

Cardioprotective effect of *Crataegus aronia* syn. *Azarolus* (L) Aqueous Extract Against Doxorubicin-Induced Cardiotoxicity and Heart Failure in Wistar Rats

Abdullah S. Shatoor¹ and Mohamed Atif A. Said Ahmed²

¹Department of Medicine, Cardiology section, College of Medicine, King Khalid University, Abha 61421, Saudi Arabia.

²Department of Anatomy, College of Medicine, King Khalid University, Abha 61421, Saudi Arabia.

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ABSTRACT

We examined whether *Crataegus aronia* (*C. aronia*) can ameliorate Doxorubicin (DOX) - induced cardiotoxicity in rats. Six groups of Wistar rats were divided as: Group I: a control group (administered saline); Group II: administered aqueous extract of *C. aronia* alone; Group III: administered DOX alone; Group IV: administered concomitant doses of both DOX and *C. aronia*; Group V: administered DOX first then followed by *C. aronia*; Group VI: administered *C. aronia* first, followed by DOX. Following treatment, serum levels of cardiac enzymes and B-type natriuretic peptide (BNP) were measured. Endogenous antioxidant evaluation, light and electron microscopy were performed on the cardiac tissues. Those of groups III and VI showed increases in oxidative stress and lipid peroxidation, accompanied by increases in the serum levels of cardiac enzymes and BNP. Histologically, groups III and VI showed destruction of myofibrils, infiltration of mononuclear cells, fibrosis and vacuolation. Ultrastructural changes included similar findings with emptying of myocytes, mitochondrial swelling, condensed or disrupted cristae. The Z-lines were also irregular, interrupted and thickened. These changes were significantly less prominent in groups IV and V. In conclusion, the aqueous extract of *C. aronia* augments the endogenous antioxidant components of the rat's heart and ameliorates DOX-induced cardiotoxicity.

KEYWORDS: *Crataegus aronia*, cardiomyotoxicity, Doxorubicin, Oxidative stress, rats

Running head: *Crataegus aronia* in Doxorubicin induced Cardiomyotoxicity.

INTRODUCTION

Cardiovascular diseases have high incidence and are leading cause of morbidity worldwide [1]. Congestive cardiac failure (CCF) is characterized by the inability of the heart to provide an adequate cardiac output to match the metabolic demands of the remaining tissues [2]. The disorder represents the terminal phase of several conditions that affect the myocardium.

Doxorubicin (DOX), an anthracycline, is effective for treating many types of cancers [3]. While DOX has clinical benefits, its use is dose-limited due to the high incidence of irreversible myocardial damage and dilated cardiomyopathy [4].

DOX-induced cardiotoxicity is believed to be induced through endogenous antioxidant deficiencies and increased lipid peroxidation [5]. The heart is particularly susceptible to free radical injury, as it contains fewer free radical detoxifying substances; e.g., superoxide dismutase (SOD), reduced glutathione (GSH) and catalases (CAT) [6]. The generation of reactive oxygen species (ROS) like superoxide anions and hydrogen peroxide; and overproduction of reactive nitrogen species by DOX, lead to the impairment of cell function and cytolysis [5, 6].

Several approaches may be taken to decrease the risk of DOX-induced cardiotoxicity while maintaining its efficacy. These include altered schedules of drug administration, modifications of the anthracycline molecule, adjunctive treatment with beta-adrenergic blockers, angiotensin-converting enzyme inhibitors (ACEi), dexrazoxane, and probucol [7, 8]. None of these have been entirely successful. A new drug to prevent or treat DOX-induced cardiotoxicity is therefore needed.

Plants are indispensable resources for the development of new cardiovascular drugs [9]. Hawthorn (*Crataegus species.*) has been used by many cultures for therapeutic purposes for centuries, and is a valuable remedy for cardiovascular diseases [10]. In addition to its potent antioxidant effects, several species are capable of increasing myocardial contraction and causing dilatation of peripheral and coronary blood vessels. This plant can therefore be used for treating hypertension, CCF, and ischemic heart disease (IHD) [11-13].

Crataegus aronia (*C. aronia*), synonym: *azarolus* (L), the dominant species in the mountains of the Mediterranean basin, has been used in traditional Arab medicine, to treat cardiovascular diseases, cancer, diabetes, hyperlipidemia and sexual weakness [14]. Furthermore, *C. aronia* was found to have the highest

Corresponding Author: Prof. Abdullah S. Shatoor, MD, ArBIM, Professor of Medicine and Consultant Cardiologist, Department of Medicine, Cardiology Section, College of Medicine, King Khalid University, Abha 61421, Saudi Arabia. Mobile.: +966505268277, Fax: +96672418201, E-mail: asshalghamdi@yahoo.com

antioxidant activity of any hawthorn species [15]. So far, there is no single study in the literature which has investigated the effect of this plant species against DOX-induced cardiotoxicity in animals or humans. Hence, the aim of the present study is to investigate whether the aqueous extracts of *C. aronia* can prevent and/or treat DOX-induced cardiotoxicity and heart failure in albino Wistar rats.

MATERIALS AND METHODS

Drugs:

DOX was obtained as a gift from Asser Central Hospital, Abha, Saudi Arabia as a 50 mg/ 25 ml bottle (2mg/ml).

Plant material and extraction procedure:

Fresh *C. aronia*, synonym *C. azarolus* (L) whole plant (stems, leaves, and flowers) was purchased from a local market in Jordan (Middle-east). The plant was identified, dried, and extracted in the Department of Pharmacognosy, College of Pharmacy, King Khalid University. The dried plant material was ground to a powder and extracted by maceration using distilled water (1 kg/L, w/v) for 3 days at 37°C. The extract was filtered and evaporated under reduced pressure in a rotary evaporator. The resulting residue (40 g) was then stored at 4°C. The residue was re-constituted in double distilled deionized (DDD) water and filtered through 0.2- μ m filters to obtain a stock solution of a strength of 1 mg/mL, which was diluted further to obtain the concentrations required in our study [16]. The chemical constituents of the water extract of *C. aronia* were identified by quantitative analysis and confirmed by thin-layer chromatography for the presence of flavonoids, terpenes/sterols, saponins and tannins, whereas anthraquinones (free and combined), glycosides and alkaloids were not detected [17].

