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Protective effect of omega-3 on Doxorubicin-induced hepatotoxicity in male albino rats

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Abstract

Doxorubicin (DOX) is an antineoplastic anthracycline used to treat various forms of cancer. Although DOX is an effective chemotherapeutic agent, it has been documented to cause oxidative damage in several body organs. The present study aimed to investigate the protective effects of omega-3 against doxorubicin-induced hepatic toxicity in adult male rats. Animals were divided into four groups. The first group was orally administered with 0.5ml corn oil and served as a control group. The second group was treated with omega-3 fatty acid (400mg/kg b.w) daily for 30 days. The third group was injected intraperitoneally with a single dose of DOX (30mg/kg b.w). Animals in the fourth group were treated with omega-3 at the same dose level as those of group 2 followed by intraperitoneal injection of a single dose of DOX as in the third group.

Injecting animals with DOX induces various histological changes in the liver. These changes include congestion and dilatation of blood vessels, leucocytic infiltration, cytoplasmic vacuolization, degenerated hepatocytes, and pyknotic nuclei. Moreover, DOX caused a significant elevation in serum ALT, AST, LDH, lipid profile, total bilirubin, total protein, albumin, and globulin after 4 weeks of treatment. It also caused an increase in malondialdehyde (MDA) and depletion of the antioxidant enzymes, catalase (CAT), superoxide dismutase (SOD), and reduced glutathione reduced (GSH). Treating animals with omega 3 fatty acids in combination with DOX led to an improvement in the histological and biochemical changes induced by DOX together with a significant decrease in the level of MDA and an increase in the activity of antioxidant enzymes. The results of the present work indicated that omega-3 fatty acid had a protective effect against liver damage induced by Doxorubicin and this is due to its antioxidant activities.

Key words: Omega 3, Doxorubicin, liver, histology, biochemical parameters

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1- Introduction

Cancer is a genetic disease that develops when one or more cells begin to grow uncontrollably, and this may be due to the activation of oncogenes, and/or inactivation of tumor suppressor genes (American Cancer Society, 2014). Chemotherapeutic drugs are widely used against a variety of human tumors and are given to most of the people, which help to sustain the completion of cancer treatment (El-Sayyad et al., 2009; Sundaram and Sangavai, 2009). However, while they generate an acceptable outcomes in the chemotherapy of some cancers, they also exhibit severe toxicity and undesirable side effects (Minami et al., 2010). Anthracycline antibiotic doxorubicin (DOX) is one of the most useful anticancer agents and is still a cornerstone in the therapy of many carcinoma types including leukemia, lymphoma, breast, lung, ovarian, and liver cancers (Simunek et al., 2009; Kambas et al., 2013). It is isolated from the fungus streptomyces peucetius (youngmok, 2003). Although DOX is an effective chemotherapeutic agent, dosage in patients is limited clinically due to severe cardiac and hepatic toxicity (Saad et al., 2001; Singal et al., 2000; El-Sayyad et al., 2009)

Although the mechanism underlying the severe cytotoxicities of doxorubicin (DOX) is not fully clear, ROS is assumed to be a key factor in the toxicity of DOX, and events controlling this oxidative injury are extensively appreciated (Reddy *et al*., 2007). Increased lipid peroxidation, reduced antioxidants, free radical trapping capacity in plasma, and a marked reduction of tissue Glutathione (GSH) levels are frequently reported during doxorubicin treatment (Goncalves *et al*., 2009).

Many natural products are used today in the therapy of different diseases. Omega-3 is an essential polyunsaturated fatty acid (PUFAs) found in large amounts in fish oil. It contains alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) (Burdge and Calder, 2006). PUFAs EPA and DHA, commonly referred to as marine fatty acids, are most efficiently obtained from fatty cold-water fish such as salmon (Moyad, 2005; Fabian et al., 2015). In fact, these compounds are produced by single-cell marine organisms that are consumed by fish. Ruzickowa et al. (2004) reported that EPA and DHA act as hypolipidemic exert prophylactic effects on cardiovascular disease, protect against insulin resistance and obesity in rodents fed high diet and insulin response to glucose in a healthy human. Polyunsaturated fatty acids (EPA and DHA) play a central role in the normal development and functioning of the brain and central system (Schuchardt et al., 2010). They also have a role in regulating the inflammation which is caused by substances called prostaglandins by producing anti-inflammatory prostaglandin. Moreover, omega-3 is considered as a strong antioxidant and its role as an anticancer agent has been extensively confirmed in most of the human malignancies (Shaikh et al., 2010).

