In vitro and In vivo study on the effect of *Scoparia Dulcis* in inhibiting the growth of urinary crystals.

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**Abstract**

Urolithiasis is a very common and highly recurring painful disease both in developing and developed nations due to the change in lifestyle and food habits. *Scoparia Dulcis* is herbal plant which has been used as a traditional medicine for dissolving urinary stones by the tribal group of people in Western Ghats, India. This experimental study gives a scientific awareness about the effect of *Scoparia Dulcis* in dissolving the urinary crystals and will confirm the drug’s effect on the organs like kidney and liver.

The invivo testing was done by developing urinary crystals using single diffusion gel growth technique and the water extract of the drug is incorporated to monitor the growth of the crystal. The statistical analysis was done for the in vitro study and has proved 2 ml dose shows the highest significance in variation.

In invivo testing, the urinary crystals were induced in Wistar Rats and the drug was fed to monitor its effect on the growth of crystals as well as on nearby organs. The urine and serum samples were tested for all the group of rats and parameters analysis proved the significance of the drug. The histopathology analysis gives the sectional view and report of diseased and treated groups. In both the invivo as well as invivo testing, the drug showed a significant effect in inhibiting the growth of urinary crystals.

**Keywords:** Urolithiasis; Scoparia Dulcis; Natural crystal growth; Western Ghats; tribal medicine.

**Introduction**

Urolithiasis is a globally concerned ailment due to its severe effect on the normal metabolic activity of a human being. Urinary calculi are more prominently detected in the areas like British isles, Northern Australia, India, Europe and Pakistan [1]. The most common types of urinary stones are brushite, struvite, whewellite, weddellite etc. [2,3]. One of the major threatening factors in kidney stone disease is its recurrence. The frequency of stone formation increases with increasing years and this is mainly due to the inefficiency of various available chemotherapy and side effects of surgeries. Hence, an alternative medicine is the important concern to be addressed in treatment of this painful disease.

Ayurvedic treatment started replacing the allopathic and homeopathic treatment for the majority of diseases in India, due to its less side effects and better results. In the Ayurvedic system of medicine, there is a group of plants named ‘Pashanabheda’ which is diuretic and are used to dissolve urinary stones [4]. Various herbal plants like *Costus Igneus* [5], *Costus spiralis*, *Herniaria hisut* [7], *Helichrysum plicatum* [8], *Tribulus Terresteris* [9], *Flos carthami* [10] were successfully proved as preventive as well as curative medicine for urolithiasis. In this study, we are studying the effect of *Scoparia Dulcis*, a Pashanabheda herb, as a preventive and curative medicine for urolithiasis. It is a folk medicine, which is abundantly found in hilly areas of South India, especially in Kerala and locally called as ‘Kalluruki’. It is being used for the treatment of Diabetes [11] as well as for hypertension. The phytochemical constituents of *Scoparia Dulcis* include scoparic acid [A, B, D], scopadulcicil [12,13] scopadulin [14] which made them as highly effective medicinal plants.

The inhibitory effect of the herbal drug *Scoparia Dulcis* in the growth of struvite crystals was monitored using both in vitro and in vivo experiments. The in vitro study was totally concentrated on struvite stones which are also known as magnesium ammonium phosphate hexahydrate [MAPH] [15]. The struvite stones are caused due to the presence of urea splitting bacteria in the urinary tract and persist due to alkaline urine [16-18]. According to the statistical studies, females are more prone to struvite stones than males with a ratio of 2:1 [19]. The herbal extract of the plant was applied to the development in vitro crystals and monitored its growth before and after the incorporation of the extract. On the other hand in vivo experiments were also performed to check the ability of the plant extract in treatment of hypercalciuria induced calcium oxalate urolithiasis. In vivo experimental studies are done by inducing kidney stones in the Wistar rats and modulation of disease progression was noted after intervention of extract.
Materials and Methods

Collection of plant material
The herb which is used for the study is Scoparia Dulcis which is commonly known as goat-weed. This plant material is collected from South Kerala during the monsoon season. These plant were made as a herbarium (Voucher no: 75151, Jawaharlal Nehru Tropical Botanic Garden and Research Institute) and identity of specimen is also confirmed.

