

# Prophylactic and Curative Activity of '*Barleria Buxifolia*. Linn' on Experimentally Induced calcium Oxalate Nephrolithiasis in Animal Model

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## ABSTRACT

**Background:** Increased level of urinary oxalate increases the risk of hyperoxaluria and followed by Nephrolithiasis. *Barleria buxifolia* having flavonoid and saponins showing more active diuretic and antioxidant property. **Objectives:** The main objective of the study is to evaluate the reduction of urinary oxalate crystal formation by treating with methanol extraction of the test drug '*Barleria buxifolia*' on experimentally induced Nephrolithiasis in animal model. **Methods:** In the present study, methanol extract of whole plant of *Barleria buxifolia* was evaluated for their Antiuro lithiatic potential in albino rats of Wistar strains (Five groups containing five animals each). Nephrolithiasis was induced by administering 0.75% Ethylene glycol supplemented with 2% Ammonium chloride. Urinary microscopy and histopathology were used as criteria for assessing the preventive or curative effect of the plants. The elevated serum creatinine levels of lithiatic rats were reduced in both groups (prophylactic and curative) by the administration of the extract treatment (once daily for 28 days by p.o route). **Result:** Assessment of Antiuro lithiatic activity was determined by the urine and blood analysis; which gave supportive evidence of Antinephrolithiatic activity, (all p values < 0.05). Experiment findings also resulted in reduction in the stones compared to the control group, but neither was significantly reduced. **Conclusion:** Prophylactic and curative property of *Barleria buxifolia* against induced Nephrolithiasis showed concurrent reduction in the formation of Lithiasis in animal. So the test drug can be used as therapeutic agent. **Key words:** Creatinine, Nephrolithiasis, Calcium oxalate, Ethylene glycol, *Barleria buxifolia*.

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## INTRODUCTION

Urolithiasis is a complex process including super saturation, nucleation, growth, aggregation and deposition of the solid non metallic minerals within the kidney and renal tubules. [1] The common Nephrolithiasis is calcium oxalate lithiasis. Hyperoxaluria is the major risk factor in calcium oxalate lithiatic disease [2] In rats, hyperoxaluria can be induced by various diets. Extra Corporeal Shock Wave Lithotripsy [ESWL] and Open renal surgery is the treatment of choice in nephrolithiasis. [3] The traumatic effects of shock waves, persistent residual stone fragments and the possibility of infection causes acute renal injury, decrease in renal function and increase in stone recurrence. Production of calcium oxalate and crystals deposition induces lipid peroxidation. The generation of lipid peroxidation due to reactive oxygen species causes renal epithelial cell injury which promotes

calcium oxalate lithiasis formation by providing cellular debris for crystal nucleation, aggregation and augments crystal attachment to other tubular cell. [4] Antioxidants prevent calcium oxalate nucleation and retention in the renal tubules by preventing oxalate mediated peroxidative injury. [5] The inhibitory effect of some herbals on calcium

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oxalate urolithiasis is claimed by antioxidant effects<sup>[6]</sup> though the drug treatment effected in many randomized trials, it is without side effect by phytotherapy. The literature survey revealed that *Barleria buxifolia* is endowed with various activities such as antibacterial and cyto-toxic activities.<sup>[8-10]</sup> The diuretic activity has been shown by other plants of the same family.<sup>[11]</sup> Earlier study has proved that the plants contain Saponins and Flavonoid have diuretic and antioxidant activities.<sup>[7, 9]</sup> The aim of the present study was to evaluate the Prophylactic and curative activity of '*Barleria buxifolia*. Linn' on experimentally induced calcium oxalate Nephrolithiasis in animal model.

## MATERIALS AND METHODS

### *Plant material and preparation of extract*

The whole plant of *Barleria buxifolia*. linn (locally called as *katti mullu*) was collected from Trivandrum district, Kerala and authenticated at the University of Kerala by the Head of botany department. A voucher specimen of the plant was deposited in the departmental herbarium for future reference. (Specimen Number KUBH 5846). The whole plants were washed with distilled water to remove dirt and soil, dried in shade and finally powdered in grinder. The powder of whole plants was defatted with petroleum ether. The powder material (500 g) was extracted thrice with 80% methanol (v/v) using Soxhlet apparatus and microwave assisted (Panasonic, Japan, Modal No: NN-CT641M) aqueous extraction (15 S power on, 15 S power off for water as solvent, and then 3 min power on for heating and 30 S power off for cooling). The extracts were filtered, pooled, evaporated at 45°C on a rotary evaporator (Dolphin-India) and then dried at room temperature. The 80% methanol extract and aqueous extract were stored at 4°C and re-suspended in double distilled water containing 1% Tween 80 at the time of administration.

