

Original Research Article

Cardio protective Effect of *Boerhaavia diffusa* against Doxorubicin-induced myocardial toxicity in Albino Rats

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Abstract: Doxorubicin is Anrthacycline derivative and most active cytotoxic agent in current use. It is been using to treat various malignancies but the clinical usefulness has been limited due to its specific cardiac toxicity. The main objective is to study the preventive role of ethanolic extract of whole plant of *Boerhaavia diffusa* (EEBD) against doxorubicin (Dox) induced myocardial toxicity in rats. Cumulative administration of Dox (2.5 mg/kg for two weeks) was injected to produce cardio toxicity. EEBD (100 and 200 mg/kg, po) was administered as pretreatment for two weeks followed by Dox on alternative days for two weeks. The general observations, mortality, histopathology, biomarker enzymes like lactate dehydrogenase (LDH), Creatine kinase (CK-MB) and cardiac troponin-I (cTnI), biochemical parameters such as aspartate aminotransferase (AST) alanine aminotransferase (ALT) and alkaline phosphatase (ALP), antioxidant enzymes such as glutathione (GSH), superoxide dismutase (SOD) catalase (CAT) and lipid peroxidation were monitored after three weeks of last dose of Dox. Histopathologic studies of heart were also carried out to evaluate myocardial toxicity. In results the repeated administration of Dox produces cardiomyopathy, which was characterized by increased level of cardiac biomarkers and deficit antioxidant enzymes. Pretreatment with the EEBD (200mg/kg) significantly protected myocardium from the toxic effects of Dox by reducing the elevated level of biomarker enzymes like LDH, CK-MB and absence of cTnI and biochemical parameters such as AST, ALT and ALP reduced to normal. EEBD increased the reduced level of GSH, SOD and CAT while decreased the elevated level of malondialdehyde (MDA) in cardiac tissue. In conclusion the biochemical and histopathological data evidently substantiate the cardio protective effect of EEBD, which could be attributed to its antioxidant property.

Keywords: Antioxidant, cardio toxicity, EEBD, doxorubicin.

INTRODUCTION

Doxorubicin (Anrthacycline drug) is a clinically well-established anti-cancer drug, which is widely used for the treatment of various human neoplastic diseases as well as wide range of solid tumours including breast cancer, acute leukemia's, Hodgkin and non-Hodgkin lymphoma, lung thyroid and ovarian cancer [1]. But the clinical use of drug is limited by an unusual and often irreversible cardiomyopathy, the occurrence of which is related to the total dose of the drug [2]. Proposed mechanisms of doxorubicin for its anti-malignancy includes intercalation into DNA, DNA cross linking, induction of apoptosis by inhibition of topoisomerase –II, interference with DNA unwinding and direct membrane damage [3,4]. Although the mechanism underlying the severe cytotoxicity of doxorubicin and other anthracycline are not fully understood, there is evidence that toxicity may ensue through drug free radical formation and subsequent redox cycle with O₂ resulting in the generation of reactive oxygen species such as superoxide anion, hydroxyl radicals and hydrogen peroxide. Tissues with less developed activities of anti-

oxidant enzymes have been reported in doxorubicin induced cardio toxicity in rats [5]. The anti-oxidant defenses of heart are particularly susceptible to injury by doxorubicin induced oxygen radicals.

Boerhaavia diffusa Linn belongs to family Nyctaginaceae has been traditionally used in culinary practices and distributed all over India. Traditionally in Ayurveda it has been used for the cardiac disorders [6, 7]. It has been reported to have analgesic and anti-inflammatory [8], Anti stress [9], antioxidant [10], Hepatoprotective [11], anticonvulsant [12] and diuretic [13]. It has been reported in scientific papers that flavonoids [14] and triterpenoids [15] are responsible for antioxidant activity. So considering the phytochemicals present in *Boerhaavia diffusa*, and in order to give a scientific background for the above traditional claim, this work has been taken.

