Augmenting Anticancer Potential of Exotoxin A By Mutating *Pseudomonas aeruginosa.*

Yehia A. O. Ellazeik[1], Samah S. Abdelgawad[2], Essam MA Elsawy[2], Ahmed M. El-Wassef[3].

[1]Department of Botany, Faculty of Science, Mansoura University.
[2]Microbiology lab, Urology and Nephrology Center, Mansoura University.
[3]Department of Chemistry, Faculty of Science, Mansoura University.
yaolazeik@mans.edu.eg

Abstract: Cancer is a group of more than 100 different and distinctive types of diseases. It is an abnormal growth of cells which tend to proliferate in an uncontrolled manner forming masses and in some cases dislodge and spread all over the body. Luckily, a number of bacteria including *Pseudomonas aeruginosa* produce some virulence factors that help in combating cancer such as exotoxin A (ETA). This toxin arrests protein synthesis and induces apoptosis in cancer cells. During this study 68 Pseudomonades were isolated from urine samples collected from patients with urinary tract infection during the period Sept. 2009- Feb. 2010. Classical bacteriological, molecular and automated methods were used to identify all them. Based on the protein banding patterns, the 68 isolates were re-grouped to 30according to the results of Sodium dedocylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), from these 30 isolates only three gave the typical PCR product for Exo A gene using its specific primers. ETA production and toxicity were enhanced by mutating the three wild-type isolates. The crude and partially purified ETA from the selected three wild-type isolates of *Pseudomonas aeroginosa* (1, 8, 15) and two UV mutants (3-1 and 16-15) showed a promising inhibitory activity against the MCF-7 cell line of breast carcinoma. The IC_{50} (inhibition concentration) of the five organisms were 14.1, 35.6, 36.8, 5.3, 3.4µg, respectively. The mutation increased the anticancer activity of ETA 2-fold for one mutant and 10-fold to the second mutant. No change in the molecular weight of the mutated protein was found and the exact nature of its anticancer activity is under further investigation.

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1. Introduction

Bacteria such as *Pseudomonas aeruginosa* have been reported to possess anticancer activity. Pseudomonades are gram negative rods, strict aerobes and are ubiquitous in the nature. They can live in diverse environments which are surpassed only by their pathogenicity towards humans. As an opportunistic pathogen, the organism infects immunocompromised individuals such as those with cystic fibrosis, burns victims and cancer patients. **(Maschmeyer and Braveny, 2000**). Most Pseudomonas infections are both invasive and toxinogenic due to the wide array of virulence factors possessed by this bacterium such as Exotoxin A.

Bacterial toxins have emerged as powerful therapeutic agents with possible applications in treating cancer. Several bacterial toxins have been used in the form of an immunotoxin composed of antibodies linked to bacterial toxins as well as purified forms. Pseudomonas aeroginosa bacterium produces several extracellular products such as proteases, hemolysins, exotoxin A(ETA), exoenzyme S, elastase, and Pyocyanin(**Demir et al 2008)** among which ETA is known to be the most toxic factor secreted.

P. aeruginosa exotoxin A (ETA) is considered one of the most virulent factors produed by *P.aeruginosa* and causes direct tissue damage and necrosis.The entire toxin molecule is comprised of three domains for receptor binding, translocation, and enzymatic activity. ETA, encoded by toxA, is synthesized as a 71kDa precursor with a 25 amino acid leader peptide. The protein is secreted to the extracellular environment through the type II secretion machinery as a 66 kDa mature toxin.

ETA belongs to a family of enzymes termed mono-ADP-ribosyl transferases, and more specifically is a NAD^+ diphthamide ADP-ribosyltransferase (ADPRT). In eukaryotic cells ETA catalyses the transfer of the ADP-ribose moiety from NAD^+ to elongation factor 2 (eEF-2). As a result, protein synthesis ceases and intoxicated cells die since elongation of polypeptide chains no longer occurs **(Iglewski et al., 1977, Yates et al., 2006).**

ETA causes cell intoxication via a three-step mechanism. The first step involves binding of ETA to a specific receptor, the α2-macroglobulin receptor/low-density lipoprotein. The second step consists of the internalization of the toxin by target cells through endocytosis. In the third step, the toxin

is cleaved by a cellular protease **(Ogata et al., 1992),** reduced, and translocated to the cytosol, where it ADP-ribosylates EF-2. A better understanding of the mechanism of action of ETA was made possible by the characterization of its three-dimensional structure. Several studies have been performed to characterize this protein and assign specific functions to its three domains **(Allured et al., 1986).**

It became apparent that the high cytotoxic potential of ETA can be used for the construction of immunotoxins against different cancers, whereby the enzymatic active domain of ETA is specifically targeted to tumor-associated antigens **(Wolf et al.,2008)** ETA irreversibly blocks protein synthesis in cells by adenosine diphosphate-ribosylating a post-translationally modified histidine residue of elongation factor-2, and induces apoptosis. **(Barlow, et al 2006).**

