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Prophylactic Role of *Boerhaavia diffusa* in Ethylene Glycol Induced Calcium Oxalate Urolithiasis

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ABSTRACT

Introduction: *Boerhaavia diffusa* Linn. (Family: *Nyctaginaceae*) is a widely used plant in India and Brazil as a traditional medicine for treatment of urolithiasis and other urinary disorders.
Objectives: The aim of this study was to evaluate the antiurolithic activity of *Boerhaavia diffusa* root aqueous extract (BDE) as prophylaxis for renal stones.
Methods: In vitro calcium oxalate (CaOx) crystallization inhibitory effect of BDE was determined by measuring change in turbidity at 620nm on addition of sodium oxalate in the synthetic urine. In a rat model of urolithiasis, induced by adding 0.75% ethylene glycol (EG) in drinking water and effect of simultaneous treatment of BDE (100-200 mg/kg) was observed for 28 days.
Results: BDE inhibited CaOx nucleation, aggregation and crystal formation in the synthetic urine in vitro on addition of NaOx. The lithogenic treatment caused polyuria, weight loss, hyperoxaluria and impairment of renal function which was prevented by BDE. Hyperoxaluria and CaOx crystal deposition in the renal tubules caused by EG intake was prevented by BDE treatment.
Conclusion: This study indicates that the antiurolithic activity of *Boerhaavia diffusa* extract possibly mediated through inhibition of CaOx crystallization, diuresis and hypo-oxaluria may justify its prophylactic use in urolithiasis.

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

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INTRODUCTION

Urolithiasis (the formation of kidney, ureteric or bladder stones) poses a severe health problem in mankind[1]. It affects 4-8% of the population in the UK, 15% in the US, 20% in Gulf countries and 11% in India. Epidemiological data suggest that 60-80% of stones are composed of calcium oxalate (CaOx). Unless prophylactic measures are taken, the stone recurrence rate is about 40% in 3 years, 75% in 10 years and during the next 25 years every patient has at least one more stone recurrence[1, 2].

Treatment procedures for renal stones such as surgical removal, percutaneous techniques and extracorporeal shock wave lithotripsy (ESWL) are prohibitively costly and with these procedures recurrence is quite common[3]. Complications include residual stone fragments, compromised renal function, acute renal injury and urinary tract infection[4]. However, herbal remedies have been found to be effective in reducing the recurrence rate of renal stones[3]. In India, in the Ayurvedic system of medicine, Pashanabheda (Pashana-stone; Bheda-break) is the Sanskrit term used for a group of plants with diuretic and antiurolithic activities. In Indian folk medicine many plants have been used to treat kidney stones, including *Boerhaavia diffusa*.

Boerhaavia diffusa Linn., commonly known as Punarnava in India and Erva tostão in Brazil is a herbaceous plant of the family *Nyctaginaceae*. The roots of *Boerhaavia diffusa* have held an important place in herbal medicine in both Brazil and India since ancient time. It is used to treat gallbladder stones, liver disorders (jaundice and hepatitis), urinary tract and renal disorders including kidney stones[5,6]. Root decoctions of this plant are used to treat kidney stones in many regions of India[7]. The plant is also included in the Indian Pharmacopoeia 2006 as a diuretic agent. The herbal extract of *Boerhaavia diffusa* inhibits the growth of struvite crystals in vitro[3], suggesting that it may have antiurolithic activity. *Boerhaavia diffusa* is also an important ingredient of a polyherbal formulation Cystone (Himalaya Health Care Pvt. Ltd) widely used for the treatment of renal stone disease. However, no scientific study has been conducted to justify the use of plants in urolithiasis.

The aim of this study was to establish the scientific validity for the prophylactic role of *Boerhaavia diffusa* root extracts in urolithiasis, using an ethylene glycol induced hyperoxaluria model in rats.

MATERIAL AND METHODS

Plant material and extraction

Boerhaavia diffusa was collected from Birla Institute of Technology, Mesra campus Ranchi, India during August. A sample of the plant material was submitted to the herbarium of the Botanical Survey of India in Kolkata. The plant was authenticated as "*Boerhaavia diffusa* Linn. variety red".

