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Original Article

GROWTH CHARACTERIZATION OF CALCIUM HYDROGEN PHOSPHATE DIHYDRATE CRYSTALS INFLUENCED BY *COSTUS IGNEUS* AQUEOUS EXTRACT

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ABSTRACT

Objective: To investigate the inhibitory effect of aqueous extract of leaves, stems and rhizome of *Costus igneus* on the growth of calcium hydrogen phosphate dihydrate (CHPD) crystals.

Methods: Calcium hydrogen phosphate dihydrate (CHPD) crystals were grown by the single diffusion gel growth technique and the inhibitory effect of aqueous extracts of leaves, stems and rhizome of *Costus igneus* on the growth of CHPD crystals has been studied. The grown crystals were characterised by Fourier Transform Infrared Spectroscopy (FTIR), Powder X-Ray diffraction (XRD) for further confirmations.

Results: With an increase in the concentration of aqueous extract of *Costus igneus*, the weight of the formed crystals were gradually reduced from 2.03 g to 0.06 g (leaves), 0.05 g (rhizome), 0.03 g (stem) for the CHPD crystals, respectively. The crystals harvested from the CHPD were characterised by Fourier Transform Infrared Spectroscopy (FTIR) to confirm the functional groups, and Powder X-Ray Diffraction technique (XRD) analyses to confirm the crystalline phases of the CHPD and hydroxyapatite (HAP) crystals. Results obtained indicated that *Costus igneus* (leaves, stems and rhizome) has the potential to inhibit the formation of calcium hydrogen phosphate dihydrate crystals.

Conclusion: This study confirms that using an aqueous extract of stem and rhizome of *Costus igneus* can promote the formation of hydroxyapatite (HAP) crystals and reduce the nucleation rate of CHPD crystals, a major component of calcium urinary stone.

Keywords: Calcium phosphate, Hydroxyapatite, *Costus igneus*, Fourier Transform Infrared Spectroscopy (FTIR), Powder X-Ray diffraction (XRD)

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INTRODUCTION

A large number of people are suffering from problems due to urinary stones [1]. Urinary stone is the formation of urinary calculi at any level of the urinary tract. It is estimated that 12% of world population experiences renal stone disease with a recurrence rate of 70-80% in male and 47-60% in female [2]. Urinary stones have been found to contain calcium phosphate, calcium oxalate, uric acid and magnesium ammonium phosphate with apatite and sruvites predominating [3, 4]. Epidemiological data collected during several decades showed that the majority of stones, up to 80%, are composed mainly of calcium oxalate (CaOx) [5]. 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%) [6, 7]. Calcium oxalate stones are found in two different varieties, calcium oxalate monohydrate or whewellite and calcium oxalate dihydrate or weddellite [8-13]. Calcium phosphate is present in urinary calculi as either apatite ($Ca_{10}(PO_4)_6(OH)_2$ or brushite ($CaHPO_{4\cdot2}H_2O$) [13-16].

These calcium oxalate and calcium phosphate chemicals are part of a person's normal diet and make up important parts of the body, such as bones and muscles [17]. Urinary stones are characterised by high recurrence rate, therefore, requiring a preventive treatment using medicinal plants [18, 19]. *Costus igneus* also known as fiery costus or spiral flag or insulin plant, belonging to the costaceae family are rich in protein (18%), iron (40 mg) and antioxidant components such as ascorbic acid, β-carotene, α-Tocopherol, glutathione, phenols, flavonoids (diosgenin, quercetin), steroids, alkaloids and terpenoids, and is traditionally used in India to control diabetes [20, 21]. Administration of the water extract of *Costus spiralis* to rats with experimentally induced urolithiasis were found reduced the growth of urinary stones [22]. The blood glucose levels were controlled in alloxan induced diabetes in rats after administration of ethanolic extract of leaves of *Costus igneus* [23]. The constant use of *Costus pictus* enhances low-density lipoprotein (LDL) to high-density lipoprotein (HDL) cholesterol ratio due to higher levels (24.51% in leaves, 28.30% in the stem and 25.26% in rhizome) of hexadecanic acid found in diethyl ether extractions [24]. Bioactive compound quercetin and diosgenin were isolated from *Costus igneus* rhizome by high-performance thin layer chromatography (HPTLC). These compounds exhibit antioxidant activity, which was sufficient to reverse oxidative stress in the liver, pancreas and kidney of diabetic rats [25]. In the present investigation, the effects of aqueous extract of leaves, stems and rhizomes of *Costus igneus* 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 and extraction of plant materials

