

RESEARCH ARTICLE

Omega-3 fatty acids ameliorate doxorubicin-induced cardiorenal toxicity: *In-vivo* regulation of oxidative stress, apoptosis and renal Nox4, and *in-vitro* preservation of the cytotoxic efficacy

Dalia Saleh¹, Marawan Abdelbaset¹, Azza Hassan², Ola Sharaf¹, Sawsan Mahmoud¹, Rehab Hegazy^{1*}

1 Department of Pharmacology, Medical Division, National Research Centre, Giza, Egypt, **2** Department of Pathology, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt

* rehab_hegazy@hotmail.com



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Abstract

This study examines the protective effects of omega-3 fatty acids (OMG), a frequently used nutritional therapy in cancer patients, against doxorubicin (DOX)-induced acute cardiorenal toxicity in rats, and evaluates the cytotoxic activity of DOX when used with OMG against breast cancer cell line. Five groups of rats were treated for 4 consecutive weeks with vehicle (groups I & II), or OMG (25, 50 or 100 mg/kg/day, po; groups III, IV & V, respectively). After twenty-four hours, the last four groups were injected with DOX (200 mg/kg, ip). In DOX-treated rats, the altered ECG, serum cardiac and renal function biomarkers, and histopathological features indicated the induction of cardiorenal toxicity. Increased oxidative and apoptotic markers in both organs was observed, with elevated renal contents of NADPH-oxidase-4 (Nox4) and renin. OMG pretreatment improved those DOX-induced impairments in a dose-dependent manner, and showed antioxidant and antiapoptotic effects with regulation of renal Nox4 expression. The *in-vitro* study showed preservation of the cytotoxic activity of DOX on MCF7 cell line in the presence of OMG. The data suggests OMG for protection against acute DOX-induced cardiorenal damage without affecting the latter anti-tumor activity. It proposes regulation of oxidative stress, Nox4 activity and apoptosis as contributing protective mechanisms.

1. Introduction

Doxorubicin (DOX) is an antineoplastic drug widely used in clinical practice to treat a wide range of malignancies [1]. However, the clinical use of DOX is restricted by its undesirable side effects, especially cardiotoxicity and nephrotoxicity [2,3]. Oxidative damage to cellular components is believed to be a major factor responsible for DOX-induced toxicity [1,4,5]. Induction of apoptosis is also among the mechanisms involved in DOX-induced toxic effects [6].

The exact mechanism by which DOX treatment differentially affects the cardiac and renal function is obscure [7]. Also, it is incomprehensible whether DOX-induced cardiotoxicity precedes or follows nephrotoxicity. Cardiotoxicity usually appears in a significant dose-dependent manner in the form of congestive heart failure, and it develops within a short period after initiation of therapy in humans. It is characterized by a progressive degeneration of heart muscle [7]. DOX-induced renal toxicity, besides being a part of a multiorgan damage mediated mainly through reactive oxygen species (ROS) formation, it may be mediated directly through accumulation of DOX preferentially in the kidney. Additionally, DOX-induced cardiotoxicity may lead to modulation of blood supply to the kidney and alter the detoxification processes, respectively, thus indirectly contributing to DOX-induced nephropathy [8]. Remarkably, although the reported NADPH oxidase-4 (Nox4)-stimulating activity of DOX [9], and the emerging evidences support the role of Nox4 in different nephropathogenesis [10,11], few data correlates this Nox4-stimulating effect of DOX with its nephrotoxic activity.

Omega-3 fatty acids (OMG) are considered immunonutrients and are frequently used in the nutritional therapy of cancer patients due to their plentiful biological effects, and for alleviation of many cancer-related complications [12]. These fatty acids, including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are incorporated in many parts of the body, including cell membranes, and play a role in cell signaling and the anti-inflammatory and anti-oxidant processes [13,14]. Moreover, they are precursors of more active metabolites that have many beneficial effects in preventing and/or treating several diseases [15], most importantly, the cardiovascular diseases [16–18]. Also, the renoprotective effect of OMG has been also established [19]. Clinical studies suggest that long-term treatment with OMG fatty acids improves renal function and lowers the risk of death or end-stage renal disease [20].

The present study investigated the potential protective effects of OMG against DOX-induced cardiorenal toxicity in rats. It evaluated some possible underlying mechanisms that may be involved in the DOX toxicity and OMG protective effects, including oxidative stress, apoptosis, and renal Nox4 pathways. The study also assessed the cytotoxic activity of DXO *in vitro* for any interaction that may hinder its anti-cancer activity when combined with OMG.

2. Materials and methods

2.1. Drugs and chemicals

Doxorubicin (DOX), Sulforhodamine-B (SRB) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (USA). Fish oil omega-3 fatty acids (OMG) were obtained from BIOVEA (Egypt); each 100 ml of this fish oil provides 95 mg of total OMG comprising 18 mg EPA, 12 mg DHA, and other fatty acids. Dulbecco's Modified Eagle Medium (DMEM medium), penicillin-streptomycin ($\times 100$), 0.25% trypsin-EDTA, phosphate buffered saline (PBS) and fetal bovine serum were purchased from Lonza Group Ltd. (Switzerland). All other chemicals were of the highest available analytical grade.

2.2. Animals

Adult female Wistar albino rats weighing 180–200 g were used in this study. They were obtained from the Animal House Colony of the National Research Center (NRC, Egypt). Animals were kept under standardized conditions with a 12-h light and dark cycle, and were allowed food and tap water ad libitum. They were treated according to the national and international ethics guidelines stated by the ethics committee of NRC. As well, this study has been approved by the ethics committee of NRC, and all procedures and experiments were performed according to a protocol approved by it.

2.3. Cell culture

Human breast cancer cell line MCF7 was obtained from VACSERA (Cairo, Egypt). Cells were maintained in DMEM media supplemented with 100 mg/mL of streptomycin, 100 units/mL of penicillin and 10% of heat-inactivated fetal bovine serum in humidified, 5% (v/v) CO₂ atmosphere at 37°C.

2.4. Experimental design

Rats were allocated into five groups each consisting of 7–8 animals. Treatments were orally administered for 4 consecutive weeks as follows: Groups I and II received distilled water, while groups III, IV and V received OMG (25, 50 and 100 mg/kg/day, p.o.), respectively. Twenty-four hours after the last doses of OMG, each rat of groups II, III, IV and V was given a single intraperitoneal injection of DOX (200 mg/kg).

2.5. Electrocardiographic (ECG) measurements

Forty-eight hours after DOX injection, the body weight for each of the animals was measured and the rats were anaesthetized with thiopental (5 mg/kg, i.p.) and kept warmed with a heating lamp to prevent the incidence of hypothermia. Subcutaneous peripheral limb electrodes were inserted for ECG recording (HPM 7100, Fukuda Denshi, Japan) for 1 min. Heart rate, P duration, QTc, and ST Height were monitored using ECG Powerlab module which consists of Powerlab/8sp and Animal Bio-Amplifier (Australia), in addition to Lab Chart 7 software with ECG analyzer [21,22].

2.6. Serum collection and tissue preparation

After ECG recording, blood samples were collected from the retro orbital sinus using heparinized capillary tubes under *ip* anesthesia with ketamine–xylazine (K, 100 mg/kg; X, 10 mg/kg) [23] for serum separation. The rats were then sacrificed by cervical dislocation under the same anesthesia, the heart and both kidneys of each animal were rapidly isolated, washed with ice-cold saline, dried and weighted. Then, weighed parts from all organs were homogenized in cold buffer, using a homogenizer (Heidolph DiAx 900, Germany) to prepare 10% homogenate. The serum and homogenates were used for biochemical assays.

2.7. Serum and tissue biochemical analysis

Sera stored at –20°C were used for the estimation of serum cardiac and renal injury biomarkers such as creatine kinase-myocardial band (CK-MB or CK for short) [24], urea [25], and creatinine [26]. A highly sensitive quantification of CRP (hsCRP) in serum was also performed using a specific rat enzyme-linked immunosorbent assay (ELISA) kit (Biovendor, Czech Republic) as per the manufacturer's instructions.

Cardiac and renal tissues homogenate were used for determination of lipid peroxidation measured as malondialdehyde (MDA) besides reduced glutathione (GSH), as oxidative stress markers, using specific diagnostic kits (Biodiagnostic, Egypt). Cardiac concentrations of atrial natriuretic peptide (ANP) and interleukin-6 (IL-6), as well as renal contents of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-4 (Nox4) and renin were measured using the specific rat ELISA kits (Biovendor, Czech Republic) according to the manufacturer's instructions. The data were normalized to tissue weight by multiplying by a dilution factor used in the preparation of the tissue homogenates.