### *Phytochemical Studies*

Standard Phytochemical screening was carried out for various phytoconstituents of the Methanol extract and Microwave assisted aqueous extract of whole plant of '*Barleria buxifolia*' according to the methods of Trease and Evans.<sup>[12-14, 29]</sup>

### *Drugs and chemicals*

Ethylene glycol (B.No:0013100500) was purchased from (Loba chemie pvt. Ltd, India), Tween 80, Formaldehyde (Himedia Laboratories Pvt. Ltd, Bombay, India) and

all other chemicals and reagents used were of analytical grade and procured from approved vendors. Biochemical estimation diagnostic kits were purchased from Agappe biosystem, India. Standard drug (cystone) was procured from Himalaya drug company, India. All drugs and solutions were freshly prepared.

### *Animals*

Thirty healthy male wistar albino rats weighing between 150 and 250 g were used for the study. The animals were acclimatized for 7 days before experiment commenced. The animals were housed in polypropylene cages and maintained at 27 ± 2°C, relative humidity 65 ± 10% under 12 h light/dark cycles. The animals were fed with standard pellet diet and clean drinking water allowed *ad libitum*. The food was withdrawn 18-24 h prior to the experiment. The experimental protocol was duly approved by the institutional animal ethical committee (IAEC) vide letter no: SKCPRC/2014-2015/IAEC/09 and the care of the animal was done as per the guidelines of the committee for the purpose of control and supervision of experiments on animals (CPCSEA) ministry of environment and forest Government of INDIA.(Reg: No; 1551/PO/a/11/CPCSEA).

### *Acute toxicity study*

Acute toxicity study was carried out as per OECD, guidelines 425. The acute toxicity of 80% methanol extract '*Barleria buxifolia*' was evaluated in female wistar albino rats (B/W 150 and 250 g) using the up and down procedure. Extract was administered orally in the escalating dosages up to 2000 mg/kg to different groups of rats (n=5 in each), the animals were observed for behavioral and physiological variations (Toxic symptoms) continually for first hour after dosing. Number of survivors was noted after 24 h, and these animals were then maintained and observed daily for next 14 days for any further toxicity. One-tenth of the median lethal dose was taken as an effective dose.<sup>[15, 16, 24, 25]</sup>

### *Anti-urolithiasis activity against ethylene glycol induced urolithiasis*

The effect of methanol extract of '*Barleria buxifolia*' was measured against ethylene glycol induced urolithiasis. Animals were divided into five groups containing five rats in each and were housed in metabolic cages individually for entire duration of the experiment. All animals had free access to regular rat chow and drinking water *ad libitum*. Renal calculi were induced in group II to V by ethylene glycol (0.75%, v/v) and ammonium chloride (2%, w/v) in drinking water *ad libitum* for 28 days.<sup>[10]</sup> Group I served

as control and group II served as lithiatic control, Group III received standard regimen (cystone 750 mg/kg, *p.o.*) from 15<sup>th</sup> day till 28<sup>th</sup> day.<sup>[9,27]</sup> Group IV were treated with methanol extract of entire plant of '*Barleria buxifolia*' starting from 15<sup>th</sup> day to 28<sup>th</sup> day (Curative regimen). Group V were treated with methanol extracts of entire plant of '*Barleria buxifolia*' starting from 1<sup>st</sup> day to 28<sup>th</sup> day (prophylactic schedule). The urine was collected (on the 14<sup>th</sup> and 28<sup>th</sup> days) from all the animals and serum samples from each rat were collected only on 28<sup>th</sup> days, 24 h after the treatments.<sup>[17,18]</sup> Urine samples were then analyzed for creatinine and uric acid concentration (Autoanalyzer, Agappe-neo), sodium, potassium, and chloride ion excretion (electrolyte analyzer, Biolyte 2000, Taiwan) and pH (pH meter, model EQ-614). On 29<sup>th</sup> day the animals were sacrificed. Their kidneys were harvested, one kidney of each animal was processed for histopathology and another kidney was used to determine  $CaOx$  deposition.

