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AMELIORATIVE EFFECT OF KALANCHOE PINNATA, EMBLICA OFFICINALIS, BAMBUSA NUTANS AND CYNODON DACTYLON ON ETHYLENE GLYCOL AND AMMONIUM CHLORIDE INDUCED NEPHROLITHIASIS

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Abstract

The study aims at the exploration of antilithiatic potential Effect of Kalanchoe pinnata, Emblica officinalis, Bambusa nutans and Cynodon dactylon ethyl acetate fraction (EACD, EAEO, EAKP, and EABN) rich in flavonoid, polyphenol on ethylene glycol (EG) and ammonium chloride (AC) induced nephrolithiasis.

Rats were treated with three graduated doses of EACD, EAEO, EAKP, and EABN per orally while given EG 0.75% (v/v) along with AC 2% (w/v) in drinking water for 10 days. On 11th day 24 hour urine was collect and after sacrifice blood was collected for estimation of biochemical parameters. Kidneys tissue was subjected to free radical scavenging ability estimation and histopathological examination.

EG and AC treated group showed reduced body weight gain whereas EACD was most effective in maintaining normal weight gain. EAKP and EAEO showed significant reversal of decreased urine volume and pH caused by EG and AC treatment. EG and AC treatment showed significant elevation of urine sodium, chloride, potassium, calcium, phosphates and protein with a decrease in magnesium, creatinine and uric acid. EACD, EAEO, EAKP, and EABN significantly ($P < 0.01$ - 0.001) reverted all the urine parameter abnormalities. EG and AC induced nephrolithiasis caused significant increase in blood sodium, chloride, potassium, calcium, phosphates, BUN, creatinine, uric acid, SGOT, and SGPT. Significant ($P<0.05–0.001$) reduction in elevated levels of serum parameters were observed following administration of EAKP (50 and 100 mg/kg), EAEO (50 and 100 mg/kg), EABN (50 and 100 mg/kg) and EACD (300 and 500 mg/kg). EAKP, EAEO, EABN, and EACD treated groups significantly ($P<0.01–0.001$) decreased malondialdehyde, superoxide dismutase and calcium level with an increase in reduced glutathione level of the kidney. EG and AC showed deposition of CaOx microcrystals along with marked dilation of tubules, interstitial fibrosis, and degeneration of epithelial cell lining with infiltration of mononuclear inflammatory cells in the kidney.

EAKP and EAEO was most effective in minimizing these histological damages. EACD, EAEO, EAKP and EABN showed dose dependent anti-nephrolithiatic activity. This study outcome substantiates excellent anti urolithiatic potential of K. pinnata and E. officinalis, and moderate protective effect of B. nutans and C. dactylon against EG and AC induced urolithiasis.

Keywords: Kalanchoe pinnata, Emblica officinalis, Bambusa nutans, Cynodon dactylon, *flavonoid, polyphenol, nephrolithiasis*

Introduction

The formation of kidney stone or calculi is serious though not life threatening disorder occour throughout the world with increasing prevalence rate due to environmental factors and genetic predisposition. In medical terminology the condition of having urinary calculi is termed as nephrolithiasis and urolithiasis where the root word "lith" means 'a stone' [1]. Renal calculi are crystalline structures composed most commonly of calcium oxalate (CaOx) salts known as hyperoxaluria condition with an excessive urinary excretion of oxalate. Renal calculi are formed when the concentrations of CaOx, hydrogen, sodium ions and uric acid are higher than usual with supersaturation of urine. The supersaturated ions are more likely to come out of solution and crystallize. Risk factors for supersaturation include dehydration, high fat diet, animal protein, high salt intake, and obesity. Typically, the crystals are formed in the distal tubule, nephron loop and/or in collecting system. Most crystals simply pass unnoticed into the urine, but some of them adhere to the epithelium of the tubules, particularly in the collecting system and form seed crystals that lead to the formation of stones [2]. The stones may remain in the collecting system or may break off and lodge in the calyces, renal pelvis, and ureter. Stones lodged within the urinary system causes occurrence of common nephrolithiasis symptom like, severe pain and renal colic that radiates from the lumbar region to the pubic region along with hematuria, nausea, and vomiting. Renal calculi are perceived as an acute disorder, but with the growing stage of urolithiasis, it became a systemic disease that can lead to end stage renal disorder [3].

Most of the renal calculi are composed of CaOx and nephrolithiasis in rats can be induced experimentally with the administration of 0.75-1% of ethylene glycol (EG) for 4-6 weeks [4]. Reactive oxygen species are the most destructive factors of stone formation [5]. Medicinal plants with well-known antioxidative and diuretic effects are useful for prevention of nephrolithiasis [6]. Many herbs are used to treat kidney stones, but the action of most of these herbs have not yet proved scientifically. In this study, Kalanchoe pinnata (Crassulaceae) leaf, Emblica officinalis (Euphorbiaceae) fruit, Bambusa nutans (Graminae) shoot, and Cynodon dactylon (Poaceae) whole plant were selected to screen for antilithiatic activity based on extensive literature search and ethnopharmacological uses.

Our previous reports signify rich presence antioxidative phytocompounds like polyphenols and flavonoids in K. pinnata and E. officinalis ethyl acetate fractions followed by C. dactylon and B. nutans. Ethyl acetate fractions of B. nutans shoot, E. officinalis fruit, and K. pinnata leaf have excellent in vitro CaOx crystal growth inhibition potential. All the plants have well known traditional and Ayurvedic uses for diuretic potential [7]. C. dactylon, E. officinalis, K. pinnata and B. nutans showed high sodium (Na^+) and potassium (K^+) excretion potential where as high chloride (Cl^-) excretion ability was observed in K. pinnata followed by C. dactylon and E. officinalis [8]. Diuretics are effective in lowering urine calcium excretion and reducing kidney stone recurrence in nephrolithiasis patients by controlling supersaturation of urine [9,10,11]. Forced diuresis showed acute symptomatic relief in patients of urolithiasis [12]. These facts indicate the possible antiurolithiatic potential of the ethyl acetate fraction of the selected plants worth to be explored. The study aims at screening ethyl acetate fraction of C. dactylon, E. officinalis, K. pinnata and B. nutan rich in flavonoid and polyphenol for anti-nephrolithiasis potential following EG and AC oral administration in drinking water.

Methods

Collection and identification of plant

The K. pinnata leaf, E. officinalis fruit, B. nutan shoot, and C. dactylon whole plant was collected in between October 2014 to April 2015 from Rewa, Raisen and Bhopal districts of M.P. Herbarium was prepared for all the four plants and authenticated by Dr. Zia Ul Hasan, Prof, and Head, Herbarium Department, Department of Botany, Saifia College of Science Bhopal, M.P. Voucher specimen no. 456/Bot/saifia/14 was allotted.

Extraction of Plant Material

Drying, processing, and enrichment of ethyl acetate fraction from hydro-methanolic extract was performed as described previously [7].

Experimental Animal

The in-vivo study was performed with due permission from the Institutional Animal Ethical Committee. Laboratory breed adult Wistar male rats (100-150 gm) were used. The animals were housed in polypropylene cages with paddy husk bedding maintained in hygienic condition at 22±2°C temperature and 12hr light-dark cycle. The animals were fed with standard pallet balanced diet and water ad libitum. All experimental procedures were conducted in accordance with the ethical guidelines of CPCSEA, New Delhi.

