



Calcium oxalate crystallization inhibition by *Pedalium murex* and *Tribulus terrestris* fruit extracts

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Abstract : Urinary calculi are one of the most common disorders of the urinary tract seen all around the world. It is mainly due to the supersaturation of urine. Although there are many medical treatments there is no satisfactory drug to treat them. Urinary calculus is mainly composed of calcium oxalate (CaOx) which is formed due to the higher concentration of stone forming salts in urine. This study is aimed to look for an alternative treatment using *Pedalium murex* and *Tribulus terrestris* fruit ethanol extracts. To confirm the antiurolithiatic activity the extracts were tested in artificially prepared urine samples. The inhibitory effect of the extracts on CaOx was measured using UV-Vis spectrophotometer at 620 nm over various concentrations of extracts 200, 400, 600, 800 and 1000 µg/ml. A significant inhibitory effect on CaOx nucleation, crystal growth and aggregation in the urine sample was found. Maximum inhibition of nucleation of 84.5 % was observed for *Pedalium murex* and 81.3 % for *Tribulus terrestris* at concentration of 2600 µg/ml.

Key words: Urinary calculi, *Pedalium murex*, *Tribulus terrestris*, antiurolithiatic activity, UV-Vis spectrophotometer, CaOx crystallization.

Introduction

Urinary Calci also known as kidney stone has victimized people all around the world and there is no specific treatment to cure or prevent this in Allopathic medicine¹. There are only techniques like Extracorporeal Shock wave treatment (lithotripsy) and the recent Biopolar Radio frequency treatment to break the kidney stones into small fragments². Generally these stones are made of oxalate of calcium, uric acid, struvite and cystine. Studies show that depending on the composition of the kidney stone the compressive strength and the dielectric property of the urinary calci vary³. Sometimes it is initiated by minute pathogens or nanobacteria namely *P.aeruginosa*, *K.pneumoniae* and *E.coli* living inside the urinary track can migrate to nephritic tissues causing the calcium oxalates to crystallize along with impurities resulting in a urinary calci^{4,5}.

Many plants have been used to treat kidney stones for years although they were rationale not well established through methodical and pharmacological studies⁶. Some herbal drugs and plant medicine are of greater importance all around the world for the health care because of their wide range of bio-medicinal active ingredients⁷. A large variety of herbal drugs and alternative treatments has been tried for prevention and treatment of kidney stones but none of them got the commercial importance⁸.

Pedalium murex and *Tribulus terrestris* are native of India and have been reported to possess promising

antiurolithiatic components. From the survey *Pedalium murex* and *Tribulus terrestris* were among the twenty three plants that found to be used as potential remedies in treatment, cure and prevention of urinary calculi. Some treatment methods used in villages were four grams of *Pedalium murex* fruit powder administered with sheep milk once a day for 7 days and fifty ml of *Tribulus terrestris* root decoction administered twice a day for 15 days⁹. Even these are marketed in the form of diuretic syrup in combination with other herbs like *Mimusops elengi*, *Crateva nurvala*, and *Andropogon muricatus*¹⁰. The Figure 1 shows the plant, fresh fruit and dried fruit of *Pedalium murex* and *Tribulus terrestris*.

