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INHIBITION OF GROWTH OF CALCIUM HYDROGEN PHOSPHATE DIHYDRATE CRYSTALS BY *COUROUPITA GUIANENSIS* LEAVES EXTRACT

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ABSTRACT

Objective: To investigate the inhibitory effect of methanol extract of *Couroupita guianensis* leaves 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 methanol extracts of *Couroupita guianensis* leaves 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. **Results:** With an increase in the concentration of methanol extract of *Couroupita guianensis* leaves, the weight of the formed crystals were gradually reduced from 2.35 g to 0.13 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 and hydroxyapatite (HAP) crystals. Results obtained are indicated that *Couroupita guianensis* (leaves) has the potential to inhibit the formation of CHPD crystals. **Conclusion:** This study confirms that using methanol extract of leaves of *Couroupita guianensis* 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, *Couroupita guianensis*, Fourier Transform Infrared Spectroscopy (FTIR), Powder X-Ray diffraction (XRD).

INTRODUCTION

A large number of people are suffering from urinary stones problems.[1] Urinary stones have been found to contain calcium phosphate, calcium oxalate, uric acid and magnesium ammonium phosphate.[2-4] Epidemiological data showed that the majority of stones are composed of calcium oxalate (CaOx).[5] Calcium containing stones are 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 ($CaHPO_{4\cdot2}H_2O$).[14-16] Brushite form a smooth (010) surface which allows the formation of hydroxyapatite (HAP) on brushite.[17] Urinary stones are characterized by high recurrence rate therefore requiring a preventive treatment by using the medicinal plants.[18,19]

Couroupita guianensis are commonly known as cannon ball tree belongs to the family of Lecythidaceae.[20] The leaves, flowers and barks of *Couroupita guianensis* are used to treat hypertension, tumor, pain, gastritis, scabies, bleeding piles, dysentery, scorpion poison, skin diseases and malaria. Medicinal uses of *Couroupita guianensis* contains anti-pyretic, anti-depressant, analgesic, anti-septic, anti-inflammatory, anti-protozoal, anti-cancer, and anti-ulcer activities.[21-23] The chemical constituents shows that the presence of α-amirin, β-amirin, β-sitosterol, nerol, tryptanthrine, indigo, indirubin, isatin, linoleic acid, carotenoids, and sterols.[24-27] In the present investigation, the effects of methanol extract of *Couroupita guianensis* leaves 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

Analytical grade of anhydrous methanol, calcium chloride, magnesium acetate, oxalic acid, sodium metasilicate, orthophosphoric acid were all purchased from sigma-aldrich, New Delhi, India. 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 leaves of *Couroupita guianensis* 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 leaves of *Couroupita guianensis* 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.[6] 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).

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 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 and 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% methanol extract)	5 ml of 1 M calcium chloride+5 ml of 0.15% of methanol extract of leaves of *Couroupita guianensis* separately
IV (0.25% methanol extract)	5 ml of 1 M calcium chloride+5 ml of 0.25% of methanol extract of leaves of *Couroupita guianensis* separately
V(0.50% methanol extract)	5 ml of 1 M calcium chloride+5 ml of 0.50% of methanol extract of leaves of *Couroupita guianensis* separately
VI(0.75% methanol extract)	5 ml of 1 M calcium chloride+5 ml of 0.75% of methanol extract of leaves of *Couroupita guianensis* separately
VII(1.00% methanol extract)	5 ml of 1 M calcium chloride+5 ml of 1.00% of methanol extract of leaves of *Couroupita guianensis* separately

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 *Couroupita guianensis* leaves 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,

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

Effect of *Couroupita guianensis* on CHPD crystals

The effect of the methanol extract of *Couroupita guianensis* leaves 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). The elongated broad needle shaped crystals were grown within the liesegang ring as observed after 96 h. In the presence of methanol extract of *Couroupita guianensis* leaves nucleation was delayed and reduced masses of the

crystals (fig. 1b-g). Morphology of the harvested CHPD crystals as shown in fig. 2. The largest single CHPD crystals having dimensions of 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 as observed in figs. 3b-g. With an increase in the concentration of methanol extracts of *Couroupita guianensis leaves* 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 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 was reduced due to the inhibitory effect of methanol extracts of *Couroupita guianensis* under *in vitro* conditions.

Table 2: ANOVA statistical analysis for harvested CHPD crystals.

