CHAPTER 5
GROWTH CHARACTERIZATION OF CALCIUM OXALATE
MONOHYDRATE CRYSTALS INFLUENCED BY COSTUS IGNEUS
AQUEOUS STEM EXTRACT

5.1 INTRODUCTION

Calcium oxalate (CaOx) is a major component of urinary stones that is generally found in two different varieties, calcium oxalate monohydrate (COM), or Whewellite, and calcium oxalate dihydrate (COD), or Weddellite. It is difficult to form urinary stones from COD because COD crystals are unstable and are easily excreted in the urine of both humans and animals (Monje and Baran, 2002; Bouropoulos et al., 2004; Sheng et al., 2005; Anjian et al., 2007; Yongtai et al., 2008; Valarmathi et al., 2010). The formation of COD crystals actually protects against stone disease because of their reduced capacity to form stable aggregates or strong adhesion contacts to renal epithelial cells (Wesson and Ward, 2007) due to the single micron-sized crystals. Urinary stones are characterized by high recurrence rates and would therefore benefit from a preventive treatment using medicinal plants (Fouad et al., 2004; Ahmed Bensatal and Ouahrani, 2008). Costus igneus, also known as Fiery Costus, Spiral Flag or Insulin Plant, belongs to the Costaceae family. It contains a range of phytochemicals (Devi and Urooj, 2010), such as flavonoids, alkaloids and terpenoids, and is traditionally used in India to control diabetes (Devi and Urooj, 2008). Administration of the aqueous extract of Costus spiralis to rats with experimentally induced urolithiasis has been found to reduce the growth of urinary stones (Viel et al., 1999).

5.2 SPECIFIC AIM

The effects of aqueous Costus igneus stem extracts on the nucleation and growth of CaOx crystals, using both gel and liquid growth methods. This study incorporates a multidisciplinary approach for the characterization of COM crystals
grown *in vitro* to facilitate the development of prevention and dissolution strategies aimed at managing urinary stone growth.

### 5.3 MATERIALS AND METHODS

#### 5.3.1 Materials and instruments

Anhydrous ethanol, calcium chloride, sodium oxalate, magnesium acetate, oxalic acid and sodium metasilicate were purchased from Sigma-Aldrich (New Delhi, India) and were labeled analytical grade. All reagents and double distilled water were used without further purification. Fourier Transform Infrared (FTIR) spectra were recorded with a nominal resolution of 4 cm⁻¹ and a wave number range from 400 to 4000 cm⁻¹ using the KBr pellet technique. Powder X-Ray Diffraction (XRD) was performed with a PW1710 diffractometer using CuKα radiation. Scanning Electron Microscopy (SEM) with an accelerating voltage of 20 kV was utilized to analyze our products.

#### 5.3.2 Collection and extraction of plant materials

The medicinal plant *Costus igneus* was collected from the nursery of the Periyar Maniammai University, Vallam, Thanjavur and identified at the Rapinat Herbarium, St. Joseph College, Tiruchirapalli, Tamil Nadu, India. The *Costus igneus* aqueous extract (A.E) was prepared by boiling 25 g of *Costus igneus* stems in 100 mL distilled water for 30 minutes and then filtering through Whatman filter paper twice (Vimal *et al.*, 2005a). The filtrate was condensed using a rotary evaporator and the obtained residue (1.4 g) was used to prepare the series of aqueous supernatant concentrations for *in vitro* studies shown in (Table 5.1).

#### 5.3.3 Growth and characterization of COM crystals with gel methods

Growth of COM crystals was prepared in the procedure as described in chapter III.
5.3.4 The nomenclature of different additive solutions on the growth of COM crystals with the gel method

An attempt was made to investigate the putative activity of the plant extracts as inhibitors of COM crystal formation in the gel method. The supernatant solutions shown in (Table 5.1) were added to the set gels and the results were noted. The experiments were repeated four times. To study the effects of the aqueous extract of *Costus igneus* on the growth of COM crystals, five different concentrations of the plant extracts (see Table 5.1) were added to equal amounts of the supernatant solution, and the average weights of the grown crystals were measured.