Preparation of herbal extract
The leaves were separated from the branches and the leaves was washed and dried for more than a week. The dried leaves are powdered [100g] and added distilled water [250ml] and kept in a Soxhlet apparatus for extraction. It was kept in a heating mantle with a temperature of 70 °C for 3 days. The crude extract of color blackish-brown was obtained and was used for the inhibition studies.

In vitro testing procedure

Preparation of additive solutions
In order to do a comparative study on the inhibition rate of different dosage of the drug in the growth of crystals, various additive solutions were prepared as shown in Table 1. The additive solutions will be used as the supernatant reactant for the growth of crystals.

<table>
<thead>
<tr>
<th>No of additive solutions</th>
<th>Volume of 1M magnesium acetate solution</th>
<th>Volume of Scoparia Dulcis extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTA</td>
<td>20ml</td>
<td>0ml</td>
</tr>
<tr>
<td>TTB</td>
<td>19ml</td>
<td>1ml</td>
</tr>
<tr>
<td>TTC</td>
<td>18ml</td>
<td>2ml</td>
</tr>
</tbody>
</table>

The test tube A [TTA] will be the control test tube where no drug will be there. In test tube B [TTB] and test tube C [TTC] 1ml and 2ml dosage of the drug is mixed respectively with the supernatant solution.

Growth of Struvite Crystals
The single diffusion gel growth technique was used for the growth of crystals. In this method, the gel was prepared by mixing sodium meta silicate solution of density 1.03 g cm -3 with 0.5M of ammonium dihydrogen phosphate solution and the pH was adjusted to 7.2 [16]. It took approximately 48 h for the gel to set. After gelling, 1M of magnesium acetate solution is added as a supernatant solution to the test tube. The test tube is kept at room temperature for the crystallization to occur.

Characterization
The crystals fromed in the control tube [TTA] were given for X-Ray Diffraction [XRD] characterization to confirm the crystalline nature of struvite crystal. The characterization was done with the instrument PANalytical Model X’pert PRO X-ray diffractometer with Cu-K radiation [of wave length 1.54060 Å]. The FT-IR characterization of the grown crystals was done with the instrument Thermo Nicolet model AVATAR 330 Spectrometer which confirms the functional groups present in the developed crystal. The optical images of the grown crystals were taken with the help of the optical microscope to view the morphology of the developed crystal.

Invivo testing procedure

Animals
Twenty four male Wistar rats, weighing approximately 150 - 200 g were used in the present experimental study. All animal experiments and maintenance were carried out according to the ethical guidelines suggested by the Institutional Animal Ethics Committee (Registration Number - 1333/c/10/PCSEA; Ethical Clearance Number - VIT/IAEC/8/F/07). The animals were housed in polypropylene cages and maintain under the standard conditions of 12 hours dark/light cycle at 27±1 °C.

Study design
The animals were divided into four groups and were kept in four different cages. The normal diet was given to the rats in the first cage, which was the control group, Group I. The drinking water was replaced by the solution of 1% ethylene glycol in water [20] for the Group II. The Group III animals were ingested with ethylene glycol solution and the extract (50mg/kg) was supplemented simultaneously for 22 days, to study the prophylactic effect of the drug. In Group IV, the animals were supplied with ethylene glycol solution for 22 days to induce the stone and the extract was administered (50mg/kg) from 22nd to 42nd day to observe the curative effect.

Biochemical Analysis
The twenty-four hour urine samples were collected by keeping the rats in the metabolic cages. The blood was collected from the rats in each group after euthanizing the animals and stored without anticoagulant to get the serum. The biochemical analyses for the urine and serum samples were done to find the quantity of calcium, magnesium, phosphate, creatine and urea using standard kits.
Histopathology Study

At the end of the study period the rats were euthanized by cervical dislocation under anesthesia. Kidney and liver were carefully removed and perfused using phosphate buffer saline (PBS). The cleaned organs were fixed in 10% formalin solution. Section were cut with 4 \( \mu \text{m} \) thicknesses using 4 Leica RM 2126 microtome and mounted on slides after staining with Haematoxylin & Eosin (H & E). The sections were focussed using a microscope of magnification 400x and the photographs were taken.

