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Growth and Characterization of Calcium Hydrogen Phosphate Dihydrate Crystals influenced by Leaves of Hibiscus Rosasinensis

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Abstract: ***To investigate the inhibitory effect of methanol extract of leaves of Hibiscus rosasinensis on the growth of calcium hydrogen phosphate dihydrate (CHPD) crystals. Calcium hydrogen phosphate dihydrate (CHPD) crystals were grown by the single diffusion gel growth technique and the inhibitory effect of methanol extracts of leaves of Hibiscus rosasinensis on the growth of CHPD crystals has been studied.***
The grown crystals were characterized by Fourier Transform Infrared Spectroscopy (FTIR), Powder X-Ray diffraction (XRD) for further confirmations. With an increase in the concentration of methanol extract of Hibiscus rosasinensis, the weight of the formed crystals were gradually reduced from 142 g to 17.8 g (leaves) for the CHPD crystals, respectively. The crystals harvested from the CHPD were characterized 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 Hibiscus rosasinensis (leaves) has the potential to inhibit the formation of calcium hydrogen phosphate dihydrate crystals. This study confirms that using methanol extract of leaves of Hibiscus rosasinensis 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, Hibiscus rosasinensis, Fourier Transform Infrared Spectroscopy (FTIR), Powder X-Ray diffraction (XRD).

I. INTRODUCTION

A large number of people are suffering from problems due to urinary stones [1]. Urinary stone is formation of urinary calculi at any level of 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 ($CaHPO_4.{}_2H_2O$) [14-16]. 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 characterized by high recurrence rate therefore requiring a preventive treatment using medicinal plants [18,19].

The herb Hibiscus rosa-sinensis Linn [Malvaceae] is a glabrous shrub widely cultivated in the tropics as an ornamental plant and has several forms with varying colours of flowers. In medicine, however, the red flowered variety is preferred [20]. Medicinal uses of *Hibiscus rosasinensis* constitute antipyretic, antidepressant, analgesic, antiseptic, anti-inflammatory, antiprotozoal, anticancer, and antiulcer activities. In Ayurveda, they are used extensively for its anti-inflammatory property [21]. The flower of *Hibiscus rosasinensis* comprises antibacterial activity and antifungal property.

Hibiscus rosasinensis was used as an ingredient in many ways for curing gastritis, scabies, bleeding piles, dysentery, and scorpion poison [22]. The various parts of *Hibiscus rosasinensis* such as leaves, flowers, and barks are used to treat hypertension, tumor, pain, and inflammatory reactions [23]. Leaves are used to make juice which is used to treat skin diseases, and South Americans use each part of the *Hibiscus rosasinensis* tree to treat malaria. The studies of chemical constituents show the presence of α-amirin, β-amirin,

β-sitosterol, nerol, tryptanthrine, indigo, indirubin, isatin, linoleic acid, carotenoids, and sterols [24-26]. Aqueous extract of *Hibiscus rosasinensis* is endowed with flavonoids, alkaloids, phlorotannins, glycosides, tannins, steroids, and terpenoids [27]. In the present investigation, the effects of methanol extract of leaves of *Hibiscus rosasinensis* 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.

II. MATERIALS AND METHODS

A. *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.

B. *Collection of Plant Material*

The leaves of *Hibiscus rosasinensis* were collected in the month of june from the srirangam, Trichy, Tamil Nadu, India. The plant was identified and leaves of *Hibiscus rosasinensis* were authenticated and confirmed from Dr. S. John Britto, Director, Rapinat herbarium, St. Joseph College, Tiruchirapalli, and Tamil Nadu for identifying the plants. The voucher specimen number KVN 001 (20.06.2018).

C. *Preparation of Methanol Extracts*

The leaves of *Hibiscus rosasinensis* were washed in running water, cut into small pieces and then shade dried for a week at 35-40ºC, after which it was grinded to a uniform powder of 40 mesh size [6]. The methanol extracts were prepared by soaking 100 g each of the dried powder plant materials in 1 L of methanol using a soxhlet extractor continuously for 10 hr. The extracts were filtered through whatmann filter paper No. 42 (125mm). The entire extracts were concentrated to dryness using a rotary evaporator under reduced pressure. The filtrate was condensed using a rotary evaporator and the residue 1.2 g (leaves) obtained were used to prepare the series (0.15, 0.25, 0.50, 0.75 and 1.0%) of supernatant concentrations for *in vitro* studies (table 1).

