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Cardioprotective Activity of Fruit of *Lagenaria siceraria* (Molina) Standley on Doxorubicin Induced Cardiotoxicity in Rats

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Abstract: The aim of present study was to investigate the cardioprotective effect of *Lagenaria siceraria* (LS) fruit powder against the cardiotoxicity of doxorubicin (Dox) in rats. Male wistar rats (250-300 g) were randomly divided into three groups. Group 1-was control (gum acacia 2%), group 2-Dox (10 mg kg⁻¹), group 3-Dox+LS (200 mg kg⁻¹ for 18 days). Dox (10 mg kg⁻¹ i.v.) was administered in group 2 and 3 on day 16. After anesthetizing the animals on the 18th day, electrocardiogram (ECG) was recorded and blood was investigated for creatine kinase-MB isoenzyme (CK-MB), lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) while determination of superoxide dismutase (SOD), reduced glutathione (GSH), lipid peroxidation (LPO) and histopathology was carried out for heart. Group 3 animals showed decreased QT and ST intervals (p<0.01 and p<0.05, respectively) and non significant increase in heart rate as compared to group 2. Significant decrease in serum CK-MB, AST (p<0.001) and LDH (p<0.05) of group 3 animals was observed as compared to group 2. There was significant increase in the level of GSH (p<0.05) and non significant increase in SOD, whereas lipid peroxidation (p<0.01) was inhibited in group 3 as compared to group 2. Histopathological study of LS treated group showed protection against myocardial toxicity induced by doxorubicin. Acute toxicity study showed that LS was safe at 5000 mg kg⁻¹. It is concluded that *Lagenaria siceraria* possessed cardioprotective effect against doxorubicin induced cardiotoxicity in rats.

Key words: *Lagenaria siceraria*, doxorubicin, electrocardiogram, cardioprotective activity

INTRODUCTION

Lagenaria siceraria (Molina) Standley fruit (bottle gourd) from Cucurbitaceae family, a commonly used vegetable in India is described as cardiogenic (Deshpande *et al.*, 2008). Doxorubicin, an anthracycline antibiotic, has a broad antitumor spectrum and has been used against a wide variety of hematopoietic malignancies and solid tumors (Ogura, 2001). Unfortunately, the cardiotoxicity of Dox, resulting in a cardiomyopathy with irreversible congestive heart failure and high mortality, is one of the main factors that limit its use. The molecular mechanisms explaining the cardiotoxicity of anthracyclines are complex, but it appears that the induction of an oxidative stress within myocardial tissue constitutes a common denominator (Vergely *et al.*, 2007). Considerable efforts have been made on using antioxidants and iron-chelators to protect the heart against Dox toxicity. Dexrazoxane, prevent Dox induced cardiotoxicity, is an iron chelator and possesses potent antioxidant properties. But, due to the high incidence of dexrazoxane-induced myelosuppression, its use has been limited to some advanced stages of malignant disorders

(Seifert *et al.*, 1994). The objective of the present study was to investigate the protective effects of LS fruit powder against acute Dox-induced cardiotoxicity in rats.

MATERIALS AND METHODS

Animals: The rats were obtained from Lupin Research Park, Pune. Male Wistar albino rats (250-300 g) were housed under standard housing conditions of 25°C, relative humidity 60% and photo period of 12 h dark/12 h light. Standardized pellet diet (Chakan Oil Mills, Pune, India) and water were provided *ad libitum*. Female Swiss albino mice (25-30 g) were purchased from National Toxicology Center (NTC) Pune and maintained under the above mentioned conditions. All experimental protocols including the animal studies were approved by the Institutional Animal Ethical Committee (IAEC) of Poona College of Pharmacy, Bharati Vidyapeeth University, Pune, India. This study was conducted during September 2007 to February 2008.

