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The Protective Effect of *Lasia spinosa* (Linn.) Dissipates Chemical-Induced Cardiotoxicity in an Animal Model

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Abstract

Lasia spinosa (L.) Thwaites is a medicinal plant of enormous traditional use with insufficient scientific evidence. This research screened the antioxidative effect of *L. spinosa* extracts by measuring the total phenolic content, total flavonoid content, DPPH free radical scavenging activity, ABTS scavenging activity, Iron-chelating activity, and Ferric reducing power followed by an evaluation of *in vivo* cardioprotective effect in doxorubicin-induced Wistar Albino rats. Phytochemical characterization was made by Gas Chromatography–Mass Spectroscopic analysis. *L. spinosa* showed an excellent antioxidative effect while methanol leaf extract (LSM) was found to be more potent than ethyl acetate leaf extract (LSE) in scavenging the free radicals. Intraperitoneal injection of doxorubicin caused a significant ($P < 0.001$) increase in lactate dehydrogenase (LDH), creatine kinase (CK-MB), C-reactive protein (CRP), and Cardiac troponin I. Pretreatment with orally administrated (LSM100 and LSM200 mg/kg b.w.) daily for 10 days showed a decrease in the cardiac markers, lipid profiles, especially triglycerides (TG), total cholesterol (TC), low-density lipoprotein (LDL), and an increase of high-density lipoprotein (HDL) compared to the disease control group. LSM200 was found to significantly ($P < 0.05$) decrease the levels of CK-MB and LDH. It also restored TC, TG, and LDL levels compared to the doxorubicin-induced cardiac control group. The protective role of LSM was further confirmed by histopathological examination. This study thus demonstrates that *L. spinosa* methanol extract could be approached as an alternative supplement for cardiotoxicity, especially in the chemical-induced toxicity of cardiac tissues.

Keywords *Lasia spinosa* · Cardioprotective · Antioxidant · Biochemical · Heart · Doxorubicin

Abbreviations

DPPH	2,2-Diphenyl-1-picrylhydrazyl
ABTS	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
LDH	Lactate dehydrogenase
CK-MB	Creatine kinase
CRP	C-reactive protein
LSM	*L. spinosa* Methanol extract
ESL	*L. spinosa* Ethyl acetate leaf extract
TG	Triglyceride

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TC	Total cholesterol
LDL	Low-density lipoprotein
HDL	High-density lipoprotein
DOX	Doxorubicin
ROS	Reactive oxygen species
ETC	Electron transport chain
NADH	Nicotinamide adenine dinucleotide
eNOS	Endothelial Nitric Oxide Synthase
NO	Nitric oxide
GC–MS	Gas chromatography–mass spectrometry
EI	Electron impact ionization
TPC	Total phenolic content
FCR	Folin–Ciocalteu Reagents
GAE	Gallic acid equivalent
TFC	Total flavonoid content
QE	Quercetin equivalent
IC_{50}	The half-maximal inhibitory concentration
OECD	Organization for Environmental Control Development
LD_{50}	Lethal Dose 50
IP	Intraperitoneal
LSM100	*L. spinosa* Methanol extract (100 mg/kg /day bw)
LSM200	*L. spinosa* Methanol extract (200 mg/kg/day bw)
LSM50	*L. spinosa* Methanol extract 50 mg/kg/day bw
LSE100	*L. spinosa* Ethyl acetate extract 100 mg/kg/day bw
LSE50	*L. spinosa* Ethyl acetate extract 50 mg/kg/day bw
NBF	10% Neutral-buffered formalin
H & E	Eosin and hematoxylin

Introduction

Anthracyclines, a broad range of antibiotic families, extensively serve as chemotherapeutics in a single dose or consonance with other antitumor agents [1]. Doxorubicin (DOX), belonging to this therapeutic class, is a hold potent, robust cytotoxic agent applied to a wide array of carcinoma and malignant tumors [2]. One of the most dreadful implications of this antineoplastic drug is cardiotoxicity which compromises their alleviative capacity resulting in cardiomyopathy. Hallmarks of DOX-mediated cardiotoxicity are functional abnormality and progressive pathophysiology of the heart due to apoptosis and necrosis of myocardiocytes [1, 3]. Unfortunately, at this juncture, no viable strategies have been brought to light in cancer treatment to mitigate this persistent myocardial insult stimulated by DOX administration [4].

A substantial number of mechanisms have been incriminated in DOX-induced cardiac injury, but the most well-known mode in which DOX affects the heart is through elevated levels of ROS [5]. Cardiomyocytes are highly susceptible to oxidative stress due to their dependence on metabolic substrate oxidation. As a result, a significant proportion of myocardial mass comprises mitochondria, opposite to other neoplastic/cancer cells [6]. As a cationic drug, DOX irreversibly binds to mitochondrial cardiolipin, messes with ETC proteins' regulatory function, and frequently generates superoxide free radicals. Forming molecular complexes with iron, DOX facilitates the transformation of hydrogen peroxide to hydroxyl radicals (Fenton reaction). In mitochondrial respiratory complex I, the transition of DOX into semiquinone by NADH dehydrogenase causes molecular oxygen to produce superoxide.

Furthermore, DOX binds to eNOS reductase and converts NO to assemble superoxide and peroxynitrite [1, 6, 7]. An excessive amount of reactive oxygen and nitrogen species catalyze lipid peroxidation, reduction of antioxidants and sulfhydryl groups, myofibrillar atrophy, and disruption of intracellular calcium. Due to this extreme vulnerability of cardiac cells to oxidative stress generated by the imbalance between free radicals and low levels of intrinsic antioxidants, DOX-mediated toxicity has received much attention [6]. To exert the efficiency of DOX as a chemotherapeutic agent next to a spectrum of different cancers, rigorous research has been driven to investigate pharmacotherapeutics, exclusively, bioactive antioxidants and metal ion chelators which could minimize and diminish the chances of complications, in this regard, cardiomyopathy [7]. Natural compounds such as flavonoids, plant sterols, stanols, and some other plant-based nutraceuticals have been reported to function as cardioprotective through diminishing or attenuating reactive oxygen species eventually inhibiting the pro-inflammatory markers and involved pathways as evident for polydatin, a recently studied "twin" molecule of resveratrol [8]. Polydatin is reported to decrease anticancer-induced cardiotoxicity by decreasing pro-oxidative stress, pro-inflammatory cytokines, and NLRP3 inflammasome expression [9]. Curcuminoids, another class of active compounds within turmeric, are polyphenolic pigments and include curcumin, demethoxycurcumin, and bisdemethoxycurcumin are reported to reduce cardiotoxicity and coadministration of curcumin with DOX impacts better outcome in cancer treatment reducing the side effects through antioxidant, anti-apoptotic, and anti-inflammatory mechanisms individually or together [10, 11]. Another very vibrant dietary nutraceutical ascorbic acid, commonly known as Vitamin C, is reported to play a pivotal role in scavenging biological free radicals and thereby greatly contribute to both cancer and cardiovascular diseases as many other potential plant-derived products do [9]. However, several other plant sources are still unexplored for their exciting cardioprotective effects.

