL - 0.162 ZnG - 0.398 GTW - 0.818 P $R^2 = 0.997^{**}$

FeG, FeUG, TGY, FeL, ZnG, GTW and P denote grain Fe content (mg/kg), Fe uptake by the grain (g/ha), total grain yield (kg/ha), leaf Fe content (mg/kg), grain Zn content (mg/kg), 1000-grain weight (g) and grain protein content (%), respectively.

3.5.2. The grain Mn content

There was a positive correlation between grain Mn content and the grain P content (R=0.35), Zn content (R=0.49*) and B content (R=0.61**); the uptake of N (R=0.38), P (R=0.52*), K (R= 0.41), Fe (R= 0.47*), Mn (R= 0.94**), Zn (R= 0.71**) and B (R= 0.66**) by the grain; total grain yield (R= 0.46*), the number of grains in the ear length (R= 0.4) and the number of grains across the ear diameter (R=0.45*), and a negative correlation with leaf B content (R= -0.3) and the percentage of grain in the ear (R= -0.37). The relevant equations were:

1) MnG = 1.192 + 0.0846 MnUG R = 0.943**
2) MnG = 3.993 + 0.113 MnUG - 0.000454 TGY $R^2 = 0.985^{**}$

MnG, MnUG and TGY are grain Mn content (mg/kg), Mn uptake by the grain (g/ha) and total grain yield (kg/ha), respectively.

3.5.3. The Fe uptake by the grain

The Fe uptake by the grain showed a positive correlation with the grain P content (R= 0.48*), Fe content (R= 0.89**), Mn content (R= 0.47*) and B content (R= 0.45*); the uptake of N (R=0.31), P (R=0.49*), Mn (R=0.52*), Zn (R=0.35) and B (R=0.49*) by the grain; and total grain yield (R= 0.33) and a negative correlation with leaf K content (R=-0.43). The equations were:

1) FeUG = -2.221 + 8.599 FeG R = 0.892**
2) FeUG = - 474.065 + 9.09 FeG + 0.0523 TGY $R^2 = 0.985^{**}$
3) FeUG = -575.283 + 9.103 FeG + 0.0549 TGY + 0.691 FeL $R^2 = 0.99^{**}$
4) FeUG = -626.702 + 9.071 FeG + 0.055 TGY + 0.683 FeL + 2.017 ZnG $R^2 = 0.994^{**}$
5) FeUG = - 697.792 + 9.081 FeG + 0.0544 TGY + 0.661 FeL+ 1.656 ZnG + 0.258 GTW $R^2 = 0.996^{**}$
6) FeUG = - 758.18 + 9.084 FeG + 0.0521 TGY + 0.458 FeL + 1.48 ZnG + 0.362 GTW + 7.45 P $R^2 = 0.997^{**}$

FeUG, FeG, TGY, FeL, ZnG, GTW and P are Fe uptake by the grain (g/ha), grain Fe content (mg/kg), total grain yield (kg/ha), leaf Fe content (mg/kg), grain Zn content (mg/kg), 1000-grain weight and grain protein content (%), respectively.

3.5.4. The Mn uptake by the grain

There was a positive correlation between Mn uptake by the grain and the leaf Zn content (R= 0.31); grain P content (R= 0.43), Mn content (R= 0.94**), Zn content (R= 0.35) and B content (R= 0.66**); the uptake of N (R= 0.63**), P (R= 0.73**), K (R= 0.61**), Fe (R= 0.52*), Zn (R= 0.76**), Cu (R= 0.41) and B (R= 0.8**) by the grain; ear weight (R= 0.48), grain weigh in the ear (R= 0.42), total grain yield (R= 0.71**), the number of grains in the ear length (R=0.57**), the number of grains across the ear diameter (R= 0.51*). The relevant equations were:

1) MnUG = -8.293 + 10.498 MnG
$R = 0.943^{**}$

2) MnUG = -35.219 + 8.673 MnG + 0.00409 TGY
$R^2 = 0.991^{**}$

3) MnUG = -32.572 + 8.279 MnG + 0.00357 TGY + 0.0427 BUG $R^2 = 0.991^{**}$

MnUG, MnG, TGY and BUG are Mn uptake by the grain (g/ha), grain Mn content (mg/kg), total grain yield (kg/ha) and B uptake by the grain (g/ha)

4. Conclusion

The effects of Zn, B (relative to no B level) and the effect of Zn-B interaction on the grain Fe content were insignificant. Zinc application had no effect on Fe uptake by the grain and among different B levels, only application of 3 kg/ha B significantly increased Fe uptake by the grain. The B effect on Fe uptake by the grain did not depend on the soil Zn content. Also, Zn application only at high B levels (6 kg/ha B) increased Fe uptake by the grain but had no effect at other levels. The B effect (relative to no B level) and the effect of Zn-B interaction on grain Mn content was not significant and among different Zn levels, only application of 24 kg /ha Zn increased grain Mn content. The main Zn, B and Zn-B interaction effects on Mn uptake by the grain, was significant. Zinc application at all levels increased Mn uptake by the grain. The minimum and the maximum Mn uptake by the grain were seen at no Zn and 24 kg/ha Zn levels, respectively. Boron application to the soil increased Mn uptake by the grain but B spraying had no effect on it. The minimum mean Mn uptake by the grain, 33.48 g/ha, was seen at no B level. Therefore, there was a synergism between Zn and Mn as well as B and Mn. Boron use at high Zn levels (16 and 20 kg/ha Zn) increased Mn uptake by the grain, but at other Zn levels, no effect was seen. Therefore, a high Zn level helped with B affecting the increase in Mn uptake by the grain. Application of Zn at 0 and 6 kg/ha B levels increased Mn uptake by the grain but had no effect at other B levels.

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Algae of Soil Surface Layer of Wadi Al-Hitan Protective Area (World Heritage Site), El-Fayum Depression, Egypt

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Abstract Four artificial solid and liquid media were recommended for cultivation, isolation and purification of algae from the soil surface layer of Wadi Al-Hitan protective area, El-Fayum depression, Egypt. Some selective physical and chemical properties of soil were also determined. These analyses indicated that this soil had sandy texture, alkaline pH, relatively high nitrogen and electeric conductivety, with low phosphorus content (which mainly charcterized the desert, saline and arid soil).Total 35 soil algal taxa were identified. These related to 3 algal divisions which dominated with cyanophycophyta (represented by 30 taxa from 15 genera), followed by Chlorophycophyta, (represented by 3 taxa from 3 genera), and Xanthophycophyta, (each by 2 taxa from 2 genera). Most of the recorded cyanophytes were xeric and found in filamentous and in heterocystous forms which correlated with the relatively high nitrogen content in this arid soil. While green algae were showed in coccal forms. Myer's and modified Chu's No.10 media were relatively the most productive algal media and they were also characterized with high cyanophytes members . While Beijernicks medium was characterized with chlorophytes. These associated with no growth for xanthophytes in Myer's and modified Chu's No.10 media. This study concluded that, pH and E.C were the most physic-chemical controlling and selective factors, which effect on the availability of several soil nutrients and in turn on the biodiversity of soil algae in the studied region. Eco-phycological analysis of the idenified algae isolated from the regarded desert arid soil was indicated that, most of the recorded algae were mainly related to xeric cyanophytes belonging to *Nostoc, Anabeana, Cylindrospermum, Calothrix, Camptylonema, and Scytonema* algal genera. [Journal of American Science 2010;6(8):243-255]. (ISSN: 1545-1003).

Keywords: soil algae; arid and semiarid regions; edaphic factors; Wadi Al-Hitan protective area; El-Fayum depression; Egypt.

1. Introduction

Althogh in the natural world, they are important foundations of terrestrial ecosystems but the knowledge of terrestrial algae is still far from being complete. The importance of the biodiversity and abundance of terrestrial algae, particularly from urban habitats have gradually appeared in the last few decades (Mike, 1982). Wherever there is soil there are algae on or near the surface, since soil prokaryotic and eukaryotic algae are dominant in a broad spectrum of terrestrial habitat and able to colonize almost all of the biotopes on earth (Friedmann and Ocampo-Friedmann, 1984 and Lukes¡ová, 2001). Rahmonov and Piatek, (2007) stated that, the degree of algael distribution on the ground and in the soil profile varies as a result, the role of algae at particular stages of soil development and in different soils is differentiated. Soil algae are well-known to play an important ecological role in terrestrial ecosystems as colonizers of soils that have become bare (DeWinder, 1990; Belnap and Gardner, 1993; Johansen and Shubert, 2001; Lukes¡ová, 2001and Chun-Xiang and Yong-Ding, 2003). The most obvious and significant role of algae in the aired, semiarid soils is in desertification control, primary humus formation and in stabilization of unconsolidated sand (Dor and Danin,2001; Chun-Xiang, *et al*., 2002 and Zhao *et al*., 2009).

Literature data on the soil algal flora of Wadi Al-Hitan in Egypt is completely lacking. In order to fill this gap in knowledge, the present work initiated to investigate the algal biodiversity of the surface soil layer of this desert protective area.

2. Material and Methods

I. Study area

Wadi Al-Hitan (Whale Valley) is the most important international site in the world, and in 2005 it located within the protected area and was designated as a world heritage sit in recognition of the 40 million year old whale skeletons found here. It represents an outstanding record of middle to late Eocene life and geological evolution. Wadi Al-Hitan provides evidences of millions of years of coastal marine life particularly the evolution of the whales. It is in an area over 10 km long there has been found an unusually large concentration of over 400 fossil skeletons of

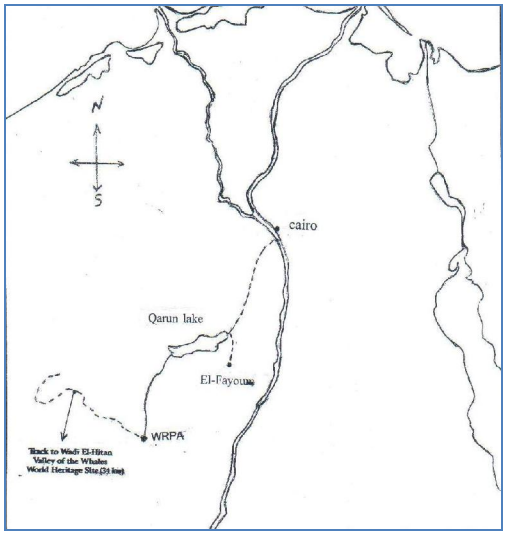
archaic whales and other vertebrates. Since the fossils of different periods lie at different levels they are valuable indicators of palaeogeologic and palaeoecologic conditions, Eocene life, and the evolution of marine mammals (Uhen, 2004).

Wadi Al-Hitan located at 29° 15’ 13" to 29° 23’ 56"N by 30° 00’ 41" to 30° 10’ 06 E in the Western desert 150 km southwest of Cairo and 80 km west of Faiyum and it is included as a special protected area within the Wadi El-Rayan protective area (Map 1). The climate is typically Saharan, hot and dry in summer and mild with scanty rain in winter. The mean winter temperature is 13.7 degrees °C with an absolute minimum of –1.2°C; the mean summer temperature is 28.5°C with an absolute maximum of 48.4°C; the average diurnal range is 15.6°C. Wadi Al-Hitan is extremely barren and was probably always rather abandoned in historical times. There is very little vegetation and no-one lives on the site, but Wadi el-Rayan 40 km away has a few thousand settled and temporary farmers and fishermen (Dolson et al., 2002).

II. Treatment of soil

Soil sampling

Soil samples of Wadi-El -Hitan were randomly collected at 27/4/2009 from seven localities in clean and sterilized air-tight polyethylene pags. Soil samples were collected from the soil surface down to a depth of about 17 cm (Gollerbach and Shtina, 1969). In a certain amounts from each locality, soil samples were mixed together to form one processed sample which was analyzed for different chemical and physical characteristics of soil as well as the culturing, isolation, identification and then purification of algae.



Map (1) indicating the investigated region of Wadi Al-Hitan protective area (1: 100000km)
WRPA = Wadi El-Rayan protective area

Soil analysis

One kg from the produced soil sample was analyzed for determination of soil texture with the hydrometer method (Brouach and Barthokur, 1997), providing quantitative data on the percentage of sand, silt and clay. The soil samples (soil: water = 1: 2.5) were analyzed with respect to their EC (Electrical Conductivity) and pH range following the methodology outlined by Black, 1992. Available phosphorus, nitrogen, organic matter % and micronutrients were determined according to Richard *et al*. (1954). While silica content was estimated according to Tan (1996).

III. Culturing, isolation and identification of algal taxa

Culturing studies are essential for isolation, purification and correct identification of soil algae (Round, 1984). In this study, four artificial (solid and liquid) media were recommended for cultivation and isolation of algae from Wadi Al-Hitan soil sample. These were Allen's, (Allen, 1968); Myer's media (Venkataraman, 1969) and Beijernicks & modified Chu's No.10 (Stein, 1979). Under aseptic conditions, liquid media in 250 Erlen Myer's flasks (one gm of the investigated soil was inoculated in 50 ml of sterilized liquid media), and solid media in 10 cm diameter Petri-dishes were used. For solid media, about 20 gm of agar-agar were added to one liter of each previously recommended, sterilized liquid media. Constant volume was poured in 10 cm diameter Petri-dishes and lifted to solidify. Dissolve 1 gm of soil in 99 ml of sterilized distilled water, then spread one ml from this solution on the surface of the previously solidified and sterilized solid media. The inoculated flasks and Petri-dishes were incubated at 25 0C under continuous light intensity of 4000 lux conditions. For each soil inoculum, the solid and liquid media were used in triplicates and under aseptic conditions. Uni- algal cultures were produced using plating and serial dilution procedures recommended by Jurgensen and Davey, (1968). These supported by microphotographs for each produced and identified common uni-algal species.

The identifications of algal taxa were principally accomplished using one fifty, Reichert binocular research microscope, after mounting the micro algal mass on the microscopic slide and covering them with a thin cover slip, for characterization of soil algal species up to morphological level. Most of microscopic characters of prominence specimens were taken into consideration and were identified according to the systematics works of: Gollerbakh *et al*. (1953); Kiselov *et al*. (1953); Korsanov *et al*. (1953); Dedesenko *et al*. (1959); Desikachary (1959); Philipose (1967); Komarenko & Vasileva (1975 & 1978).

3. Results

Physico-chemical analysis of soil

According to the physical analysis of soil (Table 1), the investigated soil of Wadi Al-Hitan showed sandy texture (92.32% sand), with alkaline pH (8.5) and realtively high electric conductivety (3 m.mohs|cm). Furthermore, chemical soil analysis indicated that, the studied soil had Calcium-Sodium cationic and Sulphate-Chloried anionic structures, with low organic matter, phpsphorous contents and relatively high nitrogen and iron ones (Table 1).

Algal growth appeared after two weeks from the start of incubation period.Total 35 soil algal taxa were isolated and identified from the concerned soil (Table 2). These related to 3 algal divisions which dominated with Cyanophycophyta (30 blue-green algal taxa from 15 genera), followed by Chlorophycophyta, (3 green algal taxa from 3 genera) and Xanthophycophyta, (2 yellow green algal taxa from 2 genera). In the material examined, most of the recorded cyanophytes were found in filamentous and in heterocystous forms (nitrogen fixing blue-green algae). While green algae were showed in coccal forms.

On the level of media used , Myer's and modified Chu's No.10 media were relatively the most productive algal media (represented by totally 23 and 16 taxa respectively), followed by Allen's (total 14 taxa) and Beijernicks (total 6 taxa) media. The results also revealed that Myer's and modified Chu's No.10 media were characterized with high cyanophytes members (21 and 15 blue green algal taxa) respectively.While Beijernicks medium was characterized with chlorophytes (3 green algal taxa). These associated with no growth for xanthophytes in Myer's and modified Chu's No.10 media.

The most common soil algal genera and species present along different media and through all over the studied region were *Stratonostoc linckia f. calcicola* (Breb.) Elenk and *Chlamydomonas atactogama.* The investigated soil algal list also recorded rare algal genera and species as *Anabeana hallensis* (Janez.) Born. Et Flah.; Scytonema *drilosphon* (Kutz.) Elenk.et V.Poljansk; Botrydiopsis *eriensis* Snow (only recorded in Allen's medium) - *Scenedesmus quadricauda var. eualternans* Proschk (only showed in Beijernicks medium) - *Nematonostoc flagelliforme* (Berk et Curt.) Elenk; *Sphaeronostoc. microscopicum* (Carm.) Elenk; *Anabeana thermalis f. rotundospora* Aptek; Calothrix *marchica* Lemm.; *Symploca muscorum* (Ag.) Gom.; and *Schizothrix tinctoria* (Ag.) Gom. (only characterized Chu's No.10 medium) - and *Amorphonostoc punctiforme* (Kutz.) Elenk; *Stratonostoc commune* (Vauch) Elenk.; *Stratonostoc linckia f. spongiaeforme* (Ag.) Kutz.; *Anabeana knipowitschii* Ussatsch; *Anabeana macrospora* Kleb; *Cylindrospermum catenatum* Ralfs ; *Symploca muralis var. minor* Grander (Kutz) ex Gemont; *Lyngbya lutea* (Ag.) Gom and *Schizothrix lutea* Fremy (recorded only in Myer's medium).

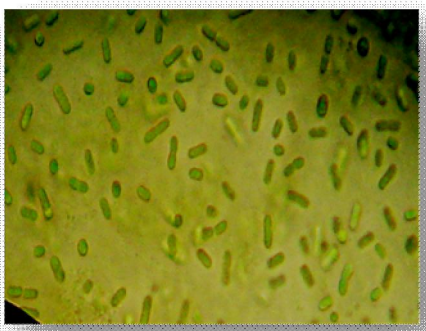
Table 1. Soil physico-chemical characters of Wadi Al-Hitan region

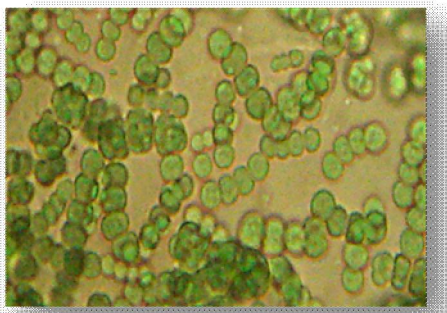
PARAMETERS	VALUES	
Mechanical analysis %	SAND	92.32
	SILT	4
	CLAY	3.68
Texture		Sandy
Soil reaction (pH)		8.5
Electerica conductivety (E.C m.mohs/cm)		3
Soluble cations (meq./l)	Ca^{++}	464
	Mg^{++}	73
	Na^{+}	334
	K^{+}	9
Total soluble cations		880
Soluble anions (meq./l)	CO_3^{--}	4
	HCO_3^{-}	30.5
	Cl^{-}	414
	SO_4^{--}	431.5
Total soluble anions		880
Calcium carbonate %		4
Organic matters %		0.34
Available macronutrients (ppm)	Nitrogen	854
	Phosphorus	10.4
Available micronutrients (ppm)	Fe	4.3
	Cu	0.4
	Zn	0.1
	Mn	0.5

Table 2. Recorded soil algal flora of Wadi Al-Hitan region.

ALGAL TAXA	USED NUTRITIVE MEDIA				MICROPHOTOGRAPHS	
	Allen	Bej	Chus no. 10	Myers		
Cyanophyta					Plate	Figer
Synechococcus elongatus Nag.	+	-	+	-	I	A
Siphonema polonicum Geitl	-	+	-	+		B
Amorphonostoc paludosum Kossinsk	+	-	-	+		D
Amorphonostoc paludosum f.longius Kossinsk	+		+	+		C
Amorphonostoc punctiforme (Kutz.) Elenk	-	-	-	+		E
Nematonostoc flagelliforme (Berk et Curt.) Elenk	-	-	+	-	II	A & B
Sphaeronostoc. microscopicum (Carm.) Elenk	-	-	+	-		C
Stratonostoc commune (Vauch) Elenk.	-	-	-	+		D
Stratonostoc linckia f. calcicola (Breb.) Elenk	+	+	+	+		E
Stratonostoc linckia f.minutum (Desmaz.) Elenk	+		+	+		F
Stratonostoc linckia f. spongiaeforme (Ag.) Kutz.	-	-	-	+	III	A
Anabeana hallensis (Janez.) Born. et Flah.	+	-	-	-		B
Anabeana knipowitschii Ussatsch	-	-	-	+		C
Anabeana macrospora Kleb	-	-	-	+		D
Anabeana oscillarioides Bory	+	-	+	+		E
Anabeana spiroides Kleb.	-	-	+	+		F
Anabeana thermalis f. rotundospora Aptek.	-	-	+	-	IV	A
Anabeana variabilies f. rotundospora Hollerb	+	-	-	+		B
Cylindrospermum alatosporum F.E.Fritsch	-	-	+	+		C & D
Cylindrospermum catenatum Ralfs	-	-	-	+		E & F
Scytonema drilosphon (Kutz.) Elenk.et V.Poljansk	+	-	-	-	V	A
Plectonema gracilllinum (Zopf) Hansgirg	-	-	+	+		B
Calothrix elenkinii Kossinsk	+	-	-	+		C
Calothrix marchica Lemm.	-	-	+	-		D
Camptylonema danilovii Hollerb			+	+		E
Symploca muralis var. minor Grander (Kutz) ex Gemont	-	-	-	+		F
Symploca muscorum (Ag.) Gom.	-	-	+	-	VI	A & B
Lyngbya lutea (Ag.) Gom	-	-	-	+		C
Schizothrix lutea Fremy	-	-	-	+		D
Schizothrix tinctoria (Ag.) Gom.			+			E
Total	**10**	**2**	**15**	**21**		
Xanthophyta						
Chlorocloster raphidoides	+	+	-	-	VII	C
Botrydiopsis eriensis Snow	+	-	-	-		A & B
Total	**2**	**1**	**0**	**0**		
Chlorophyta						
Chlorococcum humicolo (Nag.) Raben.	+	+	-	+	VII	D
Chlamydomonas atactogama Korsch	+	+	+	+		E
Scenedesmus quadricauda var. eualternans Proschk	-	+	-	-		F
Total	**2**	**3**	**1**	**2**		

(A)





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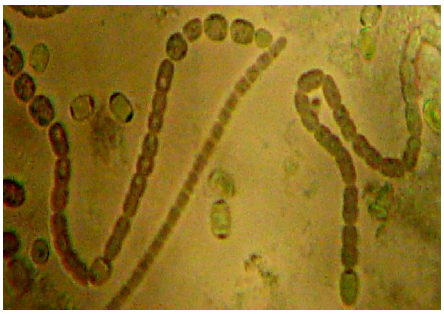

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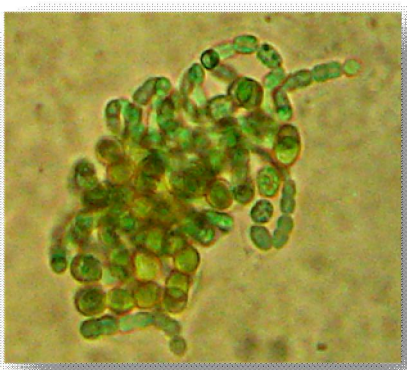

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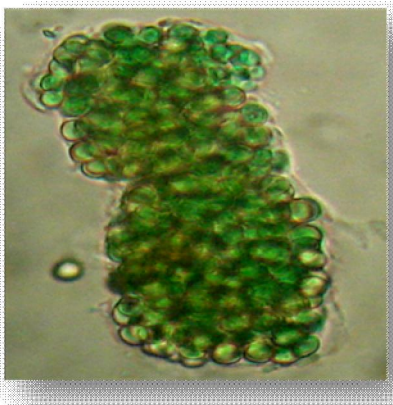
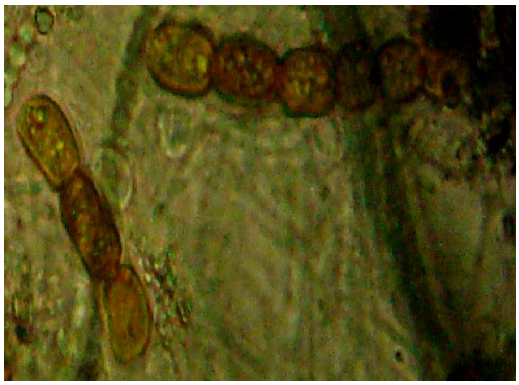
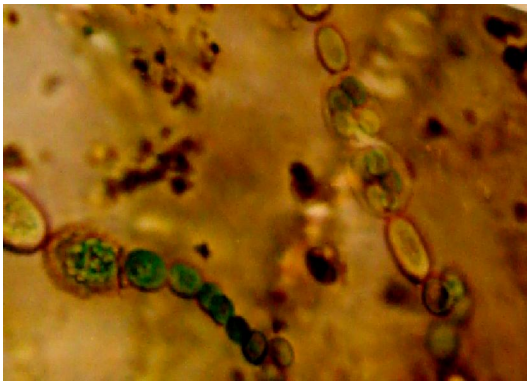


(E)

Plate I



(A) Mature stage



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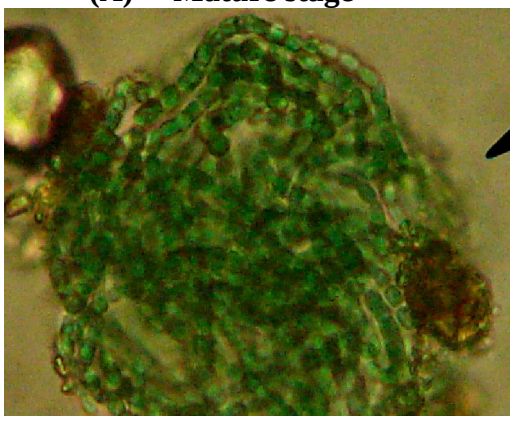

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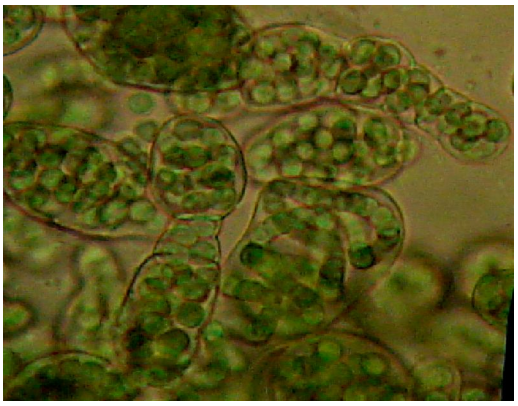

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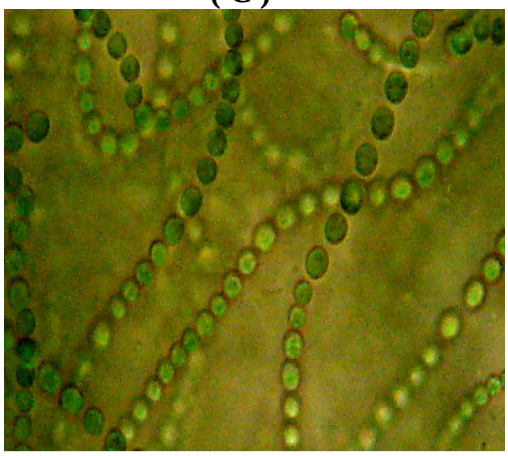

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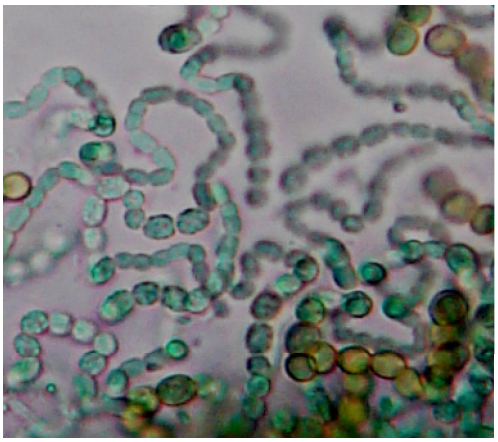

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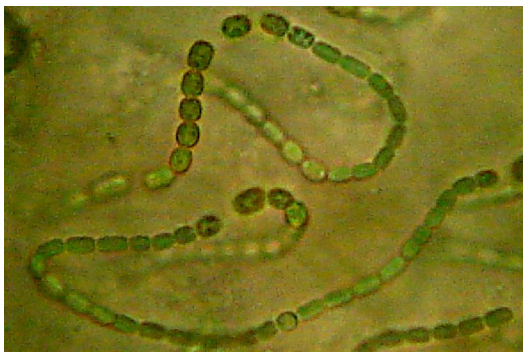

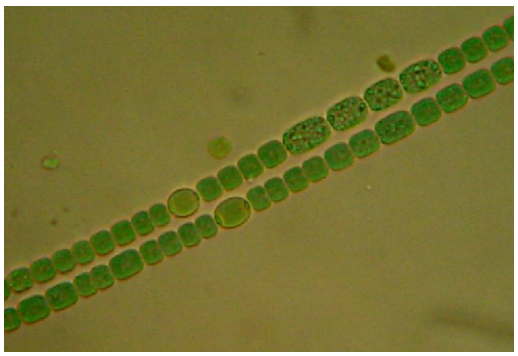

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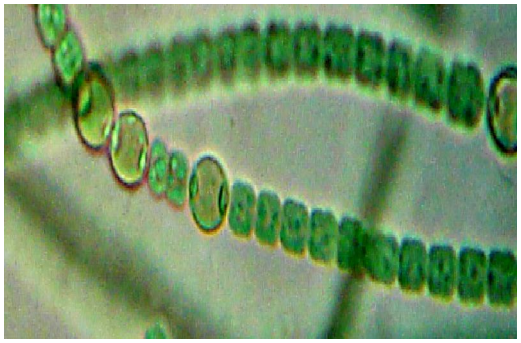

(C) (D)

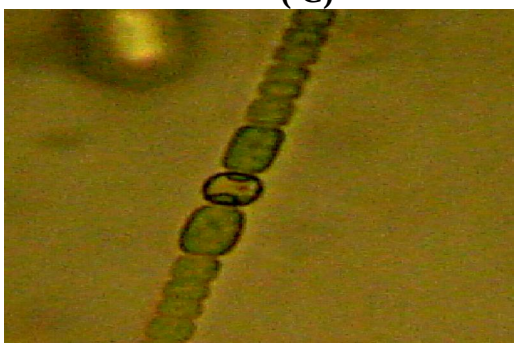

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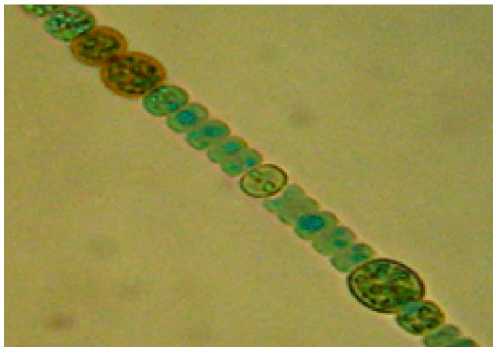
Plate III



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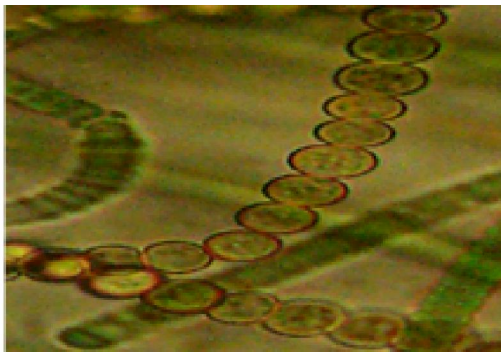

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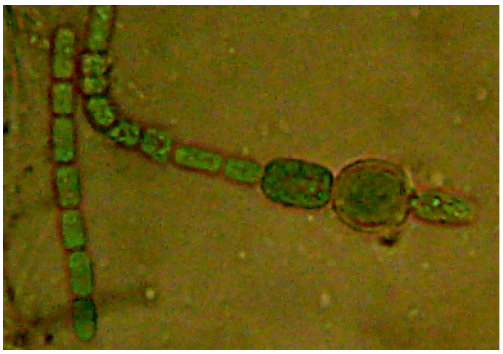
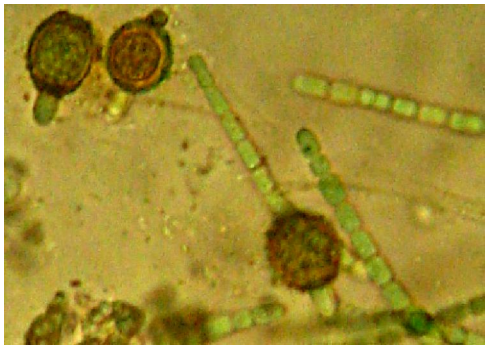
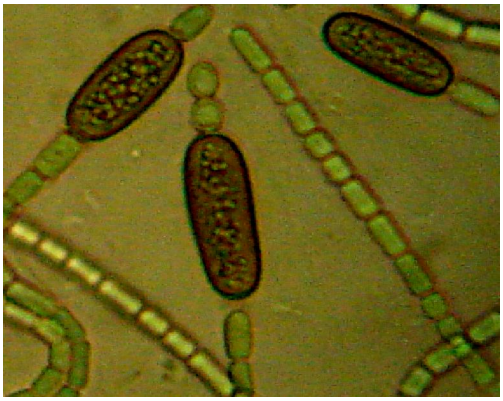


(C) Mature stage



(D) Late mature stage



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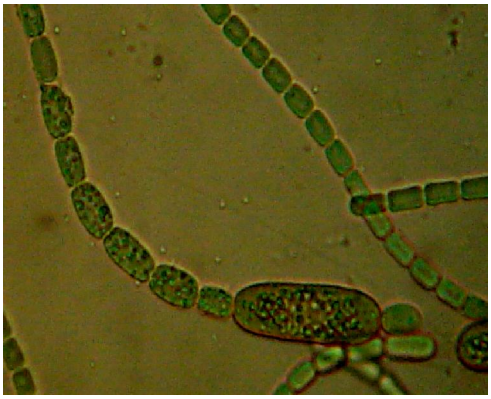
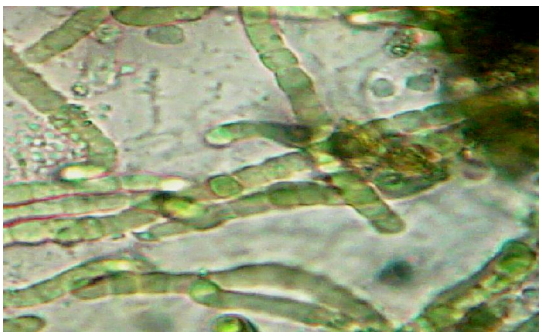


(F) With chain of spores

Plate IV



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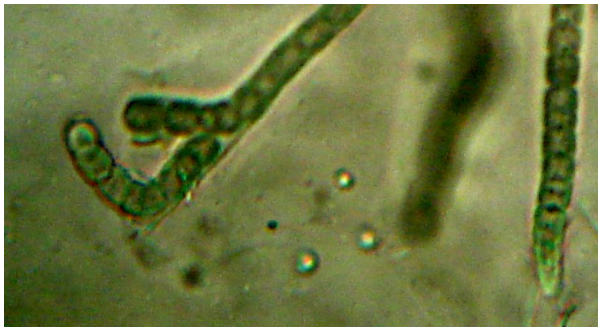

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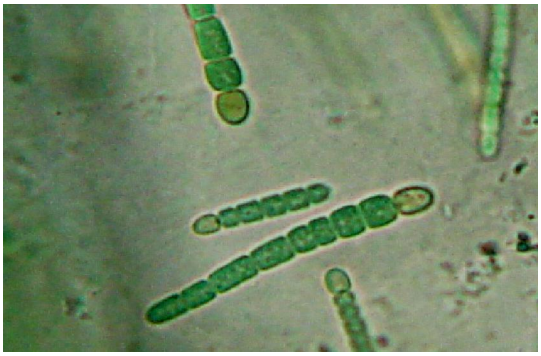

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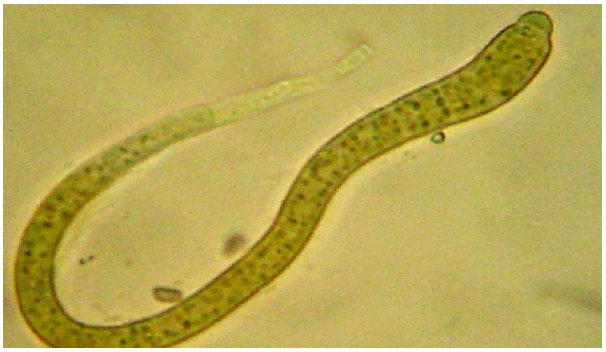

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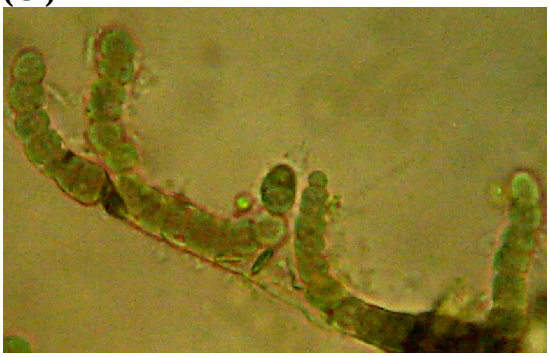

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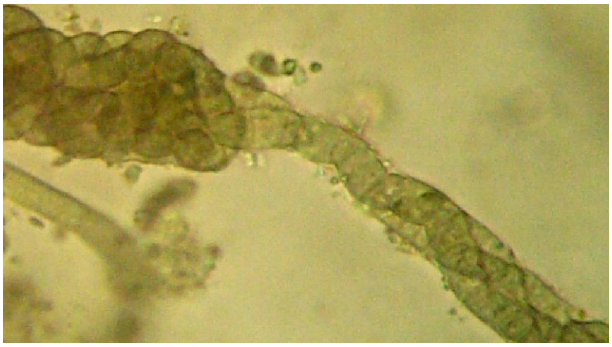
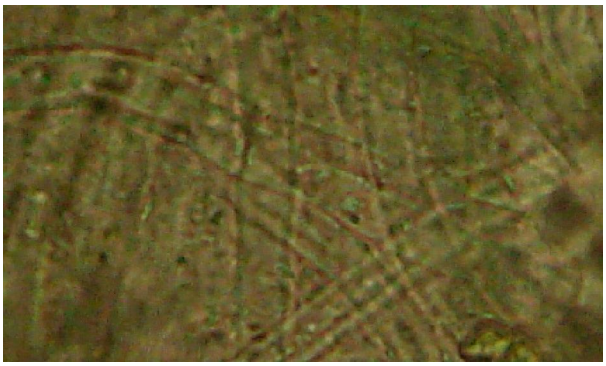


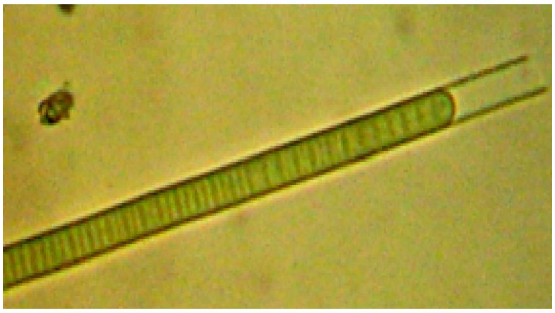
(F)

Plate V

(A) Trichome out sheath



(B) Empty sheaths

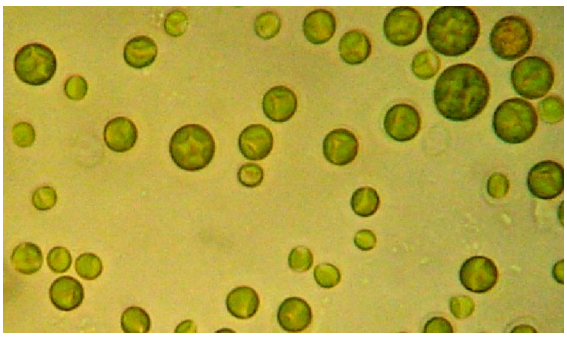


(C)

(D)

(E)

Plate VI



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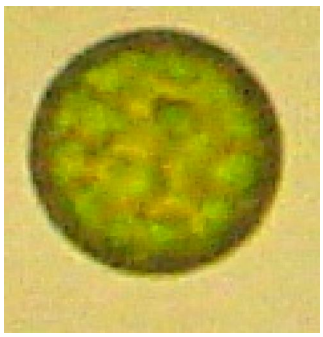

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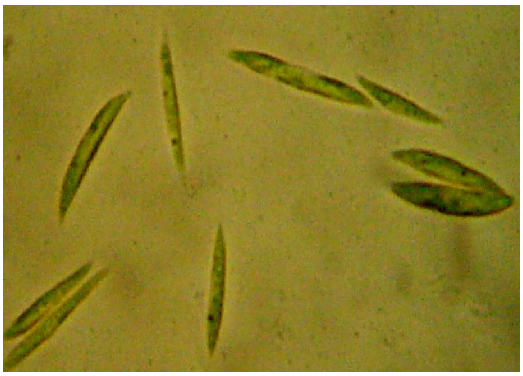

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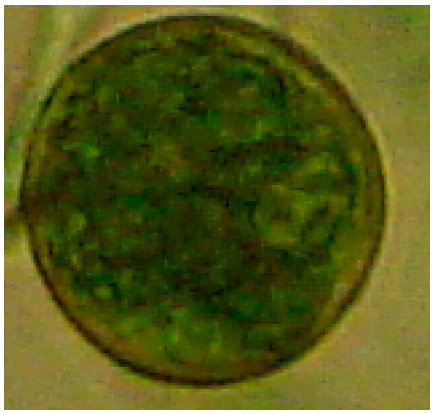

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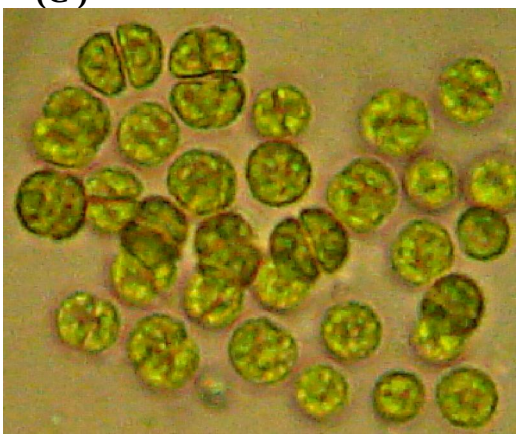

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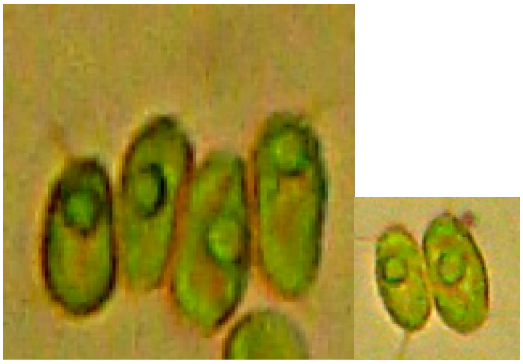

(F)

Plate VII

4. Discussion

Shields & Durrell, (1964) suggest soil algae as a first stage of succession on substrates poor in nutrients. This applies even more to arid and semiarid regions, since nitrogen-fixing bacteria here are rarer than in other climates (Loftis and Kurtz, 1980). Physico-chemical analyses of Wadi Al-Hitan soil indicated that this soil had arid character with sandy texture; alkaline pH; relatively high electric conductively and nitrogen contents, with deficient in organic matter and other nutrients as phosphorous. These were in agreement with the observation of Oldeman (1994) and Veste *et al*. (2001).

Manchanda and Kau-shik (1997); Misra *et al*. (2001) and Verma *et al. (*2002) stated that, salinity and alkalinity significantly influence the diversity of micro-algae, their number, morphology and activity in the arid and semi-aried soil.

Soil algal flora of Wadi Al-Hitan were found to be composed of 35 taxa belonging to 3 algal divisions. Blue-green algae were the most qualitative dominating division. Manchanda & Kau-shik, (1997) and Potts, (2000) observed that, cyanophytes can be survive adverse condition of arid desert, as long period of desiccation.

Patternn of algal composition that isolated from this arid desert soil was predominantly by

filamentous, heterocystous cyanophytes as well as some coccal green taxa. In accord with these Hahn and Kusserow, (1998); Chun-Xiang *et,al.*, 2002 and Nayak *et al.* (2004) pointed out that these members of blue-green and green algae usually characterized the soil algae of arid and semiarid regions. The relatively high nitrogen content was associated with the occurrence of heterocystous nitrogen fixing forms of blue-green algae. This in agreement with the observation of Hahn and Kusserow, (1998) and Singh *et al.* (2008).

Eco-phycological analysis of the idenified algae isolated from the regarded desert arid soil was indicated that, most of the recorded algae were mainly related to xeric cyanophytes belonging to *Nostoc, Anabeana, Cylindrospermum, Calothrix, Camptylonema, and Scytonema* algal genera such as, *Stratonostoc commune* (Vauch) Elenk, *Amorphonostoc palodusum f. longius*, *Stratonostoc linckia f. calcicola* (Breb.) Elenk, *Anabeana variabilies f. rotundospora* Hollerb, *Anabeana thermalis f. rotundospora* Aptek. *Cylindrospermum alatosporum* F.E.Fritsch, Calothrix *elenkinii* Kossinsk, *Camptylonema danilovii* Hollerb and *Scytonema drilosphon* (Kutz.) Elenk.et V.Poljansk.

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Management of the Root-Knot nematode, *Meloidogyne incognita* on Tomato in Egypt

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Abstract: The efficacy of carbofuran at 1 mg a.i./kg soil, *Serratia marcescens* (1 x 10^9 bacterium cells/ml water) at 2 ml of the suspension/kg soil, and three different *Trichoderma harzianum* isolates each separately added at 50 ml./kg soil against the root-knot nematode *Meloidogyne incognita* on two tomato cultivars Super Strain B and Alisa was assessed in the glasshouse. Fresh and dry weight of shoots were higher ($P \leq 0.05$) in nematode-free plants of the two cultivars than both *M. incognita*-infested plants and the above-mentioned treatments. Carbofuran followed by *S. marcescens* and *T. harzianum* generally decreased nematode development and reproduction parameters compared to the untreated control. Although chemical nematicide viz. carbofuran showed a significant effect in increase of growth parameters and in suppression of *Meloidogyne incognita* multiplication, it can be replaced to some extent by microbial antagonists viz. *Serratia marcescens* and *Trichoderma harzianum* isolates to comply with environmental issues confronting the use of chemicals. Our results revealed differences in activities of peroxidase and chitinase related to the above-mentioned treatments on both local (in roots) and systematic (in leaves) levels but late in the growing season as well. [Journal of American Science 2010;6(8):256-262]. (ISSN: 1545-1003).

Keywords: enzymatic induction, *Meloidogyne incognita*, nematode management.

1. Introduction

Root-knot nematode (*Meloidogyne* spp.) infestations on tomato (*Solanum lycopersicum*) are common in Egypt and worldwide and cause severe crop damage especially in light soils (e.g., Abd-Elgawad and Aboul-Eid, 2001; Netscher and Sikora, 1990). Root-knot nematodes can be managed effectively by chemical treatments but many of the nematicides are expensive, pose human and environmental risk or have been withdrawn from use (Greco *et al*., 1992; Abd-Elgawad, 2008). Management of root-knot nematodes with biological control agents has been receiving growing consideration (Abd-Elgawad and Aboul-Eid, 2005, Al-Hazmi *et al.*, 2010). Hence, the present study identified five treatments: a nematicide carbofuran, a bacterium *Serratia marcescens*, and three Egyptian isolates of a fungus *T. harzianum* with an end in view that a clear comparative line could be drawn distinguishing the significance between previously used chemical control with its implied dangers on man and environment and that of biocontrol measure represented by fungi and bacterium aiming at reaching the best policy to be implemented through comparing the five control methods with the untreated growing of two common tomato cultivars; in the presence or absence of the nematode. Since it is fundamental to have prior knowledge of the interaction involved especially in the biocontrol treatments, some enzymatic activities in leaves and roots of tomato plants were assayed.

2. Material and Methods

Pure culture of three Egyptian *Trichoderma harzianum* isolates were provided by The Center of Fungi, Assiut University, Egypt and maintained on Potato Dextrose Agar in Petri plates at 27 ± 5°C. The isolates were designated as f_1, f_3 and f_8 and cultured on healthy sorghum seeds as described by Haseeb *et al* (2005) so that the surface of the seeds was colonized and colony forming units (CFU) reached above to 10^8 CFU/gm culture. Also, eggs were obtained by separation with sodium hypochlorite (0.5%, 1 minute) of *Meloidogyne incognita* egg masses cultured on roots of tomato cv. Strain B plants, and active second stage juveniles (J_2) by egg mass incubation in tap water at 27 ± 1°C in the dark. J_2 were collected after 3 days and concentrated in small volumes of sterilized water by filtering through 1 µm filters (Whatman type) and collecting them after repeated washes. The effect of *T. harzianum* f_1, f_3 and f_8 on egg hatch and J_2 motility of *M. incognita* was tested by in vitro assays similar to the methodology of Suarez *et al.* (2004). Crude culture filtrates (10^6 CFU/ml) of the above-mentioned fungal isolates, compared to sterile deionized distilled water as a check, were added to equal amount (2 ml) of a suspension having about 200 eggs or J_2 of *M. incognita*. Each treatment, had 20 replicates (5-cm Petri dishes), was tested at 25 ± 1°C in the dark and gently shaken every 12 h for one week. Numbers of J_2 that emerged from eggs and immobile J_2 were counted in 1 ml sample from each

dish twice; after 3 and 7 days. Each time, J_2 were transferred in aerated distilled water and then the active nematodes were counted after a day.

Seeds of two tomato cultivars, Super Strain B and Alisa, were germinated in small (3.5-cm diameter) foam wells filled with sterilized peat and a single seedling per well of 11x19-well germination trays and allowed to grow up to the four-true-leaf stage. Afterwards, seedlings were thoroughly washed with tap water and singly transplanted into 140 20-cm-diameter earthen pots filled with a mixture of autoclaved sandy loam soil (sand 68, silt 24% and clay 8%, pH 7.6) and compost (4:1, 2.5 kg/pot) in a greenhouse (26 ± 5 °C and 61% ± 12% Relative Humidity). Plants were periodically watered with Hoagland's nutrient solution. Nematode-treated pots were inoculated with a suspension containing 1000 ± 5 of the J_2 poured in three holes around the plant stem. *T. harzianum*-treated pots received 125 ml/pot of about 10^6 germinated spores/ml of mycelial inoculum gently mixed with soil. In the early morning, ten days after transplanting and immediately after *M. incognita* inoculation, five treatments for each of the two tomato cultivars consisted of adding 4 ml of Nemaless (a commercial suspension of *Serratia marcescens* having 1 x 10^9 bacterium cells/ml water) per pot; mixing carbofuran with soil around plant roots at 1 mg a.i./kg soil; and similarly applying the three fungal isolates separately. Additional two untreated checks for each of the two tomato cultivars included only *M. incognita* inoculation and uninoculated control. The pots were arranged in a randomized complete block design with 20 replicates (pots) and the experiment was repeated once. Data were pooled together for statistical computation.

For enzymatic assays, in each experiment, three seedlings, from each treatment were uprooted 56 days after nematode inoculation. Then the leaves and roots were thoroughly rinsed with tap water, excised from the plants, and separately dried, weighed and kept at -20°C until examined. The roots or leaves were placed in chilled mortars and reduced to powder by grinding after immersion in liquid nitrogen. The powder was homogenized in 1 ml (per 1 g powder) of an acid buffer (84 mM citric acid, 32 mM Na_2HPO_4, and 14 mM 2-mercaptoethanol, pH 2.8). The homogenate was centrifuged at 10,000 rpm for 30 min at 4°C. Total protein content and enzyme activity were determined in the supernatants. Three replicates representing three tomato plants per treatment were used for each test. The method described by Bradford (1976) was adopted to determine protein content. Peroxidase activity was determined spectrophotometrically by measuring the increase in absorbance at 470 nm due to oxidation of guaiacol according to Lee (1973). One unit of enzyme activity was defined as the amount of enzyme that causes 1.0 Optical density (O.D.) min^{-1} change under standard assay conditions. Specific activity was expressed in units mg^{-1} protein. For chitinase assay, the substrate colloidal chitin was prepared from chitin powder according to the method described by Ried and Ogryd-Ziak (1981) and reducing sugar was determined in 1 ml of the supernatant by dinitrosalicylic acid (Monreal and Reese, 1969) using 1 ml of 1% colloidal chitin in 0.05 M citrate phosphate buffer (pH 6.6) in test tubes, 1 ml of enzyme extract was added and mixed by shaking. Chitinase activity was similarly assayed in crude culture filtrates, 6 replicates, of each fungal isolate. Tubes were kept in water both at 37°C for 60 minutes, then cooled and centrifuged before measuring O.D. at 540 nm. Chitinase activity was defined as mM N-acetylglucose amine equivalent released/gram fresh weight tissue/60 minutes.

For nematode-tomato interaction study, the remaining pots were maintained for 60 days after *M. incognita* inoculation; then, the tops of the tested plants were cut off and the roots gently washed free of soil. Fresh and dry weights and lengths of the shoot and root systems as well as nematode galls on roots were counted and rated on a 0—5 scale, known as gall index (Taylor and Sasser, 1978). Figures of egg hatching, J_2 motility, plant growth parameters, protein content and enzyme activities were subjected to analysis of variance and their averages were compared using Duncan's New Multiple Range Test. Final nematode population was counted (Abd-Elgawad and Mohamed, 2006) and divided by 1000 (initial nematode population) to calculate the reproduction factor (RF).

3. Results

The three fungal isolates inhibited ($P \leq 0.05$) egg hatching and J_2 motility of *Meloidogyne incognita* (Table 1). The isolate f_1 had the highest influence. The percentage of J_2 immobility increased with increase of the exposure period. When the J_2 were transferred to water and allowed to recover 13, 11, and 12% of immobile J_2 exposed to f_1, f_3, f_8, respectively were reversible. Differences among such percentages were not significant ($P \leq 0.05$).

Environmental conditions in the greenhouse during the experiment were suitable for both tomato plant growth and nematode infestation and reproduction. So, *Meloidogyne incognita* adversely affected ($P \leq 0.05$) the plant growth parameters of the two cultivars in all treatments compared to uninoculated checks (Tables 2 and 3). This was mainly reflected by fresh and dry weights of plant shoot systems. Apparently, nematode galls

Table 1. Percentage egg hatching and second stage juvenile (J_2) motility of *Meloidogyne incognita* as exposed to three *Trichoderma harzianum* isolates f_1, f_3 and f_8 and distilled water (SDDW) for one week.

Treatment	f_1	f_3	f_8	SDDW
Eggs	20 % c	27% b	31% b	38% a
J_2	59% b	62% b	61% b	76% a

*Average of 20 replicates. Averages in a row sharing a common letter are not significantly ($P \leq 0.05$) different according to Duncan's New Multiple Range Test.

Table 2. Effect of *Trichoderma harzianum*, *Serratia marcescens* and carbofuran on galling scores and reproduction factors of *Meloidogyne incognita* on tomato cv. Super Strain B and plant growth parameters*.

Treatments	Gall Score**	RF+	Fresh weight(gm)		Dry weight(gm)		Length (cm)	
			Shoot	Root	Shoot	Root	Shoot	Root
Nematode (N) only	4.5	1.9	19.6 e	3.1	12.8d	0.8	86.7 c	13.6 b
***T. harzianum* f_1+N**	2.8	0.7	40.3 c	5.2	15.0c	1.3	108.9 b	17.8 a
***T. harzianum* f_3 +N**	3.4	0.9	30.7 d	3.9	14.1cd	0.9	111.0 b	13.4b
***T. harzianum* f_8+N**	3.4	0.8	29.0 d	4.3	13.8cd	1.0	102.5 b	15.1b
Carbofuran +N	2.3	0.6	56.0 b	5.4	17.5b	1.7	116.4ab	13.8b
***S. marcescens* +N**	2.8	0.8	44.4 c	3.5	15.3c	0.9	126.4 a	13.7b
Control	0.0	-------	66.5 a	4.3	21.7a	1.0	127.3 a	18.4a

*Average of 34 replicates. Averages in a column sharing a common letter are not significantly ($P \leq 0.05$) different according to Duncan's New Multiple Range Test.
** Gall index was scored on each plant on a 0-5 basis with 0 = no galls or eggmasses (galls), 1= 1 or 2 galls, 2 = 3-10, 3 = 11-30, 4 = 31-100, and 5 = >100 galls.
+Reproduction factor (RF) = Nematode final population / Nematode initial population.

Table 3. Effect of *Trichoderma harzianum*, *Serratia marcescens* and carbofuran on galling scores and reproduction factors of *Meloidogyne incognita* on tomato cv. Alisa and plant growth parameters*. 0.05) different according to Duncan's New Multiple Range Test.

Treatments	Gall Score**	RF+	Fresh weight(gm)		Dry weight(gm)		Length (cm)	
			Shoot	Root	Shoot	Root	Shoot	Root
Nematode (N) only	3.9	1.4	31.0 d	5.1	13.6 d	2.7	83.6 c	15.5c
***T. harzianum* f_1+N**	2.9	0.8	49.9 bc	7.3	25.1bc	3.7	107ab	20.8b
***T. harzianum* f_3 +N**	3.0	0.9	48.0 c	7.2	24.8bc	3.5	104.9b	21.3b
***T. harzianum* f_8+N**	3.1	1.0	45.8 c	6.9	21.4 c	3.4	99.5b	21.8b
Carbofuran + N	2.2	0.4	58.6 b	5.4	30.8 b	2.5	115 a	19.3b
***S. marcescens* +N**	2.9	0.9	52.8 bc	5.6	27.2bc	2.6	116.2a	26.5a
Control	0.0	-----	74.5 a	10.3	41.7 a	5.5	123.9a	27.1a

*Average of 34 replicates. Averages in a column sharing a common letter are not significantly ($P \leq$
** Gall index was scored on each plant on a 0-5 basis with 0 = no galls or eggmasses (galls), 1= 1 or 2 galls, 2 = 3-10, 3 = 11-30, 4 = 31-100, and 5 = >100 galls.
+Reproduction factor (RF) = Nematode final population / Nematode initial population.

formed on infected roots could confuse their weights as a dependable growth parameter for comparison. Nematode-gall index, of untreated inoculated plants, was higher on roots of tomato cv. Super Strain B than cv. Alisa indicating that the first cultivar is probably more susceptible to the tested nematode population. Generally, maximum increase in all the growth parameters of the two tomato cultivars was found in carbofuran treated plants followed by *S. marcescens*, *T. harzianum* f_1, *T. harzianum* f_3, *T. harzianum* f_8 treated plants as compared to untreated inoculated plants. No galls were found on roots of the control plants demonstrating no nematode contamination. Nematode final population did not increase over their initial populations in all treatments but the reproduction factor exceeded one in untreated inoculated plants of the two cultivars.

Protein content, chitinase and peroxidase activities in leaves and chitinase and peroxidase activities in roots of the two tomato cultivars are shown in tables (4 and 5), respectively. Protein content in plant leaves of tomato cvs Super Strain B and Alisa ranged 4.22 - 19.31and 4.94 - 6.84 mg/gm fresh weight, respectively. It was significantly ($P \leq 0.5$) higher only in *T. harzianum* f_8-treated plants than in other treatments and controls of the cultivar Super Strain B but no significant difference was detected concerning protein contents of tomato cv. Alisa plants. Chitinase activities in plant leaves of cv. Super Strain B-treated plants were generally higher than those of Alisa plants. *T. harzianum* f_1-treated plants demonstrated the highest ($P \leq 0.05$) activity. Uninoculated Super Strain B plants had the lowest ($P \leq 0.05$) level of chitinase activity. No significant ($P \leq 0.05$) difference in chitinase activity was found among treatments of Alisa cultivar. Also, chitinases assayed in the crude culture filtrates of the three *T. harzianum* isolates f_1 f_2 and f_8 were 1.82, 0.78 and 1.98 mM N-acetylglucose amine equivalent released / gram filtrate / 60 minutes, respectively. Differences in peroxidase activities in leaves of the two tomato cultivars at 56 days after nematode inoculation were not significant. Yet, such a difference was significantly ($P \leq 0.05$) less in the roots of *T. harzianum* f_1+nematode-treated cv. Alisa plants than the control in the presence or absence of the nematode (Table 4). Chitinase in the roots of *T. harzianum* f_1+ nematode-treated cv. Alisa plants was significantly ($P \leq 0.05$) less than those of untreated inoculated plants.

Table 4. Protein content, chitinase and peroxidase activities in leaves of two tomato cultivars grown in soil treated with *Trichoderma harzianum*, *Serratia marcescens* or carbofuran and/or *Meloidogyne incognit* as compared to untreated plants*.

Treatments	Tomato cv. Super Strain B			Tomato cv. Alisa		
	Protein	Chitinase	Peroxidase	Protein	Chitinase	Peroxidase
Nematode (N) only	5.91 b	10.12 bc	0.15	5.75	7.94	0.106
***T. harzianum* f_1+N**	4.56 b	25.74 a	0.19	6.27	5.63	0.043
***T. harzianum* f_3 +N**	7.96 b	11.0 bc	0.17	4.94	7.34	0.069
***T. harzianum* f_8+N**	19.31a	12.25 bc	0.16	6.28	6.30	0.060
Carbofuran + N	8.07 b	10.12 bc	0.19	6.57	8.30	0.087
***S. marcescens* +N**	8.53 b	15.31 b	0.24	5.80	11.93	0.082
Control	4.22 b	7.83 c	0.25	6.84	9.44	0.169

*Average of six replicates. Averages in a column sharing a common letter are not significantly ($P \leq 0.05$) different according to Duncan's New Multiple Range Test.

Table 5. Chitinase and peroxidase activities in roots of two tomato cultivars grown in soil treated with *Trichoderma harzianum* f_1 and *Meloidogyne incognit* as compared to controls in the presence or absence of the nematode *.

Treatments	Tomato cv. Super Strain B		Tomato cv. Alisa	
	Chitinase	Peroxidase	Chitinase	Peroxidase
Nematode (N) only	1.90	0.042	2.41 a	0.25 a
***T. harzianum* f_1+N**	1.92	0.037	1.23 b	0.04 b
Control	1.31	0.038	1.91 ab	0.17 a

*Average of six replicates. Averages in a column sharing a common letter are not significantly ($P \leq 0.05$) different according to Duncan's New Multiple Range Test.

4. Discussion

Although chemical nematicides, like carbofuran, demonstrated high effectiveness ($P \leq 0.05$) against the root-knot nematodes (Tables 2 and 3), biological control of plant-parasitic nematodes by microorganisms such as *Serratia marcescens* and *Trichoderma harzianum* has been considered a more natural and environmentally acceptable alternative to such chemicals (Suarez *et al*., 2004, Abd-Elgawad and Mohamed, 2006). Thus, the overall goal of such biocontrol agents is the identification and deployment of highly effective strain(s) against several plant pathogenic fungi and/or nematode pests before their development into registered, ready-for-sale plant protection products. Admittedly, strains of *T. harzianum* are able to antagonize numerous phytopathogenic fungi (e.g., Harman, 2000, Hajieghrari *et al*., 2008) and suppress *Meloidogyne* spp. as the most economically important group of phytonematodes worldwide (Rao *et al*., 1996, Sharon *et al*., 2001, Spiegel and Chet, 1998, Windham *et al*., 1993, Suarez *et al*., 2004). Commercial products of *T. harzianum* are available (Harman, 2000) but local ones may be more adaptive and less expensive without any risk to Egyptian fauna and flora than imported strains. So, the relatively high efficacy demonstrated by *T. harzianum* f_1 (Tables 1-5) may nominate it for further experimentations and development. Increase in the efficacy of the fungi appears possible when such biocontrol agents are integrated with organic amendments such as oil cakes (Parvatha Reddy *et al*., 1996) and wheat bran-peat preparations (Sharon *et al*., 2001). Eventually, although chemical nematicide viz. carbofuran showed a significant effect in increase of growth parameters and in suppression of *Meloidogyne incognita* multiplication, it can be replaced to some extent by microbial antagonists viz. *Serratia marcescens* and *Trichoderma harzianum* isolates to comply with environmental issues confronting the use of chemicals.

The mechanisms by which strains of *T. harzianum* function against phytopathogenic fungi are mycoparasitism, antibiosis, competition for nutrients or space, tolerance to stress through enhanced root and plant development, induced resistance, solubilization and sequestration of inorganic nutrients, inactivation of the pathogen's enzymes or/and enzymatic hydrolysis (Sivan and Chet, 1992, Harman, 2000). All mechanisms, except competition, can potentially be involved in the nematode biocontrol process (Sharon *et al*., 2001). Understanding the possible mechanisms of this fungal activity against nematodes could lead to the development of improved biocontrol application methods and selection of active isolates. Direct interactions between *T. harzianum* and the potato cyst nematode *Globodera rostochiensis* were demonstrated in vitro by Saifullah and Thomas (1996). The fungus penetrated the cysts and the eggs in those cysts, resulting in larval death. Two mechanisms were suggested against the root-knot nematodes: effect of metabolites produced by the fungus in the soil and direct parasitism by the antagonist (Sharon *et al*., 2001). Our visible observations by the light microscopy (Table 1) support such suggestion since many non-viable and distorted eggs were found in the fungal treated dishes.

In leaves of tomato cvs Super Strain B and Alisa, significant ($P \leq 0.05$) differences were observed among treatments only in the protein content and chitinase activities of the first cultivar (Table 4). So, such differences could be cultivar specific but also could be the result of plant-microorganism interaction. Also, differences in peroxidase and chitinase activities were pronounced in roots of only Alisa cultivar (Table 5). Yet, peroxidase activities had the highest level in roots of another susceptible tomato cultivar 56 days after *M. incognita* inoculation as well (Mohamed *et al*., 2003).

It is well known that defence reactions in Solanaceae depend primarily on the type of elicitor, on the plant genotype/species, or on the type (compatible or incompatible) of interaction (Desender *et al*., 2007). Indeed, in tomato roots infected with root-knot nematodes, genes with homology to several known plant defense genes including peroxidase and chitinase are induced locally within 12 h of inoculation (Williamson and Hussey, 1996) but systematically when invaded by *T. harzianum* (Yedidia *et al*., 1999). Defence gene transcription or enzyme activity is, most of the time, delayed and lower in compatible (susceptible plant) than in incompatible (resistant plant) interactions. Our results revealed such enzyme activities on both local (in roots) and systematic (in leaves) levels but late in the growing season as well (Tables 4 and 5). Yet, the comparison of reaction patterns highlighted by Desender *et al*. (2007) suggested that these patterns are as a rule specific to each plant genotype/elicitor pair, irrespective of the compatibility/incompatibility status of the interaction. Hence, our present study characterized such patterns of two genotype/elicitor pairs (Tables 2-5). Additional studies are needed to clarify the interaction of *Meloidogyne* spp. with *T. harzianum* as a biocontrol agent in terms of the physiological roles of enzyme activities in response to nematode attack and fungal colonization.

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IR Spectroscopic Analysis of Polymorphism in Diphenyl Carbazide

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Abstract: IR analysis is used here to investigate the changes in N-N, N-H, C=O …modes of thermally treated diphenyl carbazide (DPC) during the variation of temperature from room temperature up to ≈ 160^{o}C. Polymorphism in DPC compound has been studied here by detecting the changes in some IR spectroscopic parameters (e.g., mode shift, band contour…) during the elevation of temperature. Also, DSC, X-ray, NMR and atomic mass spectra are used as confirming tools for what is obtained by IR. All of the vibrations of DPC were found to be due to ionic fundamentals 3311 cm^{-1}, 3097 cm^{-1}, 3052 cm^{-1}, 1677 cm^{-1}, 1602 cm^{-1}, 1492 cm^{-1}, 1306 cm^{-1}, 1252 cm^{-1}, 887 cm^{-1} and 755 cm^{-1}. The results revealed for the first time that the thermally treated DPC traverse four different phase transformations at 50^{o}C, 90^{o}C, 125^{o}C and 140^{o}C. The crystal structure was found to be amorphous, monoclinic, tetragonal, orthorhombic and amorphous within a temperature range (30^{o}C-160 ^{o}C). X-ray diffraction patterns support the results obtained by IR and DSC. [Journal of American Science 2010;6(8):263-270]. (ISSN: 1545-1003).

Keywords: Diphenyl carbazide; FT-IR, Phase transformation
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1. Introduction

Diphenyl Carbazide $C_{13}H_{14}N_4O$ is one of the important organic compounds mainly used as an artificial donor material. It is frequently utilized in analytical chemistry for colorimetric determination of chromium and as a sensitive reagent for metal ions (Hg and Cd) [1-6]. Diphenyl Carbazide (DPC) has an orthorhombic structure with space group pbnm with a = 5.7171 A^o, b = 8.4121 A^o and c = 25.6982 A^o. The two phenyl hydrazide groups lie on either side of a crystallographic symmetry plane passing along the bond direction of the carbonyl group [7]. The present work is considered as an extension of our previous work on DPC in which DTA, DSC and electrical measurements were carried out [8, 9]. Previous work on DPC compound showed the presence of an exothermic phase transformation at ≈ 90^{o}C. Nowadays and after more than twelve years, instrumental resolution, sensitivity and accuracy have been widely improved. Besides that, most of the techniques have been computerized. So it is very interesting and promising to perform thermal scanned programs for studying the effect of heat treatment on the spectroscopic properties of heat treated DPC. Here DPC is subjected to an IR spectroscopic analysis under conditions similar to that performed previously in thermal and electrical study [9]. The nature of the temperature dependence of the vibrating groups is of great interest as its role is fundamental in explaining many of the physical properties of this complex organic compound.

We reported here the important IR changes accompanying new polymorphic transformations in DPC during controlled heat treatment of the samples. Also, a thermal histogram will be suggested for describing what happened in DPC during transformation to several polymorphic states.

Beside these IR analysis, X-ray diffraction, NMR analysis and mass spectroscopy are used here for several purposes. The present study aims to reveal the presence of several phase transformations in DPC (not only one) during heat treatment between room temperature and 160^{o}C.

2. Material and Methods

Experimental

Diphenyl Carbazide powder (ultra pure) was obtained from the British Drug House (BDH), Laboratory Chemical Division, England. IR spectra were recorded using the FT-IR type Perkin Elmer recorded on satellite 2000 spectrometer and the experimental technique employed has been reported [10]. The samples used were in the form of discs prepared by mixing 20 mg of DPC with one gram of KBr in a cylindrical die of 10 mm diameter. The die was then evacuated to ensure dryness and the sample was pressed. The spectra were recorded in the region of 400- 4000 cm^{-1} by a computerized system attached to a monitor and a printer. NMR analysis was obtained by a Varian instrument (Gemim 20, 200 MHz) using DMSO solvent. The mass spectra of heat treated DPC were studied using a Quaderpole mass spectrometer (type GCMS – QP 1000 EX, Shimadzu, Japan). The ions are created by electron impact (EI)

in the ion source at 70 eV. The working in vacuum, which depends on the vapor pressure of the sample, was of the order 10^{-4} Torr. The temperature of the ion source was about 250^{o}C. X-ray diffraction patterns are carried out by X-ray machine type, Bruker axs D8, Germany) with Cu-K_{α} (λ = 1.5406 A^{o}) radiation and secondary monochromator in the 2θ range from 20 to 70^{o}. The program which is used in this study is crysfire 2002 interactive powder indexing support system. Crysfire assists to find possible solutions to the powder indexing problem, in the form of complete or partial unit cells.

3. Results

In a previous study on DPC (amorphous) including thermal and electrical properties, it was found that DPC thermograms clearly indicate to a probable phase changes at 50 ^{o}C, 90 ^{o}C, and 140^{o}C, Fig. 1. Also, obvious sudden change has been observed in electrical properties (dielectric constant, d.c resistively, pyroelectric current…) at 125^{o}C. So, according to both of the two techniques, probable polymorphs at 50^{o}C, 90^{o}C, 125^{o}C and 140^{o}C in DPC can be the most probable. IR spectroscopic analysis could be used here to confirm the polymorphism in thermally treated DPC. The spectral change accompanying the polymorphic transitions are obtained from 400 cm^{-1} up to 4000 cm^{-1} for original samples of DPC and thermally treated (previously melted DPC) from room temperature (R.T ≈ 30^{o}C) up to ≈ 160^{o}C melting point (m.p of DPC). IR analysis used here covered this temperature range.

Fig. 2 shows the IR spectra of DPC samples at room temperature (before heat treatment Fig. 2A), and after melting and cooled to room temperature, Fig. 2B. One can easily observe a slight change in the mode frequencies between spectra A and B. Also, there is a remarkable variation in the transmittance. This may be due to a change in the crystal structure after melting. According to our previous work on DPC [8-11], the crystal structure of the R.T phase of DPC (before heat treatment) is orthorhombic while that after heat treatment is amorphous. The normal modes of vibrations of these two phases are due to ionic fundamentals and given in Table (1). The bands obtained are due to ionic fundamentals of N-H, C-H, N-N and C=O.

The nature of temperature dependence of vibrating groups is of great interest since its role is fundamental in explaining many of the physical properties of this organic compound. The spectrum shown in Fig. 2A, indicates that the strongest bands are at 3359 cm^{-1} and 3276 cm^{-1}. In the amorphous phase Fig. 2B, the strongest band is that at 3311 cm^{-1}. It is due to the asymmetric stretching of N-H, while the mode 3097 cm^{-1} indicates the symmetric stretching of N-H. The aromatic C-H stretching mode appears here at 3044 and 3052 cm^{-1} for both orthorhombic and amorphous phases respectively. The variation of the band contours of the three modes 3311, 3097 and 3052 cm^{-1} at temperatures 30, 80, 110, 130 and 150^{o}C are those corresponds to different phase states of DPC. As shown in Fig. 3, the frequency and shape changes of these modes at the different temperatures is very good indication of the polymorphic changes in DPC during heating.

Also, it is well known that the distinct shape of a transmittance band for a particular molecule relates to the number of ions that undergo the various energy transitions. Thus, the most intense absorbance line is that which represents the largest number of ions undergoing a particular transition and the band envelope as a whole is an indication of the total number of ions involved. As the temperature of ions increases the band contour will change. Fig. 4 shows detailed variation of the band contours of bending mode of N-H, symmetric and asymmetric stretching vibrations of N-N as DPC temperature increased from 30^{o}C to 160 ^{o}C. The change in mode frequency here is very little, but the change of the mode shape is very clear. The band contour also change systematic during the temperature elevation and accompanying the successive phase variation in DPC. Fig. 5 shows the band contour variation of C-H stretching vibrations and C=O stretching vibration during polymorphic change. As clear, the mode shift is very little, but the change in band shape is obvious.

Fig. 6 shows the variation of the bond contour of the out of plane C-H bending mode during the elevation of temperature between 30^{o}C and 160 ^{o}C. Here the change in band shape is very clear. The out of plane C-H bending mode is very sharp one. Its peak height changes alternatively during phase changes. This indicates that the C-H group may be responsible for phase change, i.e., the orientational motion of this group in space is responsible for phase transition in DPC.

Fig. 7 shows the variation of the band contour of monosubstituted benzene during a temperature variation from 30^{o}C up to 160^{o}C. The change in mode frequency and band shape for this band is very limited.

The effect of heating on ten modes of vibrations of DPC is summarized in Table (2). This effect is clearly notified in the first two stretching modes of vibrations of N-H (Asymmetric and Symmetric). In the other modes of vibrations, the effect of heating seems to be little. Such behavior of N-H group indicates that N-H group may be responsible for the polymorphism in DPC. This means that the orientational motion of N-H in space may control the phase state of DPC. An important

question arises now, after these five polymorphic transitions, what is the molecular formula of the final compound? Is it still DPC or has changed to a new organic compound? To put an answer for such question, NMR and mass spectra of DPC is obtained by electron impact at 70 eV.

Based on the results of the microchemical analysis, Table 3, and Figs. 8 and 9. The general molecular formula of the compound under investigation can be given as $C_{13}H_{14}N_4O$. This means that after all polymorphic transitions, DPC at 50 ^oC, 90 ^oC, 125 ^oC and 140^oC still has the same molecular weight (242), which is the same as the original unheated DPC. But it belongs to another phase modification and new crystal structure. It seems that under the present experimental conditions the formation of this new crystal structures is an end of the thermodynamic phase transformation of the original DPC subjected to heat treatment processes. Fig. 10 showing the effect of heat and reheat treatment of DPC and the resulting polymorphic phase changes.

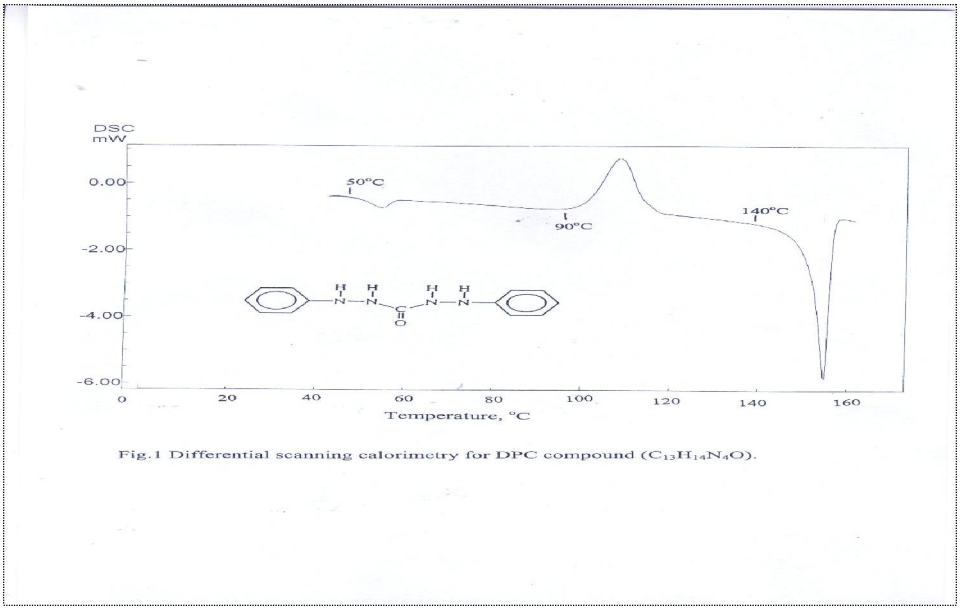

Fig.1 Differential scanning calorimetry for DPC compound ($C_{13}H_{14}N_4O$).

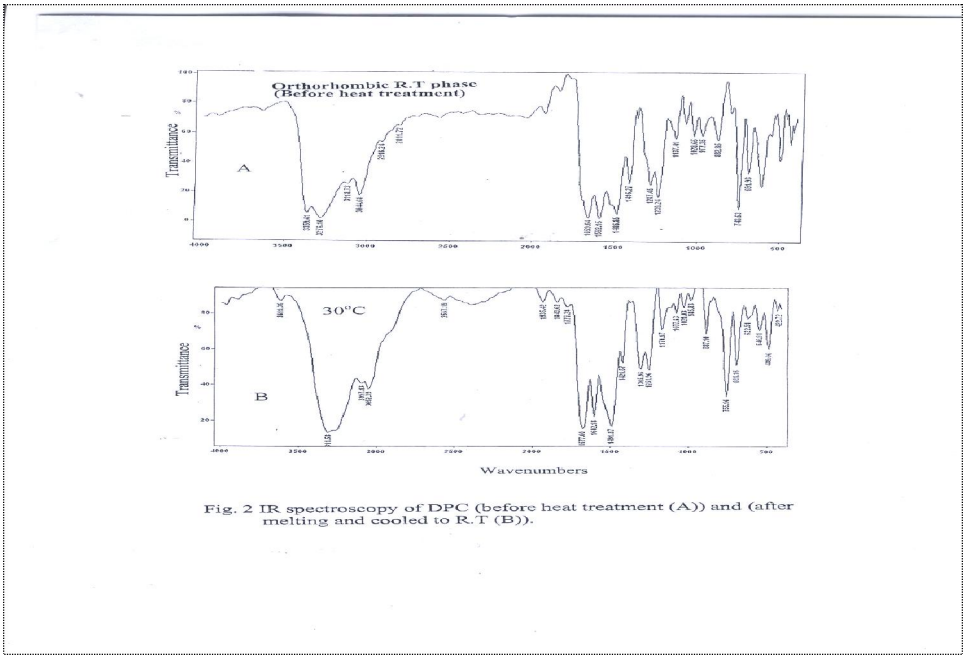

Fig. 2 IR spectroscopy of DPC (before heat treatment (A)) and (after melting and cooled to R.T (B)).

Table (1): Ionic fundamental vibrations of DPC compound before and affect melting

Normal vibrations of DPC due to ionic fundamentals	Orthorhombic DPC at R.T	Amorphous DPC at R.T
Asymmetric stretching of N-H	3359	3311
Symmetric stretching of N-H	3118	3097
Bending or deformation mode of N-H	1659	1677
Aromatic C-H stretching	3044	3053
Out of plane C-H Bending	882	887
N-N stretching symmetric vibrations	1592	1602
N-N stretching asymmetric vibrations	1486	1492
C-H stretching vibrations	1287	1306
C=O stretching vibrations	1239	1252
Monosubstituted benzene	748	756

Table (2) Normal mode variations during polymorphs in thermally treated DPC

	Thermally treated DPC					Untreated DPC
Phase number	Room temperature phase	IV	III	II	I	
Crystal Structure	Amorphous	Monoclinic	Tetragonal	Orthorhombic	Amorphous	Orthorhombic
Temperature	30 °C	80 °C	110 °C	130 °C	150 °C	R.T
Asymmetric stretching mode of N-H	3311	3280	3297	3320	3325	3359
Symmetric stretching mode of N-H	3097	3099	3107	3125	3150	3118
Aromatic C-H stretching mode	3052	3048	3053	3049	3048	3044
Bending mode of N-H	1677	1676	1679	1679	1679	1659
Symmetric stretching mode of N-N	1602	1600	1601	1600	1599	1592
Asymmetric stretching mode of N-N	1492	1488	1489	1489	1489	1486
C-H stretching mode	1306	1300	1302	1301	1299	1287
C=O stretching mode	1252	1248	1251	1249	1248	1239
Out of plane C-H Bending	887	885	886	884	884	882
Monosubstituted benzene mode	755	752	755	755	754	748

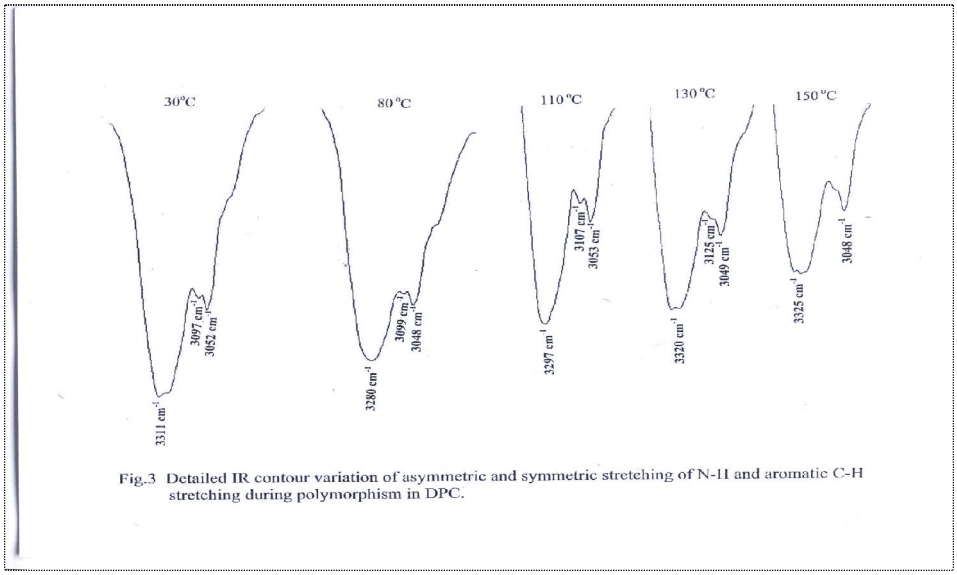

Fig.3 Detailed IR contour variation of asymmetric and symmetric stretching of N-H and aromatic C-H stretching during polymorphism in DPC.

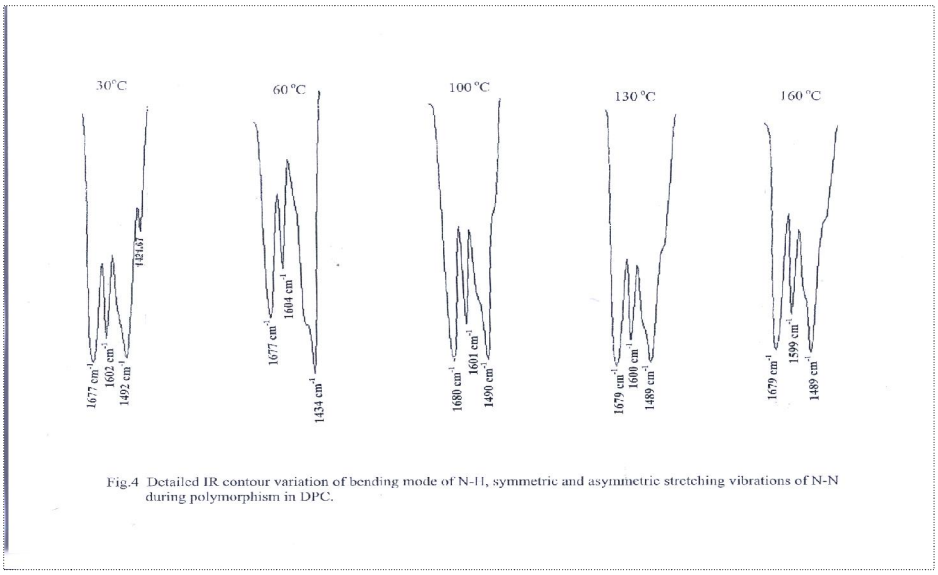

Fig.4 Detailed IR contour variation of bending mode of N-H, symmetric and asymmetric stretching vibrations of N-N during polymorphism in DPC.

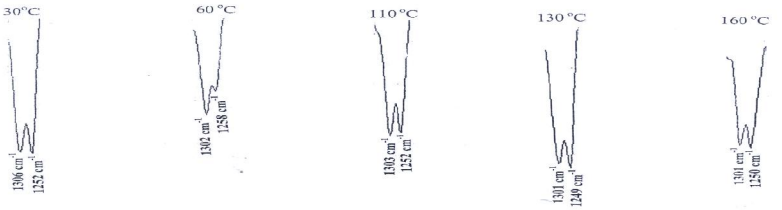

Fig.5 Detailed IR contour variation of C-H stretching vibrations and C—O stretching vibration during polymorphism in DPC.

Fig.6 Detailed IR contour variation of the out of plane C-H bending vibration during the polymorphism in DPC.

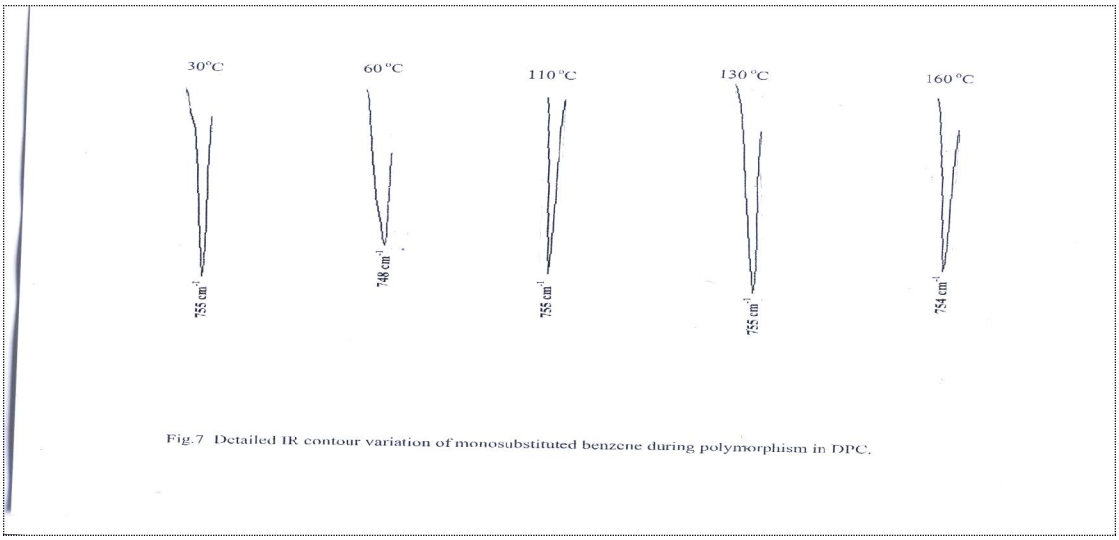

Fig.7 Detailed IR contour variation of monosubstituted benzene during polymorphism in DPC.

4. Discussion

Polymorphic transitions in organic compounds are of particular interest especially for pharmaceuticals and light sensitive compounds. However, polymorphism [12] is the property exhibited by certain substances of changing, at certain temperature, their crystal symmetry class or structure. This is accompanied by changes in physical characteristics [13]. In the framework of phenomenological theory, one can describe the anomalies in thermodynamic quantities and also, determine the nature of the changes that occur in the spectrum of the elementary excitations of the crystal of DPC in the neighborhood of the phase transitions. The order parameter is related to the change that occurs in the positions of the atoms in the crystal lattice during phase transition. The changes in the vibrational (phonon) spectrum of the crystal in the neighborhoods of the critical point of such phase transition are experimentally the most noticeable. A change in structure occurs in a displacive – type phase transition as a result of the displacement of the ions (atoms) in the crystal lattice.

Here the IR spectra of thermally treated diphenyl carbazide are due to lattice vibrations of the individual ions and internal vibrations of molecules. Since the vibrations occur through the lattice and are not concerned with a single unit cell, these vibrations are often observed as broad peaks which represent a composite band of several vibrations. In fact the stability of DPC and its critical properties depends on to what extent changes which occur in the hydrogen bonding of the amino ions (N-H) to the carbonyl (C=O) ions.

In a polyatomic molecule like DPC ($C_{13}H_{14}N_4O$) each atom has three degrees of freedom in three directions which are perpendicular to one another. Consequently a polyatomic molecule like that requires three times as many degrees of freedom as the number of its atoms. In the case of DPC and according to the character of vibration, the normal modes of vibrations of DPC can be divided into two principal groups, stretching vibration and bending vibration. In the first type the atom move essentially along the bond axis, so that the bond length increases or decreases periodically, i.e., at regular intervals. In the second type, there occurs a change in bond angles between bonds with a common atom or there occurs the movement of a group of atoms with respect to the remainder of the molecule without movement of the atoms in the group with respect to one another. The first type appears at high frequencies while the second type appears at lower frequencies. In fact, in a polyatomic molecule like DPC, the same bond can perform stretching and bending vibrations simultaneously.

According to De Ranter et al, DPC has an orthorhombic structure at room temperature. After melting (at ≈ 160°C), DPC transforms to amorphous structure. Now reheat treatment of amorphous DPC cause a set of phase transformations as follows:

(Amorphous DPC at R.T) $\xrightarrow{\textit{Heating up to } 50°C}$ Phase IV (Monoclinic with space group is P21/A)

Phase IV (Monoclinic DPC) $\xrightarrow{\textit{Heating up to } 90°C}$ Phase III (Tetragonal with space group is P4/NNC)

Phase III (Tetragonal DPC) $\xrightarrow{\textit{Heating up to } 125°C}$ Phase II (Orthorhombic with space group is PMNA)

Phase II (Orthorhombic DPC) $\xrightarrow{\textit{Heating up to } 140°C}$ Phase I (Amorphous DPC)

So the effect of heat treatment on DPC is cyclic, i.e., starts with amorphous state and ends with amorphous state. Successive reheat treatment of DPC for several times, repeat the same phase transition cycle and always ends with amorphous state whatever the number of heating cycles passed. To check a behavior like that, an X-ray diffraction pattern for DPC after first heat treatment cycle has been performed, Fig. 11 A and after second heat treatment cycle Fig. 11 B. Both of these two x-ray diffraction pattern indicate amorphous structure for the starting phase and final phase whatever the number of heating cycles passed. Such behavior is summarized in Fig. 10.

Table (3)
The relative abundance for the mass Spectra of DPC by electron impact (70 eV).

S = 39	B = 10	B_1 = 93	B_2 = 37820		RT = 1.91		CT = 133
Mass	Int (%)	Mass	Int (%)	Mass	Int (%)	Mass	Int (%)
50	10.3	51	32.0	52	11.3	58	6.6
54	2.2	55	0.8	60	0.4	57	1.0
59	0.1	60	2.3	61	1.6	62	3.1
63	6.1	64	6.1	65	31.8	66	17.4
67	4.2	69	0.4	70	0.7	71	0.2
73	0.7	74	2.1	75	0.2	76	1.8
77	70.1	78	15.5	79	5.1	80	4.4
81	1.4	82	0.6	83	0.7	84	0.5
85	0.1	87	1.0	88	0.2	90	1.1
91	8.4	92	32.7	93	100.0	94	13.3
95	1.5	97	1.2	103	9.7	106	11.3
107	27.9	108	35.0	109	2.7	110	0.5
111	0.6	117	0.4	120	0.7	121	0.4
133	0.8	134	5.9	135	1.0	149	0.8
150	2.6	151	8.5	152	6.5	153	0.4
167	0.8	169	1.5	240	1.0	242	4.8
243	1.5						

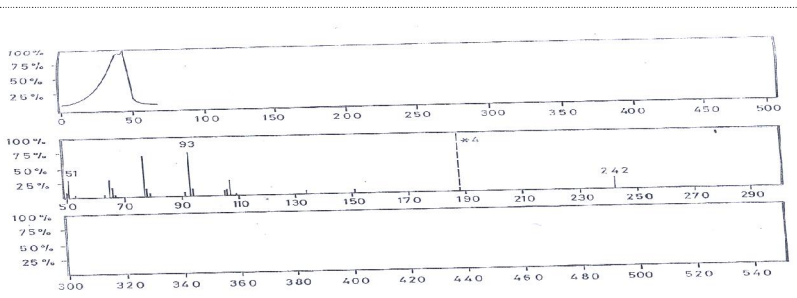

Fig. 8 Mass spectra of reheated DPC at 70 eV.

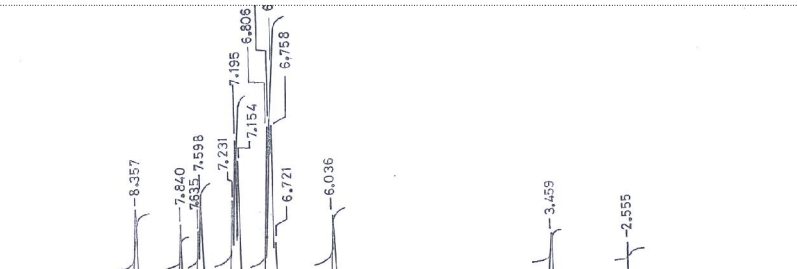

Fig. 9 NMR spectra of reheated DPC.

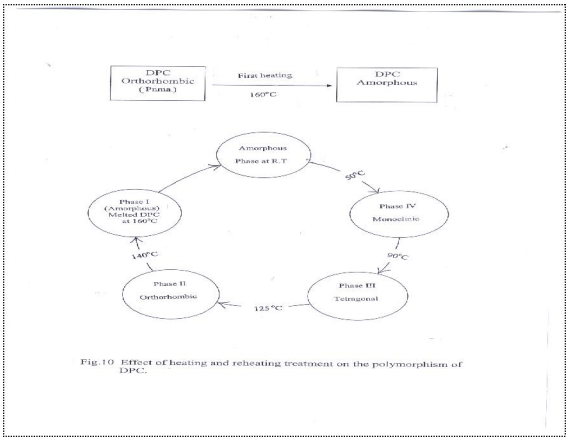

Fig.10 Effect of heating and reheating treatment on the polymorphism of DPC.

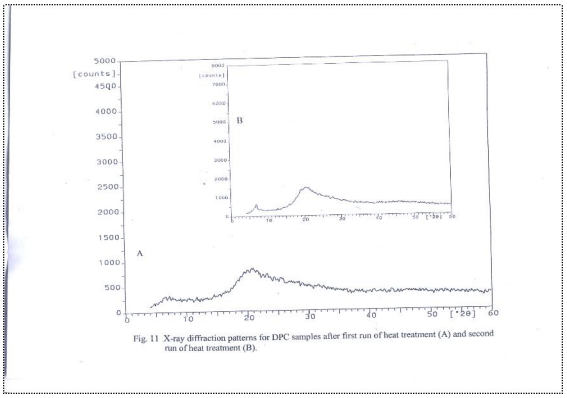

Fig. 11 X-ray diffraction patterns for DPC samples after first run of heat treatment (A) and second run of heat treatment (B).

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A Study the Phase Transformations in Amorphous Diphenyl Carbazide ($C_{13}H_{14}N_4O$)

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Abstract: Thermal and electrical properties of amorphous diphenyl carbazide (DPC) are studied between room temperature and its melting point ≈ 435 K. The study includes a set of measurements DTA, DSC, dielectric constant (ε), electrical resistance (R), the pyroelectric current (I) and X-ray diffraction analysis. The results obtained are strongly supported each other and indicate that the amorphous DPC samples undergo four different phase transitions at 323 K, 363 K, 395 K and 415 K respectively. It is found that each phase transition belongs to a certain definite crystal structure. These crystal structure variations are found to be amorphous, monoclinic, tetragonal, orthorhombic and amorphous within the temperature range of this study. Thermal analysis revealed that the thermal energies for these four different phase transitions are 10.92 J/g, 7.63 J/g, 79.3 J/g and 31.77 J/g respectively. The electrical measurements showed that the conduction in these phases is activated by energies of 0.22, 0.3, 0.16 and 0.47 eV respectively. The first phase transition is attributed to the variation in hydrogen bonding N-H to the carbonyl group C=O, which is leading to a twisted intermolecular charge transfer (TICT) in the temperature region of this phase transition. The second phase transition is mainly due to the weaking of the N-H hydrogen bonding with increasing temperature. Beside that, it may be due to the reorientational molecular motion to another equivalent position. The third phase transition is attributed to a changing in the intermolecular hydrogen bonding with –CO as well as –N-H groups. These changes can greatly affect the charge distribution and in turn affect the transition process (populated locally excited states). The fourth phase transition is due to the weaking of the hydrogen bond of N-H group. [Journal of American Science 2010;6(8):271-277]. (ISSN: 1545-1003).

Keywords: Diphenyl carbazide; Polymorphism, thermal and electrical properties.

1. Introduction

Diphenyl carbazide (DPC) is an organic compound usually used in analytical chemistry for colorimetric measurements. It exhibits many useful properties [1]. It is used as an artificial donor during charge separation in photochemical reactions and also photosynethesis electron transport [2]. It can be used for determination of trace amounts of copper (II) in water matrices [3]. It is also used for determination of trace mercury (II) in waste water [4] and to identify chlorine ions in liquid phase in mineral inclusion where DPC is used as a complexing agent [5]. It is helpful for measuring methods of chromium (VI) in leather and in dyes and determination of trace Cr (VI) and Cr (II) in water [6-8]. It is important in DNA unwinding assay's principle and application where DNA damage prevented by compounds, one of these compounds is diphenyl carbazide which is powerful one [9]. The photophysical characteristics of diphenyl carbazide (DPC) have been investigated in various protic and aprotic polar solvents in which the results showed that DPC behaves like a keto compound than a hydrazide derivative [10]. At room temperature, it has orthorhombic crystal structure and melts at ≈ 435 K and after melting and on cooling DPC transforms to an amorphous structure as detected by X-ray analysis [11] as shown in fig. 1. According to De Ranter et al [12], DPC has an orthorhombic structure with space group pbnm with a = 5.7171, b = 8.4121 and c = 25.6982 A^o.

In a previous work on amorphous DPC, an exothermic phase transition has been previously detected at ≈ 363 K by thermal analysis (DSC and DTA) and electrical measurements [1]. The presence of this phase transition in DPC is strongly confirmed by an accurate infrared study [11]. This complex compound has two phenyl hydrazide groups lie on either side of a crystallographic symmetry plane passing along the bond direction of the carbonyl group fig.1 [1]. The previous measurements which was carried out on this compound by some thermal (DTA & DSC) and electrical (d.c resistance, pyroelectric current and dielectric constants) measurements of DPC samples during the heat treatment runs to investigate the kinetics responsible for variations and the probable phase changes in this organic compound. [1].

In the present work, it is thought that the study of temperature dependence of the variation of d.c resistivity and dielectric constant as well as pyroelectric current in addition to DTA & DSC studies might help in understanding of the

mechanism of each phase transition process in this complex compound.

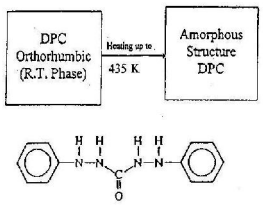

Fig. 1. The structure of the diphenyl carbazide compound.

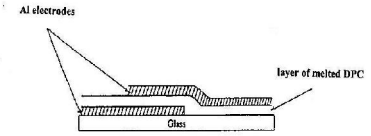

Fig. 2. Schematic diagram of cross section of the sandwich structures.

2. Experimental

The material used in the present work was ultra pure diphenyl carbazide powder $C_{13}H_{14}N_4O$ obtained from PDH, England. For thermal analysis measurements (DTA and DSC) a Shimadzu system type 50 was used to perform high precision thermal analysis. All functions are incorporated into a single compact instrument including detector, temperature, gas control and computer interface. The heating rate used for DTA is 10^oC/min while for DSC is 5^oC/min.

For electrical measurements the samples were prepared in the form of a thin layer. Thin layer of DPC was prepared by melting it on the surface of the glass substrate as shown in fig.2. Aluminum electrodes were used here as a contact terminals of the sample. The electrical measurement (the capacitance C and electrical resistance R), are carried out by an RLC bridge type (Philips 6304). Also, a picoammeter was used for measuring the pyroelectric current (type Keithley 485). X-ray diffraction patterns are carried out by X-ray machine type, Bruker axs D8, Germany with Cu-K_α (λ = 1.5406 A^o) radiation and secondary monochromator in the 2θ range from 20 to 70^o.

3. Results

The results of a set of electrical and thermal parameters measurements of DPC samples are given below in a temperature range between room temperature (303 K) and the melting point (435 K).

Fig.(3a-d) shows the DTA and DSC thermograms for amorphous DPC samples during heating runs starting from room temperature up to ≈ 433 K. The two thermograms indicate a new endothermic phase transition at ≈ 323 K. The thermal energy of transformation is found to be 10.92 J/g, as indicated in fig. (3a). In fig. (3b), one can easily see that an exothermic phase transition has been recorded near ≈ 363 K, and the energy required for such exothermic transformation is found to be 7.63 J/g. Fig.(3c) indicates another new exothermic phase transition takes place at ≈ 395 K with heat of transformation is 79.3 J/g. Finally, fig.(3d) shows a new endothermic phase transition at ≈ 415 K, with energy of transformation process equals to 31.77 J/g.

All of these phase transformations detected here by thermal analysis (DTA & DSC) are also checked by studying the variations in electric and the dielectric properties of amorphous DPC. Fig.(4a-c) shows the temperature dependence of the d.c resistance (R), the dielectric constant (ε) and the pyroelectric current of amorphous DPC during the phase transformation which takes place near ≈ 323 K, as seen in fig.(4a). It is clear that the variation of the sample resistance with temperature showed a minimum value at ≈ 323 K. But with respect to the dielectric constant and the pyroelectric current, the behavior is completely different. Both of ε & I showed maximum values at the critical temperature ≈ 323 K. The behavior of R, ε & I with temperature strongly confirm each other and indicate the presence of a phase transition at ≈ 323 K. The activation energy of such phase transformation has been calculated in the present study and found to be 0.22 eV. It must be noticed here that this phase transformation observed here at ≈ 323 K is accompanied by a change in crystal structure. According to our X-ray measurements, it is found that the crystal structure has been changed from amorphous state to monoclinic crystal structure. This phase transformation is mainly due to the variation in hydrogen bonding (N-H) to the carbonyl group C=O, which is leading to a twisted intramolecular charge transfer (TICT) in the temperature region of this phase transition [13-15].

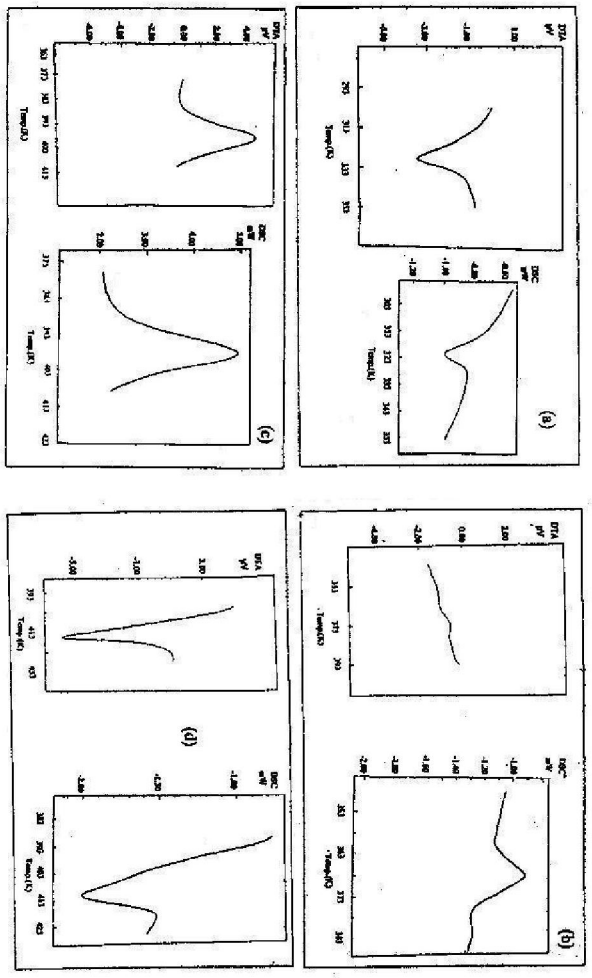

Fig.(3a-d): The DTA and DSC thermograms for reheated DPC through the polymorphism regions.

Fig. (5a-c) shows the temperature dependence of these electric parameters (R, ε and I) as the temperature elevated to ≈ 373 K. One can observe best similarity with fig.(4a-c). These

parameters showed minima for (R) and maxima for (ε &I) at a new critical temperature ≈ 363 K. Such behavior implies the presence of two different phase states before and after 363 K. This conclusion is strongly supported by the results obtained by thermal analysis as seen in fig. (3b). X-ray measurements showed that during this phase transition at 363 K, the crystal structure is changed from monoclinic to tetragonal state. The activation energy of this phase transition process is calculated and is found to be 0.3 eV. Such transition may be due to a change in the hydrogen bonding of amino ions (N-H) to the carbonyl group (C=O) ions. This means that it is possible to attribute this observed transition to the weaking of the N-H hydrogen bonding with increasing temperature. Also the thermal energy during this phase transformation is sufficient to stimulate the reorientational molecular motion to another equivalent position which resulting in the observed structural phase transition [16].

When the DPC sample temperature exceeds 373 K, the electric parameters (R, ε & I) showed new variations with temperature. Maximum and minimum values has been recorded at ≈ 395 K, fig.6 (a, b& c). This critical change with temperature can be considered as a good evidence for the presence of a structure phase change. The X-ray analysis for such phase change indicates a structural change from tetragonal system to orthorhombic state system. However, this phase transition may be mainly due to a change in the intermolecular hydrogen bonding with –CO as well as –N-H groups [17]. These changes can be strongly affect the charge distribution and in turn the transition process (populated locally excited states) and also may be assigned to a TICT state which is generated via twisting in the higher excited state [13-15]. The energy activated this high temperature phase transition is calculated and found to be 0.16 eV.

When the DPC sample temperature approaches the melting point, the three electric parameters (R, ε & I) showed clear anomalies with temperature fig.7 (a, b& c). Each of these parameters denotes a new critical temperature point at ≈ 415 K at which different behavior can be easily observed. Such trend is strongly supported by endothermic peak shown in the DTA & DSC thermograms fig. (3d). According to X-ray analysis, the crystal structure in this case has been changed from orthorhombic to amorphous state. Also, this transition may be due to the weaking of the hydrogen bond of N-H group during the elevation of temperature and hence stimulate the reorientational molecular motion to another equivalent position (due to the sufficient thermal energy) [16]. The activated energy required for this phase transformation is calculated and found to be 0.47 eV. The energy diagram for the conduction process during the different phase transformations is given in fig. 8.

4. Discussions

According to the present investigation of amorphous DPC, all of the obtained results (thermal analysis DTA & DSC and electrical measurements R, ε & I) are clearly confirmed the presence of four different independent phase transformations at 323 K, 363 K, 395 K and 415 K respectively. In fact, the phase transformation at 363 K has been previously detected [1]. But the other three transformations at 323 K, 395 K and 415 K are completely new and detected here for the first time. According to the present analysis, each of these phases belongs to a new a crystal structure. A set of X-ray diffraction curves is obtained for these phases at the corresponding temperatures and the results are given in fig. 9. The study of the heating effect on the physical properties of amorphous DPC samples can provide better understanding of the nature of transition in this organic compound. The associated changes in the interaction potential of the atoms or molecules during the transformation process play an important role in this transition. Near the phase transition point, the probability of reorientations or vibrations of the molecules is considerably changed with increasing temperature. But at the transition point itself, the nature of this motion changes qualitatively, and in turn lead to a change in the characteristic structure of the sample [18, 19].

In fact, the data presented here for the variation of different electric parameters as a function of temperature, showed two completely different regions before and after the phase transition points. This means that there are two independent mechanisms which are operative in the two phase regions. The energy activating values control the electric conduction mechanisms in these four different phases are 0.22, 0.3, 0.16 and 0.47 eV. The largest value corresponds to the highest temperature phase. With respect to the transition at 323 K, this phase changes from amorphous structure to monoclinic structure. It may be due to the variation in hydrogen bonding of N-H to the carbonyl group C=O. This leads to a twisted intramolecular charge transfer (TICT) in the temperature region of such phase transition (≈ 323 K) [13-15].

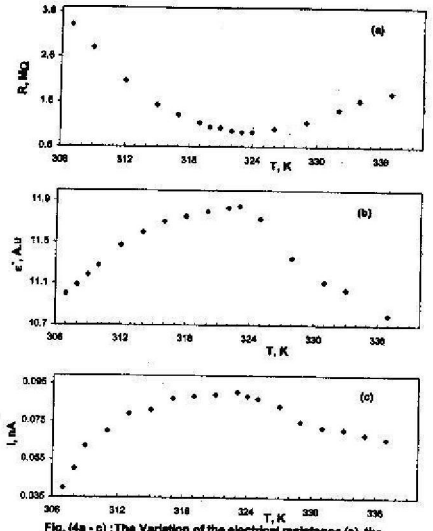

Fig. (4a - c) : The Variation of the electrical resistance (a), the dielectric constant (b) and the pyroelectric current (c) with temperature for amorphous DPC samples during the heating transformations.

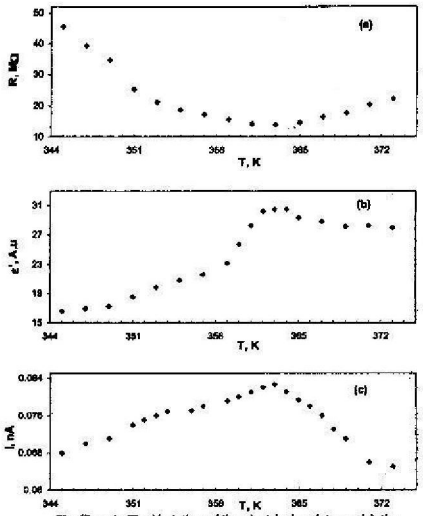

Fig. (5a - c) : The Variation of the electrical resistance (a), the dielectric constant (b) and the pyroelectric current (c) with temperature for amorphous DPC samples during the heating transformations.

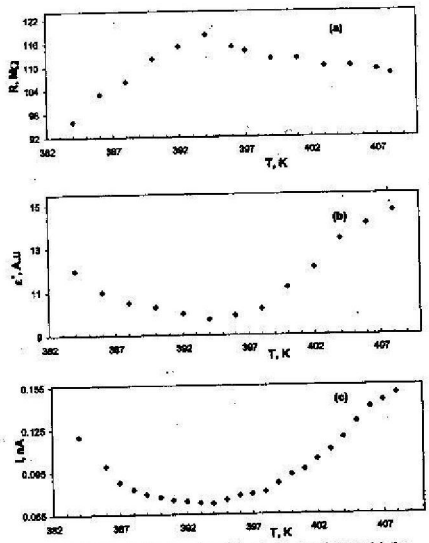

Fig. (6a - c) : The Variation of the electrical resistance (a), the dielectric constant (b) and the pyroelectric current (c) with temperature for amorphous DPC samples during the heating transformations.

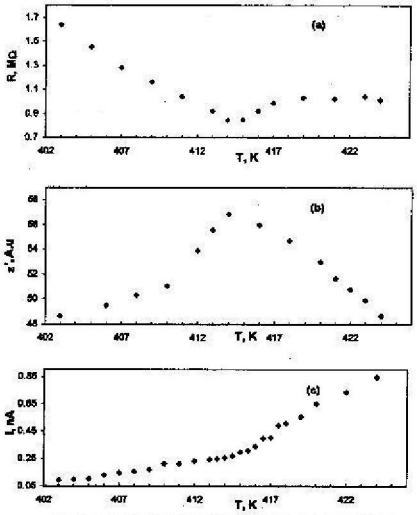

Fig. (7a - c) : The Variation of the electrical resistance (a), the dielectric constant (b) and the pyroelectric current (c) with temperature for amorphous DPC samples during the heating transformations.

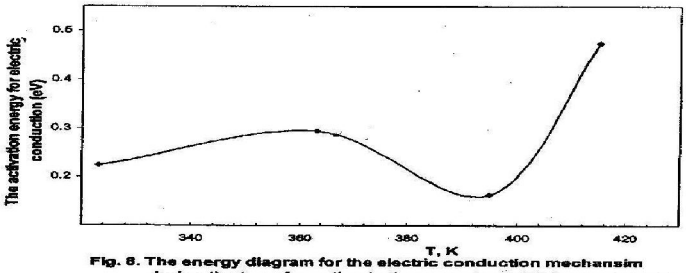

Fig. 8. The energy diagram for the electric conduction mechansim during the transformation in the amorphous DPC samples with temperature.

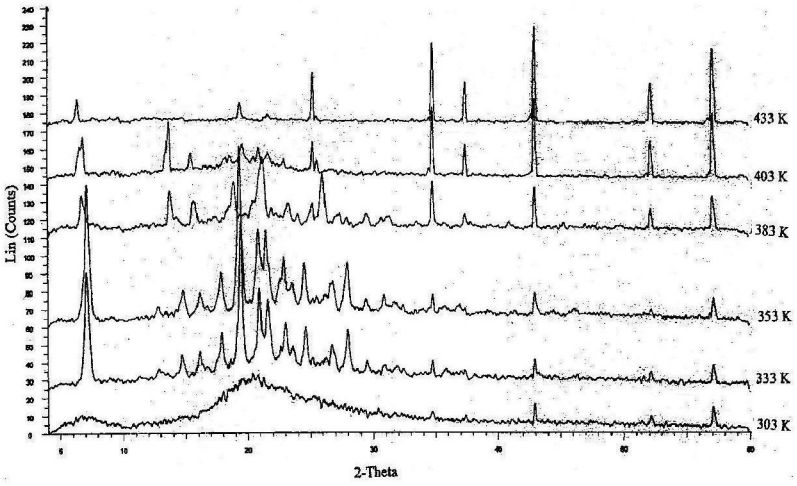

Fig. 9 X-ray diffraction patterns for the polymorphism of amorphous DPC at different temperatures (303, 333, 353, 383, 403, 433 K).

During the transition at (≈ 363 K), the crystal structure changes from monoclinc to tetragonal structure. This phase transition is probably due to a change in N-H, which is involved a change in the hydrogen bonding of amino ions (N-H) to the carbonyl group (C=O). However, it is possible to attribute this transition to a weaking of the N-H hydrogen bonding during the increasing of temperature. Also, here the thermal energy required for this phase transformation is sufficient to stimulate the reorientational molecular motion to another equivalent position which resulting in the observed structural phase transition [16].

As the sample temperature exceeds 373 K, another phase transition takes place at ≈ 395K in which the structure is changed from tetragonal to orthorhombic structure. This phase transformation is accompanied by a small thermal activated energy for conduction process. It may be explained as a large change in the intermolecular hydrogen bonding with –CO as well as –N-H groups [17]. These changes can be greatly affect the charge distribution so as to affect the transition process (populated locally excited states) and also may be assigned to a TICT state which is generated via twisting in the higher excited state [13-15].

The increase in the thermal activated energy for conduction process during the phase transition (≈ 415K), may be related to the existence of a high ordering transformation which is characterized by sudden changes in the atomic arrangements at higher temperature (Thermoplastic phenomena) [20]. This phase transition changes from orthorhombic to amorphous structure. Also this phase transformation may be related to the variation of the hydrogen bond in N-H group. The electrical resistivity of a dielectric material depends on the presence of locally excited states due to the activation of the thermal energy, which act as a source of ions in the dielectric material. Here, the conduction mechanism is complex, because it depends on the physical and chemical properties of the dielectric materials. In fact, DPC is considered as an artificial electron donor, so that the presence of electrons causes the conduction in it to be electronic process where it is easy for formation of electronic excited states. The rate of formation of (EES) depends on the change of temperature inside the material and the crystal structure transformation from state to another. The conduction mechanism which occurs in the amorphous DPC material within the temperature range from room temperature (≈ 303 K) up to the melting point (435 K) is mainly related to the electronic conduction process.

4/4/2010

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IR Study of the Low Temperature phase Transition in Amorphous Diphenyl Carbazide $C_{13}H_{14}N_4O$

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Abstract: The low temperature dependence of IR spectra of amorphous diphenyl carbazide $C_{13}H_{14}N_4O$ has been investigated below zero temperature. The data is reported here between room temperature and down to -70°C and the IR spectra are recorded in the frequency range 400-4000 cm^{-1}. This study is an extension of a recent one, which detected the presence of polymorphic character of this complex compound by IR, DSC and DTA at 50°C, 90 °C, 120 °C and 140 °C. It includes measurements and interpretation of the IR spectral band shape, frequencies of modes and band shifts as a function of temperature. Eleven different fundamental modes have been investigated explicitly. Special attention is paid to the most sensitive modes which reflect the low temperature phase transition process in detail. These modes are 3328 cm^{-1}, 1677 cm^{-1}, 1602 cm^{-1}, 1251 cm^{-1}, 750 cm^{-1} and 494 cm^{-1} in which the variations strongly support the low temperature phase transition in DPC at -47°C. This phase transition does not show structural change but just a transformation from amorphous state to amorphous state as detected by x-ray analysis. The phase transition here is suggested to be of displacive type. A low temperature phase transition has been detected here for the first time in diphenyl carbazide at - 47°C. [Journal of American Science 2010;6(8):278-287]. (ISSN: 1545-1003).

Keywords: Low temperature IR spectra; Diphenyl carbazide.

1. Introduction

Diphenyl carbazide (DPC) $C_{13}H_{14}N_4O$ is a complex organic compound frequently used in analytical chemistry. It is also used as a sensitive reagent for metal ions (mercury and cadmium). It has many applications, especially in the field of biophysics and microbiology. DPC is used in differential regulation of high light tolerance in mutant and wild-type Anacystis cells [1-3]. The crystal structure of DPC has been determined by De Ranter [4] and found to be orthorhombic at room temperature with space group pbnm with a = 5.7171 A°, b = 8.4121 A° and c = 25.6982 A°. The melting point of DPC is ≈ 162°C after that, it transforms to amorphous structure.

IR spectroscopy has been widely used to identify the presence and relative population of phase transformations in solids [5-8]. Recently, IR spectroscopy was used to measure and identify new structure formation accompanying phase transformation [9-14].

The vast majority of IR spectroscopy deals with the determination of IR fingerprint of a polymorphic modification. In the present work we wish to employ the IR technique to obtain physical information somewhat beyond the mere identification of a particular crystal modification.

Previous work on DPC showed the presence of several phase transitions above room temperature and up to its melting point. In the present work, a low temperature investigation of the IR characteristic mode changes in DPC was discussed in details.

This study aims to detect a probable low temperature phase transition below zero degrees centigrade down to -70°C. In fact there is absolutely no previous study for this compound in this low temperature range.

2. Experimental

Diphenyl Carbazide powder (ultra pure) was obtained from the British Drug House (BDH), Laboratory Chemical Division, England. IR spectra were recorded using the FT-IR type Perkin Elmer recorded on satellite 2000 spectrometer and the experimental technique employed has been reported [15]. The samples used were in the form of discs prepared by mixing 20 mg of DPC with one gram of KBr in a cylindrical die of 10 mm diameter. The die was then evacuated to ensure dryness and the sample was pressed. The spectra were recorded in the region of 400- 4000 cm^{-1} by a computerized system attached to a monitor and a printer.

3. Results

A specimen of DPC is analyzed at first at low temperature by differential scanning calorimetry

DSC. Fig. 1 shows the differential scanning calorimetry (DSC) thermogram and its derivative with time of pure DPC compound. The DPC sample is cooled at first in the DSC cell down to ≈ -70°C by using liquid nitrogen. After that, the DPC sample is slowly heated with a heating rate of 5°C/min. The DSC thermogram in fig. 1 illustrates at first very little variation, then, sudden drop at ≈ - 47°C. This is clearly indicates a probable low temperature phase transformation of DPC at - 47°C. The derivative of DSC indicate a peak at – 36°C, i.e, the transformation to the next state has been completed at this temperature. So, there is no doubt, that there is a low temperature phase transition in DPC near to ≈ - 50°C. Now, we are going to follow up what happened in such transition by studying the IR vibrating modes at selected set of low temperatures -70°C, - 40°C, - 30°C, - 20°C, - 10°C and room temperature.

Fig. 2 shows the IR spectra of a crystalline sample (orthorhombic) of DPC at room temperature (≈ 30°C). Eleven different ionic modes are shown in this figure. It showed no band which could be attribute to any mode other than those due to the ionic fundamentals of N-H, C-H, N-N, C=O, C-H and C-C groups. A quick glance at the spectrum shows that the strongest bands are at 3359 cm^{-1}, 3276 cm^{-1}, 3045 cm^{-1} (sharp), 1659 cm^{-1}, 1592 cm^{-1} (sharp), 1487 cm^{-1}, 1287 cm^{-1}, 1239 cm^{-1} (sharp), 882 cm^{-1} (sharp), 748 cm^{-1} (sharp) and 515 cm^{-1} (sharp). The broad band contains the three important modes 3359 cm-1 (asymmetric stretching of N-H), 3276 cm^{-1} (symmetric stretching of N-H) and 3045 cm^{-1} (sharp) (aromatic C-H stretching). The next modes at 1659 cm^{-1}, 1592 cm^{-1} (sharp) and 1486 cm^{-1} indicate the bending or deformation of N-H, N-N stretching symmetric vibration and N-N stretching asymmetric vibration respectively. The next two bands of the spectra at 1287 cm^{-1} and 1239 cm^{-1} (sharp) indicate the C-H stretching and C=O stretching vibrations. The sharp band at 882 cm^{-1} indicates the out of plane C-H bending. Also, the sharp bands at 748 cm^{-1} and 515 cm^{-1} are due to monosubstituted benzene and N-H oscillation respectively. All of these modes of vibrations of crystalline DPC at room temperature are tabulated in table (1). Crystalline diphenyl carbazide (DPC) can be melted at ≈ 162°C. Melted DPC transforms to amorphous structure at room temperature. Amorphous sample of DPC is subjected to IR and the obtained spectra are shown in fig. 3. The numerical values of the fundamental modes of vibration are given in table (1). One can easily observe a clear mode shift in the fundamental modes of vibrations as DPC transforms from crystalline (orthorhombic) structure to amorphous one. The mode shift corresponds to each ionic vibration of DPC is given in table (2). It is clear that the asymmetric stretching of N-H recorded the maximum mode shift ($\Delta\nu$ = - 48 cm^{-1}) when DPC changes to amorphous structure. One can deduce that the stretching of N-H is very sensitive to any structure change of DPC.

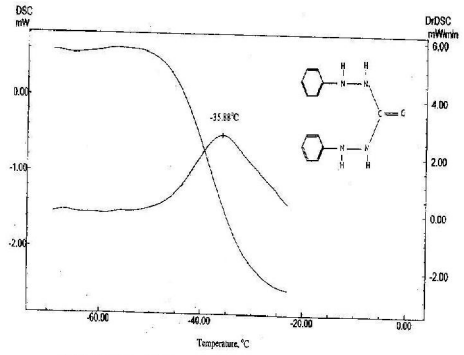

Fig.1 Low temperature DSC thermogram and its first derivative for amorphous DPC sample.

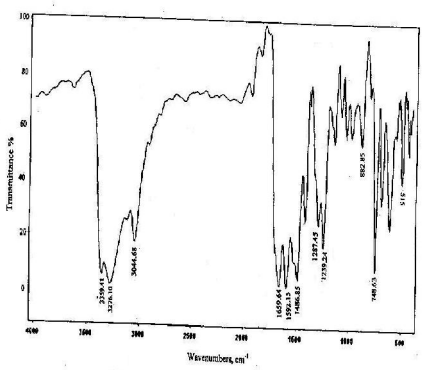

Fig.2 IR spectra of orthorhombic DPC at room temperature.

Now, amorphous DPC is studied by IR at low temperatures. A set of selected low temperatures -70°C, - 40°C, - 30°C, - 20°C, - 10°C are chosen and the spectra at these temperatures are shown in figs. 4, 5, 6, 7 & 8. Clear mode shift can be easily noticed in symmetric stretching of N-H, bending mode of N-H, N-N stretching symmetric vibrations, C-H stretching vibration, monosubstituted benzene and N-H oscillation. As an example for such mode shift is given in table (3) in which the temperature changed from - 70°C to - 30°C. Such mode shift is probably

due to a low temperature phase transition at ≈ - 47°C as previously detected by thermal analysis. Also, in these figs. 4, 5, 6, 7 & 8, the band contour (band shape) is widely varied and loses its sharpness. This means that, more cooling of DPC cause more broadening of bands. Also, noisy band contour often appears for lower temperature below zero. The main objective of the present work is to study the temperature dependence of the IR analysis of DPC below zero temperature. For a detailed investigation three important spectral regions, fig.9 will be studied at three different temperatures.

The variation of band contours during cooling below zero temperature is shown in fig. 9, for the principle ionic modes of vibrations of amorphous DPC. The band shapes in this fig.9 is completely different above - 70°C (i.e, at - 30°C & 30°C). As the sample temperature approach the room temperature (≈ 30°C) the noisy character in the vibrating mode disappeared. Also, the broadening of bands becomes little. In fact, such character accompanied the low temperature phase transition in amorphous DPC.

Table (1) Infrared spectra of DPC at a set of low temperatures and room temperature.

Modes of vibrations of DPC	-70 °C	- 40°C	- 30°C	- 20°C	- 10°C	Orthorhombic DPC at R.T	Amorphous DPC at R.T
Asymmetric stretching of N-H	3339	3328	3328	3319	3319	3359	3311
Symmetric stretching of N-H	3276	3275	3275	3269	3260	3276	3255
Aromatic C-H stretching	—	3049	3049	3052	3052	3044	3052
Bending or deformation mode of N-H	1685	1677	1677	1666	1666	1659	1677
N-N stretching symmetric vibrations	1592	1602	1602	1600	1600	1592	1602
N-N stretching asymmetric vibrations	1492	1493	1493	1490	1490	1486	1492
C-H stretching vibrations	1248	1251	1251	1247	1247	1287	1306
C=O stretching vibrations	1174	1174	1174	1169	1169	1239	1252
Out of plane C-H bending	883	887	887	882	882	882	887
Monosubstituted benzene	737	750	750	749	749	748	755
N-H oscillation	507	494	494	466	466	515	489

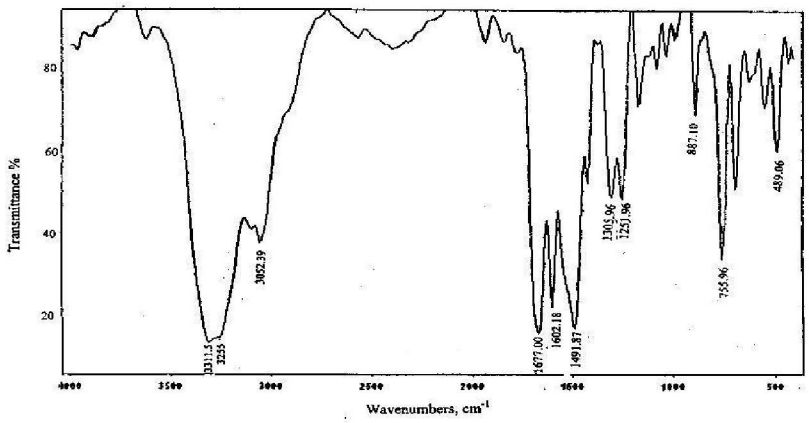

Fig.3 IR spectra of amorphous DPC at room temperature.

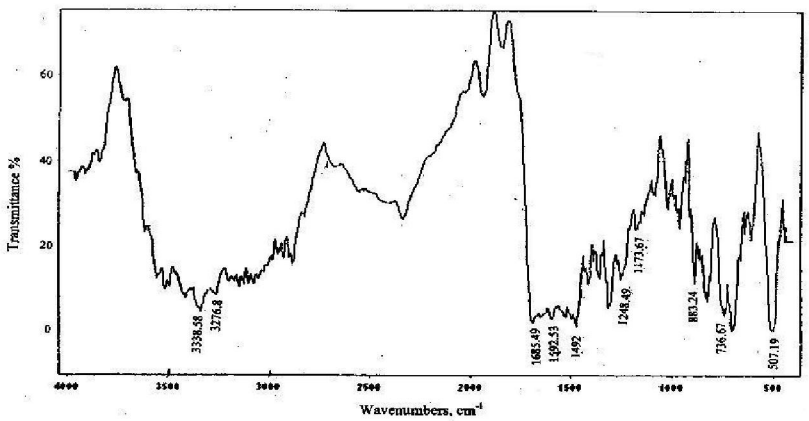

Fig.4 IR spectra of amorphous DPC at -70°C.

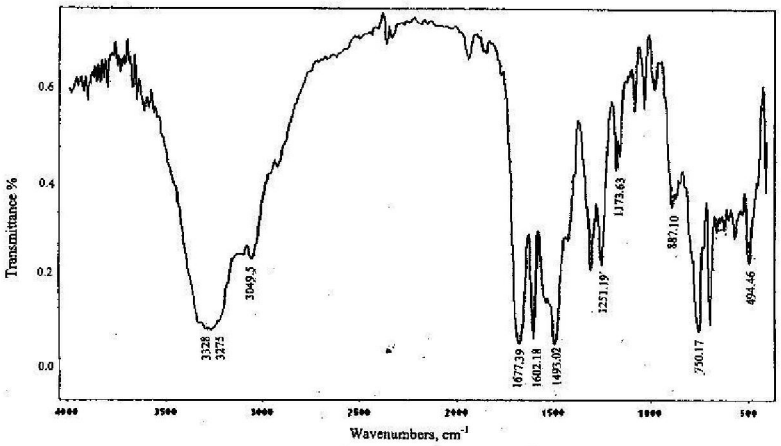

Fig.5 IR spectra of amorphous DPC at -40°C.

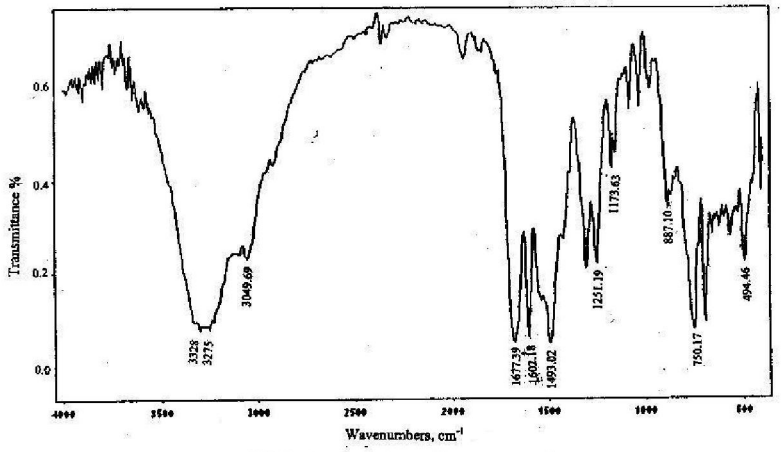

Fig.6 IR spectra of amorphous DPC at -30°C.

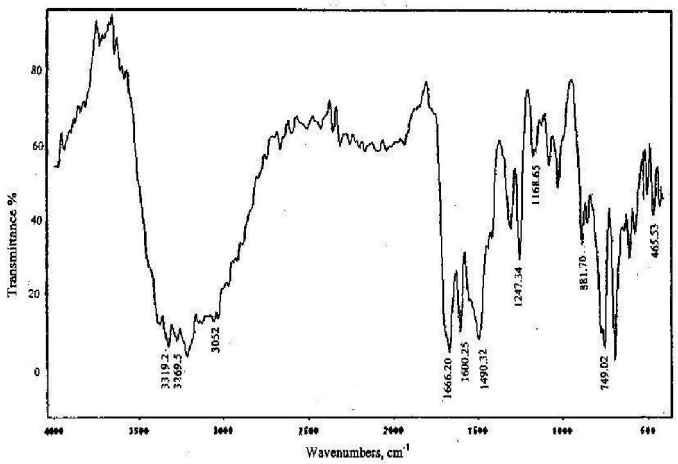

Fig.7 IR spectra of amorphous DPC at -20°C.

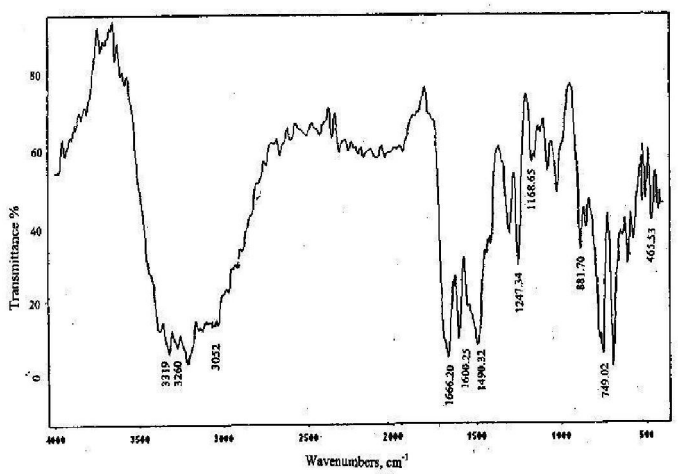

Fig.8 IR spectra of amorphous DPC at -10°C.

Table (2) Mode shifts as DPC changes from crystalline structure to amorphous structure.

Modes of vibrations of DPC	Mode shift, Δv (cm^{-1})
Asymmetric stretching of N-H	- 48
Symmetric stretching of N-H	- 21
Aromatic C-H stretching	+ 8
Bending or deformation mode of N-H	+ 18
N-N stretching symmetric vibrations	+ 10
N-N stretching asymmetric vibrations	+ 6
C-H stretching vibrations	+ 19
C=O stretching vibrations	+ 13
Out of plane C-H bending	+ 5
Monosubstituted benzene	+ 7
N-H oscillation	+ 26

Table (3) Fundamental mode shift during low temperature phase transition

Fundamental mode	-70°C	- 30°C	Mode shift, Δv
Asymmetric stretching of N-H	3339cm^{-1}	3328cm^{-1}	- 11 cm^{-1}
Bending or deformation mode of N-H	1685 cm^{-1}	1677 cm^{-1}	- 8 cm^{-1}
N-N stretching symmetric vibrations	1592 cm^{-1}	1602 cm^{-1}	+ 10 cm^{-1}
C-H stretching vibrations	1248 cm^{-1}	1251 cm^{-1}	+ 3 cm^{-1}
Monosubstituted benzene	737 cm^{-1}	750 cm^{-1}	+ 13 cm^{-1}
N-H oscillation	507 cm^{-1}	489 cm^{-1}	- 18 cm^{-1}

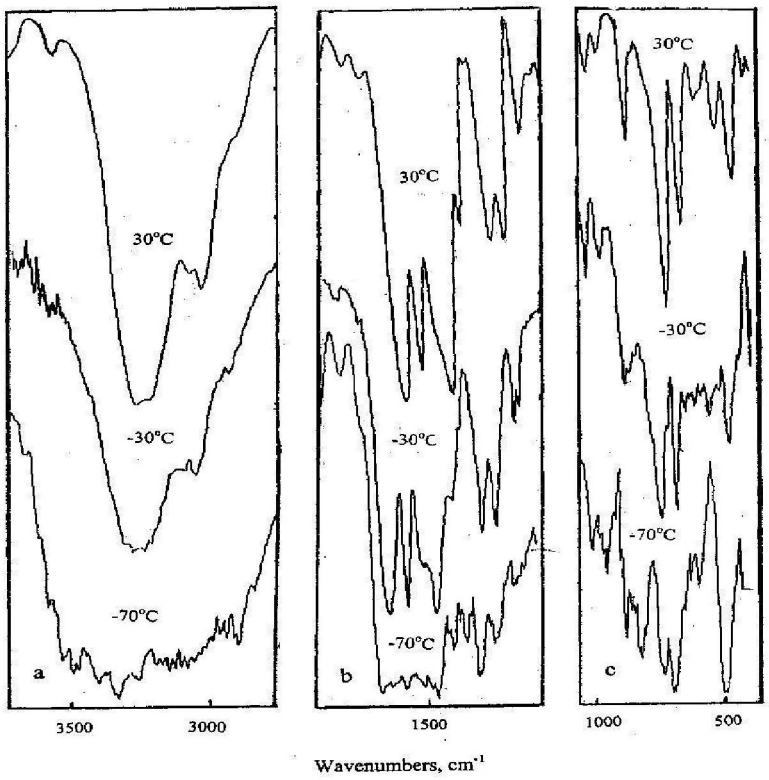

Fig.9 Variation of band shapes at a set of selected temperatures (-70°C, -30°C, 30°C) for DPC modes:-
a)Asymmetric stretching of N-H.
b)Bending or deformation mode of N-H, N-N stretching symmetric vibrations and C-H stretching vibrations.
c)Monosubstituted benzene and N-H oscillaton.

4. Discussions

The study of the low temperature phase transition in DPC by IR technique is based on the fact that after the absorption of IR radiation, DPC molecule vibrates at many rates of vibrations and forms close-packed absorption bands. Different bands observed in the present investigation are correspond to various functional groups and bonds found in DPC molecules.

The IR spectra of DPC in its solid state arise from some vibrations of the individual ions and internal vibrations of molecules. Since the vibrations occur throughout the lattice and are not concerned with a single unit cell, these vibrations are often observed as broad peaks which represent a composite band of several vibrations. The mass of ions here plays an important role in determination of the frequency of absorption. However, a regular change in frequency is observed during the variation of temperature. For those lattices containing covalent molecules, the vibrations likely to be observed in the IR spectrum arise from two sources: i) Molecular lattice vibrations which would occur at very low frequencies due to the mass of molecule; ii) Internal

vibrations of the molecules. Within this second class there are stretching and bending vibrations which involve heavy atoms and hindered rotation of ligands attached to cental atom.

