IN VITRO INHIBITION OF UROLITHIASIS USING TERPENE ISOLATED FROM SCOPARIA DULCIS.

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Abstract—Urolithiasis is the vital challenge in present scenario of medical world. About one in 20 people are affected by kidney stone in their life time. More than 80% of kidney stones are calcium oxalate stones. Scoparia dulcis, the herbal plants are used in the treatment of urolithiasis by ancient tribes. Scoparia dulcis plants consist of major constituents of terpenes that have the adverse effect on the urolithiasis. Present study to evaluate the antilithiatic potential of Scoparia dulcis under in vitro condition. Calcium oxalate stones were synthesized using in vitro techniques. The extracted sample and the calcium oxalate were characterized using FTIR analysis. Terpenes were isolated using column chromatography and analyzed through Thin Layer Chromatography. The study of release kinetics of calcium oxalate through Scanning Electron Microscope analysis reveals that terpenes from Scoparia dulcis inhibited the calcium oxalate by morphological changes with reduction in its size. The in vitro study of calcium oxalate with terpenes affirms the percentage of its affinity to dissolve the stones, which regulates further techniques. There is a great need for recurrence prevention that requires a better understanding of the mechanisms involved in stone formation to facilitate the development of effective drugs.

Keywords—Terpene, Synthesis, Calcium oxalate, Column chromatography, Thin Layer Chromatography, FTIR, SEM.

1. INTRODUCTION

Scoparia dulcis is the widely used traditional medicinal plant which belongs to the family scrophulariaceae. It is branched green color, leafy, odor taste and tough. It is commonly called as sweet broom weed, liquorice weed and it is locally named as Sarokkotthini in Tamil, Kallurukki in Malayalam, and Ghoda Tulsi, Rice Weed in other languages. It is a perninal herb, which is distributed widely in tropical and subtropical Indian regions and it grows upto 1m, which is small, 3 leaves whorled, the stamens and ovary are in green color, the petioles and pedicles are in 9mm. The plant has an rich sources Flavones, Terpenes, Steroids, Phenols, Tannis, Saponins, Aminoacids, Coumarins, Carbohydrates of a biochemical compound. The Fresh and dried plants has been used for treating diseases. It is meant famous in treating the kidney stones. The terpenoids in the herb are responsible for the medicine effects.

Terpenes are common in plants and essential oils. It is the subunits of isoterpene biosynthetically. The types of terpenes are evaluated as Hemiterpenes, Monoterpennes, Diterpenes, Sesterterpenes, Sesquiterpenes, Triterpenes, Tetraterpenes, Polyterpenes, Norisoprenoids. Terpenes are been
restricted to a basic aromatic hydrocarbon. They are volatile and non-polar compound. The importance of terpenes in the biomedical field use in the insect repellants, preparation of perfumes & also in cosmetics, it has a property of antimicrobial, anti-tumour activity, anti-hyperglycemic activity, anti-inflammatory and in neuro psychological disorder treatments. Rowatinex has been used in the treatment of kidney stone after extracorporeal shock. The capsule which consist of an active ingredients such as Anethol, Borneol, Camphene, Fenchone and Pinene.

Kidney stone are solid crystalline mineral deposition occurs in kidney. Size of kidney stone plays major role in treatment of kidney stone. The passage of stone through ureter causes severe pain in abdomen, nausea and haematuria. The causes of kidney stone depend on various parameters like gout, hyper calciuria, hyper thyroids, hyper oxaluria, diabetes, hypertension, obesity, dietary factors, medications and hereditary factor have a higher risk of kidney stone formation. The treatment various depending on size of renal calculi deposited, age of patient and their diseases. The patients may prone to multiple attacks by kidney stone. There are several types of kidney stones, calcium oxalate and calcium phosphate forms 80% kidney stone.

II. MATERIALS AND METHODS

A. Collection of Samples
The plant specimens were collected from the road side of Ambattur and Thiruvallur in Tamilnadu during the month of December. By the side of the road, abundant population of the plant *Scoparia dulcis* was present. Leaves of *Scoparia dulcis* plants were separated, washed carefully 2-3 times with running tap water, rinsed with distilled water, air dried for 1 hour, and shade dried. The air dried samples were shade dried for a period of one week. They were coarsely ground in to powder and preserved in air tight containers. The sample was then stored room temperature.

B. Solvent Extract Preparation
The extract of the samples were prepared by soaking 100gm of dried powder in 200ml of various solvents for 48 hours. Solvents such as hexane, ethyl acetate and water were selected. About 200ml of the solvent was added to the dried sample in a conical flask. The flask was then covered with cotton wool. After 48 hours the sample mixture was filtered using a muslin cloth and then through Whatman No.1 filter paper. The filtrate was then concentrated by using a rotary evaporator. The extract was stored and photochemical tests were analyzed for the presence of terpenes.

C. Qualitative Analysis
Qualitative analysis was carried out by using various phytochemical screening methods

*Test For Terpenes*
**Salkowski test:** 2 ml of the extract of the leaves, flowers and seeds was mixed with 2ml of extract was mixed with 2ml of chloroform and 3ml of concentrated sulphuric acid was added, the appearance of reddish brown color of interface indicates the presence of terpenes.