Chemicals and sample: The fruit of LS which was used for this study was authenticated by Dr. A.M. Mujumdar

of Agharkar Research Institute, Pune, India. The fruit powder was gifted by Deerghayu Pvt. Ltd., Pune. It was prepared by cutting, drying and pulverization with a yield of 4.5-5% w/w. Doxorubicin powder injection was gifted by Serum Institute of India Ltd., Pune. All solvents/chemicals used were of analytical grade and chemicals required for sensitive biochemical assays such as MDA, GSH, SOD, 5, 5'-dithiobis (2-nitro benzoic acid) (DTNB) and thiobarbituric acid (TBA) were obtained from Sigma Chemical Co., USA.

Experimental procedure: The animals were randomly divided into three groups (control, Dox and LS+Dox) containing eight rats in each group. The changes in body weight were recorded daily. Powdered LS suspended in 2% gum acacia was administered (5 mL kg^{-1}) orally to LS+Dox group daily for 18 days. The dose was 200 mg kg^{-1} which was selected on the basis of previous pilot dose response study. To the control as well as Dox groups only vehicle (gum acacia 2%, 5 mL kg^{-1}) was administered orally for 18 days. The control group received a single parenteral dose of sterile water for injection (1 mL kg^{-1}) through femoral vein on the 16th day, while on the same day the Dox and LS+Dox groups received Dox (10 mg kg^{-1}) dissolved in sterile water and injected within 1 h, similarly. After 48 h of the injection, the rats were anaesthetized under mild anesthetic ether and ECG was recorded using 8 channels Power Lab System (AD Instruments Pvt. Ltd., Unit 13, 18-22 Lexington Drive, Bella Vista NSW 2153, Australia) and the changes in QT and ST intervals and heart rate were noted from the ECG. Later, blood was withdrawn from retro orbital puncture (ROP) and serums were separately stored at 4°C till processed. The animals were finally sacrificed on the 18th day with overdose of anesthetic ether and the hearts were isolated and weighed. Four hearts from four animals were randomly selected for histopathology. The hearts from remaining four animals were cut into small pieces, placed in chilled 0.25 M sucrose solution and blotted on a filter paper. The tissues were then homogenized in 10% chilled tris hydrochloride buffer (10 mM, pH 7.4) by tissue homogenizer (Remi Motors, Mumbai 400 058, India) and centrifuged at 7500 rpm for 15 min at 4°C using Eppendorf 5810-R high speed cooling centrifuge. The clear supernatant was used for the estimation of SOD, GSH, MDA content and total protein.

Serum parameters: Serum levels of CK-MB, LDH and AST enzymes were measured by automated chemistry analyzer, Micro lab 300, Merck, using reagent kits. CK-MB kit was purchased from Randox Laboratories Ltd., Ardmore, Diamond Road, Crumlin, Co. Antrim, United Kingdom, BT29 4QY and LDH as well as AST from Ecoline, Merck Ltd., Worli, Mumbai-400 018.

Tissue parameters

Lipid peroxidation assay (MDA content): This assay was used to determine thiobarbituric acid-reactive substances (TBARS) level as described by Slater and Sawyer (1971). In 2.0 mL of the tissue homogenate (supernatant) was added 2.0 mL of freshly prepared 10% w/v trichloroacetic acid (TCA) and the mixture was allowed to stand in an ice bath for 15 min, followed by centrifugation at 2500 rpm for another 15 min at 4°C . Two milliliter of clear supernatant solution was mixed with 2.0 mL of freshly prepared 0.67% w/v TBA. The resulting solution was heated in a boiling water bath for 10 min. It was then immediately cooled in an ice bath for 5 min. The absorbance of colour developed was measured by UV/VIS spectrophotometer (JASCO-V-530, Japan) at 532 nm using 1, 1, 3, 3-tetraethoxypropane as a standard.

Estimation of GSH: The assay of GSH was determined by method described by Moron *et al.* (1979). One milliliter of tissue homogenate (supernatant) and 1.0 mL of 20% TCA were mixed and centrifuged at 2500 rpm for 15 min at 4°C . In 0.25 mL of supernatant, 2 mL of DTNB (0.6 M) reagent was added. The final volume was made up to 3 mL with phosphate buffer (pH 8.0). The colour developed was read at 412 nm against reagent blank. Different concentrations (10-50 μg) of standard glutathione were processed as mentioned above for constructing standard curve. The amount of reduced glutathione was expressed as μg of GSH /mg of protein.