Changing the temperature of cation or anion shifts the strongest absorption. This behavior appears to be characterizing the lattice vibrations in complex compounds. According to DSC and the present IR analysis, a low temperature phase transition at - 47°C take place in amorphous DPC. An x-ray structure analysis is carried out for DPC before and after the phase transition point. The obtained results are given in fig. 10.

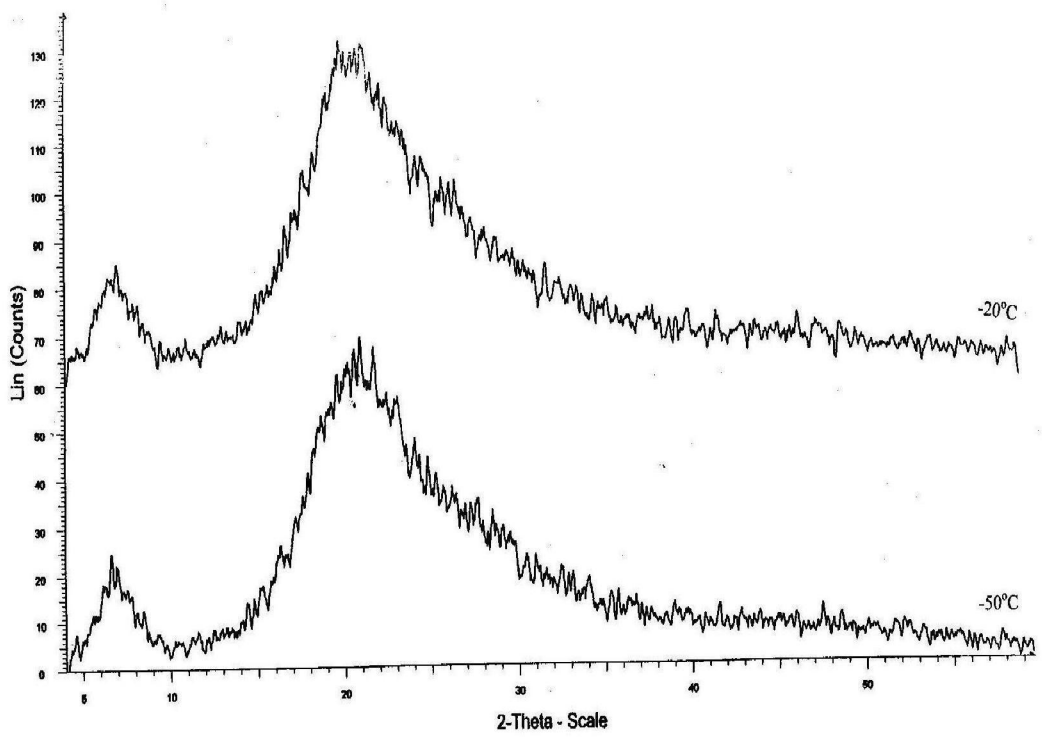

Fig.10 X-ray diffraction patterns of DPC at -20°C and -50°C.

The X-ray analysis strongly indicates that the crystal structure is amorphous before and after the phase transition process. This means that this low temperature phase transition in DPC involves no crystal structure changes. So, this type of phase transition is displacive type. According to Burger's [16, 17], in displacive transition, the initial lattice is deformed to a final lattice without breaking all bonds that link the particles within the crystal. The transition is considered displacive if a resemblance is found between the structures of the phases. It is asserted that these transitions must occur rapidly.

In order to explain the anomalous behavior shown in fig.9, it is proposed that N, H, C and O ions are oriented in a disordered fashion among the positions with nearly equal energies and symmetry. The rate of reorientation among these positions increases with temperature due to thermal agitation. The observed splitting in different band modes indicates a remarkable lowering in the site symmetry of the different ions. Also, both of the observed change in wave number and splitting in certain characteristic peaks can be linked to an overall change in the local molecular environment of vibrational dipole moments. It is hypothesized here that varying temperature will also have some effect on molecular order and thus on the IR spectrum of DPC.

In the present study, DPC complex compound contains large number of atoms, and thus many modes of vibrations will exist. When a complex with mixed ligands is formed, there is

always a decrease in symmetry. This reduction in symmetry causes an increase in the number of IR bands of the complex compound [18].

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Impact of Foliar Spray of Inorganic Fertilizer and Bioregulator on Vegetative Growth and Chemical Composition of *Syngonium Podophyllum* L. Plant at Nubaria

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Abstract: A pot experiment was carried out during 2007 and 2008 seasons at Research Production Station, of National Research Centre at Nubaria, Egypt. The aim of this work is to study the effect of foliar spray of inorganic fertilizer (Grow-more) (0.0, 1.0 and 2.0 ml/L) and bioregulator (Putrescine) (0, 100 and 200 ppm) and their interaction on vegetative growth and some chemical composition of *Syngonium podophyllum* L. plant. Most criteria of vegetative growth expressed as plant height, stem diameter, number of leaves, leaf area, fresh and dry weight of plant organs were significantly affected by application of the two factors which were used in this study. Chemical constituents i.e. Chl. (a), Chl. (b), carotenoids, nitrogen, phosphorus and potassium content in the leaves have increased in comparison control plants. Highest values of the mentioned characters were obtained from plants treated with Grow-more 2.0 ml/L combined with Putrescine 100 ppm followed by Grow-more 2.0 ml/L and Putrescine 100 ppm. [Journal of American Science 2010;6(8):288-294]. (ISSN: 1545-1003).

Keywords: Chlorophylls, Grow-more, Putrescine.

1. Introduction

Genus Syngonium includes thirty three species, native to tropical South America and Africa. *Syngonium podophyllum* L. family Araceae, commonly name arrowhead plant is one of the most well known and verstile herb. African evergreen is the most commonly produced species in the foliage industry. It is recognized by its juvenile leaves, which are simple, alternately, arranged, saggitate in shape, approximately 7.5 -17.5 cm long. Mature leaves are dramatically different they are compound with 3-11 elliptic leaf lets , and the center leaflet may attain a length of 19 cm with petiole reached 38.0 cm in long. The shape and color of the juvenile leaves in frequently used to name the cultivars that are commercially available. Syngonium is commonly used as hanging baskets, if upright growth is desired, a totem, tillers or other support is needed, and otherwise plants can be used as ground covers. The plants can be used in various such as offices, shops, hospitals, conferences, rooms and windows (Uphof, 1959).

There are many beneficial effects of micronutrients on plants and their involvement in the other processes, carbohydrate and nitrogen metabolism, as well as the resistance of plant disease and adverse environmental conditions. Micronutrients are also essential for organization and rapid alternation of nutrition compound within plant owing to their great importance in contribution to direct the enzymes way in metabolism (Massoud *et al.*, 2005). Therefore, both granular and fluid NPK fertilizers are commonly used as carriers of the micronutrients with mixed fertilizers is a convenient method of application and allows more uniform distribution with conventional application equipment. Micronutrients are essential for plant growth, but required in much smaller amount than those of the primary nutrients Brady and Weil, (2000).

Polyamines have been ascribed various roles such as that of a new class of plant growth regulators, hormonal second messengers and as one of resources of carbon and nitrogen at least polyamine synthesis from ornithine and arginine. Polyamines are essential for cell viability, and are correlates with or required for a variety of physiological events. The bound are conjugated to various secondary metabolism, these conjugated may have at least as significant function in plant development as free polyamines. In plant polyamines have been implicated in a wide range of biological processes including growth development, and biotic stress responses, cell division, differentiation, flowering, and delay senescence (Kuehn and Phillips, 2005).

Many investigators found that Putrescine has many physiological processes such as, regulation of elongation, it is correlated with cell division and increased number of roots, stimulated development and growth of economic crops (Yang et al., 1996) and Chattopadhayay *et al.*, 2002). Younghua *et al.*, (1996); Gupta *et al.*, (2003); Hussien *et al.*, (2006) reported that polyamines stimulated many physiological; processes including protein synthesis and photosynthetic activity. El-Bassiouny (2004)

found that foliar application with polyamines cause significant increase in growth characters, chlorophyll and soluble protein content, consequently increase yield of pea plants.

The objective of the present investigation was to study the effect of foliar inorganic fertilizer (Grow-more) and polyamine (Putrescine) and their interactions on growth and some chemical constituents of *Syngonium podophyllum* L. plant.

2. Material and Methods

The experiment was carried out at National Research Centre (Research and Production Station, Nubaria) during two successive seasons of 2007 and 2008, to investigate the response of *Syngonium* plant to foliar inorganic fertilizer (Grow-more) and Putrescine and their interactions on growth and chemical composition. On 20th Feb. 2007, 2008 seasons, vegetative uniform cuttings (18-22 cm long) were taken from *Syngonium* plants cuttings were treated for one minute with 1000 mg/L indole butric acid before planting in pot to enhance rooting.

Rooted cuttings were planted in black plastic pot 10 cm in diameter (one plant/pot) and grown in shaded greenhouse media formulated by combination of peatmoss and sandy soil (1:1 v/v). the seedlings were transplanted on 20th April 2007 and 2008 seasons, in plastic pots 30 cm in diameter filled with 10 kg of peatmoss and sandy soil (1:1, v/v), arranged in a complete randomized design with three replicates. Each replicate consisted of three plants. Water requirement were relative humidity maintained between 40-60 %, allow the surface of potting media to dry slightly before irrigation.

Each pot was fertilized twice with 1.5 g nitrogen as ammonium nitrate (33.3 % N) and 1.0 g potassium sulphate (48.5 % K_2O), the fertilizers were applied at 30 and 60 days after transplanting. Phosphorus as calcium superphosphate (15.5 % P_2O_5) was mixed with soil before transplanting at the rate of 3.0 g/pot. Other agricultural processes were performed according to normal practice. Plants were sprayed with different concentration of foliar inorganic fertilizer (Grow-more), Table (1) which produced by Ajemco International Company at the rate of (0.0, 1.0 and 2.0 ml/L), Putrescine was sprayed with concentrations (0, 100 and 200 ppm), interactions treatments were of the different concentrations of the two factors had been also carried out, in addition to untreated plants (control) which were sprayed with tap water. Foliar application of Grow-more and Putrescine was carried out two times of 30 days intervals, starting at 20th July at both seasons. The experiments were sit in a completely randomized design (CRD) with three replicates and two factors.

The following data were recorded on 30th Nov. 2007 and 2008 seasons, the recorded data were plant height (cm), stem diameter (mm), number of leaves, leaf area (cm^2) of 4 and 5 base leaves, fresh and dry weights (gm) of plant organs. Photosynthetic pigments i.e. chlorophyll (a and b) and caroteniods were determined exactly 0.1 g of fresh leaves of syngonium plants using the spectrophotometric method developed by Metzzner *et al*., (1965). Total nitrogen was determined by Chapman and Pratt (1961); while phosphorus determination carried out colorimetrically according to King (1951) potassium were determined photomerically by the flame photometer method as described by Brown and Lilland (1946).

Data obtained were subjected to standard analysis of various procedures. The values of LSD at 5% level as reported by Snedcor and Cochran (1980).

3. Results and Discussions

Effect of foliar fertilizer Grow-more and Putrescine:

1- Vegetative growth:

Data presented in Table (2) elucidate that foliar spray of Grow-more on *Syngonium podophyllum* L. plants significantly increased all growth parameters under study at 1.0 and 2.0 ml/L.

The highest values of plant height, stem diameter, number of leaves/plant, leaf area, fresh and dry weights of roots and leaves were obtained at 2.0 ml/L. as compared with control plants. Fresh weight of roots and leaves were exceeded by 19.73 and 16.48 %, respectively than the control plant. These results are in accordance with those obtained by Nemeat Alla and El-Geddawy (2001), they reported that used two times foliar spray of micronutrients mixture significantly increased root length, root diameter and yield of sugar beet. Mirvat and Alice (2001) reported that foliar application with micronutrients on sugar beet plant growth in reclaimed sandy soil were increased all yield components, El-Zanaty *et al.,* (2000) reported that foliar spray of Fe-EDDHA (6% Fe) tow times, significantly increased both fresh and dry weight of sugar beet plant. These results may be due to micronutrients boron, which helps transport vital sugars through plant membranes and promotes proper cell division, cell wall formation and development, also due to zinc which acts as enzyme activator in protein, hormone (i.e., IAA) and RNA / DNA synthesis, metabolism and increased growth.

Data in Table (2) showed that foliar application of Putrescine on syngonium plants significantly increased all growth characters at 100 and 200 ppm. The highest values of plant height, stem diameter, number of leaves, leaf area, fresh and dry weights of roots and leaves were obtained at 100

ppm Putrescine as compared with control plants. Fresh weight of root and leaves were exceeded by 22.41 and 22.31 %, respectively than the control plant.

These results are in agreement with those obtained by (Kuchn and Phillps, 2005), who reported that polyamines have been implicated in a wide range of biological processes including growth development and a biotic stress responses and cell division, differentiation. Talaat *et al.,* (2005) reported that the increase in shoot growth could be due to enhanced cell division activity on *Catharanthus*. Galston (1983) reported that polyamines are currently considered to be regulators of plant growth and development owing to their effects on cell division and differentiation. Polyamines have been ascribed various roles such as that a new class of plant growth regulators, hormonal second messengers and as one of the reserves of carbon and nitrogen at least polamines synthesis from ornithine and originine via ornithine decarboxylase and orginine decarboxylase (Asthir *et al.,* 2004). As regarding the interaction treatments, foliar application of Grow-more and puterscine, the data show that significantly increased all growth parameters under study. The highest values of growth parameters were obtained by Grow-more 2 ml/L combined with puterscine 100 ppm followed by Grow-more 2 ml/L and puterscine 100 ppm as compared with control plants. Data emphasized that interactions effects were significantly all growth parameters under study i.e. plant height, stem diameter, number of leaves, leaf area, and consequently fresh and dry weights of roots and leaves of *Syngonium podophyllum* L. plants.

The aforementioned results indicate that puterscine combined with Grow-more micronutrient solution favours the growth of Syngonium plants, which in turn reflect higher and good growth and consequently fresh weight which was regarded as a better indicator for foliar quality.

2-Chemical constituents:

Pigments content: Data in Fig (1) indicate that foliar application of Grow-more and puterscine separately to Syngonium plants significantly increased chl (a), chl (b) and total caroteniods content. Grow-more foliar spray induced effects on previous parameters by increasing its concentration. These results were in accordance with those obtained by Ratanarat *et al.,* (1990).

Putrescine induced effects on chlorophylls content, the highest values was recorded at put. 100 ppm. Yang *et al.,* (1996); Chattopadhayay *et al.,* (2002) reported that polyamines stimulated some physiological responses including vegetative growth and photosynthetic activity.

Concerning the interaction, foliar spray with Grow-more at 2 ml/L combined put. 100 ppm followed by Grow-more 2 ml/L and put 200 ppm, lead to significantly increase in this criterion. As regarding that spraying Syngonium plants with Putrescine at all used levels led to increase in (chl a, chl b and caroteniods) content compared with control plants.

These results may be due to iron and manganese which promote chlorophyll production and photosynthesis processes and cupper which helps in chlorophyll formation, these increments led to positive effects on growth parameters.

Minerals ions content: It is evident from the data in Fig (2) that foliar application of Grow-more or Putrescine increased the total amount of nitrogen, phosphorus and potassium ions content compared with control plants. These results were in line with those obtained by Sharma *et al.,* (2002) who found that application of organic materials either alone or in combination with chemical fertilizers caused substantial increase in total N, and available P, K as well as increased wheat and straw yield. As regarding the interaction treatments, foliar application of the two factors under study, the data show that significantly increased N, P and K contents of Syngonium plant. The highest values of mineral ions content were obtained by Grow-more 2 ml/L combined with put. 100 ppm followed by Grow-more 2 ml/L and put. 200 ppm. The highest recorded data in total protein percentage (11.19 and 10.99 %) were obtained from Grow-more 2 ml/L combined with put. 100 ppm, Grow-more 2 ml/L with put. 200 ppm. These increments led to positive effect of growth parameters and enhancing effect on plant metabolism which was regarded as a better indicator for foliar quality.

Table (1): Chemical properties of inorganic fertilizer (Grow-more) which used in this study

Grow-more content	N_2	P_2O_5	K_2O	Fe	Zn	Mg	Ca	Cu	S	B	Mo
%	11	6	8	0.15	0.15	0.14	0.02	0.20	0.02	0.01	0.01

Table (2): Effect of foliar inorganic fertilizer (Grow-more) and Putrescine on vegetative growth of *Syngonium podophyllum* L. plants (means of two seasons 2007 and 2008)

Characters / Treatments	Plant height	Stem diameter	No. of leaves	Leaf area	Root FW	Root DW	Leaves FW	Leaves DW
	cm	mm		cm^2	g/plant			
Effect of micronutrients								
Control	158.2	192.0	19.4	62.84	29.0	9.72	68.5	23.48
Grow- 1ml/L	159.2	194.0	20.7	64.10	29.1	10.37	71.1	24.05
Grow-2ml/L	194.0	2.5	23.2	73.45	34.7	12.28	79.8	26.92
LSD 5%	3.2	0.2	1.7	1.95	1.9	0.94	2.2	1.71
Effect of Putrescine								
Control	155.4	184.0	18.3	61.92	27.9	9.73	66.3	22.99
Put. 100 ppm	193.2	2.5	23.9	73.94	34.2	11.94	81.1	26.85
Put. 200 ppm	162.8	2.0	21.1	65.33	30.6	10.70	72.1	24.61
LSD 5%	3.2	0.2	1.7	1.95	1.9	0.94	2.2	1.71
Effect of interaction								
Control	115.7	1.2	13.0	48.45	19.5	6.20	49.3	18.00
Grow- 1ml/L	164.1	1.9	20.0	65.50	30.0	11.00	71.5	24.00
Grow-2ml/L	186.7	2.3	22.0	71.82	34.3	12.00	78.0	26.92
Put. 100 ppm	193.7	2.5	24.3	75.33	35.0	12.39	83.1	27.33
Put. 200 ppm	165.3	2.0	21.0	67.44	32.4	10.58	73.2	25.10
Grow 1ml/L +Put. 100 ppm	165.7	1.9	21.3	66.80	31.3	10.11	72.7	24.66
Grow1ml/L +Put. 200 ppm	147.0	1.9	20.0	60.00	26.0	10.00	69.0	23.50
Grow 2ml/L + Put. 100 ppm	220.3	2.9	26.0	79.68	36.3	13.33	87.5	28.56
Grow 2ml/L +Put. 200 ppm	175.0	2.2	21.7	68.33	33.5	11.52	74.0	25.23
LSD 5%	5.6	0.3	2.9	3.38	3.3	1.63	3.8	2.92

Micronutrients:micro, Putrescine:Put., Grow: Grow-more, FW: fresh weight, DW: dry weight.

Table (3) Effect of foliar inorganic fertilizer (Grow-more) and Putrescine on vegetative growth of *Syngonium podophyllum* L. plants (means of two seasons 2007 and 2008)

Characters / Treatments	Chlorophyll(mg/g)			Total carot.	N %	P %	K %	Total protein %
	a	b	a+b					
Effect of micronutrients								
Control	0.66	0.32	0.99	0.20	1.65	0.28	3.01	10.30
Grow- 1ml/L	0.67	0.33	1.00	0.21	1.66	0.28	3.08	10.42
Grow-2ml/L	0.75	0.39	1.13	0.25	1.74	0.33	3.65	11.00
LSD 5%	0.05	0.03	0.05	0.01	0.03	0.01	0.29	0.29
Effect of Putrescine								
Control	0.63	0.31	0.94	0.19	1.63	0.27	2.89	10.27
Put. 100 ppm	0.75	0.39	1.14	0.25	1.74	0.33	3.66	10.87
Put. 200 ppm	0.69	0.35	1.04	0.22	1.67	0.29	3.20	10.58
LSD 5%	0.05	0.03	0.05	0.01	0.03	0.01	**	0.29
Effect of interaction								
Control	0.51	0.21	0.72	0.12	1.50	0.19	2.17	9.40
Grow- 1ml/L	0.66	0.33	0.99	0.12	1.67	0.29	3.08	10.59
Grow-2ml/L	0.73	0.39	1.12	0.25	1.73	0.32	3.42	10.81
Put. 100 ppm	0.76	0.41	1.17	0.26	1.76	0.34	3.63	11.00
Put. 200 ppm	0.70	0.35	1.05	0.23	1.68	0.29	3.24	10.50
Grow 1ml/L +Put. 100 ppm	0.69	0.34	1.03	0.22	1.67	0.29	3.17	10.42
Grow1ml/L +Put. 200 ppm	0.65	0.32	0.97	0.19	1.64	0.27	3.00	10.25
Grow 2ml/L + Put. 100 ppm	0.80	0.42	1.22	0.28	1.79	0.36	4.18	11.19
Grow 2ml/L +Put. 200 ppm	0.72	0.37	1.09	0.24	1.70	0.30	3.33	10.99
LSD 5%	0.08	0.06	0.09	0.02	0.06	0.02	0.50	0.50

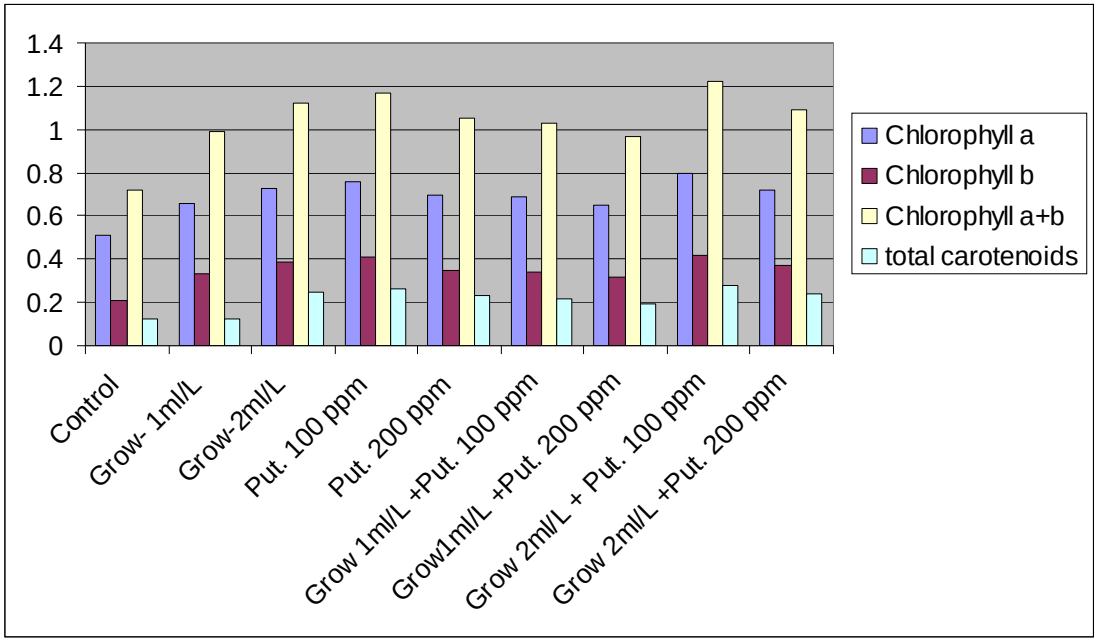

Fig (1) Effect of foliar inorganic fertilizer (Grow-more) and Putrescine on chlorophyll (a, b, a+b) and total carotenoids of *Syngonium podophyllum* L. plants

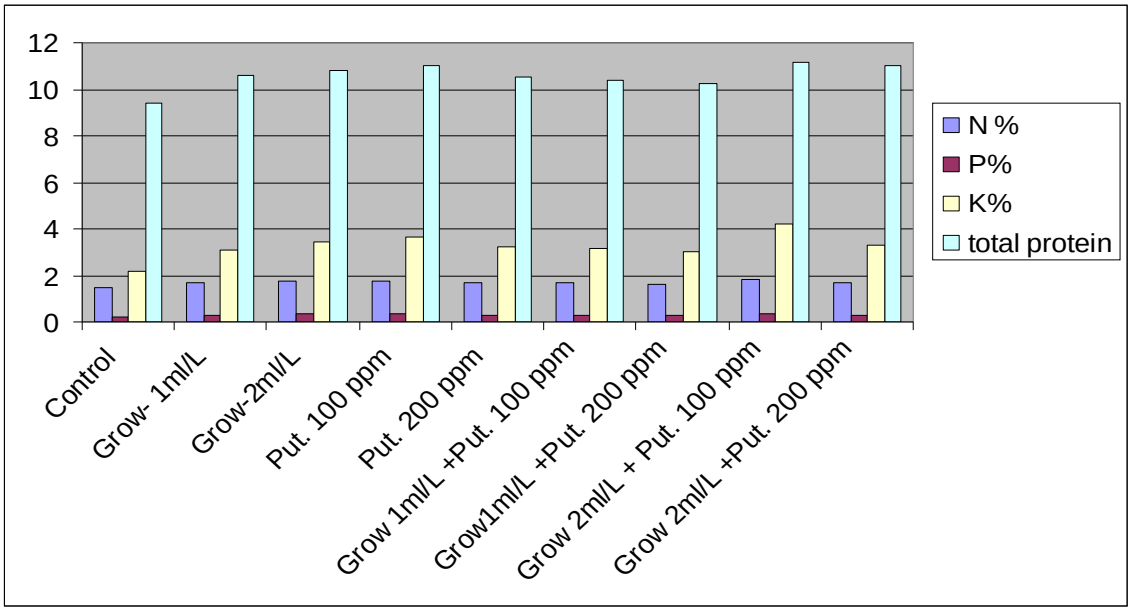

Fig (2)Effect of foliar inorganic fertilizer (Grow-more) and Putrescine N, P, K and total proteins percentage of *Syngonium podophyllum* L. plants

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Response of vegetative growth and chemical constituents of *Thuja orientalis* L. plant to foliar application of different amino acids at Nubaria.

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Abstract: A pot experiment was carried out during 2008 and 2009 seasons at Research and production Station, Nubaria of National of Research Centre, Dokki, Egypt to study the response of *Thuja orientalis* plants to foliar application of tyrosine, thiamine and tryptophan each at (0, 25, 50, and 100 ppm) on vegetative growth expressed as stem length, stem diameter, root length, fresh and dry weight of root and shoot and chemical constituents significantly were affected by the application of almost all of the three amino acids which were used in this study. Tyrosine, Thiamine and Tryptophan promoted all morphological characters. The comparison between the effect of tyrosine, thiamine and tryptophan revealed that the influence of tyrosine on increasing the growth parameters (especially at the rate of 10 ppm, which can be described as the most effective treatment) was superior to other amino acids (thiamine and tryptophan). The three amino acids increased total soluble sugar %, total free amino acid mg/g as well as essential oil %, essential oil yield / plant and N, P, K % and protein. Therefore, amino acid (Tyrosine, Thiamine and Tryptophan) at 100 ppm maybe recommended for promoted growth parameters and the best oil percentage in *Thuja orientalis* L. seedling. [Journal of American Science 2010;6(8):295-301]. (ISSN: 1545-1003).

Keywords: Tyrosine, Thiamine and Tryptophan, morphological characters.

1. Introduction

Thuja orientalis plants, Family Cupressaceae are an evergreen tree growing to 15m. at a slow rate. The seeds ripen from September to October. The flowers are monoecious (individual flowers are either male or female, but both sexes can be found on the same plant) and are pollinated by wind. It can grow in semi-shade (light woodland) or no shade. This plant is commonly used in Chinese herbalis, where it is considered to be one of the 50 fundamental herbs (Duke and Ayensu, 1985). The leaves are antibacterial, antipyretic, antitussive, astringent, diuretic, refrigerant and stomachic (Yeung, 1985).

The root bark is used in the treatment of burns and scalds (Duke and Ayensu, 1985). The stems are used in the treatment of coughs, colds, dysentery and parasitic skin-diseases.

Amino acids as organic nitrogenous compounds are the building blocks in the synthesis of proteins (Davies, 1982). Amino acids are particularly important for stimulation cell growth, they act as buffers which help to maintain favorable PH value within the plant cell, since they contain both acid and basic groups; they remove the ammonia from the cell.

This function is associated with amid formation, so they protect the plants from ammonia toxicity. They can serve as a source of carbon and energy, as well as protect the plants against pathogens.

Tyrosine is hydroxy phenyl amino acid that is used to build neurotransmitters and hormones. Hass (1975) stated that the biosyntheses of cinamic acids (which are the starting materials for the synthesis of phenols) are derived from phenylalanine and tyrosine.

The role of Tryptophan is well known: it has an indirect role on the growth via its
Influence on auxin synthesis. Phillips (1971) reported that alter native routes of IAA synthesis exist in plants, all starting from Tryptophan.
Thus, when Tryptophan was supplied to some plant tissues IAA was formed.

Thiamine (vitamin B1) could serve as coenzyme in decaboxylation of a-keto acids, such as Pyruvic acid and keto-glutamic acid which has its importance in the metabolism of carbohydrates and fats (Bidwell, 1979). Thiamine is an important cofactor for the transketolation reactions of the pentose phosphate cycle, which provides pentose phosphate for nucleotide synthesis and for the reduced NADP required or various synthetic pathways (Kawasaki, 1992).

Several authors indicated the promotive effect of amino acids on plants growth including, Mohamed and Khalil (1992) on Antirrhinum majus, Mathiola incana and Callistephus chinensis, Abou Dahab and Nahed (2006) on Philodendron erubescens, Nahed

and Balbaa (2007) on Salvia farinacea and Nahed et al, (2009) on Antirrhinum majus .

Harridy (1986) on *Catharanthus reseus* L., Abou Dahab and Nahed (2006) on Philodendron erubescens, and Nahed et al. (2009) on Antirrhinum majus found that foliar application of different amino acid treatments caused a significant increase in the content of total free amino acids. Talaat and Youssef (2002) on Ocimum basilicum L., Wahba et al., (2002) on Antholyza aethiopica, Abou Dahab and Nahed (2006) on Philodendron erubescens and Nahed et al. (2009) on Antirrhinum majus stated that application of amino acids as a foliar spray caused an increase in the contents of total soluble sugars.

The aim of this work was to enhance plant growth and chemical constituents of *Thuja orientalis* by foliar application of tyrosine, thiamine and tryptophan.

2. Material and Methods

Two pot experiments were carried out at National Research Centre (Research and production Station Nubaria), during two successive seasons 2008 and 2009 to study the effect of some amino acids (tyrosine, thiamine and tryptophan) on growth, chemical constituents and essential oil of Thuja orientalis L. Six months old seedling of Thuja were obtained from nursery of Forestry Department, Horticulture Research Institute, the seedlings were planted on the last week of March at the two seasons 2008 and 2009, in plastic pot 30cm. in diameter, filled with 10kg of sand soil, one plant / pot, the average heights of seedlings were 15-20cm. The investigated soil characterized by sand 73.33 %, silt 4.43%, clay 17.24% with PH 7.81, EC 2.18ds/m. CaCo 22.5% OM 1.55%, Ca 11.61, Mg 4.80, Na 4.64, Cl 1.80, HCo 1.40, So 8.5 meq/L, N 19.60, P 65.80, K 165.64 meq/100g. The available commercially fertilizer used through this experimental work was Kristalon (NPK 19:19:19) produced by Phayzon Company, Holand. The fertilizers rates (5.0 gm/pot) used in four equal doses after 4,8,16 and 20 weeks from transplanting. The plants were sprayed with the three amino acids tyrosine, thiamine and tryptophan (Each concentration of 25, 50 or 100ppm), in addition to the untreated plants. The amino acids foliar spray treatments were applied two month after transplanting (on May 25th in both seasons) and were repeated two times at one month intervals. At last week of November of 2008 and 2009, the following data were recorded: plant height (cm), stem diameter (mm), root length (cm), fresh and dry weights of shoots and roots (gm).

The experiment was set in a completely randomized design with ten treatments and six replicates of each treatment. The data were statistically analyzed according to Snedcor and Cochran (1980) using the least significant differences (LSD) at 5% level. The following chemical analyses were determined: total soluble sugar percentages were determined according to the method of Dubois *et al.*, (1956). Essential oil was determined in the shoots of each treatment according to Badawy *et al.* (1991), and essential oil yield / plant were determined by multiplying essential oil percentage X average of dry weight of shoots /plants. Free amino acid content was determined according to Rosen, H., (1957) Nitrogen, Phosphorus and Potassium were determined according to the method described by Cottenie *et al.*,(1982). Protein percentage in the root and shoot was determined by multiplying Nitrogen % x 6.25. (This is based in the assumption that plant proteins contain 16% nitrogen) according to the method as described by Ranganna (1978).

3. Results and Discussions

Effect o of Tyrosine, Thiamine and Tryptophan on vegetative growth:

Data presented in fig. (1, 2, 3, 4 and 5), indicated that foliar application of amino acid (tyrosine, thiamine and tryptophan) significantly promoted stem length, stem diameter, root length, fresh and dry weights of shoots and roots, as compared with the untreated plants, except the application of thiamine at 25ppm increased stem diameter root length and root fresh weight compared with control but it was non significant. Tyrosine at all level gave the greatest values of result compared with the other amino acids at the same level. The highest values of results were obtained in plants treated with Tyrosine followed by tryptophan and thiamine at 100ppm. The increase in fresh weight of shoots by 52.6%, 41.7% and 31.9%, respectively compared with control plants, as well as dry weight of shoot by 83.0%, 61.5% and 46.6% respectively. Generally, all growth parameters gradually increased by increasing amino acids concentration. The positive effect of amino acids on yield may be due to the vital effect of these amino acids stimulation on the growth of plant cells. The positive effect of amino acids on growth was stated by Goss (1973) who indicated that amino acids can serve as a source of carbon and energy when carbohydrates become deficient in the plant's amino acids are determinate, releasing the ammonia and organic acid from which the amino acid was originally formed. The organic acids then enter the Kerb's cycle, to be broken down to release energy through respiration. Waller and Nawacki (1978) reported that the regulatory effects of the amino acids could indirectly be explained since certain amino acids were suggested to affect plant development

through their influence on gibberellins biosynthesis. Thon et al., (1981) pointed out that amino acids provide plant cells with an immediately available source of nitrogen, which generally can be taken by the cells more rapidly than inorganic nitrogen. Kawasaki (1992) mentioned that thiamine is a necessary ingredient for the biosynthesis of the coenzyme thiamine pyrophosphate; in this latter from it plays an important role in carbohydrate metabolism. It is an essential nutrient for both plant and animal. In plants, it is synthesized in the leaves and is transported to the roots where it controls growth.

The results are characteristically accompanied by Mona and Talaat (2005) on Pelargonium graveolens plants , Nahed and Balbaa (2007) on Salvia farinea plants, Nahed et al., (2009) on Gladiolus grandflorum and Nahed et al., (2009)on Antirrhinum majus , they found that amino acids significantly increased vegetative growth.

Total soluble sugar contents:

Data in Table (1) showed that, the shoots and roots contents of total soluble sugar gradually increased by increasing amino acids concentration compared with the untreated plants. In addition data revealed that the amino acids treatments which were used in this study had significant effect on total soluble sugar contents.

Plant treated with thiamine at 100ppm had the highest total soluble sugar contents (7.18, 7.18%) in roots and shoots respectively. The comparison between the effect of thiamine, tyrosine, and tryptophan revealed that the influence of thiamine on increasing the total soluble sugar contents (especially at the rate of 100ppm, which can be described as the most effective treatment) was surperior to other amino acids (tyrosine, and tryptophan). The promotive affect of the amino acids on the total soluble sugars percentages may be due to their important role of the biosynthesis of chlorophyll molecules which in turn affected chlorophyll content.

In this concern, Devlin (1969) stated that there is agreement that succinyl CoA (Kerbs cycle amino acids glycin, initiate the biosynthetic leading to chlorophyll formation. Similar results have been reported in other plant species, Attoa *et al.*, (2002) on *Iberis amara* L., Yossef and Talaat (2003) on rosemary, Iman et al., (2005) on Catharanthus roseus L., Nahed and Balbaa (2007) on Salvia farinacea plants and Nahed et al., (2009) on Antirrhinum majus.

Total free amino acid: The results presented in Table (1) illustrated that total free amino acid contents were significantly increased as a result of different foliar application of amino acid. Application of the high tyrosine concentration (100ppm) was the most effective treatment in producing the highest values. The percentage of increase due to this treatment over the control reached 81.03 and 28.88% in the root and shoot respectively. The increase in the content of total free amino acids as a result of the different amino acid treatments may be attributed to amino acids as organic nitrogenous compound are the building blocks in the synthesis of proteins which formed by a process in which ribosome catalyze the polymerization of amino acids (Davies, 1982). Our results are in agreement with the findings of Abou Dahab and Nahed (2006) on Philodendron erubescens, Nahed and Balbaa, (2007) on Salvia farinacea.

Essential oil % and essential oil yield /plant (ml):

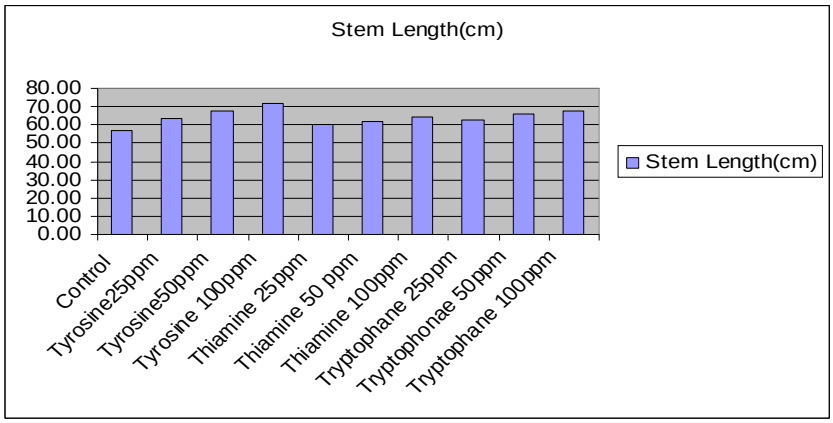
Data presented in Table (1) show that oil % and total oil yield (ml) / plant were significantly increased as a result of foliar spray of different amino acids. It is clear from the obtained data that the highest recorded value of essential oil was obtained in the herb of plants treated with 100ppm tyrosine. In this respect several investigators studied the effect of different amino acids on the total oil percentage and oil yield (ml / plant) Gamal El.Din et al., (1997) on Cymbopogon citratus hort, Talaat and Youssef (2002) on basil plant, Karima et al., (2005) on Matricaria chamomilla L.and Mona and Talaat (2005) on pelargonum graveolens L.

Minerals and total protein percentage:

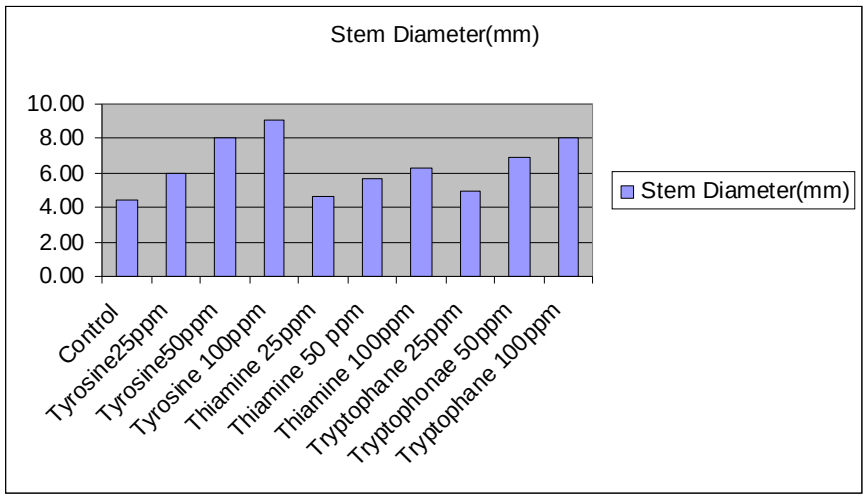
As regards the effect of different amino acids application on macronutrients (N, P and K) and total protein, it is to be from the results in Table (2) tyrosine, thiamine and tryptophan at 100ppm respectively gave the highest values in most cases (shoots, roots) as compared with untreated plants.

These results are in agreement with those obtained by Youssef and Talaat (2003) who reported that folia application of thiamine increased the total nitrogen percentage, total phosphorus % and total potassium % on rosemary plants. These increments led to quantitive changes in amino acids and specific proteins which acted positively in cell division and cell elongation (Bekheta and Mahgoub, 2005) on Carnation plants.

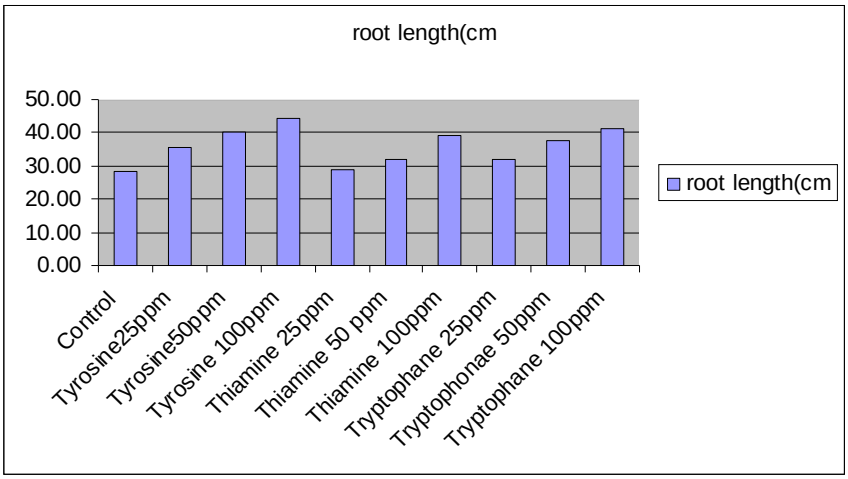
Therefore, amino acid (thiamine, tyrosine, and tryptophan) at 100 ppm maybe recommended for promoted growth parameters and best oil percentage in Thuja orientalis L., seedlings.



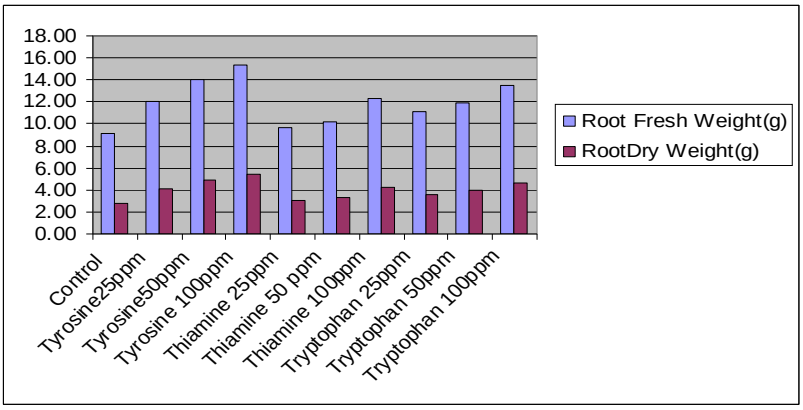
Figure(1): Effect of tyrosine, thiamine and tryptophan on Stem length(cm) of *Thuja orientalis* seedlings



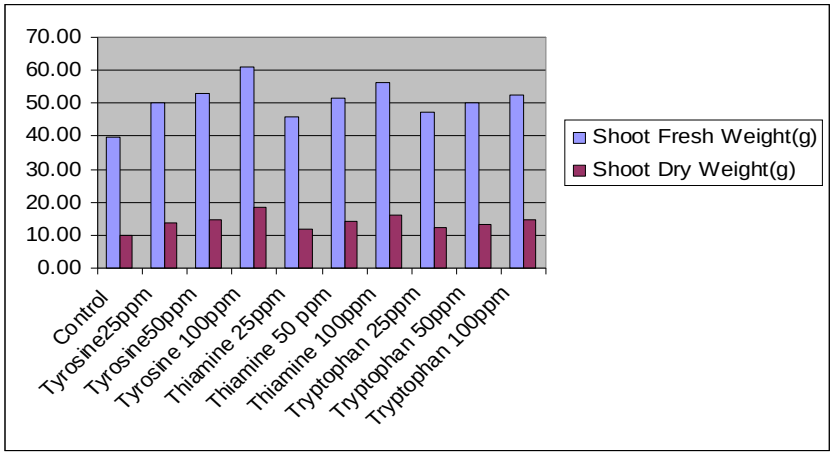
Figure(2): Effect of tyrosine, thiamine and tryptophan on Stem diameter(mm)of *Thuja orientalis* seedlings



Figure(3): Effect of tyrosine, thiamine and tryptophan on root length(cm) of *Thuja orientalis* seedlings



Figure(4): Effect of tyrosine, thiamine and tryptophan on root fresh and dry weights (g) of *Thuja orientalis* seedlings



Figure(5): Effect of tyrosine, thiamine and tryptophan on shoot fresh and dry weights of *Thuja orientalis* seedlings

Table(1) Effect of tyrosine, thiamine and tryptophan on total soluble sugar percentage in root and shoot, total free amino acid mg/g in root and shoot, essential oil percentage in shoot and essential oil yield/plant m/l. of *Thuja orientalis* seedlings(average of two seasons 2008 and 2009)

characters/treatments	Total soluble sugars %		Total free acid mg/g		Essential oil %	Essential oil yield/plant (ml)
	root	Shoot	Root	Shoot		
Control	4.10	3.59	3.11	4.46	0.259	0.026
Tyrosine25ppm	4.85	4.85	5.40	5.53	1.367	0.186
Tyrosine50ppm	5.36	5.36	5.54	5.69	1.497	0.223
Tyrosine100ppm	5.97	5.97	5.63	5.98	1.693	0.309
Thiamine 25ppm	5.89	5.93	3.73	4.83	0.447	0.053
Thiamine 50ppm	6.38	6.65	3.9	4.99	0.473	0.067
Thiamine 100ppm	7.18	7.18	4.24	5.39	0.537	0.087
Tryptophan 25ppm	4.37	4.37	4.34	4.96	0.766	0.096
Tryptophan 50ppm	4.67	4.67	4.59	5.18	0.879	0.118
Tryptophan 100ppm	5.17	5.17	4.91	5.61	0.996	0.146
L.S.D at 5%	0.06	0.05	0.14	0.08	0.11	

Table(2): Effect of tyrosine, thiamine and tryptophan on Nitrogen, phosphorus, potassium, and protein percentage in (Root and Shoot)of *Thuja orientalis* seedlings (average for two seasons 2008 and 2009)

characters	Shoot				Root			
treatments	N%	P%	K%	Protein%	N%	P%	K%	Protein%
Control	0.89	0.26	0.24	5.56	1.08	0.24	0.43	6.75
Tyrosine 25ppm	1.08	0.32	0.59	6.75	1.18	0.29	0.58	7.38
Tyrosine 50ppm	1.45	0.39	0.68	9.06	1.22	0.35	0.6	7.63
Tyrosine 100ppm	1.61	0.43	0.86	10.06	1.26	0.39	0.66	7.88
Thiamine 25ppm	1.98	0.42	0.86	12.38	1.24	0.33	0.68	7.75
Thiamine 50ppm	2.04	0.52	0.98	12.75	1.39	0.39	0.72	8.69
Thiamine 100ppm	2.26	0.58	1.12	14.13	1.67	0.53	0.88	10.44
Tryptophan 25ppm	1.65	0.35	0.76	10.31	1.2	0.31	0.61	7.5
Tryptophan 50ppm	1.72	0.44	0.86	10.75	1.29	0.38	0.63	8.06
Tryptophan 100ppm	1.88	0.49	0.93	11.75	1.36	0.43	0.69	8.5
L.S.D at5%	0.07	0.06	0.05	0.44	0.03	0.07	0.04	0.2

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Antiviral Properties of Garlic Cloves Juice Compared with Onion Bulbs Juice Against Potato Virus Y (PVY).

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Abstract: Potato y disease caused by potato virus y (PVY) is widespread wherever potato is grown. PVY affects plants and yields. The effectiveness of extracts from garlic cloves (GE) and onion stems (OE) against potato virus y (PVY) *in vitro* and *in vivo* has been evaluated. GE and OE reduced the *in vitro* and *in vivo* infectivity of PVY to a certain extent, expressed as the number of local lesions induced by PVY on *Chenopodium amaranticolor* plant as a local lesion host. The effect of these extracts and their dilutions (from 10^{-1} to 10^{-3}) was the highest in the crude extract and decreased gradually by increasing the extracts dilutions from 10^{-1} to 10^{-3}. PVY inhibition percentages induced by GE and OE varied according to the time of treatment (1, 2, 3 and 4 days). High percentages of inhibition were recorded for *in vitro* treatment. The highest effect of GE against PVY infectivity was in the crude extract and after 4 days (percentage of inhibition was 63.63 %). While, the highest percentage of inhibition of OE against PVY infectivity was 51.51 % in the crude extract and after 4 days. PVY inhibition of pre-inoculation treatment was higher than that of post-inoculation treatment. In pre-inoculation treatment, the highest effect of GE against PVY infectivity was in the crude extract and after 4 days (percentage of inhibition was 38.89 %). While, the highest percentage of inhibition of OE against PVY infectivity was 33.33 % in the crude extract and after 4 days. In post-inoculation treatment, the highest effect of GE against PVY infectivity was in the crude extract and after 4 days (percentage of inhibition was 33.33 %). While, the highest percentage of inhibition of OE against PVY infectivity was 30.56 % in the crude extract and after 4 days. So, GE was more effective in reducing the local lesions produced by PVY on *Chenopodium amaranticolor* than OE. [Journal of American Science 2010;6(8):302-310]. (ISSN: 1545-1003).

Key words: Potato virus y (PVY), potyviruses, garlic (*Allium sativum*), onion (*Allium cepa*), inhibition.

1. Introduction

Potato virus y (PVY) is one of the most damaging viruses-caused diseases in potato plant (*Solanum tuberosum* L.). PVY is widespread wherever potato is grown. The genome of PVY is 9704 nucleotides long. The flexuous particles of PVY have a model length of 740 nm and width of 11 nm (Robaglia *et al*., 1989). PVY belongs to potyviruses. Potyviruses are important pathogens and cause substantial losses in crop plants of economic importance, such as cereal, millet, fruit, vegetable, sugarcane, oilseed, ornamentals, fodder and pasture, in different parts of the world. Several distinct potyviruses are also known to infect one crop, for instance bean, clover, maize, pea, peanut, potato, soybean, sorghum, tulip, tobacco and cucurbits (Shukla *et al*., 1994). Virus diseases are the major limiting factor of potato production and cause its deterioration (Kurppa, 1983; Omer and El-Hassan, 1992; Jan *et al.,*1994; Arif *et al.,* 1995; Jan and Khan, 1995; Al-Shahwan *et al.,* 1997; Al-Shahwan *et al.,* 1998; Hord and Rivera, 1998; Hamm and Hane, 1999; and Mansour, 1999). Medicinal plants have been widely used to treat a variety of infectious and non-infectious ailments. According to one estimate, 25% of the commonly used medicines contain compounds isolated from plants. Several plants could offer a rich reserve for drug discovery of infectious diseases, particularly in an era when the latest separation techniques are available on one hand, and the human population is challenged by a number of emerging infectious diseases on the other hand. Among several other ailments, viral infections, particularly infections associated with human immunodeficiency virus type 1 (HIV-1) and 2 (HIV-2), and newly emerging infectious viruses have challenged mankind survival. Of importance, a variety of medicinal plants have shown promise to treat a number of viral infections, and some of them possess broad-spectrum antiviral activity. In the past, exploration into the antiviral activity of various promising medicinal plants was limited due to: (a) highly infectious nature of viruses and (b) lack of appropriate separation techniques for the identification of antiviral components from plants. Development of vector-based strategies, in which non-infectious molecular clone of a virus could be used for antiviral screening purposes, and advancement in separation technologies offers promise for medicinal plants usage in modern drug discovery (Muhammad *et al*., 2008). Many investigators have studied the effect of antiviral

activity of garlic or onion on different plant viruses(Dene *et al.,* 1982; Hecht, 1984; Presly *et al.,* 1986; Entwistle *et al.,* 1987 ;Pachauri and hukla, 1988 ; Cheremushkina *et al.,* 1989; Shul'-man, 1989a; Shul'-man, 1989b; Walkey and Antill, 1989; Dijk *et al.,* 1992; Kinghorn and Balandrin, 1993; Pathak, 1993; Dijk and Van, 1994; Patel *et al*., 2000 ; Lot *et al*., 2001; Gurkina, 2002; Thirumalaisamy *et al*., 2003; Cho *et al*., 2010 and Satyakumar *et al*., 2010).

2. Material and Methods:

Extracts of GE and OE were prepared separately in distilled water (1:1w/v). Extracts were diluted by distilled water to 10^{-1}, 10^{-2} and 10^{-3} before use. All experiments were repeated twice. Four replicates were used for each treatment.

1. Virus isolate:

Virus was isolated and identified by Mohamed (1999). Virus inoculum was the crude sap obtained by trituration of frozen leaves of tobacco plants (*Nicotiana tabacum* L. cv.White burley) seedlings showing mosaic and vein banding symptoms. These symptoms developed 14 days after inoculation with a single local lesion obtained from *Chenopodium amaranticolor* leaves that were inoculated with sap extracted from naturally infected potato plants (*Solanum tuberosum* L. cv. Diamond). Inoculation of leaves was carried out by rubbing with finger after their being dusted with carborandum as described by Rawlins and Tompkins, (1936).

2. Preparation of garlic and onion extracts (GE and OE):

Cloves of garlic (*Allium sativum* cv. Balady) and bulbs of onion (*Allium cepa* cv. Giza 20) plants were ground in blender using sterilized distilled water (1:1w/v). The pulp was pressed through two layers of cheesecloth, and then the fluidextract was centrifuged at 1000 rpm for 30 min. The supernatant was collected and stored at -20C (Ismail *et al*., 1989).

3. Effect of garlic and onion extracts (GE and OE) on PVY infectivity *in vitro:*

For testing the effect of garlic and onion extracts for different time intervals(1, 2, 3, and 4 days) on PVY infectivity *in vitro*, 1 ml of the expressed sap containing virus was added to 1 ml of each of garlic and onion extracts, mixed well and allowed to stand for 1, 2, 3 and 4 days. Distilled water was used as a control. Virus-GE and OE mixtures and the control were inoculated into one month old *Chenopodium amaranticolor* at previously mentioned intervals. The developed local lesions were counted and the percentage of inhibition was calculated from the following formula according to Taha and Mousa, (2000):

% Inhibition = (control -treatment) x100/ control

4. Effect of garlic and onion extracts (GE and OE) on PVY infectivity *in vivo:*

4.1. Pre-inoculation treatment:

1 ml of each GE and OE concentrations was rubbed on leaves of *Chenopodium amaranticolor,* then they mechanically inoculated with PVY infected sap (1ml/plant) at different intervals: 1, 2, 3, and 4 days respectively. Distilled water was used as a control.

4.2. Post-inoculation treatment:

The former steps in pre-inoculation were applied except that, virus infected sap was applied first followed by GE and OE treatments

3. Results and Discussion

Garlic has a long history of medicinal use, being mentioned more than 2,000 years ago in ancient Chinese medical literature. Several centuries ago the Egyptians used garlic to treat many disease entities. Aristotle and Hippocrates called attention to the healing powers of garlic, and Pasteur mentioned its medicinal and antibacterial properties. In addition to its well-documented antibacterial properties. Over the past decade, there has been a proliferation of literature on the antimicrobial properties of garlic extract. The principal antimicrobial component of garlic oil is the sulfur compound diallyl thiolsulfinate, which named allicin(Edward and Vincent, 1985).

Onion *(Allium cepa L.)* is a species of the Alinaceae family of great economic importance, and is the second most important vegetable crop in the world. Besides making a significant nutritional contribution to the human diet, onions also have medicinal and functional properties. (Lanzotti, 2006). As with other vegetables, onions contribute to the intake of certain vitamins and minerals, but carbohydrates are the major nutrient fraction, many of which are components of dietetic fibre. Non-structural and soluble carbohydrates form a substantial part of onion dry matter, mainly as fructooligosaccharides (FOS) and monosaccharides (glucose, fructose and sucrose). The non-digestible carbohydrates have recently been shown to be of particular interest for human health. They have a prebiotic effect, improving the intestinal flora, especially the bifidobacteria intestinal conditions against pathogen agents. Onions also demonstrate systemic physiological effects such as decreasing the level of fasting glycemia as well as insulinemia, triglycerides and cholestero. The onion is second only

to the tomato in terms of the agricultural surface area dedicated to its cultivation production. A large increase in onion production has taken place in recent years. The chemical composition of the onion is variable and depends on cultivar, ripening stage, environment and agronomic conditions (Rodríguez *et al.,* 2009).

1. Effect of garlic and onion extracts (GE and OE) on PVY infectivity *in vitro:*

The results obtained from Table (1) and Fig. (1) show an inhibitory effect of GE against PVY infectivity. This effect was the highest in the crude extract and decreased gradually by increasing GE dilutions from 10^{-1} to 10^{-3}. The highest effect of GE against PVY infectivity was in the crude extract and after 4 days (percentage of inhibition was 63.63 %).

Data obtained from Table (2) and Fig. (2) show an inhibitory effect of OE against PVY infectivity. This effect was the highest in the crude extract and decreased gradually by increasing GE dilutions from 10^{-1} to 10^{-3}. The highest effect of GE against PVY infectivity was in the crude extract and after 4 days (percentage of inhibition was 51.51 %).

GE was more effective in reducing the local lesions produced by PVY on *Chenopodium amaranticolor* than OE.

Table (1): Effect of crude and diluted extract from garlic (*Allium sativum* cv. Balady) plants on local lesions number produced by PVY on *Chenopodium amaranticolor in vitro* treatment at different intervals.

Time intervals	Mean number of local lesions								
	Control	Crude extract	% I*	Dilutions			% I*		
				10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}
One day	36	23	36.11	25	28	33	30.56	22.22	8.33
Two days	34	19	44.11	21	24	29	38.32	29.41	14.71
Three days	35	16	54.28	18	21	26	48.57	40.00	25.71
Four days	33	12	63.63	14	17	22	57.57	48.48	33.33

% I*: Percentage of inhibition

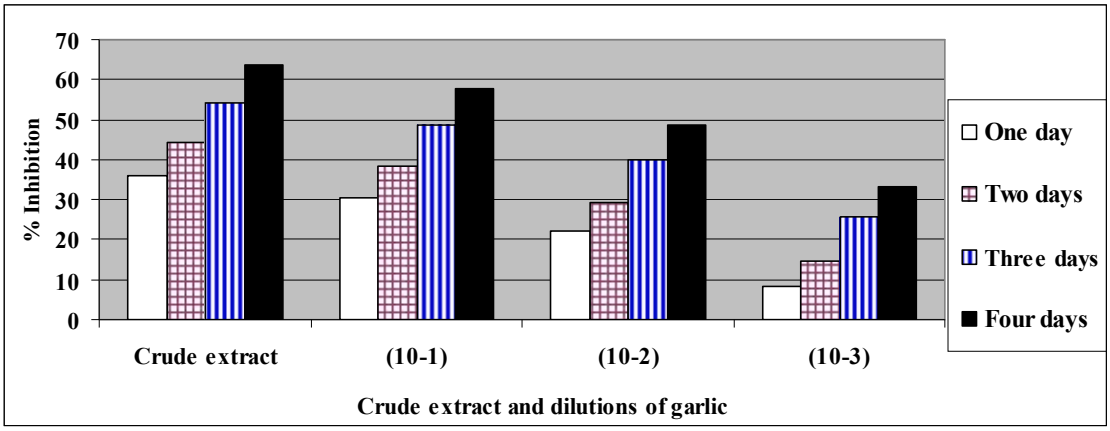

Fig. (1): % of inhibition produced by crude and diluted extract of garlic on PVY infected sap *in vitro.*

Table (2): Effect of crude and diluted extract from onion (*Allium cepa* cv. Giza 20) plants on local lesions number produced by PVY on *Chenopodium amaranticolor in vitro* treatment at different intervals.

Time intervals	Mean number of local lesions								
	Control	Crude extract	% I*	Dilutions			% I*		
				10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}
One day	36	26	27.78	28	31	34	22.22	13.89	5.56
Two days	34	22	35.29	24	27	30	29.41	20.58	11.76
Three days	35	20	42.85	22	25	28	37.14	28.57	20.00
Four days	33	16	51.51	18	21	24	45.15	36.36	27.27

% I*: Percentage of inhibition

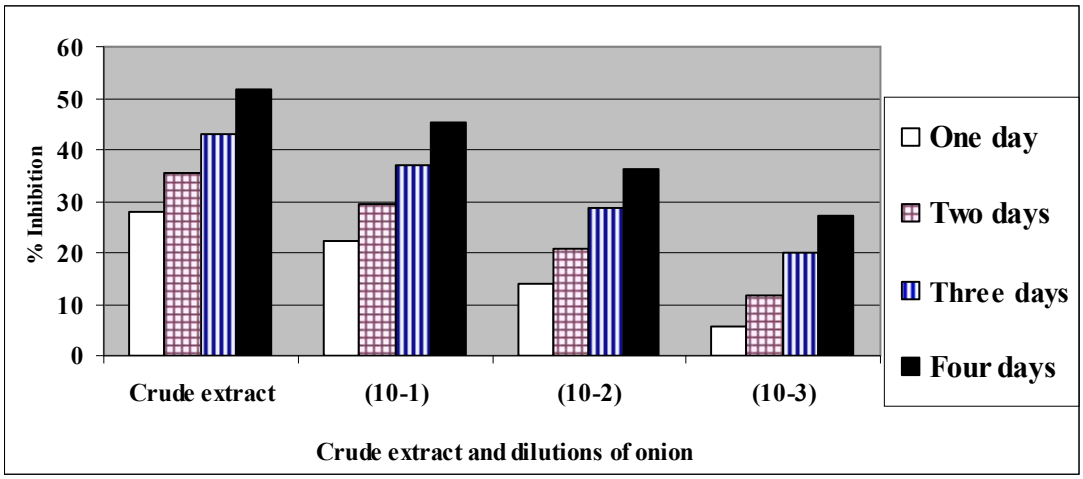

Fig. (2): % of inhibition produced by crude and diluted extract of onion PVY infected sap *in vitro*.

2. Effect of garlic and onion extracts (GE and OE) on PVY infectivity *in vivo:*

Data obtained from Table (3) and Fig. (3) Indicate that better inhibitory effect of GE was obtained by pre-inoculation treatment than post-inoculation one. Effect of GE was the highest in the crude extract and decreased gradually by increasing GE dilutions from 10^{-1} to 10^{-3}. In pre-inoculation treatment, the highest effect of GE against PVY infectivity was in the crude extract and after 4 days (percentage of inhibition was 38.89 %). Also in post-inoculation treatment, the highest effect of GE against PVY infectivity was in the crude extract and after 4 days (percentage of inhibition was 33.33 %).

Similar results were obtained in Table (4) and Fig. (4) using OE. A higher inhibitory effect of OE was obtained by pre-inoculation treatment than post-inoculation one. Effect of OE was the highest in the crude extract and decreased gradually by increasing OE dilutions from 10^{-1} to 10^{-3}. In pre-inoculation treatment, the highest effect of OE against PVY infectivity was in the crude extract and after 4 days (percentage of inhibition was 33.33 %). Also in post-inoculation treatment, the highest effect of OE against PVY infectivity was in the crude extract and after 4 days (percentage of inhibition was 30.56 %). So, GE was more effective in reducing the local lesions produced by PVY on *Chenopodium amaranticolor* than OE.

Table (3): Effect of crude and diluted extract from garlic (*Allium sativum* cv. Balady) plants on local lesions number produced by PVY on *Chenopodium amaranticolor in vivo* treatment at different intervals.

Time intervals	Mean number of local lesions																	
	Pre-inoculation									Post- inoculation								
				Dilutions			% I*					Dilutions			% I*		
	Control	Crude	% I*	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}	Crude	% I*	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}
One day	33	26	21.21	28	31	32	15.15	6.06	3.03	27	18.18	29	31	32	12.12	6.06	3.03
Two days	35	26	25.71	28	31	34	20.00	11.42	2.85	28	20	30	32	34	14.28	8.57	2.85
Three days	34	24	29.41	26	29	32	23.52	14.70	5.88	26	23.52	28	31	33	17.64	8.82	2.94
Four days	36	22	38.89	24	27	30	33.33	25.00	16.67	24	33.33	26	29	32	27.78	19.4	11.1

% I*: Percentage of inhibition

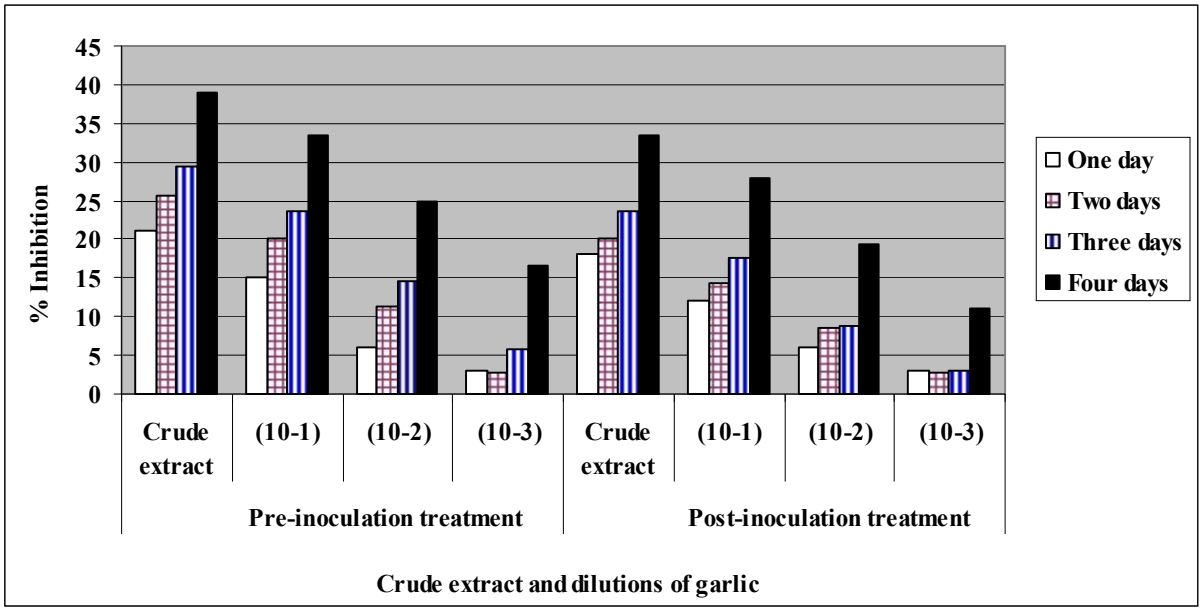

Fig. (3): % of inhibition produced by crude and diluted extract of garlic on PVY infected sap *in vivo*.

Table (4): Effect of crude and diluted extract from onion (*Allium cepa* cv. Giza 20) plants on local lesions number produced by PVY on *Chenopodium amaranticolor in vivo* treatment at different intervals.

Time intervals	Mean number of local lesions																	
	Pre-inoculation									Post- inoculation								
	Control	Crude	% I*	Dilutions			% I*			Crude	% I*	Dilutions			% I*			
				10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}			10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}	
One day	33	26	21.2	28	31	32	15.2	6.1	3.0	28	15.2	30	32	32	9.1	3.3	3.3	
Two days	35	27	22.9	29	32	34	17.1	8.6	2.9	29	17.1	31	33	34	11.4	5.7	2.9	
Three days	34	25	26.5	27	30	33	20.6	11.6	2.9	25	26.5	28	30	32	17.6	11.8	5.9	
Four days	36	24	33.3	26	29	32	27. 8	19.4	11.1	25	30.6	27	30	33	25.0	16.7	8.3	

% I*: Percentage of inhibition

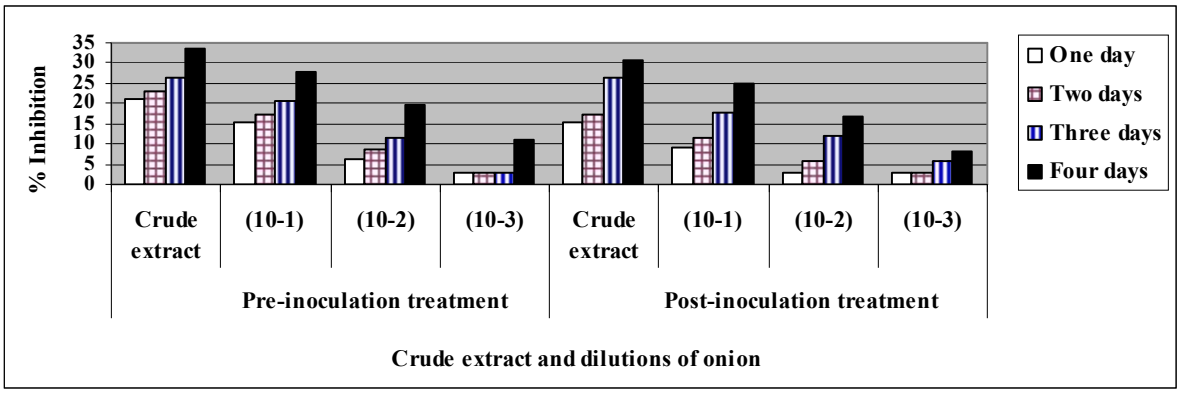

Fig. (4): % of inhibition produced by crude and diluted extract of onion on PVY infected sap *in vivo*.

These findings are compatible with that of Chowdhury and Saha (1985) who tested 15 plant extracts against virus of *Vigna mungo in vitro*, ginger extract gave the highest percentage inhibition after 1 h incubation and turmeric after 2 h. The effects of 4 extracts on the virus *in vivo* were less marked than *in vitro* and onion had the greatest effect, followed by turmeric, ginger and garlic. Othman *et al.,* (1991) investigated the antiviral effect of a crude extract of garlic bulbs on tomato mosaic tobamovirus (TMV) *in vitro*. The extract inhibited the virus when mixed with virus inoculum *in vitro* when used immediately, and after 1 or 2 hours. The extract was thermostable, inhibited local lesions produced by TMV and remained active up to a dilution of 10^{-2}. Viruses which had been treated with the extract were infectious to all varieties of tomato tested but the rate of replication within the host was lower for the extract-treated virus than for the untreated virus. Parida *et al*., (1997) reported that, four plants in traditional medicine in India were assessed for their *in vitro* potential to inhibit polio virus type-3 replication in VERO cells. The ethanolic extract of leaves inhibited polio virus type-3 replication by 99.9%; the aqueous extract was active (99.68% inhibition). Baba *et al*., (1999) showed that, diallyl pentasulfide (DPS), a constituent of aged garlic, dose-dependently inhibited Epstein-Barr virus early antigen (EBV-EA) expression induced by 12-O-tetradecanoyl-phorbol-13-acetate (TPA), a potent tumour-promoter. DPS did not affect TPA-enhanced 32Pi incorporation into phospholipids of cultured cells, although a low dose of DPS increased 32Pi incorporation. *In vivo*, DPS inhibited TPA-induced epidermal ornithine decarboxylase activity in mouse, and also suppressed the promoting effect of TPA on skin tumour formation in mice initiated with 7,12-dimethylbenz-[a]-anthracene. DPS seems to be one of the anti-carcinogenic constituents of garlic extract. Borek (2001) demonstrated that, Oxidative modification of DNA, proteins and lipids by reactive oxygen species (ROS) plays a role in aging and disease, including cardiovascular, neurodegenerative and inflammatory diseases and cancer. Extracts of fresh garlic that are aged over a prolonged period to produce aged garlic extract (AGE) contain antioxidant phytochemicals that prevent oxidant damage. These include unique water-soluble organosulfur compounds, lipid-soluble organosulfur components and flavonoids, notably allixin and selenium. Long-term extraction of garlic (up to 20 mo) ages the extract, creating antioxidant properties by modifying unstable molecules with antioxidant activity, such as allicin, and increasing stable and highly bioavailable water-soluble organosulfur compounds, such as S-allylcysteine and S-allylmercaptocysteine. AGE exerts antioxidant action by scavenging ROS, enhancing the cellular antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase, and increasing glutathione in the cells. AGE inhibits lipid peroxidation, reducing ischemic/reperfusion damage and inhibiting oxidative modification of LDL, thus protecting endothelial cells from the injury by the oxidized molecules, which contributes to atherosclerosis. AGE inhibits the activation of the oxidant-induced transcription factor, nuclear factor (NF)-kappaB, which has clinical significance in human immunodeficiency virus gene expression and atherogenesis. AGE protects DNA against free radical-mediated damage and mutations, inhibits multistep carcinogenesis and defends against ionizing radiation and UV-induced damage, including protection against some forms of UV-induced immunosuppression. AGE may have a role in protecting against loss of brain function in aging and possess other antiaging effects, as suggested by its ability to increase cognitive functions, memory and longevity in a senescence-accelerated mouse model. AGE has been shown to protect against the cardiotoxic effects of doxorubicin, an antineoplastic agent used in cancer therapy and against liver toxicity caused by carbon tetrachloride (an industrial chemical) and acetaminophen, an analgesic. Substantial experimental evidence shows the ability of AGE to protect against oxidant-induced disease, acute damage from aging, radiation and chemical exposure, and long-term toxic damage. Although additional observations are warranted in humans, compelling evidence supports the beneficial health effects attributed to AGE, i.e., reducing the risk of cardiovascular disease, stroke, cancer and aging, including the oxidant-mediated brain cell damage that is implicated in Alzheimer's disease. Masoomeh *et al*., (2003) used onion and garlic extracts to inhibit fungal growth and keratinolytic activity in *Trichophyton mentagrophytes* fungus as one of the major etiologic agents of human and animal dermatophytosis in Iran and other parts of the world. Eun *et al.* (2005) reported that, a pharmaceutical composition PENNEL comprising garlic oil (GO) and and other compounds, has been used as a curative preparation for patients with acute or chronic viral hepatitis. Chen *et al.,* (2006) illustrated that, the volatile oil was extracted from *Allium sativum*. It was diluted with distilled water to 250 micro g/ml solution and little DMSO was added to make the final concentration at 2 mg/ml. The tobacco mosaic virus (TMV) crude protein solution was added to the volatile oil and observation was done after 2 hours under room temperature. It was found that the volatile oil split the particles of TMV. It strongly inhibited TMV-CP polymerization in vitro, but did not obviously influence

the infectivity of TMV-RNA. The volatile oil also increased the in vitro activities of peroxidase (POD) and polyphenol oxidase [catechol oxidase] (PPO) when sprayed on Nicotiana tabacum . Ji *et al.,* (2007) investigate the anti-listerial effect of garlic shoot juice (GSJ) against the four strains of *Listeria monocytogenes* ATCC 19116, 19118, 19166 and 15313. Various concentrations of (1%, 2.5% and 5%) were used and applied for 0, 4, 7, 10 and 14 days at 4 °C and 0, 6, 12, 18 and 24 h at 37 °C. 5% GSJ showed the strongest anti-listerial effect against all the bacterial strains tested as compared to control at 37 °C. At 4 °C, 2.5% GSJ showed a strong growth inhibitory effect against all the bacterial strains when compared to control after 14 days.

The inhibitory effect of garlic and onion on the virus infectivity may due to many reasons. Serge and David (1991) who concluded that, allicin, one of the active principles of freshly crushed garlic homogenates, has a variety of antimicrobial activities. Allicin in its pure form was found to exhibit i) antibacterial activity against a wide range of Gram-negative and Gram-positive bacteria, including multidrug-resistant enterotoxicogenic strains of *Escherichia coli*; ii) antifungal activity, particularly against *Candida albicans*; iii) antiparasitic activity, including some major human intestinal protozoan parasites such as *Entamoeba histolytica* and *Giardia lamblia*; and iv) antiviral activity. The main antimicrobial effect of allicin is due to its chemical reaction with thiol groups of various enzymes, e.g. alcohol dehydrogenase, thioredoxin reductase, and RNA polymerase, which can affect essential metabolism of cysteine proteinase activity involved in the virulence of *E. histolytica*. Yamasaki *et al.,* (1991) examined the effect of allixin, a phytoalexin isolated from garlic, on aflatoxin B_1(AFB_1)-induced mutagenesis using *Salmonella typhimurium* TA100 as the bacterial tester strain and rat liver S9 fraction as the metabolic activation system. The effects of allixin on the binding of [^{3}H]AFB_1 to calf thymus DNA and on the formation of metabolites of [^{3}H]AFB_1 were also determined. Allixin showed a dose-related inhibition of Histidine$^+$ revertants induced by AFB_1. Allixin at 75 µg/ml inhibited [^{3}H]AFB_1 binding to calf thymus DNA and reduced formation of AFB_1-DNA adducts. In addition, allixin exhibited a concentration-dependent inhibition of the formation of organosoluble metabolites and the glutathione conjugates of [^{3}H] AFB1. The data indicate that the effect of allixin on AFB_1-induced mutagenesis and binding of metabolites to DNA may be mediated through an inhibition of microsomal P-450 enzymes. Allixin may thus be useful in the chemoprevention of cancer. Melcher *et al.* (1992) concluded that, many plant species resist systemic CaMV infection by preventing replication or local spread of CaMV, while others solely prevent systemic movement of infection. The inhibitory effect may due to the interference with the protein synthesis of the virus (Gangel, 2002), or may alter the gene transcription (mRNA) of the virus. Virginia (2006) concluded that, onion and garlic among the oldest cultivated plants, are used in the treatment and prevention of a number of diseases, including cancer. These activities are related to the thiosulfinates, volatile sulfur compounds. Besides these low-molecular weight compounds, onion and garlic are characterized by more polar compounds of phenolic and steroidal origin, often glycosilated, showing interesting pharmacological properties. Marta *et al.,* (2007) indicated that, garlic and onion possess many biological activities including antimicrobial, antioxidant, anticarcinogenic, ntimutagenic, antiasthmatic, immunomodulatory and prebiotic activities. Pin *et al.*, (2008) suggested that, the inhibitory effect of onion on the virus infectivity due to its two major flavonoids, quercetin and kaempferol. It is scientifically proven that garlic is effectively used against bacterial, viral, mycotic and parasitic infections. It's also known that garlic is a wonderful plant having the properties of empowering immune system, anti-tumour and antioxidant effects (Goncagul and Ayaz, 2010).

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Interaction Between Some Viruses Which Attack Tomato (*Lycopersicon esculentum* Mill.) Plants and Their Effect on Growth and Yield of Tomato Plants.

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Abstract: Interaction between tomato mosaic virus(ToMV), tomato yellow leaf curl virus(TYLCV) and cucumber mosaic virus(CMV) and their effect on growth and yield of tomato plants was studied. In symptoms and disease severity experiment, the most pronounced synergistic effects were caused by mixtures of ToMV+TYLCV and ToMV+TYLCV+CMV. Combination of ToMV+CMV caused slight symptoms. Generally, systemic symptoms were of the mosaic or mottling types in addition to different degrees of stunting and malformation. In interaction experiment, all virus combinations tested decreased the effect of ToMV on tomato plants, and few plants showed the characteristic pale-and dark-green mosaic symptoms of ToMV infection. In cross-protection experiment, the effect of double infection is depending on the subsequence of infections. When the plants were first inoculated with TYLCV and later with ToMV, the effect of TYLCV was prominent. On the other hand, first inoculation with ToMV suppressed the effect of the subsequent infection with TYLCV. Infection with ToMV supported the symptoms of the subsequent infection with CMV. This may be due to the weak symptoms of CMV which can be suppressed by the severe symptoms of the ToMV. On the other hand, infection with CMV suppressed the subsequent infection with ToMV. All virus treatments significantly reduced tomato height. TYLCV had the greatest effect (Mean height of plants was 27.75 cm.), while CMV was slightly reduced plant height (Mean height of plants was 34.95 cm). The tested viruses significantly reduced the yield of infected tomato plants. The greatest effect was obtained in the double infection with TYLCV + ToMV and TYLCV+CMV (Mean yield of plants was 130.15 and 139.06 gm. respectively). While CMV was slightly reduced plant yield (Mean yield of plants was 160.08 gm). [Journal of American Science 2010;6(8):311-320]. (ISSN: 1545-1003).

Key words: Tomato mosaic virus (ToMV), tomato yellow leaf curl virus (TYLCV), cucumber mosaic virus (CMV), interference, synergism and antagonism.

1. Introduction

Virus diseases are considered one of the most important problems affecting tomato production in many countries(Daniela *et al*., 2009; Murad *et al*., 2009; Salvatore *et al*., 2009; Sead *et al*., 2009; Torsten *et al*., 2009; Weimin *et al*., 2009; Akos and Ervin, 2010; and Pradeep and Masato, 2010). There are about 75 viruses infect this crop (Thornberry, 1966).

Tomato mosaic virus (ToMV) is widespread wherever tomato is grown. ToMV particles are rigid rods of 300 x 18 nm that contain single-stranded RNA (2000 kDa) and a coat protein of a single polypeptide, 21 kDa(Sutic *et al*., 1999). ToMV belongs to Tobamoviruses. Tobamoviruses contain more than a dozen rod-shaped viruses that cause serious losses in their hosts by damaging the leaves, flowers and fruits and by causing stunting of the plant (Agrios, 1997).

Tomato yellow leaf curl (TYLC) is one of the most devastating viral diseases of cultivated tomato (*Lycopersicon esculentum* Mill.) in tropical and subtropical regions worldwide, and losses of up to 100% are frequent. In many regions, TYLC is the main limiting factor in tomato production (Moriones and Navas-Castillo, 2000). TYLCV, belonging to geminiviruses, is a severe viral disease of tomato crops in the Mediterranean basin region. The disease has been reported in several countries. All commercial tomato varieties are susceptible to this disease. TYLCV is transmitted by the whitefly Bemisia tabaci. and fails to in infect plants when inoculated mechanically(Akad et al., 2004; Bosco *et al*., 2004; Jiang *et al*., 2004; Noris *et al*., 2004; and Parrella *et al*., 2004). Genome consists of DNA; single-stranded; circular; of two parts; largest (or only) genome part 2.787 kb; the 2nd largest 2.7 kb. Virions geminate; 20 nm in diameter; dimers 30 nm in length; angular in profile; without a conspicuous capsomere arrangement(Dalmon *et al*., 2003; Fekih-Hassan *et al*., 2003; Cui *et al*., 2004; and Onuki *et al*., 2004).

Cucumber mosaic virus (CMV) with its 59 strains causes damage for tomatoes production (Kaper and Waterworth, 1981). CMV infects many crops and cause huge reduction in the yield all over

the world. CMV appears to be one of the most important virus in Eastern China, Chroatia, France, Egypt, Greece, Italy, Japan, Poland, Portugal, Sweden, Australia and Northeastern United States(Tomlinson, 1987; Pares and Gunn, 1989; Mavrodieva, 1998; and Stommel et al, 1998). The percentage of crop losses in tomato was 100% in Italy and Spain in 1987 (Jorda *et al*, 1992); 80% in Australia; 20 % in melons in California (Grafton–Cardwell *et al*, 1996); and 50% in tobacco and pepper in Florida (Kucharek *et al*, 1998). Genome consists of RNA; single-stranded; linear; of three parts; largest (or only) genome parts the largest 3.389 kb; the 2nd largest 3.035 kb; the 3rd largest 2.197 kb. Virions isometric; not enveloped; 29 nm in diameter; rounded in profile; without a conspicuous capsomere arrangement. Virions contain 18 % nucleic acid; 82 % protein; 0 % lipid(Brunt, 1996).

Interference, the reduction of infection by one virus when two related viruses are used as inoculum together, has been extensively investigated. Exclusion mechanism may be operating such that when an infection is initiated with a particle of one strain of virus, a particle of a second strain cannot participate in the same infection. Interference by infectious agents occurs after attachment to host cells. Metabolic changes initiated by the interfering strain were the basis of the phenomenon. The proof of competitive exclusion at an infection site requires showing that the interfering strain does not multiply set reduces numbers of lesions produced by another strain. On the other hand, if the interfering strain does infect and multiply, then interference may involve interaction during multiplication (Cohen et al., 1983; Rochow *et al*., 1983; Sakai *et al*., 1983; Sherwood and Fulton, 1983; Sackey and Francki 1986; Ammar *et al*., 1987; and Marchoux *et al*., 1988).

Thus, the effect of ToMV, TYLCV and CMV on tomato symptoms, disease severity, plant growth and plant yield as well as the interaction of these viruses on tomato plants were the aims of the present investigation.

2. Material and Methods

1. Virus isolate:

1.1. Concerning ToMV, virus inoculum was the crude sap obtained by trituration of frozen leaves of tomato plants (*Lycopersicon esculentum* Mill.) seedlings showing mosaic symptoms. These symptoms developed 14 days after inoculation with a single local lesion obtained from *Nicotiana sylvestris* leaves that were inoculated with sap extracted from naturally infected tomato plants. Inoculation of leaves was carried out by rubbing with finger after their being dusted with carborandum.

1.2. Concerning TYLCV, virus-free colony of specific vector (*Bemisia tabaci*) raised on healthy squash seedlings were used in transmission of TYLCV from diseased tomato to healthy tomato plant. Insects were transferred to the diseased tomato plants to feed and become viruliferous. These viruliferous insects were used for transmission process.

1.3. Concerning CMV, virus inoculum was the crude sap obtained by trituration of frozen leaves of cucumber plants (*Cucumis sativus* L..) seedlings showing mosaic symptoms. These symptoms developed 14 days after inoculation with a single local lesion obtained from *Chenopodium amaranticolor* leaves that were inoculated with sap extracted from naturally infected cucumber plants. Inoculation of leaves was carried out by rubbing with finger after their being dusted with carborandum.

2. Interaction experiment:

The effect of single, double and mixed infection with ToMV, TYLCV and CMV on the symptoms, disease severity, height and yield of tomato (*Lycopersicon esculentum* Mill. cv. Cassel rock) plants was studied. ToMV and CMV were inoculated mechanically, while *Bemisia tabaci* insect was used to transmit TYLCV from diseased to healthy plants. Symptoms were recorded weekly for 3 successive weeks. Plant height was recorded after 4 weeks of inoculation. Four replicates were used in this study, each containing 5 plants. Data obtained were statistically analyzed according to Steel and Torrie, (1960). It was observed that the pale-and dark-green mosaic symptom is characteristic of ToMV infection. Therefore, to determine the interaction between this virus and other viruses of tomato, the tested viruses were inoculated on tomato seedlings, then later (15 days after symptom appearance), ToMV was inoculated to the same infected plants of each treatment. The number of plants which showed pale-and dark-green mosaic symptom of each treatment was recorded.

3. Results and Discussion

1. Effect of ToMV, TYLCV and CMV on symptoms and disease severity of tomato plants:

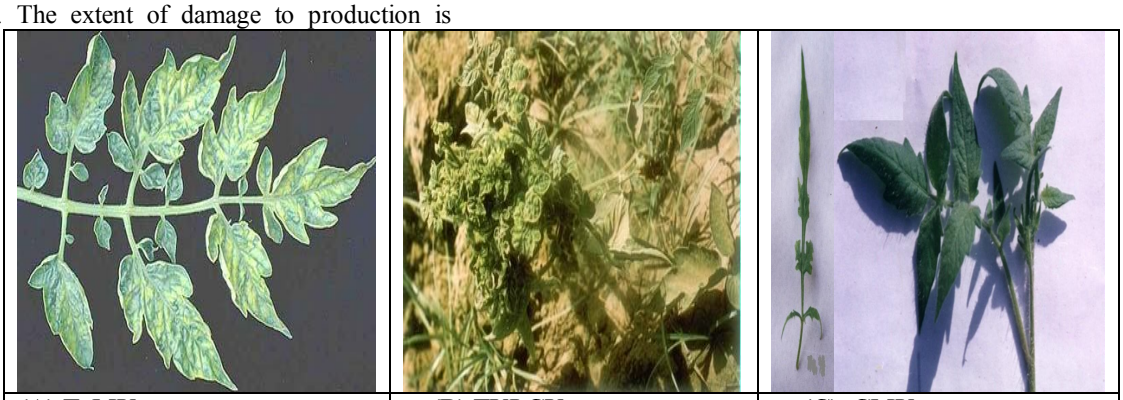
Tomato seedlings were inoculated with single, double and mixed infection with ToMV, TYLCV and CMV. Measurements on symptoms and disease severity were recorded.

It was found that, symptoms can be used to differentiate between ToMV, TYLCV and CMV during the early stages of inoculation. ToMV causes a pale-and dark-green mosaic on the young leaves which became malformed, and stunting of the plants.

The most damaging symptom is necrosis in leaves, along the stems and on the fruits. This result agrees with that obtained by Singh (1983) and Sutic *et al.* (1999).

TYLCV-infected tomato leaves are small, malformed, curled upward, and severely chlorotic. Yield losses can reach 80%. This result agrees with that obtained by Brunt (1996) and Sutic *et al.* (1999).

On the other hand, CMV-infected tomato displays pronounced pathological changes. Mild mosaic and mottle first appears in leaves and becomes more evident with development of the disease in the formation of new leaves of abnormally narrowed/elongated thread-like formations Shortened and compressed internodes alter the general plant appearance. Virulent strains cause necrosis along leaf veins, necrotic streaks along the stems, and death of shoot tips. The extent of damage to production is dictated by the number of infected plants. This result was similar with that obtained by Brunt (1996) and Sutic *et al.* (1999). The main symptoms of ToMV, TYLCV and CMV inoculated singly or in combination to tomato plants are shown in Table (1) and Fig. (1). Mixed inoculation with all three viruses caused a more severe disease than either alone. The first symptoms appeared one week after inoculation and the infected plants showed severe symptoms. Mixtures of the two viruses caused less severe symptoms. The most pronounced synergistic effects were caused by mixtures of ToMV+TYLCV and ToMV+TYLCV+CMV. Combination of ToMV+CMV caused slight symptoms. Generally, systemic symptoms were of the mosaic or mottling types in addition to different degrees of stunting and malformation.



(A) ToMV symptoms on tomato plants	(B) TYLCV symptoms on tomato plants	(C) CMV symptoms on tomato plants

Fig. (1): Symptoms of some viruses affecting tomato (*Lycopersicon esculentum* Mill. cv. Cassel rock) plants. (A) ToMV symptoms, (B) TYLCV symptoms and (C) CMV symptoms.

2. Interaction between ToMV and other viruses of tomato plants:

A pale-and dark-green mosaic symptom is characteristic of ToMV and was used to differentiate between the effects of ToMV and other viruses in tomato. Table (2) and Fig. (2) show that infection with ToMV alone caused extremely severe pale-and dark-green mosaic symptoms, whereas, pale-and dark-green mosaic symptoms on leaves with dual infection were less severe and few plant showed such symptoms. When all three viruses were inoculated simultaneously on tomato leaves, ToMV produced slight pale-and dark-green mosaic symptoms. It can be concluded that all virus combinations tested decreased the effect of ToMV and few plants showed the characteristic pale-and dark-green mosaic symptoms of infection.

3. Cross protection between ToMV and other viruses of tomato plants:

Data obtained in Table (3) and Fig. (3) show that, the effect of double infection is depending on the subsequence of infections. When the plants were first inoculated with TYLCV and later with ToMV, the effect of TYLCV was prominent. On the other hand, first inoculation with ToMV suppressed the effect of the subsequent infection with TYLCV. In this respect, partial antagonism may be suggested to be occurred between ToMV and TYLCV. Results obtained on the effect of double infection on disease symptoms indicated that infection with ToMV supported the symptoms of the subsequent infection with CMV. This may be due to the weak symptoms of CMV which can be suppressed by the severe symptoms of the ToMV. On the other hand, infection with CMV suppressed the subsequent infection with ToMV. So, antagonistic effect may be suggested to be occurred between ToMV and CMV. Generally, these results show that the extend to which antagonistic or Synergistic effects occur depends on the timing of the inoculations.

4. Effect of single, double and mixed infection with ToMV, TYLCV and CMV on the height of tomato plants:

According to our knowledge, the effect of these three viruses on plant height have not carried out before. The effect of virus infection on growth of tomato plants was measured(Table 4 and Fig. 4). Under greenhouse condition, it was found that single infection with ToMV, TYLCV or CMV significantly reduced plant height. Inhibitory effect of virus infection on tomato growth is a common phenomenon and it had reported by several investigators. It could be mentioned that TYLCV markedly reduced plant height and significant differences were detected between TYLCV-infected plants and those infected either with ToMV or CMV. When tomato plants were inoculated with ToMV or CMV and 15 days later re-inoculated with ToMV or CMV respectively, no significant differences were detected between double infected plants and those single inoculated either with ToMV or CMV. Concerning TYLCV, the effect of double infection is depending on the subsequence of infections, when the plants were first inoculated with TYLCV and later with ToMV or CMV, the effect of TYLCV was prominent. On the other hand, first inoculation with ToMV or CMV suppressed the effect of the subsequent infection with TYLCV. In this respect, partial antagonism may be suggested to be occurred between ToMV and TYLCV or CMV. Simultaneous infection with the three viruses (ToMV, TYLCY and CMV) at the same time, significantly reduced the plant height as compared by healthy plants or those inoculated with ToMV only. No significant were detected between simultaneously inoculated plants and those singly inoculated with TYLCV. Results obtained on the effect of double infection on disease symptoms indicated that infection with ToMV supported the symptoms of the subsequent infection with CMV. This may be due to the weak symptoms of CMV which can be suppressed by the severe symptoms of the ToMV. On the other hand, infection with CMV suppressed the subsequent infection with ToMV. So, antagonistic effect may be suggested to be occurred between ToMV and CMV. Generall y, these results show that the extend to which antagonistic or Synergistic effects occur depends on the timing of the inoculations. On the other hand, no significant differences were detected between plants inoculated with ToMV and those infected with CMV.

5. Effect of single, double and mixed infection with ToMV, TYLCV and CMV on the yield of tomato plants:

Regarding the yield of tomato plants, it was found that the tested viruses significantly reduced the yield of infected plants (Table 5 and Fig. 5). Previous infection with TYLCV suppressed the symptoms of the subsequent infection either with ToMV or CMV. Previous infections with ToMV elongated the incubation period necessary for TYLCY symptoms for about 2-3 weeks and then, the symptoms of TYLCY started to suppress the symptoms of ToMV. Suppression of ToMV or CMV symptoms by TYLCV infection may be due to severe symptoms and leaf curling caused by TYLCV. In this experiment, the greatest effect was obtained in the double infection with TYLCV + ToMV and TYLCV+CMV (Mean yield of plants was 130.15 and 139.06 gm. respectively).

Table (1): Interaction between three viruses in tomato (*Lycopersicon esculentum* Mill.cv. Cassel rock) plants and its effects on symptoms and disease severity.

Virus combination		Symptom	Disease severity
Control (no virus)	-	-*	-
Virus alone	ToMV	Severe Mosaic, leaf malformation	Severe
	TYLCV	Curling, leaf malformation	Severe
	CMV	Slight mosaic	Slight
Pair of viruses	ToMV+ TYLCV	Mosaic, Curling, leaf malformation	Severe
	ToMV+CMV	Mosaic	Moderate
	TYLCV+CMV	Curling, Mosaic	Moderate
All three viruses	ToMV+ TYLCV+CMV	Mosaic, Curling, leaf malformation	Severe

*(-): no symptoms.

Table(2): Interaction between tomato mosaic virus and other two viruses of tomato (*Lycopersicon esculentum* Mill.cv. Cassel rock) plants

Virus combination	Severity of pale-and dark-green mosaic symptom	Number of plants showing typical symptom	Percentage of affected plants
Control(no virus)	-	-	-
ToMV	Severe	20/20	100
ToMV+ TYLCV	Moderate	13/20	65

ToMV+CMV	Moderate	9/20	45
ToMV+ TYLCV+CMV	Severe	11/20	55

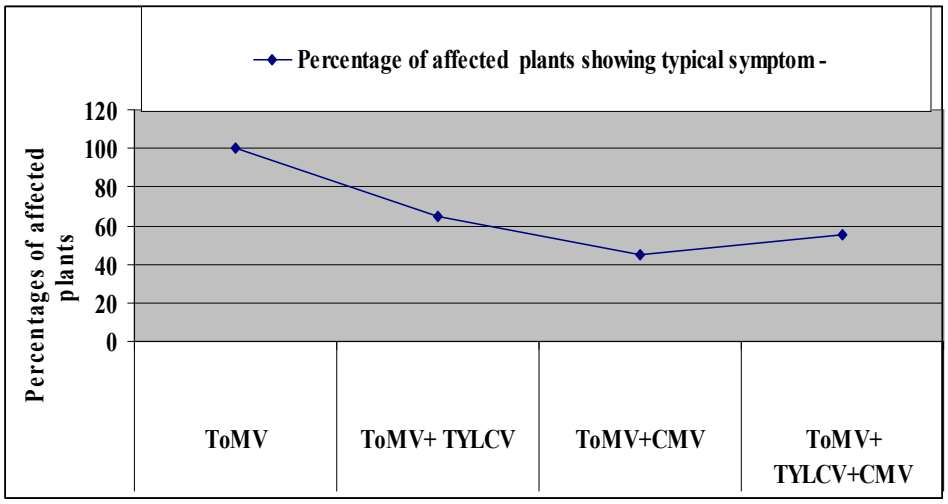


Fig.(2): Percentage of affected plants showing typical symptom(pale-and dark-green mosaic symptom) as a result of interaction between tomato mosaic virus and other two viruses of tomato (*Lycopersicon esculentum* Mill. cv. Cassel rock) plants.

Table(3): Cross protection between tomato mosaic virus and other two viruses of tomato (*Lycopersicon esculentum* Mill.cv. Cassel rock) plants.

Virus combination	Severity of pale-and dark-green mosaic symptom	Number of plants showing typical symptom	Percentage of affected plants
TYLCV	Moderate	10/20	50
CMV	Slight	7/20	35

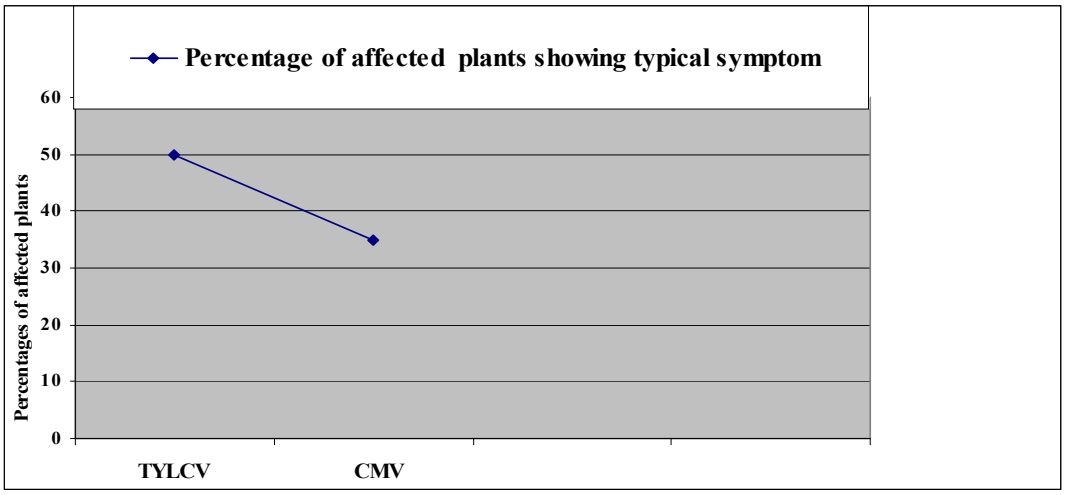


Fig.(3): Percentage of affected plants showing typical symptom(pale-and dark- green mosaic symptom) as a result cross protection between tomato mosaic virus and other two viruses of tomato (*Lycopersicon esculentum* Mill. cv. Cassel rock) plants

Table (4): Effect of single, double and mixed infection with ToMV, TYLCV and CMV on the height of tomato (*Lycopersicon esculentum* Mill.cv Cassel rock) plants after 4 weeks from the first inoculation.

First inoculation	Second inoculation	Mean length of plants(cm)
Control(no virus)	-	38.70
ToMV	-	32.25
TYLCV	-	27.75
CMV	-	34.95
ToMV+ TYLCV+CMV	-	30.75
ToMV	TYLCV	31.95
ToMV	CMV	34.50
TYLCV	ToMV	28.50
TYLCV	CMV	30.45
CMV	ToMV	33.30
CMV	TYLCV	31.50
L.S.D at 5%	3.15	

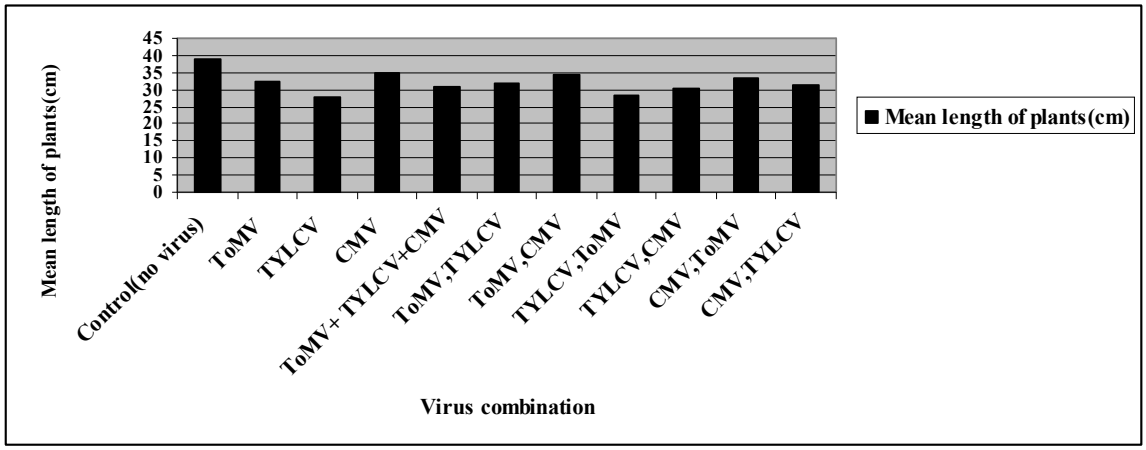


Fig.(4). Effect of single, double and mixed infection with ToMV, TYLCV and CMV on the height of tomato (*Lycopersicon esculentum* Mill. cv. Cassel rock) plants after 4 weeks from the first inoculation.

Table(5): Effect of single, double and mixed infection with ToMV, TYLCV and CMV on the yield of tomato (*Lycopersicon esculentum* Mill. cv. Cassel rock) plants.

First inoculation	Second inoculation	Mean yield of plants(gm)
Control(no virus)	-	219.00
ToMV	-	152.25
TYLCV	-	147.75
CMV	-	160.08
ToMV+ TYLCV+CMV	-	141.34
ToMV	TYLCV	145.91
ToMV	CMV	157.56
TYLCV	ToMV	130.15
TYLCV	CMV	139.06
CMV	ToMV	152.08
CMV	TYLCV	142.86
L.S.D at 5%	17.15	

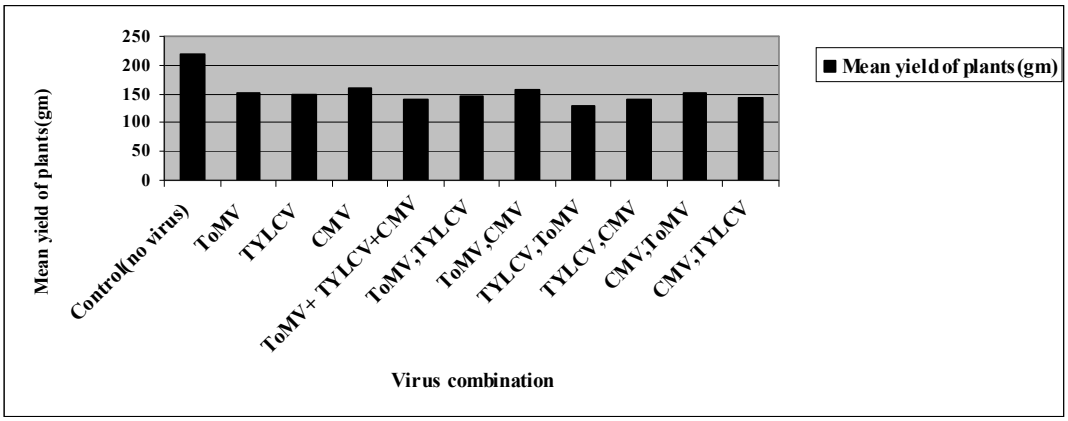


Fig.(5). Effect of single, double and mixed infection with ToMV, TYLCV and CMV on the yield of tomato (*Lycopersicon esculentum* Mill. cv. Cassel rock) plants.

Nitzany and Sela (1962) described interference between tobacco mosaic virus (TMV) and a strain of cucumber mosaic virus, causing local lesions on *Zinnia elegans*, (CMV-LL). Interference was observed on *Nicotiana repanda* and *N. glutinosa* when CMV-LL functioned as protecting virus, as well as on *Z. elegans* when the protecting virus was TMV. Simultaneous inoculations of *N. repanda* or *N. glutinosa* with the two viruses prevented the establishment or the multiplication of CMV-LL. Increasing concentrations of TMV in the challenge inocula resulted in an increased percentage of protection. This supports the hypothesis that the protecting virus occupies some cellular structures or exhausts some precursors needed for the establishment or multiplication of the challenge virus. Eastwell and Kalmar (1997) reported that, in certain cultivars of cowpea (*Vigna unguiculata*) that are operationally immune to cowpea mosaic virus strain SB (CPMV), coinoculation of CPMV with cowpea severe mosaic virus strain DG (CPSMV) reduces severity and delays expression of symptoms normally induced by CPSMV alone. In cultivars susceptible to both viruses, coinoculation delays development of symptoms in response to CPSMV. Using monoclonal antibodies for serological assays and virus-specific RNA probes for hybridization, it is demonstrated that the presence of CPMV in the inoculum yields a concomitant delay in the synthesis of CPSMV coat protein and replication of CPSMV RNA and restricts the transport of CPSMV out of infection centers. Only bottom component of CPMV containing RNA1 is required to offer protection against CPSMV. Destroying the integrity of CPMV RNA eliminates its protective capability. In cowpea cultivars that are operationally immune to CPMV, the presence of CPSMV in the inoculum is unable to compensate for events of CPMV replication that are inhibited. The lack of complementation suggests a high degree of specificity in the replication of these 2 comoviruses. Hristova and Maneva(1999) studied the effect of cucumber mosaic virus (CMV) and broad bean wilt virus (BBWV) as single and mixed infections on the quantity and quality of *Capsicum annuum* yield. It was found that the susceptibility of *C. annuum* depends on both virus type and plant cultivar, the determining factor being the timing of viral infection. CMV infection had the strongest effect on *C. annuum* followed by BBWV and mixed infection. Mixed infection shows an effect of interference. The total yield and number of marketable fruits as a percentage of total fruits produced by an infected plant was one of the most representative parameters. The proportion number of unmarketable to marketable fruits was a very important characteristic for estimating the reduction caused by a viral infection on the *C. annuum* yield quality. Aguilar *et al.,* (2000). Oilseed rape mosaic virus (ORMV) and tobacco mild green mosaic virus (TMGMV) were mechanically inoculated onto *Arabidopsis thaliana* and *Nicotiana tabacum* 15 days after transplanting and at the 4-leaf stage, respectively. The interactions between the 2 viruses were studied in 2 types of experiments. In the first experiment, ORMV and TMGMV were co-inoculated by mixing and inoculating on one leaf. In the second experiment, ORMV and TMGMV were inoculated onto different leaves at different times. One virus was inoculated as the protecting virus and the second virus was inoculated as the challenging virus 7, 14, 21 or 28 days after inoculation of the protecting virus. The viruses were detected by dot-blot hybridization. The result of co-inoculation was the same for both hosts: there was strong interference, with ORMV being the more successful of the two viruses. In tobacco, whichever virus acted as the protecting virus, it interfered significantly with the multiplication of the challenging virus, providing cross-protection. Regardless of the time elapsed between the inoculation of both viruses, the protecting virus always inhibited the

accumulation of the challenging virus. In *A. thaliana*, when TMGMV was the protecting virus, it protected the plants from ORMV infection. When ORMV was used as the protecting virus, TMGMV was not detected, but as TMGMV infection is symptomless and slow to develop in *A. thaliana*, the existence of cross-protection could not be determined. Miguel *et al.,* (2009) illustrated that, tomato rugose mosaic virus (ToRMV) and tomato yellow spot virus (ToYSV) infect tomatoes. ToYSV symptoms in tomato and *Nicotiana benthamiana* appear earlier and are more severe compared to those of ToRMV. Results indicate that ToYSV establishes a systemic infection and reaches a higher concentration earlier than ToRMV in both hosts. ToRMV negatively interferes with ToYSV during the initial stages of infection, but once systemic infection is established this interference ceases. In *Nicotiana benthamiana*, ToYSV(Tomato yellow spot virus) invades the mesophyll, while ToRMV(Tomato rugose mosaic virus) is phloem-restricted. During dual infection in this host, ToYSV releases ToRMV from the phloem.

There are many opinions for explanation of interference phenomenon; Sherwood and Fulton (1983) suggested that the specificity of interference lies at the virus-replication stage. It was concluded that both competition for infection sites and multiplication of the interfering strain are involved in the interference phenomenon Horikoshi *et al.,* (1987) suggest that the inhibitory effect is due to the interference with the binding site of replicase(necessary for RNA synthesis) by partial reassembly of nucleoprotein and that this phenomenon may be a cause of cross protection. Results obtained by Rao- and Hall (1991); Romero *et al*., (1994); Hsu-YauHeiu *et al*., (1998) and Teycheney and Tepfer (2001) show that, viral infection can interfere with post-transcriptional gene silencing (PTGS) of a native plant gene, and that this can have profound effects on symptom expression. Khan *et al.,* (1994) suggested that, suppression of bean common mosaic potyvirus (BCMV) str. NL3 symptoms by mosaic-inducing str. NY15is not caused by impeding its multiplication, but by delaying its transport to the xylem of petiole and stem. Huntley and Hall (1996) concluded that, the observed interference, with brome mosaic virus replication in transgenic rice, appeared to be mediated through viral RNAs rather than protein products, but was not proportional to detectable levels of messenger expression, suggesting the induction of a host-defense mechanism. Ranjith-Kumar *et al.,* (1998) suggested that, interference with physalis mottle tymovirus replication could be due to the formation of RNA-RNA hybrids at the 3' end of the genomic RNA. DaPalma *et al.,* (2010) concluded that, virus-virus interactions can be organized into three main categories: (1) direct interactions of viral genes or gene products[such as, Helper-dependent viruses, Pseudotype viruses, Superinfection exclusion, Genomic recombination, Embedded viruses, Heterologous transactivation], (2) Environmental interactions or indirect interactions that result from alterations in the host environment[such as, Indirect transactivation of genes, Breakdown of physical barriers, Altered receptor expression, Heterologous activation of pro-drugs, Modification of the interferon-induced antiviral state] and (3) immunological interactions[such as, Altered immune cell activation, Induction of autoimmunity, Antibody-dependent enhancement of infection, Heterologous immunity].

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Ricotta Cheese from Whey Protein Concentrate

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Abatract: Ricotta cheese was made from UF-whey protein concentrate (WPC) fortified with 2, 4 and 6 % skim milk powder (SMP), and acidified with 0.14 g/kg citric acid or 1.5 % Glucono-Delta-Lactone (GDL). All WPC mixtures were homogenized at 300 kp/cm^2 first stage and 200 kp/cm^2 second stage at 60°C. The fresh cheese yield was determined. Samples were taken from whey, WPC mixtures and cheese. The samples were analysed for moisture, nitrtogen, ash, pH, lactose and fat contents. Viscosity of WPC mixtures and cheeses were also assessed. The cheese samples were also assessed for organoleptic properties.The best results were obtained from cheese treated with citric acid , this treatment showed the higher cheese yield, higher viscosity and best organoleptic quality than those made with GDL. Ricotta cheese made from WPC showed the best organoleptic properties followed by that made from WPC fortified with 2% SMP. The results indicated that WPC ingredient can be successfully use in the manufacture of Ricotta cheese. [Journal of American Science. 2010;6(8):321-325]. (ISSN: 1545-1003).

Key words: Ricotta cheese, WPC, SMP, Citric acid, GDL

1. Introduction

The consumption and manufacture of cheese is incresing worldwide at a rate of about 2% par year. As a result, the amount of cheese whey is also incresing and is estimated to be some 130 million tonnes annually (Korhonen et al., 1998).

The disposal of whey represented a serious enviromental problems, as it contains a great deal of organic substances with a high (B.O.D). In fact whey contains (6-7%) more than half of the solids in the original milk, including about 20% of the protein, and most of lactose, minerals and water-soluble vitamines (Zall, 1992).

Now, whey is increasingly used for human consumption, the principal processes applied are concentration, grying and isolation of whey proteins by means of membrane separation, such as reverse osmosis (RO), ultrafiltration (UF) and diafiltration.

Whey proteins are known by their health effects. Boumous et al. (1988) reported the immune enhancing property of WPC. A commercial preparation of whey proteins inhibited HIV virus (Bounos, 1997). McIntosch et al. (1998) demonstrated the anticarcinogenic effect of whey proteins against colon cancer. This has been due to their high content of sulphur amino acids.

Nowadays, utilization of whey proteins and WPC to food and dairy productsis considered to be one of the most important functional foods due to their effects either on the product characteristics and/or the health promotion (Shenana, et al. 2007)

Ricotta cheese is a dairy product of Italian origin, which means “recooked”, it is produced by boiling acidified cheese whey (Maubois and Kosikowski, 1978).

Ricotta cheese is a high moisture soft cheese (Modler and Emmons, 2001). It can be produced using cheese whey or milk, or a mixture of both (Pizzillo et al.,2005). Ricotta cheese is very mild and it is used in many Italian dishes.

Several methods have been developed for utilization of whey, one of these methods is the manufacture of Ricotta cheese. Fresh Ricotta cheese had a mild and muty flavour and is used as a flavour enhance in Salad (Kosikowski, 1982). Several methods have been suggested for Ricotta cheese making (Weatherup, 1986 and Modler & Emmons, 1994). These include study the effect of type of acidulant on the quality of Ricotta cheese.

Production of Ricotta cheese has been considered to be one of the economical way for the utilization of whey. So, the manufacture of Ricotta cheese could easily be undertaken as an additional source of income (shukla et al., 1986).

Therefore, this work was carried out to study the effect of use WPC fortified with SMP on the composition and quality of Ricotta cheese.

2. Materials and Methods

Preparation of WPC:

The method of EL-Sheikh et al., (2001) for the preparation of WPC was adopted as follows:

-Ultrafiltration of Edam cheese whey obtained from Arab Dairy Company (Kaha-Kaluobia, Egypt) was carried out in National Research Centre by using a Carbosep pilot plant (Orelis-France) with 6.3 m^2 inorganic membranes at 4 bars transmembrane pressure at 50°C and pH 7 had in a batch system to volume concentration 20.

-SMP: Skim milk powder (low heat) USA origin with average chemical composition (according to supplier):

Moisture: 5.20 %
Proteins: 32.15 %
Fat : 1.20 %
Ash: 7.44 %
Lactose: 54.30 %

-Acidulants: Food grade, Citric acid and GDL were used

Preparation of WPC mixtures:

WPC was fortified with 2, 4, and 6% SMP respectively. Control cheese was made from WPC without SMP addition. Ricotta cheese making (each of three replicated) as follows:

- Control 2 Kg WPC
- Tr.1 1.96 Kg WPC + 0.04 Kg SMP
- Tr.2 1.92 Kg WPC + 0.08 Kg SMP
- Tr.3 1.88 Kg WPC + 0.12 Kg SMP

All treatments were acidified using 0.14 gm/Kg citric acid or 1.5 % GDL.

Ricotta cheese manufacture:

Ricotta cheese was manufactured as described by Mahran et al., (1999).

All WPC mixtures were homogenized (2 stage) at 300/200 Kp/cm^2 at 60°C using laboratory homogenizer (Rannie, Copenhagen). All mixtures were heated at 90°C/15-30 min. The curd was left in the whey for 10 min and then scooped in plastic frame lined with cheesecloth and placed over a drainage table and allows to achieve complete drainage in 24 hr. The curd was packed in plastic container and stored at 4°C.

Methods of analysis:

The whey, WPC mixtures and cheese samples were analysed for moisture, fat and ash as given by A.O.A.C.(1990) and total nitrogen as described in IDF Standard (1986). Lactose was determined according to Barnett and Abdel-Tawab (1957). pH value was measured using a laboratory pH meter with glass electrode.

Determination of WPC viscosity:

The viscosity of WPC mixtures were measured according to Farrag et al. (2006). A coaxial cylinder viscometer (Bohlin V88, Sweden) attached to a work station loaded with soft ware V88 viscometry programme was used. The system (C30) was filled with the WPC samples at 25 °C and measurement of shear stress and viscosity was carried out in the up mode at shear rates ranging from 21 to 144 1/s .

Measurment of Ricotta cheese viscosity:

Bohlin viscometer (Bohlin V88, Sweeden) equipped with a cone and plate system (CP 5.4/30) and attached to a workststion loaded with V88 viscosity program was used. About 1-2 gm of the cheese sample with a flat surface was placed on the plat and allowing the cone to touch the surface of the cheese sample. The viscosity was measured at 25°C with shear rate of 33 1/s virsus time. A total of 6 measurememts were carried out.

Cheese yield:

Cheese yield was calculated as kg of fresh cheese per kg of WPC mixture.

Organoleptic assessment:

Ricotta cheese samples were scored for organoleptic properties by a taste panel of 11 persons for National Research Centre staff as described by 15 Mahran et al., (1999). The panelists scored the cheese flavour (out of 40 points); body & texture (out of 50 points) and appearance (10 points).

3. Results and Discussion

Composition of WPC mixtures:

Table (1) shows the chemical composition of Edam cheese whey which used in the preparation of WPC mixtures.

Total solids content of WPC (control) was 13.95 % while in the other treatments increased from 15.65 in Tr.1 to 17.33 in Tr.2 and 18.93 % in Tr.3 this variations has been attributed to SMP addition, also protein content increased from 7.41 % in control WPC to 7.96 in Tr.1,to 8.51 in Tr.2 and 9.06 % in Tr.3. The same trend was noticed for lactose and ash contents.

Table (1) :Gross composition of Edam cheese whey, WPC and WPC mixtures used in Ricotta cheese manufacture (average of 3 replicates).

%	Whey	WPC	Tr. 1	Tr. 2	Tr. 3
Total solids	7.1	13.95	15.65	17.33	18.93
Protein	0.7	7.41	7.96	8.51	9.06
Ash	0.8	2.3	2.35	2.37	2.40
Lactose	4.8	4.1	5.11	6.13	7.15

Fat	-	0.1	0.1	0.1	0.1

Tr.1: WPC + 2% SMP
Tr.2: WPC + 4% SMP
Tr.3: WPC + 6% SMP

The low fat content of WPC mixtures due to the separation of fat by high speed separator in the company beforee ultrafiltration process.

Viscosity of WPC mixtures:

Fig (1) and (2) shows the viscosity of WPC mixtures measured at 25°C at share rate ranging from 21 to 144 1/s. The lowest intial viscosity showed with WPC without SMP addition. The intial viscosity increased with the increasing adition of SMP. All treatments showed increasing in viscosity as share rate increase.

In general, viscosity of WPC mixtures acidified with Citric acid were higher than those acidified with GDL.

Gross composition of cheese:

Tables (2 and 3) shows that the total solids contents of Ricotta cheese increased with the incresing of SMP addition. Also, protein content increased, the same trend was observed for lactose and ash contents, the pH values also inreased with SMP addition increased.

Total proteins, lactose and ash contents of Ricotta cheese made from WPC mixtures acidfied by using 1.5 % GDL were higher than that made from WPC mixture acidfied by using Citric acid while, it's pH values were lower

Table (2): Composition of Ricotta cheese made from WPC mixtures acidfied with 1.5 % GDL (average of 3 replicates).

%	Total solids	Protein	Lactose	Ash	pH value
WPC	25.34	17.55	3.78	2.46	5.35
Tr. 1	27.54	19.09	5.00	2.71	5.61
Tr. 2	29.92	20.67	5.50	3.03	5.80
Tr.3	31.08	21.30	5.90	3.31	5.90

Tr.1: WPC + 2% SMP
Tr.2: WPC + 4% SMP
Tr.3: WPC + 6% SMP

Table (3): Composition of Ricotta cheese made from WPC mixtures acidfied with 0.14% Citric acid (average of 3 replicates).

%	Total solids	Protein	Lactose	Ash	pH value
WPC	24.05	16.45	3.85	2.26	5.91
Tr. 1	26.38	18.05	4.85	2.45	5.96
Tr. 2	28.86	19.69	5.31	2.81	6.10
Tr.3	30.57	20.95	5.78	3.10	6.21

Tr.1: WPC + 2% SMP
Tr.2: WPC + 4% SMP
Tr.3: WPC + 6% SMP

Cheese yield:

Table (4), shows the fresh yield of Ricotta cheese made from different mixtures of WPC. WPC fortified with 6% SMP (Tr. 3) has the higher fresh yield followed by WPC fortified with 4% SMP (Tr. 2) and WPC fortified with 2% SMP (Tr. 1) compare to control treatment that made from WPC without SMP addition. The cheese yield increased with the increasing of SMP additiion. These resulta are in agreement with those reported by Shahani, (1979) and Mathur & Shahani, (1981).

Also, the fresh yield of Ricotta cheese made from WPC mixtures acidified with Citric acid was higher than that made from WPC mixtures acidfied with GDL

Table (4): Yield % of Ricotta cheese made from WPC mixtures (average of 3 replicates).

Treatment	Cheese from GDL	Cheese from Citric acid
WPC	20.11	23.56
Tr. 1	24.66	27.32
Tr, 2	29.09	30.18

Tr. 3	33.33	36.16

Tr.1: WPC + 2% SMP
Tr.2: WPC + 4% SMP
Tr.3: WPC + 6% SMP

Cheese viscosity:

Fig (3) and (4) Shows the rheogram for viscosity versus time for Ricotta cheese samples. The cheeses differed in their intial viscosities. The lowest intial viscosity was 12.12 Pas for cheese made from WPC acidified with 1.5% GDL, while the maximum was 25.31 Pas for cheese made from WPC fortified with 6% SMP and acidified with 0.14g/kg of Citric acid. All treatments showed a noticeable drop in viscosity at successive intervals. Generally, the cheese made from WPC acidified with 1.5% GDL were low viscosity than that made from acidified with 0.14 g/kg Citric acid.

Organoleptic assessment:

Table (5) shows the organoleptic evaluation of the different treatments of Ricotta cheese. Ricotta cheese made from WPC without SMP addition was ranked higher score than the other treatments followed by Tr. 1, Tr. 2 and Tr. 3 respectively.

Ricotta cheese made from WPC acidified with 0.14 g/kg of Citric acid was judged better than that made fromWPC acidified with 1.5% GDL.

Ricotta cheese made from WPC without SMP addition had a good quality (soft and moist-griny texture). The quality of Ricotta cheese was decreased with SMP addition increased, beside the cheese made from WPC fortified with SMP had a sweet taste especially with Tr. 2 and Tr. 3.

The results indicate that the addition of SMP (not more than 2%) to WPC was enough to obtain Ricotta cheese with a good quality near to that made from WPC without SMP addition.

Table (5): Organoleptic properties of Ricotta cheese (average of three replicates).

	Cheese acidified with GDL				**Cheese acidified with Citric acid**			
	WPC	**Tr.1**	**Tr.2**	**Tr.3**	**WPC**	**Tr.1**	**Tr.2**	**Tr.3**
Appearance								
10	9.2	8.5	7.3	7.3	9.0	8.5	7.2	7.2
Flavour								
40	36.6	33.3	32.6	32.3	37.2	36.4	33.2	32.8
Body and texture								
50	46.6	43.6	40.0	39.3	47.3	45.8	42.8	40.6
Total								
100	92.4	85.4	79.9	78.8	93.5	90.7	83.2	80.6

Tr.1: WPC + 2% SMP
Tr.2: WPC + 4% SMP
Tr.3: WPC + 6% SMP

4.Conclusion

It can be concluded that it is possible to successively use whey protein concetrate (WPC) for Ricotta cheese with good flavour and a high acceptability specially with WPC without SMP.

Production of Ricotta cheese has been considered to be one of the economical way for the utilization of whey. So, the manufacture of Ricotta cheese could easily be undertaken as an additional source of income.

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Molecular Analysis of Genetically Improved Therapeutic *Saccharomyces Cerevisiae* Strains with High Selenium Uptake

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Abstract: This study was initiated to take advantages of mutagenesis in conjunction with protoplast fusion technique to obtain hyper- polyploidy strains with high selenium uptake. After UV-mutagenesis, results showed that five mutants uptake nearly all selenium present in growing medium and six mutants uptake 3.5 fold of selenium than the original strain. Moreover, selenium uptake of superior yeast mutants in the presence of selenium oxide (100 mg/L) showed that yeast cells tolerate high selenium concentration in the growing medium and uptake percentages ranging from 46.84 to 79.38. Intra-specific protoplast fusion technique was carried out in order to obtain polyploidy isolates with high selenium uptake. Data showed that 38 fusants obtained from cross 1 and 36 fusants from cross 2. In cross No.1, 71 % of fusants uptake more selenium than the first parent (mutant 5/8) and 97.4% than the second parent (mutant 5/9). For cross 2, 36.9 % of fusants uptake more selenium than the first parent (5/8) and 100 % than the second parent (20/1). Finally, an attempt was done to evaluate the genetic effect of UV-mutagenesis and protoplast fusion on nucleotide sequence by random amplified polymorphic DNA (RAPD) analysis. Many differences were noticed in mutant and fusant strains in comparison the wild type strains. These differences in RAPD profiles confirmed the evidence of genetic variations in yeast genome after mutagenesis and protoplast fusion technique. Also, these differences could be used as genetic markers for genetic diversity of selenium uptake characteristics. [Journal of American Science. 2010;6(8):326-337]. (ISSN: 1545-1003).

Key words: Selenium uptake, UV-mutagenesis, protoplast fusion and RAPD

1. Introduction

Living organisms possess genetic responses to chemical stress triggered by a variety of chemical inducers, involving the expression of chemical metabolizing and detoxifying enzymes and antioxidants (Azevedo *et al*., 2003). These inducible responses are important to preserve cellular integrity and have been a focus of attention in the chemoprevention of mammalian carcinogenesis (Talalay *et at.*, 1995). A number of epidemiologic, experimental studies and clinical intervention trials have indicated that the micronutrient selenium has potential anticarcinogenic effects (Clark *et al*., 1996 and El-Bayoumy, 2001).

Selenium is an essential trace nutrient and selenium deficiency diseases are well known in veterinary medicine. Above trace levels, ingested selenium is toxic to animals and may be toxic to humans (Melwanki and Seetharmappa, 2000). Selenium tends to weaken the toxic action of some heavy metals in animals and human organisms (Stabnikova *et al.,* 2005).

Under appropriate conditions, yeasts are capable of accumulating large amounts of selenium, and incorporating them into organic selenium-containing compounds, mainly selenomethionine, which is the best source of selenium for organisms (Demirci *et al*., 1999; Suhajda *et al*., 2000; Schrauzer, 2001 and Petrera *et al.*, 2009).

This study is aimed to genetic improvement and construction of some therapeutic yeast. To achieve this goal, UV-mutagenesis and intra-specific protoplast fusion were carried out. Moreover, the genetic effect of UV-mutagenesis and protoplast fusion on nucleotide sequence by random amplified polymorphic DNA (RAPD) analysis is also considered.

2.Materials and Methods

A- Yeast strains

Saccharomyces cerevisiae NRRL Y-139, *Candida utilis* NRRL Y-184 , *Kluyveromyes marxianus* NRRL Y-2415 and UV-mutants isolated after different exposure times were used in this study

B- Growth media and reagents

1-YEPD medium: Stocks were maintained on this medium which consisting of 1% Bacto-yeast extract, 2% dextrose and 1.5% agar in distilled water.

2-Selenium (IV) oxide stock solution was prepared by dissolving 1 g selenium in 10 ml of hot distilled water and filter-sterilized .

3- Protoplast medium (Khattab, 1997)

Sacharomyces cerevisiae protoplasting medium containing (g/L): Malt extract, 3; glucose , 50; yeast extract, 3 and peptone , 5. The intial pH of the medium was maintained between 6.5 and 6.8 with 1M Na**O**H solution.

4-Regeneration medium (Takagi *et al* ., 1983): It was used to isolate tetraploid clones after protoplast fusion . It consists of (g/L) yeast extract, 1; peptone, 1; glucose , 20; KH_2 PO_4 , 1.5; $MgSO_4$. $7H_2O$, 1 and sorbitol, 182.2.

5- Protoplast fusion buffers:

5.1. Pre- treatment solution (Spencer *et al.*, 1980). It consist of EDTA- Na_2 pH 7.6, 20 mM ; Tris-HCl pH 7.6 200 mM and 2-mercaptoethanol, 100 mM.

5.2. Protoplasting buffer (Spencer *et al.*, 1980): It consists of KH_2 PO_4, 80 mM; sodium citrate, 16 mM and KCl, 600 mM . The final pH of this buffer was 5.8.

5.3. Polyethylene glycol (PEG) solution (Farahanak, *et al*., 1986): It consists of PEG (MW 3350), 35 %(w/v); $CaCl_2$, 10 mM and sorbitol, 800 mM.

5.4.

C- Growth conditions

Yeast strains and UV- mutants were grown in YEP broth (50ml) at 30^{0}C with shaking (150 rpm).

D- Evaluation of mutants for selenium uptake

I-Screening of superior mutants

Selenium (IV) oxide (10 mg /L) was added after medium sterilization and before inoculation. The flasks were incubated at the same above conditions for 72 h and selenium uptake was determined for each mutant and the original strain.

II-High capacity of selenium uptake by superior mutants

Selenium (IV) oxide (100 mg /L) was added after the original strain and superior mutants were gown at the same above conditions for 48 h. The flasks were incubated again under the same conditions for 24 h. Selenium uptake was determined for each superior mutant and the original strain.

E- Protoplast formation from *S. cerevisiae*

Yeast cells were grown in 50 ml of protoplasting medium and incubated on a rotary shaking incubator (150 rpm) at 30^{o}C for 18 hours. Cells were collected by centrifugation and washed twice with sterile distilled water. Washed cells were resuspended in the pre-treatment solution, then the suspension was incubated for 20 min at 35^{o}C with gentile agitation. After incubation, cells were centrifuged again and resuspended in a protoplasting buffer containing snail enzyme (1%, w/v) and incubated under shaking (120 rpm) in a water bath at 35^{o}C. Cells were checked periodically, using phase- contrast microscope for the formation of protoplasts. The conversion of cells into protoplasts was completed within one hour of incubation. Protoplasts were centrifuged at 2500 rpm for 5 min at 4^{o}C and resuspended in protoplasting buffer.

F- Porotoplast fusion

Protoplast fusion could be carried out between selected isolates which differ in either their nutrient requirements; resistance to antibiotics or selenium uptake levels as follow : Protoplasts from different cultures were mixed, centrifuged at 2500 rpm for 5 min and the supernatant was removed. Two ml of PEG solution were added and mixed gently with protoplasts. The mixture of protoplasts was incubated up to 40-60 min at 30^{o}C . Then, the mixture was diluted with 0.65 M KCl. Samples of 0.1 and 0.5 ml of the dilutions were overlayed on the regeneration medium.

G- Isolation of total DNA from yeast strains

Total DNA was isolated according to Khattab (1997). The quantity and purity of the obtained DNA were determined according to the UV-absorbance at 260 and 280 nm using spectrophotometer (Shimadzu UV-VIS model UV-240) according to Sambrook *et al.* (1989).

H- Molecular analysis polymerase chain reaction (PCR)

For PCR technique, Ready-To-Go PCR Beads (Amersham, Pharmacia Biotech. No. 27-9555-01) were used. Each bead contains all of the necessary reagents, except primer and DNA template, for performing 25 µl PCR amplification reactions. Table (1) presents the three different primers which were used in the present study. The first and third primers supplied by Metabion International AG, Germany. The second one supplied by Operon Technologies Company, Netherlands. To each Ready To Go PCR bead, 12 ng of the used random primer and 40 ng of the purified DNA sample were added. The total volume of the amplification reaction was completed to 25 µl using sterile distilled water.

Table (1): The nucleotides sequences of the applied primers.

Serial No.	Type of primer	Code No.	Nucleotides sequence
1	Random	opb-15	5'- GACGGATCAG -3'
2	Random	opa-2	5'- TGCCGAGCTG -3'
3	Random	opb-08	5'- GTCCACACGG -3'

The amplification protocol was carried out as follows:

1. Denaturation at 95^{o}C for five min.

2. Thirty-five cycles each consists of the following segments: a. Denaturation at 95^{o}C for one min; b. Primer annealing at 37^{o}C for two min. according to

GC ratio of each primer; c. Incubation at 72°C for two min. for DNA polymerization.
3. Hold at 4°C till analysis. Finally, the amplified DNA products from RAPD analysis were electrophorated on 1.5% agarose gel and 1 X TBE buffer at consistent 100 volt for about 2 hrs. The different band sizes were determined against 100 bp ladder and the separated bands were stained with 0.5 µg/ml ethidium bromide and photographed using both Polaroid Instant Camera and UV Transeleminator.

I- Determination of selenium uptake

Revanasiddappa and kumar (2001) described a rapid and sensitive spectrophotometric method for the determination of trace amounts of selenium using Variamine Blue (VB) as a chromogenic reagent. An aliquot of a sample solution containing 2 to 20 µg of selenium was transferred into a series of 10 ml calibrated flasks. Volumes of 1 ml of 2% potassium iodide, followed by 1ml of 2 M HCl, were added to it. The mixture was gently shaken until the appearance of yellow color, indicating the liberation of iodine. Then , 0.5 ml of 0.05 % Variamine Blue (VB) was added to it , followed by the addition of 2 ml of a 1 M sodium acetate solution . The contents were diluted to the mark with distilled water and mixed well. The absorbance of the colored samples was measured at 546 nm against the corresponding reagent blank. Shimadzu UV–VIS spectrophotometer model UV-240 with 1 cm matched quartz cells were used for all absorbance measurements.

3.Results and Discussion

Effect of high selenium concentration on yeast growth

Influence of different selenium (IV) oxide concentrations on yeast growth of several growing yeast cultures was shown in Table 1. Results showed negative correlation between selenium concentration and growth yield for all tested strains. *C. utilis* showed sensitivity to selenium oxide since the percentage of decreasing growth was 34.04 & 21.70 at selenium conc. of 5 & 10 mg/l, respectively. Selenite [Se (IV)] is assimilated by microorganisms and reduced to Se (II) which is covalent bound to C as selenomethionine. Further, a common metal-induced response in yeast is intracellular synthesis of cysteine-rich metal-binding proteins called methallothioneins which have functions in metal detoxification and also storage and regulation of the intracellular metal ion concentrations (Gadd, 1986). *S. cerevisiae* which exhibited the greatest tolerance to all selenium concentrations was selected and subjected to mutagenic treatment with different doses of ultraviolet light.

Evaluation of uptake of selenium (IV) oxide by yeast cells

Data in Table (2) showed that all yeast strains exhibited high selenium uptake at concentration of 5 mg/l. It was 55, 47.6 and 45 % for *S. cerevisiae, C. utilis* and *K. marximanus*, respectively. At the two level of selenium concentrations (5 and 10 mg/l), *S. cerevisiae* exhibited the high selenium uptake and accumulation. Cobo-Fernandz *et al*. (1995) suggested that Se (IV) uptake occurs along with conversion of Se (IV) into other selenium species, probably Se (II) as selenomethionine or selenocysteine, which do not generate hydrogen selenite under the same experimental conditions as Se (IV) does.

UV-Mutagenesis and selenium resistant mutants

Data presented in Table (3) showed selenium resistant mutants obtained after ultraviolet (UV) mutagenesis of *S. cerevisiae*. Results showed that the highest percentage of resistant mutants (29.63) was obtained after 20 min of UV treatment whereas the lowest percentage (4.71) was obtained after one min. exposure time. On the other hand, 5 and 10 min UV-treatments gave nearly the same percentages (15& 15.9, respectively) of resistant mutants. Neither 30 min nor zero min UV treatments produced selenium resistant mutants.

Evaluation of mutants for selenium uptake

The uptake of selenium by yeast cells was studied from 72 h samples taken from each flask. Yeast cells were centrifuged and washed three times with 1 ml of water. The incorporation of selenium into yeast cells are presented in Table (4). The results in this table showed that there occurs a clear stimulation of the selenium uptake by yeast cells after UV-irradiation.

Selenium uptake of all mutants, grown in the presence of selenium oxide (10 mg/L), was higher than the original strain which incorporate 39.76 (mg/L) of selenium. Increasing selenium uptake is ranged from 178.78 to 408.16% in comparison with the original strain (100%). Five mutants, Nos. 5-8, 15-5, 20-1, 25-1 and 25-2 uptake nearly all selenium present in growth medium which represents more than four fold selenium uptake by the original strain.

High capacity of selenium uptake by superior mutants

Table (5) shows selenium uptake of superior yeast mutants in the presence of selenium oxide (100 mg/L). Results showed that yeast cells tolerate high selenium concentration in the growing medium and uptake percentages ranging from 46.84 to 79.38. The superior mutant No. 5-8 uptake 79.38 mg/L selenium

which represents 199.65% of the original strain. However, all mutants exhibited more selenium uptake than the original strain except for mutant No. 5-5 which exhibited slight increase (17.81%) in comparison with the original strain. Nine mutants, Nos. 5-3, 5-8, 5-9, 15-5 , 15-6 , 20-1 , 20-8 and 25-1 which represents about 81.82% of the mutants, uptake more than 50% of selenium than the original strain. Our results with mutation are consistent with those of Ramsay and Gadd (1997). They observed many differences between mutants and wild type of *S. cerevisiae* to several toxic metals. Moreover, Gharieb and Gadd (1998 and 2004) found many differences between vacuolar – lacking strains and the defective mutants of S. *cerevisiae* to uptake of selenium. On the other hand, our results are not agreement with those reported by Caker *et al.* (2009). They indicated that the EMS- treatment didn't significantly increase the sensitivity or resistance of yeast cells to cobalt ions.

Response of superior mutants to different antifungal

In order to investigate the effect of protoplast fusion on selenium uptake, six induced mutants which exhibited the highest selenium uptake were selected to be used as parental isolates for protoplast fusion crosses. Moreover, an additional marker, i.e. antifungal resistance or sensitivity were determined for the selected mutants.

Data presented in Table (6) showed the resistance or sensitivity to benomyl, cycloheximide, Griseofulvin, nystatin and miconazole. Results showed that, although the original strain was resistance to benomyl, cycloheximide and miconazole and sensitive to the other antifungal (griseofulvin and nystatin), all the selected mutants exhibited different antifungal response to all of the tested antifungal. Mutant 25/1 proved to be resistant to all of the tested antifungal except griseofulvin. Three mutants (5/8, 5/9 and15/6) were resistant to two antifungal, mutants 5/8 and 15/16 were resistant to cycloheximide while mutants 5/9 and 15/6 were resistance to miconazole. One mutant (5/3) exhibited resistance to three and sensitive to two antifungal. The last mutant, i.e., 25/1 showed the highest level of antifungal resistance, since it resistant to four antifungal.

