

In Vitro* and *In Vivo* Anti-Urolithiatic Activity of Terpenoid-Rich Ethyl Acetate Extract of Rhizomes of *Curcuma zedoaria

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KEYWORDS Calcium Oxalate. Ethylene Glycol. Prophylactic. Struvite. Terpenoid

ABSTRACT *Curcuma zedoaria* is a medicinal plant belonging to *Zingiberaceae* family. The objective “of the study is to analyze the phytoconstituents of the ethyl acetate extract of rhizomes of *C. zedoaria* and to evaluate for anti-urolithiatic activity by *in vitro* single gel diffusion technique and by *in vivo* ethylene glycol induced urolithiasis model. Terpenoids were found to be predominant in both qualitative and quantitative analysis. The struvite crystals were grown in a gel medium. The extract of different concentrations was added to the gel formed and decrease in the crystal size was measured for 5 days using a travelling microscope. The *in vivo* anti-urolithiatic activity was assessed in wistar rat models. Histopathological analysis of kidney of treated rats showed a normal architecture similar to control. Results of both *in vitro* and *in vivo* studies” conclude potent antiurolithiatic activity of *C. zedoaria* which can be attributed to the presence of terpenoids.

INTRODUCTION

Urolithiasis is the stone formation process in the kidney and bladder. The kidney stone formation occurs from a series of physicochemical incidents like super saturation of urinary mineral contents, nucleation of the calculi, aggregation and withholding of the aggregated crystals. With increase in rate of occurrence of urolithiasis in humans, epidemiological reports state that the incidence of renal stones may increase from (40 % to 56 %) by 2050 (Aggarwal et al. 2017). The calcium oxalate stones are the most frequently observed stones accounting for 80 percentage of all the stones in the

clinical states (Chauhan and Joshi 2013; Sharma et al. 2017). Struvite, an infectious stone are formed by the action of urease splitting bacteria like *Proteus mirabilis* that cause persistently alkaline urine (Kasote et al. 2017; Li et al. 2015).

Treatment of urolithiasis involves administration of NSAIDS, calcium channel blockers, thiazide diuretics and alkali-citrate as well the use of nephrolithotomy and extracorporeal shock wave lithotripsy technologies. The undesirable side effects like hemorrhage and necrosis associated with the use of medications and surgical methods and also the increasing possibility for crystal reoccurrence results in extensive damage of the kidney functions (Terlecki and Triest 2007; Xue et al. 2009). Traditional method of treatment of urolithiasis using various plant extracts and as food supplements are largely prevailing in India, China and other Asian countries. The traditional medicine system like Ayurveda uses extracts of plants in the treatment of urolithiatic conditions, although there are no proven clinical evidences. Cystone is one of the ayurvedic polyherbal commercial medication currently (Himalaya Drug Company, India) pre-

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scribed for the treatment of urolithiasis (Rafiq et al. 2012). Recently studies have been reported where the crude extracts of plant source *Tribulus terresteris*, *Boerhaavia diffusa* and *Scoparia dulcis* (Aggarwal et al. 2010; Chauhan et al. 2011; Reshma et al. 2014) have shown a preventive and curative effect on *in vivo* urolithiatic models.

Curcuma zedoaria is a medicinal plant of the *Zingiberaceae* family. It is found widely in the tropical and sub-tropical regions of India, China and Japan. The rhizomes of *C. zedoaria* are used in the treatment of gastrointestinal diseases, blood stagnation syndromes and to promote menstruation (Matsuda et al. 2001), skin infections, stomach ailments, abdominal pain, leucoderma, diuretics and menstrual disorders. *C. zedoaria* is also used in polyherbal formulations used for the treatment of paralysis, arthritis and bronchitis (Lobo et al. 2009). Various extracts of *C. zedoaria* have been reported for their analgesic (Navarro et al. 2002), alcohol intoxication (Kimura et al. 2013) and antimicrobial (Wilson et al. 2005) properties. Previous studies have reported *C. zedoaria* to be a rich source of terpenoids with wide pharmacological applications (Park et al. 2012). However, no scientific evidence is available about the anti-urolithiatic effect of *C. zedoaria* and its active constituents.