Many different cancer treatments, including chemotherapy , radiotherapy, and surgical resections are effective in management of many cancer patients but for about half of the patients these treatments are ineffective, so alternative techniques are being developed to target their tumors. Also, it became apparent that the high cytotoxic potential of ETA can be used for the construction of immunotoxins against different cancers especially breast cancer and leukemia. (**Andersson 2004).** The aim of this work is the isolation of bacteria from clinical samples, identification of the isolates by their biochemical reactions, characterization of the isolated bacteria using molecular typing methods, and finally we intended to explore the anticancer potential of *P.aeruginosa* of local isolates ,specially their ETA .In this course several isolates of *P.aeruginosa* from patients in Urology and Nephrology Center were screened for their ability to produce ETA and control the growth of MCF-7 cancer cell lines.

2.Materials and methods:-

2.1. Bacterial strains: isolation and growth conditions

A total of 68 urine samples were collected from different hospitalized patients in different wards in Urology and Nephrology Center, Mansoura University in sterile tightly locked containers.

All isolates were processed and identified according to a standard laboratory protocol which includes:

1-Sample collection under complete aseptic conditions

2-Culture on suitable media for isolation of aerobic bacterial pathogen

Bacteria from samples were isolated on LB agar (Lauria Bertani) which consists of:

Tryptone	10 gm
Yeast extract	5 gm
Sodium chloride	10 gm
Distilled water	1 Liter
Adjusted to pH = 7	

The isolates morphologically examined for size, shape, color, pigment and hemolytic ability.

3-All purified isolates were stained with gram stain and examined microscopically **(Cappuccino and Sherman, 2001).**

2.2. Biochemical identification of isolates:

Biochemical identification and characterization of the isolates were performed using the automated identification system, VITEK 2 as suggested by the manufacturer (bioMerieux, Marcy I'Etoile, France).

2.3. Molecular characterization

Two major molecular tools were used in this study to identify and characterize the isolates: the total cellular protein analysis by denatured polyacrylamide gel electrophoresis (SDS-PAGE) and the virulence gene detection by polymerase chain reaction (PCR) using specific oligonucleotide primers for the exotoxin A.

2.3.1.The total cellular protein analysis: total protein of each isolate was extracted and fractionated using SDS-PAGE as described by **Laemmli (1970).**The electrophoretic mobility of proteins was compared to the total cellular protein of an authentic *P. aeurogionosa* strain obtained from the American Type Collection Center(ATCC 27853) and standard protein markers.

2.3.2. Polymerase chain reaction (PCR):

This in vitro method for amplification of Exo A, specific DNA sequences using three specific primers with the following sequences below:

S1:- F 5' GAC AAC GCC CTC AGC ACC AGC 3'
R 5' CGC TGG CCC ATT CGC TCC AGC GCT 3'
S2:- F 5' GGC CCA TAT GCA CCT GAT ACC CCAT3'
R 5' GAA TTC AGT TAC TTC AGG TCC TCG3'
S3:- F 5' GGC CCA TAT GGA GGG CGG CAG CCT GGC C3'
R 5' AGG TTC AGT TAC TTC AGG TCC TCG3'
(Khan and Cerniglia, 1994) and **(Fitzgerald, 2008).**

The final volume of the PCR consisted of 50µl of the following components: 25 µL of DreamTaq^{TM} Green PCR master mix (2x) (Fermentas), 1 to 10 µL of genomic DNA, 0.5 µl of both forward and reverse Exo A primers (stock concentrations100 µM), 1.25U Taq polymerase (Fermentas), and the final reaction volume was completed using sterile deionized

distilled water and then overlaid with a drop of mineral oil before running the reaction.

Amplication of the Exo A gene was started with a single denaturation step at 94 ºC for 5 min, followed by 35 amplification cycles and a final extension step at 72 ºC for 3 min. Each of the PCR cycles consisted of three segments: denaturation at 94 ºC for 1 min, annealing at 60 ºC (primer S1) and at 50 ºC for primers S2 anS3 followed by extension at 72 ºC for 3 min.

2.4.Mutation

Each of the three wild-type of pseudomonas isolates (no.1, 8 and15) was suspended in 5 ml of M9 minimal medium

Na2HPo4.7H2O	64 gm
KH2PO4	15 gm
NaCl	2.5 gm
NH4Cl	**5** gm

These salts dissolved in deionized water to final volume 1 liter**.(Sambrook, et al, 1989)** and then the isolates were exposed to UV light (254 nm) for 10 seconds in complete darkness from 20 cm height. The UV exposed cells were kept for one hour in complete darkness before a 20 ml of aliquot of Lauria Bertani (LB) agar was poured onto them in glass plates and the plates were incubated at 37°C for 24 h **(Witkin, 1989).** Survived colonies were screened for mutations using morphological characteristics and Exo A gene detection using specific primers and PCR.

