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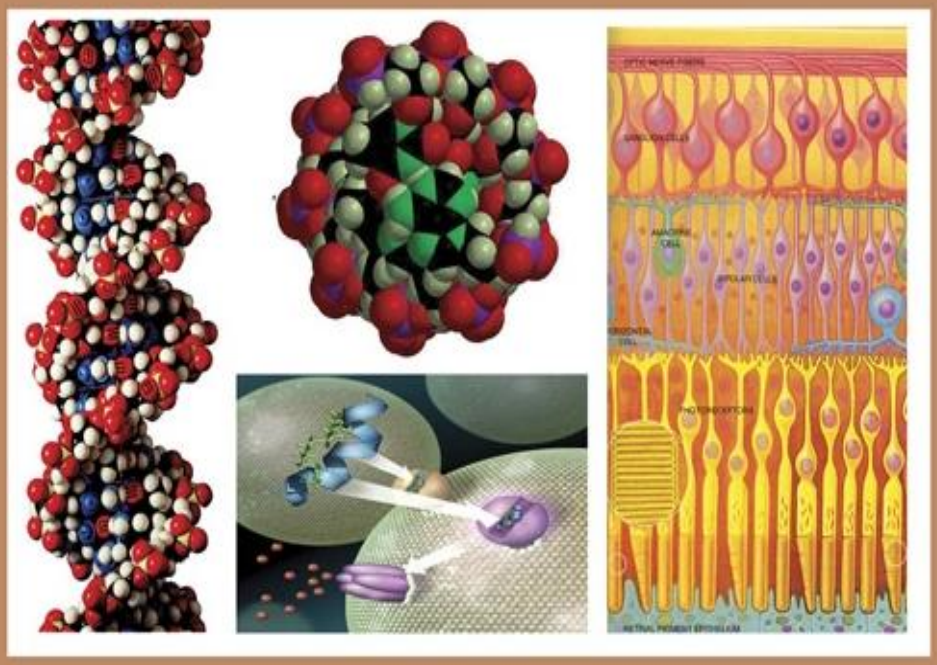

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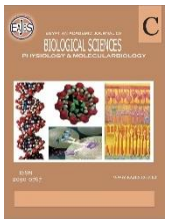

Modulation of Doxorubicin Induced Expression of Multidrug Resistance Gene in Breast Cancer cells by Astaxanthin

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ABSTRACT

Breast cancer is the most common disease in women worldwide and it is the most common cancer in the Arab world. It affects women at an early age compared with women in western countries. Doxorubicin (DOX) is a chemotherapeutic drug and is highly effective in advanced breast cancer. Unfortunately, the cytotoxic activity of DOX affects normal cells as well as cancer cells, which leads to severe heart damage. Many strategies have been investigated to protect the heart against DOX-induced cardiotoxicity one of them is the search for a natural compound with chemopreventive or anticancer properties that can be used with DOX. Recent studies proved the effect of the natural product astaxanthin (AST) as an anti-cancer. Therefore, the present study aimed to study the effect of AST co-treatment with DOX on the growth of the MCF-7 breast cancer cell line and investigate the possible mechanism to overcome the side effects and multidrug resistance of DOX treatment. To assess these effects, we investigated the DOX effect on apoptosis induction, cell cycle phase distribution, cellular uptake, ABCB1mRNA expression and p-glycoprotein (P-gp) activity in breast cancer cells in the presence and absence of AST. The addition of AST (133 and 250µg/ml) increased DOX cytotoxicity which manifested as a significant decrease in IC_{50} compared to the cells treated with DOX alone. Moreover, flow cytometric analysis of the MCF-7 cells treated with DOX (4.5µg/ml) simultaneously with AST (250µg/ml) showed the enhanced arrest of the cells in $G_0\backslash G_1$ (35.50 %). In addition, apoptotic phase cells were significantly increased to 39.13% when AST (250µg/ml) was combined with DOX compared to 27.3% in DOX alone. Also, AST (133 µg/ml and 250 µg/ml) significantly increased the cellular uptake of DOX as compared to DOX alone. The addition of AST (133 and 250µg/ml) combined with DOX resulted in a concentration-dependent decrease and almost complete reversal of DOX-induced increase in ABCB1 mRNA expression to the control value. The present study concluded that AST chemosensitizers DOX cytotoxicity against the proliferation of MCF-7 human breast cancer cells. This could be explained by induction of apoptosis, enhanced DOX cellular uptake, downregulation of ABCB1 gene expression and inhibition of P-gp activity.

INTRODUCTION

Breast cancer is now one of the most commonly diagnosed cancers and the fifth cause of cancer-related deaths, with an estimated 2.3 million new cases worldwide, according to GLOBOCAN 2020 (Ferlay *et al.*, 2021).

In Egypt, Breast cancer accounts for 33% of cancer cases in women, with more than 22,000 new cases diagnosed each year (Abdelaziz *et al.,* 2020, Ibrahim *et al.,* 2014). For the past three decades chemotherapeutic agents such as doxorubicin (DOX), have been used to increase the degree of clinical success in treating metastatic breast tumors because of the molecule's excellent antitumor activity (Danesi et al., 2002). However, DOX-based chemotherapy has poor tumor selectivity causing multi-organ toxicities in various patients (Carvalho *et al.,* 2009, Toldo *et al.,* 2013) and the development of multidrug resistance associated with DOX in such chemotherapy programs (Peetla *et al.,* 2010, Ponnusamy *et al.,* 2017). Therefore, it is of clinical interest to develop adjuvant therapy approaches that can boost the effectiveness of current medicines and/or lessen their side effects.

Astaxanthin (AST) is a potent antioxidant carotenoid with an established safety profile that is accepted as a dietary supplement (Higuera-Ciapara et al., 2006, Pashkow et al., 2008). The anti-inflammatory and anti-apoptotic properties of AST are also confirmed (Lee *et al.,* 2011**,** Lee *et al.,* 2003**,** Lee *et al.,* 2003). Previous studies reported that AST improved DOX's anticancer efficacy in several cancer cell lines (El-Agamy *et al.,* 2017, AlQahtani *et al.,* 2019, Fouad *et al.,* 2021). Moreover, a recent study in our laboratory demonstrated that AST supplementation attenuates cyclophosphamide-induced cardiotoxicity by modulating aldehyde dehydrogenase and Klotho protein expression in heart tissues, along with its downstream apoptosis effector markers (Kamel *et al.,* 2021). In order to avoid the serious side effects and multidrug resistance of DOX treatment, the current study aimed to examine the impact of AST co-treatment with DOX on MCF-7 breast cancer cells and explore potential mechanisms by examining apoptosis, cell cycle phase distribution, ABCB1 mRNA gene expression, DOX cellular uptake, and p-glycoprotein activity in breast cancer cells.

