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Original Research Article

# ANTIUROLITHIATIC ACTIVITY OF *CENTRATHERUM ANTHELMINTICUM* (L.) KUNTZE SEEDS AGAINST ETHYLENE GLYCOL INDUCED UROLITHIASIS IN RATS.

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**ABSTRACT:**
*Centratherum anthelminticum* (L.) Kuntze (compositae), commonly known as Kaligiri is traditionally used for kidney stone treatment. The study was designed to evaluate prophylactic effect of 28 days treatment of 70% methanolic extract of *C. anthelminticum* seeds (CAE) (200 mg/kg and 400 mg/kg, p.o.) against ethylene glycol induced nephrolithiasis in rats. Cystone (750 mg/kg) was used as a standard. After completion of treatment period of 28 days, parameters like urine volume, kidney weight and various urinary risk factors for urolithiasis were measured. Biochemical parameters from blood serum were also measured. Antioxidant markers (malondialdehyde and glutathione) and ionic level (calcium and oxalate) were measured in kidney homogenate. Lithogenic treatment with ethylene glycol for 28 days caused increase in urine output and weight of kidney, hyperoxaluria, hypercalciuria and hypomagnesaemia thereby contributing to renal stone formation which was prevented by simultaneous administration of CAE. Lithogenic treament caused damage of kidney and increased oxidation stress as manifested by increased malondialdehye and depleted reduced glutathione. Simultaneous treatment with CAE significantly reduced stone forming promoters (calcium, oxalate, phosphate, creatinine, urea, uric acid), enhanced stone forming inhibitors (magnesium) with significant antioxidant activity in a dose dependent manner. Antiurolithiatic effect of CAE was also confirmed by histopathological changes in kidney tissue. The protective action of CAE was comparable to Cystone. The results of the present study indicated antiurolithiatic activity of *C. anthelminticum* seeds.

**Key Words**: Antiurolithiatic activity, *Centratherum anthelminticum*, Calcium oxalate, Ethylene glycol, Nephrolithiasis

## INTRODUCTION

Urinary stone is a common disorder with a recurrence rate of 70–81% in male and 47–60% in female.[1] Urolithiasis is a complex process that is a consequence of an imbalance between promoters (calcium, sodium, oxalate, urate, cystine, low urine pH, low urine flow) and inhibitors (citrate, magnesium, pyrophosphate, Tamm Horsfall protein, urinary prothrombin fragments, glycosaminoglycan osteopontin, and high urine flow) in the kidneys.[2]

Calcium oxalate (CaOx) represents up to 80% of analyzed stone.[3] Kidney stone formation is a complex process that results from a succession of several physicochemical events including supersaturation, nucleation, growth aggregation and retention within the renal tubules.[4,5] Futhermore, calcium oxalate crystals causes oxidative stress which

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generate reactive oxygen species and free radicals leading to, damage of epithelium of kidney and bladder, thereby producing the favourable environment for crystal attachment to the surface.[6,7] This injury leads to the lipid and protein peroxidation and altered the biochemical reactions and depletes the defensive antioxidant enzyme system.[8]

