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Original Research Article

Effect of Ethanolic extract of *Boerhvia diffusa* against doxorubicin induced Nephrotoxicity in Albino Rats

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ABSTRACT

Anthracycline derivative i.e. doxorubicin (Dox) has proven efficacy in several malignancies such as breast cancer, Hodgkin and non-Hodgkin lymphoma, acute leukemia, lung, thyroid and ovarian cancer. The clinical usefulness is restricted due to its cardiotoxicity and nephrotoxicity. *Boerhaavia diffusa* belongs to family Nyctaginaceae and in Ayurveda, it is claimed for use in renal disorders. The main phytoconstituents of the plant are alkaloids, terpenoids, tannins, glycosides, flavonoids, phenolic compounds. To investigate the ameliorative role of ethanolic extract of petals of *B. diffusa* in doxorubicin-induced nephrotoxicity in rats. Nephrotoxicity was produced by administering doxorubicin (2.5 mg/kg b.w., *i.p.* alternate day) in six equal injections for two weeks to become cumulatively 15 mg/kg. Low (LEBD–100 mg/kg *p.o.*) and high (HEBD–200 mg/kg *p.o.*) dose of ethanolic extract of *Boerhhvia diffusa* was administered as a pretreatment before doxorubicin administration. The general parameters such as body weight, food, and water intake were measured throughout the study period. Serum markers such as blood urea nitrogen (BUN), serum creatinine and albumin were measured. Antioxidant enzymes such as glutathione (GSH), malondialdehyde (MDA), catalase (CAT), and superoxide dismutase (SOD) were monitored after the last dose. Histopathological studies were also carried out to evaluate nephrotoxicity. The repeated administration of doxorubicin produces several morphological changes, decreased body weight, food, and water consumption. Serum markers such as BUN and serum creatinine were increased and albumin levels decreased. The GSH, SOD, and CAT were decreased, whereas the MDA level was increased, and deteriorating changes in the histological architecture of kidney tissue were observed. The HEBD pretreated groups dose-dependently increased body weight and food and water intake ($p < 0.01$ and $p < 0.05$), whereas LEBD does not show any significant changes. The LEBD and HEBD pretreated groups decreased the BUN ($p < 0.05$ and $p < 0.01$) and serum creatinine ($p < 0.05$ and $p < 0.05$) and increased in the albumin ($p < 0.05$ and $p < 0.05$) levels, respectively. The pretreatment with LEBD and HEBD increased the level of the antioxidant enzyme i.e., GSH ($p < 0.05$ and $p < 0.01$), SOD ($p < 0.05$ and $p < 0.01$), CAT ($p < 0.05$ and $p < 0.01$) and decreased the MDA level ($p < 0.05$ and $p < 0.01$) respectively. Histopathological studies showed that the pretreatment with LEBD and HEBD groups minimized the tubular damage and reduced the inflammation as compared to doxorubicin-treated group. The biochemical and histopathological results data support the nephroprotective effect of ethanolic extract of *B. diffusa*, which might be credited to its antioxidant property.

Introduction

The anthracycline derivative Doxorubicin (Dox) was introduced in 1969 to treat various cancers like solid tumors such as breast cancer, hodgkin and non-hodgkin lymphoma, acute leukemias, lung, thyroid and ovarian cancer.[1] But, its use is restricted or reduced greatly due to multiple adverse effects such as the cardiac, kidney.[2,3] Therefore, we aimed to study the preventive role of plant extract (*B. diffusa*) in doxorubicin-induced nephrotoxicity.

