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Cytotoxic and Morphological Analysis of Azurin and Paclitaxel-Induced Apoptosis against Breast Cancer Cell line

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Abstract: Azurin is a cupredoxin produced by bacteria that is mostly utilized in electron transport processes. The anticancer properties of the Azurin and its potential use in cancer treatments have increased interest recently. In other side Paclitaxel is a chemotherapeutic agent used as therapy for several types of cancer, it can induce intrinsic apoptosis by activating caspase-3, caspase-9 and PARP.

Materials and Methods: The inhibitory dose 50 (IC50) of MCF-7 breast cancer cell line was assessed by MTT assay and then examined under inverted microscope to show the cytopathic effect against MCF-7 cell line.

Results: Azurin and Paclitaxel have cytotoxic effect on MCF-7 breast cancer cell line. In which, IC50 of the Azurin against MCF-7 cell line was 25.6 and IC50 of the Paclitaxel was 34.57. cytopathic effect under inverted microscope indicated that the Azurin was more cytopathic effect than Paclitaxel against the MCF-7 cell line.

Conclusion: Both Azurin and Paclitaxel have cytotoxic effect against breast cancer cells. But Azurin have more cytopathic effect than Paclitaxel.

Keywords: Azurin; Paclitaxel; MCF-7 cancer cell line; Pseudomonas aeruginosa.

INTRODUCTION

Cancer is a leading cause of morbidity and mortality globally. In 2020, about 19 million new cancer cases were diagnosed and almost 10 million cancer deaths were recorded. Thus, the overall demand of chemotherapeutics is rising with an estimated increase of 53 % in number of patients who need chemotherapy by 2040 compared to 2018 (Hammad *et al*., 2023)

*Pseudomonas aeruginosa is a Gram-negative bacterium that is a global threat to public health and is classified as one of the "ESKAPE" that include Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter species pathogens (*De Oliveira *et al*., 2020). *It is a group of microorganisms with a high propensity for causing problematic, nosocomial, and drug-resistant infections* (Pandey *el al*., 2021).

Azurin is a copper-containing redox protein secreted by *P. aeruginosa* that contains 128 amino acids and a copper ion. According to the Structural Classification of Proteins (SCOP) classification, the three-dimensional structure of Azurin belongs to the all-β folding class, which mainly comprises two groups of β-strands (4 strands per group) arranged in a sandwich structure (Yaghoubi *et al*., 2020).

P^{28} is a fragment (Leu50-Asp77) of the Azurin protein, encompassing a β-strand, an α-helix, a turn, and an irregular structure. It is worth noting that the structure of the p^{28} segment is separated from the sandwich structure of Azurin. Although p^{28} is folded into a stable three-dimensional structure within Azurin, it does not mean that the p^{28} fragment forms the same structure as an isolated peptide. In fact, molecular dynamics simulations showed that the α-helix of p28 was unstable after isolation (Huang, *et al*., 2020) that showed figure below (1).

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  1 AECSVDIQGN DQMQFNTNAI TVDKSCKQFT VNLSHPGNLP KNVMGHNWVL
 51 STAADMQGVV TDGMASGLDK DYLKPDDSRV IAHTKLIGSG EKDSVTFDVS
101 KLKEGEQYMF FCTFPGHSAL MKGTLTLK
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Figure 1: Amino acid sequence of Azurin. The region corresponding to p28 is shown in orange (Huang, *et al*., 2020)

Paclitaxel is a well-known anticancer agent with a unique mechanism of action. It is considered to be one of the most successful natural anticancer drugs available. This study summarizes the recent advances in our understanding of the sources, the anticancer mechanism, and the biosynthetic pathway of paclitaxel. With the advancement of biotechnology, improvements in endophytic fungal strains, and the use of recombination techniques and microbial fermentation engineering, the yield of extracted paclitaxel has increased significantly. Recently, paclitaxel has been found to play a large role in tumor immunity, and it has a great potential for use in many cancer treatments (Zhu *et al*., 2019).