2.8. Histopathological examination

Tissue specimens from the heart and kidneys of control and treated rats were fixed in 10% neutral buffered formalin and routinely processed. Paraffin embedded tissues were cut into 4 μ m thick sections and stained with hematoxylin and eosin (H&E) for routine histopathological examination. A total of ten fields per group were blindly examined. For assessment of cardiotoxicity, the cardiac tissue sections were stained with Toluidine blue for demonstration of cardiomyocyte vacuolation characteristic of doxorubicin-induced cardiomyocyte injury [27]. Cardiomyocyte vacuolation was semiquantitatively graded on a score scale ranged from 0 to 3, based on the percentage of cardiomyocytes displaying vacuolization of sarcoplasm, in which score 0 denote no vacuolation; score 1 denote minimal grade (< 5%); score 2 denote moderate grade (5–30%) and score 3 denote severe grade (>35%).

For assessment of nephrotoxicity, a scoring scale graded from 0 to 4 was used according to the method described by Herman et al, 1988, in which 0 = the tissue appeared normal, 1 = minimal lesion, 2 = mild lesion, 3 = moderate lesion and 4 = severe lesion. The histopathological criteria used in evaluation of renal lesions were tubular degeneration and /or necrosis, tubular dilatation, deposition of renal cast in the tubular lumina and interstitial inflammatory cell infiltrates.

2.9. Immunohistochemical investigation

For Caspase 3 and tumor protein P53 immune staining, the paraffin-embedded sections were dewaxed, rehydrated and incubated in 3% hydrogen peroxide for inhibition of endogenous peroxidase. The sections were then incubated with rabbit anti-cleaved caspase 3 (Abcam, 1:100 Cambridge, MA, USA) and mouse monoclonal anti-P53 (Ventana) as the primary antibodies. Staining was visualized with DAB. Cells displaying brown cytoplasm and/or nuclei were considered positive. The immunohistochemical staining was graded on three point scoring system ranging from 0 to 2, depending on the distribution of area involved, in which score 0 represent >30% of area involvement, score 1 represent 30% - 60% and score 2 represent <60% [28].

2.10. Cytotoxicity assay

To study the effect of OMG on the cytotoxic profile of DOX, the dose response curve of DOX alone was assessed relative to its combination with omega 3 in MCF7 line. Cell viability was assessed by sulforhodamine B (SRB) assay in three replicates. Aliquots of 100 μ L cell suspension were seeded at 2×10^3 – 5×10^3 cells per well in 96-well plates and incubated in complete media for 24 h. Cells were treated with another aliquot of 100 μ L media containing DOX, OMG and their equimolar combinations at various concentrations (0.01, 0.1, 1, 10 & 100 μ M). After 72 h of drugs exposure, cells were fixed by replacing media with 150 μ L of 10% TCA and incubation at 4°C for 1 h. The TCA solution was removed, and the cells were washed 5 times with distilled water. Aliquots of 70 μ L SRB solution (0.4% w/v) were added and incubated in a dark place at room temperature for 10 min. Plates were washed 3 times with 1% acetic acid and allowed to air-dry overnight. Then, 150 μ L of Tris-HCl (10 mM) was added to dissolve protein-bound SRB stain; the absorbance was measured at 540 nm using a microplate reader.

2.11. Statistical analysis

Data is presented as mean + S.E. Statistical analysis of the data was carried out using one way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test to judge the difference between the various groups. Statistical significance was acceptable to a level of

$p < 0.05$. Data analysis was accomplished using the software program GraphPad Prism, version 5 (GraphPad Software, USA).

Statistical analysis of IC50 values were calculated from concentration-response curves by Sigma Plot software, version 12.0 (System Software, USA), using an E-max model equation:

$$\% \text{Cell viability} = (100 - R) \times (1 - ((D)^m / (K^d_m + (D)^m))$$

Where (R) is the residual unaffected fraction (the resistance fraction), (D) is the drug concentration used, (K^d) is the drug concentration that produces a 50% reduction of the maximum inhibition rate and (m) is a Hill-type coefficient. IC50 was defined as the drug concentration required to reduce absorbance to 50% of that of the control (i.e., $K^d = \text{IC50}$ when $R = 0$ and $E_{\text{max}} = 100 - R$).

3. Results

Effect of OMG on heart and kidneys relative weights

DOX did not alter the body weight (BW), heart (HW) and kidneys weight (KW) significantly and, subsequently, did not alter their relative weights. Oral treatment of DOX-injected rats with OMG also did not show any alteration in the tissues' weights (Table 1).

3.2. Effect of OMG on electrocardiographic measurements

The intra-peritoneal injection of DOX induced bradycardia with elongation of R-R interval, accompanied by elongation of corrected QTc, elevation of ST height and shortening of T amplitude as compared with normal group. However, treatment of DOX-injected rats with OMG (25, 50 or 100 mg/kg; p.o.) showed a marked dose-dependent ameliorative effect on QTc, ST height and T amplitude as compared to the positive control group (Fig 1 and Table 2).

3.3. Effect of OMG on biochemical assessments

Induction of cardiac toxicity with DOX has been noticed by the marked elevation in the serum CK level as compared to the normal control group by about 2.4 folds. Oral administration of OMG (25, 50 or 100 mg/kg) for 4 consecutive weeks to DOX-injected rats showed a suppression of the CK level by 25%, 27% and 57%, respectively, in a dose dependent manner. However, renal toxicity has been observed after DOX intraperitoneal injection by significance

Table 1. Effect of OMG on body weight, heart weight, kidney weight and their relative weight in DOX-treated rats.

Parameters	BW (g)	HW (g)	KW (g)	RHW	RKW
Groups					
Normal	119.2±5.93	0.488±0.032	1.072±0.05	0.413±0.02	0.00906±0.0003
DOX	107.0±3.97	0.626±0.020	1.078±0.12	0.589±0.04	0.009913±0.0011
DOX+OMG (25mg/kg)	101.4±11.60	0.394±0.074 [@]	0.916±0.05	0.417±0.09	0.009532±0.0009
DOX+OMG (50mg/kg)	122.0±5.06	0.508±0.021	0.85±0.01	0.417±0.02	0.007061±0.0012
DOX+OMG (100 mg/kg)	123.8±3.46	0.550±0.017	1.09±0.05	0.453±0.02	0.009006±0.0006

Cardiorenal toxicity were induced in rats by a single intraperitoneal injection of doxorubicin (DOX; 200 mg/kg, i.p) after 28 consecutive days of oral administration of omega 3 (OMG; 25, 50 and 100 mg/kg) fatty acids. Forty-eight hours after DOX injection, rats were weighed (BW) and their hearts (HW) and renal (RW) were also weighed after scarification. The relative heart (RHW) and kidney (KHW) weights to BW were calculated. Results are the mean ± SEM. Statistical analyses were carried out using ANOVA followed by Tukey's multiple comparisons test.

@, $P < 0.05$ compared with the DOX treated rat.

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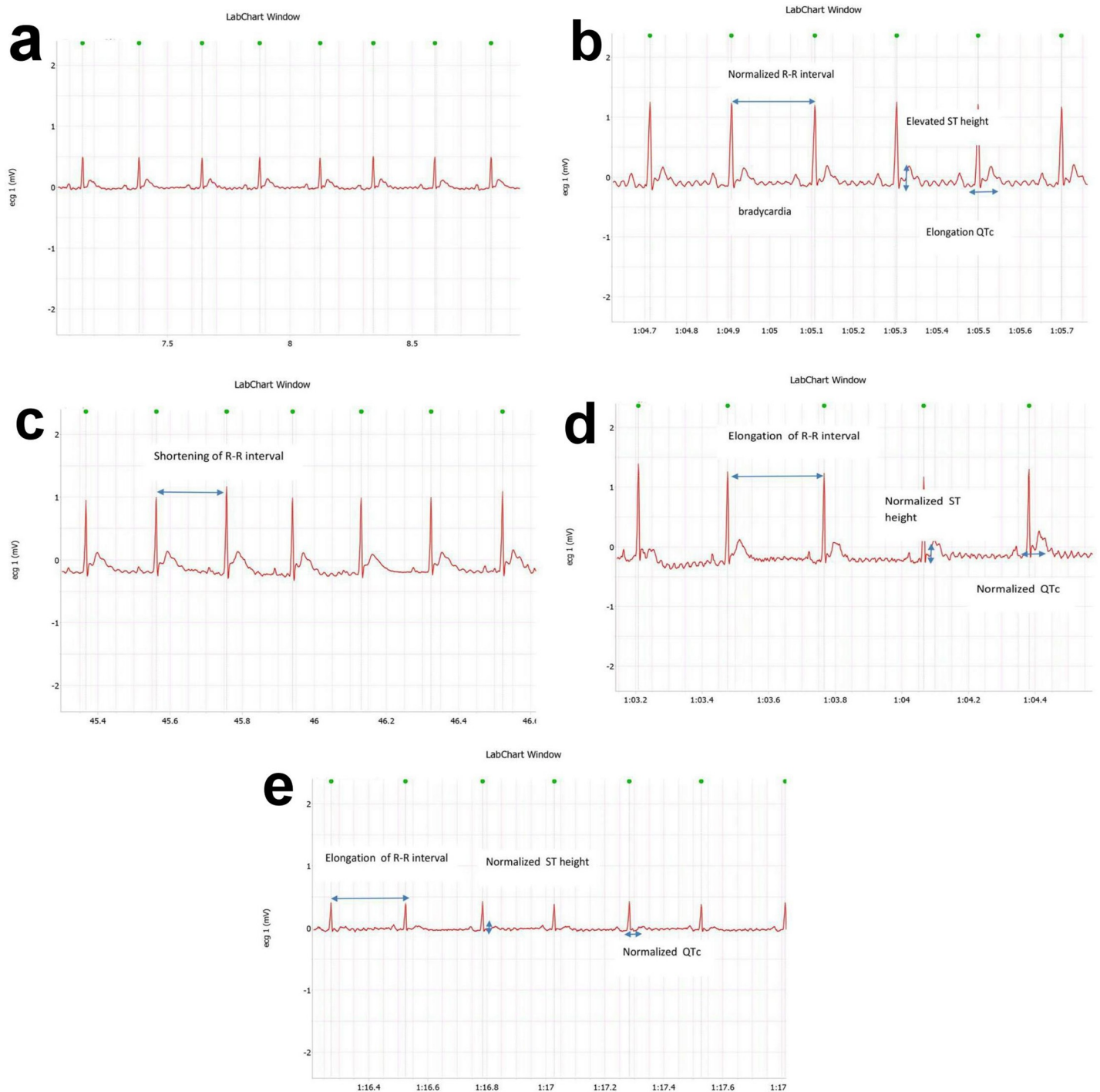