Selection of doses:

Doses were selected on the basis of the effective dose of the entire plant of *C. aronia* (200 mg/kg) [16].

Experimental animals and design:

This study was performed during May 2012, in the Research Laboratory of the Physiology Department, Medical College of King Khalid University, Abha, Saudi Arabia. All experiments were approved by the Ethical Committee of King Khalid University (REC-201-05-01). Rats were housed and kept in standard laboratory conditions under light and dark cycle (12h light/dark), and maintained at an ambient temperature ($25 \pm 2^\circ\text{C}$). The animals were fed standard rat food and water *ad libitum*.

A total of 36 adult male albino Wistar rats weighing 203.5 ± 18 g and aged 7 weeks were used in the study. After acclimatization, the rats were assigned randomly to 6 groups of 6 rats each as follows:

Group I: Control rats – were administered normal saline i.p for 28 consecutive days.

Group II: *C. aronia* group – were administered 200 mg/kg/d [16] of *C. aronia* aqueous extract for 14 consecutive days, by oral gavage, then received normal saline i.p. for the next 14 days.

Group III: DOX group – were administered an accumulative dose of 15 mg/kg DOX in 6 equal i.p. injections (2.5 mg/kg every 2 days) over 14 consecutive days [18] and received normal saline i.p. for the next 14 days.

Group IV: DOX plus *C. aronia* group (DOX + *C. aronia*) – were administered a combined treatment of *C. aronia* and DOX for 14 consecutive days with same doses and routes of administration as previously mentioned, then received normal saline i.p. for the next 14 days

Group V: *C. aronia* post-treated group (DOX then *C. aronia*) – were administered an accumulative dose of DOX (15 mg/kg) i.p. for the first 14 days then a daily dose of *C. aronia* (200 mg/kg) orally for the next 14 days.

Group VI: *C. aronia* pre-treated group (*C. aronia* then DOX) – were administered a daily dose of *C. aronia* (200 mg/kg) for 14 days then treated with an accumulative dose of DOX (15 mg/kg) i.p. for the next 14 days.

Blood collection and biochemical analysis:

At the end of the experimental period all 28 rats from all the groups were anaesthetized with diethyl ether. Blood samples were collected from the rat's eye in plain tubes and centrifuged at 5000 rpm for 10 min to obtain serum, which was stored at -20°C . The serum was used for measuring the level of cardiac enzymes and *brain natriuretic peptides* (BNP). The levels of BNP were determined using a BNP 45 Rat ELISA Kit (Abcam, Cambridge, MA, USA) and the activities of lactate dehydrogenase (LDH) and creatine kinase isoenzyme (CK-MB) were determined using the colorimetric method (Human Diagnostics, Germany).

Tissue homogenate preparation:

Immediately after blood collection, the animals were killed by decapitation. Hearts were quickly excised, washed in ice-cold isotonic saline, and blotted individually on ash-free filter paper. Samples of heart tissue (250 mg) were then homogenized separately using a Potter-Elvehjem homogenizer, at 4°C, and diluted 1:4 in 0.1 mol Tris-HCl buffer (pH 7.4). The crude tissue homogenate was centrifuged at 9000 rpm for 15 min in a cold

centrifuge. The supernatant was collected and stored at -20°C until it was analyzed to determine SOD, glutathione peroxidase (GPx), and CAT activity and GSH and thiobarbituric acid reactive substances (TBARS) levels.

Endogenous antioxidant activity assessment in cardiac homogenates:

The activity of SOD (U/mg tissue) was determined using commercial available kit obtained from Randox Laboratories Ltd, Crumlin, UK, based on the method of Sun *et al.* [19] (Ransod cat. no. SD 125). According to this method, Xanthine and xanthine oxidase were used to generate superoxide anions, which react with 2-(4-indophenyl)-3-(4-nitro-phenyl)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. Changes in the absorbance were determined at 505 nm during the first 3 min of the reaction. Enzyme activities in the samples were calculated using a standard curve. One unit of SOD was defined as a 50% inhibition of the rate of reduction in 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride, under the conditions of the assay. GPx activity (U/mg tissue) was measured by Ransel photometric kit obtained from Randox Laboratories Ltd, Crumlin, UK (Cat No: RS 505). This method is based on that of Paglia and Valentine [20]. In brief, GPx catalyses the oxidation of GSH by cumene hydroperoxide. In the presence of glutathione reductase (GR) and NADPH, the oxidized glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP⁺. The decrease in the absorbance at 340 nm is then measured. The assay of GSH levels (mg/g tissue) was performed using Biodiagnostic kit (Cat No. GR 2511) which is based on the spectrophotometric method of Beutler *et al.* [21]. This method depends on the reduction of 5, 5'-dithiobis 2-nitrobenzoic acid with glutathione to produce a yellow colour, the absorbance of which was measured at 405 nm. CAT activity (U/ml) was measured using a Catalase Colorimetric/Fluorometric Assay Kit which was purchased from Biovision Inc., Milpitas, CA, USA. (CAT, Cat No. K773-100). In the assay, CAT first reacts with H₂O₂ to produce water and oxygen, the unconverted H₂O₂ reacts with an OxiRed™ probe to produce a product, the absorbance of which can be measured at 570 nm. Enzyme activities in the samples were then calculated using a standard curve. One unit of CAT was defined as the amount that decomposed 1.0 μmol of H₂O₂ min⁻¹ at pH 4.5 and 25°C.