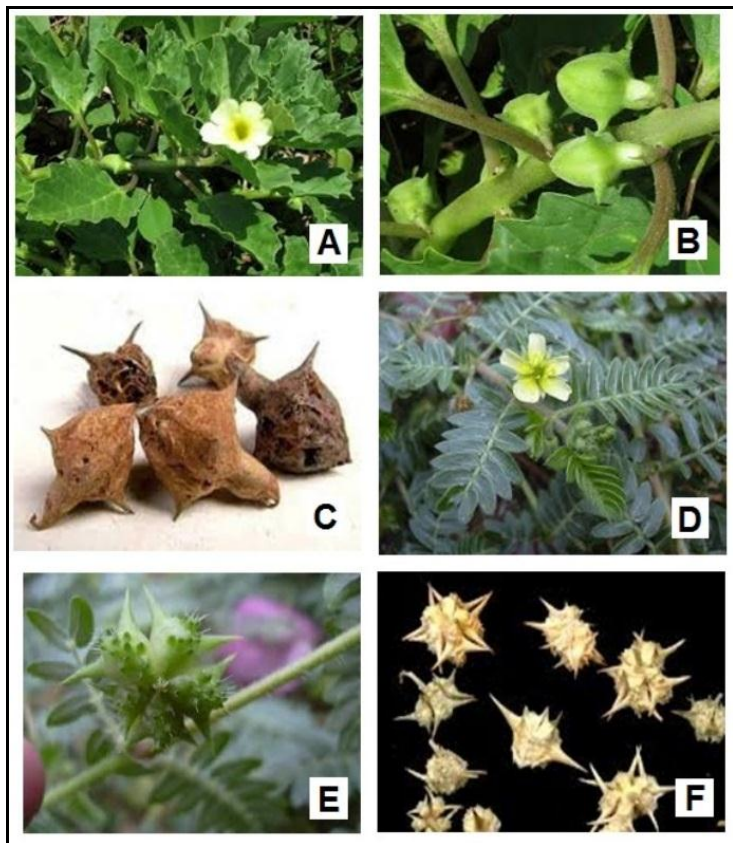


Figure 1: *Pedalium murex* A) plant B) fresh fruit C) dried fruit, *Tribulus terrestris* D) plant E) fresh fruit F) dried fruit

Pedalium murex is a flowering plant of the *Pedaliaceae* family and it is distributed in India, Sri Lanka and Tropical Africa. This plant is about 15 to 40 cm in height, having quadrangled spiny brownish colour fruits (1-2 cm). These fruits are rich in flavonoids, sapogenin (diosgenin-0.06%) and soluble proteins (20.14mg/ym)¹¹. It also contains flavonoids and several alkaloids like Pedalithin, Diosmetin, Dinatin, Pedalin dinatin-7-glucuronide¹². The decoction of the seeds obtained from it showed mild diuretic activity and the alcoholic fruit extract reduced blood pressure in dog and rat¹³.

According to Ayurveda, the fruit of *Pedalium murex* is considered to be demulcent and diuretic. It is a good antispasmodic, aphrodisiac, appetite improver and useful in strangury, urinary discharges, vesical calculi, cough, asthma, skin diseases and heart trouble. It purifies blood and removes stone in the bladder^{14,15}. It is observed that this plant extract exhibited inhibition on growth of A549 cancer cells¹⁶.

Tribulus terrestris is a flowering plant belonging to *Zygophyllaceae* family, native to tropical regions of the Southern Europe, Southern Asia, Africa and Northern Australia¹⁷. *Tribulus terrestris* is an annual herb known as puncture vine (or) small caltrops which grows upto 10 to 60 cm, with pinnate leaves and yellow flowers¹⁸. The fruit and root of *Tribulus terrestris* contains pharmacologically important metabolites such as phytosteroids, flavonoids, alkaloids and glycosides¹⁹. Various derivatives of tigogenin, neotogogenin, hecogenin, neohecogenin, diosgenin, ruscogenin, chlorogenin and sarsasapogenin are also found in this plant. Few are reported, where four sulphated furo and spiro saponins have been isolated that is used as an antifungal

against pathogens likes *Penicillium italicum*, *Aspergillus niger*, *Trichoderma harzianum* and *Botrytis cinerea*²⁰.

The fruits and seeds are of immense importance in folk medicine because they are used as an aphrodisiac, diuretic and anthelmintic. Some studies shows that an alcoholic extract of *Tribulus terrestris* is used to treat *Diabetes mellitus* in STZ induced diabetic rats²¹. It is also used to treat coughs and kidney failure²². In India *Tribulus terrestris* is used in traditional medicine in the form of tonic and it acts as aphrodisiac, analgesic, astringent, stomachic, anti-hypertensive, diuretic, lithontriptic and urinary anti-infective²³. The present study is proposed to evaluate the antiurolithiatic activity of *Pedalium murex* and *Tribulus terrestris*.

