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The Comparison Between the Cytotoxic Effect of Deinoxanthin and Mitomycin-C on MCF7 Cell Lines and the Changes on *Bax* and *BCL2* Genes After Exposure

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Research Article

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Keywords: Deinoxanthin, Apoptosis, MCF_7, Mitomycin-C, *Bax*, *BCL2*.

ABSTRACT

Background: Deinoxanthin, is a carotenoid extract isolated from *Deinococcus radiodurans*. Mitomycin-c, as a cancer chemotherapeutic agent, has been used for the treatment of wide ranges of malignancies including but not limited to uterus, lung, chest, stomach and bladder cancer for over 30 years.

Purpose: The aim of this study was to investigate the apoptotic effect of deinoxanthin on MCF_7 cell lines compared to mitomycin-c.

Method: Apoptotic effects were evaluated in MCF_7 cells and were measured through cell viability and morphological changes. Cellular viability was measured using MTT assay and the expression of apoptotic and anti-apoptotic genes was assayed by real-time PCR.

Results: The half-maximal inhibitory concentration (IC_{50}) values for deinoxanthin against the MCF_7 cell line after 24, 48 and 72 hours after treatments were 18.18, 22.18, 21.18 µg/ml and the IC_{50} values for mitomycin-c were 2.26, 0.96, 1.96 µg/ml. Moreover, *BCL2* and *Bax* expressions in MCF_7 cell line increased by treatment with mitomycin-c, deinoxanthin and both of these cytotoxic agents.

Conclusion: The present findings demonstrate the novel functional property of deinoxanthin isolated from radioresistant bacteria as a potent inducer of apoptosis in MCF_7 breast cancer cell line. These data suggest that deinoxanthin could be potentially useful as a chemopreventive agent of the ketamine.

INTRODUCTION

Deinococcus radiodurans is a well-known bacterium that is resistant to reactive oxygen species by exposure to ionizing radiation and oxidative stress. The antioxidant effect of this bacterium is highly related to carotenoids extracted from it [1]. Among the extracted carotenoids, deinoxanthin has been shown to have a very strong protective and restorative effect on DNA [2]. The structure of this chemical has several conjugated dual bonds and also a hydroxyl group at position 1c. The superior antioxidant effect of deinoxanthin can be correlated with the unique chemical structure mentioned [1,3]. Cancer can be considered as an imbalance between cell growth and death. Reduced cell death is due to the excessive expression of anti-apoptotic genes and inhibition of the expression of apoptosis genes. *BAX* and *BCL2* have been identified specifically as important proteins in the apoptosis, whose unbalanced expression, along with the presence of angiogenic agents, will exacerbate the decrementation of homeostasis in the body [4–8]. In addition, damage to the *BAX* and *BCL2* gene and the excessive expression of the anti-apoptotic genes, including *BCL2* have been observed in various cancers including melanoma, breast, prostate, and CLL, as well as in resistance to cancer treatment. [4,9,10]. In many studies, to eliminate cancer cells selectively, the use of strong antioxidants along with common chemotherapeutic agents have been proposed [2,7,11,12]. Also, in previous studies, induction of apoptosis of cancer cells by deinoxanthin was investigated and apoptotic and viability effects, morphological changes, and fragmentation of DNA were measured by oxidizing agents [2,13–15]. The results of studies showed that deinoxanthin has decreased the level of activity promoting enzyme Caspase 3 along with the expression of *BCL2*, a known gene against apoptosis in cancer cells and it increased the expression of *BAX* gene expression, the apoptotic gene [2,9]. The aim of the present study was to investigate the possibility of

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apoptotic properties of mitomycin-c and deinoxanthin. Due to the very different factors involved in the apoptosis, two genes from *BCL2* family have been considered as variables. The cytotoxic effect of mitomycin-c and deinoxanthin was evaluated on MCF7 breast cancer cell lines.

MATERIALS AND METHODS

Materials and equipment

Blue, yellow and crystal tips were purchased from QC LAB (). Samplers were obtained from Nichipet EX II. 96 well plates were purchased from SPL (). Falcon (15 and 50 ml) and flask (25T) were obtained from JetBioFil (China). Plates, microtubes, vials, Neubauer chambers, and centrifuge tubes (5 ml) were all purchased from Biologix (). MTT assay kit was obtained from Biosera (France). Deinoxanthin was purchased from Chemistry & Chemical Engineering Research Center of Iran (Tehran, Iran). MCF7 (HUMAN BREAST ADENOCARCINOMA (IRBC CODE: C10682) cell lines were obtained from the Iranian Biological Resource Center (Tehran, Iran). Fetal bovine serum (FBS), DMSO, DMEM, and ^{RPMI} culture were obtained from Bio-Idea (Tehran, Iran). PBS (Phosphate buffered saline) and EDTA-Trypsin were purchased from Biosera (France). Mitomycin- C, penicillin and streptomycin were purchased from Sigma-Aldrich (Darmstadt, Germany). DEPC Treated Water and Rnx_Plus were acquired from Cinnagen (Tehran, Iran). Laminar flow cabinet (by KimyaGen, Tehran, Iran), Incubator (SCI FINETECH, Seoul, Korea), centrifuge (CENTURION, West Sussex, United Kingdom), Invert microscope made by Sunny (Japan). Cooling system, ELISA reader, Real-Time PCR by Bioneer, autoclave, and nanodrop device were used in this study.