The medicinal plants *Costus igneus* (leaves, stems and rhizomes) and the specimen name spiral flag, fiery costus used in this experiment were collected from the herbal garden of the Periyar Maniammai University, Vallam, Thanjavur dated 10.02.2009 and identified at Rapinat herbarium St. Joseph College, Tiruchirapalli, Tamil Nadu,

India dated 1.06.2009. The aqueous extract of *Costus igneus* was prepared with 25g of the shade dried leaves, stems and rhizomes of *Costus igneus* boiled in 100 ml distilled water for 30 min and then filtered through whatmann filter paper twice [6]. The filtrate was condensed using a rotary evaporator and the residue 1.2 g (leaves), 1.5 g (stems) and 0.8 g (rhizomes) obtained were used to prepare the series (0.15, 0.25, 0.50, 0.75 and 1.0%) of aqueous supernatant concentrations for *in vitro* studies (table 1).

Growth 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 ($Na_2SiO_3 \bullet 9H_2O$) 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 d. 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 characterised using FTIR, powder XRD techniques to verify the structure and proper formation of the grown crystals.

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% Aqueous extract)	5 ml of 1 M calcium chloride+5 ml of 0.15% of aqueous extract of leaves, stems and rhizomes of *Costus igneus* separately
IV (0.25% Aqueous extract)	5 ml of 1 M calcium chloride+5 ml of 0.25% of aqueous extract of leaves, stems and rhizomes of *Costus igneus* separately
V (0.50% Aqueous extract)	5 ml of 1 M calcium chloride+5 ml of 0.50% of aqueous extract of leaves, stems and rhizomes of *Costus igneus* separately
VI (0.75% Aqueous extract)	5 ml of 1 M calcium chloride+5 ml of 0.75% of aqueous extract of leaves, stems and rhizomes of *Costus igneus* separately
VII (1.00% Aqueous extract)	5 ml of 1 M calcium chloride+5 ml of 1.00% of aqueous extract of leaves, stems and rhizomes of *Costus igneus* separately

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

An attempt was made to investigate the putative activity of the plant extracts as inhibitors of CHPD crystal formation 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 leaves, stems and rhizomes of *Costus igneus* 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 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 was made between groups. Values of $p<0.05$ was considered to be significant.

RESULTS

Effect of *Costus igneus* on CHPD crystals

The effect of the aqueous extract of the leaves, stems and rhizomes of *Costus igneus* 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 iesegang 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 the 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 aqueous leaves, stems and rhizomes of *Costus igneus*, nucleation was delayed and reduced masses of the crystals were observed after adding the supernatant solutions.

The liesegang rings formation was reduced after the addition of aqueous *Costus igneus* extracts. The liesegang (3 rings) rings were observed in (fig. 1b). The liesegang (2 rings) rings were observed in (fig. 1c). No liesegang rings formation was observed in (fig. 1d). Moreover, all of these three supernatant solutions (aqueous leaves, stems and rhizomes of *Costus igneus*).

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).

