

Rotula Aquatica Lour Aqueous Extract as Anti-Urolithiatic Agent in Experimentally Induced Urolithiatic Rat Model

Bhavaniamma Vijayakumari¹, Venkatachalam Sasikala¹, Singanallur Ramu Radha¹,
Hiranmai Yadav Rameshwar^{2*}

¹Department of Botany, Avinashilingam University for Women, Coimbatore-6410043, Tamil Nadu, India Email: drbvij@gmail.com

²School of Environment and Sustainable Development, Central University of Gujarat Gandhinagar, Sector 30, Gujarat.

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ABSTRACT

Rotula aquatica Lour is traditionally used to regulate the kidney and bladder stones and root decoction is used for diuretic activity. The present study was carried out to assess the antiurolithiatic activity of the aqueous root extract of the plant in urolithiatic rats. The extract was administered to the calcium oxalate (CaOx) urolithiatic rats orally at 100 and 200 mg/kg body weight along with ethylene glycol (EG) for 28 days. On 28 day, 24 h urine was collected from individual rats and urine was analysed for protein, calcium, phosphate, magnesium, uric acid, oxalate and creatinine. On same day the serum protein, calcium, phosphate, magnesium, uric acid, oxalate, creatinine and blood urea nitrogen levels were also estimated in each animal. The kidney homogenate was used for the estimation of calcium, oxalate and phosphate. The paraffin kidney sections were prepared to observe the CaOx deposits. The paraffin kidney sections showed significant histopathological changes. The treatment with aqueous root extract of *Rotula aquatica* at 100 and 200 mg/kg body weight doses significantly decreased the urine protein, calcium, phosphate, uric acid, creatinine and oxalate, serum protein, calcium, phosphate, uric acid, creatinine, blood urea nitrogen and oxalate and renal tissues calcium, phosphate and oxalate, in EG induced urolithiasis after 28 days. The present study indicated aqueous root extract of *Rotula aquatica* at the dose of 200 mg/kg body weight as more effective in decreasing the urolithiasis and regeneration of renal tissues in male rats. The extract has a potential to cure the urolithiasis.

Keywords: Biochemical parameters, Ethylene glycol, Cystone, *Rotula aquatica*, Urolithiasis.

INTRODUCTION

Urinary stone formation is a painful urologic disorder that is observed in global population and its re-occurrence rate is 70-81% in males and 47-60% in females¹. The tendency of stone formation is higher in men than women, which might be due to the enhancing capacity of testosterone and inhibiting capacity of oestrogen². Urine is a supersaturated liquid containing the common stone forming minerals. The crystallization inhibiting capacity of urine in nature does not allow urolithiasis in most of individuals. This natural inhibition capacity is observed to be deficient in stone forming individuals³. Stone formation is characterized by multi-factorial etiopathogenesis that involves major factors like anatomic, environmental, genetic, infections, metabolic, nutritional and socio-economic⁴. Urolithiasis is now observed in children also⁵.

The medicinal plants have played a significant role in various ancient traditional systems of medication. Indian medicine "Ayurveda" recommends several medicinal plants and uses large number of plant drugs since ancient times to treat urinary stones. Ayurveda offers vast scope for the successful treatment of urolithiasis⁶. Herbal remedy may help to prevent, dissolve and expel urinary stones

naturally. The vast Ayurvedic literature claims a number of plants to be useful in urinary stones; still many plants need to be exploited for their pharmacological actions⁷.

Now a days, surgical endoscopic stone removal and extracorporeal shock wave lithotripsy have revolutionized the treatment of urolithiasis. But these methods do not prevent the likelihood of new stone formation in individuals. The recurrence of stone formation after the treatments also is very high with no suitable medical therapy for complete cure⁸. Therefore, it is valuable to look for alternative natural and safe 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; the root of *Rotula aquatica* is also called as pashanbed, belonging to the family Boraginaceae. In the recent decades, the extracts of roots and bark of *Rotula aquatica* have been extensively studied for potential uses including anti-inflammatory effects⁹, anthelmintic¹⁰, antidiabetic¹¹, antimutagenic activity¹² and antidiarrhoeal¹³.