Among the used treatments, there are extracorporeal shock wave lithotripsy (ESWL) and drug treatment which revolutionized urological practice and almost become the standard procedure for eliminating kidney stones. However, in addition to traumatic effects of shock waves, residual stone fragments persist and infection could occur. Moreover, ESWL may cause acute renal injury, a decrease in renal function.[9] Therefore, it is worthwhile to look for an alternative to these means by using medicinal plants or phytotherapy. Ethylene glycol rat model is considered as an interesting model to evaluate renal papillary stone development; at least for those stones which genesis is linked to oxidative cell damage. Several pharmacological investigations[9-13] on the medicinal plants used in traditional antiurolithic therapy have revealed their therapeutic potential in the in vitro or in vivo models. Hence, the search for herbomineral preparations is still ongoing. *Centratherum anthelminticum* (L.) Kuntze (compositae), commonly known as Kaligiri. It is highly reputed in Hindu medicine as remedy for leucoderma and other skin diseases. The seeds have a hot sharp taste, acrid, astringent to the bowels, anthelmintic and cure ulcers. The seeds are used as purgative, for asthma, kidney troubles and hiccough, applied in inflammatory swelling, remove blood from liver, good for sores and itching of the eyes. In Punjab, it is considered as antipyretic. The seeds are also credited with tonic, stomachic, and diuretic properties.[14,15] Different organic solvent and aqueous extracts of these seeds were scientifically evaluated for antifilarial, antibacterial, larvicidal, antiviral, antifungal, anticancer, anthelmintic, antidiabetic, antioxidant, analgesic, antipyretic, anti-inflammatory, diuretic, wound healing activities.[16] There is no scientific evidence regarding nephroprotective action of this plant. Cystone is a marketed composite herbal formulation specifically developed for managing urolithiasis or renal calculi. This formulation has been approved by regulatory authorities in India as an Ayurvedic formulation, and has been available in clinical practice for the past 60 years for treating urinary calculi.[17] Based on that, aim of the present study was to evaluate antinephrolithiatic activity of *Centratherum anthelminticum* using ethylene glycol induced nephrolithiasis in rats and compared their actions with Cystone.

## MATERIALS AND METHODS

### Preparation of extract

Dried seeds of *Centratherum anthelminticum* (L.) Kuntze were procured from Anand Agriculture University, Anand. Plant material was authentified by Dr. Jina Patel, Department of Botany, Gujarat University, Ahmedabad. The voucher specimen (Authentication reference number: RRP/CA-1/7/ARGH -11-13) was deposited at the pharmacognosy department of our institute. The seeds were air-dried and ground to fine powder. About 0.5 kg powdered sample was defatted with petroleum ether (40-60°C). The remaining part was extracted with (70:30) methanol and water by cold maceration for 4 days with frequent shaking (Yield -11.49 % w/w). Freshly prepared aqueous solution of dried extract of *C. anthelminticum* (CAE) in suitable dilution was used for experimental study.

### Preliminary phytochemical screening

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The qualitative chemical investigation of hydroalcoholic extract was carried out to check the presence of various phytoconstituents.[18]

**Animals**

Wistar albino rats (150-230g) of male sex bred in Central Animal House facility of the institute were used. These animals were housed under standard conditions, maintained on a 12 h light/dark cycle and had free access to food and water up to the time of experimentation. The animals were acclimatized to the laboratory environment 1 h before the experiments. Animals were randomly distributed into groups of 6 animals each. All experiments were conducted during the light period (08.00-16.00 h). All the protocols (CPCSEA/IAEC/ARCP/12-13/06) were approved by the Institutional Animal Ethics Committee (IAEC) and conducted according to the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiment on Animals).

**Drugs and Chemicals**

All Chemicals and reagents used for study were of AR grade. Cystone (procured from Himalaya Pharmaceuticals Ltd.) was used as a standard. Diagnostic kits for various biochemical analysis were procured from Span Diagnostics Ltd., India.

**Study on ethylene glycol induced urolithiasis in rats**

Rats were divided in to five groups containing six animals in each group. Group 1 served as a vehicle treated control and maintained on regular rat food and water *ad libitum*. All remaining groups (group 2-5) received calculi inducing treatment, comprising of ethylene glycol (0.75% v/v) in drinking water *ad libitum* for 28 days to induce lithiasis. Group 3, 4, and 5 were administered CAE (200 mg/kg), CAE (400 mg/kg) and Cystone (750 mg/kg) from day 1 to 28 of calculi induction respectively. Extract and standard drug were suspended in distilled water and given orally once daily.[19]

All animals were kept in individual metabolic cages and urine samples of 24 h period were collected on 28$^{th}$ day of treatment. Animals had free access to drinking water during the urine collection period. Volume and pH of urine was measured. A drop of concentrated hydrochloric acid was added to the urine before being stored at 4ºC. Urine was analyzed for calcium, phosphorus, oxalate, magnesium, uric acid, and creatinine contents using commercially available kits (Span Diagnostics Ltd., India). After the experimental period, at the end of 28$^{th}$ day blood was collected from the retro orbital under anesthetic conditions. Serum was separated by centrifugation at 3000 rpm for 10 min and was analyzed for calcium, magnesium, phosphorus, urea, uric acid, and creatinine using commercially available kits (Span Diagnostics Ltd., India).