METHODS

Determination and preparation of deinoxanthin

The derived deinoxanthin characteristics have been compared to cytotoxic effects with pre-registered data for this compound. There are various techniques to examine the nature of this substance. One of the most commonly used techniques is UV spectroscopy and absorption spectroscopy of deinoxanthin. In this study, *Deinococcus radiodurans* culture was purchased from Chemistry & Chemical Engineering Research Center. Then, in the laboratory, it was determined by spectrophotometry technique and according to the reference; the presence of deinoxanthin was confirmed. Accordingly, the peak observed in the absorption spectrum of 220 nm proved the presence of deinoxanthin **(Figure 1).** After preparation of deinoxanthin in 15 ml Falcon, concentrations of 0.5, 1, 5, 10, 20, 30, 40 and 50 µg/ml were prepared by dilution with deionized water.

Preparation of different concentrations of mitomycin-C

A complete powder vial of mitomycin-c (2 mg) is completely dissolved in 4 ml water (concentration of 500 µg/ml). To measure the cytotoxic effect of mitomycin-C and compare it with deinoxanthin, 0.5, 1, 2, 5, 10 and 20 µg /ml concentrations of the prepared solution were prepared.

Preparation of DMEM medium

Based on the specification of the MCF7 texture obtained from Iranian Biological Resource Center, the appropriate environment for this category is DMEM, which contains essential amino acids, vitamins, and salts. To make this nutrient, 900 ml of deionized water was added into a 1 L Erlenmeyer flask and a magnet was placed in it. Then it was placed on a magnetic stirrer, and 37.16 g DMEM powder and 317 g sodium carbonate powder were weighed and added to the container as a buffer. After the solution is made, pH was adjusted in the range of 7.2-7.4 and it was sterilized with a 0.26-micron filter underneath the laminar flow cabinet. To prevent bacterial growth in this nutrient medium, 7.5 ml of penicillin-streptomycin antibiotic was added to the solution. Then 100 ml deionized water was added to the culture medium to reach a final volume of 1000 ml. In the end, underneath the laminar flow cabinet, the culture medium was filtered and transported in two 500 ml glasses and transferred to the refrigerator for storage. The stability time of the culture medium made is 2 weeks in the refrigerator.

Cell culture

DMEM medium was prepared with 10% bovine serum and penicillin-streptomycin 1% as a complete culture medium for cells. Some cells produce a lot of endogenous carbon dioxide. Every 24 to 48 hours, the cell culture medium is replaced. After covering the flask floor with bone marrow cells and achieving confluency of 70% - 87% in the initial culture, the was done accordingly: 1. culture medium in each flask was emptied, 2. the cell surface was washed with about 2 milliliter phosphate buffered saline PBS, 3.1 ml of trypsin-EDTA enzyme was added to 2 cm flask, 3. the flask was transferred to the incubator to increase the enzyme function for 3 minutes, 4. The flask was tapped a little too easily separate the cells from the flask floor, 5. trypsin effect was neutralized by adding a culture medium containing 17% FBS to 7.5 ml flask and pipetting and creating cell suspension, 6. The suspension was transferred to 15 ml Falcone and centrifuged at 1500 rpm at 25°C for 1.37 minutes. The supernatant was removed [7] cell culture medium containing 17% FBS was added to 1 ml of the precipitate. After pipetting and creating cell suspension, it was transferred to two of three new flasks in terms of cell density, 8.4 ml cell culture medium containing 17% FBS was added to each flask and the new flasks were transferred into the incubator, 9. Trypan blue was added to the suspension and the cells were counted before transferring the flasks to the incubator.

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Freezing the cells

To freeze, the supernatant in the flask must be removed and the cells must be washed with PBS. The cells were then incubated for a few minutes at 37°C and purified by pipetting. After preparing the cell suspension, cell counting was done and the viability of the cells was determined. Based on the count, the number of required cryovials was determined and the cell characteristics, including the name of the cell line, the number of cells, the passage number, and the date of freezing, were inserted onto the cryovials. Then, the cry-vials were placed in ice and with the aid of the microtiter; the medium containing DMSO, FBS, DMEM was prepared and added to the cryovials. The cryovials were transferred to a freezer-20°C for 1-2 hours. It was then frozen at 80°C for 24 hours and eventually transferred to a nitrogen tank (196°C).

Defreezing the cells

To culture the desired frozen cell line, the cryovial was removed from the nitrogen tank and proceeded with caution and speed of action: 10 ml of culture medium containing 20% bovine serum and 80% DMEM with the antibiotic mixture was added to the flask. Then, in the cryovial was opened to release the nitrogen gas. Then it was placed on bain Marie and it was melted with rotational movements. Then, it was transferred into the flask. In the end, the flask incubated at 37°C containing 0.05% carbon dioxide.

Counting cells

The cell suspension was prepared in a volume of 1 ml and pipetted 20 µl of trypan blue 25% and the same volume of cell suspension was added in a 96-well plate pit. 10 microlitres of the mixture were placed on a Neubauer chamber.

Cell treatment with mitomycin C and deinoxanthin

After counting an MCF7 cell in a 1 ml volume, three columns of 96 well plates were assigned to MCF7 cells with 3,000 cells per well and two wells from the fourth column to the negative control. After 24 hr incubation period, deinoxanthin and mitomycin C solution were added to row 1 and 2 and one row was treated with both materials. A well without treatment was considered as a negative control. Then the color of cells was changed at MTT assay and was analyzed using ELIZA reader.