Objective

The present work is aimed at identifying and quantifying the phytoconstituents present in ethyl acetate extract of *C. zedoaria* and to scientifically validate the anti-urolithiasis effect of *C. zedoaria* by *in vitro* single gel diffusion technique and by *in vivo* ethylene glycol mediated urolithiatic method.

METHODOLOGY

Chemicals

Ethylene glycol, magnesium acetate tetra hydrate and ammonium dihydrogen phosphate were obtained from SD Fine Chemicals Ltd. Sodium metasilicate pentahydrate (SMS) was purchased from Alfa Aesar. All other chemicals and solvents used were of extra pure grade, purchased from Spectro Chem Ltd., Mumbai.

Authentication and Extraction

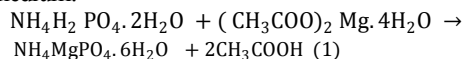
The rhizomes of *C. zedoaria* were collected during the month of July 2014, from the southern part of Orissa, India. The rhizome plant part was taxonomically identified as *C. zedoaria* at the Plant Anatomy Research Centre, Chennai. The sample with a reference number (PARC\2016\3254) was deposited in the same for future reference. The rhizomes of *C. zedoaria* were shade dried at 32-35°C for eight days. The dried rhizomes were powdered using a blender in to coarse granules. The powdered sample of 200 mg was packed in a soxhlet and was extracted with the ethyl acetate for 36 h. The concentrated extract was stored in refrigerator at -4 °C and used for the study.

Qualitative and Quantitative Analysis

The phytoconstituents present in the ethyl acetate extract were identified by qualitative phytochemical screening procedures described by Harborne (1984). The extract was quantified for the total phenol, total flavonoid and total terpenoid content.

In Vitro Anti-urolithiatic Study

The *in vitro* urolithiasis inhibitory activity of rhizomes of *C. zedoaria* was examined by single gel diffusion technique (Das et al. 2016). To 20 mL sodium metasilicate solution (S.D -1.3) appendage with 0.5M ammonium dihydrogen phosphate (ADP) in appropriate amount (pH to 6) was added to each tube. Hydro-gel formation occurred in 10 min and the gel medium was firmly set in 24 h. Different concentrations (0.5%, 1% and 2%) of ethyl acetate extracts prepared in magnesium acetate (1.0 M) solution were transferred to the gel medium. The tubes were examined every 24 h for 5 days to assess the mean decrease in size or dissolution of the crystals using a travelling microscope. The following reaction was predicted to take place in the gel medium.



The change in crystal length at different depths was measured and significance of the analysis was confirmed with ANOVA test. The crystals obtained were investigated by FTIR and X-ray diffraction analysis.

***In Vivo* Anti-urolithiatic Evaluation**

Experimental Rats

The study protocol was approved by Animal Ethical Committee and the ethical clearance number is (VIT/IAEC/10th/March 14th/N0.37). The experiment was performed following the regulations and strategies of Committee which controls the monitors the works carried out on laboratory animals. Albino male wistar rats of 200-250 gm were used for the experiment. The rats were housed in a temperature (25 ± 3°C) control room and were provided with standard diet, adequate water and day and night 'environment.

Anti-urolithiatic Activity of C. zedoaria

Calcium oxalate stones were induced in the experimental animals through ethylene glycol induced hyperoxaluria method (Kalyani et al. 2010). 'The male wistar rats used for the study were grouped as Group (I- V) with six animals in each group. The control (Group I) rats were fed with normal rat feed for a period of 28 days. Urolithiasis or the calcium oxalate stone formation in Group II rats was provoked by administration of (0.75 %) w/v ammonium chloride with ethylene glycol (1 %) for the first three days to accelerate the formation of stones. Later, for the next 25 days the rats were fed a one percent ethylene glycol in drinking water. The cystone drug treated (Group III) rats animals were administered (500 mg kg⁻¹ of b.w) of the drug from the day 15th day of initiation of calculi. Prophylactic Group IV rats were administered a dose (200 mg kg⁻¹ b.w) of ethyl acetate extract from the day one till completion of the study. The curative Group V received the ethyl acetate extract "(200 mg kg⁻¹ b.w) from the day fifteenth of initiation of calcium 'stone fromation.

