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Cardioprotective effect of *Malva verticillata* against doxorubicin -induced toxicity in rats

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Abstract

Background: *M. verticillata* (Malvaceae) is a medicinal plant used in the treatment of wounds, boils, and liver injuries. The plant leaf extracts possess anti-inflammatory and antioxidant activities. Doxorubicin (DOX) is a potent chemotherapeutic agent used in the treatment of various cancers, but its clinical use is limited by acute and chronic cardiotoxicity**.** This study aims to evaluate the possible cardioprotective role of *Malva verticillata* against doxorubicin-induced cardiotoxicity.

Method: Thirty-six male albino rats were divided into six groups, ($n = 6$): G1: normal control (was given 1 ml/kg of NaCl, 0.9%, twice a week IP), G2: cardiotoxic group (was given 1 mg/kg of DOX twice a week IP). G3 and G4 were given 250 mg/kg and 500 mg/kg of *M. verticillata,* respectively, while G5 and G6: were given 250, 500 mg/kg of *M. verticillata* PO and 1 mg/kg IP of DOX**.** for 6 weeks. Total body weight was taken weekly and Heart: body weight ratio was calculated. Blood samples were collected for determination of serum lactate dehydrogenase (LDH), creatine phosphokinase (CPK) and Troponin, the hearts were removed and processed for histopathological examination.

Results: *M. verticillata* showed a significant dose-dependent reduction in the cardiac enzyme levels, LDH, CPK activities, and Troponin levels. The histopathological studies in rat hearts also supported those findings.

Conclusion: The present study suggests that *M. verticillata* may have a novel and worthwhile cardioprotective effect against DOX-induced cardiotoxicity.

Keywords: Cardioprotective, *Malva verticillata*, Doxorubicin, Cardiac enzymes

Introduction

Medicinal plants (fruits, vegetables, medicinal herbs, etc.) are a source for a wide variety of natural products, such as phenols and flavonoids, which are very interesting for their antioxidant properties [1]. In addition to their ability to act as an efficient free radical scavenger [2], their natural origin represents an advantage of the consumer in contrast to synthetic ones, which their use is being restricted due to their carcinogenicity [3]**.** Yemen's flora is very rich. Where previous researches and studies have found that approximately 2838 species of plants; 179 families and 1068 genera are existed in Yemen [3, 4]. The most important families regarding the number of endemics are Malvaceae. Indeed, Malvaceae or mallow family is a large family of herbs, shrubs, and tree plants; containing over 200 genera comprising about 2300 species. It is a globally distributed family with the primary concentration of genera in tropical and subtropical regions [5]. Medicinally it is used in cough, ulcers in the bladders, intestinal infections, colitis, tonsillitis, gastroenteritis, cholesterol and lipid-lowering, anti-hypertensive, antioxidant, analgesics, emollient, pectoral girdle and arteriosclerosis treatment. Additionally, the plants are also used externally as antidandruff, demulcent, softening of tumors [6–12].

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The leaves of *Malva verticillata* are rich in various active constituents, particularly phenols, flavonoid (quercetin), saponin, alkaloid, resin, and tannin that possess antioxidant and anti-inflammatory effects [13]. Many studies deal with the protective effects of *Malva* spp. Ko et al., 2018 found that *M. verticillata* may become the promising antidiabetic agent. They isolated four phenolic compounds from the aerial part that recovered the size of pancreatic islets (PIs) from zebrafish larvae damaged by treatment with alloxan [14]. Additionally, the outcomes of the Shim et al., [15] study demonstrated that a water extract of *M. verticillata* seeds inhibited osteoclastogenesis and bone resorption by suppressing the RANKL signaling pathway. Therefore, they suggest that *M. verticillata* seeds may be used as a therapeutic candidate in complementary alternative medicine to treat bone diseases. Moreover, the protective effect of *Malva sylvestris* other species of Malva was approved in the study conducted by Marouane et al., [16]. They found that *Malva sylvestris* improved renal damage induced by ammonium metavanadate poisoning in rats. Another study conducted in 2018 showed that the methanol extract of leaves of *Malva verticillata* possessed considerable antinociceptive activity and can be used as a substitute for aspirin [17].