D. *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 ($Na_2SiO_3 \cdot 9H_2O$) 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

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 1% of methanol extract of leaves of *Hibiscus rosasinensis* separately
IV (0.25% methanol extract)	5 ml of 1 M calcium chloride+5 ml of 2% of methanol extract of leaves of *Hibiscus rosasinensis* separately
V(0.50% methanol extract)	5 ml of 1 M calcium chloride+5 ml of 3% of methanol extract of leaves of *Hibiscus rosasinensis* separately
VI(0.75% methanol extract)	5 ml of 1 M calcium chloride+5 ml of 4% of methanol extract of leaves of *Hibiscus rosasinensis* separately
VII(1.00% methanol extract)	5 ml of 1 M calcium chloride+5 ml of 5% of methanol extract of leaves of *Hibiscus rosasinensis* separately

E. 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 methanol extract of leaves of *Hibiscus rosasinensis* on the growth of CHPD crystals, a series of five different concentrations of 1,2,3,4 and 5% of these plant extracts were added in equal amounts in supernatant solution and the average weight of the grown crystal were measured.

F. 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.

III. RESULTS

A. Effect of Hibiscus Rosasinensis on CHPD Crystals

The effect of the methanol extract of the leaves of *Hibiscus rosasinensis* 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 leaves of *Hibiscus rosasinensis*, 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 *Hibiscus rosasinensis* extracts. Moreover, supernatant solutions (methanol leaves of *Hibiscus rosasinensis*) 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 2.6 cm as observed in (fig.3a). The sizes of the CHPD crystals were reduced from 3 cm to 1.8 cm and 1.3 cm at 1% extract, 1.2 cm and 1 cm at 2%, 0.9 cm and 0.8 cm at 3%, 0.7 cm and 0.6 cm at 4% and 0.5 cm and 0.3 cm at 5% observed in (figs. 3b-g). With an increase in the concentration of methanol extracts of *Hibiscus rosasinensis* from 1% to 5% (w/v), the weight of the formed crystals were gradually reduced from 142 g to 17.8 g (leaves) respectively. In the present work, CHPD crystals growth were reduced due to the inhibitory effect of methanol extracts of *Hibiscus rosasinensis* under *in vitro* conditions.

Table 2: Inhibition of Crystal Growth

Crystals	Supernatant Solutions (Groups and Treatments)	Weight of crystal	Percentage of inhibition of crystal
CHPD	I	142.0	0%
	II	72.6	48.8%
	III	52.7	62.8%
	IV	45.3	68%
	V	38.9	72.6%
	VI	35	75.3%
	VII	33	76.5%
	VIII	17.8	87.4%

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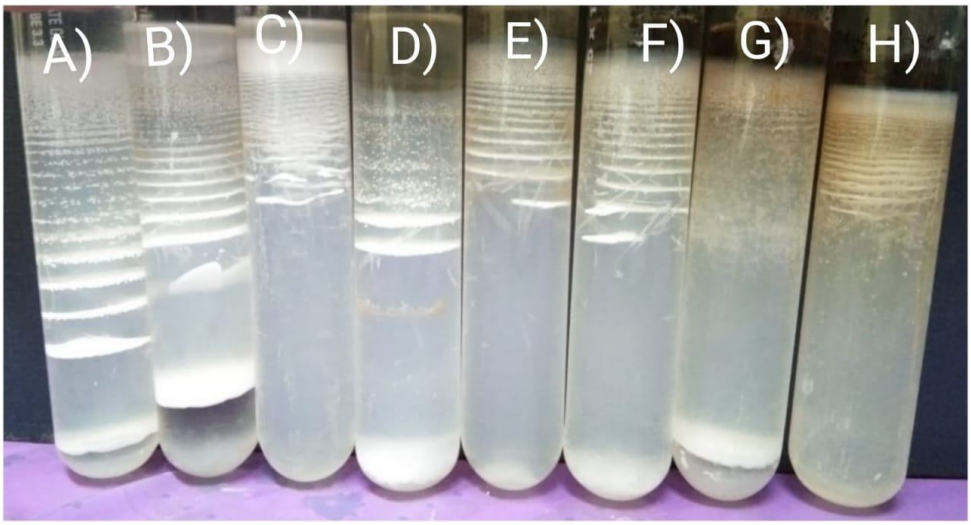