Materials And Methods

Collection of plant materials

Pedalium murex and *Tribulus terrestris* fruits were collected, washed in distilled water and allowed to shade dry. The dried material was homogenized in domestic mixture into fine powder using a mixer grinder and a ball mill and stored in plastic container at room temperature.

Preparation of extracts

The dried fruits of *Tribulus terrestris* and *Pedalium murex* were pulverized and about 15 grams of powder was extracted using ethanol in Soxhlet apparatus. Ethanol was used for the process as it is safe for ingestion and also can be evaporated after extraction. The extracts were transferred to a petri dish and concentrated to dryness at room temperature 40-45 °C to get dried extracts. The extracts of *Pedalium murex* and *Tribulus terrestris* were dissolved in distilled water, filtered and different concentrations of 200, 400, 600, 800 and 1000 µg/ml were prepared.

Experimental procedure

The effect of extract on CaOx crystallization was determined by measurement of turbidity changes due to the crystallization in artificial urine. These crystals are induced on addition of 0.01M sodium oxalate solution into the artificial urine, every minute. The precipitation of calcium oxalate at 37 °C and pH 6.8 was studied by the measurement of absorbance at 620 nm using Elico Double beam UV/Vis spectrophotometer SL 244²⁴.

Preparation of artificial urine

An artificial urine sample was prepared according to the method specified by Burns and Finlayson²⁵ and it had the following composition: Sodium chloride: 105.5 mM, Sodium phosphate: 32.3 mM, Sodium citrate: 3.21 mM, Magnesium sulfate.7H₂O: 3.85 mM, Sodium sulfate: 16.95 mM, Potassium chloride: 63.7 mM, Calcium chloride.2H₂O: 4.5 mM, Sodium oxalate: 0.32 mM, Ammonium hydroxide: 17.9 mM and Ammonium chloride: 0.0028 mM. The artificial urine was prepared fresh each time and pH adjusted to 6.0.

Study without inhibitor

A volume of 1.5 ml of artificial urine was transferred into the cuvette and 0.5 ml of distilled water added to it and blank reading was taken. The 0.5 ml of 0.01M sodium oxalate was added, to the previous volume, and the measurement was immediately started for time duration of ten minutes.

Study with inhibitor

A mixture of 1.5 ml of artificial urine and 0.5 ml of plant extract solution was transferred in the cuvette. A reading without addition of sodium oxalate was taken and then 0.5 ml of 0.01M sodium oxalate solution was added. The absorbance was measured immediately for a period of ten minutes.

Nucleation assay

The stone starts to form around nuclei; therefore the classical model of oxalate crystallization is that the CaOx start to crystallize around the nuclei²⁶. Solutions of calcium chloride and sodium oxalate were prepared at the concentration of 3 mM and 0.5 mM, respectively, in a buffer containing Tris (2-Amino-2-hydroxymethyl-propane-1,3-diol) 0.05 M and NaCl 0.15 M at pH 6.5. Both 33 mL of calcium chloride solution and the aqueous

extract of different concentrations were mixed. The crystallization was started by adding 33 mL of sodium oxalate solution. The solution was magnetically stirred at 800 rpm to measure entire potential of the given concentration of the plant extract. All the experimental procedures are carried out at 37 °C. The absorbance of the solution was monitored at 620 nm. The inhibition percentage produced by the fruit extract was calculated as:

$$\% \text{ Inhibition} = (\text{Ab Control} - \text{Ab Test}) / \text{Ab Control} \times 100$$

Where: Ab Test: Absorbance of sample in the presence of inhibitor (Extract), Ab Control: Absorbance of sample in the absence of inhibitor (Control).

Result and Discussion

The effect of extracts of *Pedalium murex* and *Tribulus terrestris* on various phases of CaOx crystallization was determined by measurement of turbidity in the urine sample with respect to time and increase in concentration of the extract. Figure 2 and 3 shows an initial detectable increase in the turbidity after initiation of the crystallization with sodium oxalate solution. In the control experiment, the initial steep rise in turbidity is due to the nucleation phase. On attaining its maximum, turbidity decreases which is the result of the aggregation phase.

