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Research Article

Chemosensitizing Effects of Marine Astaxanthin on the Anti-cancer Activity of Doxorubicin in Tumor Bearing Mice

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Abstract

Background and Objectives: Doxorubicin is an efficient anti-neoplastic drug but its clinical usefulness is limited by its serious adverse effects such as cardiotoxicity. The main objective of this research was to investigate whether natural marine astaxanthin could chemosensitize the Ehrlich ascites carcinoma cells to the anti-tumor effect of doxorubicin. **Materials and Methods:** Survival of Ehrlich ascites carcinoma bearing mice was used as model to evaluate the chemosensitizing effect of astaxanthin to doxorubicin cytotoxicity. After treatment with doxorubicin and/or astaxanthin, doxorubicin uptake was measured by spectrofluorometric assay, apoptosis and the cell cycle phase distribution were determined by flow cytometry analysis and P53 gene expression was also studied in the EAC cells. **Results:** Treatment of tumor bearing mice with doxorubicin showed a significant increase in the mean survival time to 32.5 days with 30% long-term survivor. Treatment of the tumor-bearing animals with astaxanthin and doxorubicin showed a significant increase in the mean survival time to 41.1 days with 80% long-term survivor. Doxorubicin cellular uptake was increased significantly after astaxanthin treatment. Moreover, astaxanthin treatment dramatically increased the early apoptosis and G₂/M phase accumulation after addition to doxorubicin. P53 gene expression was up regulated after doxorubicin treatment, moreover addition of astaxanthin synergized the action of doxorubicin where p53 gene expression was highly upregulated. **Conclusion:** The present study concluded that the marine astaxanthin potentiates the cytotoxic activity of doxorubicin against the growth of mammary tumor cells *in vivo* through accumulation of the tumor cells in G₂/M phase, induction of apoptosis and up regulation of the tumor suppressor p53 gene expression.

Key words: Chemosensitizing, astaxanthin, doxorubicin, Ehrlich ascites cells

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Doxorubicin (DOX) is an anthracycline antibiotic used as a chemotherapeutic drug to treat many types of cancers. Unfortunately, DOX clinical usefulness is limited by its serious cardiotoxicity which may lead to congestive heart failure¹. Chemosensitization of the tumor cells to the cytotoxic effect of anti-tumor drugs is one strategy to potentiate the cytotoxic effect of anti-tumor drugs. By modulation of the anti-cancer molecular targets of some anti-neoplastic drugs, some natural or synthetic agents have been used to sensitize the tumor cells to the anti-proliferative activity of certain anti-neoplastic drugs. These chemosensitizers may also provide a chemoprotective effect against many toxic manifestations induced by the anti-neoplastic drugs²⁻⁴. Astaxanthin is an important marine compound which is a reddish carotenoid dye belongs to the xanthophylls family. It is the compound responsible for red color in algae. It has a potent anti-oxidant, anti-tumor, anti-inflammatory, anti-lipid peroxidation and cardioprotective effects^{5,6}. Astaxanthin has shown protective effect of cells from damages through modification of pathogenesis of some diseases and toxicity induced by oxidative stress. In mammals, researchers have evaluated the effects of ATX on many kinds of cancers. Astaxanthin showed a significant decrease in the incidence of oral and colon cancer in rats^{7,8}, suppression of breast cancer⁹, inhibition of the cellular growth of prostatic carcinoma by suppression of 5- α -reductase and reduction of the liver metastasis caused by stress in mice through minimizing lipid peroxidation and scavenging the reactive oxygen species (ROS)¹⁰. From the above, ATX was nominated to be a promising chemosensitizer, the goal of this research was directed to investigate whether marine ATX can sensitize Ehrlich ascites carcinoma (EAC) cells to the anti-neoplastic effect of DOX and consequently may minimize its dose and thereby its dose dependent cardiotoxicity. This chemosensitization was studied through evaluation of the potential molecular mechanisms of ATX and DOX synergistic interaction through the evaluation of various parameters, including cytotoxicity, induction of apoptosis, DOX cellular uptake, disturbance of cell cycle phases and the expression of the tumor suppressor gene p53.

