ORIGINAL ARTICLE

Antiurolithic activity of different fractions of *Aganosma dichotoma* as folk medicine of Andhra Pradesh

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Abstract

Objective: *Aganosma dichotoma* K. Schum has been traditionally used in the treatment of urological disorder in Ayurvedic system of medicine and also used by tribals of Chittoor district of Andhra Pradesh. Therefore, this study aims to establish its traditional claims and investigate the effect of root extract and fractions of *A. dichotoma* (Apocynaceae) against ethylene glycol (EG) induced urolithiasis in male albino rats. **Materials and Methods:** Lithiasis was induced by oral administration of ethylene glycolated water (0.75% v/v) in rats for 28 days and was manifested by the high level of calcium, oxalate, phosphate, creatinine, uric acid, and blood urea nitrogen (BUN) in both urine and serum. Polarized microscopy of urine, as well as histological study of kidney, was also performed in rats. Alcoholic extract, petroleum ether, chloroform, and butanol fractions were evaluated in this study. **Result:** Supplementation with ethanolic root extract of *A. dichotoma* (EAD) and their fractions significantly ($P < 0.001$) reduced the elevated urinary oxalate, calcium, and phosphate level as well as also decreased the level of creatinine, uric acid, and BUN in serum. In urine microscopy and histology of the kidney samples, EAD and petroleum ether fraction (PF) showed maximum inhibition of crystal deposition in a dose-dependent manner as compared to control group. **Discussion and Conclusion:** The result of this study revealed that EAD and PF are endowed with antiurolithic activity and justifying folk information of the plant. Further investigations are required to illuminate the chemical constituents and mechanism liable for antiurolithic activity.

Key words: *Aganosma dichotoma*, calcium oxalate, ethylene glycol, folk medicine, urolithiasis

INTRODUCTION

Lithiasis (stone formation) is one of the most painful urologic disorders responsible for urolithiasis, acute, and chronic renal failure and associated with morbidity and end-stage renal failure in India.[1] Approximately 12% of the global population is affected with lithiasis, and its re-occurrence rate in males is 70-81% and 47-60% in female.[2] The prevalence and incidence of urolithiasis are reported to be increasing across the world and more common in children over the past few decades due to rapid variations in habits and increasing affluence.[3] Various factors such as age, sex, industrialization, socioeconomic status, diet and environment, influences urolithiasis in terms of both site, and the physico-chemical composition of the calculi.[4] Recent epidemiology studies reveals that about 80% of stones are made up of calcium oxalate and calcium phosphate; 10% of struvite (magnesium ammonium phosphate formed during infection with bacteria that possess the enzyme urease), 9% of uric acid and the remaining 1% are composed of cystine, or ammonium acid urate are diagnosed as drug-related stones.[5]

This approach for the treatment of urolithiasis is mainly associated with some allopathic agents such as thiazide diuretics, alkali, allopurinol, penicillamine, analgesic, and probiotics. But most of them are not effective in all cases and have risks of long-term fertility.[6] Despite these drugs, some

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advanced techniques such as extracorporeal shock wave lithotripsy and percutaneous nephrostolithotomy are also used, but they have some side effects such as hemorrhage, tubular necrosis, and recurrence of renal stone formation and also these methods are costly and possess a great financial burden on society.[7,8] From ancient periods, a number of medicinal plants playing an important role in treating the problem of renal calculi.[9] Therefore, contemporary research focus is shifted toward the development of novel, efficient and herbal antiurolithic agent with higher efficacy and minimum side effects.

Aganosma dichotoma K. Schum (family: Apocynaceae) is a large climber commonly called the Malati in Hindi, Jati in Sanskrit, and Adavi malathi in Telugu. The root of the plant is folk medicine and used as antiurolithic, some tribals of Chittoor District of Andhra Pradesh taken the root powder of *A. dichotoma* along with milk in the early morning for a period of 2 weeks for the removal of stone in the urinary tract and bladder.[10] Plant is widely distributed across India, China, Philippines, and Indonesia. In India it extends throughout Assam, Bihar, West Bengal, Orissa, Andhra Pradesh, and Tamil Nadu and traditionally used in various ailments, including emesis, anthelmintic, bronchitis, leprosy, skin disease, ulcers, inflammation, and disease of mouth while flowers are used in disease of eye and leaves are used in biliousness.[11] Plant is also used as antiseptic, anodyne and also used as an ingredient in massage oils for paraplegia, neuralgia, and sciatica.[12] Therefore, this study aims to scientifically validate the traditional claims of *A. dichotoma* as antiurolithic potential.