Fig 1. Effect of OMG fatty acids on ECG patterns in DOX-treated rats. Cardiorenal toxicity were induced in rats by a single intraperitoneal injection of doxorubicin (DOX; 200 mg/kg,i.p) after 28 consecutive days of oral administration of omega 3 (OMG; 25, 50 and 100 mg/kg) fatty acids. Forty-eight hours after DOX injection, rats were anesthetized and the electrocardiography (ECG) was recorded for 1 min. (a) showing part of ECG of normal control group (b) showing part of ECG of DOX group with marked bradycardia, elongation of R-R interval, elongation of QTc interval, elevated ST height and depressed T amplitude as compared to normal control group (c) showing part of ECG of DOX+OMG 25 mg/kg group with normalized R-R interval, ST height and elongation of QTc interval (d) showing part of ECG of DOX +OMG 50 mg/kg group with elongated R-R interval and ameliorated of both QTc interval and ST height (e) showing part of ECG of DOX+OMG 100 mg/kg group with elongated R-R interval and bettered of both QTc interval and ST height.

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Table 2. Effect of OMG on R-R interval, heart rate, QRS interval, QTc duration, P duration, ST height and T amplitude of DOX-treated rats.

Groups	Parameters	ECG Parameters					
		R-R	Heart Rate (bpm)	QTc duration (s)	P amplitude (mV)	ST Height (mV)	T amplitude (mV)
Normal		0.18±0.008	312.8±9.56	0.11±0.0065	0.056±0.004	0.059±0.007	0.25±0.009
DOX		0.23±0.002*	253.4±3.10*	0.19±0.0071*	0.065±0.010	0.123±0.022*	0.07±0.019*
DOX+OMG 25 mg/kg		0.17±0.006 [@]	352.7±11.87 [@]	0.18±0.010*	0.051±0.010	0.064±0.013 [@]	0.16±0.0276
DOX+OMG 50 mg/kg		0.25±0.008*	245.2±8.36*	0.13±0.009 [@]	0.053±0.037	0.0734±0.012 [@]	0.19±0.044
DOX+OMG 100 mg/kg		0.25±0.008*	242.8±8.97*	0.14±0.014 [@]	0.059±0.014	0.029±0.009 [@]	0.17±0.048

Cardiorenal toxicity were induced in rats by a single intraperitoneal injection of doxorubicin (DOX; 200 mg/kg,i.p) after 28 consecutive days of oral administration of omega 3 (OMG; 25, 50 and 100 mg/kg) fatty acids. Forty-eight hours after DOX injection, the body mass for each of the animals was measured and the rats were anaesthetized with thiopental and ECG recording for 1 minute. Heart rate, P duration, QTc, and ST Height were monitored.

Results are the mean ± SEM. Statistical analyses were carried out using ANOVA followed by Tukey's multiple comparisons test.

*, P < 0.05 compared with the normal control

@, P < 0.05 compared with the DOX treated rats.

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incrimination of serum levels of urea and creatinine by 2 and 2.6 folds respectively, as compared to normal control group. Administration of OMG (25, 50 and 100 mg/kg) for 4 consecutive weeks succeed to alleviate the serum levels of urea and creatinine by about 24%, 40% and 44%, respectively and 36%, 46% and 59%, respectively (Table 3).

An elevation in level of lipid peroxidation evidenced by MDA was noticed in both cardiac and renal tissues in DOX-treated rats by about 2 and 1.73 folds, respectively. On the other hand, the cardiac and renal levels of GSH was reduced in DOX-treated rats by about 37% and 53%, respectively. Rats treated with OMG (25, 50 and 100 mg/kg) daily for 28 consecutive days showed a reduction in the cardiac MDA level by about 26%, 45% and 40%, respectively and a reduction in the renal MDA level by about 19%, 21% and 39%, respectively as compared to DOX-treated group. Moreover, rats treated with OMG (25, 50 and 100 mg/kg) exhibited an elevation in the cardiac and renal GSH levels by about 29%, 37% and 45%, respectively and by about 31%, 56% and 76%, respectively relative to DOX-treated group (Table 4).

Doxorubicin injection was accompanied by a marked elevation of cardiac ANP levels by 3 folds as well as elevation of cardiac IL-6 levels by about 8 folds as compared to normal control

Table 3. Effect of OMG on serum cardiac and renal function parameters in DOX-treated rats.

Groups	Parameters	Serum CK-MB (U/l)	Serum Urea (g/dl)	Serum Cr (mg/dl)
Normal		28.11±2.85	13.11±1.03	17.33±1.78
DOX		67.13±0.54*	25.66±1.09*	62.66±5.26*
DOX+OMG (25mg/kg)		50.30±4.13 [@]	19.55±1.1 [@]	40.33±3.75 [@]
DOX+OMG (50mg/kg)		48.92±4.39 [@]	15.32±1.26 [@]	34.08±3.12 [@]
DOX+OMG (100 mg/kg)		35.58±2.65 [@]	14.45±0.97 [@]	25.84±2.18 [@]

Cardiorenal toxicity were induced in rats by a single intraperitoneal injection of doxorubicin (DOX; 200 mg/kg,i.p) after 28 consecutive days of oral administration of omega 3 (OMG; 25, 50 and 100 mg/kg) fatty acids. Forty-eight hours after DOX injection, blood samples were collected and sera were separated for determination of creatine kinase-myocardial band (CK-MB), urea, and creatinine (Cr). Results are the mean ± SEM. Statistical analyses were carried out using ANOVA followed by Tukey's multiple comparisons test.

*, P < 0.05 compared with the normal control

@, P < 0.05 compared with the DOX treated rats.

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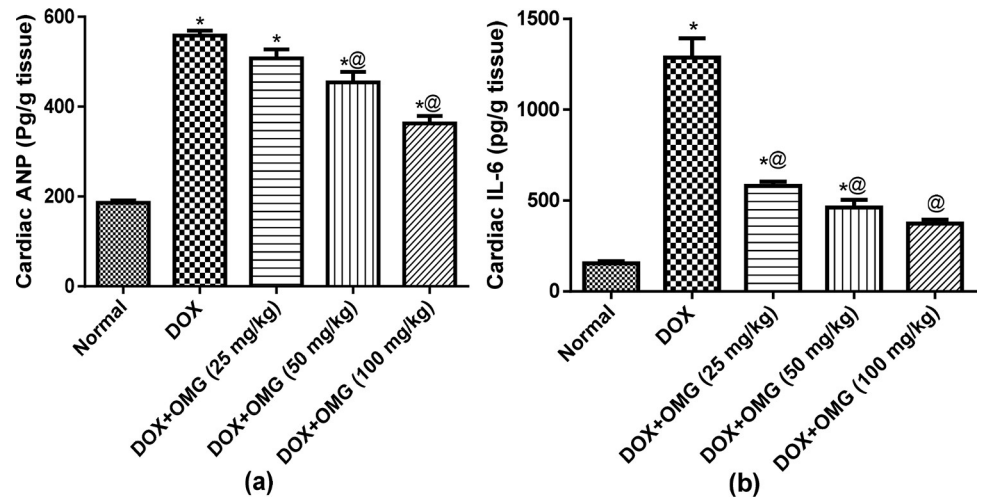


Fig 2. Effect of OMG on cardiac IL-6 (a) and ANP (b) in DOX-treated rats. Cardiorenal toxicity were induced in rats by a single intraperitoneal injection of doxorubicin (DOX; 200 mg/kg,i.p) after 28 consecutive days of oral administration of omega 3 (OMG; 25, 50 and 100 mg/kg) fatty acids. Forty-eight hours after DOX injection, the rats were scarified and the heart was isolated, homogenized, and the levels of (a) atrial natriuretic peptide (ANP) and (b) interleukin-6 (IL-6) were measured in the homogenate. Results are the mean \pm SEM. Statistical analyses were carried out using ANOVA followed by Tukey's multiple comparisons test. *, $P < 0.05$ compared with the normal control; @, $P < 0.05$ compared with the DOX treated rats.

