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IN-VITRO CALCIUM OXALATE STONE REDUCING POTENTIAL OF *Echinops echinatus Roxb.*& *Passiflora edulis Sims*

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ABSTRACT

Urolithiasis is a multistep bio-chemical process with high recurrence rate. Epidemiological studies discovered that urolithiasis is more seen in men than in women and is more widespread between the ages of 20 to 40 in both sexes. In ayurvedic system of medicine and also in herbal medicaments numerous actives/extracts are used for the management of urolithiasis. The present study was carried out with an objective to find out comparative evaluation of the kidney stone dissolving potential of some of the medicinal plants by using calcium oxalate crystals-titration method to know their actual efficacy. Two plant species were evaluated for its anti-urolithiatic activities In vitro. The inhibitory activity against calcium oxalate (CaOx) via aggregation assay and dissolution using titrimetric method were evaluated. The % dissolution of calcium oxalate stones by four formulations were estimated by redox titrations and the effects of four formulations on slope of nucleation and aggregation as well as CaOx crystal growth were evaluated spectrophotometrically. Cystone® Syrup showed the highest inhibitory activity against aggregation of CaOx crystals (25.13±1.19)and the same product had the most effective dissolution effect on CaOx crystals ((60.74 ± 1.63%)). The other promising methanolic plant extract of *Echinops echinatus Roxb* had also shown acceptable results with respect to inhibition

(78.68±1.82 %) as well as dissolution (54.82 ± 1.48%) & fruit extract of *Passiflora edulis Sims*. had also shown acceptable results with respect to Cystone (52.98 ±1.39) dissolution effect and on CaOx crystals of calcium oxalate crystals in in-vitro studies. Present study has given a fare idea about the efficacy of *Echinops echinatus Roxb. & Passiflora edulis Sims*. extract which will be used in the management of kidney stones.

Key words: In vitro, Antiurolithiatic, Dissolution, Inhibition, Comparative evaluation, Kidney stones, *Echinops echinatus Roxb., Passiflora edulis Sims*

INTRODUCTION

Nowadays stone formation is the oldest and serious painful urologic disease with significant prevalence in the population due to change in lifestyle and dietary factors. Urolithiasis is characterized by calculi formation. It has two main types such as nephrolithiasis and urolithiasis. A majority of urinary stones are composed of phosphates, oxalates, cystine, and uric acid. Almost 80% of these calculi are composed of calcium oxalate (CaOx). Calcium-containing stones may be in the form of pure calcium oxalate (50%) or calcium phosphate (5%) and a mixture of both (45%) followed by magnesium phosphate **[1]**. Calculi formation in urinary bladder, ureter or any part of urinary tract rather than kidney is known as urolithiasis while nephrolithiasis is characterized calculi formation in kidney. Generally, calcification for the formation of bone and teeth takes place in controlled biological situations Uncontrolled pathological crystallization occurs when solvent becomes supersaturate leading to the formation of precipitates in the body called as kidney stones. Urolithiasis is a multistep bio-chemical process with high recurrence rate. Globally, kidney stone disease prevalence and recurrence rates are increasing, with limited options of effective drugs. Urolithiasis affects about 12% of the world population at some stage in their lifetime. It affects all ages, sexes, and races but occurs more frequently in men than in women within the age of 20–49 years. The recurrence rate of secondary stone formations is estimated to be 70-81% in male and 47-60% in female. Therefore, prophylactic management is of great importance to manage urolithiasis. Struvite and newberyite are magnesium containing whereas ammonium acid urate, mono sodium urate monohydrate, uric acid anhydrous, uric acid mono and dihydrate are commonly existing urate stones. After urolithiasis treatment, there is 50% chance of stone formation