**Kidney homogenate analysis**

At the end of the experimental period, rats were sacrificed under ether anaesthesia and kidneys were removed quickly cleaned with ice-cold saline (0.9% w/v sodium chloride) and weighed. One kidney from each rat was homogenized in chilled phosphate buffer (pH 7.4). The homogenate was centrifuged at 800 rpm. for 10 min and resultant supernatant was used to determine malondialdehyde[20], reduced gluthathione[21], calcium and oxalate. Other kidneys from each animal were used for histopathological study.

**Histopathological study**

Fragments of the kidneys were subsequently fixed in a 10% solution of buffered formalin (pH 7.4) and enclosed in paraffin. 4µ sections were obtained and colored with the hematoxylin eosin for evaluation under optical microscope.

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**Stastical analysis**

All statistical analysis was performed using SPSS software (Version 10.0, SPSS Inc., Chicago, IL, USA). The data were expressed as mean ± S.E.M. The statistical significance of the difference between groups for the various treatments were determined by one way analysis of variance (ANOVA) followed by Dunnett's test. $P<0.05$ or $P<0.001$ was considered statistically significant as compared to control.

## RESULTS

**Preliminary phytochemical screening**

Phytochemical screening revealed the presence of flavonoids, saponins, polyphenols, tannins, carbohydrates and proteins in the 70% methanolic extract of *C. anthelminticum* seeds.

**Effect of CAE on urine parameters in ethylene glycol induced urolithiasis**

The 24 h urine volume was high in model control group as compared to those of the vehicle-control animals. In the CAE (200 and 400 mg/kg, p.o.) and Cystone (750 mg/kg, p.o.) treated groups, the urine output was significantly higher than that of calculi induced rats (Table 1).

28 days administration of 0.75% ethylene glycol in water to rats significantly increased calcium, oxalate, phosphate, uric acid and creatinine excretion in urine as compared to normal control group. While, urinary magnesium excretion was decreased significantly by stone inducing treatment. However, 28 days treatment with CAE (200 and 400 mg/kg, p.o.) and Cystone (750 mg/kg, p.o.) were found to be lowered the elevated levels of calcium, oxalate, phosphate, uric acid and creatinine in urine at significant extent. Also, subsequent treatments with CAE (200 and 400 mg/kg, p.o.) and Cystone (750 mg/kg, p.o.) significantly reverted the reduction of the excretion of the magnesium (Table 1).

**Effect of CAE on serum parameters in ethylene glycol induced urolithiasis**

The serum parameters measured from groups of animals at the end of 28 days of treatment period are shown in Table 2. Calculi was induced by administration with ethylene glycol significantly raised calcium and phosphate while reduced magnesium level in the serum. Treatments with CAE (200 and 400 mg/kg, p.o.) and Cystone (750 mg/kg, p.o.) significantly decreased these changes in serum at the administered doses. Renal stone induction caused impairment of renal functions of the untreated rats as evident from the markers of glomerular and tubular damages. Significant ($P<0.05$) elevated serum creatinine, uric acid and urea were dose dependently prevented in the animals receiving a simultaneous treatment with CAE (200 and 400 mg/kg, p.o.) and Cystone (750 mg/kg, p.o.).