### Assesment of Antirolithiatic Activity

#### *Collection and analysis of urine*

All animals were kept in individual metabolic cages and 24 h urine samples were collected in presence of sodium azide as an antibacterial agent during the urine collection days (14<sup>th</sup> and 28<sup>th</sup> day). Animals had free access to water during the study period. Urinary components, like water intake, urinary volume and pH were noted. Urine was analyzed for calcium, urea, creatinine, phosphorus, magnesium, and oxalate content. Urine and kidney calcium oxalate concentrations were determined by method described by Hodgkinson *et al.* with some modifications. In contrast, 1 ml of urine was acidified with concentrated Nitric acid ( $HNO_3$ ) to solubilize crystals and then adjusted to pH 7 by sodium hydroxide (NaOH) in the presence of color indicator Bromothymol blue. About 2 ml of saturated calcium sulphate ( $CaSO_4$ ) and 14 ml of pure ethanol were added to precipitate overnight. The sample was centrifuged at 2000 r.p.m for 20 mts and then filtered. The precipitate obtained was solubilized in 10 ml of distilled water acidified by 2 ml of concentrated Sulfuric acid. The samples were titrated by a solution of potassium permanganate.<sup>[11,19]</sup>

#### *Serum analysis*

At the end of the experimental period, rats were anaesthetized and blood was collected from the retro-orbital region. Serum separated by the centrifugation tubes was kept aside for 30 min to prevent hemolysis. The serum was estimated for calcium, potassium, urea and creatinine using the respective diagnostic kits.

#### *Kidney homogenate analysis*

The abdomen was cut open to remove the both kidneys from each animal. Isolated kidneys were cleaned and preserved in 10% neutral formalin. The kidneys were then 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  $2000 \times g$  for 10 min and the supernatant was separated. The calcium and oxalate content in the kidney homogenate was determined.<sup>[20,21]</sup>

#### *Urinary microscopy*

Clean five glass slides were collected and labeled as normal, positive control, standard, curative and prophylactic regimen. A drop of collected urine samples was placed on the appropriate slides using micro pipette and allowed the urine drops evenly to spread over the slide. Slides were allowed for drying under room temperature in a dust free place. Then observed through microscope under polarized light (100X).<sup>[22]</sup>

#### *Histopathology studies*

Rats were euthanized, abdomen was cut opened, and the kidney was removed. The kidneys were preserved in formalin (10%), processed in a series of graded alcohol and xylene (under Leica TP 1020), embedded in paraffin wax, cut at 3-5  $\mu m$  intervals, and the slices were stained using hematoxylin and eosin. Tissue slices were photographed using optical microscopy (Leica DM 2000) under polarized light.<sup>[23,26]</sup>

### Statistical Analysis

Results were expressed as mean  $\pm$  standard error of mean (SEM). Differences among mean of the groups were determined using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test.  $P < 0.05$  was considered statistically significant and  $p < 0.01$  was considered highly significant.

## RESULT

The presence of various phytoconstituents like tannins, carbohydrates, saponins, phytosterols, and phenolic compounds were observed in methanol extract. Flavonoid, amino acids, carbohydrates, saponins, phenolic compounds were observed in microwave assisted aqueous extract by preliminary photochemical screening.

From the acute toxicity study, the LD<sub>50</sub> cut off dose was found to be 2000 mg/kg body weight for *Barleria buxifolia*. Hence, the therapeutic dose was taken as 200 mg/kg body weight for the extract. The values reported in Table 1, revealed that urinary volume and pH were similar in all the groups at the beginning of the experiment. During the experiment, the values remained mostly constant for control group but increased in the groups which received 'Ethylene glycol + Ammonium chloride only' Ethylene glycol + Ammonium chloride' plus methanolic extract of *Barleria buxifolia*, and 'Ethylene glycol + Ammonium chloride' + cystone. Both the urinary pH and urinary volume were significantly decreased in untreated Nephrolithiasis rats (group II), as compared with control (group I). on the 28<sup>th</sup> day (P<0.01), Group III, IV and V showed a significant increase in urinary volume on 14<sup>th</sup> and 28<sup>th</sup> days as compared to Group II (P<0.05). Urinary pH was constant for control group throughout the experiment, while group II showed significant decrease as compared to control on 14<sup>th</sup> day (P<0.05). Treatment with methanolic extract of *Barleria buxifolia* 200 mg/kg body weight (Curative and preventive regimen) and cystone 750 mg/kg body weight (standard drug regimen) elevated the decreased urinary pH to normal. The present study stated that, chronic administration of 0.75% (V/V) Ethylene glycol