However, no studies have so far been supporting as antiurolithiatic effects of *Rotula aquatica* root. In the

absence of any scientific evidence, the present study was undertaken to assess antiurolithiatic activity of *Rotula aquatica* root in male rats.

MATERIALS AND METHODS

Plant material

The *Rotula aquatica* Lour roots were collected from Kuttiyadi river Malapuram district of Kerala State. The plant was authenticated at the herbarium of Botanical Survey of India, Southern circle, Coimbatore, Tamil Nadu. A voucher specimen BSI/SRC/5/23/2012-13 Tech/415 was deposited in the same Institute.

Extraction procedure

The sample was shade dried, 30g powder was subjected to Soxhlet extraction with various solvents viz, petroleum ether, chloroform, methanol and aqueous 300ml respectively. Each time before extracting with the next solvent, the material was dried in hot air oven. All the extracts were concentrated by distilling the solvent at low temperature. Aqueous extract was selected for antiurolithiatic activity on the basis of results from phytochemical and *in-vitro* studies.

Experimental animals

The male Wistar albino rats weighing about 150-200 g were collected from animal breeding centre, Kerala Agricultural University, Thrissur, Kerala, India. The animals were maintained in 12 hr light and dark cycle at $28^{\circ}\text{C} \pm 2^{\circ}\text{C}$ in a well ventilated animal house under natural conditions and they were acclimatized to laboratory conditions for 10 days prior to the commencement of the experiment. The animals were fed with standard pelleted diet. The experimental protocol in the study was approved by the Institutional Animals Ethical Committee (KMCRET/Ph.D/11/2012-13).

Induction of urolithiasis using ethylene glycol

The animals were divided into 5 groups, each group containing six animals.

Group I : Control rats - received normal pelleted diet

Group II : Urolithiasis induced rats - received 0.75% ethylene glycol in water for 28 days

Group III : Standard drug Cystone treated rats: urolithiasis induced rats received Cystone (100 mg/kg body weight) by oral administration for 28 days

Group IV : Plant drug treated rats - urolithiasis induced rats received *R. aquatica* aqueous root extract (100 mg / kg body weight) by oral administration for 28 days

Group V : Plant drug treated rats - urolithiasis induced rats received *R. aquatica* aqueous root extract (200 mg / kg body weight) by oral administration for 28 days.

All the animals were kept in individual metabolic cages under controlled healthy conditions required for the experiment.

Biochemical parameters

Analysis of urine samples

All the animals were kept in individual metabolic cages and the urine sample of 24 h was collected on 28th day. Animals had free access to drinking water during the urine collection period. A drop of concentrated hydrochloric acid was added to the collected urine before being stored at 4°C . The urine was analysed for protein, calcium,

phosphate, magnesium, uric acid, oxalate and creatinine using commercially available kits (Molybdate U.V, OCPC, Calmagite, Erba diagnostics Mannheim GmbH methods).

Analysis of blood samples

The blood was collected from the retro-orbital sinus under anaesthetic condition and serum was separated by centrifugation at 10,000 rpm for 10 min and analysed for protein, calcium, phosphate, magnesium, uric acid, oxalate, creatinine and blood urea nitrogen using commercially available kits (Molybdate U.V, OCPC, Calmagite, Erba diagnostics Mannheim GmbH methods).

Analysis of kidney sample

The animals were sacrificed under anesthesia after dissection; both kidneys were removed and washed with cold 0.15 M KCl. The right kidney was minced with scissors and then homogenized in 0.15 M KCl, using Remi's glass homogenizer. The homogenate was centrifuged at 1500 rpm for 10 min using refrigerated centrifuge, to remove the cell debris. The supernatant was used for estimation of calcium, oxalate and phosphate using commercially available kits (Molybdate U.V. OCPC methods).

Histopathological study

To confirm the incidence of lithiasis and its healing, the kidneys were subjected to histopathological study. The kidneys were fixed in 10% neutral buffered formalin, processed in a series of graded alcohol and xylene, embedded in paraffin wax; cut at 5 μm intervals and the slices were stained with hematoxylin and eosin. The slides were examined for renal tubular necrosis and presence of calcium oxalate crystals under binocular microscope.

Statistical analysis

All the statistical comparison between the groups were made by means of One Way Analysis of Variance (ANOVA) and followed by Dunnett's Multiple Comparison test. The values were considered significantly different at $P < 0.05$ regarded as significant using. The data expressed were Mean \pm standard error of mean (S.E.M).