Estimation of SOD activity: The SOD activity was determined by the method of Misra and Fridovich (1972). 0.5 mL of heart homogenate + 0.5 mL of cold distilled water + 0.25 mL of ice-cold ethanol and 0.15 mL of ice-cold chloroform were mixed well using cyclo mixer for 5 min and centrifuged at 2500 rpm for 15 min at 4°C . To 0.5 mL of supernatant, 1.5 mL of carbonate buffer (pH 10.2) and 0.5 mL of 0.4 M ethylenediaminetetraacetic acid (EDTA) solutions were added. The reaction was initiated by the addition of 0.4 mL of epinephrine bitartrate (3 mM) and the change in optic density/minute was measured at 480 nm against reaction blank. SOD activity was expressed as units/mg of protein. Change in optical density per minute at 50% inhibition of epinephrine to adrenochrome transition by the enzyme was taken as the enzyme unit. Calibration curve was prepared by using 10-125 units of SOD.

Determination of total proteins: Protein concentrations were determined using the method of Lowry *et al.* (1951). Diluted membrane fraction aliquots (0.1 mL) were taken in test tubes. To this, 0.8 mL of 0.1 M sodium hydroxide and 5 mL of Lowry C reagent (freshly prepared mixture of 1 mL of (0.5% w/v) Copper sulphate in 1% sodium potassium

tartarate into 50 mL of (2% w/v) sodium carbonate in 0.1 M sodium hydroxide) was added and the solution was allowed to stand for 15 min. Then 0.5 mL of 1 N Folin's phenol reagent was added and the contents were mixed well by vortex mixer. Colour developed was measured at 640 nm against reagent blank containing distilled water instead of sample. Different concentrations (40-200 µg) of standard protein bovine serum albumin (BSA) processed as mentioned above for preparation of standard curve. The values were expressed as mg of protein/g of wet tissue (mg g⁻¹).

Histopathological studies: Hearts were quickly removed, preserved in 10% formalin, processed and embedded in paraffin. Four micrometer thick paraffin sections were cut on glass slides and stained with hematoxylin and eosin (H and E) reagents and observed by light microscope to evaluate myocardium injury.

Heart weight to body weight ratio: In each group, heart weight to body weight ratio was calculated. Body weight was the weight on the day of sacrifice. Heart weight was measured after keeping the heart in cold saline and squeezing out the blood.

Acute toxicity study: Acute oral toxicity study was carried out according to OECD-423 guideline. Female Swiss albino mice (n = 3) were administered 2000 mg kg⁻¹ LS powder in 0.25% sodium carboxymethyl cellulose (CMC) as a vehicle and followed by 5000 mg kg⁻¹ orally. The animals were observed for toxic symptoms and mortality for 72 h.

Statistical analysis: The data was expressed as mean±standard error of mean (SEM). One way analysis of variance (ANOVA) was applied to test the significance of difference between average biochemical and ECG parameters of different groups with correction for multiple comparisons by the Tukey's test. p-value less than 0.05 was considered statistically significant. The entire statistical analysis was performed using statistical package called Graph pad prism version 4.03 (Graph Pad Software, Inc., USA).

RESULTS

ECG changes: Doxorubicin administration significantly increased QT (p<0.01) and ST (p<0.05) intervals and decreased heart rate (p<0.05) when compared to control group. LS administration significantly decreased QT and ST intervals (p<0.01 and p<0.05, respectively) while heart rate was non significant when compared to Dox group (Table 1).