followed by 2% Ammonium chloride aqueous solution for 28 days resulted in hyperoxaluria in both sexes of wistar rats. Calcium oxalate excretion was significantly increased (P<0.01), where as magnesium decreased in the urine of ethylene glycol treated animals group II as compared to group I ( Table 2). But treatment with methanolic extract of *Barleria buxifolia* at 200 mg/kg (curative and prophylactic) body weight and cystone 750 mg/kg significantly (P<0.05) lowered the elevated levels of oxalate and calcium in urine and kidney as compared to the untreated Group II animals (Table 2 & 4). Magnesium level in the standard and test group IV came close to normal and was comparable to the levels in rats belonging to the untreated Group II (Table 2).Serum uric acid was significantly increased in calculi-induced animals (group II) which indicated marked renal damage (Table 2). The treatment with methanolic extract of *Barleria buxifolia* (group IV and V) and cystone (group III) significantly (p<0.01) lowered the elevated serum level of uric acid as compared to group II (Table 4).

### Urinary Microscopy (X100)

Urinary microscopy revealed that there were no calcium oxalate crystals in rats on vehicle treatment Group 1, but many calcium oxalate crystal observed on the calculi induced

**Table 1: Effect of methanolic extract of *Barleria buxifolia*, on parameters of urinary excretion in control and experimental animals**

parameters	Days	Treatment groups				
		Group 1 Normal	Group II Lithiatic control (EG)	GroupIII cystone treated (standard)	Group IV curative regimen	Group V preventive regimen
Urinary volume(ml)	14	15.21 ± 1.88	16.08 ± 3.12 <sup>tb</sup>	16.16 ± 2.44	18.66 ± 2.94	19.00 ± 2.01
	28	16.50 ± 1.23	12.50 ± 1.38 <sup>ra</sup>	20.33 ± 1.96 <sup>tb</sup>	18.86 ± 1.72 <sup>tb</sup>	20.16 ± 1.13 <sup>tb</sup>
Urinary pH	14	7.00 ± 0.00	6.56 ± 0.21 <sup>ra</sup>	7.03 ± 0.25	6.83 ± 0.01	6.81 ± 0.16
	28	6.38 ± 0.20	6.17 ± 0.3	7.16 ± 0.25 <sup>tb</sup>	6.83 ± 0.01	6.83 ± 0.16 <sup>tb</sup>

All the values are expressed as mean ± S.E.M, (n=5) in each group, a comparisons are made with group I, b comparison are made with group II, \*p<0.01, † p<0.05.

**Table 2: Effect of methanolic extract of *Barleria buxifolia*, on urinary parameters in control and experimental animals**

parameters	Days	Treatment groups				
		Group1 Normal	GroupII Lithiatic control	GroupIII cystone treated.	GroupIV curative regimen	Group V preventive regimen
Oxalates (mg/24 h)	28	0.66 ± 0.05	1.62 ± 0.33	0.67 ± 0.03	0.78 ± 0.08	0.66 ± 0.02 <sup>†a</sup>
Calcium (mg/24 h)	28	5.15 ± 0.7	7.2 ± 0.12	5.2 ± 0.16 <sup>†a</sup>	6.1 ± 0.21 <sup>tb</sup>	6.2 ± 0.14 <sup>tb</sup>
Sodium (mEq/24 h)	28	142.5 ± 0.95	98.5 ± 0.9 <sup>†a</sup>	122.20 ± 1.0 <sup>tb</sup>	119 ± 2.10	139.8 ± 3.60
Potassium (mEq/24 h)	28	48.25 ± 0.2	22.4 ± 0.98 <sup>†a</sup>	43.28 ± 0.57 <sup>tb</sup>	36.08 ± 1.0	39.84 ± 1.73
Magnesium (mEq/24 h)	28	4.41 ± 0.29	1.84 ± 0.34 <sup>† a</sup>	4.20 ± 3.11 <sup>tb</sup>	3.10 ± 0.14	4.10 ± 0.25 <sup>†a</sup>

All the values are expressed as mean ± S.E.M, (n=5) in each group, a comparisons are made with group I, b comparison are made with group II, \*p<0.01, †p<0.05.



