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Apigenin attenuates doxorubicin induced cardiotoxicity via reducing oxidative stress and apoptosis in male rats

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ABSTRACT

Aims: Doxorubicin, an antibiotic belonging to anthracycline family, has been used for treatment of malignancies. Cardiotoxicity is the main adverse effect of doxorubicin. Apigenin, as a flavonoid, has antioxidant, anti-inflammatory and anti-tumoral properties. The aim of this study was the assessment of any protective effect of apigenin on cardiotoxicity induced by doxorubicin.

Main methods: 40 male Wistar rats were randomly divided into 4 groups: control, cardiotoxicity (DOX), apigenin treated group (DOX + Api 25) and apigenin group (Api 25). At the end of the experiment, the markers of cardiac function (%EF, %FS, LVIDs, LVIDd), cardiac and liver injury (LDH, CK-MB, cTn-I, ALT, and AST), cardiac apoptosis (Bax, Bcl-2 and Caspase3), cardiac oxidative stress (SOD, GSH, MDA) and cardiac fibrosis were measured.

Key findings: Apigenin improved cardiac functional parameters. The levels of cardiac and liver injury markers were significantly decreased in DOX + Api 25 compared to DOX. Treatment with apigenin caused significant decrease in percentage of cardiac fibrosis in comparison with DOX. Apigenin in DOX + Api 25 group led to significant decrease in apoptotic proteins (Casp3, Bax) and a significant increase in anti-apoptotic proteins (Bcl2). In apigenin treatment groups, SOD levels significantly increased while a significant decrease was observed in MDA. The amount of GSH in DOX + Api 25 had no significant change in comparison to control and Api 25 groups.

Significance: Apigenin reduced cardiac injuries induced by DOX through anti-fibrotic, antioxidant and anti-apoptotic properties. It seems that apigenin prevents cardiac injuries and improves cardiac function.

1. Introduction

Doxorubicin (DOX) is an anti-tumor anthracycline which is used for treatment of various malignancies such as lymphoma, solid tumors and leukemia [1]. Nevertheless, cardiotoxicity is the main adverse effect of DOX and other anthracyclines such as daunomycin, epirubicin and idarubicin. The risk of some cardiovascular diseases such as cardiac dysfunction, dilated cardiomyopathy, hypotension, tachycardia, arrhythmia and heart failure in cancer patients limits cancers treatment by DOX [2–4].

Cardiotoxicity may occur even after a single dose of drug administration or in a long period of time (from weeks to months) after cumulative dose of DOX [5]. The pathogenesis of cardiotoxicity induced by DOX isn't clear, but many studies indicate that various mechanisms such as oxidative stress, inflammation and apoptotic cell death of cardiomyocytes are involved and lead to the progression of cardiomyopathy after cumulative doses [6,7].

It is well documented that reactive oxygen species (ROS) are generated under oxidative stress conditions induced by DOX [8]. The heart is more sensitive to DOX induced lipid peroxidation, because of high energy demands. The heart also lacks the antioxidant enzymes for scavenging free radicals. Thus free radicals accumulate and cause peroxidation of lipids and destruction of cellular and mitochondrial membranes, endoplasmic reticulum, nucleic acid and intracellular macromolecules [6,9]. Several signaling pathways activated by oxidative stress can cause cardiomyocytes apoptosis [8]. In normal

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conditions, antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) detoxify free radicals. In conditions that there isn't balance between the oxidants production and the antioxidant defense systems, peroxidation of lipids occurs, as these conditions could be seen in DOX-induced cardiotoxicity [10].

Recent studies have shown that myocyte apoptosis has a major role in the pathogenesis of DOX-induced cardiotoxicity [10,11]. Apoptosis occurs via two pathways including mitochondrial (intrinsic) and death receptor (extrinsic) pathways. Cardiomyocytes apoptosis induced by DOX is probably mediated by free radicals. DOX-induced oxidative stress triggers the intrinsic mitochondria-dependent apoptotic pathway in cardiomyocytes, and in turn causes myocyte death [12].

Many studies have reported that DOX-induced cardiotoxicity has been reduced by some medicinal plants [4,13]. It seems that it is logical to research on the plant derived-natural compounds for decreasing the cardiotoxic side effects and increasing DOX chemotherapeutic effects. In recent years, a great deal of research have been conducted on the flavonoids effects in prevention and treatment of cardiovascular complications. The natural non-mutagenic plant flavone, Apigenin (Api), exists in common plants, fruits and vegetables and contains a bioactive flavonoid that has anti-inflammatory, anti-apoptotic, antioxidant and anti-carcinogenic properties (such as breast cancer, gastrointestinal cancers, skin cancer and certain hematological malignancies). Apigenin also has low levels of toxicity and it has been reported that Api exerts protective effects in other diseases related to oxidative stress process such as cardiovascular and neurological diseases [14–17]. Recent studies have shown that foods that contain more Api significantly increase antioxidant enzymes [18], and decrease apoptosis [15,19]. The incidence of cardiovascular diseases is reduced by Api and it could decrease myocardial ischemia/reperfusion injuries via its antioxidant effects [17,20].

Chen et al., reported that Api pretreatment in Anoxia/Reoxygenation-induced myocardial injury decreased the activity and down-regulated expression of proapoptotic protein caspase-3 [19]. Apigenin regulated the contractility of the heart and restored cardiac function in the isoproterenol-induced myocardial injury in diabetic rats, and reduced the lipid peroxidation in the myocardium [21].

Due to the antioxidant and anti-apoptotic effects of apigenin and based on the DOX-induced oxidative and apoptotic damage, in the present study we aimed to assess the protective effects of apigenin in DOX-induced cardiotoxicity and assess its molecular mechanism.

2. Materials and methods

2.1. Animals

Adult male Wistar rats ($n = 40$), weighing 180–230 g, were supplied from the Experimental Animal Center of Iran University of Medical Sciences. Rats were allowed to adapt for one week before beginning the study protocol. All animals had free access to the standard rodent chow and water in animal glass cages with 12-h light-dark cycles and room temperature was controlled at 22 ± 1 °C.

