Original Research Article

Cardioprotective Effect of Marine Astaxanthin on Doxorubicin-Induced Cardiotoxicity in Normal Rats

ABSTRACT

Background: Doxorubicin (DOX) is an effective antineoplastic drug indicated to treat many cancerous diseases but its clinical usefulness is limited by many side effects. The main and the most serious one is DOX induced cardiotoxicity. Many strategies have been tried to minimize this side effect such as addition of cardioprotective agent to DOX treatment protocols.
Aims: The aim of this work was directed to investigate whether marine astaxanthin (ATX), a xanthophyll carotenoid pigment with potent antioxidant effect, could protect heart against the cardiotoxicity induced by DOX.
Methodology Forty Male Wister rats were divided into four equal groups and treated for one week as follow: Group I rats were treated with normal saline (2 ml/kg, x7, i.p.) and considered as control group. Group II rats were treated with ATX (40 mg/kg, x7, i.p.). Group III rats were treated with normal saline (2 ml/kg, x7, i.p.) and a single dose of DOX (20 mg/kg, i.p.) at day 7. Finally, group IV rats were treated with ATX (40 mg/kg, x7, i.p) and with a single dose of DOX (20 mg/kg, i.p.) at day 7. After 24 and 48 hrs. of treatment, rats were anesthetized and prepared for collection of blood samples and heart isolation. The cardioprotective effect of ATX against DOX induced cardiotoxicity were evaluated by measurement of the serum level of cardiac enzymes CPK by colorimetric assay and CK-MB by Eliza. Also the levels of serum total antioxidant capacity (TAC) were measured colorimetrically. in addition, the Malondialdehyde (MDA), reduced glutathione, glutathione peroxidase (Gpx) levels and superoxide dismutase (SOD) were were determined in heart tissues homogenate by colorimetric method. In addition, Heart sample were taken for histopathology studies.
Results: The Addition of ATX to DOX significantly ($p<0.05$) decreased the serum level of cardiac enzymes (CPK, CK-MB) and increased the serum total antioxidant capacity in compare with these levels in sera of rats treated with DOX only. This addition also significantly decreased the level of malondialdehyde and increased the reduced glutathione and glutathione peroxidase and superoxide dismutase significantly in the heart tissues homogenate in compare to corresponding levels in rats treated with DOX alone. Histopathological investigation of cardiac tissues confirmed the biochemical studies, where addition of ATX to DOX treatment protocol showed that the fragmentation of the muscle fiber revealed normal with central vesicular nuclei and prevented a marked disruption of normal cardiac architecture which resulted from DOX treatment.
Conclusion: Marine astaxanthin provides excellent cardioprotective effect against doxorubicin induced cardiotoxicity in rats.

Keywords: [Doxorubicin, astaxanthin, cardioprotective effect}

1. INTRODUCTION

Doxorubicin was firstly used clinically in cancer therapy in the late 1960s. It is considered as one of the most potent antitumor anthracycline. DOX could be administered alone or with other chemotherapeutic agents in the treatment protocols of many types of cancers such as leukemias, lymphomas, soft-tissue sarcomas and solid tumors. Unfortunately, its cytotoxic effects are limited by its cardiotoxicity [1,2] ,The main side effect of DOX, which could lead to congestive heart

failure [3]. It has been reported that the cardiomyopathy and congestive heart failure after treatment with DOX is dose-dependent [4,5]. Doxorubicin induces its cardiotoxicity by many mechanisms such as DNA and RNA damages, induction of oxidative stress through liberation of reactive oxygen specious, lipid peroxidation, increase the endoplasmic reticulum-mediated apoptosis, inhibition of autophagy and interference with of calcium homeostasis [2,6]. In addition, DOX metabolism produces superoxide anion and hydroxyl radical which lead to toxic manifestation in the cellular membrane of the normal cells. Also, it has been reported that this toxicity is mediated through cardiac tissues inflammation [7]. Between the importance of DOX in cancer treatment and the increase of the incidence of its induced cardiotoxicity, it has become increasingly important to find pharmacological remedies with protective effects against this serious side effect [7,8]. Variety of approaches have been Investigated as the addition of natural compound with chemopreventive or anticancer properties to the DOX treatment protocol [9,10,11]. Astaxanthin is a natural reddish carotenoid pigment belongs to the xanthophylls family. It has a potent antioxidant, antitumor, anti-inflammatory, anti-lipid peroxidation and cardioprotective effects [12,13,14]. Intra-peritoneal administration of ATX lead to faster absorption with higher bioavailability than oral administration in oncorhynchus mykiss [15]. ATX is extensively distributed in all tissues after oral administration and metabolized by CYP1A following oral ingestion in the rat [16]. The plasma ATX elimination half-life was estimated to be 21 ± 11 hr. after oral dose in human [17]. In our laboratory, we found that ATX potentiated the cytotoxic activity of DOX against the growth of Ehrlich ascites carcinoma cells in vivo (data not shown). Therefore, the present study was undertaken to test whether ATX could protect the heart against DOX-induce cardiotoxicity in normal rats through prevention of oxidative stress.