RESULTS

Urine analysis

The concentration of urinary excretion of protein, calcium, phosphate, uric acid, creatinine and oxalate present in group I-IV, were shown in (Table 1). In the present study, administration of EG of 0.75% v/v in drinking water to male rats were caused increase in protein, calcium, phosphate, uric acid, creatinine and oxalate concentration in the urine of group-II. The protein, calcium, phosphate, uric acid, creatinine and oxalate concentration were significantly increased ($P < 0.001$ vs group-I) as compared to the normal as shown in (Table 1). However, treatment with aqueous root extract of *R. aquatica* at 100 and 200 mg/kg bodyweight respectively, reduced significantly ($P < 0.01$ vs control) protein, calcium, phosphate, uric acid, creatinine and oxalate excretion in urine. The urine magnesium level significantly decreased in EG treated group (II) when compared to control, Cystone and aqueous root extract treated groups (I, III, IV & V), (Table I). However, aqueous root extract of *R. aquatica* 200mg/kg

Table 1: Effect of *R. aquatica* aqueous root extract on urinary parameters in control and experimental animals.

Groups	Protein (g/dl)	Magnesium (mEq/l)	Calcium (mg/dl)	Phosphate (mg/dl)	Uric acid (mg/dl)	Creatinine (mg/dl)	Oxalate (mg/dl)
Group I	1.2±0.06	2.7±0.12	12.7±0.05	2.9±0.11	4.0±0.09	4.2±0.03	4.0±0.11
Group II	5.5±0.08***	1.6±0.12***	17.2±0.03***	8.8±0.15***	11.3±0.05***	15.6±0.06***	13.3±0.06***
Group III	1.3±0.05***	2.5±0.06***	13.3±0.02***	3.7±0.13***	4.6±0.02***	4.6±0.01***	4.6±0.05***
Group IV	2.5±0.06***	2.1±0.06**	14.0±0.04***	8.3±0.12**	10.9±0.06**	11.7±0.05***	10.5±0.14***
Group V	1.4±0.03***	2.4±0.15***	13.2±0.11***	3.8±0.06***	5.5±0.11***	7.2±0.06***	6.2±0.13***

Values are expressed as mean ± SEM (n=6) Statistical comparisons are made between Group II vs Group I ; Group II vs Group III, IV and V Significant status *P<0.05, **P<0.01, ***P<0.001

- Group I - Control
- Group II - Ethylene glycol (EG) (0.75%)
- Group III - EG (0.75%) + Cystone 100 mg/kg body weight
- Group IV - EG (0.75%) + Aqueous root extract 100 mg/kg body weight
- Group V - EG (0.75%) + Aqueous root extract 200 mg/kg body weight

Table 2: Effect of *R. aquatica* aqueous root extract on serum parameters in control and experimental animals.

Groups	Protein (g/dl)	Magnesium (mEq/l)	Calcium (mg/dl)	Phosphate (mg/dl)	Uric acid (mg/dl)	Creatinine (mg/dl)	Blood Urea Nitrogen (mg/dl)	Oxalate (mg/dl)
Group I	6.45±0.06	4.74±0.07	7.29±0.45	11.18±0.34	2.50±0.09	0.27±0.03	20.73±1.02	0.37±0.07
Group II	10.42±0.09###	2.05±0.14###	9.50±0.66###	12.33±0.38###	6.40±0.07###	1.85±0.08###	30.97±1.2##	5.42±0.10###
Group III	6.22±0.08**	4.71±0.09***	3.58±0.41***	4.83±0.26*	2.32±0.09***	0.25±0.02***	22.90±0.55***	0.52±0.09***
Group IV	10.02±0.08**	3.24±12*	8.17±0.35***	7.50±0.35*	6.02±0.05*	1.62±0.06*	27.92±1.00***	3.48±0.11***
Group V	7.10±0.10**	4.58±0.07***	5.48±0.70***	5.80±0.44*	3.13±0.16***	0.68±0.06***	24.13±0.45***	2.12±0.09***

Values are expressed as mean ± SEM (n=6) Statistical comparisons are made between Group II vs Group I; Group II vs Group III, IV and V Significant status *P<0.05, **P<0.01, ***P<0.001

- Group I - Control
- Group II - Ethylene glycol (EG) (0.75%)
- Group III - EG (0.75%) + Cystone 100 mg/kg body weight
- Group IV - EG (0.75%) + Aqueous root extract 100 mg/kg body weight
- Group V - EG (0.75%) + Aqueous root extract 200 mg/kg body weight

bodyweight reduced urinary excretion of protein, calcium, phosphate, uric acid, creatinine and oxalate levels, when comparable to the Cystone treated rats (group – III).

