Original Article

Modulatory Effects of Rosemary Leaves Aqueous Extract on Doxorubicin-Induced Histological Lesions, Apoptosis and Oxidative Stress in Mice

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Abstract

Background: Doxorubicin is used in treatment of many solid malignancies and lymphomas with poorly understood mechanism underlying tissue injury. Rosemary leaves or extracts were found to contain high antioxidant activity almost equivalent to BHA (Butylated Hydroxy Anesole) and BHT (Butylated Hydroxy Toluene). Thus, the possibility of aqueous rosemary leaves extract (RE) to ameliorate doxorubicin-induced histological lesions, apoptosis and oxidative stress in male mice tissues was tested.

Methods: Four doses (25, 125, 250 and 375 mg/kg b. wt.) of RE have used orally two times/ week for 15 days prior to the administration of an intraperitoneal single dose of doxorubicin (25 mg/kg b. wt.). Biochemical, histological and immunohistochemical methods were performed on liver, kidney and heart tissue sections.

Results: The positive control group (DXR alone) showed severe histological lesions in the liver, kidneys and heart, including degeneration and inflammatory response accompanied with significant increase in the apoptotic index (Bax/ Bcl-2) and oxidative stress. Rosemary extract was proved to significantly attenuate the doxorubicin-related toxic effects via more than one mechanism such as: the powerful inhibition of lipid per-oxidation, the stimulation of the synthesis of cellular antioxidants, the decrease of the inflammatory response and the reduction of the apoptotic index.

Conclusion: The efficacy of the tested doses of RE in improving doxorubicin-deteriorated effects was organ specific. The most potent dose of RE to abate the lesions in all examined tissues , was 125mg/ kg b. wt and the less effective was 375 mg/ kg b. wt.

Key words: Doxorubicin, Apoptotic Marker (Box/ Bcl-2), Oxidative Stress, Rosemary

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Introduction

Doxorubicin is one of the first anthracyclines, with efficacy in treatment of many solid malignancies and lymphomas [1, 2]. However, it's diverse cytotoxic effects, including liver, kidneys, and heart compromise its antitumour fruitful action [3-12].

The mechanism underline doxorubicin-induced tissue and cellular damage is still poorly understood and is even thought to be connected with the ability of the drug to cause severe oxidative damage mediated by oxyradical cascade involving superoxide and hydroxyl radicals [3, 10, 13-17]. Superoxide and hydroxyl radicals and hydrogen peroxide are reactive oxygen species, related to a large family of oxygen free radicals active oxygen-containing molecules, which contribute to the oxidative stress [18].

Recently, apoptosis was also considered to be one of the major processes that lead to the progressive deterioration of tissue functions leading to some tissue pathologies [1, 5, 18].

So, it was supposed that administration of a potent antioxidant together with a chemotherapeutic agent may be the proper advance to reduce the toxic side effects of ADR especially if it has the ability to modulate apoptosis [14, 18]. Though, some synthetic antioxidant agents might have cytotoxic and oxidant activity or high molecular weight that limit their therapeutic application [7]. Thus, the use of plant extracts and food supplements have been

recently preferred to struggle the oxidative stress associated with different diseases.

Rosemary (Rosmarinus officinalis, Labiatae) is a native Mediterranean small green shrub with pale-blue flowers that bloom in late winter and early spring. Rosemary and its constituents, especially caffeic acid derivatives such as rosmarinic acid, have a therapeutic potential in treatment or prevention of bronchial asthma, spasmogenic disorders, peptic ulcers, inflammation diseases, hepatotoxicity, atherosclerosis, ischemic heart disease, cataracts and cancer [21]. Rosemary leaves or extracts were found to contain high antioxidant activity [22, 23] almost equivalent to BHA and BHT [24]. This effectiveness of rosemary extracts as antioxidants have caused their commercial exploitation [25].

Thus, this study was undertaken to elucidate the possible modulating action of rosemary leaves aqueous extract on doxorubicin-induced histological lesions, apoptosis and oxidative stress in male mice.

Materials and Methods

Chemicals:

Doxorubicin (adriablastina® produced by Carlo Ebro) was purchased from a local pharmacy in a form of 10 mg/ampoule. The bcl-2 monoclonal antibody and Bax polyclonal antibodies and ultra vision detection® system were purchased from Thermo fisher Scientific (Lab vision Corporation). All other chemicals were obtained from Sigma (St. Louis, MO, USA).

Extract preparation:

Rosmarinus officinalis was identified at the Botany Department, Faculty of Science, and University of Beni-Suef. Leaves of this plant have cleaned; shade dried, powdered and extracted. The extract has prepared by refluxing with bid stilled H_2O for 36 hours (12 hours x 3). The cooled liquid extract has transformed to powder by evaporating its liquid contents. The powder has dissolved in bid stilled water just before oral administration [26].

Dosage:

Doxorubicin (DXR) dose: The dose of doxorubicin used was 25 mg/kg b. wt. This dose was previously reported to cause nephrotoxicity, cardio toxicity and hepatotoxicity [7, 27, 28]. The medicine has reconstituted to the selected dose in a sterile H_2O prior to use and has given once after 15 days of the onset of the experiment by intravenous injection.

Rosemary Extract (RE) dose: The concentration of rosemary extract has adjusted to a dose level of 25, 125, 250 or 375 mg/kg b. wt. These extract doses have given two times/week by gastric intubation for 15 days (5 times in total).