Doxorubicin is an antibiotic of the anthracycline group, which was isolated from fungal cultures of *Streptomyces peucetius* var. It has antitumor efficacy and used in combination with other agents for the treatment of sarcomas and a variety of carcinomas, including breast, ovary, endometrium, bladder, thyroid, and cancer of the lung, as well as for treatment of acute lymphocytic leukemia and lymphomas [18].

The main adverse effect is cardiotoxicity, which limits the use of this medication, the exact cardiotoxic effect of doxorubicin is not fully understood. Different mechanisms and steps are involved and end finally with cardiomyocyte death [19]. Until now the main mechanisms proposed by many researchers are oxidative stress, Ca + 2 hemostasis dysregulations, iron metabolism, sarcomeric structure alterations, gene expression modulation, and apoptosis [20]. Dox. Induces intrinsic and extrinsic apoptosis by different mechanisms, one of the accepted pathways involves the production of ROS and oxidative mechanisms that are prevented by antioxidants [19, 21]. Additionally, several studies indicated that Dox.-induced cardiotoxicity is related directly with its ability to modulate microRNAs, which in turn, have a role in all cardiac functions including conductance of electrical signals, heart muscle contraction, and growth [22]. As mentioned in the previous studies that polyphenols and flavonoids exist in *Malva verticillata* possesses antioxidants and suggest to exhibit conspicuous protective effects that may ameliorate adverse effects induced by some drugs. For this reason, the present study is designed to evaluate the possible cardioprotective effect of *Malva verticillata* against doxorubicin-induced toxicity in rats.

Materials and methods

Plant material

The fresh plant of *M. verticillata* was collected from the Bani Alharith park, Sana' a Yemen, between August and September 2018, and the botanical samples were authenticated by Dr. Mohammed Ibrahim (Head of the Biology department). Its voucher specimen has been deposited at the herbarium of Faculty of Science, Sana'a University (voucher No. BHSS: 614).

Preparation of extract

The leaves of *M. verticillata* were separated from their stem, washed with water, and then they were shaded and dried at room temperature for at least 2 weeks to be very well dried. After that, they were crushed to get a homogenous fine powder using a grinder and then kept in an airtight container in a dry place at room temperature until their use in the different study purposes.

Eight hundred grams of powdered leaves of *M. verticillata* were placed in a flask with (5 L) of methanol, and the mixture was then extracted by agitation for 5 h at 25 °C.

Then, maceration of the extracts was done overnight for 24 h. The methanolic layer containing the extract was taken. The extraction was repeated on the remaining amount of the precipitate using (1500 ml) of methanol and all extracts were filtered by using a 0.45 Millipore filter paper. The two fractions of extracts were mixed and then concentrated using a rotary evaporator at 40 °C under reduced pressure. In the final step, the extract was further freeze-dried to produce a dry powder [23, 24]. The yield was found to be equal to (124.4 g).

Phytochemical screening

Phytochemical screening and analysis of the leaves *of M. verticillata* was performed according to the methods described by Evans [25].

Alkaloids were tested by dissolving 0.2 g of the powder of *M. verticillata* in 10 ml of 1%. HCl. Then, transferred to a water bath for a few minutes. After, 1 ml of the filtrated extract was treated with 2–4 drops of Dragendorff's reagent. The presence of alkaloids is indicated by the appearance of orange-reddish precipitation.

In the test tubes containing *M. verticillata* filtered extracts were shaken for 5 min using a vortex. The appearance of big foamy indicates the presence of saponins.

Phenols were detected by taking 5 ml of methanolic extract of *M. verticillata* and 1 ml of 1% FeCl3 and 1 ml

of 1% K3 (Fe (CN)6) was added. The appearance of fresh radish blue color indicated the presence of polyphenols.

