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Original Article

Effect of bark extract of *Bauhinia purpurea linn.* on doxorubicin induced cardiotoxicity in rats

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ABSTRACT

The present study was undertaken to evaluate the effect of bark extract of *Bauhinia purpurea* Linn (BPEE) on doxorubicin induced cardiotoxicity in experimental animals. Albino wistar rats were employed in the screening technique and anticancer drug Doxorubicin (DOX) was used as cardiotoxicity inducing agent. Cardiotoxicity was induced by administering DOX intraperitoneally in six equal injections (each containing 2.5 mg/kg DOX at 48 hr interval) to a total cumulative dose of 115 mg/kg over a period of 2 weeks to produce cardiotoxicity in positive control group. Bark extract of *Bauhinia purpurea* (BPEE) was administered daily as a pretreatment for a period of 2 weeks and then alternatively with DOX for next 2 weeks in 6 equal injections (each dose containing 2.5 mg/kg body weight to make a total cumulative dose of 115 mg/kg body weight). After 36 hours of the last dose, rats were anaesthetized with light anesthetic ether, orbital blood samples were collected and the level of biomarkers estimated.

Key words: Cardiotoxicity, Doxorubicin, Free radicals, *Bauhinia purpurea*

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INTRODUCTION

Anthracycline antibiotic doxorubicin (DOX) has been one of the largely prescribed anticancer drug for the treatment of a different human cancersunfortunately, in addition to its potent antitumor property, the use of DOX is associated with a number of unwanted adverse effects, especially with serious cardiac toxicity. This drawback represents a major obstacle to prolonged use of the drug and/or cumulative dose exceeding 550 mg/m^2 body surface area.[1] However, administration of doxorubicin produses cardiotoxic effect due to free radical-induced myocardial injury, lipid peroxidation,[2] myocyte damage induced by increased intracellular calcium, oxidation of fatty acid leading to the depression of energy metabolism in cardiac cell, impairment in myocardial adrenergic signaling/regulation, and the cellular damage.[3]

Several compounds with antioxidant properties have been investigated *in-vitro* with some degree of success. However, the rate of success when performing *in-vivo* studies has been less gratifying. Molecules with antioxidant characteristics *in-vitro* such as selenium or nimesulide were found to be of limited value in counteracting DOX cardiotoxicity *in-vivo.* Additionally, traditional antioxidants, like N-acetylcysteine and tocopherol are not very successful in the prevention of DOX induced cardiotoxicity.[4] Dexrazoxane, an iron chelator with potent antioxidantproperties is the only drug approved by the US Food and Drug Administration (FDA) to prevent DOX-induced cardiotoxicity. However, it has been suggested that dexrazoxane decreases the effect of DOX on leukemia cells[5]and also due to high incidence of dexrazoxane-induced myelosuppression, its use has been limited to some advanced stages of malignant disorders. [6]

Many lines of evidence have suggested that the renin-angiotensin system (RAS) plays an important role in the development of cardiac hypertrophy, failure and reperfusion injury.[7] Suppression of the RAS ameliorates the remodeling process of heart and prolongs long-term survival in animal models and humans with cardiac hypertrophy, failure and reperfusion injury reported the non-toxic effect of doxorubicin on cardiac muscle of angiotensin II type 1_a receptor (AT1) knockout mice, indicating that AT1 mediated angiotensin II (Ang II) signaling pathway plays an important role in the doxorubicin induced cardiac impairment. Telmisartan is a nonpeptide AT1 receptor antagonist which selectively and insurmountably inhibits AT1 receptor subtype without affecting other systems involved in cardiovascular regulation. [8]

Herbal drugs / extracts / phytoconstituents with antioxidant property have shown protective effects in doxorubicin induced cardiotoxicity.[9] Cardiotoxicity seems to be a multi factorial process that leads to cardiomyocyte death as the terminal downstream event. It has long been considered that DOX exerts its anticancer and cardio toxic action by distinct mechanisms: while the anticancer response wasassociated with DNA intercalation, to poisomerase-II inhibition and apoptosis, the cardiotoxicity was mainly ascribed to oxidative stress. At present it appears that suchassociated with DNA intercalation, to poisomerase-II inhibition and apoptosis, the cardiotoxicity was mainly ascribed to oxidative stress. At present it appears that suchseparation is not fully justified. It seems that beneficial (anticancer/therapeutic) and detrimental (cardiotoxic) responses to DOX are to some extent overlapping: they share common effectors, such as oxidative stress, and both involve apoptosis.

OBJECTIVES

The present study was designed investigate the possible protective effect of bark ethanoloic extract of *Bauhinia purpurea* on doxorubicin induced cardiotoxicity and compared with positive control group. Specific objectives for the study undertaken are mentioned below. Induction of cardiotoxicity by administration of doxorubicin (2.5 mg/kg body weight, i.p.)