Animals:

Sixty (60) male albino mice (Mus musculus), each weighs 28 $\pm$ 5 gr, were used. The animals were obtained from the Research Institute of Ophthalmology, Giza. They have housed in stainless-steel cages at room temperature (25-30^{0}C) and provided with food and water ad-libitum.

Grouping:

The animals have divided into the following groups, each of six mice.

Control group (G1): administered 0.2 ml of distilled H_2O by gastric intubation for 15 days.

Doxorubicin group (G2): given a single intraperitoneal injection of DXR (25 mg/kg b. wt) 15 days after the start point of experiment. Before this dose, the animals have taken 0.2 ml distilled water by gastric intubation.

Rosemary leaves aqueous extract groups (G3-G6):

G3: taken rosemary leaves aqueous extract (RE) at a dose of 25 mg/kg b. wt.

G4: given rosemary aqueous extract at a dose of 125 mg/kg b. wt.

G5: given RE at a dose of 250 mg/kg b. wt.

G6: administered RE at a dose of 375 mg/kg b. wt.

Rosemary- doxorubicin groups (G7-G10):

G7: have used the same regime like G3 and then a single dose (25 mg/kg b. wt.) of doxorubicin.

G8: administered the same dose of RE in G4 and then a single dose of doxorubicin.

G9: given RE similarly like in G5 and then a single dose of DXR.

G10: taken RE dose of G6 and then a single dose of doxorubicin.

Sampling:

At the end of the experimental period, the animals were anesthetized with diethyl ether and after dissection, tissue samples were collected for biochemical, histopathological and immunohistochemical studies.

Biochemical analysis:

Zero point five gram (0.5g) of each examined tissue has homogenized in 5 ml 0.9% NaCl (10% w/v) using Teflon homogenizer (Glas-Col, Terr Haute, USA).

Lipid per oxidation was determined by measuring the thiobarbituric acid reactive substances (TBARS) according to method of Pressus et al. [29]. SOD activity has assayed according to method of Arthur and Boyne [30]. GSH level has measured calorimetrically as protein-free sulfhydryl content using Ellman reagent [31]. Catalase activity has determined according to method of Cohen et al.

[32]. Glutathione peroxidase has assayed according to method of Pinto and Bartley [33].

Histopathological and immunohistochemical studies:

Pieces of different tested organs have fixed in 10% neutral buffered formalin for 24 hours. After dehydration, tissue samples have embedded in paraffin wax, sectioned at 5 µm and stained with haematoxylin and eosin [34] for histopathological examination or mounted on positive slides (Thermo fisher Scientific, Lab vision Corporation) to carry on the immunohistochemical determination of apoptotic markers.

The streptavidin peroxidase method has applied [35].The Bcl-2 monoclonal antibody dilution was 1:50 while that of Bax polyclonal antibody was 1:80. The cytoplasm of apoptotic cells has stained yellow-brown using the ultra vision detection system, antipolyvalent, HRP/DAB. The apoptotic index has calculated using the image analysis program by dividing the expression rate of Bax protein on that of Bcl-2 (Bax/ Bcl-2).

Statistical analysis:

Biochemical and immune histochemical results were analyzed using PC-STAT one-way analysis of variance [36].

Results have expressed as mean ± standard error (SE). Values of $P < 0.05$ were considered significant.

Results

Histological study:

A) Liver: The liver sections of the control group have shown the normal histological architecture (Fig1). The animals have treated with doxorubicin showed cirrhosis in the portal area accompanied with activation of iron-laden macrophages, numerous lymphocytes, fibroblastic proliferation, congested portal veins and vacuolar degenerative changes of hepatocytes (Figs 2, 3).

All doses of rosemary leaves aqueous extract (RE), have revealed nearly no histological alterations except with the highest dose (375 mg/kg. b. wt.) where portal fibroblastic proliferation was noticed (Figs 4, 5, 6 , 7).

Pretreatment of doxorubicin injected mice with the lower two doses of RE (25, 125 mg/ kg b. wt.) greatly ameliorate the histological lesions (Figs 8, 9). Though, animals given the higher two doses (250, 375 mg/kg b. wt.) still have suffered from focal leucocytic aggregation, vacuolar degeneration with nuclear disintegration and dilated central veins (Figs 10, 11).

B) Kidney: All controlled animals had normal kidney histological structure (Fig 12). Doxorubicin-treated group have revealed numerous destructive changes including congested cortical vessels, perivascular leucocytic aggregation, degenerated renal tubules in the cortical region and severe medullary fibroblastic proliferation and leucocytic infiltration (Figs 13-15).

The kidney sections of RE-treated animals had almost normal appearance (Figs 16, 18) despite medullary hemorrhage (Fig 19) and degeneration of renal tubules has seen with the higher two doses (250 and 375 mg/kg b. wt.) (Figs 19, 20).

The severity of the renal histological lesions resulted from doxorubicin administration, have shown no improvement after using rosemary leaves aqueous extract in doses of 25, 250 and 375 mg/kg b. wt. (Figs 20, 22, 23) though, pretreatment of doxorubicin-injected animals with RE in a dose of 125 mg/kg b. wt greatly reduced these effects (Fig 21).

C) Heart: Examination of heart sections of control mice, have shown the normal structure (Fig 24). Administration of doxorubicin revealed hypertrophied muscle fibres with megaloblastic nuclei and scar areas of degenerated muscle fibres (Fig 25).

Mice given RE in doses of 125 and 250 mg/kg b. wt. manifested almost normal tissue structure (Figs 27, 28). Though, the other two doses (25, 375 mg/kg b. wt.) have caused vascular dilatation and congestion (Figs 26, 29) associated with lower dose hypertrophy (Fig 26).

