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ANTICANCER EFFECT OF *ACTINOMYCETES* SECONDARY METABOLITE AGAINST BREAST CANCER CELL LINE (MCF-7); CYTOLOGICAL AND MOLECULAR STUDIES

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ABSTRACT

Heliomycin is a natural compound extracted from the metabolites of *Actinomycetes* bacteria. We aim to evaluate the anticancer role of Heliomycin, against Tamoxifen as a standard drug. Flow cytometry and analysis of the cell cycle to detect the ratios of apoptosis, and cell cycle arrest were performed, and the genes expression was assayed using real-time RT-PCR. CK-19 protein was assessed using immunocytochemistry. Also, cytopathological alterations were evaluated using H&E stain and electron microscope examination. qRT- PCR showed that HER-2 expression was less elevated in tamoxifen-treated cells than in heliomycin-treated cells in the control cell. For P53 it showed significant up-regulation in heliomycin-treated cells than tamoxifen-treated cells compared to the control. In contrast, ERα, TNFα, and TLR-4 showed the reverse. The flow cytometry assay revealed a considerable increase in early and late apoptosis in the treated group with Tamox. and Helio. compared with the control. H&E stain and ultrastructure study showed cell apoptosis and chromatin degradation and cell shrinkage in treated cells against control. Immunohistochemically, CK-19 showed a severe reaction in control cells while a mild reaction in heliomycin-treated cells versus a moderate reaction in tamoxifen-treated cells. Heliomycin possesses accepted anticancer behaviour against breast cancer MCF-7 cells, as it activates cancerous cell apoptosis with upregulation of tumour suppressor genes.

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Introduction

Breast cancer is considered the most diagnosed cancer and one of the main reasons for cancer-related deaths globally [1]. Breast cancer is the most prevalent cancer in women in Egypt, representing 18.9% of all cancer patients [2].

A discharge, redness, or scariness of the nipple or breast surface, or a change in how the breast feels or looks are all common indications of breast cancer. Regular breast exams can help identify these symptoms early on to stop the spread of the disease [3].

Chemotherapy and surgery are the two most often utilized treatment techniques for breast cancer [4]. However, there are problems with chemotherapy treatments due to both drug toxicity and acquired resistance [5]. Tamoxifen is the most important chemotherapeutic anti-estrogen as it has been in use for 40 years [6].

More than 60% of approved medications are naturally derived [7]; of these, 50% are natural antibiotics made from actinomycetes, which are also known to have antifungal, antibacterial, and anti-inflammatory properties [8]. However, there aren't enough investigations on the potential actions of antineoplastic metabolites from marine actinomycetes-based drugs. Actinomycetes commonly exist as symbiotic microbes, and they are particularly associated with marine sponges [9]. To discover new leaf or seed chemicals, they are excellent sources [10]. Several natural substances from diverse marine sponge-associated were tested against many cancer cell lines for their in vitro anti-cancer effects [11]. Heliomycin is one of these beneficial secondary metabolites produced by marine bacteria [12].

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Breast cancer cell lines are widely utilized in fundamental research and have given important insights into a variety of biological features of breast cancer [13]. Cell lines are one of the most important components in determining new treatment targets for breast cancer and tumor carcinogenesis signal transduction pathways [14].

MCF7 and MDA-MB-231 cells are significant representatives in that they are both invasive ductal/breast cancer cells, and they differ greatly phenotypically and genetically: While MDA-MB-231 is triple negative, MCF7 is hormone dependent (positive for ER and PR, respectively) [15].

We aimed to evaluate the anticancer performances of heliomycin against breast cancer.

Materials and Methods

Chemicals and Reagents

RPMI-1640 medium, Trypsin 0.25%, fetal bovine serum (FBS), penicillin, streptomycin, and Ethanol were supplied from [Sigma-Aldrich, USA]. Tamoxifen was purchased from [AstraZeneca, UK]. Actinomycetes secondary metabolite was identified via the Marine Natural Products Unit (MNPRU), Faculty of Science, Helwan University according to [10]. The breast cancer cell line (MCF-7) was obtained from R&D Sector, Egyptian Organization for Biological Products and Vaccines [VACSERA Holding Company, Giza, Egypt Egypt]. Plastic utilities were purchased from (TPP –Swiss).

Cell Culture

MCF-7 cell line was supplemented by the Cell Culture Department, VACSERA. MCF-7 was cultured in RPMI-1640 medium including 10% FBS, penicillin (100 U/ml), streptomycin (100 µg/ml), HEPES (20 mM), and sodium bicarbonate (25 mM). Then, in accordance with earlier results, at 37°C cells were incubated in a humidified incubator (Jouan-France) [16].

Extraction and Isolation of Secondary Metabolite of Actinomycetes (Heliomycin)

Actinomycetes were isolated and identified according to Abdelfattah *et al*. [17].

Preparation of Compounds

The extract was redissolved in Dimethyl sulfoxide (DMSO) 10mg/1ml. Tamoxifen tablets were grained and dissolved DMSO 10 mg/ml.

Cytotoxicity

The cytotoxic and antiproliferative capability of chemical and natural materials against the MCF-7 cell line was assessed by the 3-(4,5 Dimethylthiazol-2-yl)2, 5 diphenyltetrazolium bromide (MTT) assay. Cell lines were precultured for 24 hours in 96-well tissue culture plates. A suitable concentration of the stock solution was added, serially diluted twice, and incubated at 37° C for 24 hours. The untreated cells are considered a negative control. An inverted microscope (Hund, Germany) was used to observe degenerated cells. Phosphate-buffered saline (ADWIA, Egypt) was used to wash the degenerated cells. To measure cell viability, MTT was applied to the remaining live adherent cells at a final concentration of 0.5 mg/ml for 4 hours at 37°C. In metabolically active cells, MTT is reduced to create an insoluble purple formazan product. Using an ELx-800 Plate ELISA reader from BIOTEKUSA, optical density indicative of the remaining live cells was assessed at a wavelength of 570 nm. Using the Master plex -2010 software, the IC50 of test materials was assessed.