In the kidney, Dox increases permeability in the glomerular capillary and produces glomerular atrophy.[4] Although the exact mechanism of nephrotoxicity induced by Dox has not been completely understood but there is support that they may ensue through free radical

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production and lipid peroxidation[5] and subsequent redox cycle with O_2 resulting in the formation of reactive oxygen species (ROS) like superoxide anion, OH radicals, and H_2O_2. Organs with less developed antioxidants have been reported in Dox-induced toxicity in rats.[6] Dox produces impairment in mitochondrial creatinekinase membrane binding, enzymatic activity, and assembling and also produces suppression of DNA, RNA, and protein synthesis.[7] Dox stimulates the formation of free radicals and ROS and nitrogen species by 2 pathways. First is the enzymatic pathway utilizing cellular oxidoreductases The second is the non-enzymatic pathway using complexation with iron ($Fe3+$).[8] Earlier studies reported that Dox-induced *in vivo* nephrotoxicity involves oxidative stress, and it causes an imbalance between free oxygen radicals and antioxidants.[9]

Although there are various strategies to prevent nephrotoxicity induced by Dox, herbal (natural) preparations are preferred due to their economic, effective, ease of availability, and safety.[9,10] WHO estimates that 80% of the world's developing countries' population has traditional medicines derived from plants.[11] Plants like *Solanum torvumare* and *Phoenix dactylifera* scientifically proved to be nephroprotective in Dox-induced nephrotoxicity through their antioxidant property[12] and suggested that agents that reduce free radical formation might reduce the nephrotoxcity induced by Dox.

B. diffusa Linn belongs to the family Nyctaginaceae and traditionally used in culinary practices and distributed all over India. Traditionally in Ayurveda, it has been used for various disorders including cardiac and renal.[13,14] It has been reported to have analgesic and anti-inflammatory,[15] Anti stress,[16] antioxidant,[17] Hepatoprotective,[18] anticonvulsant[19] and diuretic.[20]

Literature review of the plant reveals the presence of alkaloids, polyphenols, flavonoids, triterpenoids, lignans, steroids, rotenoids, carbohydrates, lipids, glycosides, proteins and glycoproteins as their phytoconstituents.[21] Earlier literature reported that phytoconstituents like flavonoids[22] and triterpinoids[23] present in other plants are responsible for antioxidant activity and are scientifically proven to have nephroprotective properties. The *B. diffusa* plant contains these types of antioxidant phytoconstituents and is not scientifically proven. Therefore present work was designed to evaluate the role of *B. diffusa* in Dox-induced nephrotoxicity in rats.

In current development, Herbal drugs constitute a major share of all India's officially recognized health systems. Majority of India's population has been using these non-allopathic systems of medicine, and there is an immense experiential evidence base for many of the natural drugs. These medicinal plants also provide a rich source of antioxidants known to prevent or delay different diseased states.[24] Antioxidant protection is observed at different levels. Therefore present plant study is helpful for the community after clinical investigation.

Materials and Methods

Plant Material

The authenticated plant *B. diffusa* was procured in April from the Department of Botany, Sri Venkateshwar University, Tirupati, India (Voucher Number). The plant was washed carefully, rinsed with distilled water to remove soil and foreign material, and shade dried. After complete drying, it was exposed to size reduction to obtain a uniform powder of 40 mesh size. The powder was subjected to organoleptic evaluation like color, odor and taste. The powder was stored for further use.

Ethanolic Extract Preparation

Accurately weighed 25 g of petal powder and 250 mL of ethanol added in iodine flask and allowed to stand for 1-hour with occasional shaking. A reflux condenser was attached and the contents were allowed to boil for an hour. Later the extract was cooled and concentrated using rotary flash evaporator.[25] The dried ethanolic extract was subjected to qualitative phytochemical investigation by using various tests like Molish's, Shinoda, Liebermann Burchard, Ferric chloride etc.[26]

Chemical and Drug

Dox was a gift sample from Get Well Pharmaceuticals, India. Other analytical grade chemicals and enzyme assay kits were procured from Sigma Aldrich and ERBA Mannheim.

Animals

Total 30 normal Wistar rats of either sex weighing 150–200 g and six female mice of 18–25 g were used after securing ethical approval from Institutional Animal Ethical Committee (Ref. No. KLEU's-08-IAEC.HBL-31/Aug2013). All the animals were housed in a group of six under environmentally controlled room with 12 hours light/dark cycle in polyprophylene cages and maintained at controlled room temperature (22 ± 2°C) and relative humidity of 40 to 60% with free access to standard laboratory chow (Gold Mohur Lipton India Ltd.) and water *ad libitum* was provided. Before the experiment, rats were acclimatized for seven days to the laboratory environment.

