Azurin: A Powerful Anticancer from "A" Local *Pseudomonas aeruginosa* Isolate

Yehia A. Osman[1], Douaa R. El-Deep[2] and Sadia A. Younis[1]

[1] Department of Botany, Molecular Microbial Lab, Faculty of Science, Mansoura University, Egypt.
[2] Department of Clinical Pathology, Microbiology Unit, Faculty of Medicine, Mansoura University, Egypt.
lazeikyao@gmail.com; modoy500@yahoo.com

Abstract: Microbial anticancer compounds such as azurin, constitute promising therapeutic agents against different types of human cancers. They have higher affinity and penetrate cell membranes of the target cancer cells, reaching the target sites, than other peptides used to treat cancers. Classical microbiological techniques were used to isolate 95 *Pseudomonas aeruginosa* from inpatients and outpatients attended the clinics at Mansoura University Hospitals from January 2010 to July 2012. All isolates were identified using manual biochemical tests and confirmed by the Microscan Walk away 90 systems. The gene encodes for azurin in the local *P. aeruginosa* isolates was detected using specific oligonucleotide primers in a PCR, amplifying a single 545bp DNA fragment characteristic of azurin gene. Column chromatography (superdex 75) followed by dialysis was used to purify the ammonium sulphate-precipitated azurin to near homogeneity. The crude and partially purified azurins killed the breast cancer cell line, MCF-7 cells, with an estimated IC_{50}= 37.6 µg/ml (pure) for the ATCC 15442 strain and IC_{50}= 3 µg/ml for purified azurin from local isolate II.
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Key words: Antitumor, azurin, *Pseudomonas aeruginosa*, cytotoxicity.

1.Introduction:

A significant regression of subcutaneous tumors in mice was observed by combining anaerobic bacteria with various chemotherapeutic agents **(1).** Where live or attenuated pathogenic bacteria or their products were used in the treatment of cancer **(2).**

Current anticancer drugs are either small molecules or monoclonal antibodies that target and inhibit a key important step in cancer progression pathway, thereby significantly inhibiting their proliferation **(1).** No effective drug or vaccine exists to prevent cancer initiation and drug resistance and toxicity are major problems in cancer chemotherapy **(2).** This study describes recent attempts to develop bacterial proteins that are used as weapons by certain pathogenic bacteria with long term residence in human bodies to prevent invasion of their habitat by invaders such as cancers, viruses or parasites **(3)**. In one instance, such a protein, termed azurin, has been shown not only to have entry specificity in cancer cells and prevent cancer cell growth by interfering in multiple pathways by which cancer cells grow, but also to prevent induction of pre-cancerous lesion formation triggered by a potent carcinogen **(1).**

Azurin is a small globular metalloprotein, endowed with redox activity, involved in the bacterial denitrification process **(4)**. It is acting as an electron transfer shuttle in *Pseudomonas aeruginosa* and other bacteria. The presence of the copper ion gives this protein a number of features, including an intense blue color, a high reduction potential and a small parallel hyperfine coupling in the electron spin resonance spectrum **(5).**

Azurin, a low molecular weight cupredoxin is a promising source of therapeutic peptides not only for its anticancer activity but due to its antiparasitic and anti-HIV properties **(4)**. All these biological activities are associated with different domians of the protein. In fact, studies on its anticancer activity, revealed that azurin preferentially enter breast cancer cells and induce apoptosis **(6),** cell cycle arrest **(7)** and inhibits angiogenesis **(8)** through interaction with different cellular molecules.

Azurin capability of inducing apoptosis in tumour cells by p53 stabilization makes this protein suitable for being employed as anticancer agent **(9).** The p53 tumor suppressor is involved in multiple central cellular processes, including transcription, DNA repair, genomic stability, cell cycle control, and apoptosis; it is functionally inactivated in many human cancers **(10).** Azurin appeared to form a complex with p53, somehow raising its intracellular levels **(11).** The increased amount of p53 then triggered apoptosis in the cells through enhanced Bax formation and release of mitochondrial cytochrome c in the cytosol. Many viral and mammalian proteins can modulate p53 function by physical interaction; however, azurin is the first bacterial protein reported to form a complex with p53 **(12, 13).**

2. Materials and methods:

Isolates collection and bacterial identification.