Anticancer Activity

MCF-7 untreated cells were used as a negative control. The test materials (IC50 of heliomycin and tamoxifen) were added to the precultured cell culture flasks (25 cm^2). After 24 hours of incubation at 37 °C, treated and untreated cells were examined under a microscope for detecting degenerated cells. Phosphate buffer saline (PBS) (ADWIA - Egypt) was used to discard the detached cells. The adherent cells were harvested with trypsin, collected, centrifuged at 2000 rpm (10 minutes), and washed with cold PBS. Pellets were added to 100µl PBS in Eppendorf tubes, labeled, and stored at -70 °C.

Real Time PCR

The total RNAs were extracted using RNeasy plus mini kit (Qiagen, Valencia, CA). The Revert Aid^{TM} H. Minus Reverse Transcriptase was used for cDNA synthesis (Ferments, Thermo Fisher Scientific Inc., Canada). Triplicate of the cDNA samples for rt-PCR. On the Applied Biosystems 7500 system, rt-PCR reactions were performed using Power SYBR$^{®}$ Green (Life Technologies, CA). As an internal reference, β-actin was used. The arithmetic formula $2^{-\Delta\Delta CT}$ was used to calculate relative mRNA quantification .primer sequences for the studed genes are as follow HER2 F:5′-GGT CCT GGA AGC CAC AAG G-3′.R: 5′-GGT TTT CCC ACC ACA TCC TCT-3' p53,F, 5'- CCCCTCCTGGCCCCTGTCATCTTC-3',R: 5' -GCAGCGCCTCACAACCTCCGTCAT-3' TNF-α F: 5′-CTT CAG GGA TAT GTG ATG GAC TC-3′R: 5′- GGA GAC CTC TGG GGA GAT GT -3′ ER-α F, 5'-TCCTGGACAAGATCACAGAC-3' R: 5′-GGT TTT CCC ACC ACA TCC TCT-3' ,TLR-4 F: 5'-TTGAAGACAAGGCATGGCATGC-3' R: 5'-TCTCCCAAGATCAACCGATG-3', β-actin,F;5'-TGACGGGGTCACCCACACTGTGCCCATCTA-3 'R;5'CTAGAAGCATTGCGGTGGACGATGGAGGG-3'

Flow Cytometry and Cell Cycle Analysis

All detached and adherent cells were gathered and washed twice with PBS. Annexin V, (Fluorescein isothiocyanate) and FITC conjugate/ Propidium iodide (PI) apoptosis detection kit (ab139418 Propidium Iodide Flow Cytometry Kit/BD) were used regarding the manufacturer's protocol. After incubating the labeled tube for 5 minutes with annexin V-FITC conjugate, the tube was stained with PI and incubated in the dark for 10 minutes. Scan flow cytometry (FAC) was utilized to analyze the samples, and cell quest analysis software was used for data analysis (Becton–Dickinson, USA) [18].

Cytopathology

On clean slides, 50 microliters of heliomycin and tamoxifen-exposed cells were dispensed (3 for each treatment). The slides were dehydrated in descending alcohol concentrations (100%, 90%, 75%, and 50%), air-dried, and then fixed in methanol. The slides were rinsed in distilled water for five minutes. The slides were twice rinsed with distilled water after being submerged in filtered hematoxylin (HE) stain for 3 minutes, then cleaned with distilled water after five seconds of infiltration with eosin stain. After drying, the slides were submerged in xylene then mounted with Canada balsam, and covered with coverslips. Ten microscopic fields on each slide were 400x captured. This was done by attaching a light microscope to a digital camera (Canon, Japan). The selected field is based on the proportion of apoptotic cells present. The existence of morphological apoptotic criteria was qualitatively assessed in the photomicrographs.

Immunocytochemistry

Glass slides of MCF-7 cells were fixed in 10% neutral buffered formalin immunoassays using an anti-CK19 primary antibody (Labvision, Neomarkers, USA) for 90 minutes, then by the secondary antibody (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) application using the immunoperoxidase technique.

Transmission Electron Microscopy (TEM)

MCF7 cells were centrifuged at 2000 rpm (10 minutes) before being applied 3% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.0) for 2 hours at room temperature for fixation, then rinsed in the same buffer, and rinsed 1% osmium tetroxide for 2 hours at room temperature for post-fixation, dehydrated in ethyl alcohol ascending concentrations from 10% to 100% for 15 min for each, followed by 30 minutes in absolute ethanol. Samples were impregnated in pure resin after a graded sequence of epoxy resin and acetone infiltrations. On copper grids, ultrathin sections were mounted, then stained with uranyl acetate and lead citrate. The tissues were examined using a transmission electron microscope (TEM) (80 kV, JEOL- JEM 1010) at the Regional Center for Mycology and Biotechnology (RCMB) [19].

Statistical Analysis

The numerical data of results were expressed as mean ± standard errors (SE). Statistical analysis was carried out using the "SPSS" statistical software (V23). Comparison between different groups was done using a one-way analysis of variance (ANOVA) followed by the Tukey test. The results were considered statistically significant with $P < 0.05$ [20].

Results and Discussion

Cytotoxicity

Data recorded revealed that the cytotoxicity of test compounds "heliomycin and tamoxifen" assessed using MTT assay showed variable IC50 concentrations relative to MCF-7 cell lines where toxicity varied. Bleomycin and tamoxifen were toxic in a significant way to MCF-7 **(Figures 1a and 1b)**. Also, it was found that heliomycin was significantly more toxic than tamoxifen **(Figure 1c)**. IC50 for both heliomycin and tamoxifen were 18.87 ug/ml and 29.6 ug/ml respectively.

The MTT assay was used to measure the toxicity of the test substances. Cell viability was shown to be greatly decreased as a result of the test materials' cytotoxicity, which was found to be dosage dependent. The untreated control showed typical cell shape and behavior. The cytotoxic concentration of test substances that reduced cell viability by 50%, or IC50, was discovered by interestingly detecting a distinct difference between the lethal effects of heliomycin and tamoxifen on MCF-7 cell lines even at the highest concentrations tested. After the therapy, the cells' morphology changed, taking on a thin, elongated appearance. Untreated cells were more crowded than treated ones, and tamoxifen-treated cell lines showed clusters that disappeared in heliomycin-treated cell lines after incubation with IC50 of test compounds. These changes included various morphological abnormalities, such as cell rounding, and some areas devoid of cells **(Figure 1d)**.