Acute Oral Toxicity

The toxicity studies were carried out as per OECD guidelines revised draft guideline 423. The albino mice were chosen to carry out acute toxicity studies by up and down method. Ethanolic extract of petals of *B. diffusa* was administered at a dose of 2000 mg/kg body weight orally and food was withheld for up to 4 hours after administration of the extract. The animals were observed for changes in general behavior, weight, tremor, convulsion, salivation, sleep, skin, eye, and death at 30 minutes, 1, 2, 3, 4, 24 hours, and once daily for the remaining 14 days.[27]

Experimental Design

After the end of one week of acclimatization period, the rats were divided into five groups of six animals in each as follows.[28]

- *Group I* received vehicle 5 mL/kg (saline) body weight *p.o.*
- *Group II* was treated with Dox 2.5 mg/kg body weight by *i.p*. in 6 equal injections
- on alternate days for 2 weeks.
- *Group III (ERC)* received only ethanolic extract of *B. diffusa* (200 mg/kg body weight *p.o.*) daily for 2 weeks. For next two weeks, the vehicle was administered on alternative day.
- *Group IV (LERC)* received low dose (100 mg/kg body weight *p.o*) of ethanolic extract for 2 weeks as a pretreatment followed by Dox as in group II
- *Group V (HERC)* received high dose (200 mg/kg body weight *p.o*) of ethanolic extract for 2 weeks as a pretreatment followed by Dox as in group II.

Bodyweight, Food and Water

Bodyweight, food, and water intake were regularly measured before and after the treatment for all the animals.

Serum Markers

Blood samples were collected at the end of the study period by retro-orbital route and plasma was separated by using centrifuge and concentrations of blood urea nitrogen (BUN), serum creatinine (SCr) and albumin were measured by commercially available kits using clinical chemistry analyzer (Chem7, Erbamannheim).

Enzyme Assays in Kidney Tissue

The animals were anesthetized by isoflurane and sacrificed by carotid bleeding followed by quick dissection of kidney tissue. The kidney tissue was washed with cold saline; after that, it was dried using filter paper and weighed directly. Kidney of all the animals was taken and 10% w/v of homogenate was prepared in Tris-HCl buffer (pH 7.4) and processed for the estimation of endogenous antioxidants in kidney tissue such as glutathione (GSH),[29] melonldehyde (MDA),[30] superoxide dismutase (SOD)[31] and catalase (CAT).[32] The remaining portion was used for histopathological studies.

Histopathological Studies

Kidneys were isolated after sacrificing the animals. The isolated kidneys were washed with saline, cut into pieces and preserved in 10% neutral formalin solution for two days and then pieces were washed with running water for 12 hours followed by dehydration with alcohol. The kidney tissue was cleaned by xylene two times for 15–20 minutes, subjecting to paraffin infiltration in the automatic tissue processing unit.

The hard paraffin was heated to melt and was poured in square-shaped blocks in which the kidney pieces were dropped quickly and permitted to cool. Microtome was used to cut the blocks to get 5µ thickness sections. These sections were taken on a microscopic slide to which a sticky substance was applied and the section was dried completely before staining. The acidic stain (eosin) and basic stain (haematoxyllin) were used for staining the sections followed by observing in microscope for any changes in histopathalogical characteristics.

Statistical Analysis

The experimental data were statistically analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test by using Graph Pad Prism 5.0 software. Data were expressed as Mean ± S.E.M. Differences were considered significant at $p < 0.05$.

Results

Phytochemical Constituents in Ethanolic Extract of *B. diffusa*

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

Acute Oral Toxicity

We found that acute oral toxicity studies found the lethal dose of ethanolic extract of *B. diffusa* was more than 2000 mg/kg body weight. So $1/10^{th}$ and $1/20^{th}$ of the 2000 mg/kg body weight was chosen for further studies.