MATERIALS AND METHODS

Collection of Plant Material

The roots of *A. dichotoma* were collected from Tumbura Kona Kshetram at Seshachalam hills and Tirumala hills, Chittoor District, Andhra Pradesh in the month of April 2013 and authenticated by Dr. K. Madhava Chetty, Taxonomist, S. V. University, Tirupati. A voucher specimen (COG/AD/17) has been kept in the Department of Pharmaceutics, IIT-BHU, Varanasi, for further reference.

Preparation of Extract and Fractions

After shade drying, the dried roots (1 kg) were coarsely grounded into homogenous powder using a mechanical grinder, passed through a 60 mesh sieve and exhaustively extracted with 95% ethanol (3 L) in a Soxhlet apparatus for 72 h. The extract of *A. dichotoma* (EAD) was filtered and concentrated with a rotary evaporator (IKA). The dried EAD (100 g) was made hydroalcoholic (7:3) and then subjected to successive fractionation using solvents of increasing polarity such as petroleum ether (PF), chloroform (CF), ethyl acetate (EAF), and butanol fraction (BF). The fractions were concentrated under reduced pressure in a rotary evaporator and were then kept in a desiccator until use. The yield of the fractions reported was PF - 9.49% (w/w), CF - 3.05% (w/w), EAF - 1.17% (w/w), and BF- 27.93% (w/w). After preliminary screening for antiurolithic activity, only petroleum ether, chloroform, and butanol fractions (PF, CF, and BF) were selected for the study.

Animals

Adult Charles Foster male albino rats (150 ± 10 g), were used for the study and pharmacological experiments were approved by the Central Animal Ethical Committee, Institute of Medical Sciences, Banaras Hindu University (Approval no.: Dean/2015/CAEC/983).

Acute Toxicity Study

Acute oral toxicity studies of extract (up to 2 g/kg, p.o, body weight) and fractions (up to 500 mg/kg, p.o, body weight) were performed on rats. Animals were closely observed for the initial 4 h after the administrations, then once daily up to 14 days.[13]

Ethylene Glycol (EG) Induced Urolithiasis Model

EG induced hyperoxaluria method was used to assess the antiurolithic activity in male albino rats. Animals were divided into 12 groups, each containing six animals. Group I animals served as normal control and maintained on regular laboratory diet and water *ad labium*. Groups II-XII animals were treated with 0.75% EG in drinking water to induce calcium oxalate crystals up to 28 days. All treatments were given once daily by the oral route.[14]

Group II: Disease control.
Group III: Animals received standard antiurolithiatic drug cystone (750 mg/kg, p.o.).
Groups IV-VI: Animals treated with EAD (100, 200, and 400 mg/kg, p.o.).
Groups VII and VIII: Animals treated with PF (50 and 100 mg/kg, p.o.).
Groups IX and X: Animals treated with CF (50 and 100 mg/kg, p.o.).
Groups XI and XII: Animals treated with BF (50 and 100 mg/kg, p.o.).

Collection and Analysis of Urine

All animals were kept in metallic cage separately, and urine samples of 24 h were collected on the 28th day, and a drop of concentrated hydrochloric acid was added to the urine sample before being stored at 4°C. Animals had free admittance to drinking water during the urine collection period. The collected urine sample was analyzed for urine volume, calcium, oxalate, phosphate, total protein, blood urea nitrogen (BUN), uric acid, and creatinine content using commercially available kit.[15]

Serum Analysis

On the 29th day blood was collected from retro-orbital plexus and allowed to cool centrifuge with 3000 rpm at 4°Cfor 15 min. Serum was recovered and frozen at −20°C and analyzed for creatinine, BUN, total protein, and uric acid.