MATERIALS AND METHODS

Materials: Doxorubicin was provided from King Abdulaziz University Hospital. Astaxanthin was purchased from Haihang

Industry Co., Ltd. Apoptosis Assay kits were purchased from Aviscera Bioscience, Inc. USA. Cell cycle kit was obtained from Cayman chemical, USA.

Animal and cells: In this study female Swiss albino mice (10 weeks old and weight from 25-30 g) were obtained from College of Pharmacy, King Abdul-Aziz University, Jeddah, Saudi Arabia. The mice were adapted for a week and had a free access to commercial balanced diet and water *ad libitum* throughout the experiments. The animal studies were approved by the ethical research committee unit at the College of Medicine, KAU.

The Ehrlich ascites carcinoma (EAC) cells were provided as a gift from National Cancer Institute (Cairo, Egypt) and sustained in the laboratory by continuous intra peritoneal transplantation of 2.5×10^6 cells/mouse weekly.

One hundred and eighty female Swiss albino mice were intraperitoneally injected with 2.5×10^6 cells/mouse of EAC cells, 24 h. Later, animals were randomly divided to conduct 5 experiments.

In the evaluation of anti-tumor activity, assay of apoptosis, cell cycle analysis and evaluation of p53 gene expression 40 EAC cells bearing mice were used in each experiment separately.

The 40 mice were then divided into four groups containing 10 mice each. Group I, mice were kept as a control and treated with normal saline (0.2 mL/20 g, x7, i.p.). Group II, mice were treated with ATX (40 mg kg⁻¹, x7, i.p.). Group III, mice were treated with DOX (2 mg kg⁻¹, x3 i.p.) every other day. Group IV, animals were treated with ATX (40 mg kg⁻¹, x7, i.p.) and DOX (2 mg kg⁻¹, x3 i.p.) every other day.

In the other experiment, 20 EAC cells bearing mice were used in assessment of DOX cellular uptake experiment. The 20 mice were divided equally into two groups each group contains 10 mice. Group I mice were kept as control and treated with normal saline (0.2 mL/20 g, x6, i.p.) and at day 7 a single dose of DOX (15 mg kg⁻¹, i.p.) was injected. Group II mice were treated with ATX (40 mg kg⁻¹, x6, i.p.) and at day 7 a single dose of DOX (15 mg kg⁻¹, i.p.) was injected:

- **Evaluation of anti-tumor activity:** After treatment, the survival time and anti-tumor activity were evaluated according to the method of Osman *et al.*¹¹ in which the average survival time for mice and long-term survivors were defined as the duration in which mice survived to the end of experiment (45 days) without any evidence of the tumor growth

- **Assessment of DOX cellular uptake:** Mice were sacrificed by cervical dislocation 6, 24 and 48 h after treatment. The EAC cells were collected from peritoneal cavity, washed twice with PBS, counted and 1×10^6 cells were digested by suspending the cells in 1 mL of 20% DMSO followed by centrifuge at 14000 rpm for 10 min. DOX concentration in the supernatant was determined spectrofluorometrically based on the unique intense fluorescence characteristic of DOX at wavelengths of $\lambda_{\text{ex}} = 496$ nm and $\lambda_{\text{em}} = 592$ nm, respectively (Synergy HT, Biotech, USA) according to the method of Kitagawa *et al.*¹²
- **Assay of apoptosis:** After treatment, animals were sacrificed by cervical dislocation at 6, 24 and 48 h from the end of treatment. The EAC cells were collected from the ascites fluid of each mouse, washed twice with PBS, counted and re-suspended in 100 μL annexin V incubation reagent prepared by mixing (binding buffer 10x, PI, annexin V-FITC and deionized water) for each sample. The solution was incubated in the dark for 15 min at room temperature. Then 400 μL 1x of binding buffer were added to each sample and process by flow cytometry according to the method of Van Engeland *et al.*¹³ using (Becton DICN SON (BD) FACS Calibur, USA) using annexin V-FITC-propidium iodide double staining
- **Cell cycle analysis:** After 6, 24 and 48 h of DOX treatment, mice were sacrificed by cervical dislocation and the EAC cells were collected from the ascites fluid, washed twice with PBS, counted and resuspended to a density of 10^6 cells mL^{-1} in assay buffer. After that 1 mL of fixative agent was added to each sample to fix and permeabilize the cells for at least 2 h prior to PI staining. Fixed cells were centrifuged at 500 rpm for 5 min, then the fixative decanted thoroughly. The cells pellet was suspended in staining solution which prepared by mixing 10 mL assay buffer with 200 μL RNase A solution and 200 μL PI Solution) for every 20 samples to be stained, the cells were incubated for 30 min at RT in the dark then the cell cycle phase distributions were determined according to Sulic *et al.*¹⁴ method using the flow cytometry (Becton DICN SON (BD) FACS Calibur, USA)
- **Detection of p53, mRNA expression in EAC cells with quantitative reverse-transcriptase polymerase chain reaction (RT-PCR):** The Ehrlich ascites carcinoma cells were withdrawn from the ascites fluid of mice 48 h after treatment, washed twice with PBS and counted for RT-PCR analysis. The total RNA of EAC cells were extracted

