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RESEARCH ARTICLE

Antioxidant, anti-inflammatory, and anti-apoptotic effects of crocin against doxorubicin-induced myocardial toxicity in rats

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Abstract

Doxorubicin (DOX) is a well-known chemotherapeutic drug for most malignancies including breast cancer and leukemia whilst the usage of DOX is limited owing to its cardiotoxicity. In the present study, we aimed to investigate the effects of crocin on doxorubicin-induced cardiotoxicity in rats. Forty rats were randomly divided into four groups: (a) control [received normal saline as a dose of 1 ml/kg by intraperitoneal injection (ip) for 15 days], (b) crocin (received crocin as a dose of 40 mg/kg/24h by ip for 15 days), (c) DOX (received DOX as a dose of 2 mg/kg/48h by ip in six injection, cumulative dose 12 mg/kg), and (d) DOX+ crocin (received DOX as a dose of 2 mg/kg/48h by ip in six injection, and crocin as a dose of 40 mg/kg/24h i.p for 15 days). As compared to the controls, the results showed that DOX administration caused significant increases in lipid indices [triglyseride (TG), low-dencity lipoproteins (LDL) (p<0.001), and very low-dencity lipoproteins (VLDL) (p<0.005)], oxidative stress parameters [malondialdehyde (MDA) and total oxidant status (TOS) (p<0.001)] and cardiac markers [creatine kinase-muscle/brain (CK-MB) and cardiac troponin I (cTnI) (p<0.001)]. Besides, significant decreases in antioxidant defense systems [glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), and total antioxidant status (TAS) (p<0.001)] were observed. The present study also demonstrated that co-administration of crocin with DOX significantly ameliorated the lipid profile (p<0.005), cardiac markers (p<0.005), and oxidative stress indices (p<0.001) as compared to DOX group. Histopathologically, significant increase in the mean histopathological damage score (MHDS) was found in the DOX group as compared to the controls (p<0.001). In contrast, the administration of crocin with DOX alleviated MHDS in myocardium (p<0.001). Taken together, our results reveal that crocin might be a cardioprotective agent in DOX-treated patients for cancer.

Keywords Doxorubicin · crocin · apoptosis · oxidative stress · myocardial toxicity

Introduction

Doxorubicin (DOX), one of the anthracycline glicoside antibiotic, is a frequently used chemotherapeutic drug owing to treat several malignancies in patients, such as breast cancer, leukemia, and sarcoma by causing DNA intercalation and inhibiting the DNA replication (Migrino et al. 2008; Swain et al. 2003). The chief adverse effect of DOX treatment is dose-depended cardiotoxicity that is a limiting factor for clinical usage of the drug during long-term administration in anticancer therapy (Ahmadian-Fard-Fini et al. 2018; Hong et al. 2002). The use of DOX in cancer treatment has greatly increased the survival rate of patients, but the incidence of causing heart failure (Lipshultz 2006) at DOX treatment is approximately 5% (Cardinale et al. 2015), even if the treatment dose is limited to 400 mg m^{-2}. For this reason, it is important to detect and treat patients with cardiotoxicity in order to reduce the rate of DOX-induced heart failure and improve the quality of life in DOX treatment. In addition, there are currently no imaging techniques or biochemical markers to diagnose DOX-induced heart failure at the onset of cardiac function decline, and there are no specific treatments to prevent DOX-induced cardiotoxicity (Hahn et al. 2014). There are

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several molecular mechanisms for DOX-induced cardiotoxicity and all of them result in cardiomyocyte death (Kalyanaraman et al. 2002). Although the main mechanism of DOX-induced cardiotoxicity is unknown, DOX increases inflammation and oxidative stress in the heart tissue including lipid peroxidation, mitochondrial DNA damage, and apoptosis as well as deteriorate calcium homeostasis (Li et al. 2016; Octavia et al. 2012).

Increased both calcium and reactive oxygen species (ROS) in mitochondria leads to generate lipid peroxidation and the formation of oxidative injury and cell membran of cardiyomyocytes (Abushouk et al. 2019; Salavati-Niasari et al. 2009; Zhou et al. 2001a). Also, an increase oxidative stress which raised after DOX treatment contributes to the activation of apoptotic signaling pathways and leads to apoptotic cell death of myocytes (Octavia et al. 2012; Salehabadi et al. 2018). It seems that myocardial infarction, heart failure, and cardiac dysfunction are associated with myocardial apoptosis (Abbate et al. 2006; Abdel-Daim et al. 2017; Takemura et al. 2013). Due to the high effectiveness of DOX as a chemotherapeutic drug in many cancers, recent studies are associated with preventing and treating its adverse cardiac effects of DOX by using phytochemicals or drug along with DOX (Abushouk et al. 2017; Durdagi et al. 2021; Oner et al. 2019).

In traditional medicine, saffron (Crocus sativus L.) has been used for a long time as carminative, tonic, expectorant, and sedative (Mousavi et al. 2010). Also, it is reported that saffron is used to treat for several deseases including cardiovascular disorders, urological infections, and asthma (Tavakkol-Afshari et al. 2008). Crocin, one of the most common bioactive constituents of saffron, has many pharmacological properties, such as antioxidant, free radical scavenger (Mousavi et al. 2010), anticancer (Festuccia et al. 2014), anti-inflammatory (Hong and Yang 2013), and antiatherosclerotic and cardioprotective effects (Farkhondeh and Samarghandian 2014). The structure of crocin, water-soluble carotenoid pigment, is mono- and diglycosyl esters of a polyene dicarboxylic acid, named crocetin and responsible for the red color of saffron (Thushara et al. 2013). Some studies have reported that cardioprotective effects of crocin are associated with regulation of antioxidant enzymatic activities and cardiac markers (Hariri et al. 2010; Shen and Qian 2006). It has shown that crocin protects for cardiyomyocytes against hypoxic damage by increasing the level of vascular endothelial growth factor (VEGF), which is an angiogenic protein (Wu et al. 2010).