Statistical Analysis

The results of the serum and urine analysis were expressed in terms of mean \( \pm \) S.D. The results were done One- way ANOVA test and the results are said to be significant for probability value \( p < 0.05 \).

Results and Discussion

X-Ray Diffraction [XRD] analysis

The crystalline nature was confirmed with the help of its XRD pattern as given in figure 1. The peaks in the pattern were confirmed by the standard JCPDS [Joint Committee for Power Diffraction Standard] data [96-900-7675] and is confirmed that the grown crystals were of struvite type. The highest intensity peak is obtained at 35\(^0\) which is the 100% peak value of the struvite crystals. The next intensity peak is obtained near 15\(^0\) which is a 60% peak value for the struvite crystals.

Optical image analysis

The optical image of developed struvite crystals was taken for confirming the morphology of the grown crystals and is shown in Figure 2. The grown crystal exhibits various morphologies like rectangular platelet, needle type, leaf type, dendritic etc. The approximate size of the crystals was found from the images which lie in the range of 0.5 cm to 1 cm.
In vitro studies on the effect of Scoparia Dulcis

Crystal growth rate and inhibitory effect analysis

The crystals were grown in three test tubes, namely test tube A [TTA], test tube B [TTB] and test tube C [TTC], to study the inhibitory effect of Scoparia Dulcis Figure 3. The figure clearly shows the difference in the density of crystals grown in various test tubes according to the dosage of a drug applied.

![Figure 3: The in vitro growth of struvite crystals in test tubes](image)

The growth rate of the crystals was monitored at two different sections of the test tube for different time periods. The interface section is the marginal area where the gel, outer reactant solution and the drug directly interacts. Hence the crystal growth initializes in this interface and proceeds towards the depth inside the test tube. The size of the crystals varies for different time period in gel-liquid interface as given in Figure 4.

![Figure 4: The variation of growth rate of crystals in gel-liquid interface](image)

The growth rate has been compared by incorporating different dosage of drug, namely control [TTA], 1ml dosage of drug [TTB] and 2ml dosage of drug [TTC]. It shows that initially in all the three test tubes the crystals will be growing rapidly. After 24 h the crystal growth rate will decrease in TTB and TTC and during 68 h while measuring the crystal size it was proved that the 2ml dosage incorporated in TTC reduces the crystal size to the maximum than compared to the other two test tubes. After 96 h it was observed in TTC that the crystals started dissolving in the gel medium which proves that the herbal drug used is highly effective.

The growth of crystals at different depth from the gel-liquid interface was also measured as shown in Figure 5. Despite from the gel-liquid interface, the crystal will be grown at different depths of the test tube since the gel matrix is present all over inside the test tube.

![Figure 5: The rate of crystals at the depth in test tube](image)

It was observed that the crystal size does not vary much in TTA whereas after a time period the crystals started fragmenting and dissolving in TTB and TTC. This proves the effect of drug in the fragmentation of urinary crystals.

After a specific time period, the crystals from the test tubes were removed from all the three tubes and their mass was measured as shown in Figure 6. It was observed that the crystals from TTA shows more mass compared to TTB and TTC. This is due to fragmentation and limited crystal growth in test tubes TTB and TTC with the effect of the drug during the time of crystal growth.

The crystal collected from TTA shows the higher mass or weight due to the large size of the crystal. After incorporating the drug, the crystal size decreases as well as dissolution and fragmentation take place. Hence the mass of the crystal collected will decrease in TTB and TTC respectively.
Drug Activity

The phytochemical screening was done for the drug using different assays [21] and the qualitative analysis result is given in the Table 2.