Kidney Homogenate Analysis

Both kidneys of each animal were removed and cleaned off the extraneous tissue then preserved in 10% formalin. Isolated kidneys were dried at 80°C in hot air oven. 100 mg of dried tissue was boiled in 1N hydrochloric acid and centrifuged at 2000× g for 10 min to prepare homogenate. The calcium, oxalate, and phosphate content were analyzed in the supernatant.[16]

Polarized Microscopy of Urine

The polarization microscopy of urinary crystals was performed using Nikon digital microscope (Eclipse 200) at 10× magnification.[17]

Histopathological Studies

At the end of the experiment, all rats were sacrificed by euthanasia and remove the kidney then fixed in 10% formalin and tissue were then embedded in paraffin blocks for preparing sections (1-3 µm) which were then stained using hematoxylin and eosin dye. Pathological changes were observed through photographed using a Nikon digital microscope (Eclipse 200) at 10x magnification.[18]

Statistical Analysis

Results are expressed as mean ± S.E.M. with $n = 6$ per group. Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Dunnett's posttest for multiple group comparison. The difference was considered to be significant when $P < 0.05$.

RESULT

Acute Toxicity Study

Rats did not show any abnormal behavior except for mild sedation, and no mortality was detected after extract (up to 2 g/kg., p.o.) and all fraction (up to 500 mg/kg., p.o.) administration for initial 4 h. Hence, the therapeutic dose was taken as 100 mg/kg body weight for extract and 50 mg/kg body weight for fractions. To optimize the drug therapy, we selected 3 doses for extract (100, 200, and 400 mg/kg., p.o.) and 2 doses for all fractions (50 and 100 mg/kg., p.o.) to evaluate the efficacy of extract and fraction in EG-induced nephrolithiasis.

Estimation of Urine Volume

Increased urine volume indicates the diuretic property by diluting the urinary electrolyte concentration. Cystone (750 mg/kg, p.o), EAD 400 mg/kg., p.o. and all fractions at high doses were significantly ($P < 0.001$) increases the urine output at 28 days while only PF and CF at 100 mg/kg., p.o. showed significant action at 14 days (Table 1).

Estimation of Calcium, Oxalate and Phosphate in Urine, and Kidney Homogenate

The oxalate is the main product of EG metabolism which is the important initiative factor for lithiasis, so animals of Group II showed greater excretion of oxalate. However, extract, and PF in a dose-dependent manner lower the urinary oxalate level while CF and BF showed significant action ($P < 0.01$) only at a higher dose (100 mg/kg., p.o.). Level of oxalate in kidney homogenate sample was decreased by all the dose of EAD, PF, and CF in a dose-dependent manner, while BF 50 mg/kg., p.o. did not produce any significant action on oxalate level as compare to disease control group.

Urolithic rats showed increase in calcium output while both extract and fractions treated group showed significant action ($P < 0.001$) in a dose-dependent manner on calcium level in urine and kidney homogenate samples while BF 100 mg/kg., p.o. in kidney homogenate showed lesser action ($P < 0.05$) as compare to lithiasis induced rats. Similarly, phosphate excretion was also increased in stone forming animals that favor the nucleation and precipitation of calcium oxalate. Administration of EAD and PF significantly decreased the

Table 1: Effect of EAD and its fractions on urine output

Groups	Urine output in mL at 14th day	Urine output (mL) at 28th day
Normal control	7.5±0.4	10.8±0.91
Disease control	14.38±0.51	15.5±0.34
Cystone (750 mg/kg)	14.08±0.49	22.13±0.76[c]
EAD (100 mg/kg)	13.83±0.45	15.8±0.67
EAD (200 mg/kg)	14.4±0.38	18.71±0.41[a]
EAD (400 mg/kg)	16.15±0.5	19.93±0.91[c]
PF (50 mg/kg)	13.98±0.36	14.45±0.49
PF (100 mg/kg)	18.06±0.71[c]	22.85±0.67[c]
CF (50 mg/kg)	14.21±0.6	16.28±0.5
CF (100 mg/kg)	16.98±0.64[b]	22±0.56[c]
BF (50 mg/kg)	12.7±0.63	16.4±0.83
BF (100 mg/kg)	16.45±0.48	21.98±0.66[c]