2. MATERIAL AND METHODS

2.1. Drugs and Chemicals

Doxorubicin hydrochloride was purchased from Ebewe pharma Austria. Astaxanthin was purchased from Haihang Industry Co., Ltd. CK-MB ELIZA kit (Cat No. E-EL-R1327) was purchased from Elabscience biotechnology Inc. (USA) and CPK kit (Cat No. CF13000120) was obtained from Centronic GmbH, (Germany). Total antioxidant capacity kit (Cat No. GT 2513), reduced glutathione kit (Cat. No. GR 2511), glutathione peroxidase kit (Cat No. GP 2524), glutathione -S- trasferase kit (Cat. No. 2519), malondialdehyde kit (Cat No. MD 2529), Catalase kit (Cat. No. CA 2517), Superoxide dismutase kit (Cat. No. SD 2521) were purchased from Biodiagnostic Co.(Dokki, Giza, Egypt).

2.2. Animals and housing

Male Wistar Albino rats (8-10 weeks of age, 250-300 gm. b.wt.) were provided from the animal house at College of Pharmacy, King Abduaziz University, Jeddah, Saudi Arabia. The rats were acclimatized for 7 days before experiments. A commercial balanced diet and water *ad libitum* were provided all over the experiments.

2.3. Experimental design

Forty Male Wister rat were randomly distributed into four equal groups, 10 animals in each group. Rats of group I were injected with normal saline (2 ml/kg, x7, i.p.) and considered as control group. group II animals were treated with ATX (40 mg/kg, x7, i.p.). Rats of group III were treated with normal saline (2 ml/kg, x7, i.p.) and a single dose of DOX (20 mg/kg, i.p.) at day 7. Finally, group IV rats were treated with ATX (40 mg/kg, x7, i.p) and at day 7 treated with a single dose of DOX (20 mg/kg, i.p.). After 24 and 48 hrs. of treatment, rats were anesthetized and prepared gently for collection of blood samples in non-heparinized tubes from each rat by cardiac puncture according to the IACUC recommended standard methods for blood collection. The samples were

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left to clot for 30 minutes then centrifuged for serum separation which was stored at − 80 °C to evaluate different biochemical parameters.

2.4. Evaluation of Cardiotoxicity of DOX Treatment in Presence and Absence of ATX

2.4.1 Evaluation of Serum Creatine Phosphokinase (CPK)

Creatine kinase activity was determined colorimetrically using CPK kit (Centronic GmbH, Germany) according to the method of Szasz et al [18].

2.4.2 Evaluation of Serum Serum Creatine Kinase MB Isoenzyme (CK-MB)

The serum level of CKMB was evaluated in rats' serum using Rat Creatine Kinase MB isoenzyme, CK-MB ELISA Kit, according to the manufacturer protocol.

2.4.3. Measurement of Serum Antioxidants Activities and Oxidative Stress

The Total serum antioxidant capacity was measured by colorimetric method, according to the method of Koracevic et al.[19]. In the heart tissues homogenate the reduced glutathione was measured according to the method of Beutler et al.[20].Glutathione Peroxidase (GPx) was determined by colorimetric method according to the method of Paglia et al. [21] and Malondialdehyde level was determined by colorimetric method according to the method of Ohkawa et al. [22]. Superoxide dismutase (SOD) was measured colormetrically in the cardiac tissues homogenate according to the method of Nishikimi et al.[23].

2.5 Histopathological Examination

After blood collection, rats were sacrificed by gently decapitation, chest opened and hearts were extracted. Heart sample was taken immediately and washed with saline. Part of the left ventricle of the heart was fixed in10% phosphate buffered formalin and processed for paraffin blocks. Serial histological longitudinal sections of 5-µm thickness were cut, mounted on glass slides and stained with haematoxylin and eosin (H&E) for general structure [24]. Half gm. of the remaining cardiac tissues was homogenized in 5 ml of phosphate buffer saline on ice, using an electric homogenizer (Potters, German).

2.6. Statistical Analysis

Statistical analysis of the data was carried out using computer program package (SPSS, version 21). All data are expressed as mean with their standard error of mean (SEM). One-way analysis of variance (ANOVA) was used to compare differences between experimental groups. It was followed by the least significance difference (LSD) test. However, two-sample t-test and its P-value to analyze the significance of the difference in the samples mean. Differences were considered significant at $P < 0.05$.

3. RESULTS

3.1. Effect of DOX and/or ATX on Cardiac Enzymes

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Table 1 showed the effect of DOX and/or ATX on the serum level of CPK. The level of serum CPK was significantly increased (2.14 and 2.05 fold) after 24 and 48 hrs. of DOX treatment, respectively. On the other hand, addition of ATX to DOX showed a significant decrease in CPK level in compared to DOX treated rats 24 and 48 hrs. of treatment. Table 2 showed the effect of DOX and/or ATX on the serum CK-MB level in rats. There were significant increases in CK-MB level (7.19 and 6.8 fold) in DOX treated rats compared to control after 24 and 48 hrs. treatment, respectively. While, addition of ATX to DOX nearly restored the CK-MB level to the normal at the two time points tested.

Table 1: Effect of DOX and/or ATX on CPK Activity in Rats' Serum.