Lipid peroxidation in the heart tissue homogenates was assessed using a TBARS assay described by Okhawa *et al.* [22]. In brief, cardiac tissues were homogenized in 0.1 M Tris-HCL buffer of pH 7.4 at a 1:4 weight to volume ratio, using a Potter-Elvehjem homogenizer at 4°C. A 4 mL reaction mixture containing 0.1 mL of tissue homogenate, 0.2 mL of sodium dodecyl sulfate, 1.5 mL of acetic acid with pH of 3.5 (20% acetic acid was pre-adjusted with 1 M NaOH to the desired pH), 1.5 mL of aqueous solution of TBA and 0.7 mL water was prepared. After heating at 95°C for 1 h in a hot water bath and cooling, 1 mL distilled water and 5 mL n-butanol and pyridine (15:1) mixture were added and the mixture was shaken vigorously on a vortex mixer and then centrifuged at 3,000 rpm for 10 min. The absorbance of the upper organic layer was read at 532 nm and values were obtained from a standard curve. Values were expressed as mM/100 g tissue.

Histological examination:

Light microscopy:

Left ventricular tissues were used for histological examination. In brief, tissue was fixed immediately upon termination of the experiment; using 10% neutral buffered formaldehyde solution (pH 7.0). Tissue processing was carried out using standard protocols for dehydration, clearing and paraffin infiltration. The container was filled with melted paraffin and allowed to cool to form a block of paraffin around the tissue. The block was trimmed to remove paraffin overlaying the tissue, and sections (4-μm thick) were cut and mounted on glass slides containing a drop of Mayer's egg albumin. Preparations were then dried on a hot plate at 50°C for 30 min, stained with hematoxylin and eosin and Masson's Trichrome, and examined under a compound light microscope. Myocardial damage and cellular infiltration were graded on a scale of 0 to 3, as previously described [23]. 0 = normal; 1 = mild (only a few small lesions as focal myocytes damage); 2 = moderate (multiple small lesions or extensive myofibrillar degeneration); 3 = severe (multiple moderate or large lesions or necrosis).

Electron microscopy:

Small portions (< 1mm³) of cardiac tissue were excised and fixed in 2% glutaraldehyde (dissolved in 0.1 M phosphate buffer, pH 7.4) for 3 h, followed by post-fixation for 1 h at 4°C with 1% Osmium tetroxide (OsO₄) and 0.8% potassium ferricyanide, prepared in the same buffer. The specimens were dehydrated in a graded series of acetone treatments and embedded in Epon 812. Sections were stained with uranyl acetate and lead citrate and examined using a transmission electron microscope (Jeol 1200 EX, Japan).

Statistical analysis:

Graphing and comparison between the groups were analyzed by Kruskal-Wallis test and significant pair-wise comparisons were determined by the Mann-Whitney U test, using GraphPad Prism software (version 6). All data are expressed as mean ± SD. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Macroscopic findings:

The general appearance of all animals was recorded throughout the study. The rats in groups III and VI appeared obtunded (mental blunting with mild to moderate reduction in alertness), overtly ill, and experienced

impaired movement. These observations were not seen in groups I, II, IV and V. During dissection of the rats, notable accumulation of ascetic fluid was observed in the peritoneal cavities of rats from groups III and VI. The hearts from these rats were grossly enlarged, and the atria and ventricles were dilated and hypertrophic. Similar gross morphological changes were not observed in groups IV and V (Table 1).

Table 1. Comparison of heart/body weight ratio, cardiac damage score, and mortality among treatment groups.

Treatment	Heart/Body weight ratio Mean \pm S.D.	Cardiac damage score Mean \pm S.D.	Mortality No.
Group I	0.3110 \pm 0.040	0	0/6
Group II	0.3074 \pm 0.048	0	0/6
Group III	0.4591 \pm 0.029 ^{†‡}	2.22 \pm 0.59**	1/6
Group IV	0.3480 \pm 0.052§	0.44 \pm 0.21	0/6
Group V	0.3554 \pm 0.027§	0.52 \pm 0.23	0/6
Group VI	0.4026 \pm 0.014 ^{†‡}	2.02 \pm 0.54**	1/6

[†]: significantly different from group I.

[‡]: significantly different from group II.

[§]: significantly different from group III.

** : significantly different from groups I, II, IV and V.

Lipid peroxidation, tissue antioxidant system, and biochemical alterations:

Compared to Group I, administration of *C. aronia* alone (Group II) resulted in a significant increase ($P=0.005$) in GSH but did not affect the activity of SOD, CAT and GPx or the level of TBARS (Figures 1 and 2). The levels of BNP and the activities of LDH and CK-MB also remained unchanged in Group II rats (Figure 2). In contrast, administration of DOX alone (Group III) resulted in a significant increase in lipid peroxidation, as indicated by elevated TBARS levels ($P=0.001$) (Figure 2) and the activities of SOD ($P=0.0001$), CAT ($P=0.0001$) and GPx ($P=0.002$) as well as a significant decrease in GSH ($P=0.0001$) (Figure 1). Also, in this group of rats, BNP levels and the activities of LDH and CK-MB were also significantly increased ($P=0.005$) in relative to the control group (Figure 2). When compared to DOX-treated rats (Group III), co-administered (Group IV) and *C. aronia* post-treated rats (Group V) experienced significant decreases in the activities of SOD, CAT and GPx and levels of TBARS as well as significant increases of GSH ($P=0.001$). Additionally, BNP levels and LDH and CK-MB activities were significantly reduced in these groups ($P < 0.05$) relative to the control group.

Light microscopy:

The histopathological changes observed in all groups of rats are depicted in figures 3 and 4. No abnormal cardiomyocyte pathology was detected in either the control group or the *C. aronia* treated group (group II) (Figures 3A and B, respectively). On the other hand, administration of DOX to the rats of group III resulted in many cardiomyocytes with fine granular cytoplasm without clearly defined nuclei, necrotic cardiomyocytes, disrupted myofibrils, and infiltration of mononuclear cells (Figure 3C). However, these necrotic cardiomyocytes and the infiltration of mononuclear cells were mild, and only mild disruption of myofibrils was noticed in both groups IV and V which were administered *C. aronia* with or after DOX administration, respectively (Figures 3D and E, respectively). However, destruction of myofibrils was common in group VI which were administered *C. aronia* in advance of DOX (figure 3F).

