Protective Effects of *Boerhaavia Diffusa* Against Acetaminophen-Induced Nephrotoxicity in Rats

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Summary

The current study designed to investigate the effects of pre-treatment of aqueous extract of *B. diffusa* root (200 – 400 mg/kg/day) in repeated dose acetaminophen nephrotoxic rats for 14 days. Administration of acetaminophen to rats induced marked detritions of renal function, characterized by a significant increase in blood urea nitrogen (BUN), serum creatinine ($p < 0.01$) and injured the renal cells evident from increased level of kidney malondialdehyde (MDA), protein thiol ($p < 0.01$) along with depletion of super oxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) activities and reduced glutathione (GSH) levels ($p < 0.01$), however pre-treatment with *B. diffusa* extract protected against these changes. Histopathological changes showed that acetaminophen caused significant structural damages to kidneys like tubular necrosis, degeneration of epithelial cells, glomerular damage and congestion which was reversed with *B. diffusa*. The results suggest that *B. diffusa* has the potential in preventing the acetaminophen-induced nephrotoxicity.

Keywords: *Boerhaavia diffusa,* nephrotoxicity, acetaminophen, renal function, antioxidant enzyme

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Introduction

Boerhaavia diffusa Linn. is a herbaceous plant of the family *Nyctaginaceae*. The whole plant or its specific parts (leaves, stem, and roots) are known to have medicinal properties and have a long history of use by indigenous and tribal people in India. The medicinal value of this plant in the treatment of a large number of human ailments is mentioned in *Ayurveda* [1]. Different parts of the *B. diffusa* have been widely used for the treatment of dyspepsia, jaundice, enlargement of spleen, abdominal pain, abdominal tumors, and urinary disorders used in the traditional medicine [2]. Pharmacological studies have demonstrated that *B. diffusa* exhibits a wide range of properties such as diuretic [3]; nephrotic syndrome [4]; anti-inflammatory and anti-nociceptive [5]; anticonvulsant [6]; immunomodulatory [7]; hepatoprotective [8]; antiurolithiatic [9]; antioxidant and antidiabetic activity [10]. Due to the combination of diuretic, antioxidants and anti-inflammatory activities *B. diffusa* regarded as therapeutically highly efficacious for the treatment of inflammatory renal diseases and common clinical problems such as nephrotic syndrome, oedema, and ascites. From these investigations it is believed that *B. diffusa* improves renal function and may protect renal cell against chemical induced nephrotoxicity. Therefore the present study was aimed to investigating the possible protective effect of *B. diffusa*, against acetaminophen-induced nephrotoxicity in rats.

Materials and methods

Plant material and extraction

B. diffusa was collected from Birla Institute of Technology, Mesra campus Ranchi, India in month of august. A sample herbarium of the plant material was submitted to the herbarium of Botanical Survey of India (A Central National Herbarium), Kolkata. Plant was authenticated as *Boerhaavia diffusa* Linn. variety red. (Family: Nyctaginaceae).

The dried roots of plant was cleaned and ground to coarse powder, using commercial mill. Approximately 100 g of the ground plant powder was macerated in 500 ml water at room temperature for 24 hour with occasional shaking. It was filtered through a Whatman grade 1 filter paper in a Buchner funnel under vaccum. The filtrate was evaporated to dryness on a rotary evaporator under reduced pressure to obtain a crude aqueous extract of *B. diffusa*.

Animals

Twenty four inbred male Wistar albino rats (180-200g body weight) were used in this study. Animals were procured from Central Animal House of the University approved by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). All animals were kept in polyacrylic cages and maintained under standard housing conditions (room temperature 24-27^{0}C and humidity 60-65% with 12:12 light: dark cycles). Commercial pellet diet (Amrut laboratory rat and mice feed, Sangli, India) and water were provided ad libitum. The experiments were carried out according to the guidelines approved by the Institutional Animal Ethical Committee.