within seven years if left untreated. Therefore, prophylactic management is of great importance and advisable, especially in such individual subject. Crystallogenesis is the first and essential step in stone formation which is based on three steps nucleation, growth and aggregation. Uroliths (calculi) are generally composed of calcium as calcium oxalate monohydrate and calcium hydrogen phosphate dihydrate (75-90%), magnesium and ammonium magnesium phosphate hexahydrate (10-15%), uric acid and urates (3-10%), and 0.5-1% is composed of cystine, hippuric acid, L-tyrosine and xanthine. Medicinal plants are considered as a rich source of therapeutic agents due to the belief and observations regarding their traditional use for the prevention of various ailments. Various research findings and data from different part of the globe are contributing and helping the scientific community in evaluating and establishing the pharmacological activities of these plants. In ayurvedic system of medicine and also in herbal medicaments numerous actives/extracts are used for the management of urolithiasis. Various preparations are routinely used by practitioners for management of kidney stones. The efficacy of these preparations is based on ancient knowledge of the actives. The scientific study of the various preparations based on the anti-urolithatic models is need of the hour. The present study was carried out with an objective to find out comparative evaluation of the kidney stone reducing potential of some of the medicinal plants extracts by using calcium oxalate crystals-titration method to know their actual efficacy **[2]**.

MATERIALS AND METHODS

Collection of plant materials

Plant of *Echinops echinatus Roxb*.was gathered in the month of December 2020 from hill of Manoli, sangamner, Ahemadnagar ,Maharashtra, India. Fruits of *Passiflora edulis Sims.* was gathered in the month of December 2020 from Ozar, Junnar, pune, Maharashtra, India. Authentication was done from Sangamner Nagarpalika Arts, D.J. Malpani Commerce & B. N. Sarda Science College, Sangamner, Ahemadngar, Maharashtra, Voucher No. 487,488 respectively.

Preparation of Plant Extract

Extraction process

200g of powdered Leaves of plant species *Echinops echinatus Roxb*.Methanolic was extracted by using 400 ml of pet ether, chloroform. methanol, water resp. in soxhlet apparatus at 64 °C temperature until all the compounds were extracted into the solvent.

The extract was evaporated and concentrated by using rotary evaporator at 45 °C temperature. Further, dried extract was stored in an airtight, light-resistant container at 4 °C in the refrigerator for further analysis

Fruit juice of plant species *Passiflora edulis* Sims .was dried in the sunlight and the dried extract was stored in an airtight, light-resistant container at 4 °C in the refrigerator for further analysis

Materials

Cystone® Syrup was purchased from local pharmacy shops. Hydrochloric acid, Sulphuric acid, KMnO4, Calcium Chloride dihydrate and Sodium Oxalate was purchased from S.D. Fine Chemicals Pvt Ltd Mumbai. The eggs for preparation of membranes were made available from local grocery shops. *Echinops echinatus Roxb., Passiflora edulis Sims.* were collected from western ghat of Maharashtra. All other reagents used were of analytical grade and used with further dilutions for experiments **[3]**.

Preparation of calcium oxalate by homogenous precipitation

Calcium oxalate crystals were prepared by taking equimolar solution of calcium chloride dihydrate (A.R) which was dissolved in distilled water and sodium oxalate (A.R) which was dissolved in 10 ml of 2N H_2SO_4 and distilled water, sufficient quantity was allowed to react in a beaker. The resulting precipitate was calcium oxalate which was freed from traces of sulfuric acid by addition of small amount ammonium solution. The prepared calcium oxalate crystals were washed with distilled water and dried in a hot air oven at temperature 60^{o}C for 4 hours **[4]**.

Preparation of semi permeable membrane from eggs

The outer calcified shell of the plain eggs was removed chemically by placing the eggs in to 2 ml concentrated HCL for overnight, which caused complete decalcification. Further, the membranes were washed with distilled water three times to remove the extraneous matter and then the hole was made carefully with a sharp pointer top and the contents of the eggs were squeezed out completely. The membranes were again washed thoroughly with distilled water and placed it in ammonia solution, in the moistened condition for a while and then rinsed it with distilled water. The obtained egg membranes were stored in refrigerator at a pH of 7 to 7.4 till the further experimentation **[5]**.

Estimation of CaOx by titrimetric method (hema)

The studies were carried out in five groups as per experimental design.

