The Ameliorative Effect of Ellagic Acid and Rosemarinic Acid against Cardio-nephrotoxicity Induced by Doxorubicin in Rats

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***Abstract*-** This study aimed to evaluate the possible ameliorative effect of ellagic acid, and rosemarinic acid on doxorubicin-induced cardiotoxicity and nephrotoxicity in rats. Therefore, male Sprague Dawley rats were divided into eight groups, control group, ellagic acid (10mg/kg, p.o.) group, rosemarinic acid (75mg/kg, p.o.) group, combined ellagic and rosemarinic acid for 14 days. Cardio-nephrotoxicity was induced by doxorubicin injection (5mg/kg, i.p.) every 3 days. Doxorubicin administration started on the fourth day of ellagic or/and rosemarinic acids treatment. Animals were scarified on 15th day. As a result of doxorubicin administration serum lactate dehydrogenase, creatinine kinase-MB, troponin-I, urea, and creatinine were significantly elevated. Cardiac, as well as, renal glutathione and catalase were significantly decreased, while tissue lipid peroxidation, tumor necrosis factor-α, caspase-3 were significantly increased. Administration of ellagic acid and rosemarinic acid either alone or in combination significantly improved all measured parameters. Histopathological examination confirmed the biochemical results. In conculison, ellagic acid, rosemarinic acid and their combination possess promising protective effects against doxorubicin-induced cardio-nephrotoxicity; effects that might be attributed to their antioxidant activities.

***Index Terms*-** cardiotoxicity, doxorubicin, ellagic acid, neophrotoxicity, rosemarinic acid.

I. INTRODUCTION

Doxorubicin (DOX) is an anticancer drug which belongs to anthracycline antibiotics and is being used widely for treatment of various hematological and solid tumor malignancies. The clinical use of this drug has been seriously limited by undesirable side effects especially dose-dependent myocardial injury, leading to potentially lethal congestive heart failure (**Choi et al., 2007**). In addition to limited use due to its cardiac, renal, pulmonary, testicular, and hematological toxicities (**Singal et al., 1987**, **Fadillioglu et al., 2003).**

Free radical generation and mitochondrial dysfunction iron-dependent oxidative damage of biological macromolecules, and protein oxidation are thought to contribute to DOX-induced cardiac failure and renal injury (**Hiona et al., 2011**, **Liu et al., 2007**). DOX induced renal injury in rats manifested by increased glomerular capillary permeability and tubular atrophy (**Wapstra et al., 1999**). Administration of DOX caused an imbalance between free radicals and antioxidants. The disturbance in oxidant-antioxidant systems which has been demonstrated with lipid peroxidation and protein oxidation resulted in tissue injury (**Karaman et al., 2006**). Free radicals production and/or NO release induced by DOX are entirely responsible for the DOX-induced toxicity (**Radi et al., 1991**). Mitochondria have been defined as one of the targets in DOX-induced subcellular damage in the tissue. DOX cytotoxic effect on malignant cells, as well as, its toxic effects on various organs is thought to be related to its DNA intercalation and cell membrane lipid binding activities. It has been suggested, that DOX-induced apoptosis may be an integral component of the cellular mechanism of action responsible for its therapeutic effects, toxicities, or both (**Priestman, 2008**).

Ellagic acid (EA) is a naturally polyphenolic phytonutrient found in wide varieties of fruits, berries and nuts. EA has received particular attention because of its extensive array of biological properties including potent antioxidant, anticancer and antimutagen (**Atessahin et al., 2007**). Ellagic acid showed free radical scavenging action, chemopreventive, antiapoptotic, anti-inflammatory, gastroprotective, anti-cataractogenetic, cardioprotective ulcer healing, antifibrotic, antidiabetic, hypolipidemic, antiatherosclerotic and estrogenic/antiestrogenic properties (**Warpe et al., 2015**). Although the exact molecular mechanism of EA is unknown, its potent scavenging action on OH might be responsible for these effects (**Priyadarsini et al., 2002**) polyphenols of EA have attracted considerable attention as agents that protect cells or molecules from oxidative myocardial injury.