MATERIALS AND METHODS

Plant material:

The *Boerhaavia diffusa* whole plant was authenticated and procured from Department of Botany,

Sri Venkateshwar University, and Tirupati, India. The whole plant was washed thoroughly with water, rinsed with distilled water to remove soil and foreign material if any. The plant was shed dried and subjected to size reduction to obtain uniform coarse powder of 40 mesh size. The powder of the whole plant was subjected to organoleptic evaluation like colour, taste and odour.

Preparation of the ethanolic extract:

The extract was prepared by hot extraction method. Accurately weighed 25g of powder and 250ml of ethanol in iodine flask, allowed to stand for 1h with occasional shaking and after 1h attached a reflux condenser, boiled for 1h, after 1h cooled and concentrated using rotary flash evaporator.

Chemicals and drugs:

Doxorubicin was obtained as sample gift from Get Well Pharmaceuticals, India. Other analytical grade chemicals were procured from Sigma and enzyme assay kits were purchased from ERBA.

Animals:

Healthy albino wistar rats weighing between 150-200g were used for the study after securing the ethical clearance from Institutional Animal Ethical Committee (Ref. No. KLEU's-08-IAEC.HBL-31 /Aug2013). All the animals were individually housed in polyethylene cages, maintained under standard conditions, fed with standard rat pellet diet and water ad-libitum. Animals were acclimatized for one week to laboratory conditions before starting the experiment.

Preliminary phytochemical screening of flower petals of EEED:

The ethanolic extract was investigated for the presence of various phytoconstituents like carbohydrates, proteins, amino acids, steroids, triterpenoids, glycosides, saponins, flavonoids, alkaloids, tannins and phenolic compounds.

Acute Oral Toxicity:

Acute oral toxicity studies were carried out as per OECD guidelines set by Organization for Economic Co-operation and Development (OECD, revised draft guidelines 423) received from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Govt. of India. Acute toxicity studies were performed on female albino mice weighing between 18-25gm using the up and down method. EEED was orally administered at a dose of 2g/kg body weight. After administration food was withheld for up to 4 h, animals were observed for changes in weight, general behavioral, tremor, convulsion, salivation, sleep, skin, eye and death at 30 m, 1, 2, 3, 4, 24 h and once daily for 14 days.

Experimental design:

After one week of acclimatization, the animals were randomly divided into 6 groups of 6 animals in each as follows.

Group I served as normal control, received vehicle 5ml/kg body weight.

Group II animals were treated with doxorubicin 2.5mg/kg body weight i.p. in 6 equal injections alternative day for two weeks.

Group III animals received ethanolic extract of Boerhaavia diffusa (EEED-200mg/kg body weight po.) for two weeks and then alternatively with vehicle for next two weeks.

Group IV and Group V animals received low dose (100mg/kg body weight po) of ethanolic extract of Boerhaavia diffusa (LEED) and high dose (200mg/kg body weight po) of ethanolic extract of Boerhaavia diffusa (HEED) respectively for two weeks as a pretreatment followed by doxorubicin as in group II.

Group VI served as standard (Std) and received Vitamin E 100mg/kg body weight p.o for two weeks as a pretreatment followed by doxorubicin as in group II.

ECG, Food and water:

ECG was recorded (Biopac MP35) before and after the treatment, also food consumption and water intake were regularly measured for all the animals.

Enzyme assays:

Thirty six hour after the last treatment, blood was withdrawn by retro-orbital plexus under light ether anesthesia using heparinized micro capillaries for the estimation of various biomarkers like lactate dehydrogenase (LDH) [16], creatinine phosphokinase (CPK) [17] and troponin-I (cTnI) [18]. All the animals were observed for next three weeks for the general appearance, behavior and mortality. After the three weeks, again blood was withdrawn by retro-orbital plexus under light ether anesthesia using heparinized micro capillaries for the estimation of aspartate transaminase (AST) [19], alanine transaminase (ALT)[19] and alkaline phosphatase (ALP)[20]. Animals were sacrificed under ether anesthesia and a midline incision was performed and heart tissue was quickly dissected out, washed in ice cold saline, dried by filter paper and weighed immediately. The entire animal's heart portion was taken and 10% w/v homogenate was prepared in 0.9% buffered KCl (pH 7.4) for the estimation of endogenous antioxidants such as, glutathione (GSH) [21], malonaldehyde (MDA) [22], superoxide dismutase (SOD) [23] and catalase (CAT) [24]. The remaining heart portion 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 hematoxylin and eosin method and were examined by microscopy.