General Observations

Dox-treated animals developed scruffy fur, red exudates around the eyes, and soft watery feces. At the site of Dox injection, necrosis was also observed. These conditions were more at the last days of the study period. But these changes were reduced in the pretreated extract group.

Body Weight

Fig. 1 depicts that, in the Dox-treated group, body weight was significantly reduced compared to the normal group, but the EBD ($p < 0.001$) group maintained the animal weight and. HEBD ($p < 0.01$) showed significantly improved body weight compared to the Dox-treated group, whereas LEBD showed no significant changes compared to the Dox-treated group.

Food and Water Consumption

In the Dox-treated group, food and water consumption was reduced significantly compared to the control group. In treatment groups i.e. effective biological dose (EBD) ($p < 0.001$) and herbal effective biological dose HEBD ($p < 0.01$) showed significantly improved food and water consumption as compared to Dox group. But LEBD group showed no significant changes compared to Dox-treated group. (Fig. 2)

Biochemical Parameters (Serum markers)

Dox-treated animals produced significant increase in serum enzyme markers such as BUN ($p < 0.001$) and SCr ($p < 0.001$), whereas albumin was reduced ($p < 0.001$) compared to the control group. The pretreatment groups i.e., LEBD and HEBD decreased the levels BUN ($p < 0.05$ and $p < 0.01$), SCr ($p < 0.05$ and $p < 0.05$), and increased the albumin level ($p < 0.05$ and $p < 0.05$), respectively as compared with Dox treated group. (Table 1)

Ratio of BUN to Scr

The usual ratio of BUN to serum creatinine is 10:1 to 20:1. The ratio is increased during the decrease in the flow of blood to the renal (kidneys), such as CCF (Congestive cardiac failure) or dehydration. In our study, the Dox-treated group showed an increase in the BUN/SCr ratio compared to the control group indicated damage in the kidney, but LEBD and HEBD decreased the ratio compared to the dox group (Table 2).

Antioxidant Enzymes in Kidney Tissue

Dox treated animals showed a significant increase in MDA levels ($p < 0.001$) and decrease in GSH ($p < 0.001$), SOD ($p < 0.001$), and CAT ($p < 0.001$) as compared to the control group, but pretreatment groups, i.e. LEBD and HEBD have decreased levels of MDA ($p < 0.05$ and $p < 0.01$) and increase in the levels of GSH ($p < 0.05$ and $p < 0.01$), SOD ($p < 0.05$ and $p < 0.01$) and CAT ($p < 0.05$ and $p < 0.01$) respectively as compared to Dox group (Table 3).

Histopathological Studies

Dox-treated rat renal tissue exhibited tubular damage and moderate inflammation. Whereas normal group showed normal morphological appearances but pretreated groups i.e., LEBD and HEBD showed minimal tubules damage and less inflammation as compared to Dox treated group (Fig. 3).

Discussion

In present study, the effect of ethanolic extract of *Boerhaavia diffusa* against Dox-induced nephrotoxicity was studied by general parameters, biochemical parameters and histopathological studies. It is reported that rats treated with Dox can reduce food and water intake by more than half within the few days of treatment[33] due to lack of appetite (anorexia) produced as the adverse event of Dox treatment. In the present study, the extract showed a statistically significant ($p < 0.01$) increase in body weight, food, and water intake in higher doses than the Dox-tre values are mean±S.E.M ated group. This indicated the reduction of the adverse event of Dox by *B. diffusa* plant.

Dox-induced nephrotoxicity was characterized by a decrease in glomerular filtration rate (GFR) and an increase in BUN and SCr, which are the most sensitive signs nephrotoxicity concerned in renal injury diagnosis.[34] Our results indicating the increase in BUN and SCr are completely following the earlier studies.[35 36] In the present study, the extract of Boerhaavia diffusa restored the BUN and SCr levels compared to the Dox-treated group. This might be due to its antioxidant property as earlier studies reported that recovery of nephrotoxicity induced by Dox occurs through their antioxidant properties. [36,37]

Dox-treated group showed a reduction in plasma albumin levels, resulting in hypoalbuminemia, which