Routine sampling of was performed. Samples were routinely drawn from inpatients and outpatients attending clinics at Mansoura University Hospitals from January 2010 to July 2012. Bacteriological analysis, of the collected samples included isolation and identification of pure bacterial cultures, specifically designed for *Pseudomonas aeruginosa* **(14)**. This included several tests such as Gram stain **(15),** trible sugar iron **(16)**, citrate test **(17)**, ureas test **(18)** and identification was confirmed by the Microscan Walk away 90, an automatic identification system.

Screening *P. aeuroginosa* for Azurin

The blue green isolates were selected for azurin production by cultivating them on specific differential medium **(19)**.

Molecular characterization:

The molecular characterization of azurin-producing isolates included analysis of their protein banding patterns, plasmid profiles and detection of azurin gene by using polymerase chain reaction.

1- Plasmid DNA preparation:

Total plasmid DNA was prepared from eleven selected isolates according to the alkaline lysis method **(20).** Plasmids were fractionated in agrose gel (0.8%) by electrophoresis for 1h at 100 V in TBE buffer, stained with ethidium bromide, visualized under UV illumination and photographed.

2- Total cellular protein analysis (SDS-PAGE):

Total cellular proteins were extracted and fractionated using denatured polyacrylamide gel electrophoresis **(21)**. Each run included protein markers of known molecular masses and the gel were photographed under white light illumination.

3- Detection of the gene encodes for azurin

Polymerase chain reaction (PCR) is the method of choice, it is an in vitro method for enzymatic amplification of specific DNA sequences uses two specific oligonucleotide primers for the detection of the gene encoding for azurin **(22, 23).**

Table (1): The nucleotide sequences of the primers

Primes	Nucleotide sequence	Product size & Reference
AZU-R	**5/**TGAGCCCCTGTAGGCGCCCATGAAAAAGCCCGGC **3/**	545bp
AZU-F	**5/**GCCCAAGCTTACCTAGGAGGCTGCTCCATGCTA **3/**	(12)

The PCR was performed in 25µl reaction volume containing 1x buffer (10mM tris- Hcl PH 8.3, 50mM Kcl, 2m M $Mgcl_2$), 250µM each of dGTP, dATP, dCTP and dTTP, 2.5 units of Taq DNA polymerase, 100 pmol of each primer and DNA template. Components were overlaid with a drop of mineral and DNA amplification started with denaturing the template DNA at 94^{o}C for 5 min followed by 35 cycles. Each cycle consisted of: denaturation at 94^{o}C for 1 min, annealing for at 50^{0}C45 sec and extension at 72^{o}Cfor 3 min, and a final extension step at 72 ^{o}c for 7 min. The PCR product was analyzed by agarose gel electrophoresis **(19).**

Purification of azurin :

Four local *P. aeruginosa* isolates and a standard ATCC strain were used to purify azurin from them. *P. aeruginosa* strains were prepared by growing them in LB media for overnight incubated at 37^{0}C, then used to inoculate larger volumes of azurin production specific medium which was kept shaking (150 rpm) for 21 hours. Crude azurin was precipitated from culture supernatants by 70% ammonium sulfate saturation and kept at 4^{0}C for overnight before being dialyzed. Complete purification to homogeneity was achieved by column chromatography on Superdex 75 **(24).** Azurin concentration and purity were determined by Biuret's reagent (25) and SDS-PAGE **(21),** respectively.

Antitumor activity of azurin:

The potential cytotoxicity and antitumor activity of azurin from the four local isolates and the standard ATCC 15442 strains were tested against breast cancer cell line MCF-7 **(26),** in The Regional Center for Mycology & Biotechnology, Al Azhar University, Egypt. A dose dependent curve was constructed for each and the 50% inhibitory concentration (IC_{50}) was calculated for each isolate**.**

3. Results:

Identification of bacterial isolates:

From among 95 isolates were identified as members of *P. aeuroginosa* and fitted the description in Bergey's Mannual for Determinative bacteriology. They are characterized by being Gram-negative rods (Fig 1) at the biochemical level all tested isolates were citrate utilization, urease and oxidase positive, while TSI and H_2S production were negative. The automated bacterial identification system (Microscan Walk away 90 system) not only confirmed the manual identification methods of the local isolates, but also revisable the frequency of the species belonging to each genus.

