ORIGINAL ARTICLE

Antiurolithiatic activity of *Phaseolus vulgaris* seeds against ethylene glycol-induced renal calculi in Wistar rats

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Abstract

Objective: The present study was aimed to evaluate the antiurolithiatic potential of the ethanolic extract of the seed of *Phaseolus vulgaris* (EPV). **Materials and Methods:** Calcium oxalate urolithiasis in male Wistar rats was induced by ethylene glycol (EG) (0.75% v/v) and ammonium chloride (1% w/v) administration in drinking water. Cystone (750 mg/kg, *p.o.*) was used as a standard drug, and EPV was administered at doses of 200 and 400 mg/kg, *p.o*. Both preventive and curative effects of EPV were evaluated. Urinary biochemical parameters such as calcium, oxalate, phosphate, uric acid and creatinine; deposition of calcium and oxalate in the kidney; and serum uric acid, creatinine, and blood urea nitrogen (BUN) were assessed. Creatinine clearance was calculated. Oxalate associated oxidative stress in the kidney was assessed by estimating *in vivo* antioxidant parameters such as lipid peroxidation, superoxide dismutase, catalase, and reduced glutathione. Histopathological studies of the kidney were carried out. **Results:** In the preventive and curative disease-control groups, urinary excretion of calcium, oxalate, and their deposition in the kidney were significantly increased. Elevated levels of phosphate and uric acid in urine and uric acid, creatinine, and BUN in serum were observed in both the control groups. Creatinine clearance was reduced in the control groups. On treatment with cystone and EPV, all the urinary, serum biochemical, and oxidative stress parameters were reversed to almost normal values. Cystone and EPV significantly restored the *in vivo* antioxidant enzymes by decreasing the lipid peroxidation in the kidney. This study demonstrated the antiurolithiatic activity of the ethanolic extract of *P. vulgaris* seeds against EG-induced renal calculi in Wistar rats.

Key words: Ammonium chloride, antiurolithiatic activity, ethylene glycol, *Phaseolus vulgaris*

INTRODUCTION

Urinary calculus (kidney stones) is one of the painful disorders of the urinary tract. It is estimated that 10% of the population in the industrialized areas of the world are affected by urinary tract stone disease with an incidence of 0.5–1.9%.[1,2] In India, upper and lower urinary tract stones occur frequently, but the incidence depends on regional, climatic, and socioeconomic conditions.[3] Approximately 80% are of calcium-containing stones are in the form of pure calcium oxalate (CaOx) (50%) or calcium phosphate (1%) and a mixture of both (45%), and other stones are *Struvite* (10%), uric acid (9%), and cystine (1%).[4] Although the mechanisms involved in the formation of calcific stones are not fully understood, it is generally agreed that urinary lithiasis is a multifaceted process involving a series of events such as supersaturation of urine, crystal nucleation, aggregation, and growth of insoluble particles that are finally retained in the urinary tract.[5,6] Studies have also shown that tubular cell injury facilitates CaOx crystal formation and deposition in the renal tubules.[7]

In the management of urolithiasis, combinations of medical and surgical techniques are commonly employed. Endoscopic stone removal and extracorporeal shock wave lithotripsy are commonly used methods in stone removal. These procedures are relatively costly and painful and cause undesirable side

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Received: 27-10-2017
Revised: 07-11-2017
Accepted: 13-11-2017

effects such as hemorrhage, hypertension, tubular necrosis, and subsequent fibrosis of the kidney leading to cell injury, and also, recurrence of stone is quite common.[5] Thiazide diuretics and alkali-citrate are being used to prevent the recurrence of hypercalciuria and hyperoxaluria-induced calculi, but scientific evidence for their efficacy is less reliable.[8] Hence, there is a need for the search of new drug therapy, which will be cost-effective, target multiple etiological risk factors in urolithiasis and reduce the rate of recurrence.

In India, medicinal plants in the traditional medicine have played a prominent role in the treatment of various diseases since ancient. The plants provide a cheap source of drugs for majority of the world's population. Data from *in vitro*, *in vivo,* and clinical trials reveal that phytotherapeutic agents could be used as either alternative or an adjunctive therapy in the management of urolithiasis.[9,10]

Phaseolus vulgaris, also known as kidney beans, called Raajmah in India, is a common Indian dish. The seeds of *P. vulgaris* are gaining increasing attention as a functional or nutraceutical food, due to its rich variety of phytochemicals such as proteins, amino acids, complex carbohydrates, dietary fibers, oligosaccharides, phenols, saponins, flavonoids, alkaloids, and tannins, with potential health benefits. The seeds were claimed to possess diuretic activity and were commonly used in water retention treatment in pregnant women.[11] Studies indicate that seeds of *P. vulgaris* were found to have activities such as enhancement of the bifidogenic, antioxidant, antimutagenic, anticarcinogenic, and antihyperglycemic effects.[12-16] Our previous study demonstrated *in vitro* antiurolithiatic activity of ethanolic seed extract of *P. vulgaris* on CaOx crystallization.[17] No studies were carried out on the *in vivo* antiurolithiatic activity. Hence, in the present study, *in vivo* antiurolithiatic activity of the ethanolic extract of the seed of *P. vulgaris* (EPV) was investigated in Wistar rats against CaOx urolithiasis induced by ethylene glycol (EG) and ammonium chloride (AC) in drinking water.