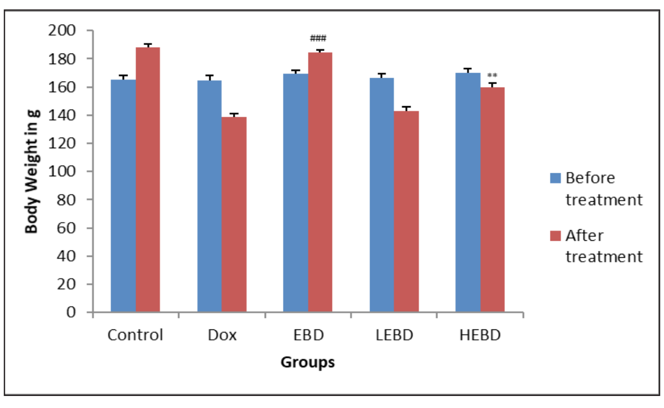

Fig. 1: Effect of ethanolic extract of Boerhaavia diffusa on Body weight
Values are Mean±S.E.M; n=6 in each group, ###p < 0.001 when compared to Control, ***p < 0.001, **p < 0.01 and *p < 0.05 when compared to doxorubicin
Dox – Doxorubicin (2.5mg/kg b.w., i.p.) alternate day in six equal injections for two weeks to become cumulatively 15 mg/kg
EBD – Only ethanolic extract of Boerhaavia diffusa (200mg/kg body weight, p.o.)
LEBD – Low dose (100mg/kg body weight, p.o.) of ethanolic extract of Boerhaavia diffusa followed by doxorubicin administration
HEBD - High dose (200mg/kg body weight, p.o.) of ethanolic extract of Boerhaavia diffusa followed by doxorubicin administration

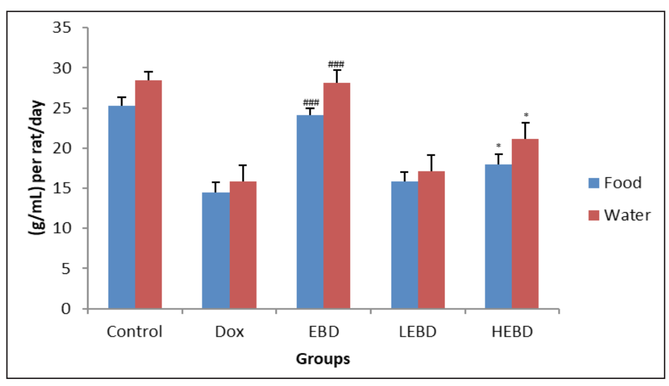

Fig. 2: Effect of ethanolic extract of ***Boerhaavia diffusa*** on Food and Water intake
Values are Mean±S.E.M; n=6 in each group, ###p < 0.001 when compared to Control, ***p < 0.001, **p < 0.01 and *p < 0.05 when compared to doxorubicin
Dox – Doxorubicin (2.5mg/kg b.w., i.p.) alternate day in six equal injections for two weeks to become cumulatively 15 mg/kg
EBD – Only ethanolic extract of Boerhaavia diffusa (200mg/kg body weight, p.o.)
LEBD – Low dose (100mg/kg body weight, p.o.) of ethanolic extract of Boerhaavia diffusa followed by doxorubicin administration
HEBD - High dose (200mg/kg body weight, p.o.) of ethanolic extract of Boerhaavia diffusa followed by doxorubicin administration

is completely following the earlier studies.[38,39] In the present study, the extract of *B. diffusa* showed a statistically significant ($p < 0.01$) increase in the albumin level as compared to the Dox-treated group.