Widespread and marked myofibril loss, with different degrees of deposition of collagen fibers and fibrosis, detected by Masson's Trichrome stain, was observed in the hearts of Group III rats (Figure 4C) compared to Groups I (Figure 4A) and II (Figure 4B). Histological samples of Group III contained groups of cardiomyocytes, occupying large areas of cardiac tissue, with numerous vacuoles and/or pale cytoplasm (Figure 4C). Fibrosis and vacuolation were also observed in Group VI (Figure 4F) but were less prominent in groups IV (Figure 4D) and V (Figure 4E).

Grading of the cardiac samples showing cytoplasmic vacuolation and/or myofibrillar loss revealed a cardiac damage score of 2.22 ± 0.59 in Group III administered DOX alone (Table 1). A score of 0 was determined for the control (Group I) and *C. aronia*-treated rats (Group II). Mean cardiac damage scores of 0.44 ± 0.21 and 0.52 ± 0.23 were determined for Groups IV and V, respectively; both were significantly lower when compared to Group III (Table 1).

Electron microscopy:

All ultrastructural changes observed in the cardiac tissue of the control and all other experimental groups of rats are depicted in figure 5. The observed cardiac ultrastructures seen in the control group (Group I) were similar to the *C. aronia* treated group (Group II) (Figures 5A and B, respectively). In both groups, cardiac myocytes contained regular and normal myofibrils, mitochondria, sarcoplasmic reticulum, T tubules and other organelles. Myocytes contained well-defined myofibrils with a normal striation pattern and numerous

interspersed globular mitochondria. Myofibrils were highly ordered in arrays of dark anisotropic A bands and light anisotropic bands. Light bands were bisected by a thick, dark Z band.

The ultrastructural alterations in the DOX treated (group III) included loss and lysis of myofibrils with loss of the normal striation pattern in the myofibrils and emptying of the myocytes, vacuolization and mitochondrial swelling, condensed or mitochondrial disruption and cristae destruction (Figures 5C and D). Cardiac myocytes from this group showed degeneration and necrosis. Also, the Z-lines were irregular, interrupted and thickened in some areas (Figure C). Mega mitochondria were also seen in this group of rats (Figure D). On the other hand, cardiomyocytes of the groups of rats which received *C. aronia* with or after DOX administration (groups IV and V, respectively) showed normal striation patterns with swollen and condensed mitochondria and mild myofibrils loss and interrupted Z-line as compared to DOX-treated rats (Figures 5E and F, respectively). However, the group of rats pre-treated with *C. aronia* and then administered DOX (Group VI) showed alterations that were similar to those in DOX-treated rats (figure F).

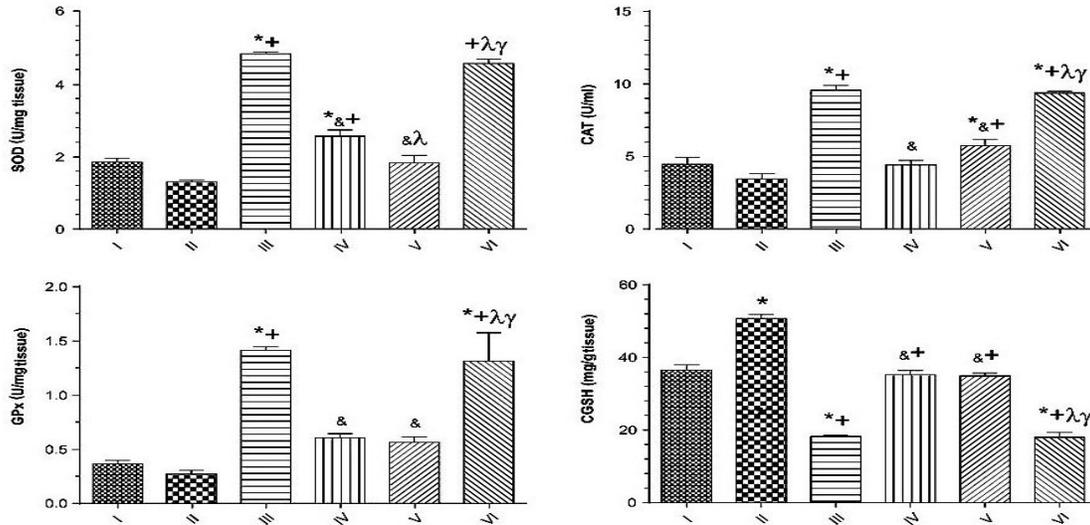


Figure 1

Figure 1. Levels and activities of non-enzymatic and enzymatic components of endogenous antioxidant defense system in the heart homogenates of treatment groups: GSH levels and the activities of SOD, CAT and GPx. Values are expressed as mean \pm SD for 6 rats in each group except for groups III and VI (n = 5 rats each). *: Significantly different when compared to group I, +: Significantly different when compared to group II, &: Significantly different when compared to group III, λ: Significantly different when compared to group IV, γ: Significantly different when compared to group V.

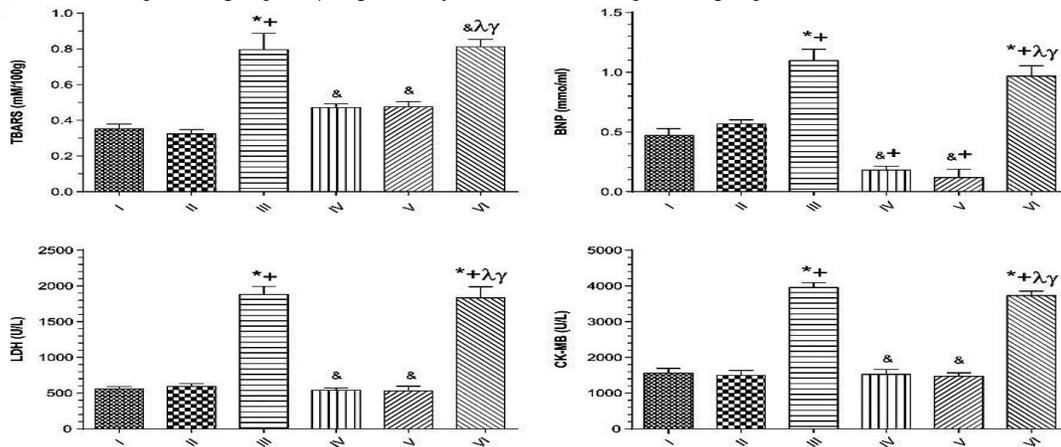


Figure 2

Figure 2. Comparison of levels of TBARS in the heart homogenate, sera levels of BNP and the activities of LDH and CK-MB among treatment groups. Values are expressed as mean \pm SD for 6 rats in each group except for DOX and *C. aronia*-pretreated groups (6 rats each). *: Significantly different when compared to group I, +: Significantly different when compared to group II, &: Significantly different when compared to group III, λ: Significantly different when compared to group IV, γ: when compared to group V.