All statistical data were expressed in mean±SEM and determined by one way ANOVA followed by Dunnett's post test, [a]$P<0.05$ statistically significant as compare to disease control, [b]$P<0.01$ statistically significant as compare to disease control, [c]$P<0.001$ statistically significant as compare to disease control

level of phosphate in kidney homogenate and urine samples while CF and BF at the higher doses showed the significant action on urine samples only (Table 2).

Estimation of Creatinine, BUN, Uric Acid and Protein in Urine, and Serum Samples

Crystal formation in renal tissue leads to obstruction in outflow of urine that reduces the glomerular filtration and causes an accumulation of waste product in blood such as creatinine, BUN, and uric acid. Marked renal damage was also seen in lithogenic rats by the elevated level of these waste products in serum. However, administration of EAD, PF, CF, and BF in dose-dependent manner significantly ($P < 0.001$) decreased the level of BUN and creatinine in both urine and serum samples as compared to untreated EG group except CF 50 mg/kg., p.o., which showed significant action ($P < 0.05$) on serum creatinine level. Similarly, level of uric acid in serum was decreased by both extract and fractions in a dose-dependent manner while in urine sample only EAD 400 mg/kg., p.o. ($P < 0.01$), EAD 200, and PF 100 mg/kg., p.o. ($P < 0.05$) showed significant action on the level of uric acid (Table 3).

As shown in Figure 1, both extract and fractions at all doses inhibit the loss of protein in serum of rats while in urine only extract and PF showed significant action while in CF and BF only CF 100 mg/kg., p.o. showed action ($P < 0.05$) on total protein loss.

Polarized Microscopy of Urine

Calcium oxalate crystals were present as monohydrate (COM) and dehydrated (COD) forms. COM crystals were generally in the form of biconcave ovals while COD were dipyramidal shape. Rat urine microscopy revealed that COM was more abundant in urine samples and also the size of crystals was observed comparatively larger in untreated urolithiatic animals. Whereas, number and size of calcium oxalate crystals were observed lesser after treatment with the different doses of extract, fractions and cystone (750 mg/kg., p.o.) as shown in Figure 2.

Histopathological Study

Histopathological evaluation revealed the deposition of calcium oxalate crystals in different groups, as well as some other histopathologic finding, was observed such as dilation of proximal tubules, tubular epithelial necrosis, and interstitial inflammation. As shown in Figure 3, normal control group [Figure 3L] possessed intact nephron structure with normal glomerular capsule and juxtaglomerular cells while in disease control group [Figure 3b] many calcium oxalate deposits and dilation of proximal tubule was observed. Cystone (750 mg/kg., p.o.), EAD (400 mg/kg., p.o.), and PF (100 mg/kg., p.o.) treated rats significantly prevented the rupture of kidney cells as compare to disease control group and close

Table 2: Effect of EAD, PF, CF and BF on oxalate, calcium and phosphate level in both urine and kidney homogenate in rats