However, three mutants (5/8, 5/9 and 20/1) were selected and introduced subsequently into protoplasting, fusion and regeneration. Two mutants, i.e., 5/8 and 5/9 were used as parental strains for cross 1 where benomyl and cyclohexamide or nystatin and miconazole was used as selective markers. On the other hand, 5/8 and 20/1 were used for cross 2 where griseofulvin and cycloheximide or griseofulvin and nystatin was used as selective markers.

Protoplast Fusion

Among the wide variety of strain improvement techniques, protoplast fusion seems to be an efficient way to induce genetic recombination and polyplodization in yeast (Gupthar and Garnett 1987and Gupthar, 1992). This method has also been proved to be valuable in the development of new industrial yeast strain. However, under the conditions mentioned under materials and methods, protoplasts can be isolated as shown in Fig(1/B).The increase of volume in comparison with the intact cells Fig.(1/A) was apparently due to osmotic enlargement of the vacuole(Svoboda,1978).

On the other hand, the addition of polyethylene glycol to the protoplast suspension resulted in intensive agglutination which leads to the formation of large aggregates which is a precondition for fusion. Furthermore, the presence of calcium ions is normally an important requirement for fusion of the aggregated protoplasts (Peberdy and Ferenczy, 1985). Any how, when the PEG-treated protoplast suspension was embedded into solid regeneration medium, some of them increased in volume and reverted to normal cells. Cell clones with normal cell morphology and prototrophic properties were selected after twice-repeated single-colony isolation and storage on slant agar medium at 4^0C for further genetic analysis. Fusants obtained will evaluate for their tolerance, uptake and accumulation of selenium in comparison with the original strain.

Evaluation of fusants for selenium uptake

Data in Tables 7 and 8 presents the obtained fusants from the crosses which were carried between different mutants. The results obtained showed that 38 fusants were obtained from the cross No. 1 and 36 fusants from the cross No. 2.

Results in Table (7) revealed that all obtained fusants exhibited higher selenium uptake than the original strain (*S. cerevisiae* NRRL Y-139). Moreover, four fusants (obtained from cross1 selected under miconazole and nystatin as selective markers and designated MN) exhibited higher selenium uptake of about 219% in comparison of the original strain. Selenium uptake of these fusants; designated MN2, MN9, MN12 and MN13, represents about 110% and 117% of parent 1 and parent 2, respectively. It's interesting to note that all fusants exhibited higher selenium uptake than its parent 2 (5/9).Only one fusants, i.e. MN11 showed a slight increasing of 1.24% of selenium uptake than parent 2 (5/9). On the other hand, fusants obtained also from cross 1 between (5/8 and 5/9 mutants) in the presence of benomyle and cycloheximide as selective markers. Data showed that 11 fusants out of 18 (designated BC)

uptake selenium more than its higher parental strain (5/8). Their selenium uptake were ranging from 80.29 to 84.58 mg/L which represents about 101 to 107%, 108 to 114% and 202 to 213% in comparison with parent 1, parent 2 and wild type, respectively. In addition, only one fusant, i.e. BC15 failed to reach the level of selenium uptake of both parents (5/8 and 5/9). However, BC12 and BC13 were considered as promising fusants which uptake more than two fold of the original strain.

Moreover, the effect of intra-specific protoplast fusion technique on selenium uptake were evaluated on 36 fusants selected from cross 2 between 5/8 and 20/1 mutants in the presence of cycloheximide, griseofulvin and nystatin. Data presented in Table (8) showed that out of 20 fusants, obtained in the presence of griseofulvin and nystatin as selective markers, ten fusants designated GN exhibited more selenium uptake than its higher parental mutant (5/8). Their selenium uptake percentages to wild type strain ranged from nearly 202 to 211 which represent about 101% to 106 % in comparison with the higher parent. Only three fusants, i.e. GN7, GN9 and GN 17 with higher selenium uptake than their higher parent strains (5/8 and 20/1), were obtained from cross 2.

On the other hand, 16 fusants obtained from cross 2 in the presence of cycloheximide and griseofulvin designated CG. Results in Table (8) showed that 62.5% of the fusants (10 fusants) exhibited more selenium uptake than its higher parent (5/8). Increasing selenium uptake is ranging from 79.60 to 82.57 which representing (100.28 to 104.02, 110.16 to 114.27) and (200.20 to 207.67) percentages in comparison with parent 1, parent 2 and the original strain, respectively. However, fusants selected from cross 2 in the presence of cycloheximide, griseofulvin and nystatin as selective markers proved to be less effective for increasing selenium uptake than fusants obtained from cross 1 in the presence of miconazoale, nystatin, benomyl and cycloheximide. In general, it could be concluded that ultraviolet mutation proved to be an effective technique to enhance selenium uptake. In addition, intra-specific protoplast fusion between higher selenium uptake mutants proved to be effective to achieve superior selenium uptake fusants. Therefore, protoplast fusion have been used successfully to enhancement the selenium uptake. The recombinants strains can be obtained by this technique (Lin *et al.*, 2005 ; Abdel Salam and Khattab 2005).

Random amplified polymorphic DNA (RAPD) analysis:

An attempt was conducted to evaluate the genetic effect of mutagenic treatment and protoplast fusion on the DNA nucleotide sequence of the mutants and protoplast fusants compared to the original strain (*S. cerevisiae* NRRL-Y-139).

Using primer No.1 (Figure 2), it was clearly noticed that six amplified bands were occurred when DNA of the original strain was used as a template. The bands sizes were 2100, 900, 650, 500,380 and 200 bp as shown in lane. Furthermore, the first band (2100bp was disappeared with the mutant No.20-1, fusants Nos. BC13 and MN (lanes 5, 6 and 7) and the second band (900bp) did not detected with the fusant BC13 (lane 6).

On the other hand, the three superior fusants No., BC13, MN9 and MN13 appeared a new amplified bands with the size of 1500 bp (lanes 6, 7 and 8) which did not detected with the original and parental strains. In addition, two new amplified bands with the size of 1250 and 1000 bp were detected with two superior fusants Nos. CG16 and GN17 (lanes 9 and 10). Furthermore, when DNA samples of the superior fusants Nos. MN9 and MN13 were used as templates with primer Nos. a new amplified band with size of 800 bp was detected. Finally, using primer No.1 with DNA templates of the fusants No., CG16 and GN17, a new amplified band with size of 250 bp was detected.

The application of primer No. 2 (Figure, 3) against strain used in RAPD analysis induced only one monomorphic amplified band with size of 2100bp . It was interested to notice that mutants Nos. 5-8 and 5-9 (lanes 3 and 4) which have about 99 % selenium uptake more than the original strain, exhibited high intensity band. The same finding was detected with the superior fusant No. CG16 (lane 9). In general all mutants and fusants exhibited high intensity band compared to the original strain.

Regarding the application of the primer No. 3 (Figure, 4), it was clearly noticed that most of the tested mutants and fusants (lanes 2-5 and 8, 9) proved to have three distinct amplified bands which identical of its original strain. With two exceptions of the fusants Nos. CG16 and GN17 (lanes 9 and 10) exhibited new amplified band with size of 500 bp. Moreover, two fusants Nos. MN9 and MN13 (lanes 7 and 8) present different banding pattern, since two distinct bands with different size were detected (700 and 1100 bp).

The above differences in RAPD profiles confirmed the evidence of genetic variations in yeast genome after the UV- mutagensis and protoplast fusion technique. Furthermore, some of these differences based on RAPD technique, could be used as genetic markers for genetic diversity of selenium uptake characteristics.

Table (1): Effect of different selenium (IV) oxide concentrations on yeast growth* ability after 72 hours incubation at 30º C .

Yeast strains	Selenium conc. (mg/L)			
	0	2	5	10
S. cerevisiae	1.004	0.846	0.713	0.526
C. utilis	1.93	1.72	0.664	0.419
K. marxianus	1.36	1.154	0.993	0.414

* Measured as optical density at 600 nm.

Table (2): Evaluation of selenium (IV) oxide uptake by different yeast cells after 72 incubation at 30º C

Yeast strain	selenium conc. (mg/L)			% Uptake
	Initial	Residual	Uptake	
S. cerevisiae	5	2.25	2.75	55
S. cerevisiae	10	7.55	2.45	24.5
C .utilis	5	2.62	2.38	47.6
C .utilis	10	7.95	2.05	20.5
K. marxianus	5	2.75	2.25	45
K. marxianus	10	9.95	0.05	0.5

Table(3):Selenium resistant mutants obtained after ultraviolet (UV) mutagenesis

Exposure time(min)	No. of tested colonies	No. of resistant colonies*	% of resistant colonies
0	192	0	0
1	85	4	4.71
5	60	9	15.00
10	44	7	15.91
15	35	8	22.86
20	27	8	29.63
25	15	3	20.00
30	8	0	0

*Mutants were screened on YEPD agar medium containing selenium oxide (20 mg /L).

Table (4): Selenium uptake of selected selenium resistant mutants grown in the presence of selenium oxide (10 mg /L).

Mutant No.	Se.*** uptake(mg/L)	% to W.T.	Mutant No.	Se. uptake (mg/L)	% to W.T.
W.T.	2.45	100.00	10-7	5.90	240.82
1*-1**	6.37	260.00	15-1	7.35	300.00
1-2	5.5	224.49	15-2	6.81	277.96
1-3	9.46	386.12	15-3	5.50	224.49
1-4	6.47	264.08	15-4	8.00	326.53
5-1	5.80	236.73	15-5	10.00	408.16
5-2	8.72	355.92	15-6	9.50	387.76
5-3	9.01	367.76	15-7	8.17	333.47
5-4	6.56	267.76	15-8	7.80	318.37
5-5	9.24	377.14	20-1	10.00	408.16

5-6	4.38	178.78	20-2	7.70	314.29
5-7	8.81	359.59	20-3	7.46	304.49
5-8	10.00	408.16	20-4	6.00	244.90
5-9	9.87	402.86	20-5	8.39	342.45
10-1	7.65	312.24	20-6	7.52	306.94
10-2	7.91	322.86	20-7	5.09	207.76
10-3	6.36	259.59	20-8	9.72	396.73
10-4	8.40	342.86	25-1	10.00	408.16
10-5	7.82	319.18	25-2	10.00	408.16
10-6	8.34	340.41	25-3	8.17	333.47

***Time of treatment (min)**

****Mutant No.**

***** Seleniu**

Table (5): Selenium uptake of superior selenium resistant mutants grown in the presence of selenium oxide (100 mg/L).

Mutant No.	Residual Se. (mg /L)	Se. uptake	
		uptake (mg/L)	% to W.T.
W.T.	60.24	39.76	100.00
1-3	45.72	54.28	136.52
5-3	34.80	65.20	163.98
5-5	53.16	46.84	117.81
5-8	20.62	79.38	199.65
5-9	25.74	74.26	186.77
15-5	39.52	60.48	152.11
15-6	28.59	71.41	179.60
20-1	27.74	72.26	181.74
20-8	37.38	62.62	157.49
25-1	36.75	63.25	159.08
25-2	41.65	58.35	146.76

Table (6): Antifungal response of the original strain (W.T) and the superior mutants selected for protoplast fusion

Mutant No.	Antifungal response				
	B	C	G	N	M
W.T	+	+	-	-	+
5-3	+	-	+	-	+
5-8	-	+	-	+	-
5-9	+	-	-	-	+
15-6	-	+	-	-	+
20-1	-	-	+	-	-
25-1	+	+	-	+	+

B**:** Benomyl (5 µg/ml), **C:** Cycloheximide (100 µg/ml), **G:** Griseofulvin (250 µg/ml) , **N:** Nystatin 10 µg/ml) , **M:** Miconazole (5 µg/ml)

(+) : Resistant , **(-):** Sensitive

Table (7): Selenium (Se) uptake of fusants obtained after protoplast fusion between 5/8 and 5/9 mutants (cross 1) .

Fusant No.	Se residual (mg/L)	Se uptake (mg/L)	% to P_1	% to P_2	% to W.T.	Fusant No.	Se residual (mg/L)	Se uptake (mg/L)	% to P_1	% to P_2	% to W.T.
W.T	60.24	39.76	50.09	53.54	100	W.T	60.24	39.76	50.09	53.54	100
P_1 5/8	20.62	79.38	100	106.89	199.65	P1 5/8	20.62	79.38	100	106.89	199.65
P_2 5/9	25.74	74.26	93.55	100	186.77	P2 5/9	25.74	74.26	93.55	100	186.77
MN 1*	18.75	81.25	102.36	109.41	204.35	BC1**	17.30	82.70	104.18	111.37	208.00
MN 2	12.90	87.10	109.73	117.29	219.06	BC 2	19.52	80.48	101.39	108.38	202.41
MN 3	15.23	84.77	106.79	114.15	213.20	BC 3	22.48	77.52	97.66	104.39	194.97
MN 4	14.61	85.39	107.57	114.99	214.76	BC 4	20.65	79.35	99.96	106.85	199.57
MN 5	17.74	82.26	103.63	110.77	206.89	BC 5	17.70	82.30	103.68	110.83	206.99
MN 6	14.28	85.72	107.99	115.43	215.59	BC 6	24.37	75.63	95.28	101.84	190.22
MN 7	22.37	77.63	97.80	104.54	195.25	BC 7	30.29	69.71	87.82	93.87	175.33
MN 8	19.08	80.92	101.94	108.97	203.52	BC 8	25.06	74.94	94.41	100.92	188.48
MN 9	12.80	87.20	109.85	117.43	219.32	BC 9	19.71	80.29	101.15	108.12	201.94
MN 10	21.76	78.24	98.56	105.36	196.78	BC 10	18.88	81.12	102.20	109.24	204.02
MN 11	24.50	75.50	95.11	101.67	189.89	BC 11	18.54	81.46	102.62	109.70	204.88
MN 12	13.19	86.81	109.36	116.90	218.34	BC 12	15.76	84.24	106.12	113.44	211.87
MN 13	12.68	87.32	110.00	117.59	219.62	BC 13	15.42	84.58	106.55	113.90	212.7
MN 14	14.94	85.06	107.16	114.54	213.93	BC 14	17.27	82.73	104.22	111.41	208.07
MN 15	23.45	76.55	96.44	103.08	192.53	BC 15	27.31	72.69	91.57	97.89	182.82
MN 16	20.21	79.79	100.52	107.45	200.68	BC 16	18.70	81.30	102.42	109.48	204.48
MN 17	19.42	80.58	101.51	108.51	202.66	BC 17	17.13	82.87	104.40	111.59	208.43
MN 18	16.55	83.45	105.13	112.38	209.88	BC 18	23.95	76.05	95.81	102.41	191.27
MN 19	18.36	81.64	102.85	109.94	205.33						
MN 20	17.40	82.60	104.06	111.23	207.75						

*Fusants obtained under miconazole and nystatin as selective markers

**Fusants obtained under benomyl and cycloheximide as selective markers

Table (8): Selenium (Se) uptake of fusants obtained after protoplast fusion between 5/8 and 20/1 mutants (cross 2).

Fusant No.	Se residual (mg/L)	Se uptake (mg/L)	% to P_1	% to P_2	% to W.T.	Fusant No.	Se residual (mg/L)	Se uptake (mg/L)	% to P_1	% to P_2	% to W.T.
W.T	60.24	39.76	50.09	53.54	100	W.T	60.24	39.76	50.09	53.54	100

P_1 5/8	20.62	79.38	100	106.89	199.65	P_1 5/8	20.62	79.38	100	106.89	199.65
P_2 20/1	27.74	72.26	91.03	100	181.74	P_2 20/1	27.74	72.26	91.03	100	181.74
GN 1*	24.89	75.11	94.62	103.94	188.91	CG 1**	21.70	78.30	98.64	108.36	196.93
GN 2	19.55	80.45	101.35	111.33	202.34	CG 2	18.32	81.68	102.90	113.04	205.43
GN 3	20.71	79.29	99.89	109.73	199.42	CG 3	23.62	76.38	96.22	105.70	192.10
GN 4	17.37	82.63	104.09	114.35	207.82	CG 4	25.38	74.62	94.01	103.27	187.68
GN 5	22.60	77.40	97.51	107.11	194.67	CG 5	19.92	80.08	100.88	110.82	201.41
GN 6	18.83	81.17	102.25	112.33	204.15	CG 6	23.65	76.35	96.18	105.66	192.03
GN 7	16.18	83.82	105.60	115.99	210.81	CG 7	17.81	82.19	103.54	113.74	206.72
GN 8	22.46	77.54	97.68	107.31	195.02	CG 8	20.40	79.60	100.28	110.16	200.20
GN 9	16.25	83.75	105.51	115.90	210.64	CG 9	24.50	75.50	95.11	104.48	189.89
GN 10	23.90	76.10	95.87	105.31	191.40	CG 10	23.47	76.53	96.41	105.91	192.48
GN 11	22.32	77.68	97.86	107.50	195.37	CG 11	20.27	79.73	100.44	110.34	200.53
GN 12	17.35	82.65	104.12	114.38	207.87	CG 12	17.86	82.14	103.48	113.67	206.59
GN 13	19.70	80.30	101.16	111.13	201.96	CG 13	19.45	80.55	101.47	111.47	202.59
GN 14	20.42	79.58	100.25	110.13	200.15	CG 14	18.29	81.71	102.94	113.07	205.51
GN 15	18.66	81.34	102.47	112.57	204.58	CG 15	20.16	79.84	100.58	110.49	200.80
GN 16	20.48	79.54	100.20	110.07	200.00	CG 16	17.43	82.57	104.02	114.27	207.67
GN 17	16.20	83.80	105.56	115.97	210.76						
GN 18	21.52	78.48	98.866	108.61	197.38						
GN 19	17.97	82.03	103.34	113.52	206.31						
GN 20	20.51	79.49	100.14	110.01	199.92						

*Fusants obtained under griseofulvin and nystatin as selective markers

**Fusants obtained under cycloheximide and griseofulvin as selective markers

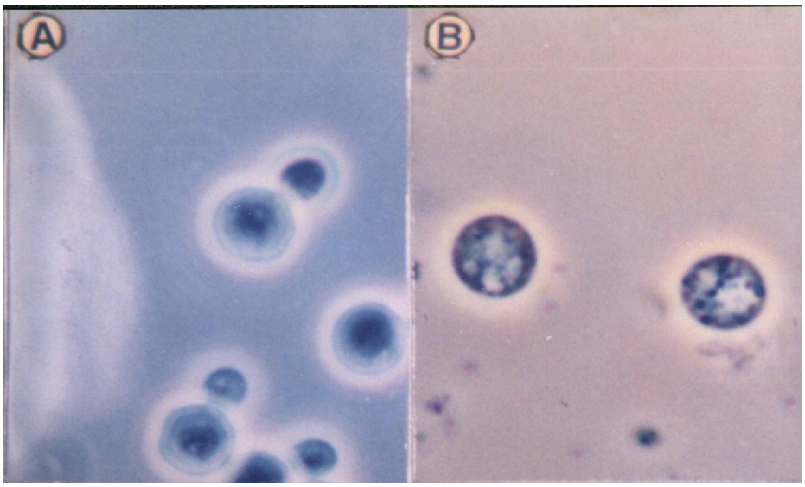

Figure (1): Micrographs represent formation of yeast protoplasts (B) in comparison with the original parent (A).

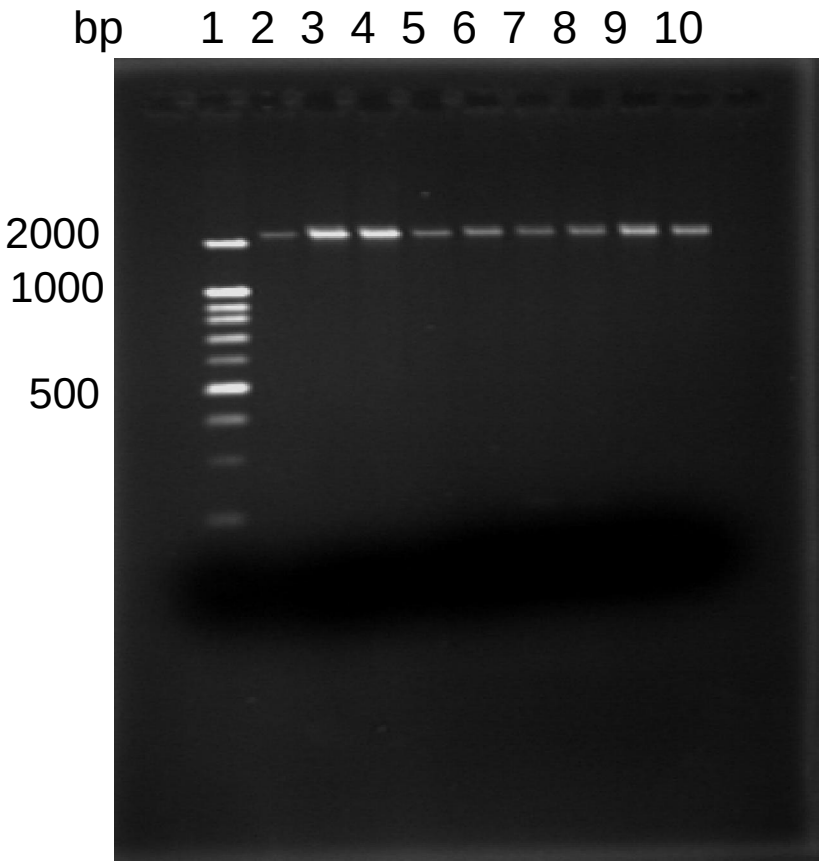

Figure (2): Photographs of DNA amplified banding patterns based on RAPD for three parental mutants and five protoplast fusants against original strain (lane 2) and 100 bp loader DNA marker (lane 1) using primer No.1. Mutant sequence as follows: (lane 3 to 5), 5-8.5-9 and 20-1.Fusant sequences (lanes 6 to 10), BC13, MN9, MN13, CG16 and GN17.

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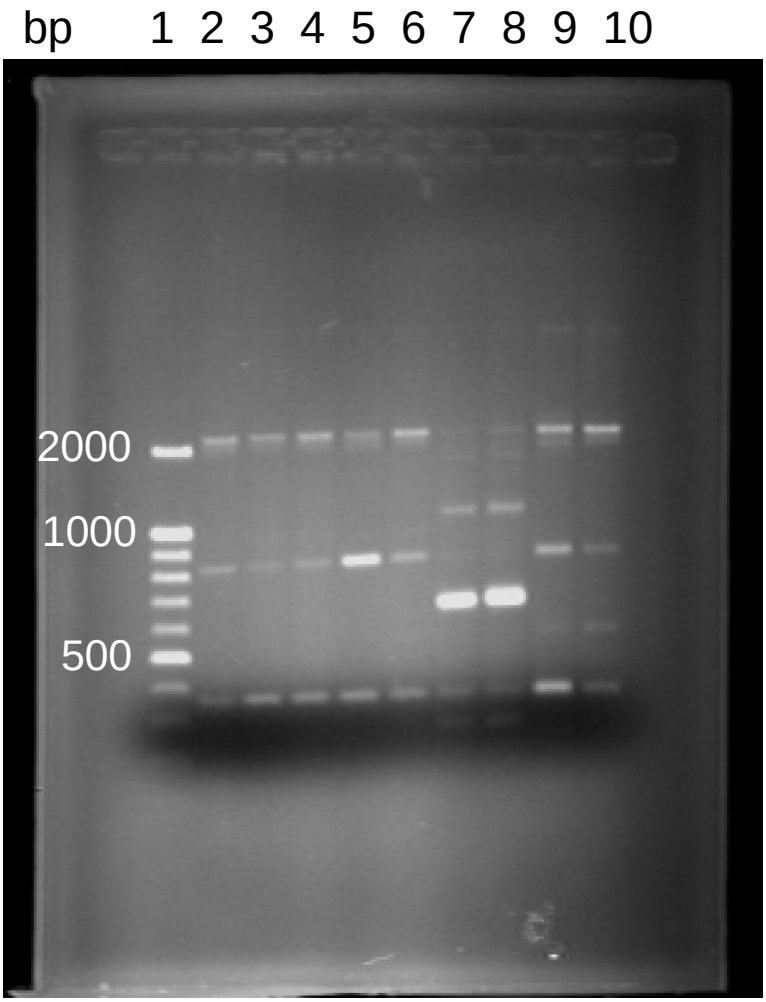

Figure (3): Photographs of DNA amplified banding patterns based on RAPD for three parental mutants and five protoplast fusants against original strain (lane 2) and 100 bp ladder DNA marker (lane 1) using primer No.3. Mutant sequences as follows: (lane 3 to 5), 5-8, 5-9 and 20-1. Fusants sequence (lanes 6 to 10), BC13, MN9, MN13, CG16 and GN17.

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Synthesis, (in vitro) Antitumor and Antimicrobial Activity of some Pyrazoline, Pyridine, and Pyrimidine Derivatives Linked to Indole Moiety

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Abstract: Aldol condensation reaction between 3-indolaldehyde 1 and 4-methoxyacetophenone 2 afforded chalcone compounds 3. This compound was reacted with some different reagents such as hydrazine hydrate, phenyl hydrazine, thiosemicarbazide, hydroxylamine, ethyl cyanoacetate, urea and thiourea to give pyrazolines 4a, 4b, 5a, 5b, 6, oxazoline 7, Michael adduct 8, pyranone 9, and oxo 14a and thiopyrimidine derivatives 14b, respectively. The structures of all the compounds were confirmed by microanalyses and various spectral data. Some of the synthesized new compounds were screened against antitumor and antimicrobial activity. [Journal of American Science. 2010;6(8):338-347]. (ISSN: 1545-1003).

Keywords: Heterocycles, cyclizations, pyrazolines, pyridines, antitumor activity.

1. Introduction

Indole nucleus is incorporated in various natural products such as alkaloids and represented a promising structural class of marine alkaloids based upon their high degree of biological activity [1-5]. Indole nucleus was reported to possess a wide variety of biological properties such as anti-tumor [6-10], anti-inflammatory [11-13], anti-convulsant [14], cardiovascular [15], and anti-bacterial [16]. In addition, the substitution at 3-position of the indole ring connecting an extra heterocyclic ring such as imidazole (topsentins [17] and nortopsentins [18]); dihydroimidazole (discodermindole 3); oxazole (martefrgin 4 amazol [19]); oxadiazine (alboinon [20]); (didemidines [21]) furnished potent agents. Also pyrazole [22, 23], pyridine [24] and pyrimidine [25] derivatives were found to possess a variety of biological activities. Considering the above observations and in connection to previous publications involving the synthesis of new biologically active hetero cycles [26-29]. I hope to report here in the synthesis of new 3-substituted indoles incorporating an extra heterocyclic rings such as pyrazole, pyridine and pyrimidine to evaluate their antitumor (anti-proliferative) activities against both of human breast cell line MCF- 7 and liver carcinoma cell line HEPG2 and their antimicrobial activities.

2. Experimental

Chemistry

Melting points were determined in open glass capillaries and are uncorrected. Elemental analyses were carried out in the Micro Analytical Laboratory, Cairo University, Cairo, Egypt, IR spectra of the compounds were recorded on a Perkin-Elmer; spectrophotometer model 1430 using potassium bromide pellets and frequencies were reported in cm^{-1}. The mass spectra were recorded using mass spectrometer HP model MS5988 EI 70eV. ^{1}HNMR spectra were measured on Varian Mercury 300 MHz spectrometer and chemical shifts were expressed in δ ppm using TMS as internal standard. Reactions were routinely followed by (TLC).

3-(1*H*-Indol-3-yl)-1-(4-methoxyphenyl)prop-2-en-1-one 3.

To a stirred mixture of 14.5 g indolaldehyde 1 (100 mmol) and 15.0 g 4-methoxyacetophenone 2 (100 mmol) in 200 mL ethanol at room temperature, 40% NaOH aqueous solution was added portion-wise after which stirring was continued for further 2 hr. The pale yellow precipitate formed was filtered and washed with 3% aqueous HCl, and crystallized from ethanol to give chalcone 3 in 75% yield, mp 124-126ºC. IR (KBr): 3162 (NH), 1633 (C=O) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): 3.83 (s, 3H, -OCH_3), 7.01-7.05 (m, 2H, Ar-H), 7.24-7.27 (m, 4H, Ar-H), 7.49-7.52 (m, 2H, Ar-H), 7.53 (d, 1H, J = 12.9 Hz, (C=O)(CH=C), 7.92 (d,1H , J = 12.9 Hz (C=O)(C=CH), 8.11-8.30 (m, 1H, Ar-H), 12.3 (s, D_2O-exchangeable, 1H, indole NH) ppm; MS (70 eV): m/z = 278 (M^++1, 4.7), 277 (M^+, 15.0), 144 (100), 116 (39.5), 89 (37.3), 62 (38,6). Anal. Calcd. for $C_{18}H_{15}NO_2$: C, 77.96; H, 5.45; N, 5.05 Found: C, 78.24; H, 5.59; N, 4.93.

3-(4,5-Dihydro-3-(4-methoxyphenyl)-1*H*-pyrazol-5-yl)-1*H*-indole 4a.

To a solution of 2.77 g chalcone 3 (10 mmol) in 50 mL of ethanol, 1.0 mL of hydrazine hydrate (80%) was added and the reaction mixture was refluxed for 4 hr., cooled to -10 °C and left over night. The solid mass separated out was filtered, washed with cold ethanol and crystallized from ethanol to afford pyrazoline derivative 4a as pale yellow crystals in 78% yield, mp 258-260ºC; IR (KBr): 3215, 3170 (2NH), 1618 (C=N) cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ = 1.97 (m, 1H), 2.64 (m, 1H), 3.39 (m, 1H), 3.75 (s, 3H, -OCH_3), 6.86-8.36 (m, 9H, Ar-H), 8.90 (s, D_2O-exchangeable, 1H, pyrazoline NH), 11.65 (s, D2O-exchangeable, 1H, indole NH) ppm; MS (70 eV): m/z = 291 (M^+, 7.3), 286 (94.9), 257 (100), 142 (36.3), 116 (45.4), 89 (47.2). Anal. Calcd. for $C_{18}H_{17}N_3O$: C, 74.20; H, 5.88; N, 14.42. Found: C, 74.42; H, 5.67; N, 14.38.

3-(4,5-Dihydro-3-(4-methoxyphenyl)-1-phenyl-1*H*-pyrazol-5-yl)-1*H*-indole 4b.

To a solution of 2.77 g of chalcone **3** (10 mmol) in 50 mL of ethanol, 5 mL of acetic acid and 1.1 g of phenyl hydrazine (10 mmol) was added. The reaction mixture was refluxed for 8 hr. and left over night. The solid mass separated out was filtered off, washed with ethanol and crystallized from ethanol to give compound 4b as pale brown crystals in 67% yield, mp 258-260ºC; IR (KBr): 3169 (NH), 1633 (C=N) cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ = 2.00 (m, 1H), 2.51 (m, 1H), 3.11 (m, 1H), 3.35 (s, 3H, -OCH_3), 6.85-7.00 (m, 2H, Ar-H), 7.18-7.21 (m, 2H, Ar-H), 7.24-7.26 (m, 1H, Ar-H), 7.27-7.30 (m, 4H, Ar-H) ,7.47-7.49 (m, 2H, Ar-H), 8.32 (s, 1H, Ar-H), 12.15 (s, D_2O-exchangeable, 1H, indole NH) ppm; MS (70 eV): *m/z* = 367 (M^++1, 3.5), 368 (M^+, 2.8), 145 (100), 221 (10.7). Anal. Calcd for $C_{24}H_{21}N_3O$: C, 78.45; H, 5.76; N, 11.44. Found: C, 78.43; H, 5.59; N, 11.48.

Synthesis of pyrazolines 5a, b

This reaction was carried out by the same procedure described in the synthesis of compound 4a by using acetic acid instead of ethanol in case of 5a and propanoic acid in case of 5b.

1-(4,5-Dihydro-5-(1*H*-indol-3-yl)-3-(4 methoxyphenyl)pyrazol-1-yl)ethanone 5a.

Pale yellow crystals, mp 288-290°C, yield 77%; IR (KBr): 3169 (NH), 1686 (C=O), 1627 (C=N) cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ = 1.93 (m, 1H), 2.24 (s, 3H, -CH_3), 2.49 (m, 1H), 3.36 (s, 3H, -OCH_3), 3.83 (m, 1H), 7.14-7.26 (m, 4H, Ar-H), 7.27-7.49 (m, 2H, Ar-H), 7.90-7.92 (m, 1H, Ar-H), 8.16-8.36 (m, 2H, Ar-H), 11.68 (s, D_2O-exchangeable, 1H, indole NH) ppm; MS (70 eV): *m/z* = 334 (M^++1, 2.4), 333 (M^+, 2.9), 286 (100), 257 (95.4), 116 (28.0). Anal. Calcd for $C_{20}H_{19}N_3O_2$: C, 72.05; H, 5.74; N, 12.60. Found: C, 71.87; H, 5.66; N, 12.49.

1-(4,5-Dihydro-5-(1*H*-indol-3-yl)-3-(4-methoxyphenyl)pyrazol-1-yl)propan-1-one 5b.

Yellow powder, mp 280-281°C, yield 73%; IR (KBr): 3165 (NH), 1675 (C=O), 1630 (C=N) cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ = 1.8-2.2 (t, 3H, -CH_3), 2.01 (m, 1H), 2.70 (m, 1H), 2.62-2.74 (q, 2H, -CH_2), 3.43 (m, 1H), 3.78 (s, 3H, -OCH_3), 6.86-6.88(m, 1H Ar-H),6.90-6.93 (m, 2H, Ar-H), 7.16-7.26 (m, 4H, Ar-H), 7.41-7.50 (m, 2H, Ar-H), 11.52 (s, D_2O-exchangeable, 1H, indole NH) ppm; MS (70 eV): *m/z* = 348 (M^++1, 1.8), 347 (M^+, 5.5), 142 (100), 131 (31.2). Anal. Calcd for $C_{21}H_{21}N_3O_2$: C, 72.60; H, 6.09; N, 12.10. Found: C, 72.44; H, 6.24; N, 12.26.

4,5-Dihydro-5-(1*H*-indol-3-yl)-3-(4-methoxyphenyl)pyrazole-1-carbothioamide 6.

To a solution of 2.77 g of chalcone **3** (10 mmol) in 50 mL of ethanol, 1.0g of sodium hydroxide (25 mmol) and 1.2 g of thiosemicarbazide (12 mmol) was added. The mixture was refluxed for 6 hr. then left to cool overnight, the formed solid product was filtered off, dried, and then crystallized from ethanol to give compound 6 as yellow powder in 69% yield, mp 228-230 °C; IR (KBr): 3316, 3227 (NH_2, NH), 1606 (C=N), 1251 (C=S) cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ = 2.22 (m, 1H),2.51 (m, 1H), 3.37 (m, 1H,), 3.80 (s, 3H, -OCH_3), 7.09-7.20 (m, 2H, Ar-H), 7.23-7.44 (m, 4H, Ar-H), 7.81-8.00 (m, 2H, Ar-H),8.24-8.31(m,1H, Ar-H), 11.11 (s, D_2O-exchangeable, 2H, NH_2), 11.60 (s, D_2O-exchangeable, 1H, indole NH), ppm; MS (70 eV): *m/z* = 351 (M^++1, 1.6), 350 (M^+, 4.0), 142 (100), 217 (61.3), 200 (54.5), 116 (56.0). Anal. Calcd for $C_{19}H_{18}N_4OS$: C, 65.12; H, 5.18; N, 15.99; S, 9.15. Found: C, 65.04; H, 5.16; N, 16.12; S, 9.07.

3-(4,5-Dihydro-3-(4-methoxyphenyl)isoxazol-5-yl)-1*H*-indole 7.

A mixture of 2.77 g of chalcone 3 (10 mmol), 0.7 g of hydroxylamine hydrochloride (10 mmol) and 1.4 g of anhydrous potassium carbonate (10 mmol) in (50 mL) of ethanol was refluxed for 8 hr., then left to cool. The reaction mixture was poured into cold water and the solid product was filtered off, washed with water, dried and finally crystallized from ethanol to afford isoxazole derivative 7 in 68 % yield as pale yellow crystals, mp 138-140 ºC; IR (KBr): 3386 (NH), 1640 (C=N) cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ = 2.11 (m, 1H), 2.52 (m, 1H), 3.47 (m, 1H) 3.72 (s, 3H, -OCH_3), , 7.12-7.16 (m, 2H, Ar-H), 7.19-7.23 (m, 4H, Ar-H), 7.43-7.50 (m,2H,

Ar-H), 8.28-8.33 (m,1H, Ar-H), 11.61 (s, D_2O-exchangeable, 1H, indole NH) ppm; MS (70 eV): *m/z* = 293 (M^++1, 1.3), 292 (M^+, 4.8), 160 (98.1), 117 (100), 89 (36.6). Anal. Calcd for $C_{18}H_{16}N_2O_2$: C, 73.95; H, 5.52; N, 9.58. Found: C, 74.11; H, 5.41; N, 9.70.

Ethyl-2-cyano-3-(1*H*-indol-3-yl)-5-(4 methoxyphenyl)-5-oxopentanoate 8.

To a stirred solution of 2.77 g of chalcone 3 (10 mmol) and 1.3 g of ethyl cyanoacetate (10 mmol) in 50 mL absolute ethanol, a sodium ethoxide solution prepared from 0.23 g sodium metal (10 mmol) and 10 mL absolute ethanol was added. The stirring was continued, at room temperature, for 12 hr. The solid product was collected by filtration, washed with water, dried and finallycrystallised from ethanol to afford compound 8 as pale yellow crystals in 80 % yield, mp 240-241 ºC; IR (KBr): 3265 (NH), 2222 (C≡N), 1715 (C=O) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 1.11-1.20 (t, 3H, CH_3), 2.50-2.56 (m, 2H (CH_2 –C=O), 3,35 (d, 1H), 3.58 (m, 1H) , 3.81 (s, 3H, -OCH_3), 4.37 (q, 2H, CH_2),6.86-6.90(m, 1H Ar-H), 6.95-7.00 (m, 2H, Ar-H), 7.18-7.23 (m, 4H, Ar-H), 7.91-7.92 (m, 2H, Ar-H), 11.69 (s, D_2O-exchangeable, 1H, indole NH) ppm; MS (70 eV): *m/z* = 391 (M^++1, 2.3), 390 (M^+, 8.0), 135 (68.5), 78 (79.8), 62 (100). Anal. Calcd for $C_{23}H_{22}N_2O_4$: C, 70.75; H, 5.68; N, 7.17. Found: C, 70.61; H, 5.60; N, 7.33.

4-(1*H*-Indol-3-yl)-6-(4-methoxyphenyl)-2-oxo-2*H*-pyran-3-carbonitrile 9.

Method A. This compound was synthesized by the same procedure described in the synthesis of compound 8 by refluxing the reaction mixture for 8 hr. The solid that formed after cooling was collected by filtration, washed with water, dried and finally crystallised from ethanol to afford compound 9 as pale yellow crystals in 68% yield, mp 208-209 ºC; IR (KBr): 3274 (NH), 2222 (C≡N), 1688 (C=O) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.31 (s, 3H, -OCH_3), 7.12-7.22 (m, 2H, Ar-H) ,7.32 (s, 1H,C5), 7.55-7.58 (m, 4H, Ar-H), 7.93-7.96 (m, 2H, Ar-H), 8.54-8.55 (m, 1H, Ar-H), 12.48 (s, D_2O-exchangeable, 1H, indole NH) ppm; MS (70 eV): *m/z* = 343 (M^++1, 2.9), 342 (M^+, 11.2), 212 (100), 168 (80.4), 140 (64.4), 113 (26.4). Anal. Calcd for $C_{21}H_{14}N_2O_3$: C, 73.68; H, 4.12; N, 8.18. Found: C, 73.74; H, 4.26; N, 8.03.

Method B. Refluxing 0.78 g of compound 8 (2 mmol) in sodium ethoxide solution prepared from 0.046 g sodium metal (2 mmol) and 50 mL absolute ethanol for 6 hr. the treatment of reaction mixture as mentioned in method A resulted in a compound identical in (mp. and mix. mp. for compound that produced by method A.

General procedure for synthesis of pyridines 11 and 13a-13d

To a solution of 0.69 g of the pyranone 9 (2 mmol) in 30 mL of ethanol, hydrazine hydrate or the appropriate sulfonamides 12a-d (2 mmol) was added. The mixture was refluxed for 6 h. Left to cool, the formed solid product was filtered off, dried, and then crystallized from ethanol/DMF to give compounds 11 and 13a-d, respectively.

1,2-Dihydro-4-(1*H*-indol-3-yl)-6-(4-methoxyphenyl)-2-oxo-1-aminopyridine-3-carbonitrile 11.

Yellow powder, yield 62%, mp 295-297 ºC; IR (KBr): 3320, 3190 (NH_2, NH), 2219 (C≡N), 1665 (C=O), cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.51(s, D_2O-exchangeable, 2H, NH_2), 3.34 (s, 3H, -OCH_3), 7.22-7.29 (m, 2H, Ar-H), 7.33-7.55 (m, 4H, Ar-H), 7.59-7.93 (m, 2H, Ar-H), 9.11 (m, 1H, Ar-H), 11.69 (s, D_2O-exchangeable, 1H, indole NH) ppm; MS (70 eV): *m/z* = 357 (M^++1, 2.5), 356 (M^+, 10.3), 281 (40.2), 78 (70.3), 62 (100). Anal. Calcd for $C_{21}H_{16}N_4O_2$: C, 70.77; H, 4.53; N, 15.72. Found: C, 70.59; H, 4.50; N, 15.86.

1,2-Dihydro-4-(1*H*-indol-3-yl)-6-(4-methoxyphenyl)-2-oxo-1-(4-aminosulfonylphenyl)pyridine-3-carbonitrile 13a.

Pale yellow crystals, yield 65%, mp 248-249 ºC; IR (KBr): 3364, 3150 (NH_2, NH), 2221 (C≡N), 1686 (C=O), 1375 (S=O, asy.) and 1140 (S=O, sym.) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.40 (s, D_2O-exchangeable, 2H, sulfonamide NH_2),3.85 (s, 3H, -OCH_3), 7.04 (s, 1H, C5), 7.24-7.27 (m, 2H, Ar-H),7.31-7.55 (m, 4H, Ar-H), 7.56-7.74 (m, 2H, Ar-H), 7.91-7,92 (m, 4H, Ar-H), 9.05 (m, 1H, Ar-H), (12.54 (s, D_2O-exchangeable, 1H, indole NH) ppm; MS (70 eV): *m/z* = 497 (M^++1, 3.8), 496 (M^+, 11.0), 212 (41.8), 168 (100). Anal. Calcd for $C_{27}H_{20}N_4O_4S$: C, 65.31; H, 4.06; N, 11.28; S, 6.46. Found: C, 65.18; H, 3.92; N, 11.33; S, 6.62.

1,2-Dihydro-4-(1*H*-indol-3-yl)-6-(4-methoxyphenyl)-2-oxo-1-(4-propylaminosulfonylphenyl)pyridine-3-carbonitrile 13b.

Pale yellow crystals, yield 65%, mp 257-258 ºC; IR (KBr): 3358, 3281 (2NH), 2224 (C≡N), 1686 (C=O), 1370 (S=O, asy.) and 1148 (S=O, sym.) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 1.03 (t, 3H, -CH_3), 1.52 (m, 2H, -CH_2-), 2.10 (s, D_2O-exchangeable, 1H, sulfonamide NH) 3.35 (t, 2H, -CH_2-), 3.85 (s, 3H, -OCH_3), 6.10 (s, 1H, C5), 6.61-

6.66 (m, 2H, Ar-H), 7.27-7.30 (m, 4H, Ar-H), 7.33-7.48 (m, 2H, Ar-H),7.94-7.97 (m, 4H, Ar-H) , 8.54-8.56 (m, 1H, Ar-H), 12.51 (s, D_2O-exchangeable, 1H, indole NH), ppm; MS (70 eV): *m/z* = 539 (M^++1, 3.3), 538 (M^+, 8.9), 212 (100), 140 (87.5), 63 (65.0). Anal. Calcd for $C_{30}H_{26}N_4O_4S$: C, 66.90; H, 4.87; N, 10.40; S, 5.95. Found: C, 66.82; H, 4.87; N, 10.29; S, 6.11

1,2-Dihydro-4-(1*H*-indol-3-yl)-6-(4-methoxyphenyl)-2-oxo-1-(4-phenylaminosulfonylphenyl)pyridine-3-carbonitrile 13c.

Pale yellow crystals, yield 71%, mp 220-221 ºC; IR (KBr): 3347, 3273 (2NH), 2222 (C≡N), 1686 (C=O), 1380 (S=O, asy.) and 1148 (S=O, sym.) cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ = 3.83 (s, 3H, -OCH_3), 6.00 (s, 1H, C5), 6.49-6.54 (m, 2H, Ar-H), 6.95-7.03 (m,1H Ar-H), 7.07-7.26 (m, 2H, Ar-H), 7.27-7.39 (m, 4H, Ar-H), 7.54-7.58 (m, 2H, Ar-H), 7.92-7.97 (m, 4H, Ar-H), 8.54-8.56 (m, 1H, Ar-H), 9.33 (s, D_2O-exchangeable, 1H, sulfonamide NH), 12.51 (s, D_2O-exchangeable, 1H, indole NH), ppm; MS (70 eV): *m/z* = 573 (M^++1, 3.6), 572 (M^+, 10.0), 212 (100), 168 (74.5), 140 (70.3). Anal. Calcd for $C_{33}H_{24}N_4O_4S$: C, 69.22; H, 4.22; N, 9.78; S, 5.60. Found: C, 69.38; H, 4.06; N, 9.70; S, 5.48.

1,2-Dihydro-4-(1*H*-indol-3-yl)-6-(4-methoxyphenyl)-2-oxo-1-[4-(4-methoxyphenyl) aminosulfonylphenyl] pyridine-3-carbonitrile 13d.

Pale yellow crystals, yield 71%, mp 235-236 ºC; IR (KBr): 3379, 3285 (2NH) 2220 (C≡N), 1684 (C=O), 1376 (S=O, asy.) and 1140 (S=O, sym.) cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ = 3.37 (s, 3H, -OCH_3), 3.65 (s, 3H, -OCH_3),5.93 (s, 1H, C5), 6.49-6.52 (m, 4H, Ar-H), 6.76-6.78 (m, 2H, Ar-H), 6.93-6.96 (m, 4H, Ar-H), 7.22-7.25 (m, 2H, Ar-H), 7.94-7.96 (m, 4H, Ar-H), 8.53-8.55 (m, 1H, Ar-H), 9.74 (s, D_2O-exchangeable, 1H, sulfonamide NH), 12.52 (s, D_2O-exchangeable, 1H, NH idnole) ppm; MS (70 eV): *m/z* = 603 (M^++1, 2.9), 602 (M^+, 13.3), 241 (100), 169 (73.7), 124 (45.2). Anal. Calcd for $C_{34}H_{26}N_4O_5S$: C, 67.76; H, 4.35; N, 9.30; S, 5.32. Found: C, 67.71; H, 4.28; N, 9.18; S, 5.40.

General procedure for synthesis of pyrimidines 14a, b

A 2.77 g of chalcone **3** (10 mmol) was added to sodium ethoxide solution [prepared from sodium metal (0.23 g, 10 mmol) and 50 mL of absolute ethanol] then urea or thiourea (10 mmol) was added. The reaction mixture was refluxed for 16 hr., then left to cool and poured into crushed ice and neutralized with diluted hydrochloric acid. The precipitated product was collected by filtration, washed with ethanol and dried. Crystallization from EtOH/DMF afforded the pyrimidine derivatives 14a, b, respectively.

6-(1*H*-Indol-3-yl)-4-(4-methoxyphenyl)pyrimidin-2(1*H*)-one 14a.

Pale yellow crystals, yield 69%, mp 198-200 ºC; IR (KBr): 3438, 3170 (2NH), 1669 (C=O), 1633 (C=N) cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ = 3.83 (s, 3H, -OCH_3), 5.46 (s, 1H, C5), 6.99-7.04 (m, 2H, Ar-H), 7.18-7.22 (m, 4H, Ar-H), 7.49-7.54 (m, 2H, Ar-H), 8.28-8.30 (m,1H, Ar-H), 9.94 (s, D_2O-exchangeable, 1H, pyrimidin NH), 12.07 (s, D_2O-exchangeable, 1H, indole NH) ppm; MS (70 eV): *m/z* = 318 (M^++1, 2.4), 317 (M^+, 9.0), 213 (100), 169 (38.4) 140 (42.5), 62 (36.8). Anal. Calcd for $C_{19}H_{15}N_3O_2$: C, 71.91; H, 4.76; N, 13.24. Found: C, 72.04; H, 4.78; N, 13.38.

6-(1*H*-Indol-3-yl)-4-(4-methoxyphenyl)pyrimidine-2(1*H*)-thione 14b.

Yellow powder, yield 73%, mp 180-182 ºC; IR (KBr): 3375, 3165 (2NH), 1630 (C=N) cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ = 3.35 (s, 3H, -OCH_3), 5.6 (s, 1H, C5),7.21-7.25 (m, 2H, Ar-H), 7.26-7.49 (m, 4H, Ar-H), 7.54-7.90 (m, 2H, Ar-H), 8.28-8.30 (m, 1H, Ar-H), 8.82 (s, D_2O-exchangeable, 1H, pyrimidin NH), 12.07 (s, D_2O-exchangeable, 1H, indole NH) ppm; MS (70 eV): *m/z* = 334 (M^++1, 2.6), 333 (M^+, 6.8), 144 (100), 116 (28.7), 89 (24.5). Anal. Calcd for $C_{19}H_{15}N_3OS$: C, 68.45; H, 4.53; N, 12.60; S, 9.62. Found: C, 68.48; H, 4.66; N, 12.49; S, 9.56.

3-(2-Chloro-6-(4-methoxyphenyl)pyrimidin-4-yl)-1*H*-indole 15.

A solution of 0.63 g of 14a (2 mmol) in 30 mL phosphorus oxychloride was refluxed for 5 hr., then left to cool and poured carefully into crushed ice. The precipitated product was collected by filtration, washed with water and dried. Crystallization from EtOH/DMF afforded compound 15 as violet powder in 68% yield, mp 160-162 ºC; IR (KBr): 3217 (NH), 1632 (C=N) cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ = 3.38 (s, 3H, -OCH_3), 7.11-7.53 (m, 6H, Ar-H), 7.87-8.31 (m, 4H, Ar-H), 12.20 (s, D_2O-exchangeable, 1H, indole NH) ppm; MS (70 eV): *m/z* = 336 (M^++1, 1.9), 335 (M^+, 5.6), 156 (92.3), 108 (57.5), 92 (100), 64 (78.7). Anal. Calcd for $C_{19}H_{14}ClN_3O$: C, 67.96; H, 4.20; N, 12.51. Found: C, 67.79; H, 4.34; N, 12.50.

N-(4-Phenylaminosulfonylphenyl)-4-(1*H*-indol-3-yl)-6-(4-methoxyphenyl)pyrimidin-2-amine 16.

This reaction was carried out by the same procedure described in the synthesis of compounds 13a-d. Crystallization of the reaction product from EtOH/DMF afforded compound 16 as faint brown

powder in 66% yield, mp 100-101 ºC; IR (KBr): 3419, 3349, 3245 (3NH) cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ = 3.83 (s, 3H, -OCH_3), 6.5-8.32 (m, 19H, Ar-H), 9.85 (s, D_2O-exchangeable, 1H, NH), 9.93 (s, D_2O-exchangeable, 1H, NH), 12.3 (s, D_2O-exchangeable, 1H, indole NH) ppm; MS (70 eV): *m/z* = 548 (M^++1, 3.7), 547 (M^+, 12.2), 248 (41.1), 156 (88.8), 108 (56.3), 92 (100), 65 (79.0). Anal. Calcd for $C_{31}H_{25}N_5O_3S$: C, 67.99; H, 4.60; N, 12.79; S, 5.86. Found: C, 68.13; H, 4.69; N, 12.76; S, 5.66.

Pharmaceutical Applications

Antiproliferative screening

Cytotoxic activity against human breast carcinoma cell line (MCF-7) and liver carcinoma cell line HEPG-2 in vitro. The method applied is similar to that reported by Skehan,P.[30] using Sulfo-Rhodamine-B stain (SRB). Cells were plated in 96-multiwell plate (104 cells/well) for 24 h before treatment with the test compound to allow attachment of cell to the wall of the plate, different concentration of the compound under test (0, 2.5, 5, and 10 µg/ml) were added to the cell monolayer in triplicate wells individual dose, monolayer cells were incubated with the compounds for 48 h at 37°C and in atmosphere of 5 % CO_2 , after 48 h, cells were fixed, washed and stained with SRB stain, excess stain was washed with acetic acid and attached stain was recovered with Tris-EDTA buffer, color intensity was measured in an ELISA reader, the relation between surviving fraction and drug concentration is plotted to get the survival curve of each tumor cell line after the specified compound and the IC50 was calculated.

Antimicrobial screening

Applying the agar plate diffusion technique [31], the compounds were screened in vitro for their bactericidal activity against Gram positive bacteria (Staphylococcus aureus) and Gram negative bacteria (Escherichia Coli and Pseudomonas aeroginosa), and for their fungicidal activity against Fusarium, Aspergillus niger and Candida albicans. In this method, a standard 5 mm diameter sterilized filter paper disc impregnated with the compound (0.3 mg/0.1 ml of DMF) was placed on an agar plate seeded with the test organism. The plates were incubated for 24 hours at 37 °C for bacteria and 28 °C for fungi. The zone of inhibition of bacterial and fungi growth around the disc was observed.

3. Results and Discussion:

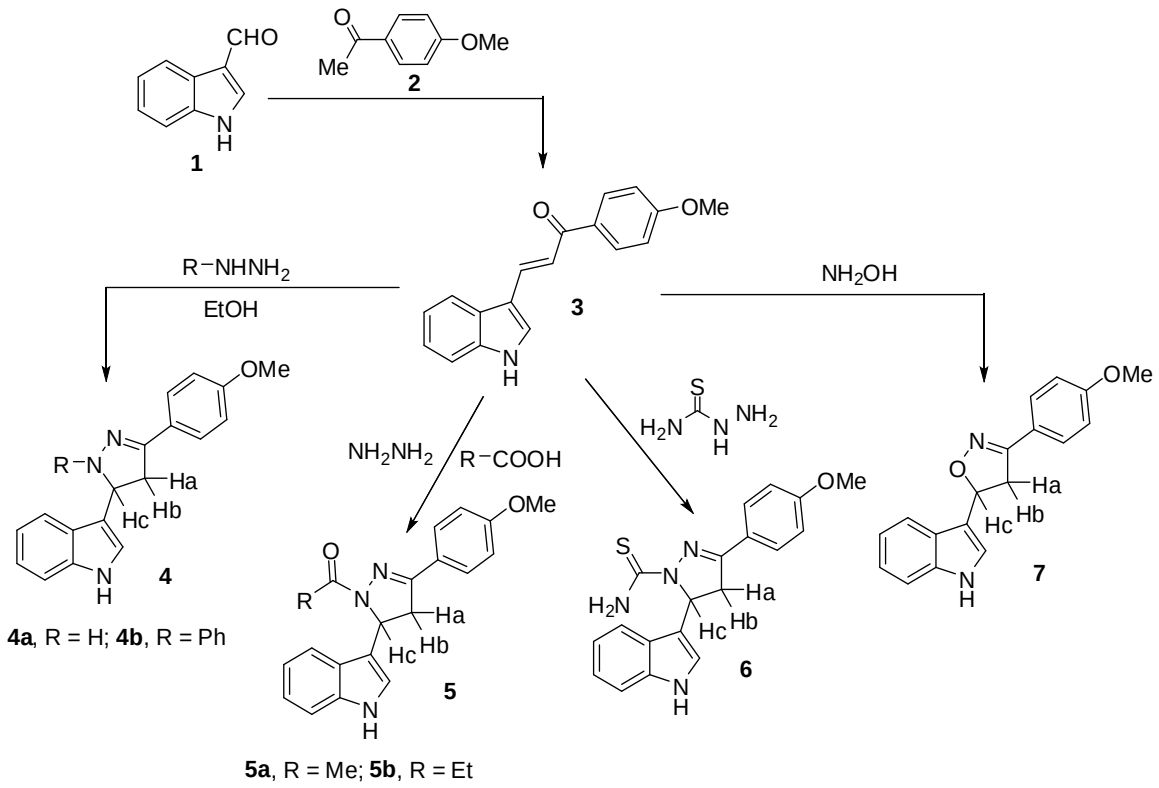
Chemistry

Aldol condensation reaction of 3-indolaldehyde 1 with 4-methoxyacetophenone 2 in ethanolic NaOH solution afforded chalcone 3. Its structure was confirmed by its IR spectrum where it showed a characteristic peak for a conjugated carbonyl group at 1633 cm^{-1}, and also by its ^1H NMR which gave signals at δ 7.53 (d, 1H, *J* = 12.9 Hz, (C=O)(CH=C), and 7.92 (d, 1H, *J* = 12.9 Hz (C=O)(C=CH). Reaction of chalcone 3 with either hydrazine hydrate or phenyl hydrazine gave the corresponding pyrazoline derivatives 4a, b respectively. Structures of 4a, and 4b were assigned by their IR and ^1HNMR Spectra. Both exhibited C=N stretching vibrations in the region 1618-1630 cm^{-1}, and compound 4a showed an additional absorption band at 3215 cm^{-1} characteristic for pyrazole NH. ^1H NMR spectra of compounds 4a and 4b showed three multiplets at δ 2.00, 2.69, and 3.40 ppm resulted from the AMX pattern displayed by two diastereotopic protons at C-4 (H_a and H_b) and one proton (H_c) at C-5 (Scheme 1).

The acyl derivatives 5a , b were prepared either by treating 4a with the corresponding acid chloride, or by reacting chalcone 3 with hydrazine hydrate by changing the reacting medium from ethanol to acetic acid or propanoic acid, respectively. Structures of 5a , b were established by their IR spectra where no characteristic band for NH was detected at 3215 cm^{-1}. The ^1HNMR of compound 5a exhibited a singlet at δ 2.24ppm (acetyl CH_3 protons), whereas 5b showed a triplet at δ 2.0 ($C\underline{H}_3CH_2$) and quartet at δ 2.68($CH_3C\underline{H}_2$) respectively.

The formation of pyrazoline 6 was achieved by refluxing chalcone 3 with thiosemicarbazide and sodium hydroxide in ethanol 30. Its structure was confirmed by its IR where absorption peaks appeared at 3316 cm^{-1} and 3227 cm^{-1}, characteristic for NH_2 and NH, respectively, and its ^1H NMR displayed two singlets at δ = 11.11ppm (D_2O-exchangeable, 2H, NH_2), and 11.60 (D_2O-exchangeable, 1H, indole NH).

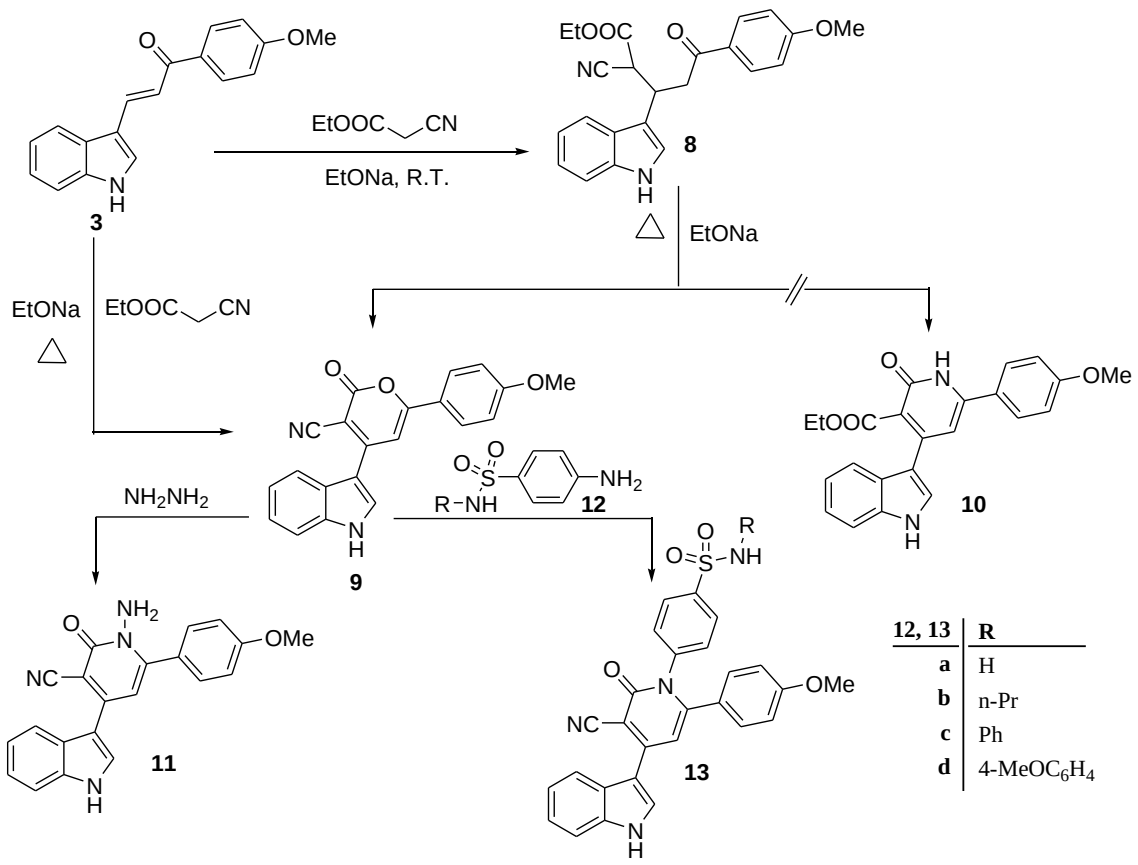
The oxazoline 7 was obtained from refluxing chalcone 3 with hydroxylamine in ethanol, its structure was ascertained by spectral data , where its IR spectrum showed 2 peaks at 3386 and 1640, characteristic for NH and C=N, respectively. Its ^1H NMR displayed a singlet at δ =11.61 (D_2O-exchangeable, 1H, indole NH). The mass spectrum revealed its molecular ion peak at *m/z* 292 (M^+) (Scheme 1).



(Scheme 1)

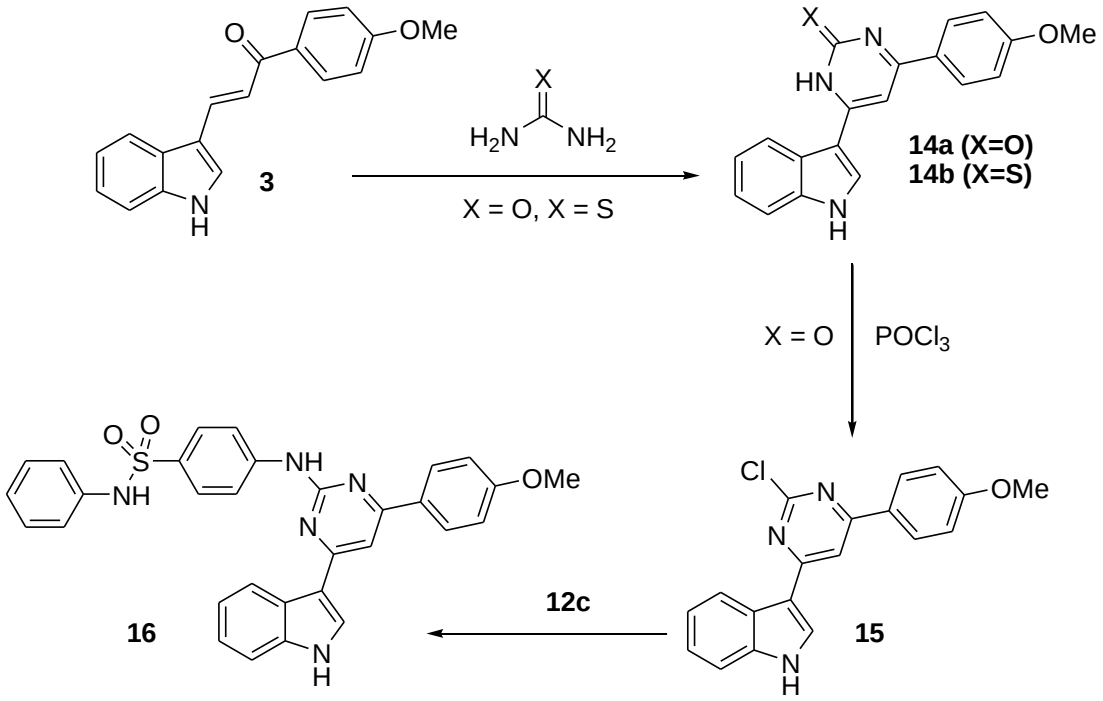
Reaction of chalcone 3 with ethyl cyanoacetate in the presence of sodium ethoxide at room temperature afforded product 8 through *Michael* addition, whereas the similar reaction gave pyranone derivative 9 under the reflux conditions [32] (Scheme 2). The elemental analysis and spectroscopic data confirmed the assigned structure for 9, where its IR displayed a peak at 2222cm^{-1} for C≡N and disappearance of the band representing the carbonyl of the COOR group. This result led to exclude the formation of an alternative compound 10. Compound 9 could also be obtained by refluxing 8 in ethanol in presence of sodium ethoxide.

Condensation of 9 with either hydrazinehydrate or sulfonamide derivatives (12a-d) [33] in refluxing ethanol gave the corresponding pyridinones 11, or 13a-d, respectively (Scheme 2). The structures of these products were ascertained by their spectral data, where their IR spectra showed absorption bands in the region 2219-2224cm^{-1} and 1665-1780cm^{-1} characteristic for C≡N and carbonyl group, respectively. In addition, compounds 13a-d displayed bands at 1375 (S=O, asy.) and 1140 (S=O, sym.) cm^{-1}. Their ^{1}H NMR spectra were agreed with the structures 11 and 13a-d.



(Scheme 2)

Reflux of chalcone **3** with either urea or thiourea in presence of sodium ethoxide in ethanol afforded the pyrimidine derivatives **14a**, **b**, respectively (Scheme 3). The structure of **14a** was substantiated by its IR, where it revealed bands at 3438, 3170 cm^{-1} for the 2NH groups, 1669 and 1633 cm^{-1} characteristic for C=O, and C=N, respectively. Its ^1H NMR, displayed two singlets at δ 9.94 for pyrimidin NH and δ 12.07 for indole NH by ppm. Reflux of compound **14a** in excess phosphorus oxychloride as a reacting medium gave compound **15** which on its reflux with sulfonamide **12c** in ethanol furnished compound **16** in a moderate yield (Scheme 3). The IR spectrum of compound **16** showed three separate absorption bands at 3419, 3349, and 3245 cm^{-1} characteristic for (3NH). Its ^1H NMR displayed three singlets at δ 9.85 (D_2O-exchangeable, 1H, NH), 9.93 (D_2O-exchangeable, 1H, NH), and 12.3 (D_2O-exchangeable, 1H, indole NH) by ppm.



(Scheme 3)

Pharmaceutical Applications

Anti-proliferative screening

Compounds 5a, 6, 7, 13a, 13b, 13d and 14b were tested against a human breast carcinoma cell line (MCF7) and a human liver carcinoma cell line (HEPG2), using 5-Fluorouracil (5-Fluoro-1*H*-pyrimidine-2,4-dione) as a reference drug (Table1). The measurements were carried out in the National Institute of Cancer, Cairo University, Cairo Egypt.

Table1. **In vitro cytotoxic activity (IC_{50}) of compounds 5a, 6, 7, 13a, 13b, 13d and 14b and 5-Fluorouracil against a human breast carcinoma cell line (MCF7) and a liver carcinoma cell line (HEPG2)**[a]

Entry	compound	MCF7 ($\mu g.mL^{-1}$)	HEPG2 ($\mu g.mL^{-1}$)
1	**5a**	1.01	-
2	**6**	2.62	2.48
3	**7**	2.55	5.17
4	**13a**	1.34	4.09
5	**13b**	0.84	5.30
6	**13d**	0.60	3.96
7	**14b**	0.85	2.68
8	5-Flurouracil	0.67	5.0[b]

[a]IC_{50} is defined as the concentration which results in a 50% decrease in cell number as compared with that of the control structures in the absence of an inhibitor.

[b]This value of IC_{50} for the reference drug 5-Fluorouracil against HEPG2 was maintained by the National Institute of Cancer, Cairo University, Cairo, Egypt. (The liver carcinoma cells were found to be more resistant to 5-Fluorouracil as a reference drug).

As shown in Tables 1, it was found that compounds 5a, 6, 7, 13a, 13b, 13d and 14b have significant anti-proliferative activities against human breast cell line MCF-7 compared to the reference standard drug 5-Fluorouracil. Compounds 13b and 13d showed high

activities which can be attributed to the presence of the sulfonamide group, where it was confirmed [34, 35] that compounds containing sulfonamide function group have potent anti-proliferative activities. Compound 13d was found to have a promising activity due to the presence of a methoxy group in the para- position to the sulfonamide group [36]. The screened results against liver carcinoma cell line (HEPG2, Table 1) revealed that the tested compounds 6, 7, 13a, 13b, 13d and 14b showed significant activities compared to the reference drug 5-Fluorouracil.

Antimicrobial screening

The synthesized new compounds 4a, 5a, 6, 7, 11, 13a, 13b, 13d and 14b were screened in vitro for their bactericidal activity against Gram positive bacteria (*Staphylococcus aureus*) and Gram negative bacteria (*Escherichia Coli* and *Pseudomonas aeroginosa*), and for their fungicidal activity against *Fusarium*, *Aspergillus niger* and *Candida albicans* (Table 2). These measurements were carried out in the Quality Control and Propagation of plants unit, Department of Botany, Faculty of Women, Ain Shams University. All the screened compounds showed high or moderate bactericidal activity against *Staphylococcus aureus and Pseudomonas aeroginosa* compared to that of ciprofloxacin. Compounds 4a, 5a, 6, 13a, 13b and 13d showed a good fungicidal activity, near to that of nystin, against *Fusarium*, all screened compounds except 11 were found to have moderate fungicidal activity against *Candida albicans*.

Table 2. Bactericidal and fungicidal activity of some of the new compounds and ciprofloxacin and nystin.[a]

No.	*Staphylococcus aureus*	*Escherichia coli*	*Pseudomonas aeroginosa*	*Fusarium*	*Aspergillus niger*	*Candida albicans*
4a	+++ (32)	-	++ (17)	+++ (31)	-	++ (16)
5a	++ (21)	-	++ (20)	++ (16)	-	++ (18)
6	+++ (30)	-	++ (19)	+++ (27)	-	++ (20)
7	++ (18)	-	++ (17)	-	-	++ (22)
11	++ (15)	-	+++ (27)	-	-	-
13a	+++ (34)	-	+++ (29)	+++ (32)	-	++ (22)
13b	+++ (28)	-	++ (17)	+++ (31)	-	++ (20)
13d	+++ (34)	-	+++ (31)	+++ (30)	-	++ (24)
14b	+++ (29)	-	++ (15)	-	-	++ (20)
Ciprofloxacin	++++	++++	++++	-	-	-
Nystin	-	-	-	++++	++++	++++

[a] The activities are based on the diameters of zones of inhibition in mm. One mL of stock solution (5 ug/mL in DMF) was applied in each hole of each paper disk.+: < 15 mm; ++: 15-24 mm; +++: 25-34 mm; ++++: 35-44 mm, etc.

4. Conclusion

Aldol condensation reaction between 3-indolaldehyde and 4-methoxyacetophenone gave a chalcone compound from which some pyrazoline, pyridine, and pyrimidine derivatives linked to indole moiety were obtained and found to have promising antitumor and antimicrobial activities.

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Increasing Health Benefits of Milk Fat by Partial Replacement with vegetable oils Fortified with Carotenoids

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Abstract: Partial replacement of milk fat with vegetable oils meet consumer demanded for reducing the intake of saturated fatty acids and hence may contribute to reduce the risk for heart disease. Different natural sources of carotenoids as fat-soluble antioxidants (green pepper, parsley and broccoli) were mixed in emulsion form with milk fat phase to protect the polyunsaturated fatty acids of vegetable oils (olive oil, sunflower oil, sesame oil, 1:1:1, v/v/v) which used in partial replacement from the oxidization, besides acting as biological antioxidants. Carotenoids solubility efficiency through the preliminary treatments of milk such as blending, pasteurization and homogenization was monitored as well as antioxidant activity using ß-carotene/ linoleic acid assay was determined in homogenized milk with (2%) carotenoids extract emulsion. Results showed that the solubility of carotenoids increased with blinding, pasteurization and homogenization. The partial substitution of milk fat was the most suitable milk fat phase as a healthy benifits. Broccoli showed the highest carotenoid content and also recorded the highest antioxidant activity. [Journal of American Science. 2010;6(8):352-360]. (ISSN: 1545-1003).

Keywords: Milk fat, vegetable oils, carotenoids solubility, antioxidant activity.

1. Introduction:

Consumer is looking for the products can offer more health benefits. Full-fat milk is rich in saturated fatty acids, which has been shown to significantly elevate total, Low-density lipoprotein (LDL-), and high-density lipoprotein (HDL)-cholesterol and apolipoprotein B and A concentrations (Kris-Etherton *et al*. 1993; Wood, *et al.* 1993) which has long been associated with increased risk of cardiovascular disease. To meet consumer becomes demand reducing the intake of saturated fatty acids from the milk fat; partial substitution of milk fat by vegetable oils leads to reduction of saturated fatty acids. From a nutritional point of view, a different fatty acids profile of the consumer dairy product has many benefits, keeping part of milk fat has positive effect due to milk fat globule membrane (MFGM) contained signaling molecules called sphingolipids which has anticancer properties (Berra *et al.* 2002; Hertervig *et al*. 2003; Lemonnier *et al*. 2003). Cholesterol in milk fat is an essential part of sex hormones, bile acids, D vitamins and steroid hormones (Whitney and Rolfes 2002), acts also as an antioxidant (Cranton and Frackelton 1984). Conjugated linoleic acid (CLA), a component of milk fat, has also potential health benefits (MacDonald 2000; Collomb et al. 2006; Salas-Salvado et al. 2006). In same time, finding more unsaturated fatty acids from vegetable oils are desirable. Vegetable oils are rich in omega. Both linoleic and linolenic acid have a lowering effect on blood cholesterol and hence may contribute to reduce the risk for heart disease.

When vegetable oils are used, in combination with milk fat or on their own, the balance between various fatty acids can be adjusted to a higher nutritional value. On other hand; more poly unsaturated fatty acids (PUFA) are more sensitive to get rancid by lipid oxidation. Oxygen can also become activated in the presence of metals or metal complexes and initiate lipid oxidation by the formation of either free radicals or singlet oxygen ($^1O_2$). Singlet oxygen is a very efficient oxidizing agent (Frankel, 2005). The oxidation stability of vegetable oils and fats is determined by their degree of unsaturation, the presence of natural or synthetic antioxidants, the presence of pro-oxidants such as metals and the availability of oxygen.

An antioxidant can eliminate potential initiators of oxidation and thus prevent reaction. It seems, that antioxidant in milk should be applied as a systems containing compounds operating according to various mechanisms (chelators of metal ions, scavengers of free radicals or other reactive forms of oxygen, compounds deactivating enzymes), because they would be able to prove synergistic effect increasing antioxidant property of other antioxidant (Decker, 1998, Zhang and Omaye, 2000). Carotenoids, fat-soluble antioxidants can be used to protect the PUFA from the oxidized (Shany *et al*. 2006) besides acting as biological antioxidants, protecting cells and tissues from the damaging effects of free radicals and singlet oxygen (Alam and Sultan

2004) and their antioxidant properties very much depend concentration (Palozza 1998).

The aim of this research was using a combination of vegetable oils with milk fat to give higher nutritional value in dairy product by partial replacement of milk fat with vegetable oils (olive oil, sunflower oil, sesame oil). Vegetable oil phase consist of olive oil which is composed mainly of the oleic acid (55-85%); sunflower oil which is high in linoleic acid (Omega 6) (67%) and sesame oil which contains 42% oleic acid, 15% saturated fat, and 43% omega- 6 linoleic acid (Alpaslan *et al.* 2001; Sankar *et al.* 2006). Many natural sources of carotenoids were mixed with different fat phases of milk and investigated for both solubility through blending, pasteurization and homogenization, and evaluate the antioxidant activity of homogenized milk with carotenoids natural sources.

2. Materials and Methods:

Materials

Preparation of carotenoids extract in emulsion forms.

Broccoli, parsley and green pepper from local market were chopped into fine pieces by a commercial blender. 10 g of fine pieces were immediately homogenized again for 30 s with the vortex blender with 2% edible oil (corn oil) and 0.5% emulsifier (tween 20) in water bath (90°C/5 min). Corn oil is exhibited as a medium stability of carotenoids and considers a very popular delivery system. Carotenoids extract emulsion was cooled in an ice bath for 5 min and filtered through a buchner funnel using Whatman filter paper No. 4.

Vegetable oils (from NRC) used in partial substitution of milk fat.

Three types of vegetable oils should have similar melting properties (melting point 58 to 60°C, Salado, et al. 2004) was chosen and used to create a balance of fatty acids by ratio (1:1:1) to get a higher nutritional value.

Methods

Preparation fat phases incorporated with carotenoids

Three milk fat phases were prepared mixed with skim milk to give 3% milk fat; 1.5% milk fat mix with 1.5% vegetable oils and 3% vegetable oils. These fat phases were mixed with carotenoids extract emulsion at different concentrate (0.5, 1.0, 1.5 and 2%), they blended, pasteurized at 65°C / 30 min and homogenized at two-stags (200, 50 Kg/cm^2) (Rannie homogenizer). Homogenization used as a mechanical emulsifying process for incorporations the emulsion of carotenoids with skim milk.

Extraction of fat phase from milk

Extraction of fat from the milk was carried out as described by (Havemose *et al.* 2004). Fat phase was extracted from milk by adding methanol and chloroform with ratio (1:1, v/v) to the milk. The mixture was shaken vigorously for 1min, and then centrifuged for 10min at 3000 × g at 4 °C. Total carotenoid content was determined in the lower phase containing the lipid fraction.

Evaluation the total carotenoid content (TC)

Carotenoids are expected to be dissolved in the dispersed fat droplets and its soluble efficiency in fat phase was evaluated by determination the total carotenoid content (TC) in fat phase after emulsion homogenization by absorption SP-2000UV UV-VIS spectrophotometer according to (Lachman *et al.* 2003). Absorbance of organic extracts was then measured in 1 cm cuvettes at λ = 444 nm and total carotenoid content in mg/kg fw of sample was expressed as lutein equivalent from the equation:

$$(K + X)L = \frac{A444.\ 25\ .\ 15}{0.259\ m}\ (\text{mg/kg fw})$$

Where:

(K + X)L is total carotenoid content (carotenes and xanthophylls)

A444 is absorbance of acetone extract at λ = 444 nm

m is sample weight (g).

Determination antioxidant activity using β-carotene bleaching assay.

Evaluation of antioxidant activity based on coupled oxidation of ß-carotene and linoleic acid was conducted as described by Taga et al. (1984). ß-carotene (6 mg) was dissolved in 20 ml of chloroform. A 3 ml of the solution was added to a conical flask with 40µl linoleic acid and 400 µl Tween 20. Chloroform was removed by using a rotary evaporator under vacuum at 35ºC. Oxygenated distilled water (100 ml) was added to the ß-carotene emulsion and mixed well. 3 ml aliquot of the ß-carotene emulsion and 0.2 ml of the diluted sample extract were placed in a test tube and mixed well. The tubes were immediately placed in a water bath and incubated at 50ºC. Oxidation of ß-carotene emulsion was monitored by measuring absorbance at 470 nm. Sample absorbance was measured at 60 min after incubation. A control consisted of 0.2 ml distilled water, instead of the extract.

The degradation rate of the extracts was calculated by first order kinetics: Sample degradation rate = ln (a/b) ×1/t, where: ln = natural log; a = initial absorbance at time 0; b = absorbance at 60 min; t = time (min). Antioxidant activity (AA) was expressed

as % inhibition relative to the control using the equation:

$$AA(\%) = \frac{\text{Degradation of control Degradation of sample X 100}}{\text{Degradation of control}}$$

Statistical Analysis

All data are presented as the mean ± SD. One-way analysis of variance (ANOVA) unpaired and paired t-tests were performed using statistical Analysis System (SAS Institute, 1988) ($P \leq 0.05$) was the level of significance.

3. Results:

Total soluble carotenoids content (TC) in fat phase (3%) supplemented with green pepper extract.

Total carotenoids content (mg/kg fw) resulted in full fat phase (3%) supplemented with different ratio of green pepper extract (0.5, 1.0, 1.5 and 2.0%) at different treatments showed at Tab. 1. The average of soluble carotenoids percentage of the green pepper in fat phases was different as showen in Fig 1. The average soluble carotenoids percentage to the absolute emulsion at different concentrate in blending extract/ full fat milk was 46.0% increased in extract/partial substitution milk fat to 60.5% and extent in increasing in extract/vegetable fat milk to 71.4%. At pasteurization stage, the average soluble carotenoids percentage from the absolute emulsion in extract/ full fat milk was also 49.25% increased also to 63.5% in extract/partial substitution milk fat and to 72.0% in the extract/vegetable fat milk. At the following stage, the average soluble carotenoids percentage in homogenized extract/ full fat milk with different concentrate was 57.7% increased to 73.0 and 82.6% in homogenized extract/partial substitution milk fat and extract/vegetable fat milk respectively.

Total soluble carotenoids content (TC) in fat phase (3%) supplemented with parsley extract

Total carotenoids content (mg/kg fw) resulted in full fat milk (3%) supplemented with different ratio of parsley extract through different treatments showed at Tab. 1. The average soluble carotenoids percentage from the absolute emulsion in blending extract/ full fat milk was 59.5% increased in extract/partial substitution milk fat to 69.5% and extent in increasing in extract/vegetable fat milk to 78.7%. At pasteurization stage, the average soluble carotenoids percentage of absolute emulsion in extract/ full fat milk was 62.82% increased also to 69.65% in extract/partial substitution milk fat and to 78.77% in the extract/vegetable fat milk. At the following stage, the average soluble carotenoids percentage in homogenized extract/ full fat milk was 67.87% increased to 76.64 and 85.73% in homogenized extract/partial substitution milk fat and extract/vegetable fat milk respectively. These gradually increasing of soluble carotenoids in different fat phase through different treatments showed in Fig 2.

Total soluble carotenoids content (TC) in fat phase (3%) supplemented with broccoli extract

It is obviously that the broccoli has the highest source of carotenoids compared to the other sources. Resulted showed that soluble carotenoids percentage from the absolute broccoli extract through different treatments showed at Tab. 1. With blending the average soluble carotenoids percentage calculated was 64.47% in extract/ full fat milk increased to 74.29% and 84.28% in extract/partial substitution milk fat and in the extract/vegetable fat milk respectively. At pasteurization the milk, the average soluble carotenoids percentage was 70.72, 79.02 and 87.19% in extract/ full fat milk, in extract/partial substitution milk fat and in the extract/vegetable fat milk respectively. The solubility of carotenoids was increased after homogenization that was 73.43, 82.28 and 95.25% in extract / full fat milk, extract/partial substitution milk fat and in the extract/vegetable fat milk respectively Fig 3.

Antioxidant activity (%)

Results showed that a positive correlation between dissolved natural source percent and its soluble carotenoids, so that we choose (2% emulsion) after homogenized stage to determination the antioxidant activity of the carotenoids soluble in fat phase

Results showed that antioxidant activity was increased significantly with increasing the carotenoids content (Table 1). Broccoli extracts dissolved in modified fat showed promotion of bleaching of the β-carotene emulsion; it was 11.46% in milk broccoli mixture (as antioxidant activity) followed by 11.05% in parsley and 10.28% in green pepper mixture as antioxidant activity as show in Table 1. A correlation coefficient (r^2) between total carotenoids (TC) and antioxidant activity was 0.95. The results revealed that the correlation between total carotenoids (TC) and antioxidant activity was strongly positive.

Table 1: Total carotenoid content (mg/Kg fw) in 3% fat phase and antioxidant activity of homogenized milk with 2% carotenoids extract emulsion.

Treatment	Conc.	Green pepper				Parsley				Broccoli			
		Milk fat	Mix*	Oil**	Absolute green pepper.	Milk fat	Mix*	Oil**	Absolute Parsley	Milk fat	Mix*	Oil**	Absolute Broccoli
Blending	0.5	173^{d}	225^{c}	265^{b}	401^{a}	238^{d}	284^{c}	341^{b}	423^{a}	252^{d}	314^{c}	370^{b}	430^{a}
	1	193^{d}	264^{c}	311^{b}	438^{a}	284^{d}	327^{c}	362^{b}	454^{a}	310^{d}	338^{c}	385^{b}	456^{a}
	1.5	216^{d}	287^{c}	334^{b}	454^{a}	297^{d}	341^{c}	379^{b}	475^{a}	322^{d}	363^{c}	407^{b}	489^{a}
	2	237^{d}	300^{c}	358^{b}	475^{a}	322^{d}	364^{c}	398^{b}	508^{a}	335^{d}	388^{c}	427^{b}	512^{a}
Pasteurization	0.5	191^{d}	238^{c}	288^{b}	418^{a}	251^{d}	305^{c}	357^{b}	431^{a}	282^{d}	335^{c}	376^{b}	432^{a}
	1	214^{d}	279^{c}	324^{b}	445^{a}	311^{d}	338^{c}	384^{b}	488^{a}	333^{d}	364^{c}	406^{b}	458^{a}
	1.5	237^{d}	304^{c}	351^{b}	475^{a}	332^{d}	363^{c}	399^{b}	513^{a}	356^{d}	391^{c}	428^{b}	491^{a}
	2	269^{d}	335^{c}	366^{b}	494^{a}	341^{d}	375^{c}	419^{b}	518^{a}	378^{d}	417^{c}	451^{b}	525^{a}
Homogenization	0.5	224^{d}	290^{c}	325^{b}	425^{a}	290^{d}	329^{c}	391^{b}	441^{a}	318^{d}	351^{c}	428^{b}	436^{a}
	1	261^{d}	338^{c}	375^{b}	454^{a}	327^{d}	362^{c}	413^{b}	492^{a}	341^{d}	388^{c}	464^{b}	486^{a}
	1.5	304^{d}	376^{c}	426^{b}	509^{a}	346^{d}	399^{c}	436^{b}	515^{a}	390^{d}	431^{c}	488^{b}	510^{a}
	2	335^{d}	418^{c}	486^{b}	522^{a}	400^{d}	449^{c}	481^{b}	530^{a}	410^{d}	466^{c}	507^{b}	553^{a}
Antioxidant activity (%)	2	8.24^{d}	10.28^{c}	11.96^{b}	13.05^{a}	9.84^{d}	11.05^{c}	11.83^{b}	13.25^{a}	10.09^{d}	11.46^{c}	12.47^{b}	13.83^{a}

The different letters means significant

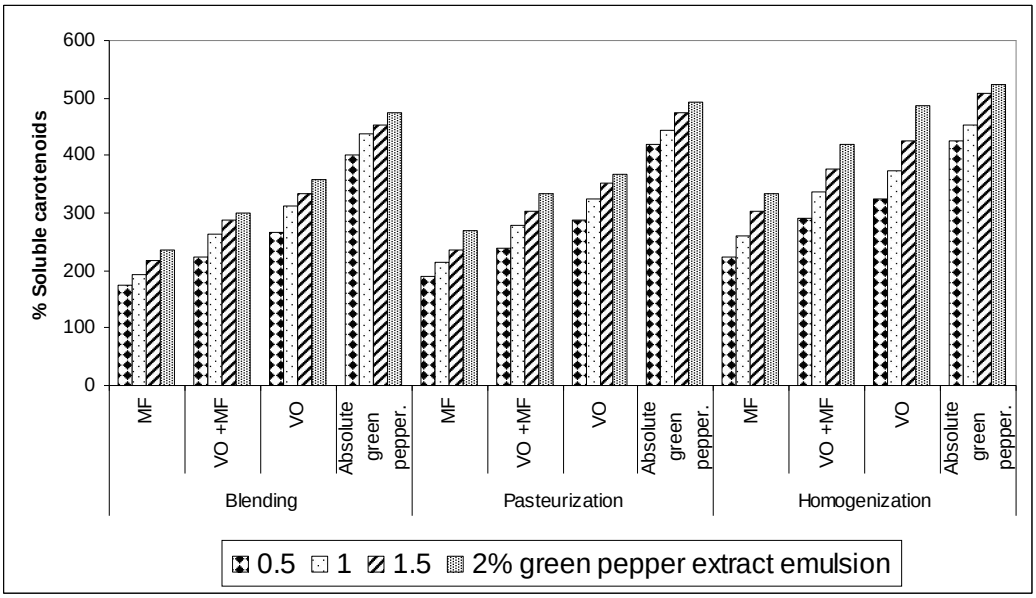

Figure 1: The average of soluble carotenoids percentage of the green pepper in different fat phases, at each technological treatments.

MF: Milk fat
VO+MF: Milk fat +vegetable oil (1:1)
VO: Vegetable oil

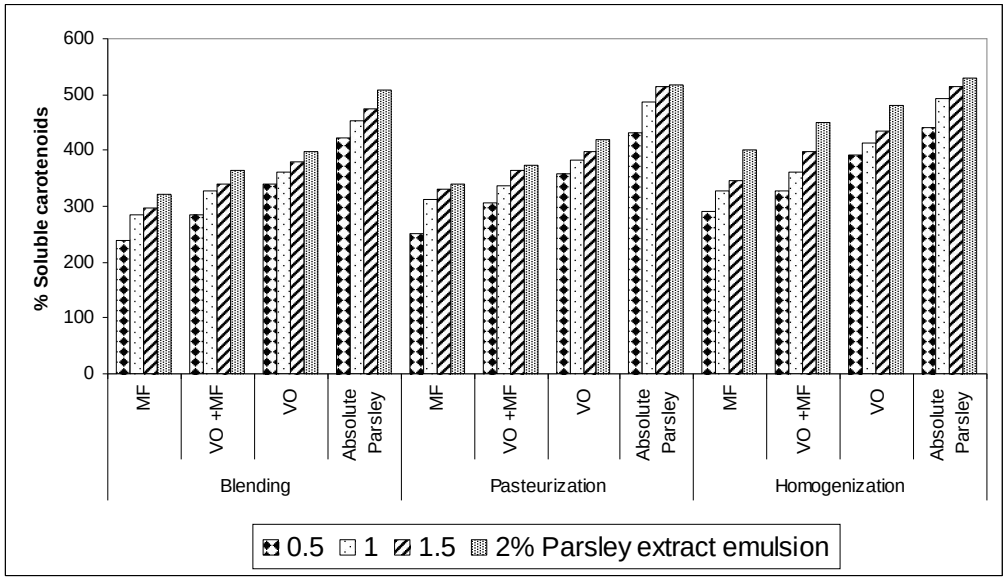

Figure 2: The average of soluble carotenoids percentage of the parsley in different fat phases, at each technological treatments.

MF: Milk fat
VO+MF: Milk fat +vegetable oil (1:1)
VO: Vegetable oil

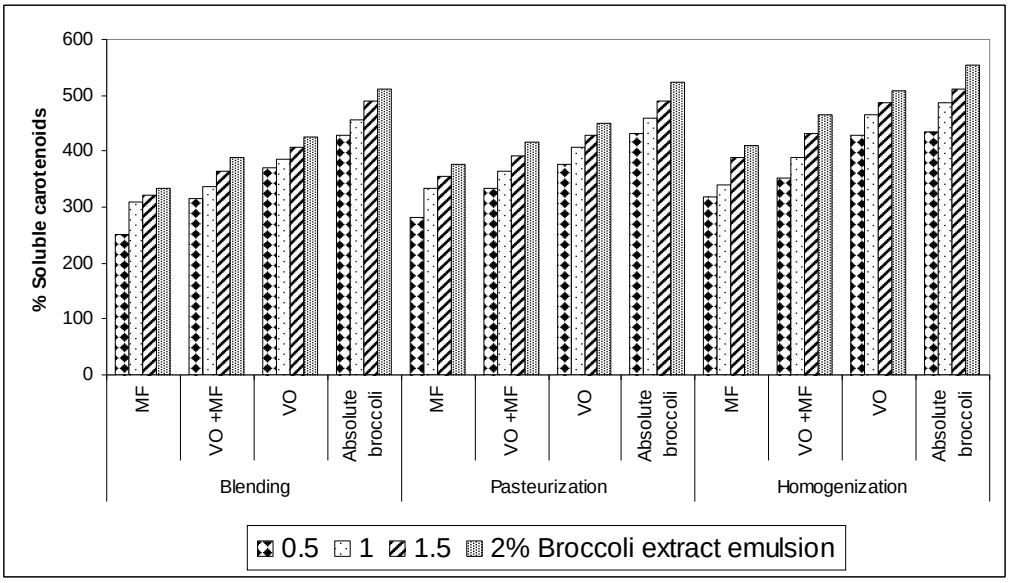

Figure 3: The average of soluble carotenoids percentage of the broccoli in different fat phases, at each technological treatments.
MF: Milk fat
VO+MF: Milk fat +vegetable oil (1:1)
VO: Vegetable oil

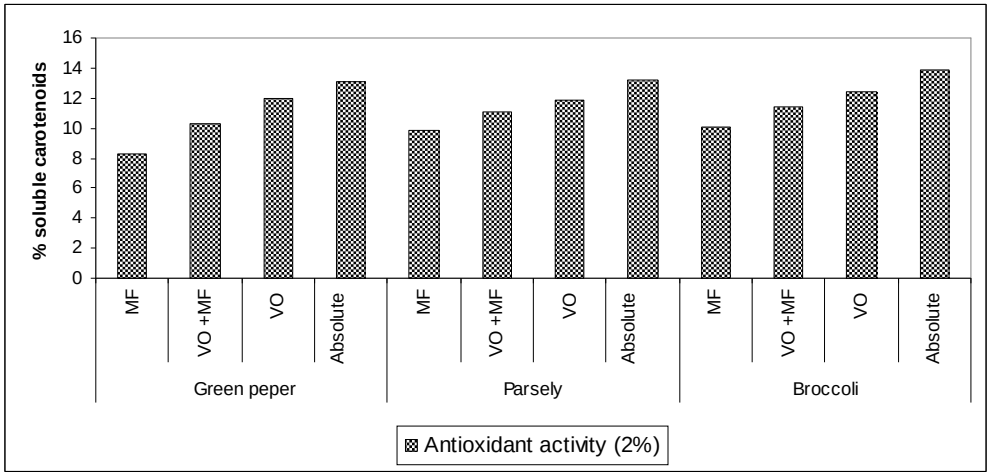

Figure 4: Antioxidant activity (%) of homogenized milk with 2% carotenoids extract emulsion from green pepper, parsley and broccoli.
MF: Milk fat
VO+MF: Milk fat +vegetable oil (1:1)
VO: Vegetable oil

4. Discussion:

It is obviously that total carotenoids in absolute emulsion were decreased after technological treatments. After blending carotenoids in the vegetable particles were transferred from the vegetable matrices into the two phases of milk; fat phase and aqueous phase, therefore total carotenoids in fat phase was decreased than in absolutely of the three different vegetable matrices extract emulsion. The carotenoids in the aqueous phase were bound to bovine serum albumin (BSA) forming the carotenoid–protein complex (Wackerbarth *et al.* 2009).

An important step is to measure and predict the solubility of target components (carotenoids) in the fat phase of milk at pasteurization and homogenization conditions, the preliminary steps towered dairy manufacture to optimize the extraction process. At pasteurization stage to inactivation of microorganisms, resulted showed increasing in the total carotenoids content although the carotenoids are sensitive to heat, and may varying percentages of desirable constituents such as nutrients, colour, aroma and texture are destroyed (Alwazeer *et al*. 2002; Blasco *et al*. 2004). This increased may relate to lower the density of fat phase that had influence increasing the solubility of carotenoids. A second hypothesis is that the different vegetable matrices (green pepper, parsley and broccoli) have different abilities to release carotenoids (due to different fiber composition or different intracellular locations of carotenoids) during heat treatment. After homogenization, the total carotenoids content was extent increased as showed in Table.1. A first hypothesis is that homogenization pressures size-reduced the fat droplets and further the carotenoids were associated with lipid portions thus enhancing the total soluble carotenoids. The solubility of natural sources of targeted carotenoids (green pepper, parsley and broccoli) is related to its physical and chemical properties such as polarity, molecular structure, and nature of the material particles and it is also related to the operating conditions such as temperature, pressure, density of fat (Shi *et al.* 2007). The degree of saturation of fat phase was also significantly affect ($P \leq 0.05$) on the carotenoids solubility. Oils rich in unsaturated fatty acids promote carotenoid solubility. Results showed that vegetable oils which are usually rich in polyunsaturated fatty acids were enhanced the release of carotenoids from vegetable matrices; whereas in partial substitution of milk fat (saturated fat milk) by vegetable oils the release of total corotenoids was more than in milk fat (saturated fat milk). Solubility of carotenoids depends on the presence of polar groups in the carotenoid and fatty acid pattern of the vegetable oil (Borel *et al.* 1996).

In dairy products, in addition to carotenoids function as the natural pigments and provitamin A precursor role, carotenoids has another function that is the role the proteins with respect to other molecules (Britton 1995). Partial substitution of milk fat is important not only because of their nutritional significance, but also due to their role in providing the required fatty acids to the body that may render health benefits to the consumer. In this condition, carotenoids present in these vegetables have a strong

ability to neutralize active oxygen species (Howard et al. 2000) and therefore can protect unsaturated fatty acids against oxidation free radical damage (Surita *et al.* 2008) and off-flavor development.

In the β-carotene/linoleic acid assay, antioxidant activity of the carotenoids extracts from fat phase of homogenized milk (2%) varied and broccoli was higher in antioxidant activity than parsley and green pepper respectively ($P<0.05$). There are positive correlation between carotenoids content and its antioxidant activity. Antioxidants play an important role in the protection of human body against damage by reactive oxygen species (Govindarajan, 2005). Furthermore previous epidemiological studies have shown that the intake of natural antioxidants has been associated with reduced risks of cancer and other diseases associated with oxidative damages (Rietveld and Wiseman, 2003).

The stability and nutritional properties can be optimized for different applications, depending on the demands on the final product.

5. Conclusion:

The reduction of saturated fatty acids and cholesterol content in the human diet has thus become an increasingly important goal in modern medicine, especially in view of the preponderance of evidence connecting these components of the diet are increased risk for heart disease, etc. Partial replacement of milk fat (saturated fatty acids) with vegetable oils and enrichment milk with natural source for carotenoids (as antioxidant) is an excellent way to develop optimum health benefits of dairy products. When vegetable oils are used, in combination with milk fat or on their own, the balance between various fatty acids can be adjusted to a higher nutritional value. Supplementation milk with natural source of carotenoids play an important role as antioxidant for protect the PUFA from the oxidation and as a fortified product. Choosing the type of carotenoids source depend on the sensory evaluation of milk supplemented and the product need to manufacture. Therefore, the consumption of the described modified dairy product may meet the demand of subjects who wish to lower their risk for atherosclerosis and cardiovascular disease.

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New Safe Methods for Controlling Anthracnose Disease of Mango (*Mangifera indica L.*) Fruits Caused by *Colletotrichum gloeosporioides* (Penz.)

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Abstract. Mango suffers from several diseases at all stages of its life. Anthracnose, caused by the fungus *Colletotrichum gleosporioides* is the most important post harvest disease of mango. The effect of various concentrations of chitosan solution on the mycelium growth and spore germination of *Colletotrichum gloeosporioides* (Penz.) the causal agent of anthracnose disease of mango fruits was studied under vitro conditions. Chitosan solution at 0.6mg/l obtained significantly reduction of *C. gloeosporioides* growth and inhibited spore germination, while, chitosan solution at 0.8mg/l resulted a complete reduction and inhibition of fungal mycelium growth and spore germination. Meanwhile, coating mango fruits with 0.2 and 0.4% (w/v) chitosan solution obtained a highly protective effect against anthracnose disease incidence of mango fruits, by 98.1% and 95.4% after 30 days of storage, respectively. At the same treatments were reducing the percentage of fruit rotted tissues by 89.3 and 95.0%, respectively. The results of this study showed that chitosan was a alternative safe coating method for prevent mango fruits against anthracnose disease which causes economic losses during transportation, marketing and storage. [Journal of American Science. 2010;6(8):361-367]. (ISSN: 1545-1003).

Key words: Chitosan – Mango fruits – Anthracnose disease –*Colletotrichum gloeosporioides.*

1-Introduction

Mango (*Mangifera indica* L.) is considered one of the most popular fruits among millions of people in the tropical area and increasingly in the developed countries (FAO STAT, 2005). Mango fruits are sensitivity to decay, low temperature and general fruit perish ability due to the rapid ripening and softening limits the storage, handling and transport potential.

Anthracnose, caused by *Colletotrichum gloeosporioides* (Penz.) Penz. And Sacc., is the major postharvest disease of mango in all mango producing areas of the world (Dodd *et al*., 1997) and (Swart *et al*., 2002). The disease occurs as quiescent infections on immature fruit and the damage it incites is more important in the postharvest period (Muirhead and Gratitude, 1986; Dodd *et al*., 1997). Fungicides, either as preharvest or postharvest treatments, form the main approach to reduce losses from anthracnose. However, their use is increasingly restricted due to public concerns over toxic residues. Moreover, fungicides are unaffordable for many mango growers in developing countries (Dodd *et al*., 1989).

Figure 1, Anthracnose disease symptoms on mango fruits

Synthetic fungicides are the primary means to control postharvest diseases (Eckert,1990 and 1991). They are used alone, combined in mixtures, or applied separately in sequence (Ismail and Zhang,2004). However, several fungicides have been removed from the market due to possible toxicological risks. In addition, repeated use of certain systemic fungicides in packinghouses has led to the appearance of fungicide-resistant pathogens. Over recent decades there has been increasing public pressure to reduce the use of synthetic fungicides in agriculture products and their presence in the environment. Moreover, concerns have been raised

about the health risk involved in the use of synthetic fungicides on fresh fruits and vegetables shortly before consumption. Chitosan (poly-β-(1→4)*N*-acetyl-d-glucosamine) derived from the outer shell of crustaceans, has become a promising alternative treatment due to its natural character, antifungal activity, and elicitation of defense responses in plant tissue (Terry and Joyce, 2004). Some research results have indicated that chitosan can inhibit the growth of *Puccinia arachidis* Speg. (Sathiyabama and Balasubramanian, 1998), *Alternaria alternata* (Fr.) (Reddy *et al.*, 1998) and *Aspergillus niger* V. Tiegh. (Plascencia- Jatomea *et al.*, 2003). Coating citrus fruit with chitosan was effective in controlling fruit decay caused by *Penicillium digitatum* Sacc. And *Penicillium expansum* Link (Chien *et al.*, 2007) and rots including gray mould and blue mould caused by *B. cinerea* and *P. expansum* in sweet cherry fruit were reduced by preharvest spraying or postharvest dipping of chitosan (Romanazzi *et al.*, 2003). The objectives of this study were to investigate the effects of chitosan on the control of anthracnose disease caused by *Colletotrichum gloeosporioides* in mango fruits, as well as to evaluate the antifungal activity of chitosan against pathogen *in vitro*, and their effect of disease incidence on mango fruits.

2-Materials and methods

2.1. Fruits

Mango (*Mangifera indica* L.) fruits Sanara cv. were harvested at the mature stage, and sorted based on size and the absence of physical injuries or disease infection. Before treatments, fruit were surfaced disinfected with 2% sodium hypochlorite for 3 min, then rinsed with tap water, and air-dried.

2.2. Pathogen culture

Colletotrichum gloeosporioides was cultured for 1–2 weeks on potato dextrose agar (PDA) at 25 ◦C. The isolate used was obtained from infected mango fruit in Egypt. Spores were harvested by adding 3–4 ml of sterile, de-ionized water (diH2O) to the Petri dish. The spores were then rubbed with a sterile glass rod to free them from the PDA medium, and the spore suspension was passed through two layers of cheese cloth. The suspension was diluted with water to obtain the spore concentrations (10^6 spores ml^{-1}) according to determination with a haemocytometer.

2.3. Chitosan solution preparation

Crab-shell chitosan, purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.), was ground to a fine powder (particle size smaller than 1 mm) by extensive grinding in a mortar, washed 3 times in distilled water (20 ml of water per g of chitosan), pelleted by low-speed centrifugation and air-dried at room temperature. The purified chitosan was prepared as described by Benhamou *et al.*, (1998). For experimental use the stock solution, (1%, w/v) of chitosan, was prepared by dissolving purified chitosan in 0.5% (v/v) glacial acetic acid (Du *et al.*,1998), under continuous stirring, and the pH was adjusted to 5.6 using 1 N NaOH.

2.4. Effect of different chitosan concentrations on linear growth of *C. gloeosporioides*

Different concentrations of chitosan solution prepared by the method described by El-Gaouth (1992). chitosan solution was added to conical flasks containing melted PDA medium to obtain final concentrations of 0.0, 0.2, 0.4, 0.6 and 0.8 mg/l and mixed gently and then dispensed in sterilized Petri plates (10 cm diameter).Plates were individually inoculated at the center with equal disks (10-mm-diameter) of the same physiological age of each *C. gloeosporioides***,** then incubated at 22-25°C. The average linear growth of fungi tested was calculated.

2.5.Effect of different chitosan concentrations on spore germination of pathogenic fungi

Conidia of 10 days old of *C. gloeosporioides* cultures were harvested in sterilized water containing 0.1% (Tween 80), aliquots of spore suspension (10^6 spore/ml) were inserted into plates containing different concentrations of chitosan. *i.e.*, 0.0, 0.2, 0.4, 0.6 and 0.8 mg/l and then PDA medium were poured into the plates before solidification. Four plates as replicates were used for each treatment. Inoculated plates were incubated at 22-25°C for 48hr. Spore germination was determined microscopically and the percent of germinated spores was calculated.

2.5.Effect of chitosan coating on anthracnose diseases incidence of mango fruits

Fresh mango fruits cv. Cenara apparently free from physical damage and diseases were used in this experiment. Fruits were surface disinfected with sodium hypocholorite (5%) and washed several times with sterilized water, fruits were gently injured with sterilized needle. Fruits were dipped in 0.05 , 0.1 ,0.2 and 0.4 % (w/v) chitosan solutions. Control fruits were dipped in sterilized water. The coated and control (uncoated) fruits were air dried for 2hr in laminar flow. Inoculation of fruits was carried out by spraying them individually with spore suspension (10^6 spore/ ml) of *C. gloeosporioides*.Coated-inoculated fruits were stored at 10°C for 30 days.

Mango fruits were examined daily for disease assessment. Each treatment was represented by 5 replicates with 10 fruits of each were used. Each experiment was repeated three times.

2.6. Disease assessment

Percentage of diseased fruits was recorded after3, 7,15 and 30 days of storage. Fresh weight of rotted tissue part and its percentage were recorded and calculated after 30 days of storage.

Statistical analysis

Tukey test for multiple comparisons among means was utilized (Neler *et al*.,1985)

3. Results

Results in Table (1) indicate that all chitosan concentrations reduced linear growth of tested fungus. Complete inhibition was obtained by 0.8m g/l and 76.6% linear growth reduction at 0.6mg/l and 54.1% fungal growth reduction at 0.4mg/l as compared with control treatment. Meanwhile, concentration of 0.2m g/l of chitosan was less effective.

Chitosan solutions in 4 concentrations *i.e*, 0.2, 0.4, 0.6 and 0.8 mg/l were tested for their inhibitory effect on *C. gloeosporioides* linear growth.

Chitosan solutions in 4 concentrations *i.e*. 0.2, 0.4, 0.6 and 0.8 mg/l were tested for their inhibitory effect on *C. gloeosporioides* spores germination.

Results in Table (2) indicate that spore germination of pathogenic fungus was inhibited by all tested chitosan concentrations. The inhibitor effect increasing with increased chitosan concentrations. The most effective concentration was 0.8mg/l, where a complete inhibition of *C. gloeosporioides* spore germination, meanwhile, it was 44.5% inhibition at 0.2mg/l of chitosan solution.

Table 1, Effect of different concentrations of chitosan on linear growth (mm) of *C. gloeosporioides.*

Chitosan concentration (mg/l)	*C. gloeosporioides.*	
	linear growth (mm)	Reduction (%)
0.2	56.5 b	37.2
0.4	41.3 c	54.1
0.6	21.0 d	76.6
0.8	0.0 e	100.0
Control (0.0)	90.0 a	----

Table 2, Effect of different concentrations of chitosan on percentage of conidia germination of *C. gloeosporioides.*

Chitosan concentration (mg/l)	*C. gloeosporioides.*	
	Conidia Germination (%)	Reduction (%)
0.2	51.3 b	44.5
0.4	31.4 c	66.0
0.6	17.8 d	80.7
0.8	0.0 e	100.0
Control (0.0)	92.5 a	----

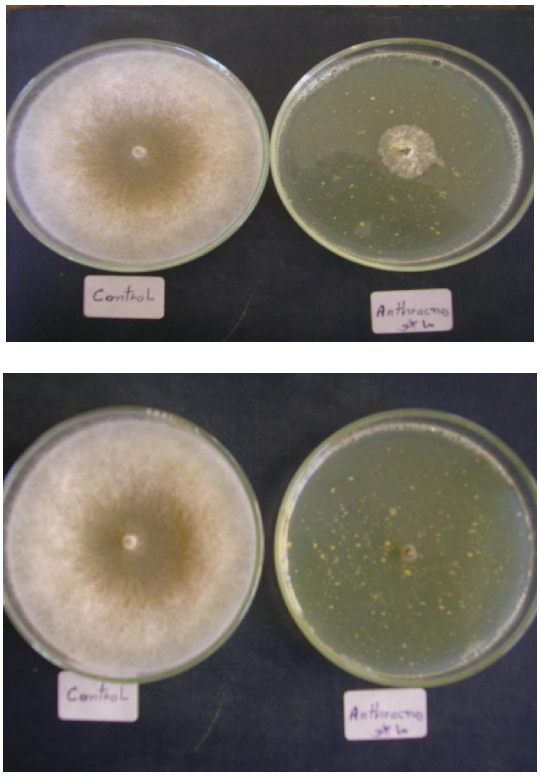

Figure 2, Effect of different concentrations of chitosan on linear growth (mm) and spore germination of *C. gloeosporioides* : 0.6mg/l (up) and 0.8mg/l (down).
Figures with the same letter in the same column are not significantly different (P=0.05). Figures with the same letter in the same column are not significantly different (P=0.05).

Results in Table (3) indicate that all concentrations tested of chitosan significantly reduced the of anthracnose diseases incidence. The percentage of decayed fruits increased by prolonging the storage period, reaching its maximum after 30 days. Chitosan concentrations of 0.05 and 0.1%

resulted in the highest reduction in disease incidence of mango fruits. At2.0% chitosan solution showed a complete protective effect of mango fruits against anthracnose incidence during 30day of storage, and after 30days of storage gave 85% reduction of disease incidence. Meanwhile, at4.0% chitosan solution showed a highly protective effect after 30days by 95.4% reduction of disease incidence, and 92.1% at the end of storage.

Results in Table (4) indicate that all concentrations tested of chitosan significantly reduced the percentage of mango fruits rotted tissue.

On the other hand, Fruits coated with chitosan solution at0.2 % and 0.4 % gave a strongly reduction of the percentage of mango fruit rotted tissue by 89.3 % and 95.0% , respectively, followed by fruit treated with chitosan solution at1.0% by 50.5 % reduction of fruit rotted tissue . At low concentration 0.5 % resulting less effect for reducing the percentage of fruit rotted tissue.

Table 3: Effect of chitosan coating on anthracnose disease incidence of mango fruits during storage period.

Chitosan Concentration (%)	Fruit rotted tissue (%)	
	Fresh weight of rotted tissue (%)	Reduction (%)
0.05	33.3 b	34.1
0.1	25.0 b	50.5
0.2	5.4 c	89.3
0.4	2.5 d	95.0
Control (0.0)	50.6 a	-----

Figures with the same letter are not significantly different (P=0.05).

Table 4, Effect of chitosan on Fruit rotted tissue of diseased mango caused by *C. gloeosporioides* after 30 days of storage.

Chitosan Concentration (w/v) %	Anthracnose disease incidence %			
	Storage period (days)			
	3	7	15	30
0.05	10.0 b	20.4 b	25.3 b	44.5 b
0.1	6.4 c	6.0 c	8.1 c	14.4 c
0.2	0.0 d	0.0 d	0.0 d	13.5c
0.4	0.0 d	0.0 d	4.1 d	7.1 d
Control (0.0)	15.8a	51.2a	60.0a	90.5a

Figures with the same letter are not significantly different (P=0.05).

Figure 3, Effect of chitosan coating on anthracnose disease incidence of mango fruits cv.canara during storage period.

4. Discussion

Among natural elicitor compounds, chitosan offers a great potential as a biodegradable substance that has antimicrobial and eliciting activities (Benhamou , 1996). Previous studies have shown that chitosan reduces decay incidence, mainly caused by *B.cinerea* in tomato fruits. (El-Ghaouth *et al*., 1992).The results of the present experiments showed that, all tested chitosan concentrations effective to reduced the linear growth and inhibited spore germination of *C. gloeosporioides* , but complete inhibition was obtained by used 0.8mg/l chitosan under vitro experiments. Meanwhile, at 0.4 and 0.6 mg/l , shown medium effect for reducing fungal mycelium growth and spore germination if compared with control treatments under the same conditions, These results were confirmed with Pongphen *et al*., 2007 , found that, the effects of chitosan on mycelial growth and spore germination of *Colletotrichum gloeosporioides* were investigated on potato dextrose agar (PDA) containing 0%, 0.5%, 1.0%, 1.5%, and 2.0% (w/v) chitosan dissolved in 0.5% acetic acid, the highest concentrations of chitosan, at 1.5% and 2.0%, were more inhibitory effect of fungal mycelia growth and spore germination than the lower concentrations (0.5% and 1.0%). Hewajulige *et al*.,2006 and 2009, reported that , chitosan solution at 0.1 % and above was inhibited effect to the radial mycelium growth and spore germination for *C. gloeosporioides* the causal agent of anthracnose disease on papaya var.Rathna during storage , at 0.1% chitosan under vitro conditions, a complete inhibition of radial mycelium growth and spore germination of the pathogen. Recently, two models have been proposed to explain the antifungal activity of chitosan, according to Leuba and Stossel ,1986, the activity of chitosan is related to its ability to interfere with the plasma membrane function. In the model of Hadwiger and Loschke,1981, the interaction of chitosan with fungal DNA and mRNA is the basis of its antifungal effect. In this study, mango fruits were coated with chitosan solution at 0.2% and 0.4% resulted in the highest reduction in anthracnose disease incidence of mango fruits and also the above treatments showed reducing the percentage of fruit rotted tissues during all storage periods. Complete protective effect was obtained when mango fruits were coated with 0.2% chitosan during 30 days of storage, while fruits coated with 0.4% chitosan showed a highly protective effect and reduction of disease incidence by 92.1% and 95.0% and reducing the percentage of fruit rotted tissues during all storage periods, these results agreement with, Zhu *et al*., 2008, reported that, disease progress in the mango fruits inoculated with *Colletotrichum gloeosporioides* was significantly inhibited by the treatment with chitosan coating. The disease incidence and lesion diameter in the fruits treated with 2.0% chitosan were 71.3 and 49.8% lower than that in the control fruits after 4 and 16 days of inoculation, respectively. Pongphen *et al*., 2007 reported that,chitosan treatment (0.5%, 1.0%, 1.5%, and 2.0%) of mangoes (*Mangifera indica*) previously inoculated with *C. gloeosporioides* resulted in a lower rate of disease progression compared with the controls and added that chitosan concentrations of 0.5% and 1.0% had stimulatory effects on chitinase and β-1,3-glucanase activities. Nadeem *et al*., 2009, found that, the decay controls of irradiated chitosan on mango fruits was better as compared with uncoated fruits. Chitosan treated fruit inhibited the growth of a wide variety of bacteria and fungi as compared to the control treatments. Various defense responses in several fruit have been induced, including the elicitation of phenylalanine ammonia lyase (PAL) activity in grape berries (Romanazzi *et al*., 2002), and chitinase and β_-1,3- glucanase in oranges, strawberries and raspberries (Fajardo *et al*., 1998; Zhang and Quantick, 1998). Jiang *et al.* (2005) observed an increase in anthocyanin levels in chitosan-coated fruit already after 6 hours of storage. Jiang and Li (2001) who investigated that, 1%, 2% and 3% chitosan coating on the activity of polyphenol oxidase on the third and sixth day of

storage of lychee fruit, Jiang *et al*. (2005) confirmed chitosan's inhibitory effect when analyzing the influence of 2% chitosan coating on the activity of polyphenol oxidase in lychee fruit stored at 25 °C. Pen and Jiang (2003) also noted that chitosan's inhibitory effect on polyphenol oxidase increases at higher concentrations of the chitosan solution which is applied to coat food products. The present study shows that chitosan, as a natural substance, could directly inhibit the growth of *Colletotrichum gloeosporioides in vitro* and potently induce defense reactions in mango fruits. This suggests that chitosan improves resistance of mango fruits against anthracnose disease and suggests that chitosan is promising as a natural fungicide to partially substitute for the utilization of synthetic fungicides in fruit and vegetables.

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The Antischistosomal Activity of *Fasciola gigantica* and *Schistosoma mansoni* Eggs is Influenced by Saponin Extracted from *Atriplex nummularia*

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Abstract: The objective of the present study was to evaluate the antischistosomal, biochemical and humoral immune response of *Fasciola gigantica* and *Schistosoma mansoni* eggs homogenate influenced with or without saponin extracted from *Atriplex nummularia*. The work was extended to study the histopathological picture of the liver before and after challenge. Total worms reduction recorded 57.14, 80.95 and 42.85% in immunized mice with *Fasciola* egg homogenate (50µg/100µl PBS/mouse), *Fasciola* egg homogenate influenced by saponin (50µg/100µl PBS/mouse) and saponin alone (50µg/100µl PBS/mouse), respectively. Immunized groups with *Schistosoma* egg antigen and *Schistosoma* egg antigen influenced by saponin showed reduction in total worms by 47.61, 52.38%, respectively. In conclusion, immunization with *Fasciola gigantica* egg homogenate possesses promising antischistosomal properties with an immunomodulatory response to saponin. Heterologous homogenate had antischistosomal activity more than homologous homogenate. In addition, heterologous homogenate influenced with saponin had more antischistosomal activity than its homologous homogenate. Moreover, *Fasciola gigantica* egg homogenate had an immunoprophylactic effects by increasing the IgM and IgG levels against *Schistosoma* egg antigen. [Journal of American Science. 2010;6(8):368-381]. (ISSN: 1545-1003).

Keywords: *Fasciola gigantica*- *Atriplex nummularia*- saponin- *Schistosoma mansoni*- egg antigen- immunoprophylactic

1. Introduction

Vaccine strategies represent an essential component for the future control of schistosomiasis, as an adjunct to chemotherapy (McManus and Loukas, 2008). Immunization of *Schistosoma mansoni* (*S. mansoni*) infected mice by *Fasciola* egg antigens (SEA) showed cross-reactivity that protecte against infection by reduction fluke burden and egg production (Hassan *et al.*, 2008a). Hassan *et al.* (2008b) demonstrated a structural homology in *Fasciola gigantica* excretory-secretory (E\S) and egg antigens. This homology was resided in the components of the similar molecular weights between both antigens. *Fasciola / Schistosoma* defined a cross-reactive antigen from *F. hepatica* worms that designated as FhSmIII. An antiserum of this antigen was developed and used as a probe to detect its presence (or its determinants) in different extracts of parasitic trematodes. In this manner, it was possible to demonstrate that FhSmIII was found in *S. mansoni, S. bovis* and *Paragonimus westermani* (Hillyer, 1984).

S. mansoni egg antigen (SEA) was found to contain numerous protein, polysaccharide, glycoprotein (Hassanein *et al.*, 1999) and enhances microsomal and mitochondrial enzymes including urea cycle enzymes (Kamel *et al.*, 1998). Vaccination of mice with egg antigen showed delayed ovulation of ova in the liver and stools (Dunn *et al.*, 1988). In addition, Hillyer *et al.*, (1988) noticed that SEA appears to be good candidate in developing a screening assay for the immunodiagnosis of schistosomiasis. Infection of mice with a multiple doses of SEA down regulated egg deposition before *S. mansoni* infection (Botros *et al.*, 1999).

Saponin was constituted as one of the most distributed classes of secondary metabolite from plant or animal domains (Al-Habori and Rahman, 1998). They have broad spectrum of biological activity; cytotoxicy, antitumor, antioxidant and anti-inflammatory (Yanamandra *et al.*, 2003). Triterpenoid and steroid saponin have been found to be immunomodulator and detrimental to several infectious protozoan such as *Plasmodium* and *Leishmanaia spp.* (Plock *et al.*, 2001). The addition of a new immunomodulator to the adjuvant-adaptation (ADAD) system with fatty acid binding proteins increased the protection against *F. hepatica* (Lopez-Aban *et al.*, 2008).

Saponin had a depressive effect against cancer cells by imitated specific cytokine inhibitors (Francis *et al.*, 2001) and detected specific toxic activity against macrophage (Kuroda *et al.*, 2001). Previous investigation on saponin revealed its role as

immunomodulator against *S. mansoni* infection (Maghraby *et al*., 2007).

The objective of this study was to evaluate the influence of saponin on the anti-schistosomal activity of *F. gigantica* and *S. mansoni* egg antigens before and post challenge with 100 cercariae of *S. mansoni* through determination of certain biochemical and humoral immune responses. The histopathological analysis of the liver was also done to support our findings.

2. Materials and Methods

Female Swiss albino mice CD-1 strain (18 – 22g) were obtained from the Animal House, National Research Centre, Cairo, Egypt. They lived in controlled environment of constant temperature and humidity with freely access of water and food. *Atriplex nummularia* (family: *Chenopodaceae*) collected from Marsa Matroh Desert, Cairo, Egypt. Extraction of plant saponin was carried out according to Maghraby *et al.* (2007).

Antigens preparation

Cercariae, worm and egg antigens of *S. mansoni* as well as *F. gigantica* eggs used for enzyme linked immunosorbent assay (ELISA) technique were obtained from Theodore Bilharz Research Institute (Giza, Egypt). Immunization protocol was done according to Maghraby *et al.* (2007).

Ethics

Anesthetic procedures complied with the ethical guidelines approved by the Ethical Committee of the Federal Legislation and National Institutes of Health Guidelines in USA were approved by the Medical Ethical Committee of the National Research Centre in Egypt.

Immunization with *Schistosoma mansoni* eggs antigen

Mice were divided into eleven groups (5 mice/ group). Group 1, served as normal control group subcutaneously injected with 100µl of phosphate buffer saline (PBS). Groups 2-4 received 50 µg protein/ 100µl PBS of *S. mansoni* egg homogenate (SEA), saponin (SAP), SEA mixed with saponin (SEA+SAP), respectively and sacrificed 15 days post the 1st immunization. Groups 5-7 immunized with 50 µg protein/ 100µl PBS of SEA, SAP and SEA+SAP. At 15th day post the 1st immunization each group was booster by 2nd immunization with 50 µg / 100µl PBS of SEA, SAP, SEA+SAP, respectively and sacrificed 15 days later. Group 8 served as positive control infected mice with 100 *S. mansoni* cercariae by tail immersion technique (Oliver and Stirewalt, 1952). Groups 9-11 immunized at 0 time with 50 µg / 100µl PBS of SEA, SAP and SEA+SAP, respectively. At 15 days post the 2nd immunization, mice were challenged with 100 *S. mansoni* cercariae and sacrificed after two months of infection.

Immunization with *Fasciola gigantica* eggs antigen

Vaccination regimens and animal grouping was carried out as described above, whereas *Fasciola* egg homogenate (FEA) and *Fasciola* egg homogenate influenced with saponin (FEA+SAP) were replaced all SEA groups.

Parasitological studies

Worms were recovered by liver perfusion as described by Smithers and Terry (1965). The percent of reduction in worm burden was calculated by the method of Tendler et al. (1968) as follows

$$P = (C - V) / C \times 100$$

Where, P was the percentage of protection; C was the mean number of parasite recovered from infected mice; and V was the mean number of parasite recovered from vaccinated mice. The relative sex ratio (RSR) was calculated by the method of Fallon *et al*. (1994) according to the formula:

RSR= [Male/ female ratio in treated group] / [Male / female ratio in untreated group]

The ratio of untreated groups was standardized as 1.

Enzyme linked Immunosorbent Assay (ELISA)

The assay was performed according to Hillyer *et al*. (1979). This assay was used for determination of IgM and IgG levels in immunized mice sera before and after infection with *S. mansoni* cercariae. Plates were coated with cercarial antigen preparation (CAP), soluble worm antigen preparation (SWAP), soluble egg antigens (SEA) and incubated at room temperature overnight. Orthophenylene diamin dihydrochloride (OPD) was used as a substrate. The reaction was read at 490 nm using Micro Well Plate Reader.

Liver tissue homogenate and biochemical analysis

The liver was removed, blotted, weight and homogenized in 4.5 volume of 0.1% hecadecyle trimethyle ammonium bromide (CTB) for urea cycle enzymes determination. For estimation of succinate and lactate dehydrogenase enzymes and antioxidant

parameters, liver tissue was homogenized in 0.9 N NaCl (1:10 w/v).

Protein was estimated by the method of Bradford (1976). Lipid peroxides was determined as malondialdehyde. Its concentration was calculated using the extinction coefficient value $1.56 < 10^5 M^{-1}$ cm^{-1} and read at 535 nm by the method of Buege and Aust (1978). Glutathione was estimated by the method of Moron *et al.* (1979) using dithiobis-2-nitrobenzoic acid (DTNB) in PBS. Catalase activity was assayed spectrophotometrically according to Nelson and Kiesow (1972). Superoxide dismutase (SOD) was estimated by the method of Nishikimi *et al*. (1972). Urea cycle enzymes; ornthine aminotransferase (OAT), argininosuccinate synthetase (ASS), argininosuccinate layase (ASL) and arginase (Arg.) were employed according to the method of Linton and Campell (1962). Estimation of lactate dehydroginase (LDH) was carried out according to the method of Babson and Babson (1973). Succinate dehydrogenase (SD) was measured colorimetrically at 490 nm (Shelton and Rice, 1957).

Liver histopathology

Sections of liver tissue from each experimental group were fixed in 10% buffered formalin solution for histopathological studies. Paraffine embedded sections (5 µm thick) were taken after fixation. Slides were stained using haemoatoxylin and eosin (H&E) according to the method by Hirsch *et al*. (1997).

Statistical analysis

Statistics is carried out by one way analysis of variance (ANOVA), Costat Computer Program accompanied by least significance difference between groups at $P<0.05$.

Antishistosomal activity and immunological determination will be carried out by independent *t*-test (Ronald *et al*., 1983) and Graph pad instat software.

3. Results

Immunized groups with *Fasciola* egg homogenate (FEA) recorded reduction in total, male and female worms by 57.14, 42.10 and 46.15%, respectively with a relative sex ratio of 1.07. *Fasciola* egg homogenate influenced by saponin (FEA+SAP) showed reduction by 80.95, 73.68 and 76.92% as compared to infected group with relative sex ratio of 1.14. On the other hand, mice immunized by SAP showed diminution in total, male and female worms by 42.85, 31.57 and 15.38 %, respectively. Immunized groups with *Schistosoma* egg antigen (SEA) recorded reduction by 47.61, 26.31 and 38.46% in total, male and female worms, respectively. Attenuation in total, male and female worms post immunization with SEA+SAP was 52.38, 36.84 and 38.46 %. The relative sex ratio recorded 0.80, 1.19 and 1.02 for the last three antigens, respectively (Table 1).

Immunoprophylactic effect of *Fasciola gigantica* egg homogenate and *Schistosoma* egg antigen

Post 2nd immunization, the IgM level against SEA showed significant elevation in immunized group with FEA+SAP (Fig.1). Post 1st and 2nd immunization with SEA+SAP, the level of IgM against CAP showed a significant stimulation. SEA and SEA+SAP showed significant stimulation against SWAP. After challenge with *S.mansoni* cercariae, the level of IgM in immunized mice by SEA against SWAP showed a significant elevation, but insignificant stimulation was recorded with SEA+SAP as compared to the infected group (Fig.1).

Post first and second immunization with FEA and FEA+SAP as well as SEA, the levels of IgG showed significant elevation in immunized mice sera against CAP and SWAP. Post 2nd immunization with SEA+SAP, IgG level against CAP was significantly elevated. IgG level in immunized mice sera of SEA+SAP against SEA showed a significant elevation as compared to the infected group (Fig.2).

Urea cycle enzyme activities

After 1st immunization with saponin, FEA and FEA+SAP, significant increase in urea cycle enzymes; OAT and ASL were recorded. Significant decrease was observed in ASS and arginase as compared to normal group. After 2nd immunization with SAP, FEA and FEA+SAP, significant increase in OAT and significant decrease in ASS were noticed. On the contrary, ASS, ASL and arginase enzyme activities showed significant increase in all immunized infected groups (Table 2). Significant increase in all urea cycle enzyme activities after 1st and 2nd immunization with SEA+SAP was recorded. A significant decrease by 29.46% was observed in ASS after 1st immunization with SAP. Arginase enzyme activity showed significant decrease after 1st immunization with SAP and significant increase after 2nd immunization with SEA+SAP compared to normal mice. Meanwhile OAT exhibited significant increase in SAP vaccinated group and insignificant change in SEA and SEA+SAP groups (Table 3).

Glycolytic enzyme activities

Significant decrease was noticed in glycolytic enzymes (SD and LDH) after 1st and 2nd immunization with SAP, FEA and FEA+SAP **(**Table 3). Total protein recorded significant increase after 1st

immunization with FEA while significant decrease was noticed with saponin. After 2nd immunization with SAP, FEA and FEA+SAP a significant increase in total protein was recorded. The glycolytic enzyme; SD showed significant reduction after 1st immunization with SAP, SEA+SAP. LDH showed a significant reduction after 1st immunization with SAP. In 2nd immunization, significant decrease was noticed.

Antioxidant parameters

S. mansoni infected mice and all immunized groups recorded significant increase in lipid peroxides and superoxide dismutase levels, while significant decrease in glutathione and catalase levels were observed. In post challenged groups, the antioxidant levels recorded significant improvement in mice immunized by saponin. In addition, FEA and FEA+SAP recorded more potent effect than SEA and SEA+SAP (Table 4).

Histopathological studies

Figures (3 and 4) showed histological profile of liver sections, arrow indicated normal hepatocytes (A). Immunized mice liver showed normal hepatic lobular structure and normal distribution of central vein and portal spaces with very mild lymphoid infiltration in few portal spaces (C, D, E, F, I, J, K, L). Liver of infected mice showed distribution of foci with acute inflammation destroying hepatic trabecular structure, portal space with biliary duct hyperplasia and chronic inflammatory infiltrations (B). Post challenged mice revealed remarkably reduced of granuloma size and count with minimal hepatic infiltrations (G, H, M, N).

Table 1: Reduction of total, male and female *S. mansoni* worms and relative sex ratio in immunized groups post challenge infection.

Groups	Total worm (TW)	Male (M)	Female (F)	%R (TW)	%R (M)	%R (F)	RSR
Infected	42.00 ± 4.52	19.00 ± 1.26	13.00 ± 1.82	-	-	-	1
***Schistosoma* eggs**	22.00 ± 1.59	14.00 ± 1.41*	8.00 ± 1.16*	47.61	26.31	38.46	1.19
Saponin	24.00 ± 1.41*	13.00 ± 1.41*	11.00 ± 1.32*	42.85	31.57	15.38	0.80
***Schistosoma* eggs + saponin**	20.00 ± 1.41*	12.00 ± 1.62*	8.00 ± 1.67*	52.38	36.84	38.46	1.02
***Fasciola* eggs**	18.00 ± 1.85*	11.00 ± 1.41*	7.00 ± 1.72*	57.14	42.10	46.15	1.07
***Fascioa* eggs + saponin**	8.00 ± 1.62*	5.00 ± 1.72*	3.00 ± 1.11*	80.95	73.68	76.92	1.14

- Values represented mean ±SD of 5 mice in each group.
- (*) Level of significance as compared to infected group at P<0.01
- % R is the percentage of reduction as compared to infected group.
- RSR is the relative sex ratio of each group, where RSR = (male / female) ^{treated} / (male / female) ^{infected}

Table (2): Change in urea cycle enzyme activities in immunized groups with *F. gigantica* and *S. mansoni* eggs homogenate influenced with and without saponin before and after challenge with *S. mansoni*

Part A

Groups	Immunized groups with *F. gigantica* eggs homogenate				Immunized groups with *S. mansoni* eggs homogenate			
	OAT	**ASS**	**ASL**	**Arg.**	**OAT**	**ASS**	**ASL**	**Arg.**
F / S 1st	6.09 ± 0.37^{c}	1.11 ± 0.08^{d}	27.72 ± 0.48^{a}	43.93 ± 0.74^{ef}	7.23 ± 0.66^{bc}	1.750 ± 0.23^{ab}	29.35 ±0.90^{b}	47.43 ±2.08^{bc}
SAP + (F/S 1st)	6.70 ± 0.24^{b}	1.19 ± 0.01^{bc}	23.97 ± 0.82^{c}	45.22 ± 1.17^{cd}	8.58 ± 0.67^{ab}	2.13 ± 0.26^{a}	31.24 ±1.78^{ab}	47.85 ±2.11^{bc}
F / S 2nd	7.31 ± 0.25^{a}	1.19 ± 0.03^{cd}	23.91 ± 0.46^{c}	44.40 ± 1.42^{de}	8.98 ± 0.69^{a}	1.83 ± 0.36^{ab}	30.15 ±1.83^{ab}	50.33 ±0.59^{b}
SAP + (F/S 2nd)	4.90 ± 0.30^{e}	1.95 ± 0.05^{a}	26.75 ± 0.36^{b}	46.67 ± 0.61^{b}	9.60 ± 0.49^{a}	2.04 ±0.12^{a}	33.15 ±0.85^{a}	55.98 ±1.77^{a}
F / S + Infection	1.94 ± 0.103^{h}	0.79 ± 0.07^{f}	19.45 ± 0.98^{e}	22.43 ± 2.31^{h}	3.98 ± 0.89^{e}	1.06 ±0.29^{bc}	21.36 ±1.48^{e}	34.65 ±3.90^{e}
SAP + F / S + Infection	3.1 ± 0.188^{g}	0.77 ± 0.06^{g}	20.35 ± 1.23^{d}	31.10 ± 1.52^{g}	5.20 ± 1.82^{d}	1.17 ±0.11^{bc}	24.40 ±3.55^{cde}	44.58 ±3.80^{cd}

Part B

Common groups	OAT	ASS	ASL	Arg.
SAP 1St	6.65 ± 0.41^{bc}	0.91 ± 0.08^{e}	26.53 ± 0.39^{b}	42.93 ± 1.05^{f}
SAP 2nd	5.87 ± 0.19^{d}	1.20 ± 0.03^{bcd}	24.30 ± 1.02^{c}	46.75 ± 1.69^{bc}
Infection	3.50 ± 0.14^{g}	0.57 ± 0.10^{h}	2.04 ± 0.25^{g}	4.79 ± 0.71^{i}
SAP + Infection	7.48 ± 0.31^{a}	0.72 ± 0.02^{g}	16.61 ± 1.37^{f}	30.61 ± 1.73^{g}
Control	4.60 ± 0.26^{f}	1.29 ± 0.04^{b}	24.73 ± 0.26^{b}	49.39 ± 1.05^{a}

- Data are mean ± SD of five mice in each group.
- Values are expressed as: nmol/mg protein for lipid peroxide and catalase, µmol/g protein for superoxide dismutase, µg/mg protein for glutathione.
- OAT: ornthine aminotransferase, ASS: argininosuccinate synthetase, ASL: argininosuccinate layase, Arg: arginase, SAP: saponin, *F*: *Fasciola gigantica*, S: *Schistosoma mansoni*, 1st: first immunization, 2nd: second immunization.
- Unshared letters between groups are the significance values at $p < 0.0001$.
- Statistical analysis is carried out by one way analysis of variance (ANOVA), Costat Computer Program.

Table 3: Change in glycolytic enzyme activities and total protein content in immunized groups with *F. gigantica* and *S. mansoni* eggs homogenate influenced with and without saponin before and after challenge with S. *mansoni* infection.

Part A.

Groups	Immunized groups with *F. gigantica* eggs homogenate			Immunized groups with *S. mansoni* eggs homogenate		
	SD	LDH	Total protein	SD	LDH	Total protein
FEA / SEA 1st	0.29 ± 0.01^{e}	96.27 ± 3.92^{d}	44.25 ± 0.95^{e}	1.17 ± 0.11^{ab}	145.25 ± 2.22^{b}	40.18 ± 0.78^{ef}
SAP+ (FEA/ SEA 1st)	0.32 ± 0.02^{d}	113.43 ± 2.33^{b}	38.25 ± 0.95^{g}	0.97 ± 0.05^{ab}	148.150 ± 1.39^{ab}	40.95 ± 0.98^{e}
FEA / SEA 2nd	0.32 ± 0.02^{d}	94.83 ±2.88^{j}	70.50 ± 1.30^{c}	1.25 ± 0.09^{ab}	150.25 ± 6.65^{ab}	47.60 ± 2.28^{d}
SAP + (FEA / SEA 2nd)	0.24 ± 0.01^{e}	102.75 ± 2.22^{c}	80.25 ± 1.25^{b}	1.39 ± 0.06^{a}	155.40 ± 5.49^{a}	59.15 ± 3.00^{b}
FEA / SEA + Infection	1.65 ± 0.01^{a}	79.37 ± 3.00^{h}	29.75 ± 0.50^{j}	1.42 ± 0.07^{a}	91.41 ± 5.40^{d}	35.88 ± 4.09^{f}
SAP + FEA / SEA+ Infection	0.75 ± 0.01^{c}	84.58 ± 4.09^{f}	38.00 ± 0.82^{g}	1.39 ± 0.13^{a}	123.70 ± 9.96^{c}	45.01 ± 4.5^{d}

Part B.

Common groups	SD	LDH	Total protein
SAP 1St	0.27 ± 0.01^{e}	92.62 ±1.36^{e}	36.75 ± 2.36^{h}
SAP 2nd	0.29 ± 0.01^{e}	93.92 ± 3.87^{e}	54.50 ± 1.29^{d}
Infection	0.10 ± 0.02^{f}	60.75 ± 0.47^{i}	83.25 ± 4.28^{a}
SAP + Infection	1.03 ± 0.06^{b}	83.08 ± 1.47^{g}	35.75 ± 0.95^{i}
Control	1.66 ± 0.04^{a}	143.93 ± 3.45^{a}	39.75 ± 1.71^{f}

- Data are mean ± SD of five mice in each group.
- Values are expressed as µl mol/mg protein / min and total protein in mg protein /ml.
- SD: succinate dehydrogenase, LDH: lactate dehydrogenase, SAP: saponin, *F*: *Fasciola gigantica*, S: *Schistosoma mansoni*, 1st: first immunization, 2nd: second immunization.
- Unshared letters between groups are the significance values at $p < 0.0001$
- Statistical analysis is carried out by one way analysis of variance (ANOVA), Costat Computer Program.