MATERIALS AND METHODS

Drugs and chemicals: DOX and AST were obtained from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). The stock solution of both agents was dissolved in phosphate-buffered saline (PBS) and preserved at -20^0C. The solutions were diluted in Dulbecco's modified Eagels medium (DMEM) or PBS immediately before each experiment to the desired final concentrations. All other chemicals and Kits were purchased from sigma Aldrich Co.

Cells and Cell Cultures:

Human breast cancer cell line MCF7 was used in this study. It was obtained from Pharmacology Unit, National Cancer Institute, Cairo University, Egypt. The adherent cells were grown as a monolayer culture in DMEM supplemented with penicillin (100 IU/ml), streptomycin (100 ug/ml) and 10% fetal bovine serum, Cells were cultured at 37^0C in humidified 5 % $C0_2$ atmosphere and were passaged every 4-5 days.

Assessment of Cytotoxic Activity:

Cytotoxic activity of DOX and/or AST was determined using the (sulforhodamine) SRB method as previously prescribed by Skehan et al., 1990. In brief, cells were planted in 96 well microtiter plates at a concentration of 5×10^3 cells/well with RPMI-supplemented medium. Following a 24hrs incubation period, cells were incubated for an additional 48hrs with various concentrations of DOX (6, 12.5, 25, and 50 ug/ml) and AST (133 and 250 ug/ml). Cells were fixed by adding 50 µl of cold 50% TCA for 1 hr at 4°C. The supernatant is then discarded and the wells were then washed five times with distilled water, air dried, stained for 30 minutes at room temperature with 0.4% SRB dissolved in 1% acetic acid and then washed four times with 1% acetic acid. The plates were then allowed to dry naturally and the dye was dissolved in 100 µl/well of 10 mM Tris base (pH 10.5) for 10 minutes. Each well's optical density (OD) was measured spectrophotometrically at 490-530 nm using an ELISA microplate reader (Bio Teck

Instruments, (Winooski VT, USA), with automated shaking taking place for 30 seconds before reading.

Surviving fraction=Optical density of treated cells/Optical density of untreated control cells.

IC_{50} (the concentration of DOX necessary to produce 50% inhibition of cell growth) was calculated from the linear regression equation of the survival fraction curve. $Y = mX + b$, where: $Y = 0.5$ (the surviving fraction when there is a 50% inhibition of cell growth), **m** = the slope, **X** = dose of DOX induces 50% inhibition, **b** = the y-intercept.

Flow-Cytometric Assay Of Apoptosis:

Apoptotic cells were quantified by Annexin V-FITC- Propidium iodide (PI) double staining, using an Annexin V-FITC apoptosis detection kit according to the method of Van Engeland *et al*., 1998. Cells were seeded in 12-well plates at a cell density of 6–8 $\times 10^5$ cells/well in DMEM supplemented medium. Twenty-four hours later, cells were incubated for an additional 48 h with DOX (4.5µg/ml) and AST (133 and 250 µg/ml). The cell medium was then removed, the wells were washed with PBS and the cells were harvested with trypsin/EDTA. Cells were washed once with PBS following trypsinization and resuspended in 1 ml of Binding Buffer. Annexin V FITC Conjugate was added to the cells according to the manufacturer's instructions for 10 min at room temperature while protected from light. The Fluorescence of the cells was read immediately by a flow cytometer (NAVIOS Beckman Coulter, U.S.A.).

Cell Cycle Analysis:

Cells were plated in 12-well plates at a cell density of 6–8$\times 10^5$ cells/well in DMEM supplemented medium. Twenty-four hours later, cells were incubated for an additional 48 h with DOX (4.5 ug/ml) and AST at varying doses (133 and 250 ug/ml). The cell medium was then removed and the wells were washed once with PBS. Cell cycle analysis was performed according to the method of Pozarowski and Darzynkiewicz (2004). The cells were harvested with trypsin/EDTA, washed once with PBS and then resuspended in 0.5 ml of 0.05% Triton X-100 for 10 min at room temperature. Staining of cellular DNA was performed by adding 1 ml of 50 µg/mL PI to each cell suspension for 20 min at room temperature. Cell cycle analysis was performed by using a flow cytometer (Becton Dicknoson (BD) FACSCalbur, USA)**.**

Determination of MDR in MCF-7 Cells:

According to the technique of Pétriz and García-López (1997), P- glycoprotein activity of MCF-7 cells was assessed in the presence of Rh123 using a spectrofluorometer with excitation and emission wavelengths of 485 nm to 590 nm, respectively. Cells in RPMI medium were planted in 6-well plates for 24hrs at 37^{o}C in a humidified CO_2 incubator.100 µl of 5 M Rh123 was added, and the mixture was then maintained in a CO_2 incubator at 37^{o}C in a dark for 60 minutes. Cells were exposed to either DOX (4.5 µg/ml) alone and\or AST (133 and 250 µg/ml). The cells' medium was aspirated, washed with PBS twice and trypsinized.

Cells were centrifuged at 1600 g for 5 minutes after being trypsinized and washed once with ice-cold PBS. Cells ($1x10^6$) were resuspended in 1 ml of ice-cold PBS, shaken and use for the P-glycoprotein analysis, using a spectrofluorometer with excitation and emission wavelengths of 485 nm to 590 nm, respectively.

ABCB1 mRNA Gene Expressions Using Real-Time Polymerase Chain Reaction (RT-PCR):

Total RNA was extracted from control and treated cell pellets with a total RNA purification kit. One or more specific sequences in a DNA sample can be found and measured using real-time PCR. Exactly, 25 µl of master mix, 0.25 µl of CXR Reference Dye, 1µl of forward and reverse primers, and a final volume of 50 µl was completed after adding 1 µl of equal concentration of cDNA. A 7500 Fast Real-Time PCR System was used for all analyses, which were done in triplicate. The procedure included an initial step of denaturing at 95 °C

for 10 minutes, followed by 40 cycles of denaturing at 95 °C for 15 seconds and annealing at 62 °C for 1 minute. (Applied Biosystems, Foster City, CA, USA). The data were analysed and amplification plots were generated using the software program, 7500 fast real-time PCR. The comparative CT method was used ($2^{-\Delta\Delta CT}$) for calculating the relative quantification of the target gene was used as follows ΔΔCT = (CT of target gene – CT of RNA18S5) for χ – (CT of target gene – CT, RNA18S5) for y, where χ = treated sample and y = control sample. After validation of the method, results for each sample were expressed in N-fold changes in χ target gene copies, normalized to RNA18S5 relative to the copy number of the target gene in control, according to the following equation: the amount of target = $2^{-\Delta\Delta Ct}$. Statistical analysis was performed using SPSS. $P \leq 0.05$ was considered significant.