MTT assay

5 ml of RPMI1640 was added to a powder vial along with 12 ml of MTT solution and it was vortexed and the suspended particles were removed by centrifugation and filtration. Then, the clear solution was equally divided into 5 vials. Each vial is sufficient to be tested on 100 wells. The vial was stable at 4°C for 4 weeks. Other prepared vials for subsequent testing were stored at -20 ° C for 12 months. After preparing the cells, 10 µl of 12 mM MTT solution was to each well and considered a well as a negative control. Then, 10 microliters of the stock solution were added to a non-cell culture medium. The plate was then transferred to a 37-degree incubator for 4 hr. After incubation, the plate was removed from the incubator and 50 µL of isopropanol solution was added to each well and it was placed in an incubator for 10 minutes. Then, the solution in each well was transferred to an ELISA reader for analysis at 570 nm.

Real-time PCR

cDNA molecules were denaturated at 95°C for 4 minutes. then, the temperature was maintained at 94°C for 30 seconds until the denaturation phase is completed, and then for 30 seconds, the temperature is reduced to 57°C until the DNA strands are paired and within 30 seconds Next, the temperature increased to 72 degrees to allow the DNA strand to elongate. The existence of different temperature steps provides for the propagation of 40 cycles.

RESULTS AND DISCUSSION

The distribution of the viability test for 3 groups of deinoxanthin, mitomycin-C and mixed after 24, 48 and 72 hrs can be seen in **Tables 1-4.** As can be seen in these tables, they all have a normal distribution.

Table 1. Normality of viability data of MCF7 cancer cells in exposure to various mitomycin-C doses at 24, 48 and 72 hr after exposure to the drug.

Group	Results	P-value (hr 72)	Results	P-value (48 hr)	Results	P-value (24 hr)
Mitomycin c (µg/cc)	Normal	0/990	Normal	1	Normal	0/998
Mitomycin c (µg/cc 5/0)	Normal	1	Normal	0/999	Normal	0/987
Mitomycin c (µg/cc 1/0)	Normal	1	Normal	1	Normal	1
Mitomycin c (µg/cc 2/0)	Normal	1	Normal	1	Normal	1
Mitomycin c (µg/cc 5/0)	Normal	1	Normal	1	Normal	1
Mitomycin c (µg/cc 10/0)	Normal	1	Normal	1	Normal	0/997
Mitomycin c (µg/cc 20/0)	Normal	0/998	Normal	1	Normal	1

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Table 2. Normality of viability of MCF7 cancer cells in exposure to different doses of deinoxanthin at 24, 48 and 72 hr after exposure to the drug.

Group	Results	P-value) hr 72)	Results	P-value)hr 48)	Results	P-value)hr 24)
Deinoxanthin (µg/cc0)	Normal	0/990	Normal	1	Normal	0/998
Deinoxanthin (µg/cc5/0)	Normal	1	Normal	1	Normal	0/979
Deinoxanthin (µg/cc1)	Normal	1	Normal	1	Normal	0/997
Deinoxanthin (µg/cc5)	Normal	1	Normal	0/995	Normal	1
Deinoxanthin (µg/cc10)	Normal	0/999	Normal	1	Normal	0/979
Deinoxanthin (µg/cc20)	Normal	1	Normal	1	Normal	1
Deinoxanthin) µg/cc	Normal	1	Normal	1	Normal	1
Deinoxanthin (µg/cc40)	Normal	0/982	Normal	1	Normal	1
Deinoxanthin (µg/cc50)	Normal	0/998	Normal	0/973	Normal	0/979

Table 3. Normality of the viability of MCF7 cancer cells in exposure to different doses of mitomycin-C and deinoxanthin at 24, 48 and 72 hr after exposure to drugs.

Group	Results	P-value (72) (hr)	Results	P-value 48 (hr)	Results	P-value 24))hr
Mitomycin-C (µg/cc0) + deinoxanthin (µg/cc0)	Normal	0/990	Normal	1	Normal	0/998
Mitomycin-C (µg/cc 5/0) + deinoxanthin (µg/cc 5/0)	Normal	1	Normal	0/999	Normal	1
Mitomycin-C (µg/cc 1) + deinoxanthin (µg/cc 1)	Normal	1	Normal	0/989	Normal	0/999
Mitomycin-C (µg/cc 2) + deinoxanthin (µg/cc 5)	Normal	0/999	Normal	1	Normal	1
Mitomycin C (µg/cc 5) + deinoxanthin (µg/cc 10)	Normal	1	Normal	1	Normal	1
Mitomycin C (µg/cc 10) + deinoxanthin (µg/cc 20)	Normal	0/999	Normal	0/999	Normal	1
Mitomycin C (µg/cc 20) + deinoxanthin(µg/cc 30)	Normal	1	Normal	1	Normal	1

Table 4. Normality of the data on the change in the expression of *bax* and 2bcl genes in exposure to Mitomycin-C-, deinoxanthin and combined with 24 hr after exposure to drugs

Group	Results	P-value (BCL2)	Results	P-value (Bax)
Mitomycin c (µg/cc1)	Normal	0.998	Normal	1
Deinoxanthin (µg/cc20)	Normal	1	Normal	0/967
Mitomycin (µg/cc 1 + Delnoxanthin (µg/cc 20)	Normal	0/907	Normal	1

Viability of cells post-exposure to mitomycin-C

Comparison of the effects of different concentrations of mitomycin-C at 24, 48, and 72 hrs after exposure, MCF7 cancer cells in comparison with the control group showed that the cells were exposed at the highest concentration at all three times. The highest viability rate was observed in 20 µg/cc and at the highest concentration of 0.5 µg/cc. The calculated IC50 for 24, 48 and 72 hr is 2.6, 0.96 and 1.96 µg /ml, respectively. The increased concentration of mitomycin-c has led to a decrease in the viability of MCF7 cell cancer cells. From the concentration of 0.5 µg/cc, the viability of the cells has a significant difference with the control group. On the other hand, by increasing the time of treatment of cancer cells with mitomycin-C, the viability of the cells is reduced. As the control-negative cells grow more after 72 hours, the viability of the cell decreases significantly compared to the other two groups in less than 72 hours