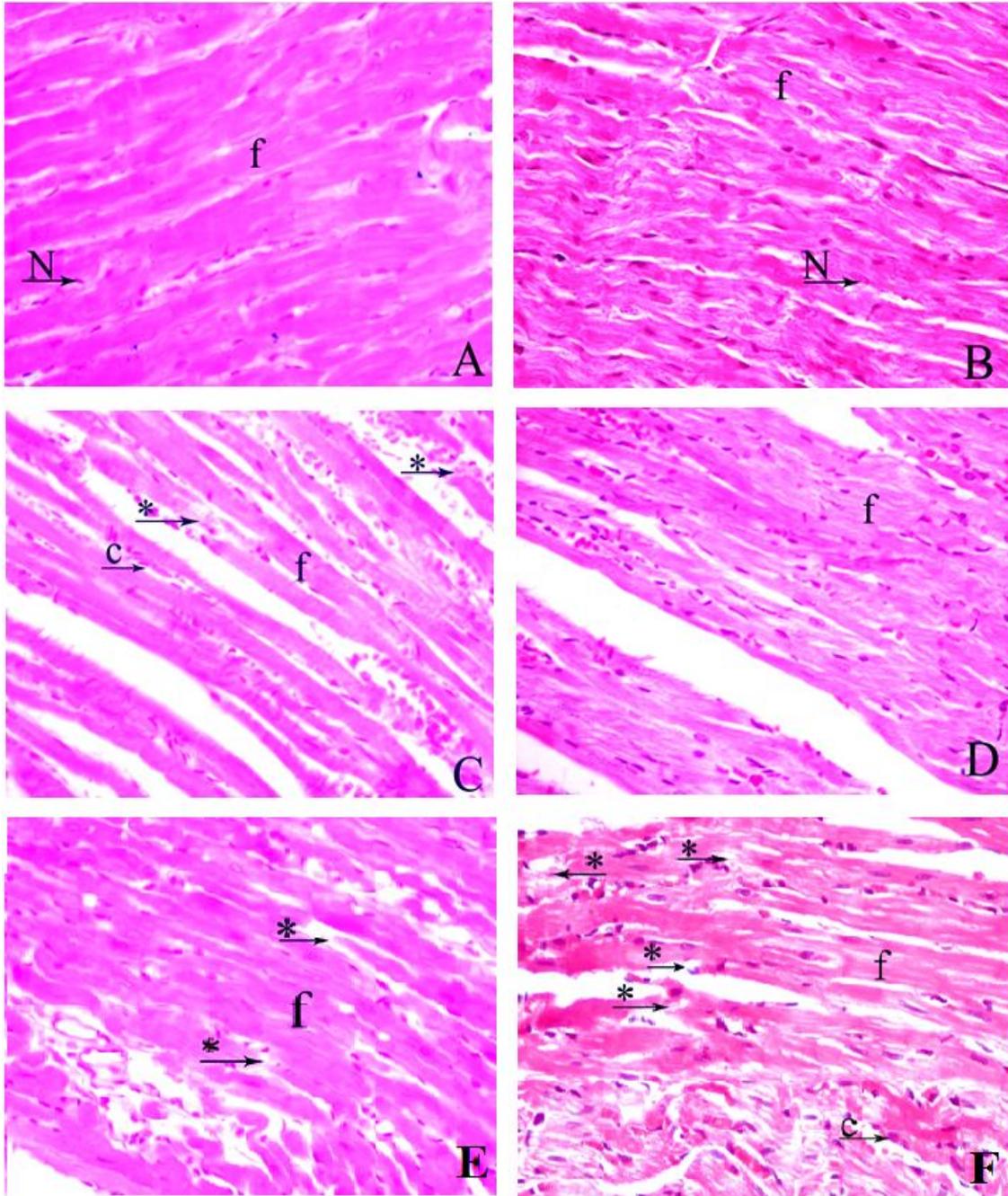


Figure 3

Figure 3: Photomicrographs of the histopathological samples of cardiac tissue stained by H&E, from all experimental groups. **A.** Control group (Group I) shows normal myocardial myofibril (f) and nuclei (N), H&E $\times 400$; **B.** *C. aronia* treated group (Group II) shows normal myocardial myofibril (f) and nuclei (N), H&E $\times 400$; **C.** DOX-treated rats (Group III) showing marked disrupted and destructed (*) myocardial myofibril (f), Mononuclear cells (c) H&E $\times 400$; **D.** Photomicrograph of a section of a heart from the group of rats administered combined doses of DOX and *C. aronia* aqueous extract (Group IV) showing mild disrupted and destructed myocardial myofibril (f), H&E $\times 400$; **E.** Photomicrograph of a section of a heart of *C. aronia* post-treated group (Group V) from the group of rats administered showing mild disrupted and destructed(*) myocardial myofibril (f), H&E $\times 400$; **F.** Photomicrograph of a section of a heart of *C. aronia* pre-treated group (Group VI) showing disrupted and destructed(*) myocardial myofibril (f) and many mononuclear cells (c), H&E $\times 400$.