2.2. Experimental design

This study was performed according to the U.S National Institutes of Health for the care and use of laboratory animals' guidelines (NIH Publication No. 85-23, revised 1996). All experimental protocols and treatments were approved by the ethics committee of Iran University of Medical Sciences (Tehran, Iran). Animals were randomly divided into four groups as the following: 1) Control group (Ctrl): received orally Carboxymethyl cellulose as a solvent of Api for 12 days. 2) Apigenin group (Api): received orally apigenin 25 mg/kg/day for 12 days via gavage (based on our results in pilot study between 25, 50 and 75 mg/kg doses). 3) Doxorubicin group (DOX): received a cumulative dose of DOX (12 mg/kg intraperitoneally (i.p)) in 6 equal 2 mg/kg doses every 48 h for 12 days. 4) Doxorubicin + Apigenin group (DOX + Api): received orally apigenin 25 mg/kg/day for 12 days via gavage, starting 1 h after the first DOX injection for 12 days. Doxorubicin and apigenin were purchased from Exir Nano Sina (Iran) and Aktin Chemical (China) companies respectively. All primary and secondary antibodies were prepared from Biorbyt Company (UK).

2.3. Echocardiography

12 days after treatment protocol and before sacrificing animals, cardiac function was monitored and assessed via echocardiography. For sedation during echocardiography, rats were anesthetized with low dose of ketamine (10–20 mg/kg i.p). The chest was shaved and then animals positioned in a supine position. M-mode transthoracic echocardiography by an echocardiographic machine equipped with 12 MHz transducer linked to an ultrasound system (SSD-5500; Aloka, Tokyo, Japan) was performed in all animals by an echocardiographist who was blind to animal treatment. The probe with gel was placed in the left parasternal position. Acquired images were used for calculation of left ventricular function. The percent of ejection fraction (%EF), percent of Fractional shortening (%FS), left ventricular inner diameter in end diastole (LVIDd) and left ventricular inner diameter in end systole (LVIDs) were calculated from three cardiac cycles.

2.4. Blood sampling

After echocardiographic recording animals were weighed and blood samples were collected from heart at day 12. The samples were centrifuged at 5000 rpm, 4 °C, for 15 min; the serum was separated and stored at −70 °C until biochemical analysis including: creatine kinase-MB (CK-MB), lactate dehydrogenase (LDH), cardiac troponin I (cTnI), alanine aminotransferase (ALT or SGPT) and aspartate aminotransferase (AST or SGOT).

2.5. Collection of heart specimens

After blood sampling animals were sacrificed under deep anesthesia. The chest was opened and the heart was excised, washed in normal saline and weighed to calculate the heart weight to body weight ratio (HW/BW mg/g). In some animals ($n = 5$ in each group) heart tissues were cut into two section, fixed in 10% formalin in phosphate buffer and processed through histological methods and then embedded in the paraffin. Several 6 μm transverse sections were prepared from paraffin embedded tissues by microtome and stained with hematoxylin and eosin (H&E) for histological examination, Masson's trichrome for measuring fibrotic area and immunohistochemical staining for determining cardiomyocytes apoptosis. In other animals ($n = 5$ in each group) the heart tissues were stored in −80 °C before they were used. Frozen samples thawed before homogenization in phosphate buffered saline (PBS) for measuring cardiac levels of malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GSH).

2.6. Measurement of LDH, CK-MB, cTn-I, ALT, and AST

At day 12, serum levels of CK-MB and LDH (markers of myocyte necrosis) were measured with specific CK-MB and LDH kits (Pars Azmoon, Tehran, Iran) by auto analyzer (Roche Hitachi Modular DP Systems; Mannheim, Germany) according to the manufacturer's instructions. The measured values are presented in IU/l. Serum levels of cardiac troponin-I (cTn-I) as a specific biomarker of cardiac injury [22] were measured by a specific kit purchased from Monobind Inc. (Lake Forest, California, USA). The measured value for cTn-I was presented in ng/ml. Serum levels of ALT and AST as a specific biomarker of liver injury were measured by a specific kit purchased from Monobind Inc. (Lake Forest, California, USA).

2.7. Measurement of myocardial fibrosis

For measuring fibrosis, 6 μm transverse sections were deparaffinized and stained with Masson's trichrome (Sigma-Aldrich Co., MO, USA). Then photographs were prepared with a digital camera. The collagen volume as a representative of myocardial fibrosis was calculated by Photoshop software in the heart sections (Ver. 7.0, Adobe System, San Jose, CA, USA) by measuring the pixels of fibrotic area. Myocardial fibrosis was expressed as a mean percentage of fibrotic area to left and right ventricular area (%) in 5 slides in each heart.

2.8. Measurement of MDA

Cardiac MDA level (marker of lipid peroxidation) was assessed by reaction with thiobarbituric acid (TBA) at 90–100 °C [23]. Reaction between MDA or MDA-like substances and TBA produce a pink pigment which has a maximum absorption at 532 nm. Briefly, 50 μl of supernatant, 1 ml of TBA and 1 ml of trichloroacetic acid (TCA) [0.75% TBA: 30% TCA] were mixed, then placed in boiling water bath for 90 min, then cooled and centrifuged at 4 °C for 10 min at 3000 rpm. The supernatant absorbance was measured against a reference blank at 532 nm by spectrophotometer. 1,1,3,3-Tetramethoxypropane (Sigma Chemicals, USA) was used as a standard MDA. The results were expressed as μmol/l.

2.9. Measurement of SOD

Cardiac superoxide dismutase (SOD) activity was detected biochemically. The method used to determine SOD activity in homogenized heart samples is based on the fact that the autoxidation of pyrogallol is inhibited by SOD. One unit of SOD is generally defined as the amount of enzyme that inhibits the autoxidation of pyrogallol by 50%. The activity of SOD was measured spectrophotometrically at 420 nm [24].

2.10. Assessment of apoptotic markers

For apoptosis assessment, immunohistochemical staining of 6 μm cardiac sections was done on day12 after treatment with Bax, Bcl-2 primary antibodies. Immunofluorescence staining was done with Caspase-3primary antibodies. Number of Bax, Bcl-2 and Caspase-3 positive cells was determined from the stained sections by counting the number of them using a light microscope under a high power field (×400), and images of each section were created by digital camera. 6 random fields within each section of 5 experimental hearts from each group were counted and averaged in a blinded fashion. The number of Bax, Bcl-2 and Caspase-3 positive cells in each field were averaged and expressed as the number of positive cells per field.