Study protocol

Dose range selected for E. officinalis, K. pinnata and B. nutans are 25, 50 and 100 mg/kg, p.o., and 100, 300 and 500 mg/kg for C. dactylon based on the acute toxicity we have reported previously [8]. Rats were divided into fifteen groups comprising five animals in each group. Each group had been treated with the doses of enriched extracts orally as per the protocol daily for 10 days. EG 0.75% (v/v) along with AC 2% (w/v) was given via drinking water as nephrolithiasis inducing agent to all the groups except group I [5,13]. The animals were grouped as follows:

GROUP 1: Fed with drinking water ad libitum for 10 days (Vehicle control)

GROUP 2: Fed with drinking water containing 0.75% EG + 2% AC (Negative control)

GROUP 3: Fed with drinking water containing 0.75% EG + 2% AC + Cystone (750 mg/kg)

GROUP 4: Fed with drinking water containing 0.75% EG + 2% AC + EAKP (25 mg/kg)

GROUP 5: Fed with drinking water containing 0.75% EG + 2% AC + EAKP (50 mg/kg)

GROUP 6: Fed with drinking water containing 0.75% EG + 2% AC + EAKP (100 mg/kg)

GROUP 7: Fed with drinking water containing 0.75% EG + 2% AC + EAEO (25 mg/kg)

GROUP 8: Fed with drinking water containing 0.75% EG + 2% AC + EAEO (50 mg/kg)

GROUP 9: Fed with drinking water containing 0.75% EG + 2% AC + EAEO (100 mg/kg)

GROUP 10: Fed with drinking water containing 0.75% EG + 2% AC + EABN (25 mg/kg)

GROUP 11: Fed with drinking water containing 0.75% EG + 2% AC + EABN (50 mg/kg)

GROUP 12: Fed with drinking water containing 0.75% EG + 2% AC + EABN (100 mg/kg)

GROUP 13: Fed with drinking water containing 0.75% EG + 2% AC + EACD (100 mg/kg)

GROUP 14: Fed with drinking water containing 0.75% EG + 2% AC + EACD (300 mg/kg)

GROUP 15: Fed with drinking water containing 0.75% EG + 2% AC + EACD (500 mg/kg)

Vehicle control rats had free access to drinking water ad libitum along with regular food. Rats of groups 2 to 15 also has free access to regular food and drinking water containing EG and AC. Group 4 to 15 animals were treated with plant extracts per orally starting from 1st day to 10th day. Body weight of all the animals was noted before extract treatment on the 1st day, 5th day and 10th day and percent change calculated. The animals were sacrificed on the 11th day by cervical decapitation under ether anesthesia. Liver, kidney, spleen, and heart were removed and washed with cold saline, pressed between filter paper pads and weighed. Relative organ weight per 100 gm of body weight was calculated.

Collection and analysis of urine

All the animals were kept in individual cages and 24 hr urine samples were collected on the 10th day after the last dosing with free access to water. Urine volume and pH was determined and stored at 4°C after adding a drop of concentrated HCl. The sample was also subjected to microscopic examination for CaOx crystals and the confirmation of urolithiasis [14]. Urine samples were analyzed for the level of sodium, chloride, potassium, calcium, phosphates, magnesium, creatinine, uric acid, and protein with the help of diagnostic kits (Span Diagnostics Ltd, India) in Autoanalyser (Star 21 Plus, Rapid Diagnostics, India).

Collection and analysis of blood

Blood was collected immediately after sacrifice in a clean, dry test tube and allowed to coagulate for

30 min in room temperature. Serum was separated by centrifugation at 3000 rpm for 10 minutes. The supernatant (serum) was collected and used for estimation of sodium, chloride, potassium, calcium, phosphates, blood urea nitrogen (BUN), uric acid, creatinine, SGOT and SGPT using diagnostic kits (Span Diagnostics Ltd., India) in Autoanalyser (Star 21 Plus, Rapid Diagnostics, India).

Estimation of free radical scavenging ability of the kidney

Promptly excised kidney tissue was kept in chilled saline, thoroughly washed with cold saline and homogenized with 10% trichloro acetic acid using a Teflon-glass tissue homogenizer (Remi, India) and centrifuged at 3000 rpm (4□C) for 10 min. The supernatant was diluted 10 fold with phosphate buffer, kept on ice and used for the assay of lipid peroxidase (LPO), reduced glutathione (GSH) and superoxide dismutase (SOD) [15,16,17]. Calcium content was measured using a calcium assay kit (Pro Lab Marketing Pvt. Ltd., New Delhi, India) following the method of Hernandez et al. [18].

Histopathology of kidney

Kidney tissue sections were stained with hematoxylin-eosin and observed for histopathological changes. A minimum of 10 fields for each kidney slide was examined for necrosis and the presence of CaOx crystals and photographed using an optical microscope at 10× magnification [19].

Statistical analysis

The values were expressed as Mean ± SEM. Statistical comparison was performed using one way analysis of variance ANOVA to assess the Statistical significance, followed by Dunnett multiple comparison tests. A P value of less than 0.05 was considered statistically significant.

Results

Body weight and relative organ weight

Animals of the vehicle control group showed average 26.15% increase in body weight on the 10th day of study. EG and AC treated group showed only 8.57% increase whereas cystone showed 31.58% increase in body weight. The EAKP, EAEO, and EABN treated groups at 100 mg/kg dose showed respectively 4.01, 2.78 and 11.87% increase in the body The EACD treated group showed 12.44% increase in body weight at 500 mg/kg dose (Figure 1).

Relative weight of liver, kidney, heart, and spleen of animals treated with EG and AC showed non-significant changes. EAKP and EABN at 100 mg/kg dose have significantly ($P < 0.05$) increased relative weight of spleen whereas has a non-significant effect on the liver, kidney and heart. The relative weight of liver, kidney, heart, and spleen of animals treated with EAEO and EACD at all three doses showed non-significant changes similar with negative control and cystone treated groups (Table 1).

Urine volume and pH

EG and AC treatment caused significant ($P < 0.05$ - 0.01) decrease in urine volume and urine pH compared to vehicle control groups. Rats treated with 50 and 100 mg/kg dose of EAKP showed significant ($P < 0.05$-0.001) increase in urine volume and pH. EAEO significantly ($P < 0.05$-0.01) increased the urine volume and urine pH compared to the negative control groups at 100 mg/kg dose. Treatment with EABN at 100 mg/kg, and EACD at 300 and 500 mg/kg dose had significantly ($P < 0.05$-0.01) increased urine volume but not the pH (Table 2).

Urine biochemical parameters

Nephrolithiasis induction by combined treatment of EG and AC was confirmed by significant ($P < 0.01$ - 0.001) elevation of the urine biochemical parameters, i.e., sodium, chloride, potassium, calcium, phosphates, and protein with a decrease in magnesium, creatinine and uric acid. Administration of EAKP at 50 and 100 mg/kg dose normalized all the disturbed urine parameters with an extremely significant ($P < 0.001$) response. EAEO showed significant ($P < 0.01$ - 0.001) decrease in urinary excretion of sodium, chloride, potassium, calcium, phosphates, and protein along with an increase in magnesium, creatinine and uric acid level. EABN at 100 mg/kg dose has significantly ($P < 0.01$-0.001) reversed all the abnormality induced by EG and AC treatment. Rats treated with 300 and 500 mg/kg dose of EACD significantly ($P < 0.05$ - 0.00) decreased urinary excretion of sodium, chloride,

potassium, calcium, phosphates, and protein along with an increase in magnesium, creatinine and uric acid level compared to negative control group (Figure 2 and 3)

Serum biochemical parameters

The serum sample of EG and AC induced nephrolithiatic control animals showed significant ($P < 0.01–0.001$) increase in sodium, chloride, potassium, calcium, phosphates, BUN, creatinine, uric acid, SGOT, and SGPT. Significant ($P<0.05–0.001$) reduction in elevated levels of sodium, chloride, potassium, calcium, phosphates, BUN, creatinine, uric acid, SGOT and SGPT was observed following administration of EAKP (50 and 100 mg/kg), EAEO (50 and 100 mg/kg), EABN (50 and 100 mg/kg) and EACD (300 and 500 mg/kg) as shown in Figure 4 and 5.