Rosmarinic acid (RA) is a naturally occurring hydroxylated compound widely distributed in Labiatae herbs, which include rosemary, sweet basil, and perilla. Rosmarinic acid is an ester of caffeic acid and 3,4- dihydroxy-phenyl acetic acid. It is commonly found in Boraginaceae species and in the Nepetoideae subfamily of the Lamiaceae. It is also found in species of other higher plant families, and in some fern and hornwort species (**Petersenand, 2003**). Rosmarinic acid has a number of interesting biological activities, for example, antioxidant, antimutagen, antibacterial, and antiviral effects (**Gamaro et al., 2011).**This work aimed at evaluation of the possible ameliorative effect of EA, RA or its combination on the cardiotoxicity and nephrotoxicity induced by DOX.

II. MATERIAL AND METHODS

Animals

Adult male Sprague-dawley rats (200-250g) were obtained from National Organization for Drug Control and Research (NODCAR). Animals were maintained under standard conditions of humidity with regular light dark cycles and free access to food and water.

Drugs and Chemicals

Doxorubicin vial (50mg/25ml) was purchased from Ebewe Pharma, Austria Company and used in dose (5mg/kg day i.p every 3 days 4 times) (**Jensen et al., 1984**). Ellagic acid (LKT Laboratories, INK) was used in dose (10mg/kg/day p.o for 14 days) (**Girish et al., 2014**). Rosemarinic acid (Carbo Synth Company, Berkshire, UK) was used in dose (75mg/kg/day p.o for 14 days) (**Tavafi et al., 2011**).

Experimental design

Eighty male adult Sprague-Dawley rats were divided randomly into eight groups as follow:

Group (A) received corn oil orally as a vehicle.

Group (B) received ellagic acid (EA) (10mg/kg, p.o.) for 14 days.

Group (C) received rosemarinic acid (RA) (75mg/kg, p.o.) for 14 days.

Group (D) received EA (10mg/kg, p.o.) and RA (75mg/kg, p.o.) for 14 days.

Group (E) received doxorubicin (DOX) injection (5mg/kg, i.p.) every 3 days for 14 days.

Group (F) received EA (10mg/kg, p.o.) for 14 days and DOX (5mg/kg, i.p.) every 3 days started in fourth day of ellagic acid administration.

Group (G) received RA (75mg/kg, p.o.) for 14 days and DOX (5mg/kg, i.p.) every 3 days started in fourth day of rosemarinic acid administration.

Group (H) received EA(10mg/kg, p.o.) with RA (75mg/kg, p.o.) for 14 days and DOX injection (5mg/kg, i.p.) every 3 days started in fourth day of ellagic and rosemarinic acids administration.

Serum and Tissue Preparation

Twenty four hours after the last treatment, the blood was collected from retero-orbital plexus of animals was allowed to collected by leaving at undistributed at room temperature (15-30 min), then centrifuged, serum were collected for biochemical investigation. The animals were sacrificed, hearts and kidneys were removed, washed in ice saline and divided into two portions, and one was kept in 10% formalin for histopathological examination while the other in -80 $^{\circ}$C for estimating the other parameters.

Cardiac and renal biomarkers

Lactate dehydrogenase (LDH) was measured according to the method of (**Heiden et al., 1994**) Creatine kinase-MB (CK-MB) was measured according to the method of Gerhardt (**Gerhardt et al., 1979)**, standards commercial kits provided from (CHRONOLAB, SPAIN). Troponin-I were determined using the method of **(Arad et al., 2005)**, a standard commercial kit from (Glory Science, USA). Serum urea (BUN) was measured according to the method of **(Tobacco et al., 1979)** and creatinine (Cr) was measured according to the method of **(Bower et al., 1980)** (Biodiagnostics, Cairo, Egypt).

Determination of oxidative stress markers in heart and kidney

Lipid peroxidation (MDA) was measured according to the method of **(Satoh, 1978**).Tissue glutathione (GSH) was measured according to the method of (**Beutler et al., 1963)**. Catalase (CAT) was measured according to the method of **(Aebi 1983)**. (Bio diagnostics, Cairo, Egypt).