### Table 2: Qualitative analysis of phytochemicals of the medicinal plant.

<table>
<thead>
<tr>
<th>Phytoconstituent</th>
<th>Present (+) or Absent (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>+</td>
</tr>
<tr>
<td>Saponin</td>
<td>+</td>
</tr>
<tr>
<td>Steroid</td>
<td>-</td>
</tr>
<tr>
<td>Flavanoid</td>
<td>+</td>
</tr>
<tr>
<td>Tannin</td>
<td>+</td>
</tr>
</tbody>
</table>

Statistical Analysis

The One-Way ANOVA test was done to check whether there is a significant difference in the growth rate of crystals in different test tubes at depth. It gives a probability value 0.0001 [p < 0.05] which proves that there is a highly significant difference in the growth rate of crystals in three test tubes.

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Urine Parameters and Analysis

The 24-hr urine has been collected from all the animals of all the groups. The urine parameters were quantitatively analyzed and given in Table 3. The calcium level has been increased in diseased group II compared to the group I, which occurs due to excessive tubular damage in the kidney, which may lead to excretion of intracellular calcium. However, during the administration of extract the calcium level was managed in Group III and IV, which proves that extract is effective in inhibiting hypercalciumia. Magnesium reduces super saturation and so, it is considered as one of the potent inhibitors of calcium oxalate crystallization [22]. Magnesium level was significantly decreased in Group II animals compared to normal animals, due to metabolic acidosis and super saturation. When the administration of the extract was started, magnesium level was improved in Group III and IV animals in order to reduce the concentration of calcium. The level of magnesium was in preventive group reached almost normal value while the curative therapy showed a mild improvement than the urolithic animals. Hypercalciumia leads to increased phosphate leakage and in the urolithic rats it was only a slight increase in comparison to control rats [23]. In treated rats decreased in level of phosphate was also at par to control rats. Formation of CaOx crystals can be well exhibited from the obvious increase in urinary oxalate level in the ethylene glycol ingested rats compared to control Group I. The effect of the extract can be well demonstrated by its potency in treating hyperoxaluria. This has been proved by the decreased level of oxalate in Group III and IV.

### Table 3: The changes in urine parameters in control and experimental animals

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium</td>
<td>3.47 ± 0.03</td>
<td>4.09 ± 0.26a**</td>
<td>3.765 ± 0.13b</td>
<td>3.595 ± 0.06bNS</td>
</tr>
<tr>
<td>Magnesium</td>
<td>5.99 ± 0.80</td>
<td>2.99 ± 0.63a</td>
<td>5.89 ± 0.37b**</td>
<td>3.746 ± 0.25bNS</td>
</tr>
<tr>
<td>Phosphate</td>
<td>3.89 ± 0.69</td>
<td>4.10 ± 0.45cINS</td>
<td>1.74 ± 0.03cNS</td>
<td>2.60 ± 0.24cNS</td>
</tr>
<tr>
<td>Oxalate</td>
<td>4.52 ± 0.18</td>
<td>6.39 ± 0.39cINS</td>
<td>5.05 ± 0.42c**</td>
<td>5.23 ± 0.16c**</td>
</tr>
</tbody>
</table>

All the parameters were expressed as mg/dL. The values are expressed as mean ± SD of four animals and results are statistically analysed by one way ANOVA with Bonferroni’s multiple comparison post test (n=6). The comparisons are made as follows: ‘a’ – Control Vs Diseased; ‘b’ – Diseased Vs Prevention; ‘c’ – Diseased Vs Treatment. *** p<0.001, ** p<0.01, * P<0.05, NS – Not Significant.