by RNA extraction kit according to the manufacturer protocol and subjected to the real time PCR reaction to evaluate the expression p53 genes. Glyceraldehyde-3-phosphatedehydrogenase (GPDH) was employed as a housekeeping gene. The real time reaction consists of 2 μL of the extracted RNA (100 ng/2 μL), 12.5 μL of 2X Quanti Fast SYBR Green PCR Kit (Qiagen, CA, USA), 0.5 μL of 10 pmol μL^{-1} forward (F) primer, 5'-CCAAGTCTGTTATGTGCACG-3', 0.5 μL of 10 pmol μL^{-1} reverse (R) primer, 5'-TGACCCACAACCTGCACAG-3', 9.5 μL of RNAase free water for a total of 25 μL . Before loading in the rotor's, samples were spun wells. The RT-PCR steps were conducted as following: Initial denaturation for 10 min at 95°C., 40 cycles of 64°C for 15 sec. Data acquisition performed through the extension step. This reaction was conducted with One-Step RT-PCR System (QIAGEN, USA)

Statistical analysis: In this research SPSS as statistical analysis program (version 21) was used. The comparison of the statistical significance was acceptable to level of $p < 0.05$. One way analysis of variance (ANOVA) followed by least significant difference (LSD) for *post hoc* analysis.

RESULTS

Survival of tumor bearing mice: The effect of intraperitoneal administration of DOX (2 mg kg^{-1}) for a total of 3 doses and/or ATX (40 mg kg^{-1} x7, i.p.) on the survival of female Swiss albino mice bearing EAC cells was represented in Table 1.

Control group showed a mean survival time of 16.9 days, whereas, treatment with DOX showed a significant increase in the mean survival time to 32.5 days with 30% long-term survivor. Treatment of the tumor-bearing animals with ATX (40 mg kg^{-1} , x7 i.p.) and DOX (2 mg kg^{-1} , i.p.) every other days for a total of three doses showed a significant increase in the mean survival time to 41.1 days with 80% long-term survivor. Although, the mean survival time of ATX treated mice showed a significant increase to 20 days, no significant difference in long term survivor between control and ATX treated groups.

DOX level in tumor cells: The represented results in Fig. 1 showed the cellular level of DOX in EAC cells after pretreatment with normal saline (0.2 mL/20 g, x6, i.p.) or (ATX 40 mg kg^{-1} , x6, i.p.) followed by a single dose of DOX (15 mg kg^{-1} , i.p.) at day 7. DOX treatment showed mean DOX cellular level of 104.64, 70.96 and 44.03 ng/10⁶ cells after 6, 24 and 48 h of treatment, respectively. Astaxanthin