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group. Oral administration of OMG in its three dose levels showed a suppression of the cardiac IL-6 level by about 55%, 64% and 71%, respectively. Similarly, OMG (50 and 100 mg/kg) decreased the cardiac ANP level by about 17% and 35% (Fig 2). Similarly, the serum level of hs-CRP has been elevated by about 3 folds in DOX-treated rats while the rats treated with OMG (25, 50 and 100 mg/kg) for 28 consecutive days showed a suppression of the serum level of hs-CRP by about 40%, 59% and 62%, respectively as compared to the DOX-treated group (Fig 3).

Induction of renal toxicity by DOX was associated with a significant elevation in renal Nox4 levels by about 50% as compared to the normal control group. However, this elevation has been ameliorated in a dose dependent manner in the groups treated daily with OMG (25, 50 and 100 mg/kg) for 28 consecutive days by about 11%, 13% and 18%, respectively (Fig 4A). On the other hand, DOX-treated group showed a prominent elevation in renal renin levels by 3 folds while treatment with OMG (25, 50 and 100 mg/kg) for 28 consecutive days declined renal renin levels by 36%, 43% and 46%, respectively in a dose dependent manner (Fig 4B).

3.4. Effect of OMG on histopathological examinations

The mean pathologic score recorded in the heart and kidneys of control and treated rats are illustrated in Table 5.

Kidneys of normal control rats revealed normal histological structure of renal parenchyma with normal morphology of renal glomeruli and tubules (Fig 4A). In contrast, kidneys of DOX-treated rats revealed severe histopathological alterations particularly in the renal cortex. Glomeruli revealed glomerular congestion associated with presence of eosinophilic flocculent material in Bowman's space (Fig 4B). Renal tubules particularly proximal convoluted one revealed tubular nephrosis with extensive necrosis of their epithelial lining (Fig 4C) and intra luminal aggregation of cellular and protein cast (Fig 4D). Mild intertubular leukocytic cell infiltration (Fig 4E) and tubular dilatation were also demonstrated. Treatment with OMG

Table 4. Effect of OMG on cardiac and renal oxidative stress biomarkers in on DOX-treated rats.

Parameters	Cardiac MDA (nmol/g tissue)	Renal MDA (nmol/g tissue)	Cardiac GSH (mg/g tissue)	Renal GSH (mg/g tissue)
Normal	26.36±1.02	33.97±0.87	61.11±1.54	18.03±0.55
DOX	54.96±1.97*	58.97±0.56*	38.56±0.89*	8.58±0.18*
DOX+ OMG (25mg/kg)	40.86±1.65* [@]	47.59±0.16* [@]	49.93±1.64* [@]	11.24±0.19* [@]
DOX+ OMG (50mg/kg)	30.12±0.95 [@]	46.09±2.22* [@]	52.68±1.71* [@]	13.38±0.45* [@]
DOX+ OMG (100 mg/kg)	33.41±2.54 [@]	35.89±2.67 [@]	56.05±1.83 [@]	15.11±0.47* [@]

Cardiorenal toxicity were induced in rats by a single intraperitoneal injection of doxorubicin (DOX; 200 mg/kg,i.p) after 28 consecutive days of oral administration of omega 3 (OMG; 25, 50 and 100 mg/kg) fatty acids. Forty-eight hours after DOX injection, the rats were scarified and the heart and kidneys were isolated, homogenized, and the malondialdehyde (MDA) and reduced glutathione (GSH) contents were measured in the homogenate. Results are the mean ± SEM. Statistical analyses were carried out using ANOVA followed by Tukey’s multiple comparisons test.

*, P< 0.05 compared with the normal control
 @, P< 0.05 compared with the DOX treated rats.

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revealed improvement at all doses level with most the significant improvement recorded in OMG (100mg/kg) +DOX group. Treatment with OMG (25mg/kg) +DOX revealed mild vacuolar degeneration of the surrounding renal tubules with intra luminal aggregation of renal cast (Fig 4F). Remarkable amelioration of the histopathological lesions was demonstrated in OMG

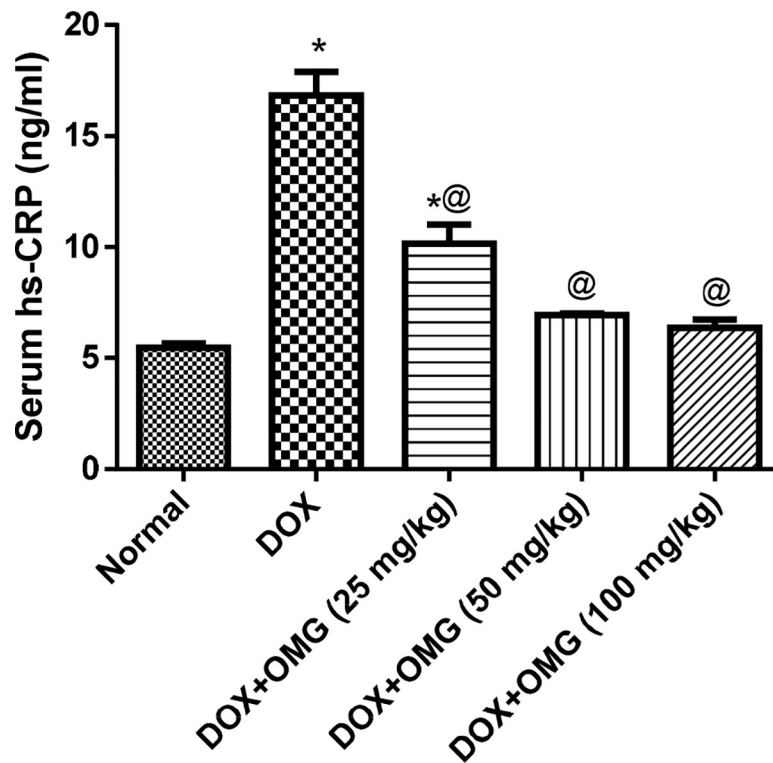


Fig 3. Effect of OMG on serum hs-CRP in DOX-treated rats. Cardiorenal toxicity were induced in rats by a single intraperitoneal injection of doxorubicin (DOX; 200 mg/kg,i.p) after 28 consecutive days of oral administration of omega 3 (OMG; 25, 50 and 100 mg/kg) fatty acids. Forty-eight hours after DOX injection, blood samples were collected and sera were separated for determination of highly sensitive quantification of CRP (hs-CRP). Results are the mean ± SEM. Statistical analyses were carried out using ANOVA followed by Tukey’s multiple comparisons test. *, P< 0.05 compared with the normal control; @, P< 0.05 compared with the DOX treated rats.

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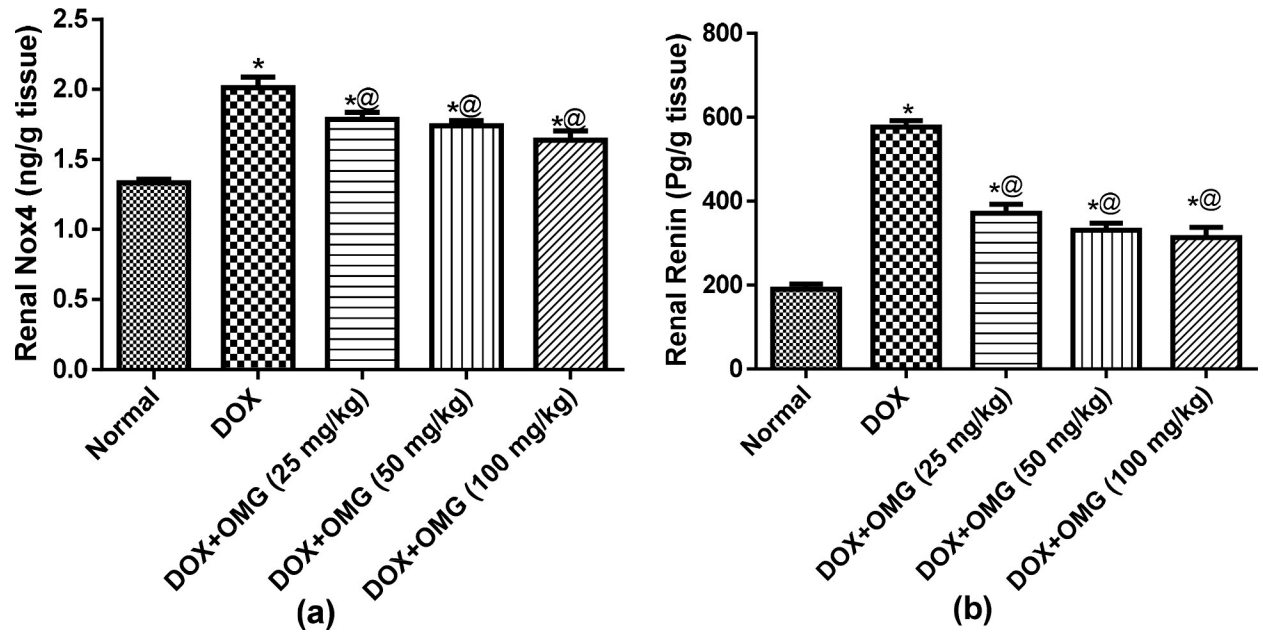