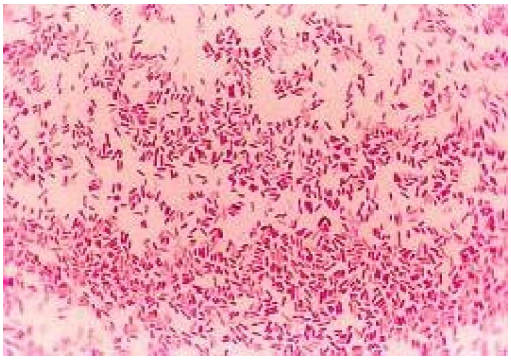


Fig. (1): Gram negative *Pseudomonas aeruginosa* (pink red rod)

Only 10 (assigned numbers: 5-8-9-12-16-24-39-43-I-II) produced azurin (blue-green) on its specific differential medium while the standard strain ATCC 15442 (57) produced creamy color i.e azurin negative (Fig. 2).

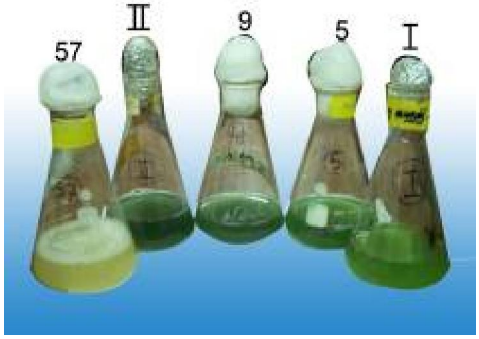


Fig. (2). Isolates no. I, 5, 9, II produced azurin (blue-green color) and standard strain ATCC 15442 (57) grown in azurin differential media.

Molecular characterization:

Molecular characterization of any living organisms depends not only on its own genome, but also upon its genome expression. Bacterial genome consists of chromosomal and extra chromosomal DNA elements present in the cytoplasm (plasmid).This included plasmid profile, total cellular protein analysis and detection of genes encoding for azurin production by PCR technology **(27)**.

Production of azurin by the local isolates was seen only when they grown at 37°C (Figs: 3&4). This indicated by the plasmids curing experiments which have shown that the gene encodes for azurin production is carried on a plasmid, not chromosomal coding. Moreover, theses isolates did not produce the characteristics PCR product (545bP) of gene encodes for azurin (Fig. 4).

Total cellular proteins extracted from 11 isolates fractionated by SDS-PAGE produce patterns with discrete bands with molecular masses ranged from 10-260 kDa. All the lanes appear to be homologous except in few bands; i.e. the high molecular weight Protein bands above 260 kDa are absent only in lane 9 isolates (I) fig.(5). Combining the whole cell protein patterns of the 11 isolates a dendrogram can be constructed using the simple matching rules of the numerical analysis to differentiate between these closely related bacterial isolates and even can help assign names for the genera and species.

Fig. (3) *Pseudomonas aeruginosa,* isolates numbers (43, 12, 9, and 56) were grown in azurin differential medium at 37°C (Flasks) 42°C, 45°C (green racks), respectively.

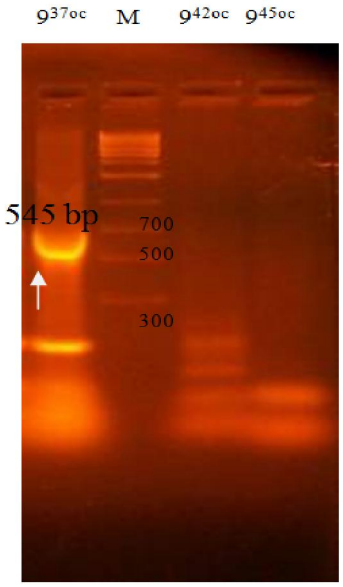


Fig.(4) PCR product the gene encoding for azurin for one of the cured isolates (No. 9) grown at different temperatures; lanes 37°C, 42°C, 45°C and lane M: DNA marker.

The dendrogram of the 11 isolates Fig.(6), a 95% degree of similarity does exist between isolates from 1-11, which qualifies the assignment of the genus to each isolate according to the numerical taxonomy rules. Any isolates assigned to the same species have a similarity close to 98%. Applying these rules to the current isolates makes their genera and species stand as they are.

Based on the above gel all isolates can be divided into 4 groups, group (1): including isolates no. 16, 43, 39, 8, 5, 24 & group(2): including isolates no. 9, II & group(3): including isolates no. 12,I and group(4): including stander strain ATCC 15442 (56).