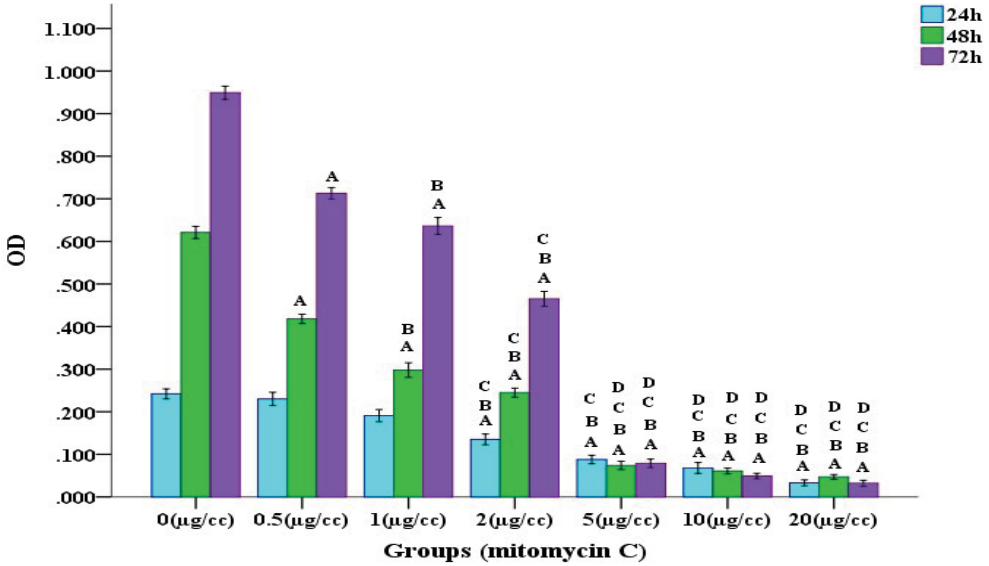

Figure 1. Comparison of MCF7 Cancer Cells with Different Doses of Mitomycin-C at 24, 48 and 72 hr after exposure to the drug. A indicates a significant difference between the mitomycin-C (B,) 0 µg/cc and the significant difference between the mitomycin-C (C/5 µg/cc) and the mitomycin-c (1 µg/cc)and D indicates a significant difference between the mitomycin-c group (2 µg/cc).

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Comparison of the effects of different concentrations of deinoxanthin at 24, 48 and 72 hrs after exposure ofMCF7 cancer cells to deinoxanthin compared to the control group showed that cells at the highest concentration at all three times (50 µg/cc) had the lowest viability percentage and the calculated IC50 for 24, 48 and 72 hr was 18.18, 22.18 and 21.18 µg/cc, respectively. Increasing the concentration of deinoxanthin up to concentrations of 25 µg/cc in the 24-hour period led to an increase in the viability of MCF7 cells, but due to an increase in deinoxanthin dosage and the time when cells were exposed to cytotoxic agents, cell death rates increased. According to the graph of concentration of 10 µg/cc, the viability of the cells in all three times was significantly reduced compared to the control group. On the other hand, with the increase in the time of treatment of cancer cells with deinoxanthin, the viability of the cells decreased so that the cells of the negative control group grew more after 72 hours **Figures 1 and 2.**

