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Development and evaluation of anti-urolithiatic herbal formulation

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Abstract

According to the WHO herbal medicine are the medicinal products that contain an active herbal ingredient in aerial or underground parts of a plant. The use of herbal formulations now-a-days is increasing rapidly and due to this it was thought to make an anti-Urolithiatic herbal formulation with a reduced dosage and enhanced onset of action. Out of three herbal plants namely; *Lycopodium clavatum*, *Berberis Vulgaris* and *Smilax Officinalis* used for Urolithiasis, it was found by *in-vitro* screening that *Lycopodium clavatum* had excellent activity. To assess the pharmacokinetics various tests were performed namely, *in-vitro* dissolution study of calcium oxalate crystals, %inhibition of calcium oxalate crystals, whereas the cytotoxicity was tested by MTT Assay.

Keywords: *Lycopodium clavatum*, anti-Urolithiasis, MTT assay, *in-vitro* screening

Introduction

Urolithiasis is a disease in which there is occurrence of stone formation anywhere in the urinary system including kidneys and bladder [1]. According to a recently carried out survey, 19% of men and 9% of women of the total world population are afflicted by this disease [2]. The reason behind Urinary stone formation is mainly due to some of the urine content which turns in to crystals. And these content are often found to be salts of calcium and uric acid or sometime minerals [3]. The cause of Urolithiasis is still unknown but probably positive family history, overweight, obesity or increased body mass index (BMI) could lead to Urolithiasis [4]. Despite lack of awareness of the stone forming procedure precisely it is assumed that crystal nucleation, aggregation and growth of insoluble particles are mainly responsible for causing Urolithiasis [5]. The real culprit behind these processes is termed "Supersaturation". Supersaturation may be defined as the formation of crystals beyond its metastable limit. Which is caused by many factors like etiopathogenesis, genetic factor, Improper nutrition, socio-economic circumstances, environmental conditions, metabolic changes, anatomic and infections factors [6].

Pathophysiology of Urolithiasis

The mechanism of stone formation is found in sequence as nucleation followed by aggregation of crystals constituting stone [7]. The urine and, presumably, the tubular fluid of stone formers is often more highly supersaturated than that of normal healthy adults, which favors nucleation and the growth of crystals [8]. Supersaturation is expressed as the ratio of urinary calcium oxalate or calcium phosphate concentration to its solubility, which is the driving force in stone formation [9]. Nucleation is the process by which free ions in solution get associated into microscopic particles. Crystallization can occur in solution micro-environments, such as those that may be present in certain points in the nephron, as well as on surfaces, such as those of cells and on extracellular matrix [10]. Aggregation is a process by which there is agglomeration of crystals that appear in free solution to form larger multicomponent particles. It may also encompass the phenomenon of secondary nucleation of new crystals on the surface of those which are already formed [11]. Growth of microscopic crystals is accomplished by the movement of ions out of the solution onto growing crystals [12, 13]. The saturation can be raised by an increase in the concentration of the reactants. They are known as promoters which can increase crystallization of stone constituents or their growth by a number of mechanisms. It is observed that tubular fluid and urine of stone patients are highly supersaturated compare to normal healthy people [14].

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Table 1: Promoters and Inhibitors of stone formation

Promoters	Inhibitors
Promoters can increase crystallization of stone constituents or their growth by a number of mechanism.	Inhibitors are the substances generally considered to inhibit stone formation or the process of stone formation.
Urine acidic pH, Uric acid	Alkaline pH, Citrate, Pyrophosphate

To get rid of the calculi normally surgical operation, lithotripsy and impacting laser beam on local calculus are used to destroy it. However, a huge amount of risk is involved in applying these techniques such as renal injury, followed by a decrease in the renal function. Moreover, it may cause increase in stone recurrence in absence of preventive treatment. Thereby, creating a need of hour to find out an effective alternative medical therapy [9, 15].

Plant Profile

There is an overwhelming hype around the globe about the use of herbal medicine now-a-days. Hence, the Indian medicinal plants are constantly being evaluated for possible Anti-Urolithiatic effects. In this research work I have studied three plants; *Lycopodium clavatum*, *Smilax Officinalis*, and *Berberis Vulgaris*.