The present work was conducted to study the effect of Omega-3 on the hepatotoxicity of the anticancer drug, doxorubicin in albino rats.

2- MATERIALS AND METHODS

Experimental animal:

Adult Male albino rats (bodyweight of 240±10 gm) were obtained from the Animal House, Medical Technology Center, Research Institute, Alexandria University, Egypt. Animals were housed in plastic cages in an environmentally controlled room (25-27°C, with 12h light/dark cycle) for two weeks prior to starting the experiments for adaptation and they were provided with tap water and a standard rat diet.

Chemicals:

- Doxorubicin(DOX) was purchased from EIMC united pharmaceuticals

- Fish oil omega-3 was purchased from Sigma-Aldrich Chemical (USA)

Experimental design:

Forty male rats were randomly divided into 4 equal groups (10 rats each).

Group 1: Control rats were administrated 0.5ml corn oil by intragastric intubation.

Group 2: The rats were administrated (400mg/kg) fish oil omega-3 fatty acid daily for 30 days by intragastric intubation (Uygur et al., 2014)

Group3: The rats were administrated with 0.5 ml corn oil for 30 days followed by intraperitoneal injection of a single dose of DOX (30mg/kg b.w)

Group4: The rats were administrated (400mg/kg) fish oil omega-3 fatty acid daily for 30 days followed by a single dose of DOX (30mg/kg b.w).

Blood serum analyses

After four weeks, the experimental animals were fasted for 12 h and then anaesthetized with diethyl ether. Blood samples were collected from the dorsal aorta in non–heparinized tubes, centrifuged at 2500 rpm for 15 min and blood sera were then collected and stored at -80^{o}C. Serum samples were used to determine the level of liver enzymes: Alanine aminotransferase (ALT), Aspartate aminotransferase (AST) and lactate dehydrogenase (LDH). The levels of serum ALT, and AST were estimated according to Tietz (1976) whereas LDH was measured using the method of Henry (1974). Determination of the level of triglycerides, total cholesterol, high-density lipoprotein (HDL-C), Low-Density Lipoprotein (LDL), and Very Low Density Lipoprotein (VLDL-C) was carried out according to the method of Fassati and Prencipe (1982), Allain et al. (1974), Fruchart (1982), Assman et al. (1984) respectively. Moreover, levels of total protein& albumin, globulin, bilirubin, and glucose were measured according to (Gornall et al., 1949), (Tietz, 2005), (Young, 2001), (Trinder, 1969) respectively.

Liver tissue collection and preparations

After blood sampling, rats were sacrificed and the Liver of control and treated animals were isolated and washed with saline solution. Portions of liver tissue from rats from each group were processed immediately for the determination of oxidative stress parameters and antioxidants in the liver. Other portions of the liver were also removed from control and treated rats and fixed in formalin 10 % for histopathological examination.

Biochemical analysis in liver tissue

Weighted portions of livers were minced and homogenized in 5-10 ml cold buffer (i.e, 50 mM potassium phosphate, Ph7.4, 1 mM ethylene diamine tetra acetic acid (EDTA). Homogenates were centrifuged at 10,000×g for 20 minutes at 4°C (Goldberg and Spooner, 1983) and the clear supernatants were used for the determination of oxidative stress and antioxidant parameters.

Malondialdehyde (MDA) was determined by the method of Ohkawa et al. (1979), reduced glutathione (GSH) was measured according to Beutler et al. (1963), superoxide dismutase (SOD) was assayed using the method of Rest and Spitznagel (1977) and Catalase (CAT) was determined according to Aebi, (1984).

