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Protective effect of ethanolic leaf extract of *Alphonsea sclerocarpa* against ethylene glycol induced urolithiasis in rats

Chandrasekar R[1] Jayanth P C[2*] and Niranjan Babu M[1]

[1]Department of Pharmacognosy, [2]Department of Pharmacology, Seven Hills College of Pharmacy, Venkataramapuram, Tirupati, Chitoor District 517561, Andhra Pradesh, India

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Alphonsea sclerocarpa Thwaites belonging to the family Annonaceae is a small tree, which grows up to 10-15 m tall the leaves are simple and alternate. Despite its medicinal properties the plant seems to be less explored and hence this research aims at exploring the antiurolithiatic activity of ethanolic leaf extract of *A. sclerocarpa* on ethylene glycol induced urolithiasis in rats. *A. sclerocarpa* leaf powder was extracted using ethanol. The effect of ethanolic leaf extract of *A. sclerocarpa* (250 and 500 mg/kg, p.o.) was studied in experimentally induced renal stone in rats by *in vivo* model. Ethylene glycol model (0.75% in drinking water, for 28 days) was used for renal stone induction. The blood, urine and kidney samples were used for various parameters. The concentration of calcium, oxalate, phosphorus, creatinine and blood urea nitrogen was observed in each group. The phytochemical analysis was carried out to detect the presence of secondary metabolites like saponins and flavonoids in the ethanolic extract of *A. sclerocarpa* leaf extract. In ethylene glycol (0.75% v/v) treated animal model ethanolic extract of *A. sclerocarpa* leaf extract showed significant results on stone promoters (calcium oxalate, inorganic phosphate and sodium), kidney function parameters (uric acid, blood urea nitrogen, creatinine). On the basis of biochemical parameters and histopathological study it was confirmed that *A. sclerocarpa* leaf extract protected the renal cells from oxidative stress and injury induce by calcium oxalate crystals. The investigation of ethanolic extract of *A. sclerocarpa* leaf has shown promising antiurolithiatic activity and support folklore claims of these plants as antiurolithiatic. The mechanism of action of these plants for antiurolithiatic is apparently related to increased diuresis and lowering of urinary concentrations of stone-forming constituents, though it should be confirmed by the extensive exploratory studies.

Keywords: *Alphonsea sclerocarpa* Thwaites, Antiurolithiatic activity, Ethylene glycol, Kidney stones.

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Introduction

Alphonsea sclerocarpa Thwaites belonging to the family Annonaceae is a small tree, which grows up to 10-15 m tall the leaves are simple and alternate. The plant was explored for its antimicrobial, antioxidant, and antifungal[1]. Antibacterial and anticancer activity[2]. The study aims to report the antiurolithiatic activity of ethanolic leaf extract of *A. sclerocarpa* on ethylene glycol induced urolithiasis in rats.

Kidney stones are one of the most painful urologic disorders. Renal stone affects 5 to 15% of adults. Epidemiological studies revealed that nephrolithiasis is more common in men (12%) than in women (6%) and is more prevalent between the ages of 20 to 40 in both sexes[3] Urinary calculi is the third prevalent disorder in the urinary system. Urolithiasis is a common disease with an increasing incidence and prevalence worldwide that appears even more pronounced in industrialized countries[4]. Once recurrent, the subsequent relapse risk is raised and the interval between recurrences is shortened[5]. Features associated with recurrence include a young age of onset, positive family history, infection stones and underlying medical conditions[5]. Urolithiasis or nephrolithiasis represents the clinical condition of kidney stone disease. Stone formation in the urinary tract has been recognized for thousands of years, but during the last few decades, the pattern and incidence of the disease have changed markedly. Urinary stones affect 10–12% of the population in industrialized countries[5,6]. The incidence of urinary stones has been increasing over the last years while the age of onset is decreasing[7]. With a prevalence of > 10% and an expected recurrence rate of ~ 50%, the stone disease has an important effect on the healthcare system[8,9]. The aetiology of this disorder is multifactorial and is strongly related to dietary lifestyle habits or practices.

*Correspondent author
Email: chandrumnrcop@gmail.com

Most calculi in the urinary system arising from a common component of urine, e.g. calcium oxalate representing up to 80% of analyzed stones. In India, 12% of the population is expected to have urinary stones, out of which 50% may end up with the loss of kidneys or renal damage. Also, nearly 15% of the population of northern India suffers from kidney stones[10].