- *Berberis Vulgaris* contains a larger number of Phytochemical materials including Ascorbic acid, Vitamin K, several triterpenoids, more than 10 phenolic compounds and more than 30 alkaloids (Berberine, Oxyacanthine, Brolicin, Columbamine etc). Therefore, *B. vulgaris* may have anti-cancer, anti-inflammatory, anti-oxidant, anti-diabetic, anti-Urolithiatic and many more uses [16, 17].
- *Smilax Officinalis* was identified with the Phytoconstituent like Saponins, glycosides, b-sterols, triterpenes, flavonoids and many more. It has a sweet taste, cooling effect, demulcent activity and used as aphrodisiac, diuretic also. *S. Officinalis* is extensively used for syphilis, rheumatism, kidney disorder, liver diseases [18].
- *Lycopodium clavatum* contains fatty oil, carbohydrate sporonin, sucrose, alkaloids like lycopodium(lycopodine, clavatin, clavatoxine) *L. clavatum* was used to stimulate appetite, in treatment of flatulence, rheumatism, gout, diarrhea, kidney ailments [19, 20].

Materials and Method

Reagents Used: Methanol, Distilled Water, Plant Active, Cystone, Calcium Oxalate, Sodium Oxalate, Calcium Chloride, Sodium Chloride, Acetic Acid, Ammonia, 0.1N Hydrochloric Acid, Sodium Acetate Trihydrate, Tris HCl 1N H₂SO₄, 0.9494N KMnO₄, Egg shell, MCC, Capsule shell #3, MTT dye, HEK cell line, RPMI media, 96 well plate, micropipette, DMSO.

Instrument and Apparatus: UV-spectrophotometer (Shimadzu-1800), magnetic stirrer, micro-pipette, filter-paper, BOD Incubator, ELISA plate reader.

In-vitro Screening of Anti-Urolithiatic activity

The effects of Methanolic solution of *Lycopodium*, *Berberis* and sarsapogenin were analyzed on *in-vitro* homogeneous system of Calcium oxalate crystal by applying 3 methods:

1. Nucleation and aggregation assay
2. *In vitro* calcium oxalate crystal growth assay
3. Evaluation of Anti-Urolithiatic activity using egg shell membrane.

Nucleation and Aggregation assay [21]

Freshly prepared solution of 10 mM calcium chloride dihydrate and 1.0 mM sodium oxalate, containing 200 mM NaCl and 10 mM sodium acetate trihydrate was prepared and the pH was adjusted to 5.7.

25 ml of sodium oxalate solution was then transferred into a beaker and placed in a hot plate magnetic stirrer, which was maintained at 37 °C and constantly stirred at 800 rpm.

Now, 1 ml of distilled water (control) / cystone (standard) / methanolic solution of drug were added and finally calcium chloride solution (25 ml) was added.

The optical density was measured at 620 nm in spectrophotometer after the addition of calcium containing solution, first after 20 seconds and then every 2 minutes over a time period of 8 minutes.

The percent inhibition in the presence of cystone or Methanolic solution was compared with the control by using the following formula.

$$\% \text{ inhibition} = 1 - \left[\frac{T_s}{T_c} \right] \times 100$$

Where, Ts = turbidity of sample, Tc= turbidity of control.

In vitro calcium oxalate crystal growth assay [22, 23]

Inhibitory activity of the solution containing these drugs was also checked on calcium oxalate crystal growth. In this assay, 1mL each of 4mM Calcium chloride and 4mM Sodium Oxalate were added to 1.5mL of Solution, containing NaCl (90mM) buffered with Tris-HCl (10mM) and adjusting the pH to 7.2.

In the above mentioned mixture 30µL of Calcium Oxalate crystal slurry (1.5mg/mL acetate buffer) was added. The consumption of oxalate begins immediately after addition of the slurry and this was monitored just like in the nucleation method and absorbance was measured at 214nm. When the drug sample is added to the solution, depletion of free oxalate ions will decrease as drug sample inhibit Calcium Oxalate crystal growth.

This inhibitory activity is calculated by following equation:

$$\% \text{ Relative Inhibitory Activity} = \left(\frac{C - S}{C} \times 100 \right)$$

Evaluation of Anti-Urolithiatic activity using egg shell membrane: 24

Preparation of semipermeable membrane:

First of all, broken eggshells are to be placed in 10% v/v acetic acid overnight for complete decalcification. Further wash with distilled water and treat with ammonia to neutralize the acidic effect. Afterwards, again rinse with distilled water and maintain the pH value between 7 - 7.5.

Estimation of calcium oxalate by titrimetry

1mg of calcium oxalate and 10mg of Drug extract (test) / cystone (standard) is packed together in semipermeable membrane of eggshell which is already prepared. This is kept suspending in a conical flask filled with 100mL of 0.1 M of tris-buffer. Here 3 differently treated groups in separate flasks are represented in the table below.