Protein banding pattern:

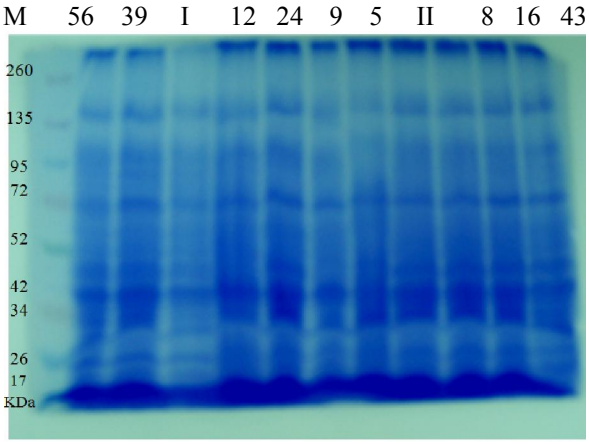


Fig. (5) SDS–PAGE protein profiles of selected *Pseudomonas aerouginosa* isolates. Lans: 1) isolates *43*, 2) isolates *16, 3)* isolates 8, 4) isolates II, 5) isolates 5, 6) isolates 9, 7) isolates 24, 8) isolates 12, 9) isolates I, 10) isolates 39, 11) isolates 56(ATCC 15442) and M) protein molecular weight marker.

Detection of the gene encoding for azurin by polymerase chain reaction.

PCR was used in this study to detect the gene encodes for azurin in the 11 local isolates. This is a powerful tool and is widely used by clinicians to diagnose difficulty to diagnose bacterial pathogens. In this discriminately reaction a PCR amplicons at 545bp, characteristic of gene encodes for azurin, existed in all 11 local isolates and the standard ATCC strain (Fig.7).

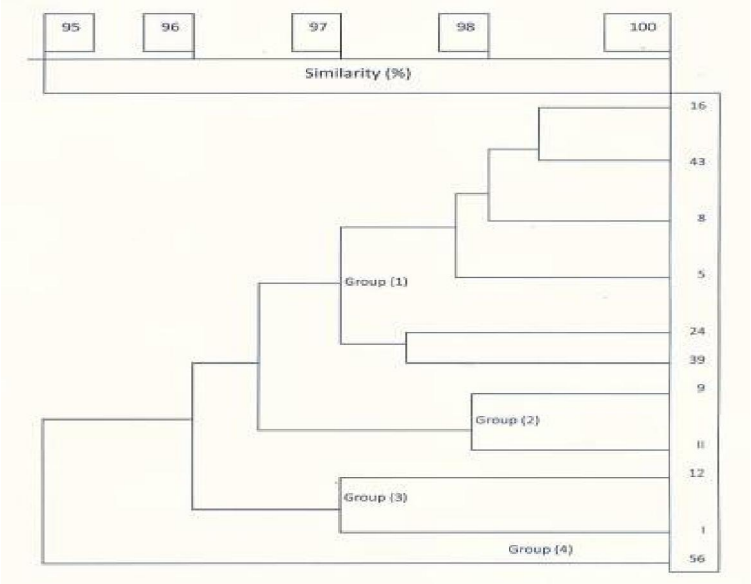


Fig. (6): The dendrogram of the 11 *Pseudomonas aerouginosa* isolates

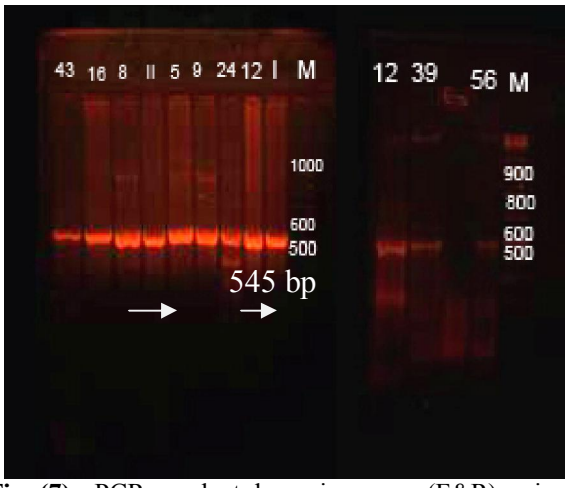


Fig (7): PCR product by using azu. (F&R) primer applied in 12 isolates no. 43, 16, 8, II, 5, 9, 24, 12, I, M lambda DNA marker, 12, 39, standard strain 56 and M DNA marker.

Antitumor or cytotoxicity of Azurin: crude azurin and purified azurin by Colum chromatography:

This experiment was carried out on isolates (I, II, 5, 9, and 56) for studying the effect of azurin (crude and purified) against breast cancer cell line MCF-7. All isolates were inoculated on azurin media over night and centrifuged. The supernatant was precipitated by ammonium sulphate (70%), then dialysis the crude azurin and determined concentration by Biuret's reagent (table(3)).