	CPK level (U/L)	
Treatment	**24 hrs.**	**48 hrs.**
Normal saline	350 ± 6.78	361.53 ± 8.62
ATX	344.19 ± 9.37	344.19 ± 3.83
DOX	748.64 ± 11.42 [a]	741.21 ± 6.60 [a]
ATX and DOX	411.05 ± 19.21 [a, b]	413.53 ± 17.67 [a, b]

DOX (20 mg/kg, i.p.) was injected in male Wistar rats pretreated either with ATX (40 mg/kg, x7, i.p.) or normal saline (2 ml/kg, x7, i.p.).Data are expressed as mean ± SEM of five male Wistar rats after 24 hrs. and 48 hrs. [a] Significantly different from control at P-value < 0.05 , [b] Significantly different from corresponding DOX at P-value < 0.05, one way ANOVA with LSD post test.

Table 2: Effect of DOX and/or ATX on Serum CK-MB Level

	CK-MB (pg/ml)	
Treatment	**24 hrs.**	**48 hrs.**
Normal saline	106 ± 2.02	106.40 ± 1.96
ATX	105.80 ± 2.58	108.80 ± 1.28
DOX	763.80 ± 5.03 [a]	727 ± 7.41 [a]
ATX and DOX	378.60 ± 5.41 [a, b]	347.60 ± 4.09 [a, b]

DOX (20 mg/kg, i.p.) was injected in male Wistar rats pretreated either with ATX (40 mg/kg, x7, i.p.) or saline (2 ml/kg,x7, i.p.).Data are expressed as mean ± SEM of five male Wistar rats 24 hrs. and 48 hrs. after treatment.[a] Significantly different from control at P-value < 0.05 ,[b] Significantly different from corresponding DOX at P-value < 0.05, one way ANOVA with LSD post test

3.2. Effect of DOX and/or ATX on the Serum Antioxidant Capacity

Table 3 showed the effect of DOX and/or ATX on the total antioxidant capacity (TAC) level in rats' serum. There was a significant increase in TAC in ATX treated rats (1.26, 1.16 fold) compared to control rats after 24 and 48 hrs. of treatment, respectively. However, there was a significant decrease (1.4 and 1.54 fold) in serum TAC level in DOX treated rats in compare with control after 24 and 48 hrs. of treatment, respectively. Combination DOX and ATX in the treatment protocol showed a significant increase (1.26, 1.11 fold) in serum TAC level compared to control after 24 and 48 hrs., respectively.

Table 3: Effect of treatment with DOX and/or ATX on Serum Total Antioxidant Capacity

	TAC (mmol/L)	
Treatment	**24 hrs.**	**48 hrs.**
Normal saline	1.23 ± 0.07	1.31 ± 0.05
ATX	1.55 ± 0.01 [a]	1.52 ± 0.01[a]

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DOX	0.88 ± 0.01 [a]	0.85 ± 0.04 [a]
ATX and DOX	1.53 ± 0.01 [a, b]	1.46 ±0.02 [a, b]

DOX (20 mg/kg, i.p.) was injected in male Wistar rats pretreated either with ATX (40 mg/kg, x7, i.p.) or saline (2 ml/kg, x7, i.p.)
Data are expressed as mean ± SEM after 24 and 48 hrs. (n=5). [a] Significantly different from control at P-value < 0.05 ,[b] Significantly different from corresponding DOX at P-value <0.05, one way ANOVA with LSD post test.

Table 4 showed the effect of DOX and/or ATX on GSH level in rat's heart homogenate. There were significant decreases (1.7 and 1.8 fold) in GSH level in DOX treated rats compared with control after 24 and 48 hrs. of treatment, respectively. While, in presence of ATX the GSH levels maintained nearly to the normal values (104.6 and 104.78 mg/g tissue) respectively.

Table 4: Effect of DOX and/or ATX on Reduced Glutathione (GSH) level in rats' heart homogenate.

	GSH (mg/g tissue)	
Treatment	**24 hrs.**	**48 hrs.**
Normal saline	118.95 ± 6.10	118.33 ± 6.07
ATX	125.39 ± 8.54	120.95 ± 5.90
DOX	71.26 ± 0.95 [a]	67.27± 1.88 [a]
ATX and DOX	104.78 ± 2.71[a, b]	104.61±1.16 [a, b]

DOX was injected (20 mg/kg, i.p.) in male Wistar rats pretreated either with ATX (40 mg/kg, x7, i.p.) or saline (2 ml/kg, x7, i.p.). Data are expressed in mean ± SEM of the experiment in male Wistar rats after 24 hrs. and 48 hrs. (n=5). [a] Significantly different from control at P-value < 0.05 [b] Significantly different from corresponding DOX at P-value <0.05, one way ANOVA with LSD post test.

3.3. Effect of DOX and/or ATX on Lipid Peroxidation in the Rats Cardiac Tissues.

Table 5 showed the effect of DOX and/or ATX on malondialdehyde (MDA) level in the rat's heart homogenate. There were significant increases (3.5 and 3.7 fold) in MDA level in DOX treated rats compared with control after 24 and 48 hrs. of treatment, respectively. While, addition of ATX to DOX showed a significant reduction in MDA levels and return it nearly to normal values (47.10 nmol/g tissue and 43.30 nmol/g tissue) after 24 and 48 hrs. of treatment, respectively.