**Effect of CAE on markers of oxidation and ionic levels in kidney homogenate**

Lithogenic treatment caused renal tissue damage by inflammatory reaction as well as by deposition of the crystalline component such as calcium and oxalate which was increased in the model control group (Table 3). Ultimately this can lead to a significantly increased wet kidney weight. However, simultaneous administration of CAE significantly decreased the wet kidney weight. Stone inducing treatment enhanced MDA ($P<0.05$) and decreased GSH level ($P<0.05$) in untreated rats. A simultaneous treatment with CAE (200 and 400 mg/kg; p.o.) and Cystone (750 mg/kg, p.o.) protected against the oxidative changes induced by lithogenic treatment in a dose dependent manner (Table 3).

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Histopathological analysis of rat kidneys revealed that tubules of normal size with single epithelial lining along the margin and no calcium oxalate deposits were observed in vehicle treatment group (Figure 1). On the other hand, presence of calcium oxalate crystals, marked dilatation of tubules and total degeneration of epithelial lining with infiltration of inflammatory cells into the interstitial space were observed in ethylene glycol treated group. Simultaneous administration with CAE (200 and 400 mg/kg; p.o.) gradually decreased deposition of calcium oxalate crystals with decreased dilatation of tubules and prevent the lithogen induced renal tissue injuries. In Cystone (750 mg/kg, p.o.) treated group, no deposition of calcium oxalate crystals were observed in any parts of the renal tubule and showed characteristics similar to the normal control group.

**Table 1. Effect of CAE on urine parameters in ethylene glycol induced urolithiasis**

| Parameter | Normal control | Model control (EG only) | EG + CAE 200 mg/kg | EG + CAE 400 mg/kg | EG+ Cystone 750 mg/kg |
|---|---|---|---|---|---|
| **Urine volume (mL)** | 5.5 ± 0.28 | 3.5 ± 0.28* | 6.3 ± 0.33** | 8.0 ± 0.28** | 9.0 ± 0.57** |
| **Calcium (mg/ 24 h)** | 4.1 ± 0.28 | 12.3 ± 0.51* | 10.6 ± 0.32** | 8.49 ± 0.57** | 8.21 ± 0.59** |
| **Oxalate (mg/ 24 h)** | 3.2 ± 0.30 | 8.43 ± 0.34* | 6.91 ± 0.33** | 5.91 ± 0.18** | 5.67 ± 0.28** |
| **Phosphate (mg/ 24 h)** | 5.3 ± 0.56 | 11.8 ± 0.30* | 10.8 ± 0.26** | 9.8 ± 0.33** | 8.84 ± 0.44** |
| **Magnesium (mEq/24hr)** | 4.23 ± 0.27 | 4.1 ± 0.23* | 6.08 ± 0.17** | 6.2 ± 0.11** | 7.78 ± 0.17** |
| **Creatinine (mg/24hr)** | 3.1 ± 0.08 | 4.2 ± 0.08* | 3.12 ± 0.41** | 2.26 ± 0.27** | 2.12 ± 0.08** |
| **Uric acid (mg/24hr)** | 2.2 ± 0.23 | 7.5 ± 0.32* | 4.58 ± 0.28** | 4.29 ± 0.33** | 3.62 ± 0.32** |

Each Value Expressed as mean ± SEM. Data were analysed by using one-way ANOVA test followed by Dunnett's test. *p<0.05 compared to control group;**p<0.05 when compared with EG (ethylene glycol) treated group.