Flavonoids were tested by taking the solution of the leaves to extract that was prepared previously. The solution consists of 5 ml of methanolic solvents added to 5 ml of 50%. KOH The presence of flavonoids is indicated by the appearance of the yellow color. For detection of ash, about 10 ml of *M. verticillata* filtered extracts were taken and 20 ml of 4% HCl were added. The appearance of turbidity indicates the presence of resins in the extracts.

Ferric chloride test was used to detect the presence of tannins. Many drops of ferric chloride reagent 1% (FeCl3) were added to the different filtered extracts. The presence of tannins is indicated by blue color.

For the detection of coumarins about 5 ml of filtered extracts were kept in a test tube and covered by a filter paper saturated in Na OH. Then the test tube was put in a water bath, boiled for 10 min. After that, the filter paper was taken and exposed to UV light. The presence of coumarin isindicated by a bright green yellow color.

To test terpenoids about 1 ml of acetic anhydride and 2 ml of concentrated sulfuric acid were added to beakers containing 1 ml of previously filtered extracts. The presence of reddish-brown on the interface indicated the presence of terpenoids.

Volatile oils were tested by adding 10 ml of previously filtered extracts were filtered by filter paper till saturation and then exposed to UV light. The presence of volatile oil was indicated by the appearance of the bright pinkish color on the filter paper.

Dose fixation

Doxorubicin hydrochloride 50 mg was used to induce cardiotoxicity, it was prepared in 25 ml solution with saline. One milligram per kilogram i.p. twice a week of DOX during 6 weeks is safe and sufficient to induce chronic cardiotoxicity in animals [26, 27], while 20 ml/kg i.p. a single dose is sufficient to induce the acute toxicity in animals. Then the animals were weighed accurately and the volume of doxorubicin hydrochloride solution was calculated and administrated to animals according to their body weight by intraperitoneal route [28].

Drugs and chemicals

Doxorubicin hydrochloride 50 mg (Adriamycin), were purchased from Sigma-Aldrich, All the chemicals used were of analytical grade. Absolute methanol, Sodium hydroxide, Dragendorff's reagent, Ferric chloride reagent (FeCl3) 1%, potassium hydroxide, Hydrochloric acids (HCL), acetic anhydrate, potassium ferric cyanide, sulfuric acid, formalin, and Chloroform were purchased from Sigma-Aldrich. Distilled water was prepared in the Department of Biochemistry, University of Science and Technology, Sana' a.

Equipment

Buchi-type Rotary evaporator (R-200-Switzerland), Centrifuge 6000 rpm (Hitachi, Germany), Super Dry Grinder-Model NO: MX-J 225 G, Panasonic, Orbital shaker (D-79219-IKA-Werke-Germany), Conical flask (V1000 ml), Freeze-dryer (Loboconco-Missouri-USA), Ultraviolet (Specord 205-Analytikjena), Electronic digital balance (Bofco-Max120g-Germany).

Experimental animals

Adult healthy male albino rats (*Rattus norvegicus Albinus*) of weighing (170 ± 30 g) and aged (8 months ±1 week) were obtained from the animal house of the Biology Department, Sana'a University. One week before the experiment was started, the animals were left to acclimatize in metal cages (30 × 30 × 50 cm). The animals were fed with clean water and food through the period of the experiment. During the study, rats were maintained at 12 h light/dark cycle. The approval of the Ethical Committee was obtained before the experiment [EAC/UST156].

Experimental design

The study comprised thirty-six adult males, albino rats and which divided into six groups. Each group contains six animals according to the following protocol:

The group I: received 1 ml/kg of NaCl, 0.9%, twice a week IP.
Group II: cardiotoxicity was induced by giving 1 mg/kg IP of doxorubicin twice a week in groups II, V, VI.
Group III: received 250 mg/kg of *M. verticillata*
Group IV: received 500 mg/kg of *M. verticillata*
Group V: cardiotoxic – induced group – treated group by giving 250 mg/kg/d of *M. verticillata* (treated group).
Group VI: cardiotoxic – induced group – treated group by giving 500 mg/kg/d of *M. verticillata* (treated group). The extract was administered to the tested animals by oral gavage once a day for 6 weeks and was injected intraperitoneally with a dose of 1 mg/kg of doxorubicin twice a week for 6 weeks [25, 26, 29–31].