Table 5: Effect of DOX and/or ATX on malondialdehyde (MDA) level in rats' heart homogenate.

	MDA (nmol/g tissue)	
Treatment	**24 hrs.**	**48 hrs.**
Normal saline	37.30 ± 1.49	37.46 ± 0.95
ATX	33.24 ± 1.79	31.56 ± 0.55
DOX	128.36 ± 2.99 [a]	139.90 ± 1.11 [a]
ATX and DOX	47.10 ± 1.11 [a, b]	43.30 ± 0.63 [a, b]

DOX (20 mg/kg, i.p.) was injected in male Wistar rats pretreated either with ATX(40 mg/kg, x7, i.p.) or saline (2 ml/kg, x7,i.p.). Data are expressed as mean ± SEM of the experiment in male Wistar rats after 24 hrs. and 48 hrs. (n=5). [a] Significantly different from control at P-value < 0.05 , [b] Significantly different from corresponding DOX at P-value <0.05, one way ANOVA with LSD post test.

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Table 6: Effect of DOX and/or ATX on GPx on level in rats' heart homogenate:

	GPx (U/mg tissue)	
Treatment	**24 hrs.**	**48 hrs.**
Normal saline	6.68 ± 0.37	6.09 ± 0.12
ATX	6.41 ± 0.21	6.76 ± 0.15
DOX	2.96 ± 0.08 [a]	3.38 ± 0.07 [a]
ATX and DOX	4.60 ± 0.08 [a, b]	5.21 ±0.05 [a, b]

DOX (20 mg/kg, i.p.) was injected in male Wistar rats pretreated either with ATX (40 mg/kg, x7, i.p.) or saline (2 ml/kg, x7, i.p.). Data are expressed as mean ± SEM of five male Wistar rats after 24 hrs. and 48 hrs. [a] Significantly different from control at P- value < 0.05 ,[b] Significantly different from corresponding DOX at P-value <0.05, one way ANOVA with LSD post test.

Table 7 represented the effect of DOX and/or ATX on superoxide dismutase (SOD) activity in rats' serum. There were significant decreases (1.6 and 1.72 fold) in SOD activity in DOX treated rats compared to control after 24 and 48 hrs. of treatment, respectively. Addition of ATX to DOX, maintaining the SOD activity nearly to the normal values (3.35 and 4.01 U/ml).

Table 7: Effect of DOX and/or ATX on SOD activity in rats' Cardiac Tissues Homogenate.

	SOD activity (U/ml)	
Treatment	**24 hrs.**	**48 hrs.**
Normal saline	3.57 ± 0.22	4.01 ± 0.39
ATX	3.81 ± 0.31	4.43 ± 0.40
DOX	2.23 ± 0.02 [a]	2.32 ± 0.22 [a]
ATX and DOX	3.35 ± 0.03 [a, b]	4.01 ± 0.22 [a, b]

DOX (20 mg/kg, i.p.) was injected in male Wistar rats pretreated either with ATX (40 mg/kg, x7, i.p.) or saline (2 ml/kg, x7, i.p.).Data are expressed as mean ± SEM of five male Wistar rats after 24 hrs. and 48 hrs.[a] Significantly different from control at P-value < 0.05 , [b] Significantly different from corresponding DOX at P-value <0.05, one way ANOVA with LSD post test.

Figure 1 showed Light photomicrographs of rat's cardiac tissues from control group treated with normal saline (2ml /kg) showing normal branching muscle fiber with central vesicular nuclei. Fibroblasts with flat nuclei are noted in the surrounding endomysium and blood capillaries are present between the cardiac muscle fibers.

Light photomicrograph (Figure 4) showed the effect of DOX (20 mg/kg) treatment on the myocardium tissues of the rats. DOX treatment showed a marked disruption of normal cardiac architecture, congestion of blood vessels and capillaries, condensed pyknotic peripheral nuclei and multiple areas of fragmented cardiac muscle fibers.

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These changes have been attenuated when pretreated with ATX. Treatment with DOX (20 mg/kg) + ATX (40 mg/kg) showed that most of cardiac muscle fibers regained its normal structure but localized areas of myocytolysis and shortening of cardiac muscle fibers are still noted (Figure)

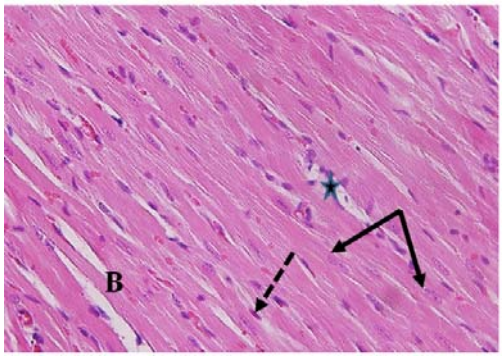

Figure 1: Photomicrograph of a section of myocardium of a rat of the control group showing branching (B) muscle fiber with central vesicular nuclei (arrows). Fibroblasts with flat nuclie are noted in the surrounding endomysium (dashed arrow). Blood capillaries are present between the cardiac muscle fibers (★). (H & E x 400).