Cytopathology

Using the H&E assay, some changes were observed, including a reduction in nuclear sizes, which is frequently associated with varying degrees of chromatin condensation in the same culture. Additionally, the apoptotic bodies featured reduced cell size and blebs formation on the cell surface **(Figures 2)**.

Immunocytochemistry

Immunocytochemistry results showed significant changes in color intensity of treated and untreated cells due to CK-19 Immuno antigen-antibody interaction as the following: decreased levels of heliomycin-treated and tamoxifen-treated cells versus the control **(Figure 3)**.

Transmission Electron Microscopy

- The ultrastructure of control cells showed an enlarged and irregular nucleus, peripheral chromatin, and enlarged nucleolus **(Figure 3D a)**.
- The ultrastructure of heliomycin-treated cells showed degenerated cells, shrunk nuclei, and fragmented chromatin **(Figure 3D b)**.
- The ultrastructure of tamoxifen-treated cells showed degenerated cells, shrunk nuclei, fragmented chromatin, and enlarged dens nucleolus **(Figure 3D c)**.

Real Time (PCR)

Gene expression of pro-apoptotic genes (TNFα, P53, and TLR4) and anti-apoptotic genes (HER-2 and ERα) of heliomycin and tamoxifen-treated cells showed statistically different ratios. HER-2 gene expression was less elevated in tamoxifen-treated cells than in heliomycin-treated cells in the control cell. For P53 it showed significant up-regulation in heliomycin-treated cells than tamoxifen-treated cells compared to positive control. In contrast, each ERα, TNFα, and TLR-4 showed significant down-regulation in heliomycin-treated cells than tamoxifen-treated cells compared to positive control **(Figure 4)**.

Flow Cytometry and Cell Cycle Analysis

Data revealed considerably elevated early and late apoptosis in the treated group with Tamoxifen and Heliomycin compared with untreated cells. Respecting the present results, treated cells percentages in early and late apoptosis for Tamoxifen were 5.66%, and 11.16% while heliomycin recorded 3.22% and 14.46% for Heliomycin treated, while this proportion for the untreated group was 0.27%, 0.15% respectively. Also, necrosis cells showed a slight increase in both two-treatment groups compared to the control MCF-7 group **(Figure 5)**.

Cell cycle showed remarkable elevation in two treated groups cells in the Sub G1 phase related to a notable reduction in G0 / G1 population after treatment with both heliomycin and tamoxifen compared to the control group. Furthermore, for S phase showed a non-significant decrease in both two groups compared to the control group. Also, G2/M showed a significant increase in the treated group with Heliomycin than Tamoxifen treated group compared to the control groups. Regarding the present findings, untreated cell percentage in the sub-G1, G0 /G1, S, and G2/M phases were assessed at 1.78%, 52.06%, 39.88%, and 8.06%, respectively. These proposition for the treated cells obtained 25.72%, 36.25%, 31.48%, and 38.27% for Heliomycin treatment, 23.14%, 38.45%, 36.99%, and 24.56% for Tamoxifen treatment, respectively **(Figures 5 and 6)**.

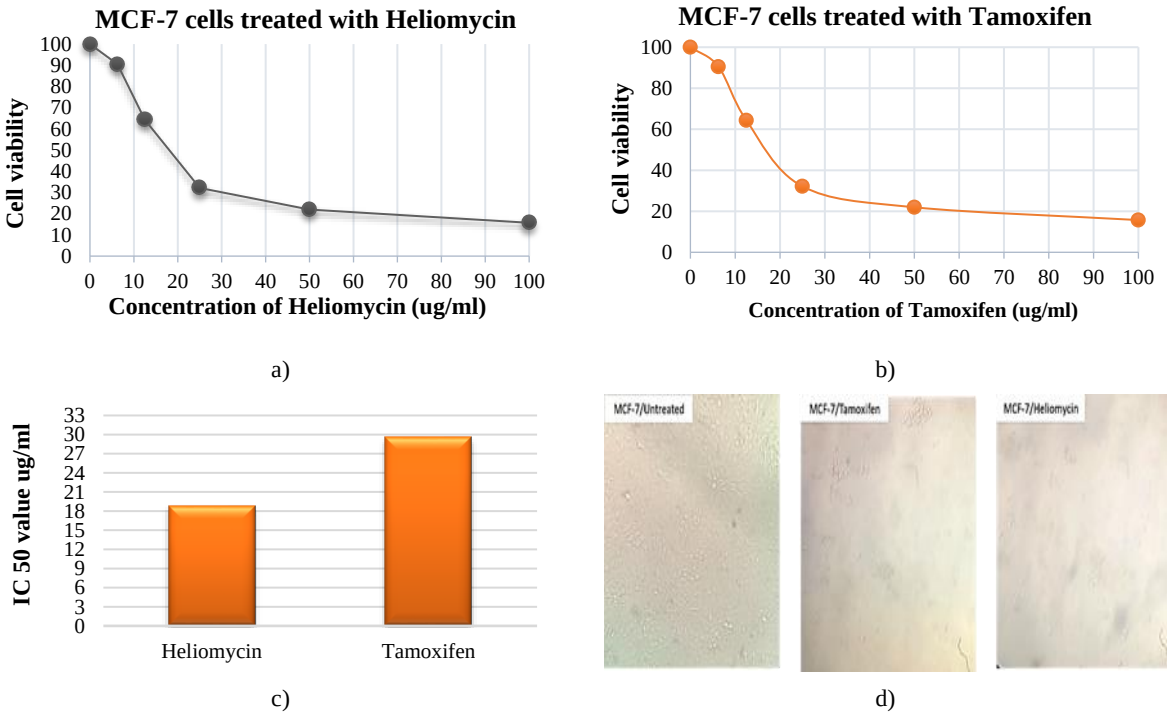
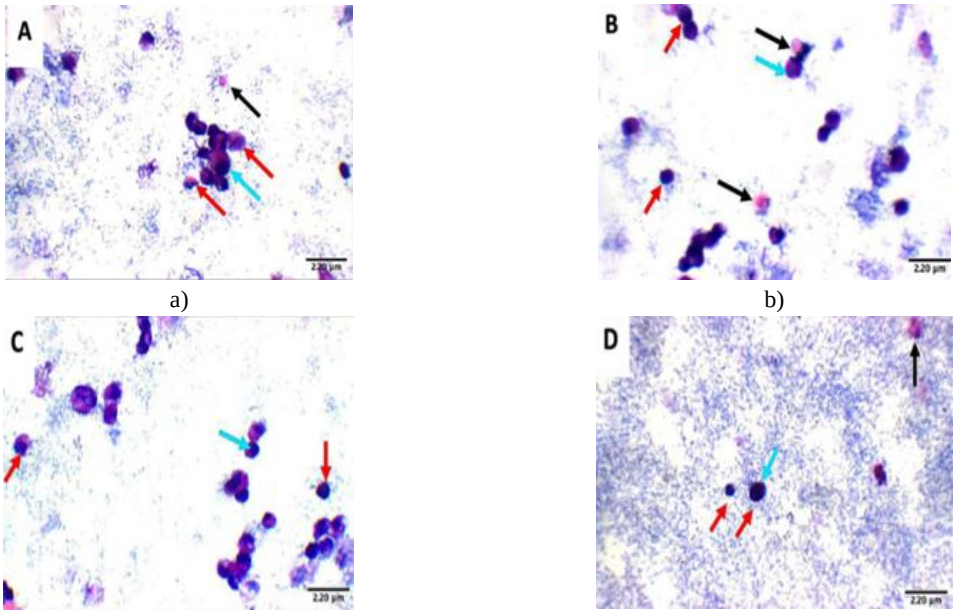