The dried roots of the plant were cleaned of dirt and ground to powder, using a commercial mill. Approximately 500 gm of the plant powder was soaked in 2.5 liter water at room temperature for 24 hours with occasional shaking. It was filtered through a single layer of muslin cloth and the final filtrate was collected by passing it through a What man grade 1 filter paper in a Buchner funnel under vacuum. The filtrate was evaporated to dryness on a rotary evaporator under reduced pressure. A thick and dark brown material (52 gm), the crude *Boerhaavia diffusa* extract (BDE) was obtained, the approximate yield was 10.4%.

Animals

Twenty four inbred male Wistar rats (180-200 gm body weight) were used. Animals were procured from the Institutional Animal House of Birla Institute of Technology, Mesra. All animals were kept in polyacrylic cages and maintained under standard housing conditions (room temperature 24-27°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 complied with the ethical standards of animal handling and were approved by the Institutional Animal Ethics Committee and CPCSEA India.

In vitro study: CaOx crystallization assay in synthetic urine

The effect of BDE on CaOx crystallization was determined by the time course measurement of turbidity changes due to crystal nucleation and aggregation in the synthetic urine on addition of sodium oxalate (NaOx). The precipitation of CaOx at 37°C and pH 6.8 was studied by the measurement of turbidity at 620 nm. A spectrophotometer UV/Vis (Shimadzu 1700) was employed to measure the turbidity of the formation of CaOx. Aliquots of 2 ml of synthetic urine were pipetted into test tubes and 0.2 ml of BDE of various strength (5%, 10% and 20%) were added and incubated at 37°C for 30 minutes. 0.2ml distilled water instead of BDE was used as control. Finally 1 ml of 4 mM NaOx was added and absorbance was measured at 620 nm. Tests were performed in triplicate[8].

Optical density (OD) was measured over 10 min and the percentage of inhibition produced by the additions was calculated by % inhibition = {1-(St / Sc)} X 100. Where; St: slope of graph in the presence of BDE, Sc: slope of graph in control experiments[9].

In vivo study: Antiurolithic activity

The rat model of ethylene glycol (EG) induced hyperoxaluria described by Atmani et al 2003[10] was used to assess antiurolithic activity in vivo. Male Wistar rats weighing 180-200 gm were housed in metabolic cages three days prior to the start of the experiment The animals were divided into four groups comprising six animals each. EG was added to their drinking water for 28 days to induce a chronic low grade hyperoxaluria and precipitate CaOx deposition in the kidneys. Simultaneously, BDE was administered to the treatment group. One tenth of LD50 was selected as the higher dose and half of this was selected as the lower dose. Group 1 was used as normal control and was given water only; Group 2 was given 0.75% v/v EG in drinking water and served as the untreated nephrolithiasis group. Group 3 was given 0.75% v/v EG in drinking water plus 100 mg/kg p.o. of BDE and served as the treated nephrolithiasis group. Group 4 was given 0.75% v/v EG in drinking water plus 200 mg/kg p.o. of BDE and served as the treated nephrolithiasis group. During the study of 28 days various urine and serum biochemical parameters as well as histopathology of the kidneys were assessed for antiurolithic activity.

General observations

During the study period body weight, water intake and animal health was observed regularly, so that stressed and unhealthy animal were excluded from study[11].

Urine analysis

All animals were kept in individual metabolic cages and 24 hour urine samples were collected daily. Animals had free access to drinking water during the urine collection period. Volume and pH were measured immediately after collection. A drop of concentrated hydrochloric acid was added to the urine before being stored at 4°C[12]. Urine was analyzed for calcium, phosphate and oxalate content using commercial kits. A 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 placed on malassez cell and examined microscopically (Leica DME)[13]. The number of crystals was expressed in crystals/mm3

Serum analysis

After the experimental period, blood was collected by cardiac puncture under anesthetic conditions and the animals were sacrificed. Serum was separated by centrifugation at 10.000×g for 10 min and analyzed for creatinine and blood urea nitrogen[12].