Urolithiasis refers to the solid nonmetallic minerals in the urinary tract. Among the several types of kidney stones, the most common are calcium oxalate. The formation of these stones involves several physico-chemical events, beginning with crystal nucleation, aggregation, and ending with retention within the urinary tract[11].

Ethylene glycol (EG) is rapidly absorbed and metabolized in the liver via alcohol dehydrogenase/ aldehyde dehydrogenase to glycolic acid. Glycolic acid is oxidized to glyoxylic acid, which, in turn, is further oxidized to oxalic acid by glycolate oxidase. High doses of EG (>2,500 mg/kg body wt), particularly when given as an oral bolus, cause the saturation dependent accumulation of glycolic acid in the plasma. So, glycolate oxidase (GO) is one of the rate-limiting enzymes in the metabolism of EG[12].

Materials and Methods

Chemicals and reagents

Ethylene glycol was purchased from Merck Ltd., Mumbai, India. Cystone was obtained from Himalaya Health Care Pvt. Ltd. Various kits for biochemical estimation of urine and serum were purchased from Light care Diagnostics, Vijayawada India. All other chemicals and reagents used were analytical grade and procured from approved chemical suppliers.

Plant collection and identification

The leaves of *A. sclerocarpa* were collected from Thalakona, Tirupati, India. The plant was authenticated (Voucher No 1168) by Dr K. Madhava Chetty, Assistant Professor, Department of Botany, Sri Venkateswara University, Tirupati (India).

Determination of physico-chemical parameters

Determination of physico-chemical parameters such as total ash, acid insoluble ash, water-soluble ash, foreign organic matter and moisture content of the crude drug was determined according to WHO guidelines on quality control methods for medicinal plant materials (WHO, 1992)[13].

Extraction procedure

The leaves were washed and dried at room temperature (35 ^{o}C) for 7 days and then crushed into a coarse powder using a mixer grinder. The coarse powder was separated using sieve number 24. The coarse powder was extracted using 100% ethanol at (45-50 ^{o}C). The coarse powder was packed in a muslin cloth and the extraction was carried out using soxhlet apparatus. The extraction process was continued for 120 hours. The ethanolic extract was concentrated on a boiling water bath and the solvent was evaporated until a semisolid mass was obtained. The wet mass was stored in a desiccator for further studies. The obtained powder was weighed and kept aside for phytochemical screening and pharmacological evaluation.

Preliminary phytochemical analysis

The ethanolic extract was subjected to phytochemical analysis using a conventional protocol like alkaloids, flavonoids, carbohydrate and glycosides gums and mucilage, fixed oils, saponins, and proteins[14].

In vivo evaluation

Animals

Healthy male Wistar rats weighing 150–200 g were obtained from the central animal house facility of Liveon Biolabs Pvt. Ltd, Tumkur. Prior to initiation of the experiment, all the protocols were reviewed and approved by the Institutional Animal Ethics Committee (IAEC) (SHCP/IAEC/17-01 dt. 25-12-2017) following guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animal (CPCSEA), Government of India. All the rats were randomly housed under hygienic conditions in polypropylene cages and allowed to acclimatize for one week. The animals were maintained at an ambient temperature of 25±2 °C with proper ventilation with 12 h light and dark cycle. The animals were acclimatized to the laboratory conditions for one week prior to the experiments and provided with standard diet and water ad libitum.

Acute toxicity studies

Acute oral toxicity study for the test extract of the *A. sclerocarpa* was carried out as per the guidelines set by Organization for Economic Co-operation and, revised draft (OECD) 425 and by the CPCSEA, Ministry of Social Justice and Empowerment, Government of India. The study revealed that

the administration of ethanolic leaf extract of *A. sclerocarpa* was safe up to a dose of 2000 mg/kg. No death was observed up to this dose, and the experimental animals were physically active. Hence 1/4th (250 mg/kg) and 1/8th (500 mg/kg), were selected as working doses for the present study[15].

Preparation of suspension of extracts

The extracts were suspended in a dilute solution of Tween-80 before dosing.

Ethylene glycol induced urolithiasis

Calcium oxalate urolithiasis was induced in experimental animals by administering ethylene glycol 0.75% (0.75 mL of ethylene glycol in 100 mL of drinking water) to rats for a period of 28 days for the production of calcium oxalate stone in rats[16,17].