Biochemical tests

Blood samples were collected into the sterilized tubes with the anticoagulant to reduce the risk of hemolysis after removing the needles from syringes, and then were centrifuged at 6000 rpm (revolutions per minute) for 10 min with Hitachi, Germany at 25 °C for separation of serum [31]. The investigations include creatine phosphate kinase

(CPK), lactate dehydrogenase (LDH), Troponin, and Heart: bodyweight ratio.

Histopathological study of heart

At the end of the experiment, the animals were sacrificed by decapitation under light anesthesia by halothane in the anesthetic desiccator. The anesthetic procedure following the International safety of the WMA Statement on Animal Use in Biomedical Research and conducted by an expert person. The rats were very calm as they were undergoing slow death, then the heart was removed and stored in 10% formalin and taken to the Department of Pathology, Hospital of University of Science and Technology for the histopathological studies. Tissue processing was embedded by autotechnicon in paraffin wax and the tissue was prepared 5-μm thickness with hematoxylin and eosin.

Statistical analysis

Data were summarized as mean ± SEM. One-way analysis of variance (ANOVA) followed by Dunnet's multiple comparison test was used to conduct the significance of association using statistical package for the social sciences (SPSS) program version 21. Differences were considered significant at P values of less than 0.05.

Results

Plant extract yield and preliminary phytochemical analysis

The percentage yield of the methanolic crude extract which was obtained by cold extraction was (15.55%). The results of the phytochemical screening revealed the presence of alkaloids, saponin, phenols, flavonoids, ash, and tannins in the *M. verticillata* extract. Coumarin, terpenoids, and volatile oil were not found. (Table 1).

Table 1 Preliminary phytochemical constituents of the leaves *M. verticillata* extract

No	Constituents	Results
1	Alkaloids	+
2	Saponins	+
3	Flavonoids	+
4	Phenols	+
5	Ash	+
6	Tannins	+
7	Coumarins	-
8	Terpenoids	-
9	Volatile oil	-

+ = presence of active compound and - = absence of active compound

Cardiotoxicity results

Methanolic extract of *M. verticillata* showed significant amelioration of Dox.-induced cardiac injury as it reduced the level of cardiac function enzymes CPK, LDH, Troponin (Fig. 1). This effect was supported by the weight of the heart (Table 2) as well as cardiac tissue improvements. Moreover, continuous administration of extract *M. verticillata* in albino rats for 6 weeks counteracted the weight loss caused by DOX. and improved the health state of animals without harmful effect as shown in Figs. 2 and 3 When compared with DOX. Induced cardiotoxicity, the methanolic extract *M. verticillata* treated groups significantly showed a dose-dependent reduction in cardiac enzymes.

Histopathological finding

Histopathological examination of heart sections also supported biochemical investigation as shown in Figs. 2 and 3. The Histopathological change of the rat's heart was significantly observed in the cardiotoxic group that showed layers of the heart wall with sever reactive change compared to the control group, vacuolization, edema, inflammatory were seen in DOX group.

However, in the other treated groups, it was significantly observed that these changes improved when treated with either extract 250 mg/kg or 500 mg/kg.