Table 4: Antioxidant parameters in immunized groups with *F. gigantica* and *S. mansoni* eggs homogenate influenced with and without saponin before and after challenge with *S. mansoni* infection.

Part A.

Groups	Immunized groups with *F. gigantica* eggs homogenate				Immunized groups with *S. mansoni* eggs homogenate			
	LPO	GSH	Cat	SOD	LPO	GSH	Cat	SOD
FEA / SEA1st	0.56 ± 0.01^{fg}	125.83 ± 3.11^{b}	22.82 ± 0.50^{c}	469.96 ± 2.72^{h}	0.56 ± 0.01^{b}	125.83 ± 3.11^{b}	22.82 ± 0.51^{c}	469.96 ± 2.72^{h}
SAP + (FEA / SEA 1st)	0.78 ± 0.05^{c}	117.67 ± 2.06	3.31 ± 0.03^{c}	10.66 ± 0.26^{e}	0.80 ± 0.02^{e}	113.04 ± 1.58	17.73 ± 0.98^{i}	481.78 ± 1.91^{e}
FEA / SEA 2nd	0.58 ± 0.02^{e}	120.51 ± 1.36^{d}	22.99 ± 0.59^{c}	474.02 ± 1.64^{g}	0.61 ± 0.02^{d}	117.82 ± 1.65^{e}	22.50 ± 0.03^{d}	476.75 ± 2.62^{f}
SAP + (FEA / SEA 2nd)	0.51 ± 0.02^{g}	125.66 ± 2.26^{b}	23.41 ± 0.56^{b}	466.68 ± 1.56^{i}	0.55 ± 0.02^{f}	122.20 ± 1.14^{c}	23.19 ± 0.02^{b}	470.46 ± 1.52^{gh}
FEA / SEA + Infection	0.75 ± 0.02^{c}	113.20 ± 2.32^{f}	20.21 ± 0.79^{f}	571.15 ± 6.08^{c}	0.78 ± 0.01^{c}	94.59 ± 1.43^{j}	20.05 ± 0.08^{g}	579.79 ± 2.33^{c}
SAP + FEA / SEA + Infection	0.56 ± 0.01^{fg}	125.83 ± 3.11^{b}	22.82 ± 0.50^{c}	469.96 ± 2.72^{h}	0.56 ± 0.01^{b}	125.83 ± 3.11^{b}	22.82 ± 0.51^{c}	469.96 ± 2.72^{h}

Part B.

Common groups	LPO	GSH	Cat	SOD
SAP 1St	0.65 ± 0.01^{d}	116.41 ± 3.48^{f}	21.63 ± 0.44^{e}	481.56 ± 4.10^{e}
SAP 2nd	0.54 ± 0.02^{g}	123.73 ± 1.86^{c}	23.47 ± 0.53^{b}	468.88 ± 2.76^{h}
Infection	0.93 ± 0.01^{a}	72.06 ± 1.55^{i}	15.81 ± 0.27^{h}	710.18 ± 6.71^{a}
SAP + Infection	0.85 ± 0.02^{b}	97.87 ± 1.54^{h}	17.98 ± 0.33^{g}	620.32 ± 6.86^{b}
Control	0.49 ± 0.02^{h}	129.18 ± 2.20^{a}	23.90 ± 0.18^{a}	463.25 ± 2.35^{j}

- Data are mean ± SD of five mice in each group.
- Values are expressed as: nmol/mg protein for lipid peroxide and catalase, µmol/g protein for superoxide dismutase, µg/mg protein for glutathione
- SOD: superoxide dismutase, GSH: glutathione, LPO: lipid peroxides, Cat: catalase, SAP: saponin, *F*: *Fasciola gigantica*, S: *Schistosoma mansoni*, 1st: first immunization, 2nd: second immunization.
- Unshared letters between groups are the significance values at $p < 0.0001$
- Statistical analysis is carried by one way analysis of variance (ANOVA), Costat Computer Program.

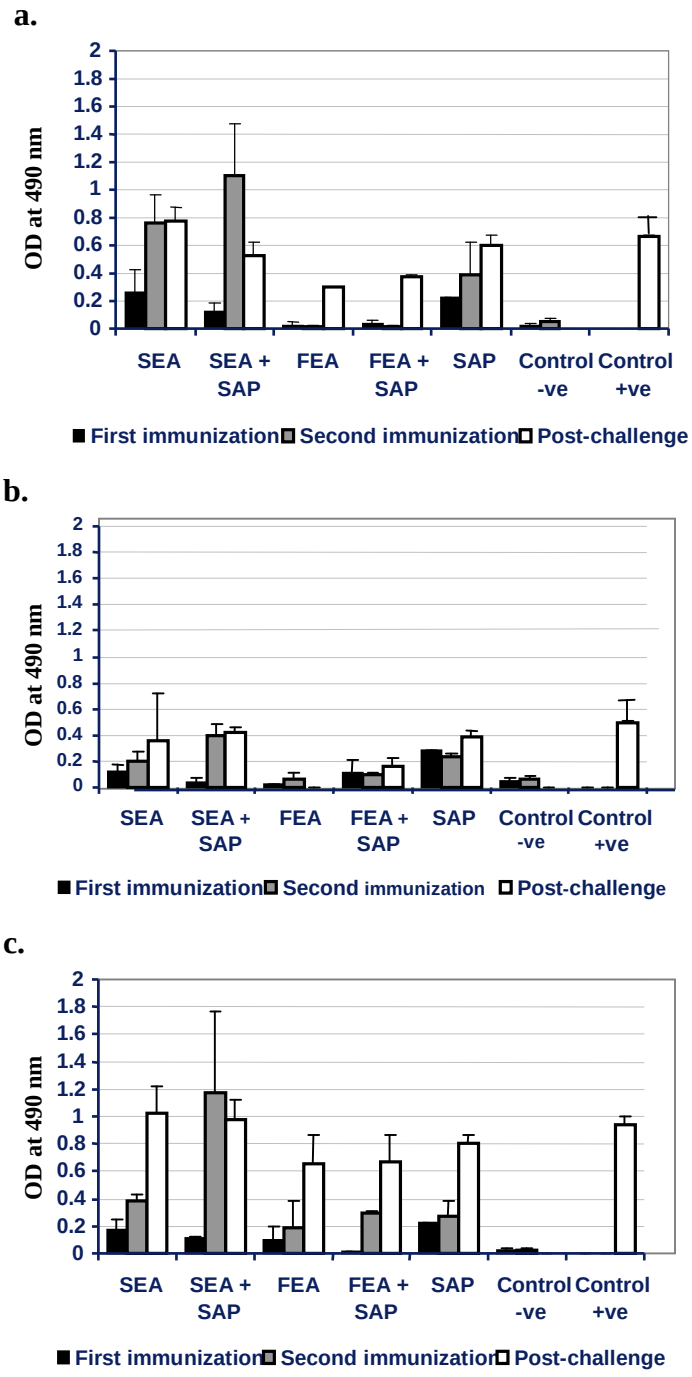

Fig. 1: Detection of IgM by ELISA in mice sera post the first immunization (black bars) and second immunization (grey bars) with *S. mansoni* or *F. gigantica* homogenates influenced with or without saponin and post challenge (white bars) with 100 *S. mansoni* cercariaea. Plates were coated with CAP (a), SWAP (b), or SEA (c).

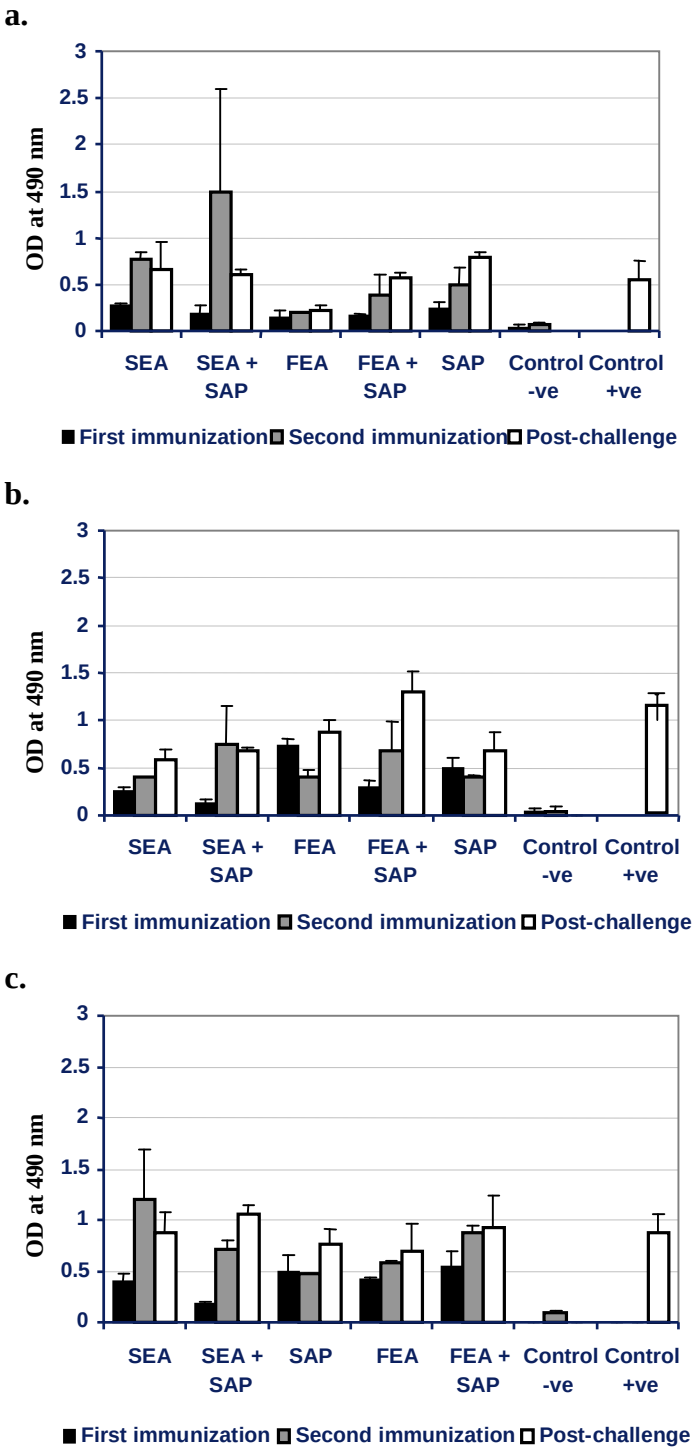

Fig. 2: Detection of IgG by ELISA in mice sera post the first immunization (black bars) and second immunization (grey bars) with *S. mansoni* or *F. gigantica* homogenates influenced with or without saponin and post challenge (white bars) with 100 *S. mansoni* cercariaea. Plates were coated with CAP (a), SWAP (b), or SEA (c).

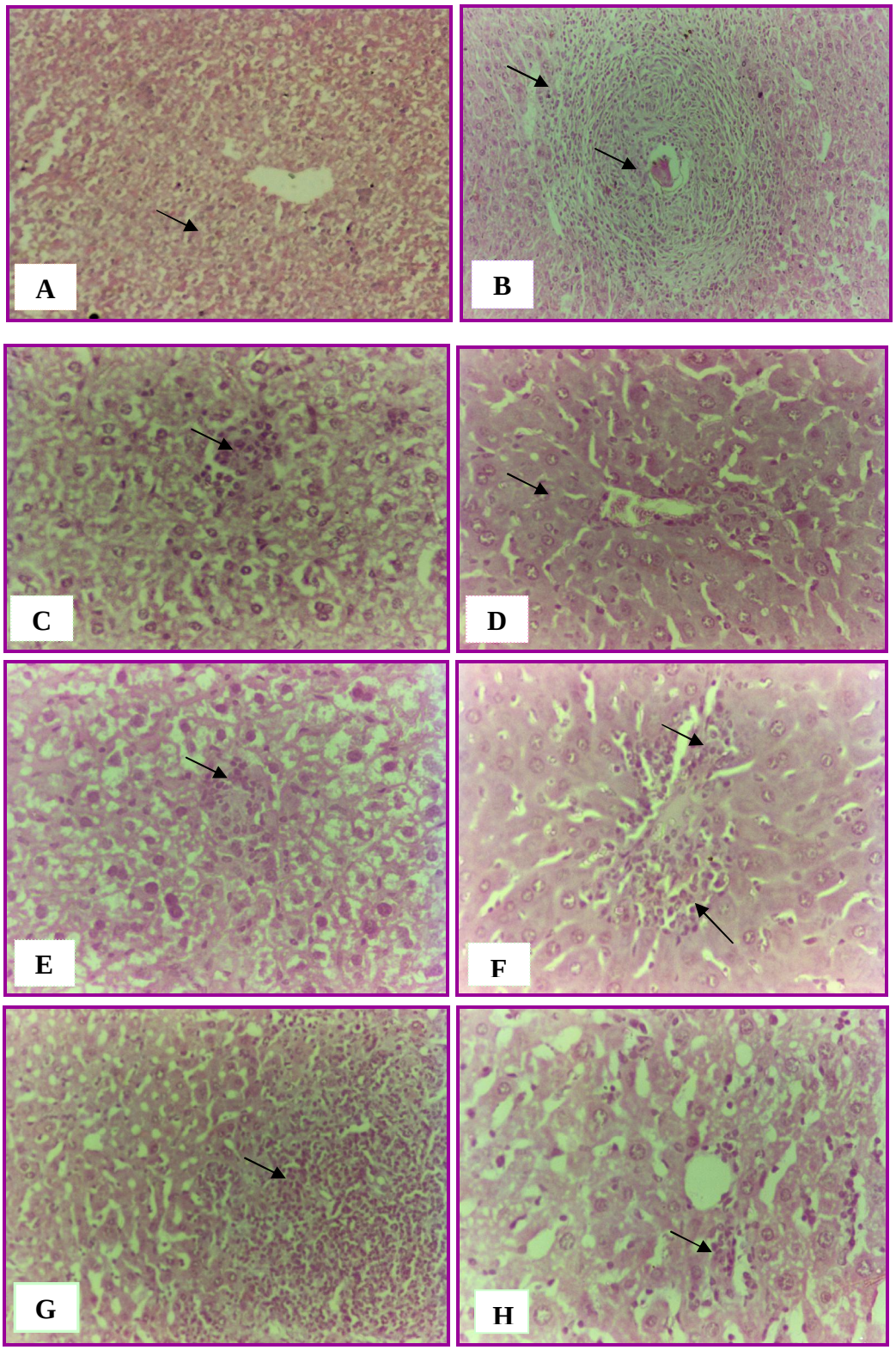

Fig. 3: Representative histological analysis photos for liver sections of different mice groups. (A) Normal structure of liver control mice [100x]. (B) infected group (positive control) [100x]. (C) FEA immunized group after 1st immunization [200x]. (D) FEA immunized group after 2nd immunization [200x]. (E) FEA and SAP immunized group after 1st immunization [200x]. (F) FEA +SAP immunized group after 2nd immunization [200x]. (G) FEA infected group [100x]. (H) infected FEA +SAP group [200x].

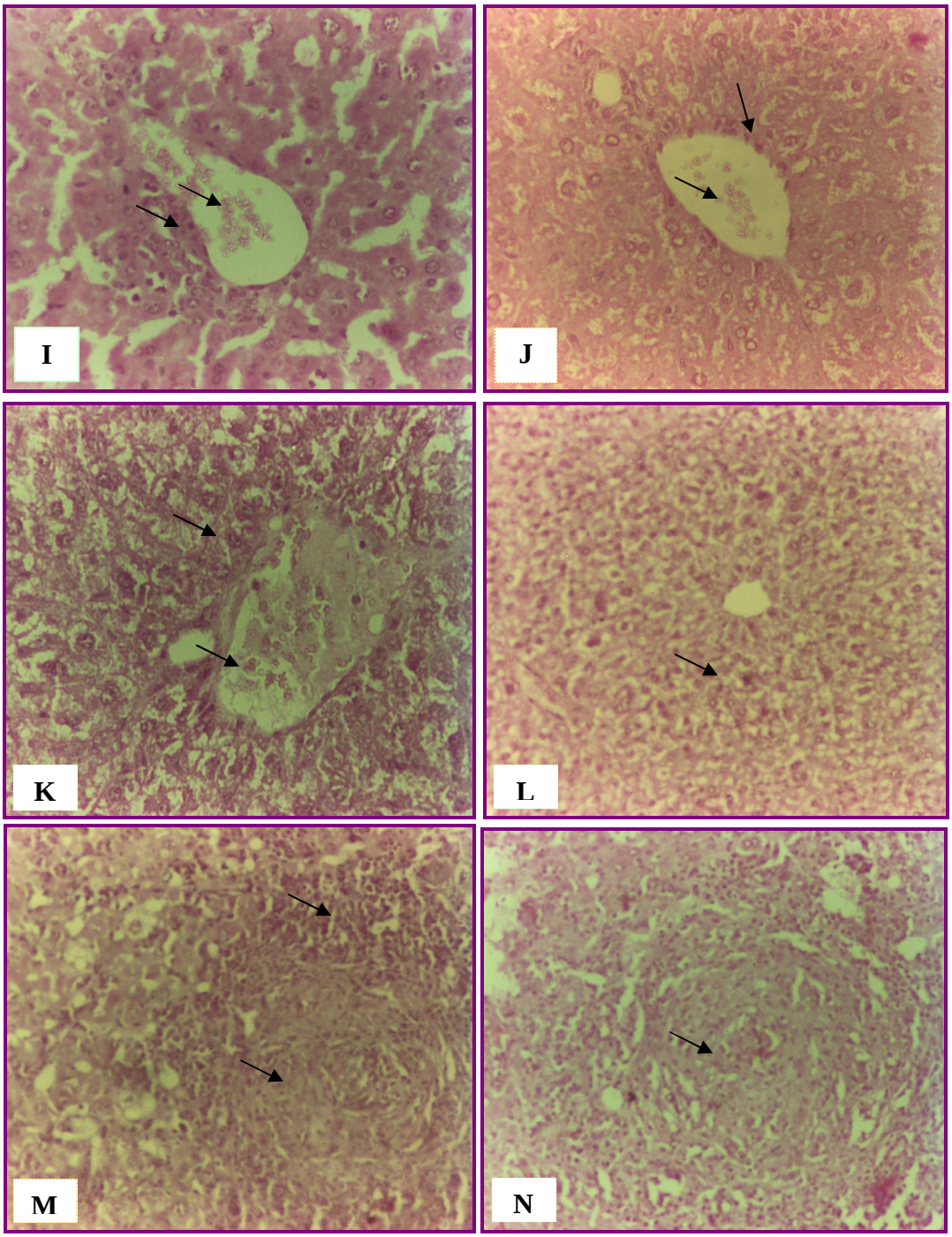

Fig. 4: Representative histological analysis photos for liver sections of different mice groups. (I) SEA group after 1st immunization [200x]. (J) SEA group after 2nd immunization [100x]. (K) SEA +SAP group after 1st immunization [200x]. (L) SEA +SAP group after 2nd immunization [100x]. (M) SEA-infected group [100x]. (N) SEA + saponin infected group [100x].

4. Discussion

Recent studies revealed that FEA enhances antibodies production supporting the hypothesis of immunomodulatory effect caused by vaccines (Schuster et al., 2007). Fasciola derived 12kd antigen developed antibodies that protected against S. mansoni, proofing its cross- reactivity (Cervi et al., 2004). Almeida et al. (2003) stated that S. mansoni (Sm14) induced high levels of protection against both S. mansoni and F. hepatica. Hillyer (2005) demonstrated that some vaccine candidates have anti-fecundity, anti-pathology, and anti-embryonation effects. El- Sayed et al. (2000); Noureldin et al. (2000) and Ruangittichai et al. (2006) reported that all cross-reactivity is ascertained by a significant protection of 57% against challenge. Our results recorded significant reductions ranged from 47.61: 80.95% in total worm of immunized mice with Schistosoma, Fasciola antigens and their combination with saponin. Yabe et al. (2008) and Maghraby et al. (2009) investigated interactions among F. gigantic and Schistosoma spp. In addition, Barduagni et al. (2008) found patients co-infected by Fasciola spp. and S. mansoni, while Abane et al. (2000) found that F. hepatica fatty acid binding protein (FABP) had been used in vaccination against S. bovis. López-Abán et al. (2008) reported that addition of a new immunomodulator to FABPs increase the protection against F. hepatica.

Our results showed that immunized mice with FEA+SAP and FEA caused a significant improvement in urea cycle enzymes, SD, LDH and total protein content. After challenged with S. mansoni infection, a significant reduction in urea cycle enzymes was observed. These results could be correlated to Mousa et al. (1975) and Senft (1976) who reported that bilharzial infection resulted in defect in protein metabolism through a defect in absorption of amino acid or defect in enzymes synthesis and derangement of many metabolic pathways which may involve detoxification mechanism in urea cycle regulation. Immunization with SEA or SEA+SAP showed a significant increase in OAT, ASS, ASL and an insignificant change was noticed in arginase, LDH and total protein content, while significant reduction was observed in SD enzyme activity. This can be explained on the basis that these antigen or metabolic product act on gene expression as a signal, so the transcription of DNA specific sequence into messenger RNA either be repressed or activated. Gene repression or activation is an effective way of changing enzyme activity (Hoek et al., 1997). Saponin showed a definite reactive immunomodulatory action and has a diuretic action causing increasing in OAT (Zhang et al., 1990; Plohmonn et al., 1997; Yamanandra et al., 2003). Al-Habori and Raman (1998); Bogoiavlenskii et al. (1999) and Francis et al. (2001) showed that saponin modulate serum glycoprotein.

Pascal et al. (2000) and Hamed (2006) reported that oxidative stress due to schistosomiasis causes an elevation in lipid peroxides. In addition, Hamed (2006) showed that liver GSH was drastically depleted in S. mansoni infected mice and Gharib et al. (1999) attributed this decrease to the increased cytotoxicity with H_2O_2 produced as a result of inhibition of glutathione reductase that keeps glutathione in the reduced state. S. mansoni infection impairs the antioxidant system since the level of GSH depletion was used as an index of oxidative stress and a sign that hepatic cells are utilizing more antioxidant defenses (Ip, 2000). Therefore, all antigens used improved the level of antioxidants due to the reduction of schistosomal toxins evolved by worms which was confirmed through the observed reduction in worm burden. Catalase activity in our results recorded significant decrease after infection. Hahn et al. (2001) attributed this decrease to the oxidative stress which leads to peroxide radicals that detoxified by catalase and thus results in decline in its activity. Our results were in accordance with Son et al. (2007) who attributed the increase in superoxide dismutase to the enhancement of its mRNA expression as a result of exposure to superoxide and hydroxil radicals.

Histopathological examination revealed that hepatic cells, central vein and portal triad were normal in immunized groups. Fibrous perihepatitis and hepatic parenchyma showed severe fibrosis and severe diffuse inflammatory infiltration, causing the distortion of hepatic lobules (Ali and Hamed 2006; EL-Banhawy et al., 2007). The current study showed that S. mansoni egg granuloma sizes were reduced after immunization with all antigens used. Saponin was maintaining liver architecture that in turn preventing the development of malignancies (Haridas et al., 2001).

In conclusion, Fasciola eggs, as a heterologous antigen, exerted more antischistosomal activity than the homologus Schistosoma one. In addition, combination of saponin enhanced the protective immunity of Fasciola eggs homogenate with more potent effect than Schistosoma eggs antigen.

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Prognostic value of a simple evolving disseminated intravascular coagulation score in patients with severe sepsis

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Abstract: Objective: to predict outcome in patients with severe sepsis using the simple evolving DIC score calculated in the first 48hrs from two readily available global coagulation markers, platelet count and prothrombin time, and comparing its accuracy with (SOFA) score. Patients and Methods: fifty patients with severe sepsis in an adult intensive care unit (ICU) in Critical Care Medicine Department Cairo University were included in the study. The SOFA score and our simple evolving DIC score were calculated in all patients just before enrollment in the study. Results: Patients with higher simple DIC score had the highest SOFA scores and were associated with worst outcome. Mortality rate increased from 0% for simple DIC score < 1 to 90,9% for simple DIC score 2 or 3 and reach 100% for simple DIC score 4. Conclusion: the simple evolving DIC score calculated in the first 48hr appears, besides its general availability and easy calculation at the bedside, to be a reliable and accurate tool in predicting patients' outcome. [Journal of American Science. 2010;6(8):382-388]. (ISSN: 1545-1003).

Keywords: Prognostic value; intravascular; coagulation; sepsis

1. Introduction

Patients with sepsis characteristically manifest an intense systemic inflammatory response that can result in activation of the coagulation system. This activation is initiated by microbial products, such as endotoxin, and amplified by proinflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-1[(1,2)]. Local thrombin generation can intensify the inflammatory response at sites of infection, but its spillover into the systemic circulation can result in disseminated intravascular coagulation (DIC) [(3)]. The presence of DIC has been associated with increasing risk of death from sepsis [(4)]. The International Society of Thrombosisn and Haemostasis (ISTH) has recognized that many patients who do not fulfill the criteria for overt DIC have an evolving coagulopathy manifest by worsening coagulation tests such as the platelet count and prothrombin time [(5,6)].

Over the past years, many scoring systems have been developed to describe the severity of illness of critically ill patients or to predict the outcome of intensive care units. As an example, the Acute Physiology and Chronic Health Evaluation (APACHE) score and the Simplified Acute Physiology Score (SAPS) are based on the first 24hr of ICU admission. Although the score on admission to the ICU provides useful information, it is clear that the pattern of change over time is a better indicator of the ultimate outcome [(7)]. The first Sepsis related Organ Failure Assessment score, later called the Sequential Organ Failure Assessment (SOFA) score, was introduced in 1994, the aim was to quantify the severity of the patients' illness based on the degree of organ dysfunction, and taking into account the time course of a patient's condition during the entire ICU stay. This enables physicians to follow the evolving disease process.

We are aiming to predict outcome in patients with severe sepsis using the simple evolving DIC score calculated in the first 48hrs from two readily available global coagulation markers, platelet count and prothrombin time, and comparing its accuracy with (SOFA) score.

2. Material and Methods

Patients &Methods

Fifty patients who had been admitted to the Critical Care Medicine Department, Cairo University during 2007 with the diagnosis of severe sepsis or septic shock were enrolled in the study. Patients included in this study were diagnosed to have severe sepsis according to the American College of Chest Physicians/Society of Critical Care Medicine (ACCP & SCCM) definitions [(8)].

Exclusion criteria:

Patients presenting with any of the following were excluded from the study:

1. Anticoagulant therapy.
2. Post cardiopulmonary resuscitation.
3. Presence of an advanced condition to withhold life-sustaining treatment e.g. (metastatic cancer).

All patients were subjected to*:* Full history taking including underling diseases, previous therapy and acute findings.

Routine monitoring and recording of heart rate HR, respiratory rate RR, temperature, mean arterial pressure MAP, urine output and Glasgow coma scale (GCS).

Daily recording of the need for: Mechanical ventilation (MV), adrenergic drugs was done.

Scoring system: The SOFA score [(9)] and the simple evolving DIC score [(10)] were recorded on admission and 48hr later. All patients were followed up until death or hospital discharge.

Routine laboratory investigations including: Complete blood count (CBC), Random blood sugar (RBS), kidney function, liver profile and arterial blood gases (ABG) was done.

Cultures: We performed Blood culture for all patients suspected to have sepsis. Tracheal aspirate from all ventilated patients and those suspected to have pneumonia was sent for sputum analysis and culture. A clean mid-stream sample of urine was sent for analysis and culture from all patients.

Statistical analysis:

Statistical Package for social science (SPSS) version 12 was used for analysis of data. For comparative purposes between groups in all continuous data unpaired t-test ANOVA test were performed.

Chi-square test for assessing association in categorical data.

Tools to assess the accuracy of diagnostic test have been calculated:

S ensitivity, specificity, positive and negative predictive values together with Odds ratio

3. Results:

This study included fifty Patients with mean Age of 61.2 ± 12.7 years, (23-87 years), thirty patients were males (60%), twenty patients were female and twenty sevens were diabetic (54%). Comparison between demographic data of septic patients in relation to outcome was shown in Table (1). 66% of our severely septic patients develop adult respiratory distress syndrome (ARDS) and managed by mechanical ventilator (MV). Patients without mechanical ventilation have 13 time chance to survive than those with mechanical ventilation (OR=13 and 95%confidence interval CI= 1.9-95; P<0.001). We also notice a trend towards increased mortality with prolonged periods of MV (P=0.001). Table (2) showed no significant difference regarding type of infecting microorganism in relation to outcome. A statistically significant difference was observed between length of stay, duration of mechanical ventilation and the mean difference in DIC score. While no significant difference was observed in age, Table (3). patients with simple evolving DIC score > 2 (calculated after 48 hours from admission) appear to have a shorter length of ICU stay, low PaO_2/FiO_2 ratio, prolonged period of MV and difficult weaning and higher values of liver enzymes (AST, ALT) in comparison to patients with simple DIC score < 2 (P < 0.05). Comparison between DIC score (calculated after 48 hours from admission) in septic patients in relation to outcome was shown in table 4. The accurate predictive value of the simple DIC score was 92% of died septic patients with DIC score > 2, and all who had score < 1 survive, while 65.7% of those with SOFA score > 5 died (table 5). Table (6) showed the high ability of the simple DIC score to predict SOFA score (calculated after 48 hours from admission), as all patients with DIC score > 2 had SOFA score > 5. While only 40% of those with DIC score < 1 had SOFA score > 5. Sensitivity, specificity, positive and negative predictive values and Odds ratio of SOFA score > 5 (calculated after 48 hours from admission) was 100 %, 55,6 %, 65,6%, 100 % and 2,9 respectively. While that of DIC > 2 (calculated after 48 hours from admission) was 100 %, 92,6 %, 92 %, 100 % and 12,5 respectively.

Table (1): Comparison between demographic data of septic patients in relation to outcome.

	Total N (%)	Survivors N (%)	Non-survivors N (%)	P. Value
Diagnosis				
Pneumonia	18 (36)	10 (55.6)	8 (44.4)	
Peritonitis	11 (22)	4 (36.4)	7 (63.6)	0.6
Skin & Soft tissue	13 (26)	8 (61.5)	5 (38.5)	
Urosepsis	8 (16)	5 (62.5)	3 (37.5)	
Culture :				
+ve organism	18 (36)	8 (44.4)	10 (55.6)	
-ve organism	26 (52)	14 (53.8)	12 (46.2)	0.4
Mixed	5 (10)	4 (80)	1 (20)	
Fungal	1 (2)	1 (100)	0 (0)	
Mechanical ventilator :				
- Ventilated	33 (66)	11 (33.3)	22 (66.7)	
- Non ventilated	17 (44)	16 (94.1)	1 (5.9)	0.001*
Diabetes :				
- Positive	23 (46)	7 (30.4)	16 (69.6)	0.002*
- Negative	27 (54)	20 (74.1)	7 (25.9)	

Table (2): Comparison between microorganisms of septic patients in relation to outcome

Organism	N (%)	Survivors N (%)	Non- Survivors N (%)	P value
+ ve organism				
• MRSA	9 (18)	4 (8)	5 (10)	
• Other staph	5 (10)	2 (4)	3 (6)	0.4
• Strepto	2 (4)	1(2)	1 (2)	
• Other (cocci, ..	2 (4)	1(2)	1(2)	
-ve organisms				
o E-coli	8 (16)	3 (6)	5 (10)	
o Pseudomonas	5(10)	3 (6)	2 (4)	
o Klebsiella	5 (10)	3 (6)	2 (4)	
o haemophilus	2 (4)	1(2)	1(2)	0.61
o Proteus	3(6)	2 (4)	1(2)	
o Actinobacter	3 (6)	2 (4)	1(2)	
Mixed	5 (10)	4(8)	1(2)	0.17
Fungal	1(2)	1(2)	none	---

MRSA: Methicillin resistant staph aureus

Table (3): Comparison between age, duration of MV and length of stay of septic patients in relation to outcome.

	Survivors mean ± SD	Non-survivors mean ± SD	P. Value
Age (years)	59.9 ± 13.0	62.8 ± 12.5	0.42
Duration of MV(Days)	2.4 ± 3.1	6.3 ± 3.7	0.001
Length of stay(Days)	11.1 ± 4.1	8.2 ± 3.5	0.01
Mean Difference in DIC*	- 0.33 ± 1.03	1.73 ± 0.915	0.001

*The change from simple DIC score calculated on admission to that calculated 48hr later.

Table (4): Comparison between DIC score (calculated after 48 hours from admission) in septic patients in relation to outcome

	Survivors N (%)	Non-survivors N (%)	P. Value
Simple DIC Score (calculated after 48 hours from admission)			
0			
1	12 (100)	None	
2	13 (100)	None	
3	1 (9.1)	10 (90.9)	0.001
4	1 (9.1)	10 (90.9)	
	None	3 (100)	

Table (5): DIC and SOFA scores of septic patients in relation to outcome

	Survivors	Non-survivors
DIC < 1	25 (100%)	None (0%)
DIC≥ 2	2 (8%)	23 (92%)
SOFA < 5	15 (100%)	None (0%)
SOFA ≥ 5	12 (34,3%)	23 (65,7%)

Table (6): The correlation between the simple DIC score & SOFA (calculated after 48 hours from admission)

	(N) of patients	
	SOFA < 5	**SOFA ≥ 5**
DIC < 1	15 (60%)	10 (40%)
DIC ≥ 2	None	25 (100%)

4. Discussion:

The aim of this present study was to predict outcome in patients with severe sepsis using the simple evolving DIC score calculated in the first 48hrs from two readily available global coagulation markers, platelet count and prothrombin time, and comparing its accuracy with (SOFA) score.

In our study, pneumonia was the most frequent cause of sepsis in ICU (36%), followed by skin and soft tissue infection (26%), then peritonitis (22%) and finally, urosepsis (16%).

The later findings go with Vincent et al, [9], who reported that in patients with severe sepsis, the lung was the most common site of infection 68% followed by the abdomen 22%. Alberti et al,[11] reported that pneumonia contributed to 62% of infections with intra-abdominal infections contributing to 15% of infections. Angus et al.,[7] reported that the lung was the site of infection for 44% of patients with severe sepsis with abdominal infections involved in only 9%. Some earlier studies reported a higher incidence of abdominal infection. Brun- Buisson et al., [12] noting abdominal infection in 32% of 1.052 patients with microbiologically documented infection however pneumonia still contributed to 40% of infections.

In our study, patients with urosepsis had the lowest mortality rate (37, 5%) compared to patients with peritonitis who had the highest mortality rate (63, 6%). Although this finding is statistically insignificant (p=0.5) it can be explained by the PIRO (Predisposing factor, Infectious organism, host Response, Organ dysfunction). Concept in which characters of Infectious insult such as, the site of infection, can influence severity of sepsis response and the patient's likely response to therapy [13].

This go with the data from PROWESS trial of patients with urinary tract infection as a cause of severe sepsis had mortality rate (21%) while patients with pneumonia had a mortality rate (34%) [14].

In our study, all cultures showed growth of an organism with slightly higher frequency of Gram-negative organisms (52%) than Gram-positive organisms (36%) and polymicrobial infection in 10%

of cases. The type of the organism had no impact on the outcome (p=0.4).

These data are comparable to the data of Vincent et al.,[10] (SOAP) study in which cultures showed almost equal frequency of Gram negative and Gram-positive organisms and 18% of infections were polymicrobial but about one third of cultures showed no growth in the SOAP study, this may be attributed to our inclusion criteria which necessitate a documented infection to diagnosis sepsis.

The most common organisms in our study were Staphylococcus aureus (28%, including 18% methicillin-resistant), Escherichia coli (16%), Pseudomonas (10%) and Klebsiella (10%). Inspite of wide variations in microorganism's virulence factors and their susceptibility to antibiotics, no organism in our study appear to be independently associated with increased mortality rate.

Similar results were obtained by Vincent et al., [10] (SOAP) study who reported that, Staphylococcus aureus (30%, including 14% methicillin-resistant), Pseudomonas (14%), Escherichia coli (13%). But in contrast to our finding, Pseudomonas species were independently associated with increased mortality rate. This could be explained by the emerging of more resistant strains of that organism.

This go with data of Antonis et al., [15] who reported that in severe sepsis, gram-positive, gram-negative and other microorganisms produce identical impairment of coagulation.

In our study, about 66% of our severely septic patients develop ARDS defined as ($PaO_2/FiO_2 \leq 200$, bilateral infiltrates on chest radiograph and pulmonary artery wedge pressure $\leq$18mmHg when measured or no evidence of left atrial hypertension) and managed by MV. 66.7% of them died while 94.1% of patients without MV survive their episode of sepsis.

Patients without mechanical ventilation have 13 time chance to survive than those with mechanical ventilation (OR=13 and 95% confidence interval CI= 1.9-95; P<0.001). We also notice a trend towards increased mortality with prolonged periods of MV (P=0.001). This could be explained by higher incidence of complication with prolonged periods as ventilator-associated pneumonia (VAP), the need for tracheostomy and deep venous thrombosis (DVT) with embolization.

Similar results were obtained by Vincent et al., [10] (SOAP) study as patients with MV has increased mortality rate (OR = 7, 95%CI=4.1-12; p<0.001).

These finding go also with the data of Danner et al., [16] and Hudson et al., [17] who reported that incidence of ARDS is 37% and 41% respectively in severely septic patients.

In our study, diabetes mellitus was among the most important risk factors for mortality in septic patients where mortality rate was 69.6% in diabetics while it was 25% in non diabetics (p=0.002).

In the contrary, Leonidou et al., [18] reported that no statistically significant difference in mortality between diabetics and non diabetic patients with severe sepsis. This may be attributed to the inclusion criteria of his study as;

- All diabetic patients included in his study suffered from type II diabetes mellitus.
- Patients with septic shock or diabetic ketoacidosis were excluded from the study.

Also our results contrasted the work of Vincent et al., [10], who reported that the effect of diabetes mellitus on septic patients was statistically insignificant and was associated with just a trend towards higher mortality (p=0.1). This controversy may be attributed to the tight glycemic control strategy applied in the SOAP study which can lessen the deleterious effects of hyperglycemia. But in our study, we still follow the sliding scale regimen.

Our study shows that non- survivors have a shorter length of ICU stay (8.2±3.5 days) compared with survivor (11.1±4.1days). This may be explained by the rapid progression that occurs in some patients.

These finding go with the data of Bertrand et al., [19] who reported that the ICU length of stay was significantly longer in surviving patients (21.8±23.5) than in non surviving patients (18.5±21.6) p<0.001.

Edbrooke et al., [20], reported an increasing length of ICU stay with increasing severity of septic process, severe sepsis versus sepsis (13.3 versus 12.7 days). However, they found a shorter duration of ICU stay in shock patients (11.6 days).

Our study stated that, the change in simple DIC score from admission to 48hr later (mean difference in DIC) was an accurate predictor of clinical course and may reflect improving or worsening septic process (p=0.001). We chose to award points even when the absolute values of PT and /or platelet count were within normal range but moving in the direction that suggest an underlying coagulopathy. This may highlight the value of change over time rather than single admission score.

This go with the data of Dhainaut et al., [21] who noted that a worsening coagulopathy augers a worse outcome in patients with severe sepsis.

In our study, patient group with simple evolving DIC score $\geq$ 2 (calculated after 48 hours

from admission) appear to have a shorter length of ICU stay which may reflect the severity of the underlying condition. Also they show significant hypoxia (low PaO2/FiO2 ratio) and those who were managed with MV suffer from prolonged period of MV and difficult weaning in comparison to patients with simple DIC score < 2 (P < 0.05).

In our study, higher values of liver enzymes (AST, ALT) were detected in patient group with simple evolving DIC score ≥ 2 (calculated after 48 hours from admission) which demonstrates the strong link between evolving DIC and liver dysfunction which can be summarized in the following points;

- The increase in α-1antitrypsin and α-2-macroglobulin inhibits protein C [(22)].
- C4-binding protein is increased, lowering the levels of free protein S [(22)].
- The synthesis of antithrombin is decreased; In addition, tissue factor expression is increased [(22)].

In our study, almost all patients with simple DIC score ≤ 1 survive their episode of sepsis, while 92% of those who had simple DIC score ≥ 2 develop multiple organ failure and died.

These results are consistent with the work of Kinasewitz et al, [(23)] who reported that a majority (55%) of those with a score ≤1 had a rapid recovery, and overall 86% of these patients survived their episode of sepsis. In contrast, 85% of those with a score of ≥ 2 developed multiple organ failure and about half of those patients died from sepsis.

In our study, we compare the SOFA score with the simple DIC score using cut level 5 for SOFA and 2 for simple DIC score

Simple DIC score ≥ 2 (calculated after 48 hours from admission) shows sensitivity 100% and specificity 92.6% while SOFA score ≥ 5 shows sensitivity 100% and specificity 55.6%. This indicate that simple DIC score is more accurate in predicting mortality in septic patients.

This high sensitivity may be explained by data from the PROWESS trial which indicated that activation of coagulation and inflammatory pathways are virtually universal phenomena in patients with severe sepsis. And this is consistent with experimental primate studies of E.coli sepsis that have demonstrated a strong link between procoagulant activities and inflammation [(24)].

A higher odds ratio 12.5 (OR=12.5, 95% CI=3.3-47.1) was observed in patients with DIC score > 2(calculated after 48 hours from admission). This indicate that patients with simple DIC score > 2 have 12.5 times increased risk to die than those who had DIC < 2, compared to 2.9 times increased risk of death in patients with SOFA score > 5 than those with SOFA score < 5 where odds ratio was just 2.9, (95% CI=1.8-4.6).

In the current study, all patients with simple DIC score ≥ 2 had SOFA score ≥ 5 (calculated after 48 hours from admission), indicating an association between the subtle evolving coagulopathy and the extent of organ dysfunction. The behavior of the coagulation system is a part of the pathophsiology of the septic process not an isolated organ which may fail or not.

This go with the data of Dhainaut et al [(21)] who have suggested that coagulopathy preceded multiple organ failure and that continued coagulopathy during the first day of severe sepsis increases the risk of new organ failure and, ultimately, death.

Shorr et al., [(25)] stated that coagulopathy results in microvascular fibrin deposition responsible for multiple organ failure in severe sepsis.

5. Conclusion:

The simple evolving DIC score calculated in the first 48hrs from two readily available global coagulation markers, platelet count and PT was an accurate predictor of clinical course and outcome in patients with severe sepsis. The power of the simple evolving DIC score may be related to that it scores for the change over time in platelet count and PT, not only for the absolute values of these markers. The simplicity of this score, as it can easily be applied at the bedside as well as the wide availability of its components may help physicians to follow up their patients on daily basis.

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Optimization of Process Parameters for the Production of Tannase and Gallic Acid by *Enterobacter Cloacae* MTCC 9125.

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Abstract: Tannase and gallic acid production by *Enterobacter cloacae* MTCC 9125 was optimized. The organism produced maximum enzyme and gallic acid at initial medium pH 4.5 and cultivation temperature of 37^0C after 48 h of incubation period. 1% of 24 h old inoculumn was found to be optimum for tannase production. However, 48 h old inoculumn showed maximum gallic acid accumulation. Supplement of carbohydrates decreased enzyme synthesis, while fructose, sucrose and glucose increases cell mass growth.1.2% tannic acid was found to be optimum for biosynthesis of tannase and gallic acid. The organism showed maximum tannase production with sodium nitrate and KH_2PO_4 as nitrogen source and phosphate source respectively. Ca^{2+} and Mg^{2+} ions were found to be stimulatory for enzyme production. [Journal of American Science. 2010;6(8):389-397]. (ISSN: 1545-1003).

Keywords: *Enterobacter cloacae*, Tannase, Gallic acid, Submerged Fermentation, Enzyme Production

1. Introduction:

Tannase (tannin acyl hydrolase, EC 3.1.1.20) catalyzes the hydrolysis of ester and depside linkages in hydrolyzable tannins like tannic acid. The products of hydrolysis are glucose and gallic acid (Lekha and Lonsane, 1997; Mohapatra et al. 2007). Gallic acid find applications in photography and printing inks, production of an anti-microbial drug trimethoprim, in manufacturing propyl gallate which is used as an antioxidant in fats and oils. Gallic acid also exhibited cytotoxic activity against cancer cells. Besides this, gallic acid possesses wide range of biological activities, such as antibacterial, antiviral, analgesic etc. (Bajpai and Patil 2008; Kar et al. 1999; Mondal et al. 2001; Pourrat et al. 1987; Trevino-Cueto et al. 2007). Other than gallic acid production, tannase is used extensively in the preparation of instant tea, wine, beer, and coffee-flavored soft drinks and also as an additive for detannification of food. A potential use of tannase is in the treatment of waste water contaminated with polyphenolic compounds such as tannic acids (Aguilar et al. 2007; Belmares et al. 2004; Mukherjee and Banerjee 2006; Seth and Chand 2000).

The world wide annual demand of gallic acid is 8000 tons. At present gallic acid is produced industrially by acid hydrolysis of naturally occurring gallotannins. Due to high cost, low yield of desired product and production of large toxic effluent by acid hydrolysis, an enzyme based eco-friendly technology for gallic acid production is urgently required. Microorganisms are known to degrade tannic acid by producing tannases (Bajpai and Patil 2008; Banerjee et al. 2007).

Microorganisms are the main source for industrial enzymes due to their biochemical diversities, ease of cultivation and amenability to genetic modifications (Trevino et al. 2007). Bacteria, yeast and filamentous fungi are known tannase producers. Most of the organisms capable of degrading tannins isolated till date are either anaerobic or facultative anaerobic bacteria from the alimentary canal of ruminating animals or fungal strains associated with the degradation of wood and forest litter. A major problem in the utilization of fungal strains for industrial applications is that degradation by fungi is relatively slow. It is also difficult to manipulate fungal strains genetically because of their complexity. Although several tannin-degrading anaerobic bacteria have been isolated but the processes based on anaerobic bacteria are slow (Chowdhury et al. 2007). Keeping these facts in view, the present communication deals with the production of extracellular tannase and gallic acid by a newly isolated aerobic strain of *Enterobacter cloacae* MTCC 9125.

2.Materials and Methods:

Microorganism: It was isolated from a compost sample and was identified as *Enterobacter cloacae* on the basis of its morphological, physiological and biochemical characteristics (Table 1). The strain has been deposited at MTCC (Microbial Type Culture Collection), IMTECH (Institute of Microbial Technology), Chandigarh, India and has been given accession number 9125.

Table 1. Characterization of *Enterobacter cloacae* MTCC 9125

Colony morphology	
Configuration	Round
Margin	Entire
Elevation	Raised
Surface	Smooth
Pigment	Cream
Opacity	Translucent
Cell Shape	Shorts rods
Size(um)	0.5-1.5u
Arrangement	Occurring singly
Spore(s)	-
Motility	+
Physiological tests	
Growth Temperature	10^0C-42^0C
Growth pH (5-9)	5-9
Growth on NaCl	8%
Anaerobic Growth	(+)
Biochemical tests	
Indole test	-
Methyl red test	-
Voges proskauer test	(+)
Citrate utilization	+
Casein hydrolysis	-
Esculin hydrolysis	(+)
Gelatin hydrolysis	-
Starch hydrolysis	-
Urea hydrolysis	-
Nitrate reduction	+
Catalase test	+
Oxidase test	-
Arginine dihydrolase	+
Tween 20 hydrolysis	+
Tween 40 hydrolysis	+
Tween 80 hydrolysis	(+)
Acid production from	
Glucose	+
Cellobiose	-
Xylose	+
Arabinose	-
Mannitol	+
Maltose	+

+: Positive; -: Negitive; (+): weak positive

Optimization of fermentation process for enzyme production: Various process parameters influencing enzyme production during submerged fermentation were optimized. The strategy followed was to optimize each parameter (cell mass, tannase activity and gallic acid), independent of the others and subsequently optimal conditions were employed in all experiments.

Effect of incubation period and incubation temperature: Fermentation was carried out at various temperatures such as 25, 30, 37, 40 and 45^0C. Samples were withdrawn at regular intervals (12, 24, 36, 48, 60 and 72 h) and analyzed for tannase activity and gallic acid production.

Effect of initial pH: While optimizing the initial pH of the selective medium, the pH of the aqueous solution was varied from 3 to 7 with 1N NaOH or 1N HCl.

Effect of inoculum age and inoculum volume: To determine the effect of inoculum age, the organism was grown at 37 °C for 12, 24, 48, and 72 h in nutrient broth medium (pH 5.0). Then the broth was centrifuged (5,000x*g*, 5 min) and the pellet washed twice in sterilized distilled water and used as inoculum. To determine the effect of inoculum volume, 50 mL of broth was centrifuged and the pellet was dissolved in 1mL of distilled water. A volume of 1, 2.5, 5 and 10 mL of inoculums was used.

Effect of substrate concentration: Various concentrations of tannic acid (0.2, 0.4, 0.6, 0.8, 1.0 and 1.2%) were used as carbon source in the production medium.

Effect of sugar additives: To study the effect of carbon source on enzyme production, simple and complex carbon source like glucose, galactose, mannose, sucrose, lactose and fructose (2.0% w/v) were incorporated in the production medium.

Effect of nitrogen source: Various nitrogen sources such as ammonium chloride, urea, creatinin, sodium nitrate, ammonium nitrate, ammonium molybedate and ammonium thiocynate were added into the medium to determine their effect on cell mass, tannase activity and gallic acid production.

Effect of Phosphate source: The effect of inorganic phosphate (KH_2PO_4) was studied in tannic acid media for tannase and gallic acid production by *E. cloacae* MTCC 9125.

Effect of divalent cations: Different divalent cations (0.01%) were added to study their effect on the enzyme and gallic acid production. These included chlorides of Ca^{2+}, Cu^{2+}, Mg^{2+} and Zn^{2+}.

Tannase assay: Tannase activity was determined colorimetrically using the method of Mondal et al. (2001). The reaction mixture contained 0.3 mL of tannic acid (0.5% in 0.2M sodium acetate buffer, pH 5.5) and 0.1 mL of enzyme, incubated at 50 ^{0}C for 20 min. The enzymatic reaction was stopped by addition of 3 mL of BSA solution, which precipitates the remaining tannic acid. The tubes were centrifuged (5000xg 10 min) and the resultant precipitate was dissolved in 3 mL SDS-triethanolamine solution. One mL of $FeCl_3$ reagent was added to each tube and was kept for 15 min at room temperature for stabilization of the color. The absorbance was read at 530nm using T80 UV/Vis spectrophotometer. One unit of enzyme activity is defined as the amount of enzyme required to hydrolyze 1 mM of tannic acid in 1 min under assay conditions.

Estimation of Gallic acid: Bacterial biomass was obtained by filtration on Whatman filter. The settled solid were then dried overnight at 60 ^{0}C for 24 h and the dry weight expressed in g/L. Gallic acid in the culture filtrate was estimated by the method of Bajpai and Patil (2008). Filtrate was diluted to 100-fold in 0.2 M acetate buffer at pH 5.0. The absorbance was recorded at two selective wavelengths of 254.6 and 293.8 nm. The concentration of gallic acid was measured using specific extinction coefficient by the following equation:

Concentration of gallic acid (µg/mL) =21.77 (A254.6)-17.17 (A293.8).

3. Results and Discussion:

Effect of incubation period and incubation temperature: Tannase and gallic acid production from *E. cloacae* MTCC 9125 was studied in tannic acid medium. Among the different temperatures such as 25, 30, 37, 40 and 45 °C tried, the maximum enzyme and gallic acid production was observed at 37 °C (Table. 2). With further increase in temperature the tannase activity was found to decrease. The organism started enzyme production after 12 h of incubation peaking at 48 h (0.38 U/mL). Optimum temperature of 35-37 ^{0}C was reported for tannase from *L. plantarum* (Ayed and Hamdi 2002) and *A. niger* (Lokeswari and Raju 2007). Maximum gallic acid (3.47 mg/mL) accumulation was also observed within 48 h of incubation period. Sharma et al. (2007), Kar and Banerjee (2000) and Trevino-cueto et al. (2007) found that 48 h of incubation period was optimum for the production of tannase and gallic acid. Rodrigues et al. (2008) reported that tannase is produced during the primary phase of growth. Tannic acid cannot penetrate the cell membrane due to its high molecular weight, but tannase produced by microorganisms can break tannic acid into gallic acid and glucose. The glucose is a readily available carbon source; therefore, it is assimilated first by the microorganism, which results in rapid growth. However, as the glucose concentration falls, gallic acid is utilized by microorganisms as a substrate for energy production.

Table.2. Effect of incubation period and incubation temperature on the production of tannase from *E. cloacae* MTCC 9125.

	Incubation Temperature				
Incubation Time	25°C	30°C	37°C	40°C	45°C
12hr	0.01 (0.41)	0.09 (0.67)	0.10 (0.58)	0.07 (0.56)	0.01 (0.23)
24hr	0.12 (0.64)	0.18 (0.89)	0.21 (1.17)	0.17 (0.85)	0.08 (0.51)
36hr	0.19 (0.82)	0.24 (1.13)	0.31 (2.32)	0.27 (2.01)	0.10 (0.59)
48hr	0.20 (0.84)	0.30 (1.96)	0.38 (3.47)	0.32 (3.18)	0.11 (0.67)
60hr	0.18 (0.77)	0.21 (0.97)	0.25 (1.97)	0.30 (2.68)	0.07 (0.46)
72hr	0.12 (0.69)	0.16 (0.87)	0.18 (1.31)	0.21 (1.17)	0.01 (0.32)

Values in the parenthesis indicating gallic acid production.

Effect of initial pH: The optimum pH for the gallic acid and tannase production was found to be 4.5 (Figure 1). Upon varying the pH of medium from 3.0 to 7.0, the enzyme activity decreased as pH approached the neutrality, whereas highest growth of the organism occurred at pH 5.5. Our results are in accordance with the finding of other reported organisms including fungi (Kar et al. 1999; Chhokar et al. 2009) and bacteria (Mondal and Pati 2000; Mondal et al. 2001).

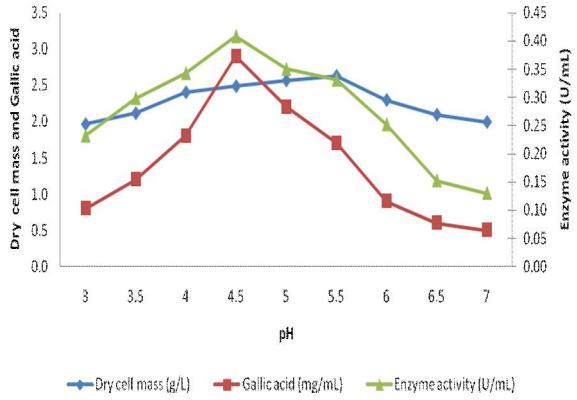

Figure.1 Effect of pH on the production of tannase, gallic acid and growth of E. *cloacae* MTCC 9125.

Naturally any changes in pH may affect the protein structure and a decline in enzyme activity beyond the optimum pH could be due to enzyme inactivation or instability. Tannases have been reported to be acidic proteins, with an optimum pH around 5.5 (Kumar et al. 2007). Effect of pH on the enzyme activity is determined by the nature of the amino acid at the active site, which undergoes protonation and deprotonation and by the conformational changes induced by the ionization of the amino acids (Natarajan and Rajendran 2009).

Effect of inoculum age and size; A 24 h old culture when used as inoculum, gave maximum tannase production in the fermentation medium (Figure 2a.), thereafter the enzyme production declined. However gallic acid and the growth of organism were found to be maximum when 48h old inoculum was used.

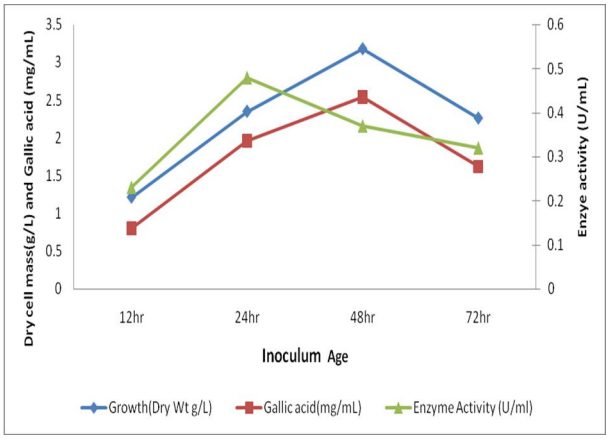

Fig.2a Effect of inoculums age on production of tannase, gallic acid and growth of E. *cloacae* MTCC 9125.

The inoculum level of 1, 2.5, 5 and 10% (v/v) was used in the cultivation medium to establish the effect of inoculum size on the enzyme production by *E. cloacae* MTCC 9125. A 1% (v/v) inoculums (Figure. 2b) was optimal for growth as well as tannase and gallic acid production. Mondal et al. (2001) reported similar results (24 h old culture inoculum) when *Bacillus cereus* KBR9 was used for the production of tannase. Inoculums of 2% (v/v) and 1% have also been reported for the production of tannase by *A. pullulans* DBS66 (Banerjee et al. 2007) and *Lactobacillus sp. ASR- S1* (Sabu et al. 2006) respectively. Lower level of inoculum may not be sufficient for initiating growth and enzyme synthesis on different substrates. An increase in the number of

cells however, ensures a rapid proliferation of biomass and enzyme synthesis. After a certain limit, enzyme production could decrease because of depletion of nutrients due to the enhanced biomass, which would result in a decrease in metabolic activity. A balance between the proliferating bacterial biomass and available substrate material would yield maximum enzyme (Sabu et al. 2006).

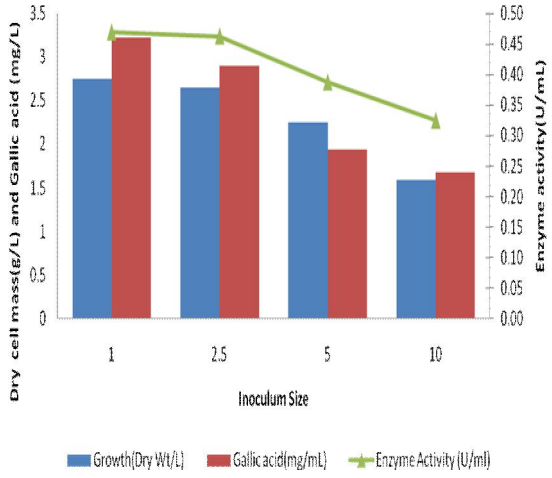

Figure 2b. Effect of inoculums size on production of tannase, gallic acid and growth of E. *cloacae* MTCC 9125.

Effect of substrate concentration: Various concentrations of tannic acid (0.2-1.4%) were used in medium to find out the optimum concentration for tannase and gallic acid production. It was observed that 1.2% tannic acid was suitable for tannase and gallic acid production. (Figure 3). Mohapatra et al. (2009) have reported maximum tannase production by *Bacillus licheniformis* KBR6 in liquid submerged fermentation containing 1% tannic acid. Tannase production from fungal strains were found to be maximum in the medium containing higher concentration of tannic acid (Banerjee et al. 2001; Seth and Chand 2000; Sharma et al. 2007; Paranthaman et al. 2009). However, at higher tannic acid concentration tannase activity was higher in SSF whereas it was repressed in submerged fermentation as tannic acid at higher concentration produces complexes with membrane protein of the organism thereby both growth and enzyme production may be inhibited (Paranthaman et al. 2009). Ayed and Hamdi (2002) reported that increase in substrate concentration induced an increase in tannase activity followed by a decrease because the enzyme synthesis is affected by deposition of gallic acid on the cell surface.

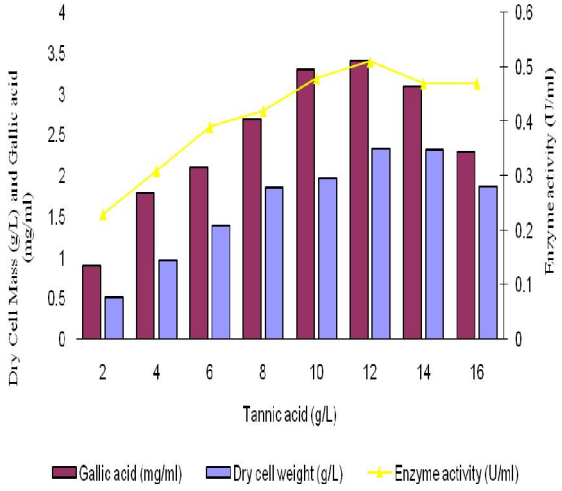

Figure 3. Effect of substrate concentration on the production of tannase, gallic acid and growth of E. *cloacae* MTCC 9125

Effect of sugar additives: Various carbohydrates were added at a concentration of 2 g l^{-1} to the medium containing tannic acid. It was observed that the supplementation of additional carbon sources (glucose, galactose, mannose, sucrose, lactose and fructose) slightly inhibited the enzyme production (Table. 3). Gallic acid synthesis was reduced significantly in the presence of all the additional carbon sources studied.

The presence of glucose decreased the gallic acid production by 54.8% followed by lactose (46.1%), mannose (44.4%), galactose (32.0%), sucrose (25.6%) and fructose (9.8%). However, fructose, glucose and sucrose favored the organism growth. Available reports on the role of carbon sources on the extracellular secretion of tannase are contradictory. Repression of tannase activity by glucose, sucrose, lactose etc. was also reported by Sabu et.al. (2006) and Kumar et al. (2007). Lokeswari and Raju (2007) found that glucose at higher concentration repressed tannase synthesis while the lower concentration is not repressive. This may be due to the fact that high concentrations of additional carbon sources such as glucose changes the carbon/nitrogen ratio as well as creates an osmotic stress, which depresses the enzyme synthesis by microorganisms (Rodrigues et al. 2008). However,

Table.3. Effect of different sugar additives (0.2%, w/v) on growth, gallic acid and tannase production by *E. cloacae* MTCC 9125

Carbo-hydrates	Enzyme Activity (U/mL)	Relative activity (%)	Gallic acid (mg/mL)	Growth (Dry Wt/L)
Control	0.43	100	3.47	1.3
Glucose	0.32	75.0	1.57	1.35
Galactose	0.35	82.0	2.36	1.25
Mannose	0.35	81.0	1.93	1
Sucrose	0.40	92.7	2.58	1.35
Lactose	0.35	80.3	1.87	1.1
Fructose	0.41	94.2	3.13	1.5

Battestin and Macedo (2007) and Bradoo et al. (1997) have reported that external carbon sources did not affect the tannase production. Van de Lagemaat and Pyle (2005) reported that the glucose if present in the media will be exhausted rapidly and this may lead to the partial induction of tannase.

Effect of nitrogen source: Seven different nitrogen sources were used at a concentration of 2 g l^{-1} in a medium containing tannic acid. Among all the nitrogen sources used, sodium nitrate was most effective in tannase biosynthesis and gallic acid production (Table 4) with the relative activity of 171.41% and 266.02% respectively. However, urea favored maximum growth of the organism. Ammonium chloride, urea, creatinin and ammonium nitrate were comparatively less significant for tannase production, presumably because of the release of ammonium ions. Tannic acid by itself could produce only a moderate increase in tannase production whereas by interacting with $NaNO_3$, the tannase activity could be increased significantly (Naidu et al. 2008). Bardoo et. al. (1997) also reported $NaNO_3$ as the preferred nitrogen source for both growth and tannase production by *Asergillus japonicus*. Paranthaman et.al. (2009) observed maximum tannase production by *Aspergillus flavus* in the medium containig $NaNO_3$.

Different concentrations (0.5–5 g l^{-1}) of sodium nitrate were used in the production medium. Optimum concentration of sodium nitrate for the production of tannase and gallic acid was 2 g l^{-1} (Figure 4). Higher concentrations of sodium nitrate in the fermentation medium did not significantly increase enzyme and gallic acid yield. However, maximum growth of the organism was observed at 3 g l^{-1}.

Table 4. Effect of nitrogen sources on the production of tannase and gallic acid from *E. cloacae* MTCC 9125.

Nitrogen Sources	Enzyme Activity (U/mL)	Relative activity (%)	Growth (Dry Wt g/L)	Gallic acid (mg/mL)
Blank	0.22	100	1	1.03
Ammonium Chloride	0.33	148.20	1.41	1.69
Urea	0.32	146.72	2.72	1.57
Creatinin	0.32	145.49	1.19	1.57
Sodium Nitrate	0.38	171.41	1.12	2.74
Ammonium nitrate	0.30	135.79	0.95	0.91
Ammonium Molybedate	0.12	54.90	0.55	0.24
Ammonium Thiocynate	0.21	95.27	1	0.46

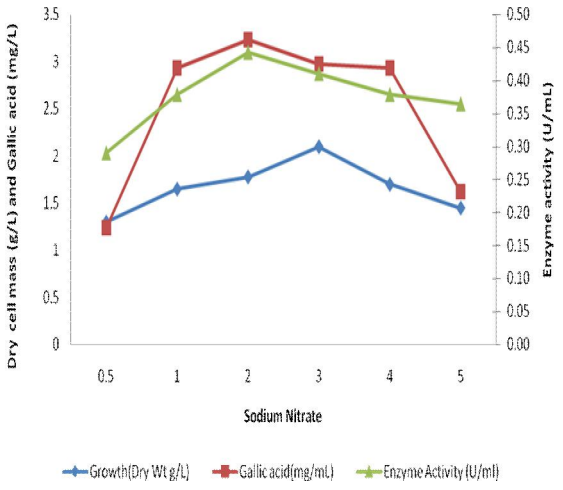

Figure.4 Effect of Sodium Nitrate on production of tannase, gallic acid and growth of E. *cloacae* MTCC 9125.

Effect of metal ions: Cu^{2+}, Zn^{2+}, Ca^{2+} and Mg^{2+} were used in the production medium to determine the effects of metal ions on growth, gallic acid and

tannase production. Cu^{2+} and Zn^{2+} did not affect the enzyme production as well as gallic acid and growth of the organism (data not shown) whereas Ca^{2+} and Mg^{2+} stimulated tannase synthesis. When the concentration of Ca^{2+} and Mg^{2+} (0.05, 0.1, 0.15 and0.2% w/v) was further varied to find out the optimum level it was observed that 0.2% (w/v) Mg^{2+} showed the maximum activity (0.60 U/mL) which accounted for 13.17% and 106.29% increase in enzyme activity and gallic acid production respectively(Table 5). Ca^{2+} at 0.05% (w/v) also supported maximal production of tannase (0.58U/mL). However, gallic acid and growth were maximum at 0.1% (w/v). Whereas at higher concentrations, a small decrease in enzyme activity, growth and gallic acid was observed. This suggests that Mg^{2+} and Ca^{2+} ions are required for the production of tannase by *E. cloacae* MTCC 9125. Natarajan and Rajendran (2009) reported the maximum production of tannase by *Lactobacillus plantarum* MTCC 1407 in the medium containing tannic acid, glucose, NH_4Cl, $MgSO_4$, KH_2PO_4, K_2HPO_4 and $CaCl_2$. Mohapatra et. al. (2009) have also observed the beneficial effect of $MgSO_4$ for tannase production by *Bacillus licheniformis* KBR6.

Effect of Phosphate source:

The results presented in Table 5 indicate that KH_2PO_4 at a concentration of 0.5 g/L enhanced maximum tannase (0.6U/mL) and gallic acid (3.71mg/mL) production with a specific activity of 107.7 and 106.9 respectively. Mohapatra et. al. (2009) observed that phosphate source has strong influence on tannase production by *Bacillus licheniformis* KBR6. Belmares et.al. (2004) reported that the presence of phosphate showed a great importance for optimization, because phosphate promoted the synthesis level and resulted in very expressive decrease in the maximum production time, from 72 to 24 h. The optimized process promoted an increase of 861% in yield and 2783% in productivity. Ayed and Hamdi (2002), Cruz-Hernandez et. al. (2006), Trevino-Cueto et al. (2007), Banerjee et.al. (2007) and Enemuor and Odibo (2009) have also reported beneficial effect of phosphate in the production medium.

Table.5. Effect of Magnesium chloride, calcium chloride and phosphate source on the production of tannase and gallic acid from *E. cloacae* MTCC 9125.

	Calcium Chloride			KH_2PO_4			Magnesium Chloride		
	Enzyme Activity (U/mL)	Gallic acid (mg/mL)	Growth (Dry Wt/L)	Enzyme Activity (U/mL)	Gallic acid (mg/mL)	Growth (Dry Wt/L)	Enzyme Activity (U/mL)	Gallic acid (mg/mL)	Growth (Dry Wt/L)
C	0.55(100.00)	3.02(100.00)	1.78	0.64(100.00)	3.47(100.00)	1.17	0.53(100.00)	1.59(100.00)	1.53
0.5	0.58(111.03)	3.25(107.62)	1.82	0.69(107.71)	3.71(106.92)	1.10	0.57(106.86)	1.68(105.66)	1.78
1	0.54(103.28)	3.30(109.27)	1.86	0.60(94.00)	2.85(82.13)	0.98	0.57(108.46)	1.74(109.43)	1.89
1.5	0.49(93.60)	2.80(92.72)	1.74	0.59(92.81)	2.64(76.02)	1.23	0.59(110.40)	2.87(180.50)	1.97
2	0.34(65.22)	2.20(72.85)	1.63	0.56(87.06)	2.61(75.22)	1.36	0.60(113.17)	3.28(206.29)	1.90

Values in the parenthesis indicating specific activity, C-Control

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Effect of Foliar Spraying with Uniconazole and Micronutrients on Yield and Nutrients Uptake of Wheat Plants Grown under Saline Condition.

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Abstract: A Pot trial experiment conducted in the green house of National Research Centre, Dokki, Giza, Egypt to study the effect of foliar spraying with Mn., Fe. and uniconazole on photosynthetic pigments and yield as well as the chemical contents of the grains of wheat plants grown under saline condition. The results showed that salinity affects most yield parameters, i.e. plant height, harvest index, number of tillers and spikes/plant, spikes weight, weight of grains and straw/plant as well as the biological yield. However, it increases photosynthetic pigments content in the leaves and potassium concentration in the grains. Concerning the foliar spraying application with Mn and Fe or uniconazole, yield parameters and photosynthetic pigments were increased in the leaves, on the other hand, Macro nutrients (NPK) as well as micro nutrients (Mn. And Fe.) increased by foliar spraying. [Journal of American Science 2010;6(8):398-404]. (ISSN: 1545-1003).

Key words: Wheat, Salinity, Foliar spray, Mn, Fe, Uniconazole, photosynthetic pigment, yield, nutrients content.

1. Introduction:

Wheat is considered as the major cereal crop in the world in respect of the cultivated area and total production. It provides an almost 20 % of food calories for people in the world as well as in Egypt. Increasing wheat production is the ultimate goal to reduce the wide gap between production and consumption.

The limited available water resources represent the main limiting factor affects the agricultural development in arid and semi arid regions. Therefore, it is urgent to fined an alternative source of water for irrigation. The application of saline water in irrigation may be one of the most appropriate solutions. Wheat is generally classified to be moderately tolerant to salinity (1).

The effect of micro nutrient elements on yield and crop performance has been reported by many investigators. Rehm and Albert Sims (2) reported that, yields were higher for the treatments with micronutrients. In this respect, Singh (3) reported that, foliar sprays of ferrous sulphate or chelates are found to be more effective and efficient than soil application in correcting Fe-chlorosis in wheat. Nevertheless, the soil and foliar application of Mn significantly increased the yields, but the rates of soil applied Mn (40-50 kg ha-1) are uneconomical than its foliar sprays due to more reversion of soil applied Mn to higher oxide in alkaline soils.

It is well known that, salinity adversely affects growth and yield of crop plants through its effect on several aspects among them ion uptake (4). Thus, foliar application of nutrients via foliage is particularly preferable under such conditions where nutrient uptake from soil is restricted. This is often the case for Fe, Zn and Mn. These nutrients are frequently fixed by soil particles under saline conditions.

One of the recent techniques used to counteract or alleviate the injurious effect of salinity is by using growth retardants (5). One of these retardants is uniconazole. Wang-Xi *et al*., (6) found that uniconazole increased grain yield, yield components and rate of photosynthesis of rice plants. Imam *et al*., (7) reported that number of tillers, leaves, yield of wheat plants increased as affected by uniconazole under water stress conditions. Moreover, Uniconazole, as a potent and active member of the triazole family, was developed for use as plant growth retardant and is increasingly used to manipulate plant growth and yield.(8).

The objective of this study was to investigate the effect of foliar application of Mn and Fe individually or combined and uniconazole on growth, yield, and nutrient uptake of wheat plants grown under saline conditions

2. Materials and Methods:

Wheat grains (*Triticum aestivum*) C.v. Sakha 92 were sown in 25 cm diameter pots filled with clay loam soil**.** The physical and chemical properties of the used soil are presented in Table (1) using the standard method described by klute (9). Pots were divided into two groups one group irrigated with tap water and the second group irrigated with artificially salinized water by adding NaCl and $CaCl_2$ (in the ratio of 1:1 by weight) to obtain 4000 ppm concentration.

Three weeks after sowing , plants were thinned to three plants per pot .Soil in each pot received 3.74

gm ammonium sulphate (20.6%N) at a rate of 60 kg N / fad split into two equal doses applied at tillering and at onset of ear emergence (i.e. after 7 and 11 weeks from sowing). At tillering stage (after 7 weeks from sowing) each treatment of water.

Table (1): Some physico-chemical properties of soil

Properties	Items
Sand %	14.8
Silt %	37.0
Clay %	48.2
Texture	Clay loam
pH (1:2.5 soil : water)	8.16
E.C.(1:2.5 soil : water) dS/m	0.23
CaCO3 %	1.4
Organic matter %	2.05
Available Macronutrients (mg/100g soil)	
P	1.8
K	48.5
Mg	276
Ca	390
Na	22.2
Available Micronutrients (mg/ Kg soil)	
Fe	4.30
Mn	3.10
Zn	1.90
Cu	0.90

At tillering stage (after 7 weeks from sowing) each treatment of water irrigation were sprayed with water or one of the following aquas solutions containing 0.1% ferrous sulphate , 0.1% manganese sulphate .mixture of ferrous sulphate and manganese sulphate with the aforementioned concentration and uniconazole with concentration of 20ppm. Each pot represent one replicate.

Two weeks after foliar fertilization (13) weeks from sowing), representative sample from two replicates was taken from every treatment for photosynthetic pigments content determination in the flag leaf on the basis of leaf area (mg/ dm^2) and calculated by means of (10).

At harvest time , wheat plants were collected and the following characters were determined, number of tillers, number of spikes / plant , spike length weight of grains / spike, the yield of both grains and straw/ plant and weight of 100 grains.

Total nitrogen content in grains was determined using the method described by (11). Phosphorus was determined calorimetrically and potassium was determined using Flame Photometer according to the methods described by (12). Fe, Mn were determined by using Perkin Elemer Atomic Absorption Spectrophotometer.

The analysis of variance of split complete randomized design was used according to the method described by (13).

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3. Results and Discussion:

Effect on chlorophyll content

Data presented in Table (2) show that photosynthetic pigments (chlorophyll a,b, a+b and carotenoids content on basis of leaf area (mg/ dm2) increased by salinity as compared with unsalinized plants . These results confirmed by (14 &15). Such increase may be attributed to the inhibition of chlorophylase enzyme activity which is known to be responsible for synthesis and destruction of chlorophyll in plant tissue (16). It can be observed also from Table 2 that foliar spraying with different micro element singly or combined increased formation of chl. a, b and total chlorophyll as well as carotenoids comparing with control treatments under both saline or non saline conditions. .The positive effect of Fe on chlorophyll synthesis has been recognized. Fe is an essential nutrient element for plant growth and development and is involved in chlorophyll synthesis, thylakoid synthesis, and chloroplast development. Therefore, when the plant suffers from Fe deficiency, the newly forming leaves develop chlorosis symptoms (17 & 18).

The data in the same table also show that the highest chlorophyll a and b and total chlorophyll was obtained by foliar spraying with $MnSO_4$. Such promoting effect of Mn may be attributed to its effect as an activator of many different enzymatic reactions and takes part in photosynthesis (19). They also added that deficiency of Mn induces inhibition of growth, chlorosis and necrosis, and the structure of chloroplasts is markedly impaired. Our results were in harmony with those obtained by (20& 19).

Uniconazole treatment tended also to increase photosynthetic pigments content. This was achieved under saline or non saline conditions as compared with control treatment Table (2). These results were in harmony with those obtained by (21& 22) who recorded an increase in such pigments due to uniconazole treatment. Such increase of chlorophyll content may be attributed to earlier cytokinins formation which stimulate chlorophyll synthesis and production (23). In this respect, Izumi *et al* (24) reported that the application of uniconazole increased cytokinin levels in treated plants.

Regarding the interaction effect between salinity treatment and foliar spraying with Fe, Mn. and uniconazole on photosynthetic pigment content the data indicate that the interaction between saline irrigation water and foliar spraying with uniconazole slightly increased pigment content as compared with other interactions.

Table (2): Effect of foiar spraying with Fe , Mn. and uniconazole on Chl.a, b, a+b and carotenoids content (mg/Dm2) in the flag leaves of wheat plants grown under saline conditions

saline treatment	Foliar spray treatment	Chl. a	Chl.b	Chl. a+b	Carotenoids
saline water	control	3.34	1.43	4.77	1.93
	Mn	3.55	1.82	5.37	2.20
	Fe	3.48	1.75	5.23	2.20
	Mn. + Fe	3.35	1.63	4.98	1.98
	Uniconazole	3.55	1.55	5.10	2.00
tap water	control	2.77	1.18	3.95	1.84
	Mn	3.22	1.42	4.64	1.98
	Fe	3.18	1.38	4.56	1.92
	Mn. + Fe	3..00	1.28	4.28	1.88
	Uniconazole	3.14	1.33	4.47	1.94

Effect on yield and yield components

Data presented in Tables (3&4) indicate that irrigating wheat plants with saline water significantly decreased plant height and harvest index and highly significant decreased number of tillers/ plant, number of spikes per plant and spikes weight/ plant and drastically and very highly significant depressed weight of grains and weight of straw/plant as well as the biological yield as compared with non saline water. These findings coincide with those obtained by (25, 26 and 27). Such depressive effect of salinity in wheat growth may be attributed to the adverse effect on enzymatic processes through some interactions of salts and some organic substances of the cell (28). Moreover, crop reduction due to salinity is generally related to the osmotic potential increase of the root – zone soil solution which leads to certain phonological changes and substantial reduction in productivity (29). The observed reduction in grain yield under salt stress has been attributed to reduction in dry matter production through a feedback effect of photosynthesis during reproductive phase of growth (30).

Regarding, the foliar spraying with Fe or Mn as well as their mixture, increased most parameters of yield components but did not reached the levels of significance for plant height, tillers, spikes No. and spikes weight as compared with the control. However, it is significantly increased grains weight, straw weight, biological yield as well as harvest index. The more pronounced effect on increasing grain yield was recorded by wheat plants foliar sprayed with either Fe or Mn. and their mixture. The superiority of Fe on grain yield may be attributed to the indirect role of Fe in chlorophyll synthesis. In addition Iron enters in many plant enzymes that play a dominant roles in oxidoredox reactions of photosynthesis and respiration (18). The superiority of Mn. treatment resulted from the fact that Manganese (Mn), is regarded as an activator of many different enzymatic reactions and takes part in photosynthesis. Manganese activates decarboxylase and dehydrogenase and is a constituent of complex PSII-protein, SOD and phosphatase. Deficiency of Mn induces inhibition of growth chlorosis and necrosis, early leaf fall and low reutilization (31) concerning the effect of uniconazole on yield and yield components , the uniconazole followed the same pattern of Fe and Mn. but to some extent irrespective type of water irrigation. Such improving effect of uniconazole in improving grain yield may be attributed to its effect on photosynthesis, hormones and antioxidant system (8).

Regarding the interaction effect of water irrigation type and foliar spraying with Fe, Mn. and uniconazole, It is obviously clear that foliar spraying with the fore-mentioned microelements and uniconazole increased most of yield parameters under both types of water irrigation as compared with controls, However, plant height , tillers No., spikes No. and spikes weight records did not reach the level of significance. The highest grain yield was obtained by wheat plants irrigated with tap water and foliar sprayed with Mn. then followed by wheat plants irrigated with saline water and foliar sprayed with Fe.

Table (3): Effect of saline treatment and foliar spraying with Fe or Mn and uniconazole on yield and yield componants of wheat plants.

Treatments	Plant height	Number of tillers /plant	Number of spikes /plants	Weight of spikes /plant	weight of grains /plant	Straw yield/fed.(Ton)	Biological yield/fed.(Ton)	Seed index
Saline treatment								
saline water	74.88	3.38	3.36	13.01	8.61	13.09	21.71	40.07
Fesh water	80.84	4.16	4.04	15.50	9.45	15.92	25.38	37.44
F- test	*	**	**	**	***	***	***	*
Foliar spray treatment								
control	78.75	3.65	3.60	14.54	8.62	16.53	25.15	34.78
Mn.	81.75	3.85	3.85	14.76	9.58	14.31	2.88	40.96
Fe.	80.90	4.15	3.85	16.44	10.45	16.41	26.86	39.18
Mn. + Fe.	77.50	3.75	3.60	14.06	9.45	13.15	22.60	42.01
Uniconazole	70.40	3.45	3.60	11.47	7.07	12.15	19.21	36.84
L.S.D.	6.33	ns	ns	2.20	1.03	2.16	2.74	3.24

Table (4): Effect of interaction between saline treatment and foliar spraying with Fe , Mn. and uniconazole on yield and yield components of wheat plants

saline treatment	Plant height	Number of tillers /plant	Number of spikes /plants	Weight of spikes /plant	weight of grains /plant	Straw yield/fed.(Ton)	Biological yield/fed.(Ton)	Seed index
saline water	74.5	3.5	3.4	12	7.67	13.63	21.3	36.27
	77.6	3.2	3.2	12.29	7.47	9.82	17.55	44.16
	80.30	3.90	3.70	15.47	10.72	18.12	28.84	37.38
	74.30	3.50	3.20	13.93	9.98	12.12	22.10	45.35
	67.70	2.80	3.30	11.34	6.96	11.78	18.74	37.18
LSD								
non saline water	83.00	3.80	3.80	17.07	9.57	19.44	29.01	33.30
	85.90	4.50	4.50	17.24	11.42	18.79	30.21	37.75
	81.50	4.40	4.00	17.41	10.18	14.69	24.87	40.98
	80.70	4.00	4.00	14.19	8.92	14.18	23.10	38.67
	73.10	4.10	3.90	11.59	7.17	12.51	19.69	36.49
L.S.D. 5%	**ns**	**ns**	**ns**	**ns**	**1.45**	**3.05**	**3.88**	**4.59**

Effect on protein percentage and nutrients concentration in wheat grains:

Data presented in Table (5) shows an obvious decrease of N, P and K percentage as well as protein percentage in grains of wheat plants as a result of irrigation with saline water as compared with tape water irrigation. The results are in good agreement with those obtained by Murat *et al*. (32) who reported that application of NaCl caused a decreases in chlorophyll, N, K, Fe and Cu concentrations.

Such effect may be attributed to the effect of salinity on nutrient absorption. Under saline condition, nutrient absorption is restricted by lack of nutrients by small water potential in the rooting medium (33& and 34). May and Muna (34) also added that salt stress induced a significant decrease in protein content along with activity of nitrase in the developed seedlings. The observed decrease in K percentage might be related to the competition between the uptake of cations Na and K. Such

competition might be due to the existence of general carrier for their absorption by the roots (35).

The same table (5) shows decreases of Fe and Mn concentration in wheat grains under saline condition. These decreases may be due to the depressive effect of salinity on root growth and distribution in soil.

Regarding the uniconazole application data in table (5), increased the macro (N,P,K) as well as micro nutrients (Fe and Mn). In this concern, May and Muna (34) reported that such effect may be attributed to the effect of uniconazole in counter acting the adverse effect of salinity and significantly increased protein levels and stimulated nitrase reductase activity particularly at lower NaCl concentrations.

Regarding the foliar application effect of Fe, and Mn on macro and micronutrient content the same table show that N,P and K percentage as well as Fe and Mn greatly increased under both types of water irrigation as compared with control. These results were confirmed by the findings obtained by (35) .In this connection Erdal *et al*. (36) suggested that foliar application is an effective way to increase Fe concentrations in strawberry cultivars.

From the obtained results, it can be concluded that, the use of foliar spraying can alleviated the harmful effect of salinity on growth of wheat plants by foliar spraying with either Mn, Fe or uniconazole .

Table (5): Effect of foiar spraying with Fe , Mn. and uniconazole on N,P,K , Fe and Mn content of wheat grains under saline condition.

saline treatment	Foliar spray treatment	Total N %	P %	K%	Protein %	Fe ppm	Mn.ppm
saline water	control	1.89	0.21	0.043	10.868	94	6.0
	Mn	2.18	0.30	0.011	12.535	102	8.5
	Fe	2.29	0.38	0.012	13.168	144	7.4
	Mn. + Fe	2.38	0.49	0.015	13.685	146	10.1
	Uniconazole	2.38	0.45	0.017	13.685	197	10.0
tap water	control	2.08	0.25	0.015	11.960	104	7.0
	Mn	2.28	0.36	0.017	13.110	103	10.1
	Fe	2.38	0.39	0.019	13.685	197	9.2
	Mn. + Fe	2.59	0.45	0.020	14.893	201	11.0
	Uniconazole	2.49	0.52	0.021	14.318	221	12.0

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Quasispecies Of Genotype 4 Of Hepatitis C Virus Genome In Egyptian Patients Treated With A Combination Therapy Of Interferon (Ifn) And Ribavirin

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Abstract: The resistance to IFN/ribavirin combination therapy in chronic hepatitis C genotype 4, the prevalent genotype in the Middle East and Africa are poorly documented. Several factors implicated in non response, viral quasispecies are one of these factors. Thus our subject aimed to investigate whether the quasispeices population could be used as a predictor of response to antiviral therapy in Egyptian patients. To analyse the implication of quasispecies of genotype 4 in this phenomenon, we studied in 25 naïve Egyptian patients at zero, three, and six months following IFN/ribavirin combination therapy. Hypervariable region 1 within the E2/NS1 gene of the virus was analyzed by the single-strand conformation polymorphism (SSCP) technique after amplification. Pretreatment DNA bands by SSCP (2-7 bands) were detected in all patients. In those who achieved a complete virological response within six months (viral load <0.2 Meq/mL; n=7), bands ranged from 2-6 (mean = 3.71±1.25). In six of these seven patients, the number of SSCP bands remained either the same or decreased sequentially. In those patients who did not respond (viral load >0.2 Meq/mL; n=18), the bands also ranged from 2-7; mean 3.77±1.73. In six of these non-responding patients, the SSCP bands remained the same or decreased sequentially. There was no significant difference between pretreatment quasispecies composition and response (P=.53). Two of the four patients with pretreatment high viral load and the same or decreased composition of quasispecies bands responded to the therapy. In conclusion: quasispecies in our studied patients cannot be used to predict non response to treatment, but may be explained the failure of most HCV-4 patients response to IFN/ ribavirin therapy. [Journal of American Science 2010;6(8):405-412]. (ISSN: 1545-1003).

Keywords: Genotype; Hepatitis C virus; Interferon; Ribavirin

1. Introduction

Hepatitis C virus (HCV) belongs to the genus *Hepacivirus* of the family Flaviviridae. HCV constitutes a major health problem globally and it is estimated that 170 to 200 million individuals are chronically infected with HCV worldwide, with more than one million new cases every year. [1] HCV has a genome of nearly 9.6 kb that encodes a single large open-reading frame that yields a 3000-amino acid polyprotein. The large polypeptide is further post-translationally processed into several structural and non-structural proteins. [2],[3] The HCV genome contains two conserved regions at its 5' and 3' ends that are essential for translation of the polypeptide and for viral replication. [4] HCV replicates at a very high rate resulting in a daily production of nearly 10 [12] virions, and the turnover of HCV virions is very short, reaching a maximum time of three hours. [5],[6]

During replication, mutations in the virus genome are generated due to the limited fidelity of the viral RNA-dependent RNA polymerase. As a result, HCV circulates in an infected individual as a heterogeneous population of different, but closely related genomes, known as quasispecies. [7], [8]

The hypervariable region 1 (HVR1) of 27 amino acids, at the N terminus of the E2/NS1 region is considered the most variable in the HCV genome with a high frequency of mutations that exhibit significant amino acid variations between different genotypes and within the same genotype, during infection. [9] HVR1 is expressed on the surface of the virus and is thought to be the major epitope for neutralizing antibodies and for specific T cells. [10],[11] It has been suggested that genetic variations in the quasispecies in HVR1 are the direct result of the selective pressure of the immune response during infection. [12], [13] Many biological and clinical properties of the virus such as selection of the host immunity escape mutants and persistence of viral infection are attributed to the presence of quasispecies. [14], [15], [16], [17] Furthermore, HCV quasispecies may influence the progression of liver diseases and the overall clinical and histological features after interferon alfa treatment. [18], [19], [20]

The current FDA-approved treatment of HCV infection is a combination of ribavirin and polyethylene glycol interferon (PEG-IFN), which includes two forms namely PEG-interferon alfa-2a (PEG-INTRON) and PEG-interferon alfa-2b (PEGASYS). [21],[22] The attachment of PEG to interferon alfa extends its half-life and this reduces the number of injections of the drug. It was suggested that the quasispecies complexity of HCV may be a possible independent predictor of response to interferon alfa therapy. This conclusion was based on several studies that showed that the existence of a highly heterogeneous population of HVR1 sequences before treatment correlates with a lower rate of response to interferon alfa, independently of viral load and HCV genotype. [23], [24] We investigated the effects of interferon and ribavirin combination therapy on the evolution of HCV quasispecies in 25 of patients at the Egyptian Hospital, with genotype 4 (HCV-4), and determined whether the quasispecies population could be a predictor of sustained virological response among Egyptian patients.

2. Material and Methods

Twenty five Egyptian patients characteristics, clinical profiles, detection, quantification (viral load), and genotyping of serum HCV RNA were used for this study. Each of them received ribavirin (Virazol, ICN Pharmaceuticals Inc., Costa Mesa, CA, USA) at 1000 mg daily, in two divided doses and interferon alfa-2a (Intron-A, Schering-Plough International, Kenilworth, NJ, USA) at three million units, three times a week, for six months. All 25 Egyptian patients had HCV-4 infection as confirmed by an InnoLiPA HCV II Genotyping Kit (Innogenetics, Belgium), which was used according to the manufacturer's instructions. HCV RNA was extracted from 100µL of serum, using the Q1Aamp Viral RNA Kit (QIAGEN Inc.

Santa Clarita, CA, USA). Primers for nested PCR were selected from E1 to E2 genes flanking the HVR1 region of the HCV genome. [27] The first round of amplification was carried out in a total volume of 50µL using 20µL of HCV cDNA preparation and 30µL of PCR master mix (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM of each of the dNTPs (dATP, dGTP, dCTP, and dTTP), 40 pmoles of outer primers QP1 and QP2). The sequences of QP1 (sense) and QP2 (antisense) were: 5′ AGAATGGCCTGGGACATGATG-3′(nt 1228-1248) and 5′-CAGTGTTTAAGCTGTCATTGC-3′ (nt 1565-1585), respectively. A 'hot start' PCR reaction was initiated by the addition of 1.25 U of Amplitaq DNA polymerase. PCR cycling parameters consisted of denaturation at 94° C for 1 minute, annealing at 55° C for 1 minute, and extension at 72° C for 1 minute, for 35 cycles. The second round was performed with 40 pmoles for each of the inner primers, with 2µl of the primary product as the template. The sequences of QP3 (sense) and QP4 (antisense) were: 5′-ACTGGGGTGTCCTCGTGGG-3′ (nt1334-1352) and 5′AACAGCAATGGGAGCTGGCA-3′ (nt 1522-1541), respectively. All primer sequences were from the GenBank database Accession Number Y11604.1. Thirty cycles of amplification were performed using the same cycling conditions as the first round except that the final extension step was increased to 7 minutes, to allow the complete formation of duplex molecules. The amplified products (10µL) were analyzed on 2% agarose gel electrophoresis, stained with ethidium bromide (1µg/mL), and visualized under UV illumination.

The concentration of the PCR products was determined by using the DNA Dip Stick Kit (Invitrogen, San Diego, CA). SSCP was performed as previously described. [28] Briefly, 20µl (15µg) of the amplicons were mixed with equal volume of a solution containing 98% formamide, 0.05% bromophenol blue, and 2% glycerol, and denatured by heating at 94° C for 5 minutes, and immediately chilled on ice. The denatured samples were applied on 12.5% neutral polyacrylamide gels and separated at 200 V constant voltages at 25° C for 2 hours. The DNA bands were visualized by silver staining (PhastGel, Pharmacia).

The results were expressed as a mean ±SD. Differences in proportions were tested by the chi-square test. Mean quantitative values were compared by the t-test. All *P* values were two-tailed and a level of less than 0.05 was considered statistically significant.

3. Results

E2/NS1 fragment containing a HVR1 of HCV-4 genome was amplified from the serum samples of egyptian patients at zero, three, and six months of interferon alfa and ribavirin combination therapy. The amplified products were used for SSCP analysis, to investigate the constitution of HVR1 quasispecies in the patients. A positive virological response (responders) was assessed by the disappearance of the viral serum RNA (viral load <0.2 Meq/mL) as long as six months of treatment. [Figure 1] a, b, and c illustrate the SSCP bands obtained.

The pretreatment number of the SSCP bands in patients who responded to the therapy ranged from

2 to 6 [mean number of SSCP bands=3.71±1.25], whereas, in the non-responding patients the range of SSCP bands was 2 to 7 [mean number of SSCP bands=3.77±1.73]. No significant difference was found between the pretreatment number of SSCP bands and response (*P*=.53). Seven patients (2, 4, 10, 13, 19, 20, and 25) were considered responders, six of them (4, 10, 13, 19, 20, and 25) were with a sustained virological response, as they had no detectable levels of HCV RNA at three and six months of treatment [Table 1]. However, their SSCP bands varied. In all except one (patient 10), the SSCP band either remained the same or decreased. One of these patients (patient 25) had a low virus load (<1.0) before initiation of treatment, whereas, two patients (2 and 4) had a high virus load (>10.0). Three patients (13, 19, and 20) showed a decrease in the number of SSCP bands during the treatment period and had medium viral loads (1-10 Meq/mL) prior to initiation of treatment. Therefore, in patients who responded to interferon alfa and ribavirin therapy within six months, the HCV-4 quasispecies population in the majority of them either remained the same or decreased during the treatment period.

Eighteen of the 25 patients with viral load >0.2 Meq/mL were considered as non-responders [Table 2]. Among them, four patients (16, 17, 23, and 24) showed the same SSCP band number during the treatment period. Three patients (5, 8, and 21) showed a decrease and four patients (1, 3, 6, and 12) showed an increase in the number of SSCP bands during the treatment period. In seven patients (7, 9, 11, 14, 15, 18, and 22), there were a variable number of SSCP bands in which there was a decrease at three months followed by an increase at six months and vice-versa. Among the patients in whom the number of SSCP bands either remained the same or decreased, a medium viral load (1-10 Meq/mL) prior to initiation of therapy was noted. Two (6 and 12) of the four patients who showed an increase in the number of SSCP bands had a medium pretreatment viral load, whereas, the other two patients (1 and 3) had a high pretreatment viral load (>10 Meq/mL). All patients with variable number of SSCP bands (7, 9, 11, 14, 15, 18, and 22) had a medium pretreatment viral load (1-10 Meq/mL). Statistical analysis revealed no significant difference between pretreatment quasispecies composition and the response (*P*=.53).

Table 1: Viral load and quasispecies population of responder patients.
(Pre T: pretreatment; M3: 3 months post treatment & M6: six months post treatment)

Patient Number	Genome Copies/ml x 10^6 (number of SSCP bands)		
	Pre T	M3	M6
2	15.2 (4)	0.22 (4)	< 0.2 (2)
4	12.9 (2)	< 0.2 (2)	< 0.2 (2)
10	4.8 (4)	< 0.2 (3)	< 0.2 (6)
13	3.8 (4)	< 0.2 (2)	< 0.2 (1)
19	1.6 (6)	< 0.2 (4)	< 0.2 (4)
20	1.6 (3)	< 0.2 (2)	< 0.2 (2)
25	0.5 (3)	< 0.2 (3)	< 0.2 (3)
Mean ± SD of SSCP bands	3.7 (1.25)	2.9 (0.89)	2.9 (1.68)

Table 2 : Viral load and quasispecies population of non-responder patients.

(Pre T: pretreatment; M3: 3 months post treatment & M6: six months post treatment)

Patient Number	Genome Copies/ml x 10^6 (number of SSCP bands)		
	Pre T	M3	M6
1	40.8 (4)	4.2 (6)	14.4 (5)
3	13.1 (3)	3.2 (3)	3.5 (4)
5	9.8 (4)	‹ 0.2 (3)	22.2 (2)
6	8.1 (2)	3.4 (4)	5.3 (6)
7	6.3 (7)	2.2 (4)	1.3 (5)
8	6.3 (5)	0.7 (4)	7.7 (4)
9	5.0 (4)	5.2 (2)	0.3 (5)
11	4.8 (3)	0.9 (1)	14.2 (3)
12	4.2 (2)	19.4 (2)	21.7 (4)
14	3.6 (2)	0.3 (4)	0.9 (2)
15	3.5 (3)	0.3 (2)	0.9 (4)
16	3.3 (3)	0.3 (3)	2.4 (3)
17	2.8 (2)	1.0 (2)	0.9 (2)
18	2.4 (7)	0.7 (2)	0.6 (5)
21	1.5 (6)	‹ 0.2 (5)	29.0 (3)
22	1.3 (6)	3.5 (3)	1.1 (5)
23	0.6 (3)	‹ 0.2 (3)	0.3 (3)
24	0.6 (3)	1.0 (3)	1.0 (3)
Mean ± SD of SSCP bands	*3.8 (1.73)*	*3.1 (1.23)*	*3.8 (1.22)*

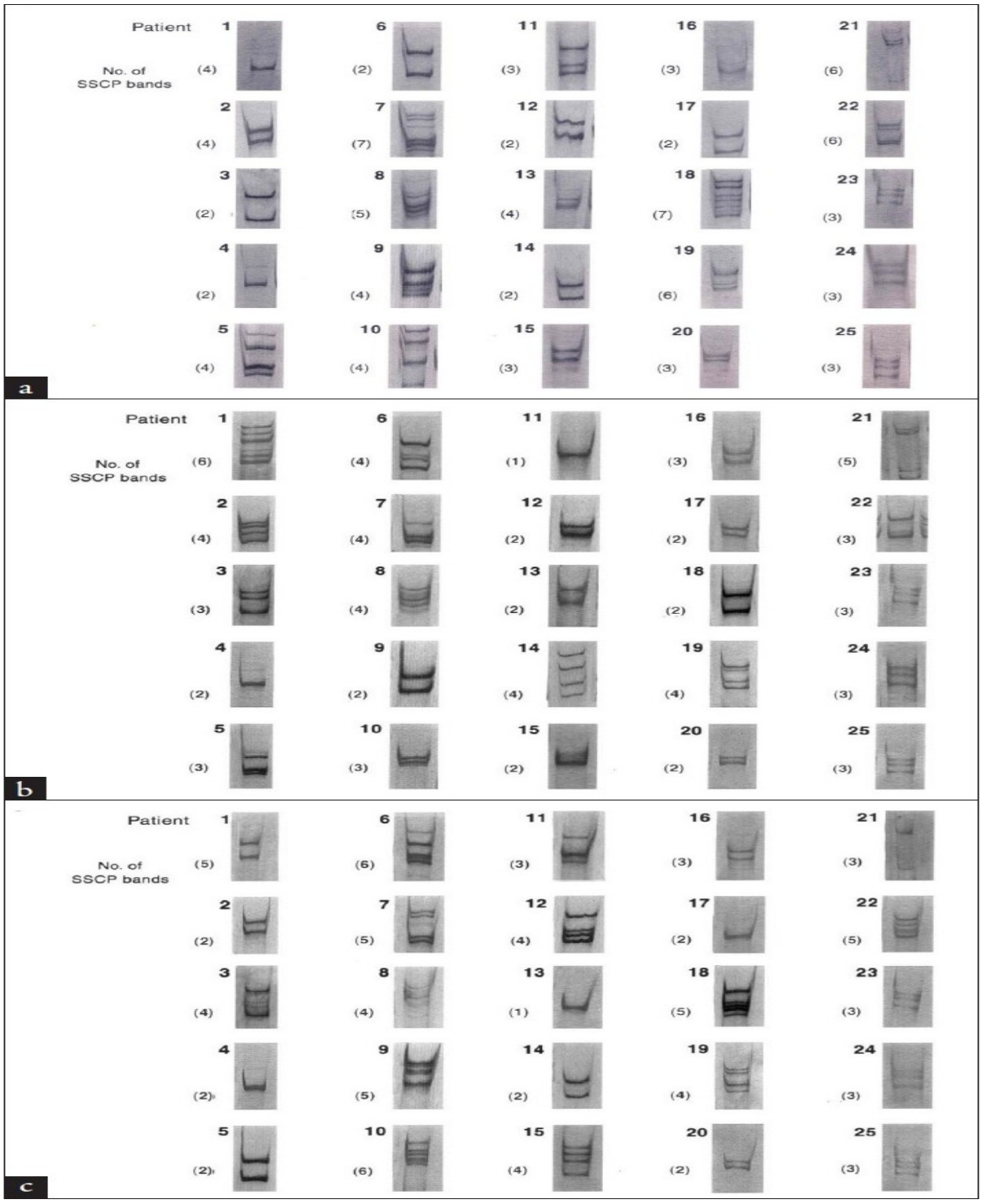

Figure 1: Quasispecies population in each of the 25-genotype 4, HCV-positive specimens. (a) Pretreatment quasispecies population for all patients, (b) quasispecies population 3-months post-treatment, and (c) 6-months post treatment. Many bands may not be clearly seen as they were faint in the original gel due to lower concentration of the variants.

4. Discussions

Patients with HCV infection show many HCV genetic variants of the same genotype, known as quasispecies, due to the frequent mutation of the virus as it replicates. Persistence, resistance to therapy, and the transformation seen in these patients have long been attributed to these variants. [29] We did not study the details and outcomes of the treatment modalities, but investigated the importance of quasispecies constituents during the therapy of HCV-4 chronic infection, to determine if it could be used to predict the treatment outcomes in our population. The treatment has been discussed elsewhere. [25] Quasispecies heterogeneity was shown in different studies to be a possible independent predictor of response to interferon alfa therapy. Responsive patients typically possess less HCV quasispecies population in the pretreatment sera than do non-responsive patients. [30],[31] A combination regimen of interferon alfa with ribavirin was demonstrated in clinical trials to produce higher rates of sustained virological, biochemical, and histological responses, particularly in patients with a relapse. [21],[32] However, the role of HCV quasispecies complexity on the outcome of interferon alfa and ribavirin combination therapy for HCV-4 infected Saudi patients, has not been studied. In a previous study to assess the effect of combination therapy of interferon alfa and ribavirin on chronic HCV-4 patients, we reported a poor response among the patients, with 23% showing a sustained biochemical response and 12% showing a sustained virological response at the end of a treatment period of 24 weeks (6 months). [25] In the present study we utilized SSCP analysis, a rapid and sensitive method for determining HCV heterogeneity, to evaluate HCV quasispecies complexity in the sera of 25 patients infected with HCV-4, before initiation of combination therapy and to assess changes in the quasispecies population after three and six months of therapy. The SSCP analysis was carried out on amplicons derived from the HVR1 region. This region is known to be highly heterogeneous in the virus genome and is therefore considered to reflect most accurately the quasispecies nature of the virus in the serum of the individual patient. [13], [33] Previous studies of HCV quasispecies, using SSCP and heteroduplex gel shift analysis, have identified two types of populations circulating in the serum of an infected individual. [34],[35] The first type consists of simple quasispecies (single HCV species) characterized by a single SSCP band; the second is comprised of complex quasispecies characterized by multiple SSCP bands. Our SSCP data showed the presence of multiple bands in the pretreatment serum of both responder and non-responder patients. Hence the pretreatment serum of all patients examined contained variable quasispecies population, although statistically no difference was found in the average number of SSCP bands between responsive and non-responsive patients. The finding that none of our patients had homogenous HCV species in the pretreatment serum is significant and appears to be a unique feature of the HCV-4 virus not related to the pretreatment viral load. Indeed, despite the fact that patients in the study group were chosen randomly, a careful examination of the virus titer in the pretreatment serum supports this observation. Of seven patients who responded to the therapy, five had a low-to-medium viral load in the pretreatment serum. Of 18 patients who were non-responders, 16 also had a low-to-Gmedium pretreatment viral load in the serum. Our findings were in agreement with previous studies carried out on patients with HCV-1b, who were also recognized to respond poorly to interferon alfa treatment. [36] Sakuma et al. [37] detected multiple SSCP bands in the pretreatment sera of seven of eight patients who did not respond to interferon alfa treatment. Gonzalez-Peralta *et al.* [38] also reported that among chronic hepatitis C patients treated with interferon alfa, HCV-1 (unspecified subtype) was associated with an increased number of quasispecies, resulting in poor response. Zekri *et al.* [39] in Egypt recently reported the effect of HCV-4 quasispecies on the treatment outcome. However, this study involved quasispecies from a less common area of the genome for this purpose (the 5' UTR) and not the usual HVR1, and the technique used was not the universally accepted SSCP. Another study reported that quasispecies were an important predictive factor for a sustained virological response for PEG-interferon alfa and ribavirin treatment. [40] However, the investigators worked on HCV-1 for 12 weeks and also reported that it was no better than a viral load as a predictive factor.

The presence of multiple HCV species in the pretreatment serum of HCV-4 patients could be one possible explanation for the resistance of HCV-4 virus to both the interferon alfa and interferon alfa and ribavirin combination regimen. The small number of subjects in our study does not allow for analysis of other possible factors such as side effects and histological grading, but the study did demonstrate that regardless of the presence of multiple HCV species in pretreatment serum, patients with stable viral populations or those with viral populations that decreased in complexity following treatment tend to respond to therapy. Nonetheless, the determination of quasispecies population cannot be used as a reliable predictor for effective therapy of HCV-4 infection.

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Study The Effects Of Radio Waves Propagation Under Sea At Pakistan Coastal Zones

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Abstract — In this communication the factors affecting the radio wave propagation at low, moderate and high salinity and temperatures conditions for different depths are studied. The propagation of radio waves under water is a severe technological task that requires specific attention for implementing suitable techniques and devices. This is because electromagnetic radiation is rather difficult to propagate to through sea water. Very low frequency (VLF) radio waves (3-30 KHz) can penetrate sea water down to depth of roughly 20 meters and extremely low frequency (ELF) waves penetrate sea to the depths of hundreds of feet. The range and quality of transmission varies with water conditions. In this communication we have selected few stations along the coastal zones of Pakistan to study the possible effects of fluctuating dynamics of water masses on the radio waves propagation by transmitting small power at VLF range. The physical problems, concerned with the issue of radio wave propagation under water at coastal environment are of great significance and accordingly the intended assessment needs to be dealt within the frame work of Ocean wave's dynamics. [Journal of American Science 2010;6(8):413-419]. (ISSN: 1545-1003).

Key words: T – S Model, Water Masses, communication between Submarines, VLF, Salinity and Temperature.

1. INTRODUCTION

The effects of natural environment on the propagation of radio waves are highly dependent on frequency used. The physical nature of the intervening paths show significant effects on the propagation such as the fluctuations in the T-S profiles of water masses expected to be affecting. Radio waves inside the sea increase with the frequency of the signal. This means that the lower the frequency a radio transmission, the deeper into the ocean a useable signal will travel. Radio waves in the very low frequency (VLF) band at frequencies about 20,000 Hertz (Hz) penetrates sea water to depths of only tens of feet. Whereas, extremely low frequency (ELF) waves penetrate sea to the depths of hundreds of feet, permitting communications with submarines while maintaining stealth [1]. Our purpose of studies is to make sure the qualification of signal to noise ratios. The performance of the communication link can be predicted on the basis of typical characteristics of the propagation path that is channeled by the fluctuation in the water masses profiles.

The assessment of the effects of water masses fluctuations on radio wave propagation are dealt in the form of the long-term variability in temperature, salinity, conductivity and density occurring in the Pakistan coastal zones. As it is well known fact that the sea water is a lossy dielectric medium characterize the different frequencies bands with relative permittivity or dielectric constant and average conductivity in S/m [2].

2. T – S REPRESENTATION OF STATIONS

In deep sea water mass is usually defined as a body of water with a common formation history. This is based on the observation that water renewal in the deep ocean is the result of water mass formation in contact with the atmosphere, spreading from the formation region without atmospheric contact, and decay through mixing with other water masses [3]. The basic tool for water mass classification and analysis is the temperature-salinity (T-S) diagram in which the two conservative properties are plotted against each other. The temperature-salinity combinations identified by the water mass points or curves are known as source water types. Coastal oceans are shallow ocean regions lying over continental shelves. They are strongly affected by nearby lands, river outflows, large human populations, and industrial and agricultural discharges. Coastal oceans are also highly variable. Their currents, water characteristics, and even marine life change over relatively short distances and short periods of time [4]. Coastal-ocean waters respond within a few hours to winds blowing over months. Coastal-ocean distances are also shorter than those involving the open ocean.

Many coastal waters are partially isolated from the open ocean [5]. For instance, parts of the Southern California Bight are partially isolated from the open Pacific Ocean by the Channel Islands, off Santa Barbara to the north Bays, harbors and fjords have restricted communication with the sea. Water characteristics commonly vary substantially in coastal waters. Rivers discharge large amounts of fresh water into coastal waters [6].

2.1. Temperature

Changes in a substance's physical form, such as a solid changing to a liquid or to a vapor, are called changes of state. Changes of state require breaking of intermolecular bonds, or forming of new ones. If bonds are broken, energy is taken up; when new bonds are formed, energy is released, usually as heat. Temperatures in coastal waters are controlled primarily by the incoming energy received from solar radiation. Thus, water temperatures are highest in the mid-latitudes, where insulation is largest, and are lowest in Polar Regions, which receive little insulation and where sea ice buffers surface waters from temperature changes [7]. Temperatures of coastal waters are also affected by oceanic currents transporting either warm or cold waters and by wind-induced upwelling of cold, subsurface waters. Cold winds off the land chill coastal waters during winter and sea ice forms in higher-latitude waters and in isolated shallow bays and estuaries [8]. The surface temperatures of northern Arabian Sea is about 29ºC and the same increase when moving from north to South. However, at depth (500 and 1000m), there was a decrease in water temperature from North to South. In 1982 research found that during the pre-monsoon and NE monsoon period the difference between minimum and maximum temperature of the surface layer of Northern Arabian Sea is about 7ºC which increases to 12ºC in SW monsoon. McGill in 1973 gave a thorough account of Oceanographic Parameters extending from the surface layer down 4000 m depth layer in the western Indian Ocean during the South-West monsoon period. He reported a temperature of 1.6ºC to 1.8ºC for the 3000-4000 m deep [10].

Salinity

Seawater is a solution of salts, of nearly constant composition, dissolved in variable amounts of water. Water, its most abundant constituent, determines most of seawater's physical properties. The presence of salt in seawater influences its density and other physical characteristics [9]. The major constituents in sea salts come primarily from three sources: volcanic eruptions, chemical reactions between seawaters and hot, newly formed crustal rocks; and weathering of rocks on land. Some properties of seawater change as salt concentration increases [11]. For instance, changing salinity from 0 to 40 causes Viscosity (internal resistance to flow) to increase about 5%. Adding sea salts to water also changes its temperatures of maximum density and initial freezing. Because salt does not fit into the ice crystal structure, it inhibits ice formation and depresses the initial freezing point. Salinity variations in coastal waters persist longer than wind effects [12]. In the Northern most Arabian Sea the surface salinity ranges from about 36.2‰ to about 36.5‰. Salinity decreases, from surface to 1000 m, from North to South and from West to East. The high salinity Arabian Sea water is formed at the surface and intensified by the outflow of salinity water from the Persian Gulf at approximately 300 m and from the Red Sea at approximately 800 m at 2000 m salinity in the Arabian Sea is still as high a 34.8‰ found similar values at 3000 m and 4000 m from the Northern Arabian Sea [13].

The graphical analysis will clearly explain and gives the easy understanding to the readers to evaluate the difference between the physical properties of coastal regions of Arabian Sea and other regions of the oceans

3. MATERIAL AND METHOD

For study and analysis water masses at Pakistan coastal regions (stations) were selected at different locations along the coastal zones of Arabian Sea. It has been observed that the basis of water mass analysis in the deep ocean is the derivation of water mass properties in the formation region are small compared to the property differences that are observed between different water masses at some distance from their formation region. The graphical views of the stations are given below:

The data used in the under study area is collected by the help of equipment provided by the Pakistan Naval Authority and Institute of Oceanography, Karachi regions. Survey Echo Sounder (Hydro Star 4900) is used to measure deep sea depth where as Echo Sounder (NJA – 193S) is utilized to determine low depth data. Temperature, pressure and conductivity parameters are computed by use current meters of models, 108,308 MKIII. Besides MTCD is utilized to quantify salinity and density of the under study area.

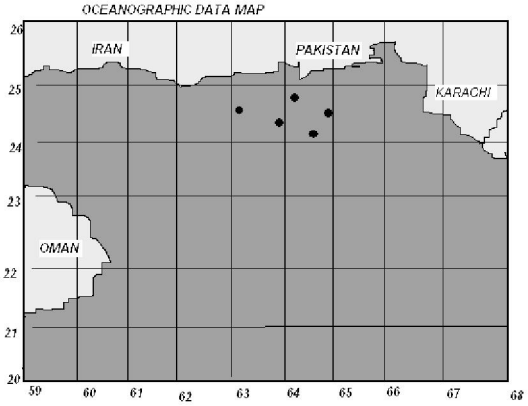


Figure 1. Graphical view of understudy stations

4. T –S MODEL CURVES OF THE STATIONS

The graphical analyses of parameters evaluated at different locations are described under figures.2 to 6.

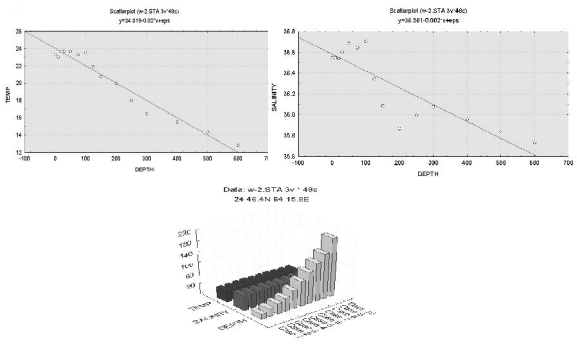


Figure 2. T-S model of station No. 1.

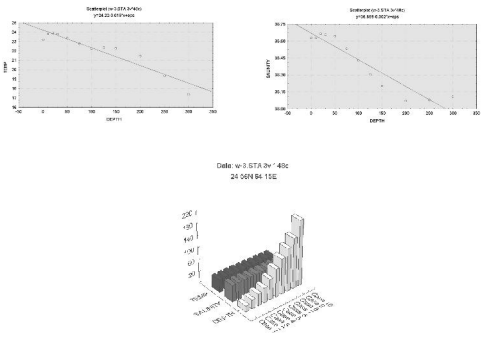


Figure 3. T-S model of station No. 2.

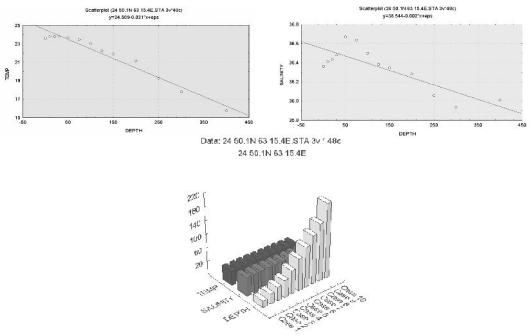


Figure 4. T-S model of station No. 3.

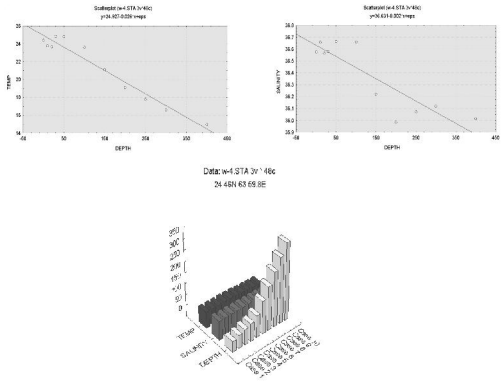


Figure 5. T-S model of station No. 4.

5. ESTIMATION OF DENSITY

Temperature, salinity and pressure control seawater density. Of the three, temperature and salinity are the most important. In the open ocean, seawater density varies only a small amount. These slight density differences cause ocean currents [14]. It is noticeable that this piece of investigation is crucially based on the utilization of observed data obtained at various stations. Figure below shows maximum water-density changes for salinities between 30 and 37 and temperatures from -3 to 30ºC [15]. This encompasses the temperature and salinity ranges of the entire ocean. At 30ºC, a change in salinity from 34 to 35 changes the density from 1.021 to about 1.022 [16]. Density is changed an equal amount by cooling water with a salinity of 37 from 27.5 to 24.3ºC, a change of 3.2ºC. Such temperature changes occur commonly at the ocean surface. Large salinity changes usually occur near land, as a result of river discharges and increased precipitation there, and in Polar Regions, because of ice formation. Specifically, a stable water column is one in which density increases monotonically with depth [17].

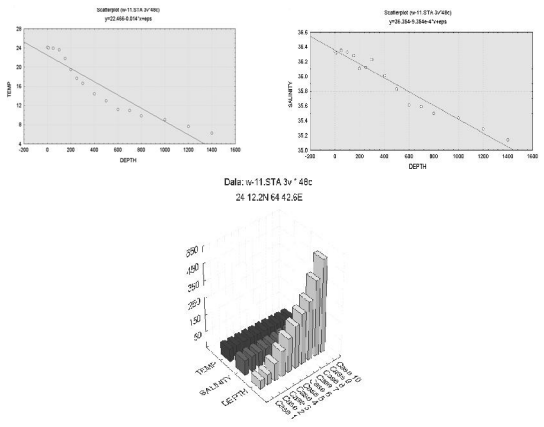
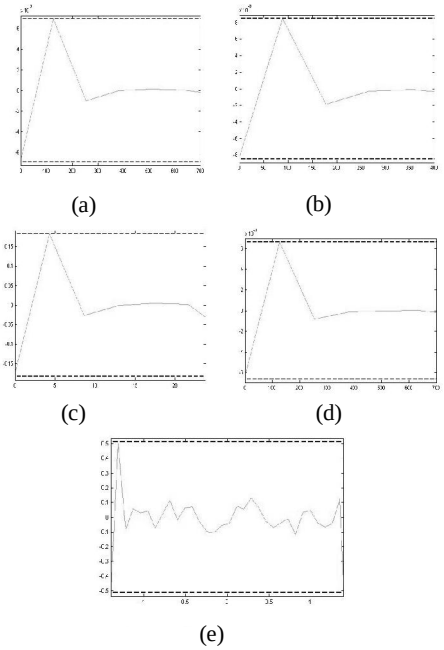


Figure 6. T-S model of station No. 5

6. ESTIMATION OF DENSITY

Temperature, salinity and pressure control seawater density. Of the three, temperature and salinity are the most important. In the open ocean, seawater density varies only a small amount. These slight density differences cause ocean currents [14]. It is noticeable that this piece of investigation is crucially based on the utilization of observed data obtained at various stations. Figure below shows maximum water-density changes for salinities between 30 and 37 and temperatures from -3 to 30ºC [15].



(a) (b) (c) (d) (e)

Figure 7. Changes in seawater density are caused by variations in salinity and temperature.

7. WAVELET CONSIDERATIONS

Wavelet technique has been used for the analysis of oceanographic and communication data and results received at different locations. It provides engineers, scientists and other technical professionals with a single interactive system that integrates numeric computation; visualization and programming. Graphical analyses of the results of density at different stations with respect to salinity and temperature are given below:

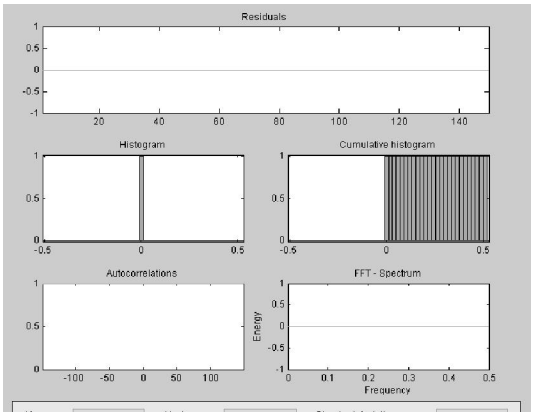


Figure 8. Graphical analysis of the results of density with respect to temperature and salinity of the understudy stations

8. BEHAVIOUR OF THE SIGNAL AT THE WATER MASSES

As a result of the high electrical conductivity of sea water, signals are attenuated rapidly as they propagate downward through thick conductors such as salt water. In many cases, the obvious solution is to surface and raise an antenna above the water surface to use standard technology. This is not sufficient, however, for nuclear-powered submarines. VLF radio waves (3-30 KHz) can penetrate sea water down to a depth of roughly 20 meters. The range and quality of transmission varies with water conditions, local noise level, and reverberation effects. Continuous wave energy radiated by antennas oscillates at radio frequencies. The associated free-space waves range in length from thousands of meters at the long-wave extreme to fractions of a millimeter at the short-wave extreme. Short radio waves and long infrared waves overlap into a twilight zone that may be regarded as belonging to both [18].

The wavelength λ of a wave is related to the frequency f and velocity υ of the wave by

$$\lambda = \upsilon / f \qquad (1)$$

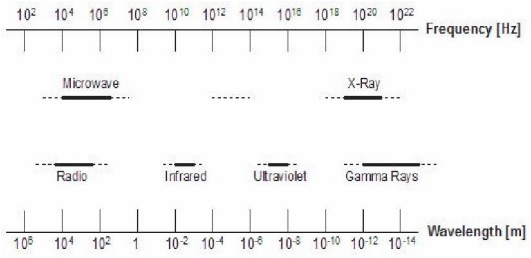


Figure 9. The electromagnetic spectrum with wavelength on a logarithm scale from shortest gamma rays to the longest radio waves.

Thus the wavelength depends on the velocity υ which depends on the medium. In this sense, frequency is a more fundamental quality since it is independent of the medium, when the medium is free space.

$$\upsilon = c = 3x10^{8} m/s \quad (2)$$

$$\lambda = c/f = 3x10^{8}/3000x10^{6} = 1m \quad (3)$$

The propagation of an electromagnetic wave is described through different parameters: the electric and magnetic fields E and H, the electric flux density

D and the magnetic induction B. Only vectors $\vec{E}$ and $\vec{B}$ generate effects allowing the determination of the electromagnetic field. The vectors $\vec{D}$ and $\vec{B}$ are linked to vectors $\vec{E}$ and $\vec{H}$ through the following linear relations:

$$\vec{D} = \varepsilon * \vec{E} \quad (4)$$

$$\vec{B} = \mu * \vec{H} \quad (5)$$

The ε and μ coefficients depend on the nature of the medium where the electromagnetic wave propagates. In the case of homogeneous, isotropic media, ε and μ are constants, whereas inside vacuum, these coefficients, respectively referred to as the permittivity and magnetic permeability of the medium, assume the following values:

$$\varepsilon_0 = 10^{-9}/36\pi = 8.842 * 10^{-12}$$

(Farad per meter) (6)

$$\mu_0 = 4\pi * 10^{-7}$$

(Henry per meter) (7)

For any material medium, these coefficients can be deduced from the previous values using the following two equations:

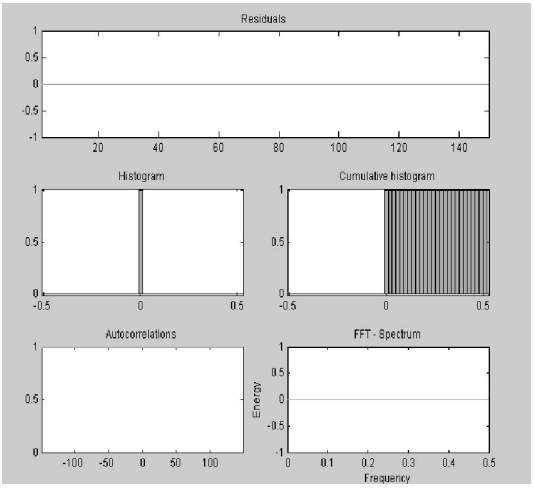
$$\varepsilon = \varepsilon_0 * \varepsilon_0 \quad (8)$$

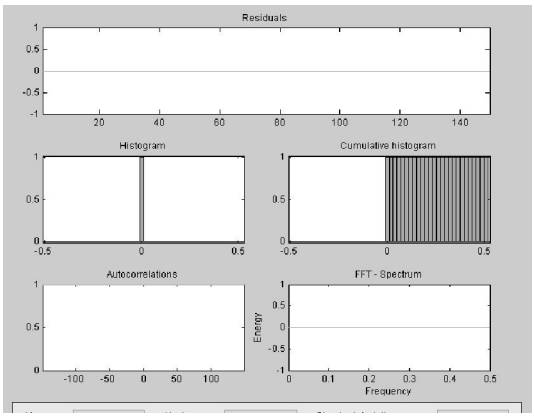
$$\mu = \mu_0 * \mu_r \quad (9)$$

Where εr and μr are the permittivity and the relative magnetic permeability of the medium respectively [18].

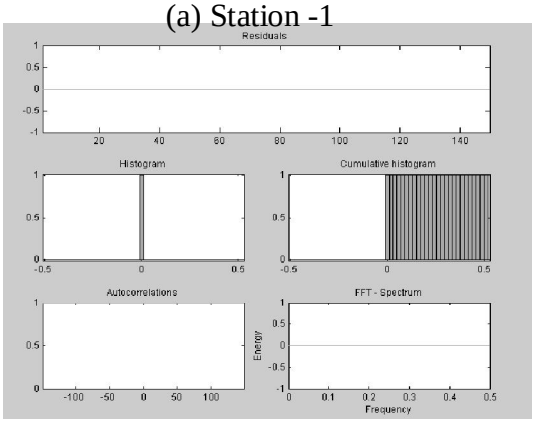
In this study we design the VLF antenna in which we used copper wires which are rewound just like a transformer core in either direction. The gap between the two windings is filled with ferrite material. In this construction we can't ignore the basic parameters without which it is very difficult to
fulfill a good and efficient antenna. The low power modulated and amplifier circuit is design to transmit the signal for short distance. This is an experimental effort to enquire the live data and with the passage of time and effort it can be used for both way communications between two submarines [19].

The graphical analyses of the results of radio waves communication through wavelets are given below:

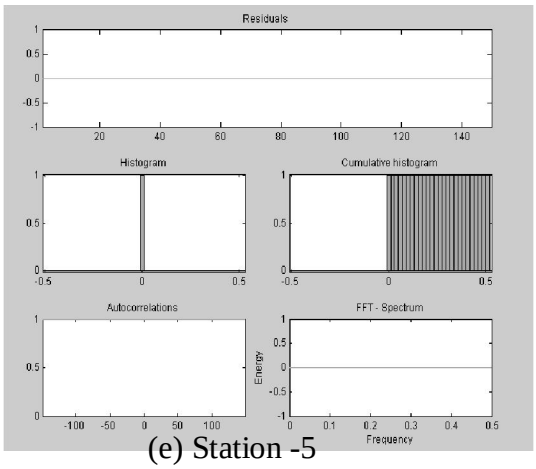




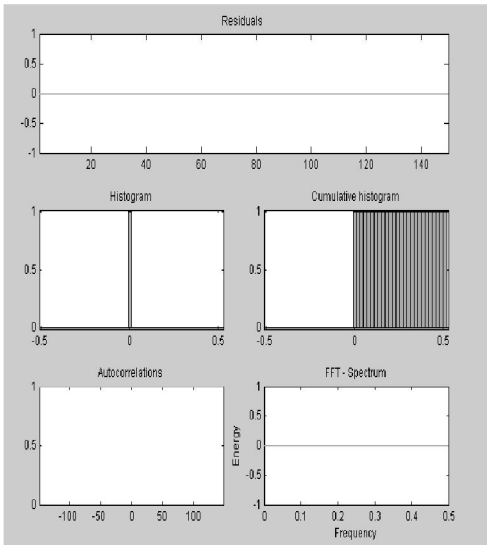
(a) Station -1



(c) Station -3



(e) Station -5



(d) Station -4

Figure 10: Wavelet analysis of radio wave communication on water masses of understudy stations.

7. CONCLUSION

In this study we have observed that physical variables i.e. temperature and salinity have some diverse effects to study the fluctuating dynamics of water masses on radio wave propagation under sea at Pakistan coastal environment. It has been examined that long wavelength can easily penetrate on those areas where the salinity and temperature are very low. In our analysis it has been observed that out 05 stations, there are 04 positions (24 46.4N 64 15.8E, 24 50.1N 63 15.4E, 24 56N 64 15E and 24 46N 63 59.8E) in which the density is not vary normally. Whereas, position 24 12.2N 64 42.6E the density is drastically change due to variation in temperature and salinity.

Geo-morphological studies are necessary to understand the physics and underlying structure of natural coastal zones and to build models that monitor some aspects of these investigations. Often this is achieved by analyzing observations of the process, extracting important features regarding its morphological and statistical structure, and using this information for model building. Wavelet technique has been used for oceanographic and communication data received at different locations. It provides engineers, scientists and other technical professionals with a single interactive system that integrates numeric computation; visualization and programming. On some occasions, physical considerations directly guide model construction and in such cases,

observations are used to validate and refine the model presented in this paper.

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06/27/2010

Nitrogen Soil Dressing And Foliar Spraying By Sugar And Amino Acids As Affected The Growth, Yield And Its Quality Of Onion Plant

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Abstract: Field experiments were conducted during the two seasons of 2007/2008 and 2008/2009 at the Experimental Farm of National Research Centre at El-Nobaria (110 km far Cairo City) to study effect of the nitrogen application (as soil dressing) at two rates, i.e. 90 and 120 N units/fed., and the supplying of amino mix compound and sugar (as foliar spraying) on onion plant productivity. The main important findings are as follows: 1. The vigor plant growth parameters, i.e. plant length, average number of leaves per plant, fresh and dry weight of whole onion plant and its different organs as well as the heaviest bulbs yield and its better physical and chemical properties, all of them were associated with addition the higher nitrogen rate, i.e. 120 N units/fed. 2.Foliar spraying by each amino mix compound and or sugar as individually or together resulted plants more vigor and heavier bulbs yield as well as better values of nutritional elements (N, P, K, Fe, Mn, Zn and Cu) if compared with that control plants. Moreover, the vigorously of plant growth and the highest bulbs yield with the best physical and chemical constituents all them were obtained with the foliar spraying by amino acids and sugar together. 3.Soil dressing by 120 N units/fed., and the foliar spraying by amino acids and sugar as one compound resulted the highest values of plant growth characters and heaviest bulbs yield with the best parameter values of nutritional elements, but the differences were not significantly at 5 % level in most cases. [Journal of American Science 2010;6(8):420-427]. (ISSN: 1545-1003).

Keywords: Field experiments; plant growth; sugar; nutritional element

1. Introduction

In Egypt, onion (*Allium cepa*, L.) is one of the most important vegetable crops. The total grown area amounted by 87.47 thousands fed., produced about 1147.6 thousands ton and by average 13.12 tons/fed. (AERI, 2006). Increasing productivity of onion with good quality is an important target by the growers for local market and exportation. The productions of the best fields require that the soil must have favorable physical, chemical nutritional and biological conditions. Now more than ever the importance of an adequate supply of plant nutrients to ensure efficient crop production is being recognized. Generally, from the horticultural point of view, the yield of any plant is the most important target from any plantation. The role of nitrogen levels has been associated with so many significant increasing in the plant growth and total yield of onion (Aisha *et al*., 2007; Shaheen *et al*., 2007) and / or garlic (Shaheen, 1999 and Ali, *et al*., 2001) and potatoes (Belonger *et al*., 2000; Shafeek *et al*., 2001; Love *et al*., 2005 and Faten *et al*., 2008).

Moreover, it is known that, every plant like any organism needs certain components for growth over or above soil. The basic component of living cells is protein, which are formed by sequence of amino acids. The requirement of amino acids in essential quantities is well known as a means to increase yield and overall quality of corps (Sanaa, *et al*., 2001; Slviero *et al*., 2001; Attoa et al., 2002; El-Shabasi *et al*., 2005; Awad *et al*., 2007; Al-Said and Kamal, 2008; Faten *et al*., 2010).

In spite of the plants require 3 essential elements, light, water, temperature and nutrients, etc. to thrive and produce optimum yield, but plants produce sugars such as glucose and sucrose. These sugars are needed to produce energy promote growth and aide in the processes of respiration and transpiration, consequently effected plant growth and its productivity. Many workers studied the response of plant to external application of sugar, and reported that, sugars have important signal functions all stages of plant's life cycle (Gibson, 2000; Riou-Khamlichi *et al*., 2000; Smeekens, 2000; Flnkelstein and Gibson, 2000; Pourtau *et al*., 2004; Filip Rolland *et al*., 2006).

Therefore, this study was conducted to elucidate the effect of soil dressing by two levels of nitrogen fertilizer and foliar application of amino acids mixture and/or sugar and their interactions on growth, bulbs yield of onion and its some physical and chemical properties under the newly sandy soil.

2. Materials and Methods:

Two field studies were carried out during the two seasons of 2007/2008 and 2008/2009 at the Experimental Farm of National Research Centre at Nobaria (Behera Governorate), Egypt to investigate effect of the application of nitrogen (as soil dressing) at two rates and supplying amino mix compound and/or sugar (as foliar spraying) on onion plant productivity. The studied used treatments were as follows:

A. Soil dressing:

1. Soil dressing of 90 N units/fed.

2. Soil dressing of 120 N units/fed.

The amount of nitrogen rates was added as urea form at two equal quantities (60 and 90 days after transplanting).

B. Foliar spraying:

1. Foliar spraying by amino mix compound (1000 ppm).

2. Foliar spraying by sugar (2000 ppm).

3. Foliar spraying by amino mix + sugar.

4. Control treatment (water foliar spraying).

The foliar spraying applied 3 times, starting at 60 days old with 15 days internals.

Table (1) shows the chemical composition of amino mix compound.

Nutritional elements g. / 100 cm.		Amino acids Mg. / 100 cm.				Vitamins mg. / 100 cm.	
Elements	Value						
Zn	2	Aspartic acid	249	Methionine	180	Vitamin B_1	0.8
Fe	1.5	Threnine	45	Iso- Leucine	52	Vitamin B_2	2.4
Mn	0.5	Serine	56	Tyrosine	38	Vitamin B_6	1.2
Mg	0.004	Glutamic acid	55	Phenylalanine	22	Vitamin B_{12}	0.82
Cu	0.004	Glycine	50	Histidine	12	Folic acid	4.2
Ca	0.025	Alanine	100	Lysine	40	Pantothenic acid	0.52
Br	0.056	Prolein	38	Arginine	20	Nicotine B_5	1.14
S	0.01	Valine	68	Tryptophan	20		
Co	0.03	Cysteine	44				

Source: Agrico international (www.agricointernational.com).

The soil texture of experiments was sandy with 95.3 % sand, 0.4 % silt and 4.3 % clay. The pH was 7.9 and E.C. was 2.0 ds/m.

All treatments were arranged in split plot design with 3 replicates. Where the two Nitrogen rates as soil dressing were assigned in the main plots and the 4 foliar spraying treatments were arranged randomly within the sub-plots. The total area of each sub-plot amounted by 14.0 sq. m. and contained 4 ridges (5 m long and 0.7 m width).

Onion seedlings cv. Giza 20 were planted on the 4th week of December in the two seasons of 2007 and 2008, where seedlings were grown on the two sides of ridge with 20 cm apart. All experiments were fertilized by phosphorus as calcium super-phosphate (16.0 % P_2O_5) at rate of 300 kgs / fed., and applied during soil preparing and before transplanting. Also, potassium sulphate (48 % K_2O) at rate of 200 kgs./fed. were divided two equal proportion and applied at 60 and 90 days after seedlings. The different agricultural practices of onion, such as irrigation, weeds, disease and pest controls were applied according the advice of Egyptian Agricultural Ministry.

A random sample of 3 plants were taken from every experimental plot at 120 days old to determine the vegetative growth characters, i.e. plant length, leaves number, length and diameter of each bulb and its neck, as well as fresh and dry weight of whole plant and its different organs.

At harvesting time, 150 days after transplanting, the total bulbs yield as tons/fed., were recorded. In the same time sample of 6 bulbs were taken randomly to record the physical onion properties, i.e. length, diameter and average weight of bulb.

In over dry weight of onion bulbs, N, P, K elements were determined according the methods described by Pregl (1945); Trough and Mayer (1939) and Brown and Lilleland (1946), respectively. However, Fe, Mn, Zn and Cu were analysis and determined by that methods of Chapman and Pratt (1978).The protein percentages in bulbs were accounted by multiplying nitrogen content by 6.25.

All obtained data were statistically analyzed according to the method described by Gomez and Gomez (1984).

3. Results and Discussion:

A. Plant growth:

Onion plant growth characters as expressed by plant length, leaves number, length and diameter of blub and neck, fresh and dry weight of whole plants and its different organs as affected by the interaction between nitrogen fertilizer as urea at two rates, i.e. 90 and 120 units of N per feddan and the application of some promotion substances (sugar and/or Amino acids) are presented in Tables (2 and 3) for the two experimental seasons.

All onion plant growth parameters recorded their highest values with that plants which received the higher rate of nitrogen fertilization, i.e. 120 N units/fed., whereas, the statistical analysis of the obtained data indicated that the differences within the two rates of nitrogen were great enough to reach the 5 % level of significant. These findings were true in both experiments for all plant growth measurements, except average leaves number per plant in two seasons and whole dry weight of plant and its leaves in 1st seasons.

With regard to the parameters of bulb and it neck dimension as affected by the different treatments its response followed the same pattern of change like that of plant growth characters.

Generally, it could be concluded that, the plant growth of onion plant was more vigor when the nitrogen application was increased up to 120 N units/fed., These superior amounted by 33.1 and 36.04 % for fresh weight of whole plant and by 3.0 and 21.8 % for dry weight of whole plant respectively in 1st and 2nd experiment.

Nitrogen fertilization increased planting growth and its measurements could be attributed to nitrogen role in enhancing plant capacity in protein synthesis leading to an increase in building up carbohydrates and this in turn resulted in increases in plant growth characters. The obtained results are in harmony with there before applied (Ali *et al*., 2001; Love *et al*. 2005; Aisha *et al*., 2007 and Shaheen *et al*., 2007).

Concerning to the response of onion plant growth measurements to the application of some plant growth promotion substances, i.e. foliar spraying by sugar and/or amino mix compound are shown in Table (2 and 3) for the two seasons of 2007/2008 and 2008/2009. However, the presented data indicated that, applied both sugar and amino mix together resulted in the most vigor plant growth, followed in descending order by that plants received amino mix, then that treated by sugar as individually. It means that, the poorest plant growth of onion plant was noticed with that plants no received any plant growth promotion substances. Also, the recorded data indicated that, no great enough difference was found within the 3 promotion substances. However, the statistical analysis for the obtained data reveals that, the significant variation only recorded within the control and the applied sugar and/or amino mix as individually or as a compound treatments with the control treatment. These findings were true in both experiments.