Kidney homogenate analysis

The abdomen incised to remove both kidneys from each animal. Isolated kidneys were cleaned of extraneous tissue. Left kidneys were preserved in 10% neutral formalin for histopathological examination and right kidneys were dried at 80°C in a hot air oven. A sample of 100 mg of the dried kidney was boiled in 10 ml of 1N hydrochloric acid for 30 min and homogenized. The homogenate was centrifuged at 2000×g for 10 min and the supernatant was separated[14]. The calcium, phosphate and oxalate content in kidney homogenates was determined.

Histopathological examination

The left kidneys were fixed in 10% formalin solution in 0.1M phosphate buffer saline. dehydrated in ascending grades of alcohol and embedded in paraffin. Sections of 6µm thickness were taken, stained with hematoxylin and eosin and examined histologically under a light microscope (Leica DME)[13].

Statistical analysis

Data are expressed as mean ± standard error of mean (SEM). Comparisons between groups were made by means of one way analysis of variance (ANOVA) with post hoc Dunnett's t-test ($p<0.01$ regarded as significant). Concentration response curves were analyzed by linear regression using Graphpad Prism 5 software.

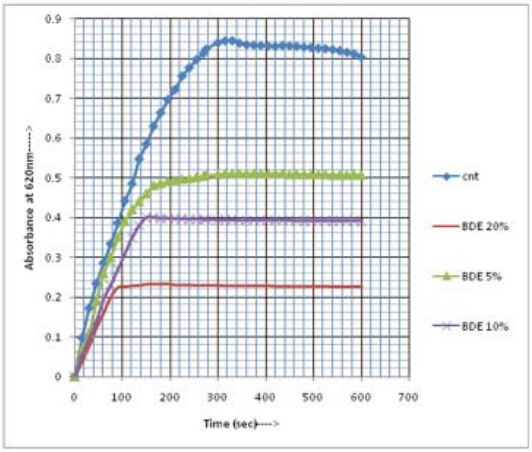

Fig. 1: Change in turbidity with time course measured by variation in absorbance at 620nm with and without inhibitor (BDE); various curves showing the percentage inhibition of calcium oxalate crystallyzation with BDE with respect to control.

RESULTS

Effects on in-vitro CaOx crystallization

The effect of BDE on various phases of CaOx crystallization was determined by time course measurement of turbidity in the urine. Fig. 1 shows an initial detectable increase in the turbidity after induction of the crystallization with NaOx. In the control experiment it shows the initial steep rise in turbidity (nucleation phase) and on attaining its maximum it was followed by a decrease in turbidity (aggregation phase). BDE decreased the slope of turbidity in a concentration dependent manner as shown in Fig. 1. Crystallization was inhibited by19.7%, 33. 8% and 57.2% at 5%, 10% and 20% BDE, respectively.

Effect observed in animal model

Body weight, water intake, urine volume and pH before the start of experiment were not significantly different among the study groups. The parameters recorded during the experimental period are shown in Table 1. The mean weight of the untreated group decreased significantly ($p<0.01$) compared to the normal and treated groups. However, the BDE treated groups did not show any significant change in body weight. Water intake significantly ($p<0.01$) increased in all groups compared to the control group.

EG treatment reduced the urine pH in the untreated group compared to that of the control group, although not significantly. Co-treatment with BDE increased urine volume ($p<0.01$) in a dose-dependent manner, although these parameters remained higher than those of the untreated animals even at 100 mg/kg ($p<0.05$). EG treatment also increased oxalate excretion ($p<0.01$) in the untreated animals. BDE at 100 and 200 mg/kg prevented the change in urinary oxalate ($p<0.05$ and $p<0.01$, respectively). Other changes in urine composition induced by the lithogenic treatment such as calcium and phosphate excretion were not statistically significant (Table 1).

Microscopy revealed that the urine of hyperoxaluric rats contained numerous CaOx monohydrate (COM) crystals (dumbbell-shaped) and CaOx dihydrate (COD) crystals (bipyramidal shaped) whereas the urine of control rats was devoid of crystals (Fig. 2a and 2b). At 100 and 200 mg/kg BDE reduced the crystal size with significant decrease in number ($p<0.01$) (Fig. 2c and 2d) and (Table 1).

Lithogenic treatment impaired renal function in the untreated rats as evident from raised BUN and serum creatinine ($p<0.01$), which was dose-dependently prevented in the animals receiving BDE (Table 1). Serum oxalate increased in untreated rats ($p<0.01$). However, serum calcium and phosphate l did not show significant changes.