Anti-oxidant enzyme level in kidney

EG and AC induced nephrolithiasis caused significant ($P < 0.001$) increase in malondialdehyde, superoxide dismutase and calcium level along with a decrease in glutathione in kidney tissue. A significant decrease in malondialdehyde, superoxide dismutase and calcium level, and increased glutathione level of kidney tissue was observed in EAKP ($P<0.001$), EAEO ($P<0.01$-0.001) and EABN ($P<0.01$-0.001) treated groups at 100 mg/kg dose. Likewise, EACD treated group significantly ($P<0.01–0.001$) decreased malondialdehyde, superoxide dismutase and calcium level with an increase in reduced glutathione level of the kidney at 300 and 500 mg/kg doses (Table 3).

Histopathology of kidney

Vehicle control group rats kidney histopathology revealed normal cellular architecture with no CaOx deposits. The kidney tissue section of EG and AC treated rat showed deposition of CaOx microcrystals along with marked histological changes like dilation of tubules, interstitial fibrosis, and degeneration of epithelial cell lining with infiltration of mononuclear inflammatory cells. Cystone treated group showed very less CaOx deposits and near normal cytology of the nephrotic tissue. Rats treated with EAKP at 50 and 100 mg/kg dosages showed absence of crystal deposition and mono nuclear cell infiltration along with reversal of interstitial fibrosis, degeneration of epithelial cell and inflammatory cell infiltration. The kidney architecture was almost similar to healthy control rats at 100 mg/kg dose. Histopathological observations of renal sections of animals treated with 50 and 100 mg/kg EAEO showed minimally dilated collecting systems with the near absence of interstitial fibrosis, degeneration of epithelial cell and inflammatory cell infiltration. EABN treated animals at 25, 50 and 100 mg/kg dose showed minimal efficacy in ameliorating the symptoms of tubular dilation, damage and degeneration of epithelial cell lining, though showed less deposition of microcrystal in tubules. EACD at 300 and 500 mg/kg treated groups showed a moderate reversal of the histopathological symptoms and less deposition of microcrystal in tubules (Figure 6).

Discussion

Urolithiasis is still a mysterious disease even after extensive research in Urology. Sophisticated instrumental analysis and investigations have failed to trace out the exact cause and mechanism of urolithiasis. The treatment for this condition in modern medicine is not only expensive but also dissatisfactory as proper drug therapy is not available in modern medicine that can dissolve the kidney stone. Patients mostly depend on alternative systems of medicine for better relief of kidney stone [20]. Plant based phytotherapeutic agents are used by the majority of patients for treating urolithiasis as they are efficacious and have lesser side effects compared to modern medicines and also reduce the recurrence rate of renal stone [21]. Unlike allopathic medicines, most plant based therapy is effective at different stages of stone formation pathophysiology [22]. Phytocompounds exert antilithogenic properties by altering the ionic composition of urine, e.g., decreasing the calcium ion concentration or increasing magnesium and citrate excretion [23]. These remedies also express additional diuretic activity or lithotriptic activity [24]. Herbal remedies containing groups of compounds are way forward in minimizing tissue injury in human disease as enabled with multiple mechanisms of protective action [25]. Ethyl acetate fraction of plants C. dactylon, E. officinalis, K. pinnata, and B. nutan were screened for anti-nephrolithiatic potential as it is rich in flavonoid and polyphenol with reported antioxidant

and diuretic activities. Studies done earlier have demonstrated presence of flavonoid and polyphenols in EACD, EAEO, EAKP and EABN with in vitro CaOx crystal dissolution potential and crystal growth inhibition properties [7]. EACD, EAEO, EAKP and EABN was also found to possess potent diuretic activity on rats [8].

The anti-nephrolithiatic potential was screened on rats induced with EG and AC treatment in drinking water. Administration of EG, a precursor for oxalate formation, is a well-known model of nephrocalcinosis induction. Rodents are not very prone to nephrolithiasis, so specific experimental models are required to induce long standing hypercalciuria or hyperoxaluria [26]. Several rodent models have been developed in order to study the pathophysiology of intrarenal crystal formation. Induction of hyperoxaluria is essentially important for the event of CaOx related urolithiasis in rat and lots of experimental models demonstrate its formation within the animal urinary organ [27,28]. Rat models of CaOx urolithiasis induced by either EG alone or in combination with AC are most commonly used to study the pathogenesis of urolithiasis. Three main precursors of oxalate are EG, glycolate and glyoxylate that are administered by suitable route for inducing experimental urolithiasis on rat and mice [29]. Urinary excretion of oxalate is exaggerated with chronic administration of EG as a 0.75% solution in drinking water to male rats. EG is also administered in combination with AC or vitamin D3 in a shot to induce CaOx crystals deposits on the urinary system of rats [30]. EG is metabolized into glycolate, glyoxylate, and oxalate leading to the formation of calcium oxalate monohydrate (COM) crystals in kidneys. Rats receiving EG-supplemented drinking water (0.75%) develop hyperoxaluria and hypercalciuria one day after initiation. Intra tubular crystal deposits are detected after the first day both in medulla and cortex altogether with tubular injury, dilatation, regeneration and interstitial inflammation [29]. Urinary excretion of calcium, magnesium, and citrate are decreased concomitantly as reported by Khan [31]. This study is an accelerated model, where rats were treated with 0.75% EG and 2% AC for 10 days following the modified method of Touhami et al. [32].

K. pinnata ethyl acetate fraction showed a moderate effect on urine volume and pH reversal of nephrolithiatic rats, whereas it has excellent action on urine and serum biochemical parameters normalization. Kidney architecture was almost similar to healthy control rats at 100 mg/kg dose of EAKP along with excellent protection from oxidative damage in kidney tissue against combined EG and AC induced nephrolithiasis. The hydroalcoholic extract of K. pinnata leaf was found to exert significant diuretic and antiurolithitic activity on male Wistar rats [33]. Juice extracted from the leaves of Bryophyllum pinnata effectively dissolved the stones despite its position, nature and former treatments when administered to patients having renal stones. B. pinnata additionally expedited decreased oxalate excretion while increasing citrate excretion suggesting prospective antilithiatic properties [34]. Gilhotra et al. [35] developed tablets of K. pinnata extracts and reported its efficacy in reducing the accumulation of CaOx crystals and preventing stone formation within the kidneys. Shukla et al. [36] reported potent anti nephrolithiatic property of aqueous extract of B. pinnata on EG-induced urinary organ calculi based on histopathological examination of the kidneys showing reduced injury and degeneration of tissue lining in the extract-treated rats. K. pinnata fresh leaf aqueous extract is reported to have potent CaOx crystal dissolving capability compare to cystone [37].

E. officinalis ethyl acetate fraction treatment does not reverse the body weight loss induced by EG and AC but increased the urine volume and urine pH. EAEO significantly reversed urinary supersaturation and normalized EG and AC treatment induced serum biochemical parameter. EAEO showed minimally dilated collecting systems with near absence of kidney histopathology damages along with antioxidant related protection on kidney tissue. Ethyl acetate fraction of E. officinalis fruit having high flavonoid and polyphenol content has shown excellent diuretic and in vitro CaOx crystal growth inhibition potential [7,8]. Amla fruits are well known for the diuretic activity in traditional literature [38,39]. The Phyllanthus niruri commonly known as Bhuiamla is also reported to have diuretic activity in rats [40].