**Table 2. Effect of CAE on serum parameters in ethylene glycol induced urolithiasis**

| Parameter | Normal control | Model control (EG only) | EG + CAE (200 mg/kg) | EG + CAE (400 mg/kg) | EG+ Cystone (750 mg/kg) |
|---|---|---|---|---|---|
| **Calcium (mg/ 24 h)** | 5.92 ± 0.22 | 12.5 ± 0.33* | 9.2 ± 0.51** | 8.03 ±0.68** | 6.13 ± 0.41** |
| **Phosphate (mg/ 24 h)** | 5.08 ± 0.31 | 7.16 ± 0.42* | 5.78 ± 0.34** | 4.4 ± 0.32** | 3.9 ± 0.53** |
| **Magnesium mEq/24hr)** | 5.03 ± 0.47 | 2.05 ± 0.24* | 2.54 ± 0.16** | 2.92 ± 0.11** | 3.52 ± 0.17** |
| **Creatinine (mg/24hr)** | 0.64 ± 0.06 | 3.21 ± 0.18* | 1.8 ± 0.33** | 1.03 ± 0.21** | 0.90 ± 0.19** |
| **Uric acid (mg/24hr)** | 4.56 ± 0.81 | 12.2 ±.268* | 7.57 ± 0.45** | 5.32 ± 0.54** | 4.7 ± 0.42** |
| **Urea (mg/24hr)** | 47.8 ± 1.99 | 54.2 ± 1.61* | 42.1 ±1.32** | 39.0 ±1.15** | 33.8 ± 0.90** |

Each Value Expressed as mean ± SEM. Data were analysed by using one-way ANOVA test followed by Dunnett's test. *p<0.05 compared to control group;**p<0.05 when compared with EG (ethylene glycol) treated group.

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**Table 3. Effect of CAE on Kidney parameters in ethylene glycol induced urolithiasis**

| Parameter | Normal control | Model control (EG only) | EG + CAE (200 mg/kg) | EG + CAE (400 mg/kg) | EG+ Cystone (750 mg/kg) |
|---|---|---|---|---|---|
| **kidney weight (g)** | 1.2 ± 0.07 | 2.04 ± 0.08* | 2.09 ± 0.05 | 1.67 ± 0.04** | 1.93 ± 0.12 |
| **Calcium (mg/ g)** | 1.67 ± 0.31 | 2.8 ± 0.43* | 1.51 ± 0.44** | 1.11 ± 0.37** | 0.49 ± 0.17** |
| **Oxalate (mg/g)** | 0.73 ± 0.1 | 1.86 ± 0.38* | 0.52 ± 0.1** | 0.68 ± 0.35** | 0.59 ± 0.34** |
| **MDA (mmoles/g)** | 1.67 ± 0.07 | 3.99 ± 0.28* | 2.18 ± 0.21** | 1.78 ± 0.19** | 1.86 ± 0.23** |
| **GSH (mmoles/g)** | 2.20 ± 0.08 | 0.35 ± 0.09* | 0.58 ± 0.10** | 0.78 ± 0.10** | 1.53 ± 096** |

Each Value Expressed as mean ± SEM. Data were analysed by using one-way ANOVA test followed by Dunnett's test. *p<0.05 compared to control group;**p<0.05 when compared with EG (ethylene glycol) treated group.