Objectives of study

The present study was designed to investigate the:

1. Induction of cardiotoxicity by administration of doxorubicin (2.5 mg/kg body weight, i.p.).
2. To monitor the cardiotoxicity by measuring heart weight, body weight, histopathological and biomarkers.
3. To evaluate the cardioprotective activity of bark ethanoloic extract of *Bauhinia purpurea* (BPEE) in doxorubicin induced cardiotoxicity
4. Measurement of biochemical parameters related to oxidative stress:
   Glutathione (GSH)
   Superoxide Dismutase (SOD)
   Catalase (CAT)
   Malondialdehyde (MDA)

MATERIALS AND METHODS

Plant Material: The stem bark of *Bauhinia purpurea* Linn was collected from Western Ghats. The location is Dandeli Forest in the month of June-July. The collected material was washed with running water. The barks were chopped in to small pieces and dried under shade. Dried parts of plant were coarsely powdered and used for extraction

Animal Selection: Wistar rats of either sex weighing 150-200 g, 6-7 weeks old were used in the study and were purchased from Venkateshwara Enterprises, Bangalore. They wereacclimatized to controlled conditions of temperature (23±2°C), 30-70% humidity and 12 hr light-dark cycles. The animals were randomized into experimental and control groups and housed four each in sanitized polypropylene cages containing sterile paddy husk as bedding. They had free assessed to standard pellets as basal diet and water ad libitum. All the studies conducted were approved by Institutional Animal Ethical Committee (IAEC), SET's College of pharmacy, Dharwad, Karnataka (REG.No.112/1999/CPCSEA). According to prescribed guidelines of Committee for the Purpose of Control and Supervision of Experiments in Animals (CPCSEA), Government of India.

Preparation of Doxorubicin and dose selection: Dosage of the DOX administered intra-peritonially was prepared in the saline (0.9% sodium chloride saline solution). Dose of the doxorubicin each dose containing 2.5 mg/kg body weight for a cumulative dose of 15 mg/kg body weight for the present study, were chosen based on previous reports.

PHARMACOLOGICAL SCREENING:

Evaluation of cardioprotective activity in doxorubicin induced cardiotoxicity: The animals were divided into 4 groups of 6 animals each. Details of eachgroup and route and dose of the treatments are mentioned in the **Table 1.** After thirty six hour of the last treatment, orbital blood samples were obtained under light ether anesthesia using heparinzed micro capillaries for the estimation of biomarkers (CPK, LDH,). After experimental period, blood waswithdrawn from retro orbital sinus; serum was separated by centrifugation and used for estimation of marker enzymes (ALT and AST).

Table: Grouping of animals and various treatments:

Groups	Treatment
GROUP I Normal Control	Animals were treated with normal saline 5ml/kg bodyweight.
GROUP II Doxorubicin Induced	Doxorubicin was administered intraperitonially in 6 equal injections (each dose containing 2.5 mg/kg body weight) alternatively for 2 weeks to make a total cumulative dose of 15 mg/kg body weight.
GROUP III BPEE (200 mg/kg) + Doxorubicin	BPEE is dispensed in distill water containing 2% CMC, given orally for 15 days and alternatively treated with doxorubicin 6 equal injection for a cumulative dose of 15 mg/kg body weight.
GROUP IV BPEE (400 mg/kg) + Doxorubicin	BPEE dissolved in distill water containing 2% CMC, given orally for 15 days and alternatively treated with doxorubicin 6 equal injection for a cumulative dose of15 mg/kg body weight.

EFFECT ON HEART WEIGHT: After collecting the blood for the biochemical parameters the heart was isolated, washed with saline and weight was determined by recording relative heart weight with respect to body weight. The isolated hearts were preserved in 10% neutral formalin solution for histopathological studies.

METHODS FOR ESTIMATION OF OXIDATIVE STATUS:

Estimation of Reduced Glutathione (GSH): Reduced glutathione was determined by the method of Ellman. 1.0 ml of homogenate was added to 1ml of 10% TCA and centrifuged. The supernatant is separated. 1.0 ml of supernatant was treated with 0.5ml of Ellmans reagent and 3 ml of phosphate buffer (pH8.0). And take the absorbance immediately at 412nm. The amount of glutathione is calculated using the absorption 13,600M-1 cm-1.

Estimation of Lipid Peroxidation (MDA): Lipid peroxidation was estimated in terms o fthiobarbituricacid reactive species (TBARS), using malondialdehyde (MDA) as standard by the method of Buege and Aust. 0.1mlof the tissue homogenatewasaddedwith2.0ml of the TCA-TBA- HCl reagent (15%w/v TCA 0.375% w/v TBA and 0.25N HCl). The contents were boiled for 15 minutes, cooled and centrifuged at 1000rpm for10 min. The absorbance of clear supernatant was read at 535nm and malodialdehyde concentration of the sample was calculated using extinction coefficient of $1.56 \times 10^5 M^{-1} cm^{-1}$