A partial purification of crude protein was made by using mille -pore filter regencreated cellulose (30.000 MW) (centre prep), then complete purification by Column chromatography (sepadex 75). Collect at least 16 fractions from each sample after running on Column (2ml/ 15min) then O.D was measured. at 280nm for each fractions separately and draw relationship between O.D. and fractions fig (8), mix between fraction no.5 (peak start) to fraction no. 11 (peak end), protein was precipitated by ammonium Sulphate (70%), dialyzed and protein concentration was determined table (4).

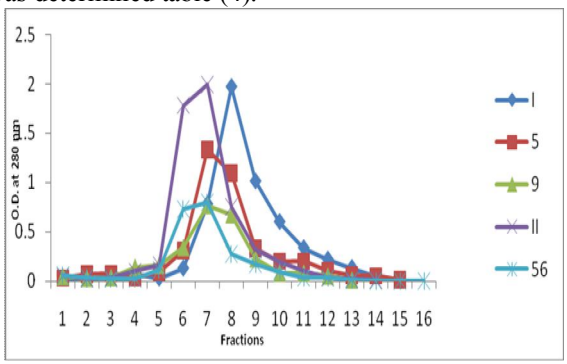


Fig.(8): relationship between O.D. at 280 nm and fractions for isolates no I, II, 5, 9, 56 after crude azurin purified on colum chromatography.

Table (3): Show the concentration of crude protein in isolates no. 9-5-I-II-56 by Biuret's reagent after precipitating the supernatant by ammonium sulphate.

Isolates no.	O.D	Dil. Factor =1/ (transfer volume/total volume)	Slop	Conc.(ug/ml)=(O.D/slop)*dil factor
9	0.501	7/2	0.11	15.94
5	0.520	7/2	0.11	16.54
II	0.554	6/2	0.11	15.1
I	0.577	5/2	0.11	13.11
56	0.304	10/2	0.11	13.81

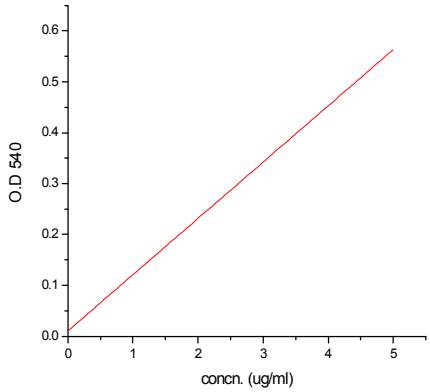
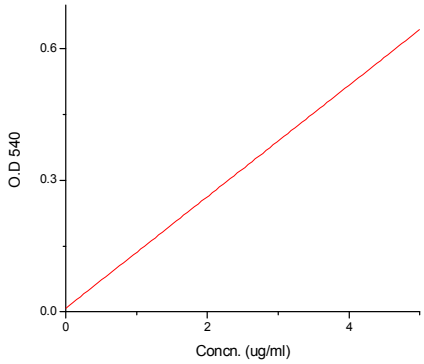


Table (4): Estimation of total purified protein concentration (azurin) after Colum chromatography isolates no. 9-5-I-II-56 by Biuret's reagent.

Isolates no.	O.D	Dil. Factor =1/ (transfer volume/total volume)	Slop	Conc.(ug/ml)=(O.D/slop)*dil factor
9	0.208	10	0.12	17.33
5	0.247	10	0.12	20.58
II	0.193	10	0.12	16.08
I	0.164	10	0.12	13.66
56	0.226	10	0.12	18.83



Azurin purified from column chromatography, purified from milipore filter and crud azurin were loaded with protein molecular weight marker at different lanes in SDS-PAGE for profiling the protein purification process to isolates no. 9, 5, I, II, 56 figs (9, 10, and 11).

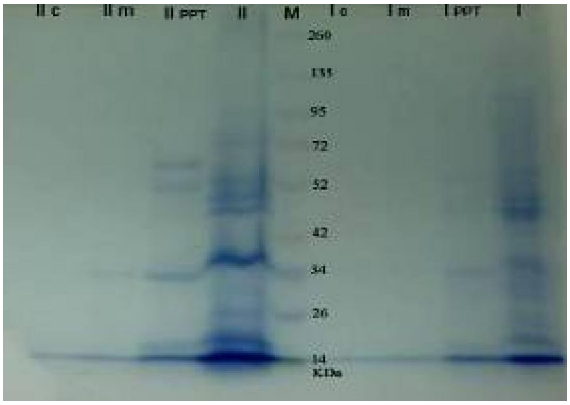


Fig. (9): SDS–PAGE protein profiles of *Pseudomonas aerouginosa* (isolates I, II). Lans: 1.isolates II^{C}, 2. isolates II^{m} , 3. isolates II^{PPT} , 4. isolates II., 5. M. protein molecular weight marker, 6. isolates I^{C}, 7. isolates I^{m}, 8. isolates I^{PPT}, 9. isolates I.