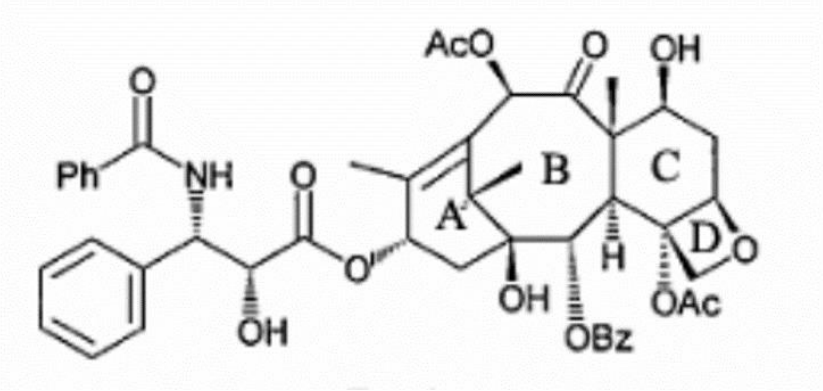

Figure 2 The chemical structure of Taxol (Kai and Tang, 2006).

MATERIAL AND METHODS

Azurin was previously purified according to (Ramachandran *et al*., 2012 and Ünver, 2021) and Paclitaxel (Zuvius, India) was purchased.

Cytotoxicity assay:

MTT stain was used to detect the cytotoxic effect of the Azurin and Paclitaxel MCF-7 cancer cell line (Iraqi Biotechnology Company ltd. Iraq.) pursuant to the following:

To determine the cytotoxic effect, the MTT cell viability assay was conducted on 96-well plates. Cell lines were seeded at 1×10^4 cells/well. After 48 hrs. or a confluent monolayer was achieved, cells were treated with tested compound. Cell viability was measured after 48h of treatment by removing the medium, adding 28 µL of 2 mg/mL solution of MTT (and incubating the cells for 1.5 h at 37 °C. the crystals remaining in the wells were solubilized by the addition of 130 µL of DMSO (Dimethyl Sulphoxide) followed by 37 °C incubation for 15 min with shaking. The absorbency was determined on a microplate reader at 492 nm (test wavelength); the assay was performed in triplicate. The inhibition rate of cell growth (the percentage of cytotoxicity) was calculated as the following equation: -

Inhibition Rate %= (Absorbance of Treated Cell/ Absorbance of Non-treated Cells) × 100

The morphology of treated or untreated cultured cells documented in microphotographic images using crystal violet stain. The untreated cells revealed the cellular properties of the related cell lines. The morphological changes of the treated cells were inclusive of damaged cell membranes and cell shrinkage. Cell damage contributes to the loss of shape and forming of small spherical bodies which are feature of apoptosis.

RESULTS AND DISCUSSIONS

The term cytotoxicity is commonly referred to the potential of a compound to induce variations in the cellular behavior and essential processes that subsequently trigger cell death or cause a large decrease in cell survival (Damiani *et al*., 2019).

To test the cytotoxic activity of Azurin and Paclitaxel on MCF-7 cell lines, MTT cell viability measurement was performed on 96 well micro-titration plates as mentioned before, in which the inhibitory dose 50 IC_{50} of Azurin against MCF-7 cell line was 25.6 (figure 3).

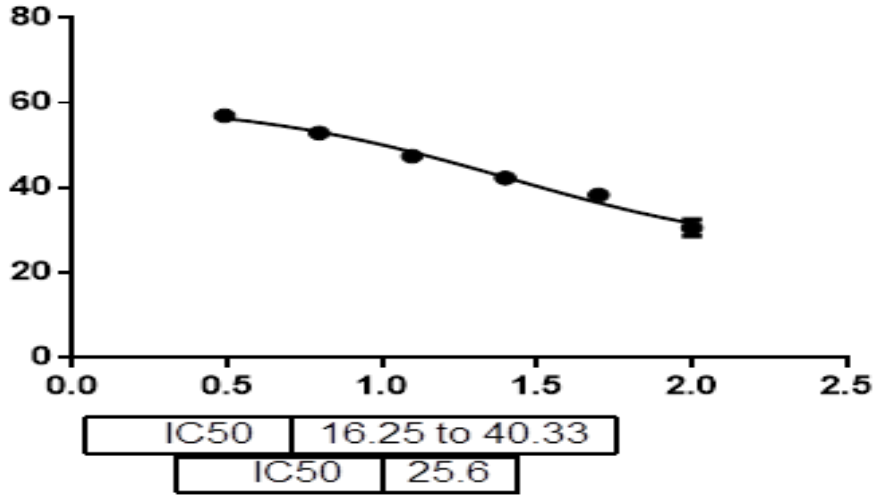

Figure (3) IC_{50} of Azurin on MCF-7 cell line

The second drug "Paclitaxel" that exposed to the same cell line was examined. In which, the results were showing their inhibitory dose (IC_{50}) was 34.57 (figure 4).

