

INOTROPIC AND CARDIOPROTECTIVE EFFECT OF *TERMINALIA PANICULATA* ROTH BARK EXTRACT IN DOXORUBICIN INDUCED CARDIOTOXICITY IN RATSDavey M.S.^{1*}, Atlee C.W.²¹Department of Pharmacology, C.L.Baid Metha College of Pharmacy, Thorraipakam, Chennai, India²Asst.Professor, Department of Pharmacology, C.L.Baid Metha College of Pharmacy, Thorraipakam, Chennai, India

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ABSTRACT

Doxorubicin (DOX) is a widely used cancer chemotherapeutic agent. However, it generates free oxygen radicals that result in serious dose-limiting cardiotoxicity. Supplementations with *Terminalia paniculata* bark extract were proven effective in reducing oxidative stress associated with several ailments. The aim of the current study was to investigate the potential protective effect of ethanolic extract of *Terminalia paniculata* (EETP) bark against doxorubicin induced cardiotoxicity in rats and to compare with vitamin E, a known cardioprotective antioxidant. Extract was given orally to rats (200mg/kg/day and 400mg/kg/day for 28 consecutive days), Vitamin E was given orally (100mg/kg/day for 28 consecutive days) and DOX (15 mg/kg; i.p.) was administered on the 29th day to induced cardiotoxicity. EETP protected against DOX-induced increased in heart weight. It significantly inhibited DOX-provoked elevation in serum lactate dehydrogenase (LDH), aspartate transaminase (AST), alanine transaminase (ALT), creatine kinase-MB (CK-MB) as well as troponin I level. The reductions in cardiac activities of catalase (CAT), superoxide dismutase (SOD), glutathione (GSH) and glutathione peroxidase (GSH-Px) were also significantly mitigated. Elevation of cardiac lipid peroxidation (LPO) activity in response to DOX treatment was significantly hampered. Pretreatment with extract significantly guarded against DOX-induced decreased in cardiac ATPase activity like Ca⁺² ATPase, Na⁺-K⁺ ATPase and Mg⁺² ATPase. EETP alleviated histopathological changes in rats heart treated with DOX. Finally we concluded that *Terminalia paniculata* bark extract exerts equipotent cardioprotective and inotropic activity in the experimental model of doxorubicin induced myocardial infarction in rats as compared to vitamin E, a known cardioprotective antioxidant.

KEYWORDS: Cardioprotective; *Terminalia paniculata*; Myocardial infarction; Doxorubicin; Vitamin E; Cardiac biomarkers; Antioxidants.

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INTRODUCTION

Doxorubicin (DOX) is a naturally occurring anthracycline that is widely used in the treatment of a variety of human malignancies including breast cancer, small cell carcinoma of the lung and acute leukemia¹. However, like most of the anticancer drugs, DOX causes various toxic effects, the commonest of which is the dose-dependent cardiotoxicity which leads to cardiomyopathy and eventually congestive heart failure². The mechanism by which DOX causes myocardial injury is not fully understood. Several explanations account for the DOX cardiotoxicity, e.g., free radical production, calcium overloading, mitochondrial dysfunction and peroxynitrite formation have been proposed^{3,4}.

Nonetheless, the oxidative stress hypothesis of DOX toxicity remains the cornerstone. Following entry into cardiomyocytes, DOX generates reactive oxygen species (ROS) via several mechanisms. The role of ROS in DOX-induced cardiac toxicity is supported by the findings that treatment of animals with a variety of antioxidants protects heart against the toxicity of DOX⁵. Nature has been a source of medicinal treatments for years and plants derived products continue to play an important role in primary health care of about 80-85% of world's population. Recently, the keen interest in medicinal plants for cardioprotection has increased because of their numerous possible cardioprotective mechanisms besides antioxidant activity⁶.