Estimation of Superoxide Dismutase (SOD): The estimation was determined by method of Kakkar et al. Briefly, to 0.1ml of the sample was mixed with 1.2 ml of sodium pyrophosphate buffer (pH 8.3, 0.052M), 0.1 ml of 186µM of phenazine methasulphate (PMS), 0.3 ml of 300 µM nitro blue tetrazolium (NBT).The reaction was started by addition of 0.2 ml of NADH (750µM). It as incubated at 30°C for 90 sec. Then reaction was stopped by addition of 0.1 ml glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4 ml of n-butanol. The mixture was allowed to stand for 10 min, centrifuged and n-butanol layer was separated. The Colour intensity

of the chromogen was measured against n-butanol at 560nm using spectrophotometer. A system devoid of enzyme activity was defined as enzyme concentration required decreasing the rate of reaction by 50% in one min under the assay conditions.

Estimation of Catalase (CAT): Catalase (CAT) was assayed calorimetrically as described by Sinha. The reaction mixture (1.5ml, vol.) contained 1.0ml of pH 7.0 phosphate buffer, 0.1mlof tissue homogenate (supernatant) and 0.4ml of 0.2M H_2O_2. The reaction was stopped by the addition of 2.0ml of dichromate-acetic acid reagent. Colour intensity was measured olorimetrically at 620 nm and expressed as µmoles of H_2O_2 consumed/min/mg protein.

HISTOPATHOLOGICAL STUDY:

Processing of Isolated Heart: The isolated organ was cut into small pieces and preserved in neutral buffered formalin (10% solution) for at least 2 days. The Heart pieces were washed in running water for about 12 hours. This was followed by dehydration with alcohol of increasing strength (70, 80 and 90 %) for 12 h each. Then the final dehydration was done using absolute alcohol 3 times for 12 h each. Againthe tissue is cleaned by using xylene 2 times for 15 to 20min. each. After cleaning the organ pieces were subjected to paraffin infiltration in automatic tissues processing unit.

Embedding in Paraffin: Hard paraffin was melted and was poured into square-shaped blocks. The liver pieces were then dropped into the liquid paraffin quickly and allowed to cool.

Sectioning: The blocks were cut using microtome to get sections of thickness 5 microns. The sections were then taken on a microscopic slide on which egg albumin (sticky substance) was applied. The sections were allowed to remain on the sticky substance for three days till it sticks firmly onto the slide. The section should be dried completely before staining.

Staining: Eosin is an acidic stain and hematoxylin is a basic stain, which is used for staining.

Observation: All the slides were observed for changes in histopathological characteristics and photographs were taken.

STATISTICAL ANALYSIS

The results were expressed as the mean ± SEM and analyzed using one-way ANOVA followed by Dunnet's multiple comparison tests. Data were computed for statistical analysis using the Graph Pad Prism Software.

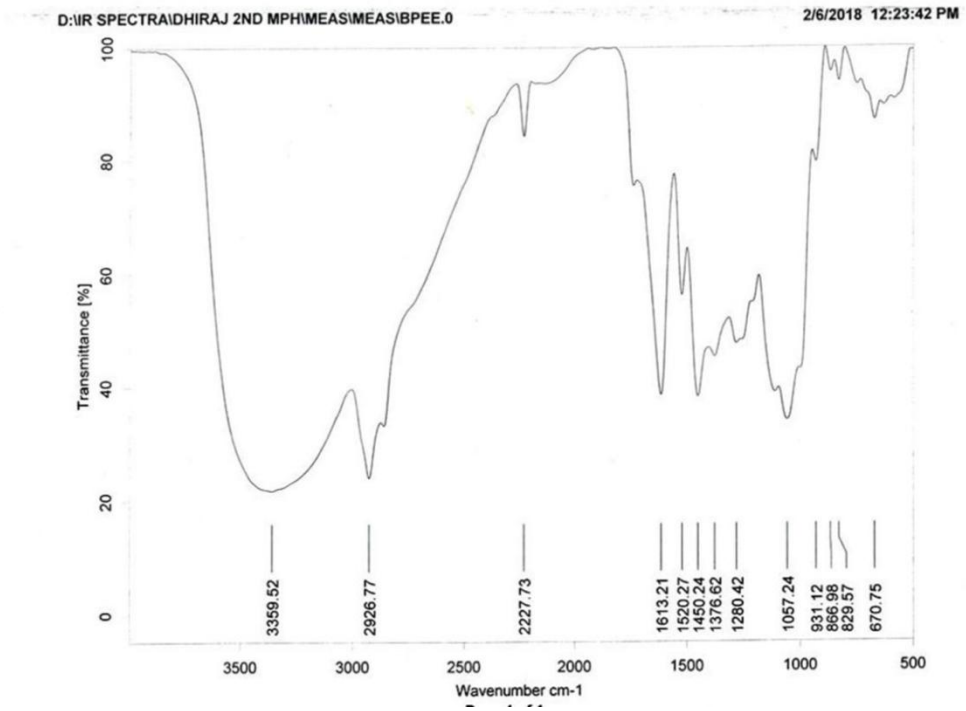
RESULTS

FT-IR ANALYSIS: The Fourier transform infrared spectroscopy (FT-IR) spectrum of ethanolic bark extract of *Bauhinia Purpurea* given in **Figure 1** The data on the peak values and the probable functional groups present in the bark extracts is presented in **Table 1.**

