Boerhaavia diffusa Linn AQUEOUS EXTRACT AS CURATIVE AGENT IN ETHYLENE GLYCOL INDUCED UROLITHIASIS

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Summary

Boerhaavia diffusa Linn. is widely used plant in India, as a traditional medicine for the treatment of renal disorders including urolithiasis as mentioned in *Ayurveda*, *Charaka Samhita*, and *Sushrita Samhita*. Present study aims to evaluate antiurolithiatic activity of *Boerhaavia diffusa* Linn. root aqueous extract (BDAE) and rationalize its use in treating renal stones. Male Wister Albino rats (180-200 g body weight) were orally administered with 0.75% v/v ethylene glycol (EG) and 1% ammonium chloride for 3 days in drinking water and then 0.75% EG only until they reached three weeks to induce the deposition of calcium (CaOx) crystal deposition in rat kidney. The lithogenic treatment caused weight loss, hyperoxaluria and impairment of renal function. However, treatment with BDAE (100 or 200 mg/kg) did not cause excessive hyperoxaluria and consequently reduced the crystal depositions in a dose dependent manner in rat kidney after one week of urolithiasis induction. *Boerhaavia diffusa* Linn. causes diuresis and hasten the process of dissolving the preformed crystals and help in mechanical expulsion of the stone, improve the renal function by increasing the removal of waste product and decreases the oxalate excretion probably by interfering with metabolism. Results of this study indicate *Boerhaavia diffusa* Linn. posses antiurolithiatic activity, that possibly mediated through diuretic and hypo-oxaluric effects.

Key Words: Urolithiasis, *Boerhaavia diffusa* Linn, Calcium Oxalate, Crystallyzation, Ethylene glycol

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Introduction

Urolithiasis (Renal stone disease, nephrolithiasis) is a recurrent disease, affects 1–5% of the population in 'developed' nations with a peak incidence between 20 and 50 years of age. Men are three times more likely to be affected than women and the existence risk of developing a calculus in a Caucasian man is nearly 20% (1), whereas in India 11% people expected to suffer this problem (2). The recent advances in treatment of renal stone like endoscopic stone removal and extracorporeal shock wave lithotripsy (ESWL) are prohibitively costly for the common man and with these procedures recurrence is quite common and the patient has to be subjected to

careful follow up for a number of years. However herbal medicines are efficacious and have lesser side effects compared to modern medicines and also reduce the recurrence rate of renal stone (3). Therefore, it is valuable to look for an alternative treatment strategies like the use of medicinal plants or phytotherapy. *Pashanabheda* (Pashana-stone; Bheda-break) is a term used in *Ayurveda* for a group of plants with diuretic and antiurolithiatic activities, in this regard, *Boerhaavia diffusa* Linn. have been used to treat kidney stones in Indian folk medicine since ancient time.

Boerhaavia diffusa Linn., commonly known as *Punarnava* is a herbaceous plant of the family Nyctaginaceae and included in *Indian Pharmacopoeia 2006* as herbal diuretic agent. Root decoction of this plant is taken by patients for one month to treat kidney stones in many regions of India (4). The herbal extract of *Boerhaavia diffusa* Linn. has been reported to inhibit *in vitro* growth of struvite crystals *in vitro* (2), suggestting that plant may exhibit antiurolithiatic activity. *Boerhaavia diffusa* is also an important ingredient of polyherbal formulation, **Cystone** (Himalaya health care Pvt. Ltd) widely used for the treatment of renal stone disease in India. Since currently, there is no *in vivo* studies have been carried out to provide scientific rationale for use of this plant in treating urolithiasis. Therefore, the present systematic pharmacological study has been carried out to assess the traditional use of *Boerhaavia diffusa* Linn. root aqueous extract in treating urolithiasis.

Materials and Methods

Plant material and extraction

Boerhaavia diffusa Linn. was collected in August, 2008 from Birla Institute of Technology, Mesra campus Ranchi, India in month of august . Taxonomic identity of the plant (Boerhaavia diffusa Linn. variety red) was confirmed at the herbarium of Botanical Survey of India (A Central National Herbarium), Kolkata.