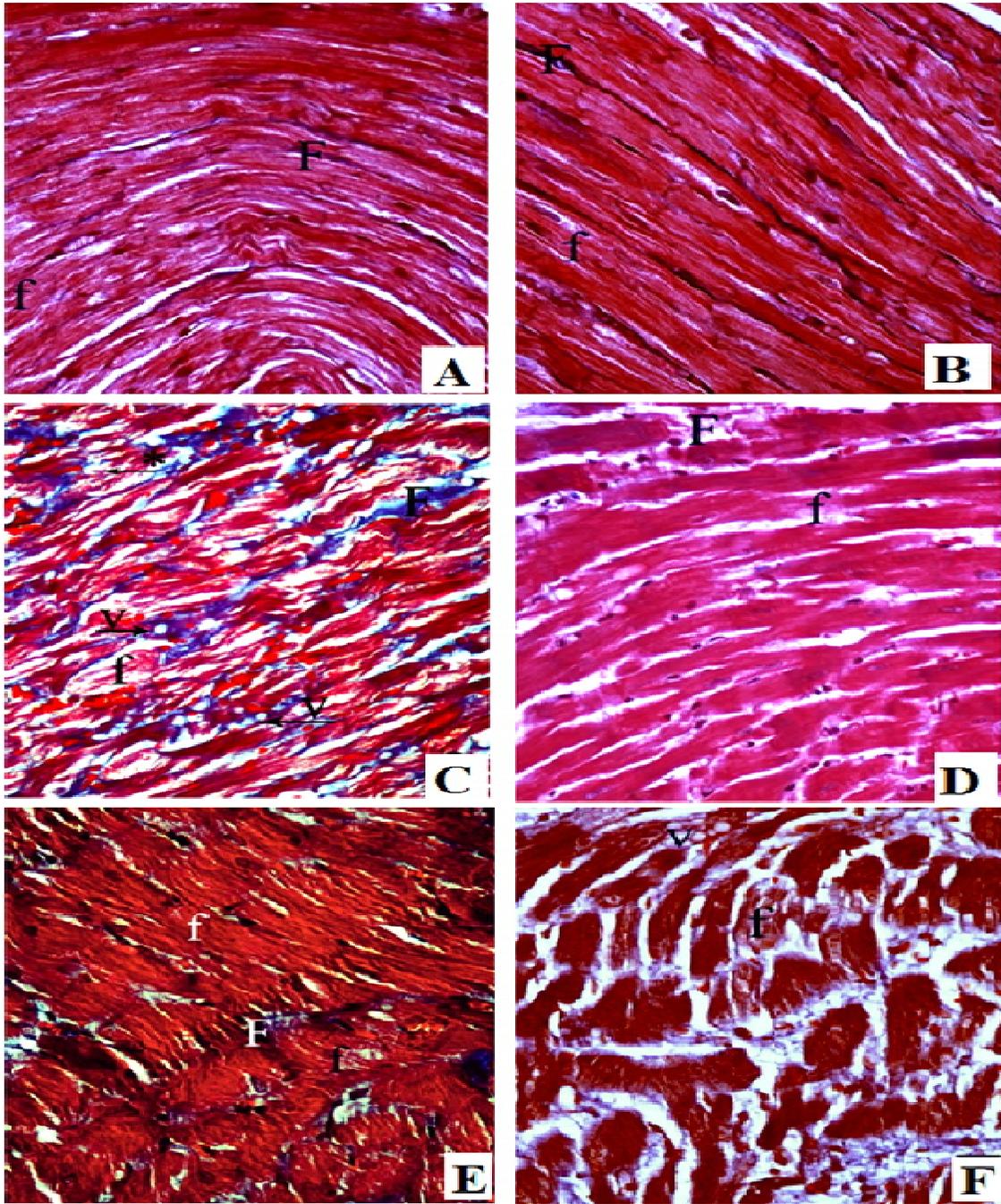


Figure 4

Figure 4: Photomicrographs, of the heart histopathological samples of cardiac tissue stained by Masson's trichrome, from the experimental groups. **A.** Control group (group I) shows normal myocardial myofibril (f) and very mild collagen fiber (F), Masson's trichrome $\times 400$; **B.** *C. aronia* treated group (group II) shows normal myocardial myofibril (f) and very mild collagen fiber (F), Masson's trichrome $\times 400$; **C.** DOX-treated rats (group III) shows marked interstitial fibrosis (f), disrupted and destructed myocardial myofibril (F) and vacuolization (v) of myocardial myofibril, Masson's Trichrome $\times 400$; **D.** (group IV) Photomicrograph of a section of the heart from the group of rats treated with *C. aronia* and DOX showing mild interstitial fibrosis (F). Mild disruption of myocardial myofibril (f) and vacuolization of myocardial myofibril (v), Masson's Trichrome $\times 400$. **E.** Photomicrograph of a section of the heart from the group of rats post treated with *C. aronia* after DOX treatment (group V) showing very mild interstitial fibrosis (F). Mild disruption of myocardial myofibril (f) and vacuolization of myocardial myofibril (v), Masson's Trichrome $\times 400$. **F.** Photomicrograph of a section of the heart from the group of rats pre-treated with *C. aronia* then treated with DOX (group VI) showing interstitial fibrosis (F). Disruption of myocardial myofibril (f) and vacuolization of myocardial myofibril (v), Masson's Trichrome $\times 400$.