Name of Gene	Forward	Reverse
ABCB1	5'-ACCCACTCCTCCACCTTTGA-3'	5'-CTGTTGCTGTAGCCAAATTCGT-3'
GAPDH	5'-ACCCACTCCTCCACCTTTGA-3'	5'- CTGTTGCTGTAGCCAAATTCGT-3'

Assessment of Doxorubicin Cellular Accumulation:

DOX cellular accumulation assessment in MCF-7 cells was performed using a spectrofluorometer (F-2000 Fluorescence spectrophotometer Hitachi, Japan) according to the method of Kitagawa *et al.,* 2004 DOX fluorescence intensity was measured at excitation and emission wavelengths of λ ex = 496 nm and λ em = 592 nm, respectively. DOX cellular accumulation ratio=DOX concentration in AST+DOX treated cells/DOX concentration in cells treated with DOX alone

Statistical Analysis:

Statistical analysis was performed using SPSS (statistical package of social sciences, version 16). Data are expressed as mean ± SD and analysed using one-way analysis of variance (ANOVA) followed by Tukey- Kramer multiple comparisons. Statistical significance was acceptable to a level of $p < 0.05$.

RESULTS

Effect of AST Treatment On The Cytotoxic Activity of DOX:

Cytotoxicity was expressed as the percentage of surviving fraction after DOX treatment compared with untreated control cells (Table 1 and Fig. 1). It was observed that AST (133 and 250µg/ml) induced a significant concentration-dependent decrease in the IC50 of DOX by 33.4 % and 44.5 %, respectively as compared to IC50 of DOX alone.

Table 1: Cytotoxic effect of DOX and/or AST treatment on the growth of MCF-7 cells.

Treatment	IC50µg/ml
DOX	4.5 ± 0.175
DOX and AST (133µg/ml)	3.00 ± 0.14[a]
DOX and AST (250µg/ml)	2.5 ± 0.066[a,b]

Cell survival was determined after 48hrs exposure to drugs by sulphorhodamine B assay. Data are presented as mean ± SD of 3 independent experiments performed in 3 replicates and analyzed using one way analysis of variance (ANOVA) followed by Tukey- Kramer multiple comparison test. IC_{50}: the concentration necessary to produce 50% inhibition of cell growth. [a] Significantly different from the corresponding DOX at $P < 0.05$. [b] Significantly different from the corresponding DOX + AST (133µg/ml) at $P < 0.05$.

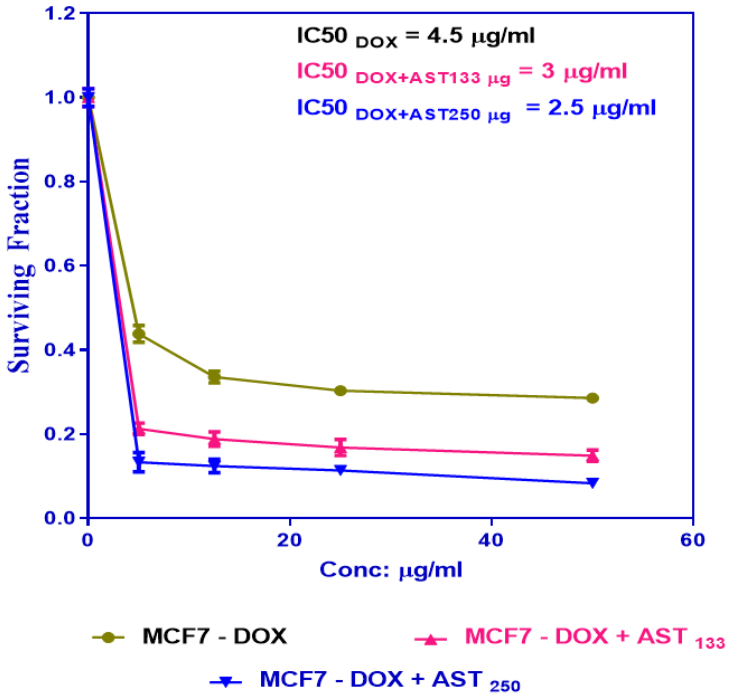

Fig. 1: Effect of DOX and\or AST on MCF-7 cells by sulphorhodamine B assay.
Cell survival was determined after 48hrs exposure to drugs by sulphorhodamine B assay. Data are presented as mean ± SD of 3 independent experiments performed in 3 replicates and analyzed using one way analysis of variance (ANOVA) followed by Tukey- Kramer multiple comparison test. IC_{50}: the concentration necessary to produce 50% inhibition of cell growth. [a] significantly different from the corresponding DOX at $P < 0.05$. [b] significantly different from the corresponding DOX + AST (133µg/ml) at $P < 0.05$.

Effect of AST and DOX Treatment On Apoptosis Induction:

Table (2) and Figure (2) showed the effect of DOX and/or AST treatment on the percentage of cells in early apoptosis. After 48hrs of treatment, it was observed that the addition of AST (133 and 250µg/ml) with DOX showed a significant increase ($P < 0.05$) in the percentage of early apoptosis (32.47%, 39.14) and respectively compared to DOX alone (27.3%).

Table 2: Effect of DOX and/or AST treatment on live cells and induction of early apoptosis in MCF-7 cells.

Groups	Early apoptosis
Control	0.53 ± 0.003
DOX (4.5µg/ml)	27.30 ± 0.424[a]
AST (133µg/ml)	0.33 ± 0.010[b]
AST (250µg/ml)	0.89 ± 0.120[b]
DOX (4.5µg/ml) +AST (133µg/ml)	32.47 ± 0.664 [a, b, c, d]
DOX (4.5µg/ml) +AST (250µg/ml)	39.14 ± 0.190 [a, c, d, e]

Early apoptosis was analysed after 48hrs exposure to drugs by staining with Annexin V FITC-A and Propidium iodide (PI). Data are presented as mean ± SD of 2 independent experiments performed in 2 replicates and analysed using one way analysis of variance (ANOVA) followed by Tukey- Kramer multiple comparison test. [a] significantly different from control cells at $P < 0.05$. [b] significantly different from the corresponding DOX at $P < 0.05$. [c] significantly different from the corresponding AST (133µg/ml) at $P < 0.05$.
[d] significantly different from the corresponding AST (250µg/ml) at $P < 0.05$. [e] significantly different from corresponding DOX + AST (133µg/ml) at $P < 0.05$.