**Table 3: Effect of methanolic extract of *Barleria buxifolia* on various physical parameters in Ethylene glycol induced urolithiasis preventive study**

Parameters	Days	Treatment groups				
		Group 1 Normal	Group II Lithiatic control (EG)	Group III cystone treated (standard)	Group IV curative regimen	Group V preventive regimen
Change in Body Weight (gm)	29	276.66 ± 6.69	216.66 ± 5.59 <sup>†</sup>	248.33 ± 4.02 <sup>‡</sup>	255 ± 6.21*	253.33 ± 4.96*
Wet Kidney Weight (gm)	29	0.82 ± 0.03	1.38 ± 0.08 <sup>†</sup>	0.77 ± 0.02*	0.78 ± 0.02*	0.76 ± 0.01*
Dry Kidney Weight (gm)	29	0.14 ± 0.01	0.36 ± 0.01 <sup>†</sup>	0.18 ± 0.01*	0.19 ± 0.01*	0.17 ± 0.01*

All the values are expressed in terms of mean ± SEM; (n=5) in each group, <sup>†</sup>highly significant difference from normal p<0.01, \*highly significant difference from control p<0.01, <sup>‡</sup>Significant difference from control p<0.05.

**Table 4: Effect of methanolic extract of *Barleria buxifolia*, on kidney and serum parameters in control and experimental animals**

Parameters	Days	Treatment groups				
		Group I Normal	Group II Lithiatic control	Group III cystone treated	Group IV curative regimen	Group V preventive regimen
Kidney (homogenate analysis) Calcium(mg/g)	29	1.73 ± 0.30	3.24 ± 0.32	1.73 ± 0.12	2.23 ± 0.14	2.12 ± 0.21
Kidney (homogenate analysis) Oxalates(mg/g)	29	0.96 ± 0.10	2.10 ± 0.12	1.17 ± 0.17	1.42 ± 0.18	1.13 ± 0.14
Serum uric acid (mg/dl)	29	1.41 ± 0.19	3.05 ± 0.33	1.40 ± 0.15	1.14 ± 0.07	1.99 ± 0.02
Serum creatinine (mg/dl)	29	0.43 ± 0.08	1.16 ± 0.15	0.5 ± 0.17	0.8 ± 0.13	0.53 ± 0.17
Serum Calcium (mg/dl)	29	3.51 ± 0.19	0.72 ± 0.14	3.46 ± 0.56	1.98 ± 0.14	2.42 ± 0.41
Serum Sodium (mEq/L)	29	148.00 ± 2.23	151.30 ± 1.88	123.00 ± 2.81	142.00 ± 0.21	146.70 ± 2.51
Serum potassium (mEq/L)	29	20.4 ± 0.08	27.2 ± 0.01	23.8 ± 0.05	16.75 ± 0.85	19.15 ± 0.12
Serum magnesium (mEq/L)	29	0.60 ± 0.08	1.77 ± 0.06	0.80 ± 0.09	1.38 ± 0.13	0.73 ± 0.21

All the values are expressed in terms of mean ± SEM; (n=5) in each group.