Here, we aimed to assess the possible effecs of crocin combined with DOX on cardiotoxicity, and the possible role of pathogenesis of DOX-induced cardiotoxicity, based on the specific cardiac biomarkers, biochemical parameters, oxidative stress markers, and histopatological and immunohistochemical evaluations in Wistar albino rats.

Materials and methods

Animals

Forty healty adult male Wistar albino rats (10 weeks age, 225 +25 g) were purchased from Zonguldak Bulent Ecevit University Faculty of Medicine Experimental Animal Production and Research Center (ZBEUN-DEHAM). All rats were housed in a well-ventilated room with temperature- and humidity-controlled conditions (an ambient temperature range of 22 °C; relative humidity of 55–60%) in rat cages with a 12 h–12 h light–dark cycle (light from 08: 00–20: 00). The study was approved by the Experimental Animals Ethics Committee of Zonguldak Bulent Ecevit University, Faculty of Medicine (Protocol No: 2020-08-07/05). All experimental procedures were carried out in accordance with the Animal Ethics Committee Guidelines for the use of experimental animals. The experimental animals were allowed access to drinking water and standard rodent diet *ad libitum*.

Study design

Doxorubicin® 10 mg was purchased from Kocak Company (Istanbul, TURKEY) and crocin was obtained from Sigma Aldrich Corporation (St. Louis, Missouri, ABD). Forty Wistar rats were randomly aliquoted into four groups. At the beginning of the study, each experimental group consisted of ten animals. In group 1 (control), animals recevied normal saline (1 ml/kg) via intraperitoneal injection (i.p.) for 15 days. In group 2 (Crocin), animals received crocin (40 mg/kg) via i.p for 15 consecutive days (Razmaraii et al. 2016c). In group 3 (DOX), animals received DOX (2 mg/kg) via i.p in six injection at 48-h intervals during the 12-day period (cumulative dose:12 mg/kg) (Razmaraii et al. 2016a). And in group 4 (DOX+crocin), animals received crocin (40 mg/kg) through 15 consecutive days (starting 4 days before first DOX administration) along with DOX teratment (with the same dose as the group mentioned above). Lyophilized DOX powder was prepared for i.p injection by dissolving with the solvent water. Crocin powder was dissolved by normal saline (0.9%). Throughout the study, all applications were carried out everyday in the same time period.

Blood and tissue collection

At the sixteenth day of the experiment, all rats were anesthetized under ketamine/xylazine anesthesia. Then, the blood samples were taken into non-heparinized tubes from the abdominal vein. Following blood collection, all tubes were kept under room condition about 30 min for allowing to clot. The serum was obtained by centrifuging at 3000 rpm for 20 min and stored at −80°C until for determination of biochemical parameters. The rats were then killed by cervical decapitation

and the heart tissues were removed immediately. The cardiac tissues were cleaned with ice-cold normal saline to remove excess blood then weighed. The tissues were divided into two equal parts. One of the parts was stored at −80°C until for measurement of biochemical parameters. The other part of cardiac tissue was fixed in 10% neutral buffered formalin for histological evaluations.

Biochemical analyzes

Preparation of samples

Rat heart tissues were removed from the freezer and rapidly weighed. Then the tissues were homogenized at 10.000 rpm for one min by an otomatic homogenizer (Bioprep-24 Homogenizer, Hangzhou Allsheng Instruments Co., Ltd., China) in a 10-fold volume ice-cold phosphate buffer (70 mM, pH 7.5). The homogenate was used for determination of MDA analysis. To obtain supernatant, the homogenates were centrifuged at +4°C and 3000 rpm for 10 min. The supernatants were used for determination of tissue biochemical parameters.

Assessment of oxidative stress markers

Heart tissue homogenate was used for assessment of lipid peroxidation by measuring cardiac MDA levels according to the method of Ohkawa et al. (Ohkawa et al. 1979). After adding 1% H3PO4 and 0.6% thiobarbituric acid with homogenate in test tubes, it was incubated in a boiling water bath for 45 min. Then, the samples were extracted by n-butanol and a pink colored product was formed. MDA level was calculated by reading this colored product in an ELISA reader at 535 nm. Results are given as nmol/gram wet tissue (gwt).

Heart tissue homogenates were centrifuged at 4000 rpm for 20 min to obtain the supernatant. These supernatants were used for other biochemical analyses. Cardiac superoxide dismutase (SOD) activity was determined with the method proposed by Sun et al. (Sun et al. 1988). In the experimental procedure, superoxide radicals are formed by the xanthine-xanthine oxidase reaction. These radicals cause the formation of blue colored formazan by reducing NBT (nitro blue tetrazolium) in the environment. Tissue SOD activities were calculated by measuring this blue formazan colour at 560 nm in an ELISA reader. Results were obtained as U/g protein.

Cardiac catalase (CAT) activity was determined by the method of Aebi (Aebi 1974). By mixing the tissue supernatants with phosphate buffer (pH: 7.5 mM) containing H_2O_2, the H_2O_2 in the medium was degraded into H_2O and O_2 by CAT activity. This degradation causes a decrease in absorbance at 240 nm. The decrease in absorbance was observed for 1 min to calculate enzyme activity. CAT activities were noted as K/g protein.

Reduced glutathione (GSH) content, one of the non-enzymatic antioxidant markers, was determined with the method illustrated by Ellman (Ellman 1959). After the tissue supernatants were deproteinized, the samples were mixed with 5,5′-dithiobis 2-nitrobenzoic acid (DTNB) to form a yellow-green product. The GSH level was calculated by measuring the colored compound at 410 nm. Data were obtained as nanomol/gwt.