Lasia spinosa is a widely used evergreen shrub of the Araceae family and is predominantly allocated in India, Sri

Lanka, southeast Asia, and Malaysia [12]. Several indigenous populations worldwide exploit different portions of the herb in the traditional medication system. The herb is used in ailments of diarrhea, rheumatism, swelling, mal-absorption, insect and snake bites, constipation, and blood purification. Due to numerous bioactive compounds, *L. spinosa* exhibits antioxidant, cytotoxic, antimicrobial, antiparasitic, antinociceptive, hypoglycemic, hypolipidemic, anti-inflammatory, and anti-inflammatory antidiarrheal activities [13, 14].

However, this plant required a more comprehensive analysis involving advanced research methods and technologies. As a result, there has been no exploration further into the cardioprotective properties of this plant. Therefore, this study was carried out to evaluate the in vitro antioxidant effects of hot methanolic and hot ethyl acetate leaf extract and in vivo cardioprotective activity of hot methanolic leaf extract in a DOX-induced cardiotoxicity model.

Methods and Materials

Chemicals and Reagents

All the reagents and chemical used in this research were included in the analytical grade. They were collected from the sources as parenthesized. Ethyl acetate (Merck, Germany), methanol (Fisher chemical, USA), n-hexane (DAE JUNG, Korea), gallic acid (Merck, Germany), Folin–Ciocalteu reagents (FCR) (Sigma-Aldrich Co., St. Louis, USA), sodium carbonate (Merck, Germany), quercetin (Sigma-Aldrich Co., St. Louis, USA), aluminum chloride (Merck, Germany), potassium acetate (BDH chemicals Ltd., UK), ascorbic acid (Sigma-Aldrich Co., St. Louis, USA), 2,2-diphenyl-1-picrylhydrazyl (Sisco research laboratories, India), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (Sigma-Aldrich Co., St. Louis, USA), potassium persulfate (Sisco research laboratories, India), ethanol (Merck, India), Trolox (Tokyo chemical, Japan), O-phenanthroline (Merck, Germany), ferrous chloride (Merck, Germany), streptokinase (Sisco research laboratories, India), doxorubicin (Beacon Pharmaceuticals Limited, Bangladesh), losartan potassium (Square Pharmaceuticals Ltd., Bangladesh), AST liquiUV kit (Human, Germany), ALT liquiUV kit (Human, Germany), Triglyceride liquicolor kit (Human, Germany), cholesterol liquicolor kit (Human, Germany), NaCl (Sigma-Aldrich Co., St. Louis, USA), Xylene (Merck, Germany), and Eosin and Hematoxylin (Merck KGaA, Germany).

Collection of Plant Materials

Plants of *Lasia spinosa* were obtained from the University of Chittagong's adjacent hilly regions. Plant Biologist Dr. Sheikh Bokhtear Uddin, Professor, Department of Botany, University of Chittagong did the identification and verification of the plant sample. A sample specimen has been preserved in the institutional Herbarium with accession no. LAMLS-A120.

Preparation of the Plant Extract

Healthy green leaves of *L. spinosa* were sorted, cleaned, and air-dried at room temperature. The dried leaves were pulverized into a fine powder using a mechanical grinder, and the powder was then extracted with ethyl acetate and methanol through a gradual increase of polarity to generate crude extract following established protocol. Briefly, the resulting powder (500 g) was steeped in n-hexane for two days, with gentle stirring to make it fat-free. The defatted residue was further extracted with ethyl acetate and methanol for 8 days, four days for each solvent, and the supernatants were filtered through Whatman filter paper no.1. The resulting filtrates were evaporated by a rotary evaporator (RE200, Bibby Sterling, UK) under reduced pressure and 40 °C. Finally, the concentrated crude extracts were collected in Petri dishes and allowed to dry at room temperature for complete solvent evaporation. Thus, the black-green semisolid mass of the leaf ethyl acetate extract (LSE) and methanolic extract (LSM) was attained.

Quantitative Phytochemical Screening

Gas Chromatography–Mass Spectroscopy Analysis

The bioactive compounds extracted from the leaves of *Lasia spinosa* were analyzed by gas chromatography (GC-2010 plus, Shimadzu Corporation, Kyoto, Japan), coupled with a mass spectrometer (GCMS- TQ 8040, Shimadzu Corporation, Kyoto, Japan). A fused silica capillary column (Rxi-5 ms; 30 m, 0.25 mm ID, and 0.25 µM) was used for GCMS analysis maintaining sample inlet temperature at 250°C. One (1.0) µL sample was injected in splitless mode. The oven temperature was programmed as 75 °C (1 min); 25 °C, 125 °C (1 min); 10 °C, 300 °C (15 min). The aux (GC to MS interface) temperature was set to 250 °C. The total run time was 36.50 min and the column flow rate was 1.5 mL/min He gas. An electron ionization (EI) type mass spectroscopy (MS) was used in Q3 scan mode. Ion source temperature 200 °C, 250 °C interface temperature, 1.17 kV detector voltage, and 50–1000 m/z mass range were set for MS. Individual compound with m/z ratio was searched in NIST-MS Library 2014. Total Ionic Chromatogram (TIC) was used to determine the peak area as well as the percentage amounts of each compound.

Determination of Total Phenolic Content (TPC)

The total phenolic content of LSM and LSE was measured according to the method provided by Ainsworth et al. with minor modifications [15]. Briefly, gallic acid was applied as a standard to draw the calibration curve. Standard and extracts of different concentrations were oxidized with 10% Folin–Ciocalteu Reagents (FCR), and the reaction was neutralized with 7.5% Na_2CO_3. The color formation was attained by incubating the reaction mixture at room temperature for 30 min with periodic shaking. The absorbance of the resulting blue color was observed at 765 nm. The total phenolic material was calculated using a linear equation based on the gallic acid standard curve. Total phenolic components in the dry extract were expressed as mg/g gallic acid equivalent (GAE).

Determination of Total Flavonoid Content (TFC)

The TFC of LSM and LSE was determined using a slightly modified version of the aluminum chloride colorimetry method reported by Chang et al. [16]. By diluting quercetin in methanol, the standard calibration curve was obtained. Diluted extracts and quercetin of different concentrations were mixed with 10% aluminum chloride. After adding 0.1 mM potassium acetate to the final mixture, the solutions were incubated at room temperature for 30 min. The absorbance of the solution was then measured using a UV–vis spectrophotometer at 415 nm. TFC was calculated in milligrams of quercetin equivalent (mg/g QAE) per gram of LSM & LSE extracts.

