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ORIGINAL ARTICLE

In-Vitro Calcium Oxalate Stone Reducing Potential of Selected Commercial Samples From Indian Market

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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 marketed preparations by using calcium oxalate crystals-titration method to know their actual efficacy. Four marketed products 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 (80.60 ± 1.75 %) and the same product had the most effective dissolution effect on CaOx crystals (56.07 ± 1.14 %).The other promising formulation UT-Star™ Syrup had also shown acceptable results with respect to inhibition (65.20 ± 1.22 %) as well as dissolution (52.47 ±1.14 %) of calcium oxalate crystals in in-vitro studies. Present study has given a fare idea about the efficacy of four marketed polyherbal liquid formulations which are used in the management of kidney stones.

Key words: *In vitro, Antiurolithiatic, Dissolution, Inhibition, Comparative evaluation, Kidney stones*

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INTRODUCTION

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. Calcium comprising uroliths are recognized as brushite, whewellite, weddellite, whitlockite and carbonate apatite. 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 [1,2]. 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 [3]. 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. Majority of the herbal actives preparations contains Combination of *Tribulus terrestris* (Gokhru), *Boerhavia diffusa* (Punarnava), *Aerva lanata* (Gorakshaganja), *Saxifraga ligulate* (Pashanabheda), cyperus rotundus (Mustaka), *Asparagus racemosus* (Shatavari), dolichos biflorus (Horse gram), *Vetriveria zizanioides* (Usher), *Curcuma zedoaria* (kachur), Saccharum Officinarum (Ikshumool), *Crataeva Nurvala* Bucj-Ham (varun), *Gentiana Kurroo Royle* (Trayamana), *Butea Frondosa* Koen (Palashpushp), *Dolichos Biflorus* Linn (Kulthi), *Cynodon Dactylon* Linn.Pers (Durva), *Solanum Nigrum* Linn (Makoi) and Potassium Nitrate [4]. Numerous dosage forms are available in Indian market such as tablets, syrups, extracts etc. 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 marketed preparations by using calcium oxalate crystals-titration method to know their actual efficacy.

MATERIAL AND METHODS

Materials

Cystone® Syrup, UTSTAR™ Syrup, Pathreena® Syrup and URI-Flush™liquid were 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. All other reagents used were of analytical grade and used with further dilutions for experiments

Preparation of calcium oxalate by homogenous precipitation [5]

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.

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.

Estimation of CaOx by titrimetric method

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 UTSTAR™Syrup (Novetra Lab Pvt Ltd)
Group 4	10 mg calcium oxalate + 0.5 ml Pathreena® Syrup (Baidyanath Pharmacy)
Group 5	10 mg calcium oxalate + 0.5 ml URIFLUSH™ Liquid (Elements India Ltd)

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.

% 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 each commercial sample i.e Cystone® Syrup, UT-Star™ Syrup, Pathreena® Syrup and URI-Flush™ Liquid was added. 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]

"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 URI-Flush™ Group. Group 5 (URI-Flush™) showed minimum dissolution of calcium oxalate stones (34.97 ± 1.20 %) compared to all the other groups tested and control (18.62 ± 0.26%). The standard drug, Cystone® (56.07 ± 1.63%) showed effectively reduced the amount of calcium oxalate stones. The efficacy of UT-Star™, Pathreena® Syrup and Uri-Flush™ Liquid shown they are less sufficient to dissolve calcium oxalate stone whereas Cystone® Syrup was very efficient. Our results have clearly indicated that the Cystone® Syrup from Himalaya Drug Company Ltd which contains the major ingredients as *Tribulus Terrestris, Boerhaavia Diffusa, Saxifraga Ligulate, Cyperus Rotundus, Asparagus Racemosus, Dolichos Biflorus Vetriveria Zizanioides* and Curcuma zedoariais quite good when compared with other market samples for anti-urolithiatic activity in this regard [8-10]. Percentage of dissolution of CaOx crystals by Negative Control and the Commercial samples was listed in table 2.