**Table 5.1** Supernatant solutions added to the set gels for COM crystals.

<table>
<thead>
<tr>
<th>(Groups and Treatments)</th>
<th>Compositions</th>
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<tbody>
<tr>
<td>I (CONTROL)</td>
<td>10 mL of 1M CaCl₂ + 10 mL of 1M (CH₃COO₂)Mg.4H₂O</td>
</tr>
<tr>
<td>II (DIST.H₂O)</td>
<td>5 mL of 1M CaCl₂ + 5 mL of 1M (CH₃COO₂)Mg.4H₂O + 10 mL of distilled water</td>
</tr>
<tr>
<td>III (0.15% A.E)</td>
<td>5 mL of 1M CaCl₂ + 5 mL of 1M (CH₃COO₂)Mg.4H₂O + 10 mL of 0.15% aqueous extract of stem of <em>Costus igneus</em></td>
</tr>
<tr>
<td>IV (0.25% A.E)</td>
<td>5 mL of 1M CaCl₂ + 5 mL of 1M (CH₃COO₂)Mg.4H₂O + 10 mL of 0.25% aqueous extract of stem of <em>Costus igneus</em></td>
</tr>
<tr>
<td>V (0.50% A.E)</td>
<td>5 mL of 1M CaCl₂ + 5 mL of 1M (CH₃COO₂)Mg.4H₂O + 10 mL of 0.50% aqueous extract of stem of <em>Costus igneus</em></td>
</tr>
<tr>
<td>VI (0.75% A.E)</td>
<td>5 mL of 1M CaCl₂ + 5 mL of 1M (CH₃COO₂)Mg.4H₂O + 10 mL of 0.75% aqueous extract of stem of <em>Costus igneus</em></td>
</tr>
<tr>
<td>VII (1.00% A.E)</td>
<td>5 mL of 1M CaCl₂ + 5 mL of 1M (CH₃COO₂)Mg.4H₂O + 10 mL of 1.0% aqueous extract of stem of <em>Costus igneus</em></td>
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</table>

5.3.5 Growth and characterization of COM crystals with liquid methods

In the liquid method, 15 mL of 5 mmol/L CaCl₂ (Calcium chloride) solution was added to 30 mL of 0.15, 0.25, 0.50, 0.75 and 1.00% (w/v) aqueous *Costus igneus* extract and was thoroughly mixed. The solution was combined with 15 mL of 5 mmol/L Na₂C₂O₄ (Sodium oxalate) solution and was thoroughly mixed again. A corresponding control experiment was carried out by reacting CaCl₂ with Na₂C₂O₄ under the same conditions, without the addition of any additives. The mixtures were
covered with Parafilm and stored at 10°C for 3 days. The products were then carefully extracted from the aqueous solution, washed three times with ethanol and double distilled water, and dried for further characterization using FTIR, powder XRD and SEM technique (Yongtai et al., 2008).

### 5.3.6 Statistical analysis

The masses of the crystals are presented as the mean ± SD for the control and treatment samples. One-way analysis of variance (ANOVA), followed by Tukey’s test for multiple comparisons, was made between the groups shown in (Table 5.2). Values of p <0.05 were considered significant.

### 5.4 RESULTS AND DISCUSSIONS

#### 5.4.1 Effect of Costus igneus extract on COM crystals

The effect of the aqueous Costus igneus stem extract on the nucleation and crystallization characteristics of COM was determined by measuring the mass of the formed crystals. We found that the aqueous extract favored the formation of the dihydrate (metastable) form of calcium oxalate crystals in both the gel and liquid methods. In both the gel and liquid methods, the control solutions led to the maximum nucleation of crystal growth within 24 h of adding the supernatant solutions (Figure 5.1). In the presence of the aqueous Costus igneus stem extract, nucleation was delayed and reduced crystal masses were observed 96 h following addition of the supernatant solutions. As the concentration of aqueous Costus igneus extract increased from 0.15% to 1.00% (w/v), the average weights of the formed crystals gradually decreased from 2.16 g to 0.08 g and from 2.89 g to 0.035 g for the gel and liquid methods, respectively. ANOVA was performed for the crystal masses, and a p <0.05 suggested that the correlation was significant as shown in (Table 5.2). The crystal masses from Group III to VII, treated with various concentrations of aqueous Costus igneus extract ranging from 0.15% to 1%, were significantly different at p <0.05 when compared to Group I, the untreated control. However, Group II, which was treated with distilled water, was not significantly different at p <0.05
compared to Group I. This result indicates that distilled water did not show any inhibitory activity with regard to crystal growth, whereas the aqueous extract of *Costus igneus* possessed inhibitory activity due to the presence of bioorganic molecules (Devi and Urooj *et al.*, 2010). Group VI and VII, treated with 0.75% and 1% aqueous extracts, respectively, were not significantly different. In general, an increase in aqueous extract concentrations resulted in a decrease in the crystal mass due to a reduction in the sizes of the crystals during treatments. Morphology of the harvested crystals changed from hexagonal (COM) to tetragonal (COD) as shown in (Figure 5.2). In the present study, COM crystal growth was reduced, and the morphology of the crystals was altered due to the inhibitory effect of aqueous *Costus igneus* extracts under *in vitro* conditions by the gel and liquid methods, as has previously been reported (Sheng *et al.*, 2005; Vimal *et al.*, 2005a; Beghalia *et al.*, 2007; Beghalia *et al.*, 2008).