In Vitro Antioxidant Activity

DPPH Free Radical Scavenging Activity of LSM & LSE

The DPPH free radical scavenging effect was determined using the established methods of Brand-Williams et al. [17]. In this experiment, ascorbic acid was utilized as a control antioxidant. The required amount of ascorbic acid (0.96 mg) and the samples were dissolved in 12 mL methanol to make the stock solution. LSM, LSE, and ascorbic acid stock solutions were diluted to 800–50 µg/mL quantities. Two (2.0) mL of each LSM/LSE and ascorbic acid solutions at various concentrations were triplicated into test tubes, followed by 2.0 mL of freshly produced DPPH solution. The reaction mixture was incubated at room temperature for 30 min in the dark, and the absorbance was measured at 517 nm with a visible spectrophotometer. The control was made the same way as the sample but without the sample.

The following calculation was used to obtain the percentage of Inhibition: percent Inhibition = $(A_0\text{-}A1)/A_0 \times 100$,

where A_0 represents the absorbance of the control and A_1 represents the absorbance of the extract/Std. The proportion of scavenging activity or inhibition was then plotted against concentration, and the IC_{50} was estimated from the graph using linear regression analysis.

ABTS Assay

The ABTS (2, 2-azino-bis (3-ethylbenzthiazoline- 6-sulfonic acid) radical cation decolorization test [18] was used to assess LSM/LSE's free radical scavenging capability. A 7.0 mM ABTS aqueous solution was mixed with 2.45 mM potassium persulfate in the dark for 12–16 h at room temperature to produce ABTS·+. This solution was diluted in ethanol (approximately 1: 49, v/v) and equilibrated at 30 ± 2 °C to yield an absorbance of 0.700 ± 0.02 at 734 nm at the start of the test. Next, the LSM/LSE extract stock solution was diluted to give a concentration range of 50–800 µg/mL. By adding 1 mL of diluted ABTS·+ solution to 62 µL of LSM/LSE sample in ethanol, the final concentration (0–15 M) was reached. The absorbance was read at 25 °C after 40 min of mixing. Positive control and a blank were employed with Trolox and ethanol. Triplicate determinations were made at each dilution of the standard and sample, and absorption was measured in the UV–Vis spectrophotometer at 734 nm (UV-1200S UV–VIS 1200, Shimadzu Corporation, Japan). The extract's ABTS·+ scavenging capacity was matched to that of Trolox. Percentage inhibition is computed as follows:

ABTS radical scavenging activity = [(Absorbance of control—Absorbance of the sample)/Absorbance of control] × 100.

Iron-Chelating Activity

The iron-chelating activity of LSM/LSE was determined using Benzie and Strain's technique [19]. The notion is based on the development of the O-phenanthroline-Fe^{2+} complex and its dissolution in the presence of chelating agents. The absorbance of a reaction mixture containing 1 mL of 0.05% O-phenanthroline in methanol, 2 mL of fresh ferrous chloride (200 mM), and 2 mL of various LSM/LSE concentrations was measured at 510 nm after 10 min at room temperature. Experiments were carried out in triplicate, and the results were compared to a standard positive control, ascorbic acid.

Iron radical inhibition (percentage) = $[A_0\text{-}A1]/A_0 \times 100$.

The test absorbance is A_0 and the control absorbance is A_1.

In vitro Thrombolytic Activity

In vitro thrombolytic activity of LSM/LSE was determined following the method described by [20]. A 4.0 mL of venous blood collected from healthy volunteers was divided into 500 mL pre-weighed sterile microcentrifuge tubes and incubated for 45 min at 37 °C. Following clot formation, serum was withdrawn entirely without disturbing the clot, and each tube containing the clot was weighed again to calculate the clot weight (clot weight = clot containing tube—tube alone). One microcentrifuge tube containing a pre-weighed clot received 100 µL of sample solution.

Positive control of 100 µL of Streptokinase (10,000 I.U) was employed, whereas a negative non-thrombolytic control of 100 µL of distilled water was used. After that, all the tubes were incubated for 90 min at 37 °C.

Percentage clot lysis = (weight of the clot after lysis by sample/weight of the clot before lysis by sample) × 100.

Statement on Informed Consent of the Donors

The whole study was accomplished following the institutional ethical guidelines of the Faculty of Biological Sciences of the University of Chittagong, Bangladesh. An informed donor consent form was filled before starting the experiment. Briefly, the volunteer donors were given a consent form that included information about the research project's title, investigators' names, and contact information, as well as the study's goal. The volume of blood to be collected, the potential inconvenience of the puncture sites, and the time required for blood sampling were all mentioned in the research description, which included a step-by-step brief of the proposed research, donor inclusion, and exclusion criteria, whether donors received any therapy or not, the volume of blood to be collected, the possible inconvenience of the puncture sites, and the time required for blood sampling. If future use of the research data beyond the current study is desired, the interpretation was produced. The donor's ability to withdraw his sample data was revealed. The consent form stated that the sample will be used just for that specific study and not for any future research projects. Informed consent statement was used to connect any adverse effects, discomforts, injuries, or inconvenience related with donors in this study. If there were any known adverse occurrences to the donors, current knowledge about the occurrence of the harm, the clinical outcome of the harm, and any relevant knowledge about the likelihood of reversibility and the fact that these discomforts were transient were also provided. A statement of confidentiality was added to the consent form, stating that "confidentiality will be observed and no information that discloses the participant's identity would be disseminated or published without agreement unless required by state law." Finally, detailed contact information for researchers was provided in case donors had any questions about the project. The consent form was completed with the donor's consent disclosure as Yes/NO and the signature of the donor.

In vivo Cardioprotective Effect

Experimental Animals

Laboratory-bred thirty adult Wistar albino rats weighing around 180–200 g were selected for the experiment. They were housed in rectangular polypropylene cages bedded with wood husk, supplied with a standard pellet diet and fresh tap water, exposed to 12-h light–dark cycle at about 25 ± 2℃ temperature, and 55–60% humidity in an animal house at the Department of Biochemistry and Molecular Biology, University of Chittagong. The study was designed following international guidelines for the care and use of laboratory animals and was endorsed by the Institutional Ethical Committee of the University of Chittagong.

Acute Toxicity Test

The acute toxicity test was carried out in a traditional laboratory setting, following the "Organization for Environmental Control Development" (OECD: Guidelines 420; Fixed-Dose Method). Each assigned animal was given a single oral dose of LSM and LSE (250 to 1000 mg/kg) ($n = 6$). The rats fasted overnight, and their meals were postponed for 3 to 4 h before receiving the extract. Likewise, food was withheld for 3–4 h after administration. Individual animals were observed for the first 30 min after dosing, then every 24 min for the next 24 h (with particular attention paid to the first 4 h), with particular attention paid to any unusual reactions, such as changes in the skin, fur, eyes, mucous membranes, respiratory, autonomic, and central nervous systems, allergic syndromes as well as behavior patterns [21].

Experimental Dose and Induction of Cardiotoxicity

Due to the better effect of LSM in vitro assay, especially in vitro clot lysis, it was chosen for in vivo experiments, and two different doses, 100 mg/kg/day bw and 200 mg/kg/day bw were selected based on LD_{50} values in acute toxicity assay. Experimental cardiotoxicity was induced in rats by a single intraperitoneal injection of Doxorubicin at 15 mg/kg bw. This experiment used Losartan potassium (100 mg/kg/day bw) as a reference drug.