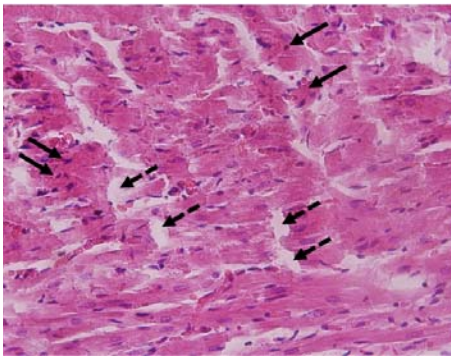

Figure 2: Photomicrograph of a section of myocardium of a rat after 24 hrs. of treatment with DOX(20 mg / kg i.p.) showing loss of normal organisation of cardiac muscle fibers revealing deeply acidophilic sarcoplasm and peripheral pyknotic nuclei. Numerous areas of muscle fibers shortening (dashed arrows) are noted. (H & E x 400).

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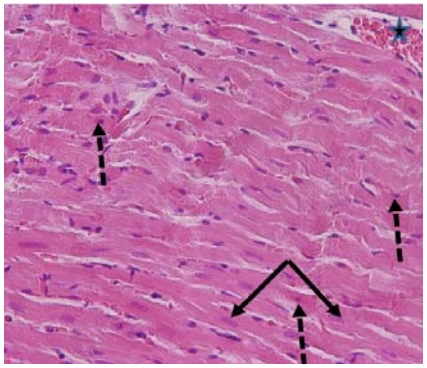

Figure 3: Photomicrograph of a section of myocardium of a rat after 24 hrs. of treatment with ATX (40mg/kg,x7,i.p.) + single dose of DOX (20 mg/kg i.p.) at day 7 showing normal structure of most of the cardiac muscle fibers (arrows). Few still reveal deeply acidophilic sarcoplasm and peripheral pyknotic nuclei (dashed arrows). Localized dilated congested capillaries are noted (★) (H & E x 400).

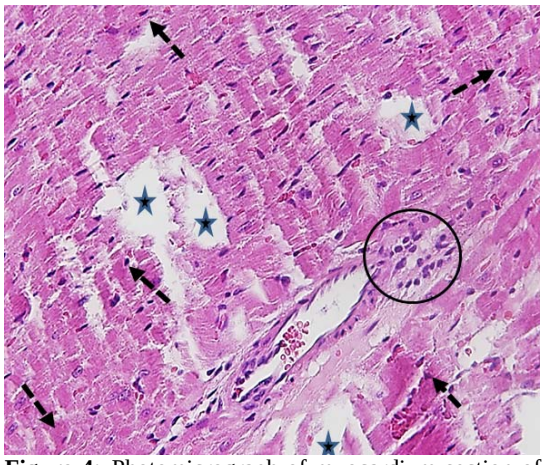

Figure 4: Photomicrograph of myocardium section of a rat after 48 hrs. of treatment with DOX (20mg/kg,i.p.) showing marked disruption of the myocardium architecture. Areas of myocytolysis (★), cardiac muscle fibers with deeply acidophilic sarcoplasm and peripheral pyknotic nuclei (dashed arrows) and perivascular polymorphnuclear cell infilteration are noted (O). (H & E x 400)

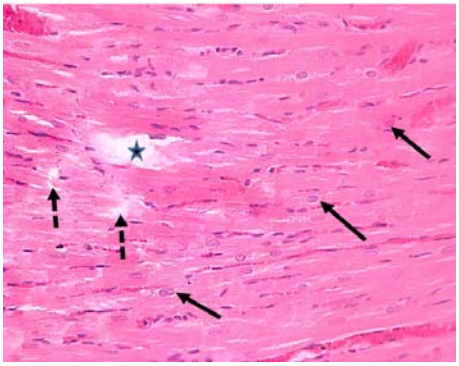

Figure 5: Photomicrograph of a section of myocardium of a rat after 48 hrs. of treatment with ATX (40mg/kg,x7,i.p.) + single dose of DOX (20 mg/kg i.p.) at day 7 showing most of cardiac muscle fibers regained its normal structure (arrows). But localized areas of myocytolysis (★) and shortening of cardiac muscle fibers are still noted (dashed arrows). (H & E x 400).

4. DISCUSSION

Doxorubicin is one of the effective and widely used antineoplastic drugs indicated for treatment of many kinds of cancers either alone or in combination with other antineoplastic drugs. However, its clinical usefulness is limited by its detrimental adverse effects as cardiotoxicity which may be exaggerated to reach heart failure [25]. Cardiotoxicity is the major and the most serious adverse effect of DOX which limit its clinical usefulness. Many strategies have been tried to minimize this serious side effects by using combination treatment with cardioprotective agent and synthesis of DOX liposomes [7,8,9,10,26,27,28].