Fig 4. Effect of OMG on renal Nox4 (a) and renin (b) in DOX-treated rats. Cardiorenal toxicity were induced in rats by a single intraperitoneal injection of doxorubicin (DOX; 200 mg/kg,i.p) after 28 consecutive days of oral administration of omega 3 (OMG; 25, 50 and 100 mg/kg) fatty acids. Forty-eight hours after DOX injection, the rats were scarified and the kidneys were isolated, homogenized, and the levels of (a) NADPH oxidase 4 (Nox4) and (b) renin were measured in the homogenate. Results are the mean \pm SEM. Statistical analyses were carried out using ANOVA followed by Tukey's multiple comparisons test. *, $P < 0.05$ compared with the normal control; @, $P < 0.05$ compared with the DOX treated rats.

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(50mg/kg) +DOX and OMG (100mg/kg) +DOX, respectively, in which the renal tubular degeneration was confined to individual cells (Fig 4G and 4H, respectively).

Heart of control rats revealed normal cardiomyocyte with compact symmetrically arranged myofibrils and no evidence of sarcoplasmic vacuolization (Figs 5A and 6A). While, heart of DOX-treated rats revealed expanded vacuolization of cardiomyocyte sarcoplasm associated

Table 5. Effect of OMG on cardiac and renal pathologic score of DOX-treated rats.

Groups	Parameters	Cardiac Pathologic score	Renal Pathologic score
Normal		0.20 \pm 0.13	0.30 \pm 0.15
DOX		2.60* \pm 0.16	3.20* \pm 0.24
DOX+OMG (25mg/kg)		2.20* \pm 0.20	2.30* \pm 0.42
DOX+OMG (50mg/kg)		1.70 \pm 0.21	1.80 \pm 0.32
DOX+OMG (100mg/kg)		1.40 \pm 0.16	1.30 \pm 0.21

Cardiorenal toxicity were induced in rats by a single intraperitoneal injection of doxorubicin (DOX; 200 mg/kg,i.p) after 28 consecutive days of oral administration of omega 3 (OMG; 25, 50 and 100 mg/kg) fatty acids. Forty-eight hours after DOX injection, the rats were scarified and the heart and renal were isolated. Tissue specimens from the kidneys and heart of control and treated rats were fixed in 10% neutral buffered formalin and routinely processed. Paraffin embedded tissues were cut into 4 μ m thick sections and stained with H&E for routine histopathological examination. A total of ten fields per group were examined. For assessment of nephrotoxicity, a scoring scale graded from 0 to 4. Results are the mean \pm SEM. Statistical analyses were carried out using ANOVA followed by Tukey's multiple comparisons test.

*, $P < 0.05$ compared with the normal control

@, $P < 0.05$ compared with the DOX treated rats.

<https://doi.org/10.1371/journal.pone.0242175.t005>

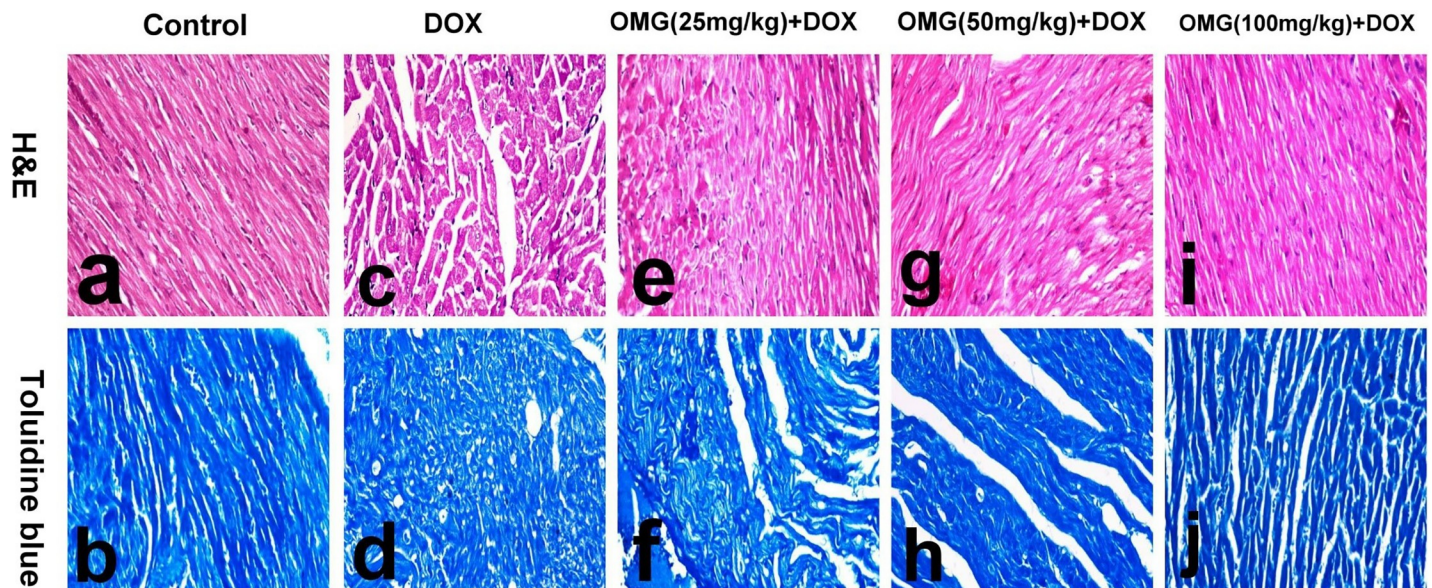


Fig 5. The findings of histopathological analysis of cardiac tissues of the rats. (a,b) normal rats showing normal cardiomyocyte (a) with no evidence of sarcoplasmic vacuolation (b), (c,d) DOX- treated rats showing vacuolization of cardiomyocyte, (e,f) OMG (25mg/kg)+DOX showing individual necrosis of cardiomyocyte with intensely eosinophilic cytoplasm (e) and cytoplasmic vacuolization (f), (g,h) OMG (50mg/kg)+DOX showing mild and focal vacuolation of cardiomyocyte and (i,j) OMG (100mg/kg)+DOX showing normal cardiomyocyte (i) with vacuolation of individual cardiomyocytes (j). (H&E for a, c, e, g, and i and Toluidine blue for b, d, f, h and j, 40X). DOX, doxorubicin, OMG, omega 3 fatty acids.

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with loss of striations and individual necrosis of cardiomyocyte (Figs 5B and 6B). Mild regression of histopathological lesions was demonstrated in OMG (25mg/kg) +DOX 0.25 group with individual necrosis of cardiomyocyte and cytoplasmic vacuolization (Figs 5C and 6C).