These results comply with literature values. It is reported that several classes of phytochemicals like waxes, triterpenes, steroids, glyceride, flavonoids, phenylpropanoids and alkyl ω-hydroxyalkanoate, have been reported in*BauhiniaPurpurea*. Secondary metabolites such as Myristic acid, Octadecanoic acid, 9,12-Octadecadienoic acid, isopropyl-24methyl-pentacosanoate, Stigmasterol, β-Amyrin, β-Sitosterol, Lupeol and ethyl 9,12-Hexadecadienoate have been isolated.

Table 1: FT-IR absorption frequencies of functional groups in barkextractof *Bauhinia purpurea*.Linn

Frequency (cm^{-1})	Functional groups
3359.52cm^{-1}	OH stretching
2926.77cm^{-1}	Aromatic C-H stretching
2227.73cm^{-1}	CN stretching
1613.21cm^{-1}	C=N stretching
1027.78cm^{-1}	C-O stretching



Doxorubicin Induced Cardiotoxicity:

Effect of Ethanolic extract of *Bauhinia purpurea* (BPEE) on Heart Weight, in Doxorubicin Induced Cardiotoxicity in Rats: Results of body weight, heart weight are presented in **Table 2** and **Figure 2-4** in doxorubicin treated group. Weight of heart was found to be 0.772 ± 0.0281 in normal group, after induction of cardiotoxicity, weight of heart was found to be weight 0.892 ± 0.0.271. There is significant increase weight of this organ indicates formation oedema and water retention. Both doses BPEE treated of animals showed significant reduction in the heart when compared with Doxorubicin treated group.

The ratio of heart weight to body weight in Doxorubicin treated rats were significantly increased as compared with normal rats. The heart weight to body weight ratio Ethanolic extract of *Bauhinia purpurea* (BPEE 200mg/kg) rats was quite significant as compared with normal rats. The ratio of heart weight to body weight in pretreatment group i.e. BPEE + Doxorubicin in post treatment group i.e. BPEE is significantly decreased as compared with Doxorubicin rats.

ESTIMATION OF BIOMARKERS:

CPK and LDH: Results of CPK and LDH is presented in **Table 3** and **Figure 5 and 6** BPEE 200mg/kg and 400mg/kg treated animals showed significant ($P<0.05$ and $P<0.01$) reduction in CPK level and ($P<0.01$ and 0.001) in LDH level.

Table 2 Effect of BPEE and DOX on Heart weight, Body weight, and ratio of heart weight to body weight
Values are mean ± SEM; n=6 in each group, compared to normal group

TREATMENT AND DOSE	BODYWEIGHT(grams) (grams)	HEART WEIGHT (grams)	HEART/BODY
NORMAL	211.44 ± 7.521	0.772 ± 0.0281	36.67
DOXORUBICIN	162.23 ± 6.210	0.892 ±0. 0.0271	55.12
BPEE(200mg/kg) + DOX	189.51 ± 6.121	0.761 ± 0.0291	40.23
BPEE(400mg/kg) + DOX	195.14 ± 7.230	0.751 ±0. 0.0264	38.65