Figure 1. a) Determination of IC50 and correlation between the Heliomycin concentrations and the cell viability against MCF-7, b) Determination of IC50 and correlation between the Tamoxifen concentrations and the cell viability against MCF-7, c) Assessment of IC50 values of Heliomycin and Tamoxifen on MCF-7 using MTT assay, d) Cytotoxic effect of heliomycin and tamoxifen on MCF-7



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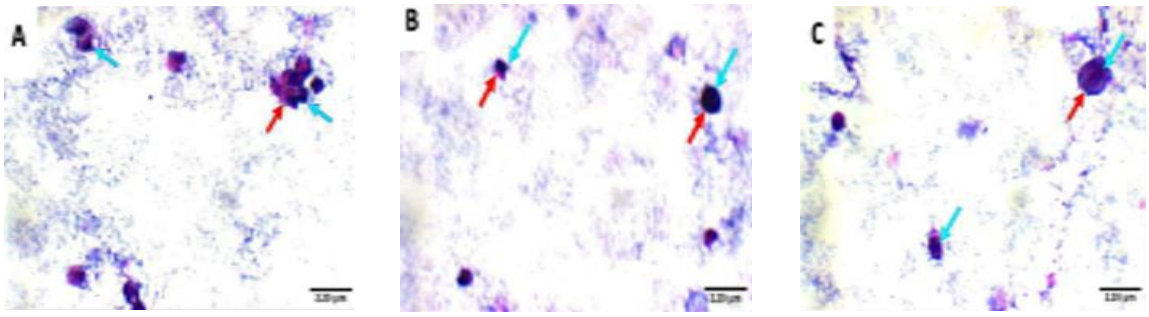

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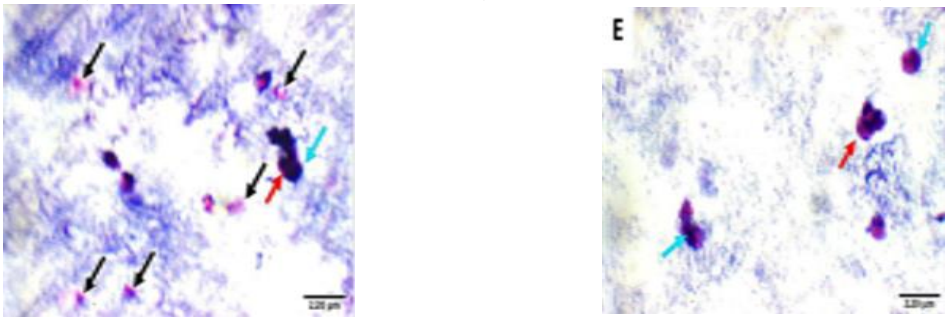

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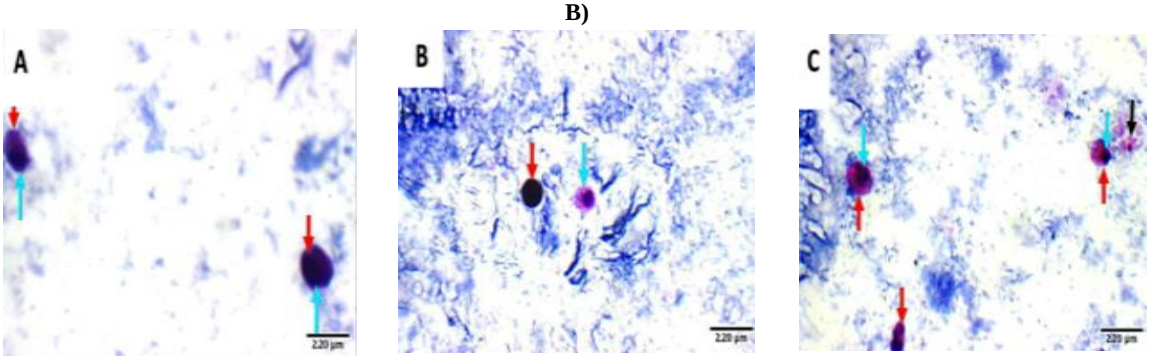

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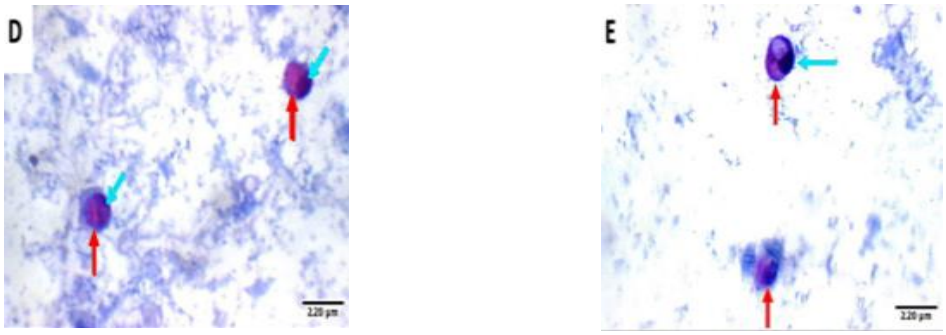

d) e)