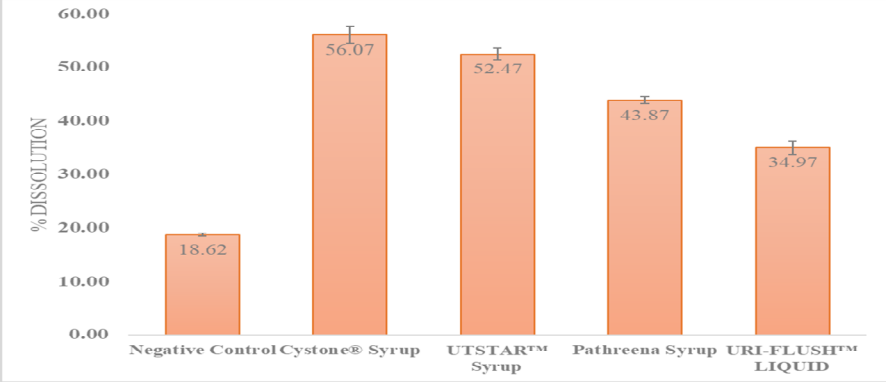

Fig. 1: CaOx Dissolution activity of negative control and Commercial samples

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 Pathreena® Syrup Group. The Pathreena® syrup marketed by Baidyanth Pharmacy, showed minimum inhibition of calcium oxalate aggregates (34.97 ± 2.15%) compared to all the other groups tested and control (24.13 ± 1.19%). The standard formulated considered by various doctors, Cystone® (880.60 ± 1.75%) showed effectively reduced the amount of calcium oxalate aggregates. The efficacy of UT-Star™, Pathreena® Syrup and Uri-Flush™ Liquid shown they are less sufficient to inhibition of calcium oxalate aggregates, whereas Cystone® Syrup was very efficient. Percentage of inhibition of CaOx aggregation shown in table 3.

Table 2: Percentage of inhibition of CaOx aggregation by Negative Control and the Commercial samples

Groups	% Inhibition	Standard Deviation	Inhibition percentage (%) (Mean ±Standard Deviation
Negative Control	24.13	1.19	24.13 ± 1.19
Cystone® Syrup	80.60	1.75	80.60 ± 1.75
UT-Star™ Syrup	65.20	1.22	65.20 ± 1.22
Pathreena® Syrup	34.97	2.15	34.97 ± 2.15
URI-Flush™ Liquid	43.50	0.87	43.50 ± 0.87

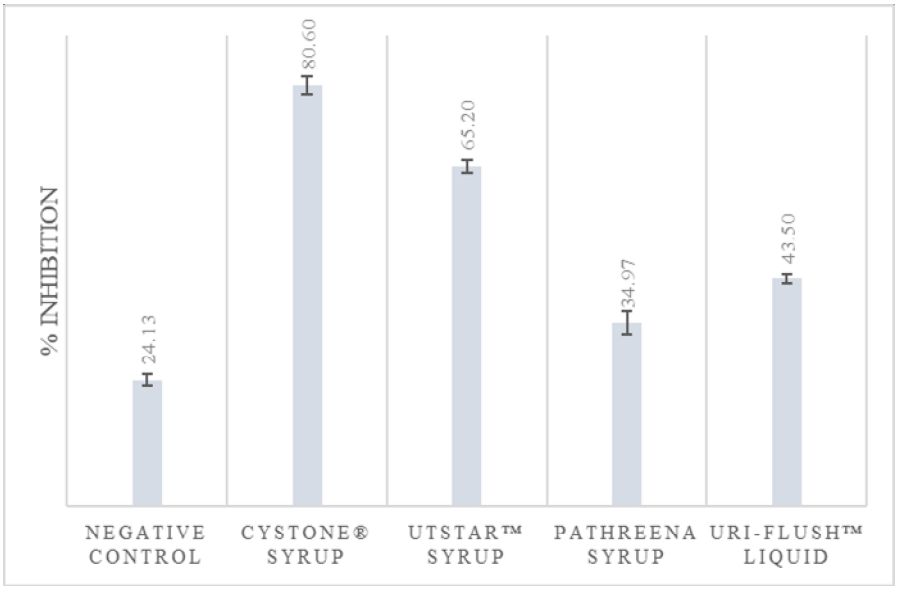

Fig. 2: CaOx inhibition activity of negative control and Commercial samples

DISCUSSION

Urinary stone disease remains to inhabit an important place in everyday urological practice. The average life time risk of stone formation has been reported in the range of 5-10 %.Calcium- containing stones, especially calcium oxalate monohydrate, calcium oxalate dihydrate and basic calcium phosphate are the most commonly occurring ones to an extent of 75-90% followed by magnesium ammonium phosphate (Struvite) to an extent of 10- 15%, uric acid 3-10% and cystine 0.5-1%.In majority of the cases the commonly occurring stones are calcium oxalate or magnesium ammonium phosphate type. 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:

- Helps in spontaneous passage of calculi by increasing urine volume, pH and anti-calcifying activity (Diuretic activity)
- Balance the Inhibitor and promoter of the crystallization in urine and affects the crystal nucleation, aggregation and growth (Crystallization inhibitioninhibition activity)
- Relieves the binding mucin of calculi (lithotriptic activity)
- Improved renal function
- Regulation of oxalate metabolism

- Regulates the crystalloid colloid imbalance and improve renal function, thus prevents recurrence of urinary calculi.
- Improve renal tissue antioxidant status and cell membrane integrity and prevent reoccurrence (Antioxidant activity).

Various marketed compositions of herbal formulations, Cystone® (Himalaya Drug Company, India), Pathreena® (Baidyanath Pharmacy, India) UT-Star™ (Novetra Laboratories Pvt Ltd Mumbai and Uri-Flush™ Liquid (Elements India Pvt Ltd) have been widely used clinically to dissolve urinary calculi in the kidney and urinary bladder. These products are liquid in nature and suggested to take with dilution with water for the treatment. The Cystone® tablets and Syrup has long history of efficacy and has documented evidences of scientific usage [11]. 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 [12].