Experimental design

Animals were divided into five groups, each group containing 6 animals. Group 1 - normal control rats received normal pelleted diet, group 2 disease control received (0.75% v/v EG in drinking water; p.o) group 3 received standard antiurolithiatic drug Cystone (750 mg/kg body weight) from 15th to 28th day, group 4 – received ethanolic extract of the leaves of *A. sclerocarpa* leaf at a dose of 250 mg/kg body weight from 15th to 28th day respectively, group 5 received ethanolic extract of the leaves of *A. sclerocarpa* leaf at a dose of 500 mg/kg body weight from 15th day to 28th day respectively.

Grouping of Animals

Animals were divided into five groups, each group containing 6 animals. Group 1 control rats received normal pelletted diet, group 2 disease control received 0.75% v/v EG in drinking water; p.o. Group 3 standard control received 0.75% v/v EG in drinking water+750 mg/kg Cystone; p.o. Group 4 received *A. sclerocarpa* ethanolic extract 250 mg/kg (0.75% v/v EG in drinking water+AsEe 250 mg/kg; p.o. Group 5 received *A. sclerocarpa* ethanolic extract 500 mg/kg (0.75% v/v EG in drinking water+AsEe 500 mg/kg; p.o.

Study of biochemical parameters

Collection and analysis of urine

For the estimation of calcium and oxalate the rats were kept separately in metabolic cages and urine samples of 24 h were collected on 28th day. The urine samples were acidified with HCl. The acidified samples were titrated with 0.9494 N of $KMNO_4$ till a light pink colour was obtained[18,19].

Serum collection and analysis

After urine collection blood was collected retroorbitally from each rat under mild anesthetic condition and animals were sacrificed by cervical decapitation and serum was separated by centrifugation at 10,000 rpm for 10 minutes and analyzed for urea, uric acid, creatinine and calcium using commercially available diagnostic kits (Light Care Diagnostics, Vijayawada)[20-22].

Histopathological study of the rat kidney

Preparation of kidney homogenate and biochemical estimation

To confirm the incidence of lithiasis, the animals were sacrificed and their kidneys were subjected to histopathological studies. The abdomen was incised and opened, both kidneys were removed from each animal. Isolated kidneys were cleaned off extraneous tissue, weighed and rinsed with ice-cold normal saline. The left kidney was fixed with 10% v/v neutral formalin and after harvesting, sliced horizontally. Histological sections were prepared by microtomy and stained with hematoxylin-eosin (H&E) dye for histological examination, (Light Care Diagnostics, Vijayawada) for Hematoxylin and Eosin staining.

Data analysis

The data obtained by the various parameters were statistically evaluated by one-way analysis of variance (ANOVA). The mean values±SEM were calculated for each parameter. P <0.05 was considered significant.

Results

Physico-chemical parameters

The determination of physico-chemical parameter is important in the determination of adulterants and improper handling of drugs. The result of the various physico-chemical parameters of powdered drug carried out using standard methods is shown in Table 1. The Phytochemical screening revealed the presence of alkaloids, saponins, tannins, steroids, anthocyanins, flavonoids and steroids.

In vivo antiurolithiatic activity

Ethylene glycol induced renal stone

There was a significant decrease in animal weight and a significant increase in dry and wet kidney weight in ethylene glycol induced model control as

compared to control animals. Similarly, a significant decrease in urine output was observed in model control animals. These changes were significantly prevented as well as reversed by the treatment of *A. sclerocarpa* and cystone respectively. In normal control animals, urinary pH was found acidic. But in model control animals, urine was found alkaline in nature. Treatment of test and standard drugs significantly reduced urinary pH level. The effect of urine parameters are shown in Table 2 and Fig. 1. The effect of serum parameters are shown in Table 3 and Fig. 2 whereas the effect of Kidney parameters are shown in Table 4 and Fig. 3.

Histopathological evaluation of kidneys

Histopathological examination of kidney section of normal control animals showed intact nephron structure. But in the model control group, there was significant damage to the kidney cells. Rupture of bowman's capsule and tubules, as well as infiltration of cells, were observed with ethylene glycol treated animals. This damage was significantly prevented by four weeks of treatment of all test drugs and standard drug. Ethylene glycol induced damage was also reversed by two weeks treatment of *A. sclerocarpa.* The results of Histopathological