2.5.Preparation of exotoxin A (ETA):-

Purification of ETA was done according to the method of **Gallant, et al (2000)** where, culture conditions and purification were based on the method described by **Liu (1973)**. In brief, the P. *aeroginosa* was grown at 37ºC for 21hrs in trypticase soy broth dialysate (TSBD) to which 1% (v/v) glycerol and 0.05M monosodium glutamate were added. The supernatant was collected by centrifugation. All proteins in the supernatant were precipitated by 60% ammonium sulfate saturation, dialyzed (cutoff 20 kDa) to get rid of ammonium sulfate and low molecular weight proteins. The partially purified ETA was used in cytotoxicity assays.

2.6.Cytotixicity viability assay:-

The breast cancer cell line MCF-7 cells used to test the cytotixicity of ETA was propagated in Dulbecco's Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum,1%L-glutamine,HEPES buffer and 50 µg /ml gentamycin. All cells were maintained at 37°C in a humidified atmosphere with 5%CO_2 and were subcultured two times a week **(Vijayan et al., 2004).**

For cytotoxicity assay, the cells were seeded in a 96-well plate at a cell concentration of 10^4 cells per well in 100µl of DMEM medium. The microtitre plates were incubated at 37°C in a humidified incubator with 5% CO_2 for a period of 48 h. After incubation of the cells for the first 24h at 37°C, various concentrations of ETA (50, 25, 12.5, 6.25, 3.125 and1.56 µg) were added, and the incubation was continued for another 24 h and the yield of the viable cells was determined by a colorimetric method. Three wells were used for each concentration of ETA. Control cells were incubated without ETA and with or without DMSO. Cell toxicity was monitored daily by determining the effect of the ETA on cell morphology (loss of monolayer, granulation and vacuolization in the cytoplasm) and cell viability. After incubation, media were aspirated and crystal violet solution (1%) was added to each well for at least 30 minutes, the stain was removed and the plates were rinsed using tap water, Glacial acetic acid (30%) was then added to all wells with through mixing and then the absorbance of the plates were measured at 490 nm after gentle shaking on a microplate reader (TECAN, inc.). The cell cytotoxic effect of each tested concentration was calculated according to the methods of **Mosmann (1983)** nd **Vijayan et al., (2004),** and the 50 per cent inhibitory (cytotoxic) concentration (IC_{50}) was determined.

3.Results

Characterization and identification of the isolates:-

The colonies were mucoid, non-pigmented, gram negative bacilli. Biochemically, they were unable to: ferment sugars (glucose, lactose, and/or sucrose), produce indole or produce urease. However, they utilized citrate and were oxidase and catalase positive, i.e belongs to the genus *Pesudomonas.* The 68 isolates were identified as *Pseudomonas earuginosa* and grouped into seven biotypes which belonged to glucose non -fermenters/slow fermenters group using the automated bacterial identification system VITEK 2 (bioMerieux, Marcy I'Etoile, France).

3.1.Molecular characterization

The protein banding patterns (SDS-PAGE) and detection of Exo A gene by PCR were used in combination to attain the great validity in characterization and identification of the bacterial isolates.

3.2.Protein analysis of Pseudomonads:-

The total cellular proteins extracted from all isolates fractionated by SDS-PAGE produce patterns with discrete bands with molecular masses ranged

from 14-116 kDa (fig.1).protein patterns appear very similar proving that all isolates belonged to the same species.

According to the few differences in the cellular protein banding patterns, the 68 isolates had been grouped into 30 isolates these isolates of pseudomonads were further analyzed by PCR.

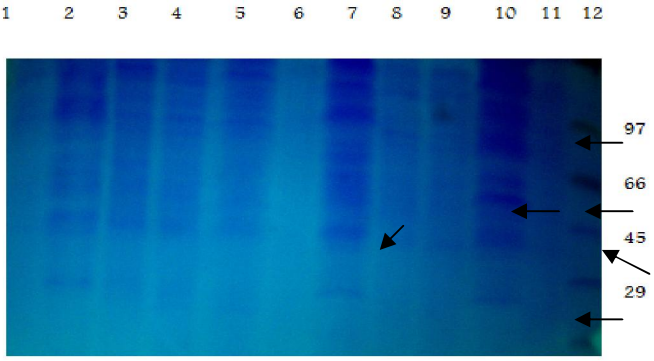


Figure 1: SDS-PAGE protein banding patterns of 11 isolates of *Pseudomonas aeroginosa* (lanes 1:11), and the molecular Weight marker (lane 12).

3.3.PCR detection of Exo A gene:

The PCR product profile of three primers (S1, S2 and S3) selected for detection and analysis of Exo A are presented in Figs2-4.