In the Dox-treated group, there was decrease in the levels of SOD, CAT, and GSH enzymes, while increase in the levels of MDA which are completely in accordance with the earlier studies.[40] Several articles reported that increased oxidative stress with decrease in antioxidant enzymes initiates the sequence of reactions responsible for Dox-induced heart muscle and kidney damage.[41] Dox produces kidney damage by accumulating in the glomerulus but the exact mechanisms are not elucidated.[42] Literature survey reveals that Dox stimulates semiquinone radical formation, which combines with O2¬ and produces other free radicals at a preliminary stage, this results in locally in-filtered neutrophils and glomerular cells mesangial cells continue free radical production.[43,44]

Table 1: Effect of ethanolic extract of *B. diffusa* on Serum Markers

Groups	*BUN (mg/dL)*	*sCr (mg/dL)*	*Albumin (g/dL)*
Control	19.12 ± 3.26	1.10 ± 3.24	2.58 ± 1.14
Dox	56.22 ± 4.82$^{\#\#\#}$	2.12 ± 3.24$^{\#\#\#}$	0.85 ± 0.54$^{\#\#\#}$
EBD	22.45 ± 4.34***	1.45 ± 4.25***	3.11 ± 1.12***
LEBD	48.55 ± 6.52*	2.02 ± 5.48*	0.95 ± 0.62*
HEBD	40.14 ± 8.45**	1.75 ± 6.25*	1.18 ± 0.46*

Values are mean ± S.E.M; n=6 in each group, ###p < 0.001 when compared to Control, ***p < 0.001, **p < 0.01 and *p < 0.05 when compared to doxorubicin
Dox – Doxorubicin (2.5 mg/kg b.w., i.p.) alternate day in six equal injections for two weeks to become cumulatively 15 mg/kg
EBD – Only ethanolic extract of *Boerhaavia diffusa* (200mg/kg body weight, p.o.)
LEBD – Low dose (100 mg/kg body weight, p.o.) of ethanolic extract of Boerhaavia diffusa followed by Dox administration
HEBD - High dose (200 mg/kg body weight, p.o.) of ethanolic extract of Boerhaavia diffusa followed by doxorubicin administration

Table 2: Effect Of ethanolic Extract of *B. siffusa* on Bun/Scr Ratio

Groups	*BUN/sCr Ratio*
Control	17.38
Dox	26.51
EBD	15.48
LEBD	24.03
HEBD	22.93

Dox – Doxorubicin (2.5 mg/kg b.w., i.p.) alternate day in six equal injections for two weeks to become cumulatively 15 mg/kg
EBD – Only ethanolic extract of Boerhaavia diffusa (200 mg/kg body weight, p.o.)
LEBD – Low dose (100 mg/kg body weight, p.o.) of ethanolic extract of Boerhaavia diffusa followed by doxorubicin administration
HEBD - High dose (200 mg/kg body weight, p.o.) of ethanolic extract of Boerhaavia diffusa followed by doxorubicin administration

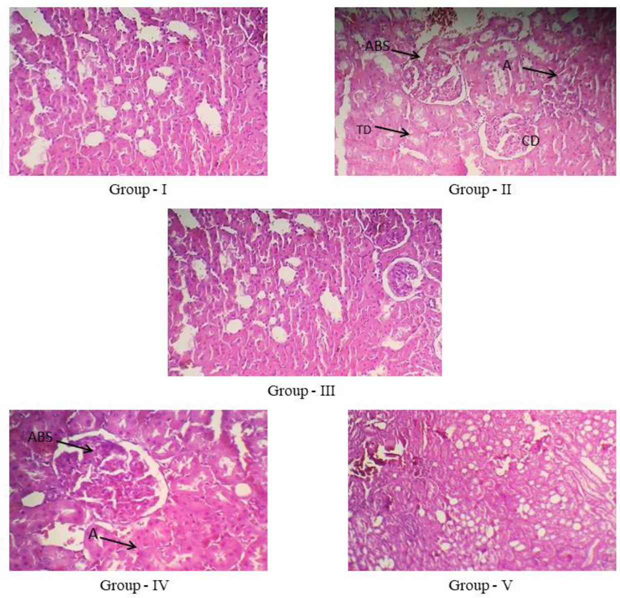

Fig. 3: Effect of ethanolic extract of Boerhaavia diffusa on histopathology of kidney tissue.