Parameter	Disease control	Cystone	EAD 100	EAD 200	EAD 400	PF 50	PF 100	CF 50	CF100	BF 50	BF 100
Urine (mg/24 h)											
Oxalate	2.37±0.07	0.83±0.06^{c}	1.73±0.07^{c}	1.32±0.12^{c}	0.91±0.06^{c}	1.78±0.12^{b}	1.22±0.11^{c}	2.2±0.07	1.76±0.12^{b}	2.06±0.2	1.82±0.11^{b}
Calcium	4.35±0.1	1.64±0.08^{c}	2.85±0.11^{c}	2.19±0.1^{c}	1.96±0.12^{c}	2.85±0.1^{c}	2.22±0.12^{c}	2.84±0.12^{c}	2.44±0.22^{c}	2.96±0.08^{c}	2.19±0.11^{c}
Phosphate	3.26±0.11	1.21±0.11^{c}	3.1±0.11	2.04±0.09^{c}	1.32±0.12^{c}	1.7±0.17^{c}	1.4±0.11^{c}	2.94±0.14	2.29±0.14^{c}	2.83±0.12	2.32±0.17^{c}
Kidney homogenate (mg/g)											
Oxalate	2.48±0.18	0.94±0.09^{c}	2.01±0.14^{a}	1.51±0.17^{c}	0.99±0.09^{c}	1.42±0.13^{c}	1.13±0.05^{c}	1.99±0.08^{a}	1.61±0.09^{c}	2.04±0.14	1.79±0.14^{c}
Calcium	4.96±0.08	3.55±0.13^{c}	4.02±0.09^{c}	3.83±0.12^{c}	3.7±0.19^{c}	4.12±0.15^{c}	3.97±0.18^{c}	4.08±0.17^{c}	4.04±0.14^{c}	4.13±0.09^{c}	4.4±0.08^{a}
Phosphate	1.83±0.15	0.84±0.09^{c}	1.35±0.08^{a}	0.99±0.17^{c}	0.86±0.08^{c}	1.14±0.14^{c}	1.07±0.09^{c}	1.51±0.08	1.47±0.13	1.55±0.11	1.38±0.09^{a}

All statistical data were expressed in mean±SEM and determined by one way ANOVA followed by Dunnett's post test, $^{a}P<0.05$ statistically significant as compare to disease control, $^{b}P<0.01$ statistically significant as compare to disease control, $^{c}P<0.001$ statistically significant as compare to disease control

Table 3: Effect of EAD and its fractions on creatinine, BUN and uric acid level in serum and urine samples

Parameter	Disease control	Cystone	EAD 100	EAD 200	EAD 400	PF 50	PF 100	CF 50	CF100	BF 50	BF 100
Urine (mg/24 h)											
Creatinine	4.06±0.15	1.87±0.08^{c}	3±0.11^{c}	2.47±0.06^{c}	1.85±0.25^{c}	2.96±0.09^{c}	2.4±0.13^{c}	2.91±0.07^{c}	2.7±0.1^{c}	2.76±0.16^{c}	2.77±0.2^{c}
BUN	23.34±0.68	10.41±0.57^{c}	16.83±0.61^{c}	14.21±0.77^{c}	13.27±0.43^{c}	17.57±0.8^{c}	14.2±0.63^{c}	16.02±0.84^{c}	13.54±0.41^{c}	16.81±0.6^{c}	15.31±0.82^{c}
Uric acid	2.1±0.08	1.41±0.04^{c}	1.89±0.08	1.72±0.11^{a}	1.66±0.06^{b}	1.77±0.07	1.72±0.11^{a}	1.99±0.07	1.88±0.1	1.9±0.09	1.82±0.13
Serum (mg/dL)											
Creatinine	3.05±0.19	0.37±0.05^{c}	1.87±0.11^{c}	1.37±0.12^{c}	0.83±0.06^{c}	2.26±0.16^{b}	1.76±0.2^{c}	2.46±0.12^{a}	2.15±0.1^{c}	2.17±0.19^{c}	2.16±0.18^{c}
BUN	25.66±0.79	11.67±0.59^{c}	17.81±1.04^{c}	16.04±1.23^{c}	13.81±0.64^{c}	16.02±0.64^{c}	16.18±0.98^{c}	15.34±0.63^{c}	14.75±0.82^{c}	15.2±0.62^{c}	13.15±0.71^{c}
Uric acid	3.13±0.17	1.52±0.18^{c}	2.15±0.14^{c}	2.08±0.16^{c}	1.99±0.09^{c}	1.95±0.2^{c}	1.76±0.09^{c}	2.27±0.16^{b}	2.11±0.15^{c}	2.24±0.16^{b}	2.25±0.19^{c}

All statistical data were expressed in mean±SEM and determined by one way ANOVA followed by Dunnett's post test, $^{a}P<0.05$ statistically significant as compare to disease control, $^{b}P<0.01$ statistically significant as compare to disease control, $^{c}P<0.001$ statistically significant as compare to disease control

to normal architecture of kidney section while CF and BF showed less protection even at higher dose.