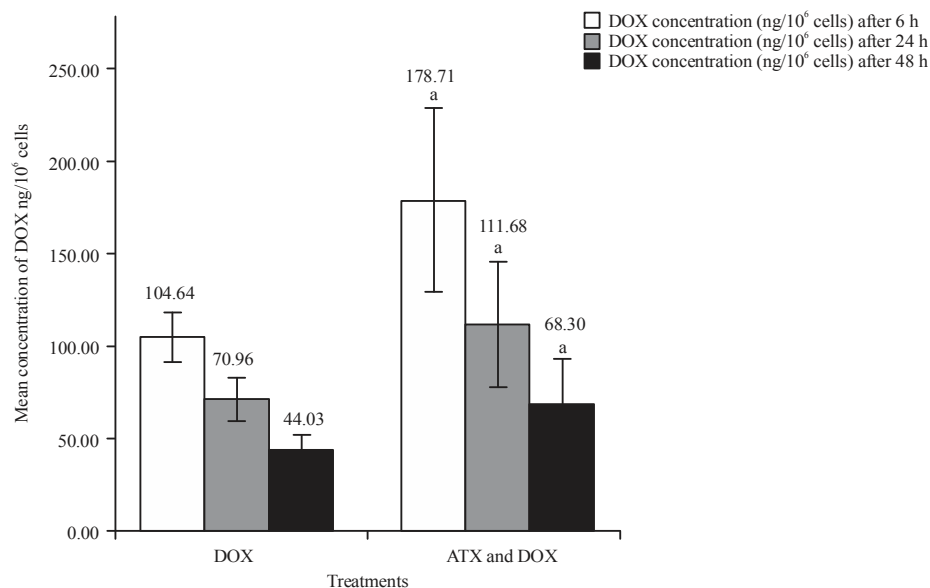


Fig. 1: Effect of ATX pretreatment on the DOX cellular uptake in to EAC cells

DOX was injected (15 mg kg⁻¹ i.p.) in tumor-bearing mice pretreated for 6 days with ATX (40 mg kg⁻¹, i.p.) or normal saline. After 6, 24 and 48 h of treatment, cells were withdrawn, washed once with PBS, counted and digested by suspending 1x10⁶ cells in 1mL of 20% DMSO followed by centrifuge at 14000 rpm for 10 min. The supernatant absorbance was measured using spectrofluorometer. Data were expressed as mean ± SEM (n = 10). ^aSignificantly different from corresponding DOX at p<0.05, one sample t-test

Table 1: Effects of doxorubicin and/or ATX on the survival time of mice bearing EAC cells

Groups	Mean survival time (days)	Long term survivor (%)
Normal saline (0.2 mL/20 g, x7, i.p.)	16.90 ± 1.85	0
ATX (40 mg kg ⁻¹ , x7, i.p.)	20.00 ± 3.53 ^a	0
DOX (2 mg kg ⁻¹ , x3 e.o.d. i.p.)	32.50 ± 11.15 ^a	30
ATX (40 mg kg ⁻¹ , x7, i.p.) and DOX (2 mg kg ⁻¹ , x3 e.o.d. i.p.)	41.10 ± 8.22 ^{ab}	80

Data were expressed as mean ± SD of 10 mice. ^a Significantly different from control at p<0.05. ^bSignificantly different from DOX at p<0.05, one way ANOVA with LSD post test

Table 2: Effect of DOX and /or ATX on apoptosis Induction in EAC Cells

Treatments	Percentage of early apoptosis after treatment		
	6 h	24 h	48 h
Normal saline	0.66 ± 0.17	0.63 ± 0.17	1.10 ± 0.38
ATX	21.66 ± 0.48 ^a	26.03 ± 2.09 ^a	24.40 ± 1.10 ^a
DOX	35.60 ± 3.13 ^a	37.90 ± 1.99 ^a	31.50 ± 3.6 ^a
DOX and ATX	51.70 ± 1.30 ^{ab}	51.00 ± 0.88 ^{ab}	54.46 ± 2.17 ^{ab}

Each data were expressed as the mean ± SEM of two experiments each one in duplicate. ^aSignificantly different from normal saline treated group at p<0.05, ^bSignificantly different from DOX treated group at p<0.05

pre-treatment significantly increased the mean DOX cellular level to 178.71, 111.68 and 68.30 ng/10⁶ cells at the same time points tested compared to DOX alone.