MATERIALS AND METHODS

Chemicals

In the present study, analytical grade chemicals (Merck India Ltd, Hi-media and Sigma Aldrich) were used. Cystone, a polyherbal formulation from the Himalaya Drug Co., Bengaluru, India, was used as a standard drug.

Plant Material

Seeds of *P. vulgaris* were purchased from the local market, authenticated by the Dr. B. Sitaram, Professor, Department of Dravyaguna, S.V. Ayurvedic Medical College, Tirupati. Voucher specimens were kept in the Sri Padmavati Mahila Visvavidyalayam (Women's University), Tirupati. The seeds were coarsely powdered and used for extraction.

Preparation of Ethanolic Seed Extract of *P. vulgaris*

The seed powder (200 g) was macerated with 95% ethanol (1 L) for 24 h at room temperature followed by Soxhlet extraction for 6 h. The extract was concentrated under reduced pressure, and the obtained semisolid mass (11 g) was stored in an airtight container in a refrigerator.

Preliminary Phytochemical Screening

Preliminary phytochemical screening of EPV was carried out for the presence of alkaloids, carbohydrates, flavonoids, phenolic compounds, saponins, sterols, and tannins using standard procedures.[18] Quantification of total polyphenolic content in the EPV was determined spectrometrically[19] and expressed as µg of gallic acid per gram of dry extract. Flavonoids content was determined by aluminum chloride colorimetric assay[20] and expressed as µg of quercetin per gram of dry extract.

Experimental Animals

Healthy male Wistar albino rats weighing between 150 and 200 g were procured from Sri Venkateswara Enterprises, Bengaluru. Animals were acclimatized to standard laboratory conditions. They were provided with standard rat food pellets and drinking water *ad libitum*. The animal care and experimental protocols were in accordance with the CPCSEA. The study was approved by the Institutional Animal Ethical Committee (IAEC) with the approval No. 1677/PO/a/12/IAEC-Feb-14/07.

Acute Toxicity Studies

Oral acute toxicity studies were carried out in accordance with the OECD guidelines 423.[21]

Experimental Design

Rats were divided into nine groups of six animals each ($n = 6$). CaOx stones were induced in rats by administering 0.75% v/v of EG and 1 % w/v of AC in drinking water for 15 days.[22] The treatment schedule was planned as follows:

Group I: Normal (untreated)
Group II: Preventive control (EG + AC + vehicle from day 1 to 15)
Group III: Preventive standard (EG + AC + cystone 750 mg/kg, orally from day 1 to 15)
Group IV: Preventive low dose (EG + AC + EPV 200 mg/kg, orally from day 1 to 15)
Group V: Preventive high dose (EG +AC + EPV 400 mg/kg, orally from day 1 to 15)
Group VI: Curative control (EG + AC from day 1 to 15, vehicle from day 16 to 30)
Group VII: Curative standard (EG + AC from day 1 to 15; cystone 750 mg/kg, orally from day 16 to 30)

Group VIII: Curative low dose (EG + AC from day 1 to 15, EPV 200 mg/kg, orally from day 16 to 30)
Group IX: Curative high dose (EG + AC from day 1 to 15, EPV 400 mg/kg, orally from day 16 to 30)

Urine and blood samples were collected from all the groups of animals after completion of their respective treatment schedules. Rats were orally hydrated with 5 ml of drinking water, placed in individual metabolic cages and 24 h urine was collected. Urinary volume was noted, and urinary pH was determined. Urine was centrifuged at 2,500 rpm at 30 ± 2°C for 5 min, and the supernatant was used to estimate calcium, phosphate, uric acid, creatinine using commercially available diagnostic kits (ERBA Diagnostic Mannheim and Span Diagnostics Ltd., India) according to manufacturer's instructions. Oxalate was estimated by the method of Hodgkinson and Williams, 1972.[23] Blood samples were collected from the retro-orbital venous plexus and serum was separated by centrifuging at 1500 rpm for 15 min and used for the estimation of uric acid, creatinine, and Blood urea nitrogen (BUN) using ERBA diagnostic kits according to the manufacturer's instructions. Fully automated autoanalyzer (Erba EM-200, Transasia Bio-medicals Ltd, Mumbai) was used for the estimations. Creatinine clearance was considered as kidney function parameter and was calculated using the formula:[24]

$$\text{Creatinine clearance}\,(\text{ml/min}) = \frac{(\text{mg creatinine/dl urine}) \times (\text{24 h urine output}\,(\text{ml}))}{(\text{mg creatinine/dl serum}) \times 1440}$$

After collecting urine and blood, rats were sacrificed by cervical decapitation; kidneys were perfused with ice-cold saline (0.9% w/v sodium chloride) and carefully isolated. One kidney from each animal was used for the estimation of calcium and oxalate as well as *in vivo* antioxidant parameters such as lipid peroxidation expressed as malondialdehyde (MDA) levels,[25] superoxide dismutase (SOD),[26] catalase (CAT),[27] and reduced glutathione (GSH).[28] Other kidney was fixed in 10% solution of buffered formalin (pH 7.4) and processed to paraffin wax. The sections were made using microtome and stained with hematoxylin and eosin and were examined under light microscope for renal tubular necrosis.