Table 1: Experimental design

Group 1	**Negative control (10 mg calcium oxalate)**
Group 2	**10 mg calcium oxalate + 0.5 ml Cystone®Syrup (Himalaya Drugs Company. Ltd.)**
Group 3	**10 mg calcium oxalate + 0.5 ml Pet ether extract of *Echinops echinatus Roxb*.**
Group 4	**10 mg calcium oxalate + 0.5 ml Chloroform of *Echinops echinatus Roxb*.**
Group 5	**10 mg calcium oxalate + 0.5 ml Methanolic of *Echinops echinatus Roxb*.**
Group 6	**10 mg calcium oxalate + 0.5 ml Aqueous extract of *Echinops echinatus Roxb*.**
Group 7	**10 mg calcium oxalate + 0.5 ml Aqueous extract of *Passiflora edulis Sims*.**

All the above groups were packed in semi permeable membrane by suturing. They were suspended in a conical flask containing 100ml of 0.1 M TRIS buffer. The conical flasks of all groups were placed in an incubator, pre heated to 37^{o} c for 2 hours. The contents of semi permeable membrane from each group were taken into test tubes. To this 2ml of 1N sulphuric acid was added to each test tube and titrated with 0.9494 N $KMnO_4$ till a light pink colour end point obtained. Consequently, each ml of 0.9494 N $KMnO_4$was equivalent to 0.1898 mg of calcium. Percentage dissolution of calcium oxalate in various groups was calculated by the formula **[6]**.

% Dissolved Calcium= [(C-T)/C] X 100

Where,

C = precipitate of CaOx remained in control (mg) and

T = precipitate of CaOx remained when test solution was used (mg).

Inhibition activity of Commercial samples against calcium oxalate (CaOx) crystals by aggregation assay

The aggregation assay was performed following a previously described method with slight modifications **[6]**. In addition, the rates of inhibition of CaOx aggregation by the extracts were compared with those of the standard drug, Cystone®. CaOx crystal solution was prepared by using 10mM calcium chloride dihydrate and 1.0mM sodium oxalate containing 200 mM NaCl and 10 mM sodium acetate trihydrate. All tests were conducted at 37°C at 5.7 pH. For crystallization of CaOx, 25 ml of CaOx solution was transferred to a beaker and stirred on a hot plate using a magnetic stirrer. Then, 0.5 ml of sample i.e Cystone® Syrup, Pet ether extract, Chloroform extract, Methanolic extract , Aqueous extract of leaves of *Echinops echinatus Roxb*.& juice extract of *Passiflora edulis* Sims. The addition of 25 ml of sodium oxalate solution

immediately caused the solution to become turbid. The turbidity formed was measured in terms of absorbance at 620 nm using UV-Vis spectrophotometer continuously for 10 min after the mixing of the chemicals. In fact, the turbidity of the solution increased indicating nucleation and then decreased after some time, which indicates aggregation. This experiment was performed in triplicate. The percentage inhibition rate of CaOx aggregation was calculated as follows: **[7, 8]**

"Inhibition % = [1- (Si/Sc)] x 100"

Where,

Sc= slope of aggregation without inhibitor (negative control) and

Si= slope of aggregation in the presence of inhibitor (Commercial sample)

Statistical analysis:

All the experiments were conducted in triplicate and the data are presented as mean values and standard deviation. One-way ANOVA was applied on data using IBM SPSS Statistics software (Version 20.0, USA) and the level of significance was kept at $p< 0.05$.

RESULTS

It is observed that highest calcium oxalate dissolution was observed in Cystone® group and lowest was recorded in Pet ether extract of Leaves of *Echinops echinatus* Roxb. Group showed minimum dissolution of calcium oxalate stones (30.12. ± 1.20 %) compared to all the other groups tested and control (20.65 ± 0.26%). The standard drug, Cystone® (60.74 ± 1.63%) showed effectively reduced the amount of calcium oxalate stones. Along with cystone Methanolic extract of leaves of *Echinops echinatus* Roxb. Group (54.82 ± 1.48) & was fruit extract of *Passiflora edulis* Sims.(52.98 ±1.39) showed effectively reduced the amount of calcium oxalate crystals. Percentage of dissolution of CaOx crystals by Negative Control and the different plant extracts of *echinatus* and fruit extract of *Passiflora edulis sims*. was listed in **Table 1**.