Histopathological examination

Small pieces of livers were preserved in 10% formalin immediately after removal from the animals for Histological examination. After 24 hours, they placed in 70% ethanol until processing. After fixation, the specimens were dehydrated in ascending grades of ethyl alcohol series until they reached the absolute alcohol. Then, they were transferred to xylene (3 changes, 5 minutes each). Specimens were

transferred to a mixture of melted wax and xylene (1:1) in an oven (60ºC) for 10 minutes, and after that they were transferred through 3 changes of paraffin wax for 2 hours. Finally, the materials were embedded in wax. Sections (5-6µ) were stained with hematoxylin and counterstained with eosin (Bancrof and Steven, 1977).

Statistical analysis

The data were analyzed using the Statistical Package for Social Sciences (SPSS for Windows, version 22.0). Each value is expressed as mean± standard deviation (S.D.) and values were analyzed using a two-way analysis of variance (ANOVA) to determine differences between the mean values of experimental groups. P-values of less than 0.05 were considered significant.

3- Results

Liver functional parameters:

Table (1) shows that the level of AST, ALT, and LDH increased significantly ($p<0.05$) in DOX treated rats as compared to the controls. Administration of omega-3 in combination with DOX resulted in a significant ($p<0.05$) reduction in the level of these enzymes compared to the DOX treated group. Also, serum total bilirubin was significantly increased ($p<0.05$) after DOX injection. Co-administration of omega-3 with DOX significantly reverted back the level of total bilirubin DOX - treated rats.

Table (1): Effect of fish oil omega-3 on liver function in doxorubicin-treated rats.

Parameters	Experimental groups			
	Control	Omega-3	DOX	Omega-3+DOX
Aspartate transaminase (AST) (u/L)	253.8±38.3	206.2±37.3	302.3±27.5[a]	228.8±20.8[ab]
Alanine transaminase (ALT)(u/L)	166.0±36.8	141.6±50.1	323.8±50.0[a]	193.6±51.7[ab]
Lactate dehydrogenx (LDH) (u/L)	1003.0±284.4	713.0±310.0[a]	2014.6±405.6[a]	887.4±380.2[ab]
Total bilirubin(mg/dl)	0.2±0.03	0.2±0.03	0.3±0.03[a]	0.2±0.03[b]

- Values are expressed as means ± SE; n= 6 for each treatment group
a- Means statistically significant compared to control group. P<0.05
b- Means statistically significant compared to DOX-treated group.

Effect of fish oil omega-3 on total protein, albumin, and globulin in the doxorubicin -treated rats

Serum level of total protein, albumin, and globulin were significantly (P<0.05) increased after DOX injection, as compared to normal control (Table2).

Supplementation of omega-3 in combination with DOX caused a significant (P<0.05) decrease in the level of total protein, albumin, and globulin in comparison to the DOX-treated group.

Table (2): Effect of fish oil omega-3 on total protein, albumin, and globulin in the doxorubicin -treated rats

Parameters	Experimental groups			
	Control	Omega-3	DOX	Omega-3+DOX
Total proteins(g/dl)	4.3±0.3	4.4±0.2	5.2±0.2[a]	4.5±0.2[b]
Albumin(g/dl)	2.4±0.3	2.4±0.2	3.4±0.1[a]	2.6±0.2[b]
Globulin(g/dl)	1.1±0.3	1.4±0.3	2.5±0.2[a]	2.1±0.21[ab]

- Values are expressed as means ± SE; n= 6 for each treatment group
a- Means statistically significant compared to control group. P<0.05
b- Means statistically significant compared to DOX-treated group.

Effect of fish oil omega-3 on serum glucose in doxorubicin-treated rats

Figure (1) showed a significant (P<0.05) increase in serum glucose in DOX- treated rats as compared to the control The administration of omega-3 in combination with DOX resulted in a significant (P<0.05) reduction of glucose level when compared to DOX treated group.