Rosemary-administration for doxorubicin-injected mice, in 125 and 250 mg/kg b. wt. highly have abated the histopathological effects of doxorubicin (Figs 31 , 32) while the cardiac muscle fibers still have suffered from hypertrophy with the lower dose (25 mg/kg b. wt.) (Fig 30) and necrosis with the higher dose (375 mg/ kg. b. wt.) (Fig 33).

Immunohistochemical study:

A) Liver: Bcl-2 antibody (anti-apoptotic marker) has shown cytoplasm reactivity. This have indicated lower reactivity, in doxorubicin-treated group compared to the tissue of the control group (Figs 34 a,b).The lower two doses of RE (25, 125 mg/kg b. wt.) have given similar results to control (Figs 34c , d), while the dose of 250 mg/kg b. wt. have highly elevated this reactivity (Fig 34e). On the contrary, the highest dose (375 mg/kg. b. wt.) has reduced the intensity of Bcl-2 reaction

Table 1*.Effect of different doses of rosemary aqueous extract on apoptotic index (Bax/ Bcl-2) in liver, kidneys and heart of different treated groups*

Group \ Parameter		*Liver*	*Kidney*	Heart
Normal (G1)		0.93 ± 0.03^{f}	0.73 ± 0.01^{f}	0.75 ± 0.01^{g}
Doxorubicin (G2)		9.31 ± 0.22^{b}	11.5 ± 0.18^{a}	5.35 ± 0.16^{a}
Rosemary extract	**(G3)**	1.21 ± 0.27^{e}	0.95 ± 0.01^{e}	0.83 ± 0.01^{d}
	(G4)	0.93 ± 0.03^{f}	0.84 ± 0.01^{ef}	0.82 ± 0.04^{e}
	(G5)	1.33 ± 0.13^{e}	0.92 ± 0.01^{e}	0.95 ± 0.01^{c}
	(G6)	2.27 ± 0.15^{d}	1.55 ± 0.19^{d}	1.17 ± 0.11^{b}
Doxorubicin treated group	**(G7)**	3.31 ± 0.14^{c}	10.82 ± 0.27^{a}	2.39 ± 0.19^{e}
	(G8)	2.35 ± 0.19^{d}	4.30 ± 0.18^{c}	1.91 ± 0.02^{f}
	(G9)	9.22 ± 0.27^{b}	10.96 ± 0.22^{a}	2.71 ± 0.09^{d}
	(G10)	11.67 ± 0.28^{a}	11.16 ± 0.23^{a}	3.42 ± 0.12^{c}
F-Probability		$P < 0.001$	$P < 0.001$	$P < 0.001$
LSD at the 5% level		0.23	0.18	0.13
LSD at the 1% level		0.30	0.24	0.17

- Means which have the same superscript symbol(s) are not significantly different.

(Fig 34f). Mice, which have given RE before doxorubicin injection, have shown decreased reactivity, with the lowest and highest doses of Bcl2 antibody (Figs 34g , j) while high intensity has noticed with the doses 125 and 250 mg/kg b. wt. (Figs 34h,i), compared to the control or doxorubicin group.

Concerning Bax antibody reactions (pro-poptotic marker) in liver tissue, which appeared cytoplasmic, all doses of rosemary leaves aqueous extracts, have shown nearly similar degree to the control group (Figs 35a, c, d, e, f). The liver sections of doxorubicin-injected mice have shown a marked increase in the intensity of brown colour specific for Bax protein, compared to the negative control group, have indicated increase in its gene expression (Fig 35b).The pretreatment with the first three doses of RE greatly have decreased the Bax gene expression rate, compared to the doxorubicin group (Figs 35g-i). Though, no changes have recorded to its expression with the higher doses, relative to the doxorubicin (Fig 35j).

The apoptotic index (Bax/ Bcl-2) have shown significant elevation in doxorubicin group ($P< 0.001$) compared to the control, while the pre-administration of the first and second RE doses significantly have abated doxorubicin-treated mice ($P< 0.001$), relative to the DXR group. Though, the higher dose, have resulted significant elevation in apoptotic index (Table 1).

B) Kidney: Regarding the anti-apoptotic Bcl-2 marker cytoplasm expression, doxorubicin administration have resulted severe reduction in the cortical region compared to the control group (Figs 36a, b). All RE doses have shown no changes in Bcl-2 reactivity in the cortex relative to the control (Figs 36c-f). Pretreatment of doxorubicin-injected mice with all doses of RE greatly increase the Bcl-2 expression compared to the doxorubicin and control groups (Figs 36g-j).Though, no changes in Bcl-2 expression rate have noticed in the medullary region of all the examined groups.

On the contrary, the cortical and medullary regions of doxorubicin-injected mice have shown increased cytoplasm intensity of brown color specific for the pro-apoptotic Bax marker compared to the control (Figs 37a-d). Administration of the higher three doses of RE (125, 250 and 375 mg. kg b. wt.) have revealed greater reactivity in the medullary region compared to the control but similar to that of doxorubicin (Figs 37e,f,h, j, l). Though, the Bax reactivity in the cortical region was nearly similar to control (Figs 37g, 37i, 37k). Animals which have given RE prior to doxorubicin, have shown a marked decrease in the kidney brown intensity of Bax compared to the doxorubicin group but similar to the control (Figs 37m-o) except for the higher dose which have shown no change on the level of Bax expression relative to the doxorubicin (Figs 37p, q) but still of higher value than the control.