DISCUSSION

According to Khan (1997), urolithiasis can be induced in rats using a variety of agents such as EG, sodium oxalate, ammonium oxalate, hydroxy-L-proline, and glycolic acid but Kidney being the principal target for EG-induced toxicity.[19] Thus, this study involves the induction of hyperoxaluria in male albino rats with the oral administration of EG which stimulates the nidus formation, calcium oxalate saturation, crystal aggregation, and retention within kidney tubules.[20] Male rats were selected for the study due to resemblance of urinary system with the human and also earlier studies have shown that frequency of stone deposition was significantly high in male as compare to female rats.[21] This study demonstrates that ethanolic root extract and petroleum ether fraction showed good antiurolithic activity in a dose-dependent manner as compared the standard drug cystone.

EG is rapidly absorbed and metabolized in the liver into glycolic acid via alcohol dehydrogenase/aldehyde dehydrogenase then glycolic acid is further oxidized into glyoxylic acid. This glyoxylic acid is again oxidized into oxalic acid by glycolate oxidase. High doses of EG (>2.500 mg/kg, body wt) through an oral bolus, cause the saturation dependent accumulation of glycolic acid in the plasma, so glycolate oxidase is one of the rate-limiting enzymes in the metabolism of EG.[22] Thus, oxalate is the main product of EG metabolism which is the important initiative factor for lithiasis.

Calcium and phosphate play a key role in renal calculogenesis and increased level of calcium and phosphate in urine could be due to defective tubular re-absorption in the kidney.[23] Urinary supersaturation is also considered to be one of the causative factors in calculogenesis. Similarly, phosphate excretion was also increased in stone forming animals that favor the nucleation and precipitation of calcium oxalate.[24] In this study, increased urinary phosphate excretion along with the oxalate stress seems to provide an environment appropriate for stone formation by forming calcium phosphate crystal, which epitaxially induces calcium oxalate depositions.[25]

However, treatments with ethanolic root extract and their fractions lower the levels of calcium as well as oxalate in urine; this is possibly by increasing/restoring the tubular reabsorption in the renal tubules.

Administration of EG causes the development of persistent crystalluria in all rats, due to agglomeration of particles. It is a crucial step in urinary stone formation because agglomerates getting trapped in renal tubules and retain in the kidney.[26] Crystal formation in renal tissue leads to obstruction in the outflow of urine that reduces the glomerular filtration and causes an accumulation of waste product in blood such as

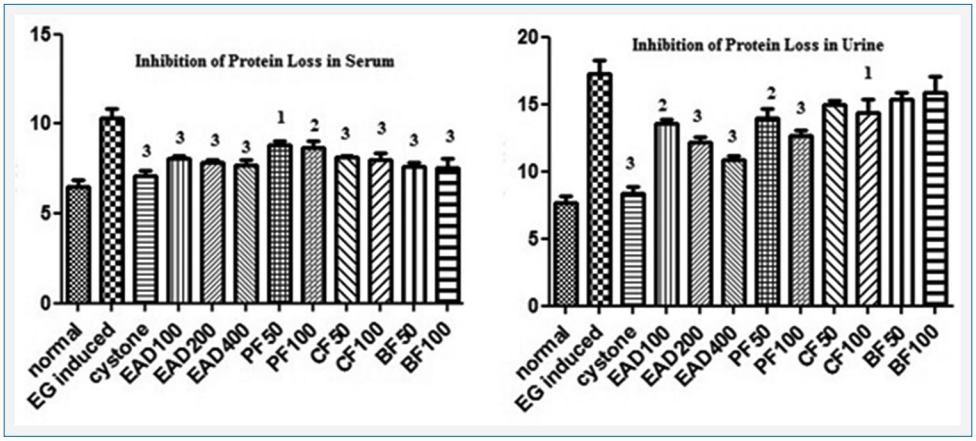

Figure 1: Effect of extract and fractions on inhibition of protein loss in serum and urine. All statistical data were expressed in mean ± SEM (*n*=6) and determined by one-way ANOVA followed by Dunnett's posttest. (1) *P*<0.05 statistically significant as compare to disease control, (2) *P*<0.01 statistically significant as compare to disease control, (3) *P*<0.001 statistically significant as compare to disease control

