4.1 Introduction

Lepidagathis genus plants are spread throughout the Western Ghats. There are hundreds of plants listed under genus lepidagathis world-wide. The genus falls under the family Acanthaceae. As the genus plants are herbs or shrubs with mostly woody root stalk, they grow on the crevices of laterite rocks. The plant of the genus lepidagathis also considered as a pashanbhed (Kapoor, 2000, Verma et al., 2014, Devkar et al., 2016, Bhat, 2003). Plants of lepidagathis genus were used traditionally for the treatment of urinary calculi, dysuria, polyuria, fever, dysentery, and uterine disorders (Madhavan et al., 2010). Other medicinal uses of lepidagathis plants include skin infections, malaria, migraine, cardiovascular diseases, and gastric problems (Hassan-Abdallah et al., 2013, Mollik et al., 2009, Ravikanth et al., 2001, Sawadogo et al., 2011). Previous studies have shown to possess excellent larvicidal, anti-inflammatory, analgesic, antipyretic, and cytotoxic properties (Charoenchai et al., 2010, Obomanu et al., 2006, Sawadogo et al., 2011). Despite having a number of traditional claims and uses in management of kidney stones, very few species of this genus have been studied for antiurolithiatic activity.

Choice of the assay to screen the herb for activity is a crucial. Assay should be sensitive, reproducible, easy to perform, fast enough to provide result for multiple samples and reliable. Other important condition is assay should answer or should give clue about mechanism of action (Hamburger and Hostettmann, 1991). In urolithiasis about 80% of cases are of calcium stones, hence assays which explore plants for inhibiting calcium oxalate crystal formation were considered (Patel et al., 2012).

In the current study four species of the genus lepidagathis from Western Ghats have been explored for antiurolithiatic activity. Plants screened were Lepidagathis cristata Willd, Lepidagathis prostrata Dalz, Lepidagathis incurva Buch.-Ham. ex D. Don., and Lepidagathis pungens Nees.

4.2 Candidate plants

L. prostrata was collected from end point, Manipal, Karnataka, India and authenticated by Dr. K. Gopalkrishna Bhat, Professor and Head (Ret.), Department of Botany, Poornaprajna College, Udupi. A voucher specimen (PP609) has been deposited in the herbarium of our institute, Department of Pharmacognosy, Manipal College of Pharmaceutical Sciences, Manipal for future reference. L. cristata (225/2013), L. incurva
(256/2014), and *L. pungens* (259/2014) were received as a gift samples from Dr. P. Santhan, Taxanomist (Pharmacognosy), Natural remedies Pvt. Ltd., Bengaluru, Karnataka, India.

*L. cristata*: Plant is a perennial herb with reduced stem. Leaves are sessile (3-6 × 0.5-1) cm, linear-lanceolate, pubscent, acute at both the ends. Flowers have globose heads, crowded at the base. Calyx are five lobed, hairy. Corolla is white with brown or purple spots. Capsules are oblong; seeds 2. Commonly found on waste lands (Plate 4.1).

Traditionally used for fever, skin infections and for wounds (Reddy and Rao, 2013).

*L. prostrata*: It is a rigid prostrate undershrub with woody rootstock. Sessile leaves (2.5×0.8) cm, plicate, rigid, oblong-lanceolate with pointed apex. Flowers are simple, erect, pubescent spikes, usually terminal on short lateral ascending branches. Corolla pinkish about 2 cm long. Capsules are ovoid-lanceolate, glabrous, 2-seeded. Commonly found on laterite rocks (Plate 4.1) (Bhat, 2003).

Traditionally used for urinary diseases, skin infections, fever (Devkar et al., 2016).

*L. incurva*: It is a perennial herb; erect or prostrate stem. Leaves are small (10×3.5 cm), lanceolate or obovate, acuminate at the apex and decurrent at the base. Flowers are in axillary or terminal 1-sided, often clustered, hairy and have spikes. Corolla is white, with pink or brown spots on lower lip. Capsules are oblong-lanceolate, pubscent at tip. (Plate 4.1) (Bhat, 2003).

Traditionally leaves of the plants are used for the cough, fruits are useful in ear complaint (Panda, 2002).

*L. pungens*: is a erect or ascending herb. It can grow up to 1-1.5 feet. Leaves have spines on the margin and on the tip. 3-4 sharp spinous teeth on either side of the leaf, tip spinious, spikes broadly ovoid, calyx lobes spinous mucronet, woody soft silky hairs inside, 4 stamens, didynamous, capsules 2 seeded. Leaves are 1-1.5 cm long. It is also called as spiny leave lepidagathis. It is found in waste land of Tamilnadu (Plate 4.1).
Plate 4.1 Candidate plants of lepidagathis genus

4.3 Experimental

Material

All the chemicals and reagent used were of analytical grade and purchased from usual sources.
4.3.1 Preparation of extract

The whole plants were shade dried and extracted (50 g) with methanol (250 mL) in a Soxhlet apparatus for 72 h at 68 °C. The extract was filtered through Whatman filter paper no 1. Filterate was dried to a dark brown sticky mass in a rotary evaporator under controlled temperature (38 °C) and reduced pressure. Percentage yield was calculated and noted.

4.3.2 In vitro antiurolithiatic activity

Inhibition of CaOx crystallization was assayed according to the methods described by Patel et al (2012) with slight modifications.