In the calcium oxalate aggregation inhibition study, it was observed that highest calcium oxalate inhibition was observed in Cystone® group and lowest was recorded in Pet ether extract Group. The, Methanolic extract of leaves of *Echinops echinatus* Roxb showed minimum inhibition of calcium oxalate aggregates (54.82 ± 1.48) & fruit extract of *Passiflora edulis* Sims. (52.98 ±1.39) compared to all the other groups tested and control (20.65 ± 0.26%). Percentage of inhibition of CaOx aggregation shown in **Table 2**.

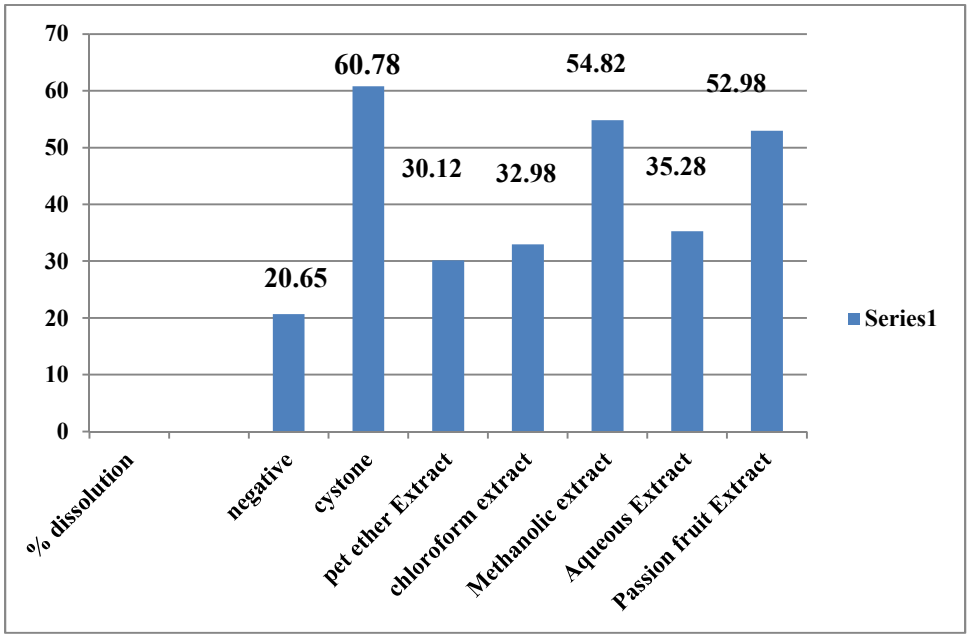


Figure 1: CaOx Dissolution activity of negative control and different extracts

Table 2: Percentage of inhibition of CaOx aggregation by Negative Control and Different extracts

Groups	% Inhibition	Standard Deviation	Inhibition percentage (%) (Mean ±Standard Deviation
Negative Control	25.13	1.19	25.13±1.19
Cystone Syrup	83.60	1.75	83.60±1.75
Pet ether Extract of *Echinops* e. leaves	32.26	1.22	32.26±1.22
Chloroform Extract of *Echinops* e. leaves	49.20	1.15	49.20±1.15.
Methanolic Extract of *Echinops* e. leaves	78.68	1.82	78.68±1.82
Aqueous Extract of *Echinops* e. leaves	52.20	1.12	52.20±1.12
Fruit Extract of Passion fruit	76.82	1.98	76.82±1.98