B. nutans ethyl acetate fraction moderately reversed body weight loss and urine volume depletion and has significantly reversed all the urine and serum parameter abnormality induced by EG and AC treatment. EABN treatment also protected the kidney from oxidative damage but showed minimal efficacy towards ameliorating kidney the kidney histopathology. B. nutans has no traditionally reported diuretic property though other plants of bamboo species, i.e., Bambusa aurundinacea and Bambusa vulgaris is reported to have diuretic activity [41]. Bamboo shoots are one of the natural sources of phenolic compounds that act as a natural diuretic and helps to get rid of excess salts [42,43]. The aqueous extract of bamboo shoot further extracted with ethyl acetate and n-butanol showed rich antioxidant capacities due to the presence of high amount of phenolic acids, such as ferulic acid and p-coumaric acid [44,45]. Consumption of hot water extract of B. nutans shoot daily for 7 days is helpful for the reduction of urinary calculus [46]. The alcoholic extract of leaves of B. vulgaris showed in vitro inhibitory effect on the formation of CaOx precipitate and crystallization [47].

Treatment C. dactylon ethyl acetate fraction showed reversal of body weight loss and urine volume decrease in nephrolithiatic rats induced by EG and AC cotreatment. EACD significantly corrected urine and serum biochemical abnormalities, kidney tissue oxidative damage along with moderate reversal of the histopathological symptoms. In consensus with previous publications C. dactylon ethyl acetate fraction has substantial diuretic and in vitro CaOx crystal dissolution and crystal growth inhibition properties [7,8]. C. dactylon n-butanol extract fractions given with drinking water showed prevention and elimination of ethylene glycol–induced kidney calculi in rats [48]. In the traditional system of medicine, C. dactylon plant is used as a diuretic in cases of dropsy. Aqueous extract of C. dactylon root given orally showed significant increase in the urine volume [49]. Aqueous extract administered at 500 gm/kg dose induced highly significant urinary water and electrolytes output [50]. C. dactylon crude extract at 2.5 ml/kg dose possessed nearly similar diuretic effect as that of standard drug hydrochlorothiazide on rats [51]. Aruna et al. [52] evaluated the diuretic activity of C. dactylon extract in guinea pigs and observed a similar response. Aqueous extract of C. dactylon rhizome was reported to reduce EG induced kidney tissue CaOx deposition especially in medullary and papillary sections of treated rats [53]. C. dactylon has shown immunomodulatory and free radical scavenging activity, in addition to anti-inflammatory and antioxidative effect on rats [54,55]. Rad et al. [6] reported preventive effects of hydroalcoholic extract of C. dactylon roots on calcium oxalate calculi in rat treated with 1% EG daily for 28 days. C. dactylon aqueous decoction reduced stone formation against ethylene glycol (1% v/v) given in drinking water for 6 weeks on rats [56].

Supersaturation of urine with CaOx is the foremost important aspect of urinary stone formation, along with other factors such as crystallization, nucleation, growth, and aggregation [57]. Therefore, if supersaturation or in later steps crystallization is prevented, then pathology of renal stone formation is ought to be avoided. This study reports noteworthy antilithiatic potential of K. pinnata ethyl acetate extract that has rich flavonoid and poly phenol content. K. pinnata extreme significantly decreased urinary excretion of sodium, chloride, potassium, calcium, phosphates and protein with increase in creatinine and uric acid reducing the urine supersaturation. Sohgaura et al. [8] has reported excellent diuretic potential of K. pinnata leaf ethyl acetate fractions with in vitro CaOx crystal growth inhibition potential. K. pinnata had the highest content of total flavonoid and polyphenol followed by E. officinalis and C. dactylon [8]. Bogucka-Kocka et al. [58] reported rich quantity of ferulic and caffeic acid in K. pinnata leaf along with antioxidant and cytotoxic activities which may be responsible for the antinephrolithistic property. This study validates the excellent antiurolithiatic potential of E. officinalis justifiable with its protective response on serum and urine parameter, antioxidant effect on kidney tissue and histopathology normalization. Acid-hydrolyzation of apple juice given with Citrus medica and E. officinalis enhances the solubility of renal stones as reported by Sinha and Tagore [59]. E. officinalis fruit contains tannins, flavonoids, phenolic compounds, especially chebulinic acid, chebulagic acid, emblicanin, gallic

acid, ellagic acid, and quercetin [60]. E. officinalis is reported to possess potent free radical scavenging, antioxidant, anti-inflammatory, immunomodulatory and nephroprotective activities which correlates with its efficacy in the prevention of urolithiasis [61,62]. This study observation indicates moderate antinephrolithiasis potential of B. nutans.

Sohgaura et al. [8] has reported nominal diuretic potential of B. nutans ethyl acetate fractions. B. nutans had low flavonoid but high polyphenol content along with excellent in vitro CaOx crystal growth inhibition potential [7]. The ethyl acetate fraction of B. nutans leaf is reported to contain seven phenolic acids, that is, caffeic acid, p-coumaric acid, sinapic acid, ferulic acid, coumaroylquinic acid, 5-feruloyquinic acid, and dihydroxybenzoic acid. Caffeic acid along with flavonoid homoorientin, luteolin, and ferulic acid were detected in high quantity in the ethyl acetate fraction of B. nutans [63]. Rich synergistic presence of caffeic and ferulic acid may be responsible for antinephrolithiatic response of B. nutans. The study observation reports moderate anti nephrolithiasis potential of C. dactylon on rats treated with 0.75% EG along with 2% AC for 10 days. Sohgaura et al. [8] has reported excellent diuretic potential of C. dactylon ethyl acetate fraction along with in vitro CaOx crystal growth inhibition property [7]. Aqueous whole plant extracts C. dactylon is reported to contain phenolic and flavonoidal compounds [64]. Ethyl acetate fraction of C. dactylon leaves protected liver from oxidative stress correlated with the rich presence of natural antioxidants like polyphenols and flavonoids are being reported by Devi et al. [65]. Phenolic fraction of the whole parts of C. dactylon has hydroquinone as the most abundant component along with isolated compounds like propanoic acid, pantolactone, pentanoic acid, vanillic acid, syringic acid and cinnamic acid [66]. HPLC–ESI MS have authenticated the presence of many flavonoids including apigenin, luteolin, apigenin and luteolin in C. dactylon [67]. The observed anti urolithiatic activity of C. dactylon ethyl acetate fraction can be correlated to the concerted effect of many flavonoids and polyphenolic compounds present.

Conclusion

This study substantiates excellent anti urolithiasis potential of K. pinnata and E. officinalis whereas B. nutans and C. dactylon has shown moderate protective effect against urolithiasis induced by EG and AC combined treatment for 10 days. K. pinnata and E. officinalis not only exerted crystal growth inhibition properties, but also showed noteworthy urine and serum abnormal parameter reversal. Phytochemical analysis revealed highest flavonoid and polyphenol content in K. pinnata followed by E. officinalis, C. dactylon and B. nutans. Though in the previous in vitro screening for the inhibition of sodium oxalate induced CaOx crystals growth. E. officinalis was the most effective one followed by B. nutans, C. dactylon, and K. pinnata but in case of in vivo screening K. pinnata was most efficacious with excellent protection kidney architecture. As flavonoid and polyphenol content in K. pinnata was highest, the observed potent antiurolithiatic activity may be due to enhanced bioavailability of phytocompounds ferulic and caffeic acid present. Further evaluations of these plants are underway in different animal models to explore the utility potential in urolithiasis treatment.