*Test For Diterpenes*
**Copper Acetate Test:** 5 ml of extract were dissolved in water and treated with 10 drops of copper acetate solution, formation of emerald green color indicates presence of diterpenes.
D. Column Chromatography

Column chromatography is one of the most useful methods for the separation and purification of both solids and liquids. The principle of column chromatography is based on differential adsorption of substance by the adsorbent. This is a solid - liquid technique in which the stationary phase is a solid and mobile phase is a liquid.

The stationary phase, a solid absorbent Silica gel of about 230-400 mesh was used to prepare the slurry. In a separate beaker, solvents mixture of hexane and ethyl acetate was measured approximately one and a half times the volume of silica. Silica gel was added to the solvent, a little at a time by constant swirling. Thus slurry of silica gel was prepared which was ready to load in the chromatography column. The packed slurry was about 10 to 12 cm about the height of the column. The sample was added about 2 cm about the height of the column. When the sample loaded was completely eluted on to the silica bed, the solvent mixture was loaded in the column. The eluent was collected in a series of test tubes. The flow rate of eluent was maintained at optimum flow rate for each particular separation. Once the composition of each fraction was known, the fractions containing the desired compounds were combined. The TLC plates were examined under the Ultra Violet cabinet to infer the spots. The spots were carefully marked for the Retention factor calculations. Based on the value of retention factor, similar compounds were collected together.

E. Thin Layer Chromatography

TLC plates used were purchased as 5 cm x 20 cm sheets. Each large sheet was cut horizontally into plates which are 10 cm tall and 5 cm width. A large beaker with a glass lid was chosen. The solvent such as the hexane and ethyl acetate were chosen in the ratio of suitable concentration. The solvent were poured into the chamber to a depth of just less than 0.5 cm. The microcap was dipped into the solution and then gently touched the end of it onto the proper location on the TLC plate. The prepared plates were placed in the developing beaker, the beaker was covered with the watch glass, and it left undisturbed on the bench top. The plate was removed from the beaker and immediately marked the solvent front with a pencil. The plate was allowed to dry in air. The spots were analyzed under the Ultra Violet cabinet at a wavelength range of about 254 to 366 nm. The mobile phases were traced by spots indicated by pink color. This coloration of spots indicates the presence of terpenes. Based on the value of retention factor, similar compounds were collected together. The $R_f$ value was calculated according to this equation:

$$R_f = \text{distance of sample} / \text{distance of solvent}.$$
and 3 to 4 drops of Methyl Orange was added. 6M of NH₃ solution was added as 1 ml at a time until the indicator is intermediate between Orange and yellow or until a persistent precipitate appears. A gravity funnel was set-up in a Ring and filtered using a fine filter paper to filter the bulk of the liquid from the solution. A few squirts of Water used to wash the solid and the beaker. A rubber policeman was used to completely transfer the remaining solid to the funnel. The solid was washed with five portions of 7 ml cold Water. The filter paper is removed from the funnel and spread it out on a watch glass so that the solid can dry.

Observation-Formation of white crystalline compound that infers the calcium oxalate stone.

**G. Examination of release kinetics of calcium oxalate in Terpenes - In Vitro Anti Lithiatic Activity Test**

The dissolution percentage of calcium oxalate was evaluated by taking exactly 1 gram of calcium oxalate at different time intervals in the organic extraction terpenes. The crystallization inhibition effect of terpene extracted from *Scoparia dulcis* was estimated by course of time measurement of turbidity changes, due to the crystallization effects of calcium oxalate. The organic extract terpene isolated from the plant was chosen at different time intervals in order to examine the release kinetics of calcium oxalate in the terpenes. Three vital extraction variables are examined such as time duration, temperature and drug-solvent release kinetics. The terpene turbidity test was carried out under the constant temperature of about 37°C.

**H. FTIR Analysis**

Fourier transform infrared spectroscopy is used to analyse the chemical constituents by obtaining the infrared spectra from various interference patterns. The organic extract and calcium oxalate was characterized by using Fourier transform infrared spectroscopy to study the preliminary chemical constituents based on the transmission versus wave number infrared spectra.

**I. SEM Analysis**

Scanning Electron Microscopy is used to study the morphological characteristics of various materials. The in vitro synthesis of calcium oxalate and the calcium oxalate suspended in the terpene from *scoparia dulcis* were analyzed through SEM analysis. The morphological changes of the calcium oxalate with various time durations in presence of terpene were used as the preliminary analysis test to study the nature and morphology of calcium oxalate.