The number of isolates which gave typical PCR products were seventeen, three and twelve with primer S1 (367bp), S2 (1800bp) and S3 (1074bp) respectively, a characteristic DNA band at 367bp was generated at samples from 3-8 using specific primer S1 and sharply appeared also in other eleven samples (Fig.2). A characteristic DNA band at 1800 bp was generated at samples in lanes 3 and 9 using specific primer S2 and sharply appeared also in another sample (Fig.3). A characteristic DNA band at 1047bp was generated at samples in lanes no.2, 4, 5 and 6 using specific primer S3 and sharply appeared also in other eight samples (Fig.4).

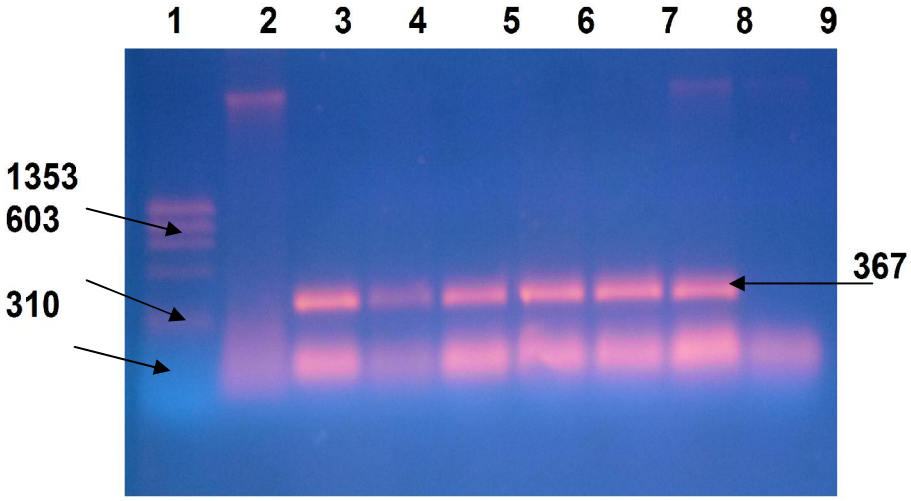


Figure 2 Polymerase Chain Reaction .Amplified DNA products of 8 isolates of *Pseudomonas aeruginosa* using primer S1 separated by gel electrophoresis and detected by ethidium bromide staining. .lane 1: DNA molecular weight Marker of III digested phage ϕx 174; lanes 2 to 9 *Pseudomonas aeruginosa* DNAs.

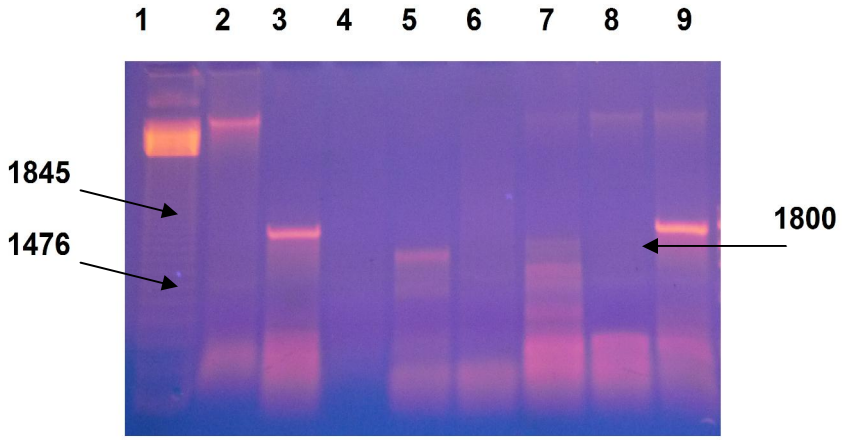


Figure 3 Polymerase Chain Reaction. Amplified DNA product of 8 isolates of Pseudomonas aeruginosa using primer S2 separated by gel electrophoresis and detected by ethidium bromide staining. Lane 1: DNA ladder Marker; lanes 2:9 *Pseudomonas aeruginosa* DNAs.

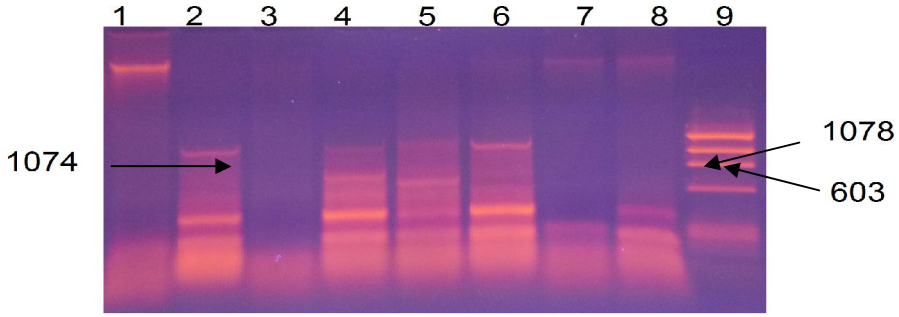


Figure 4 Polymerase Chain Reaction. Amplified product of 8 isolates of Pseudomonas aeruginosa using primer S3 separated by gel electrophoresis and detected by ethidium bromide staining. Lane 9: DNA molecular Weight marker of III digested phage ϕx 174; lanes 1:8 *Pseudomonas aeruginosa* DNAs.