Statistical Analysis

The values were expressed as mean ± standard error of mean. Data obtained were analyzed by one-way ANOVA followed by *post hoc* Bonferroni multiple comparisons test using GraphPad Prism (version 5.0).

RESULTS

Preliminary phytochemical analysis revealed the presence of flavonoids, polyphenols, saponins, and tannins in the ethanolic extract of *P. vulgaris* seeds. Total phenolic content of EPV was found to be 418.78 µg/g gallic acid equivalents and total flavonoid content of EPV was 181.47 µg/g quercetin equivalents.

In the oral acute toxicity studies, EPV was found to be safe as it did not cause any mortality up to 2000 mg/kg. Hence, 200 and 400 mg/kg doses were selected for the present study.

Deposition of Calcium and Oxalate in the Kidney

On administration of EG and AC in drinking water for 15 days, a significant increase in the deposition of calcium and oxalate levels in the kidney were observed in both the preventive-control (II) and curative-control (VI) groups when compared to the normal (I). In the preventive regimen, animals treated with cystone (III) and EPV at doses 200 and 400 mg/kg (IV and V), a significant decrease in calcium and oxalate deposition in the kidney was observed when compared to the preventive-control group (II). In the curative regimen, animals treated with cystone (VII) and EPV (VIII and IX) also showed a significant decrease in calcium and oxalate deposition in the kidney when compared to curative-control group (VI). These effects were found to be dose-dependent [Table 1].

Urinary Biochemical Parameters

A significant increase in the urinary excretion of calcium, oxalate, phosphate, and uric acid was observed in both the preventive and curative-control (II and VI) group animals when compared to the normal group (I). On treatment with cystone and EPV, there was a significant decrease in the excretion of calcium, oxalate, phosphate and uric acid in the preventive-treated (III, IV and V) and curative-treated groups (VII, VIII, and IX) compared to their respective control groups (II and VI) [Table 2].

The urinary pH in normal animals was between 6.0 and 7.0. On induction of CaOx stones, the pH reduced to 5.0-6.0 in both the preventive and curative-control (II and VI) groups whereas in preventive (III, IV, and V) and curative (VII, VIII, and IX) group animals treated with cystone, EPV low and high doses showed an increase in the urinary pH (6.5–7.5) when compared to their respective control groups.

Serum Biochemical Parameters

Administration of EG and AC in drinking water for 15 days in both the control groups (II and VI), increased serum levels of uric acid, creatinine and BUN when compared to the normal group (I). These levels were significantly decreased in both preventive and curative regimens on treatment with cystone and EPV. A significant decrease in creatinine clearance was observed in the control groups (II and VI) and this was improved in groups treated with cystone and EPV in both the preventive and curative regimens [Table 3].

Table 1: Effect of EPV on kidney weight and deposition of calcium and oxalate in the kidney

Groups	Treatment	Wet kidney weight g/100 g body weight	Calcium	Oxalate
			mg/g of kidney	mg/g of kidney
I	Normal	0.39±0.02	2.37±0.18	1.60±0.19
II	Preventive control	0.77±0.08[a]	5.83±0.47[a]	4.82±0.27[a]
III	Preventive standard	0.40±0.02[b]	2.34±0.28[b]	1.96±0.15[b]
IV	Preventive low dose	0.53±0.01[d]	4.56±0.31	3.89±0.19
V	Preventive high dose	0.49±0.03[d]	4.23±0.29[d]	3.39±0.28[c]
VI	Curative control	0.84±0.02[a]	7.28±0.59[a]	5.57±0.21[a]
VII	Curative standard	0.42±0.01[e]	3.39±0.30[e]	2.10±0.19[e]
VIII	Curative low dose	0.60±0.01[g]	5.02±0.41[g]	4.32±0.29[g]
IX	Curative high dose	0.54±0.01[f]	4.64±0.39[f]	3.98±0.31[f]

All values are expressed in mean±SEM; [a]P<0.001 when compared to Group I; [b]P<0.001, [c]P<0.01, [d]P<0.05 when compared to Group II; [e]P<0.001, [f]P<0.01, [g]P<0.05 when compared to Group VI. EPV: Ethanolic extract of the seed of *Phaseolus vulgaris*, SEM: Standard error of the mean

