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Study of Effect of Methanolic Extract of *Thevetia Peruviana* Leaves on Doxorubicin Induced Cardio Toxicity in Wistar Rats

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ABSTRACT

Doxorubicin is an anthracycline antibiotic widely used as a chemotherapeutic agent in the treatment of several tumors. However, its cardiac toxicity limits its use at maximum therapeutic doses. Most studies implicated increased oxidative stress as the major determinant of DOX cardiotoxicity. *Thevetia peruviana* known to have antioxidant activity. The aim of the current study was to explore the potential protective effects of methanolic extract of *Thevetia peruviana* against DOX-induced cardiotoxicity in rats. Methanolic extract of this plant showed significant cardioprotective effect by lowering the serum levels of various biochemical parameters like Creatinine phosphokinase (CPK), Lactate dehydrogenase (LDH), Alanine transaminase (ALT) and Aspartate transaminase (AST) in the selected model. Additionally, histopathological examination indicated a protection against DOX-induced cardiotoxicity. The results also suggests that the biologically active phytoconstituents such as flavonoids, alkaloids, glycosides, carbohydrates, triterpenoids and tannins present in the methanolic extract of plant which is confirmed from the qualitative analysis may be responsible for the significant cardioprotective activity.

Keywords: Thevetia peruviana, Cardioprotective activity, Doxorubicin, Cardiotoxicity.

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INTRODUCTION

Heart failure (HF) is a common cardiovascular condition with increasing incidence and prevalence. Several large clinical trials on use of pharmacological therapy and devices has resulted in an increasing use of evidence based therapy of heart failure. Despite these advances the morbidity and mortality of those afflicted with heart failure continues to remain high. Adherence to guidelines results in improved outcomes of heart failure patients. Education of caretakers on evidence based therapy is the cornerstone of a successful heart failure programme. Unlike western countries where heart failure is predominantly a disease of elderly, in India it affects younger age group. The important risk factors for heart failure include coronary artery disease, hypertension, diabetes mellitus, cardiotoxic drugs, valvular heart disease and obesity. In India coronary artery disease, diabetes, hypertension, valvular heart diseases and primary muscle diseases are the leading causes for heart failure. Rheumatic heart disease is still a common cause of heart failure in Indians.

Doxorubicin (DOX) is one of the most effective antitumor antibiotics belonging to the class of anthracyclines, but its use is limited by high incidence cardiotoxicity (Hortobágyi, 1997). With the increasing use of DOX, an acute cardiotoxicity has been recognized as a severe complication of DOX chemotherapy (Doroshow, 1991). Although numerous mechanisms have been proposed, most studies supported that increased oxidative stress, along with a reduction in the levels of antioxidants, plays a key role in the pathogenesis of DOX-induced cardiomyopathy (Yen et al., 1996). Therefore, the use of natural or synthetic antioxidants might protect from oxidative stress caused by DOX and other cytotoxic drugs (Bristow et al., 1981). Diets rich in fruits and vegetables have been associated with decreased risks of several chronic diseases, such as coronary heart disease (Hertog et al., 1993). These protective effects have been attributed partly to the various antioxidant compounds, e.g. vitamins C and E, β-carotene, and polyphenolics (Diplock et al., 1998). Several compounds with antioxidant activities are known to against DOX-induced toxicities. Lycopene, a carotenoid occurring in tomatoes (Yilmaz et al., 2006), and gingerols in Zingiber officinale (Ajith et al., 2008) were found to protect against DOX-induced nephrotoxicity. The antioxidant properties of flavonoids were shown to reduce DOX toxicity due to their ability to scavenge free radicals (Vaclavikova et al., 2008).

The local flora Thevetia peruviana are known to have antioxidant activity. In continuing our interest in the evaluation of the biological activities of, the present study was designed to screen the methanolic extracts of the Thevetia peruviana plant for a potential protective effect against DOX-induced cardiotoxicity in rats.

MATERIALS AND METHOD

Preparation of extract:

The coarse powdered material was subjected to sequential soxhlet extraction. The solvent used was Methanol. The dried powder was defatted by macerating the powder for 7 days in petroleum ether with occasional stirring. Then the marc was subjected to soxhlet extraction with Methanol respectively. Finally, the resultant marc was subjected to aqueous extraction. The collected extracts were then concentrated using rotary vacuum evaporator and were air dried at room temperature, weighed and percentage yield was calculated.