Discussion

Despite the wide use of doxorubicin in the treatment of cancer patients, its mechanism of action is still not well known and often has been subject to controversy. This proposed mechanism showed that is most sensitive in the S phase of the cell cycle, although cytotoxicity also occurs in other phases of the cell cycle [18]. In the present study, DOX. was used to induce cardiotoxicity in rats for 6 weeks. Different molecular hypotheses can explain this cardiac toxicity effect. Based on the pharmacokinetics it is known that DOX. reduced to semiquinone free radicals by catalyzes Cytochrome P450 reductase (present in cell nuclear membranes). These, in turn, reduce molecular O2, producing superoxide.

ions and hydrogen peroxide, which mediate single-strand scission of DNA [32]. Additionally, an increase in oxidative stress evidenced increases in the levels of ROS, and lipid peroxidation along with reductions in the levels of antioxidants and sulfhydryl group may affect the myocardial cell membrane integrity [32]. Calcium has a crucial role in the contraction of myocyte [33]. Many studies suggest that calcium homeostasis dysregulation has a major role in the pathogenesis of Dox. –induced cardiotoxicity through increasing intracellular calcium [34]. This is referred to as the metabolism of Dox. and generates a toxic metabolite, DOXol, through a

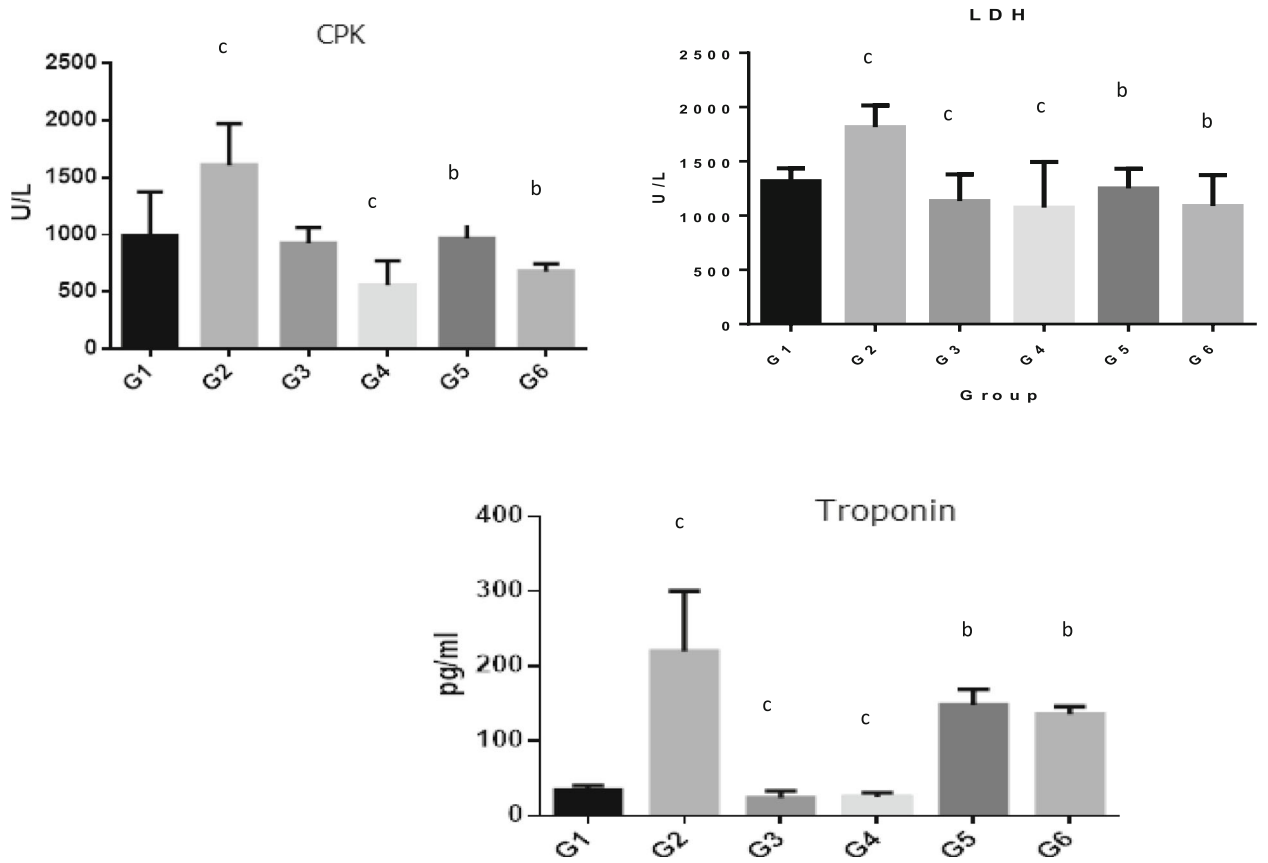

Fig. 1 Effect of extract *M. verticillata* on the Creatine phosphate kinase (CK), lactate dehydrogenase (LDH), for 6 weeks in albino rats. *Significant as compared with control at (p 0.05), Significant as compared with Doxoinduced heart damage at (p 0.05), G1: Control, G2: Dox., G3: Extract 250 mg\kg, G4: Extract 500 mg\kg, G5: Dox. + Treatment 250 mg\kg, G6: Dox. + Treatment 500 mg\kg