3.4. Virulence gene analysis of mutants

From 17 UV-mutants obtained after mutation process, three gave the typical PCR product profile to the Exo A gene, especially when tested with S2 primer. Mutation was done on these three positive samples(wild type), the number of mutants was seventeen samples, two samples of these mutants, and the three wild type samples were chosen to undergo cytotoxicity assay.

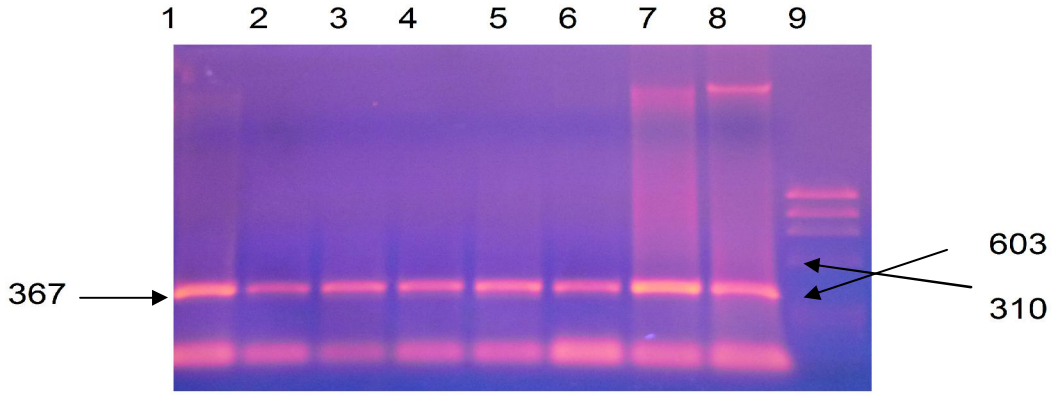


Figure 5 Polymerase Chain Reaction. Amplified DNA product of 8 isolates of *Pseudomonas aeruginosa* after mutation using primer S1.lane 9: DNA Molecular weight marker of III digested phage ϕx 174; lanes 1 - 8 Pseudomonas *aeruginosa* DNAs.

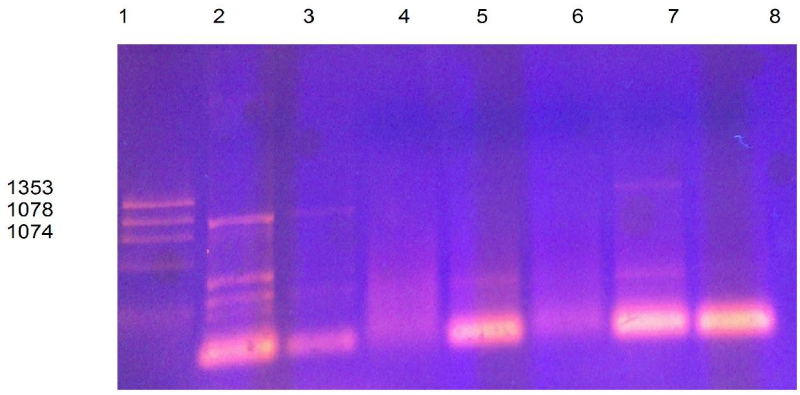


Figure 6 Polymerase Chain Reaction. Amplified DNA product of 8 isolates of *Pseudomonas aeruginosa* after mutation using primer S3.lane1: DNA Molecular weight marker of III digested phage ϕx 174; lanes2 - 8 Pseudomonas *aeruginosa* DNAs. S3 (1074bp).