Animals:

Albino Wistar rats of 125-150g of either sex were used for the study. The animals were maintained under controlled conditions of temperature (23 ± 2ºC) before the study. The animals were randomized into experimental, normal and control groups, housed individually in sanitized polypropylene cages containing sterile paddy husk as bedding. They had free access to standard pellets as basal diet and water ad libitum. Animals were habituated to laboratory conditions for 48 hours prior to experimental protocol to minimize if any of non-specific stress. All the studies conducted were approved by the revised OECD guidelines 423 and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Chemicals:

Normal saline (0.9%), Doxorubicin Hydrochloride (Cipla pvt ltd), Petroleum Ether (40- 60), Methanol (Loba cheme), Formaldehyde (Rankem), Serum ALT diagnostic kit, Serum AST diagnostic kit, Serum Creatinine diagnostic kit, Serum LDH diagnostic kit, all the chemicals used was of analytical grade.

Experimental Procedure:

The animals were divided into eight groups. Each groups had 6 rats.

1. Group I: Control rats received 1% Na CMC 2.5ml/kg/day for a period of 30 days.
2. Group II: These rats received doxorubicin (2.5 mg/kg body weight, i.p, in six divided doses alternatively for two weeks to a total cumulative dose of 15mg/kg).
3. Group III: Rats received doxorubicin (2.5 mg/kg body weight, i.p) in six divided doses for first 15 days, followed by oral treatment with 200mg/kg METP for next 15 days.
4. Group IV: Rats received doxorubicin (2.5 mg/kg body weight, i.p) in six divided doses for first 15 days, followed by oral treatment with 400mg/kg METP for next 15 days.

Determination of biological parameters:

At the end of treatment period, animals were fasted overnight for a period of 12 hr. Blood (0.5 ml) was withdrawn via retro-orbital plexes under mild ether anesthesia and was collected into micro tubes. The micro tubes were centrifuged at 4000 rpm at 4°C for 20 min to obtain clear serum. The serum was then analyzed for CPK, LDH, SGPT and SGOT in the semi auto analyzer using commercially available biochemical kits.

Later the animals were sacrificed under ether anesthesia and a midline abdominal incision was performed and the heart tissue was quickly dissected out and washed in ice cold saline.

1. A portion of each heart was taken from all the groups and a 30% w/v homogenate was prepared in 0.9% buffered KCl (pH 7.4) for the estimation of biochemical parameters (CAT and MDA).
2. The remaining portion of the heart tissue was used for histopathological studies.

Histopathological studies:

The heart 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 stained according to the haematoxylin and eosin method and were examined by light microscopy.

Statistical analysis:

The results were expressed as mean S.E.M. The results were analyzed using ANOVA followed by Dunnett's multiple comparison test. Data was computed for statistical analysis by using Graph Pad Prism 5 Software.

RESULTS AND DISCUSSION

Preliminary phytochemical screening of extract:

The results of different phytochemical tests of the methanolic extract showed that components like flavonoids, alkaloids, glycosides, carbohydrates, triterpenoids and tannins were present while components like gums, mucilage, sterols and steroids were absent. The detail results are represented in **table-1**

Table-1: Preliminary phytochemical screening of Methanolic extract of *Thevetia peruviana*

S. No	Constituents	Status
1	Alkaloids	+
2	Carbohydrates	+
3	Cardiac glycosides	+
4	Anthraquinone glycosides	+
5	Gums & mucilage	-
6	Proteins & aminoacids	+
8	Steroids & sterols	-