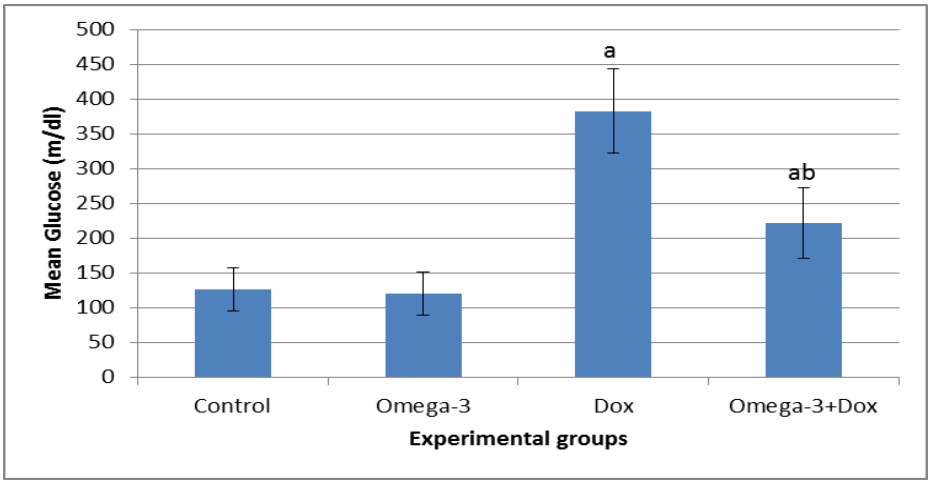

Figure (1): Serum glucose in omega-3, doxorubicin (DOX) & omega-3+doxorubicin (omega-3+DOX) treated rats

a- Means statistically significant compared to the control group. P<0.05
b- Means statistically significant compared to the DOX-treated group.

Effect of fish oil omega-3 on serum lipid profile in doxorubicin-treated rats

DOX administration was associated with a highly significant (P<0.05) increase in lipid profile, triglyceride(TG), total cholesterol (T.Ch), high-density lipoprotein (HDL), low density lipoprotein (LDL), and very low-density lipoprotein(VLDL), as shown in Table (4). Omega-3 treatment in combination with DOX improved these elevations to be decreased toward the normal level.

Table (4): Effect of fish oil omega-3on serum lipid profile in doxorubicin-treated rats.

Parameters	Experimental groups			
	Control	Omega-3	DOX	Omega-3+DOX
Triglyceride(TGs)(mg/dl)	15.2±2.9	17.6±3.5	146.2±11.8[a]	115.4±14.2[ab]
Total cholesterol (mg/dl)	65.4±5.9	54.8±4.98	133.8±17.0[a]	114.6±14.2[ab]
High density lipoprotein (HDL) (mg/dl)	19.2±2.9	18.7±4.3	24.1±4.7[a]	22.6±5.3[ab]
Low density lipoprotein (LDL) (mg/dl)	59.2±2.9	51.9±7.0	90.2±4.7[a]	71.8±5.3[ab]
Very low density lipoprotein(VLDL)(mg/dl)	3.0±0.6	5.5±1.2[a]	19.7±2.2[a]	17.2±1.7[ab]

- Values are expressed as means ± SE; n= 6 for each treatment group
a- Means statistically significant compared to the control group. – P<0.05
b- Means statistically significant compared to the DOX-treated group.

Effect of fish oil omega-3 on oxidative stress and Liver antioxidant activities in liver of doxorubicin-treated rats

The administration of DOX was associated with alterations in the level of both oxidative stress parameters and antioxidant enzyme activities in the liver tissue different experimental groups as shown in Table (5). MDA level of DOX- injected rats revealed a significant (P<0.05) elevation, however, DOX-induced a significant depletion in reduced glutathione (GSH), catalase (CAT), superoxide dismutase (SOD) concentrations. On the other hand, co-administration of DOX with omega-3 restored the values of these altered parameters toward the control group.

Table (5): Effect of fish oil omega-3 on CAT, SOD and GSH in liver of doxorubicin-treated rats.

Parameters	**Experimental groups**			
	Control	**Omega-3**	**DOX**	**Omega-3+DOX**
(MDA) (nmol/g)	16.48±1.49	11.09±1.12[a]	21.94±1.99[a]	17.97±1.34[b]
Glutathione reduced (GSH)liver Tissue (nmol/g)	2.36±0.56	2.49±0.22	1.99±0.18[a]	4.87±0.442[ab]
Catalase(CAT)liver tissue (u/gm)	5.91±0.53	4.80±0.76	3.50±0.33[a]	3.87±0.31[ab]
Superoxide dismutase (SOD)Liver Tissue(U/gm)	1820.23±165.0	1310.46±297.50[a]	1171.71±248.25[a]	1611.20±258.43[ab]

- Values are expressed as means ± SE; n= 6 for each treatment group
a- Means statistically significant compared to control group. – P<0.05
b- Means statistically significant compared to DOX-treated group.

