Chapter 5

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5.1 The study in Retrospect

The present study specifically aims to document the indigenous/traditional knowledge of herbal collectors and traditional healers of Kottayam district of Kerala state with special reference to antiurolithiatic medicinal plants. The study also aims to validate their traditional knowledge by subjecting selected medicinal plants for \textit{in vitro} crystallographic assay of CHPD crystallization by employing single diffusion gel growth technique. The details of the study are presented below.

5.1.1 Hypothesis of the study

Present study was designed to test the following hypothesis:

- Traditional practitioners and herbal collectors may have sufficient knowledge about potential antiurolithiatic medicinal plants and their indigenous uses/preparations.
- Gel method of crystallization is an effective strategy for growth dissolution studies of Calcium Hydrogen Phosphate Dihydrate (CHPD) crystals.
- The aqueous extract of \textit{Rotula aquatica} Lour (\textit{Kalloorvanchi}), \textit{Aerva lanata} (L.)Juss. ex Schult (\textit{Cherula}), \textit{Ensete superbum} (Roxb.) Cheesman (\textit{Kalluvazha}), \textit{Achyranthes aspera} L. (\textit{Vankadaladi}) and \textit{Spheranthus indicus} L. (\textit{Adakkamaniyan}) may have the potential to bring out morphological and structural changes in the CHPD under \textit{in vitro} growth conditions.

5.1.2 Objectives of the study

The major objectives of the present study are:

1. To document the traditional botanical knowledge (TBK) of antiurolithiatic medicinal plants from Traditional healers and herbal collectors of Kottayam district, Kerala, India.
2. To standardize the growth of CHPD crystals in metasilicate gel medium.


4. To characterize the gel grown CHPD crystals by FTIR, XRD, TGA/DTA and SEM / EDX.

### 5.2 Methodology in brief

An ethnobotanical survey was conducted among the traditional practitioners and herbal collectors of Kottayam district to document the traditional botanical knowledge of antiurolithiatic medicinal plants. The plant specimens were collected and identified using relevant taxonomic literatures. The voucher specimens were kept in the CMS herbarium, CMS College, Kottayam. Selected medicinal plants Viz., *Rotula aquatica* Lour, *Aerva lanata* (L) Juss. ex Schult, *Ensete superbum* (Roxb.) Cheesman., *Achyranthes aspera* L. and *Spheranthus indicus* L. were subjected to screening for their growth inhibition property by employing crystallographic techniques. Modified Single diffusion gel growth technique in sodium metasilicate gel was employed for screening the antiurolithiatic property of the selected medicinal plants. The crystallization and growth inhibition of CHPD crystals were done using the aqueous extract of selected medicinal plants. The gel grown crystals were then characterized by Fourier transform infrared spectroscopy (FTIR), X-ray powder diffraction (XRD), Thermo gravimetric Analysis (TGA/DTA), Scanning electron microscope (SEM) and Energy dispersive X-ray (EDX) analysis.

### 5.3 Major Findings

The important findings that have emerged from the study are presented below under appropriate heads.

#### 5.3.1 Ethnobotanical studies

- The ethnobotanical investigations have brought to light the potential application of 63 species of ethno-medicinal plants belonging to 42 families for the treatment of urolithiasis.
Medicinal plants viz., Kalloorvanchi [Rotula aquatica Lour.] (77.5%), Cheroola [Aerva lanata (L.) Juss. ex Schult.] (60%), Kalluvazha [Ensete superbum (Roxb.)] (37.5%), Kadaladi [Achyranthes aspera L.] (30%) and Adakkamanian [Sphenanthus indicus L.] (30%) are highly effective not only for dissolution but also for prevention of Kidney stone.

When the plants identified were subjected to family wise categorization, Fabaceae ranked as the dominant family with 5 species followed by Euphorbiaceae (4 species), Cucurbitaceae, Rubiaceae and Poaceae (3 species), Acanthaceae, Asteraceae, Lamiaceae, Lythraceae, Amaranthaceae, Musaceae and Piperaceae (2 species) and the remaining 29 families with a single representation.

Majority of the highly effective antiurolithiatic medicinal plants share identical habitat conditions, either as riverine/wetland component or as lithophytes.

Habit wise break up of identified medicinal plants revealed herbs (54%), followed by trees (19%), Creepers/runners (19%), shrubs (6%) and epiphytes (2%).

For majority of antiurolithiatic medicinal plants, root (34%) is preferred as the officinal part, followed by stem/stem bark (19%), fruits (15%), plant as a whole (12%), leaves (10%), seeds (7%) and rhizome/bulbs (3%).

Herbal medicines prescribed by traditional healers are preparations based on either single plant part or a combination of several plant parts. Generally, fresh plants/plant parts are used for the preparation of medicine. When fresh plant parts are not available, dried parts are also used.