The apoptotic index (Bax/ Bcl-2) revealed significant increase in doxorubicin group ($P< 0.001$) compared to the control, while the pre-administration of the second dose (125 mg/ kg b. wt.), only, from RE to doxorubicin-injected animals significantly have lowered ($P< 0.001$) this increase relative to the DXR group (Table 1).

Figure 1. *A photomicrograph of the liver section of a control animal showing the central vein (CV), the hepatic cords (↓) and the sinusoids (S). H & E X150* ***Figure 2.*** *A light micrograph of the liver section of a doxorubicin-injected mouse showing cirrhosis (*) in the portal area associated with activated iron-laden macrophages (↓), numerous mononuclear leucocytes (MN) and fibroblastic proliferation (F).H & E X150* ***Figure 3.*** *A photomicrograph of the liver section of a doxorubicin- injected mouse manifesting congested portal vein (PV) and vacuolar degeneration of hepatocytes. H & E X150* ***Figure 4.*** *A light micrograph of the liver section of a mouse administered 25 mg/ kg b. wt of rosemary leaves aqueous extract showing few mononuclear leucocytes (MN) in the pericentral area of the liver lobule. H & E X150* ***Figure 5.*** *A photomicrograph of the liver section of a mouse given 125 mg/ kg b. wt of rosemary leaves aqueous extract revealing normal structure of sinusoids (s), central vein (CV) and hepatic cords (↓). H & E X150* ***Figure 6.*** *A light micrograph of the liver section of a mouse given 250 mg/ kg b. wt of rosemary leaves aqueous extract illustrating normal liver architecture. H & E X150*

Figure 7. *A photomicrograph of the liver section of a mouse administered 375 mg/ kg b. wt of rosemary leaves aqueous extract showing fibrocytes in the portal area.H & E X150* ***Figure 8.*** *A light micrograph of the liver section of a doxorubicin-injected mouse given 25 mg/ kg b. wt of rosemary leaves aqueous extract showing hydroid degenerative changes of hepatocytes(↓).H & E X150* ***Figure 9.*** *A photomicrograph of the liver section of a doxorubicin-injected mouse given 125 mg/ kg b. wt of rosemary leaves aqueous extract illustrating normal liver architecture with few inflammatory cells (↓) in the pericentral area of the liver lobule. H & E X150* ***Figure 10.*** *A light micrograph of the liver section of a doxorubicin-injected mouse given 250 mg/ kg b. wt of rosemary leaves aqueous extract manifesting vacuolar degenerative changes (↓) of the hepatocytes with nuclear disintegration.H & E X150* ***Figure 11.*** *A photomicrograph of the liver section of a doxorubicin-injected mouse administered 375mg/ kg b. wt of rosemary leaves aqueous extract revealing massive number of mononuclear leucocytes (MN) with hydroid degenerative changes (↓). H & E X150*

Figure 12. *A light micrograph of the kidney section of a control mouse showing the proximal tubules (P), the distal tubules (D), the collecting ducts (C) and the Malpighian corpuscles with the basement membrane (↓), glomerulus (G) and urinary space (U). H & E X150* ***Figure 13.*** *A photomicrograph of the kidney section of a doxorubicin-injected mouse showing congested blood vessels (BV), perivascular mononuclear leucocytes (↓) and degenerated renal tubules (D). H & E X150* ***Figure 14.*** *A light micrograph of the kidney section of a doxorubicin-injected mouse manifesting severe destructive changes of renal tubules infiltrated with number of fibrocytes (↓). H & E X150* ***Figure 15.*** *A photomicrograph of the kidney section of a doxorubicin-injected mouse illustrating degenerated epithelial cells (↓) of renal tubules. H & E X150* ***Figure 16.*** *A photomicrograph of the kidney section of a mouse given 25 mg/ kg b. wt of rosemary leaves aqueous extract showing normal kidney structure. X150* ***Figure 17.*** *A light micrograph of the kidney section of a mice administered 125 mg/ kg b. wt of rosemary leaves aqueous extract manifesting nearly normal histological architecture. H & E X150*

Figure 18. *A photomicrograph of the kidney section of a mouse given 250 mg/ kg b. wt of rosemary leaves aqueous extract illustrating hyperemic blood vessels (H) with degenerated renal tubules (D). H & E X150* ***Figure 19.*** *A light micrograph of the kidney section of a mouse given 375 mg/ kg b. wt of rosemary leaves aqueous extract revealing severely degenerated renal tubules (↓). H & E X150* ***Figure 20.*** *A photomicrograph of the kidney section of a doxorubicin-injected mouse administered 25 mg/ kg b. wt of rosemary leaves aqueous extract showing no ameliorative effect of the extract on doxorubicin-induced injury where the tissue is (*) invaded with mononuclear leucocytes (↓) and fibrocytes (F). H & E X150* ***Figure 21.*** *A light micrograph of the kidney section of a doxorubicin-injected mouse administered 125 mg/ kg b. wt of rosemary leaves aqueous extract manifesting less degenerative effect and intertubular deposition of amorphous material. H & E X150* ***Figure 22.*** *A photomicrograph of the kidney section of a doxorubicin-injected mouse administered 250 mg/ kg b. wt of rosemary leaves aqueous extract illustrating severe destructive changes of the renal tubules with fibroblastic proliferation (F) and mononuclear leucocytes aggregation (↓).H & E X150* ***Figure 23.*** *A light micrograph of the kidney section of a doxorubicin-injected mouse administered 375 mg/ kg b. wt of rosemary leaves aqueous extract revealing severely degenerated renal tubules (*) invaded with fibrocytes (F).H & E X150*