Experimental Design

The experimental animals were randomly divided into 5 groups consisting of 6 animals in each group.

Group I (Normal control): Received only standard diet and tap water.
Group II (Cardiac control): Administrated (doxorubicin) DOX 15 mg/kg/day bw via IP.
Group III (Standard control): Administrated DOX + Losartan (100 mg/kg/day bw).
Group IV (LSM 100): Administrated DOX + LSM 100 (100 mg/kg/day bw).
Group V (LSM 200): Administrated DOX + LSM 200 (200 mg/kg/day bw).

Animals have been sacrificed 24 h after receiving a DOX injection. The animals were sacrificed after 10 days and the blood and hearts were collected. A 5.0 mL heparinized syringe was used to collect the blood and keep it in the heparinized blood vials. The blood was centrifuged for 15 min at 3000 rpm at 25 °C, and the serum was collected into a tube and stored at − 20 °C for further biochemical testing. After collecting the target organ hearts, they were cleaned with 0.8% NaCl, then soaked in tissue paper and stored in a vial containing 10% neutral-buffered formalin (NBF) solution. The NBF buffer was modified after two days, then every week. The heart was stored at room temperature for histopathological examination.

Serum Biochemical Indices

Cardiac markers CK-MB, Troponin I, LDH, hepatic enzymes (alanine aminotransferase, aspartate aminotransferase), lipid profile, uric acid, and creatinine were all tested in established methods using commercial kits introduced by Humalyzer 3000.

Histopathological Analysis

The tissues were fixed in 10% neutral-buffered formalin for 48 h to preserve tissue from degradation. The vertical sections of tissues were taken by a sharp blade for dehydration passing through ascending grades of ethanol (70%, 8o%, 90%, 100% vol/vol) for one hour in each solution to remove water. The tissues were then passed through xylene solution three times consecutively to make the sample alcohol-free. The tissues were then embedded in molten paraffin wax for 2 h and thus paraffin impregnation was done to remove water from tissues and replace it with a medium that solidifies to allow thin sections to be cut. External embedding of the tissues was ensured by placing them into molds along with liquid paraffin. The paraffin along with tissues in the molds was allowed to cool and harden to preserve at 4 °C until the cross-sectioning [22]. The implanted tissues were sectioned using a semi-automated rotator microtome machine (Biobase Bk-2258, Laboratory Manual Microtome, China). Tissues were then put on glass slides using an incubator at 60–70 °C for 30 min. The tissue sections were deparaffinized with xylene and rehydrated with graduated ethanol dilutions (100%, 90%, and 70%). The sections were stained with eosin and hematoxylin (H&E X40). The slides were inspected with the Olympus BX51 microscope, and the histopathological images were acquired with the help of the Olympus DP20 system at a magnification of 40X.

Results

Quantitative Phytochemical Analysis

GC–MS Analysis

The GC–MS analysis of *L. Spinosa* uncovered almost 40 phytoconstituents demonstrated in Table 1 and Fig. 1. The significant and higher concentration than other phytochemicals identified in the *L. spinosa* methanol leaf extract includes Hexanoic acid, methyl ester (9.1%), Nonadecanoic acid, methyl ester (9.1%), Pentadecanoic acid, methyl ester (9.1%), Heptadecanoic acid, methyl ester (9.1%), Phytol (3.5%), Isomenthol (3.5%), Heneicosanoic acid, methyl ester (2.5%), Methyl stearate (2.5%), etc.

Total Phenolic and Flavonoid Content Determination

The quantitative analysis of plant total phenolic and total flavonoid content is demonstrated in Table 2, while LSM contained the highest phenolic content (5.64 mg GAE/g of plant extract) than LSE (4.65 mg GAE/g of plant extract). Also, it is observed that LSM contained a higher amount of flavonoid (16 mg QE/g of plant extract) than LSE (12.54 mg QE/g of plant extract).

Antioxidative Effects of LSM and LSE

The 2, 2-diphenyl-1-picrylhydrazyl free radical scavenging effect for LSM and LSE was summarized in Fig. 2A. The LSM exhibited the highest free radical scavenging activity with the IC_{50} value of 54.95 µg/mL compared with LSE (IC_{50} 407.38 µg/mL). The IC_{50} of the ascorbic acid solution (standard) was 3.46 µg/mL with $R^2 = 0.857$.

In the $ABTS^+$ antioxidant assay, LSM showed a moderate $ABTS^+$ radical scavenging activity with the IC_{50} value of 316.25 µg/mL, while LSE showed the free radical scavenging activity with an IC_{50} value of 920.18 µg/mL (Fig. 2B). However, the ABTS free radical scavenging effect was not statistically significant compared to Trolox (the standard), with an IC_{50} value of 93.32 µg/mL ($R^2 = 0.9915$).

IC_{50} values of LSM and LSE in the iron-chelating assay are shown in Table 3, where both LSM and LSE showed

Table 1 Phytochemicals identified by GC–MS in *L. spinosa* methanol leaf extract

SL	Compound name	R. Time	m/z	Area	Concentration (%)
1	Phenol, 3,5-bis(1,1-dimethyl ethyl)-	8.665	191.00	3,193,026	2.879
2	D-erythro-pentose, 2-deoxy-	9.320	60.00	2,525,659	2.277
3	Acetic acid, 2-(1-methyl-1H-1,2,3,4-tetrazole-5)	10.795	45.00	26,602	0.024
4	Rhamnose	9.966	60.00	2,803,445	2.527
5	Dodecanol	11.125	45.00	77,402	0.070
6	2-pentadecanol	11.125	45.00	77,402	0.070
7	1-Hexadecanol	12.074	83.00	412,714	0.372
8	1-Octadecanol	12.074	83.00	412,714	0.372
9	1-Heptadecene	12.074	83.00	412,714	0.372
10	Phytol, acetate	12.588	68.00	718,673	0.648
11	Dodecanal	12.588	43.00	459,428	0.414
12	9-Eicosyne	12.855	44.00	50,215	0.045
13	Eicosanal	12.559	81.00	351,976	0.317
14	Bicyclo(3,1,1)heptan-3-ol,2,6,6-trimethyl-	13.492	44.00	125,695	0.113
15	Hexadecanoic acid, methyl ester	13.493	74.00	10,442,993	9.415
16	Nonadecanoic acid, methyl ester	13.493	74.00	10,442,993	9.415
17	Pentadecanoic acid, methyl ester	13.493	74.00	10,442,993	9.415
18	Heptadecanoic acid, methyl ester	13.493	74.00	10,442,993	9.415
19	1-(+)-Ascorbic acid 2,6-dihexadecanoate	14.195	73.00	492,520	0.444
20	n-Hexadecanoic acid	14.195	73.00	492,520	0.444
21	9,9-Dimethoxybicyclo(3,3,1)nona,2,4-dione	14.195	73.00	492,520	0.444
22	Eicosanoic acid, ethyl ester	14.195	88.00	2,407,827	2.171
23	Decanoic acid, ethyl ester	14.195	88.00	2,407,827	2.171
24	Heneicosanoic acid, methyl ester	14.530	74.00	442,150	0.399
25	Linolenic acid ethyl ester	15.215	67.00	2,493,943	2.248
26	Linoelaidic acid	15.215	67.00	2,493,943	2.248
27	Cis-9-Hexadecimal	15.278	55.00	1,365,414	1.231
28	Ethyl oleate	15.278	55.00	1,365,414	1.231
29	Phytol	15.384	71.00	3,990,208	3.597
30	(+)- Isomenthol	15.384	71.00	3,990,208	3.597
31	Heneicosanoic acid, methyl ester	15.522	74.00	2,777,136	2.504
32	Methyl stearate	15.522	74.00	2,777,136	2.504
33	Ethanol, 2-(9,12-octadecadienyloxy-(Z,Z)-	15.384	67.00	413,167	0.372
34	Z,Z-8,10-Hexadecadien-1-ol	15.522	44.00	68,003	0.061
35	RT:16.175	16.183	57.00	336,159	0.303
36	Octadecanal, 2-Bromo-	16.874	44.00	94,712	0.085
37	Oleic acid	16.874	44.00	94,712	0.085
38	1,1'-Bicyclopropyl-2-octanoic acid,2'-hexyl	16.251	73.00	336,045	0.303

