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**Research Article**

# EFFECT OF *ALPINIA GALANGA* ROOT ON CALCIUM HYDROGEN PHOSPHATE DIHYDRATE (CHPD) CRYSTALS

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**A B S T R A C T**

To investigate the inhibitory effect of methanol extract of *Alpinia galanga* root on the growth of calcium hydrogen phosphate dehydrate (CHPD) crystals. CHPD crystals were grown by the single diffusion gel growth technique and the inhibitory effect of methanol extracts of *Alpinia galanga* root on the growth of CHPD crystals has been studied. The grown crystals were characterized by Fourier Transform Infrared Spectroscopy (FTIR) and Powder X-Ray diffraction (XRD) methods for further confirmations. With an increase in the concentration of methanol extract of *Alpinia galanga* root, the weight of the formed crystals were gradually reduced from 2.84 g to 0.44 g in CHPD crystals, respectively. The crystals are harvested from the CHPD were characterized by Fourier Transform Infrared Spectroscopy (FTIR) to confirm the functional groups and Powder X-Ray Diffraction technique (XRD) analysis to confirm the crystalline phases of the CHPD crystals. Results obtained are indicated that *Alpinia galanga* root has the potential to inhibit the formation of CHPD crystals. This study confirms that using methanol extract of *Alpinia galanga* root can promote the formation of hydroxyapatite (HAP) crystals and reduce the nucleation rate of CHPD crystals, a major component of calcium urinary stone.



## INTRODUCTION

A large number of people are suffering from problems due to urinary stones (Mohamed *et al*., 2007). Urinary stones have been found to contain calcium phosphate, calcium oxalate, uric acid and magnesium ammonium phosphate with apatite and sruvites predominating (Tiwari *et al*., 2012; Beghalia *et al*., 2008; Aggarwal *et al*., 2000). Epidemiological data collected during several decades showed that the majority of stones, up to 80%, are composed mainly of calcium oxalate (CaOx) (Daudon *et al*., 1993). Calcium containing stones are the most common comprising about 75% of all urinary calculi, which may be in the form of pure calcium oxalate (50%) or calcium phosphate (5%) and a mixture of both (45%) (Joshi *et al*., 2005; El-Shall *et al*., 2004). Calcium oxalate stones are found in two different varieties, calcium oxalate monohydrate or whewellite and calcium oxalate dihydrate or weddellite (Bouropoulos *et al*., 2004; Anjian *et al*.,2 007; Monje and Baran, 2002; Valarmathi *et al*., 2010; Yongtai *et al*., 2008; Sheng *et al*., 2005). Calcium phosphate is present in urinary calculi as either apatite ($Ca_{10}(PO_4)_6(OH)_2$ or brushite ($CaHPO_{4\cdot2}H_2O$) (Rajendran and Dale keefe, 2010; Doddametikurke *et al*., 2007; Madhurambal *et al*., 2009).

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Brushite form a smooth (010) surface which allows the formation of hydroxyapatite (HAP) on brushite (Prasobh and Revikumar, 2011). Urinary stones are characterized by high recurrence rate therefore requiring a preventive treatment using medicinal plants (Fouad *et al*., 2004; Bensatal and Ouahrani, 2008).

*Alpinia galanga* root are commonly known as Golongan, Kholongan belongs to the family of Zingiberaceae (Kong and Qin, 2002). The leaves, flowers and barks of *Alpinia galanga* root are used to treat hypertension, tumor, pain, gastritis, scabies, bleeding piles, dysentery, scorpion poison, skin diseases and malaria. Medicinal uses of *Alpinia galanga* root contains anti-pyretic, anti-depressant, analgesic, anti-septic, anti-inflammatory, anti-protozoal, anti-cancer, and anti-ulcer activities (Matsuda *et al*., 2003; Zhu *et al*., 2000; Akhtar *et al*., 2002). The chemical constituents shows that the presence of volatile oil, chiefly sesquiterpene, hydrocarbons, sesquiterpene alcohols, gingerole., starch, tannins flavonoids like galangin (Cheah and Gan, 2000; Bisset and Wichtl, 2001; Altman and Marcussen, 2001; Warrier and Ramankutty, 1994). In the present investigation, the effects of methanol extract of *Alpinia galanga* root are used as additives to induce the nucleation and growth of CHPD crystals by single diffusion gel growth technique and are reported for the first time. This study incorporated a multidisciplinary approach in characterizing CHPD crystals grown *in vitro* to help formulate prevention or

dissolution strategies in controlling calcium urinary stone growth.