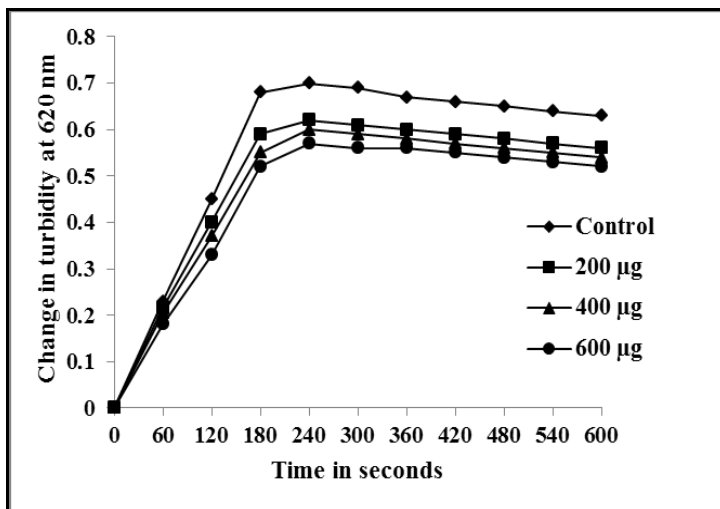


Figure 2: Change in turbidity without and with 200, 400 and 600 µg/ml of *Pedalium murex* extracts (inhibitor)

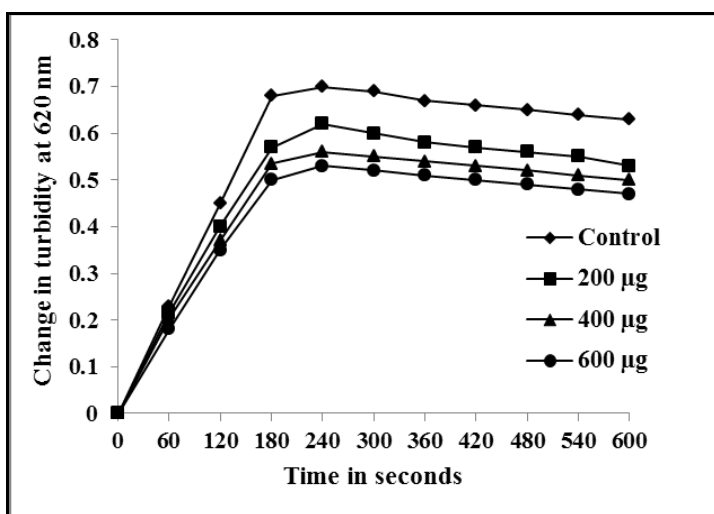


Figure 3: Change in turbidity without and with 200, 400 and 600 µg/ml of *Tribulus terrestris* extracts (inhibitor)

CaOx crystallization was inhibited 9.11%, 13.72% and 16.29% in concentration dependent manner for 200, 400 and 600 $\mu\text{g/ml}$ of *Pedaliium murex* extract respectively.

Similarly CaOx crystallization was inhibited 5.33%, 11.42% and 13.79% in concentration dependent manner for 200, 400 and 600 $\mu\text{g/ml}$ of *Tribulus terrestris* extract respectively.

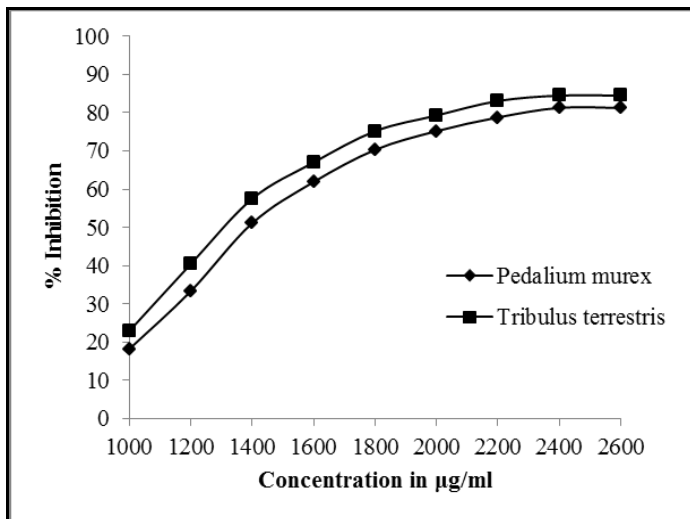


Figure 4: Effect of aqueous extract of *Pedaliium murex* and *Tribulus terrestris* of dosage in steps of 200 $\mu\text{g/ml}$ till maximum inhibition on nucleation of calcium oxalate