RT Retention Time

a similar percentage of free radical scavenging activity (Fig. 2C). It is observed that both the extracts showed a significant ($P < 0.05$) iron-chelating effect compared with ascorbic acid (IC_{50} value 4.46 µg/mL with $R^2 = 0.961$).

In vitro Thrombolytic Activity

Clot lysis percentages achieved by two different concentrations of LSM and LSE, positive control (Streptokinase) and negative control (water), are shown in Fig. 3. The clot lysis was 50.54% for 100 µL of Streptokinase (10,000 I.U.) and 4.4% for the negative control (water). The main difference in clot lysis percentage between positive and negative control was significant ($P < 0.001$). When clots were treated with 100 µL each of two different concentrations of the extracts LSM and LSE, they showed moderate % of clot lysis, i.e., LSM50 (25.54%), LSM100 (21.25%), LSE50 (20.32%), and LSE100 (19.45%). The values were compared with

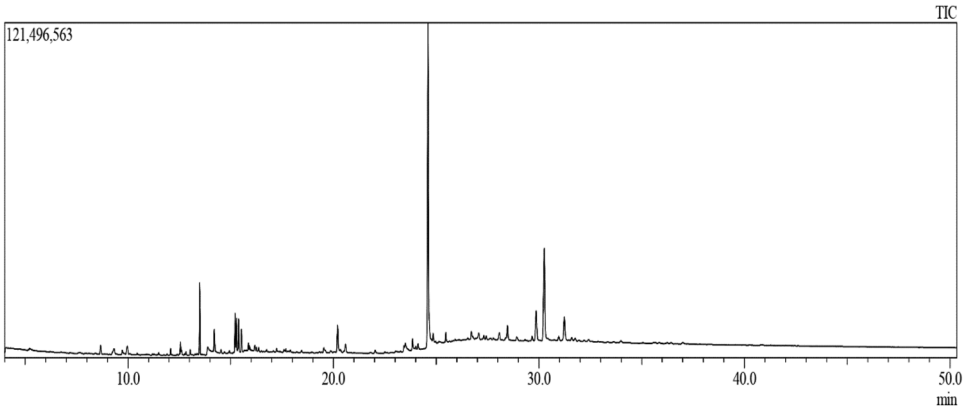

Fig. 1 GC–MS spectra of *L. spinosa* crude extract. The extract was analyzed by electron impact ionization (E.I.) with a gas chromatograph (GC-17A, Shimadzu Corporation, Kyoto, Japan) connected to a mass spectrometer (GC–MS TQ 8040, Shimadzu Corporation, Kyoto, Japan) integrated with silica capillary column (Rxi-5 ms; 0.25 m film thickness). The column flow rate was 0.6 mL/min of Helium gas, and the auxiliary temperature was fixed at 280°C. Total GC–MS running time was 35 min. Database in the GC–MS library version NIST 08-S was used to compare all peak areas

Table 2 Quantitative measurement of TPC and TFC in *L. spinosa*

Plant extract	TPC (Total phenolic content)	TFC (Total flavonoid content)
LSM	5.64 mg GAE/g plant extract	16 mg QE/g plant extract
LSE	4.65 mg GAE/g plant extract	12.54 mg QE/g plant extract

LSM L. spinosa methanol leaf extract, *LSE L. spinosa* ethyl acetate leaf extract, *GAE* Gallic Acid Equivalent, *QE* Quercetin Equivalent

the positive control, and the mean clot lysis was significant ($P < 0.05$).

Acute Oral Toxicity Test

Oral administration of 1000 mg/kg of the *L. spinosa* did not produce any anxiety or any sign of toxicity. Furthermore, there was no restlessness, diarrhea, or convulsion in the following one-week period.

Effect of LSM on Lipid Profile

Doxorubicin administration significantly ($P < 0.001$) increased TG and LDL levels in DOX-treated animal groups when compared with normal control. Both treatment groups LSM100 and LSM200 significantly decreased TG, and LDL levels when compared with a DoX-treated group of animals (Fig. 4).

Effect of LSM on Cardiac Biomarkers

The intraperitoneal administration of DOX significantly increased the serum cardiac biomarkers such as CK-MB and LDH ($P < 0.001$) compared with normal control, which is shown in (Fig. 5a). Treatment with LSM100 and LSM200 significantly ($P < 0.05$) decreased CK-MB and LDH compared with the DOX-treated group. LSM (200 mg/kg) decreased C-reactive protein (CRP) and troponin I (CTrI) level significantly compared with the DOX-treated group (Fig. 5b).

Histopathological Analysis

To evaluate the cardioprotective activity of *L. spinosa*, a histopathological examination of heart tissues was carried out. As shown in Fig. 6, it is observed that normal control animals showed the typical structure of the heart with no necrosis with slight edema. Rats treated with DOX showed congestion, necrosis, degeneration of myocardial tissue, and infiltrated inflammation. In the treatment control groups (LSM100), no necrosis, no congestion, and clear striated myocytes were observed like the typical structure of the heart, while LSM200 treated group showed no necrosis but a mild edema in histopathological analyses.