Table 2: Differently treated groups

1 st group	Blank	1mg calcium oxalate
2 nd group	Standard	1mg calcium oxalate+10mg cystone
3 rd group	Test	1mg calcium oxalate+10mg lycopodium

The above mentioned flasks were then kept in a preheated incubator at 37 °C for 2 hours. Finally removing the content from the semipermeable membranes of each group they are taken in 3 separate test-tubes. Adding 2ml 1N H₂SO₄ this is titrated with 0.9494N KMnO₄ till light pink color appears as the end point.

Factor: Each 1mL of 0.9494 N KMnO₄ is equivalent to 0.1898mg of Calcium.

MTT Assay: [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] ^[25, 26]

Cytotoxicity assays are routinely used to calculate the effectiveness of a chemotherapeutic drug against cancer cells. MTT assay is used to evaluate the cytotoxicity.

Procedure

Split the cells and determine the optimal count for seeding in 96 well plate. Seed optimum number of cells in 100µl media (5000-10000cells/well) and incubate the plate for 18-24hrs in

5% CO₂ incubator at 37 °C. Treat the cells in triplicate when they reach 40-50% confluency. Use appropriate positive and negative controls. Incubate the plates at 37 °C for 24, 48 and 72hrs depending upon the desired time point for your experiment. After the desired time point, add 10 µl of MTT to each well, the concentration of MTT is 5mg/ml PBS. Incubate for 2-3 hours at 37°C until the purple colored Formosan crystals are formed. Discard the media gently without disturbing the crystals and add 100 µl of DMSO per well to solubilize the crystals. Incubate this on a shaker in the dark for 10minutes and take the reading of absorbance between 540-595nm.

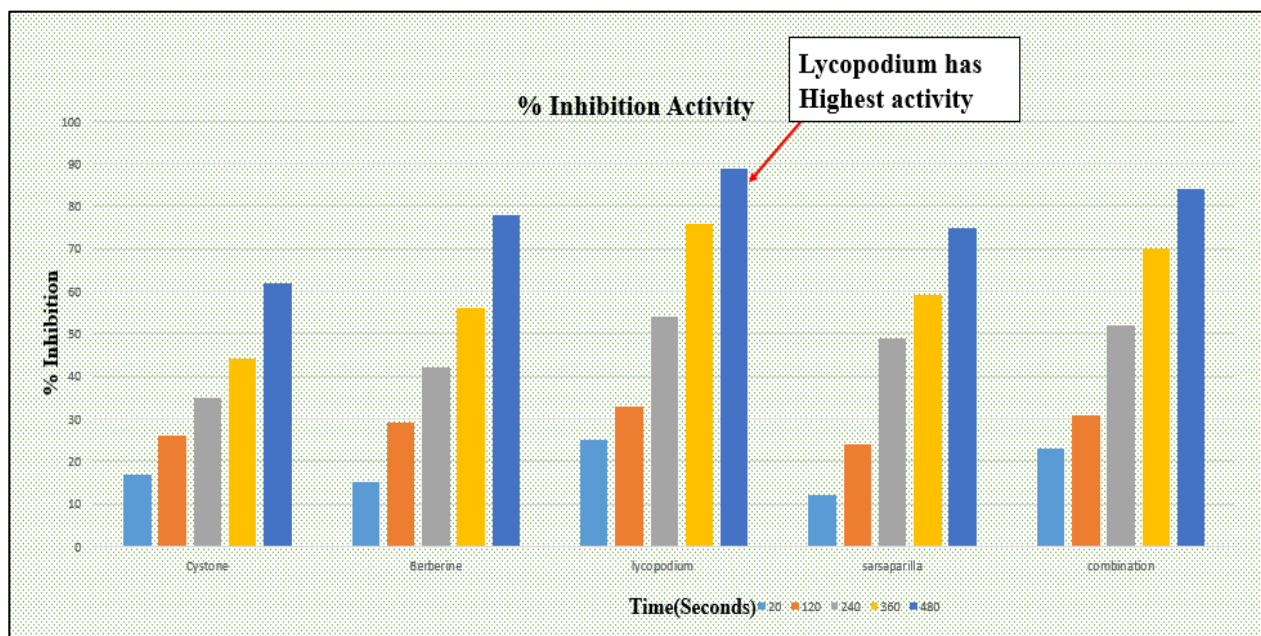
Results and Discussion

By using all of the above *In-vitro* determination methods for the treatment of Urolithiasis we found some important data which is presented in the following section.