2.11. Statistical analysis

Data are presented as mean ± SEM. Statistical analyses were performed by GraphPad Prism software (Version 5.00). One-way analysis of variance (ANOVA) was used to compare differences among the experimental groups followed by Tukey post hoc test. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Mortality and survival

There was no mortality in control group from the beginning of study to day 12. Totally four animals died during protocols; three animals in DOX-intoxicated group (3/10) and one in DOX + Api treated group (1/10). (Table 1).

3.2. Changes in body weight and heart weight

Statistical analysis showed that treatment with DOX significantly decreased body weight (BW) in DOX group compared to Ctrl and Api groups ($P < 0.001$ and $P < 0.01$, respectively, Table 1). 12 days treatment with Api 25 in DOX-intoxicated animals prevented BW reduction as compared with DOX group ($P < 0.05$). Heart weight (HW) in the DOX group decreased in comparison with Ctrl group and Api-only animals ($P < 0.001$). Treatment with Api in DOX + Api group prevents HW reduction compared with DOX group ($P < 0.05$). Our results also showed that there were, however, decreases in heart weight to body weight (HW/BW) ratio in the DOX-treated animals as compared with Ctrl animals ($P < 0.05$).

3.3. Echocardiographic results

Statistical analysis of echocardiographic results showed that in DOX group percent of EF and FS decreased significantly compared to control and Api-only groups ($P < 0.001$). Treatment of DOX-intoxicated animals with Api 25 mg/kg could reverse these changes in comparison with DOX group ($P < 0.001$). Our results also showed that LVIDd and LVIDs in DOX group increased significantly as compared to control group ($P < 0.01$ and $P < 0.001$ respectively). Treatment with Api in DOX-intoxicated animals significantly decreased LVIDd and LVIDs compared with those in DOX group ($P < 0.05$ and $P < 0.01$ respectively, Table 2 Fig. 1).

3.4. Biochemical myocardial injury markers

The serum markers of myocardial injury (LDH, CK-MB and cTn-I) and liver injury (ALT, AST) were analyzed as shown in Table 3. Serum levels of LDH, CK-MB, cTn-I, ALT and AST were significantly elevated in DOX- treated group in comparison with control and Api-only treated groups ($P < 0.001$). 12-day treatment of DOX-intoxicated animals with Api 25 mg/kg significantly reduced serum LDH, CK-MB, cTn-I, ALT and AST levels, as compared to DOX treated animals. Statistical analysis also showed that Api only-treated animals did not show any significant changes in serum levels of LDH, CK-MB, cTn-I, ALT and AST levels when compared to the control group.

3.5. Effects of Api on myocardial fibrosis

To evaluate the effect of Api in DOX-induced fibrosis, we measured ventricular collagen deposition at day 12. Staining with Masson's trichrome and statistical analysis of data showed that fibrosis significantly increased in DOX animals compared to control and Api groups ($P < 0.001$). As shown in Fig. 2A and B, 12-day oral treatment with Api significantly reduced interstitial fibrosis as compared with DOX group (20.42 ± 2.7% vs. 42.74 ± 3.79% in DOX, $P < 0.001$). Our results also showed that in DOX + Api group, fibrotic area was higher than control group ($P < 0.05$). Moreover, there was seldom any collagen in control and Api-only groups (Fig. 2).

3.6. Effect of Api on MDA, SOD and GSH levels

MDA is a product of lipid peroxidation and it used as a biomarker of cardiac oxidative injury. Our results showed that induction of cardiotoxicity with DOX significantly increased MDA levels at day 12 in comparison with control group ($P < 0.001$, Fig. 3A). Api treatment for 12 days in Dox intoxicated animals significantly decreased MDA levels in comparison with DOX group ($P < 0.001$), suggesting the possible antioxidant role of Api against myocardial oxidative injury induced by DOX. Fig. 3A also shows that there are no significant differences between Api-only treated and control animals.

SOD, an antioxidant enzyme, can be changed by a variety of oxidative damages. As shown in Fig. 3B, induction of cardiotoxicity with

Table 1
Mortality rate and changes in body weight, heart weight and heart weight/body weight.

	Ctrl	Api	DOX	DOX + Api
Mortality	0	0	3	1
HW (mg)	1.017 ± 0.021	0.921 ± 0.0347	0.74 ± 0.0159$^{***\ ###}$	0.885 ± 0.0317$^{*\$}$
BW (g)	266 ± 6.245	253 ± 9.335	207.7 ± 5.708$^{***\ ##}$	238.7 ± 8.305$^{\$}$
HW/BW (mg/g)	3.867 ± 0.117	3.483 ± 0.170	3.300 ± 0.073*	3.750 ± 0.0763

BW; body weight (g), HW; heart weight (mg), (HW/BW); heart weight to body weight, DOX; doxorubicin, Api; apigenin. Data are presented as mean ± SEM. * $P < 0.05$ and *** $P < 0.001$ vs. ctrl group, $^{\$}$ $P < 0.05$ vs. DOX group, $^{##}$ $P < 0.01$ and $^{###}$ $P < 0.001$ vs. Api group.

Table 2
Echocardiographic parameters after 12 days of treatment ($n = 8$).

	Ctrl	Api	DOX	DOX + Api
% EF	90.71 ± 1.32	84.2 ± 0.819	68.73 ± 2.204$^{***\ ###}$	85.84 ± 2.306 $^{\$\$\$}$
% FS	52.52 ± 1.073	47.34 ± 0.825	34.01 ± 1.580$^{***\ ###}$	50.99 ± 2.820$^{\$\$\$}$
LVIDd (cm)	0.465 ± 0.0243	0.513 ± 0.0348	0.620 ± 0.0169**	0.49 ± 0.0302$^{\$}$
LVISd (cm)	0.24 ± 0.028	0.281 ± 0.0249	0.381 ± 0.017***	0.27 ± 0.018$^{\$\$}$

LVIDd; left ventricular inner diameter in end-diastole (cm), LVIDs; left ventricular inner diameter in end-systole (cm). EF; ejection fraction (%), FS; fractional shortening (%), DOX; doxorubicin, Api; apigenin. Data are presented as means ± SEM. ** $P < 0.01$ and *** $P < 0.001$ vs. ctrl group, $^{\$}$ $P < 0.05$, $^{\$\$}$ $P < 0.01$ and $^{\$\$\$}$ $P < 0.001$ vs. DOX group, $^{###}$ $P < 0.001$ vs. Api group.