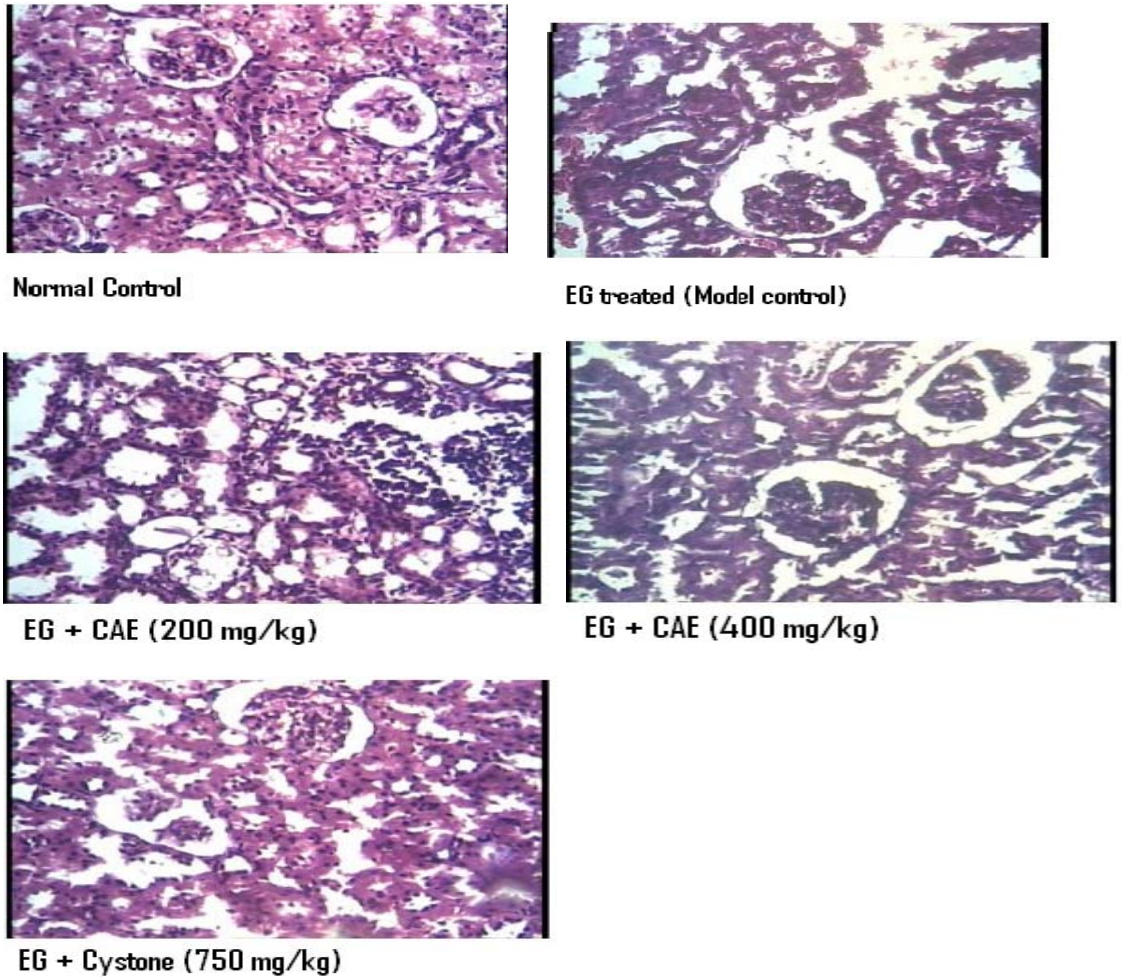


Figure 1. Histology of Rat kidney

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## DISCUSSION

Male rats were selected to induce urolithiasis because the urinary system of male rats resembles that of humans and earlier studies shown that the amount of stone deposition in female rats was significantly less.[22] Doses of the CAE was selected based on its reported diuretic activity and acute and sub acute toxicity studies.[23,24] Urinary output was markedly increased in model control group compared to normal control. Treatment with CAE (200 and 400 mg/kg; p.o.) and Cystone (750 mg/kg, p.o.) significantly prevented the polyuria associated with lithogenic treatment, although urine output remained higher than that of the normal control, which can be ascribed to its intrinsic diuretic activity.[23] Similar results were also reported in other studies.[19,22] So, this effect dilutes the concentration of the urinary electrolyte. As a result of this calcium and phosphorus flush out from the urine and there are a lesser chances of saturation and precipitation and decreased formation as well as growth of urinary stone.

Ethylene glycol disturbs oxalate metabolism by the way of increasing substrate availability.[25] Lithogenic treatment significantly increased urinary calcium excretion and serum calcium level in model control group. High urinary calcium concentrations lead to increased urinary saturation of calcium salts and reduced urinary inhibitory activity by way of complexation with negatively charged inhibitors such as citrate.[26] Positive correlation between urinary oxalate levels and renal tubular epithelial injury has been discovered in experimental urolithiasis rats[19,22] and patients with renal stones.[27] In the present study, urinary and serum oxalate was increased in ethylene glycol induced urolithicrats. It was observed that treatment with CAE (200 and 400 mg/kg; p.o.) significantly and dose dependently prevented lithogen induced hypercalciuria and hyperoxaluria thereby inhibiting calcium stone formation. An increase in urinary phosphorus excretion was observed in ethylene glycol induced urolithicrats. Increased urinary phosphate excretion along with oxalates tress seems to provide an environment appropriate for stone formation by forming calcium phosphate crystals, which epitaxially induces calcium oxalate deposition.[22] Treatment with CAE (200 and 400 mg/kg; p.o.) lowered the excretion of phosphate and reduced the risk of stone formation. In the present investigation uric acid excretion was increased in urolithic rats. Uric acid interferes with calcium oxalate solubility and reduces the inhibitory activity of glycosaminoglycans.[28] Treatment with CAE (200 and 400 mg/kg; p.o.) lowered the excretion of uric acid and reduced the risk of stone formation.