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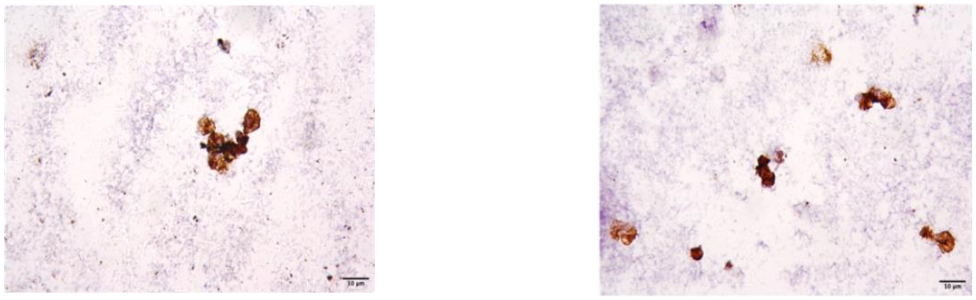
Figure 2. (A) Cytopathology of positive control MCF-7: (a) clusters and isolated viable markedly pleomorphic cells (red arrow) with increased N/C ratio and hyperchromatic nuclei (blue arrow), and one apoptotic cell/HPF (black arrow); (b) another view showing clusters and isolated viable markedly pleomorphic cells (red arrow) with hyperchromatic nuclei (blue arrow), and 2 apoptotic cells/HPF (black arrow); (c) another view showing clusters and isolated viable markedly pleomorphic cells (red arrow) with hyperchromatic nuclei (blue arrow); (d) another view showing isolated viable markedly pleomorphic cells (red arrow) with hyperchromatic nuclei (blue arrow), and 1 apoptotic cell/HPF (black arrow) (H&E X400).
(B) Cytopathology of tamoxifen treated MCF-7: (a) clusters and isolated viable markedly pleomorphic cells (red arrow) with increased N/C ratio and hyperchromatic nuclei (blue arrow); (b) another view showing clusters and isolated viable markedly pleomorphic cells (red arrow) with increased N/C ratio and hyperchromatic nuclei (blue arrow); (c) another view showing isolated viable markedly pleomorphic cells (red arrow) with increased N/C ratio and hyperchromatic nuclei (blue arrow); (d) another view showing clusters and isolated viable markedly pleomorphic cells (red arrow) with increased N/C ratio and hyperchromatic nuclei (blue arrow), and 5 apoptotic cells/HPF (black arrow); (e) another view showing isolated viable markedly pleomorphic cells (red arrow) with increased N/C ratio and hyperchromatic nuclei (blue arrow), and 1 apoptotic cell/HPF (black arrow) (H&E X400).
(C) Cytopathology of heliomycin treated MCF-7: (a) isolated viable markedly pleomorphic cells (red arrow) with increased N/C ratio and hyperchromatic nuclei (blue arrow); (b) another view showing viable cell with increased N/C ratio and hyperchromatic nuclei (red arrow) and another cell with hyperchromatic nucleus and eosinophilic cytoplasm (blue arrow); (c) another view showing isolated viable markedly pleomorphic cells (red arrow) with increased N/C ratio and hyperchromatic nuclei (blue arrow), and 1 apoptotic cell/HPF (black arrow); (d) another view showing isolated viable markedly pleomorphic cells (red arrow) with increased N/C ratio and hyperchromatic nuclei (blue arrow); (e) another view showing isolated viable markedly pleomorphic cells (red arrow) with increased N/C ratio and hyperchromatic nuclei (blue arrow) (H&E X400).



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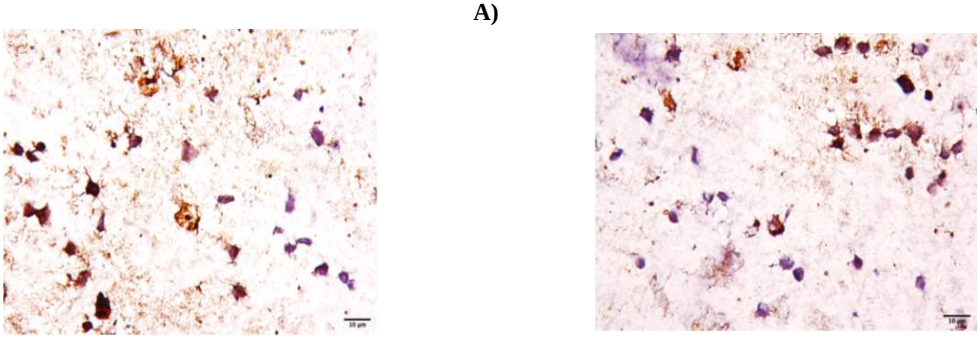

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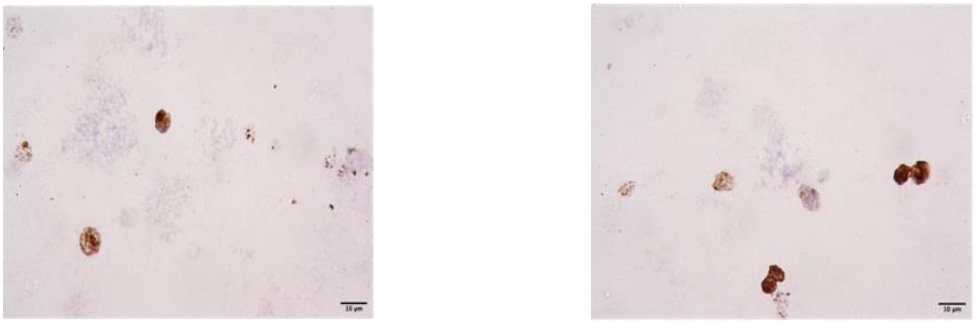