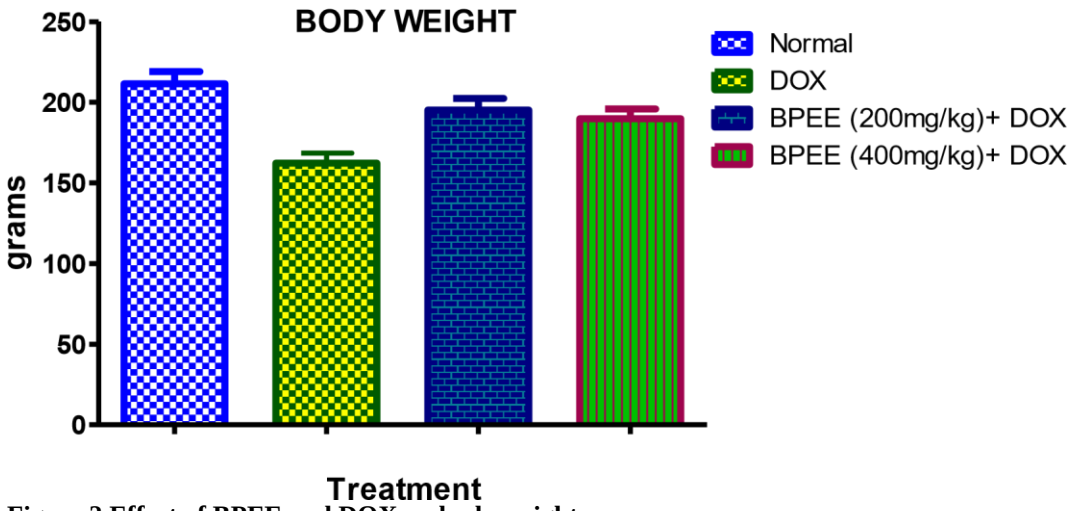


Figure 2 Effect of BPEE and DOX on body weight

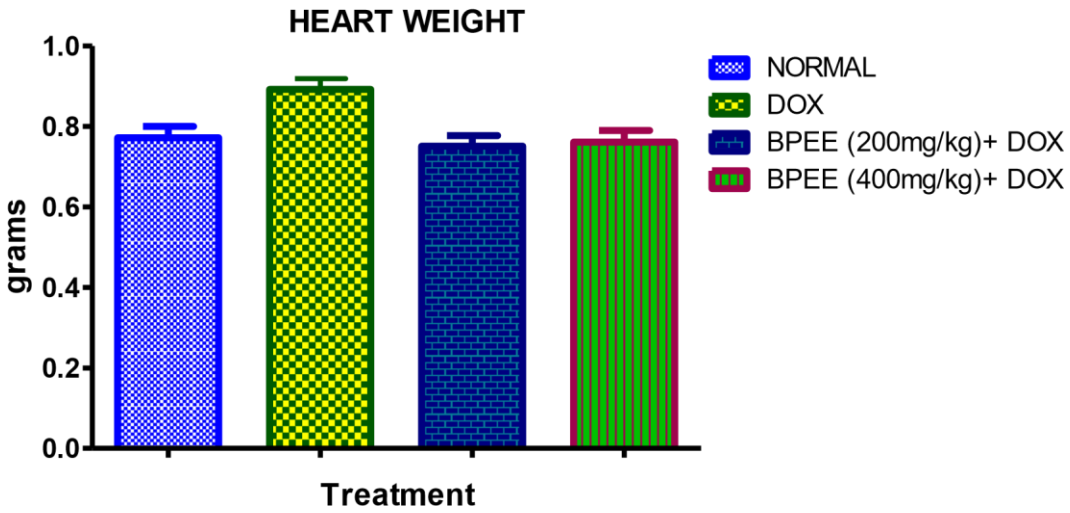


Figure 3 Effect of BPEE and DOX on Heart weight

Table 3 Data showing the effect of Ethanolic extract of*Bauhinia purpurea* (BPEE) on DOX induced changes in Serum CPK and LDH in rats:

TREATMENT AND DOSE	CPK (IU/L)	LDH (IU/L)
NORMAL (Saline 1ml/kg)	152.12 ± 9.851	145.82 ± 8.210
DOXORUBICIN 2.5mg/kg	296.23 ± 9.677	215.14 ±7.418
BPEE (200mg/kg) + DOX	244.13 ± 9.36*	181.31 ± 6.323**
BPEE (400mg/kg) + DOX	222.93 ± 8.484**	171.43 ± 6.089***

Values are mean ± SEM; n=6 in each group, *P<0.05, **P<0.01, ***P<0.001 when compared to doxorubicin treated groups.