Figure 24. *A photomicrograph of the heart tissue of control mice showing the muscle fibres (MF) and the intramuscular septa (↓).H & E X150* ***Figure 25.*** *A light micrograph of the heart tissue of a doxorubicin-injected mouse revealing hypertrophied muscle fibres (MF), megaloblastic nuclei (↓) and scar areas (S) of degenerated muscle fibers. H & E X150* ***Figure 26.*** *A photomicrograph of the heart section of a mouse given 25 mg/ kg b. wt of rosemary leaves aqueous extract showing inter-muscular congested blood vessels (BV) with hypertrophied muscle fibers (MF). H & E X150* ***Figures 27-28.*** *A light micrograph of the heart tissue of a mouse given 125 and 250 mg/kg b. wt. of RE, respectively, illustrating normal heart architecture. H & E X150* ***Figure 29.*** *A photomicrograph of the heart section of a mouse given 375 mg/ kg b. wt of rosemary leaves aqueous extract revealing congested intramuscular blood vessels (BV).H & E X150* ***Figures 30-33.*** *Photomicrographs of the heart sections of doxorubicin-injected animals administered rosemary leaves aqueous extract at doses of (25, 125, 250 and 375 mg/ kg b. wt., respectively) showing high amelioration of the doxorubicin-implications (Figs. 31 ,32) except for the hypertrophy of muscle fibers (MF) recorded with the lower dose (Fig. 30) and necrosis (N) with the higher dose (Fig. 33). H & E X150*

Figures 34 (a-j). *Photomicrographs showing the expression rate of Bax protein (Brown color) in the liver of the tested groups: control (a), doxorubicin (b), rosemary leaves aqueous extract at doses of 25 (c), 125 (d), 250 (e) and 375 (f) mg/kg b. wt. and doxorubicin pretreated with 25 (g), 125 (h), 250 (I) or 375 (j) mg/kg b. wt of RE. X150*

Figures35 (a-j).*Photomicrographs illustrating the difference in the hepatic reactivity of various examined groups: control (a), doxorubicin (b), rosemary leaves aqueous extract at doses of 25 (c), 125 (d), 250 (e) and 375 (f) mg/kg b. wt. and doxorubicin pretreated with 25 (g), 125 (h), 250 (I) or 375 (j) mg/kg b. wt of RE, towards Bcl-2 antibody (Brown color).X150*

Figures 36 (a-j).*Photomicrographs showing the expression rate of Bax protein (Brown color) in the kidney of the tested groups: control (a), doxorubicin (b), rosemary leaves aqueous extract at doses of 25 (c), 125 (d), 250 (e) and 375 (f) mg/kg b. wt. and doxorubicin pretreated with 25 (g), 125 (h), 250 (I) or 375 (j) mg/kg b. wt. of RE. X150*

Figsures 37 (a-j). *Photomicrographs illustrating the difference in the renal reactivity of various examined groups: control (a), doxorubicin (b), rosemary leaves aqueous extract at doses of 25 (c), 125 (d), 250 (e) and 375 (f) mg/kg b. wt. and doxorubicin pretreated with 25 (g), 125 (h), 250 (I) or 375 (j) mg/kg b. wt of RE, towards Bcl-2 antibody (Brown color). X150*

Figures 38 (a-j). *Photomicrographs showing the expression rate of Bax protein (Brown color) in the heart of the tested groups: control (a), doxorubicin (b), rosemary leaves aqueous extract at doses of 25 (c), 125 (d), 250 (e) and 375 (f) mg/kg b. wt. and doxorubicin pretreated with 25 (g), 125 (h), 250 (I) or 375 (j) mg/kg b. wt of rosemary leaves aqueous extract. X150*

Figures 39 (a-j). *Photomicrographs illustrating the difference in the cardiac reactivity of various examined groups: control (a), doxorubicin (b), rosemary leaves aqueous extract at doses of 25 (c), 125 (d), 250 (e) and 375 (f) mg/kg b. wt. and doxorubicin pretreated with 25 (g), 125 (h), 250 (I) or 375 (j) mg/kg b. wt of RE, towards Bcl-2 antibody (Brown color) . X150*

Table 2. *Effect of Doxorubicin and different doses of rosemary aqueous extract on reduced glutathione (GSH, nmol/100mg) and glutathione peroxidase (GPX, mU/ 100mg) content in liver, kidneys and heart of different treated groups.*