The kidneys were larger and heavier in the untreated group, but not in the BDE treated groups, compared with the control group ($p < 0.01$). The calcium and oxalate content of the kidneys was significantly ($p<0.01$) increased in the untreated group, but not in the BDE treated groups (Table 1).

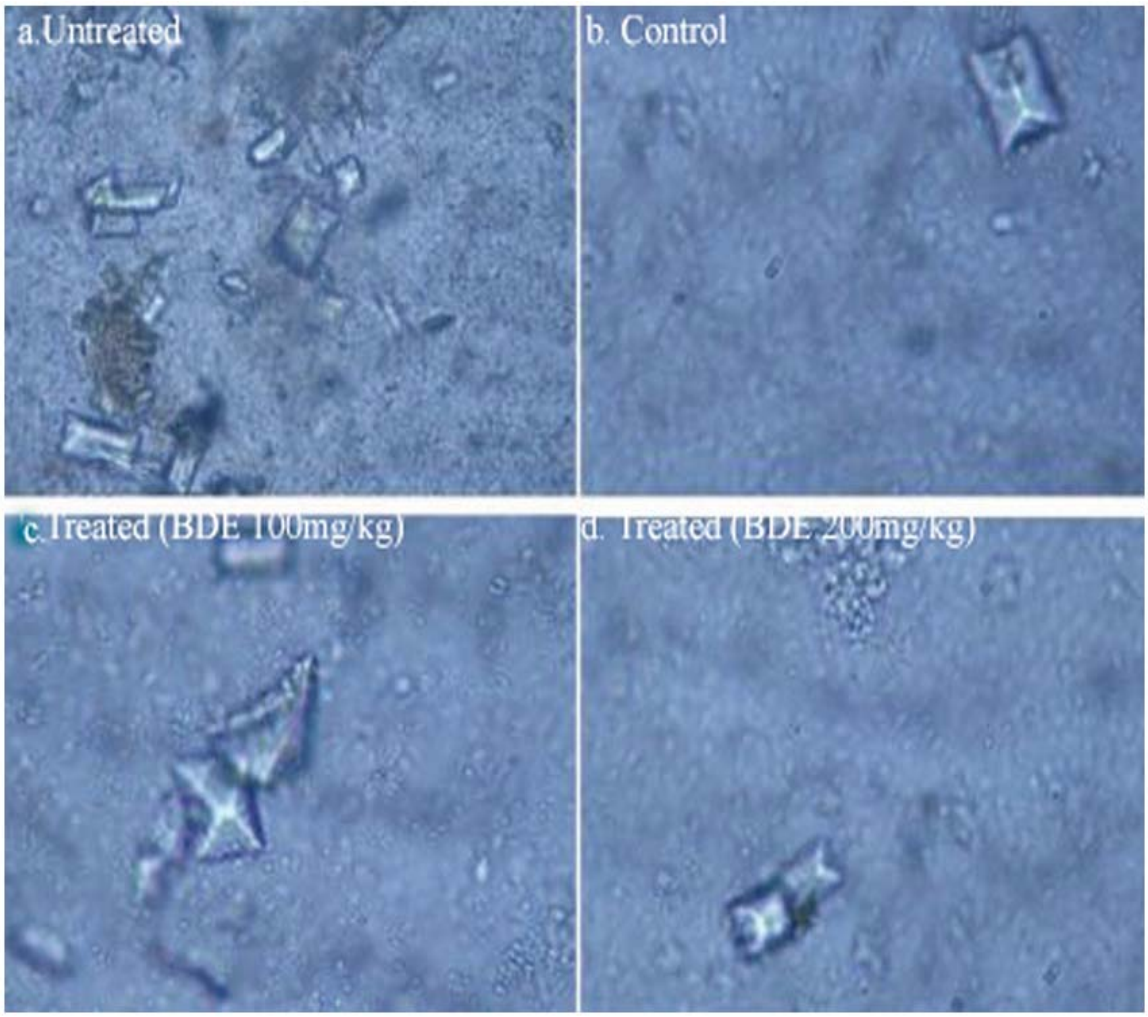

Fig. 2: CaOx crystals in the urine of different treatment groups seen under a microscope (Leica DME) at 40×10X magnification (a) Untreated rats excreted numerous oval COM and pyramidal shaped COD (b) Control rats showed very few or no crystals (c & d) Treated rats excreted significantly reduced number of COD and few COM.