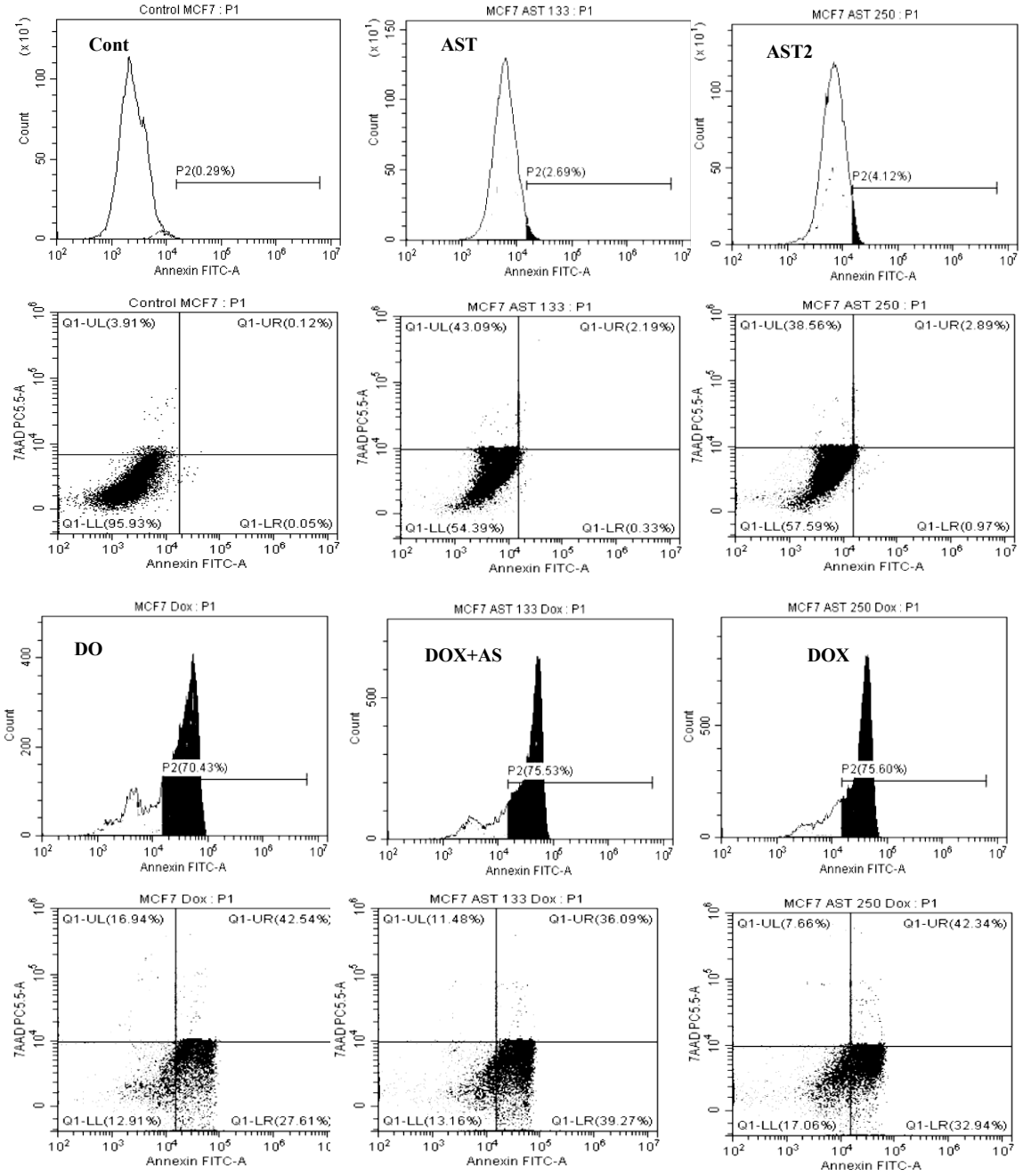

Fig. 2: Effect of DOX and /or AST treatment on induction apoptosis in MCF-7 cells.
Apoptosis was analyzed after 48hrs exposure to drugs by staining with Annexin V FITC-A and Propidium iodide (PI).

Effect of DOX and/or AST Treatment On Cycle Phase Progression of MCF-7:

Table (3) showed the effect of DOX (4.5µg/ml) and\or AST (133 and 250µg/ml) on Cell cycle distribution using the flow cytometry after staining with PI. The addition of AST (133 and 250µg/ml) to DOX showed a significant (P< 0.05) increase in the accumulation of the cells in G_0/G_1 (20.40 and 35.50 %) respectively compared to DOX alone (18.25%). A similar finding was observed in the S phase when AST (133 and 250µg/ml) was added to DOX it was shown a significant increase (P< 0.05) in the accumulation of the cells (35.30 and 25.80 %) compared to cells treated with DOX alone (21.25%).

Table 3: Effect of DOX and/or AST treatment on the Cell Cycle phase distribution of MCF-7 cells.

Treatment	G0\G1%	S%	G2\ M%
Control	83.25 ± 0.21	0.05 ± 0.00	16.70 ± 0.28
DOX (4.5µg/ml)	18.25 ± 0.21 [a]	21.25 ± 0.21 [a]	60.65 ± 0.21 [a]
AST (133µg/ml)	30.30 ± 0.14 [a, b]	34.35 ± 0.21 [a, b]	35.45 ± 0.21 [a, b]
AST (250µg/ml)	58.25 ± 0.21 [a, b, c]	2.35 ± 0.21 [a, b, c]	39.25 ± 0.21 [a, b, c]
DOX (4.5µg/ml) + AST (133µg/ml)	20.40 ± 0.14 [a, b, c, d]	35.30 ± 0.14 [a, b, c, d]	44.20 ± 0.14 [a, b, c, d]
DOX (4.5µg/ml) + AST (250µg/ml)	35.50 ± 0.14 [a, b, c, d, e]	25.80 ± 0.14 [a, b, c, d, e]	38.60 ± 0.14 [a, b, c, d, e]

Cell cycle phase distribution was analysed after 48hrs exposure to drugs by staining with Propidium iodide (PI). Data are presented as mean ± SD of 3 independent experiments performed in 2 replicates and analysed using one way analysis of variance (ANOVA) followed by Tukey- Kramer multiple comparison test. [a] significantly different from the corresponding control cell cycle phases at $P < 0.05$. [b] significantly different from the corresponding DOX at $P < 0.05$.[c] significantly different from the corresponding AST (133µg/ml) at $P < 0.05$. [d] significantly different from the corresponding AST (250µg/ml) at $P < 0.05$ and [e] Significantly different from the corresponding DOX + AST (133µg/ ml) at $P < 0.05$.

Effect of AST on Doxorubicin Cellular Accumulation:

Cells treated with DOX and AST (133 and 250µg/ml) showed a significant increase ($P < 0.05$) in DOX cellular uptake compared to DOX alone (Fig. 3). The increase in uptake was time-dependent up to 6 hours. After 6 hours there was no increase in DOX cellular uptake by increasing the time. At the same time, there was a significant increase ($P < 0.05$) in DOX uptake in MCF-7 cells treated with (DOX and AST 250µg/ml) compared to (DOX and AST (133µg/ml).