Experimental protocol

Animals were randomly divided into the following experimental groups with six animals in each group:, groups I and II rats, that served as **normal** and **control**, respectively, were orally administered 10ml/kg/day of normal saline, while groups III–IV rats were pre-treated with single daily oral doses of 200–400 mg/kg of aqueous extract of *B. diffusa,* 1 h before the intraperitoneal injection of 200 mg/kg/day of acetaminophen, for 14 days. For group II rats, in place of single daily intraperitoneal dose of 200 mg/kg of acetaminophen, 10 ml/kg/day of normal saline was administered, for 14 days. Prior to termination of the experiment on the 15th day, the rats were weighed and fasted overnight. On the 15th day, the fasted rats were sacrificed under diethyl ether. Blood samples were withdrawn immediately by cardiac puncture for biochemical assays. The kidneys were rapidly excised, weighed and kept for microscopic examination and biochemical analysis [11]. The percentage loss in body weight and degree of renal hypertrophy was expressed as the ratio of the weight of the two kidneys to total body weight [12].

Preparation of serum and tissue homogenate

Blood collected from rats was allowed to clot and serum was separated using centrifuge (Remi instruments Ltd., Mumbai) at 5000 rpm for 4 min. Serum creatinine and blood urea nitrogen (BUN) level were estimated using commercial kits (Bio In-vitro). The renal tissue was washed with ice-cold 0.9% saline and homogenized quickly with ice-cold 0.1 M Tris–HCl buffer (pH 7.5) using Remi homogenizer to give a 10% homogenate. The homogenate was centrifuged at 10,000 rpm for 20 min [13] and the supernatants were used for estimation of malondialdehyde (MDA), catalase (CAT), superoxide dismutse (SOD), reduced glutathione (GSH) and glutathione peroxidase (GPx).

Estimation of antioxidant marker

Malondialdehyde assay

According to the method of Esterbauer and Cheeseman (1990), MDA was estimated in terms of TBARS. Homogenized renal tissue (1ml) in 2 ml of normal saline was mixed with 1 ml tri-chloro acetic acid (20%), 2 ml thiobarbituric acid (0.67%) and heated for 1 h at 100°C. After cooling, the precipitate was removed by centrifugation. The absorbance of the sample was measured at 535 nm using a blank containing all the reagents except the sample [14]. As 99% TBARS are MDA, so TBARS concentrations of the samples were calculated using the extinction co-efficient of MDA (1.56×10^5 $M^{-1}cm^{-1}$).

Catalase assay

The CAT activity was measured using the method of Chance and Maehly (1955) by following the decomposition of H_2O_2. The reaction mixture consisted of 2 ml of 100mM phosphate buffer (pH 7.0), 0.90 ml of hydrogen peroxide (30 mM) and 0.1 ml of supernatant in a final volume of 3 ml. Absorbance were recorded at 240 nm at every 10 sec interval for 1 min. One unit of CAT was defined as the amount of enzyme required to decompose 1 µM H_2O_2/min, at 25°C [15].

Superoxide dismutase assay

An indirect method of inhibiting auto-oxidation of epinephrine to its adrenochrome by Misra & Fridovich (1972) was used to assay SOD activities. Kidney homogenate (0.05 ml) was added to 2.0 ml of carbonate buffer and 0.5 ml of 0.01 mM EDTA solution. The reaction was initiated by addition of 0.5 ml of epinephrine (3×10^{-4} M) at pH 10.2 and The change in optical density every minute was measured at 480 nm against reagent blank for 5 min. A graph of absorbance against time was plotted for each sample and the rate of auto-oxidation calculated. One unit of SOD activity was defined as the concentration of the enzyme (mg protein/ml) in the plasma that caused 50% reduction in the auto-oxidation of epinephrine [16].

Glutathione peroxidase assay

GPx activity was measured by using the method of Paglia and Valentine (1969). Reaction mixture contained 2.525 ml of 0.1 M/l Tris-HCl buffer (pH 7.2), 75 µl of 0.04 M/l GSH, 100µl of 0.1 M/l nicotinamide adenine dinucleotide phosphate (NADPH) and 100 µl of GSH (0.24 units). Homogenate (20 µl) was added to the reaction mixture. Reaction was initiated by adding 100µl of 0.75 mM/l hydrogen peroxide. The decrease in absorbance was measured at 340 nm for 3 min at every 30 sec. The activity was expressed as unit/mg protein using molar extinction coefficient of 6.22×103 $(mM/l)^{-1}cm^{-1}$ [17].