9	Triterpenoids	+
10	Saponins	+
11	Flavonoids	+

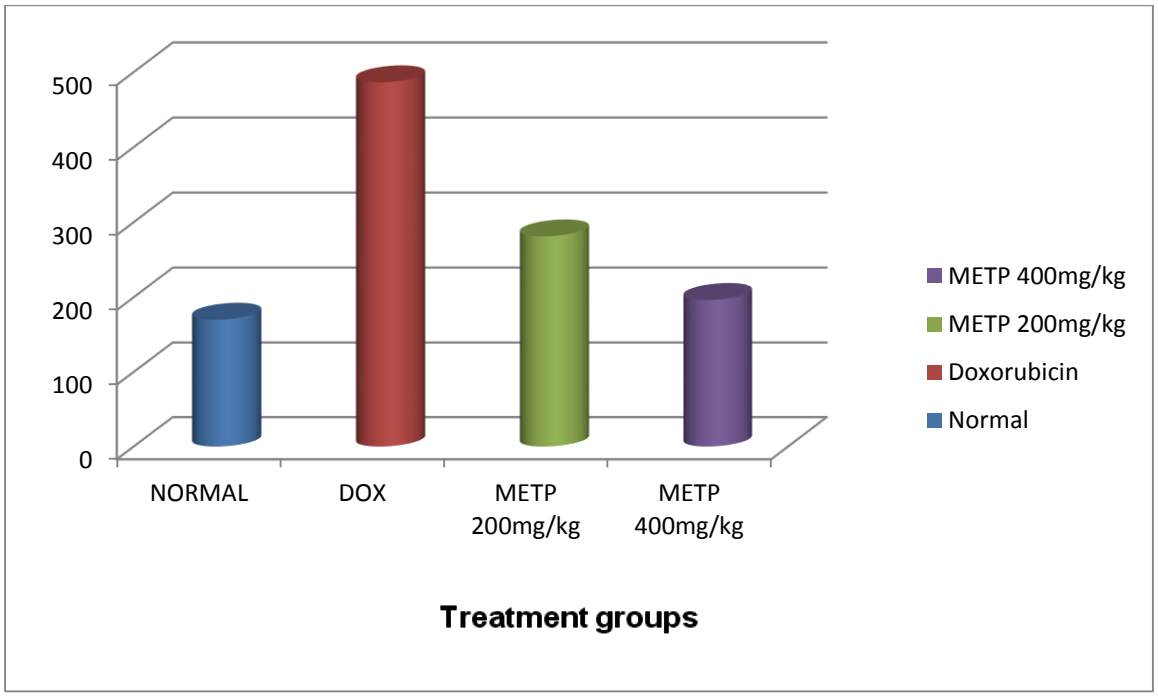
Serum and homogenate markers- CPK, LDH, ALT and AST:

Treatment with doxorubicin causes an elevation in level of these enzymes which are considered as the biomarkers of myocardial damage when compared with the normal. Our study showed decrease in the elevated levels of these enzymes. Post(curative) treatment with METP 200 and 400mg/kg showed a dose dependent significant decrease in the elevated enzymes when compared with the post treatment. **Tables: 2-7** and **Graph 1-6**

Table – 2: Creatinine Phosphokinase (CPK) (IU/L) levels in different groups of rats

Treatment Groups	ANIMALS						MEAN±SEM
	1	2	3	4	5	6	
I (NORMAL)	164	199	186	170	147	156	170.2±7.868
II (DOX)	498	591	412	435	473	510	486.5±25.812
III(DOX+METP 200mg/kg)	290	269	291	275	284	272	281.2±3.831**
IV(DOX+METP 400mg/kg)	210	201	211	189	186	192	196.5±6.39***

All values are expressed as mean ±SEM for each group (n= 6/group). Significance was determined by One-Way ANOVA followed by Dunnett comparison test; ***P*<0.001 Vs doxorubicin control;****P*<0.0001 Vs doxorubicin control.