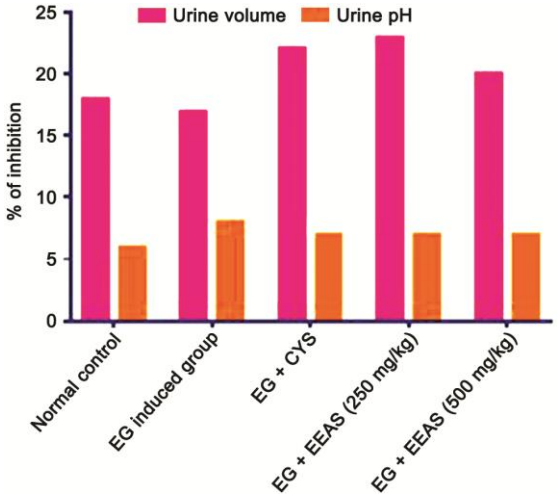

Fig. 1 — Effect of *A. sclerocarpa* treatment on urine parameters urine volume and urine pH

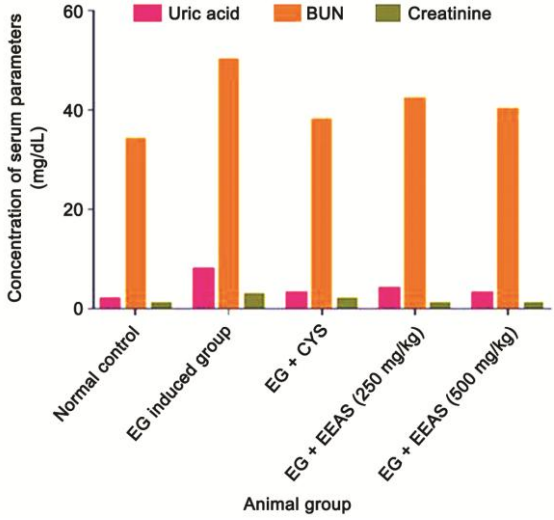

Fig. 2 — Effect of *A. sclerocarpa* treatment on serum parameters uric acid, creatinine and BUN

Table 1 — Physico-chemical analysis of *A. Sclerocarpa*

S. No.	Evaluation parameters	*A. sclerocarpa* leaf yield (% W/W)
1	Total ash	5.804±0.321
2	Water-soluble ash	1.042±0.062
3	Acid insoluble ash	1.553±0.078
4	Foreign organic matter	0.064±0.321
5	Moisture content	10.802±0.728

Table 2 — Effect of *A. sclerocarpa* on urine parameters on ethylene glycol induced urolithiasis in Wistar rats

	Group 1 Normal Control	Group 2 EG Induced	Group 3 EG + cystone Standard	Group 4 Test-1 AsEe 250 mg/kg	Group 5 Test-2 AsEe 500 mg/kg
Urine volume	18.17±0.872	16.50±0.763	21.50±0.806**	22.17±0.872 **	19.67±0.760
Urine pH	6.52±0.092	8.08±0.071$^{###}$	7.09±0.110***	7.42±0.066***	6.91±0.070***

All values are expressed as mean ± SEM (n = 6). Significance at $^{###}P$ <0.001, $^{##}P$ <0.01, $^{#}P$ <0.05 compared with normal control. ***P <0.001, **P <0.01, *P <0.05 compared with EG group

Table 3 — Effect of *A. sclerocarpa* in serum parameters on ethylene glycol induced urolithiasis in Wistar rats

	Group 1 Normal Control	Group 2 EG Induced	Group 3 EG + cystone Standard	Group 4 Test-1 AsEe 250 mg/kg	Group 5 Test-2 AsEe 500 mg/kg
Uric acid	2.21 ± 0.165	7.63 ± 0.451$^{###}$	3.12 ± 0.266***	3.96 ± 0.266***	2.65 ± 0.173***
BUN	34.37 ± 0.425	50.76 ± 1.043$^{###}$	38.36 ± 0.595***	41.18 ± 0.515***	39.35 ± 0.438***
Creatinine	1.01±0.048	2.51±0.060	1.30±0.040***	1.62±0.045***	1.19±0.045***

(BUN -Blood Urea Nitrogen)

All values are expressed as mean±SEM (n = 6). Significance at $^{###}P$ <0.001 compared with normal control. ***P <0.001 compared with model control.

Table 4 — Effect of *A. sclerocarpa* in kidney parameters on ethylene glycol induced urolithiasis in Wistar rats

	Group 1 Normal Control	Group 2 EG Induced	Group 3 EG + cystone Standard	Group 4 Test-1 AsEe 250 mg/kg	Group 5 Test-2 AsEe 500 mg/kg
Calcium	9.25±0.213	13.46±0.316$^{###}$	9.47±0.182***	10.11±0.147***	9.78±0.53***
Phosphate	3.99±0.128	9.74±0.361$^{###}$	4.35±0.187***	4.67±0.183***	4.47±0.143***
Sodium	136.3±1.282	147.0±1.033$^{###}$	140.3±0.666***	141.0±0.365***	138.8±0.477***

All values are expressed as mean±SEM (n = 6). Significance at $^{###}$ *P* <0.001 compared with normal control. ****P* <0.001, ***P* <0.01 compared with model control.

