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Research Article

EVALUATION OF INVITRO ANTI-UROLITHIASIS ACTIVITY OF *CONVOLVULUS ARVENSIS*

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ABSTRACT

Objective: The present study was carried out to evaluate the invitro anti-urolithiasis activity of leaves and flower infusions of Convolvulus arvensis.

Materials and methods: The inhibition of in-vitro calcium-oxalate crystal (a major component of most urinary stones) formation by various extracts was investigated by different methods. Synthetic urine supersaturated with calcium oxide was prepared and urolithiasis was investigated by inhibition assay, aggregation assay, and sedimentary crystal formation. Crystal formation in synthetic urine was studied at different time intervals using leaf and flower infusions at different concentrations 10, 25, 50, 75,100 mg/ml each respectively.

Results: Among the two extracts when compared to control group, the inhibitory potency of leaf extract was found to be more significant ($P<0.05$), than the flower extract.

Keywords: Calcium oxalate, Urolithiasis, Convolvulus arvensis, *In vitro*.

INTRODUCTION

Urolithiasis (from Greek *oûron*, "urine" and *lithos*, "stone") is the condition where urinary calculi are formed or located anywhere in the urinary system, or the process of formation of stones in the kidney, bladder, and/or ureters (urinary tract).Kidney stones are a common cause of blood in the urine and pain in the abdomen, flank, or groin. Kidney stones occur in one in 20 people at some time in their lives [1]. Urinary composition determines stone formation based on three factors: exceeding the formation product of stone forming components, the quantity of inhibitors (e.g., citrate, glycosaminoglycans, etc.) and promoters (e.g., sodium, urates, etc.) in the urine [2]. The stones form in the urine-collecting area (the pelvis) of the kidney and may range in size from tiny to stag horn stones to the size of the renal pelvis itself [1,3]. Kidney stone formation or urolithiasis is a complex process that results from a succession of several physicochemical events including supersaturation, nucleation, growth, aggregation, and retention within the kidneys[4]. Since it is one of the major problem that is disturbing the life style of young population herbal extract formulation had their significance in the therapy. Unlike allopathic medicines which targets only one aspect of Urolithiasis Pathophysiology, most plant based therapy have been shown to be effective at different stages of stone pathophysiology[5]. So the medicinal plant *Convolvulus arvensis* is selected for the basic study. *C. arvensis* (Field Bindweed) is a species of bindweed or morning glory family (Convolvulaceae), native to Europe and Asia. *C. arvensis* has many therapeutic benefits such as its use in tribal area as the root, is cholagogue, diuretic, laxative and strongly purgative [6]. The juice of the root is used in the treatment of fevers. Phytochemical studies on the aerial parts of this plant showed the presence of various compounds such as saponins, terpinoids, steroids, tropane alkaloids (Pseudotropine, tropine, tropinone, meso-cuscohygrine, Hygrine, calystegine and atropine), flavonoids (Kaempferol, Quercetin and rutin), phenolic acids and different quantities of essential elements. [7]. A cold tea made from the leaves is laxative and is also used as a wash for spider bites or taken internally to reduce excessive menstrual flow. However, no scientific data are available to establish the anti-urolithiatic property of *C. arvensis* Linn. In the present study, an effort has been made to establish the scientific validity of the antiurolithiatic activity of *C. arvensis* flower and leaf extract in-invitro model.

MATERIALS AND METHODS

Plant Collection and Identification

The leaves and flowers of *C. arvensis* were collected from road side area of Nellore, , Andhra Pradesh, India, during the month of January and plant was identified with the help of regional Floras [6] and taxonomists and finally confirmed with the herbarium.

Preparation of the infusion

The collected plant materials of *C. arvensis* was washed thoroughly in water ,cut into small parts and dried for two week at 35-40 ºC temp. infusion were prepared by dissolving the plant material in the boiled water for 15 min just prior to the conducting of experiments.