Terminalia paniculata Roth (Combretaceae) is a tropical tree with a large natural distribution in Western Ghats, India. Traditionally, flower juice and bark of *Terminalia paniculata* have been used as a remedy for cholera, for the treatment of inflamed parotid glands and in menstrual disorders⁷. However, till date there has been no investigation supporting the pharmacological properties of this plant. This study was therefore aimed to evaluate the cardioprotective potential of ethanolic extract of *Terminalia paniculata* bark (EETP) against doxorubicin induced cardiotoxicity in male Wistar rats.

MATERIALS AND METHODS

Collection of plant material

The bark of *Terminalia paniculata* was collected from the evergreen forest of Western Ghats under the supervision of forest officials. The plant material was identified and authenticated by, Resident Botanist, Prof. Dr. S. Jayaraman, Director of Plant Anatomy Research Centre (PARC), West Tambaram, Chennai, Tamilnadu (Voucher no- PARC/2010/595).

Preparation of extract

Freshly collected bark of *Terminalia paniculata* Roth were dried in shade and pulverized to get a coarse powder. A weighed quantity of the powder (830g) was extracted with ethanol in Soxhlet apparatus at 60-80°C. The filtrate was evaporated to dryness at 40°C under reduced pressure in a rotary vacuum evaporator. The percentage yield of ethanolic extract was 23.3 %w/w. The extract was stored at 4°C for further use.

Experimental models

Colony inbred strains of Wistar albino rats of male sex weighing 150-200g were used for the pharmacological studies. They were housed in groups in polypropylene cages one week prior to the experiments to acclimatize to laboratory conditions and were fed on standard pellet diet (Hindustan Lever Pvt Ltd., Bangalore) and water *ad libitum*. The experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC) of CPCSEA (Committee for the Purpose of Control and Supervision of Experimental Animals).

IAEC	Reference	number:
<u>IAEC/XXX/06/CLBMCP/2010 dated 22.09.2010</u>		

Acute toxicity studies

Acute toxicity tests were performed in animals of single sex. All animals were fasted overnight before treatment and were given food 1 h after treatment. A single high dose (2000 mg/kg), as recommended by the OECD guidelines, was administered orally to rats (3 no's). General behavior was also observed at 1, 3 and 24 h after administration. The number of animals that died after administration was recorded daily for 14days⁸.

Experimental protocols

The experimental protocol applied for cardioprotective effect of EETP and in DOX induced cardiotoxicity are as follows. Five groups each containing six animals were selected for the study.

Group I- Control group, animals treated with 0.9% normal saline daily for 28days.

Group II- Negative control, animals treated with 0.9% normal saline + Doxorubicin (DOX) (15 mg/kg b.w, i.p in saline on 29th day)

Group III- Animals administered with 200 mg/kg b.w, p.o of EETP dissolved in saline for 28 days + DOX (15 mg/kg b.w, i.p in saline on 29th day)

Group IV- Animals administered with 400 mg/kg b.w, p.o of EETP dissolved in saline for 28 days + DOX (15 mg/kg b.w, i.p in saline on 29th day)

Group V- 5% Tween 80 in normal saline + vitamin E (100mg/kg b.w, p.o) for 28 days and DOX (15 mg/kg b.w, i.p in saline on 29th day)

Animals were pretreated with extract and experimental drug for 28days. A single dose of DOX was administered intraperitoneal to the groups II-V on 29th day. 48 hours after DOX administration, animals were anesthetized with thiopentone sodium (50mg/kg). The blood was collected from retro-orbital sinus under light ether anesthesia and the serum separated for biochemical estimation. Plasma and serum were separated by centrifugation. Rats were sacrificed and the heart was excised immediately, weighed, immersed in physiological saline and dissected out for histopathological examination.

Plasma cardiac specific injury markers

Activity levels of serum troponin I using Enzyme Immunoassay Test Kit⁹ (Oxis International, Inc), creatine phosphokinase-MB (CK-MB)⁹, lactate dehydrogenase (LDH)¹⁰, alanine transaminase (ALT)¹¹ and aspartate transaminase (AST)¹¹ in plasma were estimated.