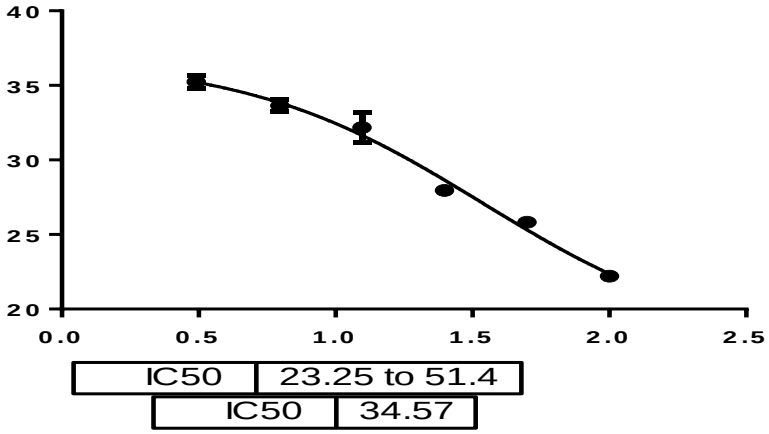

Figure (4) IC_{50} of Paclitaxel on MCF-7 cell line.

In other side, the AMJ-13 cancer cell line showed different cytopatrhic effect than MCF-7, That the inhibitory dose 50 IC_{50} of Azurin was 50.07 (figure 5).

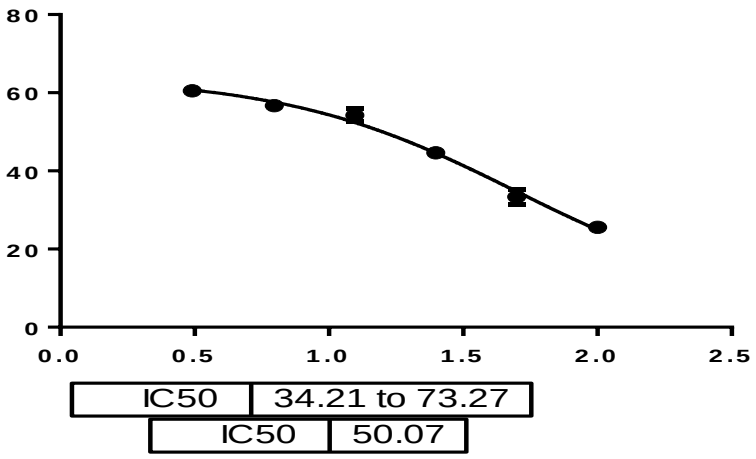

Figure (5) IC_{50} of Azurin on AMJ13 cell line

While the inhibitory dose 50 (IC_{50}) of Paclitaxel was 76.97 (figure 6).

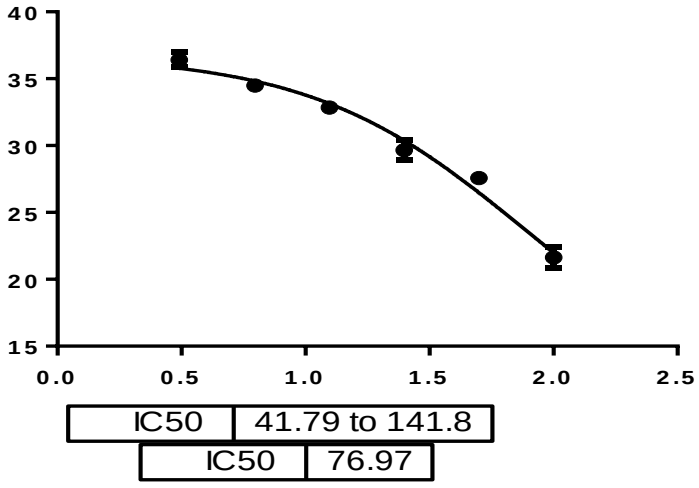

Figure (6) IC_{50} of Paclitaxel on AMJ13 cell line

According to their Inhibitory dose 50 (IC_{50}) results, the Azurin was more cytotoxic impact on cancer cell lines (MCF-7) than Paclitaxel, and Azurin had more cytotoxic effect on MCF-7 with IC_{50} (25.6) comparing with their effect on the AMJ-13 with IC_{50} (50.07). The same effect was recorded by the Paclitaxel, it was more cytotoxic impact against MCF-7 with IC_{50} (34.57) comparing less effectiveness against AMJ-13 with (76.97), and these results were summarized in table (1) bellow.