According to the respondents, the mode of medicine preparation varies in accordance with the type of plants required, their mode of action, nature and composition of kidney stone, and severity of the disease. Majority of the plants recommended by the respondents were given in the form of water based or milk based decoction (72%). One-fourth of the plants recommended were used as fresh extract and remaining 3% in the form of paste or powder.
5.3.2 Crystallographic studies

- The gel growth technique by Sodium Meta silicate gel medium is advantageous to mimic the body tissues and serve as a good *in vitro* model to study the growth of bio-materials like Calcium Hydrogen Phosphate Dihydrate (CHPD) crystals.

- Modified gel growth technique (Petri plate method) is useful as a rapid screening test for *in vitro* crystallization of CHPD. It is an effective method to screen the herbal based drugs for the dissolution and growth inhibition of urinary crystals like CHPD.

- The aqueous extract of *Rotula aquatica* Lour (ROTR4), *Aerva lanata* (L) Juss.ex Schult (roots extract, ALR4 & shoot extract, ALS4), *Ensete superbum* (Roxb.) Cheesman (EN4), *Achyranthes aspera* L. (ACHR4) and *Spheranthus indicus* L. (SP4) were found to be effective to bring out morphological and structural changes in the CHPD under *in vitro* growth conditions.

- The efficacies of herbal extracts were studied by measuring the number of Liesegang rings, their width and spacing between them. All the five medicinal plants tested were found to be effective to inhibit the growth of CHPD crystals. In all the five plants, a dose dependent reduction in the number of Liesegang rings was noted with increasing concentration of the extract.

- All the five medicinal plant extracts are effective to make morphological and structural changes in CHPD crystals. The gel grown CHPD crystals (Control) were sharp edged and transparent with sword, star, leaf like or irregular crystal morphology. On the other hand, plant extracts treated crystals (Treatments) were found to be smaller with less transparent and smooth surface, showing different morphology.

- The FTIR studies give direct solid evidence for the incorporation of organic compounds. Compared to CHPD, the FTIR pattern of all the treatments show additional bands, absence of bands or band shift which reflects the incorporation of functional groups such as carboxylic acids, phenolic compounds, chroman derivatives and alkanes.
• Characterization of standard CHPD (control) and plant extract doped CHPD crystals (Treatments) by XRD revealed the basic structural changes in all the plant extract treated crystals. Comparison of XRD pattern of pure CHPD (control) with that of plant extract treated crystals revealed shift in the peak positions, changes in peak intensity and appearance of new peaks. The above structural changes in XRD patterns of the plant extracts treated CHPD thus shows the effectiveness of these plant extracts to modify the crystal structure of CHPD. The XRD patterns in all five treatments thus provide conclusive evidence for the potent antiurolithiatic property of all the five medicinal plants tested.

• The elemental analysis using EDX revealed the incorporation of carbon element in the framework of CHPD. The percentage of carbon incorporation was found to be greater in ROTR4 (6.7%) followed by SP4 (6.57%), ACHR4 (5.86%) ALR4 (4.24%), ALS4 (3.72) and EN4 (2.97%) crystals. This forms another solid evidence for the incorporation of the bioactive compounds within the framework of CHPD.

• The TG/DTA studies of the pure CHPD and plant extract treated crystals revealed different thermal behavior and thermal stability with respect to all five medicinal plants. The total thermal degradation of all the doped crystals was found to be less than that of CHPD. This forms an indirect evidence for the incorporation of organic moiety of plant extract within the framework of CHPD crystals.

• The results of the SEM analysis also revealed the basic structural changes of the treated crystals from pure CHPD crystals. The SEM of CHPD crystals shows a continuous sheet like formations (without any intermediate space) with characteristic etch pits. The SEM of all treatments revealed dissolution of CHPD as evinced from their morphological variations with crumbled, broken or corrugated surface view with lots of space in between crystal aggregates. In all the five treatments, the crystal aggregates show distinct morphological patterns with unique unit measurements.

• The FTIR, EDX and TGA/DTA data of the treated CHPD shows incorporation of the bioactive compounds within the framework of CHPD. It is
assumed that such specific bioactive compounds in anti-urolithiatic medicinal plants either helps in dissolution or may prevent/block further growth and aggregation of CHPD crystals, which in turn alter the morphology and structure of extract treated crystals. These morphological and structural changes are well evident from macroscopic, microscopic, XRD and SEM analysis.

5.4 Tenability of the hypotheses

All the three hypotheses formulated for the present investigation were substantiated based on the results emerged from the ethnobotanical and crystallographic investigations.