Fig. 1 The effect of *Hibiscus rosasinensis* on CHPD crystals in the gel method (a) without any additive (b) with the distilled water (c) with the methanol (d) with the 1% of methanol extract of leaves of *Hibiscus rosasinensis* (e) with the 2% of methanol extract of leaves of *Hibiscus rosasinensis* (f) with the 3% of methanol extract of leaves of *Hibiscus rosasinensis* (g) with the 4% of methanol extract of leaves of *Hibiscus rosasinensis*, (h) with the 5% of methanol extract of leaves of *Hibiscus rosasinensis* after 7 days.

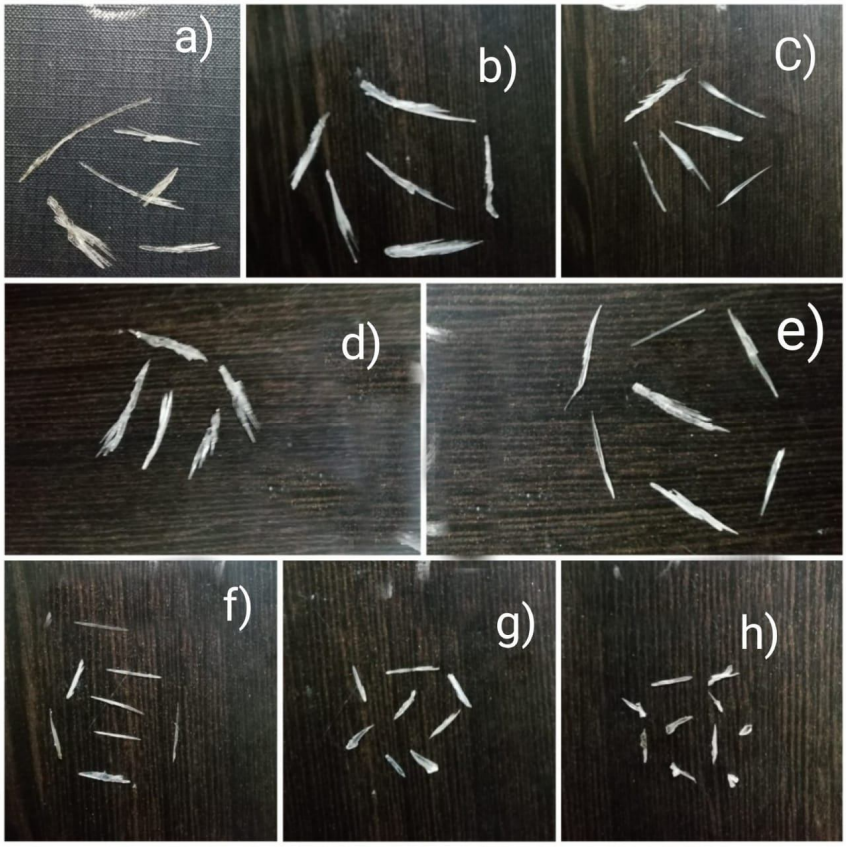

Fig. 2 The harvested crystals of CHPD in the gel method (a) without any additive (b) with the distilled water (c) with the methanol (d) with the 1% of methanol extract of leaves of Hibiscus rosasinensis (e) with the 2% of methanol extract of leaves of Hibiscus rosasinensis (f) with the 3% of methanol extract of leaves of Hibiscus rosasinensis (g) with the 4% of methanol extract of leaves of Hibiscus rosasinensis, (h) with the 5% of methanol extract of leaves of Hibiscus rosasinensis after 7 days.