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Table 1. Relative organ weight of ethylene glycol and ammonium chloride induced nephrolithiatic rats treated with ethyl acetate fraction of *K. pinnata*, *E. officinalis*, *B. nutan* and *C. dactylon*

Treatment group (mg/kg)	Relative organ weight (gm/100 gm body weight)			
	Liver	Kidney	Heart	Spleen
Vehicle control	4.07 ± 0.32	0.93 ± 0.01	0.42 ± 0.01	0.53 ± 0.01
Negative control	4.38 ± 0.06^{ns}	1.10 ± 0.31^{ns}	0.42 ± 0.08^{ns}	0.33 ± 0.08^{ns}
Cystone (750)	4.30 ± 0.07^{ns,ns}	0.95 ± 0.01^{ns,ns}	0.49 ± 0.07^{ns,ns}	0.49 ± 0.07^{ns,ns}
EAKP (25)	5.11 ± 0.01^{*,a}	1.37 ± 0.07^{ns,ns}	0.48 ± 0.02^{ns,ns}	0.38 ± 0.05^{ns,ns}
EAKP (50)	4.66 ± 0.32^{ns,ns}	0.98 ± 0.01^{ns,ns}	0.47 ± 0.04^{ns,ns}	0.45 ± 0.03^{ns,ns}
EAKP (100)	4.01 ± 0.77^{ns,ns}	0.93 ± 0.02^{ns,ns}	0.46 ± 0.23^{ns,ns}	0.57 ± 0.02^{ns,a}
EAEO (25)	6.36 ± 0.05^{**,a}	1.22 ± 0.02^{ns,ns}	0.46 ± 0.03^{ns,ns}	0.36 ± 0.04^{ns,ns}
EAEO (50)	4.87 ± 0.61^{ns,ns}	0.96 ± 0.01^{ns,ns}	0.46 ± 0.06^{ns,ns}	0.42 ± 0.02^{ns,ns}
EAEO (100)	3.44 ± 0.65^{ns,ns}	0.91 ± 0.05^{ns,ns}	0.48 ± 0.02^{ns,ns}	0.53 ± 0.05^{ns,ns}
EABN (25)	4.39 ± 0.07^{ns,ns}	1.22 ± 0.01^{ns,ns}	0.46 ± 0.02^{ns,ns}	0.39 ± 0.06^{ns,ns}
EABN (50)	4.65 ± 0.23^{ns,ns}	0.96 ± 0.01^{ns,ns}	0.46 ± 0.06^{ns,ns}	0.42 ± 0.02^{ns,ns}
EABN (100)	3.47 ± 0.69^{ns,ns}	0.91 ± 0.07^{ns,ns}	0.51 ± 0.03^{ns,ns}	0.56 ± 0.01^{ns,a}
EACD (100)	5.44 ± 0.06^{ns,ns}	1.03 ± 0.01^{ns,ns}	0.45 ± 0.01^{ns,ns}	0.34 ± 0.07^{ns,ns}
EACD (300)	4.91 ± 0.31^{ns,ns}	0.98 ± 0.01^{ns,ns}	0.45 ± 0.07^{ns,ns}	0.40 ± 0.01^{ns,ns}
EACD (500)	3.05 ± 0.71^{ns,ns}	0.90 ± 0.06^{ns,ns}	0.54 ± 0.01^{ns,ns}	0.55 ± 0.01^{ns,ns}

All the values are Mean ± SEM of five animals per group. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ and ns = not significant compared to vehicle control values. $^{c}P< 0.001$, $^{b}P< 0.01$, $^{a}P< 0.05$ and ns = not significant compared to negative control values. EAKP = ethyl acetate fraction of *K. pinnata*, EAEO = ethyl acetate fraction of *E. Officinalis*, EABN = ethyl acetate fraction of *B. nutan*, EACD = ethyl acetate fraction of *C. dactylon*.

Table 2. Urine volume and pH of ethylene glycol and ammonium chloride induced nephrolithiatic rats treated with ethyl acetate fraction of *K. pinnata, E. officinalis, B. nutan* and *C. dactylon*

Treatment group (mg/kg)	Total volume (ml/kg)	pH
Vehicle control	10.12 ± 0.60	6.72 ± 0.22
Negative control	6.80 ± 0.72**	5.02 ± 0.26*
Cystone (750)	12.10 ± 1.25^{**,b}	6.82 ± 0.38^{ns,a}
EAKP (25)	8.05 ± 0.70^{ns,ns}	6.47 ± 0.36^{ns,ns}
EAKP (50)	10.25 ± 1.04^{ns,b}	6.71 ± 0.48^{ns,a}
EAKP (100)	11.15 ± 1.11^{a,c}	6.78 ± 0.42^{ns,a}
EAEO (25)	8.45 ± 0.98^{ns,ns}	5.66 ± 0.64^{ns,ns}
EAEO (50)	9.33 ± 1.12^{ns,a}	6.23 ± 0.75^{ns,ns}
EAEO (100)	10.78 ± 1.06^{ns,b}	6.07 ± 0.83^{ns,a}
EABN (25)	7.45 ± 0.96^{ns,ns}	5.35 ± 0.67^{ns,ns}
EABN (50)	8.40 ± 1.09^{ns,ns}	5.90 ± 0.84^{ns,ns}
EABN (100)	9.33 ± 1.11^{ns,a}	6.17 ± 0.76^{ns,ns}
EACD (100)	7.55 ± 0.86^{ns,ns}	5.91 ± 0.19^{ns,ns}
EACD (300)	9.16 ± 1.06^{ns,a}	6.33 ± 0.40^{ns,ns}
EACD (500)	10.08 ± 1.22^{ns,b}	6.25 ± 0.51^{ns,ns}

All the values are Mean ± SEM of five animals per group. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ and ns = not significant compared to vehicle control values. $^{c}P< 0.001$, $^{b}P< 0.01$, $^{a}P< 0.05$ and ns = not significant compared to negative control values. EAKP = ethyl acetate fraction of *K. pinnata*, EAEO = ethyl acetate fraction of *E. Officinalis*, EABN = ethyl acetate fraction of *B. nutan*, EACD = ethyl acetate fraction of *C. dactylon*.

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Table 3. Kidney antioxidant enzyme and calcium level of ethylene glycol and ammonium chloride induced nephrolithiatic rats treated with ethyl acetate fraction of *K. pinnata, E. officinalis, B. nutan* and *C. dactylon*