Table 1: Effect of Dox and LS+Dox on ECG changes in rats on day 18

Groups	Treatment	ST interval (ms)	QT interval (ms)	Heart rate (bp m)
1	Control (n = 8)	36.00±1.95	66.88±1.56	367.9±9.13
2	Dox (n = 7)	43.14±1.86*	76.86±1.99**	308.3±12.47*
3	LS+Dox (n = 7)	36.71±0.97*	68.67±1.26**	316.7±15.66 ^{NS}

Table 2: Effect of Dox and LS+Dox on levels of CK-MB, LDH and AST enzymes in rats

Groups	Treatment	CK-MB (U L ⁻¹)	LDH (U L ⁻¹)	AST (U L ⁻¹)
1	Control	82.8±15.71	179.8±19.97	201.2±24.59
2	Dox	220.8±18.83***	393.4±38.54***	377.4±15.53***
3	LS+Dox	100.4±14.56***	283.8±4.64*	213.8±13.47***

Table 3: Effect of Dox and LS+Dox on the levels of MDA, GSH and SOD in the heart of rats

Groups	Treatment	Lipid		
		peroxidation (nM of MDA mg ⁻¹ protein)	GSH (µg of GSH mg ⁻¹ protein)	SOD (U mg ⁻¹ protein)
1	Control	2.69±0.18	27.51±0.70	9.24±0.91
2	Dox	4.69±0.19**	17.79±1.01***	4.76±0.78*
3	LS+Dox	2.50±0.27**	23.63±1.96*	7.87±0.99 ^{NS}

Values are mean±SEM. *p-value compares Dox with control group while *p-value compares Dox with LS+Dox group. *p<0.05, **p<0.01, ***p<0.001, *p<0.05, **p<0.01, ***p<0.001, NS = Non Significant

Table 4: The average heart weight to body weight ratio across three study groups

Groups	Treatment	Heart weight to body weight ratio
1	Control	(2.72±0.02)×10 ⁻³
2	Dox	(2.67±0.11)×10 ⁻³
3	LS+Dox	(2.81±0.09)×10 ⁻³

Values are mean±SEM. There was no statistical significant difference between the averages of ratio across the groups

Serum parameters: Administration of Dox (10 mg kg⁻¹) increased serum CK-MB, LDH and AST levels compared to that of control, whereas LS+Dox decreased the levels of CK-MB, LDH and AST significantly compared to that of Dox alone (Table 2).

Tissue parameters: There was a significant increase (p<0.01) in MDA contents in the Dox group when compared to the control group. Significant decrease in the level of MDA contents was observed in LS+Dox group (p<0.01) in comparison to the Dox group. Significant (p<0.001) decrease in myocardial GSH was observed in the Dox group in comparison to the control group. In LS+Dox group there was significant increase (p<0.05) of myocardial GSH as compared to Dox group. In LS+Dox group the increase in SOD was not statistically significant when compared to Dox group, whereas SOD in Dox group decreased significantly (p<0.05) when compared to control group (Table 3).

Histopathological changes: Marked tissue injury with myocardial atrophy, nuclear pyknosis, cytoplasmic

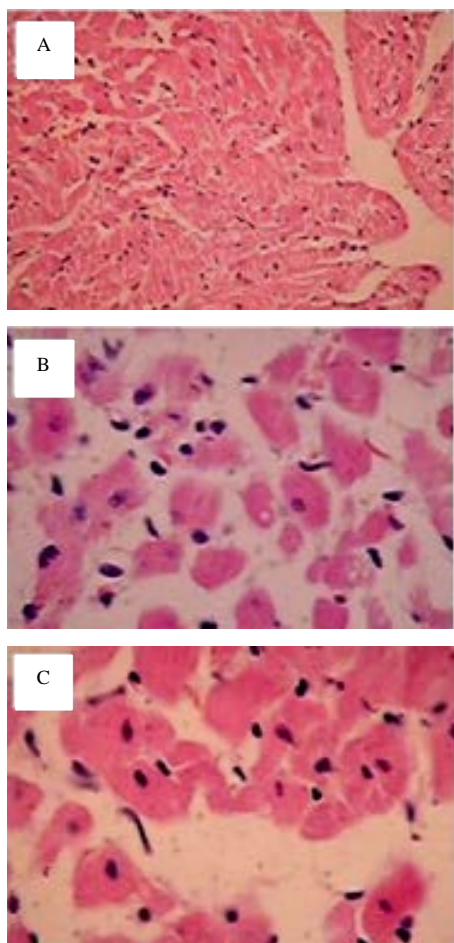


Fig. 1: Histopathological changes in heart, (A) Control: Normal histological structure of heart, (B) Treated with Dox, showing myocardial atrophy, nuclear pyknosis and cytoplasmic vacuoles in the rat heart and (C) Treated with LS+Dox, showing less myocardial atrophy, nuclear pyknosis and cytoplasmic vacuoles than Dox treated animals. Magnifications 40x

vacuoles and cytoplasmic eosinophilia were seen in Dox group (Fig. 1B). In the LS+Dox group (Fig. 1C) these changes were markedly reduced.