Table 2: Effect of EPV on urine volume and urine biochemical parameters

Groups	Treatment	Urine volume (ml/24 h/100 g)	Calcium (mg/dl)	Oxalate (mg/dl)	Phosphate (mg/dl)	Uric acid (mg/dl)
I	Normal					
	On day 15	10.71±0.63	5.20±0.21	2.27±0.35	4.48±0.33	1.45±0.31
	On day 30	10.12±0.54	5.63±0.33	2.31±0.19	4.51±0.52	1.88±0.22
II	Preventive control	4.10±0.89[a]	10.66±0.19[a]	6.51±0.31[a]	7.99±0.51[a]	3.99±0.27[a]
II	Preventive standard	9.16±1.04[b]	6.83±0.71[b]	2.60±0.56[b]	4.81±0.33[b]	2.30±0.34[b]
IV	Preventive low dose	5.91±0.40[c]	8.51±1.56[d]	5.40±0.78[d]	6.56±1.17[d]	3.58±0.38
V	Preventive high dose	7.38±0.76[b]	8.16±1.67[c]	4.99±0.70[c]	6.18±1.09[c]	3.16±0.51[c]
VI	Curative control	4.86±1.09[a]	11.17±0.35[a]	7.18±0.34[a]	7.59±0.47[a]	3.81±0.10[a]
VII	Curative standard	9.81±0.77[e]	6.11±0.86[e]	2.78±0.63[e]	4.19±0.51[e]	2.11±0.39[e]
VIII	Curative low dose	6.80±0.98[g]	8.48±1.64[f]	5.81±0.71[g]	6.48±0.83[g]	3.51±0.21
IX	Curative high dose	7.46±1.06[e]	7.98±1.47[e]	5.33±1.20[f]	5.90±0.68[e]	3.28±0.39[f]

All values are expressed in mean±SEM; [a]P<0.001 when compared to Group I; [b]P<0.001, [c]P<0.01, [d]P<0.05 when compared to Group II; [e]P<0.001, [f]P<0.01, [g]P<0.05 when compared to Group VI. EPV: Ethanolic extract of the seed of *Phaseolus vulgaris*, SEM: Standard error of the mean

In Vivo Antioxidant Parameters

A significant increase in MDA levels was observed in both the control groups (II and VI) on administration of EG and AC, when compared to the normal group (I), indicating increased lipid peroxidation. These values were significantly decreased in groups treated with cystone and EPV [Table 4]. *In vivo,* antioxidant parameters such as SOD, CAT, and GSH were significantly decreased in both preventive and curative controls, and these levels were significantly improved in groups treated with cystone and EPV [Table 4].

Histopathological Studies

Histological examination revealed marked congestion, dilation of Bowman's capsules, scattered mononuclear inflammatory infiltrations and necrotic changes in both preventive and curative groups. EG induced necrotic changes were decreased in the animals treated with cystone and EPV [Figure 1a-i].

DISCUSSION

Formation of kidney stones is a complex process and involves a series of biological events that are most likely triggered by genetic susceptibility together with dietary factors and lifestyle changes.[29] Several *in vivo* animal models have been developed to investigate the mechanisms involved in the formation of urinary stones. However, rat model has been widely used for the study of CaOx deposition in the kidneys, a process that mimics the etiology of kidney stone formation in humans.[22] In this study, male rats were selected to induce urolithiasis as the amount of stone deposition in

Table 3: Effect of EPV on serum biochemical parameters

Groups	Treatment	Uric acid (mg/dl)	Creatinine (mg/dl)	BUN (mg/dl)	Creatinine clearance (ml/min)
I	Normal				
	On day 15	2.40±0.12	0.74±0.12	18.71±0.59	0.088±0.003
	On day 30	2.54±0.27	0.66±0.19	18.10±0.77	0.082±0.005
II	Preventive control	6.54±0.27[a]	1.74±0.07[a]	37.12±1.41[a]	0.015±0.003[a]
III	Preventive standard	2.50±0.19[b]	0.79±0.19[b]	24.12±0.87[b]	0.060±0.002[b]
IV	Preventive low dose	5.48±0.29	1.01±0.07[c]	31.11±1.47[d]	0.025±0.003[b]
V	Preventive high dose	5.01±0.36[c]	0.94±0.10[b]	30.59±1.36[c]	0.037±0.004[b]
VI	Curative control	6.80±0.20[a]	1.85±0.12[a]	41.20±1.45[a]	0.016±0.002[a]
VII	Curative standard	2.71±0.40[e]	0.81±0.09[e]	26.70±1.48[e]	0.064±0.002[e]
VIII	Curative low dose	5.91±0.37	1.16±0.13[f]	35.21±1.22[g]	0.026±0.001[e]
IX	Curative high dose	5.14±0.29[e]	1.05±0.09[f]	32.20±1.41[e]	0.034±0.002[e]

All values are expressed in mean±SEM; [a]P<0.001when compared to Group I; [b]P<0.001, [c]P<0.01, [d]P<0.05 when compared to Group II; [e]P<0.001, [f]P<0.01, [g]P<0.05 when compared to Group VI. EPV: Ethanolic extract of the seed of *Phaseolus vulgaris*, SEM: Standard error of the mean, BUN: Blood urea nitrogen

Table 4: Effect of EPV on *in vivo* antioxidant parameters

Groups	Treatment	LPO (nMole of MDA/mg of protein)	SOD (units/mg of protein)	CAT (µM of H_2O_2 consumed/min/mg of protein)	GSH (nmole of GSH/mg of protein)
I	Normal	0.30±0.17	6.11±0.33	0.059±0.001	1.97±0.09
II	Preventive control	5.01±0.53[a]	2.18±0.49[a]	0.025±0.002a	0.53±0.14[a]
III	Preventive standard	0.81±0.40[b]	5.73±0.40[b]	0.054±0.002[b]	1.86±0.10[b]
IV	Preventive low dose	3.10±0.31	3.68±0.71	0.036±0.001[c]	0.79±0.21
V	Preventive high dose	1.98±0.89[c]	5.12±0.67[c]	0.040±0.003[b]	1.20±0.19[d]
VI	Curative control	5.65±0.51[a]	2.39±0.33[a]	0.018±0.001[a]	0.53±0.28[a]
VII	Curative standard	1.09±0.72[e]	5.56±0.27[e]	0.050±0.001[e]	1.77±0.14[e]
VIII	Curative low dose	2.80±0.39[g]	4.10±0.38[g]	0.027±0.002[e]	1.24±0.19
IX	Curative high dose	2.15±0.71[f]	5.11±0.55[e]	0.041±0.001[e]	1.50±0.17[g]