Serum analysis

Renal function was assessed by measuring serum protein, calcium, phosphate, uric acid, creatinine, blood urea nitrogen and oxalate in control, Cystone and aqueous root extract treated rats were shown in (Table 2). The serum protein, calcium, phosphate, uric acid, creatinine, blood urea nitrogen and oxalate levels were significantly (P < 0.001 vs group-I) increased in EG treated group (II). While aqueous root extract of *R. aquatica* treated group significantly reduced when compared to group-II. However, aqueous root extract of *R. aquatica* 200mg/kg bodyweight significantly reversed the serum excretion closer to standard drug Cystone values. Similarly, the serum magnesium were significantly (P < 0.001 vs group-I) increased in ethylene glycol treatment (group- II) compared to control, Cystone and aqueous root extract treated groups. The maximum decrease in serum excretion was found in aqueous root extract of *R. aquatica* 200 mg/kg bodyweight , showing dose dependent activity.

Kidney homogenisation

The deposition of the crystalline components namely, calcium, oxalate and phosphate in the renal tissue, were significantly (P < 0.001 vs group-I) increased in the ethylene glycol treated groups (group – II). Further, treatment with aqueous root extract of *R. aquatica* 200 mg/kg significantly reduced the renal oxalate content and other stone forming constituents in the rats of group V, were shown in Figure 1.

Histopathological results

The examinations of the paraffin kidney sections (Figure 2) revealed that in 100 and 200 mg/kg aqueous root extract of *R. aquatica* treated groups (group IV and V), no crystal was found when compared to untreated group (group –II). Fig 2 (A-E) A. Control: Arrow mark indicates normal glomerular structure and renal tubules. B. EG, arrow marks indicate Oxalate renal stone and tubular dilation and renal tubular damage. C. Cystone, arrow mark indicates little tubular dilation and normal glomeruli. D. RAAE 100 mg/kg, arrow mark indicates tubular dilation and partial atrophy of glomeruli. E. RAAE 200 mg/kg, arrow mark indicates little tubular dilation and normal glomeruli.

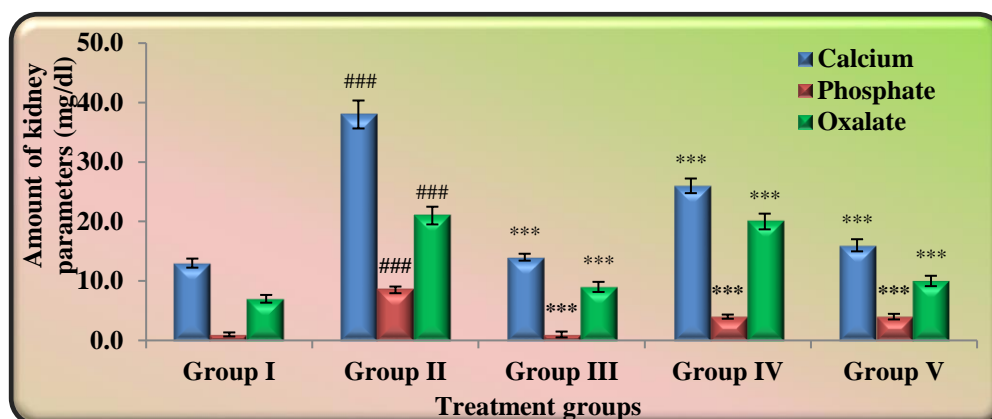


Figure 1: Effect of *R. aquatica* aqueous root extract on kidney homogenate parameters in control and experimental animals.