The cleaned and dried roots of plant were ground to powder, using commercial mill. Approximately 100 g of the powder was soaked in 500 ml water at room temperature for 24 hour with occasional shaking. Followed by initial filtration through a single layer of muslin cloth, and finally the filtrate was collected after passing through a Whatman grade 1 filter paper in a Buchner funnel under vaccum. This filtrate was evaporated to dryness on a Rotary Evaporator under reduced pressure and a thick dark brown material (10 g), the crude extract of *Boerhaavia diffusa* Linn. (BDAE) was obtained, representing the the approximate yield of 10%.

Animals

Twenty four inbred male Wister Albino rats (180-200g body weight) were used in this study. Animals were procured from Institutional Animal House (Reg no. 621/02/ac/CPCSEA) of Birla Institute of Technology, Mesra. 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). Food was provided in the form of dry pellets and water *ad libitum*. The animals were allowed to get acclimatized to the laboratory conditions for 7 days before the commencement of the experiment. All experiments involving animals complies with the ethical standards of animal handling and approved by Institutional Animal Ethics Committee.

In Vivo Study: Antiurolithiatic activity

Ethylene glycol (EG) induced hyperoxaluria model by Atmani et al; 2009 was used to assess the antiurolithiatic activity in albino rats using curative regimen protocol (5). Male Wister rats were housed in metabolic cages three days prior to the start of the experiment for acclimatization. The experiment was conducted in accordance to internationally accepted standard guidelines for use of animals. They were fed regular chow and had free access to tap water *ad libitum*. They were then divided into four groups comprising six animals each. Group 1 was used as normal control & given water only and other rats (group 2,3 & 4) were given 0.75% v/v EG and 1% ammonium chloride for 3 days in drinking water and then EG alone until they reached 3 weeks

for induction of urolithiasis. After one week of the urolithiasis induction confirmed by the crystalluria analysis following treatment protocol was followed for rest of 2 weeks.

Group 1 was given normal saline 10 ml/kg body weight p.o served as normal **control** group;
Group 2 was given normal saline 10 ml/kg body weight p.o. served as **untreated** nephrolithiasic rats group;
Groups 3 were given BDAE 100 mg/kg body weight p.o. served as **treated** nephrolithiasic rats group;
Groups 4 were given BDAE 200 mg/kg body weight p.o. served as **treated** nephrolithiasic rats group.

The doses of BDAE selected for the evaluation of antiurolithiatic activity in diuretic range (10-300 mg/kg) (6) as diuresis is preliminary requirement for antiurolithiatic activity. During the study of 21 days body weight, water intake and animal health observed regularly, so that stressed and unhealthy animal excluded from study. Various biochemical parameter of urine and serum as well as histopathological characters of kidney were considered for *assessment of antiurolithiatic activity:*

Urine Analysis:

All animals were kept in individual metabolic cages and urine samples of 24 h were collected daily. Animals had free access to drinking water during the urine collection period. **Volume** and **pH** of urine sample were measured immediately after collection and also analyzed for **calcium, phosphate** and **oxalate** content using commercial kits (Crest Biosystem Pvt. Ltd. India). A semi-quantitative microscopic crystalluria analysis was performed by counting the number of crystals. Twenty-four hour urine samples were first mixed well and then aliquots were withdrawn and put on slide and examined under microscope (Lieca DME) (7).

Serum Analysis:

After the experimental period, blood was collected by cardiac puncture under anesthetic conditions. Serum was separated by centrifugation at 10,000×*g* for 10 min. than analyzed for **creatinine** and **blood urea nitrogen (BUN)** using commercial kits (Crest Biosystem Pvt. Ltd. India) (8)**.**

Histopathological Examination:

Animals were sacrificed by cervical dislocation and kidneys were dissected out. Kidneys were fixed in 10% formalin solution in 0.1M phosphate buffer saline and then dehydrated in ascending grades of alcohol and embedded in paraffin. Sections at 6µm thickness were cut using microtome, stained with hematoxylin and eosin (5) and examined under light microscope (Lieca DME) for histological evaluation.