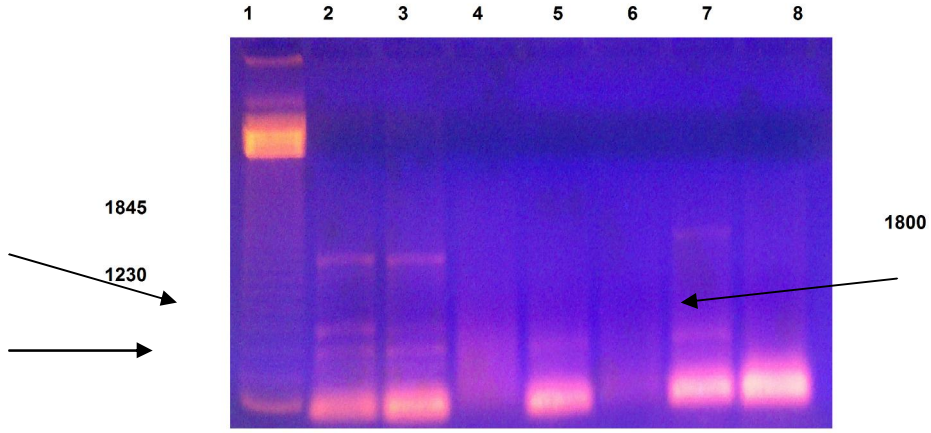


Figure 7 Polymerase Chain Reactions. Amplified DNA product of 8 isolates of *Pseudomonas aeruginosa* after mutation using primer S2 lane1: DNA ladder; lanes2 - 8 Pseudomonas *aeruginosa* DNAs. S2 (1800bp).

A characteristic DNA band at 367bp was generated at samples from 1:8 using specific primer S1(Fig.5).Also a characteristic DNA band at 1047bp was generated at samples in lanes no.2, 3, and7 using the specific primer S3(Fig.6).and a characteristic DNA band at 1800 bp was generated at the sample in lane 7 using specific primer S2 (Fig.7).

3.5.Cytotoxicity assay:

The semi-purified ETAs from different local isolates of P.aeruginosa have exerted visible cytotoxic effect on the MCF-7 cell line of breast cancer as shown in Figs. 8 and 9. All ETA preparations inhibited cell line growth and the cells became rounded and lifted off the bottom of the plates, compared to the control cells (Fig. 9). ETA from isolate no.1 showed the highest inhibition while the other three showed less inhibition. The IC50s were calculated to the four wild-type isolates and arranged in ascending order as follow: 14.1, 22.3, 35.6 and 36.8 µg for isolates 1, 57, 8 and 15, respectively . The two mutants were also assayed for their cytotoxicity (no photo is shown) and their IC50s were 3.4 and 5.3 µg for mutant 16 (derived from wild-type isolate no. 15) and 3 (derived from wild-type 1), respectively.

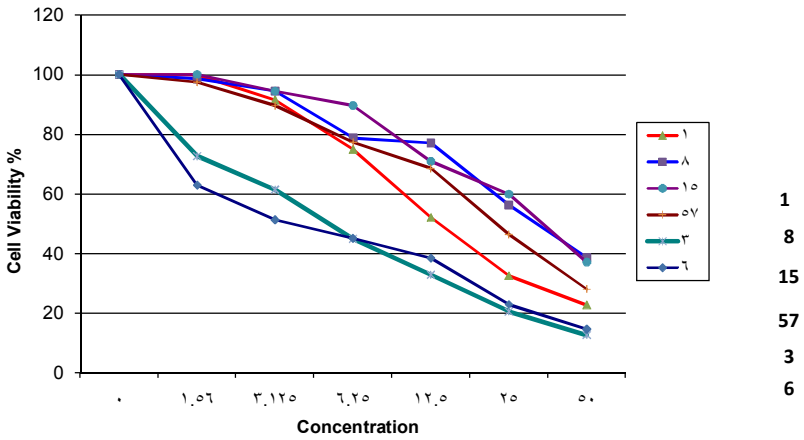
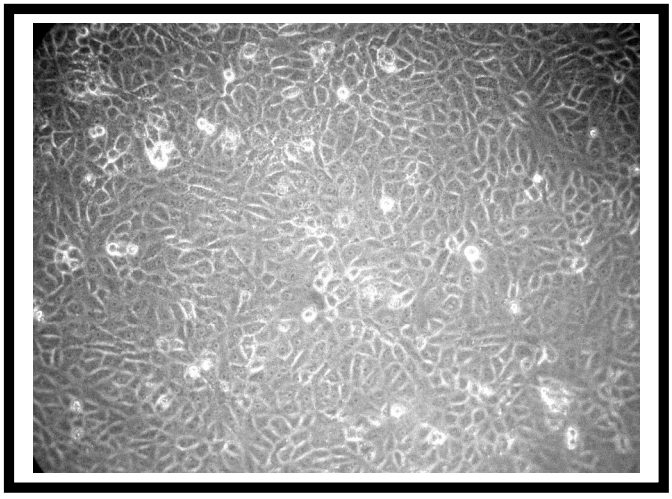
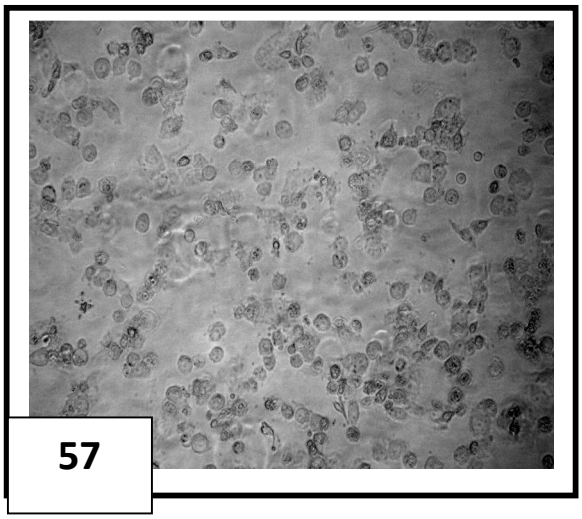