Discussion

Doxorubicin is a quinone-containing anthracycline family of drugs. DOX plays a major role in chemotherapy and is used to treat many solid organ tumors and cancers [23]. However, it is widely reported that a dose-dependent cardiotoxicity of DOX limits their clinical effectiveness and leads to myocardial dysfunction and cell death. Although multiple mechanisms are involved in DOX-induced cardiotoxicity, the generation of ROS is considered one of the major mechanisms. Cardiac tissue is more susceptible to DOX-induced

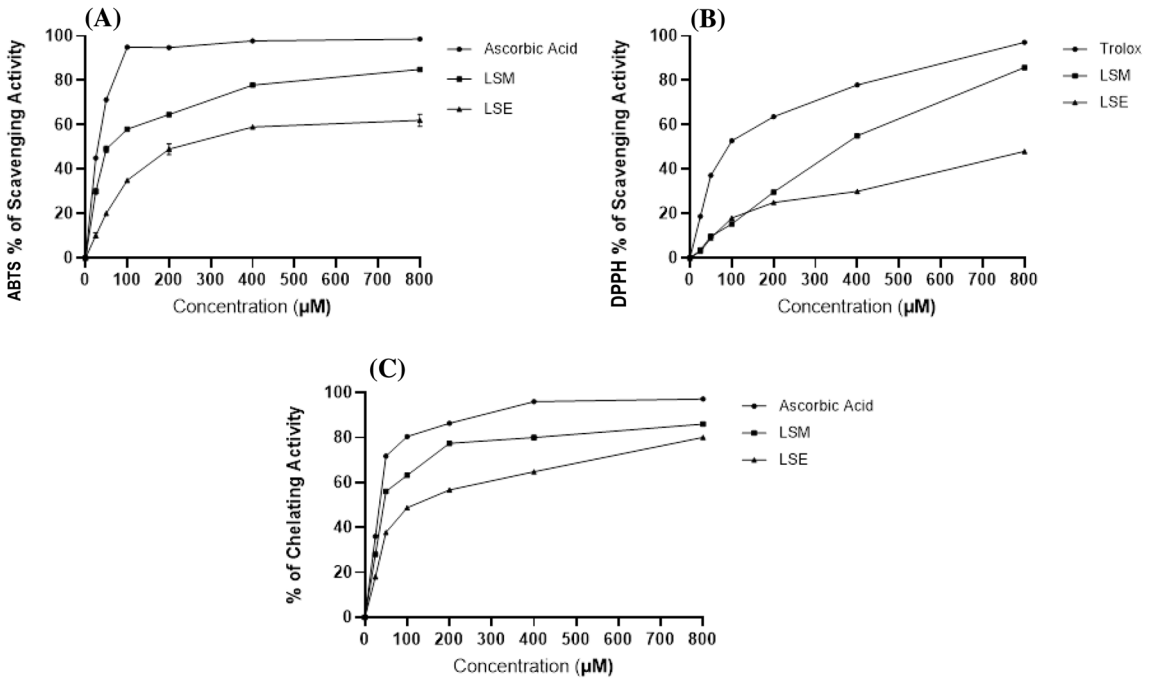

Fig. 2 Antioxidative potential of LSM and LSE. **A** ABTS scavenging effect; **B** DPPH free radical scavenging effect; **C** Iron-Chelating effect. Trolox in the ABTS assay and Ascorbic acid in two other assays were used as reference standards. Values were presented as Mean ± SD. Data were analyzed using the statistical software package for social sciences (SPSS, version 22.0), while P values were less than 0.05

Table 3 Inhibition concentrations (IC_{50}) of LSM, LSE, and respective standard in antioxidative assay

Models	Inhibition concentrations (IC_{50}) (μg/mL)		
	Standard (ascorbic acid)	Plant extract	
		LSM	LSE
DPPH free radical scavenging effect	3.46	54.95	407.38
ABTS scavenging activity	93.32	316.25	920.18
Iron-chelating activity	4.46	25.70	117.48

cardiotoxicity due to its effect on mitochondria. DOX treatment is evident to increase the mitochondrial iron levels in cardiomyocytes and mice hearts. It also increased mitochondrial lipid peroxidation, mitochondrial protein oxidation, and caused mitochondrial DNA damage [24]. Due to the formation of ROS in DOX-induced cardiotoxicity, it is suggested to be treated by plant-derived drugs because of their potential ROS neutralizing/antioxidant agents. Interestingly, recently synthetic drugs are replaced by alternative medicines which have fewer side effects and are considered as a better option [12].

Lasia spinosa (Linn.) Thwaites, known as Kattosh in the local area of Chittagong and Kohala in Bengali, is commonly used as a medicinal plant and is widely distributed in Southeast Asia. *L. spinosa* leaves contain phytochemicals including polyphenol, tannin, alkaloid, flavonoid, saponin, carotenoid, etc. [25]. Their most abundant polyphenols are flavonoids which are reported to have a strong antioxidant effect [26]. During the study on *L. spinosa* plant extract, gas chromatography coupled with mass spectrometry technique was being performed for separating and identifying the components of complex volatile plant extract. GC–MS chromatogram of the methanol extracts of *L. spinosa* leaf showed 36 peaks and indicated the presence of bioactive compounds while the major compounds Hexadecanoic acid methyl ester, Heptadecanoic acid methyl ester, Pentadecanoic acid methyl ester, Heptadecanoic acid methyl ester had the same concentration (9.42%) and same RT Time (13.493). Among the others, Phenol,3,5-bis (1,1-dimethyl)- (2.88%), D-erythro-pentose,2-deoxy- (2.28%), Rhamnose (2.53%), Phytol (3.6%), Isomenthol (3.6%), Heneicosanoic acid methyl ester (2.5%), Methyl stearate (2.5%), Linoleic acid ethyl ester (2.25%), Linoelaidic acid (2.25%), Eicosanoic acid ethyl ester (2.17%), Decanoic acid ethyl ester (2.17%), Cis-9-Hexadecanal (1.2%), Ethyl oleate (1.2%), Oleic acid, and some other alcohol derivatives such as 2-pentadecanol, 1-hexadecanoic, 1-octadecanoic, and phytol acetate were present at the lower concentrations. All those compounds are a good source of phytoconstituents like carboxylic acid,

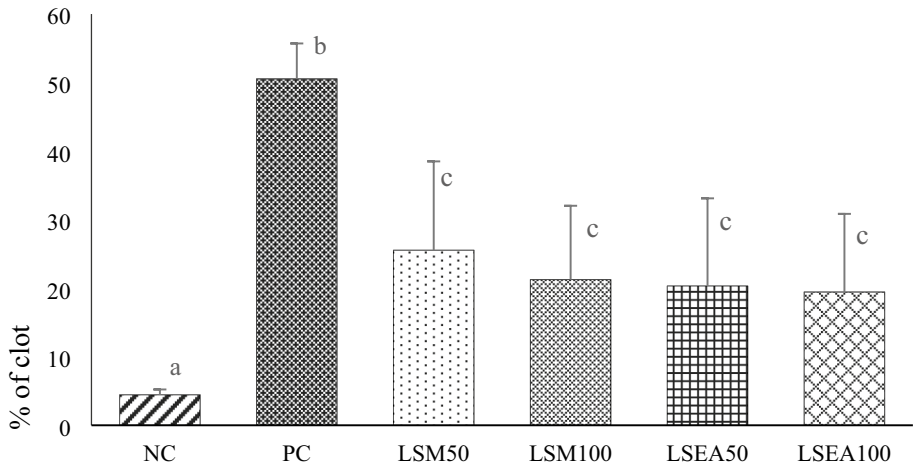

Fig. 3 Clot lysis effect of LSM and LSE in in vitro models. Data were presented as Mean ± S.D. Data were analyzed by one-way analysis of variance (ANOVA) followed by a Dunnet test using the statistical software Statistical Package for Social Sciences (SPSS, Version 22.0). Superscript letters$^{(a-c)}$ over the bars indicate the significance of the values at $P < 0.05$