All values are expressed in mean±SEM; [a]P<0.001 when compared to Group I; [b]P<0.001, [c]P<0.01, [d]P<0.05 when compared to Group II; [e]P<0.001, [f]P<0.01, [g]P<0.05 when compared to Group VI. EPV: Ethanolic extract of the seed of *Phaseolus vulgaris*, SEM: Standard error of the mean, MDA: Malondialdehyde, SOD: Superoxide dismutase, CAT: Catalase, GSH: Reduced glutathione, LPO: Lipid peroxidation

female rats was significantly less.[30] Induction of CaOx type of urolithiasis by EG is a well-validated and clinically relevant model. EG is metabolized to form acidic metabolites such as oxalic acid, benzoic acid, formic acid, and hippuric acid, which causes metabolic acidosis. Subsequently, this acidic condition favors CaOx nucleation followed by growth, aggregation and crystallization, then finally retention at the renal tissue to cause renal mitochondrial toxicity similar to clinical CaOx renal calculi.[31,32]

Urinary supersaturation with respect to stone forming constituents is considered as one of the causative factors for calculogenesis. Previous studies indicated that administration of EG (1% v/v) for 14 days in young albino rats produced renal calculi composed mainly of CaOx associated clinical symptoms of hyperoxaluria, hypercalciuria, and hyperphosphaturia.[33] Moreover, hyperoxaluria is considered as a significant risk factor in the pathogenesis of kidney stones than hypercalciuria. AC was administered to rats along with EG, as it acidifies the urine and then accelerates the process of lithiasis.[34] Hence, EG and AC in drinking water were employed to induce CaOx urolithiasis in rats.

In this study, enhanced deposition of calcium and oxalate in the kidney and their urinary excretion was observed in the disease control group animals indicating that administration of EG and AC induced CaOx urolithiasis. These results were inconsistent with the earlier reports of Patel *et al*.[35] On administration of EPV, the dose-dependent reduction in calcium and oxalate deposition in the kidneys and their urinary excretion in both the preventive and curative group animals reveals the potential of EPV in both preventing