Apoptotic effect of DOX and/or ATX: Apoptosis of EAC cells was quantified using flow cytometry by annexin V and PI double staining. The mean percentage of early apoptotic cells (Annexin V-positive cells) were significantly increased after administration of DOX (2 mg kg⁻¹, x3, i.p.) every other day to 35.60, 37.90 and 31.50% compare to the control cells after 6,

24 and 48 h of treatment, respectively (Table 2). On the other hand, pre-treatment with ATX (40 mg kg⁻¹, x7, i.p.) and DOX (2 mg kg⁻¹, x3, e.o.d., i.p.) significantly increase in the percentage of the early apoptosis in the EAC cells to 51.7, 51 and 54.46% compared with DOX treatment alone at the same time points tested (Table 2).

Effect of ATX and DOX treatment on the cell cycle progression of Ehrlich ascites carcinoma cells: The percentage of distribution of Apop, G1/G0, S and G₂/M phases

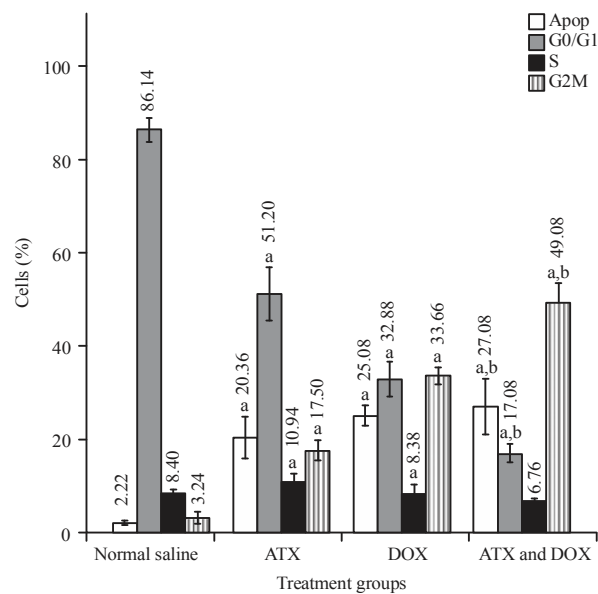


Fig. 2: Effect of DOX and /or ATX treatment on cell cycle phase distribution of EAC cells, after 6 h of treatment

EAC cells were withdrawn from peritoneal cavity of mice, washed with PBS followed by staining with PI for cell cycle analysis. Each data are expressed as mean \pm SEM of two experiments each one in duplicate. ^aSignificantly different from control in corresponding phase ($p < 0.05$), ^bSignificantly different from the DOX in corresponding phase at $p < 0.05$

Table 3: Effect of DOX and/or ATX on p53 gene expression in EAC cells quantitated by RT-qPCR after 48 h of treatment, $Rq = 2^{-\Delta\Delta Ct}$

Treatments	Relative quantification (Rq) of p53 gene
ATX	1.42 \pm 0.13
DOX	1.58 \pm 0.22
ATX+DOX	2.96 \pm 0.27 ^a

Real-time PCR relative quantification (Rq) of the EAC cells p53 gene levels in EAC cells withdrawn from DOX and/or ATX treated animals after 48 h of treatment. Each data represent the mean \pm SEM of two experiments each one in triplicate. ^aSignificantly different from DOX treated group at $p < 0.05$. Rq value above 1 indicates up regulation and below indicates down regulation where control was normalized to 1. (The $\Delta\Delta Ct$ method)

in EAC cells after treatment with DOX and/or ATX at three different time points (6, 24 and 48 h) was evaluated. DOX treatment (2 mg kg⁻¹, i.p.) every other day for 6 days accumulated the cells in G₂/M phase by 34, 33 and 29% after 6, 24 and 48 h of the treatment, respectively. Pre-treatment with ATX (40 mg kg⁻¹, x7, i.p.) and DOX (2 mg kg⁻¹, i.p.) every other day for 6 days significantly increase the mean percentage cells accumulation in G₂/M phase by 49, 52 and 53% after 6, 24 and 48 h of treatment, respectively with maximum accumulation percentage after 48 h of treatment (Fig. 2, 3 and 4). In addition, ATX treatment alone showed an increase in the G₂/M accumulation at all the time points tested.