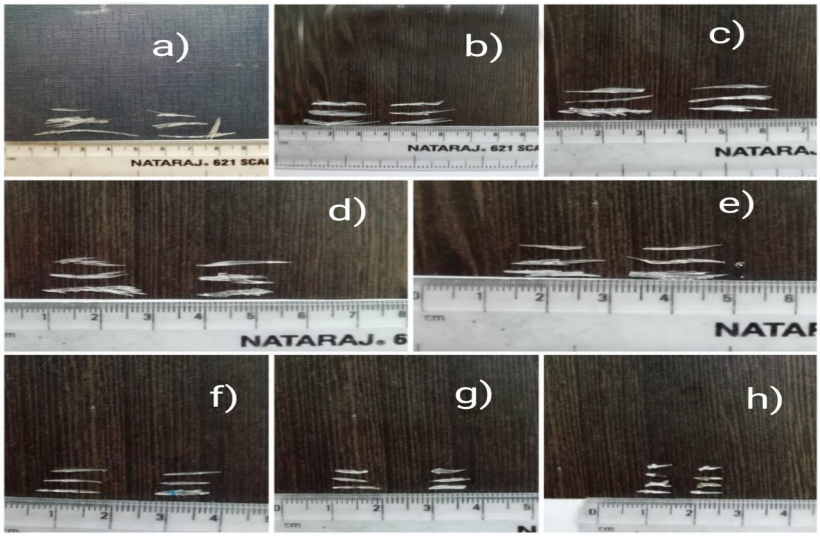

Fig. 3 The measurement of CHPD obtained in the gel method(a) without any additive (b) with the distilled water (c) with the methanol (d) with the 1% of methanol extract of leaves of Hibiscus rosasinensis (e) with the 2% of methanol extract of leaves of Hibiscus rosasinensis (f) with the 3% of methanol extract of leaves of Hibiscus rosasinensis (g) with the 4% of methanol extract of leaves of Hibiscus rosasinensis, (h) with the 5% of methanol extract of leaves of Hibiscus rosasinensis after 7 days.

B. Characterization of CHPD Crystal

The FTIR spectra of CHPD crystals obtained in the presence and absence of the methanol extract of sample. In Fig. 4a, the absorptions at 3468 cm^{-1} are due to intermolecular and weakly H bonded OH because of water of crystallization. The weak absorption at 2371 cm^{-1} is due to HPO_4^{2-}. The H-O-H bending gives rise to absorption at 1597 cm^{-1}. The absorption at 1130 cm^{-1} are due to P=O associated stretching vibrations. Whereas, the absorption at 1068 cm^{-1} is due to P=O stretching vibrations. The P-O-P asymmetric stretching vibrations give rise to absorption at 991, 871 cm^{-1}. The absorption at 666 cm^{-1} is due to (H-O-) P=O. However, the strong absorption at 575 and 528 cm^{-1} are again due to acid phosphate. In (Fig. 4b), the absorptions at 3423 cm^{-1} are due to intermolecular and weakly H bonded OH because of water of crystallization. The week absorption at 2372 cm^{-1} is due to HPO_4^{2-}. The H-O-H bending give rise to absorption at 1595 cm^{-1}. The absorption at 1012 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 882 cm^{-1}. The absorption at 762 cm^{-1} is due to (H-O-)P=O. However, the strong absorption at 568 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 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} are 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 cm^{-1} are due to P-O-P asymmetric stretching vibrations. The absorption at 666, 576 and 526 cm^{-1} are again due to acid phosphate. In (fig. 4f), 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 790cm^{-1} are due to P-O-P asymmetric stretching vibrations. The absorption at 667, 565 and 526 cm^{-1} are again due to acid phosphate. In (fig. 4g), 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 872 and 776 cm^{-1} are due to P-O-P asymmetric stretching vibrations. The absorption at 575 cm^{-1} are again due to acid phosphate. At higher concentration of methanolic extract (5%) shifting from brushite crystals band at 1068 cm^{-1} to 1601 for hydroxyapatite crystals band at 1068 to 1066 cm^{-1}. The shifting further supports that the extract favour the nucleation and or transformation of brushite into hydroxyapatite crystals. The XRD patterns of CHPD crystals obtained in the presence and absence of the methanol extract 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 extract effected the nucleation and growth of hydroxyapatite crystals.