### Table 5.2 ANOVA statistical analysis for harvested crystals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatments</th>
<th>Mean(g)±S.D</th>
<th>Gel</th>
<th>Liquid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>Control</td>
<td>2.16±0.05</td>
<td>2.89±0.04</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>Distilled water</td>
<td>2.07±0.09a-ns</td>
<td>2.8±0.08a-ns</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>0.15% extract</td>
<td>1.6 ± 0.08ab</td>
<td>1.07±0.01ab</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>0.25% extract</td>
<td>0.6 ± 0.08abc</td>
<td>0.42±0.05abc</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>0.50% extract</td>
<td>0.31±0.01abcd</td>
<td>0.19±0.02abcd</td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td>0.75% extract</td>
<td>0.11± 0.008abcd</td>
<td>0. 07±0.008abcd</td>
<td></td>
</tr>
<tr>
<td>VII</td>
<td>1.00% extract</td>
<td>0.08± 0.006abcd,f-ns</td>
<td>0.035±0.006abcd,f-ns</td>
<td></td>
</tr>
</tbody>
</table>

Values represent mean (g) ± S.D (n=4)
Comparisons between means are as follows. (a) I vs II-VII, (b) II vs III-VII, (c) III vs IV-VII, (d) IV vs V-VII, (e) V vs VI-VII, (f) VI vs VII.
a-ns, f-ns were not significantly different, Statistical significance were considered to be a p<0.05, b p<0.05, c p<0.05, d p<0.05, e p<0.05.

### 5.4.2 Characterization of harvested crystals

The changes in the morphology of CaOx crystals obtained from SEM studies in the absence and presence of the *Costus igneus* stem extract using both the gel and
liquid methods (Figure 5.3). In the gel method, the CaOx crystals that were formed in the absence of any additive were elongated hexagonal (Figure 5.3(a)) with an average size of 1874.1 x 857.8 μm. In the presence of the Costus igneus stem extract, the CaOx crystals were elongated tetragonal bipyramidal (Figure 5.3(b)) with an average size of 1075.5 x 990.5 μm. In the liquid method (Figure 5.3(c)), the CaOx crystals formed in the absence of any additive were elongated hexagonal with an average size of 0.524 μm and 0.563 μm. In the presence of the Costus igneus stem extract, the CaOx crystals were elongated tetragonal bipyramidal with an average size of 0.291 x 0.239 μm and 0.295 x 0.272 μm (Figure 5.3(d)). The COM crystals grown displayed many different morphologies, but the most prominent faces were (100), (001), (101), (021). COD crystals usually appeared as bipyramids with (101) being the dominant face as was previously reported (Jianming et al., 2003; Qiu et al., 2004).

The FTIR spectra of CaOx crystals obtained in the presence and absence of the Costus igneus extract, using both the gel and liquid methods (Figures 5.4 and 5.5). In (Figure 5.4(a)), the peaks at 1642 and 1330 cm⁻¹ are the main antisymmetric carbonyl stretching bands, and the band at 1031 cm⁻¹ shows C-O stretching. The band at 886 cm⁻¹ is due to C-C stretching, which reveals the presence of two carboxylate anions. This confirms the existence of the oxalate group in COM crystals. In (Figure 5.4(b)), shifts from 1642 cm⁻¹ for COM to 1635 cm⁻¹ for COD as well as from 1330 cm⁻¹ for COM to 1386 cm⁻¹ for COD were observed, and the band at 1007 cm⁻¹ is due to C-O stretching. The band at 877 cm⁻¹ demonstrates a C-C stretch, which reveals the existence of an oxalate group in the COD crystals. In (Figure 5.5(a)), the peaks at 1636 cm⁻¹ and 1326 cm⁻¹ were the main antisymmetric carbonyl stretching bands, and the band at 1024 cm⁻¹ is due to the C-O stretching. This result indicates the presence of a carboxylate anion in COM crystals. In (Figure 5.5(b)), shifts from 1636 cm⁻¹ for COM to 1639 cm⁻¹ for COD as well as from 1326 cm⁻¹ for COM to 1366 cm⁻¹ for COD were observed. In addition, two bands located at 925 cm⁻¹ and 654 cm⁻¹ were also assigned to COD. (Figures 5.4(c) and 5.5(c)) display the FTIR spectra of the raw stem powder of Costus igneus, which is rich in protein, iron, antioxidant components
and phytochemicals (i.e., flavonoids, alkaloids, terpenoids, steroids, saponins and phenolics). (Figures 5.4(d) and 5.5(d)) show the FTIR spectra of the aqueous extract powder of *Costus igneus* containing water-soluble phytochemicals, which were separated according to the polarity of the solvent. There were no impurities found in the *Costus igneus*-treated COD crystals (Figures 5.2(b) and 5.3(b)). The peaks at 1618 cm\(^{-1}\) and 1318 cm\(^{-1}\) are the main antisymmetric carbonyl stretching bands specific to the oxalate family and the metal carboxylate stretch for COM, respectively. The peaks shifted from 1618 cm\(^{-1}\) to 1652 cm\(^{-1}\) or 1647 cm\(^{-1}\) and from 1318 cm\(^{-1}\) to 1328 cm\(^{-1}\) or 1327 cm\(^{-1}\) for COD, as previously reported (Jianming *et al.*, 2003; Sheng *et al.*, 2005; Anjian *et al.*, 2007; Yongtai *et al.*, 2008). The shifting further supports the hypothesis that *Costus igneus* stem extracts favor the nucleation and or transformation of COM into COD.