Cardiac protein content was assessed according to the Biuret method (Gornall et al. 1949) using bovine serum albumin (BSA) as standard and used for calculating the antixiadant enzyme's activities.

Total antioxidant status (TAS) of cardiac tissue was determined with the method proposed by Erel (Erel 2004). TAS content was studied according to the manufacturer's instructions (Rel Assay Diagnostics, Gaziantep, TURKEY). Data were determined as mmol Trolox Equiv/L.

Total oxidant status (TOS) of cardiac tissue was measured according to the method of Erel (Erel 2005). TOS content was studied according to the manufacturer's instructions (Rel Assay Diagnostics, Gaziantep, TURKEY). The results were obtained as μmol H2O2 equiv/L.

Assessment of cardiomyocytes damage

The serum samples were removed from freezer and kept under room condition for bring all samples to room temperature before use. The serum content of cardiac isoenzyme creatine kinase (CK-MB) and cardiac troponin I (cTn-I) was measured by using their respective ELISA kits specific for rats, according to the principle of kits (Elabscience, USA; Catalog number: E-EL-R1327 and E-EL-R1253, respectively).

Assessment of lipid profile

The serum content of lipid profile, including triglyceride, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and very low-density lipoprotein (VLDL), was assessed by using their commercially available Architect c 1600 automatic analyzer kits by Abbott Laboratories (Illinois, USA).

Histological analysis

Histopathological evaluation

The heart tissues were fixed in 10% neutral buffered formalin for 48h. Tissues were washed in running water, and were dehydrated with increasing concentrations of ethanol (50%, 75%, 96%, and 100%). After dehydration, specimens were placed into xylene to obtain transparency and were embedded in paraffin. Paraffin blocks were cut at 5

μm, mounted on slides, and stained with hematoxylin and eosin (H-E). The tissue sections were examined under light microscopy. The evaluated parameters for severity of cardiac damage were congestion, necrosis, infiltration, and loss of myofibrillar in 10 different fields for each section. For this analysis, cardiac damage was semiquantitatively graded as absent (0), mild (1), moderate (2), and severe (3), for each criterion. The maximum damage score was 12. All sections were examined using a Nikon Eclipse 80i light microscope and Nikon Image Analysis system.

Immunohistochemical (IHC) evaluation

For immunohistochemical analysis, sections were mounted on polylysine coated slides. After rehydrating, samples were transferred to citrate buffer (pH 7.6) and heated in a microwave oven for 20 min. After cooling for 20 min at room temperature, the sections were washed with phosphate buffered saline (PBS). Then, sections were kept in 0.3% H_2O_2 for 7 min and afterward washed with PBS. Sections were incubated with an anti-TNF-α (1:100, bs-2081R, Bioss, China) and anti-caspase-3 (1:100, bs-0081R, Bioss, China) for 60 min. They then were rinsed in PBS and incubated with biotinylated goat antipolyvalent for 10 min and streptavidin peroxidase (SHP 125, ScyTek Laboratories, ABD) for 10 min at room temperature. Staining was completed with chromogen + substrate for 15 min, and slides were counterstained with Mayer's hematoxylin (M06002, Bio-optica, ITALY) for 1 min, rinsed in tap water, and dehydrated. The antibody was used according to the manufacturer's instructions. Staining for anti-TNF-α and anti-caspase-3 was identified by a brown color. The relative intensity of TNF-α and caspase-3 immunostaining was scored as follows: 0–5% (+), 6–20% (++), 21–40% (+++), 41–60% (++++), 61–80% (+++++), and 81–100% (++++++). All sections were examined using a Nikon Eclipse 80i light microscope and Nikon Image Analysis system.

Statistical analysis

Statistical analysis was carried out using the SPSS for Windows version 14.0 (SPSS Inc., Chicago, III., USA) statistical program. All data are expressed as arithmetic mean ± SE. Normality for continued variables in groups were determined by the Shapiro Wilk test. The variables did not show normal distribution ($p<0.05$). Kruskal-Wallis and Mann-Whitney *U* tests were used for comparison of variables among the studied groups. $p<0.05$ was regarded as significant.

Results

Effects of crocin on cardiac oxidative stress markers and antioxidant parameters

Tables 1 and 2 present the average cardiac oxidative stress markers and tissue antioxidant contents. The values of cardiac MDA, antioxidant enzymes (SOD, CAT), GSH, TAS, and TOS were analyzed statistically. The levels of cardiac oxidative stress markers and tissue antioxidant contents showed no pathological changes between control and crocin groups. Statistical analysis showed that DOX treatment resulted in dramatically increases ($p<0.001$) in the cardiac levels of MDA and TOS relative to values measured within the control group. In addition, rats receiving DOX also revealed significant reductions ($p<0.001$) in cardiac GSH and TAS levels as well as the activities of antioxidant enzymes CAT and SOD as compared to levels measured within the control group. In contrast, the rats receiving DOX with crocin reversed significantly the effect of DOX administration on cardiac oxidative stress markers (MDA and TOS) ($p<0.001$) as compared to the DOX group. Similarly, treatment of DOX-intoxicated rats with crocin revealed significant increases in both antioxidant contents (GSH and TAS) ($p<0.001$) and the activities of antioxidant enzymes (CAT and SOD) ($p<0.001$) relative to values measured within the DOX group.

Effects of crocin on cardiotoxicity indices

Table 3 presents the comparison of mean serum cardiac CK-MB and troponin I values. The serum cardiotoxicity markers showed no pathological changes in the control and crocin groups. The data revealed that the levels of the serum cardiotoxicity indices (CK-MB and cTnI) dramatically increased ($p<0.001$) after DOX treatment relative to values measured within their control ones (control and crocin groups). On the other hand, the rats receiving DOX with crocin reversed significantly the effect of DOX treatment on cardiotoxicity indices CK-MB ($p<0.05$) and cTnI ($p<0.001$) as compared to the DOX group. On the other hand, the rats receiving crocin with DOX reversed significantly the effect of DOX treatment on cardiotoxicity indices ($p<0.05$ for CK-MB; $p<0.001$ for cTnI) as compared to the DOX group.