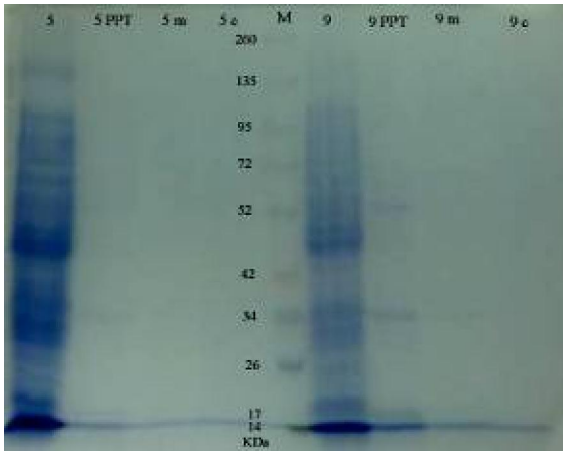


Fig. (10): SDS–PAGE protein profiles of *Pseudomonas aerouginosa* (isolates 9, 5). Lans: 1.isolates 5, 2. isolates 5^{PPT}, 3. isolates 5^{m}, 4. isolates 5^{C}, 5. M. protein molecular weight marker, 6. isolates 9, 7. isolates 9^{PPT}, 8. isolates 9^{m}, 9. isolates 9^{C}.

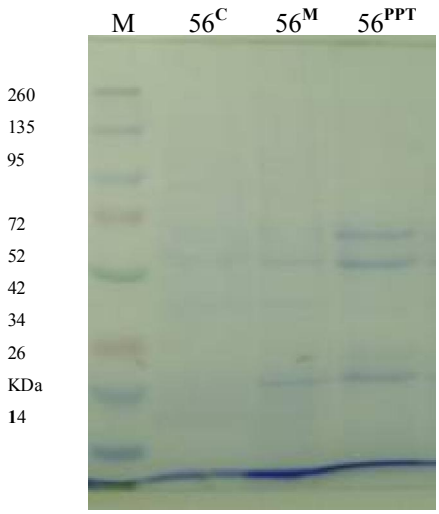


Fig. (11): SDS–PAGE protein profiles of Pseudomonas aerouginosa (stander isolates 56 (ATCC 15442)) Lans: 1. M protein molecular weight marker, 2. isolates 56 ^{C}, 3. isolates 56 ^{M} ,4. isolates 56 ^{PPT}.
Where, c: purification of crud protein by column choromatography on sephadex 75.
m: Partially purification of protein by milepore filter (30.000MW).
PPT: precipitation of protein by ammonium Sulphate (70%) from the supernatant after centrifuge.
I, II, 5, 9: pellet of isolates after grown on azurin differential media over night then precipitate by centrifuge.

Antitumor or cytotoxicity against MCF-7 cell line of crude and purified Azurin:

azurin 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. 13 and 14). All azurin preparations inhibited cell line growth and the cells became rounded and lifted off the bottom of the plates, instead of the spindle shaped cells in the control; cell density differs according to the potency of the azurin from different isolates fig (12).

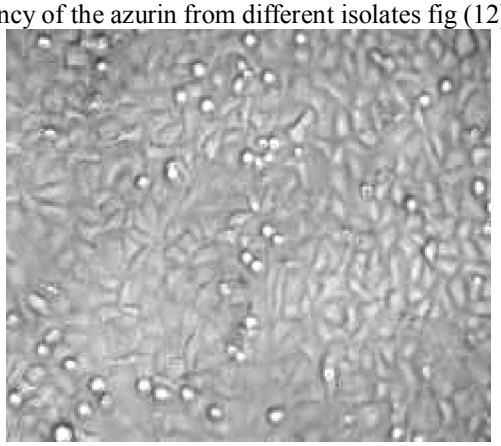
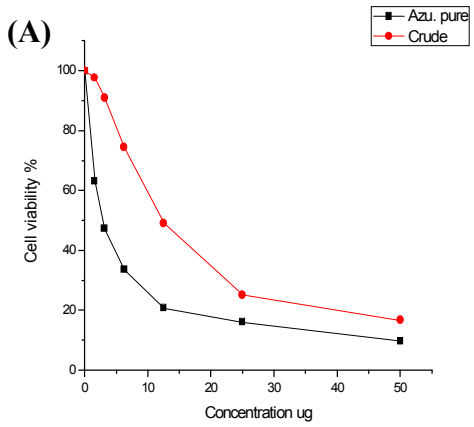


Fig (12): Light microphotograph of cancer cells morphology was observed in azurin-untreated cells.