On microscopy many birefringent crystalline deposits were seen in all regions of the kidneys of all animals in the untreated group (Fig. 3b). In the BDE treated groups such deposits were comparatively small and rare (Fig. 3c and 3d). In untreated rats the renal tubules were also markedly dilated, with widespread necrosis of the tubular epithelium and deposition of birefringent oxalate crystals.

Other findings observed in untreated rats included generalized congestion and diffuse petechial haemorrhage in internal organs. The kidneys appeared swollen with pale cortex and dark medulla.

DISCUSSION

Kidney stone formation is a physico-chemical process including various events starts with supersaturation, nucleation, growth, aggregation, and retention within renal tubules[15]. Different in-vitro models were used to study various physico-chemical events and simulate the urinary conditions by various authors[16]. We have evaluated the effect of BDE on CaOx crystallization kinetics by the time course measurement of turbidity. Since the initial events of nucleation occurs in the first few minutes, the graphs were re-plotted within the first 3-min for each of BDE concentration as well as for control[17].

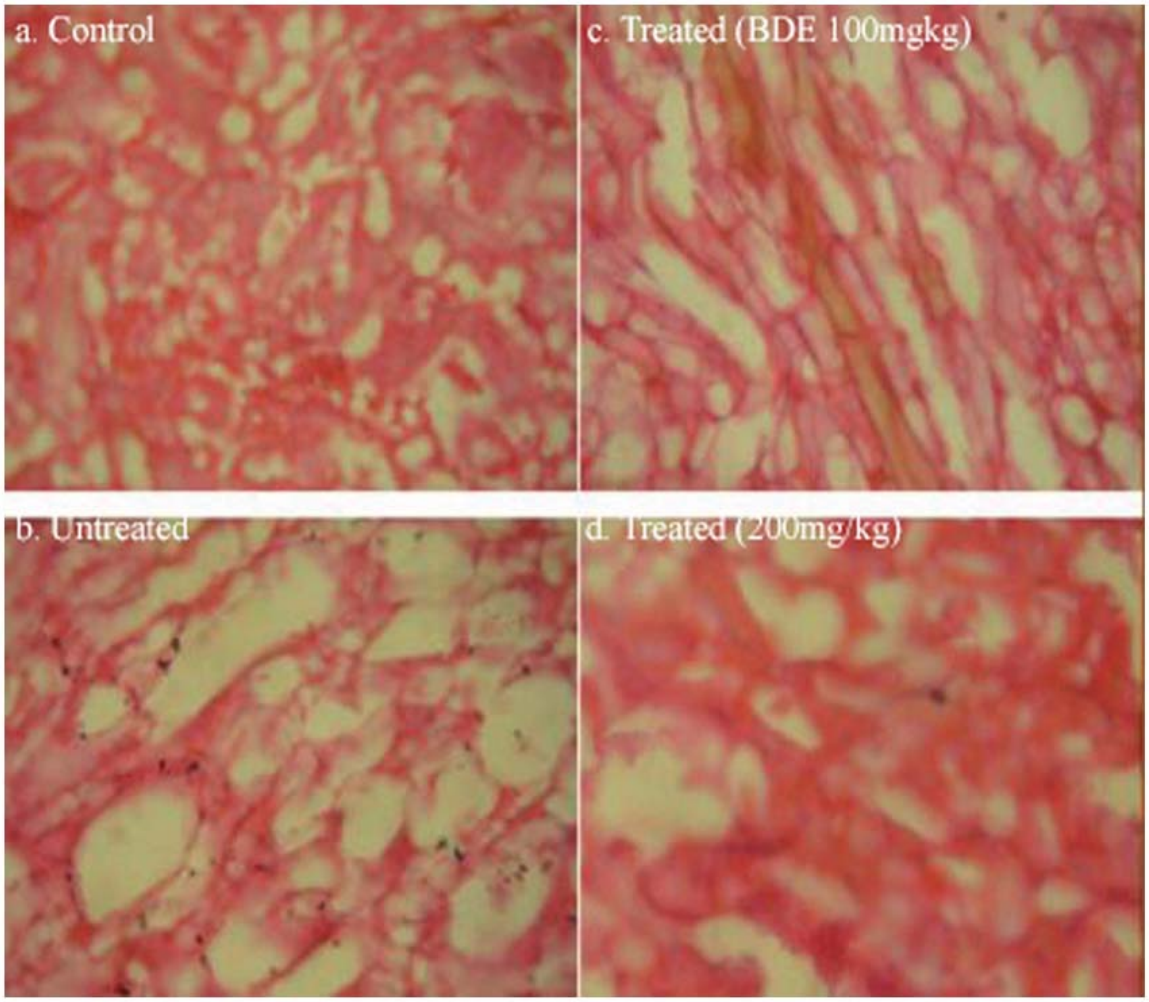

Fig. 3: Histopathological examination of kidney slides of different treatment groups seen under a microscope (Leica DME) at 40×10X magnification (a) Control rats showing normal renal architecture (b) Kidneys of untreated rats showing polymorphic irregular crystal deposits inside the tubule, renal tubules were markedly dilated along with tubular epithelium necrosis;(in the figure black spots are crystal deposits) (c) BDE (100mg/kg) treated rats showed few crystal deposits and little dilatation of tubules (d) BDE (200mg/kg) treated rats showed a very few or no crystal deposits and nearly normal renal architecture.

The initial positive slope of the turbidity curve shows increase in number of particle during nucleation phase and later plateau (negative slope) reflects the decrease in the particle number, during crystal aggregation phase[18]. In this study, BDE inhibited the CaOx crystal nucleation and aggregation in a concentration dependent-manner. Ability of BDE to reduce the nucleation increases the metastable limit of oxalate