Effects of crocin on lipid profile

Table 4 presents the comparison of mean serum lipid parameters. The data demonstrated that rats receiving DOX revealed significant increases in serum TG and LDL levels ($p<0.001$) as well as VLDL levels ($p<0.05$) compared to the control group. In contrast, the rats receiving DOX revealed significant reduction in serum HDL levels ($p<0.001$) relative to values measured within the control group. On the other hand, rats

Table. 1 Comparison of average tissue oxidant-antioxidant parameters

Group	MDA (nmol/gwt)	GSH (nmol/gwt)	SOD (U/g protein)	CAT (K/g protein)
Group 1: control	395.36±26.88	483.50±1.99	80.79±3.06	8.46±0.45
Group 2: crocin	290.39±33.03	506.80±5.70	87.97±3.96	11.10±1.00
Group 3: DOX	1405.42±226.30[a]	229.41±14.79[a]	38.00±2.00[a]	4.38±0.36[a]
Group 4: DOX + crocin	495.10±5.91[b]	345.28±13.78[b]	57.62±3.05[b]	7.09±0.62[c,d]

Data are expressed as the arithmetic mean ± SE (n = 10). *MDA*, malondialdehyde; *GSH*, reduced glutathione; *SOD*, superoxide dismutase; *CAT*, catalase; *gwt*, gram wet tissue. Superscripts represent the statistically significant difference: [a] $p < 0.001$ when compared to group 3 vs group 1 ve group 2, [b] $p < 0.001$ when compared to group 4 vs group 2 and group 3, [c] $p < 0.05$ when compared to group 4 vs group 2, [d] $p < 0.001$when compared to group 4 vs group 3

receiving DOX with crocin decreased in serum TG and VLDL levels (p<0.001) as well as LDL (p<0.05) as compared to the DOX group. In addtion, treatment of rats receiving DOX with crocin ameliorated serum HDL levels (p<0.05) relative to the DOX group.

Histopathological findings

The group 1 (control) and group 2 (crocin) were normal in histological appearance (Figure 1). There was no significant difference among these groups (p>0.05). Congestion, necrotic cells with picnotic nuclei, inflammatory cell infiltration, loss of myofibrillar, and cytoplasmic vacuolization were detected in DOX group (Figure 2a, b, and c). The mean histopathological damage score (MHDS) was 6.30±0.42 DOX group. Statistically significant increase in MHDS was found in the DOX group, when compared to the groups 1 and 2 (p<0.001, for all). The histopathological changes markedly regressed in DOX+crocin group (Figure 2d). The MHDS was 2.10±0.27 in the DOX+crocin group. When DOX group and DOX+crocin group were compared, statistically significant difference was detected (p<0.001). The MHDS for each group is given in Table 5.

Immunohistochemical findings

The expression of TNF-α in the heart tissues of the experimental groups was observed in the sarcoplasm, nuclei, and capillary vessels of cardiomyocytes. TNF-α expression is weak (0–5%) in groups 1 and 2. TNF-α expression is quite high in the DOX group (81–100%). In the DOX+crocin group, it is seen that the expression is moderate (41–60%) (Figure 3).

It was observed that the expression of caspase-3 in the heart tissues of the experimental groups was found in the sarcoplasm of cardiomyocytes and the tunica media layer (smooth muscles) of the vessels. Caspase-3 expression is weak (0–5%) in groups 1 and 2. In the DOX group, caspase-3 expression was moderate (41–60%). In the DOX+crocin group, it is seen that the expression is moderate to low (21–40%) (Figure 4). The intensity scores of TNF-α and caspase-3 immunostaining in cardiac tissue is given in Table 6.

Discussion

In the current study, we administered the cumulative dose of DOX (12 mg/kg) to observe cardiotoxicity during 2 weeks in line with previous studies (Babaei et al. 2020; Razmaraii et al. 2016b). The dose of DOX is the same as using in treatment of human cancers (Tarr et al. 2015). Based on previous evidence, DOX-induced cardiotoxicity is associated with dose-related and long-term administration of DOX (Takemura and Fujiwara 2007). To our knowledge, the main mechanism of cardiotoxicity caused by DOX administration has not been

Table. 2 Comparison of average tissue TAS and TOS values

Group	TAS (mmol Trolox eqv/L)	TOS (μmol H202 Equiv./L)
Group 1: control	1.27±0.09	7.48±0.22
Group 2: crocin	1.52±0.09	7.06±0.14
Group 3: DOX	0.67±08.08[a]	10.91±0.65[a]
Group 4: DOX + crocin	1.20±0.17[b,c]	7.33±0.26[c]

Data are expressed as the arithmetic mean ± SE (n = 10). *TAS*, total antioxidant status; *TOS*, total oxidant status. Superscripts represent the statistically significant difference: [a] $p < 0.001$ when compared to group 3 vs group 1 and group 2, [b] $p < 0.05$ when compared to group 4 vs group 2, [c] $p < 0.001$ when compared to group 4 vs group 3

Table. 3 Comparison of mean serum CK-MB and cTn-I values

Group	CK-MB (pg/ml)	cTn-I (pg/ml)
Group 1: Control	16.62±1.67	175.50±17.58
Group 2: Crocin	15.76±1.32	186.16±12.02
Group 3: DOX	32.36±2.15[a]	354.89±16.20[a]
Group 4: DOX + Crocin	20.80±0.66[b,c]	231.96±7.85[b,c]

Data are expressed as the arithmetic mean ± SE (n = 10). *CK-MB*, cardiac isoenzyme creatine kinase; *cTn-I*, cardiac troponin I