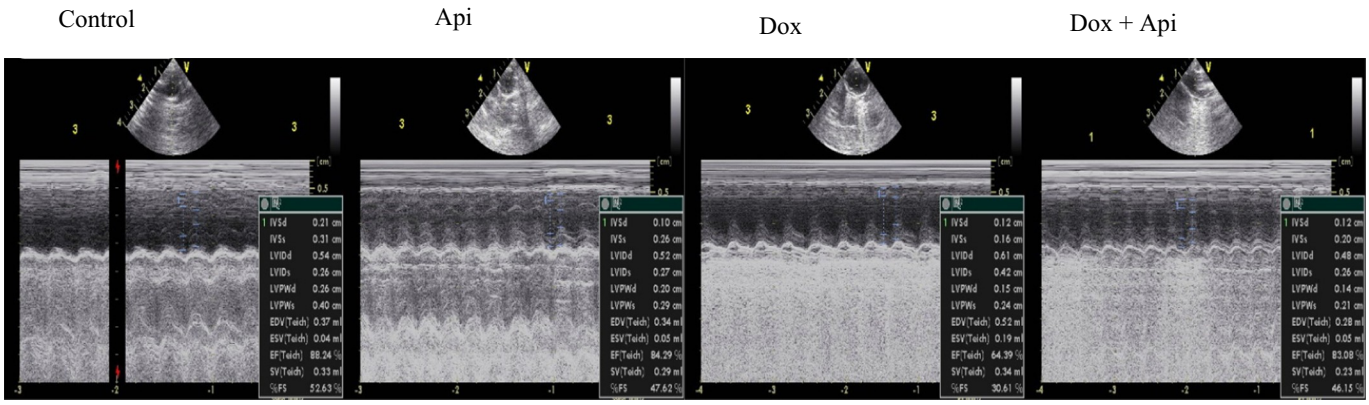

Fig. 1. Echocardiographic records in different experimental groups. DOX; doxorubicin, Api; apigenin.

Table 3
Changes in serum levels of CK-MB, LDH, cTn-I, ALT and AST at day 12 after treatment ($n = 10$).

	Ctrl	Api	DOX	DOX + Api
LDH (IU/L)	489.7 ± 40.58	525.8 ± 75.86	1033 ± 77.97$^{***\ ###}$	420.8 ± 57.23$^{\$\$\$}$
CK-MB (IU/L)	541 ± 24.64	509.7 ± 41.24	800.8 ± 32.93$^{***\ ###}$	427.5 ± 33.36$^{\$\$\$}$
cTn-I (ng/ml)	0.15 ± 0.018	0.272 ± 0.0684	2.529 ± 0.397$^{***\ ###}$	0.550 ± 0.105$^{\$\$\$}$
ALT (IU/L)	42.57 ± 2.92	42.71 ± 3.69	69.57 ± 4.91$^{***\ ###}$	47.14 ± 4.19$^{\$\$}$
AST (IU/L)	84.00 ± 6.68	105.4 ± 11.80	214.3 ± 28.20$^{***\ ###}$	93.29 ± 9.85$^{\$\$\$}$

DOX, doxorubicin; Api, apigenin. Data are presented as means ± SEM. *** $P < 0.001$ vs. control group, $^{\$\$}$ $P < 0.01$ and $^{\$\$\$}$ $P < 0.001$ vs. DOX group and $^{###}$ $P < 0.001$ vs. Api group.

DOX significantly decreased SOD levels at day 12 ($P < 0.05$) in comparison with control and Api groups. Treatment with Api in DOX-intoxicated animals for 12 days significantly increased SOD levels in comparison with DOX treated animals ($P < 0.001$). Statistical analysis also shows that there is no significant difference among Api-only treated and control animals.

Data analysis also showed that induction of cardiotoxicity with DOX significantly increased GSH levels at day 12 ($P < 0.01$) in comparison with. Treatment with Api in DOX-intoxicated animals for 12 days significantly increased GSH levels in comparison with control and Api groups ($P < 0.01$). Fig. 3C also shows that there are no significant differences between Api-only treated and control animals.

3.7. Effects of apigenin on apoptotic markers

To evaluate the effect of Api in the DOX-induced apoptosis, we examined apoptosis using immunohistochemical staining with Bax, Bcl-2 and immunofluorescence stain with Casp-3 antibodies at day 12 after starting the first treatment. Our results showed that the number of Bcl-2 positive cells increased in Dox + Api treated animals compared to Dox group (Fig. 4B). Fig. 4C shows that the number of Bax-positive cells increased in Dox group compared with control group. After treatment with Api, the number of Bax-positive cells were significantly reduced. Moreover, as shown in Fig. 4D, the number of Casp3 positive cells also increased in Dox group and 12-day treatment with Api decreasing in comparison with Dox group.