Histological observations

Figure (2A) showed the histological structure of the liver of the control rat. Sections of the control liver demonstrated normal hepatic architecture. The polyhedral hepatocytes jointed to one another in anastomosing cords radiating from the central vein. The hepatocytes contained central nuclei and some were binucleated. The cords of hepatocytes were separated by blood sinusoids which lined by endothelial cells and contained phagocytic kupffer cells in between. Animals administered with omega 3 for 4 weeks showed the same histological observations as in the liver of control animals (Fig 2B).

In animals treated with doxorubicin, liver sections reflected signs of injury as indicated by disrupted histological architecture, degeneration of hepatic cords, irregular cell membrane, congestion and dilation of central vein and blood sinusoids, and some hepatocytes appeared with pyknotic nuclei and vacuolated cytoplasm (Fig2 C&D).

Co-administration of omega-3 with doxorubicin showed a marked improvement in liver architecture (Fig.2E). Slight congestion of blood vessels and few hepatocytes with pyknotic nuclei were observed.

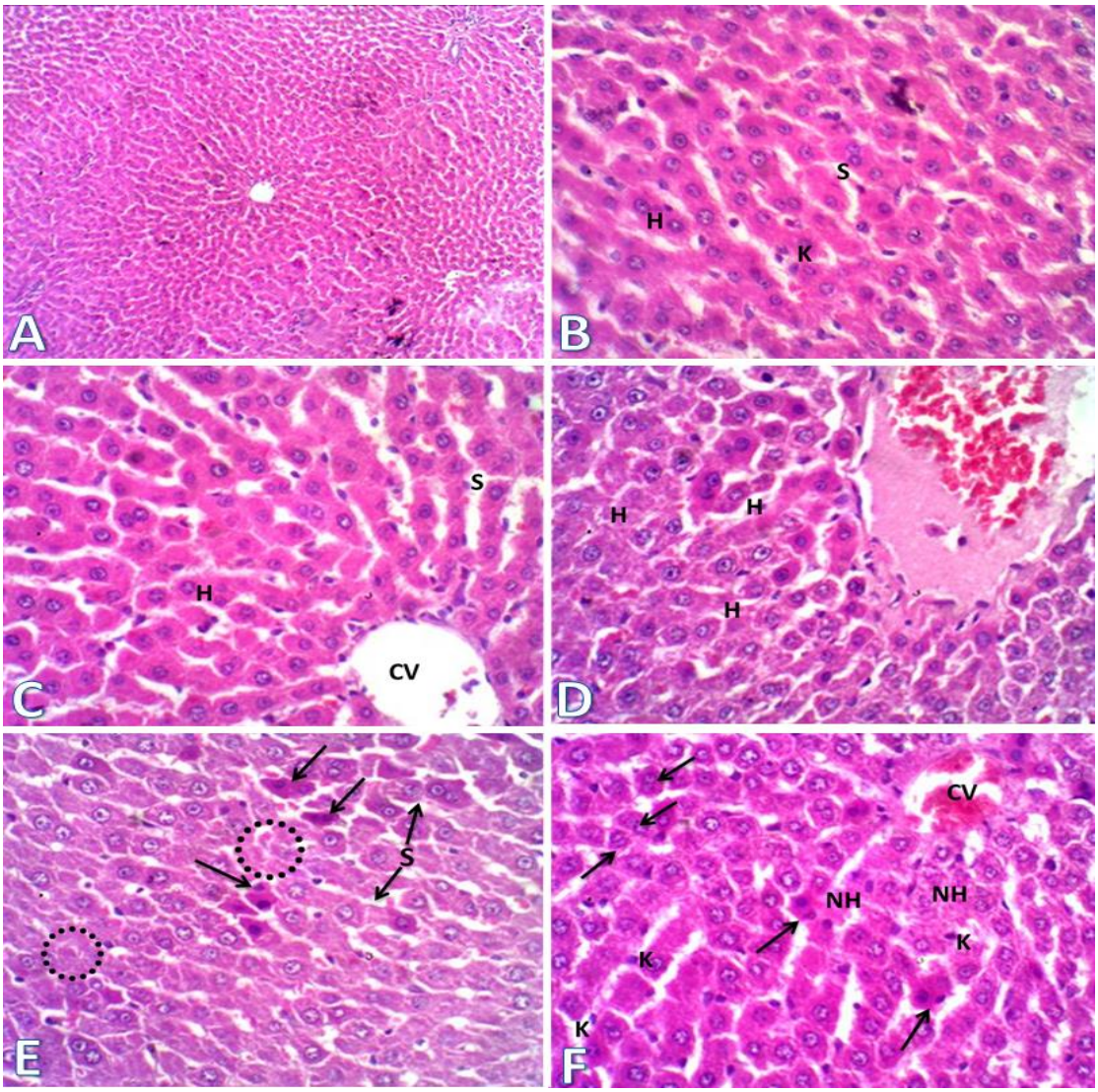

Figure 2 A-F): Light micrographs of sections of the liver of rats. A, Section of control liver showing a normal architecture of hepatic strands and hepatocytes and central vein(X 100). B, Higher magnification of section (A) showing Polyhedral hepatocytes (H) jointed to one another in anastomosing sheathes, their borders face either sinusoids (S) or adjacent cells kupffer cells (K) (×400). C, a section of liver from omega-3 treated rat, showing central vein (CV), normally branched strands of hepatocytes (H) separated by sinusoids (S) (X 400). D&E, sections from DOC- treated rats, showing congested central vein (CV) degenerated hepatocytes (H), hepatic cells with pyknotic nuclei (→), necrotic cells (○), and dilated sinusoids (S) (X 400). F, Section of liver from a rat treated with DOX + omega-3, showing normal structure with numerous binucleated hepatocytes (→) kupffer cells (K) and few necrotic hepatocytes (NH) (X 400).

4- Discussion

The present study indicated the protective effect of fish oil (Omega-3) fatty acids against doxorubicin (DOX) induced hepatic toxicity in male albino rats. Aminotransferases liver enzymes (AST, ALT, LDH) are a marker for clinical diagnosis of DOX liver injury (Hozayen et al., 2014).