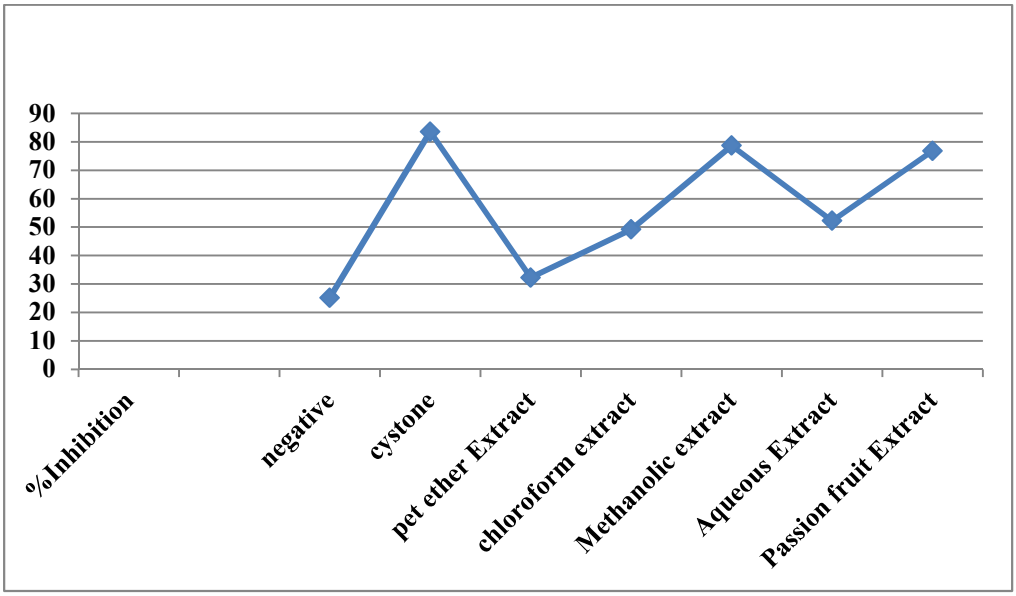
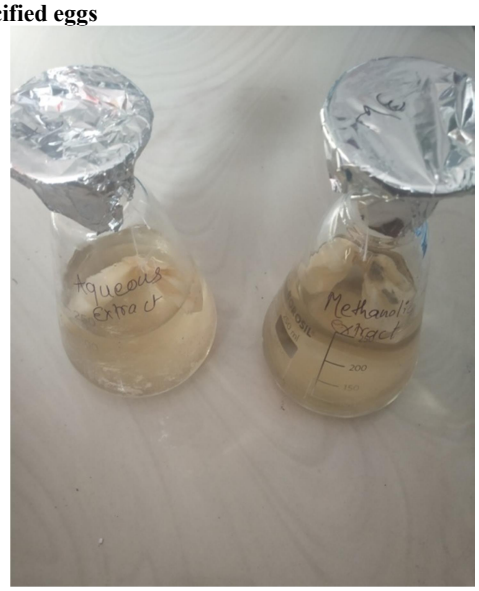
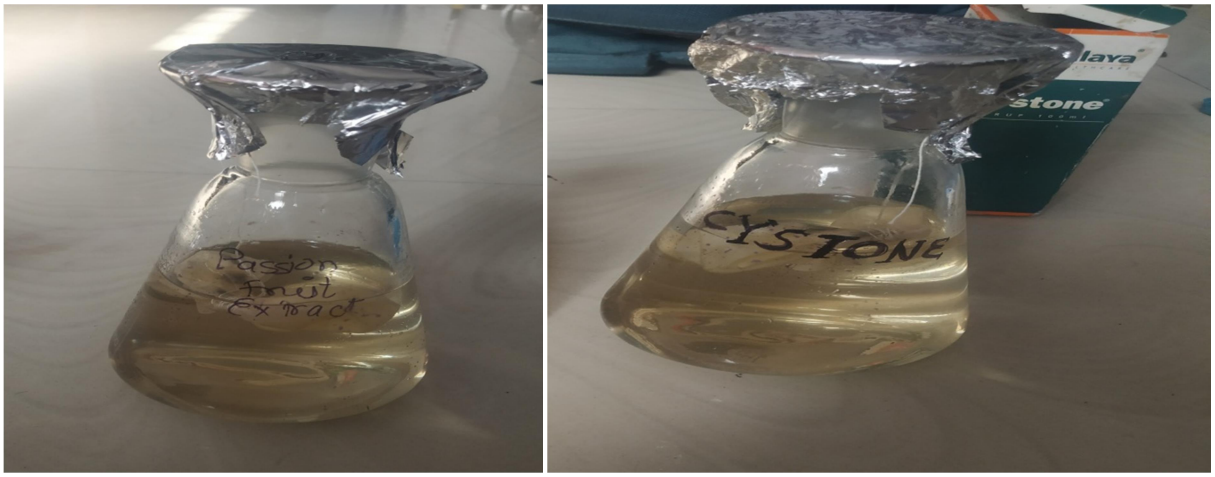