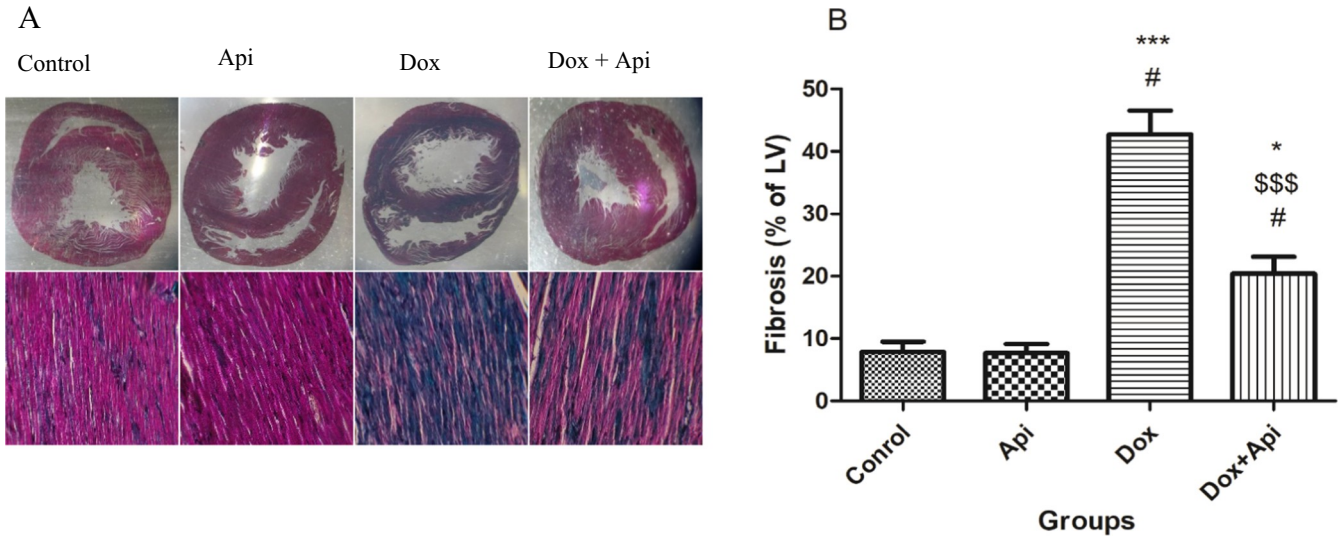

Fig. 2. Histological analysis of fibrosis at day 12 after treatment ($n = 5$). (A) Transverse sections of the hearts from apex to base stained with Masson's trichrome to detect interstitial fibrosis (upside pictures shows heart sections with 4 x magnification and downside pictures was taken by 400 X magnifications). The green areas indicate collagen fibers deposition (as marker of fibrosis) in cardiac tissue. DOX, doxorubicin; Api, apigenin. (B) Quantification of interstitial fibrosis. Data are presented as mean ± SEM. * $P < 0.05$ and *** $P < 0.001$ vs. control group, $^{\$\$\$}P < 0.001$ vs. DOX group, $^{\#}$ $P < 0.05$ vs. Api group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

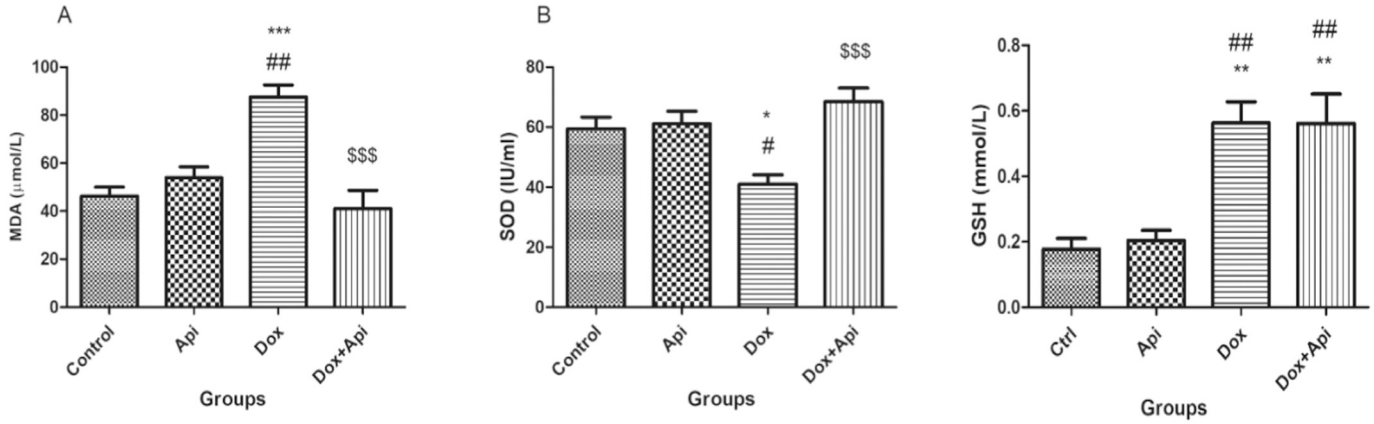

Fig. 3. Myocardial content of MDA (A), SOD (B) and GSH (C) at day 12 after treatment ($n = 5$). DOX, doxorubicin; Api, apigenin. Data are presented as means ± SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. control group, $^{\$\$\$}$ $P < 0.001$ vs. DOX group.

4. Discussion

In the present study, we provide evidence for cardioprotective effects of apigenin after induction of cardiotoxicity with doxorubicin (Dox) in male rats. Our results showed that cardiotoxicity induced by 12 mg/kg Dox is manifested by elevation of serum levels of LDH, CK-MB and cTnI, myocardial tissue damages, myocardial dysfunction, induction of cardiomyocytes apoptosis and elevation of oxidative stress in heart tissue. These results are in line with previous studies [25,26]. The body weight (BW), heart weight (HW) and HW/BW ratio also decreased in Dox treated animals. In addition, we have demonstrated that Api treatment of Dox intoxicated animals significantly reduced myocardial fibrosis, improves myocardial function and prevented the progression of myocardial damages by decreasing oxidative stress and apoptosis in cardiomyocytes, suggesting that Api exerts cardioprotective effects against cardiotoxicity induced by Dox.

Dox is one of the most commonly used anti-tumor anthracycline agents. Nevertheless, the cardiotoxicity of Dox is a major limiting factor in cancer treatment [2,27]. Previous studies have demonstrated that Dox induced cardiotoxicity occurs at 12 mg/kg [28,29]. In this study, we used this dose of Dox for induction of cardiotoxicity. The results showed that cumulative dose of Dox causes cardiotoxicity as it is manifested by heart weight and body weight loss, myocardial fibrosis, elevation of serum biomarkers of myocardial damages like LDH, CK-MB and cTn-I, and myocardial dysfunction as it was showed by echocardiography. Our results also showed that Dox causes cardiomyocytes apoptosis and elevation of oxidative stress.