It could be concluded that, the onion plants which treated with the amino mix + sugar gained the vigor plant growth. This might be attributed to that; amino acid mix contains more amino acids, vitamins as well as some growth regulators (Table, 1). Whereas, the previous studies have proved that, amino acids, can directly or indirectly influenced the physiological activities of the plants (El-Shabase *et al*., 2005, Awad *et al*., 2007; Al-Said and Kamal, 2008 and Faten *et al*., 2010). Also, the vigorously of plant growth of onion plants might be attributed to the role of sugar which needed to produce energy, promote growth, consequently affected plant growth (Flnkelstein and Gibson, 2002 and Filip Rolland *et al*., 2006).

Regarding the interaction within the soil dressing by nitrogen at rate of 120 units/fed., and the foliar spraying by the compound of sugar with amino mix the obtained data for the two experimental seasons are presented in Tables 2 and 3.

It is clear that, under the higher levels of nitrogen fertilizer (120 N units/fed.), the foliar application of sugar and amino mix together resulted the strongest plant growth in both experiments. The statistical analysis of the recorded data reveals that the interaction treatments had no significant differences for all plant growth measurements in two experiments, except fresh and/or dry weight of leaves.

Table (2): Effect of the application by sugar and amino compound under two rates of nitrogen fertilizers on plant growth of onion grown in newly lands during 2007/2008 season.

Treatments				Bulb (cm)		Neck (cm)		Fresh weight (g.)				Dry weight (g.)			
Urea	Compounds	Plant length (cm)	No. of leaves/ plant	Length	Diameter	Length	Diameter	Leaves	Neck	Bulb	Total	Leaves	Neck	Bulb	Total
90 Unit N. /fed.	Control	37.97	10.00	4.47	5.37	9.23	1.57	11.57	8.83	68.13	88.53	3.37	2.53	15.03	19.43
	Sugar (S)	41.67	11.00	5.13	5.80	10.30	1.70	12.10	9.80	110.53	132.43	4.37	3.37	15.47	23.70
	Amino(A)	41.83	11.00	5.43	5.97	10.93	1.80	17.17	8.80	113.07	139.03	4.33	3.57	20.73	28.47
	S.+ A.	44.97	11.00	5.57	6.10	11.90	1.97	19.00	10.67	129.73	159.40	4.33	5.50	19.90	27.93
Mean		**41.61**	**10.75**	**5.15**	**5.81**	**10.59**	**1.76**	**14.96**	**9.53**	**105.37**	**129.85**	**4.10**	**3.74**	**17.78**	**24.88**
120 Unit N. /fed.	Control	39.90	10.33	5.03	5.90	9.90	1.70	12.87	9.03	86.27	108.17	3.67	2.93	12.83	20.93
	Sugar (S)	45.33	10.33	5.80	6.30	10.30	2.23	16.77	10.33	121.93	149.03	4.43	4.23	15.03	23.20
	Amino(A)	46.27	11.33	6.30	6.60	12.10	2.07	18.03	11.23	133.70	162.97	4.33	3.90	20.23	28.63
	S.+ A.	47.60	13.00	6.53	7.37	12.10	2.20	20.70	11.03	141.10	172.83	5.60	5.10	17.23	29.73
Mean		**44.78**	**11.25**	**5.92**	**6.54**	**11.10**	**2.05**	**17.09**	**10.41**	**120.75**	**148.25**	**4.51**	**4.04**	**16.33**	**25.63**
Average	Control	38.93	10.17	4.75	5.63	9.57	1.63	12.22	8.93	77.20	98.35	3.52	2.73	13.93	20.18
	Sugar (S)	43.50	10.67	5.47	6.05	10.30	1.97	14.43	10.07	116.23	140.73	4.40	3.80	15.25	23.45
	Amino(A)	44.05	11.17	5.87	6.28	11.52	1.93	17.60	10.02	123.38	151.00	4.33	3.73	20.48	28.55
	S.+ A.	46.28	12.00	6.05	6.73	12.00	2.08	19.85	10.85	135.42	166.12	4.97	5.30	18.57	28.83
L.S.D. at 5%	Fertilizers	**1.03**	**N.S.**	**0.75**	**0.52**	**0.04**	**0.22**	**0.78**	**0.77**	**15.26**	**14.45**	**N.S.**	**0.22**	**1.30**	**N.S.**
	Compounds	**3.05**	**N.S.**	**0.58**	**0.73**	**1.73**	**0.30**	**3.30**	**1.09**	**13.89**	**15.14**	**0.78**	**0.48**	**3.01**	**3.09**
	Interactions	**N.S.**	**N.S.**	**N.S.**	**N.S.**	**N.S.**	**N.S.**	**N.S.**	**N.S.**	**N.S.**	**N.S.**	**N.S.**	**N.S.**	**N.S.**	**N.S.**

Table (3): Effect of the application by sugar and amino compound under two rates of nitrogen fertilizers on plant growth of onion grown in newly lands during 2008/2009 season.

Treatments				Bulb (cm)		Neck (cm)		Fresh weight (g.)				Dry weight (g.)			
Urea	Compounds	Plant length (cm)	No. of leaves/ plant	Length	Diameter	Length	Diameter	Leaves	Neck	Bulb	Total	Leaves	Neck	Bulb	Total
90 Unit N. /fed.	Control	65.67	9.00	3.87	3.67	7.83	0.90	15.79	15.40	74.63	105.82	3.15	7.52	8.05	18.72
	Sugar (S)	66.67	8.33	4.03	3.80	8.57	1.27	21.31	19.37	84.10	124.78	3.95	8.15	12.37	24.47
	Amino(A)	78.33	10.00	4.17	4.20	9.63	1.53	23.25	26.43	96.63	146.32	4.52	7.71	16.37	28.60
	S.+ A.	83.00	9.67	4.63	4.27	10.77	1.70	30.44	46.43	129.67	206.54	4.34	8.53	18.87	31.74
Mean		**73.42**	**9.25**	**4.18**	**3.98**	**9.20**	**1.35**	**22.70**	**26.91**	**96.26**	**145.86**	**3.99**	**7.98**	**13.91**	**25.88**
120 Unit N. /fed.	Control	71.33	8.67	4.30	3.73	7.63	1.17	17.11	17.13	75.20	109.44	3.51	5.57	10.13	19.21
	Sugar (S)	72.67	12.00	6.33	4.33	9.07	1.70	25.27	48.83	113.03	187.14	4.03	6.73	19.37	30.13
	Amino(A)	88.93	11.67	5.80	4.90	12.37	1.50	31.50	49.57	141.57	222.63	4.24	5.87	25.27	35.37
	S.+ A.	91.50	10.67	8.03	5.97	14.23	1.73	35.77	54.16	184.60	274.53	4.52	8.57	28.40	41.49
Mean		**81.11**	**10.75**	**6.12**	**4.73**	**10.83**	**1.53**	**27.41**	**42.42**	**128.60**	**198.44**	**4.08**	**6.68**	**20.79**	**31.55**
Average	Control	68.50	8.83	4.08	3.70	7.73	1.03	16.45	16.27	74.92	107.63	3.33	6.54	9.09	18.97
	Sugar (S)	69.67	10.17	5.18	4.07	8.82	1.48	23.29	34.10	98.57	155.96	3.99	7.44	15.87	27.30
	Amino(A)	83.63	10.83	4.98	4.55	11.00	1.52	27.38	38.00	119.10	184.48	4.38	6.79	20.82	31.99
	S.+ A.	87.25	10.17	6.33	5.12	12.50	1.72	33.11	50.30	157.13	240.53	4.43	8.55	23.63	36.61
L.S.D. at 5%	Fertilizers	**N.S.**	**N.S.**	**1.70**	**0.71**	**0.84**	**0.16**	**1.78**	**15.06**	**18.73**	**30.58**	**N.S.**	**0.31**	**4.46**	**4.37**
	Compounds	**5.67**	**N.S.**	**1.15**	**0.73**	**1.48**	**0.21**	**2.36**	**11.28**	**21.74**	**23.89**	**0.51**	**1.37**	**2.99**	**3.28**
	Interactions	**N.S.**	**N.S.**	**N.S.**	**N.S.**	**N.S.**	**N.S.**	**3.34**	**N.S.**	**N.S.**	**33.79**	**N.S.**	**N.S.**	**N.S.**	**N.S.**

B. Total bulbs yield and its physical properties:

Table (4) shows clearly that, total bulbs yield as tons/fed., as well its some physical properties (average wt. and length and diameter of bulb)are affected by the application of rates of nitrogen fertilizers as soil dressing and some plant growth promotion substances as foliar spraying in both experiments.

Nitrogen fertilizer in the form of urea at 120 kg N/fed., as soil dressing resulted the heaviest tonnage of onion bulbs yield in both experiments if compared with applying the lower rate, i.e. 90 kg. N/fed., whereas, the amounted total yield recorded 17.07, 14.84 and 18.39 and 15.72 tons/fed., respectively for applied 120 and 90 N units in 1st and 2nd seasons. By short words, the higher nitrogen application rate increased total bulbs yield by 15.0 and 17.0 % over applied the lower rate respectively in 1st and 2nd experiment.

It could be summarized that, the higher total bulbs yield as tons/fed., was associated with the higher rate of nitrogen fertilizer.

The vigorously of plant growth under the application of higher nitrogen rate may be attributed to that nitrogen stimulated stem elongation throughout the increment in the number and size of cells. It is noteworthy to mentioned that nitrogen is essential for plant growth as it is a constituents of all proteins and nucleic acids and hence of all protoplasm. As the level of nitrogen supply increases compared with the lower rate, the extra protein produced allows the plant leaves to grow larger and hence to have a larger surface available for photosynthesis proportional to the

amounts of nitrogen supply (Russel, 1982). The obtained results are in good agreement with the results of Shaheen, 1989; Yildirim *et al*., 2007; Aisha *et al*., 2007 and Shaheen *et al*., 2008).

Regarding to the some physical properties of onion bulb at harvesting time, i.e. length, diameter, and average weight, as affected by the application of nitrogen fertilizers during the two experiments of 2007/2008 and 2008/2009) the obtained data are showed in Table (4). It is obvious that, the better physical properties of onion bulbs were correlated with the higher nitrogen (120 N units /fed.) application. Whereas, the superiority in average bulb weight, length and diameter amounted by 14.0, 15.1 and 11.6 % respectively in 1st season and by 12.9, 5.4 and 9.4 % for the same respective in 2nd season.

Generally, the presented data of Table (4) clearly shows that, the higher bulbs yield with the better physical properties were produced with supplying nitrogen fertilizer at higher rate, i.e. 120 N units/fed. These findings are in good accordance with those which obtained by Shaheen, 1999; Ali *et al*., 2001; Love *et al*., 2005 and Shaheen *et al*., 2007.

Foliar spraying by sugar, Amino mix as individually and/or as compound with sugar significantly affected total bulbs yield of onion and its some physical properties, these were true in both experiments. However, foliar spraying of onion plant with sugar and amino mix together resulted the heaviest tonnage yield (19.5 and 19.06 tons/fed., for 1st and 2nd season respectively) followed in descending order by that plants which received amino mix as individual, then by that treated with sugar. Generally, the shown data of Table (4) indicate that, using sugar, amino mix as individual and/or as compound gained the higher bulbs yield if compared with the control treatment. Moreover, the statistical analysis of the obtained data reveals that, the differences within using the 3 promotion substances were not great enough to be significantly, for most measurements. These findings were true in the two experimental seasons.

The physical properties of onion bulbs i.e. average weight, length and diameter followed the same trend of direction for their response to the foliar application treatments by sugar and/or amino mix, like total bulbs yield as mentioned before.

It could be concluded that, total bulbs yield as ton/fed., as well as its physical properties recorded their higher and best values when supplied by sugar and/or amino mix as individually or as together, if compared the untreated plants.

This superiority might be due to that amino mix compound contains many amino acids as well as some growth regulators and Vitamins which stimulate and enhancement the metabolism processes in plant tissues. Moreover, the application of sugar as foliar, it supply the plant organism by the energy which promote synthesis of plant organs consequently, it could be summarized that, onion plants which received amino mix with sugar together as foliar spraying gained the heaviest bulbs yield and its physical measurements. The obtained results are in some direction with that of Paul *et al*., 2001; Sanaa *et al*., 2001; Pourtan, *et al*., 2004; Awad *et al*., 2007 and Al-Said and Kamal, 2008.

The interaction within addition two rates of nitrogen fertilizer as soil dressing and foliar application of sugar, amino mix had no significance effect on the total bulbs yield and/or its physical properties. This indicated that, each interaction factor may be act as independently the total bulbs yield and its properties.

Table (4): Effect of the application by sugar and amino compound under two rates of nitrogen fertilizers on total bulbs yield and its some physical properties of onion grown in newly lands during 2007/ 2008 and 2008/2009 seasons.

Treatments		Yield (ton/ fed.)	Average weight of bulb	Bulb (cm)		Yield (ton/ fed.)	Average weight of bulb	Bulb (cm)	
Urea	Compounds			Length	Diameter			Length	Diameter
		2007/2008				2008/2009			
90 Unit N. /fed.	Control	8.85	80.30	6.11	5.26	10.73	92.70	3.43	4.51
	Sugar (S)	14.40	129.45	6.76	6.24	16.68	146.33	6.00	6.20
	Amino(A)	17.60	154.95	7.30	6.90	17.58	164.83	7.33	6.90
	S.+ A.	18.50	160.11	7.42	7.14	17.88	168.20	7.05	7.11
Mean		**14.84**	**131.20**	**6.90**	**6.39**	**15.72**	**143.01**	**5.95**	**6.18**
120 Unit N. /fed.	Control	12.24	111.62	6.93	6.17	13.80	110.77	3.50	5.50
	Sugar (S)	16.50	151.67	7.86	7.03	19.65	173.43	7.01	6.92
	Amino(A)	19.03	167.15	8.33	7.21	19.84	178.21	7.40	7.45

	S.+ A.	20.50	168.28	8.63	8.11	20.25	183.79	7.15	7.16
Mean		**17.07**	**149.68**	**7.94**	**7.13**	**18.39**	**161.55**	**6.27**	**6.76**
Average	Control	10.54	95.96	6.52	5.72	12.27	101.73	3.47	5.01
	Sugar (S)	15.45	140.56	7.31	6.64	18.17	159.88	6.51	6.56
	Amino(A)	18.32	161.05	7.82	7.06	18.71	171.52	7.37	7.18
	S.+ A.	19.50	164.19	8.03	7.63	19.06	176.00	7.10	7.14
L.S.D. at 5%	Fertilizers	**2.00**	**5.32**	**0.61**	**0.51**	**1.70**	**13.37**	**0.30**	**0.19**
	Compounds	**2.57**	**17.72**	**1.11**	**0.97**	**1.35**	**11.87**	**0.57**	**0.35**
	Interactions	**N.S.**	**N.S.**	**N.S.**	**N.S.**	**N.S.**	**N.S.**	**N.S.**	**N.S.**

C. The nutritional values:

The response of elemental nutrients values, i.e. N, P, K, Fe, Mn, Zn and Cu as well as protein content of onion bulbs as affected by the application of N at rate of 90 and 120 units/fed., and the foliar spraying with sugar and Amino mix as stimulate substances are presented in Table (5 and 6) for the two experimental seasons.

As a general, the higher rates of nitrogen application as soil dressing, the higher nutritional values were obtained. Moreover, the statistical analysis of the obtained data reveals that, the differences within the two rates of nitrogen supplying were great enough to reach the 5 % level. These results held well in the two experiments. This superiority might attributed to that urea at high rate resulted a promotion effect on plant growth measurements as well as total bulbs yield and its physical properties, consequently, these might be reflected on the chemical properties of onion bulb.

The obtained results are in good accordance with those of Shaheen, 1999; Ali *et al*., 2001; Aisha *et al*., 2007 and Shaheen *et al*., 2007.

The foliar spraying by the mixture of sugar with amino mix compound results the highest values of N, K, Fe, Zn and Cu as well as protein content. However, P and Mn values recorded their highest values when onion plants were received amino mix compound, but the variation between this treatment and that plants which treated with the mixture of sugar + amino mix as together was not great enough to reach the significant level.

Generally, it could be concluded that, the foliar spraying by the mixture of sugar + amino mix compound resulted the rich nutritional value of N, P, K, Fe, Mn, Zn and Cu as well as protein content in bulb tissues.

The soil dressing by nitrogen fertilizer at higher rate, i.e. 120 units/fed., and the foliar application by mixture of sugar + amino mix resulted the best chemical consistent by expressed as N, P, K, Fe, Mn, Zn and Cu as well as protein. The statistical analysis of the collected data indicated that the variation within the different interaction treatments were no great to be significantly for most nutritional elements in both seasons. These findings might be attributed to that each factor of the interaction acts independently.

Similar trend was obtained by Paul *et al*., 2001; El-Shabasi *et al*., 2005; Filip Rolland *et al*., 2006; Awad *et al*., 2007 and Al-Said and Kamal, 2008. Whereas a they studies reported that, each amino acids and sugar application caused a promotion effected directly or indirectly the physiological activities in building the essential substances such as carbohydrates, protein, fats, vitamins as well as the nutritional elements (N, P, K, Fe, Mn, Zn, Cu …etc.).

Table (5): Effect of the application by sugar and amino compound under two rates of nitrogen fertilizers on chemical content of onion in newly lands during 2007/2008 season.

Treatments		%				ppm			
Urea	Compounds	Protein	N	P	K	Fe	Mn	Zn	Cu
90 Unit N. /fed.	Control	8.60	1.38	0.230	0.480	254.00	26.00	30.67	10.33
	Sugar (S)	8.85	1.42	0.310	0.540	261.33	27.00	34.00	11.33
	Amino(A)	9.31	1.49	0.400	0.610	271.67	30.00	36.00	12.00
	S.+ A.	9.65	1.54	0.347	0.640	278.00	28.67	36.67	13.00
Mean		**9.10**	**1.46**	**0.329**	**0.578**	**266.25**	**27.92**	**34.33**	**11.67**
120 Unit N. /fed.	Control	8.71	1.39	0.250	0.510	251.67	27.67	36.00	9.67
	Sugar (S)	9.04	1.45	0.363	0.717	289.00	31.00	38.33	12.00

	Amino(A)	9.60	1.54	0.463	0.733	305.00	35.67	40.33	12.67
	S.+ A.	10.23	1.64	0.437	0.770	314.67	33.33	42.33	14.67
Mean		**9.40**	**1.50**	**0.386**	**0.680**	**290.08**	**31.92**	**39.25**	**12.25**
Average	Control	8.66	1.39	0.240	0.495	252.83	26.83	33.33	10.00
	Sugar (S)	8.95	1.43	0.347	0.623	275.17	29.00	36.17	11.67
	Amino(A)	9.46	1.51	0.432	0.672	288.33	32.83	38.17	12.33
	S.+ A.	9.94	1.59	0.382	0.705	296.33	31.00	39.50	13.83
L.S.D. at 5%	Fertilizers	**0.12**	**0.02**	**0.026**	**0.006**	**2.80**	**1.08**	**1.56**	**0.36**
	Compounds	**0.19**	**0.03**	**0.014**	**0.014**	**2.61**	**0.78**	**0.91**	**0.91**
	Interactions	**N.S.**	**N.S.**	**N.S.**	**0.020**	**3.69**	**N.S.**	**N.S.**	**N.S.**

Table (6): Effect of the application by sugar and amino compound under two rates of nitrogen fertilizers on chemical content of onion in newly lands during 2008/2009 season.

Treatments		%				ppm			
Urea	Compounds	Protein	N	P	K	Fe	Mn	Zn	Cu
90 Unit N. /fed.	Control	8.50	1.36	0.250	0.470	251.33	28.33	33.67	10.67
	Sugar (S)	9.00	1.44	0.393	0.553	257.00	29.33	35.33	12.33
	Amino(A)	9.15	1.46	0.420	0.610	259.00	33.00	37.33	14.33
	S.+ A.	9.67	1.55	0.360	0.633	264.33	30.00	38.33	15.33
Mean		**9.08**	**1.45**	**0.356**	**0.567**	**257.92**	**30.17**	**36.17**	**13.17**
120 Unit N. /fed.	Control	8.96	1.43	0.287	0.483	255.67	28.33	35.33	11.67
	Sugar (S)	9.15	1.46	0.390	0.717	265.67	34.00	40.00	14.67
	Amino(A)	10.06	1.61	0.467	0.743	275.67	37.67	42.33	16.00
	S.+ A.	10.29	1.65	0.443	0.790	298.33	34.67	44.67	16.67
Mean		**9.61**	**1.54**	**0.397**	**0.683**	**273.83**	**33.67**	**40.58**	**14.75**
Average	Control	8.73	1.40	0.268	0.477	253.50	28.33	34.50	11.17
	Sugar (S)	9.07	1.45	0.392	0.635	261.33	31.67	37.67	13.50
	Amino(A)	9.60	1.54	0.443	0.677	267.33	35.33	39.83	15.17
	S.+ A.	9.98	1.60	0.402	0.712	281.33	32.33	41.50	16.00
L.S.D. at 5%	Fertilizers	**0.04**	**0.01**	**0.034**	**0.018**	**2.59**	**0.62**	**0.36**	**1.56**
	Compounds	**0.12**	**0.02**	**0.039**	**0.019**	**1.82**	**1.35**	**0.92**	**0.90**
	Interactions	**0.18**	**0.03**	**N.S.**	**0.0**	**2.57**	**N.S.**	**N.S.**	**N.S.**

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Hypercholesterolemia enhances the release of proinflammatory cytokines in obese Egyptian adolescents

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Abstract: The interrelation between hypercholesterolemia, proinflammatory cytokines, C-reactive protein (CRP) and body mass index (BMI) were not yet established in obese Egyptian adolescents .The aim of this work is to study the relation between hypercholesterolemia; as a major determinant of serum inflammatory cardiovascular risk marker; TNF- α and IL-1 β as proinflammatory cytokines; and CRP with body mass index (BMI); as indicator for obesity and waist circumference; as indicator for central obesity . The study was carried out on obese Egyptian adolescents with high lipid profile levels (Group I) and obese one with lipid profile within normal levels (Group II) from both sexes. TNF –α, IL- 1β and CRP were significantly higher in Group I than Group II ($p<0.001$). There were positive highly significant correlations between cholesterol, and each of BMI, IL1-β, TNF-α and CRP ($p<0. 001$) with a negative significant correlation between it and HDL ($p<0.029$). A significant negative correlation between HDL and both LDL ($p<0.001$), and TNF-α ($p<0.01$) was recorded. LDL have shown a significantly positive correlation with TNF-α, IL-1B and CRP ($p<0.001$). There were significant positive correlations between waist circumference (central obesity) with TNF-α, IL-1β and CRP ($p<0.001$). Conclusion: The positive association of obesity with elevated cytokines levels suggests the importance of reducing obesity to prevent elevation in cytokines levels which are risk factors for future cardiovascular diseases. [Journal of American Science 2010;6(8):428-435]. (ISSN: 1545-1003).

Keywords: hypercholesterolemia, obesity, CRP, Proinflammatory cytokines, cardiovascular risk factor, adolescents.

1. Introduction

Obesity is a pathological condition accompanied by an excessive fat deposition as compared to expected values for a given stature, sex and age which is often estimated by a body mass index (BMI) (kg⁄m2) , BMI percentiles equal to or more than 95% considered obese . The best way to estimate obesity in clinical practice is to measure waist circumference (central obesity) this is because an excess of abdominal fat is most tightly associated with the metabolic risk factor, (National institute of health., 1998). According to the obesity and health risk, 2000**,** obesity has been related to hypercholesterolemia, hypertension and cardiovascular disease. Three of the most proinflammatory cytokines, Tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) and C-reactive protein (CRP), have been implicated in atherogenesis (Cesari et al., 2003).

The immunological process involved in the collaborative defense of organisms are affected by nutritional state (Marti et al., 2001), thus a positive chronic imbalance between energy intake and expenditure leads to situations of obesity (Ulizaszek, 2008). Coppack (2001), stated that the importance of the immune system in whole body energy balance and in providing a rationale for the links between cytokines and adipose tissue which could be a major contributor to the biochemical and clinical features of the metabolic syndrome and central obesity. Fain (2006), reported that the white adipose tissue especially of humans is recognized as the central player in the proinflammatory state .As the adipose tissue was primarily a reservoir for excess calories that were stored in the adipocytes the expansion of adipose tissue seen in obesity results in more blood vessels, more connective tissue fibroblasts, and especially more macrophages. These proliferate and amplify the inflammatory response through the secretion of numerous growth factors and cytokines including tumor necrosis factor alpha (TNF-α) and interleukin (IL-1β) (Swirski et al.,2007, and Tacke et al., 2007).

IL-1β is a multipotent proinflammatory cytokines that affects most cell type and cooperates with other cytokines chemokines and a variety of cellular mediators.IL-1β is produced by a variety of inflammatory cells that release a cascade of inflammatory signals (Dinarello, 2005). Eizirik and his colleagues, 2001; stated that IL1-β is proatherogenic and appears to have procoagulant activity, stimulates monocytes-endothelial cell adhesion and cholesterol esterification in

macrophages. Blake and Ridker (2003), showed that CRP is an important marker of vascular inflammation and a predictor of atherosclerosis . A recent study of Ridker (2009) revealed also that an increase in the concentration of CRP indicate a greater risk for acute coronary syndrome .This finding showed that evaluation of CRP carry predictive power for the development of major cardiovascular events, led to the concept that advanced and unstable atherosclerotic plaques are in an even higher state of inflammation than stable plaque.

Tzoulaki and others, 2008, demonstrated that obese persons have elevated levels of CRP, which is the most sensitive circulating marker for cytokines in obese subjects. This finding let, Harris and his colleagues, 2008, to suggest that obesity is a proinflammatory state and is somehow connected with the development of unstable atherosclerotic plaques . Pai (2008) stated that CRP as an acute phase reactant and marker of inflammation has been shown to predict risk of incident cardiovascular events.

TNF- α is a multifunctional circulating proinflammatory cytokine derived from endothelial and smooth muscle cells, as well as macrophages .TNF-α plays a major role in the cytokine cascade as it stimulates the synthesis of other cytokines like interleukin 6 (IL-6) which is a central mediator of the acute phase response and the primary determinant of C-reactive protein (CRP) production (Heinrich,Castell and Andus.,1990;Van Snick .,1990). Thereby, it contribute to the maintenance of chronic low grade –inflammation state involved in the progression of obesity and its associated co-morbidities (Harris et al., 2008).

The aim of this work is to study the relation between hypercholesterolemia; as a major determinant of serum inflammatory cardiovascular risk marker; TNF- α and IL-1 β as proinflammatory cytokines; and CRP with body mass index (BMI); as indicator for obesity and waist circumference; as indicator for central obesity.

2. Material and Methods

This study was conducted by the National Research Centre, Egypt, to estimate the prevalence of obesity and metabolic syndrome among school children and adolescents, and the potential risk factors for these diseases**.** It was a cross-sectional survey. Four local public schools situated in Giza governorate were enrolled in this study regarding adolescents (two secondary schools and two high schools). The study included boys and girls during the period of October, 2007 to April 2009. Permission to perform the study was granted by the Ministry of Education, and the directors of the school included in the research. The protocol was approved by the "Ethical Committee" of the "National Research Centre".

Of the total sample, one hundred and three adolescents (32 boys and 81 girls) with the complaint of obesity were included in the current research after obtaining written informed consent from their parents. Student assent was also obtained.

These adolescents were required to meet the following inclusion criteria: age, 13–18 years and BMI, greater than the 95th percentile for age and gender based on the Egyptian Growth Reference Charts 2002 (2008). Adolescents were excluded if they had a prior major illness, including type 1 or 2 diabetes, took medications or had a condition known to influence body composition, insulin action or insulin secretion (e.g. glucocorticoid therapy, hypothyroidism and Cushing's disease).

Method:

Each adolescent underwent a complete physical examination, including anthropometric measures. The height and the weight were measured. The height was measured to the nearest 0.5 cm on a Holtain portable anthropometer, and the weight was determined to the nearest 0.1 kg on a Seca scale Balance with the subject dressed minimum clothes and no shoes. Body mass index (BMI) was calculated as weight (in kilograms) divided by height (in meters) squared. Waist circumference was measured at the level of the umbilicus and the superior iliac crests at the end of normal expiration with patient standing and breathing normally using non-stretchable plastic tape to the nearest 0.1 cm. Each measurement was taken as the mean of three consecutive readings following the recommendations of the International Biological program (Hiernaux and Tanner 1969). After a verified 10- hour fast, subject had a blood draw for laboratory assays. According to their lipid profile results the participating adolescents were divided into two groups; Group I: with high triglycerides [>110 mg/dL], low HDL-cholesterol [<40 mg/dL], high total cholesterol [>210 mg/dL] or High LDL–Cholesterol [> 130 mg/dL] (defined according to modified WHO criteria adapted for children); Group II: with normal lipid profile.

Biochemical assays:

Serum CRP levels were determined with an enzyme- linked immunosorbant assay (Eliza) method using commercial kits (BioCheck, Inc 323 Vintage Park Drive Foster City, CA 94404) and the sensitivity of detection level was 0.1 mg\l. Serum concentrations of cytokines , IL-1 β and TNF- α were measured using commercially available Elisa kits (Ani Biotech

Oy Orgenium Laboratories Business Unit Finland; Bender MedSystems GmbH Campus Vienna Biocenter and the sensitivity of detection for IL-1β and TNF-α was <4 pg⁄ml and 2.3 pg⁄ml respectively. Triglycerides, Total cholesterol, HDL, were measured using Quantitative- Enzymatic – Colorimetric Determination in serum (STANBIO laboratory.1261 North Main Street. Boerne Texas 78006 U.S.A

LDL was calculated as follow: LDL=Total Cholesterol-HDL-TG/5.

Statistical analysis

All values are expressed as mean±SD. Differences among the two groups were calculated by student´s t test, correlation were done between different parameters using pearson correlation. A p value ‹ 0.05 was considered significant. All analyses were carried out using SPSS 9.0 statistical software.

3. Results

Serum level of Cholesterol, low density lipoprotein(LDL) and triglycerides (TG) were used to classify obese adolescent into two groups .The levels of serum cholesterol, LDL and TG were very high significantly increased (P<0.001) in obese subjects group I, as compared to group II while the level of HDL was significantly decreased (P<0.01) as shown in table(1).

The level of proinflamatory cytokines; TNF-α and IL-1β, and CRP were all significantly higher in the obese Egyptian adolescents group I in comparison with the obese adolescents group II with p<0.001, table (2) fig (2).

There were significant positive correlations between each of BMI and waist circumference with TNF-α , IL-1β, and CRP (p<0.001) as shown in table(3).

Table (1) Comparison of lipid profile between Group I and Group II

Lipid profile	**Group I (N=35) Mean ±SD**	**Group** II **(N=78) Mean ±SD**
Triglycerides mg\dl	161.27±44.45	131.79±37.84***
Cholesterol mg\dl	255.65±35.86	150.10±34.56***
HDL mg\dl	40.19±19.35	49.23±32.04**
LDL mg\dl	178.3±54.7	81.64±34.7***

p<0.01= highly significant difference;*p<0.001= very highly significant difference

Table (2) Comparison of proinflammatory cytokines between Group I and Group II

Proinflammatory cytokines	**Group I (N=35) Mean ±SD**	**Group** II **(N=78) Mean ±SD**
TNF- α pg/ml	15.11±9.95	0.72±3.83***
IL-1 β pg/ml	51.24±38.77	10.68±17.7***
CRPμg/dl	8.49±3.49	1.68±0.90***

***p<0.001= very highly significant difference

Table (3): Correlation between anthropometric parameters, and TNF-α,IL-1B ,CRP of obese group I

Proinflammatory cytokines⁄ Anthropometric measurements	TNF-α pg⁄ml	IL-1β pg⁄ml	CRP μg/dl
Waist circumference	r=0.773**	r=0.901**	r=0.822**
Body mass index (BMI)	r=0.925**	r=0.901**	r=0.901**

**p<0.01= highly significant difference

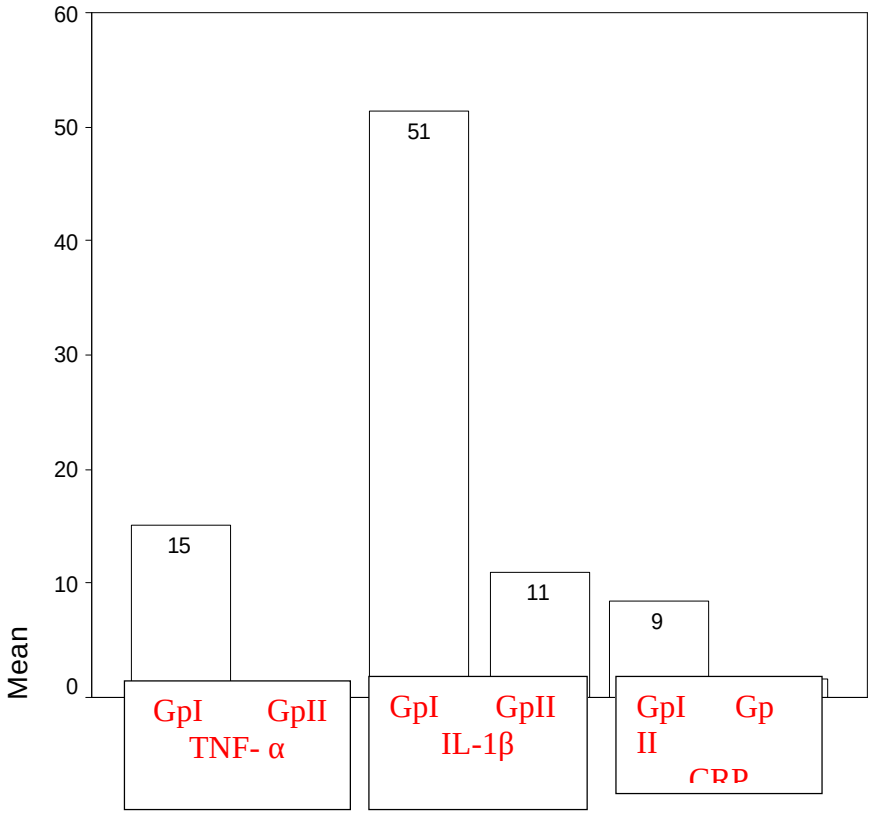

Fig :(2) Histogram of proinflammatory cytokines showing the comparison between Group I and Group II

The results of the current work also, illustrated the following: There was a highly significant positive correlation between each of BMI and waist circumference, with cholesterol, LDL ($p<0.001$), but such correlation was not found with triglycerides and no correlation between HDL and waist circumference. Moreover, BMI has recorded negative correlation with HDL ($p<0.04$). There was very highly significant positive correlation between cholesterol and TNF-α, IL-1β and CRP ($p<0.001$), a negative correlation between cholesterol and HDL at ($p<0.029$) have also been shown with no significant correlation between cholesterol and triglycerides. Negative significant correlations were found between HDL and both LDL, and TNF-α ($p<0.001$, $p<0.01$; respectively), with no significant correlation between HDL and either IL-1β or CRP was observed. LDL has shown a very highly significant positive correlation with TNF-α, IL-1B and CRP at ($p<0.001$) table (4).

Table (4): Correlations Between The Different Variables

	waist	BMI	Triglyceridesmg\dl	Cholesterol mg\dl	HDLmg\dl	LDLmg\dl	TNF-α pg\ml	IL-1β pg\ml	CRP
Waist		r=0.796** a***	r=0.031 a NS	r=0.826 a***	r=-0.091 a NS	r=0.602** a***	r=0.773** a***	r=0.901** a***	r=0.822** a***
BMI			r=0.037 a NS	r=0.935** a***	r=-0.221* a=0.041	r=0.731** a***	r=0.925** a***	r=0.901** a***	r=0.901** a***
Triglyceridesmg\dl				r=0.080 a NS	r=0.010 a NS	r=-0.123 a NS	r=0.101 a NS	r=0.025 a NS	r=0.104 a*

Cholesterol mg\dl	r=0.826 a***	r=0.935** a***			r=-0.236* a*	r=0.763** a***	r=0.979** a***	r=0.912 a***	r=0.982** a***
HDL mg\dl	r=-0.091 a NS	r=-0.221* a=0.041	r=0.010 a NS			r=-0.708** a***	r=-0.271* a**	r=-0.141 a NS	r=-0.160 a NS
LDL mg\dl	r=0.602** a***	r=0.731** a***	r=-0.123 a NS	r=0.763** a***			r=0.770** a***	r=0.681** a***	r=0.688** a***
TNF-α pg\ml	r=0.773** a***	r=0.925** a***	r=0.101 a NS	r=0.979** a***	r=-0.271* a=0.011			r=0.862** a***	r=0.969** a***
IL-1 β pg\ml	r=0.901** a***	r=0.901** a***	r=0.025 a NS	r=0.912 a***	r=-0.141 a NS	r=0.681** a***			r=0.876** a***
CRP	r=0.822** a***	r=0.901** a***	r=0.104 a NS	r=0.982** a***	r=-0.160 a NS	r=0.688** a***	r=0.969** a***		

a** Correlation is significant at 0.01 level (2 tailed),a*Correlation is significant at 0.05 level (2 tailed),aNS=non significant

4. Discussions

Because the prevalence of obesity has increased dramatically in recent years, one of the key targets of public health is obesity and its associated pathological conditions. Obesity occurs as a result of white adipose tissue enlargement, caused by adipocyte hyperplasia and/or hypertrophy. Recently, endocrine aspects of adipose tissue have become an active research area and these adipose tissue-derived factors are referred to as adipokines. These adipokines interact with a range of processes in many different organ systems and influence a various systemic phenomena (Inadera, 2008)Obesity leads to increased circulating levels of proinflammatory cytokines that are secreted by adipocytes thus increasing cardiovascular risk (Ikeoka et al., 2010).

In this study we have shown that in Group I there is a strong interrelation between hypercholesterolemia and the increased levels in proinflammatory cytokine TNF-α , IL-1β and CRP which are risk factors of cardiovascular disease.High cholesterol levels are frequently associated with increased soluble markers of systemic inflammation(Ferroni et al., 2003) such as CRP (Ridker et al., 2009) and IL-1β (Ferroni et al., 2003), which support our results. Gomes and his colleagues (2009) reported that hypercholesterolemia is associated with peripheral blood (PB) leucocytes and increased platelet levels , generally attributed to cholesterol – induced circulating proinflammatory cytokines and increased soluble markers of systemic inflammation such as IL-1 β (Ferroni et al.,2003). Studies also done by Domenico and his team (2000) suggested that cholesterol biosynthesis is associated with proinflammatory cytokines formation and that adipose tissue is acknowledged to be a source of cytokines such as TNF-α (Hostamisliglil et al.,1993.) The current work has shown that CRP concentration was higher in Group I than Group II .In obese Egyptian adolescent with high lipid profile obesity measured by BMI was significantly correlated with CRP concentration which was previously reported by (Park and co- workers 2005) and coincide with the studies of (Rexrode et al., 2003 and Tzoulaki et al., 2008) who found that BMI was the adiposity parameter that is strongly correlated with CRP concentration. Such acute phase reactant (CRP), suggest that obesity may be a state of low grade inflammation (Ikeoka et al., 2010) that indicates a greater risk for acute coronary syndrome (Ridker et al., 2009). These results may be related to the finding that adipose tissue is a dynamic endocrine organ that secretes a number of factors that contributes to systemic inflammation (Lyon et al., 2003).

The significant correlation between BMI and proinflammatory cytokines can be interpreted by the suggestion of Cousin et al., 1999, that preadipocytes could function as macrophages like cells supporting the idea of a direct involvement of adipose tissue in inflammatory process and that inflammatory cytokines as TNF –α seem to be produced by the infiltrating macrophage in the adipose tissue (Weisberg et al., 2003). These events may perpetuate a vicious cycle of macrophage recruitment and production of proinflammatory cytokines (Janeway et al., 2005).

In addition the study of Fain (2006) revealed that obesity markedly elevates the total release of TNF- α which was explained by Landry et al., (1997) as TNF-α activates the transcription factor nuclear factor kβ which organize inflammatory changes in vascular tissue . The increased TNF- α mRNA expression in adipose tissue from obese humans (Hostamisliglil., 1993)causing increase in circulating concentration of TNF-α in such people (Hostamisliglil et al.,1995). In our study the increase in TNF- α and IL-1β in Group I in comparison with Group II can be explained by the fact that adipocytes

can synthesize both TNF-α and several interleukins notably IL-1β (Fain ., 2006 ; Swirski et al., 2007,Tacke et al., 2007).

IL-1β is one of the major proinflammatory cytokines that is produced by monocytes and macrophages (Martin and Wesche., 2002). Expression of both IL-1β and its receptor is increased in visceral adipose tissue of obese subjects (Juge –Aubry et al., 2004) an observation that we confirmed in this study as we found a positive correlation between central obesity and IL-1β .

Previous studies of (Festa et al., 2001 , Pannecciulli et al., 2001) have shown a positive association between CRP and obesity.The strong correlation between serum levels of CRP and TNF-α in our study can be understood by the explanation that the increase in the concentration of TNF-α induce IL-6 secretion which promotes the production of CRP which is raised in obese individuals with high lipid profile (Petersen and Pedersen ., 2005) this fact may explain the positive association between CRP and obesity .

In this study there was also a strong correlation between CRP and lipid profile and this was in agreement with (Tamakoski et al., 2003) who found that hypertriglyceridemia, hypo- HDL cholesterolemia, hyper LDL cholesterolemia were significantly associated with elevated CRP and there is a strong association of CRP with obesity. The addition of CRP with traditional cholesterol enhances cardiovascular risk prediction independently of LDL concentration (Ridker et al .,2009) suggesting that increased CRP concentration in particular may identify asymptomatic individuals with average cholesterol concentrations at high risk for future cardiovascular events.

To sum up we agreed with the concept that obesity leads to increased circulating levels of proinflammatory cytokines that are secreted by adipocytes thus increasing cardiovascular risk (Ikeoka et al., 2010). Moreover, circulating levels of adipokines can be used as high-throughput biomarkers to assess the obesity-related health problems, including low grade inflammation.

In conclusion: Obesity is recognized as a worldwide public health problem that contributes to a wide range of disease conditions. The development of a method for convenient prediction of obesity-related health problems represents a major challenge for public policy makers facing the epidemic of obesity. We believe that Markers of inflammation have been proposed for use in clinical practice to aid in the identification of asymptomatic patients at high risk for CVD. Thus, measurement of inflammatory markers could be used to assess the risk of developing CVD. Elucidation of the significance of circulating cytokines may provide a therapeutic target for adipokine-based pharmacological and/or interventional therapies in obesity and related complications.

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Attenuation of some Metabolic Deterioration Induced by Diabetes Mellitus using *Nepeta cataria* Extracts.

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Abstract: The present research is design to evaluate the pharmacological effects of successive as well as 70% ethanolic extracts of *Nepeta cataria* on some biochemical parameters in Streptozotocin diabetic rats compared to the currently used Glicalized drug. The investigated parameters included, glucose, insulin, carbohydrate hydrolyzing enzymes; α –amylase, α- glucosidase, β- galactosidase, liver steatosis markers ; total cholesterol, HDL-cholesterol, LDL–cholesterol, triglycerides, total lipid, liver function enzymes; alanin aminotransferase(ALT), aspatrate aminotransferase (AST), alkaline phosphatase (ALP) and total protein; antioxidant activity of extracts using nitric oxide (NO) .In addition , histopathological investigations were performed. The results obtained revealed ,anti-glycemic, antioxidant, antilipidemic effects of chloroform, petroleum ether as well as 70% ethanolic extracts in comparison with Gliclazide as reference antidiabetic drug . Moreover, these extracts have principle role in treatment and normalized liver and pancreas architecture. Hence, it could be concluded that *Nepeta cataria* extracts may be applied clinically for reducing complications against diabetes mellitus paralleling with the ideal anti- diabetic Glicalized drug.

Key words: Diabetes mellitus, *Nepeta cataria*, oxidative stress, liver function enzymes , carbohydrate hydrolyzing enzymes, lipid profile.

1. Introduction:

Diabetes mellitus (DM) is a serious health problem being the third greatest cause of death all over the world, and if not treated, it is responsible for many complications affecting various organs in the body. Diabetes mellitus is a disease results from abnormality of carbohydrate metabolism and characterized by absolute (type I) or relative (type II) deficiencies in insulin secretion or receptor insensitivity to endogenous insulin, resulting in hyperglycemia . Hyperglycemia that is initiating from unregulated glucose level is widely recognized as the causal link between diabetes and diabetic complications. It was found that , hyperglycemia cause tissue damage by mechanisms involving repeated changes in cellular metabolism. One of the key metabolic pathways as being major contributors to hyperglycemia induced cell damage, is the non enzymatic reaction between excess glucose and several proteins (as hemoglobin and albumin) to form advanced glycosylated end product (AGE). Production of AGE interferes with cell integrity by modifying protein function or by inducing receptors mediated production of reactive oxygen species (ROS) (Thornalley , 2002).

Hyperglycemia-evoked oxidative stress plays a crucial role in the development of diabetic complications, including nephropathy, neuropathy, retinopathy and hepatopathy, which are considered to result from both augmented reactive oxygen species generation and decreased antioxidant defenses (Tepe *et al*., 2007).

Hypoglycemic plants are still prevalent in developing countries, where they have been used to treat diabetes for many centuries .More than 1200 species of plants have been used empirically for their alleged hypoglycemic activity .This fact is attributed to the high cost and the lack of availability of current therapies for the majority of patients in developing countries. Nevertheless, many medicinal plants claimed effective by folk medicine require scientific investigation to ascertain their effectiveness, toxicity and then provide alternative drugs and therapeutic strategies (Marles ; Farnsworth, 1994).

Nepata cataria L.(family , Limiaceae; order Lamiales) , comprises about 400 species, most of which found in the Eastern Mediterranean, Southern Asia and China, is commonly known as Catnip or Catmint because of its irresistible action on cats. Due to lemony mint flavor it finds the ways in the herbal teas as well as in cooking. Medicinally, the plants are used in gastrointestinal and respiratory hyperactive disorders such as, colic, diarrhea, cough, asthma and bronchosis (Miceli *et al.*, 2005). A limited number of studies exists on its biological activities include antibacterial (Kalpoutzakis *et al.*, 2001), antifungal (Nostro *et al*., 2001) and analgesic (Aydin *et al*., 1998). Various compounds have been identified by different groups of workers in the essential oil of *Nepeta cataria.* The main constituents so far identified, include β-caryophyllene, caryophyllene oxide, 1,8 –cineol, citronellol , geraniol , elemol , nerol (Mortuza –Semmani and Saeedi ,2004 ; Schultz *et al.*, 2004 ; Sajjadi , 2005).Also, urosolic acid, β–sisoterol, campesterol, α -amyrin, β-amyrin , and sitosterol β-glucopyranoside have been reported previously (Miceli *et al* ., 2005). In addition the plant also contained neptalactones and alkaloids, such as actinidine and iridomyecine (Kalpoutzakis *et al*., 2001).

So, the present study is design to demonstrate the hypoglycemic efficiency of petroleum ether, chloroform as well as 70% ethyl alcohol extracts of *Nepeta cataria* [compared with antidiabetic Gliclazide (diamicron) reference drug] in Streptozotocin induced diabetes mellitus in rats through measuring glucose, insulin, carbohydrate hydrolyzing enzymes, nitric oxide, liver function enzymes , total protein and lipid profile . Moreover histological examination of liver and pancreas was performed.

2. Materials and Methods:

Chemicals:

All chemicals in the present study were of analytical grade, product of Sigma, Merck and Aldrich. All kits were the products of Biosystems (Spain), Sigma Chemical Company (USA), Biodiagnostic (Egypt).

Plant materials:

Seeds of catnip (*Nepeta cataria* L.) was obtained from company of Jelitto staudensamen, Schwarmstedt, Germany). The seeds of the plant were cultivated in the experimental farm of the Cultivation and Production of Aromatic Plants Department of the National Research Center, Giza, during two successive seasons of 2006 and 2007.

The seeds of catnip were sown in nursery on 15th of October in the two seasons. Two months later after sowing, the seedlings were transplanted in 8 cm pots in medium of 1:1:1(by volume) loam, sand and peat moss. The seedlings were planted in the field on 15th of March in hills 25 cm apart on rows 60 cm in-between. The flowering aerial parts of *Nepeta cataria* were collected from the plants during two successive seasons of 2006 and 2007 and raised from seeds obtained from company of (Jelitto staudensamen , Schwarmstedt, Germany) .

Preparation of extracts and fractions:

The powdered air-dried aerial parts of *Nepeta cataria* (720 g) were extracted with petroleum ether (60-80 %) and chloroform in succession, to afford 35 (4.8%) and 28 g (3.88%) respectively. In addition, 400g of the same dried parts were extracted with 70% ethanol to yield 26 g (6.5%) of total ethanolic extract.

Determination of hypoglycemic activity of *Nepeta cataria extracts:*

Animals:

Male Wister albino rats (120-150g.) were obtained from animal house of National Research Centre, Dokki, Giza, Egypt. Rats were fed on a standard diet and free access to tap water. They were kept for one week to be acclimatized to the environmental conditions.

Doses:

All plant extracts were orally administrated with a dose of 50 mg/kg body weight for 30 consecutive days according to Miceli *et al.* (2005) ; Rabbani *et al*. (2007).

Experimental design:

98 male albino rats were selected for this study and divided to fourteen groups (seven rats in each group) as follows:

Group 1**:** normal healthy control rats.

Groups 2-4: normal healthy rats orally administrated different *Nepeta cataria* extracts (50 mg/kg body weight daily for 30 days, each rat received 7.5 mg /0.5 ml bidistilled water).

Groups 5-7: considered as diabetic groups; where type 1 diabetes was induced by Streptozotocin, each rat was injected intraperitoneally with a single dose of Streptozotocin (65mg/Kg body weight dissolved in 0.01M citrate buffer immediately before use, each rat received 9.75 mg /0.5ml citrate buffer) (Vats *et al*.., 2004). After injection, animals had free access to food and water and were given 5% glucose solution to drink overnight to counter hypoglycemic shock (Bhandari *et al*., 2005). After 2 days of STZ injection fasting blood samples were obtained and fasting blood sugar was determined (>300 mg/dl). Hyperglycemic rats were used for the experiment and classified as follows:

Group5: Diabetic +ve control group sacrificed after 2 days of STZ injection

Group 6: Diabetic +ve control group sacrificed after 10 days of STZ injection

Group 7: Diabetic +ve control group sacrificed after 40 days of STZ injection and considered as recovery group.

Group 8: Diabetic animals treated with petroleum ether extract 50mg/kg body weight for30 days, each rat received 7.5 mg /0.5 ml bidistilled water)

Group 9: Diabetic animals treated with chloroform extract (as previously group).

Group 10: Diabetic animals treated with 70% ethanol extract (as previously group).

Group11: Diabetic animals treated with Glicalized (diamicron) 10mg /kg body weight (each rat received 1.5 mg/0.5 bidstilled water) and considered as reference drug.

Group 12-14: healthy control groups sacrificed after 2, 10 and 40 days and considered as normal control groups for diabetic groups at the same times.

Sample preparations:

Serum sample: each animal was weighed, blood collected by puncture the sub-tongual vein in clean and dry test tube, left 10 minutes to clot and centrifuged at 3000 rpm for serum separation. The separated serum was stored at -80ºC for further determinations of lipid profile, liver function tests, carbohydrate metabolizing enzymes and serum total protein.

Tissue sample: liver tissue was weighed and homogenized in ice cold 0.9 N NaCl, centrifuged at 3000 rpm for 10 min, separated the supernatant and stored at -80ºC for further estimation. For determination of NO in liver tissue ,the extraction method was carried out using 1g tissue and homogenized in 10 ml 10% trichloroacetic acid (to give 10% homogenate 1%w/v).Then the sample was centrifuged at 3,000 rpm for 10 min and the supernatant was separated.

Blood biochemical analysis:

i-Determination of blood glucose:-

Glucose was determined in serum by colorimetric assay according to Trinder (1969).

ii- Human insulin enzyme immunoassay :

Insulin was determined by quantitative test kit according to the method of Sacks (1994) .

iii- Determination of total cholesterol and cholesterol - HDL in rat serum:

Total cholesterol and HDL-cholesterol were determined by the method of Stein (1986).

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v- Determination of cholesterol- LDL:

Cholesterol- LDL was calculated according to Friedewald *et al*. (1972).

vi- Determination of triglyceride:

Triglyceride was measured in rat serum by the method of Wahelfed (1974).

vii- Determination of total lipid:

Total lipid was measured in rat serum by the method of Zollner and Kirsch (1962) .

viii- Determination of alkaline phosphatase enzyme activity :

Alakaline phosphatase enzyme activity was measured in rat serum by the method of Belfield and Goldberg (1971).

x- Determination of aspartate and alanine aminotransferases (AST and ALT) enzyme activities:

AST and ALT were measured in rat serum by the method of Reitman and Frankel (1957).

xi- Determination of total protein :

Total protein was assayed in rat serum according to the method of Bradford (1976).

xii- Determination of nitric oxide (NO): NO was measured in liver tissue homogentes according to Moshage *et al.* (1995).

xiii- Determination of carbohydrate hydrolyzing enzymes :

- α- Amylase enzyme activity was performed in liver tissue homogenates according to the method of Caraway (1959).
- Glucosidase and β-galactosidase enzyme activities were performed in liver tissue homogenates by the method of Sanchez and Hardisson (1979).

Histopathology :

Liver and pancreas specimens were fixed in 10% formalin, processed to paraffin blocks, sectioned (4 µm thick) and stained with Hematoxyline and Eosin .They were examined using light microscope (Gomori , 1941).

Statistical analysis :

Data were analyzed by comparing values for different treatment groups with the values for individual controls. Results are expressed as mean ± S.D. The significant differences among values were analyzed using analysis of variance (one-way Anova)

coupled with post-Hoc, least significance difference (LSD). Anova at $p \leq 0.05$ using Co-stat computer program.

3. Results:

The present results demonstrate the biochemical effects, mechanism(s) of the hypoglycemic actions of *Nepeta cataria* extracts and their possible hepatoprotective roles against liver disorders induced by reactive oxygen species associated with diabetic complications in diabetic rats. The investigated parameters included blood glucose, insulin ,total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides, total lipid, ALT, AST, ALP enzyme activities and total protein content as well as hepatic nitric oxide , α - amaylase, α- glucosidase and β- galactosidase enzyme activities .

Blood glucose and insulin levels in serum of control, normal - treated, diabetic and diabetic – treated groups are demonstrated in Table (1) and Fig (1). It is obvious that, there is no significant change between control and different normal –treated groups either in blood glucose or in insulin levels except normal – treated with 70% ethanolic extract which exhibits significant reduction in blood glucose levels amounted 94.50±16.05 mg/dl at P≤ 0.05 with percentage of reduction -13.69%. Concerning diabetic groups, significant increase is noticed in blood glucose levels which is concomitant with significant reduction in insulin levels at day 2, 10 and 40 post STZ injection recorded 373.00±2.94, 363.00±2.94 and 364.25±3.77 mg/dL with percentage increase +240.64, +231.51 and +232.62% for glucose respectively and 0.59±0.01, 0.34±0.05 and 0.35±0.01 µIU/ml with percentage of reduction reached to -88.97, -93.64 and -93.45% for insulin respectively. Significant amelioration is noticed in blood glucose and insulin levels in all diabetic – treated groups recorded the most pronounced effect for 70% ethanolic extract (-5.94% for glucose and -14.21%.for insulin) followed by petroleum ether extract (+17.12 and -22.8%, respectively) then Glicalized as a reference drug (+20.55and -27.66% respectively) and finally chloroform extract (+21.46 and -28.41%, respectively).

Lipid profile, total cholesterol, HDL- cholesterol, LDL-cholesterol, triglycerides and total lipid in control, normal - treated, diabetic and diabetic – treated groups is shown in Table(2) and Fig (2). It can be easily noticed that, there is no significant change in total cholesterol, HDL- cholesterol , LDL- cholesterol and total lipid between different normal – treated groups as compared to untreated control one. While triglycerides show significant reduction in 70% ethanolic extract treated – normal group reached to 93.32±22.07 mg/dl with percentage of reduction amounted -15.84% as compared to untreated control group. With regard to diabetic groups, significant elevation in lipid profile is observed 2 days post STZ injection as compared to control group , recorded 202.50±4.5, 81.62 ±3.35, 163.22±4.57, 211.75 ±8.22 and 1687.19 ±202.10 mg/dl with percent of elevation +54.17, +21.75 , +94.75, +90.97 and +72.05% for total cholesterol, HDL-cholesterol, LDL–cholesterol, triglycerides and total lipid, respectively. On the other hand, nearly simultaneously elevated levels in lipid profile is recorded 10 and 40 days post STZ injection with percentage of increase +82.66 , +35.37 ,+151.46 ,+177.57 and +84.12% at 10 day and+82.54,+35.37, +151.46, +178.45 and +84.45% at 40 day respectively. All diabetic – treated groups (G8- G11) show significant enhancement in lipid profile level as compared to the normal control group, since in petroleum ether extract –treated diabetic group, total cholesterol recorded 145.99± 10.97mg/dl with percentage increase amounted +11.15%.While in both 70% ethanolic and chloroform extracts , total cholesterol reached to 150.81±6.46 and 158.15±8.20 mg /dl, respectively with percent +14.82 and +20.41%, respectively. With respect to Gliclazide as reference drug, total cholesterol amounted 72.75±0.96 mg/dl with percent of reduction - 44.61% as compared to control group. Concerning HDL –cholesterol nearly similar results is obtained for different extracts-treated diabetic groups and Gliclazide as reference drug, where insignificant change is observed either compared to each other or to control group. In addition, significant improvement is noticed in LDL- cholesterol level post treatment of diabetic rats with different extracts where , insignificant change is observed as compared to each other and significant increase as compared to normal control group amounted 107.89±14.17, 118.02±11.50 and 111.25±2.15 mg /dl with percentage of elevation + 28.73, +40.82 and +32.74% for petroleum ether, chloroform and 70% ethanol extracts, respectively. Controversy Gliclazide -treated diabetic group exhibits insignificant change as compared to normal control and significant decrease as compared to other extracts. Remarkable, significant enhancement is noticed in triglycerides level post different types of treatments, where insignificant change is recorded as compared to normal control except for petroleum ether extract-treated diabetic rats, significant increase is noticed amounted 125.74± 10.83 mg/dl (+13.40%) as compared to normal control group. Total lipid reveals an enhanced significant mean value of 1373.17±41.65, 1414.58±195.92 , 1325±99.71 and 1237.50±33.04 for petroleum ether , chloroform , 70% ethanol extracts and Gliclazide drug as compared to normal control(980.62±30.71) with percent +40.03, +44.25.,+35.12 and +26.20%, respectively.

Table (3) and Fig (3) demonstrate the level of liver function enzyme activities AST, ALT, ALP and total protein in serum of control, normal- treated, diabetic and diabetic- treated groups. It is obvious that insignificant change is recorded in AST level in serum of normal control treated either with chloroform or 70% ethanolic extracts. On the contrary significant inhibition is noticed in AST activity in normal rats treated with petroleum ether extract amounted 1.97±0.25 u mole /mg protein /min (-12.83 %.). With respect to ALT and total protein, insignificant change is observed in their levels in different normal - treated groups as compared to untreated control one. Concerning ALP, significant inhibition is noticed in all normal –treated groups recorded 2.94±0.40, 2.74±0.25 and 2.78±0.26 umole/mg protein/min with percentage decrease amounted -17.99 ,-13.27 and -19.17% for chloroform, 70% ethanol and petroleum ether extracts respectively as compared to the normal control group. With regard to diabetic condition, significant increase in all enzyme activities is noticed at day 2,10 and 40 post STZ injection reached to 2.73±0.24, 3.23±0.23, 3.37±0.11 µmole / mg protein/min for AST with percentage increase +20.80,+42.92 and +49.12% respectively. While , ALT recorded 1.97±0.09, 2.44 ±0.17 and 2.48±0.08 µmole/ mg protein /min with percentage increase +23.13,+52.50 and +55.00% respectively. ALP shows a value of 4.51± 0.36, 5.55±0.60 and 5.61±0.13 µmole/mg protein/min with percentage of elevation +33.04, +63.72 and +65.49% respectively. Total protein content shows insignificant change at day 2 and 10 post STZ injection, while marked significant reduction at day 40 amounted 84.3 ±1.74 mg / ml (-22.12%). The curative effect of petroleum ether, chloroform, 70% ethanol extracts and Gliclazide drug on diabetic rats can easily be noticed through the normalization of all enzymes tested returned more or less to the level of normal control, where an insignificant change is observed. While total protein content still recorded significant reduction post petroleum ether, chloroform and total ethanol extracts treatments (although, it shows normalized level with Gliclazide drug) amounted 83.25±5.37, 89.25±4.92 and 83.50±5.06 mg/ml with percentage decrease -23.09, -17.55 and -22.86% respectively.

NO level in hepatic tissue of control, normal- treated, diabetic and diabetic–treated groups is manipulated in Table (4) and Fig (4). It can be easily noticed that NO level is insignificantly affected post various extracts- treated normal rats as compared to untreated control one. In response to diabetic state, NO shows significant increase of a value 62.96±2.32, 72.55±1.87 and 72.55 ±2.00 µg/g tissue with percentage increase +42.86,+64.62 and +64.62 %.at day 2, 10 and 40 post STZ injection respectively. The level of NO is significantly improved as a result of different treatments, shows the best pronounced effect for 70% ethanol and petroleum ether extracts, where insignificant change is recorded either as compared to normal control or diabetic– Gliclazide treated groups. In spite of, significant elevation in NO level is

noticed in diabetic –chloroform extract treated group amounted 51.30 ±2.69µg /g tissue with percent +16.41 %.

The level of carbohydrate hydrolyzing enzymes, α - amylase, β- galactosidase and α -glucosidase in liver tissue homogenates of the different studied groups is recorded in Table (5) and Fig (5) . Careful inspection of the data would reveal that , α - amylase and β- galactosidase show insignificant change in different normal - treated groups as compared to the untreated control one. While α-glucosidase shows significant increase as a result of treatment with both 70% ethanolic (0.342±0.04 µmole/mg protein/min) and petroleum ether extracts (0.370±0.06 µmole/mg protein/min) with percentage increase amounted +25.74 and +36.03 %, respectively. On the other hand, total protein content reveals a significant reduction with a value of 39.50±8.22, 36.25±10.30 and 41.25±8.54 mg/g tissue in response to treatment of normal rats with petroleum ether, chloroform and 70% ethanol extracts with percent -21.0, -27.5 and -17.5 %, respectively. It can be deduced that , carbohydrate metabolizing enzymes are strongly affected with diabetic condition show significant inhibition at day 2, 10 and 40 post STZ injection reached to 20.07 ±1.40, 17.79 ±0.68 and 18.32 ±0.69 µmole/ mg protein/min for α-amylase with percent of reduction -38.27, - 45.28 and -43.65% respectively. While, β-galactosidase enzyme activity recorded 0.052±0.02, 0.037±0.01 and 0.050±0.01 µmol/mg protein / min with percent - 68.86, - 77.84 and -70.06 %, respectively. In addition, α-glucosidase shows a value of 0.192± 0.01, 0.187±0.01 and 0.190± 0.01 µmole/ mg protein /min with percent -29.41, -31.25 and - 30.15 % respectively. On the contrary, total protein content shows insignificant change at the different durations post STZ injection.

Treatments of the diabetic rats with the different extracts of *Nepeta cataria* produce obvious enhancement in all carbohydrate hydrolyzing enzymes tested. This can be easily seen through normalization of α-amylase level to show insignificant change as a result of diabetic rats treatments with 70% ethanol extract and as compared to either normal or Glicalized- treated diabetic groups .While α-amylase shows significant increase post treatments with petroleum ether (52.90±6.22 µmole /mg protein/min) and chloroform extracts (40.94±2.2 µmole/mg protein/min) with percentage increase +62.72 and +25.93 % respectively. The curative effect of the different extracts can be also seen through improvement in β- galactosidase enzyme activity that is returned to its normal value as compared to both normal control and reference drug. In addition, α -glucosidase activity shows insignificant change post chloroform treatment, while it recorded significant increase post petroleum ether, 70% ethanol extracts and Gliclazide drug(0.335±0.02, 0.357±0.02 and 0.372.±0.03 µmole/ mg protein / min respectively) with percentage increase +23.16, +31.25 and +36.76 % respectively as compared to normal control. Concerning total protein content, insignificant change is observed in all diabetic –treated groups except in petroleum ether extract, significant reduction is obtained amounted 35.00 ±8.16 mg /g tissue with reduction percent -30 % as compared to normal control.

Table (6) and Fig (6) illustrate body weight, liver weight and liver weight / body weight ratio in control and different treated groups. It is obvious that body weight of normal control rats recorded significant increase (181.00 ±2.58 gm) with concomitant increase in liver weight (6.90±0.297) at day 40 post experiment as compared to body weight and liver weight at zero time with percentage increase amounted +19.47 and +16.50 % respectively. While, body weight and liver weight show insignificant change at other durations (2 and 10 days). Moreover, normal control liver weight/body weight ratio shows insignificant change at different durations as compared to zero time. On the other hand, body weight and liver weight /body weight ratio of normal rats treated - chloroform, 70% ethanol and petroleum ether extracts show insignificant change either as compared to normal control at 40 day or as compared to each other. Normal liver weight of rats treated with 70% ethanolic extract exhibits significant increase amounted 8.50 ±0.37 g with percentage reached to + 23.14 %.While, it shows insignificant change in other treatments. It is clearly noticed from the present study that, diabetic condition is always associated with a significant reduction in body weight. This result is ascertained through the degradable , remarkable significant reduction in body weight at day 2, 10 and 40 post STZ injection amounted 142.25±4.34, 112.75 ±8.77 and 98.50±5.68 g with percentage of reduction -5.79, - 28.86 and - 45.58% respectively. On the contrary, liver weight and liver weight / body weight ratio manipulated significant increase of 7.67 ± 0.87, 9.56±1.86 and 8.25±0.42 g with percent +26.96, +53.49 and +22.06 % for liver weight and 0.0525±0.005, 0.085±0.013 and 0.088±0.005 for liver weight / body weight ratio with percentage increase +31.25, +112.50 and +118.75 % respectively. Treatment of diabetic rats with different extracts clearly produces improvement in body weight, liver weight and their ratios, while chloroform extract- treated diabetic rats shows significant increase in body weight amounted 123.50 ±6.02 g with percent +25.38 % as compared to untreated diabetic group at day 40 (recovery group), although, with respect to normal control rats at day 40, significant reduction is recorded (-31.77%). Nearly the same results are achieved for 70% ethanolic extract treated– diabetic rats. In addition, diabetic rats –treated with petroleum ether extract and Gliclazide drug show significant increase in body weight amounted 131.00±4.24 and 153.25 ± 2.36 g with percent +32.99 and +55.58 % respectively as compared to diabetic - untreated one ,while as compared to normal control, significant reduction is noticed (-27.62 and -15.33 %, respectively). Concerning liver weight, insignificant change is observed in chloroform extract treated-diabetic rats and Gliclazide drug, while significant reduction is recorded in petroleum ether and total ethanol extracts amounted 5.00±0.47 and 5.26±0.50 g with percentage -27.56 and -23.83% respectively as compared to normal control rats (40 days). Liver weight /body weight ratio exhibits insignificant change in all diabetic –treated groups except chloroform extract which shows significant increase as compared to normal control group at day 40 (0.055±0.02) with percentage of increase +37.5%.In addition, significant reduction is observed in liver weight and liver weight / body weight ratio in all diabetic –treated groups as compared to diabetic untreated one(40 days).

Histological studies on pancreas and liver:

Normal architecture in liver and pancreas of treated – normal groups at the cellular level as compared to the control un-treated one. Gradual cellular changes include imperfection, reduction in β-cells numbers, degeneration and atrophic changes are appears in pancreas of Streptozotocin - treated groups at different durations (Figs 11-13). On the other hand, sections of diabetic liver at day 2, 10 and 40 show degeneration of hepatocytes, necrosis and congestion of central vein (Figs 22-24). Successive as well as total ethanolic extracts of *Nepeta cataria* and Gliclazide drug appear to regulate diabetes at the cellular level resulting in , restoration of normal architecture of pancreatic islets (Figs 14-17) and hepatocytes (Figs 25-28) in the diabetic- treated groups. These suggested a possible regeneration or repair of the cells in diabetic –treated rats.

Table (1): Evaluation of successive and 70% ethanol extracts of *Nepeta cataria* on blood glucose and insulin levels in control, normal- treated, diabetic and diabetic-treated groups

Treatments / Parameters	Glucose	Insulin
Normal control	109.5 ±6.65 de	5.35± 0.62 a
Normal treated chloroform extract	105.25± 7.36 de	5.21 ±1.07 ab
Normal treated 70% ethanol extract	94.50 ±16.05 f	5.07 ±1.47 ab
Normaltreated petroleum ether extract	113.75 ±1.25 d	5.03 ±1.66 abc
Diabetes after 2 days	373.00± 2.94 a	0.59± 0.01 e
Diabetes after 10 days	363.00± 2.94 b	0.34± 0.05 e
Diabetes after 40 days	364.25± 3.77 ab	0.35 ±0.01 e
Diabetes treated chloroform extract	133.00± 5.35 c	3.83 ±0.59 d
Diabetes treated 70% ethanol extract	103.00± 2.44 ef	4.59± 0.59 abcd
Diabetes treated petroleum ether extract	128.25± 2.87 c	4.13± 0.57 bcd
Diabetes treated gliclazide (Ref.Drug)	132.00± 2.44 c	3.87± 0.05cd
LSD 5%	9.08	1.19

Blood glucose is expressed in mg /dl while insulin level is expressed in uIU/ml
Data are mean ±SD of 7 rats in each group.
Statistical analysis is carried out using one way analysis of variance (ANOVA) using Co-Stat computer program.
Unshared superscript letters between treatments are significance values at P<0.001.

Table (2): Evaluation of successive as well as 70% ethanol extracts of *Nepeta cataria* on lipid profile in serum of control, normal- treated, diabetic and diabetic-treated groups.

Treatments / Parameters	T- cholesterol	HDL- Cho	LDL- Cho	Tri glycride	Total lipid
Normal control	131.34± 6.26 e	67.04±3.00 cd	83.81± 2.27 d	110.88±7.36 de	980.62± 30.71 d
Normal treated chloroform extract	126.72± 6.16 e	65.45 ±3.89 cd	81.99± 5.27 d	103.61± 7.55 ef	1033.50± 23.70 d
Normal treated 70% ethanol extract	128.79± 7.54 e	69.00 ±4.54 c	80.01± 7.29 d	93.32 ±22.07 f	931.94± 145.94 d
Normal treated petroleum ether extract	125.50± 6.40 e	65.00 ±4.16 cd	83.30± 6.08 d	114.06 ±11.09 cde	1049.00± 11.04 d
Diabetes after 2 days	202.50± 4.51 b	81.62± 3.35 b	163.22± 4.57 b	211.75± 8.22 b	1687.19 ±202.10 a
Diabetes after 10 days	239.91 ±10.89 a	90.75 ±1.89 a	210.72 ±9.96 a	307.77± 8.60 a	1805.55 ±103.93 a
Diabetes after 40 days	239.75 ±4.11 a	90.75±0.95 a	210.75± 4.98 a	308.75 ±1.25 a	1808.75± 8.54 a
Diabetes treated chloroform extract	158.15± 8.20 c	64.37±4.53 cd	118.02± 11.50 c	121.16± 4.65 cd	1414.58 ±195.92 b
Diabetes treated 70% ethanol extract	150.81± 6.46 cd	61.92 ±6.46 d	111.25± 2.15 c	112.72± 5.57 cde	1325.00 ±99.71 bc
Diabetes treated petroleum ether extract	145.99± 10.97 d	63.25 ±7.36 cd	107.89± 14.17 c	125.74±10.83 c	1373.17± 41.65 bc
Diabetes treated Gliclazide (Ref.Drug)	72.75 ±0.96 f	61.25± 0.95c	81.90± 1.68 d	124.50± 4.20 cd	1237.50± 33.04 c
LSD 5%	10.27	6.06	10.7	14.07	153.84

Lipid profile (total chloesterol,HDL-cholesterol,LDL-cholesterol,Triglycrides and Total Lipid) are expressed in mg/dL.
Data are mean ±SD of 7 rats in each group.
Statistical analysis is carried out using one way analysis of variance (ANOVA) using Co-Stat computer program.
Unshared superscript letters between treatments are significance values at P<0.001.

Table (3) Evaluation of successive and 70% ethanol extracts of Nepeta *cataria* on liver function enzyme activities in serum of control, normal -treated , diabetic and diabetic-treated groups

Parameters \ Treatments	AST	ALT	ALP	Total Protein
Normal control	2.26 ±0.02 c	1.60± 0.09 cd	3.39± 0.13 c	108.25± 5.67 ab
Normal treated chloroform extract	2.48 ±0.08 bc	1.72± 0.14 c	2.94± 0.40 d	112± 1.41 a
Normal treated 70% ethanol extract	2.50 ±0.07 bc	1.65 ±0.03 cd	2.74± 0.25 de	109± 6.21 ab
Normal treated petroleum ether extract	1.97± 0.25 d	1.64± 0.19 cd	2.78± 0.26 de	102± 7.70 b
Diabetes after 2 days	2.73± 0.24 b	1.97 ±0.09 b	4.51± 0.36 b	104.5± 8.42 ab
Diabetes after 10 days	3.23 ±0.23 a	2.44 ±0.17 a	5.55 ±0.60 a	108.75 ± 6.29 ab
Diabetes after 40 days	3.37 ±0.11 a	2.48 ±0.08 a	5.61 ±0.13 a	84.3± 1.74 c
Diabetes treated chloroform extract	2.48 ±0.36 bc	1.72± 0.04 c	2.41 ±0.08 ef	89.25± 4.92 c
Diabetes treated 70% ethanol extract	2.40± 0.27 c	1.66± 0.04 cd	2.52 ±0.31 def	83.5± 5.06 c
Diabetes treated petroleum ether extract	2.41± 0.13 c	1.59±0.11 cd	2.18± 0.17 f	83.25± 5.37 c
Diabetes treated Gliclazide (Ref.Drug)	2.53 ±0.03 bc	1.55±0.04 d	2.19 ±0.08f	101.25 ± 5.05 b
LSD 5%	0.28	0.16	0.42	8.11

AST,ALT and ALP are expressed in µ mole/mg protein/min
Total protein is expressed in mg /ml
Data are mean ±SD of 7 rats in each group.
Statistical analysis is carried out using one way analysis of variance (ANOVA) using Co-Stat computer program.
Unshared superscript letters between treatments are significance values at P<0.001.

Table (4) Evaluation of successive as well as 70% ethanol extracts of *Nepeta cataria* on nitric oxide level in liver of control, normal-treated, diabetic and diabetic-treated groups

Parameters \ Treatments	NO
Normal control	44.07 ±1.37 d
Normal treated chloroform extract	43.32 ±0.70 d
Normal treated 70% ethanol extract	44.03± 0.96 d
Normal treated petroleum ether extract	43.45 ±1.25 d
Diabetes after 2 days	62.96 ±2.32 b
Diabetes after 10 days	72.55 ±1.87 a
Diabetes after 40 days	72.55 ±2.00 a
Diabetes treated chloroform extract	51.30± 2.69 c
Diabetes treated 70% ethanol extract	43.92± 1.47 d
Diabetes treated petroleum ether extract	43.12 ±0.83 d
Diabetes treated gliclazide (Ref.Drug)	44.75 ±1.70 d
LSD 5%	2.4

Nitric oxide (NO) is expressed in µg/g tissue
Data are mean ±SD of 7 rats in each group.
Statistical analysis is carried out using one way analysis of variance (ANOVA) using Co-Stat computer program.
Unshared superscript letters between treatments are significance values at P<0.001.

Table (5) Evaluation of successive and 70% ethanol extracts of *Nepeta cataria* on carbohydrate hydrolyzing enzymes in liver of control, normal - treated, diabetic and diabetic-treated groups.

Parameters \ Treatments	α- amylase	β- glactosidase	α- glucosidase	Total protein
Normal control	32.51 ±4.49 de	0.167± 0.02 bc	0.272± 0.02 c	50.00±10.80 abc
Normal treated chloroform extract	32.36± 4.06 e	0.167± 0.03 bc	0.305± 0.01 bc	36.25± 10.30 d
Normal treated 70% ethanol extract	38.08± 5.43 bc	0.142± 0.03 c	0.342± 0.04 ab	41.25± 8.54 bcd
Normal treated petroleum ether extract	35.83 ±2.20 bcde	0.167± 0.01 bc	0.370± 0.06 a	39.50 ±822 cd
Diabetes after 2 days	20.07± 1.44 f	0.052± 0.02 d	0.192± 0.01 d	55.00 ±9.13 a
Diabetes after 10 days	17.79 ±0.68 f	0.037± 0.01 d	0.187± 0.01 d	48.75± 11.08 abc
Diabetes after 40 days	18.32 ±0.69 f	0.050± 0.01 d	0.190± 0.01 d	56.50± 2.38 a
Diabetes treated chloroform extract	40.94± 2.29 b	0.197± 0.02 a	0.282± 0.04 c	51.25±8.54 abc
Diabetes treated 70% ethanol extract	34.12 ±4.18 cde	0.150± 0.02 c	0.357± 0.02 a	52.50 ±6.45 ab
Diabetes treated petroleum ether extract	52.90 ±6.22 a	0.165± 0.01 bc	0.335± 0.02 ab	35.00± 8.16 d
Diabetes treated Glicalized (Ref drug)	37.60 ±2.06 bcd	0.180± 0.01 ab	0.372± 0.03 a	53.92± 1.78 a
LSD 5%	5.13	0.029	0.044	11.95

Enzymes (α-amylase,β- glactosidase and α- glucosidase) are expressed in µmole/mg protein /min
Total protein is expressed in mg /g tissue
Data are mean ±SD of 7 rats in each group.
Statistical analysis is carried out using one way analysis of variance (ANOVA) using Co-Stat computer program.
Unshared superscript letters between treatments are significance values at P<0.001.

Table (6):Evaluation of successive and 70% ethanol extracts of *Nepeta cateria* on body weight, liver weight and liver weight/body weight ratio in control, normal -treated, diabetic and diabetic-treated groups

Parameters \ Treatments	Body weigh	Liver weight	Liver weight/ body weight
Normal control zero time	151.50 ± 1.29 b	5.925 ± 0.25 def	0.04± 0 c
Normal control after 2 days	151.00 ± 5.47 b	6.0375± 0.149 def	0.04 ± 0 c
Normal control after10-days	158.50 ± 3.10 b	6.225 ± 0.22 def	0.04 ± 0 c
Normal control after 40-days	181.00± 2.58 a	6.9025± 0.297 cd	0.04 ± 0.008 c
Normal treated chloroform extract	186.5.00± 10.96 a	7.81± 0.64 bc	0.04 ± 0 c
Normal treated 70% ethanol extract	187.75 ± 3.5 a	8.50± 0.37 ab	0.0475± 0.005 bc
Normal treated petroleum ether extract	184.00± 4.69 a	7.7925± 0.80bc	0.04 ± 0 c
Diabetes after 2 days	142.25± 4.34 c	7.665± 0.87 bc	0.0525± 0.005 b
Diabetes after 10-days	112.75± 8.77 e	9.555 ± 1.86 a	0.085 ± 0.0129 a
Diabetes after 40-days	98.50± 5.68 f	8.425± 0.419 ab	0.0875± 0.005 a
Diabetes treated chloroform extract	123.50± 6.02 d	7.0075± 2.248 cd	0.055± 0.017 b
Diabetes treated 70% ethanol extract	123.75 ± 3.68 d	5.2575± 0.50 ef	0.0425± 0.005 c
Diabetes treated petroleum ether extract	131.00± 4.24 d	5.00± 0.467 f	0.0425± 0.005 c
Diabetes treated Gliclazide (Ref.Drug)	153.25± 2.36 b	6.30± 0.29 de	0.04± 0 c
LSD 5%	7.66	1.29	0.009

Body weight, liver weight and liver weight/body weight ratio are expressed in g
Data are mean ±SD of 7 rats in each group.
Statistical analysis is carried out using one way analysis of variance (ANOVA) using Co-Stat computer program.
Unshared superscript letters between treatments are significance values at P<0.001.

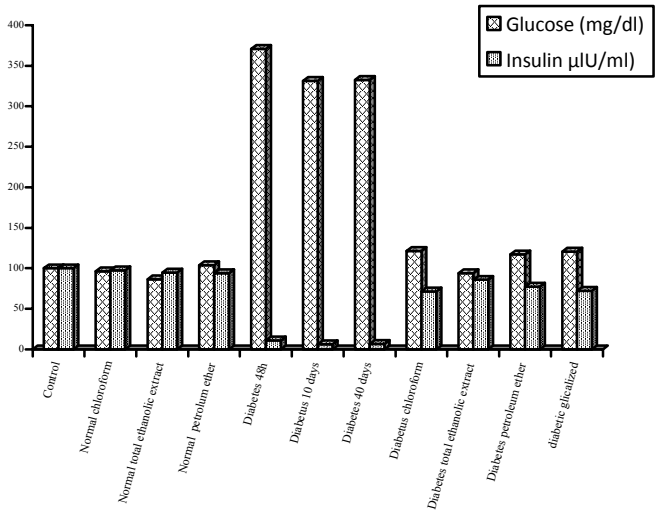


Fig. (1): % change of *Nepeta cataria* extracts on blood glucose and insulin levels in normal control and various treated groups.

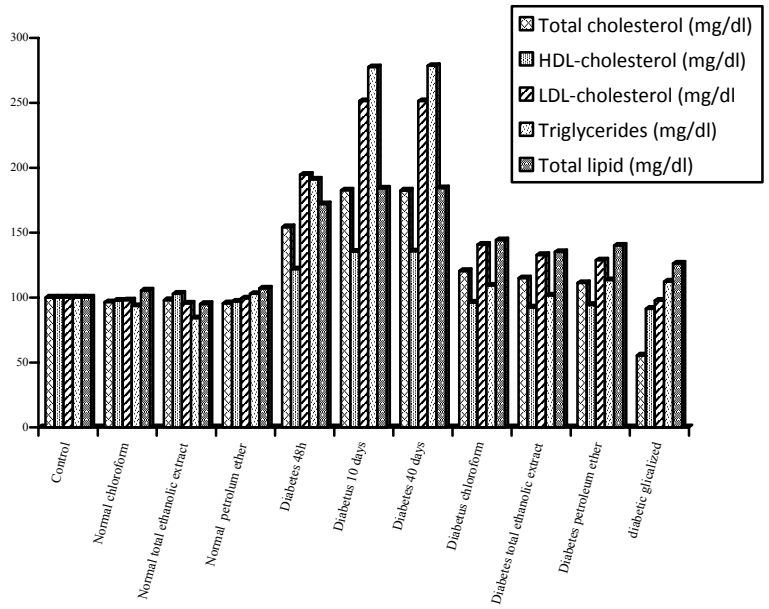


Fig. (2): % change of *Nepeta cataria* extracts on lipid profile in serum of normal control and various treated groups.

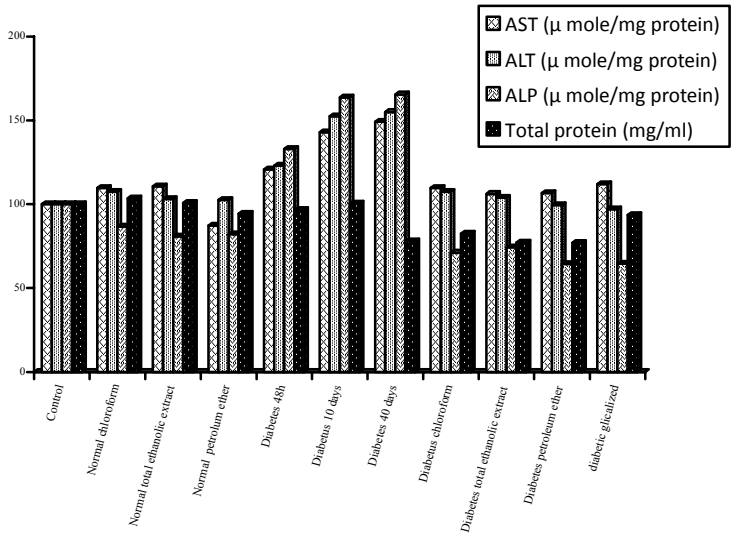


Fig. (3): % change of *Nepeta cataria* extracts on AST,ALT , ALP and total protein in normal control and various treated groups.

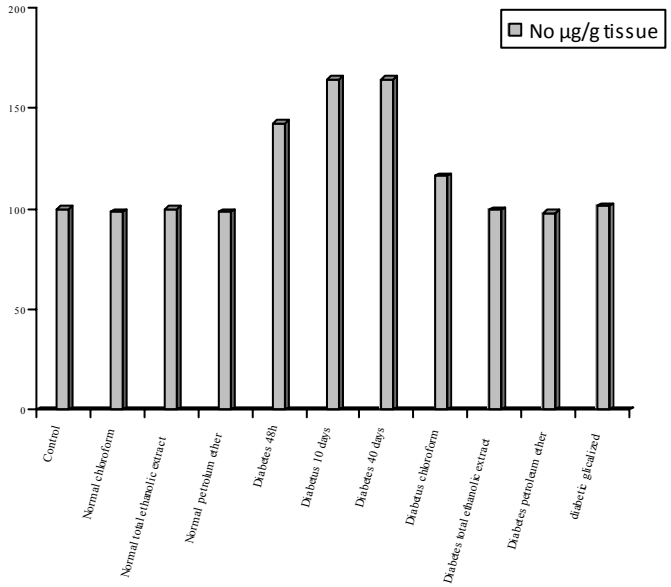


Fig. (4): % change of *Nepeta cataria* extracts on nitric oxide (NO) level in liver of normal control and various treated groups.

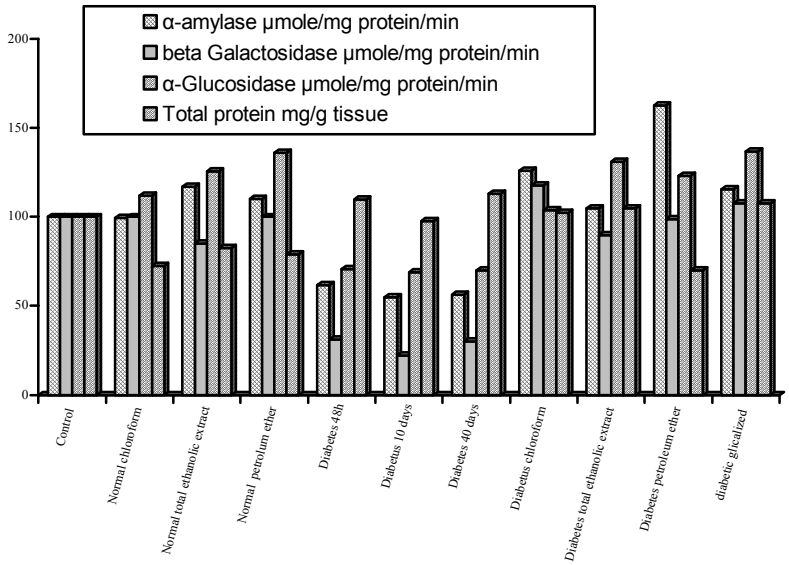


Fig. (5): % change of *Nepeta cataria* extracts on α-amylase , β-galactosidase and α- glucosidase in normal control and various treated groups.

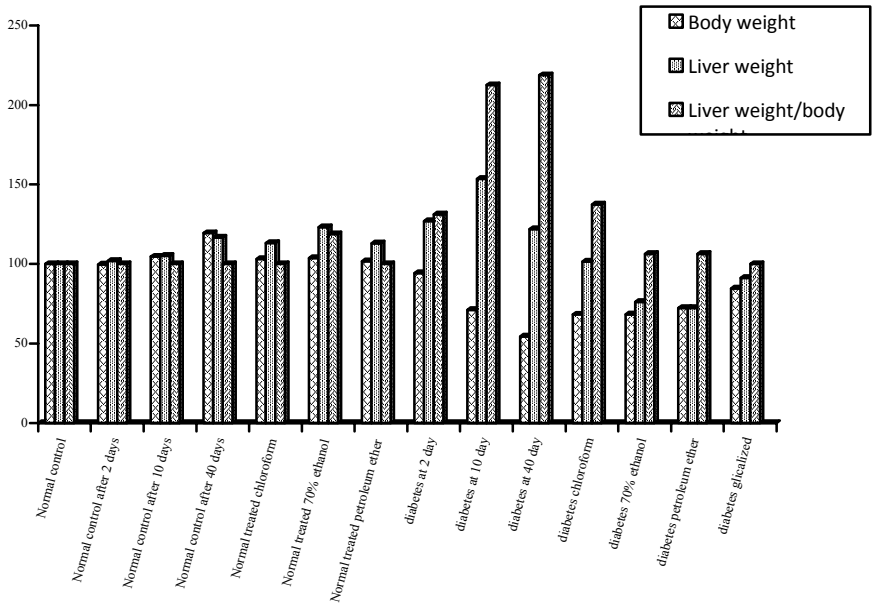


Fig. (6): % change *Nepeta cataria* extracts on body weight, liver weight and liver weight/body weight ratio in control , different normal-treated, diabetic and diabetic- treated groups.

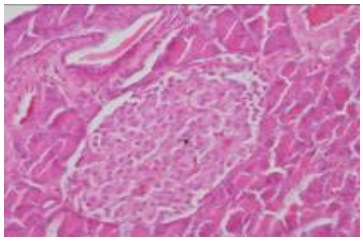


Fig 7: Photomicrograph in the islet of Langerhans of normal control rats showing normal cellular elements (Hx. E stain X200)

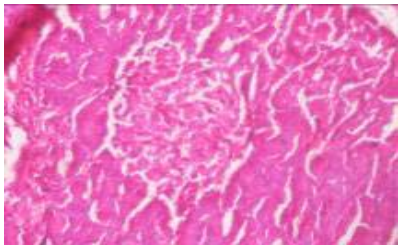


Fig 8: Photomicrograph in the islet of Langerhans of normal- treated petroleum ether showing normal different cellular elements with a typical morphology and without any lymphoid infiltration (Hx. E stain X200)

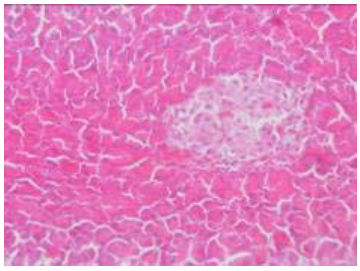


Fig 9: Photomicrograph in the islet of Langerhans of normal -treated chloroform extract showing normal different cellular elements (Hx. E stain X200)

Fig10: Photomicrograph in the Islet of Langerhans of normal treated -70%ethyl alcohol showing normal different cellular elements with a typical morphology and without any lymphoid infiltration (Hx. E stain X200).

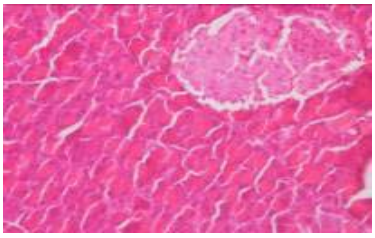


Fig 11: Photomicrograph of section in the islet of Langerhans after 2days of s STZ injection showing degenerative changes and decrease in number of β- cells (Hx. E stain X200)

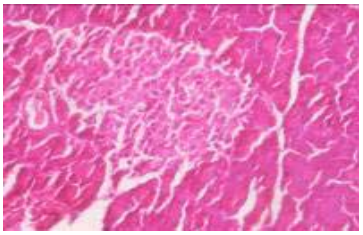


Fig 12a: Photomicrograph in the Islet of Langerhans at day 10 post STZ injection showing degenerative cells (Hx. E stain X200)

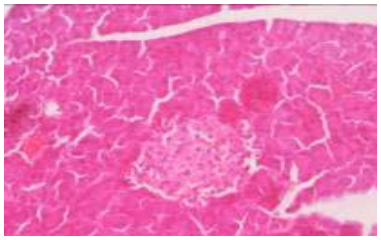


Fig 12b:Photomicrograph in the islet of Langerhans after 10 days of STZ injection showing degenerative changes and decrease in islet size and β-cells number (Hx. E stain X200).

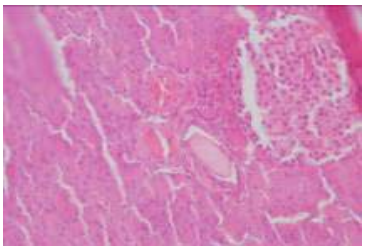


Fig 13a: Photomicrograph in the islet of Langerhans of diabetic rats (after 40 days of STZ injection) showing imperfections with lymphoid infiltration , atrophic changes and only small regions with preserved structure..(Hx. E stain X 200).

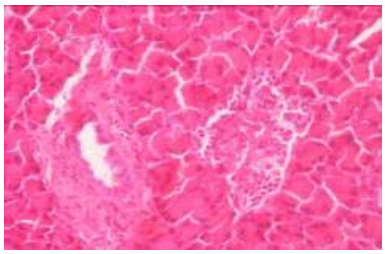


Fig13b: Photomicrograph in the islet of Langerhans after 40 days of STZ injection showing degenerative cells decrease in β-cells number (Hx. E stain X200).

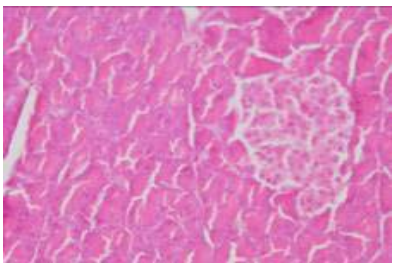


Fig 14: Photomicrograph in the islet of Langerhans of cholorophorm extract- treated diabetic rats showing less vaculation and more healthy beta cells (Hx. E stain X200)

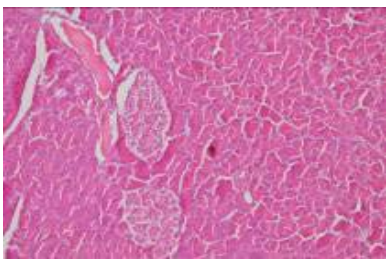
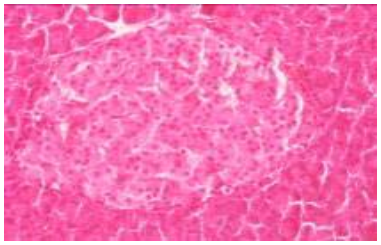


Fig 15: Photomicrograph in the islet of Langerhans of petroleum ether treated diabetic rats showing no observed cellular changes (Hx. E stain X 200)



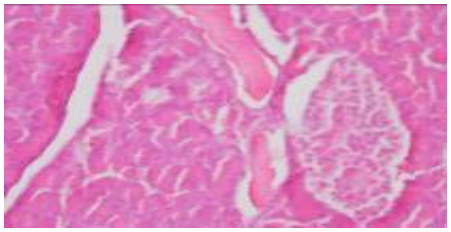


Fig 16: Photomicrograph in the islet of langerhans of diabetic rats treated with 70% ethyl alcohol extract showing no observed cellular changes (Hx. E stain X200)

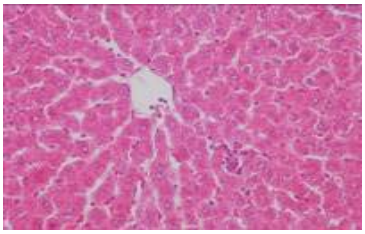


Fig 17: Photomicrograph section in the islet of langerhans in diabetic rats treated with Glicalized antidiabetic drug , showing less cellular vaculation and more healthy beta cells (Hx. E stain X200)

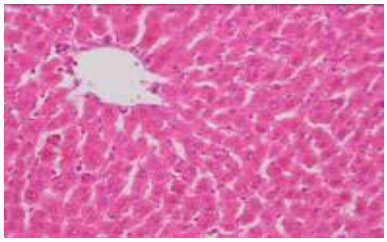


Fig 18: A photomicrograph of control rat liver section showing normal hepatic cells, sinusoidal space and central vein. (HX & E x200).

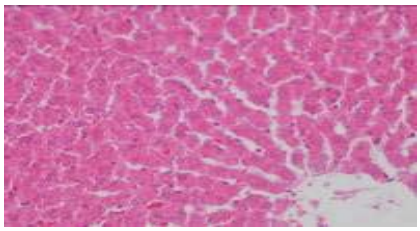


Fig 19: A photomicrograph of normal rat liver section treated with petroleum ether showing normal hepatic cells , sinusoidal space and central vein . (HX & E x200**).**

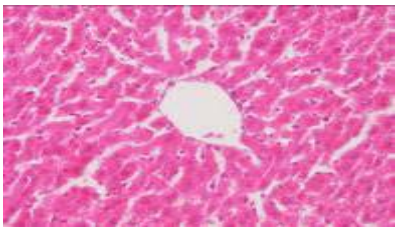


Fig 20: A photomicrograph section of normal rat liver section treated with chloroform showing normal hepatic cells , sinusoidal space and central vein . (HX & E x200).

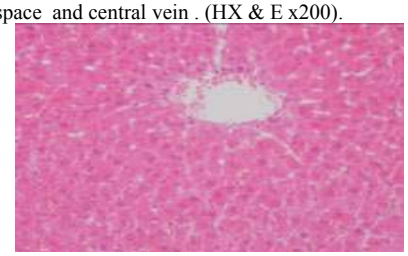


Fig 21 a : A photomicrograph section of normal rat liver section treated with total extract showing normal hepatic cells , sinusoidal space and central vein . (HX & E x200).

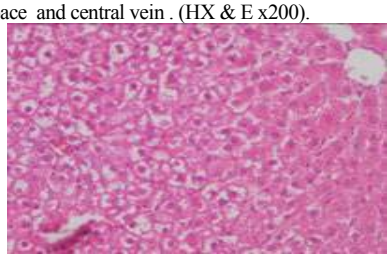


Fig 21 b: A photomicrograph of normal rat liver section treated with 70% ethanol extract, showing normal hepatic cells , sinusoidal space and central vein . (HX & E x200).

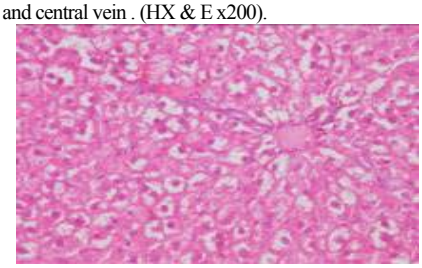


Fig 22 a : A photomicrograph of rat liver section after 2 days of STZ injection , exhibited hepatocyte degeneration and necrosis (HX & E x200).

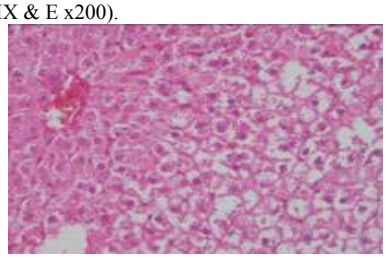


Fig 22b : Photomicrograph of rat liver section after 2 days of STZ injection showing degeneration of hepatocytes (Hx. E stain X200).

Fig23 a : Photomicrograph of rat liver section after 10 days of STZ injection showing degeneration of hepatocytes and congestion of central vein (Hx. E stain X 200) .

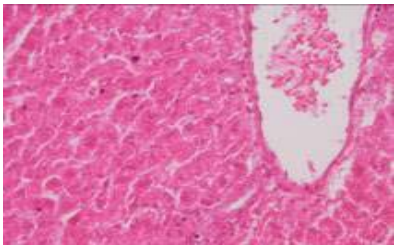


Fig 23 b: A photomicrograph section of rat liver t section after 10 days of STZ injection (Hx & E X 200) exhibited hepatocyte degeneration, necrosis and congestion of central vein.

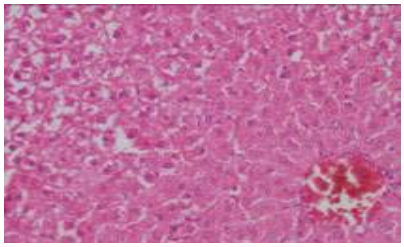


Fig 24: A photomicrograph section of rat liver after 40 days of STZ injection exhibited severe hepatocyte degeneration, necrosis and congestion of central vein (Hx & E X 200)

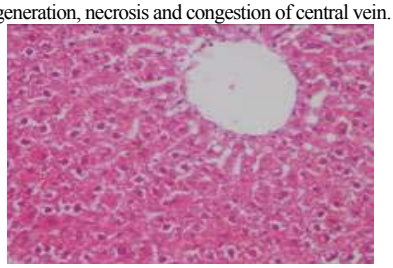


Fig 25: A photomicrograph section of diabetic rat liver treated with petroleum ether, showing moderate degeneration of hepatic cells and un – congested central vein . (Hx & E X 200).

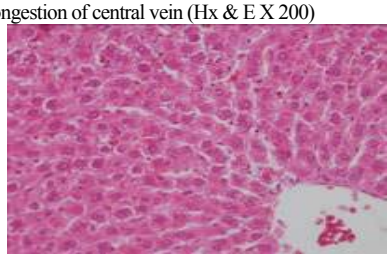


Fig 26:Aphotomicrograph section of diabetic rat liver treated with chloroform extract, showing mild hepatocyte degeneration less congested and dilated central vein and blood sinusoids (Hx & E X 200).

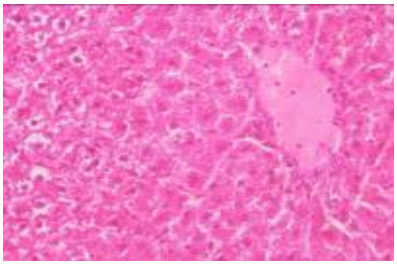


Fig27 a: A photomicrograph of diabetic rat liver section treated with 70% ethanol extract, showing moderate degeneration of hepatic cells. (Hx & E X 200)

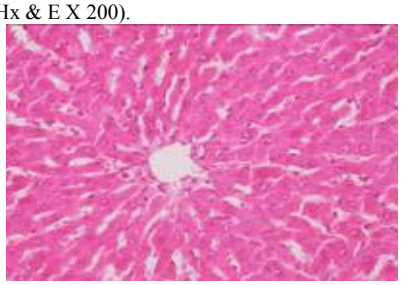


Fig 27b: A photomicrograph of diabetic rat liver section treated with 70% ethanol extract, showing, almost normal cell architecture with dilated blood sinusoids (Hx & E X 200).

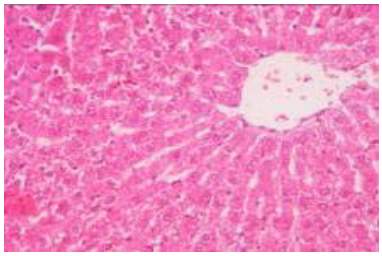


Fig 28 : Aphotomicrograph of diabetic rat liver section treated with Gliclazide drug, showing near normal hepatic cells, ,sinusoidal space and central vein ((Hx& E X200) .

4. Discussion:

Diabetes mellitus (DM) is a chronic disease caused by inherited or acquired deficiency in insulin secretion(IDDM) or by decreased responsiveness of the organs to secreted insulin(Non IDDM), resulting in increased blood glucose level, This , in turn ,can damage many of the body's systems, including blood vessels, nerves and causes oxidative tissue damage (Matsui *et al .,* 2007).

Reactive oxygen species (ROS), superoxide anion, hydrogen peroxide and hydroxyl, nitric oxide and peroxynitrite radicals, play an important role in oxidative stress related to the pathogenesis of various important diseases as diabetes. In healthy individuals, the production of free radicals is balanced by the antioxidative defense system, while defense system is greatly affected during diseases (Shyur *et al.*, 2005).

The potential role of the medicinal plants as hypoglycemic agents has been reviewed by several authors, supported by the ethnobotanical surveys and traditional medicines of different cultures (Yeh *et al.,* 2003; Biesalski , 2004 ; Li *et al .,* 2004).

The current study has demonstrated insignificant change between normal- control and different normal –treated groups (G2-G4) either in blood glucose or insulin levels except for normal rats treated with 70% ethanol extract that, recorded significant decrease in blood glucose level (13.69%). Injection of rats with STZ (G5-G7) induced significant elevation in fasting blood glucose with concomitant reduction in insulin levels at day 2, 10 and 40 post STZ treatment as compared to control untreated group(G1) (from109.5± 6.65 mg/dl to 373.00±2.94, 363.00±2.94 and 364.25±3.77 mg /dl,

respectively for glucose and 5.35µIU/ml to 0.59 ±0.01, 0.34±0.05 and 0.35±0.01 µIU/ml for insulin, respectively).

The present histological examinations at the cellular level, reveal atrophy, necrosis and degenerative changes in both hepatocytes and β-cells of pancreas, indicating establishment of diabetic state (Figs 11-13 ;.22-24 pancreas and liver respectively). Holemans *et al.* (1997) demonstrated that, Streptozotocin induced beta cells destruction by necrosis; it is an antibiotic and anticancer agent which is widely used for inducing diabetes (Type 1 IDDM) in a variety of animals. It interferes with cellular metabolic oxidative mechanisms (Bagri *et al.,* 2008). It selectively induces degenerative alterations and necrosis of pancreatic β-cells resulting in insulin deficiency and impairment in glucose oxidation (DeCarvalho *et al.,* 2005). Ikebukuro *et al* . *(*2002) have reported that, the use of lower dose of Streptozotocin produced an incomplete destruction of pancreatic beta cells even though rats became permanently diabetic. In accordance to the present study, Mitra *et al.* (1996) earlier reported that, the diabetic liver showed degeneration and congestation two hours after injection of STZ, hyperglycemia is observed with a concomitant drop in blood insulin level. The changes in blood glucose and insulin concentrations reflect abnormalities in beta cell functions. The fluctuation in the blood sugar might also be attributed to the sensitivity to STZ that varies with species, strain, sex and nutritional state and there are batch differences in activity (Mir *et al.,* 2008). In a good agreement with the present results, Akbarzadeh *et al.* (2007) confirmed the destruction of islet cells in pancreatic biopsy of diabetic rats due to the effect of Streptozotocin and added that 60 mg /kg dose of STZ ensured induction of diabetes in rats and hyperglycemia, hypoinsulinemia, polyphagia, polyuria and polydipsia were seen in adult rats within 3 days of STZ treatment and the amounts of these relevant factors were almost stable , which indicates irreversible destruction of langerhans islets cells. Previous studies have reported that, Streptozotocin enters the beta cells via a glucose transporter and causes alkylation of DNA. DNA damage induces activation of poly ADP ribosylation, a process that is more important for the diabetogenecity of Streptozotocin than DNA damage itself. Poly ADP- ribosylation leads to depletion of cellular NAD and ATP. Enhanced ATP dephosphorylation after Streptozotocin treatment supplies a substrate for xanthine oxidase resulting in the formation of super oxide radicals. Consequantly, hydrogen peroxide and hydroxyl radicals are generated. Furthermore Streptozotocin liberates toxic amounts of nitric oxide that inhibits aconitase activity and participates in DNA damage (Fiordaliso *et al.,* 2000).

In diabetes state, degradation of liver glycogen and gluconeogensis are increased while glucose utilization is inhibited. Glucose -6- phosphatase increases in the liver, facilitating glucose release into the blood. The opposing enzyme which phosphorlyates glucose, i.e hexokinase, is unaffected by insulin while glucokinase is decreased in diabetes. As a result, the liver continues to produce glucose even with severe hyperglycemia. Under these circumstances the normal liver would shut off and deposit glycogen (Shelia and James, 1993).

The present results reveal , significant amelioration in blood glucose and insulin levels post treatment of diabetic rats with the 70% ethanol, petroleum ether and chloroform extracts of *Nepeta cataria* with percent of improvement amounted 239.68, 215.29 and 211.19 % for glucose and 79.25, 70.65 and 65.05% for insulin, respectively. The current investigation also showed that ,treatment of diabetic rats with Gliclazide (reference drug) modulated the alterations in blood glucose and insulin within its normal levels (212.10 and 65.79%, respectively). In addition, the histological examination showed improvement in hepatocytes and pancreas β- cells (Figs 14-17 ;25-28). In line with the present study , Vats *et al.* (2004) found that the 70% ethanol extract of *Ocimum sanctum*(Labiatae) leaves significantly improve β–cells function and enhances insulin secretion leading to lowering blood glucose level. These authors added that the antihyperglycemic effect of *Ocimum sanctum* is at least partially dependent upon insulin release from the pancreas and significantly increased the activity of three key enzymes involved in carbohydrate metabolism, namely phosphofructokinase, glucokinase and hexokinase (PFK, GK, HK) towards normal levels. In addition ,it increases glycogen in muscle and liver by stimulating glycogen synthase, suggesting that, the antihyperglycemic action is the result of increased glucose utilization at the level of skeletal muscle as well as liver. The authors added that inhibition of disaccharide enzymes sucrase and maltase seems to be one of the factors which explain the hypoglycemic action of many antidiabetic plant extracts. Immune-stimulation also might be one of the mechanisms contributing towards the protective actions of *Ocimum sanctum* (Sembulingam *et al.,* 2005). In addition, Zheng *et al.* (2007) studied the hypoglycemic effect of the aqueous ethanol extract of *Prunella vulgaris L.* (Labiatae) in STZ diabetic mice and attributed the hypoglycemic effect to increase the insulin sensitivity of target tissues and the antihyperglycemic effect of insulin is enhanced and prolonged, which could result from increased tissue metabolism or from suppressed levels of non- esterified fatty acids (NEFA). Excess plasma NEFA can inhibit insulin – stimulated glucose utilization in muscle and promote hepatic production of glucose .Whereas, reduction of plasma NEFA concentration improves glucose utilization and enhances the suppression of hepatic glucose production by insulin. Stimulation of glucose uptake by peripheral tissues and inhibition of endogenous glucose production may be involved in hypoglycemic mechanisms of Labiatae family. Some constituents in the *Prunella vulgaris L.* have been identified such as phenolic acids (rosmarinic, caffeic), triterpenoids (methyl oleanolate, methyl ursolate, methyl maslinate), flavonoides (quercetin, campherol, rutin), tannis and polysaccharide . The antihyperglycemic activity of *Prunella vulgaris L.* (Labiatae) may be due to any one or more of the constituents in the extracts. Based on this findings , the hypoglycemic action of the 70% ethanol, petroleum and chloroform extracts of *Nepeta cateria* may be insulin –mediated by mechanism(s) in common with Gliclazide. Another explanation for the hypoglycemic action of *Nepeta cataria* extracts is depend on the presence of antioxidants such as flavonoides which may prevent the progressive impairment of pancreatic beta-cell function due to oxidative stress and may thus reduce the occurrence of type 2 diabetes (Li *et al.,* 2004; Bhandari *et al.,* 2008). *Nepeta cataria* was found to possess relatively high percentage of essential oil (nerol, geraniol and citral as well as ursolic acid) and polyphenols (falvonoids, phenolic acid) and the antihyperglycemic, antisplasmodic and myorelaxant effects may be related to these constituents (Gilani *et al.,* 2009).

With respect to lipid profile, the current results demonstrate, insignificant change in total cholesterol, HDL-cholesterol, LDL-cholesterol and total lipid in different normal-treated groups, with significant reduction in triglycerides in normal – 70% ethanolic extract treated group. Meanwhile, significant elevation in lipid profile was noticed at day 2, 10 and 40 post STZ injections. In addition, to abnormal glucose metabolism, DM often involves abnormal lipid metabolism which is considered as additional metabolic disorder, in diabetic complications. The same results were achieved by Sethi *et al.* (2004) who found significant elevation in lipid profile in serum of diabetic rats. In a good agreement with the present data, some authors revealed that hyperglycemia produced marked increased level of serum triglycerides, total –cholesterol and LDL- cholesterol (LDL-C), while in contrast to the present data HDL- cholesterol (HDL-C) showed reduced concentration in diabetic rats (Abou –Seif and Youssef ,2004 ; Jurgonski *et al.,* 2008). Levinthal and Tavill (1999) reported that, hepatic fat accumulation is a well – recognized complication of DM. The most common clinical presentation in DM is hepatomegaly. This hyperlipidemia associated with DM may be attributed to insulin deficiency (Morel and Chisolm, 1989) and elevated cortisol level, which have an important role in the process of fat accumulation (Hristova and Aloe, 2006). Under normal circumstances insulin activates lipoprotein lipase which hydrolyzes triglycerides. Insulin

deficiency results in failure to activate the enzyme, thereby causing hyper-triglyceridemia (Shirwaikar *et al.,* 2004). On the other hand, in insulin deficiency, the plasma free fatty acids concentration is elevated as a result of increased free fatty acids outflow from fat depots, where the balance of the free fatty acids estrification , triglycerides lipolysis is displaced in favors of lipolysis (Shirwaikar *et al.,* 2004). Also elevated cortisol promotes the liberation of free fatty acids from adipose tissue into blood stream by inducing and maintaining the synthesis of the hormone sensitive lipase , thus increasing free fatty acids level which contribute to cardio- vascular risk (Lundberg, 2005).

The elevation in cardioprotective HDL-C means increase of cholesterol afflux from the tissues, the first step in reverse cholesterol transport from the peripheral tissues to the liver. The antioxidant and antiatherogenic activities of HDL-C are enhanced when its circulating level is increased. LDL- C particles become small and dense which undergo oxidative modification, thus leading to a diabetic complication (Kalousova *et al*., 2002). In addition, Mir *et al*. (2008) reported hypercholesterolemia, hypertriglyceridemia associated with DM and explained these increments at the basis of Streptozotocin induced diabetes. There is excess of fatty acids in the serum , which promotes conversion of excess fatty acids into phospholipids and cholesterol in liver. These two substances along with excess triglycerides formed at the same time in liver may be discharged into blood in the form of lipoproteins.

In parallel results, Mir *et al*. (2008) found high concentration of total lipid in serum of diabetic rabbits and attributed this elevation mainly to increase mobilization of free fatty acids from the peripheral fat depots , since insulin inhibits the hormone sensitive lipase.

Significant amelioration in lipid indices was observed after treatment of diabetic rats with the test extracts (petroleum ether , chloroform and 70% ethanol) with percentage of amelioration amounting to 71.51, 62.13 , 67.75 %, respectively for total cholesterol(as compared to 127.15% in Gliclazide-treated diabetic group). HDL-cholesterol recorded enhanced level of 41.02, 39.35, 43.00 %, respectively as compared to 44.00% for Gliclazide. The improved level of LDL- cholesterol reached to 122.73, 110.64, 118.72 %, respectively (153.74 %for Gliclazide). Triglycerides showed normalized level of 165.05, 169.18, 176.77% respectively and 166.17 % for Gliclazide. Total lipid recorded percent of improvement amounted to 40.19, 44.42, 49.33%, respectively (58.25% for Gliclazide). In concomitant with the present results, El - Hilaly and Lyoussi (2002); Brinker *et a*l. (2007) found relative high percentage of essential oil with nerol, geraniol and citrol as well as ursolic acid, polyphenols (flavonoids , phenolic acid) and steroids in *Nepeta cataria* (Lbiatae) which may related to hypolipidemic and cholesterol –lowering effect. The mechanism(s) of hypolipidemic effect of many medicinal plants such as *Ajuga iva (L.) Schreber L.* (Liabiatae) was mediated through insulin –enhancing lipolytic activity by inhibition of hormone –sensitive lipase (Al- Shamaony *et al.*, 1994) or lipogenic enzymes (Pari ;Venkates, 2004) , and / or activation of lipoprotein lipase (Ahmed *et al.,* 2001). In addition, the hypolidimic lowering effect may be related to several active constituents extracted such as diglycerides , ecdysones , ecdysterones , iridoides , phenylcarboxylic acids , steroid compounds which is considered as anti-inflammatory agents (Brinker *et al*., 2007) , thus the mechanism(s) of action of such family (Labiatae) as antihyperlipidemia may involve insulin – like effect (Khushbaktova *et al*., 2001).

However ,Sethi *et al*. (2004) found that, leaves of *Ocimum sanctum* (Labiatae) significantly reduce lipid profile in serum and tissue in normal and diabetic rats through inhibition of oxygen free radical incorporated in pathogenesis of diabetes and enhancement of cellular enzymatic (SOD) and non enzymatic antioxidants (GSH).

The present results demonstrate, insignificant change in AST level in serum of normal treated rats either with chloroform or total ethanol extracts, while significant inhibition was noticed with petroleum ether extract. Serum total protein content and ALT showed insignificant change in different normal –treated groups. Considering ALP, significant inhibition was noticed in all normal treated groups. With respect to different diabetic- groups, high serum levels of these enzymes at day 2, 10 and 40 post STZ treatment was recorded which are associated with inflammation and /or injury to liver cells, a condition known as hepatocellular liver injury and apopotosis (Fiordaliso *et al.*,2000). Histological examination of diabetic rat liver showed congestion, destruction of cells by necrosis and hepatocytes degeneration. In parallel with the present work, Hickman *et al.* (2008) revealed significant increased activities of serum enzymes relative to their normal levels. Supporting our findings, it has been found that hyperglycemia resulted in hepatolysis reflected by histopathological investigation and increased blood serum aminotransferases as one of the consequences of diabetic complication. The increment of such serum markers may be due to the leakage of these enzymes from the liver cytosol into the blood stream as a result of hepatomegaly (fatty liver) (Muhammad *et al.,* 2008).

The present results reveal also, insignificant change in serum total protein content at day 2, 10 post STZ injection and significant reduction at day 40. In concomitant with the present results , Otsuki ; Williams (1982) found significant reduction in serum total protein concentrations in diabetic rats and this may be due to reduction in the three major phases in protein secretion, intracellular transport and discharge. Also, Alderson *et al.* (2004) demonstrated a significant increase of total protein excretion, albuminuria, glucosuria and urinary urea levels indicating impaired renal function. The reduction in serum total protein content in the present results may be related to reduction in albumin which is the most abundant blood plasma protein (70%) produced in liver. Non –enzymatic glycation of albumin was found the potential to alter its biological structure and function (Mendez *et al.*, 2005). It is mainly due to the formation of a Schiff base between amino – group of lysine (and sometimes arginine) residues and excess glucose molecules in blood to form glycoalbumin. Elevated glycoalbumin was observed in diabetes mellitus accompanied by decrease in albumin and this is confirmed with early studies suggested that the level of glycosylated albumin may indeed be a sensitive indicator of moderate hyperglycemia and of early glucose intolerance (Miwa *et al.*, 2005). Hypoalbuminemia is one of the factors responsible for the onset of ascites related to liver fibrosis (Horie *et al*., 1998).

Significant improvement in liver function enzyme markers was noticed in treatment of diabetic rats with petroleum ether , chloroform as well as 70% ethanol extracts with percent of amelioration amounted to 42.48, 39.38, 42.92%, respectively for AST as compared to 37.17% for Gliclazide .While ALT recorded 55.63, 47.5, 55.63 %, respectively (58.13 % for reference drug). ALP showed ameliorated level amounted to 94.39, 101.18, 91.15% respectively as well as 100.88% for Gliclazide drug. It was shown that administration of successive extracts and 70% ethanol one to diabetic rats reflect an improvement of cellular damage as determined in the current research by histopathological examination of liver and pancreas (Figs 25-28;14-17) and as shown by normalization of altered liver enzymes in response to diabetic complications. Our results are consistent with previous studies that administration of some antioxidants (as zinc , selenium , vitamin C and E) to diabetic rats , normalized the elevated activities of liver function enzymes AST, ALT, ALP induced in response to diabetes mellitus (Abdel Mageed, 2005). The mechanism of hepatoprotective ability of extracts may be attributed to numerous bioactive compounds such as terpenoids, flavonoids , sterols , essential oil, alkaloids and polysaccharides. Most of them (especially falvonoids, triterpenoids such as ursolic acid) showed a mechanism to improve the function of liver and pancreas cells and hence normalization of liver enzymes (El Hilaly and Lyoussi , 2002; Li *et al.*, 2004 ; Zheng *et al*. 2007 ; Gilani *et al.,* 2009).

Significant decrease was observed in serum total protein content in different–diabetic treated groups as compared to both normal control and Gliclazide -treated diabetic group .In contrast, Otsuki ; Williams (1982); Sethi *et al.* (2004) demonstrated enhanced level of serum proteins post treatment of diabetic rats with aqueous extract of *Ocimum sanctum* (Labiatae) and attributed this effect to insulin –like factors contained in the extract , since insulin is reported to increase protein synthesis. In addition, the total thiols in Labiatae family play a vital role in the structure, activity and transport function of proteins, membranes and enzymes.

With respect to oxidative stress marker NO, insignificant change was observed in normal control treated group as compared to untreated one, while a significant increase is noticed in various diabetic groups. It was reported that NO over production has been linked to a variety of clinical inflammatory diseases (Kim *et al*., 2002). Experimental studies suggested that NO may be responsible for increased liver injury (Ma *et al*., 1995). The direct toxicity of NO is enhanced by reacting with superoxide radical to give powerful secondary toxic oxidizing species, such as peroxynitrite (ONOO) which is capable of oxidizing cellular structure and causes lipid peroxidation (Sayed Ahmed *et al.,* 2001), a process leading to membrane damage and considered the proximal cause of cell death . Lipids peroxidation can damage protein, lipid , carbohydrates and nucleic acids . Also, it has been found that lipid peroxidation is one of the risk factor of protein glycation. The present results indicate, significant elevation in NO in liver of diabetic rats. This increment may be due to oxidative stress which is considered as one of the necessary causative factors that link diabetes with the pathogenic complications of several tissues (Anwar and Meki , 2003) .

Significant improvement in NO level post various treatments with percent amounted 146.15, 176.92 , 169.23% for chloroform, petroleum and 70% ethanol extracts respectively and 169.23% for Gliclazide . In concomitant with the present research , Sethi *et al*. (2004) ; Vats *et al*. (2004) found that treatment of diabetic rats either with aqueous or ethanolic extracts of *Ocimum sanctum* (OS -Labiatae) significantly increased activity of two antioxidant enzymes in liver namely , superoxide dismutase (SOD) and catalase . The protective effect of the plant extracts can be brought about directly by scavenging free radicals or indirectly by elevating glutathione levels (GSH). GSH protects the cell against oxidative stress by reacting with peroxiodes and hydroperoxides . SOD detoxifies superoxide radicals and converts them to H_2O_2 which is further converted to H_2O by catalase. Thus, the antihyperglycemic activity of OS supplemented with its adaptogenic and antioxidant activity will be an ideal multi-prolonged treatment for managing diabetes as it will target the stress , catabolism and glycemic effects associated with disease . Moreover, Sembulingam *et al*. (2005) ascertained the reduction in the NO level by the component ursolic acid separated from OS. Essential oil in many species in the Labiatae family are composed of mono –and – sesquiterpenes in addition to phenolic compounds and favonoids such as cinnamic acid , caffeic acid , sinapic acid , ferulic acid and rosmarinic acid have also been reported as antioxidants , free radical scavengers and metal chelators (Manosroi *et al*.,2006). In this context , Tepe *et al*. (2007) proved that , *Nepeta flavida(Nepeta species)* essential oils have various biological effects , including antioxidant activity due to the presence of 1,8-cineole , phenolic compounds especially terpenoids and phenolic acids. Furthermore, Souri *et al*. (2008) found that, different antioxidant and radical scavenging activity of several array of medicinal plants may partly be due to wide variety of antioxidant constituents such as phenolics, ascorbate and carotenoids .

Concerning carbohydrate hydrolyzing enzymes, ά-amylase, ά- glucosidase , β-galactosidase as well as liver total protein content ,insignificant change was observed in ά- amylase, β-galactosidase post different normal -treated groups as compared to untreated one . While significant increase in ά- glucosidase post treatment of normal rats with petroleum ether and total ethanol extracts (70%). Liver total protein content exhibited significant reduction in all normal –treated groups. Significant inhibition of all carbohydrate hydrolyzing enzymes was noticed in diabetic rats at the different durations post STZ treatment. However, total protein content showed insignificant change. Messer and Dean (1975) reported that liver and serum amylases are immunological identity and both are very similar to parotid gland and their differences from pancreatic amylase strengthens previous suggestion that liver is the main source of serum amylase and , further , eliminates the possibility of the pancreas being a source . In the same context, Terada ; Nakanuma (1995) found pancreatic enzymes in bile ducts and hepatocytes due to common cell lineage. The results of present study are in agreement with the previous reports that indicated a decreased in pancreatic amylase activity in diabetic rats (Otsuki and Williams, 1982). Moreover, the pancreatic content of ribonuclease is also significantly reduced in diabetic acini's. The fall in amylase content either in pancreas or in liver is may be due to increased secretion or intracellular degradation *in vivo* and a decreased rate of synthesis. In addition ,the reduction in amylase content is paralleled by a change in specific messenger RNA content suggesting that, insulin regulates the synthesis of amylase at the level of transcription (Otsuki and Williams, 1982). Kim *et al*. (1990) has reported that STZ induced diabetes resulted in reductions in glandular contents of DNA, RNA and amylase protein . However, the changes in amylase protein and its mRNA levels did not exactly parallel each other during diabetogenesis or subsequent insulin treatment. One reason for this discrepancy might be related to fluctuations in glands contents of amylase protein due to variations in the secretary activity of the glands in diabetic rats. The possibilities exists that the lower level of parotid amylase was related to an elevated rate of secretion due to increased mastication associated with hyperphagia in diabetic rats (Anderson , 1983). However, it is unlikely that the level of a secretary protein (amylase) mRNA will be reduced in glands with an increased secretary activity. The increase in secretary activity is likely to affect the glandular levels of all secretary proteins equally. Furthermore, Roy *et al*. (2005) found a decrease in amylase level in liver, parotid glands and pancreas during STZ induced diabetes and this is due to a decrease in the gene expression of amylase RNA.

With regard to α- glucosidase and β- glactosidase in liver of diabetic rat, significant inhibition was demonstrated that is parallel with the results of Otsuki and Williams (1982) who noticed reduced maximal amounts of digestive enzymes released from acini of diabetic rats and explained this inhibition at the basis of , reduced secretary capacity , alterations in nutritional or other hormones states , a decrease of secretagogues or a combination of these factors.

Significant improvement in all carbohydrate hydrolyzing enzymes post chloroform, petroleum ether as well as 70% ethanol extracts amounted 69.58, 106.37, 48.60%, respectively for amylase (as compared to 59.30 % for Gliclazide) ; 33.82, 53.31, 61.39 % respectively for α- glucosidase comparing to 66.91% in Gliclazide- treated diabetic group and 88.02, 68.88 , 59.88% respectively for β-galactosidase (77.84% for Gliclazide). The enhanced levels of carbohydrate hydrolyzing enzymes α-amylase , α- glucosidase and β-galactosidase may be related to the phenolic compounds inhibited the disaccharide enzyme activities as mucosal sucrase and maltase .The inhibition of glycolytic activity of brush border enzymes by polyphenolic compounds seems to be one of the factors which explain the discussed hypoglycemic action of *Nepeta cataria* (Jurgonski *et al*., 2008). The improvement in the level of carbohydrate metabolizing enzymes can be also explained at the basis of *Nepeta cataria* extracts contained fluctuated level of flavonoids . Flavonoids , like antioxidants may prevent the progressive impairment of pancreatic beta cell function due to oxidative stress and may thus reduce the occurrence of type 2 diabetes (Bhandari *et al*., 2008).

The present results demonstrate, insignificant change in normal -treated body weight, liver weight (except for liver weight treated with 70% ethanol extract which recorded significant increase as compared to normal control at day 40) and liver weight/ body weight ratio post treatment with chloroform ,petroleum ether and 70% ethanol extracts. Gradual significant reduction is noticed in body weight with concomitant increase in liver weight and liver weight / body weight ratio at day 2, 10 and 40 post STZ injection .In concomitant with the present results , several authors (Vats *et al*., 2004; Akbarzadeh *et al*. ,2007;Mir *et al*. ,2008) reported that diabetes state is usually accompanied by weight loss and increase in liver weight were seen in adult rats within three days of Streptozotocin induction. The literature regarding the effect of diabetes on liver weight is contradictory as some workers have shown an increase in hepatic weight in animals (Chen ; Ianuzzo, 1982;Sadique *et al*., 1987) as well as human (Van Lancker , 1976) while others have reported no change (Gupta *et al*., 1999) .Exact reasons of hepatic hypertrophy are not known , however fat deposition has been proposed to be the cause. The pattern of increase in liver weight /body weight ratio was manifested by the reduction in body weight and increase in liver weight of diabetic rats (Vats *et a*l., 2004).

Significant amelioration in body weight, liver weight and liver weight /body weight ratio post treatment of diabetic rats with chloroform , petroleum ether and 70% ethanol extracts as well as Gliclazide as reference drug amounted 13.81, 13.95 , 17.96, 30.25%, respectively for body weight 20.54, 49.62, 45.89 , 30.79% respectively for liver weight and 81.25, 112.5, 112.5 , 118.75% respectively for liver weight /body weight ratio .The enhancement in body weight may be attributed to anabolic action of ecdysones and ecdysterones found in Lbiatae family (EL Hilaly and Lyoussi, 2002). In concomitant with the present results , Vats *et al*. (2004) and Sembulingam *et al*.(2005) reported that ethanol extract of *Ocimum sanctum* treated rats showed higher and significant gain in body weight in comparison to diabetic controls but was lower than in the normal controls. In addition, it prevents increase in organ weight due to the protective action of urosolic acid concerned with free radical inhibition . Based on this findings, the ameliorative effect of *Nepeta cataria* may be due to various investigated phytochemicals compounds(as polyphenols, urosolic acid , essential oils, terpenoids , flavonids and phenolic acids) that can ameliorate physiological response to stress (Gilani *et al*.,2009).

In conclusion, the present study demonstrate the anti-glycemic, antioxidant, antilipidemic effects of chloroform, petroleum ether as well as 70% ethanol extracts in comparison with Gliclazide as reference antidiabetic drug. The present data reveal that ,these extracts have significant beneficial glycemic control, scavenging free radical, normalized liver function , inhibited lipid synthesis associated with diabetic complication, as well as they have principle role in treatment and amelioration liver damage at the cellular level. Thus, the safely promising therapeutic dose used in the current study, can be effective in treatment and enhanced liver tissue from the damage induced by diabetes and may candidate as natural antidiabetic drugs.

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The Mutagenic effects of Insecticide Telliton and Fungicide Dithane M-45 on Meiotic Cells and Seed Storage Proteins of *Vicia faba.*

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Abstract: The genotoxic effects of insecticide Telliton and fungicide Dithane M-45 were examined on meiotic cell divisions and changes in the M2 seed storage protein banding pattern of *Vicia faba* plants. The percentage of abnormal pollen mother cells, (PMCs) increased as the concentration of both pesticides increased. All concentrations and treatment periods of both pesticides, induced a number of chromosomal aberrations in PMCs as stickiness, bridges, laggards, disturbed, micronuclei and multinucleate. A marked change was observed in the M2 *V. faba* seed storage protein banding pattern. These changes included alterations in band intensity, relative mobilities, disappearance of some bands and appearance of new other ones. These results showed that Telliton has more mutagenic effects than Dithane M-45. [Journal of American Science 2010;6(8):456-462]. (ISSN: 1545-1003).

Key words: *Vicia faba,* chromosomal abnormalities, insecticide, fungicide and SDS -PAGE protein.

1. Introduction:

Pesticides are used all over the world, their use has increased spectacularly because it has greatly improved agricultural yield through inhibition of diseases by acting against pests in the field and during storage of agricultural products Taylor *et al.,* (1997). A number of pesticides are used to protect agricultural products from diseases, weeds and insects, but residues of these chemicals lead to environmental pollution and pose threat to people and animals. Although chemical control creates several problems, use of pesticides is still maintaining its popularity for obtaining effective results. Now, after increased application of many new agrochemicals on a large scale in the egyptian agriculture and other countries has led some workers to investigate the possible genetic material and storage proteins alterations Badr (1988), Abdel Salam *et al*., (1993a & b), Hassan (1996), George and Ghareeb (2001) Asita and Makhalemele (2009). Telliton known as profenophos is the insecticide commonly used in delta Egypt region in agricultural fields. Dithane M-45, also known as mancozed fungicide belongs to a class of chemicals as ethylene bisdithiocarbamate (EBDC).The EBDC is fungicide used to prevent crop damage in the field and to protect harvested crops from deterioration during storage or transport. Chromosomal aberrations have been considered as a reliable indicator of mutagenic activity, since there have been evidence for a correlation between chromosomal damage and toxic effects of a number of pesticides Badr (1983), Askin (2006), Shehata *et al*., (2008), Ozturk (2008) and Fisun and Goc Rasgele (2009)

on the other hand, many authors used electrophoretic banding patterns of seed storage proteins for monitoring the mutagenic effects of pesticides and other chemicals Abdelsalam *et al.,* (1993b) , Hassan *et al*., (2002) and George and Ghareeb (2001).

The present work was planned to study the mutagenic effects of the insecticide Telliton and fungicide Dithane M-45 as revealed by meiotic abnormalities and changes in M2 seed storage protein banding patterns of *V. faba* as a biological system.

2. Materials and Methods:

Vicia faba L. variety Giza 3, kindly procured from Crop Research Institute, Agricultural Research Center, Giza, Egypt.

1- Meiosis

Vicia faba plants at the flowering stage were sprayed with different concentrations of the insecticide Telliton, (*0*-4-bromo-2-chlorophenyl *0*-ethyl s-propyl phosphorothioate) 1.5, 3, and 6 ml/L and the fungicide Dithane M-45(ethylene-bis dithiocarbamate) 150, 300 and 600 mg/L. These pesticides selection were purely on the basis of the frequent use in the agricultural fields by the farmers of Delta, Egypt. A negative control plants were sprayed with distilled water. Eight flower buds from eight different plants were gathered through durations of 24 h., 48 h. and 10 days.

For meiotic studies the appropriate flower buds were collected and fixed in Carnoy's solution (ethyl alcohol absolute and glacial acetic acid in the ratio 3:1) for 24 h. and then transferred to 70% ethyl alcohol and kept in refrigerator. The cytological analysis were carried out by using 2% aceto-carmine stain as described by Darlington and La Cour(1976). The data recorded for different treatments were statistically analyzed using *t*-test for determine significant differences between these treatments.

2-Electrophoresis of water soluble and non soluble proteins:

Water soluble and non soluble proteins were performed on vertical slab (20 cm x 20 cm x 0.2 cm) using the gel electrophoresis apparatus (Manufactured by LABCONCO) according to Laemmli (1970). The dry M2 seeds of *V. faba* plants, whose parents were sprayed with these pesticides, were decoated and milled to fine powder. Soluble proteins were extracted overnight using 0X Tris-Hcl buffer of pH 6.8. Centrifugation was performed at 10000 rpm for 10 min., then the non soluble proteins were extracted from the belt by add IX Tris-Hcl buffer pH 6.8 for 24 h. and then centrifuged at 10000 rpm for 10 min., then 40 µl supernatant of soluble and non-soluble proteins were loaded in SDS-slab gel of 15% acrylamide containing 10% SDS. Gel was run at a current of 15 mA for 1 hour followed by 25 mA for 4-5 h. Molecular weights of different bands were calibrated using the wide range protein marker ranged from 10 -200 KDa according to Matta *et al.,* (1981).

3. Results and Discussion:

I-Cytological studies:

A wide spectrum of chromosomal abnormalities were recorded in eight flower buds from different plants after treatment with different concentrations of Telliton (1.5, 3 & 6 ml/L) and Dithane (150, 300 & 600 mg/L). The number of meiotic cells of treated and control plants are presented in Tables (1& 2). The insecticide Telliton give the number of chromosomal abnormalities higher than fungicide Dithane. Both pesticides caused a hollow range of meiotic abnormalities. The number of abnormal pollen mother cells (PMCs) formed in the flower buds of *V. faba* plants was obvious with all concentrations of pesticides and in all stages and durations.

Data in Tables 1 & 2 shows that the percentages of abnormal PMCs in the first division were greater than those recorded in the second division after spraying with both pesticides. The most frequent types of abnormalities were observed stickiness, laggards, bridges, disturbed, micronuclei and multinucleate after being treated with all concentrations of both pesticides. These results demonstrated in Tables (1&2) and Fig.1 revealed that the abnormalities were present in metaphase, anaphase and telophase stages of the meiosis with all treatments. The induction of meiotic abnormalities appears to be a common effect of most pesticides (Fisun & Goc Rasgele, 2009).

The stickiness and disturbed stages were the most common abnormalities found in all phases of the meiosis after treatments with all doses of both pesticides (Fig.1). The number of sticky cells increased in all stages of meiotic divisions as the concentration of both pesticides increased during durations of 24 h, 48 h and 10 days. Our results are in agreement with the results of Badr (1988); Pandey *et al.,* (1994); Singh *et al.* (2007) and Srivastava & Singh (2009). Abdelsalam *et al.,* (1993b) suggested that the chromosome stickiness may results from breakage and exchange between chromatin fibers on the surface of adjoining chromosomes.

The second type of abnormalities is the laggard that occurred at metaphase cells. They could be attributed to the failure of the spindle apparatus to organize and function in a normal way Pickett-Heaps *et al*., (1982). These laggards may be distributed randomly to either poles at anaphase I or II which result ultimately in aneuploidy (Amer & Mikhael, 1987; Amer & Ali, 1988) or may give for micronuclei at telophase II (Abdelsalam *et al*., 1993 a). The induction of laggard chromosomes could be attributed to irregular orientation of chromosomes (Patil and Bhat, 1992).

In addition to the previous common abnormalities, it was observed more on meiotic division including bridges, micronuclei and multinucleate. Bridges were induced under the treatment with both pesticides. They could be due to the breakage and reunion (El-Khodary *et al*., 1990) or due to the general stickiness of chromosomes (Haliem, 1990).While, micronuclei and multinucleate were also recorded with low percentages after treatment with both pesticides and our results are in agreement with the results of Badr (1988) and Pandey *et al.*,(1994). Finally, the induction of these chromosomal abnormalities were pointed to the mutagenic potential of the applied concentrations of these pesticides.

II-Biochemical studies:

At the biochemical genetic level, water soluble and non-soluble protein, Table (3 & 4) and Fig. (2) represent the mutagenic effects of both pesticides, Telliton and Dithane on the banding pattern of M2 seed storage proteins of *V. faba* plant. These changes include alterations in band intensity, relative mobilities, disappearance of some bands and appearance of some new other bands.

Alterations in bands intensity could be attributed to change in the structure or performance of genes and thus they produce changes in the gene expression of the regulator genes used in the regulatory system of the structural genes Hassan (1996). The increase in band(s) intensity could be attributed to gene(s) duplication that resulted from cytological abnormalities induced by applied pesticides. The presence of laggards and bridges support this conclusion. This conclusion is in agreement with Gamal El-Din *et al*., (1988).Also, they noticed that increasing the number of genes encoding for

the different protein subunits through doubling of chromosome number from 12 to 24 in *V. faba* caused an increase in band intensity.

Changes in relative mobility of these bands are probably due to point mutation that leads to production of shorter or longer polypeptide chains. These changes in the soluble proteins are probably due to the occurrence of gene duplication mutation more than point mutation that takes place in one or more of the duplicated genes that encoding the protein subunit of that band Abdelsalam *et al*., (1993b). Also, these alterations in bands intensities or densities and relative mobility are in agreements with Hassan (1996), George and Ghareeb (2001) and Hassan *et al*., (2002).