(B)

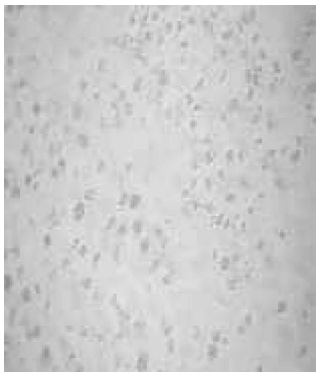


Fig (13): (A) cytotoxicity effects of isolate II, IC_{50} after azurin purification 3µg/ml. (B) Light microphotograph show condensed nuclei and apoptotic bodies were observed in azurin-treated cells for isolate II.

Pure azurin from isolate no. II showed the highest inhibition while the other showed less inhibition fig (13). The IC_{50} were calculated to the crud azurin for four isolates arranged in ascending order as follow: 12.2, 12.2, 15.8 and 21.8 µg/ml for isolates II, 9, I and 5, respectively, after azurin purification also assayed their cytotoxicity and their isolates, IC_{50} 3, 5.3, 20.7 and 14.3 µg/ml. The standard strain (56) before azurin purification weak inhibitory activity against breast carcinoma cells, meaning that all proteins above 14 KDa inhibitory the effectiveness of azurin so after azurin purification IC_{50}= 37.6 µg/ml fig (14).