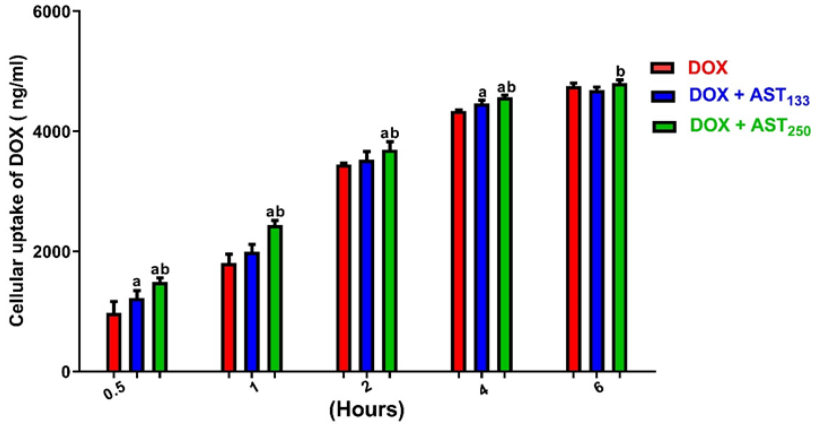

Fig. 3: Effect of AST treatment on the cellular uptake of DOX in MCF-7 cells.
The growing cells were incubated with DOX and /or AST for (0.5, 1, 2, 4, and 6 hrs). After incubation time, 250 µl of medium was transferred to a glass tube containing 750 ul of methanol. An injection volume of 10 µL from the clear supernatant was transferred into HPLC autosampler vial for LC–MS-MS analysis (AB SCIEX 3200 QTRAP, Germany). Data are presented as mean ± SD of 3 independent experiments and analysed using one way analysis of variance (ANOVA) followed by Tukey- Kramer multiple comparison test. [a] significantly different from the corresponding DOX alone at $P < 0.05$. [b] significantly different from the corresponding DOX + AST (133 µg/ml) at $P < 0.05$.

Effect of DOX and\or AST on the MDR activity:

As shown in Figure (4), DOX decreased dye accumulation in MCF-7 to (61.22/ %) compared to the control cell. The addition of DOX with AST (133 and 250µg/ml) increased dye accumulation to (124.41 and 133.69%) respectively compared to DOX alone.

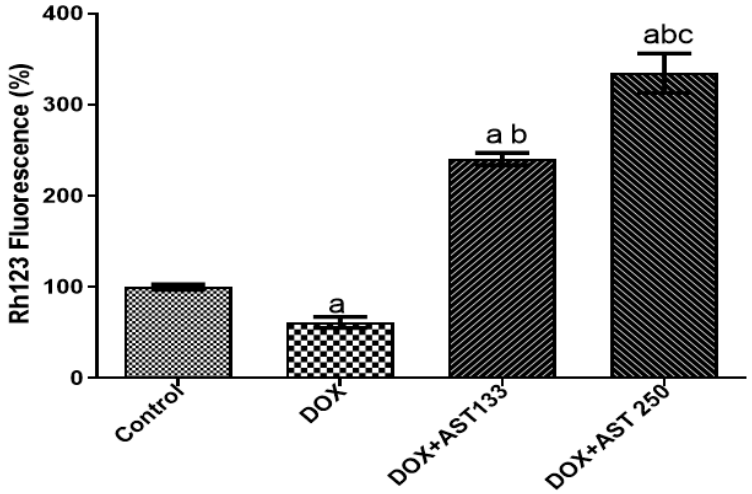

Fig. 4: Effect of DOX and/or AST on MDR activity**.**
The MCF-7 cells were exposed to Rh 123 for 60 minutes at 37°C. DOX and /or AST were added for 48hrs at 37°C. P- glycoprotein activity of MCF-7 cells was measured in the presence of Rh123 by using spectrofluorometer with excitation and emission wavelengths of 485 nm to 590 nm, respectively. Data are presented as mean ± SD of 3 independent experiments performed in 3 replicates and analyzed using one way analysis of variance (ANOVA) followed by Tukey- Kramer multiple comparison test. [a] significantly different from untreated cells at $P < 0.05$. [b] significantly different from the corresponding DOX at $P < 0.05$. [c] significantly different from the corresponding DOX + AST (133µg/ml) at $P < 0.05$.

Effect of DOX and\or AST on ABCB1 mRNA Gene Expression of MCF7:

Figure (5) showed the effect of DOX and\or AST on the gene expression of ABCB1. Cells treated with DOX alone showed a significant 74 %-fold increase ($P < 0.05$) in gene expression of ABCB1 compared to an untreated control cell. The addition of AST (133 and 250µg/ml) combined with DOX resulted in a concentration-dependent decrease and almost complete reversal of DOX-induced increase in ABCB1 expression ($P < 0.05$) to the control value.

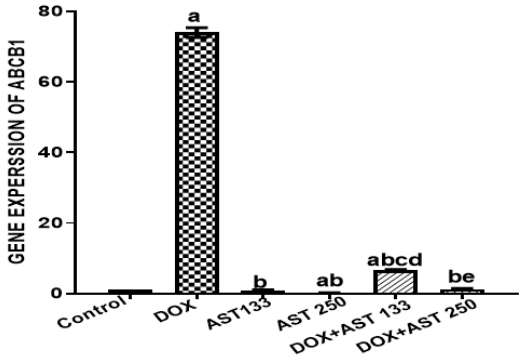

Fig. 5: Effect of DOX and\or AST after 48hrs treatment on mRNA expression of ABCB1 in MCF-7 by RT-PCR.
1µl of equal concentration of cDNA was add to 25 µl master mix, 0.25µl CXR Reference Dye, 1µl of forward and reverse primers and the volume was completed to 50 µl**.** All analyses were carried out in triplicate using a 7500 Fast Real-Time PCR System using a protocol that involved an initial denaturation step at 95 °C for 10 minutes, followed by 40 cycles of denaturing at 95 °C for 15 seconds and annealing at 62 °C for 1 minute**.** Data are presented as mean ± SD and analysed using one way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test. [a] significantly different from untreated control cells at $P < 0.05$.[b] significantly different from the corresponding DOX at $P < 0.05$.[c] significantly different from the corresponding AST (133µg/ml) at $P < 0.05$. [d] significantly different from the corresponding AST (250µg/ml) at $P < 0.05$. [e] significantly different from the corresponding DOX + AST (133µg/ml) at $P < 0.05$.