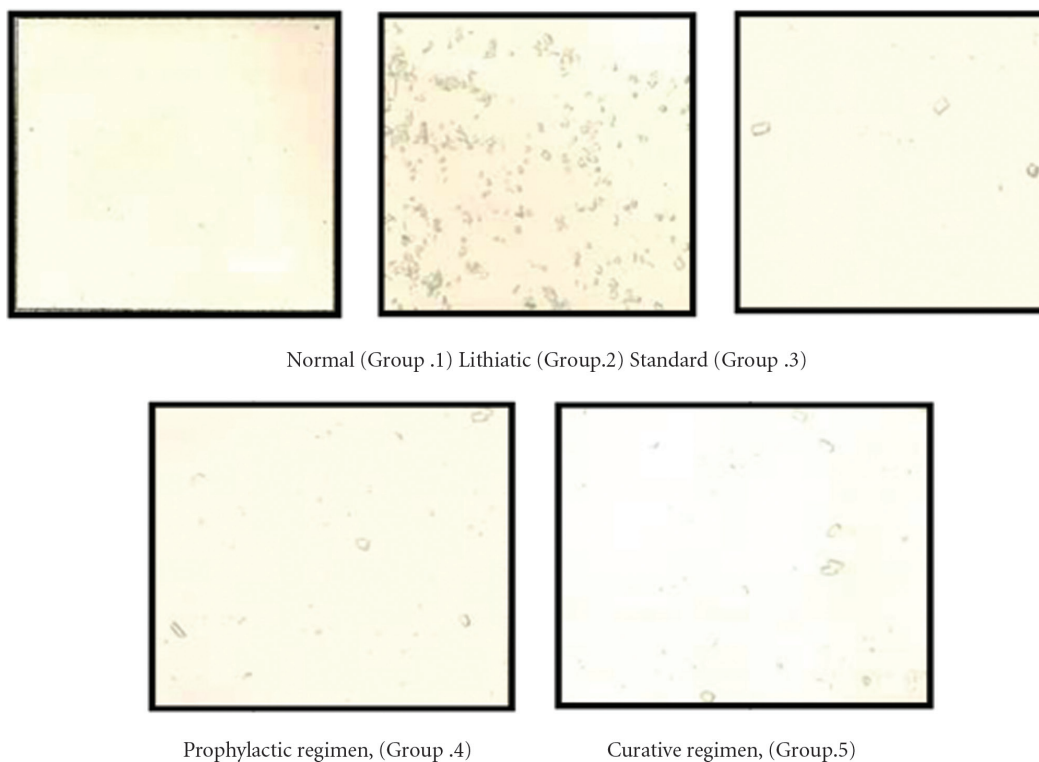
Group 2. Administration with standard drug (cystone) and methanolic extract of *Barleria buxifolia*, gradually decreased the crystals seen in the urinary microscopic examination. (Figure1).

### Histopathology of Rat's kidney (X400)

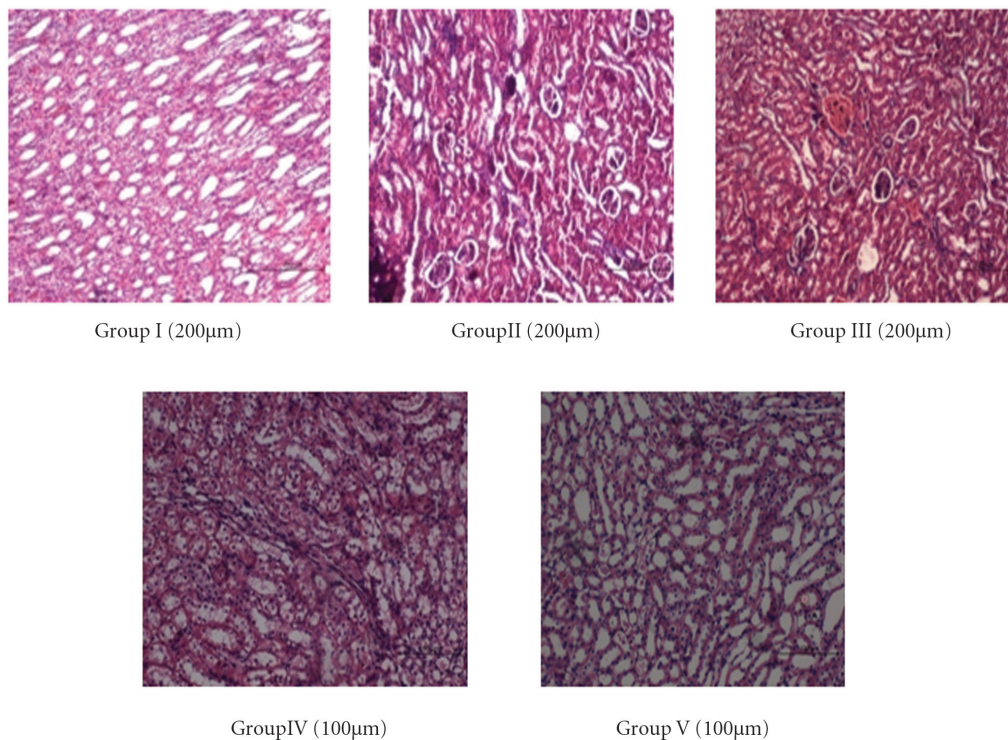
Histopathological analysis (Figure 2) revealed no calcium oxalate deposits or other abnormalities in the nephron segment of vehicle treatment group (Group.1). On the other hand, many calcium oxalate deposits were observed inside the glomeruli and tubules. Glomerular congestion, peritubular congestion, epithelial desquamation, blood Vessel congestion and deposition of inflammatory cells were observed in calculi induced group (Group. 2). Simultaneous administration with cystone and methanolic extract of *Barleria buxifolia*, gradually decreased deposition as well as damage from lithogenic treatment (Group. 3–5)) and prevented the lithogen induced renal tissue injuries.