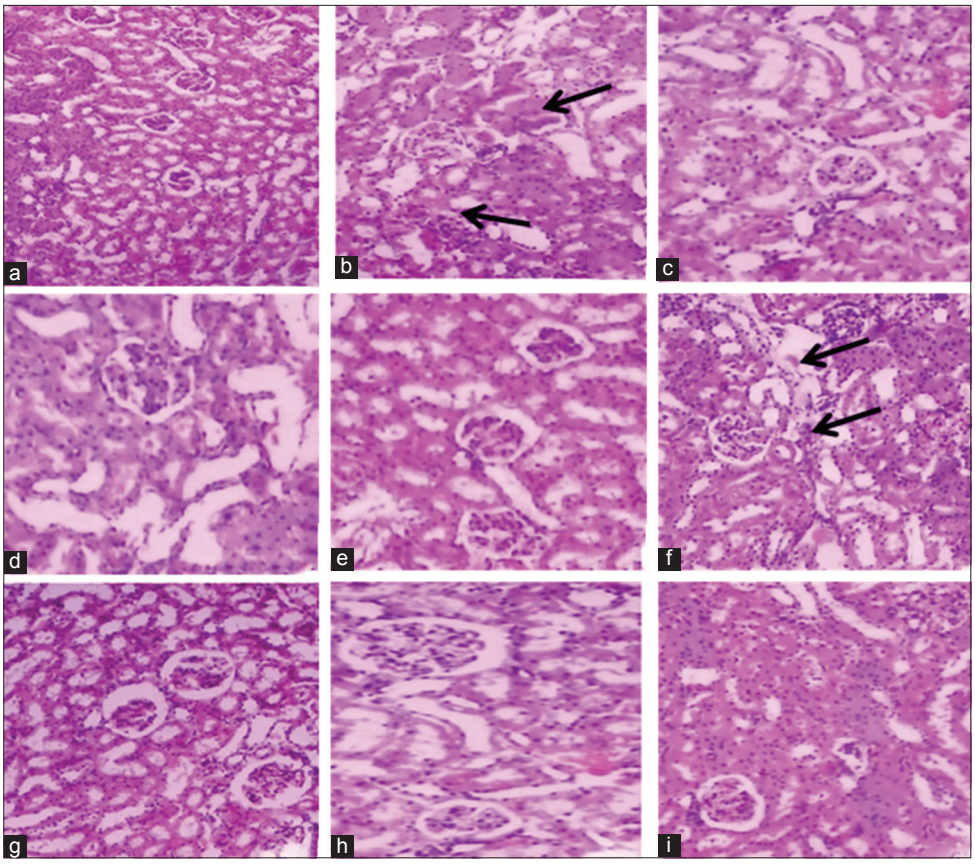

Figure 1: Histopathological findings of kidney under a light microscope (×40). (a): Normal: Normal glomerular structure and renal tubules. (b): Preventive control: Oxalate renal stone and tubular dilation and renal tubular damage. (c): Preventive standard (cystone 750 mg/kg): Dilation in the renal tubules and with normal glomerular structure. (d): Preventive low dose (EPV 200 mg/kg): Glomerular atrophy and tubular dilation. (e): Preventive high dose (EPV 400 mg/kg): Regenerated to normal glomerular structure with partial glomerular atrophy. (f) Curative control: Congested blood vessels and damage in the renal tubules. (g): Curative standard (cystone 750 mg/kg): Normal renal glomerular structure compared to normal. (h): Curative low dose (EPV 200 mg/kg): Renal tubular dilation and glomerular atrophy. (i) Curative high dose (EPV 400 mg/kg): Congested blood vessels and partial renal tubular dilation

the formation and dissolving the preformed CaOx stones. Although the exact reason was not clear for these diminished levels of calcium and oxalate in EPV treated groups, it might be due to inhibition of the synthesis of oxalate or interrupting the oxalate metabolism. In addition to this, EPV might have increased the bioavailability of nitric oxide to sequester calcium through the cGMP (3', 5' cyclic guanosine monophosphate) pathway.[36]

An increase in urinary phosphate excretion was observed in both the disease control group animals. Elevated urinary phosphate excretion along with oxalate induced stress appears to provide a suitable environment for stone formation by forming calcium phosphate crystals, which epitaxially causes CaOx deposition.[37] Treatment with EPV lowered the excretion of phosphate and reduced the risk of stone formation.

On treatment with EG and AC, an increase in urinary uric acid excretion was observed in the disease control group animals. Uric acid interferes with CaOx solubility and also reduces the inhibitory activity of glycosaminoglycans on CaOx crystallization.[38] Groups treated with EPV, lowered the uric acid concentration in the urine and reduced the risk of stone formation. These results were inconsistent with the earlier reports of Selvam *et al*.[38]

In addition to urinary stone forming constituents, urinary pH and volume play a prominent role in the process of stone development. The type of stones formed in humans depends on the pH of urine. In general, CaOx stones occur at a pH 5.0–6.5.[39] In this study, in both the disease control groups, a decrease in urinary pH to 5.0–6.0 was observed compared to normal animals (pH of 6.0–7.0) indicating the induction of CaOx stones. Moreover, dissolution of calculi can be achieved by an alteration in urinary pH. Treatment with EPV reversed the acidic pH to normal. This increase in urinary pH might be responsible for dissolving the complexes of calcium and oxalate. Urine volume also played a major role in the CaOx stone formation. In this study, a decrease in urine output was observed in both disease controls, indicating an obstruction in the urinary flow due to the presence of the CaOx stones.

An increase in the urinary output was observed on treatment with EPV, indicating its diuretic action. Further, it dilutes the urinary electrolytes concentration and might decrease the chance of stone development.

In both the preventive and curative controls, elevated levels of serum uric acid, creatinine and BUN with a concurrent decrease in creatinine clearance indicates marked renal impairment. In both disease controls, the elevated level of nitrogenous waste (creatinine, uric acid, and BUN) in the serum was accompanied by the decreased glomerular filtration rate (GFR) due to obstruction of stones in the Bowman's capsule.[40] Groups treated with cystone and EPV significantly reversed these levels through improved GFR.

In this study, EG and AC administration caused a significant increase in MDA levels through increased lipid peroxidation activity in the kidneys. This is further supported by earlier studies that exposure to high levels of oxalate and CaOx crystals produce cellular injury mediated by membrane lipid peroxidation through intracellular ROS (Reactive oxygen species) generation.[7,41] In this study, groups treated with EPV decreased the elevated MDA levels, indicating the effect of EPV in reducing oxalate associated lipid peroxidation through diminishing the oxalate levels and/or by scavenging the free radicals produced during the process of stone formation. In both the disease controls, a marked reduction in the levels of enzymatic antioxidants (SOD and CAT) and non-enzymatic antioxidant (GSH) levels were observed. These reductions in the levels, impaired antioxidant protection against oxidative stress, which might have further favored the accumulation and retention of oxalate and subsequent deposition of CaOx. Earlier studies suggested that treatment with antioxidants prevent CaOx deposition in the kidney.[42] In this study, groups treated with EPV improved these enzyme levels to normal, and this could be attributed due to the antioxidant principles/ phytoconstituents present in EPV. Preliminary phytochemical screening of EPV revealed the presence of flavonoids, polyphenols, tannins, and saponins. These phytochemicals may be responsible for the anticrystallization and antioxidant activities of EPV. Plant flavonoids are reported to possess antiurolithiatic activity through its antioxidant property.[43] Several reports suggest that saponins are having antiurolithiatic activity through its diuretic and disaggregating the suspension of mucoproteins.[34,44] An *in vitro* study suggests that phytic acid process anti-crystallization property by its chelating nature with the calcium.[45] *P. vulgaris* beans are rich in phytic acid, which combines with calcium to form calcium-phytate complex that reduces the availability of calcium for stone formation by reducing its intestinal absorption.[46]

Histopathological studies revealed congested blood vessels, tubular changes, irregular eosinophilic material, and scattered mononuclear inflammatory infiltration in both the diseased control groups. These remarkable histological changes might be due to oxalate induced lipid peroxidation and renal tissue damage. Treatment with EPV prevented these histological changes causing a significant decrease in the damage index of renal tissue.