Analysis of Biochemical Parameters

Body Weight

The decrease or increase in the total weight of the experimental animal groups are reported in percentage. The major variations in the body weight observed with of control, 'stone induced, cystone and *C. zedoaria* treated groups are shown in Table 2.

Analysis of Biochemical Parameters of Urine and Serum

The urine sample (24 h) of all the group rats was collected by placing them in separate metabolic cages on final day of study. The urine samples of all the groups were evaluated for the amount of phosphorous, magnesium and calcium contents using commercially available kits. The serum of the blood samples collected after the completion of the study was examined for the change in urea, creatinine, calcium and phosphorous content.

Histopathology Examination

The experimental wistar rats were sacrificed after collection of the blood. Kidney of each group of animals was preset in 10 percent phosphate buffered formalin solution were sectioned. The sections were examined for the abnormalities in their architecture as well as the deposits of calcium oxalate crystal using a light microscope.

Statistical Study

The significance of the parameters was evaluated by ANOVA method in Graph pad prism software (version 6.0). The results examined are provided in the table with their mean ± standard error mean value. The data containing a p value (< 0.05) was found to be significant.

RESULTS

Extraction, Qualitative and Quantitative Analysis

The ethyl acetate extract of rhizomes of *C. zedoaria* was obtained as a semisolid mass of yield (24.5 %) using soxhlet method. The qualitative phytochemical screening of the ethyl acetate extract of rhizomes of *C. zedoaria* revealed the presence of phenols, flavanoids and terpenoids. The rhizome of *C. zedoaria* contained a total phenol content of 45.9 ± 3.3 mg g⁻¹ of gallic acid equivalent. Flavonoid content of 44.6 ± 2.4 mg g⁻¹ equivalent to quercetin was present in moderate quantity. The terpenoid content of 101.9 ± 1.2 mg g⁻¹ of linalool equivalents was higher in the rhizome of *C. zedoaria*. The study results demonstrated the ethyl acetate extract of

Table 1: Rate of inhibition struvite crystal growth by *C. zedoaria* extract

| | Time (h) | Control | Ethyl acetate extract (%) | | |
|--------------------------|----------|--------------|---------------------------|--------------|--------------|
| | | | 0.5 | 1.0 | 2.0 |
| Growth rate (cm/ day) | 24 | 1.8095± 0.2* | 1.3450± 0.9* | 1.2120± 0.5* | 0.8900± 1.3* |
| | 48 | 1.8400± 1.3* | 1.4300± 0.5* | 1.2380± 0.9* | 0.8030± 0.5* |
| | 72 | 1.9695± 0.5* | 1.1500± 0.4* | 0.8800± 0.8* | 0.7600± 0.3* |
| | 96 | 1.9955± 1.2* | 1.1250± 0.1* | 0.8450± 0.1* | 0.7200± 0.8* |

Note: one-way analysis of variance. The * $p < 0.001$ shows the significance level. Each value represented as mean± SD (n=3).

C. zedoaria to be a rich source of terpenoids as compared to phenols and flavonoids.

Struvite Inhibitory Activity of *C. zedoaria*

The struvite crystals of different morphologies like (feather, X-shaped dendritics, dendritics) shapes were observed in the gel medium. The maximum decrease in crystal length (0.7200 ± 0.8) was observed at the highest concentration of (2%) ethyl acetate extract. Table 1 explains the urolithiasis inhibitory effect of *C. zedoaria* extract and also its dose dependant effect on the average size of the crystals. The crystal growth rate decreases with increase in the dose of the ethyl acetate extract of *C. zedoaria*. The struvite crystals started to dissolve with ethyl acetate extract (2%) treated group from the

day five and the measurement of the crystal was not achievable due to the disintegration of the crystals. The results were found to be noteworthy from the analysis carried out using ANOVA. The obtained p value ($p < 0.001$) shows the results obtained to be significant. Figures 1(A-B) illustrate the different morphologies of crystals obtained in the gel medium.