Cardiac endogenous antioxidant milieu

Heart of control and treated groups were weighed and homogenized (10%w/v) in chilled Tris buffer (10mM, pH7.4), centrifuged at 10,000 rpm for 20min in high speed cooling centrifuge (0 °C). Clear supernatant was used for assaying reduced glutathione (GSH)¹², glutathione peroxidase (GSH-Px)¹³, superoxide dismutase (SOD)¹⁴, catalase (CAT)¹⁵ and lipid peroxidation (TBARS)¹⁶.

Cardiac ATPases

Tissue homogenate pellet obtained after centrifugation was resuspended in ice-cold Tris buffer (10mM, pH7.4) to get a final concentration of 10% and was used for the

estimation of Na⁺-K⁺ ATPase¹⁷, Ca²⁺ ATPase¹⁸ and Mg²⁺ ATPase¹⁹.

Histopathology

After draining the blood heart samples were excised, washed with normal saline and processed separately for histological observations. The materials were fixed in 10% buffered neutral formalin for 48hrs. Paraffin sections were taken at 5µm thickness processed in alcohol-xylene series and was stained with haematoxylin-eosin dye. The sections were examined microscopically for histopathological changes.

Statistical analysis

The statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Dunnett's test using Graph pad. P values <0.05 were considered as statistically significant condition.

RESULTS

Evaluation of general toxicity

Two rats died in DOX-only-treated group (33.3%) two days after DOX administration. However, no mortality was observed in all other groups including the combined EETP + DOX-treated group as well as Vit E+DOX treated group. Rats in the DOX-only-treated group showed scruffy fur and developed a light yellow tinge. These animals showed, also, brown exudates around the eyes and nose, soft watery feces, colored urination and enlargement of abdomen, which appeared to sicker, weaker and lethargic as compared to EETP and Vit E treated group.

Plasma cardiac specific injury markers

DOX treated rats (Group II) exhibits significantly (p<0.001) higher levels of serum myocardial biomarker enzymes such as cardiac troponin I, CK-MB and LDH levels when compared to control group (Group I; **Table no.1**). DOX treated groups also shows a significant (p<0.001) increased in serum AST and ALT levels when compared to control rats (**Figure no.1**). Rats pretreated with EETP (200mg/kg and 400mg/kg) resulted in a significant (p<0.001) decreased in those enzymes. Whereas, Vit E administered group showed no significant effect upon cardiac troponin I levels and significant (p<0.01) decreased on other enzymes levels when compared to Dox group. The results indicate that *T.paniculata* bark extract has the tendency to reduce the elevated cardiac marker enzymes proving its Cardioprotective effect.

Cardiac endogenous antioxidant milieu

The levels of antioxidant enzymes such as GSH, GSH-Px, SOD and CAT were found to be significantly (p<0.001) decreased in Dox alone administered group when compared with control animals (**Table no.2**). Rats

pretreated with *T.paniculata* bark extract 200mg/kg and 400mg/kg as well as Vit E 100mg/kg resulted in a significant (p<0.001) elevation of the antioxidants in heart tissue. Lipid peroxidation levels in the form of TBARS shows a significant (p<0.001) elevation in the Dox alone treated rats when compared to control group (Group I; **Table no.3**). Rats pretreated with EETP and Vit E resulted in a significant (p<0.001) decreased in TBARS levels when compared to Dox group.

Heart weight and Cardiac ATPases

Dox group recorded significant (p<0.001) increased in mean heart weight compared to control animals. Pretreatment with EETP and Vit E recorded significant (p<0.001) decrement in heart weight compared to DOX group (**Table no.3**). Moreover, DOX treated group also recorded significant (p<0.001) decrement in the activity levels of cardiac Ca²⁺ ATPase, Na⁺-K⁺ ATPase & Mg²⁺ ATPase compared to control group. Pretreated EETP group recorded significant (p<0.001) increased, whereas, Vit E recorded (p<0.01) increment in activity of cardiac ATPase enzymes compared to Dox group (**Figure no.2**). The results indicate that *T.paniculata* bark extract has the tendency to reduce the elevated cardiac ATPase enzymes proving its inotropic effect.