The disappearance of some bands in soluble and non soluble proteins of *V. faba* to the inherited effects of the both pesticides, Telliton and Dithane could be explained on the basis of mutational event at the regulatory genes that prevent or attenuate transcription (Muller &Gottschalk, 1973). Induction of laggards, bridges and micronuclei by these pesticides may lead to the loss of genetic materials. Therefore, some electrophoretic bands were disappeared due to the loss of their corresponding genes (Abdelsalam *et al.,* 1993b). They also reported that the reduction of chromosome complement in *V. sativa* (2n=6) lead to the complete disappearance of the convicilin like band. The present results therefore may point out a mutagenic potential of both pesticides, Telliton and Dithane as indicated by observing a large number of the meiotic abnormalities and the heritable changes in the M2 seed storage protein banding patterns.

Table (1): Numbers and percentages of abnormal PMCs in the1st & 2nd meiotic divisions, percentages of types and mean of meiotic abnormalities after spraying of *V.faba* plants with Telliton insecticide for (24, 48 hours & 15 days).

Time	Conc. In Ml/L	No. of counted PMCs	No. of abnormal PMCs	% abnormal In 1st division	% abnormal In 2nd division	Types and percentages of meiotic abnormalities						Mean of % abnormal PMCs ± SE
						Stick.	Lag	Brid.	Dist.	Micronuclei	Multinuclei	
24 h	Cont.	7684	12.00	0.19	0.11	25.00	16.66	8..33	50.00	-	-	0.16±0.03
	1.5	5761	479	8.15	8.49	29.23	13.36	7.72	47.59	0.83	1.25	8.31±1.04
	3	4677	718	18.14	12.68	35.93	12.39	6.82	42.06	1.11	1.67	15.35±1.32
	6	3471	924	28.43	24.89	42.09	11.04	6.17	35.60	1.62	3.46	26.62±1.12
48h	1.5	3023	567	10.55	8.20	31.75	13.76	7.41	43.56	1.59	1.94	9.38±1.12
	3	2968	656	13.00	9.10	31.09	14.18	7.32	42.84	1.98	2.59	11.05±1.22
	6	2741	798	17.10	12.01	35.34	14.16	6.52	39.59	1.50	2.88	14.55±1.34
10 days	1.5	4672	390	4.64	3.70	28.97	12.05	7.43	50.25	0.51	0.77	4.17±0.94
	3	4195	506	7.44	4.67	33.00	13.04	6.32	44.66	1.78	1.19	6.06±1.01
	6	3841	565	8.25	6.46	35.75	12.92	6.55	41.42	1.42	1.95	7.36±1.32

Stick: stickiness Lag.: laggards Brid.: bridge Dist.: disturbed PMCs : pollen mother cells

Table (2): Numbers and percentages of abnormal PMCs in the1st & 2nd meiotic divisions, percentages of types andmean of meiotic abnormalities after spraying *V. faba* plants with Dithane fungicide for (24, 48 hours & 15 days).

Time	Conc. In mg/L	No. of counted PMCs	No. of abnormal PMCs	% Abnormal In 1st division	% Abnormal In 2nd division	Types and percentages of meiotic abnormalities						Mean of % abnormal PMCs SE
						Stick.	Lag	Brid.	Dist.	Micronuclei	Multinuclei	
24 hr	Cont.	7684	12.00	0.19	0.11	25.00	16.66	8.33	50.00	-	-	
	150	6142	384	3.58	2.67	25.26	14.06	10.67	47.14	0.52	1.82	0.16±0.03
	300	5639	463	4.58	3.64	24.62	16.41	11.66	43.84	1.08	2.37	3.12±0.90
	600	5173	628	6.57	5.56	29.30	18.78	11.46	36.15	1.91	2.39	4.11±1.10
48hr	150	5672	298	2.96	2.29	26.17	14.42	12.08	43.28	1.67	2.35	6.07±1.20
	300	5113	315	3.23	2.93	26.66	16.50	13.33	40.63	0.95	1.90	2.63±0.80
	600	4370	362	4.53	3.75	26.79	16.85	13.81	37.56	1.65	4.42	3.08±1.02
10 days	150	6176	256	2.14	2.01	23.04	14.84	12.11	45.31	0.78	3.91	4.14±1.32
	300	6214	291	2.43	2.25	24.39	15.81	13.05	41.24	1.72	3.78	2.07±1.00
	600	5365	310	3.31	2.46	26.45	16.45	15.81	36.77	1.94	2.58	2.34±1.36

Stick: stickiness, Lag.: laggards, Brid.: bridge, Dist: disturbed, PMCs : pollen mother cells.

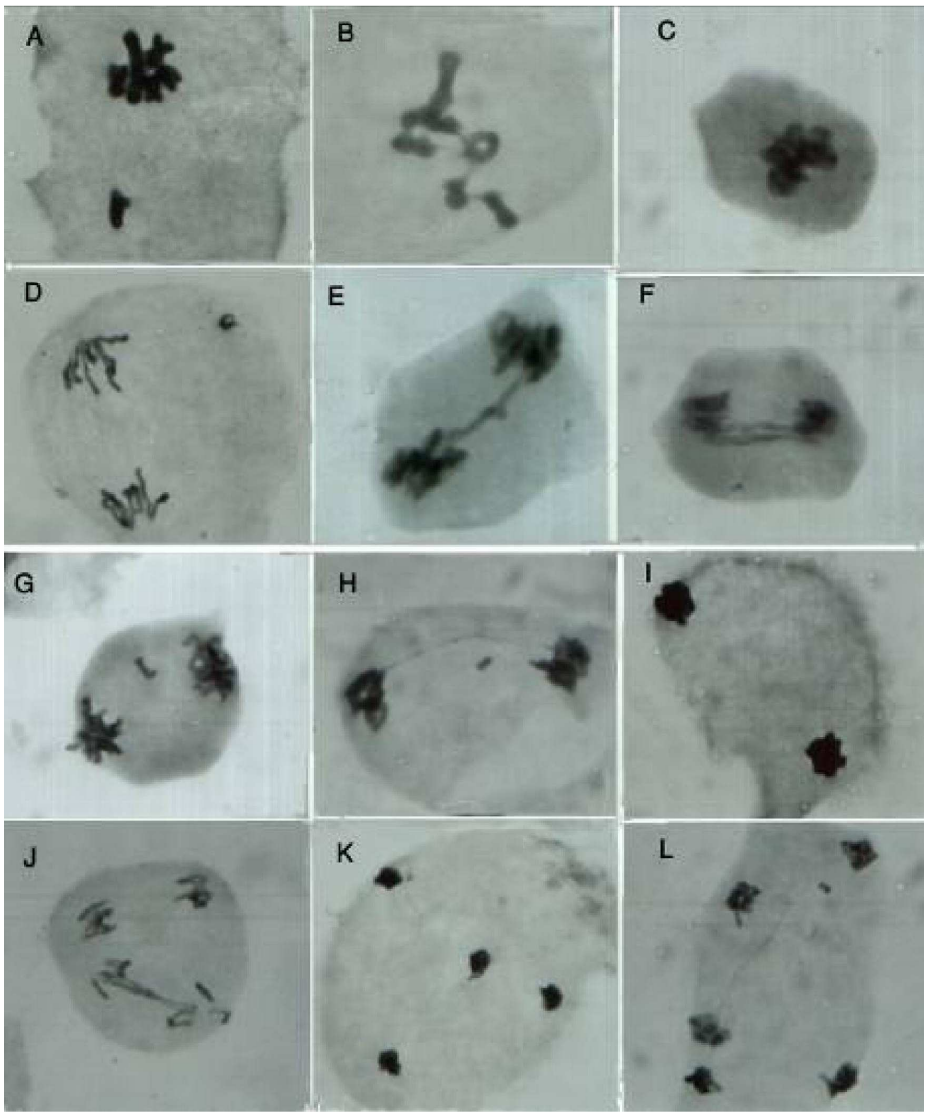

Fig (1): Types of chromosomal abnormalities produced after treatments with different concentrations of Telliton and Dithane. A- metaphase I with laggard. B- metaphase I with ring. C- metaphase I with sticky. D- anaphase I with laggard. E- anaphase I with bridge. F- anaphase I with double bridges. G- telophase I with laggard. H- telophase I with broken bridge and laggard. I- telophase I with sticky .J- anaphase II with double bridges. K- disturbed telophase 2. L- telophase II with multinucleate and micronucli.

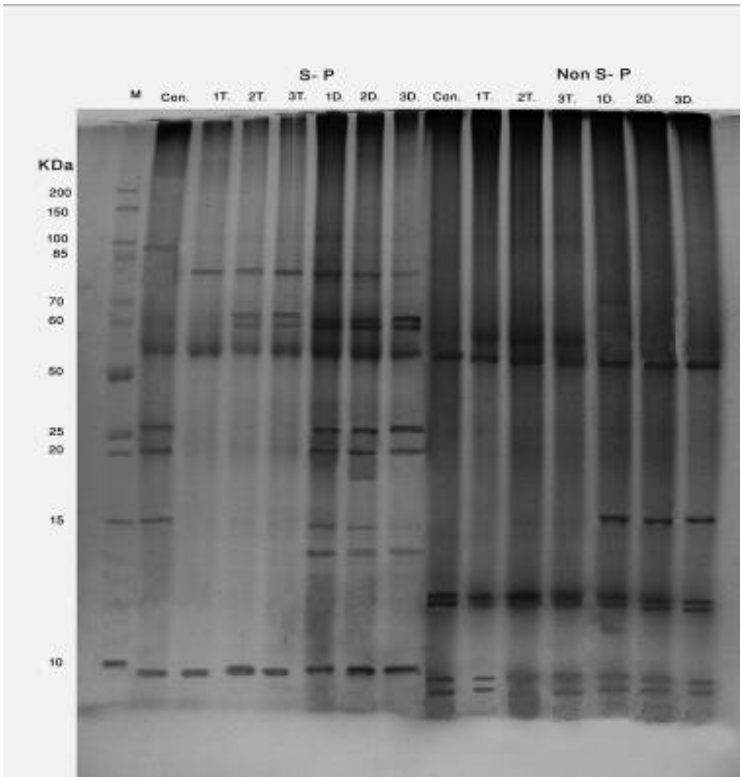

Fig.(2): SDS–PAGE banding patterns of water soluble (s-p) & non-soluble (non s-p) proteins for *V. faba* after sprayed with three concentrations of Telliton (T) and Dithane (D) pesticide.

Table (3): Electrophoretic of water soluble protein banding patterns of *V. faba* seed storage protein showing the effects of three concentrations of Telliton (T) & Dithane (D) pesticides.

No. of band	MW	RF	Con.	1T	2T	3T	1D	2 D	3 D
1	102.939	0.139	+2	-	-	-	-	-	-
2	92.218	0.172	-	+2	+2	+3	+2	+2	+
3	69.238	0.258	-	-	+2	+2	+3	+3	+3
4	65.207	0.276	-	-	+2	+2	+3	+3	+3
5	56.501	0.319	+2	+2	+	+2	+3	+3	+2
6	34.965	0.463	+2	-	-	-	+2	+2	+2
7	30.196	0.507	+2	-	-	-	+2	+2	+2
8	19.320	0.641	+	-	-	-	+	+	-
9	14.171	0.734	-	-	-	-	+	+	+
10	7.325	0.932	+2	+	+2	+2	+2	+2	+2

- : Missed +: Fait +2: Dark +3: Very dark

Table (4): Electrophoretic of water non-soluble protein banding patterns of *V. faba* seed showing the effects of three concentrations of Telliton (T) & Dithane (D) pesticides.

No. of band	MW	RF	Con.	1 T	2 T	3T	1D	2D	3D
1	63.107	0.287	-	+2	+2	+2	-	-	-
2	55.478	0.327	+2	+2	+2	+2	+2	+2	+2
3	19.982	0.644	-	-	-	-	+3	+3	-
4	13.359	0.769	+2	+2	+2	+2	+2	+2	+2
5	12.485	0.790	+2	+2	+2	+2	+2	+2	+2
6	8.056	0.926	+2	+2	+	+	+	+	+
7	7.505	0.948	+2	+2	+	+	+	+	+

- : Missed + : Fait +2: Dark +3: Very dark

4. Conclusion

From the present data it may be concluded that treatment of *V. faba* plants with different concentrations of Telliton and DithaneM-45 showed positive chromotoxic effects in PMCs and changes in the M2 seed storage protein banding patterns. These effects included chromosomal abnormalities such as stickiness, laggards, bridges, disturbed, micronuclei and multinucleate. While at the biochemical level, the obtained data showed several changes in M2 seed storage protein banding pattern included alterations in band intensity, alterations in the relative mobilities of some bands, disappearance of some bands and appearance of new other bands of protein banding patterns, as compared with the negative control.

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Synthesis, (in vitro) Antitumor and Antimicrobial Activity of some Pyrazoline, Pyridine, and Pyrimidine Derivatives Linked to Indole Moiety

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Abstract: Aldol condensation reaction between 3-indolaldehyde 1 and 4-methoxyacetophenone 2 afforded chalcone compounds 3. This compound was reacted with some different reagents such as hydrazine hydrate, phenyl hydrazine, thiosemicarbazide, hydroxylamine, ethyl cyanoacetate, urea and thiourea to give pyrazolines 4a, 4b, 5a, 5b, 6, oxazoline 7, Michael adduct 8, pyranone 9, and oxo 14a and thiopyrimidine derivatives 14b, respectively. The structures of all the compounds were confirmed by microanalyses and various spectral data. Some of the synthesized new compounds were screened against antitumor and antimicrobial activity. [Journal of American Science 2010;6(8):463-471]. (ISSN: 1545-1003).

Keywords: Heterocycles, cyclizations, pyrazolines, pyridines, antitumor activity.

1. Introduction

Indole nucleus is incorporated in various natural products such as alkaloids and represented a promising structural class of marine alkaloids based upon their high degree of biological activity 1-5. Indole nucleus was reported to possess a wide variety of biological properties such as anti-tumor 6-10, anti-inflammatory 11-13, anti-convulsant 14, cardiovascular 15, and anti-bacterial 16. In addition, the substitution at 3-position of the indole ring connecting an extra heterocyclic ring such as imidazole (topsentins 17 and nortopsentins 18); dihydroimidazole (discodermindole 3); oxazole (martefrgin 4 amazol 19); oxadiazine (alboinon 20); (didemidines 21) furnished potent agents. Also pyrazole 22, 23, pyridine 24 and pyrimidine 25 derivatives were found to possess a variety of biological activities. Considering the above observations and in connection to previous publications involving the synthesis of new biologically active hetero cycles 26-29. I hope to report here in the synthesis of new 3-substituted indoles incorporating an extra heterocyclic rings such as pyrazole, pyridine and pyrimidine to evaluate their antitumor (anti-proliferative) activities against both of human breast cell line MCF- 7 and liver carcinoma cell line HEPG2 and their antimicrobial activities.

2. Experimental

Chemistry

Melting points were determined in open glass capillaries and are uncorrected. Elemental analyses were carried out in the Micro Analytical Laboratory, Cairo University, Cairo, Egypt, IR spectra of the compounds were recorded on a Perkin-Elmer; spectrophotometer model 1430 using potassium bromide pellets and frequencies were reported in cm^{-1}. The mass spectra were recorded using mass spectrometer HP model MS5988 EI 70eV. ^{1}HNMR spectra were measured on Varian Mercury 300 MHz spectrometer and chemical shifts were expressed in δ ppm using TMS as internal standard. Reactions were routinely followed by (TLC).

3-(1*H*-Indol-3-yl)-1-(4-methoxyphenyl)prop-2-en-1-one 3.

To a stirred mixture of 14.5 g indolaldehyde 1 (100 mmol) and 15.0 g 4-methoxyacetophenone 2 (100 mmol) in 200 mL ethanol at room temperature, 40% NaOH aqueous solution was added portion-wise after which stirring was continued for further 2 hr. The pale yellow precipitate formed was filtered and washed with 3% aqueous HCl, and crystallized from ethanol to give chalcone 3 in 75% yield, mp 124-126ºC. IR (KBr): 3162 (NH), 1633 (C=O) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): 3.83 (s, 3H, -OCH_3), 7.01-7.05 (m, 2H, Ar-H), 7.24-7.27 (m, 4H, Ar-H), 7.49-7.52 (m, 2H, Ar-H), 7.53 (d, 1H, J = 12.9 Hz, (C=O)(CH=C), 7.92 (d,1H , J = 12.9 Hz (C=O)(C=CH), 8.11-8.30 (m, 1H, Ar-H), 12.3 (s, D_2O-exchangeable, 1H, indole NH) ppm; MS (70 eV): *m/z* = 278 (M^++1, 4.7), 277 (M^+, 15.0), 144 (100), 116 (39.5), 89 (37.3), 62 (38,6). Anal. Calcd. for $C_{18}H_{15}NO_2$: C, 77.96; H, 5.45; N, 5.05 Found: C, 78.24; H, 5.59; N, 4.93.

3-(4,5-Dihydro-3-(4-methoxyphenyl)-1*H*-pyrazol-5-yl)-1*H*-indole 4a.

To a solution of 2.77 g chalcone 3 (10 mmol) in 50 mL of ethanol, 1.0 mL of hydrazine hydrate (80%) was added and the reaction mixture was refluxed for 4 hr , cooled to -10 °C and left over night. The solid mass separated out was filtered, washed with cold ethanol and crystallized from ethanol to afford pyrazoline derivative 4a as pale yellow crystals in 78% yield, mp 258-260ºC; IR (KBr): $\bar{\nu}$ = 3215, 3170 (2NH), 1618 (C=N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 1.97 (m, 1H), 2.64 (m, 1H), 3.39 (m, 1H), 3.75 (s, 3H, -OCH_3), 6.86-8.36 (m, 9H, Ar-H), 8.90 (s, D_2O-exchangeable, 1H, pyrazoline NH), 11.65 (s, D2O-exchangeable, 1H, indole NH) ppm; MS (70 eV): m/z = 291 (M^+, 7.3), 286 (94.9), 257 (100), 142 (36.3), 116 (45.4), 89 (47.2). Anal. Calcd. for $C_{18}H_{17}N_3O$: C, 74.20; H, 5.88; N, 14.42. Found: C, 74.42; H, 5.67; N, 14.38.

3-(4,5-Dihydro-3-(4-methoxyphenyl)-1-phenyl-1*H*-pyrazol-5-yl)-1*H*-indole 4b.

To a solution of 2.77 g of chalcone **3** (10 mmol) in 50 mL of ethanol, 5 mL of acetic acid and 1.1 g of phenyl hydrazine (10 mmol) was added. The reaction mixture was refluxed for 8 hr and left over night. The solid mass separated out was filtered off, washed with ethanol and crystallized from ethanol to give compound 4b as pale brown crystals in 67% yield, mp 258-260ºC; IR (KBr): $\bar{\nu}$ = 3169 (NH), 1633 (C=N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.00 (m, 1H), 2.51 (m, 1H), 3.11 (m, 1H), 3.35 (s, 3H, -OCH_3), 6.85-7.00 (m, 2H, Ar-H), 7.18-7.21 (m, 2H, Ar-H), 7.24-7.26 (m, 1H, Ar-H), 7.27-7.30 (m, 4H, Ar-H) ,7.47-7.49 (m, 2H, Ar-H), 8.32 (s, 1H, Ar-H), 12.15 (s, D_2O-exchangeable, 1H, indole NH) ppm; MS (70 eV): *m/z* = 367 (M^++1, 3.5), 368 (M^+, 2.8), 145 (100), 221 (10.7). Anal. Calcd for $C_{24}H_{21}N_3O$: C, 78.45; H, 5.76; N, 11.44. Found: C, 78.43; H, 5.59; N, 11.48.

Synthesis of pyrazolines 5a, b

This reaction was carried out by the same procedure described in the synthesis of compound 4a by using acetic acid instead of ethanol in case of 5a and propanoic acid in case of 5b.

1-(4,5-Dihydro-5-(1*H*-indol-3-yl)-3-(4 methoxyphenyl)pyrazol-1-yl)ethanone 5a.

Pale yellow crystals, mp 288-290°C, yield 77%; IR (KBr): $\bar{\nu}$ = 3169 (NH), 1686 (C=O), 1627 (C=N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 1.93 (m, 1H), 2.24 (s, 3H, -CH_3), 2.49 (m, 1H), 3.36 (s, 3H, -OCH_3), 3.83 (m, 1H), 7.14-7.26 (m, 4H, Ar-H), 7.27-7.49 (m, 2H, Ar-H), 7.90-7.92 (m, 1H, Ar-H), 8.16-8.36 (m, 2H, Ar-H), 11.68 (s, D_2O-exchangeable, 1H, indole NH) ppm; MS (70 eV): *m/z* = 334 (M^++1, 2.4), 333 (M^+, 2.9), 286 (100), 257 (95.4), 116 (28.0). Anal. Calcd for $C_{20}H_{19}N_3O_2$: C, 72.05; H, 5.74; N, 12.60. Found: C, 71.87; H, 5.66; N, 12.49.

1-(4,5-Dihydro-5-(1*H*-indol-3-yl)-3-(4-methoxyphenyl)pyrazol-1-yl)propan-1-one 5b.

Yellow powder, mp 280-281°C, yield 73%; IR (KBr): $\bar{\nu}$ = 3165 (NH), 1675 (C=O), 1630 (C=N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 1.8-2.2 (t, 3H, -CH_3), 2.01 (m, 1H), 2.70 (m, 1H), 2.62-2.74 (q, 2H, -CH_2), 3.43 (m, 1H), 3.78 (s, 3H, -OCH_3), 6.86-6.88(m, 1H Ar-H),6.90-6.93 (m, 2H, Ar-H), 7.16-7.26 (m, 4H, Ar-H), 7.41-7.50 (m, 2H, Ar-H), 11.52 (s, D_2O-exchangeable, 1H, indole NH) ppm; MS (70 eV): *m/z* = 348 (M^++1, 1.8), 347 (M^+, 5.5), 142 (100), 131 (31.2). Anal. Calcd for $C_{21}H_{21}N_3O_2$: C, 72.60; H, 6.09; N, 12.10. Found: C, 72.44; H, 6.24; N, 12.26.

4,5-Dihydro-5-(1*H*-indol-3-yl)-3-(4-methoxyphenyl)pyrazole-1-carbothioamide 6.

To a solution of 2.77 g of chalcone **3** (10 mmol) in 50 mL of ethanol, 1.0g of sodium hydroxide (25 mmol) and 1.2 g of thiosemicarbazide (12 mmol) was added. The mixture was refluxed for 6 hr then left to cool overnight, the formed solid product was filtered off, dried, and then crystallized from ethanol to give compound 6 as yellow powder in 69% yield, mp 228-230 °C; IR (KBr): $\bar{\nu}$ = 3316, 3227 (NH_2, NH), 1606 (C=N), 1251 (C=S) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.22 (m, 1H),2.51 (m, 1H), 3.37 (m, 1H,), 3.80 (s, 3H, -OCH_3), 7.09-7.20 (m, 2H, Ar-H), 7.23-7.44 (m, 4H, Ar-H), 7.81-8.00 (m, 2H, Ar-H),8.24-8.31(m,1H, Ar-H), 11.11 (s, D_2O-exchangeable, 2H, NH_2), 11.60 (s, D_2O-exchangeable, 1H, indole NH), ppm; MS (70 eV): *m/z* = 351 (M^++1, 1.6), 350 (M^+, 4.0), 142 (100), 217 (61.3), 200 (54.5), 116 (56.0). Anal. Calcd for $C_{19}H_{18}N_4OS$: C, 65.12; H, 5.18; N, 15.99; S, 9.15. Found: C, 65.04; H, 5.16; N, 16.12; S, 9.07.

3-(4,5-Dihydro-3-(4-methoxyphenyl)isoxazol-5-yl)-1*H*-indole 7.

A mixture of 2.77 g of chalcone 3 (10 mmol), 0.7 g of hydroxylamine hydrochloride (10 mmol) and 1.4 g of anhydrous potassium carbonate (10 mmol) in (50 mL) of ethanol was refluxed for 8 hr, then left to cool. The reaction mixture was poured into cold water and the solid product was filtered off, washed with water, dried and finally crystallized from ethanol to afford isoxazole derivative 7 in 68 % yield as pale yellow crystals, mp 138-140 ºC; IR (KBr): $\bar{\nu}$ = 3386 (NH), 1640 (C=N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.11 (m, 1H), 2.52 (m,

1H), 3.47 (m, 1H) 3.72 (s, 3H, -OCH_3), , 7.12-7.16 (m, 2H, Ar-H), 7.19-7.23 (m, 4H, Ar-H), 7.43-7.50 (m,2H, Ar-H), 8.28-8.33 (m,1H, Ar-H), 11.61 (s, D_2O-exchangeable, 1H, indole NH) ppm; MS (70 eV): *m/z* = 293 (M^++1, 1.3), 292 (M^+, 4.8), 160 (98.1), 117 (100), 89 (36.6). Anal. Calcd for $C_{18}H_{16}N_2O_2$: C, 73.95; H, 5.52; N, 9.58. Found: C, 74.11; H, 5.41; N, 9.70.

Ethyl-2-cyano-3-(1*H*-indol-3-yl)-5-(4 methoxyphenyl)-5-oxopentanoate 8.

To a stirred solution of 2.77 g of chalcone 3 (10 mmol) and 1.3 g of ethyl cyanoacetate (10 mmol) in 50 mL absolute ethanol, a sodium ethoxide solution prepared from 0.23 g sodium metal (10 mmol) and 10 mL absolute ethanol was added. The stirring was continued, at room temperature, for 12 hr. The solid product was collected by filtration, washed with water, dried and finallycrystallised from ethanol to afford compound 8 as pale yellow crystals in 80 % yield, mp 240-241 ºC; IR (KBr): $\overline{\nu}$ = 3265 (NH), 2222 (C≡N), 1715 (C=O) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 1.11-1.20 (t, 3H, CH_3), 2.50-2.56 (m, 2H (CH_2 –C=O), 3,35 (d, 1H), 3.58 (m, 1H) , 3.81 (s, 3H, -OCH_3), 4.37 (q, 2H, CH_2),6.86-6.90(m, 1H Ar-H), 6.95-7.00 (m, 2H, Ar-H), 7.18-7.23 (m, 4H, Ar-H), 7.91-7.92 (m, 2H, Ar-H), 11.69 (s, D_2O-exchangeable, 1H, indole NH) ppm; MS (70 eV): *m/z* = 391 (M^++1, 2.3), 390 (M^+, 8.0), 135 (68.5), 78 (79.8), 62 (100). Anal. Calcd for $C_{23}H_{22}N_2O_4$: C, 70.75; H, 5.68; N, 7.17. Found: C, 70.61; H, 5.60; N, 7.33.

4-(1*H*-Indol-3-yl)-6-(4-methoxyphenyl)-2-oxo-2*H*-pyran-3-carbonitrile 9.

Method A. This compound was synthesized by the same procedure described in the synthesis of compound 8 by refluxing the reaction mixture for 8 hr. The solid that formed after cooling was collected by filtration, washed with water, dried and finally crystallised from ethanol to afford compound 9 as pale yellow crystals in 68% yield, mp 208-209 ºC; IR (KBr): $\overline{\nu}$ = 3274 (NH), 2222 (C≡N), 1688 (C=O) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.31 (s, 3H, -OCH_3), 7.12-7.22 (m, 2H, Ar-H) ,7.32 (s, 1H,C5), 7.55-7.58 (m, 4H, Ar-H), 7.93-7.96 (m, 2H, Ar-H), 8.54-8.55 (m, 1H, Ar-H), 12.48 (s, D_2O-exchangeable, 1H, indole NH) ppm; MS (70 eV): *m/z* = 343 (M^++1, 2.9), 342 (M^+, 11.2), 212 (100), 168 (80.4), 140 (64.4), 113 (26.4). Anal. Calcd for $C_{21}H_{14}N_2O_3$: C, 73.68; H, 4.12; N, 8.18. Found: C, 73.74; H, 4.26; N, 8.03.

Method B. Refluxing 0.78 g of compound 8 (2 mmol) in sodium ethoxide solution prepared from 0.046 g sodium metal (2 mmol) and 50 mL absolute ethanol for 6 h. the treatment of reaction mixture as mentioned in method A resulted in a compound identical in (mp. and mix. mp. for compound that produced by method A.

General procedure for synthesis of pyridines 11 and 13a-13d

To a solution of 0.69 g of the pyranone 9 (2 mmol) in 30 mL of ethanol, hydrazine hydrate or the appropriate sulfonamides 12a-d (2 mmol) was added. The mixture was refluxed for 6 h. Left to cool, the formed solid product was filtered off, dried, and then crystallized from ethanol/DMF to give compounds 11 and 13a-d, respectively.

1,2-Dihydro-4-(1*H*-indol-3-yl)-6-(4-methoxyphenyl)-2-oxo-1-aminopyridine-3-carbonitrile 11.

Yellow powder, yield 62%, mp 295-297 ºC; IR (KBr): $\overline{\nu}$ = 3320, 3190 (NH_2, NH), 2219 (C≡N), 1665 (C=O), cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.51(s, D_2O-exchangeable, 2H, NH_2), 3.34 (s, 3H, -OCH_3), 7.22-7.29 (m, 2H, Ar-H), 7.33-7.55 (m, 4H, Ar-H), 7.59-7.93 (m, 2H, Ar-H), 9.11 (m, 1H, Ar-H), 11.69 (s, D_2O-exchangeable, 1H, indole NH) ppm; MS (70 eV): *m/z* = 357 (M^++1, 2.5), 356 (M^+, 10.3), 281 (40.2), 78 (70.3), 62 (100). Anal. Calcd for $C_{21}H_{16}N_4O_2$: C, 70.77; H, 4.53; N, 15.72. Found: C, 70.59; H, 4.50; N, 15.86.

1,2-Dihydro-4-(1*H*-indol-3-yl)-6-(4-methoxyphenyl)-2-oxo-1-(4-aminosulfonylphenyl)pyridine-3-carbonitrile 13a.

Pale yellow crystals, yield 65%, mp 248-249 ºC; IR (KBr): $\overline{\nu}$ = 3364, 3150 (NH_2, NH), 2221 (C≡N), 1686 (C=O), 1375 (S=O, asy.) and 1140 (S=O, sym.) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.40 (s, D_2O-exchangeable, 2H, sulfonamide NH_2),3.85 (s, 3H, -OCH_3), 7.04 (s, 1H, C5), 7.24-7.27 (m, 2H, Ar-H),7.31-7.55 (m, 4H, Ar-H), 7.56-7.74 (m, 2H, Ar-H), 7.91-7,92 (m, 4H, Ar-H), 9.05 (m, 1H, Ar-H), (12.54 (s, D_2O-exchangeable, 1H, indole NH) ppm; MS (70 eV): *m/z* = 497 (M^++1, 3.8), 496 (M^+, 11.0), 212 (41.8), 168 (100). Anal. Calcd for $C_{27}H_{20}N_4O_4S$: C, 65.31; H, 4.06; N, 11.28; S, 6.46. Found: C, 65.18; H, 3.92; N, 11.33; S, 6.62.

1,2-Dihydro-4-(1*H*-indol-3-yl)-6-(4-methoxyphenyl)-2-oxo-1-(4-propylaminosulfonylphenyl)pyridine-3-carbonitrile 13b.

Pale yellow crystals, yield 65%, mp 257-258 ºC; IR (KBr): $\overline{\nu}$ = 3358, 3281 (2NH), 2224 (C≡N), 1686 (C=O), 1370 (S=O, asy.) and 1148 (S=O, sym.) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 1.03 (t, 3H, -CH_3), 1.52 (m, 2H, -CH_2-), 2.10 (s, D_2O-exchangeable, 1H, sulfonamide NH) 3.35 (t, 2H, -CH_2-), 3.85 (s, 3H, -OCH_3), 6.10 (s, 1H, C5), 6.61-6.66 (m, 2H, Ar-H), 7.27-7.30 (m, 4H, Ar-H), 7.33-

7.48 (m, 2H, Ar-H),7.94-7.97 (m, 4H, Ar-H) , 8.54-8.56 (m, 1H, Ar-H), 12.51 (s, D_2O-exchangeable, 1H, indole NH), ppm; MS (70 eV): *m/z* = 539 (M^++1, 3.3), 538 (M^+, 8.9), 212 (100), 140 (87.5), 63 (65.0). Anal. Calcd for $C_{30}H_{26}N_4O_4S$: C, 66.90; H, 4.87; N, 10.40; S, 5.95. Found: C, 66.82; H, 4.87; N, 10.29; S, 6.11

1,2-Dihydro-4-(1*H*-indol-3-yl)-6-(4-methoxyphenyl)-2-oxo-1-(4-phenylaminosulfonylphenyl)pyridine-3-carbonitrile 13c.

Pale yellow crystals, yield 71%, mp 220-221 ºC; IR (KBr): $\bar{\nu}$ = 3347, 3273 (2NH), 2222 (C≡N), 1686 (C=O), 1380 (S=O, asy.) and 1148 (S=O, sym.) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.83 (s, 3H, -OCH_3), 6.00 (s, 1H, C5), 6.49-6.54 (m, 2H, Ar-H), 6.95-7.03 (m,1H Ar-H), 7.07-7.26 (m, 2H, Ar-H), 7.27-7.39 (m, 4H, Ar-H), 7.54-7.58 (m, 2H, Ar-H), 7.92-7.97 (m, 4H, Ar-H), 8.54-8.56 (m, 1H, Ar-H), 9.33 (s, D_2O-exchangeable, 1H, sulfonamide NH), 12.51 (s, D_2O-exchangeable, 1H, indole NH), ppm; MS (70 eV): *m/z* = 573 (M^++1, 3.6), 572 (M^+, 10.0), 212 (100), 168 (74.5), 140 (70.3). Anal. Calcd for $C_{33}H_{24}N_4O_4S$: C, 69.22; H, 4.22; N, 9.78; S, 5.60. Found: C, 69.38; H, 4.06; N, 9.70; S, 5.48.

1,2-Dihydro-4-(1*H*-indol-3-yl)-6-(4-methoxyphenyl)-2-oxo-1-[4-(4-methoxyphenyl) aminosulfonylphenyl] pyridine-3-carbonitrile 13d.

Pale yellow crystals, yield 71%, mp 235-236 ºC; IR (KBr): $\bar{\nu}$ = 3379, 3285 (2NH) 2220 (C≡N), 1684 (C=O), 1376 (S=O, asy.) and 1140 (S=O, sym.) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.37 (s, 3H, -OCH_3), 3.65 (s, 3H, -OCH_3),5.93 (s, 1H, C5), 6.49-6.52 (m, 4H, Ar-H), 6.76-6.78 (m, 2H, Ar-H), 6.93-6.96 (m, 4H, Ar-H), 7.22-7.25 (m, 2H, Ar-H), 7.94-7.96 (m, 4H, Ar-H), 8.53-8.55 (m, 1H, Ar-H), 9.74 (s, D_2O-exchangeable, 1H, sulfonamide NH), 12.52 (s, D_2O-exchangeable, 1H, NH idnole) ppm; MS (70 eV): *m/z* = 603 (M^++1, 2.9), 602 (M^+, 13.3), 241 (100), 169 (73.7), 124 (45.2). Anal. Calcd for $C_{34}H_{26}N_4O_5S$: C, 67.76; H, 4.35; N, 9.30; S, 5.32. Found: C, 67.71; H, 4.28; N, 9.18; S, 5.40.

General procedure for synthesis of pyrimidines 14a, b

A 2.77 g of chalcone **3** (10 mmol) was added to sodium ethoxide solution [prepared from sodium metal (0.23 g, 10 mmol) and 50 mL of absolute ethanol] then urea or thiourea (10 mmol) was added. The reaction mixture was refluxed for 16 h, then left to cool and poured into crushed ice and neutralized with diluted hydrochloric acid. The precipitated product was collected by filtration, washed with ethanol and dried. Crystallization from EtOH/DMF afforded the pyrimidine derivatives 14a, b, respectively.

6-(1*H*-Indol-3-yl)-4-(4-methoxyphenyl)pyrimidin-2(1*H*)-one 14a.

Pale yellow crystals, yield 69%, mp 198-200 ºC; IR (KBr): $\bar{\nu}$ = 3438, 3170 (2NH), 1669 (C=O), 1633 (C=N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.83 (s, 3H, -OCH_3), 5.46 (s, 1H, C5), 6.99-7.04 (m, 2H, Ar-H), 7.18-7.22 (m, 4H, Ar-H), 7.49-7.54 (m, 2H, Ar-H), 8.28-8.30 (m,1H, Ar-H), 9.94 (s, D_2O-exchangeable, 1H, pyrimidin NH), 12.07 (s, D_2O-exchangeable, 1H, indole NH) ppm; MS (70 eV): *m/z* = 318 (M^++1, 2.4), 317 (M^+, 9.0), 213 (100), 169 (38.4) 140 (42.5), 62 (36.8). Anal. Calcd for $C_{19}H_{15}N_3O_2$: C, 71.91; H, 4.76; N, 13.24. Found: C, 72.04; H, 4.78; N, 13.38.

6-(1*H*-Indol-3-yl)-4-(4-methoxyphenyl)pyrimidine-2(1*H*)-thione 14b.

Yellow powder, yield 73%, mp 180-182 ºC; IR (KBr): $\bar{\nu}$ = 3375, 3165 (2NH), 1630 (C=N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.35 (s, 3H, -OCH_3), 5.6 (s, 1H, C5),7.21-7.25 (m, 2H, Ar-H), 7.26-7.49 (m, 4H, Ar-H), 7.54-7.90 (m, 2H, Ar-H), 8.28-8.30 (m, 1H, Ar-H), 8.82 (s, D_2O-exchangeable, 1H, pyrimidin NH), 12.07 (s, D_2O-exchangeable, 1H, indole NH) ppm; MS (70 eV): *m/z* = 334 (M^++1, 2.6), 333 (M^+, 6.8), 144 (100), 116 (28.7), 89 (24.5). Anal. Calcd for $C_{19}H_{15}N_3OS$: C, 68.45; H, 4.53; N, 12.60; S, 9.62. Found: C, 68.48; H, 4.66; N, 12.49; S, 9.56.

3-(2-Chloro-6-(4-methoxyphenyl)pyrimidin-4-yl)-1*H*-indole 15.

A solution of 0.63 g of 14a (2 mmol) in 30 mL phosphorus oxychloride was refluxed for 5 hr, then left to cool and poured carefully into crushed ice. The precipitated product was collected by filtration, washed with water and dried. Crystallization from EtOH/DMF afforded compound 15 as violet powder in 68% yield, mp 160-162 ºC; IR (KBr): $\bar{\nu}$ = 3217 (NH), 1632 (C=N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.38 (s, 3H, -OCH_3), 7.11-7.53 (m, 6H, Ar-H), 7.87-8.31 (m, 4H, Ar-H), 12.20 (s, D_2O-exchangeable, 1H, indole NH) ppm; MS (70 eV): *m/z* = 336 (M^++1, 1.9), 335 (M^+, 5.6), 156 (92.3), 108 (57.5), 92 (100), 64 (78.7). Anal. Calcd for $C_{19}H_{14}ClN_3O$: C, 67.96; H, 4.20; N, 12.51. Found: C, 67.79; H, 4.34; N, 12.50.

N-(4-Phenylaminosulfonylphenyl)-4-(1*H*-indol-3-yl)-6-(4-methoxyphenyl)pyrimidin-2-amine 16.

This reaction was carried out by the same procedure described in the synthesis of compounds 13a-d. Crystallization of the reaction product from EtOH/DMF afforded compound 16 as faint brown

powder in 66% yield, mp 100-101 ºC; IR (KBr): $\overline{\nu}$ = 3419, 3349, 3245 (3NH) cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ = 3.83 (s, 3H, -OCH_3), 6.5-8.32 (m, 19H, Ar-H), 9.85 (s, D_2O-exchangeable, 1H, NH), 9.93 (s, D_2O-exchangeable, 1H, NH), 12.3 (s, D_2O-exchangeable, 1H, indole NH) ppm; MS (70 eV): *m/z* = 548 (M^++1, 3.7), 547 (M^+, 12.2), 248 (41.1), 156 (88.8), 108 (56.3), 92 (100), 65 (79.0). Anal. Calcd for $C_{31}H_{25}N_5O_3S$: C, 67.99; H, 4.60; N, 12.79; S, 5.86. Found: C, 68.13; H, 4.69; N, 12.76; S, 5.66.

Pharmaceutical Applications

Anti-proliferative screening

Cytotoxic activity against human breast carcinoma cell line (MCF-7) and liver carcinoma cell line HEPG-2 in vitro. The method applied is similar to that reported by Skehan et al. 36 using Sulfo-Rhodamine-B stain (SRB). Cells were plated in 96-multiwell plate (104 cells/well) for 24 h before treatment with the test compound to allow attachment of cell to the wall of the plate, different concentration of the compound under test (0, 2.5, 5, and 10 µg/ml) were added to the cell monolayer in triplicate wells individual dose, monolayer cells were incubated with the compounds for 48 h at 37^{o}C and in atmosphere of 5 % CO_2 , after 48 h, cells were fixed, washed and stained with SRB stain, excess stain was washed with acetic acid and attached stain was recovered with Tris-EDTA buffer, color intensity was measured in an ELISA reader, the relation between surviving fraction and drug concentration is plotted to get the survival curve of each tumor cell line after the specified compound and the IC50 was calculated.

Antimicrobial screening

Applying the agar plate diffusion technique 37, the compounds were screened in vitro for their bactericidal activity against Gram positive bacteria (Staphylococcus aureus) and Gram negative bacteria (Escherichia Coli and Pseudomonas aeroginosa), and for their fungicidal activity against Fusarium, Aspergillus niger and Candida albicans. In this method, a standard 5 mm diameter sterilized filter paper disc impregnated with the compound (0.3 mg/0.1 ml of DMF) was placed on an agar plate seeded with the test organism. The plates were incubated for 24 hours at 37 °C for bacteria and 28 °C for fungi. The zone of inhibition of bacterial and fungi growth around the disc was observed.

3. Results and Discussion:

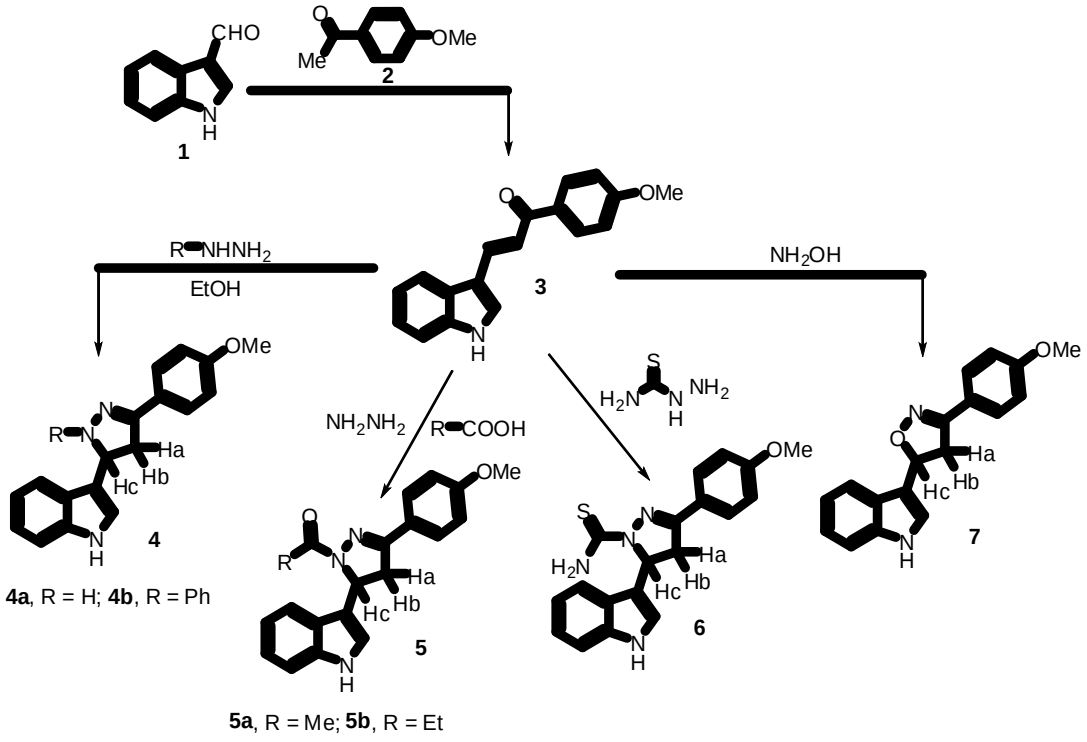
Chemistry

Aldol condensation reaction of 3-indolaldehyde 1 with 4-methoxyacetophenone 2 in ethanolic NaOH solution afforded chalcone 3. Its structure was confirmed by its IR spectrum where it showed a characteristic peak for a conjugated carbonyl group at 1633 cm^{-1}, and also by its ^1H NMR which gave signals at δ 7.53 (d, 1H, *J* = 12.9 Hz, (C=O)(CH=C), and 7.92 (d, 1H, *J* = 12.9 Hz (C=O)(C=CH). Reaction of chalcone 3 with either hydrazine hydrate or phenyl hydrazine gave the corresponding pyrazoline derivatives 4a, b respectively. Structures of 4a, and 4b were assigned by their IR and ^{1}HNMR Spectra. Both exhibited C=N stretching vibrations in the region 1618-1630 cm^{-1}, and compound 4a showed an additional absorption band at 3215 cm^{-1} characteristic for pyrazole NH. ^1H NMR spectra of compounds 4a and 4b showed three multiplets at δ 2.00, 2.69, and 3.40 ppm resulted from the AMX pattern displayed by two diastereotopic protons at C-4 (H_a and H_b) and one proton (H_c) at C-5 (Scheme 1).

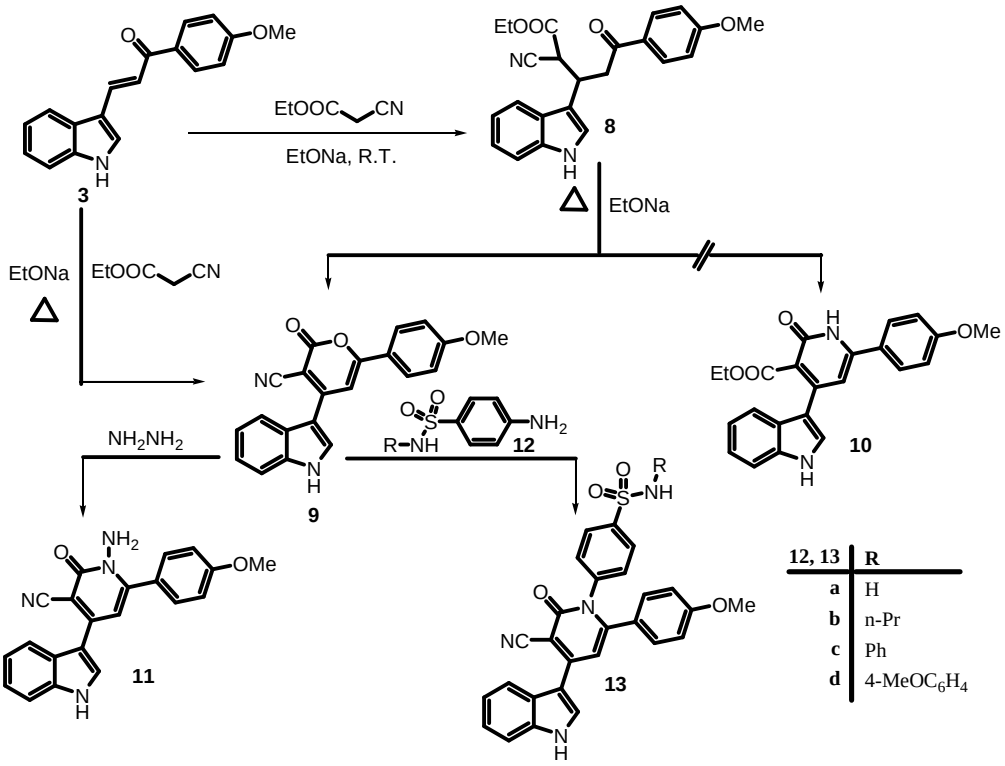
The acyl derivatives 5a , b were prepared either by treating 4a with the corresponding acid chloride, or by reacting chalcone 3 with hydrazine hydrate by changing the reacting medium from ethanol to acetic acid or propanoic acid, respectively. Structures of 5a , b were established by their IR spectra where no characteristic band for NH was detected at 3215 cm^{-1}. The ^{1}HNMR of compound 5a exhibited a singlet at δ 2.24ppm (acetyl CH_3 protons), whereas 5b showed a triplet at δ 2.0 ($C\underline{H}_3CH_2$) and quartet at δ 2.68($CH_3C\underline{H}_2$) respectively.

The formation of pyrazoline 6 was achieved by refluxing chalcone 3 with thiosemicarbazide and sodium hydroxide in ethanol 30. Its structure was confirmed by its IR where absorption peaks appeared at 3316 cm^{-1} and 3227 cm^{-1}, characteristic for NH_2 and NH, respectively, and its ^1H NMR displayed two singlets at δ = 11.11ppm (D_2O-exchangeable, 2H, NH_2), and 11.60 (D_2O-exchangeable, 1H, indole NH).

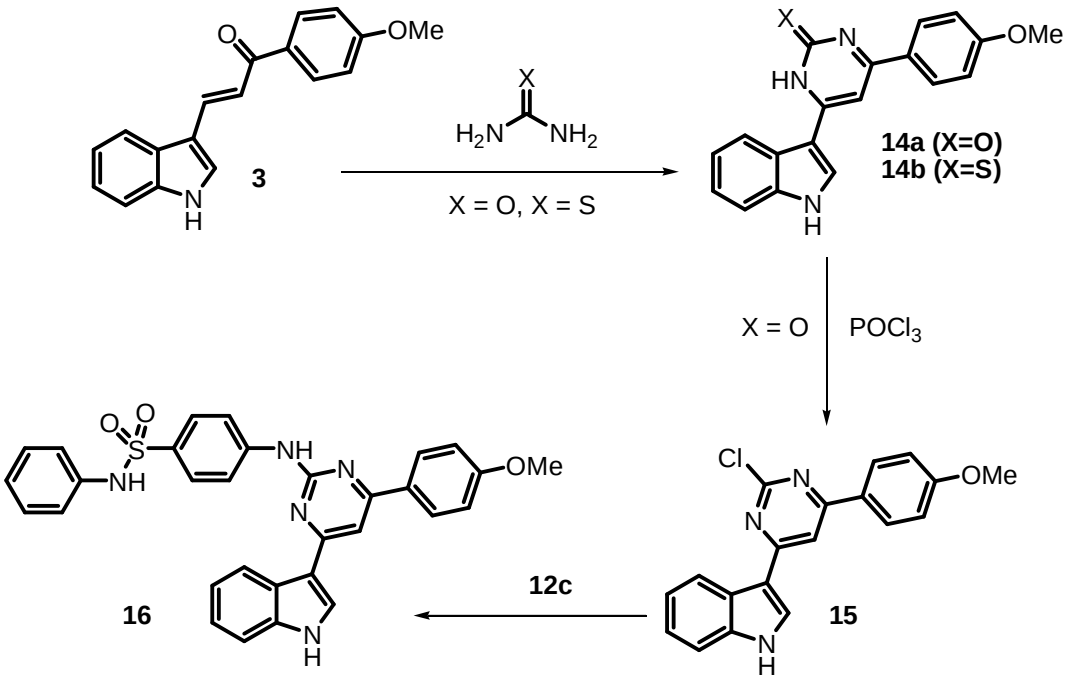
The oxazoline 7 was obtained from refluxing chalcone 3 with hydroxylamine in ethanol, its structure was ascertained by spectral data , where its IR spectrum showed 2 peaks at 3386 and 1640, characteristic for NH and C=N, respectively. Its ^1H NMR displayed a singlet at δ =11.61 (D_2O-exchangeable, 1H, indole NH). The mass spectrum revealed its molecular ion peak at *m/z* 292 (M^+) (Scheme 1).



(Scheme 1)



(Scheme 2)



(Scheme 3)

Table1. In vitro cytotoxic activity (IC_{50}) of compounds 5a, 6, 7, 13a, 13b, 13d and 14b and 5-Fluorouracil against a human breast carcinoma cell line (MCF7) and a liver carcinoma cell line (HEPG2)[a]

Entry	compound	MCF7 ($\mu g.mL^{-1}$)	HEPG2 ($\mu g.mL^{-1}$)
1	**5a**	1.01	-
2	**6**	2.62	2.48
3	**7**	2.55	5.17
4	**13a**	1.34	4.09
5	**13b**	0.84	5.30
6	**13d**	0.60	3.96
7	**14b**	0.85	2.68
8	5-Flurouracil	0.67	5.0[b]

[a]IC_{50} is defined as the concentration which results in a 50% decrease in cell number as compared with that of the control structures in the absence of an inhibitor.

Reaction of chalcone 3 with ethyl cyanoacetate in the presence of sodium ethoxide at room temperature afforded product 8 through *Michael* addition, whereas the similar reaction gave pyranone derivative 9 under the reflux conditions 31 (Scheme 2). The elemental analysis and spectroscopic data confirmed the assigned structure for 9, where its IR displayed a peak at 2222cm^{-1} for C≡N and disappearance of the band representing the carbonyl of the COOR group. This result led to exclude the formation of an alternative compound 10. Compound 9 could also be obtained by refluxing 8 in ethanol in presence of sodium ethoxide.

Condensation of 9 with either hydrazinehydrate or sulfonamide derivatives 32 12a-d in refluxing ethanol gave the corresponding pyridinones 11, or 13a-d, respectively (Scheme 2). The structures of these products were ascertained by their spectral data, where their IR spectra showed absorption bands in the region 2219-2224cm^{-1} and 1665-1780cm^{-1} characteristic for C≡N and carbonyl group, respectively. In addition, compounds 13a-d

displayed bands at 1375 (S=O, asy.) and 1140 (S=O, sym.) cm^{-1}. Their ^1H NMR spectra were agreed with the structures 11 and 13a-d.

Reflux of chalcone **3** with either urea or thiourea in presence of sodium ethoxide in ethanol afforded the pyrimidine derivatives **14a**, **b**, respectively (Scheme 3). The structure of **14a** was substantiated by its IR, where it revealed bands at 3438, 3170 cm^{-1} for the 2NH groups, 1669 and 1633 cm^{-1} characteristic for C=O, and C=N, respectively. Its ^1H NMR, displayed two singlets at δ 9.94 for pyrimidin NH and δ 12.07 for indole NH by ppm. Reflux of compound **14a** in excess phosphorus oxychloride as a reacting medium gave compound **15** which on its reflux with sulfonamide **12c** in ethanol furnished compound **16** in a moderate yield (Scheme 3). The IR spectrum of compound **16** showed three separate absorption bands at 3419, 3349, and 3245 cm^{-1} characteristic for (3NH). Its ^1H NMR displayed three singlets at δ 9.85 (D_2O-exchangeable, 1H, NH), 9.93 (D_2O-exchangeable, 1H, NH), and 12.3 (D_2O-exchangeable, 1H, indole NH) by ppm.

Pharmaceutical Applications

Anti-proliferative screening

Compounds 5a, 6, 7, 13a, 13b, 13d and 14b were tested against a human breast carcinoma cell line (MCF7) and a human liver carcinoma cell line (HEPG2), using 5-Fluorouracil (5-Fluoro-1*H*-pyrimidine-2,4-dione) as a reference drug (Table1). The measurements were carried out in the National Institute of Cancer, Cairo University, Cairo Egypt.

[b]This value of IC_{50} for the reference drug 5-Fluorouracil against HEPG2 was maintained by the National Institute of Cancer, Cairo University, Cairo, Egypt. (The liver carcinoma cells were found to be more resistant to 5-Fluorouracil as a reference drug). As shown in Tables 1, it was found that compounds 5a, 6, 7, 13a, 13b, 13d and 14b have significant anti-proliferative activities against human breast cell line MCF-7 compared to the reference standard drug 5-Fluorouracil. Compounds 13b and 13d showed high activities which can be attributed to the presence of the sulfonamide group, where it was confirmed 33, 34 that compounds containing sulfonamide function group have potent anti-proliferative activities. Compound 13d was found to have a promising activity due to the presence of a methoxy group in the para- position to the sulfonamide group 35. The screened results against liver carcinoma cell line (HEPG2, Table 1) revealed that the tested compounds 6, 7, 13a, 13b, 13d and 14b showed significant activities compared to reference drug 5- Fluorouracil.

Antimicrobial screening

The synthesized new compounds 4a, 5a, 6, 7, 11, 13a, 13b, 13d and 14b were screened in vitro for their bactericidal activity against Gram positive bacteria (*Staphylococcus aureus*) and Gram negative bacteria (*Escherichia Coli* and *Pseudomonas aeroginosa*), and for their fungicidal activity against *Fusarium*, *Aspergillus niger* and *Candida albicans* (Table 2). All screened compounds showed high or moderate bactericidal activity against *Staphylococcus aureus and Pseudomonas aeroginosa* compared to that of ciprofloxacin. Compounds 4a, 5a, 6, 13a, 13b and 13d showed a good fungicidal activity, near to that of nystin, against *Fusarium*, all screened compounds except 11 were found to have moderate fungicidal activity against *Candida albicans*.

Table 2. Bactericidal and fungicidal activity of some of the new compounds and ciprofloxacin and nystin.[a]

No.	Staphylococcus aureus	Escherichia coli	Pseudomonas aeroginosa	Fusarium	Aspergillus niger	Candida albicans
4a	+++ (32)	-	++ (17)	+++ (31)	-	++ (16)
5a	++ (21)	-	++ (20)	++ (16)	-	++ (18)
6	+++ (30)	-	++ (19)	+++ (27)	-	++ (20)
7	++ (18)	-	++ (17)	-	-	++ (22)
11	++ (15)	-	+++ (27)	-	-	-
13a	+++ (34)	-	+++ (29)	+++ (32)	-	++ (22)
13b	+++ (28)	-	++ (17)	+++ (31)	-	++ (20)
13d	+++ (34)	-	+++ (31)	+++ (30)	-	++ (24)
14b	+++ (29)	-	++ (15)	-	-	++ (20)
Ciprofloxacin	++++	++++	++++	-	-	-
Nystin	-	-	-	++++	++++	++++

[a] The activities are based on the diameters of zones of inhibition in mm. One mL of stock solution (5 ug/mL in DMF) was applied in each hole of each paper disk**.**+: < 15 mm; ++: 15-24 mm; +++: 25-34 mm; ++++: 35-44 mm, etc.

4. Conclusion

Aldol condensation reaction between 3-indolaldehyde and 4-methoxyacetophenone gave a chalcone compound from which some pyrazoline, pyridine, and pyrimidine derivatives linked to indole moiety were obtained and found to have promising antitumor and antimicrobial activities.

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7/11/2010

Structural Effect on the Base-Catalyzed Hydrolysis of (E) Methyl 3-Carboxy-4-Aryl-3-Butenoate Hemiesters, and the Isomeric (E) 3-Methoxycarbonyl-4-(2-Naphthyl)-3-Butenoic Acid

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Abstract: The structural effect on the base-catalyzed hydrolysis of (E) methyl 3-carboxy-4-phenyl-3-butenoate (1), (E) methyl 3-carboxy-4-(1-naphthyl)-3-butenoate (2), (E) methyl 3-carboxy-4-(2-naphthyl)-3-butenoate (3), and (E) 3-methoxycarbonyl-4-(2-naphthyl)-3-butenoic acid (4), at different temperatures (35-50°C) in 50% aqueous dioxane (v/v), show that the rate of reaction follows the overall second order kinetics, first order with respect to each of the hemiester and base. It decreases in the order: (1) > (2) > (3) > (4). Ratios between the values of k_2 (k_1/C_w), and also between k_3 (k_1/C_w^2) in different mixtures are less than 1, which means that the reaction is apparently independent of C_w. The activation parameters $E^{\#}$, $\Delta H^{\#}$, $\Delta S^{\#}$, $\Delta G^{\#}$, and Arrhenius frequency factor (A), confirmed the structural effect on their rates of hydrolysis. [Journal of American Science 2010;6(8):472-475]. (ISSN: 1545-1003).

Keywords: Hemiesters, Hydrolysis, Molecularity, Reactivity

1. Introduction

Although extensive work on the kinetics of esters hydrolysis has been investigated, hydrolysis of unsaturated esters has not been much explored. Further investigations of the hydrolytic cleavage of some esters and comparison of the results with those for their isomers may provide additional insight into mechanisms which can serve as models for biological systems [1] and enzymatic processes where they are helpful in elucidating the effects of structural variation on the character of intramolecular catalysis [2]. However, Jones and Watkinson [3] found that in the hydrolysis of 28 nuclear substituted ethyl cinnamate derivatives as the ethoxy carbonyl group remains conjugated with the ring, the polar effects of nuclear substituents are qualitatively similar to those operating in the hydrolysis of benzoates.

This investigation is planned to study the structural effect of hemiesters, (E) methyl 3-carboxy-4-phenyl-3-butenoate (1), (E) methyl 3-carboxy-4-(1-naphthyl)-3-butenoate (2), and (E) methyl 3-carboxy-4-(2-naphthyl)-3-butenoate (3) on their rates of hydrolysis. Also to compare the hydrolysis of esters which contain an olefinic linkage either conjugated with the carboxyl group (hemiester 3), or with the carboxylate; (E) 3-methoxycarbonyl-4-(2-naphthyl)-3-butenoic acid (4), and extended its conjugation with the naphthyl group. The comparison between the results of hydrolysis of unsaturated and isomeric hemiesters may provide additional insight into mechanisms which can serve as models for biological systems and enzymatic processes.

2. Experimental:

The hemiesters, (E) methyl 3-carboxy-4-phenyl-3-butenoate (1) [4]; m.p. 99°C, (E) methyl 3-carboxy-4-(1-naphthyl)-3-butenoate (2); 141°C, (E) methyl 3-carboxy-4-(2-naphthyl)-3-butenoate (3) [5]; m.p. 125°C, and its isomeric (E) 3-methoxycarbonyl-4-(2-naphthyl)-3-butenoic acid (4) [5]; m.p. 136°C, were prepared and their structures confirmed.

Kinetic Measurements

The kinetic procedure involved the hydrolysis of 0.015M hemiester with 0.150M NaOH in 50% purified dioxane [6] and doubly distilled CO_2-free water (v/v), in order to assure the reaction to become pseudo-first order. The reaction was followed by a titremetric procedure at different time intervals [7] at 35-50°C, using Diphenol Purple as an indicator until a constant reading was obtained which was always concordant with that theoretically calculated. The structure of the produced dicarboxylic acid after complete hydrolysis was extracted was found to be identical with that given in the literature. The thermostatic bath was used where the temperature could be adjusted to and controlled within ± 0.02°C. The rate constants (k_1) were obtained graphically by using the integrated form of the first order rate equation, and found to be reproducible within ± 0.01. However the concordance of the

authentically prepared dicarboxylic acids by the complete hydrolysis of hemiesters (1-4) in 50% aqueous dioxane under reflux for 4 hours, assured the proposed mechanism of the reaction.

3. Results and Discussion:

The specific rates of the base-catalyzed hydrolysis of hemiesters (1-4) follow the overall second order kinetics equation, first order with respect to each of the hemiester and the base, and takes place by $B_{Ac}2$ mechanism. A carbonyl addition mechanism involving a tetrahedral intermediate is proposed. In this mechanism, the positively charged carbonyl carbon of the ester group is supposed to be attacked by the hydroxide ion where an anchimeric assistance takes place by the neighboring carboxylate group to form transition state (I), in the rate determining step, which is less polar than one or two of the reactants (charge distribution).

Activation Parameters

The specific rate constants (k_1) of the base-catalyzed hydrolysis of hemiesters (1-4) at temperatures 35-50°C allow the calculations of the activation parameters $E^{\#}$, $\Delta H^{\#}$, $\Delta S^{\#}$, $\Delta G^{\#}$, and also the Arrhenius frequency factor (A). The results obtained are represented in Table 1.

Table 1: **Rate constants and activation parameters for the hydrolysis of the hemiesters (1-4) in 50% aqueous dioxane**

Hemiester	Temp. K	$10^3 \times k_2$ l mole^{-1} sec^{-1}	-log k_2	$E^{\#}$ K cal mole^{-1}	$\Delta H^{\#}$ K cal mole^{-1}	$-\Delta S^{\#}$ cal deg^{-1} mole^{-1}	$\Delta G^{\#}$ K cal mole^{-1}	Log A
1	308	2.9854	3.4750	13.357	12.721	29.239	22.019	6.909
	313	4.3501	3.6385					
	318	6.1404	3.7882					
	323	8.1884	3.9132					
2	308	1.8408	3.2650	14.279	13.653	27.342	22.211	7.314
	313	2.5586	3.4080					
	318	3.4514	3.5380					
	323	4.3152	3.6350					
3	308	1.5667	3.1950	14.509	13.873	26.792	22.418	7.441
	313	2.3878	3.3780					
	318	3.4119	3.5330					
	323	4.8641	3.687					
4	308	1.4962	3.1750	14.739	14.103	26.147	22.932	7.579
	313	2.3015	3.3620					
	318	3.1281	3. 5160					
	323	4.7424	3.6760					

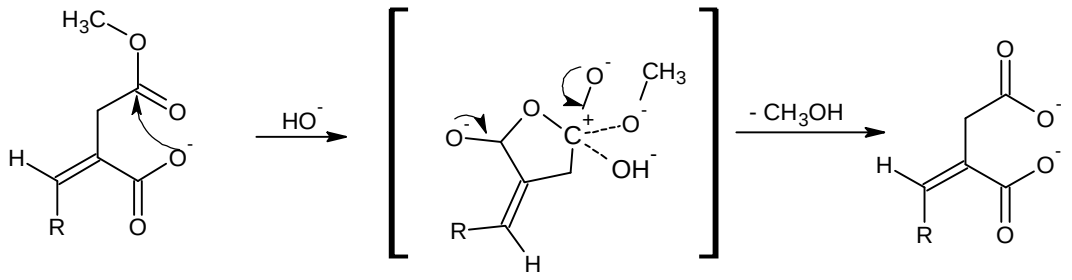
The magnitude of the frequency factor and the large negative entropies of activation substantiate the bimolecular mechanism in which the rate-determining step involves the formation of the anhydride as a cyclic intermediate [7, 8]. The values of $\Delta H^{\#}$, $\Delta S^{\#}$, for the hemiesters (1-4) change as the structures are changed, and the compensation effect between $\Delta H^{\#}$ and $T\Delta S^{\#}$ plays an important role in keeping $\Delta G^{\#}$ more or less constant. This is substantiated by the linear relationship between them with a slope equals to unity.

The lowest entropy of activation of hemiester (1) more than (2), (3), or (4), indicates the greatest solvation of the activated complex produced during its hydrolysis than the other hemiesters.

Structure and Reactivity

The present work aims to describe the relationship between structure of the hemiester and its reactivity towards base-catalyzed hydrolysis in 50% aqueous dioxane at different temperatures. It includes the effect of aromatic ring size of phenyl versus naphthyl (hemiesters 1-3), location of the hemiester moiety on the naphtyl ring (hemiesters 2 and 3), and the conjugation of the olefinic double bond with carboxyl versus carboxylate group (hemiesters 3 and 4). The results obtained show that reactivity falls in the order: 1> 2> 3> 4.

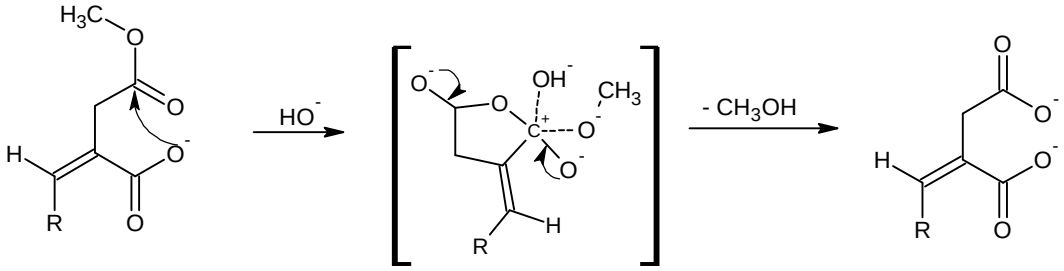
The higer reactivity of phenyl hemiester (1) more than (2) or (3) can be ascribed to the less conjugation exerted by the carbonyl group of the carboxylate ion with the phenyl ring compareable to that in (2) or (3), where the conjugation takes place with the naphthyl group. Such a conjugation in (1) leads to a more susceptibility of the carboxylate oxygen atom towards intramolecular nucleophilic attack on the carbonyl carbon of the ester group to form the anhydride as a cyclic intermediate. (Scheme1).



Transition State I

Hemiester	R
1	Phenyl
2	1-Naphthyl
3	2-Naphthyl

Scheme 1



Transition State II

Hemiester	R
4	2-Naphthyl

Scheme 2

The higher rate of hydrolysis of isomeric hemiester (2) than (3) can be attributed to the higher reactivity of position 1 rather than position 2 in the naphthyl group. The reactivity for position 1 over 2 can be rationalized in terms of the stability of the intermediate resonating structures where for the position 1, seven resonance structures are produced, of which four preserve the aromatic ring, comparable to position 2, where the intermediate has only six resonance structures, and only two of them are aromatic. Moreover, a Dreiding model for both intermediates show that no steric hinderence exists, i.e., the electronic factor outweighs the steric factor.

The higher reactivity of hemiester (3) than (4) towards base catalysed hydrolysis can be explained on the basis of hemiester structure where in hemieser (4) the conjugation of carbomethoxy group with the olefinic double bond extended to the naphthyl group decreases the susceptibility of the carbonyl carbon in the ester group to be attcked by the carboxylat anion.

Effect of the Dielectric Constant of the Medium

The rate of base catalyzed hydrolysis of hemiester (3) was followed up, at 40°C using aqueous purified dioxane (40, 50, and 60% v/v), in order to study the effect of the dielectric constant (D) [9] of the medium on the rate of hydrolysis of the

hemiester. The results obtained show that the rate follows the overall second order kinetics; first order in both of the hemiester and hydroxide ion and the rate constants decrease with the increase of the dielectric constant (D) of the medium. However, to study the role of water in the base catalyzed hydrolysis, the mole fraction of water was calculated in different dioxane-water mixtures and the values of k_1/C_w, and k_1/C_w^2, were calculated to give the rate constants k_2 and k_3, respictevly. Ratios between the values of k_2 and also between k_3 in different mixtures were found to be less than 1, which means that the reaction is apparently independent of C_w. The results obtained are given in Tables 2 and 3.

Table 2: Relation between dielectric constant of the medium and mole fractions of water on the rate of hydrolysis of hemiester (3) at 40°C

Dioxane-Water Mixture (v/v)	D 40°C	10^3 x k_1 sec^{-1}	10^4 x k_2 $l\ mole^{-1}\ sec^{-1}$	10^5 x k_3 $l^2\ mole^{-2}\ sec^{-1}$
60:40	35.39	0.614	0.279	0.126
50:50	37.41	0.384	0.139	0.051
40:60	39.45	0.192	0.058	0.018

Table 3: Relation mole fractions of water in different dioxane-water mixtures (v/v) on the rate of hydrolysis of hemiester (3) at 40°C

Ratio	Value	Ratio	Value	Ratio	Value
k_1 (50:50)/ k_1 (60:40)	0.625	k_2 (50:50)/ k_2 (60:40)	0.500	k_3 (50:50)/ k_3(60:40)	0.406
k_1 (40:60)/ k_1 (50:50)	0.500	k_2 (40:60)/ k_2 (50:50)	0.413	k_3 (40:60)/ k_3 (50:50)	0.347
k_1 (40:60)/ k_1 (60:40)	0.313	k_2 (40:60)/ k_2 (60:40)	0.208	k_3 (40:60)/ k_3 (60:40)	0.139

4. Conclusion

- The rates of base-catalyzed hydrolysis of hemiesters (1-4), show that it follows the overall second order kinetics, first order with respect to both hemiester and base. The order of the reactivity which falls in the order 1>2>3>4 indicating that the electronic factor outweighs the steric factor.
- The highest rate of (1) is attributed to the less conjugation exerted by the carbonyl group of the carboxylate ion with the phenyl ring.
- The higher rate of hydrolysis of isomeric hemiester (2) than (3) is attributed to the higher reactivity of position 1 rather than position 2 in the naphthyl group. Dreiding models for both intermediates show that no steric hinderence exists.
- The lowest rate of base-catalyzes hydrolysis of hemieser (4) is ascribed to the conjugation of carbomethoxy group with the olefinic double bond and extended to the naphthyl group.
- The calculated values of different ratios between k_2 and between k_3 in different dioxane-water mixtures which found to be less than 1, indicae that the reaction is apparently independent of C_w.

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Corrosion Behavior of Aluminum Electrode in Absence and in Presence of Sodium Chloride at Different pH Solutions using Toludine as Inhibitor

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Abstract: The electrochemical behavior and the corrosion rate of aluminum electrode have been evaluated in different pH solutions in absence and presence of aggressive anion, using open-circuit potential measurements and potentiodynamic polarization technique. Also, the corrosion inhibiting effects of ortho, meta and para toludine in a solution of pH4 containing 0.1M sodium chloride was investigated using potentiodynamic polarization measurements. [Journal of American Science 2010;6(8):476-486]. (ISSN: 1545-1003).

Keywords: corrosion; aluminum electrode; pH; anion; sodium chloride; potentiodynamic polarization

1. Introduction

Aluminum and aluminum alloys represent an important category of materials due to their high technological value and wide range of industrial applications especially in aerospace and household industries. The use of these materials in light weight installation is widespread. Aluminum and its alloys, however, are reactive materials and porn to corrosion. Passivation and activation can occur depending on the potential, the pH and the Cl^- concentration. Rather than the potential for pitting initiation, the pitting potential of aluminum appears to be the point at which the passivation phenomena become very low [1,2].

The variation of OCP of aluminum with time of immersion in NaCl solution has been also studied[3,4]. The electrochemical behavior of aluminum and some of its alloys was investigated[5] in buffer solution of different pH. The inhibition of corrosion of these materials using inorganic passivators like sulphates, molybdates was discussed showing that inhibition efficiency is independent on the concentration of the passiviting anion in the solution. The corrosion and passivation behavior of aluminum in alkaline solution in pH range from 2-11 has attracted the attention of many investigators [6-10]. Results indicated that increasing pH changing the cathodic reaction, and increasing Cl^- ion concentration decreased the cathodic reaction rate. On the other hand, the anodic reaction rate increased with increase in Cl^- concentration. The open circuit corrosion potential and the pitting potential shifted in the active (negative) direction with increasing pH and Cl^- ion concentration. The electrochemical and surface properties of Al in citric acid solutions of pH2-8 by OCP, potentiodynamic polarization and potentiostatic current-time transient measurements were investigated[11]. The corrosion kinetic parameters E_{corr} , I_{corr} and b_a suggest that surface processes are involved in the dissolution kinetics, especially in the pH range 3-6. Corrosion inhibition of Al in HCl solution was investigated in the presence of different concentration of tetrazole derivative. The adsorption of these compounds on the Al surface obeys a langmuir adsorption isotherm and has a mixed physisorption and chemisorption mechanism[12]. The aim of the present work is to study the electrochemical behavior of aluminum metal in different pH of aggressive NaCl solution and also to study the inhibitive effect of adding o,m and p toludine with different percent**.**

2. Experimental

The aluminum sample selected for the study was of high purity (99.99 %). Prior to each electrochemical experiment, the electrode surface was polished with emery paper [from 400-1200 grade], degreased with acetone and finally rinsed with doubly distilled water. For the OCP against time measurements, pH-meter-millivoltameter type WGPYE model 290 was used. Electrochemical polarization measurements were made using EG&G PARC model 350A corrosion measurement system, in all measurements the reference electrode was a saturated calomel electrode (SCE). The exposed area of the working electrode was $1cm^2$.Two carbon electrodes were used as counter electrodes. The experiments were carried out in different pH using buffer acetic and sodium acetate in acid medium and buffer boric acid and sodium borate in alkaline medium, free and containing NaCl with concentration ranging between 0.01M and 0.5 M. The effect of toludine as inhibitor was studied using different percent of o^-, p^- and m^- toludine.

3. Results and Discussion:

III.1-OCP measurements:

The aluminum electrode was immersed in aerated different pH solutions at 30°C and the variation of OCP was followed as a function of time till attainment of the steady state value (E_{st}). Two main trends can be seen for the variation of OCP with time. The first one includes solutions of pH 2-7 (some representative examples are given in Fig.1.

The OCP of the aluminum electrode was generally similar in acetic acid – sodium acetate solutions of pH 4 and 5 but was different from boric acid and sodium borate of pH 8 and 9. In Fig.1 example of OCP-time curves, which illustrate both types of behavior are shown. These results indicate a significant change of the surface oxide film during the immersion time. In this respect a similar behavior were reported for aluminum electrode in acetate solutions and in mixtures of acetate and oxalate solutions[13-14], for Al- 7075 alloy in oxalate solutions[15] and for Al- 3003 and Al-5052 alloys in citrate and acetate buffer solutions[16]. On the other hand the marked increase in the OCP with decrease of pH denotes an ongoing surface oxide film growth and ageing of the aluminum oxide film, therefore in solutions of pH4 and pH5 the very slow reaching of steady- state potential of aluminum electrode means that beside the electrochemical process, surface coordination processes also play an important role.

$$Al^{3+}+H_2O=AlOH^{2+}+H^+$$
$$Al^{3+}+2H_2O= Al(OH)_2^{+}+2H^+$$
$$Al^{3+}+3H_2O= Al(OH)_3+3H^+$$

The second shape of potential- time curves observed in the solutions of pH8 and pH9 is quite different from those recorded in solutions of pH4 and pH5. It is clear from Fig.1 that the potential of aluminum electrode decreases in a relatively short time and reaching a steady- state value after about one hour from the moment of immersion. This behavior indicates the instability of the pre-immersion air-formed aluminum oxide film on the metal surface.

3.2- Potentiodynamic polarization measurements:

Fig.2 show some examples of the potentiodynamic polarization curves performed on aluminum electrode in different pH solutions at 30°C with a scan rate of 0.5 mV/sec starting from- 0.250 V with respect to SCE (in the so called Tafel region).

All the potentiodynamic polarization curves have similar shapes. The corrosion kinetic parameters E_{corr}, i_{corr}, b_c and b_a determined from polarization curves in the Tafel region are summarized in Table (1).

The cathodic reaction occurring at the Al-oxide/acetate solution interface was hydrogen evolution. Table (1) shows that the cathodic slopes (b_c) are much greater than that expected for H_2 evolution according to Volmer-Tafel mechanism[17] (-118mV (decade)$^{-1}$at 30°C). Such large cathodic Tafel slopes are not unexpected for aluminum, and have previously been reported for H_2 evolution reaction on Al oxide- covered electrodes[16,18]. The presence of the oxide film can markedly influenced the surface reduction process, by affecting the energetics of the reaction at the double layer, or by imposing a barrier to change transfer through the oxide film, or both.

The anodic Tafel slopes (b_a) are much greater than the expected 40mV (decade)$^{-1}$ corresponding to the uniform anodic dissolution of aluminum via hydrated Al^{+3} ions. The observed (b_a) values are in agreement with the b_a values reported for an Al oxide- covered electrode[1314], which clearly indicates the presence and growth of a passive oxide film. However the b_a is greater than those for the simple aluminum dissolution mechanism ($Al \rightarrow Al^{+3}+3e^-$), it can also be attributed to the precipitation of some side reactions (e.g., surface coordination reactions) in anodic oxide film growth.

Table (1): Corrosion kinetic parameters E_{corr}, i_{corr}, b_c and b_a for aluminum electrode at different pH solutions, determined from the Tafel plot.

pH	E_{corr} (V)	i_{corr}(μA/cm^2)	b_c(mV)	b_a(mV)
2	-0.415	1.75	190	137
3	-0.420	1.80	185	141
4	-0.451	1.92	175	144
5	-0.517	2.15	173	150
6	-0.562	2.23	170	153
7	-0.620	2.35	156	162
8	-0.720	4.24	146	173
9	-0.828	6.40	140	187

3.3- Effect of solution pH on corrosion potential and corrosion current:

It is evident from Fig.3 that the values of corrosion potential (E_{corr}) of aluminum electrodes decreases with increase in pH. On the E_{corr} vs pH curve three different regions were distinguished. The first are corresponds to the pH range 2-3 in which E_{corr} changed relatively slowly towards more negative values. It is apparent therefore, that at this range of pH the concentration of the H^+ ions is sufficiently high and oxygen is eliminated from solutions, the dissolution of aluminum is under the control of electrochemical processes. This means that the main cathodic reaction is a reduction of H^+ ions whereas

the main anodic reaction is the dissolution of aluminum in the form of Al^{+3} aqueous complexes. The second region extending from pH3 to pH6, where E_{corr} changes approximately linearly with pH, and the slope of E_{corr} against pH curve was- 59.66 mV/ unit pH. This behavior can be explained as follows: In aqueous solutions Al- oxide surface is significantly hydrated. The hydration of aluminum-oxygen bonds on the oxide film leads to the formation of surface-OH groups. Subsequent amphoteric dissociation of -OH groups leads to the development of a charge on the oxide surface[19]. Therefore, an acid- base equilibrium is established on the surface which controls the surface charge and the surface processes on an oxide covered Al-electrode. E_{corr} against pH behavior in the pH region 6-9 was characterized by a strong negative shift of E_{corr} with increase in pH. At the same time, the corrosion current density i_{corr} increased significantly with increase in pH Fig. [3], especially in solution of pH8. Thus the dissolution of aluminum increases significantly from pH7 to pH9. Such behavior is in agreement with the fact that aluminum dissolves relatively strongly at these pH values as aluminate AlO_2^- or $Al(OH)^-_4$ ions [23] .It is seems that the electrochemical corrosion mechanism (evolution of hydrogen, dissolution of aluminum as aluminate ion) proceeds in this pH range, especially at pH8 and pH9.

3.4- Influence of chloride ion concentration at different pH solutions:

The variation of the OCP with time of immersion for aluminum electrode at various pH (4 and 5 (acetic acid +sodium acetate and pH 8 and 9 (boric acid and sodium borate solutions) solutions containing (0.01 M – 0.5 M) sodium chloride at 30°C are shown in Fig.4(a-d). In each experiment the potential was followed at intervals for 4 hours. The steady-state potential (E_{st}) obtained were plotted against the logarithm of the molar chloride concentration at different pH solutions, and are represented in Fig.5.

As evident from Figs.4(a,b) the OCP values were changed rapidly at the beginning towards positive potential then reached stationary potential after about one hour in absence of Cl^- ion , but in presence of different chloride concentrations the OCP values were changed toward negative potential and reached steady- state value after about half an hour and the same behavior is observed in pH8 and pH9 as shown in Figs.4(c,d).

Fig.5 shows the effect of variation of salt concentration on steady-state potential of aluminium electrode at different pH solutions. The OCP is shifted in the negative direction with increase in pH and chloride ion concentration. The shift in the OCP towards more negative values is attributed to the adsorption of negative chloride ion on the surface of the metal[20], (the adsorption of ions on the metal surface is responsible for change in its potential).

Fig.6(a-d) show potentiodynamic polarization curves for aluminum electrode in various pH 4, 5, 8 and 9 solutions free and containing various chloride ion concentrations. The behavior of aluminum in chloride containing solution of pH4 and pH5 is different from those recorded at pH8 and pH9.

In case of pH4 and pH5 free and containing different chloride ion concentrations we observed the absence of pitting potential due to the presence of acetic acid which resists the pitting corrosion[21].

In case of pH8 and pH9 in absence of chloride ions there is no pitting potential because the presence of OH^- ions dissolved aluminum metal, but in the presence of chloride ions the curves exhibit an apparent active region with passivity at intermediate value of potential and pitting at and beyond the pitting potential due to the presence of chloride ions.

The values of the corrosion potential (E_{corr}), anodic Tafel slope, (b_a), cathodic Tafel slope (b_c) are given in Table (2), (3) and (4) respectively.

Table (2): Effect of various chloride concentrations on the corrosion potential of aluminum in different pH solutions.

Conc. of NaCl (M)	Corrosion Potential, E_{corr}, (V)			
	Acetic acid+ sodium acetate		Boric acid +sodium borate	
	pH4	pH5	pH8	pH9
0	-0.515	-0.530	-0.720	-0.828
0.01	-0.535	-0.560	-0.770	-0.901
0.05	-0.598	-0.613	-0.780	-0.918
0.1	-0.628	-0.647	-0.800	-0.992
0.5	-0.690	-0.708	-0.828	-0.957

Table (3): Effect of various chloride concentrations on the anodic Tafel slope of aluminum in different pH solutions

Conc. of NaCl (M)	Anodic Tafel Slope ,$b_{a,}$ (mV)			
	Acetic acid+ sodium acetate		Boric acid +sodium borate	
	pH4	pH5	pH8	pH9
0	143	150	173	187
0.01	140	154	170	180
0.05	142	152	166	176
0.1	148	150	160	170
0.5	146	156	165	177

Table (4): Effect of various chloride concentrations on the cathodic Tafel slope of aluminum in different pH solutions

Conc. of NaCl (M)	Cathodic Tafel Slope ,$b_{c,}$ (mV)			
	Acetic acid+ sodium acetate		Boric acid +sodium borate	
	pH4	pH5	pH8	pH9
0	-190	-180	-165	-146
0.01	-195	-175	-160	-154
0.05	-201	-170	-164	-152
0.1	-204	-182	-168	-162
0.5	-205	-187	-160	-150

Increase in pH from 4 to 9 and /or chloride ion concentration shifted E_{corr} in the active direction. This shift is a result of the shift in the cathodic curve as hydrogen ion concentration decreases. Increase in pH of the solution changed the slope of the cathodic curve. This change is attributed to the change in the cathodic reaction at different pH levels. Increase in chloride ion concentration decreased the cathodic reaction rate, but the slope of the curve remained almost the same.

At pH8 and pH9 the increase in chloride ion concentration shifted the passive region of the anodic curve to higher current density. The height of the passive region also varied with pH but was not affected by increasing in cholride concentration (e.g. for pH8 passive region extends for about ~0.102 mV, corresponding value for pH9 is ~ 0.212 mV). Also, the increase in chloride concentration shifted E_{pit} to more negative values.

The passive region observed in all cases is attributed to the formation of a thin oxide film on the surface of the metal which causes the metal to stop interaction with the surrounding media[21].

Fig.7 shows the plot of corrosion rate (MPY), calculated using I_{corr} values obtained from polarization curves, against the logarithm of molar chloride concentration at different pH. At all pH values the corrosion rate increase with increasing chloride ion concentration and it was found to be significance at pH9.

The increase in corrosion rate with increasing chloride ion concentration is due to the participation of this ion in the metal dissolution reaction. This kind of mechanism has been predicted by[22-24].

The corrosion rate was found to be enhanced at acidic pH values; this result was in agreement with the enhanced solubility of aluminum oxide at low pH values.

At pH 9 the oxide film is highly soluble and this leads to a higher corrosion rate, the dissolution equilibrium is governed by the equation.

$$Al^{+3} + 4H_2O \longleftrightarrow Al(OH)_4^- + 4H^+$$

$Al(OH)_4.2H_2O^+$ is equivalent to AlO_2^-

The results obtained for increasing amount of sodium chloride in aqueous solution were also consistent with the suggestion that the chloride ions are responsible for the attack and subsequent dissolution of the oxide layer. Thus the rate of corrosion increases rapidly with increasing chloride concentration in solution. This is attributed to the adsorption of chloride to weak parts of the oxide film leads to the formation of film / solution interface of transitional chloride ion containing complex by the reaction

$$Al + nCl^- \longrightarrow AlCl^{(n-3)} + 3e^-$$

These complexes are much more soluble than the complex formed in the absence of chloride

ion. The dissolution of the complex leads to the breakdown of passivity and hence pitting.

3.5-Toludine as Inhibitor for Acidic Chloride Corrosion of Aluminum.

Examples of potentiodynamic polarization curves for aluminum in a solution of pH4 containing 0.1 M sodium chloride without and with various para-toludine concentrations are shown in Fig.8. Ortho and meta toludine show similar behavior as para toludine. In all cases, addition of toludine induced a negligible decrease in the anodic current and a significant decrease in the cathodic current. The observed decrease in the cathodic current was the greatest for 0.1% m-toludine. The value of E_{corr} was not significantly affected by the addition of inhibitors. None of the inhibitors affected the anodic Tafel slope which was around 64 mV. The cathodic Tafel slope of approximately (190 – 197mV) was observed in presence and in absence of inhibitors i.e is independent of inhibitor concentration Table (5). This fact can be classified the inhibitor as cathodic inhibitor.

Table (5) contains the values, corrosion potential (E_{corr}) cathodic Tafel slope (b_c) ,corrosion (i_{corr}) and exchange (i_o) current densities, percentage decreases in each, polarization resistance (R_p), degree of surface coverage (θ) and the percentage inhibition (P) as a function of inhibitors concentrations. The percentage decreases in the exchange current density equals that in the corrosion current density within the limits of experimental error.

The cathodic Tafel slopes (b_c) were above 180 mV, this indicate that hydrogen evolution reaction takes place at the metal covered by a surface layer, probably an oxide or an oxide-inhibitor complex, which acts as a potential energy barrier to the charge carriers[18,25]. Polarization resistances, Rp, for aluminum in a solution of pH4 containing 0.1 M sodium chloride free and in the presence of different toludine (o, m, and p) at various concentrations were determined. Generally R_p values increased with increasing inhibitor concentration and were greatest in 0.1% m-toludine.A comparison of the inhibiting efficiencies and corrosion rate obtained using the polarization technique shows that (NH_2) group in meta position is much more effective than in the para and ortho position.

Fig.9a shows the plot of corrosion rate (MPY), calculated using i_{corr} values obtained from polarization curves, of aluminum electrode versus the logarithm of different toludine (o, m and p) of various concentrations in a solution of pH4 containing 0.1M sodium chlodride. The corrosion rate decreases as the inhibitors concentrations increases and the corrosion rate follows the sequence

ortho > para > meta. The efficiency of a certain corrosion inhibitor is measured by the percentage inhibition (P%)[26].

$$P = 100\left(1 - \frac{W_2}{W_1}\right)$$

Where W_1, W_2 are the corrosion rates in absence and presence of inhibitor, respectively. Fig.9b shows a plot of percentage protection P versus the logarithm of different concentrations of various toludine in a solution of pH4 containing 0.1 M sodium chloride. It is seen that the percentage protection increase with increasing inhibitor concentration approaching 88% in case of meta, 86% in case of para and 83% in case of ortho. This is may be due to the adsorption of a complete monolayer of toludine on the surface. The degree of surface coverage θ was calculated from the shift in the cathodic Tafel lines caused by the presence of inhibitors. For inhibitors which causes a parallel shift of the cathodic Tafel line, a comparison can be made at constant potential thus,

$E = a_1 - b \log (i_c)_1$ Without inhibitor
$E = a_2 - b \log (i_c)_2$ With inhibitor
and $(i_c)_2 = (1- \theta)\ (i_c)_1$

By comparing the current densities in presence $(i_c)_1$ and in absence $(i_c)_2$ of a blocking adsorption inhibitor , the degree of coverage can be calculated[27]. The only requirment for the above equation to hold is that the presence of inhibitor does not change the Tafel slope, a condition which is fullfilled here.

It is now commonly believed that the first step in the corrosion inhibition is an adsorption process. Therefore, it is of interest to determine the type of adsorption isotherm which best explains the adsorption data.

The Langimuir isotherm is

$$\frac{\theta}{1 - \theta} = KC$$

Where θ is the degree of coverage, K is the absorbability and C is the concertration of inhibitor. Taking logarithms of the above equation gives.

$$\log \frac{\theta}{1-\theta} = \log k + \log C$$

Alternatively, a plot of log θ /(1- θ) versus log C should be a straight line with unit slope.

Fig.10a shows such a plot of $\log \frac{\theta}{1-\theta}$ versus the logarithm of different concentrations of various toludine for aluminium in a solution of pH4 containing 0.1 M sodium chloride. The figure shows that a good straight line relation is obtained which indicates that the adsorption of inhibitor on aluminium follows langimuir isotherm . However the slope of the straight line are about 0.62 , 0.72 and 0.82 , for meta , para and ortho respectively, which is less than unity this may be attributed to the effects of lateral interaction between the adsorbed inhibitor molecules.

Fig.10b shows the relation between log P / (1-P) versus inhibitors concentrations. Again the relation is a straight line with a slope of 0.64, 0.77 and0.93 for meta, para and ortho respectively. These values are sufficiently close to the values of 0.62, 0.72 and 0.82. It is then concluded that the adsorption does not change the mechanism of the hydrogen evolution reaction, even though, it significantly reduced its rate. This supports the view that toludine blocks a fraction of the electrode surface hence it reduces the surface area avaliable for hydrogen evolution, this reduce the rate of hydrogen evolution and consequently the rate of the overall corrosion reaction. From Table (5) it is clear that the percentage decreases in i_{corr}, i_o and θ and P are all equal within experimental error. It can be concluded that the cathodic (hydrogen evolution) reaction is the rate determining step and the fractional of the surface area covered with toludine does not participate in the corrosion reaction. This is strongly suggested that toludine is a cathodic corrosion inhibitor of a blocking adsorption type. The general conclusion is that toludine is an excellent inhibitor for the corrosion of aluminum in acidic chloride solution and meta-toludine shaving marked efficiency than para and ortho toludine at comparable concentration. The reactivity of toludine follows the order:

m – toludine > p – toludine > o – toludine

This order of reactivity can be explained on the basis of basicity of toludine as aromatic amine. The low basicity of the amino group ($-NH_2$) in ortho and para position results from the resonance (+R) effect where nitrogen atom shares its lone pair of electrons with the aromatic ring and consequently acquires a positive charge. The lower reactivity of o–toludine attributed to the lower basicity of the amino group results from both its + R effect and to the ortho effect where steric hindrance is exerted by the bulky methyl group located at the ortho position to the amino group.

4. Conclusion

The OCP for pH 4-5 varies in the noble direction at the moment of immersion then decrease slowly after 50 minute till steady state values. In pH 8&9 the OCP decrease in a relatively short time reaching a steady-state value after one hour.

In the presence of Cl^- ion the OCP are shifted towards negative direction with increasing pH and Cl^- concentrations.

Potentiodynamic polarization of Al electrode in different pH solutions similar shapes with shift of the passive region to higher current density with increasing pH. In presence of Cl^- we observed the absence of pitting potential at pH4 and pH5 due to presence of acetic acid which resist the pitting corrosion.

At pH8 and 9 all the curves exhibit an active region with passivity at intermediate value of potential and pitting at and beyond the pitting potential due to presence of Cl^- ion.

The variation of the corrosion rate as a function of the logarithm of molar chloride concentration show that the corrosion rate increases with increasing Cl^- ion concentration and pH of the solutions.

The corrosion rate decrease as the inhibitor concentrations increase following the sequence o,m and p and the percentage protection with increasing inhibitor concentrations due to the toludine on the surface.

Table (5): The effect of inhibitor concentration on corrosion potential, cathodic Tafel slope, corrosion and exchange current densities, percentage decreases in each, polarization resistance, degree of surface coverage and percentage protection.

Conc (Wt %)	Log C	- Ecorr(V)			bc (m V)			i_o (nA/cm^2)			% decrease in i_o			i_{corr} (nA/cm^2)			% decrease in i_{corr}			Rp			θ			P		
		o	p	m	*o*	p	m	*o*	p	m	*o*	p	m	*o*	p	m	*o*	p	m	*o*	p	m	*o*	p	m	*o*	p	m
0.00	-	**0.672**			**190**			**$7.638x10^4$**			-			**$4.59x10^4$**			-			**$9.2x10^2$**			-				-	
0.01	**-2**	0.646	0.632	0.626	143	186	190	$4.53x10^4$	$3.6x10^4$	$2.78x10^4$	41	50.4	64.6	$2.8x10^4$	$2.19x10^4$	$1.6x10^4$	39	52	65	$1.86x10^3$	$2x10^3$	$3.6x10^3$	41	50	62	37	52	64
0.025	**-1.6**	0.648	0.635	0.652	187	195	198	$3.05x10^4$	$2.6x10^4$	$1.58x10^4$	60	66	79	$1.76x10^4$	$1.5x10^4$	$1x10^4$	61	67	78	2.3x103	3.19x103	4.2x103	60	66	79	59	67	78
0.05	**-1.3**	0.660	0.642	0.656	195	180	190	$2.14x10^4$	$1.66x10^4$	$1.38x10^4$	72	78	82	$1.2x10^4$	$1x10^4$	$7.68x10^3$	74	78	83	2.9x103	3.67x103	4.9x103	72	78	82	74	78	83
0.1	**-1**	0.666	0.648	0.667	196	197	196	$1.37x10^4$	$1.23x10^4$	$1x10^4$	82	84	85.7	$7.8x10^3$	$6.25x10^3$	$5.55x10^3$	83	86	88	3.5x103	4.2x103	5.7x103	82	84	86	83	86	88

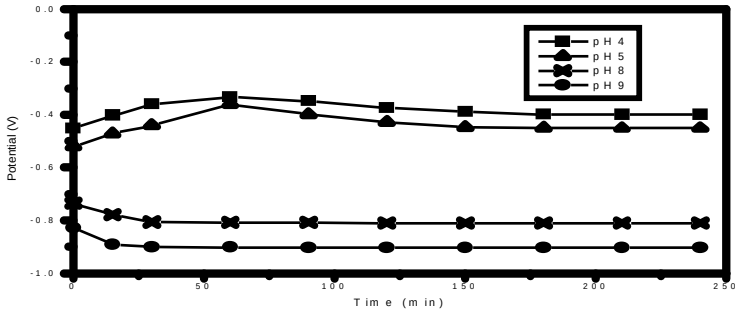

Fig. (1): Variation of the OCP with time for aluminium electrode in different pH solutions.

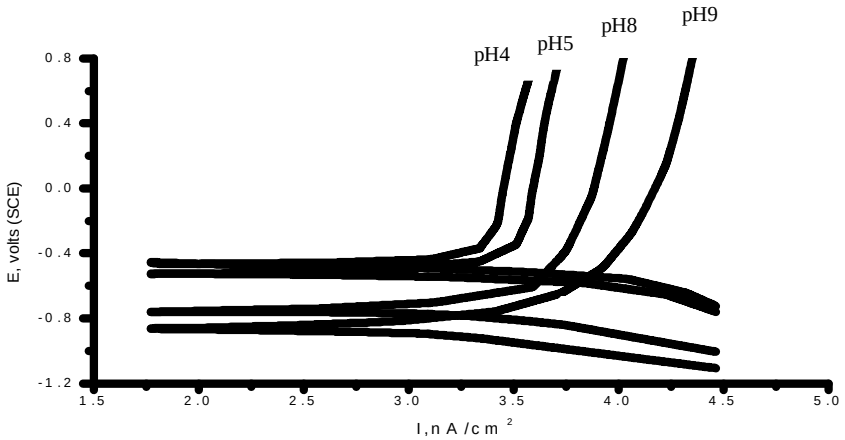

Fig.(2): Potentiodynamic polarization curves of aluminum electrode in different pH solutions.

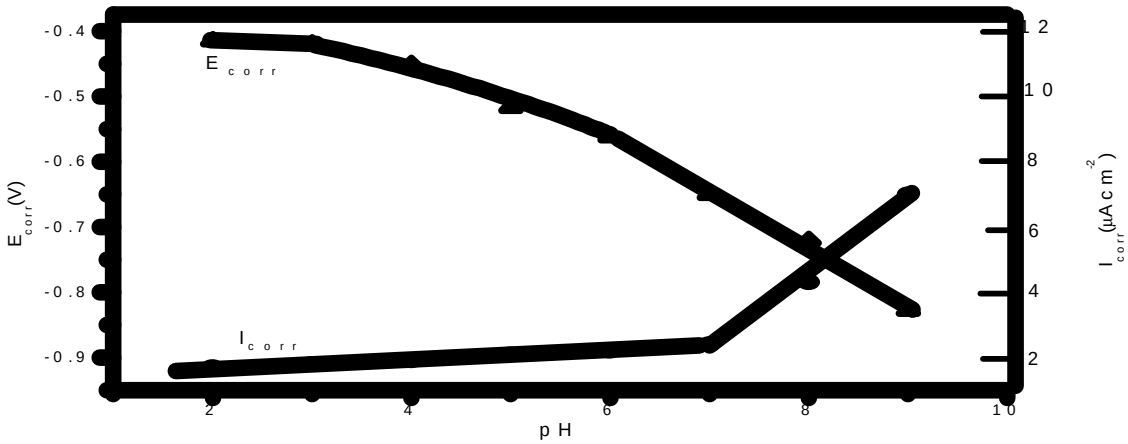

Fig. (3): Dependence of corrosion potential (E_{corr}) and corrosion current densities (i_{corr}) of aluminium electrode, determined from Tafel plots, on various pH values.

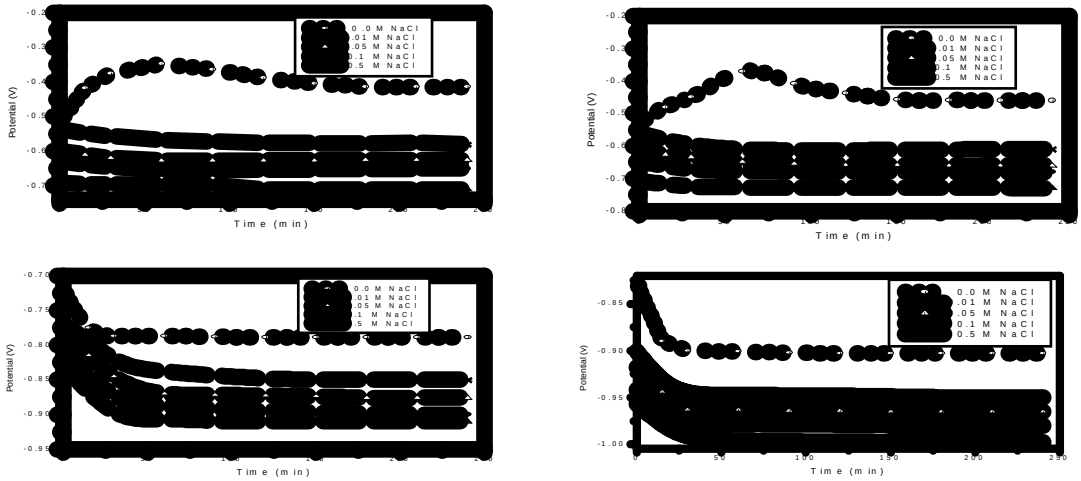

Fig. (4): Variation of the OCP with time for aluminium electrode in a solution of a) pH4 b) pH5 c) pH8 d) pH9 free and containing different concentrations of sodium chloride

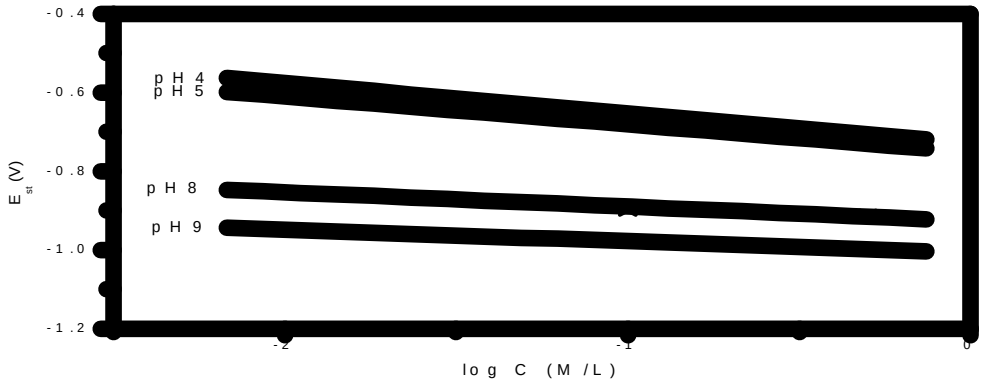

Fig. (5): Effect of variation of salt concentration on steady-state potential of aluminium electrode at different pH solutions.

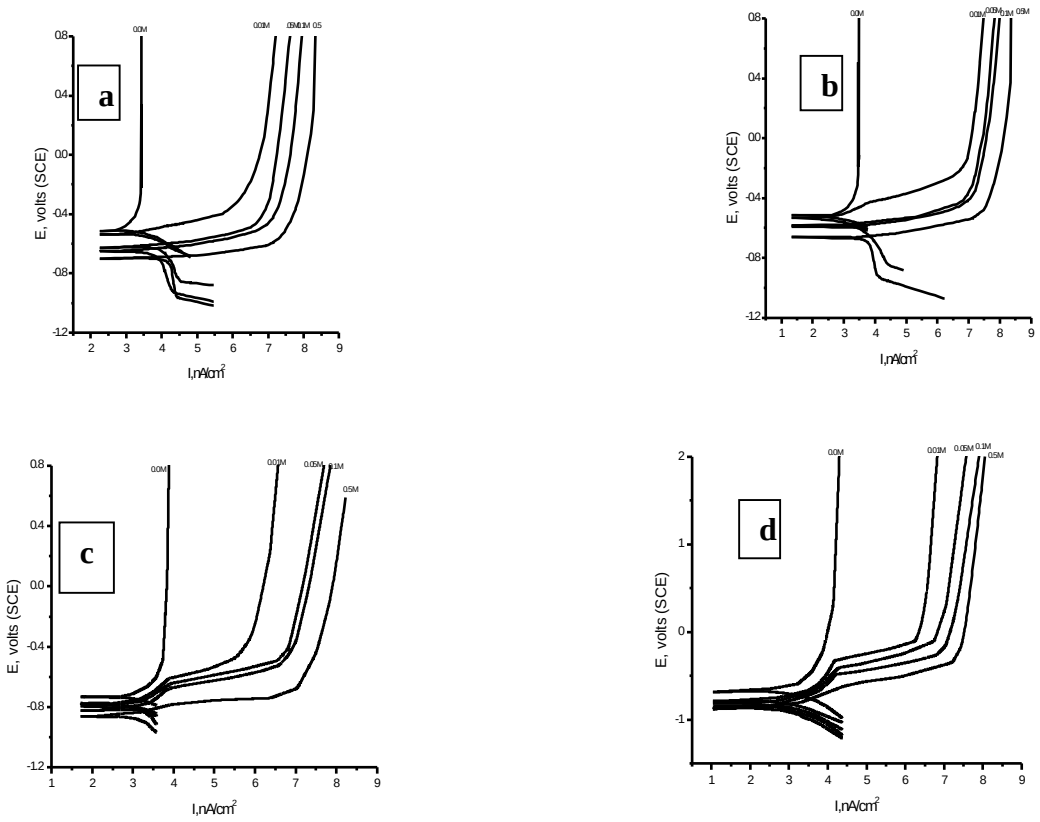

Fig. (6): Potentiodynamic polarization curves of aluminium electrode in a solution of (a) pH4 (b) pH5 (c) pH8 (d) pH9 free and containing various sodium chloride concentrations.

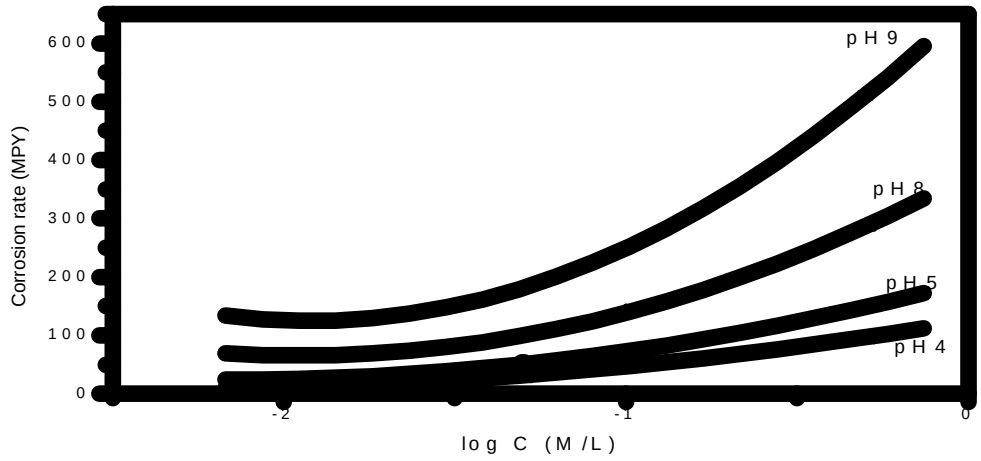

Fig. (7): Variation of the Corrosion rate of aluminium electrode with logarithm of molar chloride concentration at different pH values.

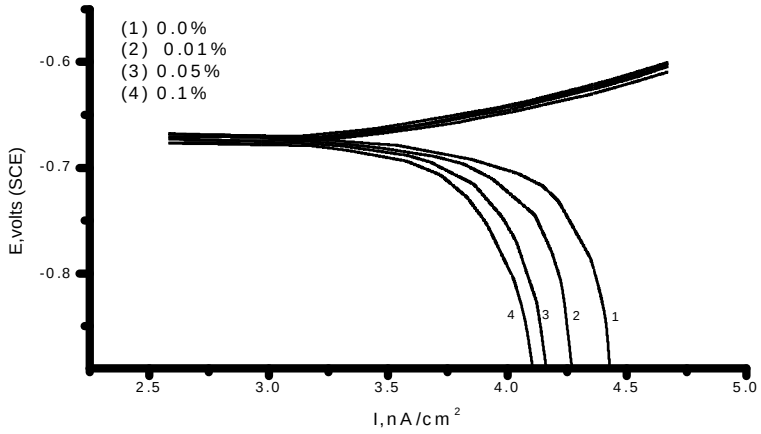

Fig.(8): Potentiodynamic polarization curves of aluminium electrode in a solution of pH4 containing 0.1M sodium chloride in absence and in presence of various o-toludine concentrations .

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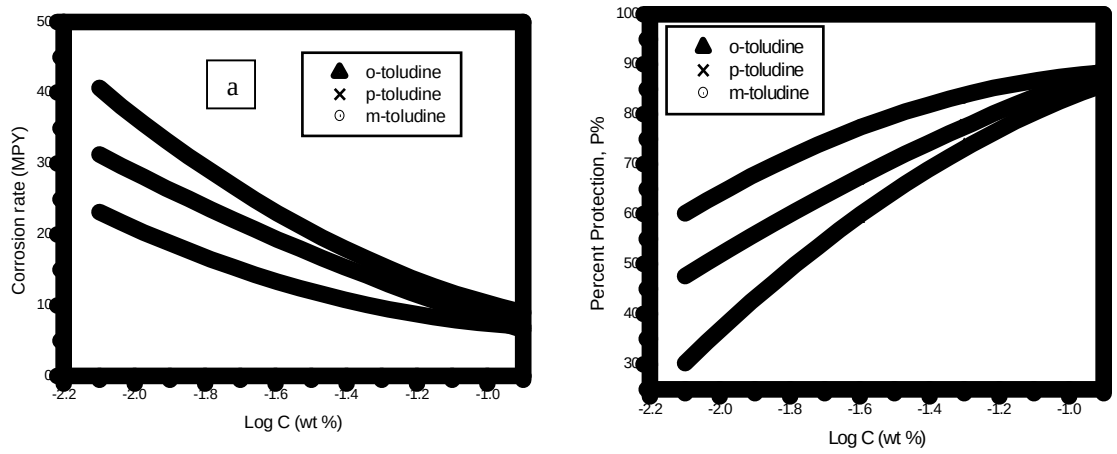

Fig. (9): Effect of o,p and m-toludine concentrations as inhibitors on a) corrosion rate b) percentage protection of aluminium in a solution of pH4 containing 0.1 M sodium chloride.

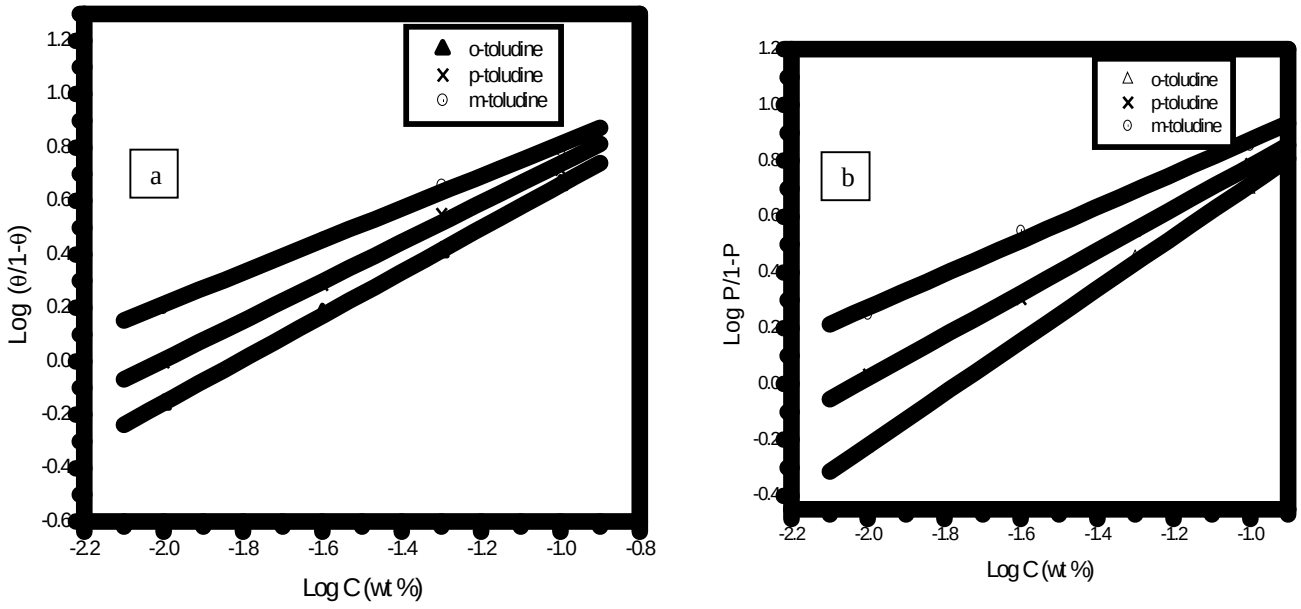

Fig.(10):Plot of a) $\log\frac{\theta}{1-\theta}$ **b)** $\log\frac{P}{1-P}$ **vs. log concentrations of o,p and m toludine in a solution of pH4 containing 0.1 M sodium chloride.**

Corrosion Inhibition of Copper and Copper Alloy in 3M Nitric Acid Solution using Organic Inhibitors

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Abstract: The effect of the addition of organic compounds containing an amino group, such as ethylamine (EA), ethylenediamine (EDA) and butanediamine (BDA) , on the corrosion of copper and copper alloy in nitric acid was studied by weight loss, open circuit potential and potentiodynamic polarization techniques. The explored methods gave almost similar results. Results obtained revealed that butanediamine is the best inhibitor and the protection efficiency (p%) follows the sequence butanediamine > ethylenediamine > ethylamine. The effect of temperature on the corrosion behavior of copper and copper alloy in nitric acid solution in presence and absence of inhibitor were studied in the temperature range 30-60°C. The associated activation corrosion and free adsorption energies have been determined. SEM examination of the copper and copper alloy surface revealed that these compounds prevented copper and copper alloy from corrosion by adsorption on its surfaces. [Journal of American Science 2010;6(8):487-498]. (ISSN: 1545-1003).

Keywords: Copper; Copper alloy; Nitric acid; Corrosion inhibition; Amines

1. Introduction

Copper and its alloys have many industrial applications such as electronics because of their excellent corrosion resistance properties as well as their superior electrical and thermal performance [1]. Although copper is malleable and machinable, it is not very durable. If a high strength material is desired, copper has to be alloyed with other metals, such as zinc, tin, aluminum or nickel [1]. Doping with divalent cations is an effective way of improving the corrosion resistance of copper. Chemical dissolution and electrolytic plating are the main processes used in the fabrication of electronic devices. The most widely used corrosive solution contains nitric acid, so this medium has induced a great deal of research on copper corrosion [2-6]. One of the most important methods in the corrosion protection of copper is the use of organic inhibitors [7-9]. Most of the excellent acid inhibitors are organic compounds containing nitrogen, sulfur and oxygen [10-18], and heterocyclic compounds with polar functional groups and conjugated double bonds [19-23]. The inhibiting action of these organic compounds is usually attributed to their interactions with the copper surface via their adsorption. Polar functional groups are regarded as the reaction center that stabilizes the adsorption process [24]. In general, the adsorption of an inhibitor on a metal surface depends on the nature and the surface charge of the metal, the adsorption mode, its chemical structure, and the type of the electrolyte solution [25]. Amines are known to be very effective inhibitors for metals and alloys in different corrosion media. Srivastafa et al [26] found that 0-, m- and p-phenylenediamine show effective inhibition efficiencies. The aim of the present investigation was to study the inhibitive action of some aliphatic amines on the corrosion of copper in the aerated 3M nitric acid solution.

2. Experimental

The investigation of inhibiting properties of butanediamine (BDA), ethylenediamine (EDA) and ethylamine (EA) (Aldrich) were performed using the weight loss method and electrochemical studies, including open-circuit potential and potentiodynamic polarization techniques, along with scanning electron microscopy (SEM). Both copper (99.9% Cu) and copper alloy (65% Cu- 10% Ni- 25% Zn) were tested in 3 M nitric acid solution. The latter is of analytical grade and has been used without further purification. Double distilled water was used in the preparation of solutions. Prior to all measurements, the substrates were etched in a 6 M nitric acid solution for 20 sec, then washed thoroughly with distilled water, degreased with ethanol, washed again with distilled water and finally dried at room temperature. The specimens were weighted and immersed in the corrosive medium.

The weight loss measurements were carried out at 30, 40, 50 and 60°C using sheets of the substrates with dimensions 2 x 2.5 cm^2. The sheets were suspended in 20 mL solution of 3 M nitric acid with and without the different inhibitors concentrations (0.001-1 M) for 30 minute. The loss in weight per area A (=10 cm^2) in mg/cm^2 (W_t), the corrosion rate (R_{corr}), and the percentage of protection efficiency (P%) were calculated over different inhibitors concentrations according to the following equations:

$$W_t = \frac{W_o - W_1}{A} \quad (1)$$

$$R_{corr} = \frac{W_t}{t} \quad (2)$$

$$P\% = \left[1 - \left(\frac{R'_{corr}}{R_{corr}}\right)\right] \times 100 \quad (3)$$

where W_o is the original weight (mg) and W_1 the weight after immersion in the test electrolyte, t the immersion time (sec), and R'_{corr} and R_{corr} are corrosion rates with and without an inhibitor, respectively.

Two measurements were performed in each case and the mean value of the weight loss was reported. The open circuit potential (OCP) was measured using pH-meter-millivoltameter type WGPYE model 290. The OCP of copper and the copper alloy were measured versus (SCE) saturated calomel electrode as a function of immersion period in 3 M nitric acid in the presence and absence of amine derivatives till steady state values were reached.

Potentiodynamic polarization studies were carried out with the test specimens having an exposed area of 1 cm^2 using EG&G PARC model 350A (console) in the range from -250 mv to 1000 mv using a scan rate of 0.5 mV/sec. The cell consisted of test specimens as working electrode and (SCE) as reference electrode. Two carbon electrodes were used as counter electrodes.

Scanning electron microscope (SEM) unit "JEOL JSM5410" was used to demonstrate the surface morphology of different samples employing an accelerating voltage of 30 kv. Samples were mounted using carbon paste to ground them. The results were obtained as computer print out.

3. Results and Discussion:

3.1- Weight loss measurement

Fig.1 and Fig. 2 show the average corrosion rate (R_{corr}) of copper and copper alloy sheets, expressed as mg sec^{-1} cm^{-2}, as a function of the logarithmic concentration of BDA, EDA and EA in 3 M HNO_3 solution at 30°C, respectively. They demonstrate that the addition of the organic inhibitors decreases the corrosiveness of the acid. The corrosion rate of copper and copper alloy depends on the concentration of inhibitors. Their action depends on the nature of substituent. Fig. 3 and Fig. 4 show the variation of the protection efficiency of copper and copper alloy respectively as a function of the logarithmic concentration of BDA, EDA and EA in 3 M HNO_3 solution at 30°C. The protection efficiency increases with increasing inhibitors concentrations. Also, the protection efficiency increases from EA to EDA to BDA as shown in Table (1).

Fig. 5 and Fig. 6 show the variation of corrosion rate of copper and copper alloy, respectively, as a function of the logarithmic concentrations of BDA at different temperatures. Fig. 7 and Fig. 8 show the effect of the concentration of BDA on the protection efficiency of copper and copper alloy, respectively, at various temperatures. The same behaviour is observed for EDA and EA .In general, the protection efficiency increases with decreasing temperature. This indicates that the adsorption of these compounds on copper and copper alloy surfaces is a physical adsorption.

3.2- Adsorption isotherm

In order to obtain a better understanding of the electrochemical process on the metal surface, adsorption isotherms at 30˚C were drawn. The degree of surface coverage (θ) at different concentration of each inhibitor in acidic medium has been evaluated from weight loss measurement according to equation (4).

$$\theta = \left[1 - \left(\frac{R'_{corr}}{R_{corr}}\right)\right] \quad \ldots\ldots\ldots\ldots \quad (4)$$

Data related to the degree of the surface coverage (θ) were tested graphically in order to determine the most suitable adsorption isotherm.

As shown in Fig.9 and Fig.10 the plot of C / θ versus C for copper and copper alloy in 3M HNO_3 with different inhibitors concentrations, yields a straight line showing that the adsorption of these inhibitors is well described by the Langmuir isotherm. These isotherms are described by equation 5.

$$\frac{C}{\theta} = \frac{1}{k} + C \quad \ldots\ldots\ldots\ldots \quad (5)$$

$$k = \frac{1}{55.5} \exp\left(\frac{-\Delta G^{\circ}}{RT}\right) \quad \ldots\ldots\ldots\ldots \quad (6)$$

where C is the inhibitor concentration, k the adsorption constant, and ΔG° the standard free energy of adsorption.

The calculated values of k and ΔG° of the adsorption reaction for copper and copper alloy are shown in Table (2). The negative value of the standard free energy of adsorption indicates spontaneous adsorption of these inhibitors on copper and copper alloy. This means that the inhibitive action of these substances results from the physical adsorption [27] as mentioned before.

3.3- Activation Energy

According to the Arrhenius equation, the apparent activation energy, E_a; for the corrosion process can be determined from the slope of logarithm corrosion rate, log R_{corr}, against 1/T.

$$\ln R_{corr} = \ln A + \frac{-Ea}{RT} \quad (7)$$

where A is the pre-exponential parameter of Arrhenius. Fig. 11 and Fig.12 show the logarithmic plot of the corrosion rate of copper and copper alloy in 3M HNO_3 as a function of (1/T) in free and inhibited solutions respectively. For the inhibitor free solution, the energy of activation, evaluated from these graphs, is found to be equal to 5.733 and 7.644 KJ mol-1 for copper and copper alloy, respectively. These values are in agreement with the literature data [28]. In the presence of anyone of the studied inhibitors, the energy of activation increases. For a 0.3M concentration of BDA, EDA and EA, the calculated values of the apparent activation energies are 15.28, 12.74 and 9.55 KJ/mol for copper respectively, and 22.93, 19.11 and 15.28 for copper alloy, respectively. This indicates that the formation of the adsorption film occurs by a physical mechanism [29].

By plotting log (R_{corr}/T) vs. (1/T) at 0.3M of the tested inhibitors, straight lines are obtained for copper and copper alloy as shown in Fig.13 and Fig.14 respectively. The values of ΔH° and ΔS° can be calculated from the slopes and intercepts of the straight lines, respectively, Table (3).

These results indicate that copper metal suffers from more corrosion than copper alloy. This observation may be attributed to the relatively high resistance of copper alloy due to the presence of nickel. ΔS in the presence of additives is larger, meaning that a decrease in disorder takes place in going from reactant to the activated complex [30, 31].

3.4. Open circuit potential (OCP) measurements:

Copper and copper alloy sheets were kept for 2 hour in 3 M HNO_3 solution in the absence and presences of the tested inhibitors. The variation of the steady state potential (E_h) with the logarithm concentration of the tested inhibitors for copper and copper alloy is represented by Fig. 15 and Fig. 16. It is clear from these curves that, as the inhibitor concentration increases, there is a shift in the steady state potential to less positive or more negative direction (relative to the blank).

3.5. Potentiodynamic polarization measurements:

Fig. 17 and Fig. 18 show the potentiodynamic polarization curves of copper and copper alloy in 3M HNO_3 in the absence and in the presence of the different inhibitors under investigation. The figures show that the cathodic current potential curves give rise to parallel Tafel lines indicating that the hydrogen evolution reaction is activation controlled and the addition of the studied inhibitors does not modify the mechanism of this process (The adsorbed molecules of studied inhibitors have no effect on the mechanism of either copper or copper alloy dissolution or hydrogen evolution reaction).

The value of corrosion potential (E_{corr}) is modified by the addition of compounds studied. The addition of compounds studied decreases the current densities in a large domain of anodic and cathodic potentials.

3.6. Scanning electron microscope:

Fig. 19 and Fig. 20 show the morphological images of copper and copper alloy after 1 hour in 3M HNO_3 solution free and with 1 % of EA, EDA or BDA. It is clear that the corrosion attack was more pronounced in absence of additives, while by the addition of different inhibitors the film formed on copper and copper alloy surfaces becomes more protective. The protective film is more pronounced for BDA › EDA › EA.

Table 1: The protection efficiency of different inhibitors in 3M HNO_3 solution calculated from equation 1, at 30°C

Inhibitors	**Protection efficiency**	
	Copper	**Coppr alloy**
Butandiamine	99.66	99.90
Ethylenediamine	99.19	99.27
Ethyl amine	87.18	88.10

Table 2: The calculated K and ΔG˚ of the adsorption reaction for copper and copper alloy in presence of different inhibitors in 3M HNO_3 solution, at 30°C

Inhibitors	Copper		Coppr alloy	
	K (L mol^{-1})	ΔG˚ (kJ mol^{-1})	K (L mol^{-1})	ΔG˚ (kJ mol^{-1})
Butandiamine	2750.00	-30.02	2000.00	-29.22
Ethylenediamine	1100.10	-27.71	1000.00	-27.47
Ethyl amine	613.40	-26.24	546.40	-25.95

Table 3: Thermodynamic parameters for the adsorption of different inhibitors on copper and copper alloy surfaces in 3M HNO_3 solution.

Inhibitors	Copper		Coppr alloy	
	ΔH_{ads}, KJ mol^{-1}	$-\Delta S_{ads}$, J $mol^{-1}K^{-1}$	ΔH_{ads}, KJ mol^{-1}	$-\Delta S_{ads}$, J $mol^{-1}K^{-1}$
Butandiamine	15.288	8.881	24.843	10.292
Ethylenediamine	11.466	8.051	19.110	8.47
Ethyl amine	9.555	7.138	15.288	7.47

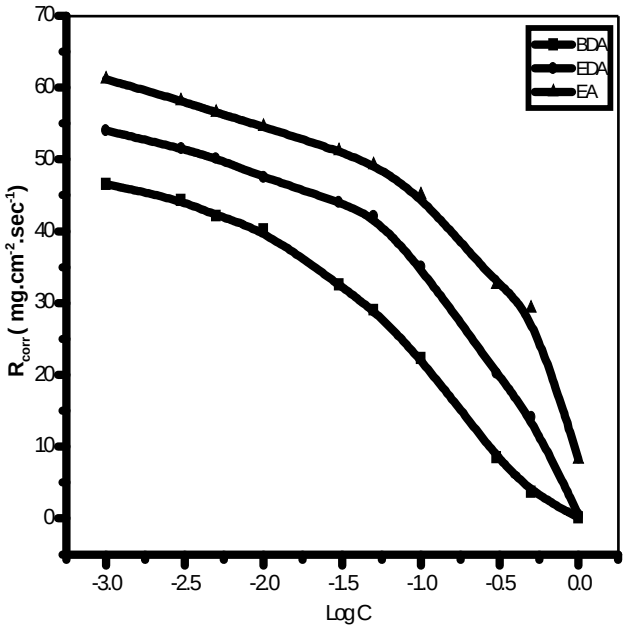

Fig.(1): The corrosion rate of copper in 3M HNO_3 solution in presence of different concentration of BDA,EDA and EA.

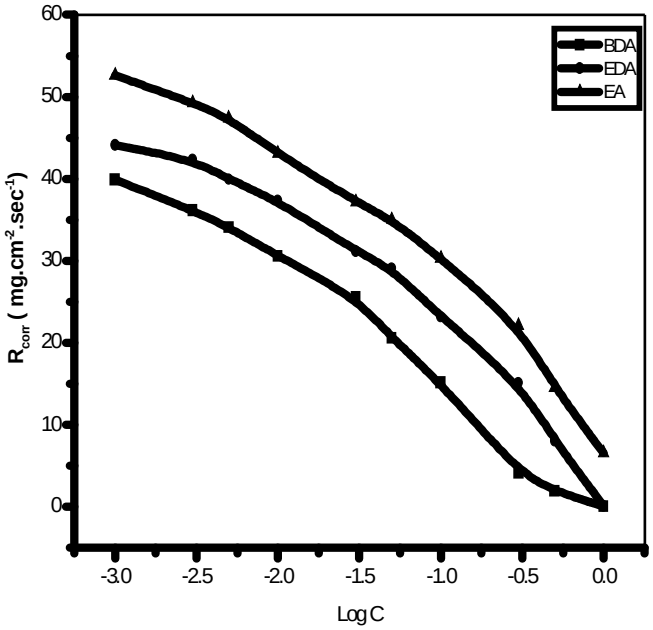

Fig.(2): The corrosion rate of copper alloy in 3M HNO_3 solution in presence of different concentration of BDA,EDA and EA.

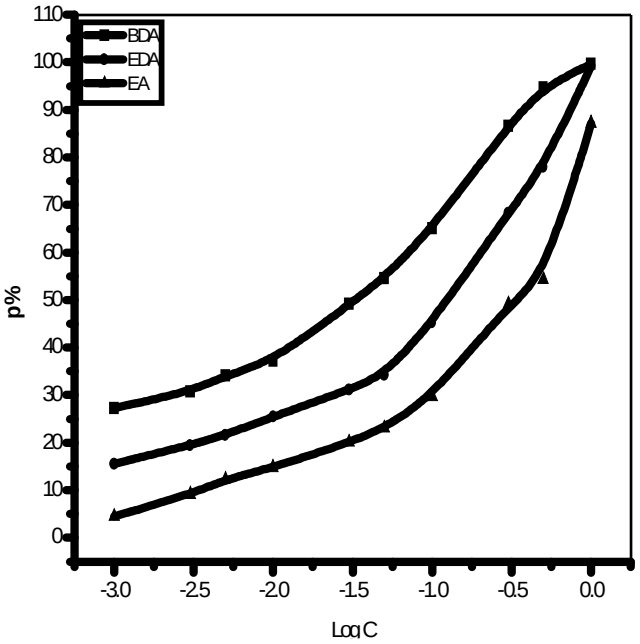

Fig.(3): The protection efficiency of copper in 3M HNO_3 solution in presence of different concentration of BDA,EDA and EA.

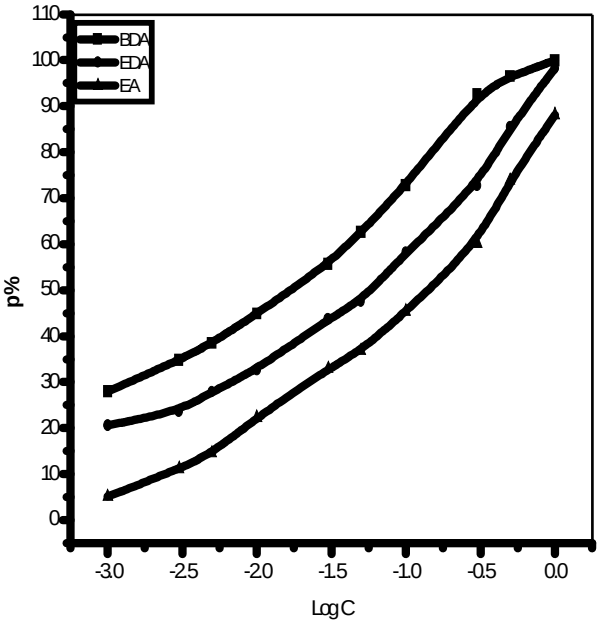

Fig.(4): The protection efficiency of copper alloy in 3M HNO_3 solution in presence of different concentration of BDA,EDA and EA.

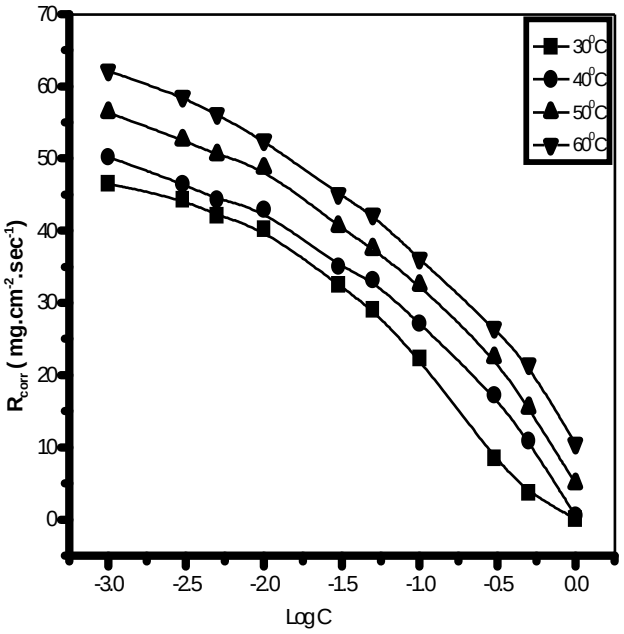

Fig.(5): The corrosion rate of copper in 3M HNO_3 solution in presence of different concentration of BDA, at different temperature

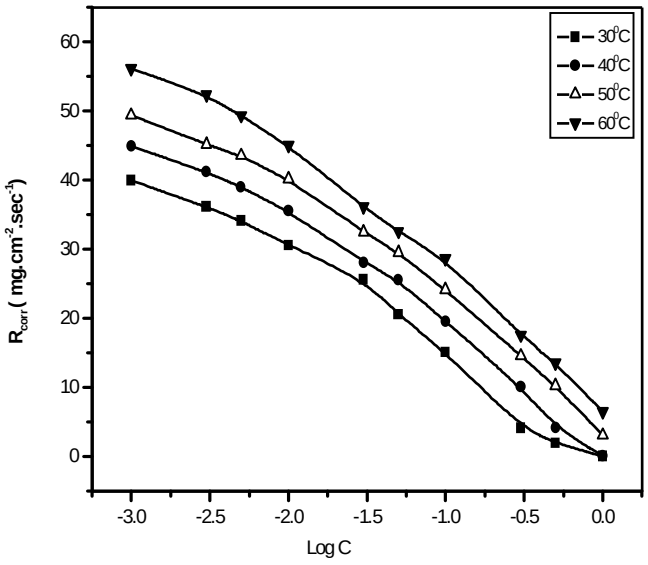

Fig.(6): The corrosion rate of copper alloy in 3M HNO_3 solution in presence of different concentration of BDA at different temperature.

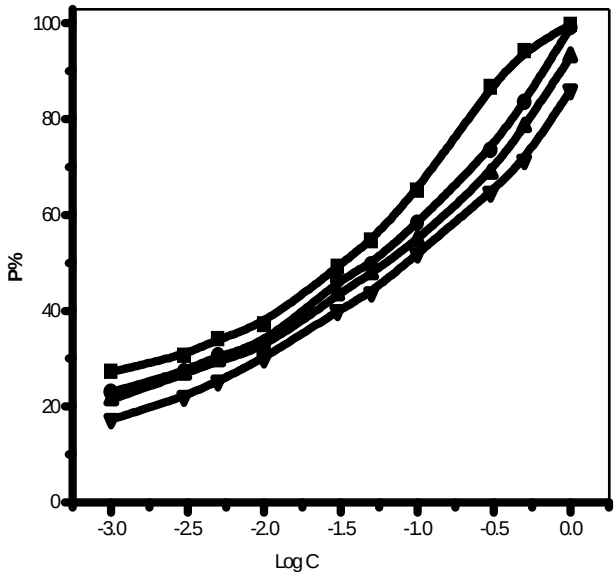

Fig.(7): The protection efficiency of copper in 3M HNO_3 solution in presence of different concentration of BDA at different temperature.

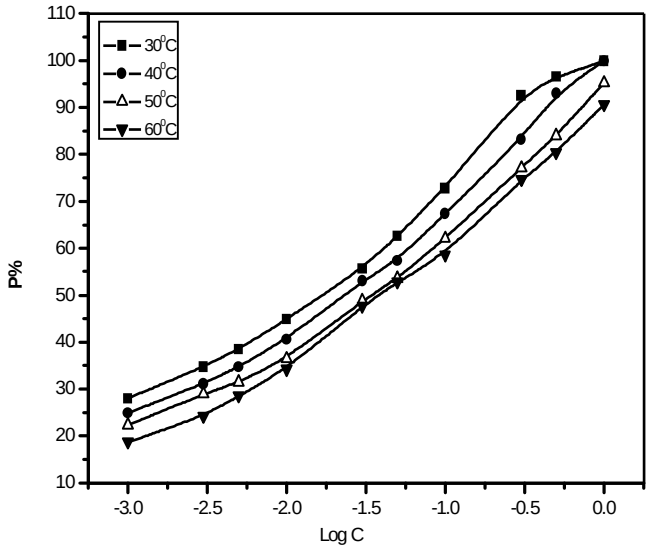

Fig.(8): The protection efficiency of copper alloy in 3M HNO_3 solution in presence of different concentration of BDA at different temperature.

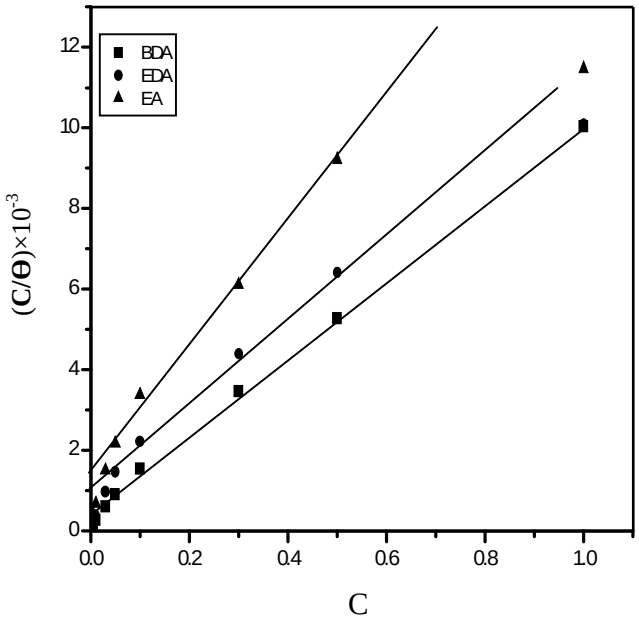

Fig. (9):Langmiur isotherm adsorption of BDA,EDA and EA on the surface of copper in 3M HNO_3 at 30°C.