In this study, statistical analysis of data showed that 12-day treatment with Dox caused body and heart weight loss, which is in line with other studies [26]. It seems that cardiomyocytes and myofibrils loss might be the reason for heart weight reduction. It has also been suggested that cytoplasmic vacuolization because of dilation or distention of T-tubules and sarcoplasmic reticulum in cardiomyocytes and food intake reduction as gastrointestinal side effects of Dox might cause heart and body weight reduction [26,30]. Our results showed the same findings and that 12-day treatment with Api could prevent heart and body weight loss. Therefore, it seems that Api may exert protective effects and prevent cytoplasmic vacuolization, myofibrillar and

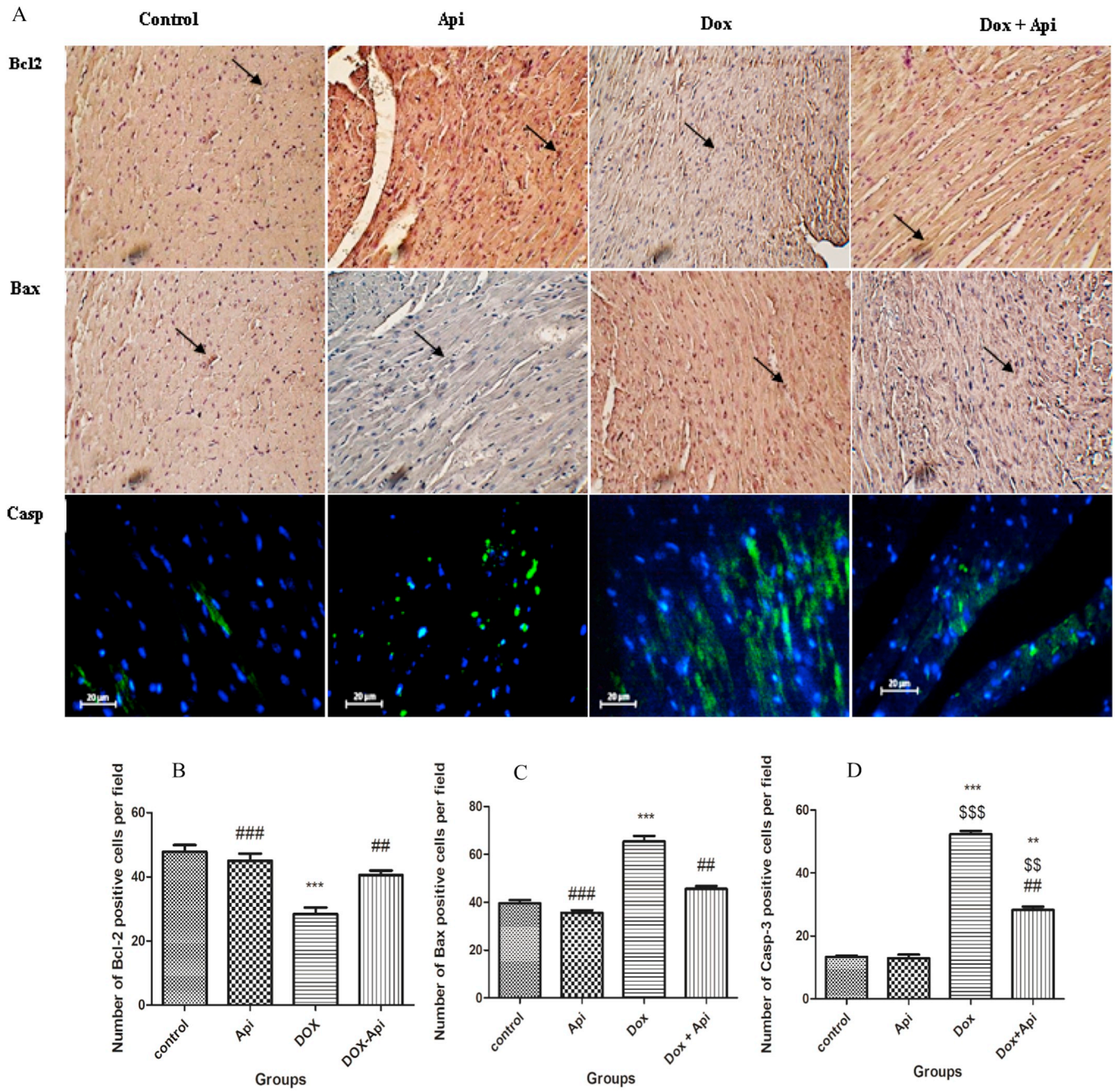

Fig. 4. Immunohistochemical (for MDA and SOD) and immunofluorescence staining (for GSH) (A) at day 12 after treatment ($n = 5$). Quantification of positive cell numbers of Bcl-2 (B), Bax (C) and (D) casp-3. The arrows represent Bcl-2 and Bax positive cells (brown color) (40× magnification). Blue points represent nuclei of cells and green points represent Casp-3 positive cells. DOX, doxorubicin; Api, apigenin. Data are presented as mean ± SEM. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. control group, $^{\$\$}$ $P < 0.01$, $^{\$\$}$ $P < 0.01$ and $^{\$\$\$}$ $P < 0.001$ vs. DOX group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cardiomyocytes loss (as it could decrease cardiomyocytes loss).

It is well-documented that oxygen and nutrients deficiency to the myocardium may cause cardiac muscle cell membrane damages and LDH, CK-MB and cTn-I release from the heart. Thus, serum levels of these enzymes are considered as biomarkers of cardiac damages [31]. We showed that 12-day treatment with Api in Dox intoxicated animals prevented the expansion of myocardial damages induced by Dox. Api prevented LDH, CK-MB and cTn-I elevation as well as structural changes in the myocardial tissues that is shown in Fig. 1. Therefore it seems that Api by preserving structural integrity could decrease myocardial damages and elevation of serum markers of cardiac injuries.