Histopathology

Hearts from control showed regular cell distribution and normal myocardium architecture (**Figure no.3a**). Histological examination of the rat hearts from DOX-only treated animals revealed myocardial fibers with degenerative changes, myofibrillar loss and cellular infiltration (**Figure no.3b**). Myocardial lesions were significantly reduced in animals from EETP (200mg/kg & 400mg/kg) + DOX group (**Figure no.3c & d**). Histological examination of the rat hearts from vitamin E treated groups showed slightly changes of fibrous tissue (**Figure no.3e**).

DISCUSSION

Doxorubicin continues to be an effective and widely used broad spectrum chemotherapeutic agent. However, its clinical use is limited because of its serious dose dependent cardiotoxicity. Clinical and experimental investigations suggested that increased oxidative stress plays a critical role in subsequent cardiomyopathy and heart failure associated with DOX treatment²⁰. The present study was designed to investigate the potential protective effects of the ethanolic extract of *Terminalia paniculata* bark against DOX-induced cardiotoxicity in rats.

In the present study, animals administered with doxorubicin accompanied by the high mortality rate compared to control group. Live animals showed

excessive degree of pericardial, pleural and peritoneal effusion. These findings are in line with those observed by previous investigators²¹. The ability of EETP to protect against DOX-induced high mortality was considered an early sign of cardioprotection. Cardiotoxicity due to Dox is also characterized by increased in the heart weight²². The results of the present study confirmed the earlier findings that animals treated with Dox caused a significant increase in the heart weight. The observation showed that EETP and Vit E treatment caused lesser increment in the heart weight following DOX administration, when compared to ischemic control animals.

DOX administration to rats significantly elevated serum AST, ALT, LDH activity, CK-MB and troponin I levels; which are released from damaged myocytes and sensitive indicators of cardiac injury. The serum enzymes viz AST and ALT serve as sensitive indices to assess the severity of myocardial infarction²³. CK-MB, LDH and troponin I, cardiac enzymes found primarily in the myocardium are used to evaluate the existence and extent of myocytes injury²⁴. The increase of these biomarkers in serum and extracellular fluid suggests an increased leakage of these enzymes from mitochondria as a result of toxicity induced by the treatment with doxorubicin. EETP significantly inhibited DOX-induced elevations in serum activity of AST, ALT, LDH, and CK-MB as well as troponin I levels.

Since oxidative stress is a cornerstone in DOX-induced cardiotoxicity, it was reasonable to investigate the oxidant/antioxidant status of the rats. Current data showed that cardiac levels of GSH, CAT, SOD and GSH-Px were significantly reduced while LPO levels in the form of TBARS was elevated following DOX administration as compared to control group. Such data clearly indicate an overt oxidative stress. Lipid peroxidation plays a major role in the myocardial cell damage and the accumulated lipid peroxides reflect the various stages of diseases and its complications. Significant elevation observed in the level of lipid peroxidation with a concomitant decline in the level of GSH, GSH-Px and antiperoxidative enzymes activity (CAT and SOD) in heart of negative control group, which is in correlation with previous investigations reflected the high vulnerability to oxidative deterioration in ischemic conditions³. Depletion of glutathione is known to result in enhanced LPO and excessive LPO can cause increased glutathione consumption. Inhibition in the activities of antioxidant enzymes may lead to the increased generation of O²⁻ and H₂O₂, which in turn can form hydroxyl radical (OH.) and bring about a number of

reactions harmful to structural and functional integrity²⁵. The finding that cardiac LPO activity was increased in DOX group is important because it denotes leukocyte accumulation in cardiac tissue. This is in line with previous reports implicating inflammation in DOX cardiotoxicity²⁶. Based on our data, it may be suggested that inhibition of CAT and GSH-Px activities would channel the produced H₂O₂ to the LPO pathway with the resultant cardiac injury. Pretreatment of rats with EETP and Vit E significantly guarded against the oxidative stress observed in the DOX group.