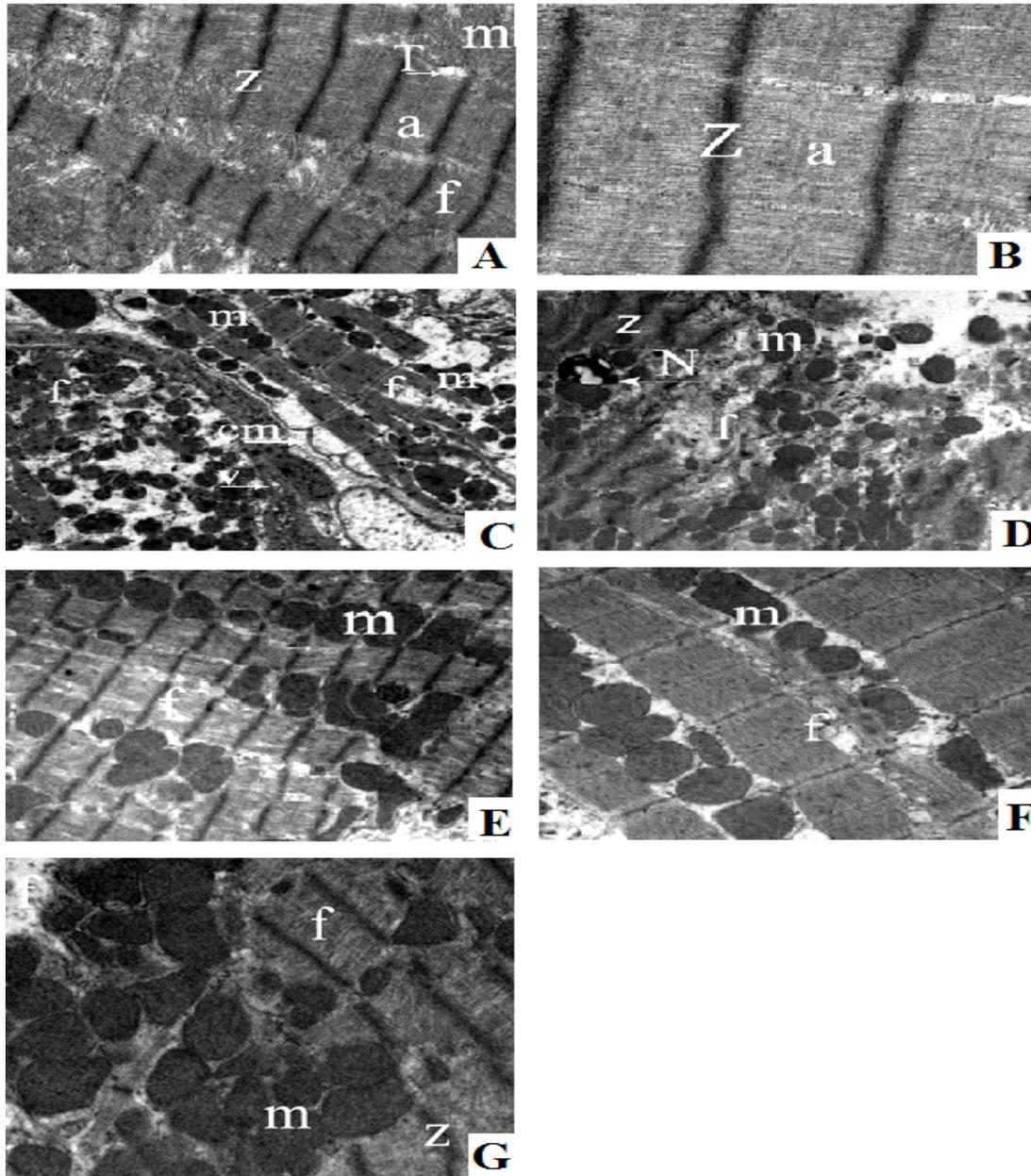


Figure 5

Figure 5. Transmission electron micrographs of the cardiac tissue of rats from the treatment groups. **A.** Control group (Group I): showing normal striation pattern and myofibrils (f). Z- line (z) and A- band (a) of myofibrils. Plenty of mitochondria (m) and T tubule (T) are also seen (magnification = 8000x); **B.** *C. aronia* treated group (Group II) showing normal striation pattern and myofibrils (f). Z- line (z) and A- band (a) of myofibrils (magnification = 25000x); **C.** DOX-treated group (Group III) showing degenerated cardiac muscle cells with damaged mitochondria (m) with condensed or loss cisternae. Myofibrils are disrupted or lost (f) with irregular cell membrane (cm) and some vacuoles (v) and degenerated area (D) (magnification = 5000x); **D.** DOX-treated group (Group III) showing degenerated cardiac muscle cells with damaged condensed mitochondria (m). Myofibrils are disrupted or loosed (f) with atrophied nucleus (N) and degenerated area (D) with thickened Z line (z) in some areas and interrupted in others (magnification = 4000x); **E.** Photomicrograph of cardiac muscle from rat treated with DOX and *C. aronia* (Group IV), showing condensed and swollen mitochondria (m) in between myofibrils. Myofibrils (f) very mild disrupted (magnification = 4000x). **F.** Photomicrograph of cardiac muscle from rat post-treated with *C. aronia* (Group V), showing condensed and swollen mitochondria (m) in between myofibrils. Myofibrils (f) mild disrupted (magnification = 20000x). **G.** Photomicrograph of cardiac muscle from rat pre-treated with *C. aronia* (Group VI), showing condensed and swollen mitochondria (m) in between myofibrils. Myofibrils (f) disrupted.

DISCUSSION

The histological and ultrastructural changes, as well as a marked improvement and boosting of the cardiac endogenous enzymatic and non-enzymatic antioxidant defence system of rats, demonstrate that an aqueous extract of *C. aronia* can protect and treat DOX-induced cardiomyotoxicity and heart failure.

DOX-induced cardiomyotoxicity has been well documented [24-26], and is caused mainly by oxidative stress and possibly also through damage and thrombosis of the coronary circulation [5, 6, 27, 28]. Indeed,

DOX-induced cardiotoxicity and oxidative stress relationship has been confirmed in many experimental models. As the role of reactive oxygen species (ROS) including hydroxyl radical in DOX-induced cardiotoxicity have been well documented [5,6,18, 27,28]. DOX is converted in the cardiac tissue into its semiquinone form, which is a toxic, short-lived metabolite that can interact with molecular oxygen initiating a cascade of reaction leading to ROS generation [29]. Another reported mechanism of DOX-induced oxidative stress cardiac damage is the formation of an anthracycline-iron (Fe²⁺) free radical complex [30]. The latter reacts with hydrogen peroxide to produce hydroxyl (OH•) radical. ROS react with lipids, protein and other cellular constituents causing damage to mitochondria and cell membranes of the heart muscle cells [31]. As a consequence of increases DOX induced cardiac oxidative damage, a progressive reduction of left ventricular function is observed in a significant proportion of patients during the course of DOX therapy [32, 33]. Furthermore, calcium imbalance, mitochondrial damage, and apoptosis were reported in heart failure induced by DOX therapy [34].