reduction of its carbonyl group, capable of inhibiting the sodium-calcium exchanger channel.

The sodium/potassium pump of the sarcolemma is also affected by DOXol, which disrupts the sodium gradient needed for calcium to flow into the sarcolemma of a cardiomyocyte [35]. Consequently, there is an imbalance in the energetics of the myocardium and diminished systolic function [36]. Furthermore, it is reported that this secondary metabolite is more difficult to eliminate from the cardiomyocyte than the parent drug. Thus, DOXol accumulation contributes significantly to the dysregulation of calcium homeostasis, leading to myocardial damage.

Moreover, normal calcium homeostasis is altered by ROS and hydrogen peroxide via disruption of normal sarcoplasmic reticulum function. This is accomplished by inhibiting the Ca2 + −ATPase pumps, caused by reducing the expression of SERCA2a mRNA levels and/or the direct activation of the ryanodine calcium-release channels themselves [29, 37]. Other reported study

Table 2 Effect of extract *M. verticillata* on the (Mean ± SEM) body weight (g) for 6 weeks in albino rats (*n*=6)

Parameter	Mean ± SEM					
	G1	G2	G3	G4	G5	G6
Initial body weight (g)	171.3±5.84	169.5±5.70	171.3±6.81	182.0±4.68	175.8±8.20	185.6±5.42
Final body weight (g)	200.0±11.8	154.1±9.03[abc]	207.1±9.91[ab]	221.5±8.23[ab]	169.3±13.3[ab]	186.7±3.33[b]
Weight of heart (g)	0.793±.04	0.533±.043[c]	0.668±.033[b]	0.742±.0361[b]	.668±.0317[b]	.640±.019[b]
Relative weight of heart	0.39	0.34	0.32	0.34	0.39	0.34

[a]Significant as compared with control value at (*p* 0.05)
[b]Significant as compared with Doxo- induced heart damage at (*p* 0.05)
[c]Significant as compared with control, G1: Control, G2: Dox., G3: Extract 250mg\kg, G4: Extract 500mg\kg, G5: Dox.+ Treatment 250mg\kg, G6: Dox.+ Treatment 500mg\kg