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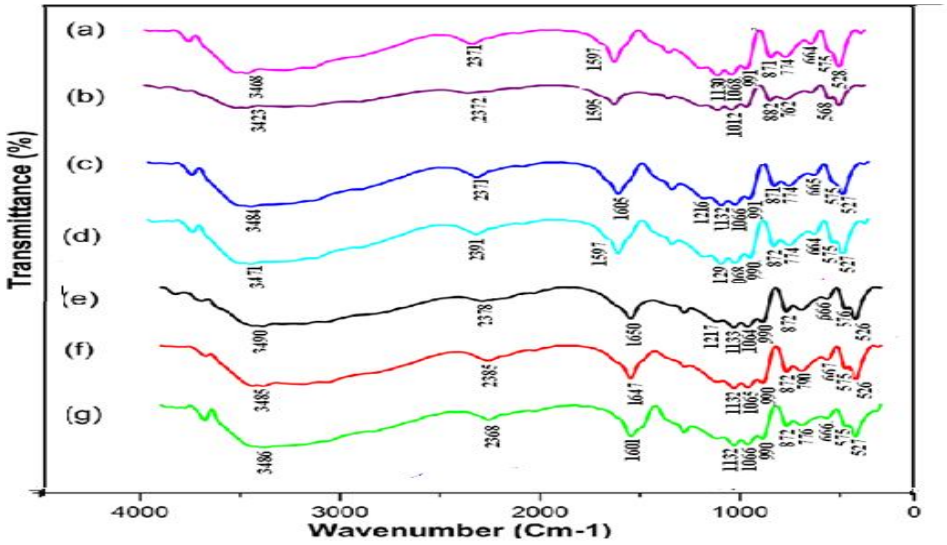

Fig. 4: The FTIR spectra of CHPD in the gel method (a) without any additive (b) with the distilled water (c) with the 0.15% of methanol extract of leaves of Hibiscus rosasinensis (d) with the 0.25% of methanol extract of leaves of Hibiscus rosasinensis (e) with the 0.50% of methanol extract of leaves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

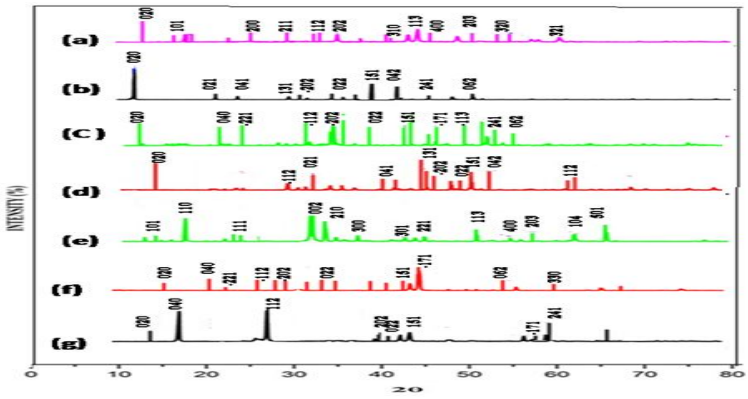

Fig. 5 The XRD pattern of CHPD in the gel method (a) without any additive (b) with the distilled water (c) with the 0.15% of methanol extract of leaves of Hibiscus rosasinensis (d) with the 0.25% of methanol extract of leaves of Hibiscus rosasinensis (e) with the 0.50% of methanol extract of leaves of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

IV. DISCUSSION

The single diffusion gel growth technique has found to be promising method to grow CHPD crystals. This technique provides much simplified method to understand the growth of urinary crystal in vitro. It can be seen from the above results that the methanol extracts of leaves of i Hibiscus rosasinensis nhibit 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 inhibitive solution containing Hibiscus rosasinensis extracts. This reduction in the average apparent length is minimum in case of the supernatant solution containing 4% and 5% extracts of Hibiscus rosasinensis followed by 1% and 2% extracts of Hibiscus rosasinensis. 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 [28-30]. This Group II indicates that distilled water has not contained any inhibitory activity on crystal growth whereas methanol extract of Hibiscus rosasinensis has inhibitory activity due to the presence of natural substances such as α-amirin, β-amirin, β-sitosterol, nerol, tryptanthrine, indigo, indirubin, isatin, linoleic acid, carotenoids, sterols, flavonoids (stigmasterol, quercetin), steroids, alkaloids and terpenoids [20,21]. Group VI and VII (treated with 3% and 4% 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 [31] citric acid and lemon juice along with human urine and artificial reference urine [32], citric acid [14], tartaric acid and tamarind solution [33] 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 methanol extracts of leaves of Hibiscus rosasinensis under in vitro conditions. Several researchers [14,16, 34-36] have reported crystallization characterization of CHPD crystals using FTIR techniques. The formation of hydroxyapatite in brushite crystals due to leaves of Hibiscus rosasinensis (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 Hibiscus rosasinensis 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 Hibiscus rosasinensis on CHPD crystals by gel method showed that the leaves 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.