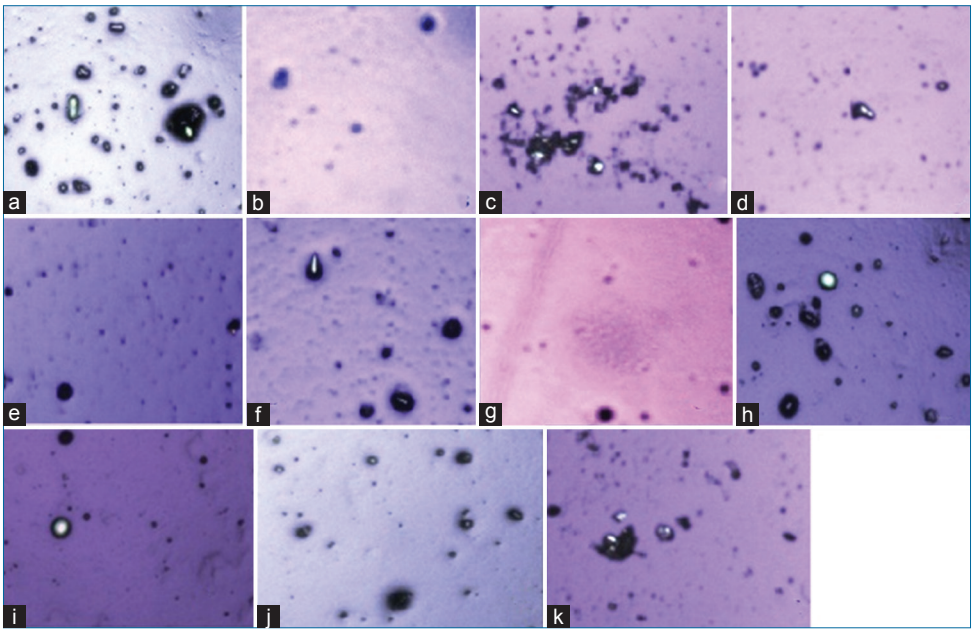

Figure 2: Polarized microscopy of rat urine. (a) Lithiasis induced, (b) cystone (750 mg/kg, p.o.) treated rat, (c-e) EAD (100, 200, and 400 mg/kg, p.o.) treated, (f-g) PF 50 and 100 mg/kg treated, (h-i) CF 50 and 100 mg/kg treated, and (j-k) BF 50 and 100 mg/kg treated rats

creatinine, BUN, and uric acid.[27] However, extract, and fractions at all doses decreased the concentration of these waste products and inhibits the loss of protein in serum and urine of rats. This data clearly support that the ethanolic extract of root of *A. dichotoma* and their bioactive fractions can reduce supersaturation of urine with calculogenic ions such as calcium and oxalate while decreased urinary creatinine reflects improved renal function. According to Selvam *et al*., uric acid interfere the solubility of calcium oxalate by binding with it and decreased the inhibitory activity of glycosaminoglycans.[16] The predominance of uric acid crystals in calcium oxalate stones improve the capability of calcium oxalate binding with uric acid binding protein and modulates its crystallization. Treatment of EAD and PF reduced the excretion of uric acid and thereby reduces the risk of stone formation.