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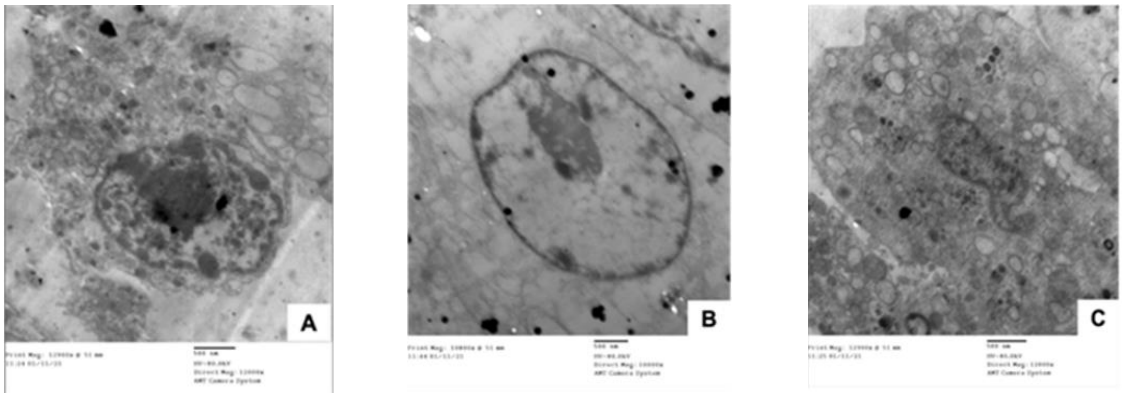

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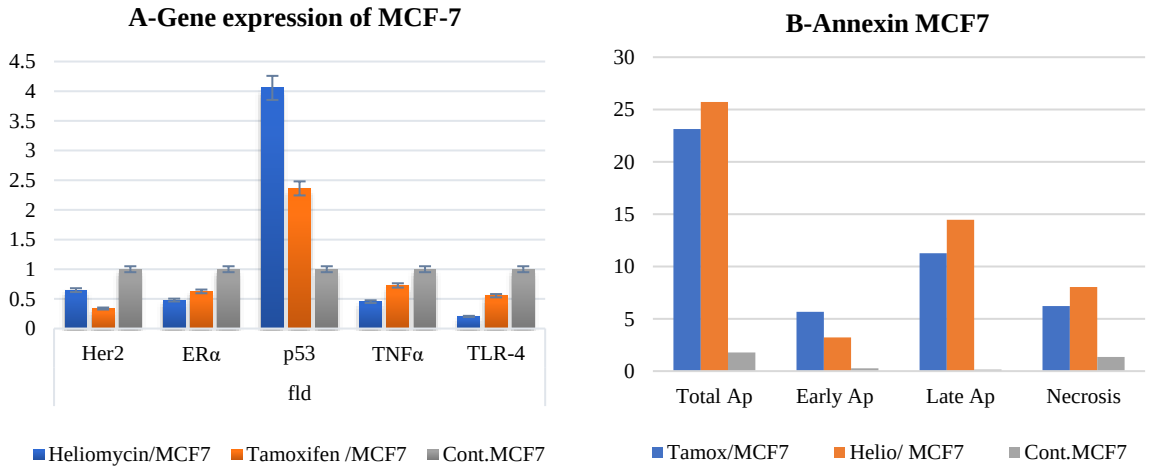
Figure 3. Immunocytochemistry of MCF-7, **(A)** MCF-7 adenocarcinoma cell cluster showing strong positivity for cyrokeratin 19 (CK-19), **(B)** MCF-7 adenocarcinoma cells treated with tamoxifen showing moderate positivity for cyrokeratin 19 (CK-19), **(C)** MCF-7 adenocarcinoma cells treated with heliomycin showing mild positivity for cyrokeratin 19. **(D)** Electron micrograoh for MCF-7: (a) control cells showing enlarged and irregular nucleus, peripheral chromatin and enlarged nucleolus. (b) tamoxifen-treated cells showing degenerated cell, shrinked nucleus, fragmented chromatin and enlarged dens nucleolus. (c) heliomycin-treated cells showing degenerated cell, shrinked nucleus and fragmented chromatin



a) b)

Figure 4. a) HER-2, ERα, P53, TNFα and TLR-4 expression in MCF-7 post treatment with heliomycin and tamoxifen compared to positive control. **b)** Assessment of apoptosis and necrosis using flowcytometry assay against MCF-7

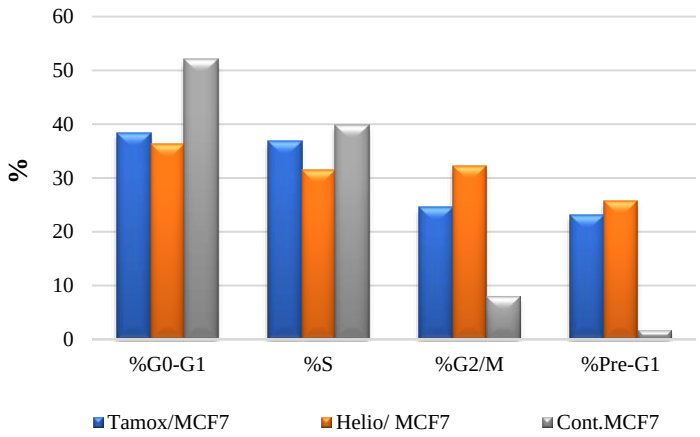


Figure 5. Assessment of flow cytometric cell cycle analysis for MCF-7 cells.