In the present study, treatment with DOX has an acute devastating effect on the liver, as exemplified by the increase in the liver enzymes, AST, ALT, LDH that released into the bloodstream as a result of the damage of hepatocytes (Olufunke et al., 2016). **This finding is** in agreement with many authors who showed that DOX-induced significant increases in ALT, AST, and LDH serum levels in rats (Anandakumar *et al*., 2007; Ajith *et al*., 2008). The higher activities of AST and ALT recorded in the present study, could be a result of drastic conditions caused by the toxic activity of DOX accumulation in the liver which provokes cellular destruction and increases the permeability of hepatic cells (Hozayen et al., 2014).

Membrane permeability and transport function are altered by injured hepatocytes, which lead to the leakage of enzymes from the cells to the serum (Mohan et al., 2010). On the other hand, Raskovic et al. (2011) demonstrated an elevation in LDH activity in rats treated with a single cumulative dose of DOX. The increase of LDH concentration may be due to excess production of ROS and less antioxidant defenses.

Moreover, exposure to DOX resulted in a significant increase in total protein, albumin, and globulin in the serum of rats. The finding is in agreement with Olufunke et al., (2016) who found that rats administered with DOX showed a significant increase in serum total protein, albumin, and globulin. Gilleron et al. (2009) reported that, chemotherapeutic treatment with DOX-induced liver and kidney damage and generated peroxynitrite through the mitochondrial production of ROS.

In the present study, DOX-administered rats showed a very highly significant increase in the serum level of glucose concentration. Increasing the level of glucose in DOX-treated rats may be due to hepatotoxicity which affect glucose metabolism (Strigun et al., 2012). The results of Junior et al. (2016) exhibit the strong association between oxidative stress and an increase in glucose uptake in skeletal muscle.

Results of the present study indicated that DOX-administered rats showed a significant increase in serum level of total bilirubin. This is in agreement with Ahmed (2001) who showed that the increase in serum total bilirubin may be due to blockage of bile ducts as a result of the inflammation and fibrosis in the portal triads and/or due to regurgitation of conjugated bilirubin from the necrotic hepatocytes to sinusoids.