Statistical Calculations

The data expressed are mean ± standard error of mean (SEM). All statistical comparisons between the groups are made by means of One Way Analysis of Variance with post hoc Dunnett's test or by Student's t-test using Graphpad Prism 5 software. The p value less than 0.01 is regarded as significant.

Results

Body weights, water intake, urine volume and pH recorded during 21 days of study period. Weight of untreated rats decreased significantly ($p<0.01$) and BDAE treated rats did not show any significant change in body weight as compared to control rats. However Water intake significantly ($p<0.01$) increased in all groups compared to the control group.

Lithogenic (EG) treatment also reduced pH of urine in the untreated group as compare to that of the control group, although not to a significant extent. Treatment with BDAE at both the

doses increased urine volume (p<0.01) in a dose-dependent manner. In parallel with crystalluria, there was an increased oxalate excretion (p<0.01) in untreated animals. BDAE treatment (100 & 200 mg/kg) prevented the change in urinary oxalate. Other changes in the urine composition, induced by the lithogenic treatment like calcium and phosphate excretion were not found to be statistically significant (**Table 1**).

Microscopic observation revealed that urine of control rats was devoid of any crystal and the presence of numerous aggregated calcium oxalate monohydrate (COM) crystals (dumbbell-shaped) and calcium oxalate dihydrate (COD) crystals (bipyramidal shaped) in hyperoxaluric rat's urine. Only few COD crystals were observed in BDAE treated animals. At 100 & 200 mg/kg BDAE visibly reduced the crystal size with significant decreased in number of crystal as well as crystal size. Raised BUN and serum creatinine (p<0.01) observed in hyperoxaluric rats, while, this was reduced in a dose-dependent manner in animals receiving a simultaneous treatment with BDAE (**Table 1**).

Table 1: Various parameters recorded for assessment of Antiurolithiatic Activity during 21 days study

Observational Parameter	Control	Untreated	Treated BDE 100mg/kg	Treated BDE 200mg/kg
General Observations				
Body weight change (gm)	5.34±0.38	-8.78±1.76[b]	3.98±0.96[d]	4.18±0.54[d]
Water Intake (ml/24hr)	10.82±1.67	15.67±1.54	22.34±2.81[b,c]	28.23±2.94[b,d]
24 hour Urinalysis				
Volume (ml/24hr)	6.85±0.97	11.52±1.80	16.40±2.65[b,d]	22.30±2.87[b,d]
pH	6.8±0.03	5.4±0.06[b].	6.7±0.04[d]	6.7±0.05[d]
Crystalluria	-	+++	++	+
Calcium (mg)	0.624±0.15	0.710±0.27	0.645±0.24	0.638±0.32
Oxalate (mg)	0.481±0.07	1.940±0.08[b]	0.620±0.24[d]	0.570±0.06 [d]
Phosphorus (mg)	2.970±0.69	3.150±0.46	2.853±1.23	2.761±0.56
Serum Values (mg/dl)				
Blood Urea Nitrogen	38.46±1.45	54.78±1.76[b]	36.24±2.32[d]	32.14±2.43[d]
Creatinine	0.564±0.07	0.948±0.08[b]	0.678±0.07[c]	0.640±0.05 [d]
Histopathology of Kidney				
Kidney Weight (gm)	0.64±0.02	1.45±0.03 [b]	0.78 ±0.04[a,d]	0.68±0.03[d]
Crystal Deposits	-	+++	+	-

Values are expressed in mean ± SEM (n=6), - Nil; +++ Numerous; ++ Few; + Rare
[a] p<0.05 compared with control group; [b] p<0.01 compared with control group;
[c] p<0.05 compared with untreated group; [d] p<0.01 compared with untreated group.