Through several mechanisms are involved in the endogenous stone formation, in the present study, the antiurolithiatic activity of EPV may be due to its property of lowering stone forming constituents and by its antioxidant property. Further studies are needed to isolate the active principle/s responsible for the antiurolithiatic activity of *P. vulgaris* and to probe the mechanism/s of action.

ACKNOWLEDGMENTS

The authors are thankful to University Grants Commission (UGC), for providing financial assistance through UGC-SAP-DRS-I programme to the Department of Pharmaceutical Technology, Sri Padmavati Mahila Visvavidyalayam, Tirupati. The authors are also thankful for DST-CURIE facilities.

REFERENCES

1. Stamatelou KK, Francis ME, Jones CA, Nyberg LM, Curhan GC. Time trends in reported prevalence of kidney stones in the United States: 1976-1994. Kidney Int 2003;63:1817-23.
2. Lieske JC, Peña de la Vega LS, Slezak JM, Bergstralh EJ, Leibson CL, Ho KL, *et al*. Renal stone epidemiology in rochester, minnesota: An update. Kidney Int 2006;69:760-4.
3. Colobawalla BN. Incidence of urolithiasis in India: 1971. ICMR Tech Rep Ser 1971:8:42-51.
4. Coe FL, Evan A, Worcester E. Kidney stone disease. J Clin Invest 2005;115:2598-608.
5. Bouanani S, Henchiri C, Migianu-Griffoni E, Aouf N, Lecouvey M. Pharmacological and toxicological effects of paronychia argentea in experimental calcium oxalate nephrolithiasis in rats. J Ethnopharmacol 2010;129:38-45.
6. Baumann JM. Stone prevention: Why so little progress? Urol Res 1998;26:77-81.
7. Khan SR, Hackett RL. Retention of calcium oxalate crystals in renal tubules. Scanning Microsc 1991;5:707-11.
8. Pak CY. Prevention and treatment of kidney stones. Role of medical prevention. J Urol 1989;141:798-801.
9. Tang X, Lieske JC. Acute and chronic kidney injury in nephrolithiasis. Curr Opin Nephrol Hypertens 2014;23:385-90.
10. Barros ME, Lima R, Mercuri LP, Matos JR, Schor N, Boim MA, *et al.* Effect of extract of *Phyllanthus niruri* on crystal deposition in experimental urolithiasis. Urol Res 2006;34:351-7.
11. Khare CP. Indian Medicinal Plants: An Illustrated