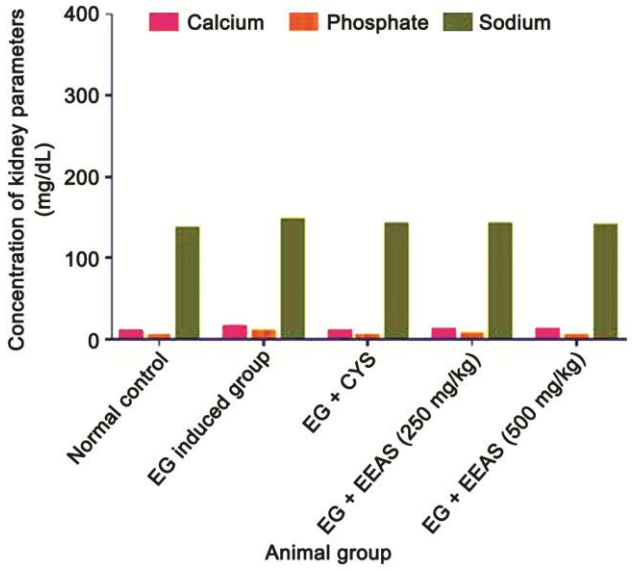

Fig. 3 — Effect of *A. sclerocarpa* treatment on kidney parameters calcium, phosphate and Sodium

Evaluation of Kidney tissue of different groups are shown in Plate. 1.

Discussion

In the present study, male rats were selected to induce urolithiasis because the urinary system of male rats resembles that of humans. Urinary supersaturating with respect to stone-forming constituents is generally considered to be one of the causative factors in calculogenesis. Evidence in previous studies indicates that in response to 28 days period of ethylene glycol (0.75%, v/v) administration. Stone formation in ethylene glycol fed animals causes hypercalciuria, which causes increased renal retention and excretion of calcium. The glomerular filtration rate (GFR) is an important parameter for ensuring renal function and it gets decreased in urolithiasis due to the obstruction to the outflow of the urine by stones in the urinary system, which leads to a rise in nitrogenous waste products like urea, creatinine, and uric acid in blood[23].

In the present study, calcium excretion was progressively increased in calculi induced animals. Since, it is accepted that hypercalciuria is the significant risk factor in the pathogenesis of stone formation. Increased urinary calcium is a factor favouring the nucleation and precipitation of calcium phosphate from urine and subsequent crystal growth. The increased urinary excretion of calcium and oxalate levels in urolithiatic rats similar to that of uroltihiatic rats increased the excretion of calcium and oxalate[24].

However, supplementation with ethanolic extract of *A. sclerocarpa* at 500 mg/kg in group V significantly lowered the elevated levels of blood urea, uric acid in serum and calcium and oxalate in urine as compared to the group II and standard control[25].

The antiurolithiatic effect was further confirmed by kidney histopathological studies. Indeed, kidney sections of disease control showed abundant crystal deposition. Furthermore, renal epithelial cells had more tubular dilatation and damage shown by large spaces in the tissue. In groups III, IV and V fewer crystal depositions were seen compared to untreated animals and the necrosis, as well as the tubule dilatation, was very limited**.** Renal stone deposition damages the renal tissue and deteriorates the renal function[26].

Lithogenic treatment caused impairment of renal functions of the untreated rats. It was lowered in animals receiving treatment with *A. sclerocarpa* leaf extract. Tissue injury and inflammation in these animals is due to exposure to calcium oxalate crystals. The renal epithelial injury further promotes crystal retention, as epithelial injury exposes a variety of crystal adhesion molecules on epithelial surfaces and promotes stone formation. The constituents of *A. sclerocarpa* restored the renal function and prevented renal cell injury.