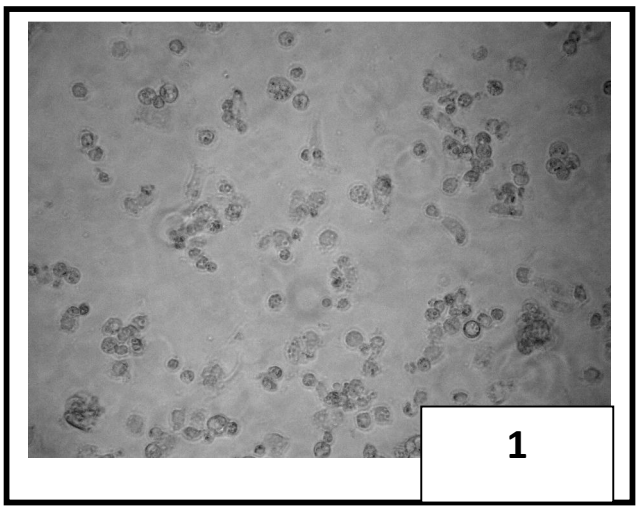
Figure 8 Relation between tumor cell viability and concentration of ETA produced from different *P.aeruginosa* strains.

1.56 3.125 6.25 12.5 25 50

Control "breast cancer tissue culture"







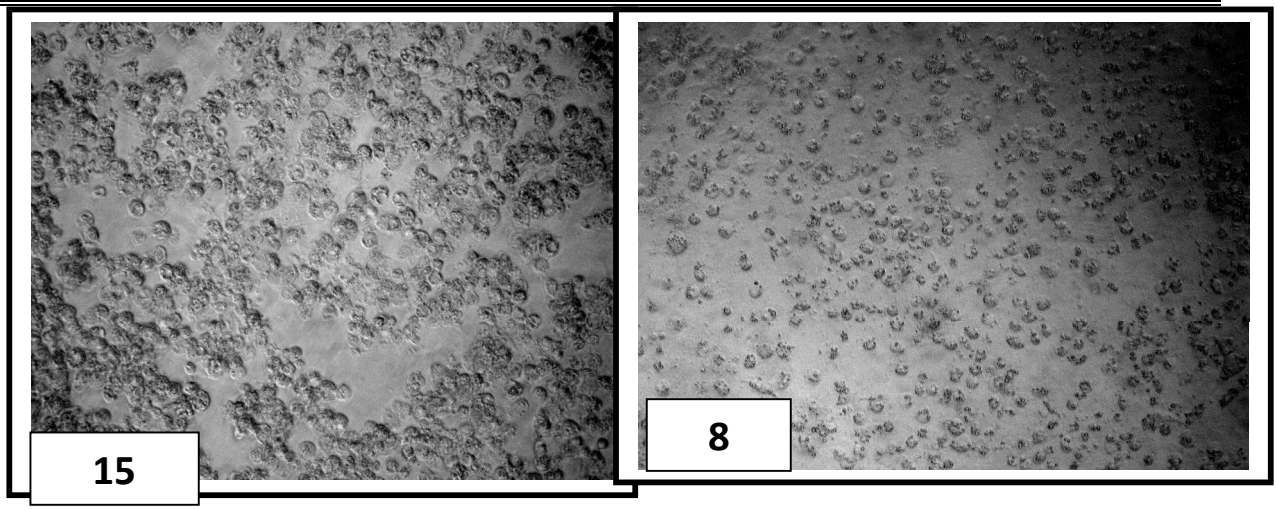


Figure 9: Cytotoxicity assay. Control cell line: breast cancer tissue culture, 1, 8, 15: breast cancer tissue culture after treatment with exotoxin A of wild Type strains of*P.* Aeruginosa, 57: breast cancer tissue culture after Treatment with exo.A of *P.* aeruginosa (ATCC 27853)**.**

Cells became rounded instead of the spindle shaped cells in the control; cell density differs according to the potency of the ETA from different samples.

