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 staghorn 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 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, terpenoids, steroids, tropaeol alkaloids (Pseudotropane, tropein, tropinine, 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-urolithic property of C. arvensis Linn. In the present study, an effort has been made to establish the scientific validity of the antiurolithic activity of C. arvensis flower and leaf extract 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 37ºC 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.

1. Inhibition assay

Antilithic activity in different extracts of leaves and flowers of Convolvulus 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.
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 (85 mM) and Na₂C₂O₄ (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 

\[ \text{Percentage inhibition} = \left( 1 - \frac{\text{Sm}}{\text{Sc}} \right) \times 100 \]

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 crystalization in vitro The classical model for the study of oxalate crystalization was chosen because of its simplicity and satisfactory reproducibility. According method reported by Atmani and Khan [11] which involves crystalization 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°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:

\[ \text{CaCl}_2 + \text{Na}_2\text{C}_2\text{O}_4 \rightarrow \text{CaC}_2\text{O}_4 \text{2NaCl} \]

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 equalized to 60C in a water bath for 1 h and then cooled to 37°C overnight. The crystals were harvested by centrifugation and then evaporated at 37°C. CaOx crystals were used at a final concentration of 0.8 mg/ml, buffered with Tris–HCl 0.05 mol/l and NaCl 0.15 mol/l at pH 6.5. Experiments were conducted at 37°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:

\[ \% \text{ inhibition} = \frac{\text{Turbidity sample} - \text{Turbidity control}}{\text{Turbidity control}} \times 100 \]

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 only after 48 hours (Grases et.al.). Both the infusions of Convolvulus arvensis has significantly reduced the size of calcium oxalate crystals (dihydrate variety). 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

<table>
<thead>
<tr>
<th>Concentration mg/ml</th>
<th>10</th>
<th>25</th>
<th>50</th>
<th>75</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf extract</td>
<td>32.83±0.83*</td>
<td>43.63±1.16*</td>
<td>46.33±1.38*</td>
<td>55.33±0.88*</td>
<td>82±2.38*</td>
</tr>
<tr>
<td>Flower extract</td>
<td>25.5±0.09</td>
<td>35.17±0.87</td>
<td>44.17±0.47</td>
<td>50.17±0.60</td>
<td>68.17±0.94</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SEM, n=6, where, *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 crystalization using turbidimetric measurement without inhibition in the supersaturated solution of calcium oxalate in synthetic urine was shown below.

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 crystalization in the absence of plant extract 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

<table>
<thead>
<tr>
<th>Time</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>45</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.of coc/mm³ in absence of inhibitors</td>
<td>120</td>
<td>660</td>
<td>720</td>
<td>790</td>
<td>860</td>
</tr>
<tr>
<td>In presence of leaf infusion 100 mg/ml</td>
<td>45</td>
<td>51</td>
<td>63</td>
<td>82</td>
<td>84</td>
</tr>
<tr>
<td>In presence of flower infusion 100 mg/ml</td>
<td>51</td>
<td>59</td>
<td>69</td>
<td>86</td>
<td>90</td>
</tr>
</tbody>
</table>

Effect on nucleation and aggregation assay

Incubating the metastable solutions of CaCl₂ 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 (100 mg/ml) respectively.

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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REFERENCES