From various reports, it was also observed that calcium oxalate crystals cause direct oxidative stress which damage the glomerulus and tubules due to the generation of reactive oxygen species. From the histopathological study of kidney samples, it was also confirmed that EG damage the kidney. Lipid peroxidation represents oxidative tissue damage due to hydrogen peroxide (H_2O_2) and superoxide anion (O_2) and hydroxyl radicals (OH), which causes the structural alteration of membrane due to loss of essential fatty acid with the formation of cytosolic aldehydes and peroxide products.[28] Catalase enzyme also regulates the H_2O_2 level which can lead

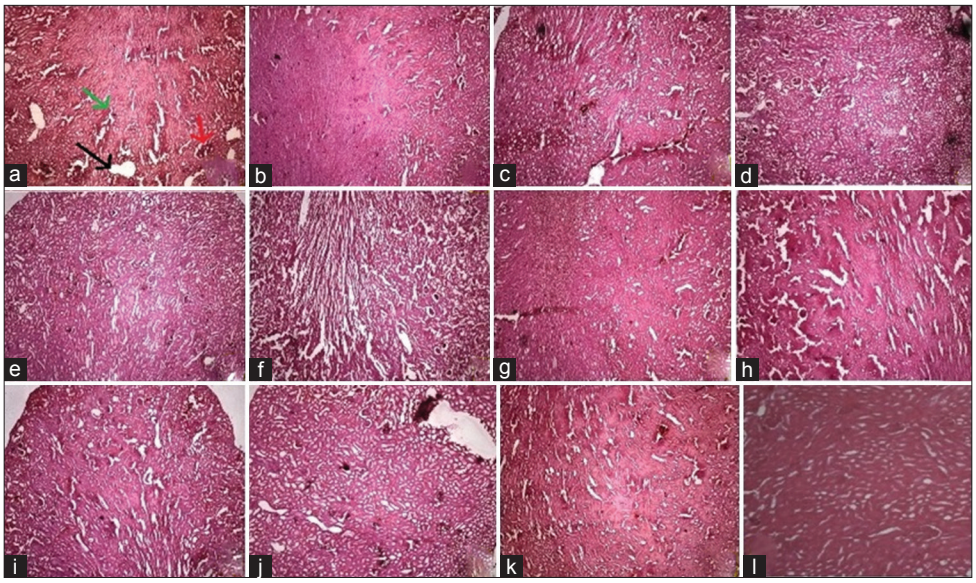

Figure 3: Histology of rat kidney. (a) Lithiasis induced, (b) cystone (750 mg/kg, p.o.) treated rat, (c-e) EAD (100, 200, and 400 mg/kg, p.o.) treated, (f-g) PF 50 and 100 mg/kg treated, (h-i) CF 50 and 100 mg/kg treated, and (j-k) BF 50 and 100 mg/kg treated rats. (Indication of arrow) black arrow indicates the dilation of tubule, red arrow indicates the ruptured glomeruli, and green arrow indicates the hemorrhage

to hydroxyl radical surplus through the metal-catalyzed fenton (Fe/Cu) and Haber-Weiss reactions. The release of xanthine oxidase during uric acid formation is the main factor for the release of hydrogen peroxide. Catalase is the only enzyme that regulates the hydroxyl radical formation and its decreased activity in urolithiasis may causes excessive accumulation of H_2O_2 in kidney. Hence, elevated antioxidant enzymes in the kidney can remunerated the oxidative stress.[29] Thus, *in-vitro* and *in-vivo* antioxidant activity have been also studied in our previous study which further act as a supporting evidence that extract would also be producing protective effect on urolithiasis due to its antioxidant potential.[30,31]

Various phytoconstituents such as lupeol, ursolic acid, quercetin, and beta-sitosterol have been identified in our high-performance thin layer chromatography study of extract and fraction in which quantity of lupeol was high in the ethanolic extract as compared to other bioactive fractions, but this study is under consideration in some other journal. According to Anand *et al*., lupeol was acting as antiurolithic agent and helps in the removal of lithogenic substances and prevent their deposition in the urinary bladder.[32] Thus, lupeol may contribute in antiurolithic activity. This study demonstrates that mainly EAD and PF showed good antiurolithic activity in a dose-dependent manner while CF and BF possessed lesser action as compared the standard drug cystone. The mechanism underlying this effect is still unknown, but the possible mode of action includes increased diuresis, lowering the urinary concentration of stone-forming salts and prevent the supersaturation of the crystallizing salts, decreasing the crystal size and restore normal kidney architecture by reducing the renal tissue injury. Further, studies are required to illuminate the chemical constituents and mechanism liable for pharmacological activities.

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