Statistical analysis:

The experimental data were statistically analyzed using one-way analysis of variance (ANOVA) followed by Dunnet’s multiple comparison test using Graphpad Prism 5.0. Data were expressed as Mean±S.E.M. P<0.05 was considered as significant.

RESULTS

Phytochemical constituents in EEBD:

The preliminary phytochemical investigation revealed the presence of carbohydrates, steroids, triterpenoids, glycosides, saponins, flavonoids, alkaloids, tannins and phenolic compounds.

Acute oral toxicity:

By acute oral toxicity studies, we found that lethal dose of ethanolic extract of *Boerhaavia diffusa* was more than 2000mg/kg body weight. So 1/10th and 1/20th of the 2000mg/kg body weight was chosen for further studies.

General observations:

Doxorubicin treated animals developed a scruffy fur. These rats also had red exudates around the eyes and soft watery faeces. Necrosis was also observed at the site of doxorubicin injection. These conditions were more severe at the end of study period. But these changes were less in extract pretreated animals.

Fig. 1 depicts that, in doxorubicin treated group, food and water consumption was significantly reduced as compared to normal but only extract treated group showed no significant changes in food and water consumption as compared to normal. In treatment groups i.e. HEEBD (p<0.01) and Vit-E (p<0.01) but not LEEBD showed significantly improved food and water consumption as compared to doxorubicin treated group.

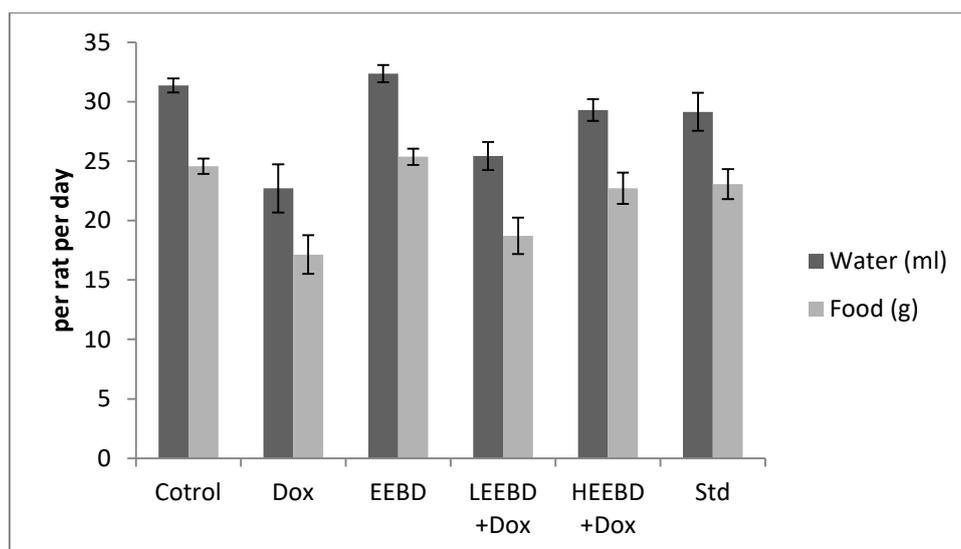


Fig 1: Effect of EEBD on water and food consumption

Body weight, heart weight and ratio of heart weight to body weight:

Table No. 1 represents the body weight was significantly decreased in doxorubicin treated group compared to normal. The only extract treated group shown no significant changes in the body weight as compared to normal. The treatment groups showed significantly increased body weight HEERC (p<0.01) and Std (p<0.001) compared to doxorubicin treated

group. The heart weight and ratio of heart weight to body weight in doxorubicin treated rats were significantly increased as compared to normal group rats, whereas only extract treated group showed similar ratio as that of normal group. The pretreatment groups HEEEC (p<0.01) and Std (p<0.01) but not LEEBD showed significantly decreased ratio of heart weight to body weight as compared to doxorubicin treated group.