DISCUSSION

Doxorubicin is the most widely used drug in the treatment of a variety of human neoplasms, However, with the increasing use of DOX, acute as well as chronic cumulative dose-dependent cardiomyopathy has been recognized as the major limiting factor for DOX chemotherapy (Buzadar *et al.,* 1981, Kantrowitz and Bristow, 1984). A number of strategies have been investigated in order to improve the cytotoxic effects of chemotherapeutic drugs while lowering their toxicity. Chemosensitization is one technique that could be utilized to reduce anti-tumor dose and toxicity. Among the prospective chemosensitizers is AST (a carotenoid pigment anti-inflammatory inhibitor), a natural product that may inhibit the proliferation of certain cancer cells (Ambati *et al.,* 2014). Additionally, Fouad *et al.* (2021) suggested that AST triggered the beneficial epigenetic pathways to mitigate the chemotoxicity of DOX cytotoxicity in hormonal-positive breast cancer cells. Therefore, in this study, we investigated the modulatory effect of the natural compound AST on DOX cytotoxicity in MCF-7 human breast cancer cell line as well as the underlying mechanisms of cell cycle disruption, apoptosis, DOX cellular uptake, P-glycoprotein activity, and gene expression.

Treatment of MCF-7 cells with different DOX doses alone was observed to be cytotoxic to the cells. The cytotoxicity of DOX has been confirmed by the results of induction of apoptosis and cell cycle progression, where After 48hrs of treatment it was observed that the addition of AST (133 and 250µg/ml) with DOX showed a significant increase ($P < 0.05$) in the percentage of early apoptosis (32.47%,39.13) respectively compared to DOX alone (27.3%). These findings are in agreement with AlQahtani et al. (2019) who found that AST increases the cytotoxic activity of DOX which leads to suppression of the growth of Ehrlich ascites carcinoma (EAC) cells. In the same line Meiyanto *et al.* (2011)**.** Observed that DOX treatment alone caused MCF-7 cells to undergo apoptosis. While evidence came from the Annexin-V test of apoptosis, which revealed that MCF-7 cells treated with AST and DOX had much higher percentages of early apoptosis and lower percentages of live cells than cells treated with DOX alone. When DOX is coupled with AST, many ways of AST inducing apoptosis have recently been discovered (Vijay et al., 2018, AlQahtani et al., 2019). According to several reports, rather than normal breast epithelial cells (MCF-10A), the pro-oxidant property of AST was the primary catalyst for the selective death of MCF7 cells when growth inhibition was increased in a synergistic manner (Vijay *et al.,* 2018). The apoptotic impact of AST has also been demonstrated in colon cancer cell lines which is similar to the current findings (Liu et al., 2016). In 2016 Shao et al. showed that AST therapy resulted in a decrease in tumor weight of H22 hepatoma cells relative to untreated controls, most likely as a result of an enhanced rate of apoptosis. AST treatment increased the percentage of Ehrlich cells in G_2/M, the best phase for the activity of DOX due to the maximum expression of its target enzyme topoisomerase II (AlQahtani et al., 2019). They concluded that AST caused up-regulation of the tumor suppressor p53 gene, potentiating DOX cytotoxicity and apoptosis against mammary tumor cells and accumulating them in the G_2/M phase of the cell cycle. It has been found that p388 leukemia cells synchronized in S and G_2\M phases were more sensitive to DOX than cells in G_1 phase (Oktem *et al.,* 2012). These previous studies may explain why the cytotoxic activity of DOX was enhanced in presence of AST. Chemotherapeutic drugs, such as DOX, must be present in cellular compartments and transported to the site of action in order to exert their cytotoxic activity (Jiang *et al.,* 2014). As a result, it is hypothesized that cells with more DOX uptake will have greater sensitivity to the drug. In the present investigation, the addition of AST resulted in considerable increases in DOX cellular uptake. When cells were treated

with DOX and AST (133 and 250µg/ml), DOX cellular uptake was increased with increasing concentration and time compared to cells treated with DOX alone (figure 4). So, AST not only improved DOX's efficacy by triggering apoptosis, but it also raised DOX availability within the cells. This may explain why the combination of AST and DOX was more cytotoxic than DOX alone. In the same line, another natural agent has been used for increasing the cytotoxic effect of DOX. In 2012, Osman *et al.* showed high DOX levels in MCF-7 cells when concomitantly administered with resveratrol with the more cytotoxic activity of DOX.

In the present study, the P-gp experiment revealed that treatment with AST (133 and 250µg/ml) resulted in an increase in fluorescent dye accumulation inside the cells, respectively. This was due to the suppression of P-gp activity, which resulted in greater DOX accumulation in the presence of AST. So, the rise in DOX cytotoxicity seen by AST treatment coincided with an increase in its cellular uptake. In the present study, the accumulation of rhodamine-123 in the cells was used to determine the role of P-gp in the uptake of DOX. These findings imply that AST treatment may impair the catalytic activity of MDR proteins, resulting in an increase in DOX accumulation. So, the inhibition of P-gp and multidrug resistance (MDR) by AST could explain the increase in DOX cellular uptake in MCF-7 cells (Rogalska *et al.,* 2014). This finding is consistent with those reported by van der Sandt *et al.* (2000) who investigated the influence of eugenol and AST on DOX's anti-tumor activity in hormonally positive breast cancer cells. Using rhodamine uptake and efflux experiments, they reported that AST has the potential to block the function of P-gp, resulting in an increase in DOX anti-tumor activity. In our study we demonstrated that after DOX treatment, ABCB1 mRNA was overexpressed in MCF-7 cells, presumably contributing to the development of drug resistance (figure 5). However, ABCB1/P-gp-mediated MDR was shown to be reversed by AST therapy as demonstrated by a reduction in ABCB1 mRNA expression following the addition of AST. In fact, this downregulation restored the sensitivity of MCF-7 cells to DOX treatment and demonstrated that AST administration reduced ABCB1 protein expression with the consequent increase in DOX uptake and cytotoxicity. In a study similar to ours, Komoto *et al.* (2008) evaluated the effects of 12 Ca2+ antagonists, including diltiazem (DIL), on MDR1 mRNA expression in human cervical carcinoma (HeLa and Hvr100-6 cells) that had previously been treated with chemotherapeutic agents in an effort to develop a novel method to inhibit MDR1-mediated MDR (Didziapetris *et al.,* 2003, Genovese *et al.,* 2017). They showed a significant decrease in MDR1 mRNA expression following treatment with diltiazem (DIL), which is in line with our findings, but inconsistent outcomes for other Ca2+ antagonists, proving that the down-regulation of MDR1 mRNA expression contributed to the decrease in resistance via increases in the intracellular concentrations of anticancer drugs. In conclusion, AST chemosensitizers DOX cytotoxicity against the proliferation of MCF-7 human breast cancer cells. This could be explained by the induction of apoptosis, enhanced DOX cellular uptake, downregulation of ABCB1 gene expression and inhibition of P-gp activity.