Kidneys excised from untreated group were larger and heavier than the control animals (p< 0.01), whereas in BDAE treated group kidneys were not significantly different from those of the control animals (**Table 1**). When observed under microscope, many birefringent crystalline

deposits in the histological preparations were seen in tubules of all regions of kidneys: cortex, medulla and papilla, of all the animals in the untreated group (**Fig.1b**). The renal tubules were also markedly dilated along with wide spreaded necrosis of tubular epithelium in the entire kidney of the in untreated rats. In BDAE treated groups, such deposits were found visibly small and rare with decreased renal cell injury and normalization of renal architecture (**Fig.1c&d**) as compared to those in the untreated rats

Discussion

A wide range of plants are used traditionally for their purported effect on the elimination of urinary calculi including *Boerhaavia diffusa* Linn. Root decoction of this plant is used traditionally in India by many lithiasis patients. Therefore, we evaluated the plant extract for its ability to eliminate the pre-existing CaOx deposition in kidneys.

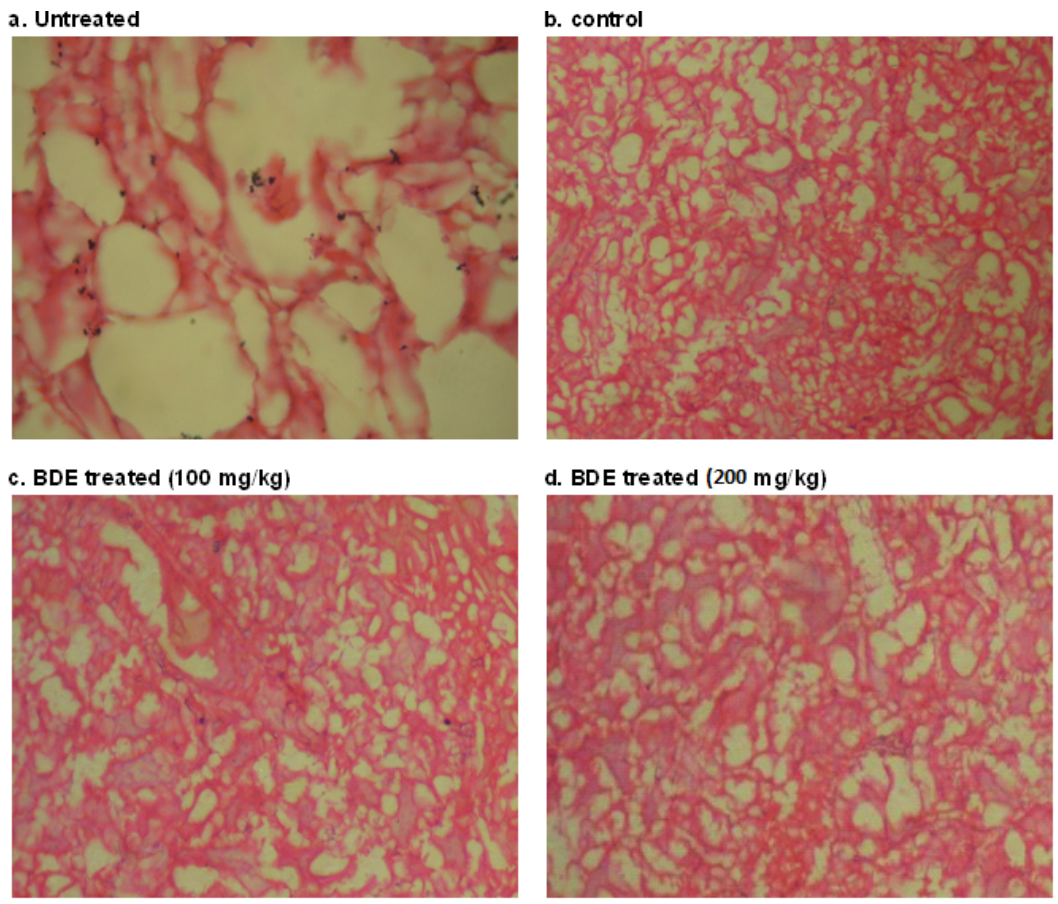

Figure 1: Histopathological examination of Kidney slides of different treatment groups groups seen under microscope (Leica DME) at 40×10X magnification (a) Kidney of Untreated rats showing polymorphic irregular crystal deposits inside the tubule, renal tubules were markedly dialated along with tubular epithelium necrosis;[in figure black spots are crystal deposits] (b) Control rats showing normal renal architecture (c) BDE treated [100mg/kg] showed few crystal deposits and little dilation of tubules (d) BDE treated [200mg/kg] showed a very few or no crystal deposits and nearly normal renal architecture.