## MATERIALS AND METHODS

### *Materials and instruments*

Anhydrous ethanol, calcium chloride, magnesium acetate, oxalic acid, sodium metasilicate, orthophosphoric acid were all purchased from sigma-aldrich (New Delhi, India) analytical grade. 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 based type set up using CuKα radiation.

### *Collection of plant material*

The *Alpinia galanga* roots were collected in the month of June from the bishop heber college, Trichy, Tamil Nadu, India. The plant was identified and confirmed by Dr. S. John Britto, Director, Rapinat herbarium, St. Joseph College, Tiruchirapalli, Tamil Nadu. The voucher specimen number PP001 dated 14.07.2016.

### *Preparation of methanol extracts*

The *Alpinia galanga* root were washed in running water, cut into small pieces and then shade dried for a week at 35-40ºC, after that it was grinded to a uniform powder of 40 mesh size (Joshi *et al*., 2005). The methanol extracts were prepared by soaking 100 g of the dried powder plant materials in 1 L of methanol by using a soxhlet extractor continuously for 10 hr. The extracts were filtered through whatmann filter paper No. 42 (125mm). The filtered extract was concentrated and dried by using a rotary evaporator under reduced pressure. The obtained residue 1.2 g (leaves) was used to prepare the series (0.15, 0.25, 0.50, 0.75 and 1.0%) of methanol supernatant concentrations for *in vitro* studies (table 1).

**Table 1** Supernatant solutions added to the set gels for CHPD crystals

| Supernatant Solutions (SS) (Groups and Treatments) | Compositions |
|---|---|
| I (Control) | 10 ml of 1 M calcium chloride |
| II (Distilled water) | 5 ml of 1 M calcium chloride +5 ml of distilled water |
| III (0.15% methanol extract) | 5 ml of 1 M calcium chloride +5 ml of 0.15% of methanol extract of *Alpinia galanga* root separately |
| IV (0.25% methanol extract) | 5 ml of 1 M calcium chloride +5 ml of 0.25% of methanol extract of *Alpinia galanga* root separately |
| V(0.50% methanol extract) | 5 ml of 1 M calcium chloride +5 ml of 0.50% of methanol extract of *Alpinia galanga* root separately |
| VI(0.75% methanol extract) | 5 ml of 1 M calcium chloride +5 ml of 0.75% of methanol extract of *Alpinia galanga* root separately |
| VII(1.00% methanol extract) | 5 ml of 1 M calcium chloride +5 ml of 1.00% of methanol extract of *Alpinia galanga* root separately |

### *Growth and characterization of CHPD crystals*

Glass test tubes were used as a crystallization apparatus and the single diffusion reaction technique was employed. 1M Ortho phosphoric acid was mixed with the sodium metasilicate ($Na_2SiO_3$•$9H_2O$) solution (density 1.04g/$cm^3$ at pH 9.4), so that the pH of the mixture was maintained at 5 and left undisturbed for 2-3 days. After gelation took place, a supernatant solution of 1 M calcium chloride ($CaCl_2$) was gently poured onto the set gel. After adding the supernatant solution, the test tubes were capped airtight. All experiments were conducted at a temperature of 37 ± 2°C. The grown CHPD crystals were characterized using FTIR, powder XRD techniques to verify the structure and proper formation of the grown crystals. The grown CHPD crystals were characterized using FTIR to verify the compound and structure of the grown crystal. FTIR was performed by Hitachi 570 FT-IR spectrophotometer technique to verify the proper formation of crystal and their purity (Joshi *et al*., 2007).