(Figure 5.6) shows the XRD patterns of CaOxa crystals obtained in the presence and absence of the *Costus igneus* extract using both the gel (Figure 5.6(a) and 5.6(b)) and liquid methods (Figure 5.6(c) and 5.6(d)). The diffraction peaks obtained in both methods correlate well to the (hkl) indices of the COM phase (JCPDS card number 20-231) and the COD phase (JCPDS card number 17-541). As reported previously (Vimal *et al.*, 2005a; Sheng *et al.*, 2005), the diffraction peaks 14.95, 24.39, 30.12 and 33.13 for COM as well as 14.26, 20.01, 24.15, 32.17, 37.21 and 40.00 for COD were assigned. It is inferred from the above results that the *Costus igneus* stem extract affected the nucleation and growth of COD crystals.

5.5 CONCLUSION

COM crystals were grown using both gel and liquid growth methods and were characterized by SEM, FTIR and Powder XRD techniques. The COM crystal growth was reduced, and the morphology of the crystals changed from a hexagonal (COM) to a bipyramidal (COD) form due to the inhibitory action of the aqueous extracts of *Costus igneus* under *in vitro* conditions. FTIR and Powder XRD techniques confirmed the functional groups and crystalline phases of the COM and COD
crystals. SEM studies confirmed the morphology of the changed crystals. It also confirmed that the average size of the crystals was reduced from 1874.1 x 857.8 μm to 1075.5 x 990.5 μm and from 0.524 μm to 0.291 μm for the gel and liquid methods, respectively. One-way ANOVA performed with treated and untreated crystal growth data obtained from both the gel and liquid methods showed significant differences (p < 0.05). This study confirmed that aqueous *Costus igneus* stem extracts promote the formation of COD crystals and may possibly treat urinary stones by inhibiting the formation of COM crystals, which are a major component of urinary stones. Hence, isolation, purification and characterization antiurolithiatic compound from the stem of *Costus igneus* extracts has been carried out in further study.
Figure 5.1 Effect of *Costus igneus* extract on COM crystals (a) without any additive after 24hrs, 48hrs and 7\textsuperscript{th} days (b) with the *Costus igneus* extract after 24hrs, 48hrs and 7\textsuperscript{th} days.
Figure 5.2 Morphology of the harvested COM crystals (a) without any additives, (b) with the 0.15% (w/v) *Costus igneus* extract obtained using the gel methods.
**Figure 5.3** SEM images of CaOxa crystals (a) and (c) without any additives, (b) and (d) with the 0.15% (w/v) *Costus igneus* extract obtained using the gel and liquid methods.
Figure 5.4 FTIR spectra of CaOxa crystals obtained from the gel method (a) without any additives, (b) with 0.15% (w/v) *Costus igneus* extract, (c) raw stem powder of *Costus igneus* and (d) aqueous extract powder of *Costus igneus* stem.
Figure 5.5 FTIR spectra of CaOxa crystals obtained from the liquid method (a) without any additives, (b) with 0.15% (w/v) Costus igneus extract, (c) raw stem powder of Costus igneus and (d) aqueous extract powder of Costus igneus stem.
Figure 5.6 XRD patterns of CaOxa crystals (a) and (c) without any additives, (b) and (d) with the 0.15% (w/v) *Costus igneus* extract obtained using the gel and liquid methods. * denotes COD crystals.