Determination of TNF-α and caspase-3 in heart and kidney

Tumor necrosis factor-α (TNF- α) was measured according to the method of **(Taylor, 2001)** (Assaypro, LLC). Caspase-3 was measured according to the method of **(Chang et al., 2003)** (Glory Science, USA).

Histopathological Examination of heart and kidney

Autopsy samples were taken from the heart and kidney of rats in different groups and fixed in 10% formaldehyde saline for 24 hours. Washing was done in tap water then serial dilutions of alcohol (methyl, ethyl and absolute ethyl) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56 degree in hot air oven for twenty four hours. Paraffin bees wax tissue blocks were prepared for sectioning at 4 microns thickness by slidge microtome. The obtained tissue sections were collected on glass slides, deparaffinized and stained by hematoxylin & eosin stain for routine examination through the light electric microscope (**Bancroft et al., 1996**).

Statistical analysis

Data were expressed as the mean ± SEM, statistical analysis was carried out by one-way ANOVA followed by Tukey Kramer multiple comparison test using Graph Pad Prism version 5 (GraphPad Software Inc., La Jolla, CA, USA). The p value was considered significant when $p< 0.05$.

III. RESULTS

Effect on cardio-renal biomarkers

Rats treated with DOX showed a significant ($p < 0.05$) increase in serum levels of LDH, CK-MB, and troponin-I, compared to the corn oil control rats. On the other hand, oral treatment with either EA, RA or their combination significantly ($p < 0.05$) decreased the elevated levels of serum LDH, CK-MB and troponin-I when compared to DOX group (Table 1). DOX treated rats showed a significant ($p < 0.05$) increase in serum levels of BUN and Cr, when compared to the corn oil control rats. On the other hand, oral treatment with EA, RA, and their combination significantly ($p < 0.05$) decreased the elevated level of BUN and Cr when compared to DOX group (Table 1).

Effect on tissue oxidative stress parameters

Table 2 showed that DOX treated rats showed a significant increase ($p < 0.05$) in tissues lipid peroxidation when compared to the corn oil control rats. On the other hand, oral treatment with EA, RA, and their combination significantly ($p < 0.05$) decreased the elevated level of MDA when compared to DOX group. DOX treated rats showed a significant reduction ($p < 0.05$) in GSH content, as well as, CAT activity in tissue homogenates when

compared to the corn oil control rats. Treatment with EA and RA significantly ($p < 0.05$) elevated both GSH and CAT when compared to DOX group (Table 2).

Effect on tissue TNF-α and caspase-3

Doxorubicin treated rats showed a significant increase ($p < 0.05$) in TNF-α and caspase-3 levels in the heart, as well as, kidney compared to the corn oil control rats. Oral treatment with EA, RA, or their combination significantly ($p < 0.05$) decreased the elevated level of TNF-α and caspase-3 levels in both organs compared to DOX group (Figure 1).

Histopathological finding

Concerning heart, corn oil control, EA, RA, and their combination treated rats showed normal histopathological structure (A, B, C & D). Section of heart of DOX treated rat (E) showed focal necrotic hyalinization in some myocardial bundles with lose of striation and appeared as homogenous deeply eosinophilic. Heart microscopic section of DOX group treated with EA (F) showed some improvement with mild congestion in the blood vessels. Administration of RA in DOX-treated group resulted in normal histopathological structure (G). Finally, EA and RA combination in DOX treated rats (H) showed some improvement in myocardial bundles appearance (Figure 2).

Concerning kidney, corn oil control, EA, RA, and their combination treated rats showed normal histopathological structure (A, B, C & D). Microscopic section of heart of DOX treated rat (E) showed degenerative change and noticed necrosis in the lining epithelium of the tubules at the cortex associated with focal haemorrhage in between. Kidney microscopic section of DOX group treated with EA (F) showed mild congestion was noticed in the glomerular tufts. Administration of RA in DOX-treated group resulted in cortical tubules showed degeneration in the lining epithelium (G). Finally, EA and RA combination in DOX treated rats (H) improved the histological appearance of kidney except for mild congestion in the cortical blood vessels (Figure 3).

IV. DISSCUSION

Doxorubicin is a widely used anticancer. Its chief dose-limiting side effects are myocardial and renal injuries which require a reduction of dose or discontinuation of the treatment. The present study was designed to investigate whether EA or/& RA administration before DOX could afford a protection against DOX induced cardio-nephrotoxicity.