Table-1: Effect of EEED on heart to body weight ratio

Group	Body Weight (g)	Heart Weight (g)	Heart / Body weight Ratio(10 ⁻³)
Control	195.8±1.79	0.8443±0.01	4.306±0.78
Dox	155.3±2.01	0.7983±0.01	5.142±0.88 ^{###}
EERC	193.2±2.89	0.8350±0.01	4.323±0.45 ^{***}
LEEED+Dox	163.7±2.66	0.8133±0.02	4.975±0.01 ^{ns}
HEEED+Dox	171.0±3.39	0.7817±0.02	4.576±0.12 ^{**}
Std	185.3±1.40	0.8383±0.02	4.521±0.12 ^{**}

Values are Mean±S.E.M; n=6 in each group, ^{###}p<0.001 when compared to normal, ns=not significant, ^{***}p<0.001, ^{**}p<0.01, ^{*}p<0.05 when compared to Dox.

Dox- Doxorubicin, EEED- Ethanolic extract of Boerhaavia diffusa, LEEED- Low dose (100mg/kg) of ethanolic extract of Boerhaavia diffusa, HEEED- High dose (200mg/kg) of ethanolic extract of Boerhaavia diffusa, Std- Standard treated with Vitamin-E (100mg/kg).

Cardiac markers:

Table No. 2 depicts the doxorubicin treated animals produced significant increase in LDH (p<0.001) and CK-MB (p<0.001) and also the presence of Troponin I (Table No.3), as compared to normal group, whereas

pretreatment groups i.e. HEEED and Std but not LEEED significantly decreased the levels of LDH (p<0.01 and p<0.001) and CK-MB (p<0.05 and p<0.01) respectively and also the absence of Troponin I (Table No.3) as compared with doxorubicin treated group.

Table-2: Effect of EEED on biochemical parameters

Group	LDH (IU/L)	CK-MB (IU/L)	ALP (IU/L)	AST (IU/L)	ALT (IU/L)
Control	123.3±13.73	27.60±4.484	44.15±3.873	64.85±14.63	38.00±10.48
Dox	275.0±27.47 ^{###}	124.4±18.73 ^{###}	183.3±19.65 ^{###}	204.6±41.39 ^{##}	99.83±12.22 ^{##}
EERC	133.9±14.64 ^{***}	30.73±6.358 ^{***}	59.32±10.19 ^{***}	66.17±13.47 ^{**}	40.33±7.740 ^{**}
LEEED+Dox	217.7±18.32 ^{ns}	80.98±14.49 ^{ns}	134.6±20.05 ^{ns}	178.8±19.63 ^{ns}	60.17±16.73 ^{ns}
HEEED+Dox	180.2±23.82 ^{**}	73.07±15.29 [*]	102.5±17.69 ^{**}	109.7±27.00 [*]	53.50±13.61 [*]
Std	131.5±10.06 ^{***}	51.17±13.39 ^{**}	79.65±11.90 ^{***}	79.13±19.97 ^{**}	53.17±10.29 [*]

Values are Mean±S.E.M; n=6 in each group, ^{###}p<0.001 when compared to normal, ns=not significant, ^{***}p<0.001, ^{**}p<0.01, ^{*}p<0.05 when compared to Dox.

Dox- Doxorubicin, EEED- Ethanolic extract of Boerhaavia diffusa, LEEED- Low dose (100mg/kg) of ethanolic extract of Boerhaavia diffusa, HEEED- High dose (200mg/kg) of ethanolic extract of Boerhaavia diffusa, Std- Standard treated with Vitamin-E (100mg/kg).

Table-3: Effect of EEED on cardiac troponin I

Group	Troponin I
Control	--
Dox	+++
EEED	--
LEEED+Dox	+
HEEED+Dox	--
Std	--

‘+’ Present and ‘-’ Absent

Dox- Doxorubicin, EEED- Ethanolic extract of Boerhaavia diffusa, LEEED- Low dose (100mg/kg) of ethanolic extract of Boerhaavia diffusa, HEEED- High dose (200mg/kg) of ethanolic extract of Boerhaavia diffusa, Std- Standard treated with Vitamin-E (100mg/kg).