The chemoprotective effect of Omega-3 on liver tissue was confirmed by the reduction of the activities of serum AST, ALT, and LDH in addition to the normalization of serum protein and albumin contents. These results are compatible with the results of Attia et al. (2011) who mentioned that the mode of action of Omega-3 can be intercepted pharmacologically at different levels with agents that scavenge free reactive oxygen, block their generation, or heighten endogenous antioxidant capabilities. Meganathan et al. (2011) showed that Omega-3-Fatty acids improved AST, ALT, ALP, and LDH levels, and decreased the production of pro-inflammatory cytokines due to its anti-inflammatory effect.

Also, the treatment of DOX administered rats with omega-3 caused a highly significant decrease in the glucose level in serum as compared to DOX-treated rats. Shariati et al (2011) found that fish oil omega-3 reduced the level of glucose by 50.09%.

In the current work, DOX-treated rats showed a significant increase in serum lipid profile. This result is in agreement with other previous studies carried out by Chen et al. (2007) and Olufunke et al (2016).

They mentioned that serum total cholesterol and triglyceride concentration was significantly increased in the DOX-treated rats. DOX has the ability to modify the chemical composition, structure, and function of biological membranes, mainly at the mitochondrial, fundamentally due to the peroxidation of membrane lipids, leading to the release of protein and cholesterol from the cytosol into the bloodstream (Chen et al., 2007).

Malabade et al. (2014) found that doxorubicin significantly increases the level of triglyceride, HDL, LDL, VLDL. Injury induces by DOX could be mainly mediated through the free radical generation and iron-dependent oxidative damage to the biological macromolecules and membrane lipid peroxidation).

Fish oil omega-3 fatty acids have antioxidants, anti-inflammatory, and antiapoptotic properties Zararsiz et al. (2006). Several studies have demonstrated apparent beneficial effects of fish omega-3 fatty acids on the plasma level of triglycerides, LDL, and HDL cholesterol. The triglyceride-lowering effect of omega-3 fatty acids has been mainly described to reduce the hepatic synthetic of VLDL (Strolien et al. 2007). Omega-3 fatty acids suppressed hepatic lipogenesis and reduced circulating triglyceride levels (Caterina et al., 2007). The decreased hepatic cholesterol content was accounted for higher cholesterol secretion into bile thus leading to a depletion of the intrahepatic pool of cholesterol (Vijaimohan et al., 2006).

In the current study, DOX administered rats showed free radical generation and antioxidant deficit. This is in agreement with Kalender et al. (2005) who studied the effect of doxorubicin on major enzymes participating in free radical metabolism. They found that Superoxide dismutase and catalase activity decreased while malondialdehyde levels increased in the doxorubicin-treated group compared to controls.

On the other hand, our results indicated that fish oil omega-3 has a protective effect against oxidative stress induced by DOX and this is mediated by its antioxidant activities.

Our results corroborate with the data presented by Takayama et al. (2010) who found that hepatic antioxidant enzymes increased, while hepatic lipid peroxide concentrations prominently reduced in rats that were fed with an essential omega-3 rich diet.

Histopathological results showed that DOX-induced many alterations in the liver tissue. These are hepatocyte degeneration, blood vessel congestion, as well as apoptosis, and necrosis. Similar observations were obtained by some investigators. Gokcimen et al. (2007) reported that DOX increased mononuclear cell infiltration, congestion of blood vessels, and necrosis. Also, Atif (2013) found vascular congestion, sinusoidal dilatation, and hepatocyte degeneration after DOX treatment.

On the other hand, treating rats with DOX and omega-3 improved the histopathological changes induced in the liver by DOX alone. This indicated the effectiveness of omega-3 in the prevention of DOX hepatotoxicity. Abdel-Moneim et al. (2011) studied the beneficial effects of the administration of omega-3 in amelioration of DOX hepatotoxicity and showed that the liver of rat treated with omega-3 retained its normal architecture and was also able to diminish congestion and hepatocyte vacuolation and degeneration.

The results of the present work indicated that omega-3 has a protective effect against hepatoxicity induced by DOX and this is mediated by its antioxidant activities.

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