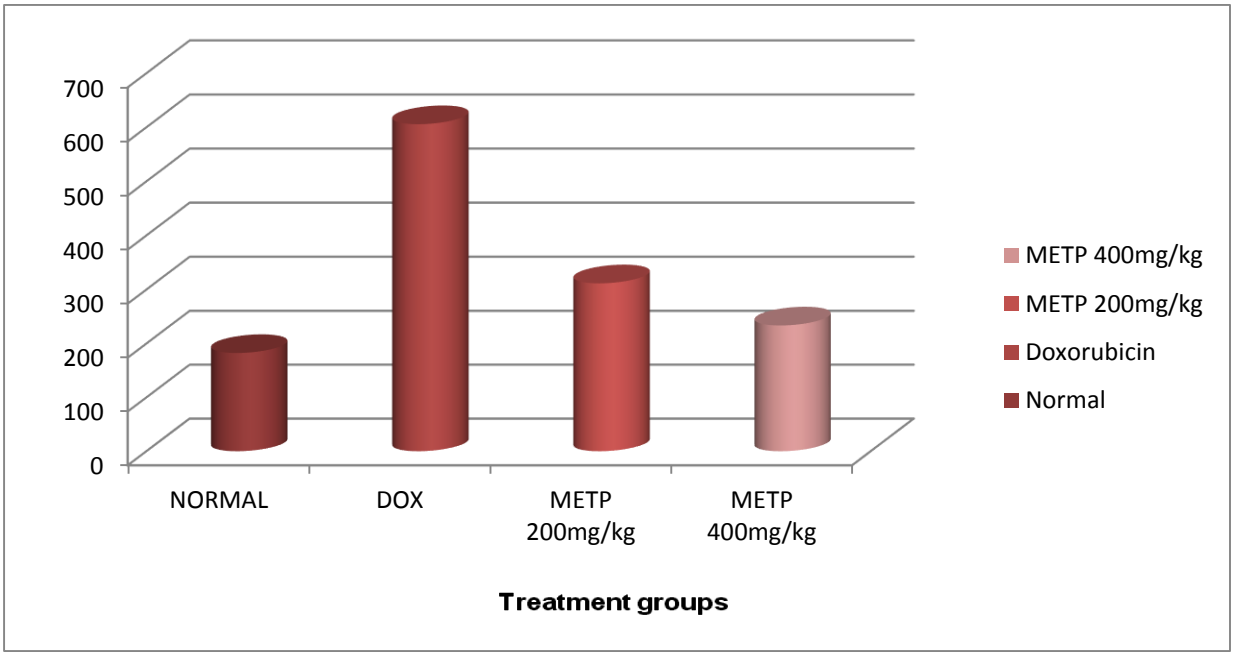


Graph 1: Effect of *METP* on CPK levels in different groups of rats

Table 3: Effect of METP on Lactate dehydrogenase (LDH) (IU/L) levels in different groups of rats

Treatment Groups	Animals						Mean±SEM
	1	2	3	4	5	6	
I (NORMAL)	198	163	219	159	176	182	182.5±9.25
II (DOX)	610	621	685	530	569	620	605.8±21.46
III(DOX+METP 200mg/kg)	301	312	310	290	321	333	311.5±6.12**
IV(DOX+METP 400mg/kg)	219	275	233	211	229	232	233.6±9.056***

All Values are expressed in mean ± SEM of six rats in each group. Significance was determined by One-Way ANOVA followed by Dunnett comparison test; ***P*<0.001 Vs doxorubicin control;****P*<0.0001 Vs doxorubicin control.

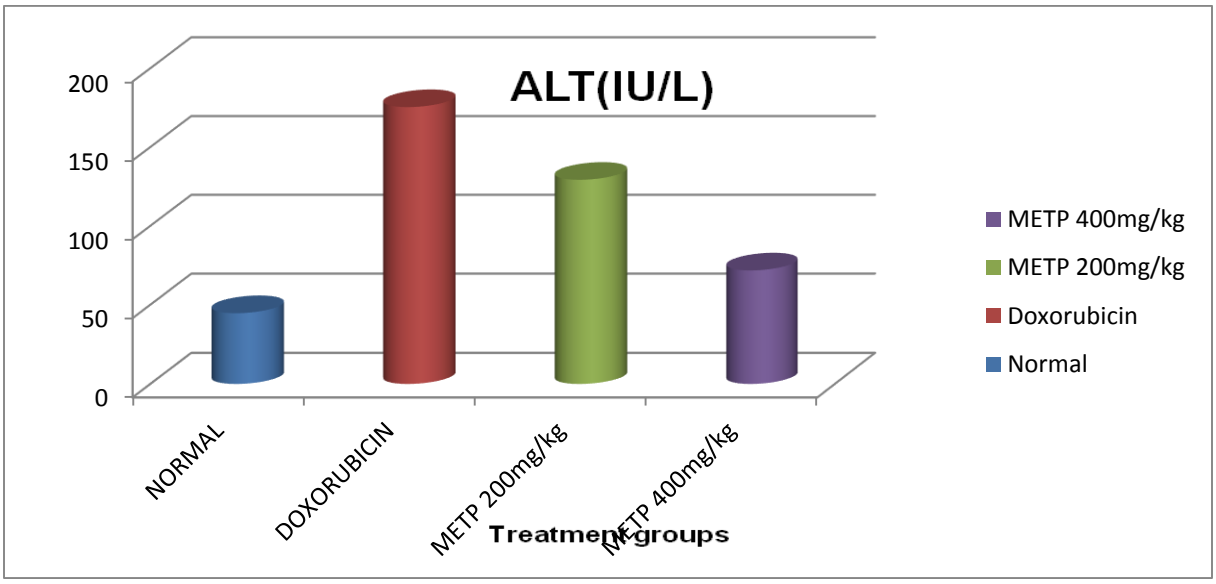


Graph 2: Effect of *METP* on LDH levels in different groups of rats

Table 4: Effect of METP on Alanine Transaminase (ALT)(IU/L) levels in different groups of rats

Treatment groups	ANIMALS						MEAN±SEM
	1	2	3	4	5	6	
I (NORMAL)	46	41	43	37	49	52	44.66±2.23
II (DOX)	186	180	171	162	169	185	175.33±3.98
III(DOX+METP 200mg/kg)	137	122	136	131	123	129	129.2±3.16 **
IV(DOX+METP 400mg/kg)	84	71	69	63	74	69	71.7±2.88***

All Values are expressed in mean ± SEM of six rats in each group. Significance was determined by One-Way ANOVA followed by Dunnett comparison test; ***P*<0.001 Vs doxorubicin control;****P*<0.0001 Vs doxorubicin control.