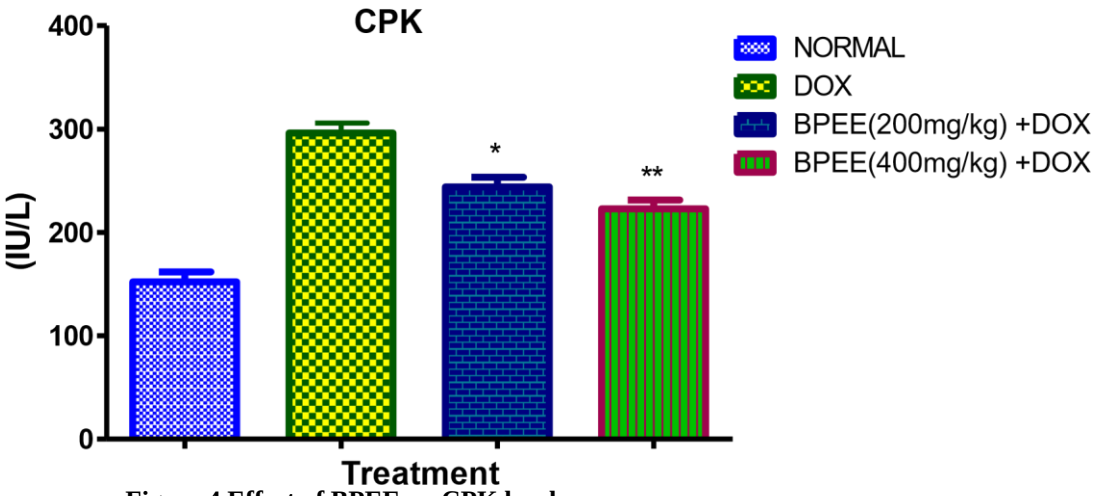


Figure 4 Effect of BPEE on CPK levels

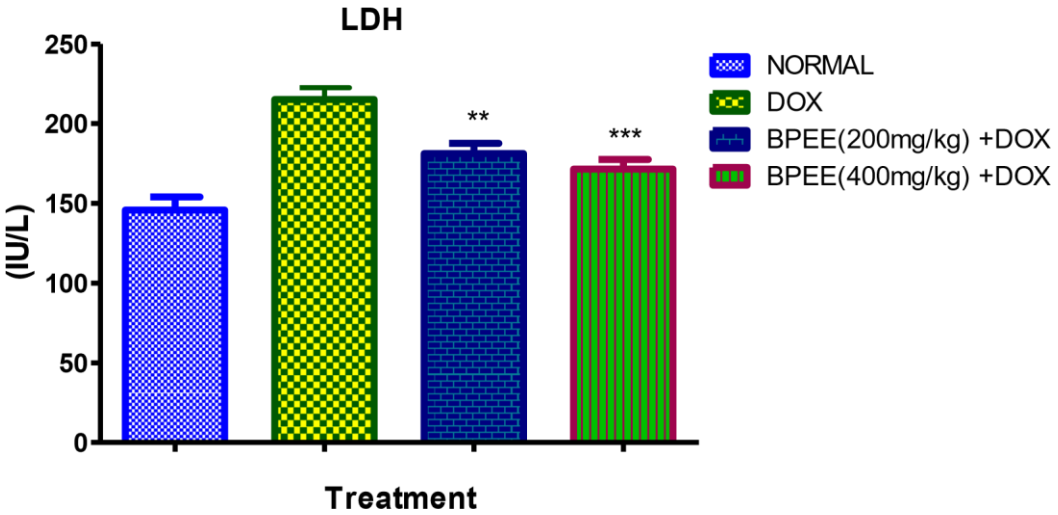


Figure 5 Effect of BPEE on LDH levels

SERUM ENZYME LEVEL: The effect of BPEE on serum AST and ALT level are summarized in **Table 4** and **Figure 7 and 8** animals treated with BPEE 400mg/kg showed significant decrease in AST ($P<0.01$) and ALT ($P<0.001$) level compared to DOX treated group. Similarly BPEE 200mg/kg showed significant decrease in AST ($P<0.05$) and ALT ($P<0.05$) level compared to treated group.

Table 4 Data showing the effect of Ethanolic extract of*Bauhinia purpurea* (BPEE)on Doxorubicin induced changes in Serum enzyme levels [AST and ALT] in rats.

TREATMENT AND DOSE	AST (IU/L)	ALT (IU/L)
NORMAL (Saline 1ml/kg)	67.92 ± 5.51	48.52 ± 4.63
DOXORUBICIN 2.5mg/kg	168.18 ± 6.50	156.71 ± 4.77
BPEE (200mg/kg) + DOX	141.39 ± 7.34*	131.68 ± 5.58**
BPEE (400mg/kg) + DOX	108.24 ± 7.39**	99.73 ± 5.31***