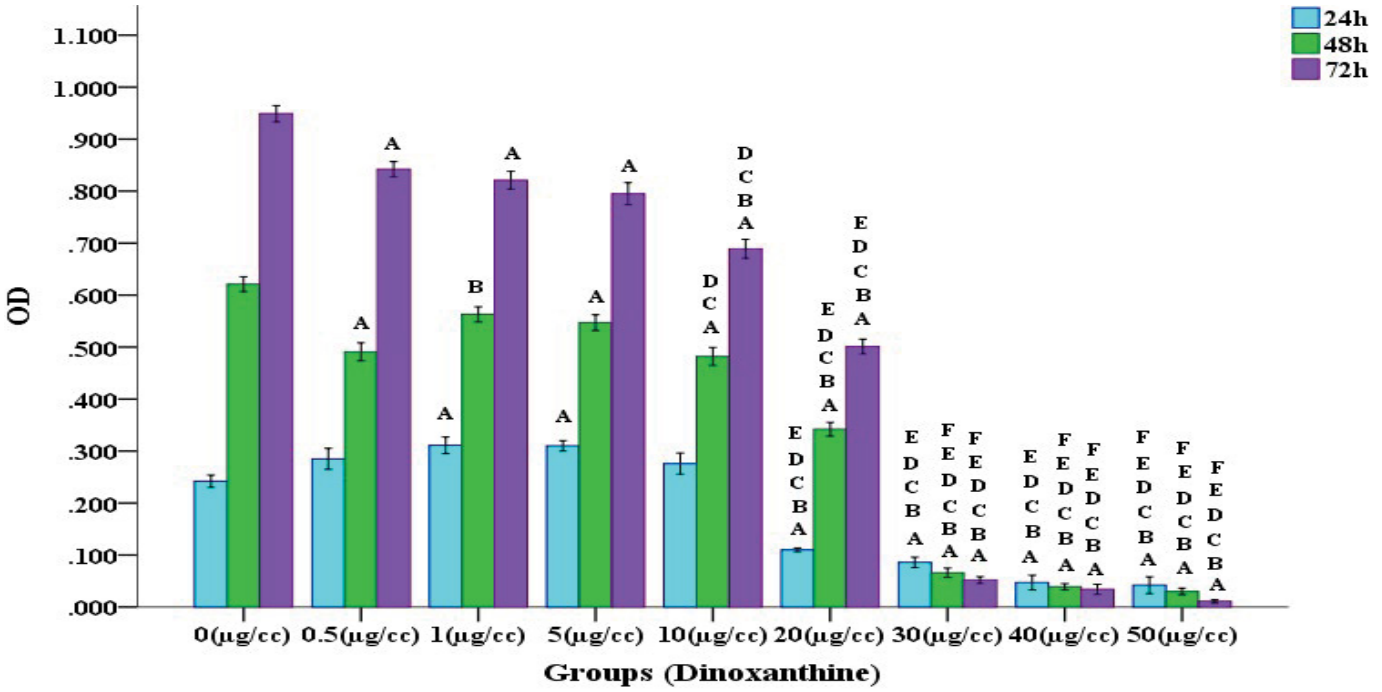

Figure 2. Comparison of the viability of MCF7 cancer cells after exposure to different deinoxanthin doses at 24, 48 and 72 hr after exposure to the drug. A indicates a significant difference compared to the deinoxanthin group (0 µg/cc), B indicating a significant difference compared to the deinoxanthin group (5 µg/cc), C indicating a significant difference compared to the deinoxanthin group (1 µg/cc), D showed a significant difference compared to the deinoxanthin group (5 µg/cc),E indicating a significant difference between the deinoxanthin group (10 µg/cc) and F showed a significant difference compared to the deinoxanthin group (20 µg/cc)

Effect of treatment with both mitomycin-C and deinoxanthin on the viability of MCF7

The cells were exposed to the highest concentration of both cytotoxic agents (20 µg/cc mitomycin-c, and 30 µg/cc deinoxanthin) at the highest concentration at all three times and the calculated IC50 for 24 hours after exposure was 10.9 µg/ml of the bacterial extract and 5.45 µg/ml of the drug. For 48 hours after exposure IC_{50} was 9.4 µg/ml of the bacterial extract and 4.28 µg/ml of the drug. At the time of 72 hours after exposure IC50 was 10.88 µg/ml of the bacterial extract and 5.44 µg/ml of the drug. Increasing the concentration of deinoxanthin to concentrations of 25 µg/cc in the 24-hour period led to an increase in the viability of MCF7 cells and reduced the effects of mitomycin-C. However, due to an increase in the dose of bacterial extract and drug and exposure time, cytotoxicity increased the mortality rate of the cells. According to the graph, from the concentration of 10 µg/cc of deinoxanthin and 5 µg/cc of mitomycin-c, the viability of the cells in all three times reduced significantly compared to the control group.

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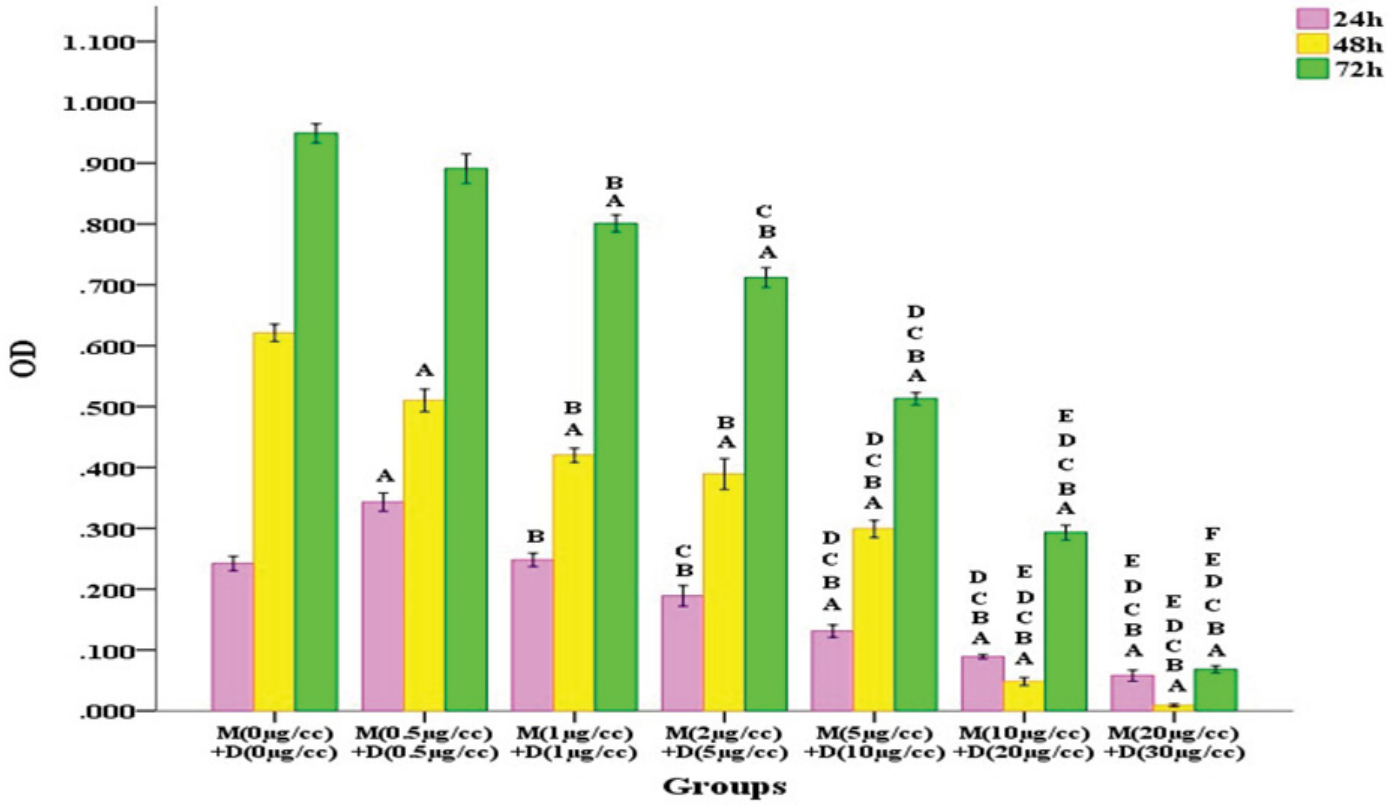