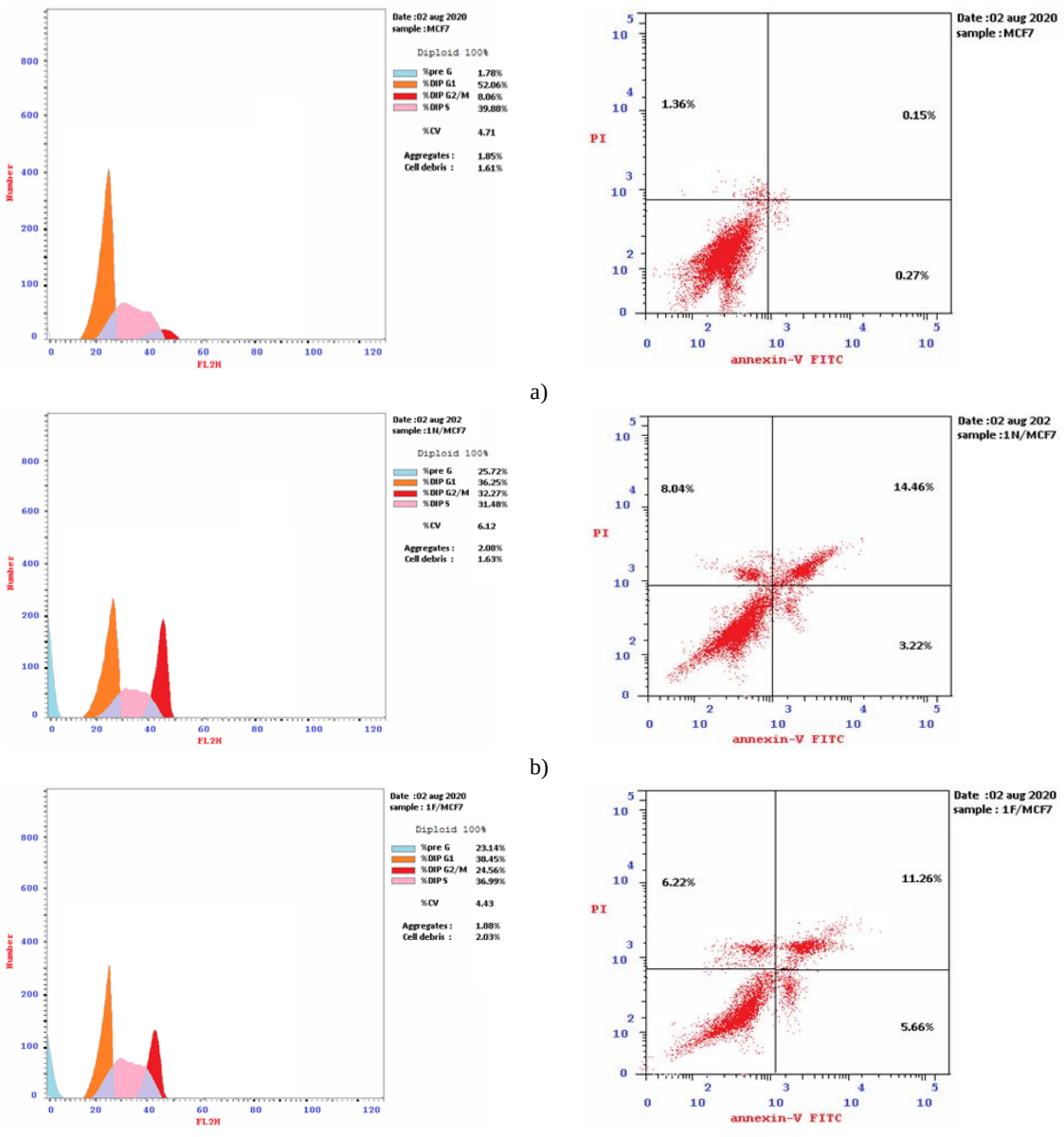


Figure 6. Flow cytometric cell cycle analysis of MCF-7 and related % of arrested cells in different cell cycle phases, as well as the apoptotic % post treatment. a) Post. control, b) Helio. treated and c) Tam. treated.

The most prevalent type of cancer in women globally is breast cancer, which is also the second largest cause of death after lung cancer [21]. In this context, extracts from natural products remain a promising source of novel cancer treatments.
In the current work, we looked at how tamoxifen and heliomycin affected breast cancer (MCF-7) cells. The effect was investigated by cell cycle analysis, apoptotic flow cytometry analysis, real-time RT-PCR for ER-α, HER-2, p53, TNFα, and TLR4, cytopathology, and immunocytochemistry.
Heliomycin is an unusual aromatic polyketide that possesses anticancer [22], antibacterial and antiviral activities. Several studies revealed that heliomycin inhibits RNA polymerase [23], and translation. Also, it inhibits histone deacetylase [24]. Histone deacetylase is an enzyme that regulates the deacetylation mechanism of DNA and once it is inhibited, DNA replication will be stopped which leads to stopping the cell cycle and enhancing the mechanisms of apoptosis.
Tamoxifen is a nonsteroidal agent with anti-estrogenic properties that bind to oestrogen binding sites in breast tissue [25]. It is frequently prescribed in clinics as hormonal therapy for oestrogen positive breast cancer. Tamoxifen competes with oestrogen receptors in neoplastic cells, as a consequence a nuclear complex was formed inhibiting the synthesis of DNA and actions of estrogen.
The IC50 was first determined to be used as a base for subsequent investigations. The present study showed that the Actinomycete isolate "heliomycin" recorded IC50 values of 18.87 ug/mL concentration in MCF-7 compared to higher IC50 values of 29.6 ug/mL of "tamoxifen" which adheres with Suffness and Pezzuton [26] who indicated that The IC50 values less than 30 ug/mL in cancer cell lines can be considered significant for the development of anticancer drugs.
Cell-free extracts from Actinomycete isolates have been shown to have anticancer properties, caused by quinine and alkaloids in their metabolites [27]. Actinomycete extracts can induce apoptosis, the degradation of fusion transcripts, and partial cellular differentiation as well as reduce angiogenesis and proliferation [28].
CK19 is formed in the epithelial cells as cytoskeleton protein, it is also formed in the form of full-length protein in active tumor cells. This feature could be relevant in metastatic breast cancer [29]. It is widely used as a diagnostic marker for breast epithelial cells [30].
The present study showed a reaction of CK-19 with MCF-7 cells giving mild reaction with heliomycin-treated cell line, moderate reaction with tamoxifen-treated cell line, and strong reaction with positive control. The results of our study are in agreement with Stathopoulou *et al*. [31] who concluded that CK-19 expression was found primarily in cases with stages II and III breast cancer. CK-19 expression upregulated as the disease progresses and in agreement with Fogh *et al*., [32] who reported that CK19 marker expression was found in two T47D and MCF7 cell lines, indicating that they are derived from luminal epithelial cells.
We can explain that the expression of CK-19 is related to the cancer activity of MCF-7 cells and both heliomycin and tamoxifen showed a significant anticancer activity which limited CK-19 expression in cells compared to the positive control cell line which gave a strong reaction with CK-19 where is no anticancer activity of treatment.
TNF-α is a pleiotropic cytokine that, although it was initially discovered to cause apoptosis in some cancer types, may also have tumor-promoting properties. These include the ability to induce transformation, proliferation, angiogenesis, invasion, and metastasis [33]. Many malignant tumors, especially breast cancers, have high levels of TNF-α, which has repeatedly been linked to aggressive activity and a poor prognosis [34].
Supporting this line of evidence, we have reported a significant down-regulation of TNF-α in MCF-7 cells treated with heliomycin and tamoxifen as compared with the control. These results agreed with Katerinaki *et al*., [35] who reported that in human epithelial malignancies, TNF-α is typically increased. It has been demonstrated that TNFR1, which upregulates JNK and PI3K/AKT, both of which boost NF-kB, mediates TNF-α proliferation potential in MCF-7. TNF-α triggers the expression of cyclin D1, which results in elevated proliferation [35, 36]. Remarkably, estradiol treatment showed similar effects [35]. TNF-α also induces cancer cells' growth by the p42/p44 MAPK pathway, which is influenced by TNFR1 and TNFR2 and operates independently of NF-kB, providing a different route for the spread of breast cancer [36]. JNK pathway activation makes the MCF-7 cell line vulnerable to TNF-α-induced apoptosis, but p42/p44 MAPK is only minimally activated [37].
Human epithelium and immune cells are the main sources of expression for toll-like receptors (TLRs) [38]. TLRs' primary purpose is to stimulate the production of inflammatory cytokines, which sets off the inflammatory response [39]. In metastatic ductal carcinoma, TLR 4 expression has also been shown to be substantially correlated with clinical indications [40].
The present study showed down-regulation of TLR4 in MCF-7 cells treated with heliomycin and tamoxifen compared with control cells which are in agreement with Haricharan and Brown [41] who observed that TLR4 expression changes the ratio of growth and apoptotic cytokines in the extracellular space in TP53 mutant breast cancer cells, ultimately leading to an increase in proliferation and growth and with Yang *et al*., [42] who found that TLR4 was expressed in MDA-MB-231 and MCF-7.
P53 is a remarkable protein with many different functions. Numerous biological processes were influenced by P53 such as cell cycle control, DNA repair, differentiation, inflammatory response, metabolism, senescence, immunological response, hormone-induced processes, transcription, autophagy, and epigenome are influenced by it [43].
The present study showed a significant up-regulation of p53 in MCF-7 cells treated with heliomycin and tamoxifen compared with control which is in line with Van Slooten *et al.* [44] who reported that given that p53 acts as an inhibitor for