Table 2: ANOVA statistical analysis for harvested CHPD crystals

Groups and treatments	Mean weight of the CHPD crystals (gm)±SD		
	Leaves	Stems	Rhizomes
I (Control)	2.0323±0.05377	2.0323±0.05377	2.0323±0.05377
II (Distilled water)	$2\pm0.03742^{a\text{-ns}}$	$2\pm0.0374^{a\text{-ns}}$	$2\pm0.03742^{a\text{-ns}}$
III (0.15% Aqueous extracts)	$1.185\pm0.02646^{a,b}$	$1.0375\pm0.06292^{a,b}$	$1.1975\pm0.05123^{a,b}$
IV (0.25% Aqueous extracts	$0.5875\pm0.01708^{a,b,c}$	$0.3975\pm0.02217^{a,b,c}$	$0.45\pm0.04082^{a,b,c}$
V (0.50% Aqueous extracts	$0.2825\pm0.00957^{a,b,c,d}$	$0.1975\pm0.00957^{a,b,c,d}$	$0.2325\pm0.01258^{a,b,c,d}$
VI (0.75% Aqueous extracts	$0.1225\pm0.01708^{a,b,c,d}$	$0.08\pm0.00816^{a,b,c,d}$	$0.1125\pm0.0095^{a,b,c,d}$
VII (1.00% Aqueous extracts	$0.0625\pm0.00957^{a,b,c,d,e,f\text{-ns}}$	$0.035\pm0.00577^{a,b,c,d,e,f\text{-ns}}$	$0.0575\pm0.005^{a,b,c,d,e,f\text{-ns}}$

Values represent mean (gm)±SD (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 $^{a}p<0.05$,$^{b}p<0.05$,$^{c}p<0.05$,$^{d}p<0.05$,$^{e}p<0.05$.

The largest single CHPD crystals having dimensions of 4 cm as observed in (fig. 3a). The sizes of the CHPD crystals were reduced from 4 cm to 2.2 cm (leaves), 1.3 cm (rhizomes) and 0.7 cm (stems) were observed in (figs. 3b, 3c and 3d). With an increase in the concentration of aqueous extracts of *Costus igneus* from 0.15% to 1.00% (w/v), the weight of the formed crystals was gradually reduced from 2.03 g to 0.06 g (leaves), 0.05 g (rhizome), 0.03 g (stem) 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 aqueous extracts of *Costus igneus* under *in vitro* conditions.

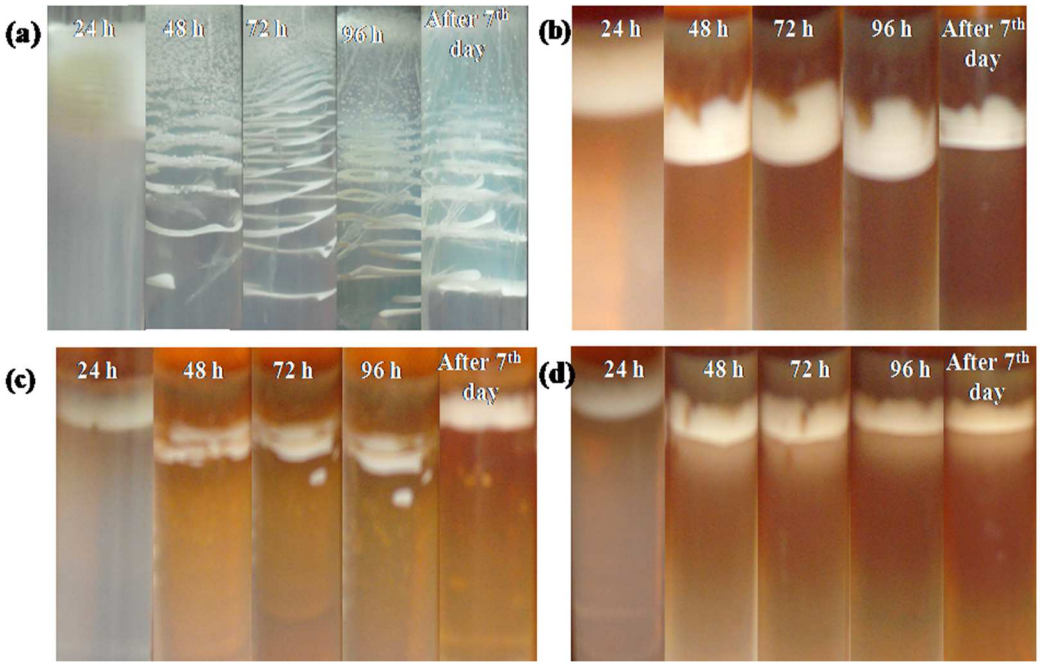

Fig. 1: The effect of *Costus igneus* extract on growth of CHPD crystals in the gel method (a) without any additive and (b) with the extract of leaves of *Costus igneus* (c) with the extract of rhizomes of *Costus igneus* (d) with the extract of stems of *Costus igneus* after 24h, 48 h, 72 h, 96h and 7d