Synthetic urine

We chose the classical model for the study of oxalate crystallisation because of its simplicity and reproducibility. Synthetic urine supersaturated with calcium oxide was prepared according to a previously described method [8] at a constant temperature of 37°C in capped vessels. It was prepared by dissolving all the composition described by [9] Chemicals of reagent-grade purity were dissolved in deionised and redistilled water. The artificial urine was prepared immediately before use by mixing in a T-type mixing chamber. For determination of the effects of plant extracts on crystal formation, preparation of the synthetic urine was performed in their presence at various different concentrations

Preparation of reagents and solution

All the chemicals used were of AR grade. Crystalloid forming solutions, *viz*., solution of calcium acetate and sodium oxalate (for calcium oxalate) were prepared in distilled water.

I. Inhibition assay

Antilithic activity in different extracts of leaves and flowers of *C.arvensis* was investigated as per the method of N. A. M. farrook et.al.,[10]. with minor modifications. The whole amount of extract solutions (50 mL) was placed in the beaker in the beginning itself and the two salt forming solutions were allowed to run into it drop wise through burettes. Thus, a reservoir of extract solutions was created into which the salt forming solutions ran down. At the end the mixture was boiled on a heating mantle (Elite scientific instruments co.) for 10 min., cooled to room temperature and the precipitate was collected into a pre-weighed centrifuge tube by centrifuging (Remi equipments, Bombay) small volumes at a time and rejecting the supernatant liquid. Next, the tube with the precipitate was dried in a hot air oven, cooled to room temperature and weighed till constant weight using a weighing balance. Weight of the precipitate was determined. Simultaneous blank experiments with water in place of extracts were also carried out for evaluating the inhibition efficiency of inhibitors compared to water. All the experiments were conducted at room temperature. Data were expressed as mean values of three independent experiments as Mean ±STDEV. Percentage efficiency of both leaf and flower extracts were calculated using the following formula.

Percentage = Wt of precipitate in blank set- wt of precipitate in experimental set ×100 Inhibition / Wt of precipitate in blank set

II. Kinetic study

The effect of the test material on kinetics of calcium oxalate (CaOx) crystallization was determined by the time course measurement of turbidity changes due to the crystal nucleation and aggregation after mixing meta stable solutions of calcium (Ca++) and oxalate (Ox). Stock solutions of CaCl2 (8.5 mM) and $Na_2C_2O_4$ (1.5 mM), containing 200 mM NaCl and 10 mM sodium acetate were adjusted to pH 5.7 [13]. An aggregometer devised for platelet aggregation studies based on the measurement of optical density at 620 nm was used to investigate the event of CaOx crystallization [14]. The slopes of nucleation (SN) and aggregation phases (SA) were calculated using linear regression analysis. Using the slopes, the percentage inhibition was calculated as [(1-Sm/Sc) × 100], where Sm is slope in the presence of modifier; K.Cit or Ov.Cr, and Sc is slope of the control experiment. Evaluation of CaOx crystallization in vitro The classical model for the study of oxalate crystallization was chosen because of its simplicity and satisfactory reproducibility. According method reported by Atmani and Khan [11] which involves crystallization without inhibitors and with it, in order to assess the inhibiting capacity of test material used was suitably modified for the study.

III. Nucleation assay

Solution of calcium chloride (5 mmol/l) and sodium oxalate (7.5 mmol/l) were prepared in a buffer containing Tris–HCl 0.05 mol/l and NaCl 0.15 mol/l at pH 6.5. Nine milliliter of calcium chloride solution was mixed with 1 ml of herb extracts at different concentrations (10, 25, 50, 75, and 100 mg/ml). Crystallization was started by adding 950 ml of sodium oxalate solution. The temperature was maintained at 37^{0}C. The OD of the solution was monitored at 620 nm after 30 min. The rate of nucleation was estimated by comparing the induction time in the presence of SXS with that of control [12]. The growth of crystals was expected due to the following reaction:

$$CaCl_2 + Na_2C_2O_4 \longrightarrow CaC_2O_4 + 2NaCl$$

IV. Aggregation assay

The method used for aggregation described by [11,12] was modified. 'Seed' calcium oxalate (CaOx) crystals were prepared by mixing calcium chloride and sodium oxalate at 50 mmol/l. Both solutions were equilibrated to 60C in a water bath for 1 h and then cooled to 37^{0}C overnight. The crystals were harvested by centrifugation and then evaporated at 37^{0}C. CaOx crystals were used at a final concentration of 0.8 mg/ml, buffered with Tris–HCl 0.05mol/l and NaCl 0.15 mol/l at pH 6.5. Experiments were conducted at 37^{0}C in the absence or presence of the plant extract. The percentage aggregation inhibition was calculated by comparing the turbidity in the presence of SXS at different concentrations of both leaf and flower infusions (10–100 mg/ml) with that obtained in the control using following formula:

$$\% \text{ inhibition} = \frac{\text{1-Turbidity sample} \times 100}{\text{Turbidity control}}$$

V. Simulation of the sedimentary crystal formation

Mixture agitation was maintained to prevent sedimentation. The crystal size development was monitored by polarized microscopy at different time intervals. Sample drops were examined at every five minutes by polarising optical microscopy. Crystals were identified using a microscope of the Zeiss type with 40 x magnifying lens, equipped with a WINDER M 476079 camera[15].

RESULTS

Effect of inhibition assay

Most of the crystals measured in this study were calcium oxalate (dihydrate variety) since 90% of monohydrate variety were formed only after 48 hours (Grases et.al.,). Both the infusions of Convolvulus arvensis has signifcantly reduced the size of calcium oxalate crystals (dihydrate variety) (P<0.05). The higher the concentrations of extract the more will be the size reduction.

Table 1: Effect of leaf and flower infusions of *Convolvulus arvensis* on CaO crystals

Concentration mg/ml	10	25	50	75	100
Leaf extract	32.83±0.83[a]	43.63±1.16[a]	46.33±1.38[a]	55.33±0.88[a]	82±2.38[a]
Flower extract	25.50±0.09[a]	35.17±0.87[a]	44.17±0.47[a]	50.17.83±0.60[a]	68.17±0.94[a]

Values are expressed as mean±sem n=6, where, [a] statistical significance P<0.05

Kinetic study

The mean induction time was more than 120 min. The turbidity slope was determined with good reproducibility (n =6 CV = 5%) given in figure no.1. A typical curve of calcium oxalate crystallization using turbidimetric measurement without inhibition in the supersaturated solution of calcium oxalate in synthetic urine was shown below.

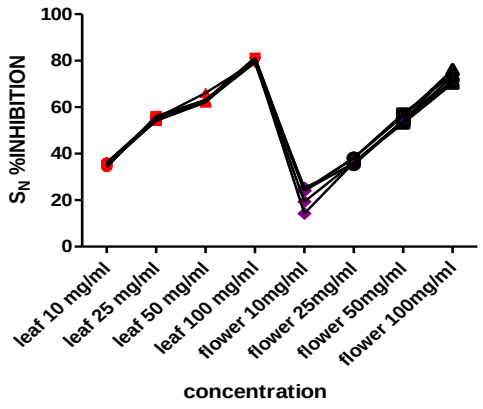

Fig. 1: Effect of infusions on kinetic study where S_N refers to slope of nucleation inhibition.

Simulation of the sedimentary crystal formation

Kidney oxalate stone is the result of supersaturation of urine with certain urinary salts such as calcium oxalate. The process of calcium oxalate crystallisation in the absence of plant extracts is summarised in Table 2 and formation of calcium oxalate crystals(COC) crystals and their aggregation are shown in Figure 2. Maximum numbers of CO crystals were apparently detected after 15 minutes of incubation.