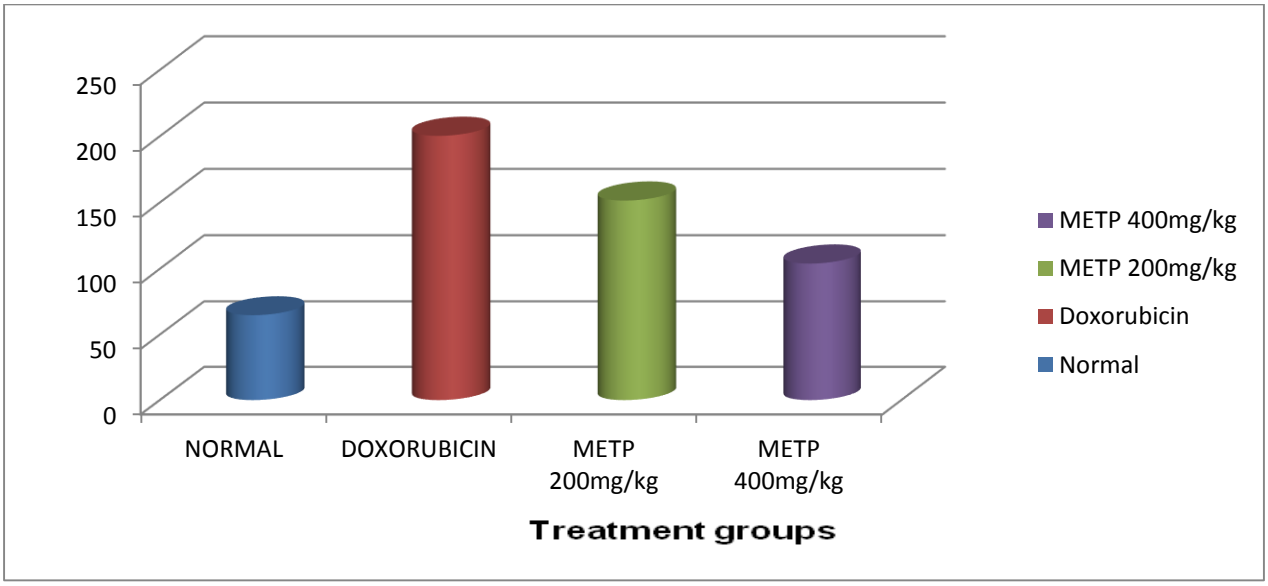


Graph 3: Effect of METP on ALT (SGPT) levels in different groups of rats

Table 5: Effect of METP on Aspartate Transaminase (AST) (IU/L) in different groups of rats

Treatment Groups	ANIMALS						MEAN±SEM
	1	2	3	4	5	6	
I (NORMAL)	81	52	69	45	79	63	64.83±5.89
II (DOX)	200	203	198	189	216	196	200.33±3.67
III(DOX+METP 200mg/kg)	150	159	139	141	159	161	151.5±9.71**
IV(DOX+METP 400mg/kg)	106	109	98	110	103	98	104.0±2.14***

All Values are expressed in mean ± SEM of six rats in each group. Significance was determined by One-Way ANOVA followed by Dunnett comparison test; ***P*<0.001 Vs doxorubicin control;****P*<0.0001 Vs doxorubicin control.