Figure 3. Comparison of the viability of MCF7 cancer cells in the presence of mitomycin-C and deinoxanthin at three periods of 24, 48 and 72 hours after exposure to drugs.

The *bax* and *BCL2* gene expression can be seen in **Figures 3- 5**.

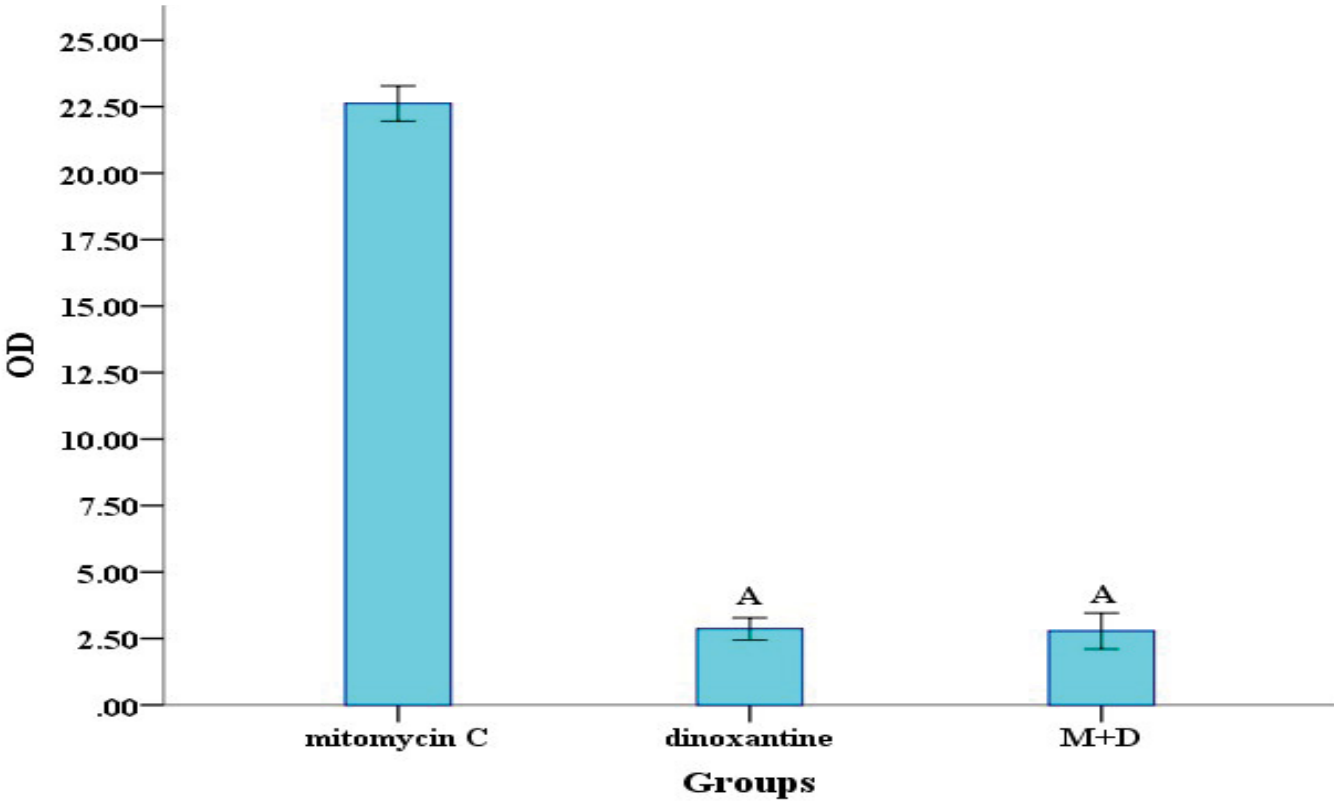

Figure 4. Comparison of expression of *bax* gene in mitomycin-C (2 µg/cc), deinoxanthin (18 µg/cc) and combined, 24 hours after exposure to drugs (A) showed a significant difference between the mitomycin-c group (2 µg/cc).