It has also been shown that after cumulative dose of DOX, cardiotoxicity occurs in a long period of time from weeks to months [5]. In this study, therefore, we used cumulative dose of Dox for inducing cardiotoxicity. The exact pathogenic mechanisms of cardiotoxicity by DOX aren't clear. Many researchers have reported that inflammation of cardiomyocytes, oxidative stress induced damages and programmed cell death of cardiomyocytes (apoptosis) are the main involved mechanisms and causes progression of cardiomyopathy after cumulative doses [6,7]. Under oxidative stress conditions induced by DOX production of reactive oxygen species (ROS) increase [8]. The heart also lacks the antioxidant enzymes for scavenging free radicals. Therefore

oxygen free radicals accumulation causes peroxidation of membrane lipids and degeneration of cellular and mitochondrial membranes, endoplasmic reticulum, nucleic acid and intracellular macromolecules [6,9]. Oxidative stress causes activation of many signaling pathways and leads to cardiomyocytes apoptosis [8]. In normal conditions, antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) clear free radicals. In abnormal conditions that there isn't balance between the oxidants production and the antioxidant defense systems, peroxidation of lipids and alteration of homeostasis occurs, as these conditions could be seen in DOX-induced cardiotoxicity [10]. Our results showed that MDA as a marker of lipid peroxidation increased and antioxidant enzymes, SOD and GSH decreased in Dox treated animals. Thus Dox by increasing oxidative damages could increase myocardial injuries as shown in myocardial tissues. Data analysis showed that Api treatment in Dox intoxicated animals could decrease MDA, and increase SOD in myocardium. It seems that Api by antioxidant effects prevented Dox-induced damages and improves myocardial function.

It has also been reported that one of the most important mechanisms for the pathogenesis of DOX-induced cardiotoxicity is cardiomyocytes apoptosis which is probably mediated by free radicals formation [10,11,31]. DOX-induced oxidative stress triggers the extrinsic and intrinsic mitochondria-dependent apoptotic pathways in cardiomyocytes, and in turn, by this mechanisms, causing cell loss in myocardium [12,30]. It is well-documented that in the process of apoptosis, anti-apoptotic proteins such as Bcl-2 decreases and pro-apoptotic proteins such as Bax and Casp-3 increase [26,32]. Our results also showed that in Dox treated animals, expression of Bcl-2 decreased and Bax and Casp-3 expression increased as shown in immunohistochemical staining in myocardial tissues. 12-day treatment by Api in Dox intoxicated animals could increase Bcl-2 expression and decrease Bax and Casp-3 expression. We can, thus, suggest that Api by anti-apoptotic effect could preserve myocardial integrity and decrease cardiac damage. By these effects, it could decrease serum levels of myocardial injury biomarkers, prevent myocardial tissue changes and expansion of fibrosis, and finally improve myocardial function. The latter can be shown by improvement of echocardiographic recording of EF, FS, LVIDd and LVIDs. In line with our study, previous studies also showed that apigenin has an anti-apoptotic effect which supports its cardioprotective effects via suppression of myocardial apoptosis [31,33].

5. Conclusion

In summary, our results showed that oral administration of Api for 12 days along with induction of cardiotoxicity by Dox can decrease myocardial damages and serum levels of cardiac injury biomarkers. Further, it can decrease cardiomyocytes oxidative stress and apoptosis via increasing Bcl-2 expression and decreasing the expression of Bax and Casp-3. Thus, Api through its antioxidant and anti-apoptotic effects preserves structural integrity and improves myocardial function.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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References