4.3.2.1 Nucleation assay

A solution of 10 mmol/L calcium chloride and 10 mmol/L sodium oxalate were prepared in a buffer containing 0.05 mmol/L Tris-HCl and 0.15 mol/L sodium chloride solution at pH 6.5. Calcium chloride solution (500 µL) was mixed with 200 µL of extract of plants at final concentrations in the range of 0.04–3 mg/mL. Crystallization was started by addition of 500 µL of sodium oxalate solution. Temperature was maintained at 37 ºC and absorbance monitored at 620 nm (0–30 min every 3 s) by kinetic method. The rate of nucleation was calculated by comparing the induction time of CaOx crystallization in the presence or absence of inhibitors. All samples were assayed in triplicate. Cystone® (Himalaya Herbal Healthcare) was used as a positive control (Devkar et al., 2016). The percentage inhibition of nucleation was calculated using the formula: \[1 - \left(\frac{\text{Slope}_{\text{sample}}}{\text{Slope}_{\text{control}}}\right)\] \times 100.

4.3.2.2 Aggregation assay

CaOx crystals were prepared by mixing calcium chloride and sodium oxalate at 50 mmol/L. The solutions were equilibrated to 60 °C in water bath, cooled to 37 °C and kept overnight. The solution was centrifuged to yield CaOx crystals and evaporated at 37 °C. The reaction mixture consisted of CaOx crystals at a concentration of 0.8 mg/mL, 0.05 mol/L Tris-HCl and 0.15 mol/L sodium chloride at pH 6.5. The experiment was conducted at 37 °C in the presence of plant extract at final concentrations in the range of 0.04–3 mg/mL and incubated for 30 min (Patel et al., 2012). All samples were assayed in triplicate. Cystone® was used as positive control. The percentage inhibition of
aggregation was calculated using the formula: 
\[
\left( \frac{\text{Turbidity}_{\text{control}} - \text{Turbidity}_{\text{sample}}}{\text{Turbidity}_{\text{control}}} \right) \times 100.
\]

**Statistical analysis**

The results are expressed as mean ± standard error of mean (SEM) of three determinations. Graph pad prism 5 was used to analyze the data. One way analysis of variance (ANOVA) and post-hoc Tukey’s multiple comparison tests were used to determine the difference between means.

**4.4 Results and discussion**

The nucleation of CaOx crystals in solution was inhibited by the addition of plant extract at different concentrations. The absorbance values were found to decrease with the increase in concentration of extract indicating the decrease in nucleation of CaOx crystals. As shown in (Table 4.1) and (Figure 4.1, 4.2), *L. prostrata* demonstrated highest inhibitory activity with an IC$_{50}$ of 807.27 ± 69.63 µg/mL in nucleation among the screened plants. The *L. prostrata* showed significantly (P < 0.05) higher inhibition of CaOx nucleation than the standard drug Cystone®.

**Table 4.1. In vitro antiurolithiatic activity by inhibition of CaOx crystallization**

(A) Nucleation (B) Aggregation by extract of plants

<table>
<thead>
<tr>
<th>Sample</th>
<th>% w/w yield MeOH</th>
<th>CaOx nucleation IC$_{50}$ (µg/mL)</th>
<th>CaOx aggregation IC$_{50}$ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. prostrata</em></td>
<td>8.1</td>
<td>807.27 ± 69.63$^a$</td>
<td>627.97 ± 39.14$^a$</td>
</tr>
<tr>
<td><em>L. cristata</em></td>
<td>7.3</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><em>L. incurva</em></td>
<td>8.6</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><em>L. pungens</em></td>
<td>9.1</td>
<td>1967.54 ± 61.33$^b$</td>
<td>1859 ± 43.29$^b$</td>
</tr>
<tr>
<td>Cystone</td>
<td>--</td>
<td>1891.67 ± 54.74$^b$</td>
<td>1738.00 ± 15.37$^b$</td>
</tr>
</tbody>
</table>

All values are expressed as mean ± SEM (n=3). $^a$$^b$ Column wise values with different superscripts of this type indicate significant difference (P < 0.05). NA denotes not active.
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Screening for bioactive plant

Figure 4.1: Percentage inhibition of *L. prostrata* and *L. pungens* by Nucleation assay.

Figure 4.2: Percentage inhibition of *L. prostrata* and *L. pungens* by Aggregation assay.
It has been explored previously (Chaudhary et al., 2010) that aggregation is an important contributing factor in CaOx stone formation. Urine from normal people contains few segregated forms of crystals while stone formers urine contains aggregates of CaOx crystals. Hence, aggregation is a critical step, aids in the progression and worsening of the disease (Fleisch, 1978). Incubation with the extract with CaOx crystals (generated from the metastable solutions of Ca\(^{2+}\) and oxalate) resulted in reduced aggregation of CaOx crystals indicated by lower absorbance as compared to the control (absence of extract) in a dose-dependent manner. *L. prostrata* showed the highest percent inhibition of aggregation IC\(_{50}\) of 627.97 ± 39.14 µg/mL that was significantly (\(P < 0.05\)) better than Cystone® (Table 4.1).

Other candidate plants were either found not effective or significantly (\(p<0.05\)) not effective than Cystone in inhibiting calcium oxalate crystals in both the assay. Therefor *L. prostrata* was considered for further detailed study for its antiurolithiatic activity. *L. prostrata* was the candidate plant for bioactivity guided fractionation (BAGF). Nucleation and Aggregation were the choice of assay to assess the antiurolithiatic activity of the plant extract/fractions.
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