Table 2: Effect of *Convolvulus arvensis* on no. of CaO crystals

Time	0	15	30	45	60
No.of coc/mm^3 in absence of inhibitors	120	660	720	790	860
In presence of leaf infusion 100 mg/ml	45	51	63	82	84
In presence of flower infusion 100 mg/ml	51	59	69	86	90

Effect on nucleation and aggregation assay

Incubating the metastable solutions of $CaCl_2$and oxalate resulted in the formation of CaOx crystals. The respective crystals, observed under the light microscope (100x), in solutions incubated with SXS at 10–100 mg/ml also caused a morphological change in calcium oxalate dihydrate crystals, which was not fully grown as bipyramid CaOx crystals that were inhibited in nucleation phase. The OD decreased with the increase in concentration of plant infusions

indicating that decreased the nucleation of CaOx particles. The OD was highest (0.73±0.052) of positive control i.e. in the absence of herb extract and it was lowest (0.31±0.046) at the highest concentration of leaf infusion(100 mg/ml). The crystals formed in the presence of both leaf and flower infusions were less than that in the control, showing that crystals were less aggregated. The percent inhibited aggregation associated with the leaf infusion and flower at concentration of 10 mg/ml was found to be 62.5% and 57.5% respectively, while percent was maximum i.e. 92.27% and 90.41% at highest concentration of leaf infusion (100 mg/ml) and flower infusion (100mg/ml) respectively.

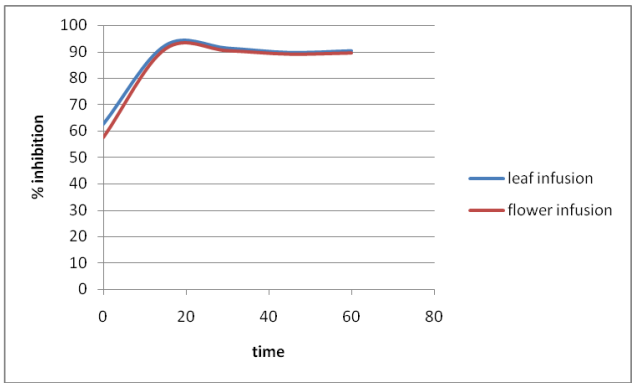

Fig. 2: Effect of *Covolvulus arvensis* at 100 mg/ml on crystal formation inhibition.

DISCUSSION

UL is a common disease with an increasing incidence and prevalence worldwide. Urolithiasis is an extremely painful disease that afflicts the human population since ancient times [16]. The mechanism of calcium oxalate renal calculi formation has attracted the attention of medical scientists because of its widespread clinical occurrence and the difficulty of treatment.life style and dietary choices are implicated in the complex of this disease [17]. Hyperoxaluria is one of the main risk factors of human idiopathic calcium oxalate disease.

An in vitro crystallization study was performed, since nucleation is an important first step for the initiation of crystals, which then grow and form aggregates, this may be due to presence of saponin rich fraction from plants inhibited the crystallization by inhibiting nucleation of CaOx in solution; less and smaller particles were formed with increasing concentrations of the fraction. The results of the nucleation assay confirmed that the extract contained nucleation-preventing agents. The limiting factors in stone formation could be those processes that affect crystal growth, because particles may become large enough to occlude the urinary tract, leading to stone formation [18]. The herb extracts may contain substances that inhibit the growth of CaOx crystals. This property of plants may be important in preventing the growth of kidney stone. Aggregation may be an important factor in the genesis of stones [19]. Recurrent calcium stone formers excrete clusters of crystals in their urine, caused by aggregation, also named agglomeration, whereas urine from normal people contains mainly single crystals [19].

The reviewed studies showed that some possible mechanisms of action of plant extracts include an increased excretion of urinary citrate, decreased excretion of urinary calcium and oxalate or could be attributable to diuretic, antioxidant or antibacterial effects. Future scientific and clinical studies about the efficacy of herbal extracts would highly benefit from an adequate phytochemical description of the extract.

CONCLUSION

We conclude that *Convolvulus arvensis* plant part infusions have shown statistically significant inhibition on the crystal formation when compared to the standard crystal formation using supersaturated calcium oxalate, the leaf infusion has shown significant urolithiolytic activity than that of the flower infusion. Thus infusions of *Convolvulus arvensis* could be further analyzed in vivo and further characterization of its active compound could lead to the discovery of a new candidate drug for the patients suffering with urolithiasis..

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