Table (1**)** The inhibitory dose 50 (IC_{50}) of Azurin and Paclitaxel on AMJ-13 and MCF-7 cancer cell line comparing with NHF normal cell line.

Anticancer Drug	AMJ-13 Cancer Cell-line IC_{50}	MCF-7 Cancer Cell-line IC_{50}
Azurin	50.07	25.6
Paclitaxel	76.97	34.57

As mentioned before, breast cancer (AMJ-13) resist to Azurin as well as Paclitaxel more than the breast cancer cell line (MCF-7). It is may be related to origin of the each one. Since the cell type sources was different, AMJ-13 is infiltrating ductal carcinoma cells (Al-Shammari *et al*., 2015) whereas, MCF-7 is glandular cells (adenocarcinoma). In which, breast tumor cells heterogeneity progression is a dynamic process encompassing the entire clinical history of tumor progression and metastasis, tumor cell heterogeneity is the driving force of therapeutic resistance and distant

metastasis, as it provides endless derivative variant cells to ensure that some will always survive antitumor insults, while some will always bear migration and invasion advantages (Wang *et al*., 2022). The same study revealed that the mechanism of tumor cell heterogeneity seems multifaceted, Genomic damage, genetic mutation, and epigenetic anomalies may all induce phenotypic changes among tumor cells. Therefore, the sensitivity of the cancer cell-lines to the Azurin and Paclitaxel were influenced by the type of their cells origin and morphology that determined the interaction between the cancer cells and the anticancer therapies.

In a prior study, Jadoo N.T.M. (2009) discovered that Azurin had an impact on the Hep-2 and AMN-3 cancer cell lines. This study showed that Azurin has anti-cancer activity on both cancer cell lines, and that this effect was dose- and time-dependent. The Hep-2 cancer cell line had a statistically significant inhibition rate, especially at high doses, and this effect would significantly diminish over time as the Azurin concentration was decreased, with the results being recorded after 24 hrs. while the inhibition effect of the Azurin was increased gradually reaching the most effective inhibition rate after 48hrs. followed by decreased again significantly in inhibition rate at 72 hrs., but in other side, AMN-3 cancer cell line was not only inhibited at low concentration of Azurin but also significantly enhanced and proliferated it. However, extending exposure to 48 hours will increase Azurin's inhibitory effects on the same cancer cells. This effect would be decreased significantly in increased of the exposure time

It is worthy to mentioned that previous studies were focused on the effect of Azurin on Lymphocytes as normal cells, found that it was concentration-depended effect since it was the low concentration was not led to chromosomal disorders like break, gap or ring chromosomal outcome; and the number of chromosomes was stay unchanged, and this indicate has not cytopathic effect at low concentrations. The studies found that phytohemagglutinin (PHA) was activate mature T-cells at arrest cells (G_0 phase) to divide by effecting on proto-oncogene leads to enter the cell cycle phases (M, G2, S and G1) producing daughter cells (Panda *et al*., 2019) therefore, many studies attributed the effect of Azurin at high concentration on PHA function, other studies belong to effect on genes and proteins that worked on cell division not on the cell itself (Gammuto *et al*., 2021 and Ghasemi-Dehkordi *et al*., 2020). In other side, previous study, found the low concentration of Azurin did not show any a notable effect on blast index (BI) as well as mitotic index (MI) comparing with control group, this indicate the Azurin was have not Synergistic effect with PHA at low concentrations (Jadoo N.T.M, 2009).