ROS impairs the function of isolated mitochondria and subsequently result in adenosine (ATP) depletion. ROS have been shown to reduce calcium stimulated ATPase activity and slow down calcium transport in the sarcolemma. Hence, the disturbances in calcium homeostasis occurs due to the ROS interactions with cellular membranes could explain some of the contractile abnormalities associated with Ischemia/Reperfusion²⁷. Several factors are known to modify the levels of ATPase, especially lipid peroxidation and membrane fluidity. It has been reported that doxorubicin treatment resulted in a decreased in the activities of membrane-bound ATPases resulting in the enhanced calcium influx into ventricular cell through stimulation of the voltage dependent calcium sodium slow channels; which in turn result in the intracellular accumulation of calcium ions²⁸. The failure in ATPase activity in the ischemic condition may be responsible for causing not only functional damage but also irreversible necrotic changes in the involved myocardial cell. Peroxidation of membrane lipids could inactivate Na⁺K⁺ATPase and Ca²⁺ATPase because of the oxidation of 'SH' groups present in its active site resulting in the conformational changes in the enzymes²⁹. Decreased in the activity of Ca²⁺ATPase can increase intracellular concentration of free Ca²⁺ and alter the signal transduction pathways and cellular fluidity³⁰. The present study shows that the altered activity of the membrane-bound ATPases by doxorubicin was protected by pretreatment with EETP. This could be due to the anti-oxidative effect of experimental drugs against ROS induced by doxorubicin. Thus the above observations suggest that the bark extract of *Terminalia paniculata* possess inotropic activity.

Histopathological studies showed severe degeneration of the myofibrils with focal necrosis and vacuolated cytoplasm was seen clearly in DOX treated animals. DOX intoxication also induced eosinophilic cytoplasmic and focal hemorrhage with inflammatory cell infiltration. Similar observations have also been made in previous studies on acute DOX induced cardiotoxicity³¹. The

severity of the histological changes was much less in sections from animals pretreated with EETP.

In conclusion, our data indicate that EETP protects against DOX-induced cardiotoxicity in rats as evidenced by improved mortality, mitigation of cardiac injury markers, improved ATPase activity and restoration of the oxidant/ antioxidant status as well as lessening histopathological changes. This can be attributed, at least in part, to its antioxidant activity. Thus *Terminalia paniculata* bark extract possess cardioprotective and inotropic activity against doxorubicin induced cardiotoxicity.

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Table 1: THE EFFECT OF *T.paniculata* BARK EXTRACT ON CARDIAC TROPONIN I, CREATINE KINASE-MB (CK-MB) AND LACTATE DEHYDROGENASE (LDH).

Groups	Troponin I ng/ml	CK-MB IU/litre	LDH IU/litre
Control	0.403±0.019	126.1±1.981	106.2±3.510
DOX group	0.981±0.21 ^{a***}	292.4±4.34 ^{a***}	216.8±2.70 ^{a***}
EETP 200mg/kg	0.633±0.064 ^{b***}	192.3±2.56 ^{b***}	144.0±3.70 ^{b***}
EETP 400mg/kg	0.525±0.014 ^{b***}	157.9±4.86 ^{b***}	125.7±1.82 ^{b***}
Vit E 100mg/kg	0.893±0.045 ^{bNS}	226.7±4.94 ^{b**}	178.4±3.36 ^{b*}

SIGNIFICANT: *P<0.05, **P<0.01, ***P<0.001, ^{NS} nonsignificant.
Values are expressed as mean ±SEM of 6 animals; Compared with ^acontrol, ^bDOX treated rats.