Values are mean ± SEM; n=6 in each group,*P<0.05, **P<0.01, ***P<0.001

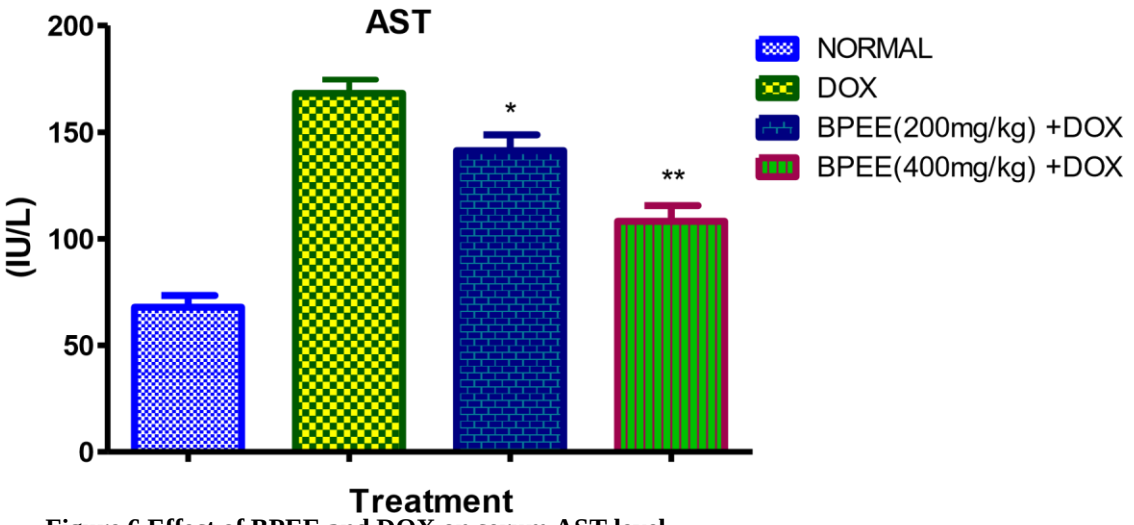


Figure 6 Effect of BPEE and DOX on serum AST level

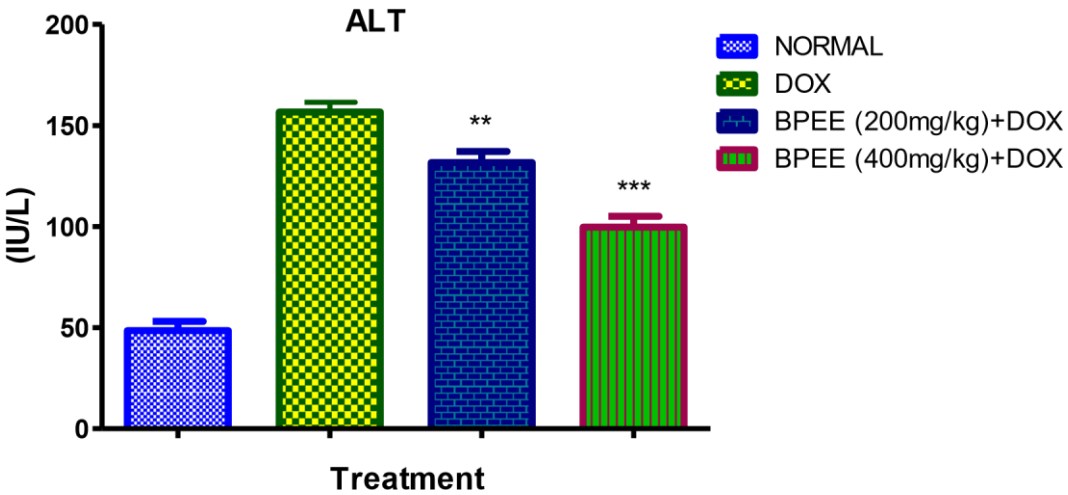


Figure 7 Effect of BPEE and DOX on serum ALT level

EVALUATION OF ANTIOXIDANT ACTIVITY: The heart antioxidant enzyme levels in DOX treatment group showed a significant increase in lipid peroxidation (nmol MDA/min/mg of wet tissue) while a significant decrease in reduced Glutathione (GSH) (nmol/min/mg of wet tissue), Superoxide dismutase (SOD) (Unit x/mg protein) and Catalase (CAT) (Unit y/mg protein) are seen when compared to normal group.

Glutathione and Superoxide dismutase: Effect of BPEE on GSH and SOD are summarized in **Table 5**. In DOX treated animals three is significant decrease in both GSH and SOD level when compared to normal animals. However, animals pre-treated with BPEE (400mg/kg) has shown significant increase in GSH ($P<0.001$) and SOD ($P<0.01$) level when compared to DOX treated animal.

Catalase and Lipid Peroxidation: Animals treated with DOX has shown significant decrease in CAT and increase in lipid peroxidation when compared to normal animals. Animals pre-treated with BPEE (400mg/kg) has showed significant ($P<0.01$)restorationof CAT and lipid peroxidation to normal level when compared to DOX treated animals.

Table No. 5 Effect of ethanolic extract of*Bauhinia purpurea* (BPEE) on **DOX induced changes in Reduced Glutathione and SOD levels in heart of rats:**

TREATMENT AND DOSE	GSH (n mole/min/mg of wet tissue)	SOD (Unit y/mg of Protein)
NORMAL (Saline 1ml/kg)	2.64 ± 0.15	10.91 ± 0.81
DOXORUBICIN 2.5mg/kg	2.08 ± 0.19	5.84 ± 1.23
BPEE (200mg/kg) + DOX	2.38 ±0.31**	7.55 ± 1.38*
BPEE (400mg/kg) + DOX	2.51 ± 0.23***	8.96 ± 1.70**

Values are mean ± SEM; n=6 in each group, *P<0.05, **P<0.01, ***P<0.001 when compared DOX treated animals. y – one unit of SOD activity is amount of protein required to give 50% inhibition of epinephrine auto-oxidation.

Table 6 Effect of ethanolic extract of*Bauhinia purpurea* (BPEE)on DOX induced changes in CAT and MDA levels in heart of tissue.

TREATMENT AND DOSE	CAT (Unit x/mg protein)	LIPID PEROXIDATION (n mol of MDA/min/mg of wet tissue)
NORMAL (Saline 1ml/kg)	18.36 ± 0.66	6.21 ± 1.51
DOXORUBICIN 2.5mg/kg	10.18 ± 1.44	10.95 ± 1.43
BPEE (200mg/kg) + DOX	13.84 ± 1.11*	9.13 ± 1.56
BPEE (400mg/kg) + DOX	14.99 ± 1.23**	7.94 ± 1.68**

Values are mean ± SEM; n=6 in each group,**P<0.01, ***P<0.001 when compared to normal, DOX treated animals. x – µmoles of H_2O_2 decompose/min/mg/protein.