Figure 2: CaOx inhibition activity of negative control and Commercial samples

Figure 3: Eggs with HCl

Figure 4: Decalcified eggs





Set up for *In vitro* anti-urolithiatic assay

DICUSSION

Urinary stone disease remains to inhabit an important place in everyday urological practice. Despite of the available numerous treatment options available to management of kidney stones, use of medicines are the primary options to cure the kidney stones. The use of herbal remedies produced various pharmaceutical companies with claimed uses in the traditional systems of medicine assumes much importance. In India, in the Ayurvedic system of medicine, 'Pashanabheda' group plants, claimed to be useful in the treatment of urinary stones. 'Pashanabheda'. Herbal medicines have several phytoconstituent and exercise their beneficial effects on urolithiasis by multiple mechanisms like:

- Diuretic activity
- Increasing urine formation rate.
- Showing analgesic action.
- Balance the Inhibitor and promoter of the crystallization in urine and affects the crystal nucleation, aggregation and growth.
- Relieves the binding mucin of calculi (lithotriptic activity)
- Improved renal function
- No side effect
- Regulates the crystalloid colloid imbalance and improve renal function, thus prevents recurrence of urinary calculi.
- Antioxidant activity.
- Recurrrence rate is low

The primary objective of our study was to evaluate the efficacy other few selected polyherbal formulations in scientific way in selected in-vitro model. Cystone® which is a polyherbal formulation was chosen because it has proven its efficacy in burning micturition and acute urinary tract infection and been

used for long-term therapy (for four to six months and even longer) in urolithiasis and various other urinary disorders without significant side-effects **[9]**.

Exhaustive literature survey revealed that calcium oxalate inhibition aggregation and calcium oxalate dissolution in-vitro model are widely used models to study various herbal actives as well as the polyherbal formulations. In the calcium oxalate dissolution study, the highest dissolution of stone was observed with Cystone syrup (60.74 ± 1.63%) when compared with the negative control. The probable reason for getting such results may be the formulation which consists of various constituents in the product. In one of the scientific studies it was reported that the Cystone® (polyherbal formulation) maintains crystalloid-colloid balance by decreasing excretion of urinary calcium, oxalate, uric acid, phosphorus and protein in urolithiasis. The increasing practices and interest of herbal remedies for the treatment of urolithiasis is due to the limited choice in pharmacotherapy. Numerous medicinal plants have been evaluated continuously and reputed with positive results. However, the rationale behind their use is not well established except for few ones.In our study, The methanolic extract of leaves of *Echinops echinatus Roxb*.& juice extract of *Passiflora edulis* Sims. has also shown a positive effect as anti-urolithiatic agent. The different solvent extract of plants has also shown the ability to dissolve calcium oxalate stone compared to the control group **[10]**.

Herbal medicine may also refer to phytomedicine, phytotherapy, or paraherbalism, which are alternative and pseudoscientific practices of using unrefined plant or animal extracts as supposed medicines or health-promoting agents. In general, Herbal medicines are used for cure, mitigation, treatment and prevention of diseases especially those endemic to the local environment of the herbs. Plants have been the basis for medical treatments through much of human history, and such traditional medicine is still widely practiced today. Modern medicine makes use of many plant-derived compounds as the basis for evidence-based pharmaceutical drugs. Introduction of herbal medicines extracted from various medicinal plants improved health and quality of people's life.

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