- Group I - Control
 Group II - Ethylene glycol (EG) (0.75%)
 Group III - EG (0.75%) + Cystone 100 mg/kg body weight
 Group IV - EG (0.75%) + Aqueous root extract 100 mg/kg body weight
 Group V - EG (0.75%) + Aqueous root extract 200 mg/kg body weight

DISCUSSION

A number of models using rats have been studied for calcium oxalate urolithiasis¹⁴. Calcium oxalate crystals and high oxalate levels in nephrons damage epithelial cells, inducing heterogeneous crystal nucleation and causing aggregation of crystals^{15,16}. Male rats are being selected to induce urolithiasis as their urinary system resembles that of humans and earlier studies have shown that the amount of stone deposition in female rats was significantly less¹⁷.

In the present study, the aqueous root extract of *R. aquatica* was found to have ability to inhibit calcium oxalate crystallization. The root of *R. aquatica* has shown significant inhibitory potential of calcium oxalate crystallization *in vitro*. The aqueous extract of *R. aquatica* root have inhibitory effect on CaOx crystallization this may be beneficial in the treatment of urolithiasis¹⁸.

The present observation showed increased protein excretion in ethylene glycol induced urolithiatic rats. Proteinuria in animals reflects proximal tubular dysfunction. Supersaturation of urinary colloids may result in precipitation as crystal initiation particle which when trapped acts as a nidus leading to subsequent crystal growth¹⁹. Administration of *R. aquatica* aqueous root extract (RAAE) to male rats exhibited profound effects on minimizing the excretion of protein and thus might have prevented the nidus formation of crystal nucleation.

Urinary stone formation is the result of super saturation of urine with certain urinary salts such as calcium oxalate. Administration of 0.75% ethylene glycol aqueous solution to male Wistar rats resulted in hyperoxaluria. Lower level of calcium, oxalate and phosphate in urine and kidney reduces the risk of stone formation²⁰. The aqueous root extract of *R. aquatica* significantly lowered the levels of oxalate and calcium in urine and kidney.

In urolithiasis, the glomerular filtration rate is affected by obstruction in flow of urine by stones in urinary system.

This obstruction result in accumulation of nitrogenous waste products like urea, creatinine and uric acid in blood. In addition calculi-producing diet of rats increased lipid peroxidation and decreased levels of antioxidant potential. Elevated oxalate concentration in urine has been reported to induce lipid peroxidation and cause renal damage by reacting with polyunsaturated fatty acids in cell membrane²¹. In calculi-induced rats, marked renal damage was seen as individual by the elevated serum levels of creatinine and uric acid which are markers of Glomerular and tubular damage. Administration of aqueous root extract of *R. aquatica* to the stone induced rats prevented the elevation of serum levels and inhibited the lipid peroxidation.

Normally, urine is characterised by many inorganic and organic inhibitors of crystallization. Magnesium is one such well-known inhibitor and low levels of it are encountered in stone formers as well as in stone-forming rats. The magnesium levels return to normal after drug treatment for stone²². Different studies have shown that diets with high magnesium protect against deposition of calcium oxalate in the kidneys. Magnesium reduces the growth and nucleation rate of calcium oxalate crystals as it complexes with oxalate^{23,24}. Urinary and serum magnesium was significantly diminished in EG induced urolithiatic rats. The aqueous root extract of *R. aquatica* treatment restored the magnesium excretion and thus reduces the growth of calcium oxalate crystals in groups IV & V.

EG induced urolithiatic rats showed an increase in urinary phosphorus excretion along with oxalate stress seems to provide an environment appropriate for stone formation by forming calcium phosphate crystals, which epitaxially induces calcium oxalate deposition^{25,26,27}. Treatment of *R. aquatica* aqueous root extract lowered the excretion of phosphorus and reduces the risk of stone formation.