Group	Parameter	GSH			GPX		
		Liver	***Kidney***	**Heart**	***Liver***	***Kidney***	**Heart**
Normal (G1)		47.88 ± 1.43^{c}	42.39 ± 1.7^{c}	41.54 ± 0.42^{b}	169.09 ± 2.8^{d}	132.9 ± 0.79^{b}	151.85 ± 0.49^{cd}
Doxorubicin (G2)		28.11 ± 1.75^{g}	31.90 ± 1.23^{e}	32.1 ± 0.29^{f}	128.98 ± 3.9^{g}	116.25 ± 8.38^{d}	115.70 ± 2.53^{h}
Rosemary extract	**(G3)**	51.78 ± 4.2^{b}	45.61 ± 1.75^{b}	38.93 ± 0.49^{c}	191.74 ± 0.46^{a}	132.47 ± 0.36^{b}	151.2 ± 0.36^{d}
	(G4)	57.13± 2.4^{a}	54.29 ± 1.79^{a}	46.86 ± 0.59^{a}	182.16 ± 0.66^{b}	143.97 ± 2.45^{a}	177.35 ± 0.74^{a}
	(G5)	35.55 ± 1.61^{ef}	37.26 ± 5.7^{d}	34.28 ± 0.40^{d}	163.49 ± 0.18^{d}	136.39 ± 7.52^{b}	156.6 ± 0.42^{b}
	(G6)	32.98 ± 0.63^{f}	34.72 ± 3.2^{de}	34.46 ±0.89^{d}	144.89 ± 0.14^{e}	129.08 ± 3.54^{c}	135.7 ± 0.58^{f}
Doxorubicin treated group	**(G7)**	36.21 ± 1.6^{de}	43.15 ± 1.07^{c}	33.65 ± 0.25^{e}	160.83 ± 0.32^{d}	130.58 ± 2.44^{b}	133.13 ± 0.58^{f}
	(G8)	38.41 ± 0.98^{d}	45.74 ± 0.19^{bc}	42.17 ± 0.24^{b}	175.19 ± 0.48^{c}	132.87 ± 1.84^{b}	153.87 ± 0.62^{bc}
	(G9)	32.95 ± 0.98^{f}	41.11 ± 1.45^{f}	37.95 ± 0.98^{c}	143.22 ± 0.24^{e}	126.82 ± 3.09^{c}	142.09 ± 0.62^{e}
	(G10)	24.16 ± 1.5^{h}	37.62 ± 1.41^{e}	32.45 ± 0.22^{f}	139.36 ± 0.21^{f}	128.98 ± 2.69^{c}	129.44 ± 0.25^{g}
F-Probability		P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001
LSD at the 5% level		2.66	2.89	1.36	2.59	4.97	2.74
LSD at the 1% level		3.58	3.89	1.83	3.49	6.69	3.69

- Means which have the same superscript symbol(s) are not significantly different.

Table 3. *Effect of Doxorubicin and different doses of rosemary aqueous extract on catalase activity (CAT, K.10^{-2}) and super-oxide dismutase (SOD, U/100mg) in liver, kidneys and heart of different treated groups*

Group	Parameter	CAT			SOD		
		Liver	***Kidney***	**Heart**	***Liver***	***Kidney***	**Heart**
Normal (G1)		31.85 ±1.02^{e}	31.12 ± 0.69^{c}	33.13 ± 0.51^{cd}	418.09 ± 1.12^{c}	330.04 ± 0.94^{c}	332.19 ± 0.84^{c}
Doxorubicin (G2)		26.24 ± 0.98^{f}	20.82 ± 0.25^{g}	26.6 ± 1.22^{e}	345.92 ± 0.78^{g}	244.69 ± 1.14^{i}	246.23 ± 1.13^{i}
Rosemary extract	**(G3)**	35.75 ± 0.56^{bc}	32.57 ± 0.43^{b}	32.46 ± 0.69^{cd}	445.07± 0.78^{b}	336.75 ± 1.10^{b}	336.94 ± 1.16^{b}
	(G4)	39.54 ± 0.29^{a}	35.21 ± 0.38^{a}	36.67 ± 0.49^{a}	480.98 ± 10.01^{a}	345.36 ± 0.96^{a}	344.37 ± 0.57^{a}
	(G5)	34.24 ± 0.79^{cd}	31.25 ± 0.39^{c}	35.16 ± 0.01^{ab}	396.03 ± 1.31^{d}	295.69 ± 0.98^{f}	293.95 ± 0.83^{f}
	(G6)	31.9 ± 0.75^{e}	30.46 ± 0.23^{d}	27.22 ± 0.59^{e}	375.82 ± 10.69^{e}	266.89 ± 1.46^{h}	264.42 ± 0.87^{h}
Doxorubicin treated group	**(G7)**	33.84 ± 0.25 ^{d}	29.88 ± 0.5 ^{e}	32.15 ± 0.71^{d}	382.91 ± 0.69^{de}	304.83 ± 2.25^{e}	304.94 ± 1.02^{e}
	(G8)	36.75 ± 0.93 ^{b}	32.36 ± 0.42^{b}	34.05 ± 0.33^{bc}	441.13 ± 0.19^{b}	325.17 ± 3.03^{d}	317.53 ± 1.25^{d}
	(G9)	30.29 ± 0.85 ^{e}	28.59 ± 0.41^{f}	33.19 ± 0.49^{cd}	361.15 ± 0.39^{f}	274.22 ± 1.14^{g}	272.58 ± 0.78^{g}
	(G10)	30.77 ± 2.36 ^{e}	28.08 ± 0.75^{f}	26.23 ± 0.64^{e}	345.92 ± 1.23^{g}	254.46 ± 0.73^{i}	253.77 ± 1.05^{i}
F-Probability		P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001
LSD at the 5% level		1.63	0.563	1.85	13.58	4.40	2.79
LSD at the 1% level		2.19	0.758	2.45	18.28	5.92	3.76

- Means which have the same superscript symbol(s) are not significantly different.