40X magnifications)
Group I: Control rat's renal section showing normal morphology.
Group II: Dox treated rat's renal sections showing degenerative changes, cellular infiltrations (CI), capsule distortion (CD), and atrophy (A).
Group III: EBD alone treated rat's renal section.
Group IV: LEBD treatment showing slight tubular dilations.
Group V: HEBD treatment showing significant protection against doxorubicin induced renal injury.

Table 3: Effect of ethanolic extract of *B. diffusa* on antioxidant enzymes

Groups	*MDA (n mole/mg of wet tissue)*	*GSH (n mole/mg of wet tissue)*	*SOD (Unit/mg protein)*	*CAT (Unit/mg protein)*
Control	21.45 ± 3.25	22.59 ± 2.88	60.41 ± 3.26	60.11 ± 2.95
Dox	62.12 ± 4.36$^{\#\#\#}$	12.89 ± 3.54$^{\#\#\#}$	28.55 ± 3.15$^{\#\#\#}$	31.44 ± 3.26$^{\#\#\#}$
EBD	23.55 ± 3.68***	24.11 ± 4.14***	60.14 ± 2.59***	59.18 ± 3.54***
LEBD	55.85 ± 4.56*	18.42 ± 3.45*	42.52 ± 2.98*	38.22 ± 2.57*
HEBD	46.82 ± 5.18**	19.54 ± 3.12**	46.88 ± 3.54**	43.28 ± 3.24**

Values are Mean±S.E.M; n=6 in each group, ###p < 0.001 when compared to control, ***p < 0.001, **p < 0.01 and *p < 0.05 when compared to doxorubicin
Dox – Doxorubicin (2.5 mg/kg b.w., i.p.) alternate day in six equal injections for two weeks to become cumulatively 15 mg/kg
EBD – Only ethanolic extract of Boerhaavia diffusa (200 mg/kg body weight, p.o.)
LEBD – Low dose (100 mg/kg body weight, p.o.) of ethanolic extract of *B. diffusa* followed by Dox administration
HEBD - High dose (200 mg/kg body weight, p.o.) of ethanolic extract of *B. diffusa* followed by Dox administration

Superoxide dismutase enzyme catalyzes the dismutation of molecular oxygen to hydrogen peroxide and molecular oxygen (O_2), while glutathione peroxidase and catalase enzymes catalyze the degradation of hydrogen peroxide to O_2 and H_2O.[45] In detoxifying xenobiotic compounds, these enzymes play an important role in producing anti-oxidation of free radicals. Low levels of these are associated with excessive oxidative stress.[46] In the present study, the extract of *B. diffusa* showed a statistically significant increase in the superoxide dismutase (p < 0.01), glutathione (p < 0.01), and catalase (p < 0.01) enzymes, indicated the nephroprotective property might be due to degradation of the free radicals. As Deman et al[44] reported, enhanced concentrations of reduced glutathione, particularly in the renal cortex, supported the clue of free radicals in Dox nephrotoxicity.

In the Dox-treated group, the malondialdehyde level was increase Group I: Controled compared to the control group, resulting in increased lipid peroxidation. It is the end products of polyunsaturated fatty acids peroxidation in the cells. Increased production of malondialdehyde occurs when there is an increase in free radicals and it is commonly considered as a marker of oxidative stress.[47] In the present study, the extract of B. diffusa showed a statistically significant (p < 0.01) decrease in the malondialdehyde level compared to the Dox-treated group. It indicated the extract was reduced the lipid peroxidation and reduced the free radical generation and oxidative stress, which might be responsible for its nephroprotective activity.

In histopathologic studies of kidney tissue, the Dox-treated group showed tubular damage, inflammation, and degenerative changes in the renal tubules and glomeruli of the Dox group compared to the Dox group control group and these changes are following the earlier studies.[48] The present study extract of B. diffusa ameliorated the histopathological damage caused by Dox.

The present study signifies the ethanolic extract of petals of *B. diffusa* reduced the nephrotoxicity induced by cumulative administration of Dox in rats. The study revealed that extract of *B. diffusa* may be considered as useful in combination with Dox. However, further elucidation of the cellular and molecular mechanisms would provide robust evidence for the nephroprotective effects of extract.

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