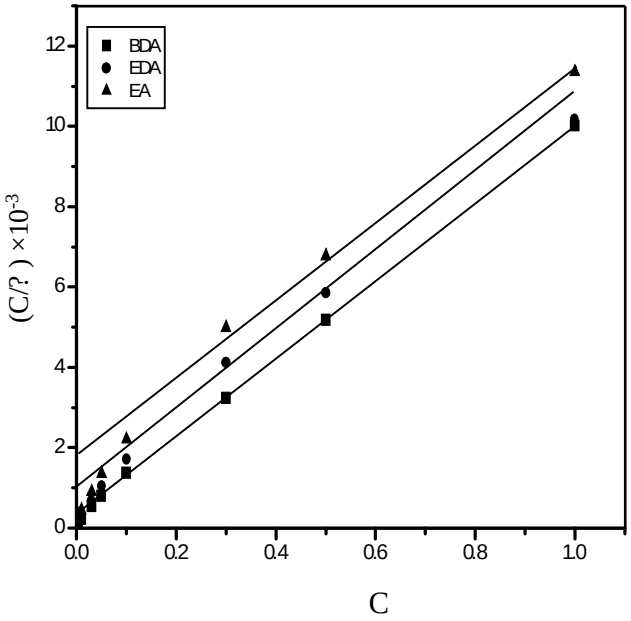

Fig.(10): Langmiur isotherm adsorption of BDA,EDA and EA on the surface of copper alloy in 3M HNO_3 at 30°C.

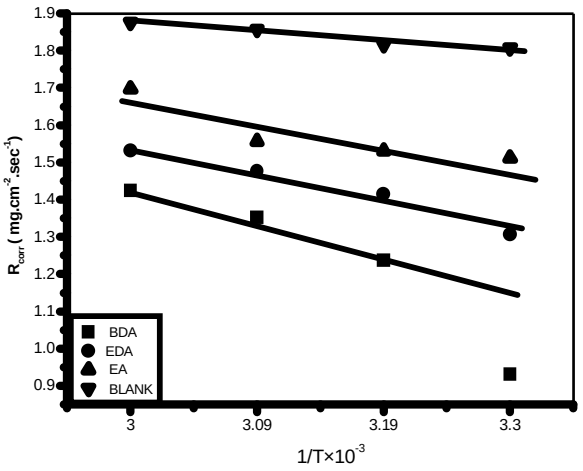

Fig.(11):Arrhenuis plot of copper in 3M HNO_3 with and without BDA,EDA and EA.

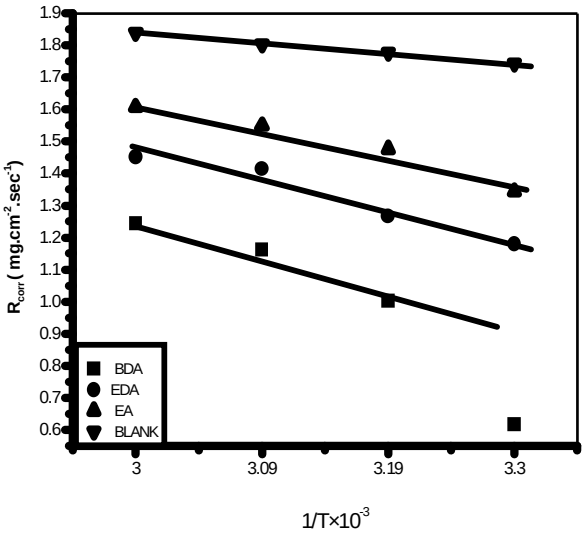

Fig.(12): Arrhenuis plot of copper alloy in 3M HNO_3 with and without BDA,EDA and EA.

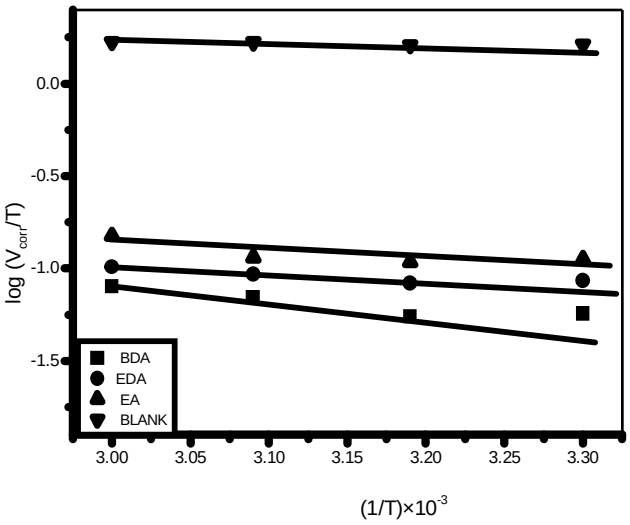

Fig.(13): log(V_{corr}/T) vs. (1/T)of copper in 3M HNO_3 in presence and absence of BDA, EDA and EA.

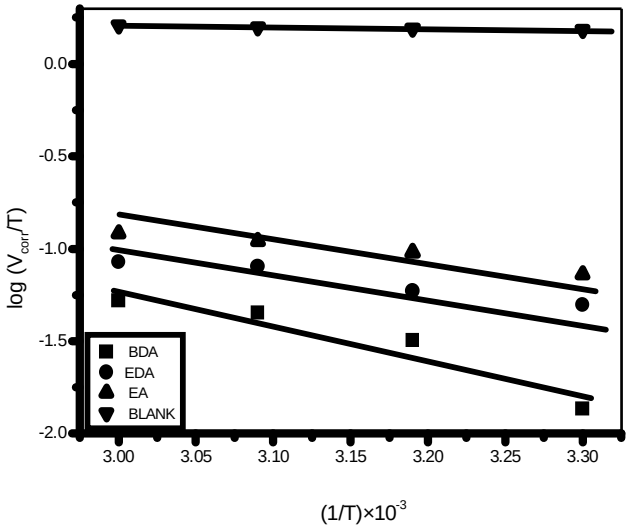

Fig.(14): log(V_{corr}/T) vs.(1/T)of copper alloy in 3M HNO_3 in presence and absence of BDA, EDA and EA.

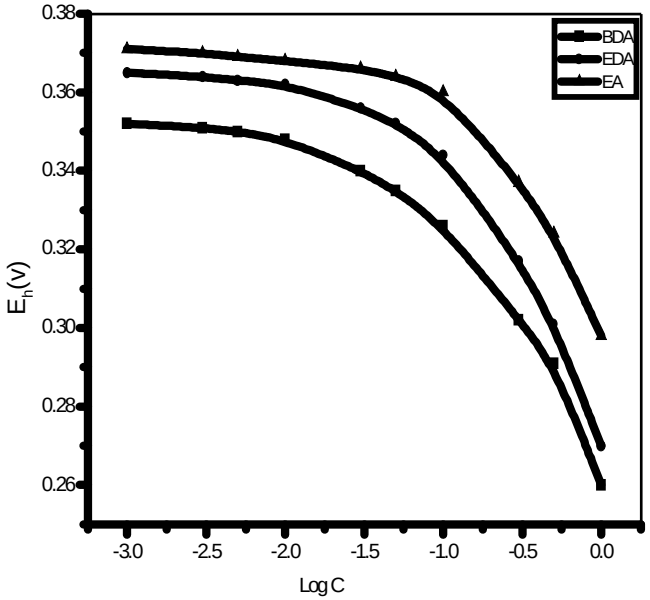

Fig.(15):the steady state potential of copper E_h vs. the logarithm of different concentrations of tested inhibitor in 3M HNO_3.

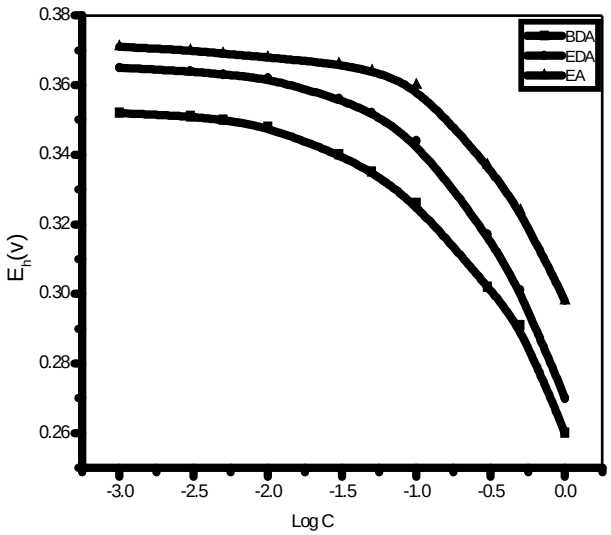

Fig.(16): the steady state potential of copper alloy (E_h) vs. the logarithm of different concentrations of tested inhibitor in 3M HNO_3.

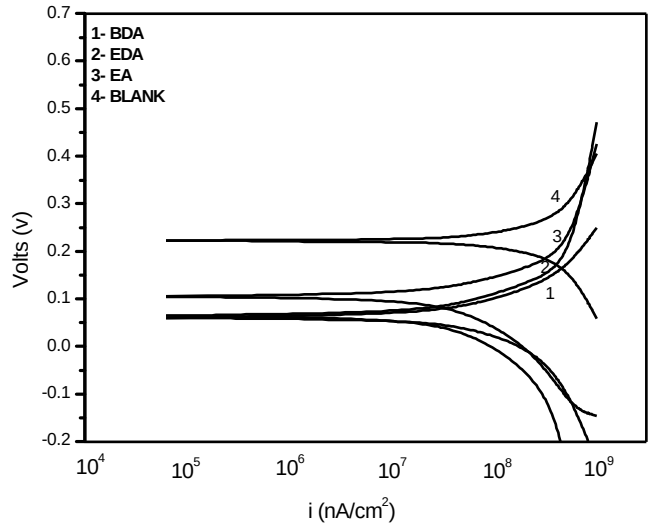

Fig.(17): Potentiodynamic polarization curves of copper in 3M HNO_3 in absence and presences of the tested inhibitors.

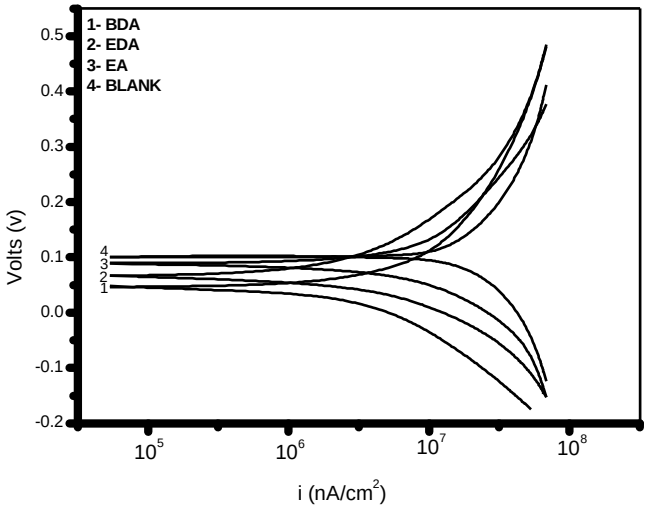

Fig.(18): Potentiodynamic polarization curves of copper alloy in 3M HNO_3 in absence and presences of the tested inhibitors.

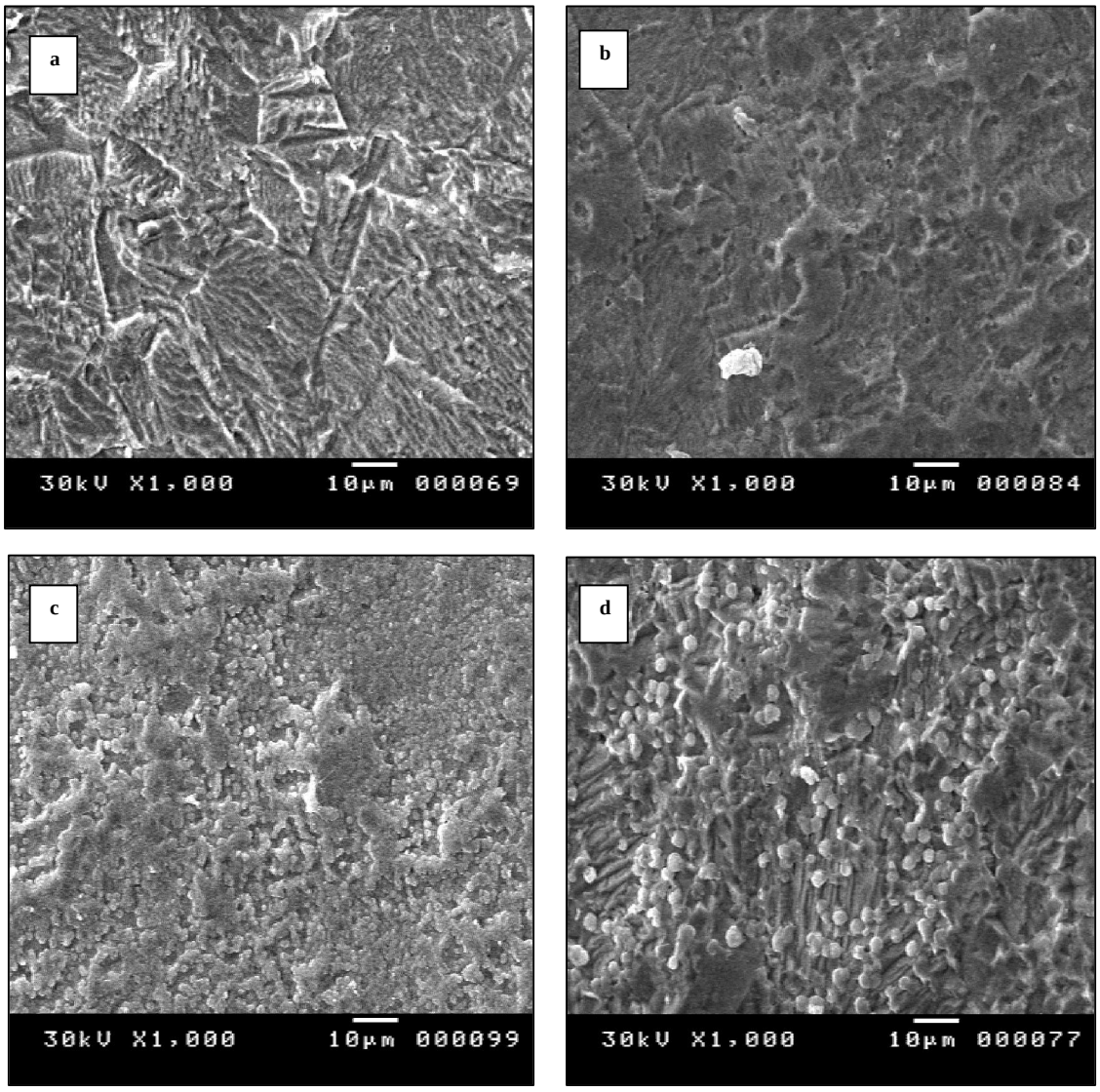

Fig.(19): Scanning electron microscope of copper in free acid (a) and in presence of BDA (b), EDA (c) and EA(d).

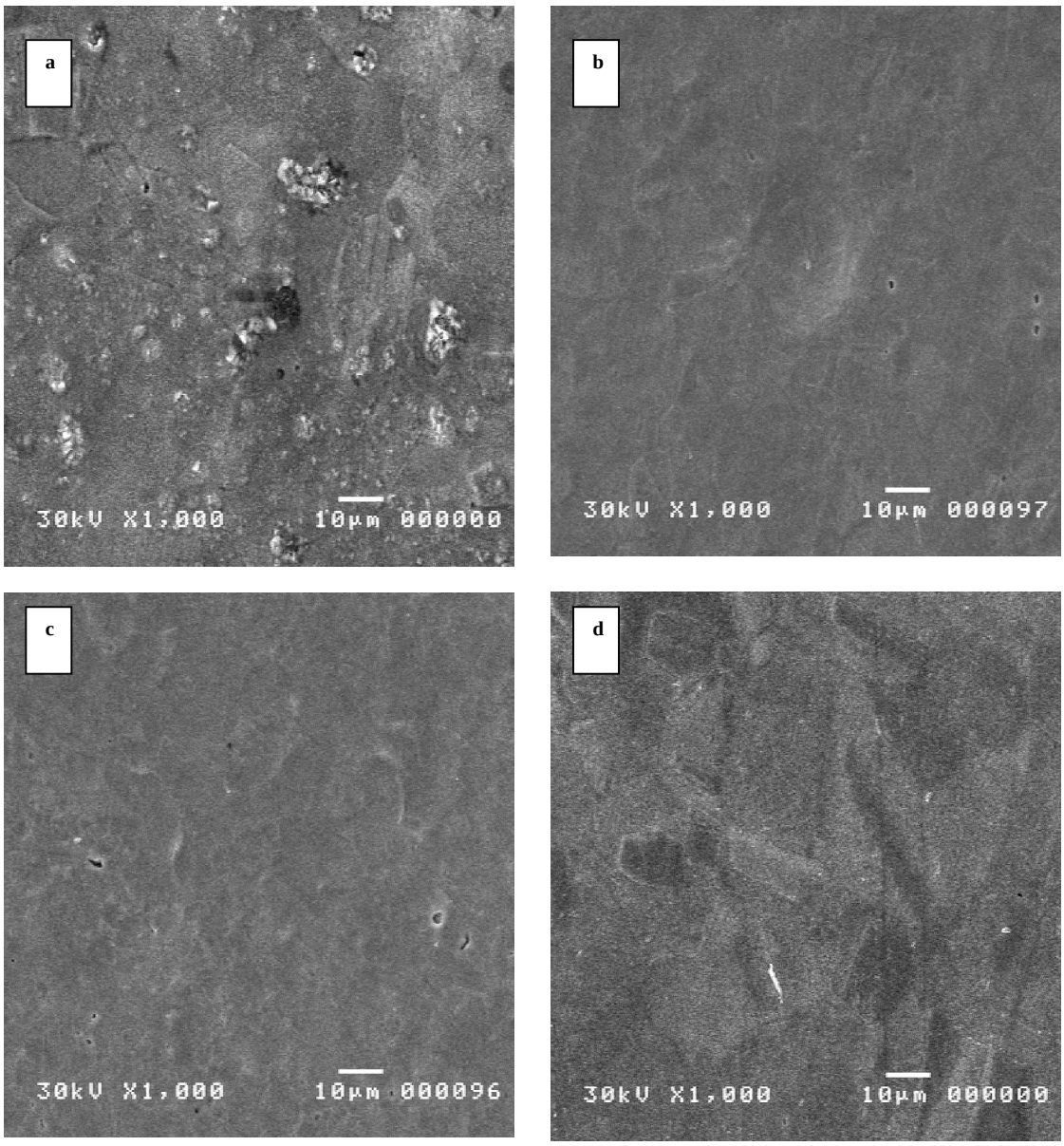

Fig.(20): Scanning electron microscope of copper alloy in free acid (a) and in presence of BDA (b), EDA (c) and EA(d).

4. Conclusion

The corrosion rate of copper is greater than copper alloy in the corrosive medium with or without the addition of the inhibitor.

The addition of BDA, EDA or EA has strong inhibiting effect for copper and copper alloy dissolution in 3M nitric acid solution, and the protection efficiency follows the order BDA › EDA › EA.

The protection efficiency of BDA and EDA are very similar, at higher inhibitor concentration, 99.6% and 99.07% respectively.

The adsorption of BDA, EDA and EA on copper and copper alloy followed a physical adsorption.

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Variation of tree species in response to biotic stress in a fresh water swamp of Doon valley

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Abstract: The present communications deals with the changes in tree structure at Golatappar 7b block range in DehraDun Forest division, Uttarakhand India in response to biotic pressure.Golatappar Fresh water swamp, lies between 30^0 4' 30'' to 30^0 5' 15'' North latitude and 78^0 12' 00'' to 78^0 12' 25'' East longitude. In present the Golatappar Fresh water facing a lot of biotic pressure the swamp is encroached by the near by villagers to full fill their daily requirements viz food, fodder, fuel, timber etc. Lopping of trees for fodder, felling of trees for timber and fuel is the main cause of the extinction of important tree species of swampy area. In this paper we try to assess the variation of tree species in Ridge, slope and Swamp proper of Golatappar fresh water swamp. The ridge and slope of Gola tappar fresh water swamp is dominated by the tree species with maximum IVI (110.07), meanwhile the Swamp proper is dominated by tree species *Diospyros peregrine* and *Trewia nudiflora* with highest IVI value (56.91). A comparison of the tree species of Swamp proper were made by the (Somdeva & Srivastava, 1978). From this study a major decline of important value index was recorded in the dominant species of Gola tappar fresh water swamp. The biotic interference has vanished a number of plant species representatives of swampy area. [Journal of American Science 2010;6(8):499-502]. (ISSN: 1545-1003).

Key words: Golatappar; Biotic stress; Doon valley; Swamp

1. Introduction

Swamps are the marshy area where water oozes out in perennial streams at constant level throughout the year. These are transitional ecosystems located at the interface of terrestrial and aquatic ecosystems. Their position makes them recipients of susceptible to losses to downstream. Swamp plays a vital role in landscaping with a wide variety of flora and fauna.

Doon valley has many fresh water swamps due to its unique topography. There was a time when low lying areas of the valley were having a chain of swamps but human interference are started in the name of "malarious climate" (King, 1871) still persists. The trees were cut at that time and the opening created, which result in the extinction the most of the swamps. Most of the swamps have been cleared off their original forests, but a few still remain with virgin vegetation. Golatappar is one of such fresh water swamp forest of Doon valley, where the present study was carried out.

Gola tappar fresh water swamp is the source of the fuel, food, fodder medicine etc and have been exploited by the nearby villagers to full fill their daily requirements. Due to this unscientific exploitation of the swamp by the local people, the swamp is degrading, depleting and reducing day by day. The vegetation structure of tree species of the swamp is also changing as consequences of these disturbances. In present paper we have attempted to assess the Variation in tree species in response to biotic stress of Golatappar fresh water swamp and compare it with the work of Som deva and Srivastava (1978).Gola tappar swamp had been regularly studied by various workers Srivastava et al 2000, Chauhan 2001,& Kandwal et al 2007).

2. Material and Methods

The present study has been carried out in Golatappar (7b block of Barkot range) near village Khiri in DehraDun forest division, Uttarakhand, India. It is a typical, tropical valley fresh water swamp. It lies at an altitude of $30^0$6' North latitude and $78^0$19' East longitudes at an altitude of 370m and is located 35 km. east of DehraDun. It occupies an area of approximately 2 km^2. The Tawa river flows along its southern limit which is one of the tributaries of Song-Suswa-Jakhan complex that fans out here in innumerable streams over a wide area before it meet the river Ganga at Satyanarayan. The swampy zone lies in a depression and is surrounded on all sides except the south by a clay bed of immense thickness overlying the great mass of the gravel deposit. Physiographically the surrounding area is so constructed that all the water brought down by Himalayan Rivers that they received from the annual rainfall, infiltrates deep in the stable strata and reappears in the form of innumerable springs at various depression, sites of Doon valley.

The study area was surveyed frequently surveyed to assess the tree diversity. Three community were made viz Ridge, Slope and Swamp

proper for study. At each site the presence of all tree species were recorded by quadrate method. The quadrates measuring $1\times1m^2$ were laid periodically in case of swamp proper and once $10\times10m^2$ in case of swamp forest (ridge and slope). The quadrates were laid randomly. The data obtained for frequency, density, abundance and basal area were calculated to determine Importance Value Indices (IVI) of each species during the study period. The method employed was followed as given by Misra (1968). The diversity index (H') was computed by using Shannon-Wiener information index (Shannon & Wiener 1963). The concentration of dominance (CD) was computed by Simpson's index (Simpson 1949). Similarity index (Community coefficient) among different sites was calculated as per Jacard (1912). The variation of tree species was studied by compare the present data with Somdeva and Srivastava (1978).

3. RESULTS

The Important Value Index (IVI) of Tree species of Ridge, slope, and Swamp proper of GolaTappar Fresh water swamp revealed that *Shorea robusta* was the dominant tree species of ridge and slope (Table 1). Maximum IVI value (110.07) was recorded for ridge and (79.68) for slope. The swamp proper is dominated by the tree species *Diospyros peregrine* and *Trewia nudiflora* with highest IVI value (56.91).

Table 1: Important value Index of the tree species of GolaTappar Fresh Water Swamp forest

Tree species	Ridge	Slope	Swamp Proper
Diospyros peregrine (Gtn.) Gurke	22.65	55.80	56.91
Trewia nudiflora Linn.	12.84	-	56.91
Pterospermum acerifolium Willd.	22.65	31.92	25.92
Bischofia javanica Bl.	9.45	23.70	29.20
Syzygium cumini (Linn.) Merr.	12.84	6.07	8.04
Shorea robusta Gaertn.	110.07	79.68	53.63
Phoebe lanceolata Nees.	4.73	11.55	-
Mallotus philippinensis Muell.-Arg.	16.23	20.37	14.06
Toona ciliataRoem.	13.20	6.07	-
Xylosma longifolia Clos.	6.42	-	8.04
Alstonia scholaris	9.81	6.07	8.04

Table (2) reveals the variation in important value index (IVI) of Tree species of swamp proper in last three decades. A major decline have been recorded in the Important Value Index of tree species in last three decades. During the study it reveals that the Important value index of the dominant tree of swamp proper showed decline 76.88to 56.91in case of tree species *Diospyros peregrine*, 36.64 to 25.92 in case of *Pterospermum acerifolium*, and 32.02 to 29.20 in case of *Bischofia javanica*.

Table 2 variation in importance values of tree species in last three decade

TREE SPECIES	1978	2006
Diospyros peregrine (Gtn.) Gurke	76.88	56.91
Trewia nudiflora Linn.	51.05	56.91
Pterospermum acerifolium Willd.	36.64	25.92
Bischofia javanica Bl.	32.02	29.20
Carallia brachiata (Lour) Merr.	17.87	-
Syzygium cumini (Linn.) Merr.	13.94	8.04
Shorea robusta Gaertn.	13.73	53.63
Phoebe lanceolata Nees.	10.39	-
Glochidion assamicum Hook.f.	9.40	-
Acronychia pedunculata (Linn.) Miq.	7.81	-
Persea gamblei(King ex Hook.f.) Kosterm.	6.45	-
Ficus lucescens Bl.	6.01	-
Ficus racemosa L.	5.93	-
Mallotus philippinensis Muell.-Arg.	5.05	14.06
Toona ciliata Roem.	3.36	-
Xylosma longifolia Clos.	3.29	8.04
Mangifera indica Linn.	2.75	-
Ficus oligodon Miq.	2.14	-
Anthocephalus indicus A.Rich	1.98	-
Alstonia scholaris	1.88	8.04
Schleichera oleosa (Lour.) Oken.	1.77	-
Cyclostemon assamicus Hook.f.	1.36	-

Various indices for measuring diversity and evenness of the selected community of the tree species of study area are given in (Table 3). A maximum number of tree species (11) were recorded in ridge meanwhile (9) species of trees were recorded in slope and swamp proper. The values Species diversity (H') ranged between (1.83-1.94). Maximum species diversity (1.94) recorded for the tree species of Swamp proper and minimum species diversity (1.83) recorded for the tree species of Slope. The value of evenness of the tree species of selected community ranged between (0.78-0.88). Minimum value of evenness (0.78) recorded for ridge and maximum value of evenness (0.88) was recorded for Swamp Proper.

Table 3 Species richness, Concentration of dominance, Diversity and Evenness of tree species of Gola Tappar Fresh water Swamp.

Diversity indices	Ridge	Slope	Swamp proper
Richness	11	9	9
H′	1.88	1.83	1.94
cd	0.24	0.19	0.16
J	0.78	0.83	0.88

The concentration of dominance (Cd) of all the communities of study area are ranged between (0.16) swamp proper and (0.24) ridge. The values of Cd shows that Ridge was dominated by higher number of tree species (lower Cd). Gosselink and Turner (1978) & Chaneton and Facelli (1991) found that as the flooding increases, the dominance of a very few species increased.

4. Discussion

Doon valley with its unique topographical set up has a large number of swampy areas. Most of them have been cleared off their original forests, but a few still remain with their characteristics vegetation. Golatappar is one of such swamp. Champion and Seth (1968) cited this as an example of the tropical valley fresh water swamp. Bischofia javanica, Trewia nudiflora, *Ficus racemosa* are the characteristic tree species of swamp proper. These trees have peculiar surface roots and are suited to colonise low-islands, consolidated by successional seral community of herbaceous and bush fern swamp stages. During the present study it was found that swamp proper is dominated by *Diospyros peregrine* and *Trewia nudiflora* with maximum IVI value (56.91) this value is more less the values of Somedeva and Srivastava (1978). A major variation have been recorded in the dominant trees species of swamp proper. This is resulted due to lot of biotic pressure in swamp proper by their local people. The Golatappar swamp is being converted into agricultural fields and other land use systems whereas the water of the streams is diverted to a watershed from where a network irrigation canals has been drawn which irrigates these agricultural fields. Unscientifically loping of medicinal plants, loping of fodder trees and exploitation of trees for timber is another reason for the extinction of tree species. All these consequences are affecting the vegetation structure of the swamp. From the above study it was found that the tree species of the swamp are facing a lot of biotic pressure and loss their status in swampy area. This is a preliminary study and not sufficient to give the real picture of the swamp. The study is suggested that there is need of detailed study. Only then we can ensure the proper management and conservation of this fragile ecosystem.

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Prophylatic Potential of Lemon Grass and Neem as Antimalarial Agents

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Abstract: Prophylactic activities of methanol, ethanol and aqueous extracts of neem and lemon grass against plasmodium development in mice were investigated. Various extracts of the plants were prepared with soxhlet apparatus. Growth and reproduction of malarial parasite in the treated animals was delayed 3 days after treatment. The mean % parasitaemia obtained in mice administered with methanolic, ethanolic and aqueous extracts of lemon grasss were 43.01%, 50.21% and 48.08% while those treated with methanol, ethanol and aqueous extracts of neem displayed 59.54%, 61.50% and 13.4% respectively indicating the anti – plasmodial activity of both plants. . It is therefore, concluded that the activities of these plants depend neither on weight of the mice nor dosage but on the solvent used. The parasitaemia development in the group treated with standard drug (Malariech) was significantly minimal having 2.47% and 88.23% % parasitamia and average % suppression recorded. Aqueous Neem extract exhibited highest suppressive effect 76.21% followed by Lemon grass in respect of the methanolic(43.67%) and aqueous(38.07%) extracts as compared with methanolic(25.47%) and ethanolic(23.32%) extracts of Neem.. The suppressive value of aqueous neem extract 76.21%, was considered significant and could serve as sufficient replacement for conventional antimalarial drugs that easily loose their potency with the impending development of resistance. [Journal of American Science 2010;6(8):503-507]. (ISSN: 1545-1003).

Keywords: Lemon grass, Malaria, Neem, *Plasmodium*, Prophylactic.

1. Introduction

Before the advent of orthodox medicine in the treatment of ailments which include malaria, the traditional African society had devised various means of combating such ailments. One of the major ailments that is of concern in the world today is malaria. Malaria is the single most important cause of ill health, death and poverty in the Sub-Saharan Africa. About 200 to 300 million new cases of malaria occur worldwide each year, and about one to three million deaths occur of which 2 / 5 occur in Africa (WHO, 1999). The disease is believed to be a major obstruction to social and economic development in Africa, causing enormous misery and suffering through the pain of fevers and the anguish of bereavement. Ninety percent of the deaths caused by malaria occur in children aged less than five year old (WHO, 2004)

However, the problems militating against the effective management of malaria have been enumerated. The most important problem is that *Plasmodial* parasites are resistant to most widely available, affordable and safest first line treatments such as Chloroquine and Fansidar(Kisame,2005; Sendegive et al 2005) or secondly , the overall control of mosquitoes which transmits malaria is made difficult by their resistance to a wide range of insecticides. The third which is a new and rapid developing problem is the wide production of fake antimalarial drugs. In Southeast Asia 32% and 53% of artesunate blister packs sampled contained no active ingredients (Newton et al, 2006). Lastly, most countries in Africa lack the necessary infrastructure and resources to manage and control malaria (WHO, 2004).

Most times, accessibility and the affordability to health facilities and drugs are not within the reach of most people as a result of the location of the health centers and cost of antimalarial drugs. This could be seen as a factor to high death rates recorded in malarial cases. As a viable remedy, there is a continuous search for alternative low cost drugs and herbal preparations to replace those that lost their activities to drug resistance. A number of traditional herbs have been tested and used in the prevention and also treatment of malaria including *Artemisia annua* (Akininyi *et a*l, 1986), old leaves of *Carica papaya*, roots and leaves of *Guinensis*, unripe fruit of *Capsicum frutescence* and *Azadirachta indica* popularly called Dangoyaro in Nigeria. Recent studies indicate that lemon grass can be successfully used to treat drug resistant malarial and typhoid fever (Madu, 2007). It has been discovered that several drugs most of which are used for the treatment of

malaria can be taken for prevention. This study is aimed at determining the prophylactic effect of neem and lemon grass in malarial cases in order to ascertain the extent of the effectiveness of these plants as antimalarial agents and to verify the activities of the extracts from different solvent against malarial *Plasmodium.*

2. Materials and Methods

(a).Preparation of plant materials/ extractions and drug suspension

The leaves of *Cymbopogon citratus*(lemon grass) and *Azadirachta indica*(neem)were collected from and around Army barracks, Bori Camp Port Harcourt and Federal University of Technology, Owerri(FUTO). They were then taken to the laboratory of the Department of Pharmacology, College of Health Sciences, University of Lagos. The leaves were sorted to remove insects, variegated leaves and debris. They were then rinsed in water to remove dust particles after which they were allowed to air dry, with further drying in a Gallenkam oven at 40^0C for 6 hours, the leaves were then powdered using a blender for 15 minutes.

Methanolic, ethanolic and aqueous extraction of the lemon grass and Neem leaves were carried out by using soxhlet apparatus. Six extracts were obtained which were then heated to dryness using hot water bath. The concentrated extracts were scraped out with spatula. 500mg of each of the concentrates was weighed, then dissolved in 10ml of water and poured into sample bottles. Also, six tablets of malariech were dissolved in 10% sugar solution according to Isah *et al*, (2003). Both preparations were stored at 4^0C in the refrigerator in the laboratory till use.

(b). Sample Animal and weight determination

Swiss albino mice used in this research were collected from the Department of Pharmacology College of Health Sciences, University of Lagos and then transferred to the laboratory in the Department of Parasitology, Nigeria Institute of Medical Research (NIMR) Yaba, Lagos. A total of 37 mice were used, 22 female and 15 male mice weighing between 11g and 28g were fed on standard diets manufactured by Pfizer Livestock Feeds LTD, Nigeria. The same environmental conditions of 12 hours light and 12 hours darkness, normal room temperature of 27^0C were maintained for all the animals throughout the period of investigation. They were separated into 8 groups according to their weights as follows: 4, 6, 6, 6, 5, 5, 3, and 3 in each 3.5cm x 6.5cm cage.

(c). Malaria Parasite

Plasmodium berghei obtained from the Department of Parasitology. Nigeria Institute of Medical Research (NIMR), Yaba, Lagos was used.

(d). Standard Drug - Malariech

This drug was obtained from Maryland Pharmacy, Surelere, Lagos Nigeria

(e). Dosage determination

The dosage of each extract was determined according to Marcia *et al* (1992)

(f). Administration of Extracts

The drugs were administered to all the mice except those in group 8 by forceful feeding using an oral canular for 7 consecutive days.

Group 1 received methanolic extract of lemon grass
Group2 received ethanolic extact of lemon grass
Group3 received aqueous extractof lemon grass
Group4 received methanolic extract of neem
Group 5 received ethanolic extract of neem
Group 6 received aqueous extracts of neem
Group 7 received a standard antimalarial drug – Malariech
Group 8 received no drugs and served as control group.

All the animals were left for eight days after treatment, and then infected with *P. berghei* and left for three days after which thick blood smear was prepared from their tail veins.

(g). Inoculation of the mice

Each of the Swiss albino mice was intraperitonally administered with a standard inoculum of *P berghei*. Stored parasitized blood in liquid Nitrogen was allowed to thaw. The content was injected into 3 donor mice and left for five days so as to allow for the development of parasitaemia. The parasitized blood was collected from each donor mouse and diluted appropriately with Phosphate buffer saline to make 1×10^7 parasitized red blood cells, as described by Isah *et al* (2003). 0.2ml of the diluted inoculum was then injected into each of the sample mouse after which they were left for 3 days. The aim was to achieve a high level of parasitaemia. However, thick blood smear were prepared from their tail veins and viewed under the × 40 microscope after five days. The parasitaemia count was carried out by the use of a tally counter and the average percentage parasitaemia calculated using the approach of Obih and Makinde (1985) as thus:

Average % Parasitaemia = No of parasitaemia / No of WBC × 100.

Average % Suppression =
Av. % Parasitaemia in control − Av. % Parasitaemia in treated /Av. % Parasitaemia in Control × 100

3. Result

Parasite development was observed only in group 8 that received no drugs, with the average mean parasitaemia of 75.09% which increased to 90.94% 6 days after (Table 1&5). Since there was no development of parasitaemia in group 1- 7 after 3 day's infection, blood samples were then collected from each mouse, and smear made to check for parasite development in day 6. The mean % parasitaemia obtained in Gp1- 3 mice administered with methanolic, ethanolic and aqueous extracts of lemon grasss were 43.01%, 50.21% and 48.08% while in Gp 4 - 6, 59.54%, 61.50% and 13.4% for the methanol, ethanol and aqueous extracts of neem respectively(Table 2&3).The parasitaemia development in group 7 treated with standard drug(Malariech) was signicantly minimal with 2.47s and 88.23% as% mean parasitaemia average suppression recorded Aqueous Neem extract exhibited the highest suppressive effect 76.21% followed by Lemon grass in respect of the methanolic(43.67%) and aqueous(38.07%) extracts as compared with methanolic(25.47%) and ethanolic(23.32%) extracts of Neem(Table 6). The growth and reproduction of malarial parasite was evidently delayed after 72 hours for groups 1-7 which received treatment indicating the prophylactic activities from the extracts of the both plants

Table 1: Paristaemia count in group 8 mice 3 days after infection

Sample	Parasite	WBC	Av. % Parasitaemia
1	161	201	80.09
2	139	203	68.67
3	153	200	76.50

Table 2: Parasitaemia count in groups 1, 2 and 3 mice that were administered with methanolic, ethanolic and aqueous extracts of lemon grass after 6 days of infection

Since there was no development of parasitaemia in other groups after three days of passaging, blood samples were collected from each mouse and smear were made on the sixth day to check for parasite development.

solvent	Sample No	Average weight (g)	Average dose/ day	Parasite count	WBC	Average % Parasitaemia
Methanol / GP 1	1	11	0.3	91	203	44.8
	2	11	0.3	82	201	40.7
	3	11	0.3	77	200	38.5
	4	11	0.3	98	204	48.0
Ethanol / Gp2	1	15	0.4	102	200	51.0
	2	15	0.4	99	201	49.4
	3	15	0.4	107	201	53.0
	4	15	0.4	100	202	49.5
	5	15	0.4	97	200	48.5
	6	15	0.4	102	205	49.8
Aqueous / Gp3	1	15	0.4	98	200	49.0
	2	15	0.4	100	200	50.0
	3	15	0.4	99	201	49.3
	4	15	0.4	97	200	46.1
	5	15	0.4	94	202	46.3
	6	15	0.4	98	205	47.8

Table 3: Parasitaemia count in group 4 – 6 mice administered with methanolic, ethanolic and aqueous extracts of Neem after 6 days of Infection

Solvent	Sample No	Average weight(g)	Average dose/ day	Parasite count	WBC	Average% Parasitaemia
Methanol / GP 4	1	20	0.53	127	205	61.8
	2	20	0.53	115	202	56.1
	3	20	0.53	120	202	60.0
	4	20	0.53	122	203	60.0
	5	20	0.53	125	207	59.8
Ethanol / Gp5	1	20	0.53	150	208	72.1
	2	20	0.53	131	203	63.9
	3	20	0.53	123	207	59.4
	4	20	0.53	147	201	43.1
	5	20	0.53	139	202	68.1
Aqueous / Gp6	1	24	0.64	20	202	9.0
	2	25	0.64	25	201	12.4
	3	24	0.64	41	205	20.0
	4	24	0.64	30	203	14.8
	5	24	0.64	22	203	10.8

Table 4: Parasitaemia count in group 7 mice treated with standard drug (Malariech) after 6 days

Sample No	Average weight	Dose/day	Parasites	WBC	Average % parasitaemia
1	28	0.74	--	201	
2	28	0.74	15	202	7.4
3	28	0.74	—	200	

Table 5: Parastaemia count in group 8 in the Control group after 6 days of infection

Sample No	Average weight	Parasites	WBC	Average % parasitaemia
1	28	191	205	93.17
2	28	179	201	89.05
3	28	183	202	90.59

Table 6: Average % Suppression observed at 8 days after infection

Group	Average Suppression
1	43.67
2	35.74
3	38.07
4	25.47
5	23.32
6	76.21
7	88.23
8	—

4. Discussion

The extracts of the two plants, *Cympobogo citratus* and *Azadirachta indica* showed a high activity similar to results of Iwalewa et al (1992). From the results, the growth and reproduction of malarial parasite in the treated animals was delayed after 3 days. It showed that the pretreatment of mice with the extracts interrupted the establishment of parasitaemia against the control.

There were observed variations in the degree of prophylactic activities of the extracts with regards to the solvent used. Aqueous extract of Neem exhibited highest prophylactic effects (13.40%) on the development of malarial parasites followed by the methanolic and aqueous extracts of lemon grass that showed 43.20% and 48.09% prophylactic effect on mice 6 days after pretreatment. On the other hand, methanol extracted more active ingredients than ethanol; this may be due to the higher volatility of methanol than ethanol. According to Dutta (1993), different solvents dissolve different active compounds from the same plants. The aqueous extracts of neem had the greatest suppression / reduced parasitaemia value of 76.21% while methanolic extracts of neem had the least suppressive effect (23.32%) indicating that water extract of neem the most effective (Obaseki and Jegede, 1986). Although previous studies have shown that water extract of neem are less active than leaf extract obtained by water/ acetone combination (Udeinya, 1993). This result also corroborates Devi *et al* (2001) who reported that the aqueous extract of leaves of *Azadirachta indica* showed significant activity at 125 – 500mg/kg against *P. berghei.* . It is concluded that the activities of these plants depend neither on weight nor dosage but on the solvent used.

The average % parasitaemia by group 7 that received the standard drug (Malareich) was 2.47%, showing little parasite development. Also the average suppressive effect of neem from all the groups that were administered with neem was 47.10% while that of lemon grass extract was 44.81%, making the standard drug the most suppressive drug at a percentage of 96.71%. Although, the orthodox antimalarial drugs prove most effective but with regards to numerous problems associated with management of malaria such as the development of resistance to most drugs as well as substandard antimalarial drugs, coupled with fact that these drugs are neither affordable nor available to the reach of common man, use of these plant extracts should encouraged. Again, with 76.21% as the suppressive value of aqueous neem extract, definitely means that this plant could serve as sufficient replacement for conventional antimalarial drugs that may loose their potentials with the impending development of resistance. Obih and Makinde(1985) showed neem extracts to be effective even against chloroquine resistant strains sof the malarial parasite.

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Evaluation Of Yield And Yield Attributes Of Some Cowpea (*Vigna Unguiculata (L) Walp)* Varieties In Northern Guinea Savanna

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Abstract: Five cowpea varieties were evaluated in 2009 to determine their grain yield attributes. The experiment was conducted at the Research and Teaching Farm of Department of Crop Science, Adamawa State University Mubi laid in randomized complete block design in three replicates. Data were collected on plant height, number of leaves per plant, number of branches per plant, number of days to flowering, pod filling period, days to physiological maturity, pods per plant, pod length, number of seeds per pods, number of seeds per plant, 1000 seed weight and yield per hectare. Data collected were subjected to analysis of variance. Most of the yield and yield determining attributes recorded significant (P = 0.05) difference due to treatment effects. These varieties flowered between 38.02 days to 50.12 days after planting and the maturity period did not exceed 71 days. Average grain yield ranged between 14,000.3kg/ha to 20, 000.20kg/ha. V_2 (Brown Kananado) recorded the highest yield of 20,000.20kg/ha with V_5 (Ife - Brown) giving the least yield of 14,000.30kg/ha. The need to develop varieties with different attributes and resistance to major biotic and abiotic constraints to suite the needs of different agro – ecological zones can not be over emphasized. This is because varietal requirements in terms of plant type, seed type, maturity, yield for cowpea varies from one agro – ecological region to another. [Journal of American Science 2010; 6(8):508-511]. (ISSN: 1545-1003).

Key Words: Cowpea, Genotypes, Optimum, Evaluation, Yield Components

1. Introduction

Cowpea (*Vigna unguiculata* (L) Walp) is a nutritious legume crop that is of considerable importance in Nigeria and other Sub sahelian countries. Cowpea constitutes a significant proportion of the total dietary protein and energy intake of Nigerians (Davio *et al*; 1976 and Ologhbo and Fetuga, 1987). The two types of cowpea cultivated in Nigeria are the grain and vegetable cowpea. Legumes like cowpea serves as alternatives or supplements to animal proteins, particularly in parts of the world where there is paucity of animal proteins due to socio economic constraints (Ojimelukwe, 2002). The grain cowpea is a crop predominantly of drier regions, but with advances in the crop development has given opportunities for its production in other agro – ecological zones of the country. This can be achieved through more efficient manipulation of the crop duration, reduction in the severity of pests and diseases. . Yield evaluation of some cowpea cultivars has continued to generate interest among researchers in Nigeria (Ndon and Ndaeyo, 2001). Yield evaluation usually involves the consideration of other characters that determine the overall of the genotypes. This is important because yield is a quantitative character and therefore influenced by a number of traits acting singly or interacting with each other. Earliness (Number of days to flowering, pod filling period and number of days to physiological maturity), number of branches per plant, pod length, number of seeds per pod and 1000 seed weight are the necessary agronomic traits of cowpea that contribute to seed yield (Ogunbodede, 1989; Okeleye *et al*; 1999). When the aim is to increase seed yield in cowpea these traits and their inter – relationship are important factors to consider.

Cowpea tolerate heat and dry conditions but intolerant to frost. The ability of cowpea to tolerate heat and dry conditions makes it unique in fixing nitrogen.Cowpea is adapted to warm weather and requires less rainfall than most crops ; therefore, it is cultivated in the semi-arid regions of lowland tropics and subtropics, where soils are poor and rainfall is limited [Mortimore et al; 1997]. Under developed parts of the world attach importance to cowpea cultivation as the crop is drought resistant and do well under various kinds of cropping systems (Singh *et al*; 1997). Yield potential of cowpea is high averaging 1.5 – 3t/ha (Rosalind *et al*; 2000). The fast growth and spreading habit of traditional cowpea varieties suppress weeds and soil nitrogen increased. Agronomic practices such as date of planting, plant

populations, maintenance of physical and chemical properties of the soil, weed control and manipulation of cropping systems strongly influence cowpea yields.

2. Materials and Methods

This experiment was conducted in the experimental field of the Department of Crop Science, Faculty of Agriculture Adamawa State University Mubi which is located (10^o 11' N, 13^o 19' E) at an altitude of 696m. The experiment was conducted in 2009 cropping season. The mean annual rainfall of Mubi is about 1016mm with sandy loam soil texture (Rayar, 1986). Treatments consisted of five varieties of cowpea which were laid in randomized complete block deign (RCBD) with three replicates. Two varieties were obtained from ADADP Yola and the other three were local genotypes obtained from Mubi market. Each experimental plot measured 4m x4m where appropriate varieties were planted at 50cm x 50cm. Three seeds were planted per hole and later thinned to two per hole one week after .seedling emergence. Weeding was done twice using manual labour at 4 and 8 weeks after sowing. Spraying against insect pests were done twice commencing the onset of seed formation and repeated after 4 weeks. Data were collected on plant height, number of leaves per plant, number of branches per plant, number of days to flowering, pod filling period, number of days to physiological maturity, number of pods per plant, pod length, number of seeds per pod, number of seeds per plant, 1000 seed weight and yield per hectare. The data was subjected to analysis of variance (ANOVA) using the statistical analysis system (SAS). Means were separated using the Least Significant Difference (LSD) at P = 0.05

3. Results and Discussion

The physio-chemical characteristics of the experimental areas are shown in Table 1.The result from the chemical analysis show that the soil was slightly acidic. Although the organic carbon (0.65%) and available nitrogen (0.40%) values were low there was a high concentration of available phosphorus 6.6 (ppm). The particle size analysis show that, the soil type of the experimental area was sandy loam with a high proportion of sand (56.5%) and silt (40.5%) and less clay (3.0%). The soil had a high water holding capacity with a maximum of (39.7%).

Table 2 summarizes the effect of varietal difference on plant height, number of leaves per plant, number of branches per plant, number of days to flowering, pod filling and number of days to physiological maturity.

Table 1: Physioco–Chemical Characteristics of the Soil From the Experimental Area

Chemical analysis	
P^H in water	6.80
Organic Carbon (%)	0.65
Carbon to Nitrogen ratio (C:N ratio)	1: 40
Available Nitrogen (%)	0.40
Available Phosphorus (PPM)	6.60
Available Calcium (Me/100g)	4.20
Available Sodium (Me/100g)	0.35
Available Potassium (Me/100g)	0.49
Particle Size Analysis	
Clay (%)	3.00
Sand (%)	56.50
Silt (%)	40.50
Soil Texture	Sandy Loam
Maximum water Holding Capacity (%)	39.7

Table 2: Plant height, number of leaves per plant, number of branches per plant, number of days to 50% flowering, pod filling period, number of days to physiological maturity of some cowpea varieties.

Treatment	*Plant Height (cm)*	*Number of Leaves Per Plant*	*Number of Branches Per Plant*	*Number of Days to Flowering*	*Pod Filling*	*Number of Days to Physiological Maturity*
V_1	190.41	65.02	5.62	45.03	22.01	63.27
V_2	181.30	71.31	5.27	44.27	22.03	62.09
V_3	56.710	38.91	3.72	38.02	19.00	53.18
V_4	65.240	41.20	3.40	38.71	21.49	53.00
V_5	170.02	66.07	4.19	50.12	24.00	70.49
SD(P=0.05)	33.43	6.790	NS	3.030	1.250	4.370

V_1 = White Kananado
V_2 = Brown Kananado
V_3 = White Borno Local
V_4 = Brown Borno Local
V_5 = Ife – Brown

The result showed a significant (P=0.05) difference in plant height. Highest means of 190.41cm was recorded with V_1 (White Kananado), followed by 181.30cm with V_2 (Brown Kananado). V_3 (White Borno Local) significantly recorded the least plant height of 56.71cm compared to other treatments. Significant (P=0.05), difference was recorded on the number of leaves. The highest mean number of leaves per plant (71.31) was obtained with V_2 (Brown Kananado) followed by 66.07 with V_5 (Ife-brown). The least mean of 38.91 was recorded with V_3 (White Borno Local). The variations in plant height, number of leaves and the mean were very high. Plant height and number of leaves were evaluated and determined by the vegetative growth of the 5 cowpea varieties. This is in conformity with

suggestion by Pfeiffer and Harris (1990) who observed and reported that measurement are used as indicator of vegetative growth.The soil texture of the experimental area based on the particle size analysis is sandy loam. The good performance of the cowpea in the study area with sandy loam soil is in total conformity with the findings of Rayar[1986] who observed that legumes can do well on sandy loam soils which can help in producing an extensive root system that extracts moisture from lower depth of soil.

The result revealed that no significant (P=0.05) difference was recorded among the treatments although there were higher number of branches with V_1 (White Kanando) compared to other treatments. Number of branches ranged from 3.40 to 5.62. Days to flowering statistically differ in all the treatments. Longer days to flowering 50.12 were recorded with V_5 (Ife- Brown). These varieties flowered between 38.02 days to 50.12 days; while the period between flowering and maturity implies that these varieties must fill their seeds very fast and this is an important trait in areas where water availability is very low. It is worth interesting to note that the number of days to physiological maturity did not exceed 71 days. Another interesting fact about this result is that $V_3$and V_4 matured before 54 days making them more adaptable in drought –prone areas. Similar result were reported by (Ndaeyo *et al*; 1995) who found that seed colour preference and use differed from region to region and the maturity, growth habit and photosensitivity requirement depends upon the cropping systems. Furthermore, this result is corroborated by [Grema,1995 and IITA,1998] who found that no single variety of cowpea can be suitable for all conditions.

Table 3:Number of pods per plant , pod length, number of seeds per pod , number of seeds per plant, 1000 seed weight and yield per hectare of some cowpea varieties.

Treatment	*Number of Pod Per Plant*	*Pod Length (cm)*	*Number of Seeds Per Pod*	*Number of Seeds Per Plant*	*1000 Seed Weight*	*Yield Per Hectare (Kg/Ha)*
V_1	33.40	13.23	17.11	415.08	160.13	19,012.10
V_2	35.72	14.81	16.24	420.16	168.04	20,000.20
V_3	30.90	19.00	14.28	312.41	120.00	16,000.00
V_4	32.41	20.03	12.41	310.10	121.40	15,214.80
V_5	23.34	17.50	13.14	258.21	140.91	14,000.30
LSD (0.05)	8.38	1.70	1.18	40.49	12.01	315.1000

The mean number of pods per plant pod length number of seeds per pod, number of seeds per plant, 1000 seed weight and yield per hectare of some cowpea varieties are presented in Table 3. Significant (P=0.05) difference was observed for all the yield components. The result showed that number of pods per plant ranged 23.34 in V_5 to 35.77 in V_2. Significantly longer pods 20.03 were recorded with V_4 while the least 13.23 was recorded with V_1. There was significant difference in the number of seeds per pod which ranged from 12.41 to 17.11. The number of seeds per plant was significantly higher 420.16 with V_2 and also significantly lower 258.21 with V_5. Significant difference was observed for all the treatments which ranged from 120.00g to 168.04g for 1000 seed weight. Grain yield (kg/ha) was significantly (P = 0.05) superior 20,000.20 with V_2, although V_5 recorded the lowest yield 14,000.30 in the mean where significant difference was observed due to treatment effects. This result showed that where there were more pods per plant and more number of seeds per plant the yield was increased. Based on the result from the present study cowpea yield in Nigeria is low. The use of early maturing varieties as adopted in this study can help in minimizing these problems. This study agrees with the earlier views of [Ofori and Djagbletey 1995; Okeleye *et al*; and Ndon and Ndaeyo, 2001] who reported that early maturing Cowpea genotypes have been shown to yield as much as or more than the late maturing varieties with added advantage of being suitable in areas with unreliable rainfall in terms of total amount,distribution and duration where crop failure is often attributed to early cessation of rains and there by making it adaptive to different agro-ecological environments in Nigeria.Varietal requirements in terms of plant type, seed type, maturity, yield for cowpea varies from region to region thus the importance of selection of varieties.

4. Conclusion

It can be concluded from this study that early maturing cowpea can perform very well in Northern Guinea Savanna. V_1 (White Kananado) and V_2 (Brown Kananado) recorded the highest yield of 19,012.10kg/ha and 20,000.20kg/ha respectively.The yield ranged from 14,000.30kg/ha to 20,000.20kg/ha. The earliness character (days to flowing, pod filling and days to physiological maturity enables them to flower, pod fill and mature early and therefore escape the dryness of November and December). From the findings of this study the yield of V_1 and V_2 were quite high and encouraging. Since V_1 and V_2 performed better, they are recommended to farmers of this area based on their optimal performance for adoption.

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7/11/2010

Synthesis and Modification of some Heterocyclic Compounds with Potential Biological Activity Coupled on Poly (Maleic Anhydride – Methyl Methacrylate)

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Abstract: This paper presents some results concerning the immobilization through chemical bonds of some biologically active compounds on the maleic anhydride- methyl methacrylate) copolymer. The high reactivity of the anhydride cycle of the polymer has allowed us to obtain conjugates in which the biologically active compounds of some heterocyclic compounds are immobilized on the polymeric support through amide bonds. The reaction products were characterized through elemental analysis, mass spectra, FTIR and ^{1}HNMR spectroscopy. The antimicrobial activity of the modified copolymer was tested against various microorganisms (Staphylococcus aureus, Escherichia coli, pseudomonas aeroginosa, Klebsiella and fungal, Aspergillus niger, Aspergillus Flavus and Fusarium oxyspoium). In general, the copolymers showed good antimicrobial activity against the previously mentioned microorganism. [Journal of American Science 2010; 6(8):512-524]. (ISSN: 1545-1003).

key words : Synthesis , Copolymer, Heterocyclic, Antibacterial activity.

1. Introduction:

Many polymers with reactive functional groups are now synthesized, tested and used not only for their macromolecular properties, but also for the properties of their functional groups. These functional groups provide an approach to a subsequent modification of the polymer for specific end applications (Vogl et al. 1985). Nowadays a strong demand prevails for functional polymers with very specific properties. Functional groups give the polymer structure of special characters substantially different from the inherent properties of the basic polymer chain (Vogl 1996). In recent years some comprehensive work has been published on functional monomer and their polymers (Godwin et al. 2004, Erol and Soykan 2003, Akelah and Moel 1990, Erol et al. 2001). The antimicrobial property of the polymers plays an important role for many of its applications. Contamination by microorganisms is of great concern in several areas such as medical devices, health care products, water purification systems,hospital and dental equipments, etc. One possible way to avoid the microbial contamination is to develop materials possessing antimicrobial activities. Consequently, biocidal polymers have received much attention in recent years (Worley and Sun 1996).

Thiosemicarbazones and the corresponding metal complexes are widely known as having a large range of biological applications, such as antiviral, antimalarial, antifungal, etc (West et al. 1991, El-Sawaf et al. 1997). The compound p-acetamidobenzaldehyde thiosemicarbazone, commercially available as thiacetazone, has long been employed in the treatment of tuberculosis (Beraldo et al. 1999). The activity of these compounds is strongly dependent upon the nature of the heteroaromatic ring and the position of attachment to the ring, as well as the form of the thiosemicarbazone moiety.

Some thiazole, thiosemicarbazole, imidazole, benzothiazole derivatives (Muthusubramanian et al. 2001, Chevica et al. 2003, Tolkova et al. 2001, Shaha et al. 2002, Paramashivappa et al. 2003) and isatin (Pandeya and Sriram 1998, Sarangapani and Reddy 1994, El-Sawi et al. 1998, Aanandhi et al. 2008) are mentioned in literature to show antimicrobial (Bartlett et al. 1992, Sunel et al. 2001, Basu et al. 2002, Koci et al., 2002) antifungal (Gbadamassi et al. 1988), antihelmitic (Hazelton et al., 1995) pesticide and herbicidal properties. The high reactivity of anhydride ring in the copolymers based on maleic anhydride toward the nucleophilic reagents (Angelescuu-Dogaru et al. 1999, Spiridn et al. 1997, Kysela et al. 1992, Staudner et al. 1992) would suggest the possibility of its opening by various nucleophilic compunds in order to obtain new products with potential biological activity. There are many systems mentioned in the literature (Jeong et al. 2002), obtained throught the opening of the anhydride ring of maleic anhydride copolymer with some vinyl monomers, especially with the methyl methacrylate,under the action of heterocyclic compounds. It must be mentioned that

these copolymers are biocompatible, and some of them present an important intrinsic biological activity (Stauner et al. 1997, Uglea et al. 1996).

In this work, some heterocyclic compounds have been prepared. These compounds were allowed to react with methylmethacrylate-maleic anhydride copolymer to obtain higher biological active products. Several studies have demonstrated the antimicrobial and antifungal action of these compounds using four bacterial and three fungi strains Staphlococcus aureus, Escherichia coli, pseudomonas aeroginosa, Klebsiella Aspergillus niger, Aspergillus Flavus and Fusarium oxysprium respectively.

2. Materials and Methods:

Methylmethacrylate(MMA) was supplied by Aldrich and was deinhibited by using sodium hydroxide. Maleic anhydride(MAN),purity 99.9% Merck,Germany) Thiosemicarbazide, 2-iminothiazolidine -4 –one, Thiophene aldehyde , 2 -acetyl thiophene,Isatin ,other compounds such as solvents, and other reagents were reagent grade and used without further purification.

Spectrophotometric measurements:

FTIR was carried out using Mattson 1000 FTIR spectrophotometer,Unicam,England in the range 400-4000 cm^{-1}.

NMR spectra were recorded on a Varian Mercury VX-3oo NMR spectrometer 1H spectra were run at 75.46 MHz in dimethylsulphoxide(DMSO-d6).

Mass spectra were measured on a GCMS-Gp 1000 EX spectrometer at 70 eV.

Elemental analyses carried out at the Microanalytical Center of Cairo University.

Synthesis of poly (methyl methacrylate- maleic anhydride) copolymer (p MMA/MAN):

The MMA-MAN used in this study was synthesized by a modified solution polymerization of the monomers methyl methacrylate,maleic anhydride, (MMA/MAN). The polymerization reaction was carried out in a reactor under intensive mixing and nitrogen atmosphere at 80 ^{0}C for 8 h. The copolymers were synthesized toluene benzoyl peroxide as initiator. Appropriate volume of the solvent was added to maintain the monomer concentration at 2 mol /L. The obtained copolymers were fractionated by precipitation in excess methanol to remove all the toluene and the non-reacted maleic anhydrid (MAN). The synthesis procedure used in this work is a modified version of similar methodologies used in the literature (Spridon wt al. 1997, Popa et al. 1997, Wilde and Smets 1950).

Synthesis of (1a)

To around bottom flask contaning 150 ml of boiling ethanol under stirring, 4.5 gm (50.2m mol) 0f thiosemicarbazide ,and 5.5 ml (50 .2 m mol) of 2-acetyl thiophene were added and the solution was allowed to cool at room temperature; then 2,4-drops of concentrated H_2SO_4 were added causing the precipitation of a pale yellow solid. After 24 hours of stirring the solid was separated by filtration and washed with cold ethanol and hexane (yield 65% (6.5 gm, 32.7 m mol) mp=132-133 ^{0}C).

Synthesis of (1b)

A mixture of compound 1a (0.01 mol) with $ClCH_2COOH$ (0.011mol) and KOH(0.02 mol) in ethyl alcohol was refluxed for 2-hours,cooled diluted with H_2O and stirred with KOH solution, filtered wased with H_2O dry, crystals from ethyl alcohol were obtained.

Synthesis of (1c):

In a reaction flash provided with a refluxing condenser 100 ml absolute ethyl alcohol were introduced and then 2.3 gm (0.01 mol) metallic sodium were added. To the obtained solution of natrium ethoxide 16.7 gm (0.01 mol) Ib were added in small portians under strring and gentle heating on a water bath for the solution homogenisation. 13.5 gm (0.11 mol) of benzoyl chloride were added as several doses to the hot alcoholic solution of the Ib sodium derivate the sodium chloride was then removed by filtration under vacuum and the obtained yellow solution is cooled to the room temperature and poured as thin thread, under strring, into cold water . Acream coloured precipitate is separated and filtered, dried and crystallized.

Preparation of compound (Id)

To a suspension of Ic (o.01mol) in absolute ethanol (20 Cm^3), hydrazine hydrate (0.015 mol; w=98%) was added and the reaction mixture was refluxed for 2h. The mixture was then cooled, treated with one drop HCl and the separated pale yellow product was filtered off,dried and crystallized.

Reaction of the compound (1d) with copolymer :

In a flash provided with a refluxing cooler 1 gm (0.005 mol) poly (MMA/MAN), 0.70 gm (0.005 mol) from Id and 50 ml anhydrous acetone were introduced. The mixture was refluxed on a water bath for 2-hours, where a homogeneous solution resulted. after removing the excess acetone by distillation under normal pressure until a volume of 4-5 mL, a mixture of anhydrous petroleum ether and ethyl ether (1:1) was added where an oily product separates. It

was submitted to repeated washings with ethyl acetate and then with anhydrous ethyl ether where it turned into a fine yellow powder, the final product was obtained in a yield (76%) after recrystallization from ethyl acetate and drying. (1e)

Synthesis of Compound (IIe):

In a reaction flash provided with a refluxing cooler l00 ml absolute ethyl alcohol were introduced and then 2.3 gm (0.01 mol) metallic sodium added to the obtained solution of natrium ethoxide 16.7 gm (0.01 mol) IIa were added as small portians under strring and gentle heating on a water bath for the solution homogenisation. 13.5 gm (0.11 mol) from ethyl chloro acetate were added as several doses to the hot alcoholic solution of the IIa sodium derivate the sodium chloride was them removed by filtration under vacuum and the obtained yellow solution was cooled to the room temperature and poured as thin thread, under strring, into cold water, a paleyellow coloured precipitate was separated and filtered off under vacuum and dried The crude product was purified by recrystallization from boiling ethyl alcohol .

Synthesie of compound (IIIe).

The schiff bases of isatin were synthesized by condensation of the keto group with thiosemcarbazide to give the compound(IIIa)and then reaction with monochloro acetic acid gives the structure(IIIb) and these when reacted with p-chlorobenzaldehyde give the structure(3c) which with addition to the copolymer gives the compound (IIIe) .

Synthesis of (IIIf).

Isatin (2gm, 13.6 m mol) and 3,4 diphenylether (1.36gm) (6.8 m mol) were dissolved in 35 ml warm ethanol containing 0.45 ml of acetic acid for 10 hours washed with ethanol ,recrystalized from ethanol (Schiff base) m.p > 260 ^{O}C.

Preparation of 2- iminothiazolidine – 4- one . (IV):

6 gram of thiourea with 7.2 gm of monochloro acetic acid in glacial acetic acid, are refluxed for half an hours, to give a solid, filter, and wash with methyl alcohol. Then dissolve in lest amount of water, then basified with sodium bicarbonate (3%) solution to give a white crystal. m.p=212 ^{0}C

This compound was reacted with thiosemicarbazide in ethanol (30 Cm^3) and refluxed for 3h. The formed yellow solid product was filtered off, drided and crystallized.

Preparation of(IVd).

A mixture of the acetic anhydride (0.01 mol) and compound (IVa) (0.012) in ethanol (30 Cm^3) was refluxed for 3 hours. The formed yellow solid product was filtered off, dried and crystallized.

Preparation of (IVg)

The reaction of 2-iminothiazolidine -4- one with formaldlehyde and primary aliphatic amine in molar ratio (1:2:1) under Mannich - type condition gave thiazolo (3,2-a) s-triazine . (IVf)

Mannich reaction

0.92 gm (8 m mole) of (IV) in 1.92 ml (0.0 24 mole) of formalin add dropwise with stirring 1gm (8m mol) of 25 % aqueous solution of aliphatic amine at 0-5 ˚C . After the addition is complete .The reaction mixture is stirred without cooling for another 15 min.after which it is treated with worm (30 -40 ˚C) benzene (four portions 20 ml each) .The benzene layer is drided with magnesium sulphate,the benzene is removed by distillation to one fifth of its original volume, to give ppt.(IVf)

The compound of (IVf) reacts with the copolymer gives the structure (IVg).

Procedure for the aldehyde with amine):

Amixture of the appropriate aldehydes (10 m mol) and (4-amino) (12 m mol) in glacial acetic acid ,the reaction mixture was left to stand at room temperature for overnight and the resulting crystalline material was collected by filtration washed with cold ethanol, and recrystallized from the proper solvent.

Evaluation of antimicrobial activity

The antibacterial and antifungal activities were carried out at the Regional Centre for Mycology, and Biotechnology at Microanalyticl Center, Cairo University, Egypt

Some of the newly synthesized compounds were screened for their antimicrobial activity using the diffusion agar techniques (EL-Merabani et al. 1972) were tested against four bacterial species namely. (Staphylococcus aureus, ,Escherichia coli,pseudomonas aeroginosa and Klebsiella, as well as against three fungal species namely Aspergillus niger , Aspergillus flavus and Fusarium oxysporium) for their antimicrobial activity using 5 mg /ml.of each compound in dimethylformamide. Inhibition Zone diameter (IZD) in cm was taken as the criterion for antimicrobial activity. The antimicrobial activity was assayed biologically using a spore suspension of the fungal pecies (1 ml of sterile water containing approximately 108 conidia) or spreading bacterial suspension over a solidified malt agar (Dawson 1957, Kucheria et al. 2005). The layer was allowed to set for 30 min. A solution of each of the tested

compounds (5 mg/ml) was placed onto sterile 5 mm filter paper discs and allowed to dry, and then the discs were placed onto the center of the malt agar plate and incubated at the optimum incubation temperature, 28 ºC. A clear zone around the disc was taken as an indication of the inhibition growth of the test organism. The size of the clear zone is proportional to the inhibitory action of the compound under investigation. The fungicide terbinafin and the bactericide Chloramphenicol were used as references to evaluate the potency of the tested compounds under the same conditions (Elamin et al. 2005, Abdel-Aziz et al. 2008). Measurements were considered after 72 h for fungi and 24h for bacteria. The results are contained in Table (1)

3. Results and Discussion:

The conversion of maleic anhydride moiety in heterocyclic compound to malemide was monitored by FTIR, ^{1}HNMR spectra and elemental analyses. This may be attributed to the improvement of rigidity of polymer chains because the imide ring was formed in the chemical modification.

The compound (1a) was prepared by reacting thiosemicarbazide with 2-acetylthiophene in ethanol following the addition of sulphuric acid. A yellow crystalline, air–stable compound in 65% yield was isolated. The compound solubility is in ethanol and other polar solvents.

Compound (I-Ie):

By heating the p (MMA/MAN) copolymer with compound (Id) in anhydrous acetone the amidlic compund (Ie) resulted according to the following reaction (in scheme 1) the reaction mechanism is supposed to be similar to that oxazolone decyclization by the nucleophilic reagents **(**Chevica et al. 2003, Sunel et al. 2001) the compound (Ie) derivative reacts by means of the NH_2 group due to the unshared p electrons on the nitrogen atom. the product purified by recrystallization from ethyl alcohol was obtained as yellow crystals. The FTIR, ^{1}HNMR spectral measurements along with the nitrogen analysis data confirm the structure of the reaction product as the found nitrogen content is 14:01 % in comparison with the calculated value, of 14:55%.m.p>300 ^{o}C. The FTIR spectrum of the synthesized compound (Ie) shows the characteristic absorbtion band : 1724.7 ,1626 cm^{-1} C=O, CO-NH, 2995 Cm^{-1} C-H aliphatic, 3407 cm^{-1} OH in COOH ,3245Cm^{-1} of NH group,1390Cm^{-1}CH_3 bend C=N at (1519) Cm^{-1} and 3046Cm^{-1}CH aromatic.

The ^{1}HNMR(δ,DMSO) also confirms the structure of the synthesized compound; δ(ppm) 3.0(s,3H,CH_3)),9.8(s,1H,NH) 7.2(s, 5H,aromatic), 5.5(m,1H,CH).

MS(Int.%):210(100),216(1.8),215(2,28)212(8.16)524 (0.87),523(1.17),521(2,14).Anal.Calcd.for: $C_{25}H_{26}O_6N_5S$(524):C,57.25,H,4.96,N,13.35,S,6.1.Fou nd:C,56.5,H,4.1,N,12.9,S,5.5. In scheme(1)

In order to obtain sulpha-drug (MMA /MAN) copolymer derivatives,the amino derivatives of such compounds were introduced to the anhydride ring of the copolymer(1:1 mol/mol) by heating them in DMF /acetone (1:1 v/v) at 70 0C for 12 h.The reaction of copolymer anhydride groups with such different functional compound is shown in (1g)In scheme(2).

The FTIR spectrum of the synthesized compound shows the following characteristic absorption bands : at 3410-3313 Cm^{-1} for NH_2 ,1585 Cm-1 of C=N, 1655 Cm-1 C=O, Ar-H st.at 3100 Cm-1 and SO_2 at 1370 Cm^{-1}.The ^{1}NMR(∂,DMSO),3.94 (s,3 CH_3),5.1(bs,2H,NH_2).Anal.Calcd for : $C_{22}H_{24}O_7N_4S_2$ (520):C,50.76,H,4.6,N,10.76,S,12.3.Found:C,51.2,H, 4.3,N,10.1,S,11.8.

Compound (IIe):

The studies in the present paper resulted also in the development of a new synthesis method of a compound (IIe) by using p(MMA / MAN). The reaction was carried out by adding the copolymer to (IIc) solution in an anhydrous solvent. The chemical equation is given in scheme (3). The structure was elucidated by means of,the FTIR ,MS and ^{1}HNMR spectral measurement, The FTIR spectum shows an 2996 ,2932 Cm^{-1} may be attributed to the asymmetric and symmetric C-H stretching of methylene group.1455 cm^{-1} CH group,1394 Cm $^{-1}$ may be bending ,1710 Cm^{-1} of C= O ,1148 Cm^{-1} C-O ,of group ,1630 Cm^{-1} methyl group, peak at N=C 1690 Cm^{-1},amides (1500-1600 Cm^{-1}) ,NH_2 at 3250 Cm^{-1} 2980 Cm^{-1} aliphatic ,SO_2 at 1430 Cm^{-1} and C=N at 1665 Cm^{-1}.The MS(Int.%):Base peak 361(100),433(4.23),408(5.47),302(20.83),317(5.3),30 0(15.52).

The structure of the synthesized compound was also confirmed by ^{1}HNMR spectrum PPm δ 2.4 (m,1H ,CH) ,δ 3.5(m,2H CH_2) , δ 2.1(s ,3H,CH_3) and δ 9.7 (s,1H,NH).

Anal.Calcd.for'$C_{19}H_{21}O_7N_5S$(463),C,49.24,H,4.54,N, 15.12,S,6.9.found:C,48.8,H,5.1,N,15.2,S,6.5.

Compound (IIIe):

The target compounds were prepared by using the reaction sequence in Scheme(4) The schiff bases of isatin were synthesized by condensation of the keto group with thiosemcarbazide to give the compound(IIIa)and then the reaction with monochloro acetic acid gives the structure(IIIb) and these when reacted with p-chlorobenzaldehyde give the structure(IIIc),and then react with hydrazine

hydrate, which adds to the copolymer and give the compound (IIIe) The chemical structures of these synthesized compounds were confirmed by means of FTIR, and ^{1}HNM spectra. In scheme (4).

The FTIR spectram showed peaks at 3300 Cm^{-1} (NH), 1680 Cm^{-1}(C=O), 1620 Cm^{-1}(C=N), and 798 Cm^{-1} for (C-Cl).and 669-603Cm^{-1} for (CH_2) aromatic.

The^{1}NMR(∂,DMSO):11.9(bs,1H,NH),4.2(s,3H,CH_3).

Anal.Calcd.for $C_{27}H_{23}O_7N_6Cl$(578.5) C,56,H,5.5,N,14.5,Cl,6.13. Found: C, 55.5, H, 6.1 N, 14.3, Cl, 5.83.

The compound (III) reacting with 3, 4, diaminodiphenyl ether gave the compound (IIIf) and when reacted with the copolymer gave the structure (IIIg).In scheme(5).

The structure of the synthesized compound was also confirmed by FTIR and ^{1}HNMR spectral measurements.

The FTIR spectrum shows an absorption peak at 1719 Cm^{-1} attributed to the carbonyl group, 3151 Cm^{-1} for N-H group and 1350 $Cm^{-1}$for C=S.

The ^{1}HNMR also confirms the structure of the synthesized compound: δ (ppm) 6.9-7.6(m,4H,Ar), and δ 11.3(s,1H, NH). Anal.Calcd.for $C_{29}H_{25}O_7N_3$ (527).C,66,H,4.74,N,7.96. Found C,65.4,H,4.1,N,7.2.

Compound (IVd):

The reaction of 2-iminothiazolidine 4-one (IV) with thiosemicarbazide gave the compound (IVa): FTIR specrum shows (IVa): 1370 for C=S, 1590-1605 for (C=N),1610 for (NH_2 def),3180 (NH) and ^{1}HNMR spectrum, δ 8.4(s 1H, CH=N), δ11.7 (br, 2H, NH_2) and when reacted with acetic anhydride gave the compound (IVb). The FTIR spectrum shows 1580 Cm^{-1} (C=N), 1663 (C=O), and 1350 Cm^{-1} (C=S): by treating the (IVb) through condensation. These compounds react with the copolymer according to scheme (6).

In the FTIR spectrm the frequency of the valence vibration of the structure shows the following characteristic absorption bands : C=O group is found at 1724 Cm^{-1}, that of N-H band at 3350-3400 Cm^{-1} and C=N at 1585 Cm^{-1}. And the ^{1}HNMR spectrum shows: δ (ppm) 11.3(s, 1H, NH), and 2.3 (3H, CH_3). Anal. Calcd. For $C_{17}H_{23}O_7N_7S$ (469) C, 43.49, H, 4.9, N, 20.9, S, 6.8. Found C, 44, H, 4.2, N, 21.2, S, 7.3.

The reaction of 2- iminothiazolidine -4-one (IV) with formaldehyde and primary aliphatic amines in molar ratio (1:2:1) under Mannich-type condensation gave thiazolo(3,2-a) s- triazine (IVg).The reaction was carried out by adding the copolymer to the compound (IVh) in an anhydrous solvent, the chemical equation is given in scheme (7).

The structure of the synthesized compound was also confirmed by FTIR and ^{1}HMNR spectral measurements.

The FTIR spectrum shows absorpation peaks at(1500-1600) Cm^{-1} for amides, 2921Cm^{-1} for (C-H) in (-CH3), 1712 Cm^{-1} for (-COOCH3), and 1657 Cm^{-1} for(C=N). The ^{1}NMR shows: δ(ppm):10.9(s,1H,NH), 2.4(3H,CH_3),and 9.6(H,OH).

Anal. Calcd. for $C_{14}H_{19}O_5N_5S$.(369) C,45.5, H,5.15,N,18.9,S,8.67.Found $C_{46}H_{4.8}N_{19.3}S_{7.8}$.

Notes:

N ratio If clar zone of inhibition is a) 15 mm = + b) 15-24 mm = + + c) 25-34 mm = +++ d) 35-44 mm = ++++ e) 45-54 = +++++

Antifungal agent is flucoral (150 mg / 10 ml H_2O).

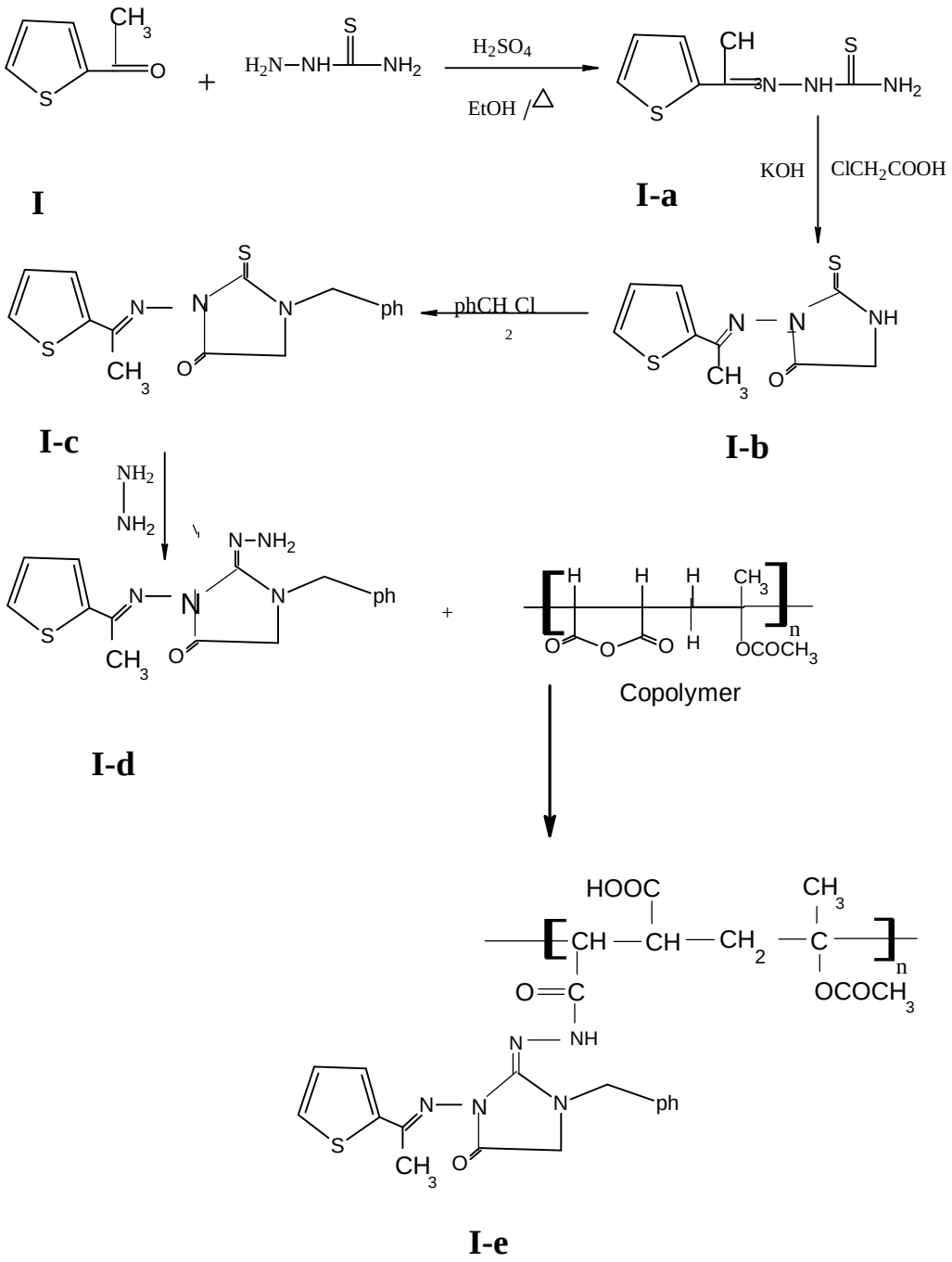
Antibacterial agent is Duricef (250 mg / 10 ml H_2O).

When we used the antibiotics tested with concentrations equal to that of the chemicals tested, No inhibition of growth for any microorganism was noted so we had to use a concentration fraction of antibiotics (standard) higher than that of the tested chemicals to ensure inhibition.

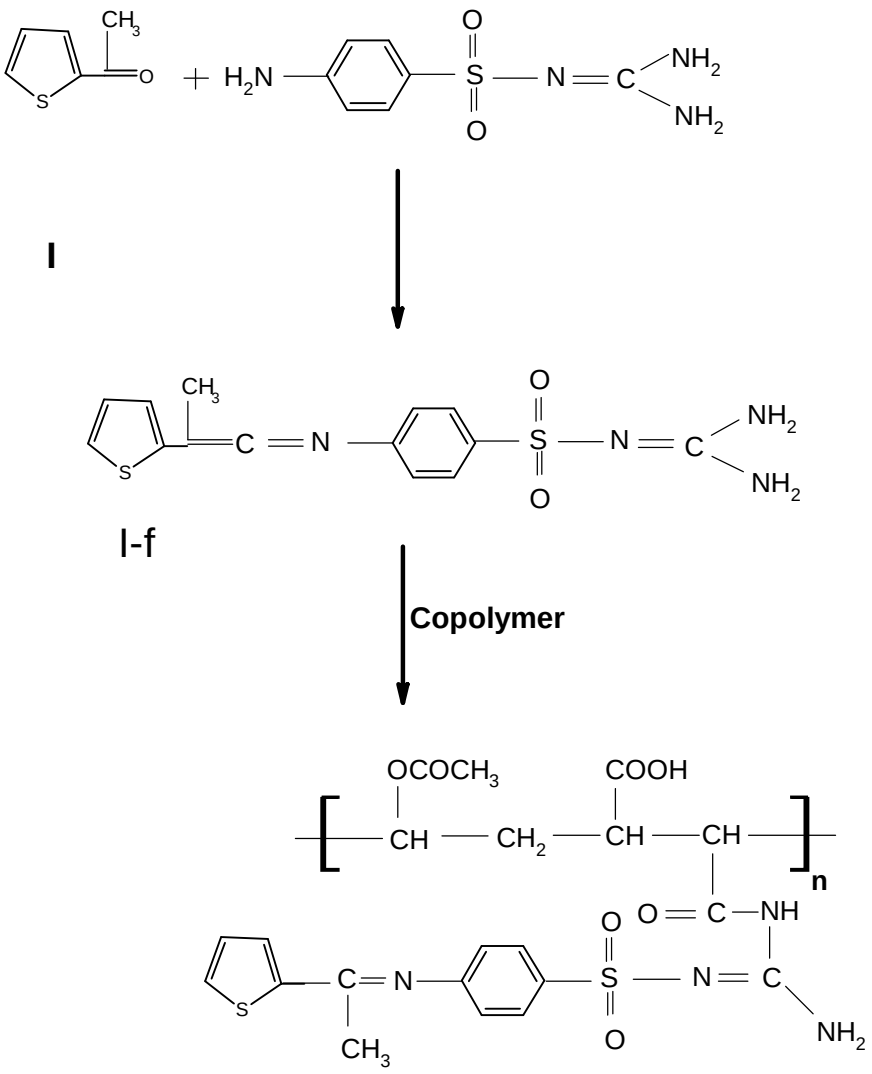
All the synthesized compounds were evaluated for their antimicrobial activities. The Zone of inhibition found for the compounds against gram positive, gram negative bacteria and fungi are presented in Table(1):

The results of antimicrobial activity showed that the heterocyclic compound (Id) was found to have exhibited effect against, (St),(Es),(Ps),(Kl) and (Fu).When reacted with copolymer to give the compound ((Ie) and found to have activity towards (Es),(Ps),(Kl) and (Fu); but the compound (Ig) with sulph-drug exhibited a highly effect against (St),(Es),(Ps),(Kl),(As-flavus) and(Fu).

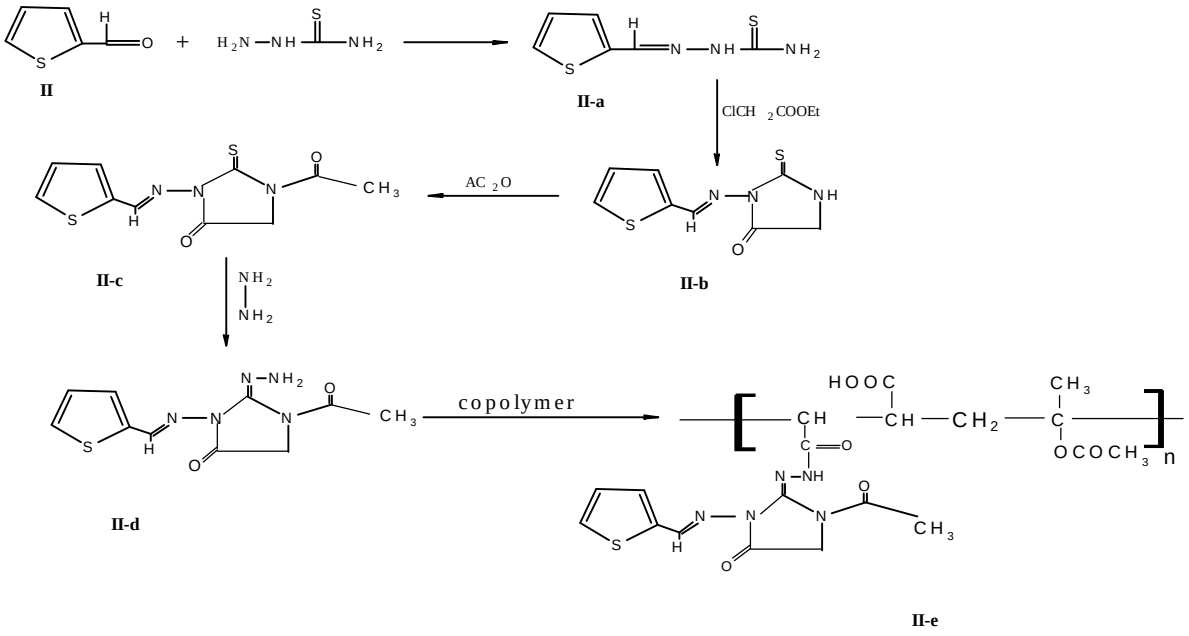
Also the compound (IId) was found to have an effect against (Es) and (Kl) only but when the compound reacted with the copolymer gave the compound (IIe), which has an effect against (Es), (Ps),(Kl) and (Fu).The compound (IIId) exhibited a moderate effect against (Ps),(Kl),(As-niger) and (Fu).but the compound (IIIe) has an effect against (St),(Es),(Ps),(Kl) and (Fu); and the compound (IIIg) effect on (Ps),(kl) and (As-niger),and then the compound (VId), highly effect against (St),(Es),(Kl),(As-flavus) and (Fu), but the compound (IVc) exhibited a moderate effect against (Ps),(Kl) and (Fu). And while (IVg) has a highly effect toward (St),(Es),(Ps) and (Kl).



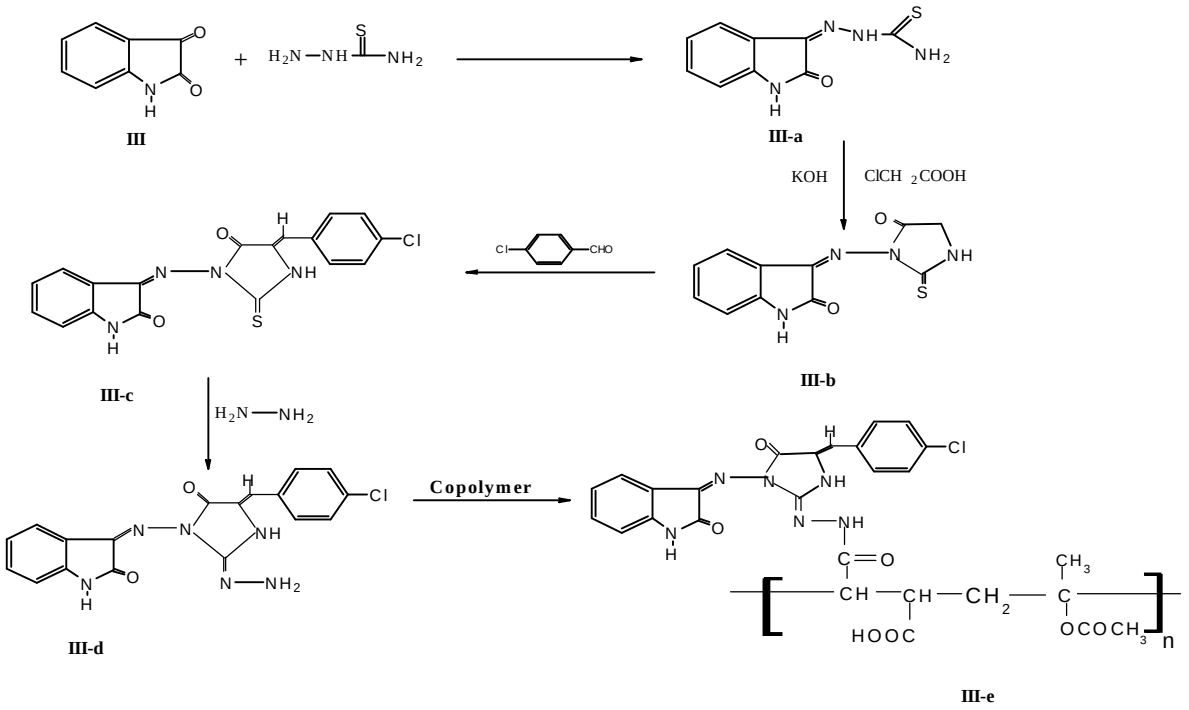
Scheme 1



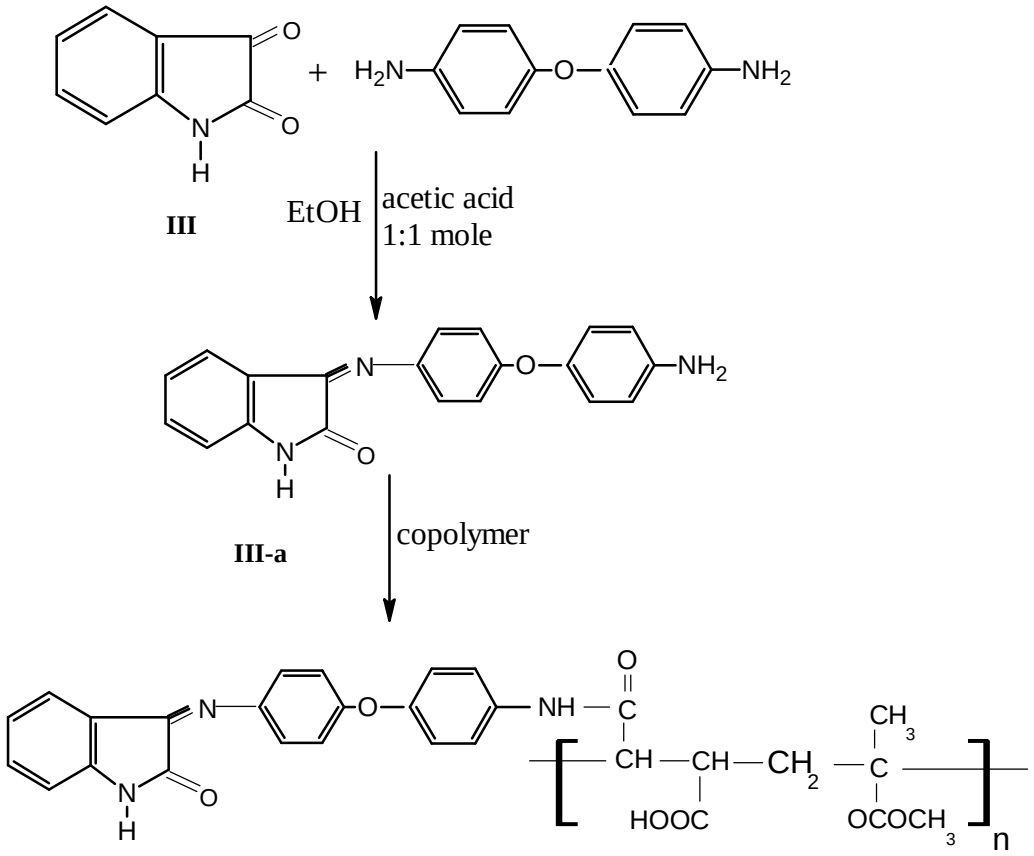
Scheme 2



Scheme 3

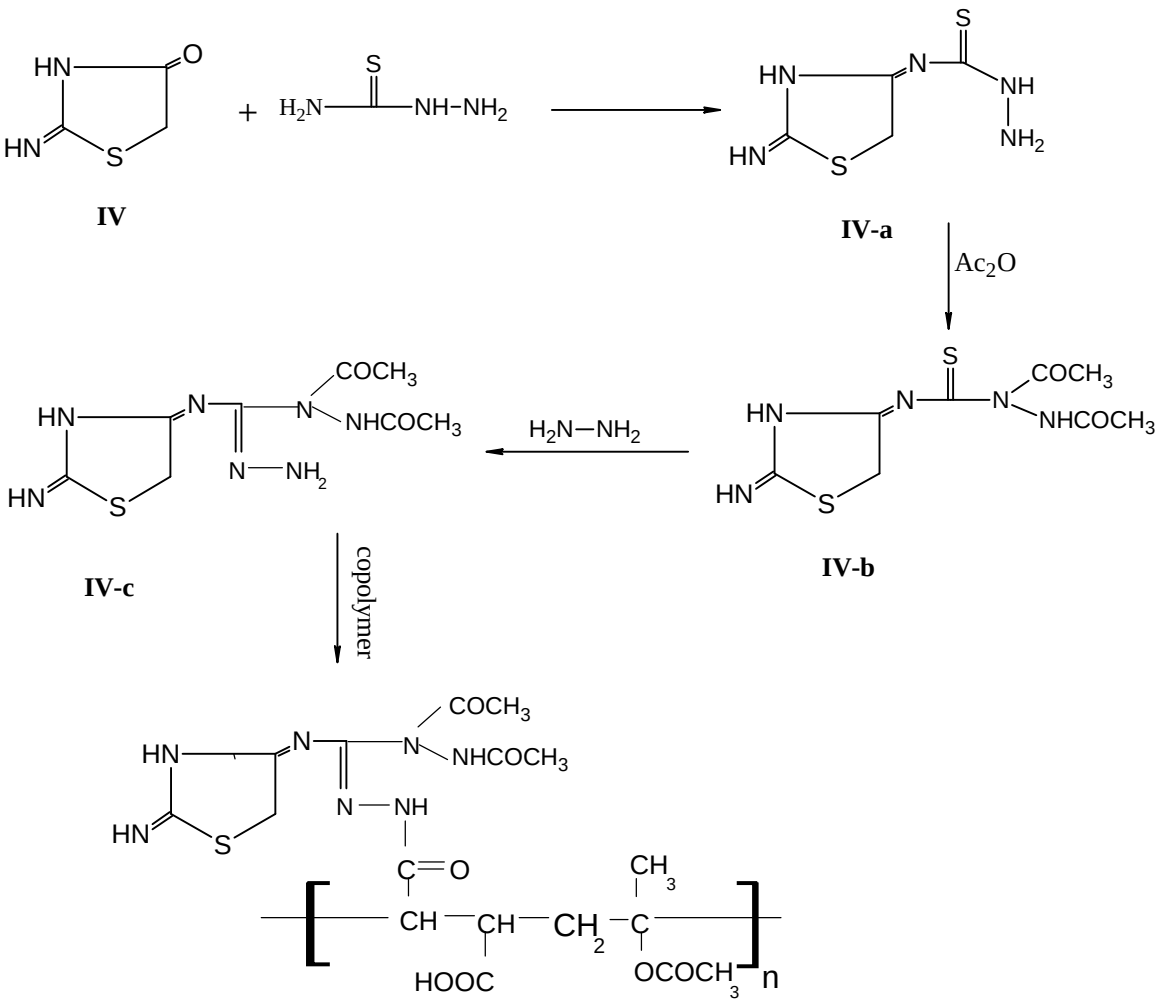


Scheme 4



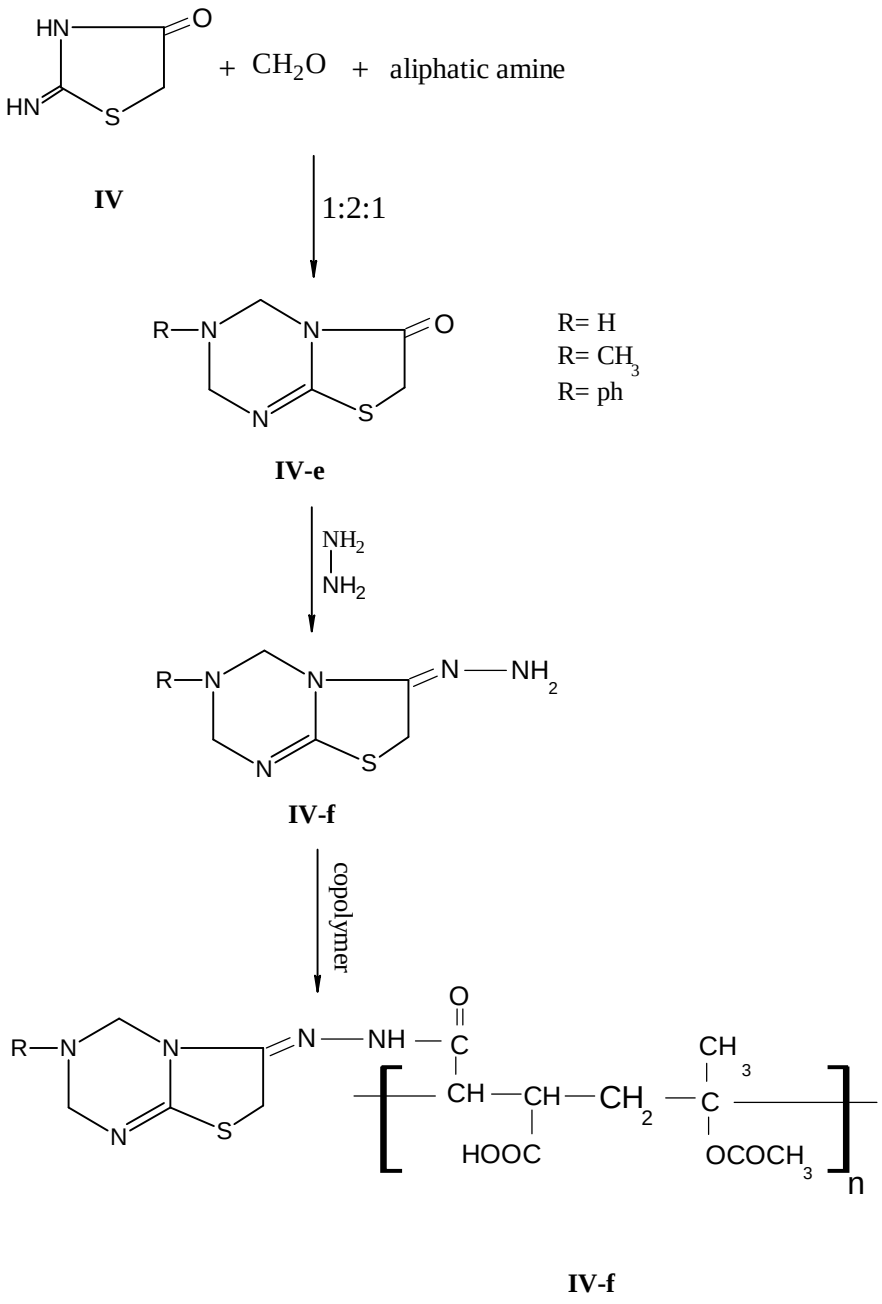
III **III-a** **III-b**

Scheme 5



IV **IV-a** **IV-b** **IV-c** **IV-d**

Scheme 6


HN
O
HN
S
+ CH2O + aliphatic amine
IV
1:2:1
R
N
N
O
N
S
R= H
R= CH3
R= ph
IV-e
NH2
NH2
N
NH2
IV-f
copolymer
NH
C
CH
CH
CH2
C
CH3
HOOC
OCOCH3
n

IV-f

Scheme 7

Table 1:Antimicrobial activity of chemical substances tested

Diameter of clear zone (mm)							Compound No.
Antifungal			Antibacterial				
Fusarium oxysporium	Aspergillus Flavus	Aspergillus niger	Klebsiella	Pseudomonas aeroginosa	Escherichia coli	Staphylococcus aureus	
20	-	-	14	19	18	19	1d
30	-	-	16	23	18	-	1e
-	-	-	30	28	17	29	1g
-	-	-	18	-	16	-	IId
25	18	-	35	26	40	24	IIe
30	-	21	15	15	-	-	IIId
30	-	-	13	19	16	15	IIIe
-	-	24	19	17	-	-	IIIf
20	-	-	15	12	14	12	IVc
50	20	-	32	-	21	13	IVd
40	-	-	19	21	17	-	IVh
-	-	-	50	33	No growth	No growth	Standard antibacterial agent
No growth	21	38	-	-	-	-	Standard antifungal agent

4. Conclusions:

The new synthesized compounds have been immobilised through amidic bonds, through the opening of the anhydride cycle of the poly (methyl methacrylate –maleic anhydride);the spectral analysis proved the obtaining poadation 3 of the polymer – biologically active principle conjugates.

The antimicrobial activity of the modified copolymers were tested against four bacterial and three fungi strains Staphylococcus aureus, Escherichia coli, pseudomonas aeroginosa ,Klebsiella, Aspergillus niger , Aspergillus Flavus and Fusarium oxysporium, respectively.

It was found that the diameter of inhibition zone varied according to the type of active group in the copolymer and also the examined microorganism.In general,the copolymer was further modified and showed antimicrobial activity against the tested microorganisms. However, the compound of the modified copolymer was found to be the most effective on bacteria and fungi species.

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Cytogenetics Changes on Cancer Cells as Affected by Ginger Extracts

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Abstract: A wide variety of phenolic substances derived from spice possess potent antimutagenic and anticarcinogenic activities**.** Some of the phenolic substances are present in ginger, possessing strong anti-inflammatory and anti-oxidative properties as well as exert substantial anti-carcinogenic and anti-mutagenic activities. The present study was conducted to examine the in vivo cytogenetic effect of ginger extract on Ehrlich ascites cell inoculated in female mice. This study was performed on two groups of female mice. The first, one was inoculated intraperitoneally (*i.p.*) with 2.5x 10^6 Ehrlich ascites cells. However, the second one received oral daily ginger (100 mg /Kg body wt.) on day two of inoculating animals with Ehrlich ascites cells. Results revealed that various types of chromosomal aberrations in Ehrlich ascites cells were detected. These aberrations were manifested in either numerical or structural aberrations. Ehrlich ascites cells contain different number of chromosomes ranging form 26 to 125 with an increase in micronuclei cells and incidence of mitotic index**.** In addition, a reduction in micronuclei cells and mitotic index in Ehrlich ascites cells were detected. Also, a reduction in chromosomal aberration of Ehrlich ascites cells was achiveved. The reduction of abnormalities in tumor cells by the extracts may stimulate the cells to divide normally or go to die (through apoptosis) if they cannot remove chromosomal abnormalities. It was concluded that ginger extract may have a chemotherapeutic effect on Ehrlich ascites cells. The extract greatly changed tumor cells to diploid normal cells. The use of dietary agents such as ginger may have potency for the treatment and prevention of cancer. [Journal of American Science 2010; 6(8):525-539]. (ISSN: 1545-1003).

Key words: *Zingiber officinale*, Ehrlich ascites cell, chromosomal aberration, micronuclei cells.

1. Introduction:

Epidemiologic evidence suggested that regular consumption of fruits, vegetables, and whole grains may reduce cancer risk in some individuals. This association has been attributed to these foods being rich sources of numerous bioactive compounds (Milner,2004). Bioactive components present in fruits and vegetables can prevent carcinogenesis by blocking metabolic activation, by increasing detoxification, or by providing alternative targets for electrophilic metabolites. Numerous constituents of plant foods, including flavonoids (such as quercetin, rutin, and genistein), phenols (such as curcumin, epigallocatin-3-gallate and resveratrol), isothiocyanates, allyl sulfur compounds, indoles, and selenium have been found to be potent modulators of detoxification enzymes *in vitro* and in preclinical models (Milner ,2001 and Keum *et al.*, 2004).

The effect of plant extracts as antitumors was widely studied due to their low toxicity and side effect. The inhibition of ascites tumor cells by garlic extracts was investigated (Aboul-Enein, 1986). Soybean seed extracts showed antitumor activity which is due to the presence of trypsin inhibitor (Aboul-Enein *et al.*, 1986). The tumor inhibitors of plant origin depend upon the type of cancer cells and plant species as well as the extract used. Different plant species (from eight families) growing in Egypt showed anticancer activity (EL-Mrezabani, *et al.*, 1979a). The principles separated from these plants were also studied such as alkaloids (EL-Mrezabani, *et al.*, 1979b; Pokorny, *et al.*, 1983), terpenes (Nozaki, *et al.*, 1990), flavonoids (Hirano, *et al.*, 1994 and Duthie, *et al.*, 1997), or chlorophyll (Sarkar, *et al.*, 1996).

The metaphase of tumor cells was highly arrested by vincristine and vinblastine as alkaloids which have antimitotic effect (EL-Mrezabani, *et al.*, 1979b).The abnormalities in chromosomes of tumor cells such as sister chromatid change and aberrations (Duthie, *et al.*, 1997;Gonalez, *et al.*, 1997) and DNA fragmentation (Royman and Ruddon, 1995) by natural extracts were illustrated. The induction of apoptosis in the cancer cells by natural plant extracts was studied by (Mapara, et al., 1993; Neubauer, et al., 1996; Mehta, et al., 1997; Pirianov, et al., 1998 and Filion, et al., 1998).

Mutagenicity, clastogenicity, cytotoxicity and carcinogenicity are inhibited by antioxidant compounds found in abundance in plants (Hochstein and Atallah, 1988). Most chemopreventive compounds and their analogs or derivatives are initially of plant origin and inhibit spontaneous and

chemical mutagenesis in a variety of in vitro and in vivo test systems (Xifeng *et al.*, 2007).

Many herbs and spices are known to possess an array of biochemical and pharmacological activities including antioxidant and anti-inflammatory properties that were believed to contribute to their anticarcinogenic and antimutagenic activities. Tumor promotion is closely linked to inflammation and oxidative stress; so compounds that exhibit anti inflammatory and/or antioxidant properties could acts as anticarcinogenic agent (Masuda et al. 2004).Some phenolic substances present in ginger(Zingiber officinale Roscoe, Zingiberaceae), generally, possess strong anti-inflammatory and anti-oxidative properties and exert substantial anti-carcinogenic and anti-mutagenic activities (Surh, 2002 Bode, 2003; Kim *et al.*,2005a and Vijayapadma *et al.*, 2007).

This study amid to evaluate the effect of ginger extract on characteristics of ascitic Ehrlich tumor cells by using chromosome aberration assay, mitotic index and micronucleus test.

2. Materials and Methods:

Preparation of plant extract

It is conducted according to Mothana et al (2009) where air-dried and powdered plant materials (10 g) were extracted with 400 ml methanol (CH_3OH) by Soxhlet extraction for 8 hours. Residue was dried overnight and then extracted with 250 ml water (H_2O) by using a shaking water-bath at 70°C for 2 hours. The obtained methanolic and water extracts were filtered and evaporated by using a rotary evaporator and freeze dryer. The dried extracts were stored at -20°C until used .

Tumor Cell Line

A line of Ehrlich ascites carcinoma (EAC) obtained from Egyptian National Cancer institute, Cairo University. The parent line was supplied through the courtesy of Dr. Gklein , Amsterdam, Holland. The tumor line was maintained in female Swiss albino mice by weekly interperitoneal injection of 2.5x 10^6 cells/mouse according to the method recommended by the Egyptian National Cancer institute, Cairo university.

Such developed tumor is characterized by its moderate rapid growth which could not kill the animal due to the accumulation of ascites before injection using the bright line haemocytometer and dilutions were made by physiological saline and desired numbers of cells were injected in a volume of 0.5 ml.

Animals:

Eighty female mice (18-20 g.) were obtained from experimental animal house found in National Research Center, Cairo, Egypt. Animals were kept under normal healthy laboratory conditions. Inbred colony was used in the present study. Animals were provided with standard rat feed (procured from Animal Nutrient Co., Cairo) with water *ad libitum* and were maintained under controlled conditions of temperature and light (Light: dark, 10 hrs: 14 hrs.). Five animals were housed in a polypropylene cage with locally procured paddy husk (Oryza sativa) as bedding throughout the experiment. Tetracycline-containing water (0.13 mg/ml) was provided once a fortnight and was given as a preventive measure against infections. Animal care and handling were performed according to the guidelines set by the World Health Organization (WHO), Geneva, Switzerland and the ETC (Ethical Committee National Research Center), Cairo, Egypt.

Experimental Design:

Mice were divided into two groups:

1-Ehrlich group: mice were intraperitoneally (*i.p.*) inoculated with 2.5x 10^6 Ehrlich ascites cells.

2-Ehrlich and ginger group: animal received oral daily ginger (100 mg /Kg body wt.) on day two after inoculation with Ehrlich ascites cells.

Collection and preparation of Ehrlich ascites cells

After 7 and 14 days of tumor implantation, animals were necropsied. Ascitic liquid was collected in order to chromosome preparation and micronucleated cells. Smears were carried out from the cell suspension obtained from each animal and then they were submitted to Giemsa stain in order to determine mitotic index.

Chromosome preparation: was conducted according to Evans (198)7:

Animals were sacrificed and Ehrlich ascites cells were collected. Ehrlich ascites cells was subjected to colchicine treatment (0.5 solution, 0.1 ml /culture), hypotonic treatment (KCl, 5.6 g/l), fixed in acetomethanol, spread and stained with Giemsa stain

Mitotic Index:

1000 cells per animal were counted and the number of dividing cells, including prophases and metaphases, was determined.

The micronucleus test: (according to the method of Salamone *et al*. 1980).

Ascitic liquid was collected from five animals of both groups and smear preparations were made by using fetal calf serum.

Statistical analysis:

Obtained data were subjected to analysis of PRIMER Ver 5.0 according to Bary-curtis Similarity Inedex. and ANOVA-test according to Snedecor & Cochran (1980) at probability 0.01

3. Results and Discussion:

Chromosome number aberrations

As shows in Table (1); on day 7& 14; in Ehrlich group, Ehrlich ascites cells contain different number of chromosomes ranging form 26 to 125 chromosomes[Plate 1,2&3].. The stem cells of this tumor had 46 rod –shaped chromosome [Plate3 (a)]. In Ehrlich and ginger group, ginger extract significant decrease the variations of chromosome number in Ehrlich ascites tumor cells. The ginger extract a significant increase diploid Ehrlich ascites cells (46 chromosomes).

Structural Chromosomal aberrations

Data cited in table (3) show the percentage of the Structural chromosome aberration in diploid Ehrlich ascites cells (46 chromosomes) on day 14. These aberrations included breaks, deletions, rings, end to end association, centric fusion and fragment [Plate4&5]. Centric fusion type observed in Ehrlich group with percentage more than all types of chromosome aberration (25%). Addition of ginger extract reduced chromosome aberration in Ehrlich ascites cells. Centric fusion type highly decreases in Ehrlich ascites cells treated with ginger extract (8%). Also the percentage of other type of chromosome aberration were decreased except in break type weree increased (Fig. 3).

Mitotic Index

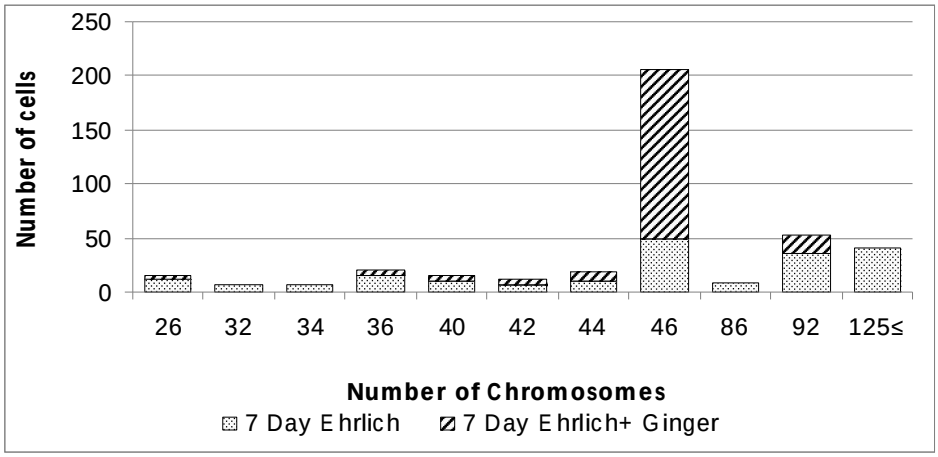
Cytological studies using Giemsa staining methods revealed a significant increase in number of mitotic cells in Ehrlich ascites cells at 7 and 14 days. While ginger extract induces significant decrease in mitotic cells in Ehrlich ascites tumor cells (Table 4& **Fig. 4**).

The micronucleus test:

On analyzing the frequency of micronucleated cells in Ehrlich ascites tumor cells (Table 4), it was found that significant increase in the frequency of micronucleated cells in Ehrlich group. When Ehrlich ascites cells treated with ginger extrac significant decrease in the micronucleated cells was observed (Fig. 5).

Table (1): Distribution of chromosome numbers in Ehrlich ascites cells treated with orwithout ginger extract after 7 and 14days

Groups		number of chromosomes										
		26	32	34	36	40	42	44	46	86	92	125≤
Ehrlich 7days	total	12	6	6	16	10	6	10	50	8	36	40
	%	6	3	3	8	5	3	5	25	4	18	20
Ehrlich+	total	4	0	0	4	6	6	8	156	0	16	0
Ginger 7days	%	2	0	0	2	3	3	4	78	0	8	0
Ehrlich 14days	total	6	3	5	7	30	0	19	117	0	13	0
	%	3	1.5	2.5	3.5	15	0	9.5	58.5	0	6.5	0
Ehrlich+	total	5	0	0	6	8	0	8	164	0	9	0
Ginger 14days	%	2.5	0	0	3	4	0	4	82	0	4.5	0



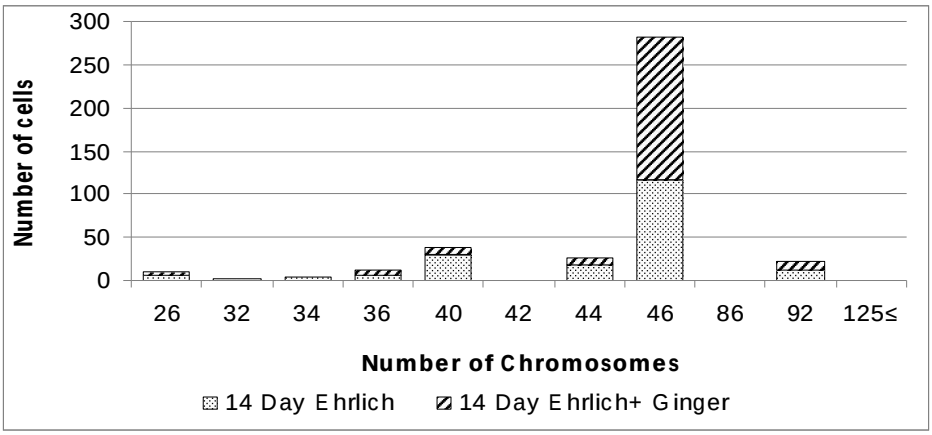


Fig. 1: Distribution of chromosome number in Ehrlich ascites cells treated with ginger extract after 7 and 14days.

Table (2): Distribution of chromosome number in Ehrlich ascites cells (normal and abnormal) treated with or without ginger extract after 14days

Groups	number of chromosomes							
		26	32	34	36	40	44	46	92
Ehrlich 14days	normal	5	2	3	3	17	13	54	4
	abnormal	1	1	2	4	13	6	63	9
	total	6	3	5	7	30	19	117	13
	%	3	1.5	2.5	3.5	15	9.5	58.5	6.5
Ehrlich+ Ginger 14days	normal	4	0	0	3	6	5	120	4
	abnormal	1	0	0	3	2	3	44	5
	total	5	0	0	6	8	8	164	9
	%	2.5	0	0	3	4	4	82	4.5

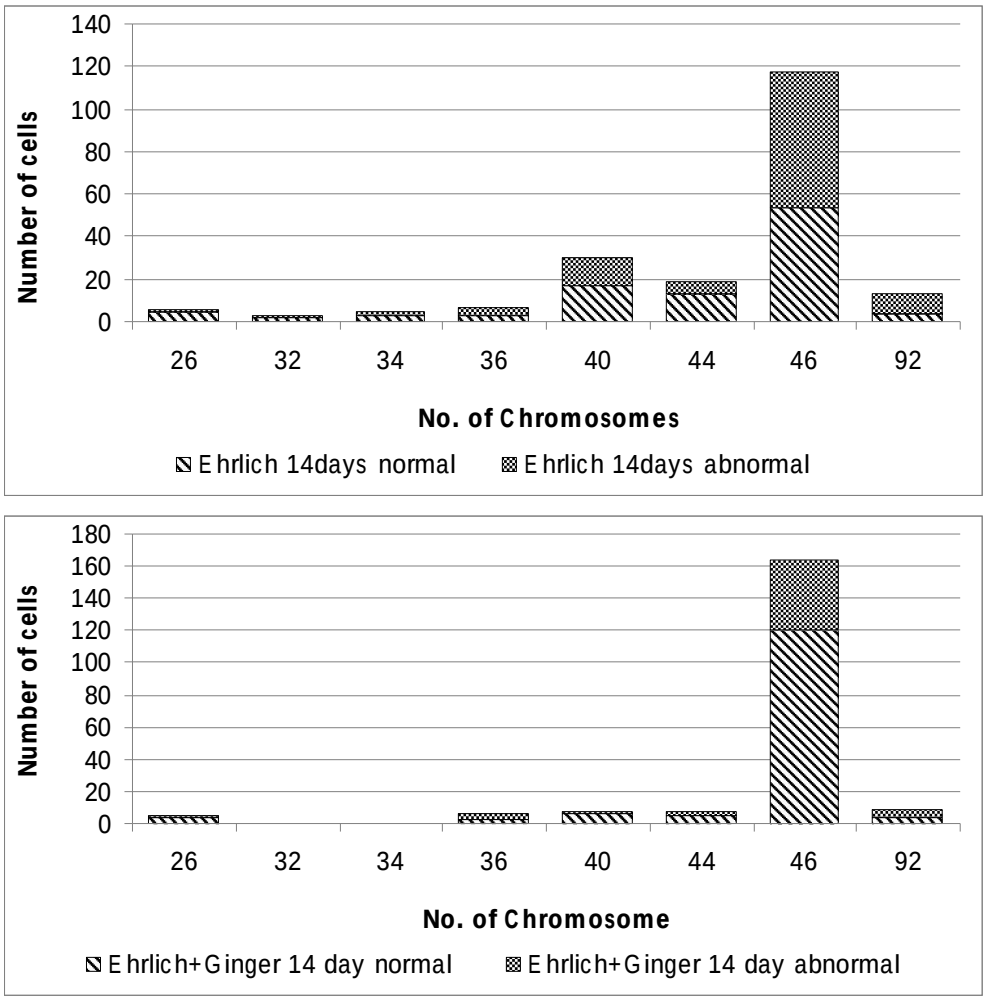


Fig. 2: Distribution of chromosome number in Ehrlich ascites cells (normal and abnormal) treated with or without ginger extract after 14days

Table 3: Structural chromosomal aberration in diploid Ehrlich ascites cells (46 chromosomes) treated with or without Ginger extract.

Groups	Abnormal cells							Normal cell
	Structural chromosomal aberrations							
	Break	Deletion	Ring	End to End association	Centric fusion	fragment	More aberrations in one cell	
Ehrlich (14 days)	18	8	4	2	50	14	12	92
	9%	4%	2%	1%	25%	7%	6%	46%
Ehrlich+ Ginger(14 days)	22	2	2	2	16	2	8	146
	11%	1%	1%	1%	8%	1%	4%	73%

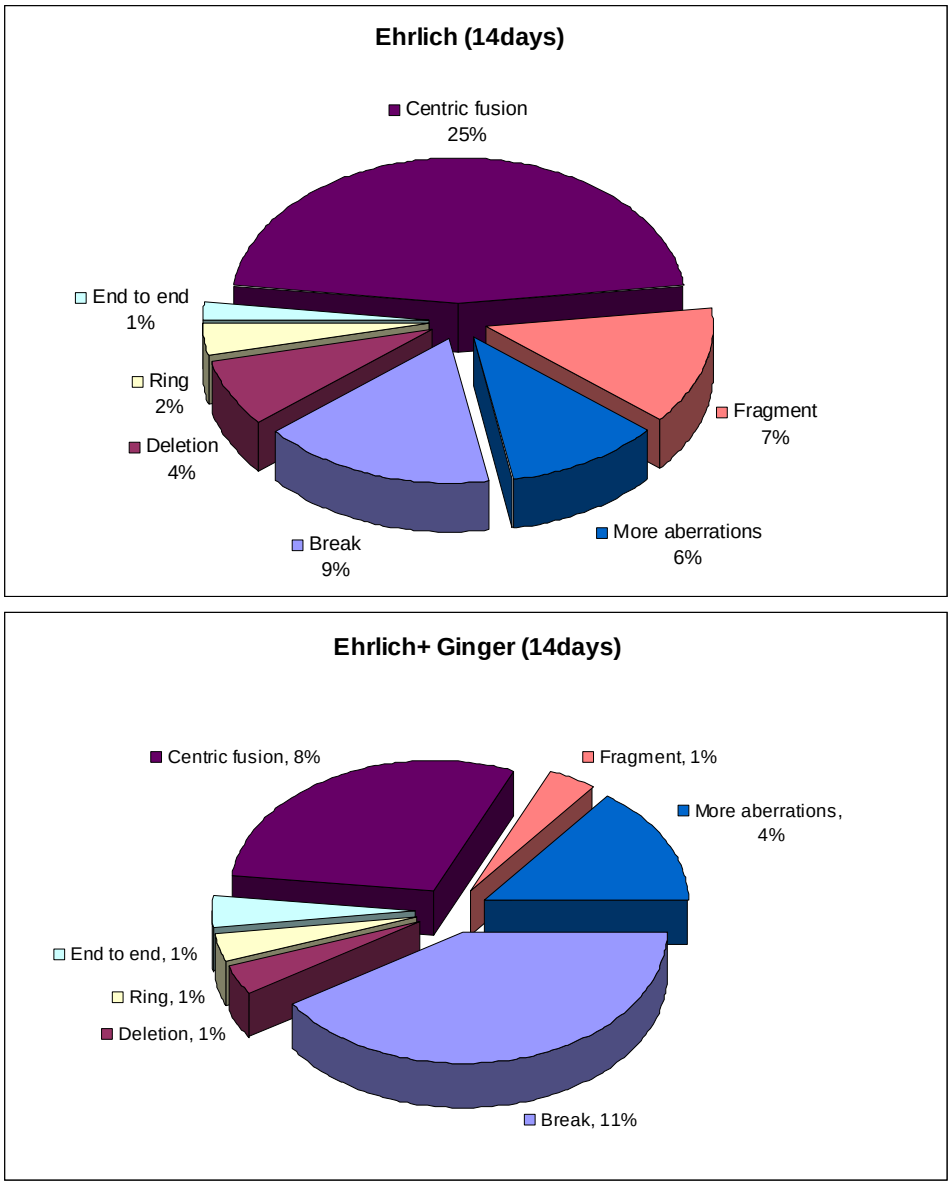


Fig. 3: Structural Chromosomal aberration in diploid Ehrlich ascites cells (46chromosomes) treated with or without Ginger extract after 14days

Table 4: Mean of mitotic index and micronuclei cell in Ehrlich ascitic tumor cells treated with or without Ginger extract after 7 and 14days.

Groups	Mean	
	Mitotic index	Micronuclei cell
Ehrlich (7days)	216	78
Ehrlich +Ginger (7days)	33	19
Ehrlich (14days)	238	91
Ehrlich +Ginger (14days)	74	23

Each point on the chart is 1000 cell counted from each animal.

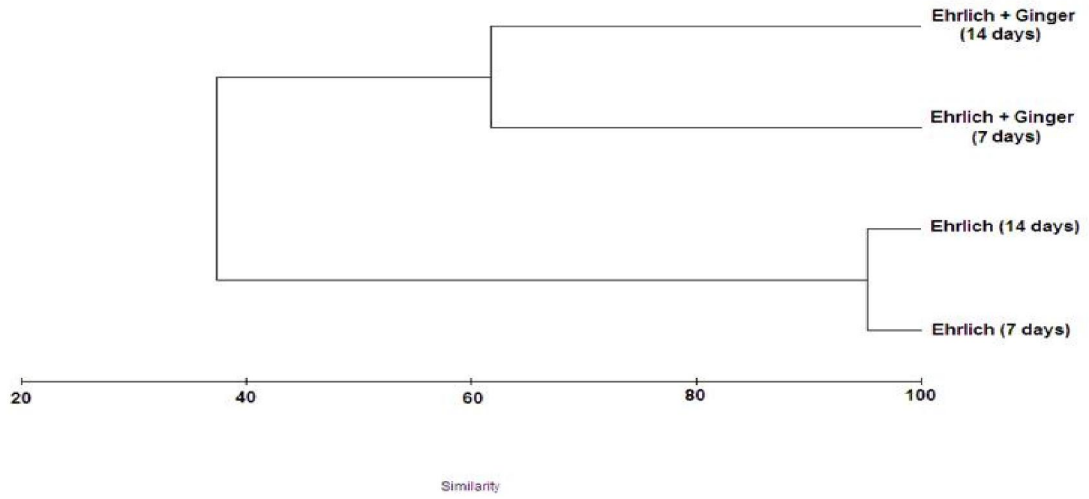


Figure 4: Dendrogram represent similarity of mitotic index between different groups after 7 and 14 days

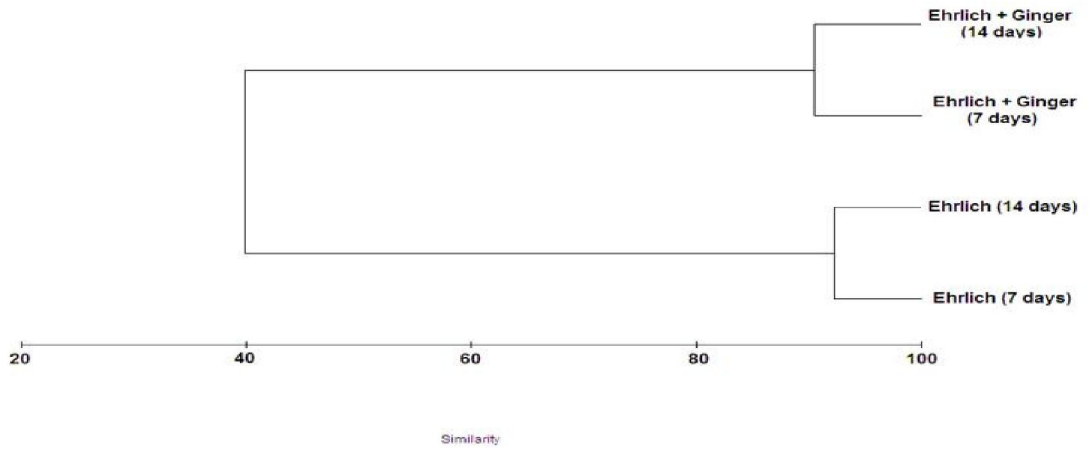
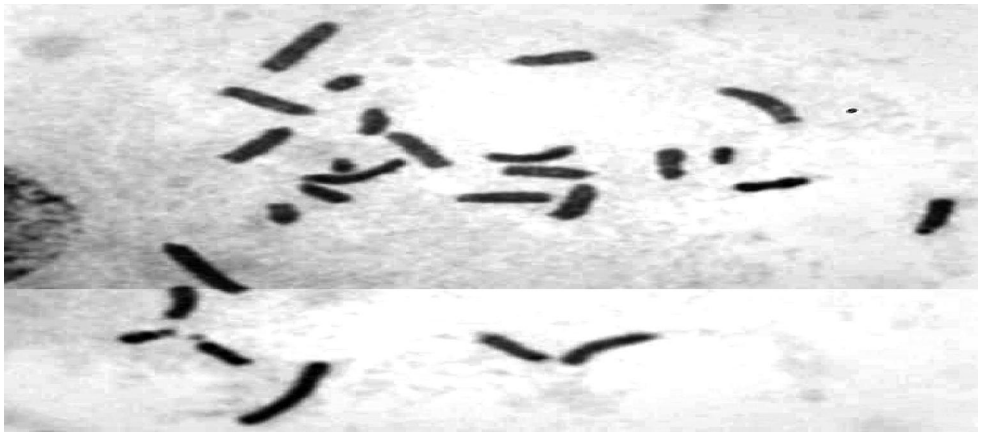
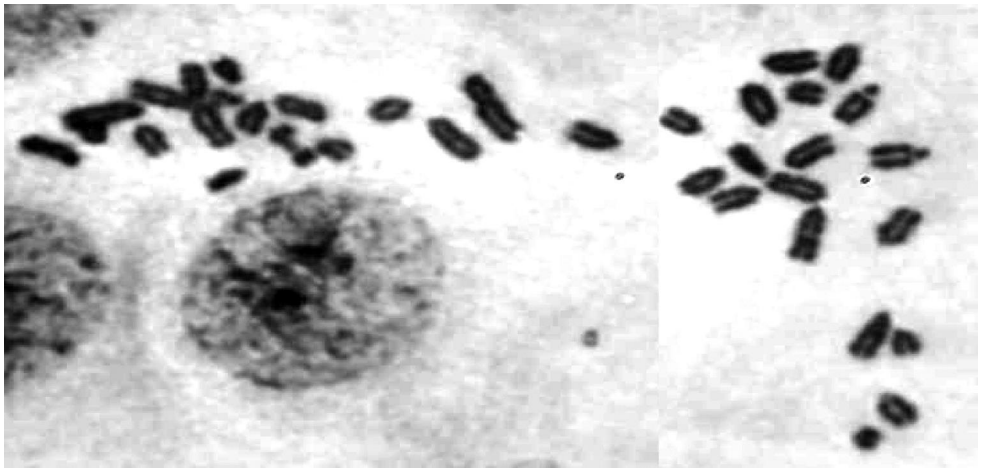


Figure 5: Dendrogram represent similarity of micronuclei cell between different groups after 7 and 14 days



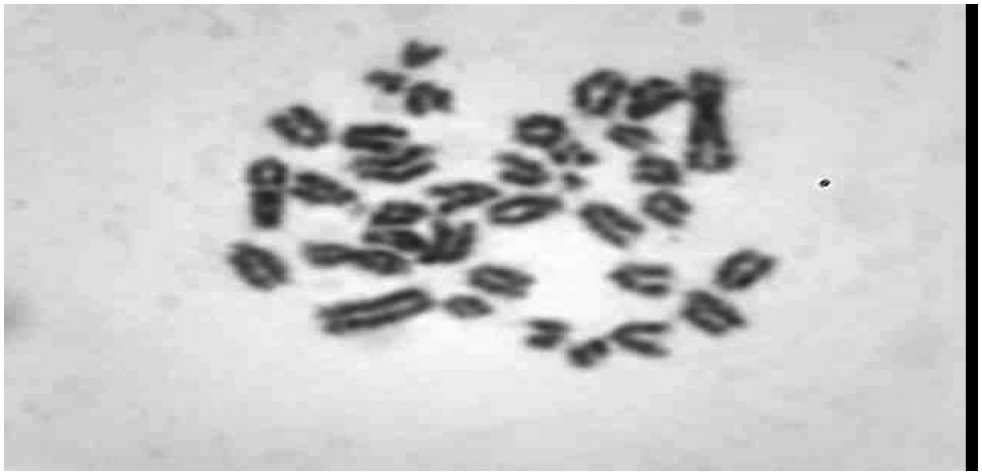
a) **26 Chromosomes**



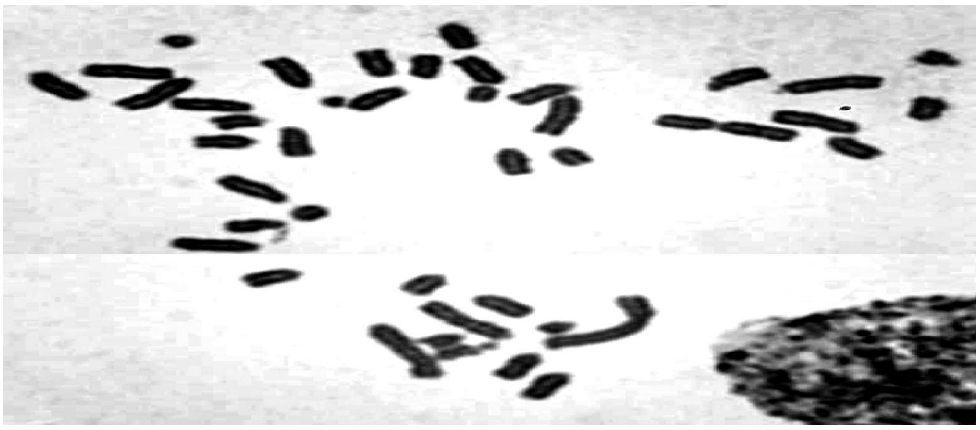
b) **34 Chromosomes**

c) **36Chromosomes**

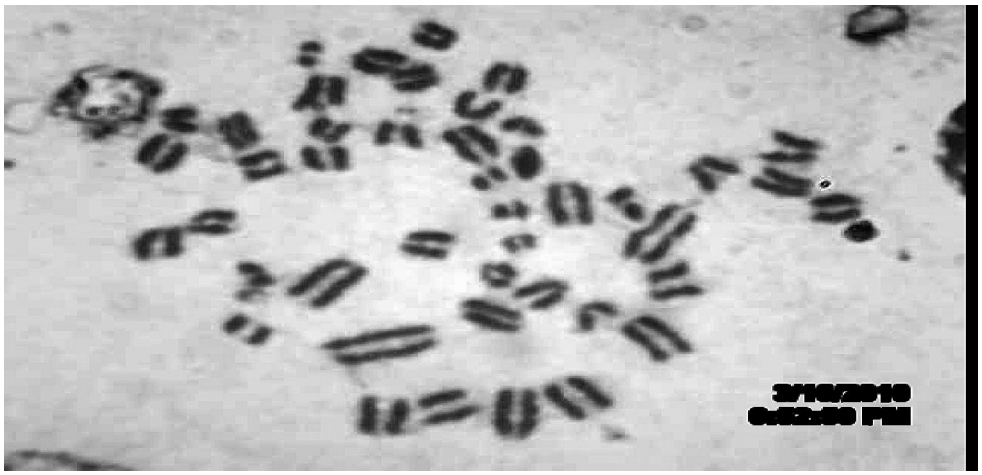
Plate (1): Metaphases showing different chromosome number in Ehrlich ascites cells



a) **40 Chromosomes**



b) **42Chromosomes**



c) **44Chromosomes**

Plate (2): Metaphases showing different chromosome number in Ehrlich ascites cells

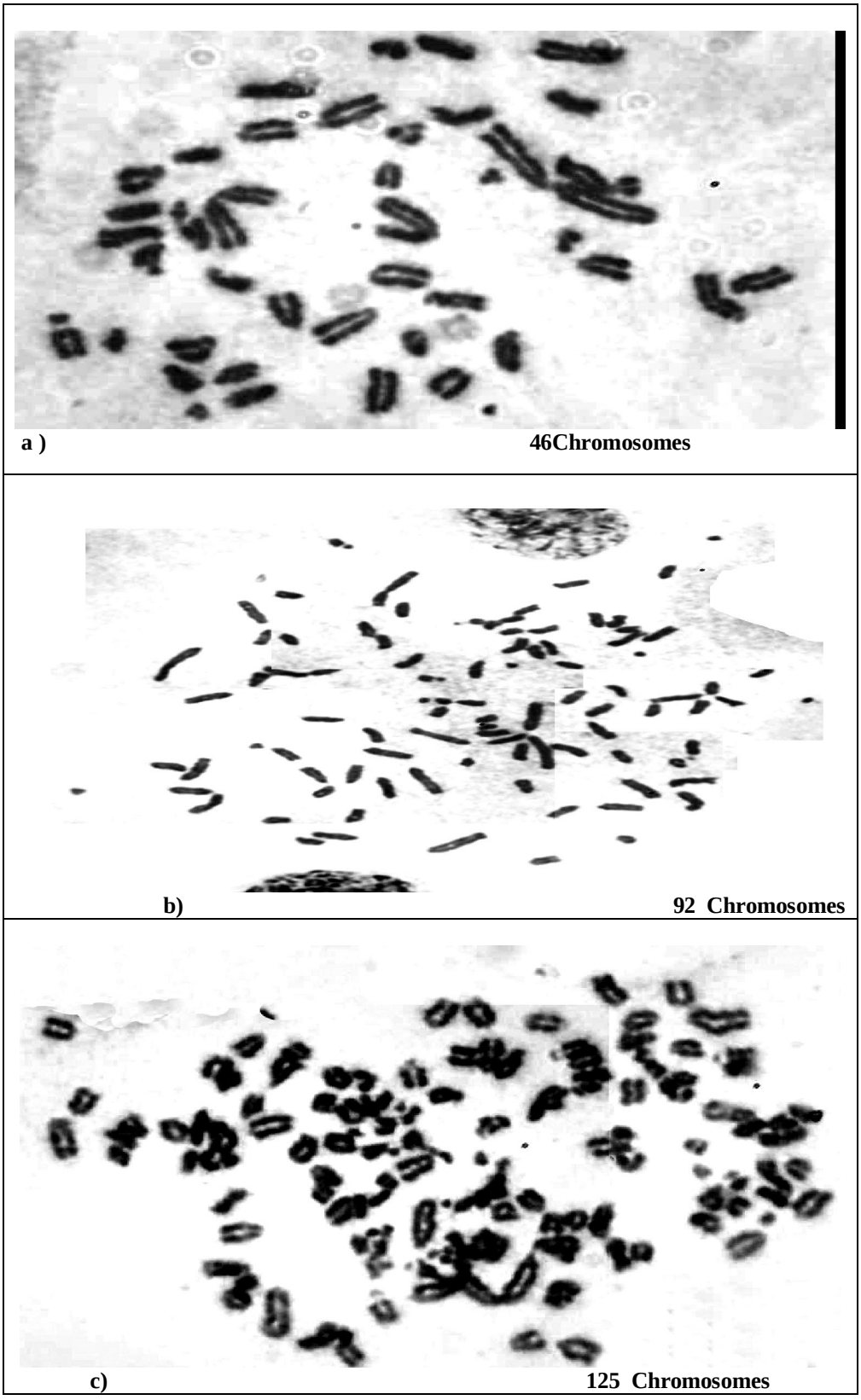
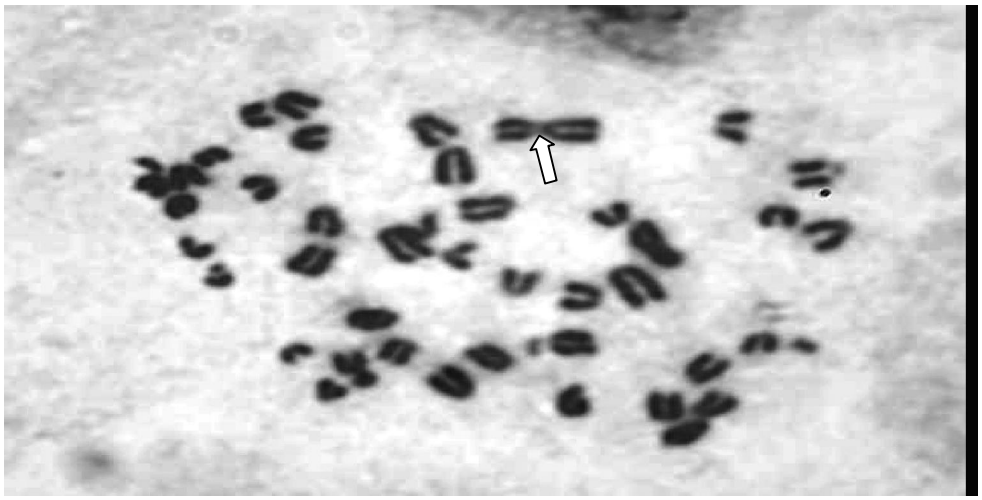
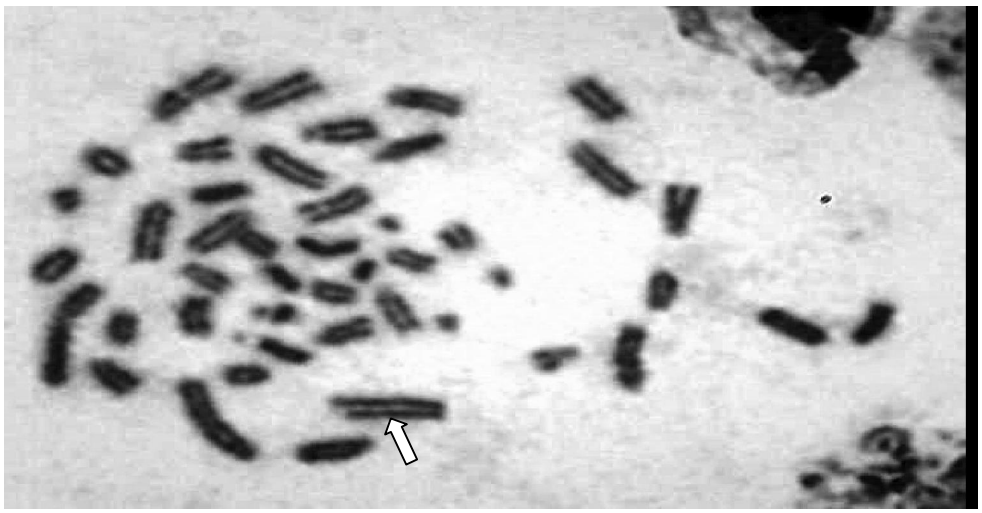


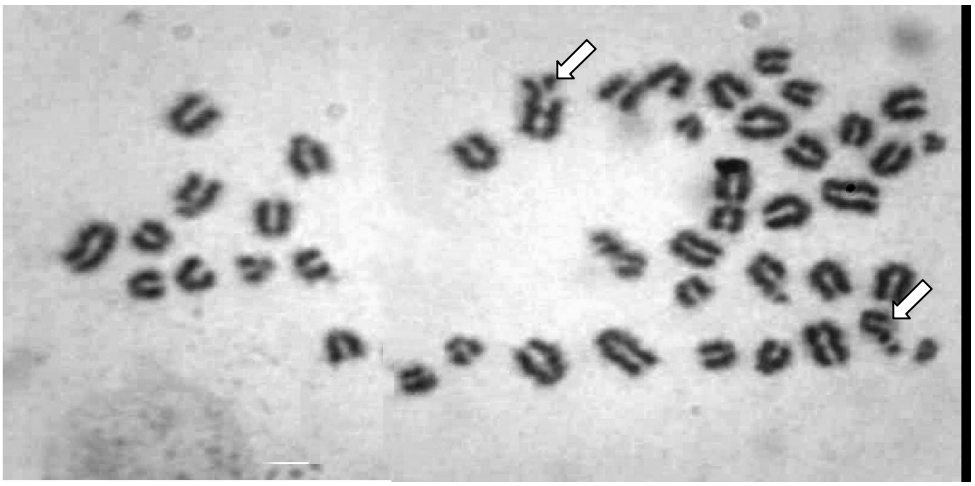
Plate (3): Metaphases showing different chromosome number in Ehrlich ascites cells



a) Centric fusion

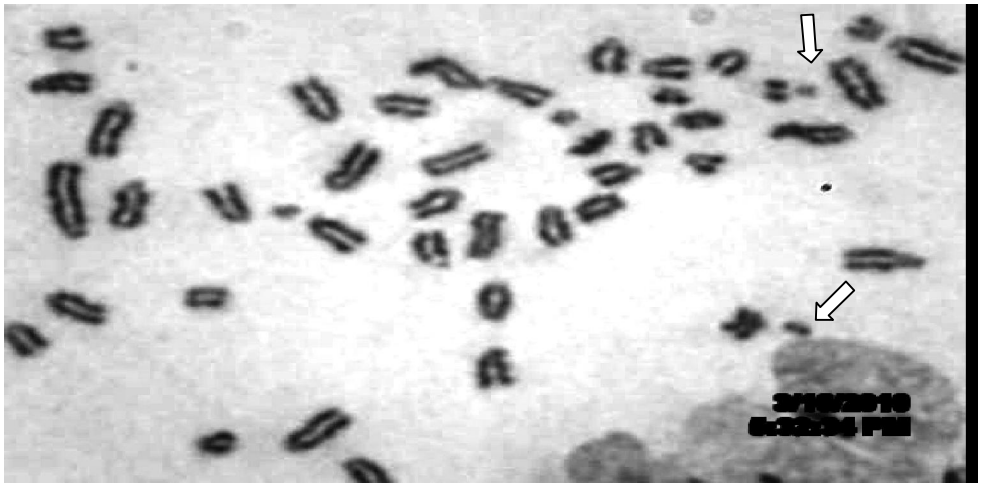


b) Deletion

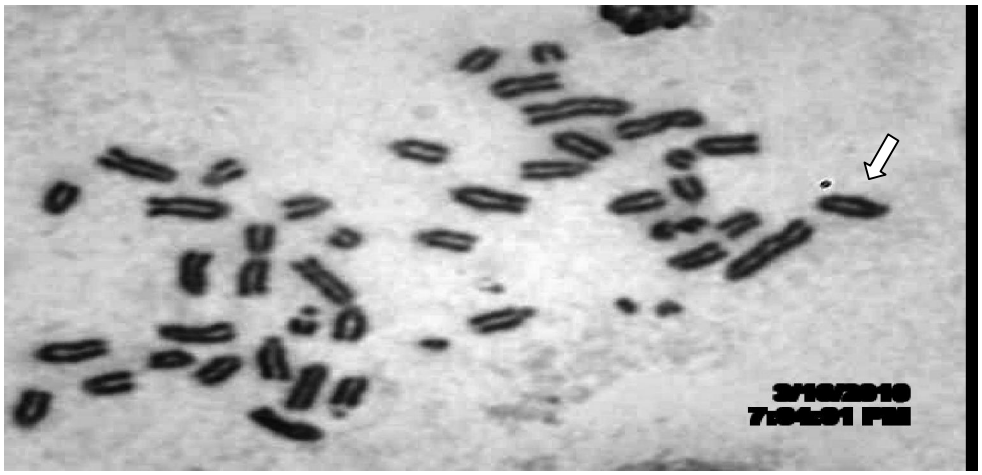


c) Break

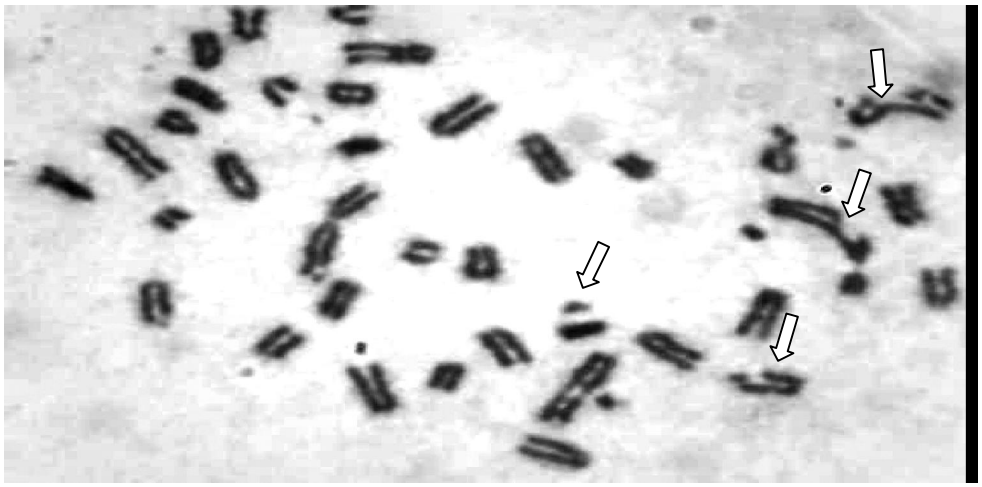
Plate (4): Ehrlich ascites cells metaphases showing, structural chromosomal aberrations



a) Fragment



b) Ring



c) Break, end to end association and fragment

Plate (5): Ehrlich ascites cells metaphases showing, structural chromosomal aberrations

4. Discussion:

Ginger has long been used in traditional medicine as a cure for some diseases including inflammatory diseases (Afzal *et al.,* 2001). Ginger contains active phenolic compounds such as gingerol, paradol and shogoal that have antioxidant (Jeyakumar *et al*., 1999), anti-cancer (Shukla and Singh 2007), anti-inflammatory (Hudson *et al.,* 2006), anti-angiogenesis (Huang *etal*., 2000) and anti-artherosclerotic properties (Coppola and Novo 2007). It has also been shown to down-regulate NF-κB-regulated gene products involved in cellular proliferation and angiogenesis, including IL-8 (Nonn *etal*., 2007).