### *The nomenclature of different additive solution on the growth of CHPD crystals*

An attempt was made to study the effect of the methanol extract of *Alpinia galanga* root on the growth of CHPD crystals in gel method. The supernatant solutions as given in (table 1) were added to the set gels and the results were noted. The experiments were repeated four times, to study the effect of the aqueous extract of five medicinal plants on the growth of CHPD crystals, a series of five different concentrations of 0.15, 0.25, 0.50, 0.75 and 1.00% of these each plant extracts were added in equal amounts in supernatant solution and the average weight of the grown crystal were measured.

### *Statistical analysis*

The masses of the crystals (gm) are presented as the mean ±standard deviation for the control and treatment samples. One-way analysis of variance (ANOVA) followed by tukey's test for multiple comparisons were made between groups. Values of $p<0.05$ was considered to be significant.

## RESULTS AND DISCUSSIONS

### *Effect of Alpinia galanga root on CHPD crystals*

The effect of the methanol extract of the *Alpinia galanga* root on nucleation and crystallization characteristics of CHPD crystals is determined by measuring the weight of the formed crystals. The control using pure calcium chloride led to the nucleation of crystal growth within 24 h of adding the supernatant solutions. The liesegang ring was observed after 48 h of pouring the supernatant solution. The formation of liesegang (5-10 rings) rings which have promoted crystals growth as observed in the present study (fig. 1a).

However, at the same time the first few liesegang rings started diffusion. The distance between two consecutive liesegang rings was found to be increased towards bottom of the test tubes. The elongated broad needle shaped crystals were grown within the liesegang ring as observed after 96 h. In the presence of methanol extract of *Alpinia galanga* root, nucleation was delayed and reduced masses of the crystals were observed after adding the supernatant solutions (fig. 1b-g). The liesegang rings formation was reduced after the addition of methanol *Alpinia galanga* root extracts. Moreover, supernatant solutions (methanol *Alpinia galanga* root) exhibited an inhibitive effect compared to control (pure calcium chloride), and a minimum apparent length of growing crystals was observed. CHPD growth habit was observed during and after harvesting crystals from the gel systems. Morphology of the harvested CHPD crystals as shown in (fig. 2). The largest single CHPD crystals having dimensions of 3 cm and 2.3 cm as observed in (fig.3a). The sizes of the CHPD

crystals were reduced from 3 cm to 0.6 cm at 1.00% concentration of extracts observed in (figs. 3b-g). With an increase in the concentration of methanol extracts of *Alpinia galanga* root from 0.15% to 1.00% (w/v), the weight of the formed crystals were gradually reduced from 2.35 g to 0.13 g (leaves) respectively. The ANOVA statistical analysis was performed for masses of CHPD crystals have been evaluated, and $p<0.05$ has suggested that the correlation is significant as shown in (table. 2).In the present work, CHPD crystals growth were reduced due to the inhibitory effect of methanol extracts of *Alpinia galanga* root under *in vitro* conditions.

Recently, growth inhibition studies of Struvite crystals in the presence of some of the herbal extracts (Prasobh and Revikumar, 2011; Fouad *et al*., 2004, Habib *et al*., 2007) [17,18,31] were attempted in literature. In the present work, CHPD crystals growth was reduced due to the inhibitory effect of *Alpinia galanga* root under *in vitro* conditions. This result indicates that distilled water did not show any inhibitory activity with regard to crystal growth, whereas the methanol extract of *Alpinia galanga* root possessed inhibitory activity due to the presence of bioorganic molecules volatile oil, chiefly sesquiterpene, hydrocarbons, sesquiterpene alcohols, gingerole., starch, tannins flavonoids like galangin (Cheah and Gan, 2000; Bisset and Wichtl, 2001; Altman and Marcussen, 2001; Warrier and Ramankutty, 1994)