CONCLUSION

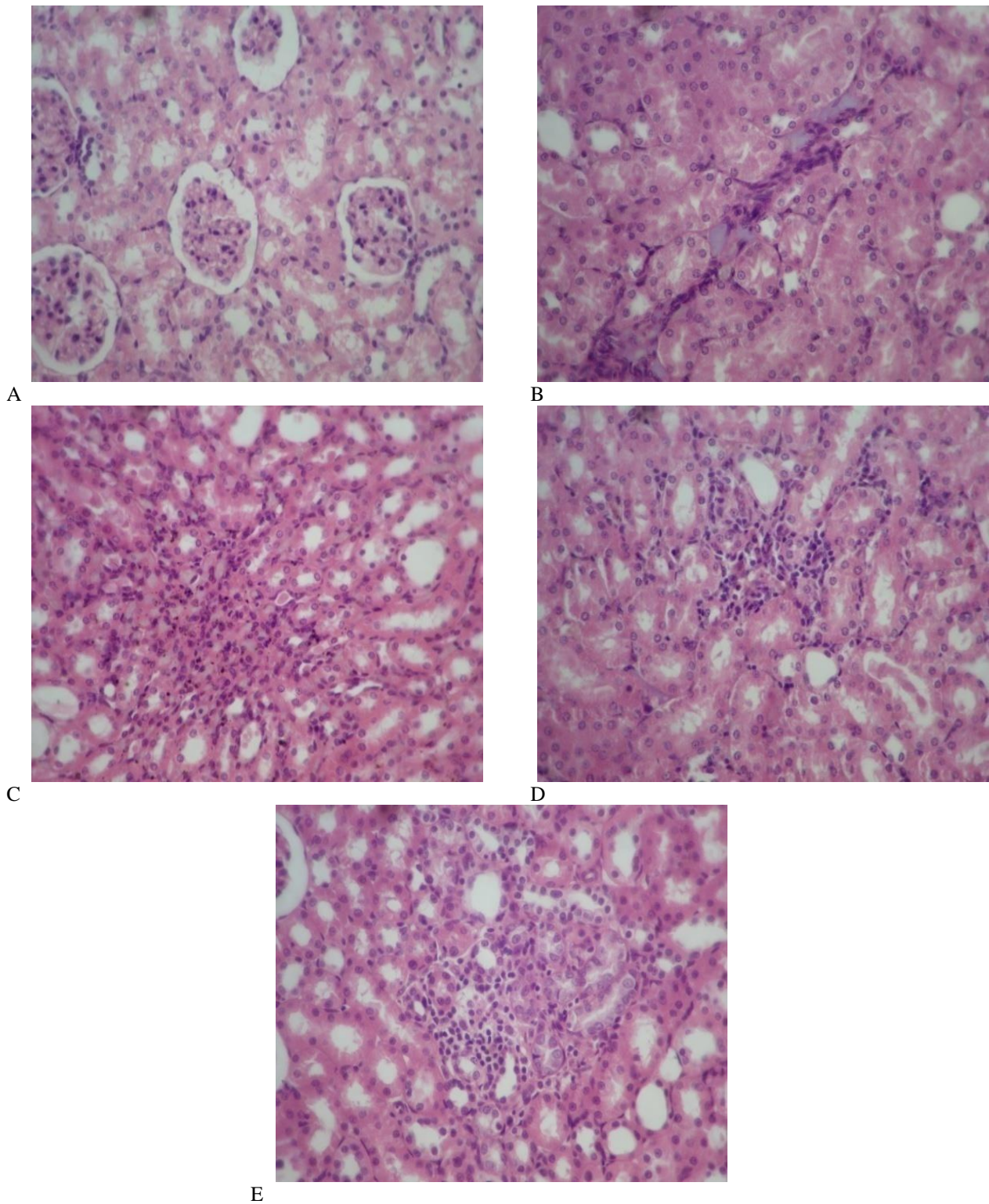


Figure 2: Histopathological examination of kidney in the different treatment groups at 400X magnification.

The aqueous root extract of *R. aquatica* suppressed renal calcium accumulation and urinary CaOx levels in a rat model of EG induced urolithiasis. The histopathological analysis also showed the efficacy of the aqueous root extract. This study showed *R. aquatica* aqueous root extract as a remedial measure for the treatment of urolithiasis.

Ethical committee approval

This study obtained the approval of the Institutional Animals Ethical Committee (KMCRET/Ph.D/11/2012-13).

CONFLICT OF INTEREST

The authors have no conflicts of interest to disclose.

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AUTHORS CONTRIBUTION

Professor Vijayakumari, B is the major investigator of the project and guided the conduct of experiments.

Dr. Sasikala, V and Dr. Radha, S.R were the project staffs who carried out the experimental study.

Dr. Hiranmai Yadav R. helped in writing and editing works of the article.

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