[1] A.A. Hamza, M.M. Ahmed, H.M. Elwey, A. Amin, Melissa officinalis protects against doxorubicin-induced cardiotoxicity in rats and potentiates its anticancer activity on MCF-7 cells, PLoS One 11 (11) (2016) e0167049.
[2] J.V. McGowan, R. Chung, A. Maulik, I. Piotrowska, J.M. Walker, D.M. Yellon, Anthracycline chemotherapy and cardiotoxicity, Cardiovasc. Drugs Ther. 31 (1) (2017) 63–75.
[3] J.F. An, P.F. Li, J.C. Li, R. Dietz, S. Donath, ARC is a critical cardiomyocyte survival switch in doxorubicin cardiotoxicity, J Mol Med-Jmm 87 (4) (2009) 401–410.
[4] D.M. Ibrahim, R.R. Radwan, S.M. Abdel Fattah, Antioxidant and antiapoptotic effects of sea cucumber and valsartan against doxorubicin-induced cardiotoxicity in rats: the role of low dose gamma irradiation, J. Photochem. Photobiol. B 170 (2017) 70–78.
[5] R. Uygur, C. Aktas, F. Tulubas, S. Alpsoy, B. Topcu, O.A. Ozen, Cardioprotective effects of fish omega-3 fatty acids on doxorubicin-induced cardiotoxicity in rats, Human & Experimental Toxicology 33 (4) (2014) 435–445.
[6] E.M. Mantawy, W.M. El-Bakly, A. Esmat, A.M. Badr, E. El-Demerdash, Chrysin alleviates acute doxorubicin cardiotoxicity in rats via suppression of oxidative stress, inflammation and apoptosis, Eur. J. Pharmacol. 728 (2014) 107–118.
[7] K.A. Wouters, L.C. Kremer, T.L. Miller, E.H. Herman, S.E. Lipshultz, Protecting against anthracycline-induced myocardial damage: a review of the most promising strategies, Br. J. Haematol. 131 (5) (2005) 561–578.
[8] J. Sun, G. Sun, Myricitrin protects against doxorubicin-induced cardiotoxicity by counteracting oxidative stress and inhibiting mitochondrial apoptosis via ERK/P53 pathway, 2016 (2016) 6093783.
[9] M.T. Kelleni, E.F. Amin, A.M. Abdelrahman, Effect of Metformin and Sitagliptin on doxorubicin-induced cardiotoxicity in rats: impact of oxidative stress, inflammation, and apoptosis, Journal of Toxicology 2015 (2015) 424813.
[10] S. Alpsoy, C. Aktas, R. Uygur, B. Topcu, M. Kanter, M. Erboga, et al., Antioxidant and anti-apoptotic effects of onion (Allium cepa) extract on doxorubicin-induced cardiotoxicity in rats, Journal of Applied Toxicology: JAT 33 (3) (2013) 202–208.
[11] J.L. Reeve, E. Szegezdi, S.E. Logue, T. Ni Chonghaile, T. O'Brien, T. Ritter, et al., Distinct mechanisms of cardiomyocyte apoptosis induced by doxorubicin and hypoxia converge on mitochondria and are inhibited by Bcl-xL, J. Cell. Mol. Med. 11 (3) (2007) 509–520.
[12] J. Kluza, P. Marchetti, M.A. Gallego, S. Lancel, C. Fournier, A. Loyens, et al., Mitochondrial proliferation during apoptosis induced by anticancer agents: effects of doxorubicin and mitoxantrone on cancer and cardiac cells, Oncogene 23 (42) (2004) 7018–7030.
[13] A. Hamza, A. Amin, S. Daoud, The protective effect of a purified extract of Withania somnifera against doxorubicin-induced cardiac toxicity in rats, Cell Biol. Toxicol. 24 (1) (2008) 63–73.
[14] S. Shukla, S. Gupta, Apigenin: a promising molecule for cancer prevention, Pharm. Res. 27 (6) (2010) 962–978.
[15] H. Du, J. Hao, F. Liu, J. Lu, X. Yang, Apigenin attenuates acute myocardial infarction of rats via the inhibitions of matrix metalloprotease-9 and inflammatory reactions, Int. J. Clin. Exp. Med. 8 (6) (2015) 8854–8859.
[16] D. Patel, S. Shukla, S. Gupta, Apigenin and cancer chemoprevention: progress, potential and promise (review), Int. J. Oncol. 30 (1) (2007) 233–245.
[17] X. Yang, J. Yang, J. Hu, X. Li, X. Zhang, Z. Li, Apigenin attenuates myocardial ischemia/reperfusion injury via the inactivation of p38 mitogenactivated protein kinase, Mol. Med. Rep. 12 (5) (2015) 6873–6878.
[18] B.H. Jin, L.B. Qian, S. Chen, J. Li, H.P. Wang, I.C. Bruce, et al., Apigenin protects endothelium-dependent relaxation of rat aorta against oxidative stress, Eur. J. Pharmacol. 616 (1–3) (2009) 200–5.
[19] C. Chen, H. He, Y. Luo, M. Zhou, D. Yin, M. He, Involvement of Bcl-2 signal pathway in the protective effects of apigenin on anoxia/reoxygenation-induced myocardium injury, J. Cardiovasc. Pharmacol. 67 (2) (2016) 152–63.
[20] J. Hu, Z. Li, L.T. Xu, A.J. Sun, X.Y. Fu, L. Zhang, et al., Protective effect of apigenin on ischemia/reperfusion injury of the isolated rat heart, Cardiovasc. Toxicol. 15 (3) (2015) 241–9.
[21] U.B. Mahajan, G. Chandrayan, C.R. Patil, D.S. Arya, K. Suchal, Y.O. Agrawal, et al., The protective effect of apigenin on myocardial injury in diabetic rats mediating activation of the PPAR-gamma pathway, Int. J. Mol. Sci. 18 (4) (2017).
[22] P.J. O'Brien, D.E. Smith, T.J. Knechtel, M.A. Marchak, I. Pruimboom-Brees, D.J. Brees, et al., Cardiac troponin I is a sensitive, specific biomarker of cardiac injury in laboratory animals, Lab. Anim. 40 (2) (2006) 153–71.
[23] Y. Azizi, M. Faghihi, A. Imani, M. Roghani, A. Nazari, Post-infarct treatment with [Pyr1]-apelin-13 reduces myocardial damage through reduction of oxidative injury and nitric oxide enhancement in the rat model of myocardial infarction, Peptides 46 (2013) 76–82.
[24] M. Khalili, Z. Kiasalari, M. Roghani, Y. Azizi, Anticonvulsant and antioxidant effect of hydro-alcoholic extract of Cyperus rotundus rhizome on pentylentetrazole-induced kindling model in male mice, J Med Plants Res 5 (7) (2011) 1140–6.
[25] J. Gu, Z.P. Song, D.M. Gui, W. Hu, Y.G. Chen, D.D. Zhang, Resveratrol attenuates doxorubicin-induced cardiomyocyte apoptosis in lymphoma nude mice by heme oxygenase-1 induction, Cardiovasc. Toxicol. 12 (4) (2012) 341–9.
[26] B.D. Sahu, J.M. Kumar, M. Kuncha, R.M. Borkar, R. Srinivas, R. Sistla, Baicalein alleviates doxorubicin-induced cardiotoxicity via suppression of myocardial oxidative stress and apoptosis in mice, Life Sci. 144 (2016) 8–18.
[27] L. Rochette, C. Guenancia, A. Gudjoncik, O. Hachet, M. Zeller, Y. Cottin, et al., Anthracyclines/trastuzumab: new aspects of cardiotoxicity and molecular mechanisms, Trends Pharmacol. Sci. 36 (6) (2015) 326–48.
[28] N. Razmaraii, H. Babaei, A. Mohajjel Nayebi, G. Assadnassab, J. Ashrafi Helan, Y. Azarmi, Crocin treatment prevents doxorubicin-induced cardiotoxicity in rats, Life Sci. 157 (2016) 145–51.
[29] N. Razmaraii, H. Babaei, A. Mohajjel Nayebi, G. Asadnasab, J. Ashrafi Helan, Y. Azarmi, Cardioprotective effect of phenytoin on doxorubicin-induced cardiac toxicity in a rat model, J. Cardiovasc. Pharmacol. 67 (3) (2016) 237–45.
[30] F.S. Carvalho, A. Burgeiro, R. Garcia, A.J. Moreno, R.A. Carvalho, P.J. Oliveira, Doxorubicin-induced cardiotoxicity: from bioenergetic failure and cell death to

cardiomyopathy, Med. Res. Rev. 34 (1) (2014) 106–35.
[31] W. Yu, H. Sun, W. Zha, W. Cui, L. Xu, Q. Min, et al., Apigenin attenuates adriamycin-induced cardiomyocyte apoptosis via the PI3K/AKT/mTOR pathway, Evidence-based Complementary and Alternative Medicine : eCAM 2017 (2017) 2590676.
[32] Y. Azizi, A. Imani, H. Fanaei, S. Khamse, M.R. Parvizi, M. Faghihi, Post-infarct treatment with [Pyr1]apelin-13 exerts anti-remodelling and anti-apoptotic effects in rats' hearts, Kardiol. Pol. 75 (6) (2017) 605–613.
[33] T. Zhang, T. Yan, J. Du, S. Wang, H. Yang, Apigenin attenuates heart injury in lipopolysaccharide-induced endotoxemic model by suppressing sphingosine kinase 1/sphingosine 1-phosphate signaling pathway, Chem. Biol. Interact. 233 (2015) 46–55.