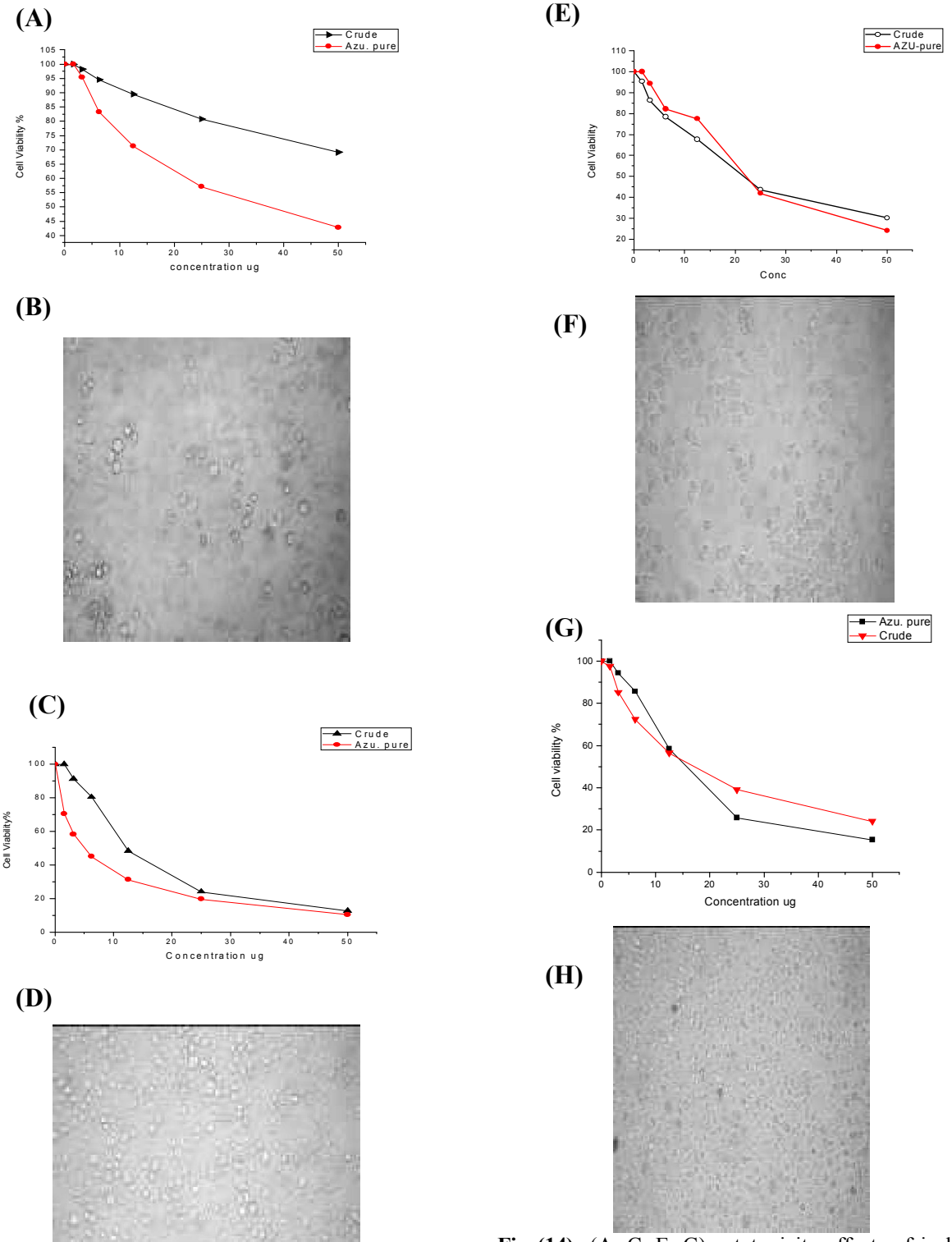


Fig (14): (A, C, E, G) cytotoxicity effects of isolates (56, 9, I, 5), IC_{50} after azurin purification (37.6, 5.3, 20.7, 14.3 µg/ml). (B, D, F, H) Light microphotograph shows condensed nuclei and apoptotic bodies were observed in azurin-treated cells for isolate (56, 9, I, 5) respectively.

4. Discussion

Malignancy is a complex disease with a network of multiple metabolic pathways that are interlinked to promote growth and resist immune surveillance. Such a network is efficiently maintained through acquisition of multiple mutations in the human genome that result in the escape from normal cellular growth regulation and formation of lumps of fast growing cells known as tumors. The varied pathways through which cancer cells grow and inhibit their own cell death have made it difficult to develop effective drugs either to prevent the emergence of tumors or to check their rapid growth **(28)**.

The secondary metabolites from microorganisms play a vital role in developing new chemotherapeutics. Azurin a blue copper protein have redox properties plays a role in electron transfer as donors **(11).** Azurin was reported as a potential anticancer protein against breast cancer cell lines which evoked biomedical researchers to develop an alternate method for enhanced production of azurin **(6).** Early researchers revealed the synthesis of blue copper protein azurin from different microbial sources, especially from *Pseudomonas aeruginosa* **(12)**.

Pseudomonas aeruginosa is known to secrete the protein azurin as a weapon against invaders as cancers, parasites and viruses. The production of such weapons by pathogenic bacteria could provide important insights into how a pathogen responds in the postcolonization state to impede other intruders for its own survival. Moreover, these molecules might find use in the pharmaceutical industry as next-generation therapeutics **(9).**

The researchers have shown that *P. aeruginosa* preferentially enters human melanoma and breast cancer cells, triggering apoptotic cell death. They further discovered that azurin sets off this death sequence by forming a complex with the well-known tumor suppressor protein p53, stabilizing it, and activating caspases that induces apoptosis in cancer cells. p53 normally stops cells that are damaged from reproducing and encourages them to commit apoptosis, but a majority of cancer cells have damaged or missing p53 **(29).** Now, they demonstrate that a smaller, 28 amino-acid fragment of azurin also enters cancer cells selectively, but not in any of the normal cells tested. This small molecule could potentially be used as a vehicle for cancer-targeted chemotherapy **(30).** So in this study an attempt was done to isolate, clone and study the effects of azurin on proliferation of human breast cancer cell line (MCF7) **(9)**.

Azurin also inhibits growth of cancer cells as by interfering in the signaling pathways and angiogenesis **(31)**, demonstrating the multiple pathways through which it exerts its inhibitory action. Azurin is not only a potential anticancer drug candidate, but it also forms complexes with many surface proteins, there by interfering in their entry to the host cells and significantly suppressing their growth **(32).** Thus azurin could potentially be used as a drug in the treatment of such unrelated diseases as cancer.

The cytotoxicity assay of the crude and purified azurin from the local isolate of *P. aeruginosa* showed significant differences compared to the standard strain. Moreover, the IC_{50} of the local isolate increased 4-fold as a result of purification; 3 ug/ml. Genetic modification of the promoter region is expected to improve azurin production and may enhance its activity **(10).**

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