Among the possible potential chemosensitizer is ATX which has cytotoxic activity [29,30,31] and chemoprotective effect against chemotherapy adverse effects [12,29,32]. In our laboratory, it has been proven that ATX sensitized DOX cytotoxic activity against the growth of Ehrlich ascites carcinoma cells *in-vivo* (data not shown).Therefore, our current study focus on the protective effect of ATX against DOX-induced cardiotoxicity. The mechanisms by which DOX exerts its cardiotoxicity are not clear enough and still under investigation. Although there are several cellular pathways involved in DOX induce cardiotoxicity such as release of vasoactive substances, mitochondrial deteriorations, lipid peroxidation and depletion of the cellular antioxidants such as glutathione. As ROS liberation plays an essential role in the DOX induced cardiotoxicity we and other researchers focused on the potential involvement of ROS in DOX induced cardiotoxicity [2,3,4,6,33].

It is well known that the heart tissues are highly susceptible to oxidative stress due to its inherent decreased detoxifying natural antioxidants [8,11].

In animal's studies, the acute cardiotoxicity induced by DOX was associated with high level of ROS liberation and lipid peroxidation. Moreover cardiac tissues injuries are associated with elevation of the level of CPK and CK-MB enzymes. It is well known that these enzymes are released from the heart muscle cells when they are injured and their activities in the blood after myocardial injury reflect the extent of damage in its musculature [34,35]. Our results showed that there was a significant reduction in serum total antioxidant capacity, reduced glutathione level, glutathione peroxidase level and superoxide dismutase activity in the cardiac tissues after DOX treatment (tables 3, 5, 6, 7). In addition, there was a significant increase in the lipid peroxidation in term of malondialdehyde level in the cardiac tissues which was significantly increased to (3.4 and 3.7 fold) 24 and 48 hrs. after DOX treatment, respectively (Table 5).

These results are in a good agreement with others who reported the cardiac toxicity after DOX treatment. Their findings reported the decrease in the serum level of TAC and increase in the level of MDA after DOX administration in the rats' cardiac tissues [11,36,37,38]..

Addition of ATX to DOX maintained the serum total antioxidant capacity, superoxide Dismutase, malondialdehyde level and glutathione peroxidase level in rats hearts tissues nearly to the normal values in compare with animal treated with DOX alone.

It has been reported that the treatment with DOX increase CPK level and Ck-MB as a sequences of DOX induced cardiotoxicity as a good marker to evaluate the toxic deterioration in cardiac tissues [7,39].

In the present study, there was a significant increase in CPK level after 24 and 48 hrs. of DOX treatment, respectively in compare with control (table 1).This result was confirmed by a significant increase of the specific cardiac marker CK-MB at the same two time points tested (Table 2).

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Addition of ATX showed a cardioprotective effect against DOX induced cardiotoxicity. These findings were confirmed by a significant reduction of the total CPK levels in ATX + DOX treated rats compared with rats treated with DOX alone. This cardioprotective effect of ATX were further confirmed by a significant reduction in CK-MB level in ATX + DOX treated rats in compare with animals treated with DOX alone (Table 2).

These results agree with Gross et al.[40], Monroy-Ruiz et al.[41] and Binu et al.[42] who reported that ATX has a cardioprotective effect through scavenging of free radicals involved in deterioration and remodeling of cardiomyocytes and tissues such as superoxide anion and reduction of oxidative stress markers involved in cardiotoxicity from the arachidonic acid and linoleic acid pathways.

In harmony with our results, Nakao et al.[43] reported that ATX protects heart tissues damage through its antioxidant properties. Moreover, Nishigaki et al.[44] stated that ATX minimize the glycated protein/iron chelate-induced toxicity through suppression of lipid peroxidation and protein oxidation and enhance the activity of antioxidant enzymes in human umbilical vein endothelial cells.

The current study showed that DOX-induced cardiotoxicity is minimized by quenching of ROS and hydrogen peroxide which is one of the proposed molecular mechanisms involved in the DOX induced cardiotoxicity and induction of apoptosis in cardiomyocytes [45].

Our results are in a good agreement with Wang et al.[46] who concluded that quenching of H_2O_2 or over expression of glutathione peroxidase decreased DOX-induced apoptosis in endothelial cells and cardiomyocytes but not in tumor cells. This may explain that the ATX provides a cardiomyocytes protective effect with potentiation of DOX cytotoxicity in EAC cells.

In contrary to our results, one of the molecular mechanisms of DOX induced cardiotoxicity is induction of apoptosis in endothelial cells and cardiac cells through activation of p53 protein. In our results ATX upregulated the expression of p53 gene in tumor cells (data not shown) as synergistic mechanism to potentiate the DOX cytotoxic effects which may be falsely explain that ATX increase the DOX-induced cardiotoxicity.

This discrepancy could be refuted as reported by Wang et al. [46] who found that DOX caused early activation of p53 in tumor cells that was followed by caspase-3 activation and DNA fragmentation. These findings suggest that the transcriptional activation of p53 in DOX-induced apoptosis in endothelial and cardiac cells may not be as crucial as it is in tumor cells. Therefore, the cytotoxicity of DOX is potentiated through over expression of p53 gene by ATX in EAC cells but not in cardiomayocytes.

Histopathological studies confirmed the biochemistry results where DOX causes loss of normal organization of cardiac muscle fibers revealing deeply acidophilic sarcoplasm and peripheral pyknotic nuclei. Moreover, numerous areas of muscle fibers shortening are noted. While, rats treated with ATX + DOX have less histopathological deteriorations (Figures 1, 2, 3, 4 and 5).