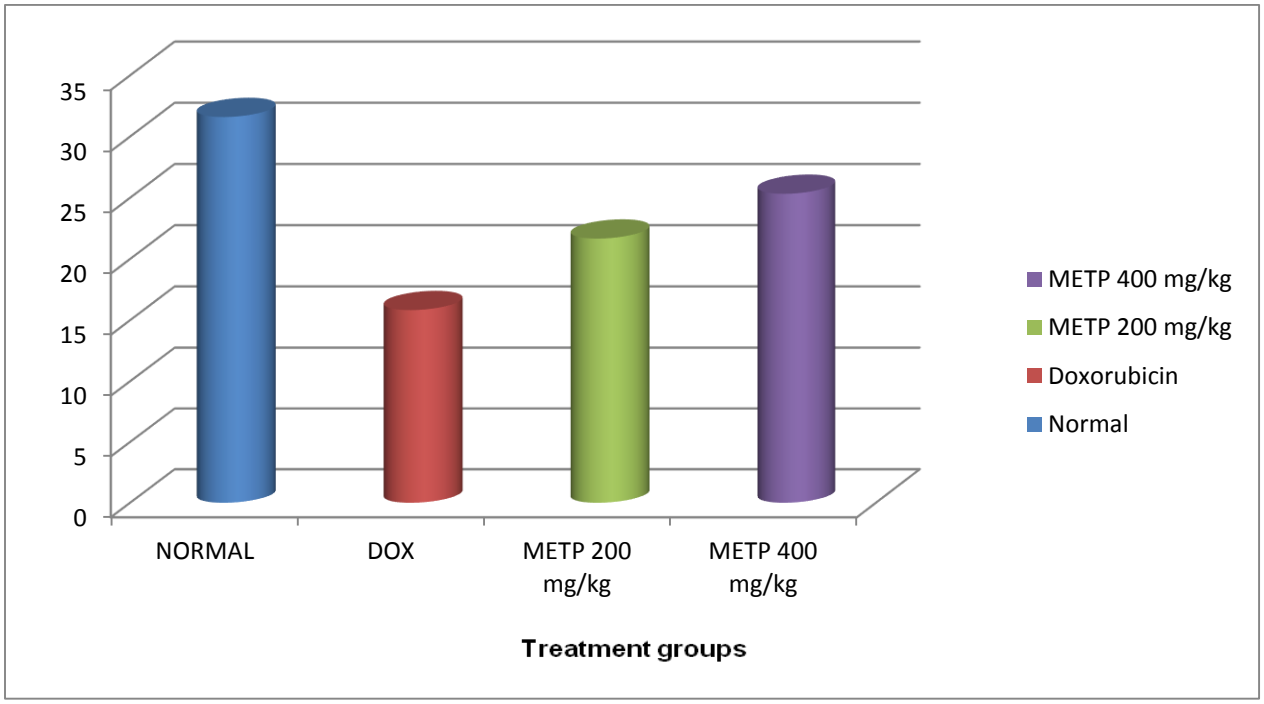


Graph 4: Effect of *METP* on AST levels in different groups of rats

Table 6: Catalase (CAT) (units/mg protein) levels in different groups of rats

Treatment Groups	ANIMALS						MEAN±SEM
	1	2	3	4	5	6	
I (NORMAL)	31	33	35	32	30	29	31.66±0.881
II (DOX)	13	16	17	19	14	16	15.83±0.87
III(DOX+METP 200mg/kg)	20	21	23	21	22	23	21.67±0.78 **
IV(DOX+METP 400mg/kg)	24	26	27	24	25	26	25.33±0.89 ***

All Values are expressed in mean ± SEM of six rats in each group. Significance was determined by One-Way ANOVA followed by Dunnett comparison test; ***P*<0.001 Vs doxorubicin control;****P*<0.0001 Vs doxorubicin control.