Table 4. *Effect of different doses of rosemary aqueous extract on lipid peroxidation (LPO, nmol TBARS/ 100mg tissue/hr) in liver, kidneys and heart of different treated groups.*

Group \ Parameter		*Liver*	*Kidney*	Heart
Normal (G1)		47.18 ± 4.44^{e}	34.2 ± 0.70^{e}	31.86 ± 0.49^{g}
Doxorubicin (G2)		67.68 ± 0.63^{a}	54.19 ± 0.82^{a}	54.62 ± 0.36^{a}
Rosemary extract	**(G3)**	48.28 ± 0.53^{de}	37.14 ± 0.65^{d}	42.79 ± 0.48^{d}
	(G4)	45.55 ± 0.65^{e}	33.36 ± 0.78^{e}	37.52 ± 0.56^{e}
	(G5)	51.92 ± 0.24^{cd}	42.95 ± 0.58^{c}	47.01 ± 0.58^{c}
	(G6)	53.56 ± 0.85^{c}	45.39 ± 0.40^{b}	50.55 ± 1.09^{b}
Doxorubicin treated group	**(G7)**	46.82 ± 0.72^{e}	34.71 ± 0.87^{e}	36.73 ± 0.35^{e}
	(G8)	44.99 ± 0.53^{e}	32.85 ± 0.80^{e}	34.26 ± 0.3^{f}
	(G9)	58.12 ± 0.77^{b}	41.85 ± 0.76^{c}	44.05 ± 0.54^{d}
	(G10)	63.77 ± 0.37^{a}	45.21 ± 0.35^{b}	47.09 ± 0.36^{c}
F-Probability		P < 0.001	P < 0.001	P < 0.001
LSD at the 5% level		4.41	1.99	1.61
LSD at the 1% level		5.93	2.69	2.16

- Means which have the same superscript symbol(s) are not significantly different.

C) Heart: Examination of heart tissue has revealed low reactivity for Bcl-2 antibody, which have appeared cytoplasm, and in doxorubicin-injected mice compared to the control (Figs 38a , b). This reactivity has increased with all doses of RE (Figs 38c, d ,f) except the third one (250 mg/kg b. wt.) (Fig 38e). Administration of RE before doxorubicin, almost increased the anti-apoptotic Bcl-2 gene expression in cardiomyocytes relative to the doxorubicin and control groups (Figs 38g, i, j) except with the dose of 125 mg/kg b. wt. which have shown similar degree of expression to the control (Fig 38h).

On the other hand, the pro-apoptotic Bax antibody have shown lowered cytoplasm reactivity, in most examined groups compared to the control (Figs 39a-g ,j). Though, it expressed higher reaction at a doxorubicin group treated with RE at a dose of 250 mg/kg b. wt. (Fig 39h-i). The expression has seemed to be vascular.

The apoptotic index (Bax/ Bcl-2) have revealed significant increase in doxorubicin group (P< 0.001) compared to the control, while the pre-administration of the second dose (125 mg/ kg b. wt.) of RE to doxorubicin-injected animals significantly have lowered (P< 0.001) this increase relative to the DXR group (Table 1).

Biochemical study:

Administration of doxorubicin have induced highly significant increase (P< 0.001) in the level of lipid per oxidation and a significant decline (P< 0.001) in the antioxidant system content including catalase, SOD, GSH and GPX relative to the control, in all the examined tissues (Tables 2, 3 , 4).

Pretreatment of doxorubicin animals with all doses of RE, have revealed reduced level of lipid per oxidation and increased contents of the antioxidant system molecules compared to those recorded with doxorubicin. Though, the higher dose failed in some cases (SOD in liver, CAT in heart, GSH in liver and kidney and LPO in liver) to abate the doxorubicin effects. In liver and kidney tissues the RE efficacy could be arranged in the following order 125> 25> 250>375 mg/Kg b. wt. while in heart this order is 125>250>25>375 mg/kg b. wt. In all tested tissues, the 125 mg/kg b. wt. of RE always resulted in the best ameliorative effect where the values recorded always returned to the normal (Tables 2, 3, 4).

Discussion

Most hypothesis believed that the primary pathogenic mechanisms of doxorubicin-induced cytotoxicity are mediated via its ability to generate lipid peroxides, super oxide anions, hydroxyl radicals and hydrogen peroxides [9, 28, 37].

Super-oxide ($O_2^{\cdot}$) radical acts as an oxidant and reductant that is reduced to hydrogen peroxide (H2O2) by super oxide dismutase (SOD), which is then detoxified to water by catalase (CAT) or glutathione peroxidase (GPX). Glutathione peroxidase, detoxifying lipid peroxides also, needs glutathione as a substrate [28, 38, 39]. Glutathione (GSH) is a versatile protector exerting its free

radical scavenging restoration by hydrogen donation or reduction of peroxides and maintenance of protein thiols in a reduced state [26].

The balance between lipid peroxidation level, resulted from reactive oxygen species attack polyunsaturated fatty acids, proteins and genetic material, and antioxidant factor affecting tissue damage level.

The oxidative stress role as a major factor in tissue toxicity of doxorubicin was further supported by many authors [7, 40-42].

Yagmurca et al. [7] found that administration of DXR caused an increase in MDA level, an important indicator of lipid per oxidation, and reduction in CAT and GPX activities with no effect on SOD activity accompanied with numerous renal lesions including marked glomerular sclerosis, tubule desquamation, loss of brush border and untidiness of basement membranes of the urinary and glomerular tubules. Also, Quin et al. [18] registered increased level of malondialdehyde (MDA), nitrogen oxide (NO) and decreased activities of super-oxide dismutase (SOD), catalase and glutathione reduced form (GSH). However, Kalendar et al. [9] have reported hepatic vacuolization and swelling of the mitochondria and pyknosis of nuclei of hepatocytes associated with elevated MDA level, SOD, GPX and CAT enzyme activities, after doxorubicin injection.

Another accepted radical producing mechanism in DXR-toxicity might be the NO producing system [43, 44]. Nitric oxide is one of the reactive mediators released through endothelial cells, macrophages, hepatocytes, leucocytes and Kupffer cells in response to different stimuli [7, 28, 45].