III. RESULTS AND DISCUSSION

The calcium oxalate crystals that have been produced in this in vitro study were similar to the crystals in the urine of patient with calcium oxalate crystals. In this study hexane extract of *scoparia dulcis* has been used since many of the active compounds of terpenes in the plants, were dissolved in it and extracted through solvent extraction method. Because the polarities of three different solvents like hexane, ethyl acetate and aqueous are different, the active compound in them is also different. Terpenes were isolated using the column chromatography with various concentrations of solvents such as hexane and ethyl acetate. The solvents in the ratio 4:1 were proven to be the most effective in terms of terpenes isolation. The isolated components were analyzed through Thin Layer Chromatography techniques under the Ultra Violet cabinet to infer the presence of terpenes. The analysis of various fractions provided the different Rf values which was shown in table 1. The results of the in vitro inhibition effects of different concentrations of terpenes from *Scoparia dulcis*, on the area of calcium oxalate crystal formation were shown in the table 2.
**FTIR analysis on organic extract**

FTIR analysis of extract of *Scoparia dulcis* shows peaks at 2924 cm⁻¹, 2950 cm⁻¹, 2405 cm⁻¹, 1734 cm⁻¹, 1589 cm⁻¹, 1361 cm⁻¹, 1222 cm⁻¹, 1011 cm⁻¹, 665 cm⁻¹. FTIR analysis of organic extract shows peaks that represent the presence of. These peaks represent the functional groups such as nitro groups, acids, alkyl halides, alcohols, cyano group, amines and hydrocarbons.

**FTIR Analysis on calcium oxalate**

FTIR analysis of calcium oxalate indicates the presence of its chemical constituents with presence of various bonds. The FTIR analysis graph have two peaks at 775 cm⁻¹ and at 658 cm⁻¹ as a off-water bending band. Two transmission bands were observed at 1611 cm⁻¹ and 1314 cm⁻¹. The first is attributed to the stretching band anti symmetric carbonyl and second is symmetric stretch band, this two bands emphasis on the formation of calcium oxalate.

**SEM Analysis**

SEM (Scanning electron microscopy) analyses the surfaces of materials, particles and fibers so that fine details can be measured and analyzed via image analysis. Also SEM analysis attest the morphological changes in the calcium oxalate suspended in terpene. The morphology of the calcium oxalate is crystalline in nature. Micro scale study at the size of 10 micrometer and magnification at 6.0K was used as the basic study tool. Thus crystalline nature of the calcium oxalate get truncated and eroded when it dissolves in the terpene from *scoparia dulcis*. This provides difference between the two Calcium oxalate stones. Fig.1 displays a crystalline structure of calcium oxalate and Fig.2 displays the amorphous structure of calcium oxalate. As the duration increases, weight of the stone decrea
TABLE I
Retention factor values of fractions under TLC Screening.

<table>
<thead>
<tr>
<th>FRACTIONS</th>
<th>MOBILE PHASE [Solvents]</th>
<th>R_f VALUE</th>
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</thead>
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<tr>
<td>1</td>
<td></td>
<td>0.20</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>0.30</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>0.44</td>
</tr>
<tr>
<td>4</td>
<td>Hexane : Ethyl acetate [4:1]</td>
<td>0.50</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>0.67</td>
</tr>
<tr>
<td>6</td>
<td></td>
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</tr>
<tr>
<td>7</td>
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</tr>
<tr>
<td>8</td>
<td></td>
<td>0.81</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>0.73</td>
</tr>
</tbody>
</table>
**In vitro Analysis of calcium oxalate**

TABLE II
Effects of terpenes of *Scoparia dulcis* on calcium oxalate with various time durations.

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>DURATIONS (hours)</th>
<th>WEIGHT OF CALCIUM OXALATE (Initially in gram)</th>
<th>WEIGHT OF CALCIUM OXALATE (In Terpene)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24</td>
<td>1</td>
<td>0.90</td>
</tr>
<tr>
<td>2</td>
<td>48</td>
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<tr>
<td>3</td>
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</tr>
<tr>
<td>4</td>
<td>96</td>
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<td>0.75</td>
</tr>
<tr>
<td>5</td>
<td>120</td>
<td>1</td>
<td>0.69</td>
</tr>
</tbody>
</table>

Fig.2 FTIR Analysis of in vitro synthesized calcium oxalate.
Fig. 1 FTIR Analysis of leaves sample of *Scoparia dulcis*

Fig. 1 Analysis of Calcium oxalate.
III. CONCLUSION

Evaluation of release kinetics of calcium oxalate infers the effective dissolving concentration of terpenes. The test to study the dissolving ability of the kidney stone reveals the percentage of its affinity to dissolve the stones, which regulates further techniques. The product yield from the organic extracted from *Scoparia dulcis* can be used as the oral solution to dissolve the calcium oxalate. Further study the effect of compounds in *Scoparia dulcis* extract on urine of urolithiatic patients to see how effective it on crystals of lithiatic patients and also clinical trials are needed on the human beings with calcium oxalate urolithiasis. There may be a potential for using alternative medicine for patients with urolithiasis apart from surgery and western medicines.

IV. REFERENCES


ACKNOWLEDGEMENT

We, the students of VEL TECH MULTI TECH ENGINEERING COLLEGE from the department of BIOMEDICAL ENGINEERING, are extremely grateful to company for the confidence bestowed in us and entrusting our project paper, we also extend our gratitude to project guide Mr.M.Sethuram, faculty who assisted us in compiling the project, we would also like to thank all the faculty members of our department for their advice, motivation & guidance.
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