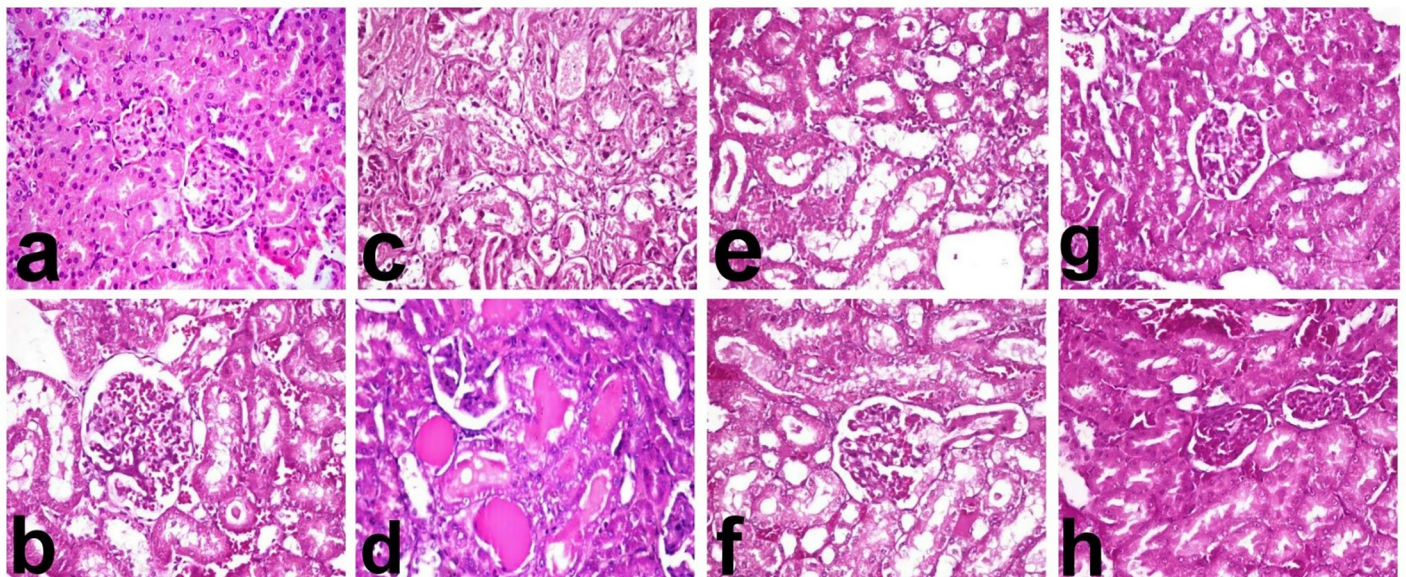


Fig 6. The findings of histopathological analysis of renal tissues of the rats. Kidneys of (a) normal rats showing normal renal glomeruli and tubules, (b, c, d) DOX- treated rats showing glomerular congestion (b), extensive necrosis of their epithelial lining (c), intra luminal aggregation of cellular and protein cast (d), and mild intertubular leukocytic cell infiltration (e), (f) OMG (25mg/kg)+DOX showing mild vacuolar degeneration of the surrounding renal tubules with intra luminal aggregation of renal cast, (g) OMG (50mg/kg)+DOX showing vacuolar degeneration of individual cells and (h) OMG (100mg/kg)+DOX showing normal renal tubules. (H&E, 40X). DOX, doxorubicin, OMG, omega 3 fatty acids.

<https://doi.org/10.1371/journal.pone.0242175.g006>

Table 6. Effect of OMG on cardiac and renal activated caspase 3 and P53 in DOX-treated rats.

Groups	Parameters	Activated caspase 3 (% of stained area)		P53 (% of stained area)	
		Heart	kidneys	Heart	kidneys
Normal		0.00±0.00	0.00±0.00	0.00±0.00	0.00 ^a ±0.00
DOX		2.00*±0.00	1.60*±0.24	1.60*±0.24	1.60*±0.24
DOX+OMG (25mg/kg)		1.40* [@] ±0.24	1.40* [@] ±0.37	1.20* [@] ±0.37	1.24* [@] ±0.37
DOX+OMG (50mg/kg)		1.00 [@] ±0.31	1.00 [@] ±0.37	0.60 [@] ±0.24	0.80 [@] ±0.37
DOX+OMG (100 mg/kg)		0.60 [@] ±0.24	0.20 [@] ±0.20	0.40 [@] ±0.24	0.40±0.24

Cardiorenal toxicity were induced in rats by a single intraperitoneal injection of doxorubicin (DOX; 200 mg/kg,i.p) after 28 consecutive days of oral administration of omega 3 (OMG; 25, 50 and 100 mg/kg) fatty acids. Forty-eight hours after DOX injection, the rats were scarified and the heart and renal were isolated. Tissue specimens from the kidneys and heart of control and treated rats were fixed in 10% neutral buffered formalin and routinely processed. Paraffin embedded sections were dewaxed, rehydrated and incubated in 3% hydrogen peroxide for inhibition of endogenous peroxidase. The sections were then incubated with rabbit anti-cleaved caspase 3 (Abcam, 1:100 Cambridge, MA, USA) and mouse monoclonal anti-P53 (Ventana) as the primary antibodies. Staining was visualized with DAB. Results are the mean ± SEM. Statistical analyses were carried out using ANOVA followed by Tukey's multiple comparisons test.

*, P< 0.05 compared with the normal control

[@], P< 0.05 compared with the DOX treated rats.

<https://doi.org/10.1371/journal.pone.0242175.t006>

On the other side, marked attenuation of histopathological lesions was recorded in OMG (50mg/kg) +DOX group with mild and focal vacuolization of cardiomyocyte (Figs 5D and 6D). The cardiomyocyte restored its normal structure and appeared as normal as the control in OMG (100mg/kg) + DOX group (Figs 5E and 6E).

3.5. Effect of OMG on immunohistochemical analysis

The results of immunohistochemical analysis of activated caspase-3 and P53 demonstrated in the kidneys and heart of the control and treated rats are illustrated in Table 6 and Figs 7 and 8.

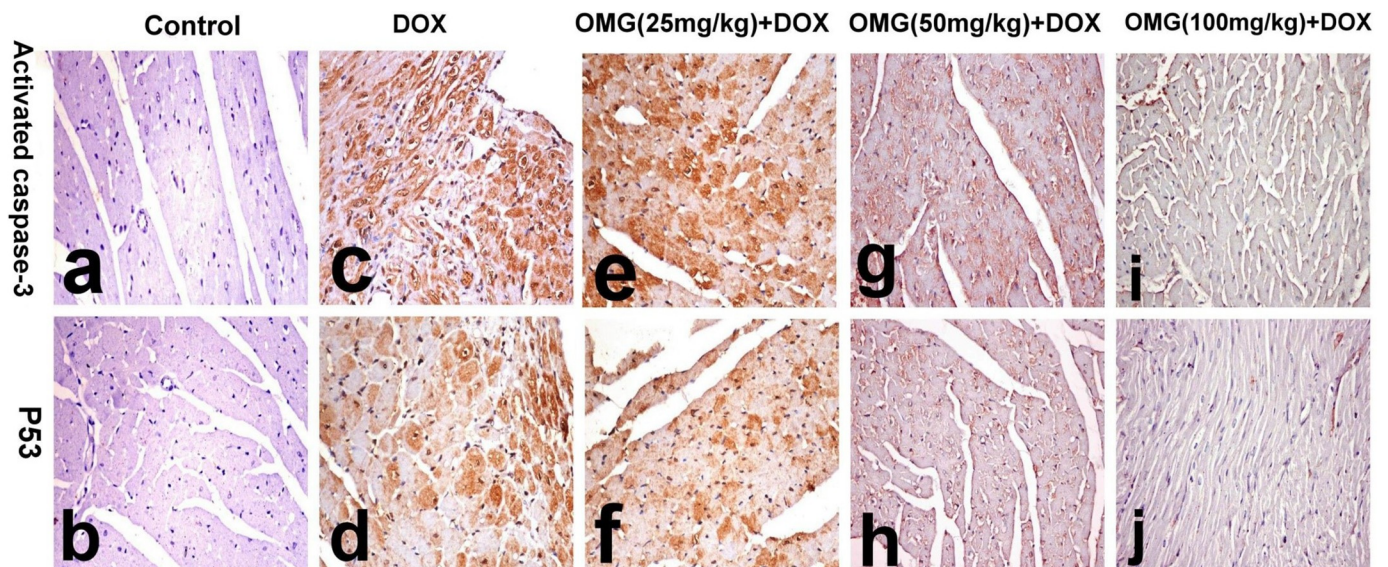


Fig 7. Immunohistochemical staining of activated caspase 3 and P53 in heart tissues of the rats. (a, b) normal rats showing no staining, (c, d) DOX-treated rats showing diffuse intensely stained cytoplasm and/or nuclei, (e, f) OMG (25mg/kg)+DOX showing diffuse positive reaction in the cardiomyocytes, (g, h) OMG (50mg/kg)+DOX showing faint immune reaction in the cardiomyocytes, and (i, j) OMG (100mg/kg)+DOX showing very faint immune staining of individual cardiomyocytes. (activated caspase 3 immunohistochemical staining for a, c, e, g, and i and P53 immunohistochemical staining for b, d, f, h and j. 40X). DOX, doxorubicin, OMG, omega 3 fatty acids.