High NO production might be the reason of inflammatory reaction, have reported in the present study, where many leucocytes have noticed in liver and kidney and so were the iron-laden macrophages in hepatic tissue. Agoura et al. [28] have mentioned: if a large amount of NO is produced, it reacts with super oxide anion to produce peroxynitrite, which is also a toxic radical to tissue. This could explain the decrease in both catalase and super oxide dismutase, reported herein.

Recently, DXR cytotoxicity is also attributed to its capability to induce apoptosis [46]. Apoptosis is an important mechanism regulating cell number and their development in different organs and tissues [47].

In the current study, DXR administration resulted in increased level of apoptotic index (Bax/ Bcl-2), compared to the control mice. The high level of apoptotic index could be attributed to the increase in Bax expression and decrease in Bcl-2 level in liver and kidney, while it could be the result of decreased expression of pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) proteins in heart.

In mammalian cell, two main ways of apoptotic signals have involved, the external and internal (mitochondria) ways. The internal way is a response to factors causing DNA damage and is often connected to pro-apoptotic proteins including Bax protein [47]. Bax protein promotes apoptosis by enhancing susceptibility to apoptotic stimuli while Bcl-2 oncoprotein regulates programmed cell death [48] by providing a survival advantage to rapidly proliferating cells.

Wang et al. [49] have reported cardiomyocyte apoptosis after DXR administration and related it to Bax induction and caspase 3 activations. Also, Redrycz et al. [47] registered high level of apoptotic index (Bax/ Bcl-2) in the liver of rat foetuses whose mothers have treated with a single dose of Adriamycin along time before pregnancy. They have attributed this increase to the lack of Bcl-2 protein expression and the intensive expression of the Bax protein. Furthermore, Quin et al. [18] have recorded Bcl-2 down-regulation in a liver of a rat tumour model have treated with Adriamycin (doxorubicin).

As it has pointed out in previous studies, it has known that the combination of any anthracycline together with a potent antioxidant [18, 50, 51], and anti-inflammatory compounds [7, 30] may be the appropriate approach to reduce its toxic side effects.

Another strategy for the prevention of anthracycline-induced toxicity is based on the use of compounds with anti-apoptotic activity [3, 52-54]. Therefore, novel therapeutic agents with improved antioxidant, anti-inflammatory and anti-apoptotic efficacy seemed to be considerable for clinical approach.

Rosemary (Rosmarinus officinalis), a common used spice and flavouring agent, has been known for many years as an antioxidant against oxidant conditions that cause tissue injury [55-58].

Rosmarinic acid, diterpenoids, carotenoids and alpha-tocopherol [59-61] have been documented as principal antioxidant constituents of water rosemary extract.

The rosemary extracts have been shown to inhibit lipid peroxidation and free radicals generation in vitro [61] and in vivo [58, 62] in addition to their abilities to scavenge peroxy radicals [26, 55-57, 63]. Moreover, it has been proposed that rosemary and its constituents have a therapeutic potential in treatment or prevention of inflammatory diseases,

hepatotoxicity, renal toxicity and heart diseases [21, 64].

Recently, rosemary leaves aqueous extract has proved significant efficacy towards decreasing apoptosis in crypts of small intestine of irradiated mice [26].

In the present work, administration of rosemary leaves aqueous extract prior to doxorubicin at doses of 25, 125, 250 and 375 mg/ kg b. wt greatly abated the oxidative stress, compared to the doxorubicin group, which has evidenced by a significant depletion in lipid per-oxidation (LPO) and a significant elevation in endogenous antioxidant system. The depletion of LPO level was non-significant in liver with the higher dose (375 mg/ kg b. wt.) of RE and so was the elevation in GSH and SOD level, while in heart the change in catalase value was non-significant.

The reduced oxidative stress have reported, herein, has accompanied with significant decrease in the apoptotic index in liver with the lower two doses (25 and 125 mg/ kg b. wt.) of RE and a significant increase with the higher dose (375 mg/ kg b. wt.). The lowered apoptotic index in this organ was due to the decrease in Bax protein expression and increased in Bcl-2 protein level, while its increase was a result of the lack of changes in Bax protein level and down-regulation of Bcl-2 expression.

In kidney, the RE administration prior to DXR showed significant decreased in the apoptotic index only with the second dose (125 mg/kg b. wt.), compared to the doxorubicin-injected mice. This ameliorative effect of the second dose has related to the decrease in Bax activity and increase in Bcl-2 regulation.

In heart, all doses of RE caused significant decrease on the apoptotic index, relative to the doxorubicin group. This decrease could be attributed to the down-regulation of Bax protein and up-regulation of Bcl-2 except in the case of the third dose (250 mg/ kg b. wt.) where up-regulation of both proteins has noticed. The expression rate of Bcl-2 may exceed that of Bax.

From the current above-mentioned results, it could be concluded that the histological lesions related to doxorubicin in liver, kidneys and heart tissues have mainly related to the increased apoptotic index and inflammatory response and then to the oxidative stress. RE has the ability to significantly attenuate the doxorubicin-related toxic effects via more than one mechanism including the powerful inhibition of lipid per-oxidation, the stimulation of the synthesis of cellular antioxidants, the decrease of the inflammatory response and the reduction of the apoptotic index. The efficacy of the tested doses of RE in improving doxorubicin-deteriorated effects has organ-specific. The most potent dose of RE to abate the lesions in all the examined tissues was 125mg/ kg b. wt and the less effective was 375 mg/ kg b. wt.

Acknowledgement

None

Conflicts of Interest

The authors declare that they have no conflicts of interest in this article.

Author's Contribution

The design, biochemical part and writing the manuscript were done by both authors, while the histological and immunohistochemical studies were done by ARR.

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