Biochemical parameters (Serum markers):

Doxorubicin treated animals produced significant increase in serum enzyme markers such as ALP (p<0.001), AST (p<0.001) and ALT (p<0.001) as compared to normal group, whereas pretreatment groups i.e. HEEED and Std but not LEEED significantly decreased the levels ALP (p<0.01 and p<0.001), AST (p<0.05 and p<0.01) and ALT

(p<0.05 and p<0.05) respectively as compared with doxorubicin treated group (Table No. 2).

ECG parameters:

Table No. 4 represents that doxorubicin treated animals showed significantly increase in QT interval (p<0.001) and decrease in R wave amplitude (p<0.001) as compared to normal group, whereas pretreatment

groups i.e. HEERC and Std but not LEERC significantly decreased in QT interval ($p < 0.05$ and $p < 0.001$) and increased in R wave amplitude ($p < 0.01$

and $p < 0.001$) respectively as compared to doxorubicin treated group.

Table-4: Effect of EEBD on ECG parameters

Group	QT interval (sec)	R wave amplitude (mV)
Control	0.07667±0.0033	0.3883±0.0040
Dox	0.1050±0.0042 ^{###}	0.1767±0.0111 ^{###}
EEBD	0.07833±0.0030 ^{***}	0.3833±0.0084 ^{***}
LEEBD+Dox	0.09833±0.0030 ^{ns}	0.1917±0.0079 ^{ns}
HEEBD+Dox	0.0900±0.0036 [*]	0.2383±0.0107 ^{**}
Std	0.08167±0.0030 ^{***}	0.2967±0.0190 ^{***}

Values are Mean±S.E.M; n=6 in each group, ^{###} $p < 0.001$ when compared to normal, ns=not significant, ^{***} $p < 0.001$, ^{**} $p < 0.01$, ^{*} $p < 0.05$ when compared to Dox.

Dox- Doxorubicin, EEBD- Ethanolic extract of Boerhaavia diffusa, LEEBD- Low dose (100mg/kg) of ethanolic extract of Boerhaavia diffusa, HEEBD- High dose (200mg/kg) of ethanolic extract of Boerhaavia diffusa, Std- Standard treated with Vitamin-E (100mg/kg).

Antioxidants:

Table No.5 represents that doxorubicin treated animals showed significantly increased the MDA levels ($p < 0.001$) and decreased the levels of GSH ($p < 0.001$), SOD ($p < 0.001$) and CAT ($p < 0.001$) as compared to

normal group, but pretreatment groups i.e. HEERC and Std decreased the levels of MDA ($p < 0.01$ and $p < 0.001$) and increase in the levels of GSH ($p < 0.01$ and $p < 0.01$), SOD ($p < 0.01$ and $p < 0.001$) and CAT ($p < 0.05$ and $p < 0.01$) respectively as compared to doxorubicin group.

Table No. 5: Effect of EEBD on antioxidant enzymes

Group	MDA (n mole/mg of wet tissue)	GSH (n mole/mg of wet tissue)	SOD (Unit/mg protein)	CAT (Unit/mg protein)
Control	14.54±2.49	10.40±1.26	62.41±3.92	54.16±4.90
Dox	54.96±5.61 ^{###}	3.49±0.57 ^{###}	24.99±3.38 ^{###}	27.98±2.91 ^{###}
EEBD	16.56±1.49 ^{***}	10.56±1.03 ^{***}	61.06±3.56 ^{***}	53.17±3.99 ^{***}
LEEBD+Dox	43.48±4.91 ^{ns}	6.06±0.84 ^{ns}	41.06±4.66 ^{ns}	33.84±3.31 ^{ns}
HEEBD+Dox	35.85±4.80 ^{**}	8.66±1.15 ^{**}	49.28±4.91 ^{**}	43.95±4.97 [*]
Std	31.10±3.01 ^{***}	9.20±1.36 ^{**}	52.37±5.24 ^{***}	47.66±3.81 ^{**}