Reduced glutathione

GSH was measured by using the method described by Dringen and Hamprecht (1996) with slight modifications. Tissue homogenate 50 µl was diluted with 50 µl of 100 mM phosphate buffer containing 1 mM EDTA. To this mixture, 100 µl of reaction buffer (295 µM 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) made in 10 ml of phosphate buffer) was added and change in absorbance measured at 412 nm for 5 min. Reduced pure GSH was used to obtain a standard curve. Reduced GSH was expressed as µM GSH/mg tissue [18].

Protein estimation

The protein carbonyl content was estimated by the protein derivatization with dinitrophenyl hydrazine (DNPH) into chromophoric dinitrophenyl hydrazones by the method of Levine et al. (1990) [19].

Histopathological studies

The left kidney excised from animal immediately fixed in 10% buffered formalin (pH 7.0). The tissues were dehydrated with ascending grade of alcohol and embedded with paraffin wax (M.P. 55°C). Six micrometer thick paraffin sections were cut and finally stained with hemotoxylin and eosin staining. The sections were examined under light microscope (Lieca EZ-4D) to evaluate pathological changes and photomicrographs were taken. A semiquantitative scoring method was used to analyze the renal tissue damage [13].

Results

Effects of treatment on body weight and relative weight of kidney

A significant ($P < 0.01$) loss in body weight and increase in relative kidney weight was observed in rats treated with acetaminophene only as compared to normal rats. The pre-treatment with extract prevented the change in body and kidney weight. Increase size and relative kidney in control rats shown significant renal hypertrophy rats as compared to the normal rats ($p < 0.01$). The pre-treatment with extract prevented renal hypertrophy significantly ($p < 0.01$) (data shown in table 1).

Table 1: Effect of *B. diffusa* treatment on body and kidney weight in rats

Observational Parameter	Normal (normal saline)	Control (normal saline + acetamenophen)	Treated (200 mg/kg extract)	Treated (400 mg/kg extract)
Change in Body weight (gm)	4.8±0.9	-7.2±1.9[a]	12.1±1.5[a,b]	12.6±1.4[a,b]
Kidney weight (gm)	1.48±0.04	2.4±0.07[a]	1.56±0.03[b]	1.54±0.04[b]
Degree of renal hypertrophy*	7.21±0.2	12.5±1.30[a]	7.35±0.6[b]	7.23±0.8[b]

Values are expressed in mean ± SEM (n=6)

[a] p<0.001 compared with normal group; [b] p<0.01 compared with control group;

**(kidney weight × 1000/Body weight)*

Effect on renal function

Single daily intraperitoneal administration of 200mg/kg/day of acetaminophen for 14 days induced significant ($p < 0.01$) rise in serum creatinine and BUN in the control rats (group II). However, elevations in the serum creatinine and BUN were significantly ($p < 0.01$) attenuated by extract pre-treatments, in dose related fashion (Figure 1A & 1B).

Changes in antioxidant marker in kidney

Nephrotoxic treatment enhanced MDA and protein thiol content ($p < 0.01$), decreased GSH level ($p < 0.01$) and activities of the antioxidant enzymes including SOD ($p < 0.01$), GPx ($p < 0.01$) and catalase ($p < 0.01$) in kidneys of the control rats as compare to the normal animals. A pre-treatment treatment with extract protected against the oxidative changes induced by acetaminophene in a dose-dependent manner (data shown in Table 2).