Figure 4 displays the effect of the different concentration of the extracts of *Pedaliium murex* and *Tribulus terrestris* respectively on the nucleation of calcium oxalate crystals. The increase in the concentration of extract showed decrease of nucleation. Maximum inhibition of nucleation of 84.5 % was observed for *Pedaliium murex* and 81.3 % for *Tribulus terrestris* at concentration of 2600 $\mu\text{g/ml}$.

The population of CaOx crystal when observed under light microscope (400X magnification) with ethanol extract at its higher concentration (2600 $\mu\text{g/ml}$) showed a greater potential towards crystal growth inhibition.

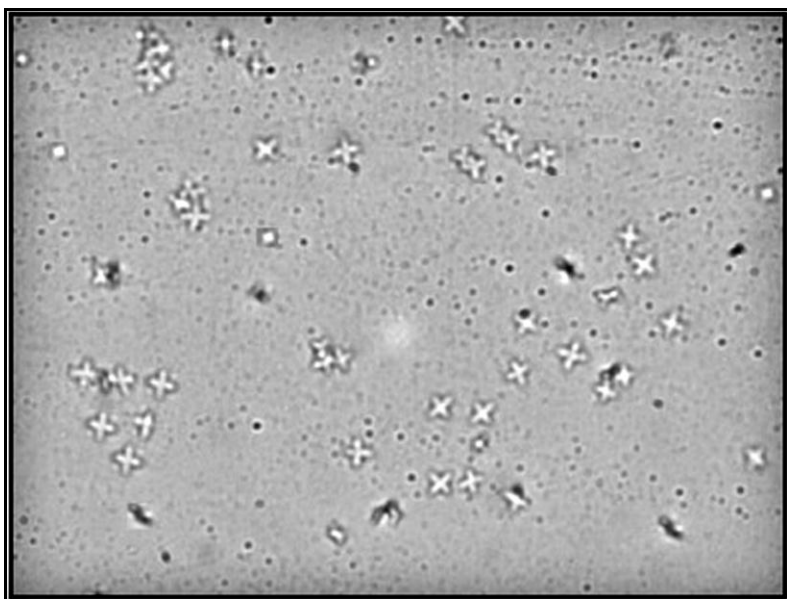


Figure 5: CaOx nucleation without ethanolic plant extract (Light microscopy 400x)

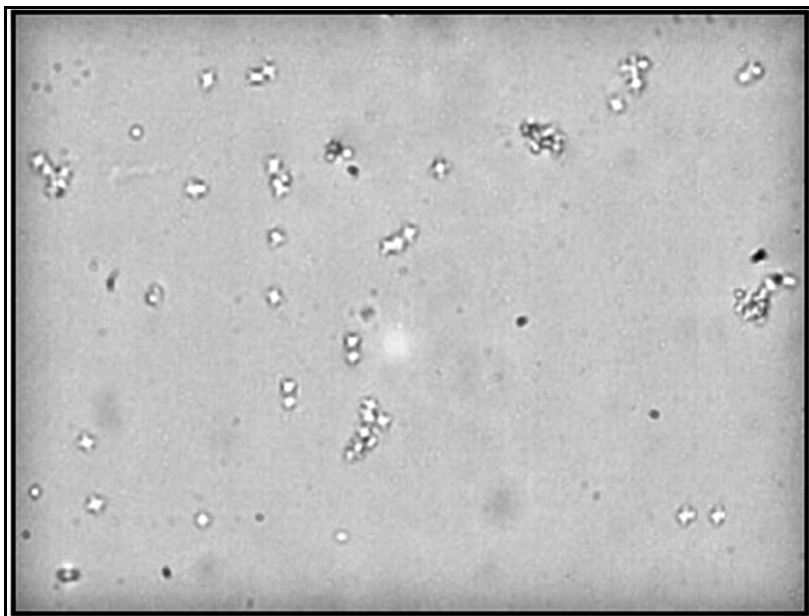


Figure 6: CaOx nucleation with ethanolic plant extract of concentration 2600µg/ml (Light microscopy 400x)