HISTOPATHOLOGICAL INVESTIGATION:

The tissue sections were fixed in 10% formalin. The specimens were processed by standard procedure and embedded in paraffin wax. The blocks were sectioned from the ventricular portion and 5-micron thick sections were stained according to the hematoxylin and eosin method given by Smith and Burton the sections were examined by light microscopy.

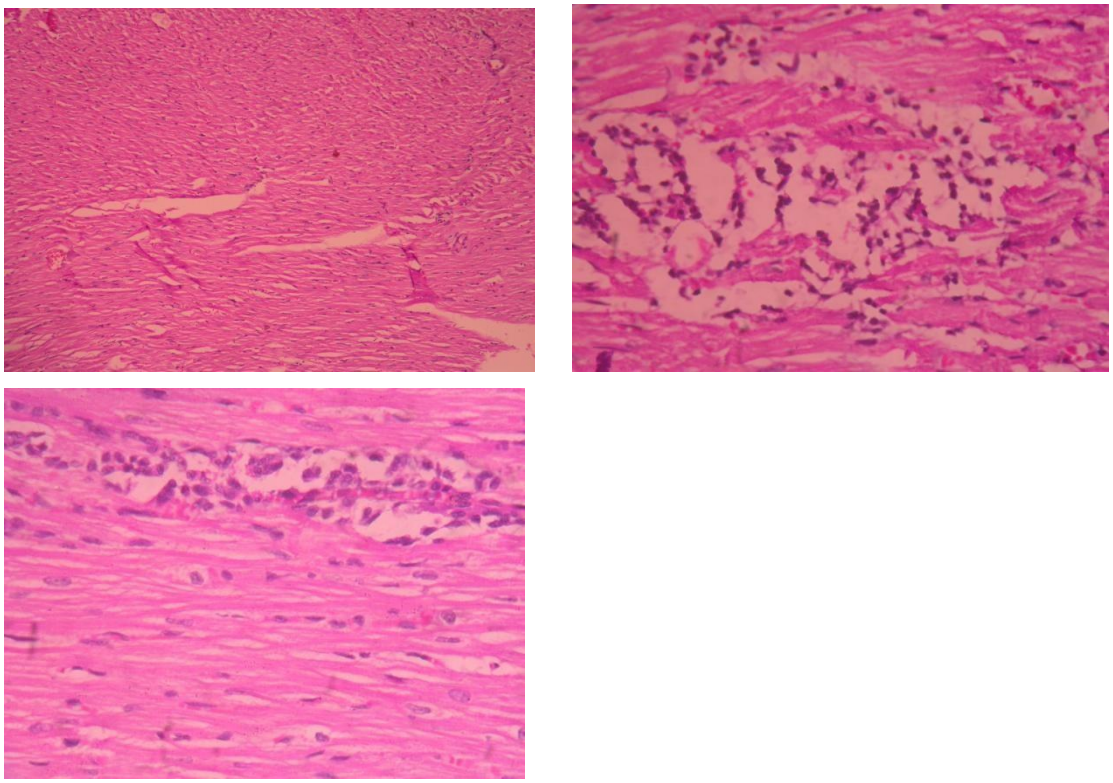


Figure No 8 Histopathological studies of heart in treated groups

Histopathological results are depicted in **Figure 9** Image (A) Normal control rat heart showing normal myocardial fiber, vacualation, nacrosis and inflammation are absent, Image (B) Represent DOX alone treated rats, showing hypertrophic myocardial cells, vacuation and narcotic cells. Image (C) Represent BPEE 400mg/kg + DOX treated rats showing cardiac muscle fiber of normal shape and size with few cells damage. Their shown cardiac protection of BPEE against DOX induced cardiac
toxicity.

CONCLUSION

It may be concluded that pre administration of Ethanolic extract of *Bauhinia purpurea* protects cardiotoxicity induced by Doxorubicin- in rats. Administration of Ethanolic extract of *Bauhinia purpurea* showed a less cardiac injury and restoration of the oxidant/ antioxidant status as well as lessening histopathological changes. This can be attributed, at least in part, to antioxidant activity of the extract used in the rats. It may be concluded that the cardiotoxicity induced by Doxorubicin is in relationship with oxidative stress. Ethanolic extract of *Bauhinia purpurea* has to be most effective in the functional recovery of the heart and restoration of biochemical and histopathological alteration which may be associated with its cardioprotective property. Our study suggests that Ethanolic extract of *Bauhinia purpurea* may be considered as a potentially useful candidate in the combination with doxorubicin to limit cardiotoxicity. Further molecular level of investigation is to be done using different animal model and using different biochemical parameters to assess the possible mode of action of Ethanolic extract of *Bauhinia purpurea* as cardio protective drugs. It is worthwhile to consider this aspect for clinical application in patient of cardiac injury.

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