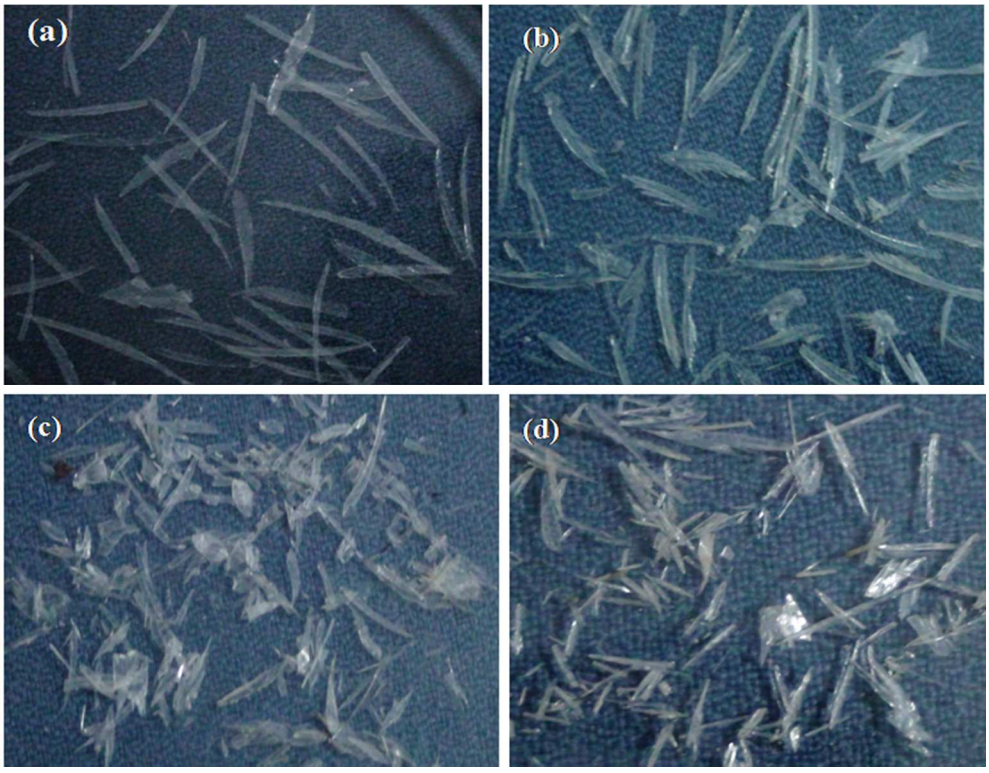

Fig. 2: The harvested crystals of CHPD obtained in the gel method (a) without any additive and (b) with the extract of leaves of *Costus igneus* (c) with the extract of rhizomes of *Costus igneus* (d) with the extract of stems of *Costus igneus*