Effect of DOX and/or ATX on p53 gene expression in EAC cells: Treatment of EAC cells bearing mice with DOX (2mg kg⁻¹, x6, i.p.) every other day for 6 days up regulated the p53 gene expression in the tumor cells 48 h after treatment

(relative quantification (Rq) of p53 gene was 1.58). Pre-treatment with ATX (40 mg kg⁻¹, x7, i.p.) and DOX (2 mg kg⁻¹, x3, e.o.d., i.p.) showed a significant increase in p53 expression (Rq of p53 gene was 2.96) compare to DOX treated cells (Table 3).

DISCUSSION

Chemotherapy considers the main frequently used regimens in the majority of cancer therapy. An important anti-neoplastic drugs indicated in treatment of many types of cancer is DOX. It is either used alone or in combination with other type of anti-neoplastic drugs. Unfortunately, its clinical usefulness is limited by serious adverse effects especially cardiotoxicity which is the most harmful one and may lead to congestive heart failure¹⁵. Chemosensitization is one strategy to potentiate the cytotoxic effect of anti-tumor drugs by using some natural or synthetic agents which have the ability to potentiate the anti-cancer activity of some anti-neoplastic drugs and as a sequence decrease its dose and thereby minimized its side effects. Astaxanthin is an important marine compound which belongs to the xanthophylls family. One of natural sources of marine ATX is the green microalgae haematococcus pluvialis. It has a potent anti-oxidant, anti-tumor, anti-inflammatory, anti-lipid peroxidation and cardioprotective effects^{16,5,6}. Therefore, in this study the modulatory effects of marine ATX on the cytotoxic effect of

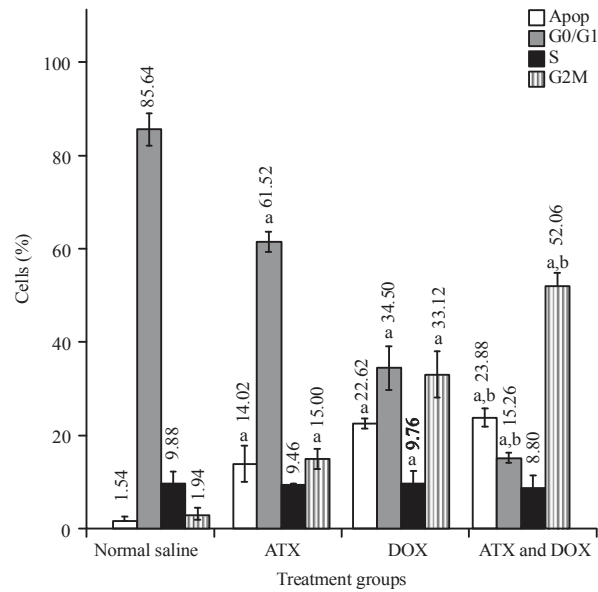


Fig. 3: Effect of DOX and/or ATX treatment on cell cycle phase distribution of EAC cells, After 24 h of treatment

EAC cells were withdrawn from peritoneal cavity of mice, washed with PBS followed by staining with PI for cell cycle analysis. Each data are expressed as mean \pm SEM of two experiments each one in duplicate. ^aSignificantly different from control in corresponding phase ($p < 0.05$), ^bSignificantly different from the DOX in corresponding phase at $p < 0.05$