Nucleation and Aggregation assay

Table 3: % Inhibition of differently treated groups

% Inhibition						
Sr. No.	Time (sec.)	Cystone	Berberine (3ppm)	Lycopodium (3ppm)	Sarsaparilla (3ppm)	Combination (3ppm)
1	20	17	15	25	12	23
2	120	26	29	33	24	31
3	240	35	42	54	49	52
4	360	44	56	76	59	70
5	480	62	78	89	75	84

**Fig 1:** Graphical representation of above % inhibition

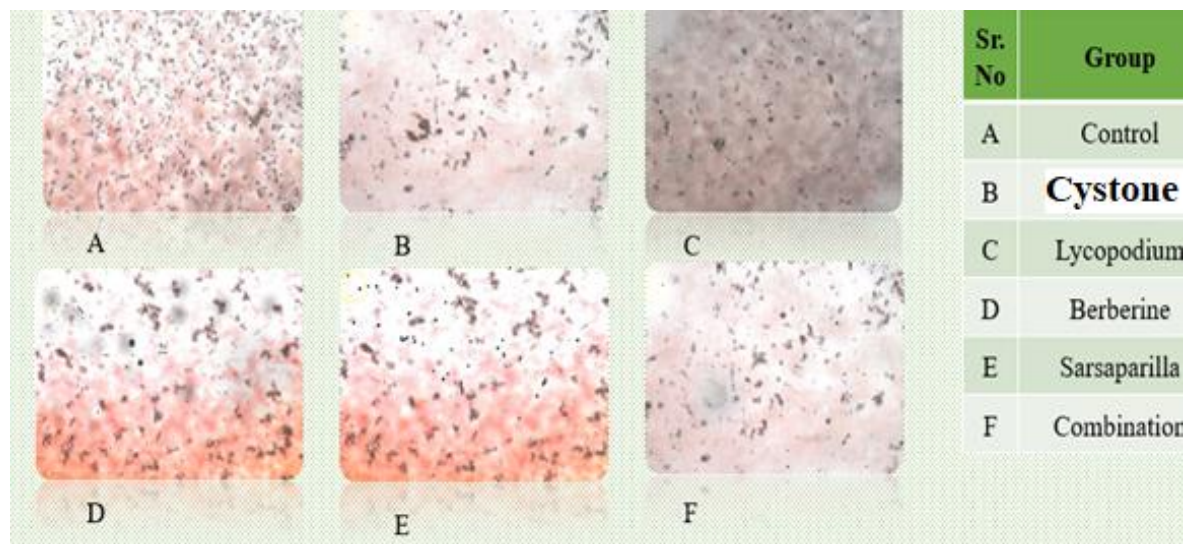


Fig 2: Microscopic view of crystal inhibition

***In vitro* calcium oxalate crystal growth assay**

Table 4: % Relative Inhibition of differently treated groups

% Relative Inhibitory Activity						
Sr. No.	Time (sec.)	Cystone	Berberine (3ppm)	Lycopodium (3ppm)	Sarsaparilla (3ppm)	Combination (3ppm)
1	20	35	30	58	38	54
2	120	42	38	62	44	61
3	240	52	54	73	53	70
4	360	65	76	85	67	82
5	480	77	89	97	76	90