[a] p< 0.001 when compared to group 3 vs group 1 and group 2

[b] p< 0.05 when compared to group 4 vs group 2

[c] p< 0.001 when compared to group 4 vs group 3

Table. 5 The mean histopathological damage score (MHDS) of all groups

Groups	**Histopathological damage score**
Group 1: control	0.30±0.15
Group 2: crocin	0.50±0.16
Group 3: DOX	6.30±0.42[a]
Group 4: DOX+crocin	2.10±0.27[a,b]

Data are expressed as the arithmetic mean ± SE (n = 10)

[a] p<0.001 when compared to group 3 vs group 1 and group 2

[b] p<0.001 when compared to group 4 vs group 3

exactly known yet. However, many researchers examining doxorubicin's cardiotoxic effects have reported that the overproduction of ROS (Ahmed et al. 2005; Berthiaume and Wallace 2007) and the stimulation of inflammation (Deepa and Varalakshmi 2005) are responsible for DOX-induced adverse effects on myocardium. Owing to its lower antioxidant capacity in myocardium, the heart tissue is thought to be the main target tissue for DOX-induced oxidative damage relative to other tissues (De Beer et al. 2001; Zhou et al. 2001b). Also, myocardium contains cardiolipin, which is considered to have an attractive for DOX, resulting in DOX accumulation in the heart mitochondria, disturbance of the respiratory chain, and stimulation of apoptotic pathways (Ascensão et al. 2005).

Documentation for this hypothesis about ROS as the primary cause of DOX-induced cardiotoxicity has been demonstrated by numerous studies in transgenic animals, with the expression of antioxidant enzymes playing important protective roles against myocardial damage as a result of DOX administration (Kang et al. 1997; Sun et al. 2001). The formation of ROS and cardiac oxidative stress have been demostrated in Wistar rats receiving DOX (Oner et al. 2019). The increase in ROS production with DOX administration results in oxidative stress and is one of the major causes of myocardial damage (Berthiaume &Wallace 2007). In view of these facts, researchers have suggested trials on antioxidant therapy on DOX-induced toxicity. Antioxidants ameliorate DOX-caused oxidative damage by scavenging free radicals and the regulation of the activities of antioxidant enzymes without inhibiting DOX's anticancer properties. According to all these evidence, we studied the cardioprotective effects of crocin by biochemically and histologically.

Previous studies have reported that several antioxidants including coenzyme Q, N-acetylcysteine, vitamin E, and vitamin C have cardioprotective activities to prevent myocardial

Table. 4 Comparison of mean serum lipid parameters

Group	TG (mg/dl)	VLDL (mg/dl)	HDL (mg/dl)	LDL (mg/dl)
Group 1: control	55.40±3.44	10.40±0.44	45.82±2.05	8.31±0.85
Group 2: crocin	41.50±1.98[a]	8.21±0.33[a]	50.96±2.68	7.22±0.67
Group 3: DOX	88.40±5.17[b,c]	16.50±1.35[e,c]	37.40±1.67[f,c]	16.06±1.04[b,c]
Group 4: DOX + crocin	49.70±4.21[d]	10.04±0.84[d]	44.46±1.95[g]	12.46±0.92[h,j,g]

Data are expressed as the arithmetic mean ± SE (n = 10). *TG*, triglyceride; *VLDL*, very low-density lipoprotein; *HDL*, high-density lipoprotein; *LDL*, low-density lipoprotein

[a] p< 0.05 when compared to group 2 vs group 1

[b] p< 0.001 when compared to group 3 vs group 1

[c] p< 0.001 when compared to group 3 vs group 2

[d] p< 0.001 when compared to group 4 vs group 3

[e] p< 0.05 when compared to group 3 vs group 1

[f] p< 0.01 when compared to group 3 vs group 1

[g] p< 0.05 when compared to group 4 vs group 3

[h] p< 0.05 when compared to group 4 vs group 1

[j] p< 0.001 when compared to group 4 vs group 2

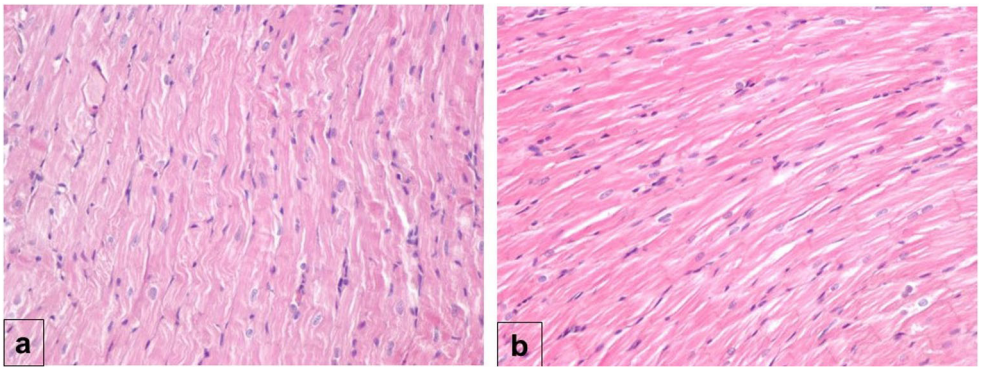

Figure 1 The control and crocin groups were normal in histological appearance. **a** Group 1 (control), H-E; ×20. **b** Group 2 (crocin), H-E; ×20

damage induced by antracyclines in animals (Songbo et al. 2019; Zamorano et al. 2016). Recently, several studies have demonstrated that crocin can be used as a new therapeutic drug, owing to its anticancer (Fernández 2006; Konoshima et al. 1998) and antioxidant properties (Assimopoulou et al. 2005; Hosseinzadeh et al. 2005). Whereas the mechanism of crocin, a water soluble carotenoid of saffron, is not yet fully established, it has been recognized that the effect of crocin on pathways may be similar to well-known carotenoids (Pham et al. 2000). Therefore, it has been accepted that crocin is able to ameliorate intracellular oxidative stress by increasing the activities of the antioxidant enzymes (Rahaiee et al. 2015).