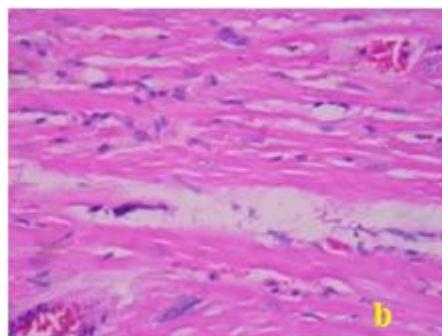
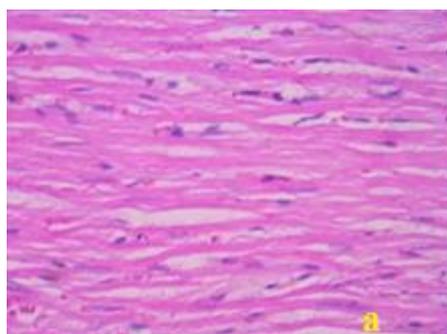
Values are Mean±S.E.M; n=6 in each group, ^{###} $p < 0.001$ when compared to normal, ns=not significant, ^{***} $p < 0.001$, ^{**} $p < 0.01$, ^{*} $p < 0.05$ when compared to Dox.

Dox- Doxorubicin, EEBD- Ethanolic extract of Boerhaavia diffusa, LEEBD- Low dose (100mg/kg) of ethanolic extract of Boerhaavia diffusa, HEEBD- High dose (200mg/kg) of ethanolic extract of Boerhaavia diffusa, Std- Standard treated with Vitamin-E (100mg/kg).

Histopathological studies:

There was myofibril loss, cytoplasm vacuolization, patchy necrosis and inflammatory cells in doxorubicin treated animal's heart tissue, whereas normal group

showed normal morphological appearances but pretreated groups showed less loss of myofibrils and vacuolization of the cytoplasm as compared to doxorubicin treated group (Fig. 2).



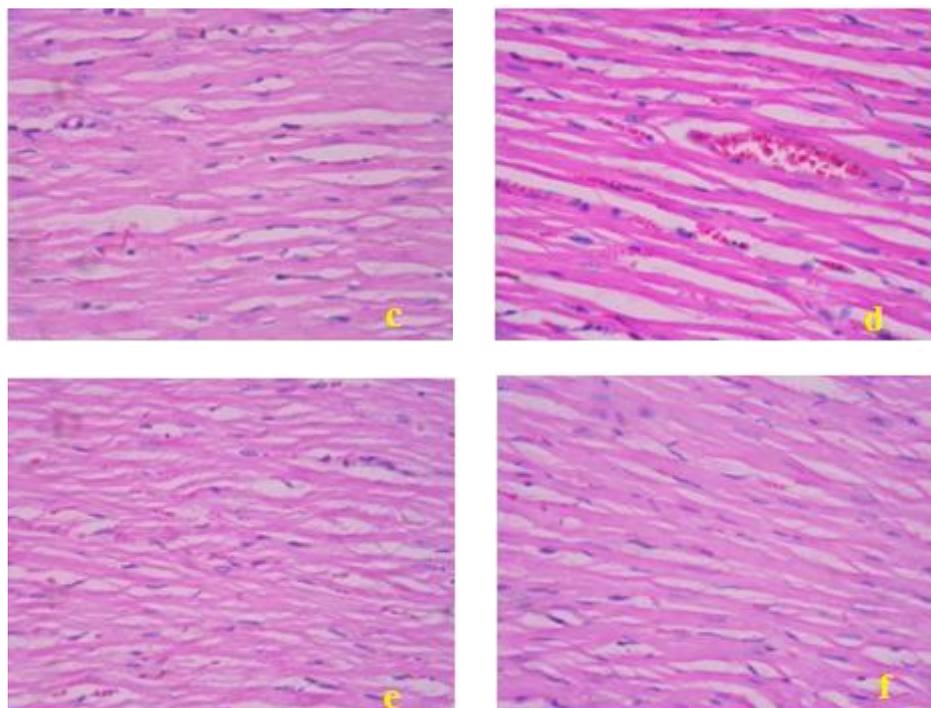


Fig 2:Histopathological studies by hematoxylin and eosin staining. (All the figures were captured under 40 x magnifications)

- (a) Photomicrograph of normal group heart showing normal myocardial fibers and architecture. (b) Doxorubicin treated group showing loss of myocardial fibers and vacuolated cells.
(c) Only EEBD treated group showing no myocardial fiber loss and vacuolated cells.
(d) LEEBD+Dox treated group have less loss of myocardial fibers and vacuolated cells.
(e) and (f) HEEBD+Dox and Std+Dox group respectively showing scanty myocardial fibers loss and vacuolated cells.