Table 2: EFFECT OF *T.paniculata* BARK EXTRACT ON ANTIOXIDANTS LEVELS IN HEART HOMOGENATE

Treatment	SOD Units/mg protein	CAT µmol/H ₂ O ₂ /decomposed/ mg/min	GSH µg/mg protein	GSH-Px U/min/mg protein
Control	6.80±0.239	8.16±0.183	5.227±0.143	4.365±0.114
DOX group	2.717±0.15 ^{a***}	3.290±0.10 ^{a***}	2.010±0.10 ^{a***}	1.83±0.13 ^{a***}
EETP 200mg/kg	5.060±0.10 ^{b***}	5.358±0.17 ^{b***}	3.550±0.14 ^{b***}	3.120±0.12 ^{b***}
EETP 400mg/kg	4.957±0.09 ^{b***}	6.808±0.27 ^{b***}	4.680±0.19 ^{b***}	3.768±0.22 ^{b***}
Vit E 100mg/kg	5.135±0.11 ^{b***}	6.905±0.24 ^{b***}	4.673±0.16 ^{b***}	3.505±0.28 ^{b***}

SIGNIFICANT: *P<0.05, **P<0.01, ***P<0.001, ^{NS} nonsignificant.
Values are expressed as mean ±SEM of 6 animals; Compared with ^acontrol, ^bDOX treated rats.

Table 3: THE EFFECT OF *T.paniculata* BARK EXTRACT ON HEART WEIGHT AND LIPID PEROXIDATION LEVELS IN HEART HOMOGENATE.

Groups	Heart weight (g)	LPO nmoles TBARS/mg protein
Control	0.692±0.009	2.323 ±0.159
DOX group	0.898±0.010 ^{a***}	5.133±0.161 ^{a***}
EETP 200mg/kg	0.740±0.008 ^{b***}	4.177±0.051 ^{b***}
EETP 400mg/kg	0.704±0.011 ^{b***}	3.268±0.127 ^{b***}
Vit E 100mg/kg	0.709±0.010 ^{b***}	3.058±0.096 ^{b***}

SIGNIFICANT: *P<0.05, **P<0.01, ***P<0.001, ^{NS} nonsignificant.
Values are expressed as mean ±SEM of 6 animals; Compared with ^acontrol, ^bDOX treated rats.

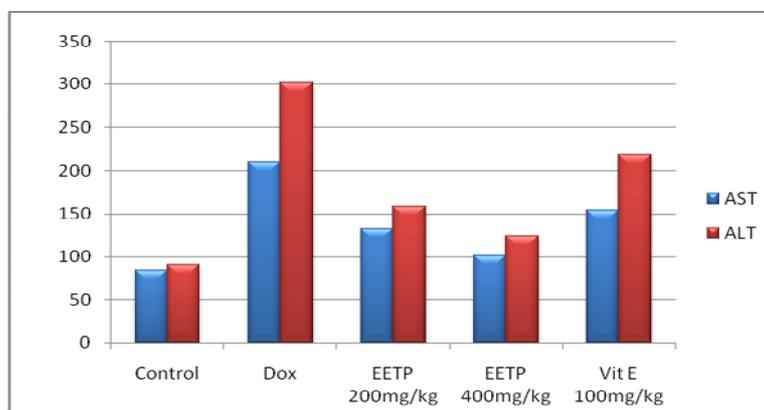


Figure 1: The effect of *T.paniculata* bark extract on serum aspartate transaminase (AST) and alanine transaminase (ALT)