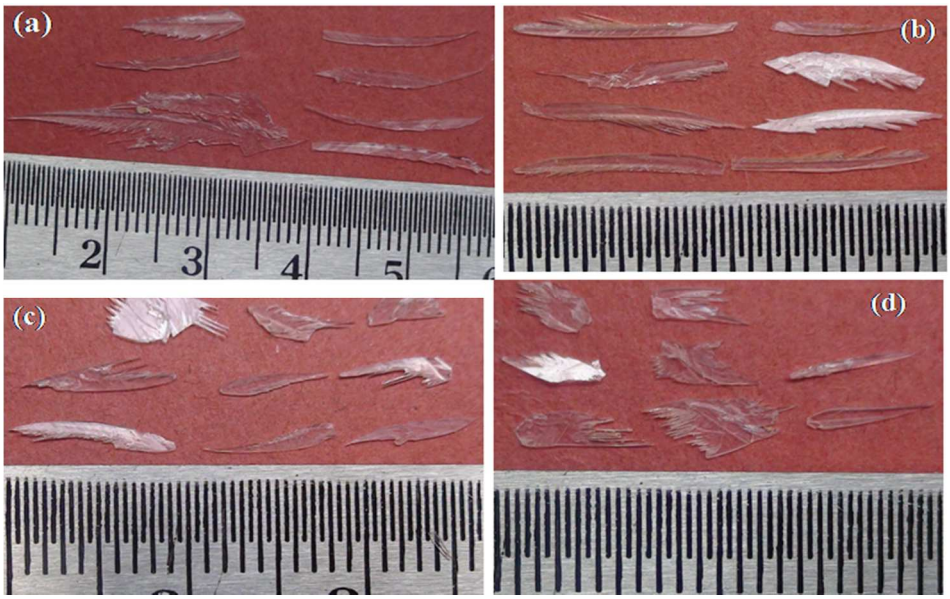

Fig. 3: The measurement of CHPD crystals obtained in the gel method (a) without any additive and (b) with the extract of leaves of *Costus igneus* (c) with the extract of rhizomes of *Costus igneus* (d) with the extract of stems of *Costus igneus*

Characterization of CHPD crystals

The FTIR spectra of CHPD crystals obtained in the presence and absence of the aqueous extract of *Costus igneus* are shown in (fig. 4). In fig. 4a, the absorptions at 3928, 3796 and 3491 cm^{-1} are due to intermolecular and weakly H-bonded OH because of water of crystallization. The weak absorption at 2394 cm^{-1} is due to HPO_4^{2-}. The H-O-H bending gives rise to absorption at 1651 cm^{-1}. The absorption at 1218 and 1134 cm^{-1} are due to P=O associated stretching vibrations. Whereas, the absorption at 1065 cm^{-1} is due to P=O stretching vibrations.

The P-O-P asymmetric stretching vibrations give rise to absorption at 991, 871 and 765 cm^{-1}. The absorption at 668 cm^{-1} is due to (H-O-) P=O. However, the strong absorption at 575 and 527 cm^{-1} are again due to acid phosphate. In (fig. 4b), the absorptions at 3924 and 3486 cm^{-1} are due to intermolecular and weakly H-bonded OH because of water of crystallization. The week absorption at 2368 cm^{-1} is due to HPO_4^{2-}. The H-O-H bending gives rise to absorption at 1601 cm^{-1}. The absorption at 1132 cm^{-1} is due to P=O associated stretching vibrations. Whereas, the absorption at 1066 cm^{-1} is due to P=O stretching vibrations. The P-O-P asymmetric stretching vibrations give rise to absorption at 990, 872 and 776 cm^{-1}. The absorption at 666 cm^{-1} is due to (H-O-)P=O. However, the strong absorption at 575 and 527 cm^{-1} are again due to acid phosphate. In (fig. 4c), the absorption at 3485 cm^{-1} is due to OH ions. The absorption at 1065 cm^{-1} is due to PO_4 stretching vibrations. Whereas, the absorption at 990, 872 and 790 cm^{-1} are due to P-O-P asymmetric stretching vibrations. The absorption at 667, 575, 527 and 414 cm^{-1} are again due to acid phosphate. In (fig. 4d), 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, 8721and 774 cm^{-1} are due to P-O-P asymmetric stretching vibrations. The absorption at 667, 575, 527 and 414 cm^{-1} are again due to acid phosphate. The shifting further supports that the stems and rhizomes of *Costus igneus* favour the nucleation and or transformation of brushite into hydroxyapatite crystals.