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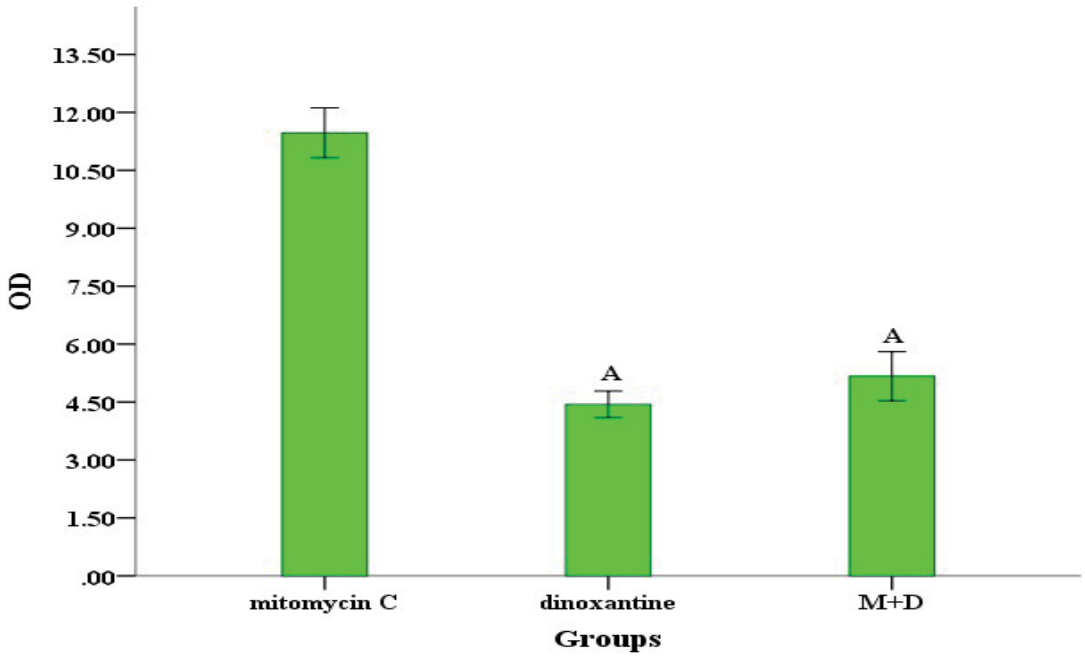

Figure 5. Comparison of changes in the expression of *BCL2* gene expression in exposure to different doses of mitomycin-c (2 µg/cc), deinoxanthin (18 µg/cc) and combined with 24 hrs after exposure to drugs (A) showed a significant difference between the mitomycin-c group (2 µg/cc).

Evaluation of morphological changes of cells

In order to investigate the apoptotic properties of MCF7 cells 24 hours after exposure to cytotoxic materials, microscopic changes in the cell were evident from cellular morphology of the cell from the spindle to the round shape and the presence of vesicles containing small cells. Also, unlike the normal state of the cells that grow like epithelial tissue in a regular manner, after apoptosis, the cells had formed an irregular dispersion in a compact and clinging manner. Also, changes such as cell shrinkage, buckling, and cytoplasmic loss, membrane contamination, and pigmentation occur at IC_{50} concentrations **(Figure 6).**

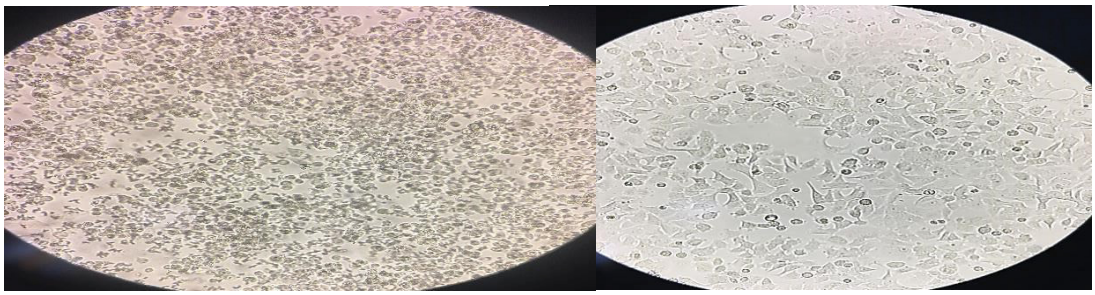

Figure 6. Changes in the morphology of MCF7 cells 24 hours after exposure to deinoxanthin

Quantitative RNA assay

This part is done with the help of Nanodrop machine. The 230 nm wavelength is related to phenol, ethanol and isopropanol contamination, and the wavelength of 260 nm is related to nucleic acid contamination and 280 nm wavelengths is associated with protein contamination, which means that the molecules in the corresponding wavelengths have the most optical absorption. In ideal conditions, the amount of contamination with protein, phenol, and ethanol should be very low. The concentration of 800 ng/ µl of extracted RNA at 260 nm wavelength resulted in **Table 5.**

Table 5. Quantitative RNA assay for both deinoxanthin and mitomycin-C

Associated RNA	ratio 280/260
Deinoxanthin	1.6
Mitomycin-C	2.1
Both	2.2
Control	1.9

Melting curve

The melting curve begins with a gradual increase in temperature from a lower temperature than the melting point of products and continues to a temperature above its melting point. The range of temperature variations in the melting curve is adjustable and the products multiply at different temperatures based on the length of the multiplicative part and the amount of guanine-cytosine

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they will melt (denatured). When the melting products are denatured, the fluorescence decreases. Then, It's measured by the device. The melting curve data will qualitatively result in the display of products at the end of the cyberrine test. The temperature separation analysis (melting curve) at the end of the PCR determines whether only a single product is multiplied by plotting a negative first derivative curve (using a curve of fluorescence intensity against the temperature; that is plotted by the software of the machine). The precise Tm product of PCR is determined. This value should be close to the expected Tm for the product PCR. Below the results of the melting curves for the gene derived from the cell line compared to this study,The existence of several melting curves with only a single courier indicates a specificity of the PCR product. It should be noted that the melting point measured for the *BAX* gene is 14/78 and the melting point for the *BCL2* gene is equivalent to 22.27 **(Figures 7-9).**