In the present study, DOX induced significant elevation in serum CK-MB, LDH, troponin-I, as well as, BUN and Cr. These results were in agreement with **(Mantawy et al. 2014**, **Saparano et al., 2002)** who mentioned that the magnitude of CK-MB, LDH and troponin-I activities in blood after myocardial injury reflects the extent of damage in its musculature. The elevated serum BUN and Cr levels suggested the reduction of glomerular filtration rate (**Hassan et al., 2014**).

In the present work, DOX administration led to a significant increment in MDA level, reduction in GSH content and CAT activity in both organs. These results agreeed with (**Jadhav et al., 2013)** who mentioned that, DOX induced heart and kidney damage was caused by disturbing antioxidants defense mechanism, augmenting membrane lipid peroxidation and free radicals formation. Moreover the impairment of oxidant-antioxidant system, initiates peroxidation of membrane bound polyunsaturated fatty acid and protein oxidation, lead to alterations in permeability of myocytes and nephrons, intracellular calcium overload causing finally irreversible damage to tissue. Two different mechanisms have been identified; the first one is formation of semiquinone type free radical molecules leading to ROS generation (**Jadhav et al., 2013**). The second pathway includes a non-enzymatic reaction, involving DOX-iron reaction **(Injac et al., 2008**). The reduced GSH level might be due to GSH consumption in DOX-induced lipid peroxidation **(Ashour et al., 2011)**. In the present study, the TNF-α and caspase-3 elevation in the target organs by DOX treatment are in agreement with **(Mantawy et al. 2014, Hassan et al., 2014)**. The production of ROS induces apoptosis and cytochrome-c release in the cytosol of the DOX-treated animals which interacts with procaspase-9, leading to the generation of active caspase-9, then, caspase-3 (**Green et al., 1998).** Herein, cardiotoxicity and nephrotoxicity induced by DOX is manifested by altered histopathological features including focal necrotic hyalinization in some myocardial bundles, as well as, degenerative change in the lining epithelium of the cortex tubules, these findings agreed with **(Jadhav et al., 2013, Argun et al., 2015).**

In the current study, concomitant administration of EA and DOX significantly reduced serum LDH, CK-MB, troponin-I. Decreased levels of serum troponin-I by EA indicates its protective effect on the myocardium by reducing the extent of the myocardial damage **(Kannan and Quine, 2011, Yüce et al., 2007**). Serum BUN, Cr, as well as, tissue MDA, TNF-α and caspase-3 were also decreased by EA, confirming the studies of **(Atessahin et al., 2007, Lin et al., 2013**, **El-Garhy et al., 2014**). In contrast, it caused significant rise in GSH and catalase activities and histopatological examinations, therefore, protect cells against cardiotoxicity and nephrotoxicity.

The mechanism of EA responsible for these effects may be due to its potent scavenging action on both superoxide anion and hydroxyl anion as mentioned by Iino et al., 2001. The antioxidant effect of EA may be involving dual actions: direct action on free radical scavenging and indirect action through the induction of antioxidant enzymes (Palani et al., 2015). In addition, EA could inhibit PGE2 produced and reduced the COX-2, thus, decreased TNF-α level (Mansouri et al., 2015).

In the same manner, concomitant administration of RA with DOX significantly adjust cardio-nephrotoxicity biomarkers, these results were supported by **Chlopcíková et al. 2004**, **Azab et al. 2014** and **Rasha et al. 2010**. In addition, RA improved the histological alterations caused by DOX in both heart and kidney. The observed results with RA were shown by (**Rasha and Abdella, 2010**) in heart and kidney. The major proposal for action of RA is to intercept free radicals and protect cellular molecules from oxidative damage through stimulation of the endogenous antioxidant defense system and the ability to donate electrons to reactive radicals, converting them to more stable form (**Sancheti et al., 2007**, **Moreno et al., 2006**). RA can inhibit lipoxygenase, cyclooxygenases, and interfere with the complement cascade, thus, inhibiting the expression of inflammatory cytokines **(Gamaro et al., 2011).** The

antiapoptotic activity of RA is through the reduction of active caspase-3 expression (**Rasha and Abdella, 2010**).