One of the end product of lipid peroxidation, MDA, is an indicator of oxidative stress induced by free radicals. DOX causes to increase ROS, impairs membrane function, and exposes cardiac dysfunction via myocardial apoptosis. Elevations in the levels of lipid peroxidation of the heart tissue by increasing MDA and TOS levels following DOX administration were determined in the present study. In addition, co-administration of crocin with DOX resulted in significant amelioration of DOX-induced lipid peroxidation in rat myocardium. Protection of the heart by strengthening the cardiac antioxidant defense system against DOX-induced cardiac injury caused by ROS formation plays an important role in protection against DOX-induced myocardial damage (Arafa et al. 2014; Oner et al. 2019). Previous studies reported that DOX application caused impairment in antioxidant defense such as SOD, CAT, and GSH (Qi et al. 2020; Sadek et al. 2021). As antioxidant enzymes, SOD and CAT scavenge superoxide anion and hydrogen peroxide. GSH, non-enzymatic antioxidant, is a tripeptide formed by the combination of glutamate, cysteine, and glycine. GSH cleans ROS from the body with its powerful antioxidant properties. Our data suggested that the activities of cardiac SOD and CAT as well as the

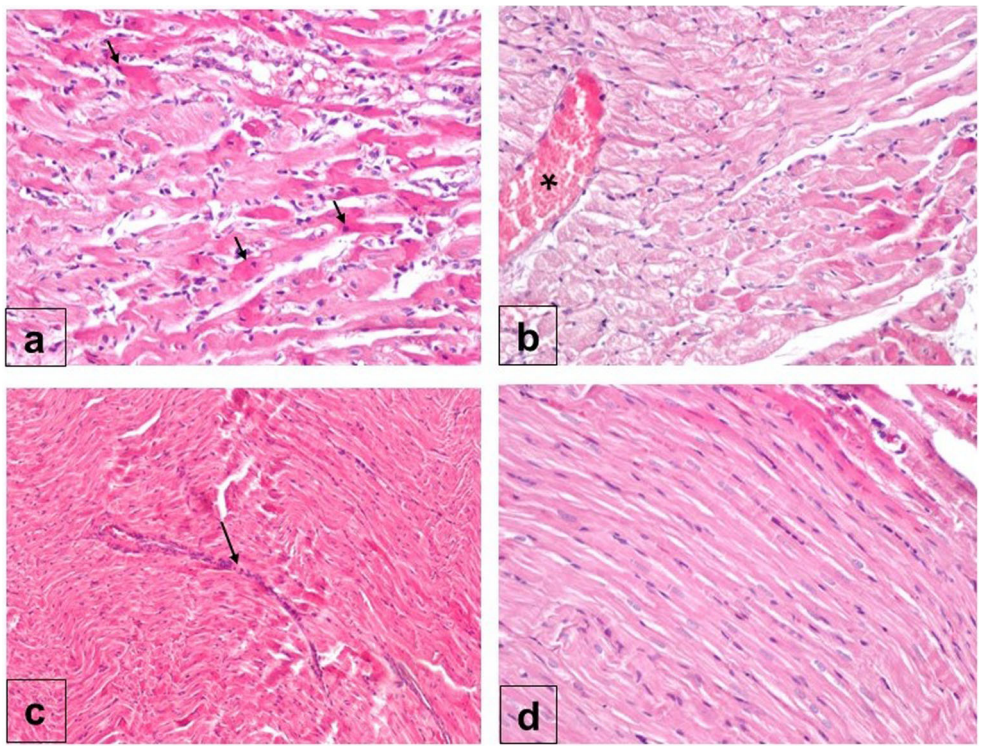

Figure 2 DOX group showed cardiac damage including (a) necrotic cells with picnotic nuclei (arrows), (b) congestion (asterisk), loss of myofibrillar, and cytoplasmic vacuolization (c) inflammatory cells infiltration (arrow). **a** Group 3 (DOX), H-E; ×20. **b** Group 3 (DOX), H-E; ×20. **c** Group 3 (DOX), H-E; ×10. The histopathological changes markedly regressed in the DOX+ Crocin group. **d** Group 4 (DOX+ crocin), H-E; ×20