4.Discussion

The role of bacteria as anticancer agent was recognized almost hundred years back. The German physicians W.Buch and F.Fehleisen separately observed that certain types of cancers regressed following accidental erysipelas (streptococcus pyogenes) infections for hospitalized patients. The American physician William Coley noticed that one of his patients suffering from neck cancer began to recover following an infection with erysipelas. He began the first well documented use of bacteria and their toxins to treat end stage cancers .He developed a safer vaccine in the late of 1800's composed of two killed bacterial species S.pyogenes and Serratia marcescens to stimulate an infection with accompanying fever without the risk of an actual infection **(Zacharski and Sukhatme, 2005).**

In this study we identified 68 isolates of bacteria.The morphological and physiological studies indicated that these isolates could be classified as the genus pseudomonas according to the protocol described in Bergey's manual **(Palleroni, 1989)** and this classification was confirmed by automated system Vitek 2 and molecular biology tools. The isolates were identified as Pseudomonas aeruginosa. Molecular characterization of the samples included both the protein banding patterns and PCR .The total cellular protein analysis (SDS-PAGE),not only helped in determining the identity of the studied microbes by showing the degree of similarity between the studied bacteria ,but also it helped to show the extra protein bands for the same isolates.

The significance of association of pesudomonads with cancer tissue represented a puzzle in the beginning, but these ambiguities were clarified after realizing the initial reports about the use of bacteria to treat cancer. The local pesudomonads showed great promise in vitro studies against the breast cancer cell line MCF-7. The crude and partially purified ETA from the selected three wild-type (1, 8, 15) isolates and two UV mutants (1-3 and 15-16) of the wild types exhibited a promising inhibitory activity against the MCF-7 cell lines of breast carcinoma. The IC50 of the five organisms were 14.1, 35.6, 36.8, 5.3, 3.4µg, respectively. The mutation increased the anticancer activity of ETA 2-fold for one mutant and 10-fold to the second mutant. No change in the molecular weight of the mutated protein was found and the exact nature of the anticancer activity is underway. The arrest of the growth of MCF-7 cells is indicative of the presence of the ETA protein to interfere with the eEF-2 and hence preventing protein synthesis. This mechanism is in agreement with that published by **Wolf et al, (2008)**. Moreover; several reports had talked about the preferential replication and accumulation within tumors for some bacterial species. These bacterial species possessed certain advantageous features such as motility, capacity to simultaneously carry and express multiple therapeutic proteins, and elimination by antibiotics, thus making bacterial treatment a promising new strategy in cancer treatment (**Nauts, et al, 1953)**.

For the local isolates, PCR was used to amplify the target DNA (Exo A), using specific oligonucleotide primers and the PCR product profile showed extra DNA bands. The appearance of these extra DNA fragments can be attributed to either genetic variability in our local isolates, or the location of the binding sites to the primers were different in

our strains and hence produced a concontower (adjacent repeats) with different migration rates. This is usual with bacterial isolates because of the continuous mutation and pressure of existing in new environment. But existance of the DNA fragment encoding for the Exo A gene is confirmed not only by the PCR but also by the toxicity assays against the MCF-7 breast cancer cell line.

Bacterial toxins can be used for tumor destruction and cancer vaccines can be based on immunotoxins of bacterial origin and it can be exploited as delivery agents for anticancer drugs, and as vectors for gene therapy. Protein toxins such as pseudomonas exotoxins, diphtheria toxin, and ricin may be useful in cancer therapy because they are among the most potent cell-killing agents. Although they are lethal yet for therapeutic efficacy these toxins need to be targeted to specific sites on the surface of cancer cells. This process is accomplished by eliminating binding to toxin receptors by conjugating the toxins to cell-binding proteins such as monoclonal antibodies or growth factors. These conjugates bind and kill cancer cells selectively thus sparing normal cells, which don't bind the conjugates.

A large varity of antibodies and ligands to surface antigens overexpressed in different tumors have been conjugated to ETA. Some important ones tested in clinical trials are IL-4,IL-13 monoclonal antibody recognizing a carbohydrate antigen, Lewis Y reacting with metastatic adenocarcinoma cells (Mab B3) and transforming growth factor (α - TGF)**(Fan et al, 2002).**

Two examples of targeted cytotoxins using pseudomonas exotoxin are interleukin (IL)-13 fused with pseudomonas exotoxin (TP-38). They were safe and produced responses in patients with malignant gliomas in early phase 1/11 studies **(Kunwars et al,2003)**. A large number of ETA based immunotoxins directed against various surface antigens overexpressed in different tumors were constructed and tested in pre-clinical trials. They were characterized with respect to antigen binding on primary tumor cells and tumor cell lines ,thermostsbility , possible cross reativities towards normal tissues ,cytotoxicity to target tumor cells, and induction of apoptosis in vitro .The majority of these studies additionally examined antitumor effects and maximal tolerated doses (MTD) in animals bearing tumors xenografts,e.g. the immunotoxin A5-PE40 whose its target antigen is EGFR (Elongation Growth Factor Receptor) in breast carcinoma (**Andersson et al,2004)**

Breast cancer is the target tumor of the immunotoxin Sc Fv (MUCI)-ETA, which showed target –specific killing of breast cancer cells and on primary breast tumors samples. **(Singh et al., 2007).**

In conclusion, the local P. aureoginosa produced exotoxin A protein with potential anticancer activity as demonstrated on MCF-7 cell line.

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