V. CONCLUSION

In conclusion, EA or/and RA protected heart and kidney tissues in rats through their antioxidant, anti-inflammatory and antiapoptotic activities. Thus, they represent a potential candidate to protect against cardio-nephrotoxicity, which is a major dose-limiting problem during DOX therapy.

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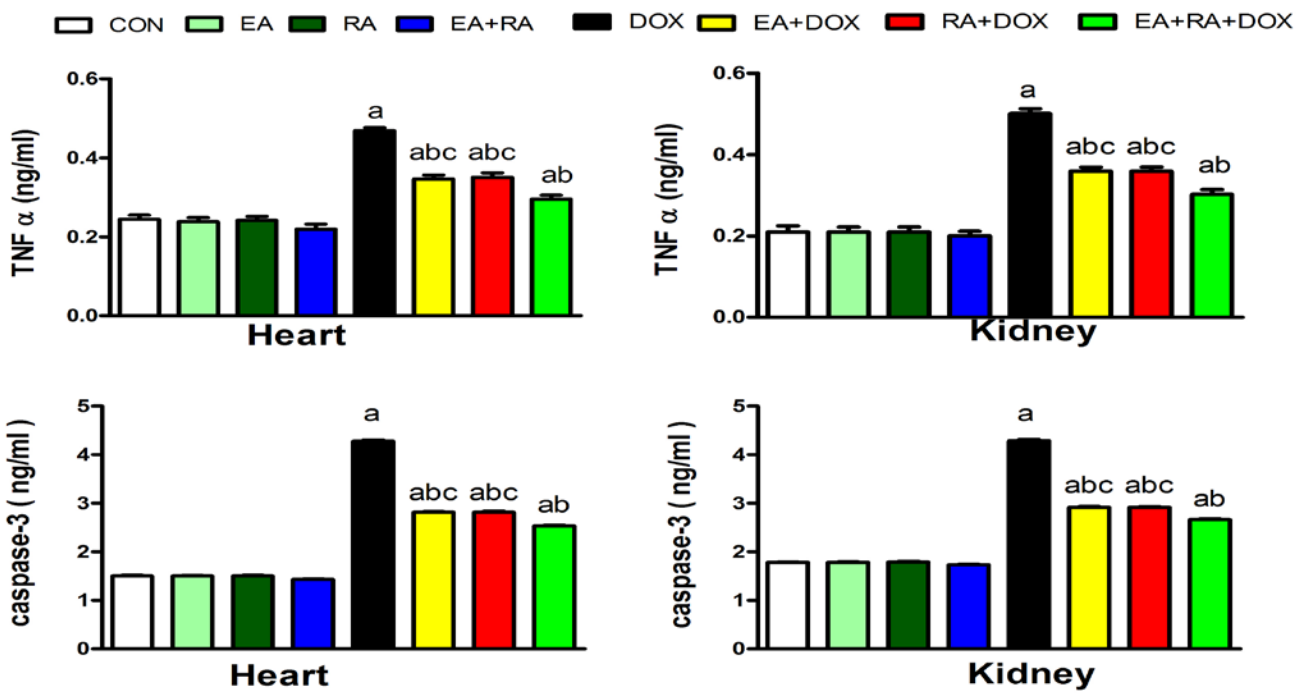

FIGURE 1. Effect of Ellagic acid (EA), Rosemaneric acid (RA) and their combination on tissue TNF-α and caspase-3 in rats treated with Doxorubicin (DOX). Data were expressed as the mean ± SEM, a: means significant with control, b: mean

significant with DOX, c: mean significant with DOX +EA+ RA ($p < 0.05$). Statistical analysis was carried out by one-way ANOVA followed by Tukey's multiple comparison test (n=10).