5. CONCLUSION

This research concluded that astaxanthin has the ability to reduce the cardiotoxic effect of DOX through inhibition of oxidative stress.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

CONSENT OF PUPLICATION

All authors approved the publication of this article

ETHICAL APPROVAL (WHERE EVER APPLICABLE)

All the animal studies were approved by the ethical research committee unit at the College of Medicine, King Abdulaziz University (Reference No.112-18).

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

REFERENCES

1. Lefrak EA, Pit'ha J, Rosenheim S, Gottlieb JA. A clinicopathologic analysis of adriamycin cardiotoxicity. Cancer 32 1973 302–314.
2. Renu K, Abilash VG, B TPP, Arunachalam S. Molecular mechanism of doxorubicin-induced cardiomyopathy – An update. Eur J Pharmacol 2018; 818: 241–253.
3. Octavia Y, Tocchetti CG, Gabrielson KL, Janssens S, Crijns HJ, Moens AL. Doxorubicin-induced cardiomyopathy: From molecular mechanisms to therapeutic strategies. J Mol Cell Cardiol 2012; 52: 1213–1225.
4. Mitry MA, Edwards JG. Doxorubicin induced heart failure: Phenotype and molecular mechanisms. Int J Cardiol Hear Vasc 2016; 10: 17–24.
5. Barry E, Alvarez JA, Scully RE, Miller TL, Lipshultz SE. Anthracycline-induced cardiotoxicity: course, pathophysiology, prevention and management. Expert Opin Pharmacother 2007; 8: 1039–1058.
6. Mobaraki M, Faraji A, Zare M, Manshadi HRD. Molecular Mechanisms of Cardiotoxicity: A Review on Major Side-effect of Doxorubicin. Indian J Pharm Sci 2017; 79: 335–344.
7. Abushouk AI, Ismail A, Salem AMA, Afifi AM, Abdel-Daim MM. Cardioprotective mechanisms of phytochemicals against doxorubicin-induced cardiotoxicity. Biomed Pharmacother 2017; 90: 935–946.
8. Yu J, Wang C, Kong Q, Wu X, Lu J-J, Chen X. Recent progress in doxorubicin-induced cardiotoxicity and protective potential of natural products. Phytomedicine 2018; 40: 125–139.
9. Osman A-MM, Al-Harthi SE, AlArabi OM *et al.* Chemosensetizing and cardioprotective effects of resveratrol in doxorubicin- treated animals. Cancer Cell Int 2013; 13: 52.
10. Alkreathy H, Damanhouri ZA, Ahmed N, Slevin M, Ali SS, Osman A-MM. Aged garlic extract protects against doxorubicin-induced cardiotoxicity in rats. Food Chem Toxicol 2010; 48: 951–956.
11. Kwatra M, Kumar V, Jangra A *et al.* Ameliorative effect of naringin against doxorubicin-induced acute cardiac toxicity in rats. Pharm Biol 2016; 54: 637–647.
12. Pashkow FJ, Watumull DG, Campbell CL. Astaxanthin: A Novel Potential Treatment for Oxidative Stress and Inflammation in Cardiovascular Disease. American Journal of