Characterization of Struvite Crystal

Powdered X-Ray Diffraction Analysis

The powdered XRD pattern with the assigned plane index of struvite crystals is shown in Figure 2. The XRD pattern goes well with the reference 'JCPDS (Joint Committee on Powder Diffraction Standards No. 96-900-7675)' of a

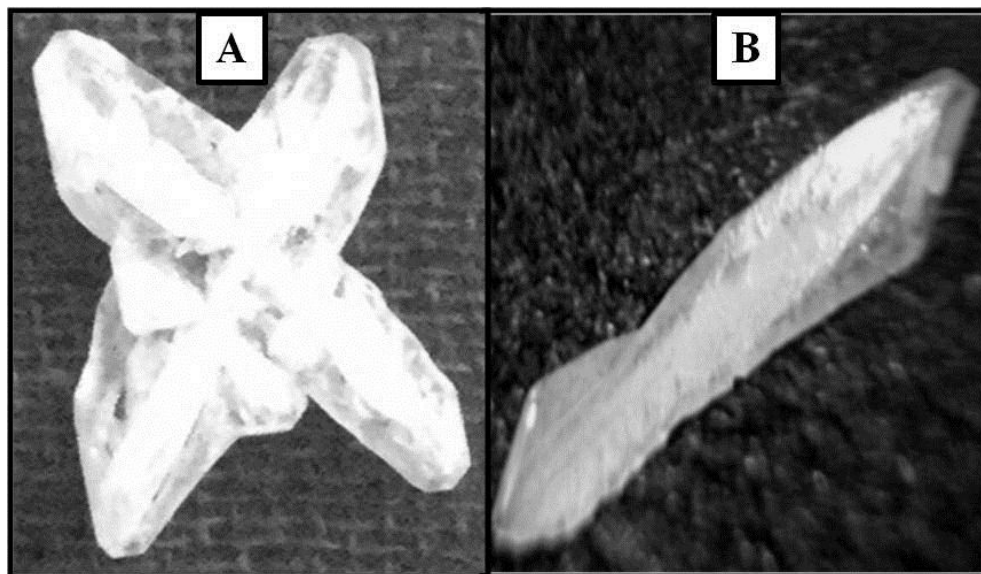


Fig. 1. Struvite crystals of different morphology A) X shaped dendritic and B) Dendritic type.
Source: Author

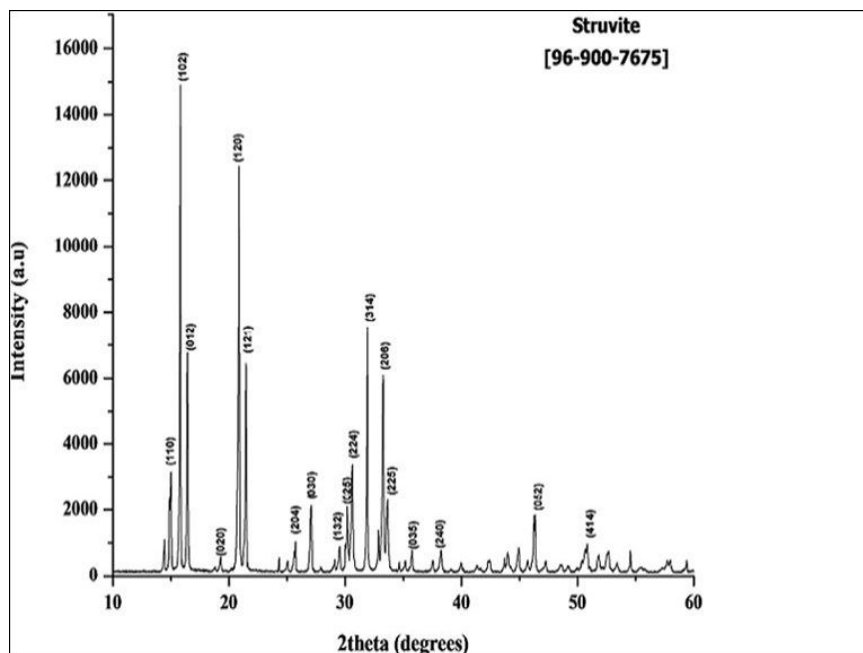


Fig. 2. XRD pattern of struvite crystal
Source: Author

struvite stone. Struvite stones formed in the gel medium were found to be crystallized in the orthorhombic $Pmn2_1$ system with the space lattice parameters assigned for struvite crystals. The values matched greatly with the values reported earlier (Chauhan and Joshi 2013) confirming the structure to be struvite crystal.