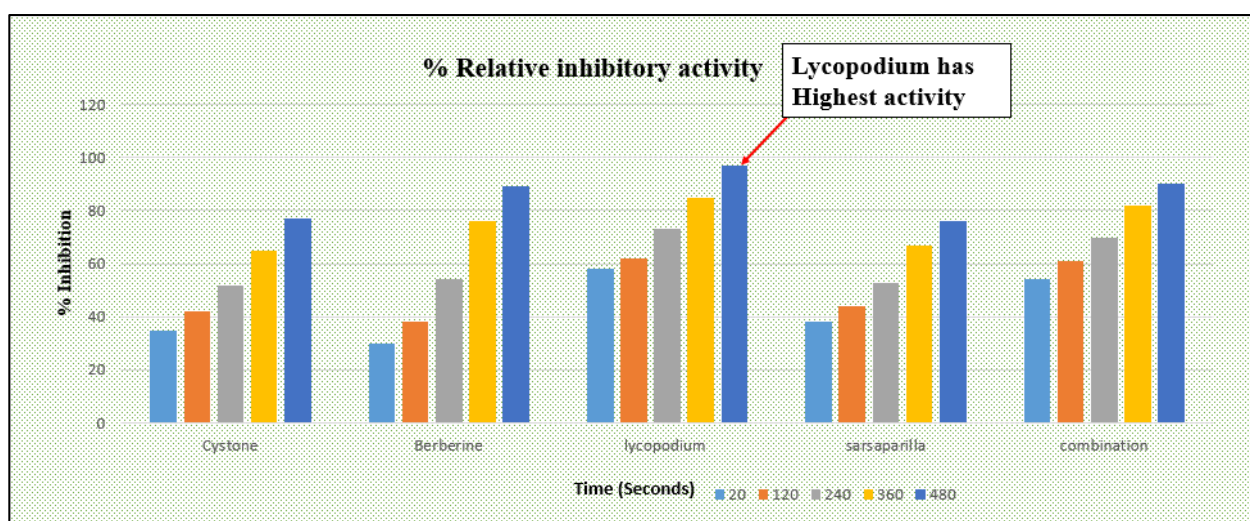


Fig 3: Graphical representation of above % Relative inhibition

Evaluation of Anti-Urolithiatic activity using egg shell membrane:

Table 5: Titrimetric Results of Different Drug Group

Sr. No.	Group	% Dissolution
1.	Blank	0%
2.	Cystone (Standard)	56%
3.	Lycopodium (Test)	89.94%
4.	Berberine (Test)	80.43%
5.	Sarsaparilla (Test)	60.87%



Fig 4: Titration Setup

MTT ASSAY Results

Table 6: Cell Viability Results

Sr. No.	Name	Average cell counting	Cell Viability
1	Control	0.335	100%
2	Berberine	0.307	91.64%
3	Sarsaparilla	0.310	94.62%
4	Lycopodium	0.325	97.01%
5	Combination	0.204	60.89%

Capsule Formulation

Keeping all above mentioned results in view it is quite apparent that *Lycopodium clavatum* is the most impactful herbal drug on Urolithiasis. Hence, we chose to formulate the same in capsule form. As in the subject matter it is found more economical, thus making it affordable for common people. We took 3mg Lycopodium as active ingredient and 100mg MCC as a diluent, this was then mixed using geometric mixing procedure. The final mixture is filled in to the capsules ensuring each capsule contains 300µg of Lycopodium. Now it comes to the evaluation stage of prepared capsule. Further evaluation of this capsule was done by weight variation and *in vitro* dissolution study and results of both the parameter are within the limits. In this we have also performed *in-vitro* dissolution study for the final formulation where we have kept a capsule in 0.1N HCl solution for 2hrs and further transferred in a calcium oxalate crystal solution. And it shows that with acid our drug doesn't degrade but it will give better effect, which was shown in Figure 5.

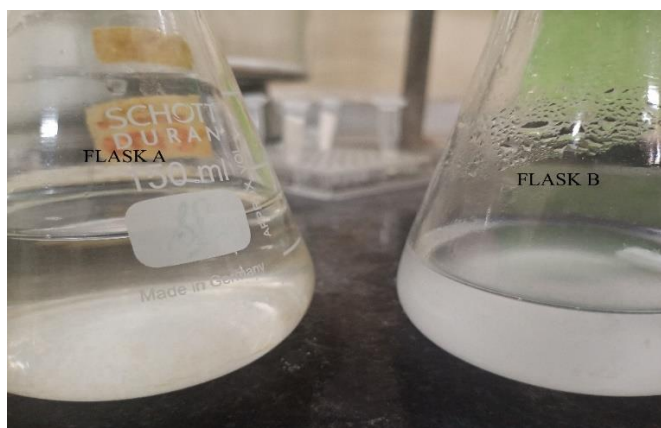


Fig 5: Effect of final formulation in calcium oxalate crystal growth study

Flask A: Treated with 0.1N HCl for 2hrs and further calcium Oxalate crystal solution

Flask B: Directly with Calcium Oxalate crystal Solution

Conclusion

The study has given primary evidence for *Lycopodium clavatum* as the plant which possesses anti-Urolithiatic property. Here, *In-vitro* Urolithiasis has been performed on selected plants *Lycopodium clavatum*, Berberine and Sarsapogenin by using the Cystone as the standard drug. Amongst all *Lycopodium clavatum* has the highest activity with lowest dosage. The work has performed by *in vitro* anti-urolithiatic model for calculating percentage dissolution of kidney stone, percentage inhibition of crystal growth. Hence, final formulation is made in form of capsule as it is easily available for common public.

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