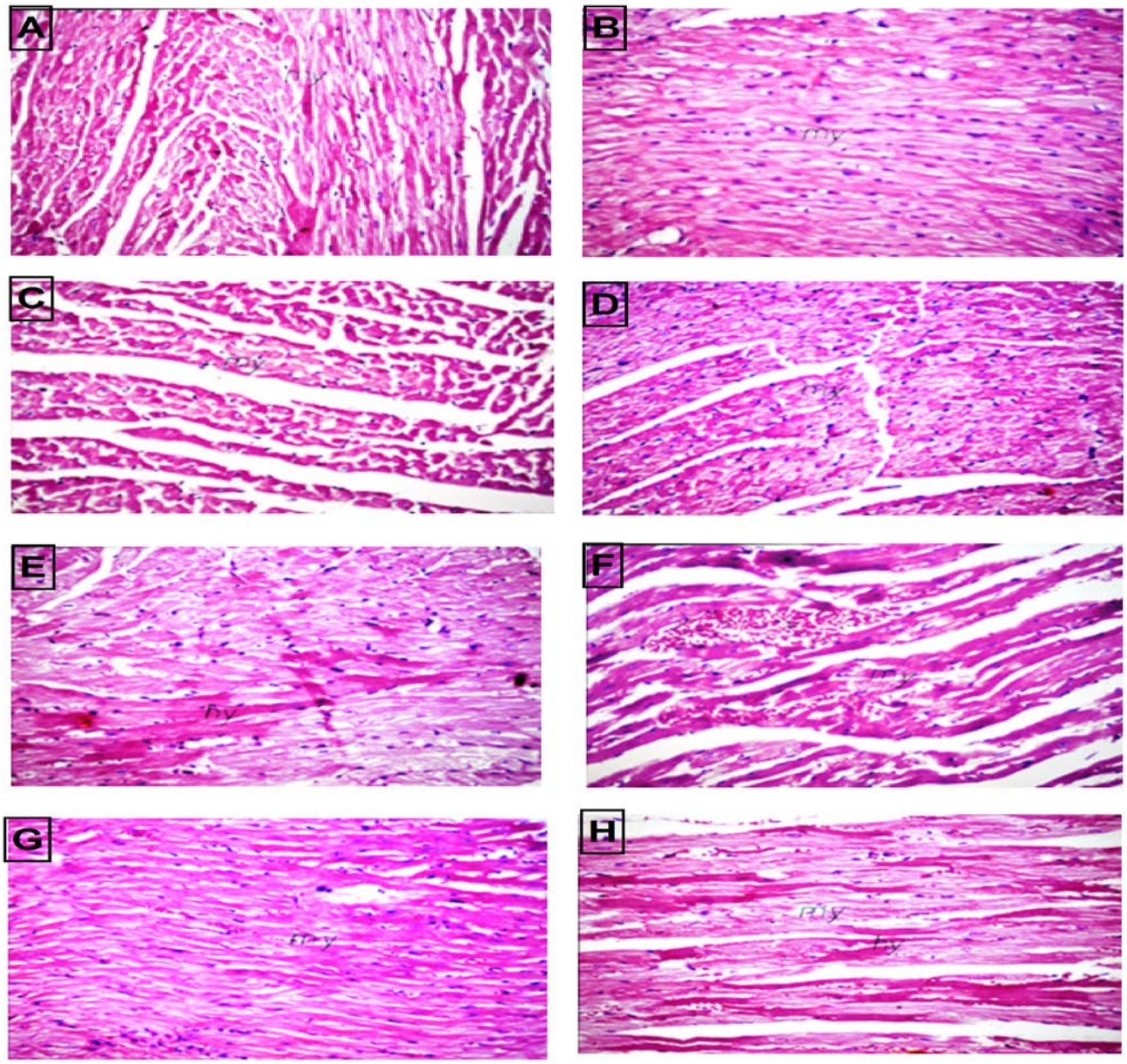

FIGURE 2. Heart samples of the groups (A,B,C,D), shows normal histological structure, (E) showing focal necrosis in some myocardial bundles with lose of striation, (F) showing congestion in the blood vessels, (G) showing normal histopathological structure, (H) Zenkers hyalinization was detected in focal manner of some myocardial bundles.