5.5 Conclusions

The major conclusions and suggestions based on the present ethnobotanical and crystallographic investigation of selected antiurolithiatic medicinal plants are noted below:

The present study had brought to light the potential application of a total of 63 species of ethno-medicinal plants belonging to 42 families. As revealed in the present investigation, *Rotula aquatica* Lour, *Aerva lanata* (L) Juss.ex Schult, *Ensete superbum* (Roxb.) Cheesman., *Achyranthes aspera* L. and *Sphaeranthus indicus* L. were ranked as highly useful indigenous medicinal plants for the treatment of kidney stones. The antiurolithiatic potential of aqueous extract of these medicinal plants were assessed by studying their effect on growth inhibition of CHPD crystallization under *in vitro* conditions employing single diffusion gel growth technique. All the tested plants (*Rotula aquatica*, *Ensete superbum*, *Aerva lanata*, *Sphaeranthus indicus* and *Achyranthes aspera*) have shown remarkable results with potent antiurolithiatic property. The plant extract added treatments showed a gradual reduction in the number of Liesegang rings and reduction in growth of CHPD crystals with increasing concentration of the extract. Also, the crystal morphology got transformed leading to the formation of a large number of smaller crystals with less transparent and smooth surface. Moreover, the frequency of the sharp edged and larger crystals or crystal aggregates were much reduced in treatments. These
findings are in agreement with the results of earlier studies based on a glycoprotein inhibitor of calcium oxalate crystal growth (Nakagawa et al., 1987; Coe et al., 1991). As pointed out by Parekh et al. (2007), change in morphology of crystal is an important phenomenon because, if the painful star type or spiky, needle, irregular stones were converted into smooth spherical or oval grain like ones, then, their passage through the urethra is less painful.

The FTIR studies of the control CHPD and plant extract treated crystals provide substantial evidence for the incorporation of organic compounds. Compared to CHPD, the FTIR pattern of all the plant extract treated crystals shows addition, deletion or shift of bands which revealed the incorporation of Carbon containing compounds (Carboxilic acids, phenolic compounds, chroman derivatives and alkanes) from antiurolithiatic medicinal plants within the framework of CHPD. The EDX results of plant extract treated CHPD crystals give further evidence for the incorporation of carbon moiety. The EDX results of the doped crystals shows the incorporation of Carbon (ROTR4 crystals 6.7%, ALR4 crystals 4.24%, ALS4 crystals 3.72%, EN4 crystals 2.97%, ACHR4 crystals 5.86% and SP4 crystals 6.57%) elements in the framework of CHPD.

The XRD pattern of the gel grown CHPD crystals (control) matches well with JCPDS data (72-0713). However, compared to XRD pattern of CHPD, the XRD pattern of all the treatments shows shift in the peak positions, change in peak intensity and appearance of new peaks which shows the effectiveness of the plant extracts to inhibit/ reduce the growth of CHPD crystals. The XRD patterns confirm the structural modification of the treated CHPD crystals.

The TG/DTA studies provide the thermal degradation pattern of the control and treated crystals and thereby forms indirect evidence for the incorporation of carbon compounds. The TG/DTA pattern of CHPD (control) obtained during the present investigation matches with the standard TG/DTA pattern of CHPD, whereas, the TG/DTA pattern of all the plant extract treated crystals showed altered thermal behavior and thermal stability. The total thermal degradation of all the doped crystals was found to be less than that of CHPD.
The results of the SEM analysis revealed the basic structural changes of the treated crystals from that of pure CHPD crystals. The SEM of CHPD crystals shows a continuous sheet like formations with characteristic etch pits. The SEM of all CHPD treatments shows a structure with irregular crystal aggregates with lots of gaps or spaces in between crystal units. The surface morphology of the crystal units is unique with unit measurements different with respect to different medicinal plants. Earlier researchers had studied the growth morphology of CHPD crystals and stated it to be typical in form of thin plates with prominent (010) and lateral (401) faces. The structure within the (010) plane is composed of two corrugated rows of Ca2+ and HPO₄²⁻ that are offset in the <010> direction. Between these calcium and phosphate containing sheets, are layers of water molecules bound to the calcium ions above and below the (010) plane (Legeros and Legeros, 1971). It is assumed that when the interconnecting layers of water molecules, that bound to the calcium ions above and below the (010) plane, is unavailable for bonding due to incorporation of bioactive compounds in plant extract, further growth of CHPD crystals may get arrested. This explains one of the possible mechanisms for the efficacy of the extracts of plants on growth inhibition of CHPD crystals under in vitro conditions. It is assumed that this incorporation of the bioactive compounds within the framework of CHPD might have blocked further growth, arresting the aggregation of CHPD crystals.

It is expected that this multidisciplinary approach for in vitro crystallization and characterization of CHPD crystals will provide a better explanation to develop novel strategies for prevention or dissolution of urinary stones. The results of the crystallographic investigation confirmed the traditional antiurolithiatic property of all the five indigenous medicinal plants. However, further studies are needed to isolate, characterize and also to develop suitable drugs for urolithiasis based on clinical trials.