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REFERENCES

Abdelaziz, A., Shawki, M., Shaaban, A., Albarouki, S., Rachid, A., Alsalhani,

O., & Jomaa, M. (2020). Breast Cancer Awareness among Egyptian Women and the Impact of Caring for Patients with Breast Cancer on Family Caregivers' Knowledge and Behaviour. *Research in Oncology*, *0*(0), 1–8. https://doi.org/10.21608/resoncol.2020.42340.1114

AlQahtani, A. A., Moneim M. Osman, A. -, Al-Kreathy, H. M., Al-harthy, S. E., S. Al-malky, H., S. AL Nasser, M., O. Kamel, F., N. Alaama, M., & Damanhouri, Z. A. (2019). Chemosensitizing Effects of Marine Astaxanthin on the Anti-cancer Activity of Doxorubicin in Tumor Bearing Mice. *International Journal of Cancer Research*, *15*(1), 1–8. https://doi.org/10.3923/ijcr.2019.1.8

Ambati, R., Phang, S.-M., Ravi, S., & Aswathanarayana, R. (2014). Astaxanthin: Sources, Extraction, Stability, Biological Activities and Its Commercial Applications—A Review. *Marine Drugs*, *12*(1), 128–152. https://doi.org/10.3390/md12010128

Buzdar, A. U., Marcus, C., Blumenschein, G. R., & Smith, T. L. (1985). Early and delayed clinical cardiotoxicity of doxorubicin. *Cancer*, *55*(12), 2761–2765. https://doi.org/10.1002/1097-0142(19850615)55:12%3C2761::aid-cncr2820551206%3E3.0.co;2-p

Carvalho, C., Santos, R., Cardoso, S., Correia, S., Oliveira, P., Santos, M., & Moreira, P. (2009). Doxorubicin: The Good, the Bad and the Ugly Effect. *Current Medicinal Chemistry* , *16*(25), 3267–3285. https://doi.org/10.2174/092986709788803312

Danesi, R., Fogli, S., Gennari, A., Conte, P., & Del Tacca, M. (2002). Pharmacokinetic-pharmacodynamic relationships of the anthracycline anticancer drugs. *Clinical Pharmacokinetics*, *41*(6), 431–444. https://doi.org/10.2165/00003088-200241060-00004

Didziapetris, R., Japertas, P., Avdeef, A., & Petrauskas, A. (2003). Classification Analysis of P-Glycoprotein Substrate Specificity. *Journal of Drug Targeting*, *11*(7), 391–406. https://doi.org/10.1080/10611860310001648248

El-Agamy, S. E., Abdel-Aziz, A. K., Wahdan, S., Esmat, A., & Azab, S. S. (2017). Astaxanthin Ameliorates Doxorubicin- Induced Cognitive Impairment (Chemobrain) in Experimental Rat Model: Impact on Oxidative, Inflammatory, and Apoptotic Machineries. *Molecular Neurobiology*, *55*(7), 5727–5740. https://doi.org/10.1007/s12035-017-0797-7

Ferlay, J., Colombet, M., Soerjomataram, I., Parkin, D. M., Piñeros, M., Znaor, A., & Bray, F. (2021). Cancer statistics for the year 2020: an overview. *International Journal of Cancer*, *149*(4). https://doi.org/10.1002/ijc.33588

Fouad, M. A., Sayed-Ahmed, M. M., Huwait, E. A., Hafez, H. F., & Osman, A.-M. M. (2021). Epigenetic immunomodulatory effect of eugenol and astaxanthin on doxorubicin cytotoxicity in hormonal positive breast Cancer cells. *BMC Pharmacology and Toxicology*, *22*(1). https://doi.org/10.1186/s40360-021-00473-2

Genovese, I., Ilari, A., Assaraf, Y. G., Fazi, F., & Colotti, G. (2017). Not only P-glycoprotein: Amplification of the ABCB1- containing chromosome region 7q21 confers multidrug resistance upon cancer cells by coordinated overexpression of an assortment of resistance-related proteins. *Drug Resistance Updates*, *32*, 23–46. https://doi.org/10.1016/j.drup.2017.10.003

Higuera-Ciapara, I., Félix-Valenzuela, L., & Goycoolea, F. M. (2006). Astaxanthin: A Review of its Chemistry and Applications. *Critical Reviews in Food Science*

and Nutrition, *46*(2), 185–196. https://doi.org/10.1080/10408690590957188

Ibrahim, A. S., Khaled, H. M., Mikhail, N. N., Baraka, H., & Kamel, H. (2014). Cancer Incidence in Egypt: Results of the National Population-Based Cancer Registry Program. *Journal of Cancer Epidemiology*, *2014*, 1–18. https://doi.org/10.1155/2014/437971

Jiang, T., Mo, R., Bellotti, A., Zhou, J., & Gu, Z. (2014). Gel-Liposome-Mediated Co-Delivery of Anticancer Membrane-Associated Proteins and Small-Molecule Drugs for Enhanced Therapeutic Efficacy. *Advanced Functional Materials*, *24*(16), 2295–2304. https://doi.org/10.1002/adfm.201303222

Kamel, S. S., Baky, N. A. A., Karkeet, R. M., Osman, A. M., Sayed-Ahmed, M. M., & Fouad, M. A. (2021). Astaxanthin extenuates the inhibition of aldehyde dehydrogenase and Klotho protein expression in cyclophosphamide-induced acute cardiomyopathic rat model. *Clinical and Experimental Pharmacology and Physiology*, *49*(2), 291–301. https://doi.org/10.1111/1440-1681.13598

Kantrowitz, N. E., & Bristow, M. R. (1984). Cardiotoxicity of antitumor agents. *Progress in Cardiovascular Diseases*, *27*(3), 195–200. https://doi.org/10.1016/0033-0620 (84) 90004-5

Kitagawa, S., Nabekura, T., & Kamiyama, S. (2004). Inhibition of P-glycoprotein function by tea catechins in KB-C2 cells. *Journal of Pharmacy and Pharmacology*, *56*(8), 1001–1005. https://doi.org/10.1211/0022357044003

Komoto, C., Nakamura, T., Yamamori, M., Ohmoto, N., Kobayashi, H., Kuwahara, A., Nishiguchi, K., Takara, K., Tanigawara, Y., Okamura, N., Okumura, K., & Sakaeda, T. (2008). Reversal effects of Ca2+ antagonists on multidrug resistance via down-regulation of MDR1 mRNA. *The Kobe Journal of Medical Sciences*, *53*(6), 355–363. https://europepmc.org/article/med/18762730

Lee, D.-H., Kim, C.-S., & Lee, Y. J. (2011). Astaxanthin protects against MPTP/MPP+- induced mitochondrial dysfunction and ROS production in vivo and in vitro. *Food and Chemical Toxicology*, *49*(1), 271–280. https://doi.org/10.1016/j.fct.2010.10.029