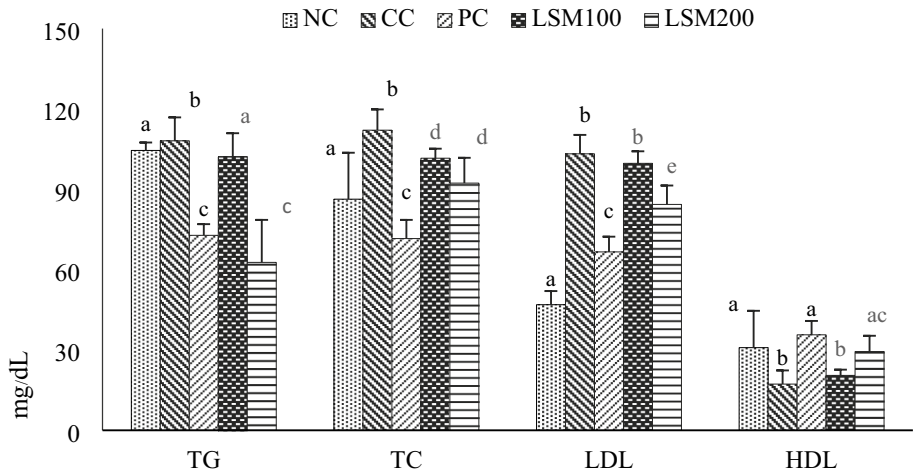

Fig. 4 Effect of LSM on the lipid profiles of experimental animals ($n = 6$). Data were presented as Mean ± S.D. Data were analyzed by one-way analysis of variance (ANOVA) followed by a Dunnet test using the statistical software Statistical Package for Social Sciences (SPSS, Version 22.0). Superscript letters$^{(a-e)}$ over the bars indicate the significance of the values at $P < 0.05$

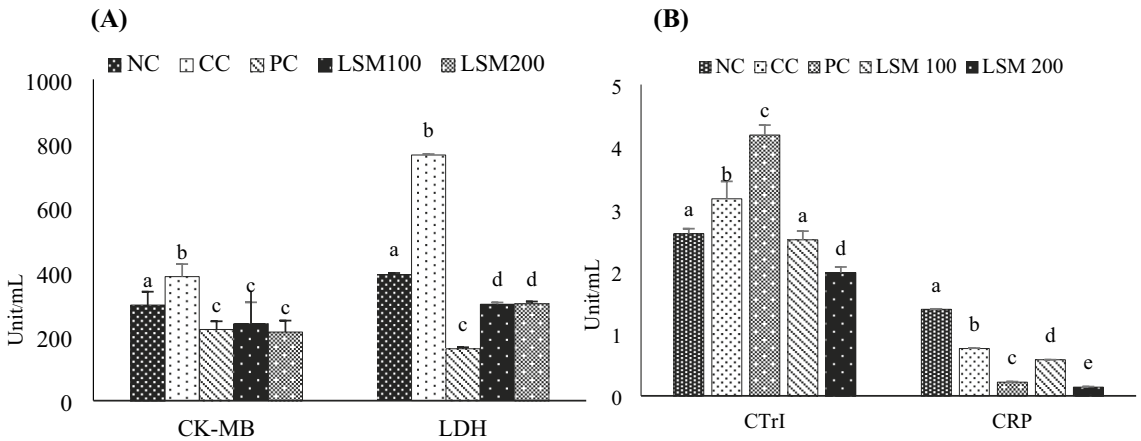

Fig. 5 Effect of LSM on the cardiac biomarkers **a** creatine kinase (CK-MB) & lactate dehydrogenase (LDH); and **b** cardiac troponin I (CTrI), and C-reactive protein (CRP) of experimental animals ($n = 6$). Data were presented as Mean ± S.D. Data were analyzed by one-way analysis of variance (ANOVA) followed by a Dunnet test using the statistical software Statistical Package for Social Sciences (SPSS, Version 22.0). Superscript letters $^{(a-e)}$ over the bars indicate the significance of the values at $P < 0.05$

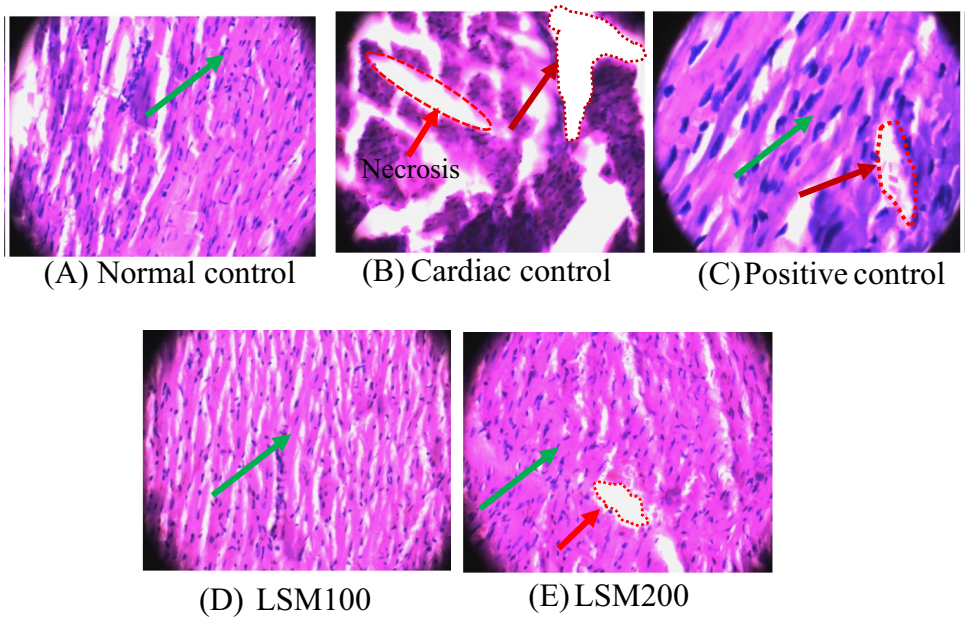

Fig. 6 Histopathological analysis of cardiac tissue of different groups (H&E; ×40). Histopathological changes in the heart tissue treated with methanol leaf extract of *Lasia spinosa* (LSM100 and LSM200)**. A** Normal control group (normal structure of the heart). **B** Cardiac control (necrosis, degeneration of myocardial tissue, congestion, infiltrated inflammation). **C** Positive control (mild necrosis). **D** LSM100, methanol leaf extract at 100 mg/kg (clear striated myocyte near-normal structure). **E** LSM200, methanol leaf extract at 200 mg/kg (no congestion, no necrosis, edema). Green arrow indicates normal or recovery of cardiac tissues; red arrow indicates necrosis and edema