Fourier Transform Infra-Red (FTIR) Analysis

The FTIR characterization spectrum of the crystal is shown in Figure 3. The regions depicting the absorption due to water of crystallization matched with several inorganic hydrated compound peaks. Similarly, the vibration peaks of NH_4^+ cation, PO_4^{3-} anions also correspond to several inorganic compounds reported (Khalil et al. 2007). The result confirms the presence of N-H bond, water of hydration and P-O bonding of the struvite crystal.

In Vivo Anti-urolithiatic Activity of *C. zedoaria*

The urolithiasis condition led to the decrease in body weight of Group (II-V) rats. On treat-

ment both the prophylactic and curative groups demonstrated a significant increase in the body weights on the 28th day (Table 2).

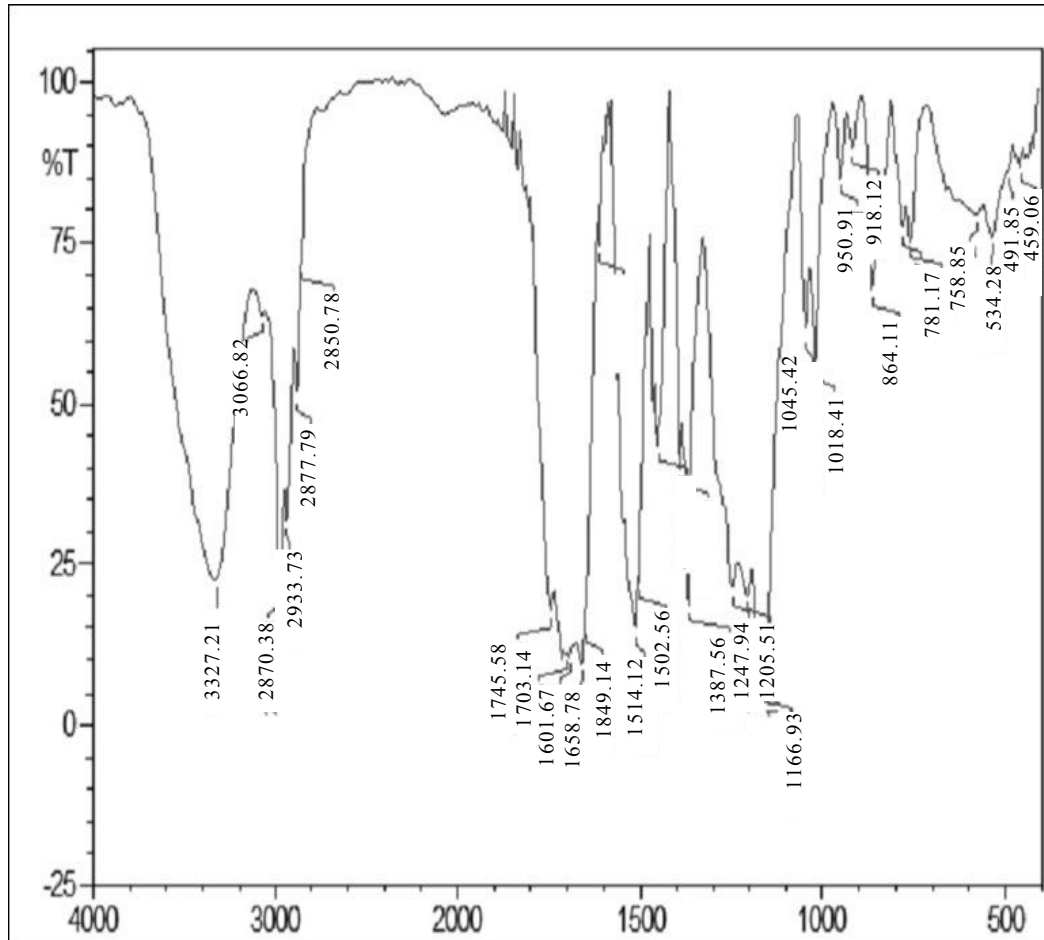
The urinary (calcium and phosphorous content) had increased in the urine of Group II urolithiatic group (Table 2). However, when treated with ethyl acetate extract of *C. zedoaria* (curative and prophylactic), it was found that the calcium and phosphorous content was restored to normal levels ($p < .01$, $p < .001$) comparable to cystone Group II. Magnesium which is known as an inhibitor of crystal growth decreased in urolithiatic group rats (Selvam et al. 2001). However, administration of ethyl acetate extract of *C. zedoaria* significantly restored ($p < .001$) the magnesium levels close to normal.