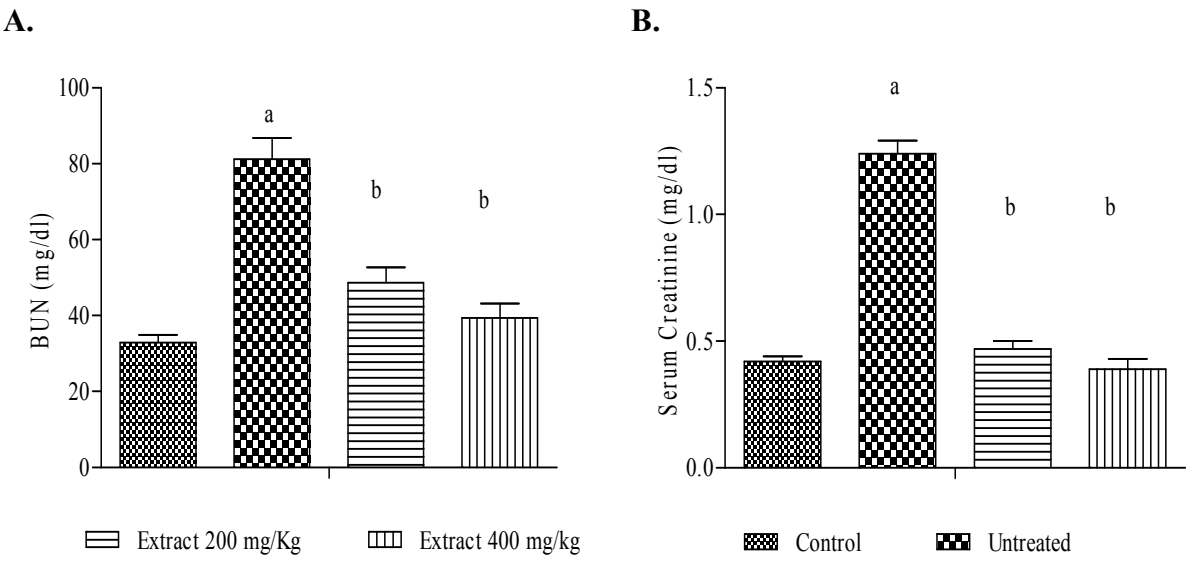

Figure 1: Effect of *B. diffusa* aqueous extract on renal function **A.** Blood urea nitrogen **B.** Serum creatinine

Table 2: Effect of *b. Diffusa* aqueous extract treatment on renal enzyme and other antioxidant marker in rat kidney homogenate analysis

Antioxidant Marker /Enzyme	Normal (normal saline)	Control (normal saline + acetamenophen)	Treated (200 mg/kg extract)	Treated (400 mg/kg extract)
Malondialdehyde (µM/mg protein)	43.24±1.8	98.35±5.1[a]	65.38±2.2[b]	54.70±3.5[b]
Catalase (µMH_2O_2 decomposed/min)	38.2±1.2	23.5±1.6[a]	32.6±1.5[b]	35.4±0.9[b]
Super Oxide Dismutase (U/mg protien)	9.64±0.2	3.82±0.9[a]	7.15±0.6[b]	7.98±0.5[b]
Reduced Glutathione (nM /mg protien)	28.45±0.7	10.80±1.3[a]	24.40±0.9[b]	25.90±0.4[b]
Glutathione Peroxidase (U/mg protien)	16.75±0.8	9.46±1.3[a]	13.54±0.2[b]	14.70±0.3[b]
Total Protien Content (mg/dl)	58±2.5	215± 7.8[a]	80±3.8[b]	63± 4.1[b]

Values are expressed in mean ± SEM (n=6)
[a] *p<0.001 compared with normal group;* [b] *p<0.01 compared with control group*

Histopathology

Histological findings showed glomeruli with loss of surrounding Bowman's capsule, glomerular damage and varying degrees of tubular epithelium necrosis (Fig. 2B) in control rats, when compared to normal (group I) rats which showed normal glomerulus with intact Bowman's capsule and tubular brush borders (Fig. 2A). Overall kidney of control rats were severely damaged whereas pre-treatment with the extract significantly attenuated the damage of renal cells and shown mild damage to normal renal architecture (Fig. 2 C & D).