**Table 2** ANOVA statistical analysis for harvested CHPD crystals

| Crystals | Group | Treatents | Mean (gm)±SD |
|---|---|---|---|
| Struvite | A | Control | 2.84±0.014 |
| | B | Control+ Distilled water | 2.63±0.057 |
| | C | Control+0.15% extracts | $1.47\pm0.014^{a,b}$ |
| | D | Control+0.25% extracts | $0.68\pm0.014^{a,b,c}$ |
| | E | Control+0.50% extracts | $0.41\pm0.014^{a,b,c,d}$ |
| | F | Control+0.75% extracts | $0.38\pm0.014^{a,b,c,d,e}$ |
| | G | Control+1.00% extracts | $0.44\pm0.014^{a,b,c,d,e,f\text{-ns}}$ |

Values represent mean (gm) ± S.D (n=4) Comparisons between means are as follows. a: A *vs* B-G, b: B *vs* C-G, c: C *vs* D-G, d: D *vs* E-G, e: E *vs* F-G, f: F *vs* G. Statistical significance were considered to be $^{a}p<0.05$,$^{b}p<0.05$, $^{c}p<0.05$, $^{d}p<0.05$, $^{e}p<0.05$.

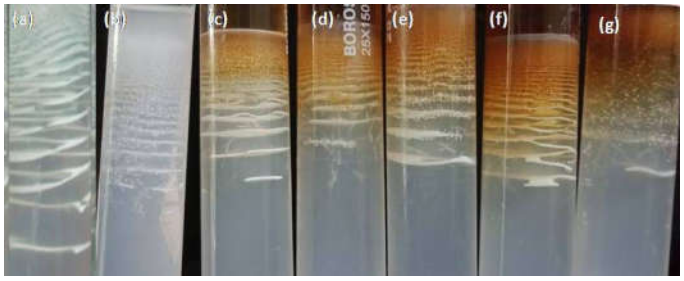


**Fig 1** The effect of *Alpinia galanga* root on CHPD crystals in the gel method (A) without any additive (B) with the distilled water (C) with the 0.15% methanol extract (D) with the 0.25% methanol extract (E) with the 0.50% methanol extract (F) with the 0.75% methanol extract (G) with the 1.00% methanol extract after 7 days.

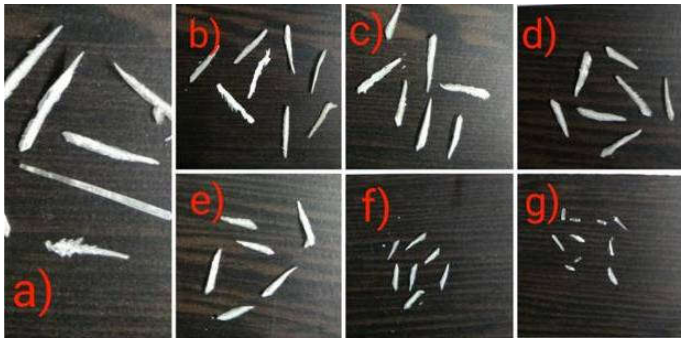


**Fig 2** The harvested crystals of CHPD obtained from *Alpinia galanga* root in the gel method (a) without any additive (b) with the distilled water (c) with the 0.15% of methanol extract (d) with the 0.25% of methanol extract (e) with the 0.50% of methanol extract (f) with the 0.75% of methanol extract (g) with the 1.00% of methanol extract.