V. 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 Hibiscus rosasinensis, the weight of the formed crystals were gradually reduced from 142 g to 17.8 g (leaves) for the CHPD crystals, respectively. The formation of hydroxyapatite was observed in brushite crystals due to inhibitory action by the methanol extracts of leaves of Hibiscus rosasinensis under in-vitro conditions. The leaves of Hibiscus rosasinensis 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 leaves of Hibiscus rosasinensis 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 to find new alternative medicine for the treatment of calcium oxalate urinary stone.

VI. ACKNOWLEDGEMENT

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VII. 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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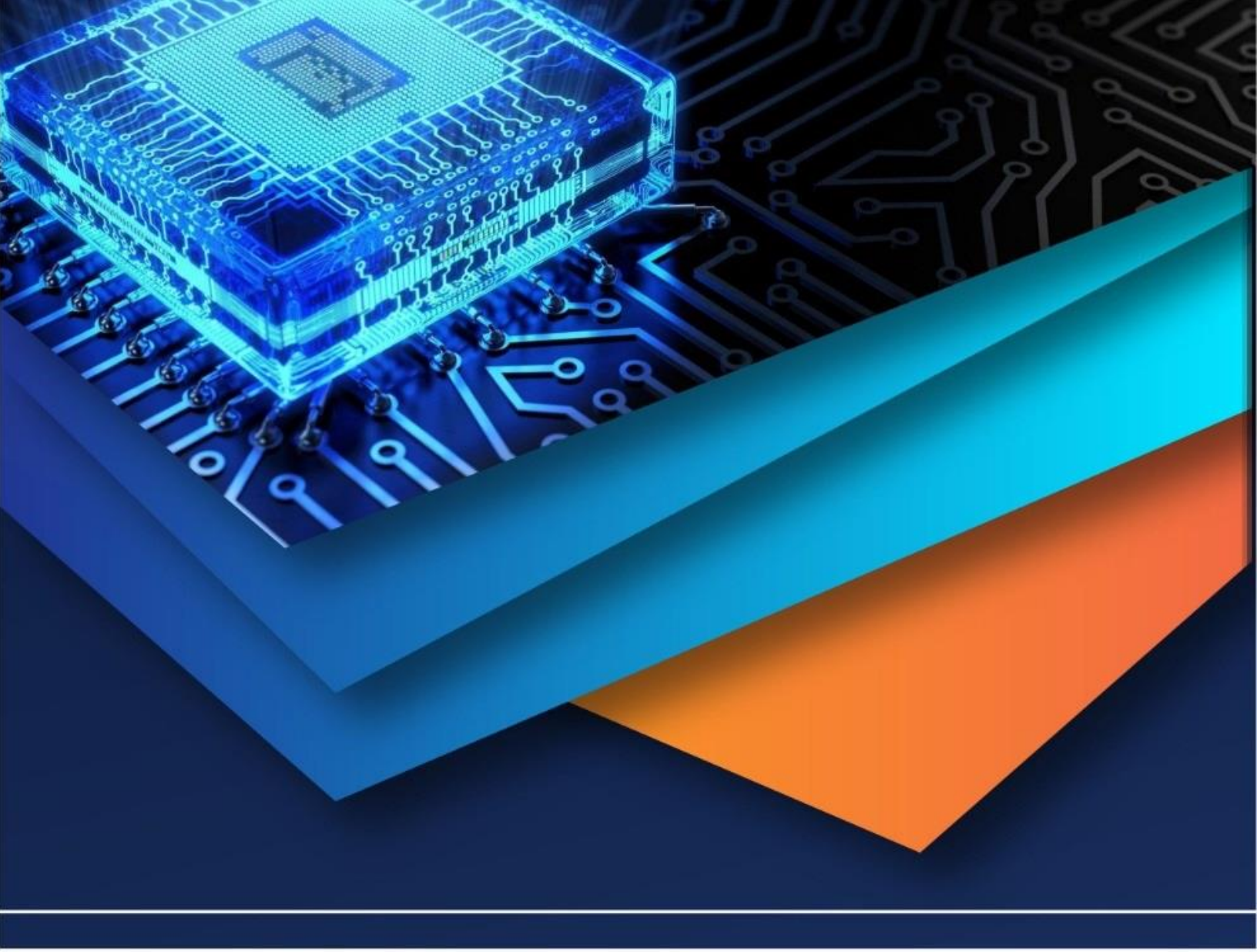

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