Since oxidative stress is implicated in DOX-induced cardiotoxicity [27]. We investigated the therapeutic and cardioprotective potential of a powerful antioxidant, *C. aronia*. Lipid peroxidation, GSH content, SOD, CAT, and other antioxidant enzyme activities have been used as markers of tissue injury and oxidative stress in cardiac tissue. Here, DOX-induced cardiomyotoxicity and oxidative damage were indicated by significant increases in the activities of antioxidant enzymes, SOD, GPX and CAT, the cardiac content of TBARS (lipid peroxidation marker), and a significant decrease of GSH in the heart homogenate. The heart is highly susceptible to oxidative stress due to decreased levels of detoxifying natural antioxidants [5,6]. The observed GSH deficiency and rise in all enzymatic antioxidant parameters and TBARS levels caused by DOX administration, together with the protective effect of *C. aronia*, strongly support the view that the oxidative damage is due to the generation of free radicals. This is consistent with other studies, which found that the cardiac content of TBARS increased and GSH decreased in rodents after DOX administration [35].

DOX-induced myocardial damage in rats was also indicated by increased levels of serum biomarker enzymes (LDH and CK-MB). This increase suggests a leakage of these enzymes from the cardiomyocytes secondary to DOX administration, which is supported by other studies [36,37].

Recent advances in BNP physiopathology and improved assay methods have clarified its diagnostic and prognostic role in CCF [38]. The BNP and the amino-terminal fragment of its precursor (NT-proBNP) are rapidly produced and secreted by the heart muscles in response to ventricular wall distention. Accordingly, increased blood concentrations of these markers are associated with the onset of DOX-induced cardiotoxicity [39]. Consistent with previous findings, levels of BNP were significantly elevated in Groups III and VI rats which also showed accumulation of ascites, indicating ventricular dysfunction and CCF compared to Groups IV and V rats.

Many studies have discussed the modulation of oxidative stress, lipid peroxidation and cardiac damage following DOX administration [40-42]. Interestingly, co-administration or post-treatment of the animals with *C. aronia* ameliorated the oxidative damage and normalized the enzymatic and non-enzymatic defence activities and significantly reduced lipid peroxidation in the cardiac tissues. Subsequently, cardiac enzymes and BNP levels reached normal levels in these groups of rats. The modulating effect of *C. aronia* is likely to be, at least in part, due to its antioxidant scavenging potential, as significant reductions in GSH levels were observed in the group of rats administered only *C. aronia*.

Similar to previous reports [43], these findings were supported by the histopathological examination of the cardiac tissue. Vacuolizations observed in the cardiac muscle are autophagic in nature and may increase because of inefficient clearance of autophagosomes rather than up-regulation of macroautophagy. Autophagy has been considered an adaptive mechanism for degradation and recycling of cytoplasmic components [44, 45]. Much less vacuolization was seen in rats that were co-administered or post-treated with *C. aronia* (Groups IV and V).

The increase in cytoplasmic injury, including loss, disruption, and disassembly of myofibrils after DOX treatment was also observed by Chaiswing et al.[46]. Myofibril loss is a known characteristic of myocardial injury caused by DOX treatment and is associated with reduced expression (mRNA) of cytoskeletal genes [47]. An early step in the breakdown of sarcomeres after DOX exposure is the degradation of the myofilament protein, titin [48]. Mihm et al.[49], showed that cardiac myofibrils are a primary site of protein nitration following DOX treatment.

Myocyte cell loss, through apoptosis, has been reported in DOX-induced cardiomyopathy [50, 51]. Apoptotic cell death correlates with deteriorating myocyte morphology and is considered essential to progress to

CCF [52]. Recently a study confirmed the protective effect of *Crataegus oxyacantha* against isoproterenol-induced inflammation and apoptosis-associated myocardial infarction (MI) in rats [53]. Here, we observed mitochondrial swelling, disruption of cristae, and condensed or mega mitochondria after DOX treatment (Group III and VI). These ultrastructural changes are consistent with those observed following DOX therapy and are correlated with decreased function of mitochondrial respiratory complexes I and II [54]. Our results also show interruption, broadening, and irregularity of Z lines in the cardiac tissue of DOX-treated rats. Again, these changes are similar to those observed by electron microscopy in the ischemic myocardium of dogs [55].

Different species of hawthorn has been used to successfully treat CCF and IHD through multiple actions, including the vasodilatation of both coronary and peripheral vessels by enhancing the endothelial release of nitric oxide and inhibition of angiotensin converting enzyme (ACE), enhancement of the inotropy of the heart and possibly through the antiplatelet and anticoagulant effects [11,13,16, 17, 56-59]. Furthermore, we demonstrated that *C. aronia* has in addition to the direct cholinergic receptor agonist, beta-adrenergic receptors blocking effect [60]. Preliminary reports have shown that concomitant use of ACEi and beta-blocking agents with DOX improve the outcome of DOX-induced cardiomyopathy [61, 62]. These favorable multiple actions, together with the potent antioxidant effects of *C. aronia*, administered over short period (14days), in a single fixed dose (200mg/kg), conferred clear protection against DOX induced cardiotoxicity. However, extreme caution has to be exercised, since supplementation with antioxidant could influence the response to chemotherapeutic agents [63].

CONCLUSION:

Our results show that oxidative stress underlies DOX-induced cardiotoxicity. Administration of the aqueous extract of *C. aronia* with or following DOX treatment ameliorated damage to cardiac tissue by modulating the pathways that trigger cardiotoxicity. Additional animal and human trials are needed to confirm these findings and determine the active ingredient present in the aqueous extract of *C. aronia* which is responsible for these effects for future drug development.

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