In other side, a study done by Sheppard and his colleagues on colon carcinoma as well as normal colonic cell found that the administration of the Paclitaxel on the normal cells for a period has an effect on these cells, this study found that Low paclitaxel doses had little effect on normal colonic cell growth over 7 days. Higher paclitaxel doses ($>1 \times 10^{-8}$–10^{-5} M) produced a dose-dependent inhibitory effect on the growth of normal human colonic epithelial cells over 7 days but had no effect on the growth of polyposis, Caco-2, and LoVo cells over 3–7 days of treatment (Sheppard *et al*., 1999).

Study of Morphological Characterization of the Treated and Untreated cell lines

The morphology of treated and untreated cultured cells documented in microphotographic images using Hematoxylin and eosin stain. The untreated cells revealed the cellular properties of the related cell lines. The morphological changes of the treated cells were inclusive of damaged cell membranes and cell shrinkage. Cell damage contributes to the loss of shape and forming of small spherical bodies which are feature of apoptosis.

The morphological changes in population of cells, or the cytopathic effect (CPE) of the current study caused by treatment with Azurin and Paclitaxel were easy to observe. Azurin and Paclitaxel were showed high efficiency for killing breast cancer cells. Many alterations in morphological characteristics of cancer cells after 48 hrs. of exposure representing by cell rounded shrinkages, cell aggregation, formation of syncytia and hollow spaces with cell debris due to cell lysis and death, whereas uninfected cells showed no morphological alterations.

Azurin have reported by several *in vitro* and *in vivo* studies as the multi-targeting anticancer (Yaghoubi *et al*., 2020; Dharmawickreme and Witharana 2021) one of them as apoptotic protein and it is related by Complex formation with the DNA-binding domain (DBD) of tumor suppressor protein P^{53}, stabilizing it and enhancing its intracellular level (both nuclear and cytoplasmatic fractions), which then allows induction of apoptosis. Azurin is expected to boost the mRNA levels of pro-apoptotic molecules via P^{53}, such as the levels of BAX, which would result in an imbalanced level of BCL2-BAX and accelerated cell death or growth arrest.

As Azurin as, Paclitaxel also described as multifactorial proteins. One of these effect as apoptotic drug. In which, PTX causes cellular death, which has been associated with the activation of the transcription factor P^{53} (Xu *et al*., 2018). The previous studies observe the capacity both Azurin and Paclitaxel to treat cancerous cells by more than one way including apoptosis, and this effect was clearly showed by the current study. The figure below showed the anticancer affect them on MCF-7 as well as AMJ-13 and this was confirmed by using H&E stain as illustrated by the figures below (4. A, B and C), (Figure 4. A, B and C) and (Figure 4. A, B and C).

The figure below (7) showing MCF-7 cancer cell line treated by Paclitaxel (7 B) and by Azurin (7 C) comparing with untreated AMJ-13 cells (7 A), it is revealing the Azurin has more destructive effect than Paclitaxel and it is agreed with results of the cytotoxicity effect by MTT assay.