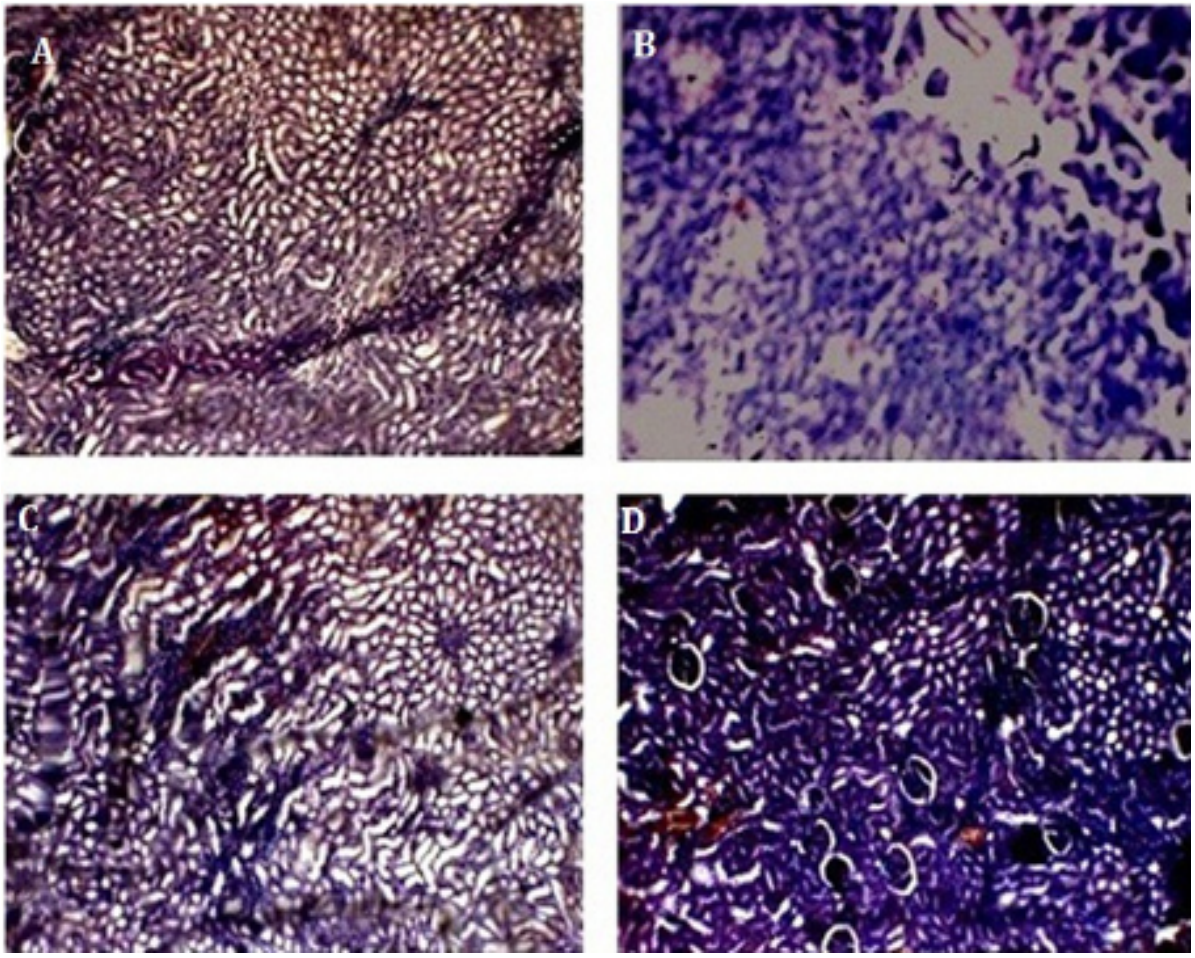

Figure 1: Hemotoxylin and eosin-stained sections of normal rat kidney showing effect of various treatments. (a) Kidney section of normal rats showing normal renal architecture (b) Kidney section of control rats showing glomerular degeneration, tubular brush border loss, tubular dilatation, necrosis of epithelium and interstitial oedema. (c) Kidney section of extract of *B. diffusa* (200 mg/kg)-treated rats showing mild tubular damage. (d) Kidney section extract of *B. diffusa* (400 mg/kg)-treated rats showing almost normal renal architecture.