In this study, *Tribulus terrestris* and *Pedalium murex* extracts inhibited the CaOx crystal nucleation and aggregation with respect to increase in concentration. The extract reduces the nucleation thereby increasing the metastable limit of oxalate in urine and preventing the precipitation of the CaOx crystal²⁷. Figure 6 shows a significant inhibition in nucleation due to the presence of fruit extracts, whilst Figure 5 shows instantaneous CaOx crystal nucleation in the absence of fruit extracts.

After nucleation, crystal growth is the next major step of stone formation. The potential energy of the atoms or molecules when they form bonds to each other is the driving force for crystallization and it is reduced by the plant extracts. It is observed from Figures 7 and 8.

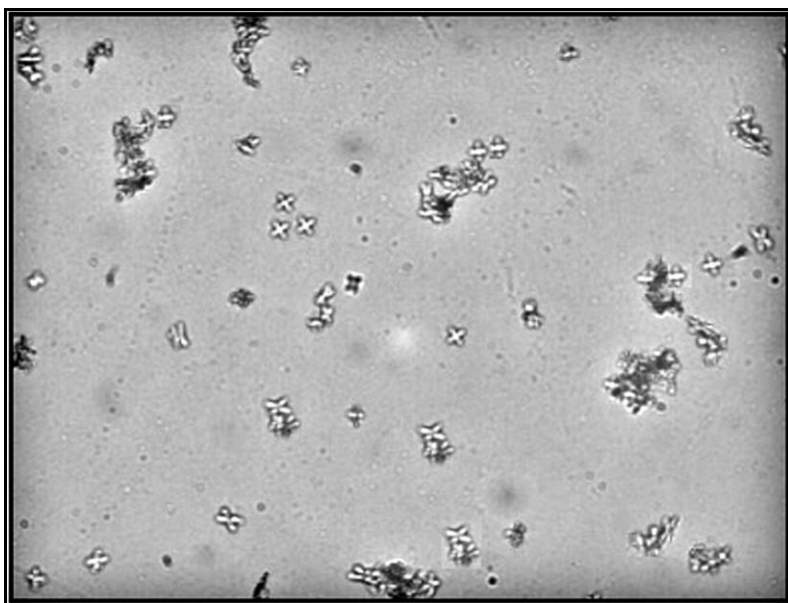


Figure 7. CaOx crystal growth without ethanolic extract (Light microscopy 400x)

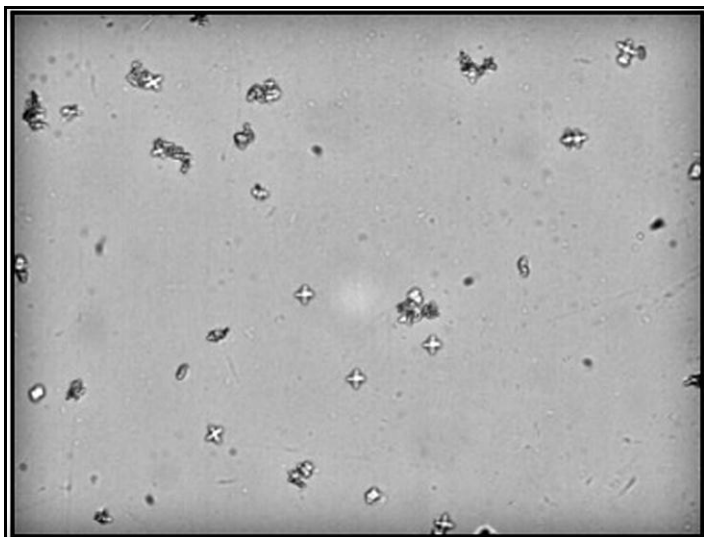


Figure 8: CaOx crystal growth with ethanolic plant extract of concentration 2600µg/ml (Light microscopy 400x)