Dictionary. New York: Springer Science and Business Media; 2008. p. 268-9.
12. Hangen L, Bennink MR. Consumption of black beans and navy beans (*Phaseolus vulgaris*) reduced azoxymethane-induced colon cancer in rats. Nutr Cancer 2002;44:60-5.
13. Heimler D, Vignolini P, Dini MG, Romani A. Rapid tests to assess the antioxidant activity of *Phaseolus vulgaris* L. Dry beans. J Agric Food Chem 2005;53:3053-6.
14. Pari L, Venkateswaran S. Effect of an aqueous extract of *Phaseolus vulgaris* on plasma insulin and hepatic key enzymes of glucose metabolism in experimental diabetes. Pharmazie 2003;58:916-9.
15. Sharma SB, Nasir A, Prabhu KM, Murthy PS, Dev G. Hypoglycaemic and hypolipidemic effect of ethanolic extract of seeds of *Eugenia jambolana* in alloxan-induced diabetic rabbits. J Ethnopharmacol 2003;85:201-6.
16. da S Queiroz-Monici K, Costa GE, da Silva N, Reis SM, de Oliveira AC. Bifidogenic effect of dietary fiber and resistant starch from leguminous on the intestinal microbiota of rats. Nutrition 2005;21:602-8.
17. Sreelakshmi N, Sujatha D, Bharathi, Prasad KV. Effect of *Phaseolus vulgaris* on *in vitro* calcium oxalate crystallization. J Glob Trends Pharm Sci 2015;6:2817-24.
18. Kokate CK, Purohith AP, Ghokale SB. Text Book of Pharmacogonosy. 36th ed. Pune: Nirali Prakashan; 2003. p. 593-7.
19. Singleton VL, Orthofer R, Lamuela-Raventos RM. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. Methods Enzymol 1999;299:152-78.
20. Quettier-Deleu C, Gressier B, Vasseur J, Dine T, Brunet C, Luyckx M, *et al*. Phenolic compounds and antioxidant activities of buckwheat (*Fagopyrum esculentum* moench) hulls and flour. J Ethnopharmacol 2000;72:35-42.
21. OECD. Guideline 423 Acute Oral Toxicity. Environmental Health and Safety Monograph Series on Testing and Assessment No. 24. Paris: OECD Environment, Health and Safety Publications; 2000.
22. Atmani F, Slimani Y, Mimouni M, Hacht B. Prophylaxis of calcium oxalate stones by herniaria hirsuta on experimentally induced nephrolithiasis in rats. BJU Int 2003;92:137-40.
23. Hodgkinson A, Williams A. An improved colorimetric procedure for urine oxalate. Clin Chim Acta 1972;36:127-32.
24. Cockcroft DW, Gault MH. Prediction of creatinine clearance from serum creatinine. Nephron 1976;16:31-41.
25. Niehaus WG Jr, Samuelsson B. Formation of malonaldehyde from phospholipid arachidonate during microsomal lipid peroxidation. Eur J Biochem 1968;6:126-30.
26. Misra HP, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem 1972;247:3170-5.
27. Aebi H. Catalase. In: Bergmeyer HU, editor. Methods of Enzymatic Analysis. New York and London: Academic Press; 1984. p. 673-7.
28. Jollow DJ, Mitchell JR, Zampaglione N, Gillette JR. Bromobenzene-induced liver necrosis. Protective role of glutathione and evidence for 3,4-bromobenzene oxide as the hepatotoxic metabolite. Pharmacology 1974;11:151-69.
29. Menon M, Resnick MI. Urinary lithiasis: Etiology, diagnosis and medical management. In: Walsh PC, Retik AB, Vaughan ED Jr, Wein AJ, editors. Campbell's Urology. 7th ed. Philadelphia, PA: WB Saunders; 2002. p. 2661-733.
30. Prasad KV, Bharathi K, Srinivasan KK. Evaluation of *Musa* (*Paradisiaca* linn. Cultivar)—"Puttubale" stem juice for antilithiatic activity in albino rats. Indian J Physiol Pharmacol 1993;37:337-41.
31. Marshall RW, Cochran M, Hodgkinson A. Relationships between calcium and oxalic acid intake in the diet and their excretion in the urine of normal and renal-stone-forming subjects. Clin Sci 1972;43:91-9.
32. McMartin KE, Wallace KB. Calcium oxalate monohydrate, a metabolite of ethylene glycol, is toxic for rat renal mitochondrial function. Toxicol Sci 2005;84:195-200.
33. Huang HS, Ma MC, Chen J, Chen CF. Changes in the oxidant-antioxidant balance in the kidney of rats with nephrolithiasis induced by ethylene glycol. J Urol 2002;167:2584-93.
34. Fan J, Glass MA, Chandhoke PS. Impact of ammonium chloride administration on rat ethylene glycol urolithiasis model. Scanning Microsc 1999:13:299-306.
35. Patel PK, Patel MA, Vyas BA, Shah DR, Gandhi TR. Antiurolithiatic activity of saponin rich fraction from the fruits of *Solanum* xanthocarpum schrad. & wendl. (*Solanaceae*) against ethylene glycol induced urolithiasis in rats. J Ethnopharmacol 2012;144:160-70.
36. Pragasam V, Kalaiselvi P, Sumitra K, Srinivasan S, Varalakshmi P. Counteraction of oxalate induced nitrosative stress by supplementation of l-arginine, a potent antilithic agent. Clin Chim Acta 2005;354:159-66.
37. Karadi RV, Gadge NB, Alagawadi KR, Savadi RV. Effect of *Moringa oleifera* lam. Root-wood on ethylene glycol induced urolithiasis in rats. J Ethnopharmacol 2006;105:306-11.
38. Selvam R, Kalaiselvi P, Govindaraj A, Bala Murugan V, Sathish Kumar AS. Effect of A. Lanata leaf extract and vediuppu chunnam on the urinary risk factors of calcium oxalate urolithiasis during experimental hyperoxaluria. Pharmacol Res 2001;43:89-93.
39. King JS Jr. Etiologic factors involved in urolithiasis: A review of recent research. J Urol 1967;97:583-91.
40. Rathod NR, Biswas D, Chitme HR, Ratna S, Muchandi IS, Chandra R, et al. Anti-urolithiatic effects of *Punica granatum* in male rats. J Ethnopharmacol 2012;140:234-8.
41. Khan SR. Reactive oxygen species, inflammation and

calcium oxalate nephrolithiasis. Transl Androl Urol 2014;3:256-76.
42. Selvam R. Calcium oxalate stone disease: Role of lipid peroxidation and antioxidants. Urol Res 2002;30:35-47.
43. Mayee R, Thosar A. Evaluation of *Lantana camara* Linn. (*Verbenaceae*) for antiurolithiatic and antioxidant activities in rats. Int J Pharm Clin Res 2011;3:10-4.
44. Gürocak S, Küpeli B. Consumption of historical and current phytotherapeutic agents for urolithiasis: A critical review. J Urol 2006;176:450-5.
45. Ahmed S, Hasan M, Mahmood Z. Inhibition and modulation of calcium oxalate monohydrate crystals by phytic acid: An *in vitro* study. J Pharmacog Photochem 2016;5:91-5.
46. Akond AG, Crawford JB, Talukder ZI, Hossain K. Minerals (Zn, Fe, Ca and Mg) and anti-nutrient (phytic acid). Am J Food Technol 2011;6:235-43.

Source of Support: Nil. **Conflict of Interest:** None declared.