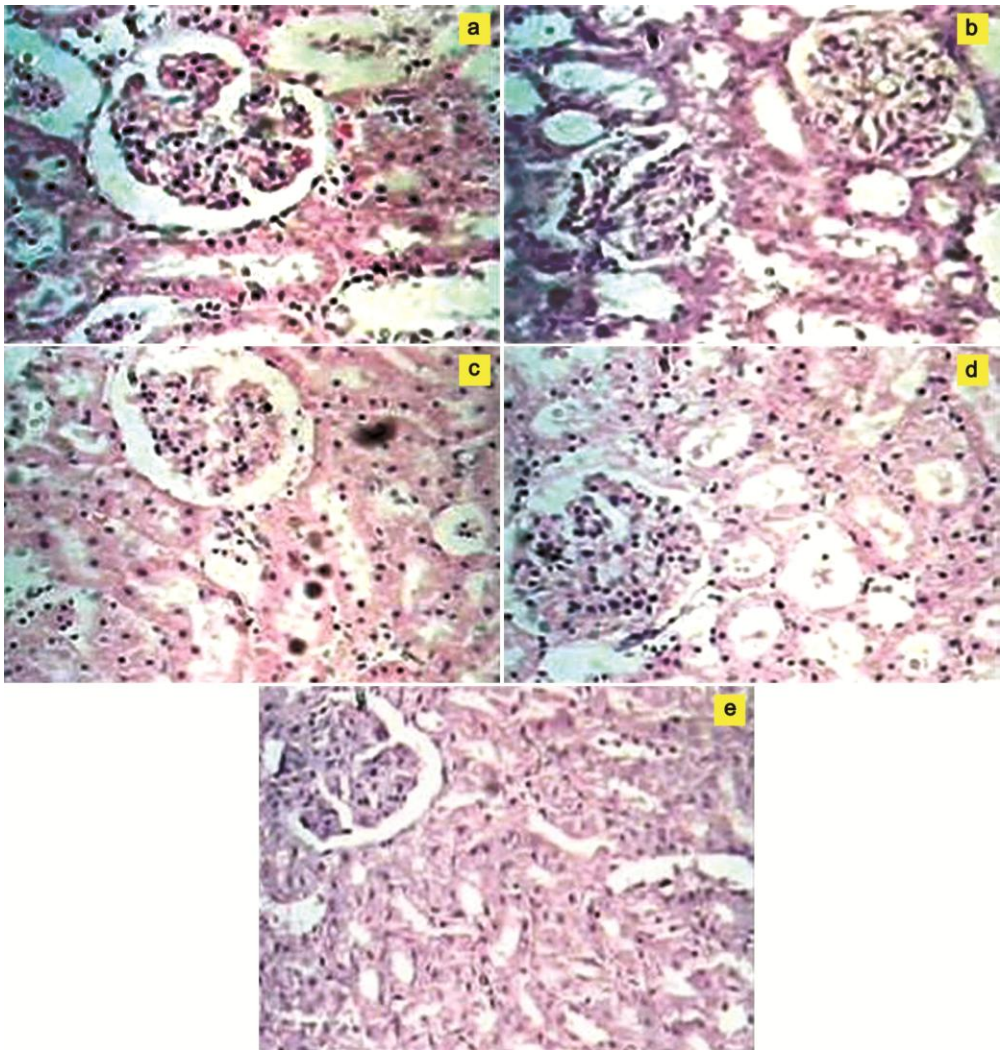

Plate 1 — Hematoxylin and eosin staining from animals of (a) normal control, (b) EG induced group, (c) EG+ cystone treated, (d) EeAs 250 mg/kg, (e) EeAs 500 mg/kg.

Conclusion

In the present investigation, the medicinal plant *Alphonsea sclerocarpa* was explored for its phytochemical nature and antiurolithiatic properties. The conclusion drawn from this investigation can be summarized as: Preliminary phytochemical screening of the drugs was carried out for the detection of different plant constituents. In ethylene glycol treated animal model, *A. sclerocarpa* (500 mg/kg, p.o.) showed significant results when compared with standard drug - cystone. It was also found to have significant prevention and reverse alteration in the kidney function parameters (uric acid, blood urea nitrogen and creatinine), when compared with cystone.

In vivo treatment of ethanolic extract of *A. sclerocarpa* leaf extract has shown significant anti urolithiatic activity in ethylene glycol induced urolithiasis in rats. In histopathological examinations of the kidney also shows a reduction in renal damage with the treatment of ethanolic extract of *A. sclerocarpa* leaf extract. Hence, selected plant *A. sclerocarpa* leaf extract has shown promising antiurolithiatic activity and support folklore claims of these plants as antiurolithiatic. Urolithiasis is apparently related to increased diuresis and lowering of urinary concentrations of stone-forming constituents, though it should be confirmed by the extensive exploratory studies. Finally, future research is recommended on these leaves for authentication in human beings.

Conflict of interest

No conflict of interest among the authors.

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