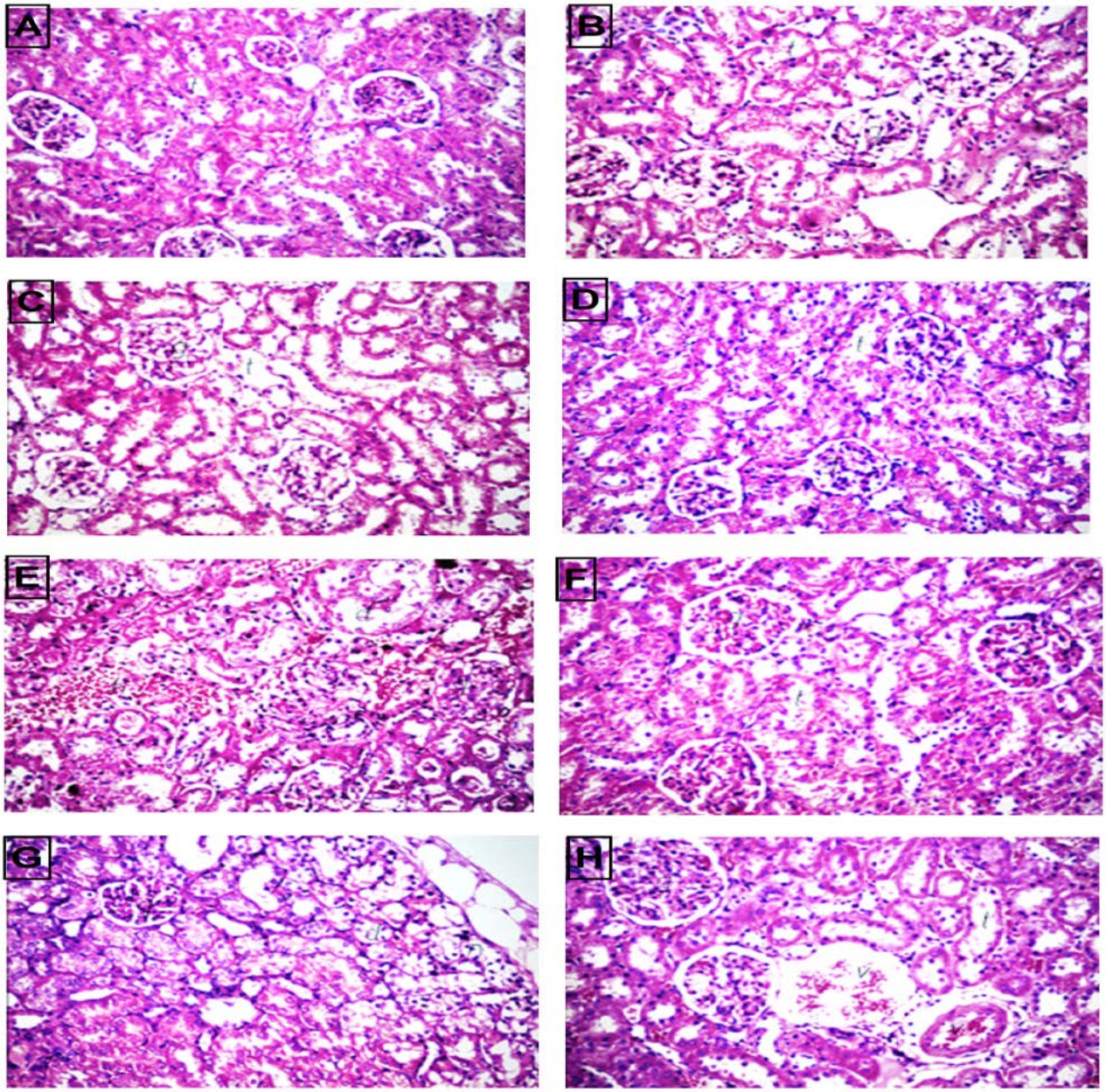

FIGURE 3. Kidney samples (groups A,B,C,D), shows normal histological structure, (E) showing degeneration and necrosis in lining epithelium of the tubules associated with focal haemorrhage in between (F) Mild congestion in glomerular tufts (G) cortical tubules showed degeneration in the lining epithelium (H) mild congestion in the cortical blood vessels.

TABLE 1. Effect of administration of Ellagic acid, Rosemaneric acid and their combinations on serum levels of LDH, CK-MB, Troponine-I, urea and creatinine in rats treated with Doxorubicin.