Discussion

Acetaminophen remains one of the most popular, effective, over-the-counter analgesic-antipyretic agents belonging to the non-steroidal anti-inflammatory drugs (NSAIDs). Acute large doses or chronic use of acetaminophen is commonly associated with nephrotoxicity and hepatotoxicity in humans as well as animals [20]. Thus, acetaminophen-induced nephrotoxicties are well established experimental models of drug-induced renal injury ([21]. Acetaminophen nephrotoxicity occurs due to its highly reactive metabolite*N*-acetyl-*para*-amino-benzoquinoneimine, which arylates proteins in the proximal tubule, initiating cell death of renal tubular cells [21]. Drug-induced nephrotoxicities are often accompanied with marked elevations in BUN, serum creatinine and acute tubular necrosis and depletion of antioxidant enzyme [22].

In the present study, acetaminophen-induced nephrotoxicity was characterized by marked elevations in the circulating levels of BUN and serum creatinine in control (group II) rats. However, these changes were attenuated by pre-treatment with extract. In nephrotoxicity and renal disease, the serum urea and creatinine accumulates because the rate of production exceeds the rate of clearance due to compromised renal function [23]. The acute elevations in the measured biochemical parameters in group II rats, could also be attributed to increased catabolic state of the rats due to the prolonged anorexia associated with acetaminophen nephrotoxicity [24]. The anorexic state of the control nephrotoxic rats could account for the significant and progressive weight losses recorded for the acetaminophen treated rats. The weight gain in extract treated rats probably due to restoration of normal feeding pattern by the extract and its nutritious value, as *B. diffusa* contains a large amount of amino acids.

Many animal experiments have reported the positive correlation between oxidative stress and nephrotoxicity [25]. Acetaminophen induced oxidative stress results in lipid peroxidation, protein thiols oxidation, depletion of antioxidant enzyme and mitochondrial endoplasmic reticulum injury [26]. Several antioxidant systems occur in the body, which include superoxide dismutase, glutathione peroxidase, catalase, glutathione, vitamin C and vitamin E which maintain the redox balance [27]. While enzymatic and non-enzymatic systems preserve the antioxidant status, these defense systems become overwhelmed during oxidative stress, leading a metabolic derangement due to an imbalance caused by excessive generation of ROS and diminished antioxidant capacity followed by renal cell injury and inflammation [28]. In acetaminophen treated animals the MDA levels are increased significantly, when compared to normal rats. On administration of extract, the levels of MDA decreased significantly when compared to acetaminophen induced control rats. During renal injury and inflammation, superoxide and peroxide radicals are generated at the site of damage, resulting in the depletion of SOD and CAT activity. SOD and CAT are the most important enzymes involved in ameliorating the effects of oxygen metabolism [29]. The present study also demonstrated that acute acetaminophen overdose resulted in a decrease in the SOD, CAT and GPx activities, when compared with normal control rats. It is due to enhanced lipid peroxidation or depletion of the antioxidative enzymes. When rat was treated with the extract, the activity of SOD, CAT and GST was restored significantly. A significant reduction in the renal tissue concentrations of glutathione has been associated with drug induced nephrotoxicity including acetaminophen nephrotoxicity as evident in untreated rats. It is also possible for the extract to be mediating its renal antioxidant activities by enhancing the antioxidant defence enzymes mediated through SOD, CAT and replenishing renal glutathione storage.

The biochemical results were also confirmed by the histological findings which showed damaging glomeruli, mildly dialated tubules and necrosis of tubular epithelium in control rats which were protected by extract (Fig. 1B). The protection offered by the *B. diffusa* aqueous extract could have been due to the presence of any of the active principles contained in it. Literature has shown *B. diffusa* contains a large number of compounds such as flavonoids, alkaloids, steroids, triterpenoids, lipids, lignins, carbohydrates, proteins, and glycoproteins [1]. Flavonoids and other antioxidant constituent of medicinal plants have been reported to inhibit xenobiotic induced nephrotoxicity in experimental animal models due to their potent anti-oxidant effects [25]. Any of these or their combination could be responsible for the nephroprotective effect of *B. diffusa*.

Conclusion

Results of this study indicate the *B. diffusa* have nephroprotective effect in acetaminophine induced nephrotoxicity possibly through improving the renal function and its antioxidant status.

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