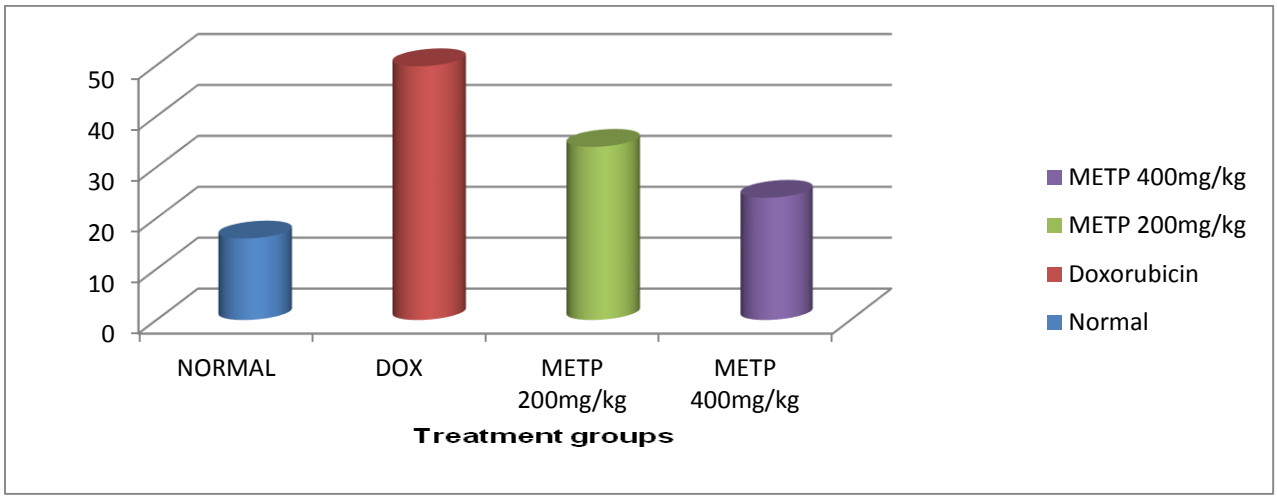


Graph 5: Effect of METP on Catalase levels in different groups of rats.

Table 7: Malondialdehyde (MDA) (nmol/g tissue) levels in different groups of rats

Treatment groups	Animals						Mean± SEM
	1	2	3	4	5	6	
I (NORMAL)	14	13	19	15	18	17	16±0.956
II (DOX)	50	48	53	51	51	46	49.83±1.013
III(DOX+METP 200mg/kg)	34	37	33	30	34	36	34.0±1.00 **
IV(DOX+METP 400mg/kg)	24	26	24	21	27	22	24.0±0.93 ***

All Values are expressed in mean ± SEM of SIX rats in each group. Significance was determined by One-Way ANOVA followed by Dunnett comparison test; ***P*<0.001 Vs doxorubicin control;****P*<0.0001 Vs doxorubicin control.



Graph 6: Effect of METP on MDA levels in different groups of rats

Histopathological studies:

The sections of heart of normal rats (vehicle treated rats) showed normal morphological appearance **(Fig – 1).** The cardiac muscle fibers were found to be of uniform size, shape ,without any inflammation. The sections of heart obtained from the doxorubicin treated animals showed severe congestion of blood vessels. Degenerative changes and areas of necrosis in cardiac muscle fibers were observed along with moderate infiltration of mononuclear cells **(Fig – 2).** In the rats, which were subjected to post-treatment with low dose of METP (200mg/kg), the heart sections showed congestion of blood vessels with few patchy areas of myocardial degeneration and mononuclear infiltration **(Fig-3).** In the rats, which were subjected to post treatment with high dose of METP (400mg/kg), the heart sections showed almost normal architecture. with few areas of congestion **(Fig-4).** All the sections were shown below from **(Fig 1-4)**

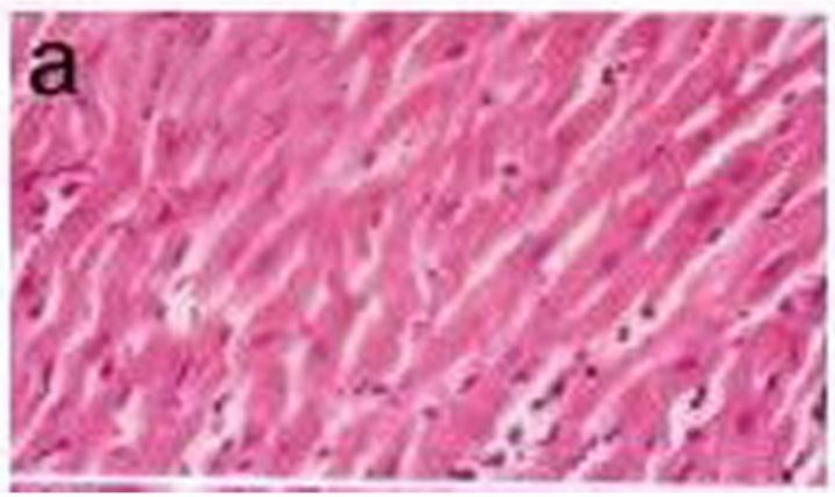


Figure-(1) Group I (Normal) rat heart, section showing Normal morphological appearance. H&E 400X