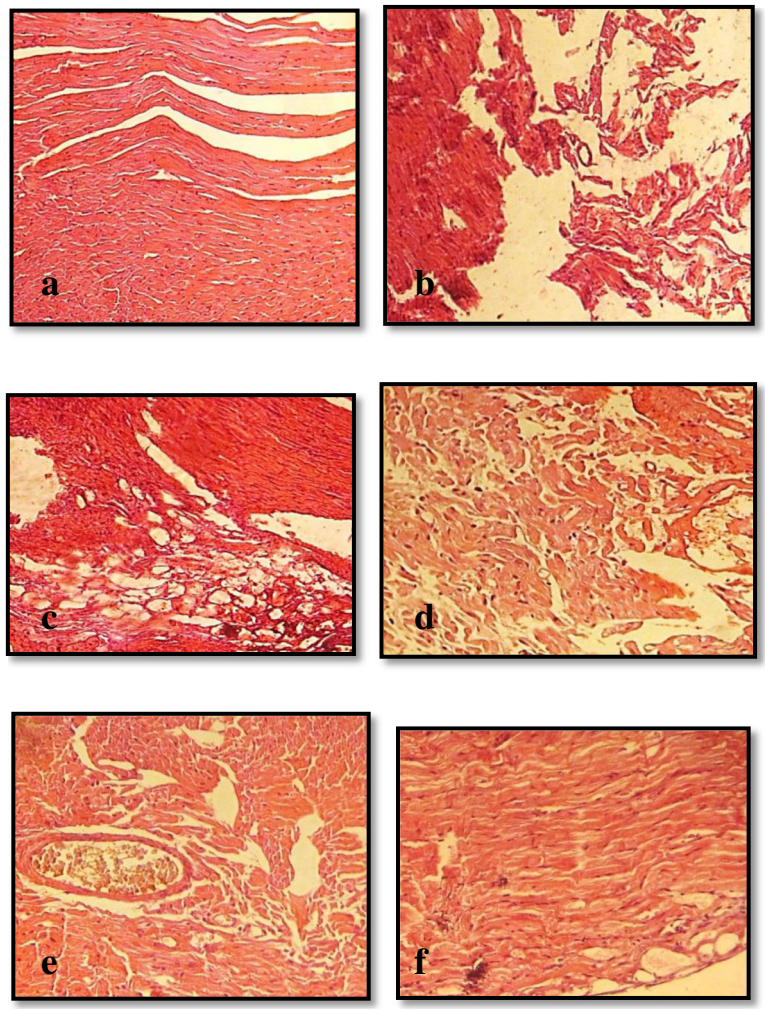

Fig. 2 Histopathology of the rat heart from different treated groups. **a**: control group heart showing the normal architecture of cardiac muscle. **b, c, d, e, f**: doxorubicin-induced group heart showing disruption of cardiac muscle architecture, vacuolation, necrosis, congestion and inflammatory cell infiltration, respectively

showed that Dox. Can alter iron metabolism as it has a strong affinity to this metal to form Iron-Dox complexes, thus react with oxygen and triggers ROS production [38]. Most researchers believed that only oxidative stress was responsible for the cardiotoxicity induced by iron-DOX complexes. Furthermore, recent work suggested that DOX can also interact with iron-responsive elements (IREs) of the ferritin heavy and light chains. It is known that ferritin operates as an iron transporter, reducing free iron within the cell. Accordingly, disruption of this protein eventually results in increased free iron, which in turn causes myocardium injury [39]. Another work showed iron-overload, mitochondrial damage, and mortality after DOX treatment in mice depleted of the iron regulatory gene HFE (also known as human hemochromatosis protein). The HFE protein is responsible for the regulation of circulating iron uptake [40]. Therefore, free iron accumulation within the myocardium after DOX treatment seems to be the major determinant of Dox.-induced cardiotoxicity [41–43]. All these effects disrupt the cardiac muscle resulted in leakage of cardiac enzymes. Since Dox. –induced cardiotoxicity by different mechanisms, different strategies are being performed to prevent the cardiomyopathy adverse effects of DOX. One of these powerful strategies is the role of antioxidants in counteracting the Dox.-induced cardiotoxicity. Antioxidants are suggested to beneficially interfere with diseases or drugs-related oxidative stress [44]. *M. verticillata* contains a higher total content of flavonoids compared with phenolic components according to the previous studies [9, 45]. Flavonoids, phenolic compounds and alkaloids possess potent antioxidants