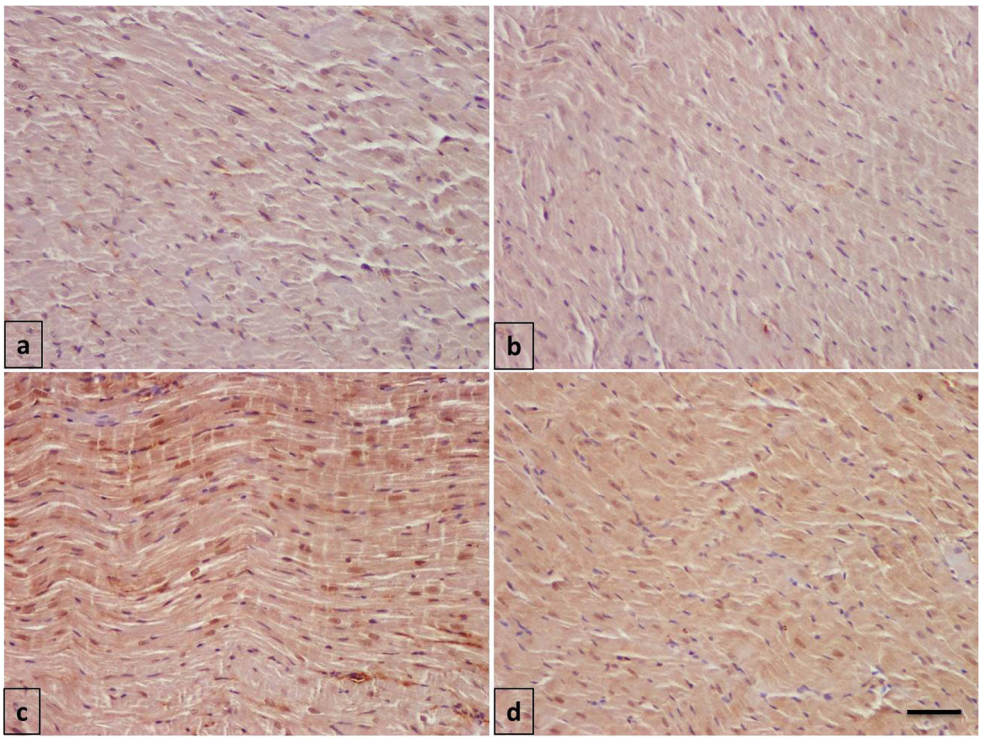

Figure 3 The intensity of TNF-α immunostaining in cardiac tissue. Control and crocin groups were similar. In the DOX group, apoptosis was most evident. The intensity of TNF-α was reduced in DOX+crocin group. **a** Group 1 (control), **b** Group 2 (crocin), **c** Group 3 (DOX), **d** Group 4 (DOX+crocin); ×20

levels of TAS and GSH remarkably decreased in the heart tissue of rats receiving DOX. The results also revealed that co-administration of crocin with DOX ameliorated antioxidant defense including SOD, CAT, GSH, and TAS. As a potent antioxidant, crocin shows its possible mechanism by directly scavenging ROS (Hosseinzadeh et al. 2005) and upregulating antioxidant enzyme genes (Deng et al. 2018). However, the regulatory pathway is still unclear. More research is needed for the future. In line with our findings, previous studies showed that crocin elevates the antioxidant capacity in cardiac tissue in different conditions such as isoprenaline-induced myocardial fibrosis and arsenic

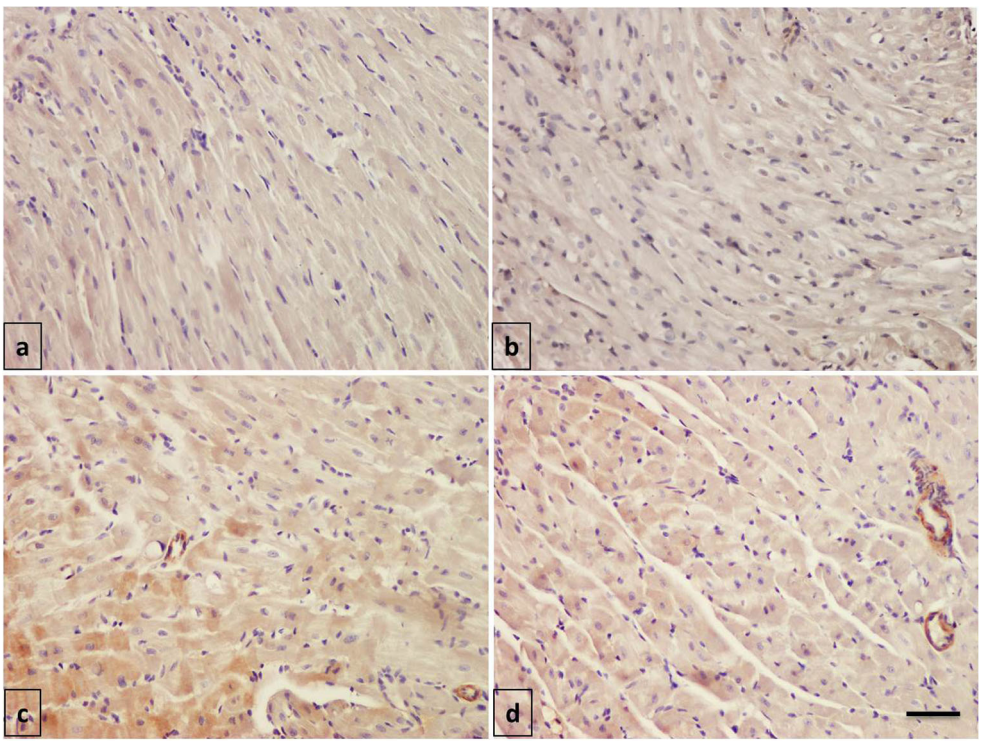

Figure 4 The intensity of caspase-3 immunostaining in cardiac tissue. Control and rocin groups were similar. In the DOX group apoptosis was most evident. The intensity of caspase-3 was reduced in DOX+crocin group. **a** Group 1 (control), **b** group 2 (crocin), **c** group 3 (DOX), **d** group 4 (DOX+crocin); ×20

Table 6 The intensity of TNFα and caspase-3 immunostaining in cardiac tissue

Immunoreactivity	Control Group	Crocin Group	DOX Group	DOX+Crocin Group
TNF-α	+	+	++++++	+++
Caspas 3	+	+	+++	++

0−5% = score (+); 6−20% = score (++); 21−40% = score (+++); 41−60% = score (++++); 61−80% = score (+++++); and 81−100% = score (++++++)

trioxide-induced cardiotoxicity (Chu et al. 2020; Liang et al. 2020). Also, Chu et al. (Chu et al. 2020) have demonstrated that crocin ameliorates increased ROS levels induced by DOX in H9c2 cells.