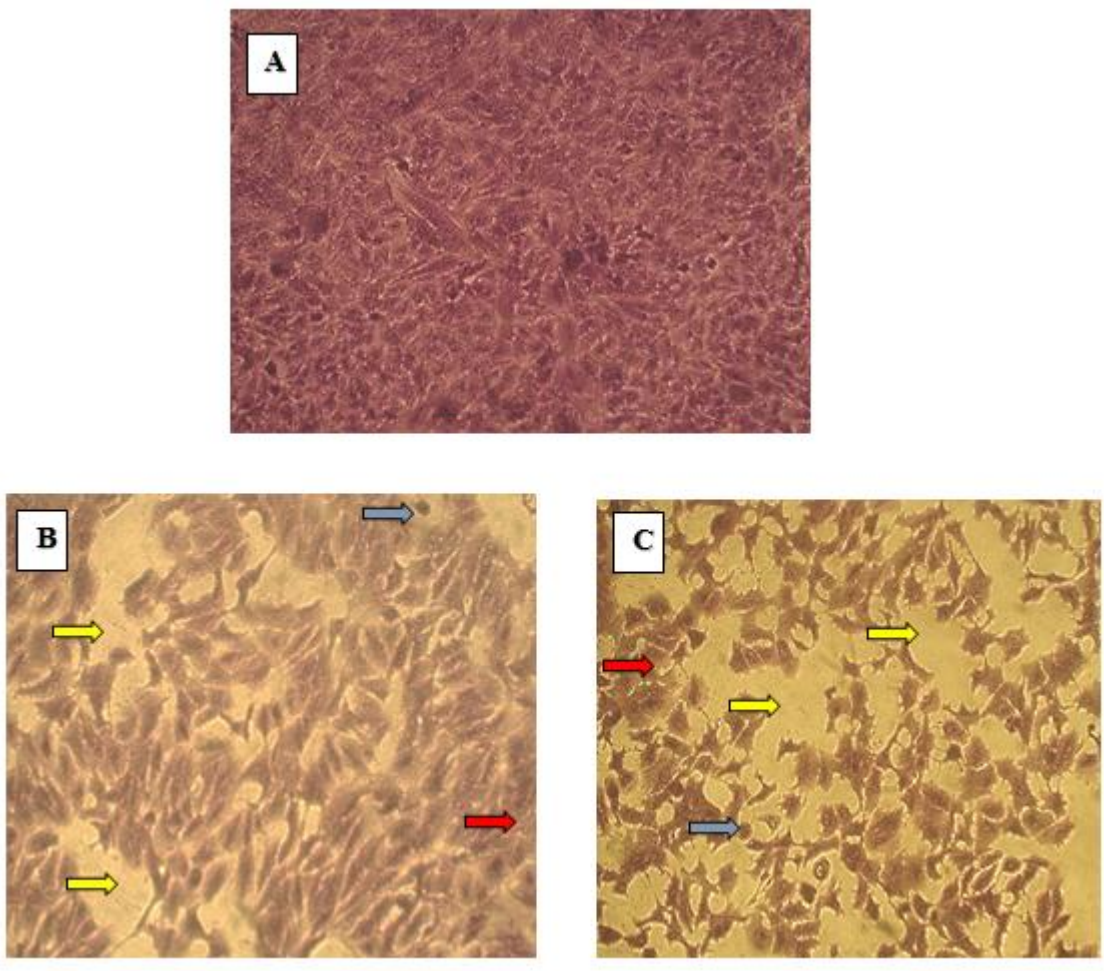

Figure (7) Azurin and Paclitaxel on MCF-7 breast cancer cell line. (A) Untreated control cells retained their distinct morphology. (B) Showing treated MCF-7 by Paclitaxel (C) showing treated MCF-7 by Azurin. The degradation of the breast cancer cells with hollow spaces formation (yellow arrow) and syncytia formation (red arrow) apoptotic bodies (blue arrow). Crystal violet stain, 20x under inverted microscope

The figure below (8) showing MCF-7 cancer cell line treated by Paclitaxel (8 B) and by Azurin (8 C) comparing with untreated AMJ-13 cells (8 A), it is revealing the Azurin has more destructive effect than Paclitaxel and it is agreed with results of the cytotoxicity effect by MTT assay.