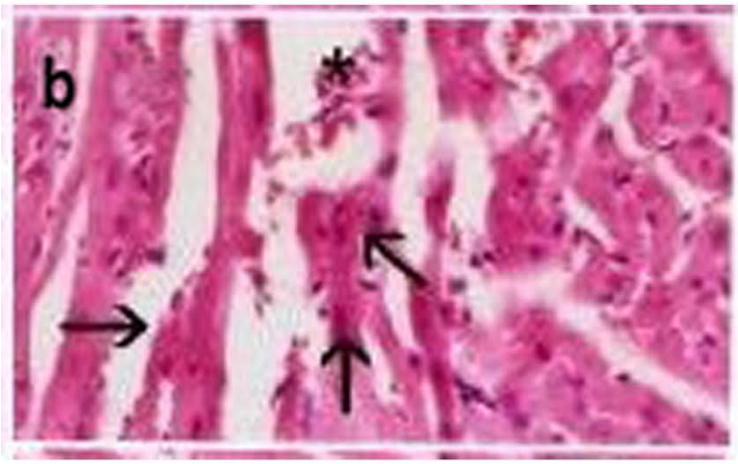


Figure-(2) Group II (DOX treated) rat heart section showing severe congestion of blood vessels. Degenerative changes and areas of necrosis of Cardiac muscle fibers were observed. H&E 400X

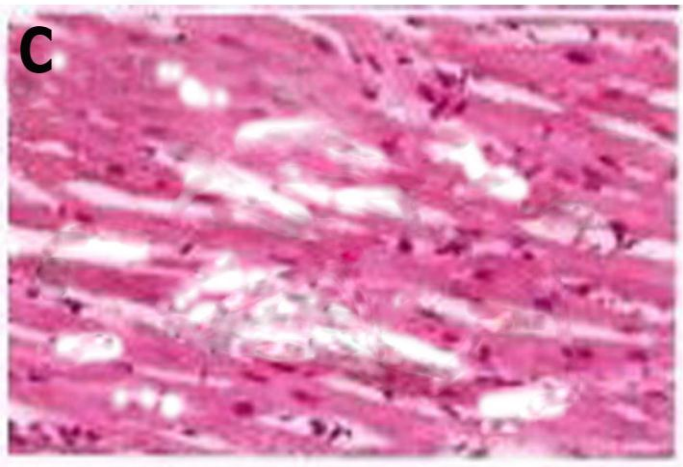


Figure-(3) Group III (DOX +METP 200mg/kg) Section showing mild congestion of blood vessels Section showing mild congestion of blood vessels Only few patchy areas of myocardial degeneration With mononuclear infiltration were observed H&E 400X

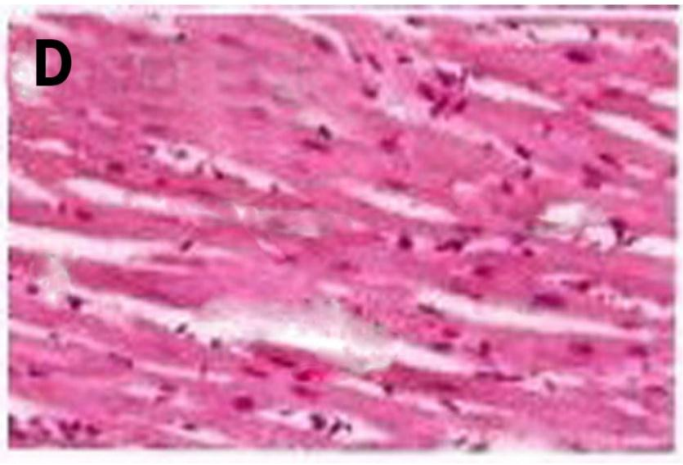


Figure-(4) Group IV (DOX + METP 400mg/kg) rat heart section showing near normal architecture with few areas of congestion were observed H&E:400X

DISCUSSION

Alterations in serological and homogenate parameters:

Rats treated with the doxorubicin show the increased levels of CPK, LDH, ALT and AST in the serum and homogenate preparation. Doxorubicin treatment had also elevated the levels of MDA in the homogenate, but the levels of catalase are decreased.

Treatment of doxorubicin exposed rats with METP show the decline in the increased levels of biomarkers and biochemical parameters (only MDA).

The catalase levels are increased after the treatment with METP.

Histopathological changes:

The doxorubicin treated animals showed ventricular dilation, inflammation, cardiac hypertrophy and overall enlargement.

There is loss of myofibrils and formation of vacuoles in the heart tissue.

These changes are lowered in case of low and high doses of METP.

CONCLUSION

Cardiotoxicity induced by doxirubicin is due to the oxidative stress, which can be observed by the elevated levels of biomarkers (CPK, LDH, AKT, AST) and biochemical parameter (MDA); whereas the other biochemical parameter, catalase (CAT) levels were decreased. The toxicity can also be confirmed through the histopathological changes, which can be treated with the methanolic extract of *Thevetia peruviana* leaves. However the methanolic extract of *Thevetia peruviana* leaves shows significant protective effect.

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