Low levels of magnesium are also encountered in stone formers as well as in stone-forming rats. Magnesium complexes with oxalate, reduce the supersaturation of calcium oxalate and as a consequence reduce the nucleation rate and growth of crystals.[29] The CAE (200 and 400 mg/kg; p.o.) and Cystone treatments restored the magnesium excretion and thus reduced the growth of calcium oxalate crystals in drug treated animals.

In urolithiasis, the glomerular filtration rate decreases due to the obstruction of the outflow of urine out flow by stones in urinary system. Due to this, the waste products particularly nitrogenous substances such as urea, creatinine, and uric acid accumulate in blood.[30] In this study, ethylene glycol administration induced nephrotoxicities which were characterized by marked elevation of serum urea, creatinine and uric acid. Treatment with CAE (200 and 400 mg/kg; p.o.) significantly decreased the nephrotoxicity indicated by reducing amount of nitrogenous waste products. Increase in

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calcium and oxalate levels in the renal tissue of ethylene glyol treated rats were observed due to deposition of crystalline material as calcium oxalate. Oxalate toxicity is developed as inflammatory reaction. This view is strengthened by the fact that the relative weights of the kidneys in model control show evidence of toxicity. However, these elevations of calcium and oxalate were attenuated by treatment with the CAE (200 and 400 mg/kg; p.o.) and thereby decreasing calcium oxalate crystal induced cell injury. Stone inducing treatment caused hypertrophy and extensive calcium oxalate crystal deposition in kidneys, oxidative damage as reflected from increased MDA and decreased activities of antioxidant enzymes like GSH in kidneys and deteriorated renal functions. CAE (200 and 400 mg/kg; p.o.) significantly prevented all these effects of lithogenic treatment, thus confirming antiurolithic effect, as well as, antioxidant potential in vivo.[31]

Histopathological studies showed ethylene glycol induced crystal deposition in the renal cells and most of crystal deposition took place in the renal tubules, which corroborates the results of other studies reporting that crystals deposition mainly occur in tubules.[19,22,28] Several studies have shown that, crystal formation results in cell damage and cell detachment from the basement membrane and the released degradation products further promote nucleation of crystals.[32] Administration of CAE to ethylene glycol exposed rats, prevents supersaturation of calcium oxalate and thus decreased their deposition in renal tubules. These results further confirm the antiurolithiatic effect of CAE in ethylene glycol induced urolithiatic model.

Phytochemical analysis revealed the presence of flavonoids and saponins in the *C. anthelminticum* seeds. Saponins and flavonoids are reported to have anti-oxidant and anti-urolithiatic potential.[19,33] However, the contribution of other phytochemicals on the reported activities cannot be excluded. Our results suggested that diuretic, anti-oxidant, antiinflammatory and antiurolithiatic potential of the seeds of *C. anthelminticum* could be attributed to the nephroprotective action of this plant.

## CONCLUSION

It is concluded that 28 days oral treatement with 70% methanolic extract of *C. anthelminticum* seeds has potential antiurrolithiatic activity against ethylene glycol induced nephrolithiasis, mediated possibly through a combination of diuretic, antioxidant and hypermagnesuric effects, rationalize its medicinal use for various kidney disorders. Further experimental and clinical studies are required to elucidate the chemical constituents and the mechanism(s) that are responsible for the pharmacological activities.

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