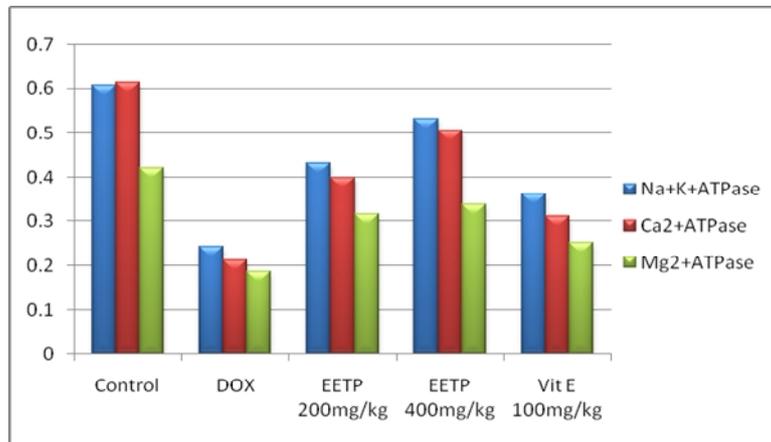
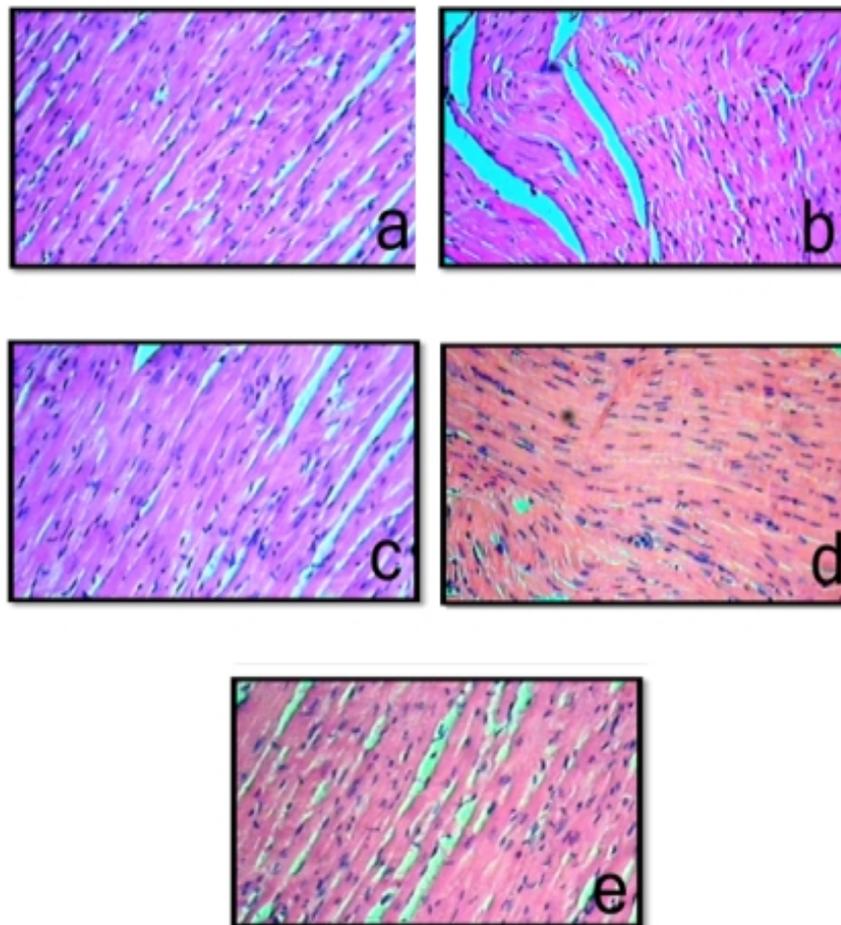


Figure 2: The effect of *T.paniculata* bark extract on membrane bound Ca²⁺ATPase, Na⁺K⁺ATPase and Mg²⁺ATPase



Histopathological evaluation of cardiac tissue for protective effect of EETP against doxorubicin induced cardiotoxicity.

Figure 3 (a) Photomicrograph showing normal architecture of control rat heart. (b) Photomicrograph section of rat heart subjected to doxorubicin showing degenerative changes, hyalinization of muscle fibers, myofibrillar loss and cellular infiltration. (c) Photomicrograph of rat heart of EETP (200mg/kg) treated group showing lesser degree of myonecrosis and infiltration of inflammatory cells. (d) Microscopic section of rat heart treated with EETP (400mg/kg) showing the maximum protective effect by reduced histopathological changes showing apparently normal architecture. (e) Photomicrograph section of heart tissue treated with Vitamin E (100mg/kg) showing slightly changes of fibrous tissue.

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