It is well-known that DOX administration causes imbalance on the serum lipid and fatty acid profiles (Hong et al. 2002). Hyperlipidemia induced by DOX leads harmful effects on heart function and is one of the most known reasons for DOX-induced cardiotoxicity. Therefore, agents with lipid lowering properties might have beneficial effects on DOX-induced cardiac damage (Iliskovic and Singal 1997). Recent studies have shown the evidence that crocin have protective effects against cardiovascular-related disorders including atherosclerosis, hiperlipidemia, and cardiac dysfunction (Alavizadeh and Hosseinzadeh 2014; Li et al. 2018). In this study, we observed increases in triglyceride, LDL, and VLDL levels as well as decrease in HDL levels. On the other hand, we demonstrated that co-administration of crocin with DOX resulted in a significant improvement in serum lipid profiles. According to our results, heart damage caused by DOX induction is associated with increases in the serum lipid profile and crocin might occur its cardioprotective effect by improving these biochemical indices because of its anti-hyperlipidemic properties. In line with our findings, Li et al. (Li et al. 2018) reported that crocin decreased total cholesterol, triglyceride, and LDL, and increased HDL in a coronary atherosclerosis rat model. Also, Haybar et al. (Haybar et al. 2019) demonstrated that DOX administration caused to increase in total cholesterol, TG, and LDL, and decreased in HDL whilst gemfibrozil improved the lipid panel in DOX-treated rats.

Normally, CK-MB and cTn-I are located in the cytoplasm of cardiomyocytes and pass through cardiomyocytes into the systemic circulation following cell membrane injury (Goudarzi et al. 2018). Thus, CK-MB and cTnI are cardiac markers and accepted as an indicator to evaluate cardiac dysfunctions. In our study, DOX-induced myocardial toxicity by the formation of ROS caused to elevate CK-MB and cTn-I whilst co-administration of crocin with DOX resulted in a significant improvement in elevated cardiac biomarkers. There is an evidence showing that crocin exhibits cardioprotective impact by decreasing serum cardiac markers (Elsherbiny et al. 2016). According to previous study, DOX-induced myocardial toxicity is associated with oxidative stress (Oner et al. 2019). It is well established that crocin could be potent antioxidant agent through downregulation of apoptotic and inflammatory pathways and/or scavenging free radicals (Elsherbiny et al. 2016).

On the other hand, the evidence suggested that the other mechanisms in the pathogenesis of DOX-induced cardiotoxicity except oxidative stress involve apoptosis and inflammation (Sun et al. 2016). Numerous literatures indicate that inflammation has a key role in DOX-induced myocardial injury (Chularojmontri et al. 2013; Imam et al. 2018; Shaker et al. 2018). Also, there are many documents confirming that DOX triggers a series of inflammatory responses in cardiomyocytes by upregulating the NF-kB and provides cascading release of some pro-inflammatory cytokines, including TNF-α and IL-1 (Abd El-Aziz et al. 2012). Recently, it has been demonstrated that the intensive increase of pro-inflammatory cytokines in the heart tissue may be the pathological basis of DOX-induced cardiovascular damage (Pecoraro et al. 2016). In compliance with previous studies, our results demonstrated significant increases in caspase-3 and tumor necrosis factor alpha (TNF-α) reactivities in myocardium after DOX injection (Dash et al. 2015; Ma et al. 2019). Also, Shaker et al. (Shaker et al. 2018) reported that DOX administration caused to increase in inflammatory markers such as TNF-α and interleukin-1 beta (IL-1β) and apoptotic markers such as caspase-3. Moreover, caspase-3, known as a cell death protease, plays an important role in cell apoptosis (Müller et al. 1998). Activated caspase-3 can alter cell morphology and degrade DNA, ultimately triggering apoptosis in the cell. DOX administration causes disruption of the respiratory chain function in the mitochondria of myocardial cells. The elevation of ROS production results in a significant disruption of the permeability of the mitochondrial membrane. These events ultimately trigger the apoptosis cascade reaction of caspases in the cell (Oliveira et al. 2006). The elevation of oxidative stress induced by DOX administration stimulates certain signaling pathways involving caspase-3 activation, resulting in myocardial cell apoptosis (Dash et al. 2015). In line with the literature, we found that DOX toxicity caused serious increases in TNF-α and caspase levels in myocardium. In contrast, our results revealed that increased inflammatory cytokines and apoptotic markers evoked by DOX induction improved by crocin treatment because of its anti-inflammatory and anti-apoptotic effectiveness. Moreover, these data are supported by our histopathological evaluation showing improvement in the histological architecture of myocardium through crocin treatment. Consistent with the results of the present study, Razavi et al. (Razavi et al. 2013) showed that crocin might have benefical effects against diazinon-induced cardiotoxicity through regulating signaling pathways associated with oxidative stress and cell apoptosis. Also, Chu et al.

(Chu et al. 2020) reported that there were significant decreases in TNF-α and IL-6 levels with different doses of crocin treatment in DOX toxicity.

Taken together, present study demonstrated that crocin protected rat myocardium against DOX-induced cardiotoxicity by modulating oxidative stress, reducing apoptosis, and inhibiting hiperlipidemia because of its antioxidant, anti-inflammatory, and antihiperlipidemic properties. Overall, we suggest that crocin may be a very good choice against DOX-induced myocardial damage. However, before moving into clinical practice in patients with DOX-induced cardiomyopathy, it is essential to better understand the mechanisms involved and the factors that govern the crocin therapy.

Author contribution Sara Asaad ABDULKAREEM ALJUMAILY and Yasemin Bicer studied biochemical analysis, Mehmet Demir designed the study and collected the tissues, Hulya Elbe and Gurkan Yigitturk performed the histological examination of the hearth tissues, and Eyup Altınoz designed the study and calculated the biochemical results, and was a major contributor in writing the manuscript.

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Availability of data and materials The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical approval and and consent to participate The study was approved by the Experimental Animals Ethics Committee of Zonguldak Bulent Ecevit University, Faculty of Medicine (Protocol No: 2020/04).

Consent to publish Not applicable

Conflict of interest The authors declare no competing interests.

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