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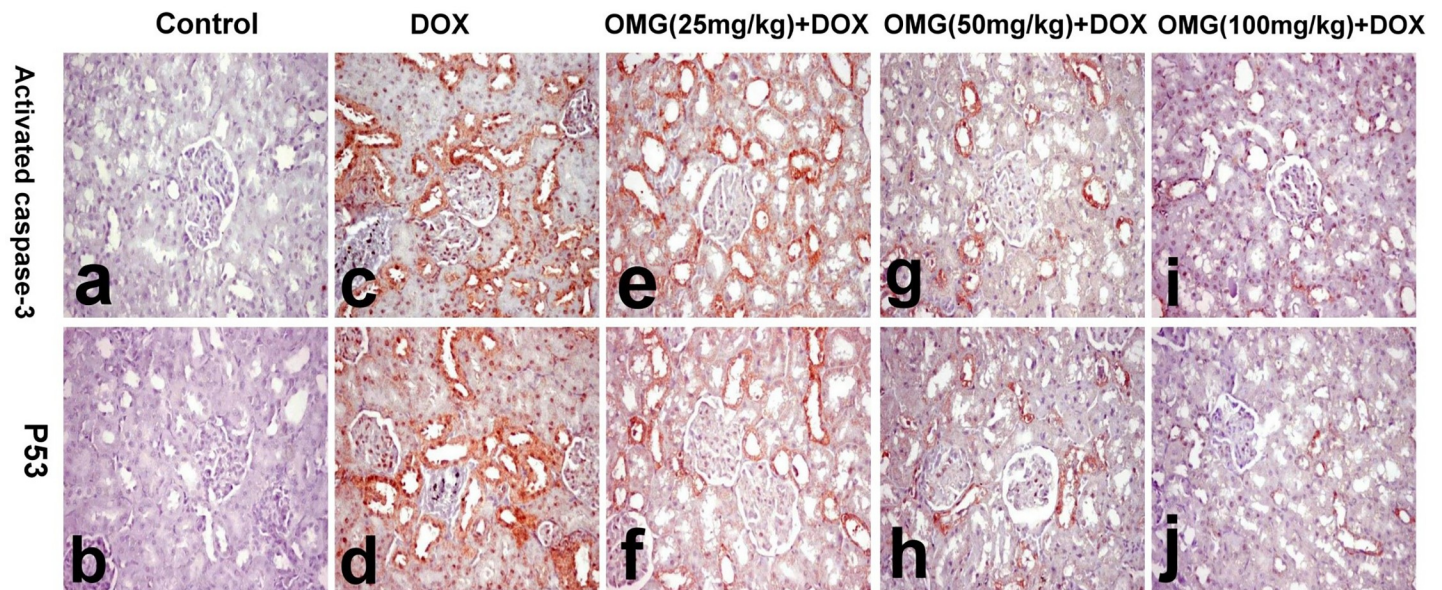


Fig 8. Immunohistochemical staining of activated caspase 3 and P53 in the in heart tissues of the rats. (a, b) normal rats showing no staining, (c, d) DOX-treated rats showing diffuse positive reaction in the glomerular and renal tubular epithelium, (e, f) OMG (25mg/kg) + DOX showing diffuse positive reaction in the renal tubular epithelium, (g, h) OMG (50mg/kg) + DOX showing focal immune staining in the glomerular and renal tubular epithelium, and (i, j) OMG (100mg/kg) + DOX showing faint and focal immune staining. (activated caspase 3 immunohistochemical staining for a, c, e, g, and i and P53 immunohistochemical staining for b, d, f, h and j. 40X). DOX, doxorubicin, OMG, omega 3 fatty acids.

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Immunohistochemical staining for activated caspase 3 and P53 revealed no staining in the kidneys and heart of control rats (grade 0). While, marked and diffuse positive reaction for activated caspase 3 and P53 were demonstrated in the glomerular and renal tubular epithelium as well as cardiomyocytes of DOX-treated rats (grade 2). Similarly, intense positive reaction was demonstrated in OMG (25mg/kg) + DOX group (grade 2). On the contrary, the renal tubular epithelium and cardiomyocytes of OMG (50mg/kg) + DOX and OMG (100mg/kg) + DOX groups revealed faint and focal immune staining (grade 1) which is significantly different from DOX- treated group (Figs 7 and 8).

3.6. Cytotoxicity assay findings

DOX exerted gradient cytotoxic activity against MCF7 with increasing concentration; viability started to drop significantly at a concentration of 1 μ M. Cellular log kill was gradual in profile with IC₅₀ of $0.3 \pm 0.02 \mu$ M. Similarly, OMG single treatment exerted gradual cytotoxic activity with increasing concentration; viability started to drop significantly at concentration of 10 μ M. OMG has steep cellular log kill profile with IC₅₀ of $1.2 \pm 0.02 \mu$ M. Equimolar combination of OMG with DOX significantly improved the cytotoxic profile of OMG. IC₅₀ of OMG after combination with DOX was significantly decreased from $1.2 \pm 0.02 \mu$ M to $0.33 \pm 0.01 \mu$ M. However, this combination showed no effect on the cytotoxic profile of DOX (Fig 9).

4. Discussion

In this study, single intraperitoneal injection of DOX (200 mg/kg) induced toxic effects on the heart and kidneys of rats. The cardiotoxicity was reflected by the recorded ECG in DOX-treated rats on both conductivity and rhythmicity of cardiac muscle. Both QTc and R-R intervals were elongated with a marked bradycardia, in addition to the elevation of ST height and shortening of T- wave amplitude, indicating the delay in both conductivity and rhythmicity of

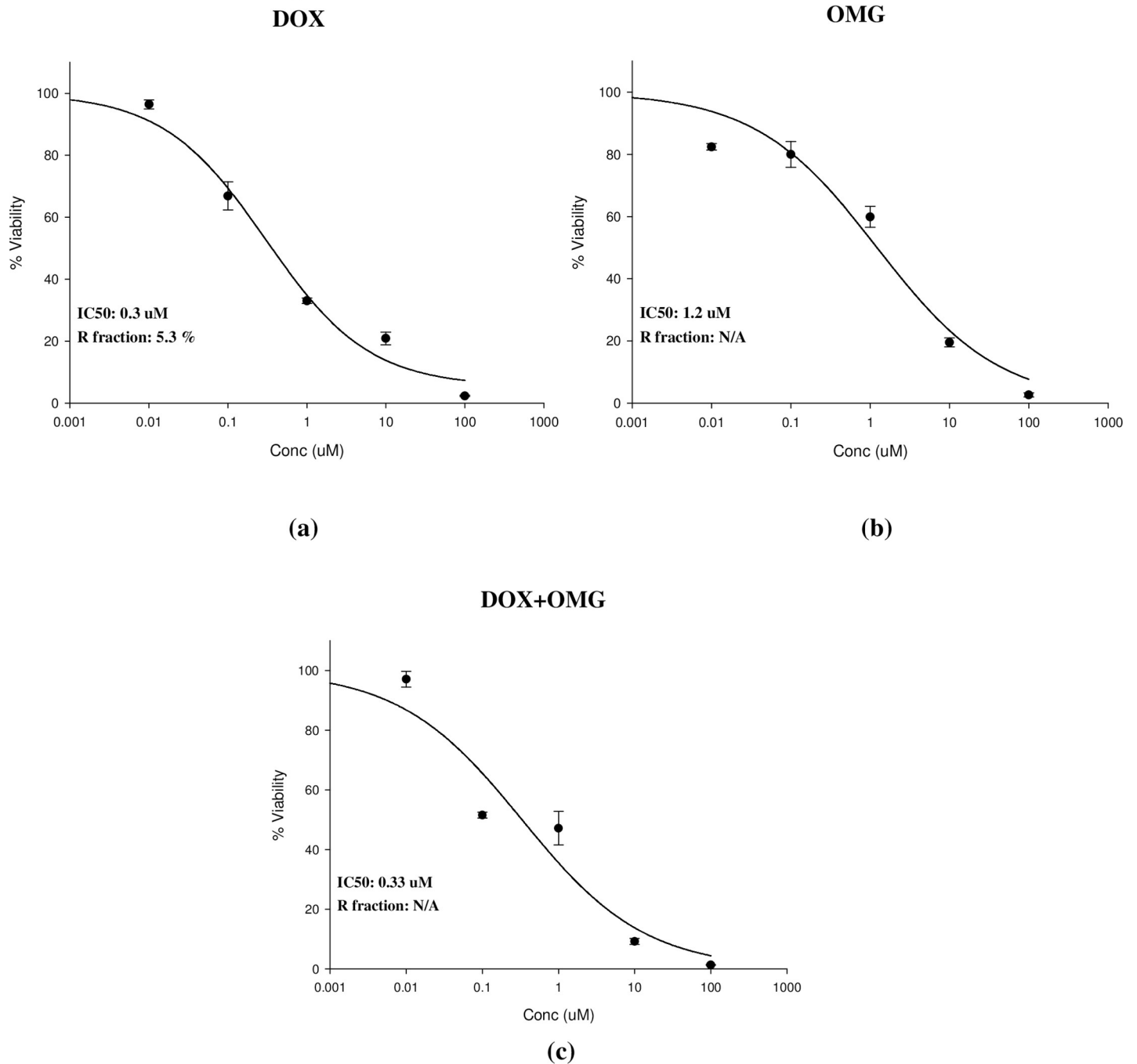


Fig 9. Cytotoxicity of DOX, OMG, and their combination as assessed *in vitro* against human breast cancer MCF7 cell line. DOX, doxorubicin, OMG, omega 3 fatty acids; Statistical analysis of IC50 values were calculated from concentration-response curves by Sigma Plot software, version 12.0 (System Software, USA).