Serum Parameters and Analysis

The serum collected from the experimental animals was analyzed and the quantity of various parameters has been calculated and noted in Table 4. The value of urea and creatinine is significantly (p< 0.05) increasing in Group II, III and IV compared to the control Group I, this shows that a renal function has been during the calcium oxalate crystallization. The urea and creatinine level has been shown a significant decrease in treating Group III and IV compared to diseased group II, which shows the ability of the extract to offer nephro protection which is mainly due to various antioxidants and anti-inflammatory compounds present in it [13].
Table 4: The changes in serum parameters in control and experimental animals

<table>
<thead>
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<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>29 ± 0.94</td>
<td>53 ± 0.85&lt;sup&gt;a***&lt;/sup&gt;</td>
<td>31.1 ± 1.12&lt;sup&gt;b***&lt;/sup&gt;</td>
<td>30.3 ± 0.75&lt;sup&gt;c***&lt;/sup&gt;</td>
</tr>
<tr>
<td>Creatinine</td>
<td>0.35 ± 0.06</td>
<td>0.65 ± 0.06&lt;sup&gt;a*&lt;/sup&gt;</td>
<td>0.4 ± 0.07&lt;sup&gt;b*&lt;/sup&gt;</td>
<td>0.35 ± 0.04&lt;sup&gt;c*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Calcium</td>
<td>9.6 ± 0.24</td>
<td>8.4 ± 0.38&lt;sup&gt;NS&lt;/sup&gt;</td>
<td>9 ± 0.40&lt;sup&gt;NS&lt;/sup&gt;</td>
<td>9.4 ± 0.16&lt;sup&gt;NS&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

All the parameters were expressed as mg/dL. The values are expressed as mean ± SD of four animals and results are statistically analysed by one way ANOVA with Bonferroni’s multiple comparison post test (n=6). The comparisons are made as follows: ‘a’ – Control Vs Diseased; ‘b’ – Diseased Vs Prevention; ‘c’ – Diseased Vs Treatment. *** p<0.001, ** p<0.01, * P<0.05, NS – Not Significant.

Serum calcium level was mildly decreased in the Group II animals compared to the control rats, corresponds to excessive urinary excretion. This was very well improved in group III and IV animals. The calcium level seems to be increasing in the treated groups compared to group II, which proves the effect of inhibition of calcium crystal formation by the extract.

Histopathology studies

The microscopic images of kidney and liver sections of various groups are given in Figure 7. According to the histopathology observations, the intake of ethylene glycol has induced polymorphic irregular crystals in the kidneys of Group II animals (Figure 7c) with early tubular necrosis.
Figure 7: The microscopic images of the histopathological sections of (a) Group I control kidney section (b) Group II control liver section (c) Group II ethylene induced kidney section with crystals marked (d) Group II diseased liver section (e) Group III prophylactic drug effect kidney section (f) Group III liver section (g) Group IV curative effect of drug tested kidney section (h) Group IV liver section.

While the liver section of the diseased animal shown in Figure 7 (d), demonstrates sinusoidal and venular dilation. In prophylactic treatment, kidney section Figure 7(e) shows significantly reduced tubular vacoulization and crystals deposition compared to the diseased group due to the intake of drug whereas the liver section Figure 7(f) shows a central venular congestion. The curative effect study shows that there is a reduction on number of crystals in the kidney section as shown in Figure 7 (g) and a mild liver degeneration is being detected in the curative treated liver section. The pathological observations in the liver and kidney show the efficacy of the extract in restoring the normal function of liver and kidney, which can be one of the signs in management of hyperoxaluria and urolithiasis. The significant contribution of the bioactive compounds from the plant can be the sole reason for reduction in the disease progression [14].

Conclusion

The results suggest that *Scoparia Dulcis*, an Indian Ayurvedic plant can dissolve the urinary crystal and significantly reduce the risk factors of stone formation. The growth rate of the crystals was significantly diminished by the plant extract and proved that the higher rate is in TTA. Based on the in vitro studies we can conclude that, the formation of struvite stone has been inhibited by the extract, while prevention and treatment of calcium oxalate crystals was potentially attenuated in vivo. The urinary and serum parameters of rats offered with prophylactic and curative therapy showed notable improvement and it was well supported by the histopathological reports. Hence, the study was able to exhibit that the plant extract and its bioactive constituents have the ability to reduce both struvite and calcium oxalate crystallization. Further studies on bioactive compound isolation and its mechanism of action may help in formulation of a potential anti-urolithic drug.
Conflict of Interest

There is no conflict of interests between the authors in submitting and publishing this research article.

References