in urine and prevents the precipitation of the CaOx crystal in kidney. Various physiological inhibitors of urolithiasis found in urine including citrate, glycosaminoglycans and other macromolecules. Citrate preferentially binds to calcium to form soluble salt and inhibit nucleation, whereas glycosaminoglycans and other macromolecule inhibit aggregation and growth by adsorbing over the surface of crystals[19]. Interference with crystal growth and aggregation therefore seems a possible therapeutic strategy for the prevention of recurrent stone disease. The medicinal plants contain chemical compounds which themselves possess an inhibitor effect in the crystallization of CaOx. Macromolecule of higher molecular weight of plant extract excerpts their action similar to natural urinary inhibitors and inhibits crystal aggregation and growth. It is reported that *Boerhaavia diffusa* have some macromolecular constituent (>20KD) which may attribute this property[6].

Table 1: Various parameters recorded for assessment of antiurolithic activity during 28 days of study.

	Parameter	Control	Untreated	Treated BDE 100 mg/kg	Treated BDE 200 mg/kg
General	Change in body weight (gm)	4.66±1.49	-7.33±5.21[b]	2.17±1.33	2.51±1.69
	Water intake (ml/24hr)	10.9±0.13	14.67±0.38[b]	18.42±0.57[b,d]	29.92±1.08[b,d]
24 hour Urinalysis	Volume (ml/24hr)	6.77±0.48	12.15±0.34[b]	16.04±0.36[b,d]	24.03±1.29[b,d]
	pH	6.8±0.03	6.4±0.06.	6.8±0.04	6.8±0.05
	Crystalluria (crystals/ mm^3)	4.33±1.15	125.0±7.30[b]	14.0±1.21[d]	23.67±1.15[d]
	Calcium (mg)	0.56±0.12	0.71±0.21	0.61±0.06	0.54±0.11
	Oxalate (mg)	0.46±0.06	1.85±0.61[b]	0.72±0.29[a,d]	0.55±0.05[d]
	Phosphorus (mg)	2.87±1.7	3.01±0.46	2.83±1.53	2.61±0.57
Serum Values	Blood urea nitrogen (mg/dl)	37.21±1.91	50.67±2.28[b]	36.13±2.01[d]	27.91±1.68[c,d]
	Creatinine (mg/dl)	0.57±0.08	0.87±0.03[b]	0.58±0.06[c]	0.36±0.09[d]
	Phosphorus (mg)	2.45±0.25	2.08±0.36	2.23±0.92	2.14±0.59
	Oxalate (mg)	0.29±0.03	1.82±0.46[b]	0.34±0.08[d]	0.36±0.37[d]
	Calcium (mg)	3.12±1.05	3.32±0.49	3.26±0.46	2.34±0.82
Kidney homogenate analysis	Weight (gm)	0.62±0.03	1.42±0.23[b]	0.74 ±0.04	0.64±0.03
	Calcium (mg)	0.20±.026	1.29±0.17[b]	0.49±0.07[d]	0.30±0.08[d]
	Oxalate (mg)	0.27±0.05	1.58±0.15[b]	0.56±0.21[d]	0.24±0.05[d]
	Phosphorus (mg)	2.38±0.17	2.52±0.01	2.17±0.67	2.89±0.79