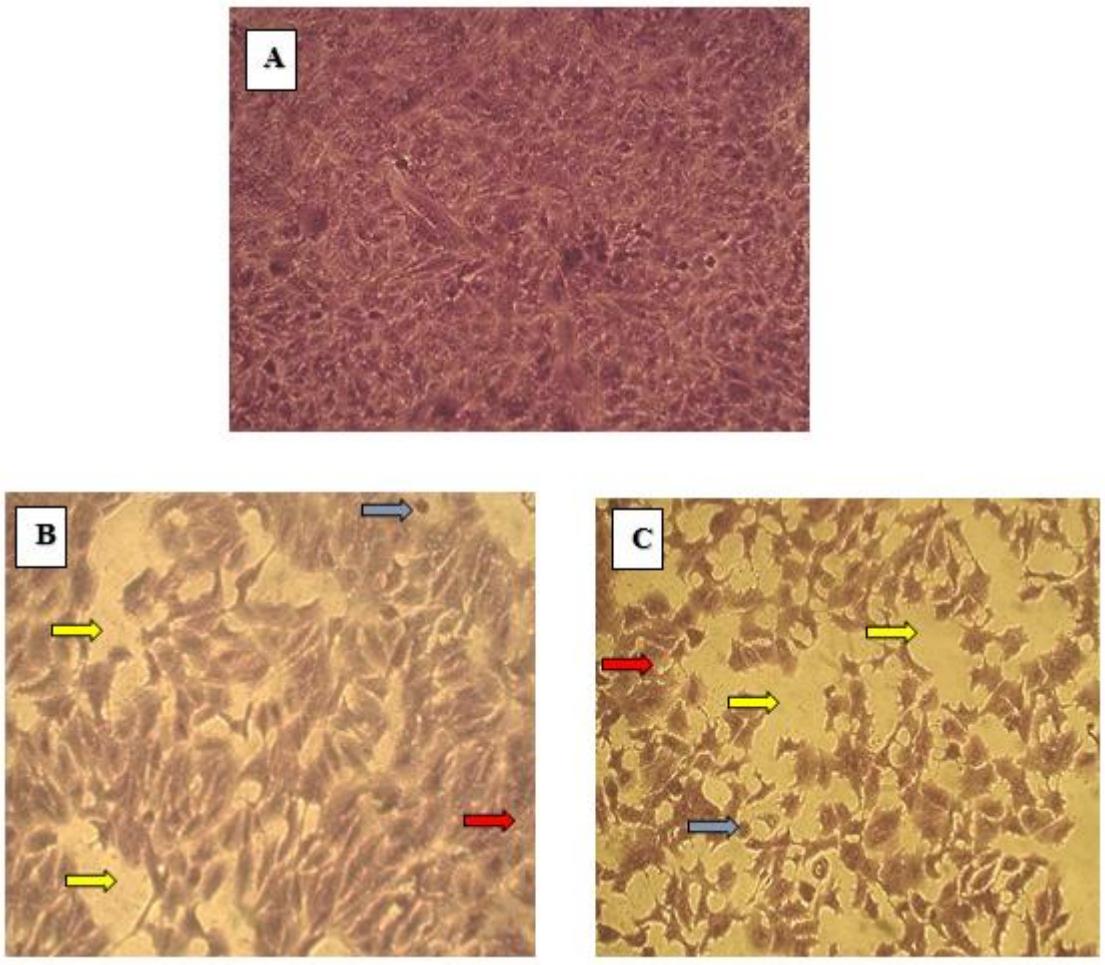

Figure (8) Azurin and Paclitaxel on MCF-7 breast cancer cell line. (A) Untreated control cells retained their distinct morphology. (B) Showing treated MCF-7 by Paclitaxel (C) showing treated MCF-7 by Azurin. The degradation of the breast cancer cells with hollow spaces formation (yellow arrow) and syncytia formation (red arrow) apoptotic bodies (blue arrow). Crystal violet stain, 20x under inverted microscope

The typical CPE of Azurin as well as Paclitaxel is the formation of syncytia which appear as large multi-naculated cells (giant cells) due to cell- cell fusion. In parallel to syncytial development, distinct membrane-enclosed vesicles as results of apoptosis, termed apoptotic bodies or, more recently, apoptosome (Battistelli *et al*., 2020) was formed in addition to a hollow space making by degradation of the breast cancer cells. Garizo *et al*., (2021) showed these peptide act efficiently at the plasma-membrane level, in which the fluorescence microscopy images of the cells showed that treated cells suffered a variety of morphological modifications; i.e., the cell shape became irregular and the fragmentation of the plasmatic membrane and the nucleus was visible. Another study by Ghasemi-Dehkordi *et al*., (2020) showed that Azurin induces apoptosis and necrosis in human MCF-7 breast cancer cells by up-regulation of *BAK*, *FAS*, and *BAX* genes.

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Declaration of Interest

The authors have no conflicts of interest to declare

REFERENCES

1. Al-Shammari, A. M., Alshami, M. A., Umran, M. A., Almukhtar, A. A., Yaseen, N. Y., Raad, K., & Hussien, A. A. (2015). Establishment and characterization of a receptor-negative, hormone-nonresponsive breast cancer cell line from an Iraqi patient. Breast Cancer: Targets and Therapy, 223-230.