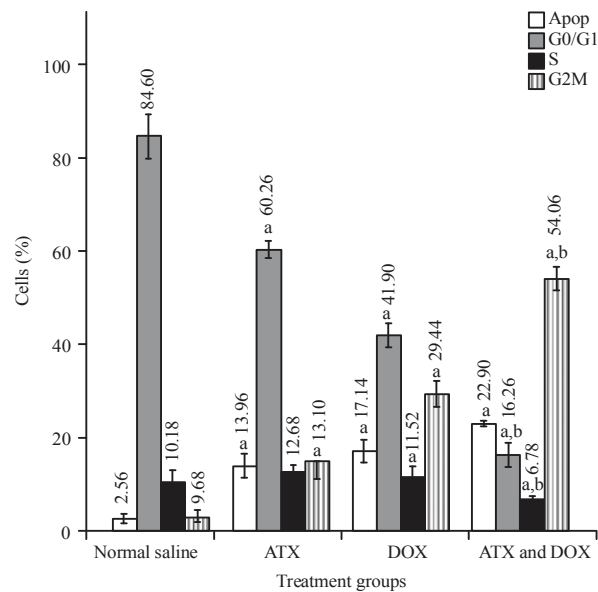


Fig. 4: Effect of DOX and /or ATX treatment on cell cycle phase distribution of EAC cells. After 48 h of treatment

EAC cells were withdrawn from peritoneal cavity of mice, washed with PBS followed by staining with PI for cell cycle analysis. Each data are expressed as mean \pm SEM of two experiments each one in duplicate. ^aSignificantly different from control in corresponding phase ($p < 0.05$), ^bSignificantly different from the DOX in corresponding phase at $p < 0.05$

DOX against the growth of EAC cells in mice were studied. ATX increase the cytotoxic effect of DOX which lead to suppression of the growth of EAC cells. Treatment of tumor-bearing mice with ATX+DOX showed 80% long term survivor compare to 30% in DOX treated mice alone (Table 1). This increase in MST indicate a synergistic interaction between ATX and DOX, which has been confirmed by observing an increase the level

of DOX in tumor cells after addition of ATX in treatment protocol (Fig. 1). This increase in the DOX cellular uptake inside Ehrlich ascites carcinoma cells after addition of ATX may be clarified based on inhibition of P-glycoprotein pump that play an essential function in the absorption, distribution and elimination of DOX and thus regulate its efficacy and toxicity^{17,4}. It is known that ATX up regulate connexin-43 gene

expression which is down regulated in cancer cells¹⁸. Moreover, Zhang and Wang¹⁹ reported that ATX up regulate p53 gene expression and induce apoptosis through p53 regulated modulators on lung adenocarcinoma cell lines and human lung fibroblast. This is consistent with the results of this work where more expression of p53 gene after addition of ATX to DOX protocol had been observed (Table 3). Moreover, synchronization of the tumor cells in G₂/M phase by ATX had been observed (Fig. 2,3 and 4) which is a good phase for the action of DOX^{20,21} due to maximum expression of DOX target enzyme TOPO II in G₂/M²¹. DOX induces its anti-neoplastic effect through inhibition of DNA replication and inhibition of topoisomerases II (TOPO II) enzyme which leads to induction of apoptosis^{22,23}. This has been observed in the result of this study through increase in early apoptosis in the tumor cells after DOX treatment at all the time point tested. Addition of ATX potentiated the action of DOX in induction of apoptosis (Table 2).

CONCLUSION

This study concluded that marine ATX treatment efficiently enhances the cytotoxic activity of DOX leading to suppression of the growth of mammary tumor *in vivo* through accumulation of the tumor cells in G₂/M, induction of apoptosis and upregulation of the p53 gene expression. Therefore, addition of ATX to DOX is promising cancer treatment and could be of a potential practical value in cancer therapy and open the field for preclinical study to evaluate the protective effect of marine ATX against the DOX severe side effects which will lead to protect the patient and reduce the chemotherapy cost and complications of DOX chemotherapy.

SIGNIFICANCE STATEMENT

This research investigates the roles of marine astaxanthin in the potentiation of doxorubicin cytotoxic effect *in vivo*. It introduces a key for the researchers to study other molecular targets of the marine astaxanthin which may be involved in the cytotoxic potentiation of the chemotherapeutic drugs. The findings of the research work encourage the utilization marine astaxanthin in many aspects of different cancer researches which may provide a promising progression in the cancer prevention and therapy.

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Only the authors listed on the manuscript contributed towards the article others not applicable.

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