The present study, showed an increase in frequency of aberrant cell and micronuclei cells. Various pictures of chromosomal aberrations appeared in Ehrlich ascites cells. These aberrations were manifested in numerical (haploid and polyploidy) and structural aberrations. Treatment with ginger extract reduced aberrant cells and micronuclei cells. Also ginger extract greatly changed the polyploidy and haploid of tumor cells to diploid (normal morphology). The reduction of abnormalities in tumor cells by the extracts may stimulate cells to divide normally or go to die (through apoptosis) if they cannot remove chromosomal abnormalities. These results confirm those obtained by Vijayapadma, et al (2007), which show that saline extract prepared from ginger extract caused suppression of cell proliferation and marked morphological changes including cell shrinkage and condensation of chromosomes. This is attributed to its anticancer properties due to presence of certain pungent vallinoids, viz. [6]-gingerol and [6]-paradol, as well as some other constituents like shogaols, zingerone etc in its structure (Shukla and Singh 2007).

Results obtained in the present study demonstrate that ginger extract can reduce the mitotic index in Ehrlich ascites cells. These results may be attributed to that gingerdione is one of the components from ginger that has been demonstrated to be an effective anti-tumor agent in human leukemia cells (Hsu, et al., 2005). Also, gingerdione induces G1 arrest in human leukemia HL 60 cells. B-Elemene is a novel anticancer drug, which is extracted from the ginger plant. It triggers apoptosis in non-small-cell lung cancer cells through a mitochondrial release of the cytochrome c-mediated apoptotic pathway (Wang *et al.,* 2005). Also, 6-shogaol exhibited the most potent cytotoxicity against human A549, SK-OV-3, SK-MEL-2, and HCT15 tumor cells (Kim *et al*., 2008).

6-shogaol inhibited proliferation of the transgenic mouse ovarian cancer cell lines. In addition 6-gingerol has two types of antitumor effects: 1) direct colon cancer cell growth suppression, and 2) inhibition of the blood supply of the tumor via angiogenesis (Brown *et al*,. 2009).

5. Conclusion:

Ginger extract may have a chemotherapeutic effect on Ehrlich ascites cells. It is greatly changes tumor cells to diploid normal cells .The use of dietary agents such as ginger may have potential in the treatment and prevention of cancer.

Color evaluation showed significant differences between Egyptian Gouda cheese related to ripening time, although due to the similar structure the cheeses, the more identical values were found for Water activity and sensory evaluation in Egyptian Gouda cheese.

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Detecting Municipal Solid Waste Leachate Plumes Through Electrical Resistivity Survey And Physio-Chemical Analysis Of Groundwater Samples

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Abstract: A Direct Current electrical resistivity survey was carried out in Unguwan Dosa open dumpsite in Kaduna metropolis, North Western Nigeria. The dumpsite is the typical non-controlled waste facility that lack bottom liner. 8 vertical electrical soundings (VES) employing the Schlumberger electrode array were conducted with maximum electrode spacing of 100 m. Interpreted resistivities were obtained by iterative computer modeling of the apparent resistivity data. The VES data were plotted as pseudo and resistivity cross-sections in order to look at the spatial distribution of the contaminant plumes. The interpreted VES data measured inside the dumpsite showed contamination plumes as low zones with resistivity values ranging between 1 and 12.9 ohm-m extending from the surface down to the aquifer of shallow groundwater of less than 5 m. Calculated hydraulic conductivity ranges between 5.9×10^{-2} m/s and 3.7 m/s for shallow subsurface layers of interpreted VES points located inside and outside the dumpsite. This moderately conductivity value of the subsurface materials is believed to facilitate movement of the leachate plume through the soils and migration of the contaminants outside the dump and into the shallow aquifer in the study area. Elevation in concentrations of the measured parameters of the physio-chemical analysis of water samples from existing hand dug wells indicate contamination of the groundwater as a result of solid waste leachate accumulation, consequently, complimenting the geophysical data.
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Keywords: Open dump; electrical resistivity; Schlumberger; leachate; contamination; physio- chemical

1. Introduction

Groundwater resources are very important for public water supply most especially in the arid regions. Solid wastes are being produced every day by urban societies and in an attempt to dispose these materials; man has carelessly polluted the environment. The problems associated with municipal non-controlled dumpsites are of general concern, especially because of the hydraulic contact between the hazardous contents of the leachate plume and the groundwater. Poor water quality is a significant problem in many parts of the world and Kaduna metropolis with about 342 unsanitary open dumpsite(Ministry of Environment and Natural resources, Kaduna state, personal communication, 2008) is not immune from this environmental problem. Generally, electrical resistivity method provides economic and non destructive means to identify and delineate leachate contaminant plumes from dumpsite because the electrical conductivity of leachate tends to be higher than that of natural water. This is as a result of the leachate diminishing the electrical resistivity of the formation containing them (Martinho and Almeida, 2006). The use of resistivity method applied to landfills studies are well documented (Porsani et al., 2004; Karlik et al., 2000; Mukhtar et al., 2000;).The objective of the present work is to apply the Electrical resistivity technique to detect and delineate leachate plume from non-controlled solid waste dumpsite in Kaduna municipal, North- West, Nigeria. The addition of physio-chemical analysis of water sample is to compliment the results of the geophysical data and measure the level of accumulation of the contaminants.

2. Material and Methods.

Geologic and Hydrogeologic Settings of the study area

The survey area (Fig.1) is located on Abdullahi Bello road at co-ordinates $10^0 34^{''}$ N and $07^0 27^{''}$ E, in Unguwan Dosa, Kaduna North Local Government Area of Kaduna State, North West Nigeria. Geologically, the survey area lies entirely within the Basement Complex rocks of Northern Nigeria. These are undifferentiated basement rocks comprising mainly granites, gneisses, migmatite,

quartzite and amphibolites. These basement rocks are mechanically competent and therefore respond to imposed strains by brittle fracture. Surface water percolates down through the fractures and the process of chemical weathering proceeds. In general, gneiss and migmatite weather more easily than granite (Olorunfemi et al., 1991). However, the weathering products of the gneiss and migmatite are richer in clay minerals and hence less permeable with attendant lower groundwater yield. The top soil varies in composition, colour and texture and in most places they are predominantly clayey sand and quartz grains (deep brown or reddish brown soil). GSN Unpublished report No. 1539. The study area has a typical Savannah climate with distinct wet and dry seasons. The dry season normally begins in October and ends in April while the rainy season occurs between May and September. Average annual rainfall for Kaduna is 1270mm Eduvie (2003). Rainfall generally reaches a peak in August. Temperatures vary between less than $15^{o}C$ around December/January and $32^{o}C$ in March/April. Concerning groundwater occurrence, geophysical investigations and borehole drilling reports have identified two major aquifers. These are the overburden weathered aquifer (clayey sand/sand) and the fractured crystalline aquifer (Eduvie, 2003; Dan-Hassan and Olorunfemi, 1999). The overburden aquifers usually contain great quantity of water largely exploited by hand dug wells for domestic water supply. At some locations, these aquifers are interconnected and form a hydrogeological unit of water table surface.

Electrical resistivity survey and data processing

Eight Schlumberger vertical electrical soundings (VES) measurements were conducted with maximum electrode spacing (AB/2) of 100 m. Four of the VES points (1- 4) were located inside the dump. Based on the local groundwater flow and topography of the area (Fig 1), the remaining four (5-8) were located at the southern margin of the dump to observe any migration of the leachate outside the dump. The VES points were separated by 5 m intervals. The ABEM SAS 300C Terrameter was used to collect the VES data. Field resistivity structures of sounding data were determined by the software, IPIWIN (version 3.0.1) developed by the Geophysics Group Moscow State University for inverse interpretation. Data were interpreted in terms of three and four layer structure (Fig 2). The fit between model response and the field data for VES points outside the dump were generally lower than 10% while a maximum fit of 24. 4% was obtained for VES points located inside the dump. This high discrepancy is attributed to the large lateral variations between the materials on top of the dump and those surrounding the dump (Porsani et al., 2004). The interpretation of the VES curves aided by lithological logs from boreholes (Fig.3) enabled the derivation of maximum of four geologic sections. The topmost layer consists of clayey sand and lateritic clay. This formation is followed in succession by sandy clay/clayey sand/sand, weathered transitions zone/fractured layer, and the fresh basement. Qualitative interpretation indicates that the weathered/fractured basement constitutes the main aquifer unit. From the VES results, the depth of water table in the study area varies between 1.21 and 5.68 m. This is due to variations in the thickness of the weathered zone and intensity of weathering. The VES data were subsequently plotted as pseudo and resistivity cross-sections in order to look at the spatial distribution of the leachate plume (Figs 4 and 5).

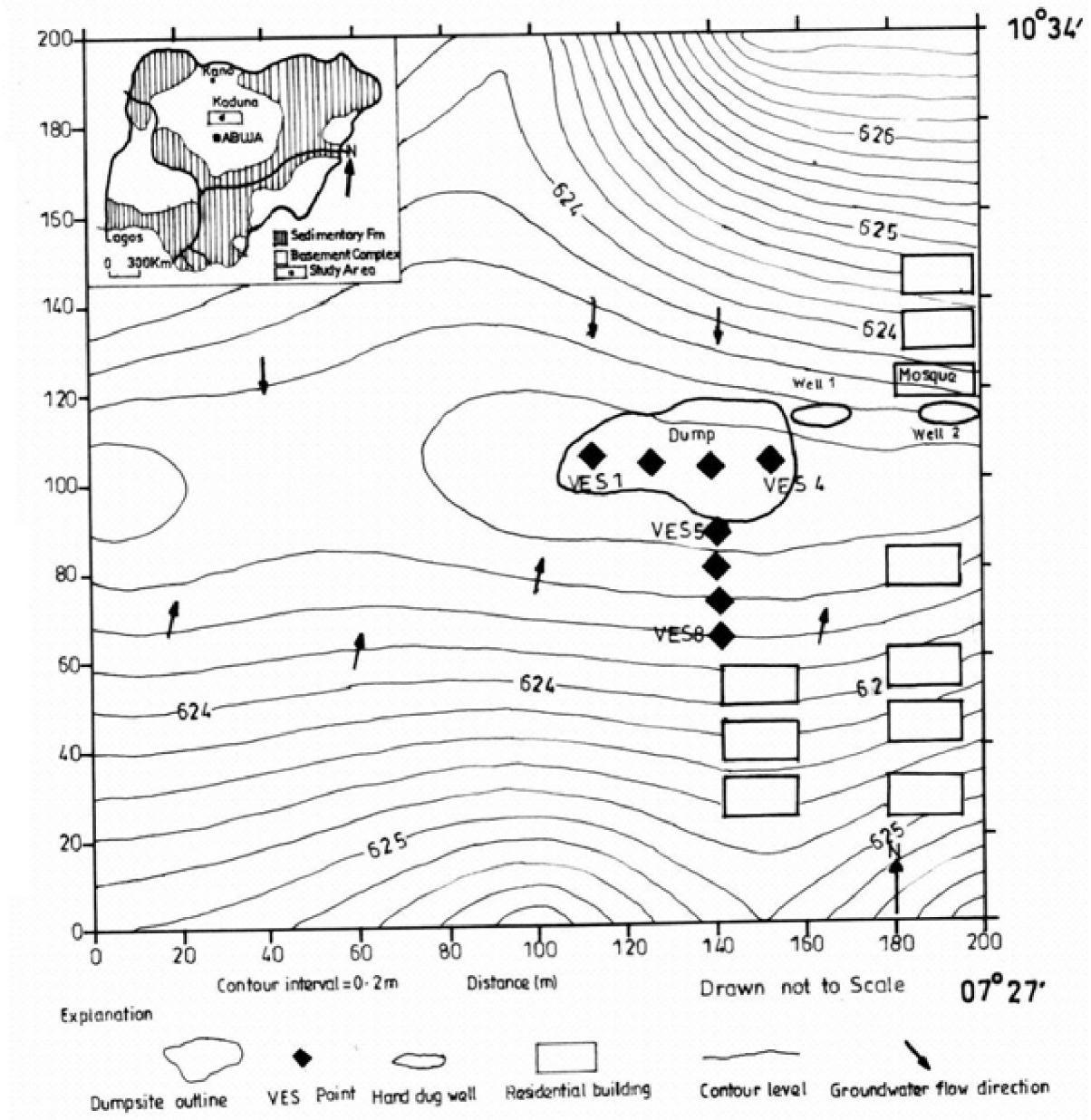

Fig.1: Genealised map of the study area(Modified after Osazuwa and Abdullahi,2008)

Physio- chemical survey

In order to assess the level of groundwater contamination by the solid waste leachate, water quality analysis was conducted on water samples from two hand dug wells, one located 2 m from the dumpsite(Named well1) and the other at 16 m from the dumpsite near a mosque (Named well 2). The two hand dugs are located at the northern margin of the dump and the static water level of the hand dug wells are 2.55m for well 1 and 2.44m for well 2.The water samples were analysed for physical parameters{Temperature, pH, Conductivity, Biochemical oxygen demand (BOD), Chemical oxygen demand (COD), Total dissolved solids (TDS), and Chloride (Cl^-)} at the Water Resources Department, Ahmadu Bello University, Zaria while the chemical analysis was conducted for five (5) trace elements at National Research Institute for Chemical Technology (NARICT), Basawa, Zaria. Water samples collected were stored in well-drained clean polythene bottles already rinsed out with same water samples from each hand dug well. The water samples collected for chemical analysis were acidified with

dilute nitric acid and stored in a refrigerator prior to analysis. Analysis of all the physical parameters were done using the various standard methods for water analysis (APHA, AWWA & WPC, 1985) while preserved water samples were analysed for cations and anions using Atomic Absorption Spectrometer (AAS) method.. The results of the physio-chemical analysis are presented in table1.

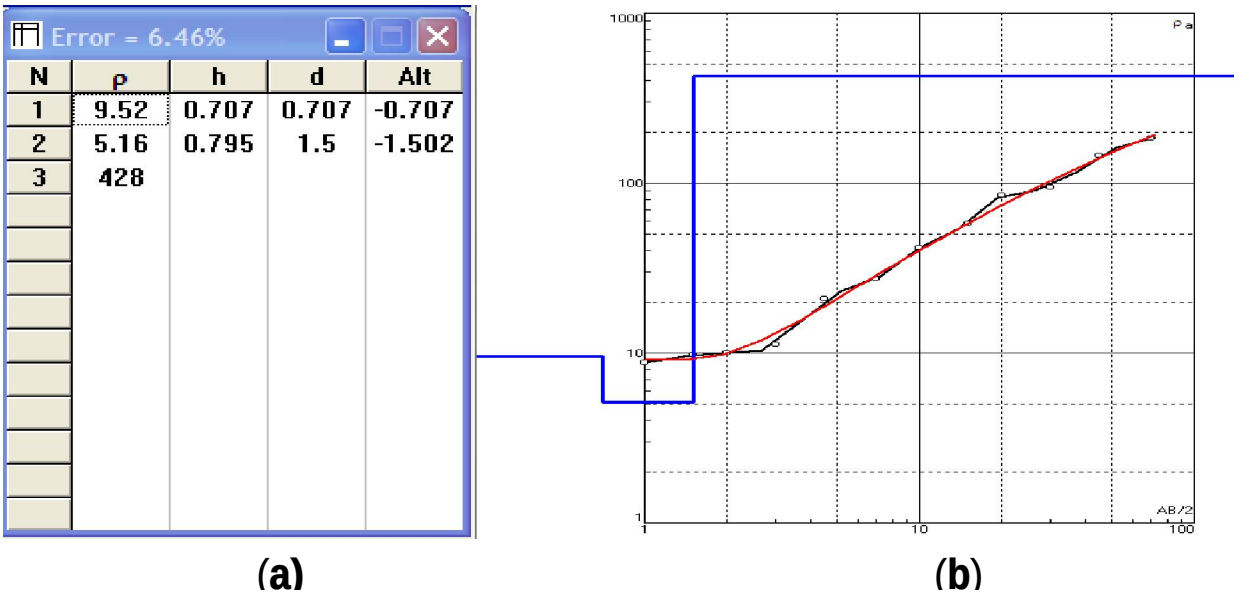

Fig 2: (a) Model description and (b) DC Curve interpretation for VES 1

Table 1. Physio-chemical analysis of Hand dug wells

Parameter	Unit	Well 1 (Dump)	Well 2 (Mosque)	WHO (1992)
Conductivity	µs	1024	283	100
pH		6.63	6.72	6.5-8.5
TDS	mg/l	1000	400	500
COD	mg/l	902	742	80.0
BOD	mg/l	480	395	<40
Chloride	mg/l	433	298	250
Chromium	mg/l	0.4970	0.1778	0.0500
Cadmium	mg/l	0.0330	0.0270	0.0030
Lead	mg/l	0.929	0.0618	0.0100
Iron	mg/l	1.5561	0.7754	0.3000

3. Results

Figs 4a and 4b show the pseudo and the resistivity cross-sections for VES points located inside the dump (VES 1 – 4). The pseudo cross-section (Fig 4a) shows a low resistivity zone (1 – 12.9 ohm-m) for AB/2 spacing of 1- 4.5 m extending vertically at VES 3 and narrowing laterally at VES 4. This is attributed to contamination of the top soil as a result of accumulation of leachate. The lower limit (black color scaling /2 ohm-m) established around VES 3 indicates that most of the waste are dumped there. A horizontal horizon made up of green, grey, yellow and pink colour scaling observed at AB/2 spacing of 4.5 m - 15 m (equivalent to maximum depth of 6 m) is the water bearing zone. The resistivity of this layer depends on the sand to clay ratio and saturation,

hence the wide range of colours. The low resistivity end (< 80 ohm-m) could be attributed to contamination of the groundwater as a result of invasion of the leachate. The resistivity cross – sections (Fig 4b) revealed a sequence of three geo-electric sections of H (VES 3) and A (VES1, 2 & 4) types. The low resistivity value of the second layer at VES 3 is an indication of downward migration of the leachate. The bedrock has a resistivity that exceeds 1000 ohm-m but where it is fractured and saturated (VES2); the resistivity reduces to less than 1000 ohm-m (Olayinka and Olorunfemi, 1992). The fractured zone constitutes a major aquifer component in a Basement Complex area. The hydraulic conductivity of the first and second layer was estimated in order to provide an insight into the mode of contaminant movement in the formations. Sing (2005) established a non–linear relationship between hydraulic conductivity (K) and apparent resistivity (ρ) given by $K = 0.0538e^{-0.0072\rho}$ where ρ equals the apparent resistivity of the formation. The calculated hydraulic conductivity of the first and second layer is 5.9×10^{-2}m/s and 3.3×10^{-1}m/s respectively. These moderately low conductivity values are characteristic of the resistivities of the layers which vary between 1.89 and 326 ohm-m corresponding to leachate accumulation or due to clay material.

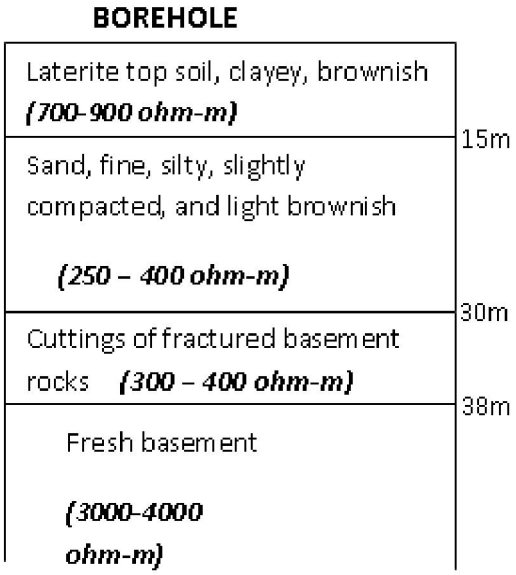

Fig.3**:** Borehole lithology and interpretation modified from Aboh (2001).

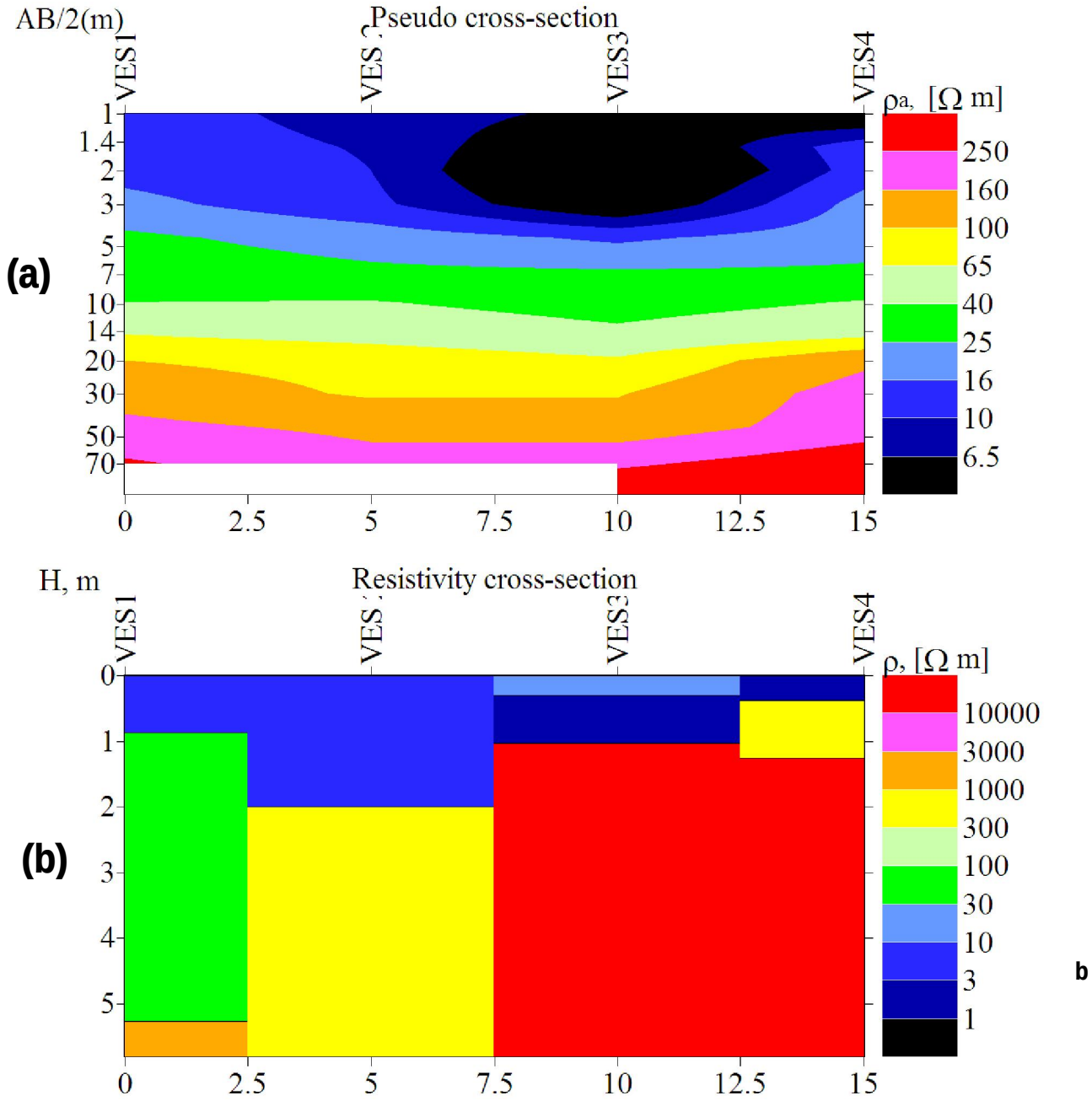

Fig.4: (a) pseudo cross-section and (b) resistivity cross-section of VES 1- 4.

Fig 5a shows the pseudo cross-section for VES points 5-8. The resistivity at the AB/2 spacing of 1- 4.5 m varies between 168 ohm-m and 367 ohm-m and is representative of clayey sand. The water bearing zone has colour scaling consisting of blue and green with a maximum resistivity of 263 ohm-m. This zone occurs at AB/2 spacing of 4.5 m to 20 m. The low resistivity values recorded in this zone could be an indication of accumulation of leachate and the contamination of the saturated sand. Fig.5b is the corresponding resistivity cross-section. The curve type consists of H (VES 1, 2 and 3) and HA (VES 4) and the basement rocks are competent (resistivity > 1000 ohm-m). The second layer for all the VES points, show low resistivity values (26 – 121 ohm-m) which could be attributed to contamination of the groundwater as a result of leachate accumulation or the presence of in situ weathered clay material. The former is favoured as the dumpsite is situated in a seasonally water

surplus climate and therefore, leachate generation and migration from the dumpsite can be expected. Furthermore, the high calculated hydraulic conductivity of the second layer, 3.7m/s is representative of sand and this may possibly be responsible for the migration of the leachate outside the dump. The calculated hydraulic conductivity of the topmost layer is 4.4×10^{-1} m/s which is typical of sandy clay.

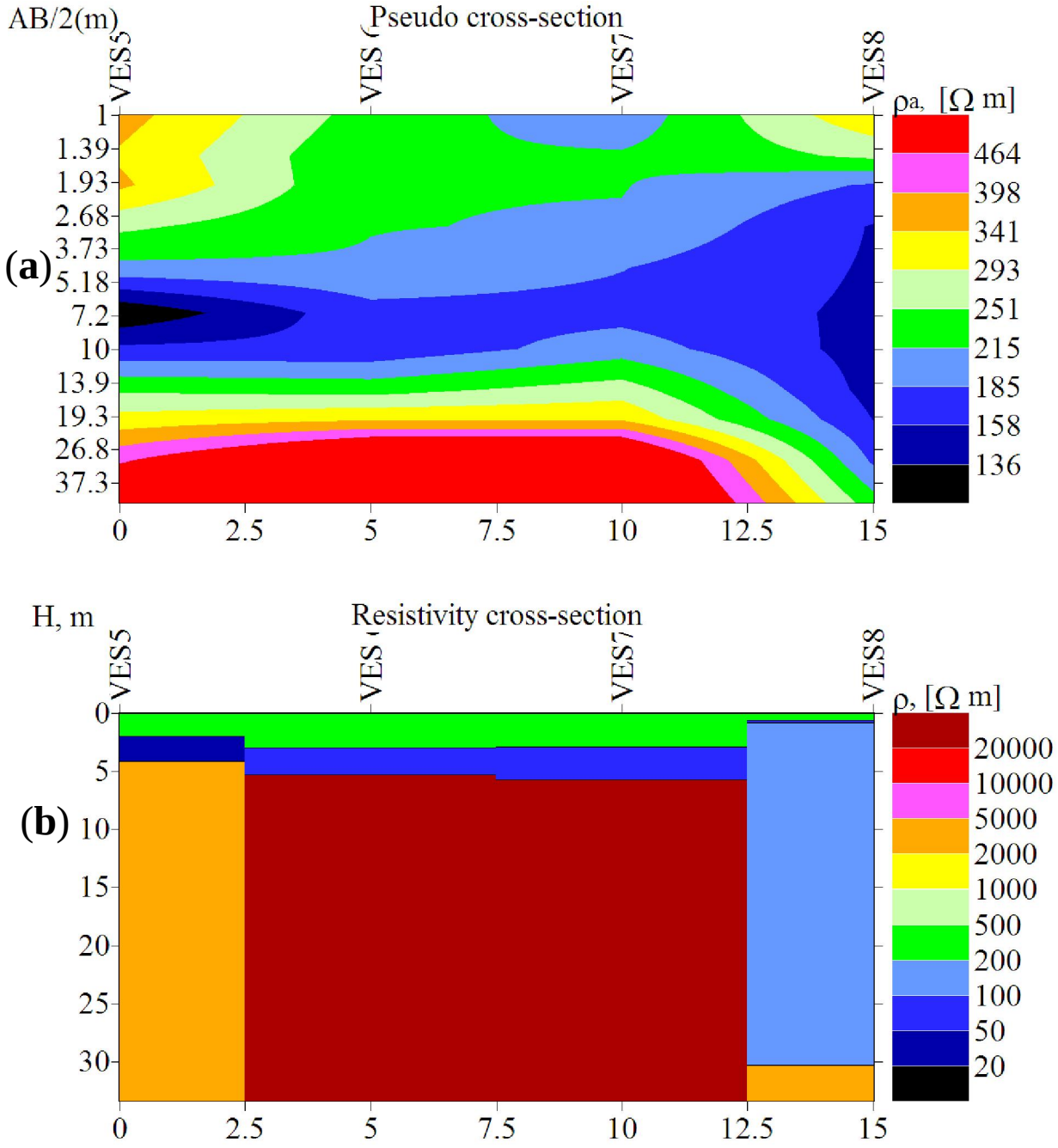

Fig.5: (a) pseudo cross-section and (b) resistivity cross-section of VES 1- 4.

In the light of WHO standards, it could be inferred from the results of the physiochemical analysis (Table 1) from the two laboratories, the values of the different parameters showed pollution of the groundwater. High electrical conductivities are attributed to contaminant fluids rich in total dissolved solids. High BOD concentration is an indication of

high concentration of biodegradable organic substances from the dumpsite while elevated COD concentration indicates pollution from both oxidizable organic and inorganic pollutants. High concentrations of the trace metals are possibly due to the effect of the leachate migrating from the waste body facilitated by the high conductivity of the geo-electric layers. High concentration of iron in the groundwater is probably due to the leaching of iron scraps which constitute a reasonable part of the waste. The high concentration of chloride, iron and zinc ions is an indication of toxic or hazardous substances in solid forms in the leachate Meju (2000). The high concentrations of these substances (chloride, iron and zinc) and detrimental substances (Lead, Chloride and Chromium) observed in both wells call for urgent concern because according to Bashir. (2001) this may cause central nervous system and kidney/liver effects.

4. Discussions

The main aim of the present work is to determine contamination of groundwater as a result of municipal solid waste leachate accumulation within the study area. The integration of vertical electrical soundings and physio-chemical analysis of water samples from existing hand dug wells; geologic logs and the local geology have been successfully used for the detection of groundwater contamination due to municipal solid waste in the study area. From the VES data, the thickness and resistivity values of the layers were determined. The VES interpretation revealed a maximum of four geologic units beneath the VES stations. The geologic sections revealed the various lithological compositions of various layers delineated. The generated pseudo and resistivity cross-sections showed leachate plumes extending below the water table, thus polluting the groundwater: a conclusion supported by the water quality analysis from existing hand dug wells which showed concentrations of organic/inorganic parameters exceeding permissible limits. The high concentration of detrimental heavy metals (lead, cadmium and chromium) is an indication of toxic or hazardous substances in the leachate. This is a major threat to human population, especially those within the area. The calculated hydraulic conductivity of the topmost layers of the subsurface outside the dump (4.4×10^{-1} m/s & 3.7 m/s) are higher than those inside the dump (5.9×10^{-2} m/s & 3.3×10^{-1} m/s).This signifies that the uppermost layers of the resistivity models in the study area consist of loose permeable sediments. Hence, the contamination of the hand dug wells indicated by the water quality analysis is believed to be as a result of transportation of the leachate plume outside the dumpsite through the pore spaces of the subsurface materials which are interconnected.

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Impact of different soil media on growth and chemical constituents of *Jatropha curca* L.seedlings grown under water regime

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Abstract: Two pot experiments were carried out at Research and Production Station, Nubaria of National Research Centre, Egypt, during 2008 and 2009 seasons. The purpose of this study is to investigate the influence of some soil media (sand, clay and sand + clay) under different water regimes (500, 1000, 1500 and 2000 cm^3/pot) on growth and chemical constituents of *Jatropha curcas* L. Results showed that, increasing water supply gradually increased significantly plant height, stem diameter, number of leaves/plant, leaf area, fresh and dry weight of leaves and stem. The same behavior was noticed concerning chlorophyll a, b, a+b, carotenoids content as well as N, P and k uptake in shoots. On the contrary, root length, fresh and dry weight of roots as well as N, P, K and proline content increased as water level decreased. Data also observed that all growth parameters, three pigments content, N, P, K and proline content tended to increase by using clay media followed by sand + clay media as compared with sand media. Clay media can be used to reduce the effect of water stress up to 500 cm^3/pot. These applications may be recommended for overcoming the harmful effect on growth and chemical constituents of *Jatropha curca* L. Seedlings under water stress. [Journal of American Science 2010; 6(8):549-556]. (ISSN: 1545-1003).

Keywords: soil media; *Jatropha curca* L; water regime; seedling

1. Introduction

Jatropha is a genus of approximately 175 succulent plants, shrubs and trees (same are deciduous, like *Jatropha curcas* L.*),* from the family Euphorbiaceae. *Jatropha curcas* L. is a drought-resistant perennial, growing well in marginal/poor soil. It is easy to establish , grows relatively quickly and lives, producing seeds for 50 years. Jatropha the wonder plant produces seeds with an oil content of 37%. The oil can be combusted as fuel without being refined. It burns with clear smoke-free flame, tested successfully as fuel for simple diesel engine. The by-products are press cake a good organic fertilizer, oil contents also insecticide. Medically it is use for diseases like cancer, piles, snakebite, paralysis, dropsy etc.. Stress has been defined as any environmental factor capable of inducing a potentially injurious strain in plants. Water is a major constituent of tissue, a reagent in chemical reaction, a solvent for and mode of translocation for metabolites and minerals within plant and is essential for cell enlargement through increasing turgor pressure. With the occurrence of water deficits many of the physiological processes associated with growth are affected and under severe deficits, death of plants may result. Farahat (1990) in study on *Schinus molle*, *Schinus terbinthifolius* and *Myoporum ocminatum*,Metwally *et al.* (2002) on roselle Metwally and Azza *et al.* (2007) on *Bauhinia variegata* seedlings, found that plant height, stem diameter and fresh and dry weight of leaves, stem and root decreased with prolonging the water intervals. Shehata (1992) working on *Cupressus sempervirens* and *Eucalyptus camaldulensis*, El-Tantawy *et al.* (1993) on *Eucalyptus camaldulensis*, Azza *et al.* (2006) on *Melia azedarach* seedlings and Azza *et al.* (2006) on *Taxodium distichum*, supplied seedlings with three soil moisture content (40, 60 and 80% of water holding capacity. They observed that plant height, stem diameter, fresh and dry weight of leaves, stem and roots were increased by increasing soil moisture but root length and fresh and dry weight of roots were decreased. Also, Uday *et al.* (2001) studied the effect of irrigation at (field capacity 10.4% w/w) 0.2 F.C., 0.5 F.C. and 10 F.C. levels) on growth of *Simmondsia chinensis* and found that growth was increased with increasing irrigation levels. Sayed (2001) on *Khaya senegalensis* and Soad (2005) on *Simmondsia chinensis* , Azza *et al.* (2006) on *Taxodium distichum*, supplied seedling with three soil moisture content (40, 60 and 80% of water holding capacity. They observed that plant height, stem diameter, fresh and dry weight of leaves, stem and roots were increased by increasing soil moisture but root length and fresh and dry weight of roots were decreased. Also, Uday *et al.* (2001) studied the effect of irrigation at (field capacity 10.4% w/w) 0.2 F.C., 0.5 F.C. and 10 F.C. levels) on growth of *Simmondsia chinensis* and found that growth was increased with increasing irrigation levels. Sayed (2001) on *Khaya sensegalensis* and Soad (2005) on *Simmondsia chinensis*, Azza *et al.* (2006) on *Melia*

azedarach and Azza *et al.* (2007) on *Bouhinia variegata*, irrigated seedling with different soil moisture content. They found that chlorophyll (a, b and carotenoid content) were increased as soil moisture content decreased. In addition to that total sugars, N, P and K concentration in the leaves were also stimulated gradually by decreasing water supply. While, leaf content of nitrogen, phosphorus and potassium were increased by increasing water supply. Azza *et al.*, (2006) on *Taxodium distichum* found that increasing water supply gradually increased N, P and k uptake in shoot. Data also revealed that proline tended to increase by decreasing water level.

Successful greenhouse and nursery production of container-grown plants is largely dependent on the chemical and physical properties of the growing media. An ideal potting medium should be free of weeds and diseases, heavy enough to avoid frequent tipping over and yet light enough to facilitate handling and shipping. The media should also be well drained and yet retain sufficient water to reduce the frequency of watering. Other parameters to consider include cost, availability, consistency between batches and stability in the media overtime. Selection of the proper media components is critical to the successful production of plants. (James and Michael, 2009).

Sandy soil: soils with a high proportion of sand drain easily, so water logging is not a problem unless a "pan" or impervious layer has formed below the surface. Their open structure means that they are easy to work and quick to warm up in the spring, allowing earlier sowing and plating. However sandy soils do dry out very quickly and nutrients are easily washed through the soil. Clay soil: These are heavy and difficult to work. They are slow to warm in the spring, sticky when wet and very hard when dry. Clay sails hold moisture and nutrients well and remain warm, in the autumn because they are slow to cool down. Mach of the water they contain will not be available to plants and in winter they are prone to water logging. (James and Michael, 2009). El-Sallami (2003) on *Leucaena leucocephala* seedlings, El-Khalifa (2003) on *Dalbergia melanoxylon*, Mohmood (2005) on *Caesalpinia pulcherrima* and *Thevetia peruviana* and Kathiravan *et al.* (2008) on *Jatropha curcas*, found that, clay soil was superior to sandy one in stimulating the morphological growth. Abd-El-Razek (2002) on *Gasteria verrucosa* and *Haworthia fasciata* plants and El-Sallami (2003) on *Lucaena leucocephala* seedlings, found that clay medium gave the highest chlorophyll (a and b), N, P, K percentages and total carbohydrates compared with sandy medium. Mahmood (2005) showed that N, P and K content in leaves exhibited maximum values under the clay + sand medium of *Caesalpinia pulcherrima* and *Thevetia peruviana.*

The aim of the present work is the evaluate the influence of different soils media (sand,clay and mix. soil) grown under several irrigation levels.

2. Material and Methods

This study was carried out at Research and Production Station, Nubaria of National Research Centre during two successive summer seasons of 2008-2009. The main objective of this study was to investigate the effect of different soil types under different water regimes on growth and chemical constituents of *Jatropha curcas.* The physical properties of the different media are shown in Tables (1a& 1b).

(1a)- Mechanical analysis

Soil sample	Coarse sand%	Fine sand %	Silt + Clay %
Clay	43.0	13.0	44.0
Sand	72.5	16.5	11.0

(1b) Chemical analysis:

Soil sample	E.C. m.mohs/cm^3	pH	SP%	Anion (meq/L.)			Cation (meq/L.)		
				HCO_3^-	Cl^-	SO_4^{--}	Ca^{++}	Mg^{++}	Na^++K^+
Clay	2.9	7.9	33.0	4	21	5	21	5	3
Sand	2.4	7.1	23.0	2	23	-	4	3	20

One year-old seedlings of *Jatropha curcas* L. were obtained from nursery of Forestry Department Horticulture Research Institute, Agriculture Research Centre. The seedlings were planted on 15th March in plastic pots 30 cm diameter (one plant/pot, the average height of seedlings were 12-15cm), each pot filed with three different soil media according to treatments i.e. sand, clay and the mixture of them 1: 1 (v/v). The irrigation schedule was four treatments (500, 1000, 1500 and 2000 cm^3/ pot). After one month from transplanting, the irrigation regime was started and was terminated in 15th November. The available commercially fertilizer used through this experimental work was kristalan (NRK 19: 19: 19) produced Phayzen campany, Halland. The fertilizer rates were 20.0gm/pot in four equal does after 4,8, 16 and 20 weeks from transplanting.

The following data were recorded: Plant height (cm), leaves number/ plant, root length (cm), stem diameter (mm), leaf area (cm^2), fresh and dry weight of leaves, stems and roots (gm). The experiment was sitting in completely Randomized experiment Design with four irrigation rates and three types of soil to give 12 treatments with 6 replicats. The obtained results was subjected to statistical analysis of variance according to the method described by Snedecor and Cochran (1980) and the combined analysis of the two seasons was calculated according to the method of Steel and Torrie (1980). The following chemical analysis was determined: Chlorophyll a, b and carotenoids contents were determined according to Saric *et al.,* (1967). The proline concentration was determined using fresh material according to Bates *et al.,* (1973). Nitrogen, phosphorus, potassium and sodium contents of leaves were determined according to the method described by Cottenie *et al.,* (1982). The physical and chemical properties of the soil were determined according to Chapman and Pratt (1961).

3. Results and Discussion

Vegetative growth: The results obtained in Tables (2-5) showed that the above-ground vegetative growth including plant height, leaves number/ plant, stem diameter, leaf area and fresh and dry weight of shoot were gradually increased as the level of water irrigation was sloping upward. The highest values for all these characters were obtained due to the use of the high irrigation level (2000cm^3).

The reduction in these growth parameters under low irrigation level (500cm^3) conditions may be attributed to losses of tissue water which inhibiting cell division and enlargement. El – Monayeri *et al.* (1985) reported that the vital roles of water supply at adequate amounts for different physiological processes such as photosynthesis respiration, transpiration translocation, enzyme reaction and cells turgidity occurs occurs simultaneously. Such reduction could be attributed to a decrease in the activity of meristemic tissues responsible for elongation, as well as the inhibition in photosynthetic efficiency under insufficient condition (Siddique *et al.,* 1999). Ali *et al.,* (1999) indicated that soil drying decreased leaf growth thereby reducing leaf water status in addition to accumulation of organic solutes to osmotic adjustment which in turn inhibit the incorporation of small substrate molecules into the polymers needed to grow new cell. On contarary, all water prementioned vegetative growth parameters root length and fresh and dry weight of roots took on opposite trend as they were gradually decreased with the irrigation levels was sloping upward. The lower water supply causes the root system to penetrate deeper and extending wider in the soil with higher root system researching for moisture in lower. This results were on line with those reported by Burman *et al.* (1991)on *Simmondsia chinensis* Uday *et al.* (2001) and Azza *et al.,* (2006) on *Taxodium distichum.*

Data in Tables (2-5) showed that clay and mix. media significantly increased all growth parameters in the two seasons compared with the sandy soil which gave the lowest values. Clay minimized this reductory effects ,while mix. medium gave a moderate values in this respect. This effect may be attributed to the physical properties of the soil. Sand soil is parous, the ions absorption is more easy while some of ions adhere on the clay soil particles. Also, sandy soils do dry out very quickly and nutrients are easily washed through the soil while, clay soils hold moisture and nutrients well.

These results were confirmed by El-Mesiry and Azza (2001) on *Melia azedarach* L., El-Sallami (2002) on *Chorisia speciosa, Leucaena leucocephala* and *Prosopis Juliflora,* El-Khalifa (2003) on *Dalbergia melanoxylon* plant and Kathiravan *et al.* (2008) on *Jatropha curcas* L.

The results presented in Tables (2-5) declared that clay and mix. media minimized the reductory effect of water irrigation levels compared with the sandy soil. Therefore,plant grown in clay media and irrigated with 2000 cm^3 treatment relatively increased the all morphological growth followed by mix. media compared with the sandy soil under irrigated with 500cm rate which produced the lowest values in the two seasons.

Chemical constituents:

Pigments content : From the given data in Table (6) it can be concluded that, increasing irrigation rates caused an increase in the content of photosynthetic pigments (chlorophyll a, b, a+b and carotenoids). Accordingly it can be stated that irrigation with 2000 cm^3 was the most effective irrigation treatment for promoting the synthesis and accumulation of the three photosynthetic pigments. In harmony with these results were those obtained by Soad (2005) on jojoba seedlings and Azza *et al.,* (2006) on *Taxodium distichum.*

Clay media tended to increase chlorophyll a, b, a+b and carotenoids as comparing with the sandy and mix.media in fresh leaves. These results are in accordance with those obtained by Sayed (2001) on khya and El-Sallami (2003) on *Leucaena leucocephala* plant .

Seedling grown in clay soil and irrigated with 2000cm^3 rate produced the greatest photosynthetic pigments of fresh leaves . The increment effect on chlorophyll a ,b, a+b and carotenoids by 38.3,50.3,42.8 and 40.8% respectively compared with sand soil that was irrigated with 500cm^3 rate which produced the lowest value.

Proline content: From the given data in Table (7) it can be concluded that decreasing irrigation level caused an increase of proline content. This may be due to the proline metobdism which is a typical mechanism of biochemical adaptation subjected to stress condition . The catabolism of proline involves its conversion to glutamic acids via. Pyrroline-s-carboxylate reduction and subsequent metabolism of glutamate by Kreb cycls veactin that release CO_2 as the end product (Armestrong, 1993). Obtained results were in harmony with the finding of Azza *et al.* (2006) on *Taxodium distichum.*

Soil media results on proline content had a similar trend as that in the photosynthetic pigments. Clay and mix.med increased the average proline content of leaves in both seasons compared with sandy soil. Seedlings grown in clay media which was irrigated with 500cm^3 gave the highest proline content followed by mix.media + 500cm^3 and sand +500 cm^3 respectively.

Minerals content : It is evident from the data in Tables (7 - 9) that minerals content under investigation (N,P and K)were gradually increase with decreasing the water supply. Regarding irrigation level from 2000cm^3 and up to 500cm^3 caused an increase in pervious minerals concentrations. This may be due to the leaching of the minerals from soil. In agreement with these results concerning irrigation were the results of Sayed (2001) on some woody trees, Soad (2005) on jojoba and Azza *et al.,* (2007) on *Bauhinia variegata.*

Furthermore, sodium content increased by increasing irrigation levels in plant, these results run parallel with those obtained by Farahat (1990) on *Scinus molle* L. *Myoprum acumination* and El-Tantawy *et al.,* (1993) on *Eucalyptus camaldulensis.*

Concerning the effect of soil media, it is evident from data that the previous minerals in plant in the tow growing seasons, were increased by using clay media, following by mix media. But, sodium content took an opposite menner, it decreased by using clay media. This effect was found in the two seasons and it may be attributed to the ability of such soil to supply the plant with its needs from nutrients. These results were confimed by El-Sallami (2003) on *Leucaena leucocephala.* Concerning ineraction between clay media + irrigation by 500 cm^3 showed that maximum values for each element.

On the contrary of N, P and K uptake as influenced by irrigation treatments, plant uptake of such four nutrients were gradually increased by increasing irrigation level. These results could be explained in high of the considerable increase in leave dry weight by the increase in irrigation level. In harmony with the prementioned results were finding of Soad (2005) on Jojoba seedlings and Azza *et al.,* (2007) on *Bauhinia variegata.*

Leaves uptake of each of nitrogen, phosphorus and potassium were increased due to the use of the clay soil than mix media and sand media. Similar results were obtained by El-Sallami (2003) on *Leucaena leucocephala.*

In the interaction between clay soil and irrigation by 2000 cm^3 showed the maximum uptake of each elements, except Na uptake, it obtaine the lowest uptake.

The results in this study indicated that clay soil media can be used to overcome the reduction effect of water regimes.

Table (2) Effect of soil types on plant height (cm), number of leaves/plant and root length (cm) of *Jatropha curcas.*L.plant grown under water regime condition (Average of two seasons 2008 and 2009).

Characters	Plant height (cm)					Number of leaves/plant					Root length (cm)				
Treatments	Water regime (cm^3/pot)					Water regime (cm^3/pot)					Water regime (cm^3/pot)				
Soil Types	500	1000	1500	2000	Mean	500	1000	1500	2000	Mean	500	1000	1500	2000	Mean
Sand	75.0	74.3	79.0	86.3	78.7	27.7	32.0	33.0	35.0	31.9	30.0	29.6	28.3	25.3	28.3
Clay	131.3	132.3	147.0	161.7	143.1	48.7	52.0	58.0	63.3	55.5	48.0	47.6	44.0	39.3	44.7
Sand+ Clay	86.7	89.7	94.0	96.3	91.6	36.7	39.6	41.7	46.0	40.8	37.7	36.6	32.6	31.0	34.5
Mean	97.6	98.7	106.6	114.7		37.3	41.0	44.2	48.1		38.5	37.9	34.9	31.8	
L.S.D at 5%															
Soil types (S)	7.3					4.7					1.8				
Water regime (W)	9.5					6.7					2.6				
(S)*(W)	12.11					7.3					3.1				

Table (3) Effect of soil types on leaves, stems and roots fresh weight (gm) of *Jatropha curcas* L. plant grown under water regime condition (Average of two seasons 2008 and 2009).

Characters	Leaves fresh weight (gm)					Stem fresh weight (gm)					Roots fresh weight (gm)				
Treatments	Water regime (cm^3/pot)					Water regime (cm^3/pot)					Water regime (cm^3/pot)				
Soil Types	500	1000	1500	2000	Mean	500	1000	1500	2000	Mean	500	1000	1500	2000	Mean
Sand	62.42	72.74	74.95	86.68	74.20	165.62	182.68	195.10	224.13	191.88	69.35	56.09	44.20	43.83	52.86
Clay	130.17	177.57	210.40	247.04	191.29	349.37	381.91	410.95	447.95	397.54	158.99	122.49	115.79	99.33	124.15
Sand+ Clay	80.88	89.23	103.67	117.32	97.77	237.47	261.25	285.96	315.05	274.93	93.11	73.12	70.22	69.35	76.45
Mean	91.16	113.18	129.67	150.34		250.82	275.28	297.33	329.04		106.48	83.90	76.73	70.83	
L.S.D at 5%															
Soil types(S)	7 13					11.62					7.35				
Water regime(W)	8.92					13.53					9.33				
(S)*(W)	11.31					15.71					11.93				

Table (4) Effect of soil types on leaves, stems and roots dry weight (gm) of *Jatropha curcas* L. plant grown under water regime condition (Average of two seasons 2008 and 2009).

Characters	Leaves dry weight (gm)					Stem dry weight (gm)					Roots dry weight (gm)				
Treatments	Water regime (cm^3/pot)					Water regime (cm^3/pot)					Water regime (cm^3/pot)				
Soil Types	500	1000	1500	2000	Mean	500	1000	1500	2000	Mean	500	1000	1500	2000	Mean
Sand	9.05	10.83	11.54	13.78	11.30	62.45	69.75	77.78	86.95	74.23	20.74	17.00	13.12	12.80	15.91
Clay	23.56	33.38	40.32	49.16	36.60	98.17	109.61	120.00	133.49	115.31	55.32	42.01	38.90	32.97	42.30
Sand+ Clay	13.10	14.99	17.86	20.64	16.64	72.45	79.75	87.78	96.95	84.23	30.45	23.69	22.18	21.56	24.47
Mean	15.23	19.73	23.24	27.86		77.69	86.37	95.19	105.80		35.50	27.56	24.73	22.49	
L.S.D at 5%															
Soil types(S)	2.60					4.35					2.86				
Water regime(W)	3.01					6.75					4.53				
(S)*(W)	4.63					7.88					6.12				

Table (5) Effect of soil types on stem diameter (mm) and leaf area (cm^2) of *Jatropha curcas* L. Plant grown under water regime condition (Average of two seasons 2008 and 2009).

Characters	Stem diameter (mm)					Leaf area (cm^2)				
Treatments	Water regime (cm^3/pot)					Water regime (cm^3/pot)				
Soil Types	500	1000	1500	2000	Mean	500	1000	1500	2000	Mean
Sand	2.60	2.60	3.00	3.20	2.85	37.32	41.05	41.99	42.48	40.78
Clay	3.70	3.80	4.20	4.50	4.05	53.29	58.42	60.38	68.60	60.18
Sand+ Clay	3.30	3.50	3.60	3.70	3.52	49.32	49.52	51.77	52.08	50.67
Mean	3.20	3.30	3.60	3.80		46.64	49.66	51.38	54.48	
L.S.D.at 5%										
Soil Typs(S)			0.04					1.31		
Water regime(W)			0.06					2.07		
(S)*(W)			0.10					2.71		

Table (6) Effect of soil types on photosynthetic pigments (mg/g F.W.) of *Jatropha curcas* L. plant grown under water regime condition (Average of two seasons 2008 and 2009).

Characters	Chlorophyll (a)					Chlorophyll (b)					Chlorophyll (a+b)					Carotenoids				
Treatments	Water regime (cm^3/pot)					Water regime (cm^3/pot)					Water regime (cm^3/pot)					Water regime (cm^3/pot)				
Soil Types	500	1000	1500	2000	Mean	500	1000	1500	2000	Mean	500	1000	1500	2000	Mean	500	1000	1500	2000	Mean
Sand	0.732	0.769	0.791	0.822	0.778	0.431	0.467	0.485	0.528	0.477	1.163	1.236	1.276	1.350	1.256	0.235	0.243	0.255	0.264	0.249
Clay	0.968	0.984	0.986	1.013	0.987	0.604	0.624	0.632	0.648	0.627	1.572	1.608	1.618	1.661	1.614	0.308	0.318	0.323	0.331	0.320
Sand+ Clay	0.841	0.891	0.934	0.959	0.906	0.534	0.549	0.585	0.596	0.566	1.375	1.440	1.519	1.555	1.472	0.270	0.278	0.285	0.297	0.282
Mean	0.847	0.881	0.903	0.931		0.523	0.546	0.567	0.590		1.370	1.428	1.471	1.522		0.271	0.279	0.287	0.297	

Table (7) Effect of soil types on prolin content, sodium percentage and uptake/plant leaves of *Jatropha curcas* L. plant grown under water regime condition (Average of two seasons 2008 and 2009).

Characters	Proline (µmg $^{-1}$)					Sodium %					Sodium – uptake mg/plant				
Treatments	Water regime (cm^3/pot)					Water regime(cm^3/pot)					Water regime (cm^3/pot)				
Soil Types	500	1000	1500	2000	Mean	500	1000	1500	2000	Mean	500	1000	1500	2000	Mean
Sand	5.7	4.59	3.73	2.91	4.23	1.92	1.76	1.69	1.49	1.72	17.38	19.06	19.50	20.53	19.12
Clay	8.31	7.12	5.32	4.42	6.29	1.49	1.39	1.33	1.15	1.34	45.23	46.40	53.63	56.53	50.45
Sand+ Clay	7.00	5.73	4.93	3.76	5.36	1.64	1.47	1.31	1.23	1.41	21.48	22.04	23.40	25.39	23.08
Mean	7.00	5.81	4.66	3.70		1.68	1.54	1.44	1.29		28.03	29.17	32.18	34.15	

Table (8) Effect of soil types on nitrogen, phosphorus and potassium percentage of *Jatropha curcas* L. plant grown under water regime condition (Average of two seasons 2008 and 2009).

Characters	Nitrogen %					Phosphorus %					Potassium %				
Treatments	Water regime (cm^3/pot)					Water regime (cm^3/pot)					Water regime (cm^3/pot)				
Soil Types	500	1000	1500	2000	Mean	500	1000	1500	2000	Mean	500	1000	1500	2000	Mean
Sand	0.94	0.90	0.82	0.76	0.83	0.45	0.44	0.38	0.32	0.40	0.85	0.78	0.59	0.53	0.69
Clay	1.23	1.19	1.12	0.99	1.13	0.79	0.71	0.64	0.60	0.69	1.35	1.26	1.20	1.14	1.24
Sand+ Clay	1.07	0.95	0.84	0.80	0.92	0.54	0.52	0.50	0.48	0.51	1.09	1.06	1.02	0.99	1.04
Mean	1.08	1.01	0.93	0.85		0.59	0.56	0.51	0.47		1.10	1.03	0.94	0.89	

Table (9) Effect of soil types on nitrogen, phosphorus and potassium uptake mg / plant leaves of *Jatropha curcas* L. plant grown under water regime condition (Average of two seasons 2008 and 2009).

Characters	Nitrogen –uptake mg/plant					Phosphorus –uptake mg/plant					Potassium –uptake mg/plant				
Treatments	Water regime (cm^3/pot)					Water regime (cm^3/pot)					Water regime (cm^3/pot)				
Soil Types	500	1000	1500	2000	Mean	500	1000	1500	2000	Mean	500	1000	1500	2000	Mean
Sand	85.1	97.5	94.6	104.7	93.8	281.0	306.9	295.6	278.2	296.9	108.8	102.3	100.3	109.9	109.8
Clay	289.8	397.2	451.6	486.7	413.6	775.5	778.2	768.0	800.9	795.6	445.1	490.1	504.1	630.6	524.5
Sand+ Clay	140.2	142.4	150.0	165.1	153.1	391.2	414.7	476.0	507.8	429.6	235.0	235.1	241.6	301.5	254.5
Mean	164.5	199.3	216.1	236.8		458.4	483.7	485.5	497.3		247.4	254.7	259.1	316.0	

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Serum resistin levels and haemostatic changes in experimentally induced diabetic and high fat fed rats

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Abstract: Adipose tissue is considered as an active endocrine gland that affects many aspects of body homeostasis. Adipose tissue derived molecules ‘‘adipokines” regulate energy homeostasis, dietary behavior, as well as insulin sensitivity and immunity; it refers to leptin, adiponectin, resistin, apelin, visfatin and omentin. Resistin is a cysteine-rich adipokine that is released by adipocytes and macrophages and has been involved in the development of insulin resistance in rodents. Moreover a strong link between diabetes, hypercoagulability and thrombogenesis, had been recognized for decades. Aim: In a trial to identify any possible relationship between resistin levels and some haemostatic changes in streptozotocin-induced diabetic and high fat diet-fed rats (HFD); the present work had been carried out. Design: A total number of 40 adult male albino rats were divided into 2 main groups: Group I (n= 24): To study the effect of streptozotocin-induced type 1 diabetes and was further divided into 3 equal subgroups (n= 8 in each) and survived for 30 days: Ia: (control group), Ib: (experimental diabetic non-treated group (by a single i.p. injection of streptozotocin (65mg/Kg B.W), Ic (experimental diabetic group treated with insulin). Group II (n= 16) : To study the effect of high fat diet and was further divided into 2 equal subgroups (n= 8 in each) and survived for 7 weeks: IIa: (control group),IIb (high fat diet fed (58% fat). In all groups, serum levels of glucose, insulin, resistin, total cholesterol(TC), triglycerides (TG), HDL, LDL, BT, WBCT, PT, aPTT, plasma fibrinogen level, plasma D-dimmers level and platelet count were measured. Results: The results of this study showed a significant decrease in serum resistin levels ($p<0.001$) in streptozotocin-induced diabetic group in comparison with its control group and insulin-treated group. Moreover, no significant correlation could be detected between resistin levels and any of measured parameters in these groups except the significant positive correlation with body weight at the end of experimental period. In addition, our study revealed a significant increase in serum resistin levels ($p<0.001$) in HFD-fed group in comparison with its controls, which was correlated positively and significantly with body weight, serum glucose levels, insulin levels and HOMA-IR index ($p<0.001$), atherogenic lipid profile and markers of hyper-coagulability (except for platelet count). Conclusion: No role for resistin in metabolic and haemostatic changes in type 1 diabetic rats was detected. Although, hyperresistinemia may represent a link between metabolic signals, atherogenesis, and hypercoagulability in type 2 diabetic rats. However, further studies are needed to clarify this relationship in human cardiovascular diseases. [Journal of American Science 2010; 6(8):557-567]. (ISSN: 1545-1003).

Keywords: Resistin, Streptozotocin, high fat, diabetes, heamostasis

1. Introduction

Obesity is associated with an array of health problems in adult and pediatric population. Understanding the pathogenesis of obesity and its metabolic sequelae has advanced rapidly over the past decades (American Diabetes Association, 2007).

Adipose tissue represents an active endocrine organ that, in addition to regulating fat mass and nutrient homeostasis, releases a large number of bioactive mediators (adipokines) that signal to organs of metabolic importance including brain, liver, skeletal muscle, and immune system, thereby modulating haemostasis, blood pressure, lipid and glucose metabolism, inflammation, and atherosclerosis (Rabe et al., 2008).

In (2001), Steppan et al. discovered a novel adipocyte–derived hormone called resistin, which was expressed exclusively in white adipose tissue as a member of a family of cysteine-rich proteins called resistin–like molecules.

Intra-peritoneally administered resistin augments blood glucose and plasma insulin levels and limits the hypoglycemic response to insulin infusion, furthermore, resistin suppresses insulin–stimulated glucose uptake in cultured adipocytes, and this effect is prevented by exposure to anti-resistin antibodies (steppanet al., 2001).

Hence, these data suggest that resistin could contribute to the insulin resistance observed in obesity by decreasing insulin sensitivity (Rajala et al., 2003, Muse et al., 2007).

In fact, obese subjects show a reduced insulin-stimulated skeletal muscle glucose uptake as well as an impaired insulin-evoked vasodilatation

(Baron, 1994) and these observations have suggested that the pathophysiological mechanisms linking obesity to the development of cardiovascular diseases could go beyond the classical metabolic derangements, so, much effort has been made to understand the interaction between insulin resistance and vascular function, with particular emphasis on adipocyte-derived hormones and their effects on vascular homeostasis (verma et al., 2003).

Resistin has been shown to selectively impair the effect of insulin on endothelial nitric oxide synthase (eNOS) enzymatic activity and through this mechanism resistin can reduce insulin-evoked vasorelaxation (Gentile et al., 2008). Moreover, in high fat-fed rats, resistin levels correlate negatively with vascular nitric oxide (NO) levels even after correction of insulin measurements, which suggests a direct inhibitory role of resistin on NO secretion (Li et al., 2007).

Also, plasma resistin levels were reported to be associated with many inflammatory markers including C-reactive protein, tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) (Silswal et al., 2005, Stofkova, 2010). Considering the crosstalk between inflammatory pathways and the insulin signaling cascade, resistin may represent a link between metabolic signals, inflammation and atherosclerosis (Lehrke et al., 2004, Daniel et al., 2010).

In general, diabetes is associated with an excessive risk of cardiovascular events (Williams et al., 2002), in that the coagulation system is switched towards a pro-thrombotic state involving, increased blood coagulation, decreased endothelial thrombo-resistance and pro-inflammatory state (Palomo et al., 2006), thereby increasing the risk of micro-vascular disease as well as macro-vascular diseases (Lender and Sysko, 2007).

So, this study was designed in a trial to clarify any possible relationship between resistin levels and some haemostatic changes in streptozotocin-induced diabetic rats and high fat diet-fed rats.

2. Material and Methods

This study was conducted on 40 healthy, adult, male albino rats weighing 200–260 gm (animals were obtained from faculty of medicine animal house and the animal experiments were approved by the local ethics committee). The rats had free access to water and chow and are kept at room temperature. All rats received standard chow (25.8 % protein, 62.8 % carbohydrate and 11.4 % fat (Ahren and Scheurink, 1998) except the rats in high fat-fed group, which received high-fat chow (16.4% protein, 25.6% carbohydrate, and 58.0% fat (a total 23.4 kJ/g) in the form of cotton seed oil added to the laboratory chow diet (Cha et al., 2000), (Diets were obtained from faculty of agriculture, Zagazig university).

The animals were divided into 2 main groups:

Group I: To study the effect of streptozotocin-induced type 1 diabetes on the measured parameters. This group was further divided into 3 equal subgroups and survived for 30 days:

Group Ia: "Control group (n=8)". Each rat was intra-peritoneally (i.p.) injected with 0.2 mmol/L Na citrate (0.1 mL).

Group Ib: "Experimental diabetic non-treated group (n=8) ". Diabetes was induced by single intra-peritoneal injection of freshly prepared solution of streptozotocin (sigma) 65 mg/kg of body weight dissolved in 0.2 mmol/L sodium citrate, at PH 4.5 (Lutz and Pardridge, 1993) and maintained for 30 days (Toba et al., 2009).

Three days later, diabetes induction was confirmed through measurement of blood glucose level in each animal (from blood sampled from the tail vein) with the One Touch Ultra Glucometer (Yves and Theo, 2007) and rats with blood glucose levels more than 250 mg/dl were selected for experiments (Coskun et al., 2004). The rats were provided with 10% glucose solution after 6 hours of streptozotocin administration for the next 48 hours.

Group Ic: "Experimental diabetic group treated with insulin (n=8)". These animals were treated with regular (R) and NPH (N) insulin (2UR at diagnosis of diabetes and then 1R/3N at 6 P.M and 1R/1N at 9 A.M daily subcutaneously for 30 days after induction of diabetes (Sivitz et al., 1998).

Group II: To study the effect of high fat diet (HFD) on the measured parameters. This group was further divided into 2 equal subgroups:

Group IIa: " control group (n=8)", which was fed a standard chow for 7 weeks.

Group IIb: "High fat diet fed group (n=8)". These rats were fed a high-fat chow for 7 weeks.

For all groups, body weight was recorded per week, and at the end of the study period.

Heamostatic measurements:

-Determination of bleeding time (BT) according to Martin, (1981).

-Determination of whole blood clotting time (WBCT) according to Quick, (1966).

-Determination of prothrombin time (PT) according to Ansell, (1992).

-Determination of activated partial thromboplastin time (aPTT) according to Ansell, (1992).

-Estimation of plasma fibrinogen levels according to Cooper and Douglas, (1991).

-Estimation of plasma D-dimmers levels according to Declerck et al. (1987).

-Determination of platelet count according to Brecher et al. (1953).

Biochemical and Hormonal measurements:

- Estimation of serum glucose levels according to Trinder, (1969).
- Estimation of serum insulin levels according to Reaven, (1991).
-Estimation of serum resistin levels: by enzyme-linked immunoassay kits from Linco Research Inc., (USA) according to Steppan and Lazar, (2004).
-Estimation of serum total cholesterol (TC) levels according to and Allain et al. (1974).
--Estimation of serum triglycerides (TG) levels according to Naito, (1989).
-Estimation of serum high density lipoproteins (HDL) levels according to Warnick et al. (1983)
-Estimation of serum low density lipoproteins (LDL) levels according to Friedwald et al. (1972).

Calculations:

The homeostasis model of assessment of insulin resistance (HOMA-IR) = fasting blood glucose (mmol/l) x fasting insulin (uIU/ml)/22.5) was calculated as an index of insulin resistance (Matthews et al., 1985) in group II.

Statistical analysis:

Data were presented as mean ± SD. Statistical significance was determined by unpaired Student's "t" test, P values less than 0.05 were considered to be significant. The correlations between parameters were analyzed using Pearson,s correlation.

In statistical analysis, SPSS version 10.0 programs for Windows (SPSS Inc. Chicago, IL, USA) was used.

3. Results

Table 1: Body weight and the metabolic parameters of the studied groups:

	Group Ia	Group Ib	Group Ic	Group IIa	Group IIb
Initial BW (gr	222.5±16.1	219.4±18.9	224.6±21	213.7±14.8	225.5±19.3
Final BW (gm	252.3±16.5 r=0.73*	192±14.8*** r=0.86**	251.3±17.5 r=0.82*	242.5±15.1 r=0.92**	339±19.4*** r=0.97***
Glucose (mg/dl)	75.6±6.6 r=0.31	411.5±97.6*** r=0.19	79.4±5.5 r=0.12	86.1±8.7 r=0.65	262.5±37.4*** r=0.98***
Insulin (uIU)/ml	19.7±2.5 r=0.55	1.24±0.36*** r=0.5	66±9.2*** r=0.64	18.4±3.1 r=0.03	46.1±4.8*** r=0.97***
HOMA-IR				4.13±0.47 r=0.03	30.28±7.26*** r=0.99***
Resistin (ng/ml)	7.2±0.77	3.99±0.2***	7.4±0.6	7.6±0.46	14±0.78***
TC (mg/dl)	115.25±6.5 r=0.32	223.6±24.2*** r=0.45	110.5±8.5 r=0.39	113.75±7.8 r=0.04	208.6±26*** r=0.73*
TG (mg/dl)	49.75±5.3 r=0.35	82.6±6.5*** r=0.37	51.5±7.5 r=0.33	54.25±7.5 r=0.25	188.25±12.5*** r=0.9**
HDL (mg/dl)	64.4±10.2 r= -0.52	35.5±4.2*** r= -0.25	54.9±10.2 r= -0.22	40.9±4.2 r= -0.05	36.1±4.4* r= -0.91**
LDL (mg/dl)	40.9±13.3 r=0.53	171.6±24.3*** r=0.47	47.4±13.7 r=0.25	62.03±7.4 r=0.01	134.9±27.7*** r=0.75*

r=correlation coefficient versus resistin levels.
*=significant (P<0.05).
**=significant (P<0.01).
***=significant (P<0.001).

Table 2: The haemostatic parameters of the studied groups:

	Group Ia	Group Ib	Group Ic	Group IIa	Group IIb
BT (sec)	214.4±26.5 r= -0.33	150±19.4*** r= -0.52	202.5±35.6 r= -0.38	212.5±25.2 r= -0.31	170.6±14.5*** r= -0.97***
WBCT (sec)	236.1±21.6 r= -0.002	165.1±27*** r= -0.16	220.75±32.4 r= -0.69	228.3±28.7 r= -0.28	166±14.2*** r= -0.88**
PT (sec)	13.3±.0.85 r= -0.06	10.25±1.7*** r= -0.17	13.55±0.65 r= -0.25	13.2±1 r= -0.29	10.6±1.28*** r= -0.8*
aPTT (sec)	24.1±2.7 r= -0.2	13.6±4.5*** r= -0.37	23±2.4 r= -0.22	25.6±3.8 r= -0.02	14.7±4.5*** r= -0.93**
Fibrinogen (mg/dl)	272.2±44.6 r=0.52	512.4±89.6*** r=0.19	287.2±46.2 r= -0.59	286.6±56.1 r=0.17	438.9±66.2*** r=0.84**
D-Dimmers (mg/dl)	171.7±34.6 r=0.49	255.1±20.6*** r=0.6	170.8±15.9 r=0.28	147.75±27.6 r= 0.02	216.87±27*** r=0.73*
Platelet cou (1000/mm$^{3)}$	211.9±17.8 r=0.55	302±21.7*** r=0.05	226.4±19.1 r=0.02	220.4±20.4 r=0.08	216.1±13.8 r= 0.43

r=correlation coefficient versus resistin levels.
*=significant (P<0.05).
**=significant (P<0.01).
*** =significant (P<0.001).

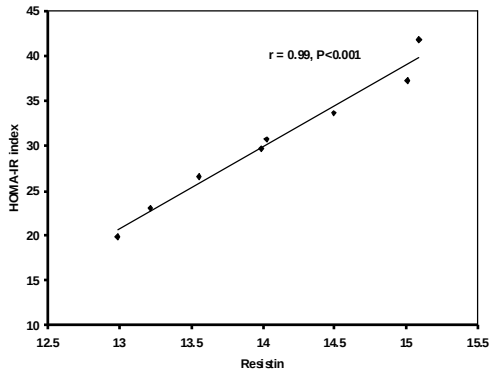

Figure (1): Correlation between serum resistin levels and HOMA IR index in group IIb.

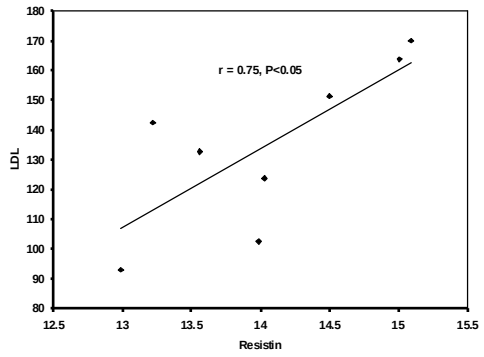

Figure (2): Correlation between serum resistin levels and serum levels of LDL in group IIb**.**

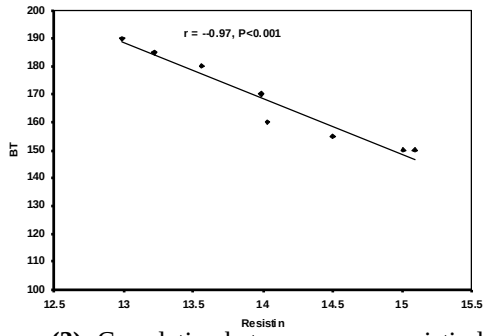

Figure (3): Correlation between serum resistin levels and bleeding time in group IIb.

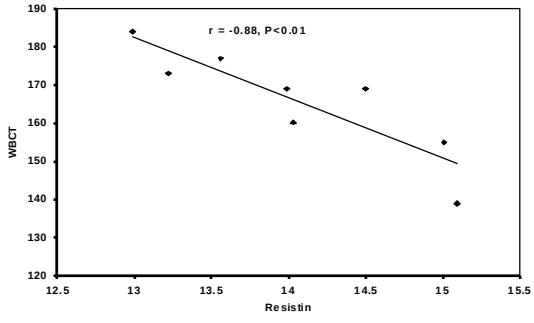

Figure (4): Correlation between serum resistin levels and WBCT time in group IIb.

The metabolic, hormonal and the haemostatic parameters of the groups are summarized in table 1&2, respectively. There was a significant positive correlation between the final body weight and serum resistin levels in all groups ($p<0.05$, $p<0.01$, $p<0.05$, $p<0.01$, $p<0.001$, respectively).

As regards group I, the results of this study showed a significant decrease in serum insulin and resistin ($p<0.001$) levels in group Ib in comparison with that of control group (Ia) in association with increased blood glucose levels ($p<0.001$).In addition, there was a significant increase ($p<0.001$) in the serum levels of TC, TG and LDL while the serum levels of HDL were significantly decreased ($p<0.001$) in the same group. Concerning the haemostatic parameters in group Ib, there was a significant decrease ($p<0.001$) in BT, WBCT, PT, aPTT while plasma fibrinogen levels, D-dimmers and platelets count were significantly increased ($p<0.001$). Moreover, all these metabolic and haemostatic disturbances were normalized in insulin treated group.

Finally, no significant correlation could be detected between serum resistin levels and metabolic, hormonal or haemostatic measured parameters in this group.

As regards group II, group IIb showed a significant increase($p<0.001$) in body weight, serum glucose levels, insulin levels, HOMA-IR , TC levels, TG levels, and LDL levels, in addition to the significant decrease($p<0.001$) in HDL levels in comparison with that of controls (IIa). Moreover, our study revealed a significant increase ($p<0.001$) in serum resistin levels in this group in comparison with that of controls, which was correlated positively and significantly ($p<0.001$) with body weight, serum glucose levels, insulin levels and HOMA-IR. Also, a significant positive correlation was found between serum resistin levels and serum levels of TC ($p<0.05$), TG ($p<0.01$) and LDL ($p<0.01$) while a significant negative correlation ($p<0.05$) between its levels and serum levels of HDL was reported.

Moreover, BT, WBCT, PT and aPPT were found to be significantly decreased ($p<0.001$), while, plasma fibrinogen and D-dimmers levels were found to be significantly increased ($p<0.001$) However, no significant difference in the platelets count was found. Furthermore, serum resistin levels were found to be correlated negatively and significantly with BT ($p<0.001$), WBCT ($p<0.01$), PT ($p<0.05$), aPTT ($p<0.01$) and positively with plasma fibrinogen ($p<0.01$) and D-dimmers ($p<0.05$) levels.

4. Discussions

The results of this study showed a significant decrease in serum insulin and resistin levels in streptozotocin-induced diabetic group in comparison with that of control group and insulin-treated group, in association with increased blood glucose levels. This decrease in serum resistin levels could be attributed to the weight loss that occurs in type1 diabetes, as resistin levels were positively and significantly correlated with the body weight in this study, which is in agreement with that of Stroubini et al. (2009).

In addition, our results indicated that there was a significant increase in the serum levels of total cholesterol, triglycerides and LDL while the serum levels of HDL were significantly decreased in streptozotocin-induced type 1 diabetes which is defined as an atherogenic lipid profile (Vergè, 2009).

Concerning the haemostatic parameters in group Ib, there was a significant decrease in BT, WBCT, PT, aPTT while D-dimmers, platelets count and plasma fibrinogen levels were significantly increased, denoting a hyper-coagulable state (Khatun et al., 1999). Moreover, all these metabolic (Sivitz et al., 1998) and haemostatic (Sobel, 2003, Nishikawa et al., 2008) disturbances were normalized in insulin treated group.

In addition, no significant correlation could be detected between serum resistin levels and either metabolic or haemostatic measured parameters in this group.

So, it can be concluded that resistin has no role in type 1 diabetes as regards heamostatic changes, however, these changes could be attributed to the presence of high levels of LDL particles, as these particles have atherogenic properties (Skyrme-Jones et al., 2000). Also, lowered HDL may play a role as HDL has antioxidative, anti-inflammatory, anti-thrombotic and vasorelaxant properties, all of which are potentially anti-atherogenic (Link et al., 2007). Therefore, it can be concluded that this dyslipidemia is associated with an increased cardiovascular risk in type 1 diabetes (Vergè, 2009).

As regards group HFD group, it was found to show marked increase in body weight, insulin resistance and dyslipidemia in rats. These results are in accordance with those of Willett, (2002) and Schaalan et al. (2009).

Moreover, our study revealed a significant increase in serum resistin levels in this group in comparison with that of controls, which correlate positively and significantly with body weight, serum glucose levels, insulin levels and HOMA-IR index, while no significant correlation could be found between its levels and those measured parameters in controls except a significant positive correlation with body weight.

These results are supported by that of Azuma et al. (2003) and Silha et al. (2003) who

reported that the mean circulating resistin levels in obese subjects is increased about four folds compared with lean subjects and by Stroubini et al. (2009), who reported that resistin levels were elevated in many experimental models of obesity and decreased after weight loss.

Moreover, de Luis et al. (2009) demonstrated that resistin concentrations were related to the total fat mass in patients with metabolic syndrome.

Concerning resistin-insulin relationship, our results are supported by those who concluded that, administration of antiresistin antibody improved insulin action and glucose metabolism in mice with diet-induced obesity (Steppan et al., 2001). While, infusion of recombinant resistin to rats rapidly induces hepatic IR and increases hepatic glucose production (Rajala et al., 2003). Also, ablation of the resistin gene in mice decreases fasting glucose through reducing gluconeogenesis, while resistin administration in these resistin-deficient mice increases hepatic glucose production (Banerjee et al., 2004).

Resistin primarily exerts its glucoregulatory effect by stimulating hepatic glucose output (Rangwala et al., 2004). As elevation of circulating resistin in rodents, either acutely (Muse et al., 2007) or chronically (as following diet-induced obesity) (Muse et al., 2004), leads to marked decreases in hepatic insulin sensitivity.

Moreover, the findings of this study are in line with that of Kushiyama et al. (2005), who found that transgenic mice with hepatic resistin over-expression exhibit significant hyperglycemia, hyperlipidemia, fatty liver, and pancreatic islet enlargement, when fed an HFD. These effects may be due to resistin-induced impairment of glucose homeostasis and insulin action, thus modulating one or more steps in the insulin signaling pathway and possibly playing a role in the pathogenesis of insulin resistance (Muse et al., 2004).

The majority of in vivo studies showed that resistin has a negative effect on insulin signaling in the liver (Qi et al., 2006).

In contrast with our results, no significant correlation was found between resistin levels and glucose levels in high fat-fed rats (Li et al., 2007), and also in patients with type 2 diabetes mellitus (T2DM) (Mojiminiyi and Abdella, 2007). Also, some studies have observed significant low resistin mRNA levels in adipose tissue in different obese mouse models, such as db/db, or high-fat-diet-induced obesity, and in rat models characterized by IR (Way et al., 2001).

The mechanism whereby resistin decreases insulin sensitivity involves several impacts. First, resistin reduces adenosine 5′-monophosphate-activated protein kinase activity in skeletal muscle, adipose tissue, and liver. In addition, insulin receptor substrate-1 (IRS-1) and IRS-2 protein levels and phosphorylation states, as well as protein kinase B activity, were decreased in hyperresistinemic animal tissues. These alterations decrease tissue insulin sensitivity that results in glucose intolerance, hyperinsulinemia, elevated free fatty acid (FFA) levels, and hypertriglyceridemia (Rajala et al., 2003).

Secondly, the resistin-induced reduction in IRS-1 and IRS-2 elevates mRNA levels of gluconeogenetic enzymes, such as glucose-6-phosphatase (G-6-Pase) and phosphoenolpyruvate carboxykinase, thus suggesting a direct resistin induction of insulin resistance in the liver (Moon et al., 2003).

Thirdly, it was found that resistin decreased glycogen synthase (GS) activity both in the presence or absence of insulin, this suggests that resistin directly down-regulates GS activity (Ferrer et al., 2003).

Insulin signaling in pancreatic islets plays an important role in the maintenance of β-cell functions and glucose-induced insulin secretion in islets of pancreas (Otani et al., 2004). Therefore, the inhibition of insulin signaling could underlie the impairment of glucose-induced insulin secretion by resisitn (Nakata et al., 2007).

Moreover, it was observed that there is a positive correlation between resistin levels and C - reactive protein (CRP) (Kunnari et al., 2006). Accordingly, the correlation between fasting glucose and resistin levels might be explained by this inflammatory state (Bo et al., 2005).

As regards the lipid profile in the high fat diet-fed group, our results revealed a significant increase in serum levels of total cholesterol, triglycerides and LDL, while serum levels of HDL were significantly decreased (atherogenic lipid profile). Also, a significant positive correlation was found between serum resistin levels and serum levels of total cholesterol, triglycerides and LDL while a significant negative correlation between its levels and serum levels of HDL was reported.

These results are supported by those of Mojiminiyi and Abdella (2007), who concluded that resistin was correlated positively and significantly with atherogenic lipid profile in type 2 diabetic patients, and in patients with metabolic syndrome (de Luis et al., 2009).

On the contrary to our results, Qi et al. (2008), found no significant correlation between resistin levels and lipid profile parameters except a negative correlation with HDL levels only in patients with metabolic syndrome.

Moreover, it was reported that resistin directly increases the endothelial expression of adhesion molecules, vascular wall adhesion molecule-1 (VCAM-1) and intracellular adhesion moleculer-1(ICAM-1) that play central roles in the early stage of the atherogenic processes (Burnett et al., 2005), it also promotes lipid accumulation in human macrophages by up-regulating CD36 cell surface expression, which is one of the scavenger receptors in macrophages involved in the uptake of modified LDL (Xu et al., 2006). Based on these data, resistin is supposed to induce atherosclerosis by mediating endothelial hyperactivity in response to the systematic inflammatory condition in human (Tsukahara et al., 2009).

At the cellular level, resistin has also been shown to exert potent pro-inflammatory properties by up-regulating pro-inflammatory cytokines, probably via the nuclear factor-κβ (NF-κβ) pathway, and resistin can induce inflammation in animal models (Stofkova, 2010). Besides this inflammatory induction, resistin also promotes proliferation and activation of human smooth muscle cells and endothelial cells, (Calabro et al., 2004) and induces angiogenic responses in endothelial cells, in part via phosphorylation and activation of different phosphate-kinase pathways (Mu et al., 2006).

Accordingly, it was reported that resistin may influence angiogenesis, not only in adipose tissue but also at other sites, so that systemic concentrations of resistin may contribute to the development of vascular disease (Robertson et al., 2009).

Hence, considering the expression of resistin by mononuclear cells and that obesity and T2DM are states of low-grade inflammation with activated inflammatory cascades, resistin may indeed present a molecular link between metabolic signals, inflammation and atherosclerosis (Kadoglou et al., 2007).

As insulin resistance is associated not only with hyperinsulinemia and hyperglycemia but also with other disorders such as abnormal lipid profile (Ding et al., 2005). These findings indicate a link between lipid profile and insulin sensitivity, since systemic excess of FFAs impairs the ability of insulin to stimulate glucose metabolism, contributing to whole-body insulin resistance (Schaalan et al., 2009).

In addition, several studies reported that resistin is implicated in the control of lipolysis (Rae et al., 2007). Also, in the humanized resistin mice, resistin was found to increase hormone sensitive lipase (HSL) activity by inducing white adipose tissue (WAT) inflammation and enhance the phosphorylation of HSL at its activating protein kinase-A (PKA) site (Qatanani et al., 2009).

Taken together, it could be postulated that the significant relationship between serum resistin levels and atherogenic lipid profile may be due to a direct effect in addition to induction of insulin resistance.

In relation to the haemostatic changes in the same group (group IIb), BT, WBCT, PT and aPTT were found to be significantly decreased, while, plasma fibrinogen and D-dimmers levels were found to be significantly increased (indicating hypercoagulable state). However, no significant difference in the platelets count was found. Furthermore, serum resistin levels were found to be correlated negatively and significantly with BT, WBCT, PT and aPTT and positively with plasma fibrinogen and D-dimmers levels.

Our findings are in line with those of other investigators who concluded that, resistin is an emerging cardiovascular risk factor implicated in T2DM (Kershaw and Flier, 2004). Furthermore, the patients with myocardial infarction showed higher plasma resistin levels especially those with coronary heart disease (CHD) when compared with the controls (Burnett et al., 2006).

Hyperfibrinogenaemia is associated with an increased prevalence and incidence of primary and recurrent CHD and thrombosis (Mc Dermott et al., 2003) and correlates with measures of obesity in several studies (Woodward et al., 1997).

Also, Menzaghi et al. (2006) reported a significant positive correlation between resistin levels and fibrinogen levels in insulin resistant patients.

Furthermore, it was found that, resistin has been shown to selectively impair the effect of insulin on endothelial nitric oxide synthetase (eNOS) enzymatic activity and indicate a mechanism through which resistin can reduce insulin-evoked vasorelaxation (Gentile et al., 2008).

Moreover, resistin induces serine protease (Akt)-dependent endothelial NO dysfunction through the inhibition of IRS-1 signaling pathway and IRS-1 itself is present in a lower amount in cells challenged with insulin and pretreated with resistin suggesting that resistin interferes with the insulin-stimulated IRS-1-dependent signaling pathway, acting both on the IRS-1 protein and on its ability to activate phosphatidyl inositol tri-phosphate kinase (PI3K) (Palanivel et al., 2006).

Moreover, in high fat diet-fed rats, resistin levels correlate negatively with vascular NO levels even after correction of insulin levels, which suggests a direct inhibitory role of resistin on NO secretion (Li et al., 2007). And since endothelial NO has a crucial role not only in modulating vascular tone but also in anti-atherogenic protection (Myazaky et al., 2003) by inhibiting inflammation, oxidation, vascular smooth

muscle cell proliferation and migration, it can be speculated that endothelial NO dysfunction induced by resistin could also participate to the enhanced atherosclerotic process that occurs in obese subjects (Gentile et al., 2008).

In vitro studies of Takahashi et al. (2006), who described that resistin activates endothelial cell directly by promoting endothelin-1 (ET-1) release and expression of ET Jung et al. (2006). In addition, Li et al. (2007) reported a significant positive correlation between resistin levels and ET levels in high fat-fed rats.

Furthermore, Li et al. (2007) concluded that chronic administration of resistin in rats produced a significant increase in plasminogen activator inhibitor-1 (PAI-1) and Von Wellebrand factor (VWF). Moreover, they also reported that, diet-induced hyperresistinemia in rats correlated positively with levels of PAI-1 and VWF even after correction of insulin levels. In addition, Qi et al. (2008) reported that, resistin was correlated positively with PAI-1 levels in women with metabolic syndrome. In fact, the increased levels of PAI-1 found in obesity may predispose to micro- and macro-vascular, arterial and venous thrombosis (Lundgren et al., 1996).

5. Conclusion:

No role for resistin in metabolic and haemostatic changes in type 1 diabetic rats was detected. Although, hyperresistinemia may represent a novel link between metabolic signals, atherosclerosis, and hypercoagulability in type 2 diabetic rats. However, further studies are needed to clarify this relationship in human cardiovascular diseases.

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Assessment of DNA Sensitivity and Heat Stress Protein Response (HSP70) in Male Wistar Rat Blood After Exposure to Microwave Radiation

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Abstract: Because of the increasing use of mobile phones, the possible risks of non-ionizing radiofrequency of electromagnetic fields (RF-EMF) adverse effects on human health was evaluated. The present study aims to assess possible DNA damaging effects induced by microwave radiation of mobile phone type in male Wistar rat blood in *vivo*. A number of 30 male Wistar rats (118±20g) was divided into 3 groups (10 animals in each group) exposed for (15, 30 and 60 min.) to a working Global System for Mobile Communication (GSM) cell phone rated at a frequency of 900 MHz, at non-thermal specific absorption rate (SAR) of 2.9 W/Kg. Concurrent control animals (n=10) were also included in the study. After the exposure periods five animals from each group were sacrificed immediately while the other five animals were sacrificed after 7 days (recovery period). DNA sensitivity in rat blood leukocytes was assessed by using the alkaline comet assay method. The heat shock protein stress response (HSP70) in serum samples of the rats was also investigated. The results showed significant increased DNA damage in blood leukocytes after the exposure times 15 and 30 min and after the three exposure times of the recovery period (7 days) as detected by the comet assay method. Serum HSP70 levels were also significantly increased in the exposed animals and in the animals at the recovery period as compared to the control animals. The present study indicates that RF-EMF represents a potential DNA-damaging hazards and using the alkaline comet assay is a sensitive tool in the measurement of DNA damage after exposure to 900 MHz microwave radiation in *vivo*. The increased HSP70 stress response to RF-EMF exposure might involved in protecting cells from DNA damage induced by microwave radiation. [Journal of American Science 2010; 6(8):568-575]. (ISSN: 1545-1003).

Key words: comet assay – DNA damage – microwave radiation – HSP70

1. Introduction

Microwave radiation is a type of non-ionizing electromagnetic radiation present in the environment and of potential threat to human health (Croft *et al.,* 2002). Today, non-ionizing radiation has increasingly been used in industry, commerce, medicine and for private purposes, especially in mobile telephone usage. Although the average exposure levels are low compared to the exposure limits, there is a growing public concern about the potential hazard of exposure to these frequencies for human health (BreckenKamp *et al.,* 2003; ICNIRP, 2004; Jauchem, 2008 and Garaj-Vrhovac *et al.,* 2009).

Genotoxic studies on microwave radiation in *vivo* and in *vitro* have yielded contradictory and often intriguing experimental results (Vijayalaxmi and Obe, 2004; Verschaeve, 2005). Some reports suggest that exposure of human cells to radiofrequency radiation does not result in increased genetic damage (Vijayalaxmi *et al.,* 2000; McNamee *et al.,*2003). In addition a number of studies have been conducted on animal models. These studies have also given contradictory results regarding exposure to microwave radiation (Witt *et al.,*2000; Vijayalaxmi *et al.,*2003; Paulrai and Behari, 2006).

Epidemiological studies have demonstrated that, there is a link between microwave exposure and excess of cancer, leukemia and brain tumors (Maskarinec *et al.,* 1994; Szmigielski, 1996). Other investigated health outcomes include spontaneous abortions, lenticular changes, neurological and sensitivity reactions, haematological or chromosome changes occurring in certain populations exposed to microwave radiation (Zotti-Martelli *et al.,* 2000; Trosic *et al.,* 2004). On the other hand, there is a range of studies showing that radiofrequency radiation can induce genetic alteration after exposure to microwave radiation (Garaj-Vrhovac *et al.,* 1990 and 2009; Fucic *et al.,* 1992; Maes *et al.,* 1993; Zotti-Martelli *et al.,* 2000; and Tice *et al.,*2002).

The comet assay is now a well-established genotoxicity test for the estimation of DNA damage at the individual cell level both in *vivo* and in *vitro.* The comet assay has widely been used to detect primary biological effects on the level of DNA molecule in human and animal cells exposed to

several environmental or occupational substances (Collins *et al.*, 1997; ESCODD, 2003; Kumaravel *et al.*, 2009 and Garaj-Vrhovac and Oreščanin, 2009). Therefore, this method makes it possible to evaluate the level of primary DNA damage even after short-term of exposure to irradiation.

Living cells have mechanisms to maintain homeostasis and the activation of heat shock proteins (heat shock/stress protein) as a normal defense response to cellular stress (Santoro, 2000; Nollen and Morimoto, 2002) such as the non-thermal response to microwave radiation (Goodman and Blank, 1998 and 2002). Also, certain previous studies have shown stress response proteins and particularly HSP70 to be activated by the electromagnetic radiation emitted from mobile phones (de Pomerai *et al.*, 2000 and Weisbrot *et al.*, 2003).

The aim of the present study, was to assess possible DNA damaging effects induced by 900 MHz microwave radiation of mobile phone type in male Wistar rat blood in *vivo*. For this purpose the alkaline comet assay method as a sensitive tool in detecting primary DNA damage is used. In addition, the cell stress response to microwave radiation was determined by measuring the HSP70 in blood of male Wistar rats.

2. Material and Methods

Animals

Male Wistar rats weighing about 118±20g were obtained from Helwan Farm for Vaccine and Biological Preparations. The animals were housed in cages 10 animals in each for one week before the beginning of the experiment. The animals were maintained on 12h dark/light cycle and were given food and water *ad libitum*.

Method of exposure

After the acclimation period, animals were randomly divided into four groups, 10 animals per group. The 1st group was used as a control group. The 2nd, 3rd,and 4th groups were exposed to EMF for 15, 30 and 60 minutes respectively. Five animals from each group were sacrificed immediately after exposure while the rest of the animals were sacrificed after a recovery period of 7 days. Control animals were housed in separate cages with the same conditions as the exposure groups but without power input.

During irradiation, each animal was placed in its own Plexiglas cage (25 cm x 7.5 cm x 7.5 cm). For EMF exposure, a cell phone in the "on" mode was placed with its antenna over the center of the cage. The cell phone was manufactured by Nokia (model 6300 type RM-217, GSM 900MHz). The power density of the field was 0.35 W/m^2, corresponding to a whole-body specific absorption rate (SAR) of 2.9 W/Kg. Control animals were performed similarly as the exposed group and the cell phone is in the switched off´ mode.

Blood sampling

After the exposure period of 15, 30 and 60 min blood samples were collected immediately and after recovery period of 7 days. One rat at a time was anesthetized by placing it in a glass jar containing cotton dipped in anesthetic ether. The rats were then decapitated and blood samples were collected from dorsal aorta under sterile conditions in heparinzed tubes and kept at -4°C until analysis. A part of the blood (2ml) were centrifuged for 15 min at 4000 r.p.m. and serum was stored at -4°C until analysis.

Single cell gel electrophoresis (comet assay)

To measure the potential DNA damaging effect of microwave radiation in single Wistar rat leukocytes, the comet assay was carried out as described by Jaloszynski and Szyfter (1999). A total of 50 cells were examined in this experiment. For each cell, the length of DNA migration (comet tail length) was measured in micrometers from the center of nucleus to the end of the tail. The percentage of damaged DNA concentration in the comet tail was determined by measuring the total intensity of ethidium bromide fluroscence in the cells, which was taken as 100% and determining what percentage of this total intensity correspond to the intensity measured only in the tail.

Determination of serum HSP70 levels

The levels of HSP70 (pg/ml) in the serum samples were determined according to the method described by Oc *et al.* (2008) using ELISA Kit (DUOSEt® IC, US). One hundred µL of each 1:5 (v/v) diluted serum samples and prepared standards were applied to each well precoated with a mouse monoclonal antibody specific for inducible HSP70, the plate was sealed and incubated at room temperature for 2 h on an orbital shaker. After washing six times with wash buffer, the captured HSP70 was detected by 100 µL HSP70-specific, biotinylated rabbit polyclonal antibody, the plate was sealed and incubated at room temperature for 1 h on an orbital shaker. After washing six times with wash buffer, the biotinylated detector antibody was subsequently bound by 100 µL avidin-horseradish peroxidase conjugate. The colour was developed by an addition of 100 µL of tetramethylbenzidine substrate and allowed to react for 15 min. To terminate reaction, 100 µL stop solution was added and the intensity of the colour was measured at 450 nm.

Statistical Analysis

Data were expressed as a mean ± standard error (SE). Differences between the control and treated groups were tested with unpaired student t-test. Statistical differences between control and exposed rats are as follows ⋆P<0.05, ⋆⋆P<0.01 and ⋆⋆⋆P0.001.

3. Results

The results of the comet assay parameters (mean tail length and mean DNA%) as well as the mean serum levels of HSP70 performed on rat blood at the three different times of exposure to 900 MHz EMF as compared to that of the control animals are summarized in (Table 1)and (Fig.1).

By comparing the values of mean tail length of the comet assay of the exposed and control animals, it is clear that, mean ±SE of tail length of the comet assay showed significantly increased levels (P<0.05 and P<0.00) after 15 and 30 min. of exposure respectively (Fig.1 B). While, the mean tail length of the comet assay showed insignificantly increased values after 60 min. of the exposure period.

On the other hand, the damaged DNA% showed significantly (P<0.05) and (P<0.01) increased levels after 15 and 30 min. of the exposure period, while it increased insignificantly after 60 min. of the exposure period.

After exposing male Wistar rats to 900 MHz EMF for (15, 30 and 60 min.), the mean levels of serum HSP70 increased significantly in all the three times of exposure (Table 1).

When comparing both comet assay parameters (mean tail length and damaged DNA%) in the mobile phone exposed rats and the control ones after 7 days recovery period (Table 2) and (Fig.2), it showed significantly increased levels (P<0.001 and P<0.01) after the three exposure times (15, 30 and 60 min.) respectively (Fig.2 C), except for the damaged DNA% at the exposure time 30 min. which showed insignificantly increased levels as compared to the control animals.

The mean levels of serum HSP70 increased significantly (P<0.001 and P<0.05) after the exposure times 15, 30 and 60 min in animals sacrificed immediately and after the recovery period (Table 2).

Table 1. Comet assay parameters (mean tail length and mean % of DNA damaged ±SE) and mean ±SE of HSP70 levels performed on rat blood at different exposure times to 900 MHz microwaves as compared to the control group.

Parameter	control	15 min	30 min	60 min
Tail length(µm)	0.645±0.06	⋆2.752±0.28	⋆⋆⋆2.085±0.13	1.456±0.35
% of DNA damaged	0.414±0.05	⋆1.410±0.13	⋆⋆1.321±0.15	0.990±0.58
Hsp70(Pg/ml)	115.630±0.54	⋆⋆⋆156.188±0.97	⋆⋆⋆126.908±0.98	⋆123.859±2.06

Results are means ± SE of 5 animals.
Statistical differences between control and exposed rats as follows:⋆ P< 0.05 (significant), ⋆⋆P<0.01 (highly significant), and ⋆⋆⋆P<0.001 (more highly significant).

Table 2. Comet assay parameters (mean tail length and mean DNA% ±SE) and mean ±SE of HSP70 levels performed on rat blood at different exposure times to 900 MHz microwaves and kept for 7 days recovery period as compared to the control group.

Parameter	control	15 min	30 min	60 min
Tail length(µm)	0.820±0.12	⋆⋆⋆7.998±0.23	⋆⋆3.269±0.23	⋆⋆4.249±0.50
% of DNAdamaged	0.520±0.07	⋆⋆⋆3.383±0.07	1.615±0.41	⋆.2.351±0.44
Hsp70(Pg/ml)	115.636±0.54	⋆⋆⋆173.974±05.26	⋆120.734±1.32	⋆⋆⋆171.877±4.50

Results are means ± SE of 5 animals.
Statistical differences between control and exposed rats as follows:⋆ P< 0.05 , ⋆⋆P<0.01, and ⋆⋆⋆P<0.001

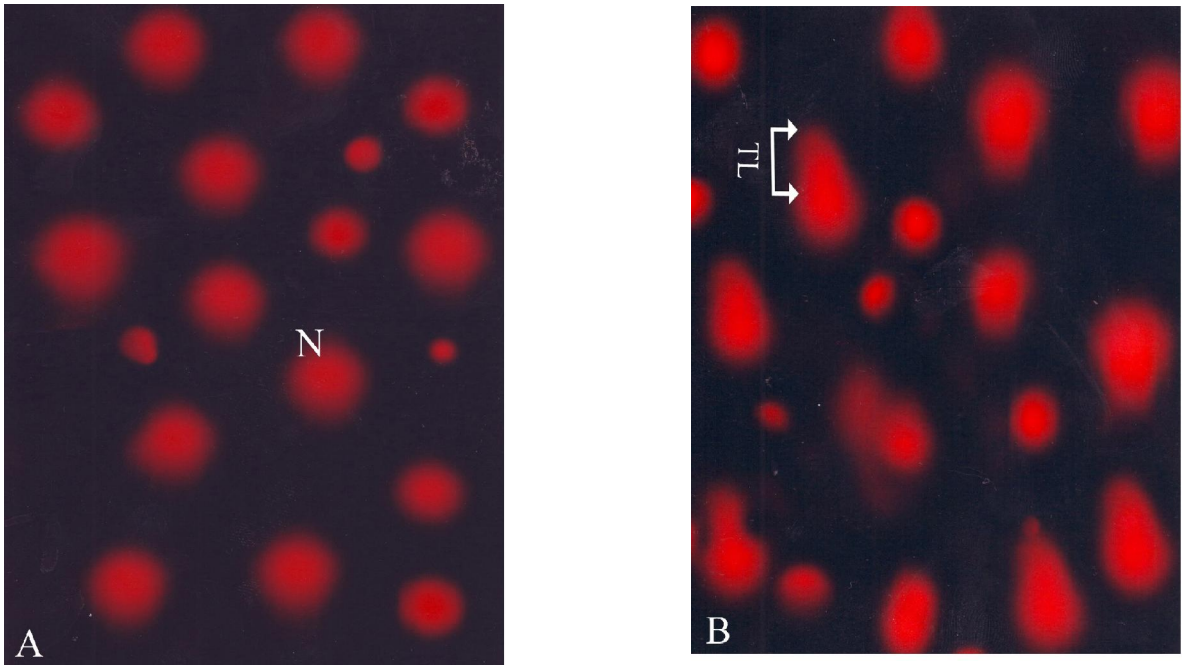

Fig.1. Photograph of male Waster rat leukocytes showing DNA damage (tail length) after exposure to 900 MHz microwave radiation at three different times (15, 30 and 60 min.) as compared to the control group. (A) Leukocyte nuclei from control as established by comet assay method. (B): Damaged spot of DNA. N= Normal nuclei. TL= Comet tail length of migrated DNA.

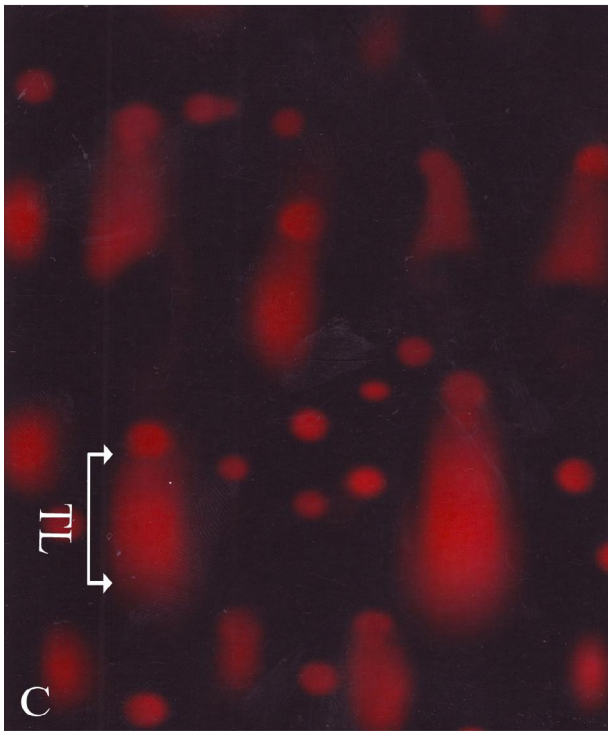

Fig.2. Photograph of male Waster rat leukocytes sacrificed after 7 days recovery period showing DNA damage (tail length) after exposure to 900 MHz microwave radiation as compared to the control group time. (C) Strong damaged spot of DNA. TL= comet tail length of migrated DNA.

4. Discussion:

A large number of experimental and epidemiological studies have been carried out to elucidate the possible health hazards associated within human exposure to RF-EMF. Many studies have provided evidence suggesting that EMF with relatively low intensity are capable of interacting with many molecular, cellular and systemic processes associated with carcinogenesis and teratogenesis (Juutilainen and Lang, 1997). Biologically detri mental effects of the EMF has not yet been sufficiently explored, and the existing data are often contradictory. Because DNA damage is closely related to every aspect of physical and pathological activity of cells, one of the most active areas RF-EMF investigation is the assessment of direct and indirect effects on DNA (Brusick *et al.,* 1998). The single-cell gel electrophoresis (comet assay) is the simplest and most sensitive method that can measure and identify DNA damage at the cellular level (Tice *et al.,*2000). This technique has been widely used for investigations of the possible DNA damage induced by RF-EMF but the results have been contradictory.

In the present study, the alkaline comet assay method was used to test whether microwave radiation can induce DNA damage in male Wistar rat leukocytes after in *vivo* exposure to 900 MHz microwave radiation for 15, 30 and 60 min of exposure. The results showed a significant increase in the comet assay parameters after the exposure times (15 and 30 min) which indicate the presence of DNA damage. Thus the results of the present study demonstrated that the alkaline comet assay can be successfully applied to study microwave-induced DNA damage in rat leukocytes. By using the alkaline comet assay in exposed animals, a lot of DNA single-strand breaks and alkali-labile sites were detected. Similar results were reported by Lai and Singh (1995 and 1996) on rat brain cells immediately and at 4 h of acute exposure to RF-EMF. Their results indicated that acute exposure to microwave radiation at an average body SAR of 1-2 W/Kg caused a significant increase in both single and double-strand DNA breaks and suggested that this could be due to either a direct effect on the DNA and/or an effect of the radiation on DNA repair mechanisms. However, the study of Malyapa *et al.*(1998) did not obtain the same results as Lai and Singh. The poor replicability and contradictory findings may result from the different specifications of EMF, both of which influence the interactions with living cells (Roti *et al.,* 2001). The ability to repair DNA lesions is a ubiquitous defense mechanism that is essential for cell survival and the maintenance of cell functions. Different cells have different DNA repair capacities due to genetic differences (Schmezer *et al.,* 2001).

It should be emphasized that the type of DNA damage measured by alkaline comet assay is rather continuously and efficiently repaired. Therefore, the increased DNA damage observed in the present study after one week of exposure at the different three times is as a result of equilibrium between damaged infliction and repair. It is known that DNA single-strand breaks are rapidly repaired; however, other lesions such as oxidized bases, may persist longer and be misrepaired (Collins *et al.,* 1997). On the other hand, apurinic/apyrimidinic sites (alkali-labile sites) may not be readily repaired and without DNA replication, they may prove to be "silent" lesions (Gichner *et al.,* 2000). It was reported that DNA damage accumulates with time and its repair capacity decreases over time. When DNA damage is not repaired or is improperly repaired, biological effects result. Although majority of DNA lesions are repaired in few hours and days after their infliction, a part of DNA damage induced persisted over time. It can be considered that this elevated level reflects an accumulation of non-repaired DNA damage.

Extensive damage to DNA can lead to cell death. Large number of cells dying can lead to organ failure and death for the individual. Damaged or improperly repaired DNA may develop into cancers. Data on the possible cancer-related effects of microwave radiation are still controversial. However, it is possible that in complex cellular processes involved in carcinogenesis it could have co-carcinogenic effects (Verschaeve and Maes, 1998).

The results of the present study are in agreement with those of (Gichner *et al.,*2000; Trosic *et al.,* 2002 and 2004; Zotti-Martelli *et al.,* 2005; Lixia *et al.,* 2006; Garaj-Vrhovac and Oreščanin, 2009 and Garaj-Vrhovac *et al.,* 2009).

Cells respond to abnormal physiological conditions by producing protective heat shock (or stress) proteins. Heat shock proteins are an important group of cell response proteins. They act primarily as molecular chaperones to eliminate unfolded or miss-folded proteins, which can also appear from cellular stress. This stress response can be induced by many different external factors including temperature, oxidative stress, ionizing and non-ionizing radiation (Ramage and Guy, 2004). It has been reported that HSP70, one of the most studied HSP families is induced with exposure of human epithelial cells to GSM signal of 960 MHz at a SAR of 0.0021 W/Kg for 20 min (Kwee *et al.,* 2001).

Some investigators described an increased HSP level after RF-EMF exposure (de Pomerai *et al.,* 2000; Leszczynski *et al.,* 2002), however, these results are controversial, because of some other negative findings (Cotgreave,2005). The present study indicated that there was a significant increase in

serum HSP70 after the three times of exposure to RF-EMF at SAR 2.9 W/Kg. This increase was persisted also after one week of exposure. Thus the current study conformed a cellular protective response as a result of microwave exposure. Fritze *et al.*(1997) examined the levels of HSP70 m-RNA in rat brain after exposure of animals to EMF from mobile phones and found that SAR of 7.5W/Kg induced HSP70 m-RNA in cerebellum and hippocampus. However, there is also a contrasting reports that radiofrequency radiation were not able to induce the HSP70 response and others speculated that temperature increase not microwave radiation may cause the induction of heat shock gene expression (de Pomerai *et al.*, 2000 and Dawe *et al.*,2006). So, the increase of HSP70 observed in this study might be involved in protecting the cells from DNA damage induced by microwave radiation of the mobile phone exposure.

The results of the present study, are in agreement with those of Fritze *et al.*(1997); Danilles *et al.*(1998); de Pomerai *et al.*(2000); Kwee *et al.*(2001) Lixia *et al.* (2006) and Simkó *et al.* (2006).

In conclusion, the results obtained in the current study demonstrate the presence of DNA damage and heat shock response in male Wistar rat blood due to acute non-thermal exposure to 900MHz RF for GSM. The results also indicated that the alkaline comet assay, as a reliable biomarker of exposure, can be successfully applied in the study of DNA-damaging effects. From a public health standpoint, it is important to re-evaluate the present RF-EMF safety limits proposed by the International Commission on Non-Ionizing Radiation Protection (ICNIRP). Moreover, longer and chronic exposures should be tested to see if the effect could evolve in a permanent effect on the cells or not.

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Effect of Soil Water Content and Salinity on Daily Evaporation from Soil Column

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Abstract: The paper was presented in order to demonstrate the performance of the maximum surface temperature model (MSTM) developed for estimating soil evaporation based on surface energy balance and the periodic various in soil surface temperature. In this study, to minimize the influence of meteorological factors on evaporation, a relative evaporation (*RE*) was used to demonstrate the change in the estimated evaporation from non-saline and saline soils with differ initial soil water content. It was a positive correlation between the *RE* and soil water content, which indicated that the MSTM may be affected by the soil water. And the evaporation from saline soil was less than that from non-saline soil, which was attributed to the effect of salt accumulating on soil surface reducing the evaporation. At the same time, the MSTM can also show the stage of soil evaporation during entire observation. Therefore, it is indicated that the MSTM may be used to predict the daily evaporation by using only soil surface temperature. [Journal of American Science 2010; 6(8):576-580]. (ISSN: 1545-1003).

Keywords: Non-saline and saline soils; Soil surface temperature; Soil evaporation; Soil water content

1. Introduction

In order to maintain the sustainable development of irrigated agriculture in arid region, an accurate knowledge of water loss from soil is necessary. In general, the main routes of soil water loss are evaporation and transpiration. The soil evaporation and crop transpiration are often considered together as the evapotranspiration (ET), yet each part is variable portion to water balance in farmland scale. In many parts of the world the available water resources are presently being trapped close to the limit, so that an accurate knowledge of the consumptive use through evaporation is indispensable (Brutsaert, 1982).

In arid irrigated regions, such as an agricultural area of Hetao, which is located in a cold and arid region in China, there is a contradiction of water supply and demand owing to the rapid development of regional economy and shortage of water resources in recent years. And most of saline lands are distributed in the district. The saline soils are considerably resulted from the irrigated water containing salinity and the high evaporation in the district. However, the evaporation not only makes water loss but also may induce secondary salinization in the district. Also, plant growth is considered as a function of the total soil moisture stress. These are the sum of the soil moisture tension and the osmotic pressure of the soil solution (U.S. Salinity Laboratory Staff, 1954).Thus, such as regions will have to face an important challenge for increasing food production in order to realize food security for a growing population while optimizing the use of limited water resources (FAO, 1994) and controlling the salinization of soil.

To avoid the salt accumulation in soil, the leaching irrigation (locally named autumn irrigation) needs to be implemented at October every year in the district. Now, the district is facing the water resources shortage and the soil salinization problems. Some studies (Shi, H. B., Akae, T., et al. 2002; Akae, T., et al. 2004, 2008) have concerned with these problems for sustainable irrigated agriculture in the district.

Soil evaporation is affected by most of factors in natural environment, which includes mainly two aspects: climate aspect (net radiation, wind speed, air temperature, etc) and soil aspect (soil types, soil water content and temperature, hydrological properties, soil salinity, etc). The processing of soil evaporation is often divided into two or three stages based on the evaporative capacity of atmosphere and available water in soil for evaporation (Lei, et al. 1988; Jury, W. A., Horton, R. 2004). For soil temperature aspects to evaluate of soil evaporation, some studies (Ben-Asher, 1983; Evett, 1994; Qiu, 1999) have been addressed. To the larger scale, using the soil temperature to estimate of evaporation may be concerned greatly in the future due to the available temperature data from satellite image. In general, the soil water is considered as a major of factor affecting evaporation directly. In fact, the surface temperature may be considered as the indirect factor on soil evaporation. Thus, the aim of this study is carried out to show the performance of

the developed model for estimating evaporation only with surface temperature.

2. Theoretical background

Under neglecting the energy stored in soil, the soil surface energy balance can be expressed as

$$R_n = H + G + \lambda E \quad (1)$$

Soil energy balance for each soil column can be written based on the equation (1) as follows:

Control soil column: $R_{no} = H_o + G_o$ (2)

Wet soil column: $R_{nd} = H_d + G_d + \lambda E_d$ (3)

where, R_n, H, G and λE are the flux density of net radiation, sensible heat, soil heat and latent heat. The subscripts o and d refer to the control and the wet soil columns, respectively. The λ is the latent heat of vaporization (2.45 MJkg-1).

The net radiation term (Brutsaert, 1982) is also given by:

Control soil column: $R_{no} = R_s(1-\alpha_{so}) + R_{ld} - R_{luo}$ (4)

Wet soil column: $R_{nd} = R_s(1-\alpha_{sd}) + R_{ld} - R_{lud}$ (5)

where R_s is global short-wave radiation, αis albedo of soil surface, R_{ld} is the downward long-wave radiation, R_{lu} is the upward long-wave radiation.

The long-wave radiation can be written by:

$$R_l = \varepsilon_a \sigma T_a^4 \text{ (or } \varepsilon_s \sigma T_s^4) \quad (6)$$

where ε_a is the emissivity of air, ε_s is the emissivity of soil surface, σ is the Stefan-Boltzmann constant (5.67×10-8 W m-2K-4), T_a and T_s are air and soil surface temperature, respectively.

Sensible heat flux in energy balance can be written as follows:

Control soil column: $H_o = \rho C_p c_h (T_o - T_a)$ (7)

Wet soil column: $H_d = \rho C_p c_h (T_d - T_a)$ (8)

where ρ is air density, cp is specific heat of air at constant pressure, T_o, T_d and T_a refer to surface temperature for control and wet soil column and air temperature (Kelvin). The c_h is the exchange coefficient for sensible heat flux (m/s).

Soil surface temperature is supposed as the change in sine function in equation (9):

$$T(0,t) = 0.5(T_{\max} + T_{\min}) + 0.5(T_{\max} - T_{\min})\sin(\omega t - \theta) \quad (9)$$

here $T_{\max}$ and $T_{\min}$ are the maximum and minimum surface temperature of soil column.

Soil evaporation was estimated based on a maximum surface temperature model (MSTM) in equation (10), the detail procedures of developed model is not presented here:

$$E_d = 7.2(\rho C_p c_h + 4\varepsilon\sigma T_{\min}^3)\Delta T_{\max}/\lambda \quad (10)$$

where E_d is soil evaporation estimated (mm/d), $T_{\min}$ is the minimum surface temperature for both of control and wet soil columns (from observed temperature, the $T_{\min}$ is almost equal for the control and wet soil columns), in this study, the c_h given 69.45 m/h, $T_{\min}$ taken 281.3 K. $\triangle T_{\max}$ is the difference in maximum surface temperature of control and wet soil columns.

3. Material and Methods

The experiment was conducted in the Water Cycle Facility (34˚41′ N, 133˚55′ E, and 4 m elevation), Okayama University Japan. The experiment site is located in the 21 m distance from south of the weather station. A loamy soil was collected from Hetao Irrigation District, China. The undisturbed soil were sampled by excavation in field, and the disturbed soil materials were also collected and then brought to laboratory for air dry and pass a 2 mm sieve. The loamy soil was divided into two parts based on the level of salinity ($EC_{1:5}$) in 1:5 air-dry soil/distilled water extracts, one was non-saline loam (LN, $EC_{1:5}$=0.6mS/cm); another was a saline loam (LS, $EC_{1:5}$=4.9mS/cm). The air-dry soil was moistened to desired initial gravimetric soil water content by adding distilled water. The initial soil water content was 30% for higher moisture (LNW, LSW), 12% for lower moisture (LND, LSD), and next to 0% (CT) for control treatments, respectively. These soil materials were packed into plastic buckets (15 cm length, 9.8 cm top inside diameter and 9.2 cm basal inside diameter) with the wall thickness of 0.2 cm in the bulk density of 1.46g/cm^3 (non-saline loam) and 1.48g/cm^3 (saline loam), respectively. The plastic buckets were packed in 2.5 cm increments for uniform distribution along the whole soil column length. At the same time, the soil temperature cables were carefully embedded at 12.5 cm, 10 cm, 7.5 cm, 5 cm, and 2.5 cm soil depth and surface layer to measure the soil profile temperature and then temperature recorders (TR-52S, T&D Co.) were packed together in a plastic box. These plastic buckets were called soil columns or the mirco-lysimeter (ML).

The surface temperature was determined by using infrared thermometry (Raynger ST, Raytek, Co.). The 5 points were randomly taken on each surface soil column. The surface temperatures were measured and recorded the average of five readings as the surface temperature.

Soil columns were put into cylinders (15.5 cm length, 10-cm diameter) with 0.5 cm thickness of foam sheet for insolating heat conduction. The tops of the soil columns and cylinders were maintained the same level with field surface. Weigh of soil columns were weighed daily at about 9:00 by using a precision of 0.1g with electronic balance (ELB 3000, Shimadzu Co.) Soil water content was calculated based on water balance with the daily weigh of soil column. Soil water retention curves (pF-moisture curve) of saline and non-saline loam were measured

by pressure plate (DIK-3400, Daiki Rika Kogyo Co., LTD)

4. Results and discussions

The change in soil water content in each soil column was decreased with time under theoretical considerations. Thus, the soil water content in each soil column based on water balance is presented in Figure 1.

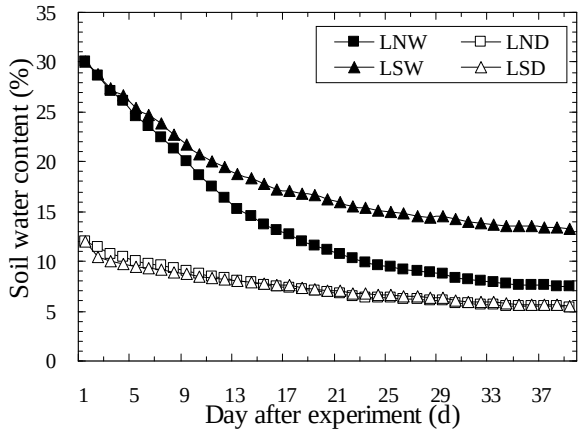

Figure 1. Change in Soil Water Content during Observation

Figure 1 gives that the change in soil water content under daily evaporation conditions from the saline and non-saline soils with different initial soil water levels. The curves in Figure 1 shows that the soil water content rapid decreases with time under high initial water contents at the begin periods of experiment, when the water contents reached to certain values, the change trend becomes smaller and reaches to steady state at the end of experiment, these values were about 10% for LNW, 15% for LSW; at low water content conditions, the decrease in soil water content is smaller for LND and LSD. The differences in soil water content between LNW and LND, LSW and LSD, the main reasons were attributed to different initial water content. The difference in soil water content between LNW and LSW was owed to the impact of salinity on soil water movement.

In general, the range of pF value from1.5 to 4.2 is often considered available water for plants (Yahata, T., et al. 1983). The available water may influence the soil evaporation. In order to demonstrate the event, we made the pF-moisture curves of studied soils, which are presented in Figure 2.

Figure 2 indicates that the water content is slightly larger for saline soil than non-saline soil during the range of the available water, which results to the reducing in soil evaporation from saline soil. It was also indicated that the water hold capacity in saline soil was larger than non-saline soil, which may affect the soil evaporation. These are consistent with observation of the experiment. The water hold capacity of soil is demonstrated by soil water retention curves in next section.

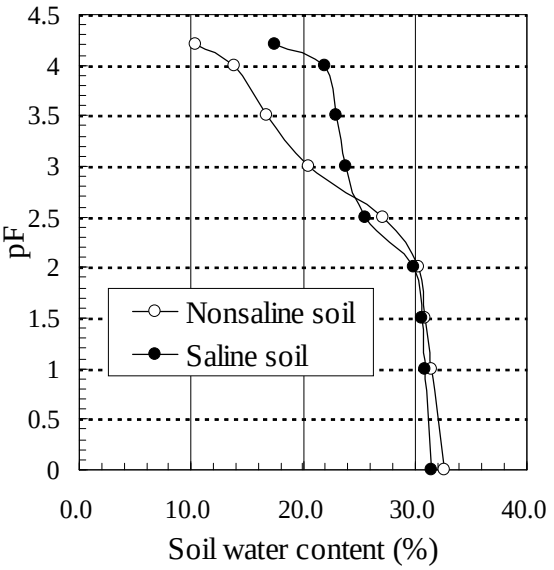

Figure 2. Soil Water Retention Curves for Non-saline and Saline Soils

In order to quantify water content effects on evaporation, we used the Van Genutchen's equation (Van Genutchen, M. T., 1980):

$$\theta = \theta_r + \frac{(\theta_s - \theta_r)}{\left[1+(\alpha h)^n\right]^m} \qquad (11)$$

where θ_r and θ_s are residual water content and saturated water content, h is pressure head, α, n and m are curve-fitting parameters. The parameters were fitted by using RETC (Van Genutchen, M. T., 1991).

Due to the same soil type for nonsaline and saline soil, the fitting parameters are similar, which are listed in Table 1.

Table 1. The Results of Fitting Soil Water Retention Curves

	θ_r kg kg^{-1}	θ_s kg kg^{-1}	α cm^{-1}	n	m#
Loam	0.078	0.43	0.036	1.56	0.35

m =1-1/n

In order to minimize the influence of meteorological factors on evaporation, we used the relative evaporation (*RE*) to demonstrate the change in the estimated evaporation under different soil water content. It is shown in Figure 3.

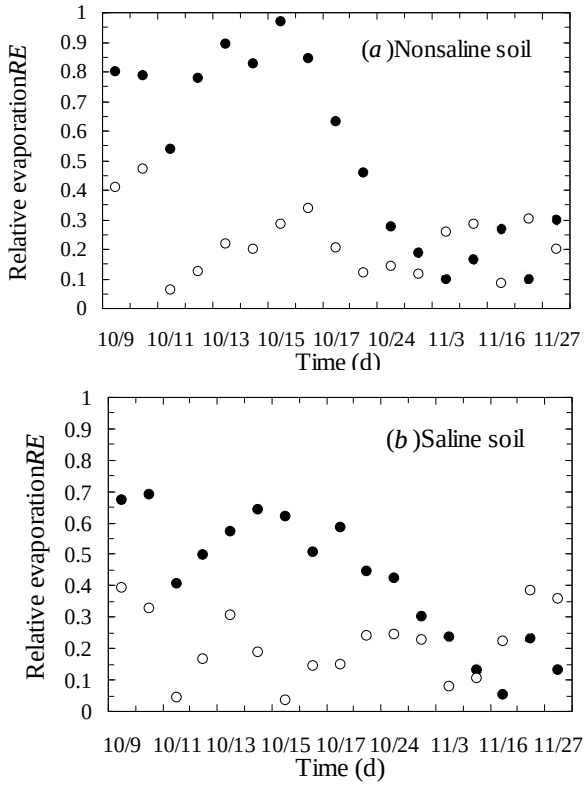

Figure 3. Change in Relative Evaporation under Different Soil Water Content with Non-saline (*a*) and Saline (*b*)

Figure 3 shows the change in *RE* estimated from the MSTM under different initial soil water content for non-saline and saline soil. The closed and open circles in Fig.3 refer to the evaporation under high initial soil water (30%, pF=1.8) and low soil water content (12%, pF=4.2) conditions, respectively. The difference in evaporation rate was attributed to the effect of salinity under the same water content and meteorological conditions. In Figure 3 can be seen the processing of evaporation. The dashed made off the different stage of soil evaporation based on change performance of estimated *RE*. At the first one week period of experiment, the evaporation of soil was in the first stage, this period the soil evaporation is major affected by the meteorological factors. After then, when the evaporation come into the second stage (falling stage), this period the soil evaporation is mainly controlled by soil-self water content. It is indicated that the developed model can determine the evaporation stages: the first stage of evaporation was during the first 7 day period of experiment (in Figure 3*a*) corresponding the soil water content was more than 22.5% (pF=2.0) (in Figure1), the second stage was from 7th to13th day after experiment (in Figure 3*a*) corresponding soil water content was from 22.5% to 10.3% (pF from 2.0 to 2.8)(in Figure 1) for non-saline soil; the first stage was the first 6-day period of experiment (in Figure 3*b*) corresponding soil water content was more than 25.4% (pF=1.9) (in Figure 1), the second stage of evaporation was from 6th to 14th day after experiment (in Figure 3*b*) corresponding soil water content was from 25.4to 15.5% (pF from 1.9 to 2.3) for saline soil.

For further demonstrate the developed model can described the effect of soil water content on evaporation, the relationships of the relative evaporation and soil water content are presented in Figure 4.

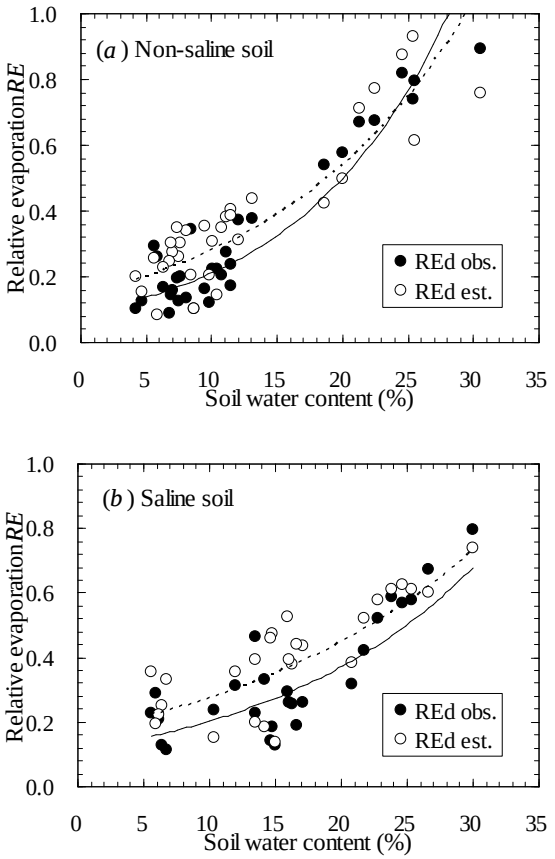

Figure 4. The Effect of Soil Water Content on Relative Evaporation under Nonsaline Soil (*a*) and Saline Soil (*b*)

Figure 4 shows the agreement in the observed and estimated *RE* along with the change in soil water content. The *RE* is decreasing with the reducing in soil water content, which means the evaporation rate decreasing with the reducing soil water content. At the higher soil water content, the *RE* estimated was close to the *RE* observed. At lower soil water content, however, the *RE* estimated overestimated the *RE* observed. The reasons may be that the $\bar{c}_h$ was used for the all of soil water range and the small evaporation itself at lower soil water content.

Especially, the efficiency of developed model was greater the non-saline soil than the saline soil. Therefore, the developed MSTM can describe the effect of soil water content on evaporation and be hopeful to be used to large scale for estimating soil evaporation only using surface temperature.

5. Conclusion

Some results obtained from this study concluded that are 1) the evaporation from salt-affected soil was less than that of non-saline soil under the same climate condition, the main reasons may be due to the effect salt accumulating on soil surface reducing the soil evaporation, 2) the developed model (MSTM) also can reveal the stage of soil evaporation and, 3) the simple model developed can describe the effect of soil water content on evaporation.

The simple MSTM is hopeful to be used to estimate soil evaporation for large scale only by using surface temperature.

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