2. Battistelli, M., & Falcieri, E. (2020). Apoptotic bodies: particular extracellular vesicles involved in intercellular communication. Biology, 9(1), 21.
3. Damiani, E., Solorio, J. A., Doyle, A. P., & Wallace, H. M. (2019). How reliable are in vitro IC50 values? Values vary with cytotoxicity assays in human glioblastoma cells. Toxicology letters, 302, 28-34.
4. De Oliveira, D. M., Forde, B. M., Kidd, T. J., Harris, P. N., Schembri, M. A., Beatson, S. A., ... & Walker, M. J. (2020). Antimicrobial resistance in ESKAPE pathogens. Clinical microbiology reviews, 33(3), e00181-19.
5. Dharmawickreme, R. B. L., & Witharana, C. (2021). Bacterial protein azurin and tumour suppressor p53 in cancer regression. Advances in Human Biology, 11(2), 147-151.
6. Gammuto, L., Chiellini, C., Iozzo, M., Fani, R., & Petroni, G. (2021). The azurin coding gene: Origin and phylogenetic distribution. Microorganisms, 10(1), 9.
7. Garizo, A. R., Coelho, L. F., Pinto, S., Dias, T. P., Fernandes, F., Bernardes, N., & Fialho, A. M. (2021). The azurin-derived peptide ct-p19lc exhibits membrane-active properties and induces cancer cell death. Biomedicines, 9(9), 1194.
8. Ghasemi-Dehkordi P, Doosti A, Jami MS. (2020). The functions of azurin of *Pseudomonas aeruginosa* and human mammaglobin-A on proapoptotic and cell cycle regulatory genes expression in the MCF-7 breast cancer cell line. Saudi J Biol Sci. ;27(9):2308-2317.
9. Hammad, A., Mohamed M, S. A., Khalifa, M., & El-Daly, M. (2023). Mechanisms of Paclitaxel-induced peripheral neuropathy. *Journal of advanced Biomedical and Pharmaceutical Sciences*, *6*(1), 25-35.
10. Huang F., Shu Q., Qin Z., Tian J., Su Z., Huang Y. & Gao M. (2020). Anticancer Actions of Azurin and Its Derived Peptide p28. The Protein Journal. 39: 182–189.
11. Jadoo, N.T.M. (2009). Cytogenetic Effect of Partial Azurin from Pseudomonas aeruginosa on Cancer and Normal Cells. M.Sc. thesis, College of Science / Baghdad University.
12. Panda, S. K., Ray, S., Nayak, S., Behera, S., Bhanja, S., & Acharya, V. (2019). A review on cell cycle checkpoints in relation to cancer. J. Med. Sci, 5, 88-95.
13. Pandey R, Mishra SK, Shrestha A. (2021). Characterisation of ESKAPE Pathogens with Special Reference to Multidrug Resistance and Biofilm Production in a Nepalese Hospital. Infect Drug Resist.;14: 2201-2212
14. Ramachandran S., Singh M. and Mandal M. (2012). Purification of Azurin from Pseudomonas Aeuroginosa. Chromatography. Leonardo de Azevedo Calderon (ed.).
15. Sheppard, B. C., Rutten, M. J., Meichsner, C. L., Bacon, K. D., Leonetti, P. O., Land, J., ... & Deveney, C. W. (1999). Effects of paclitaxel on the growth of normal, polyposis, and cancerous human colonic epithelial cells. Cancer: Interdisciplinary International Journal of the American Cancer Society, 85(7), 1454-1464.
16. Ünver Y., Yildiz S and Acar M. (2022). Extracellular production of azurin from Pseudomonas aeruginosa in the presence of Triton X-100 or Tween 80. Bioprocess and Biosystems Engineering vol. 45:553–561.
17. Wang, R., Wang, X., Yin, L., Yin, L., Chu, G. C. Y., Hu, P., ... & Pandol, S. J. (2022). Breast Cancer MCF-7 Cells Acquire Heterogeneity during Successive Co-Culture with Hematopoietic and Bone Marrow-Derived Mesenchymal Stem/Stromal Cells. Cells, 11(22), 3553.
18. Yaghoubi, A., Khazaei, M., Avan, A., Hasanian, S. M., Cho, W. C., & Soleimanpour, S. (2020). p28 bacterial peptide, as an anticancer agent. Frontiers in Oncology, 10, 1303.
19. Yaghoubi, A., Khazaei, M., Avan, A., Hasanian, S. M., Cho, W. C., & Soleimanpour, S. (2020). p28 bacterial peptide, as an anticancer agent. Frontiers in Oncology, 10, 1303.
20. Zhu, L., & Chen, L. (2019). Progress in research on paclitaxel and tumor immunotherapy. *Cellular & molecular biology letters*, *24*, 1-11.