### DISCUSSION

In the present study, male rats were selected to induce urolithiasis, because the urinary system of male rats resembled that of humans, and previous studies have shown that the amount of stone deposition in female rats were significantly less.<sup>[28]</sup> The antiurolithiatic effect of methanolic extract of '*Barleria buxifolia*' was studied in experimentally induced urolithiasis in male rats. In rat model, calcium oxalate urolithiasis induced by ethylene glycol is commonly used to study the pathogenesis of urolithiasis. In this study, body weight, serum concentrations of calcium, phosphorus, urea, creatinine, urinary microscopy and histopathology of the kidneys were analyzed. The possible mechanism action of *Barleria buxifolia* may be due decrease in the urinary concentration of the urinary salt that prevented super saturation of the crystallizing salts. This property favored anti urolithiasis by hastening the process of dissolving or by flushing of the preformed stones or by preventing the



**Figure 1:** Urinary microscopy of the experimental rats: **(A) Group .1;** Normal animal urine showed the absence of crystals; **(B) Group 2: Calculi induced groups:** urine sample showed numerous crystals;**(C) Group 3: standard drug treated groups:** showed very less or almost dissolved crystals;**(D) Group 4: Prophylactic regimen:** Better prevention of stones formation; **(E) Group 5: Curative regimen:** showed better dissolution in preformed crystal formation.



**Figure 2:** Histopathology of rat kidney section. **(A) Group I** (normal): Normal renal tubules with no dilation and inflammation; **(B) Group II** (EG +Ammonium chloride): Marked tubules dilation with interstitial inflammatory infiltrate due to crystal deposits; **(C) Group III** (standard): less tubules dilation with interstitial inflammatory infiltrate compared to lithiatic model group; **(D) Group IV**(curative): same changes as seen with std group; **Group V** (Prophylactic): same as normal group; (original magnification X400 under polarized light). **(Leica DM 2000)**.

new stones formation in urinary system on prophylactic treatment. Calcium oxalate crystals and high oxalate levels in nephrons can produced damages in the epithelial cells, and consequently. The renal epithelial cells release free radicals, which leads to heterogeneous crystal nucleation and causing aggregation of crystals.

The other possible mode of action of *Barleria buxifolia* may be due to its high antioxidant effect. From the earlier studies it had been reported that flavonoid, saponins have diuretic activity.<sup>[7,9]</sup> The present study reveals that the plant possesses flavonoid, saponins and polyphenols with antioxidant property. Besides the earlier studies showed that the antibacterial activity<sup>[8,30]</sup> study of the *Barleria buxifolia* probably contributed for its antiurolithiatic activity as bacterial infection also promoted urolithiasis.<sup>[28,31]</sup> In urolithiasis, the glomerular filtration rate decreased due to the obstruction to the outflow of urine by stones in the urinary system and also due to the damage to renal parenchyma. Due to this, the waste products, and uric acid, got accumulated in the blood. The decreases in the serum levels of these were due to the antiurolithiatic effect of methanolic extract of *Barleria buxifolia* linn.

It may be concluded that the administration of methanolic extract of '*Barleria buxifolia*' significantly decreased the development of urolithiasis in rats.

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