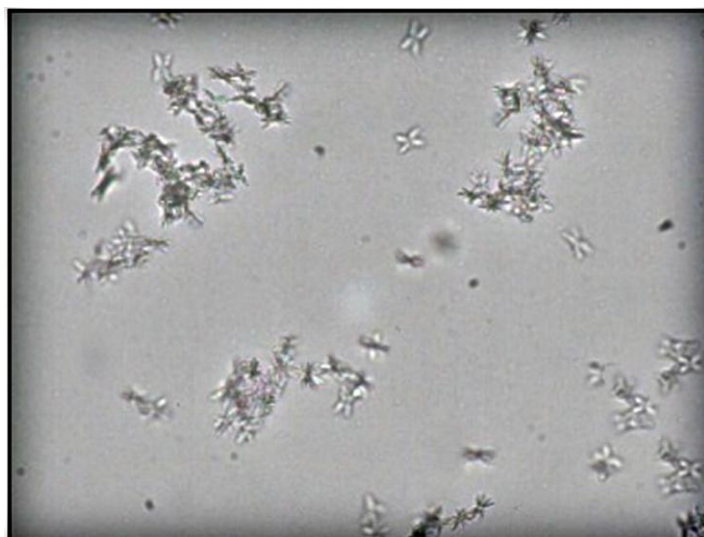


Figure 9: CaOx aggregation without ethanolic plant extract (Light microscopy 400x)

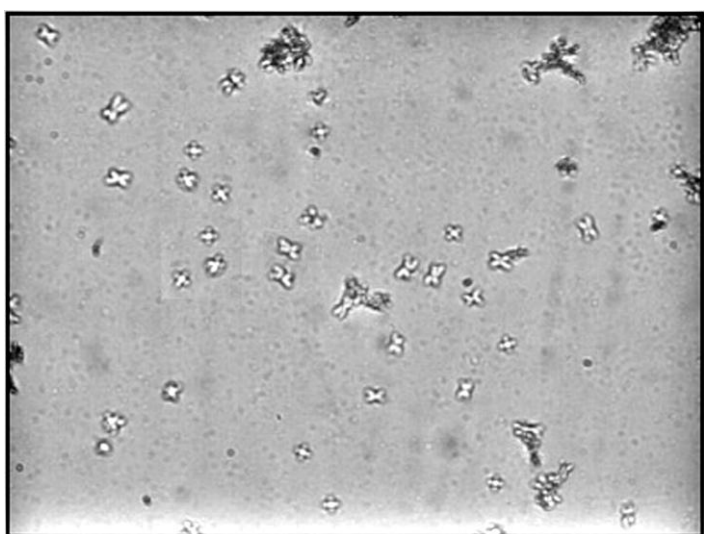


Figure 10: CaOx aggregation with ethanolic plant extract of concentration 2600µg/ml (Light microscopy 400x)

Aggregation is followed by crystal growth and aggregated CaOx crystals adhere to the cells in the renal papilla leading to renal calculi. This process also depends on the urinary pH and the concentration of CaOx in the urine. *Pedalium murex* *Tribulus terrestris* and contain chemical compounds, which themselves possess ability to inhibit the crystallization of calcium oxalate thereby preventing crystal growth and aggregation. The aggregation of CaOx crystals in Figure 10 is also very much reduced compared to that of CaOx crystal aggregation as shown in Figure 9. Microscopic observation revealed that the extracts visibly reduced the crystal size with significant decrease in population of CaOx crystals.

The present in-vitro study provides a scientific proof for traditional claim of *Pedalium murex* and *Tribulus terrestris* as antiurolithiatic herbs. These fruit extracts have inhibitory effect on crystal nucleation and aggregation but a detailed preclinical and clinical study is to be carried out in future to confirm its activity.

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