Groups	Cardiotoxicity			Nephrotoxicity	
	LDH (u/l)	CK-MB (u/l)	Troponine-I (ng/l)	Urea (mg/dl)	Creatinine (mg/dl)
Control	652.6 ± 6.39	49.00 ± 0.96	19.99± 0.66	26.62 ± 0.07	0.650 ± 0.011
EA	651.0 ± 1.00	47.40 ± 1.05	18.99± 0.45	25.06 ± 0.47	0.642 ± 0.020
RA	651.6 ± 1.25	48.68 ± 0.75	19.74± 0.27	25.45 ± 0.17	0.632 ± 0.022
EA +RA	644.1 ± 1.48	45.81 ± 0.63	18.56± 0.32	24.56 ± 0.12	0.611 ± 0.015
DOX	1335 ± 1.11[a]	144.1 ± 1.11[a]	36.71± 0.20[a]	52.33 ± 0.77[a]	3.206 ± 0.054[a]
DOX + EA	979.9 ± 2.60[a,b,c]	85.49 ± 1.96[a,b,c]	26.99± 0.31[a,b,c]	39.99 ± 0.37[a,b,c]	1.954 ± 0.031[a,b,c]
DOX + RA	987.3 ± 1.19[a,b,c]	89.11 ± 0.45[a,b,c]	27.80± 0.16[a,b,c]	40.41 ± 0.70[a,b,c]	1.893 ± 0.039[a,b,c]
DOX+EA+RA	902.4 ± 1.36[a,b]	69.64 ± 1.82[a,b]	24.97± 0.05[a,b]	37.62 ± 0.50[a,b]	1.624 ± 0.016[a,b]

Data are expressed as means ± SEM of eight rats per group. a: means significant compared to control, b: means significant compared to DOX, c: means significant compared to DOX+EA+RA ($p < 0.05$). Statistical analysis was carried out by one-way ANOVA followed by Tukey's multiple comparison test.

TABLE 2. Effect of administration of Ellagic acid, Rosemaneric acid and their combinations on tissue levels of MDA, GSH and CAT in rats treated with Doxorubicin

Groups	Cardiotoxicity			Nephrotoxicity		
	MDA (nmol/g.tissue)	GSH (mg/g.tissue)	CAT (u/g)	MDA (nmol/g.tissue)	GSH (mg/g.tissue)	CAT (u/g)
Control	9.26±0.42	23.64±0.54	0.584±0.014	9.14± 0.34	23.77±0.79	0.587±0.014
EA	8.97±0.08	23.77±0.11	0.586±0.013	8.64± 0.22	23.69±0.24	0.588±0.013
RA	9.01±0.03	24.01±0.16	0.585±0.011	8.99 ± 0.08	24.02±0.23	0.587±0.015
EA+RA	8.40±0.11	24.32±0.05	0.591±0.012	8.53 ± 0.07	24.92±0.22	0.595±0.014
DOX	20.30±0.71[a]	11.12±0.02[a]	0.131±0.012[a]	17.32± 0.16[a]	11.90±0.76[a]	0.143±0.015[a]
DOX+EA	13.96±0.75[a,b,c]	18.31±0.14[a,b,c]	0.349±0.011[a,b,c]	12.99± 0.29[a,b,c]	18.22±0.21[a,b,c]	0.348±0.015[a,b,c]
DOX+RA	14.01±0.52[a,b,c]	18.88±0.37[a,b,c]	0.339±0.010[a,b,c]	13.17± 0.17[a,b,c]	18.68±0.37[a,b,c]	0.339±0.000[a,b,c]
DOX+EA+RA	11.93±0.16[a,b]	20.01±0.07[a,b]	0.405±0.015[a,b]	11.84± 0.04[a,b]	20.68±0.13[a,b]	0.410±0.010[a,b]

Data are expressed as means ± SEM of ten rats per group. a: means significant compared to control, b: mean significant compared to DOX, c: mean significant compared to DOX+EA+RA ($p < 0.05$). Statistical analysis was carried out by one-way ANOVA followed by Tukey's Kramer multiple comparison test.