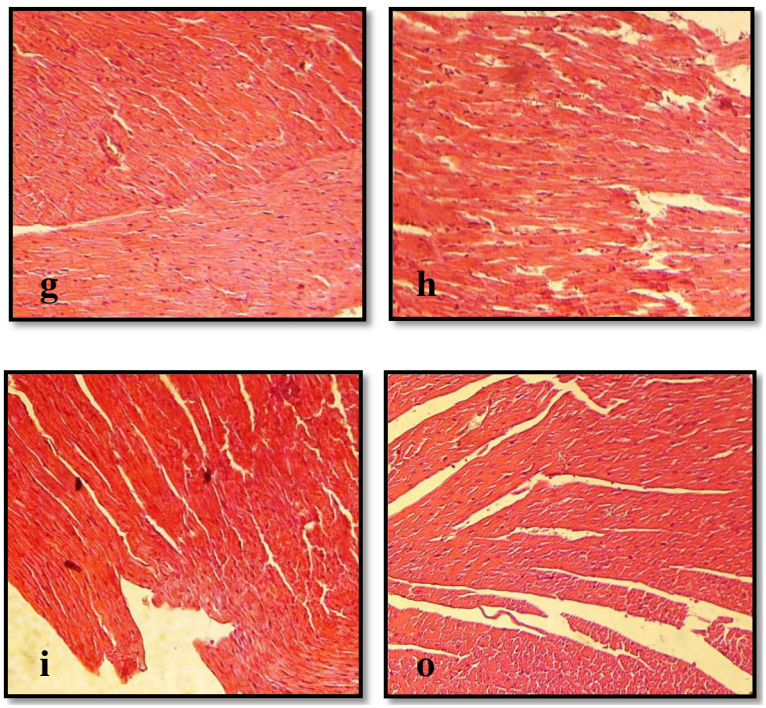

Fig. 3 Histopathology of the rat heart from different treated groups. **g**: Plant-treated group heart (250 mg/kg) showing the normal architecture of cardiac muscle. **h**: Plant-treated group heart (500 mg/kg) showing the normal architecture of cardiac muscle. **i**: Doxorubicin and plant-treated group heart (250 mg/kg) showing mild disruption of the cardiac muscle architecture. **o**: Doxorubicin and plant-treated group heart (500 mg/kg) preserved cardiac muscle structure

naturally existing in some plants, and abundantly in *M. verticillata* [46]. They can prevent the cellular and genetic damage caused by Dox-induced ROS formation and dramatically inhibited mitochondrial superoxide production. Moreover, Flavonoids (Quercetin) inhibits Ca^{+2} dependent ATP hydrolysis, ATP-dependent Ca^{+2} uptake and chelator induced Ca^{+2} release, so this effect contradicts Dox. –induced cardiotoxicity [47]. Additionally, flavonoids have a powerful anti-inflammatory effect that causes intact and prevent degeneration and inflammation of myocardial cells and preserve the cardiac enzymes, thus obviously appeared on the reduction of CPK, troponin, and LDH levels, in the plant treated groups compared with untreated one in the current study. All these findings were supported by histopathological studies of cardiac tissues that showed a dose-dependent preserved cardiac muscle structure, all these were in agreement with other previous findings [46, 48].

Conclusion

From the outcomes of this study, it suggests that *M. verticillata* can be used as complementary medicine, particularly for the patients who treated with doxorubicin as it showed the worthwhile cardioprotective effect. It contains various natural antioxidants, including flavonoids, phenols, and alkaloids. Further studies are needed to focus on the quantitative screening of the important antioxidant contents particularly flavonoids, phenols and alkaloids taken into consideration their molecular pharmacology as well as the protective mechanism of the action.

Abbreviations

ROS: Reactive oxygen species; LDH: Lactate dehydrogenase; CPK: Creatine phosphokinase; DCM: Dilated cardiomyopathy; DOX: Doxorubicin

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Authors' contributions

Both authors DA and MA were contributed and revised the article and approved the final manuscript. RB supervised on the pharmacognosy part. The author(s) read and approved the final manuscript.

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Availability of data and materials

All data and analyzed outcomes are available with the corresponding author if requested.

Declarations

Ethics approval and consent to participate

All procedures of the experiment were approved by the University of Science and Technology.
Research Ethics Committee NO. [EAC/UST156].

Consent for publication

Not applicable.

Competing interests

There was no competing interest.

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