Treatment group (mg/kg)	Calcium (mg/100 gm wet tissue)	Malondialdehyde (nmol/gm wet tissue)	Reduced glutathione (mmol/gm wet tissue)	Superoxide dismutase (mmol/gm wet tissue)
Vehicle control	3.44 ± 0.25	7.33 ± 0.27	2.71 ± 0.04	30.09 ± 1.38
Negative control	6.34 ± 0.61***	13.50 ± 1.27***	1.29 ± 0.08***	72.92 ± 2.27***
Cystone (750)	3.21 ± 0.14^{ns,c}	6.67 ± 0.17^{ns,c}	2.62 ± 0.07^{ns,b}	34.72 ± 1.25^{ns,c}
EAKP (25)	4.11 ± 0.12^{ns,b}	12.00 ± 1.15^{***,ns}	2.97 ± 0.12^{ns,c}	29.03 ± 1.76^{ns,c}
EAKP (50)	3.45 ± 0.2^{ns,c}	8.98 ± 0.42^{ns,c}	3.88 ± 0.06^{***,c}	23.62 ± 1.34^{ns,c}
EAKP (100)	3.01 ± 0.7^{ns,c}	6.78 ± 0.16^{ns,c}	4.02 ± 0.07^{***,c}	16.47 ± 0.96^{***,c}
EAEO (25)	4.22 ± 0.37^{ns,a}	11.54 ± 1.04^{**,ns}	2.74 ± 0.02^{ns,c}	28.03 ± 1.47^{ns,c}
EAEO (50)	3.66 ± 0.9^{ns,b}	9.54 ± 0.35^{ns,b}	3.54 ± 0.05^{***,c}	21.62 ± 1.25^{**,c}
EAEO (100)	3.12 ± 0.15^{ns,b}	6.97 ± 0.52^{ns,c}	3.97 ± 0.07^{***,c}	15.47 ± 0.86^{***,c}
EABN (25)	4.29 ± 0.33^{ns,b}	11.48 ± 0.88^{ns,ns}	2.11 ± 0.08^{***,c}	27.04 ± 1.47^{ns,c}
EABN (50)	3.90 ± 0.24^{ns,c}	9.98 ± 0.45^{ns,a}	2.96 ± 0.05^{ns,c}	25.73 ± 1.33^{ns,c}
EABN (100)	3.87 ± 0.32^{ns,c}	8.79 ± 0.12^{ns,b}	3.12 ± 0.06^{**,c}	22.52 ± 0.86^{*,c}
EACD (100)	4.36 ± 0.20^{ns,b}	10.67 ± 0.72^{*,a}	2.88 ± 0.03^{ns,b}	26.04 ± 1.38^{ns,c}
EACD (300)	3.92 ± 0.2^{ns,c}	9.03 ± 0.29^{ns,b}	2.96 ± 0.04^{ns,b}	23.73 ± 1.32^{ns,c}
EACD (500)	3.69 ± 0.17^{ns,c}	8.83 ± 0.41^{ns,b}	3.22 ± 0.09^{***,c}	18.52 ± 0.97^{***,c}

All the values are Mean ± SEM of five animals per group. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ and ns = not significant compared to vehicle control values. $^{c}P< 0.001$,$^{b}P< 0.01$, $^{a}P< 0.05$ and ns = not significant compared to negative control values. EAKP = ethyl acetate fraction of *K. pinnata*, EAEO = ethyl acetate fraction of *E. Officinalis*, EABN = ethyl acetate fraction of *B. nutan,* EACD = ethyl acetate fraction of *C. dactylon*.

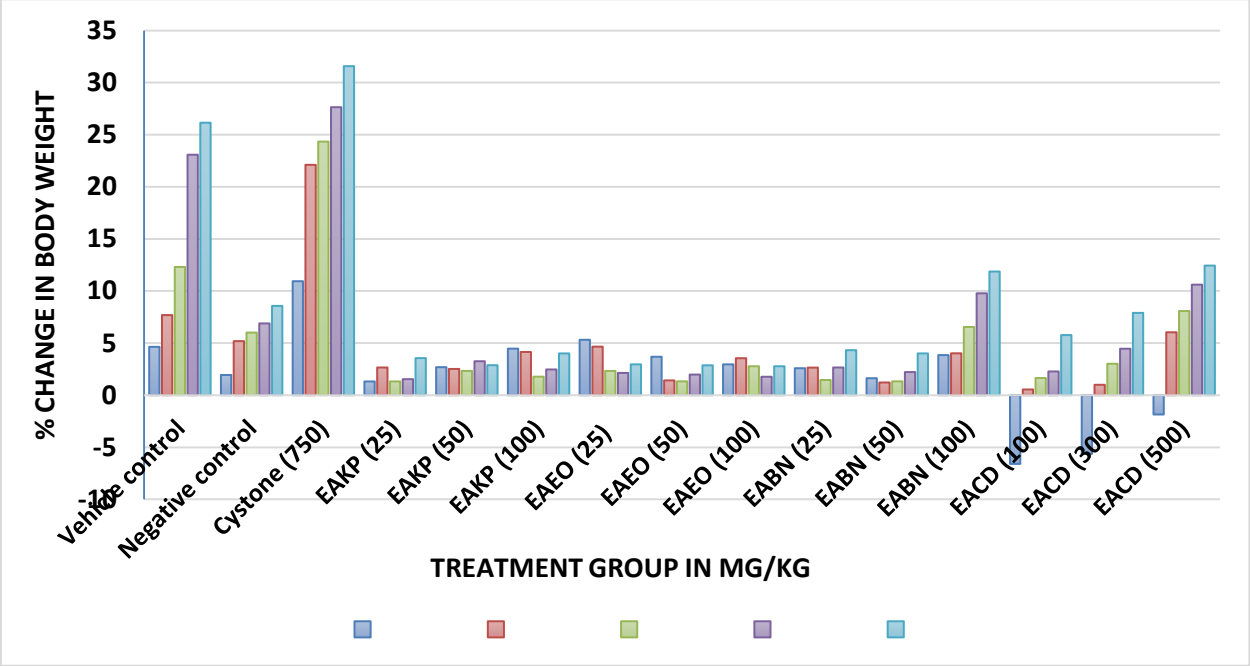

Figure 1. Percentage change in Body weight of ethylene glycol and ammonium chloride induced nephrolithiatic rats treated with ethyl acetate fraction of *K. pinnata, E. officinalis, B. nutan* and *C. dactylon.*

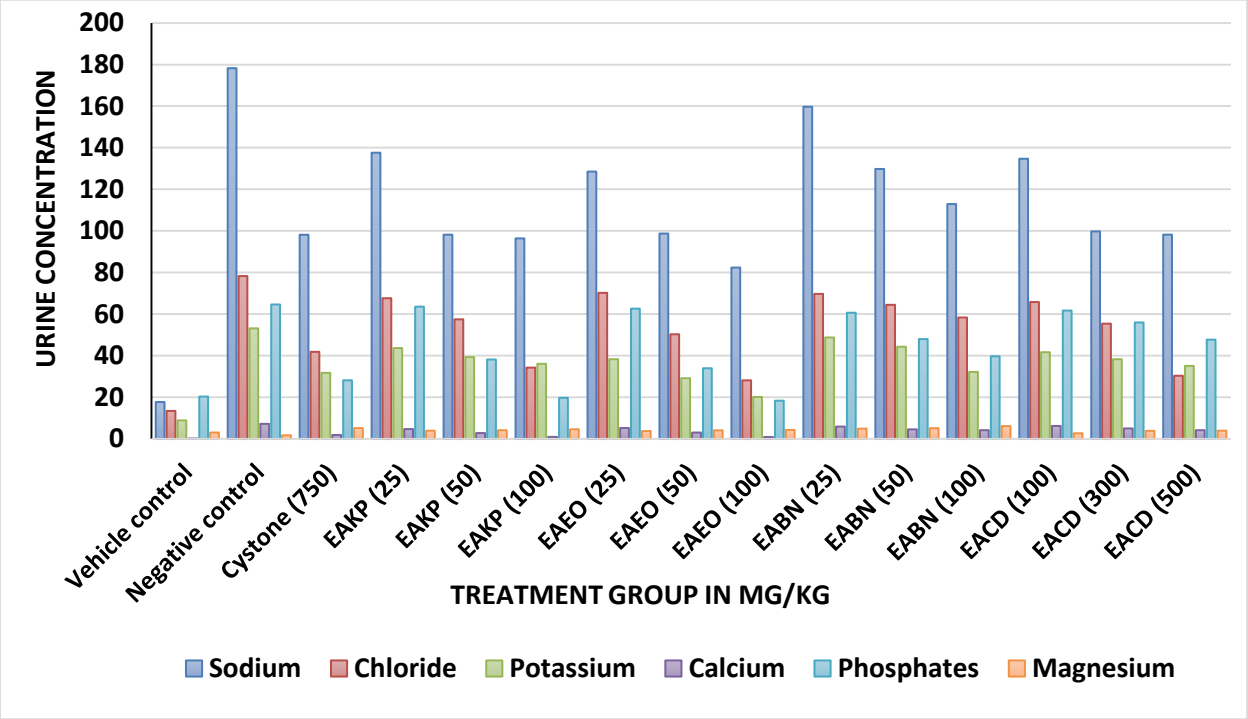

Figure 2. Urine ionic concentration of ethylene glycol and ammonium chloride induced nephrolithiatic rats treated with ethyl acetate fraction of *K. pinnata*, *E. officinalis*, *B. nutan* and *C. dactylon.*