Renal CaOx deposition induced by EG and ammonium chloride in rats and various parameter recorded for the assessment of antiurolithiatic activity are shown in **Table 1**. Oxalate is a natural byproduct of metabolism and in normal individuals is harmlessly excreted. However, increased urinary excretion of oxalate can be toxic largely because of its propensity to crystallize at physiologic pH and form calcium oxalate (9). EG metabolized to oxalate in the body with

subsequent increased oxalate excretion and ammonium chloride lowered the urine pH that accelerates the precipitation and deposition of CaOx crystals in the kidney of untreated rats during the first week. BDAE treatment after induction of urolithiasis lowered the oxalate excretion in a dose-dependent manner in treated rats probably by interfering the metabolism. However BDAE did not affected the level of calcium and phosphorus to a significant extent and no correlation can be established in excretion. BDAE at both the doses increased the urine output in a dose-dependent manner along with normalization of urine pH. Increased urine volume decreases the saturation of the oxalate and prevents the precipitation of the CaOx at physiological pH. Diuresis also increase the amount of fluid going through the kidneys and flush out the deposits (10).

The important change worth to be mentioned concerning crystalluria which was characterized by abundant excretion of crystals in untreated rats indicating an elimination of crystals already deposited and attached to renal lining cells when compared to treated ones. Microscopic observation revealed that BDAE visibly reduced the crystal size with significant decrease in number of crystal. COM and COD particles were observed in the urine from the first day of EG usage. Where treated rats excreted more COD crystals of a reduced size than COM .It is important to emphasize here, that the crystal structure changes in treated rats have several positive virtues. First, it shows that substances from the plant end up in the urine and excerpt their action directly or indirectly on crystals. Second, the appearance of more COD than COM particles is beneficial since COM crystals have high adhesion affinity to renal epithelial cells as compared to COD particles (5).

The antiurolithiatic effect was further confirmed by kidney histopatholological analysis. Indeed, kidney sections of untreated rat showed abundant CaOx crystal depositions. Furthermore, renal epithelial cells had more tubular dilatation and damage shown by large spaces in the tissue **(Fig. 1a)**. In treated rats, less CaOx crystal depositions were seen compared to untreated animals and the necrosis as well as the tubule dilatation was very limited **(Fig.1c&d)**.Renal stone deposition damages the renal tissue and detoriate the renal function. Lithogenic treatment caused impairment of renal functions of the untreated rats as evident from the markers of glomerular and tubular damage: raised BUN and serum creatinine (8), that was lowered in a dose-dependent manner in animals receiving a treatment with BDAE. Tissue injury and inflammation in these animals is due to exposure to oxalate and CaOx crystals, leading to the generation of reactive oxygen species, development of oxidative stress, lipid peroxidation and depletion of antioxidant enzymes (9). Renal epithelial injury further promotes crystal retention, as epithelial injury exposes a variety of crystal adhesion molecules on epithelial surfaces and promotes stone formation (11). Probably antioxidant constituents of *Boerhaavia diffusa* Linn restore the renal antioxidant enzyme and prevent renal cell injury (12).

Boerhaavia diffusa Linn. caused diuresis and hasten the process of dissolving the preformed crystal deposits, improved the renal function by increasing the removal of nitrogenous waste product and decreased the oxalate excretion probably by interfering with metabolism. All these activities synergistically attribute to the antiurolithiatic activity to *Boerhaavia diffusa* Linn.

Conclusion

Results of this study indicating the presence of antiurolithiatic effect of *Boerhaavia diffusa* Linn. against calcium oxalate stones. Diuretic effect of BDAE may be responsible for the mechanical expulsion of renal stone and hypooxaluric effect prevents further risk of renal stone. Thus, the present study offers scientific pharmacological evidence on the traditional use of *Boerhaavia diffusa* Linn for the treatment of urolithiasis.

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