Formation of calcium oxalate stone was confirmed from the marked impairment of renal tubular and glomerular functions which further lead to increased levels of calcium, creatinine, phosphorous and uric acid in serum. On the 28th day *C. zedoaria* treated group IV and V revealed a substantial reduction ($p < 0.05$) in the creatinine, urea, calcium and phosphorous levels near to normal (Table 2).

Table 2: Effect of *C. zedoaria* extract on biochemical parameters in wistar rat models

| Parameter (unit) | Group I (Control) | Group II (Stone-induced) | Group III (Standard) | Group IV (Prophylactic) | Group V (Curative) |
|---------------------------|-------------------|--------------------------|------------------------------|------------------------------|------------------------------|
| Change in body weight (%) | 20.1± 0.03 | -3.2 ± 0.05 ^a | 12.8 ± 0.03 ^{a, *b} | 14.4± 0.01 ^{a, *b} | 10.7± 0.1 ^{a, *b} |
| Urine (mg/dl) | | | | | |
| Phosphorus | 6.0± 0.03 | 16.3± 0.08 ^a | 9.4± 0.1 ^{a, *b} | 7.5± 0.1 ^{a, *b} | 8.3± 0.04 ^{a, *b} |
| Calcium | 4.1± 0.1 | 6.8± 0.1 ^a | 5.7± 0.1 ^{a, ‡b} | 5.0± 0.1 ^{a, ‡b} | 4.5± 0.03 ^{a, ‡b} |
| Magnesium | 2.6± 0.03 | 0.7± 0.1 ^a | 1.7± 0.1 ^{a, *b} | 2.1± 0.04 ^{a, *b} | 2.4± 0.03 ^{a, *b} |
| Serum (mg/dl) | | | | | |
| Phosphorus | 1.8 ± 0.03 | 6.0 ± 0.1 ^a | 3.3 ± 0.03 ^{‡a, ‡b} | 2.4 ± 0.03 ^{‡a, ‡b} | 3.0 ± 1.03 ^{‡a, ‡b} |
| Calcium | 8.0 ± 0.1 | 14.0 ± 0.05 ^a | 11.1 ± 0.1 ^{a, *b} | 8.6 ± 0.1 ^{a, *b} | 9.9 ± 0.1 ^{a, *b} |
| Urea | 17.1 ± 0.1 | 25.5 ± 0.1 ^a | 20.3 ± 0.2 ^{a, *b} | 17.9 ± 0.1 ^{a, *b} | 19.3 ± 0.1 ^{a, *b} |
| Creatinine | 0.5 ± 0.02 | 1.9 ± 0.03 ^a | 1.2 ± 3.1 ^{a, *b} | 0.6 ± 0.02 ^{*b} | 0.99 ± 0.03 ^{a, *b} |

Note: Values for urine parameters are measured in 24 h urine sample. All values are stated as mean ± standard deviation. a = Comparisons are made with Group I; b = Comparisons are made with Group II; * = $p < 0.001$; ‡ = $p < 0.01$; † = $p < 0.05$.