cell proliferation, it is not surprising that increased proliferative potential of cancer cells is correlated with the increased p53 mutations (decreased expression).

HER2 is a member of the epidermal growth factor (EGF) family of receptors (erbB4). A 185-kDa transmembrane protein is encoded by the HER2 gene, which is found on chromosome 17 [45].

The present study showed a significant down-regulation of HER2 in MCF-7 cells treated with tamoxifen and heliomycin compared with control which is in agreement with Hudis, [46] who illustrated that in about 20–30% of breast cancer, HER2 is overexpressed. Shorter survival, more aggressiveness, and higher recurrences are all linked to HER2 overexpression [47].

Its ligand, oestrogen or estradiol (E2), activates the ER signaling pathway, which is essential for the development of the mammary gland [48]. Consequently, a key therapeutic target for luminal breast tumors is the ER. Antiestrogen medications including the pure antagonist fulvestrant and partial antagonist tamoxifen (Tam.) directly target the ER [49].

The present study showed a significant down-regulation of ERα in MCF-7 cells treated with heliomycin and tamoxifen compared with the control. This result is on the same line with [50] who reported that it has been noticed that breast cancer cell lines showed increased development with elevated estrogen.

The present study shows the ability of both heliomycin and tamoxifen to form apoptotic bodies and inhibit cell growth. Clusters and isolated viable markedly pleomorphic cells with increased N/C ratio and hyperchromatic nuclei, and 1-6 apoptotic cells/HPF are the results of treating MCF-7 with tamoxifen while heliomycin treated MCF-7 cells show isolated viable markedly pleomorphic cells with increased N/C ratio and hyperchromatic nuclei, and other cells with hyperchromatic nuclei and eosinophilic cytoplasm, and 1 apoptotic cell/HPF, while positive control cells show clusters and isolated viable markedly pleomorphic cells with hyperchromatic nuclei, increased nucleo/cytoplasmic (N/C) ratio, 1-2 apoptotic cells/HPF, and few large cells with hyperchromatic nuclei and abundant eosinophilic cytoplasm. This result is in agreement with Vijayabharathi *et al*., [21] who found that heliomycin had a significant antiproliferative effect on cervical carcinoma (HeLa), hepatic carcinoma (HepG2), and gastric carcinoma (HMO2) cell lines.

According to [50], Heliomycin was expected to influence HER2 tyrosine kinase, which is overexpressed in tumor cells, according to molecular docking studies. Additionally, research has shown that heliomycin has the unique ability to control the pro- or anti-apoptotic actions of other substances [47].

Apoptosis is featured by a string of morphological phenomena that are common to it, like cell shrinkage and fragmentation into membrane-bound apoptotic bodies [49]. Internucleosomal and fragmentation of genomic DNA [48]. Chromatin condensation into clearly defined masses that are peripherally located [49]. In our study, transmission electron microscopy ultrastructure of control MCF7 cells showed enlarged and irregular nucleus, peripheral chromatin, and enlarged nucleolus, and ultrastructure of heliomycin-treated cells showed degenerated cell, shrunk nucleus and fragmented chromatin where ultrastructure of tamoxifen-treated cells showed degenerated cell, shrunk nucleus, fragmented chromatin, and enlarged dens nucleolus. TEM results are in line with both findings of cytopathology and flow cytometry and we can explain this according to the apoptotic pathway by the action of anticancer activity of heliomycin and tamoxifen [50].

Conclusion

Actinomycetes bacteria metabolite Heliomycin possesses an anticancer effect against breast cancer MCF-7 cells, as it activates cancerous cells apoptosis with upregulation of tumor suppressor genes, so it may be a promising anti-cancer drug besides chemotherapy tamoxifen.

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