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

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.

Renal CaOx deposition induced by EG in rats is frequently used by various researchers to mimic the urinary stone formation in humans[10]. Oxalate is produced during metabolism and excreted harmlessly in normal individuals. However increased concentration of oxalate in urine can be highly toxic because it crystallize at physiological pH to form CaOx[20]. EG metabolise to oxalate in body and increase its excretion in the untreated group, which was prevented by BDE in a dose-dependent manner. However it did not affect the level of calcium and phosphorus to a significant extent and no correlation can be established in excretion. Since it is accepted that hyperoxaluria is a far more significant risk factor in the pathogenesis of renal stones than hypercalciuria. Increased oxalate concentration is responsible for precipitation of CaOx crystals. BDE increased urine volume in treated animals as compared to untreated animals. Increase urine volume decreases the saturation of the oxalate and prevents the precipitation of the CaOx at physiological pH. Diuresis also flushing out the renal system and help in mechanical expulsion of the stone.

The analysis of crystalluria after 28 days of treatment with CaOx stone inducing agents showed that untreated animals excreted abundant and larger crystals than the treated animals. BDE significantly prevented the crystalluria associated with lithogenic

treatment. Patients with renal stone excrete larger and aggregated particles than the healthy individuals[21]. Change in crystal morphology have thereupatic virtues; first, it shows that substances from the plant excerpt their action directly or indirectly on crystal morphology. Second, the appearance of more COD than COM particles is advantageous since COM crystals have high adhesion affinity to renal epithelial cells[22].

In the present study, oxalate and calcium level found to be increased in untreated rats in kidney homogenate which is also evident from abundant CaOx crystal deposit in kidney. Increased calcium level is a factor favouring the nucleation and precipitation of CaOx or apatite (calcium phosphate) from urine and subsequent crystal growth[23].

CaOx crystal agglomerates, tends to retain in kidney by trapping in renal tubules and develop into renal stones. Renal stone deposition damages the renal tissue and detoriate the renal function. Treatment with EG leads to decrease in renal function, characterized by elevated level of BUN and serum creatinine and fall in creatinine clearance[12], which were prevented dose-dependent manner in the animals receiving a simultaneous treatment with BDE. Stone inducing treatment caused hypertrophy and extensive CaOx crystal deposition in kidneys of untreated rats, accompanied by oxidative damage as reflected from glomerular and tubular damage seen in the histopathological examination of kidney tissue (Fig. 3b). Renal cellular exposure to oxalate and CaOx crystals leads to the production of reactive oxygen species, development of oxidative stress followed by injury and inflammation. Renal injury and inflammation appear to play a significant role in stone formation[21]. BDE treatment significantly protected the kidney from crystal induced renal cell injury and other damage thus inhibited the CaOx crystal deposition in kidneys of rats.

CONCLUSIONS

Results of this study indicate the presence of antiurolithic effect of *Boerhaavia diffusa* aqueous extract against CaOx stones in a dose depending manner. It inhibits the CaOx crystal deposition and protect kidney from crystal induced oxidative stress and renal cell injury, possibly mediated through a combination of CaOx crystal inhibitory, hypoxaluric and diuretic activity. The study rationalizes its medicinal use for treatment of recurrent CaOx urolithiasis as a prophylactic agent.

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