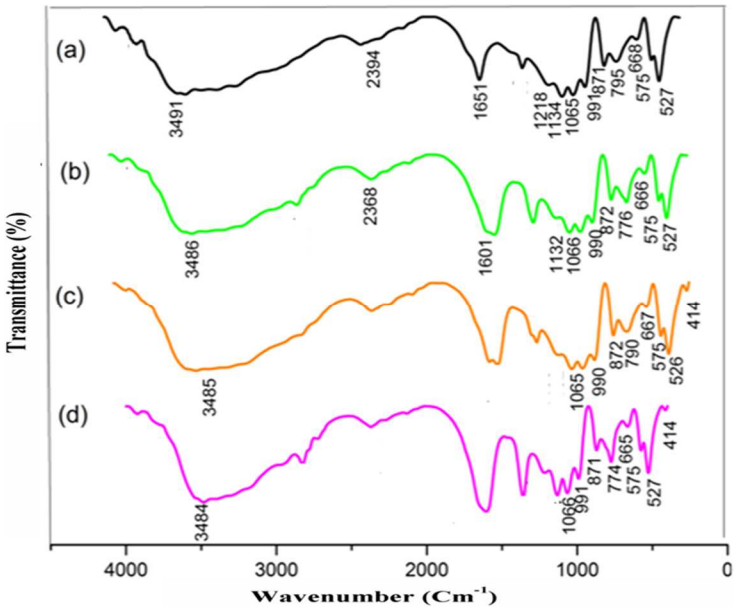

Fig. 4: The FTIR spectra of CHPD crystals obtained in the gel method (a) without any additive and (b) with the extract of leaves of *Costus igneus* (c) with the extract of rhizomes of *Costus igneus* (d) with the extract of stems of *Costus igneus*

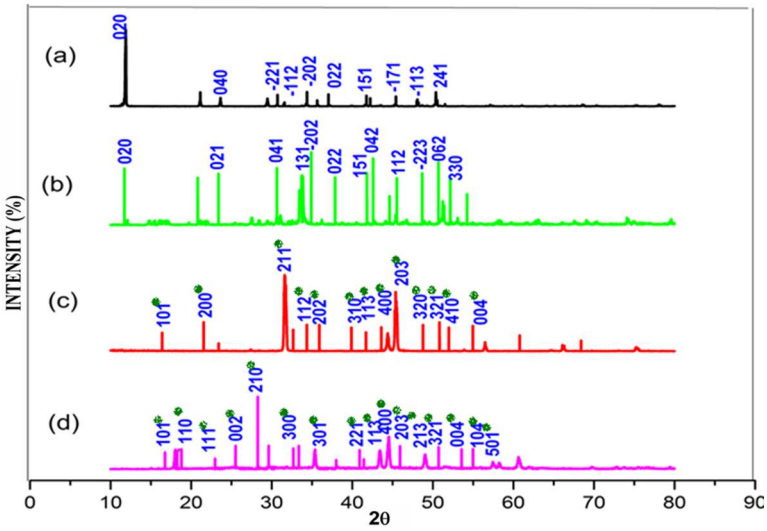

Fig. 5: The XRD pattern of CHPD crystals obtained in the gel method (a) without any additive and (b) with the extract of leaves of *Costus igneus* (c) with the extract of rhizomes of *Costus igneus* (d) with the extract of stems of *Costus igneus*

The XRD patterns of CHPD crystals obtained in the presence and absence of the aqueous extract of leaves, stems and rhizomes of Costus igneus 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 stem and rhizomes extract of Costus igneus effected the nucleation and growth of hydroxyapatite crystals. The leaves of Costus igneus can reduce the formation of brushite crystals.