Acute toxicity: The acute oral toxicity in mice indicated that LS powder was nontoxic to the mice at 5000 mg kg⁻¹ body weight.

DISCUSSION

Doxorubicin, which possesses potential for generating free radicals, causes an unusual and often irreversible cardiomyopathy. It has quinone and

hydroquinone moieties that can form semiquinone radical intermediates, which in turn can react with oxygen to produce superoxide anion radicals. These can generate both hydrogen peroxide and hydroxyl radicals ([•]OH), which attack DNA and oxidize DNA bases (Serrano *et al.*, 1999). The production of free radicals is significantly stimulated by the interaction of Dox with iron (Hardman and Limbird, 2001). Doxorubicin treatment changes ECG and causes prolongation of QT interval in rats (Cirillo *et al.*, 2000).

The results of present study clearly demonstrated that there was increase in myocardial injury as indicated by increase in QT and ST intervals of ECG pattern in acute Dox treated group. Administration of LS along with Dox (10 mg kg⁻¹) restored QT and ST intervals close to control level.

The results also demonstrated that acute administration of doxorubicin induced cardiotoxicity manifested by a significant increase in serum CK-MB, AST and LDH. These results are consistent with previous studies (Sayed-Ahmed *et al.*, 2001; Saad *et al.*, 2001; Mostafa *et al.*, 1999). In the present study the serum levels of CK-MB, LDH and AST in LS treated group decreased significantly.

The most abundant ROS (Reactive Oxygen Species) generated in living cells are superoxide anion and its derivatives, particularly highly reactive and damaging hydroxyl radical, which induces peroxidation of cell membrane lipid (Hemnani and Parihar, 1998). In this respect, any increase in SOD activity of the organ appears to be beneficial in the event of increased free radical generation. A single dose administration of Dox (10 mg kg⁻¹) caused a significant increase in MDA content (an index of lipid peroxidation) in cardiac tissues and decrease in myocardial GSH and SOD as compared to control group indicating an increase in oxidative stress. These results correlate with previous studies which have demonstrated the involvement of oxidative stress and lipid peroxidation in doxorubicin induced cardiomyopathy (Patil and Balaraman, 2005; Fadillioglu *et al.*, 2004). Administration of LS improved the biochemical marker levels indicating decrease in oxidative stress as evident by increased level of GSH and SOD with decreased lipid peroxidation. LS contain polyphenolic compounds. The total amount of polyphenolic compounds in LS powder was 25.27 mg g⁻¹ by Folin Cio-calteu method using Gallic acid as a standard. The antioxidant activity of polyphenolic compounds from others plants have been reported earlier (Karou *et al.*, 2005; Maisuthisakul *et al.*, 2007). Further studies are required to explore the role of pure polyphenols isolated from LS in contributing to the cardioprotective activity.

Histopathological studies revealed myocardial atrophy, nuclear pyknosis, cytoplasmic vacuoles and cytoplasmic eosinophilia in the Dox treated rat hearts. Similar observations have also been made in earlier studies on acute doxorubicin induced cardiotoxicity (Saad *et al.*, 2001; Morishima *et al.*, 1998). Histopathological observations showed less damage in LS+Dox group than Dox group.

Doxorubicin also causes decrease in heart weight to body weight ratio, which indicates loss of myofibrils and cytoplasmic vacuolisation in myocytes (Mukherjee *et al.*, 2003). In this study the average of heart weight to body weight ratio in Dox group was not statistically significant when compared to control group as well as LS+Dox treated group. It is thus concluded that LS administration may prevent acute doxorubicin-induced cardiotoxicity and decrease myocardial injury by preservation of endogenous antioxidants and reduction of lipid peroxidation in rat heart.

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