Groups and Treatments	Mean weight of the CHPD crystals (gm)±S.D
	Leaves
I (Control)	2.35±0.057
II (Distilled water)	2.3±0.081
III (0.15% methanol extracts)	$1.22\pm0.014^{a,b}$
IV (0.25% methanol extracts)	$0.54\pm0.014^{a,b,c}$
V (0.50% methanol extracts)	$0.37\pm0.014^{a,b,c,d}$
VI (0.75% methanol extracts)	$0.22\pm0.014^{a,b,c,d}$
VII (1.00% methanol extracts)	$0.13\pm0.014^{a,b,c,d,e}$

Values represent mean (gm) ± 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. Statistical significance were considered to be $^{a}p<0.05$, $^{b}p<0.05$, $^{c}p<0.05$, $^{d}p<0.05$, $^{e}p<0.05$.

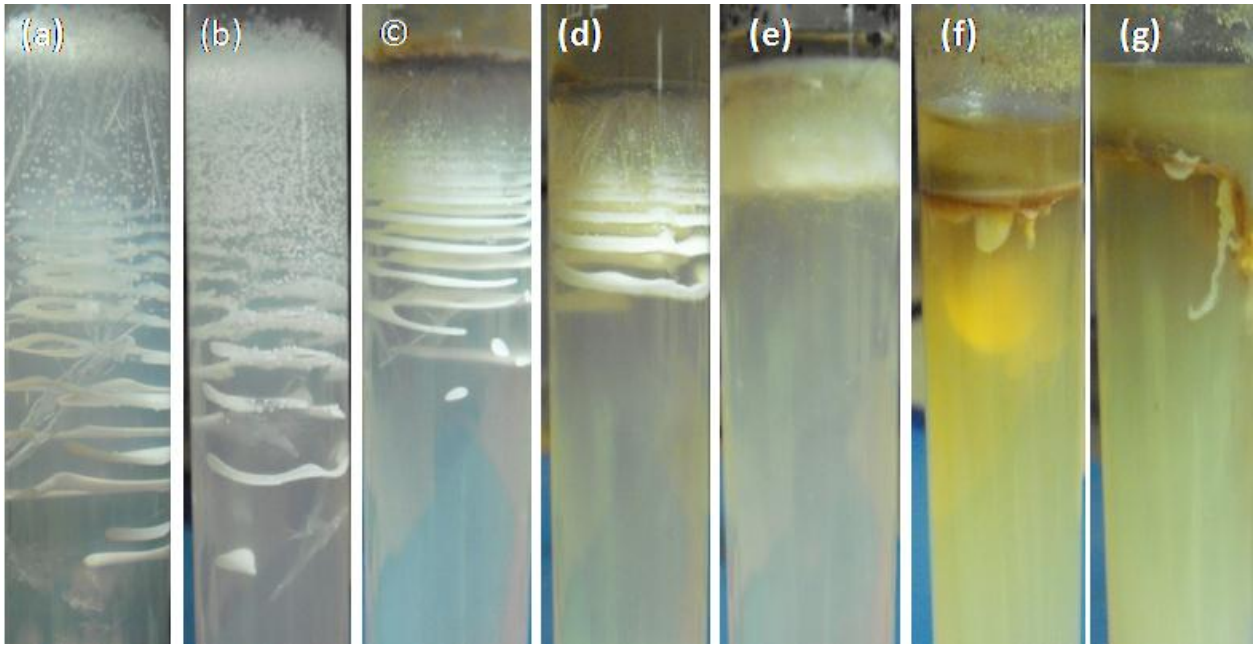

Fig. 1: The effect of *Couroupita guianensis* leaves on CHPD crystals in the gel method (a) without any additive (b) with the distilled water (c) with the 0.15% methanol extract (d) with the 0.25% methanol extract (e) with the 0.50% methanol extract (f) with the 0.75% methanol extract (g) with the 1.00% methanol extract after 7 days.

Characterization of CHPD crystals

The FTIR spectra of CHPD crystals obtained in the presence and absence of the methanol extract of *Couroupita guianensis* leaves 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 absorptions at 3485 cm^{-1} are due to intermolecular and weakly H bonded OH because of water of crystallization. The weak absorption at 2385 cm^{-1} is due to HPO_4^{2-}. The H-O-H bending gives rise to absorption at 1647 cm^{-1}. The absorption at 1132 cm^{-1} is 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 990, 872 and 790 cm^{-1}. The absorption at 667 cm^{-1} is due to (H-O-)P=O. However, the strong absorption at 575 and 526 cm^{-1} are again due to acid phosphate. In (fig. 4c), 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} is 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, 871 and 774cm^{-1} are due to P-O-P asymmetric stretching vibrations. The absorption at 665, 575 and 527 cm^{-1} is again due to acid phosphate. In (fig. 4e), 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 774cm^{-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. 4f), 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 774cm^{-1} are due to P-O-P asymmetric stretching vibrations. The absorption at 664, 575 and 526 cm^{-1} is again due to acid phosphate. In (fig. 4g), 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 leaves of *Couroupita guianensis* (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 leaves of *Couroupita guianensis* favour the nucleation and or transformation of brushite into hydroxyapatite crystals.