DISCUSSION

The single diffusion gel growth technique has found to be a promising method to grow CHPD crystals. This technique provides a much simplified method to understand the growth of a urinary crystal *in vitro*. It can be seen from the above results that the aqueous extracts of leaves, stems and rhizomes of *Costus igneus* inhibit the nucleation and growth of CHPD crystals. The reduction of the length of crystals and the number of liesegang rings are due to the presence of an inhibitive solution containing *Costus igneus* extracts. This can be seen from (fig. 1b-d) in comparison with pure calcium chloride solution (fig. 1a) This reduction in the average apparent length is minimum in case of the supernatant solution containing stems and rhizomes of *Costus igneus* followed by leaves of *Costus igneus*. The formation of liesegang rings was observed in the present study. The effect of various parameters such as, the gel pH, the concentration of reactants and the formation of liesegang rings were previously reported [26-28]. With an increase in the concentration of aqueous extracts of *Costus igneus* from 0.15% to 1.00% (w/v), the weight of the formed crystals were gradually reduced as shown in (tables 2). 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. Group III to VII (treated with aqueous *Costus igneus* extract at various concentration 0.15% to 1%) of crystal masses were significantly different at $p<0.05$ when compared to Group I (untreated control), whereas Group II (treated with distilled water) was not significantly different at $p<0.05$ compared to Group I. This indicates that distilled water has not contained any inhibitory activity on crystal growth whereas aqueous extract of *Costus igneus* has inhibitory activity due to the presence of natural substances such as protein(18%), iron(40 mg) and antioxidant components such as ascorbic acid, β-carotene, α-Tocopherol, glutathione, phenols, flavonoids (diosgenin, quercetin), steroids, alkaloids and terpenoids [20, 21]. Group VI and VII (treated with 0.75% and 1% extracts) were not significantly different. Recently, growth inhibition studies of CHPD crystals in the presence of some of the herbal extracts *Tribulus terrestris* and *Bergenia ligulata* [26], *Terminalia arjuna* [29] citric acid and lemon juice along with human urine and artificial reference urine [30], citric acid [14], tartaric acid and tamarind solution [31] were attempted in literature. In the present work, CHPD crystals growth were reduced and the morphology of the crystals changed from hydroxyapatite in brushite crystals due to the inhibitory effect of aqueous extracts of stems and rhizomes of *Costus igneus* under *in vitro* conditions. Several researchers [14, 16, 32-34] have reported crystallization characterization of CHPD crystals using FTIR techniques. The formation of hydroxyapatite in brushite crystals due to stems and rhizomes of *Costus igneus* (fig. 4). Further it has been reported for the CHPD crystals [14, 34], the diffraction peaks 11.69, 21.0, 23.44, 29.32, 30.54, 34.18, 37.10, 41.6, 42.0, 45.28, 48.49 and 50.25 for brushite crystals and for the hydroxyapatite crystals, the diffraction peaks 16.87, 18.84, 21.75, 22.84, 25.86, 28.92, 32.18, 32.90, 34.04, 35.44, 39.79, 40.43, 43.84, 44.36, 45.29, 48.58, 49.46, 50.47, 51.25, 53.16, 54.43, 58.03 were attempted in the literature are well correlate in (fig. 5). Altogether, Crystal growth and inhibition in the presence of herbal extracts exhibits interesting results, *in vitro* study on the growth and inhibition of these CHPD crystals under the influence of herbal extracts *Costus igneus* has been reported first time in the present study. The inhibition of Brushite crystals increases as the concentration of herbal extracts increases; consequently, the number of grown crystals and their average size decrease. The influence of the extracts of *Costus igneus* on CHPD crystals by gel method showed that the stems and rhizomes can promote the formation of hydroxyapatite crystals and reduce the nucleation rate of CHPD crystals. Although the stone formation process occurring in the human body is quite complex and takes place in a dynamic environment, the present study provided basic information, under laboratory conditions, which led us to identify new inhibiting herbal extracts for stone growth.

CONCLUSION

CHPD crystals were grown by single diffusion gel growth techniques and were characterized by FTIR and Powder XRD techniques for the experimental confirmations of the grown crystal. With an increase in the concentration of aqueous extract of *Costus igneus*, the weight of the formed crystals was gradually reduced from 2.03 g to 0.06 g (leaves), 0.05 g (rhizome), 0.03 g (stem) for the CHPD crystals, respectively. The formation of hydroxyapatite was observed in brushite crystals due to inhibitory action by the aqueous extracts of stems and rhizomes of *Costus igneus* under *in vitro* conditions. The leaves of *Costus igneus* can reduce the nucleation rate of CHPD crystals. FTIR and Powder XRD techniques confirmed its functional groups and crystalline phases of CHPD 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 stems and rhizomes of *Costus igneus* 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. This study is focused on finding new alternative medicine for the treatment of calcium oxalate 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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