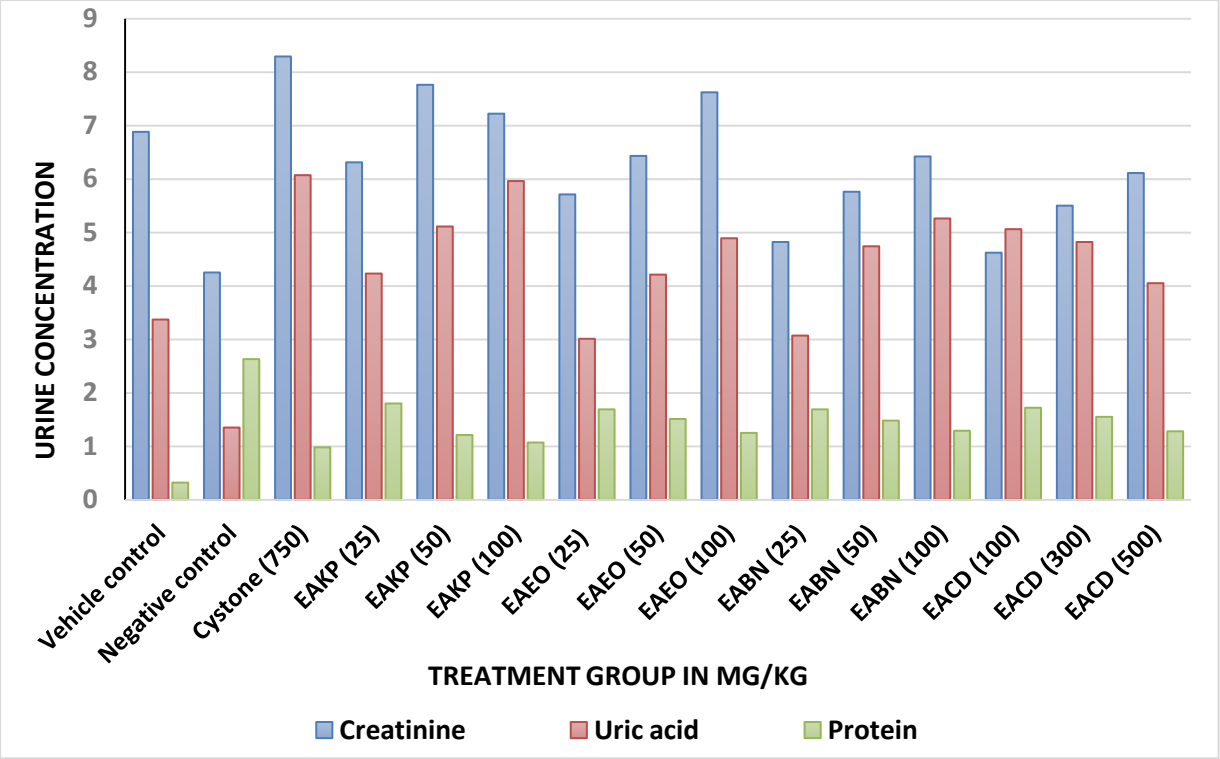

Figure 3. Urine biochemical parameters of ethylene glycol and ammonium chloride induced nephrolithiatic rats treated with ethyl acetate fraction of *K. pinnata*, *E. officinalis*, *B. nutan* and *C. dactylon.*

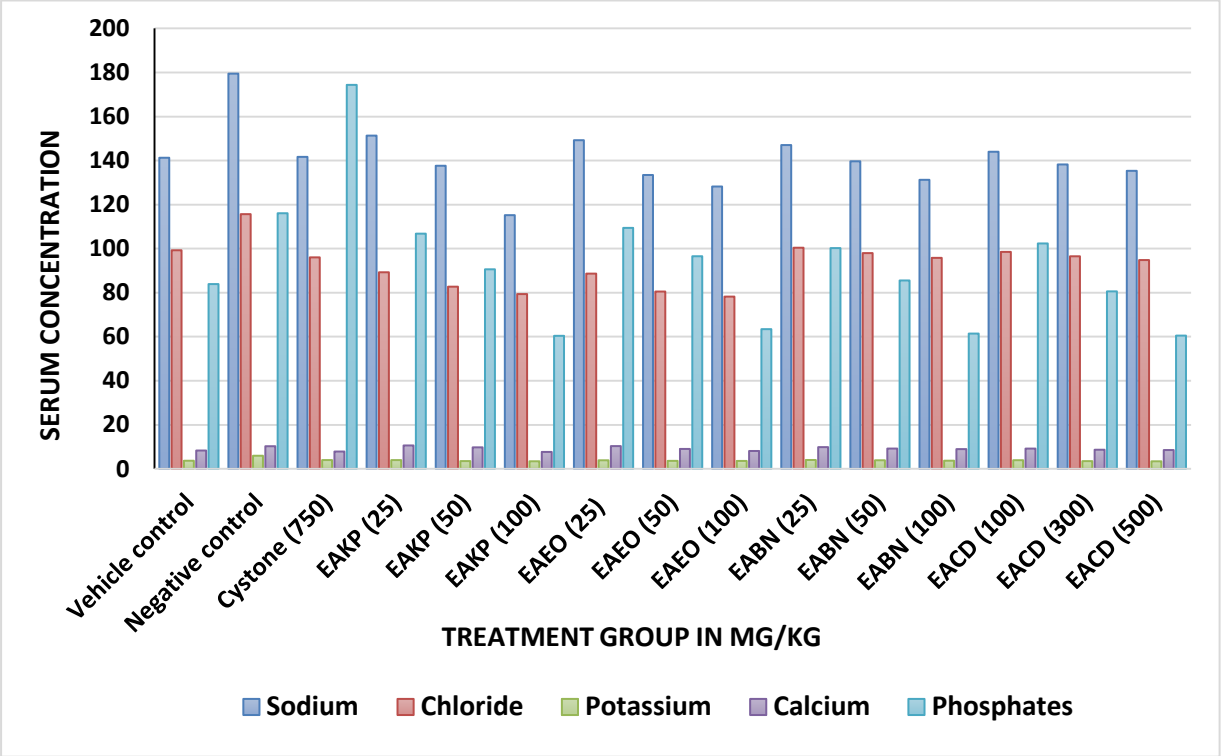

Figure 4. Serum ionic concentration of ethylene glycol and ammonium chloride induced nephrolithiatic rats treated with ethyl acetate fraction of *K. pinnata*, *E. officinalis*, *B. nutan* and *C. dactylon.*

