Chapter 4

*In vitro* studies on the effect of *Scoparia Dulcis* in the growth struvite urinary crystals and the study on its antimicrobial activity

4.1 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 (Menon et al., 1998). The most common types of urinary stones are brushite, struvite, whewellite, weddelite etc. (Pak, 1998; Kok, 2002). One of the major threatening factors in kidney stone disease is its recurrence. The frequency of stone formation increases as year passes and this is mainly due to the inefficiency of various available medications 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 (Chow et al., 1974). Various herbal plants like *Costus Igneus* (Manjula et al., 2012), *Costus spiralis* (Veil et al., 1999), *Herniaria hisut* (Grasses et al., 1995), *Helichrysum plicatum* (Bayir et al., 2011), *Tribulus Terresteris* (Aggarwal et al., 2010), *Flos carthami* (Lin et al., 2012) were successfully proved as preventive as well as curative medicine for urolithiasis.

In this study, we are studying different effects of *Scoparia Dulcis*, a Pashanabhedha 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 (Friere et al., 1993) as well as for hypertension previously. The phytochemical constituents of *Scoparia Dulcis* include scoparic acid [A, B, D], scopadulciol (Hayashi et al., 1990; 1991) scopadulin (Hayashi et al., 1993) which made them as highly effective medicinal plants. The inhibitory effect and the anti microbial property of *Scoparia Dulcis* is been discussed in this chapter.

The inhibitory effect of the herbal drug *Scoparia Dulcis* in the growth of struvite crystals was monitored using *in vitro* experiments. Struvite stones which are also known as magnesium ammonium phosphate hexahydrate [MAPH] (Chauhan et al., 2011). The struvite stones are caused due to the presence of urea splitting bacteria in the urinary tract and persist due to alkaline urine (Griffith, 1978; Coe et al., 1992; Hesse and Heimbach, 1999). According to the statistical studies, females are more prone to struvite stones than males with a ratio of 2:1 (Takasake, 1975). The herbal extract of the plant was applied to the solutions which is used for the development *in vitro* crystals and monitored its growth before and after the incorporation of the extract.

The antimicrobial activity of the struvite crystal is also been studied and discussed in this chapter. The antimicrobial activity determines whether the agent itself can kill the micro organisms. The struvite crystal has been checked for its antimicrobial activity in various microorganisms like *Escherchia coli*, *Pseudomonas spp* etc which proves us whether the drug has its effect in reducing the presence of microbes which can lead to urinary tract infection.

4.2 MATERIALS AND METHODS

4.2.1 *IN VITRO* INHIBITION STUDY

4.2.1.1 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 which is shown in Fig 4.1. 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.

![Image of Scoparia Dulcis](image)

**Fig 4.1: Scoparia Dulcis**

4.2.1.2 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 [100 g] and added distilled water [250 ml] 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 colour blackish-brown was obtained and was used for the inhibition studies.

4.2.1.3 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 4.1. The additive solutions will be used as the supernatant reactant for the growth of crystals.
Table 4.1: Composition of the supernatant solution

<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>20 ml</td>
<td>0 ml</td>
</tr>
<tr>
<td>TTB</td>
<td>19 ml</td>
<td>1 ml</td>
</tr>
<tr>
<td>TTC</td>
<td>18 ml</td>
<td>2 ml</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 2 ml dosage of the drug is mixed respectively with the supernatant solution.

4.2.2 ANTIMICROBIAL EFFECT OF *SCOPARIA DULCIS*

4.2.2.1 DISSOLVING THE STRUVITE CRYSTALS

The crystal was developed using the gel growth technique as described in the previous chapter. The struvite crystal was separated by filtering method from the test tube and was allowed to dry. Later the crystal was crushed using mortar and pestle. The powdered crystals were dissolved in 5ml of solvents like DMSO (dimethyl sulfoxide), DMF (dimethylformamide), THF (tetrahydrofurone), ethanol and acetone. These dissolved solvents were used for the antimicrobial sensitivity test with different concentrations such as 50ppm, 100ppm, 200 ppm and 300ppm.

4.2.2.2 PREPARATION OF MHA

Muller Hinton agar (MHA) was weighed for 21.6gms and agar agar weighed for 20 gm, and was dissolved in 600ml of distilled water. They were autoclaved at 121\(^\circ\)C at 15lbs pressure for 15minutes. At hand bearable condition the media was poured into 9 sterile petri-plates and they were set aside for solidification.
4.2.2.3 ANTIMICROBIAL ACTIVITY

After solidification each agar plates was scrapped with the respective cultures like *Staphylococcus aureus*, *Echerchia coli*, *pseudomonas spp* and *acetobacter spp* by immersing sterile cotton swab in the bacterial suspension and then rotate and compress against the wall of test tubes so as to rinse the excess fluid and 1 plate was maintained as control. Each plate was then cut with 5 wells using cork borer or well cutter and were added with dissolved crystal solvents at specific concentration such as 0.05ml, 0.1ml, 0.2ml and 0.3 ml. In each plate 1 well was maintained as control with no dissolved solvents in it. All the plates were replicated and incubated for 24h at 37°C. They were examined later for the zone of inhibition.

4.3 RESULTS AND DISCUSSION

4.3.1 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*

![Fig 4.2](image)

Fig 4.2 The invitro growth of struvite crystals in test tubes as shown in Fig 4.2. This figure clearly shows the difference in the density of crystals grown in various test tubes according to the dosage of a drug applied.
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 gel matrix of the test tube. The size of the crystals varies for different time period in gel-liquid interface as given in Fig 4.3.

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 positions inside the gel matrix was also measured and its average value was used to plot a graph as shown in Fig 4.4. Despite from the gel-liquid interface, the crystal will be grown more at depths of the test tube since the gel matrix is present all over inside the test tube.

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 Fig 4.5. 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.
Fig 4.3 The variation of growth rate of crystals in gel-liquid interface

Fig 4.4 The growth rate of crystals inside the gel matrix of test tube
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.

4.3.2 DRUG ACTIVITY

The phytochemical screening was done for the drug using different assays and the qualitative analysis result is given in the Table 4.2. The absence of steroid in the drug is the main attractive factor and advantage in giving to the human being. The medicines which are available in market have high content of steroid, which is highly dangerous for health and results in lot of side effects in long term.

Fig 4.5 The mass of the crystals in various test tubes
Table 4.2: 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>Flavonoid</td>
<td>+</td>
</tr>
<tr>
<td>Tannin</td>
<td>+</td>
</tr>
</tbody>
</table>

4.3.3 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.

4.3.4 ANTIMICROBIAL ACTIVITY

The antimicrobial activity of struvite crystals were studied to monitor the effect of struvite crystals in inhibiting the growth of micro-organisms. The study showed an inhibitory zone of 12 mm in Staphylococcus aureus at concentration of 0.1 ml in the solvent DMF and 10 mm in Escherichia coli at concentration of 0.05 ml in the solvent ethanol. The inhibitory zone formation is shown in Fig 4.6.
Fig 4.6 Inhibitory zone formation for (a) *Escherichia coli* and (b) *Staphylococcus aureus*
4.4 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. From the in vitro experimental procedure it proved that the test tube which contains 2ml dosage of the drug significantly reduced the growth of urinary crystals. Based on the in vitro studies we can experimentally prove that the drug have its inhibitory effect, whereas the intake of drug in human body can be only confirmed by a detailed in vivo drug testing. The antimicrobial activity shows that the struvite crystals have developed a zone of inhibition for Escherichia coli and Staphylococcus aureus.