Lee, S.-J., Bai, S.-K., Lee, K.-S., Namkoong, S., Na, H.-J., Ha, K.-S., Han, Jeong-A., Yim, S.-V., Chang, K., Kwon, Y.-G., Lee, S. K., & Kim, Y.-M. (2003). Astaxanthin inhibits nitric oxide production and inflammatory gene expression by suppressing I(kappa)B kinase-dependent NF-kappaB activation. *Molecules and Cells*, *16*(1), 97–105. https://pubmed.ncbi.nlm.nih.gov/14503852

Liu, X., Song, M., Gao, Z., Cai, X., Dixon, W., Chen, X., Cao, Y., & Xiao, H. (2016). Stereoisomers of Astaxanthin Inhibit Human Colon Cancer Cell Growth by Inducing G2/M Cell Cycle Arrest and Apoptosis. *Journal of Agricultural and Food Chemistry*, *64*(41), 7750–7759. https://doi.org/10.1021/ acs.jafc.6b03636

Meiyanto, E., Hermawan, A., Junedi, S., Fitriasari, A., & Susidarti, R. A. (2011). Nobiletin increased cytotoxic activity of doxorubicin on MCF-7 cells but not on T47D cells. *International Journal of Phytomedicine*, *3*(1), 129.

Oktem, G., Uysal, A., Oral, O., Sezer, E. D., Olukman, M., Erol, A., Akgur, S. A., & Bilir, A. (2012). Resveratrol attenuates doxorubicin-induced cellular damage by modulating nitric oxide and apoptosis. *Experimental and Toxicologic Pathology*, *64*(5),

471–479. https://doi.org/10.1016/j.etp.2010.11.001

Osman, A.-M. M., Bayoumi, H. M., Al-Harthi, S. E., Damanhouri, Z. A., & ElShal, M. F. (2012). Modulation of doxorubicin cytotoxicity by resveratrol in a human breast cancer cell line. *Cancer Cell International*, *12*(1), 47. https://doi.org/10.1186/1475-2867-12-47

Pashkow, F. J., Watumull, D. G., & Campbell, C. L. (2008). Astaxanthin: A Novel Potential Treatment for Oxidative Stress and Inflammation in Cardiovascular Disease. *The American Journal of Cardiology*, *101*(10), S58–S68. https://doi.org/10.1016/j.amjcard.2008.02.010

Peetla, C., Bhave, R., Vijayaraghavalu, S., Stine, A., Kooijman, E., & Labhasetwar, V. (2010). Drug Resistance in Breast Cancer Cells: Biophysical Characterization of and Doxorubicin Interactions with Membrane Lipids. *Molecular Pharmaceutics*, *7*(6), 2334–2348. https://doi.org/10.1021/mp100308n

Ponnusamy, L., Mahalingaiah, P. K. S., & Singh, K. P. (2017). Treatment schedule and estrogen receptor-status influence acquisition of doxorubicin resistance in breast cancer cells. *European Journal of Pharmaceutical Sciences*, *104*, 424–433. https://doi.org/10.1016/j.ejps.2017.04.020

Pozarowski, P., & Darzynkiewicz, Z. (2004). Analysis of cell cycle by flow cytometry. *Methods in Molecular Biology (Clifton, N.J.)*, *281*, 301–311. https://doi.org/10.1385/1-59259-811-0:301

Pétriz, J., & García-López, J. (1997). Flow cytometric analysis of P-glycoprotein function using rhodamine 123. *Leukemia*, *11*(7), 1124–1130. https://doi.org/10.1038/sj.leu.2400659

Rogalska, A., Szwed, M., & Rychlik, B. (2014). The Connection between the Toxicity of Anthracyclines and Their Ability to Modulate the P-Glycoprotein-Mediated Transport in A549, HepG2, and MCF-7 Cells. *The Scientific World Journal*, *2014*, 1–10. https://doi.org/10.1155/ 2014/ 819548

Shao, Y., Ni, Y., Yang, J., Lin, X., Li, J., & Zhang, L. (2016). Astaxanthin Inhibits Proliferation and Induces Apoptosis and Cell Cycle Arrest of Mice H22 Hepatoma Cells. *Medical Science Monitor*, *22*, 2152–2160. https://doi.org/10.12659/msm.899419

Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J. T., Bokesch, H., Kenney, S., & Boyd, M. R. (1990). New Colorimetric Cytotoxicity Assay for Anticancer-Drug Screening. *JNCI Journal of the National Cancer Institute*, *82*(13), 1107–1112. https://doi.org/10.1093/jnci/82.13.1107

Toldo, S., Goehe, R. W., Lotrionte, M., Mezzaroma, E., Sumner, E. T., Biondi-Zoccai, G. G. L., Seropian, I. M., Van Tassell, B. W., Loperfido, F., Palazzoni, G., Voelkel, N. F., Abbate, A., & Gewirtz, D. A. (2013). Comparative cardiac toxicity of anthracyclines in vitro and in vivo in the mouse. *PloS One*, *8*(3), e58421. https://doi.org/10.1371/journal.pone.0058421

van der Sandt, I. C. J., Blom-Roosemalen, M. C. M., de Boer, A. G., & Breimer, D. D. (2000). Specificity of doxorubicin versus rhodamine-123 in assessing P-glycoprotein functionality in the LLC-PK1, LLC-PK1:MDR1 and Caco-2 cell lines. *European Journal of Pharmaceutical Sciences*, *11*(3), 207–214. https://doi.org/10.1016/s0928-0987(00)00097-x

van Engeland, M., Nieland, L. J. W., Ramaekers, F. C. S., Schutte, B., & Reutelingsperger, C. P. M. (1998). Annexin V-Affinity assay: A review

on an apoptosis detection system based on phosphatidylserine exposure. *Cytometry*, *31*(1), 1–9. https://doi.org/10.1002/(sici)1097-0320(19980101)31:1%3C1::aid-cyto1%3E3.0.co;2-r

Vijay, K., Sowmya, P. R.-R., Arathi, B. P., Shilpa, S., Shwetha, H. J., Raju, M., Baskaran, V., & Lakshminarayana, R. (2018). Low-dose doxorubicin with carotenoids selectively alters redox status and upregulates oxidative stress-mediated apoptosis in breast cancer cells. *Food and Chemical Toxicology*, *118*, 675–690. https://doi.org/10.1016/j.fct.2018.06.027

Zhang, L., & Wang, H. (2015). Multiple Mechanisms of Anti-Cancer Effects Exerted by Astaxanthin. *Marine Drugs*, *13*(7), 4310–4330. https://doi.org/10.3390/md13074310