DISCUSSION

Doxorubicin induced myocardial damage was used as a model in this study and doxorubicin induced myocardial lesions have been well documented in patients as well as in experimental animals [25].

The present study investigated the influence of ethanolic extract of *Boerhaavia diffusa* against doxorubicin induced cardio toxicity. Following lines of evidence can be emphasized from the present study.

Rats treated with doxorubicin alone developed a pink tinge, fur become scruffy, alopecia, red exudates around the eyes and nose and also necrosis at the site of injection. But these changes were reduced in EEBD and vitamin-E pretreated groups, which accounts for the effective cell protecting property with anti-inflammatory, antioxidant and anti-fibrotic effect [26].

Water and food consumption were decreased in doxorubicin alone treated group, also increase in heart weight, which may be attribute to the enlarged, dilated and hyper tropic atrium and ventricles, swelling of mitochondria [25] and decrease in body weight due to reduced food intake. This was determined by ratio of heart weight to body weight. However all of the above changes were inhibited in pretreated groups.

Administration of doxorubicin alone at a cumulative dose of 15mg/kg body weight showed the biochemical changes and oxidative damage in the cardiac tissue. As a result of destruction of myocardial cells, CK-MB, LDH and cTnI were released into blood stream and these serve as the diagnostic markers of cardiac damage [27, 28]. The amount of these enzymes present in the blood reflects the alteration in plasma integrity and/or permeability.

Doxorubicin administered rats showed significant elevation in the levels of these diagnostic markers, which are an indicators of the severity of doxorubicin induced myocardial damage, which are in agreement with earlier reports [29, 30]. The pretreatment with EEBD showed significant reduction in doxorubicin induced elevated serum diagnostic markers. This evidently confirms that EEBD is responsible for maintenance of normal structural and architectural integrity of cardiac myocytes, thereby restricting the leakage of these enzymes, which can be accounted for its membrane stabilizing property.

For myocardial injury electrocardiograph (ECG) recordings are the main criteria generally considered for the diagnosis. In doxorubicin administered rats, the characteristic findings were prolongation of QT interval and decrease in R wave amplitude. These changes are signs of doxorubicin induced myocardial damage,

which is line with an earlier report [31]. Pretreated with EEED showed a protective effect (reduced abnormalities) in doxorubicin induced altered ECG patterns.

In present study, doxorubicin treated rats also showed an increase in MDA levels suggesting increased lipid peroxidation and decreased in levels of GSH, SOD and CAT. It is accepted that SOD protects against injury by converting O_2^- radical to H_2O_2 and prevent the formation of OH radicals through O_2^- driven Fenton reaction [32] and H_2O_2 can be removed by CAT. Pretreatment with EEED improved the antioxidant status and thereby preventing the damage to the heart, mainly because of its anti-oxidant property.

Doxorubicin administration produced changes in heart, mainly in the form of swollen mitochondria, vacuoles within cytoplasm, also vacuolar changes in cardiac muscle fibers like degeneration of myocardial tissue, vacuolization of the cardiomyocytes, myofibrillar loss, and myocardial hypertrophy and fragmentation of the nuclei. Histopathological reports suggested EEED attenuates the doxorubicin induced cardiac toxicity by fewer and less extensively swollen cardiac mitochondria and myofibrils loss.

CONCLUSION

We conclude that the cardio toxicity induced by doxorubicin is related mainly with oxidative stress and anti-oxidant properties of EEED may boost myocardial integrity and attenuate the cardiac toxicity. Our study revealed that EEED may be considered as a potentially useful in combination with doxorubicin to limit free radical mediated cardiac injury.

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