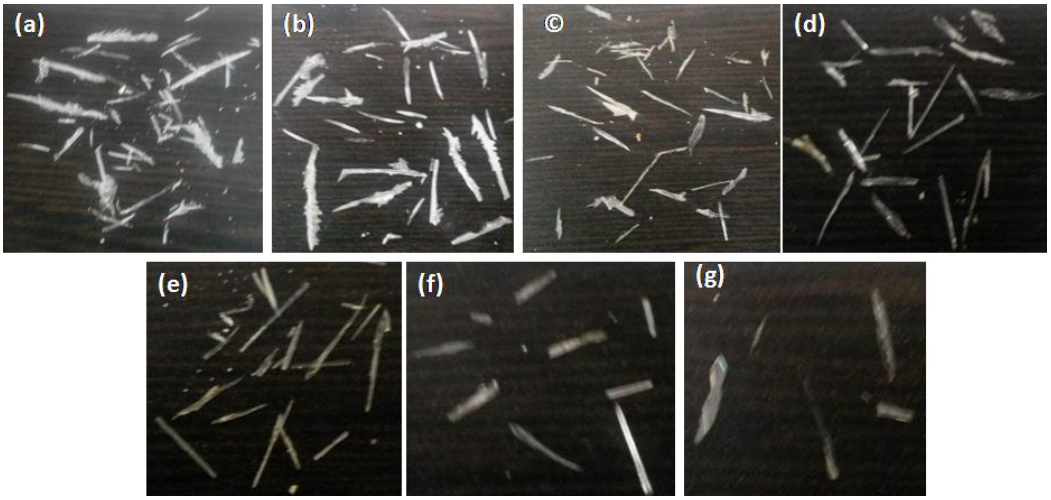

Fig. 2: The harvested crystals of CHPD obtained from *Couroupita guianensis* leaves in the gel method (a) without any additive (b) with the distilled water (c) with the 0.15% of methanol extract (d) with the 0.25% of methanol extract (e) with the 0.50% of methanol extract (f) with the 0.75% of methanol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

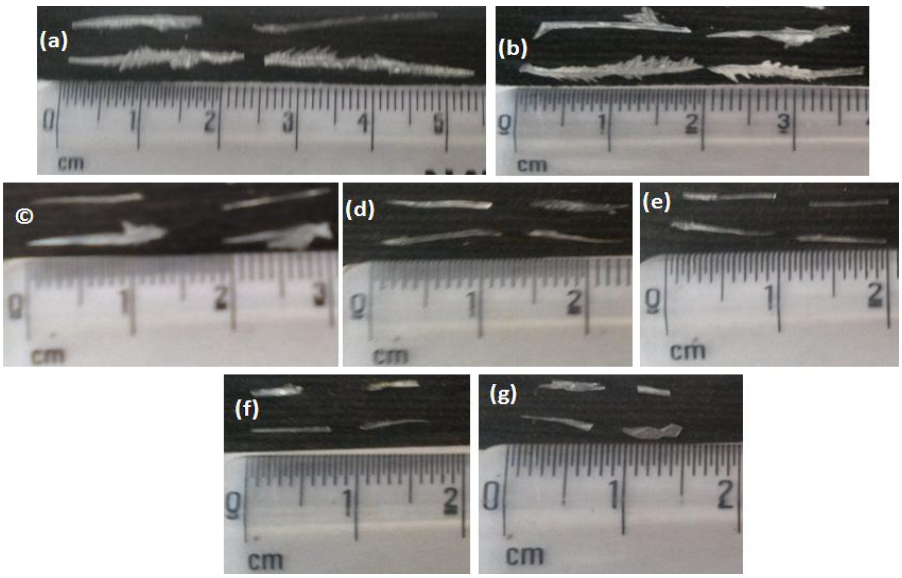

Fig. 3: The measurement of CHPD obtained from *Couroupita guianensis* leaves in the gel method (a) without any additive (b) with the distilled water (c) with the 0.15% of methanol extract (d) with the 0.25% of methanol extract (e) with the 0.50% of methanol extract o (f) with the 0.75% of methanol (g)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

The XRD patterns of CHPD crystals obtained in the presence and absence of the methanol extract of *Couroupita guianensis* leaves 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). The *Couroupita guianensis* leaves effected the nucleation and growth of hydroxyapatite crystals.