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cardiac cells. These current findings in the DOX group were in agreement with previous studies [29,30], and they resemble the typical characteristics of acute DOX-induced cardiotoxicity in human [31]. Moreover, induction of cardiotoxicity by DOX was also confirmed by the observed elevation in serum levels of CK and hsCRP as well as cardiac content of ANP and IL-6. It was also established by the observed histopathological alterations of cardiac tissues. CK

and ANP are myocardium-specific protein reported as useful biomarkers for heart diseases [32]. Previous studies reported the increased release of CK and ANP from myocardial tissues with DOX treatment [33–35]. IL-6 is an important cardiac cytokine that is upregulated in the acute phase of cardiac injury [36], and mediates adverse myocardial remodeling [37]. Its elevated levels in relation with acute DOX-induced cardiotoxicity has been demonstrated in other studies [38,39]. Also, hsCRP is a strong indicator of cardiac stress that is accompanying increased rates of cardiovascular diseases [40]. Elevation in hsCRP levels has been reported before in association with DOX-induced cardiotoxicity [41].

In the same way, DOX-induced nephrotoxicity was manifested by increasing serum levels of kidney function biomarkers, urea and creatinine, as well as alteration of histopathological features of renal tissues in DOX-treated rats. Similar findings have been recorded before [1,42,43]. Furthermore, upregulation of renal Nox4 was observed in the current study. NADPH-oxidase (Nox) is an important source of ROS that has many isoforms including Nox1, Nox2, Nox3, and Nox4. In the kidney, Nox4 is highly expressed and plays an important role in nephropathologies through ROS-mediated and non-mediated pathways [10,11]. By stimulating several signaling pathways, Nox4 leads to redox processes including diabetic nephropathy, acute kidney injury, obstructive nephropathy, hypertensive nephropathy, carcinoma of the renal cells and other renal diseases [44]. In line with our findings, upregulation of renal Nox4 with DOX was reported before [9,45]. Previous studies suggested that ROS generated by NADPH oxidases can activate Nrf2 [46,47], which is the redox homeostasis modulator [48]. The expression of Nrf2-dependent genes is upregulated by overexpression of Nox4. This Nox4-directed upregulation was completely attenuated in an Nrf2-null inherited background, indicating the involvement of Nrf2 in the molecular pathway [49]. Moreover, Nrf2 regulation has been recently related to the chemosensitivity of cancer cells, thus this approach is a promising therapy for chemoresistant neoplastic cells [50]. Ryoo et al. [51] has also revealed the involvement of Nrf2 signaling in DOX resistance of cancer stem cell.

Also, increased levels of renal renin was demonstrated in the present study. According to literatures, elevated levels of renal renin are best attributed to reduced renal perfusion and impair glomerular filtration [52]. Decreased renal perfusion releases large quantities of renin as a provoking factor to induce compensatory renal vasoconstriction. This action occur entirely intrarenally, and the vasoconstriction appears to be limited to this vascular bed. Therefore, evaluation of renal the renin, rather than peripherally, gives more accurate indication of renal problem [53]. The current increase in renal renin levels assumes that a part of DOX-induced nephrotoxicity might be secondary to a preceding cardiotoxicity that results in decreasing the cardiac output, as evidenced by ECG in the current study, and consequently the renal perfusion. Supporting this hypothesis, previous data suggested that DOX-induced cardiotoxicity might lead to modulation of blood supply to the kidney and alter xenobiotic detoxification processes, respectively, thus indirectly contributing to DOX-induced nephropathy [8].

On the other hand, our study evidenced that DOX nephrotoxic effect is also a part of a multiorgan damage mediated by DOX mainly through free radical formation. This was demonstrated by deterioration of oxidative stress biomarkers as indicated by a reduction of GSH content and elevation of lipid peroxidation end product, MDA, in both cardiac and renal tissues of DOX-treated rats. These results that are concordant with those from other studies [54,55]. Exertion of DOX-induced oxidative stress occurs as a result of DOX reduction by the mitochondrial enzymes following its cellular uptake; a reaction that results in generation of abundant ROS [33,56]. This may explain that DOX-induced toxicity mostly affects cells with a huge number of mitochondria, including cardiac and renal cells [30]. Furthermore, DOX also decreases the levels of endogenous antioxidant, such as GSH. Therefore, the low

concentrations of such proteins in heart and kidney tissues gives another explanation of the high susceptibility of both organs to DOX toxicity [57].

Moreover, the current immunohistochemical findings revealed increased expression of apoptotic markers such as caspase-3 and P53 in the heart and kidneys of DOX-treated rats. Accumulating evidence supports induction of apoptosis as a key mechanism for DOX-induced cardiorenal toxicity [58,59]. DOX-induced elevated levels of free radicles cause intercalation into DNA leading to apoptosis [60–64]. These findings were empathized by Mizutani *et al* who exhibited activation of caspase-3 in human promyelocytic leukemia (HL60) cells treated with DOX resulting in cell death [65]. Interestingly, in the study of Mizutani *et al*, apoptosis was identified to be mediating through different mechanisms including Nox activation. This explains our finding where renal apoptosis was accompanied by elevated expression of Nox4, suggesting the latter as one of the driving mechanism leading to DOX-induced apoptosis.

However, pretreatment of DOX-injected rats with with OMG (25, 50 or 100 mg/kg; p.o.) showed a marked ameliorative effect of the acute cardiorenal toxicity in a dose-dependent manner. In agreement, the protective effect of OMG against DOX-induced cardiac and renal toxicities has been described in other studies [66,67]. The data of the current study revealed an improvement in ECG, cardiac and renal function biomarkers and histopathological findings by OMG in DOX-treated rats. Several mechanisms of cardio- and reno- protective effects of OMG against different heart and kidney diseases have been reported, including their advantage on platelet function, cytokine production, fibrinolysis, and inflammatory factors [68–71]. Moreover, the present study confirmed the established anti-oxidant and anti-apoptotic activity of OMG [72–74] by showing an improvement in oxidative and apoptotic biomarkers in both heart and kidney tissues of DOX-injected rats when pretreated with OMG. These findings indicated those activities as protective mechanisms of OMG. Moreover, a decline of Nox4 expression was observed with OMG pretreatment. Interestingly, this points out a role of Nox4 inhibition in the protective effect of OMG against DOX-induced renal toxicity, especially, when considered the direct effect of OMG on Nox in different organs that has been described [75,76]. Moreover, this finding indicates a protective role of OMG against Nox4-Nrf2 redox imbalance, suggesting it as a potential agent to be used in conjunction with the chemotherapeutic agent, DOX, to reduce the probability of chemoresistance. It has been previously found that OMG chemosensitize multidrug resistant colon cancer cells [77]. Further investigation is still required to assess this effect of OMG and this would be of potential importance because pharmacological inhibition of Nox4 has received massive consideration as a therapeutic strategy for many kidney diseases [78].

An important point in using a protective agent against DOX-induced deleterious effects is to confirm that administration of this protective does not interfere with DOX antitumor activity [79]. In this study, preservation of the cytotoxic activity of DOX, when tested on MCF7 cell line in the presence of OMG, was observed. In line with our findings, data reveals that OMG do not only have any negative effect on the antitumor activity of conventional cancer therapies, however, they can also increase the sensitivity of tumor cells to these drugs and improve their efficacy, especially against cancers resistant to treatment [80]. Stimulatingly, a cytotoxic activity of OMG alone has been observed in the current in-vitro study. This result is in agreement with the studies reported a selective cytotoxic activity of OMG towards cancer cells with little or no toxicity on normal cells [81,82].

5. Conclusion

In conclusion, the findings of the current study revealed induction of acute cardiorenal toxicity in rat by a single intraperitoneal injection of DOX (200 mg/kg) that is characterized by

oxidative stress and induction of apoptosis in both heart and kidney tissues. Activation of Nox4 that observed in the renal tissues suggested its role in DOX oxidative and proapoptotic effects. Elevation of renal renin levels that was accompanied by defective cardiac functions hypothesized that part of DOX-induced renal toxicity occurs secondary to impaired renal perfusion due to DOX-cardiotoxic outcome. The study also showed that pretreatment with OMG protected the rats against DOX-induced cardiorenal toxicity. It demonstrated the anti-oxidant and anti-apoptotic activities of OMG as underlying mechanisms for this reported protective effect, and proposed the involvement of Nox4 inhibition. The study confirmed the preservation of DOX cytotoxic activity *in vitro* when used with OMG. Therefore, this study suggests OMG as a protective agent against acute DOX-induced cardiorenal damage, with no negative influence on the latter required antitumor activity.

Author Contributions

Conceptualization: Dalia Saleh, Rehab Hegazy.

Data curation: Rehab Hegazy.

Formal analysis: Dalia Saleh, Marawan Abdelbaset, Azza Hassan, Rehab Hegazy.

Investigation: Dalia Saleh, Marawan Abdelbaset, Azza Hassan, Rehab Hegazy.

Methodology: Dalia Saleh, Rehab Hegazy.

Project administration: Sawsan Mahmoud.

Resources: Dalia Saleh, Ola Sharaf, Sawsan Mahmoud, Rehab Hegazy.

Supervision: Ola Sharaf, Sawsan Mahmoud.

Writing – original draft: Dalia Saleh, Rehab Hegazy.

Writing – review & editing: Rehab Hegazy.

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