UNDER PEER REVIEW

Cardiology 101 2008.
13. Ambati RR, Moi PS, Ravi S, Aswathanarayana RG. Astaxanthin: Sources, extraction, stability, biological activities and its commercial applications - A review. Marine Drugs 12 2014 128–152.
14. Galasso C, Orefice I, Pellone P *et al.* On the Neuroprotective Role of Astaxanthin : New Perspectives ? 2018; 1–16.
15. Ytrestoyl T, Bjerkeng B. Intraperitoneal and dietary administration of astaxanthin in rainbow trout (Oncorhynchus mykiss)--plasma uptake and tissue distribution of geometrical E/Z isomers. Comp Biochem Physiol B Biochem Mol Biol 2007; 147: 250–259.
16. Choi HD, Kang HE, Yang SH, Lee MG, Shin WG. Pharmacokinetics and first-pass metabolism of astaxanthin in rats. Br J Nutr 2011; 105: 220–227.
17. Østerlie M, Bjerkeng B, Liaaen-jensen S. <TypeOut Quote 4920.pdf>. 2000; 2863: 482–490.
18. Szasz G, Gerhardt W, Gruber W. Creatine kinase in serum: 3. Further study of adenylate kinase inhibitors. Clin Chem 1977; 23: 1888–1892.
19. Koracevic D, Koracevic G, Djordjevic V, Andrejevic S, Cosic V. Method for the measurement of antioxidant activity in human fluids. J Clin Pathol 2001; 54: 356–361.
20. BEUTLER E, DURON O, KELLY BM. Improved method for the determination of blood glutathione. J Lab Clin Med 1963; 61: 882–888.
21. Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med 1967; 70: 158–169.
22. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979; 95: 351–358.
23. Nishikimi M, Appaji N, Yagi K. The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. Biochem Biophys Res Commun 1972; 46: 849–854.
24. Monnet E, Orton EC. A canine model of heart failure by intracoronary adriamycin injection: hemodynamic and energetic results. J Card Fail 1999; 5: 255–264.
25. Abdel-Daim MM, Kilany OE, Khalifa HA, Ahmed AAM. Allicin ameliorates doxorubicin-induced cardiotoxicity in rats via suppression of oxidative stress, inflammation and apoptosis. Cancer Chemother Pharmacol 2017; 80: 745–753.
26. Swain SM, Whaley FS, Gerber MC *et al.* Cardioprotection with dexrazoxane for doxorubicin-containing therapy in advanced breast cancer. J Clin Oncol 1997; 15: 1318–1332.
27. Xiong C, Wu Y-Z, Zhang Y *et al.* Protective effect of berberine on acute cardiomyopathy associated with doxorubicin treatment. Oncology Letters 15 2018 5721–5729.
28. Tanaka T, Morishita Y, Suzui M, Kojima T, Okumura A, Mori H. Chemoprevention of mouse urinary bladder carcinogenesis by the naturally occurring carotenoid astaxanthin. Carcinogenesis 15 1994 15–19.
29. Tanaka T, Makita H, Ohnishi M, Mori H, Satoh K, Hara A. Chemoprevention of Rat Oral Carcinogenesis by Naturally Occurring Xanthophylls, Astaxanthin and Canthaxanthin. Cancer Research 55 1995 4059–4064.
30. Zhang L, Wang H. Multiple Mechanisms of Anti-Cancer Effects Exerted by Astaxanthin. 2015; 4310–4330.
31. Cho KS, Shin M, Kim S, Lee SB. Review Article Recent Advances in Studies on the Therapeutic Potential of Dietary Carotenoids in Neurodegenerative Diseases. 2018; 2018.
32. Cappetta D, Rossi F, Piegari E *et al.* Doxorubicin targets multiple players: A new view of an old problem. Pharmacol Res 2018; 127: 4–14.
33. Zhang S, Liu X, Bawa-Khalfe T *et al.* Identification of the molecular basis of doxorubicin-induced cardiotoxicity. Nat Med 2012; 18: 1639–1642.
34. Sterba M, Popelova O, Vavrova A *et al.* Oxidative stress, redox signaling, and metal chelation in anthracycline cardiotoxicity and pharmacological cardioprotection. Antioxid Redox Signal 2013; 18: 899–929.
35. Ren X, Bo Y, Fan J *et al.* Dalbergioidin Ameliorates Doxorubicin-Induced Renal Fibrosis by Suppressing the TGF-beta Signal Pathway. Mediators Inflamm 2016; 2016: 5147571.

UNDER PEER REVIEW

36. Kosoko AM, Olurinde OJ, Akinloye OA. Doxorubicin induced neuro- and cardiotoxicities in experimental rats: Protection against oxidative damage by Theobroma cacao Stem bark. Biochem Biophys reports 2017; 10: 303–317.
37. Shaker RA, Abboud SH, Assad HC, Hadi N. Enoxaparin attenuates doxorubicin induced cardiotoxicity in rats via interfering with oxidative stress, inflammation and apoptosis. BMC Pharmacol Toxicol 2018; 19: 3.
38. Preus M, Bhargava AS, Khater AE, Gunzel P. Diagnostic value of serum creatine kinase and lactate dehydrogenase isoenzyme determinations for monitoring early cardiac damage in rats. Toxicol Lett 1988; 42: 225–233.
39. Gross GJ, Hazen SL, Lockwood SF. Seven day oral supplementation with Cardax (disodium disuccinate astaxanthin) provides significant cardioprotection and reduces oxidative stress in rats. Mol Cell Biochem 2006; 283: 23–30.
40. Monroy-Ruiz J, Sevilla MÁ, Carrón R, Montero MJ. Astaxanthin-enriched-diet reduces blood pressure and improves cardiovascular parameters in spontaneously hypertensive rats. Pharmacol Res 2011; 63: 44–50.
41. Binu P, Varghese M V, Alex M, Abhilash S, Vineetha RC, R HN. The Antioxidant Potential of Astaxanthin on Arsenic Trioxide Induced Cardiac Damage in Male Wistar Rats. 2016; 2: 42–48.
42. Nakao R, Nelson OL, Park JS, Mathison BD, Thompson PA, Chew BP. Effect of astaxanthin supplementation on inflammation and cardiac function in BALB/c mice. Anticancer Res 2010; 30: 2721–2725.
43. Nishigaki I, Rajendran P, Venugopal R, Ekambaram G, Sakthisekaran D, Nishigaki Y. Cytoprotective role of astaxanthin against glycated protein/iron chelate-induced toxicity in human umbilical vein endothelial cells. Phytother Res 2010; 24: 54–59.
44. Tsang WP, Chau SPY, Kong SK, Fung KP, Kwok TT. Reactive oxygen species mediate doxorubicin induced p53-independent apoptosis. Life Sci 2003; 73: 2047–2058.
45. Wang S, Konorev EA, Kotamraju S, Joseph J, Kalivendi S, Kalyanaraman B. Doxorubicin Induces Apoptosis in Normal and Tumor Cells via. 2004; 279: 25535–25543.

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