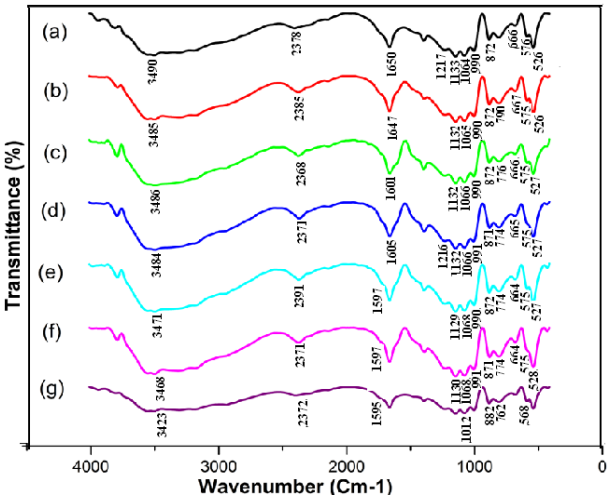

Fig. 4: The FTIR spectra of CHPD obtained from *Couroupita guianensis* leaves in the gel method (a) without any additive (b) with the distilled water (c) with the 0.15% of methanol extract (d) with the 0.25% of methanol extract (e) with the 0.50% of methanol extract (f) with the 0.75% of methanol extract (g) with the 1.00% of methanol extract.

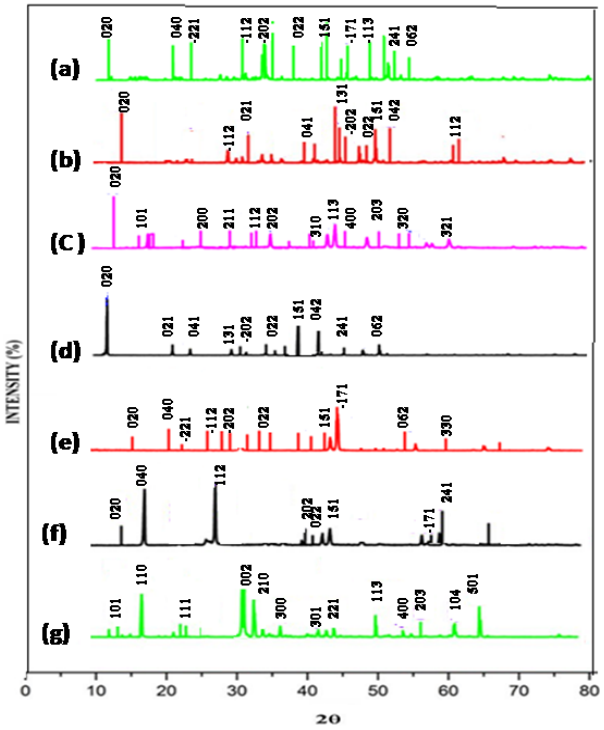

Fig. 5: The XRD pattern of CHPD obtained from *Couroupita guianensis* leaves in the gel method (a) without any additive (b) with the distilled water (c) with the 0.15% of methanol extract (d) with the 0.25% of methanol extract (e) with the 0.50% of methanol extract (f) with the 0.75% of methanol extract (g) with the 1.00% of methanol extract.

DISCUSSION

The single diffusion gel growth technique has been used to understand the growth of CHPD crystal *in vitro*. The effect of various parameters such as the gel pH, the concentration of reactants and the formation of liesegang rings were previously reported.[28-30] In the present study the reduction of the length of crystals and the number of liesegang rings are due to the presence of inhibitive solution containing *Couroupita guianensis* leaves extracts as shown in tables 2. Recently, growth inhibition studies of CHPD crystals in the presence of some of the herbal extracts *Tribulus terrestris* and *Bergenia ligulata*,[26] *Terminalia arjuna*[31] citric acid and lemon juice along with human urine and artificial reference urine,[32] citric acid,[14] tartaric acid and tamarind solution,[33] *Costus igneus*[37] were attempted in literature. Several researchers[14,16,34-37] have reported that the crystallization characterization of CHPD crystals using FTIR techniques. 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 II indicates that distilled water has not contained any inhibitory activity on crystal growth whereas methanol extract of *Couroupita guianensis* leaves has inhibitory activity due to the presence of natural substances and the various medicinal activities.[20,21,38,39] The influence of the methanol extracts of *Couroupita guianensis* leaves on CHPD crystals by gel method has been reported first time in the present study showed that the leaves can promote the formation of hydroxyapatite crystals and reduce the nucleation rate of CHPD crystals.

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 *Couroupita guianensis* leaves the weight of the formed crystals were gradually reduced from 2.35 g to 0.13 g in CHPD crystals, respectively. 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 leaves of *Couroupita guianensis* 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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