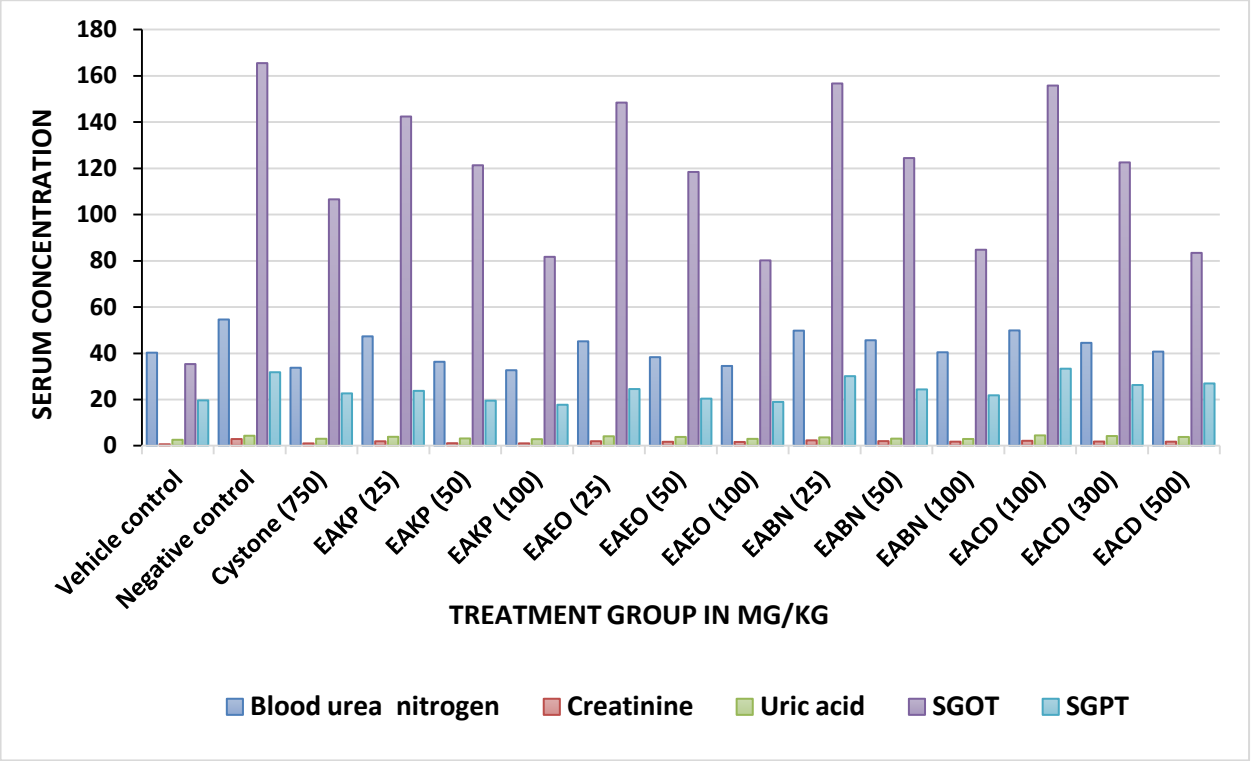

Figure 5. Serum biochemical parameter of ethylene glycol and ammonium chloride induced nephrolithiatic rats treated with ethyl acetate fraction of *K. pinnata, E. officinalis, B. nutan* and *C. dactylon.*

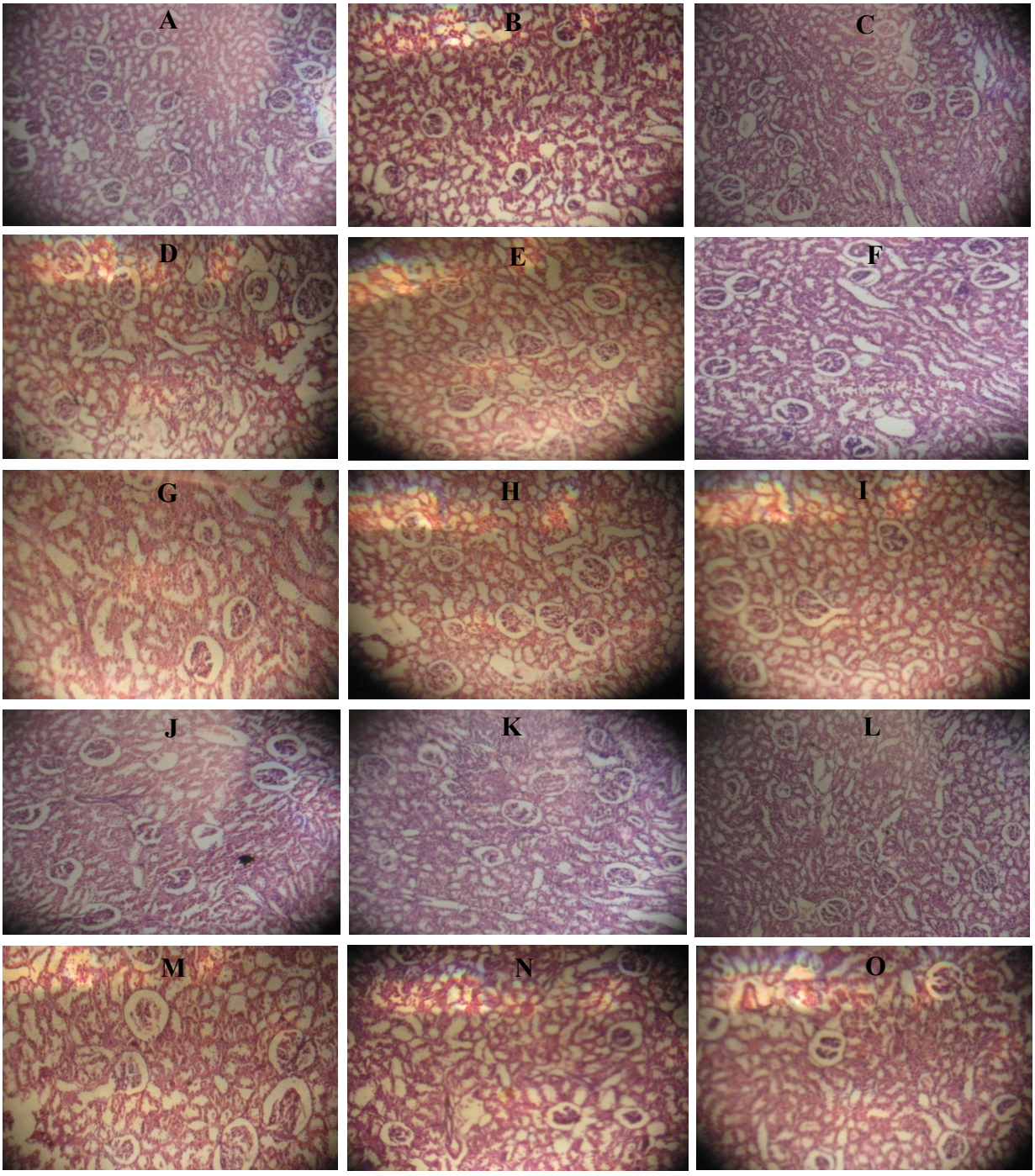

Figure 6. Kidney histopathology of ethylene glycol and ammonium chloride induced nephrolithiatic rats treated with ethyl acetate fraction of *K. pinnata, E. officinalis, B. nutan* and *C. dactylon*. Vehicle control (A), ethylene glycol (B), cystone 750 mg/kg (C), EACD 100 mg/kg (D), EACD 300 mg/kg (E), EACD 500 mg/kg (F), EAEO 25 mg/kg (G), EAEO 50 mg/kg (H), EAEO 100 mg/kg (I), EAKP 25 mg/kg (J), EAKP 50 mg/kg (K), EAKP 100 mg/kg (L), EABN 25 mg/kg (M), EABN 50 mg/kg (N) and EABN 100 mg/kg (O). EACD = *C. dactylon* ethyl acetate fraction, EAEO = *E. officinalis* ethyl acetate fraction, EAKP = *K. pinnata* ethyl acetate fraction and EABN = *B. nutan* ethyl acetate fraction.