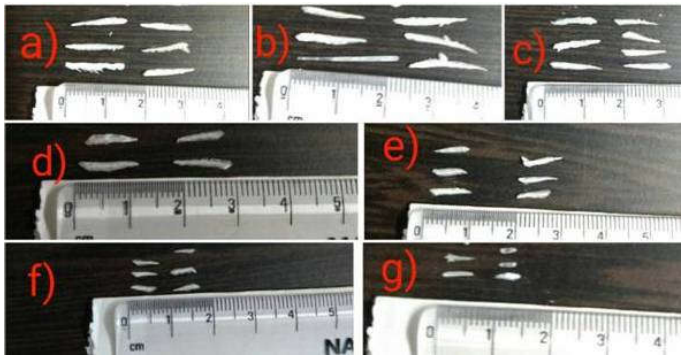


**Fig 3** The measurement of CHPD obtained from *Alpinia galanga* root in the gel method (a) without any additive (b) with the distilled water (c) with the 0.15% of methanol extract (d) with the 0.25% of methanol extract (e) with the 0.50% of methanol extract (f) with the 0.75% of methanol (g) with the 1.00% of methanol extract.

### *Characterization of CHPD crystals*

The FTIR spectra of CHPD crystals obtained in the presence and absence of the methanol extract of *Alpinia galanga* root are shown in (fig. 4). In Fig. 4a, the absorptions at 3490 $cm^{-1}$ are due to intermolecular and weakly H bonded OH because of water of crystallization. The weak absorption at 2378 $cm^{-1}$ is due to $HPO_4^{2-}$. The H-O-H bending gives rise to absorption at 1650 $cm^{-1}$. The absorption at 1217 and 1133 $cm^{-1}$ are due to P=O associated stretching vibrations. Whereas, the absorption at 1064 $cm^{-1}$ is due to P=O stretching vibrations. The P-O-P asymmetric stretching vibrations give rise to absorption at 990, 872 $cm^{-1}$. The absorption at 666 $cm^{-1}$ is due to (H-O-) P=O. However, the strong absorption at 576 and 526 $cm^{-1}$ are again due to acid phosphate. In (fig. 4b), the absorption at 3486 $cm^{-1}$ is due to OH ions. The absorption at 1066 $cm^{-1}$ is due to $PO_4$ stretching vibrations. Whereas, the absorption at 990, 872 and 776 $cm^{-1}$ are due to P-O-P asymmetric stretching vibrations. The absorption at 666, 575 and 527 $cm^{-1}$ are again due to acid phosphate. In (fig. 4c), the absorption at 3484 $cm^{-1}$ is due to OH ions. The absorption at 1066 $cm^{-1}$ is due to $PO_4$ stretching vibrations. Whereas, the absorption at 991, 871 and 774$cm^{-1}$ are due to P-O-P asymmetric stretching vibrations. The absorption at 665, 575 and 527 $cm^{-1}$ are again due to acid phosphate. In (fig. 4d), the absorption at 3471 $cm^{-1}$ is due to OH ions. The absorption at 1068 $cm^{-1}$ is due to $PO_4$ stretching vibrations. Whereas, the absorption at 990, 872 and 774$cm^{-1}$ are due to P-O-P asymmetric stretching vibrations. The absorption at 664, 575 and 527 $cm^{-1}$ are again due to acid phosphate. In (fig. 4e), the absorption at 3468 $cm^{-1}$ is due to OH ions. The absorption at 1068 $cm^{-1}$ is due to $PO_4$ stretching vibrations. Whereas, the absorption at 991, 871 and 774$cm^{-1}$

are due to P-O-P asymmetric stretching vibrations. The absorption at 664, 575 and 526 $cm^{-1}$ are again due to acid phosphate. In (fig. 4f), the absorption at 3423 $cm^{-1}$ is due to OH ions. The absorption at 1012 $cm^{-1}$ is due to $PO_4$ stretching vibrations. Whereas, the absorption at 882 and 762 $cm^{-1}$ are due to P-O-P asymmetric stretching vibrations. The absorption at 568 $cm^{-1}$ are again due to acid phosphate. At higher concentration of methanolic extract of *Alpinia galanga* root (1.00%) shifting from brushite crystals band at 1064 $cm^{-1}$ to hydroxyapatite crystals band at 1012 $cm^{-1}$. The shifting further supports that the *Alpinia galanga* root favour the nucleation and or transformation of brushite into hydroxyapatite crystals (Habib et al., 2007; Joshi and Joshi, 2003).