**Fig. 3. FTIR spectrum of the struvite crystal**

Source: Author

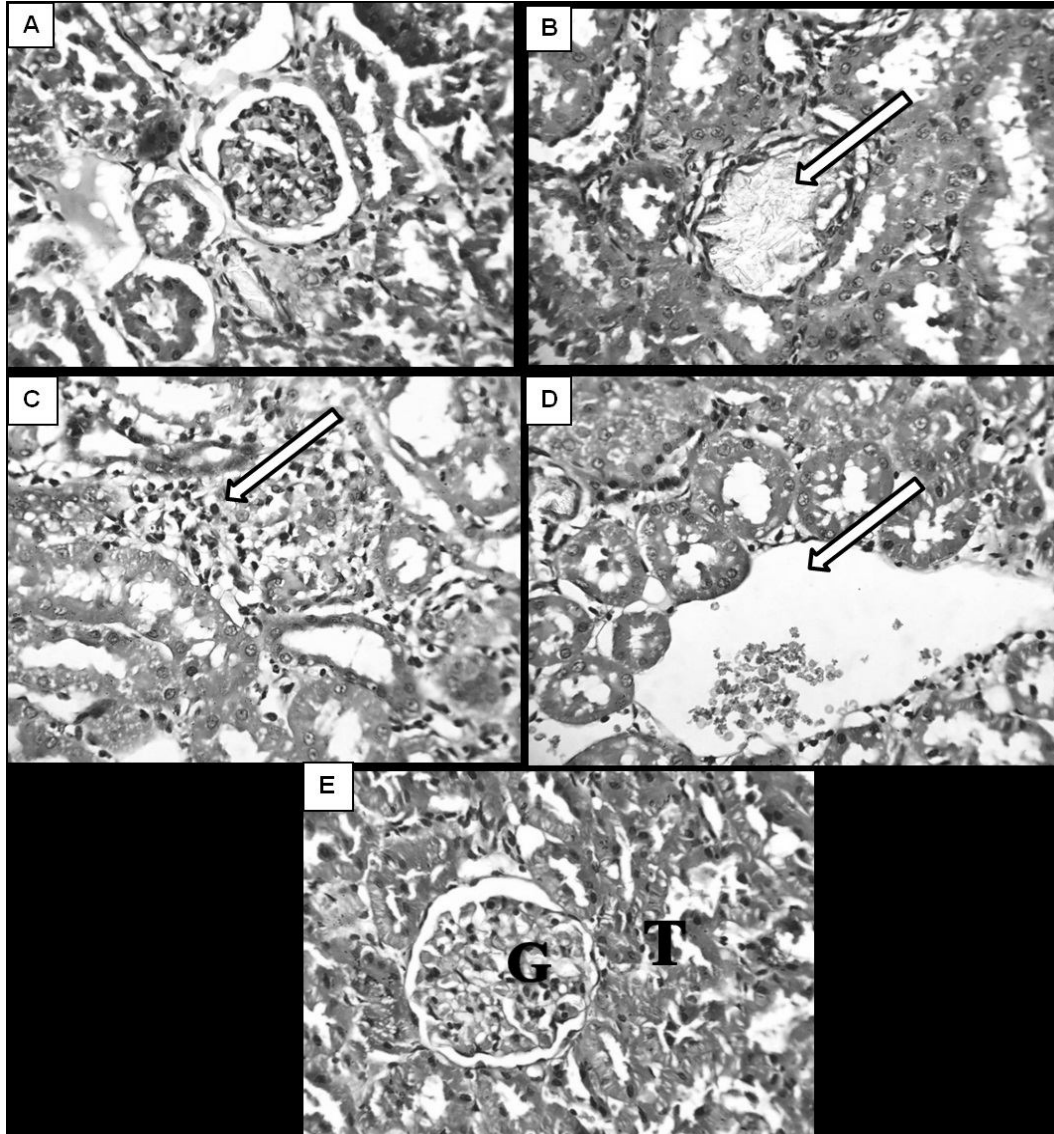


Fig. 4. Kidney tissue analysis; A) Glomerulus and proximal convoluted tubules with normal morphology in control (Group I); B) Renal tubules showing refractory crystals (arrow) in their lumen (Group II) ethylene glycol induced model; C) Group II stone induced group with minimal interstitial inflammation (arrow); D) Kidney showing normal architecture with occasional blood vessels showing dilation and congestion (arrow) in Group III; E) Glomerulus and renal tubules of kidney showing normal morphology in Group V.

Source: Author

Histopathology Study

The kidney histopathology analysis was performed for all the group of rats. The glomeru-

lar and the tubular renal cells of control rats had a healthy normal architecture. Kidney cells contained no calculi development and deposition in case of the Group I (control) as shown in Figure 4A.