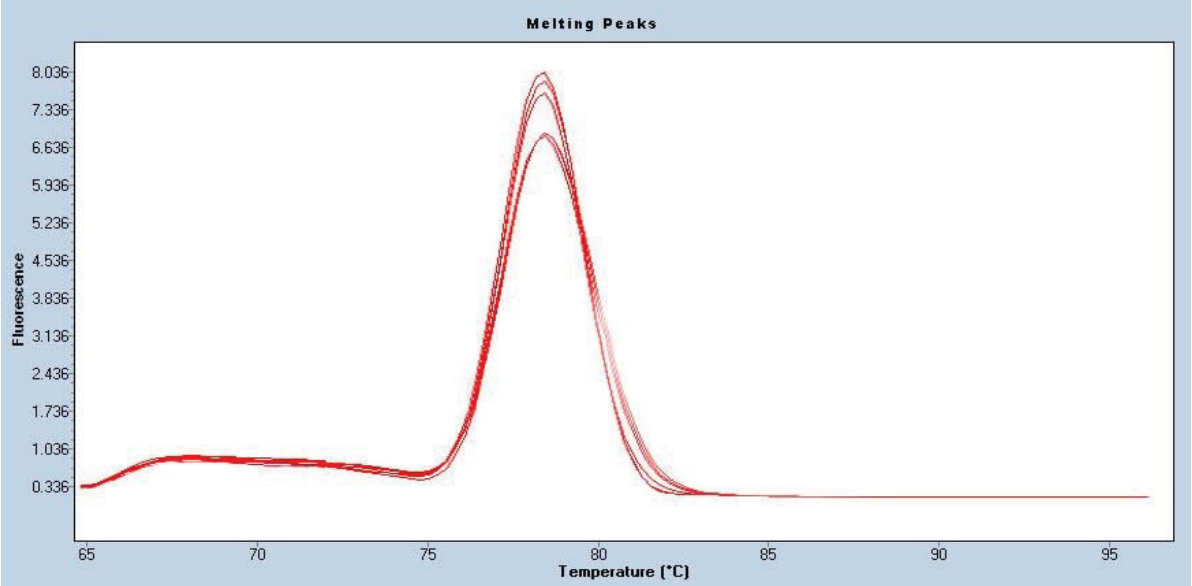

Figure 7. The melting curve of the standard gene considered (GAPDH) in the MCF7 cell line

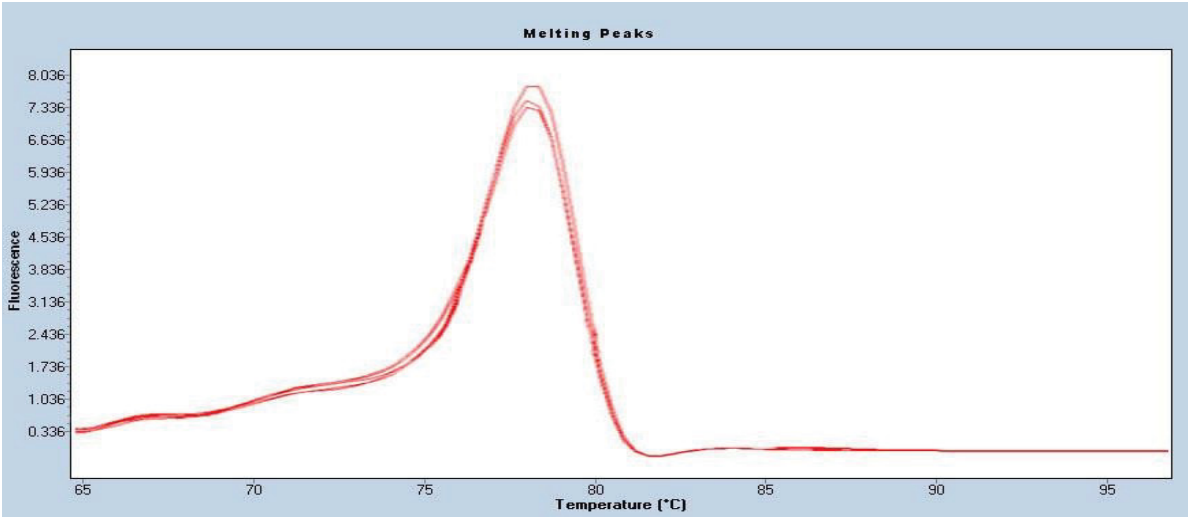

Figure 8: *BAX* gene melting curve in MCF7 cell line

Figure 9: *BCL2* gene melting curve in MCF7 cell line.

CONCLUSION

Depending on the results of the study, it was found that deinoxanthin has a cytotoxic nature that significantly decreases the viability of MCF7 cells, which reduces the viability rate completely dependent on the dose and duration of exposure so that In doses higher than 30 µg/ml, viability was reduced to less than 20% during exposure at different times. Mitomycin-C at all concentrations has led to a decrease in cell viability and in doses of 10 and 20 µg/ml, the observed effect of using both substances together and mitomycin alone is similar. Thus, it can be seen that the cytotoxicity of both substances depends on the dose and duration of exposure. In examining the expressed genes, the *BAX* gene was induced by both materials but, contrary to the expectation, *BCL2* has not decreased. The results of the expression of *BCL2* and *BAX* in mitomycin indicate that the induction of apoptosis of mitotic-c is more effective than deinoxanthin and the use of Combination of it with deinoxanthin reduces the severity of induction

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of apoptosis by this gene, and this is important because it can create a new way to treat malignancy and reduce the incidence of complications due to the high and selective cytotoxicity of chemotherapy drugs.

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