The XRD patterns of CHPD crystals obtained in the presence and absence of the methanol extract of *Alpinia galanga* root are shown in (fig. 5). The diffraction peaks obtained were well correlated to the (hkl) indices of CHPD phase (JCPDS card number 09-0077) and the hydroxyapatite phase (JCPDS card number 9-432). It is inferred from the above results that the *Alpinia galanga* root effected the nucleation and growth of hydroxyapatite crystals.

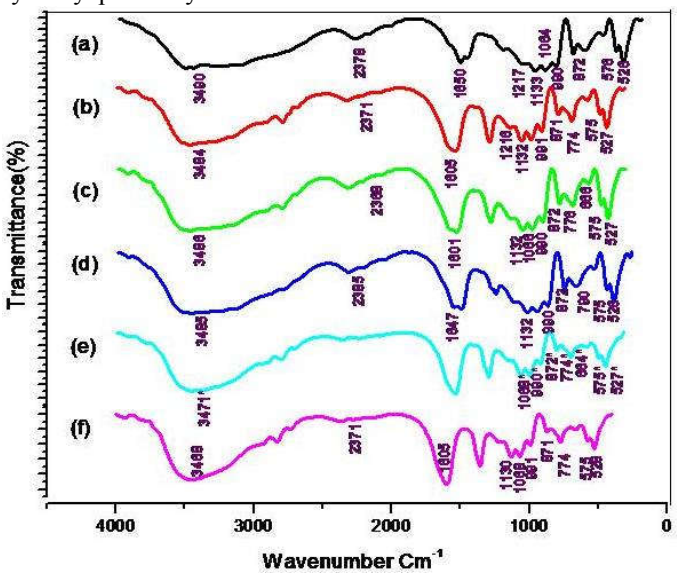


**Fig 4** The FTIR spectra of CHPD obtained from *Alpinia galanga* root in the gel method (a) without any additive (b) with the 0.15% of methanol extract (c) with the 0.25% of methanol extract (d) with the 0.50% of methanol extract (e) with the 0.75% of methanol extract (f) with the 1.00% of methanol extract.

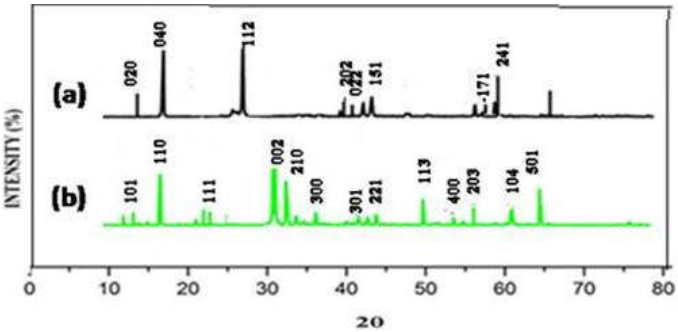


**Fig 5** The XRD pattern of CHPD obtained from *Alpinia galanga* root in the gel method (a) without any additive (b) with the 1.00% of methanol extract.

## CONCLUSION

CHPD crystals were grown by single diffusion gel growth techniques and characterized by FTIR and Powder XRD techniques for the experimental confirmations of the grown crystal. With an increase in the concentration of methanol extract of *Alpinia galanga* root the weight of the formed crystals were gradually reduced from 2.84 g to 0.34 g in CHPD crystals, respectively. FTIR and Powder XRD techniques confirmed its functional groups and crystalline phases of struvite crystals. One way ANOVA performed with treated and untreated crystal growth data obtained from CHPD crystals showed significant differences ($p<0.05$). This study confirmed that the *Alpinia galanga* root extracts can promote the formation of hydroxyapatite crystals and treat urinary stone by inhibiting the formation of CHPD crystals, a major component of calcium urinary stone.

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**Conflicts of interests**

The authors declare that they have no conflict of interest. It has not been published elsewhere. That it has not been simultaneously submitted for publication elsewhere. All authors agree to the submission to the journal.

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