The renal cell of group II rats in which the calculi was induced was found to contain depositions of refractile crystals and cells were inflamed due to diseased condition (Figs. 4B-D). Histopathological images of prophylactic group showed no refractile crystals and the kidney cells possessed normal architecture (Fig. 4E).

DISCUSSION

The struvite inhibitory activity of *C. zedoaria* was examined by the distinctive single gel diffusion technique. The Struvite crystals of different morphology were formed in the gel medium and they were mainly feather shaped, dendritic shaped and X-shaped dendritics. Apart from these shapes the coffin and rectangular platelet shaped were found to be present least in the gel medium. The decreased crystal growth rate followed by their dissolution observed in the gel medium suggested the potential preventive and curative anti-urolithiatic activity of ethyl acetate extract of *C. zedoaria*. A correlation was carried out to determine the relation between the phytoconstituents and the urolithiasis inhibitory activity of *C. zedoaria*. A positive correlation ($r^2=0.89$) was observed between the terpenoid content and the activity, suggested that the anti-urolithiatic activity might have been contributed by the rich terpenoids content of *C. zedoaria* (Ghelani et al. 2016; Sharifa et al. 2012; Shu et al. 2016; Varicola et al. 2017). The result obtained were supported by the previous reports (Barros et al. 2006; Malini et al. 2000; Nagal and Singla 2013) were plants extracts with high terpenoid contents exhibited potent anti-urolithiatic activity.

The growth and recurrence rate of the kidney stones is higher (70%) in male than the female rats hence, male rats were chosen to perform the study. Similar to the previous reports (Bahuguna et al. 2009), an increased mineral (calcium and phosphorus) excretion levels in the urine of urolithiatic group was observed on the 15th day. On treatment, the curative (Group IV) and prophylactic (Group V) rats demonstrated an appreciable decrease in the mineral contents to normal levels compared with cystone group III.

The level of magnesium ion plays an important role in crystal formation. As with increase in the number of crystal deposits in the kidney cells, the magnesium content of urine decreases

(Soundararajan et al. 2006). However, when treated with *C. zedoaria* (Groups IV and V) the magnesium content was restored to normal suggesting its significant urolithiasis inhibitory activity.

The glomerular filtration rate (GFR) of the kidney is important for the normal urinary output. This filtration rate was affected under urolithiatic condition and led to the accumulation of minerals (calcium and phosphorous) and nitrogenous substances such as urea and creatinine which, further led to the initiation of crystal nuclei growth causing renal tissue damage. This was supported by the histopathological reports wherein the renal tissues of the urolithiatic (Group II) rats demonstrated presence of refractory calcium oxalate stones. The marked interstitial inflammation of the renal cells in stone induced (Group II) rats was because of the development and growth of crystals. The group IV (prophylactic group) illustrated better activity compared with the curative Group V. The potential urolithiasis preventive effect of *C. zedoaria* might have been contributed by the rich terpenoid content of the extract which prevented the formation of the calcium oxalate crystals by either of the mechanisms; increased bioavailability of nitric oxide that suppresses the influx of calcium by cGMP pathways (Matsuda et al. 2001) and its nephroprotective effect.

CONCLUSION

The ethyl acetate extract of *C. zedoaria* was obtained by soxhlet method. The qualitative and quantitative analysis revealed the presence of phenols, flavonoids and terpenoids. The rhizomes *C. zedoaria* was observed to be rich in terpenoid content. The potent urolithiasis inhibitory activity and nephroprotective effect of rhizomes of *C. zedoaria* was proven from the study. Potent anti-urolithiatic activity of the rhizome might be because of the high terpenoid content.

RECOMMENDATIONS

The study provides a platform for further analysis and identification of the individual phytoconstituent essential for the activity. Since the rhizomes of *C. zedoaria* are well known for their medicinal use orally as well as topically, the rhizomes can be used for the treatment of urolithiasis without any toxicity concerns.

ACKNOWLEDGMENTS

The authors gratefully thank the VIT University, India for providing the grant to perform the research. The authors also thank the P.B Histopathology Laboratory and Ravichandran Medical Centre, India for analysis of the serum and urine samples.

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