aldehyde, fatty acid ester, and fatty alcohol. They have great importance in pharmacological activity such as anti-inflammatory, antioxidant, antifungal, antibacterial, antipyretic, hepatoprotective, anticancer, hypocholesterolemic, therapeutic, and cure cardiovascular, cerebrovascular diseases [26]. From the result of the phytochemical analysis of LSM and LSE, it is observed that LSM contains more total phenolic content (TPC) and total flavonoid content (TFC) than LSE using the gallic acid curve as standard in TPC and quercetin curve as standard in TFC. The role of antioxidants in the prevention and treatment of disease has brought the attention of the scientists. There are some commonly used techniques to determine the antioxidant activity in vitro for rapid screening of plant extracts speculating that the plants which have low antioxidant activity in vitro will probably show little in vivo activity. To evaluate the antioxidant activity of LSM and LSE, the established DPPH assay, ABTS assay, and Iron-chelating activity screening methods were used. In the DPPH assay, DPPH accepted an electron donated by an antioxidant, it is decolorized from purple to yellowish color. The more yellow color, the more free radical scavenging activity of the standard and test samples which can be quantitatively measured from the changes in absorbance [26]. The standard (ascorbic acid) and test samples (LSM and LSE) were compared based on their % of scavenging activity and their IC_{50} (minimum inhibition at 50% concentration). LSM exhibited more % of scavenging activity than LSE with their IC50 value. However, the standard ascorbic acid solution showed a strong antioxidant effect than LSM and LSE. In the ABTS assay, the generation of a blue ABTS + radical can be reduced by antioxidants, plant extract, beverages, body fluids, etc. [27]. In ABTS assay, LSM and LSE could not scavenge the ABTS radical appropriately that is why it shows a very poor percentage of scavenging activity when compared with the standard Trolox. It may be due to the poor solubility of the compounds of *L. spinosa* in the respected solution. Iron chelators play a great role in cardioprotection and chelate stable and soluble complexes. Chelation reduces iron-related complications and iron chelators have antioxidant and free radical scavenging activities. LSM and LSE showed a better percentage of scavenging activity like ascorbic acid and their IC_{50} values are close to the standard. As we know that the lower the IC_{50} value means the higher antioxidant effect. In context of all in vitro experiments, LSM performed better activity than LSE, probably the compounds of LSM have better solubility in methanol. Various plant sources having anticoagulant, and fibrinolytic activity result to prevent coronary risks and stroke. Before evaluating in vivo cardioprotective activity, we checked clot lysis potentiality of the plant extract LSM and LSE by performing in vitro thrombolytic activity established method. The comparison of the positive control streptokinase (50% clot lysis) with negative control water (only 4.44% clot lysis) and plant extract of two different concentrations LSM4 (25%), LSM2 (21%), and LSE4 (20%) and LSE2 (19%) exerted significant thrombolytic activity [17]. Also, it is observed that two different concentrations of methanol leaf extract LSM4 and LSM2 showed slightly better percentage of clot lysis than ethyl acetate leaf extract LSE4 and LSE2, respectively. This could be explained by the better plasmin activating capacity of methanol extract of *L. spinosa* because the plasmin is a natural fibrinolytic agent which lyses clot by breaking down the fibrinogen and fibrin contained in a clot [20]. Therefore, the further research was

carried out only on LSM to evaluate in vivo cardioprotective activity against DOX-induced cardiotoxicity. In the presence study, 15 mg/kg bw i.p administration of DOX significantly increased the level of cardiac markers including CK-MB and LDH. Increased levels of these markers were associated with heart damage such as cardiomyopathy, heart failure, and myocarditis [28]. Oral administration of LSM100 and LSM200 of *L. spinosa* leaves significantly decreased the level of cardiac markers CK-MB and LDH when compared with normal control and disease control. This might be because of the demolition or attenuation of ROS production in DOX-induced cardiotoxicity [29–32]. Some putative pathways and markers, such as NLRP3, are also reported to be involved not only to protect plain cardiotoxicity but also some anticancer-induced Cardiotoxicity in the presence of hyperglycemia in triple-negative breast cancer cells [33]. Also, both LSM100 and LSM200 significantly decreased the lipid profile including TG, TC, and LDL levels of experimental rats when compared with disease control suggesting the active participation of the antioxidants phytochemicals (carotenoids, alkaloids, flavonoids, and saponins) previously reported to protect cardiotoxicity [34, 35]. The mechanism of these phytochemicals for their cardioprotective activity is basically due to their free radical scavenging activity because free radicals are responsible for oxidative stress and cardiac injury in DOX-induced cardiotoxicity. Apart from that, phenolic compounds are reported to inhibit low-density lipoprotein peroxidation, and maintain cell membrane stability or protect the leakage of the cell membrane due to damage by DOX-metabolites as evident in the cardiac biomarkers reduction [36]. The heart section of normal control rats showed normal morphology and the treatment control (LSM100 mg/kg and LSM200 mg/kg) rats also showed clear striated myocyte similar to normal control rats, no congestion of blood vessels, no inflamed infiltration. While disease control rats showed necrosis, degeneration of myocardial tissue, and infiltrated inflammation as a confirmation of doxorubicin-induced cardiotoxicity. After all, the regeneration of cardiac tissues and healing of necrosis and edema in histopathological images of heart tissues further confirmed the potential of LSM in amelioration of DOX-induced cardiotoxicity [37–39].

Conclusion

Cardiovascular disease is one of the most common noncommunicable illnesses and the leading cause of mortality worldwide. *Lasia spinosa* (Linn.) was shown to have cardioprotective action against doxorubicin-induced cardiotoxicity in this investigation. Biochemical markers particularly creatinine kinase, lactate dehydrogenase, and troponin I were positively affected to normalize the cardiotoxicity. This finding suggests that *L. spinosa* methanol leaf extract might be an effective cardioprotective drug in the treatment of cardiovascular diseases and their consequences. However, HPLC-guided bioactive compounds are necessary to be isolated for verifying the action for further therapeutic use of *Lasia spinosa*.

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Author Contribution The research idea was proposed and designed by MAR. The investigation, formal analysis, and data curation were carried out by RA, MKJR, TAS, FYB, and SA. The initial manuscript was authored by MAR, RA, and MKJR, who also contributed to the data analysis. Visualization, validation, and writing—review and editing were done by FYN, MANK, and FS. All authors have gone through the manuscript and agreed to submit it to Cardiovascular Toxicology.

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Data Availability Data will be available upon request.

Declarations

Conflict of interest The authors declare that they do not have any conflict of interest.

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