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 (56.07 ± 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. The Cystone® syrup contains *Tribulus terrestris, Boerhaavia diffusa, Saxifraga ligulate, Cyperus rotundus, Asparagus racemosus, Dolichos biflorus, Vetriveria zizanioides* and *Curcuma zedoaria* in a syrup base. The composition reported to relieve the conditions by relaxing the detrusor muscles and promotes diuretics by virtue of its high content of natural mineral salts. *Boerhaavia diffusa* (varuna) and T*ribulus terrestris (*Ghokhru) were found to be effective in preventing the deposition of the stones in experimental rates. 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 [12].

In urolithiasis, the Glomerular Filtration Rate (GFR) declines due to the obstruction to the outflow of urine by stones in urinary system, due to these the nitrogenous waste productssuch as urea, creatinine and uric acid are accumulated in blood. So, another function to do these polyherbal formulations is to improve the GFR. The polyherbal formulations by improving this GFR ultimately improves the renal function by increasing the excretion of urea and creatinine.

Hyperoxaluria is a most significant risk factor in the pathogenesis of renal stone. In numerous scientific studies it has been reported that oxalate play very important role in stone formation and has about 15-fold greater effect than urinary calcium. Increased oxalate concentration is responsible for precipitation and deposition of CaOx crystals. *Tribulus terrestris* interfere with the metabolism of oxalate and removes from the human body.

The other groups, UT-Star™ has shown % dissolution 52.47 ± 1.14. The product is composed of promising newer ingredients which consist of Ikshumool, Palashpushp, Durva, Shevat Parpati Kalshimora, Makoi along with traditional ingredients such as *Boerhavia Diffusa*, Gokhru, varun and pashanbhed. The product shown equivalent results in dissolving the calcium oxalate stone in-vitro when compared to Cystone® vs the negative control. The Ikshumool is one of the easily available medicine mentioned as diuretic in Ayurvedic literature. The sugarcane plant root is reported to have various properties such as sooths and protects the urinary tract and relieves the inflammation. It also promotes the urine flow and has a cooling effect. The promising results of this formulation need further evaluation on in-vivo model.

The other two groups, Pathereena® and URI-Flush™ has showed 43.87 ± 0.65% and 34.97 ± 1.20% dissolution of calcium oxalate stones. The probable reason for these lower results might be few formulation aspects such as absorption of ingredients, stability of ingredients in syrup formulation or the lower concentrations of major required ingredients to show the anti-uroliathic activity.

Due to the high cost and adverse effects of minimally invasive techniques, and recurrence alternative treatment modalities with phytotherapeutic agents have become the mainstay of medical therapy.

Figure 2and table 2 show that the Cystone® group show the highest percentage inhibition of CaOx at 80.60 ± 1.75%. This is due to the presence of various bioactive compounds including saponins, tannins, terpenoids and steroids in the herbal ingredients present in the formulations. Similar types of phytochemicals were found in the UT-STAR™, Pathreena® and URI-Flush™ liquid but the in quantitative less amount. The difference in inhibition percentage value between Cystone® syrup and UT-Star™ syrup might be influenced by the amount of terpenoids and steroids present in the ingredients and also presence of newer ingredients whose efficacy is not yet studied. Based on another study, the presence of

terpenoids was proven to be useful in the inhibition activity against kidney stone crystals. The beneficial actions of polyherbal formulation (Cystone®) is due to the complex spectrum of actions with anti-inflammatory, antimicrobial, diuretic, antispasmodic, litholytic, and anti-calcifying activities of its ingredients. D. pedicellate-diuretic activity, S. lingulate-astringent properties also as antimicrobial agent, R. cordifolia- anti-inflammatory activity as well as dose dependent increase in urine volume and electrolyte excretion, C. scariosus-spasmolytic action and A. aspera on inhibition ofmineralization of urinary stone [13]. This multifactional ingredients resulted in highest inhibition of calcium oxalate stones. These pharmacological actions were confirmed by a previous study on the anti-urolithiatic activity of the polyherbal formulations which indicated that multifunctional formulations could be one of the best formulations that reduces and expels the kidney stones [14].
In comparison with Pathreena® and URI-Flush™ liquid showed lower inhibition percentage (34.97 ±2.15 %) and (43.50 ± 0.87 %), probably attributable to the less amount of alkaloids, terpenoids and steroids as compared to that in the other groups.

CONCLUSION

Present study has given a fare idea about the efficacy of four marketed polyherbal liquid formulations which are used in the management of kidney stones. Cystone® Syrup has showed effective calcium oxalate inhibition as well as dissolution studies. The other promising formulation UT-Star™ Syrup had also shown acceptable results with respect to inhibition as well as dissolution of calcium oxalate crystals in in-vitro studies. Additional in-vivo studies are needed on these formulations to get detailed information about efficacy in human volunteers.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

CaOx: Calacium Oxalate; **$KMnO_4$**:Potassium Permanganate; **GFR:** Glomerular Filtration Rate.

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